Reticulospinal and corticospinal axon regeneration after complete spinal cord injury

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Régénération des voies réticulospinale et corticospinale au travers d'une lésion complète de la moelle épinière

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Summary

Neuroprosthetic rehabilitation demonstrated that significant functional benefit could be achieved with lumbosacral neuromodulation in both human and animal models of spinal cord injury. It promoted the recovery of voluntary leg movements through the reorganization of residual reticulospinal and propriospinal projections pathways. However, in case of complete spinal cord injuries (SCI), which isolate the circuits under the lesion from any supraspinal control, the outcome of neuroprosthetic rehabilitation is still not sufficient. Indeed, it will require the restoration of robust regrowth and sprouting of several types of axons across the injury. Axons fail to regrow across spinal lesions because of different inhibitory mechanisms. It has been demonstrated that this spontaneous axon regeneration failure can be reversed by i) stimulating the neuronal intrinsic growth capacity using viral technology, ii) remodeling the lesion core with growth factors, in order to create a more permissive environment, and iii) guiding axons with chemo-attractive molecules across and beyond the SCI site. It was thus demonstrated that propriospinal axons are able to regrow and build a robust descending bridge across complete SCIs when the needed facilitators are provided. However, this robust propriospinal bridging failed to promote functional recovery by itself. It might be explained by an insufficient descending motor control partly supported by other systems such as the reticulospinal tract (RtST) and the corticospinal tract (CST). Therefore we wanted to study the regenerative potential of the RtST and CST pathways. The RtST arises from the brainstem and reaches for the spinal cord acting as relay for descending motor cortical commands. The CST is the main descending motor cortical command arising from the primary motor cortex.

In the present study, we applied the same strategy to enhance sprouting and regrowth of reticulospinal and corticospinal neurons across anatomically complete SCI. We first activated the neuronal intrinsic growth capacity of both tracts using viral technology. The lesion environment was then remodeled with growth factors, delivered using a biocompatible hydrogel. Finally, we established chemical axon guidance using chemo-attractant molecules. These interventions were delivered with a spatiotemporal profile corresponding to the axon growth sequence during development. We did not obtain any CST regeneration, due to the severe crush injury model inducing extensive CST axons degeneration probably caused by ischemic phenomenon. Regarding the RtST, we obtained significant reticulospinal regeneration into the lesion core with some fibers growing across the lesion reaching the healthy caudal tissue. This regeneration remained limited though as compared with the propriospinal results indicating the importance of identifying complementary strategies to increase the density of the regenerated tract and to attract the axons in the healthy tissue below the SCI. Our ultimate goal is to restore anatomical communications across complete SCI and promote their functional integration using neuroprosthetic rehabilitation program.

Key words: Spinal cord injury, Reticulopsinal tract, Corticospinal tract, Axon regeneration, Neuronal growth program

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Introduction

According to the World Health Organization between 250'000 and 500'000 people are affected by spinal cord injury (SCI) each year worldwide. Traffic accidents and falls are the primary causes of SCI and the age of peak incidence is typically between 15 and 30 years with a greater percentage of males than females (Singh, Tetreault, Kalsi-Ryan, Nouri, & Fehlings, 2014). Spinal cord injuries result in a disruption of the normal motor, sensory and autonomic functions below the lesion. The neurological deficits depend on the location and the extent of the lesion and can vary from mild sensitive and motor defects, bladder and bowel dysfunction to tetraplegia. Depending on the extent of the lesion, the SCI can be either complete or incomplete and is classified according to the ASIA impairment scale published by the American Spinal Injury Association (1982) and revised in 2011 (Kirshblum et al., 2011). Most of the time, the spinal cord is compressed leaving some neurological tissue intact. SCIs result in chronic neurological defects associated with a decrease of autonomy, life expectancy and social well-being leading to a drastic decrease of the quality of life. The management of SCI requires significant amount of health care resources and is associated with considerable costs for the patients, their families and society (Singh et al., 2014). The actual care relies on acute surgical decompression and stabilization of the spinal cord, prevention of the secondary damages and neurorehabilitation (Mothe & Tator, 2012). Incomplete injuries spare some functional tissue across and around the lesion allowing spontaneous reorganization of circuits associated with partial recovery of function. Activity-based therapy, which attempts to reactivate muscles below the injury, has shown significant improvement of neurological functions and leads to walking recovery in some patient with incomplete motor spinal cord injury (Behrman, Ardolino, & Harkema, 2017; Jones et al., 2014). Recently, it was reported that epidural spinal cord stimulation associated with intense locomotor training in standing and stepping allowed the recovery of intentional walking over ground in patient with chronic motor complete paralysis (Angeli et al., 2018; Rejc, Angeli, Atkinson, & Harkema, 2017). In addition, it was demonstrated that epidural electrical stimulation (EES) delivered in a spatiotemporal manner associated with intense over ground locomotor training enabled restoring voluntary control of walking in patients with complete spinal cord paralysis. The functional improvement was persistent over time even without EES (Wagner et al., 2018). In these approaches the recovery is supported by spared fibers within the spinal cord. In case of complete SCI, in which all the connections are lost, recovery is hardly conceivable without restoring some descending pathways such as the Corticospinal tract (CST), the Reticulospinal tract (RtST) and the Propriospinal system (PrSp). However, the spontaneous axons regrowth ability within the adult central nervous system (SNC) is quite limited. Potential mechanisms include a low intrinsic growth capacity of mature CNS neurons, the presence of external inhibitory factors associated with fibrotic tissue or myelin and the absence of external growth stimulation and environmental supportive cues (Anderson et al., 2016). Several strategies are being investigated, but restoring motor function after complete SCI remains to this date a

challenge. Our objective is to induce axon regrowth throughout complete lesions, in order to support the establishment of new synapses with coherent targets and restore organized descending circuitry needed for functional recovery. For this purpose, it is necessary to create a more permissive lesion environment, increasing the regeneration capacity of the injured axons as well as attracting them across the lesion into targeted networks below the injury.

Descending motor pathways

Voluntary movement involves numerous brain regions interacting as a circuit to generate motor commands. Different descending motor pathways further process and conduct these signals for the creation a purposeful movement. Roger Lemon described in 2008 these descending pathways base on the work of Hans Kuypers. They are composed of i) brainstem pathways, ii) the "emotional motor system", iii) the Corticospinal and the Corticobulbar pathways". The ventromedial brainstem pathways are composed of the tectospinal and the vestibulospinal systems, as the reticulospinal and bulbospinal projections arising from the pontomedullary reticular formation (PMRF). These tracts reach for the ventromedial part of the intermediate zone (IZ), a white matter structure located between the grey dorsal and ventral horns in the spinal cord. The reticulospinal system, arising mainly from the gigantocellular reticular nucleus, is involved in the control of proximal synergies in the forelimb and hindlimb, and gates postural changes required for locomotion. The dorsolateral brainstem pathways regroup the rubrospinal tract and the pontospinal tract, both projecting to the dorsal part of the IZ and control short propriospinal neurons. The "emotional system" is composed of a number of serotoninergic (5-HT) and noradrenergic (NA) projections that further influence movement in the spinal cord (e.g. 5-HT influences the spinal reflexes). The corticospinal (CST) and corticobulbar (CB) pathways regroup axons arising from different cortical regions (mainly motor areas) that innervate all regions of the spinal gray matter including motor neurons (MN) at all levels of the spinal cord (CST) and the brain stem (CB). It has been shown that direct corticomotoneuronal (CM) projections are only present in primate and support the fine manual dexterity required for prehensile purpose. In rodent, the information transmitted from the CST to the motoneurons is made via interneurons and reticulospinal neurons. In these species, it seems that the reticulospinal control of movement exerts a high relevance (Alstermark & Ogawa, 2004; Lemon, 2008; Lemon & Griffiths, 2005) (*fig.1*).
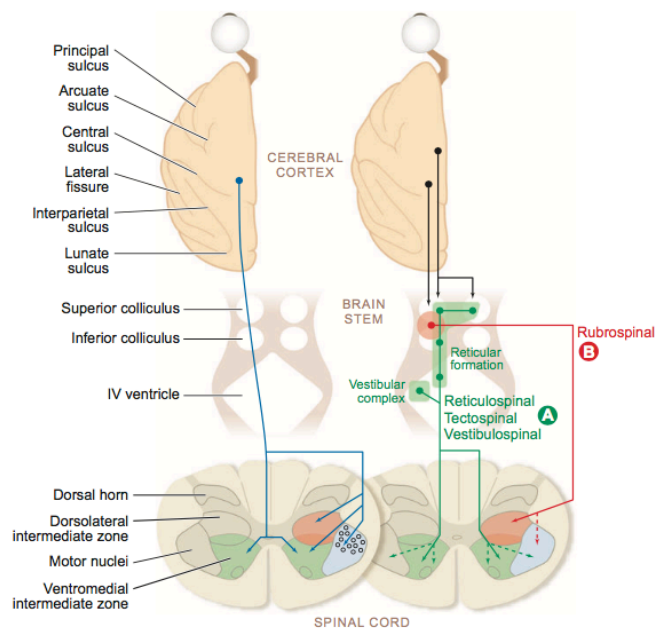
Fig. 1. Schematic representation of the descending pathways. The corticospinal tract reaching for motoneurons in the ventral horn (blue). The corticobulbar tract reaching for the brainstem (in black). The ventral brainstem pathways, encompassing the reticulospinal, tectospinal and vestibulospinal tracts (green). The dorsolateral brainstem pathway, (the rubrospinal, in red). The intermediate zone (IZ) is represented in red.

In addition to long descending motor pathways, propriospinal neurons (PSNs) are know to link different spinal segments, coordinate spinal activity and support both excitatory and inhibitory on motor neurons. In addition they project to lateral reticular nucleus neurons and spinocerebellar neurons giving information to the cerebellum about the upcoming command signal (Alstermark, Isa, Pettersson, & Sasaki, 2007). Different types of PSNs have been described; the so-called long PSNs and short PSNs. The long PSNs originate in the cervical enlargement (C3-C5) and travel in the ventral and lateral funiculi to reach the neurons in the ventral horn of the lumbosacral enlargement. They are responsible for forelimb and hindlimb coupling. Some short PSNs have been described arising from the C3-C4 cervical segment and reaching C6-T1 segments. They are involved in visually guided forelimbs movements (Bareyre et al., 2004). Recent studies demonstrated the potential of the propriospinal pathway to support motor recovery in incomplete spinal cord injuries.

Physiopathology of SCI

The spinal cord is well protected in the middle of the spine. Nevertheless, when the integrity of the spine is compromised, the spinal cord can undergo different insults such as contusion, laceration, transection, etc. In the acute phase, the primary injury mechanism causes direct death of the different cells at the impact site and plasma membranes compromise leading to ionic homeostasis modifications and neurotransmitter accumulation (Thuret, Moon, & Gage, 2006). The initial impact also leads to vascular damage with local rupture of the blood-spinal cord barrier rapidly accompanied by the infiltration of blood-derived cells and blood components infiltrating the lesion to resolve hemorrhage phenomenon. In addition, platelet influx signals to

local cells to produce extracellular matrix component such as collagen, laminin and fibrin allowing neutrophils, macrophages and other leukocytes infiltrating the lesion to monitor for pathogens, remove debris and provide wound repair signals (Burda & Sofroniew, 2014). Local rupture of the blood-spinal cord barrier causes tissue swelling through accumulation of fluid (edema) at the damaged site exacerbating tissue damage. After the initial mechanical damage, a cascade of vascular, cellular and chemical events arises and causes further tissue loss and dysfunction (secondary injuries). Vascular damages reduce blood flow resulting in tissue ischemia leading in nutrient deprivation, metabolic stress, liberation of reactive oxygen species (ROS) causing neuronal and glial apoptosis in the vicinity of the lesion (Hagg & Oudega, 2006). Permeabilisation of the blood-spinal cord barrier promotes the infiltration of systemic inflammatory cells and proinflammatory molecules (Il-6, TNF-alpha...), which increase the extent of the inflammatory response necessary for the clearance of debris but, at the same time, can exert a toxic effect on neurons and glial cells exacerbating the injury (Oyinbo, 2011). In a second phase of the inflammatory response, a shift toward anti-inflammatory cells (M2 macrophages) and cytokines (IL-10) occurs and promotes tissue repair (David & Kroner, 2011). Immune suppression experiment has failed to exert any benefit suggesting that both inflammatory phase responses are likely crucial for homeostasis within immunity (Schwab, neurobiology, 2014, n.d.). Another aspect of the acute phase is the glutamate concentration increase that causes glutamate-induced excitotoxicity affecting especially neurons and oligodendrocytes - that express a lot a GLUT receptors – through apoptotic process leading to demyelination of healthy axons (Oyinbo, 2011). Severed axons then undergo the so-called Wallerian degeneration process, in which axons die-back towards the cell body. They generally survive but fail to regrowth due to the lack of trophic support and growth program (Plunet, Kwon, & Tetzlaff, 2002). After the acute inflammation phase, the lesion enters a phase of proliferation and cell replacement. Different types of cells including fibroblast lineage cells, inflammatory cells, endothelial cells, neural and glial lineal progenitor cells and scar-forming astrocytes migrate toward the lesion. Astrocytes encounter a functional and morphological transformation and migrate at the periphery of the lesion in the so-called astrogliosis process. During this process the lesion segregates into three compartments: i) a core of non-neural tissue in the center of the lesion (= fibrotic scar) ii) a dense astrocyte scar (= the glial scar) surrounding the lesion core iii) a transition peri-lesion perimeter. The fibrotic lesion core does not enable conduction of information across the SCI site, because of its cellular composition (fibroblast lineage cells, inflammatory cells and endothelial cells). In addition, it expresses different molecules such as collagen-1 and chondroitin sulfate proteoglycans (CSPGs) that are thought to inhibit regrowth of damaged axons. In the other hand, the fibrotic scar appears to contribute to restore tissue homeostasis by preventing the extension of the lesion to surrounding tissue. In addition to regrowth inhibition the fibrotic scar has been considered as a physical barrier to growing axons (Soderblom et al., 2013). In some areas of the fibrotic scar,

fluid cystic formations of variable size can growth (Burda & Sofroniew, 2014). The second part of the lesion, the glial scar, is composed of scar-forming astrocyte tightly assembled around the fibrotic lesion core. Acutely, they exert a neuroprotective function by restricting the spread of inflammatory cells into the surrounding healthy tissue. The peri-lesion perimeter is composed of a viable neural tissue containing reactive gliosis that gradually decreases when reaching healthy neural tissue (Anderson et al., 2016; Burda & Sofroniew, 2014). Different phenotypes of astrocytes can arise depending on the inflammatory conditions. For example, in the context of neuroinflammatory and neurodegenerative disease reactive astrocytes express a cytotoxic A1 phenotype, which is detrimental to support and maintain new synapses and to phagocyte myelin debris. In addition, this A1 phenotype appears to be toxic toward injured neurons and existing synapses (Liddelow et al., 2017). In case of traumatic axotomy astrocytes express a A2 transformation, which appears to support neuron survival and axon regeneration (Anderson et al., 2016). These works suggest that the cellular mechanisms involved in response to SCI are complex and that the glial scar is primordial for balancing the necessity to clear the debris of dead cells and sparing the maximal of healthy tissue.

Spontaneous plasticity and recovery

The spontaneous repair capacity of the mammalian adult spinal cord is very limited. Indeed, almost no neurogenesis can occur and long-distance regeneration of CNS fibers does not take place. However, compensatory reorganization occurs in multiple descending motor systems, which sprout and innervate denervated spinal target promoting some extent of recovery that relies on the density of spared fibers (Filli & Schwab, 2015). In addition, new glial precursor cells can arise, axon sprouting takes place (Beattie et al., 1997), and compensatory rewiring can rely on spinal and cortical plasticity (Raineteau & Schwab, 2001; Weidner, Ner, Salimi, & Tuszynski, 2001) It has been show that new spinal circuits can bypass the lesion via sprouting of the CST tract and promote functional recovery after incomplete SCI. Indeed, Bareyre and colleagues demonstrated that after a mid-thoracic dorsal spinal cord hemisection in rat, CST axons sprout and connect with long PSNs, which in turn increase their arborization and control over lumbar motor neurons. These newly formed propriospinal relays restore CST electrophysiological conduction below the injury, as well as functional recovery (Bareyre et al., 2004). In addition, in non-human primate model of spinal cord hemisection, it was demonstrated that the contra lesional corticospinal tract can sprout and reconnect lumbar circuits below the injury (Rosenzweig et al., 2010). The implication of propriospinal relays in spontaneous functional recovery was confirmed in rodent model of incomplete spinal cord injury. After performing two staggered double hemisections performed at different time points, it was shown the animals spontaneously recovered full weight-bearing locomotion, while stepping was restored (Courtine et al., 2008). These studies demonstrated the pivotal role of spared neural tissue, such as the propriospinal system, in the reorganization of circuits to bypass incomplete injury site after SCI.

Spinal cord repair strategies

Cell therapies

Cell replacement after SCI have been investigated as a therapeutic approach for decades. As previously described, the non-neural lesion core fails to support axon regrowth. The idea is thus to replace dead neurons and/or astrocytes with exogenous cells in order to create a more suitable environment for axon regrowth (Thuret et al., 2006). At the same time, the cellular material is used to replace cystic formations and form bridges across the lesion site. Other mechanisms are hypothesized to mediate functional improvement such as neuroprotection, neuronal relays formation, axons sprouting, myelin regeneration, immunomodulation, glial scar modulation (Assinck, Duncan, Hilton, Plemel, & Tetzlaff, 2017). When transplanted cells are able to survive and form synapses with host neurons, they can relay descending information to infra-lesional circuits. Prof Tuszynski's group demonstrated that embryonic neural stem and progenitor cells (NSPCs) grafted into transected spinal cord of rat could differentiate into neurons and glial cells, fill lesion's cavity, extent long-distance axons into the host tissue that support conduction of action potential across the lesion and support some functional benefits (Lu et al., 2012). The same strategy demonstrated that human spinal cord neural progenitor cells (NPCs) could improve forelimb motor function after cervical (C7) hemisection in Rhesus monkey (Rosenzweig et al., 2018). Furthermore they demonstrated that spinal cord NPCs could self-organize in partially normal spinal cord cytoarchitecture and receive appropriate innervation from regenerating host axons projections when transplanted in SCI in rat (Dulin et al., 2018). Another recent study reported robust corticospinal axon regeneration associated with recovery in forelimb function after grafting NPCs into SCI's site of rats (Kadoya et al., 2016). Relay grafts appear to be an interesting strategy in restoring connectivity in the vicinity of SCI. However, NSCPs are multipotent cells in the neural lineage that can self renew and proliferate rapidly raising some safety concern related with tumoral transformation. Various other candidate cell types have now been explored in different models of SCI, including Schwann Cells (SCs), Oligodendrocyte Progenitor Cells (OPCs), Olfactory Ensheating Cells (OECs), mesenchymal stem cells (MSCs) for their ability to provide neuroprotection, deliver trophic factors and promote axon regeneration to endogen tissue (Assinck et al., 2017). For example Schwann cells have been demonstrated to increase the spared fibers and tissue integrity, reduce cystic formation and enhance functional recovery in contusion model of SCI in rat (Barbour, Plant, Harvey, & Plant, 2013). They are known to produce BDNF, a neuroprotective agent. In addition to their neuroprotective effect SCs, MSCs and OECs have also been demonstrated to bridge SCI. It is likely that the trophic factors secreted by these non-neuronal cells contribute to the regenerative effect (Lu et al., 2012). Recently a new potential source of NSC has been described in the Filum terminal (FT), which is a vestigial developmental structure

binding the medullary cone with the coccyx. This structure has been shown to contain neuronal progenitor cells that can proliferate, and slowly differentiate in vitro, making them less prone to induce malignancy (Chrenek, Magnotti, Herrera, Jha, & Cardozo, 2016; Jha, Liu, Chrenek, Madsen, & Cardozo, 2013). We tested this approach and reported that FT cells can survive in vitro and form neurospheres composed of neurons, oligodendrocytes and astrocytes. However, when transplanted into SCI sites in inbred rats (autologous-like approach), their survival rate and ability to promote axon regrowth through the SCI was very limited despite co-injection of survival factor and lesion remodeling factors.

In summary, numerous strategies based on cell replacement have been focusing attention on exogenous tissue to obtain functional benefits by rewiring with the neighboring healthy tissue and showed great potential but remain empirical approaches. Indeed, the complexity of interactions between transplanted cells and a host spinal environment renders both circuits control and mechanistic understanding difficult. In addition, many of these approaches have been involving stem or progenitor cells, addressing concerns such as iatrogenic development of malignant mass, immunological rejection and rising ethical debate. Finally, it seems clear that combined repair strategies are more prone to provide functional benefits, compared to cell grafting alone.

Myelin-associated inhibitors

As previously exposed, axonal regeneration within the adult CNS is very limited. Various environmental factors have been described as axonal growth limiting factors associated with myelin debris, the glial scar and axonal component after SCI (He & Jin, 2016). Chondroitin sulfate proteoglycans (CSPGs) are molecules that as been described to inhibit axon growth in both in-vitro experiment (SNOW, BROWN, of, 1996, n.d.) and in in-vivo experiment. Indeed, CSGPs have been suggested to be upregulated by reactive astrocytes after SCI supporting the idea that the glial scar constitutes the major obstacle to regeneration in the CNS after SCI (Davies, Goucher, Doller, & Silver, 1999). CSGPs inactivation strategies have been performed with chondroitinase ABC (enzymatic digestion), which promoted regeneration of some axonal tracts (Grimpe & Silver, 2004; Mckeon, Höke, neurology, 1995, 1995). More recent work has shown that the proper sensory axon regeneration can occurs after SCI without inhibiting CSCPs. level of chondroitin sulfate proteoglycan (CSPGs) was not reduced by ablating scar-forming astrocytes showing that a large amount of CSPGs is produced by non-astrocyte cells and that CSPGs inhibition can be overridden when correct growing cues are provided (Anderson et al., 2016). Contrary to the CNS, the peripheral nervous system (PNS) supports axon regeneration. This difference led to the discovery of a myelin-associated molecule, NOGO, which is expressed in oligodendrocytes but not in Schwann (GrandPré, Nakamura, Vartanian, Nature, 2000, n.d.). In addition two other molecules have been identified as inhibitor in the CNS: Myelin-associated glycoprotein (MAG) and oligodendrocyte-myelin-glycoprotein (OMgp). Different groups studied the potential of

NOGO, OMps and MAG inhibition, but results were inconsistent in between studies (Geoffroy & Zheng, 2014). It was for instance demonstrated that antibodies against NOGO (anti-NOGO) allowed some degree of axonal regeneration after SCI (Thallmair et al., 1998). Moreover, by deleting the three inhibitors (NOGO, MAG and OMgp) some limited CST axon regeneration was also demonstrated (Cafferty, Duffy, Huebner, & Strittmatter, 2010). However, another group reported that the suppression of the three inhibitors failed in enhancing CST regeneration (Lee et al., 2010). This disparity between studies about CSPGs as well as myelin-associated inhibitors highlights our partial understanding of the mechanisms involved. Nevertheless, modulation of growth inhibitors remains a good candidate to promote axonal regeneration in the CNS but will require to be combining with others strategies (boosting the axonal intrinsic growth capacity for instance) to elicit robust functional recovery.

Neuronal intrinsic growth capacity

The growth potential of axons in the adult CNS is very limited after developmental completion of axogenesis. Indeed, neuronal growth programs are upregulated during development until the axons, attracted and repelled by guidance molecules, reach their final target. Different intracellular signaling pathways control neuronal growth control in a tract-dependent manner. In 2008, Zhigang He's lab analyzed potential candidate genes for axon growth control, using a virus-knockdown approach. They found that a deletion of PTEN – known as a negative regulator of the mTOR pathway - enhances axon regeneration after optic nerve injury (Park et al., 2008). In addition, they demonstrated that a deletion of SOCS3, a negative inhibitor of the growth-controlling STAT3 cascade, further supports axon regeneration after optic nerve lesion (Smith et al., 2009). In a combinatory approach, they demonstrated that a co-activation of mTOR and STAT3, performed with a co-suppression of PTEN and SOCS3, promotes robust axonal regrowth after optic nerve crush (Sun et al., 2011). Exploring other axon tracts, Professor He's group established that a deletion of PTEN also promotes CST regeneration past SCI (K. Liu et al., 2010). However, a main concern regarding this approach relies on PTEN being a tumor suppressor. Its inhibition might lead to tumor formation, rendering its clinical applications less realistic. Interestingly, it has been shown that the activation of three specific genes can induce a robust axonal growth in a mTOR-dependent manner without deleting PTEN. These three genes code for the growth factors insulin-like growth factor 1 (IGF-1), ciliary neurotrophic factor (CNTF) and osteopontin (OPN). Osteopontin has also been shown to sensitize the CNS to growth factors (He & Jin, 2016). This approach enabled robust regrowth of retinal ganglion cells following axotomy (Duan et al., 2015) as well as recovery of visual function (Bei et al., 2016). In the future, this manipulation could replace genetic-base deletion of PTEN with a temporal ligand-receptor signaling activation of mTOR and STAT3 signaling pathways, making clinical applications more realistic. In 2017, by overexpressing IGF1 and OPN Prof. He's group demonstrated

robust CST regrowth, together with recovery of CST-dependent behavioral task after SCI (Y. Liu et al., 2017).

Glial scar (supportive substrate)

A major aspect of CNS regeneration relies on modulations of the glial scar. For decades, it was accepted that axon regeneration failure in the adult mammalian CNS was due to the reactive astroglial scar. Indeed, it was reported that axon regeneration is inhibited exactly at the border of the glial scar, where the level of chondroitin sulphate proteoglycans (CSPGs) in higher. CSGPs inhibit axons growth both in-vitro and in-vivo. It was argued that the glial scar acts as a dense physical and chemical barrier to axon regeneration (Davies et al., 1999). However, our better understandings of the glial scar process slowly challenge the detrimental role in regeneration of this structure. As previously explained, the scaring process after spinal cord injury organize in a fibrotic lesion core corresponding to the fibrotic scar and a dense astroglial layer surrounding the lesion core corresponding to the glial scar. Both the astroglial and fibrotic components appears to contribute on restoring tissue homeostasis by preventing the extension of the lesion in acute phase (Burda & Sofroniew, 2014). However, the fibrotic scar have been shown to support detrimental effect on axons regrowth and to be a physical barrier to growing axons in chronic stage (Soderblom et al., 2013). Recently, it has been demonstrated, conversely to the persisting dogma, that the astroglial component of the SCI scar supports axon regeneration, depending on the astrocytes phenotype. Indeed, astrocytes expressing A2 phenotype transformation appears to support neuron survival and axon regeneration (Anderson et al., 2016; Liddelow et al., 2017). Anderson and colleagues demonstrated that upregulating laminin levels (a permissive matrix molecule produced by astrocytes and pericytes) in combination with neurotrophic factor delivery (NT3 and BDNF) allowed robust axon regrowth trough and beyond the glial scar. In addition, they demonstrated that preventing astrocyte scar formation reversed the beneficial effect of the treatment on ascending sensory axons regrowth. These results provide strong evidence that astroglial component of the lesion supports axon regrowth, as long as the growth signaling is provided (Anderson et al., 2016) and that scar modulation may create a more permissive environment to promote axon regeneration. For instance, laminin concentration in the lesion's environment is crucial for axon regeneration. It has been shown that a combination of epithelial growth factor (EGF) and fibroblast growth factor (FGF) modulates the SCI lesion core and increases the extracellular matrix density, which is largely composed of laminin and collagen-1 (Anderson et al., 2018; Kashpur, LaPointe, Ambady, Ryder, & Dominko, 2013).

Axonal guidance (chemoattraction)

Lesion core remodeling by itself does not induce axon regrowth. Indeed, chemo-attractant factors are required to guide axons into and beyond the lesion. Several molecules have been documented as growth and plasticity stimulants, such as brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT3), glial cell-derived

neurotrophic factor (GDNF). NT3 and BDNF have been shown to exert guidance on sensory fibers. In addition, some evidence grants BDNF with attractant abilities on the reticulospinal tract (RtST) (Alto et al., 2009; K. Liu, Tedeschi, Park, & He, 2011; Madhavan & Collier, 2010; Tuszynski & Lu, 2008).

Neuroprosthetics

As previously described, axon regrowth interventions or cell-based therapies are currently insufficient to yield recovery after complete SCI. Different paradigms have been developed for a more immediate patient care in case of SCI. In most of the cases, SCI are incomplete, leaving some extent of spared tissue. In addition, the spinal networks coordinating leg movements, known as locomotor central pattern generators (CPG) located within the lumbar spinal cord (Gerasimenko, Roy, & Edgerton, 2008), remain intact in a "dormant" state. These circuits are modulating by brainstem descending pathways mediated by different molecules such as noradrenaline, serotonin (5-HT), dopamine and glutamate (Asboth et al., 2018; Hentall, Mesigil, Pinzon, & Noga, 2003; Zaporozhets, Cowley, & Schmidt, 2011). It has been demonstrated that the delivery of 5-HT and dopaminergic agonists can stimulate these circuits and potentiate gait pattern (Musienko, van den Brand, Maerzendorfer, Larmagnac, & Courtine, 2009), making monoaminergic agents good candidates in potentiating (increasing excitability) infralesional spared circuits after SCI. Moreover, the recruitment of quiescent circuits below SCI can also be achieved by targeted epidural electrical stimulation (EES). It has been shown that EES associated with serotoninergic agonists stimulation and treadmill motor training can restore a functional state of lumbosacral circuits enabling full weight-baring treadmill locomotor capacity after complete SCI (Courtine et al., 2009). Nevertheless, this electropharmacollogical circuit's recruitment associated with locomotor treadmill training allowed generating involuntary movements meaning that supraspinal inputs were not involved. Prof. Grégoire Courtine and his lab went further by building a postural robotic interface that provide rodents with body weight support, enabling volitional bipedal training on a treadmill or overground. Rats were submitted to staggered double hemisections (T7 and T10) and were treated subsequently with systemic serotoninergic (5-HT) agonists, combined with electric epidural stimulation below the injury. Rats underwent an intensive robotic-assisted training overground associated with motivational cues to promote an active participation of the animals. This training resulted in the restoration of supraspinal (voluntary) leg movements, arising from cortical projections remodeling and the formation of new propriospinal relays, in an activity-dependent manner (van den Brand et al., 2012). The same group also demonstrated that electrochemical neuromodulation of lumbar circuits after severe spinal cord contusion enabled a supraspinal control over paralyzed legs. They showed that glutamatergic reticulospinal neurons in the VGI relay cortical commands and support functional recovery after incomplete SCI. This so-called cortico-reticulo-spinal circuit allowed rats with a severe spinal contusion to walk, swim and climb stairs

(Asboth et al., 2018) after hard training. Furthermore, walking involves alternating activation of flexor and extensor muscles synergies restricted in different hot spots in the spinal cord. It was demonstrated that a precise spatiotemporal neuromodulation of these hot spots improved important gait and balance features after complete SCI in rat (Wenger et al., 2016), non-human primates (Capogrosso et al., 2016) by recruiting proprioceptive circuits within the posterior roots of the spinal cord. It was demonstrated that spatiotemporal EES during gravity-assisted overground intensive locomotor training enabled restoring voluntary control of walking in patients with incomplete SCI. Patients improved their walking capacities after this neuroprosthetic rehabilitation program and the functional improvement was persistent over time even without EES probably through propriospinal circuits activation (Wagner et al., 2018). These studies highlighted the pivotal role of both propriospinal and reticulospinal systems in the recovery after incomplete spinal cord injuries. After such assaults, the CNS reorganize through spared descending motor pathways. Indeed, propriospinal circuits were shown to be critical in recovery after staggered double hemisections experiment (van den Brand et al., 2012), probably du to their centromedullary location. In the other hand, in contusions experiments where ventrolateral reticulospinal circuits are spared (Asboth et al., 2018), the reticulospinal neurons supported the recovery. However, in case of complete spinal cord injuries (SCI), which isolate the circuits under the lesion from any supraspinal control, the outcome of neuroprosthetic rehabilitation is still not sufficient. Ideally, it will require the restoration of both propriospinal and reticulospinal across the injury, and/or the replacement of the loss neural tissue with exogenous cells that can either support regrowth, or relay information.

The project

Neuroprosthetic rehabilitation demonstrated that significant functional benefit could be achieved with lumbosacral neuromodulation after of spinal cord injury. It promoted the recovery of voluntary leg movements through the reorganization of residual reticulopinal and propriospinal projection pathways (Asboth et al., 2018; van den Brand et al., 2012). However, in case of complete spinal cord injuries (SCI), which isolate the circuits under the lesion from any supraspinal control, the outcome of neuroprosthetic rehabilitation is still not sufficient. Indeed, it will require the restoration of robust regrowth and sprouting of several types of axons across the injury. However, adult mammalian injured axons fail to regrow into complete SCI because of i) the low intrinsic growth capacity of adult CNS neurons, ii) a lack of supportive intralesional environment and iii) a lack of guidance cues. These three essential components are required for proper axon extension during development but are attenuated in adults. Anderson and colleagues demonstrated that all three components are individually necessary and sufficient in combination to promote robust regrowth across complete SCI in rodents. They reactivated the intrinsic growth capacity of descending propriospinal neuron with viral activation of IGF1, CNTF and OPN prior complete SCI (Bei et al., 2016; Duan et al., 2015); induced growth-supportive environment with FGF2 and EGF and attracted

propriospinal axons with GDNF within and below the injury. They reported a robust propriospinal axon regeneration, that regrew a full spinal segment, formed terminal-like contact within healthy tissue below the injury and conveys electrophysiological conduction capacity through anatomically complete SCI (Anderson et al., 2018). However, this robust propriospinal bridging failed to promote by itself functional recovery, which is consistent with accumulative evidences that new projections formed after complete SCI require active rehabilitation to support their integration into functional neural networks. In addition, it might be partly explained by an insufficient descending motor control partly supported by other systems such as the reticulospinal tract (RtST) and the corticospinal tract (CST). We propose here to translate the established procedure to the regeneration of both the corticospinal and reticulospinal tract after a complete spinal cord injury. We first activated the neuronal intrinsic growth capacity of both tracts using viral activation of IGF1, CNTF and OPN, that co activate mTOR, STAT and probably others pathways. The lesion environment was then remodeled with delivery of FGF2 and EGF that demonstrated to increase the extracellular matrix density mainly composed of laminin, which crucial for axons regeneration (Anderson et al., 2018). Finally, we established chemical axon guidance using chemo-attractant molecules. These interventions were delivered with a spatiotemporal profile corresponding to the axon growth sequence during development. GDNF delivery was demonstrated to decrease reticulospinal axons retraction axons after SCI (Dolbeare & Houle, 2003). BDNF was reported to promote survival axotomized axons and promote their elongation in synergy with IGF-1. NT3 was argued to promote sprouting of CST axons (K. Liu et al., 2011) and allowed attraction of sensitive fibers in combination with BDNF (Anderson et al., 2016). We hypothesize that IGF-1 itself may exert guidance on selected axon tracts, when delivered at the severed axon extremity. The present work aims at investigating the ability of NT3, BDNF, GDNF and IGF-1 to properly attract severed CST and RtST axons. To enable proper delivery of these factors in a sustained and reliable manner, a biocompatible hydrogel was used as vehicle. This material – termed diblock copolypeptide hydrogel (DCH) – was developed by our colleagues from the Deming Laboratory [University of California, Los Angeles, UCLA] as an amphiphile carrier for a broad variety of cargo molecules and/or cells (Anderson et al., 2016; 2018; Nowak et al., 2002; Zhang et al., 2014).

Research question:
Does neuronal overexpression of the factors IGF 1 - CNTF – OPN, associated to lesion remodelling and chemoattraction enable robust regeneration of corticospinal and reticulospinal bridges across complete spinal cord injuries?

Methods

Experiments were conducted on six young adult female Lewis rats ranging from 2 to 4 months old and between 180 g to 220 g body weight at the beginning of the experiment. They were housed in a pathogen-free facility, three animals per cage, with unlimited access to food and water. Four animals received the full treatment and two control animals received empty vehicle only. The experiment was conducted over 42 days, as shown in the following timeline (*fig. 2*). All procedures were conducted according to the Swiss Veterinary Law guidelines and approved by the Veterinary Office of the canton Geneva.
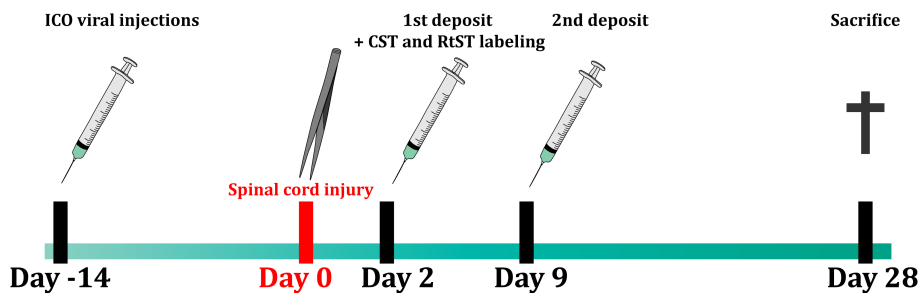

Fig. 2. Timeline of the experiment. Day 0 corresponds to the time point for the spinal cord injury. Two weeks before (d -14) the ICO viral injection is performed. On day 2 post-lesion, the first depot was performed into the lesion site as well as BDA tracing of the motor cortex and AAV5-RFP tracig of the reticular formation. On day 9 the second depot was performed into the healthy spinal cord located below the level of injury. On day 28, animals were sacrificed and the CNS was recovered for histological analysis.

Experimental design

The activation of the three genes of interest (IGF-1, CNTF and OPN) relied on Adeno-Associated-Virus 2/9 (i.e. AAV2/9 IGF-1, AAV2/9 CNTF and AAV2/9-OPN). Fourteen days prior to spinal cord injury (SCI) (Day -14 = D-14), the three viruses were combined and injected into both the motor cortex and the ventral gigantocellular nucleus (VGI). Two weeks were respected for gene expression of the signaling factors (IGF-1, CNTF and OPN) (*fig.3*).
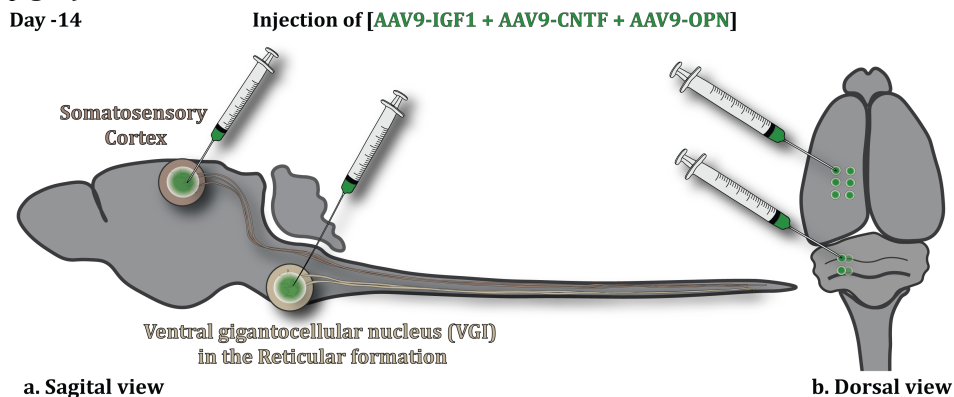

Fig. 3 . Day -14: injection of (AAV9-IGF1+AAV9-CNTF+AAV9-OPN) in the left somatosensory cortex (6 injections sites) and in the ventral gigantocellular nucleus (VGI) (2 injections sites)
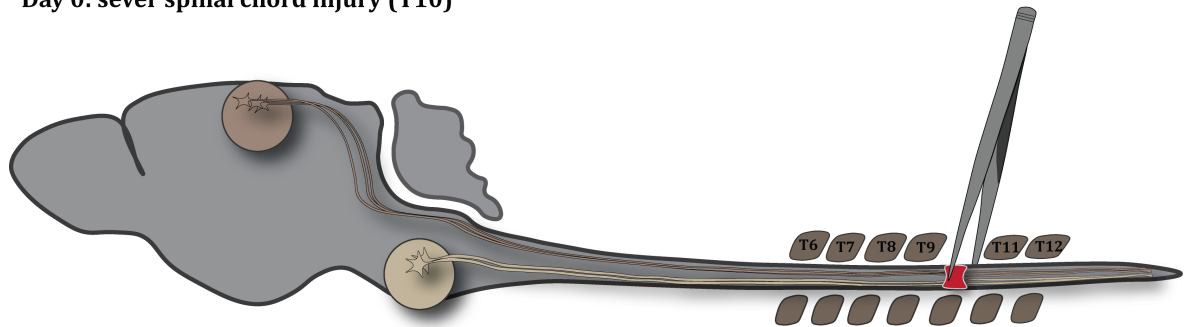
16

Day 0: sever spinal chord injury (T10)

Fig 4. Day 0: Complete crush spinal cord injury (SCI) at thoracic level 10 (T10)

Fourteen days after viral activation of the targeted signaling cascades, a complete crush SCI (corresponding to day 0 = DO) was performed at thoracic level 10 of the spinal cord (T10) (*fig.4*). Two days after SCI (Day 2 = D2) a first growth factor depot was performed into the lesion site for core remodeling. Diblock copolypeptide hydrogel (DCH) was loaded with cargo EGF and FGF (lesion core modulators), as well as BDNF, GDNF, IGF-1 and NT3 (guidance cues). During the same surgical procedure, the CST was labeled with BDA and the RtST was traced using AAV5-TurboRFP (*fig.5*).
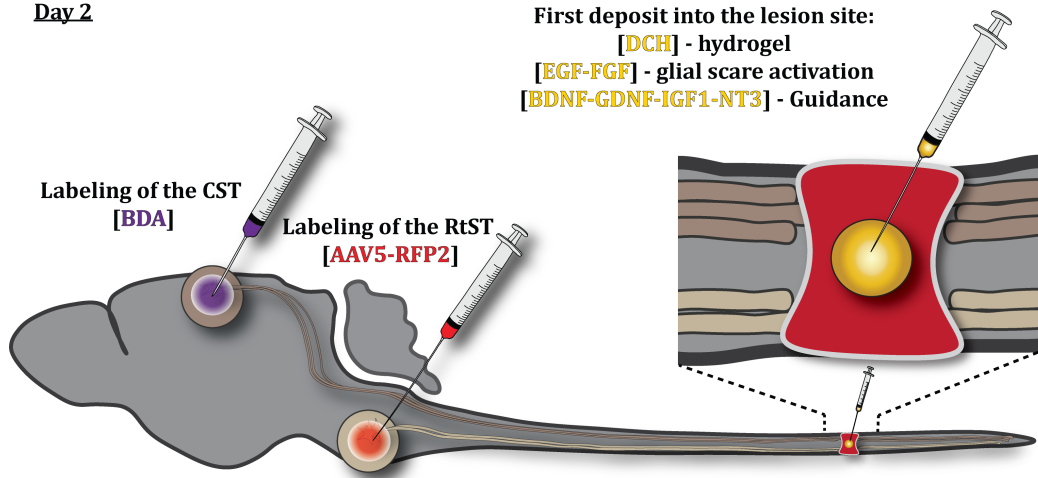

Fig. 5. Day 2: First depot into the lesion site (EGF-FGF-BDNF-GDNF-IGF1-NT3). Labelling of the CST with BDA and tracing of the RtST with RFP2.

One week later (Day 9= D9) staggered bilateral depots were performed i) 2 mm below the lesion on the right hemi-cord and ii) 3 mm below the lesion on the left side. For the second depot, DCH was loaded with guidance molecules (BDNF, GDNF, IGF-1 and NT3) alone (*fig.6*). Four weeks after SCI (Day 28 = D28) the animals were scarificed with a pentobarbital overdose and the CNS was collected for anatomical analysis. In parallel control animals only underwent complete crush SCI and labeling of both tract, without any further therapeutic treatment. Control animals were involved to quantify any spontaneous axon regeneration.

Day 9: Second deposits below the lesion

2mm below the lesion
on the right side

3mm below the lesion
on the left side

Dorsal view

Deposits: 2mm and 3mm below the lesion
[DCH] - Hydrogel
[BDNF-GDNF-IGF1-NT3] - Guidance

CST labeled with [BDA]

Sagital view
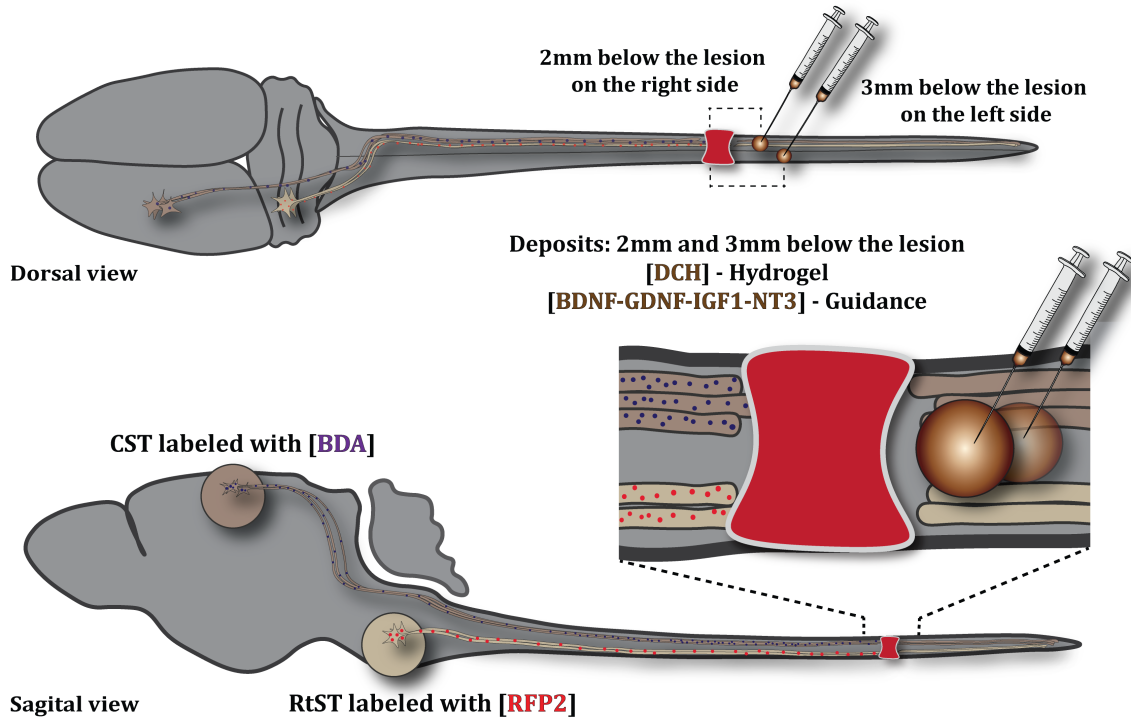
RtST labeled with [RFP2]

Fig. 6. *Day 9: Second deposits below the lesion. One deposit was performed 2mm below the lesion site on the right side and one deposit was performed 3mm below the lesion site on the left side.*

Specific signaling and gene activation

Specific signaling and gene activation was performed using adeno-associated virus (AAV) to infect neurons with plasmids carrying the genes of interests. In the present study AAV2/9 vectors were used as vectors to co-express the growth factors IGF-1, CNTF and OPN (*fig.7*). These recombinant vectors are composed of a packaging vector AAV2 rep, AAV9 cap and our gene of interest with the following dilution: AAV2/9 IGF-1: 5×10^{12} genome copies per ml, AAV2/9-OPN: 1×10^{13} genome copies per ml and AAV2/9 CNTF: 5×10^{12} genome copies per ml. In addition, labeling of the RtST was performed using a red-fluorescent protein (RFP) reporter, which was administered with an AAV2/5-RFP vector (2.612×10^{13} genome copies per ml).
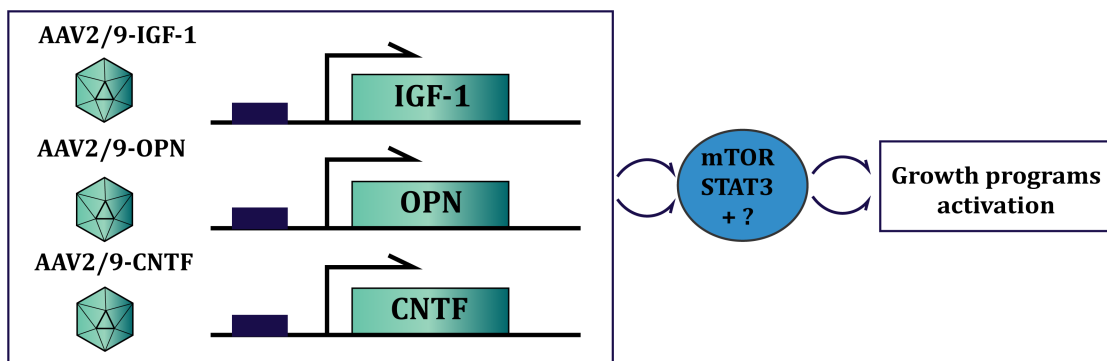

Fig. 7. *Molecular mecanisms. The three adeno-associated viruses were used as pictured with green hexagones. Expression of the three corresponding genes (e.g. IGF-1, CNTF and OPN) is enhanced in both targeted regions. Expression of these genes aimed at reactivating axon growth capacities via mTOR, STAT3 and probalby others signaling pathways .*

Hydrogel and growths factors

Biomaterial depots were performed using diblock copolypeptide hydrogel K_{180}-L_{20} (DCH), known as a well characterized CNS biocompatible vehicle that enables prolonged delivery of bioactive molecules to the CNS and self-degrades in three weeks (Anderson et al., 2016; 2018; Nowak et al., 2002; Zhang et al., 2014). Freeze-dried K_{180}-L_{20} polymer (provided by our collaborators from the Deming Laboratory, UCLA) was reconstituted to a 3% hydrogel in phosphate-buffered saline (PBS) and cargo growth factors were added when applicable: EGF, FGF, BDNF, NT3, GDNF, IGF-1 (1µg/µl each).

Surgical procedures

All surgical procedures were performed under general anesthesia. Before surgery, animals were sedated with a mixed atmosphere of 5% isoflurane, O_2 (1,5 L/min) and ambient air (1,5 L/min) for 5 minutes. In addition and to complete analgesia, animals received 0,05 mg of medetomidine hydrochloride (Dorbene®). After anesthetic induction, head and back were shaved and disinfected with a 1:1 combination of Betadine and hydrogen peroxide (H_2O_2). Animals were kept stable under anesthesia with 1,5% to 2,5% isoflurane in oxygen-enriched air.

Brain surgery

For motor cortical and brainstem injections, rats were placed in a stereotaxic frame (Kopf Instruments). A midline skin incision was performed to expose the skull bone. Bregma and Lambda were used as anatomical references. As Bregma is the anatomical intersection between the sagittal suture and the coronal suture, Lamba is the junction of the sagittal suture with the lambdoid suture (***fig.8***). Correct animal positioning in the frame and surgical coordinates were derived from these two anatomical references. After delimitation of the targeted area for injection, a craniotomy was performed through the left parietal bone or through the occipital bone to access respectively the forebrain and the cerebellum. All the coordinates were calculated based on an anatomical atlas (Paxinos and Watson), previously piloted in previous experiments and are shown in ***fig.9***. For signaling activation in the CST, six injections sites were performed in the left somatosensory cortex (***fig.8***). Injection sites were reached vertically with a glass micropipette and injections took place using a nanolitric pump. To activate growth programs in the reticular formation, four virus injections were performed at two different anteroposterior sites, with two distinct dorsoventral coordinates. The ventral gigantocellular nucleus (vGI) was reached vertically through the cerebellum. Due to their fragility, glass micropipettes were replaced with a 33G Hamilton syringe for vGI injections. For each site in both the motor cortex and in the vGI, 250nl of viral suspension containing all three virus (1:1:1, AAV2/9 IGF-1, AAV2/9 OPN and AAV2/9 CNTF) were injected using a nano-injector. Before careful withdrawal of the needle, 2 minutes were respected to avoid viral reflux. Following the same

procedure, CST and RtST were labeled with 300nl of 10% BDA per injection site in the motor cortex and 200nl of AAV5-TurboRFP in the VGI. After completion of the injections skin was closed using non-resorbable suture (Ethilon 4.0).
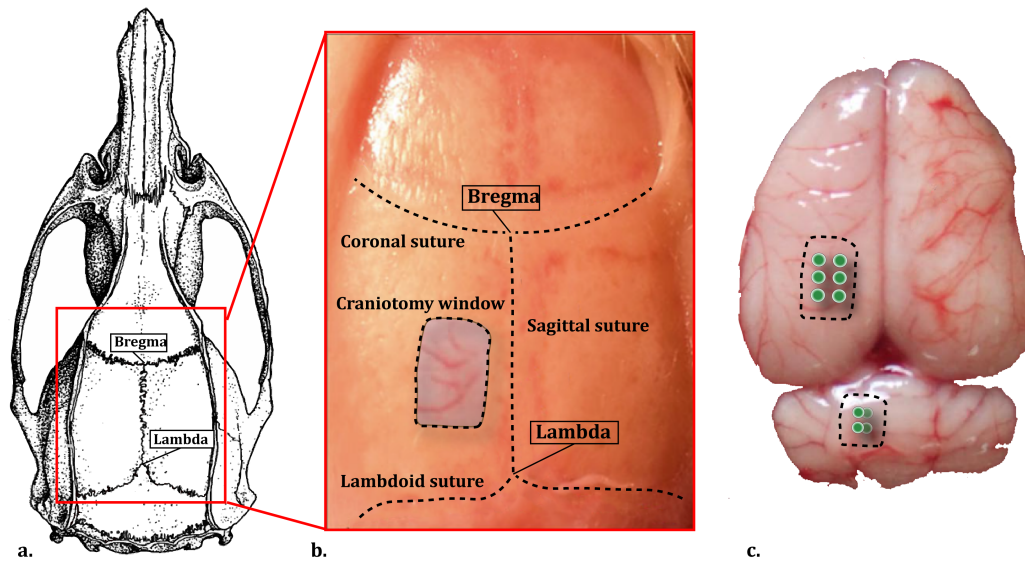

Fig. 8. a) Schematic view of a rat bone skull showing Bregma, Lamba and the skull sutures. b) Anatomical representation of stereotaxic references. A craniotomy window is depicted over the motor cortex. c) Rat brain showing both craniotomies (dashed rectangles) in the left parietal bone and in the left part of the occipital bone. Green dots represent the cortical and VGI injections.[1]

Brain region	Reference point	Antero-posterior (A/P)	Medio-lateral (M/L)	Dorso-ventral (D/V)	Injection volume Nanoliter (nl)	Injection rate (nl/min)
Motor cortex	Bregma (0, 0, 0)	*First site:* - 0.5 mm	- 2 mm	- 1.45 mm	250 nl each	200 nl/min
		Second site: - 0.5 mm	- 3 mm	- 1.45 mm		
		Third site: - 1.5 mm	- 2 mm	- 1.45 mm		
		Fourth site: - 1.5 mm	- 3 mm	- 1.45 mm		
		Fifth site: - 2.5 mm	- 2 mm	- 1.45 mm		
		Sixth site: - 2.5 mm	- 3 mm	- 1.45 mm		
Ventral gigantocellular nucleus (VGI)	Bregma (0, 0, 0)	*First site:* - 11 mm	- 1 mm	- 9 mm	250 nl each	200 nl/min
		Second site: - 11 mm	- 1 mm	- 8.8 mm		
		Third site: - 12 mm	- 1 mm	- 9 mm		
		Fourth site: - 12 mm	- 1 mm	- 8.8 mm		

Fig. 9, Stereotaxic coordinates used for the D-14 virus injections as well as the D2 axons tracing injections (BDA and AAV5-RFP). Bregma was used as reference for both motor cortex and VGI injections. Six different sites were targeted in the motor cortex with a dorsoventral coordinate located - 1.45mm from the brain surface. Two injections site were located in the VGI with two different dorsoventral depths, giving total of four injections. The injection rate *and volume were conserved for all virus injections.*

[1] Skull's schema: *George Paxinos and Charles Watson: The Rat Brain in Stereotaxic Coordinates, Academic Press, 1998.* Brain's picture: *Hui-Feng Zhu et al. 2010 - International Journal of Biological Sciences - Catalpol Increases Brain Angiogenesis and Up-Regulates VEGF and EPO in the Rat after Permanent Middle Cerebral Artery Occlusion.*

Spine surgery

Complete crush spinal cord injuries were performed under complete anesthesia with isoflurane and Dorbene. The back was shaved and disinfected, and a dorsal midline skin incision was performed. Muscles were carefully detached to reach the thoracic vertebra column. A single T10 laminectomy was performed to expose the spinal cord, and the dura matter was kept intact. The spinal cord was then crushed laterally with n°2 forceps at the spinal T10 level. Compression was conducted laterally from both sides for 5 seconds, creating a transversally complete spinal lesion. In order to label the injured site for subsequent intralesional injections, bilateral injections of dark dye (100 nl per side) were performed at the injured level. After bleeding control was achieved, muscles were sutured (Vicryl 5.0) and the skin was closed (Ethilon 4.0). For spinal cord growth factor depots, a similar procedure was repeated to reopen the first surgical wound. Injection sites were reached vertically with a glass micropipette. DCH hydrogel, either alone or loaded with cargo molecules, was injected 1.1 ml below the surface of the spinal cord to reach centromedullary layers.

## Histology and immunohistochemistry

At the end of experiments, animals were deeply anesthetized with an intraperitoneal overdose of pentobarbital. Perfusion took place with 4% paraformaldehyde (PFA) delivered transcardially to the aorta. The central nervous system was then dissected, postfixed in 4% PFA and saturated in 30% sucrose. Spinal cord segments were embedded and frozen in a cryoprotective matrix. Using a Cryostat microtome (Leica), 30µm-thick longitudinal sections were obtained from the perilesional tissue, mounted on histologic slides. Rabbit anti-GFAP was used as primary antibodies and Alexa-488 (green) as secondary antibodies. DAPI was used as nuclear non-specific maker. Biotinylated dextran amines (BDA) were reveled with biotin-avidin-peroxidase complex and diaminobenzidine as developing agent and revealed using a Cy5 conjugated dye. Bright-field and fluorescence microscopy (Zeiss) was used to examine and acquire histologic images from the stained sections.

Results

Propriospinal axon regeneration

The following data is reported from the work of Anderson and colleagues, who explored the requirements of propriospinal axon regeneration (Anderson et al, 2018). We show here RFP-traced propriospinal fibers after severe SCI in both treated and control conditions (*fig.10*). We observe under *10b* a longitudinal spinal cord section from a T10-injured rat that received successful regenerative interventions combining i) AAV-[IGF1-CNTF-OPN] administered two segment rostral to the SCI two week before the lesion, ii) remodeling of the lesion with EGF-FGF and iii) guidance with GDNF provided

within and below the lesion site. The descending propriospinal axons were traced with red fluorescent protein (RFP), appearing in red.

Fig.10

Anderson et al., Nature 2018
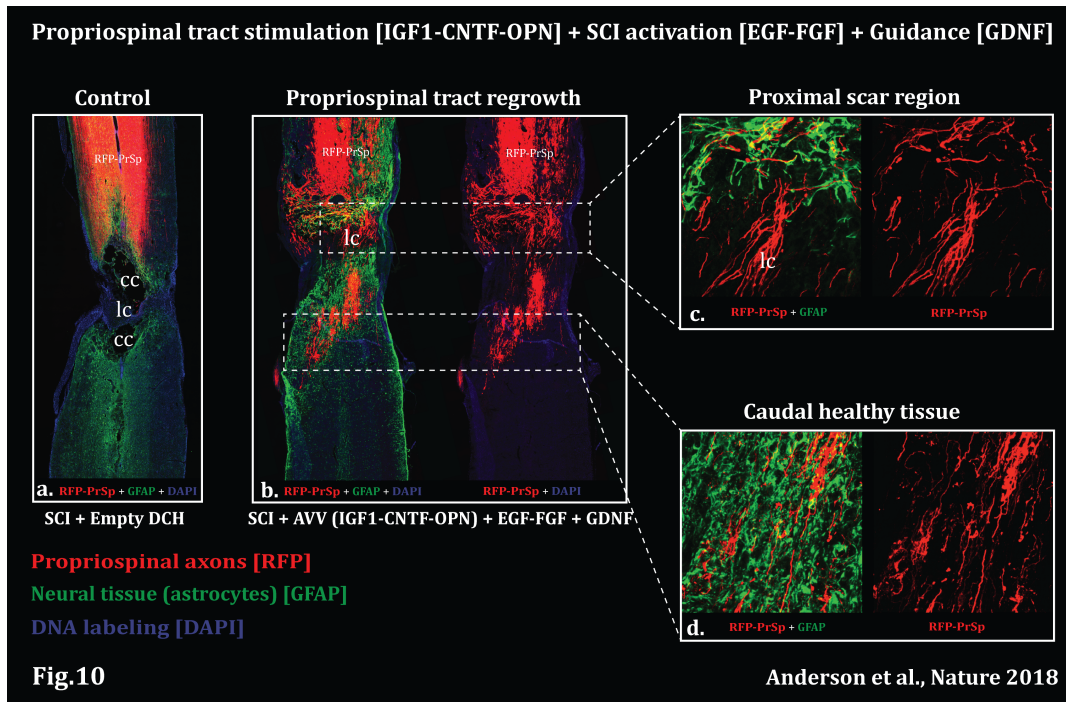
Astrocytes were labeled with an immunostaining against glial fibrillary acidic protein (GFAP, green) that reliably delimits the non-neural lesion core. In addition all tissues were conter-stained for DAPI (purple on the figure). Control animals (*10a*) only received a complete crush SCI followed by a single intralesional injection of empty DCH vehicle without any additional factor. GFAP-labeled astrocytes showed that the SCI was anatomically complete across the entire transverse segment of the spinal cord (*10a,b,c*), with large lesion core (*lc*) in both control (*10a*) and treated animals (*10b,c*). RFP-tracing demonstrates satisfying labeling of the PrSp in both the control and the treated animal. We note in the control animal (*10a*) that no PrSp axon was able to penetrate the non-neural lesion core. In addition we observe the presence of a cystic cavity (*cc*), which appears attenuated after lesion remodeling with EGF/FGF. In treated animals, robust axon regrowth through the lesion core could be achieved (*10c*), that successfully reach healthy neural tissue beyond the lesion (*10d*). In addition, we showed that less axon was found at lesion center without propriospinal growth program activation and that no more axon were found at the lesion center without EGF-FGF remodeling of the lesion and/or without GDNF guidance cues delivery, demonstrating that all three components of this regenerative intervention are individually necessary but sufficient only in combination to promote robust propriospinal axons regrowth across complete SCI in rodents.

Corticospinal axon regeneration

We demonstrate here below (*fig.11*) a longitudinal spinal cord section from a T10-injured rat with BDA-labelling of corticospinal axons after complete SCI. The candidate procedure for CST axon regeneration was performed as follows. Animals received i) a

combined injection of the growth modulating AAV2/9-[IGF1-CNTF-OPN] into the motor cortex two weeks priori to the lesion, ii) remodeling of the SCI site using EGF-FGF administered into the lesion core and iii) axon guidance with BDNF-GDNF-IGF1-NT3 delivered within and caudally to the lesion site.

Fig.11
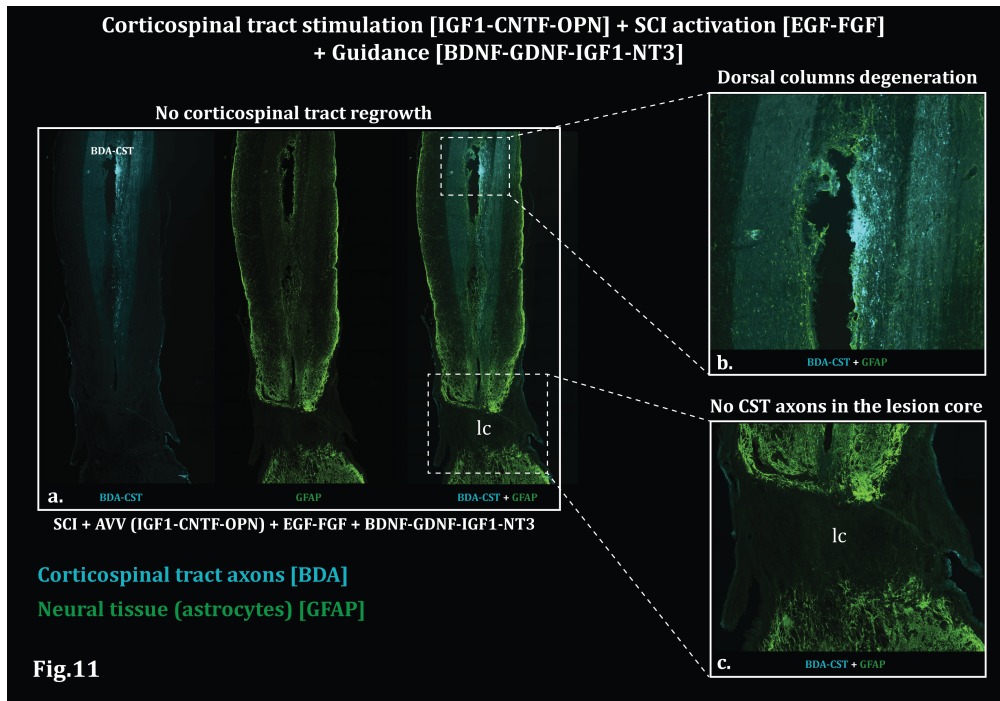
The descending corticospinal axons were labeled using biotinylated dextran amine (BDA) revealed using a Cy5 conjugated dye, appearing in turquoise blue on the histologic acquisition (**11a**). The surrounding neural tissue was highlighted with glial fibrillary acidic protein (GFAP) (**11b**) showing reactive astrogliosis bordering the non-neural lesion core (green). Astroglial immunostaining confirms that SCIs were anatomically complete across the entire transverse section of the spinal cord (**11a,c**), revealing broad lesion cores (**lc**). BDA-staining showed satisfying labeling of the CST on the rostral part of the spinal cord. However, no CST axon was left in the vicinity of the lesion center. Conversely, corticospinal axons dyed back and degenerated towards the cell body (**11a,b**). This extensive CST degeneration could be explained by its the dorsal localization making these fibers more susceptible to ischemic phenomenon. Another hypothesis could be the different axonal survival program of CST neurons, rendering CST less prone to plastic reorganization, as compared to other tracts. However, CST regeneration associated with functional recovery after SCI was reported in both cell transplantation approach (Kadoya et al., 2016) and intrinsic growth control upregulation (Y. Liu et al., 2017) suggesting that CST regeneration is feasible.

Reticulospinal axon regeneration

We show here below (*fig.12*) RFP-traced reticulospinal axons after complete SCI under both treated and control conditions. The histologic acquisitions (*12b*) depict longitudinal spinal cord sections from rats following complete T10 crush injury. Animals received reticulospinal-adapted regenerative interventions with i) injection of AAV2/9 (IGF1-CNTF-OPN) into the VGI (reticular formation) two weeks before the lesion, ii) SCI remodeling with EGF-FGF delivered to the lesion core, and iii) axon guidance using BDNF-GDNF-IGF1-NT3 administered within and caudally to the lesion site. The descending reticulospinal axons were labeled with red fluorescent protein 2 (RFP-2, red). Astrocytes were immunostained and revealed via glial fibrillary acidic protein expression (GFAP, green). For comparison, control animals (*12a*) received only received a complete crush SCI, followed by an administration of empty DCH vehicle, without any remodeling factor or guidance cue. No viral signaling-activation was performed in control animals.

Fig.12
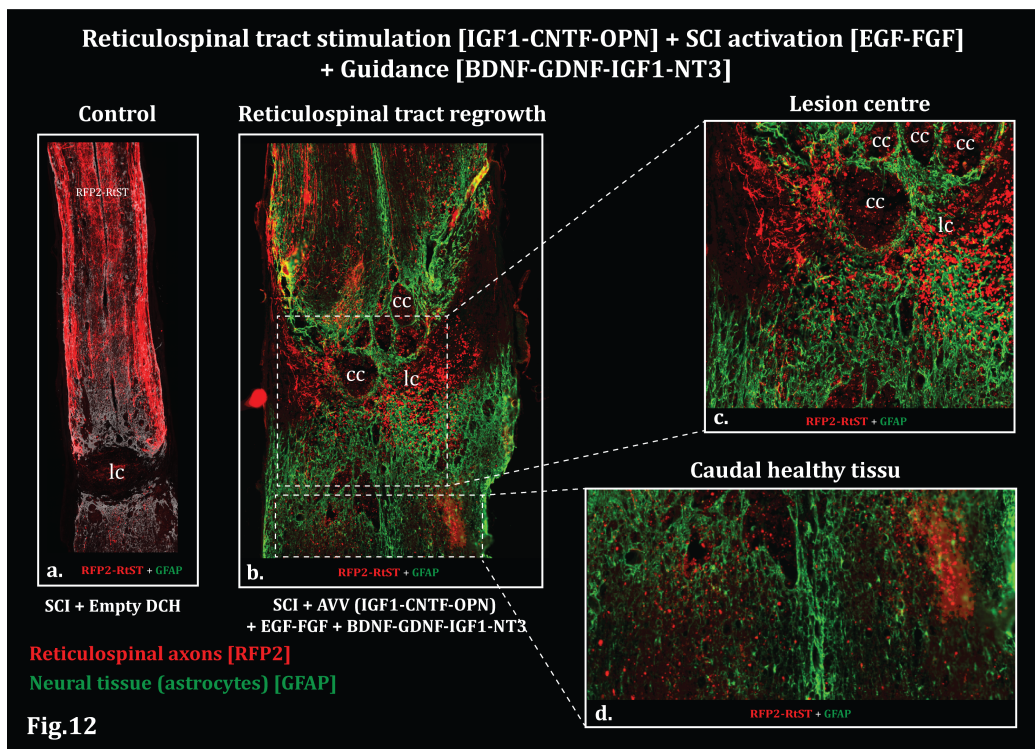
GFAP-labeled astrocytes showed that the SCI was anatomically complete across the entire with of the spinal cord (*12a,b,c*), with large lesion core (*lc*) in the control (*12a*) and in the treated animal (*12b,c*). RFP2-staining shows a good labeling of the RtST in both the control and the treated animal. In the control animal (*12a*) almost no RtST axons reach the lesion center. In the treated animal some axons regrew through the lesion core (*12b,c*) and reached the healthy tissue beyond the lesion into the caudal healthy tissue (*12d*). Some cystic cavities (cc) are present into the lesion site and on the scar proximal border (*12b,c*). We demonstrated, that viral activation of (IGF1-CNTF-OPN) into the VGI (reticular formation), SCI remodeling with EGF-FGF and axon guidance using BDNF-GDNF-IGF1-NT3 allowed reticulospinal axon regrowth through complete SCI and that some fibers successfully reach healthy neural tissue beyond the

lesion (**12d**). However, the RtST regeneration demonstrated here, is less important as compared with the robust axon regeneration demonstrated in propriospinal system (Anderson et al., 2018). We hypothesized that VGi viral activation with (IGF1-CNTF-OPN) may have cross-activated other signaling pathways, which should be identified and more specifically targeted in order to optimize reticulospinal axons regrowth strategy.

Discussion

Severed axons fail to regrow spontaneously into complete spinal injuries in adult mammals. Recent work - that we evoke here above - demonstrated that propriospinal axons can robustly regrow across complete crush lesions, penetrate healthy caudal circuits, form new synaptic-like contacts and conduct descending electrophysiological input past the lesion. However, propriospinal axon regeneration itself does not induce improvement of motor functions (Anderson et al. 2018). This is consistent with growing evidence that new projections formed after complete SCI cannot spontaneously acquire function but require active rehabilitation to support their integration into functional neural networks through use-dependent plasticity, that was demonstrated to promote functional recovery in incomplete models of SCI (Asboth et al., 2018; van den Brand et al., 2012). In addition, recovery might require extra descending tracts to relay motor commands, such as corticospinal and reticulospinal projections. Indeed it was demonstrated that the activation of IGF-1 and OPN led to robust CST regeneration that restored CST-dependent functions after spinal cord hemisection (Y. Liu et al., 2017). On the other hand, reticulospinal projections were shown to support functional recovery after severe spinal cord contusion. Indeed glutamatergic reticulospinal neurons in the VGi were shown to relay cortical commands downstream and enable the motor cortex to regain adaptive control over paralyzed legs (Asboth et al., 2018). Depending on the SCI model, CST projections recruit either RtST or PrSp neuron to bypass the lesion. In the present study, we precisely explored the regenerative potential of both these essential tracts, known to be involved in the voluntary control of locomotion. We directly transferred the successful propriospinal-targeted intervention to the corticospinal tract and to the reticulospinal tract. We tested whether CST and RtST axons are sensitive to neuronal overexpression of the factors IGF1-CNTF-OPN, associated to remodeling of the lesion environment using EGF-FGF and chemo-attraction using NT3, BDNF, GDNF and IGF-1. Further, we assessed the possible formation of new axon bridges from long descending tracts across complete spinal cord injury. Our results regarding corticospinal axon regeneration demonstrated that our treatment failed to induce any axon regeneration. Conversely, the CST underwent strong Wallerian degeneration, in which axons died back towards their cell body. Different hypotheses may explain this extensive CST degeneration. For instance, the severe crush model of injury performed here, might cause ischemic phenomenon against which CST fibers are more susceptible

because of their dorsal localization. Another hypothesis could be weaker axon survival programs in CST neurons, rendering CST less prone to plastic reorganization, as compared to other tracts. Further, the CST has been remaining refractory to most therapeutic strategies attempted in the field of spinal repair for long (Tuszynski & Steward, 2012). More recently, robust CST axon regeneration associated with functional recovery was reported after neural progenitor cells transplantation in murine model of SCI (Kadoya et al., 2016). In addition it was demonstrated, that overexpressing IGF1 and OPN supported robust CST regrowth, together with recovery of CST-dependent behavioral task after incomplete SCI (Y. Liu et al., 2017). As CST axons regeneration is feasible, it might be interesting to protect CST fibers from dieing back using neuroprotective approaches before trying regenerating them. Our goal is to increase the direct descending motor commands through complete SCI either directly via CST or indirectly via RtST and PrSp relays. Given that die-back is less important in the latter (RtST and PrSp), it highlights the importance of regenerating both these tracts. Our results regarding reticulospinal axon regeneration demonstrated that some extent of reticulospinal axons was present in the non-neural lesion core and beyond the injury in treated animals. In control animals, almost no RtST axons were found, neither in the lesion nor beyond it. These results strongly suggest that the RtST was responsive to our treatment, and that IGF1-CNTF-OPN do enhance reticulospinal regeneration, even though the density of regrown axons remained very limited. Indeed, as compared to the robust propriospinal regrowth shown in previous work (see above), the axonal density of regenerated RtST axons was negligible. This might be due to an inappropriate combination of guidance cues. Further, we cannot exclude that among the combination of guidance cues, some are detrimental for reticulospinal axons regrowth. Indeed, chemoattractant molecules for one axon tract may become repulsive for another. Nevertheless, among our guidance cocktail there are some appropriate cues without which no regeneration could occurs. Lesion remodeling with EGF-FGF induced a semi-permissive environment as some RtST axons were found in the lesion core. Both lesion remodeling and guidance cues promoted suboptimal RtST regeneration, and could thus probably be refined to increase the regenerative potential. Furthermore, intrinsic growth programs vary between neuron subtypes (He & Jin, 2016), so that a strategy working in the propriospinal system will not necessarily work for reticulospinal neurons. Thus identifying the specific molecular profile of each pathway will be required to maximize our regrowth effect. The activation of IGF1-CNTF-OPN is known to co-activate different regenerative pathways, such as mTOR and STAT3 (Bei et al., 2016; Duan et al., 2015; Y. Liu et al., 2017). We hypothesized that vGI viral activation with [IGF1-CNTF-OPN] may have cross-activated other signaling pathways, which should be identified and more specifically targeted in order to optimize reticulospinal axon regrowth strategy. We should perform RNA sequencing of brainstem during development to understands mechanisms of axogenesis in brainstem-spinal neurons as well as during neurorehabilitation to understand intracellular mechanisms of reticulospinal reorganization. Regarding functional aspects, we did not expect any functional recovery based on axon regrowth alone. Indeed, as previously explained,

functional recovery would certainly require activity-based neurorehabilitation to integrate the news fibers into functional networks. Further perspectives would be to combine such strategies with propriospinal regeneration and neuroprosthetic rehabilitation to increase our chances to restore functional motor command after complete SCI. In addition, we keep in mind that somatosensory feedback to the cortex should be addressed.

Conclusion / Perspectives of the study

To this date, rehabilitating complete spinal cord injuries remains a challenge. Neuroprosthetic rehabilitation demonstrated that significant functional benefit could be achieved through lumbar electrochemical neuromodulation in both human patients and animal models after incomplete SCI. However, following complete SCI, neuroprosthetic rehabilitation still fails to yield any functional benefit. Growing evidence put into light the pivotal role of both propriospinal and reticulospinal tracts in relaying information around incomplete SCIs. In order to transfer such relay reorganization in complete models of SCI, intrinsic mechanisms of axon regeneration failure have been explored and successfully reversed. Indeed, propriospinal axons do regrow and build a robust descending bridge across complete SCIs when the required growth facilitators are provided. However, this robust propriospinal bridging still fails to promote functional recovery by itself. We hypothesize that regrown propriospinal axons may transfer only an insufficient fraction of the descending motor input, and therefore do not suffice to restore voluntary hindlimb movements. In incomplete models, motor commands are relayed either by reticulospinal, or by propriospinal neurons. Inducing reticulospinal regeneration across complete SCI may increase the fraction of motor control transferred to lumbosacral circuits, when combined to propriospinal regrowth after complete SCI. In parallel and based on previous work, promoting direct regrowth of CST axons across complete lesions would contribute to bring direct and non-relayed motor control past injuries. We assessed the feasibility of RtST and CST regeneration based on previous successful regeneration paradigms and demonstrated that RtST axon regrowth can be achieved but needs to be optimized, whereas CST neurons were not responsive to our regrowth strategy. In our approach, the specific activation of axonal growth-related genes associated with specific growth and guidance factors enables precise tuning of the administered components, in order to promote better understanding of the necessity and efficiency of each component. Mechanistic approaches are a paradigm shift in the field of spinal cord regeneration, so that targeted and incrementally-built therapies are currently replacing empirical constructs. A coming challenge will be to identify specific cues for each neuronal subtype, in order to enable multi-tract regeneration across severe spinal cord injuries. But regrowth itself may not be sufficient to promote functional recovery. Indeed, axons need to establish coherent synaptic contacts within the healthy structures located below the lesion in order to induce behavioral benefits.

Our data confirms and provides crucial information for the development of future regenerative therapies. However, combinatorial strategies will need to be designed, in order to address multi-disciplinary solutions to the very complex spinal cord injury challenge.

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