Aus dem Institut für Klinische Neuroimmunologie

der Ludwig-Maximilians-Universität München

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Die Rolle des Signal Transducer and Activator of Transcription 3 (STAT3) bei der axonalen Regeneration im zentralen Nervensystem

Dissertation

zum Erwerb des Doktorgrades der Medizin

an der Medizinischen Fakultät der

Ludwig-Maximilians-Universität München

vorgelegt

von Natalie Garzorz

aus Aalen

2013

Mit Genehmigung der Medizinischen Fakultät

der Universität München

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Tag der mündlichen Prüfung:	**14. März 2013**

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List of Abbreviations

5-HT	5-Hydroxytryptamine = Serotonin
AAV	Adeno-associated virus
aCSF	Artificial mouse cerebrospinal fluid
ad	Adde (fill up to)
ATF-3	Activating transcription factor 3
Bcl-xL	B cell lymphoma extra large protein
BDNF	Brain-derived neurotrophic factor
BSA	Bovine serum albumin
C (+ number)	Cervical spinal cord level (+ number)
$CaCl_2 \cdot 2H_2O$	Calcium chloride dihydrate
CaM kinase II, IV	Ca^{2+}/calmodulin-dependent protein kinase II, IV
cAMP	Cyclic adenosine monophosphate
CAP-23	Cytoskeleton-associated protein 23
CD	Cluster of differentiation
C/EBP	CCAAT-enhancer-binding protein
ChABC	Chondroitinase ABC
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
CREB	cAMP response element-binding protein
CSPGs	Chondroitin sulfate proteoglycans
d/D	Day
dH_2O	Distilled water
DLK	Dual leucine zipper-bearing kinase
DNA	Deoxyribonucleic acid
DRG	Dorsal root ganglion
eCFP	Enhanced cyan fluorescent protein
EDTA	Ethylenediaminetetraacetic acid
e.g.	Exempli gratia = for example
Erk	Extracellular signal-regulated kinase
etc.	Et cetera
g	Gram
GAP-43	Growth-associated protein 43
GDNF	Glial cell-derived neurotrophic factor
GFP	Green fluorescent protein
gp130	Glycoprotein 130
h	Hour(s)
H_20	Water/aqua
IGF-I, IGF-II	Insulin-like growth factor I, Insulin-like growth factor II
IL-6	Interleukin-6
IRF1	Interferon regulatory factor 1
JAK	Janus kinase (or "Just another kinase")
JNK	c-Jun N-terminal kinases
KCl	Potassium chloride
kDa	Kilodalton
kg	Kilogram

KLFs	Krüppel-like factor family of transcription factors
l	Liter
L (+ number)	Lumbar spinal cord level (+ number)
L1CAM	L1 cell adhesion molecule
LIF	Leukemia inhibitory factor
M	Molar mass
MAG	Myelin-associated glycoprotein
MAP	Microtubule-associated protein
MAPK	Mitogen-activated protein kinase
mg	Milligram
$MgCl_2 \cdot 6H_2O$	Magnesium chloride hexahydrate
min	Minutes
ml	Milliliter
mol	Mole
mTOR	mammalian Target of Rapamycin
n	Number of samples
$Na_2HPO_4 \cdot 2H_2O$	Disodium hydrogen phosphate dihydrate
$Na_2HPO_4 \cdot 7H_2O$	Disodium hydrogen phosphate heptahydrate
NaCl	Sodium chloride
$NaH_2PO_4 \cdot H_2O$	Sodium dihydrogen phosphate monohydrate
N-cadherin	Neural cadherin
N-CAM	Neural cell adhesion molecule
NF-κB	nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
NGF	Nerve growth factor
NT-3; NT-4	Neurotrophin-3; Neurotrophin-4
OES	Olfactory ensheathing cells
OMgp	Oligodendrocyte myelin glycoprotein
PB	Phosphate buffer
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
PIAS	Protein inhibitor of activated STAT
PKA	Protein kinase A
PKC	Protein kinase C
PNS	Peripheral nervous system
P-STAT3	Phosphorylated STAT3
PTEN	Phosphatase and tensin homolog
RAGs	Regeneration-associated genes
RhoA	Ras homolog gene family, member A
RNA	Ribonucleic acid
ROCK	Rho-associated protein kinase
RT	Room temperature
s.	See
SCI	Spinal cord injury
SEM	Standard error of the mean
SH2	Src Homology 2
SOCS	Suppressor of cytokine signaling
Sox11	Sex determining region Ybox containing gene 11
SPRR1A	Small proline-rich repeat protein 1a
STAT3	Signal transducer and activator of transcription 3
STAT3c	Constitutively active STAT3

TBS	Tris buffered saline
TF	Transcription factor
T (+ number)	Thoracic spinal cord level (+ number)
Th1	T-helper cell 1
U	Unit
VEGF	Vascular endothelial growth factor
wk	Week(s)
WT	Wildtype
YFP	Yellow fluorescent protein
µg	Microgram
µl	Microliter

Zusammenfassung

Jedes Jahr erleiden weltweit circa 22 Menschen pro eine Million Einwohner eine Querschnittslähmung, die bei den Betroffenen zu dauerhaften Behinderungen und erheblichen Einschränkungen im Alltag führt. Die schwerwiegenden Defizite nach einer Querschnittslähmung, darunter Lähmungen und chronischer Schmerz, sind darauf zurückzuführen, dass geschädigte Axone im Rückenmark kaum regenerieren und es auch nur in geringem Ausmaß zur funktionellen Reorganisation der noch erhaltenen Nervenverbindungen kommt. Im Unterschied zum peripheren Nervensystem, wo zerstörte Nervenfasern erfolgreich regenerieren, ist die axonale Regenerationskapazität des zentralen Nervensystems (ZNS) spärlich ausgeprägt. Zwar konnte durch intensive Forschung im Verlauf der letzten Jahrzehnte eine Anzahl von „extrinsischen" wachstumshemmenden Molekülen von Gliazellen und der extrazellulären Matrix des ZNS identifiziert werden. Es gibt jedoch zunehmend Hinweise darauf, dass zahlreiche dieser „extrinsischen" Signale letztlich in „intrinsische" Signalwege der Neuronen selbst einmünden um schließlich die Transkription neuronaler Gene zu verändern. Einer der interessantesten intrinsischen Regulatoren axonaler Regeneration ist der Transkriptionsfaktor *´Signal transducer and activator of transcription 3´* (STAT3). In dieser Arbeit habe ich mithilfe moderner In-vivo-Mikroskopie sowie viraler Gentherapie in Spinalganglien genetisch veränderter Mäuse zum ersten Mal die entscheidende Rolle von STAT3 in der intrinsischen Regulation axonaler Regeneration *in vivo* identifizieren können. So konnte nachgewiesen werden, dass die nur rudimentär ausgeprägte Regeneration der zentralen Fortsätze der Neuronen in den Spinalganglien mit einer fehlenden Induktion von STAT3 in den entsprechenden Ganglien einhergeht. Durch Überexpression von STAT3 mittels rekombinanter Adeno-assoziierter Viren in zervikalen Spinalganglien konnte zwei Tage nach Läsion das Auswachsen von Axonen sowie das Aussprießen von Kollateralen um mehr als das Vierfache gesteigert werden. Darüber hinaus konnte mittels repetitiver Multiphotonenmikroskopie einzelner fluoreszenzmarkierter Axone gezeigt werden, dass die Überexpression von STAT3 nur in der Frühphase (2-4 Tage) die axonale Wachstumsgeschwindigkeit erhöhen konnte, nicht aber zu einem späteren Zeitpunkt (4-10 Tage) nach Läsion. Um die Hypothese zu überprüfen, dass die fehlende Aufrechterhaltung des axonalen Wachstums durch Kontakt der aussprossenden Axone mit

einem zunehmend inhibitorischen ZNS-Milieu bedingt ist, wurde die Überexpression von STAT3 zusätzlich mit der Applikation von Chondroitinase ABC, einem Enzym, das die inhibitorischen Moleküle der glialen Narbe neutralisieren kann, kombiniert. Dabei konnte ich zeigen, dass das durchschnittliche Wachstum von Axonen um mehr als das Zweifache gesteigert werden konnte.

Aus den Ergebnissen meiner Versuche konnte ich mehrere Schlussfolgerungen ziehen: Erstens konnte ich STAT3 als effektiven Initiator axonalen Wachstums nach Rückenmarksläsion identifizieren. Zweitens wurde nachgewiesen, dass STAT3 selektiv Wachstum in der frühen Phase reguliert, nicht jedoch zu späteren Zeitpunkten. Daraus folgt, dass das axonale Regenerationsprogramm aus mindestens zwei verschiedenen, molekular distinkten Phasen besteht. Mit STAT3 wurde zum ersten Mal ein phasenspezifischer Regulator der axonalen Regeneration entdeckt. Abschließend konnte gezeigt werden, dass synergistische Therapien - wie hier durch die Kombination von STAT3 und Chondroitinase ABC belegt wurde - axonales Wachstum zusätzlich verbessern. Die gewonnenen Einblicke in die Mechanismen axonaler Regeneration geben Grund zur Hoffnung, dass in der Zukunft effektive Kombinationstherapien für Querschnittsgelähmte entwickelt werden können.

Summary

Worldwide each year twenty-two in one million people experience a spinal cord injury (SCI) leading to permanent disability and tremendous restrictions in daily life activities. The severe and persistent functional deficits resulting from SCI such as paralysis and chronic pain are due to the limited repair of damaged axonal connections and the poor reorganization of spared nerve connections in the central nervous system (CNS). Whereas damaged axons in the peripheral nervous system (PNS) regenerate successfully, CNS axons fail to regrow. Intensive research over the last decades has identified a number of growth inhibitory molecules in the CNS environment and there is evidence that many of these extrinsic cues converge on intrinsic neuronal signaling pathways that eventually alter gene transcription. One of the most interesting intrinsic regulators of axonal regeneration is the transcription factor '*signal transducer and activator of transcription 3*' (STAT3).

In this study, I used time-lapse *in vivo* imaging as well as viral gene therapy in dorsal root ganglia (DRG) of genetically modified mice to identify STAT3 as a key regulator of axonal regeneration in the CNS. I could show that the failure of central DRG branches to regenerate is paralleled by the lack of STAT3 induction in the corresponding DRG neurons. Overexpression of STAT3 by recombinant adeno-associated viruses in DRG neurons increased outgrowth and collateral sprouting of central DRG branches more than fourfold at two days after lesion. Furthermore, repetitive multiphoton imaging of single fluorescently labeled CNS axons revealed that STAT3 overexpression selectively increased the speed of axonal growth at early (two to four days) but not at later time points (four to ten days) after lesion. As the lack of perpetuation of axonal growth might be due to the fact that regenerating axons encounter an increasingly growth inhibitory CNS environment, I combined viral STAT3 supplementation with the intrathecal application of chondroitinase ABC, an enzyme neutralizing the inhibitory molecules of the glial scar. I could show that the average axonal outgrowth ten days after lesion was increased more than twofold. Several conclusions can be drawn: First, STAT3 is a potent initiator of neurite outgrowth *in vivo*. Second, STAT3 selectively regulates early growth initiation but not later axon elongation implying that axonal regeneration consists of at least two molecularly distinct phases. With STAT3, I identified for the first time a phase-specific regulator of axonal outgrowth. Last,

combination of STAT3 as an “initiator of regeneration” with synergistic treatments such as chondroitinase ABC that improve axon elongation raises the hope for promising combination therapy approaches in the future treatment of spinal cord injury.

1 Introduction

1.1 Epidemiology, etiology and clinical picture of spinal cord injury

The earliest known description of spinal cord injury was found in an ancient Egyptian text from the Edwin Smith Surgical Papyrus stating that: “When you examine a man with a dislocation of a vertebra of his neck, and you find him unable to move his arms and his legs…..Then you have to say: A disease one cannot treat” (Case et al., 2005).

Until now, spinal cord injury (SCI) has remained a life changing event commonly resulting in irreversible neurological impairment affecting motor, sensor and autonomous functions. The worldwide incidence of SCI is about 22 per million population (source: International Campaign for Cures of Spinal Cord Injury Paralysis [ICCP]) and most cases result from accidents in sports and traffic, falls and violence (Devivo 2012). Young people, particularly men in their mid twenties, are affected which generates – apart from the personal consequences for the patient – immense costs in terms of medical care and social welfare.

Upon spinal trauma, when the spinal cord is locally damaged and bundles of axons are sheared, the neuronal flow of information from the periphery to the brain and vice versa is interrupted. Dependent on the level and the completeness of the injury, patients present with motor paresis (mostly para- or tetraplegia), abrogated sensation and autonomous dysregulation (e.g. postural hypotension, poikilothermia, sexual dysfunction and bowel and bladder paralysis). Moreover, patients suffer from chronic pain (s. Fig. 1-1).

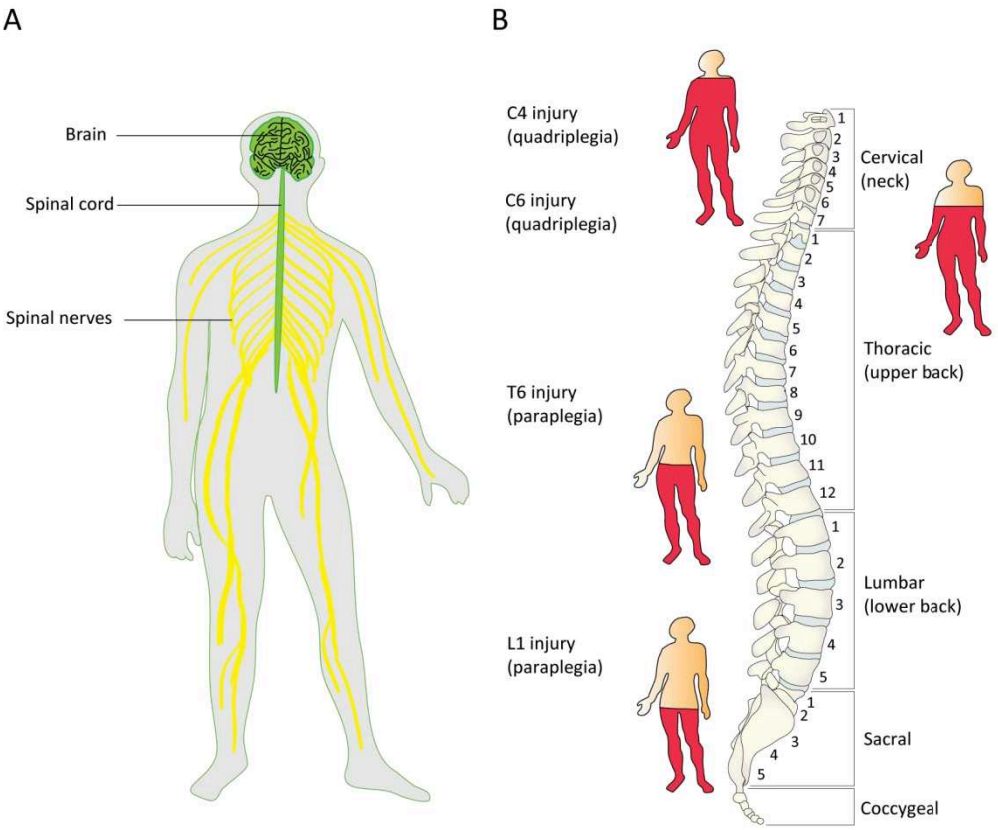

Fig. 1-1 Spinal cord injury

(*A*) The spinal cord as part of the central nervous system (green) transmits motor signals from the brain to the periphery via spinal nerves and sends information received from the peripheral nervous system (yellow) to the brain. (*B*) Motor deficits depending on the level of spinal cord injury. *B* modified from Thuret et al., 2006.

1.2 Pathophysiology and clinical management of SCI

The mechanical tissue damage upon trauma of the spinal cord is followed by numerous, only incompletely understood mechanisms such as local ischemia, edema formation, disturbance of ion homeostasis, release of free oxygen radicals and excitotoxins as well as recruitment of peripheral inflammatory cells (Schwab et al., 2006; Oyinbo 2011). Astrocytes and microglia become reactive and form a scar around the lesion site, together with extracellular substances such as chondroitin sulfate proteoglycans (CSPGs) and laminin. Within several weeks, macrophages clear the tissue debris and a fluid-filled cyst surrounded by scar tissue cells develops (Schwab et al., 1996; Bareyre et al., 2003; Schwab et al., 2006).

The acute spinal cord injury initially requires intensive medical care, as it can lead to cardiovascular, pulmonary and gastrointestinal complications dependent on the level of the

lesion. For example, high cervical lesions can cause pulmonary problems that might require rapid-sequence intubation and lesions above level T6 result in impaired sympathetic innervation of the heart leading to bradycardia. Urinary catheterization is needed to avoid bladder distension and secondary bladder infections. Furthermore, patients should receive thromboprophylaxis due to immobilization and pain killers should be applied early enough to avoid chronification of pain (source: http://www.uptodate.com). Besides, according to a recent meta-analysis, surgical stabilization and decompression of the spinal cord should be considered in all patients from eight to twenty-four hours following acute traumatic SCI (Furlan et al., 2011) to promote neurologic improvement. Moreover, in many therapeutic centers, acute interventions involve application of methylprednisolone as a standard treatment. Methylprednisolone has been found to improve clinical outcome by reducing the secondary damage including cytotoxic edema, inflammation and release of free radicals. Ideally, it is administered at high-dose (30 mg/kg) for twenty-three hours and started within the first eight hours of injury (Bracken 2002; Bracken 2012). After this early pharmacological intervention, further therapy is limited to physiotherapy, which the patient should undergo as early as possible (Edgerton et al., 2006; Mehrholz et al., 2008). In this context, robot-assisted walking training might turn out to significantly ameliorate motor function (Dietz 2010; Wirz et al., 2011).

1.3 Preclinical and clinical trials in the field of SCI

To improve future treatment of SCI in humans, many preclinical and clinical trials have been performed which target different physiological mechanisms (Fehlings et al., 2005; Schwab et al., 2006; Hawryluk et al., 2008; Kwon et al., 2011). The table below (s. Tab. 1-1) summarizes some well investigated targets. However, none of these approaches has so far achieved resounding therapeutic success. The modest or missing effects of these studies on clinical outcome emphasize even more the importance of better understanding the underlying mechanisms of SCI to provide better treatment in the future.

Non-invasive approaches			
Substance/ Method	**Mechanism**	**Status of the study and results**	**Reference**
Erythropoietin (EPO)	Neurons and astrocytes both express and respond to EPO. Amongst others, EPO drives intracellular anti-apoptotic signaling cascades and reduces ischemic damage by autoregulation of blood flow.	Studies investigating behavioral and histological outcome after EPO treatment in rats have yielded inconsistent results. The only human trial found on databases has recently been suspended (Italian multicenter Phase 3 study).	(Goldman et al., 2002; Carelli et al., 2011; Kwon et al., 2011) http://www.clinicaltrials.gov
Systemic hypothermia	Hypothermia has neuroprotective effects as it maintains intracellular ATP concentrations by slowing down enzymatic activity.	In rats, reduced apoptosis and tissue damage have been found. Results for hindlimb motor function are contradictory. Clinical trials and case reports show trend towards functional improvement, randomized multicenter trials are still pending.	(Kwon et al., 2008; Dietrich et al., 2011; Kwon et al., 2011)
Minocycline	Minocycline exerts anti-inflammatory and anti-apoptotic actions.	The results of a phase II placebo-controlled randomized trial of minocycline in an acute spinal cord injury model could not establish the efficacy of minocycline that has been shown in the majority of animal studies.	(Kim et al., 2009; Kwon et al., 2011; Casha et al., 2012)
Riluzole	Riluzole decreases excitotoxicity by blocking voltage-sensitive sodium channels and antagonizing presynaptic calcium-dependent glutamate release.	In rats, there is preclinical evidence for improved tissue protection and ameliorated locomotor scores as well as temporally limited effect on spasticity. Clinical studies are not yet open for patients.	(Cadotte et al., 2011; Kwon et al., 2011) http://www.clinicaltrials.gov
Invasive approaches			
Substance/ Method	**Mechanism**	**Status of the study and results**	**Reference**
Chondroitinase ABC	Enzyme digesting growth inhibitory chondroitin sulfate proteoglycans in the scar after SCI. More information s. section 1.4.1.	Intrathecal application of chrondroitinase ABC improves axonal regeneration and neuroprotection of injured fibers, promotes sprouting/plasticity of uninjured tracts and ameliorates motor function. Clinical trials have not yet been started.	(Bradbury et al., 2011; Kwon et al., 2011)
Anti-Nogo approaches	Mainly antibodies that target Nogo-A which is present in growth-inhibitory myelin of the CNS. More information s. section 1.4.1.	Intrathecal application of anti-Nogo-A antibody in animal models showed upregulation of growth-specific proteins, enhanced sprouting of fibers and the formation of new functional connections. A Phase II trial is currently ongoing.	(Bareyre et al., 2002; Zorner et al., 2010)
Anti-Rho approaches	The Rho signaling pathway regulates the cytoskeleton and exerts an important inhibitory role in neuronal growth	In animal models there is increased axonal sprouting and motor improvement; A phase I/II clinical trial of a recombinant Rho protein antagonist in acute spinal cord injury suggested slightly increased neurological recovery.	(Dergham et al., 2002; Fehlings et al., 2011; Kwon et al., 2011)
Activated macrophages	Activated macrophages eliminate growth	The results of a phase 2 randomized controlled multicenter trial could not	(Rapalino et al., 1998; Schwab et

	inhibitory myelin debris; They exert neuroprotective and anti-excitatory functions	support the promising results from animal models.	al., 2006; Lammertse et al., 2012)
Olfactory ensheathing cells	These cells have growth permissive properties; Together with regenerating axons they seem to migrate through an unfavorable CNS environment.	No or little effects concerning functional recovery in a phase 1 trial could be found.	(Ramon-Cueto et al., 1998; Dobkin et al., 2006; Mackay-Sim et al., 2008; Chhabra et al., 2009; Lima et al., 2010)
Stem cells (neuronal and mesenchymal stem cells)	Stem cells integrate into the damaged host spinal cord to replace glia and neurons.	Various studies in rodents and primates showed that stem cells reduce apoptosis, promote regeneration by directly inhibiting growth inhibitors in scar and myelin and replace lost cells by differentiating into mature oligodendrocytes. However, the first clinical trial with human embryonic stem cells has been suspended.	(Tetzlaff et al., 2011; Frantz 2012; Sakai et al., 2012; Snyder et al., 2012)

Tab. 1-1 Non-comprehensive overview of selective non-invasive and invasive therapeutic approaches in SCI therapy

1.4 The failure of successful axonal regeneration in the central nervous system

Although SCI patients can have long life expectancies due to improved intensive medical care and early mobilization, they still suffer from life-long neurological deficits as the spinal cord lacks the capacity to repair injured nerve connections. In contrast to nerve fibers in the spinal cord, peripheral nerves, however, can recover successfully from damage: After peripheral nerve injury, lesioned axons form so called sprouts at the proximal stump and keep growing until they enter the distal stump. They show impressive growth rates from 1-4 mm/d (Hoke 2006).

Thus, the question of why axons regenerate in the peripheral nervous system (PNS), but not in the central nervous system (CNS) has been in the focus of intensive research over the last decades. It has been found that two main obstacles define the poor regeneration in the CNS:

a) The **growth inhibitory CNS environment** and

b) The **insufficient activation of the intrinsic neuronal growth program** upon lesion.

1.4.1 The growth inhibitory CNS environment

The environment after a CNS lesion strongly contrasts with the situation seen after axonal damage in the PNS: After lesion of a peripheral nerve, axons distal to the injury degenerate (Wallerian degeneration) and macrophages infiltrate the area of injury to remove myelin and axonal debris. Besides, Schwann cells dedifferentiate within twenty-four to twenty-eight hours from myelinating to non-myelinating cells to help macrophages in clearing up myelin. In addition, they proliferate and form the so called bands of Büngner to guide sprouting axons from the proximal stump into and through the endoneural tubes of the distal nerve stumps. Once, the axon tip has reached the stump and thus successfully re-innervated an end organ, the surrounding Schwann cells start synthesizing myelin sheaths (Fenrich et al., 2004; Johnson et al., 2005). Upon axon injury, Schwann cells switch their gene expression program to promote a pro-regenerative environment. This includes downregulation of myelin genes and upregulation of regeneration-associated genes (RAGs) transcribing for growth-associated protein-43 (GAP-43), neurotrophic factors including neurotrophins (NGF, NT-3, NT-4 and BDNF) and their receptors, neuropoeitic cytokines (e.g IL-6, LIF), IGF-I, IGF-II and GDNF. Also, they enhance expression of cell adhesion molecules such as L1CAM, N-CAM and N-cadherin which guide growth towards the distal nerve stump by enabling adhesion between axons and Schwann cells (Fenrich et al., 2004). A recent study demonstrated that also fibroblasts play a key role when accumulating at the lesion side to sort Schwann cells into cords (Parrinello et al., 2010). Upon this interaction, the Schwann cell cords then guide axonal regrowth across the wound site. Moreover, extracellular matrix proteins including laminin, fibronectin and collagens are secreted to promote the function of the basement membrane (Fu et al., 1997; Fenrich et al., 2004; Johnson et al., 2005). All together, a permissive physical matrix can form around the side injury enabling axonal attachment and growth.

Not so in the CNS (s. Fig. 1-2): Oligodendrocytes do not dedifferentiate the way Schwann cells do. They don’t form bands of Büngner to bridge the lesion and to guide the axon. Moreover, oligodendrocytes and microglia sequester myelin debris not only much slower but also much more incompletely than macrophages and Schwann cells do in the PNS. Instead, oligodendrocytes continue to synthesize myelin and its associated growth inhibitors

(Filbin 2003; Fenrich et al., 2004). The most prominent myelin inhibitors are Nogo-A, myelin-associated glycoprotein (MAG) and the oligodendrocyte myelin glycoprotein (OMgp).

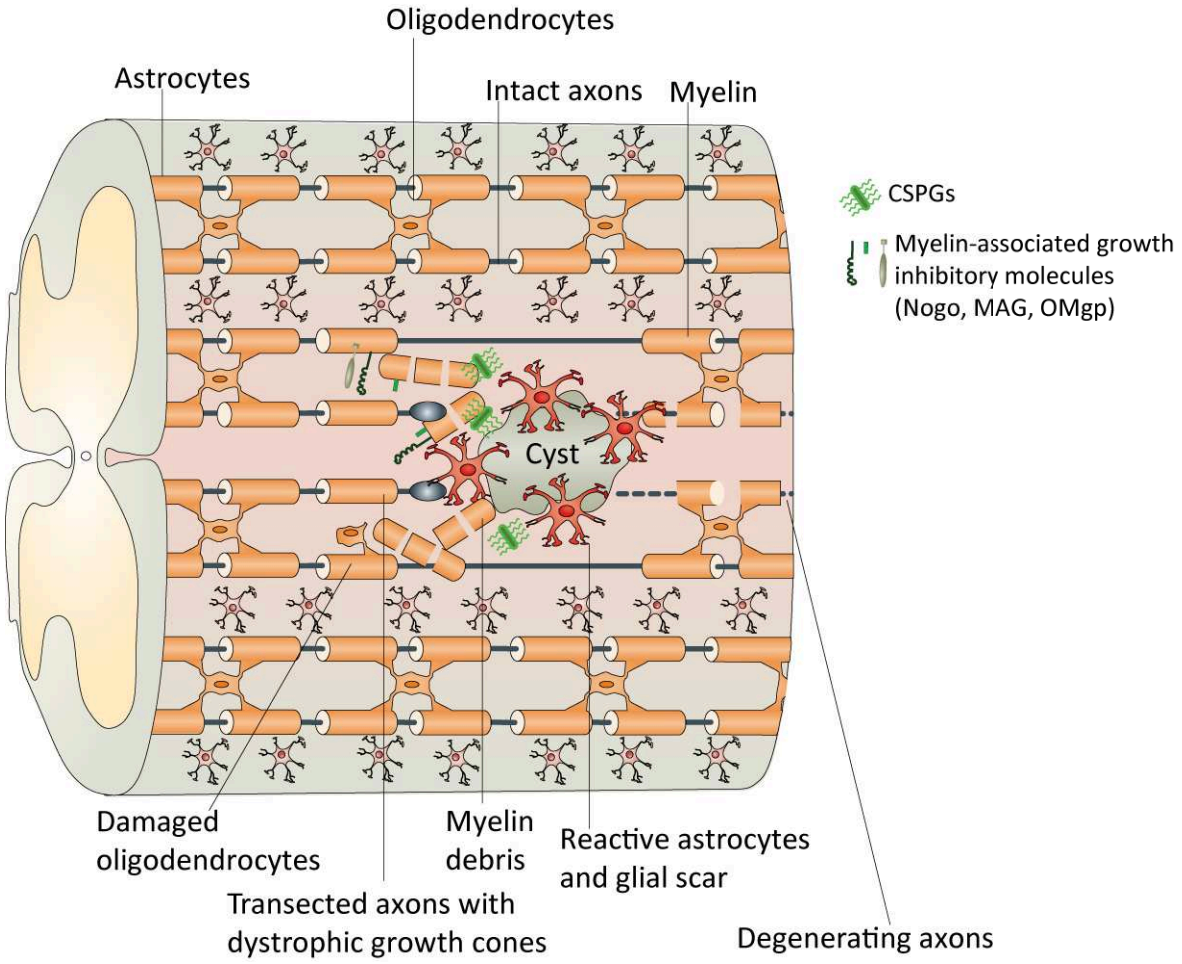

Fig. 1-2 The inhibitory CNS environment

Schematic representation of the injury site in the spinal cord showing main growth inhibitors such as myelin associated inhibitors (e.g. Nogo) and Chondroitin sulfate proteoglycans (CSPGs) as part of the extracellular matrix of the scar. Illustration modified from Yiu et al., 2006.

In line with this, blockade of Nogo-A by IN-1 antibody has been shown to allow axonal growth on myelin *in vitro* and *in vivo* (Caroni et al., 1988; Schnell et al., 1990) moving treatment strategies targeting Nogo-A into promising clinical trials in patients with acute SCI (s. Tab. 1-1).

Apart from persisting myelin, the formation of glial scar is the main barrier for regeneration in the CNS. Ineffective removal of growth inhibitory myelin proteins stimulates the proliferation of astrocytes. Latter ones gradually form a glial scar which is not only a strong

physical but also chemical barrier for growing axons. The molecules of the extracellular matrix of the glial scar have been shown to be highly growth inhibitory. Particularly the family of chondroitin sulfate proteoglycans (CSPGs) produced by astrocytes have been shown to be highly growth inhibitory (Silver et al., 2004; Bradbury et al., 2011): *In vitro* by repelling growing axons and inhibiting growth promoting molecules including laminin, fibronectin and L1 (Dou et al., 1994; Snow et al., 1996) as well as *in vivo* where development of dystrophic growth cones in regenerating axons was correlated with upregulation of CSPGs (Davies et al., 1997; Davies et al., 1999).

Addressing the growth inhibitory potential of CSPGs, their degradation has been in the focus of investigation (s. Tab. 1-1). The enzyme chondroitinase ABC extracted from the bacterium Proteus vulgaris has turned out to remove the chondroitin sulfate glycosaminoglycan (CS-GAG) chains from the CSPG core protein and thus making the CSPGs less inhibitory (Silver et al., 2004). After promising experiments in vitro, Lemons and colleagues first showed that chondroitinase ABC (ChABC) can degrade CSPGs in scar tissue when applied to spinal cords of contusion injured rats (Lemons et al., 1999). In 2002, Bradbury and colleagues demonstrated that intrathecal injection of chondroitinase ABC into a lesioned spinal cord promoted axonal sprouting and elongation in dorsal columns and corticospinal tracts. More importantly, a significant improvement in functional locomotor and proprioceptive behavior was observed (Bradbury et al., 2002).

1.4.2 The insufficient activation of the intrinsic neuronal growth program upon injury in the CNS

Apart from the growth permissive environment, successful axonal regeneration is also dependent on the growth capacity of the injured neuron. Eventually, neurons need to convert from the “transmitting” into the “growth” mode.

In contrast to neurons in the CNS, lesioned neurons in the PNS undergo this “switch” which is carried out by altered gene expression: In the PNS, nerve injury not only leads to glial but also to neuronal upregulation of regeneration-associated genes (RAGs) including genes encoding for cytoskeletal proteins like tubulin and actin and the well studied growth-associated proteins GAP-43 and CAP-23 (cytoskeleton-associated protein-23). The latter two are involved in actin accumulation and dynamics and are predominantly found in growth

cones where they mediate growth cone elongation (Skene 1989; Benowitz et al., 1997; Frey et al., 2000; Caroni 2001).

Besides, peripherally injured neurons start expressing proteins that are crucial for interactions between growth cones and Schwann cells: They upregulate receptors for neurotrophic factors released by Schwann cells and the neurons themselves release proteins such as neuregulin that – by binding to the erb receptor of Schwann cells – allow close interactions between neurons and glia. Moreover, adhesion molecules and integrins in the growth cone are upregulated to enable its extensions on the surfaces of Schwann cells and basal lamina (Fenrich et al., 2004; Raivich et al., 2007).

The following sections now explain

1) how the intrinsic neuronal growth program is induced in the PNS

2) how the understanding of 1) has already helped to develop approaches that aim to mimic the intracellular growth permissive situation in the CNS and eventually support lesioned CNS axons to regrow.

3) how all the injury signals described in 1) eventually contribute to axonal outgrowth

1.4.2.1 Induction of the intrinsic neuronal growth program after a PNS lesion

As previously mentioned, neurons alter their gene expression pattern when switching to the growth mode. To eventually initiate this transcription program, injury induced signals from the lesion site need to be transmitted to the nucleus (s. Fig. 1-3). In the following the complex mechanisms of injury induced retrograde signaling are briefly outlined (Ambron et al., 1996; Rossi et al., 2007; Rishal et al., 2010):

1.4.2.1.1 Early injury induced signaling

Immediately upon injury, a burst of voltage-gated sodium channel dependent action potentials is generated at the injury site propagating to the cell body where calcium channels are opened. The calcium influx then activates among others protein kinases A (PKA) and C (PKC) and Ca^{2+}/calmodulin-dependent protein kinases II and IV (CaM kinases II and IV)

which in turn regulate the actin cytoskeleton as well as growth related transcription factors such as CREB (Ambron et al., 1996; Swulius et al., 2008). Spira and colleagues could show that intracellular increase of calcium at the lesion site itself induced localized proteolysis of the membrane skeleton (spectrin) and cytoskeleton (actin and microtubules) by calpain proteases. The localized proteolysis is a prerequisite to restructure the cytoskeleton which in turn marks an important step during growth cone formation where post-Golgi vesicles supply membrane material (Spira et al., 2001; Bradke et al., 2012). In line with this, tetrodotoxin, a sodium channel blocker, eventually leads to reduced calcium influx and its related processes (s. above) which in turn goes along with reduced neurite regeneration (Mandolesi et al., 2004). Conversely, electrical stimulation accelerates neurite outgrowth after nerve transection (Brushart et al., 2002). However, the role of calcium and electrical stimulation is complex and remains ambiguous (s. section 1.4.2.2). Apart from calcium, also cAMP – activated by peptide hormones from interneurons and also by calcium – is an integral molecule that determines the regenerative potential already at early phases of regeneration. After peripheral nerve injury, cAMP levels are elevated and by activating the transcription factor CREB via PKA they contribute transcription-dependently to growth initiation (Ambron et al., 1996).

1.4.2.1.2 Interruption of constitutive retrograde signaling (“negative signals”)

Once a neuron is connected with its target, signals originating from the target travel retrogradely to the cell body to turn down axonal growth programs in order to enable establishment of synaptic connections (Abe et al., 2008). After lesion, the retrograde signaling from the peripheral targets is interrupted and these “negative signals” cannot longer reach the cell body. They are called “negative signals” as they are present in intact neurons and normally suppress the cell body response, but when interrupted they signalize injury to the cell body and thus induce regeneration (Zigmond 2011). The molecular mediators of this mechanism, however, are not securely identified. Neurotrophins, particularly nerve growth factor (NGF), have been suggested (Raivich et al., 2007; Rishal et al., 2010): For example, reduction of NGF with an antiserum induced axotomy-like changes in neuropeptide expression in intact sympathetic and sensory neurons (Shadiack et al., 2001). In line with this, continuous infusion of NGF to injured neurons delayed regeneration (Gold 1997). Interestingly, neurotrophins have been broadly shown to promote axonal

regeneration (Cui 2006; Lu et al., 2008). These contradictory findings might be based on different roles of neurotrophins at different time points. Loss of neurotrophic support might help inducing growth, whereas gradual increase in neurotrophins and their receptors stimulate growth in the later course of regeneration (source: Tuszynski, Mark H., Kordower, Jeffrey H.,Ed. CNS Regeneration: *Basic Science and Clinical Advances*. London: Academic Press, 2nd edition 2008.).

1.4.2.1.3 Induction of growth-promoting signals ("positive signals")

Following the early phase of injury induced changes (s. above), a large group of "positive retrograde injury signaling" molecules is activated by calcium and other still unknown mechanisms. They are named "positive signals" as they are not active in intact neurons but produced upon axotomy to trigger the cell body response. The activated molecules such as the transcription factor NF-κB (nuclear factor 'kappa-light-chain-enhancer' of activated B-cells) and mitogen-activated protein kinases (MAPKs) are microtubule-dependently transported towards the cell body where they initiate transcriptional processes. The MAPKs have become interesting candidate regulators of axonal regeneration, as exemplified by the following: Activation of extracellular signal-regulated kinase (Erk) and c-Jun N-terminal kinase (JNK) – two members of the MAPKs – and their interactions with the dynein/dynactin retrograde transport seem to be crucial for axonal regeneration (Abe et al., 2008). In addition, phorphorylated Erk e.g. is detected in axons after peripheral injury (Sheu 2000) and inhibition of Erk prevents adult DRG neurons from spontaneously initiating neurites in culture (Chierzi et al., 2005). Erk - by turning on the transcription factor CREB [cAMP response element-binding protein, (Johannessen et al., 2004)] - and JNK - by activating the transcription factors ATF-3 (Activating transcription factor 3) and c-Jun (Lindwall et al., 2005) - are directly involved in activating growth promoting transcription factors. Recently, dual leucine zipper-bearing kinase 1 (DLK 1), a kinase of a conserved MAPK cascade has been found to positively regulate growth cone formation and axonal regeneration (Hammarlund et al., 2009; Yan et al., 2009; Ghosh-Roy et al., 2010).

The molecules are transported towards the cell body in complexes of the retrograde motor dynein and proteins such as vimentin as well as the nuclear transport factors importin α and β which regulate entering of kinases to the nucleus. Interestingly, importin β and vimentin

are synthesized locally upon injury (Rossi et al., 2007). The role of this local *de novo* protein synthesis remains unclear but might contribute to the rapid injury response.

At later phases of injury signaling consists of pathways triggered by a number of cytokines such as IL-6 (interleukin-6) and CNTF (ciliary neurotrophic factor). They are released around the lesion sites from surrounding cells but also from the neurons themselves (Cheng et al., 1997; Zigmond 2011). They trigger intracellular downstream signaling via the JAK/STAT pathway resulting in the activation of the transcription factor signal transducer and activator of transcription 3 (STAT3). The key role of STAT3 in this context will be discussed later. Moreover, neurotrophic factors and neurotrophins are upregulated at the lesion site to maintain the neuron in a regenerative mode, initiating intracellular signaling cascades. Neurotrophins are released by Schwann cells and macrophages that have become recruited to the lesion site as well as by the lesioned neurons themselves, thus exerting autocrine and paracrine effects (Cui 2006). Upon binding to their cognate receptors, neurotrophins activate PKA and subsequently elevate cAMP levels leading to above described pro-regenerative activities. In addition, they activate the Ras/Raf cascade resulting in activating Erk (Ramer et al., 2000; Cui 2006). Thus it's not surprising that many studies have proven growth-supportive effects of the neurotrophic family, especially when applied with other substances in combination (Tuszynski et al., 1995; Lu et al., 2004; Alto et al., 2009).

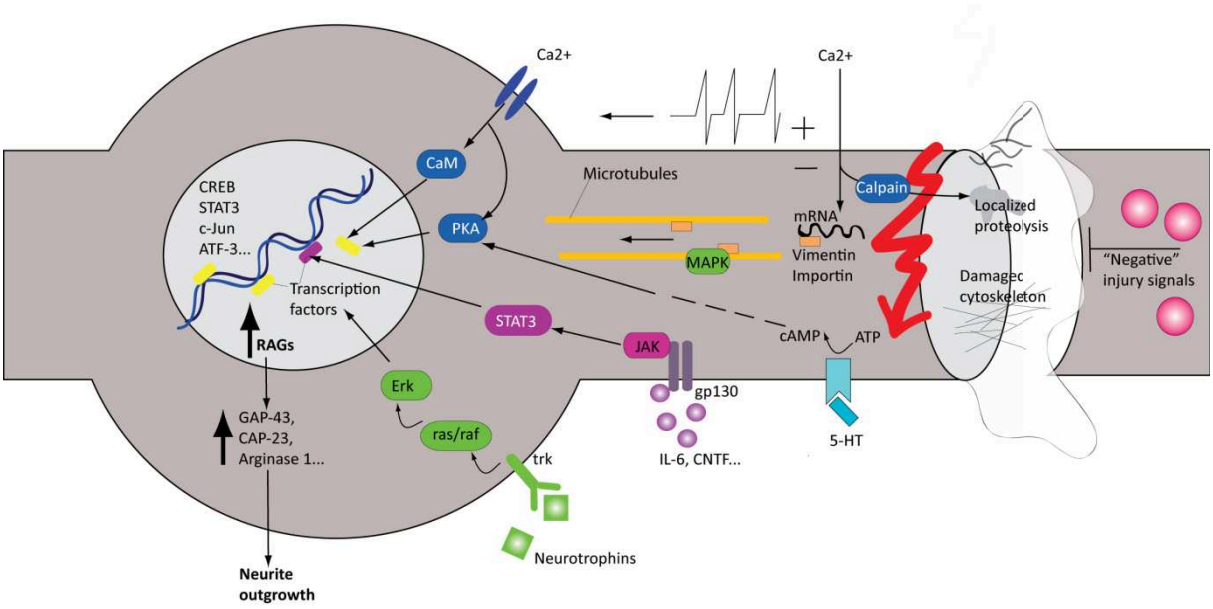

Fig. 1-3 The intrinsic neuronal growth program activated upon peripheral injury

The early phase of neuronal response upon nerve damage is characterized by injury induced action potentials and release of neuropeptides (such as 5-HT) from the environment that act via calcium and cAMP. Subsequently, retrograde MAPK and other components activated at the injury site are retrogradely transported to the nucleus. Later on, signals from cytokines and growth factors released by the neuron itself as well as by surrounding cells such as glia and macrophages arrive at the nucleus. Ultimately, all pathways activate transcription factors and alter gene expression resulting in upregulation of growth-associated proteins which in turn orchestrate neurite outgrowth.

1.4.2.2 Failed induction of the intrinsic growth program after a CNS lesion

In contrast to the PNS, where upon injury a cascade of injury signals are relayed to the nucleus and RAGs are upregulated as they are during development (Hoffman 2010), there is no upregulation of RAGs in the CNS (Plunet et al., 2002; Fenrich et al., 2004). It still remains speculative if this lack is due to neuronal inability of activation or transport of signals, failure of overcoming growth inhibition due to maturation (Liu et al., 2011) or reduced response to injury induced cytokines. However, studies on DRG neurons extending one branch to the periphery and the other to the CNS have yielded interesting findings: Lesion of the peripheral branch prior to a central branch lesion promotes regeneration of the central branch (Richardson et al., 1984; Neumann et al., 1999; Ylera et al., 2009). This phenomenon is also termed “conditioning lesion effect”, suggesting that CNS neurons still respond to

injury signals; however, only when the intrinsic program is additionally “primed” by a peripheral lesion via injury related processes such as retrograde travel of injury signals etc.

Thus, many efforts have been undertaken to mimic the conditioning lesion effect by specifically modifying one of the above described mechanisms.

Influencing early injury signals has so far led to ambiguous results: Whereas Udina and colleagues could show that electrical stimulation of the sciatic nerve promotes regeneration of injured central axons of the stimulated neurons (Udina et al., 2008), others have not found growth promotion when directly stimulating transected rubrospinal tract axons (Harvey et al., 2005). These differences might be explained by the fact that in contrast to Harvey’s experiment electrical activity in Udina’s experiment mimicked the “conditioning lesion effect”. Addressing cAMP, however, has led to more promising findings: cAMP levels can be therapeutically elevated by conditioning lesion, by administration of cAMP analogues or by treatment with the phosphodiesterase inhibitor rolipram. Interestingly, it does not only increase the neuron’s intrinsic growth capacity (s. section 1.4.2.1.1), but does also overcome myelin inhibition both *in vitro* and *in vivo,* allowing axons to growth through a spinal cord lesion (Qiu et al., 2002; Hannila et al., 2008).

1.4.2.3 Molecular anatomy of the intrinsic neuronal growth program

Experiments trying to mimic the growth pattern seen after peripheral lesions by modulating cascades during early injury induced signaling have been performed (s. above). However, more recent attempts aim to directly alter the neuronal growth program on the transcriptional and translational level where different injury induced signals eventually converge.

Studies investigated gene expression during neuronal development or compared gene expression between regenerating neurons and those that did not show regeneration (Bareyre et al., 2003; Moore et al., 2011). Not only genes but also some of the transcription factors (TFs), that regulate their expression, have been identified. Among them are c-Jun, CCAAT-enhancer-binding protein (C/EBP), CREB, STAT3, ATF-3, Sex determining region Ybox containing gene 11 (Sox11) transcription factor, Smad1 and the Krüppel-like family of transcription factors [KLFs (Moore et al., 2011)]. TFs are proteins that bind to enhancer or promoter regions of their target genes and thus regulate gene expression. As a single TF can bind to many locations of the DNA, activation of each of the TFs leads to complex alteration

in gene expression. A selection of TFs and their role for axon regeneration is exemplified in the following (s. Fig. 1-4).

Mice lacking c-Jun in the CNS showed severe defects upon injury and reduced expression of CD44, galanin, and α7β1 integrin, molecules known to be involved in axonal regeneration (Broude et al., 1997; Raivich et al., 2004). Knockout phenotype of the C/EBP transcription factor leads to decreased expression of the growth-associated genes for GAP-43 and tubulin-α1, thus attributing C/EBP an essential role for the neuronal injury response (Nadeau et al., 2005). Another factor, CREB, has been shown to overcome inhibitors in myelin and promote spinal axon regeneration *in vivo* (Gao et al., 2004). CREB activated by PKA and Erk (Johannessen et al., 2004) upregulates Tubulin, IL-6 and Arginase, whereas the latter one mediates synthesis of polyamines (Gao et al., 2004; Han et al., 2004; Hannila et al., 2008). Polyamines interact with cytoskeletal tubulin and might affect as such axonal growth (Savarin et al., 2010). Upregulation of Arginase also results in increased synthesis of spermidine, a polyamine, which has turned out to promote optic nerve regeneration *in vivo* (Deng et al., 2009). Another study investigated ATF-3 and could demonstrate that its overexpression in adult DRG neurons enhances neurite outgrowth in vitro (Seijffers et al., 2006) and ATF-3 is associated with the induction of growth promoting genes encoding for heat shock protein 27 (Hsp27), the small proline-rich repeat protein 1a (SPRR1A) and transcription factor c-Jun (Seijffers et al., 2007). Interestingly c-Jun and ATF-3 have been shown to co-localize in neurons after injury and co-immunoprecipitate suggesting that they act synergistically or even form heterodimers (Moore et al., 2011). Furthermore, Sox11 transcription factor seems to regulate neurite growth and neuron survival inducing the RAGs Microtubule-associated protein (MAP) and β-III tubulin (Bergsland et al., 2006). Also, knockdown of Sox11 goes along with increased expression of the pro-apoptotic gene BNIP3, decreased expression of the anti-apoptotic gene TANK and a decrease in Arpc3, an actin organizing protein that may be involved in axon growth (Jankowski et al., 2006). Another group has identified Smad1 to promote axonal growth in adult sensory neurons (Zou et al., 2009). Very recently, KLFs were identified to regulate intrinsic axon regeneration ability. The KLFs are zinc-finger transcription factors that suppress (e.g. KLF4) or enhance (e.g. KLF6) the regenerative capacity of CNS neurons (Moore et al., 2009; Tedeschi et al., 2010). A couple of their gene targets that are involved in axon regeneration have been identified and include

GAP-43 gene and genes important for synaptogenesis and cytoskeletal dynamics (Moore et al., 2011).

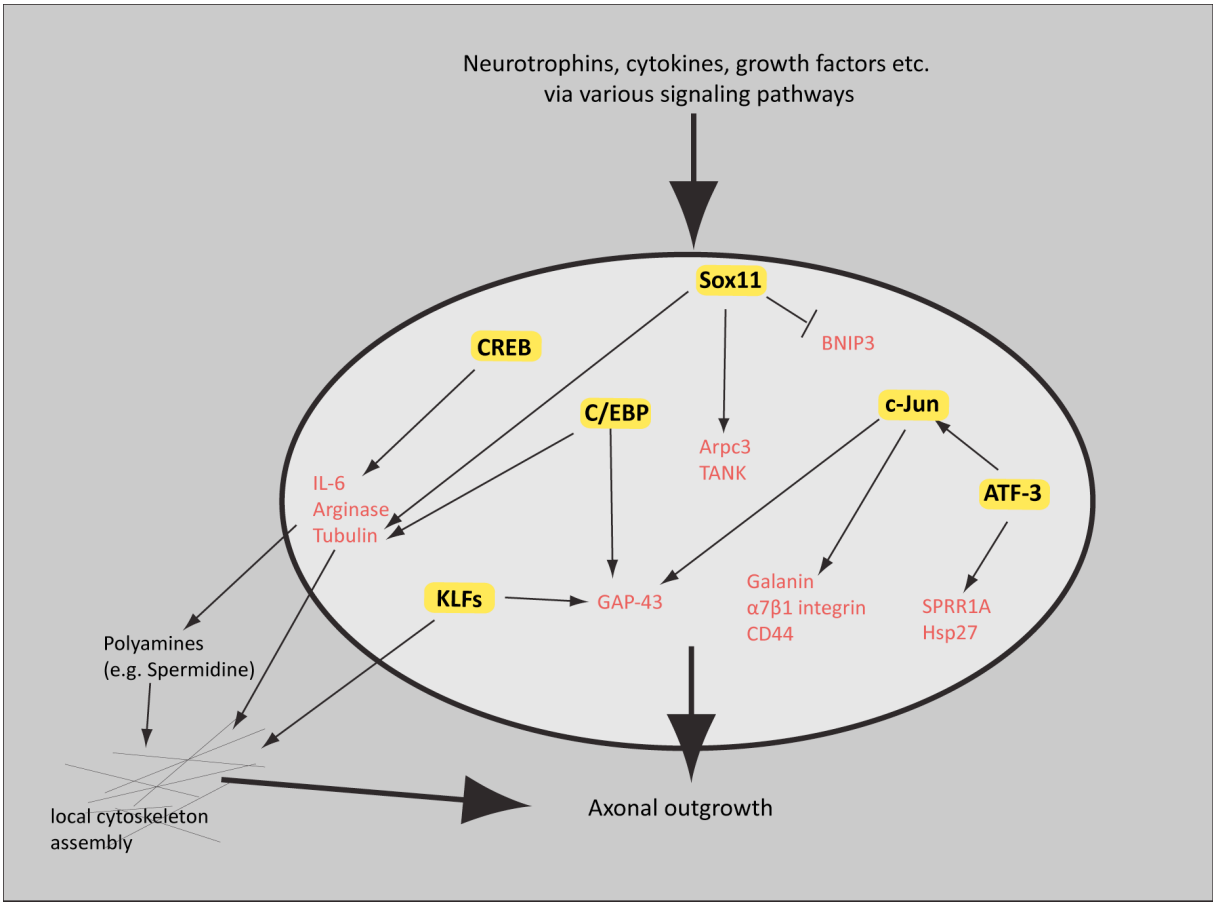

Fig. 1-4 Transcription factors involved in axonal regeneration and their target genes.

A variety of intracellular signaling molecules implicated in axon regeneration lead to activation of transcription factors (in yellow boxes). The transcription factors activate target genes (red) in the nucleus and thus promote axonal outgrowth. For example, CREB activates IL-6, Arginase and Tubulin, whereas latter ones are involved in local cytoskeleton assembly. More information as well as references about the transcription factors shown in the illustration can be taken from the text. Image adapted from Tuszynski, Mark H., Kordower, Jeffrey H., Ed. CNS Regeneration: *Basic Science and Clinical Advances*. London: Academic Press, 2nd edition 2008.

On the level of protein translation, the PTEN/mTOR pathway has been found to influence protein synthesis and cell growth: Deletion of the phosphatase and tensin homolog (PTEN) has been shown to allow robust axon regeneration of the optic nerve, whereas its downstream molecule mammalian Target of Rapamycin (mTOR) is positively associated with axonal regeneration. mTOR controls protein translation and can regulate microtubule assembly and axonal transport (Park et al., 2010). Furthermore, post-translational processes

seem to influence axonal regeneration as exemplified by the anaphase-promoting complex (APC) which might degrade pro-regenerative molecules (Lasorella et al., 2006).

1.5 STAT3

Apart from the above mentioned transcription factors, STAT3 has recently emerged as a key candidate regulator of axonal regeneration. Before presenting STAT3 in the context of axonal regeneration, the STAT family, the JAK/STAT pathway as well as the role of STAT3 outside the nervous system is briefly outlined.

1.5.1 The STAT family

STAT3 belongs to a family of transcription factors comprising seven members: STAT1, STAT2, STAT3, STAT4, STAT6 and the closely related STAT5A and STAT5B. The signal transducers and activators of transcription (STATs) range in size from 750 to 950 kDa and share several structurally and functionally conserved domains (Becker et al., 1998; Kisseleva et al., 2002; Levy et al., 2002): The amino-terminal domain (NH2) is involved in stabilizing dimers and importing of the STAT molecule to the nucleus (s. Fig. 1-5); the coiled-coil domain is implicated in receptor binding; the DNA-binding domain mediates binding of the STAT molecules to the corresponding DNA sequences; the linker domain is involved in nuclear export and transcriptional activities and is followed by the Src Homology 2 (SH2) domain, the most highly conserved domain which - through its capacity to bind to specific phosphotyrosine residues – plays an important role for STAT activation. The transactivation domain interacts with proteins that are necessary for binding of RNA-polymerase to DNA.

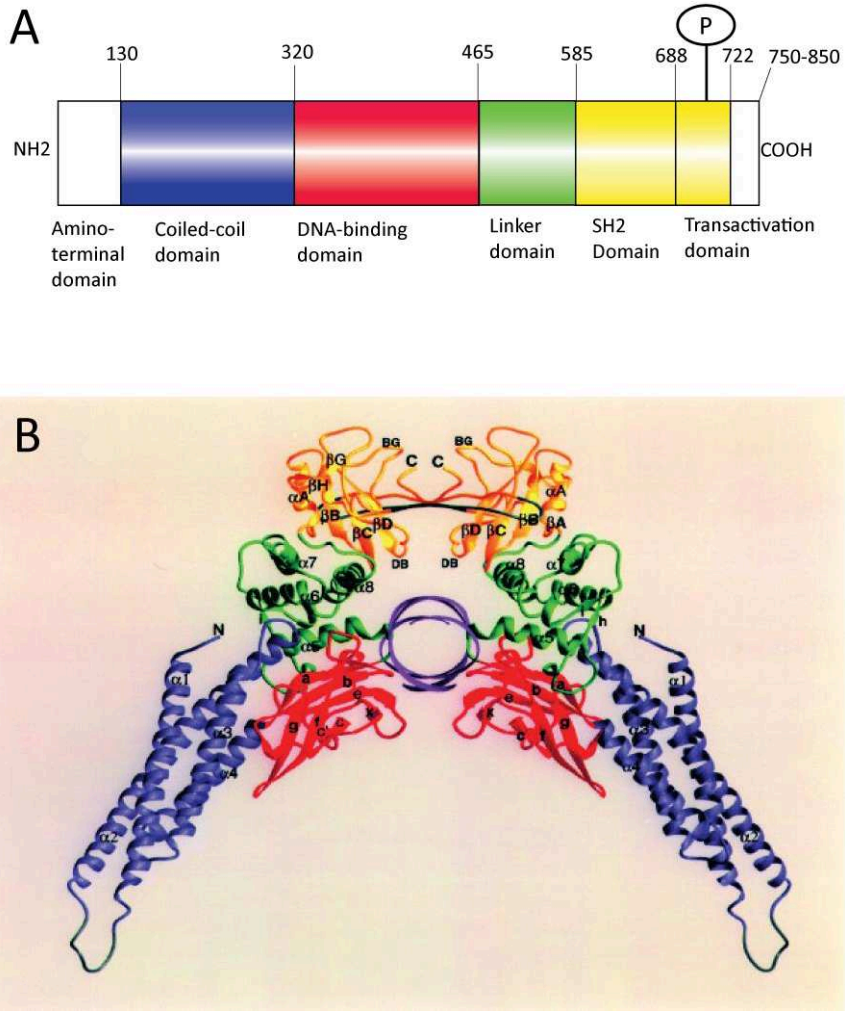

Fig. 1-5 Structure of the STAT3 molecule

(*A*) Linear structure of STAT3 and (*B*) three-dimensional structure of the STAT3 homodimer bound to DNA showing the different domains of the STAT3 molecule: Amino-terminal domain, coiled-coil domain (blue), DNA-binding region (red), linker domain (green), SH2-domain (yellow) with phosphorylation at a tyrosine residue and the transactivation domain. *A* adapted from Levy et al., 2002. *B* adapted from Becker et al., 1998.

1.5.2 The JAK/STAT pathway

STAT3 as well as the other STATs are part of the JAK/STAT signaling pathway (s. Fig. 1-6) transmitting extracellular signals to the nucleus (Schindler et al., 1995; Aaronson 2002; Kisseleva et al., 2002):

Neuropoetic cytokines such as IL-6, CNTF, leukemia inhibitory factor (LIF) and oncostatin M bind to the ligand binding chain of the gp130 receptor which subsequently dimerizes with the signal transducing chain of the receptor unit (Schindler et al., 1995). This configuration brings receptor associated Janus kinases (JAKs) mostly JAK1 (Guschin et al., 1995) and JAK2 into apposition, which can now transphosphorylate each other and thus release their

catalytic activity: JAKs phosphorylate a distal cytoplasmic tyrosine residue on the receptor thereby creating docking sites for the SH2 domain of STAT3 proteins. Subsequently, STAT3 proteins which are present in the cytoplasm in inactive form are drawn into the receptor complex and are phosphorylated on a single tyrosine residue by the JAKs (Kisseleva et al., 2002). In doing so, also in STAT3 molecules, docking sites for SH2 domains are created. When STAT3 proteins subsequently dissociate from the receptor, the phosphotyrosine residues of two STAT molecules are reciprocally recognized: The SH2 domain of one STAT3 molecule binds to the phosphotyrosine of the other STAT3 molecule, thus forming homodimers. These dimers arerapidly transported from the cytoplasm to the nucleus where they bind to promotor sequences containing the GAS (gamma activated site) element, the pallindromic DNA sequence 5'-TTTCCNGGAAA-3', and induce gene transcription (Aaronson 2002; Kisseleva et al., 2002).

The JAK/STAT pathway is regulated at several levels (Aaronson 2002; Kisseleva et al., 2002): MAPK e.g. have been shown to amplify STAT3 signaling by additional phosphorylation on serin residues. But there is also negative regulation of the pathway, e.g. phosphatases deactivating STATs and JAKs as well as suppressors of cytokine signaling (SOCS) that inhibit receptors and JAKs. Besides, nuclear regulators control STAT import and export to and from the nucleus and PIAS (Protein inhibitor of activated STATs) prevent STAT3 dimers from binding to the DNA.

A study in human breast cancer cells found that the STAT3 pathway is positively regulated by the above mentioned mTOR signaling pathway, whereas PTEN (s. above) served as a negative regulator of both STAT3 and mTOR signaling (Zhou et al., 2007).

Interestingly, upon peripheral nerve injury, it could be shown that STAT3 can be locally activated in the axon and retrogradely transported to the nucleus (Sheu 2000; Lee et al., 2004; Ben-Yaakov et al., 2012). However, gp130/JAK complexes might also be internalized and transported to the cell body to activate STAT3 (O'Brien et al., 2007).

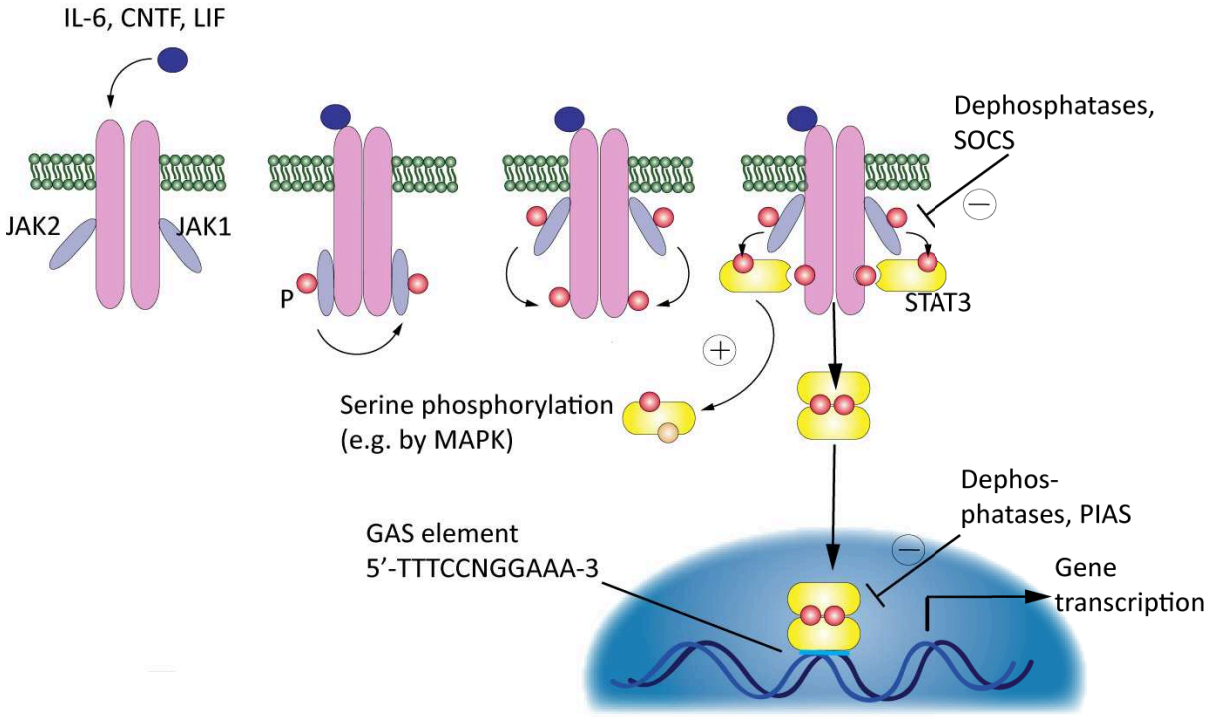

Fig. 1-6 Activation of the JAK/STAT3 pathway

The JAK/STAT3 pathway is activated by cytokines e.g. IL-6, CNTF and LIF. In the course of activation, latent cytoplasmic STAT3 proteins are recruited and phosphorylated by JAKs. Phosphorylated STAT3 molecules dimerize and are translocated into the nucleus where they modulate target genes.

1.5.3 The role of STAT3 outside the nervous system

STAT3 was initially identified in 1994 as an acute-phase response factor activated by IL-6 and other gp130- receptor dependent cytokines (Akira et al., 1994; Zhong et al., 1994). To learn more about the physiological functions of STAT3, STAT3 knockout mice were generated soon after (Takeda et al., 1997): It turned out that these mice differed from all other STAT knockouts and died already around embryonic day six indicating an important role of STAT3 for embryonic development. To study STAT3 mediated processes at later stages of development, tissue-specific knockouts were created using the cre-loxP recombination system (s. Tab. 1-2): Put under the control of a tissue-specific promotor, the cre recombinase is only expressed in the cell population of interest. Wherever it is expressed, the enzyme recognizes the loxP sites flanking the STAT3 gene on both sides and excises the DNA sequence of STAT3 (s. Fig. 4-12). In most tissues the functions affected by STAT3 deletion involve cell survival and proliferation (Levy et al., 2002).

Promotor for cre	Target tissue/cells	Phenotypes	Reference
K5	keratinocytes	Altered migration: disrupted wound repair, impaired keratinocyte migration and hair cycle; spontaneous development of ulcers. However, normal proliferation and development of epidermis and hair follicles.	(Sano et al., 1999; Sano et al., 2008)
K5	Thymus epithelium	Altered survival: severe thymic hyoplasia, increased susceptibility to apoptosis-inducing agents such as steroids	(Sano et al., 2001)
Blg	Mammary epithelium	Altered apoptosis: delayed mammary involution	(Chapman et al., 1999).
Mx	Liver	Impaired acute-phase response, similar to the phenotype of IL-6 knockout mice	(Alonzi et al., 2001)
Lck	T-lymphocytes	Altered survival/proliferation: impaired IL-6 dependent T-cell survival and reduced IL-2 induced proliferation	(Akaishi et al., 1998; Takeda et al., 1998).
Mlys	Monocytes/ Neutrophils	Hypersensitivity to endotoxin shock resulting in augmented production of inflammatory cytokines such as IL-1, IL-6 and IFNγ as a consequence of impaired IL-10 function; chronic colitis; imbalanced T-cell differentiation towards the Th1 type	(Takeda et al., 1999)

Tab. 1-2 Tissue-specific STAT3-deficient phenotypes outside the nervous system

Tissue-specific STAT3 deletion was achieved by taking advantage of the cre/loxP system. Table modified by Levy et al., 2002. In most tissues the functions affected by STAT3 deletion involve cell survival and proliferation

1.5.3.1 STAT3 in cancer

Along with its permissive role for survival and proliferation, STAT3 is also closely associated with oncogenesis (Bromberg 2002): While STAT1 is ascribed a tumor suppressor role, both STAT3 and STAT5 were found to increase tumor cell proliferation, survival and invasion. Constitutive activation of STAT3 has been found in many solid tumors such as breast cancer, prostate cancer and melanoma as well as in leukemias and lymphomas. Moreover, STAT3 is required for the transformation into malignant cells in some malignancies e.g. breast cancer and thyroid cancer.

Already in 1999, it could be demonstrated, that in patients with multiple myeloma, STAT3 was constitutively activated in bone marrow mononuclear cells where it induced the expression of Bcl-xL (B cell lymphoma extra large protein), an anti-apoptotic protein (Catlett-Falcone et al., 1999). Until now, many other tumor-associated genes encoding for proteins such as survivin and VEGF have been found to be induced by STAT3 (Yu et al., 2009).

1.5.4 STAT3 in the context of neuronal survival and regeneration

The role of STAT3 for the nervous system appears very similar to its effects outside the nervous system where it supports survival and proliferation. STAT3 is an important player during neurodevelopment, where it is e.g. required for axon pathfinding, neurite outgrowth and glial cell differentiation (Dziennis and Alkayed 2008). In rodents, STAT3 expression is detected in neurons and glia from embryonic day fourteen on with increasing levels up to postnatal day twenty-one, when levels start declining (Gautron et al., 2006).

In addition to its role in the developing nervous system, STAT3 has turned out to be an interesting transcription factor in the context of axon regeneration. Several studies demonstrate that increased levels of STAT3 expression and phosphorylation are associated with axonal regeneration in the PNS: After axotomy in rats, STAT3 phosphorylation is induced in facial and hypoglossal neurons already three hours after lesion, whereas this response is not found in non-regenerating Clarke's nucleus neurons (Schwaiger et al., 2000). A transient activation of STAT3 is also associated with sprouting of septal neurons after entorhinal cortex lesion in rats (Xia et al., 2002). Moreover, a study investigating the different spatial and temporal pattern of STAT3 and Erk phosphorylation in rats could show that nerve transection induced rapid STAT3 phosphorylation particularly in the proximal site adjacent to the injury which decreased to almost baseline when regeneration was complete at six weeks (Sheu 2000). Interestingly, STAT3 is activated at the injury site and then retrogradely transported to the nucleus (Lee et al., 2004).

After transection of the sciatic nerve in rats, but not after dorsal column lesion, STAT3 is activated in the corresponding DRG (Qiu et al., 2005). Qui and colleagues assumed that STAT3 activation was not only necessary for regeneration in the PNS, but also in the CNS. This was based on the finding that blockade of STAT3 phosphorylation by the JAK2 inhibitor AG490 impaired neurite outgrowth in vitro and compromised dorsal column regeneration after a pre-conditioning sciatic nerve transection. Not only in regeneration, but also in neuronal survival, STAT3 seems to be a key player: tissue-specific deletion of STAT3 in motoneurons from embryonic day twelve on results in reduced facial motoneuron survival (Schweizer et al., 2002). Further, a recent study using microarray approaches and subtractive hybridization identified STAT3 as a transcription factor that is selectively upregulated after PNS injury and a potent promoter of cultured cerebellar granule neurons (Smith et al., 2011).

Apart from regeneration, STAT3 is associated with axonal remodeling in the CNS: Unilateral lesion of the CST and application of IN-1 antibody directed against the growth inhibitor protein Nogo-A triggered reorganization of the remaining CST fibers. Interestingly, IN-1 antibody treatment was among others associated with upregulation of STAT3 expression (Bareyre et al., 2002).

Apart from STAT3 itself, various studies investigating molecules that exert their function via JAK/STAT pathway such as the neuropoietic cytokines IL-6, CNTF, LIF and SOCS3 highlight STAT3 as a good candidate in terms of axonal growth regulation. One study showed that LIF activated STAT3 after nerve injury (Rajan et al., 1995) and that sensory neurons of LIF deficient mice showed a significantly attenuated growth response upon a conditioning injury (Cafferty et al., 2001). Three years later, Cafferty and colleagues demonstrated that in IL-6 deficient mice, a pre-conditioning injury of the sciatic nerve was followed by a total failure of regeneration of dorsal column axons, thus identifying IL-6 as a keyplayer for mediating conditioning lesion-induced increase of regeneration of lesioned axons in the CNS (Cafferty et al., 2004). This goes along with the finding that sensory axons in IL-6 deficient mice show delayed regeneration after crush lesion (Zhong et al., 1999). Interestingly, also hormones such as Erythropoietin (Kretz et al., 2005) and IGF-I (Yadav et al., 2005) as well as neurotrophins such as NGF (Donnerer et al., 2005) and BNDF (Ng et al., 2006) activate STAT3 and have been shown to be involved in axonal regeneration and neurite outgrowth.

Recently, it could be demonstrated that deletion of SOCS3 (an intracellular downregulator of STAT3) in adult retinal ganglion cells promotes robust regeneration of injured optic nerve axons. This pro-regenerative response could be even enhanced by exogenously delivered CNTF to SOCS deficient mice (Smith et al., 2009). Latter finding confirmed previous data suggesting a neuroprotective and axon growth promoting role of CNTF (Muller et al., 2007).

1.6 Research question

In contrast to the PNS, where damaged axons can regenerate successfully, CNS axons fail to regrow. One of the reasons underlying this failure is the insufficient activation of the intrinsic neuronal growth program after a central lesion. To jump-start the intrinsic growth program after CNS injury we first need to identify its molecular key regulators. Especially transcription factors are promising candidate regulators as they can alter the expression of multiple downstream genes. The transcription factor STAT3 is a particularly promising candidate regulator as its expression correlates with the regenerative response (s. section 1.5.4). However, its direct role for axonal outgrowth *in vivo* has not yet been determined. This study now aimed to directly show a) whether and b) when STAT3 affects central axonal regeneration *in vivo* by answering the following specific questions (s. Tab. 1-3):

1. Is endogenous STAT3 activation induced after a CNS lesion?
2. Is STAT3 overexpression by viral gene therapy sufficient to induce outgrowth of CNS axons?
3. How does STAT3 affect different phases of axonal outgrowth in the CNS?
4. In case STAT3 overexpression positively influences axonal outgrowth in the CNS, can axon regeneration even be amplified when viral overexpression of STAT3 is combined with the application of chondroitinase ABC?

Tab. 1-3 Research questions to be answered in the course of my study

To address these questions, I used *in vivo* timelapse fluorescence microscopy and viral gene therapy in transgenic and wildtype mice to investigate the regeneration process of lesioned central branches emerging from dorsal root ganglia (DRGs).

2 Material and Methods

2.1 Material

1. Materials for surgical experiments		
1.1 Reagents		
Ketamine hydrochloride 10% (Ketamine)	Bremer Pharma GmbH, Warburg, Germany	
Xylariem 20 mg (Xylazine)	Riemser Arzneimittel AG, Greifswald-Insel Riems, Germany	
Forene (Isoflurane)	Abbott AG, Baar, Switzerland	
Metacam 1,5 mg/ml Oral Suspension	Boehringer Ingelheim, Ingelheim am Rhein, Germany	
Sterile artificial mouse cerebrospinal fluid (aCSF)	Solution A: 8,66 g NaCl (Merck) 0,224 g KCl (Merck) 0,206 g $CaCl_2 \cdot 2H_2O$ (Sigma-Aldrich) 0,163 g $MgCl_2 \cdot 6H_2O$ (Sigma-Aldrich) dH_20 ad 500 ml	Solution B: 0,214 g $Na_2HPO_4 \cdot 7H_2O$ (Merck) 0,027 g $NaH_2PO_4 \cdot H_2O$ (Merck) dH_20 ad 500 ml Mixture of solutions A and B in a 1:1 ratio
Bepanthen Augen- und Nasensalbe 5 g (eye cream)	Bayer Vital GmbH, Leverkusen, Germany	
Ringerlösung Fresenius KabiPac (Ringer's solution)	Fresenius KaBI Dtl., Bad Homburg, Deutschland	
Cutasept F Lösung 250 ml (disinfectant spray)	Bode Chemie GmbH & Co, Hamburg, Germany	
Chondroitinase ABC	Sigma-Aldrich, St.Louis (Missouri), USA	
Bovine serum albumin (BSA)	Sigma-Aldrich, St.Louis (Missouri), USA	

1.2 Tools and materials	
Wella contura W7807 (Hair clipper)	Wella, Darmstadt, Germany
Micro Pipettes intraMARK Blaubrand (micropipettes/glass capillaries)	Brand GmbH und Co. KG, Wertheim, Germany
Syringe 3pc 20 ml Omnifix™ luer slip (syringe for virus injection)	B. Braun Melsungen AG, Melsungen, Germany
Syringe 3pc 5 ml Omnifix™ luer slip (syringe for injection of Ringer's solution)	B. Braun Melsungen AG, Melsungen, Germany
BD Plastipak Hypodermic luer slip syringe 1 ml (syringe for Ketamine/Xylazine injection)	Becton, Dickinson and Company, Franklin Lakes (New Jersey), USA
Safety-Multifly-Set (blood collection system with needle, butterfly, tube and connector to syringe)	SARSTEDT AG & Co., Nümbrecht, Germany
Fine Iris Scissors (for dorsal column lesion)	Fine Science Tools GmbH, Heidelberg, Germany
Feather stainless steel blade (surgical blade)	pfm medical ag, Cologne, Germany
Noyes Spring Scissors (Large spring scissors)	Fine Science Tools GmbH, Heidelberg, Germany
Vannas-Tübingen Spring Scissors (Small angled spring scissors)	Fine Science Tools GmbH, Heidelberg, Germany
Dumont Mini Forceps – Inox Style 3 (Small forceps)	Fine Science Tools GmbH, Heidelberg, Germany
Dumont Mini Forceps – Inox Style 5 (Small forceps, smaller tip than Inox style 3)	Fine Science Tools GmbH, Heidelberg, Germany
Hypodermic Needles BD Microlance 3 30 Gauge (0,3 mm, yellow) for subcutaneous injection of Ringer's solution and anesthesia	Becton, Dickinson and Company, Franklin Lakes (New Jersey), USA
Hypodermic Needles BD Microlance 3 23 Gauge (0,6 mm, blue) for central lesion	Becton, Dickinson and Company, Franklin Lakes (New Jersey), USA
Olsen-Hegar Needle Holder	Fine Science Tools GmbH, Heidelberg, Germany
Ethicon Ethilon monofil 6-0 size, 667H (skin suture)	Johnson & Johnson Medical GmbH, Norderstedt, Germany
Ethicon Vicryl 4-0 size, MIC101H (intracorporal suture)	Johnson & Johnson Medical GmbH, Norderstedt, Germany

Autoclip Wound Clip Applier (stapler)	Becton, Dickinson and Company, Franklin Lakes (New Jersey), USA
Autoclip Wound Clips, 9 mm (staples)	Becton, Dickinson and Company, Franklin Lakes (New Jersey), USA
Sugi (absorbent triangles)	Kettenbach GmbH & Co. KG, Eschenburg, Germany
Metal plate	Custom-made
Cast Alnico Button Magnets (magnets)	Eclipse Magnetics Ltd, Sheffield, UK
Rubber bands	
Support cushion	Custom-made
Osmotic minipump (Model 1007B)	Alzet, Cupertino (California), USA
Brain Infusion kit 3	Alzet, Cupertino (California), USA
1.3 Technical devices	
Olympus KL 1500 LCD (cold light source for stereo microscopy)	Olympus UK Ltd KeyMed House, Southend-on-Sea, Great Britain
Olympus Stereo Microscope SZ51	Olympus UK Ltd KeyMed House, Southend-on-Sea, Great Britain
FST 250 Hot Bead Sterilizer (sterilizer for surgical instruments)	Fine Science Tools GmbH, Heidelberg, Germany
Vertical Micropipette Puller P-30	Sutter Instrument Company, Novato (California), USA
Small Animal Stereotactic Instrument	David Kopf Instruments, Tujunga (California), USA
T/Pump (Heating pad)	Gaymar Industries, Orchard Park (New York), USA

2. Materials for perfusion and immunohistochemistry	
2.1 Reagents	
PFA (paraformaldehyde)	8% PFA (Sigma-Aldrich) in dH_2O, heated up to 55 °C and stirred additional 10 min, filtrated and mixed in a 1:1 ratio with 0,2 M PB (Phosphate buffer), pH adjusted to 7,2-7,8
Agarose	Sigma-Aldrich, St.Louis (Missouri), USA
Triton X-100	Sigma-Aldrich, St.Louis (Missouri), USA

Sucrose	Sigma-Aldrich, St.Louis (Missouri), USA
EDTA (Ethylenediaminetetraacetic acid)	Merck KGaA, Darmstadt, Germany
Sodium dihydrogen phosphate monohydrate ($NaH_2PO_4 \cdot H_2O$, M=137,99 g/mol)	Merck KGaA, Darmstadt, Germany
Disodium hydrogen phosphate dihydrate ($Na_2 HPO_4 \cdot 2H_2O$,M=177,99 g/mol)	Merck KGaA, Darmstadt, Germany
PBS 10x (phosphate buffered saline), pH= 7,2/7,4	2,6 g $NaH_2PO_4 \cdot H_2O$ 14,4g $Na_2 HPO_4 \cdot 2H_2O$ 87,5g NaCl (Merck) dH_2O ad 1l
TBS 10x (Tris buffered saline), pH=7,6	61 g Tris base (121,14 g/mol), (Sigma-Aldrich) 90 g NaCl dH_2O ad 1l
O,2 M PB (phosphate buffer)	27,598 g $NaH_2PO_4 \cdot H_2O$, 35,598 g $Na_2 HPO_4 \cdot 2H_2O$ dH_2O ad 1l
Anti-STAT3 antibody	Cell signaling Technology, Danvers (Massachusetts), USA
Anti-P-STAT3 antibody	Cell signaling Technology, Danvers (Massachusetts), USA
Goat-anti-rabbit 594 antibody	Jackson ImmunoResearch Laboratories, West Grove (Pennsylvania), USA
Goat-anti-mouse 594 antibody	Jackson ImmunoResearch Laboratories, West Grove (Pennsylvania), USA
Gibco goat serum	Invitrogen GmbH, Darmstadt, Germany
NeuroTrace 435	Invitrogen GmbH, Darmstadt, Germany
NeuroTrace 488	Invitrogen GmbH, Darmstadt, Germany
Anti-Proteoglycan mouse IgG1, clone 2-B-6 antibody	SEIKAGAKU BIOBUSINESS Company, Tokyo, Japan
2.2 Tools and materials	
Microscope slides 76x26 mm	Gerhard Menzel Glasbearbeitungswerk GmbH & Co. KG, Braunschweig, Germany
Microscope cover slips 24x60 mm	Gerhard Menzel Glasbearbeitungswerk GmbH & Co. KG, Braunschweig, Germany
Parafilm	Brand GmbH & Co. KG, Wertheim Germany

Pipettes, pipette tips and tubes (2ml and 1,5 ml)	Eppendorf AG, Hamburg, Germany
12-well and 96-well cell culture plates	Becton, Dickinson and Company, Franklin Lakes (New Jersey), USA
Tissue Tek Cryomold Standard, 25x20x5 mm	Sakura Finetek Europe B.V. , Alphen aan den Rijn, The Netherlands
Tissue Tek Cryomold Biopsy, 10x10x5 mm	Sakura Finetek Europe B.V. , Alphen aan den Rijn, The Netherlands
Tissue Tek optimal cutting temperature (O.C.T.)	Sakura Finetek Europe B.V. , Alphen aan den Rijn, The Netherlands
Vectashield Mounting Medium	Vector Laboratories, Inc., Burlingame (California), USA
Paper filters (185 mm Ø circles)	Whatman Schleicher & Schuell GmbH, Dassel, Germany
50 ml centrifuge tubes	Greiner Bio-One GmbH, Frickenhausen, Germany
Erlenmeyer flasks (1l; 0,5 l)	Schott, Elmsford (New York), USA
2.3. Technical devices	
Leica CM1850 cryostat	Leica Microsystems GmbH, Wetzlar, Germany
Vibratome 1000Plus	Intracel LTD, Shepreth, Royston, Great Britain
Vortex-Genie 2	Scientific Industries, Inc., Bohemia (New York), USA
KERN EW 150-3M (scales)	Kern & Sohn GmbH, Balingen-Frommern, Germany
Laboratory pH meter inoLAB	WTW Wissenschaftlich-Technische Werkstätten, Weilheim, Germany
Magnetic stirring hotplate MR 3001K and stirring bars	Heidolph Instruments GmBH & Co. KG, Schwabach, Germany
Ismatec IP high precision multichannel pump (pump for perfusions)	ISMATEC SA, Labortechnik - Analytik, Glattbrugg, Switzerland
Olympus IX71 inverted fluorescence microscope (evaluation of immunohistochemistry)	Olympus GmbH, Hamburg, Germany
Olympus SZX16 fluorescence stereomicroscope (Dissection microscope)	Olympus GmbH, Hamburg, Germany

3. Imaging	
Olympus BX51WI upright microscope (Wide-field epifluorescence microscope) for *in vivo* imaging equipped with x4/0.13 dry, x10/0.3 dry and x20/0.5 dipping cone water-immersion objectives	Olympus GmbH, Hamburg, Germany
Fast shutter and filer wheel	Sutter Instruments, Novato (California), USA
Height-adjustable stage with attachment for metal plate	Custom-made
Cooled SensiCam QE CCD camera	The Cooke Corporation, Romulus (Michigan), USA
Standard filter for fluorescent proteins	Chroma Technology Corporation, Bellows Falls (Vermont), USA
MetaMorph®software	Molecular Devices, Orleans (California), USA
FV1000 confocal system mounted on an upright BX61 microscope, equipped with an x10/0.4 water immersion objective and x20/0.85 and x60/1.42 oil immersion objectives (confocal microscopy)	Olympus GmbH, Hamburg, Germany
Custom-built multiphoton imaging setup based on an Olympus FV 300 scanner equipped with a femto-second pulsed Ti:Sapphire laser (Mai Tai HP, Newport/Spectra-Physics)	Olympus GmbH, Hamburg, Germany Newport Corporation, Irvine (California), USA
Olympus FV1000 MPE multiphoton microscope	Olympus GmbH, Hamburg, Germany

2.2 Mice

In situ and *in vivo* experiments on central axonal regeneration were performed in *Thy1*-GFP^{S} mice (Feng et al., 2000). In this line, the *Thy1*-promotor drives expression of GFP in only a small subset of sensory neurons, thus allowing the investigation of axonal regeneration of single axons. Animals were adult (between approximately six and twenty weeks of age and a weight of 20-30 g) and both sexes were equally distributed to both experimental and control

groups. For the evaluation of STAT3 expression in DRG neurons ten, twelve and fourteen days after viral therapy both wildtype (WT) mice on a C57/bl6 background and *Thy1*-GFP^{s} mice were used. To delete STAT3 I used STAT3 $^{fl/fl}$ mice, maintained on a bl6 background (Takeda et al., 1998). For the nerve crush experiment we used *Thy*1-YFP^{16} mice (s. section 4.1). Animals were kept in Eurostandard Type II long cages 365x207x140 mmH (Tecniplast, Hohenpreißenberg, Germany) stored in IVC rack system with a maximum of five mice per cage. Autoclaved food (regular food "Maus" from Ssniff, Soest, Germany) and autoclaved tap water were supplied ad libitum. Mice were held at a 12 h light⁄12 h dark cycle. Animals were kept at the breeding room with their parent until day twenty-one postnatal and afterwards weaned and separated by sex. All animal experiments were performed in accordance with regulations of the animal welfare act and protocols approved by the Regierung von Oberbayern.

2.3 Methods

2.3.1 Plasmid constructs

The pAAV-MCS vector from Stratagene has been used to produce the viral vectors pAAV-STAT3, pAAV-STAT3c and the control viral vectors pAAV-eCFP. For the pAAV-STAT3, the STAT3 gene was taken from pcDNA3 STAT3 (Adgene plasmid 8706) by excising it with the restriction enzymes BamHI and XhoI, whereas the STAT3c gene was cut with NotI and SwaI from the pRc/CMV STAT3c Flag (Addgene plasmid 8722). Then, both genes were respectively inserted into the pAAV-MCS at the HincII site. pAAV-eCFP was produced by excising the eCFP gene from the peCFP N1 plasmide at BamHI and NotI and consecutively cloning it in the pAAV- MCS at the HincII site. All cloning was performed by Dr. Florence Bareyre.

2.3.2 Vector production and purification

AAV serotype 2 particles of pAAV-STAT3, pAAV-STAT3c, pAAV-eCFP and pAAV-cre were produced in HEK293 cells by the adenovirus-free AAV production method (Zolotukhin et al., 1999; Zhang et al., 2000). Genomic titers were as follows: rAAV-STAT3, 9x10^{12} genome

copies/ml; rAAV-STAT3c, $5.5x10^{12}$ genome copies/ml; rAAV-eCFP, $9.2x10^{12}$ genome copies/ml; rAAV-cre , $9x10^{12}$ genome copies/ml. Vector production as well as vector purification was performed by Joshua Sanes and In-Jung Kim (Harvard University). rAAV-cre was a kind donation from I.J. Kim (Harvard University).

2.3.3 Tissue processing and immunohistochemistry

Animals were deeply anesthetized with isofluorane and perfused transcardially with 20 ml of saline solution followed by 60 ml of 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer. Spinal cords including the injected DRGs were dissected out and tissues were post-fixed in 4% PFA at 4 °C for 24 h. For consecutive immunofluorescence analysis, DRGs were transferred to 30% sucrose for at least 24 h and embedded/blocked in Tissue-Tek optimal cutting temperature (O.C.T.) compound. Then 20 µm thick coronal sections were cut on a cryostat and mounted on glass slides.

Before immunostaining, sections were dried at room temperature (RT) for at least 2 h and subsequently rinsed 3 times for 10 min in TBS at room temperature. To improve antigen retrieval, slides were heated up to 60 °C in the microwave in Tris/EDTA followed by 15 min at a sub-boiling temperature. Without cooling down the slides, sections were blocked with 5% goat serum in 0,1% Triton X-100 in TBS for 1 h. Afterwards, sections were incubated for 1 h at RT and then overnight at 4 °C with either anti-STAT3 antibody (dilution 1:500) or anti-P-STAT3 antibody (dilution 1:50) diluted in 0.1% Triton X-100-TBS and 2,5% goat serum. Slides were rinsed again 3 times for 10 min with 1xTBS and subsequently incubated with fluorescent secondary antibody (goat-anti-rabbit 594) used at a dilution of 1:500 in TBS for 2 h. Furthermore, nuclei were counterstained using NeuroTrace 435 (dilution 1:500). For the STAT3 deletion experiment in the CNS (s. section 4.6) I used NeuroTrace 488. Before mounting the tissue in Vectashield mounting media, slides were rinsed 3 times for 10 min with TBS.

In the last experiment of this study (s. section 4.7) delivery of chondroitinase ABC was confirmed by immunohistochemistry using the Anti-Proteoglycan mouse IgG1 (clone 2-B-6) antibody which recognizes 4-sulfated and 6-sulfated chondroitin and dermatan sulfates resulting from ChABC enzymatic digestion. Spinal cords were embedded in 2% Agarose and

subsequently cut in 50 µm thick sections by a vibratome. Afterwards, sections were rinsed for 10 min in PBS and blocked with 5% goat serum in 0,1% Triton X-100 in PBS for 1 h. Then, sections were incubated for 1 h at RT and then overnight at 4 °C with Anti-Proteoglycan mouse IgG1 (diluted in 0.1% Triton X-100-PBS and 2,5% goat serum). Sections were rinsed 3 times for 10 minutes with PBS and subsequently incubated overnight with fluorescent secondary antibody (Goat-anti-mouse 594) used at a dilution of 1:500 in 0,1% Triton X-100 in PBS. Eventually, sections were rinsed 3 times for 10 minutes and mounted on glass slides in Vectashield.

2.3.4 Quantification of the expression of P-STAT3 and STAT3

STAT3 and P-STAT3 immunoreactivity was analyzed in DRGs of animals perfused at 10 d, 12 d and 14 d after the injection of rAAV-STAT3 and rAAV-STAT3c into cervical DRGs (C3-C6) of C57/bl6 wildtype or *Thy1*-GFP^{s} mice. DRG sections were stained for STAT3 and P-STAT3, respectively, and all sections were counterstained with NeuroTrace 435 to reveal the total number of neuronal nuclei in the DRG. Then, images in the red (P-STAT) and blue (NeuroTrace) channel of an Olympus IX71 inverted fluorescence microscope were taken and the proportion of DRG neurons showing STAT3 or P-STAT3 immunoreactivity was determined.

2.3.5 Gene therapy with recombinant adeno-associated viral vectors

To assess the efficiency of STAT3 gene transfer via viral vectors in C57/bl6 wildtype mice (s. section 0), and to determine the outcome of viral STAT3 deletion on central axon outgrowth in STAT3 $^{fl/fl}$ mice (s. section 4.6), animals were deeply anesthetized. A dorsal cervical laminectomy was performed on the cervical part of the spinal column (C3-C6) and the DRGs were surgically exposed as described in the protocol below (s. section 2.3.7.1). Then 1 µl of rAAV (rAAV-STAT3, rAAV-STAT3c, rAAV-cre or rAAV-eCFP) was slowly injected into the DRG with a glass capillary that was thinly drawn using a micropipette puller. Injections were performed using a stereotactic frame and a 20 ml syringe that was connected to the glass capillary via butterfly of a common blood collection set. All capillaries were filled by aspirating the virus with a 20 ml syringe before starting surgeries and kept on ice during the

surgical procedure. For those experiments in which the effects of axonal outgrowth after viral STAT3 supplementation in *Thy1*-GFP^{s} were investigated (s. sections 4.4, 4.5, 4.7) *in vivo* imaging was performed prior to injection to identify suitable single fluorescently labeled axons with clear visible origin from a DRG. Then, selected DRGs were surgically exposed and injected with rAVV (s. Fig. 2-3).

2.3.6 Confocal microscopy

Confocal images of fixed tissue were obtained on a FV1000 confocal system mounted on an upright BX61 microscope (Olympus) and equipped with an x10/0.4 water immersion objective and x20/0.85 and x60/1.42 oil immersion objectives. Stacks of 12 bit images were recorded and processed using the freeware ImageJ (http://rsbweb.nih.gov/ij).

2.3.7 *In situ* analysis of central axonal outgrowth

Central branches of fluorescently labeled DRG neurons were identified *in vivo* and imaged according to the *in vivo* imaging protocol by the Kerschensteiner and Misgeld labs (Kerschensteiner et al., 2005; Misgeld et al., 2007). The protocols describes how to image individual fluorescently labeled axons in the spinal cord of living transgenic mice expressing the green fluorescent protein (GFP) in a subset of sensory neurons. The decisive steps from the surgical exposure of the spinal cord until the recovery of the animal are briefly outlined in the following.

2.3.7.1 Surgical access to the dorsal surface of the spinal cord

Before starting surgical procedures, mice were given 5 µl Metacam (analgesic) per os by a pipette. Then, mice were intraperitoneally anaesthetized with ketamine-xylazine (ketamine 87 µg per g body weight, xylazine 13 µg per g body weight). Fur was clipped on the upper back and animals were positioned on a metal plate. Rubber bands connected to button magnets were used to attach all four extremities to the plate. A support pillow was put under the thorax to flex the vertebral column backwards and thus widen the interlaminar spaces (s. Fig. 2-1).

The skin in the surgery area was disinfected by Cutasept. Under the stereomicroscope a midline incision in the skin extending from the occipital bone to the upper thoracic part of the back was performed by a scalpel. Dorsal neck muscles were exposed and cut in the midline using large spring scissors. Customized magnet-mounted retractors were inserted to remove the muscles to the side. Sterile triangles (Sugi) were used to stop bleeding. Subsequently, a dorsal laminectomy was performed by removing the cervical vertebrae from C3 to C6 using spring scissors with angled blades. The dura was kept intact.

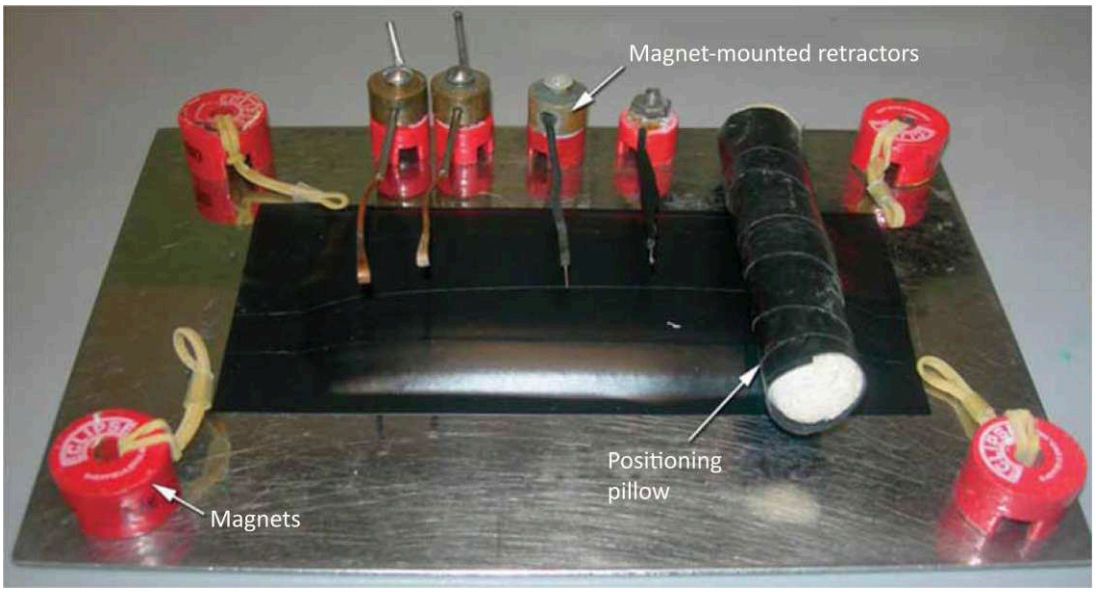

Fig. 2-1 Equipment for positioning of the animal

When the surface of the dorsal spinal cord is exposed (not shown), muscles are removed to the side by customized magnet-mounted retractors. A support pillow allows widening of the interlaminar space and rubber bands connected to button magnets attach the animal to the metal plate. Adapted from Misgeld et al., 2007.

2.3.7.2 Imaging of fluorescently labeled axons and viral injection

After surgical exposure of the spinal cord, *Thy*1-GFP^{s} mice were transferred to the imaging stage consisting of an Olympus BX51WI upright microscope, a filter wheel and a cooled CCD camera. The low-power x4/0.13 dry objective of the microscope and fluorescent excitation was used to visualize GPF positive axons of the dorsal spinal cord. Single fluorescently labeled axons emerging from a DRG and running in the superficial dorsal spinal cord were identified and documented by acquiring image streams of twenty-five to fifty image (frames) with a cooled Sensicam QE CCD camera controlled by MetaMorph® software (s. Fig. 2-2).

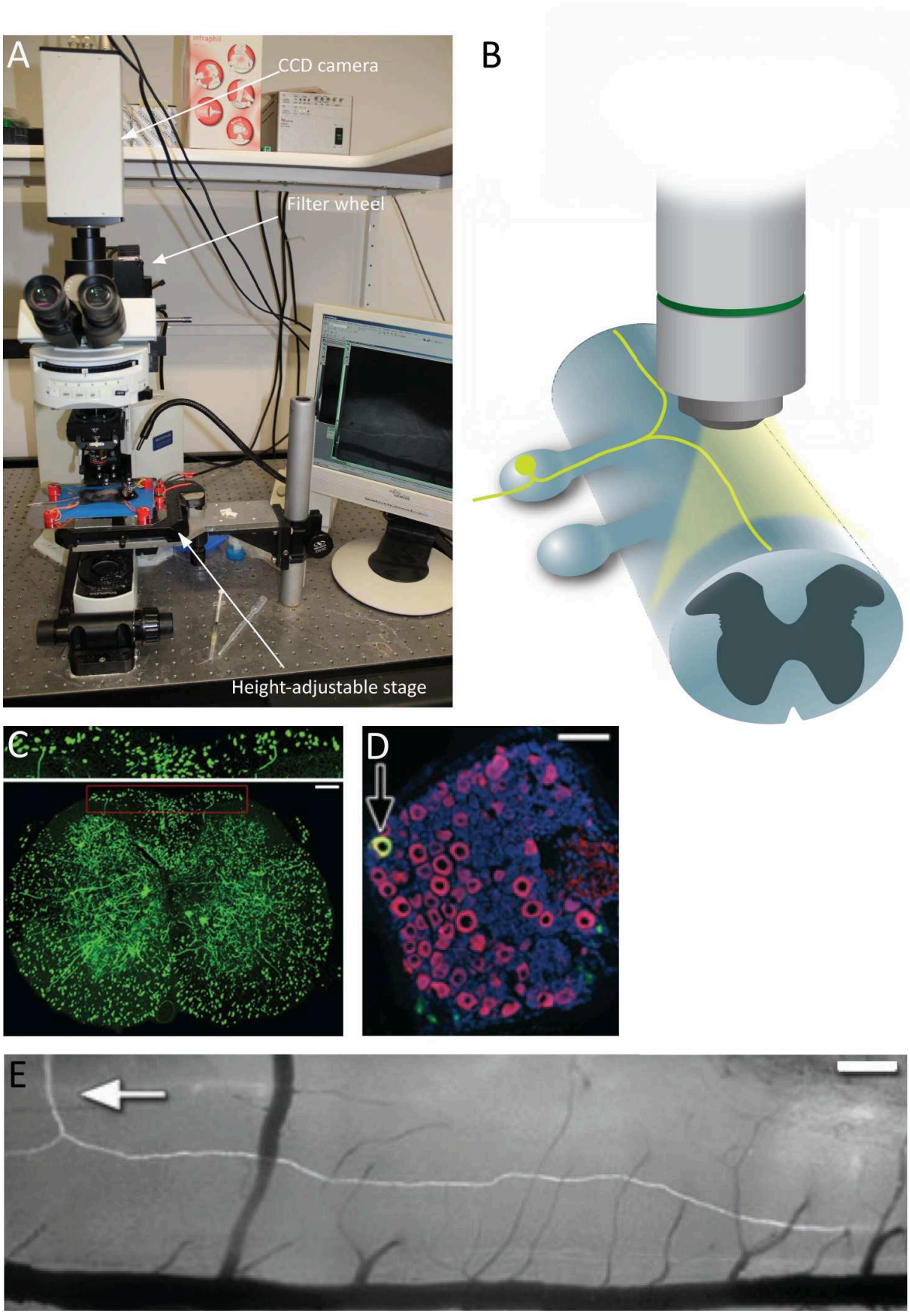

Fig. 2-2 *In vivo* imaging of fluorescent sensory DRG axons in *Thy*-GFP^{s} mice

Fig. 2-2 (*A*) *In vivo* microscopy setup consisting of a BX51WI upright microscope, a filter wheel and a CCD camera. (*B*) Schematic representation of the *in vivo* imaging of a single GFP positive DRG axon branching into a descending and an ascending branch. (*C*) Cross-section of the spinal cord of a *Thy*-GFP^{s} mouse. (D) Cross-section of a DRG of a *Thy*-GFP^{s} mouse stained with NeuroTrace. The arrow shows the soma of the fluorescent soma. (E) Black-and-white image showing an *in vivo* imaged single GFP-labeled DRG axon of a *Thy*-GFP^{s} mouse. Scale bars in *C* and *E* 200 µm and in *D* 100 µm; *C*, *D* and *E* adapted from Kerschensteiner et al., 2005.

The DRGs from which the selected axons emerged were then surgically prepared using a rongeur and 1 µl of the rAAVs was injected as described above (s. Fig. 2-3).

In the experiment to determine the effect of endogenous STAT3 expression on axonal sprouting after a CNS lesion (s. section 4.6), STAT3$^{fl/fl}$ mice were used. Here, the spinal cord and cervical DRGs were exposed and directly afterwards injected with a combination of 1 µl of rAAV-cre and rAVV-eCFP. Mice serving as control animals only received rAVV-eCFP. The rAVV- cre encodes for a cre recombinase which recognizes the two loxP-sites flanking the STAT3 gene in STAT3$^{fl/fl}$ mice and subsequently excises STAT3. The rAVV-eCFP encodes for enhanced cyan fluorescent protein and allows fluorescent identification of treated axons.

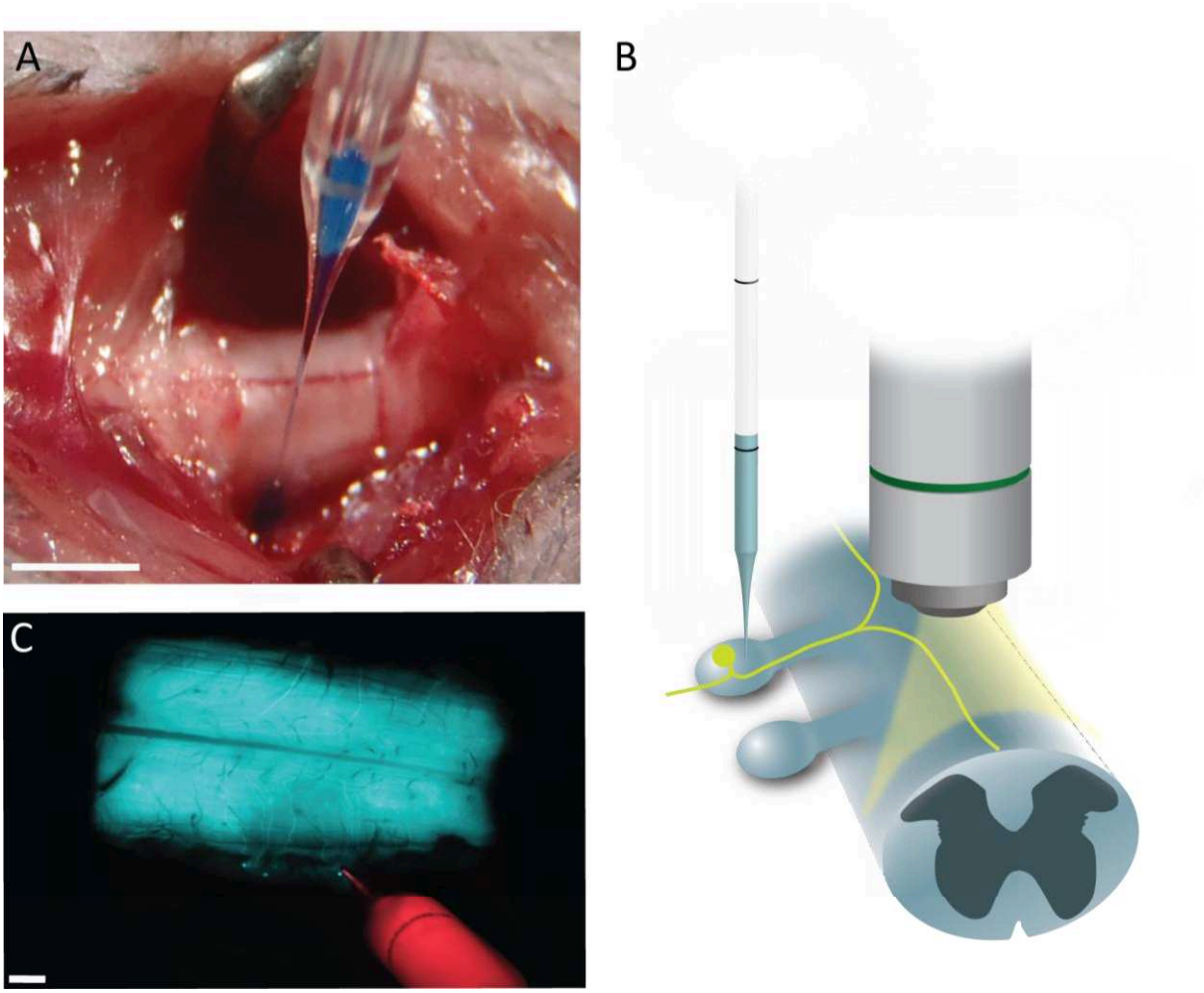

Fig. 2-3 Injection of rAAV into the DRG

(*A*) View of the surgically exposed spinal cord and injection of virus into a DRG with finely pulled glass capillaries. Muscles are displaced to the side by retractors. (*B*) Schematic representation of the injection process. (*C*) Reconstructed image from several frames acquired by *in vivo* imaging showing injection of a red fluorescent dye into a cervical DRG of a *Thy*-GFP^{s} mouse. Scale bars in *A* 2 mm and in *C* 500 µm. *B* was courteously provided by Fabian Laage-Gaupp.

2.3.7.3 Recovery of the animal

After having imaged the animal, dorsal neck muscles were adapted and sutured, whereas the skin was closed using clips. To rehydrate the animal 1 ml of Ringer's solution was subcutaneously injected postoperatively. Approximately two hours after surgery as well as one day after surgery mice were administered 5 µl of Metacam orally.

2.3.7.4 Lesion of the axon

Ten to twelve days after the DRG injection the dorsal surface of the spinal cord was surgically re-exposed and the previously selected fluorescent axon was re-identified under

the widefield microscope and transected using a hand-held 23-gauge hypodermic needle. The lesioned axon end was then documented *in vivo* (s. Fig. 2-4).

In the experiment of STAT3 deletion (s. section 4.6), viral application of rAVV-eCFP resulted in fluorescent labeling of axons, thus allowing identification of treated axons with a high probability. Ten days after injection, the spinal cord was re-exposed. Fluorescently labeled axons were identified under the fluorescent microscope and lesioned by a small diameter needle (s. above).

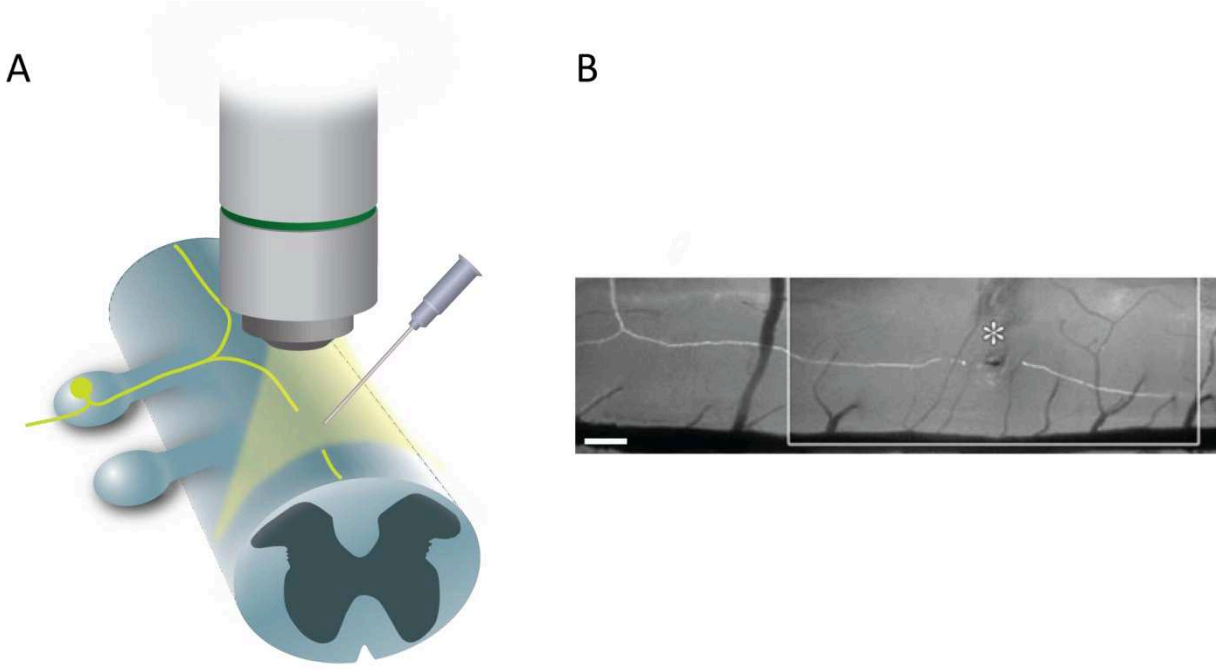

Fig. 2-4 Lesion of the axon emerging from a DRG previously injected with rAAV

(*A*) Schematic representation of the lesion process with a hand-held small diameter needle. (*B*) Black-and-white image showing a frame of an *in vivo* imaged single GFP-labeled DRG axon of a *Thy-GFP*^{s} mouse directly after lesion with a small diameter needle (asterix). Scale bar in *B* 200 µm. Image *B* modified from Kerschensteiner, et al. 2005.

2.3.7.5 Evaluation of the axonal outgrowth after lesion

To determine the frequency of axonal sprouting after rAAV-treatment, the animal was perfused transcardially with 4% PFA two days after the lesion and the previously imaged segment of the spinal cord was dissected (s. Fig. 2-5). At first, the spinal cord was imaged *in situ* mounting it in a customized imaging chamber consisting of a glass slide with a well of parafilm. The transected axon was documented using high resolution confocal microscopy as

described above. Then, some spinal cords were afterwards embedded in 2% Agarose and subsequently cut in 100 – 250 µm thick sections by a vibratome. The sections were mounted in Vectashield and re-imaged again to achieve better imaging quality. Confocal image stacks were processed as described above and the transected axons were reconstructed from the DRG root to the lesion site.

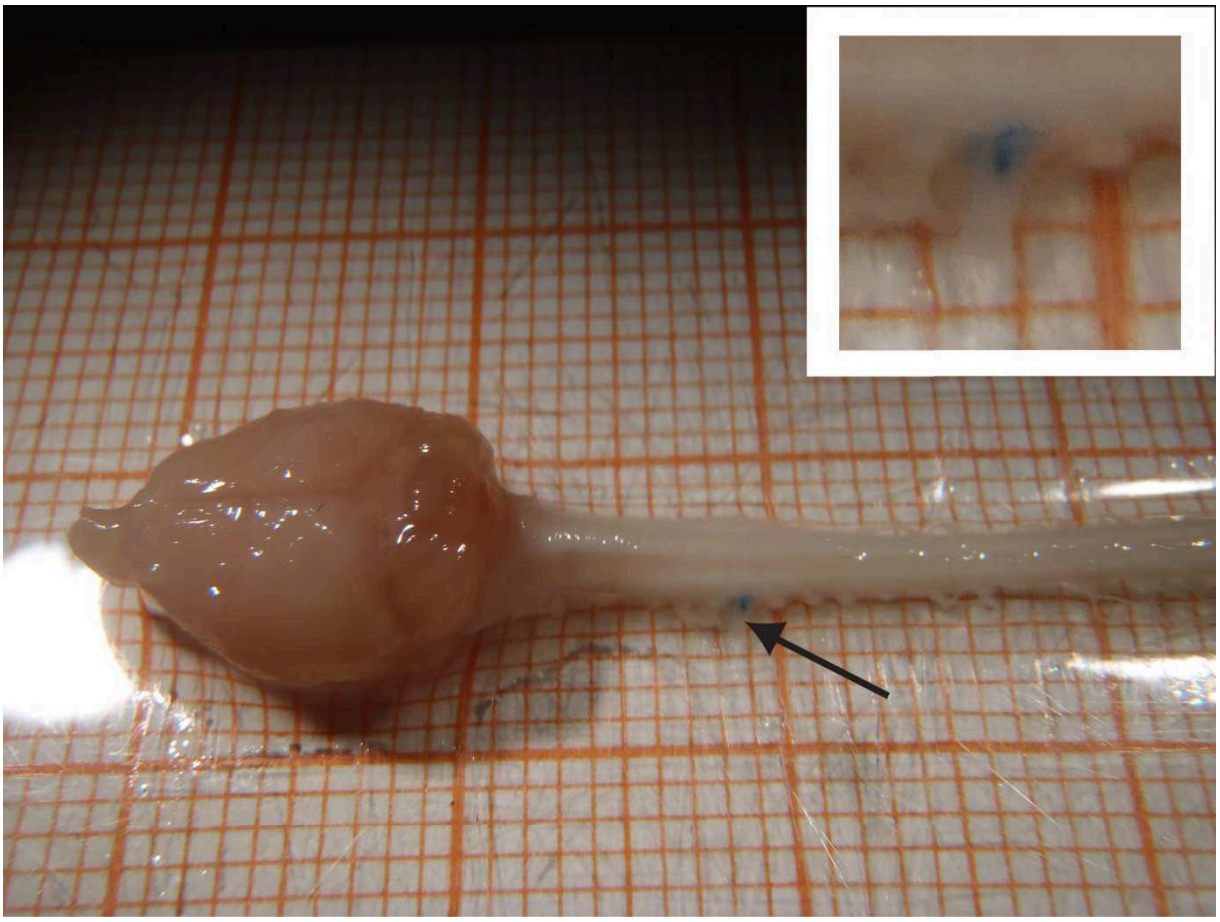

Fig. 2-5 Dissected spinal cord

Dissected spinal cord put on millimeter paper. The black arrow points to the injected DRG (blue dot). The inset shows the DRG and the root of the spinal nerve at a higher magnification. The photo was courteously provided by Fabian Laage-Gaupp.

Two blinded observers evaluated the axons by classifying their terminal ends as either “sprouts” or “bulbs” based on their characteristic morphological appearance. Furthermore, the number of collateral sprouts emerging from the transected axons was counted. In the experiment where I combined rAAV-STAT3 therapy and application of chondroitinase ABC

the lengths of axonal sprouts was measured. Axonal terminals ending in tips or bulbs were classified as non-growing axons and their outgrowth was set to 0 µm.

2.3.8 *In vivo* analysis of central axonal outgrowth

To determine the speed of axonal outgrowth after STAT3 supplementation in *Thy1*-GFP^{s} mice, transected axons were re-imaged *in vivo* at day 2 and 4 after lesion. For these purposes, a custom-build multi-photon imaging setup based on an Olympus FV 300 scanner equipped with a femto-second pulsed Ti:Sapphire laser (Mai Tai HP, Newport/Spectra-Physics) was used. Image stacks of 50 - 200 images per stack (spaced at 1 µm in z) were acquired for each frame with a 20x dipping cone water-immersion objective. During the imaging session the spinal cord was superfused with aCSF. After *in vivo* imaging at day 2 and day 4, animals recovered and at day 10 they were perfused transcardially with 4% PFA. The previously imaged part of the spinal cord was dissected out, the transected axons were re-identified in the fixed tissue and imaging of the axons in the intact spinal cord using an Olympus FV1000 MPE multiphoton microscope was performed. Image stacks of 20 - 100 frames were acquired with a 25x objective. Imaging stacks were processed using ImageJ software. Frames containing the axon in focus were selected and the transected axons were reconstructed using Photoshop. To determine the growth of the transected axon ends 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 two, four and ten days after lesion was measured. To compensate for tissue changes due to fixation in the perfused samples, a "constant" distance e.g. between two branch points in the same unlesioned axon both *in vivo* and in the corresponding fixed tissue was measured 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.

2.3.9 Combination therapy with viral gene transfer and chondroitinase ABC

Ten days following injection of either rAVV-STAT3 or rAVV-cre (control) into cervical DRGs of *Thy*1-GFP^{s} mice, the axons were lesioned and a first bolus of either 5 µl chondroitinase ABC (10 U/mL in saline with 0,01% BSA) or vehicle only (saline with 0,01% BSA) were

administered with a pipette to the lesion on the surface of the spinal cord. Directly after lesion, an osmotic minipump filled with either chondroitinase ABC or vehicle only (control animals) was connected to the brain infusion kit. The latter one was inserted into the lateral ventricle and the pump was positioned between cervical muscles and skin. The pumps then started delivering 0,5 µl/h of either chondroitinase ABC or saline for seven days. Ten days after lesion, animals were perfused with PFA, the spinal cords were imaged *in situ* by confocal microscopy (s. above) and the outgrowth was evaluated as described above. To confirm delivery of chondroitinase ABC, spinal cords were embedded in 2% Agarose and subsequently cut in 100 – 250 µm thick vibratome sections. Then, free floating immunohistochemistry using then Anti-Proteoglycan mouse IgG1 (clone 2-B-6) antibody was performed as described above. In addition, loaded pumps were weighed before implantation and the weight was compared to the weight the pumps had at the end of the experiment to confirm delivery.

2.3.10 Statistical analysis

Results are given as mean ± SEM unless indicated otherwise. Statistical significance was determined using GraphPad Prism (GraphPad Software). All data were analyzed using either a one-way ANOVA followed by a Tukey post-hoc test for multiple comparisons or a t-test for single comparisons. To evaluate the proportion of terminal sprouts after rAAV treatment, a frequency analysis was made using a Chi square test. Significance levels were chosen as follows: * = $p<0.05$; ** = $p<0.01$ *** = $p<0.001$.

3 Experiments in the Course of this Study prior to my own Work

First experiments of this study defining the role of STAT3 for axonal regeneration in the PNS were performed by Dr. Florence Bareyre. As the results of these investigations marked the starting point of my own subsequent research, the main findings are briefly outlined in the following.

STAT3 depletion impairs the regeneration of PNS axons

First, it could be demonstrated that after bilateral saphenous nerve lesion, STAT3 expression was highly upregulated already one hour after injury until two weeks after transection. Second, selective deletion of STAT3 in DRG neurons of $STAT3^{fl/fl}$ mice by adeno-associated viral delivery of cre recombinase impaired regeneration of lesioned peripheral axons: At day 4, STAT3-deficient mice showed impaired regeneration compared to STAT3-competent axons. However, at day 14, sprouting and axonal regeneration was comparable between both groups, indicating that STAT3-deficient axons could still initiate axonal outgrowth, however, with a delay.

STAT3 deletion impairs initiation but not perpetuation of PNS axon regeneration *in vivo*

To then define if impaired regeneration of STAT3-deficient peripheral axons was due to a) delayed growth induction or b) to reduced elongation of regenerating axons, axonal outgrowth was observed by repetitive *in vivo* imaging. Whereas many STAT3-competent DRG axons initiated growth within two days after lesion and progressed with an average speed of 132 ± 23 µm/day from day 2 to day 3, the vast majority of STAT-3 deficient axons failed to initiate growth during that time. Their regeneration speed was only 33 ± 6 µm/day. However, during the phase of axon elongation at seven to eight days after lesion, there was no difference between the regeneration speed of the two groups (411 ± 48 µm/day in STAT3-competent axons versus 341 ± 53 µm/day in STAT3-deficient axons). This indicated that STAT3 is crucial for initiation but not for elongation of axonal regeneration.

4 Results

4.1 Consequences of a peripheral crush lesion on STAT3 expression

As described in the previous section (s. section 3), it could be shown that STAT3 expression is significantly upregulated after a peripheral nerve cut lesion. Moreover, it could be demonstrated that STAT3 is crucial for the induction of axonal outgrowth but not for subsequent elongation of PNS axons. These results not only suggest that the neuronal growth response to the lesion is divided into at least two different phases but also allow the hypothesis that components of the neuronal environment, such as Schwann cells forming the nerve sheath might influence the distinct phases of axonal regeneration. To address this question, saphenous nerve crush lesions (performed by Dr. Florence Bareyre) that do not interrupt Schwann cell nerve sheaths were performed in *Thy*1-YFP[16] mice. Wallerian degeneration of crushed nerves was observed resulting in approximately 85% of axonal loss distal to the lesion indicating that most axons were interrupted by the crush lesion. I consecutively examined the DRGs that corresponded to the crushed nerves for STAT3 activation by immunohistochemistry at different time points after lesion (s. Fig. 4-1).

The results show that STAT3 is not significantly upregulated at any of the time points. At maximum, two days after lesion 30 ± 3% of DRG neurons are positive for P-STAT3. These results suggest that if Schwann cell guidance is maintained STAT3 is not needed for initiation of axonal regeneration.

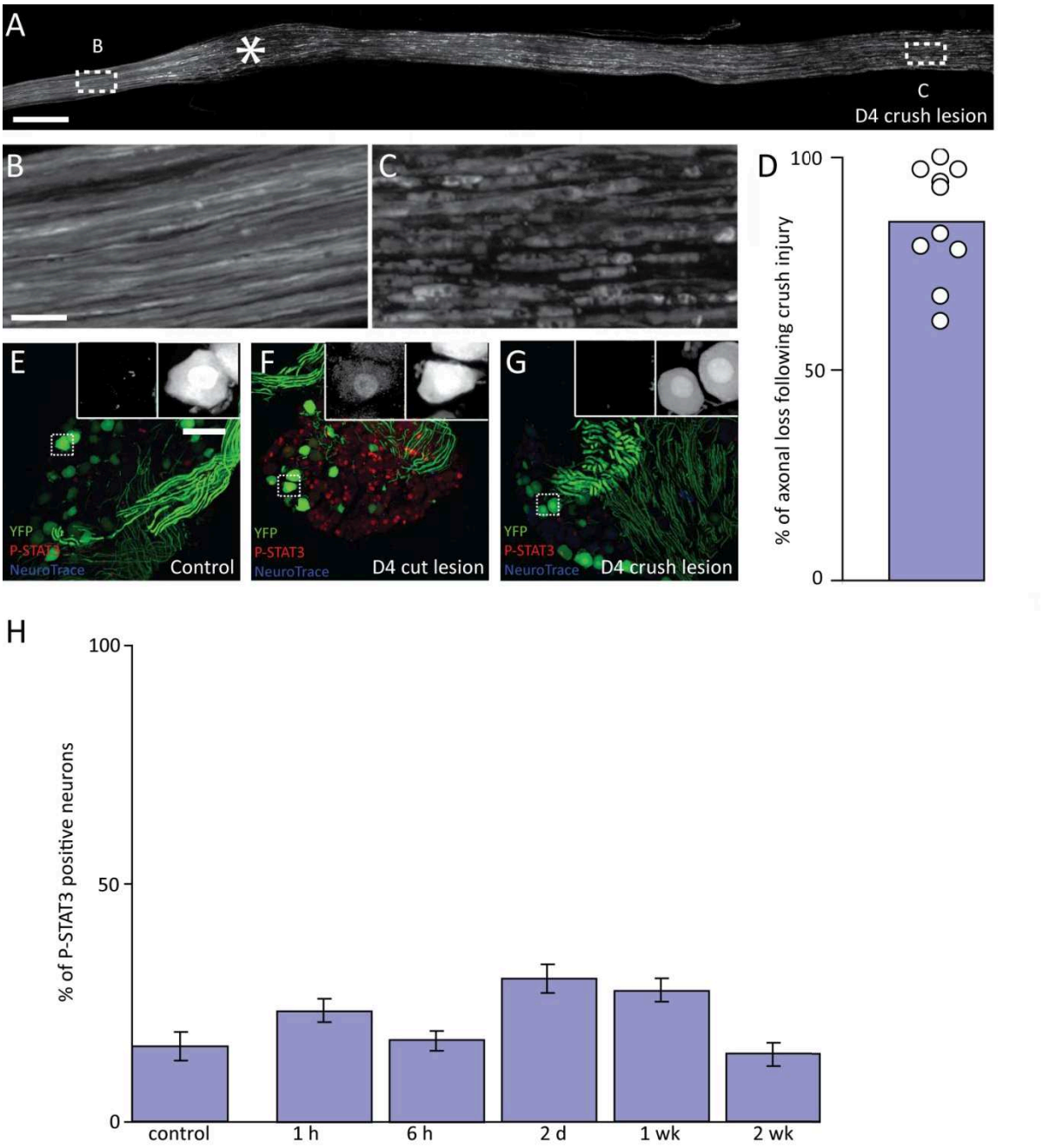

Fig. 4-1 Wallerian degeneration and STAT3 expression after peripheral nerve crush lesion

(*A*) Confocal image showing Wallerian degeneration of axons in the distal stump of a saphenous nerve four days following saphenous nerve crush (indicated by asterisk) in *Thy*1-YFP[16] mice. The areas boxed in *A* show intact axons rostral to the lesion (*B*) and axonal fragments distal to the lesion (*C*) at a higher magnification. (*D*) Quantification of the loss of continuous axons distal to the lesion at four days after injury. (*E-G*) Confocal images show STAT3 activation in an unlesioned control DRG (*E*), in a DRG four days following saphenous nerve cut (*F*) and in a DRG four days following saphenous nerve crush (*G*). (*H*) Quantification of the number of P-STAT3–positive DRG neurons (which were identified by NeuroTrace counterstaining at different time points following saphenous nerve crush). n = 5-8 DRGs for each time point. Scale bars in *A* 400 µm, in *B* 40 µm and in *E* 100 µm. Figure modified from Bareyre, Garzorz et al., 2011.

4.2 STAT3 expression after dorsal column lesion

As previously outlined, a lesion of the peripheral branches of DRG neurons is followed by successful regeneration as well as significant upregulation of STAT3 activation. To first determine if there is a correlation between the weak growth response of neurons after a lesion of their central projections and endogenous STAT3 levels, the expression of phosphorylated STAT3 in DRG neurons was detected at one hour, six hours, two days, one week and 2 weeks after bilateral dorsal column transection. It could be found that P-STAT3 immunoreactivity in DRG neurons after lesion of the central branch did not show significant changes from STAT3 activation in unlesioned control animals: Baseline STAT3 activation, represented by the percentage of P-STAT3 positive neurons in uninjured animals was 15 ± 3% (s. Fig. 4-2, Fig. 4-3). In mice having undergone bilateral column lesion 29 ± 6% of neurons were P-STAT3 positive after one hour of injury and only 12 ± 3% of neurons were P-STAT3 positive two days after injury. These findings contrast strongly with those in the PNS, where a lesion of the saphenous nerve leads to sustained STAT3 activation in more than 70% of L3 DRG neurons.

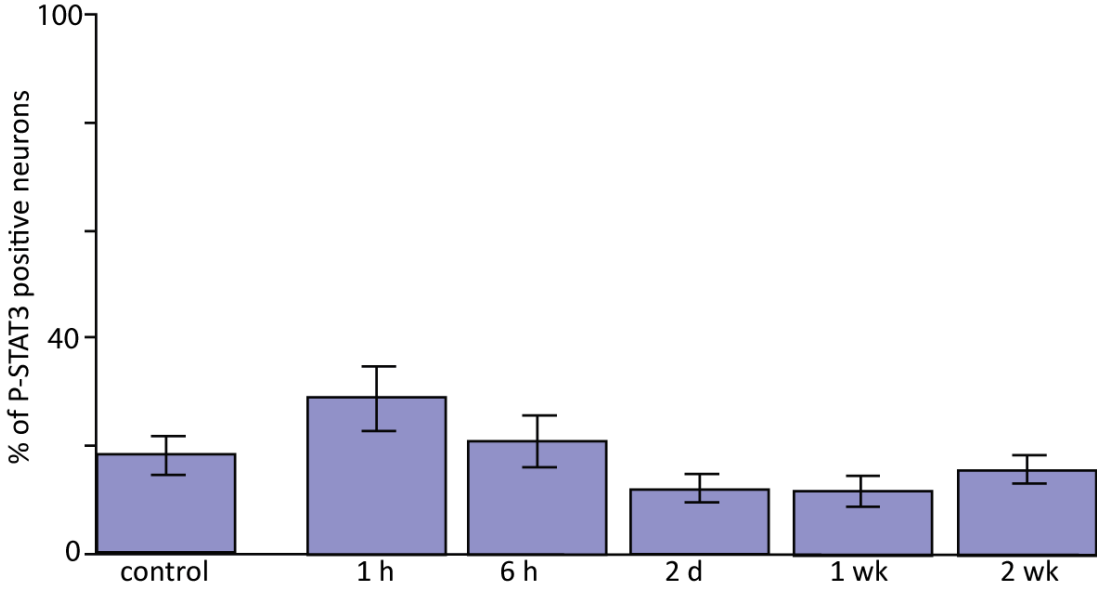

Fig. 4-2 STAT3 expression after dorsal column lesion

Quantification of the number of P-STAT3 positive neurons identified by NeuroTrace at different time points following dorsal column lesion in WT mice and in control WT mice. n = 6 DRGs per time point.

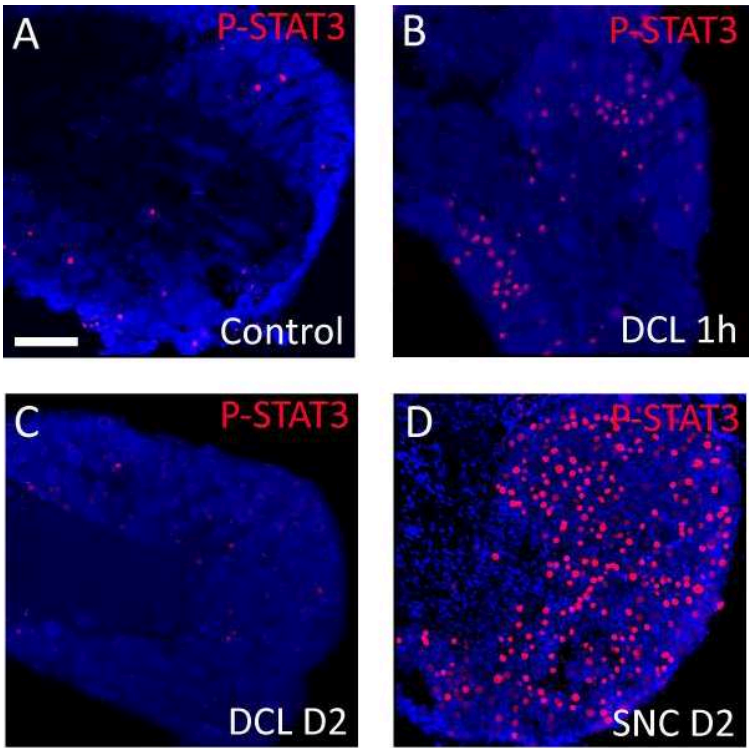

Fig. 4-3 STAT3 expression after dorsal column lesion (Immunohistochemistry)

Confocal images of cervical DRGs immunostained for P-STAT3 (red) and counterstained with NeuroTrace 435 in Wt unlesioned control mice (*A*) and in Wt mice one hour (*B*) and two days (*C*) after dorsal column lesion (DCL). (*D*) In comparison P-STAT3 expression in L3 DRGs of Wt mice after saphenous nerve cut lesion (SNC) two days after injury. Scale bar in *A* 100 µm. Figure modified from Bareyre, Garzorz et al., 2011.

4.3 Efficiency of the viral gene therapy

To determine whether activation of STAT3 in DRGs is sufficient to induce central axonal regeneration I first assessed if STAT3 expression could exogenously be increased by viral gene therapy. Thus, rAAVs were produced expressing STAT3 (rAAV-STAT3) or a constitutively active variant of STAT3 (rAAV-STAT3c) and the efficiency of the viral gene transfer was investigated by injection of the viruses into cervical DRGs of adult C57/bl6 WT mice. STAT3 activation was then assessed for three time points (ten, twelve and fourteen days after injection) by immunohistochemistry with anti-STAT3 and anti-P-STAT3-antibodies on cryostat-cut DRG sections (s. Fig. 4-4). To rule out that virus injection might itself trigger an increase of endogenous STAT3 levels, I injected rAAV-eCFP as control viruses. Quantification of STAT3 activation in DRGs injected with control viruses was performed in context of the following experiment (s. section 4.4).

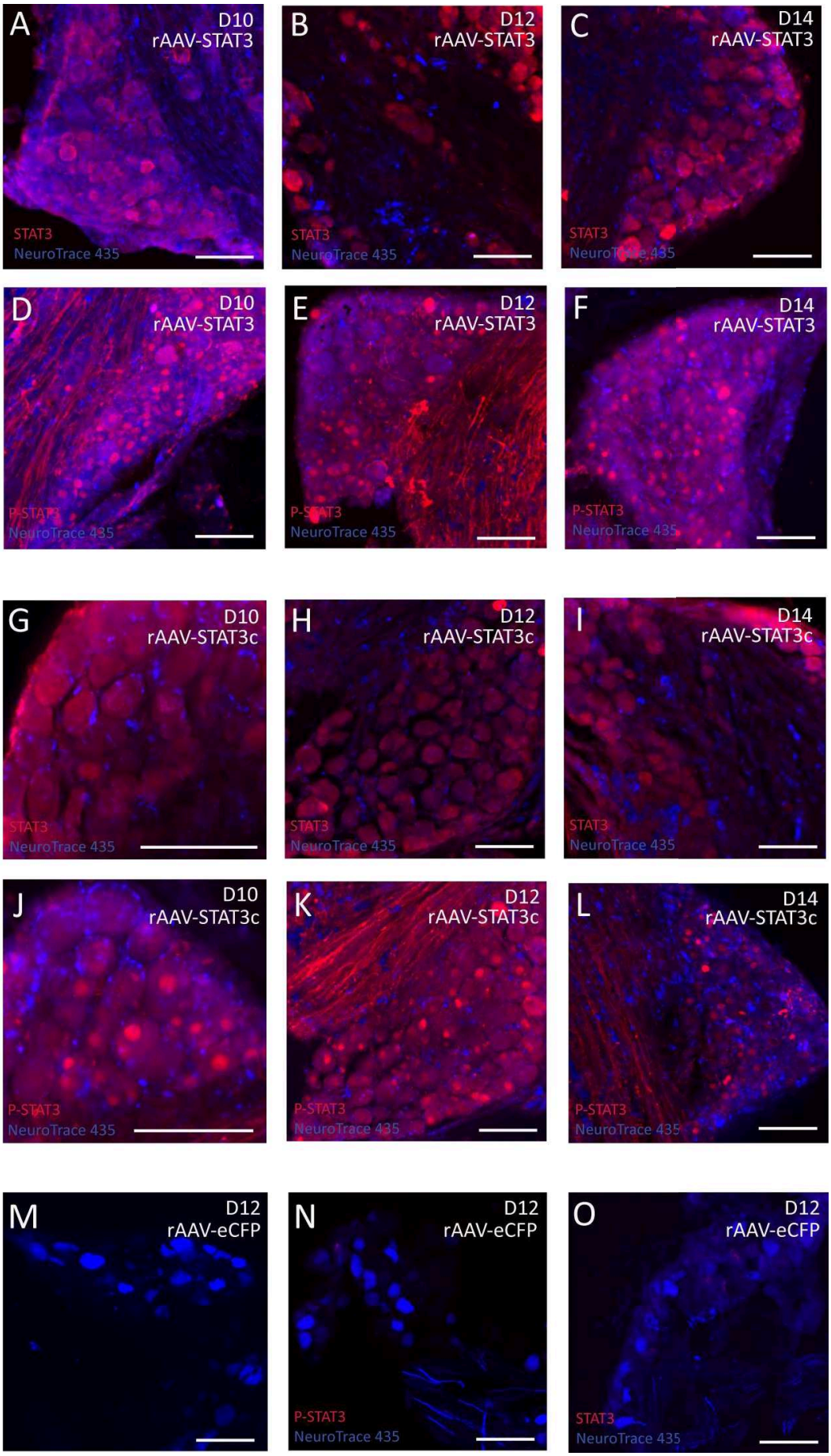

Fig. 4-4 Evaluation of the efficiency of the viral gene therapy by immunohistochemistry

Fig. 4-4: rAAV-STAT3 and rAAV-STAT3c are effective tools to deliver STAT3 to cervical DRG neurons. Images taken with the Olympus IX71 inverted fluorescence microscope illustrating STAT3 and P-STAT3 immunoreactivity at ten days (*A,D,G,J*), twelve days (*B,E,H,K,N,O*) and fourteen days (*C,F,I,L*) after injection of rAAV-STAT3 (*A-F*), rAAV-STAT3c (*G-L*) or rAVV-eCFP as conrol virus (*M-O*). To quantify the percentage of STAT-3 positive neurons, neurons were identified by NeuroTrace 435 counterstaining (*A-L, N and O*). In contrast to rAAV-STAT3 and rAAV-STAT3c, rAAV-eCFP treatment is not resulting in increased levels of STAT3 activation. Scale bars in *A-O* 100 µm.

On average 80% of DRG neurons are transduced by rAAV-STAT3 and rAAV-STAT3c which can result in high levels of P-STAT3 expression at all three time points analyzed (s. Fig. 4-5).

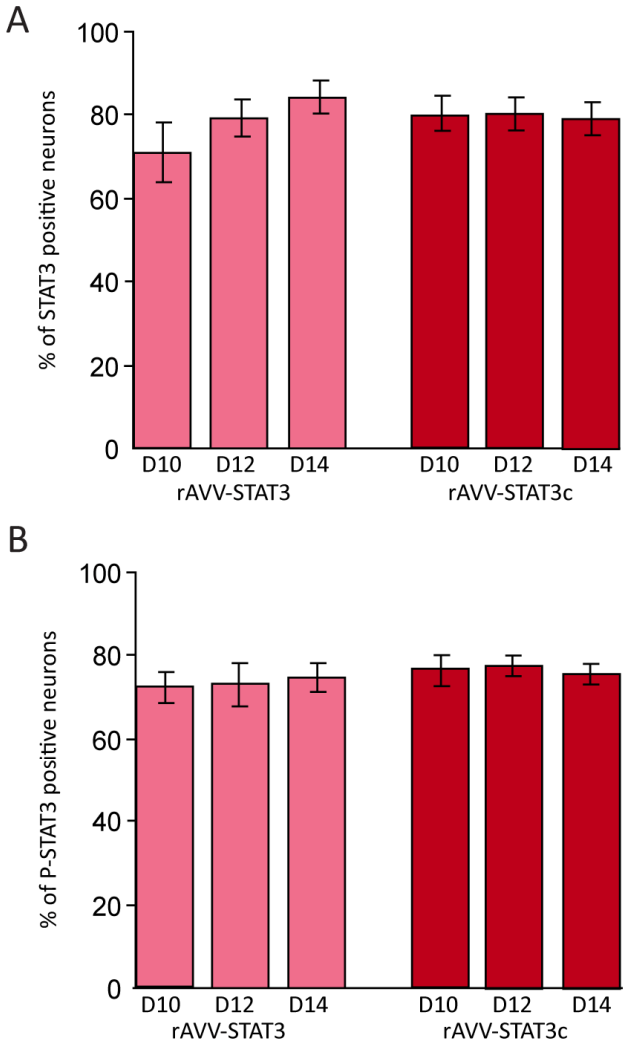

Fig. 4-5 Quantification of the Efficiency of the viral gene therapy)

On average 80% of DRG neurons show STAT3 (*A*) and P-STAT3 (*B*) expression ten, twelve and fourteen days after injection of rAAV-STAT3 and rAAV-STAT3c which shows that rAAV are effective tools to deliver STAT3 to cervical DRG neurons. n = 3-5 DRGs per group and time point.

Interestingly, both STAT3c and STAT3 expression led to a similar percentage of P-STAT3 positive neurons. Transduction with rAAV-STAT3 is thus sufficient to induce cytoplasmatic STAT3 expression as well as its phosphorylation and translocation to the nucleus. The finding that overexpressed STAT3 is readily phosphorylated and transported into the nucleus suggests that at least in this case STAT3 activation is regulated on the expression and not on the phosphorylation level. In conclusion, STAT3 expression can be successfully substituted by viral gene therapy and viral gene transfer is thus a suitable tool to investigate the effects of STAT3 overexpression on axonal outgrowth in the following experiments.

4.4 Effects of viral gene therapy with STAT3(c) on axon outgrowth after CNS lesion

For the investigation of central axonal regeneration *Thy1*-GFP^{s} mice were used. In these animals the green fluorescent protein is expressed stochastically in only a small subset of neurons allowing imaging of individual central projections from cervical DRG neurons. To examine the effect of STAT3 gene therapy on the outgrowth of lesioned central DRG branches, I identified suitably labeled central branches of DRG neurons in the dorsal funiculus of the cervical spinal cord and injected rAAV either expressing STAT3 (rAAV-STAT3), a constitutively active form of STAT3 (rAAV-STAT3c) or a control protein (eCFP or cre recombinase) into the corresponding DRGs. Efficiency of viral gene transfer was confirmed by immunohistochemistry (s. Fig. 4-6).

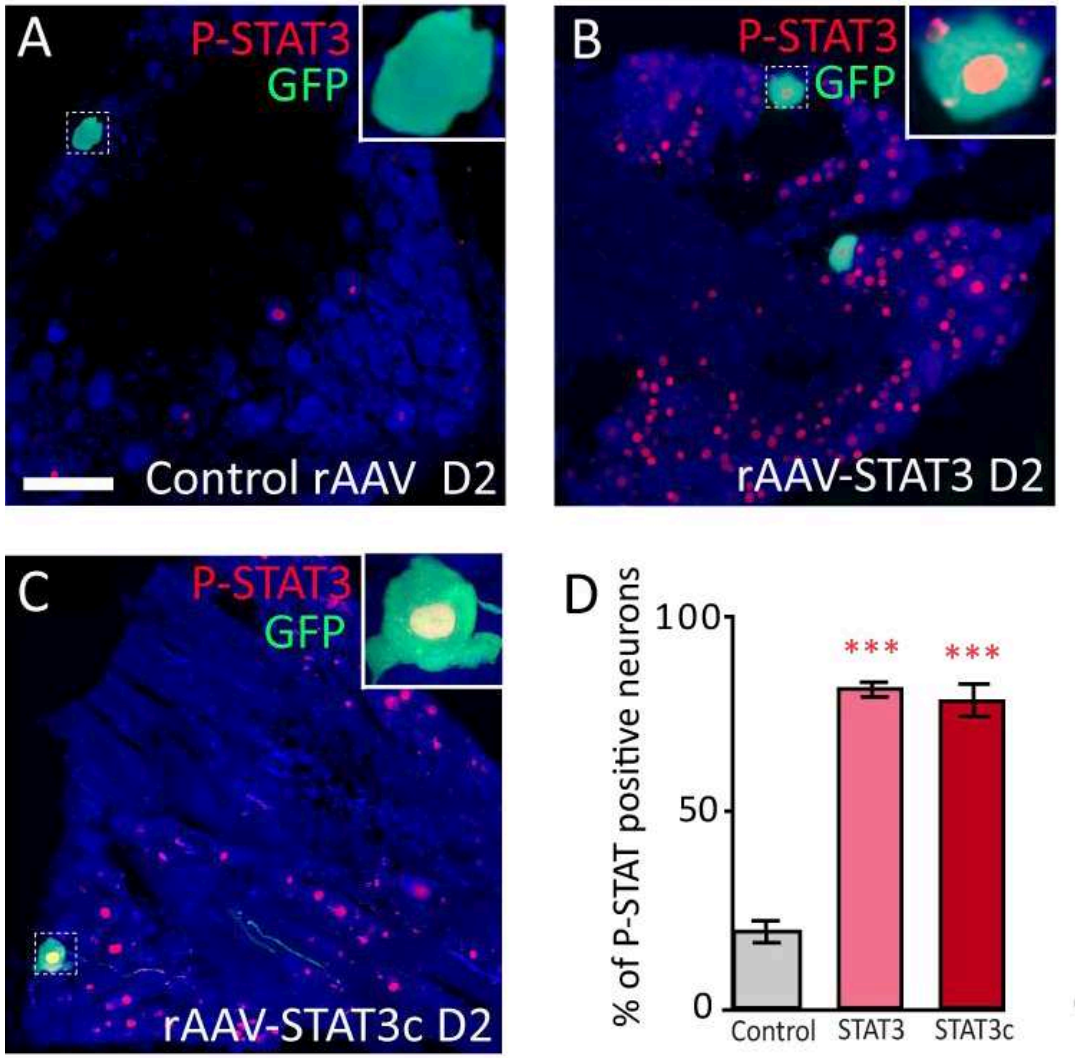

Fig. 4-6 Effects of viral gene therapy on CNS outgrowth

Confocal images of cervical DRGs immunostained for P-STAT3 and counterstained with fluorescent NeuroTrace 435 in lesioned *Thy1*-GFP mice injected with control rAVV (*A*), rAAV-STAT3 (*B*) or rAVV-STAT3c (*C*). Insets: Higher magnification (x3) of the GFP positive neurons boxed in the images. (*D*) Quantification of the number of P-STAT3 positive DRG neurons in mice previously injected with either control rAAV (grey column), rAAV-STAT3 (pink column) or rAAV-STAT3c (red column) at two days after a central lesion. n = 6 animals per group. Scale bar in *A* 100 µm. Figure modified from Bareyre, Garzorz et al., 2011.

Ten to twelve days after the injection of the virus, the spinal cord was surgically re-exposed, and the axons emerging from the injected DRGs were re-identified and selectively lesioned with a small-diameter needle. Two days after lesion, the axons were re-imaged *ex vivo* by high–resolution confocal microscopy and their endings were classified as „bulbs“ (non-growing axons) or „sprouts“ (growing axons). The time course of this experiment is shown in Fig. 4-7 and the results of the experiment in Fig. 4-8 and Fig. 4-9.

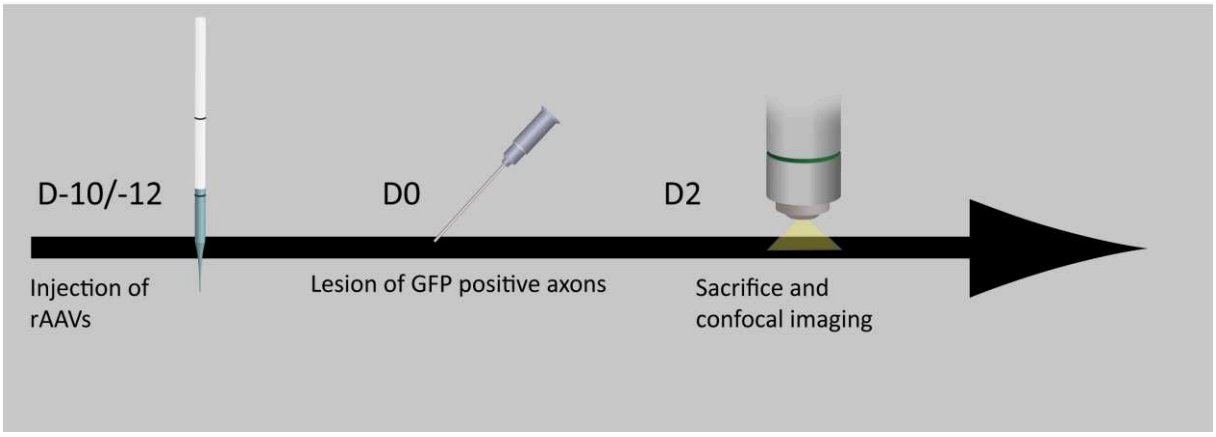

Fig. 4-7 Time course of the experiment

Ten to twelve days after injection of the virus, the axons were lesioned and the regenerative response was imaged 2 days after lesion.

As expected, two days after lesion only 8% of axons emerging from DRGs injected with control rAAV have formed sprouts (Fig. 4-8 A and B; Fig. 4-9 A). In contrast, 50% of the axons emerging from DRGs injected with rAAV-STAT3 (Fig. 4-8 C and D; Fig. 4-9 B) and 54% of the axons emerging from DRGs injected with rAAV-STAT3c (Fig. 4-8 E and F; Fig. 4-9 C) showed an early growth response. Another interesting finding is that STAT3 expression not only increased terminal sprouting, but also collateral sprouting along the axon. When testing viral efficiency in WT mice (s. section 0) it could be found that STAT3c and STAT3 overexpression led to a similar percentage of P-STAT3 immunoreactivity suggesting that substitution of STAT3 alone is sufficient to induce its phosphorylation and transport to the nucleus. In line with this finding, the similar growth responses of DRG neurons injected with rAAV-STAT3 and rAAV-STAT3c furthermore suggest that overexpression of STAT3 alone is not only sufficient to induce its phosphorylation and import to the nucleus but also its down-stream effects on regeneration.

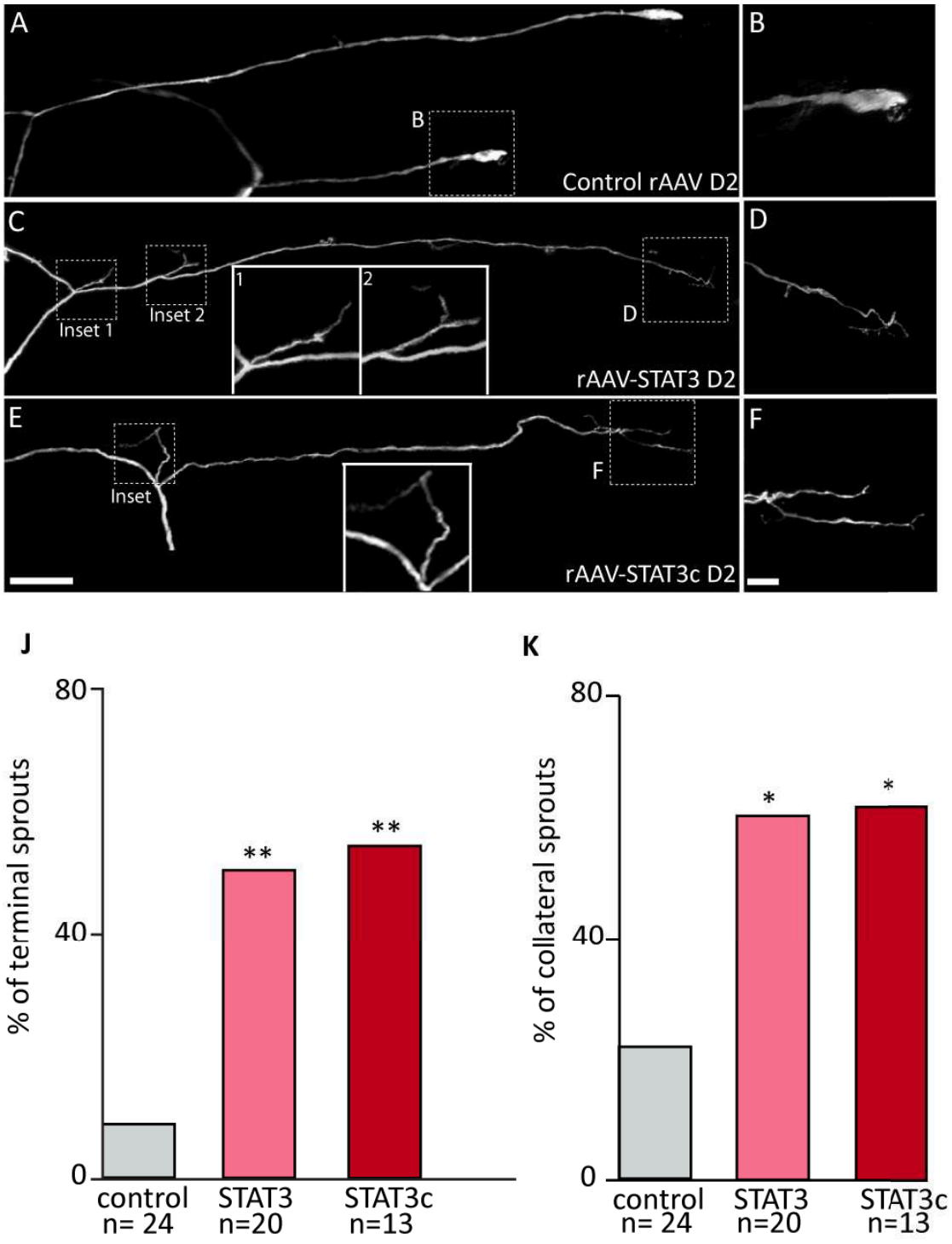

Fig. 4-8 Effects of viral gene therapy on CNS outgrowth – confocal microscopy

Confocal images of lesioned central branches derived from DRGs injected with control rAAV (*A, B*), rAVV-STAT3 (*C, D*) or STAT3c (*E, F*). *B, D* and *F*: Higher magnification of axonal endings boxed in *A, C* and E. Boxed collateral sprouts are shown at higher magnification x2 in additional insets (*C, E*). Quantification of terminal (*G*) and collateral (*H*) sprouting of rAAV-STAT3, rAVV-STAT3c and control rAAV transduced central DRG branches two 2 days after transection. Scale bars: E 100 µm, F 25µm. Figure modified from Bareyre, Garzorz et al., 2011.

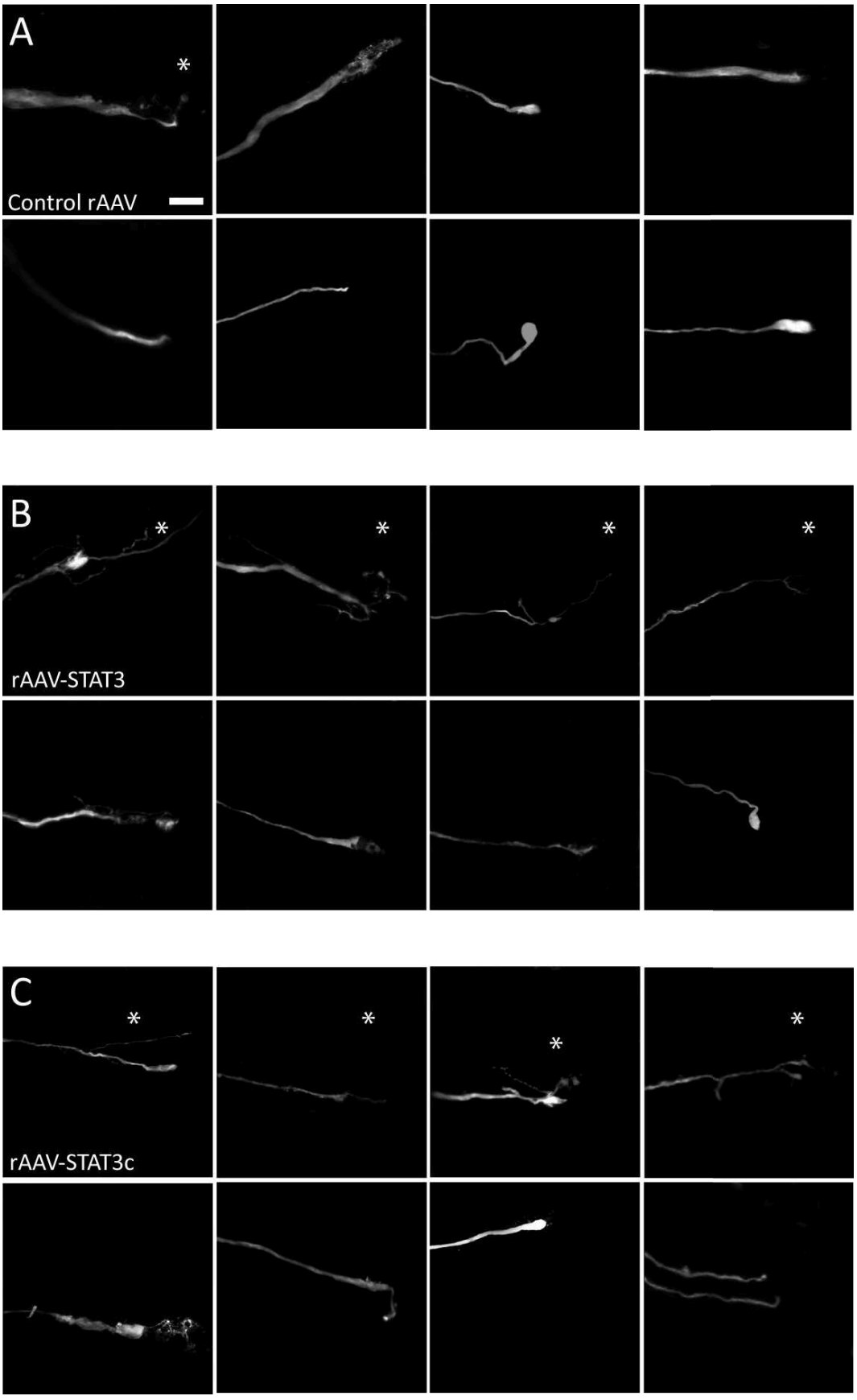

Fig. 4-9 Gallery of axonal endings

Fig. 4-9: Representative selection of axon endings two days after lesion. Axons emerged from DRG neurons that were injected with either control rAAV (*A*), rAAV-STAT3 (*B*) or r-AAV-STAT3c (*C*). Axonal sprouts (asterisks) are rarely found in control axons but are frequent in axons emerging from DRG neurons expressing STAT3 or STAT3c. Scale bar in *A* 25 µm. Figure modified from Bareyre, Garzorz et al., 2011.

4.5 The phase-specific role of STAT3 for axon regeneration in the CNS

The previous experiment revealed that overexpression of STAT3 increased the growth response in a substantial fraction of transected central DRG neurons. However, in line with the investigations in the PNS where STAT3 has turned out to initiate but not perpetuate axon regeneration, I wanted to determine if overexpression of STAT3 affected different phases of axon outgrowth. To show the growth pattern of individual axons in the same animal at distinct time points I used repetitive *in vivo* and *ex vivo* imaging (s. Fig. 4-10)

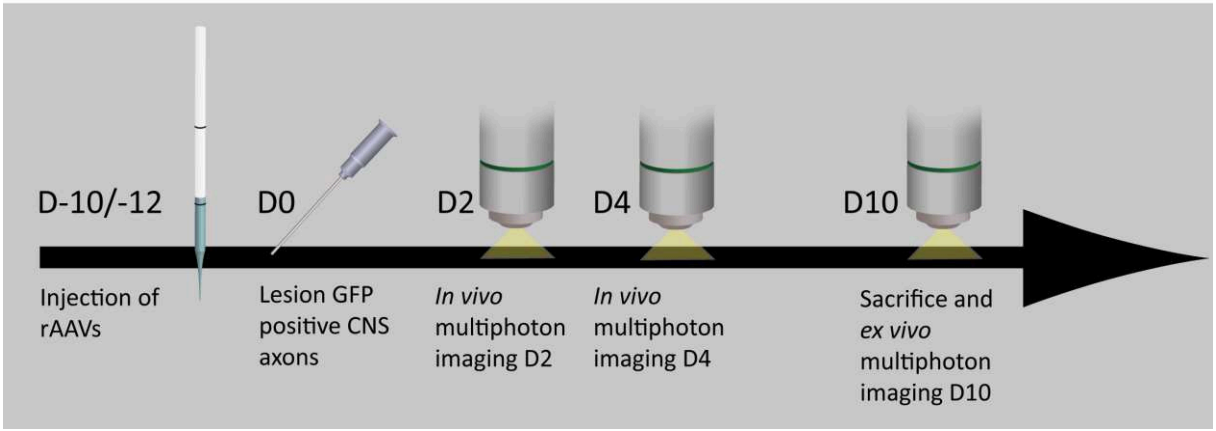

Fig. 4-10 Timecourse of the experiment

Ten to twelve days after injection, axons were lesioned and *in vivo* imaged at two and four days after lesion. Ten days after lesion the axon was imaged *ex vivo*.

To determine if STAT3 can not only initiate but also perpetuate axonal outgrowth, DRGs of *Thy1*-GPFs mice were either injected with control rAAV, rAAV-STAT3 or rAVV-STAT3c. As described above axons emerging from the injected DRG were re-identified and lesioned ten to twelve days after gene therapy. Two and four days after lesion, the spinal cord was surgically re-accessed and the selected axon was re-imaged by *in vivo* multi-photon

microscopy. After lesion at D10, animals were perfused and the axons were imaged by *ex vivo* multi-photon microscopy. Axonal lengths at each time point were measured and the speed of growth between D2 and D4 after lesion as well as between D4 and D10 was measured.

I could show that viral STAT3 treatment increases the speed of axonal growth in the early phase (two to four days) of regeneration (33 ± 8 µm/day in rAAV STAT3 axons and 36 ± 17 µm in rAAV STAT3c transduced axons vs. 7 ± 2 µm/day in control axons; Fig. 4-11 A,B and C). However, this early growth cannot be sustained and only limited axonal extension can be observed in all three groups between D4 and D10 after lesion (1,2 ± 0,7µm/day in rAAV-STAT3 transduced axons and 10 ± 8 µm in rAVV-STAT3c transduced axons vs. 1 ± 0,5 µm/day in control axons, Fig. 4-11 A, B and D). In line with what has been shown in the PNS, STAT3 controls the initiation but not the elongation of axonal outgrowth.

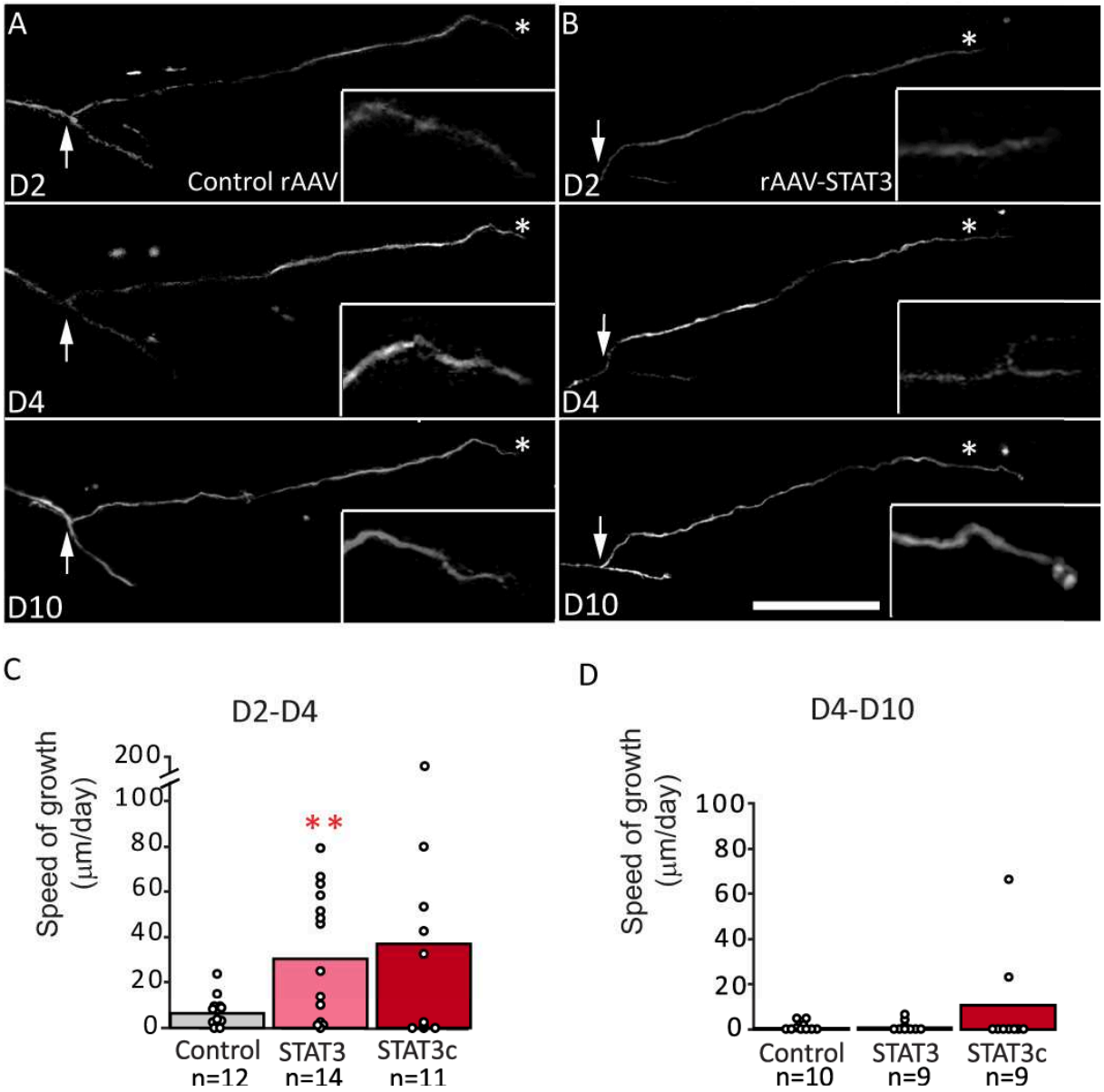

Fig. 4-11 Phase specific role of STAT3 for axonal regeneration

Multiphoton images of the lesioned DRG axons emerging from DRGs injected with control rAAV (*A*) or rAVV-STAT3 (*B*) imaged at two, four and ten days after lesion. Insets show magnification of axon ends. (*C* and *D*) Quantification of axonal growth speed *in vivo* between D2 and D4 (*C*) and between D4 and D10 (*D*) after injection of control rAAV (grey bars), rAVV-STAT3 (red bars) or rAVV-STAT3c (orange bars). Scale bar in B 200 µm. Figure modified from Bareyre, Garzorz et al., 2011.

4.6 Effects of STAT3 deletion on axonal outgrowth after CNS lesion

In the PNS, STAT3-deficient axons show minimal sprouting and extension during the initial phase of regeneration compared to STAT3-competent neurons. However, at later time points, sprouting around the lesion site and the speed of axonal regeneration between STAT3-deficient and STAT-3 competent axons is comparable. This suggests that after a lag phase other regulators can initiate regeneration. To investigate if STAT3 deficiency would influence sprouting in the same way it does in the PNS, STAT3 was deleted in cervical DRG neurons of STAT3$^{fl/fl}$ mice (Takeda et al., 1998). In these mice, STAT3 is flanked by two loxP-sites that can be recognized by a cre recombinase which subsequently deletes STAT3 (s. Fig. 4-12).

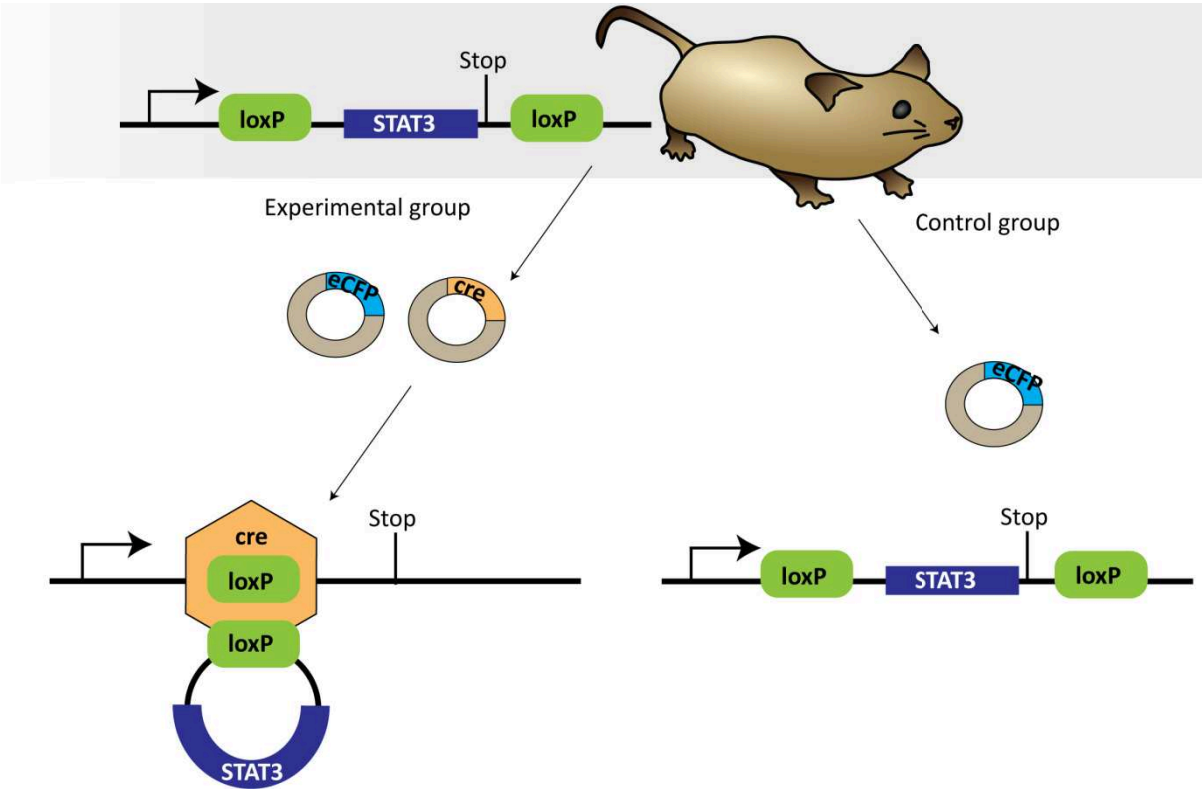

Fig. 4-12 Schematic illustration of STAT3 deletion in STAT3 $^{fl/fl}$ mice via cre recombinase

Schematic illustration of the cre/loxP system (Kuhn et al., 1997) used in this experiment to specifically delete STAT3 in DRG neurons. The cre recombinase recognizes the two loxP sites flanking the STAT3 gene and subsequently excises the sequence coding for STAT3. In control animals where the cre recombinase is absent, the STAT3 gene stays in the genome.

DRGs were injected with 1 µl of rAAV-eCFP (control animals) or rAAV-eCFP and rAVV-cre (experimental animals) encoding for cre recombinase. Ten days after viral application the eCFP fluorescently labeled axons were identified under the fluorescent widefield microscope and lesioned by a small diameter needle. Two days after injection, animals were perfused and axonal sprouting was evaluated. Immunohistochemisty on control and experimental animals was performed to determine the level of STAT3 activation after viral treatment. As expected, in animals receiving the combination of rAAV-eCFP and rAAV-cre, the percentage of P-STAT3 positive DRG neurons was significantly reduced compared to those who received eCFP only (23 ± 4% in STAT3-competent DRGs vs. 8 ± 2% in STAT3-depleted DRGs).

I found that the anyhow low baseline sprouting of CNS neurons is not influenced by STAT3 deletion: 15% of axons emerging from STAT3-deficient DRGs show sprouts compared to 12% sprouts in STAT-3 competent axons (s. Fig. 4-13). This might suggest that in analogy to the findings in the PNS, additional factors can initiate axonal outgrowth independent of STAT3.

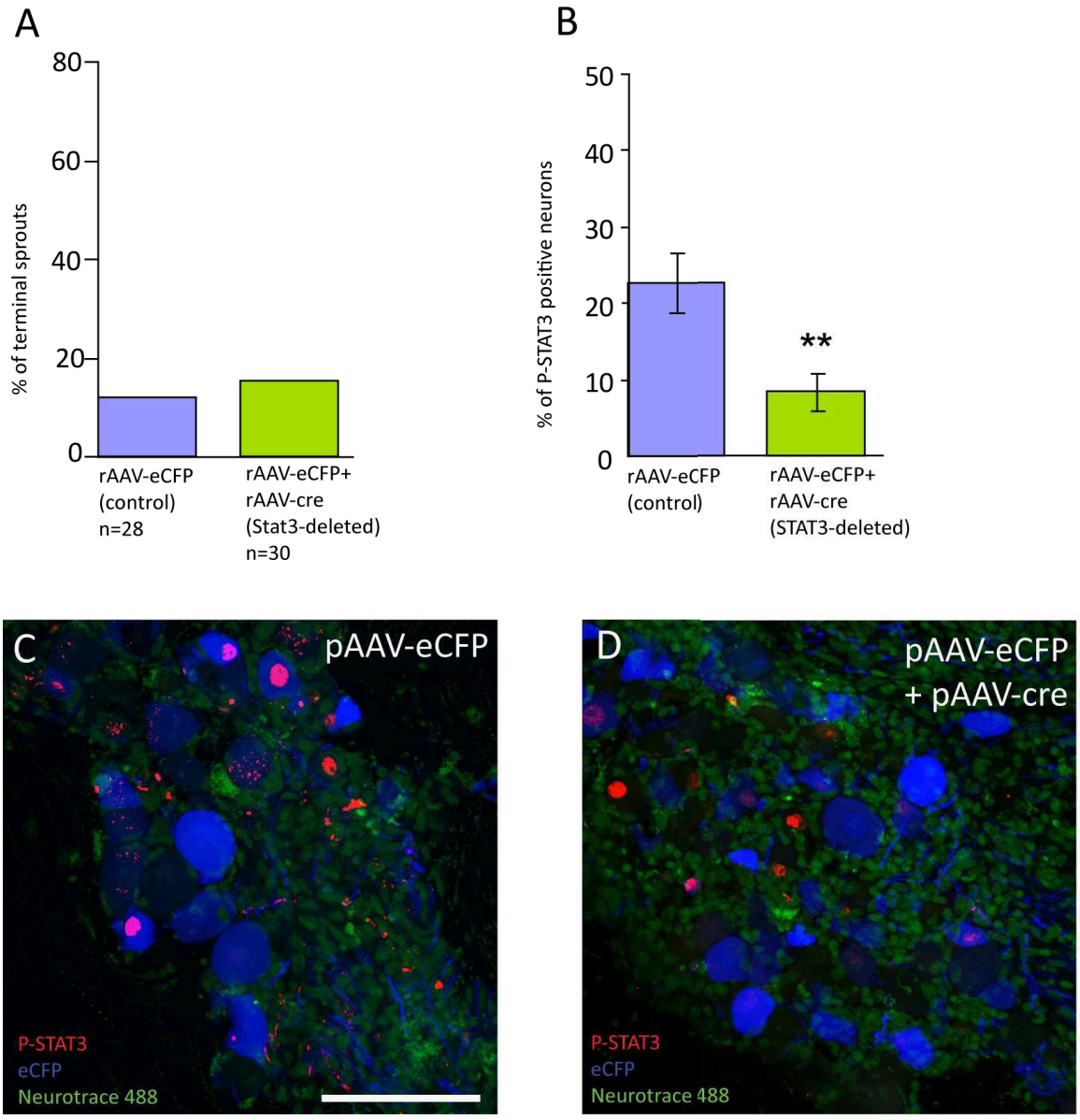

Fig. 4-13 Sprouting response of STAT3-competent and STAT3-depleted DRG neurons after a CNS lesion and evaluation of P-STAT3 immunoreactivity in the corresponding DRGs.

A) Quantification of the number of terminal sprouts in STAT3-competent DRGs (treated with rAAV-eCFP only) and STAT3-deficient DRGs (treated with rAAV-cre and rAAV-eCFP) showing that STAT3 deletion does not influence the sprouting response at baseline. (*B*) Quantification of P-STAT3 immunoreactivity in STAT3-competent and STAT3-deficient DRGs verifies successful deletion of STAT3 in STAT3 $^{fl/fl}$ mice via cre recombinase. (*C, D*) Confocal images illustrating P-STAT3 immunoreactivity in control DRGs (*C*) and in STAT3 depleted DRGs (*D*). Scale bar in *C* 200 µm.

4.7 Combination therapy with rAAV-STAT3 and chondroitinase ABC for central axon regeneration

In the PNS (s. section 3), as well as in the CNS (s. section 4.4), it could be shown that STAT3 successfully initiates axonal outgrowth but does not support elongation of growing axons at later time points. To determine if axonal growth at later time points could be improved by additional treatment regimens, the induction of STAT3 by viral gene transfer of rAAV-STAT3 was combined with the application of chondroitinase ABC, an enzyme neutralizing inhibitory scar components (Silver et al., 2004). Ten days after injection of rAVV-STAT3 or control rAVV, axons emerging from the injected DRGs were lesioned, 5 µl of chondroitinase ABC (chABC) was locally applied to the lesion site and an intrathecal pumps delivering either chondroitinase ABC or saline into the cerebrospinal fluid were installed (Installation of pumps was performed by Dr. Claudia Lang). Ten days after lesion of the axon, the outgrowth in the four groups (STAT3 + chondroitinase ABC, STAT3 alone, chondroitinase ABC+ control rAAV and control rAVV alone) was analyzed *ex vivo* by confocal imaging (s. Fig. 4-14). Immunohistochemistry analysis on spinal cords treated with ChABC (or saline as control) subsequently confirmed successful delivery of ChABC to the spinal cord (s. Fig. 4-15).

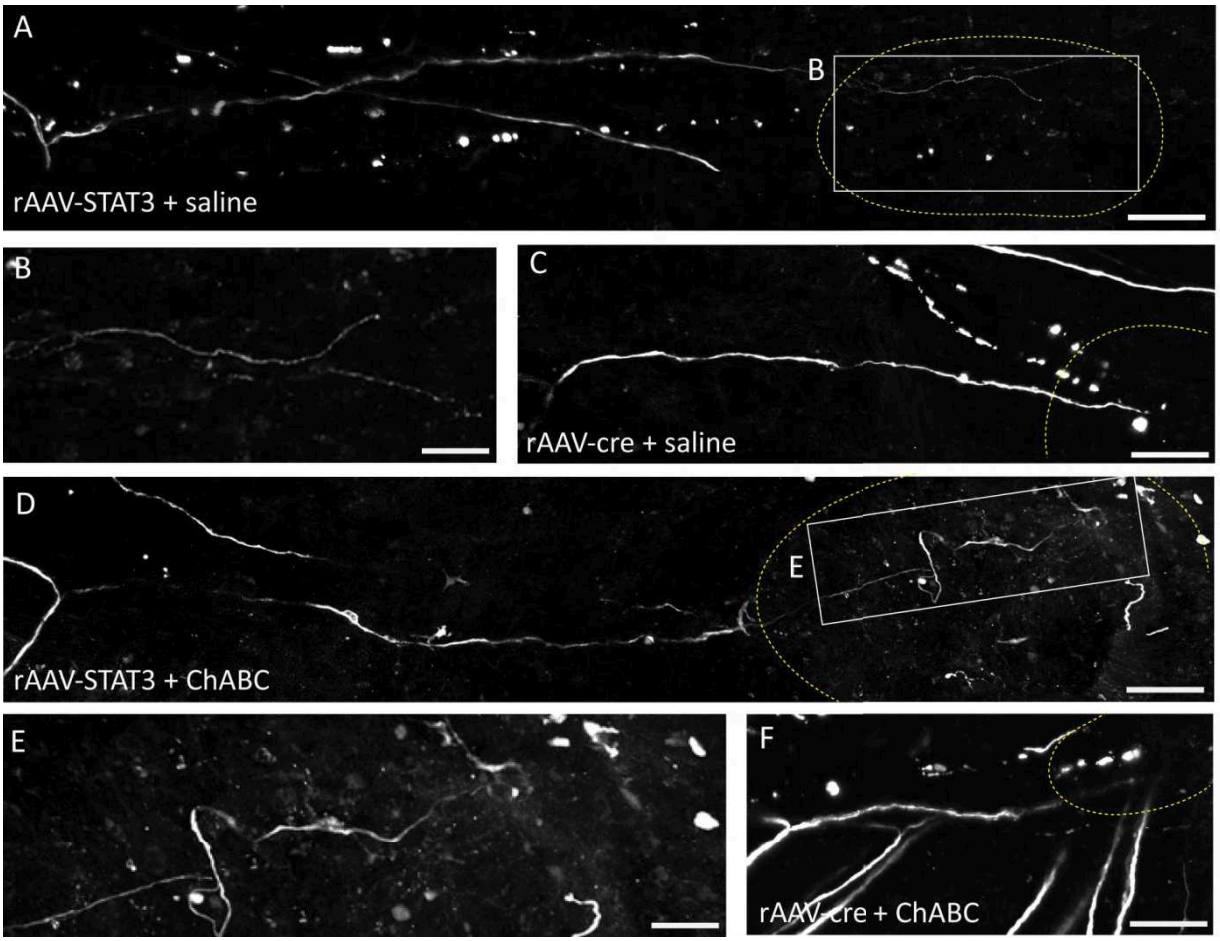

Fig. 4-14 Confocal images of axonal outgrowth after combination therapy with rAAV-STAT3 and chondroitinase ABC

Confocal images illustrating central axonal outgrowth ten days after lesion of DRG axons treated with rAVV-STAT3 plus saline (*A*), rAAV-cre plus saline (*C*), rAAV-STAT3 plus chondroitinase ABC (*D*) and rAAV-cre plus chondroitinase ABC (*F*). (*B* and *E*) Higher magnification view of axonal sprouts boxed in *A* and *D*. The yellow lines indicate the lesion site. Scale bars in *A, C, D* and *F* 100 µm; Scale bars in *B* and *E* 25 µm.

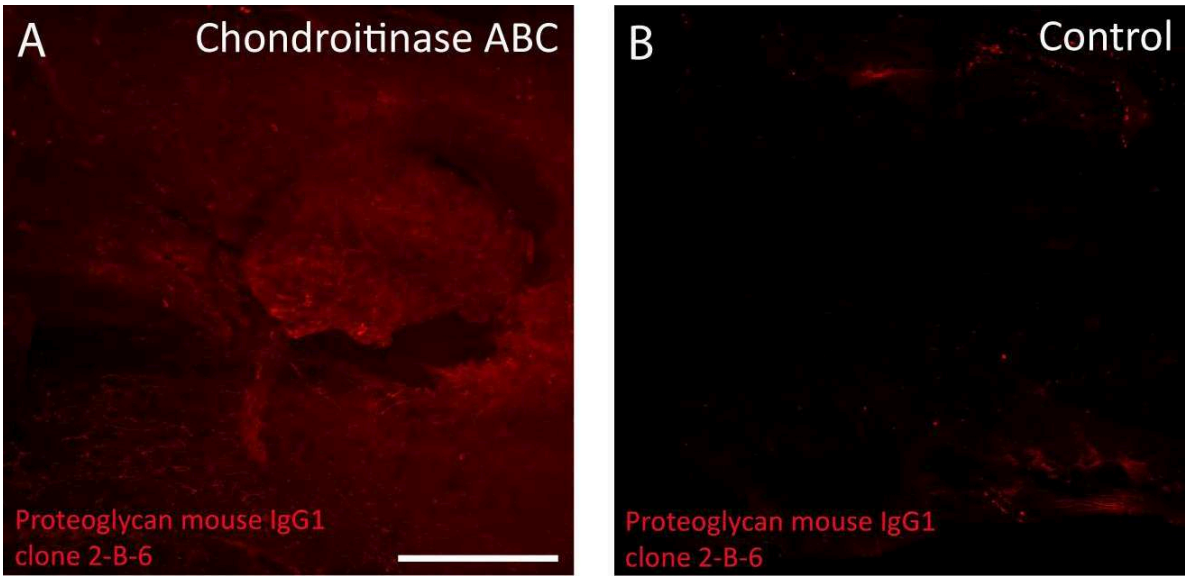

Fig. 4-15 Immunohistochemistry on spinal cords confirming delivery of chondroitinase ABC

Confocal images of spinal cords treated intrathecally with chondroitinase ABC (*A*) or saline as control (*B*). Spinal cords were immunostained for 4-sulfated and 6-sulfated chondroitin and dermatan sulfates resulting from ChABC enzymatic digestion with the Proteoglycan mouse IgG1 clone 2-B-6. Scale bar in *A* 200 µm.

I found that axonal outgrowth was increased by more than twofold in axons treated with rAAV-STAT3 + chondroitinase ABC (141 ± 53 µm) compared to those treated with rAVV-STAT3 and saline (42 ± 26 µm) and those treated with rAAV-cre and saline (30 ± 18 µm). Axons treated with chondroitinase ABC + rAVV-cre showed axonal outgrowth of 64 ± 28 µm, suggesting improved growth compared to the rAAV-cre + saline group and the rAAV-STAT3 + saline group. This trend as well as the trend of improved outgrowth in axons treated with rAAV-STAT3 + condroitinase ABC compared to those treated with chondroitinase ABC plus rAVV-cre or rAVV-STAT3 alone, were, however, not statistically significant (Fig. 4-16).

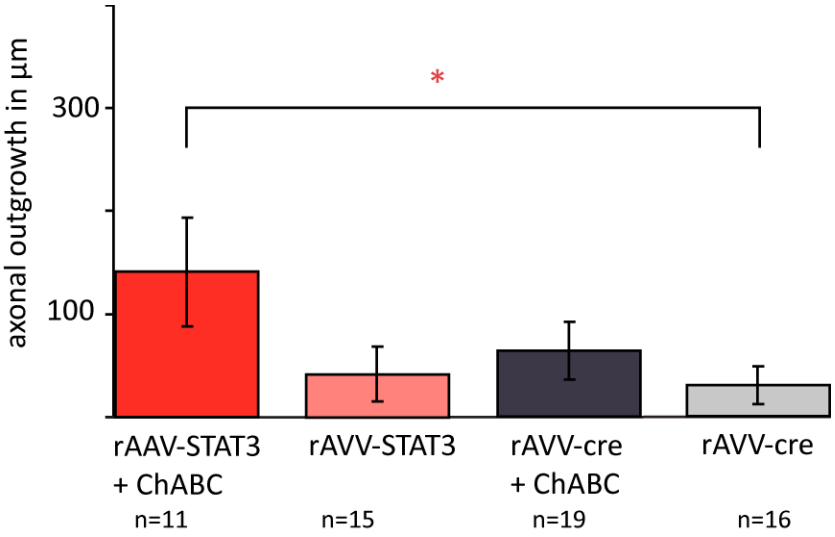

Fig. 4-16 Combination therapy with rAAV-STAT3 and chondroitinase ABC

Central axonal outgrowth ten days after lesion of DRG axons treated with rAAV-STAT3 plus chondroitinase ABC (red bar), rAAV-STAT3 plus saline (light red bar), control rAVV plus chondroitinase ABC (dark grey bar) and control rAVV plus saline (light grey bar).

5 Discussion

5.1 STAT3 is a key component of the intrinsic growth program

STAT3, as shown in this study, can initiate early stages of axon growth: In the PNS, its deletion leads to impairment of axonal regeneration by affecting the timely initiation of outgrowth. In the CNS, overexpression enables axonal sprouting of transected axons even in the hostile CNS environment. The finding that modulating the expression of a single intracellular molecule can change the growth response of a neuron highlights the importance of intrinsic mediators of axonal outgrowth. In the past, most research has been focused on the extrinsic growth inhibitory signals; however, several lines of arguments indicate that the intrinsic growth properties of neurons are equally important determinants of axon regeneration.

First, therapies only targeting the CNS environment have not yet been shown to sufficiently allow the majority of axons to regenerate for long distances and allow functional recovery: For example, there are studies on two of the most intensively investigated therapies, namely chondroitinase ABC – digesting inhibitory CSPGs in the extracellular CNS matrix – and Nogo-A antibody – targeting inhibitory Nogo-A in CNS myelin, that demonstrate the limitations of the two approaches in terms of successful regeneration (Bartsch et al., 1995; Davies et al., 1997; Davies et al., 1999; Lemons et al., 2003; Zheng et al., 2003; Silver et al., 2004; Yiu et al., 2006; Lee et al., 2009; Tom et al., 2009).

Second, when talking about “extrinsic therapies”, it needs to be clarified that intrinsic and extrinsic pathways do not work independently of each other as neuronal cells are firmly embedded in a nonneuronal environment. Due to this neuronal-nonneuronal cell interaction it is very likely that a variety of inhibitory extrinsic signals from different sources eventually converge on similar neuronal pathways. In this context, identifying common intracellular pathways in neurons might open new perspectives in the field of axonal regeneration. Ideally, we would be able to kill many birds with one stone by targeting one or few single common downstream mediators. For example, two of the extrinsic key inhibitors, Nogo-A and CSPGs have been shown to bind to neuronal receptors and consecutively turn on the cytoplasmatic RhoA/ROCK pathway. After activating this signaling cascade, the cytoskeleton

is rearranged and the growth cone collapses (Filbin 2003; Yiu et al., 2006; Bradbury et al., 2011; Tan et al., 2011). Interestingly, a recent review elucidated the close relationship between STAT3 and the Rho family of small GTPases including RhoA by showing that Stat3 is activated by Rac1, Cdc42 and RhoA (Raptis et al., 2011). It remains speculative at this point to deduce that both Nogo-A and chondroitinase ABC as key inhibitors in myelin and scar eventually alter STAT3 expression via RhoA/ROCK signaling in neurons.

Third, as the neuronal intrinsic growth program has attracted increasing scientific interest, a couple of promising molecular pathways and some of their components have recently been identified (s. introduction). Among these are cAMP and its downstream mediators (Qiu et al., 2002; Deng et al., 2009), a couple of transcription factors (Moore et al., 2011), the growth cone-associated proteins GAP-43 and CAP-23 (Bomze et al., 2001) and members of the PTEN/mTOR pathway (Park et al., 2008; Liu et al., 2010). The results of our study have identified STAT3 as an important mediator of axonal growth initiation and future research needs to determine how STAT3 interacts with the other intrinsic components found to play an important role for axonal regeneration. As eventually signals related to regeneration end up activating transcription factors to alter gene expression, a better understanding of the intrinsic neuronal growth program – especially of the transcription factors and their various interactions will – be required. Thus, as mentioned above, many extrinsic and intrinsic factors could be once bundled and allow only targeting some few, pivotal downstream mediators.

5.2 STAT3 is an initiator of neurite outgrowth *in vivo*

Previous studies have already suggested a role of STAT3 for axonal regeneration. It could be demonstrated that increased levels of STAT3 expression and phorsphorylation are associated with axonal regeneration in the PNS (Schwaiger et al., 2000; Sheu 2000; Xia et al., 2002). Moreover, molecules activating STAT3 such as IL-6, CNTF and intracellular inhibitors of STAT3 such as SOCS3 can influence axonal regeneration (Zhong et al., 1999; Cafferty et al., 2001; Qiu et al., 2005; Smith et al., 2009) and most recently, Smith and colleagues revealed that STAT3 expression promotes neurite outgrowth in cultured cerebellar granule neurons (Smith et al., 2011).

In this study, we could demonstrate for the first time that STAT3 expression is not only associated with regeneration but essential for the timely induction of axon outgrowth *in vivo*. STAT3 deletion in lumbar DRG neurons led to impaired sprouting and fewer and shorter regeneration of the lesioned saphenous nerve fibers compared to STAT3-competent neurons four days after lesion. Two weeks after lesion, regeneration speed between STAT3-competent and STAT3-deficient axons reached similar levels. However, – due to the delay of initiating regeneration in the STAT3-deficient group – long-distance regeneration of STAT3-deficient axons was still impaired, while many STAT3-competent axons have already re-entered the distal stump.

In the CNS, I found similar effects of STAT3: Two days after lesion, approximately half of the axons emerging from DRGs that had undergone STAT3 and STAT3c gene therapy formed sprouts after lesion. In contrast, the number of sprouts in control axons was fourfold lower, indicating that the failure of CNS axons to start growing is directly associated with the failure of neurons to upregulate STAT3 expression when their central neurites are lesioned. These findings corroborate very well the study of Qiu and colleagues suggesting that STAT3 expression is involved in the conditioning lesion paradigm (Qiu et al., 2005). This paradigm characterizes the phenomenon that a peripheral lesion of DRG neurons one to two weeks prior to a dorsal column lesion can condition the neuron and leads to extensive outgrowth after a subsequent lesion of its central branch (Neumann et al., 1999; Hoffman 2010). A peripheral injury can “prime” the DRG neurons by increasing their intrinsic growth capacity enabling subsequent regeneration of lesioned central branches. The term “conditioning lesion” indicates that mature neurons can be reprogrammed to enhance growth capacity in both a permissive and a restrictive extracellular environment. Thus, it suggests that there is a common intrinsic neuronal growth program that is in principle able to initiate both PNS and CNS outgrowth but which is, however, only induced after a PNS lesion.

The underlying molecular mechanisms of this phenomenon are only partly understood; however, it could be clearly demonstrated that gene transcription is crucial for the neurons’ regenerative ability (Smith et al., 1997). In line with this, Qiu and colleagues could demonstrate that perineural infusion of JAK2 inhibitor AG490 which blocks phosphorylation of STAT3 significantly reduces dorsal column axonal regeneration in the adult spinal cord after a pre-conditioning sciatic nerve transection. As in our study we could show that a) a peripheral lesions upregulates STAT3 expression and b) overexpression of STAT3 is sufficient

to induce sprouting of CNS axons, the following can be concluded: A peripheral lesion – where STAT3 is persistently upregulated - mimics the effect of STAT3 supplementation and thus ascribes STAT3 a pivotal role for the underlying mechanisms of the effect of conditioning lesions.

However, it also needs to be mentioned at that point that STAT3 is not the only factor which is in the position to initiate neuronal outgrowth of lesioned axons. We could show, that in the PNS, STAT3-deficient axons can still start a growth response, indicating that compensatory mechanisms can, albeit a delay of several days, initiate the neuronal growth program when STAT3 is not available. In the CNS, a comparable phenomenon can be seen: When STAT3 is deleted in DRG neurons, the baseline sprouting of central branches is comparable to that of STAT3-competent neurons suggesting that additional factors seem to induce CNS outgrowth in the absence of STAT3. One can assume that other transcription factors might help out and act as “second line” troop, when the “first line” growth starter STAT3 is not available. Especially the fact that the growth-associated transcription factors strongly co-operate backs up this hypothesis. The following selection of findings exemplifies this complex interaction: *In vivo* knockdown of Sox11 reduces expression of ATF-3 (Jankowski et al., 2009). ATF-3 has been shown to activate c-Jun and form functional heterodimers with c-Jun and CREB. Moreover, it is assumed that ATF3 may as well interact with STAT3 (Seijffers et al., 2007). In the context of pancreatic cancer, CREB has been demonstrated to enhance IL-6 transcription which in turn activates STAT3 (Zhang et al., 2010). C-Jun and STAT3 interact to co-operate in transcriptional activities (Zhang et al., 1999) and STAT3 and Smad1 form complexes that are involved in the fetal brain development (Nakashima et al., 1999).

Considering the close interaction of transcription factors, a question of particular interest arises: What are the (common) downstream mechanisms that mediate the effects of STAT3 and of possible “second line” transcription factors, for example which common genes are induced or repressed?

Answering this question will provide us with a better understanding of the molecular machinery that initiates axonal growth and thus help us developing more specific therapeutic interventions. As STAT3 is a transcription factor and thus, downstream effects are mediated by altering gene expression, the focus lies on identifying neuroprotective and pro-neuroregenerative genes that are directly upregulated by STAT3. Among the genes that

were found to be induced by STAT3, the bcl-2 family turned out to play a critical role in this context. Bcl-2 and bcl-xL were demonstrated to be crucial for neuron survival after injury and protection of neurons from toxic insults (Merry et al., 1997). Reg-2, another survival gene, and bcl-xL were found to be less upregulated in STAT3 conditional knockouts after nerve lesion (Schweizer et al., 2002). Furthermore, other anti-apoptotic genes such as survivin may be involved in axonal regeneration. STAT3 induces survivin gene expression (Gritsko et al., 2006) and a recent study demonstrated that survivin is gradually upregulated in the distal and proximal parts of the sciatic nerve after injury (Amiri et al., 2009).

Apart from these molecules, other downstream targets have become identified as pro-regenerative: STAT3 is closely linked with growth-associated protein 43 (GAP-43), as blockade of STAT3 phosphorylation leads to reduced levels of GAP-43 resulting in compromised neurite outgrowth (Qiu et al., 2005). SPRR1A, a regeneration-associated protein, is activated upon IL-6 and gp130 signaling as well as by transcription factor Sox11 (Pradervand et al., 2004; Jing et al., 2012). SPRR1A is induced by more than sixtyfold after peripheral axonal damage and its overexpression augments axonal outgrowth in vitro (Bonilla et al., 2002). Also, the cell cycle inhibitor p21/Cip1/Wafl is directly targeted by STAT3 and contributes to neurite outgrowth by inhibiting the Rho kinase activity (Coqueret et al., 2000; Tanaka et al., 2002). More recently, a transcriptional profiling study has identified a particularly interesting gene target of STAT3, the interferon regulatory factor 1 (IRF1). Overexpression of this transcription factor enhanced neurite outgrowth in cultured cerebellar neurons to a similar extend as overexpression of STAT3 itself (Smith et al., 2011).

In my study, I could demonstrate that the amount of sprouting axons in animals treated with rAAV-STAT3 is comparable to those treated with rAAV-STAT3c. Immunohistochemistry showing approximately the same percentage of P-STAT positive neurons after gene therapy with both rAAV-STAT3 and rAAV-STAT3c supports this observation. In contrast to STAT3, STAT3c dimerizes spontaneously and independently of prior phosphorylation by JAKs. Therefore, one could presume that STAT3c should have stronger and more prolonged effects on axonal outgrowth than STAT3. However, overexpression of STAT3 alone turned out to be sufficient to induce its phosphorylation and import to the nucleus. This might indicate that STAT3 action – at least in DRG neurons – is primarily regulated at the expression and not at the phosphorylation level.

Smith and colleagues (Smith et al., 2011), however, only detected significant neurite outgrowth in cells transfected with STAT3c and not STAT3. Also the first paper describing the generation of STAT3c conclusively showed that STAT3c is indeed more effective than STAT3 (Bromberg et al., 1999): Though STAT3c dimerizes spontaneously, even more STAT3c-dimers formed under additional stimulation of tyrosine phosphorylation by tyrosine kinases (which is the case when STAT3c is transferred to a normally functioning cell). Moreover, the DNA binding activity in STAT3c transformed cells was higher than the one in cell lines where endogenous STAT3 was constitutively activated by v-src (a tyrosine kinase) that had been transferred to the cell.

My findings might differ from the results of Smith and Bromberg, as I used adeno-associated viruses in living animals instead of cells in culture which were transfected with plasmids. In my model, DRG neurons were embedded in a real environment of glial cells and fibroblasts. Upon injection of rAAV, most likely an inflammatory milieu was created attracting immune cells that together with the local tissue released cytokines such as IL-6 (Akira et al., 1992) . These in turn might have strongly and continuously activated the JAK/STAT pathway which maintained the phosphorylation of STAT3 molecules provided by viral gene therapy. In line with this, a maximal saturation of STAT3 phosphorylated molecules could be achieved in both rAAV-STAT3c and rAAV-STAT3 transduced cells. As cells in culture which are solely transfected with plasmids are neither exposed to stimuli of other cell populations nor to inflammatory processes as triggered by rAAV-STAT3(c), one can assume that in these cells STAT3, but not STAT3c underlies the various downregulating mechanisms (s. introduction).

As outlined, my results are in favor of a model proclaiming that an optimal level of activated STAT3 can be achieved in both rAAV-STAT3 and rAAV-STAT3c-transduced cells. Smith's and Bromberg's results - not taken into account that the results from their cell culture experiments are not directly comparable to my *in vivo* work- rather argue for a model in which STAT3c is more effective than STAT3.

Considering both models when evaluating the results of my experiments, one might suggest that whenever a certain (unknown) optimal level of activated STAT3 is reached, an additional increase of STAT3 activity does not further improve axonal outgrowth. In line with this hypothesis we found that overexpression of either STAT3 or STAT3c by viral gene transfer did not further improve PNS regeneration indicating that a PNS lesion alone is sufficient to

induce optimal STAT3 levels or in other words: A PNS lesion is sufficient to induce the optimal level of activated STAT3 which is needed to drive the regenerative response.

5.3 STAT3 is a phase-specific regulator of axonal regeneration

To clearly show how STAT3 would affect different phases of axonal outgrowth in the CNS we used an *in vivo* imaging approach. Traditionally, research on axonal regeneration has been performed studying fixed tissue in which axons were stained by tracing techniques or immunohistochemistry. However, these methods are limited as regeneration can only be assessed after having sacrificed the animal. Thus, it's not possible to evaluate the dynamics of the regenerative process during at least more than one distinct time point. Also, it is difficult to distinguish regenerated axons from those that were never lesioned as well as from collateral sprouting of undamaged fibers (Steward et al., 2003; Misgeld et al., 2006). *In vivo* microscopy, however, allows the investigation of single cells in the living animal (Misgeld et al., 2006). By visualizing individual fluorescent axons repeatedly, I could directly assess the effect of rAAV-STAT3(c) treatment in individual axons over time and thus, gain direct insights into the growth dynamics which I might not have gained by other methods.

In this project, *in vivo* imaging revealed that STAT3 deletion in lumbar DRG neurons impaired the regeneration of saphenous nerve fibers in the PNS at both four and fourteen days after injury. However, at day 14, sprouting around the lesion and regeneration at short distances from the proximal stump were not significantly different between STAT3-deficient and STAT3-competent axons and at twenty-eight days after lesion, similar amounts of STAT3-competent and STAT3-deficient axons have re-approached their targets.

This indicates that STAT3-deficient axons can still start the regeneration process. Subsequent repetitive *in vivo* imaging then disclosed that impaired regeneration of STAT3-deficient axons is not due to reduced elongation but due to delayed growth induction: The speed of axon growth during the early phase (two to three days following injury) of regeneration is significantly reduced in STAT3-deficient mice compared to STAT3-competent mice. However, in the later phase (seven to eight days after injury) of the regeneration process, the speed of growth does not differ between the two groups. In the CNS, repetitive *in vivo* multiphoton

imaging showed a similar pattern: STAT3 and STAT3c overexpression increased the speed of axonal growth in the early phase (two to four days after lesion) of regeneration more than threefold compared to control axons. At late (4-10 days after lesion) time points, however, the growth speeds did not differ between the control and the STAT3(c)-treated groups. Taken together, these results identified STAT3 as a phase-specific regulator of neuronal outgrowth. STAT3 is needed for the timely initiation of the growth program after lesion. But once these processes have been implemented, STAT3 is not longer required for the perpetuation of axonal regeneration. Thus, there is evidence that the growth response *in vivo* is divided into at least two distinct phases, initiation and elongation, that are probably regulated by distinct molecular mechanisms.

The idea of a multi-phasic growth program has previously been proposed based on research in Aplysia californica. Studying the reaction upon nerve injury in Aplysia, a model was suggested that proposed several temporally distinct phases (Ambron et al., 1996): Phase 1 is characterized by injury induced action potentials acting via calcium and cAMP. In phase 2 MAP kinases are activated and retrogradely transported to the nucleus. Phase 3 is characterized by signals from the environment such as growth factors and cytokines. All phases eventually lead to alteration of gene expression. The last phase then stops growth by inhibitory target-derived factors.

In the concept of a multi-phasic growth regulation, the transcription STAT3 contributes to regeneration in the early phase likely by inducing a variety of genes such as IRF1 and SPRR1A (s. above). In line with the early action of STAT3, we could detect increased P-STAT3 levels already at one hour after saphenous nerve cut that started to decrease slowly after two weeks, indicating that STAT3 is rapidly activated after peripheral injury. Subsequent investigations should now aim to identify factors involved in the elongation phase. The results of Sheu and colleagues might already provide evidence for a candidate regulator of axonal elongation: After peripheral nerve transection, they found that Erk phosphorylation persisted longer than STAT3 phosphorylation and was, in contrast to STAT3, also intense at the distal nerve stump. They concluded that Erk and its downstream mediators are crucial for establishing and maintaining an ideal extracellular environment in the distal nerve stump for axons growing towards them (Sheu 2000).

More generally, the idea of a phase-specific regeneration program suggests that environmental cues are decisive for shaping the distinct phases of axonal regeneration. In

the PNS, the switch from growth initiation to elongation could be initiated by the interaction of Schwann cells with growing axons. As previously described, Schwann cells form the "bands of Büngner" guiding axons to their targets (Hoke 2006). The role of STAT3 in this context could be to induce neurite outgrowth before contacts with Schwann cells are established. Once a re-growing axon has contacted a Schwann cell, the cellular program shifts to the elongation mode and no longer requires STAT3. The idea that axonal growth along glial support structures does not require STAT3 can be corroborated by our results from the peripheral crush lesion: After a peripheral crush lesion which does not interrupt Schwann cell guidance, STAT3 is not activated. Obviously, crush and cut lesions implicate different injury signals and cellular responses that need to be further elucidated.

In the CNS, changes in the environmental circumstances might as well explain the transition between the different growth phases, namely the switch from the at least partially successful initiation of axonal outgrowth to the failure of axonal elongation: In the CNS, there are no Schwann cells that support regenerating axons after the first growth initiation. In contrast, STAT3-transduced axons attempting to grow encounter a hostile CNS environment containing myelin inhibitors that becomes even more growth repelling when the glial scar develops. These changes in the lesion environment could explain why overexpression of STAT3 can only initiate early growth but fails to support it later. In line with this idea, combination of viral STAT3 treatment together with chondroitinase ABC increased the length of axonal outgrowth. STAT3 can thus "jump-start" the intrinsic growth capacity and prime axons for therapies such as chondroitinase ABC that can sustain elongation.

5.4 STAT3 is a promising target for therapeutic interventions

My project identifies STAT3 as a potent initiator of axonal outgrowth. Hence, STAT3 might become an interesting candidate to use – either alone or in combination with other reagents – for therapeutic interventions that aim to promote central axonal regeneration. To be once able to make the step from "bench to bedside", a couple of questions and problems need to be addressed:

1. When is the optimal timeframe after spinal cord injury to start therapy with STAT3 and is there a “too late” from a therapeutical point of view?
2. Should we directly target STAT3 or rather its upstream or downstream mediators? What component of the JAK/STAT might be therapeutically interesting?
3. How can we manage to induce STAT3 in a way that is both targeted to neurons and well tolerated by the receiving organism?

5.4.1 Being in the right place at the right time

In this study, rAAV-STAT3 and rAAV-STAT3c were injected ten to twelve days prior to the lesion allowing sufficient upregulation of STAT3 before the injury was set. However, this application scheme is clearly not feasible for spinal cord injured patients. Future clinical application of STAT3 would only become a realistic goal if proven that growth can also be induced when it is applied immediately after the injury or – to offer therapy to patients with chronic SCI – even when it is applied at a later time point.

So far there is no direct data for STAT3 itself, however, there is strong evidence from the conditioning lesion paradigm: A recent study demonstrated that DRG neurons that were conditioned *after* lesion of the central branch initiate an intrinsic growth program with upregulation of RAGs that is similar to neurons conditioned *before* CNS injury. Moreover, significant central axonal regeneration was observed in these “post-conditioned axons”. This, however, occurred only in the absence of growth-repelling traumatic tissue which is the case when single axonal lesions were performed by minimal tissue traumatic two-photon laser injury (Ylera et al., 2009). Another study in which different therapeutical approaches were combined was tested one year after spinal cord injury and came to similar results: Grafts of marrow stromal cells together with NT-3 application and a peripheral nerve conditioning lesion allow axon regeneration bridging the lesion site. The gene profile seen in the post-conditioned neurons remarkably resembles the one in pre-conditioned neurons (Kadoya et al., 2009). A recent study comparing the efficacy of peripheral sciatic nerve crush lesions to cAMP elevations on central sensory axonal regeneration could amongst others demonstrate that regenerative effects persisted whether conditioning lesions were applied prior to or shortly after spinal cord injury (Blesch et al., 2012)

As STAT3 induction has been shown to mimic the effect of a conditioning lesion, there is hope that pro-regenerative effects of STAT3 can also be detected when STAT3 is delivered post injury. However, it needs to be considered that the more time passes after injury the more likely a strong hostile environment will develop and very likely undo STAT3 effects. In particular for later time points, a combination with reagents mitigating the extracellular matrix such as Nogo-A-antibody or chondroitinase ABC will be needed (s. below).

5.4.2 In search of the ideal target in the JAK/STAT pathway

5.4.2.1 Upstream and downstream player in the JAK/STAT pathway and their potential for therapy

STAT3 has been turned out to be a potent initiator of neurite outgrowth. However, as STAT3 is a pleiotropic mediator embedded in a complex signaling cascade, it might also induce unwanted side effects (s. below). Thus, the question arises if in the long run it would be also possible to target other mediators downstream or upstream of STAT3 that are either more convenient to target or safer when applied to patients.

In this context, the neuropoietic cytokine IL-6 that can activate the JAK/STAT pathway (Schindler et al., 1995) has emerged as an interesting candidate: Hiruta and colleagues found accelerated regeneration of lesioned peripheral nerves in transgenic mice constitutively expressing both IL-6 and IL-6 receptor (Hirota et al., 1996). In line with this, sensory impairments and delayed regeneration of peripheral sensory axons in interleukin-6-deficient mice was found (Zhong et al., 1999). Eventually, the pro-regenerative role of IL-6 could be also shown in the CNS in the context of traumatic brain injury (Swartz et al., 2001). A recent study showed that intrathecal delivery of IL-6 after cortical spinal tract injury in rats promoted regeneration and functional recovery by inducing the expression of growth-associated genes (Yang et al., 2012). In contrast to STAT3, IL-6 could be easily delivered by direct intrathecal application which would be of great advantage. However, there is evidence that more upstream interventions apart from promoting growth simultaneously induce effects abrogating the growth effect by activating different cell populations and signaling pathways. The first *in vivo* study to show the effect on IL-6 after SCI revealed that IL-6 as an upstream mediator of the JAK/STAT impaired axonal regeneration (Lacroix et al., 2002). In accordance with this, blocking the IL-6 receptor was shown to ameliorate functional

recovery in spinal cord injury (Okada et al., 2004; Mukaino et al., 2010). These negative results are not extremely surprising as IL-6 is a strong pro-inflammatory cytokine and activates *in vivo* many cell populations around the lesion side which in turn create an overwhelming pro-inflammatory response hindering successful regeneration.

Thus, more downstream interventions in the STAT3 cascade might allow more precise targeting of pro-regenerative genes. Pleiotropic effects of upstream players such as IL-6 leading to activation of genes not being involved in axonal outgrowth or – in the worst case – even induce cell harming effects could be avoided. It's also interesting to note that even STAT3 itself does not only upregulate pro-regenerative genes but also genes encoding for proteins such as SOCS3 which are negative regulators of the JAK/STAT pathway (Croker et al., 2008; Sun et al., 2010).

So far, the genetic alterations upon STAT3 activation that promote axonal regeneration are not sufficiently examined. The future will show if genes targeted by STAT3 such as IRF1 and SPRR1a (s. above) will have the potential to be considered as target molecules for axonal regeneration. Also deletion of negative regulators of the JAK/STAT pathway may be interesting approaches (Smith et al., 2009; Sun et al., 2011).

5.4.2.2 Harnessing the pleiotropic effects of STAT3 for therapy

As described above, using activators of STAT3 for therapy might result in pleiotropic effects. Also focusing on genes activated by STAT3 might narrow down the therapeutic effect as the entire set of genes responsible for the growth-promoting effect of STAT3 are not yet defined. Thus, as of now, STAT3 itself should be considered as a target of clinical endeavors. In this context, however, it is important to note that the effects of STAT3 are not only confined to neuronal cells. The role of STAT3 for inflammation and cancer has been well-documented and is of high clinical significance. Also there is evidence that STAT3 is involved in astrogliosis and scar formation after injury. The following paragraphs give an overview of the nonneuronal effects of STAT3 that help understanding why a possible STAT3 therapy needs to be target-oriented and carefully designed.

5.4.2.2.1 Effects of STAT3 on glial cells

Very rapidly upon spinal cord injury, reactive astrocytes migrate to the lesion and set up a dense barrier around the injury side, the glial scar. The function of reactive astrocytes after spinal cord injury is largely dependent on STAT3 signaling (Okada et al., 2006). Though it could be shown that STAT3 activation in glial cells after SCI is necessary to organize the scar, preventing unorganized inflammation and consecutively reducing functional deficits (Okada et al., 2006; Herrmann et al., 2008), it remains unclear if STAT3 activation in astrocytes is also beneficial during chronic phases. Especially if using STAT3 therapy over a long time to promote neuronal outgrowth it could – when applied in a non-selective way – in the same time continue activating astrocytes that will in particular during later phases impair axonal regeneration as a physical and chemical barrier.

5.4.2.2.2 Effects of STAT3 on inflammation and cancer

First discovered as an acute-phase response factor (Akira et al., 1994) and activated by pro-inflammatory molecules such as IL-6, STAT3 has been shown to be closely linked with pro-inflammatory processes (Alonzi et al., 2001; Kisseleva et al., 2002; Pfitzner et al., 2004; Adamson et al., 2009; Aggarwal et al., 2009).

More importantly, STAT3 is crucial for tumorigenesis and - to build the bridge to its role in inflammation – involved in inflammation-induced cancer, as has been shown for Helicobacter pylori associated gastric cancer (Yu et al., 2009). Apart from extrinsic signals such as bacteria and chemical agents, also intrinsic factors such as overexpression of growth factor receptors and oncogenic mutations in JAKs can lead to constitutive activation of STAT3 and thus to tumorigenesis (s. below). As previously described, constitutively activated STAT3 has been found in many tumor entities such as breast cancer, prostate cancer and melanoma (Bromberg 2002). Eventually, cancer related effects of STAT3 are carried out on the transcription level: tumor “survival gene products” - which are in the same time pro-regenerative genes in axonal regeneration - such as bcl-xl, bcl-2, survivin, Mcl-1 and cIAP2 are upregulated and lead to suppression of apoptosis (Yu et al., 2009). These tumor promoting properties would clearly restrict the use of STAT3 as therapeutic agent in the treatment of SCI.

At this point, however, it needs to be emphasized that STAT3 per se is not an oncogene. It has to be categorized as proto-oncogene fulfilling completely physiological functions within the cell: The first study to directly demonstrate the intrinsic oncogenic potential of STAT3 was performed in 1999, when Bromberg and colleagues created a constitutively active variant of STAT3, the STAT3c, that was capable of transforming fibroblasts resulting in subsequent tumor formation (Bromberg et al., 1999). In the STAT3c molecule, two cysteine residues are substituted within the C-terminal loop in the SH2 domain, allowing the molecule to spontaneously dimerize by disulfide bonds without requiring tyrosine phosphorylation. However, there is so far no evidence of naturally occurring mutations transforming STAT3 molecules into constitutively active variants. In naturally occurring tumors, constitutive STAT3 activation depends on dysregulated processes within the JAK/STAT signaling pathways such as overexpression of receptors and cytokines, methylation-induced silencing of the SOCS1-gene as found in hepatocellular carcinomas (Bromberg 2002) and mediators (e.g. IL6) activated by STAT3 itself that create a feed-forward loop eventually activating STAT3 (Yu et al., 2009). Of great clinical importance is the vast range of JAK mutant myeloproliferative neoplasms including Polycythemia vera and essential thrombocytosis (Stein et al., 2011).

In my project, I injected rAAV-STAT3 as well as rAAV-STAT3c to neuronal cells. As described above, the two different STAT3 viruses showed a similar effect on axonal regeneration. This indicates that the constitutive, oncogenic form is not necessary for therapeutic success which makes a clinical application less risky. Furthermore, I investigated STAT3 levels on rAAV-STAT3 injected DRGs of mice that had been perfused approximately three months after viral injection. Preliminary data showed, that STAT3 level declined to baseline indicating that at this late time point cell-intrinsic mechanisms are turned on to silence STAT3 expression. The therapeutic success is thereby not hindered, as STAT3 exerts its effect exclusively during initiation of growth. However, as STAT3 is involved in a broad palette of nonneuronal cell activities that are only partly understood, selective neuronal targeting of therapeutic STAT3 induction might be necessary.

5.4.3 Bringing STAT3 to neuronal cells: Maximal efficacy and minimal side effects

The two main methodical approaches to manipulate STAT3 expression are gene therapy as used in our study and pharmacological approaches. Until today, latter ones would not be able to directly transport STAT3 into the cells but they could target upstream mediators in the JAK/STAT pathway that activate STAT3 such as IL-6. Though IL-6, e.g., can be well delivered intrathecally (Yang et al., 2012), it would imply an intolerable risk due to its strong pro-inflammatory properties that are able to produce an overwhelming inflammatory milieu endangering a patient with SCI (s. above). As summarized above, therapy would need to be selective to neurons and the most efficient method in this context would be gene therapy.

Successful gene therapy is mainly dependent on powerful methods to express genes of interest into target cells. Particularly efficient tools in this context are viruses representing a natural vector for delivery and expression of exogenous genes. Among the many different virus families, adeno-associated viruses (AAV) have become increasingly popular as they are apathogenic, available in many serotypes that allow tissue-specific targeting, stable in their host organism over time and modest concerning the immune response they elicit (Daya et al., 2008). Also, recombinant AAV (rAVV) vectors have the tendency to rather stay in episomal form than integrating into the host genome which reduces the risk of insertional mutagenesis (Nakai et al., 2001). As rAAV have also been successfully transferred into neurons (Xiao et al., 1997; Glatzel et al., 2000), we used rAAV for our STAT3 gene therapy. In line with others we could yield a high transfection rate of DRG neurons: On average 80% of DRG neurons show P-STAT3 expression ten, twelve and fourteen days after injection of rAAV-STAT3, respectively rAAV-STAT3c.

It's thus not surprising that currently more than 80 trials are performed using AAV as vectors (source: http://www.abedia.com/wiley/vectors.php). To name just a few examples of successful clinical trials with AAV: Kaplitt and colleagues could show that injection of AAV carrying a transgene encoding glutamatic acid decarboxylase (GAD) into the subthalamic nucleus of patients with Parkinson's disease was not only safe and well tolerated by patients but also led to clinical improvement. Another trial investigated the effect of gene therapy with AAV in patients suffering from Leber's congenital amaurosis (LCA), a group of inherited

blinding diseases. All patients treated with AVV injected subretinally showed evidence of improvement of retinal function (Maguire et al., 2008; Maguire et al., 2009).

However, there are also obstacles in AAV therapy that need to be overcome. Very importantly, it could be shown that transgene-expressing cells are eliminated by the adaptive and innate immune system (Mingozzi et al., 2011; Rogers et al., 2011). In the first clinical trial in which AAV were introduced into the liver of hemophilia B patients to transfer human blood coagulation factor IX, two subjects developed transient elevation of liver enzymes, indicating destruction of hepatocytes most likely mediated by AAV capsid-specific CD8(+) T-cells (Manno et al., 2006). Even when adding an immunosuppressive regimen to dampen immunologic processes there was no improvement in AAV5-based liver gene transfer in non-human primates (Unzu et al., 2012). In the previously described study on congenital amaurosis, the therapeutic success might be not least due to the fact that the eye is an immunoprivileged organ. In contrast, after acute spinal cord injury, there is a strong inflammatory response recruiting a variety of immune cells (Bareyre et al., 2003). Especially in this milieu, AAV therapy could be in danger of being counteracted by a strong immune response which in the worst case would eliminate transduced neuronal cells.

But there is hope, that in the future, AAVs can be engineered to overcome the reactions of the immune system. For example, there have been efforts to increase the transduction efficiency of vector particles by changing the capsid structure. This enables a fewer amount of AAV to achieve the same expression levels as higher titers of wild-type AAV (Li et al., 2010; Bartel et al., 2011). Others pre-treated mice with glucocorticoids before delivering adenoviral vectors and could demonstrate that innate toxicity is ablated (Seregin et al., 2009). As corticosteroids are anyhow applied in SCI, a possible combination therapy with rAAV-STAT3 and corticosteroids raises hopes that rAAV-STAT3 can once be transferred to spinal cords in humans.

5.5 STAT3 in combination with other therapeutic targets

As described in previous chapters, various mechanisms including inflammation, the absence of permissive molecular and cellular bridging, myelin and extracellular matrix inhibitors,

reduced neurotrophic stimulation and an insufficient induction of the intrinsic neuronal growth program avoid successful regeneration after spinal cord injury. Thus, combination therapies that target more than one parameter seem to promise greater potency in eliciting regeneration than single therapies. Lately, several studies confirmed this hypothesis: Combination of stimulating intrinsic responses by cAMP application with a growth permissive gradient of NT-3 has been shown to promote axonal regeneration beyond spinal cord injury sites (Lu et al., 2004). Similarly, combination of cAMP with Schwann cells grafts promotes axonal growth and functional recovery after spinal cord injury (Pearse et al., 2004). Other studies combined chondroitinase ABC with cell therapy (Fouad et al., 2005), peripheral nerve grafts (Houle et al., 2006) and growth factors (Massey et al., 2008), respectively, and found improved axonal regeneration and functional recovery. Sun and colleagues double deleted two components of the intrinsic growth program, namely SOCS3 inhibiting the STAT3 pathway and PTEN (s. introduction), resulting in sustained axonal regeneration of the optic nerve compared to a single deletion of either the one or the other factor. (Sun et al., 2011). In a recent study, an electrochemical neuroprosthesis consisting of epidural electrical stimulation as well as a mixture of serotonin and dopamine receptor agonists was applied in rats after hemisection of the spinal cord. The research group could demonstrate that a robotic postural interface forcing the rats to actively use their paralyzed hindlimbs during electrochemically favorable states as created by the neuroprosthesis could remodel spared neuronal circuitries and regain supraspinal control of locomotion (van den Brand et al., 2012).

In this project, I investigated STAT3 in combination with chondroitinase ABC and demonstrated that average axonal outgrowth over ten days after lesion was increased more than twofold in animals treated with both chondroitinase ABC and rAAV-STAT3 compared to animals treated with rAAV-STAT3 alone or chondroitinase ABC alone or control animals. Though my results were only significant for the group of animals treated with rAAV-STAT3 plus chondroitinase ABC compared to the group of control animals (saline plus rAAV-cre), there is a clear trend towards increased axon regeneration when therapies are combined compared to single therapy approaches. The fact that rAAV-STAT3 treatment alone e.g. did not result in significantly better regeneration than treatment with rAAV-cre alone highlights the fact that STAT3 only induces outgrowth, but during later time points, when axons get

into contact with the increasingly hostile environment of the CNS, they stop regenerating. The results of my combination experiment are consistent with a previous study, in which functional regeneration of central DRG branches through the dorsal root entry zone in the spinal cord was improved by combining chondroitinase ABC with a pre-conditioning peripheral lesion (Steinmetz et al., 2005). Interestingly, the similarity between the results from this study and the ones from my combination therapy with chondroitinase ABC and rAVV-STAT3, suggest that indeed STAT3 overexpression can mimic the effect of a peripheral conditioning lesion.

Furthermore, the improved outcome of combined application of STAT3 and chondroitinase ABC compared to single therapy with rAAV-STAT3 alone or chondroitinase ABC alone, respectively, demonstrates that outcome can be improved when targeting both extrinsic and intrinsic factors. The need for combining different strategies can be figuratively compared to driving a car: Taking one's foot off the brake is not enough to drive a car. One also needs to step on the accelerator (Steeves et al., 1998). In this study, we identified STAT3 as a key intrinsic regulator of axonal outgrowth, thus representing the accelerator. However, as shown by multiphoton imaging in this project, the initial growth cannot be sustained between four and ten days after lesion. However, when chondroitinase ABC is applied in addition, axons can elongate further in the hostile CNS environment. In this way, chondroitinase ABC releases the brakes and paves the way for growing axons. Although CSPGs are already upregulated within twenty-four hours after injury and hence suggesting that they might prevent axon regeneration across acutely forming scar tissue, the highest peaks of CSPGs and thus inhibition are detected at later time points ranging between eight and fourteen days (Jones et al., 2003; Tang et al., 2003). Therefore, chondroitinase ABC might amplify STAT3 effects in the early induction phase of growth, but more significantly, support elongation at later stages. This shows as well that in terms of combination treatment, one would ideally think of components that also target the moleculary distinct phases during axonal growth (s. above).

5.6 Concluding remarks and outlook

In my thesis I could identify STAT3 as a potent initiator of axonal outgrowth in the CNS. Overexpression of STAT3 in DRG neurons significantly increased sprouting of the central branches after lesion, thus successfully mimicking the effect of a pre-conditioning peripheral lesion. Moreover, repetitive imaging of fluorescently labeled axons in living animals enabled me to directly observe the phase-specific action of the therapeutic intervention on the regeneration process *in vivo*: For the first time, I could show that STAT3 supplementation was sufficient to induce axonal growth but unable to sustain it during later periods. Together with similar findings in the PNS, it can be suggested that the intrinsic growth response can be at least divided into two distinct phases of regeneration, namely initiation and elongation. Latter one is most probably shaped by environmental cues, such as myelin proteins and scar in the CNS, that stall outgrowing axons. In line with this theory, combination of STAT3 with intrathecal delivery of chondroitinase ABC that neutralizes inhibitory scar components increased the length of growing axons.

The insights gained in this study add an important puzzle piece to the understanding of the complex intrinsic growth program. As it becomes more and more obvious that extrinsic approaches in enhancing regeneration are not sufficient alone and – interestingly, might even converge in part on similar intracellular pathways, scientific efforts have nowadays been increasingly focused on elucidating the intrinsic mechanisms that control neuronal growth. Many components of intracellular signaling have been found to drive axonal regeneration and further investigations are now needed to determine how these different factors act in temporal and spatial concert to develop target-specific therapies with maximal outcome and minimal side effects.

Currently, a number of therapies targeting different mechanisms in the pathology of spinal cord injury are being developed and some have even been applied to humans (s. Tab. 1-1). However, none of these therapies has so far led to groundbreaking success. It becomes more and more clear that the disappointing results in humans might be attributed to differences in species as well as to the experimental designs which do not match realistic clinical situations of SCI (Dietz 2010). For example, whereas in animals application of olfactory ensheathing cells (OEC) promoted long-distance axonal regeneration in the transected adult

rat spinal cord (Ramon-Cueto et al., 1998), human trials with OEC have only shown little to no effect (Mackay-Sim et al., 2011). Thus, it's not surprising that especially results from cell culture experiments - which are even more insufficient in mimicking the complex processes in human SCI - cannot necessarily be translated to humans. In addition, it needs to be taken into account that even if there is successful axon regeneration, there is not automatically function improvement. In particular non-directional growth can even lead to aberrant regeneration causing pain, paresthesia and other unwanted effects (Thoenen et al., 2002).

That's why in recent years not only axon regeneration but also axon reorganization has been in the focus of spinal cord injury research. As spinal cord injuries rarely present as complete lesions, improving reorganization of the spared fibers might help to establish functional bypasses and promoting functional remodeling of existing circuits. The most successful therapies in rodents namely chondroitinase ABC and Nogo-A antibody have been shown to not only positively influence regeneration but also reorganization. There is even evidence that inhibitors of myelin rather remodel existing circuits than promote long-distance regeneration (Bareyre et al., 2004; Silver et al., 2004).

In humans, marked improvements of functional recovery could be mainly achieved by improving reorganization. Especially locomotor training which facilitates stepping movements of the legs using body weight support on a treadmill to provide appropriate sensory cues has been shown to improve walking (Harkema 2001; Dietz et al., 2004). When combined with electrical stimulation of the spinal cord, a 23-year-old patient with paraplegia was even able to elicit full weight-bearing standing and to voluntarily execute some motor functions (Harkema et al., 2011). This provides an interesting parallel to a recent rodent study in which a cocktail of excitatory agents was applied in combination with electrical stimulation and training. In doing so, voluntary control of locomotion could be restored (van den Brand et al., 2012).

In my study, I could demonstrate that STAT3 supplementation did not only increase terminal sprouting. In addition, significant numbers of collaterals formed along the lesioned axons, indicating that STAT3 might also positively influence reorganization. Recent research in our lab could eventually prove that overexpression of STAT3 via viral gene therapy not only improved regeneration of lesioned CST axons but also positively influenced reorganization of neuronal connections after unilateral CST lesion. STAT3 triggered the formation of new collaterals emerging from the unlesioned side and both behavioral and electrophysiological

assessments displayed that new intraspinal circuits formed, thus contributing to functional recovery (Lang et al., submitted).

In summary, these findings indicate that STAT3 is not only a key initiator of axonal regeneration but can also support the reorganization of spared neuronal circuits after spinal cord injury. The pro-regenerative properties of STAT3 and its ability to increase reorganization might – combined with training to integrate regenerating and intact fibers into functional neuronal circuits – in the future help to improve functional recovery of patients suffering from SCI.

Though our insight concerning the underlying cellular and biochemical mechanisms of spinal cord injury needs to be broadened and translational medicine has to meet many challenges such as finding applicable forms of delivering genes like STAT3, there is increasing reason to believe that spinal cord injury might at some point be a disease one can treat.

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7 Acknowledgments

I would like to thank all members of the Institute of Clinical Neuroimmunology of the LMU Munich for their support, ideas and all the fruitful discussions we had.

In particular, I would like to express my sincere gratitude to my supervisors Dr. rer. nat Florence Bareyre and Prof. Dr. med Martin Kerschensteiner for giving me the unique opportunity of working on an extremely exciting project and sharing their enormous scientific enthusiasm and knowledge with me. Thanks to both of them for all the advice, the time and the encouragement throughout my project.

I would like to thank the German National Academic Foundation for their financial support during my research period as well as the scientific experiences I could make during conferences and seminars in the field of neuroscience.

A big thank you goes out to Julius Wehrle for his help whenever a computer problem occurred….

I would like to thank Marc Weidenbusch for his ideas and all-encompassing support during the last two years.

I would like to thank all my friends. In particular my thank goes to Claudia Lang for being an exceptional friend who has continuously given me emotional and scientific support.

Last but not least, I would like to express my gratitude towards my sister and my parents who have always provided me with everything they could offer.