Axon regeneration and circuit reorganization after complete spinal cord injury

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SUMMARY

First human trials involving neuroprosthetic rehabilitation demonstrated recently that significant functional benefits can be achieved with lumbosacral neuromodulation and reorganized spared projections. However, complete spinal cord injuries (SCI) wholly isolate infralesional circuits from any supraspinal control, leading to irreversible motor deficits that neuroprosthetics still fails to address. As axons fail to regrow across the lesion site, neuroregenerative research after SCI has been dogmatically focusing attention on minimizing the environmental inhibitions to axon regeneration. In the present work, we demonstrate that spontaneous axon regeneration failure can be reversed, and that propriospinal axons are able to grow across complete non-neural lesion cores when the required facilitators are provided. We identified three mechanisms needed for propriospinal regrowth : i) enhancing neuronal intrinsic growth capacities using viral technologies, ii) remodeling the lesion core with growth factors, in order to densify permissive substrates, and iii) guiding axons chemically across the SCI site.

Propriospinal neurons - that coordinate different spinal segments in healthy conditions - are known to support recovery of locomotion in incomplete models of spinal cord injury. However, restoring a robust descending bridge of propriospinal axons does not by itself promote any functional benefit. We hypothesize that a lack of somatosensory feedback to the motor cortex (M1), together with insufficient descending motor control, may prevent coherent exchange of information between lumbosacral and cortical motor centers. Therefore, we explored the neuroregenerative potential of reticulospinal axons - which are projected from the brainstem to the spinal cord - as a second relay for the descending motor cortical command. Together with axon guidance and lesion remodeling, our viral manipulation of intrinsic growth programs induced limited yet significant reticulospinal regeneration into the lesion core. In order to enhance this reticulospinal regrowth, we explored activity-dependent mechanisms of neurite growth control. As we observed that activity does not recruit growth programs after SCI, we revealed a possible divergence between the mechanisms that underly reticulospinal axon regrowth and neurite sprouting. In the mean time, we are developing a somatosensory interface that aims at restoring sensory feedback to the motor cortex after complete SCI. Based on hindlimb electromyographic activity, we linked locomotion to optogenetic neuromodulation of the motor thalamus, and were able to evoke responses in M1 by selectively activating thalamocortical projections, both before and after complete spinal lesion. Such technology enables a direct recruitment of thalamic nuclei that gate motor learning and motor planning at a cortical level. Coming experiments will test wether a strategy combining propriospinal regeneration with locomotion-dependent thalamocortical modulation is sufficient to restore voluntary stepping after complete SCI.

Key words : Spinal cord injury, Axon regeneration, Propriospinal axons, Reticulospinal tract, Neuromodulation, Motor thalamus, Cell grafting, Filum Terminale

RÉSUMÉ

Des premiers essais cliniques de réhabilitation neuroprothétique chez l'homme ont récemment démontré que des bénéfices fonctionnels significatifs peuvent être obtenu par neuromodulation lombo-sacrée et réorganization des projections nerveuses épargnées. Toutefois, des lésions complètes de la moelle épinière isolent totalement les circuits infra-lésionnels de tout contrôle volontaire, causant des déficits moteurs irréversibles que pour l'heure les approches neuroprothétiques ne parviennent pas à traiter. Alors que la repousse axonale est tenue en échec au site de lésion, la recherche en neurorégénération après lésion médullaire a porté dogmatiquement son attention sur l'entrave des inhibitions environnementales à la re-croissance des fibres. Dans le présent travail, nous démontrons que l'échec naturel de la régénération axonale peut être réversé, et que des fibres propriospinales sont capables de croître au travers du tissu cicatriciel non-nerveux qui compose la lésion. Nous avons identifié trois méchanismes facilitateurs requis et suffisants à la repousse propriospinale : i) l'activation des programmes de croissance intrinsèques des neurones, ii) la densification des substrats permissifs par remodelage du tissu lésionnel et iii) le guidage des axones à travers la lésion.

Les neurones propriospinaux - qui coordonnent différents segments de la moelle épinière en conditions normales - sont connus pour promouvoir la récupération motrice lors de lésions médullaires incomplètes. Cependant, la seule restauration d'un pont descendant propriospinal à travers une lésion complète ne fournit pas de bénéfice fonctionnel. Nous supposons - lors de lésions médullaires complètes - qu'un manque de retour somatosensoriel vers le cortex moteur primaire (M1), ainsi qu'un contrôle moteur descendant désorganisé, préviennent l'échange cohérent d'information entre les centres corticaux et les circuits lombo-sacrés. Pour y pallier, nous avons exploré le potentiel régénératif des axones reticulospinaux - lesquelles sont projetés dans la moelle épinière depuis le tronc cérébral - comme second relai pour les commandes corticales. Associé au remodelage du site lésé et au guidage des fibres, notre manipulation des programmes intrinsèques de croissance a permis une régénération réticulospinale faible mais significative dans la lésion. En explorant les mécanismes de croissance des projections neuronales associés à l'activité électrophysiologique, nous avons révélé de probables divergences entre les déterminants signalétiques de la repousse axonale et de l'arborescence neuronale. En parallèle, nous développons une interface neuroprothétique somatosensorielle, qui tend à restaurer un retour sensitif au cortex moteur après lésion médullaire complète. Fondé sur l'activité électromyographique des membres postérieurs, nous avons appairé à la locomotion une neuromodulation optogénétique du thalamus moteur qui nous permet d'évoquer des réponses corticales en M1, ceci en conditions saines et après lésion médullaire complète. Cette technologie nous permet de recruter spécifiquement les noyaux thalamiques qui contrôlent l'apprentissage moteur et la plannification des tâches motrices au niveau cortical. Les expériences à venir détermineront si une approche combinant neuromodulation thalamique et régénération propriospinale permet de restaurer un contrôle moteur volontaire après lésion complète de la moelle épinière.

Mots clés : Lésion de la moelle épinière, Régénération axonale, Axones propriospinaux, Tract réticulospinal, Neuromodulation, Thalamus moteur, Transplantation cellulaire, Filum Terminale

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ABBREVIATIONS

A1/A2 astrocytes - Subtypes of reactive astrocytes (cell subtypes)

AAV - Adeno-associated virus (virus)

AIS - American spinal injury association impairment scale (score)

AMPA - Amino-hydroxy-methyl-isoxazoleproprionic acid receptor for Glutamate (membrane receptor)

ASIA - American spinal injury association (association)

BDA - Biotin dextran amin tracer (non-viral axon tracer)

BDNF - Brain derived neurotrophic factor (growth factor)

cAMP - Cyclic adenosine monophosphate (signaling molecule)

ChR2 - Channelrhodopsin-2 (membrane receptor)

CNO - Clozapine-N-O (drug)

CNTF - Ciliary neurotrophic factor (growth factor)

CNS - Central nervous system (organ)

CPG - Central Pattern Generator (concept)

CREB - cAMP response element binding protein (signaling molecule)

CSPG - Chondroitin sulfate proteoglycan (inhibitory extracellular molecule)

CST - Cortico-spinal tract (axon tract)

DBS - Deep brain stimulation (neuromodulation technique)

DCH - Dicopolypeptide Hydrogel (drug vehicle)

DNA - Deoxyribonucleic acid (genetic component)

DREADD - Designer receptor exclusively activated by designer drug (technic / receptor)

E14 - Embryonic day-14 (time point)

EES - Electric epidural stimulation (neuromodulation technique)

EGF - Epidermal growth factor (growth factor)

EMG - Electromyography (electrophysiological technique)

ESC - Embryonic stem cells (cell type)

FES - Functional electrical stimulation (muscle modulation technique)

FGF - Fibroblast growth factor (growth factor)

FT - Filum Terminale (CNS structure)

GABA - gamma-aminobutyric acid (membrane receptors)

GAG - Glycosaminoglycan (extracellular molecule)

GDNF - Glial-derived neurotrophic factor (growth factor)

GFAP - Glial fibrillary acidic protein (astrocyte marker)

GFP - Green fluorescent protein (fluorescent marker)

HGF - Hepatocyte growth factor (growth factor)

IFN - Interferon (drug)

IGF - Insulin-like growth factor (growth factor)

IL - Interleukin (cytokine)

iPSC - Induced pluripotent stem cells (cell type)

KCC2 - Potassium-chloride transporter (membrane channel)

KLF - Kruppel like factor (growth factor)

M1 cortex - Primary motor cortex (CNS structure)

M1/M2 macrophages - Subtypes of activated macrophages (cell subtypes)

MAG - Myelin associated glycoprotein (inhibitory extracellular molecule)

MAI - Myelin associated inhibitiory molecules (inhibitory extracellular molecules)

MAP2 - Microtubule associated protein 2 (neuronal marker)

MAPK - Mitogen activated protein kinases (signaling molecule)

MDL28170 - Calpain inhibitor (survival factor)

MG - Medial gastrocnemius muscle (muscle)

MMP - Metalloproteinase (extracellular molecules)

mTOR - Mammalian target of Rapamycin (signaling molecule)

mRNA - Messenger ribonucleic acid (genetic component)

NGF - Nerve growth factor (growth factor)

NLI - Neurological level of injury (scoring index)

NMDA - N-methyl-D-aspartic acid receptor for Glutamate (membrane receptor)

NSPC - Neural stem and progenitor cells (cell type)

NT - Neurotrophin (growth factor)

OIC - Osteopontin-IGF1-CNTF (growth factor combination)

OMgp - Oligodendrocyte-myelin-glycoprotein (inhibitory extracellular molecule)

OPN - Osteopontin (growth factor)

PDGF - Platelet derived growth factor (growth factor)

PKA - Protein kinase A (signaling molecule)

PNS - Peripheral nervous system (organ)

PTEN - Phosphatase and tensin homolog (signaling molecule)

RFP - Red fluorescent protein (fluorescent marker)

RNA - Ribonucleic acid (genetic component)

RtST - Reticulo-spinal tract (axon tract)

SCI - Spinal cord injury (the problem...)

TA - Tibialis anterior muscle (muscle)

TDT® - Tucker Davis Technology (hardware brand)

TNF - Tumor necrosis factor (cytokine)

Tuj-1 - Class III beta-tubulin (neuronal marker)

VA - Ventralis anterior thalamic nucleus (thalamic nucleus)

VEGF - Vascular endothelial growth factor (growth factor)

vGI - Ventralis gigantocellular reticular nucleus (brainstem nucleus)

vGLUT - Vesicular glutamate transporter (membrane receptor)

VL - Ventralis lateralis thalamic nucleus (thalamic nucleus)

VPL - Ventroposterior lateralis thalamic nucleus (thalamic nucleus)

4-AP - 4-aminopyridine (drug)

5HT - Serotonin or Serotoninergic receptor (neurotransmitter / receptors)

Chapter 1

INTRODUCTION TO SCI : NATURAL COURSE & THERAPEUTIC LEADS

OF A DUALITY BETWEEN REGENERATIVE INHIBITION AND TISSUE INTEGRITY

1.1 GENERAL INTRODUCTION
SPINAL CORD INJURY

Spinal Cord Injuries (SCI) impact suddenly and often irreversibly the life of previously healthy patients. Every year, approximately 40 people out of a million are victim of SCI in the United States, so that in 2013 ca. 276'000 patients were living with deficits after a spinal lesion (Center, 2014). As the average age at injury of alive SCI victims seems to become slightly older (28.7 years in the 1970's and 42.6 years in the 2010's), traumatic spinal cord injuries still mainly affect the physical and psychosocial integrity of young adults, and represent an economic challenge for health care systems (Singh et al., 2014; Center, 2014).

Clinically, spinal lesions cause acute sensorimotor deficits - which are more or less extended depending on the level of injury - as well as autonomic sphincter impairments and sexual dysfunctions. On a transverse axis, SCIs can be anatomically classified as incomplete or complete injuries. Incomplete injuries spare some extent of functional neural tissue across or around the lesion core, which still conducts information from the decisional centers to the infralesional circuits. Significant sparing tends to yield spontaneous reorganization of circuits associated to partial recovery of function. As the current clinical gold-standard to chronically treat SCI victims, rehabilitation relies on spared fiber tracts and enhances the neuronal reorganization and recovery of motor functions (Harkema et al., 2011; Asboth et al., 2018; van den Brand et al., 2012; Wagner et al., 2018). On the contrary, complete SCIs fail to spare any functional bridge past the lesion, causing a complete and irreversible interruption of control on the sensorimotor and autonomic system located below the level of injury.

Functionally, several clinical assessment methods have been developed to quantify and characterize SCI-induced impairments (Kalsi-Ryan et al., 2014), however the American Spinal Injury Association (ASIA) Impairment Scale (AIS) remains the more convenient and widely used. The ASIA score requires fine examination of sensory and motor functions and categorizes SCIs into 5 degrees of severity as follows (Kirshblum et al., 2011) :

- **ASIA A - Complete**. No sensory or motor function is preserved in the last sacral segments.
- **ASIA B - Sensory incomplete**. Sensory function is preserved below the neurological level of injury (NLI), but no motor function is preserved more than three levels below the NLI.
- **ASIA C - Motor incomplete**. Motor function is preserved below the neurological level, but less than half of key muscle functions below the NLI are preserved against gravity.
- **ASIA D - Motor incomplete**. Motor function is preserved below the neurological level, and half or more of key muscle functions below the NLI are preserved against gravity.
- **ASIA E - Normal**. No sensorimotor impairment in a patient who used to have prior deficits.

As mentioned, spontaneous recovery of sensorimotor functions can take place after incomplete SCI, so that ASIA grading can improve for patients over their clinical evolution, especially for

patients graded ASIA-B and ASIA-C (Kalsi-Ryan et al., 2014). Successfully, recent efforts addressed at incomplete spinal cord injuries tend to maximize functional recovery beyond the slight improvements that can take place spontaneously (Asboth et al., 2018; Harkema et al., 2011; van den Brand et al., 2012). Unfortunately, anatomically and functionally complete spinal cord injuries fail to provide sufficient sparing to yield any extent of functional improvement. As mature neurons fail to regenerate after Central Nervous System (CNS) injuries, several biological and technological options are currently explored to restore coherent conduction of information past complete SCIs.

In the absence of axon sparing, deep biological modulations of the lesion environment are required to restore neural integrity and voluntary input across complete SCIs. Such modulations could theoretically rely on coherent axon regrowth across the injured site, which requires i) precise targeting of neuronal populations, ii) controlled activation of growth programs and iii) adequate extracellular support. As an alternative, exogenous neuronal relays grafted into the SCI site could in theory conduct information across the lesion, provided that grafted neurons need both to receive and provide coherent downstream input.

Different classes of molecular targets have been identified within the lesion core as inhibiting or supporting axon regrowth. By neutralizing inhibiting molecules and providing facilitating factors, some interesting extent of axon regrowth has been documented. Similarly, cell replacement therapies have shown promising anatomical results involving endogenous axon regeneration into neural grafts (Kadoya et al., 2016). New genetic tools offer nowadays the possibility to directly manipulate the intrinsic growth capacity of endogenous neurons and promote functionally-significant axon regrowth (Bei et al., 2016; Liu et al., 2017). However, beyond axon regeneration and/or cell replacement, it becomes clear that successful biological therapies applied to complete SCIs will require a strongly coherent reorganization of circuits. As they yield such coherent reorganization in the context of incomplete lesions, neuro-prosthetic strategies may become interesting candidates to promote relevant rewiring after regeneration.

1.2 SCI ENVIRONMENT
PATHO-PHYSIOLOGY OF SPINAL CORD INJURIES

1.2.1 COURSE OF SPINAL CORD INJURIES

From a biological point of view, spinal cord injuries cause a tissue insult that are time-wise distributed between immediate primary neural lesions and secondary damages. In the first phase of injury, immediate mechanical lesions take place, involving axonal shearing and neural compression from dural and extradural origins (Saadoun et al., 2016). The spinal cord insult is progressively consolidated with a local rupture of the blood-spinal cord-barrier that enables an infiltration of platelets and blood-derived extracellular components, inducing a reorganization of the extracellular matrix at the site of injury. Laminin, fibrin and collagen form locally a new scaffold to facilitate the inflammatory response and the acute remodeling of the injury by macrophages. Over the following weeks, this inflammatory response further insults the peri-SCI area through mechanisms involving edema and ischemia due to vascular incompetence, as well as excitotoxicity, DNA-damages through reactive oxygen species and delayed apoptosis in the vicinity of the lesion (Rowland et al., 2008). During the first two weeks of this second phase, a simultaneous histologic reorganization takes place within the SCI site, giving birth to a fibrotic lesion core made of fibroblasts, pericytes and endothelial cells (Fernandez-Klett and

Priller, 2014). This fibrotic lesion core is surrounded by a reactive astroglial scar, that acts as an interface between healthy neural tissue and the fibrotic non-neural epicenter (Faulkner et al., 2004). During and after the post-SCI remodeling, the lesion eventually stabilizes to minimize the volume of the scar tissue, as cystic cavities appear within the lesion core (Burda and Sofroniew, 2014; Rowland et al., 2008). In parallel, axons retract away from the lesion and endure Wallerian degeneration, while late-stage apoptotic events take place within the lesion core (Burda and Sofroniew, 2014). Demyelination and abnormal remyelination of peri-lesional spared axons have also been documented, which decreases the conduction of information across the injury via spared fiber tracts (McDonald and Belegu, 2006).

1.2.2 ACUTE AND SUBACUTE TOXICITY

VASCULAR DAMAGES AND INFLAMMATORY REACTION

The immediate response to traumatic spinal cord injury induces ischemia and disruption of the blood-spinal cord barrier, further causing swelling at the damaged site and a neuro-immunologic reaction. Very acute events induce neuronal and glial cell loss based on two neurovascular mechanisms : i) immediate necrosis due to the primary hemorrhage and ii) acute apoptosis caused by nutrients deprivation and local metabolic stress in ischemic conditions (Hagg and Oudega, 2006). As ischemia resolves, reoxygenation causes a production of reactive oxygen species, which exert - together with a mitochondrial release of pro-apoptotic molecules - acute cytotoxicity (Xu et al., 2005; Tanaka et al., 2005).

Besides these immediate damages, traumatic SCIs induce vascular shearing that promotes permeability of the blood-spinal cord barrier. Together with a cleavage and activation of extracellular metalloproteinases (Noble et al., 2002), these vascular changes contribute to local swelling and secondary neural compression (Tator and Fehlings, 1991). In the same time, permeabilization of the blood-spinal cord barrier promotes infiltration of systemic inflammatory components that follow the resident microglial reaction, especially neutrophils in an early stage (Beck et al., 2010). The initial innate immunity acutely releases cytokines (e.g. TNF-alpha, IL-1-beta) and metalloproteinases (e.g MMP-9), which further enhance vascular permeability (Donnelly and Popovich, 2008) and can have a direct toxic effect on neurons and glial components (David and Kroner, 2011). This first inflammatory reaction is followed by the recruitment of systemic monocytes - which IFN-gamma induces into pro-inflammatory M1 macrophages at the site of injury, together with resident microglia (Kigerl et al., 2009) - and circulating lymphocytes. The adaptive lymphocyte-based reaction has been shown to recognize myelin-derived components in traumatic SCI contexts, yielding an auto-immune reaction (Ankeny and Popovich, 2009). Both innate and adaptive inflammatory reactions contribute in a subacute phase to secondary damages, causing neuronal loss and glial reactivity. However, this first phase of neuro-immunologic response is likely crucial to restore immediate tissue homeostasis (Schwab et al., 2014).

At a delayed stage, a second phase of the inflammatory response - mostly guided by anti-inflammatory M2 macrophages - promotes tissue repair (David and Kroner, 2011; Kigerl et al., 2009). A shift in the cytokine constellation, and especially an up-regulation of IL-4, appears to promote the M2 phenotype, that subsequently attenuates damaging immunity by the release of anti-inflammatory extracellular mediators (e.g. IL-10) (David and Kroner, 2011). This secondary phase of reactivity seems to restore homeostasis within immunity, after neuro-inflammation itself promoted immediate integrity of tissue in the very acute context.

Specific loss-of-function studies would help define a precise role for single components of the immune response after SCI. Better understanding of the compromise made between secondary damages and immediate integrity restoration might provide acute therapeutics options, as generic immune suppression has so far failed to provide real benefits (Schwab et al., 2014). Early polarization of the immune reaction towards M2 macrophage-induced recovery may constitute a lead to enhance neural tissue sparing.

EXCITOTOXICITY, DEGENERATION AND MYELIN ABNORMALITY

Due to the primary trauma, molecules are liberated from lysed and necrotic cells, and particularly glutamate (Park et al., 2004). With such a sudden increase in glutamate, AMPA, NMDA and kainate receptors get over-activated (Matute, 2011), likely contributing to an early-phase neuronal cell death. Glutamate levels remain high after the first pathologic release, due to three mechanisms : i) glutamate re-uptake deficiency (Mills et al., 2001b,a), ii) depolarization-induced reversal of the glutamate transporters' activity (McAdoo et al., 2000; Li and Stys, 2001) and iii) vesicular release after intracellular Ca2+ increase caused by collapsing ionic gradients (Li and Stys, 2001). Beyond neuronal damages, glutamate-induced excitotoxicity has been shown to affect glial cells - and especially oligodendrocytes - through apoptotic events that contribute to white matter disruption and demyelination after SCI (Park et al., 2004).

Mechanical shearing during traumatic SCIs sections or damages axons. In the case of axotomized neurons, distal parts of the axons are subject to Wallerian degeneration as proximal parts retract away from the lesion in a process termed 'die-back'. The severed neurons generally survive, but without the ability to regenerate into or around the lesion core (Becerra et al., 1995; Schwab and Bartholdi, 1996). Causes for this regenerative failure include i) a lack of neuronal ability to upregulate growth-associated genes (Plunet et al., 2002; Mason et al., 2003), ii) trophic support deprivation (Plunet et al., 2002) and iii) a down-regulation of cytoskeletal proteins associated to soma atrophy (Tetzlaff et al., 1991). After retraction from the SCI site and regenerative failure, some extent of sectioned axons will form terminal bulbs containing intracellular compartments and enzymes susceptible - in case of rupture and release - to cause further damages to environing neural tissue (Hagg and Oudega, 2006).

Amongst spared neural tissue, some axons may as well be damaged, even though they did not undergo complete axotomy. Initial ischemic conditions after SCI and secondary glial disruption can both cause axon degeneration or further damages to these incompletely severed fibers, preventing them from conducting information (Hagg and Oudega, 2006). Besides neuronal lesions, damages to oligodendrocytes can as well compromise conduction along spared or incompletely damaged axons (Sun et al., 2012). Indeed, acute demyelination has been documented after spinal cord injury (Crowe et al., 1997; Guest et al., 2005; Totoiu and Keirstead, 2005) and contributes to axonal degeneration in other pathological contexts (Irvine and Blakemore, 2008). After anatomically incomplete spinal cord injuries and beyond initial neuronal damages, it appears that glial - and especially oligodendroglial - disruption can cause secondary lesions and put in jeopardy the competence of spared neural tissue to yield spontaneous circuit reorganization and recovery of function.

1.2.3 ASTROGLIAL SCAR FORMATION

As part of the post-inflammation reorganization of the lesion, astrocytes located at the periphery of the injury react by modifying their morphological and functional features. Astrocytes

used to be considered for long as a cellular substrate for neurons, deprived of a real part in neural integration or in the conduction of information. However, it is now clearly documented that astrocytes are a key component for the formation of new synapses, for the development and stability of neuronal circuits, for neurotransmitter re-uptake, as well as for homeostasis within the CNS and for the competence of the blood-brain-barrier (Sofroniew and Vinters, 2010).

In response to CNS injuries, astrocytes encounter morphologic and functional transformations to gather at the periphery of lesions into a layer of astroglia. This process - termed astrogliosis - gives birth to astrocyte scars with features that are dependent on the type of CNS insult or disease. According to the mechanism and severity of the CNS insult, astrogliosis is indeed heterogenous in terms of intensity and histologic outcome, so that reactive astrocytes can form matrices of variable density, gaining or losing astroglial functions (Anderson et al., 2014; Wanner et al., 2013). Within the first weeks after severe injuries - such as SCI - a dense matrix of reactive astrocytes gaining function is formed (Anderson et al., 2016), with a gradient of reactivity decreasing when going from the SCI epicenter to healthy tissue where baseline astroglial functions are preserved (Wanner et al., 2013).

As determinants and functions of astrogliosis are being explored, it appears that several phenotypes of astrocytes can arise depending on the inflammatory constellation of the CNS injury or disease. Indeed, in the context of neuroinflammatory and neurodegenerative CNS diseases, reactive astrocytes express a cytotoxic A1 phenotype. This A1 reactivity involves a loss of normal astroglial functions, so that the astrocytes' capacity to support and maintain new synapses is decreased, as well as their property to phagocyte myelin debris. Moreover, the A1 phenotype appears to be toxic towards severed neurons and existing synapses in the periphery of the injury (Liddelow et al., 2017). In the context of ischemia (Zamanian et al., 2012) or after traumatic axotomy (Liddelow et al., 2017), astrogliosis tends to express an A2 transformation which appears to promote neuron survival and support axon regeneration (Anderson et al., 2016). This duality of reactivity phenotypes draws attention on the fact that astrogliosis may play very different roles after spinal cord injury, depending on the neuroinflammatory (e.g. multiple sclerosis, amyotrophic lateral sclerosis, Devic's neuromyelitis optica) or traumatic etiology of the lesion.

In traumatic models of SCI even - and long before the A1-A2 discrepancy was shown - astrogliosis has been considered as detrimental and targeted for therapeutic considerations. Negative opinions about SCI-induced astrogliosis are mainly based on the production or inhibitory molecules by reactive astrocytes, such as chondroitin sulfate proteoglycans (CSPG), that have been shown to impede axon regeneration (Davies et al., 1997, 1999; Silver and Miller, 2004). However, parallel work proposes that astrogliosis after traumatic SCI plays a role in restricting the spread of inflammation from the epicenter of the injury. Selective ablation of proliferating astrocytes (Faulkner et al., 2004), as well as attenuation of astrocyte reactivity (Herrmann et al., 2008), both induce increased inflammation, enhanced demyelination and an extended size of the lesion site with worsened functional outcome compared to spontaneous astrogliosis. Also, preventing astrocyte reactivity increases die-back of descending corticospinal and serotoninergic fibers, as well as ascending sensory axons (Anderson et al., 2016). Moreover and in spite of a strong expression of CSPGs, astrogliosis after traumatic SCI has been shown to produce a supportive extracellular matrix and help ascending sensory axons to regenerate into the lesion core when proper growth stimuli are provided (Anderson et al., 2016).

Nevertheless and as described above (see 1.2.2), the acute inflammatory response to SCIs appears to involve microglial reactivity and auto-immune features, which theoretically should

induce A1 astrogliosis. Unveiling how ischemic and traumatic insults promote in facts A2 astrocyte reactivity may provide novel therapeutic leads, in order to manipulate inflammation and optimize the role of astroglial scars. Overall, as astrocytes may have detrimental activities in other contexts, it appears that traumatic spinal cord injury - which represent a majority of spinal cord insults - cause the astrocyte component of the scar to exert a balanced effect on the spinal environment. Preventing astrogliosis after SCI has yet failed to yield reliable and reproducible benefits, and new therapeutic options may arise based on specific and optimized manipulations of the glial scar.

1.2.4 FIBROTIC SCAR FORMATION

As astrogliosis has long been seen as the main barrier to axon regeneration (Davies et al., 1999), reactive astrocytes have raised more questions than the fibrotic lesion core, so that the intra-lesional fibrotic component is so far less understood. Nevertheless, recent work demonstrated that the fibrotic scar strongly inhibits axon regeneration (Soderblom et al., 2013; Zukor et al., 2013).

After acute inflammation is resolved and as astrocytes react to the insult, stromal cells migrate into the lesion core from locally damaged meninges or peri-vascular regions. In traumatic injuries that involve local tearing of the meningeal sheets, the fibrotic scar is progressively formed from meninges-derived fibroblasts, as well as pericytes, endothelia and inflammatory cells. However, in blunt traumatic SCIs that leave the dura intact - such as contusion injuries - fibroblasts originating from perivascular areas have been demonstrated as a main cellular source within the fibrotic core (Soderblom et al., 2013). Similarly to the initial inflammatory response to traumatic CNS insults, fibrosis appears to play both beneficial and detrimental roles. On the one hand, the organization of a fibrotic lesion core appears to contribute to restoring homeostasis, by protecting surrounding tissue and restoring integrity of the blood-spinal cord barrier (Kawano et al., 2012). Because inhibiting pericytes' reactivity after SCI causes larger lesion compartments, loss-of-function studies have established the fibrotic scar as a key process to seal the lesion site (Goeritz et al., 2011). On the other hand, this same fibrotic scar clearly inhibits regeneration by releasing molecules established to be detrimental to axon regrowth. Amongst these molecules are listed i) axon growth-inhibitory chondroitin sulfate proteoglycans (CSPGs, e.g. NG2 and phosphacan) (Tang et al., 2003), ii) tenascin-C, which is inhibitory to neurite extension (Tang et al., 2003), iii) axon-repellent semaphorin 3 (Pasterkamp et al., 1999) and iv) astrocyte-repellent EphB2 (Bundesen et al., 2003).

Either derived from meningeal or vascular origins, intra-lesional fibroblasts have been shown to express collagen-1α1 (Col1α1). In both contexts, the fibrotic scar is stabilized by 14 days post-injury and presents with stromal cells embedded in a dense deposition of extracellular matrix components. In addition to the chemical regrowth inhibition, the physical structure of the fibrotic scar has been considered as a mechanical barrier to growing axons (Soderblom et al., 2013).

At a subacute and chronic stage post-injury, both astroglial and fibrotic components of the SCI lesion have been held responsible for the lack of spontaneous regeneration. However, while immediate benefits of fibrosis on tissue homeostasis have been shown, evidence gathers to support its detrimental impact on axon regrowth. On the contrary, reactive astrocytes seem to support axon regeneration *in vivo* (Anderson et al., 2016), and neurons tend to prefer astrocytes to stromal cells when co-cultured *in vitro* (Shearer and Fawcett, 2001). In order

to promote axon regeneration, fine manipulations of the scar formation may promote the organization of permissive endogenous cell substrate. Recent findings suggest that regrowth may be improved by i) enhancing A2 astrogliosis and ii) reducing fibrosis to the minima required for proper wound sealing.

1.2.5 SPONTANEOUS PLASTICITY AND RECOVERY

In spite of detrimental inflammatory and histologic reactions, functional recovery can spontaneously occur after anatomically incomplete SCI. Clinical statistics show in human patients that recovery takes place essentially over the first 3 months post-injury, but can still positively evolve over a period of 18 months (Fawcett et al., 2007).

At an early stage, remyelination of demyelinated axons have been proposed as a mechanism to support spontaneous recovery (Hagg and Oudega, 2006). In addition, animal models of incomplete injuries have demonstrated a correlation between spontaneous recovery and plasticity of spared corticospinal projections, involving the recruitment of relay propriospinal neurons (Weidner et al., 2001; Courtine et al., 2008; Rosenzweig et al., 2010). Reinforcing the evidence of spontaneous plasticity, studies have shown that cortical reorganization of sensorimotor representations occurs after SCI. Indeed, sprouting of the motor corticospinal tract has been shown to reach spontaneously for new target-levels within the spinal cord after incomplete SCI, thus modifying the motor homunculus (Fouad et al., 2001) and making connexions with intra-spinal relays located above the injury (Bareyre et al., 2004). Similarly, sprouting of subcortical structures and spared sensory fibers appears to progressively rewire portions of the sensory cortex that became silent after spinal lesion. This sensory reorganization can either restore a same representation as prior to injury (Kaas et al., 2008), or modify the cortical sensory homunculus(Jain et al., 2008).

Spared neural tissue around incomplete SCIs provides anatomical support to plastic events that aim at restoring motor supra-spinal input below the injury. In rodents, sprouting of spared ventral components of the corticospinal tract (CST) was shown to mediate functional recovery after section of the major dorsal CST, which contains 95% of all corticospinal axons (Weidner et al., 2001). Moreover, minor ventral and dorsolateral components of the corticospinal projections were shown to sprout and reach for spared propriospinal neurons at a cervical level. With a spared access to infra-lesional circuits, these propriospinal fibers restore cortical input to the lumbar motor centers (Bareyre et al., 2004). In non-human primates with spinal hemisection, sprouting of the contralateral corticospinal tract was observed, rewiring ipsilateral lumbar circuits by decussating below the SCI level (Rosenzweig et al., 2010). Similarly, reorganization and recruitment of decussating propriospinal fibers has been documented to mediate motor recovery in a rodent model of staggered bilateral hemisections - which is shown as functionally complete, yet anatomically incomplete. In this model, propriospinal neurons decussate and bypass the SCI to conduct reorganized supraspinal input across the injury (Courtine et al., 2008).

In contrast - and likely because of an intrinsic down regulation of growth capacity in adult neurons (Liu et al., 2011; Sun et al., 2011), combined to a lack of facilitating factors (Richardson and Issa, 1984; David and Aguayo, 1981) - severed axons fail to regrow into and across anatomically complete SCIs. This phenomenon, together with the absence of spared neural tissue, impedes circuit reorganization and prevents recovery of function. While reorganization does occur in incomplete SCIs, some extent of neural substrate needs to be spared, in order

for compensatory mechanisms to yield spontaneous recovery of function. Nevertheless, recovery often remains limited or incomplete in patients victim of severe spinal cord injury. Novel therapeutic strategies - discussed in the following paragraphs - have been shown to promote endogenous reorganization of circuits after incomplete lesions. In the future, such strategies - that enhance endogenous plasticity - may become useful after anatomically complete SCIs, in order to integrate regenerated axons or grafted neurons into functional circuits.

1.3 RECRUITING SPARED CIRCUITS

Mechanisms for axon regeneration remain partially understood and are currently being investigated. As regenerative candidate therapies have failed for long to provide consistent and reproducible recovery of function, strategies have been developed to extract maximum benefit from spared neural tissue and spontaneous reorganization of circuits. As neuronal excitability relies on both electrical and chemical phenomenons, pharmacological and electrophysiological paradigms have been explored to enhance functional recovery after SCI.

1.3.1 PHARMACOLOGICAL MODULATION OF SPINAL SUBSTRATES

Studies have shown in healthy conditions that gait is tuned by Central Pattern Generators (CPG) located within the lumbar spinal cord (Gerasimenko et al., 2008; Kiehn, 2016), and is remotely modulated by descending tracts extended from the brainstem (Hentall et al., 2003). After incomplete SCI, in spite of long tracts' Wallerian degeneration, propriospinal fibers and local interneurons can contribute to relay functionally-significant supraspinal input to the lumbosacral circuits (O'Shea et al., 2017; Zaporozhets et al., 2011; Chen et al., 2018), suggesting that post-synaptic receptors within the spinal cord remain expressed and available for pharmacological modulation. In the intact rodent CNS, brainstem control over the lumbosacral circuitry is mediated by serotoninergic, noradrenergic, dopaminergic and glutamatergic input (Hentall et al., 2003; Zaporozhets et al., 2011; Asboth et al., 2018), so that monoaminergic pharmacology has provided candidate molecules to restore or modulate deafferented infralesional circuits after spinal cord injury. Serotoninergic 5HT-2 (e.g. quipazine) and 5HT-1A/7 (e.g. 8-OHDPAT) agonists, as well as dopaminergic agonists (e.g. SKF-82197), have been shown to recruit lumbosacral centers and potentiate different features of the gait pattern (Musienko et al., 2009; Gerasimenko et al., 2007; Landry et al., 2006). In combination, these molecules seem to further improve locomotion and act synergistically with electrical modulation of the infralesional spinal cord (Musienko et al., 2009; Courtine et al., 2009). Based on such evidence, monoaminergic agonists are used by the Courtine Laboratory on a daily basis to further explore the effects of adjuvant neuroprosthetic therapies.

Independently, in order to restore supraspinal input around incomplete SCIs, improving the conduction of action potentials may counterbalance demyelination and insufficient remyelination of spared neural bridges (see 1.2.2). As potassium channel-blocking molecules (e.g. 4-aminopyridine (4-AP) and its derivatives) were shown to enhance conduction of demyelinated axons in patients with multiple sclerosis (Smith et al., 2000), 4-AP - together with successful axon regeneration - recently promoted recovery of function in rodent models of spinal hemisection (Liu et al., 2017) and optic nerve crush (Bei et al., 2016). In addition, inhibiting specific inhibitory interneurons at a local level was shown to improve functional outcomes after staggered bilateral hemisections of the spinal cord (Chen et al., 2018). While a majority

of parallel strategies aim at restoring excitability and promoting adequate depolarization, this recent evidence operates a mechanistic shift, as functional recovery arose from a release - via KCC2 signaling or selective hyperpolarization - of the peri-lesional inhibition mediated by GABA-ergic interneurons.

Increasing lumbosacral excitability and conduction of action potentials along spared demyelinated axons may become part of a solution to restore supraspinal input around incomplete SCIs. Nevertheless, in a context of complete spinal cord injuries, both strategies remain catalytic and are meant to support other paradigms that specifically aim at restoring voluntary input across or above the lesion.

1.3.2 MOTOR NEUROPROSTHETICS

As a second option to restore excitability into infralesional circuits, targeted electrostimulation has been shown to successfully recruit quiescent circuits after incomplete spinal lesion. Indeed, epidural electrical stimulation (EES) - combined with a pharmacological increase of neuronal excitability - provides a functional state to the dormant lumbosacral circuits after complete spinal cord injury (Courtine et al., 2009). Hotspots over the L2 (Ichiyama et al., 2005; Lavrov et al., 2008) and S1 (Gerasimenko et al., 2007) segments have been identified in rodents and induce hindlimb movements when electrically stimulated. Pharmacological and EES interventions appear to act synergistically, so that these interventions alone fail to promote an infra-lesional functional state (Asboth et al., 2018). Nevertheless, this electropharmacologically restored functional state essentially generates involuntary hindlimb motion, which differs from voluntary locomotion at both kinematic and electromyographic levels (Ichiyama et al., 2008; Antri et al., 2003). In addition to EES and monoaminergic modulation, specific motor rehabilitation has since been proved to yield reorganization of supraspinal input and restore voluntary stepping after staggered double hemisection SCI (van den Brand et al., 2012) or severe spinal contusion (Asboth et al., 2018). Indeed, motor training appears to recruit propriospinal neurons and induce sprouting of spared reticulospinal axons. Such reorganization supports supraspinal input to bypass the SCI site and regain access to the potentiated locomotor circuitry at a lumbosacral level (Asboth et al., 2018).

Obviously, such electrochemical and neurorehabilitative strategies restore voluntary locomotion after incomplete SCI based on spared bridges of neural tissue. After complete SCI, and while robust axon regeneration still fails to restore hindlimb motor function, progresses in brain decoding have enabled the development of wireless voluntary control over the motor system. Direct stimulation of muscles (functional electrical stimulation, FES) based on cortical activity have enabled restoration of grasping (Ethier et al., 2012; Bouton et al., 2016) in a context of tetraplegia in humans. To recover hindlimb gait patterns, modulation of neural circuits based on cortical decoding has recently successfully restored weight-bearing locomotion in non-human primates (Capogrosso et al., 2016) and enabled adjusted hindlimb motor functions in rodents (Bonizzato et al., 2018) after SCI. Due to complex and sequential activation of muscle groups during voluntary stepping, modulating neural circuits seems more susceptible to restore natural gait patterns that FES. Indeed, hotspots are being identified within the lumbosacral circuitry that tend to unveil a task-related topography involving muscle synergies (Wenger et al., 2016; Capogrosso et al., 2016), more than the representation of individual muscles. Nevertheless, functional electrical stimulation may remain a useful strategy in contexts that compromise the functionality of the lumbosacral pattern generators (e.g. neuro-degenerative spinal cord diseases, spinal cord infarcts).

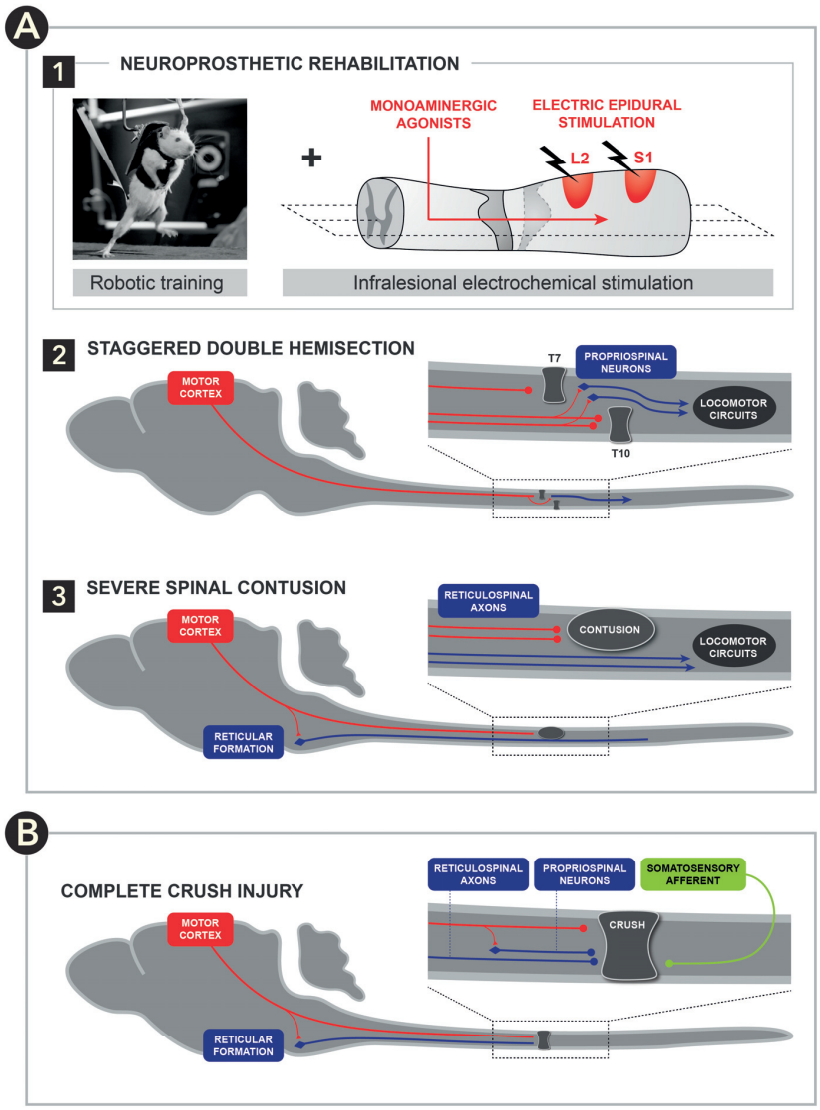

Figure 1.1: **Descending neuronal systems relay motor commands after incomplete SCI.** **A,** Neuroprosthetic rehabilitation enables recovery of voluntary stepping in incomplete models of SCI, such as staggered double hemisections or severe spinal contusions. **A1,** Therapeutic paradigm : robotically enabled locomotor training engages and reorganizes descending neural networks neuron. A combined delivery of i) infralesional electric neuromodulation and ii) systemically administered monoaminergic agents restores activity in lumbosacral motor circuits. **A2,** In a paralyzing staggered double hemisection model, neuroprosthetic rehabilitation induces the corticospinal tract to reach for propriospinal neurons (van den Brand et al, 2012). **A3,** After severe, yet incomplete spinal contusion, the ventrolaterally located reticulospinal tract remains spared. Neuroprosthetic training increases cortical projections to the reticular formation in the brainstem (Asboth et al, 2018). Reticulospinal axons act as a descending relay for recovery in contusion models, where propriospinal neurons are severed. **B**, After complete spinal cord injury, the descending corticospinal command, as well as all relay axons and ascending somatosensory tracts are interrupted. Neuroprosthetic training alone does not suffice to induce motor benefits.

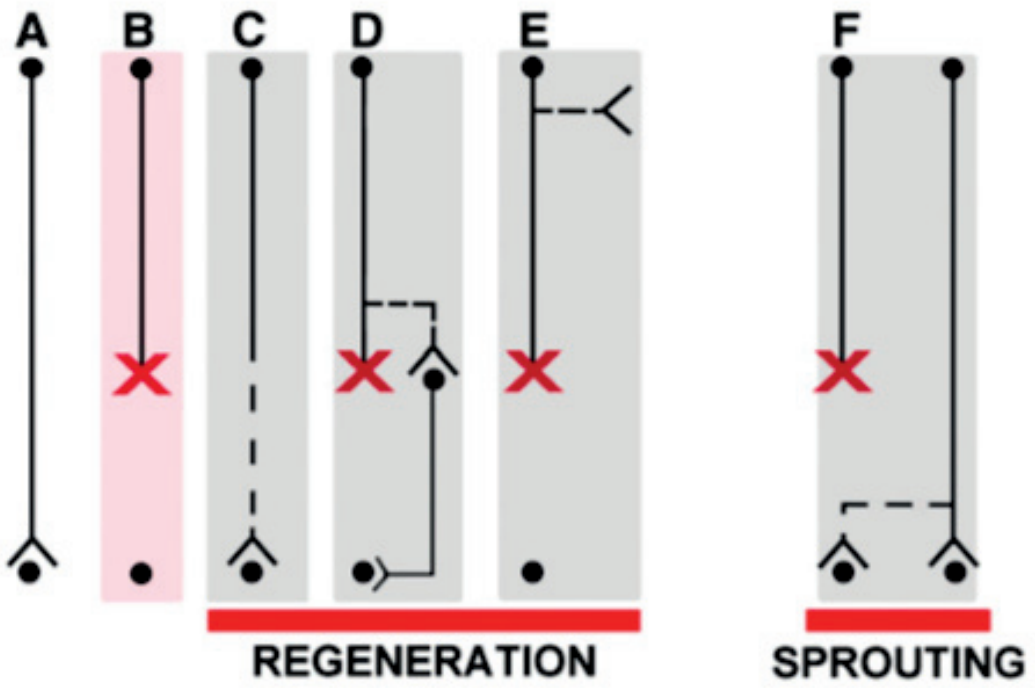

Figure 1.2: **Regeneration and sprouting after spinal cord injury : definition.** **a**, Intact axon. **b**, Axotomized neuron. **c**, Axon regeneration : regrowth occurs from the extremity of the severed axon. **d**, Regeneration : new neurites grow from severed axons, in a region located close to the SCI site (also termed 'regenerative sprouting'). **e**, Regeneration : new neurites grow from severed axon in a proximal region that is remote from the injured site (also termed 'regenerative sprouting'). **f**, Sprouting : new neurites grow from spared axons to reinnervate the deafferented targets. (Adapted from Tuszynski and Steward, 2012).

1.4 CURRENT VIEWS ABOUT AXON REGENERATION

Engineering strategies that modulate circuits after complete spinal cord injury remain invasive and involve implanted material, which can induce inflammatory and fibrotic reactions (Karumbaiah et al., 2013). Neuroprosthetics has been providing key strategies and concepts to recruit circuits after SCI. However, unveiling the mechanisms that underly axon regeneration in the adult spinal cord may promote the design of combined biological & engineering therapies, and eventually promote endogenous reorganization to wean patients of any implanted electronics.

While neuroregeneration is widely used as a generic term, a distinction needs to be made between sprouting and axon regeneration that may rely on different mechanisms. Axon sprouting has been defined as compensatory extension of new neurites from spared axons (Tuszynski and Steward, 2012), and may take part to mechanisms of rewiring below incomplete SCIs from undamaged fibers. However, regeneration includes i) proper axon regrowth from the axotomized ending, ii) extension of new neurites from severed axons in the vicinity of the lesion, and iii) development of new neurites from injured axons in areas that are remote from the SCI (Tuszynski and Steward, 2012).

Neuroregenerative hypotheses and strategies after spinal cord injury have mainly relied on

identifying i) molecular and cellular discrepancies between pre- and post-injury environments, ii) determinants of axonal growth during development, and iii) biochemical changes that underly axonal maturation and stabilization. The following sections discuss the general concepts expected to establish permissive requirements for axon regeneration.

1.4.1 AXON REGENERATION : INHIBITING THE INHIBITORS

CHEMOREPULSION

Evidence has now established that axonal growth and fiber tracts' extension during the CNS's development involve attractive and repellent molecular gradients. Following SCI, developmental extracellular molecules and membrane receptors are upregulated and may interfere negatively with the guidance of spontaneously regenerating axons. Two families of chemorepellent molecules in particular - semaphorins and ephrins - have been explored and shown as key guidance cues to pattern axonal tracts during development. Semaphorins have been identified as chemorepellents that shape the sensory system through the developing spinal cord (Messersmith et al., 1995; Behar et al., 1996). Within this molecular family, Semaphorin-IIIA has been specifically proposed as a chemorepellent molecule impeding axon regeneration after SCI. Indeed, Semaphorin-IIIA is produced by the fibrotic component of the SCI scar, and its inhibition is suggested to promote axon regrowth (Kikuchi et al., 2003). On their side, ephrins and their Eph receptors (i.e. EphB3 and EphA) - which generate a repellent action on growth cones during development to promote axonal pathfinding - are upregulated after spinal lesion (Miranda et al., 1999; Willson et al., 2002). Similarly to Semaphorin-IIIA, chemical or genetic inhibitions of receptors EphA (Goldshmit et al., 2004; Fabes et al., 2007) and/or EphB3 are suggested to support axonal regeneration (Duffy et al., 2012). Manipulating chemorepellent molecules does appear to enhance axon regrowth after SCI, even though the density of regenerated axons remains limited. Whereas the functional benefit of experimental therapies that target strictly chemorepulsion is debatable, these studies clearly define a role for chemical guidance in the design of regenerative strategies after SCI. Such approaches may tend to inhibit or optimize the effects of chemorepellent agents, but can as well aim at providing gradients of strong chemoattractive molecules to lead regenerating fibers.

PROTEOGLYCANS

Another specific class of molecules, termed proteoglycans, has been extensively studied to unveil inhibitory mechanisms after SCI. With a suggested chemorepulsion activity during development (Snow et al., 1990b), proteoglycans are a large family of molecules sharing common structural features that include a core protein carrying covalent glycosaminoglycan (GAG) side chains. Further specificities of these side chains define subfamilies of proteoglycans (e.g. chondroitin sulfate, keratin sulphate, heparan sulphate, etc.). Whereas most of these proteoglycans are acutely and chronically up-regulated following CNS lesion (Fitch and Silver, 1997; Jones et al., 2003a; Ito et al., 2010), chondroitin sulfate proteoglycans (CSPG) have been specifically addressed due to their strong and well-documented inhibition of axon regeneration (Silver and Miller, 2004; Yiu and He, 2006; Jones et al., 2003b; Galtrey and Fawcett, 2007; Andrews et al., 2012). CSPGs have been suggested to be upregulated by reactive astrocytes following spinal cord injury, therefore contributing to the belief that astrogliosis opposes both a physical

and a chemical barrier to axon regrowth (Sharma et al., 2012; Silver and Miller, 2004; Fitch and Silver, 2008).

The inhibitory CSPG interaction with growing axons has been shown both *in vitro* - where CSPGs impede normal growth cone dynamics (Snow et al., 1996) and inhibit neurite outgrowth in spite of permissive laminin (Snow et al., 1990a) - and *in vivo* where transplanted sensory neurons fail to extend axons past perilesional proteoglycans (Davies et al., 1997, 1999). Mechanisms for CSPG-mediated inhibition of axon regeneration have been proposed as direct or indirect. Growth cones were shown to present different potential receptors for CSPGs (e.g. protein tyrosine phosphatase sigma, leucocyte common antigen-related phosphatase, Nogo receptors 1 and 3) that induce signaling for cone collapse (Sharma et al., 2012; Fisher et al., 2011; Shen et al., 2009). Complimentary indirect mechanisms are suggested, that involve proteoglycan competitive binding with integrins at a growth cone level, thus preventing supportive signaling from extracellular components (Tan et al., 2011). Similarly, competitive binding with permissive extracellular substrates (e.g. laminins, neural cell adhesion molecule) can be proposed, thus hiding them to regenerating axons (Zuo et al., 1998; McKeon et al., 1995). May as well be involved chelation of different growth factors (Nandini and Sugahara, 2006; Deepa et al., 2002) that therefore fail to promote axonal regeneration.

Obviously, research efforts have been focused for several decades on inhibiting proteoglycans, in order to alleviate the negative CSPG drive on axon regeneration after spinal lesion. Different strategies have been developed to disrupt CSPG activity, including neutralization via function-blocking antibodies (Brown et al., 2012), or targeting downstream molecules and receptors in the proteoglycan signaling cascade (Shen et al., 2009; Fry et al., 2010; Fisher et al., 2011). However, the more commonly described inhibition of CSPGs relies on its enzymatic digestion with chondroitinase ABC or similar agents (McKeon et al., 1995; Bradbury et al., 2002; Grimpe and Silver, 2004). Such blocking interactions with chondroitin sulfate proteoglycans have been suggested to promote regeneration of both afferent and efferent axonal tract (Brown et al., 2012; Fry et al., 2010; Grimpe and Silver, 2004), and induce behavioral benefits (Fisher et al., 2011; Bradbury et al., 2002). However, it is worth noting that the functional outcomes were quantified using subjective scores and were unfortunately not replicated. Furthermore, a model of specific dorsal column injury was involved (Bradbury et al., 2002), leaving most of the descending reticulospinal tracts intact and available for circuit reorganization and spontaneous functional recovery.

Whereas the inhibitory impact of CSPGs relies on strong evidence, the documented regeneration following CSPG inactivation remains debatable. Indeed, the studies described here above address either anatomically incomplete injury models, or lesions that are unconvincingly shown as complete. This leads to possible confusion between real regeneration and sprouting of spared axonal tracts. As perineuronal networks below spinal lesions include proteoglycans, they are likely to be partly digested and loosen by chondroitinase, rendering the infralesional tissues more permeable to neurite outgrowth, i.e. sprouting. Moreover, recent data demonstrate that proper sensory regeneration can occur into complete SCIs even in the vicinity of proteoglycans (Anderson et al., 2016), suggesting that CSPG-mediated inhibition may be less definitive than believed and can be overridden by other growth-inducing strategies. The same report showed as well that reactive astrogliosis is not the sole source of CSPGs after SCI, and appears to be more beneficial to regenerating axons than the chemical-physical barrier previously described. Nevertheless, CSPG's inactivation may as well promote real axon regeneration, as it diminishes proteoglycan interactions with supportive extracellular components and endogenous growth factors, both elements that are exogenously administered in growth-inducing

strategies (see 1.4.2). Enzymatic disruption of proteoglycans appears as a promising strategy to support sprouting and rewiring in incomplete models of SCI, and as a potential paradigm - in combination with other approaches - to promote axonal regrowth across complete spinal lesions.

MYELIN-ASSOCIATED INHIBITORS

In parallel of the exploration of proteoglycan-mediated inhibition, oligodendrocyte-derived debris have been the focus of intense investigations and accused of driving toxic signaling onto growth cones after SCI (Schwab and Bartholdi, 1996). As axon regeneration is known to occur in the peripheral nervous system (PNS) after nerve injury (David and Aguayo, 1981), glial differences between the PNS and the CNS have been explored, leading to the hypothesis that CNS-myelin may inhibit regrowth, while PNS-myelin is permissive to regeneration (Caroni and Schwab, 1988a; Schwab and Thoenen, 1985). In a first place, two minor myelin proteins - of 35kD and 250kD - were described *in vitro* as required and sufficient to generate inhibitory white-matter substrates (Caroni and Schwab, 1988a). Further, a specific IN-1 antibody targeting these specific 35kD and 250kD proteins yielded enhanced neural outgrowth *in vitro* (Caroni and Schwab, 1988b), and was proposed to promote corticospinal and brainstem-spinal axon regeneration *in vivo* (Schnell and Schwab, 1990; Bregman et al., 1995). A decade later, the 250kD myelin inhibitor was identified as Nogo and three Nogo-isoforms were documented as Nogo-A, Nogo-B and Nogo-C (GrandPrÃ© et al., 2000). Subsequently, two other major CNS-myelin associated components have been identified as inhibitory : Myelin-Associated-Glycoprotein (MAG) (Mukhopadhyay et al., 1994) and Oligodendrocyte-Myelin-glycoprotein (OMgp) (Kottis et al., 2002).

To support the evidence of myelin-associated inhibition, biochemical mechanisms were explored and identified a common neuronal receptor - the Nogo receptor NgR1 - for Nogo , MAG and OMgp (Wang et al., 2002b; Fournier et al., 2001; Domeniconi et al., 2002). Nogo-66 - a loop of 66 amino acids conserved between Nogo isoforms - appears as the site of interaction for Nogo with NgR1 (Fournier et al., 2001). Downstream signaling of this Nogo receptor - after recruitment of NgR1 membrane co-receptors LINGO-1 together with P75 or TROY (Mi et al., 2004; Park et al., 2005; Wang et al., 2002a) - induces growth cone inhibition via the Rho/Rho-associated-kinase (ROCK) pathway (Geoffroy and Zheng, 2014; Domeniconi et al., 2005). Interestingly, CSPGs where shown to recruit Nogo receptors NgR1 and NgR3 to mediate inhibition of growth cones (Dickendesher et al., 2012), suggesting cross-talks and common signaling pathways for proteoglycans (CSPG) and myelin-associated inhibitors (MAI). However, loss-of-function studies showed that whereas the NgR1 receptor does inhibit acutely the cytoskeletal dynamics of growth cones when interacting with Nogo-66, MAG or OMgp, chronic growth cone inhibition by myelin components appears as NgR1-independant (Chivatakarn et al., 2007; Zheng et al., 2005).

Whereas evidence for CNS-derived myelin inhibition remains strong *in vitro*, the previous regenerative successes obtained *in vivo* with the IN-1 antibody have been confronted by more recent studies, in the light of the biochemical understanding of myelin-associated inhibition. Independent works have addressed the potential of Nogo, MAG and/or OMgp suppression for axon regeneration, and two distinct groups showed different *in vivo* effects from myelin-associated inhibitors (Geoffroy and Zheng, 2014). Addressing the injured corticospinal tract (CST), the first study did not show regeneration after deletion of Nogo, MAG, OMgp or all three proteins (Lee et al., 2010). Conversely, the second demonstrated that Nogo-deleted animals experi-

enced CST regeneration after Nogo-deletion, that was potentiated by concomitant MAG and OMgp deletion, but with no regeneration induced by MAG and OMgp deletion only (Cafferty et al., 2010). Additionally, the first study quantified CST sprouting, which was enhanced by Nogo deletion, and interestingly diminished by MAG deletion. Regarding serotoninergic descending axons, the second study showed sprouting induced by deleting Nogo alone or in combination with MAG and OMgp (Cafferty et al., 2010), while the first documented serotoninergic sprouting after MAG or OMgp deletion alone, but without synergistic effects (Lee et al., 2010). Deletion of all three myelin-associated inhibitors did not yield serotoninergic regeneration in the first study (Lee et al., 2010). These discrepancies between studies, between *in vitro* and *in vivo* effects of myelin suppression, between effects of single myelin inhibitors and between responses from specific axonal tracts highlight our likely incomplete understanding of the biochemistry that underlies myelin-associated inhibition. As for CSPG inhibition, regeneration and sprouting need to be well recognized as Nogo, MAG and OMgp appear to act differently on both phenomenons. Indeed, suppressing effects of CSPGs or myelin-associated inhibitors appear to promote sprouting of descending axonal tracts, while their effect on regeneration remains debatable. Targeting CSPGs and MAIs may promote sprouting, circuit reorganization and functional benefits in incomplete models of SCI. However, inducing robust and convincing functionally-significant axon regeneration will likely require more than inhibiting inhibitors.

1.4.2 **AXON REGENERATION :** INDUCING AXON REGROWTH

During development, growth programs control the extension of axons throughout the CNS. Guidance cues act as chemo-attractants or chemo-repellents and direct axons to their expected target, together with physical barriers (e.g. CSPGs). After neuronal growth is completed, developmental programs are downregulated, trophic gradients disappear. At the end of neuronal development, growth cones are morphologically and functionally transformed into pre-synaptic buttons. Thus, it can be hypothesized that the establishment of synaptic contacts requires a loss of growth - and possibly regrowth - capacities in adult neurons (Liu et al., 2011). As differences between CNS and PNS permissivity to axonal regeneration suggest that environmental factors inhibit axonal regrowth (see 1.4.1), axons still fail to regenerate in models of injury that cause minimal scarring (Canty et al., 2013; He and Jin, 2016). This further supports the assumption of an intrinsic incapacity of mature neurons to upregulate growth programs, in addition to the rise of inhibitory mechanisms after CNS lesion (Goldberg et al., 2002; Chen et al., 1995). Based on these elements, mechanisms that underly axon growth and regrowth have been explored, aiming at re-awakening developmental programs and providing development-like trophic support after axotomy. The field of neural regeneration has been proposing and testing strategies to i) modulate neuronal growth-relevant genes, ii) provide permissive substrates to regenerating axons and iii) guide axons through trophic factors and chemoattractive gradients.

SIGNALING DURING AXON DEVELOPMENT

Obviously, axon regeneration can only occur if the axotomized neuron does not undergo apoptosis in the first place. While inflammatory and acute stress mechanisms after injury may lead to neuronal necrosis or apoptosis, it has been described that axotomy itself can cause

neuronal death (Huntwork-Rodriguez et al., 2013). Following axotomy, survival and regeneration appear however to rely on different intracellular mechanisms, as promoting neuronal survival by deleting tumor-suppressor genes such as p53 or Bcl2 does not promote regeneration (Park et al., 2008; He and Jin, 2016). In order to unveil mechanisms susceptible to induce axonal regrowth, molecular understanding of axonal extension during development points towards neurotrophins and neuronal activity (Liu et al., 2011). Neurotrophins are a family of molecules that include Nerve-Growth-Factor (NGF), Brain-Derived-Neurotrophic-Factor (BDNF), Neurotrophin-3 (NT3) and Neurotrophin-4 (NT4). Interaction of neurotrophins with the neuronal receptors TrkA, TrkB and TrkC has been shown to activate signaling cascades that control axon growth during development (Liu and Snider, 2001; Markus et al., 2002; Glebova and Ginty, 2005). In the CNS, neuronal activity appears to contribute synergistically to the neurotrophin-induced axon extension, as electric activity - trough cyclic adenosine monophosphate (cAMP) upregulation - seems to densify membrane receptors to neurotrophins (Meyer-Franke et al., 1998; Goldberg et al., 2002; Liu et al., 2011). To address the bases of the reduced growth capacity following developmental completion, genetic studies propose several mechanisms. Interestingly, post-mitotic neurons express a molecular complex - known as the anaphase-promoting complex - while they exit the cell cycle. Inhibition of this complex has been shown to increase axonal growth in cerebellar neurons (Konishi et al., 2004). The neuron survival gene Bcl2 is proposed also as a modulator of axon extension during development, that is down-regulated at a mature stage (Chen et al., 1997). In addition, modifications of gene-expression patterns within the Kruppel-Like-Factors (KLF) family are suggested as a regulatory mechanism for the reduction of growth capacity in post-mitotic neurons (Veldman et al., 2007). Within the KLF family, subgroups of factors appear to exert converse effects on neurite extension (Veldman et al., 2007; Moore et al., 2009). As a specifically interesting pathway for the present work, the mammalian Target-Of-Rapamycin (mTOR) has been shown to decline at the end of development in both the visual system (Park et al., 2008) and the motor cortex (Liu et al., 2010).

LESSONS FROM CONDITIONING LESIONS

Following axotomy, the sensory system provides a robust model to explore axonal regrow since "conditioning lesions" were described as a trigger for regeneration. Indeed, studies have been showing since the 1980s that lesioning the peripheral branch of a dorsal root ganglion neuron potentiates the regeneration of the injured central branch of the neuron (Richardson and Issa, 1984; Anderson et al., 2016). Comparing the effect of neurotrophins and conditioning lesions, it appears however that the signaling required for axon regeneration differs from the developmental cascade (Liu and Snider, 2001), but recruits gene transcription that may reawaken developmental patterns of protein-expression associated with growth (Liu et al., 2011). Regeneration likely occurs based on peripheral signals given from the periphery to the soma, such as an interruption of axonal trafficking or new signaling derived from axonal debris (Zhang and Ambron, 2000). Such retrograde signaling enlightens the role of the local environment at the site of injury that can potentially modulate intrinsic neuronal growth abilities. Local cytokines at the site of injury - including ciliary neurotrophic factor (CNTF) (Sendtner et al., 1992) - have been shown to recruit the growth-associated JAK-STAT pathway, which is required for conditioning lesion-induced regeneration (Miao et al., 2006). In addition, cAMP-related variations post-conditioning lesion may promote growth-related gene transcription by i) converting myelin-associated inhibition into attractive signaling (Song et al., 1997; Cai et al., 2001) and ii) direct activation of growth-related gene patterns via the cAMP Response Element-Binding

protein (CREB) (Cao et al., 2006). Moreover, appears that electrical activity at the target of the peripheral branch exert an inhibition over intrinsic growth programs in sensory neurons that is alleviated by conditioning lesions (Enes et al., 2010). Further supporting this hypothesis, an activation of the cAMP/CREB pathway by electrical activity has been demonstrated *in vitro* (Fields et al., 1997), whereas electrical activity induces growth cone collapse in sensory neurons (Fields et al., 1990).

PROMOTING INTRINSIC REGENERATION CAPACITIES

Better understanding of the mechanisms that underly conditioning lesion-induced regeneration and axonal development have provided candidate molecules and pathways to achieve regrowth of descending CNS tracts. Extensive attention has been addressed to the mTOR pathway, since manipulations increasing mTOR levels were shown to induce regeneration in both the visual system (Park et al., 2008; Bei et al., 2016) and the corticospinal tract (Liu et al., 2010; Zukor et al., 2013; Liu et al., 2017). Beyond actual regrowth of severed axons, mTOR targeting, either via Insulin-like-Growth-Factor-1 (IGF1) overexpression or Phosphatase-and-Tensin-homolog (PTEN) deletion promoted recovery of function (Bei et al., 2016; Liu et al., 2017). PTEN acts indeed as an inhibitor of the growth-inducing mTOR pathway, whereas IGF1 actives mTOR signaling. Similarly, activation of the Jak-STAT pathway - either genetically in the sensory system (Bareyre et al., 2011) - or via exogenous application of CNTF in retinal ganglial cells (Smith et al., 2009) promotes regeneration of axotomized neurons. Synergistic co-activation of different pathways may as well further enhance the robustness of axon regrowth (He and Jin, 2016). Interestingly, IGF1, together with CNTF and osteopontin - a molecule believed to potentiate the role of IGF1 on the mTOR cascade - could be substituted to PTEN deletion and further promote regeneration (Bei et al., 2016). While co-activating different cascades, this manipulations replaced a genetics-based manipulation with a ligand-receptor signaling activation, which is presented as more prone to clinical translation (He and Jin, 2016).

Additionally, it appears that electrical activity - suggested as detrimental for the regeneration of sensory axons (Fields et al., 1990) - plays a beneficial role on the extension of neurites from spared axons (Asboth et al., 2018; van den Brand et al., 2012; Wahl et al., 2014). With known recruitment of the CREB pathway by activity, cAMP upregulating procudures, electrical activity or neurorehabilitative training appear as particularly relevant procedures to promote sprouting, or even axon regeneration. Acute cAMP delivery has indeed been proposed as a beneficial contribution to reticulospinal regeneration (Lu et al., 2012a), while activity appears to potentiate mTOR-dependant regeneration after optic nerve lesion (Li et al., 2016). Obviously other cascades are being investigated to promote CNS axon regrowth, such as KLFs signaling or the Mitogen-Activated Protein Kinases (MAPK) pathway (He and Jin, 2016). While our understanding the biochemical requirements for axon regeneration after CNS injury is effectively progressing, strategies that succeed in activating intrinsic axonal growth machinery remain for now at a proof-of-concept stage. Indeed, some of the signaling cascades that regenerative neuroscientists aim at activating are also crucial targets to inhibit for researchers in oncology (Duzgun et al., 2016). Obviously, inducing organized and controlled growth would be ideal and safe, and the development of novel viral technologies - including inducible viral vectors - may bring neuroregeneration a step closer to clinical translation.

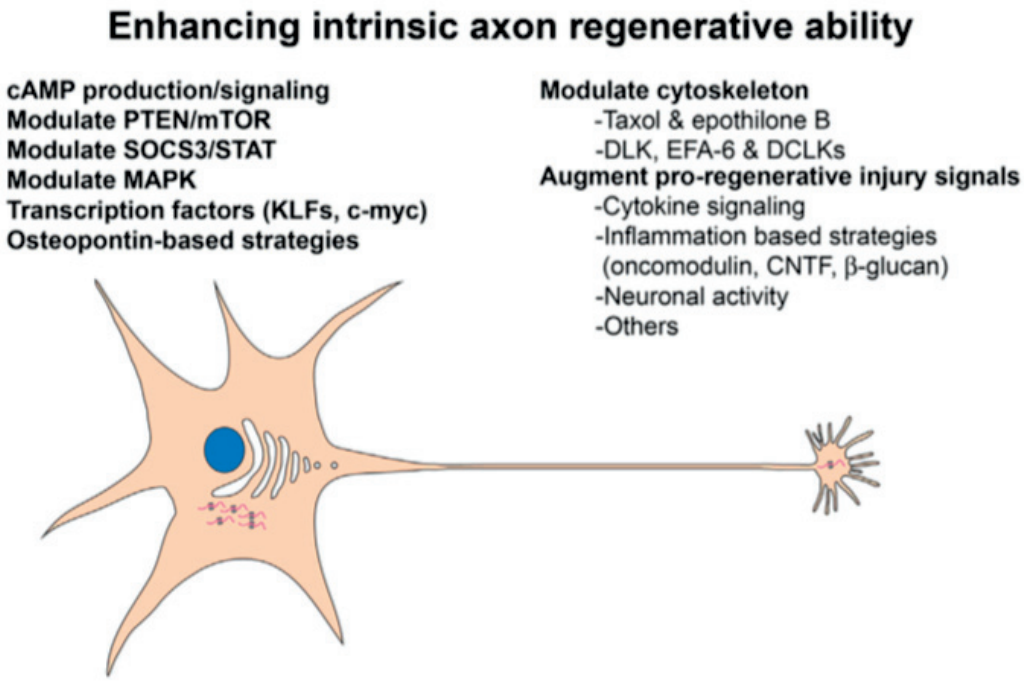

Figure 1.3: **Intrinsic mechanisms regulating axon regenerative properties.** Different mechanisms have been unveiled, that provide candidate intracellular pathways susceptible to enhance intrinsic growth capacities of axotomized neurons. These mechanisms appear to be neuron subtype-dependant. Combined manipulations are more likely to induce significant axon regeneration than single interventions, as coordinated events are required for neurite extension. (Adapted from He and Jin, 2016).

1.5 CELL REPLACEMENT
FACILITATING MATRIX AND RELAYS

Inducing robust axon regeneration has remained elusive and difficult to apprehend, due to the broad heterogeneity of mechanisms that induce and/or inhibit growth between neuronal tracts. Instead of exploring incrementally the molecular requirements for endogenous axon regeneration, a parallel field of research has been addressing cell replacement therapies as supra-molecular manipulations over the SCI site and over the endogenous neural tissue. A broad variety of cell types has been tested, including Schwann cells, neural stem (or progenitor) cells, olfactory ensheathing cells, oligodendrocyte progenitor cells and stromal cells (Assinck et al., 2017). The mechanisms that underly the observed or claimed benefits after SCI and cell grafting remain however incompletely understood and have been reported as : neuronal relay formation, neuroprotection, endogenous axon regeneration, myelin regeneration and immunomodulation (Assinck et al., 2017). In the following subsections, we discuss briefly the benefits and limitations of providing exogenous neuronal relays or supporting cell grafts into SCI sites.

1.5.1 **RELAY GRAFTS :** PROMOTING REORGANIZATION

Already back in the 1970's, Andres Björklund and colleagues assessed the feasibility of grafting fetal neurons into the injured rat CNS, showing neuronal survival, integration and connectivity within the injured forebrain (Bjoerklund et al., 1976; Bjoerklund and Stenevi, 1977). Similar results were obtained a few years later with the successful grafting of fetal spinal cord tissue into SCI sites (Bregman et al., 1993), opening the door to further developments in neuronal replacement and first human trials (Wirth et al., 2001). Grafting neurons into complete spinal cord lesions tends to mimics the reorganization of propriospinal neurons and the reach for new functional relays after incomplete SCIs. Exogenous neurons are expected to provide targets to sprouting propriospinal axons, in order to further conduct action potentials beyond the complete injury. Following Andres Björklund's work, studies have been exploring neural stem and progenitor cells (NSPC) derived from the embryonic E14 spinal cord as a source of exogenous neuronal relays. Whereas early work already suggested that endogenous axons can grow into NSPC grafts and reach for transplanted neurons (Reier, 1985; Bregman and Reier, 1986; Jakeman and Reier, 1991), Mark Tuszynski's group has been providing extensive evidence that E14 NSPCs can contribute to restore spinal cord integrity after complete transection. In a these reports, E14 NSPCs - supplemented with a broad cocktail of growth factors and embedded into a fibrin matrix - were shown to fill successfully the SCI cavity. Grafted cells differentiated into both neurons and glia, and extended long-distance axons in both rostral and caudal directions from the grafted site (Lu et al., 2012c). Moreover, the newly grafted tissue was able to restore reliable conduction of action potentials across complete transections, as well as some extent of functional benefits (Lu et al., 2012c). Transitioning to non-human primates was performed recently, as human spinal cord-derived neural progenitor cells integrated cervical circuits in rhesus monkeys after being grafted into C7 hemisection lesions(Rosenzweig et al., 2018). In the same report, cell grafts were shown to improve forelimb motor functions, even though the score-based assessments and the incomplete model of injury render these benefits debatable (Rosenzweig et al., 2018). In a context of ethical questioning and with interest into translational perspectives, effort has been put on replacing NSPCs with human induced pluripotent stem cells (iPSC), yielding anatomically successful graft integration in rats (Lu et al., 2014).

Interestingly, Mark Tuszynski's group has been showing very clearly that grafts derived from caudalized sources of NSPCs - i.e. stem and progenitor cells derived from the developing spinal cord - are permissive to the regenerating corticospinal tract (Kadoya et al., 2016). Moreover, NSPCs appear to self-organize into organotypical dorsal and ventral elements, that are preferentially and respectively innervated by regenerating sensory vs. motor axons (Dulin et al., 2018). While endogenous axons are able to penetrate the grafts, robust bridging of the injured site has yet not been achieved with relay-based cell replacement therapies.

Relay grafts appear as a promising strategy to restore anatomical integrity at the SCI site. However, reproduction studies and corrections have highlighted potential limitations and safety concerns related with proliferating cell grafts. Indeed, phenomenons such as ectopic metastases of NSPCs (Steward et al., 2014; Tuszynski et al., 2014) and collagenous partitions of the graft (Sharp et al., 2014) have been observed after transplantation of E14 neural progenitor cell in spinal cord lesions. The investigation of cell replacement therapies remains empirical, so that the mechanisms underlying endogenous regeneration into NSPC grafts are still incompletely understood. As these grafts are supplemented with survival-promoting factors, unveiling the effects of the grafted cells, respectively the added growth factors, may allow refining and optimizing molecule-based neuroregeneration paradigms. Nevertheless, promoting relay-based reorganization of circuits after SCI - either via cell replacement or with neuroprosthetics 1.3 - has been yielding robust electrophysiological or functional benefits. Restoring voluntary stepping after complete lesion will likely require supraspinal recruitment of coherent relays, in a way that is relevant for the work presented in the following chapters.

1.5.2 **SUPPORT GRAFTS :** PROMOTING ENDOGENOUS REGENERATION

Besides neuronal transplants, different cell types have been explored to provide trophic support and neuroprotection to endogenous tissues, and promote endogenous regeneration (Assinck et al., 2017). Neuroprotection has been proposed as a mechanism of action for a number of cell graft sources, including Schwann cells, Oligodendrocyte Progenitor cells, mesenchymal progenitors and Olfactory Ensheathing cells (Tetzlaff et al., 2011; Raisman, 2001). In the absence of grafted neurons, neuroprotection remains essentially relevant in incomplete models of SCI. Nevertheless, Glial progenitors and Schwann cells have been suggested to induce higher tissue sparing, promote remyelination of spared axons and enhance functional recovery after spinal contusion (Barbour et al., 2013; Pearse et al., 2004). Besides neuroprotective benefits and in coherence with the spontaneous myelin abnormalities that occur over the natural course of SCIs (see 1.2.2), remyelination induced by grafted Schwann cells has been considered as a mechanism for functional recovery in incomplete SCI models (Biernaskie et al., 2007; Takami et al., 2002).

On a molecular basis, Schwann cells and bone marrow derived progenitors are known to produce neuroprotective agents - such as BDNF - *in vitro*. A loss-of-function study showed that neuroprotection from grafted bone marrow mesenchymal cells is impeded when the graft is knocked down for *bdnf*, suggesting a possible trophic mechanism (Ritfeld et al., 2015). However, the reduction of BDNF production is correlated with a reduced graft survival that may decrease the levels of other unidentified neuroprotective agents secreted by the graft. In addition, mesenchymal cells have been shown to polarize the post-SCI immune response towards neuroprotective M2 macrophages, suggesting that non-neuronal cell grafts play a role as immunomodulators (Nakajima et al., 2012). Beyond neuroprotection and similarly to

NSPCs (see 1.5.1), grafted Schwann cells, mesenchymal cells and Olfactory Ensheathing cells are described as supporting tissue able to bridge SCI sites and promote axon regrowth (Lu et al., 2012b; Xu et al., 1995a; Takeoka et al., 2011). Trophic factors secreted by the grafts likely contribute to the regenerative effect, which appears to be enhanced by supplemented and targeted growth agents (Lu et al., 2012b).

While different reports have been describing functional benefits after non-neuronal cell grafting (Assinck et al., 2017), the possible underlying mechanisms are numerous and remain incompletely understood. Beyond the empirical exploration of functional outcomes following cell grafting into SCI lesions, cell replacement - along with viral manipulations and local administration of defined molecules - enables to manipulate endogenous tissues in order to identify the requirements for axon regeneration. Obviously, grafted cells do not only produce the molecules of interest, but they deliver in addition a constellation of other factors that can be beneficial or detrimental to regenerating neural tissue. These extra and potentially unidentified factors provide an appreciable bias to the identification of the minimal and sufficient facilitators that promote SCI wound healing and induce axon regrowth. From a translational point of view, safety concerns arise due to cell sources that are often stem or progenitor and may keep proliferative properties after transplantation. Nevertheless, unveiling beneficial cell populations may enable in the future to identify non-neuronal trophic grafts to bridge large SCI cores and promote robust and safe axon regrowth across complete lesions. Reactive astrocyte subpopulations may become of interest in this perspective (see 1.2.3 and Liddelow et al. 2017; Anderson et al. 2016).

1.6 COMBINING PARADIGMS TO RESTORE INTEGRITY

The natural course of spinal cord injuries often leaves patients with chronic sensorimotor and autonomic deficits. A mechanistic understanding of SCIs provides candidate time points and processes that therapeutic approaches can interfere with, in order to improve the functional outcome. Nevertheless, background literature strongly suggests that synergistic strategies combining multi-pronged interventions are more susceptible to yield robust and reproducible benefits than single manipulations. Most likely, rehabilitation after SCI should already start in the first hours after the trauma with strategies aiming at optimizing the immune reaction and reverting the vascular compromission. Such early strategies may enhance axon sparing and myelination, in order to help the subsequent and spontaneous circuit reorganization. At a subacute stage, reorganizing the SCI site may render the lesion core more friendly to neuroregenerative interventions, such as cell replacement and/or endogenous axon regrowth. Chronically, neuroregeneration can aim at restoring a conductive bridge across the injury, that will further need reorganization to promote coherent and functional rewiring of both afferent and efferent tracts. When endogenous neuronal relays exist, neuroprosthetics and rehabilitation - which already combine neuromodulation, pharmacology and robotics - may then support activity-based reorganization of circuits. While neuromodulation can restore lost inputs, pharmacology may rise excitability in dormant circuits and improve conduction along demyelinated tracts. In the absence of recruitable relays, neuromodulation may restore long-distance conduction of information by detecting activity at origin and stimulating accordingly the relevant target regions.

Obviously, associating such a broad range of interventions would be strongly invasive and

morbid for SCI animal models and patients. Effort needs to be devoted to the identification of the minimal and relevant manipulations susceptible to improve significantly the functional outcome and the quality of life. The following chapter relates a significant neuroregenerative breakthrough that guides robust efferent axon regrowth across complete spinal lesions. The rest of this thesis describes our attempts and current leads to promote functional rewiring of these regenerated fibers.

Chapter 2

PROPRIOSPINAL AXON REGENERATION

INDUCING GROWTH ACROSS COMPLETE SPINAL CORD INJURIES

Remark : This chapter is adapted from the published study "Required growth facilitators propel axon regeneration across complete spinal cord injury", which is referenced here below. I, Sabry L. Barlatey, contributed to this project by conducting experiments, performing surgical procedures and completing anatomical analyses.

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Required growth facilitators propel axon regeneration across complete spinal cord injury

Anderson MA., O'Shea TM., Burda JE., Ao Y., Barlatey SL., Bernstein AM., Kim JH., James ND., Rogers A., Kato B., Wollenberg AL., Kawaguchi R., Coppola G., Wang C., Deming TJ., He Z., Courtine G., Sofroniew MV.

2.1 ABSTRACT

Transected axons fail to regrow across anatomically complete spinal cord injuries (SCI) in adults. Diverse molecules can partially facilitate or attenuate axon growth during development or after injury (Tessier-Lavigne and Goodman, 1996; He and Jin, 2016; O'Shea et al., 2017), but efficient reversal of this failure remains elusive (Sofroniew, 2018). Here, we show that three mechanisms, which are essential for developmental axon growth but are attenuated or lacking in adults, (i) neuron intrinsic growth capacity (He and Jin, 2016; Goldberg et al., 2002; Bradke et al., 2012; Tedeschi et al., 2016; Geoffroy et al., 2016; Puttagunta et al., 2014), (ii) growth-supportive substrate (Letourneau, 1975; Gundersen, 1987) and (iii) chemoattraction (Campenot, 1977; Sperry, 1963), are all individually required and are in combination sufficient to stimulate robust axon regrowth across anatomically complete SCI lesions in adult rodents. We reactivated the growth capacity of mature descending propriospinal neurons with osteopontin, IGF1 and CNTF prior to SCI (Duan et al., 2015; Bei et al., 2016), induced growth-supportive substrates with FGF2 and EGF, and chemoattracted propriospinal axons with GDNF (Siebert et al., 2010; Deng et al., 2013) delivered via spatially and temporally controlled release from biomaterial depots (Nowak et al., 2002; Anderson et al., 2016) placed sequentially after SCI. We show in both mice and rats, that providing these three mechanisms in combination, but not individually, stimulated robust propriospinal axon regrowth through astrocyte scars and across non-neural lesion core tissue that was over 140-fold greater than controls. Stimulated, supported and chemoattracted propriospinal axons regrew a full spinal segment beyond lesion centers in both species, passed well into spared neural tissue below injuries, formed terminal-like contacts exhibiting synaptic markers, and conveyed a significant return of electrophysiological conduction capacity across lesions. Thus, overcoming the failure of axons to regrow across anatomically complete SCI lesions after maturity required the combined sequential reinstatement of multiple developmentally essential axon-growth facilitating mechanisms. Providing these mechanisms in combination, but not individually, was sufficient to achieve robust regrowth of physiologically active axons across such lesions. These findings identify a mechanism-based biological repair strategy for complete SCI lesions to deploy with rehabilitation paradigms designed to augment functional recovery of remodeling circuits.

PERSONAL CONTRIBUTIONS

In this multicentric collaboration, I - Sabry L. Barlatey - performed stereotaxic spinal surgeries in rats, contributed to electrophysiological acquisitions, contributed to neuroprosthetic training and behavioural assessments. I processed CNS tissue and contributed to histological analyses. I took part in conceiving figures for the published manuscript and adapted the article (Anderson et al., 2018) into the chapter presented here below.

2.2 INTRODUCTION
LESION ENVIRONMENT AFTER SPINAL CORD INJURY

Numerous studies on different models of incomplete SCI have shown the relevance of recruiting propriospinal relays to restore functional downstream input after SCI (Weidner et al., 2001; Courtine et al., 2008; Rosenzweig et al., 2010). However, in cases of complete injuries that leave no neural bridge across the lesion core, propriospinal neurons fail to regenerate and reorganize into the deafferented caudal circuits. We propose here to explore the requirements for propriospinal axon regrowth across complete SCI, in order to reafferent lumbosacral tissues and enable caudal circuit reorganization.

Recent neuroregenerative work has been orienting focus on the determinants that may upregulate growth programs in axotomized neurons. In particular, Zhigang He's group has been showing evidence that upregulating the Jak-STAT (Bareyre et al., 2011; Smith et al., 2009) and mTOR pathway (Park et al., 2008; Bei et al., 2016; Liu et al., 2010; Zukor et al., 2013; Liu et al., 2017) can promote axon regrowth in defined subpopulations of severed neurons. However, mTOR activation in corticospinal neurons mainly promotes axon regeneration through severe injuries along astroglial bridges (Zukor et al., 2013), so that fibroblast- and macrophage-filled regions of the lesion are bypassed. In cases of complete SCIs that do not spare glial bridges, enhancing intrinsic growth programs has yet failed to promote axon regrowth across the non-neural inhibitory lesion core.

As the glial scar has for long been considered a physical and chemical barrier to axon regrowth (Davies et al., 1997, 1999; Silver and Miller, 2004), new insight strongly suggests that reactive astrocytes can take different phenotypes - either beneficial or detrimental to regenerative processes - depending on the type of insult (Liddelow et al., 2017). A loss-of-function study demonstrated that reactive astrogliosis in the context of spinal cord injuries exerts a neuroprotective effect and supports axon regrowth (Anderson et al., 2016). The spontaneously repellent lesion core remains nevertheless non-permissive to regenerating axons. Debate is still strong about the regenerative benefits of neutralizing known molecular inhibitors, such as Myelin-Associated-Inhibitors (MAI) (Lee et al., 2010; Cafferty et al., 2010; Geoffroy and Zheng, 2014) and Chondroitin Sulfate Proteoglycans (CSPG) (Fitch and Silver, 2008; Sharma et al., 2012; Anderson et al., 2016). Interestingly, genetic analyses confirmed that reactive astrogliosis expresses CSPGs in spite of its clear support to regenerating fibers (Anderson et al., 2016). As support molecules such as laminins are required for axon extension (Letourneau, 1975; Gundersen, 1987; Anderson et al., 2016; Plantman et al., 2008), failure of axon regrowth after spinal axotomy may rely on a lack of supportive extracellular matrix within the lesion core, more than on an excessive molecular repulsion. As astrogliosis and other non-neural cells after SCI are able to secrete permissive components of the extracellular matrix, we explore here an opportunity to upregulate the presence of such molecules within the lesion core. In addition to an increase in intrinsic neuronal growth capacities and to the reinforcement of permissive extracellular components, we propose here to investigate the requirements for exogenous guidance cues.

2.3 RESULTS
PROPRIOSPINAL REGENERATION ACROSS COMPLETE SCI

2.3.1 A SUPPORTIVE MATRIX, AXON GUIDANCE & GROWTH INDUCTION ARE SUFFICIENT FOR REGROWTH

We tested the hypothesis that failure of adult CNS axons to regrow across complete SCI lesions was due to a combined lack of multiple mechanisms required for developmental axon growth. We targeted descending propriospinal neurons because after incomplete SCI they spontaneously form new intraspinal circuits that relay functionally meaningful information past lesions (Courtine et al., 2008; van den Brand et al., 2012; Jacobi et al., 2015). Thus, short distance regrowth of transected propriospinal axons across complete SCI lesions has the potential to find new neuronal targets and form new relay circuits. To reactivate intrinsic propriospinal neuronal growth capacity, which is attenuated in adult CNS neurons (He and Jin, 2016; Goldberg et al., 2002; Bradke et al., 2012; Tedeschi et al., 2016; Geoffroy et al., 2016; Puttagunta et al., 2014), we tested two approaches previously successful with retinal and corticospinal neurons by using adenoassociated viral vectors (AAV) to deliver either PTEN knockdown (AAV-shPT) (Zukor et al., 2013), or to express osteopontin, IGF1 and CNTF (AAV-OIC) (Duan et al., 2015; Bei et al., 2016). To increase axon growth-supportive substrates such as laminin, which are required for growth of developing and adult axons (Letourneau, 1975; Gundersen, 1987; Anderson et al., 2016; Plantman et al., 2008), we delivered fibroblast growth factor 2 (FGF) (Kashpur et al., 2013) and epidermal growth factor (EGF) (White et al., 2008). To sequentially chemoattract (Sperry, 1963; Campenot, 1977) propriospinal axons, we delivered glial-derived growth factor (GDNF) because propriospinal neurons express GDNF receptors (GDNFR), increase GDNFR expression after SCI (Siebert et al., 2010) and regrow axons into cell grafts that secrete GDNF (Deng et al., 2013), and because GDNF is lacking from SCI lesions (Anderson et al., 2016). To provide temporally controlled and spatially targeted delivery of growth factors or function-blocking antibodies, we used biomaterial depots comprised of synthetic hydrogels (Nowak et al., 2002; Anderson et al., 2016) placed sequentially into SCI lesion centers and into spared neural tissue caudal to astrocyte scar borders (Fig 2.1a). AAV efficiently targeted a majority of propriospinal neurons in the injected area one segment rostral to SCI lesions, including neurons expressing GDNFR. These manipulations were tested alone and in combinations first in adult mice and then in adult rats by using a severe crush model of SCI that generates anatomically complete lesions across which there is no spontaneous regrowth of descending or ascending axons (Anderson et al., 2016).

2.3.2 A SUPPORTIVE MATRIX, AXON GUIDANCE & GROWTH INDUCTION ARE REQUIRED FOR REGROWTH

Propriospinal axon regeneration was quantified as tract-tracer-labeled axons that regrew to lesion centers or beyond (Fig2.1b-d). Mice with SCI-only or SCI plus empty hydrogel depots exhibited few or no axons at lesion centers. Individual interventions, AAV-shPT or AAV-OIC alone, or depots with FGF+EGF alone or GDNF alone, did not significantly increase this number. Combined delivery of all three growth factors, FGF+EGF+GDNF in one or two depots, but without AAV, led to modest but significant increases in axon regrowth. Combined delivery

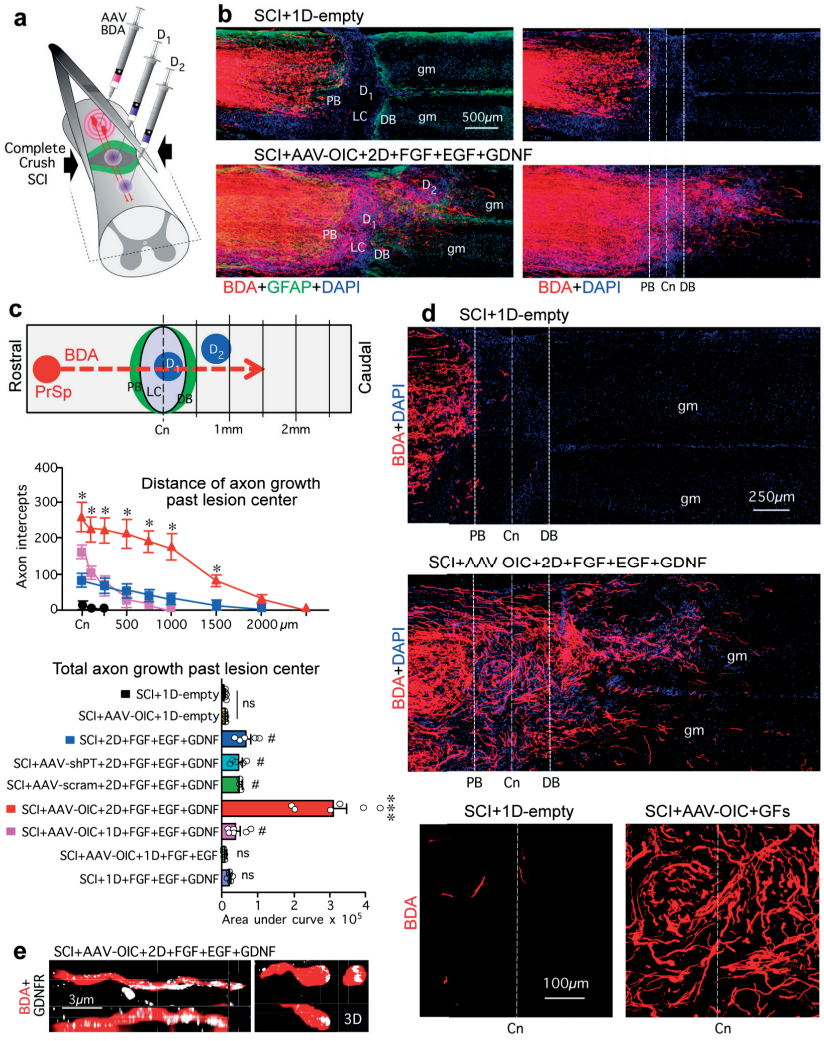

Figure 2.1: **Stimulated and chemoattracted propriospinal axons regrow robustly across anatomically complete SCI lesions in mice receiving combined delivery of AAV-OIC plus FGF, EGF and GDNF in two sequentially placed hydrogel depots.** **a**, Experimental model. D1 and D2, hydrogel depot 1 and 2, respectively. **b**, BDA-labelled axons in composite tiled scans of horizontal sections also stained for astrocytes (anti-GFAP (glial fibrillary acidic protein), left) and cell nuclei (DAPI). Dotted lines demarcate astrocyte proximal (PB) and distal (DB) border around the lesion core (LC). Dashed line demarcates lesion centre. 1D, one depot; 2D, two depots; GM, grey matter. **c**, Top, schematic of axon intercept. Middle, axon intercepts at specific distances past lesion centers (colour coding and n as in the graph below). Bottom, areas under axon intercept curves. Dots show n mice per group. NS, not significant versus SCI + 1D empty; *$P < 0.01$ versus SCI + 1D empty, two-way ANOVA with Bonferroni; **$P < 0.01$ versus all other groups and not significant versus each other, ***$P < 0.0001$ versus all other groups, one-way ANOVA with Bonferroni. Data are mean ± s.e.m. PrSp, propiospinal. **d**, Surveys (top) and details (bottom) of BDA-labelled axons. **e**, Three-dimensional detail of BDA-labelled axon and growth cone expressing GDNFR in the lesion core.

of AAV-shPT plus FGF+EGF+GDNF did not significantly increase axon numbers compared with FGF+EGF+GDNF alone or AAV-scrambled plus FGF+EGF+GDNF. In striking contrast, combined delivery of AAV-OIC plus FGF+EGF+GDNF synergistically facilitated robust propriospinal axon regrowth. In mice with two sequentially placed depots, this regrowth passed through all lesion compartments, including non-neural lesion core and its proximal and distal astrocyte scar borders, and penetrated well into spared grey matter (Fig2.1, 2.2, 2.3). In these mice, total axon regrowth past lesion centers was over 140-fold greater than SCI-only or SCI plus empty depots (Fig 2.1c). Regrowing propriospinal axons expressed detectable levels of GDNFR (Fig 2.1e) and followed irregular paths (Fig 2.1d) meeting criteria for appearance of regrowing, as opposed to spared, axons (Tuszynski and Steward, 2012). BDA tract-tracer did not label axons of passage, such as serotonin axons. In all cases, no BDA-labeled axons were present at 3mm past lesion centers confirming that lesions were complete (Fig 2.1b-d).

2.3.3 INDUCING A SUPPORTIVE EXTRACELLULAR MATRIX WITHIN THE SCI

To dissect cellular and molecular mechanisms underlying this robust axon regrowth, we determined the impact on axon-substrate interactions of molecular and cellular changes induced by our interventions (Fig 2.2). FGF+EGF significantly increased known axon-supportive substrate molecules (Gundersen, 1987), laminin, fibronectin and collagen within SCI lesions (Fig 2.2a), whereas potentially inhibitory chondroitin sulfate proteoglycans (CSPG) (Cregg et al., 2014) were not significantly altered (Fig 2.2g). FGF+EGF significantly increased the proliferation and density of scar-forming astrocytes in borders around non-neural lesion cores (Fig 2.2b), yet in spite of a near doubling of these cells, stimulated and chemoattracted propriospinal axons regrew robustly through and beyond proximal (Fig 2.2c) and distal (Fig 2.3a) astrocyte scar borders in a manner consistent with previous observations for sensory axons (Anderson et al., 2016). FGF+EGF also increased the perceptible density of stromal cells in lesion core (Fig 2.2a). Axons transitioned readily from growing along astrocytes in proximal scar borders to growing along non-neural stromal cells in lesion core (Fig 2.2c), often orientated along stromal cells or blood vessels and circumventing clusters of inflammatory cells (Fig 2.2c). Some regrowing axons in lesion core were in partial contact with cells expressing Schwann cell markers. Thus, appropriately stimulated and attracted axons were able to regrow in contact with multiple cell types. Notably, over 98% of regrowing propriospinal axons in SCI lesions had at least one surface continually in contact with laminin, whereas axon surfaces in mature uninjured tissue were rarely in contact with laminin (Fig 2.2d,e). Many regrowing axons also had contacts with fibronectin or collagen. Simultaneous in vivo delivery of anti-CD29, an integrin-function-blocking antibody (Anderson et al., 2016; Plantman et al., 2008) prevented the majority of axon regrowth (Fig 2.2f), demonstrating that axon regrowth required integrin-dependent axon-substrate interactions with laminin, fibronectin or collagen. Nevertheless, upregulation of permissive substrate alone was not sufficient to attract activated axon regrowth into lesion core, such that AAV-OIC plus depots of only FGF+EGF exhibited no significant axon regrowth, whereas AAV-OIC+FGF+EGF+GDNF did (Fig 2.1c), demonstrating that axon regrowth also required chemoattraction. Remarkably, AAV-OIC+FGF+EGF+GDNF stimulated axons regrew robustly through dense areas of CSPGs, including in direct contact with brevican or CSPG4 in both astrocyte scars and non-neural lesion cores. This growth occurred along surfaces with high expression of laminin (Fig 2.2h,i), consistent with observations that CSPG inhibition is relative rather than absolute, such that increasing laminin

overrides CSPG presence in vitro (Tom et al., 2004). To probe more broadly the effects of prolonged FGF+EGF treatment on astrocytes and other cells in SCI lesions, we conducted genome-wide sequencing of astrocyte-specific ribosome-associated RNA and RNA from non-astrocyte cells (Anderson et al., 2016) at two weeks after SCI. At this time point, FGF+EGF treatment persisted in significantly altering substantial numbers of genes relative to SCI only. The most significantly altered gene networks were associated with astrocyte proliferation and development, and with non-astrocyte inflammatory responses, in a manner consistent with our histological and biochemical evaluations.

2.3.4 CHEMOATTRACTIVE GDNF GUIDES REGENERATING AXONS ACROSS THE LESION

We next identified mechanisms required to achieve propriospinal axon regrowth beyond lesion cores and distal scar borders into spared grey matter. In our early experiments with one depot of AAV-OIC plus FGF+EGF+GDNF into lesion cores, we noted that propriospinal axons regrew robustly through proximal astrocyte scar borders and then concentrated around and encircled depots, but did not pass far beyond. We therefore placed a second depot of GDNF into neural tissue beyond the distal astrocyte scar. This second depot was injected one week after the first depot so as to sequentially chemoattract propriospinal axons that had regrown into the lesion core. In mice with two such spatially and temporally separated depots, axons regrew robustly across lesion cores and distal astrocyte borders, and penetrated well into spared grey matter beyond the injury, and routinely regrew a full spinal segment past lesion centers (Fig 2.1b-d), demonstrating that chemoattraction is required to draw regrowth of mature endogenous axons into spared neural tissue beyond injuries. Notably, the second depot was placed at 9 days after SCI, indicating that GDNF efficiently chemoattracted regrowing axons across already formed distal astrocyte scar borders without altering CSPG levels (Fig 2.3a). Starting just beyond lesion borders (Fig 2.3b,c), regrowing propriospinal axons that entered spared grey matter intermingled with NeuN-positive neurons, some of which formed terminal-like swellings that contacted neurons, colocalized with the pre-synaptic marker, synaptophysin, and were in apposition with the post-synaptic marker, homer (Fig 2.3b-d). Such contacts were found distally wherever regrowing axons were present in grey matter, up to a full spinal segment (1500μm) or more beyond lesion centers. As expected and discussed below, observer scored over ground locomotion did not improve in these experiments that were focused on dissecting mechanisms required to achieve axon regrowth across lesions.

2.3.5 REGENERATED PROPRIOSPINAL AXONS CONDUCT ACTION POTENTIALS

We next tested whether our findings could be extended to rats, whose lesion core pathophysiology has been proposed to be more similar to that of humans. As expected, rats exhibited little or no propriospinal axon regrowth after SCI plus empty hydrogel. Axon regrowth was not increased by AAV-OIC alone, and only minimally by FGF+EGF+GDNF alone. In striking contrast, and consistent with our observations in mice, rats given combined delivery of AAV-OIC plus two depots of FGF+EGF+GDNF and GDNF exhibited robust propriospinal axon regrowth through all lesion compartments, and this regrowth routinely reached a full spinal segment or more past lesion centers and penetrated well into spared grey matter around and beyond the

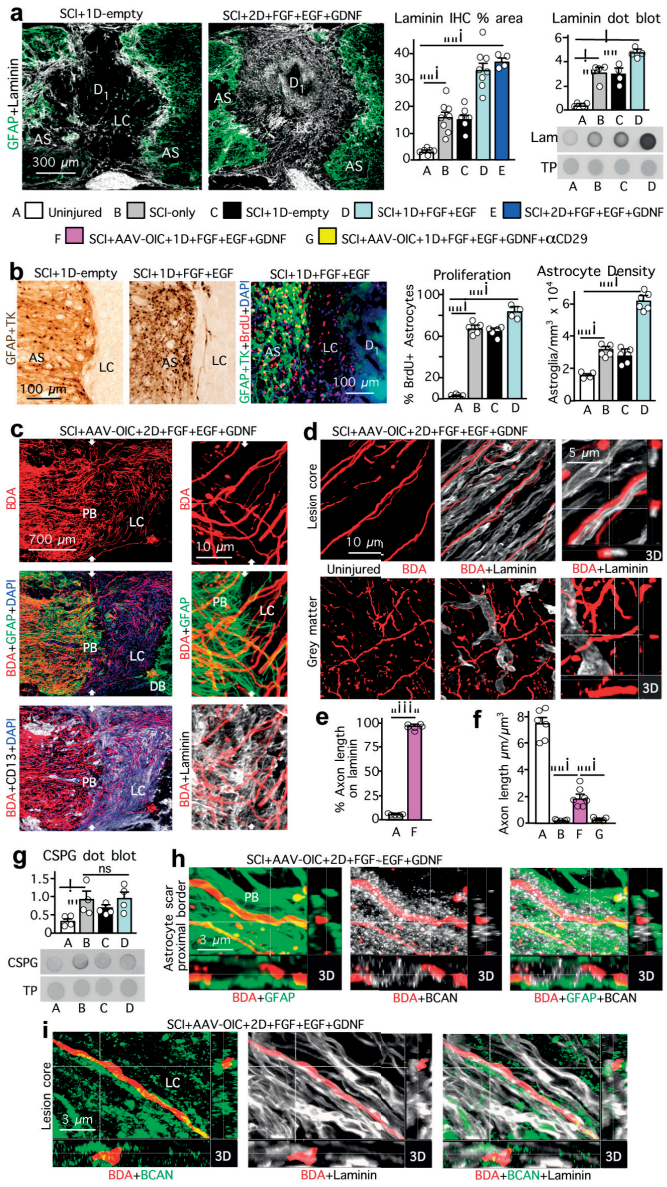

Figure 2.2: **Stimulated, supported and chemoattracted mouse propriospinal axons regrow through the proximal borders of astrocyte scars and lesion core stromal cells along laminin that has been upregulated by delivered growth factors, in spite of CSPG presence.** **a**, Laminin immunohistochemistry (IHC) images (left) plus quantification (middle, percentage stained area), and dot blot plus quantification of density (right). *P < 0.01, one-way ANOVA with Bonferroni. TP, total protein. The colour key is used throughout all panels. **b**, IHC images and quantification (cell number) of astrocyte proliferation and density. *P < 0.0005, one-way ANOVA with Bonferroni. **c**, BDA-labelled axon regrowth past proximal borders and in the lesion core among CD13+ stromal cells (left) and along laminin (right). White arrows denote proximal borders. **d–f**, IHC images (**d**), quantification of axon contact with laminin (**e**, ***P < 0.0001; Student's two-tailed t-test, t (9) = 107.4) and axon length per tissue volume (**f**, *P < 0.0005 one-way ANOVA with Bonferroni). **g**, CSPG dot blot and quantification of density. *P < 0.05, one-way ANOVA with Bonferroni. For all graphs, data are mean ± s.e.m. and dots show n mice per group. **h**, **i**, BDA-labelled axon regrowth through astrocytes of proximal borders (**h**) and along laminin in the lesion centre (**i**) in spite of dense brevican (BCAN).

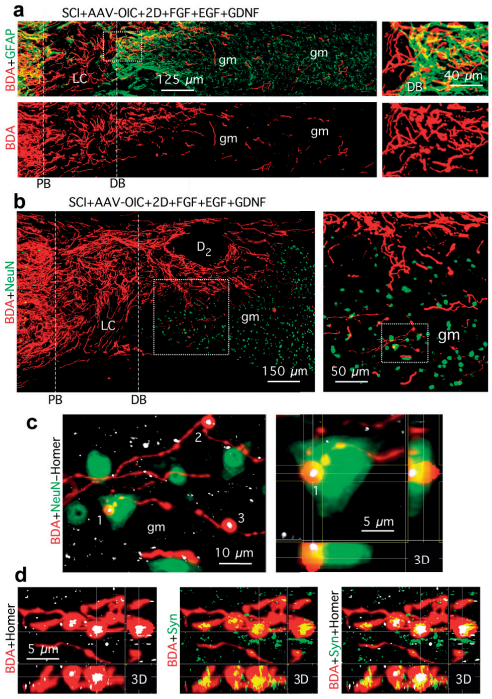

Figure 2.3: **Stimulated and chemoattracted mouse propriospinal axons regrow past astrocyte scar distal borders into grey matter and form synapse-like contacts with neurons.** **a, b**, Surveys and details (boxed areas) of BDA-labelled axon regrowth across distal borders and into grey matter. **c**, Detail of **b** (outlined region) and 3D view of synapse-like contact of BDA-labelled terminal with the post-synaptic marker, homer, on NeuN+ neuron. **d**, Synapse-like BDA-labelled terminals with overlapping pre- and post-synaptic markers, synaptophysin (Syn) and homer.

second depot (Fig 2.4a,b). Total axon regrowth past lesion centers was over 140-fold greater in rats with AAV-OIC plus FGF+EGF+GDNF versus SCI with empty hydrogel (Fig 2.4b). The AAV-derived tract-tracer, RFP, was not expressed in serotonin axons, indicating that labeling was restricted to axons of the targeted propriospinal neurons. Moreover, in striking contrast with the robust regrowth of RFP-labeled propriospinal axons, serotonin axons were not attracted to regrow into lesion cores, indicating that our growth factor depots did not simply alter lesion core environment in a manner that on its own broadly enabled regrowth of all axon types. Regrowing propriospinal axons that reached spared grey matter intermingled with NeuN-positive neurons, and some axons formed terminal-like swellings that contacted neurons, colocalized with pre-synaptic marker, synaptophysin, and were in apposition with post-synaptic marker, homer (Fig 2.4c). As in mice, observer scored over-ground locomotor score did not improve in these experiments that probed mechanisms required to achieve axon regrowth, but which did not involve additional rehabilitation programs to provide use dependent plasticity. Nevertheless, to look for potential basic functionality of regrown propriospinal axons, we studied their ability to conduct electrophysiological signals across SCI lesions. Rats with SCI-only exhibited essentially no conduction above background levels across lesions, whereas rats with AAV-OIC plus FGF+EGF+GDNF exhibited conduction at about 25% of control levels at 2mm past lesions, which disappeared by 5mm caudal to lesions (Fig 2.4d), indicating that regrowth of propriospinal axons across lesions was associated with a significant return of conduction capacity across lesions that correlated with the distance of axon regrowth and approximate locations of synapse-like contacts.

2.3.6 NEUROPROSTHETICS AND PROPRIOSPINAL REGENERATION FAILS TO INDUCE FUNCTIONAL RECOVERY

As mentioned above (see 2.3.4 and 2.3.5), no spontaneous recovery of hindlimb movements occurs in spite of robust axon regeneration. Indeed, the regrowth of propriospinal fibers that we obtain remains caudally limited by our second depot of guidance cue, suggesting that regenerated axons are spatially forced by the GDNF gradient and fail to reach functionally significant relays. In the mean time, convincing evidence proposes that propriospinal relays can be recruited in incomplete models of SCI to support recovery of function (van den Brand et al., 2012; Courtine et al., 2008). As neuro-rehabilitation seems to increase propriospinal plasticity and induce reorganization of circuits, we hypothesized that will-powered rehabilitation combined with infra-lesional circuits' activation may induce regenerated propriospinal axons to sprout and reach coherent targets susceptible to yield recovery of motor functions. We therefore repeated our strategy involving AAV-OIC+FGF+EGF+GDNF in rats after complete crush injury, and added to the regenerative paradigm the gold-standard neuroprosthetic rehabilitation established by the Courtine Laboratory (van den Brand et al., 2012; Asboth et al., 2018). Chronically over three months post-injury, the animals were trained daily for 30 minutes in a robotic postural interface designed to provide body-weight-support. During these training sessions, infralesional tonic stimulation was delivered over L2 and S1 to recruit respectively the flexion and extension lumbar hotspots. Systemic monoamines were added as a pharmacological facilitation of lumbar circuits' activity : quipazine to modulate extension muscle groups and 8-OH-DPAT to facilitate flexion components. Due to the high complexity of this strategy, technical limitations arose. Indeed, several animals could not undergo proper training after the effects of electric epidural stimulation (EES) were loss during the experimental course. This pilot test left us with a non-statistically significant group of 3 animals,

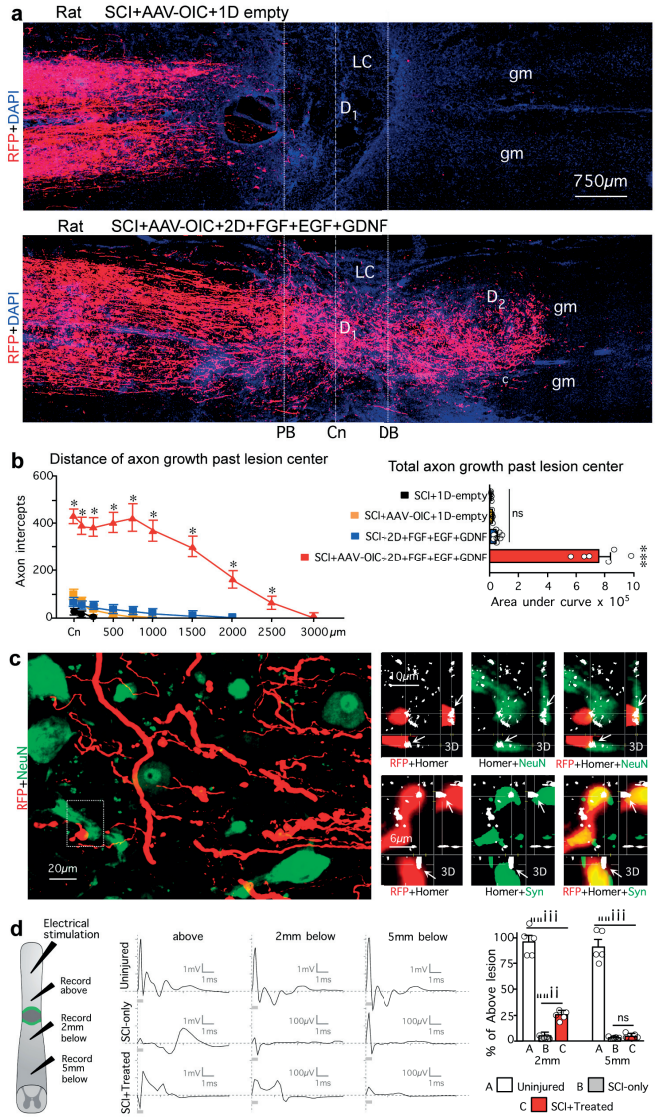

Figure 2.4: **Stimulated and chemoattracted propriospinal axons regrow robustly and conduct electrophysiological signals across anatomically complete SCI lesions in rats after combined delivery of AAV-OIC plus FGF, EGF and GDNF in two sequentially placed hydrogel depots.** **a**, RFP-labelled axons in composite tiled scans of horizontal sections. Dotted lines demarcate astrocyte proximal and distal borders around lesion core. Dashed line demarcates lesion centre (Cn). **b**, Left, axon intercepts at specific distances past lesion centers (colour coding and n as in bar graph). Right, areas under axon intercept curves. *$P < 0.01$ versus all other groups, ***$P < 0.0001$ versus all other groups, one-way ANOVA with Bonferroni. **c**, Detail images from the region indicated with an asterisk in **a**. Left, RFP-labelled axons among NeuN+ neurons in spared grey matter 2,000 μm past the lesion centre. Top right, 3D detail of the outlined area in the left panel shows synapse-like contact of BDA-labelled terminal with the post-synaptic marker, homer, on a neuron. Bottom right, RFP-labelled terminal co-labelled with the presynaptic marker, synaptophysin (Syn) in synapse-like contact with the post-synaptic marker, homer. **d**, Left, spinal-cord stimulation and recording sites. Middle, representative individual electrophysiological traces after spinal cord stimulation. Right, peak-to-peak amplitude of the evoked potential at 2 and 5 mm below lesions relative to the potential above the lesion in SCI only or SCI + treatment (SCI + AAV-OIC + FGF + EGF + GDNF) or the equivalent distance in uninjured controls. **$P < 0.005$, ***$P < 0.0001$, one-way ANOVA with Bonferroni. For all bar graphs, data are mean ± s.e.m. and dots show n rats per group.

including two rats trained without EES. After 3 months of rehabilitation, even though limitations render our preliminary data non-conclusive, no functional benefit could be obtained from associating propriospinal axon regrowth and neuroprosthetic training.

2.4 DISCUSSION

TRACT SPECIFIC GROWTH PROGRAMS AND GUIDANCE

Biological repair of anatomically complete SCI will require axon regrowth across non-neural lesion cores and their astrocyte limitans borders into spared neural tissue to form new circuits (O'Shea et al., 2017). A mechanistic understanding of why spontaneous axon regrowth across such lesions fails in adults is fundamental to creating interventions that augment regrowth (Sofroniew, 2018). Our findings, in both mice and rats, strongly support the hypothesis that regrowth of adult propriospinal axons across anatomically complete SCI lesions fails primarily because of the simultaneous absence or inadequate presence of three types of mechanisms essential for facilitating developmental axon growth, (i) neuron intrinsic growth capacity (He and Jin, 2016; Goldberg et al., 2002; Bradke et al., 2012; Tedeschi et al., 2016; Geoffroy et al., 2016; Puttagunta et al., 2014), (ii) supportive substrate (Letourneau, 1975; Gundersen, 1987), and (iii) chemoattraction (Sperry, 1963; Campenot, 1977). We show that each of these mechanisms is required, and that providing them in combination, but not individually, is sufficient to achieve robust axon regrowth across such lesions in spite of the continued presence of putative growth inhibitors. Our findings markedly extend previous observations that providing individual growth-facilitating mechanisms on their own is not sufficient to achieve meaningful axon regrowth across complete SCI lesions and that combining certain mechanisms can partially improve axon regrowth (O'Shea et al., 2017; Sofroniew, 2018; Anderson et al., 2016; Zukor et al., 2013; Richardson and Issa, 1984; Alto et al., 2009). Importantly, our findings demonstrate that chemoattraction is critically required to achieve robust axon regrowth through and beyond complete SCI lesions into spared neural tissue, and point towards the need to identify chemoattractants effective for other axon populations desirable to target after SCI. The difference in response of propriospinal neurons to AAV-shPT and AAV-OIC is consistent with previous observations of neuron-type specific activation requirements (Duan et al., 2015) and points towards the need to dissect specific molecular mechanisms regulating growth capacity of different neuronal populations, and to identify means to achieve such activation at subacute or chronic times after SCI. Although regrowth of propriospinal axons across lesions was associated with a significant return of electrophysiological conduction across lesions, there was, as expected (Sofroniew, 2018), no detectable improvement of locomotor function, consistent with accumulating evidence that new relay circuits formed after complete SCI cannot be expected to acquire function spontaneously, but will require targeted rehabilitation procedures that foster their integration into functional networks through use-dependent plasticity (Sofroniew, 2018; Courtine et al., 2008; van den Brand et al., 2012; Asboth et al., 2018). Our findings provide proof-of-concept evidence that robust and physiologically active descending propriospinal axon regrowth can be achieved across anatomically complete SCI lesions, and identify a mechanism-based biological repair strategy for such lesions to test in conjunction with targeted rehabilitation paradigms (Asboth et al., 2018) designed to augment synapse remodeling and functional recovery of remodeling circuits. Nevertheless, we hypothesize that functional recovery will require remodeling both afferent and efferent connectivity, based on

i) reinforced descending input supported by the regeneration of other supraspinal tracts and ii) restored somatosensory feedback to supraspinal structures. The following chapters address both these questions individually.

2.5 MATERIALS AND METHODS

2.5.1 ANIMALS

Mice. All experiments using mice were conducted at UCLA using C57/BL6 female and male mice. RNA sequencing experiments used C57/BL6 mice expressing an mGFAP-RiboTag transgene generated and characterized as described 19. All mice used were young adults between 10 weeks and four months old at the time of spinal cord injury. Mice receiving AAV injections were between 6 and 9 weeks old at the time of AAV injection. All mice were housed in a 12-hour light/dark cycle in a specific pathogen-free facility with controlled temperature and humidity and were allowed free access to food and water. Animal care, including manual bladder voiding, was performed at least twice daily or as needed following SCI for the duration of the experiment. All experiments were conducted according to protocols approved by the Animal Research Committee of the Office for Protection of Research Subjects at University of California Los Angeles.

Rats. All surgical procedures in rats were done at EPFL. Experiments were conducted on young adult female Lewis rats between two and four months of age (180-220g body weight) housed three to a cage on a 12-hour light/dark cycle with access to food and water ad libitum. Housing, surgery, and euthanasia were performed in compliance with the Swiss Veterinary Law guidelines. Animal care, including manual bladder voiding, was performed twice daily following SCI for the duration of the experiment. All procedures and experiments were approved by the Veterinary Office of the canton of Vaud and the Veterinary Office of the canton of Geneva (Switzerland).

2.5.2 SURGICAL PROCEDURES

REGENERATIVE SURGICAL PROCEDURES FOR MICE

All surgeries on mice were performed at UCLA under general anesthesia with isoflurane in oxygen-enriched air using an operating microscope (Zeiss, Oberkochen, Germany), and rodent stereotaxic apparatus (David Kopf, Tujunga, CA). AAV injections were made two weeks prior to SCI to allow time for molecular expression and were targeted at propriospinal neurons between one and two segments rostral to planned locations of SCI lesions after laminectomy of a single vertebra. AAV (see below) were injected into 2 sites (1 on each side of the cord) x 0.25µl (AAV2/9 OPN: 1x10^13, IGF: 5x10^12, CNTF: 5x10^12 gc/ml in sterile saline) 0.6 mm below surface at 0.1µl per minute using glass micropipettes (ground to 50 to 100 µm tips) connected via high-pressure tubing (Kopf) to 10µl syringes under control of microinfusion pumps. Severe crush SCI were made at the level of T10 after laminectomy of a single vertebra 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 seconds19,33-35 (Extended Data Fig. 1). Hydrogel depots were injected stereotaxically into the center of SCI lesions 0.6 mm below surface at 0.15µl per minute using glass micropipettes (ground to 50 to 100 µm tips) connected via high-pressure tubing (Kopf) to 10µl syringes under control of microinfusion pumps, two days after SCI 36. Tract-tracing of propriospinal neurons was performed by injection of biotinylated dextran amine 10000 (BDA, Sigma) 10% wt/vol in sterile saline injected 2 x 0.4µl into the same rostral segments targeted with AAV injections as described above. In animals receiving two hydrogel depots, the second depot was placed 1mm below the SCI 9 days post SCI. Timelines of all injections are provided in Extended Data Figure 1. All mice received analgesic prior to wound closure and every 12 hours for at least 48 hours post-injury. Animals were randomly assigned numbers and evaluated thereafter blind to experimental condition.

REGENERATIVE SURGICAL PROCEDURES FOR RATS

All surgeries on rats were performed at EPFL under general anesthesia with isoflurane in oxygen-enriched air using an operating microscope (Zeiss, Oberkochen, Germany), and rodent stereotaxic apparatus (David Kopf, Tujunga, CA). AAV injections were made two weeks prior to SCI to allow time for molecular expression and were targeted at propriospinal neurons one and two segments rostral to planned locations of SCI lesions after laminectomy of a single vertebra. AAV were injected into 4 sites (2 on each side of the cord) x 0.25µl (AAV2/9 OPN: 1x10^13, IGF: 5x10^12, CNTF: 5x10^12 gc/ml in sterile saline) 1.1 mm below the surface at 0.2 l per minute using glass micropipettes connected via high pressure tubing (Kopf) to 10l syringes under control of microinfusion pumps. Severe crush SCI were made at the level of T10 laminectomy of a single vertebra by using No. 2 Dumont Forceps (Fine Science Tools, Foster City, CA) without spacers and with a tip of 0.5mm to completely compress the entire spinal cord laterally from both sides for 5 seconds (Extended Data Fig. 1). Hydrogel depots were injected stereotaxically into the center of SCI lesions 1.1 mm below the surface at 0.2µl per minute using glass micropipettes connected via high pressure tubing (Kopf) to 10µl syringes under control of microinfusion pumps, two days after SCI. One week later, a hydrogel depot was placed 2mm

below the SCI. During the same surgery, tract-tracing of propriospinal neurons was performed by injection of AAV2/5 red fluorescent protein (RFP, University of Pennsylvania Vector Core, 2.612x10^13 gc/ml) injected 4 x 0.25 µl into the same rostral segments targeted with AAV injections as described above. Timelines of all injections are provided in Extended Data Figure 1. All rats received analgesia (buprenorphine Temgesic, ESSEX Chemie AG, Switzerland, 0.01-0.05 mg per kg, s.c.) and antibiotics (Baytril 2.5%, Bayer Health Care AG, Germany, 5-10 mg per kg, s.c.) were provided for 3 and 5 days post-surgery, respectfully. Animals were randomly assigned numbers and evaluated thereafter blind to experimental condition.

NEUROPROSTHETIC SURGICAL PROCEDURES FOR RATS

All surgeries on rats were performed at EPFL under general anesthesia with isoflurane in oxygen-enriched air using an operating microscope (Zeiss, Oberkochen, Germany). A skin incision is made along the sagittal suture of the skull and the connective tissue and the muscles covering the skull are separated. The skull is thoroughly dried and four stainless steel screws are firmly inserted into the exposed bone. An amphenol-recording unit is placed between the screws and rigidly affixed to the bone using dental cement. Multi-stranded, teflon-coated stainless steel wires connected to gold plated amphenol pins in the recording unit are passed subcutaneously to the thoracic column. Chronic electrodes were conceived as windows in the teflon coating and were attached to the dura mater (8.0 Ethilon) in order to elicit spinal cord reflexes. At the end of the procedure, the muscle fascia (5.0 Vycril) and the skin (4.0 Ethilon) were sutured in layers. All rats received analgesia (buprenorphine Temgesic, ESSEX Chemie AG, Switzerland, 0.01-0.05 mg per kg, s.c.) and antibiotics (Baytril 2.5%, Bayer Health Care AG, Germany, 5-10 mg per kg, s.c.) were provided for 3 and 5 days post-surgery, respectfully. Animals were randomly assigned numbers and evaluated thereafter blind to experimental condition.

2.5.3 ADENO-ASSOCIATED VIRUSES (AAVs)

Various adeno associated viral vectors (AAV) were used to deliver either scrambled control AAV (AAV2/1-scrambled: 5x10^12 gc/ml) or PTEN knockdown (AAV2/1-shPTEN: 5x10^12 gc/ml)23; or to express the growth factors osteopontin (OPN), IGF1 and CNTF (AAV2/9 OPN: 1x10^13 gc/ml; AAV2/9 IGF-1: 5x10^12 gc/ml; AAV2/9 CNTF: 5x10^12 gc/ml)14,15; or to express green fluorescent protein (GFP) as a reporter protein (AAV2/9 GFP: 2x10^13 gc/ml) or red fluorescent protein (RFP) as an axonal tract-tracer (AAV2/5-RFP: 2.612x10^13 gc/ml)(University of Pennsylvania Vector Core).

2.5.4 HYDROGEL DEPOTS WITH GROWTH FACTORS & FUNCTION-BLOCKING ANTIBODIES

Biomaterial depots were prepared using well characterized diblock copolypeptide hydrogels that are CNS biocompatible, biodegrade over several weeks in vivo and provide prolonged delivery of bioactive growth factors in CNS tissue for two or more weeks after injection18,19,37,38. Diblock copolypeptide hydrogel K180L20 was fabricated, conjugated with blue fluorescent dye (AMCA-X) and loaded with growth factor and antibody cargoes as described36-38. Cargo molecules were as follows: Human recombinant FGF2, EGF and GDNF were purchased from PeproTech (Rocky Hill, NJ): (i) Human FGF2 (FGF-basic) (154 a.a.) 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. Integrin-function-blocking Hamster anti-rat CD29 monoclonal antibody was purchased from BD Bioscience (San Diego, CA) as a custom order at 10.3mg/ml (product #624084; lot#7165896). Freeze dried K180L20 powder was reconstituted to 3.0% or 3.5% wt/vol in sterile PBS without cargo or with combinations of FGF2 (1.0µg/µl), EGF (1.0µg/µl), GDNF (1.0µg/µl) and anti-CD29 (5µg/µl). Diblock copolypeptide hydrogel formulations were prepared to have G´ (storage modulus at 10 rad s-1) between 75 and 100 Pascal (Pa), somewhat below that of mouse brain at 200 Pa37,38.

2.5.5 HINDLIMB LOCOMOTOR EVALUATION & ANIMAL INCLUSION CRITERIA

Two days after SCI, all mice or rats were evaluated in open field and all animals exhibiting any hindlimb movements were not studied further. Rodents that passed this inclusion criterion were randomized into experimental groups for further treatments and were thereafter evaluated blind to their experimental condition. At 2,7,14 days and 28 days after SCI, hindlimb movements were scored using a simple six-point scale in which 0 is no movement and 5 is normal walking34.

2.5.6 HISTOLOGY & IMMUNOHISTOCHEMISTRY

After terminal anesthesia by barbiturate overdose mice or rats were perfused transcardially with 4% paraformaldehyde and spinal cords processed for immunofluorescence as described19,33-35. Primary antibodies were: rabbit anti-GFAP (1:2000; Dako, Santa Clara, CA); rat anti-GFAP (1:1000, Thermofisher, Grand Island, NY); chicken anti-GFAP (1:1000, Novus Biologicals, Littleton, CO); rabbit anti NeuN (1:1000, Abcam, Cambridge, MA); rabbit anti-GDNFR-a (GDNF-receptor alpha) (1:1000, Abcam, Cambridge, MA); sheep anti-BrdU (1:300, Maine Biotechnology Services, Portland, ME); rabbit anti-HSV-TK (1:1,000, 35,39); goat anti-CD13 (1:1000, R&Dsystems, Minneapolis, MN); rabbit anti-Laminin 1 (1:100, Sigma, St.Louis, MO); rabbit anti-Fibronectin (1:500, Millipore, Burlington, MA); rabbit anti-Collagen 1a1 (1:300, Novus Biologicals, Littleton, CO); mouse anti-NeuN (1:2000, Millipore, Burlington, MA); mouse anti-CSPG40 (1:100, Sigma); rabbit anti-Brevican (BCAN) (1:300, Novus Biologicals, Littleton, CO); guinea pig anti-NG2 (CSPG4) (Drs. E.G. Hughes and D.W. Bergles41, Baltimore, MA); rat anti-PECAM-1 (1:200, BD Biosciences, San Jose, CA); guinea pig anti-homer1 (1:600, Synaptic Systems GmbH, Germany); rabbit anti-Synaptophysin (1:600,Dako, Santa Clara, CA); rabbit anti-RFP (1:1000, Rockland,Limerick,PA); chicken anti-RFP (1:500, Novus Biologicals, Littleton, CO); goat anti-GFP (1:1000, Novus Biologicals, Littleton, CO). Fluorescence secondary antibodies were conjugated to: Alexa 488 (green) or Alexa 405 (blue) (Molecular Probes), or to Cy3 (550, red) or Cy5 (649, far red) all from (Jackson Immunoresearch Laboratories). BDA tract-tracing was visualized with streptavidin-HRP plus TSB Fluorescein green or Tyr-Cy3 (Perkin Elmer). Nuclear stain: 4',6'-diamidino-2-phenylindole dihydrochloride (DAPI; 2ng/ml; Molecular Probes). Sections were coverslipped using ProLong Gold antifade reagent (InVitrogen, Grand Island, NY). Sections were examined and photographed using deconvolution fluorescence microscopy and scanning confocal laser microscopy (Zeiss, Oberkochen, Germany). Tiled scans of individual whole sections were prepared using a x20 objective and the scanning function of a Leica Aperio Versa 200 Microscope (Leica, Wetzlar, Germany) available in the UCLA Translational Pathology Core Laboratory. Composite survey images were prepared from tiled scans of multiple sections from the same animals oriented and overlaid using Imaris ® software (9.1.2 64 Bit, Bitplane, Oxford Instruments,Abingdon, U.K.).

2.5.7 AXON QUANTIFICATION

Axons labeled by tract tracing using BDA or RFP were quantified using image analysis software (NeuroLucida®, 9.14.5 32 Bit, MicroBrightField, Williston, VT) operating a computer-driven microscope regulated in the x, y and z axes (Zeiss) by observers blind to experimental conditions. Using NeuroLucida ®, lines were drawn across horizontal spinal cord sections at SCI lesion centers and at regular distances beyond (Fig. 1c, Extended Data Fig. 3a,b) and the number of axons intercepting lines was counted by observers blind to experimental conditions. Multiple sections through the middle of the cord where propriospinal axons were densest, were counted per mouse or rat and expressed as total intercepts per location per animal. To determine efficacy of axon transection after SCI, we examined labeling 3mm distal to SCI lesion centers in mice and 5mm distal to lesion centers in rats, with the intention of eliminating animals that had labeled axons at this location on grounds that these mice may have had incomplete lesions. However, essentially all mice or rats that had met the strict behavioral inclusion criterion of no hindlimb movements two days after severe crush SCI, exhibited no detectable axons 3mm or 5mm, respectively, distal to SCI lesions regardless of treatment group.

2.5.8 QUANTIFICATION OF IMMUNOHISTOCHEMICALLY STAINED AREAS

Sections stained for laminin 1, fibronectin, collagen 1a1 or CSPG were scanned using constant exposure settings. Single channel immunofluorescence images were converted to black and white and thresholded (Fig. 2a) and the amount of stained area measured in different tissue compartments using NIH Image J (1.51) software19.

2.5.9 QUANTIFICATION OF ASTROCYTE PROLIFERATION & DENSITY

To quantify astrocyte proliferation and the number of astrocytes in the immediate scar border previously defined as zone 135, we used a well characterized transgenic mouse line that expresses thymidine kinase (TK) in astrocyte cell bodies and thereby facilitating quantification of cell number and co-localization with other markers35. To quantify the proportion of newly proliferated astroglia in the scar boarder, we injected daily single doses of the cell division marker, bromodeoxyuridine (BrdU, Sigma), 100 mg/kg/day dissolved in saline plus 0.007N NaOH on days 2-7 following SCI. Newly proliferated astrocytes quantified by

determining the percentage of astrocytes stained for both GFAP,TK and BrdU in zone 135. Total astrocyte numbers in the immediate scar border (zone 1)35 were determined by counting the number of cells per defined tissue volume. Cell counts were performed using stereological image analysis software (StereoInvestigator®, 9.14.5 32 Bit, and NeuroLucida®, 9.14.5 32 Bit, MicroBrightField, Williston, VT) operating a computer-driven microscope regulated in the x, y and z axes (Zeiss).

2.5.10 DOT BLOT

For dot blot immunoassay of laminin 1, fibronectin, collagen 1 or CSPG, spinal cord tissue blocks were lysed and homogenized in standard RIPA (radioimmuno-precipitation-assay) buffer. LDS (lithium dodecyl sulfate) buffer (Life Technologies) was added to the post-mitochondrial supernatant and 2µL containing 2µg/uL protein was spotted onto a nitrocellulose membrane (Life Technologies), set to dry and incubated overnight with primary antibodies: rabbit anti-Laminin 1 (1:4000, Sigma, St.Louis, MO); rabbit anti-Fibronectin (1:7000, Millipore, Burlington, MA); rabbit anti-Collagen 1a1 (1:7000, Novus Biologicals, Littleton, CO); mouse anti-chondroitin sulfate antibody (CS-56, 1:3000, Sigma Aldrich), an IgM-monoclonal antibody that detects glyco-moieties of all CSPGs40. Immunoreactivity was detected on X-ray film with horse radish peroxidase (HRP)-conjugated secondary antibody (1:5000) and chemiluminescent substrate (ThermoFisher, Grand Island, NY). Densitometry measurements of immunoreactivity were obtained using ImageJ software (NIH) and normalized to total protein (Ponceau S) density43. Raw images of dot blots are provided as Supplementary figure 1.

2.5.11 ISOLATION & SEQUENCING OF RNA FROM ASTROCYTES AND NON-ASTROCYTE CELLS

Using mice expressing an mGFAP-RiboTag transgene, RNA was evaluated as previously described19 from uninjured mice, and mice two weeks after SCI after treatment with hydrogel depots that either contained no cargo (empty depots) or delivered FGF+EGF (Extended Data Fig. 1a). Briefly, spinal cords were rapidly dissected out of the spinal canal and the central 3mm of the lower thoracic lesion including the lesion core and 1mm rostral and caudal were rapidly removed and snap frozen in liquid nitrogen. Hemagglutinin (HA) immuno-precipitation (IP) of astrocyte ribosomes and ribosome-associate mRNA (ramRNA) was carried out as described44. The non-precipitated flow through (FT) from each sample was collected for analysis of non-astrocyte total RNA. HA and FT samples underwent on-column DNA digestion using the RNase-Free Dnase Set (Qiagen) and RNA purified with the RNeasy Plus Micro kit (Qiagen). Integrity of the eluted RNA was analyzed by a 2100 Bioanalyzer (Agilent) using the RNA Pico chip, average RIN = 7.9 + 1.4. RNA concentration determined by RiboGreen RNA Assay kit (Life Technologies). cDNA was generated from 5ng of IP or FT RNA using the Nugen Ovation® 2 RNA-Seq Sytstem V2 kit (Nugen). One µg of cDNA was fragmented using the Covaris M220. Paired-end libraries for multiplex sequencing were generated from 300 ng of fragmented cDNA using the Apollo 324 automated library preparation system (Wafergen Biosystems), enriched over 10 cycles of PCR cycles and purified with Agencourt AMPure XP beads (Beckman Coulter). All samples were analyzed by an Illumina NextSeq 500 Sequencer (Illumina) using 75-bp pair-end sequencing. The number of reads obtained are between 38.0 to 71.0M (avg 52.1M). Sequences were aligned to mouse mm10 genome using STAR aligner (2.4.0j). Uniquely aligned reads were between 57.1 to 87.0% (avg 73.9%). Read counts were determined using HT-seq (0.6.0). Differential expression analysis was conducted using Bioconductor EdgeR package (3.20.1) after removal of low count genes (5 counts for at least two samples). Three samples from astrocyte samples were excluded due to low RIN values (<5.7), no samples were excluded for non-astrocyte samples.

2.5.12 RAT ELECTROPHYSIOLOGY

Terminal electrophysiological assessments were carried out as previously described45 four weeks after SCI. Briefly, animals were anesthetized with urethane (1.5 g/kg; i.p.) and core body temperature was maintained at 37°C using a self-regulating heated pad connected to a rectal probe. Depth of anesthesia was continually monitored by assessing withdrawal reflexes and respiratory rate. After laminectomy to expose the injury site and 7mm rostral and caudal to the lesion site by removal of one vertebra on either side of the injury, the dura was removed, and the exposed spinal cord was covered with warm mineral oil to prevent drying. For stimulation, a tungsten bipolar concentric electrode was positioned intraspinally at the rostral-most point of the laminectomy. A silver ball electrode was used to record any evoked activity from the surface of the exposed spinal cord at various locations (above, 2mm below, and 5mm below lesion). Stimulation was delivered in 200 µs square wave pulses at the maximum amplitude possible before large motor responses were evoked (typically between 600 µA and 800 µA) and at a frequency of 0.75 Hz using a STG 4004 stimulus generator (Multi Channel Systems). Evoked activity was

amplified and recorded using an A-M systems differential amplifier, PowerLab and LabChart Pro acquisition and analysis system (AD Instruments). For analysis, 30 traces from each recording site were averaged and the peak to peak amplitude of the evoked potential was quantified.

2.5.13 RAT NEUROREHABILITATION PROCEDURES

After surgery, rats recovered for 7 days before neurorehabilitation began. Locomotor neurorehabilitation was conducted bipedally on a treadmill or overground with vertical robotic support. Standing and stepping enabling agents were used as facilitators and associated epidural L2 and S1 tonic stimulation and administration of pharmacological agents as a combination of quipazine (5-HT2A/C, 0.2-0.3 mg/kg) and 8-OH-DPAT (5-HT1A/7, 0.05-0.2mg/kg). The tasks were performed on a daily basis for approximately 30 minutes per day, over a period of 8 weeks. Epidural stimulation was delivered tonically at 40 Hz with a pulse duration of 0.2ms, injecting an intensity of current chosen individually for each animal depending on the observed muscle twitch tresholds (ca. 100-300uA). A robotic body weight support system was used to allow rats to adopt a comfortable bipedal or quadrupedal position. Step training is conducted using sophisticated robotic systems developed at the University of California, Los Angeles (Robomedica, Inc) and at the EPFL. The animal was restrained in a comfortable harness that was attached to a half-tube at one end of the body weight support device. A robotic control system counterbalanced inertia and enabled to determine precisely the amount of weight that the subject had to carry.

2.5.14 BEHAVIORAL ASSESSMENTS

Assessments of functional recovery were conducted during bipedal and quadrupedal locomotion overground. The bipedal task was supported by our robotic interface (Robomedica, Inc, UCLA) that delivers adapted bodyweight support. Both tasks were pharmacologically enabled with quipazine (5-HT2A/C, 0.2-0.3 mg/kg) and 8-OH-DPAT (5-HT1A/7, 0.05-0.2mg/kg), and complimentary L2-S1 tonic stimulation (40Hz, 0.2ms, 100-300uA) was delivered. Retro-reflective markers were placed on the relevant joins (iliac crest, hip, knee, tarse, toe) to enable locomotion tracking and motion analysis. A system of 12 infrared cameras (Vicon motion analysis) was used to capture recordings of the motor tasks. Runs were recorded and a three-dimensional reconstruction of the movements was obtained, in order to analyze gait parameters.

2.5.15 STATISTICS, POWER CALCULATIONS, GROUP SIZES & REPRODUCIBILITY

Statistical evaluations of repeated measures were conducted by one-way ANOVA with post hoc independent pair wise analysis as per Bonferroni, or by Student's t-test (Prism®, 7.0c, GraphPad, San Diego, CA). For one-way ANOVA statistical evaluations, F-values are reported in the format F(df1, df2)=X. The degrees of freedom are computed as, df1=k-1, where k is the number of compared treatments and df2=n-k where n is the total number of samples across the treatment groups. For Student's two-tailed t-test (Prism®, 7.0c, GraphPad, San Diego, CA), t value and degrees of freedom (df) are reported in the format t(df)=X. Power calculations were performed using G*Power Software V 3.1.9.242. For quantification of histologically-derived neuroanatomical outcomes such as numbers of axons or percent of area stained, group sizes were used that were calculated to provide at least 80% power when using the following parameters: probability of type I error (alpha) = .05, a conservative effect size of 0.25, 3-10 treatment groups with multiple measurements obtained per replicate. All graphs show mean values plus or minus standard error of the means (S.E.M.) as well as individual values as dot plots. All bar graphs are overlaid with dot plots where each dot represents the value for one animal to show the distribution of data and the number (n) of animals per group. Files of all individual values are provided as source data. The main experiments testing propriospinal axon regrowth across SCI lesions in animals treated with SCI+AAV-OIC+2D+FGF+EGF+GDNF and the main control groups (SCI+2D+FGF+EGF+GDNF, SCI+1D-empty) were repeated independently three times in mice and three times in rats with similar results. Other experiments testing propriospinal axon regrowth across SCI lesions in animals in additional control groups (SCI+AAV-OIC+1D-empty, SCI+AAV-OIC+1D+FGF+EGF+GDNF, SCI+AAV-shPT+2D+FGF+EGF+GDNF, SCI+AAV-OIC+1D+FGF+EGF, SCI+1D+FGF+EGF+GDNF, SCI-only) were repeated independently at least twice in mice with similar results.

Chapter 3

THALAMOCORTICAL NEUROMODULATION

RESTORING SOMATOSENSORY FEEDBACK TO CLOSE THE LOOP

3.1 ABSTRACT

Required mechanisms have been identified for robust and excitable axon regeneration across complete spinal cord injuries. However, we hypothesize that the persistent interruption of any somatosensory feedback to the motor cortex contributes to the absence of spontaneous functional benefits in spite of propriospinal regrowth. Restoring such feedback would enable directional control and recruit the cortical and subcortical structures involved in motor planning and motor learning. While mechanisms of sensory axon regrowth are quite well documented (Anderson et al., 2016; Kadoya et al., 2009; Lu et al., 2004), functional regeneration of the sensory system remains at the moment irrealistic. Intra-spinal sensory relays have yet not been identified and precise long-distance guidance of regenerating afferent to forebrain structures remains technically challenging. Evidence suggests that rapid somatosensory feedback to the motor cortex (M1) - integrated with outputs from the basal ganglia and from the cerebellum - occurs via rostral thalamic nuclei (Lemon and van der Burg, 1979; Gao et al., 2018; Anderson and DeVito, 1987). The motor thalamus has recently been shown as a key structure for motor learning (Tanaka et al., 2018), suggesting the rostral thalamic nuclei as potential targets for somatosensory neuromodulation after spinal cord injury. Following the concepts of a brain-spine-interface that restored wireless supraspinal control over SCIs (Capogrosso et al., 2016; Bonizzato et al., 2018), we propose here to design a muscle-brain-interface to restore thalamo-cortical feedback to M1 when muscle activity is detected. During neuroprosthetic rehabilitation after complete spinal lesion, thalamic neuromodulation triggered by automated stepping may cause cortical reorganization and promote the recruitement of regenerated axon bridges.

PERSONAL CONTRIBUTIONS

In this promising yet preliminary study, I - Sabry L. Barlatey - contributed to design experiments and piloted the feasibility of the project. I performed stereotaxic brain injections, implanted optic fibers for optogenetic modulation, took part in acute electrophysiological recordings, performed surgical spinal cord injuries and contributed to all other surgeries. I designed and contributed to behavioral training and assessment, both in healthy conditions and after spinal cord injury. I processed CNS tissue, performed histological analyses and acquired microscopy images. I supervised the analysis of chronic cortical recordings. I wrote the chapter presented here below and conceived the figures.

3.2 INTRODUCTION
CLOSING THE LOOP

Regenerative approaches have been able to restore electrophysiological conduction of information across complete SCIs (Anderson et al., 2018; Lu et al., 2012c). However, functional recovery of hindlimb movements has remained absent or insufficiently robust. We hypothesize that such a lack of behavioral benefits can arise either i) from an irrelevant integration of lesion-bridging axons within caudal circuits, ii) from an insufficient supraspinal control across the injury or iii) from a lack of sensitive feedback to the volitional locomotor centers. As we successfully propelled propriospinal axons across complete lesions (Anderson et al., 2018), current paradigms have been showing that functional reorganization of circuits can be achieved with neuroprosthetic rehabilitation (van den Brand et al., 2012; Asboth et al., 2018). However, combining propriospinal regrowth and neurorehabilitation still fails to promote motor recovery (see2.3.6), suggesting that additional input - either downstream control or upstream feedback - needs to be restored.

As dense propriospinal regrowth and rewiring is expected to provide robust and relevant substrate for downstream control after injury (Courtine et al., 2008; Weidner et al., 2001; Rosenzweig et al., 2010), we explored options to add relevant sensory feedback to the motor cortex after complete SCI. Early studies in cats suggest that the rostral thalamus - and especially the nuclei ventralis lateralis (VL) and ventroposterior lateralis (VPL) - acts as a relay for rapid somatosensory input to the motor cortex (M1) (Mackel and Miyashita, 1992, 1993; Lemon and van der Burg, 1979). Further, thalamo-cortico-thalamic loops of motor control were unveiled, that are modulated by inputs from the cerebellum, from the basal ganglia and from the hypothalamus (Anderson and DeVito, 1987). Recently, motor control has been shown in rodents to arise both from frontal cortical regions and cerebellar nuclei. A co-dependance between the frontal cortex and the cerebellum was demonstrated to maintain motor preparatory activity in both structures, and a thalamic relay was confirmed on the cerebello-cortical output (Gao et al., 2018). Further dissecting the thalamo-cortical interactions, it is now established that a significant population of axons originating in the VL thalamic nucleus reach directly for pyramidal neurons in the primary motor cortex (Yamawaki and Shepherd, 2015; Hooks et al., 2013; Tanaka et al., 2018).

Beyond relaying somatosensory, striatal and cerebellar inputs towards the primary motor cortex during motor tasks, rostral thalamic areas are also involved in motor learning (Tanaka et al., 2018). Afferent from the dorsal striatum and from the deep cerebellar nuclei recruit thalamo-cortical projections and shape layers L1 and L3 of the primary motor cortex during motor learning(Tanaka et al., 2018). Functional subdivisions of the motor thalamus have been anatomically established with a first area relaying basal ganglia input to layer L1 in M1 (Kuramoto et al., 2015; Arbuthnott et al., 1990; Rubio-Garrido et al., 2009) and a second region relaying cerebellar input to layers L3 and L5 (Shigematsu et al., 2016; Kuramoto et al., 2009). As rostral components of the motor thalamus appear to exert inhibitory activity on the motor cortical layer L1, the caudo-lateral glutamatergic portion tends to project to deeper layers Kuramoto et al. (2009). This thalamocortical circuit organization clearly suggests that the motor thalamus provides M1 with both positive and negative feedbacks, depending on the circumstances. However, the precise interplay of somatosensory afferent within the complex cortico-subcortical motor circuitry remains incompletely understood.

As the rostral thalamus acts as a key structure for multiple input integration and feedback to the motor cortex, we propose here to appose optogenetic neuromodulation to the motor

thalamus after complete SCI. When correctly timed with automated muscle activity, such technology would enable the restoration of locomotion-based feedback to M1. Further neuroprosthetic rehabilitation combining axon regeneration and motor thalamic neuromodulation can potentially allow M1 to reorganize reticulospinal relay neurons (Asboth et al., 2018) and regenerated propriospinal axons (Anderson et al., 2018; Courtine et al., 2008) into functional motor circuits.

3.3 RESULTS
THALAMOCORTICAL RECRUITMENT AFTER COMPLETE SCI

3.3.1 CRUSH SCI INTERRUPTS PERIPHERAL FEEDBACK TO THE MOTOR CORTEX

In order to confirm somatosensory input to the M1 region, our first interest was to test electrophysiologically the primary motor cortex response to peripheral and spinal electrical stimuli. In naive healthy rats anesthetized under urethane, we successfully evoked somatosensory responses in the motor cortex by stimulating either i) the contralateral sciatic nerve or ii) the dorsal columns at a lumbar level (Fig 3.1e-f). We measured a delay of response included in a range from 10ms to 12ms when stimulating respectively the dorsal columns and the sciatic nerve. As direct conduction (ca. 10 [m/sec]) would tend to elicit faster evoked potentials, the measured latencies suggest that evoked responses are indirect and involve chemical neurotransmission. During the same procedure, we performed a thoracic T10 crush SCI. After resolution of the acute spinal shock, sciatic nerve and dorsal column stimulation were repeated and showed a complete abolition of all any somatosensory response in M1. Sciatic stimulation still induced a fast afferent response at the caudal border of the lesion, whereas no evoked activity could be elicited above the SCI, confirming the complete interruption of lumbosacral input with a crush model of spinal cord injury (Fig 3.1f).

3.3.2 LUMBOSACRAL CIRCUITS PROVIDE DIRECT FEEDBACK TO THE ROSTRAL THALAMUS

Next, we sought to identify potential targets for feedback neuromodulation after spinal cord injury. Our first interest was to determine the somatosensory targets that are afferented by lumbosacral locomotor centers in rats. We injected an AAV-DJ-hSyn-mGFP-synaptophysin-mRuby viral vector to label all anterogradely all ascending tracts from the lumbosacral L1 to S1 spinal levels (Fig 3.1a). The viral technology involved enabled in addition the labelling of synapses within the afferented regions. Broad anatomical analysis unveiled four somatosensory relays : i) the rostral thalamus (Fig 3.1b4-c-d), ii) the periaqueductal gray matter, iii) the lateral parabrachial nucleus, iv) diffuse relay neurons in the brainstem (Fig 3.1b1-3). The periaqueductal gray matter has been shown as a key structure in pain- and fear-related neuronal circuits (Basbaum and Fields, 1978; Qi et al., 2018), while the lateral parabrachial nucleus has been associated to sleep/wake behavior and metabolic functions (Kroeger et al., 2017; Yahiro et al., 2017). Amongst all four somatosensory relays, the motor thalamus appeared as the more promising target for neuromodulation, due to its know interplay with the M1 area.

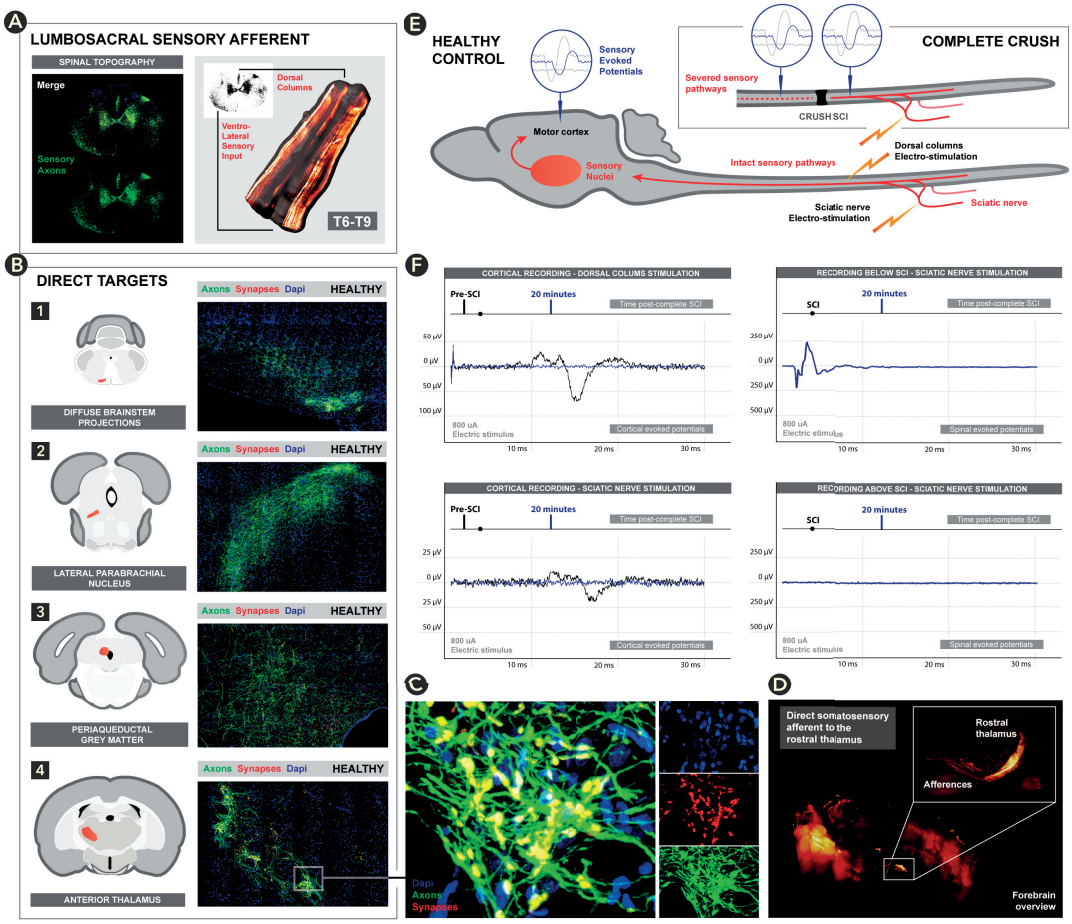

Figure 3.1: **Lumbosacral afferent reach brainstem and thalamic relays, and provide rapid somatosensory feedback to the motor cortex.** **A**. CLARITY overview and cross-section topography of somatosensory afferences at a thoracic spinal cord level. Axon labelling with AAV-DJ-hSyn-mGFP-synaptophysin-mRuby reveals somatosensory afferences (green) located in the dorsal columns and in the ventrolateral white matter. **B**. Brainstem and forebrain analysis of primary relays show multiple somatosensory relays. **B1**. Diffuse axon terminations in the brainstem. **B2**. Projections to the lateral parabrachial nucleus. **B3** Dense axon termination matrix in the periaqueductal grey matter. **B4**. Somatosensory relay in the rostral thalamus. **C**. Confocal aquisition at a higher magnification (20x) of lumbosacral projections to the rostral thalamus in B4 showing GFP labelling of axons (green), mRuby labelling of presynaptic buttons (red) and nuclei staining with Dapi. **D**. CLARITY aquisition of the forebrain somatosensory projections, with a higher magnification confirming direct axonal afferences to the rostral thalamus. **E**. Electrophysiological design. Electrical stimuli were applied to the sciatic nerve and to the dorsal columns. Recordings were performed with a silver ball electrode placed on the motor cortex and on the dorsal columns, before and acutely after complete SCI. **F**. Electrophysiological traces confirmed motor cortical responses to electrostimulation before injury, that were minimally delayed from sciatic nerve stimulation compared to dorsal colum stimulati. Acute recordings after complete thoracic T10 SCI showed a complete abolition of motor cortex responses to the infralesional stimuli. With sciatic nerve stimulation, lumbosacral responses were preserved after SCI, while thoracic T8 responses were abolished, confirming an interruption of conduction at the injured level.

3.3.3 THALAMO-CORTICAL PROJECTIONS CAN BE RECRUITED AFTER COMPLETE CRUSH SCI

As we confirmed direct innervation of the motor thalamus by somatosensory lumbosacral axons, we next explored anatomically the thalamocortical projections that originate in the rostral thalamus. To do so, retrograde AAV9-Cre was injected throughout the motor cortex and the red anterograde viral tracer AAV8-flex-rComet was delivered to the motor thalamus. As expected, direct thalamic afferent to the layer L5 of the motor cortex were observed (Fig 3.2c, Fig 3.2d1). Acute optogenetic recruitment of these thalamo-cortical axons confirmed the motor thalamic input into M1. Relying on the retrograde AAV9-Cre viral vector administered into the motor cortex in healthy rats, we selectively transduced Channelrhodopsin-2 (ChR2) into motor thalamo-cortical neurons. Under terminal and acute anesthesia with urethane, we stimulated optically the rostral thalamus and recorded evoked field potentials from the motor cortex using a silver ball electrode (Fig 3.2a). Single optic pulses (10 [ms], 20 [ms], 30 [ms], 40 [ms]) successfully elicited cortical responses, that appeared as stereotypical biphasic evoked potentials involving a short early response and a second broader potential. Interestingly, variations of the light pulse duration did not interfere with the length of the cortical response (Fig 3.2d2). However, variations of the light intensity appeared to impact the latency of the cortical response without interfering with its amplitude (Fig 3.2d3). We next tested if a somatosensory deafferentation of the motor thalamus causes acute modifications on the thalamo-cortical feedback. During the same procedure, a complete T10 crush injury was performed, and thalamocortical optic stimulations and recordings were repeated 10, 30, 45 and 60 minutes post-injury. An acute and sustained post-injury CNS shock strongly diminished the amplitude of the cortical evoked responses, which started to recover progressively between 45 and 60 minutes post-SCI (Fig 3.2d4). Acutely after complete SCI, thalamo-cortical projections appear to remain functional and available for neuromodulation after complete SCI.

3.3.4 CHRONIC NEUROMODULATION OF THALAMO-CORTICAL PROJECTIONS

As we confirmed that thalamocortical afferent can selectively be recruited using optogenetic tools, we further investigated the feasibility and effects of rostral thalamic stimulation during locomotion. Again, AAV9-Cre was injected into the motor cortex and AAV-Flex-ChR2 was infused into the motor thalamus. Eight weeks later and during a single surgery, rats received i) an optic fiber on the ChR2-expressing thalamus, ii) a 32-channel micro-electrode array in the motor cortical layer L5, and iii) electromyographic electrodes in muscles tibialis anterior (TA) and medial gastrocnemius (MG) (See Fig 3.3a). In healthy conditions and during steady walking on a treadmill, we first applied single optic pulses at a 3Hz frequency on the motor thalamus. This gait-independent stimulation pattern aimed at recognizing stereotypical responses in M1 following optic stimulation. Treadmill locomotion was chosen as it enables long periods of cyclic and stable cortical activity. Neural data was filtered, and firing rate per electrode was computed and averaged over a 50ms period, starting from the beginning of the stimulus. A stereotypical increase in the firing rate (ca. 20ms) was observed starting from the beginning of the stimulus, directly followed by a depression in the overall motor cortical activity (ca. 20ms) (See Fig 3.3b). We compared this response to representative 50ms of non-stimulated activity, extracted from the same recording as an average between all 50ms intervals without thalamic stimulation. The averaged firing rate without stimulation did not show any stereotypical M1

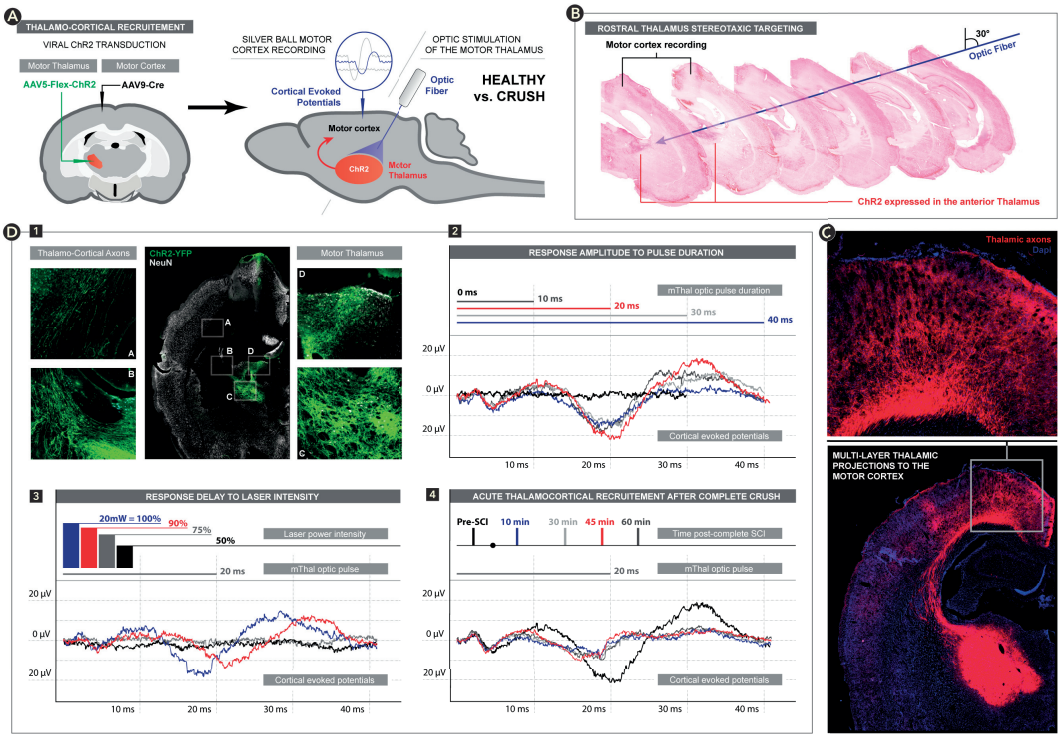

Figure 3.2: **Direct projections from the rostral thalamus can be selectively recruited to evoke afferent potentials in the primary motor cortex.** **A**. Experimental design. AAV-Flex-Channelrhodopsin2 was injected into the rostral thalamus for selective expression of ChR2, dependent on the retrograde AAV9-Cre delivered to the primary motor cortex. A custom optic fiber was implanted with a 30° angle on the rostral thalamus and cortical evoked potentials were acutely recorded under terminal anesthesia with a silver ball electrode. **B**. Histological sections showing the tract followed by the optic fiber onto the rostral thalamus. **C**. Anatomical exploration of direct thalamic afferent to multiple layers in the motor cortex. Selective AAV-Flex-rComet was delivered into the rostral thalamus, relying on a retrograde AAV9-Cre delivered to the motor cortex. **D**. Acute electrophysiological recruitement of thalamocortical projections. **D1**. Histological expression of ChR2 in the rostral thalamus with axonal projections to the motor cortex. D1A and D1B show the ChR2-YFP expressing thalamocortical projections, while D1C and D1D show the optogenetically targeted somas located in the rostral thalamus. **D2**. Recordings from the motor cortex with silver balls electrodes, correlated to the length of the single optic pulses delivered to the rostral thalamus. Biphasic responses were observed, with a maximal amplitude correlated to 20ms pulses. 100% of the laser power (ca. 30mW) was applied. **D3**. Variations in the delay of response for the second phase of the biphasic cortical response, depending on the laser intensity. **D4**. Responses in the motor cortex observed at 10, 30, 45 and 60 minutes post-complete crush SCI. As the first component of the response was preserved, a recovery of the second component took place from 45 minutes post-SCI on, while the CNS shock was resolving.

activity, confirming that the cortical response is caused by optogenetic stimulation of thalamo-cortical afferent. Interestingly, 3Hz stimulations with pulse durations of 10ms or 20ms elicited similar cortical modulations, causing in both cases a primary 20ms rise in activity, followed by a 20ms refractory phase.

Still in healthy conditions, we then sought to link optic stimulation in the motor thalamus with hindlimb activity. Real-time acquisition of EMG activity in the tibialis anterior muscle was computed and a detection threshold was set at 60% of the mean TA peak-activity. EMG detection then triggered thalamic optic stimulation during steady locomotion on a treadmill. Concomitant neural data recordings were computed and compared between runs, either without stimulation, with 20Hz stimulation, or with 40Hz thalamic stimulation. Based on our previous gait-independent observations, the pulse duration was set at 10ms to avoid tetany at a 40Hz frequency. We then extracted and averaged the neural activity during the trains of stimuli, or over the corresponding time intervals on control runs without stimulation. Stereotypical activity was observed in the three conditions, that - we assume - corresponds to the alternation between volitional flexion and extension. However, thalamocortical stimulation appeared to pace the M1 activity, as a clear synchrone refractory period follows the first stimulus on most of the channels, in both 20Hz and 40Hz optic stimulation conditions. Such synchrone depression in the firing rate was not observed in the non-stimulated control runs (See Fig 3.3c).

As no macroscopic modification of the healthy gait pattern was observed with motor thalamic stimulation, we confirmed here that gait-dependent thalamo-cortical recruitment can take place during locomotion. Gait-Independent stimulations cause a synchrone increase in the motor cortex layer L5, that is conserved when stimulation is linked to locomotion. The M1 modulations caused by thalamic stimuli share conserved patterns between the different stimulation patterns that we tested. For further applications in post-SCI conditions, we propose EMG-linked thalamocortical stimulation, with 10ms pulses delivered at a 20Hz frequency.

3.3.5 RESTORING SOMATOSENSORY FEEDBACK AFTER COMPLETE SCI

In order to further establish the somatosensory neuroprosthetic paradigm, animals implanted with a motor cortical TDT array, an ipsilateral rostro-thalamic optic fiber and EMG electrodes received a complete crush spinal cord injury. Functional training for bipedal treadmill stepping took place daily for two weeks, until automated spinal walking was restored. In order to restore infralesional functionality of circuits during training, monoaminergic drugs were associated to electric epidural simulation applied to the lumbosacral levels L2 and S1. Neurophysiological assessments were repeated as described under 3.3.4, and cortical activity was compared in the presence or absence of rostrothalamic neuromodulation linked to TA electromyographic detection. Similarly to the cortical pacing observed in healthy conditions, trains of stimulation successfully restored feedback to M1, recognized with a presence of synchrone neuronal firing, followed by synchrone cortical depression (See Fig 3.4). These observations confirm the availability of thalamocortical projections following complete SCI, and offer the perspective of combinations with regenerated descending axon tracts, in order to close a functional loop between motor and somatosensory inputs during locomotion after complete spinal lesion.

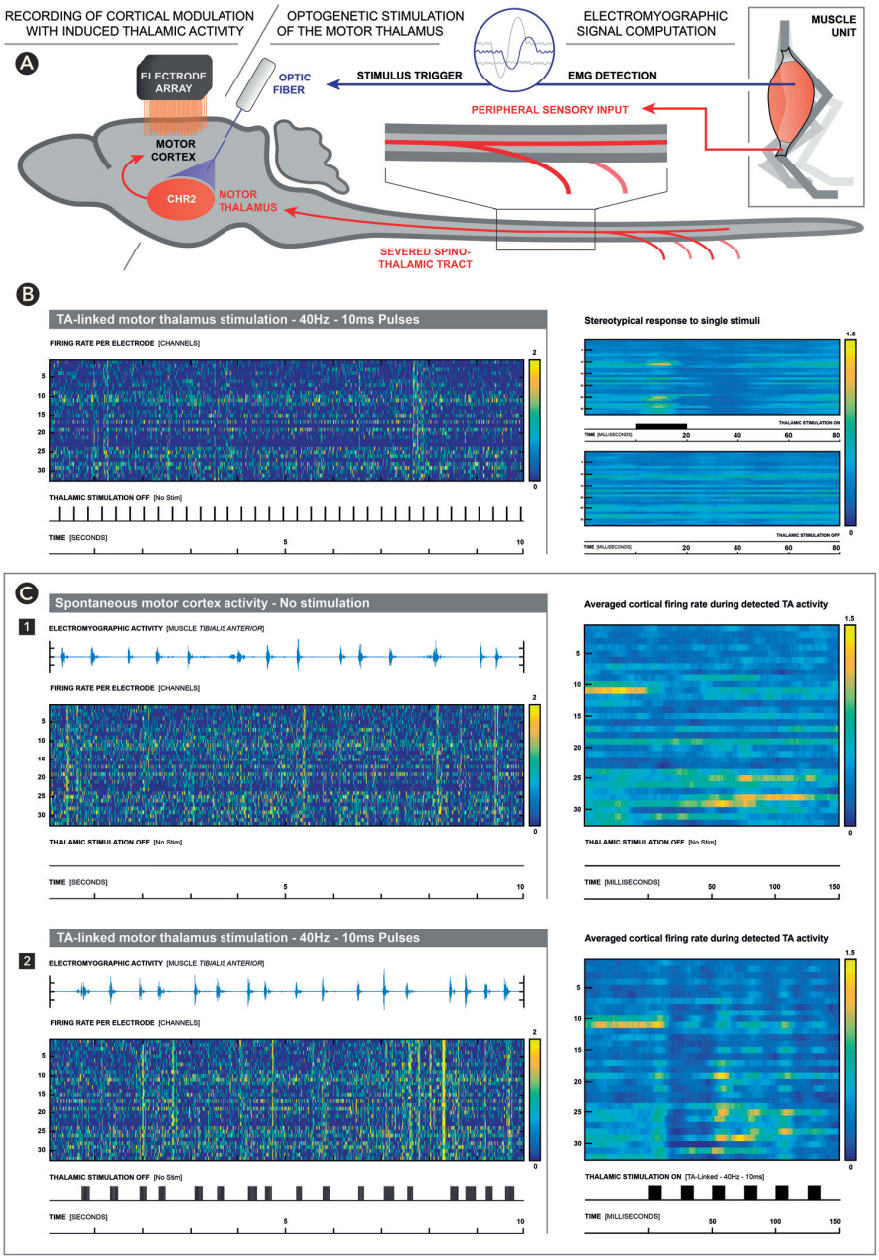

Figure 3.3: **Rostral thalamus stimulation paces the motor cortex during normal healthy walking.** **A**. Experimental design. Thalamocortical projections are selectively activated by optogenetics, in an electromyographic dependent manner during normal walking. **B**. [Left] Continuous 3Hz stimulation was delivered during steady treadmill walking and neural activity was recorded. A spike detection software extracted neuronal firing at each electrode of the TDT array. The firing rate was calculated using a sliding window of 20ms. [Right] Neuronal firing was isolated at all optic stimulations and averaged, revealing a stereotypical modulation of M1 following motor thalamus activation. This synchrone initial increase was followed by a synchrone depression in neuronal activity. **C**. Recordings were obtained without thalamocortical stimulation, or with optic stimulation timed with detected electomyographic activity in the muscle tibialis anterior **C1**. Without stimulation,the averaged M1 firing rate per electrode follows a continuum with stereotypically increased activity on selected channels, coordinated in time with TA activity. **C2**. Superposed to basic cortical activity linked to locomotion, optic trains of stimulation in the rostral thalamus induce synchrone neuronal firing, followed by synchrone depressions in firing rate. Overall, this data demonstrates our ability to induce feedback in M1 using rostral thalamus neuromodulation.

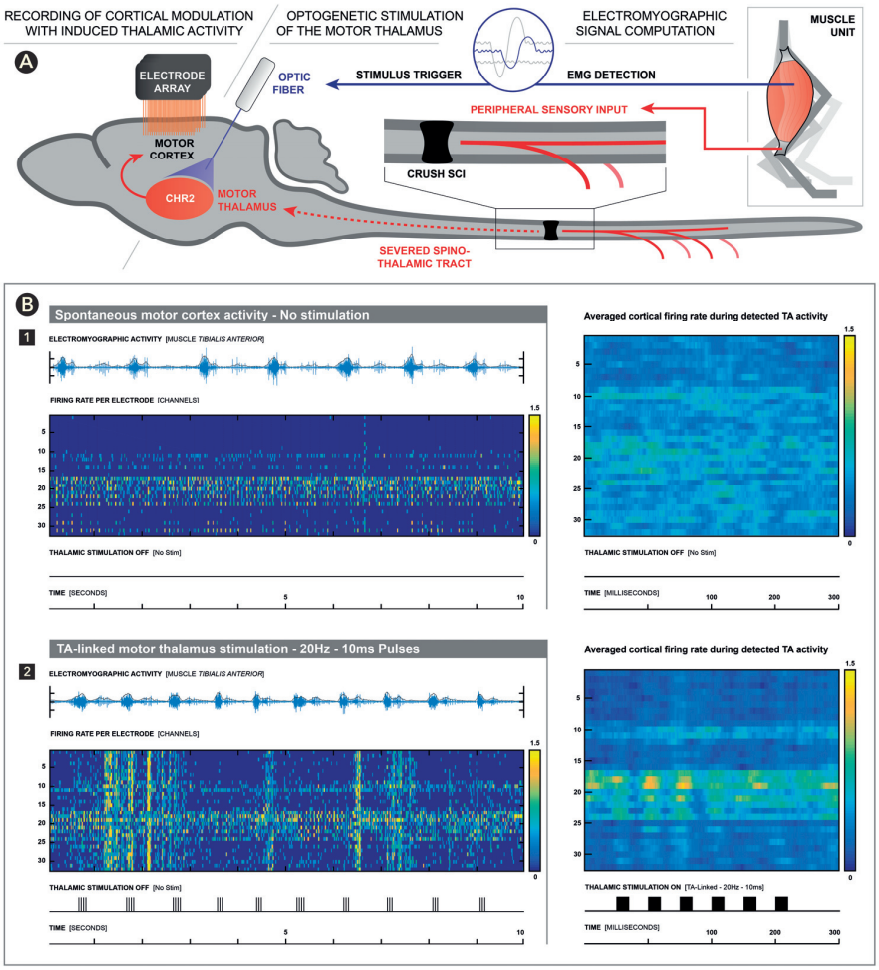

Figure 3.4: **Thalamic neuromodulation evokes gait-related cortical activity after complete SCI.** A. Experimental design. Thalamocortical projections are selectively activated by optogenetics after complete SCI, in an electromyographic dependent manner, during automated stepping on a treadmill. **B**. Cortical recordings were acquired with or without optic stimulation, timed with detected electomyographic activity in the muscle tibialis anterior (TA). Note that channels 17 to 24 show activity that is comparable to pre-SCI conditions. Other channels were likely silenced over time due to well-known progressive fibrosis around the electrodes. **B1**. Without stimulation, the averaged M1 firing rate does not show any stereotypical pattern during TA activity, as post-SCI stepping is automated and spinal. **B2**. After SCI, optic trains of stimulation in the deafferented rostral thalamus still induce synchrone neuronal firing in M1, followed by synchrone depressions in firing rate. These observations demonstrate that EMG-based thalamic neuromodulation is able to provide properly timed somatosensory feedback to the motor cortex above complete spinal cord injuries.

3.4 DISCUSSION
SPECIFIC THALAMOCORTICAL FEEDBACK

The promising data that we demonstrate in the present chapter remain for now preliminary. Nevertheless and beyond previous literature, our anatomical tracings, our electrophysiological experiments and our successful neuromodulation of thalamocortical projections suggest that the rostral thalamus may become a relevant target for restoring feedback in M1 after complete spinal cord injury.

In healthy conditions, no macroscopic behavioral modification was observed from stimulating optically the motor thalamus. However, broad analysis of cortical neural activity demonstrates that the applied stimuli exert a pacing activity on the motor cortex. In healthy conditions, the absence of functional effects can derive from different other afferent sources involved in motor planing and motor control. While a clear enhancement in cortical firing rate was observed as a response to rostral thalamic stimulation, the depression that follows the initial response is extended beyond the expected refractory period of neurons (ca. 1-3ms). Interplays between M1 and the sensory cortex, the premotor cortex and/or other subcortical structures can contribute to inhibit functional effects of thalamocortical recruitment, and prevent macroscopic modulations of the gait cycle.

As a priority, we intend in a near future to combine thalamocortical neuromodulation - applied as described in the result section above - with propriospinal axon regeneration (Fig 3.5). This procedure would theoretically enable to close the loop of afferent and efferent inputs between the motor cortex and infralesional circuits. As previous work in severe incomplete injuries has shown recovery of function from propriospinal sprouting (Courtine et al., 2008; van den Brand et al., 2012), we hope to obtain with this strategy a conduction of cortical activity to the infralesional circuits after complete SCI, and potentially muscle responses. However, we need as a preamble to demonstrate electrophysiologically that thalamocortical stimulation is able to elicit responses in the healthy spinal cord, in order to hypothesize a potential recruitment of propriospinal neurons 3.3.4.

The motor cortex has been shown to contribute to restoring movement after SCI (Asboth et al., 2018; van den Brand et al., 2012; Liu et al., 2017). One could propose, instead of inducing thalamo-cortical modulation, to directly stimulate M1 in phase with muscle activity in order to provide feedback to the primary motor cortex. However, stimulating M1 would generate whole cortical column activity and directly recruit cortico-spinal projections. Rational for targeting the rostral thalamus relies in its integration of multiple afferent, computed into fine output to specific layers of the cortex. Beyond activation of the corticospinal tract, thalamocortical neuromodulation may enable reorganization of layer-dependent functions, in a manner that can mimic motor learning. In order to reinforce this rational and as the mapping of entering inputs to the motor thalamus remain - to our knowledge - incomplete, exhaustive anatomical and functional dissection of these inputs is required. Retrograde viral vectors delivered to the rostral thalamus will enable whole CNS anatomical analysis, in order to identify the structures involved in thalamocortical activation and inhibition. Progress has been reported in our understanding of subcortical and cerebellar interplays controlling motor functions (Gao et al., 2018; Tanaka et al., 2018). In order to fulfill this understanding, studies based on *in vivo* calcium imaging, behavioral assessments, and optogenetic modulations of thalamic afferent can demonstrate finely the relevant inputs that modulate motor functions and link specific cortical modulations to each source of input (e.g. somatosensory, cerebellar, striatal). Subsequently, functional mapping of the rostral thalamus itself and functional dissection of its inputs to M1 will enable

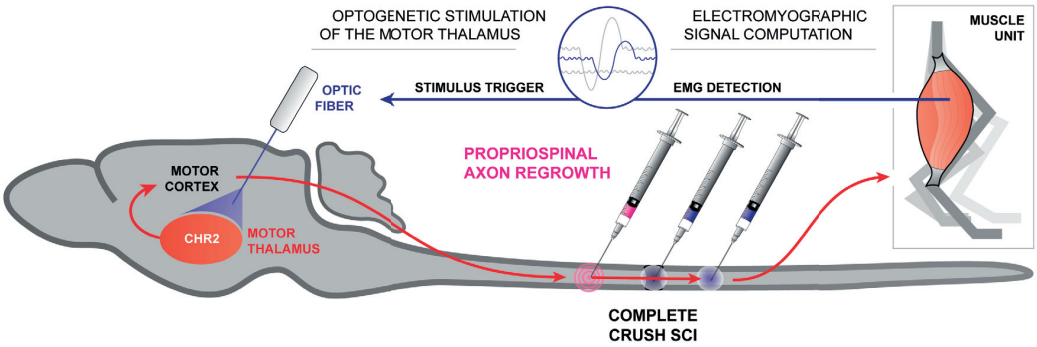

Figure 3.5: **Propriospinal axon regrowth combined with motor thalamus neuromodulation.** IGF1-CNTF-Osteopontin, lesion remodeling and propriospinal axon guidance will be applied after complete crush spinal cord injury. Thalamocortical optogenetic neuromodulation will be implemented in order to provide M1 with somatosensory feedback over the lesion. Neuroprosthetic training, enabled with electric epidural stimulation, monoaminergic pharmacology and a robotic interface will assess the functional benefits induced by this combined sensorimotor strategy.

targeted neuromodulation of selected neuronal populations that project to the motor cortex, depending on the wanted modulations (e.g. direct corticospinal firing, motor planning, motor learning reinforcement).

Our primary aim in the current pipeline is to restore thalamus-dependent feedback from the lumbosacral circuits to the motor cortex. However, the sensory modality of this feedback (i.e. proprioceptive, touch or pain) remains uncertain. Retrograde anatomical tracing from the motor thalamus would enable topographic analysis of the thalamic afferent in the spinal cord, in order to confirm the type of sensory input. As rapid somatosensory feedback to M1 has been demonstrated in early studies, our tracings and acute electrophysiological recordings suggest potential monosynaptic afferent from lumbosacral circuits to the motor cortex. Herpes viral technologies - that enable anterograde labelling of axon tracts and their targets by jumping one synapse - will test this hypothesis in a short term. Currently, the protocol applied in the present chapter recruits all outputs from the rostral thalamus to M1. Doing so, we recruit - along with lumbosacral, striatal and cerebellar targets - neurons that receive afferent from cervical and thoracic segments. In order to selectively recruit somatosensory feedback associated to hindlimb locomotion, sophisticated viral combinations may enable to recognize the specific afferent contribution of lumbosacral circuits. As an example, Herpes viral vectors carrying Cre could be delivered to the lumbar and sacral cord, while a Flex-Channelrhodopsin2 vector is administered into the motor cortex. An optic implant in the rostral thalamus would then only recruit neuronal targets of lumbosacral somatosensory axons.

In order to provide coherent feedback to M1, we need to map the topography of activity in the rostral thalamus during gait, in order to recognize potential hotspots respectively associated to hindlimb flexion and extension. Calcium imaging - a technic that enables live microscopic visualization of neuronal firing with an implanted lens - would provide mapping information during normal walking in healthy animals. This method would require the infection of lumbosacral circuits with a non-G-deleted rabies vector carrying Ca2+ dependent fluorescent markers. Such functional mapping would enable real-time and alternate neuromodulation of neuronal populations associated to flexion/extension in the motor thalamus. To do so, dual expression of

blue-light and red-light responding opsins in the rostral thalamus could enable, with a single optic fiber, using a split optic cable and two distinct lasers (blue and red), to provide alternate thalamocortical stimulation depending on electromyographic detection of function and extension.

3.5 MATERIALS AND METHODS

3.5.1 ANIMALS

Lewis rats were involved in the reported experiments in accordance with the swiss veterinary legislation applied in Geneva. Local veterinary committees accredited the procedures under Licence #GE28/17. All wild-type Lewis rats were obtained from Charles River [Les Oncins, Saint Germain sur l'Arbresle, France].

3.5.2 ADMINISTERED VIRUSES

OPTOGENETIC VIRUSES

Adeno-Associated Viral (AAV) vectors were combined to induce selective membrane expression of Channelrhodopsin-2 (ChR2) - a blue light-sensitive natrium channel. ChR2 was used as a fusion-protein with eYFP, in order to trace the successfully infected axon tracts. Retrograde AAV9-CMV-Cre [1x10^14vg/mL, kindly provided by Prof. Aebischer's group, EPFL] was infused into the middle and deeper layers of the motor cortex [AP -0.5, -1.5, -2.5 / ML 1.5, -3.0, DV -1.5 / alpha=0° / 500nL per site] for retrograde expression of the Cre-dependance. Neurotropic AAV5-Ef1a-DIO-ChR2-eYFP [4x10^12vg/mL, UNC vector core] was injected into the ipsilateral rostral thalamus [AP -1.7 / ML +2.2 / DV -5.6 / alpha=0° / 250nL per site] for selective expression of ChR2-eYFP in direct thalamocortical neurons that project to the motor cortex.

TRACING VIRUSES

Adeno-Associated Viral (AAV) vectors were combined to anterogradely label sensory input to the forebrain that originate in lumbo-sacral segments. Neurotropic AAV5-CMV-Cre [1x10^13vg/mL, kindly provided by Prof. Aebischer's group, EPFL] and AAV-DJ-hSyn-Flex-mGFP-2A-synaptophysin-mRuby [Stanford core facility] were mixed 1:1 and co-injected over 6 sites per side over the T13 and L1 vertebral levels [1mm AP spacing / ML -0.65 / DV -0.9 / 250nL per site]. This viral combination enables global labeling of anterograde somatosensory fibers projecting to forebrain areas. Axons appear in fluorescent green and synapses are shown in fluorescent red.

3.5.3 SURGICAL PROCEDURES

CORTEX AND THALAMUS INJECTIONS

All rats were maintained under anesthesia with Isoflurane in a rodent cranial stereotaxic apparatus (David Kopf, Tujunga, CA). A midline skin incision was performed to expose the skull and recognize Bregma and Lambda. Both Bregma and Lambda were vertically aligned and a craniotomy was performed using a fine drill to expose the fronto-parietal cortex. Injections took place using micro-glass pipettes (50-100um tip width) with six sites of injection in the left motor cortex (M1) and one single site in the motor thalamus (mThal). The motor cortex was stereotaxically and vertically targeted using the previously described coordinates for rats : -0.5mm, -1.5mm and -2.5mm caudal from Bregma, 1.5mm and 3mm lateral from midline and -1.5mm ventral from the cortical surface. The motor thalamus was stereotaxically and vertically targeted using following established coordinates for rats : -1.7mm caudal from Bregma, 2.2mm lateral from midline and -5.6mm ventral from the cortical surface. Injected viral suspensions were prepared to enable the concentrations and injection volumes specified under 3.5.2. Infusions were performed at 2nL/sec and a two minutes delay was respected before slow withdrawal of the pipette. The skin was finally closed using non-resorbable 4-0 sutures.

CRUSH SPINAL CORD INJURY

Rats were maintained under anesthesia with Isoflurane. A dorsal midline incision was performed and muscles were carefully detached to access the posterior aspects of the spine. Using a surgical microscope (Zeiss, Oberkochen, Germany), the inferior interspinous ligament was removed with n°5 Dumont forceps (Fine Science Tools, Foster City, CA) at the targeted thoracic level (T8 or T10). A complete laminectomy was performed with n°2 forceps (Fine Science Tools, Foster City, CA) and the cord was laterally compressed for five seconds using tweezers with a tip width of 0.5mm. The back muscles and skin were finally closed with respectively resorbable 6-0 and non-resorbable 6-0 sutures.

THALAMUS OPTIC FIBER IMPLANTATION

Optic implants were custom made to optimize the fiber length and access the targeted rostral thalamus. Multimodal optic fiber (200 µm core diameter, 0.22 NA, Thorlabs) was mounted inside 200um wide ferrules (Thorlabs, Newton, NJ) and the contact surface for light input was finely polished. Optic fiber implantation was performed in rats during a single surgery, together with implantations of an ipsilateral cortical electrode array, of lumbosacral L2-S1 spinal electrodes and of contralateral electromyographic electrodes. Eight weeks were respected between the AAV-ChR2 injection and the optic fiber implantation, in order to ensure proper expression of the ChR2 channel.

All rats were maintained under anesthesia with Isoflurane in a rodent cranial stereotaxic apparatus (David Kopf, Tujunga, CA). The headskin and the previous left-sided craniotomy were re-exposed and four stainless steel screws were firmly inserted into the exposed bone to stabilize and fix the implanted devices. To enable concomitant implantation of an ipsilateral cortical electrode array, the optic fiber was brought on the motor thalamus with a 30° angle back tilt reported to the vertical axis. A small burr hole was performed caudally to the fronto-parietal craniotomy on the left side (-5.27mm caudal from Bregma, 2.2mm lateral from midline). The path to the rostral thalamus was pre-punctured using a micro-glass pipette (50-100um tip width) and the optic fiber was implanted with a 30° angle (DV -6.1mm from the cortex surface). The exposed cortical surface was protected using biocompatible glue and the optic fiber was fixed with dental cement.

CORTICAL ELECTRODE ARRAY IMPLANTATION

32-channel micro-electrode arrays were obtained from Tucker-Davis-Technologies (ZIF2010-32, Tucker-Davis-Technologies TDT, Alachua, FL). During the same surgery and after thalamus optic fiber implantation (see 3.5.3), the left motor cortex was re-exposed and the dura mater was open. The TDT array was brought to the cortical surface (centered at -1.5mm caudal from Bregma and 2.3 lateral from midline) and lowered into the cortical layer L5 (-1.4mm from the cortical surface). After test recordings, the 32-channel micro-electrode array was fixed together with the optic fiber using dental cement.

NEUROPROSTHETIC ELECTRODE IMPLANTATION

This procedure was conducted during a single surgery together with the implantation of a cortical TDT array (see 3.5.3) and a thalamic optic fiber (see 3.5.3). An amphenol-recording unit (Omnetics Connector Corporation, USA) was placed on the right fronto-parietal bones and rigidly affixed using dental cement. Multistranded, teflon-coated steel wires connected to the amphenol pins in the recording unit were passed subcutaneously to the thoracic column. Using an operating microscope (Zeiss, Oberkochen, Germany), chronic electrodes were conceived as windows in the teflon coating and were attached to the dura mater (8.0 Ethilon) over lumbosacral levels L2 and S1 in order to elicit spinal cord reflexes. In order to implant electromyographic electrodes (EMG), incisions were performed over the *tibialis anterior* muscle (TA) and over the *medial gastrocnemius* (MG) muscle. Pairs of wires were subcutaneously passed to the incisions and windows were performed in the teflon coating to conceive chronic EMG electrodes. Electrodes were passed into the respective TA and MG muscles and fixed to the fascia. Finally, the muscle fascia (5.0 Vycril) and the skin (4.0 Ethilon) were sutured in layers.

SPINAL INJECTIONS

Spinal injections in rats were performed under general anesthesia with Isoflurane using an operating microscope (Zeiss, Oberkochen, Germany) and rodent stereotaxic apparatus (David Kopf, Tujunga, CA). A midline incision was performed to access the dorsal column and laminectomies were carried out to expose the spinal cord under the vertebral levels T13 and L1. Injections took place using micro-glass pipettes (50-100um tip width). AAV5-CMV-Cre and AAV-DJ-hSyn-Flex-mGFP-2A-synaptophysin-mRuby were co-injected into the spinal cord at 6 sites on each side, using the following coordinates : 1mm anteroposterior spacing, 0.65mm lateral from midline and -0.8 dorsoventral from the cord surface. 250nL were injected per site at 3nL per second and 2 minutes were respected before careful withdrawal of the glass pipette. At the end of the procedure, the muscle fascia (5.0 Vycril) and the skin (4.0 Ethilon) were sutured in layers.

3.5.4 ACUTE ELECTROPHYSIOLOGY

SENSORY EVOKED POTENTIALS

Terminal electrophysiological assessments were carried out in naive rats. Briefly, animals were anesthetized with urethane (1.5 g/kg; i.p.) and core body temperature was maintained at 37°C. A craniotomy was drilled over the motor cortex as described under 3.5.3 and laminectomies were performed to expose the thoracic levels T8, T10 and T12. A hindlimb incision was performed on the contralateral side to the craniotomy ; the sciatic nerve was isolated, punctured with a silver needle electrode and sectioned below the site of stimulation. The dura was then removed on all CNS exposed sites and tissues were protected using mineral oil. For spinal stimulation, a tungsten concentric electrode was positioned intraspinally on the level of interest. A silver ball electrode was used to record any evoked activity from the surface of the motor cortex or from the dorsal spinal cord. Sciatic nerve stimulation was performed using a silver needle electrode. Healthy recordings were obtained, followed by a T10 complete crush SCI (performed as described under 3.5.3) and a repetition of the evoked potentials in acute post-lesional conditions. Stimulation was delivered in 200 µs square wave pulses at the maximum

amplitude possible before large motor responses were evoked (typically between 600 µA and 800 µA) and at a frequency of 0.75 Hz using a STG 4004 stimulus generator (Multi Channel Systems). Evoked activity was amplified and recorded using an A-M systems differential amplifier, PowerLab and LabChart Pro acquisition and analysis system (AD Instruments). For analysis, 30 traces from each recording site were averaged and the peak to peak amplitude of the evoked potential was quantified.

ACUTE THALAMO-CORTICAL RECRUITMENT

Eight weeks after AAV-ChR2 injection (see 3.5.2), acute electrophysiological assessments were performed to assess the feasibility of recruiting thalamocortical projections optogenetically. Animals were anesthetized with urethane (1.5 g/kg; i.p.) and core body temperature was maintained at 37°C. An optic fiber was implanted on the rostral thalamus as described under 3.5.3 and fixed using dental cement. The ipsilateral motor cortex was re-exposed and the dura mater was removed. A silver ball electrode was used to record evoked activity from the surface of the motor cortex. Motor thalamic optic stimulation was performed using an optic cable transmitting pulses from a blue laser (Laserglow, 473 nm Blue DPSS Laser System) to the implanted fiber. Intensity of the stimuli is estimated to range from 35 to 50 mW/mm2. Laser pulses were provided by an external stimulator (A-M Systems) to enable precise control of stimulation parameters. Recordings were obtained in healthy conditions, before a T10 complete crush SCI was performed (see 3.5.3). Thalamus optogenetic stimuli and cortex surface recordings were then repeated in acute post-lesional conditions.

3.5.5 HISTOLOGY & IMMUNOHISTOCHEMISTRY

After terminal anesthesia by barbiturate overdose, rats were perfused transcardially with 4% paraformaldehyde and the CNS was retrieved and processed for immunofluorescence. Primary antibodies were: rabbit anti-GFAP (1:2000; Dako, Santa Clara, CA); guinea-pig anti-NeuN (1:100, Millipore, Burlington, MA); chicken anti-GFP (1:500, Abcam, Cambridge, MA). Fluorescence secondary antibodies were conjugated to: Alexa 488 (green), 555 (red) or 647 (far red) (Molecular Probes). Nuclear stain was added using 4',6'-diamidino-2-phenylindole dihydrochloride (DAPI; 2ng/ml; Molecular Probes). Sections were coverslipped using Mowiol mounting medium (Sigma, Saint Louis, MO). Tiled scans of individual whole sections were prepared using a x20 objective and the scanning function of a Leica Aperio Versa 200 Microscope (Leica, Wetzlar, Germany). Sections were examined and photographed using deconvolution fluorescence microscopy and scanning confocal laser microscopy (Zeiss, Oberkochen, Germany).

3.5.6 CLARITY AND 3D-IMAGE ACQUISITION

Rats were perfused with 4% paraformaldehyde and tissue was postfixed overnight. CLARITY was then applied to clear the tissue. Brain hemispheres were incubated for 2 days at 4°C in bis-free acrylamide solution with 4% paraformaldehyde. Nitrogen gas was flushed in the incubation tubes for 5 seconds to remove oxygen, and the containing tube was incubated in a 37°C water bath until polymerization (ca. 3 hours). The gel was processed and tissue was immersed in a clearing solution for 4 days, with daily medium exchange. Samples were then rinsed with PBS (0.1M) and triton (0.1%) overnight and incubated in Histodenz for 24 hours. Imaging finally took place using a custom-made light-sheet fluorescence microscope.

3.5.7 NEUROREHABILITATION PROCEDURE

After spinal cord injury, rats recovered for 7 days before neurorehabilitation began. Locomotor neurorehabilitation was conducted bipedally on a treadmill with vertical robotic support. Standing and stepping enabling agents were used as facilitators, combining epidural L2 and S1 tonic stimulation and the pharmacological association of quipazine (5-HT2A/C, 0.2-0.3 mg/kg) and 8-OH-DPAT (5-HT1A/7, 0.05-0.2mg/kg). The task was performed on a daily basis for approximately 30 minutes per day, over a period of 2 weeks. Epidural stimulation was delivered tonically at 40 Hz with a pulse duration of 0.2ms, injecting an intensity of current chosen individually for each animal depending on the observed muscle twitch tresholds (ca. 100-300uA). The animal was restrained in a comfortable harness that was attached to a half-tube at one end of the body weight support device.

3.5.8 BEHAVIORAL & NEUROPHYSIOLOGICAL ASSESSMENTS

Combined recordings of EMG activity, TDT neural activity and locomotion were conducted in two conditions. Quadrupedal locomotion was recorded on a

treadmill (ca. 16cm/s speed) in healthy conditions prior to injury, in order to obtain baseline cortical activity and establish behavioral and neurophysiological effects of motor thalamic stimulation. A system of 12 infrared cameras (Vicon motion analysis) was used to record the motor task. Retro-reflective markers were placed on the relevant joins (iliac crest, hip, knee, tarse, toe) to enable locomotion tracking and correlate neural activity with gait events. Similar assessments were performed at the end of the neurorehabilitation period (see 3.5.7) and automated bipedal locomotion was recorded on a treadmill, pharmacologically enabled with quipazine (5-HT2A/C, 0.2-0.3 mg/kg) and 8-OH-DPAT (5-HT1A/7, 0.05-0.2mg/kg). Complimentary L2-S1 tonic stimulation (40Hz, 0.2ms, 100-300uA) was delivered. Neural data and EMG activity were acquired in concomitance, and optic thalamus stimulation was delivered following relevant patterns of stimulation. Cortical activity was acquired as intracortical voltage signals sampled at 24 kHz, pre-amplified, digitalized and filtered using a real-time BioAmp processor (Tucker-Davis Technologies, USA). EMG signals and Laser firing activity were recorded as analogic events, together with neural data.

Chapter 4

RETICULOSPINAL REORGANIZATION

REINFORCING DOWNSTREAM CONTROL OVER INFRALESIONAL CIRCUITS

4.1 ABSTRACT

Propriospinal axon regeneration across complete spinal cord injuries does not promote recovery of function by itself. In addition to our exploration of somatosensory feedback to the motor cortex, we propose here to investigate other descending tracts on a regenerative aspect. Different tracts have been shown to reorganize in different models of incomplete SCI, so that corticospinal projections are able to reach for new relays, either at a spinal level, or in the brainstem. we hypothesize that regenerating reticulospinal fibers, in addition to propriospinal axons, would enhance the conduction of motor commands to the lumbosacral circuits by providing a second possibility of relay for the corticospinal tract. Indeed, recent evidence demonstrated that neuroprosthetic rehabilitation causes the motor cortex to recruit spared reticulospinal projections as descending relays after severe spinal contusion. Based on our mechanistic understanding of propriospinal axon regeneration, we first combined lesion core manipulation with mTOR activation to achieve reticulospinal axon regrowth. We documented limited, yet significant reticulospinal regeneration into the SCI site. As neuroprosthetic training was shown to promote reticulospinal neurite extension, we next tested wether chronic activity by itself can recruit neuronal growth programs in the brainstem after complete SCI. We aimed at activating the CREB and MAPK pathways by either i) evoking activity in the reticular formation using optogenetics or ii) enhancing intracellular cAMP levels with viral DREADD technology. The growth permissive matrix was densified within the lesion core using a combination of EGF and FGF. BDNF and GDNF were associated as guidance cues. As reticulospinal axon regrowth could not be observed following activity-based modulation, significant sprouting of reticulospinal fibers was obtained into the healthy grey matter located above the injury. These observations suggest that requirements and/or facilitators for sprouting and regrowth are distinct in reticulospinal neurons. Independent identification of the mechanisms underlying sprouting and axon regrowth will be necessary in order to reinforce downstream conduction of information across complete SCI.

PERSONAL CONTRIBUTIONS

In this study, I - Sabry L. Barlatey - designed experiments and piloted the feasibility of the project. I performed stereotaxic brainstem injections, implanted optic fibers for optogenetic modulation, and performed surgical spinal cord injuries. I designed and contributed to behavioral assessments. I processed CNS tissue, performed histological analyses, acquired microscopy images and quantified axon regeneration. I wrote the chapter presented here below and conceived the figures.

4.2 INTRODUCTION
REINFORCING BRAINSTEM CONTROL ACROSS COMPLETE SCIs

Spinal neuromodulation and rehabilitation have been able to promote functional benefits after incomplete spinal cord injury in both rodents (Asboth et al., 2018; van den Brand et al., 2012) and humans (Angeli et al., 2018; Wagner et al., 2018). After incomplete spinal cord injury, sprouting of spared axons supports re-establishment of downstream control over lumbosacral circuits. The extend and location of spare tracts relies on the model of injury. Indeed, a significant population of propriospinal neurons remains spared in a staggered double-hemisection model, and their recruitment and reorganization contribute to functional recovery (van den Brand et al., 2012; Courtine et al., 2008). In both the staggered double-hemisection and the dorsal contusion models, brainstem neuronal relays undergo neurorehabilitation-induced plasticity. During neuroprosthetic training in the staggered double-hemisection model, part of the supraspinal motor control reaches for spared and reorganizing propriospinal neurons via serotoninergic brainstem neurons (van den Brand et al., 2012). With a dorsal spinal contusion, some extent of reticulospinal glutamatergic control located ventrolaterally in the spinal cord remains spared and sprouts both above and below the injury during neurorehabilitation (Asboth et al., 2018). However and after complete crush SCI, whereas our recent work enabled the restoration of a robust propriospinal bridge across the lesion (Anderson et al., 2018), no spontaneous recovery of hindlimb movements could be observed. Therefore, we hypothesize that reinforcing downstream control across complete injuries with brainstem-derived axon regrowth may facilitate the conduction of motor commands to lumbosacral circuits. Complimentary neurorehabilitation may then promote reticulospinal sprouting both above and below the crush injury, promoting interactions with regenerated propriospinal neurons to encourage coherent motor control as described in the dorsal contusion model (Asboth et al., 2018).

Evidence has been gathered that different axonal tracts respond to different growth program activators (He and Jin, 2016). As corticospinal, propriospinal and alpha-retinal-ganglial axons have been demonstrated to regrow when mTOR signaling is upregulated (Ozdinler and Macklis, 2006; Liu et al., 2017; Anderson et al., 2018; Duan et al., 2015) no such manipulation has yet been experimented on brainstem-spinal structures to our knowledge. So far, intrinsic growth programs in reticulospinal neurons have been suggested to respond to the activity-related signaling pathway cAMP-CREB (Lu et al., 2012a). The role of CREB signaling in neurons has been extensively studied for its sensitivity to activity and neurotrophins, and for its implication in neurite outgrowth (Moore and Goldberg, 2011). Indeed, deficiencies for CREB were shown both *in vitro* and *in vivo* to decrease neurite growth in both peripheral (Lonze et al., 2002) and cortical neurons (Redmond et al., 2002). Additionally, increasing CREB activity was shown to enhance sensory axon regeneration after dorsal column lesion (Gao et al., 2004). Exploring activity-related signaling in sensory neurons, CREB activation was shown to respond to cAMP elevation induced by electrical activity (Fields et al., 1997). Interestingly, CREB downstream signaling includes transcription of the neurotrophin-gene *bdnf* (Moore and Goldberg, 2011). Among other interesting signaling cascades, alterations in the MAPK signaling have been demonstrated after injury to the visual system (Belin et al., 2015), while the MAPK cascade is involved in neuronal survival and axonal growth after lesion (He and Jin, 2016). Further, MAPK was shown *in vitro* to be recruited by electrical activity together with CREB (Fields et al., 1997), based on cAMP and Protein kinase A (PKA) signaling (Waltereit and Weller, 2003).

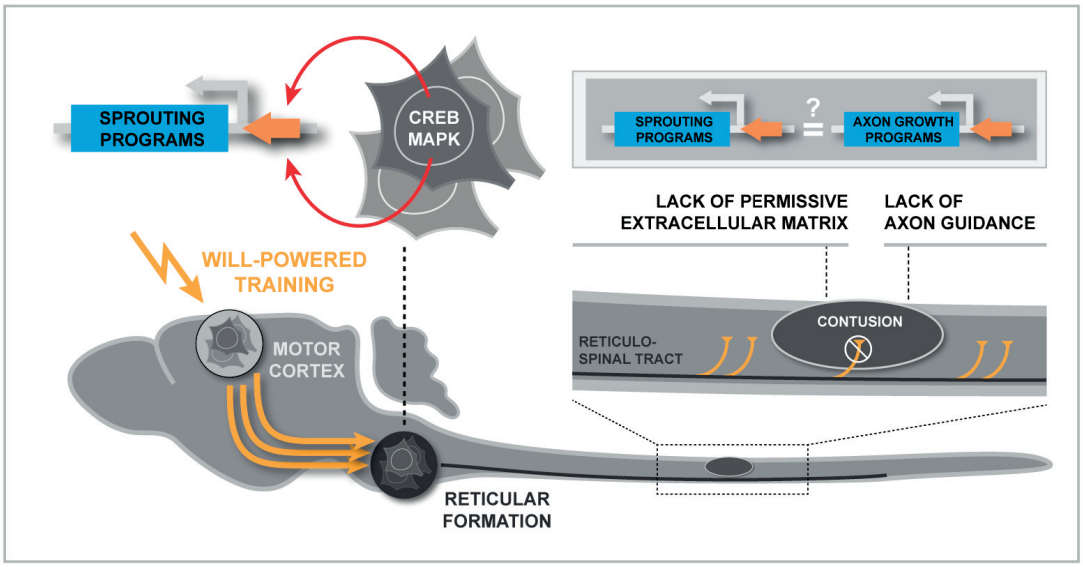

Figure 4.1: **Neuroprosthetic training induces reticulospinal sprouting and functional recovery.** Neuronal activity promoted by neuroprosthetic rehabilitation has been shown to induce sprouting from spared axon tracts, e.g. reticulospinal projections in a model of dorsal contusion. We hypothesize that reticulospinal neurites fail to penetrate the injured site in spite of activity, due to the lack of a permissive extracellular matrix and guidance. By remodeling the injured site and providing axon guidance, we further hypothesize that chronic activity may allow reticulospinal projections to grow into the lesion core, by activating sprouting and/or axon growth programs.

As much as growth-related signaling varies from a tract to another, axon guidance appears to be tract-dependent. Only little evidence of reliable cues or molecular agents has been gathered in order to provide reticulospinal axons with guidance. Combined to brainstem activation of CREB signaling with a single cAMP injection, reticulospinal fibers were shown to regrow into a mesenchymal graft on a BDNF-dependant manner (Lu et al., 2012b). Further, combined delivery of BDNF and NT-3 within the lesion - in addition to Schwann cell grafting - was proposed as a robust distant activator of brainstem-originating axons Xu et al. (1995b). Additionally, GDNF delivered at the SCI site was suggested to reduce die-back from the reticular formation (Dolbeare and Houle, 2003). We propose here to explore reticulospinal regeneration by applying a multi-pronged regenerative approach that combines i) upregulation of intrinsic reticulospinal growth programs, ii) lesion site remodeling iii) axon guidance. As previously described, we propose to remodel the SCI site using a combination of EGF and FGF, in order to enhance permissive extracellular matrix components within the injury. Based on previous work, we chose to combine the neurotrophins BDNF and GDNF as chemoattractants within the lesion site. In order to activate axon growth programs, we investigated a virus-based mTOR upregulation that was previously shown to generate robust regeneration in other severed tracts. Finally, due to the sprouting induce by neurorehabilitation, we explored neuronal activity and camp-dependent signaling as potential candidates for intrinsic growth activation (Fig 4.1).

4.3 RESULTS
SIGNALING DIFFERS BETWEEN SPROUTING AND AXON REGROWTH REGENERATION

4.3.1 LOW RETICULOSPINAL RESPONSE TO mTOR SIGNALING

As a first reticulospinal manipulation, we tested wether combined mTOR and Jak-STAT activation using the viral association [AAV-IGF1, AAV-CNTF and AAV-OPN] can yield robust axon regrowth of the reticulospinal system into complete SCI sites. Two weeks prior to injury, we co-injected IGF1-CNTF-OPN into the gigantocellular reticular nuclei (vGI) in wild-type mice. The animals then received an anatomically complete crush spinal cord injury that wholly interrupts descending axon tracts. In order to support axon regeneration at the SCI level, we primed lesion core remodeling on the second day post-injury using EGF and FGF embedded in biocompatible Dicopolypeptide Hdrogel (DCH). This growth factor association was previously shown to enhance laminins within the injured site, as a requirement for propriospinal axon regeneration (see 2). In order to attract potential regenerating axons into the lesion, BDNF and GDNF were added as chemoattractant molecules to the DCH hydrogel, together with the molecular remodeling association of EGF and FGF (Fig 4.2d).

As we sought here to identify the requirements for reticulospinal growth, we did not add any chemoattractant below the injury. Nevertheless, the described multi-pronged strategy did induce some extent of reticulospinal regrowth into the lesion core, which did not extend beyond the caudal SCI border due to the absence of caudal chemotropic guidance (Fig 4.2a-b). However, even though the obtained regeneration is significant, we estimate that the density of axons that penetrate the lesion core remains too sparse to support any functional benefit. Indeed, the absolute number of axons crossing the lesion center was ten folds lower that the propriospinal regrowth obtained with the same strategy (Fig 4.2c). This anatomical experiment study suggests that IGF-1/CNTF/OPN recruits part of the intracellular signaling required for reticulospinal axon regrowth. However, proper intracellular targets will need to be identified in order to promote dense reticulospinal regeneration across complete SCIs.

4.3.2 DREADD-INDUCED cAMP ELEVATION FAILS TO INDUCE RETICULOSPINAL AXON REGROWTH

Previous evidence suggests that a single cAMP injection on its own recruits reticulospinal axon growth programs after spinal cord injury (Lu et al., 2012b). However, concomitant guidance with BDNF was provided in this study by genetically-modified mesenchymal cells. In order to exclude side-contributions from the graft, we aimed at reproducing similar axon regeneration using molecular and controlled axon guidance, together with SCI remodeling. On day-2 following complete crush spinal cord injury, vGLUT2-Cre mice received an intralesional depot of EGF, FGF, GDNF and BDNF, embedded in DCH hydrogel (Fig 4.3d). As described under 4.3.1, EGF/FGF aimed at densifying the permissive extracellular matrix within the lesion core, while BDNF and GDNF were used as reticulospinal guidance cues. In order to promote sustained and chronic cAMP elevation in glutamatergic reticulospinal neurons, the clozapine-N-O (CNO) dependent virus AAV5-hsyn-DIO-rM3Ds-mCherry virus was injected into the vGI two weeks before SCI (Fig 4.3a). Such DREADD (designer receptors exclusively activated by designer drug) Gs-protein-targeted virus was previously shown to enhance intracellular cAMP

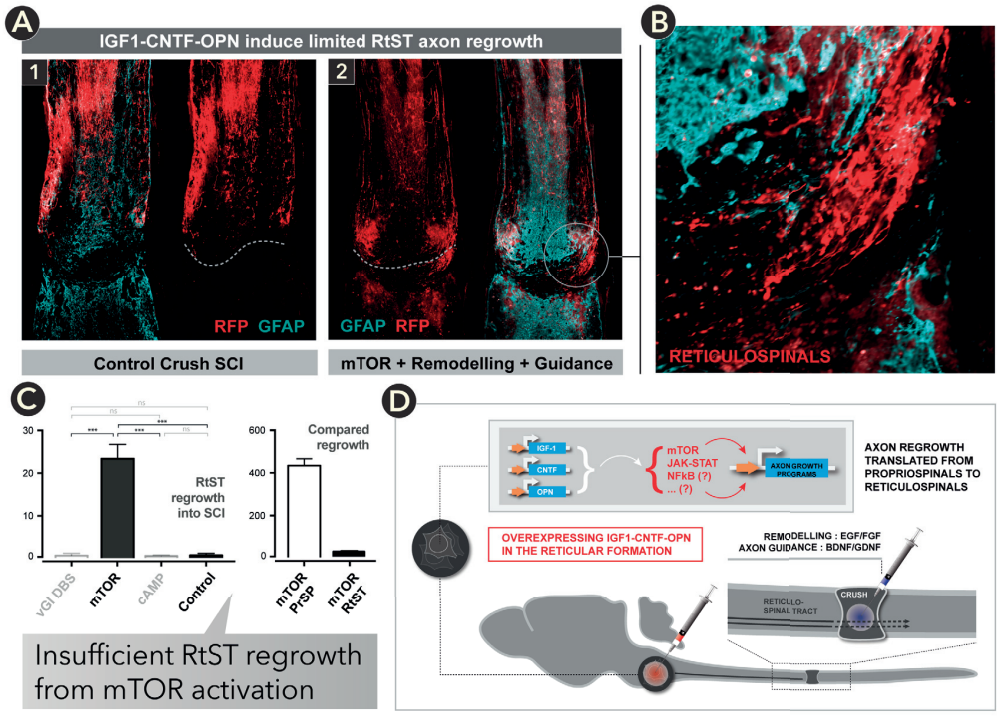

Figure 4.2: **IGF1-CNTF-Osteopontin induce significant, yet insufficient axon regrowth after complete crush spinal cord injury.** **A**, Tile scans showing descending reticulospinal axons (red) labelled with non-Cre-dependant AAV-RFP and astroglial GFAP (cyan) to delimit the lesion core. **A1**. Complete absence of reticulospinal regeneration in a control crush spinal cord injury, after lesion remodeling with EGF/FGF and axon guidance with BDNF/GDNF, but without growth program induction. **A2**. Tile Scan showing limited, yet significant reticulospinal regeneration when AAV-IGF1-CNTF-OPN is added to lesion remodelling and axon guidance. **B**. 10x magnification of the regenerated reticulospinal axons into the lesion core. Viral tracing of reticulospinal fibers with ubiquitous AAV5-RFP, immunostaining for GFAP (cyan). **C**. [Left] Quantification of regenerated axons that cross the lesion center (absolut number of axons). [Right] Direct comparison of regenerated propriospinal and reticulospinal axons with IGF1-CNTF-OPN overexpression, showing insufficient regrowth from reticulospinals. ***$P < 0.0001$, one-way ANOVA with Bonferroni. **D**. Experimental design. AAV-IGF1-CNTF-OPN were co-injected into the reticular formation without Cre-dependance to upregulate mTOR and Jak-STAT signaling. EGF/FGF were used as lesion remodelling agents and BDNF/GDNF were added as guidance cues.

levels and signaling (Ferguson et al., 2013). As a pharmacological enhancement of electrical activity-like signaling, CNO was given twice daily to recruit the DREADD-Gs pathway.

Conversely to previously published work, we did not obtain any reticulospinal axon regrowth with DREADD-induced cAMP elevation (Fig 4.3b-c). However, limitations reside in the mCherry reporter added to the viral construct, which is known for being of poor fluorescent intensity and of poor reliability. Proper injection and activation of the DREADD-Gs virus could not be confirmed, in spite of immunohistochemical enhancement of mCherry. Nevertheless, additional tracing of the reticular formation with Biotin-Dextran-Amine (BDA) confirmed the absence of regenerative effects, that we impute to a deficient activation of growth programs.

4.3.3 CHRONIC ACTIVITY FAILS TO INDUCE RETICULOSPINAL AXON REGROWTH

Viral and pharmacological elevation of cAMP fails to promote reticulospinal axon regrowth. In a context of neuroprosthetic rehabilitation, activity was shown to promote reticulospinal sprouting (Asboth et al., 2018). Therefore, we further tested the hypothesis that intracellular mechanisms may be shared between axon regrowth and sprouting when proper lesion remodeling and axon guidance are provided. To do so and to activate activity signaling that may be camp-independent, we replaced the previous DREADD-rM3Ds modulation with chronic stimulation of the reticular formation using optogenetics. Transgenic mice expressing Channelrhodopsin2 under the neuronal promoter Thy1 (Thy1-ChR2) were implanted two weeks before injury with a single midline optic fiber to co-activate both vGI nuclei in the reticular formation (Fig 4.4a). The Thy1 genetic background was chosen due to the abundant Thy1 expression from neuronal structures involved in motor functions. Stimulation parameters where chosen based on a previously established pattern *in vitro*, which demonstrated that 10Hz action potentials induce intracellular cAMP elevation in sensory neurons, as well as CREB and MAPK upregulation (Fields et al., 1997). Before injury, baseline recordings of optogenetically evoked motor responses were obtained and showed tonic hindlimb extension correlated with the trains of stimulation (Fig 4.4b). The animals were then injured with a complete thoracic T10 crush SCI. As mentioned above, mice received a depot of EGF/FGF on the second day post-injury, that was supplemented with BDNF and GDNF as reticulospinal chemoattractants. After injury, chronic vGI stimulation was conducted for four weeks on a daily basis, as two sessions of 90 minutes per day [10Hz, 5ms pulses, 5-seconds-ON / 5-seconds-OFF].

As expected, terminal recordings of optogenetically evoked motor responses showed in all animals that the lesions remain functionally complete in spite of chronic vGI stimulation (Fig 4.4c). Reticulospinal tracing with AAV2/5-RFP revealed that chronic stimulation, together with lesion remodeling and axon guidance, fails to induce regrowth into the lesion core (Fig 4.4d, Fig 4.5d).

4.3.4 CHRONIC ACTIVITY RESCUES RETICULOSPINAL PROJECTIONS ABOVE THE LESION

In order to establish that sprouting and axon regeneration rely on distinct mechanisms, we evaluated the density of reticulospinal projections at a cervical level. Reticulospinal projections to the grey matter were quantified over the cervical C6 to C9 segments and compared between i) the vGI-stimulated group, ii) healthy control mice, and iii) crush injury control animals traced

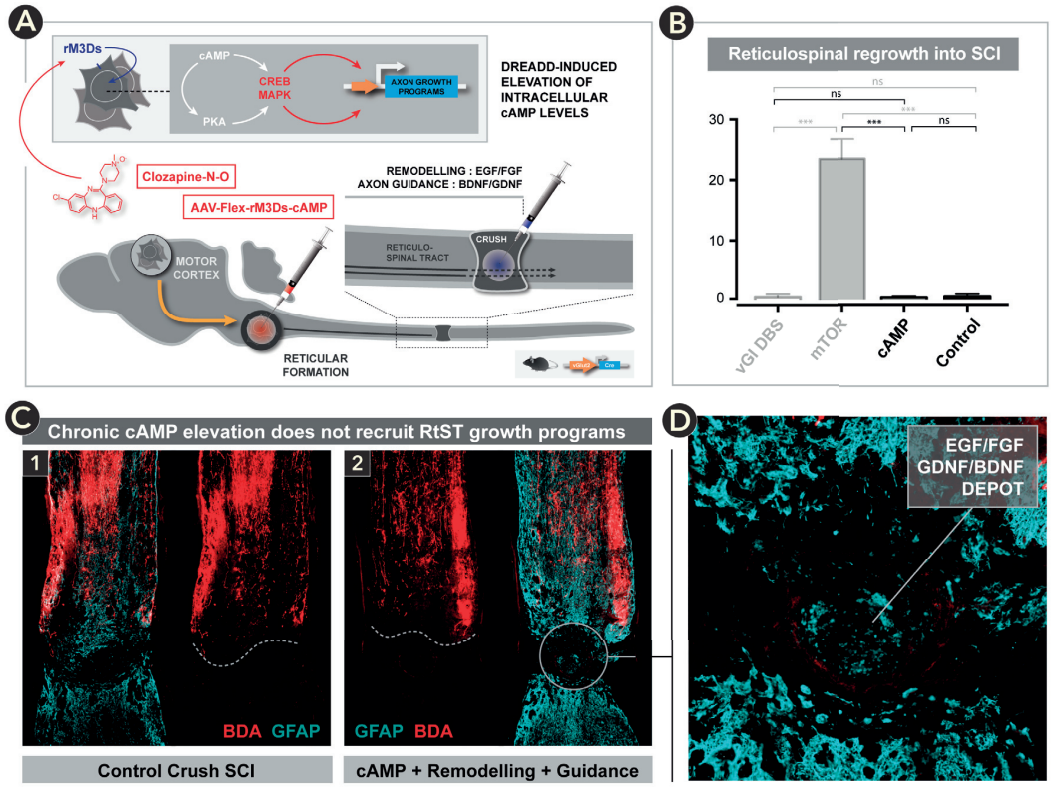

Figure 4.3: **Chronic cAMP elevation fails to recruit growth programs in spite of lesion remodeling and axon guidance.** **A**. Experimental design. DREADD viral vectors were administered in vGlut2-Cre mice to induce selective expression of the protein Gs-dependant receptor rM3Ds in glutamatergic neurons. Clozapine-N-O was given twice daily to activate the rM3Ds receptor, induce intracellular cAMP elevation, and upregulate the cAMP downstream signaling. EGF/FGF were administered into the injury to remodel the complete crush lesion, supplemented with BDNF and GDNF for reticulospinal axon guidance. **B**. Quantification of regenerated axons that cross the lesion center (absolut number of axons), showing no significant regrowth with pharmacological cAMP elevation. One-way ANOVA with Bonferroni. **C**. Tile scans showing descending reticulospinal axons labelled with Biotin Dextran Amine and revealed in Cy3 (red), together with an immunohistochemical revelation for astroglial GFAP (cyan) to delimit the lesion core. **C1**. Complete absence of reticulospinal regeneration in a control crush spinal cord injury, after lesion remodelling with EGF/FGF and axon guidance with BDNF/GDNF, but without cAMP elevation. **C2**. Tile Scan showing no reticulospinal regeneration in spite of chronic cAMP elevation, lesion remodelling and axon guidance. **D**. 10x magnification of the lesion core, showing no axon regrowth but a clear rest of the molecular depot.

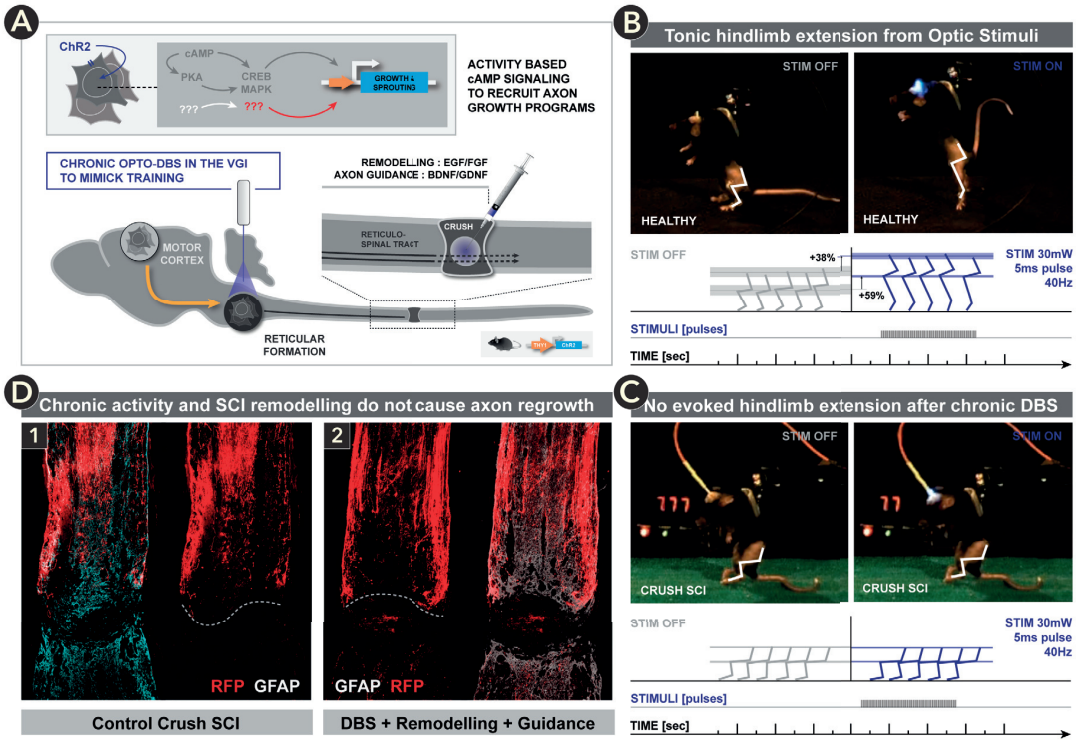

Figure 4.4: **Optogenetic targeting of the reticular formation recruits motor circuits, but fails to induce reticulospinal regrowth after complete SCI.** **A**. Experimental design. Mice expressing Channelrhodopsin2 constitutively under the Thy-1 neuronal promoter were implanted with an optic fiber on the vGI nuclei. Chronic optic stimulation was delivered on a daily basis to recruit activity-driven signaling. EGF/FGF were administered into the injury to remodel the complete crush lesion, supplemented with BDNF and GDNF for reticulospinal axon guidance. **B**. Pre-SCI evoked motor responses. Trains of stimulation provoke tonic bilateral extension in the hindlimbs. **C**. At 4 weeks post-injury and after chronic stimulation in the vGI, optic stimulation does not evoke motor responses in the hindlimbs. **D**. Tile scans showing descending reticulospinal axons traced with an ubiquitous AAV2/5-RFP viral vector (red), together astroglial GFAP (cyan) revealed immunohistochemically to delimit the lesion core. **D1**. Complete absence of reticulospinal regeneration in a control crush spinal cord injury, after lesion remodeling and axon guidance, but without vGI stimulation. **D2**. Tile Scan showing no reticulospinal regeneration in spite of chronic optogenetic stimulation in the reticular formation, lesion remodelling and axon guidance.

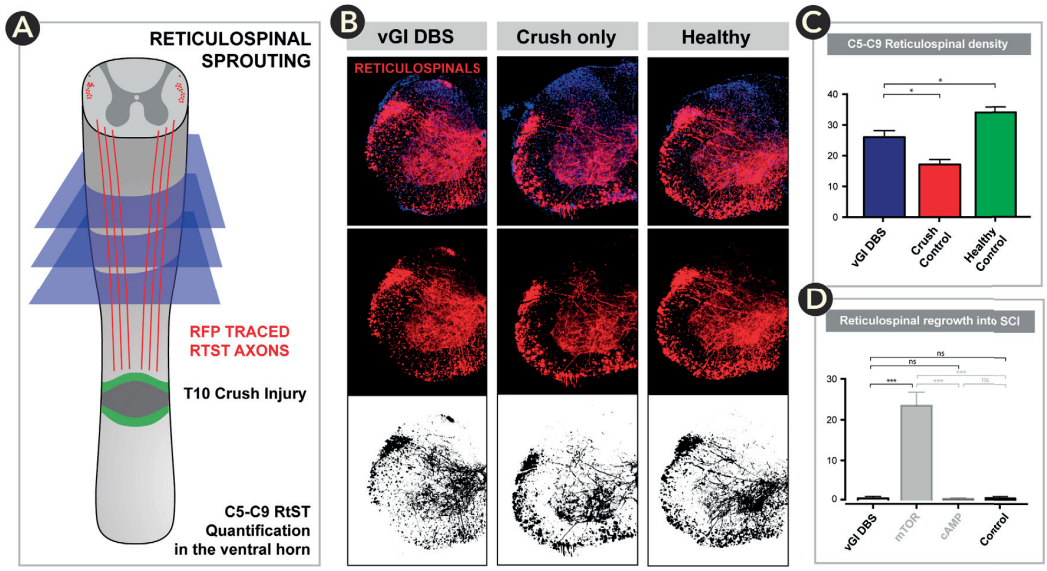

Figure 4.5: **Chronic stimulation in the reticular formation rescues reticulospinal projections to the grey matter above the lesion.** **A**. Schematic showing the quantification method for sprouting or neurite protection. Transverse sections were taken from C6 to C9 and reticulospinal coverage was quantified as a percentage of the grey matter expressing the RFP. **B**. Representative histologic sections from the vGI-stimulated group [left], the injured control group [middle] and the healthy control group [right] showing RFP-labelled reticulospinal axons (red), counterstained with nucleus-labelling Dapi (blue) for grey matter delimitation. Black/white sections depict the used thresholding for RFP measurements. **C**. Quantification of reticulospinal coverage in the grey matter, cumulated from C6 to C9, showing an increased density of reticulospinal projections in vGI stimulated animals, compared to injured controls. One-way ANOVA with Bonferroni. **D**. Quantification of regenerated axons that cross the lesion center (absolut number of axons), showing no significant regrowth with chronic vGI stimulation. One-way ANOVA with Bonferroni.

for reticulospinal axons with AAV2/5-RFP (Fig 4.5a-b). As reticulospinal projections into the grey matter were strongly reduced above the injured level in the injured control group, chronic vGI stimulation exhibited a significantly higher afferentation from reticulospinal axons at a cervical level. This anatomical data suggests that chronic activity either recruits sprouting or regenerative sprouting programs, or exerts a protective effect on neurites after axotomy (Fig 4.5c).

4.4 DISCUSSION
DISSOCIATED PATHWAYS FOR SPROUTING AND AXON REGROWTH

Axon regrowth after spinal cord injury has now been established as a problem of multiple variables that requires i) proper intrinsic activation of growth programs, ii) adequate remodeling of the SCI lesion core and iii) specific axon guidance (see 2 and Anderson et al., 2018). In order to focus our effort on intrinsic induction of growth, we chose here to apply lesion remodeling with EGF/FGF and chemoattraction with BDNF/GDNF, and consider these strategies

as established. In this context, over-expression of IGF1-CNTF-Osteopontin were able to induce some extent of regrowth into the SCI site, suggesting that EGF/FGF and BDNF/GDNF are able to act as reliable manipulations to play their respective role. A concern arises when regrowth is compared between reticulo- and propriospinal axon with lesion remodeling and guidance only. Indeed, and in the absence of any external upregulation of growth programs, laminin densification and chemoattraction induce a low but significant extent of propriospinal regrowth into the lesion core (Fig 2.1c). No similar anatomical benefit could be obtained from the reticulospinal system. This suggests that either i) optimal extracellular substrates for regrowth differ between axon types, so that EGF/FGF induce a semi-permissive environment for reticulospinal axons that can be refined, or ii) BDNF/GDNF is a suboptimal combination of reticulospinal guidance cues. The second option appears more likely, as laminin exerts conserved effects over neuron survival and/or neurite support between different types of neurons *(Plantman et al., 2008; Anderson et al., 2018; Hall et al., 2008)*. On the other hand and based on developmental studies, guidance and repulsion mechanisms to vary between neuronal subtypes, in order to form distinct axonal tracts.

Similarly to axon guidance, intrinsic growth programs are known to be neuron subtype-dependent, so that specific and targeted signaling will be required to induce axon regrowth (He and Jin, 2016). Activating the mTOR pathway only by silencing PTEN has yielded robust regeneration of the corticospinal tract (Zukor et al., 2013; Liu et al., 2010), but failed to induce propriospinal axon regrowth (Anderson et al., 2018). The benefit of using IGF1-CNTF-Osteopontin co-expression relies on the co-activation of different regenerative pathways, including mTOR and Jak-STAT. Our data in the present chapter suggests that signaling induced by IGF1-CNTF-Osteopontin likely recruits axon growth programs via cross-talks with other intracellular cascades. mRNA analysis of the exact intracellular modulations produced by these three growth factors in the reticular formation may be interesting, in order to identify overlaps with other growth-relevant pathways that can subsequently be explored. As another option, mRNA sequencing of the developing reticular formation during axogenesis can provide candidate intracellular cascades to recruit after injury. However, signaling requirements may differ between fetal development and post-lesional regeneration.

Our exploration of neuronal activity as a potential neurite growth inductor was motivated by recent work from our laboratory that associated reticulospinal sprouting to volitional motor training (Asboth et al., 2018). As a first insight, our results suggest that cAMP-signaling alone fails to induce axon regrowth in reticulospinal neurons, in spite of lesion remodeling and axon guidance that promote low but significant regeneration with IGF1-CNTF-Osteopontin overexpression. However, these findings remain non-conclusive, due to a limited reliability of the mCherry fluorescent reporter, that prevented us to confirm robust activation of the DREADD-cAMP virus with Clozapine-N-O.

cAMP levels are known to be enhanced by electrical activity (Fields et al., 1997), but neuronal firing may as well recruit other molecular pathways involved in neurite extension. Optogenetic activation of the reticular formation evoked robust neuronal activity that was confirmed with kinematic recordings. One could argue that Thy1-ChR2 mice express Channelrhodopsin2 in the corticospinal tract located close below the reticular formation, in a range that our optic implant may stimulate. Nevertheless, activation of the corticospinal tract would also recruit cortical projections to the reticular formation. Such a mechanism would induce neuronal activity at the desired location, in a manner that mimics volitional neurorehabilitation on the reticulospinal tract. As an experimental limitation, intracellular signaling may vary between direct optogenetically-induced depolarization or synaptic neuronal activation. Nevertheless and

in spite of chronic activity, the regenerative failure observed in the lesion core demonstrates that neuronal firing induces intracellular signaling that is not a key determinant for reticulospinal axon regrowth. Considering published evidence, these pathways include camp-dependent cascades (Fields et al., 1997). Interestingly, previous work demonstrated axon regrowth with a single injection of cAMP into the reticular formation (Lu et al., 2012a). However, this study combined cAMP signaling with mesenchymal cell grafting that secreted BDNF in the lesion cavity, involving a mechanistic bias that currently suggests pro-regenerative effects from the graft.

As chronic activity rescued reticulospinal projections to supralesional segments of the spinal cord, different mechanisms may be involved. Activity may either i) protect reticulospinal neurites in axotomized neurons, ii) induce proper sprouting in axotomized neurons, or iii) activate neurite extension programs in intact reticulospinal neurons that reach for segments located above the injury. This last possibility prevents the definitive conclusion that sprouting programs or neurite-protective programs differ from axon regrowth signaling. Labelling severed reticulospinal axons selectively with a Cre-Flex viral combination and repeating our chronic activity experiment could determine the anatomical mechanism that underlies neurite rescue. At an intracellular level and as neuroprosthetic rehabilitation is known to induce reticulospinal sprouting, mRNA sequencing applied in an incomplete model of SCI after volitional motor training would provide candidate pathways that can be explored and modulated in the future.

4.5 MATERIALS AND METHODS

4.5.1 ANIMALS

B6 mice were involved in the described experiments in accordance with the swiss veterinary legislation applied in Vaud and Geneva. Local veterinary committees accredited the experiments under Licenses #VD2771 and #GE28/17. All wild-type B6 mice were obtained from Charles River [Les Oncins, Saint Germain sur l'Arbresle, France]. Transgenic mice line were obtained from the Jackson Laboratory [Bar Harbor, ME, USA] and bred in our own animal facilities in Lausanne and Geneva.

4.5.2 ADMINISTERED MOLECULES AND VIRUSES

DREADD VIRUSES & CNO-MEDIATED ACTIVATION

Adeno-Associated Viral (AAV) vectors were used to induce selective expression of the Gs-coupled rM3Ds receptor : neurotropic rAAV5-hsyn-DIO-rM3Ds-mCherry [10^12vg/mL, Addgene] was injected for intracellular cAMP elevation into the vGI nucleus of B6.vGlut2-Cre mice. The mCherry reporter was meant to label in red fluorescence the successfully infected neurons. Bilateral injections were performed in the vGI nuclei [AP -5.6, -5.8 / ML +0.3, -0.3 / DV -5.6, 200nL per site]. Activation of the DREADD receptor was conducted with intraperitoneal injections of Clozapine-N-O, prepared in NaCl 0.9% (5mg/mL), twice daily (0.03mL i.p.).

TRACING VIRUSES AND DYES

The neurotropic AAV2/5-CAG-RFP [1x10^13vg/mL, University of Pennsylvania Vector Core] Adeno-Associated Viral (AAV) vector was used to anterogradely label reticulospinal axons. Bilateral injections were performed in the vGI nuclei [AP -5.6, -5.8 / ML +0.3, -0.3 / DV -5.6, 200nL per site]. Axons appear in fluorescent red during anatomical analysis. Alternatively, biotinylated dextran amine (BDA, 10'000MW, Invitrogen) was used as a non-viral tracer. BDA was prepared as a 10% solution in sterile NaCL and injected into the vGI nuclei [AP -5.6, -5.8 / ML +0.3, -0.3 / DV -5.6, 400nL per site]. During tissue analysis, BDA was enhanced and revealed by conjugation with fluorescent dyes.

VIRAL EXPRESSION OF GROWTH FACTORS

Three different Adeno-Associated Viral vectors (AAV) were combined to express the growth factors Osteopontin (OPN), IGF1 and CNTF (AAV2/9-OPN: 1x10^13 gc/ml; AAV2/9-IGF-1: 5x10^12 gc/ml; AAV2/9-CNTF: 5x10^12 gc/ml, kindly provided by Professor Zhigang He's group, Harvard). The viral association (1:1:1) was injected into the vGI nuclei [AP -5.6, -5.8 / ML +0.3, -0.3 / DV -5.6, 200nL per site] in wild-type mice. This combination has been shown to recruit axon growth programs that rely on mTOR and Jak-STAT upregulation.

HYDROGEL DEPOT WITH GROWTH FACTORS AND GUIDANCE CUES

For sustained delivery, growth factors and guidance cues were embedded in a biocompatible hydrogel. Biomaterial depots were prepared using well characterized diblock copolypeptide hydrogels (DCH) that are CNS biocompatible, biodegrade over several weeks *in vivo* and provide prolonged delivery of bioactive growth factors in CNS tissue for two or more weeks after injection. Diblock copolypeptide hydrogel (kindly provided by Pofessor Deming's group, UCLA) was loaded with growth factors and guidance molecules. Cargo molecules were as follows: Human recombinant FGF2, EGF, BDNF and GDNF (PeproTech, Rocky Hill, NJ). Freeze dried DCH powder was reconstituted to 3.0% or 3.5% wt/vol in sterile PBS with a combination of FGF2 (1.0µg/µl), EGF (1.0µg/µl), BDNF (1.0µg/µl) and GDNF (1.0µg/µl). The combined biomaterial with cargo was then injected into the SCI lesion core [DV -0.6, 1uL] as a single site depot.

4.5.3 SURGERIES

CRUSH SPINAL CORD INJURY

Mice were maintained under anesthesia with Isoflurane and Ketamine. A dorsal midline incision was performed and muscles were carefully detached to access the posterior aspects of the spine. Using a surgical microscope (Zeiss, Oberkochen, Germany), the inferior interspinous ligament was removed with n°5 Dumont forceps (Fine Science Tools, Foster City, CA) at the targeted thoracic level (T8 or T10). A complete laminectomy was performed with n°2 forceps (Fine Science Tools, Foster City, CA) and the cord was laterally compressed for five seconds using tweezers with a tip width of 0.5mm. For SCI site labelling, the mice were then suspended in a rodent stereotaxic frame (David Kopf, Tujunga, CA) and 100nL of dark dye was microinjected using glass pipettes (50-100um

tip width) controlled by micro-infusion pumps. The back muscles and skin were finally closed with respectively resorbable 6-0 and non-resorbable 6-0 sutures.

SCI REMODELING AND AXON GUIDANCE

A single depot of DCH-EGF/FGF/BDNF/GDNF was performed on the second day post injury. Mice were maintained under anesthesia with Isoflurane. A midline skin incision was performed and the previous surgical wound was reopened using n°5 Dumont forceps (Fine Science Tools, Foster City, CA) to re-expose the SCI site. The animal was suspended in a rodent stereotaxic frame (David Kopf, Tujunga, CA) and a single midline site was injected with 1uL (2nL/sec) of DCH loaded with bioactive components (cf. 4.5.2) (-0.6mm ventral from the cord surface). The back muscles and skin are finally closed with respectively resorbable 6-0 and non-resorbable 6-0 sutures.

BRAINSTEM INJECTIONS AND TRACING

All mice were maintained under anesthesia with Isoflurane and Ketamine. A midline skin incision was performed to expose the skull and recognize Bregma and Lambda. Both Bregma and Lambda were vertically aligned and a craniotomy was performed using a thin drill to expose the dorsal aspects of the cerebellum. Injections took place using micro-glass pipettes (50-100um tip width) with two targets on each side over the ventral gigantocellular nucleus (vGI). The vGI nucleus was stereotaxically addressed using the previously described coordinates for mice : -5.6mm and -5.8mm caudal from Bregma, +/-0.3mm lateral from midline and -5.6mm ventral from the cerebellar surface. Injected viral suspensions or solutions were prepared to allow for the desired volume as listed under 4.5.2. Injection took place at 2nL/sec, with respect of a two minutes delay before slow withdrawal of the micro-glass. For injections-only surgeries (cf. 4.5.3), the skin was finally closed using non-resorbable 4-0 sutures.

BRAINSTEM OPTIC FIBER IMPLANTATION

Optic implants were custom made to optimize the fiber length and access the targeted rostral thalamus. Multimodal optic fiber (200 µm core diameter, 0.22 NA, Thorlabs) was mounted inside 200um wide ferrules (Thorlabs, Newton, NJ) and the contact surface for light input was finely polished. Optic fiber implantation was combined with RtST tracing and performed in Thy1-ChR2 mice, which express constitutively the Channelrhodopsin2 membrane protein in motor-related neuronal structures.

All mice were maintained under anesthesia with Isoflurane in a rodent cranial stereotaxic apparatus (David Kopf, Tujunga, CA). Skull exposure and craniotomy were conducted as described under 4.5.3, and three stainless steel screws were firmly inserted into the exposed bone to stabilize and fix the implanted optic fiber. After puncturing the dura mater at the site of entry, a single optic implant located on the midline was lowered ventrally to the vicinity of both vGI nuclei (-5.7mm from Bregma, 0mm from midline and -5.6mm ventral from the cerebellar surface). The exposed cerebellar surface was then protected using biocompatible silicon and the optic implant was fixed with dental cement.

4.5.4 BEHAVIORAL ASSESSMENTS

Evoked motor responses were conducted prior to injury and at a terminal stage after chronic optogenetic stimulation in the reticular formation. The animal was restrained in a bipedal position overground, in a comfortable harness that was attached to a half-tube at one end of a body weight support device. A system of 12 infrared cameras (Vicon motion analysis) was used to record the motor response. Retro-reflective markers were placed on the relevant joins (iliac crest, hip, knee, tarse, toe) to enable postural tracking and correlate motor responses with optic stimuli. Trains that corresponded to the chronic pattern of stimulation were given and postural responses were recorded and analyzed.

4.5.5 CHRONIC OPTOGENETIC STIMULATION

Mice were kept free-moving in the cage and optic stimulation took place using blue lasers (470nm wavelength) and optic cables connected to the implanted optic fibers using ferrule-to-ferrule sleeves. The correct implantation of the fiber was confirmed prior to SCI via optic motor-evoked potentials (MEPs), showing hindlimb extension and forelimbs tremor as a response to rhythmic 5ms optic pulses. Chronic optogenetic stimulation in the reticular formation was performed daily for two weeks prior and four weeks after spinal cord injury. Two 90-minute-long sessions were conducted daily, during which the mice were exposed to 5-second-long optic bursts (5ms pulses, 10Hz) with a 5-second-long pre-train delay. Based on previous work, 5ms pulses were delivered to efficiently recruit reticulospinal circuits. A frequency of 10Hz was chosen for stimulation, as such frequency ranges were shown to upregulate CREB and MAPK signaling in sensory neurons *in vitro* Fields et al. (1997).

4.5.6 IMMUNOHISTOCHEMISTRY AND HISTOLOGY

After terminal anesthesia by barbiturate overdose, mice were perfused transcardially with 4% paraformaldehyde and the CNS was retrieved and processed for immunofluorescence. Primary antibodies were: rabbit anti-GFAP (1:1000 - Dako, Carpinteria, CA); chicken anti-RFP (1:500, Novus Biologicals, Littleton, CO). Fluorescence secondary antibodies were conjugated to: 555 (red) or 647 (far red) (Molecular Probes). The non-viral BDA tracer was revealed using Tyr-Cy3 (Jackson Immunoresearch Laboratories). Nuclear stain was added using 4',6'-diamidino-2-phenylindole dihydrochloride (DAPI; 2ng/ml; Molecular Probes). Sections were coverslipped using Mowiol mounting medium (Sigma, Saint Louis, MO). Tiled scans of individual whole sections were prepared using a x10 objective and the scanning function of a Leica Aperio Versa 200 Microscope (Leica, Wetzlar, Germany).

Chapter 5

AN ADULT SOURCE FOR CELL REPLACEMENT

NEW RELAYS TO SUPPORT INFORMATION

5.1 ABSTRACT

The field of spinal cord regeneration has been interested for decades in replacing the scarred lesion core with exogenous neural tissue. Previous work aimed at grafting modified stromal tissue to express facilitating factors and induce endogenous tracts' regeneration. In parallel, cell engineering enabled other groups to restore spinal cord integrity after complete injury using Embryonic Stem Cells (ESCs) and induced Pluripotent Stem Cells (iPSCs). However, apart from the ethical questions that arise from the use ESCs, these approaches have failed so far to yield significant and reproducible recovery of stepping after Spinal Cord Injury (SCI).

Some effort has been provided by different groups to propose autologous neural cell replacement options in different models of brain disorders. Autologous grafting of cortex-derived neural progenitor cells enabled significant recovery of function in non-human primates after toxic stroke and in the context of Parkinson's disease. At the caudal end of the CNS, *in vitro* studies revealed the Filum Terminale (FT) - an embryonic rest located within the *cauda equina* - as a source of adult neurogenesis, providing both astrocytes and neurons when processed and cultured.

In the present study, our strategy aims at explanting the FT from inbred adult rats, culture the obtained biopsy and develop neurospheres *in vitro*, that are subsequently grafted into the lesion core after complete SCI in an autologous-like approach. Satisfying neural tissue was obtained *in vitro*, showing free-floating neurospheres developed over 3-4 weeks of culture. When plated, these neurospheres formed new neural networks combining mature MAP2-positive neurons and GFAP-positive astrocytes. However, grafting of full neurospheres failed to promote the formation of neural bridges across complete SCIs. We hypothesize that a low cell dynamic, together with macrophage-related toxicity and failure to integrate endogenous circuits, are responsible for poor graft survival in spite of co-injected survival factors and lesion remodeling.

PERSONAL CONTRIBUTIONS

In this study, I - Sabry L. Barlatey - designed experiments and piloted the feasibility of the project. I performed surgical spinal cord injuries, Filum Terminale biopsies and spinal stereotaxic surgeries. I established and performed the *in vitro* development of adult Filum Terminale derived neural tissue. I conducted *in vitro* tissue processing and histological analyses. I processed *ex vivo* CNS tissue, performed histological analyses, acquired microscopy images and quantified graft survival. I wrote the chapter presented here above and conceived the figures.

5.2 INTRODUCTION
ADULT SOURCES OF NEURAL PROGENITOR CELLS

Neuroprosthetic rehabilitation applied after incomplete SCI has enabled robust and sustained recovery of function in rodents (Asboth et al., 2018; van den Brand et al., 2012), non-human primates (Capogrosso et al., 2016) and human patients (STIMO). However, the occurrence and extent of functional recovery has been shown to rely on the reorganization of spared supraspinal input (Asboth et al., 2018) around the lesion core. As mentioned in the previous chapters, functional recovery after more severe SCI will require the re-establishment of a tissue bridge that can be recruited by robotic neuroprosthetic rehabilitation. We demonstrated recently that axon regeneration can be achieved across non-neural lesion cores and conduct action potentials into caudal tissues (see 2 and Anderson et al. (2018)). However, the model of complete crush SCI that we used remains very controlled and only causes an infra-millimetric gap between supra- and infralesional tissues. In large and severe models of spinal cord injury, molecular-based manipulation of the lesion core may not suffice to produce the required dense bridge of permissive extracellular matrix that is required for axon regrowth. Cell replacement therapies offer the opportunity to replace wide lesion cores with growth permissive cell substrates (Assinck et al., 2017; Kadoya et al., 2016; Dulin et al., 2018).

Neural stem and progenitor cells have been studied for decades with a successful integration into host tissues and permissive effects over endogenous axon regeneration (Reier, 1985; Bregman and Reier, 1986; Jakeman and Reier, 1991). Systematic studies in rats have recently established E14 neural stem and progenitor (NSPCs) cell grafting as an efficient strategy to replace non-neural complete lesion cores with both exogenous neurons and glia. Further, NSPC grafts extend long-distance axons in both rostral and caudal directions from the lesion site and restored electrophysiological conduction across the SCI (Lu et al., 2012c). Host axons regenerating into the graft support also the hypothesis that grafted NSPC cells may get involved as neuronal relays after maturation Dulin et al. (2018); Kadoya et al. (2016)Similar and successful results were obtained with grafts of induced pluripotent stem cells (iPSCs) into complete injury sites in rats (Lu et al., 2014). Transitioning to non-human primates, human spinal cord derived progenitor cells grafted to a cervical hemisection improved forelimb motor functions (Rosenzweig et al., 2018). Our objective was at first to combine E14 NSPC grafts with neuroprosthetic rehabilitation (van den Brand et al., 2012) in a rat model of complete SCI, in order to evaluate the synergistic potential of both approaches combined for the recovery of voluntary locomotion. However, our first attempts to replace lesion sites with NSPCs produced similar results as reproduction studies. Indeed, reevaluation of the NSPC-based therapy demonstrated that the grafts tend to keep high proliferative properties, extend anarchic axonal projections and form ectopic deposits throughout the CNS (Steward et al., 2014; Tuszynski et al., 2014). Additionally, a non-negligeable number of rat subjects showed collagenous partitions of the graft, susceptible to impede action potential conduction across the lesion (Sharp et al., 2014).

Due to the undesired behavior of NSPC transplants, we focused our interest on establishing a novel cell replacement strategy based on an adult source of progenitor cells with a lower proliferation kinetics. Different groups have reported adult progenitor cells in the rodent and non-human primate CNS. Jocelyne Bloch's group has been demonstrating that a population of neural progenitor cells - identified as Doublecortin-positive cells (Bloch et al., 2011) - is distributed across the primate neocortex and can be expanded *in vitro* into neurospheres and neural tissue that combine neurons and astrocytes (Arsenijevic et al., 2001). This adult cell source enables cortical biopsies from non-dominant areas to be grown *in vitro* and grafted in the

vicinity of a lesion site in the same individual. Such autologous transplantations bypass ethical and immunological concerns of other cell replacement therapies. When grafted in the periphery of motor cortex injuries induced with ibotenic acid, Doublecortin progenitors were reported to colonize the injured site, integrate into the damaged regions, differentiate into mature neural tissue and promote functional benefits (Kaeser et al., 2011). In a Parkinsonian model in non-human primates, Doublecortin cells grafted into the caudate nucleus and appeared to ease Parkinsonian functional signs and promote local striatal and distant neuroprotective effects on the *substantia nigra* (Brunet et al., 2009; Bloch et al., 2014). So far, no tumorous transformation or aggressive behavior have been reported with this source of cells (Kaeser et al., 2011; Brunet et al., 2005). However, adult neocortex-derived tissue has only been successfully grown from human and non-human primate biopsies, as rodents seem to lack of similar progenitor cells.

Caudalized progenitor cells - i.e. stem and progenitor cells that originate from the developing spinal cord - have been shown to survive and integrate better than forebrain NSPCs in host spinal tissues (Kadoya et al., 2016; Lu et al., 2012c). Therefore, neocortex-derived progenitor cells may be of a suboptimal origin for grafting into SCI sites. Several groups have described the adult Filum Terminale (FT) - a quiescent embryonic rest located at the caudal extremity of the adult spinal cord (Arvidsson et al., 2011; Jha et al., 2013b) - as a source of progenitor cells. *In vitro* neurospheres could be obtained from Filum Terminale explants and included combined neuronal and astroglial phenotypes (Jha et al., 2013a). Because FT-derived biopsies provide a source of autologous progenitor cells with caudal properties, our aim in the present chapter is to evaluate the technical feasibility and the relevance of grafting FT-derived neural tissue for spinal cord repair in rodents.

5.3 RESULTS
POOR INTEGRITY OF FT-DERIVED CELL GRAFTS *IN VIVO*

5.3.1 NEURAL TISSUE ARISES FROM ADULT RODENT FILUM TERMINALE

Previous work from other groups documented the Filum Terminale as a niche of neural progenitor cells (Chrenek et al., 2017) and *in vitro* development of neural tissue from rodent neonatal Filum Terminale (Arvidsson et al., 2011; Jha et al., 2013a; Varghese et al., 2009). To respect clinically relevant time points, we assessed the feasibility of growing neural ecosystems from adult rats and mice *in vitro*. Whereas poor results were obtained in cultures grown out of post-mortem FT explants from adult rats, we successfully cultured neurospheres from the FT of adult subjects obtained during a live terminal surgical procedure. Whole Filum Terminale structures were explanted, enzymatically and mechanically dissociated and successfully grown free-floating *in vitro* (Fig 5.1a). Over the first 48 hours of culture, cell aggregates self-assembled and showed high metabolic activity resulting in a rotation spin. After a first week of culture, each Filum Terminale biopsy consistently induced the formation of 40-60 neurospheres, both in rats and mice. The newly formed neurospheres could grow progressively over 3-4 weeks - with mechanical passages every two weeks - before reaching an apparent developmental plateau. *In vitro* characterization of plated tissue was performed, showing

a combined expression of late stage Tuj-1 and MAP2-positive neurons, embedded in GFAP astroglial networks (Fig 5.1b-c).

To further characterize cell maturation, we plated neurospheres on a laminin-coated surface. Both rodent and non-human primate derived spheres formed planar neural networks over 10 to 21 days, extending processes. At an early stage (day post-plating 12), most cells expressed the GFAP marker, which we consider to indicate a neural progenitor state, more than astroglial maturation and reactivity. Further supporting this progenitor state, a co-expression of GFAP with the early neuronal marker Tuj-1 was documented, suggesting a pattern of neuronal differentiation for a subpopulation of the plated cells (Fig 5.1c). At a later stage, we observed fully differentiated MAP2-positive neurons *in vitro*, that lost the GFAP phenotype and demonstrated morphological features of neurons forming networks. These networks were embedded in a GFAP-positive astrocyte population.

With the perspective of *in vivo* grafting experiments, we tested cell-labelling options and were able to infect full neurospheres *in vitro* with an [AAV2/dj-CBA-GFP] viral vector. Green fluorescent labelling was documented in free-floating neurospheres, which remained after cell plating on a Laminin/Poly-L-Ornithine surface. Furthermore, FT explants were performed in ubiquitously expressing GFP Lewis rats. *In vitro* expansion of progenitor cells was successful and GFP ubiquitous neurospheres were derived from the GFP animals.

5.3.2 FT-DERIVED GRAFTS FAIL TO RESTORE SCI SITE INTEGRITY

FT-DERIVED CELLS ALONE FAILS TO COLONIZE SCI SITES

FT explantation and *in vitro* cell expansion to neurospheres were performed from GFP-positive Lewis rats or wild-type Lewis rats with subsequent labelling using the [AAV2/dj-CBA-GFP] viral vector. Our choice of involving Lewis the rat line was motivated by their inbred genetic background, that is expected to reduce risks of rejecting foreign cells. Wild-type Lewis rats received a complete crush SCI and full neurospheres from one Filum Terminale were pooled, combined as a suspension in culture medium with 3% Dicopolypeptide Hydrogel (DCH) and grafted into the lesion core without any additional factor. Cells were developed *in vitro* for 8 days, in order to obtained an optimized amount of neural progenitor cells, while preserving immaturity for further spontaneous maturation *in vivo*. Cell grafting took place on day-14 post-injury, in order to enable resolution of acute and subacute inflammatory events. Tissue analysis took place after four weeks *in vivo* showing poor graft survival, but sparse GFP-expressing cells. Strong green fluorescence was observed across the lesion core, that we associated to macrophages, based on their morphological aspect. We hypothesize that grafted cells were in a large majority phagocyted by immune cell components within the lesion core. Obviously, no neural bridging of the injured site could be obtained with Filum Terminale-derived progenitor cells.

ESC-INSPIRED COCKTAIL OF TROPHIC FACTORS

Based on the poor survival of grafted cells deprived of exogenous trophic support, we next explored options to support Filum-Terminale cell persistence into the lesion core. Previous work on embryonic neural progenitor cells from the Tuszynski laboratory [UC San Diego, California] relied on supplementing the graft with a cocktail of numerous growth factors as follows :

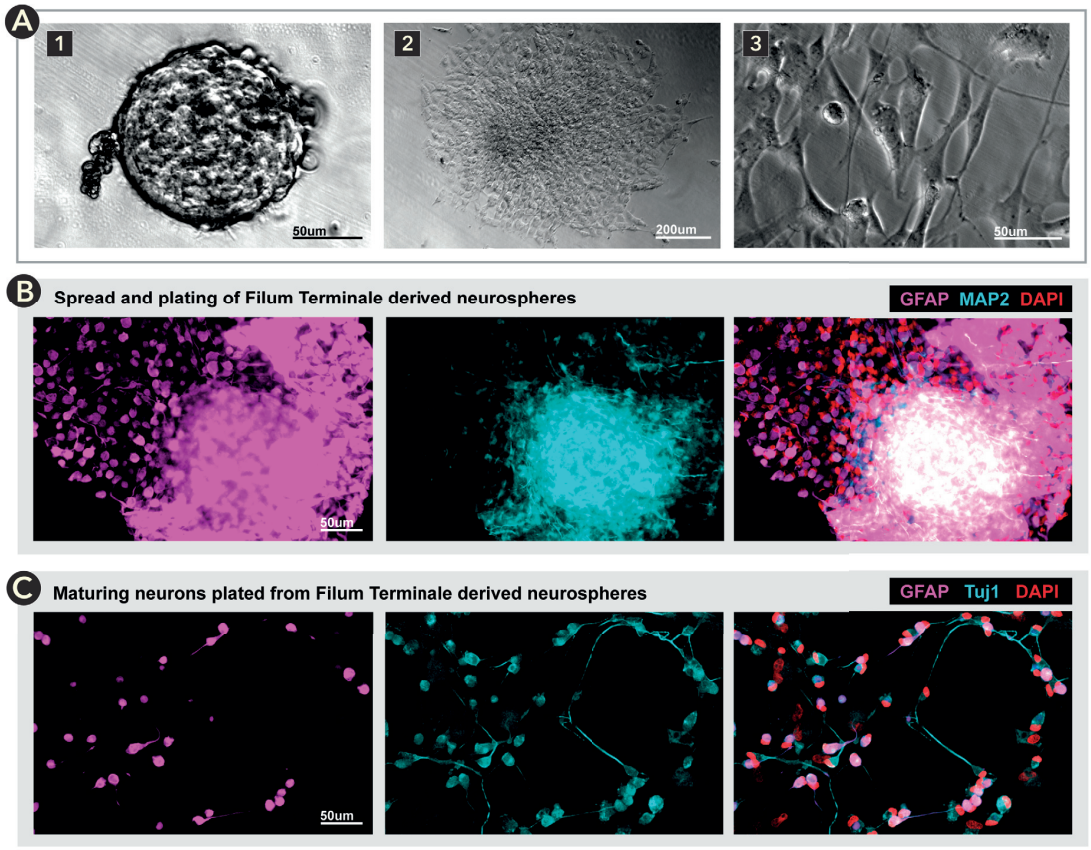

Figure 5.1: **In vitro expansion of Filum Terminale derived neural progenitor cells into neurospheres and mature neural tissue.** **A**. Transmitted light microscopy of Filum Terminale derived tissue incubated in vitro. **A1**. Representative rat neurosphere obtained at day-8 of culture. **A2**. Precipitated neurosphere spreading progenitor cells on a laminin-coated substrate. **A3**. Plated neural tissue in vitro, showing morphological features of mature neurons or late-stage neuronal progenitor cells. **B**. Plated neurosphere expressing in combination the astroglial marker GFAP (magenta) and the mature neuronal marker MAP2 (cyan). This neurosphere was plated after 14 days of free-floating culture. The phenotype and morphology of the tissue suggest self-differentiation into mature neurons within the sphere **C**. Co-expression of the astroglial and early neuronal marker GFAP (magenta) and the neuronal progenitor marker Tuj-1 (cyan). Morphological and phenotypical features correlate with a late stage process of neuronal differentiation.

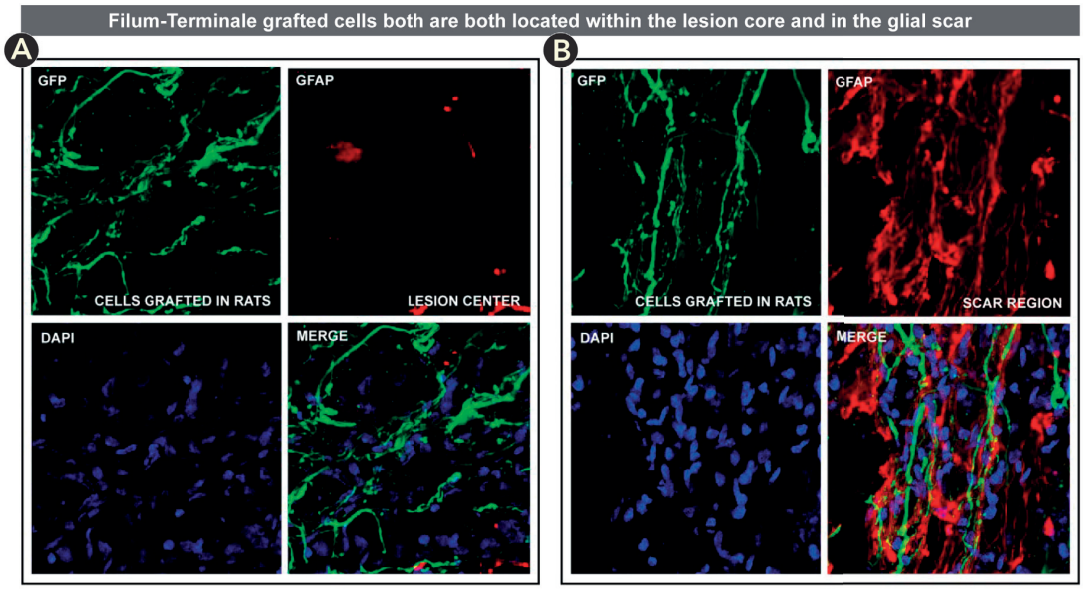

Figure 5.2: **Filum Terminale derived grafts poorly survive in vivo, but surviving cells are both located in the lesion core and among the glial scar.** **A**. Grafted cells located within the lesion core, as confirmed by the absence of colocalization between the graft (GFP, green) and the astroglial marker GFAP (red). We note a disorganized pattern of the GFP-expressing neurites, as well as a dense presence of non-neural cells suggested by nuclear labelling with Dapi. **B**. Grafted cells (GFP, green) located within the glial scar, as demonstrated by the dense astroglial matrix (GFAP, red). We note that neurites from grafted cells tend to follow the pattern of endogenous astrocytes.

brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), platelet-derived growth factor (PDGF), insulin-like growth factor (IGF-1), epidermal growth factor (EGF), basic and acidic fibroblast growth factor (respectively aFGF and bFGF), glial derived neurotrophic factor (GDNF), hepatocyte growth factor (HGF) and calpain inhibitor (MDL28170). After discussion with Professor Tuszynski's group, this cocktail was reduced to BDNF, bFGF and MDL28170, and vascular endothelial growth factor (VEGF) was added to the molecular combination. Rational for such a supplementation of the graft with exogenous growth factors was i) to extend the period of cell division in vivo with bFGF, in order to increase the occupation of the lesion cavity with grafted cells, ii) to promote neuronal survival and differentiation with BDNF, iii) to diminish apoptotic signaling in neural progenitor cells with calpain inhibitor and iii) to enhance vascular supply with VEGF and avoid ischemic events within the depot of exogenous cells. This modified graft composition slightly enhanced cell survival, showing groups of GFP-expressing cells inside the lesion core, located mainly at the proximal border of the lesion core. However, Filum Terminale cell grafts still failed to fill the lesion cavity, in spite of exogenous trophic support.

LESION REMODELING SLIGHTLY ENHANCES SURVIVAL

In a last attempt to obtain satisfying survival of grafted FT-derived progenitor cells, we tested wether - as for regenerating propriospinal axons (see 2) - a lack of permissive extracellular matrix causes an apoptotic depletion of the grafts. Rational for this hypothesis was that surviving cells in our previous experiment were mainly located near the glial scar. It was previously

demonstrated that remodeling the injured site early after injury induces protective astrogliosis and enhances extracellular substrates such as laminins (Anderson et al., 2016, 2018). Further, laminins are known as key extracellular components that i) promote neurogenesis and neuronal survival *in vitro* (Hall et al., 2008; Heaton and Swanson, 1988), and ii) support axonal growth and maturation *in vivo* (Plantman et al., 2008; Gundersen, 1987; Anderson et al., 2016). Therefore, we repeated Filum Terminale derived cell grafting - supplemented with BDNF, GDNF, VEGF and calpain inhibitor - but primed the injured site in a first place, in order to remodel the lesion core into a more permissive environment for neural tissue. EGF and bFGF were embedded in a biocompatible hydrogel and injected into the injury on day-2 post-SCI. Again, cell grafting took place two weeks after the lesion. Tissue was analyzed four weeks after grafting and demonstrated that areas that exhibit a denser laminin matrix within the lesion core allow for better survival of grafted FT-derived cells. Still, Filum Terminale progenitor cells failed to bridge the SCI site and did not extend neuronal projections into healthy tissue past the glial scar (Fig 5.3).

5.4 DISCUSSION
INTEGRATING EXOGENOUS CELLS IN A LESION ENVIRONMENT

Neural cell replacement therapies have been encountering converse limitations between cell survival and excessive proliferation. Indeed, both issues are well illustrated by the numerous and different trophic factors that were combined to support embryonic NSPC grafts (Lu et al., 2012c), that were likely chosen in order to enable *in vivo* survival and proper filling of the lesion cavity. However, such a strong trophic stimulus, associated to an intrinsic proliferative state, caused ectopic metastatisation of grafted embryonic NSPCs (Steward et al., 2014). Conversely and in spite of trophic support and intralesional laminin enhancement, cell survival of Filum Terminale-derived grafts remained minimal. Mechanisms for cell depletion likely rely on an immune response from sustained inflammatory components within the lesion core towards grafted cells. Our trophic supplementation aimed at increasing *in vivo* proliferation, promoting maturation and reducing apoptotic signaling, but did not address the immunologic response. Our aim by using an adult-derived progenitor source was to develop a cell replacement paradigm that can be applied in an autologous manner. In this circumstance, the receiver individual is grafted with its own cells, in order to bypass graft rejection concerns. An option to increase cell survival would be to immunosuppress the animal, in order to avoid phagocytation of FT-derived cells by intralesional macrophages. However, by doing so we lose the benefit of using an autologous source of cells, which is independent from any anti-rejection treatment. If we consider immunosuppressing animals with Cyclosporine-A or similar agents, involving well-established embryonic NSPCs or iPSCs would remain the more promising approaches in a functional perspective, compared to adult-derived neural progenitor grafts that struggle to survive *in vivo*.

As another option to increase survival, we could revise our cocktail of trophic factors and supplement the grafts with the exact same combination as the one applied by Mark Tuszynski's group : BDNF, NT-3, PDGF, IGF-1, EGF, aFGF, bFGF, GDNF, HGF and MDL28170. However and interestingly, we note that this cocktail includes EGF, bFGF and GDNF, that are the components that promote propriospinal axon regrowth via lesion remodeling and axon

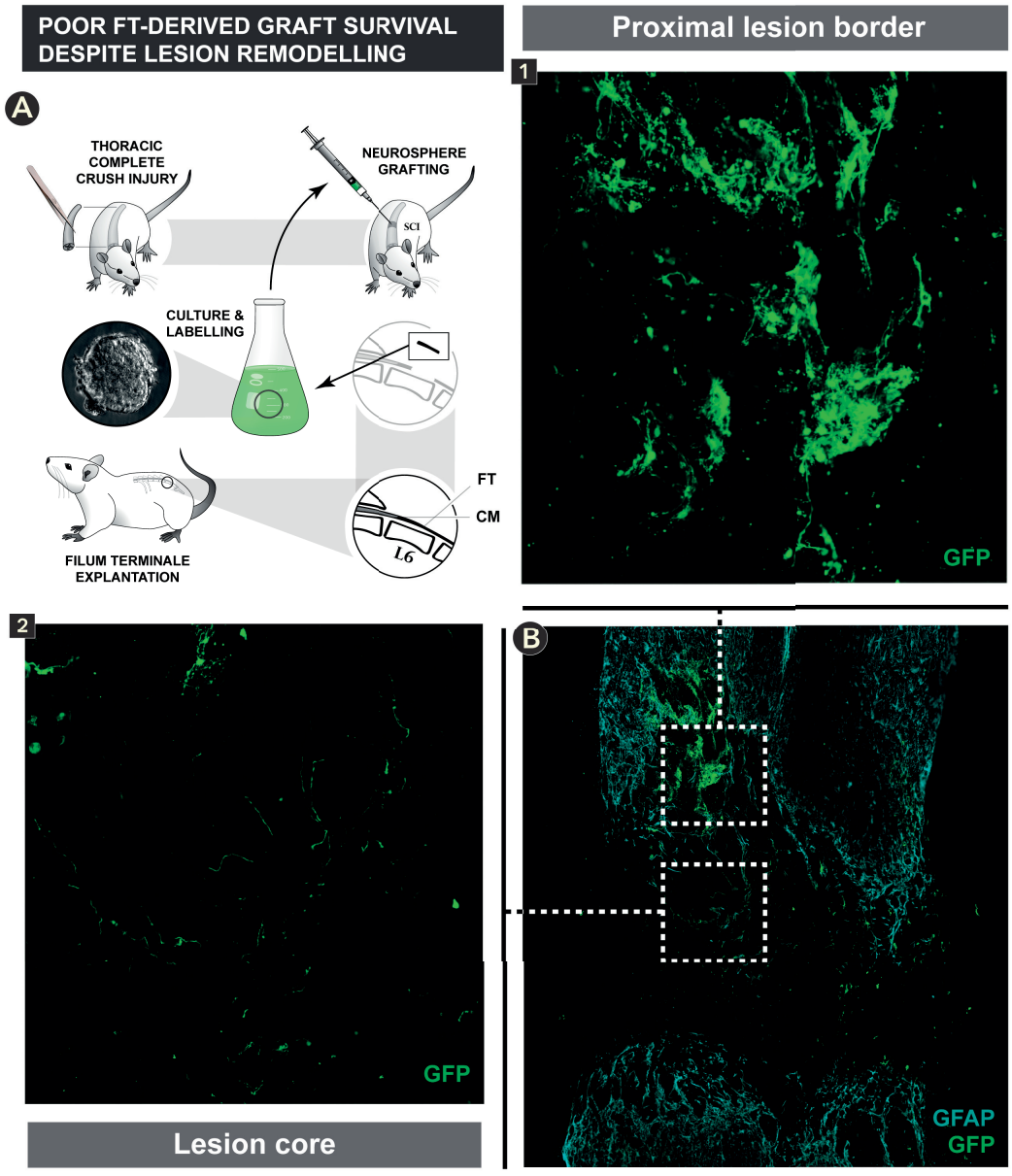

Figure 5.3: **In spite of enhanced intralesional permissive substrate, Filum Terminale derived grafts fail to bridge complete SCI sites.** **A**. Experimental design. Lewis rats received a complete spinal crush SCI. The injured site was remodeled on day-2 post-lesion, in order to enhance permissive extracellular components. In parallel, Filum Terminale biopsies were obtained from a second group of Lewis animals. The biopsies were developed in vitro for 8 days, labelled with AAV-GFP when applicable, and grafted into the injured site on day-14 post-SCI. **B**. Histologic observation of the SCI site, four weeks after grafting. Poor survival and no lesion bridging could be obtained. Grafted cells express GFP (green). SCI site is delimited with immunohistochemistry for astroglia (GFAP, cyan). **B1**. Focus of GFP-expressing grafted cells, integrated at the proximal border of the lesion, in an area located near the glial scar. **B2**. Sparse neurites from grafted cells, located at the lesion center.

guidance. Further, IGF-1 provided for NSPC graft survival is also part of our propriospinal regeneration paradigm as an intrinsic growth inductor (see 2 and Anderson et al. 2018). Rational for grafting neuronal progenitors is to provide relays for descending motor input, as theoretically ascending relays are inexistent for somatosensory input (see 3.1). As propriospinal axons are able to grow across non-neural lesion cores trophic molecular manipulations alone, cell replacement becomes less relevant for information conduction. One could argue that propriospinal axons alone may not suffice to carry supraspinal locomotor inputs to lumbosacral circuits, as no recovery of function has yet been achieved. Indeed, NSPCs were shown to enable corticospinal regrowth into - but not across - the graft (Dulin et al., 2018). Nevertheless, having corticospinal axons reach for grafted cells appears similar to promoting corticospinal reorganization that reaches for endogenous propriospinal neurons, as obtained with neuroprosthetic rehabilitation (van den Brand et al., 2012). The benefit of propriospinal regrowth over relay cell grafting is the characterized phenotype of neuronal targets for reorganizing endogenous axons. With propriospinal regeneration, supraspinal circuits - helped by volitional training - will reach for propriospinal neurons, whereas grafted embryonic NSPCs or iPSCs likely provide various neuronal subtypes as targets for regrowing or reorganizing corticospinal axons. Eventually, with the objective of restoring functional circuits after SCI, neural relay grafts are known to extend axons in both the rostral and caudal directions from the lesion center (Lu et al., 2012c). However, evidence suggests that undirected extension of neurites correlates with worsened functional outcome after SCI (Beauparlant et al., 2013). Therefore and in the light of recent developments in endogenous axon regeneration, we tend to consider neuronal relay cell replacement as an obsolete paradigm for spinal cord repair.

In a context of large-volume SCIs, glial cell replacement - more than neuronal grafts - remain an interesting approach. As propriospinal axons regenerate across complete, yet thin spinal core injuries, we currently have no insight about their behavior when regrowth is required over a long distance across non-neural tissue. In such circumstances and for translation to non-human primates, it may become interesting to support regenerating axon with exogenous reactive astrocytes and/or oligodendroglial progenitor cells. Considering the positive role of endogenous astrogliosis after axotomy (Liddelow et al., 2017; Anderson et al., 2016), A2 reactive astrocytes can contribute to permissive trophic support for propriospinal fibers. Because increased conduction was required for functional benefits of regrown axons in other systems (Bei et al., 2016), myelination of regenerated propriospinal axon may be crucial for restoring motor control. As a last remark and as increased laminin improved Filum-Terminale derived graft survival, priming the injured site with EGF/FGF before grafting may become a useful tool for other groups developing cell replacement therapies after SCI.

5.5 MATERIALS AND METHODS

5.5.1 ANIMALS

Inbred Lewis rats were involved in the described experiments in accordance with the swiss veterinary legislation applied in Vaud and Geneva. Local veterinary committees accredited the experiments under Licenses #VD3091 and #GE157/16. GFP-expressing Lewis rats were obtained from Rat Resource & Research Center (RRRC) [University of Missouri, Columbia, MO] and bred in our own facility. Wild-type Lewis rats were obtained from Charles River [Les Oncins, Saint Germain sur l'Arbresle, France].

5.5.2 SURGERIES

CRUSH SPINAL CORD INJURY

Rats were maintained under anesthesia with Isoflurane and Ketamine. A dorsal midline incision was performed and muscles were carefully detached to access the posterior aspects of the spine. Using a surgical microscope (Zeiss, Oberkochen, Germany), the inferior interspinous ligament was removed with n°5 Dumont forceps (Fine Science Tools, Foster City, CA) at the targeted thoracic level T10. A complete laminectomy was performed with n°2 forceps (Fine Science Tools, Foster City, CA) and the cord was laterally compressed for five seconds using tweezers with a tip width of 0.5mm. For SCI site labelling, the rats were then suspended in a rodent stereotaxic frame (David Kopf, Tujunga, CA) and 100nL of dark dye were microinjected using glass pipettes (50-100um tip width) controlled by micro-infusion pumps. The back muscles and skin were finally closed with respectively resorbable 6-0 and non-resorbable 6-0 sutures.

FILUM TERMINALE BIOPSY

Rats and mice were maintained under terminal anesthesia with Isoflurane and Ketamine. A dorsal midline incision was performed over the lumbar L4–L5-L6 vertebral levels. Muscles were carefully detached to expose dorsal aspects of the lumbosacral column and three-level laminectomies were performed at lumbar L4-L5-L6 levels to reveal the dural sac. Using a surgical microscope (Zeiss), the *dura mater* was incised with microsurgical scissors to access the *cauda equinae*. Lumbo-sacral roots were dissected with fine n°5 Dumont forceps (Fine Science Tools) to enable identification of the Filum Terminale extended from the *conus medullaris*. A 5mm-long section of the Filum Terminale was then explanted using microsurgical scissors and the biopsy was immediately transferred to dissociating medium. Animal were finally euthanized with a barbiturate overdose.

INTRALESIONAL DEPOT OF GROWTH FACTORS

When applicable, a single depot of DCH-EGF/FGF was performed on the second day post injury for SCI remodeling. Rats were maintained under anesthesia with Isoflurane and Ketamine. A midline skin incision was performed and the previous surgical wound was reopened using n°5 Dumont forceps (Fine Science Tools, Foster City, CA) to re-expose the SCI site. The animal was suspended in a rodent stereotaxic frame (David Kopf, Tujunga, CA) and a single midline site was injected with 1µl (2nL/sec) of DCH loaded with bioactive components (-1.1mm ventral from the cord surface). The back muscles and skin were finally closed with respectively resorbable 6-0 and non-resorbable 6-0 sutures.

CELL GRAFTING

Cell grafting was performed as an intralesional depot of biocompatible hydrogel loaded with full neurospheres, culture medium, and exogenous trophic factors when applicable. Rats were maintained under anesthesia with Isoflurane and Ketamine. A midline skin incision was performed and the previous surgical wound was reopened to expose the SCI site labelled with dark dye. The animal was suspended in a rodent stereotaxic frame (David Kopf, Tujunga, CA) and bilateral injections of graft suspension were performed into the lesion (2 sites, -1.1mm ventral from the cord surface, +/-0.4mm from the cord midline, 0.75uL/site, 2nL/sec). Injections took place using custom 30G needles mounted on a Hamilton syringe (Hamilton, Bonaduz GR, Switzerland) controlled by a microinfusion pump. The back muscles and skin were finally closed with respectively resorbable 4-0 and non-resorbable 4-0 sutures.

5.5.3 IN VITRO DEVELOPMENT OF FILUM TERMINALE-DERIVED NEURAL TISSUE

Following explantation, individual biopsies were incubated and incubated overnight at 37°C in dissociating medium combining : DMEM/F12 (1:1, Sigma), fetal bovine serum (10%, Sigma), Collagenase type II (100U/mL, Gibco) supplemented with CaCl2 (3mM,

Sigma). After 24 hours, biopsies were rinsed with culture medium made of : DMEM/F12 (1:1, Sigma), N2 supplement (1%, Gibco), B27 supplement (2%, Gibco), epidermal growth factor (20ng/mL, Peprotech), bFGF (20ng/mL, Peprotech). FT fragments were then mechanically triturated with broad-end pipette tips to complete dissociation. The obtained cell suspension was transferred to ultra low attachment culture plates (Corning) and kept for culture in humid incubators at 37°C with 5% CO2. Passages took place every two weeks by mechanical trituration and re-suspension in a 1:1 combination of old and fresh culture medium. For cell plating, neurospheres were transferred on laminin-coated coverslips and kept until cell confluence.

5.5.4 GRAFT SUSPENSION

For homogeneity, neurospheres and tropic factors were embedded in a biocompatible hydrogel. Biomaterial depots were prepared using amphiphile diblock copolypeptide hydrogels (DCH) that are CNS biocompatible, biodegrade over several weeks *in vivo* and enable volume distribution of grafted cells and cargo molecules. Diblock copolypeptide hydrogel (kindly provided by Professor Deming's group, UCLA) was reconstituted to 6.0% in culture medium and, when applicable, loaded with the following growth factors : BDNF (final conc. 50ng/µl, Peprotech), bFGF (final conc. 10ng/µl, Peprotech), VEGF (final conc. 10ng/µl) and calpain inhibitor MDL28170 (final conc. 50uM). After 8 days *in vitro*, neurospheres from single biopsies were pooled and centrifuged. Supernatant was removed to enable a 1:1 combination with the hydrogel. The graft suspension was the injected into the SCI lesion core (2 sites, DV -1.1mm, 1µl per site).

5.5.5 HYDROGEL DEPOT WITH GROWTH FACTORS

For sustained delivery, growth factors and guidance cues were embedded in a biocompatible hydrogel. Biomaterial depots were prepared using well characterized diblock copolypeptide hydrogels (DCH) that are CNS biocompatible, biodegrade over several weeks *in vivo* and provide prolonged delivery of bioactive growth factors in CNS tissue for two or more weeks after injection. Diblock copolypeptide hydrogel (kindly provided by Professor Deming's group, UCLA) was loaded with growth factors and guidance molecules. Cargo molecules were as follows human recombinant FGF2 and EGF (PeproTech, Rocky Hill, NJ). Freeze dried DCH powder was reconstituted to 3.0% or 3.5% wt/vol in sterile PBS with a combination of FGF2 (1.0µg/µl), EGF (1.0µg/µl). The combined biomaterial with cargo was then injected into the SCI lesion core [DV -1.1, 1µl] as a single site depot.

5.5.6 *EX VITRO* IMMUNOHISTOCHEMISTRY AND HISTOLOGY

Laminin-coated coverslips carrying neural tissue were recovered and fixed with 4% paraformaldehyde for 10 minutes. Tissue was rinsed with PBS and immunohistochemistry was performed using the following primary antibodies: rabbit anti-GFAP (1:1000, Dako), mouse anti-GFAP (1:500, Sigma) mouse anti-MAP2 (1:500, Sigma), rabbit anti-Tuj1 (1:500, Abcam). Fluorescence secondary antibodies were conjugated to: 488 (green), 555 (red) or 647 (far red) (Molecular Probes). Nuclear stain was added using 4',6'-diamidino-2-phenylindole dihydrochloride (DAPI; 2ng/ml; Molecular Probes). Coverslips were mounted on slides with Mowiol mounting medium (Sigma) and imaged using a fluorescence illumination microscope or a scanning confocal microscope (Zeiss).

5.5.7 *EX VIVO* IMMUNOHISTOCHEMISTRY AND HISTOLOGY

After terminal anesthesia by barbiturate overdose, rats were perfused transcardially with 4% paraformaldehyde and the CNS was retrieved and processed for immunofluorescence. Primary antibodies were: rabbit anti-GFAP (1:1000 - Dako), mouse anti-GFAP (1:500, Sigma), chicken anti-GFP (1:500, Abcam), rabbit anti-laminin (1:80, Sigma). Fluorescence secondary antibodies were conjugated to: 488 (green), 555 (red) or 647 (far red) (Molecular Probes). Nuclear stain was added using 4',6'-diamidino-2-phenylindole dihydrochloride (DAPI; 2ng/ml; Molecular Probes). Sections were coverslipped using Mowiol mounting medium (Sigma). Tiled scans of individual whole sections were prepared using a x10 objective and the scanning function of a Leica Aperio Versa 200 Microscope (Leica, Wetzlar, Germany).

Chapter 6

DISCUSSION : CIRCUIT REORGANIZATION

RESTORING UP- AND DOWN-STREAM INPUTS AFTER COMPLETE SCI

6.1 BUILDING BRIDGES
FACILITATING GROWTH IN SPITE OF INHIBITIONS

Intralesional inhibitory molecules and inflammatory processes have long been considered as the main barrier to spontaneous and therapeutic spinal repair after SCI. Effort has long been focused on inhibiting this detrimental environment, providing limited yet undebatable effects on axonal regrowth. Beyond the specific manipulations that lead to robust propriospinal regeneration, the real breakthrough in our data is to shift interest to facilitating factors that can override inhibition. Indeed, propriospinal regeneration occurs through reactive astrocytes, into the fibrotic scar, in spite of a strong presence of CSPGs that were still considered lately as a highly promising molecular target to inhibit for axon regrowth.

As discussed above 1.4.1, CSPGs have been proposed to interact with permissive extracellular substrates, thus minimizing their disponibility for axons (Zuo et al., 1998; McKeon et al., 1995), and with growth cone integrins as a competitive binder (Tan et al., 2011). Further, CSPGs may exert chelation effects on endogenous growth promoting factors (Nandini and Sugahara, 2006; Deepa et al., 2002). Beyond upregulating intrinsic growth programs, our regenerative approach relies on a saturation of the lesion core with growth factors and permissive extracellular components. Therefore, it is likely that mechanisms overlap between inhibitors degradation and lesion remodelling associated to guidance. Our recent findings likely induced more robust regeneration than previous strategies due to a larger enhancement of facilitating factors.

6.2 REORGANIZING INPUTS AFTER COMPLETE SCI

6.2.1 DEFINING RELEVANT INPUTS AND TARGETS

Locomotor circuits within the healthy CNS are conceived with an extraordinary complexity. Evidence has been gathered that gait patterns' control is plural, involving cortical structures,subcortical nuclei, brainstem modulators and spinal circuits. Within the spinal cord, long propriospinal neurons appear to coordinate fore- and hindlimbs, whereas sensory input modulates stepping via local interneurons. After incomplete SCI, these complex circuits show plastic abilities, so that therapeutic strategies can enhance spontaneous recovery of locomotion by training spared efferent - and likely afferent - in order to optimize the interactions between functional hotspots. It has been suggested that after incomplete spinal lesion, spared tracts' activity can be recruited and reoriented to support recovery.

After complete SCI, even though bridging the injury is now possible with cell replacement therapies or axon regeneration, a fundamental functional-anatomical exploration remains to be performed. Research in incomplete models has been suggesting candidate structures to rewire in order to restore functional control of locomotion. However, minimal requirements remain to be defined - in terms of anatomical rewiring between CNS structures - in order to induce defined behavioral benefits after complete SCI. A first challenge here will tend to identify, with a minimum of relays, pathways that can sustain a required and functionally sufficient exchange of efferent and afferent inputs following complete SCI. Such studies would provide specific targets, by answering the questions *where*, *how much* and *what*. *Where* - below

and above the lesion - are located the relevant centers to be reconnected in order to conduct locomotor inputs ; *how much* neuronal density is required to restore functionally significant efferent or afferent control over the identified targeted regions - without overflowing it with information ; and *what* neurotransmitter modalities are required to provide adequate control over each of the targeted regions.

Coming developmental studies may provide new tools to help neuroscience mimic parts of the CNS genesis after injury, and thus refine and optimize axon regeneration, cell replacement and chemotropic guidance. In the mean time, while biological options are being explored to tune growth properties and reestablish molecular gradients, reconnecting locomotion-relevant centers throughout the CNS will at first rely on neuroprosthetics. Especially for interrupted exchanges between distant ends of severed tracts, in neuronal pathways that offer poor anatomical options for relay recruitment, neuroprosthetics already offers neuromodulatory technology that can be combined with neuroregeneration and restore part of the required inputs. Future progress in the engineering of biocompatible materials and implantable devices might even wholly fulfill the 'post-SCI neuromodulation' contract and render neuroregeneration obsolete.

6.2.2 INTEGRATING NEURONAL CELL GRAFTS

After decades of exploration, recent procedures have enabled significant replacement of anatomically complete SCI sites with endo- or exogenous neural tissue. Nevertheless, both our and other regenerative paradigms (see 2), as well as cell replacement therapies, have failed to produce consistent and reproducible behavioral benefit. Whereas ESC and iPSC cell grafting approaches have successfully filled SCI lesion cores with neurons *de novo*, endogenous axonal tracts appear to penetrate the graft in an organotypic pattern, but fail to bridge the injury towards functionally-relevant targets. Moreover, cell grafts were claimed to extend long-distance axons throughout the CNS, away from the SCI/graft epicenter, that reach targets that may not be involved in locomotor neural pathways. It has been shown that an excessive presence of multiple inputs after SCI can prevent recovery of function, due to a disorganized input that disrupts coherence within locomotor circuits. In that sense, grafted exogenous neurons may offer, within the SCI site, confounding targets to regenerating endogenous tracts - as suggested by the failure of endogenous axons to exit and bridge ESC and iPSC grafts. So far, relay grafts have not proven to conduct information to targeted structures, as their axonal projections will require new strategies to follow pre-defined patterns and build organized fiber tracts *de novo*. Future developmental studies may provide molecular strategies to properly guide exogenous grafts to the wanted targets.

6.2.3 INTEGRATING REGENERATED AXONS

Compared to relay cell replacement, axon regeneration appears more susceptible to restore coherent conduction of information. Meanwhile, even though propriospinal axons have been shown as a key component for functional recovery in selected models of incomplete SCI, guiding them across the lesion to the vicinity of healthy infralesional circuits does not suffice to restore hindlimb movements. Next research questions and mechanistic investigations will need to define - or at least suggest - minimal requirements for neuro-regeneration to support voluntary stepping. A first concern addresses the recruitment of regenerated propriospinal neurons by descending volitional inputs. Electrophysiological experiments will need to be conducted, in

order to demonstrate evoked potentials below complete lesions from supraspinal stimulation. Subsequently, chronic motor cortex recruitment may contribute to reinforcing reorganization of infralesional propriospinal projections.

One could wonder wether the extent of propriospinal regeneration that we documented suffices to conduct information to muscle synergies. Considering our electrophysiological, signal is propagated three millimeters below the injury, corresponding to the level were the second depot of guidance cues is performed, but does not reach further caudal circuits. Therefore, functional information would rely on the recruitment of secondary endogenous relays below the lesion. We are currently investigating the effects of delivering delayed additional guidance caudally to the first two depots, in order to induce more distant regeneration. Evoked motor recruitment can then assess the potential presence of restored muscle responses to cortical stimulation with translesional conduction of descending input. It is however likely that neuroprosthetic training will be required for reafferented lumbosacral circuits to receive coherent input from supraspinal structures.

6.2.4 RECRUITING MUSCLE SYNERGIES VIA REGENERATED AXONS

It is possible that propriospinal neurons alone do not suffice to support descending motor commands through complete injuries. In such circumstances, promoting regeneration of complimentary descending tracts may reinforce the coherence of information reaching infralesional circuits. Reticulospinal axons appear as a promising source of descending input, in the light of recent mechanistic developments about the functional benefits induced by neuroprosthetic rehabilitation in an incomplete contusion model of SCI (Asboth et al., 2018). Our attempts to promote reticulospinal regrowth have for now yielded limited regeneration, but nevertheless are encouraging and suggest that the field of spinal cord repair will need to troubleshoot the requirements for tract specific axon regeneration. Identifying proper determinants will likely enhance the density of regrown reticulospinal axons.

However, the complete absence of any somatosensory feedback to the motor cortex after complete SCI appears as a major obstacle to functional recovery. In the context of our current exploration, motor thalamus neuromodulation is a robust candidate to restore gait-related feedback to M1. Indeed, recent work has been confirming the role of the rostral thalamus in motor planning and motor learning (Tanaka et al., 2018; Gao et al., 2018). Recruiting motor thalamic nuclei based on hindlimb muscle activity may enable the motor cortex to reorganize around and reach for regenerated propriospinal neurons after complete lesion. Complimentary neuroprosthetic activation of infralesional circuits may then enable rewiring of the locomotor centers, in a similar manner to the effects observed in an incomplete double hemisection model of injury (van den Brand et al., 2012).

6.3 CLINICAL SIGNIFICANCE

Obviously, the successful regenerative work presented in the present document (see 2) remains for now at a 'proof-of-concept' state, and is neither functional nor safe enough for clinical translation. In order to develop a clinically translatable paradigm for propriospinal regeneration, functional benefits will first need to be achieved. Subsequently, the safety of manipulating intrinsic growth programs will need to be addressed and optimized. To do so, temporary

induicible viral vectors would enable an activation of signaling pathways, such as mTOR and Jak-STAT, that relies on pharmacological agents. Such technology would avoid uncontrolled and excessive growth that are susceptible to lead to tumorous transformation.

Conversely, if strong benefits are induced by motor thalamus neuromodulation in animal models of incomplete or complete SCI, translation to clinics is already realistic. Indeed, commercial Deep Brain Stimulation (DBS) electrodes currently enable the targeting of thalamic and subthalamic nuclei in human conditions of movement disorders. Such DBS technology could as well be applied to the motor thalamus with spinal cord injury indications.

6.4 CONCLUSION
FINAL WORDS

Spinal cord injuries, as well as other neuroscientific challenges, have seen over the last century numerous empirical suggestions to restore integrity of tissues and promote functional recovery. In the light of the present work and considering the coming challenges, neuroscientists will need to build new methodological constructs in order to i) identify all dimensions of the SCI problem, ii) investigate hypotheses and iii) develop technics and technologies aiming at answering questions incrementally. Recent and current developments have been shifting paradigms from empirism to mechanistic approaches, in order to unveil key therapeutic leads and establish the minimal requirements to reach scientific landmarks - step by step - towards clinically successful therapies. However, the complexity of both the neuroregenerative workflow and reorganization requirements is such that expertise from most neurobiological subfields will need to interact in a collaborative manner, in order to recognize relevant questions, hypotheses and solutions.

Eventually, as neuroregenerative mechanisms are only starting to be unveiled, biological strategies might well be late to yield the first functionally significant breakthroughs in the field of complete SCIs. Indeed, current neuroengineering technologies already enable modulations of circuits that may be applied after anatomically complete spinal lesions. Nevertheless, sustained benefits in human patients will likely require biological therapies to modulate tissues and reorganize circuits, with potential contributions from temporary-applied neuroprosthetics. Such therapies would enable neuroprosthetic-based benefits and bypass long-term histologic reactions to neuromodulatory foreign bodies.

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