

Structural analysis and therapeutic modulation of axonal remodeling following spinal cord injury

Dissertation

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"Research should not be reckless, but it does need to be fearless"
Christopher Reeve (2003)

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List of Abbreviations

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5-HT	– Serotonin
Akt	– Protein Kinase B (PKB), serine-threonine proteine kinase
AAV	– Adeno-associated virus
Actb	– Actin beta
Adcyap1	– Adenylate cyclase activating polypeptide 1
AF	– Alexa fluor
ASIAIS	– American Spinal Injury Association Impairment Scale
Atf-3	– Cyclic AMP-dependent transcription factor
BDNF	– Brain-derived neurotrophic factor
BOLD MRI	– Blood-Blood-Oxygen-Level Dependence Magnetic Resonance Imaging
C#	– Cervical cord level#
cAMP	– Cyclic adenosine monophosphate
CFP	– Cyan fluorescent protein
CNS	– Central nervous system
CPG	– Ccentral pattern generator
CREB	– cAMP response element binding
ChABC	– Chodroitinase ABC
CSPGs	– Chondroitin sulfate proteoglycans
CST	– Corticospinal tract
hCST	– Hindlimb corticospinal tract
fCST	– Forelimb corticospinal tract
dlCST	– Dorsolateral corticospinal tract
vCST	– Ventral corticospinal tract
mCST	– Main corticospinal tract
DNA	– Deoxyribonucleic acid
DRG	– Dorsal root ganglia
E	– Embryonic Day
eGFP	– Enhanced green fluorescent protein
EMGs	– Electromyograms
FES	– Functional Electrical Stimulation
fMRI	– Functional magnetic resonance imaging
Gadd45a	– Growth arrest and DNA-damage-inducible protein
GAP43	– Growth-associated protein 43 kDa
GFP	– Green Fluorescent protein
GlyT2	– Glycine Transporter 2
gp130	– Glycoprotein 130
GSK-3	– Glycogen synthase kinase 3
Hspb1	– Heat shock 27kDa protein 1
HSP-27	– Heat shock protein 27
ICCP	– International Campaign for Cures of Spinal Cord Injury Paralysis
IGF-1	– Insulin-like growth factor-1
IRF1	– Regulatory factor 1
Jak	– Janus kinase (JAK, or "Just another kinase")
LTD	– long-term depression
MAGs	– Myelin associated glycoproteins
mTOR	– Mammalian target of rapamycin
n	– Number of samples

List of Abbreviations

NCS1	– Neuronal calcium sensor 1
NgR	– Nogo receptor
NMDAR	– N-methyl-D-aspartate receptor
NR2d	– Expressing NMDA-receptor2d
NT3	– Neurotrophin 3
OECs	– Olfactory ensheathing cells
OMgp	– Oligodendrocyte myeline glycoprotein
P	– Post natal day
PB	– Phosphate buffer
PBS	– Phosphate buffer saline
PCR	– Polymerase chain reaction
PFA	– Paraformaldehyde
PI3K	– Phosphatidylinositol 3-kinases
PIP3	– Phosphatidylinositol (3,4,5)-triphosphate
PSNs	– Propriospinal neurons
LPSNS	– Long propriospinal neurons
SPSNS	– Short propriospinal neurons
PTEN	– Phosphatase and tensin homolog
Ptx	– Pyramidotomy
RNA	– Ribonucleic acid
RNAi	– RNA interference
ROS	– Reactive oxygen species
RAGs	– Regenerative associated genes
RST	– Reticulospinal tract
SEM	– Standard error of the mean
Ser	– Serine amino acid
SCI	– Spinal cord injury
SOCS3	– Suppressor of cytokine signaling 3
SPRR1A	– Small proline rich protein 1a
STAT3	– Signal transducer and activator of transcription 3
STPYFP	– Stop Yellow fluorescent protein (<i>mouse line</i>)
T#	– Thoracic cord level# (eg T8= thoracic cord level 8)
TF	– Transcription factor
Trk	– Tyrosine kinase
TSA	– Tyramide Signal Amplification
Tubb3	– Tubulin beta-3 chain
TYC9	– Brainbow mouse line
Tyr	– Tyrosine
VSD	– Voltage-sensitive dye imaging

Abstract

Functional recovery following spinal cord injury (SCI) depends on the remodeling of the preserved neuronal circuits. Injury-induced remodeling can be studied using the corticospinal tract (CST), an important descending motor tract that is involved in fine-skilled limb movements, as a model system. Previously it has been shown that the CST responds to a thoracic lesion by the formation of an intraspinal detour circuit that contributes to functional recovery. However the underlying principles that govern this CST remodeling are not fully understood. By reconstructing single CST collaterals after lesion, we reveal that CST remodeling occurs in three distinct phases. Initially following lesion, newly formed collaterals undergo a growth phase that is then followed by a collateral formation phase where newly formed collaterals develop a more mature and complex structure. Finally there is a maturation phase during which there are small scale refinements of the contact pattern.

While such endogenous remodeling processes can lead to some degree of functional recovery, in many cases severe deficits persist. To this date there is no effective therapeutic treatment that restores sensorimotor function following SCI. In the injured peripheral nervous system (PNS), the activation of the intrinsic growth response can support axonal regeneration and functional recovery. In this thesis we investigated whether and how the initiation of the intrinsic neuronal growth program can improve axonal remodeling and functional recovery after injury. To initiate the intrinsic neuronal growth response we targeted the transcription factor STAT3, the expression of which had been shown to be associated with axonal regeneration. In a collaborative study, we used conditional genetics, viral gene transfer and *in vivo* timelapse imaging to show that sustained STAT3 expression is essential for the timely initiation of axonal regeneration in the PNS. In contrast to the PNS, STAT3 expression is only transiently induced following a central nervous system (CNS) lesion. Therefore we next investigated whether and how intrinsic growth initiation by STAT3 expression can be used to support the regeneration and remodeling of corticospinal fibers after spinal cord injury. Sustained expression of STAT3 induced by viral gene transfer was found to cause an increase in CST axonal sprouting and regeneration following a thoracic lesion. Interestingly, STAT overexpression could also stimulate axonal growth in the absence of any lesion. This led us to utilize a unilateral lesion pyramidotomy model to investigate whether sustained STAT3 expression can recruit the unlesioned tract to compensate for the loss of innervation in the lesioned side. Indeed STAT3 overexpression

was found to induce compensatory sprouting and remodeling of the unlesioned tract. Fibers from the unlesioned tract exited the unlesioned CST in the cervical spinal cord and grew across the midline into the denervated side of the cord. In addition these crossing collaterals were found to form contacts onto the interneurons and motoneurons responsible for forelimb movement. Behavioral and electrophysiological assessment validated that a new intraspinal circuit that was formed enabled functional recovery.

Taken together, our results show that axonal remodeling occurs in defined stages. Targeting the initial growth phase by viral gene transfer of STAT3, a transcription factor that can initiate the intrinsic neuronal growth program, is an effective strategy to enhance axonal remodeling and thereby promote functional recovery following injury. In this thesis, we were able to contribute to the further understanding of the mechanisms that underlie axonal remodeling. In addition we have identified a promising strategy to improve axonal remodeling following injury.

Zusammenfassung

Die Wiederherstellung motorischer und sensibler Funktionen (oder die funktionelle Erholung) nach Rückenmarksverletzung (SCI) ist abhängig von der Reorganisationsfähigkeit der noch unversehrten neuronalen Schaltkreise. Die auf eine Verletzung folgende neuronale Umstrukturierung kann modellhaft am kortikospinalen Trakt (CST) untersucht werden. Es handelt sich hierbei um eine wichtige absteigende motorische Bahn, die vor allem an der Ausführung feinmotorischer Bewegungen beteiligt ist. Unsere Arbeitsgruppe konnte in vorangegangenen Studien zeigen, dass der CST nach einer thorakalen Rückenmarksläsion einen intraspinalen Umgehungsschaltkreis ausbildet, der maßgeblich zur funktionellen Erholung beiträgt. Jedoch hat man bislang die Prinzipien, auf denen diese Fähigkeit zur Reorganisation beruht, noch nicht vollständig verstanden.

Durch Rekonstruktion einzelner axonaler Kollateralen des CST nach Läsion konnte ich nun zeigen, dass der Umbau des CST in drei Phasen erfolgt. Initial durchlaufen neugebildete Kollateralen eine Wachstumsphase. Diese wird dann von einer Phase gefolgt, in der Kollateralen ausgebildet werden, welche zunehmend eine reife komplexe Struktur einnehmen. Die letzte Phase ist charakterisiert durch Verfeinerungen auf Ebene der interneuronalen Kontaktmuster.

Während diese endogenen Reorganisationsprozesse zu einem gewissen Grad funktionelle Erholung herbeiführen, bleiben in vielen Fällen schwerwiegende Defizite zurück. Bislang gibt es keine effizienten Therapien, die nach einer Querschnittslähmung sensible und motorische Funktionen wiederherstellen. Im peripheren Nervensystem (PNS) hingegen kann durch die Aktivierung des intrinsischen neuronalen Wachstumsprogrammes axonale Regeneration und funktionelle Erholung erfolgreich induziert werden. In einem weiteren Abschnitt meiner Arbeit untersuchte ich, ob und in wie weit die Induktion des neuronalen Wachstumsprogramms auch im zentralen Nervensystem (ZNS) axonales Remodeling und die Wiederherstellung von Funktionen verbessern würde. Um das intrinsische neuronale Wachstumsprogramm zu initiieren, konzentrierte ich mich auf den Transkriptionsfaktor STAT3, dessen Expression während der axonalen Regeneration im peripheren Nervensystem induziert wird. Mithilfe konditioneller knock-out Mäuse, viralen Gentransfers und *in vivo* timelapse Mikroskopie konnten wir zeigen, dass die anhaltende Expression von STAT3 essentiell für den zeitgemäßen Beginn axonaler Regeneration im PNS ist. Im Gegensatz zum PNS wird die STAT3 Expression nach einer Läsion im ZNS nur

transient gesteigert. Daher untersuchte ich im letzten Abschnitt meiner Arbeit, ob und wie die Initiierung des intrinsischen neuronalen Wachstumsprogramms durch STAT3 genützt werden könnte, um die Regeneration und Reorganisation kortikospinaler Fasern nach Rückenmarksläsion anzukurbeln. Ich konnte zeigen, dass die Überexpression von STAT3 mittels viralen Gentransfers eine Zunahme des axonalen Aussprossens und der Regeneration des CSTs nach einer thorakalen Läsionen bewirkte. Interessanterweise konnte die Überexpression von STAT 3 dabei auch das axonale Aussprossen in Abwesenheit einer Läsion fördern. Infolge führte ich dann eine einseitige Verletzung des CST auf der Höhe der Medulla oblongata (Pyramidotomie) durch und untersuchte, ob die Überexpression von STAT3 in kortikalen Neuronen in der Lage ist, die unverletzte Seite des CST dazu zu stimulieren, den Innervationsverlust der lädierten Seite auszugleichen. Tatsächlich konnte ich zeigen, dass STAT3-Überexpression eine kompensatorische Reorganisation des unverletzten CST bewirkte. Der unverletzten CST bildete zunächst neue Kollateralen im zervikalen Rückenmark aus. Diese wuchsen dann über die Mittellinie auf die denervierte Seite des Rückenmarks, wo sie Kontakte mit kurzen propriospinalen Neuronen und ventralen Motoneuronen – welche die Bewegung der oberen Extremitäten kontrollieren – ausbildeten. Verhaltensexperimente und elektrophysiologische Messungen konnten bestätigen, dass diese neuen intraspinalen Verschaltungen die funktionelle Erholung verbesserten.

In der Gesamtschau meiner Ergebnisse kann man schlussfolgern, dass axonales Remodeling nach einer Rückenmarksverletzung in distinkten sukzessiven Phasen abläuft. Ich konnte weiterhin zeigen, dass der virale Gentransfer von STAT3 – einem Transkriptionsfaktor, der das intrinsische neuronale Wachstumsprogramm aktiviert – eine effektive Strategie ist, diesen axonalen Umbau weiter zu verbessern und auf diese Weise die funktionelle Erholung nach Verletzung zu fördern. Ich hoffe, dass diese Arbeit dazu beitragen konnte, das Verständnis der Mechanismen, die axonale Umbauprozesse regulieren, zu erweitern. Aufbauend auf diesen Befunden konnten wir weiterhin eine vielversprechende Strategie identifizieren, welche axonalen Umbau und funktionelle Erholung nach ZNS-Verletzungen weiter verbessern kann.

General Introduction

The worldwide incidence of spinal cord injury (SCI) is estimated to be 22 people/million population in the western and developing world (source: International Campaign for Cures of Spinal Cord Injury Paralysis [ICCP]). In Germany, an estimated 18.5 cases/million of the population suffer from SCI, with 1500 new cases reported per year (ICCP). SCI results from either direct or indirect trauma to the cord. Spinal cord lesions are commonly due to acute contusion caused by the displacement of bone fragments into the spinal cord (Schwab and Bartholdi 1996; Kraus 1996; Schwartz and Flanders 2006). A majority of injuries are caused by motor vehicle and sport accidents, while other causes include falls, acts of violence from stabbings or gunshot wounds, and sport-related injuries (Figure 1). SCI patients are commonly young males, therefore SCI presents an economical burden for society. Due to the organization of the spinal cord and the poor capacity of the central nervous system (CNS) to repair following injury, SCI disrupts descending and ascending motor pathways, causing a transient or permanent loss of sensorimotor and/or autonomic function below the level of the injury.

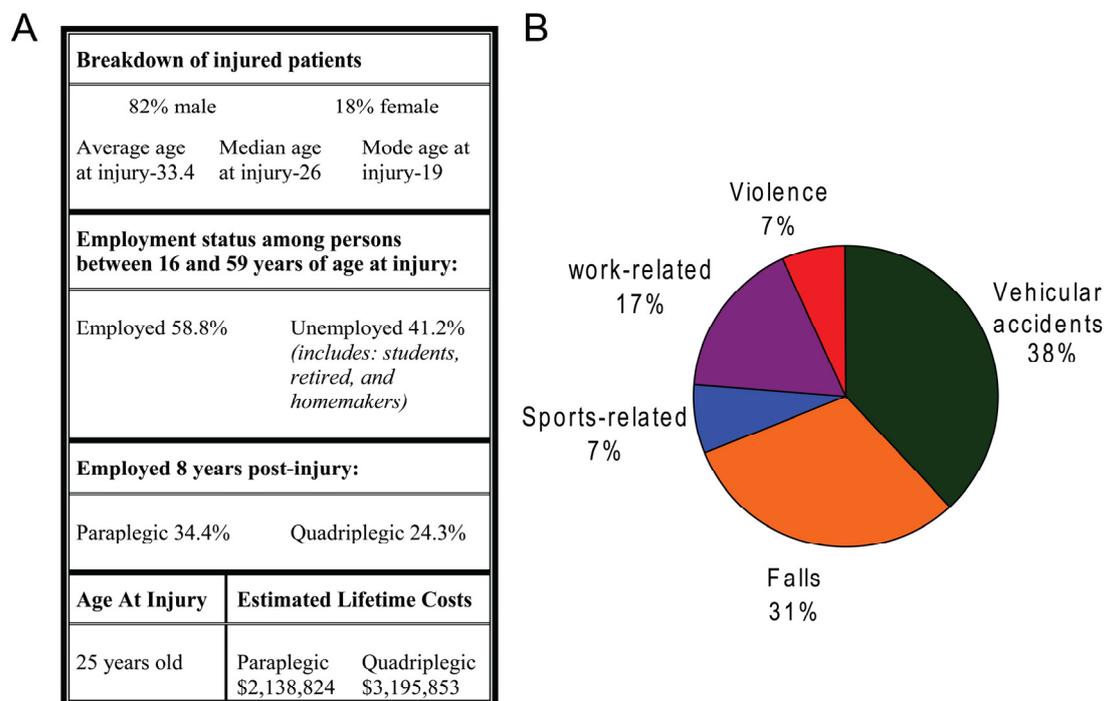


Figure 1. Spinal Cord Injury facts and figures for the North American and European populations. (A) The economical impact of traumatic spinal cord injury. Data compiled from the National Spinal Cord Injury Statistical Center 2005 and 2011, Birmingham, Alabama, USA. **(B)** Etiology of traumatic spinal cord injuries for the European population (including Germany) by WHO 1959–2008 (Cripps et al., 2011)

Over 130,000 people worldwide are affected by a traumatic spinal cord injury resulting in paralysis and loss of sensory-motor function below the level of injury (source: ICCP). Depending on the severity of the injury, the ability to control a majority of autonomous bodily functions that includes bowel, bladder and sexual function can be lost to various degrees (Figure 2; Ditunno et al., 1994; American Spinal Injury Association 2000; Rhee et al., 2006). Human spinal cord injury is classified clinically by the segmental level of injury, the completeness of the injury, and the mechanism of injury (Figure 2). An injury above the C4 cervical level leads to tetraplegia where there is paralysis in both the arms and legs. Conversely, patients with an injury at the lower thoracic to lumbar level can experience paralysis or reduced movement of their legs. In such cases of severe traumatic injury, the SCI patient will require long term care with elevated lifetime cost (Figure 1; The University of Alabama National Spinal Cord Injury Statistical Center 2002). In addition, the quality of life of many SCI patients is severely affected as they are both paralyzed and bound for life to a wheelchair (Westgren and Levi 1998; Krause 2003; Budh and Osteråker 2007).

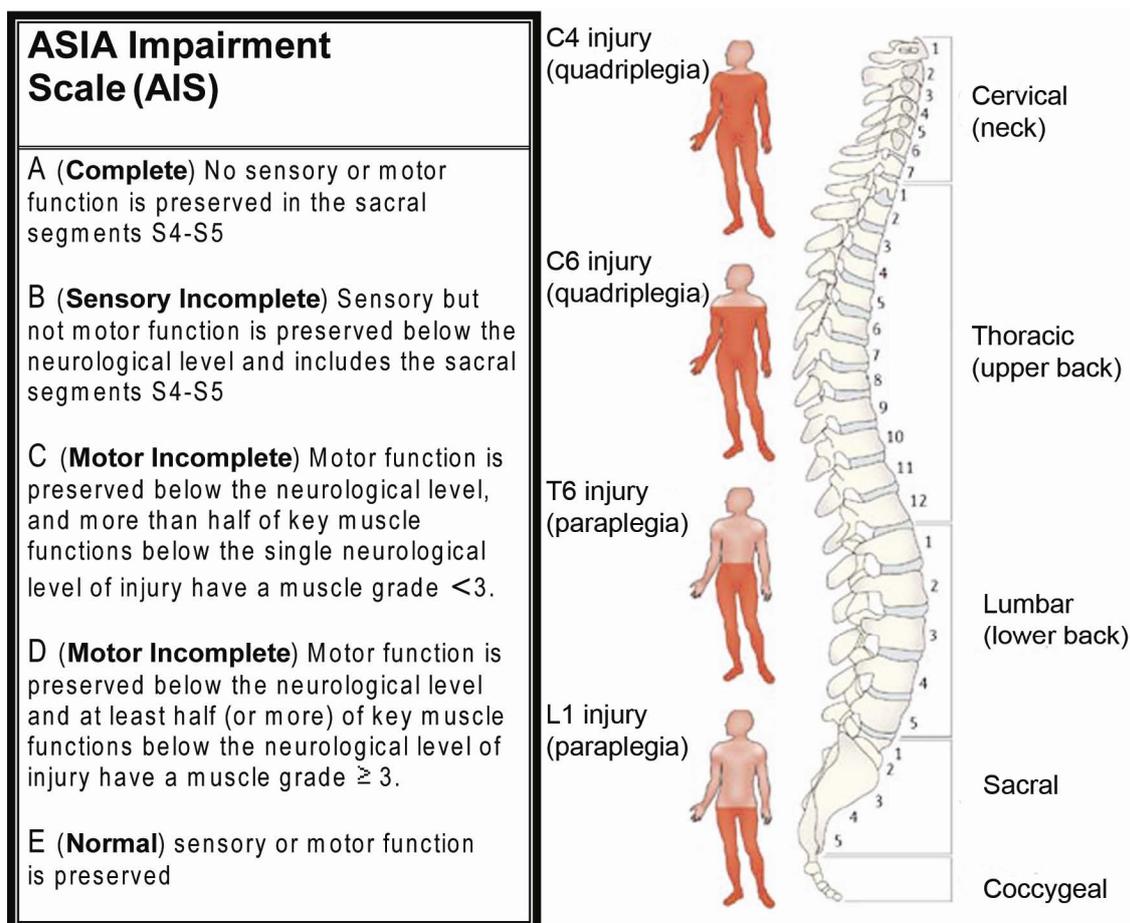


Figure 2. Spinal cord injury severity classification using the American Spinal Injury Association (ASIA) Impairment Scale (Modified from Thuret et al., 2006, Copyright © Nature Publishing Group 2006).

1.1. The pathophysiological response of the cord to injury

Information regarding the pathology of human SCI is limited, though the available data indicates there are strong similarities between humans and rodent spinal contusion injuries (Kakulas 1984; Bunge et al., 1993; Kalb and Strittmatter 2010). In response to injury, the spinal cord undergoes three time-dependent phases (Tator 1995; Schwab and Bartholdi 1996; Tator 1998; Bareyre and Schwab 2003; Schwartz and Flanders 2006): The acute, sub-acute and chronic phase.

1.1.1. The acute phase: 3-6hrs post injury

The acute phase happens immediately to a few hours after injury. Injury to the cord can be caused by the displacement of bone fragments or direct compression of the cord. During this phase the cord undergoes biochemical and structural changes. Interneuronal tracts are damaged, blood flow is reduced, intracellular calcium levels rise, and there is cellular death and degeneration of axons (Tator 1995; Martirosyan et al., 2011). The edema develops and there is a change in electrolyte levels, with an increase of extracellular potassium (Schwab and Bartholdi 1996). The biochemical changes lead to a state of spinal shock, where there is temporary flaccid paralysis and loss of tendon reflexes below the level of the lesion (Hirsemenzel et al., 2000; Ditunno et al., 2004).

1.1.2. The sub-acute phase 6-72 hrs post injury

During this phase there is an increase in free radical production and the release of excitatory neurotransmitters, such as glutamate and aspartate, up to cytotoxic levels (Park et al., 2004). Within hours and lasting for several days following injury, an inflammatory response develops (Balentine 1978; Dusart and Schwab 1994), with endothelial damage, release of inflammatory mediators, invasion of peripheral inflammatory cells and activation of microglia. Secondary damage is caused by immune cells such as neutrophils (appearing 6-24h post-injury) macrophages (appearing 24h-2wks post-injury), and T-cells (Blight 1992; Schnell et al., 1999; Bethea and Dietrich, 2002; David and Kroner 2011).

At the lesion site a cavity and scar tissue are formed, consisting of extracellular matrix (ECM) proteins, mainly collagen and chondroitin sulphate proteoglycans (CSPGs), which secrete inhibitors hindering axonal regrowth (Fitch and Silver 2008; Fehlings and Hawryluk 2010). Fibroblast, Schwann cells and macrophages form the scar tissue by the deposit of ECM laminin, fibronectin and collagen. The sealing of the scar is dependent on pericytes, perivascular cells that associate with the endothelial cells of capillaries and are the source of scar-forming cells (Göriz et al., 2011). The glial/fibrotic scar is a hindrance to regeneration as it is an inhibitory environment that is made up of fibroblast-like cells, collagen surrounded by reactive astrocytes and microglial cells (Silver and Miller 2004; Xu et al., 2011; Leal-Filho 2011). The reactive astrocytes are responsible for the upregulation, at the site of injury, of molecules such as semaphorin 3 (Pasterkamp et al., 2001), ephrin-B2 (Bundesen et al., 2003), slit proteins (Hagino et al., 2003), and chondroitin sulfate proteoglycans (McKeon et al., 1995; Jones et al., 2003; Rhodes and Fawcett 2004). The scar however does serve a function as it separates the damaged tissue from healthy, hence protecting viable tissue from necrosis (Lindsay 1986; Reier and Houlse 1988; Schwab and Bartholdi 1996; Fitch and Silver 2001; Leal-Filho 2011).

1.1.3. The late phase: weeks to months post injury

In the late phase, there is the disappearance of phagocytic macrophages from the lesion area and what remains is a fluid-filled cyst (Figure 3). There is also the formation of cavities, which are filled with cerebrospinal fluid (Balentine 1978; Zhang et al., 1997). Examination of damaged human tissue found that necrosis is similar to animal models, including the cavities formed (Hughes 1974; Bunge et al., 1993). The dense network of reactive astrocytes makes up the major component of the scar, another albeit minor component is the reactive microglial cell and macrophages. At 3 weeks there is Wallerian degeneration (Blight and Descrecito 1986; Zhang et al., 1997), and to some extent, particularly in smaller lesions, remyelination by oligodendrocytes (Gledhill and McDonald 1977; Harrison and McDonald 1977; Schwartz and Flanders 2006). There is some attempt of CNS axons to sprout after injury but the newly formed growth become dystrophic (Li and Raisman 1995; Kerschensteiner et al., 2005; Misgeld et al., 2007) after exposure to a gradient of inhibitory extracellular matrix molecules (Fitch and Silver 2008). Growth-associated inhibitors such as myelin associated glycoproteins (MAGs), Nogo and oligodendrocyte myelin glycoprotein (OMgp) are expressed in the vicinity of the lesion area hindering any attempt of growth (Schwab and Bartholdi 1996; Sekhon and Fehlings 2001;

Filbin 2003). Near the vicinity of the lesion site, chronic demyelination occurs in both humans (Bunge et al., 1993; Guest et al., 2005) and experimental animals (Blight, 1983; Blight 1993; Cao et al., 2005; Totoiu and Keirstead 2005). Overall there is loss of myelin in the white matter and conduction deficits due to the biochemical and molecular changes that occur, interruption of tracts and demyelination (Waxman 1989; Waxman 1992; Taoka and Okajima 1998; Sekhon and Fehlings 2001).

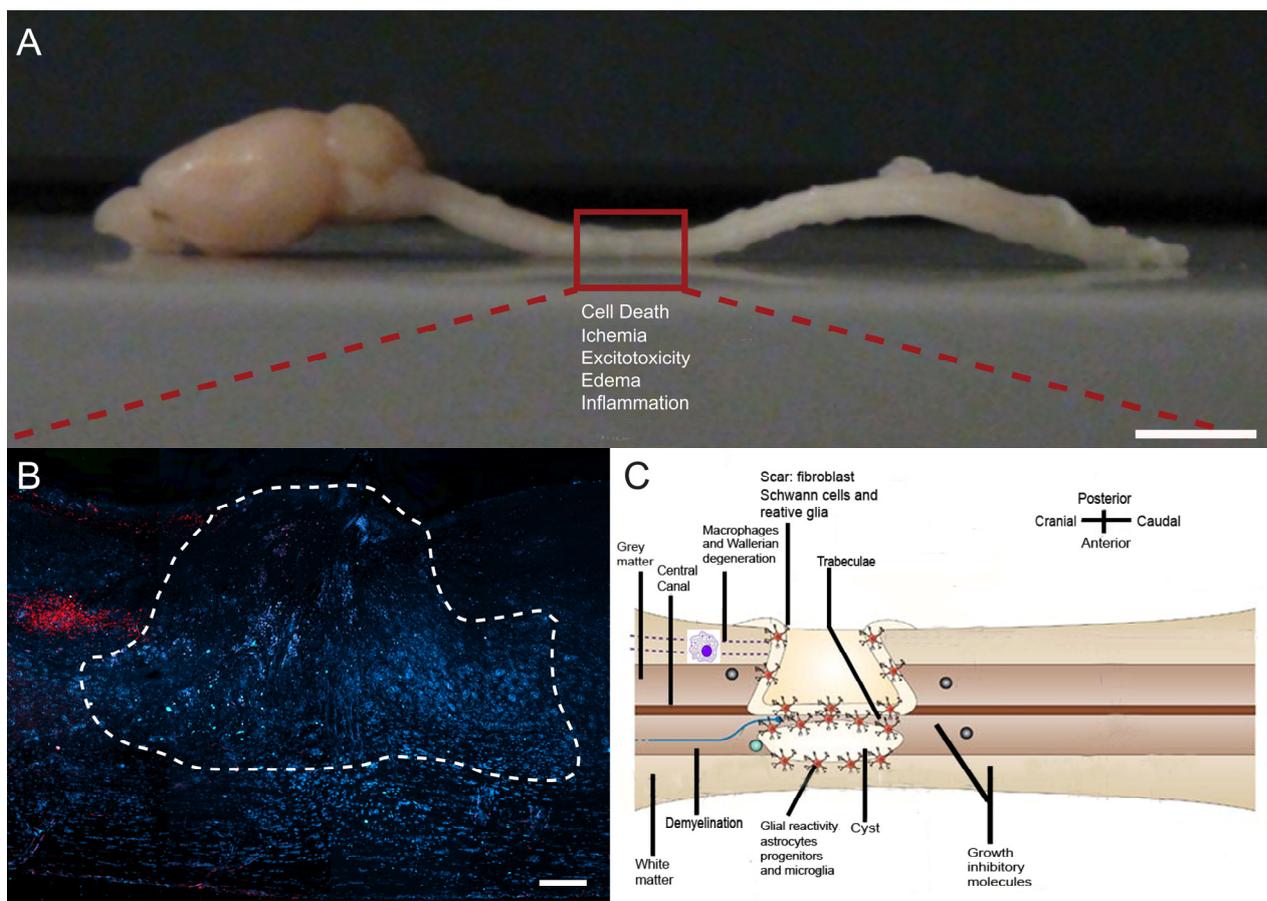


Figure 3. Response of the spinal cord following injury. (A) Dissected mouse spinal cord highlighting the thoracic spinal cord (red box). The events mentioned occur in the acute and subacute phase following a lesion. Scale bar represents 1mm (B) Sagittal section of a thoracic spinal cord 3 weeks following a dorsal T8 hemisection (neurons in blue and the corticospinal tract (CST) in red). Scale bar represents 100µm. (C) After spinal cord injury there is damage of ascending and descending tracts, upregulation of growth and inhibitory factors, accumulation of immune cells in the lesion site and the formation of the glial scar. The damaged axonal tracts can respond by sprouting new collaterals and by the formation of new circuits (Image C is modified from Thuret et al., 2006, Copyright © Nature Publishing Group 2006).

1.2. Clinical care and perspectives

At present, in humans there is no treatment that is able to fully restore sensorimotor function following injury. Patients admitted with injury undergo surgery to remove bone fragments and have their spine stabilized. After surgery care involves the prevention of secondary damages and rehabilitation in the form of physiotherapy (Dietz 2002; Edgerton et al., 2006; Mehrholz et al., 2008; Markandaya et al., 2012). The acute standard of care involves the administration of corticosteroids such as methylprednisolone, which works by decreasing inflammation thereby limiting secondary damage (Bracken et al., 1985; Bracken et al., 1997; Bracken 2002; Hurlbert and Hamilton 2008). The use of this drug is somewhat controversial with some studies highlighting the benefits (Bracken et al., 1985; Bracken et al., 1997; Bracken and Holford 2002) and others finding the effects modest in light of associated side effects that can include glaucoma, hyperglycemia, depression, psychosis and the cessation of the natural production of cortisol (Nesathurai 1998; Sayer et al., 2006).

Strategies for future treatment and management of spinal cord injury in humans are based on the pathophysiological changes that occur in the acute, sub-acute and late phases following injury. Research aims at understanding the different pathophysiological mechanisms that arise following injury in experimental models and that could be potentially targeted for therapeutic treatments. For example neuroprotective strategies have been developed by preventing excitotoxicity (Feldblum et al., 2000; Abdelkarim et al., 2001; Mazzone and Nistri 2011), by controlling the inflammatory response (Popovich et al., 1999; Fitch et al., 1999; Alexander and Popovich 2009; Pajooohesh-Ganji and Byrnes 2011), or by preventing apoptosis (Nicholson 2000; Nestic et al., 2001; Demjen et al, 2004). Other lines of work aim at promoting axonal growth or regeneration through the use of cell grafts and scaffolds that can act as bridges, through the application of growth promoting factors (Xu et al., 1995; Li et al., 1997; Li et al., 1998; Menei et al., 1998; Ramon-Cueto et al., 2000; Bamber et al., 2001; Takami et al., 2002; Li et al., 2003; Bradbury and McMahon 2006) or through the manipulation of the intrinsic growth program of neurons (Qiu et al., 2002; Yip et al., 2010; Liu et al., 2010, Bareyre et al., 2011; Sun et al., 2011). Laboratory research has provided useful information in expanding the knowledge and contributing to the treatment of spinal cord injury.

1.3. Experimental models for SCI research

In SCI research the use of appropriate animal models is important. In humans, spinal cord injuries can be diverse and it can be difficult to reproduce the same sort of injury in an experimental setting. The two main commonly used models of spinal cord injury employed by most laboratories are (Figure 4A):

(1) The contusion injury performed using a NYU-MASCIS (New York University - Multicenter Animal Spinal Cord Injury Study) impactor device, which drops a weight from specific heights, can perform standardized grades of spinal cord injuries (Gruner 1992; Agrawal et al., 2010). This injury model is based on the Allen technique, developed in 1911, whereby a weight dropped through a tube onto the exposed thoracic cord of a dog resulting in a reproducible model. The contusion model is the most similar to the histological mechanics of human spinal cord injury (Schwab and Bartholdi 1996), however in this type of injury there is haemorrhage and variable tissue damage making the comparison of neuronal damage between different animals more challenging (David and Kroner 2011).

(2) The dorsal hemisection injury involves transection of the dorsal half of the spinal cord at the thoracic level with fine iridectomy scissors. This model produces a smaller inflammatory response, is highly reproducible and allows for defined parts of the cord to be spared. This model is thus best suited for the precise investigation of axonal remodeling and was used in our studies on CST regeneration and remodeling.

In addition to spinal lesion models that focus on the analysis of the corticospinal tract, we also used the dorsal root ganglia (DRG) model system to study the growth initiation of axons (Figure 4B). The DRG model system is often employed in outgrowth studies as it provides access to both the PNS, (known for its regenerative capabilities), and to the CNS, (where there is no successful regeneration) (Richardson and Issa 1984; Richardson and Verge 1987; Sjoberg and Kanje 1990; Chong et al., 1994; Neumann and Woolf 1999).

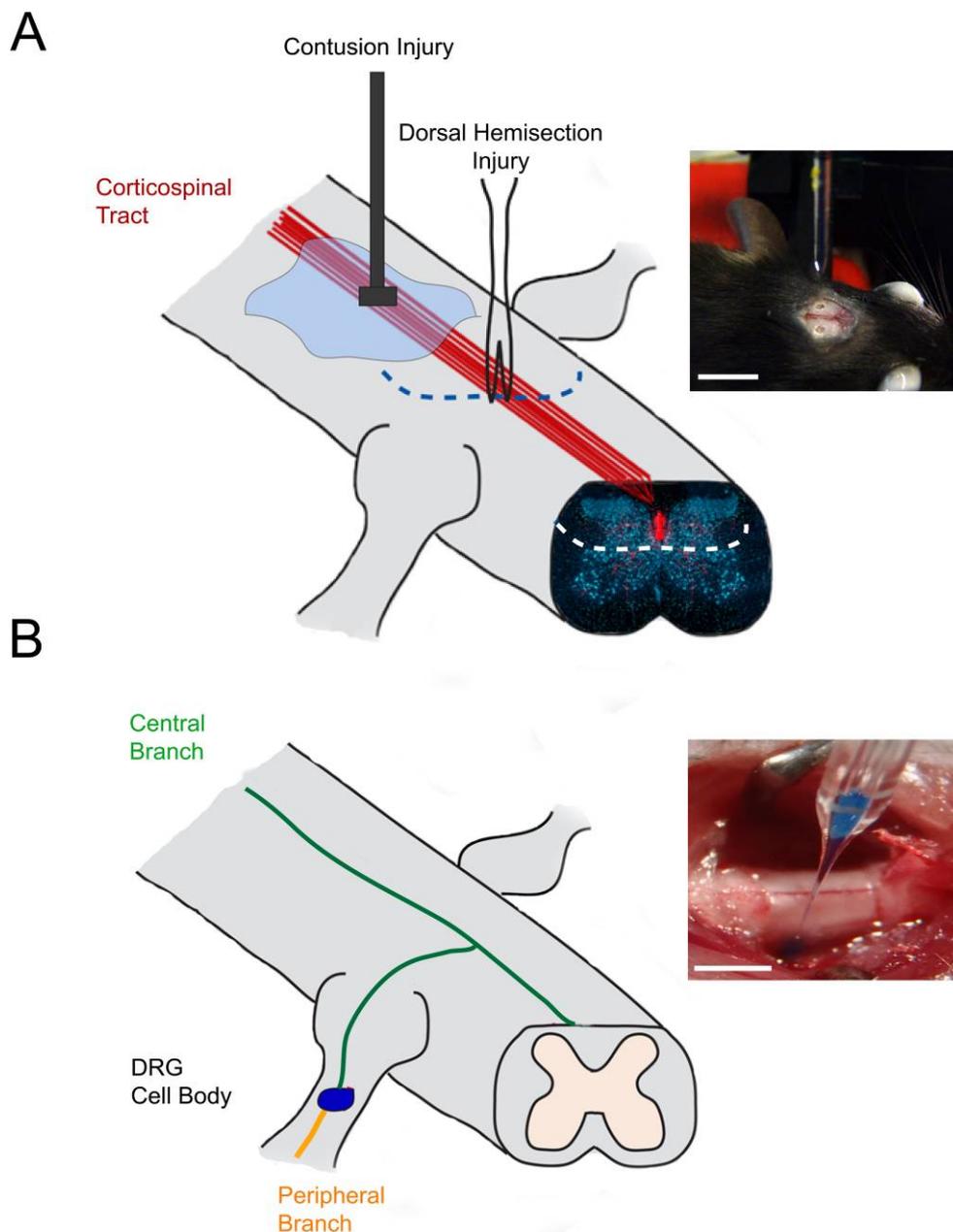


Figure 4. Localization of the Corticospinal tract (CST) and Dorsal root ganglia (DRG) neuron with its peripheral and central branch. (A) The corticospinal tract (red) can be labeled or manipulated via stereotaxic injection of a compound (either a tracer or virus) of interest into defined coordinates in the cortex. In a dorsal T8 hemisection, only half of the cord is transected (dashed line) sparing fiber tracts and interneuronal pools located in the ventral grey and white matter. For a contusion injury, an impactor drops a weight from a fixed distance onto the cord enabling standardized grades of injuries. **(B)** The DRG can be exposed through surgery and also injected with a finely pulled micropipette. The neuronal cell body of the DRG, located in the ganglia, sends one branch into the periphery (peripheral branch) and another branch into the spinal cord (central branch). The peripheral branch is known for its regenerative capabilities. Regeneration of the central branch can occur only if the peripheral branch is first injured, an effect known as a conditioning lesion. The DRG injection image is courtesy of Fabian Laage-Gaupp. Scale bar equals 1mm in A and B.

1.4. Spontaneous corticospinal outgrowth and remodeling after SCI

The corticospinal tract (CST), as one of the most important descending motor tract for skilled movements in all mammalian species (Nudo and Masterton 1988, 1990; Maier et al., 2008), has been a frequent model used to investigate axonal growth, regeneration and remodeling within the adult CNS (Schnell et al., 1994; Weidner et al., 2001; Zhou and Shine 2003; Bareyre et al., 2004; Liu et al., 2010; Rosenzweig et al., 2010). In the mammalian system the CST is responsible for fine skilled movements, for example grasping and handling (Whishaw et al., 1998) and locomotor functions such as stride length (Bregman et al., 1995). In humans the CST has an even more important role and controls locomotion, posture as well as voluntary skilled movements (Ferguson et al., 2001; Hutson et al., 2011). The pyramidal neurons of the corticospinal tract originate in lamina V of the cortex. The fibers of this tract descend from the cerebral cortex sending axons via the internal capsule to the spinal cord (Dottori et al., 1998). The CST is composed of two components: a main component also known as the main CST which comprises of 95% of all CST axons; and a minor component constituted of the ventral and dorsolateral CST (Vahlsing and Feringa 1980; Joosten et al., 1992; Brösamle and Schwab 1997; Weidner et al., 2001; Steward et al., 2004). The tract in rodents decussates at the spinomedullary junction with the main component crossing over in the dorsal funiculus. In humans the CST fibers descend contralaterally in the lateral funiculus (Harel and Strittmatter 2006; Hutson et al., 2011). The remaining minor components of the CST do not decussate and the fibers run in the white matter of the ipsilateral dorsolateral side (Hutson et al., 2011). At 4 weeks of age the rodent CST is matured and establishes contacts with the appropriate interneurons and with a subset of motoneurons (Ghosh et al., 2009). The topological organization of the CST is such that the forelimb CST (fCST) projects its collaterals in the cervical cord, while hindlimb CST (hCST) axons innervate the appropriate target in the lumbar cord (Akintunde and Buxton 1992; Ghosh et al., 2009).

The CNS for a long time has had the reputation for being a “static” system in adulthood, where successful axonal growth, sprouting and regeneration does not occur. As research progresses, we are beginning to now realize that in the adult mammal the CNS has the capability for some spontaneous recovery of function in rodents and to some extent in humans (Schwab and Bartholdi 1996; Burns et al., 1997; Dietz et al., 1998; Rossignol et al., 1999) Many examples of injury-induced plasticity stem from the study of lesioned CST

connections (Bernstein and Stelzner 1983; Kuang and Kalil 1990; Schnell et al., 1994; Terashima 1995; Bregman et al., 1995; Li et al., 1997; Weidner et al., 2001; Bareyre et al., 2002; Bareyre et al., 2005; Demjen et al., 2004). Prominent findings have found that the reorganization of neural circuits plays a key role in spontaneous recovery of function (Bareyre et al., 2004; Courtine et al., 2008; Ghosh et al., 2009; Alilain et al., 2011). In 2001, Fouad et al., (2001) observed that following an incomplete thoracic lesion in rats there were rearrangements in the cortical motor map. Stimulation of what was previously an area responsible for hindlimb muscle response instead invoked forelimb responses. The shift in the cortical map was accompanied by spontaneous growth of hindlimb corticospinal axons in the cervical cord. However it was unknown what the neuronal targets of these newly formed collaterals were.

Bareyre et al., (2004), then made a seminal discovery whereby functional recovery after SCI was attained via the formation of an intraspinal detour circuit (Figure 4). This study revealed that neural circuits are able to undergo spontaneous functional reorganization that lead to the reconnection of lesioned CST fibers with their original targets. Following a partial lesion of the CST, lesioned hindlimb CST axons sprouted newly formed collaterals in the cervical cord rostral to the lesion site. These collaterals were found to form contacts with a pool of excitatory interneurons known as propriospinal neurons. Propriospinal neurons were first described in 1902, as a network of axons extending from the proximal to distal spinal cord (Sherrington and Laslett 1902, 1903). These neurons are an important part of an intraspinal network of interneurons involved in motor reflexes, voluntary movement and sensory processing (Kostyuk and Vasilenko 1979; Jankowska 1992; Foreman 2000; Pierrot-Deseilligny and Burke 2005; Alstermark et al., 2007; Conta and Stelzner, 2009; Cowley et al., 2010; Flynn et al., 2011). There are two main populations of propriospinal neurons. Short propriospinal neurons (SPSNs), involved in the fine tuning of forelimb movement, have their cell bodies in C4 and their axons terminate at T2. Long propriospinal neurons (LPSNS) are known to coordinate forelimb and hindlimb movement. This population of PSNs has their cell bodies in C5/6 and their axons extend till T12/T13 (Nicolas et al., 2001; Dietz 2002). In this study, it was found that initially, hCST collaterals contacted both long and short PSNs equally (Bareyre et al., 2004). Overtime, contacts onto SPSNs were removed while contacts onto LPSNs were maintained (Figure 5). Long PSNs are an ideal target as they are able to bypass the lesion site and contact motoneurons in the lumbar cord. Further examination revealed that the LPSNs had increased their contacts onto lumbar motoneurons, hence forming a new intraspinal circuit (Bareyre et al., 2004). Behavioral and electrophysiological

experiments further proved that this circuit was functional and responsible for the spontaneous functional recovery seen following SCI.

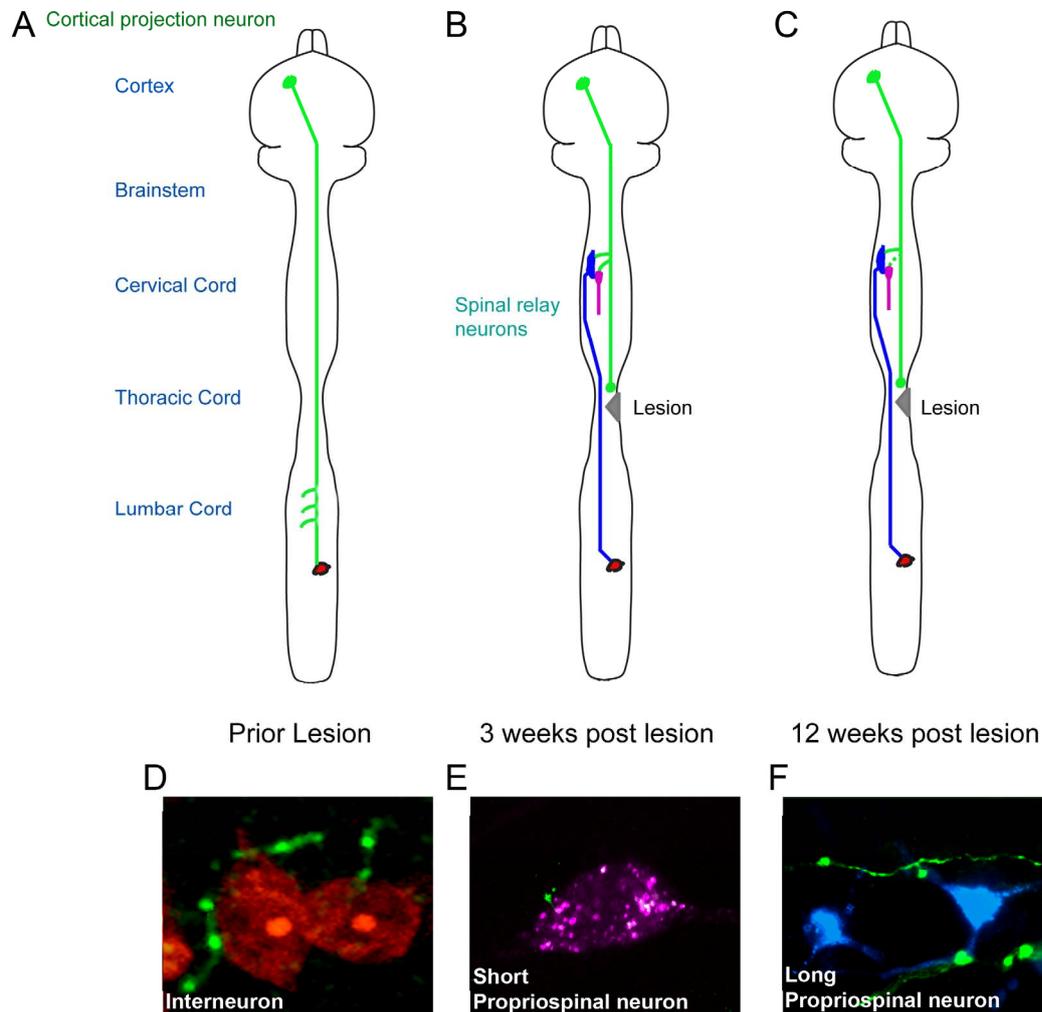


Figure 5. Reorganization of hindlimb CST collaterals following injury. Timeline of the formation of a new intraspinal detour circuit following a dorsal T8 hemisection. **(A)** Normal connectivity, in absence of a lesion, in which the hindlimb CST (hCST) sprouts its collaterals into the lumbar cord to contact interneurons or motoneurons. **(B)** Three weeks post lesion, hCST collaterals are induced to sprout in the cervical cord where they were seen to form equal contacts onto both short and long propriospinal spinal relay neurons. **(C)** By 12 weeks post lesion contacts onto long propriospinal neurons (LPSNs) that bypass the lesion site and are involved in coordinating hindlimb and forelimb movement are strengthened and maintained. While contacts onto short propriospinal neurons that do not directly aid in functional recovery are pruned. LPSNs were seen to form contacts with lumbar motoneurons, thereby creating a new intraspinal circuit that can enable functional recovery. **(D-F)** Confocal photomicrographs showing contacts of hCST collaterals to **(D)** interneurons at 3 weeks following injury, **(E)** to short propriospinal neurons 3 weeks following injury, and to **(F)** LPSNs 3 weeks following injury. **(D)** is taken from Lang et al., 2012.

Further examining the importance of this newly formed intraspinal circuit for functional recovery after incomplete injuries, Courtine et al., (2008) performed a series of elegant experiments to examine the effects of eliminating the propriospinal relay connection. They demonstrated that severing the newly formed propriospinal relay connections whether

by a staggered hemisection or a high dose of N-methyl-D-aspartate (NMDA), which in high doses can act as an excitotoxin, abolishes recovery. By severing the supraspinal axon connection, kinematic and physiological analyses showed that this results in permanent paralysis of the hindlimbs. The various lesion paradigms performed in this study, where lesions were separated both temporally and spatially, in combination with refined kinematic analysis, illustrated that the propriospinal relay circuit is responsible for the restoration of full, weight-bearing hindlimb locomotor function seen following even a severe injury. In summary, these studies revealed upon electrophysiological, behavioral and anatomical examination that a new detour circuit involving LPSNs can be formed and that this axonal remodeling contributed to functional recovery (Fouad et al., 2001; Bareyre et al., 2004; Courtine et al., 2008).

After injury, intraspinal circuits are spontaneously created and able to transmit descending supraspinal input to the lumbar motor circuits (Bareyre et al., 2004; Kerschensteiner et al., 2004; Ballermann and Fouad 2006; Courtine et al., 2008). These studies not only highlight the positive impacts that injury can induce in the form of remodeling, they can also help to identify the mechanisms that govern spontaneous remodeling and functional recovery. Exploiting this innate phenomenon by reinforcing beneficial axonal remodeling would be an advantageous strategy in facilitating functional recovery.

1.5. Therapy-induced modulation of corticospinal remodeling

About half of SCI patients, approximately 54%, suffer from an incomplete injury where at an anatomical level there is some sparing of tissue and descending fibers (Raineteau and Schwab 2001; Spinal injury network 2011). In such cases, under the right conditions extensive remodeling can occur due to the preservation of the cortical, subcortical and remaining intact fibers and spinal circuitry. Molecular interventions to take advantage of spared fibers and circuits are one of the main types of therapies that are researched in the field of SCI (Thuret et al., 2006). In the search for successful therapeutic strategies one needs to consider the extrinsic and the intrinsic barriers that limit this process in the CNS.

Extrinsic cues that limit axonal remodeling appear to accumulate over time in the CNS. For example, in young animals the CST displays great plasticity and if injured the CST axons are able to pass the lesion site to innervate the appropriate area (Bregman et al., 1989; Liu et al., 2011). As the animal matures the regenerative and plastic capability of the CNS is reduced, coinciding with the production of myelin and of inhibitory proteins such as associated neurite growth inhibitory proteins (Kapfhammer and Schwab 1994a,b; Steeves et al., 1994; Harel and Strittmatter 2006). The glial scar forms an inhibitory environment and acts as a barrier to axonal regeneration. Injured axons when faced with such a barrier are unable to grow further and instead form retraction bulbs (Fitch and Silver 1997; Silver and Miller 2004; Leal-Filho 2011). Neutralization of the inhibitory environment that is formed following injury is one strategy that has been used to enhance regeneration of lesioned axons. For example, the removal or neutralization of oligodendrocytes or myelin can improve regeneration (Bregman et al., 1995). When an antibody aimed against neutralizing inhibitory factors like Nogo-A (IN-1 antigen) is used, regenerative sprouts and long distance elongation is seen (Schnell and Schwab, 1990; Brosamle et al., 2000; Chen et al., 2000). Likewise treatment with the bacterial enzyme chondroitinase ABC (chABC), which has the ability to digest CSPG in the scar, also has beneficial effects in spinal cord injury models from contusion (Caggiano et al., 2005) hemisections (Bradbury et al., 2002; Yick et al., 2003; Barritt et al., 2006; Houle et al., 2006) and transections (Fouad et al., 2005). While some beneficial effects on axonal remodeling have been reported as well (Thallmair et al., 1998; Z'Graggen et al., 1998; Z'Graggen et al., 2000; Bradbury et al., 2002; Bareyre et al., 2002), the removal of extracellular inhibitory molecules has so far proven to be insufficient for extensive axonal remodeling and complete functional recovery. This might at least partially be due to the fact that this approach targets growth inhibitors at the lesion site, whereas axonal remodeling commonly occurs remote from this area.

The intrinsic barrier, attributed to the decline in the intrinsic growth competence of a CNS neuron as it matures, affects the capabilities of injured CNS neurons to both regenerate and remodel. To promote axonal growth and remodeling, one commonly used strategy is the application of factors such as neurotrophins. When a unilateral lesion of the CST is performed at the pyramidal decussation (pyramidotomy lesion) in the medulla oblongata, the unlesioned CST tract can be recruited to compensate for the loss of the original input and enable functional recovery. This model system can be used to evaluate the effectiveness of potential molecules to promote remodeling. Following a pyramidotomy lesion, when motoneurons were transduced to express NT3, unlesioned CST axons were found to sprout

across the midline towards the transduced motoneurons (Zhou et al., 2003). Sustained concomitant expression of neurotrophic factors BDNF and NT3 in the cortex and spinal cord, respectively, further enhanced the axonal sprouting effect from the intact tract (Zhou and Shine 2003). An alternative and more direct approach is the manipulation of key regulatory genes involved in the intrinsic growth program and known to be activated in regenerating PNS neurons. The forced upregulation of molecules that contribute to axonal growth and that are normally downregulated in the CNS after injury can induce compensatory sprouting from the unlesioned tract. Genetically overexpressing factors such as neuronal calcium sensor-1 (NCS1) (Yip et al., 2010) and mTOR (Liu et al., 2010) has been reported to stimulate spared fibers to send their axons into to the denervated side leading to functional recovery of the injured limb. Remodeling requires axonal growth, as it has been shown that manipulation of the PI3K-Akt (of which NCS1 is a member) and PTEN/mTOR pathways can induce growth and lead to recovery following CNS injuries (Park et al., 2010; Yip et al., 2010; Liu et al., 2010). Hence the approach to induce growth through exploiting the intrinsic growth potential of a neuron would be an effective strategy to promote axonal remodeling.

1.6. The Jak/STAT Pathway: A component of the intrinsic growth program

In the adult mammalian system, it is well known that only in the peripheral nervous system (PNS) there is successful regeneration of lesioned axons. Following injury, even though successful regeneration does not occur in the CNS, CNS neurons are capable of mounting a transient regenerative response as evidenced by the expression of regeneration associated proteins and genes (RAGs) (Mikucki and Oblinger 1991; Tetzlaff et al., 1994; Fournier and McKerracher 1997; Neumann and Woolf 1999; Bulsara et al., 2002; Storer et al., 2003; Kruse et al., 2011). This indicates that the intrinsic growth program inherent to neurons is activated in response to injury, however it is not sustained. This is in contrast to what has been observed in the PNS, where following injury there is regeneration and a high expression of transcription factors and proteins associated with regeneration and growth. In the classical 'conditioning lesion' paradigm, regeneration of both the peripheral and central branch of the DRG neuron can occur, although regeneration of the central branch is dependent on prior injury of the peripheral branch (Richardson and Issa 1984; Richardson and Verge 1987; Sjoberg and Kanje 1990; Chong et al., 1994; Neumann and Woolf 1999;

Cai et al., 2002). Injury of the peripheral branch activates the intrinsic growth program and is thus the key for regeneration in both branches (Cai et al., 2002; Yang and Yang 2011) as this program is not activated in the mature CNS following an injury (Stam et al., 2007). Targeting factors involved in the intrinsic growth program would therefore be an effective strategy to promote axonal growth and regeneration following CNS injuries. Most commonly upregulated genes and transcription factors after injury that are found to be associated with regeneration are: c-Jun, Atf-3, Hspb1, HSP-27, Adcyap1, Gadd45a, Gap43, Actb, Tubb3 and in particular the Signal transducer and activator of transcription 3 (STAT3) (Broude et al., 1997; Qiu et al., 2005; MacGillavry et al., 2009; Sun and He 2010; Smith et al., 2011).

STAT3 has dual roles as a signal transducer and a transcription factor. The STAT3 protein is essential during development as a complete knockout results in embryonic lethality (Takeda et al., 1997; Aaronson and Horvath 2002). In adulthood it is known to participate in various functions including neuronal cell survival, axonal growth, protection and remodeling (Levy and Darnell 2002). In the immune system, STAT3 plays a key role in the signal transduction of anti-inflammatory responses mediated via macrophages and neutrophils, in turn regulating the inflammation process (Kühn et al., 1993; Akira 2000; Shuai and Liu 2003). The Janus kinase (Jak/STAT) pathway has also been implicated to be involved in synaptic plasticity, with pharmacological inhibition or knockdown of STAT3 blocking the induction of NMDAR (N-methyl-D-aspartate receptor)-LTD (long-term depression) (Nicolas et al., 2012). In normal conditions, STAT3 exists in the cytoplasm of a cell in an inactive form where it is associated with the glycoprotein 130 (gp130). The Jak/STAT3 signaling pathway is activated by cytokines interleukin 6 (Il6) (Zhong et al., 1994), ciliary neurotrophic factor (CNTF) (Rajan et al., 1996) and the leukemia inhibitory factor (LIF) (Kunisada et al., 1996). While the intensity and duration that the Jak/STAT pathway is activated for is tightly regulated and controlled by members of the suppressor of cytokine signaling (SOCS) family (Crocker et al., 2008). Once activated by cytokines, JAK kinases phosphorylates the tyrosine residue (Tyr-705) of STAT3, leading to homodimerization or STAT1/3 heterodimerization (Figure 5). In its activated phosphorylated form, the dimerized complex is translocated to the nucleus where it binds to specific DNA-response elements activating the transcription of specific genes (Zhong et al., 1994; Akira 2000; Ng et al., 2006). STAT3 can also be phosphorylated at serine 727 (Ser-727), and it has been suggested that Ser-727 phosphorylation enables STAT3 to achieve its maximal transcriptional activity (Ceresa and Pessin 1996; Lim and Cao 1999; Ng et al., 2006).

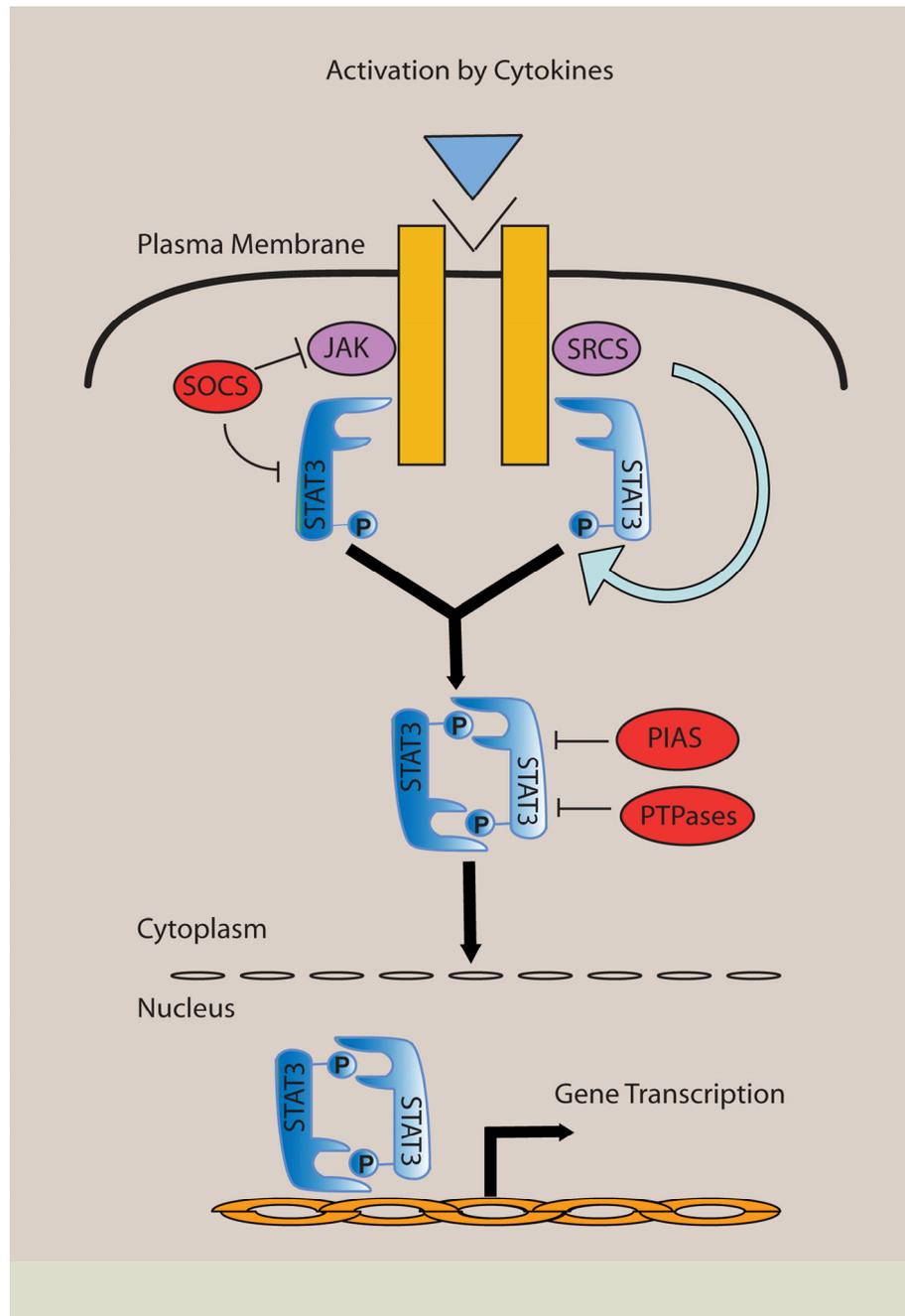


Figure 6. Signal transducer and activator of transcription 3 (STAT3) is part of the Janus-family kinases (JAKs)/STAT3 pathway. This pathway can be activated in response to autocrine or paracrine signals, including cytokines. Upon activation, JAKs and SRC tyrosine kinases are able to phosphorylate STAT3 at its tyrosine residue. Following phosphorylation, STAT3 is able to dimerize and translocates to the nucleus where it will transcribe target genes. STAT3 signaling is tightly regulated by inhibitory molecules that include suppressor of cytokine signaling (SOCS) proteins, protein inhibitor of activated STAT (PIAS) proteins and protein tyrosine phosphatases (PTPases).

The following findings suggest that STAT3 signaling could be a key event in the regulation of axonal outgrowth:

(i) The expression levels of phosphorylated STAT3 is increased in regenerating axons (Schwaiger et al., 2000; Sheu et al., 2000; Xia et al., 2002) and is associated with axonal remodeling (Bareyre et al., 2002).

(ii) Molecules that have an influence on axonal regeneration and are expressed following a peripheral nerve injury, for example cytokines IL-6, ciliary neurotrophic factor, and leukemia inhibitory factor have an effect on STAT3 signaling (Curtis et al., 1994; Zhong et al., 1999; Cafferty et al., 2001; Cafferty et al., 2004).

(iii) In cultured CNS neurons, STAT3 has been found to promote neuronal outgrowth (Smith et al., 2011) and in the DRG 'conditioning lesion' paradigm STAT3 is activated in the cell bodies of the DRG neurons only when the peripheral branch is injured (Schwaiger et al., 2000; Qiu et al., 2005). Taken together these findings suggest that STAT3 is an interesting candidate regulator of the intrinsic neuronal growth program.

Aims of the Thesis

The overall goals of this thesis were to contribute to a better understanding of the structural principles underlying axonal remodeling following spinal cord injury and to determine whether we could enhance axonal growth, regeneration and remodeling of the corticospinal tract through the genetic manipulation of intrinsic neuronal growth pathways. Therefore the following questions were investigated within this thesis:

1. What are the processes that underlie the maturation and remodeling of newly formed collaterals following injury?

In aim I of this thesis we examined the manner in which the corticospinal tract remodels its axons following spinal cord injury. Our laboratory has previously shown that in response to injury, the CST can spontaneously sprout collaterals rostrally to the lesion site. In turn these collaterals contact interneuronal populations in the cervical cord creating a “detour circuit” to mediate functional recovery. Through the use of transgenic mice, genetic tracing methods and dye tracers, we selectively labeled the CST as well as different interneuronal populations in the spinal cord. Using bulk analysis and reconstructions of single CST collaterals we were able to follow the formation, maturation and refinement of CST collaterals over several months following lesion.

2. How does the regenerative-associated transcription factor STAT3 regulate axonal regeneration in the PNS and CNS?

In aim II of this thesis, we wanted to investigate whether the transcription factor STAT3 is a suitable tool to manipulate the intrinsic neuronal growth response. To reveal the role of STAT3 during PNS and CNS regeneration we focused on the DRG system which provides access to both the CNS and the PNS branches of the same neuron. The expression levels of STAT3 were manipulated in both the PNS (where STAT3 was ablated) and CNS (where STAT3 was overexpressed) through the combined use of transgenic mice and adeno-associated viral (AAV) viruses. With confocal and repetitive in vivo timelapse

microscopy, we were able to identify sustained STAT3 expression is a key requirement for the timely initiation of PNS axon regeneration.

3. Can the sustained expression of STAT3 enhance axonal regeneration, remodeling and functional recovery following a CNS injury?

In aim III of the thesis, we expanded on our previous studies in order to elucidate whether the initiation of an intrinsic neuronal growth response by STAT3 expression could also promote outgrowth of CNS axons following spinal cord injury. To address this question we performed two sets of experiments. First we deleted endogenous STAT3 expression in cortical projection neurons and analyzed the effects on endogenous CST axonal outgrowth. Second, through gene therapy we overexpressed STAT3 in upper corticospinal motorneurons and used several CST-lesion paradigms, along with behavioral and electrophysiological assessments to reveal the role of STAT3 in axonal remodeling, regeneration and functional recovery after spinal cord injury.

Results

The work during this doctoral thesis has resulted in two peer-reviewed publications and one submitted manuscript. They are included in the thesis and constitute Chapter 3.

- **Lang C***, Guo, X*, Kerschensteiner, M., and Bareyre, F.M. (2012). Single Collateral Reconstructions Reveal Distinct Phases of Corticospinal Remodeling after Spinal Cord Injury. *PLoS ONE*. 7:e30461.
- Bareyre FM, Garzorz N, **Lang C**, Misgeld T, Büning H, Kerschensteiner M. (2011). In vivo imaging reveals a phase-specific role of STAT3 during central and peripheral nervous system axon regeneration. *Proc Natl Acad Sci U S A*. 108(15):6282-7.
- **Lang C**, Bradley P, Kerschensteiner M, Bareyre FM. (2012). STAT3 promotes corticospinal remodeling, regeneration and functional recovery after spinal cord injury. *An article submitted to the Journal of Neuroscience*

Single collateral reconstructions reveal distinct phases of corticospinal remodeling after spinal cord injury

Lang C, Guo X, Kerschensteiner M, Bareyre FM

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Single Collateral Reconstructions Reveal Distinct Phases of Corticospinal Remodeling after Spinal Cord Injury

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Abstract

Background: Injuries to the spinal cord often result in severe functional deficits that, in case of incomplete injuries, can be partially compensated by axonal remodeling. The corticospinal tract (CST), for example, responds to a thoracic transection with the formation of an intraspinal detour circuit. The key step for the formation of the detour circuit is the sprouting of new CST collaterals in the cervical spinal cord that contact local interneurons. How individual collaterals are formed and refined over time is incompletely understood.

Methodology/Principal Findings: We traced the hindlimb corticospinal tract at different timepoints after lesion to show that cervical collateral formation is initiated in the first 10 days. These collaterals can then persist for at least 24 weeks. Interestingly, both major and minor CST components contribute to the formation of persistent CST collaterals. We then developed an approach to label single CST collaterals based on viral gene transfer of the *Cre recombinase* to a small number of cortical projection neurons in *Thy1-STP-YFP* or *Thy1-Brainbow* mice. Reconstruction and analysis of single collaterals for up to 12 weeks after lesion revealed that CST remodeling evolves in 3 phases. Collateral growth is initiated in the first 10 days after lesion. Between 10 days and 3–4 weeks after lesion elongated and highly branched collaterals form in the gray matter, the complexity of which depends on the CST component they originate from. Finally, between 3–4 weeks and 12 weeks after lesion the size of CST collaterals remains largely unchanged, while the pattern of their contacts onto interneurons matures.

Conclusions/Significance: This study provides a comprehensive anatomical analysis of CST reorganization after injury and reveals that CST remodeling occurs in distinct phases. Our results and techniques should facilitate future efforts to unravel the mechanisms that govern CST remodeling and to promote functional recovery after spinal cord injury.

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Introduction

Injury to the spinal cord leads to a disruption of ascending and descending fiber tracts followed by loss of sensation and voluntary movements below the level of the lesion [1]. Whereas a complete transection of the spinal cord often leads to permanent disabilities, incomplete injuries can be followed by spontaneous functional recovery [2–4]. An important anatomical feature underlying this functional recovery is the remodeling of damaged axonal connections [5–8]. Many insights into how axons remodel after lesion stems from the study of the corticospinal tract (CST). The CST is a major descending motor pathway that mediates skilled movements in all mammalian species [9], [10]. The CST in rodents consists of a main component that runs at the base of the dorsal funiculus and minor components in the dorso-lateral and ventral funiculus [11–13]. In recent years we and others have studied how the hindlimb portion of the CST responds to a

thoracic dorsal hemisection. Using a combination of anterograde, retrograde and trans-synaptic tracing techniques we have previously shown that the formation of intraspinal detour circuits are a key component of CST remodeling after injury [6], [14]. Detour circuits are formed in the following steps: First, the lesioned CST fibers sprout new collaterals in the cervical spinal cord above the level of lesion. These collaterals then extend to the intermediate layers of the cervical gray matter. There they form contacts with different populations of spinal interneurons, including long propriospinal neurons, a population of interneurons that are involved in coupling of forelimb and hindlimb movement [15–18]. These long propriospinal neurons, the axons of which bypass the lesion in the ventral funiculus, in return increase their projections to hindlimb motoneurons in the lumbar spinal cord. Electrophysiological and detailed behavioral and kinematic analysis show that this and similar detour circuits play a key role for the recovery of CST function [6], [7].

While it is thus established that the formation of CST collaterals is a key step of axonal remodeling after injury, we still know very little about how long these collaterals persist, from which CST components they originate and how their complexity and projection pattern evolves over time. Analysis of mice traced by injection with the anterograde tracer BDA (Biotin Dextran Amine) in the hindlimb motor cortex and perfused at 10 days to 24 weeks after a dorsal hemisection of the mid-thoracic spinal cord now revealed the following findings: CST collaterals primarily started to grow in the first 10 days after injury. Both major and minor CST components contributed to this emergence of collaterals. Once emerged, the majority of CST collaterals persisted at least for up to 24 weeks after lesion. To study how these collaterals evolve over a long period of time (for up to 12 weeks after lesion), we labeled single CST collaterals by viral gene transfer of *Cre recombinase* to a small number of cortical projection neurons in *Thy1-Stp-YFP* [13] and *Thy1-Brainbow* mice [19]. The reconstruction of single collaterals emerging from main and minor CST components showed that dramatic changes in collateral length and complexity occur between 10 days and 4 weeks after injury. These parameters remain largely stable between 4 weeks and 12 weeks after lesion. Analysis of the CST contacts onto interneurons however indicated that while the morphology of the collaterals remained largely unchanged during the late stage of the remodeling process, their synaptic projections were still refined. We can further show that while the overall timing of the remodeling is similar in main and minor CST collaterals their individual complexity differed depending on their origin. Taken together our result suggest that CST remodeling after SCI occurs in 3 subsequent phases: a growth initiation phase (within the first 10 days after injury), which is followed by a collateral formation phase (between 10 days and 3–4 weeks after injury) and a later maturation phase (between 3–4 weeks and 12 weeks after injury).

Results

Cervical CST collaterals primarily emerge in the first 10 days after lesion and persist over time

Injection of BDA 10,000 into the hindlimb motor cortex revealed three components of the hindlimb CST in the spinal cord (**Fig. 1A**). The main CST component runs at the base of the dorsal funiculus and contains $97.6 \pm 0.27\%$ ($n = 8$ mice) of labeled CST fibers. The minor CST components run in the dorso-lateral and ventral funiculus and contain $2.1 \pm 0.23\%$ ($n = 8$ mice) and $0.3 \pm 0.04\%$ ($n = 8$ mice) of labeled CST fibers, respectively.

In unlesioned adult mice, axons arising from all hindlimb CST components sent only very few collaterals into the gray matter of the cervical spinal cord (level C3–C5, **Fig. 1A, D**). However, as early as 10 days following a mid-thoracic lesion, the number of CST collaterals in the cervical cord gray matter increased more than 4-fold (**Fig. 1B, D**). Over the following weeks the number of cervical collaterals slowly decreased. Still the majority of the collaterals persisted long-term and was still detected as late as 24 weeks after injury (**Fig. 1C, D**). Over this timeframe the collaterals, which in most cases have just started to enter the spinal gray matter at 10 days after lesion (**Fig. 1B**), extended further and mainly projected to the intermediate layers of the spinal cord (**Fig. 1C**). When we analyzed the contribution of different CST components to the formation of cervical collaterals, we found that, while in absolute number most of the collaterals arose from the main CST, the relative number of new collaterals that emerge per labeled fiber was several-fold higher for the minor dorso-lateral and ventral CST components (**Fig. 1E–P**). Notably, while the number of newly formed CST collaterals emerging from the main

CST significantly declined over time (**Fig. 1H**), the number of collaterals derived from the minor CST components remained stable for the entire observation period (**Fig. 1L, P**).

Complex CST collaterals form between 10 days and 4 weeks after lesion

To label single CST collaterals, we took advantage of *Thy1-Stp-YFP* [13] and *Thy1-Brainbow* [19] mice. In these mouse lines the presence of *Cre recombinase* either starts (in the case of *Thy1-Stp-YFP*) or changes (in the case of *Thy1-Brainbow* mice) the expression of fluorescent proteins in the affected neurons. Expression of *Cre recombinase* was restricted to a small number of cortical projection neurons by stereotactically injecting small amounts of a recombinant Adeno-Associated Virus expressing *Cre recombinase* (rAAV-Cre) into the hindlimb motor cortex (**Fig. 2A, B**). Single collaterals emerging from the axons of transduced cortical projection neurons could then be identified based on their unique labeling in the cervical spinal cord and reconstructed from serial cross-sections (**Fig. 2C–G**).

We used this approach to analyze the structure of cervical collaterals emerging from main and minor CST components at 10 days, 4 weeks and 12 weeks after a mid-thoracic hemisection of the spinal cord (**Fig. 3**). At 10 days following the injury, CST collaterals emerging from all CST components were fairly short (**Fig. 3A–C, J**), had a simple, mostly unbranched structure (**Fig. 3K**) and showed very few, if any, boutons (**Fig. 3L**). At 4 weeks after lesion, the collaterals were substantially longer (**Fig. 3D–F, J**), had a complex often highly branched structure (**Fig. 3K**) and a higher number of boutons (**Fig. 3L**). At this time, the anatomical structure of a collateral depended on its white matter origin. Compared to main CST collaterals, collaterals emerging from the ventral CST were long but showed a relatively simple structure with few branch points and boutons (**Fig. 3E, J–L**). In contrast, collaterals emerging from the dorso-lateral CST component had a highly complex structure and significantly more branchpoints and boutons compared to both ventral and main CST collaterals (**Fig. 3J–L**). While the structure of CST collaterals thus evolved substantially between 10 days and 4 weeks after lesion, collaterals emerging from all CST components remain largely unchanged between 4 weeks and 12 weeks after injury (**Fig. 3G–L**). Consequently, at 12 weeks after lesion dorso-lateral CST collaterals still had significantly more branchpoints than main CST collaterals and more branchpoints and boutons than ventral CST collaterals (**Fig. 3J–L**).

Synaptic differentiation of newly formed CST boutons

To determine the synaptic differentiation of the newly formed CST boutons we traced the hindlimb CST and then stained cervical and lumbar spinal cord sections with antibodies against two synaptic markers: bassoon, a marker of the presynaptic active zone and synapsin I, a protein that regulates neurotransmitter release at the synapse (**Fig. 4**). We first determined the percentage of boutons that are immunoreactive for the synaptic markers in the lumbar spinal cord of unlesioned mice ($n = 2$ mice). Of these “control” boutons 51% were immunoreactive for synapsin I and 52% were immunoreactive for bassoon. As these values likely represent the mature expression pattern, this value was set as 100% and the immunoreactivity in newly formed boutons was expressed as a percentage of the mature expression pattern. The analysis of CST boutons in the cervical spinal cord at 10 days and 3 weeks after lesion then showed that the expression of both bassoon (**Fig. 4A–C**) and synapsin I (**Fig. 4D–F**) is low at 10 days after lesion but is comparable to the expression pattern observed in the lumbar spinal cord of unlesioned mice by 3 weeks. Double-

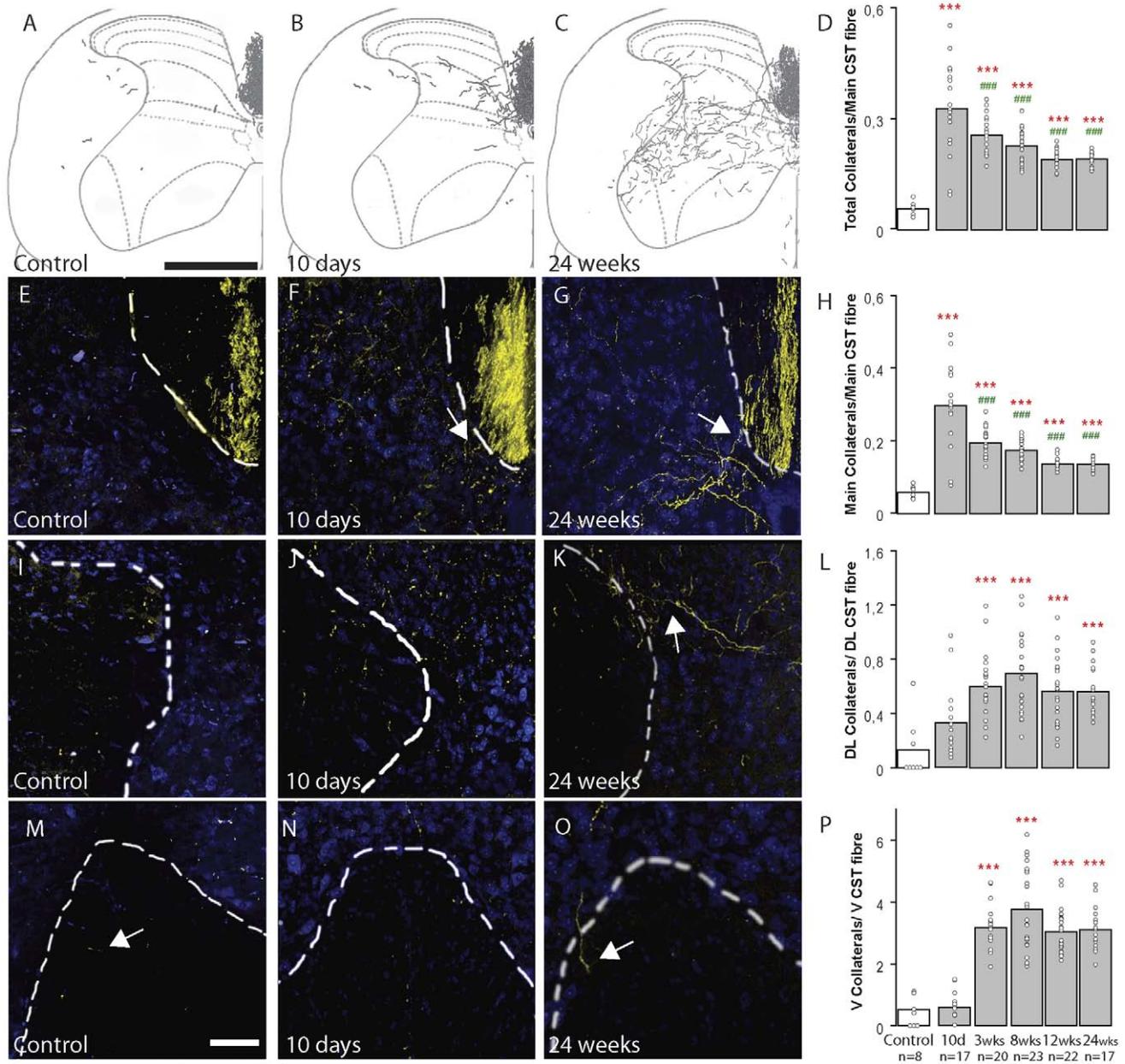


Figure 1. Population analysis of hindlimb CST collateral formation at different timepoints after SCI. (A–C) Reconstruction of hindlimb CST collaterals (black) from 5 consecutive sections in the cervical spinal cord of control mice (A) and of mice perfused 10 days (B) and 24 weeks (C) following SCI. (D) Quantification of the total numbers of collaterals emerging from all CST components in the cervical gray matter of control mice and of mice at different timepoints following SCI. (E–G) Confocal images of main CST (BDA, yellow) and the adjacent gray matter (Neurotrace, blue; border shown by dashed white line) in control mice (E) and in mice perfused 10 days (F, arrow indicates CST collateral emerging from main CST) and 24 weeks (G, arrow indicates CST collateral emerging from main CST) following SCI. (H) Quantification of the number of collaterals emerging from the main CST component at different timepoints following SCI. (I–K) Confocal images of the minor dorso-lateral CST (BDA, yellow) and the adjacent gray matter (Neurotrace, blue; border shown by dashed white line) in control mice (I) and in mice perfused 10 days (J) and 24 weeks (K, arrow indicates CST collateral emerging from dorso-lateral CST) following SCI. (L) Quantification of the number of collaterals emerging from the minor dorso-lateral CST component at different timepoints following SCI. (M–O) Confocal images of the minor ventral CST (BDA, yellow) and the adjacent gray matter (Neurotrace, blue; border shown by dashed white line) in control mice (M, arrow indicates ventral CST fiber) and in mice perfused 10 days (N) and 24 weeks (O, arrow indicates collateral emerging from ventral CST) following SCI. (P) Quantification of the number of collaterals emerging from the minor ventral CST component at different timepoints following SCI. Asterisks indicate significance compared to the unlesioned controls. Pound signs indicate significance compared to the 10-day timepoint. Scale bar in A (also for B,C), 500 μ m; in M (also for E–O), 100 μ m.
doi:10.1371/journal.pone.0030461.g001

immunostaining experiments further showed that 3 weeks after lesion $80.5 \pm 4.5\%$ of the immunoreactive CST boutons are double positive for synapsin I and bassoon while comparably few of them

showed the expression of only one marker ($8 \pm 2\%$ are only immunoreactive for synapsin I and $11.5 \pm 6.5\%$ are only immunoreactive for bassoon, **Fig. 4 G**).

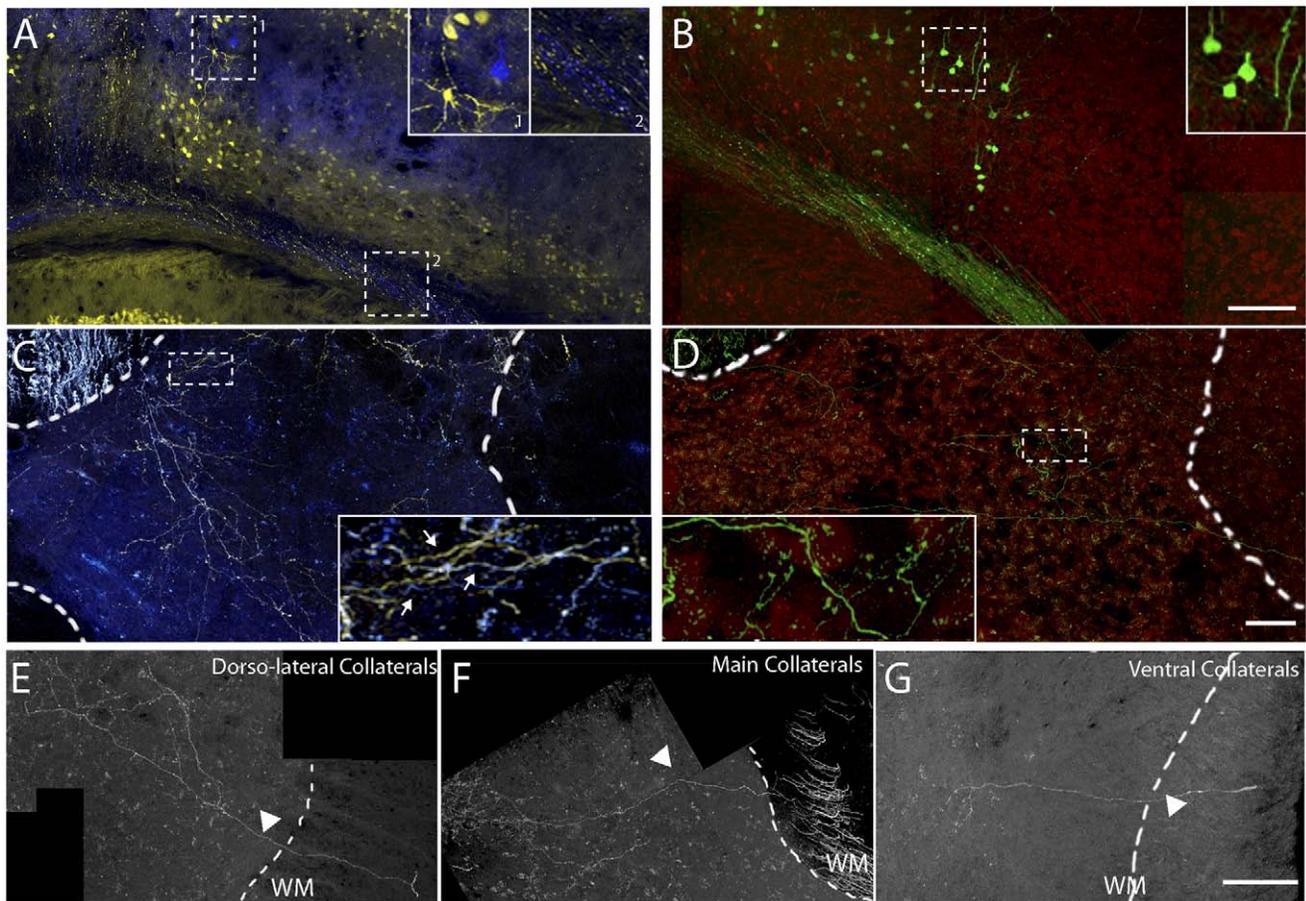


Figure 2. Strategies for labeling individual CST collaterals. (A, B) Confocal images of the cortex of a *Thy1-Brainbow* (A; YFP, yellow; CFP, blue) and *Thy1-Stp-YFP* mouse (B; YFP, green; Neurotrace, red) after local injection of rAAV-Cre. Boxed areas are magnified 2 times in insets. (C, D) Confocal images of CST collaterals in the cervical spinal cord of a *Thy1-Brainbow* (C; YFP, yellow; CFP, blue) and *Thy1-Stp-YFP* (D; YFP, green; Neurotrace, red) mouse after injection of rAAV-Cre in the cortex. Boxed areas are magnified 2 times in insets. Arrows in inset in C indicate different collaterals expressing either CFP (blue), YFP (yellow) or a combination of both (white). Dashed white line indicates the outline of the spinal gray matter. (E–G) Confocal images of individual collaterals (white) emerging from the main CST (F), the dorso-lateral CST (E) and the ventral CST (G) following SCI. Arrows indicate individual collaterals. Dashed white lines indicate the outline of the spinal gray matter. Scale bar in B (also for A), 100 μ m; Scale bar in D (also for C), 100 μ m; Scale bar in G (also for E, F), 50 μ m. doi:10.1371/journal.pone.0030461.g002

CST collaterals refine their contacts on interneurons between 3 and 12 weeks after lesion

To investigate how the projection pattern of CST collaterals evolves over time, we analyzed the number of contacts that an individual collateral formed with the cell bodies of spinal interneurons (Fig. 5). We first determined the mature projection pattern by evaluating contacts of hindlimb CST collaterals onto interneurons in the lumbar spinal cord. Here, we found that in most cases ($85.5 \pm 4.7\%$, $n = 2$ animals and 59 collaterals) a CST collateral forms 1 and in some cases ($14.5 \pm 4.7\%$) 2 contacts on spinal interneurons (Fig. 5 C). In contrast, the majority of newly emerging CST collaterals in the cervical spinal cord displayed multiple (up to 4) contacts on spinal interneurons at 10 days after lesion (Fig. 5 C). This “multiple contact” pattern still persisted at 3 weeks after lesion (Fig. 5 A, C). The mature contact pattern was only present at 12 weeks after lesion and at this time more than 80% ($81.1 \pm 3.1\%$, $n = 3$ animals and 168 collaterals) of collaterals only showed 1 contact per interneuron (Fig. 5 B, C). The mature pattern then persisted over time and was still present at 24 weeks after injury (Fig. 5 C). The analysis of the individual CST components showed that at 3, 8 and 12 weeks after lesion most of

the contacts on interneurons were formed by collaterals emerging from the main CST tract (Fig. 5 D).

Discussion

The plastic reorganization of axonal connections is an important element of the recovery process after CNS damage. This is exemplified by the remodeling of lesioned CST fibers after spinal cord injury. Previous work has shown that the sprouting of new CST collaterals above the level of the lesion is a key step in the formation of intraspinal detour circuits that contribute to functional recovery after traumatic and inflammatory lesions of the spinal cord [6], [7], [14], [20]. Here, we can show that these collaterals form and mature in distinct phases (Fig. 6). In the growth initiation phase that encompasses the first 10 days after lesion, CST collaterals emerge and, at least in the case of the main and dorso-lateral CST, start to enter the cervical gray matter. In the collateral formation phase that covers the ensuing weeks, these collaterals elongate, branch and form synaptic contacts in the cervical gray matter. The final maturation phase, 12 weeks after injury, is then characterized by the small-scale refinements of the projection pattern that includes the removal of excessive inputs

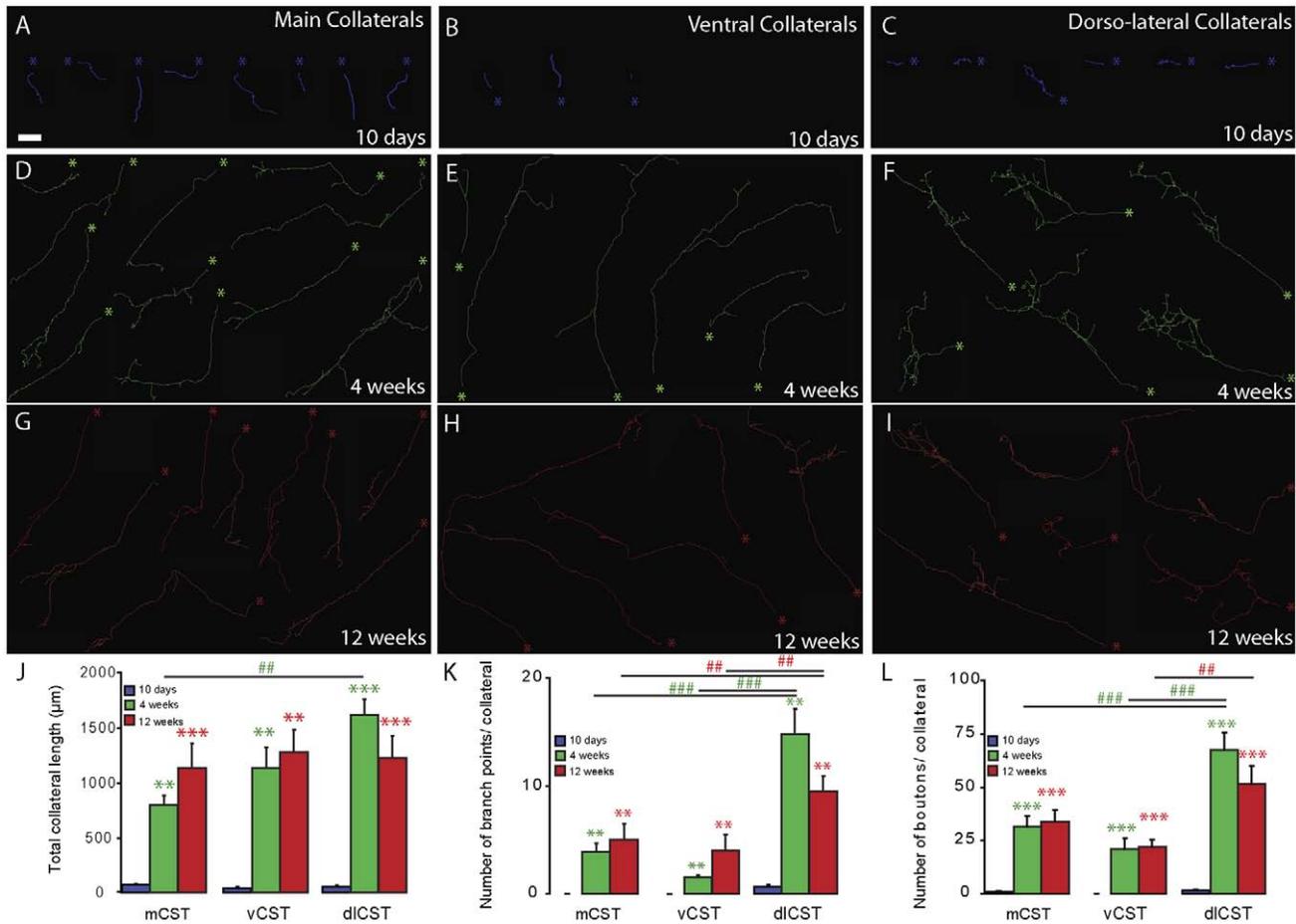


Figure 3. Reconstruction of individual hindlimb CST collaterals at different timepoints after spinal cord injury. (A–C) Reconstruction of individual collaterals (blue asterisks indicate the entry point of the collateral in the gray matter) emerging from the main dorsal (A) and the minor ventral (B) and dorso-lateral (C) CST components at 10 days following SCI. (D–F) Reconstruction of individual collaterals (green) emerging from the main dorsal (D) and the minor ventral (E) and dorso-lateral (F) CST components at 4 weeks following SCI. (G–I) Reconstruction of individual collaterals (red) emerging from the main dorsal (G) and the minor ventral (H) and dorso-lateral (I) CST components at 12 weeks following SCI. (J–L) Quantification of the total collateral length (J), the number of branchpoints/collateral (K) and the number of boutons/collateral (L) measured in individually reconstructed collaterals at different timepoints after SCI. Blue bars, 10-day timepoint; green bars, 3-week timepoint; red bars, 12-week timepoint. Asterisks indicate significant differences compared to the 10-day timepoint. Pound signs indicate significant differences between collaterals emerging from different CST components at 3 weeks (green) and 12 weeks (red) after injury. Scale bar in A (also for B–I), 50 μm. doi:10.1371/journal.pone.0030461.g003

from interneurons. Another example of this refinement process is provided by our previous analysis [6] of CST contacts onto two distinct interneuronal populations, the long propriospinal neurons which connect the cervical spinal cord to the lumbar spinal cord [15–18] and the short propriospinal neurons that form intracervical connections [21], [22]. The cell bodies of these interneurons are located side by side in the cervical gray matter. Indeed, at 3 weeks after lesion - at the end of the formation phase - similar fractions of long and short propriospinal neurons are contacted by CST collaterals. However at 12 weeks - at the end of the maturation phase - many of the contacts on short propriospinal neurons have been removed while contacts on long propriospinal neurons persisted [6]. Taken together with the results of this study, it seems that the main aim of the maturation phase is the removal of excessive connections and the strengthening of pertinent connections. The emergence and selection of CST collaterals thus shows interesting parallels to the initial formation and refinement of neuronal connections in development. In the neuromuscular system it has been shown that initially exuberant connections between motor neurons and muscle fibers are formed,

leading to the innervation of single neuromuscular junctions by multiple axons [23]. Over time, most of these inputs are then removed and only a single axon remains to innervate the junction [24], [25].

Similarly, during the development of the CNS initially excessive connections are formed that are later pruned. A classical example for the removal of excessive connections is the pruning of early corticospinal projections that originate from the occipital cortex [26], [27]. The refinement of CST connections during development however extends beyond this large scale pruning. For example, it is known that in different species the initial termination pattern of the CST encompasses the entire gray matter from dorsal to ventral horn and becomes more restricted later on [28–31]. In addition, while most corticospinal fibers in adults terminate contralateral to their origin in the cortex, during development CST axons also show extensive projections to the ipsilateral spinal gray matter. This can be at least partially explained by the findings that during development a larger proportion of axons appear to descend ipsilaterally without decussating in the pyramid while other projections cross not only in the decussation but again in the

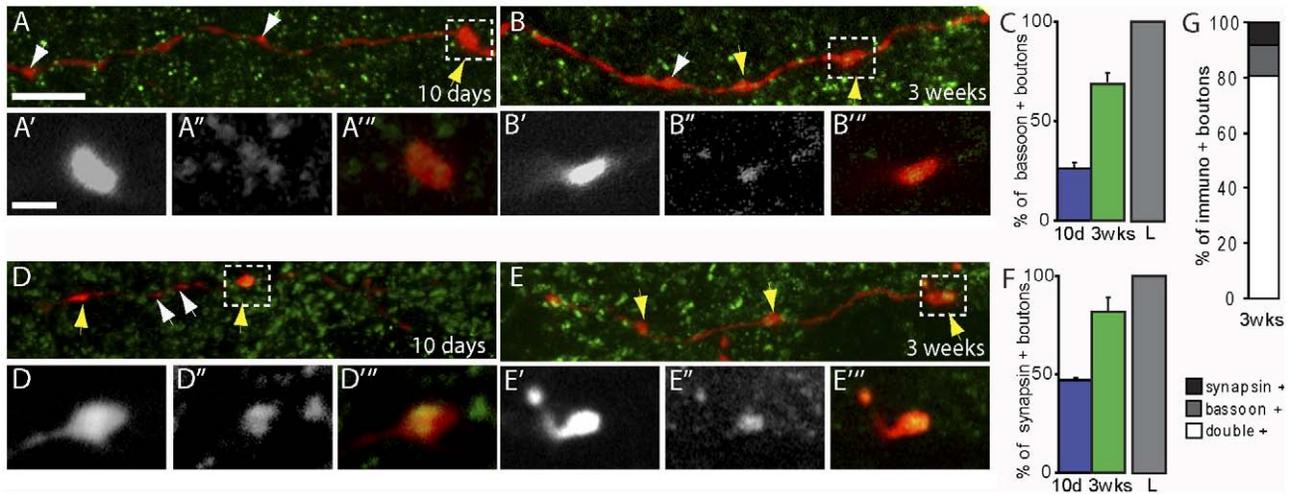


Figure 4. Synaptic differentiation of newly formed CST boutons. (A–B) Confocal images of bassoon immunostaining (green) in the cervical spinal cord of mice with a traced hindlimb CST (BDA, red) perfused 10 days (A) and 3 weeks following SCI (B). Yellow arrows indicate boutons that were immunoreactive for bassoon, white arrows indicate those that were not. (A'–A''') Single plane confocal image of the boutons boxed in A showing the collateral (A'; BDA, white), bassoon immunostaining (A''; white) and the overlay (A'''; BDA, red; bassoon, green) at 10 days after SCI. (B'–B''') Single plane confocal image of the bouton boxed in B showing the collateral (B'; BDA, white), bassoon immunostaining (B''; white) and the overlay (B'''; BDA, red; bassoon, green) at 3 weeks after SCI. (C) Quantification of the number of boutons on hindlimb CST collaterals that were immunopositive for bassoon at 10 days and 3 weeks following SCI in the cervical cord. The percentages were normalized to the expression pattern in the lumbar cord (L) of control animals (which was set to 100%). (D–E) Confocal images of synapsin I immunostaining in the cervical spinal cord of mice with a traced hindlimb CST (BDA, red) perfused 10 days following SCI (D) and at 3 weeks post-injury (E). Yellow arrows indicate boutons that were immunoreactive for synapsin I, white arrows indicate those that were not. (D'–D''') Single plane confocal image of the bouton boxed in D showing the collateral (D'; BDA, white), the synapsin I staining (D''; white) and the overlay (D'''; BDA, red; synapsin I, green) at 10 days after SCI. (E'–E''') Single plane confocal image of the bouton boxed in E showing the collateral (E'; BDA, white), the synapsin I staining (E''; white) and the overlay (E'''; BDA, red; synapsin I, green) at 3 weeks after SCI. (F) Quantification of the number of boutons on hindlimb CST collaterals that were immunopositive for synapsin I at 10 days and 3 weeks following SCI in the cervical cord. The percentages were normalized to the expression pattern in the lumbar cord (L) of control animals (which was set to 100%). (G) Quantification of the co-expression of bassoon and synapsin I in boutons of CST collaterals of animals perfused at 3 weeks after injury (expressed as percentages of all immunoreactive boutons). Scale bar in A (also for B, D, E), 10 μ m and in A' (also for A''–E'''), 3 μ m.
doi:10.1371/journal.pone.0030461.g004

spinal cord [32]. At least some of these initial CST connections, including many of the double-crossed or uncrossed collaterals appear to be transient and are removed during the maturation of the CST [32], [33]. Further work will be necessary to determine to what extent these structural commonalities between developmental and post-injury remodeling also imply common regulatory mechanisms. For example, it will be interesting to see if neuronal activity patterns, that are important determinants of competition

at the neuromuscular junction [34–36] or during CST development [37] also regulate the fate of newly formed CST collaterals. Likewise it will be important to explore to what extent molecules that affect pruning such as the semaphorins/plexins [38], [39], ephrins [40] or components of the wlds pathway [41] also influence the removal of CST collaterals.

The excessive formation and subsequent sorting of connections is one way how the specificity of new CST connections

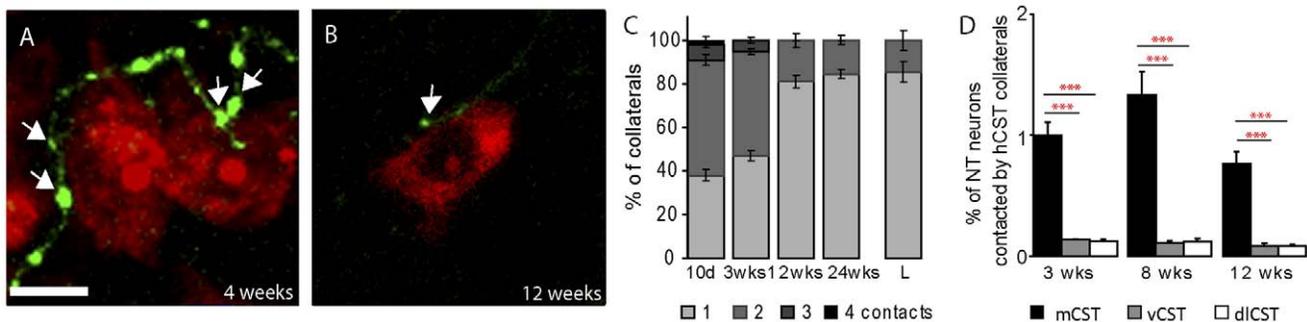


Figure 5. Analysis of CST contacts onto cervical interneurons after SCI. (A,B) Confocal images of contacts (arrows, defined as boutons in apposition to neuronal cell bodies) between hindlimb CST collaterals (YFP, green) and the cell bodies of cervical interneurons (Neurotrace, red) at 4 weeks (A) and 12 weeks (B) following SCI. (C) Quantification of the number of contacts a given hindlimb CST collateral makes with the cell body of a single interneuron at different timepoints after SCI as well as in the lumbar spinal cord of unlesioned animals. (D) Quantification of the percentage of Neurotrace (NT)-stained interneurons contacted by collaterals emerging from the different CST components at multiple timepoints following the lesion. Asterisks indicate significant difference compared to main CST collaterals. Scale bar in A (also for B), 15 μ m.
doi:10.1371/journal.pone.0030461.g005

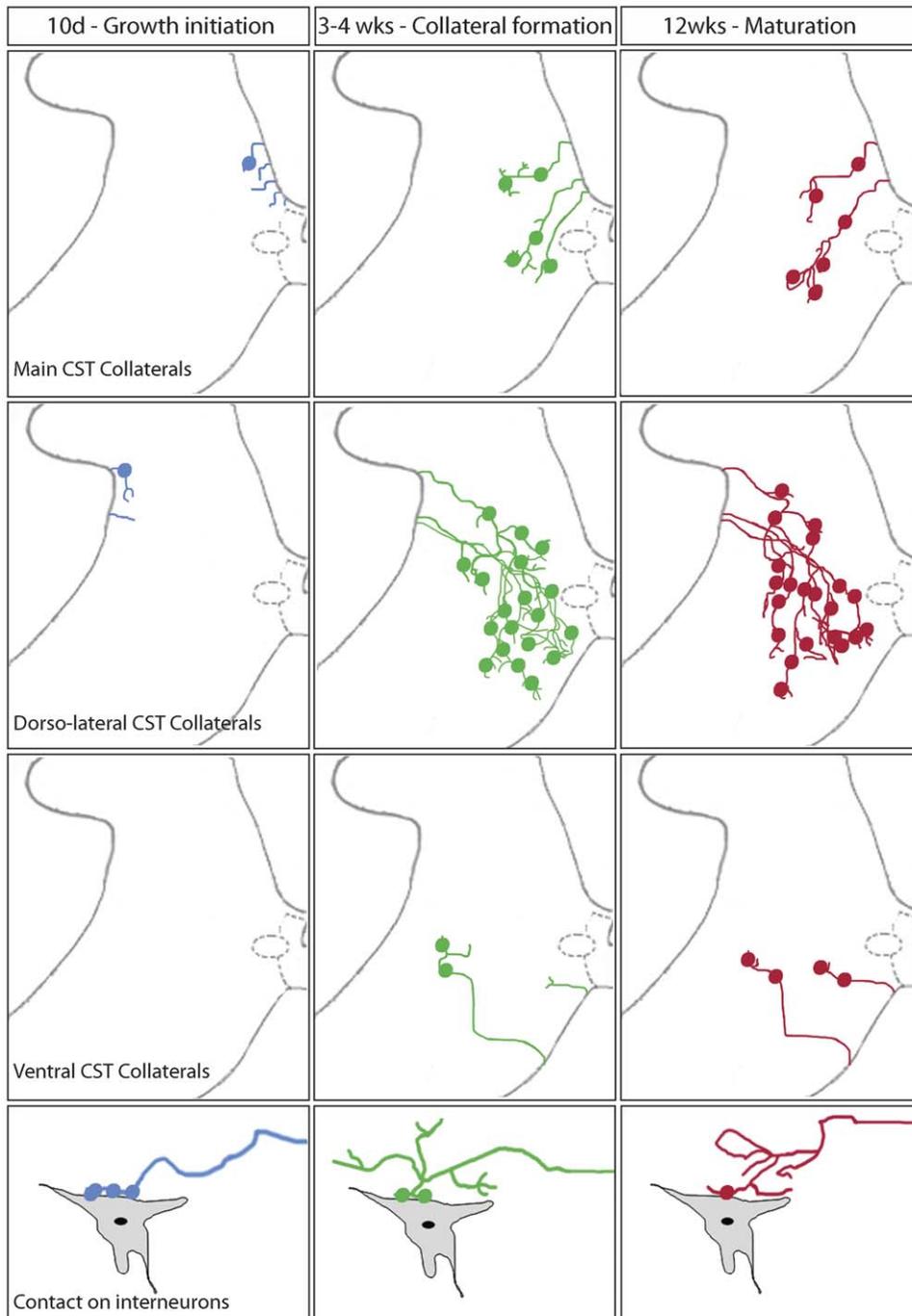


Figure 6. Schematic representation of hindlimb CST remodeling following SCI. Scheme illustrating the formation of cervical collaterals derived from the main CST (upper row) and the minor dorsolateral (2nd row) and ventral (3rd row) CST components at 10 days (blue), 3–4 weeks (green) and 12 weeks (red) after SCI. Bottom row illustrates the refinement over time of the contacts between CST collaterals and cervical interneurons.
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can be established. Another measure to ensure specificity is the targeting of axons to specific neurons or regions of the spinal cord. In the case of the newly formed CST connections, the refinement of initial connections during the maturation phase suggests that initial targeting is not established by specific recognition of single neurons. On the other hand, the distribution of newly formed CST collaterals in the spinal cord gray matter indicates that specific regions of the spinal

cord, in particular the intermediate layers V–VII are preferentially targeted by CST collaterals (see **Fig. 1C**). Taken together our findings thus suggest that a combination of “region-specific” targeting that guides collaterals to the intermediate layers of the spinal cord and a subsequent refinement process that removes excessive connections collaborate to ensure specific targeting of newly formed CST connections to intraspinal relay neurons.

It should be noted however, that while most newly formed CST collaterals end in the intermediate layers of the spinal cord, some fibers reach the ventral horn and might form contacts with ventral motoneurons [42]. These direct connections of hindlimb CST axons to forelimb motoneurons could be one anatomical substrate that underlies the shift of motor maps that occurs both in animal and humans in response to spinal cord injury [43–45].

A second important finding of our study is that different CST components contribute to the remodeling of the CST. Antero-grad tracing revealed three distinct localizations of CST fibers in the spinal cord, with the majority of fibers located in the main CST component at the bottom of the dorsal funiculus and a smaller proportion of fibers located in the dorso-lateral funiculus. Only very few fibers were observed in the ventral funiculus, contralateral to the main and dorso-lateral CST. These ventral fibers together with the fibers in the dorso-lateral funiculus form the minor CST components. This structure of the CST is in accordance with previous reports in mice, where the ventral CST component is relatively small [13], [46], [47], as well as in other rodents [11], [12], [48–50]. We further observed that following injury the number of fibers in the ventral component that is spared by the lesion is increased. This is probably due to sprouting of additional CST collaterals that can enter the ventral white matter tract as previously described [51]. Our analysis of collateral formation reveals both commonalities and differences between the distinct CST components. For example, the overall timecourse of collateral initiation, formation and maturation appears mostly similar in major and minor CST components. The comparably lower number of CST collaterals derived from the minor CST tracts detected in the gray matter at 10 days after injury likely does not reflect a different growth initiation but rather the longer distance between the parental axons and the gray matter border. It is interesting to note that while the overall timecourse of collateral formation is similar, the number of collaterals an individual CST axon sends to the gray matter differs substantially between the CST components. A CST axon running in the ventral funiculus, for example, extends more than 10-fold more collaterals into the gray matter at 3 weeks after lesion than a main CST axon (**Fig. 1 H, P**). These findings suggest an important role for ventral CST fibers in the CST remodeling process. As after a midthoracic lesion, the ventral funiculus consists of both pre-existing ventral fibers as well as newly sprouted collaterals likely derived from other CST components, it is possible that both unlesioned fibers and new collaterals emerging from lesioned CST fibers contribute to this response. An important role of ventral fibers is in line with previous experiments in rats that have demonstrated that ventral CST fibers can play an important role for the recovery of CST function [52]. However, it appears that not only the relative number of collaterals emerging from a given CST component but also their complexity differs between CST components. For example, collaterals emerging from the dorso-lateral CST contain several-fold more branch points and boutons at 4 weeks after lesion than collaterals emerging from main or ventral CST. This might suggest that dorso-laterals CST contact different target cell populations. The idea that distinct CST components target distinct cell populations is consistent with our previous observation that dorso-lateral CST collaterals appear to be primarily responsible for direct contacts on motoneurons in the lumbar spinal cord [13]. Taken together the characteristic differences between main and minor CST components strongly suggest that individual components might play distinct roles in the recovery process. The single collateral tracing techniques established in this study can in the future help to further define these distinct roles in different lesion paradigms. This is of interest as the remodeling process after a

spinal lesion likely extends beyond the corticospinal tract to other supra- and intraspinal tract systems. For example, the reticulospinal tract has been shown to spontaneously sprout after SCI [53], [54] and the spontaneous restoration of serotonergic activity, likely mediated by the remodeling of serotonergic circuits, was found to contribute to functional recovery [55], [56].

Finally our study demonstrates that once collaterals from all CST components are formed they by and large persist long-term - in our experiments at least up to the end of the observation period (24 weeks after lesion for the population analysis and 12 weeks after lesion for the analysis of individual collaterals). Our analysis further shows that after early formation and refinement of the collaterals, very little changes to the collateral number, structure and contact pattern are observed beyond 12 weeks after lesion. This suggests that an early critical period for CST remodeling exists during which the formation or refinement of collaterals can be influenced. However after this period, newly formed connections appear to remain stable. This defines a time-window for therapeutic interventions that are likely most effective in the first 10 days after lesion if the aim is to improve collateral initiation, between 10 days and 3–4 weeks if they aim to support collateral formation and between 3–4 and 12 weeks if the aim to modulate target connections. This is of interest as despite the spontaneous remodeling of axonal connections substantial functional impairments often remain following experimental and clinical spinal cord injuries. It will therefore be important to develop therapeutic strategies that can enhance the remodeling process. One promising approach could be to foster the intrinsic neuronal growth response of cortical projection neurons targeting, for example, c-AMP and its downstream mediators [57], the growth cone-associated proteins GAP43 and CAP23 [58], components of the PTEN/mTOR pathway [59], [60] or the JAK-STAT pathway [60], [61]. Another possible way to enhance axonal remodeling is through rehabilitation. Several studies [43], [62–64] have already demonstrated the positive effect of rehabilitation on axonal sprouting following spinal cord injury. Care needs to be taken however not to favor task-specific rewiring at the cost of other tasks. Several studies have indeed shown that experimental rehabilitation schemes in which one task is trained repetitively will lead to improvement in this task to the detriment of other tasks [43], [65–67]. To our mind, the analysis techniques introduced in this study can in the future help to evaluate whether and how these therapeutic approaches can improve axonal remodeling after injury.

Materials and Methods

Ethics Statement

All animal experiments conformed to the institutional guidelines and were approved by the Animal Study Committee of the Regierung von Oberbayern. Approval ID: 55.2-1-54-2531-127-05.

Animals

Adult C57BL/6 female mice 6–8 weeks old, *Thy1-Stp-YFP* and *Thy1-Brainbow* mice (line TYC9, kindly provided by J. Livet, INSERM) were used in this study. C57BL/6 mice were used for all conventional CST tracing experiments. *Thy1-Stp-YFP* mice express yellow fluorescent protein (YFP) in neurons after *Cre*-mediated excision of a floxed STOP-sequence [13], [68]. *Thy1-Brainbow* mice show a combinatorial expression of different fluorescent proteins after *Cre*-mediated excision of *Lox* sites [19]. Briefly, in the brainbow-1.0 mice used in this study, *lox P* sites alternate with incompatible *lox* variant (*lox2272*) sites. The *Cre*

recombinase thus randomly chooses between different initial excision events. As the initial excision between a pair of identical lox sites removes one of the other pair, it prevents multiple recombination. Before *Cre* action, only the gene following the promoter is expressed (RFP). The recombination then switches expression to either YFP or M-CFP depending on the site of the initial recombination event. Further, the presence of multiple copies of the brainbow construct that recombine independently can lead to the co-expression of different fluorescent proteins [19]. *Thy1-Stp-YFP* and *Thy1-Brainbow* mice were used for single collateral analysis.

Spinal Cord Injury

Mice were anesthetized with a subcutaneous injection of Ketamin/Xylazine (Ketamine 150 mg/kg, Xylazine 10 mg/kg). A laminectomy was performed at thoracic level 8 (T8) and a dorsal hemisection of the spinal cord was performed with fine iridectomy scissors. This lesion interrupts the main dorsal and the minor dorso-lateral CST components but not the minor ventral CST component (Fig. 7). After surgery animals were heated, rehydrated and treated with analgesics (which were also administrated immediately before surgery).

rAAV-Cre

AAV1/2-CAG-HA-NLS-Cre-WPRE-BGH-polyA expression vectors were used to generate viral particles in which the CAG promoter consists of the chicken β -actin promoter hybridized with the CMV immediate early enhancer sequence. The CAG promoter drives the expression of the P1 *Cre recombinase*, the N-terminus of which is fused to an HA-tag followed by a nuclear localization signal (NLS). The woodchuck post-transcriptional regulatory element (WPRE) and the presence of the bovine growth hormone (BGH) polyadenylation sequence ensure high transcription following transduction. AAV1/2 particles were generated by GeneDetect.com Ltd.

Anatomical tracing of hindlimb corticospinal tract (CST)

Population analysis of CST collaterals. To study reorganization of both the major and minor CST components we traced the hindlimb CST in adult C57BL/6 mice by bilateral pressure injections as previously described [14]. For this purpose,

1 μ l of a 10% solution of biotinylated dextran amine (BDA 10 000, Molecular Probes) was slowly injected with a glass capillary (tip diameter of about 20 μ m) into lamina V of the hindlimb motor cortex (coordinates: -1.3 mm posterior to bregma, 1 mm lateral to bregma, 0.6 mm depth). The micropipette remained in place for 2 minutes after completion of the injection to minimize backflow and diffusion of the tracer.

Single CST collateral analysis. To study the projection pattern of individual collaterals, we first determined the amount of rAAV necessary to label single hindlimb CST collaterals by varying the injected volume. We then performed bilateral pressure injections of 0.3 μ l of a rAAV-Cre (titer: 1×10^{12} genomic particles/ml) into lamina V of the hindlimb motor cortices of *Thy1-Stp-YFP* and *Thy1-Brainbow* mice. The micropipette remained in place for 2 minutes after completion of the injection to minimize backflow and diffusion of the virus.

Tissue preparation and immunohistochemistry

Animals were perfused transcardially with 4% paraformaldehyde (PFA). Brains and spinal cords were dissected, postfixed overnight and cryoprotected in 30% sucrose for 3 days. For the population analysis of CST collaterals we analyzed the cervical spinal cord of C57BL/6 mice between the spinal level C3 and C5, where the cell bodies of long propriospinal neurons are located. For this purpose coronal sections (50 μ m thickness) were cut on a vibratome and processed as described previously [69]. The hindlimb CST was revealed after BDA tracing using 0.4% ammonium nickel sulfate (Sigma), 0.015% DAB (Sigma), 0.004% H_2O_2 in 50 mM Tris buffer (pH 8) resulting in a black reaction product. For the analysis of individual CST collaterals consecutive coronal sections (100 μ m thickness) of the cervical spinal cord of *Thy1-Stp-YFP* and *Thy1-Brainbow* mice were cut on a vibratome and mounted on gelatinized glass slides. Sections were then incubated with a rabbit anti-GFP antibody (diluted 1:500, Invitrogen) overnight at 4°C and on the next day with a goat-anti-rabbit secondary antibody conjugated to Alexa Fluor 488 (Invitrogen). Finally, sections were counterstained with Neurotrace 435 (diluted 1:500, Invitrogen) to identify the cell bodies of spinal interneurons.

For analysis of synaptic maturation 20 μ m thick cryostat sections derived from animals, in which the hindlimb CST was

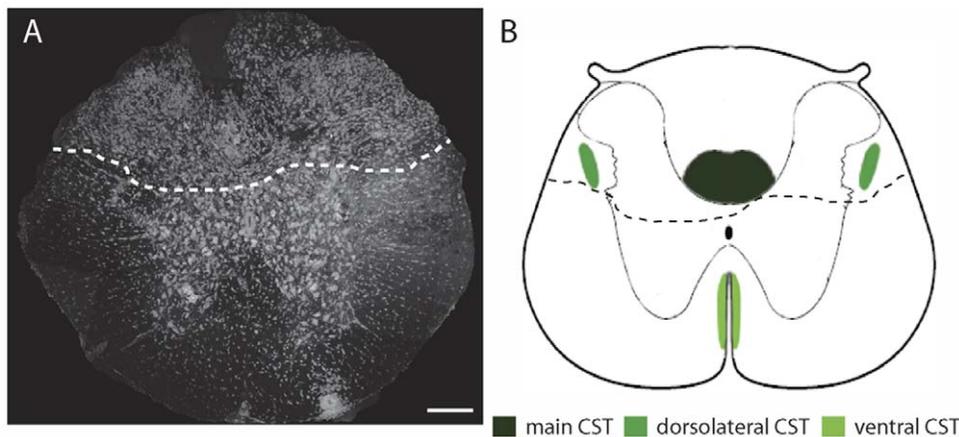


Figure 7. Illustration of a dorsal hemisection of the thoracic spinal cord. (A) Confocal image of a cross-section of the thoracic (T8) spinal cord of a mouse perfused 12 weeks after dorsal hemisection (counterstained with Neurotrace). Dashed line indicates lesion border. (B) Schematic representation of the location of the different CST components (highlighted in different shades of green) in relation to this lesion (outlined by dashed line from A). Scale bar in A, 200 μ m. doi:10.1371/journal.pone.0030461.g007

labeled with BDA, were immunostained for synapsin I or bassoon as follows. Sections were incubated with ABC (Vector Laboratories) and primary polyclonal antibodies reactive against either synapsin I (Millipore, 1:500) or bassoon (Synaptic System, 1:500) in Tris buffer containing 0.3% Triton X-100 (Sigma) and 2.5% goat serum (Invitrogen) overnight at 4°C. For double immunostaining the polyclonal anti-synapsin I antibody (dilution same as above) was combined with a mouse monoclonal anti-bassoon antibody (dilution 1:100, Enzo Life Sciences). After a 20 min tyramide amplification (Biotin-XX, TSA Kit #21, Invitrogen) to detect BDA, the sections were incubated overnight with Streptavidin conjugated to Alexa Fluor 594 (1:500, Invitrogen) and a goat-anti-rabbit antibody conjugated to Alexa Fluor 488 (1:500, Invitrogen). Counterstaining was performed using NeuroTrace 435 (1:500, Invitrogen) and sections were mounted in Vectashield (Vector Laboratories).

Quantification of anatomical reorganization

Population analysis of CST collaterals. Fibers exiting from main and minor CST components and entering the grey matter were counted in 30 consecutive coronal sections of the cervical spinal cord using a IX71 microscope (Olympus) with a $\times 40$ (NA 0.65) objective. To correct for inter-animal differences in tracing efficiency, the number of CST collaterals was divided by the number of traced fibers in the respective CST component and expressed as a ratio of collaterals per CST fiber.

Single collateral analysis. Consecutive coronal sections of the cervical spinal cord of *Thy1-Stp-YFP* and *Thy1-Brainbow* mice were imaged on an Olympus FV1000 confocal microscope. Image stacks were acquired with a $\times 20$ oil objective and processed using ImageJ (<http://rsbweb.nih.gov/ij/>) and Adobe Photoshop software. Alignment and tracing of collaterals in consecutive sections was performed in Adobe Photoshop. Collateral properties (collateral length, number of branch points) were measured using the NeuronJ plugin in ImageJ.

Contacts on interneuronal cell bodies. To quantify the contacts onto interneuronal cell bodies 20 μm sections of the cervical spinal were scanned with a $\times 20$ (NA 0.85) oil immersion objective. Single hindlimb CST collaterals (labelled with BDA) were followed and the number of boutons in contact with the cell body of an interneuron (labelled with Neurotrace) was counted.

Expression of synaptic markers. To determine the percentage of boutons that express the synaptic markers

synapsin I and bassoon image stacks were acquired with an Olympus FV1000 confocal microscope equipped with standard filter sets and a $\times 60$ (NA 1.45) oil immersion objective. Tissue from the population analysis was used for this analysis as the high number of BDA-labelled collaterals in this tissue allowed us to analyse sufficiently large numbers of CST boutons. The total number of boutons as well as the number of these boutons that expressed synapsin I or bassoon were counted. To analyze the co-expression of synapsin I and bassoon, sections from the cervical spinal cord of animals perfused 3 weeks after lesion ($n = 3$ mice) were used. The number of CST boutons immunoreactive for either bassoon, synapsin I or both was determined and expressed as percentage of all immunoreactive boutons. All quantifications were performed by a blinded observer.

Image processing

Image stacks obtained with confocal microscopy were processed using ImageJ software to generate maximum intensity projections. To obtain final images, these maximum intensity projections were processed in Adobe Photoshop using gamma adjustments to enhance visibility of intermediate gray values and median filtering to suppress noise when necessary. For the representation of CST collaterals (**Fig. 1 A–C**) 5 consecutive sections were reconstructed and overlaid.

Statistical evaluation

Results are given as mean \pm SEM unless indicated otherwise. For paired comparison data were analyzed by the Student's *t* test. For multiple comparisons a two-way ANOVA followed by a Tukey's or a Bonferroni *post hoc* was performed using Graphpad Prism 5.01 for Windows (GraphPad Software). Significance levels are indicated as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

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Author Contributions

Conceived and designed the experiments: FMB MK. Performed the experiments: CL XG. Analyzed the data: FMB CL XG MK. Wrote the paper: FMB MK.

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In vivo imaging reveals a phase-specific role of STAT3 during central and peripheral nervous system axon regeneration

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In vivo imaging reveals a phase-specific role of STAT3 during central and peripheral nervous system axon regeneration

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In the peripheral nervous system (PNS), damaged axons regenerate successfully, whereas axons in the CNS fail to regrow. In neurons of the dorsal root ganglia (DRG), which extend branches to both the PNS and CNS, only a PNS lesion but not a CNS lesion induces axonal growth. How this differential growth response is regulated in vivo is only incompletely understood. Here, we combine in vivo time-lapse fluorescence microscopy with genetic manipulations in mice to reveal how the transcription factor STAT3 regulates axonal regeneration. We show that selective deletion of STAT3 in DRG neurons of STAT3-floxed mice impairs regeneration of peripheral DRG branches after a nerve cut. Further, overexpression of STAT3 induced by viral gene transfer increases outgrowth and collateral sprouting of central DRG branches after a dorsal column lesion by more than 400%. Notably, repetitive in vivo imaging of individual fluorescently labeled PNS and CNS axons reveals that STAT3 selectively regulates initiation but not later perpetuation of axonal growth. With STAT3, we thus identify a phase-specific regulator of axonal outgrowth. Activating STAT3 might provide an opportunity to “jumpstart” regeneration, and thus prime axons in the injured spinal cord for application of complementary therapies that improve axonal elongation.

in vivo microscopy | spinal cord injury | peripheral nerve lesion | intrinsic growth program

Lesioned peripheral nervous system (PNS) axons regenerate successfully, whereas lesioned CNS axons fail to regrow. This differential behavior is exemplified by neurons located in the dorsal root ganglia (DRG), which extend one branch into the PNS and another into the CNS. In these neurons, a cut in the PNS but not in the CNS is followed by neuronal outgrowth (1). If, however, the DRG neuron is “conditioned” by a transection of its peripheral branch, a subsequent central lesion can be followed by extensive outgrowth (2, 3). This suggests the existence of a common intrinsic neuronal growth program that can, in principle, support both PNS and CNS growth but is normally initiated only after a PNS lesion. In recent years, several intracellular components that might regulate this intrinsic growth program have been identified (4, 5). They include a number of transcriptional regulators such as the transcription factors cJun (6), SMAD1 (7), ATF3 (8), AKRD1 (9), NFIL3 (10), and several KLF family members (11). One particularly interesting transcriptional regulator is STAT3, which is activated as part of the JAK–STAT signaling pathway (12). The following findings make STAT3 a good candidate for regulating axon growth: first, increased levels of STAT3 expression and phosphorylation are associated with axonal regeneration (13–15) and axonal remodeling (16). Second, molecules that can affect STAT3 signaling such as the neurotrophic cytokines IL-6, ciliary neurotrophic factor, and leukemia inhibitory factor, as well as the intracellular regulator SOCS3, have been shown to influence axonal regeneration (17–20). Third,

STAT3 expression promotes neuronal outgrowth in cultured CNS neurons (21) and increased STAT3 expression is directly involved in the conditioning response of DRG neurons (22).

The identification of STAT3 and other transcription factors indicates that multiple transcriptional programs exist that can, in principle, influence the neuronal growth response to injury. Whether they operate in concert or in succession, e.g., by affecting specific phases of the growth response, such as growth initiation or elongation, is not known. A direct way to elucidate how a given factor affects different phases of axonal growth is to visualize progress of regenerating axons in vivo (3, 23–25) in the presence or absence of such a factor.

Here we use in vivo imaging in combination with selective genetic manipulations to address whether and when the transcription factor STAT3 influences the divergent growth pattern of lesioned PNS and CNS axons. We show that deletion of STAT3 is sufficient to impair PNS axon regeneration. By comparing the in vivo growth pattern of regrowing STAT3-competent and STAT3-deficient axons, we discovered that STAT3 regulates the timing of growth induction but not subsequent axon elongation. In line with this finding, viral gene delivery of either STAT3 or its constitutively active version, STAT3c, to DRG neurons significantly improves terminal and collateral sprouting after a CNS lesion by promoting growth induction but not elongation. Thus, STAT3 acts as a phase-specific regulator of axonal regeneration that selectively controls the timing of growth induction after CNS and PNS lesions.

Results

STAT3 Deletion Impairs the Regeneration of PNS Axons. To confirm that STAT3 is activated after a PNS lesion, we studied the expression of STAT3 and its active, phosphorylated form P-STAT3 in DRG neurons by immunohistochemistry at different time points after creation of bilateral lesions of the saphenous nerves, which contain the axons of the third lumbar (L3) DRGs (26). Starting within a few hours and lasting for weeks after transection, we observed a significant increase in the number of P-STAT3-positive nuclei in L3 DRG neurons (Fig. 1 *A, B*, and *E*). STAT3 expression in DRG neurons overall followed a similar time course (Fig. S1). However, at early time points, hours after the lesion,

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The authors declare no conflict of interest.

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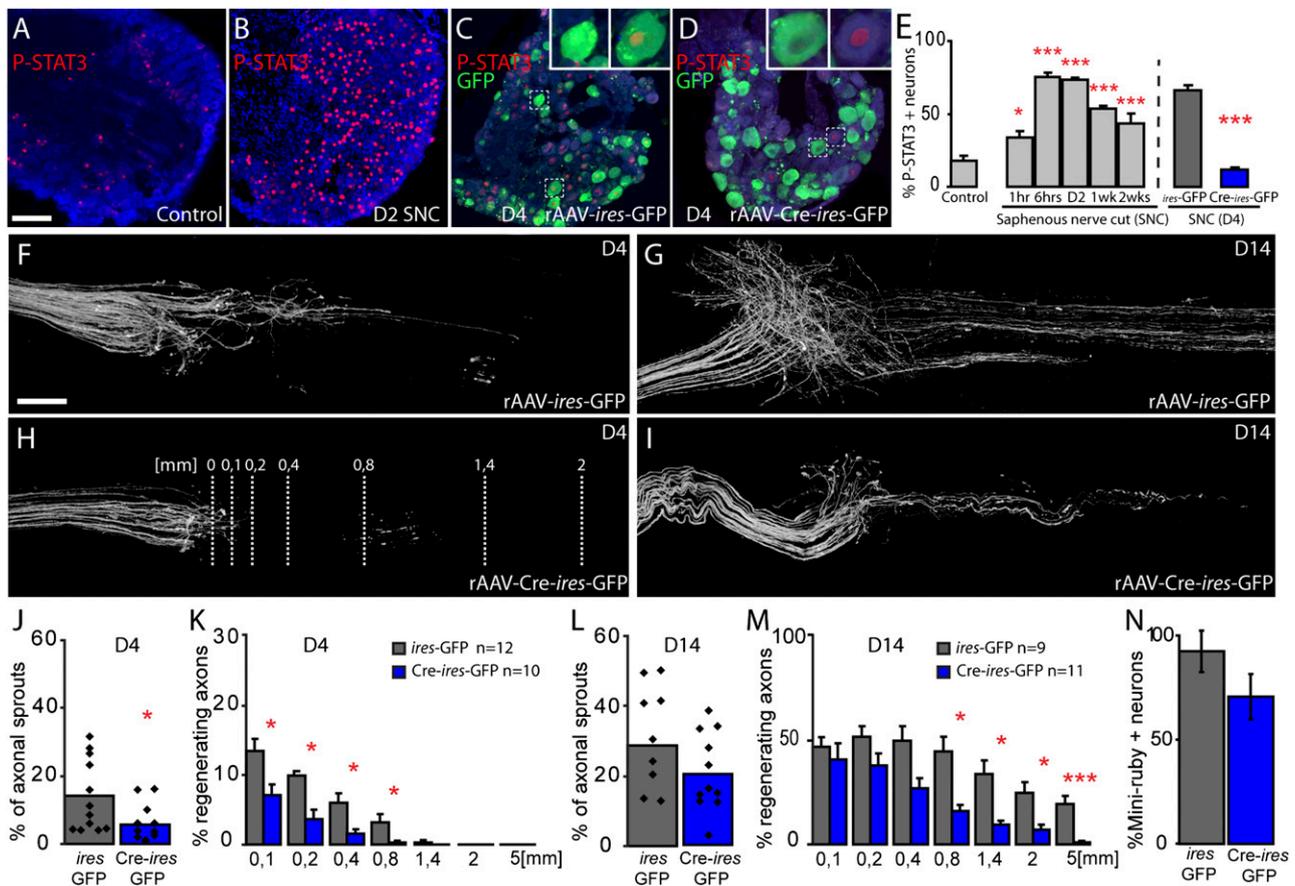


Fig. 1. Deletion of STAT3 impairs regeneration of peripheral DRG axons after a saphenous nerve cut (SNC; A and B). Confocal images of L3 DRGs immunostained for P-STAT3 (red) and counterstained with fluorescent Nissl-like stain (NeuroTrace, cyan) in a control (unlesioned) WT mouse (A) and 2 d following an SNC (B). (C and D) L3 DRGs of STAT3^{fl/fl} mice 4 d after SNC previously injected with rAAV-ires-GFP (C) or rAAV-Cre-ires-GFP (D; NeuroTrace, cyan; P-STAT3, red; GFP, green). GFP-positive DRG neurons (insets, C and D) are at a magnification of $\times 3$. (E) Quantification of the number of P-STAT3-positive DRG neurons (identified by NeuroTrace counterstaining) at different time points following SNC in WT mice and in STAT3^{fl/fl} mice previously injected with rAAV-ires-GFP (gray column) or rAAV-Cre-ires-GFP (blue column) at 4 d following a SNC ($n = 6$ animals per group). (F–I) Confocal images taken at 4 d (F and H) and 14 d (G and I) after SNC display the proximal stump of STAT3^{fl/fl} saphenous nerves that receive fibers from L3 DRGs injected with control rAAV-ires-GFP (F and G) or rAAV-Cre-ires-GFP (H and I). (J–M) Quantification of axonal sprouting at the site of lesion (J and L) and regeneration ratios (K and M) at different distances from the cut site (lines, H) of axons derived from STAT3-competent DRG neurons (ires-GFP, gray columns) and STAT3-deficient DRG neurons (Cre-ires-GFP, blue columns) at 4 d (J and K) and 14 d (L and M) after SNC. (N) Quantification of the percentage of L3 DRG neurons retrogradely labeled with the tracer Miniruby from distal STAT3-competent (ires-GFP, gray columns; $n = 21$ sections, $n = 6$ DRGs) and STAT3-deficient (Cre-ires-GFP, blue columns; $n = 11$ sections, $n = 4$ DRGs) saphenous nerves 28 d following SNC (values were normalized to the percentage of Miniruby-positive DRG neurons traced from the same anatomical localization in unlesioned mice). (Scale bars: A, 100 μm ; F, 250 μm .)

STAT3 phosphorylation appears to precede increased STAT3 expression. Together these findings indicate that, after a PNS lesion is created, STAT3 activity is regulated on both the phosphorylation and expression levels.

To assess the contribution of STAT3 activation to PNS regeneration, we selectively deleted STAT3 expression in DRG neurons. We constructed recombinant adeno-associated viral vectors (rAAVs) expressing either a bicistronic combination of Cre recombinase and GFP (rAAV-Cre-ires-GFP) or just GFP (rAAV-ires-GFP; *SI Materials and Methods*). After injection of rAAV-Cre-ires-GFP into the DRGs of STAT3-floxed (STAT3^{fl/fl}) mice (27), transduced DRG neurons become depleted of STAT3, while at the same time their axons can be readily identified by GFP expression. Injection of the control vector (rAAV-ires-GFP) labels axons without affecting STAT3 expression. The efficiency of STAT3 deletion was confirmed by analysis of P-STAT3 immunohistochemistry 4 d after lesion creation (Fig. 1 C–E and Fig. S2). We then performed bilateral saphenous nerve transections in STAT3^{fl/fl} mice that had been injected 10 d earlier with rAAV-Cre-ires-GFP in the right L3 DRG and rAAV-ires-GFP in

the left L3 DRG. We compared the growth response of STAT3-deficient and STAT3-competent GFP-positive axons over time (Fig. 1 F–M). At 4 d after lesion, STAT3-competent axons showed substantial sprouting and regeneration along the nerve for as much as several hundred micrometers (Fig. 1 F, J and K). In contrast, STAT3-deficient axons showed only minimal sprouting (Fig. 1 H and J), as well as fewer and shorter regenerating axons (Fig. 1 H and K). Two weeks after lesion, long-distance axonal regeneration in STAT3-deficient axons was still impaired (Fig. 1 I and M), while many STAT3-competent axons had grown several millimeters to reenter the distal stump of the saphenous nerve (Fig. 1 G and M). However, at this time, sprouting around the lesion and axonal regeneration close to the lesion was comparable between STAT3-deficient and STAT3-competent axons (Fig. 1 L and M). This suggests that STAT3-deficient axons can still initiate the regeneration process. Indeed, retrograde labeling from the distal saphenous nerve performed at 28 d after lesion showed that similar proportions of STAT3-competent and STAT3-deficient axons had reapproached their termination zone (Fig. 1 N).

STAT3 Deletion Affects Initiation but Not Perpetuation of PNS Axon Regeneration in Vivo. Impaired regeneration of STAT3-deficient PNS axons could be caused by delayed growth induction or reduced elongation of regenerating axons. To differentiate these possibilities, we followed the outgrowth of fluorescently labeled STAT3-competent and STAT3-deficient axons by repetitive in vivo imaging. We first imaged regrowing axons on consecutive days when axonal regeneration is induced (day 2–4 after lesion). Many STAT3-competent DRG axons initiate growth within 2 d after lesion and progress with an average speed of $132 \pm 23 \mu\text{m/d}$ from day 2 to day 3 (Fig. 2*A* and *C*). In contrast, the vast majority of STAT3-deficient axons fail to initiate growth during the first 2 d after lesion. Accordingly, the speed of regeneration is reduced more than threefold in STAT3-deficient axons in this initiation phase ($33 \pm 6 \mu\text{m/d}$; Fig. 2*B* and *C*). Notably during the axon elongation phase 7 to 8 d after lesion, when regeneration speed has increased to approximately $400 \mu\text{m/d}$, there is no difference between axons derived from STAT3-competent and STAT3-deficient DRG neurons ($411 \pm 48 \mu\text{m/d}$ for axons from rAAV-*ires*-GFP-injected DRGs vs. $341 \pm 53 \mu\text{m/d}$ for axons from rAAV-*Cre-ires*-GFP-injected DRGs; Fig. 2*D*). Taken together, these results indicate that STAT3 is crucial for the timing of growth initiation but not for subsequent elongation of PNS axons.

STAT3 and STAT3c Gene Therapy Can Initiate but Not Perpetuate CNS Axon Outgrowth. The failure to initiate growth is a key impediment to successful regeneration in the CNS. Therefore, we examined whether STAT3 overexpression is sufficient to induce outgrowth of CNS axons in vivo. We first evaluated the activation of endogenous STAT3 after a CNS lesion by P-STAT3 and STAT3 immunostaining of cervical DRGs after a bilateral dorsal column transection, which interrupts central projections from cervical DRG neurons. In contrast to the sustained activation of STAT3 after a PNS lesion (cf. Fig. 1), a dorsal column lesion induces no significant changes in the P-STAT3 and STAT3 immunoreactivity in DRG neurons (Fig. 3*A* and *D* and Fig. S1). To exogenously increase STAT3 expression, we produced rAAVs expressing either a control protein (enhanced CFP or Cre recombinase; control rAAV), STAT3 (rAAV-STAT3), or a constitutively active variant of STAT3 (rAAV-STAT3c) and confirmed the efficiency of viral gene transfer by immunofluorescence (Fig. 3*B–D*). We then injected the respective rAAVs into the DRGs of *Thy1*-GFP^s

mice, which express GFP in a subset of DRG neurons (28). Ten to 12 d later we surgically reexposed the spinal cord, lesioned individual GFP-positive axons emerging from the injected DRGs in the dorsal funiculus using a hand-held small-diameter needle, and imaged their growth response over time. As expected, 2 d after lesion, only 9% of axons emerging from DRGs injected with control rAAV had formed sprouts (Fig. 3*E, F*, and *K* and Fig. S3). In contrast, 53% of r-AAV-STAT3-transduced axons (Fig. 3*G, H* and *K* and Fig. S3) and 46% of the rAAV-STAT3c-transduced axons (Fig. 3*I, J*, and *K* and Fig. S3) showed an early growth response. Interestingly, STAT3 expression not only increased terminal sprouting, but also collateral sprouting along the axon (Fig. 3*G, I*, and *L*). The finding that a similar growth induction was observed after injection of rAAV-STAT3 and rAAV-STAT3c indicates that overexpression of STAT3 alone is sufficient to induce downstream effects on regeneration.

To determine how STAT3 overexpression affects different phases of axonal outgrowth, we used repetitive multiphoton imaging to follow the growth pattern of individual GFP-labeled DRG axons emerging from DRGs injected with control-rAAV, rAAV-STAT3, or rAAV-STAT3c at 2, 4, and 10 d after lesion. STAT3 and STAT3c overexpression increased the speed of axonal growth in the early phase (2–4 d) of regeneration (Fig. 4*A–C*). However, this early growth cannot be sustained, and only very limited axonal extension can be observed in all groups between 4 and 10 d after lesion (Fig. 4*A, B*, and *D*). Thus, in the CNS, as in the PNS, STAT3 regulates the initiation of axonal growth but not the elongation of regenerating axons.

Discussion

The present study identifies the transcription factor STAT3 as a phase-specific regulator of neuronal outgrowth in both the PNS and CNS. In DRG neurons, the endogenous expression of STAT3 parallels the regenerative response. By using conditional deletion of STAT3 in combination with in vivo imaging, we now also show that STAT3 expression is not only associated with axonal regeneration, but is in fact crucial for the timing of axonal growth initiation after a PNS lesion. It is interesting to note, however, that axons from STAT3-deficient neurons can still mount a growth response, albeit with a prolonged “lag” phase compared with their STAT3-competent counterparts. This suggests that in the PNS compensatory mechanisms are in place that can induce neuronal

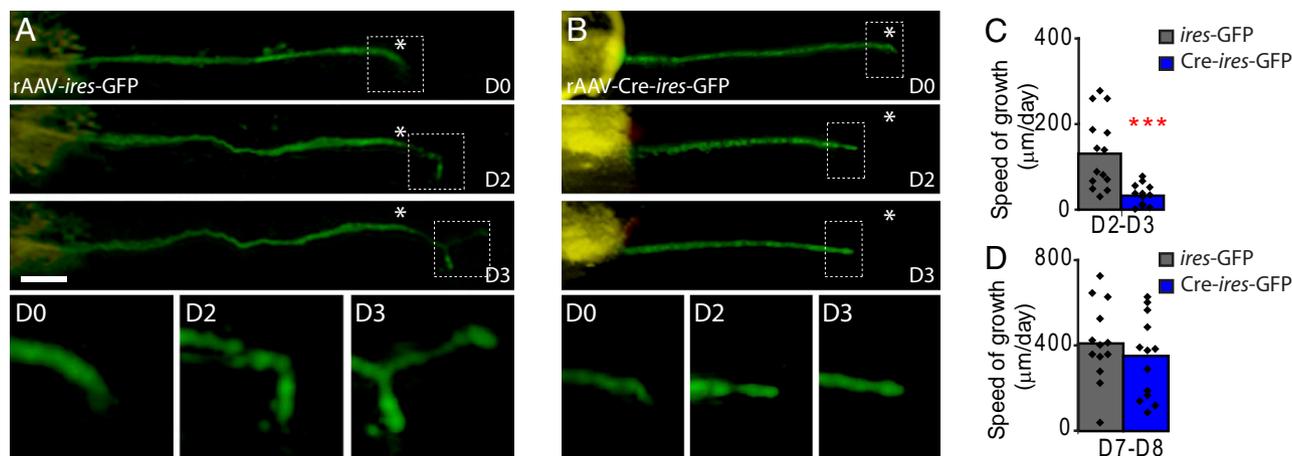


Fig. 2. In vivo imaging reveals that growth of STAT3-deficient axons is reduced in the initial but not the later stages of regeneration. (*A* and *B*) In vivo time-lapse images of saphenous nerve axons (GFP, green) in sparsely labeled animals emerging from DRGs that were injected with control rAAV-*ires*-GFP (*A*) or rAAV-*Cre-ires*-GFP (*B*). Axons were imaged immediately after transection (D0) as well as 2 and 3 d after transection (asterisk indicates lesion site, orange latex beads visible on the left were used as fiduciary markers). Lower row of boxes show the boxed axon tips at magnifications of $\times 3$ over time. (*C* and *D*) Quantification of the growth speed of axons from STAT3-competent DRG neurons (*ires*-GFP, gray columns) and STAT3-deficient DRG neurons (*Cre-ires*-GFP, blue columns) during the early (*C*, 2–3 d after transection) and the late phase (*D*, 7–8 d after transection) of the regeneration process. (Scale bar: *A*, 100 μm .)

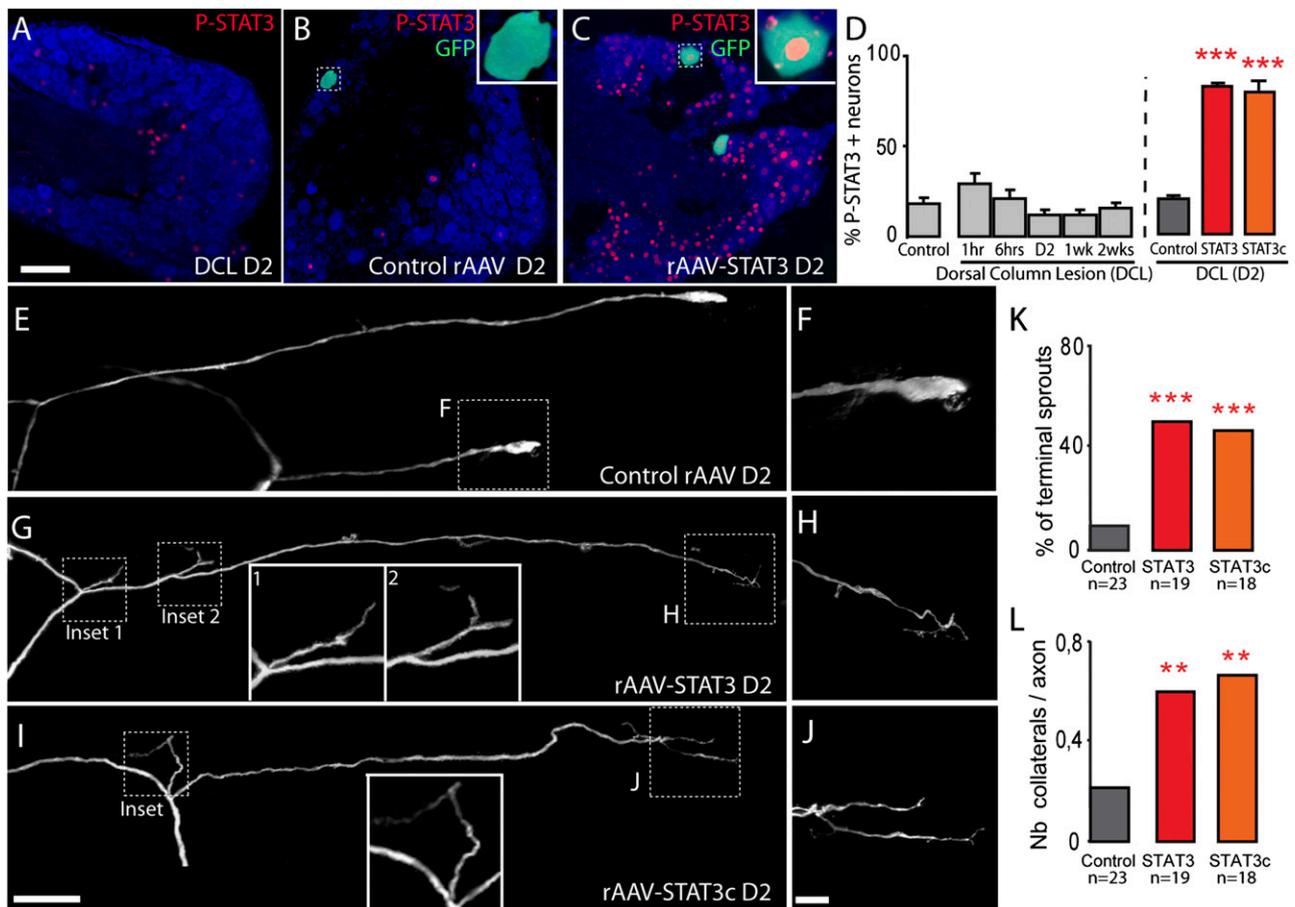


Fig. 3. Viral vector gene transfer of STAT3 and STAT3c induces terminal and collateral sprouting of DRG branches after a central lesion. (A–C) Confocal images of cervical DRGs immunostained for P-STAT3 (red) and counterstained with fluorescent Nissl-like stain (NeuroTrace, cyan) in a WT mouse 2 d following a dorsal column lesion (DCL, A) and in lesioned *Thy1-GFP⁺* mice (GFP, green) injected with control rAAV (B) or rAAV-STAT3 (C). Insets: Higher-magnification ($\times 3$) of the GFP-positive neurons boxed in the images. (D) Quantification of the number of P-STAT3-positive DRG neurons (identified by NeuroTrace counterstaining) at different time points following DCL in WT mice and in mice previously injected with control rAAV (gray column), rAAV-STAT3 (red column), or rAAV-STAT3c (orange column) at 2 d after a central lesion ($n = 6$ animals per group). (E–J) Confocal images of lesioned spinal axon endings derived from DRGs injected with control rAAV (bulbs, E and F), rAAV-STAT3 (terminal sprout, G and H), or rAAV-STAT3c (terminal sprout, I and J). (F, H, and J) Higher-magnification views of details boxed in E, G, and I. Additional insets (G and I) show a magnification $\times 2$ of the boxed collateral sprouts. (K and L) Quantification of terminal (K) and collateral (L) sprouting of axons derived from DRGs injected with control rAAV, rAAV-STAT3, or rAAV-STAT3c and analyzed 2 d after transection. (Scale bars: A and I, 100 μm ; J, 25 μm .)

growth in the absence of STAT3. Notably, additional overexpression of STAT3 or STAT3c did not further improve PNS regeneration (Fig. S4), indicating that a PNS lesion alone is sufficient to induce optimal levels of STAT3 activation for regeneration. When regeneration has been initiated, STAT3-deficient axons grow with the same speed as STAT3-competent axons. Thus, STAT3 is primarily needed to induce a neuronal growth program. When the growth program has been initiated, STAT3 is no longer required for perpetuation of axonal outgrowth. Given this role of STAT3 in PNS regeneration, it is tempting to speculate that the failure of CNS lesions to up-regulate STAT3 expression is directly linked to the failure of CNS axons to initiate outgrowth. Indeed, we can show that overexpression of STAT3 by viral vector gene transfer alone is sufficient to initiate axonal growth initiation in more than half the lesioned CNS axons. However, as the baseline sprouting response of CNS neurons is not affected by STAT3 deletion (12% of axons with sprouts are emerging from STAT3-competent DRGs, compared with 15.4% emerging from STAT3-depleted DRG axons; $n = 28$ –30 axons per group), it is likely that, as in the PNS, additional regulators can induce CNS outgrowth independent of STAT3.

The observation that early stages of axon growth can be initiated in many transected axons, even in the hostile CNS environment, by expression of a single intracellular molecule highlights the importance of intrinsic mediators of axonal growth. During recent years, a number of molecules that can influence the intrinsic neuronal growth response have been identified. These include c-AMP and its downstream mediators (29), the growth cone-associated proteins GAP43 and CAP23 (30), components of the PTEN/mTOR pathway (31), as well as a number of transcription factors (6–11). Further, a number of recent studies in *Caenorhabditis elegans* have demonstrated an essential role of the DLK-1 MAP kinase pathway for axon regeneration, and in particular growth cone formation and migration (32–34). As more and more components of the intrinsic growth response are emerging, it becomes increasingly important to understand how they act in concert to regulate the complex process of axonal outgrowth.

The present study provides evidence that, in vivo, this intrinsic growth response can be divided into at least two distinct phases: initiation and elongation. The concept of a multiphasic growth response suggests a number of conclusions. One is that the distinct phases of axonal growth are likely regulated by distinct molecular mechanisms. STAT3, for example, controls the timely initiation of

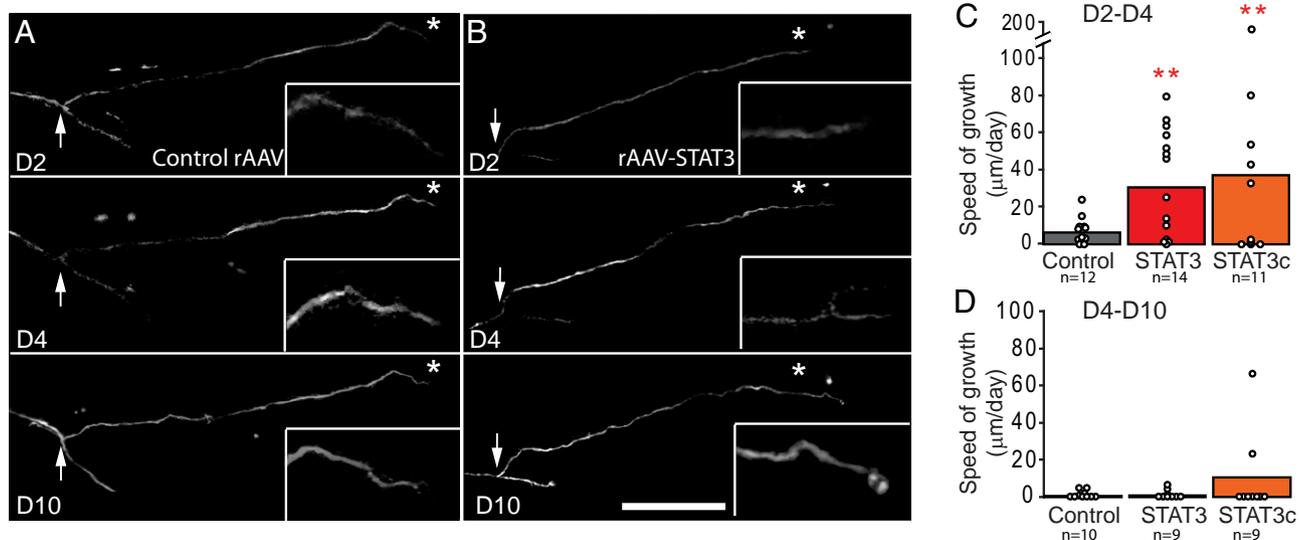


Fig. 4. In vivo imaging reveals successful initiation but not elongation of CNS axons after STAT3 and STAT3c gene therapy. (A and B) Multiphoton images of the growth pattern of spinal DRG axons emerging from DRGs injected with control rAAV (A) or rAAV-STAT3 (B) imaged 2, 4, and 10 d following a central lesion. Insets: Magnifications ($\times 2$) of boxed axon ends. (C and D) Quantification of axonal growth speed in vivo after injection of control rAAV (gray bars), rAAV-STAT3 (red bars), or rAAV-STAT3c (orange bars) analyzed early (C, 2–4 d) and late (D, 4–10 d) after transection. (Scale bar: B, 200 μm .)

axonal growth but does not affect axonal elongation. The molecular mechanisms by which STAT3 initiates this axonal growth program are currently not known. However, as STAT3 is a transcription factor, it is likely that the downstream effects are mediated by induction of gene expression. A large number of genes that are affected by STAT3 have already been identified. Some of these downstream targets like the cell cycle inhibitor P21/Cip1/Waf1 (35) or the small proline rich protein 1a (SPRR1A) (36) can directly affect neuronal outgrowth (37, 38). Notably, a recent transcriptional profiling study has identified several additional genes that are specifically regulated by STAT3 in DRG neurons. At least one of these genes, the IFN regulatory factor 1 (IRF1), is sufficient to increase neuronal outgrowth in cultured cerebellar neurons (21).

Our results further suggest that it is likely that environmental cues play key roles in shaping the distinct stages of axonal regrowth. For example, in the PNS, it is conceivable, that the transition from growth initiation to elongation is induced by the interaction of axons with Schwann cells. Schwann cells align after injury to form tubes, also called bands of Büngner, that guide the axons to their target cells (39). Axons could require STAT3 to initiate growth independently of Schwann cell guidance; however, when a regrowing axon has contacted the Schwann cell tube, it shifts to the elongation mode and no longer requires STAT3. In line with this scenario, we can show that PNS crush lesions that do not interrupt Schwann cell guidance do not induce STAT3 expression in the corresponding DRG neurons (Fig. S5). Although the reasons behind this lack of STAT induction are not yet understood and might include the induction of differential injury signals by crush and cut lesions (40), the rapid outgrowth of crushed axons (41) indicates that axonal growth along glial support structures does not require STAT3. Like in the PNS, changes in the environment may also help to explain the transition (or lack thereof) between different growth phases after a CNS lesion. Although, in the PNS, the regrowing axon can reach Schwann cell support at some point, CNS axons that initiate growth in response to STAT3 continue to encounter a growth-inhibitory CNS environment that becomes even more hostile with the development of a glial scar. These changes in the lesion environment might help to explain why STAT3-transduced axons can initiate growth early but fail to support it later. In line with this assumption, when we combined the induction of STAT3 (by viral vector gene

transfer of rAAV-STAT3) with the neutralization of inhibitory scar components (by application of chondroitinase ABC) (42), the average axonal outgrowth over a period of 10 d after a CNS lesion was increased more than twofold ($141 \pm 53 \mu\text{m}$ for axons treated with rAAV-STAT3 and chondroitinase ABC vs. $42 \pm 26 \mu\text{m}$ for axons treated with rAAV-STAT3 alone and $64 \pm 28 \mu\text{m}$ for axons treated with chondroitinase alone; $n = 14\text{--}20$ axons per group). These results underline the importance of developing combined therapeutic strategies that target the molecularly distinct phases of the axonal growth response. In this concept, phase-specific regulators of axonal growth initiation such as STAT3 would be used to “jumpstart” the regeneration process and prime axons in the injured spinal cord for application of complementary therapies that can sustain axonal elongation in the growth-inhibitory CNS environment (43).

Materials and Methods

Mice. Animals used in this study were adult female WT mice on a C57BL/6 background (weight 20–30 g, 6–12 wk of age) with the following exceptions: STAT3 was deleted by using STAT3^{fl/fl} mice, which are maintained on a BL6 background (27). Further, central axon regeneration was investigated in *Thy1-GFP⁵* mice (28), which express GFP in a subset of neurons and are maintained on a mixed background. All animal experiments were performed in accordance with regulations of the animal welfare act and protocols approved by the Regierung von Oberbayern.

AAV Vector Construction, Production, and Purification. The adeno-associated viral vectors used in this study have been cloned into the pAAV-MCS vector from Stratagene and were produced by the adenovirus-free AAV production method as detailed in *SI Materials and Methods*.

Tissue Processing, Immunohistochemistry, and STAT Expression Analysis. Animals were deeply anesthetized with isoflurane and perfused transcardially with saline followed by 4% paraformaldehyde in 0.01 M phosphate buffer. DRGs were dissected out, immunostained for P-STAT3 and STAT3 and analyzed as detailed in *SI Materials and Methods*.

Gene Therapy with Recombinant Adeno-associated Viral Vectors. For analysis of PNS regeneration, the left and right L3 DRGs of anesthetized STAT3^{fl/fl} mice were surgically exposed after a dorsal laminectomy. Then, 1 μL of rAAV-ires-GFP was slowly injected into the left L3 DRG with a thinly drawn glass capillary and the same amount of rAAV-Cre-ires-GFP was injected into the right L3 DRG of the same animal. Ten days after the injection, the sa-

phenous nerve was bilaterally transected at the midhigh level using fine iridectomy scissors as previously described (23).

For analysis of CNS regeneration, a cervical dorsal laminectomy was performed in *Thy1-GFP^S* mice anesthetized by an i.p. injection of ketamine/xylazine (ketamine 87 mg/kg, xylazine 13 mg/kg) as previously described (24). DRGs, from which suitably labeled axons emerged, were identified by in vivo imaging (*SI Materials and Methods*) and surgically exposed. Then, 1 μ L of rAAV-STAT3, rAAV-STAT3c, rAAV-eCFP, or rAAV-Cre (control rAAV) was slowly injected into the DRG with a thinly drawn glass capillary. Ten to 12 d later, *Thy1-GFP^S* mice were reanesthetized, the spinal cord laminectomy site was reaccessed, and selected fluorescently labeled axons were transected with a hand-held 32-gauge hypodermic needle.

Confocal Microscopy. We obtained confocal images of fixed tissue on a FV1000 confocal system mounted on an upright BX61 microscope (Olympus) and equipped with 20 \times /0.85 and 60 \times /1.42 oil immersion objectives. We recorded stacks of 12-bit images that were processed using MetaMorph software (Universal Imaging) or the freeware ImageJ/Fiji (<http://rsbweb.nih.gov/ij>).

In Situ and in Vivo Analysis of Axon Regeneration. The regeneration of transected peripheral and central DRG axons was evaluated as detailed in *SI Materials and Methods*.

Statistical Analysis. Results are given as mean \pm SEM unless indicated otherwise. Statistical significance was determined using GraphPad Prism software (GraphPad). All data were analyzed by using a one-way ANOVA followed by a Tukey post-hoc test for multiple comparisons or a *t* test for single comparisons. For the statistical evaluation of the proportion of terminal sprouts following rAAV treatment after CNS lesion, a frequency analysis was made using a χ^2 test.

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Supporting Information

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SI Materials and Methods

AAV Vector Construction, Production, and Purification. The adeno-associated viral vectors used in this study have been cloned into the pAAV-MCS vector from Stratagene as follows. Briefly, for the control pAAV-*ires*-GFP vector, a strong modified internal ribosome entry site (*ires*) of the encephalomyocarditis virus, which permits the translation of two genes of interest from a single bicistronic mRNA, was cut with BamHI and BstXI from the pires2-DsRed2 plasmid (Clontech) and inserted at the HincII site of pAAV-MCS. Then the DNA coding for GFP was inserted at the XhoI site of pAAV-MCS to create the pAAV-*ires*-GFP construct. The pAAV-Cre-*ires*-GFP construct was cloned by excising the coding sequence for Cre recombinase from pBS185 (gift of Thomas Hughes, Montana State University, MT) with XhoI and MluI and inserting it into the EcoRI site of pAAV-*ires*-GFP. For the pAAV-STAT3, the STAT3 gene was excised from pcDNA3 STAT3 (Addgene plasmid 8706) with BamHI and XhoI and cloned in the pAAV-MCS at the HincII site. For cloning the pAAV-STAT3c, the STAT3c gene was excised from pRc/CMV STAT3c Flag (Addgene plasmid 8722) with NotI and SwaI and inserted in the HincII site of pAAV-MCS. Control pAAV-eCFP was engineered by excising the eCFP gene from the pCFP N1 plasmid at BamHI and NotI and cloning it in the pAAV-MCS at the HincII site.

AAV serotype 2 particles were then produced in HEK293 cells by the adenovirus-free AAV production method (1, 2). Briefly, HEK293 cells were seeded at 80% confluence and cotransfected using the calcium phosphate method with 7.5 μ g of pRC (3), 7.5 μ g of transgene plasmid, and 22.5 μ g of pXX6 (1, 2). After 48 h, cells were harvested and pelleted by low-speed centrifugation. Cells were resuspended in 150 mM NaCl/50 mM Tris-HCl (pH 8.5), freeze/thawed three times, and treated with Benzonase (50 U/mL lysate) for 30 min at 37 °C. To purify viral vector preparations, cell debris was removed by centrifugation at 3,700 \times g for 20 min at 4 °C, and the supernatant was loaded onto a discontinuous iodixanol gradient (3). Following harvesting of the 40% phase, a heparin affinity chromatography was conducted. Briefly, a heparin affinity column (HiTrap heparin HP; GE Healthcare) was equilibrated with 1 \times PBS solution/MgCl₂ (1 mM)/KCl (2.5 mM). The vector solution was diluted 1:10 in the same buffer and applied to the column. After a washing step with 1 \times PBS solution/MgCl₂ (1 mM)/KCl (2.5 mM), the vector was eluted with 1 \times PBS/MgCl₂ (1 mM)/KCl (2.5 mM) plus 1 M NaCl in 500- μ L steps.

To determine the genomic particle titer, vector genomes were isolated by DNeasy Tissue Kit (Qiagen) and subjected to quantitative PCR analysis by using the LightCycler rapid thermal cycler system (Roche Diagnostics) and the SYBR Green kit (Roche Diagnostics). Genomic titers were as follows: rAAV-STAT3, 9×10^{12} genome copies/mL; rAAV-STAT3c, 5.5×10^{12} genome copies/mL; rAAV-eCFP, 9.2×10^{12} genome copies/mL; rAAV-Cre, 9×10^{12} genome copies/mL; rAAV-Cre-*ires*-GFP, $5.7\text{--}7.1 \times 10^{11}$ genome copies/mL; and rAAV-*ires*-GFP, $1.4\text{--}3.8 \times 10^{12}$ genome copies/mL.

Tissue Processing, Immunohistochemistry, and STAT Expression Analysis. Animals were deeply anesthetized with isoflurane and perfused transcardially with saline solution followed by 4% paraformaldehyde in 0.01 M phosphate buffer. Tissues were postfixed in 4% paraformaldehyde at 4 °C for 24 h. For immunofluorescence analysis, DRGs were dissected out, transferred to 30% sucrose for at least 24 h, and embedded in Tissue-Tek optimal cutting temperature compound (Sakura Finetek). Then, 20- μ m-thick coronal

sections were cut on a cryostat. Immunofluorescence staining of DRG sections was performed as described previously (4). Before immunostaining, sections were heated in a microwave to improve antigen retrieval. Antibodies were diluted in a working solution of PBS containing 0.2% Triton X-100 (Sigma) and 10% normal horse serum (Jackson Laboratory), and incubation was performed at room temperature or at 4 °C overnight. Details of the primary antibodies are as follows: STAT3 (dilution 1:500; Cell Signaling), P-STAT3 (dilution 1:50; Cell Signaling), and cJun (dilution 1:500; Santa Cruz Biotechnology). Secondary antibodies (goat anti-rabbit 594, goat anti-rabbit 488) were obtained from Jackson Laboratory and used at a dilution of 1:500. Nuclei were counterstained using NeuroTrace 435 (Invitrogen) and mounted in Vectashield (Vector Laboratories).

P-STAT3 and STAT3 immunoreactivity was analyzed in L3 DRGs of animals perfused at 1 h, 6 h, 2 d, 1 wk, and 2 wk after a bilateral saphenous nerve transection. P-STAT3 immunoreactivity was also analyzed in L3 DRGs of mice perfused 1 h, 6 h, 2 d, 1 wk, and 2 wk after the saphenous nerve was double-crushed with hand-held forceps. Immunofluorescence analysis of cJun induction in DRG neurons and confocal microscopy analysis of Wallerian degeneration in distal nerve segments of Thy1-YFP¹⁶ mice were used to confirm the success of the crush lesion (Fig. S5). P-STAT3 and STAT3 immunoreactivity was further analyzed in C6 DRGs of animals perfused at 1 h, 6 h, 2 d, 1 wk, and 2 wk after a bilateral dorsal column lesion. For the dorsal column lesion, animals were anesthetized with isoflurane, and a dorsal laminectomy was performed at the C4 to C6 spinal cord level as previously described (4). A small incision was made in the dura, and the dorsal column was then cut with fine iridectomy scissors. Unlesioned animals were used as controls. All DRG sections were counterstained with NeuroTrace 435 (as described earlier) to reveal the total number of neuronal nuclei in the DRG, and the proportion of DRG neurons showing STAT3 or P-STAT3 immunoreactivity was determined.

In Situ Analysis of Peripheral Axon Regeneration. To evaluate the regeneration of peripheral DRG branches, STAT3^{fl/fl} mice (5) injected with rAAV-Cre-*ires*-GFP or rAAV-*ires*-GFP were transcardially perfused with 4% paraformaldehyde at 4 or 14 d after saphenous nerve transection. Cross-sections of the saphenous nerve were cut rostrally to the injury site to quantify the number of GFP-labeled axons above the lesion. The distal part of the nerve containing the lesion site was dissected with the surrounding muscular tissue and flat-mounted in Vectashield (Vector Laboratories). High-resolution image stacks of the axons were taken on a FV 1000 confocal microscope system (Olympus). Then lines were positioned on the confocal maximum intensity projections at 0.1 mm, 0.2 mm, 0.4 mm, 0.8 mm, 1.4 mm, 2 mm, and 5 mm distal from the lesion site (Fig. 1H). The number of axons crossing these lines was counted. A ratio of regenerating axons was calculated by dividing the number of regenerating axons at a given distance from the lesion by the number of labeled axons rostral to the lesion.

To determine the percentage of DRG neurons that reach the proximity of their original target area, retrograde tracing experiments were performed as follows: At 28 d following the SNC, a gel foam impregnated with the tracer Miniruby (Invitrogen) was applied to the distal-most part of the saphenous nerve (located approximately 10 mm distal to the cut nerve). Three days later, mice were deeply anesthetized and transcardially perfused with PFA. Then, L3 DRGs were dissected and 20- μ m sections

were cut on the cryostat as described earlier. Confocal images of the sections were acquired on an Olympus FV 1000 confocal microscope and the number of Miniruby-positive DRG neurons and total DRG neurons (identified by costaining with NeuroTrace 435) were counted. The percentage of Miniruby-positive DRG neurons was calculated and normalized to the percentage of Miniruby-positive neurons that were retrogradely traced from the same anatomical localization in unlesioned mice ($45 \pm 6\%$ of Miniruby-positive L3 DRG neurons; $n = 14$ sections, $n = 2$ mice). Mice traced from the same anatomical localization immediately after the saphenous nerve was cut showed no labeling, indicating that the labeling at 28 d is caused by long-distance regeneration.

To determine whether the overexpression of STAT3 or STAT3c could increase peripheral axon regeneration, we injected 1 μ L of rAAV-STAT3, rAAV-STAT3c, or rAAV-Cre (control rAAV) into the L3 DRG of *Thy1-YFP*¹⁶ mice (6), in which a large proportion of DRG neurons are fluorescently labeled with the YFP. We then performed an SNC and evaluated axonal sprouting and regeneration at 4 d after the lesion as described earlier.

In Vivo Analysis of Peripheral Axon Regeneration. For in vivo imaging experiments, we used sparsely labeled animals, lesioned the saphenous nerve as described earlier, and marked the position of the axons proximally and distally from the lesion site with a suspension of 1- μ m-diameter orange FluoSpheres (Invitrogen). Before each imaging session, animals were anesthetized and placed directly under a fluorescence dissection microscope (Olympus). An incision was made on top of the lesion site and the axons in the saphenous nerve were imaged with 20 \times magnification at day 2 and 3 or at day 7 and 8 after lesion. Images were captured with a cooled Senciscam QE CCD camera (pco.imaging). To determine the growth of the transected axon ends, we measured the distance from the axon end to the FluoSpheres proximal to the lesion site at 2 and 3 d or 7 and 8 d after lesion and calculated the speed of growth per day.

In Vivo and in Situ Analysis of Central Axon Outgrowth. To image central branches of fluorescently labeled DRG neurons, we adapted our previously established spinal in vivo imaging approach (4). Briefly, *Thy1-GFP*^S mice were anesthetized by an i.p. injection of ketamine/xylazine. To access the cervical spinal cord, a laminectomy was performed and the dorsal surface of the spinal cord was exposed. During the imaging session, the spinal cord was superfused with mouse artificial cerebrospinal fluid. To follow the outgrowth of individual axons in vivo, we first identified single fluorescently labeled axons emerging from a DRG using a widefield set-up based on an Olympus BX51 microscope equipped with $\times 4/0.13$ dry, $\times 10/0.3$ dry, and $\times 20/0.5$ dipping cone water-immersion objectives. To document these axons in vivo, image streams of 25 to 50 images were acquired with a cooled Senciscam QE CCD camera controlled by MetaMorph software as previously described (4). The DRG from which the selected axon was emerging was then surgically prepared and 1 μ L of one of the rAAVs was injected by using a glass capillary as described earlier. Then, 10 to 12 d after the DRG injection, the dorsal surface of the spinal cord was surgically reexposed. The previously identified axon was transected by using a hand-held small-diameter needle and documented in vivo. Two different imaging protocols were used as follows.

To determine the frequency of axonal sprouting, the animal was perfused transcardially with 4% PFA 2 d after the lesion and the previously imaged spinal cord segment was dissected. The transected axon was then documented by using high-resolution confocal microscopy as described earlier, first in the intact spinal cord and then in 100- to 250- μ m-thick vibratome sections. Confocal stacks were processed as described earlier and the

transected axons were reconstructed from the DRG root to the lesion site. Axons were then evaluated by two blinded observers and terminal axon ends were classified as “sprouts” or “bulbs” based on their characteristic morphological appearances. Further, the number of collateral sprouts emerging from the transected axons was counted.

To determine the speed of axonal outgrowth we reimaged the transected axon ends at 2 and 4 d after lesion in vivo using a custom-built multiphoton imaging setup based on an Olympus FV 300 scanner equipped with a femtosecond pulsed Ti:Sapphire laser (Mai Tai HP; Newport/Spectra-Physics). We acquired image stacks of 50 to 200 images per stack (spaced at 1–2 μ m in *z* dimension) for each frame with a $\times 20/0.5$ dipping cone water-immersion objective. Animals were then perfused transcardially with 4% PFA 10 d after the lesion. The imaged part of the spinal cord was dissected out. The transected axons were reidentified in the fixed tissue and imaged in the intact spinal cord by using an Olympus FV1000 MPE multiphoton microscope or an Olympus FV1000 confocal microscope. Image stacks of 20 to 100 frames were acquired with a $\times 25/1.05$ water-immersion or a $\times 20/0.85$ oil-immersion objective. Imaging stacks were processed by using MetaMorph or ImageJ/Fiji software. Frames containing the axon were selected, and the transected axons were reconstructed using Photoshop. To determine the growth of the transected axon ends, we measured the distance from the axon end to a characteristic proximal structure (in most cases the Y-branch point in the dorsal root; in some cases a crossing point with another axon) at 2, 4, and 10 d after lesion. To compensate for tissue changes caused by fixation in the perfused samples, we measured a “constant” distance e.g., between two branch points in the same unlesioned axon both in vivo and in the corresponding fixed tissue to determine a sample-specific “correction factor.” The length of the transected axon end measured in fixed tissue was then multiplied by this correction factor. Axons that showed substantial die-back were excluded from the analysis.

To determine the influence of endogenous STAT3 expression on axonal sprouting after a CNS lesion, we injected 1 μ L of rAAV-eCFP or a combination of rAAV-eCFP and rAAV-Cre into the cervical DRGs of STAT3^{fl/fl} mice. Ten days after the injection, fluorescently labeled spinal DRG axons were lesioned by a pin as described earlier. Then, 2 d following the lesion, animals were perfused and axonal sprouting was evaluated based on confocal stacks of the fixed intact spinal cord as described earlier.

To determine the effects of a combination therapy with STAT3 and chondroitinase ABC, we performed the following experiment. We first injected 1 μ L of rAAV-STAT3 or rAAV-Cre into the cervical DRGs of *Thy1-GFP*^S mice as described earlier. Ten days after this injection, we then lesioned individual GFP-positive spinal axons and administered a first bolus of 6 μ L of chondroitinase ABC (10 U/mL in saline solution with 0.01% BSA; Sigma-Aldrich) or vehicle only (saline solution with 0.01% BSA) immediately following lesion creation. At the same time, an osmotic minipump (1007B; Alzet), which was connected to a brain infusion kit (Alzet) inserted into the lateral ventricle, was installed and started to deliver 0.5 μ L/h of chondroitinase ABC (10 U/mL in saline solution with 0.01% BSA) or vehicle only for 7 d. At 10 d following the lesion, the animals were perfused with PFA, the spinal cords were dissected, and the transected axons were imaged in the intact fixed spinal cord by confocal microscopy as described earlier. On confocal image stacks, we then measured the length of axonal sprouts to determine the axonal outgrowth (axonal terminals that ended in tips or bulbs were considered to be nongrowing axons and their outgrowth was set to 0 μ m).

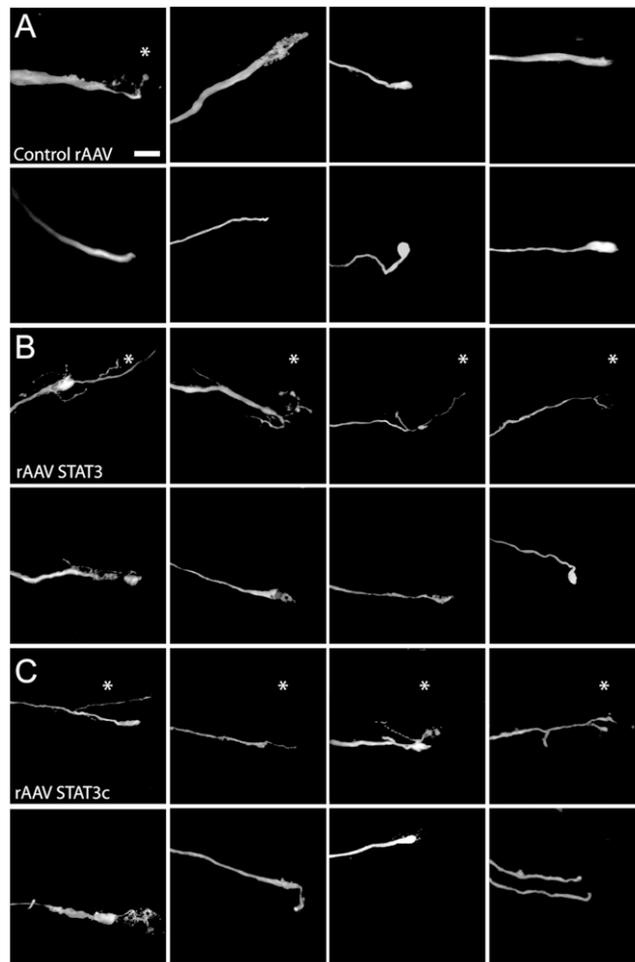


Fig. 53. Gallery of central DRG axon endings after treatment with control rAAV, rAAV-STAT3, and r-AAV-STAT3c. (A–C) Representative images of axon endings 2 d after a central lesion derived from DRG neurons treated with control rAAV (A), rAAV-STAT3 (B), or rAAV-STAT3c (C). Axonal sprouts (asterisks) are rarely found in control axons (A) but are frequent in axons emerging from DRG neurons expressing STAT3 (B) or STAT3c (C). (Scale bar: A, 25 μ m.)

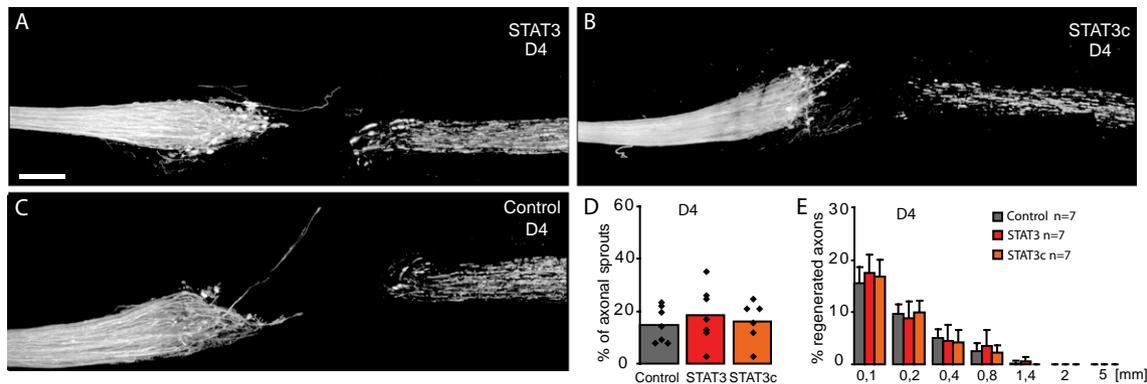


Fig. 54. Overexpression of STAT3 or STAT3c does not increase peripheral axon regeneration following a SNC. (A–C) Confocal images taken at 4 d after SNC displaying the proximal stump of saphenous nerve that receives fibers from L3 DRGs injected with rAAV-STAT3 (A), rAAV-STAT3c (B), or control rAAV (C). Axons are fluorescently labeled with YFP (white) in *Thy1-YFP¹⁶* mice. (D and E) Quantification of axonal sprouting at the site of the lesion (D) and regeneration ratios (E) at different distances from the cut site (Fig. 1H) of axons derived from DRG neurons that express normal levels of STAT3 (gray columns) or overexpress STAT3 (red columns) or STAT3c (orange columns) at 4 d after SNC. (Scale bar: A, 250 μ m.)

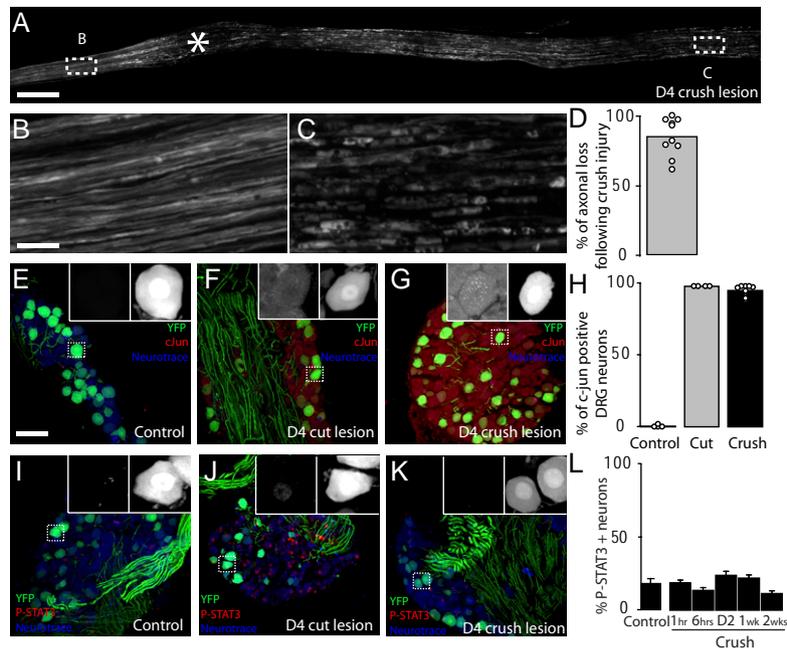


Fig. S5. A peripheral crush lesion leads to Wallerian degeneration and induces cJun expression but not STAT3 activation in DRG neurons (A–C) Wallerian degeneration of axons (white) in the distal stump of a saphenous nerve 4 d following saphenous nerve crush in *Thy1-YFP*¹⁶ mice. Magnification of the areas boxed in A shows intact axons rostral to the lesion (B) and axonal fragments distal to the lesion (C). (D) Quantification of the loss of continuous axons distal to the lesion at 4 d after injury. (E–G) Confocal images illustrating cJun immunoreactivity (red) in an unlesioned control DRG (E), and in DRGs following saphenous nerve cut (F) or crush (G; YFP, green; NeuroTrace, cyan). (H) Quantification of the number of cJun-positive L3 DRG neurons (identified by NeuroTrace counterstaining) in unlesioned controls and 4 d following saphenous nerve cut or crush. (I–K) Confocal images illustrating P-STAT immunoreactivity (red) in an unlesioned control DRG (I) and in DRGs following saphenous nerve cut (J) and crush (K; YFP, green; NeuroTrace, cyan). (L) Quantification of the number of P-STAT3-positive DRG neurons (identified by NeuroTrace counterstaining) at different time points following saphenous nerve crush in WT mice. (Scale bars: A, 400 μ m; B, 40 μ m; E, 100 μ m.)

STAT3 promotes corticospinal remodeling, regeneration and functional recovery after spinal cord injury

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STAT3 promotes corticospinal remodeling, regeneration and functional recovery after spinal cord injury

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Abstract

In contrast to peripheral neurons, neurons of the central nervous system (CNS) fail to grow after injury. As a result axonal regeneration and remodeling after CNS lesions remain limited and functional deficits persist. The transcription factor *Signal Transducer and Activator of Transcription 3* (STAT3) has recently been identified as a key element of axonal growth induction and its sustained expression is required for timely growth initiation in the peripheral nervous system.

Here we use the corticospinal tract (CST) to investigate the role of STAT3 during axon regeneration and remodeling in the CNS. We show that cortical projection neurons only transiently overexpress STAT3 after spinal cord injury. This transient STAT3 expression is insufficient to initiate axonal outgrowth and its conditional deletion in CST projection neurons affects neither axon regeneration nor sprouting at or remote from the lesion. If however sustained expression and activation of STAT3 is induced in these neurons by viral gene transfer, their axons show increased terminal and collateral sprouting as well as regeneration after spinal cord injury. Furthermore, after a unilateral pyramidotomy, sustained STAT3 expression and activation initiates the *de novo* formation of collaterals from unlesioned CST fibers that cross the spinal midline and contact interneurons and motoneurons that control forelimb function. Behavioral and electrophysiological recordings indicate that these newly formed ‘midline crossing circuits’ establish ipsilateral forelimb activation and contribute to forelimb function recovery. These findings identify intrinsic growth induction by STAT3 as a promising approach to promote regeneration and remodeling and improve functional recovery after CNS injury.

Introduction

Traumatic, inflammatory or ischemic lesions to the spinal cord lead to the transection of axonal tract systems and as a result are often followed by devastating motor and sensory deficits (1). If the lesion of the spinal cord is complete, i.e. if the entire spinal cord is transected, severe deficits persist. If the lesion is however incomplete, some functional recovery can occur in rodents (2-5) as well as in humans (6,7). Over recent years a number of studies have investigated the anatomical basis of this recovery process often using the corticospinal tract (CST), a major descending motor tract, as a model system (2,3, 8-10). The results show that – while long-distance regeneration of transected CST fibers fails – lesioned CST connections spontaneously attempt to remodel after injury (2,3,11). We have previously identified sprouting of CST collaterals into the cervical cord and their contacts to long propriospinal neurons as key components of endogenous CST remodeling (2,12). Despite the formation of this detour circuit, however, spontaneous functional recovery in most cases remains incomplete. To further improve functional recovery we thus need to devise strategies that can not only enhance axonal regeneration but also improve endogenous remodeling.

How the induction of axonal remodeling is regulated is however so far only incompletely understood. One of the reasons is that so far many studies have focused on the induction of axonal regeneration based on the neutralization of extrinsic growth-inhibitory signals at the lesion site (13-16). These strategies are however less likely to affect axonal remodeling, which often requires the formation of new collaterals remote from the lesion site. Here, strategies that affect the intrinsic growth capability of the entire neuron are conceptually more suited (17). We have previously identified the sustained expression of the transcription factor STAT3 (*Signal Transducer and Activator of Transcription 3*) is a key requirement for the timely induction of the intrinsic growth

program in DRG neurons (18). A key role for STAT3 in the induction of the intrinsic growth program is supported by the following findings: (i) The expression and phosphorylation of STAT3 correlates with the regenerative response of the neuron after injury; neurons that express STAT3 start to regenerate while those that don't generally fail to grow (18-21). (ii) Deletion of STAT3 impairs the timely initiation of PNS regeneration (18) and STAT3 inhibition blocks the growth-promoting effect of a conditioning lesion in the CNS (22). (iii) STAT3 over-expression or the deletion of its inhibitor, SOCS3, can improve sprouting of central DRG projections (18) and promote optic nerve regeneration *in vivo* (23,24). Whether and how STAT 3 can initiate the remodeling of central neurons and their projections is so far not known.

Here, we investigate how growth initiation by STAT3 affects CST regeneration and remodeling as well as functional recovery in different spinal cord injury paradigms. We use conditional genetics to delete endogenous STAT3 expression and viral gene transfer to induce sustained STAT3 expression. Stereotactic tracing of hindlimb CST fibers then allowed us to reveal the effects of STAT3 deletion or over-expression on CST regeneration and remodeling, while behavioural testing and electrophysiological recordings were used to assess the resulting functional recovery.

Results

Cortical projection neurons only transiently express and activate STAT3 after spinal cord injury

To examine whether STAT is expressed and activated in lesioned cortical projection neurons, we investigated the expression of STAT3 and its activated, phosphorylated form

(p-STAT3) immunohistochemically in the hindlimb motor cortices of mice perfused at different timepoints from 6 hrs to 3 weeks following a dorsal midthoracic hemisection. In unlesioned animals, only very few layer V pyramidal neurons in the motor cortex (that were identified based on their typical morphology after neurotrace labeling) expressed either p-STAT3 (Fig. 1 A, C) or STAT3 (Supplementary Fig. 1A, C). The number of STAT3 and p-STAT3-positive cortical projection neurons was then significantly increased at 24 hrs after lesion (Fig. 1 B, C and Supplementary Fig. 1 B, C). However, even at this time only a subset of cortical projection neurons expressed STAT3 (Supplementary Fig. 1 C) or p-STAT (Fig. 1 C). Moreover, in these neurons STAT3 expression and phosphorylation is only transiently induced and has returned to baseline levels at 1 week after injury (Fig. 1 C and Supplementary Fig. 1 C).

Transient expression of STAT3 does not contribute to endogenous axonal regeneration and remodeling after spinal cord injury

To assess to what extent the transient STAT expression supports endogenous attempts of axonal regeneration and remodeling, we selectively deleted STAT3 expression in cortical projection neurons. For this purpose we crossed *Emx*-Cre mice, which express Cre recombinase in the forebrain (25) with STAT3^{fl/fl} mice. As expected no STAT3 or p-STAT3 expression is detected in cortical projection neurons of Cre-positive STAT3^{fl/fl} mice (Fig. 1 D-F and Supplementary Fig. 1 D-F). We then performed midthoracic dorsal hemisections in STAT3 competent (Cre-) and conditional STAT3 deficient (Cre+) mice and examined the effects of STAT3 deletion on CST sprouting, regeneration, remodeling and functional recovery. Our analysis revealed no differences between STAT3 competent and conditional STAT3 deficient mice in all parameters analyzed: Axonal sprouting and regeneration at the lesion site was comparable between both groups both at 1 week

(Supplementary Fig. 1 G-K) as well as at 3 weeks after lesion (Fig. 1 G, H). Likewise, no differences in the formation of intraspinal detour circuits were detected as similar numbers of cervical collaterals form, which do not differ in length, complexity and number of boutons and contact similar proportions of long propriospinal neurons (Fig. 1 I, J Supplementary Fig. 1 L-O and *data not shown*). Consistent with these observations no differences in the recovery of hindlimb locomotion assessed by BMS and rotarod tests were detected between STAT3 competent and conditional STAT3 deficient mice (Supplementary Fig. 2 A-C).

Sustained STAT3 expression induces corticospinal sprouting and regeneration

As the transient induction of STAT3 expression after central lesions is thus insufficient to contribute to axonal regeneration and remodeling, we next investigated whether the exogenous induction of sustained STAT3 expression would be sufficient to promote axonal outgrowth. To induce sustained STAT3 expression in cortical neurons, we injected a recombinant adeno-associated viruses (rAAV) expressing STAT3 into the cortical hindlimb motor area (Fig. 2 A-C). Immunohistochemical analysis of STAT3 expression and phosphorylation demonstrated that transfer of rAAV-STAT3 is sufficient to induce sustained expression and phosphorylation of STAT3 for at least 3 weeks after the injection of the virus.

To assess the effect of sustained STAT3 expression on regeneration and remodeling of lesioned CST fibers, we injected rAAV-STAT3 or Control rAAV into the hindlimb motor cortex, lesioned the main dorsal and the minor dorso-lateral component of the CST by a midthoracic hemisection and performed the following analyses: First, to assess effects on CST regeneration we counted the number of sprouts at the lesion site as well as the number of regenerating CST fibers that extend for up to 500 μm beyond the lesion site

(Fig. 2 A, E). Our analysis showed that sustained STAT3 expression is sufficient to induce a significant increase in CST sprouting at the lesion site (Fig. 2 E-H, J). Moreover significantly more regenerated CST fibers were detected in mice injected with rAAV STAT3 at 200 – 400 μ m from the lesion site (Fig. 2 E-I). Sustained expression of STAT3 is thus sufficient to induce CST sprouting at and regeneration beyond the lesion site.

To assess to what extent sustained STAT3 expression can also support the remodeling of CST fibers distant from the lesion site, we quantified the formation of intraspinal detour circuits after midthoracic dorsal hemisections in mice injected with rAAV-STAT3 and Control rAAV (Fig. 3 A). Indeed mice injected with rAAV-STAT3 showed an increased formation of cervical CST collaterals at 3 weeks after injury (Fig. 3 B-D). However the intraspinal targeting of these collaterals is not affected and thus a similar proportion of long propriospinal neurons are contacted in mice injected with rAAV-STAT3 and Control rAAV (Fig. 3 E, F). Notably, mice injected with rAAV-STAT3 displayed the formation of cervical collaterals (Fig. 3 G-J) and contacts onto long propriospinal neurons (Fig. 3 K, L) even in the absence of a spinal lesion. This indicates that sustained STAT3 expression can also induce the remodeling of fibers that have not been primed to grow by their previous transection.

Sustained expression of STAT3 induces the de novo formation of midline-crossing CST circuits after pyramidotomy

To further investigate the capability of STAT3 to recruit unlesioned fibers to the remodeling process, we induced a unilateral lesion of the left CST at the level of the medulla oblongata ('unilateral pyramidotomy', Fig. 4 A). We then assessed whether and how unlesioned fibers from the contralateral, right forelimb portion of the CST remodel in response to the unilateral denervation. In animals injected with Control rAAV no

significant increase in the number of CST fibers that exit the right CST is detected at 1, 3 or 12 weeks after pyramidotomy (Fig. 4 B, E). Further CST fibers that exit the CST rarely crossed the spinal midline (Fig. 4 B, F). In contrast in animals injected with rAAV-STAT3 additional CST collaterals exit the CST at 3 weeks after injury (Fig. 4 C, E). These newly formed collaterals extend towards the denervated side of the spinal cord resulting in a significant increase in the number of midline-crossing fibers that is first detected at 3 weeks (Fig. 4 C, F) and persist for at least 12 weeks after lesion (Fig. 4 D, F). We next examined the projection pattern of these newly formed midline crossing CST collaterals and found that in animals injected with rAAV-STAT3, CST collaterals extended significantly further into the denervated spinal cord and often projected to the intermediate and ventral laminae VI-IX of the spinal cord (Fig. 4 G, H). As the cell bodies of short propriospinal neurons and spinal motoneurons that control forelimb movement are located in these laminae we next assessed to what extent these neurons are targeted by midline crossing CST collaterals at 12 weeks after pyramidotomy. Our results show that the proportions of short propriospinal neurons and spinal motoneurons that are contacted by CST collaterals are increased more than 4- and 20-fold, respectively, in animals injected with rAAV-STAT3 (Fig. 4 I-L).

De novo formation of midline crossing circuits improves behavioral and electrophysiological recovery after injury

To investigate whether the newly formed midline crossing CST circuits induced by sustained STAT3 expression can foster functional recovery we performed the following analyses after unilateral pyramidotomy. First, we used the staircase test which measures the capability of mice to remove sugar pellets placed on different stairs of a staircase to evaluate skilled forelimb grasping as described in *SI Materials and Methods* (Fig. 5 A,).

While mice from both experimental groups showed similarly impaired forelimb grasping immediately after injury, mice injected with rAAV-STAT3 recovered quicker and performed significantly better in the staircase reaching task compared to mice injected with Control rAAV from 5 weeks after pyramidotomy onwards (Fig. 5 B). To study the contribution of midline-crossing circuits to this functional recovery, we recorded forelimb flexor electromyographs (EMGs) after intracortical stimulations (Fig. 5 C, D). Unlesioned animals elicited EMG responses in 100% of the cases (n=24 stimulations, Fig. 5 E). In the days following pyramidotomy, this response was basically abolished (Fig. 5 E). When we investigated the response to stimulation twelve weeks following pyramidotomy, we observed that animals treated with the Control rAAV exhibited 53±9% while animals treated with rAAV-STAT3 exhibited 94±4% of responsive sites in the ipsilateral cortex (Fig. 5 E). The finding that cortical stimulation in animals injected with rAAV-STAT3 evoked an increased ipsilateral EMG response is consistent with the idea that newly formed midline crossing CST fibers mediate this recovery. To confirm the contribution of new CST connections below the level of the pyramids to the recovery we performed an additional pyramidotomy of the intact side in mice that had previously recovered responsiveness to stimulation at 12 weeks after lesion and injection with rAAV-STAT3. In these mice the second pyramidotomy completely abolishes the response to cortical stimulation (Fig. 5 E). Further analysis of the cortical stimulation parameters revealed lower stimulation thresholds and shorter latencies to an EMG response in mice 12 weeks after injection with rAAV-STAT3 compared with mice injected with Control rAAV (Fig. 5 F, G). Taken together our electrophysiological and behavioral analysis strongly suggests that the midline crossing CST circuits induced by sustained expression of STAT3 are functional and contribute to improved recovery of forelimb function after injury.

Discussion

The sustained expression of p-STAT3 is required for the timely initiation of neuronal growth in the lesioned peripheral nervous system (18). Here, we show that in response to a central lesion STAT3 and p-STAT3 expression are only transiently upregulated in a subset of cortical projection neurons. This transient upregulation is consistent with previous reports that investigated STAT3 expression in layer V cortical projection neurons following transient cerebral ischemia in rats (26) and the expression of JAK-STAT family members (JAK1 and STAT3 in particular) in the injured spinal cord (19). The conditional deletion of STAT3 expression now shows that this transient expression of STAT3 does not contribute to the induction of endogenous attempts of axonal regeneration and remodeling. This suggests that at least in cortical projection neurons other pathways might be responsible for endogenous induction of axonal sprouting.

While the transient STAT3 expression induced by central lesions can thus not influence axonal growth, we can now show that sustained expression of STAT3 induced by viral gene transfer is sufficient to substantially improve the neuronal growth induction of transduced cortical projection neuron and affect multiple aspects of the CST response to injury: First, sustained STAT3 expression improves axonal sprouting and regeneration at the lesion site. This pro-regenerative effect of STAT3 expression is consistent with previous reports that have shown that STAT3 over-expression can induce sprouting of central DRG projections (18) and that deletion of its negative regulator SOCS3 can improve optic nerve regeneration (23,24). The molecular mechanisms by which STAT3 initiates this axonal growth are currently not fully understood. However, several genes that are induced by the transcription factor STAT3 have already been identified and include the cell cycle inhibitor P21/Cip1/Waf1 (27), the small proline rich protein 1a (SPRR1A, 28)

and the interferon regulatory factor 1 (IRF1; 21), all of which can directly affect neuronal outgrowth (21,29,30). When evaluating the effects of STAT3 in regeneration, it should be noted that axonal growth initiation by STAT3 appears to primarily increase the number of CST axons that attempt to regenerate after a lesion. As these axons still have to grow through the growth-inhibitory environment of the lesioned CNS (13-16,31) it is probably not surprising that even after STAT3 induction most regenerating fibers terminate within the first 500 μm from the lesion. By itself this pro-regenerative effect of STAT3 is thus unlikely to contribute to functional recovery, however improved growth induction by STAT3 can be an ideal therapeutic complement to strategies that counteract growth-inhibitory signals in the glial scar and central myelin (13-16,31,32). In line with this concept we could previously show synergistic effects on axonal outgrowth of central sensory connections when we combined STAT3 induction with the neutralization of growth-inhibitory chondroitin sulfate proteoglycans (18).

While the contribution of axonal regeneration to functional recovery is thus limited, at least in the untreated spinal cord, the remodeling of axonal connections can promote some recovery of function even in the absence of therapeutic support. We and others have previously shown that the formation of intraspinal detour circuits is a key component of the endogenous recovery process following spinal cord injury (2,3,12,33). For detour circuits to be formed lesioned CST fibers first extend new collaterals distant from the lesion site. These collaterals enter the intermediate layers of the cervical spinal cord and there contact different interneurons including long propriospinal neurons. Long propriospinal neurons act as relay neurons and increase their projections to the denervated target area of the transected hindlimb CST thereby completing the detour circuit. We can now show that sustained STAT3 expression can further increase the number of cervical CST collaterals that form after lesion. However increased collateral formation does not result in significant

changes to the CST connections to the long propriospinal relay neurons. This might indicate that the endogenous growth response of lesioned CST projection neurons is sufficient to promote the formation of detour circuits at an “optimal” rate that is not improved further by the presence of additional CST collaterals.

While lesioned CST fibers thus spontaneously reorganize after injury, unlesioned fibers generally do not adapt in response to injury. Our results now indicate that the sustained expression of STAT3 is sufficient to recruit unlesioned fibers to the remodeling process. After a unilateral lesion of the CST at the level of the pyramids, sustained expression of STAT3 induces the formation of collaterals from the unlesioned CST. These collaterals enter the cervical gray matter, cross the spinal midline and form contacts to the previously denervated spinal interneurons and motoneurons that control forelimb function. The findings that neuronal growth initiation by STAT3 is sufficient to induce the *de novo* formation of these midline crossing circuits indicate that the guidance signals that attract newly formed collaterals are endogenously present in the denervated spinal cord. It is interesting to note in this context that the remodeling of unlesioned CST fibers can not only be induced by sustained STAT3 expression. Indeed previous studies have, for example, shown that activation of the mTOR pathway through deletion of PTEN leads to sprouting of uninjured CST axons and regeneration of injured CST axons past the lesion site (10) and that the over-expression of the neuronal calcium sensor1 (NCS1), which acts via the PI3K/Akt pathway, induces sprouting and midline crossing of unlesioned CST fibers (9).

The therapeutic potential of interventions that promote the remodeling of unlesioned connections is illustrated by our behavioral and electrophysiological analyses that demonstrate meaningful recovery of forelimb function in animals treated with rAAV-STAT3 following pyramidotomy. One caveat of strategies that promote axonal remodeling is the induction of “unwanted” new connections that could in principle augment pain or

spasticity after spinal cord injury. Our results from this and previous studies however suggest that endogenous regulatory mechanisms might be in place that prevents the formation of functionally “unwanted” connections. For example, we could previously show that the formation of collaterals is followed by a phase of sprout sorting and refinement during which excessive connections e.g. to short propriospinal neurons that do offer a detour around the lesion are at least partially removed (2,33). Further, as discussed above our current experiments provide evidence for the existence of endogenous guidance cues that target sprouting collaterals to denervated areas of the spinal cord. Finally, the combination of strategies that promote axonal reorganization with neurorehabilitation approaches might further help to enhance desired and limit unwanted consequences of nervous system remodeling (34). Thus we believe that the support of endogenous axonal remodeling, for example by the induction of sustained expression of STAT3 or selective manipulation of its downstream targets, is a promising therapeutic avenue that can help to improve functional recovery in many neurological conditions in which trauma, inflammation or ischemia cause permanent axon damage.

Materials and Methods

Animals:

To delete STAT3 expression in cortical projection neurons, we crossed STAT3^{fl/fl} mice, in which deletion of the STAT3 gene depends on Cre-mediated excision of loxP sites, and *EMX*-Cre mice (25) in which regulatory elements of the *Emx1* gene drive Cre expression in the forebrain. Adult female mice homozygous for the floxed STAT3 allele and either expressing Cre (STAT3-deficient group) or Cre-negative (control group) were used for experiments. For all other experiments we used adult female C57/Bl6 mice (6-8

weeks old). All animal experiments were performed in accordance with regulations of the animal welfare act and protocols approved by the Regierung von Oberbayern.

Generation and production of AAV vectors

We cloned and produced rAAV-STAT3 and Control rAAV as previously described (25). Additional details can be found in *SI Materials and Methods*. Genomic titers were as follows: rAAV-STAT3, 9×10^{12} genome copies/ml; Control rAAV-eCFP, 9.2×10^{12} genome copies/ml; Control rAAV-mbYFP, 8.6×10^{12} genome copies/ml.

Surgical procedures

We performed midthoracic hemisections and pyramidotomies and labelled CST fibers and propriospinal neurons as described in detail in *SI Materials and Methods*.

Tissue processing and histological analysis

Animals were perfused transcardially with 4% paraformaldehyde (PFA). Brains and spinal cords were dissected and postfixed overnight in PFA. The tissue was then cryoprotected in 30% sucrose for 3 days. Coronal sections (50 μ m thick) were cut on a cryostat. To visualize CST collaterals, BDA detection was performed as described in *SI Materials and Methods*.

Confocal microscopy and image processing

Stained sections were scanned using an Olympus FV1000 confocal microscope equipped with x20/0.85 and x60/1.42 oil immersion objectives. Image stacks were then later processed using the freeware ImageJ/Fiji (<http://rsbweb.nih.gov/ij>) to generate maximum intensity projections. To obtain final representations, these maximum intensity projections were further processed in Photoshop (Adobe) using gamma adjustments to enhance visibility of intermediate gray values.

Quantification of CST regeneration and remodelling

The extent of CST regeneration and remodelling was quantified as detailed in *SI Materials and Methods*.

Behavioral analysis and Electrophysiology

To assess behavioural recovery we monitored the BMS score and performed Food pellet grasping and the Rotarod tests as described in *SI Materials and Methods*. We stimulated the forelimb motor cortex and performed electrophysiological recordings as described in *SI Materials and Methods*.

Statistical evaluation

Data were analyzed by the Student's *t* test in case of comparisons of two groups or two-way ANOVA with Tukey's *post hoc* test in case of multiple comparisons using Graphpad Prism 5.01 for Windows (GraphPad Software).

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Figure Legends

Figure 1. Transient upregulation of p-STAT3 expression in cortical neurons does not contribute to endogenous CST regeneration and remodeling after injury.

(A,B) Confocal images of the expression of the activated form of STAT3, p-STAT3 in lamina V cortical neurons (green, NeuroTrace 435; red, p-STAT3) of an unlesioned mouse (A) and a mouse perfused 24hrs following a midthoracic hemisection (B). (C) Quantification of p-STAT3 expression in lamina V cortical neurons of unlesioned mice (white bar) and mice perfused at different timepoints following thoracic hemisection (grey bars). (D,E) Confocal images of p-STAT3 expression in lamina V cortical neurons (green, NeuroTrace 435; red, p-STAT3) of a STAT3 competent (D) and a conditional STAT3 deficient mouse (E) perfused 3wks following a midthoracic hemisection. (F) Quantification of p-STAT3 expression in lamina V cortical neurons in STAT3 competent and conditional STAT3 deficient mice perfused 3wks following a midthoracic hemisection. (G,H) Quantification of axonal regeneration at different distances distal from the lesion site (G) and of axonal sprouting (H) at the site of the lesion in STAT3 competent (grey bars) and conditional STAT3 deficient (blue bars) mice perfused 3 wks following midthoracic hemisection. (I,J) Quantification of axonal sprouting in the cervical spinal cord (I) and of the percentage of long propriospinal neurons contacted by CST fibers (J) in STAT3 competent (grey bars) and conditional STAT3 deficient (blue bars) mice perfused 3 wks

following thoracic hemisection. Scale bars equal 50 μ m B (also for A), 50 μ m in E (also for D).

Figure 2. Sustained STAT3 expression promotes axonal sprouting and regeneration following spinal cord injury.

(A) Schematic representation of the analysis of CST sprouting and regeneration following spinal cord injury. (B,C) Confocal images of p-STAT3 expression in lamina V cortical neurons (green, NeuroTrace 435; red, p-STAT3) of mice injected with Control rAAV (B) or rAAV-STAT3 (C) and perfused 3 wks following a midthoracic hemisection. (D) Quantification of p-STAT3 expression in lamina V cortical neurons of mice injected with Control rAAV (grey bars) or rAAV-STAT3 (red bars) and perfused 3wks following midthoracic hemisection. (E-H) Confocal image of a longitudinal section of the spinal cord (asterisk, indicates lesion site) illustrating sprouting and regeneration of the transected CST (BDA, white) in mice injected with rAAV-STAT3 (E-G) and in mice injected with Control rAAV (H). The dotted lines in (H) represent the distances at which regenerating CST axons were counted. Boxed areas in (E) are magnified 2-times in (F) and (G). (I,J) Quantification of axonal regeneration at different distances distal from the lesion site (I) and of axonal sprouting (J) at the site of the lesion in control (grey bars) and STAT3 overexpressing (red bars) mice perfused 3 wks following midthoracic hemisection. Scale bars equals 100 μ m in C (also for B). Scale bar in H (also for E) equals 60 μ m.

Figure 3. Sustained STAT3 expression induces sprouting of lesioned and unlesioned fibers in the cervical spinal cord following injury.

(A) Schematic representation of the analysis of cervical CST sprouting and remodeling following a midthoracic spinal cord injury. (B,C) Confocal images of sprouting hindlimb

CST collaterals in lesioned mice injected with Control rAAV (**B**) or rAAV-STAT3 (**C**) and perfused 3 wks following injury. (**D**) Quantification of the number of collaterals exiting the hindlimb CST tract in the cervical spinal cord in mice injected with Control rAAV (grey bar) or rAAV-STAT3 (red bars) 3 wks following spinal cord injury. (**E**) Confocal image (single plane) of a contact between a hindlimb CST collateral (red) and a long propriospinal neuron (green) in a mouse injected with rAAV-STAT3 and perfused 3 wks following the lesion. (**F**) Quantification of the percentage of long propriospinal neurons contacted by hindlimb CST collaterals 3 wks following the lesion (grey bar: mice injected with Control rAAV, red bar: mice injected with rAAV-STAT3). (**G**) Schematic representation of the analysis of cervical CST sprouting and remodeling in unlesioned mice. (**H,I**) Confocal images of sprouting hindlimb CST collaterals in unlesioned mice injected with Control rAAV (**H**) or rAAV-STAT3 (**I**). (**J**) Quantification of the number of collaterals exiting the hindlimb CST in the cervical spinal cord of unlesioned mice injected with Control rAAV (grey bar) or rAAV-STAT3 (red bar). (**K**) Confocal image (single plane) of a contact between a hindlimb CST collateral (red) and a long propriospinal neuron (green) in an unlesioned mouse injected with rAAV-STAT3. (**L**) Quantification of the percentage of long propriospinal neurons contacted by hindlimb CST collaterals in unlesioned mice (grey bars: mice injected with Control rAAV, red bars: mice injected with rAAV-STAT3)., Scale bar equals 50 μ m in **C** (also for **B**, **H**, **I**), 25 μ m in **E** (**K**).

Figure 4. Sustained STAT3 expression induces *de novo* formation of midline crossing circuits following pyramidotomy.

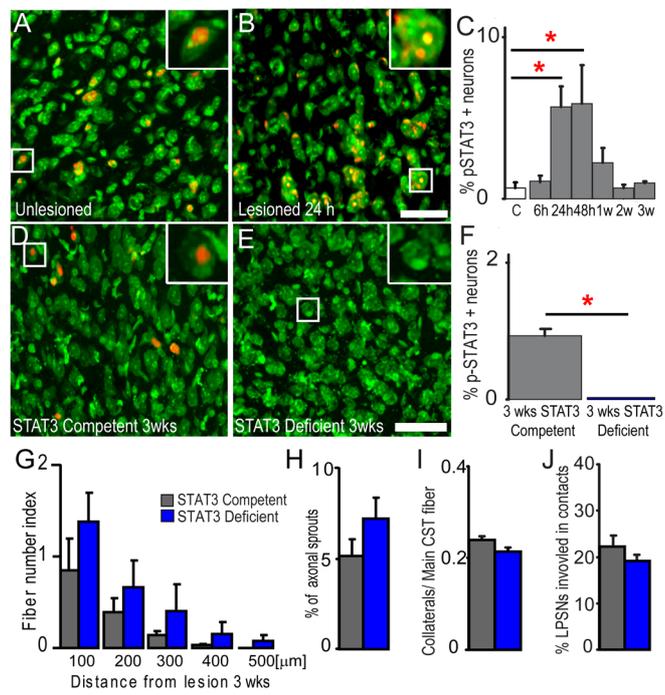
(**A**) Schematic representation of the analysis of CST remodeling after unilateral pyramidotomy and injection of Control rAAV or a rAAV-STAT3. (**B-D**) Confocal images of midline crossing fibers in mice injected with Control rAAV (**B**) or rAAV-STAT3 (**C**, **D**)

and perfused 3 wks (**B, C**) or 12 wks (**D**) following pyramidotomy. (**E, F**) Quantification of the number of fibers exiting ipsilateral from the main CST (**E**) and crossing the spinal midline (**F**) in mice injected with Control rAAV (grey bars) or rAAV-STAT3 (red bars) and perfused 1, 3 or 12 wks following pyramidotomy. (**G**) Quantification of the percentage of midline crossing fibers that project to the contralateral (denervated) laminae VI to IX in mice injected with Control rAAV (grey bars) or rAAV-STAT3 (red bars) and perfused 3 and 12 weeks following pyramidotomy. (**H**) Quantification of the density of midline crossing fibers in the contralateral (denervated) side of the spinal cord at different distances from the midline in mice injected with Control rAAV (grey line) or rAAV-STAT3 (red line) and perfused 12 weeks following pyramidotomy. (**I, K**) Confocal images (single planes) of contacts between midline crossing forelimb CST collaterals (red) and a short propriospinal neuron (**I**, green) or a motoneurons (**K**, green) in mice injected with rAAV-STAT3 and perfused 12 wks following the pyramidotomy. (**J, L**) Quantification of the percentage of short propriospinal neurons (**J**) and motoneurons (**L**) contacted by midline crossing forelimb CST collaterals in mice injected with Control rAAV (grey bars) or rAAV-STAT3 (red bars) and perfused 12wks following injury. Scale bars equal 200 μm in D (also for B, C), 50 μm in K (also for I).

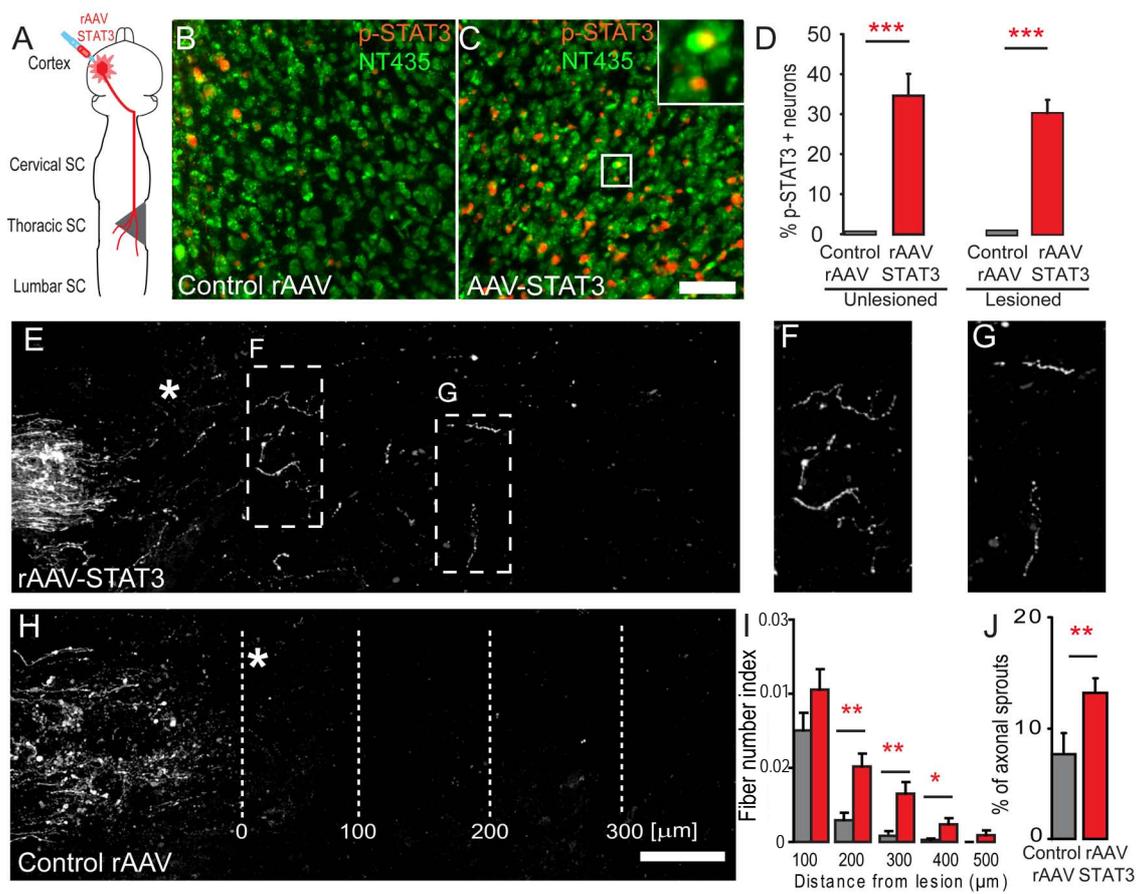
Figure 5. Sustained STAT3 expression promotes functional recovery following pyramidotomy.

(**A**) Picture of a mouse in a “staircase test” which we used to evaluate forelimb reaching and grasping abilities in mice. (**B**) Quantification of the number of pellets eaten by mice injected with Control rAAV (grey line) or rAAV-STAT3 (red line) at different test intervals up to 10 weeks following pyramidotomy. (**C**) Schematic representation of the cortical electrostimulation and EMG recording that we used to quantify circuit

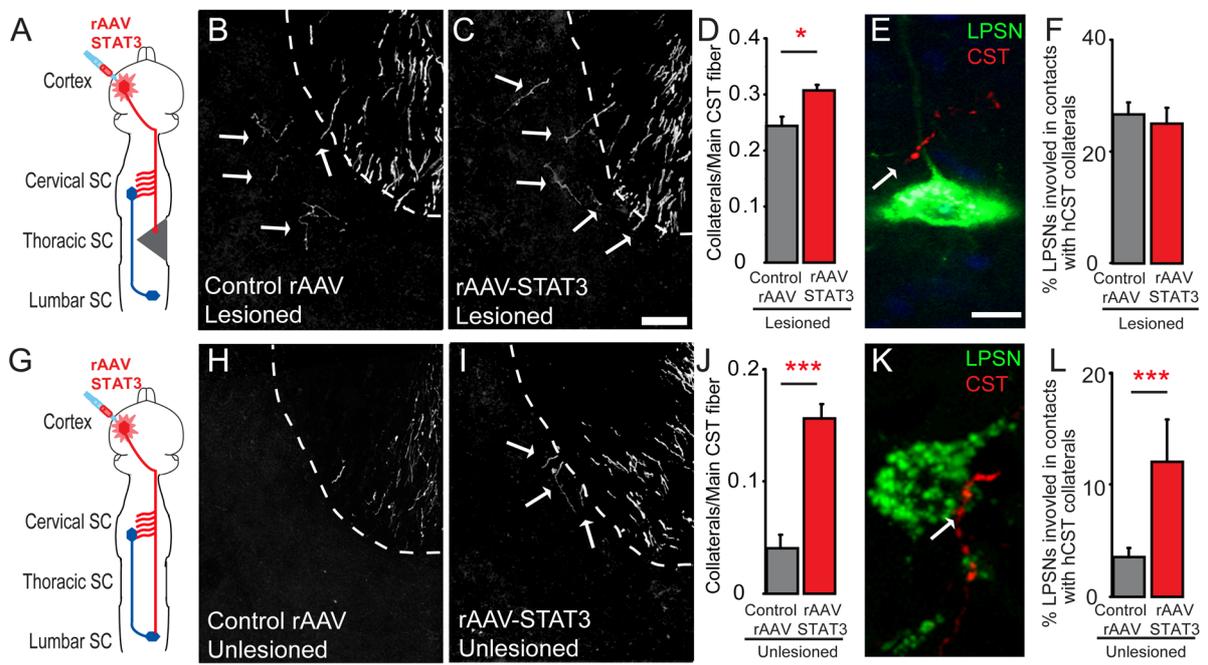
reconnection after pyramidotomy. **(D)** Trace of a forelimb EMG recording after cortical stimulation. **(E)** Quantification of the percentage of responsive sites contralateral to the lesion in unlesioned mice (black bar) and ipsilateral to the lesion acutely following pyramidotomy (white bar) and 12 wks following pyramidotomy in mice injected with Control rAAV (grey bar) or rAAV-STAT3 (red bar). A second pyramidotomy 12 weeks following pyramidotomy and injection of rAAV-STAT3n abolishes the ipsilateral responses (red bar, re-lesion). **(F, G)** Quantification of the stimulation thresholds **(F)** and latencies **(G)** of the forelimb responses in unlesioned mice (black bars, contralateral to lesion) in mice injected with Control rAAV (grey bar, ipsilateral to the lesion) or rAAV-STAT3 (red bar, ipsilateral to the lesion) at 12 weeks following pyramidotomy.



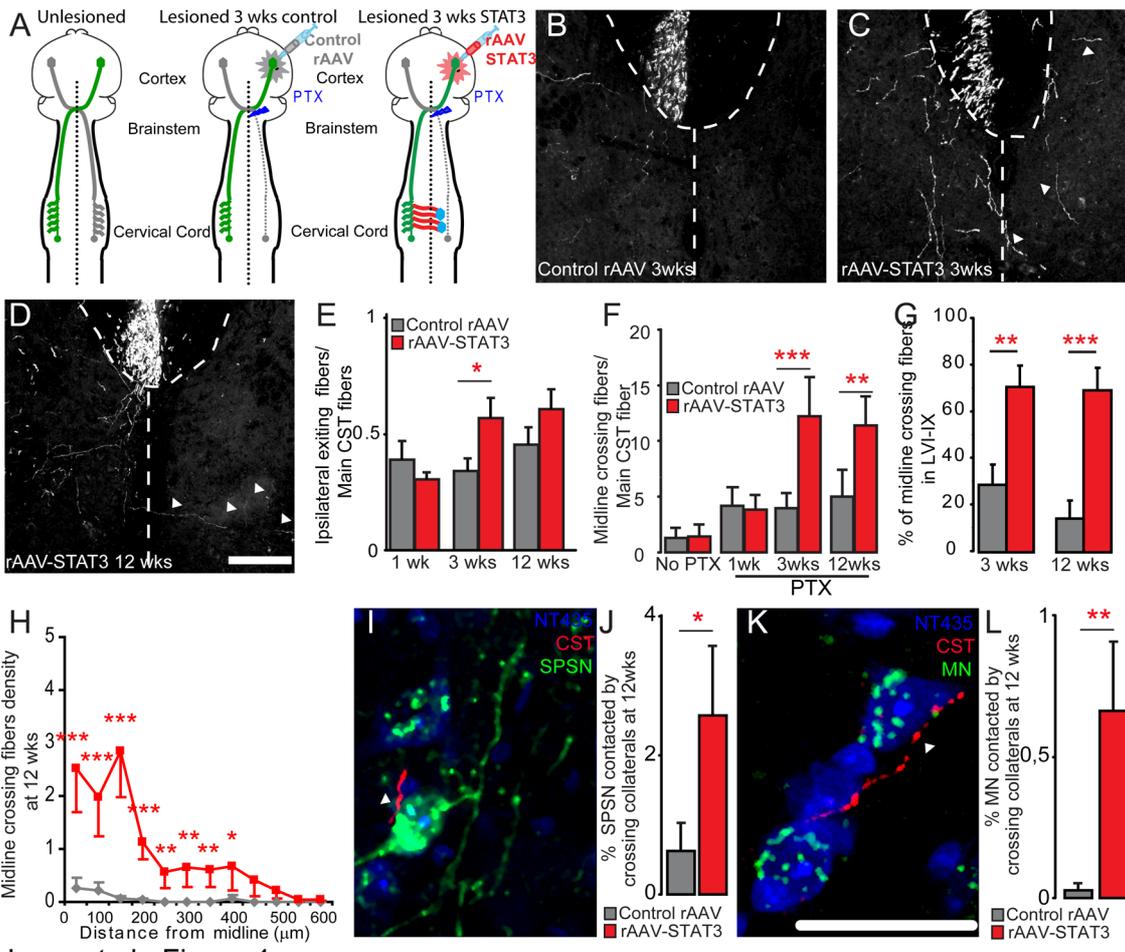
Lang et al., Figure 1



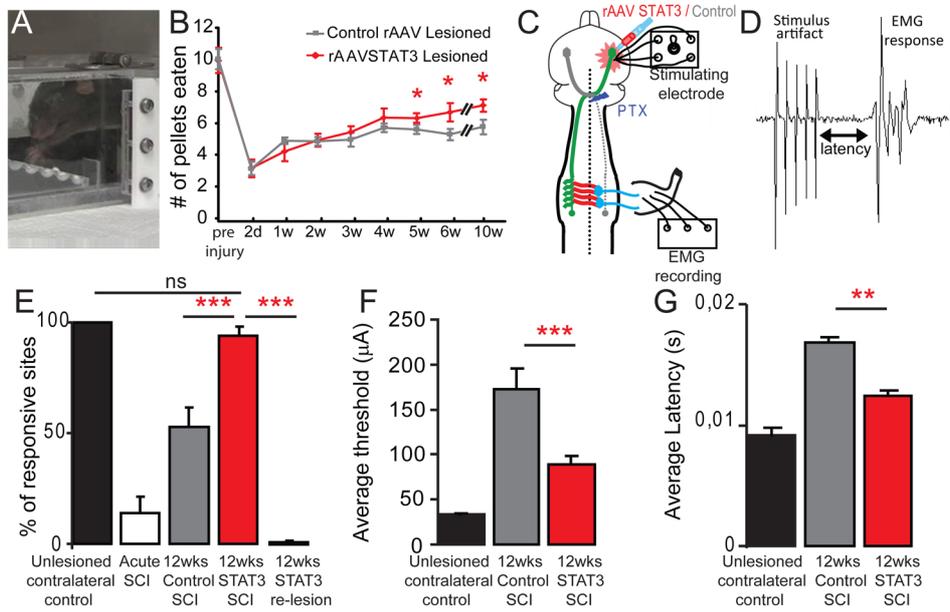
Lang et al., Figure 2



Lang et al., Figure 3



Lang et al., Figure 4



Lang et al., Figure 5

Discussion

4.1. Structural remodeling of corticospinal tract collaterals

4.1.1 Summary of key findings

Following a dorsal hemisection, the corticospinal tract (CST) sprouts newly formed collaterals and remodels its axons rostral from the lesion site. The ability of the CST tract to remodel its connections to form new intraspinal detour circuits is a key feature in enabling spontaneous functional recovery following injury (Bareyre et al., 2004; Courtine et al., 2008).

The reconstruction of single CST collaterals at different time points after injury allowed us to reveal that axonal remodeling occurs through three orchestrated phases (Figure 7; Lang et al., 2012):

- (1) A growth initiation phase, where there is growth of newly formed collaterals that are simple in structure (10 days following injury),
- (2) A collateral formation phase, where collaterals develop a complex structure (occurs between 10 days-4 weeks after injury),
- (3) A maturation phase where there is refinement and maturation of synaptic connections onto spinal interneurons (occurs 3-12 weeks after injury).

Throughout these phases, newly formed collaterals undergo structural and synaptic changes before reaching a mature projection pattern. Once they reach a mature pattern, newly formed CST collaterals persist long-term for at least up to 6 months after injury. The reconstruction of single collaterals originating from different CST components further revealed that although the timing of the remodeling phases is similar between the main and minor CST components, their structural complexity differed depending on their tract of origin.

In this study we were able to provide a detailed anatomical analysis and the time course of CST remodeling following injury. This improved understanding of the characteristics of the different phases will aid in the design and timing of therapeutic interventions that specifically target the individual phases of the remodeling process.

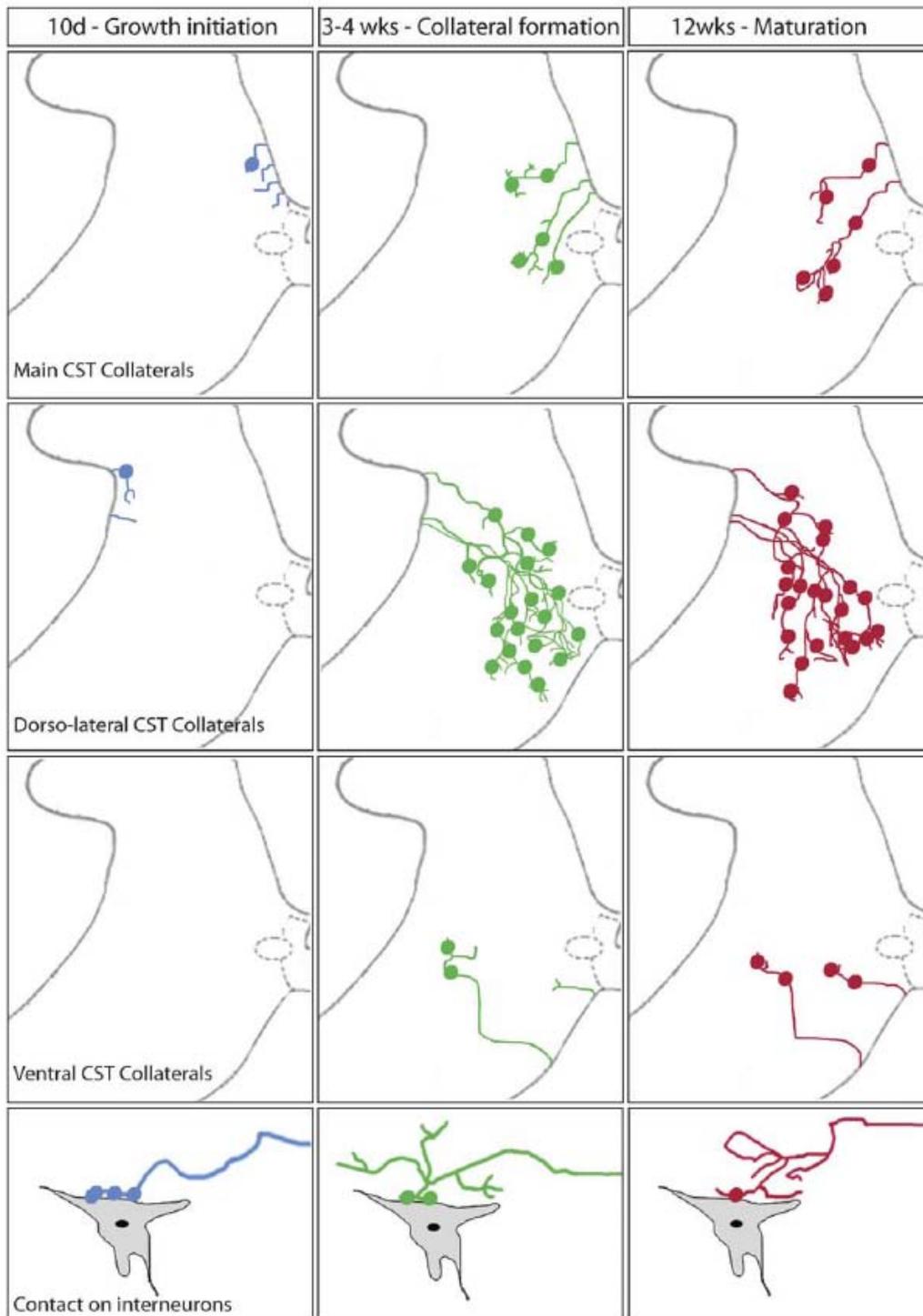


Figure 7. Schematic representation of hindlimb CST remodeling following SCI. Scheme illustrating the formation of cervical collaterals derived from the main CST (upper row) and the minor dorsolateral (2nd row) and ventral (3rd row) CST components at 10 days (blue), 3–4 weeks (green) and 12 weeks (red) after SCI. Bottom row illustrates the refinement over time of the contacts between CST collaterals and cervical interneurons (Taken from Lang et al., 2012; doi:10.1371/journal.pone.0030461.g006)

4.1.2 Stages of collateral remodeling

A key feature of CST remodeling that emerged from our analysis is that initially extensive collaterals and contacts are established. However over time only some of these contacts and collaterals are maintained while others are eliminated. This maturation and refinement of the circuit following injury is reminiscent of similar mechanisms that occur during development. For example, during development, in systems such as the visual system (Sretavan and Shatz 1986), auditory system (Leake et al., 2002) and the neocortex (Portera-Cailliau et al., 2005), there is an excess of immature axonal projections. This is then followed by a refinement phase which eventually leads to a mature adult topographical pattern. In the developing CST, aberrant sprouts are also formed though these are then later pruned during maturation (Stanfield et al., 1982; Stanfield and O'Leary 1985; Chakrabarty and Martin 2000; Li and Martin 2002), indicating that this process is a common feature necessary for circuit formation. Molecules such as semphorins/plexins (Bagri et al., 2003; Low et al., 2008) and ephrins (O'Leary and Wilkinson 1999) have been implicated to be players in this refinement process. In our study we found that the injured CST undergoes a similar process of sprouting followed by pruning, which raises the possibility that similar molecular mechanisms may also regulate the refinement of neuronal circuits after injury.

Our work further shows that during CST remodeling that not only the number of CST collaterals but also the CST contact pattern on interneurons evolves over time. Collaterals formed 10 days post injury emerging from all tracts were short in length and had very few boutons and branch points. These collaterals were immature in both structure and the synaptic machinery present in their boutons (Lang et al., 2012). Comparative observations from our population study revealed that at 10 days post injury, approximately 10% of newly formed collaterals formed more than 3 contacts onto one interneuronal cell body, with around 38% observed to form only one contact. At a late time point when a mature circuit is established, 12 weeks post injury, 80% of collaterals were found to make one contact onto a single neuronal cell body (Figure 7), while the remaining 20% were observed to form a maximum of 2 contacts. A majority of the CST collaterals boutons at this time point expressed the mature synaptic marker synapsin I (Lang et al., 2012). We considered this a "mature" contact pattern as a comparable synaptic marker expression was observed in the lumbar cord of unlesioned mice. Monosynaptic input appears to be the typical form of innervation as this is reminiscent of the innervation pattern at the neuromuscular junction. Here in this system, initially multiple axons innervate one junction. Competition between the

axons then occurs and in the end only a single input remains (Balice-Gordon and Lichtman 1993; Balice-Gordon et al., 1993).

The mechanisms that determine which inputs are maintained and which are removed during CST remodeling after injury are currently only partly understood. In the developing nervous systems, where initial connections made by invading axons are often inaccurate, neural activity has emerged as the deciding factor in the formation of a precise neural circuit (Shatz 1996; Chakrabarty and Martin 2010; Kerschensteiner et al., 2009; Morgan et al., 2011). In the case of the CST, it has been shown that inhibition of activity with an intramuscular injection of botulinum toxin A, which causes muscle paralysis, results in the development of immature CST axons (Martin et al., 2004). At maturity, CST axons are known to be morphologically complex when compared to the immature CST axons seen in development (Chakrabarty and Martin 2000; Li and Martin 2002). The complex structure of the mature CST axons, mainly due to an increase in the number of branches, has been noted to coincide with the establishment of the cortical motor map (Li and Martin 2002). To produce the mature projection pattern that is required for skilled movements, incorrect CST connections are eliminated and essential functional connections are maintained. In the CST system, motor experiences are known to be important for the development of CST terminations and function (Martin et al., 2004; Chakrabarty et al., 2009; Chakrabarty and Martin 2010).

In general, synapse formation and elimination appears to be a recurrent occurrence throughout the lifetime of an animal as even the adult system, in response to injury can undergo cell type-specific plasticity and rearrangements. The observed parallels between post-injury remodeling and developmental circuit formation suggest that a combination of guidance cues and activity-dependent mechanisms governs the formation and refinement of specific connections during corticospinal remodeling (Martin et al., 1999; Martin et al., 2000; Li and Martin 2002). Collectively, the combination influences the permanency of a collateral, its structure, a synapse, and synaptic strength (Colicos et al., 2001; Nikonenko et al., 2003; De Paola et al., 2003; De Paola et al., 2006).

4.1.3 Contribution of main and minor CST components

At maturation, the newly formed collaterals emerging from the different components of the CST, the main dorsal and the minor dorsolateral and ventral components, have distinct structures (Lang et al., 2012). Collaterals sprouting from different white matter locations displayed differences in branching and bouton number, suggesting that their contacts and their role in the *de novo* neural circuit may differ (Lang et al., 2012). Although the signals that govern the distinct structure of CST collaterals are unknown, one explanation is that structure is related to function. Newly sprouted collaterals emerging from different anatomical locations would have access to distinct interneuronal pools. Dorsolateral and main CST collaterals with their complex branching patterns are able to project into a larger area of the spinal grey matter, mainly into the intermediate and ventral horn near the vicinity of propriospinal interneurons and motoneurons (Steward et al., 2004). In contrast, the projection field of the simple-in-structure ventral CST collaterals is more confined to the region where the motoneurons are located. Given their extensive projection pattern, newly formed main and dorsolateral CST collaterals after lesion are likely to have a more prominent role in the formation of the detour circuit. In particular, the contribution from the main component is more significant as this component sprouts the majority of fibers, followed by the dorsolateral component. As a result we found that collaterals emerging from the main and to a lesser extent from the dorsolateral CST, were responsible for the majority of contacts onto spinal interneurons. The complex structure of dorsolateral and main CST collaterals could be a requirement for creating strong functional connections and for the integration of different interneuronal pools to create an operational communicative neural circuit to enable the CST to regain control of skilled limb movement.

This raises questions about the simple ventral CST collaterals and their role in the new neural circuit. While their simple structure might suggest an insignificant role, the following findings suggest that ventral CST collaterals also play an important role during the remodeling process. First, despite the fact that the ventral CST fibers are unlesioned in our model system they sprout, relative to the tract size, a high number of new collaterals. Second, previous work in rats has shown that the collaterals that sprout from this tract are important for functional recovery. Weidner et al., (2001) found that when the ventral CST component was lesioned there was no sprouting or functional recovery. Furthermore, lesion of the ventral component (at cervical level C2) 5 weeks after a dorsal column transection (at cervical level C3), eliminated the functional recovery that was previously observed with only

a dorsal lesion. In addition it was reported that the proportion of fibers remaining in the ventral or lateral funiculus after a T8 lesion in rats was proportional to the open field locomotor score (Schucht et al., 2002).

4.1.4 Axonal remodeling following SCI and the implications for functional recovery and therapy

The ability of newly formed CST collaterals for spontaneous axonal remodeling is a key mechanism that mediates functional recovery after spinal cord injury. Exploiting the natural tendency for collaterals to form compensatory detour circuits is an attractive strategy in terms of enhancing recovery following CNS lesions. Bareyre et al., (2004), illustrated that following a dorsal thoracic lesion, CST collaterals remodel their connections rostral from the lesion site. Contacts onto interneuronal pools that are not beneficial were lost while functional contacts were strengthened. Comprehensive behavioral and electrophysiological assessments validated that the CST remodeling, observed in this study, is a crucial step for the recovery of CST function (Bareyre et al., 2004).

The CST is not the only tract that is known to spontaneously remodel following injury. Studies have observed that other tract systems such as the rubrospinal and reticulospinal tract and the serotonergic circuit respond to injury. For example, the rubrospinal tract that cooperates with the corticospinal tract in controlling fine skilled motor movements (Whishaw et al., 1998) has been observed to sprout in the spinal cord following a CST transection at the brainstem (Raineteau et al., 2001). The reorganization of reticulospinal axons and the re-establishment of serotonergic activity can also contribute to functional recovery after injury. The reticulospinal tract (RST) has its axons running in the lateral and ventral white matter and is often spared in a contusion lesion (Hill et al., 2001). The RST tract, which is involved in the initiation of walking, has been observed to sprout below the injury site (Ballermann and Fouad 2006). Spinal motor neurons require neuromodulators such as serotonin (5-HT) for the generation of rhythmic movement (Jordan et al., 2008; Fouad et al., 2010). Constitutive serotonergic activity following SCI can be beneficial in terms of enabling motorneuron recovery and hence walking. However there are detrimental effects should there be no cortical regulation as constant expression of serotonergic activity can lead to spasms (Murray et al., 2010).

Remodeling processes are likely not limited to descending tract systems as circuit refinement can also happen at the level of the spinal interneurons. Proprio-spinal interneurons are part of the central pattern generator (CPG). The CPG is a self-sustained spinal motor pattern able to produce neural activity that is modulated by sensory afferent and supraspinal commands (Grillner and Wallen 1985). Courtine et al., (2008) found that elimination of proprio-spinal neurons causes a loss of functional recovery after injury. Using a staggered hemisection model they could show that when the proprio-spinal circuit was destroyed all recovered movement was abolished. Additional studies show that the CPG as a whole can spontaneously adapt and reorganize its connections following injury (Lovely et al., 1989; Rossignol et al., 1999; de Leon et al., 1999; Edgerton et al., 2004). For example in cats, when the supraspinal input is cut and the muscles are paralyzed, thereby depriving the cord of sensory cues, the animals can still be trained to walk on a treadmill. Continuous locomotor training further improves the stepping pattern. Cats with extensive treadmill training for 12 weeks are capable of full weight support and efficient stepping (Hodgson et al., 1994), demonstrating that the CPG circuits responds to task related plasticity (Raineteau and Schwab 2001). However without constant training the cats have poor locomotor performance, though with retraining the stepping ability could be relearned (Hodgson et al., 1994). In relation to humans, functional MRI studies have revealed that the supraspinal locomotor control between humans and quadruped animals is similar (Jahn et al., 2008; Fillingim et al., 2011). CPG circuits are also known to exist in humans, and can be therapeutically targeted to improve the recovery of SCI injured patients (Dietz et al., 1994; Barrière et al., 2008; van Hedel and Dietz 2010).

Extensive reorganization following SCI not only occurs in the spinal cord, it additionally transpires in the cortex. Intact areas of the cortex have been found to expand into de-afferented regions with various methods including intracortical microstimulation (Fouad et al., 2001; Emerick et al., 2003; Martinez et al., 2009), electrophysiology (Aguilar et al., 2010), trans-synaptic tracing (Bareyre et al., 2004), functional magnetic resonance imaging (fMRI) (Endo et al., 2007; Ghosh et al., 2009; Nishimura and Isa 2009; Ghosh et al., 2010), voltage-sensitive dye imaging (VSD) (Ghosh et al., 2009; Ghosh et al., 2010) and retrograde tracing (Ghosh et al., 2010). For example, Ghosh et al., (2010) found through VSD and BOLDfMRI imaging techniques that following a thoracic lesion there is expansion of the forelimb sensory representation into the affected hindlimb sensorimotor area. Input from the forelimb region could be a form of compensation or due to extensive reliance on the forelimb after injury. Even the human brain has been reported to undergo such

reorganization, utilizing these newly formed connections to regain functional recovery (Freund et al., 2011; Lu et al., 2011).

Taken together, extensive axonal remodeling processes can be observed on multiple anatomical levels and likely form the basis for the spontaneous improvement in motor, sensory or other neurological function that can be observed in approximately 40% of SCI patients (Bracken et al., 1992; Frankel 1998; Weidner et al., 2001; Freund et al., 2011). Despite the fact that there is some spontaneous remodeling, in most cases functional impairments still persist following CNS injuries. The further support of axonal remodeling processes is thus an attractive target for therapeutic strategies. The improved understanding of the remodeling process can now help to guide the development of new therapeutic strategies. Our investigations revealed that remodeling occurs in timely phases and these phases offer windows of opportunities to influence the remodeling process. For example, to improve growth, one would target treatment within the first 10 days following lesion. Alternatively interventions that aim to modulate connectivity would be effective 3 weeks following injury.

4.2. Enhancing and modulating axonal outgrowth and remodeling

4.2.1 Summary of key findings

In the first study we investigated the role of STAT3 during the regeneration of lesioned peripheral and central DRG branches (Bareyre et al., 2011). The main findings of this study were:

- Sustained STAT3 expression is induced by a peripheral but not a central lesion. Selective deletion of STAT3 impairs the regeneration of the peripheral branch following injury. While viral gene transfer of STAT3 promoted axonal outgrowth and sprouting of the DRG central branch.
- *In vivo* time-lapse imaging revealed that STAT3 is a phase-specific regulator of the intrinsic growth program and that it is essential for the initiation of axonal regeneration but does not affect axonal elongation.

In a second study we then examined whether and how the induction of STAT3 expression can be used to improve axonal regeneration, remodeling and functional recovery after spinal cord injury (Lang et al., Chapter 3 submitted). The main findings of this study were:

- Following spinal cord injury, STAT3 expression in cortical projection neurons is only transiently induced and the conditional deletion of this expression affects neither spontaneous regeneration nor remodeling of CST axons.
- Sustained expression and activation of STAT3 in cortical projection neurons can be induced by viral gene transfer leading to improved regeneration and remodeling of lesioned CST axons.
- The forced upregulation of STAT3 can recruit unlesioned CST fibers to the remodeling process. In a unilateral CST lesion (pyramidotomy) paradigm, sustained STAT3 expression triggers the formation of new collaterals that emerge from the unlesioned tract. These collaterals were observed to cross the spinal midline and contact interneurons and motoneurons on the denervated side of the spinal cord (Figure 8). Moreover, behavioral and

electrophysiological assessments validated that a new intraspinal circuit is formed that contributes to the improved recovery of the impaired forelimb.

In summary, the results from these studies identify STAT3 as a key regulator of axonal growth initiation and demonstrate that the induction of sustained STAT3 expression, for example through viral gene transfer, is a promising strategy to enhance axonal regeneration, remodeling and functional recovery after spinal cord injury.

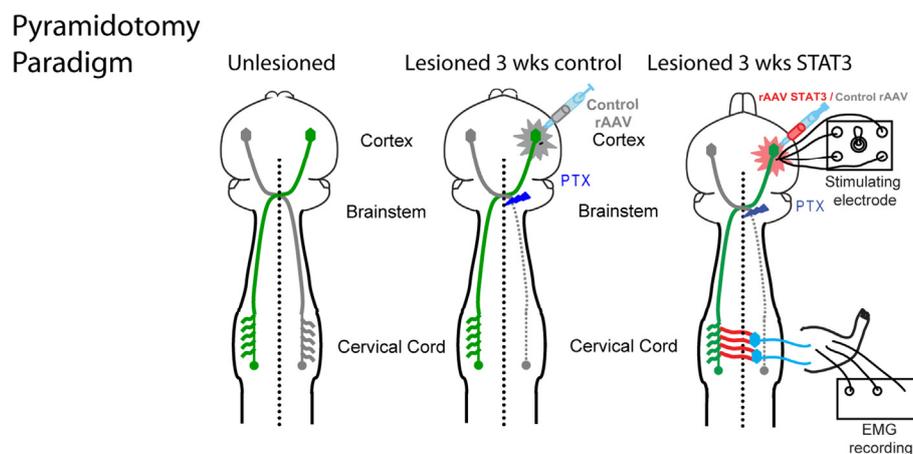


Figure 8. Schematic representation of the pyramidotomy paradigm used in this experiment in conjunction with treatment using a control rAAV or a rAAV-STAT3. STAT3 overexpression induces compensatory midline sprouting of fibers from the unlesioned tract following a pyramidotomy lesion. (adapted from Lang et al., Chapter Three, manuscript submitted)

4.2.2 STAT3 as an initiator of the intrinsic neuronal growth program

Peripheral and central injuries can trigger differential changes in gene expression, hence explaining the difference in regenerative responses (Broude et al., 1997, Herdegen et al., 1997, Herdegen and Leah 1998, Schwaiger et al., 2000, Sheu et al., 2000, Snider et al., 2002, Kruse et al., 2011, Mason et al., 2011). Regenerative associated genes (RAGs) and transcription factors are found to be upregulated in regenerating PNS neurons after a peripheral nerve injury (Richardson and Verge 1987, Mason et al., 2002). STAT3 is a regenerative associated transcription factor part of the intrinsic growth program present in a neuron. Injury to the peripheral branch of the DRG sensory neuron results in the increased expression of activated STAT3, even at two weeks post injury, and the activation of the regeneration program (Neumann and Woolf 1999; Qiu et al., 2005; Bareyre et al., 2011). The conditional deletion of STAT3 in the peripheral branch was found to only delay the regenerative response, thereby verifying the role of this transcription factor in the intrinsic neuronal growth program as an initiator (Bareyre et al., 2011). STAT3 was revealed to have a significant regulatory role in the PNS following injury, as the endogenous expression and activation of the transcription factor can influence the initiation of axonal growth.

In the CNS, the intrinsic growth machinery has been shown to be activated in CNS neurons after axotomy (Richardson and Verge 1987; Benowitz and Routtenberg 1997; Smith et al., 2011), and in CNS neurons that have axons regenerating through a peripheral nerve graft (Benfey et al., 1985). However due to inhibitory factors and feedback pathways, the effect is only transient (Sun and He 2010; Kruse et al., 2011). In upper cortical neurons, following lesion, there is only a transient upregulation of activated STAT3 with return to baseline levels one week following injury (Lang et al., Chapter Three submitted). This is due to the fact that STAT3 activation is tightly regulated and controlled by SOCS3, which itself is also upregulated after injury (Crocker et al., 2008; Smith et al., 2009). The lack of sustained activation of factors in this program in injured mature CNS neurons is one of the main reasons why regeneration in the PNS is successful, while in the CNS there is failure. This is in accordance with not only our studies but with others who have found impaired growth and regeneration when STAT3 levels were indirectly negatively affected via pharmacological means. Inhibition of the pathway with a Janus kinase 2 (JAK2) inhibitor AG490 (Qiu et al., 2005) or by blockade of IL6 signaling, in essence inhibiting activation of the Jak/STAT3 pathway (Zhong et al., 1999; Cafferty et al., 2001) resulted in regeneration failure of 'conditioned' dorsal column axons, which usually have regenerative capabilities. Alternatively, a number of studies, ours included, have found that sustained activation of

STAT3 either by genetic deletion of its inhibitor SOCS3 (Smith et al., 2009; Sun et al., 2011) or through an rAAV-STAT3 (Bareyre et al., 2011; Lang et al., Chapter Three submitted) can promote axonal growth and regeneration after a CNS lesion.

The upregulation of STAT3 following injury indicates that it is required for inducing axonal growth, but its deletion will not affect the spontaneous recovery system that the injured CST has in place in the form of spontaneous axonal remodeling and the formation of *de novo* detour circuits. The presence of axonal growth in both the PNS and CNS when STAT3 is deleted (Bareyre et al., 2011; Lang et al., Chapter Three submitted) indicates that there are other systems able to compensate for its deletion. Therefore there are likely to be substitute candidate factors that are able to initiate the growth response in the absence of STAT3. Members of the PTEN/mTOR pathway (Park et al., 2010; Liu et al., 2010) are potential candidates as they have been reported to influence the intrinsic growth response. Another such potential candidate is the cAMP signaling pathway, which is important for neuronal growth (McQuarrie et al., 1977; Qiu et al., 2000; Cai et al., 2001; Qiu et al., 2002). cAMP levels have been found to be up-regulated after sciatic nerve transection and administration of cAMP into the DRG can promote the regeneration of dorsal column axons (Neumann et al., 2002; Qiu et al., 2002).

Additionally, the identification of STAT3 as a phase-specific regulator that only affects growth initiation implies that there are distinct phases of axonal growth modulated by different factors. What are the possible regulators that will switch the axon from a growing state to an elongation state? Axonal elongation, for example, could be modulated by the interaction with Schwann cells, which following injury align to form tubes and are able to guide axons to their target cells (Höke 2006). Potentially, the Schwann cells are responsible for the shift from the growth phase to the elongation phase. In a crush injury, which leaves Schwann cell guidance intact, the absence of STAT3 has no effect on the elongation of injured PNS axons. This suggests that once axons enter the axonal elongation phase, STAT3 is no longer required. Nevertheless, sustained activation of pathways that enhance and promote growth, such as STAT3, in selective neuronal populations is a promising strategy that can further promote functional recovery. Indeed our studies (Bareyre et al., 2011, Lang et al., Chapter Three manuscript submitted) found that direct overexpression of STAT3 through gene therapy is a strong therapeutic strategy that can trigger further growth, remodeling, regeneration compensatory sprouting and functional recovery following CNS lesions.

4.2.3 Integration of STAT3 into the signaling pathways that regulate neuronal growth

How STAT3 initiates the neuronal growth program is an interesting question that is so far still only incompletely understood. However, as STAT3 is a transcription factor it is likely that the downstream effects are mediated by induction of gene expression. A large number of genes that are affected by STAT3 have already been identified. The small proline rich protein 1a (SPRR1A), the cell cycle inhibitor P21/Cip1/ and the regulatory factor 1 (IRF1) are known to be downstream targets of STAT3 (Coqueret and Gascan 200; Pradervand et al., 2004; Smith et al., 2011). All of these molecules have also been implicated as regulators of axonal regeneration (Bonilla et al., 2002; Tanaka et al., 2004; Smith et al., 2011). For example, in cultured cerebellar neurons, IRF1 was found to increase neuronal outgrowth (Smith et al., 2011). In addition to these downstream targets, STAT3 also increases the expression of immediate early gene c-Jun. C-Jun expression has been previously identified as a key step in the induction of axonal regeneration and mice that lack c-Jun expression in the nervous system have impaired regeneration of motoneurons after injury (Raivich et al., 2004).

Another important question is which factors are responsible for the activation of STAT3 after injury. Referring to the Jak/STAT pathway, we know that the pathway is activated by cytokines that include CNTF (Rajan et al., 1996), IL-6 (Zhong et al., 1994) and LIF (Kunisada et al., 1996). Following injury the expression of these neuropoetic cytokines factors are increased at the lesion site and can influence STAT3 signaling (Bourde et al., 1996; Kurek et al., 1996). At the lesion site, the secretion of cytokines would lead to the activation of STAT3, which is then transported retrogradely along the axon (Curtis et al., 1994; Lee et al, 2004; Ben-Yaakov et al., 2012). Preventing expression of these classical inducers of the Jak/STAT pathway, results in impaired axonal regeneration and functional recovery (Zhong et al., 1994; Cafferty et al., 2001; Cafferty et al., 2004). Overall these studies indicate that the activation of STAT3 by cytokines is a key step in activating the intrinsic growth program and thus facilitating axonal regeneration.

Furthermore it appears that STAT3 expression can also be affected by classical neurotrophin signaling. In particular the interaction between STAT3 and BDNF has been reported (McAllister et al., 1999; Bramham and Messaoudi 2005; Ng et al., 2006). Knockout of STAT3 by siRNA in hippocampal cells decreases the neurite outgrowth effect that BDNF

can elicit (Bramham and Messaoudi 2005). In PC12 cells, crosstalk between the BDNF and STAT3 signaling pathways was observed with STAT3 acting as a signal transducer for tyrosine kinase A (TrkA). STAT3 activation is downstream from TrkA activation, with TrkA able to phosphorylate STAT3 at Ser-727 thereby enhancing transcription (Ng et al., 2006).

In addition it is likely that other intracellular signaling pathways of the intrinsic neuronal growth program can interact with STAT3. As mentioned previously, Liu et al., (2010) and Yip, et al., (2010) illustrated that through genetic manipulation that leads to the inactivation of PTEN (Liu et al., 2010) or the overexpression of NCS1 (Yip et al., 2010), it is possible to induce axonal growth and regeneration. These experiments identified PTEN and NCS1 as key components of the intrinsic neuronal growth response. Inactivation of PTEN is known to activate Akt and mTOR signaling and inhibit signaling molecules such as GSK-3 (Zhou et al., 2004; Ma and Blenis 2009; Park et al., 2010; Liu et al., 2010) and PIP3 (Zhao et al., 2006). The Akt pathway provides a link between PTEN signaling and NCS1 as it has been shown that overexpression of NCS1 increases the levels of phosphorylated Akt, thereby activating the pathway (Yip et al., 2010; Figure 8). The activation of the PI3K/Akt pathway is associated with enhanced neurite outgrowth in the DRG, perinatal cortical neurons (Markus et al., 2002; Ozdinler and Macklis 2006) and embryonic cortical neurons (Nakamura et al., 2006). Notably, STAT3 has also been reported to interact with the Akt pathway via PI3K (Figure 8; Park et al., 2006; Lu et al., 2008; Blando et al., 2011). In cell culture experiments, when STAT3 is deleted there is reduced expression of Akt (Park et al., 2006; Lu et al., 2008). Further, in cancer cells interaction between the Jak/STAT3 and PTEN signaling pathways has been observed (Sun and Steinberg 2002; Yang et al., 2003; Saxena et al., 2007; Zhou et al., 2007; Blando et al., 2011). The Jak/STAT3 pathway was found to be positively regulated by PI3K/mTOR signaling, whereas PTEN served as a negative regulator of both Jak/STAT3 and mTOR signaling (Zhou et al., 2007). Sun et al., (2011) found that PTEN and SOCS3 independently regulate two different pathways that can act synergistically to promote axonal regeneration. Double deletion of the STAT3 inhibitor pathway, SOCS3, and PTEN resulted in enhanced and sustained axonal regeneration in the optic nerve, compared to a single deletion of either factor (Figure 8).

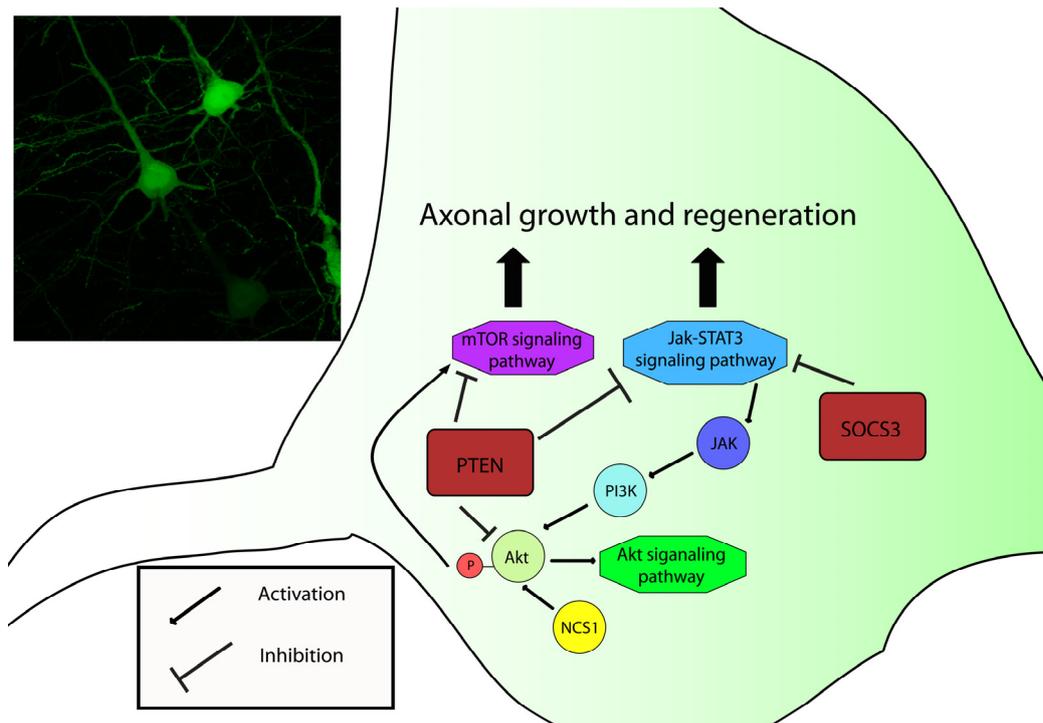


Figure 9. Simplified scheme of selected pathways that have been identified to be important for enhancing axonal growth following injury. Overexpression of neuronal calcium sensor 1 (NCS1) increases the levels of phosphorylated Akt, a serine/threonine protein kinase. Activation of the Akt signaling pathway is known to increase neurite outgrowth. Inhibition of PTEN, which functions both as a dual specificity protein phosphatase and an inositol phospholipid, activates the Akt, and mTOR signaling pathway. PTEN is also known to negatively regulate the Jak/STAT3 signaling pathway. Deletion of both SOCS3 (an inhibitor of the Jak/STAT3 pathway) and PTEN was found to enhance axonal regeneration and growth in the optic nerve. While a single knockout of either will still result in axonal growth and regeneration following injury, the effect is not as great. Insert is of a layer 5 pyramidal cortical neuron.

4.2.4 STAT3 as a therapeutic target

Our results identify STAT3 as an attractive target for therapeutic strategies that aim at improving functional recovery after spinal cord injury. One resulting action of sustained STAT3 expression is that there is the induction of sprouting of lesioned CST axons at the lesion site, as well as regeneration up to 400 μ m into the lesion site (Lang et al., Chapter Three submitted). The finding that STAT3 can induce regeneration of corticospinal fibers is important as the CST projection has a reputation of being one of the difficult axonal systems to experimentally elicit regeneration (Blesch and Tuszynski 2009). Factors and proteins that elicit regeneration in other tracts may not have an effect on the CST. For example, insulin-like growth factor-1 (IGF-1) or BDNF are able to promote regeneration of raphespinal axons or rubrospinal axons respectively but not of corticospinal axons (Kobayashi et al., 1997; Lu et al., 2001; Jin et al., 2002; Liu et al., 2002; Kwon et al., 2002; Hollis et al., 2009). However

it should be noted that although the treatment of STAT3 resulted in the regeneration of CST fibers up to 400µm from the site of the lesion, long-distance regeneration that extends substantially past the lesion site was not observed. This indicates that even though the stimulation of growth can push CST neurons into a regenerative state, alone it is still not enough to enable successful regeneration across the lesion site. The failure of successful long-distance regeneration is due in part to the inhibitory environment formed after injury. It is difficult to override the inhibitory environment with the presence of growth inhibitory molecules that include chondroitin sulphate proteoglycans (CSPGs) (Fawcett and Asher 1999; Silver and Miller 2004) and myelin-associated neurite growth inhibitors (McGee and Strittmatter 2003; Filbin 2003). In combination with the down-regulation of the CNS regenerative response (Schwab and Bartholdi 1996; Neumann and Woolf 1999), and the lack of trophic factor support (Widenfalk et al., 2001; Jones et al., 2001) it is not a surprise that it is difficult for axons to fully extend past the lesion site without additional assistance. For long-distance regeneration to be successful it is thus important to combine the induction of the intrinsic growth program with strategies that target extrinsic growth inhibition. Such strategies that target extrinsic inhibitors in the glial scar and myelin have been developed in the recent years. For example, the removal or neutralization of myelin can improve axonal regeneration. When an antibody aimed against neutralizing inhibitory factors like Nogo-A (IN-1 antigen) is used, regenerative sprouts and long distance elongation is seen (Schnell and Schwab 1990; Brosamle et al., 2000), as well as growth and sprouting of damaged and intact fibers (Schnell and Schwab 1990; Liebscher et al., 2005; Freund et al., 2007; Müllner et al., 2008; Maier et al., 2009; Gonzenbach et al., 2010). Chondroitinase ABC is a bacterial enzyme that is often used in SCI research due to its property to digest extracellular chondroitin sulfate proteoglycans (CSPGs) and the perineuronal nets (Carulli et al., 2010). Following injury it has been found to enhance axonal regeneration (Zuo et al., 1998; Yick et al., 2000; Bradbury et al., 2002) and reactivate plasticity in adult CNS by promoting sprouting and an increase in bouton number (Pizzorusso et al., 2002; Wang et al., 2011). In theory, the selective initiation of neuronal growth by sustained STAT3 expression is an ideal therapeutic complement to strategies that then counteract the growth-inhibitory signals in the inhibitory lesion environment that the regenerating axons encounters. In line with this concept we could show synergistic effects on axonal outgrowth of central sensory connections when we combined STAT3 induction with the neutralization of growth-inhibitory CSPGs (Bareyre et al., 2011). Combined treatments that include STAT3 induction may thus be promising strategies that might eventually enable successful regeneration of CST axons.

At least equally important as the initiation of axon regeneration, are the effects of sustained STAT3 expression on the remodeling of CST fibers. Our laboratory and other groups have previously shown that lesioned CST fibers can spontaneously remodel after injury and that the formation of detour circuits using intraspinal relay neurons is a key mechanism that mediates recovery of CST function (Bareyre et al., 2004; Courtine et al., 2008). We can show that sustained STAT3 expression can further increase the number of cervical CST collaterals that form after lesion. However increased collateral formation does not result in a significant increase of the CST connections to the long propriospinal relay neurons. This might indicate that the endogenous growth response of lesioned CST projection neurons is sufficient to promote the formation of detour circuits at an “optimal” rate and that is not improved further by the presence of additional CST collaterals. In line with this assumption, it was previously shown that the application of antibodies directed against the growth inhibitor NogoA does not further enhance detour circuit formation (Bareyre et al., 2004).

In contrast to lesioned fibers, unlesioned fibers mostly do not remodel after injury. Our results now indicate that sustained STAT3 expression can also recruit unlesioned fibers to the remodeling process. Through the use of the pyramidotomy model, we were able to investigate the effects of STAT3 on unlesioned fibers. This model is advantageous as it allows for a specific unilateral lesion of the CST, interrupting the direct cortical input (Thallmair et al., 1998). Pyramidotomy is known to affect voluntary movement of the forepaws (Steward et al., 2004; Lacroix et al., 2004), locomotion (Metz et al., 1998; Fanardjian et al., 2001), somatosensory sensation (Thallmair et al., 1998), fine-skilled paw reaching (Whishaw et al., 1998; Thallmair et al., 1998; Z'Graggen et al., 1998; Weidner et al., 2001), and cause asymmetrical limb use (Thallmair et al., 1998; Z'Graggen et al., 1998; Starkey et al., 2005). When STAT3 is overexpressed in cortical projection neurons, we found that the unlesioned tract sprouted more collaterals and sends these collaterals across the midline to the denervated side of the spinal cord, where they were found to contact both SPSNs and motoneurons responsible for forelimb movement. Our results thus show that after STAT3 overexpression the unlesioned tract can be recruited to the remodeling process and compensate for the loss of innervation by forming a new intraspinal circuit (Figure 10) that leads to functional recovery assessed electrophysiologically and behaviorally.

It should be noted that sustained STAT3 expression is not the only way to improve recovery in the pyramidotomy model. For example, sprouting across the midline from the

intact CST can be induced by neurotrophic factors (Zhou and Shine 2003), inhibition of the neurite outgrowth inhibitor NOGO (Thallmair et al., 1998; Bareyre et al., 2002; Wiessner et al., 2003), and even electrical stimulation (Brus-Ramer et al., 2007). There are also other factors that if manipulated can also induce midline sprouting in the pyramidotomy model. Yip et al., (2010) demonstrated that overexpression of the protein neuronal calcium sensor1 (NCS1) promotes midline sprouting and supports functional recovery. Thus, over the recent years a number of therapeutic targets and tools have been identified that can, in principle, improve axonal remodeling and functional recovery after spinal cord injury. STAT3 is a particularly attractive target as it can induce axonal regeneration and remodeling of spared fibers and due to its phase specific action it can selectively induce the initiation of axonal outgrowth.

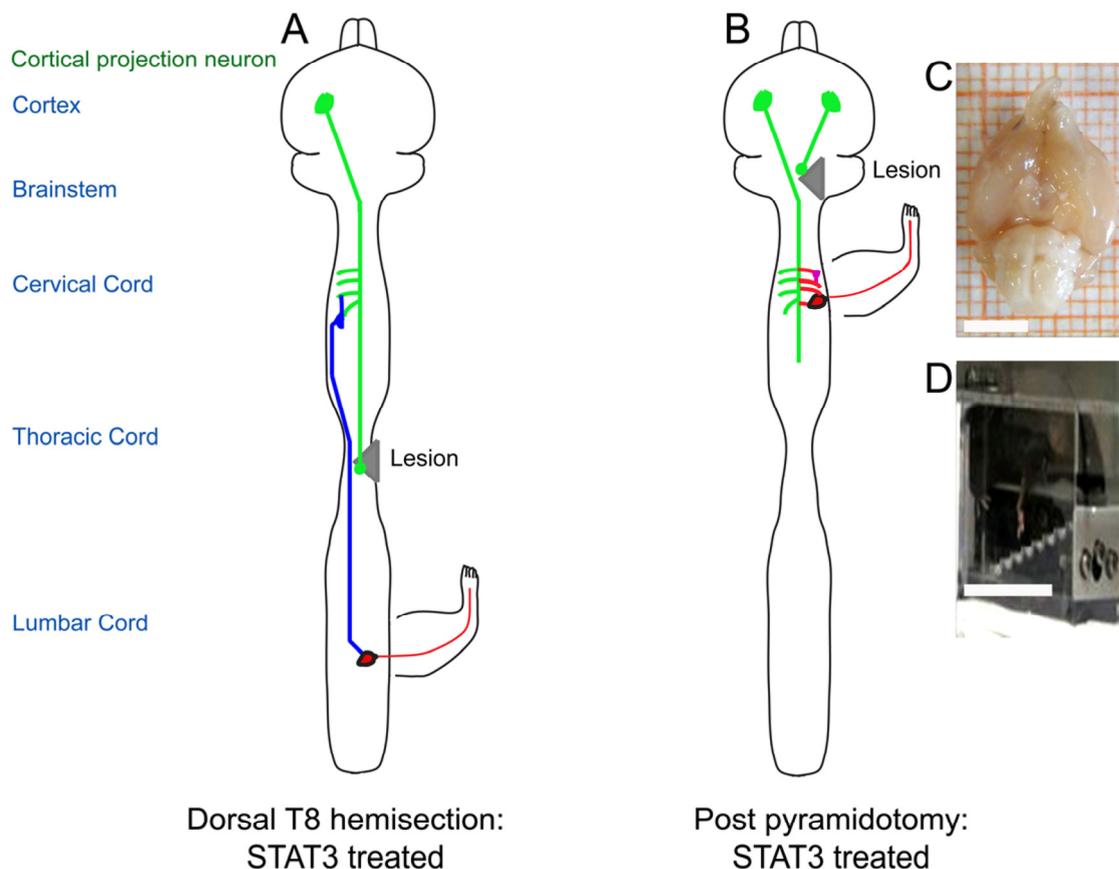


Figure 10. The effect of overexpression of STAT3 in layer 5 cortical neurons. (A) Overexpression of STAT3 promotes growth and regeneration of lesioned hindlimb corticospinal tract (hCST) fibers 3 weeks following a thoracic dorsal hemisection. (B) In the pyramidotomy paradigm, the overexpression of STAT3 induces compensatory sprouting and remodeling of forelimb CST (fCST) fibers from the unlesioned tract. Fibers from the intact fCST tract cross the midline into the denervated side of the cord, where they are found to contact both SPSNs and motoneurons responsible for forelimb movement. A new intraspinal circuit is formed enabling substantial functional recovery of the forelimb. (C) Image of pyramidotomy lesion performed at the level of the medulla and (D) of a mouse performing the pellet grasping test which assesses fine forelimb grasping ability. Scale bar equals 0.5mm in (C) and 5mm (D).

The use of pharmacological inhibitors to manipulate STAT3 expression is not ideal as STAT3 signaling is not limited to selective neuronal populations, thereby presenting a risk of activating STAT3 action in other cell types. STAT3 signaling plays an essential part in many biological processes such as regulating the immune response (Shuai and Liu 2003) and astrogliosis (Okada et al., 2006; Herrmann et al., 2008). Additionally, sustained STAT3 expression can cause uncontrolled cell growth and is linked to diseases such as cancer (Turkson and Jove 2000; Silver and Montell 2001; Sano et al., 2005). Therefore the use of viral gene therapy to specifically target STAT3 to particular neuronal cell population is a more suitable strategy. However with persistent expression the risk of tumor induction remains, hence in the long run it will be important to develop strategies to limit the duration of STAT3 overexpression. From our investigations, we have seen that through efficient genetic delivery of STAT3 to specific neuronal populations it is possible to manipulate axonal remodeling and regeneration. In terms of clinical implications, success with gene therapy has been seen in leukemia where T-cells were genetically engineered with lentiviruses to recognize and destroy leukemia tumor cells (Porter et al., 2011; Kalos et al., 2011). Recombinant adeno-associated viral vectors (rAAV) are well characterized in terms of safety profiles and are also used for gene therapy and clinical trials for neurodegenerative diseases such as Alzheimer's disease and Parkinson (Kaspar et al., 2002; Kaplitt et al., 2007; Lim et al., 2010). The vectors ability to infect CNS neurons and provide long term expression of the gene of interest without causing an immune response makes them attractive tools (Papale et al., 2009; Hutson et al., 2011; McCown 2001).

4.3. Outlook and future directions

The crucial step in the establishment of detour circuits is the formation of contacts between cortical projection neurons and intraspinal relay neurons (Bareyre et al., 2005, Lang et al., 2012). Newly formed dICST and mCST collaterals display a complexity suggesting they are capable of contacting different population of spinal relay neurons. The spinal cord is full of excitatory and inhibitory interneuronal populations that could in principle be targeted by the newly formed CST collaterals. From our study (Lang et al., 2012), we observed contacts onto PSNs, an excitatory population of interneurons, and then wondered whether the targeting of newly sprouted CST collaterals was equal on all interneuronal populations. To address this question, we examined whether CST collaterals also formed contacts onto glycinergic neurons. To selectively label glycinergic neurons we used the GlyT2-GFP mouse

line that expresses enhanced green fluorescent protein (eGFP) in neurons under the Glycine Transporter 2 promoter (Zeilhofer et al., 2005). Surprisingly, we found that compared to excitatory interneurons such as long and short propriospinal neurons, only a very small percentage of GlyT2 interneurons were contacted by newly formed CST collaterals. This indicates that not all interneuronal populations are equally targeted by CST collaterals (Figure 11).

This raises the question in what are the factors that determine which interneuronal populations are contacted by growing CST collaterals. This is an important question as the formation of functionally “meaningful” connections is key for functional recovery (Bareyre et al., 2004) and the formation of “incorrect” connections can lead to undesired symptoms that include neuropathic or phantom pain (Kaufmann and Moser 2000). The fact that propriospinal interneurons are preferentially contacted following injury suggests that this population of neurons express factors that attract the collaterals. Adhesive and repulsive interactions have been shown to guide cell-target recognition (Tessier-Lavigne and Goodman 1996; Sanes and Yamagata 1999; Matsuoka et al., 2011). Understanding the molecules at play in guiding these new CST sprouts onto their target will be an important future challenge that will help us to better understand and therapeutically enhancing post-injury circuit formation.

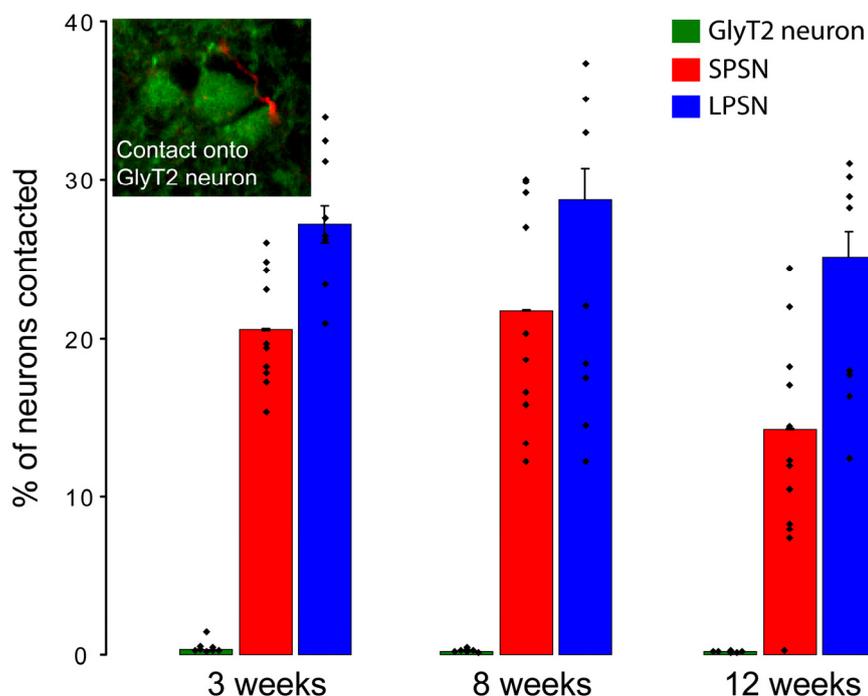


Figure 11. Differential targeting of newly formed hCST collaterals following a dorsal thoracic hemisection. Newly formed hCST preferentially contact target short and long propriospinal interneurons (SPSN and LPSN), while only a small percentage of glycinergic (GlyT2) neurons were found to be contacted (insert).

The pyramidotomy model in combination with sustained STAT3 expression can now provide an experimental approach to address the identity of the molecules that guide newly sprouted CST collaterals to their targets. Here, we could show that STAT3 expression induces the emergence of CST collaterals from the unlesioned tract both with and without a lesion of the contralateral tract. However these newly emerging collaterals crossed the spinal midline only if the contralateral side was previously denervated. This indicates that denervation induces the expression of molecules that attract the CST collaterals across the midline. In order to identify these molecular cues that might be responsible for attracting the collaterals across the midline, we plan to perform quantitative gene expression profiling. Previous gene chip studies have provided evidence that the molecular composition of the denervated spinal cord changes in response to injury (Bareyre et al., 2002; Maier et al., 2008). The denervated side was found to upregulate factors important for growth, adhesion, and synapse formation (Bareyre et al., 2002; Maier et al., 2008). In our current analysis we will focus the expression of guidance molecules that are differentially expressed following different treatment paradigms. The denervated and innervated sides of the spinal cord of mice lesioned at different timepoints and in which CST sprouting was induced by injection of rAAV-STAT3 will be analyzed and compared with untreated mice (Figure 12). From the data that will be collected we wish to identify candidate guidance molecules that attract fibers across the spinal midline. Then the manipulation of genes of interest could be envisaged using RNAi and viral gene transfer techniques to respectively knock out or overexpress candidate molecules and examine the effects on midline crossing of CST fibers.

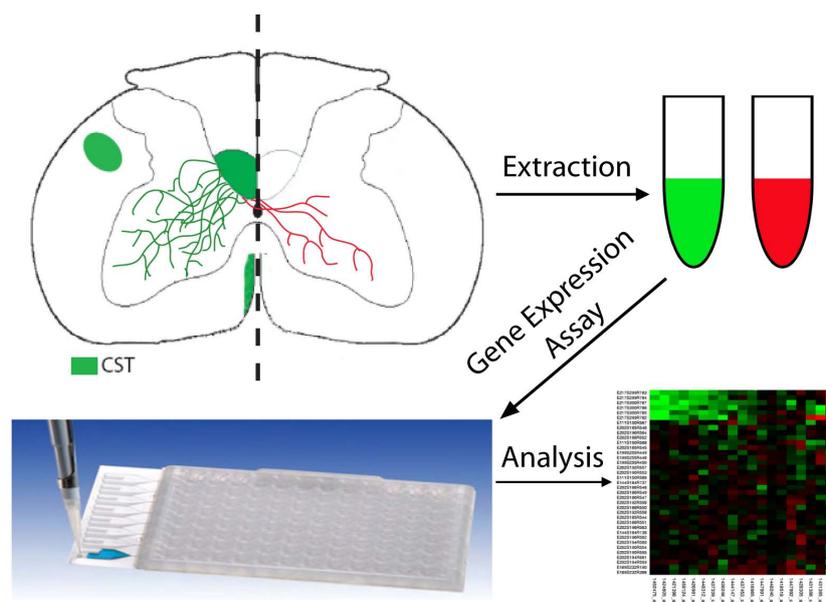


Figure 12. Scheme of proposed microarray gene expression protocol to analyze the innervated (green) and denervated (red) side of the cord following a pyramidotomy lesion. rAAV-STAT3-treated and untreated mice lesioned at different timepoints will be evaluated.

4.4. Conclusions

Spontaneous remodeling after injury has been illustrated to be an important feature that contributes to functional recovery after CNS injuries (Fouad et al., 2001; Bareyre et al., 2004; Courtine et al., 2008). Incomplete spinal cord injuries are susceptible to such remodeling because the cortical, subcortical and some of the local spinal cord circuitry remains largely intact and still partially interconnected by unlesioned fibers. In SCI, remodeling is characterized by three phases that consists of a growth initiation phase, which is then followed by a collateral formation phase, and finally there is a refinement period. In the process of anatomical remodeling, new circuits are established through the sprouting of axonal branches and dendrites, and reorganization of connections. A better understanding of this temporal sequence can help to guide future studies targeting the molecular regulation and therapeutic support of axonal remodeling.

Despite the fact that there is spontaneous remodeling, functional impairments still persist following CNS injuries. Studies have indicated that supporting growth in the CNS is an attractive strategy to enhance axonal remodeling (Zhou et al., 2003; Liu et al., 2010; Yip et al., 2010). Therefore targeting the initial growth phase is an attractive and direct approach. In our studies we have identified that the regenerative-associated transcription factor STAT3 is a key phase-specific regulator of the initial phase and is necessary for the initiation of regeneration in the PNS (Bareyre et al., 2011). Further investigations revealed that sustained activation of STAT3 after CNS injuries not only enhances axonal regeneration, it improves axonal growth and remodeling of both lesioned and unlesioned fibers, and functional recovery. This approach to enhance axonal remodeling by stimulating growth through the direct manipulation of the intrinsic growth program is a promising strategy for the future. Nevertheless, from the published research it can be inferred that a successful strategy would be expected to utilize a combination of therapies to overcome both the intrinsic and extrinsic barriers. In particular, intrinsic growth induction can be ideally combined with already established strategies to neutralize the extrinsic growth-inhibitory environment of the lesioned CNS.

In the future, it is hoped that studies such as ours will lead to better understandings of the mechanisms that underlie axonal remodeling after injury and help to devise effective therapies to prevent the devastating consequences of spinal injuries. Recent groundbreaking

work showed that improving functional recovery after spinal cord injury might not be an unrealistic therapeutic goal. In 2011, Harkema et al. (2011) revealed that it is possible for a human after spinal cord injury to achieve full weight-bearing standing, take steps on a treadmill and regain other key functions. The human subject was a 23 year old man with paraplegia from a C7–T1. A 16-electrode device was implanted in his lower back and in combination with intensive training the patient was able to walk on a treadmill as long as his cord was stimulated. The field of SCI research is continually evolving and over the last few years encouraging results have been published and with them bringing hopes to long suffering patients. The range of techniques available that include *in vivo* imaging, transgenic mice, RNAi, electrophysiology, and optogenetics are further advancing the field, making it an exciting time for scientific research and enabling us to further investigate, manipulate and understand the complicated vast intriguing system that is the CNS network.

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只想让你知道,放不下也忘不掉,你的笑你的好,是我温暖的依靠



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Eidesstattliche Versicherung

Eidesstattliche Versicherung

Ich versichere hiermit an Eides statt, dass die vorgelegte Dissertation von mir selbständig und ohne unerlaubte Hilfe angefertigt ist.

München, den 31.07.2012


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Claudia Lang

Erklärung

Hiermit erkläre ich, dass die Dissertation nicht ganz oder in wesentlichen Teilen einer anderen Prüfungskommission vorgelegt worden ist und dass ich mich anderweitig einer Doktorprüfung ohne Erfolg nicht unterzogen habe.

München, den 31.07.2012


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