University of Alberta

Promoting Neuroplasticity To Repair The Injured Spinal Cord

by

Nina Weishaupt

A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Centre for Neuroscience

Fall 2013
Edmonton, Alberta

Abstract

This thesis explores strategies to promote neuronal plasticity in a rat model of cervical spinal cord injury (SCI) in an effort to achieve improved recovery of skilled forelimb use. I focused on investigating how motor pathways disrupted by an SCI may connect to spared, lesion-bridging relay pathways to re-establish communication with target regions below the injury level.

In chapter 2, I attempted to promote a detour for the cervically injured corticospinal tract (CST) via spared reticulospinal tract (RtST) axons with a combined treatment including the neurotrophins BDNF, NT-3 and rehabilitative training. Although anatomical evidence for the desired rewiring was not obtained, I found a synergistic effect of BDNF treatment and training on recovery of skilled forelimb reaching. No effect of NT-3 administered rostral to the SCI was evident.

The experiment in chapter 3 was designed to answer the question whether NT-3-induced CST collateral growth rostral to an SCI can be facilitated by systemic immune activation. Results indicate that NT-3 expression can promote collateral growth from the injured CST, irrespective of immune activation.

Since results from chapter 2 did not shed light on the previously suggested role of the RtST in recovery of hand/paw function after cervical SCI, I next examined whether the mostly spared RtST responds to SCI with changes in its anatomical projection pattern. While collateral projections

were unchanged rostral to the SCI, I observed a marked withdrawal of collaterals from grey matter regions directly caudal to the SCI.

Results from chapter 2 combined with previous reports indicate that task-specific training does often not translate into untrained tasks and may even result in undesired side effects. To elucidate the functional relationship between trained tasks further, I next investigated how training the primarily affected forelimb (PAF) and/or the less affected forelimb (LAF) after unilateral cervical SCI influences performance outcomes for each limb. Results point towards training tasks competitively recruiting available neuronal "hardware".

This work identifies promising leads for promoting plasticity of important motor tracts after SCI, and also points out targets for optimization of strategies employed. These new insights contribute to the exploration of urgently needed repair strategies for SCI.

Acknowledgements

I would like to take this opportunity to sincerely thank my supervisor Karim Fouad. His guidance, mentorship, support and passion for his work contributed significantly (***) to developing and shaping this young scientific mind. What I learned these past five years through serious Syrian criticism, bread-and-butter optimism and encouragement to accept research as a step-dancing quest with multiple directions will serve me well in the future.

I also owe a great deal of thanks to Romana Vavrek-LeCerf, whose help in all lab matters I could always count on and whose team spirit and quality of animal care are second to none. Thanks to fellow graduate students Aleksandra Krajacic and Caitlin Hurd for their collaboration and friendship over the years, it was a great pleasure to work with you. Thank you Vivian Mushahwar and Bradley Kerr for providing constructive criticism, for supporting scholarship applications and for guidance along the way. I am also grateful for assistance with my projects from technicians Lisa Li and Aja Mason, who brightened up many days with their humor and good spirits, and from summer students Ken Ta-ala, Erin Nachtigal and David Zmyslowski. Thanks to the many dedicated people in the Centre for Neuroscience, such as Carol-Ann Johnson, Megan Airmet and Dave Collins for their guidance in all things administrative and especially for repeatedly supporting my scholarship applications. I appreciate collaboration with Dr. David Bennett, Dr. Armin Blesch, Dr. Simonetta Sipione and Dr. Fred Colbourne.

My sincere gratitude and appreciation goes to those people whose love and support I could always count on and who deserve credit for their vital contributions to making these past five years a success and a pleasure in every regard. Many thanks to my parents, Karin and Rolf Weishaupt, for their unfailing and unconditional support from across the ocean. Thanks to my partner Erwin Jands for making sure my graduate student life was never short on love, culture or adventure. This would have been considerably harder to accomplish without all of you.

I would like to extend my appreciation to funding bodies that have helped to support me and my work in Karim's laboratory over the years: International Spinal Research Trust (ISRT), Canadian Institutes of Health Research (CIHR), Alberta Innovates Health Solutions (AIHS), The University of Alberta Faculty of Graduate Studies and Research (FGSR) and the Centre for Neuroscience.

Thank you, Dr. Michael Sofroniew, for agreeing to be an external examiner for the defense of this thesis, and for traveling so far north from UCLA.

Table of Contents

List of Tables

2. Synergistic effects of BDNF and rehabilitative training on recovery after cervical spinal cord injury

List of Figures

1. General introduction to spinal cord injury repair

2. Synergistic effects of BDNF and rehabilitative training on recovery after cervical spinal cord injury

3. Vector-induced NT-3 expression promotes collateral growth of injured corticospinal tract axons in a rat model of spinal cord injury

4. Reticulospinal plasticity after cervical spinal cord injury in the rat involves withdrawal of projections below the injury

5. Training following unilateral cervical spinal cord injury in rats affects the contralesional forelimb

6. Critical discussion of findings

Abbreviations

AAV – Adeno-associated virus

AAV-2 – Adeno-associated virus serotype 2

ABC – Avidin-biotin complex (unless in "chondroitinase ABC")

ANOVA – Analysis of variance

BDA – Biotinylated dextran amine

BDNF – Brain-derived neurotrophic factor

C – Cervical level of spinal cord

CF – Contralesional forelimb

CNS – Central nervous system

CSPG – Chondroitin sulfate proteoglycan

CST – Corticospinal tract

DAB - Diaminobenzidine

DNA – Desoxyribonucleic acid

DT – Double trained group (trained in pellet reaching and on ladder)

ELISA – Enzyme-linked immunosorbent assay

FGF – Fibroblast growth factor

GFP – Green fluorescent protein

HL – Horizontal ladder only trained group

i.p. – Intraperitoneal

IF – Ipsilesional forelimb

LAF – Less affected forelimb

LPS – Lipopolysaccharide

MAG – Myelin-associated glycoprotein

MOI – Multiplicity of infection

NGF – Nerve growth factor

NT-3 – Neurotrophin 3

NT-4/5 – Neurotrophin 4/5

OMgp – Oligodendrocyte myelin glycoprotein

p.i. – Post injury

PAF – Primarily affected forelimb

PBS – Phosphate-buffered saline

PCR – Polymerase chain reaction

PNS – Peripheral nervous system

RNA – Ribonucleic acid

RST – Rubrospinal tract

RT-PCR – Real-time polymerase chain reaction

RtST – Reticulospinal tract

s.c. – Subcutaneous

SCI – Spinal cord injury

SEM – Standard error of the mean

SP – Single pellet only trained group

T – Thoracic level of spinal cord

TBS – Tris-buffered saline

TBS-TX – Tris-buffered saline containing 0.5% Triton X

TrkB – Tropomyosin-related kinase B

TrkC – Tropomyosin-related kinase C

VEGF – Vascular endothelial growth factor

CHAPTER 1

Introduction to spinal cord injury repair

1.1. Spinal cord injury

Spinal cord injury (SCI) is a devastating condition that usually leaves affected individuals with serious disabilities for the rest of their lives. It is estimated that close to 270,000 individuals are living with SCI in the US today, with approximately 12,000 new cases annually (NSCISC, 2012). Recent statistics reported almost 118,000 people living with SCI in Canada (Statistics Canada, 2010/2011). In both countries, motor vehicle accidents are the most common cause of traumatic SCI, followed by falls (Rick Hansen Registry Canada, 2001/2002, NSCISC, 2012,). Unfortunately, safe therapies for individuals with SCI that can restore lost motor function are still not within reach. Although advancements in technology, such as functional electrical stimulation and robotics (reviewed in Stein and Mushahwar, 2005, and in del-Ama *et al.*, 2012, Ethier *et al.*, 2012) promise to enhance the quality of life for people living with SCI, hopes to find effective therapeutics to restore function after SCI are high. This thesis is a contribution to exploring urgently needed 'repair' strategies that are aimed at re-establishing disrupted communication among neuronal networks above and below the injury.

1.2. Why is spinal cord injury such a devastating condition?

The spinal cord does not only carry nerve fibers through which bidirectional communication between the brain and the body is maintained, but it also has computing power of its own. When the spinal cord is injured, its significant

contribution to bodily functions including motor control and sensation becomes evident. Depending on injury location and severity, individuals who experience a traumatic SCI are often left with debilitating conditions such as loss of sensory and motor function (Kirshblum *et al.*, 2011), spasticity (reviewed in Elbasiouny *et al.*, 2010), pain (reviewed in Yezierski, 2009), autonomic dysreflexia (reviewed in Baguley, 2008, and in Krassioukov *et al.*, 2009), bladder and bowel dysfunction (reviewed in Craggs *et al.*, 2006, Abrams *et al.*, 2008), and deficits in sexual function (reviewed in Brown *et al.*, 2006, and in Sipski and Arenas, 2006).

The location and severity of any damage to axons that carry motor input from the brain down the spinal cord determine the extent and degree of the resulting paralysis or loss of sensori-motor control. While injury to the thoracic spinal cord frequently results in paraplegia of the legs, traumatic injury to the cervical spinal cord may result in tetraplegia involving arms and legs to differing degrees (Kirshblum *et al.*, 2011). My research is aimed at repairing the incompletely injured cervical spinal cord, as the majority of SCIs fall into the category "motor incomplete" (Pickett *et al.*, 2006, NSCISC, 2012). The focus of my research will be to promote recovery of lost hand function, which is one of the most desired functions that people suffering from cervical SCI wish to regain (Hanson and Franklin, 1976, Anderson, 2004, Snoek *et al.*, 2004). One of the motor tracts essential for skilled hand function in the human, and often disrupted by SCI, is the corticospinal tract (CST; Lawrence and Kuypers, 1968, reviewed in Lemon and Griffiths, 2005, and in

Brochier and Umilta, 2007). In order to develop a promising strategy for CST repair, a thorough knowledge of the challenges we are faced with when trying to repair the injured spinal cord is necessary.

1.3. Challenges of repairing the injured spinal cord

Most organs of our body have great potential to heal, because new cells are constantly born and replace aged or injured cells. Unfortunately, the central nervous system (CNS) is unique in that very few neurons are generated in the mature brain and spinal cord (reviewed in Lie *et al.*, 2004). Additionally, the complexity of the mature CNS makes proper integration of new neurons extremely challenging. Because injured neurons are not easily replaced, neither endogenously nor by grafting, acute therapeutic interventions for SCI focus on neuroprotection and tissue preservation. This is important because "secondary damage", an exacerbation of the primary injury due to toxic factors released by activated glia and inflammatory cells, commonly occurs in the early post SCI period (reviewed in Hausmann, 2003, in Hagg and Oudega, 2006, in Sofroniew, 2009, and in Oyinbo, 2011). Oxidative metabolites like nitric oxide and pro-inflammatory chemokines such as tumor-necrosis-factor-alpha (TNF-α), interferon-gamma (IFN-γ) and interleukin-1 (IL-1) create an environment that induces 'bystander' damage to cells and axons in the vicinity of the primary injury (reviewed in Hausmann, 2003, Weishaupt *et al.*, 2010). In an attempt to limit processes leading to secondary damage,

early treatment with the immune-modulating, anti-inflammatory steroid methylprednisolone was widely adopted for acute traumatic SCI (Tsutsumi *et al.*, 2006, Bracken, 2012). However, administering high dose methylprednisolone comes at the risk of increased infection (Galandiuk *et al.*, 1993, Suberviola *et al.*, 2008), and its cost/benefit ratio is controversial (Galandiuk *et al.*, 1993). Today, the use of high dose methylprednisolone is not considered the standard of care for acute SCI, and is not recommended routinely (Canadian Association of Emergency Physicians, Fehlings, 2001). While limiting the spread of damage early after an SCI is an important area of research, it is equally important to find ways to repair axonal pathways that have been severed by an SCI in an effort to restore essential connectivity between the brain, the spinal cord, spinal motor neurons, and muscles. This endeavor is particularly challenging because injured axons in the CNS do not regenerate spontaneously (reviewed in Zurn and Bandtlow, 2006). The question is, can regeneration of CNS axons be promoted to an extent where lost connectivity can be restored?

1.3.1. Can axonal regeneration in the central nervous system be achieved?

In individuals with SCI, a small degree of spontaneous functional improvement usually occurs depending on the location and extent of the injury (Steeves *et al.*, 2011, Steeves *et al.*, 2012). The main reason for this

limited recovery is the fact that central nerve fibers do not regenerate spontaneously, in contrast to peripheral nerve fibers (reviewed in Zurn and Bandtlow, 2006, Chen *et al.*, 2007). Injured peripheral axons successfully maintain growth cones at their severed end to probe the environment for growth cues (reviewed in Song and Poo, 1999, and in Zheng and Poo, 2007), and effectively regenerate to re-innervate distal targets (reviewed in Chen *et al.*, 2007). Unlike peripheral axons, severed mature CNS axons usually retract from the injury site, and most growth cones collapse soon after their formation (reviewed in Fawcett *et al.*, 1989, and in Schwab, 1996). This fundamental difference in readiness to regenerate between nerve fibers of the CNS and PNS led many to conclude that central axons inherently lack the ability to regenerate altogether.

In 1981, David and Aguayo provided hope that regeneration in the CNS may be achievable by conducting a pioneering experiment that "kick started" the field of spinal cord injury (David and Aguayo, 1981). The team provided evidence that the failure of the spinal cord to regenerate is not due to an intrinsic inability of central nerve fibers to grow, but due to local environmental factors. David and Aguayo used a peripheral nerve graft to bridge a spinal lesion between the medulla (a ventral brainstem structure connecting to the spinal cord) and the cervical spinal cord. Under this condition, both descending and ascending central axons were able to grow in the graft environment over considerable distances. This was the first time that outgrowth of central nerve fibers to this extent was observed.

The elegant experiment by David and Aguayo spurred a cascade of investigations into which factors in the peripheral nerve graft were beneficial (reviewed in Bosse, 2012), and which factors in the CNS might, in contrast, be inhibitory to the outgrowth of neurons (reviewed in Yang and Schnaar, 2008). From 1981 until today, we have learned a great deal about why neurons are so reluctant to grow in the spinal cord.

1.3.2. Impediments to axonal regeneration in the spinal cord

Myelinating glia cells (Schwann cells in the PNS and oligodendrocytes in the CNS) account for one major difference between the overall growth promoting PNS environment and the generally growth-inhibitory CNS environment (reviewed in Quarles, 2005). In the years following the discovery by David and Aguayo, Schwann cells earned a reputation for their strong growth-promoting capacities (Bixby *et al.*, 1988) and have frequently been grafted into the injured spinal cord of experimental animals (Paino *et al.*, 1994, Martin *et al.*, 1996, Wiliams and Bunge, 2012). In contrast, examination of CNS myelin led to the discovery of a group of oligodendrocyte-associated neurite outgrowth inhibitors (Schwab and Caroni, 1988, Schnell and Schwab, 1990). These myelin-associated inhibitors include Nogo A, oligodendrocyte myelin glycoprotein (OMgp), and myelin-associated glycoprotein (MAG; reviewed in Filbin, 2003, Domeniconi and Filbin, 2005). Neutralizing these growth inhibitors, blocking their receptors or targeting the inhibitory

signaling pathways downstream of receptor activation has been shown to elicit limited regeneration of selected fiber populations (Schnell and Schwab, 1990, reviewed in Fouad *et al.*, 2001, and in Kubo *et al.*, 2007). While this is a step towards promoting regeneration, the achievements are still overall underwhelming in regards to the goal of promoting long distance regeneration as a means to ultimately restore lost connectivity after SCI.

Non-myelinating CNS glia cells, such as astrocytes and microglia, were also investigated for their potential to contribute to a growth-inhibitory CNS environment. The presence and activation of glia cells create a micro-environment of signaling factors such as antigens, growth factors and chemokines. These molecules are involved in cross-talk with all neighboring cells and axons, including immune cells in the course of Wallerian degeneration of the distal axon or nerve stump (reviewed in Gaudet *et al.*, 2011, in Allodi *et al.*, 2012, in Bosse, 2012, and in Patodia and Raivich, 2012). As a result, hallmarks of injury in the CNS include the formation of scar tissue and lesion exacerbating inflammatory actions ("secondary damage"; reviewed in Hausmann, 2003, in Hagg and Oudega, 2006, in Sofroniew, 2009, and in Oyinbo, 2011), whereas in the PNS, efficient clearance of debris and spontaneous constructive reorganization prevails (reviewed in Vargas and Barres, 2007). Further, CNS glia cells were found to produce chondroitin-sulfate-proteoglycans (CSPGs), which inhibit neurite outgrowth by inducing growth cone collapse in the healthy mature CNS and particularly after CNS injury (reviewed in Sharma *et al.*, 2012). In the intact mature CNS, CSPGs

form the perineuronal net (reviewed in Wang and Fawcett, 2012), which is important for stabilizing established neuronal networks. In the course of a traumatic SCI, astroglia enter an activated state in which CSPG synthesis is increased, presumably in an effort to seal off the affected site with a scar-like structure (reviewed in Fawcett and Asher, 1999, and in Morgenstern *et al.*, 2002). The resulting CSPG-rich glial scar in the vicinity of the injury presents both a mechanical and chemical barrier for axonal growth (reviewed in Fitch and Silver, 2008). Breakdown of CSPGs in scarred regions with the bacterial enzyme chondroitinase ABC has proven to be somewhat effective in facilitating regeneration, and seems to be an intervention with potential for future therapeutic use (Bradbury *et al.*, 2002, reviewed in Bradbury and Carter, 2011).

While neutralizing numerous inhibitory molecules may ultimately provide a growth-permissive environment (reviewed in Fawcett and Asher, 1999), cells of the central nervous system are often reluctant to enter into growth mode without being exposed to active growth stimulating factors (reviewed in Sun and He, 2010). Some examples of such trophic factors are glial cell line-derived neurotrophic factor (GDNF; Fine *et al.*, 2002), ciliary neurotrophic factor (CNTF; Muller *et al.*, 2007) and the classical neurotrophins nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3) and neurotrophin 4/5 (NT-4/5; reviewed in Sofroniew *et al.*, 2001, in Skaper, 2012, and in Weishaupt *et al.*, 2012). The production and release of these trophic factors can be up-regulated under

various conditions including increased neuronal activity (Cote *et al.*, 2011, reviewed in Udina *et al.*, 2011). Neuronal activity can be maintained or increased via training/exercise or via electrical stimulation, both of which have been shown to enhance axonal growth and regeneration (Al-Majed *et al.*, 2000, reviewed in Vaynman and Gomez-Pinilla, 2005, Huie *et al.*, 2012). It is conceivable that training and exercise, which recruit complex networks of neurons to program and control a movement, may also be able to facilitate connections between cells in a target-oriented, meaningful manner. Taken together, a permissive environment, the availability of relevant growth factors and neuronal activity can promote fiber growth and regeneration to a certain degree.

Unfortunately, attempts to employ these interventions are complicated by the fact that various neuronal populations respond to environmental factors to varying extents (Vavrek *et al.*, 2007). For example, certain neurons, such as those forming the corticospinal tract (CST), still do not regenerate to a noteworthy extent following treatment with trophic factors (Hiebert *et al.*, 2002, Hollis *et al.*, 2009). Although we now have a promising array of tools in our growth-promoting toolbox, effective and functionally meaningful regeneration in the CNS, such as long distance regeneration, new synaptic connections with appropriate target cells and myelination of newly grown axons (Alto *et al.,* 2009, reviewed in Wu and Ren, 2009), is still not a reality. The relative reluctance of important fiber tracts

such as the CST to respond with regeneration to growth promoting treatments may be a good reason to explore different strategies.

One alternative approach to promote new connectivity within the injured spinal cord is to graft stem cells into the lesion site, which may then differentiate into neurons or glia cells, including myelinating oligodendrocytes (Sandner *et al.*, 2012). Efforts to improve the survival (Oh *et al.*, 2011, reviewed in Wu *et al.*, 2011), to control the differentiation (Hofstetter *et al.*, 2005) and to promote the integration (Lu *et al.*, 2012, Zhao *et al.*, 2013) of cellular grafts are ongoing. For details on the use of stem cells for spinal cord injury repair, which are beyond the scope of this thesis, the reader is referred to recent reviews on the topic (e.g., Tsuji *et al.*, 2011, Sandner *et al.*, 2012).

Another strategy to establish new circuitry after SCI is based on the observation that although spontaneous regeneration of CNS axons is basically non-existent, limited spontaneous recovery is regularly seen in individuals with SCI and in animal models of SCI. What then is the mechanism of this naturally occurring spontaneous recovery?

1.4. Plasticity

The traditional view of the mature CNS as a static, unchanging entity has experienced radical changes in the last decades. Neuroscientific investigations have uncovered an astonishing ability of the mature nervous

system to re-arrange in response to new demands, for example following CNS injury (Courtine *et al.*, 2008), or in response to intensive use of certain neuronal circuitries in the course of training (Karbach and Schubert, 2013). These adaptations, collectively called "plasticity", occur on multiple levels of the nervous system, ranging from molecular to anatomical. Hence, plastic changes may include modulation of transmission efficacy (reviewed in Lu and Chow, 1999, Sallert *et al.*, 2009), modification of excitability (reviewed in Frigon and Rossignol, 2006, Murray *et al.*, 2010), new fiber growth (Fouad *et al.*, 2001, Weidner *et al.*, 2001, Ballermann and Fouad, 2006) and ultimately adaptations in overall network connectivity (reviewed in Frigon and Rossignol, 2006, Courtine *et al.*, 2008, reviewed in Rossignol *et al.*, 2011).

The ability of the CNS to adapt to changing demands is of crucial importance when the system is injured. How powerful such plasticity can be for the recovery of stepping following SCI for example is elegantly shown in staggered lesion experiments (Courtine *et al.*, 2008). In these experiments, animals' stepping ability improved to a certain degree after two opposing hemisections at different spinal levels had interrupted all direct connections from the brain and brainstem to the cord below the lesions. In fact, most of the recovery we observe following CNS injuries in general, and improvements in locomotor ability after SCI in particular, is now thought to be largely due to plasticity in spared networks (de Leon *et al.*, 2001, reviewed in Edgerton *et al.*, 2004, and in Rossignol *et al.*, 2011). Investigating plasticity after SCI has uncovered a multitude of injury-induced changes, basically

wherever one looks (reviewed in Rossignol, 2006, and in Onifer *et al.*, 2011). On a molecular level, for instance, neurons within the caudal stump after a sacral transection have been reported to adapt to the sudden loss of excitatory serotonergic input from brainstem centres by expressing constitutively active receptors (Murray *et al.*, 2010). The resulting restoration of excitability is remarkable. On a micro-anatomical level, injured as well as uninjured nerve fibers have been shown to respond to injury with outgrowth of collateral branches (hereafter also referred to as "sprouting") that may restore connectivity either with target cells directly or via detour circuitries (Weidner *et al.*, 2001, Bareyre *et al.*, 2004, Ballermann and Fouad, 2006, Vavrek *et al.*, 2006). A direct link between axonal sprouting and functional changes in motor pathways has been demonstrated in several studies including CST sprouting following cervical and lower thoracic injury (Fouad *et al.*, 2001, Weidner *et al.*, 2001), and collateral growth of the reticulospinal tract following thoracic injury (Ballermann and Fouad, 2006). Enhancing these injury-induced re-arrangements has become a promising target for therapeutic strategies. However, not all plasticity is beneficial (reviewed in Brown and Weaver, 2012). Some re-arrangements may result in maladaptive changes, which can lead to increased autonomic dysreflexia (Weaver *et al.*, 2001), spasticity (Tan *et al.*, 2012, reviewed in Weishaupt *et al.*, 2012), and pain (reviewed in Deumens *et al.*, 2008, and in Weishaupt *et al.*, 2012). This poses a serious caveat when exploring strategies to promote plasticity for SCI repair.

My research is aimed at restoring lost motor function by promoting plasticity of the injured CST, a pivotal tract for voluntary motor control and skilled hand use. In particular, the experiments reported in this work investigate how the injured CST may be rewired via lesion-bridging spared neurons to potentially restore lost connectivity following incomplete SCI. This leads to the question of how one can promote plasticity in general, and in particular the outgrowth of new axon collaterals that can connect to specific target neurons.

1.5. Strategies to promote plasticity

The likelihood that rewiring of the injured CNS will occur can be increased by promoting the outgrowth of axonal collaterals. From a research standpoint, it is advantageous that concepts to promote regeneration (neutralizing inhibitory factors, making growth factors available and keeping networks active) are also effective at promoting axonal sprouting (reviewed in Fouad *et al.*, 2001, in Vaynman and Gomez-Pinilla, 2005, in Fouad and Tetzlaff, 2012, and in Weishaupt *et al.*, 2012).

Recently, a controversial strategy to facilitate a growth-/plasticity-promoting environment in the injured spinal cord has received a great deal of attention. Evidence has emerged that certain components of the immune response may actually contribute to repair and plasticity after SCI (reviewed in Hagg and Oudega, 2006, Hossain-Ibrahim *et al.*, 2006, reviewed in

Donnelly and Popovich, 2008). For example, whereas so-called M1 macrophages mainly contribute to a toxic environment, macrophages of phenotype M2 were found to induce an environment favourable for repair (Lazarov-Spiegler *et al.*, 1996, Rapalino *et al.*, 1998, Kigerl *et al.*, 2009). Beneficial actions of immune cells include the rapid clearance of debris from the primary injury or from Wallerian degeneration of distal axon segments (reviewed in Vargas and Barres, 2007), which may help clear inhibitory substances. Additionally, certain immune cells are known to release chemokines to regulate toxic factor production, and growth factors to stimulate new growth (reviewed in Hagg and Oudega, 2006). The debate on whether immune responses, long regarded as detrimental, can actually be harnessed to promote plasticity while minimizing undesired side effects is ongoing (Crutcher *et al.*, 2006, reviewed in Popovich and Longbrake, 2008, Kigerl *et al.*, 2009).

While there are numerous potentially valuable approaches to rewire the injured CST, I will focus here in greater detail only on what is relevant for the following chapters: rehabilitative training, the neurotrophins brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) as well as viral vectors to express neurotrophins over a prolonged period of time.

1.5.1. Rehabilitative training

Rehabilitative training is the most widely used and the safest therapy available to individuals with SCI to date (reviewed in Lam *et al.*, 2007, and in Scholtes *et al.*, 2012, and in Yang and Musselman, 2012). Its efficacy to promote functional recovery has been demonstrated repeatedly in animal models of SCI as well as in humans (Goldshmit *et al.*, 2008, Musselman *et al.*, 2009, reviewed in Battistuzzo *et al.*, 2012). In fact, animal studies demonstrating the benefits of treadmill training were the forerunners of treadmill training for people with SCI that is so widely used today (Lovely *et al.*, 1986, Barbeau and Rossignol, 1987, de Leon *et al.*, 1998a/b, reviewed in Dietz, 2009). Further investigations in animal models discovered training-induced up-regulation of growth factor production such as increases in BDNF levels (reviewed in Vaynman and Gomez-Pinilla, 2005, Ying *et al.*, 2005, Beaumont *et al.*, 2008, Cote *et al.*, 2011). Increased collateral sprouting from axons was also observed, suggesting that training contributes to axonal plasticity (Girgis *et al.*, 2007, reviewed in Fouad and Tetzlaff, 2012). It is conceivable that training may also provide adapting networks with direction, preferentially promoting those new connections that are functionally meaningful. This is not a trivial advantage, since random plasticity may potentially result in functionally useless or even in mal-adaptive changes (reviewed in Nava and Roder, 2011, in Ferguson *et al.*, 2012, and in Pape, 2012). Because training is such a potent promoter of plasticity and functional

recovery, I will use task-specific training in a combinatorial treatment strategy with neurotrophins in an effort to rewire the cervically injured rat spinal cord in chapter 2.

A closer look at rehabilitative training has also revealed that, although it has been widely practiced for a long time, we still do not know enough about the neural mechanisms of training to optimize rehabilitation. Important parameters of rehabilitative training after SCI such as the optimal time of onset, the most beneficial intensity and the ideal degree of task-specificity lack a profound scientific basis. What is well established so far is that task-specific training often translates poorly into untrained but related motor task performance. For example, rats that underwent swim training displayed enhanced swimming performance but did not show improvements in overground walking compared to untrained rats (Smith *et al.*, 2006). Similarly, forward step training in individuals with SCI did not have beneficial effects on backward stepping (Grasso *et al.*, 2004). Of even greater concern, undesired side effects of task-specific training have emerged that call for serious questioning of the previously unchallenged safety of rehabilitative training (De Leon *et al.*, 1998a/b, Girgis *et al.*, 2007, Garcia-Alias *et al.*, 2009). In these studies, animals trained in one task were found to perform worse in a second task compared with their untrained counterparts. It seems that the question as to what training protocol can maximize motor function after SCI for every day life situations has received insufficient attention in scientific investigations (Musselman *et al.*, 2009). As evidence for

limitations of task-specific training keeps accumulating, it becomes clear that a thorough understanding of how training influences plasticity is warranted in order to be able to design optimal, customized training regimes for individuals with SCI. I will contribute to the investigation of this by addressing the question of how task-specific training may impact the less affected forelimb versus the primarily affected forelimb in a rat model of incomplete cervical SCI in chapter 5.

1.5.2. Brain-derived neurotrophic factor

Brain-derived neurotrophic factor (BDNF), a member of the neurotrophin family, is an "all-rounder" in the CNS and a promising tool for repairing the injured spinal cord (reviewed in Weishaupt *et al.*, 2012). BDNF is a diffusible peptide, that, once released, can signal to enhance neuronal survival (Kobayashi *et al.*, 1997, Lu *et al.*, 2001), synaptic plasticity (reviewed in Mendell *et al.*, 2001, and in Poo, 2001), cell excitability (reviewed in Rose *et al.*, 2004, and in Blum and Konnerth, 2005), and axon growth (Ye and Houle, 1997, Hiebert *et al.*, 2002). BDNF's growth promoting actions in particular make it a useful tool for enhancing the growth of axon collaterals to achieve functional rewiring.

Encouraging advances in promoting collateral sprouting of the CST, the most important motor tract for hand function, have been made by delivering BDNF to motor cortex neurons. Following this treatment,

axotomized CST neurons were reported to sprout within healthy tissue rostral to the injury, but did not grow into a peripheral nerve graft that bridged a thoracic lesion (Hiebert *et al.*, 2002). Our laboratory was able to confirm this result for sprouting and additionally reported an increase in contacts between CST fibers and propriospinal interneurons above the lesion with the same treatment (Vavrek *et al.*, 2006). These effects of BDNF are mostly mediated by BDNF's binding to and signaling through its tyrosine kinase receptor TrkB (reviewed in Weishaupt *et al.*, 2012). It is therefore not surprising that intrathecal delivery of a TrkB agonist was similarly successful in enhancing CST sprouting above a lesion (Fouad *et al.*, 2010).

Beyond anatomical changes, BDNF may also influence the excitability of neurons and alter synaptic transmission (Kafitz *et al.*, 1999, reviewed in Poo, 2001, Rivera *et al.*, 2004, reviewed in Carvalho *et al.*, 2008, Madara and Levine, 2008). For example, by enhancing excitability of spinal neurons, BDNF can improve locomotor function after a complete spinal transection at level T10 (Boyce *et al.*, 2012). Unfortunately, the excitatory actions of BDNF may also lead to some serious undesired side effects, such as increased pain and hyperreflexia (reviewed in Pezet *et al.*, 2002, in Merighi *et al.*, 2004, and in Merighi *et al.*, 2008, Boyce *et al.*, 2012, Lu *et al.*, 2012). BDNF and neuronal activity seem to be intricately connected, as rehabilitative training is known to up-regulate BDNF levels (reviewed in Vaynman and Gomez-Pinilla, 2005, Ying *et al.*, 2005, Beaumont *et al.*, 2008, Cote *et al.*, 2011), and the release as well as the maturation of the neurotrophin are activity-dependent as well

(reviewed in Lu *et al.*, 2005, Nagappan *et al.*, 2009). Considering these reciprocal interactions between BDNF and training, and their common effect on CST sprouting, I will address the question whether BDNF treatment combined with rehabilitative training can maximize rewiring and functional recovery after incomplete cervical SCI in the rat in chapter 2.

1.5.3. Neurotrophin 3

Neurotrophin 3 (NT-3) has potent neuroprotective (Novikova *et al.*, 2000, Tobias *et al.*, 2003), growth-stimulating and chemo-attractive properties that make it a valuable tool for rewiring the CNS in a targeted manner. These effects of NT-3 are primarily mediated by NT-3 binding to its high-affinity receptor tropomyosin-receptor-kinase C (TrkC; reviewed in Patapoutian and Reichardt, 2001). The growth-promoting effects of NT-3 are frequently evidenced by enhanced regeneration of sensory fibers (Schnell *et al.*, 1994, Ramer *et al.*, 2002, Hou *et al.*, 2012), but effects on lesioned (Schnell *et al.*, 1994) and spared corticospinal fibers have been reported as well (Zhou *et al.*, 2003, Zhou and Shine, 2003). Furthermore, it is well established that NT-3, more so than BDNF, can act as a potent guidance factor (Zhou and Shine, 2003, Genc *et al.*, 2004, Alto *et al.*, 2009). Gradients of NT-3 have been created in the injured spinal cord to guide growing axons along increasing NT-3 availability towards a target area, for example from a graft environment

into host tissue (Taylor *et al.*, 2006). The potency of NT-3 as a chemo-attractant has elegantly been shown by offering regenerating sensory axons a "choice" between an appropriate target and an inappropriate target (Alto *et al.*, 2009). When NT-3 was expressed in the appropriate target, the nucleus gracilis, a dose-response relationship was shown for sensory axons growing into the target area, where they formed functional synapses. When NT-3 was expressed in the medullary reticular formation, an inappropriate target, sensory axons grew towards the source of NT-3 into the false target. Notably, motor axons have also been demonstrated to follow the lead of NT-3. NT-3 expressed by lumbar motoneurons has repeatedly been reported to promote growth of collaterals from spared CST fibers across the spinal cord midline toward its source (Zhou *et al.*, 2003, Zhou and Shine, 2003, Chen *et al.*, 2006). Recent evidence suggests that immune activation involving CD4+ T-cells, for instance in association with active Wallerian degeneration, is necessary for this effect of NT-3 to occur (Chen *et al.*, 2008).

In an attempt to make use of the growth-promoting and chemo-attractive properties of NT-3, I will try to promote targeted rewiring of the injured rat CST in chapter 2. In chapter 3, I will investigate whether motor tracts can respond to NT-3 expressed in an environment devoid of degeneration- or injury-induced inflammation, and whether activating a systemic immune response can facilitate a growth-promoting effect of NT-3 in that region.

1.5.4. Expression of neurotrophins with viral vectors

In order to increase survival of injured cells and to promote collateral growth from axons, neurotrophins like BDNF and NT-3 have to be available to bind their specific high affinity receptor (TrkB and TrkC, respectively) on the target cell or target axon over a prolonged period of time. This is why these neurotrophins have frequently been introduced into the injured spinal cord either by cell grafts that constantly produce and release neurotrophins (Liu *et al.*, 2002, Tobias *et al.*, 2003, Sasaki *et al.*, 2009, Schnell *et al.*, 2011), or by bio-materials such as lipid microtubes or hydrogel scaffolds that act as slow-release systems (Burdick *et al.*, 2006, Piantino *et al.*, 2006, Park *et al.*, 2010, Conova *et al.*, 2011, Jain *et al.*, 2011). These techniques, however, are not the methods of choice when neurotrophin action is desired in intact parts of the nervous system. Here, osmotic minipumps can be implanted to deliver the neurotrophin at a fixed concentration and infusion rate to the region of interest (Kobayashi *et al.*, 1997, Bamber *et al.*, 2001, Coumans *et al.*, 2001, Hiebert *et al.*, 2002). Unfortunately, osmotic minipumps have their limitations. First, they have to be implanted under the skin for a prolonged time period, which harbors the risk of complications such as build-up of tissue fluid and wound infection. Also, depending on the desired period of treatment, the pumps may have to be replaced with newly filled pumps. Additionally, the need for a chronically implanted delivering cannula makes the use of pumps rather impractical for infusion to deep brain or brainstem

regions, as well as to the spinal cord. An alternative method that overcomes many of the limitations of infusion by minipumps is to express neurotrophins in the area of interest by viral vectors (Zhou and Shine, 2003, Blits *et al.*, 2004, Kwon *et al.*, 2007). Such viral vectors are constructed by stripping the viral genome of any genes involved in viral replication and inserting a plasmid coding for a neurotrophin and a promoter region. The replication-deficient viral vector then infects target cells, delivers its plasmid into the cell and thereby facilitates the expression of the coded neurotrophin by the target cell over a prolonged period of time (reviewed in Heilbronn and Weger, 2010). Adeno-associated viruses (AAV) are among the virus species commonly used for vector construction, primarily because of their safety and potential for clinical use (reviewed in Grimm and Kay, 2003, in Mandel and Burger, 2004, and in Wu *et al.*, 2006). Several serotypes of AAV (subtypes with different proteins on their surface) have been well characterized in terms of their tropism for different cell types, among them serotypes that preferentially infect neurons or glia cells (Burger *et al.*, 2004, Blits *et al.*, 2010, Mason *et al.*, 2010). AAV of serotype 2, for example, has been demonstrated to have a tropism for neuronal cells in the nervous system. An additional advantage of AAVs is that they only elicit a minor immune response, with little local gliosis or inflammation in the vicinity of the injection site (reviewed in Jooss and Chirmule, 2003, own observations).

In chapter 2 and 3, I will use AAVs of serotype 2 to express either BDNF, NT-3 or the pharmacologically inactive control substance green

fluorescent protein (GFP) to investigate whether neurotrophin treatment can promote targeted rewiring of descending motor tracts in a rat model of SCI. As mentioned in the beginning, the focus of my research is the rewiring of motor tracts interrupted by an incomplete cervical SCI with the goal of improving recovery of hand function. In order to develop a strategy for targeted rewiring, a detailed look at the motor tracts involved in hand function is necessary.

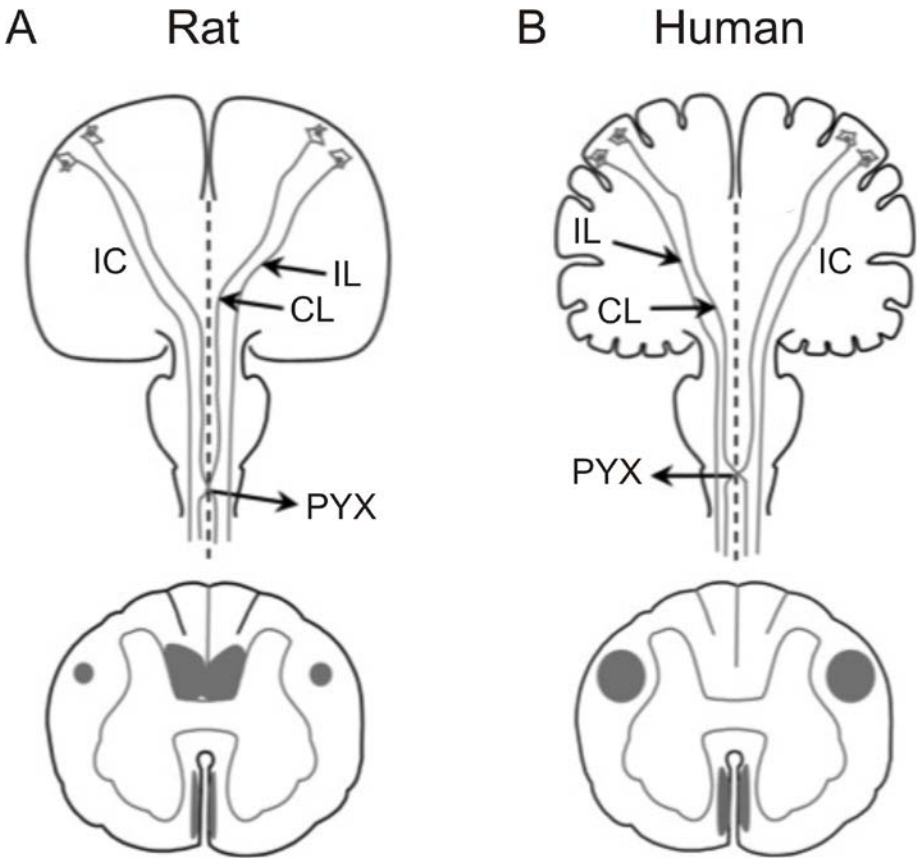
1.6. Incomplete cervical SCI: a look at motor tracts involved in fine motor control of the hand in humans and of the paw in rats

Skilled movement of the hand in non-human primates and humans largely relies on descending control from the motor cortices in the left and right hemisphere of the brain (Lawrence and Kuypers, 1968, reviewed in Lemon and Griffiths, 2005, and in Brochier and Umilta, 2007). A number of other structures at the level of the brain and brainstem are involved in fine-tuning motor programs by integrating incoming sensory feedback, which I will not discuss in detail here. I will instead concentrate on how descending motor inputs from the brain and from brainstem areas reach neurons in the cervical spinal cord to control hand movement. Species differences in the control of hand or paw movements between human and rat are important to consider as well. For example, disparities in the dominance of certain motor tracts and in the neuroanatomy pose limitations for translation between species and

warrant careful experimental design when using rat models of SCI (reviewed in Lemon and Griffiths, 2005, in Lemon, 2008, and in Oudega and Perez, 2012).

1.6.1. The corticospinal tract

In humans and rats alike, pyramidal neurons within the motor cortices send their axons out to project past deeper structures of the brain (within the internal capsule), and through the midbrain and brainstem (within the pyramidal tract; Fig. 1.1.), where some axons innervate structures such as the red nucleus, the cerebellum, and the reticular formation (Pettersson *et al.*, 2007, reviewed in Canty and Murphy, 2008). When the pyramidal tract reaches the end of the medulla, the majority of axons cross over to the opposite side before entering the spinal cord and projecting within the white matter along the length of the spinal cord (Fig. 1.1.; reviewed in Canty and Murphy, 2008). The majority of the rat CST projects in the ventral part of the dorsal funiculus (Fig. 1.1.; Fig. 1.2.; Weidner *et al.*, 2001, Oudega and Perez, 2012), while the major portion of the human CST projects in the lateral funiculus (Fig. 1.1.; Oudega and Perez, 2012). In the rat, similar to primates and humans, CST axons originating from the specialized forelimb region of the motor cortex preferentially innervate the grey matter of the cervical spinal cord to orchestrate forelimb movements (Rasmussen and Penfield, 1947a/b, Asanuma and Rosen, 1972, Kleim *et al.*, 1998).



Modified from Oudega M, and Perez MA, J. Physiol. 2012;590:3647-3663.

Figure 1.1. Corticospinal tract anatomy in rats and humans.

Schematic drawings of the brain and spinal cord illustrating the course of the corticospinal tract (CST) in adult rodents **(A)** and humans **(B)**. Corticospinal axons originate from neurons in the motor cortex and descend through the internal capsule (IC) to the brainstem, where they form the pyramidal tract. Contralaterally projecting axons (CL) cross the midline (dashed line) at the pyramidal decussation (PYX). Ipsilaterally projecting axons (IL) proceed uncrossed into the spinal cord. **(A)** In rodents, the corticospinal tract projects predominantly in the ventral part of the dorsal columns. Smaller portions of the tract project in the dorsal aspect of the lateral columns, and in the medial aspect of the ventral columns. **(B)** In humans, the majority of CST axons project in the dorsal aspect of the lateral columns, while the remaining axons travel in the medial aspect of the ventral columns (adapted from Oudega and Perez, 2012).

While CST input at cervical level is crucial for executing fine hand and finger movements in the human and non-human primate (Lawrence and Kuypers, 1968), skilled paw function in the rat is preserved to a surprisingly high degree when CST input is abolished (Whishaw *et al.*, 1998, Kanagal and Muir, 2008, Kanagal and Muir, 2009). After severance of the CST, neurons below the level of injury rely on functional reorganization of the CST itself (reviewed in Oudega and Perez, 2012), or on whatever motor input may be spared. In the rat, the spared rubrospinal tract (RST), originating from neurons within the red nucleus in the midbrain, may compensate for loss of CST input (Fig. 1.2.; Whishaw *et al.*, 1998, Raineteau *et al.*, 2001, Kanagal and Muir, 2009). One reason for this is that the motor cortex innervates the red nucleus and the rubrospinal pathway may therefore be considered as a relay for cortical signals. The fact that the RST is comparatively underdeveloped in humans and is not believed to play a major role in the control of hand movement (Nathan and Smith, 1982, Yang *et al.*, 2011) is an important caveat to keep in mind when using the rat as a model to study CST repair and recovery of hand/paw function after SCI.

Reports of reorganization of the injured CST itself or of the spared contralateral CST are plentiful (Fouad *et al.*, 2001, Hiebert *et al.*, 2002, Zhou and Shine, 2003, Bareyre *et al.*, 2004, Vavrek *et al.*, 2006, Oudega and Perez, 2012). Axonal sprouting into the grey matter from the injured CST at a spinal level rostral to a lesion occurs most commonly. It has been suggested that following thoracic SCI, these newly developed axon collaterals can connect to

long projecting interneurons located in the cervical grey matter (Bareyre *et al.*, 2004, Vavrek *et al.*, 2006), which in turn pass the signal on across several segments to denervated regions of the spinal cord. In intact rodents and cats, such relays via interneurons, though not necessarily via neurons that project across long distances, are the most common CST connections. In contrast, in intact non-human primates and humans, direct connections between the CST and motoneurons have been described to occur more frequently (reviewed in Schieber, 2007).

Taken together, a rewiring of the injured CST to areas of the spinal cord caudal to the injury site is conceivable via spared motor tracts as well as via circuitries within the spinal cord.

1.6.2. The reticulospinal tract

The reticulospinal tract is a rather heterogeneous bundle of axons originating mostly in the gigantocellular zone of the reticular formation in the brainstem (reviewed in Wang, 2009). In the spinal cord, RtST axons project broadly within the ipsi- and the contralateral ventral and lateral white matter (Fig. 1.2; Waldron and Gwyn, 1969, Zemlan *et al.*, 1984). This projection pattern is the reason why the RtST has a high probability of being at least partially spared after injury to the dorsal CST projection. Furthermore, reticular neurons are innervated by the motor cortex in humans as well as in rats, which makes the RtST a good candidate for a lesion-bridging relay for CST

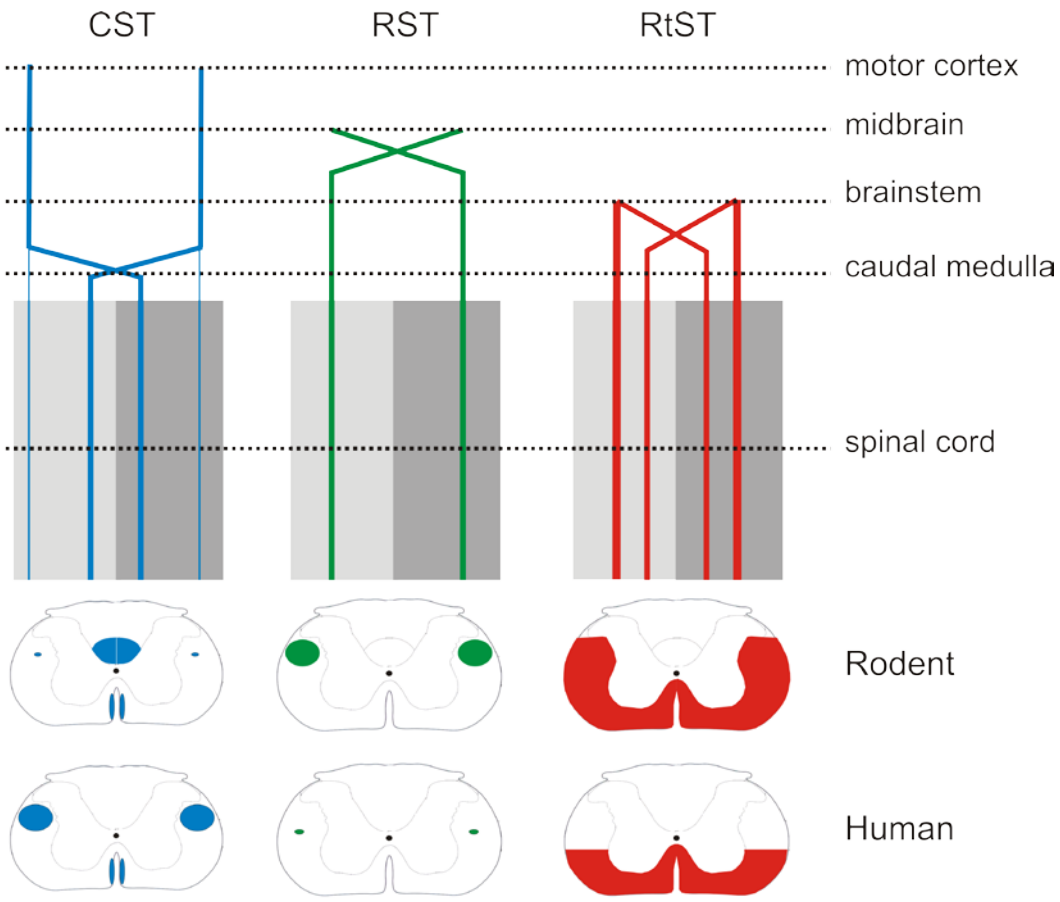


Fig. 1.2. Projections of the 3 major motor tracts in rats and humans

Corticospinal tract (CST) axons originate in the motor cortex, and the majority of axons cross the midline at the pyramidal decussation (caudal medulla). In the rat, crossed CST axons project in the dorsal white matter, whereas uncrossed axons descend either in the lateral or in the ventral white matter (blue in schematic cross section). In humans, crossed corticospinal fibers project in the lateral white matter (blue in schematic). **Rubrospinal tract (RST)** axons originate in the red nucleus (midbrain), cross the midline and project in the dorsolateral white matter in rats and humans (green in schematic cross section). **Reticulospinal tract (RtST)** axons originate in the reticular formation (brainstem), where some axons cross the midline. RtST axons project broadly within the ventral white matter (rats and humans) and within the lateral white matter (in the rat only, red in schematic cross section). Light and dark grey shaded areas represent the left and the right side of the spinal cord, respectively.

signals. To investigate this potential of the RtST, I will try to promote targeted rewiring of the unilaterally injured rat CST via the spared RtST in an effort to enhance recovery of fine motor control of the ipsilesional paw in chapter 2.

The RtST was long thought to be exclusively involved in the initiation of locomotion (Drew *et al.*, 1986, Matsuyama and Drew, 2000), in the control of posture by influencing axial core muscles (Prentice and Drew, 2001, Schepens and Drew, 2004), and in the control of reaching movements by influencing proximal muscles of the extremities (Schepens and Drew, 2004, Schepens and Drew, 2006). However, this view has recently been challenged as evidence for a role of the RtST in the control of wrist and hand/paw muscles is continuously accumulating (Alstermark *et al.*, 1987, Pettersson *et al.*, 2007, reviewed in Baker, 2011). Studies in the cat and the rat for example suggest that preserved motor function during grasping tasks following a spinal injury may be mediated by ventrally projecting pathways, such as the RtST (Alstermark *et al.*, 1987, Pettersson *et al.*, 2007, Krajacic *et al.*, 2010). Supporting such a role for the RtST, electrophysiological recordings show that distal arm muscles receive input from RtST pathways in primates (Davidson and Buford, 2006, Riddle *et al.*, 2009). While a role for the RtST in grasping function after SCI is likely, it is currently unknown whether this involvement of the RtST is accompanied by anatomical plasticity of its projections. In chapter 4, I will therefore investigate whether spontaneous

improvement in grasping is accompanied by injury-induced changes of the RtST projection pattern in the rat spinal cord.

1.7. References

Abrams P, Agarwal M, Drake M, El-Masri W, Fulford S, Reid S, et al. A proposed guideline for the urological management of patients with spinal cord injury. BJU Int. 2008;101(8):989-94.

Al-Majed AA, Neumann CM, Brushart TM, Gordon T. Brief electrical stimulation promotes the speed and accuracy of motor axonal regeneration. J. Neurosci. 2000;20(7):2602-8.

Allodi I, Udina E, Navarro X. Specificity of peripheral nerve regeneration: interactions at the axon level. Prog. Neurobiol. 2012;98(1):16-37.

Alstermark B, Lundberg A, Pettersson LG, Tantisira B, Walkowska M. Motor recovery after serial spinal cord lesions of defined descending pathways in cats. Neurosci. Res. 1987;5(1):68-73.

Alto LT, Havton LA, Conner JM, Hollis ER, 2nd, Blesch A, Tuszynski MH. Chemotropic guidance facilitates axonal regeneration and synapse formation after spinal cord injury. Nat. Neurosci. 2009;12(9):1106-13.

Anderson KD. Targeting recovery: priorities of the spinal cord-injured population. J. Neurotrauma. 2004;21(10):1371-83.

Asanuma H, Rosen I. Topographical organization of cortical efferent zones projecting to distal forelimb muscles in the monkey. Exp. Brain Res. 1972;14(3):243-56.

Baguley IJ. Autonomic complications following central nervous system injury. Semin. Neurol. 2008;28(5):716-25.

Baker SN. The primate reticulospinal tract, hand function and functional recovery. J. Physiol. 2011;589(Pt 23):5603-12.

Ballermann M, Fouad K. Spontaneous locomotor recovery in spinal cord injured rats is accompanied by anatomical plasticity of reticulospinal fibers. Eur. J. Neurosci. 2006;23(8):1988-96.

Bamber NI, Li H, Lu X, Oudega M, Aebischer P, Xu XM. Neurotrophins BDNF and NT-3 promote axonal re-entry into the distal host spinal cord through Schwann cell-seeded mini-channels. Eur. J. Neurosci. 2001;13(2):257-68.

Barbeau H, Rossignol S. Recovery of locomotion after chronic spinalization in the adult cat. Brain Res. 1987;412(1):84-95.

Bareyre FM, Kerschensteiner M, Raineteau O, Mettenleiter TC, Weinmann O, Schwab ME. The injured spinal cord spontaneously forms a new intraspinal circuit in adult rats. Nat. Neurosci. 2004;7(3):269-77.

Battistuzzo CR, Callister RJ, Callister R, Galea MP. A systematic review of exercise training to promote locomotor recovery in animal models of spinal cord injury. J. Neurotrauma. 2012;29(8):1600-13.

Beaumont E, Kaloustian S, Rousseau G, Cormery B. Training improves the electrophysiological properties of lumbar neurons and locomotion after thoracic spinal cord injury in rats. Neurosci. Res. 2008;62(3):147-54.

Bixby JL, Lilien J, Reichardt LF. Identification of the major proteins that promote neuronal process outgrowth on Schwann cells in vitro. J.

Cell. Biol. 1988;107(1):353-61.

Blits B, Carlstedt TP, Ruitenberg MJ, de Winter F, Hermens WT, Dijkhuizen PA, et al. Rescue and sprouting of motoneurons following ventral root avulsion and reimplantation combined with intraspinal adeno-associated viral vector-mediated expression of glial cell line-derived neurotrophic factor or brain-derived neurotrophic factor. Exp. Neurol. 2004;189(2):303-16.

Blits B, Derks S, Twisk J, Ehlert E, Prins J, Verhaagen J. Adeno-associated viral vector (AAV)-mediated gene transfer in the red nucleus of the adult rat brain: comparative analysis of the transduction properties of seven AAV serotypes and lentiviral vectors. J. Neurosci. Methods. 2010;185(2):257-63.

Blum R, Konnerth A. Neurotrophin-mediated rapid signaling in the central nervous system: mechanisms and functions. Physiology (Bethesda). 2005;20:70-8.

Bosse F. Extrinsic cellular and molecular mediators of peripheral axonal regeneration. Cell. Tissue Res. 2012;349(1):5-14.

Boyce VS, Park J, Gage FH, Mendell LM. Differential effects of brain-derived neurotrophic factor and neurotrophin-3 on hindlimb function in paraplegic rats. Eur. J. Neurosci. 2012;35(2):221-32.

Bracken MB. Steroids for acute spinal cord injury. Cochrane Database Syst. Rev. 2012;1:CD001046.

Bradbury EJ, Carter LM. Manipulating the glial scar: chondroitinase ABC

as a therapy for spinal cord injury. Brain Res. Bull. 2011;84(4-5):306-16.

Bradbury EJ, Moon LD, Popat RJ, King VR, Bennett GS, Patel PN, et al. Chondroitinase ABC promotes functional recovery after spinal c ord injury. Nature. 2002;416(6881):636-40.

Brochier T, Umilta MA. Cortical control of grasp in non-human primates. Curr. Opin. Neurobiol. 2007;17(6):637-43.

Brown A, Weaver LC. The dark side of neuroplasticity. Exp. Neurol. 2012;235(1):133-41.

Brown DJ, Hill ST, Baker HW. Male fertility and sexual function after spinal cord injury. Prog. Brain Res. 2006;152:427-39.

Burdick JA, Ward M, Liang E, Young MJ, Langer R. Stimulation of neurite outgrowth by neurotrophins delivered from degradable hydrogels. Biomaterials. 2006;27(3):452-9.

Burger C, Gorbatyuk OS, Velardo MJ, Peden CS, Williams P, Zolotukhin S, et al. Recombinant AAV viral vectors pseudotyped with viral capsids from serotypes 1, 2, and 5 display differential efficiency and cell tropism after delivery to different regions of the central nervous system. Mol. Ther. 2004;10(2):302-17.

Canadian Association of Emergency Physician: Position Statement on "Steroids in Acute Spinal Cord Injury". http://caep.ca/resources/position-statements-and-guidelines/steroids-acute-spinal-cord-injury

Canty AJ, Murphy M. Molecular mechanisms of axon guidance in the developing corticospinal tract. Prog. Neurobiol. 2008;85(2):214-35.

Carvalho AL, Caldeira MV, Santos SD, Duarte CB. Role of the brain-derived neurotrophic factor at glutamatergic synapses. Br. J. Pharmacol. 2008;153 Suppl 1:S310-24.

Chen Q, Smith GM, Shine HD. Immune activation is required for NT-3-induced axonal plasticity in chronic spinal cord injury. Exp. Neurol. 2008;209(2):497-509.

Chen Q, Zhou L, Shine HD. Expression of neurotrophin-3 promotes axonal plasticity in the acute but not chronic injured spinal cord. J. Neurotrauma. 2006;23(8):1254-60.

Chen ZL, Yu WM, Strickland S. Peripheral regeneration. Annu. Rev. Neurosci. 2007;30:209-33.

Conova L, Vernengo J, Jin Y, Himes BT, Neuhuber B, Fischer I, et al. A pilot study of poly(N-isopropylacrylamide)-g-polyethylene glycol and poly(N-isopropylacrylamide)-g-methylcellulose branched copolymers as injectable scaffolds for local delivery of neurotrophins and cellular transplants into the injured spinal cord. J. Neurosurg. Spine. 2011;15(6):594-604.

Cote MP, Azzam GA, Lemay MA, Zhukareva V, Houle JD. Activity-dependent increase in neurotrophic factors is associated with an enhanced modulation of spinal reflexes after spinal cord injury. J. Neurotrauma. 2011;28(2):299-309.

Coumans JV, Lin TT, Dai HN, MacArthur L, McAtee M, Nash C, et al. Axonal regeneration and functional recovery after complete spinal cord transection in rats by delayed treatment with transplants and neurotrophins. J. Neurosci. 2001;21(23):9334-44.

Courtine G, Song B, Roy RR, Zhong H, Herrmann JE, Ao Y, et al. Recovery of supraspinal control of stepping via indirect propriospinal relay connections after spinal cord injury. Nat. Med. 2008;14(1):69-74.

Craggs MD, Balasubramaniam AV, Chung EA, Emmanuel AV. Aberrant reflexes and function of the pelvic organs following spinal cord injury in man. Auton. Neurosci. 2006;126-127:355-70.

Crutcher KA, Gendelman HE, Kipnis J, Perez-Polo JR, Perry VH, Popovich PG, et al. Debate: "is increasing neuroinflammation beneficial for neural repair?". J. Neuroimmune Pharmacol. 2006;1(3):195-211.

David S, Aguayo AJ. Axonal elongation into peripheral nervous system "bridges" after central nervous system injury in adult rats. Science. 1981;214(4523):931-3.

Davidson AG, Buford JA. Bilateral actions of the reticulospinal tract on arm and shoulder muscles in the monkey: stimulus triggered averaging. Exp. Brain Res. 2006;173(1):25-39.

De Leon RD, Hodgson JA, Roy RR, Edgerton VR. Full weight-bearing hindlimb standing following stand training in the adult spinal cat. J. Neurophysiol. 1998a;80(1):83-91.

De Leon RD, Hodgson JA, Roy RR, Edgerton VR. Locomotor capacity

attributable to step training versus spontaneous recovery after spinalization in adult cats. J. Neurophysiol. 1998b;79(3):1329-40.

De Leon RD, Roy RR, Edgerton VR. Is the recovery of stepping following spinal cord injury mediated by modifying existing neural pathways or by generating new pathways? A perspective. Phys. Ther. 2001;81(12):1904-11.

Del-Ama AJ, Koutsou AD, Moreno JC, de-los-Reyes A, Gil-Agudo A, Pons JL. Review of hybrid exoskeletons to restore gait following spinal cord injury. J. Rehabil. Res. Dev. 2012;49(4):497-514.

Deumens R, Joosten EA, Waxman SG, Hains BC. Locomotor dysfunction a nd pain: the scylla and charybdis of fiber sprouting after spinal cord injury. Mol. Neurobiol. 2008;37(1):52-63.

Dietz V. Body weight supported gait training: from laboratory to clinical setting. Brain Res. Bull. 2009;78(1):I-VI.

Domeniconi M, Filbin MT. Overcoming inhibitors in myelin to promote axonal regeneration. J. Neurol. Sci. 2005;233(1-2):43-7.

Donnelly DJ, Popovich PG. Inflammation and its role in neuroprotection, axonal regeneration and functional recovery after spinal cord injury. Exp. Neurol. 2008;209(2):378-88.

Drew T, Dubuc R, Rossignol S. Discharge patterns of reticulospinal and other reticular neurons in chronic, unrestrained cats walking on a treadmill. J. Neurophysiol. 1986;55(2):375-401.

Edgerton VR, Tillakaratne NJ, Bigbee AJ, de Leon RD, Roy RR. Plasticity of

the spinal neural circuitry after injury. Annu. Rev. Neurosci. 2004;27:145-67.

Elbasiouny SM, Moroz D, Bakr MM, Mushahwar VK. Management of spasticity after spinal cord injury: current techniques and future directions. Neurorehabil. Neural Repair. 2010;24(1):23-33.

Ethier C, Oby ER, Bauman MJ, Miller LE. Restoration of grasp following paralysis through brain-controlled stimulation of muscles. Nature. 2012;485(7398):368-71.

Fawcett JW, Asher RA. The glial scar and central nervous system repair. Brain Res. Bull. 1999;49(6):377-91.

Fawcett JW, Rokos J, Bakst I. Oligodendrocytes repel axons and cause axonal growth cone collapse. J. Cell. Sci. 1989;92 (Pt 1):93-100.

Fehlings MG. Editorial: recommendations regarding the use of methylprednisolone in acute spinal cord injury: making sense out of the controversy. Spine. 2001;26(24 Suppl):S56-7.

Ferguson AR, Huie JR, Crown ED, Baumbauer KM, Hook MA, Garraway SM, et al. Maladaptive spinal plasticity opposes spinal learning and recovery in spinal cord injury. Front. Physiol. 2012;3:399.

Filbin MT. Myelin-associated inhibitors of axonal regeneration in the adult `mammalian CNS. Nat. Rev. Neurosci. 2003;4(9):703-13.

Fine EG, Decosterd I, Papaloizos M, Zurn AD, Aebischer P. GDNF and NGF released by synthetic guidance channels support sciatic nerve regeneration across a long gap. Eur. J. Neurosci. 2002;15(4):589-

601.

Fitch MT, Silver J. CNS injury, glial scars, and inflammation: Inhibitory extracellular matrices and regeneration failure. Exp. Neurol. 2008;209(2):294-301.

Fouad K, Dietz V, Schwab ME. Improving axonal growth and functional recovery after experimental spinal cord injury by neutralizing myelin associated inhibitors. Brain Res. Brain Res. Rev. 2001;36(2-3):204-12.

Fouad K, Pedersen V, Schwab ME, Brosamle C. Cervical sprouting of corticospinal fibers after thoracic spinal cord injury accompanies shifts in evoked motor responses. Curr. Biol. 2001;11(22):1766-70.

Fouad K, Tetzlaff W. Rehabilitative training and plasticity following spinal cord injury. Exp. Neurol. 2012;235(1):91-9.

Fouad K, Vavrek R, Cho S. A TrkB antibody agonist promotes plasticity following cervical spinal cord injury in adult rats. J. Neurotrauma. 2010, in press.

Frigon A, Rossignol S. Functional plasticity following spinal cord lesions. Prog. Brain Res. 2006;157:231-60.

Galandiuk S, Raque G, Appel S, Polk HC, Jr. The two-edged sword of large-dose steroids for spinal cord trauma. Ann. Surg. 1993;218(4):419-25; discussion 25-7.

Garcia-Alias G, Barkhuysen S, Buckle M, Fawcett JW. Chondroitinase ABC treatment opens a window of opportunity for task-specific

rehabilitation. Nat. Neurosci. 2009;12(9):1145-51.

Gaudet AD, Popovich PG, Ramer MS. Wallerian degeneration: gaining perspective on inflammatory events after peripheral nerve injury. J. Neuroinflammation. 2011;8:110.

Genc B, Ozdinler PH, Mendoza AE, Erzurumlu RS. A chemo-attractant role for NT-3 in proprioceptive axon guidance. PLoS Biol. 2004;2(12):e403.

Girgis J, Merrett D, Kirkland S, Metz GA, Verge V, Fouad K. Reaching training in rats with spinal cord injury promotes plasticity and task specific recovery. Brain. 2007;130(Pt 11):2993-3003.

Goldshmit Y, Lythgo N, Galea MP, Turnley AM. Treadmill training after spinal cord hemisection in mice promotes axonal sprouting and synapse formation and improves motor recovery. J. Neurotrauma. 2008;25(5):449-65.

Grasso R, Ivanenko YP, Zago M, Molinari M, Scivoletto G, Lacquaniti F. Recovery of forward stepping in spinal cord injured patients does not transfer to untrained backward stepping. Exp. Brain Res. 2004;157(3):377-82.

Grimm D, Kay MA. From virus evolution to vector revolution: use of naturally occurring serotypes of adeno-associated virus (AAV) as novel vectors for human gene therapy. Curr. Gene Ther. 2003;3(4):281-304.

Hagg T, Oudega M. Degenerative and spontaneous regenerative processes

after spinal cord injury. J. Neurotrauma. 2006;23(3-4):264-80.

Hanson RW, Franklin MR. Sexual loss in relation to other functional losses for spinal cord injured males. Arch. Phys. Med. Rehabil. 1976;57(6):291-3.

Hausmann ON. Post-traumatic inflammation following spinal cord injury. Spinal Cord. 2003;41(7):369-78.

Heilbronn R, Weger S. Viral vectors for gene transfer: current status of gene therapeutics. Handb. Exp. Pharmacol. 2010(197):143-70.

Hiebert GW, Khodarahmi K, McGraw J, Steeves JD, Tetzlaff W. Brain-derived neurotrophic factor applied to the motor cortex promotes sprouting of corticospinal fibers but not regeneration into a peripheral nerve transplant. J. Neurosci. Res. 2002;69(2):160-8.

Hofstetter CP, Holmstrom NA, Lilja JA, Schweinhardt P, Hao J, Spenger C, et al. Allodynia limits the usefulness of intraspinal neural stem cell grafts; directed differentiation improves outcome. Nat. Neurosci. 2005;8(3):346-53.

Hollis ER, 2nd, Lu P, Blesch A, Tuszynski MH. IGF-I gene delivery promotes corticospinal neuronal survival but not regeneration after adult CNS injury. Exp. Neurol. 2009;215(1):53-9.

Hossain-Ibrahim MK, Rezajooi K, MacNally JK, Mason MR, Lieberman AR, Anderson PN. Effects of lipopolysaccharide-induced inflammation on expression of growth-associated genes by corticospinal neurons. BMC Neurosci. 2006;7:8.

Hou S, Nicholson L, van Niekerk E, Motsch M, Blesch A. Dependence of regenerated sensory axons on continuous neurotrophin-3 delivery. J. Neurosci. 2012;32(38):13206-20.

Huie JR, Garraway SM, Baumbauer KM, Hoy KC, Jr., Beas BS, Montgomery KS, et al. Brain-derived neurotrophic factor promotes adaptive plasticity within the spinal cord and mediates the beneficial effects of controllable stimulation. Neuroscience. 2012;200:74-90.

Jain A, McKeon RJ, Brady-Kalnay SM, Bellamkonda RV. Sustained delivery of activated Rho GTPases and BDNF promotes axon growth in CSPG-rich regions following spinal cord injury. PLoS One. 2011;6(1):e16135.

Jooss K, Chirmule N. Immunity to adenovirus and adeno-associated viral vectors: implications for gene therapy. Gene Ther. 2003;10(11):955-63.

Kafitz KW, Rose CR, Thoenen H, Konnerth A. Neurotrophin-evoked rapid excitation through TrkB receptors. Nature. 1999;401(6756):918-21.

Kanagal SG, Muir GD. Effects of combined dorsolateral and dorsal funicular lesions on sensorimotor behaviour in rats. Exp. Neurol. 2008;214(2):229-39.

Kanagal SG, Muir GD. Task-dependent compensation after pyramidal tract and dorsolateral spinal lesions in rats. Exp. Neurol. 2009;216(1):193-206.

Karbach J, Schubert T. Training-induced cognitive and neural plasticity. Front. Hum. Neurosci. 2013;7:48.

Kigerl KA, Gensel JC, Ankeny DP, Alexander JK, Donnelly DJ, Popovich PG. Identification of two distinct macrophage subsets with divergent effects causing either neurotoxicity or regeneration in the injured mouse spinal cord. J. Neurosci. 2009;29(43):13435-44.

Kirshblum SC, Burns SP, Biering-Sorensen F, Donovan W, Graves DE, Jha A, et al. International standards for neurological classification of spinal cord injury. J. Spinal Cord Med. 2011;34(6):535-46.

Kleim JA, Barbay S, Nudo RJ. Functional reorganization of the rat motor cortex following motor skill learning. J. Neurophysiol. 1998;80(6):3321-5.

Kobayashi NR, Fan DP, Giehl KM, Bedard AM, Wiegand SJ, Tetzlaff W. BDNF and NT-4/5 prevent atrophy of rat rubrospinal neurons after cervical axotomy, stimulate GAP-43 and Talpha1-tubulin mRNA expression, and promote axonal regeneration. J. Neurosci. 1997;17(24):9583-95.

Krajacic A, Weishaupt N, Girgis J, Tetzlaff W, Fouad K. Training-induced plasticity in rats with cervical spinal cord injury: effects and side effects. Behav. Brain Res. 2010;214(2):323-31.

Krassioukov A, Warburton DE, Teasell R, Eng JJ, Spinal Cord Injury Rehabilitation Evidence Research T. A systematic review of the management of autonomic dysreflexia after spinal cord injury.

Arch. Phys. Med. Rehabil. 2009;90(4):682-95.

Kubo T, Hata K, Yamaguchi A, Yamashita T. Rho-ROCK inhibitors as emerging strategies to promote nerve regeneration. Curr. Pharm. Des. 2007;13(24):2493-9.

Kwon BK, Liu J, Lam C, Plunet W, Oschipok LW, Hauswirth W, et al. Brain-derived neurotrophic factor gene transfer with adeno-associated viral and lentiviral vectors prevents rubrospinal neuronal atrophy and stimulates regeneration-associated gene expression after acute cervical spinal cord injury. Spine. 2007;32(11):1164-73.

Lam T, Eng JJ, Wolfe DL, Hsieh JT, Whittaker M, the SRT. A systematic review of the efficacy of gait rehabilitation strategies for spinal cord injury. Top. Spinal Cord Inj. Rehabil. 2007;13(1):32-57.

Lawrence DG, Kuypers HG. The functional organization of the motor system in the monkey. II. The effects of lesions of the descending brain-stem pathways. Brain. 1968;91(1):15-36.

Lazarov-Spiegler O, Solomon AS, Zeev-Brann AB, Hirschberg DL, Lavie V, Schwartz M. Transplantation of activated macrophages overcomes central nervous system regrowth failure. FASEB J. 1996;10(11):1296-302.

Lemon RN. Descending pathways in motor control. Annu. Rev. Neurosci. 2008;31:195-218.

Lemon RN, Griffiths J. Comparing the function of the corticospinal system in different species: organizational differences for motor

specialization? Muscle Nerve. 2005;32(3):261-79.

Lie DC, Song H, Colamarino SA, Ming GL, Gage FH. Neurogenesis in the adult brain: new strategies for central nervous system diseases. Annu. Rev. Pharmacol. Toxicol. 2004;44:399-421.

Liu Y, Himes BT, Murray M, Tessler A, Fischer I. Grafts of BDNF-producing fibroblasts rescue axotomized rubrospinal neurons and prevent their atrophy. Exp. Neurol. 2002;178(2):150-64.

Lovely RG, Gregor RJ, Roy RR, Edgerton VR. Effects of training on the recovery of full-weight-bearing stepping in the adult spinal cat. Exp. Neurol. 1986;92(2):421-35.

Lu B, Chow A. Neurotrophins and hippocampal synaptic transmission and plasticity. J. Neurosci. Res. 1999;58(1):76-87.

Lu B, Pang PT, Woo NH. The yin and yang of neurotrophin action. Nat. Rev. Neurosci. 2005;6(8):603-14.

Lu P, Blesch A, Graham L, Wang Y, Samara R, Banos K, et al. Motor axonal regeneration after partial and complete spinal cord transection. J. Neurosci. 2012;32(24):8208-18.

Lu P, Blesch A, Tuszynski MH. Neurotrophism without neurotropism: BDNF promotes survival but not growth of lesioned corticospinal neurons. J. Comp. Neurol. 2001;436(4):456-70.

Lu P, Wang Y, Graham L, McHale K, Gao M, Wu D, et al. Long-distance growth and connectivity of neural stem cells after severe spinal cord injury. Cell. 2012;150(6):1264-73.

Madara JC, Levine ES. Presynaptic and postsynaptic NMDA receptors mediate distinct effects of brain-derived neurotrophic factor on synaptic transmission. J. Neurophysiol. 2008;100(6):3175-84.

Mandel RJ, Burger C. Clinical trials in neurological disorders using AAV vectors: promises and challenges. Curr. Opin. Mol Ther. 2004;6(5):482-90.

Martin D, Robe P, Franzen R, Delree P, Schoenen J, Stevenaert A, et al. Effects of Schwann cell transplantation in a contusion model of rat spinal cord injury. J. Neurosci. Res. 1996;45(5):588-97.

Mason MR, Ehlert EM, Eggers R, Pool CW, Hermening S, Huseinovic A, et al. Comparison of AAV serotypes for gene delivery to dorsal root ganglion neurons. Mol. Ther. 2010;18(4):715-24.

Matsuyama K, Drew T. Vestibulospinal and reticulospinal neuronal activity during locomotion in the intact cat. I. Walking on a level surface. J. Neurophysiol. 2000;84(5):2237-56.

Mendell LM, Munson JB, Arvanian VL. Neurotrophins and synaptic plasticity in the mammalian spinal cord. J. Physiol. 2001;533(Pt 1):91-7.

Merighi A, Carmignoto G, Gobbo S, Lossi L, Salio C, Vergnano AM, et al. Neurotrophins in spinal cord nociceptive pathways. Prog. Brain Res. 2004;146:291-321.

Merighi A, Salio C, Ghirri A, Lossi L, Ferrini F, Betelli C, et al. BDNF as a pain modulator. Prog. Neurobiol. 2008;85(3):297-317.

Morgenstern DA, Asher RA, Fawcett JW. Chondroitin sulphate proteoglycans in the CNS injury response. Prog. Brain Res. 2002;137:313-32.

Muller A, Hauk TG, Fischer D. Astrocyte-derived CNTF switches mature RGCs to a regenerative state following inflammatory stimulation. Brain. 2007;130(Pt 12):3308-20.

Murray KC, Nakae A, Stephens MJ, Rank M, D'Amico J, Harvey PJ, et al. Recovery of motoneuron and locomotor function after spinal cord injury depends on constitutive activity in 5-HT2C receptors. Nat. Med. 2010;16(6):694-700.

Musselman KE, Fouad K, Misiaszek JE, Yang JF. Training of walking skills overground and on the treadmill: case series on individuals with incomplete spinal cord injury. Phys. Ther. 2009;89(6):601-11.

Nagappan G, Zaitsev E, Senatorov VV, Jr., Yang J, Hempstead BL, Lu B. Control of extracellular cleavage of ProBDNF by high frequency neuronal activity. Proc. Natl. Acad. Sci. U S A. 2009;106(4):1267-72.

Nathan PW, Smith MC. The rubrospinal and central tegmental tracts in man. Brain. 1982;105(Pt 2):223-69.

Nava E, Roder B. Adaptation and maladaptation insights from brain plasticity. Prog. Brain Res. 2011;191:177-94.

Novikova LN, Novikov LN, Kellerth JO. Survival effects of BDNF and NT-3 on axotomized rubrospinal neurons depend on the temporal pattern of neurotrophin administration. Eur. J. Neurosci.

2000;12(2):776-80.

NSCISC, Spinal Cord Injury Facts and Figures at a Glance, 2011. https://www.nscisc.uab.edu/PublicDocuments/fact_figures_docs/Facts%202012%20Feb%20Final.pdf

Oh JS, Kim KN, An SS, Pennant WA, Kim HJ, Gwak SJ, et al. Cotransplantation of mouse neural stem cells (mNSCs) with adipose tissue-derived mesenchymal stem cells improves mNSC survival in a rat spinal cord injury model. Cell Transplant. 2011;20(6):837-49.

Onifer SM, Smith GM, Fouad K. Plasticity after spinal cord injury: relevance to recovery and approaches to facilitate it. Neurotherapeutics. 2011;8(2):283-93.

Oudega M, Perez MA. Corticospinal reorganization after spinal cord injury. J. Physiol. 2012;590(Pt 16):3647-63.

Oyinbo CA. Secondary injury mechanisms in traumatic spinal cord injury: a nugget of this multiply cascade. Acta. Neurobiol. Exp. (Wars). 2011;71(2):281-99.

Paino CL, Fernandez-Valle C, Bates ML, Bunge MB. Regrowth of axons in lesioned adult rat spinal cord: promotion by implants of cultured Schwann cells. J. Neurocytol. 1994;23(7):433-52.

Pape KE. Developmental and maladaptive plasticity in neonatal SCI. Clin. Neurol. Neurosurg. 2012;114(5):475-82.

Park J, Lim E, Back S, Na H, Park Y, Sun K. Nerve regeneration following

spinal cord injury using matrix metalloproteinase-sensitive, hyaluronic acid-based biomimetic hydrogel scaffold containing brain-derived neurotrophic factor. J. Biomed. Mater Res. A. 2010;93(3):1091-9.

Patapoutian A, Reichardt LF. Trk receptors: mediators of neurotrophin action. Curr. Opin. Neurobiol. 2001;11(3):272-80.

Patodia S, Raivich G. Downstream effector molecules in successful peripheral nerve regeneration. Cell. Tissue Res. 2012;349(1):15-26.

Penfield W, Rasmussen T. The Cerebral Cortex of Man: a Clinical Study of Localization of Function. New York: Hafner, 1968.

Pettersson LG, Alstermark B, Blagovechtchenski E, Isa T, Sasaski S. Skilled digit movements in feline and primate--recovery after selective spinal cord lesions. Acta. Physiol. (Oxf). 2007;189(2):141-54.

Pezet S, Malcangio M, McMahon SB. BDNF: a neuromodulator in nociceptive pathways? Brain Res. Brain Res. Rev. 2002;40(1-3):240-9.

Piantino J, Burdick JA, Goldberg D, Langer R, Benowitz LI. An injectable, biodegradable hydrogel for trophic factor delivery enhances axonal rewiring and improves performance after spinal cord injury. Exp. Neurol. 2006;201(2):359-67.

Pickett GE, Campos-Benitez M, Keller JL, Duggal N. Epidemiology of traumatic spinal cord injury in Canada. Spine 2006;31(7):799-805.

Poo MM. Neurotrophins as synaptic modulators. Nat. Rev. Neurosci.

2001;2(1):24-32.

Popovich PG, Longbrake EE. Can the immune system be harnessed to repair the CNS? Nat. Rev. Neurosci. 2008;9(6):481-93.

Prentice SD, Drew T. Contributions of the reticulospinal system to the postural adjustments occurring during voluntary gait modifications. J. Neurophysiol. 2001;85(2):679-98.

Quarles RH. Comparison of CNS and PNS myelin proteins in the pathology of myelin disorders. J. Neurol. Sci. 2005;228(2):187-9.

Rainetau O, Fouad K, Noth P, Thallmair M, Schwab ME. Functional switch between motor tracts in the presence of the mAB IN-1 in the adult rat. Proc. Natl. Acad. Sci. USA. 2001;98(12):6929-34.

Ramer MS, Bishop T, Dockery P, Mobarak MS, O'Leary D, Fraher JP, et al. Neurotrophin-3-mediated regeneration and recovery of proprioception following dorsal rhizotomy. Mol. Cell. Neurosci. 2002;19(2):239-49.

Rapalino O, Lazarov-Spiegler O, Agranov E, Velan GJ, Yoles E, Fraidakis M, et al. Implantation of stimulated homologous macrophages results in partial recovery of paraplegic rats. Nat. Med. 1998;4(7):814-21.

Rasmussen T, Penfield W. Further studies of the sensory and motor cerebral cortex of man. Fed. Proc. 1947a;6(2):452-60.

Rasmussen T, Penfield W. The human sensorimotor cortex as studied by electrical stimulation. Fed. Proc. 1947b;6(1 Pt 2):184.

Rick Hansen Spinal Cord Injury Registry Canada, Spinal Cord Injury Facts

and Statistics 2001/2002. http://sci-bc-database.ca/wp-content/uploads/SCI-Facts-and-Stats-2005.pdf

Riddle CN, Edgley SA, Baker SN. Direct and indirect connections with upper limb motoneurons from the primate reticulospinal tract. J. Neurosci. 2009;29(15):4993-9.

Rivera C, Voipio J, Thomas-Crusells J, Li H, Emri Z, Sipila S, et al. Mechanism of activity-dependent downregulation of the neuron-specific K-Cl cotransporter KCC2. J. Neurosci. 2004;24(19):4683-91.

Rose CR, Blum R, Kafitz KW, Kovalchuk Y, Konnerth A. From modulator to mediator: rapid effects of BDNF on ion channels. Bioessays. 2004;26(11):1185-94.

Rossignol S. Plasticity of connections underlying locomotor recovery after central and/or peripheral lesions in the adult mammals. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 2006;361(1473):1647-71.

Rossignol S, Frigon A, Barriere G, Martinez M, Barthelemy D, Bouyer L, et al. Chapter 16--spinal plasticity in the recovery of locomotion. Prog. Brain Res. 2011;188:229-41.

Sallert M, Rantamaki T, Vesikansa A, Anthoni H, Harju K, Yli-Kauhaluoma J, et al. Brain-derived neurotrophic factor controls activity-dependent maturation of CA1 synapses by downregulating tonic activation of presynaptic kainate receptors. J. Neurosci. 2009;29(36):11294-303.

Sandner B, Prang P, Rivera FJ, Aigner L, Blesch A, Weidner N. Neural stem cells for spinal cord repair. Cell. Tissue Res. 2012;349(1):349-62.

Sasaki M, Radtke C, Tan AM, Zhao P, Hamada H, Houkin K, et al. BDNF-hypersecreting human mesenchymal stem cells promote functional recovery, axonal sprouting, and protection of corticospinal neurons after spinal cord injury. J. Neurosci. 2009;29(47):14932-41.

Schepens B, Drew T. Independent and convergent signals from the pontomedullary reticular formation contribute to the control of posture and movement during reaching in the cat. J. Neurophysiol. 2004;92(4):2217-38.

Schepens B, Drew T. Descending signals from the pontomedullary reticular formation are bilateral, asymmetric, and gated during reaching movements in the cat. J. Neurophysiol. 2006;96(5):2229-52.

Schieber MH. Chapter 2 Comparative anatomy and physiology of the corticospinal system. Handb. Clin. Neurol. 2007;82:15-37.

Schnell L, Hunanyan AS, Bowers WJ, Horner PJ, Federoff HJ, Gullo M, et al. Combined delivery of Nogo-A antibody, neurotrophin-3 and the NMDA-NR2d subunit establishes a functional 'detour' in the hemisected spinal cord. Eur. J. Neurosci. 2011;34(8):1256-67.

Schnell L, Schneider R, Kolbeck R, Barde YA, Schwab ME. Neurotrophin-3 enhances sprouting of corticospinal tract during development and

after adult spinal cord lesion. Nature. 1994;367(6459):170-3.

Schnell L, Schwab ME. Axonal regeneration in the rat spinal cord produced by an antibody against myelin-associated neurite growth inhibitors. Nature. 1990;343(6255):269-72.

Scholtes F, Brook G, Martin D. Spinal cord injury and its treatment: current management and experimental perspectives. Adv. Tech. Stand. Neurosurg. 2012;38:29-56.

Schwab ME. Molecules inhibiting neurite growth: a minireview. Neurochem. Res. 1996;21(7):755-61.

Schwab ME, Caroni P. Oligodendrocytes and CNS myelin are nonpermissive substrates for neurite growth and fibroblast spreading in vitro. J. Neurosci. 1988;8(7):2381-93.

Sharma K, Selzer ME, Li S. Scar-mediated inhibition and CSPG receptors in the CNS. Exp. Neurol. 2012;237(2):370-8.

Sipski ML, Arenas A. Female sexual function after spinal cord injury. Prog Brain Res. 2006;152:441-7.

Skaper SD. The neurotrophin family of neurotrophic factors: an overview. Methods Mol. Biol. 2012;846:1-12.

Smith RR, Shum-Siu A, Baltzley R, Bunger M, Baldini A, Burke DA, et al. Effects of swimming on functional recovery after incomplete spinal cord injury in rats. J. Neurotrauma. 2006;23(6):908-19.

Snoek GJ, MJ IJ, Hermens HJ, Maxwell D, Biering-Sorensen F. Survey of the needs of patients with spinal cord injury: impact and priority for

improvement in hand function in tetraplegics. Spinal Cord. 2004;42(9):526-32.

Sofroniew MV. Molecular dissection of reactive astrogliosis and glial scar formation. Trends Neurosci. 2009;32(12):638-47.

Sofroniew MV, Howe CL, Mobley WC. Nerve growth factor signaling, neuroprotection, and neural repair. Annu. Rev. Neurosci. 2001;24:1217-81.

Song HJ, Poo MM. Signal transduction underlying growth cone guidance by diffusible factors. Curr. Opin. Neurobiol. 1999;9(3):355-63.

Statistics Canada: Neurological conditions 2010/2011. http://www5.statcan.gc.ca/cansim/pick-choisir?lang=eng&p2=33&id=1051300

Steeves JD, Kramer JK, Fawcett JW, Cragg J, Lammertse DP, Blight AR, et al. Extent of spontaneous motor recovery after traumatic cervical sensorimotor complete spinal cord injury. Spinal Cord. 2011;49(2):257-65.

Steeves JD, Lammertse DP, Kramer JL, Kleitman N, Kalsi-Ryan S, Jones L, et al. Outcome Measures for Acute/Subacute Cervical Sensorimotor Complete (AIS-A) Spinal Cord Injury During a Phase 2 Clinical Trial. Top. Spinal Cord Inj. Rehabil. 2012;18(1):1-14.

Stein RB, Mushahwar V. Reanimating limbs after injury or disease. Trends Neurosci. 2005;28(10):518-24.

Suberviola B, Gonzalez-Castro A, Llorca J, Ortiz-Melon F, Minambres E.

Early complications of high-dose methylprednisolone in acute spinal cord injury patients. Injury. 2008;39(7):748-52.

Sun F, He Z. Neuronal intrinsic barriers for axon regeneration in the adult CNS. Curr. Opin. Neurobiol. 2010;20(4):510-8.

Tan AM, Chakrabarty S, Kimura H, Martin JH. Selective corticospinal tract injury in the rat induces primary afferent fiber sprouting in the spinal cord and hyperreflexia. J. Neurosci. 2012;32(37):12896-908.

Taylor L, Jones L, Tuszynski MH, Blesch A. Neurotrophin-3 gradients established by lentiviral gene delivery promote short-distance axonal bridging beyond cellular grafts in the injured spinal cord. J. Neurosci. 2006;26(38):9713-21.

Tobias CA, Shumsky JS, Shibata M, Tuszynski MH, Fischer I, Tessler A, et al. Delayed grafting of BDNF and NT-3 producing fibroblasts into the injured spinal cord stimulates sprouting, partially rescues axotomized red nucleus neurons from loss and atrophy, and provides limited regeneration. Exp. Neurol. 2003;184(1):97-113.

Tsuji O, Miura K, Fujiyoshi K, Momoshima S, Nakamura M, Okano H. Cell therapy for spinal cord injury by neural stem/progenitor cells derived from iPS/ES cells. Neurotherapeutics. 2011;8(4):668-76.

Tsutsumi S, Ueta T, Shiba K, Yamamoto S, Takagishi K. Effects of the Second National Acute Spinal Cord Injury Study of high-dose methylprednisolone therapy on acute cervical spinal cord injury-results in spinal injuries center. Spine (Phila Pa 1976).

2006;31(26):2992-6; discussion 7.

Udina E, Cobianchi S, Allodi I, Navarro X. Effects of activity-dependent strategies on regeneration and plasticity after peripheral nerve injuries. Ann. Anat. 2011;193(4):347-53.

Vargas ME, Barres BA. Why is Wallerian degeneration in the CNS so slow? Annu. Rev. Neurosci. 2007;30:153-79.

Vavrek R, Girgis J, Tetzlaff W, Hiebert GW, Fouad K. BDNF promotes connections of corticospinal neurons onto spared descending interneurons in spinal cord injured rats. Brain. 2006;129(Pt 6):1534-45.

Vavrek R, Pearse DD, Fouad K. Neuronal populations capable of regeneration following a combined treatment in rats with spinal cord transection. J. Neurotrauma. 2007;24(10):1667-73.

Vaynman S, Gomez-Pinilla F. License to run: exercise impacts functional plasticity in the intact and injured central nervous system by using neurotrophins. Neurorehabil. Neural Repair. 2005;19(4):283-95.

Waldron HA, Gwyn DG. Descending nerve tracts in the spinal cord of the rat. I. Fibers from the midbrain. J. Comp. Neurol. 1969;137(2):143-53.

Wang D. Reticular formation and spinal cord injury. Spinal Cord. 2009;47(3):204-12.

Wang D, Fawcett J. The perineuronal net and the control of CNS plasticity. Cell. Tissue Res. 2012;349(1):147-60.

Weaver LC, Verghese P, Bruce JC, Fehlings MG, Krenz NR, Marsh DR. Autonomic dysreflexia and primary afferent sprouting after clip-compression injury of the rat spinal cord. J. Neurotrauma. 2001;18(10):1107-19.

Weidner N, Ner A, Salimi N, Tuszynski MH. Spontaneous corticospinal axonal plasticity and functional recovery after adult central nervous system injury. Proc. Natl. Acad. Sci. USA. 2001;98(6):3513-8.

Weishaupt N, Blesch A, Fouad K. BDNF: the career of a multifaceted neurotrophin in spinal cord injury. Exp. Neurol. 2012;238(2):254-64.

Weishaupt N, Silasi G, Colbourne F, Fouad K. Secondary damage in the spinal cord after motor cortex injury in rats. J. Neurotrauma. 2010;27(8):1387-97.

Whishaw IQ, Gorny B, Sarna J. Paw and limb use in skilled and spontaneous reaching after pyramidal tract, red nucleus and combined lesions in the rat: behavioral and anatomical dissociations. Behav. Brain Res. 1998;93(1-2):167-83.

Wiliams RR, Bunge MB. Schwann cell transplantation: a repair strategy for spinal cord injury? Prog. Brain Res. 2012;201:295-312.

Wu B, Ren X. Promoting axonal myelination for improving neurological recovery in spinal cord injury. J. Neurotrauma. 2009;26(10):1847-56.

Wu W, Zhao H, Xie B, Liu H, Chen Y, Jiao G, et al. Implanted spike wave electric stimulation promotes survival of the bone marrow mesenchymal stem cells and functional recovery in the spinal cord injured rats. Neurosci. Lett. 2011;491(1):73-8.

Wu Z, Asokan A, Samulski RJ. Adeno-associated virus serotypes: vector toolkit for human gene therapy. Mol. Ther. 2006;14(3):316-27.

Yang HS, Kwon HG, Hong JH, Hong CP, Jang SH. The rubrospinal tract in the human brain: diffusion tensor imaging study. Neurosci. Lett. 2011;504(1):45-8.

Yang JF, Musselman KE. Training to achieve over ground walking after spinal cord injury: a review of who, what, when, and how. J. Spinal Cord Med. 2012;35(5):293-304.

Yang LJ, Schnaar RL. Axon regeneration inhibitors. Neurol. Res. 2008;30(10):1047-52.

Ye JH, Houle JD. Treatment of the chronically injured spinal cord with neurotrophic factors can promote axonal regeneration from supraspinal neurons. Exp. Neurol. 1997;143(1):70-81.

Yezierski RP. Spinal cord injury pain: spinal and supraspinal mechanisms. J. Rehabil. Res. Dev. 2009;46(1):95-107.

Ying Z, Roy RR, Edgerton VR, Gomez-Pinilla F. Exercise restores levels of neurotrophins and synaptic plasticity following spinal cord injury. Exp. Neurol. 2005;193(2):411-9.

Zemlan FP, Behbehani MM, Beckstead RM. Ascending and descending

projections from nucleus reticularis magnocellularis and nucleus reticularis gigantocellularis: an autoradiographic and horseradish peroxidase study in the rat. Brain Res. 1984;292(2):207-20.

Zhao J, Sun W, Cho HM, Ouyang H, Li W, Lin Y, et al. Integration and long distance axonal regeneration in the central nervous system from transplanted primitive neural stem cells. J. Biol. Chem. 2013;288(1):164-8.

Zheng JQ, Poo MM. Calcium signaling in neuronal motility. Annu. Rev. Cell. Dev. Biol. 2007;23:375-404.

Zhou L, Baumgartner BJ, Hill-Felberg SJ, McGowen LR, Shine HD. Neurotrophin-3 expressed in situ induces axonal plasticity in the adult injured spinal cord. J. Neurosci. 2003;23(4):1424-31.

Zhou L, Shine HD. Neurotrophic factors expressed in both cortex and spinal cord induce axonal plasticity after spinal cord injury. J. Neurosci. Res. 2003;74(2):221-6.

Zurn AD, Bandtlow CE. Regeneration failure in the CNs: cellular and molecular mechanisms. Adv. Exp. Med. Biol. 2006;557:54-76.

CHAPTER 2

Synergistic effects of BDNF and rehabilitative training on recovery after cervical spinal cord injury

Adapted from N. Weishaupt, S. Li, A. Di Pardo, S. Sipione, K. Fouad (2012)

Behavioural Brain Research

2.1. Preface

The injured corticospinal tract (CST) generally responds to growth promoting treatments not necessarily with regenerative growth, but more likely with collateral sprouting. Previous work has shown that such sprouting can result in new synaptic connections between CST axons and interneurons in the spinal grey matter (Bareyre *et al.*, 2004, Vavrek *et al.*, 2006), which may mediate some recovery of hand function when rehabilitative training is performed (Girgis *et al.*, 2007). The experiment described in this chapter was designed to answer the question whether we can further promote recovery following incomplete cervical SCI by rewiring the injured CST via a different relay target, the reticular formation. To promote rewiring via the spared reticulospinal tract we investigated a combinatorial treatment of BDNF to motor cortex neurons, NT-3 to reticular neurons, and rehabilitative reaching training (Hiebert *et al.*, 2002, Zhou and Shine, 2003, Girgis *et al.*, 2007). The choice of experimental groups also allowed us to investigate the interaction between BDNF and training in this setting, which promised to be interesting considering the evidence for an intricate relationship between the neurotrophin and neural activity (Vaynman and Gomez-Pinilla, 2005, Ying *et al.*, 2005, Cote *et al.*, 2011).

2.2. Introduction

A promising strategy to repair the injured spinal cord is to make use of the inherent ability of the nervous system to adaptively change in response to injury (Fouad and Tse, 2008, Onifer *et al.*, 2011, Rossignol *et al.*, 2011). Although such plasticity occurs on many physiological levels, re-arrangements in the anatomical structure of neurons are especially valuable in spinal cord injury (SCI) as they offer the unique opportunity to rewire injured fiber tracts. How functionally meaningful such re-arrangements can be is shown in staggered lesion experiments where a thoracic spinal hemisection is followed by a delayed hemisection of the contralateral cord at a distance of a few spinal segments. Here, descending tracts readily form relay connections via interneurons to circumvent both lesion sites, promoting functional recovery (Kato *et al.*, 1984, Courtine *et al.*, 2008). Following SCI, the lesioned as well as the spared corticospinal tract (CST) have been reported to increase the formation of collaterals in animal models of incomplete SCI (Fouad *et al.*, 2001, Weidner *et al.*, 2001, Bareyre *et al.*, 2004). While these plastic changes occur spontaneously to a certain degree, they have also been shown to be modulated by rehabilitative training (Krajacic *et al.*, 2010, Girgis *et al.*, 2007) and pharmacological interventions, including neurotrophins (Hiebert *et al.*, 2002, Zhou *et al.*, 2003, Barritt *et al.*, 2006, Vavrek *et al.*, 2006), making rewiring of injured tracts a promising treatment target.

Recent work suggests that, following CST injury, ventrally projecting tracts may be involved in mediating recovery in forelimb use (Krajacic *et al.*, 2010, Pettersson *et al.*, 2007). Because of its wide-spread ventral and ventrolateral projection (Waldron and Gwyn, 1969, Zemlan *et al.*, 1984), its ability to moderately regenerate (Schaal *et al.*, 2007, Vavrek *et al.*, 2007) and to respond to a spinal injury with increased collateral sprouting (Ballermann and Fouad, 2006), the reticulospinal tract (RtST) may be a promising candidate for relaying CST signals following a cervical spinal cord lesion.

The aim of the present study is to investigate the potential of the injured CST to form a functional detour around a cervical lesion via the spared RtST (Fig. 2.1.A,B). Connections between the injured CST and the RtST at the level of the brainstem are promoted by a combination of neurotrophins and training. First, we intend to stimulate CST sprouting by delivering brain-derived neurotrophic factor (BDNF) to cortical motor neurons, a treatment that has previously been demonstrated to successfully increase sprouting of CST collaterals above a spinal lesion (Hiebert *et al.*, 2002, Vavrek *et al.*, 2006). Second, CST collaterals are encouraged to grow toward the gigantocellular division of the reticular formation by expressing the chemo-attractant neurotrophin 3 (NT-3) in reticular neurons whose axons contribute to the RtST (Zhou *et al.*, 2003, Taylor *et al.*, 2006, Alto *et al.*, 2009). Third, rehabilitative training supplements our neurotrophin treatment as training has been shown to be of vital importance for promoting functionally meaningful plasticity in models of spinal cord injury (Fouad and Tetzlaff,

2012, Girgis *et al.*, 2007) as well as for maximizing recovery following drug treatment (Boyce *et al.*, 2007, Garcia-Alias *et al.*, 2009). We therefore hypothesize that training will act synergistically with neurotrophin treatment to achieve maximal recovery.

2.3. Materials and methods

2.3.1. Animals and experimental groups

46 female Lewis rats (Charles River Laboratories, Montreal, QC, Canada) weighing 180–200 g were randomly assigned to 3 different treatment groups. At the time of SCI, adeno-associated viral vectors (AAV) of serotype 2 expressing either a neurotrophin or the pharmacologically inactive green-fluorescent protein (GFP) were injected as follows: (1) BDNF-expressing AAV-2 into the motor cortex and NT-3-expressing AAV-2 into the reticular formation (combined treatment group, n = 14). (2) BDNF-expressing AAV-2 into the motor cortex and GFP-expressing AAV-2 into the reticular formation (BDNF-only group, n = 16). (3) A control group received injections of GFP-expressing AAV-2 into both locations (control group, n = 16). These groups were then each split in two equally sized subgroups (n = 7 or 8, respectively) of which one underwent reaching training starting at post-injury (p.i.) day 7 (Fig. 2.1.C). Because animal numbers were too high for a single experiment, we performed two consecutive experiments with identical methods, including about half the animals of each treatment condition in each

experiment. Table 2.1 offers an overview of the number of animals included in individual analyses. All animals were group housed and kept at a 12 h:12 h light/dark cycle. On the day before each reaching session, animals were food deprived at 8 g/rat. Weights were closely monitored to ensure weight stability over time. The experimental timeline in Fig. 2.1. offers an overview of the study design (Fig. 2.1.C). All procedures were approved by the Health Sciences Animal Care and Use Committee of the University of Alberta.

2.3.2. Spinal cord injury

All animals received a dorsal quadrant spinal lesion unilateral to their preferred paw (according to reaching behaviour). Rats were anesthetized by an injection of Fentanyl (Hypnorm, Janssen Pharmaceutics, Beerse, Belgium, 0.2 mg/kg s.c.) mixed with Midazolam (Versed, Sabex, Boucherville, QC, Canada, 4 mg/kg). The surgical area was shaved and disinfected with Chlorhexidine Digluconate solution (Sigma, Oakville, ON, Canada) and the animal's head mounted into a stereotactic frame (Kopf Instruments, Tujunga, CA, USA). Throughout the surgery, body temperature was maintained at 37 °C with a heating blanket. Following a skin incision, the spinal cord between vertebrae C3 and C4 was exposed without laminectomy. A custom-made microblade was lowered 1 mm into the spinal cord at the midline, then moved laterally to create a unilateral dorsal quadrant lesion (Fig. 2.1.A). Finally, muscle layers were sutured and the skin was closed with staples.

2.3.3. Injection of viral vectors

AAV-2 (Vector Laboratories, Chapel Hill, NC, USA) were stored at −80 °C until thawed on the day of surgery and were kept on ice after thawing. While animals were still in surgical plane after lesion surgery, a skin incision was made above the skull and a small window was drilled though the skull at the cortex and brainstem locations for AAV-2 injection to expose the underlying dura. AAV-2 were drawn into glass micro-electrodes and pressure injected with a picospritzer at pulses of 15 ms duration at 15–25 psi. One µl of AAV-2 expressing BDNF was injected into the forelimb motor cortex contralateral to the spinal lesion at 2 x 10e12 particles/ml at stereotactic coordinates 1.5 mm lateral and 1.5 mm anterior to bregma, 1.3 mm beneath the dura. One µl of NT-3-expressing AAV-2 was injected into the gigantocellular division of the reticular formation ipsilateral to the spinal lesion at 1.6 x 10e13 particles/ml at coordinates 2.8 mm posterior and 0.8 mm lateral to lambda, 9.0 mm underneath the dura. One µl of GFP-expressing AAV-2 was injected into the same locations depending on treatment group at 2.5 x 10e12 particles/ml. Muscles and skin were closed with stitches. Post-operatively hydration was restored by s.c. injection of 4 ml saline, and pain was managed by s.c. injections of buprenorphine (Temgesic, Schering-Plough, Kirkland, QC, Canada, 0.05 mg/kg). Animals were kept on a heating blanket until fully awake.

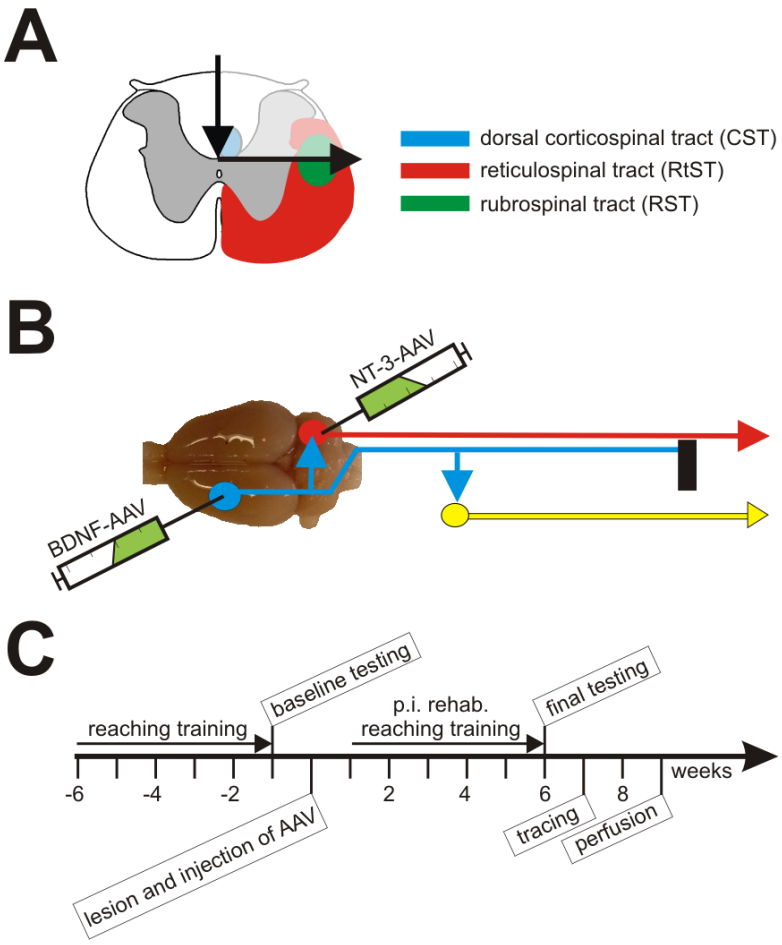


Figure 2.1. Experimental design.

(A) Schematic cross section of the spinal cord at lesion level (C4) demonstrates that a unilateral dorsolateral quadrant lesion ablates the dorsal CST completely, injures part of the RST and spares most of the RtST. **(B)** Schematic shows location of drug application (BDNF-expressing adeno-associated viral (AAV-2) vectors into the motor cortex and NT-3-expressing AAV-2 into the reticular formation) and potential sites of collateral sprouting following lesion (black bar indicates lesion site). Blue arrows indicate proposed rewiring of the injured CST via the reticular formation (red) or via spared interneurons (yellow). Red arrow indicates potential increase in reticulospinal collateral projection below the injury level. **(C)** The timeline of the two identical experiments.

2.3.4. Single pellet reaching

Before lesion surgery, all animals were trained to reach through a slot (1.5 cm wide) in a Plexiglass box (15 cm x 36 cm x 30 cm) to grasp flavored sugar pellets, 45 mg each (TestDiet, Richmond, IN, USA), offered to them in a small indentation on a tray (pellet 2 cm away from front wall at a height of 3 cm above the elevated grid floor). In addition, the rats were taught to go back to the other end of the box before the next pellet was offered. Success rates per session were calculated as the amount of pellets successfully grasped and eaten out of 20 pellets offered. Starting on day 7 p.i., animals receiving training were offered 25 pellets during one session per day, 5 days per week for 5 weeks. To prevent injured animals from using their contralateral paw, and to reinforce use of the handicapped paw, the pellet was initially placed at a slightly lateral position on the tray and reaching movements with the ipsilateral paw were rewarded with sugar pellets. Animals in the untrained groups were offered the same amount of pellets on the floor of the reaching box where they could easily be eaten without use of the paws. Untrained animals were reintroduced to the reaching task in two sessions before final testing. Baseline and final testing consisted of 3 consecutive sessions and the best performance for each animal was taken for statistical analysis.

2.3.5. Horizontal ladder

Rats were encouraged to cross an elevated horizontal ladder with rungs (1.5 mm in diameter) randomly spaced between 2 cm and 5 cm apart (Bolton *et al.*, 2006). An error made by the preferred/ipsilateral forepaw was defined as a fall or a deep slip with either the rat losing balance or the paw dropping underneath the rung level up to the point of the carpal/tarsal joint. Error rate was calculated by averaging the score of 3 ladder crossings and is expressed as percentage of erroneous steps made by the preferred/ipsilateral paw out of the total number of steps taken to cross the ladder. Before lesion surgery, animals were familiarized with the task and then 3 ladder crossings were videotaped with a JVC digital camera and analyzed frame-by-frame on a computer screen for baseline scores. Final testing was done the same way at the end of the recovery period.

2.3.6. Cylinder test

Before surgery and at the end of the training period, rats were filmed with a JVC digital camera as they explored the walls of a clear Plexiglas cylinder (24 cm high, 19 cm inner diameter, Schallert *et al.*, 2000). Spontaneous usage of either forepaw was analyzed on a computer screen by counting how many times either paw touches the wall of the cylinder during 10 rearings. To calculate the percentage that the ipsilateral paw was engaged in during exploration, the sum of all paw touches was used (a score of 50 %

representing symmetry between both forelimbs).

2.3.7. Plantar heater test

Before surgery and at the end of the training period, rats were assessed for heat sensitivity using the plantar test apparatus (Ugo Basile, Collegeville, PA, USA). It consists of a Plexiglas box (22 cm long, 17 cm wide and 14 cm high) and a movable heating unit placed directly under the floor of the box, which emits an infrared light. As soon as the rats remained still after exploring the box, their preferred forepaw was pointedly heated from beneath the Plexiglas floor. The time until the stimulated forelimb was removed from the heat source was measured 3 times with a motion detector and values were then averaged. A time gap of at least 1 min was kept between individual measurements.

2.3.8. Tracing

At the end of the recovery period, animals were anesthetized with isoflurane and the forelimb motor cortex contralateral to the spinal lesion was injected 3 times with 1 µl Alexa Fluor 488 (10 %, Molecular Probes, Eugene, OR, USA) using a Hamilton syringe. Neurons of the reticular formation and their axons were traced by pressure injection (picospritzer pulses of 15 ms duration and 15–25 psi) of 1 µl Fluororuby (10 %, Molecular Probes, Eugene, OR, USA) adhering to the same coordinates used for viral injection. The wound was closed with stitches and post-surgical care was the same as described in

section 2.3.3.

2.3.9. Perfusion and tissue collection

Two weeks following tracer injection, animals were euthanized with an overdose of pentobarbital (Euthanyl, Biomeda-MTC, Cambridge, ON, Canada) and then perfused with saline followed by 4 % paraformaldehyde containing 5 % sucrose. Brains and spinal cords were dissected, kept in formalin solution for 24 h and subsequently transferred into a cryoprotective 30 % sucrose solution for 2–4 days. Pieces of tissue to be cut on a cryostat were mounted on filter paper using Tissue Tek (Sakura Finetek, Torrance, CA, USA) and frozen in Methylbutane at −50 °C. Tissue was stored at −80 °C until sectioned.

The brainstem was cross sectioned at 30 µm and 3 consecutive pieces of the spinal cord covering the cervical enlargement (lesion area, one piece rostral and caudal to lesion) were cross sectioned at 25 µm. Tissue sections were mounted on coated slides (Fisher Scientific, Ottawa, ON, Canada) and stored at −20 °C until processed further.

2.3.10. Cresyl violet staining and lesion assessment

Slides were incubated at 37 °C for 1 h to ensure attachment of sections to the slides. They were then rehydrated 3 x 10 min in TBS, followed by incubation in 0.1 % Cresyl Violet for 3 min. Excess stain was removed by five dips in ddH_2O. Tissue was serially dehydrated in ascending concentrations of

ethanol for 1 min each, cleared 1 min in Xylene and then coverslipped using Permount. Lesions were reconstructed by examining cresyl violet stained cross sections spanning the full length of the lesion. Exclusion criteria were sparing of the dorsal CST as well as lesions covering more than 3 quarters of the ipsilateral cord.

2.3.11. Immunohistochemistry

Cross sections of the brainstem and spinal cord to be analyzed for quantification of CST tracing and collaterals were stained with DAB (Vector Laboratories) against AF488. Slides were incubated at 37 °C for 1 h prior to washing 2 x 10 min in TBS. After two additional washes in TBS-TX, slides were incubated with blocking solution containing 10 % NGS for 2 h at room temperature. Then, anti-AF488 antibody (rabbit IgG, Molecular Probes, Eugene, OR, USA) was applied at 1:750 in TBS and left at 4 °C overnight. The next day, slides were washed 2 x 10 min in TBS and the Vecta Kit goat-anti-rabbit antibody (Vector Laboratories) was applied according to manufacturer's instructions and left incubating at 4 °C overnight. On the third day, slides were again washed 2 x 10 min in TBS before Vectastain ABC solution was applied according to instructions for an incubation of 2 h at room temperature. Following 3 x 10 min washes in TBS, DAB was applied on each slide and the reaction was stopped in distilled water as soon as the desired colour intensity was reached. Slides were again washed 3 x 10 min in TBS, dehydrated in ascending concentrations of alcohol (2 min each),

immersed 2 x 2 min in Xylene and finally coverslipped using Permount (Fisher Scientific).

2.3.12. Quantification of tracing

For normalization purposes, traced CST axons within the pyramid were counted in picture collages taken at 400 x in a brainstem cross section rostral to the area analyzed for collateral projection. Traced axons within the spinal dorsal CST were counted the same way in one cross section rostral to the area of interest (Fig. 2.3.B). Quantification of traced reticulospinal axons was performed in picture collages taken under fluorescent light at 100 x with a Leica microscope (Fig. 2.3.D). Only traced axons ipsilateral to the lesion in a section rostral to the area of interest for reticulospinal collateral analysis were counted.

2.3.13. Quantification of CST collaterals in the brainstem

All CST fibers crossing the ventral portion of the vertical midline were counted along a height of 850 μm per section in ten sections at intervals of 315 μm. Values for each animal are expressed as percent of midline-crossing fibers (sum of ten sections) out of all fibers traced.

2.3.14. Quantification of CST collaterals in the spinal cord

Collaterals emerging from the dorsal CST and crossing the adjacent grey-white matter border were counted in ten sections per animal at intervals of

250 µm immediately rostral to the spinal lesion. Values are expressed as percent of fibers crossing from white into grey matter (sum of ten sections) out of the number of traced fibers at this level.

2.3.15. Quantification of RtST collaterals in the spinal cord

All RtST collaterals crossing into the grey matter across the length of the grey-white matter interface below the lesion level on the side ipsilateral to lesion were counted at 200 x using a fluorescent light microscope (Leica Microsystems). The number of fibers crossing was added for 10 sections analyzed at intervals of 300 µm. Values are expressed as percent of fibers crossing from white into grey matter out of the total number of fibers traced.

2.3.16. Analyses of AAV-2 activity in cell culture

To confirm expression of neurotrophins from the adeno-associated viral vector constructs to be used *in vivo*, we infected immortalized rat striatal derived ST14A cells with human BDNF-expressing AAV-2 at a multiplicity of infection (MOI) of 250,000, or with human NT-3-expressing AAV-2 at 80,000 and 200,000 MOI. Control cultures received PBS (vehicle). Seventy-two hours after infection, cells were collected and total RNA was extracted using RNeasy kit (Qiagen, Toronto, ON, Canada) according to the manufacturer's instructions. One µg of total RNA was reverse transcribed using Superscript II reverse transcriptase (Invitrogen, Burlington, ON, Canada) and oligo-d(T) primers, and the resulting cDNAs were amplified using Power SYBR Green

PCR Master Mix (Applied Biosystems, Streetsville, ON, Canada) following the manufacturer's instructions, and primers specific for human BDNF and NT-3. Primer sequences were: hBDNF FW: CTA CCG CTG GGA ACT GAA AG; hBDNF REV: TGC ATC CCC AGA GAC TAA CC; hNT-3 FW: CGG ATG CCA TGG TTA CTT TT; hNT-3 REV: TGA GGG AAT TGA GCG AGT CT. RT-PCR products were resolved on 1.0 % agarose gel and visualized by ethidium bromide staining.

In a second set of experiments, cells were infected with NT-3-expressing AAV-2 and the incubation medium was collected at 80 h post infection for ELISA analysis. Cell medium was immediately renewed and collected 40 h later (i.e., 120 h post infection). Human NT-3 protein expression in cell lysate and medium was analyzed using a commercial ELISA kit (RayBiotech, Norcross, GA, USA), following manufacturer's instructions.

2.3.17. Statistical analyses

All behavioural and histological values were compared among all groups by two-way ANOVA followed by Bonferroni's Multiple Comparison test if data passed the Kolmogorov–Smirnov normality test. If data did not pass the normality test, comparisons among groups were made using the Kruskal–Wallis test followed by Dunns Multiple Comparison Test. For analyses of weight differences over time, a two-way repeated measures ANOVA was employed followed by Bonferroni's post-test. A t-test was used to compare differences in protein content in the NT-3 ELISA. All data are presented as mean ± SEM, the median is lined in dot plots. A p value ≤0.05 was considered

Analysis	GFP/GFP	GFP/GFP training	BDNF/NT-3	BDNF/NT-3 training	BDNF/GFP	BDNF/GFP training	Excluded
Lesion	8	7	7	7	8	8	1
CST tracing	6	7	7	7	8	7	4
RtST tracing	8	7	7	7	8	7	2
CST collaterals brainstem	7	7	6	7	7	7	5
CST collaterals above lesion	6	7	6	7	8	6	6
RtST collaterals below lesion	7	7	7	7	8	7	3
Single pellet reaching	8	8	7	7	8	8	0
Horizontal ladder	8	8	7	7	8	8	0
Zylinder	8	8	7	7	8	8	0
Plantar heater	5	4	3	4	4	4	0
Weight	4	3	4	3	3	4	0

Table 2.1. Animals per group included in different analyses.

The plantar heater test was only performed in the first experiment whereas weight development is shown only for the second experiment. All animals underwent behavioral testing, however, some were excluded from histological assessments if processed tissue did not meet the quality or quantity necessary for analyses as per sections 2.3.13-2.3.15.

significant.

2.4. Results

2.4.1. Protein expression by viral vectors

To assess the ability of our AAV-2 constructs to drive expression of transgenic BDNF or NT-3 protein, we infected rat striatal cells in culture and analyzed expression of human BDNF and NT-3 mRNA. In rat striatal cells infected with human BDNF-expressing AAV-2, human BDNF mRNA was detected 72 h after viral infection. No signal was detected in uninfected cells (Fig. 2.2.A). Similarly, human NT-3 mRNA was detected in rat cells 120 h after infection with the corresponding AAV-2 (Fig. 2.2.B). To assess NT-3 protein production and secretion, we performed an ELISA assay on cell medium, at 80 h post infection, then added fresh medium and analyzed it 40 h later. As expected, the medium of uninfected cell cultures did not show any traces of human NT-3. NT-3 protein was detected in different concentrations only in cultures that had been infected with NT-3-expressing AAV-2 (Fig. 2.2.C). At both time points, NT-3 protein content was proportional to the number of AAV-2 particles used to infect the cells. Together, these results validate the use of our BDNF- and NT-3-AAV-2 to drive expression of these neurotrophins in our *in vivo* experiments.

To confirm viral protein expression in the appropriate target location *in vivo*, signal generated by the control protein green-fluorescent protein

(GFP) in the reticular formation following respective AAV-2 injection was analyzed under a fluorescent microscope. The target area, the gigantocellular division of the reticular formation, is located just dorsal to the pyramidal tract on either side of the midline in a brainstem cross section (Fig. 2.2.D). Several cells in this area were found to fluoresce in green, indicating not only successful vector delivery to the target structure, but also successful transfection of cells in a defined location as well as expression of the protein encoded by viral DNA. Although this analysis allows no inference as to the onset of expression, we know that protein expression was evident at the time of euthanasia, 9 weeks post injection.

Additional evidence for successful BDNF expression and release after respective AAV-2 injection is provided by the emergence of differences in weight gain between BDNF treated and control animals (Fig. 2.2.E). Starting at about 3 weeks p.i., both groups that received BDNF-expressing AAV-2 displayed a significantly lower gain in body mass than rats treated with GFP-expressing AAV-2. This observation continued for the length of the experiment while all animals were in mixed-group cages with the same degree of food restriction. Animals that received both BDNF and NT-3 showed the least weight gain, with weights being significantly different from GFP-only treated animals at the time points indicated (greatest difference on day 41 p.i., BDNF/NT-3: 239.14 ± 8.73 g vs. GFP/GFP: 255.25 ± 7.4 g, $p < 0.01$). Although significance was not reached when BDNF-only treated animals were compared to control animals, we could still find a significant

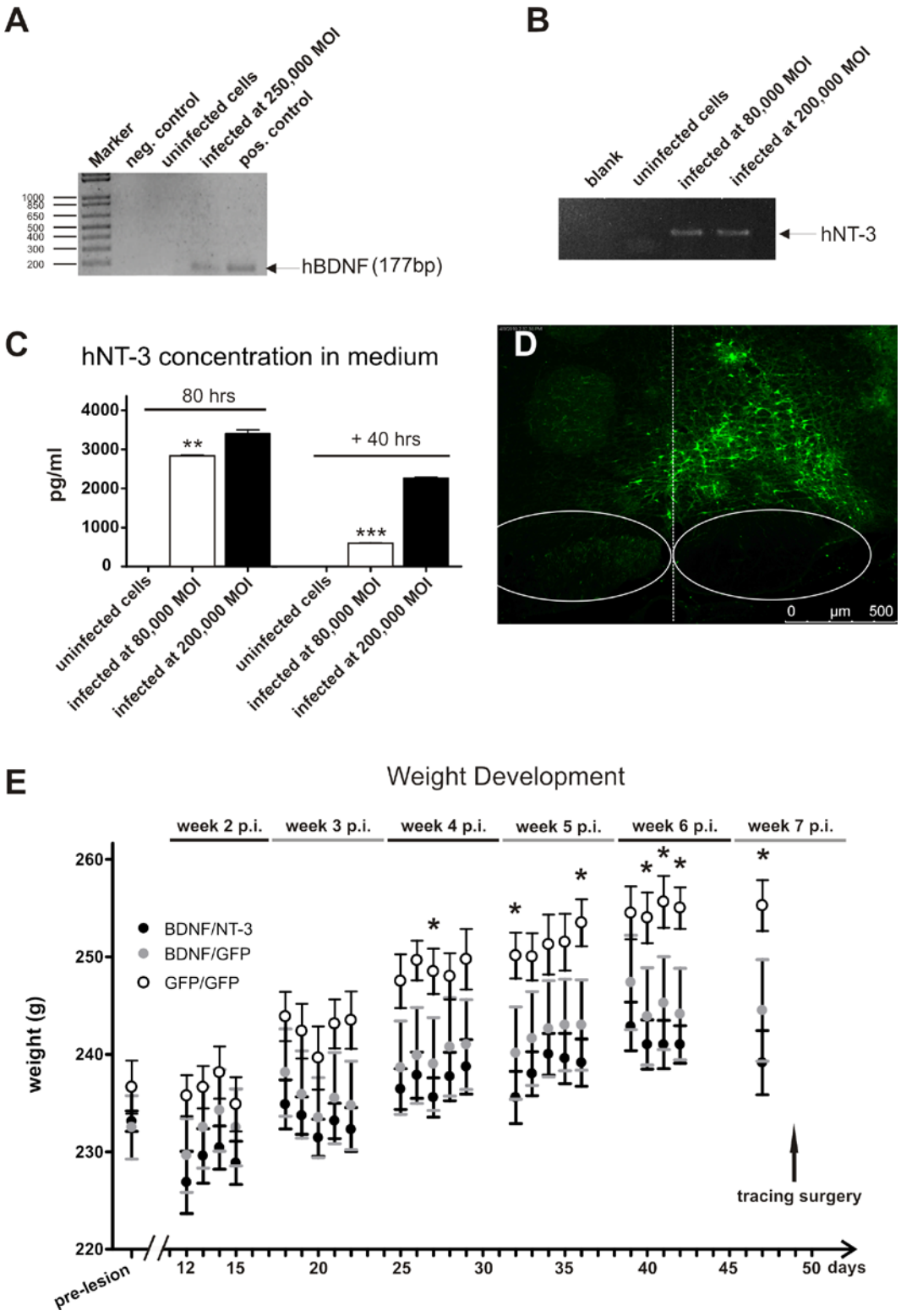


Figure 2.2. Evidence for AAV-2-driven expression of transgenic BDNF or NT-3.

(A) The RT-PCR product for human BDNF-RNA (hBDNF, 177 base pairs) is

detectable already at 72 h following infection of rat striatal cells with human BDNF-expressing AAV-2. DNA ladder (marker) indicates product sizes in base pairs (bp). **(B)** Similar to A, human NT-3 RNA (hNT-3) is detected by RT-PCR in rat striatal cultures 120 h following infection with NT-3-expressing AAV-2. **(C)** Human NT-3 (hNT-3), detected by ELISA, is expressed and secreted into the culture medium by striatal cells infected with human NT-3-expressing AAV-2. Please note that the initial cell culture medium was collected at 80 h, and the fresh medium was then collected 40 h later. Double asterisks indicate $p < 0.01$, triple asterisks indicate $p < 0.0001$. **(D)** GFP expression in the reticular formation in the ventral part of a brainstem cross section at 9 weeks following injection of GFP-expressing AAV-2. Ovals indicate location of the pyramids, and the vertical dashed line marks the section midline. **(E)** BDNF treated animals were found to gain weight slower than animals that did not receive BDNF. Differences in weight become significant at day 27 post-injury (p.i.) between BDNF/NT-3 and GFP/GFP treated animals. This observation is a good indicator for expression of BDNF in the brain. Asterisks indicate $p < 0.05$ between BDNF/NT-3 treated and GFP treated control animals. Error bars represent SEM.

BDNF effect regardless of NT-3 expression at five time points when both BDNF treated groups were pooled. Because an appetite-reducing effect of BDNF has previously been reported (Fouad *et al.*, 2010, Rosas-Vargas *et al.,* 2011), this finding is another indication of successful BDNF expression by AAV-2 injection. Also, the emergence of this effect roughly coincides with the expected onset of substantial protein expression at about 14 days following injection of AAV-2 and the continuing difference in weight development suggests ongoing BDNF expression over the course of the experiment.

2.4.2. Variability of lesion and tract tracing

Reconstruction of spinal lesions revealed a variability of the total lesion area between 16.33 % and 48.64 % of the complete spinal cross section. Importantly, there was no significant difference in lesion size among all treatment groups as lesion severities were equally distributed among the six groups (GFP/GFP: 27.28 ± 3.83 %, GFP/GFP plus training: 27.53 ± 2.53 %, BDNF/NT-3: 34.25 ± 3.31 %, BDNF/NT-3 plus training: 31.21 ± 2.27 %, BDNF/GFP: 31.67 ± 3.15 %, BDNF/GFP plus training: 24.65 ± 1.83 %; Fig. 2.3.A). Thus, behavioural outcomes in different treatment groups can be confidently attributed to an effect of treatment rather than to a difference in lesion severity.

Tracing of the injured CST yielded between 143 and 2108 traced axons within the dorsal CST at level C2 with statistically insignificant

differences among the groups (lowest tracing in the BDNF/NT-3 group at 452 ± 104.2, highest tracing in the control group at 1071 ± 157.4; Fig. 2.3.B, C). The number of traced fibers was used for normalization of CST collateral counts for each animal.

Tracing of reticular neurons that project mainly ipsilateral to the spinal lesion yielded between 48 and 614 traced axons within the ipsilateral white matter at level C5 (Fig. 2.3.D, E). Although there was no difference in tracing efficacy among the treatment groups, these numbers were also used for normalization of RtST collateral counts (lowest tracing BDNF/NT-3: 134.3 ± 19.66, highest tracing BDNF/GFP: 219 ± 62.12).

2.4.3. Single pellet reaching

The single pellet reaching task is a demanding paradigm, especially following a dorsal quadrant lesion because success heavily relies on fine motor control that is mediated especially by the CST. Consistent with the challenges this task poses after CST injury, neither the combined neurotrophin group nor the BDNF-only group performed different from the GFP-only control group in the reaching task at final assessment (GFP/GFP: 14.38 ± 5.21 %, BDNF/NT-3: 12.86 ± 4.48 %, BDNF/GFP: 11.88 ± 5.90 %; Fig. 2.4.A). All of these untrained groups performed significantly poorer than baseline (36.41 ± 1.664 %), whereas the addition of rehabilitative reaching training lead to a performance level not statistically different from baseline in all treatment groups. Of notice, even after weeks without practice, untrained rats

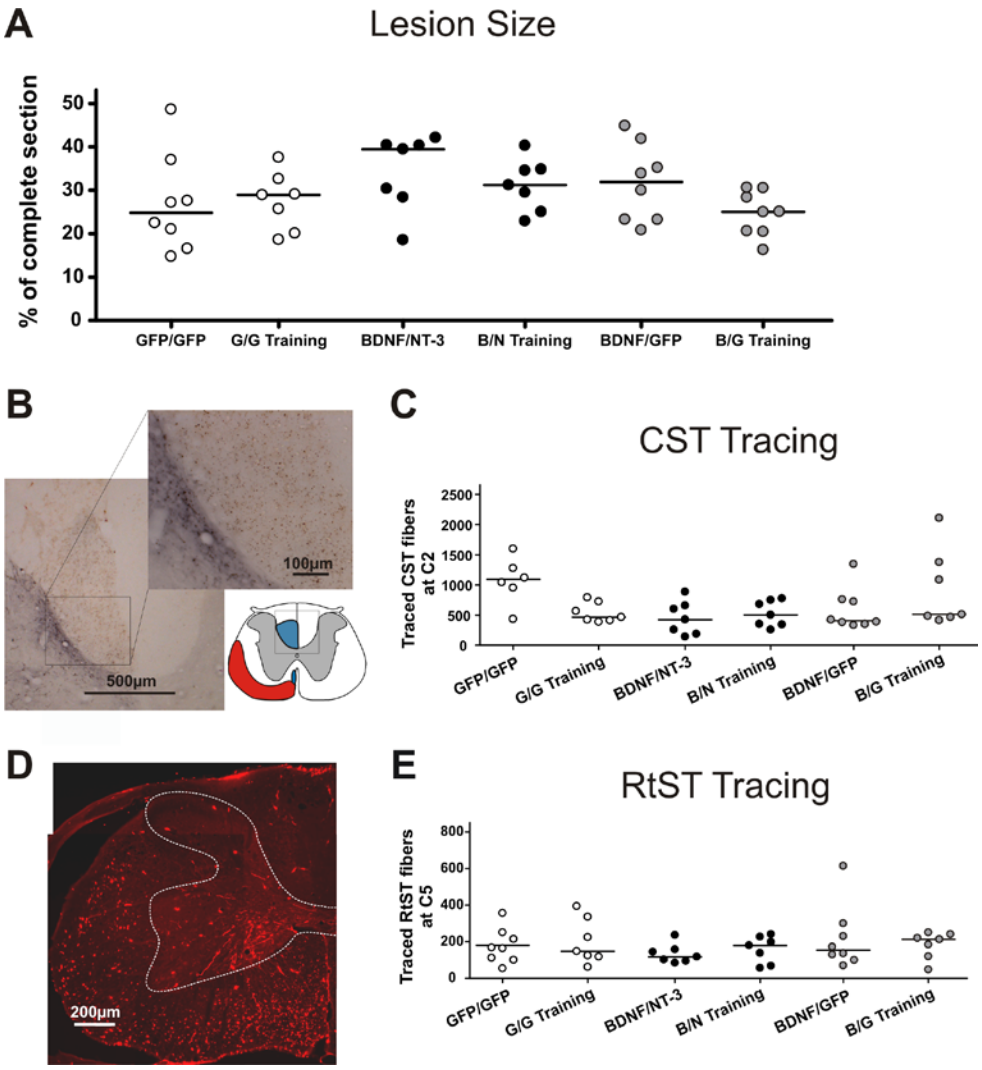


Figure 2.3. Lesion size and tracing efficacy.

(A) Lesion sizes are not significantly different among the groups. **(B)** For normalization of collateral sprouting counts, differences in tracing efficacy were assessed rostral to the area of interest. Therefore, following tracing with AF 488, the number of labeled axons of the injured CST (blue in schematic) was quantified. **(C)** The number of labeled fibers within the injured dorsal CST is comparable among the groups. **(D)** Labeled RtST fibers in the lateral, ventrolateral and ventromedial white matter (red in schematic) were quantified below the injury level following tracing of the ipsilateral reticular formation with Fluororuby. This number is similar among all groups **(E)**. Dashed white line marks the grey-white matter interface. Horizontal lines mark median.

remembered the task well and attempted to reach immediately. Overall there was no difference in attempt rates between any of the treatment groups. Training had the greatest effects in combination with neurotrophin treatment, resulting in a considerably improved recovery in the reaching task (BDNF/NT-3: 12.86 ± 4.48 %, plus training: 32.14 ± 11.44 %, BDNF/GFP: 11.88 ± 5.90 %, plus training: 46.25 ± 10.43 %). Animals that received BDNF in the motor cortex, GFP in the brainstem and reaching training stood out with an average success rate slightly higher than baseline, performing significantly better than BDNF/GFP treated animals that were not trained. In summary, only the combination of BDNF delivery and training promoted significant recovery of fine motor skill in the trained task.

2.4.4. Horizontal ladder

Animals were tested on the horizontal ladder not only to have an additional read-out for motor recovery, but also to investigate to what extent training can make a difference for performance in an untrained task in neurotrophin treated animals. Although statistical significance was not reached, there was a slight improvement for trained animals compared to those that did not receive reaching training in the control condition (GFP/GFP: −20.28 ± 4.89 %, plus training: −8.46 ± 3.84 %; Fig. 2.4.B). Interestingly, although BDNF-only treatment plus training proved beneficial in the trained task, it had no obvious effect on ladder scores. However, the additional delivery of NT-3 in the brainstem raised performance close to baseline level (BDNF/GFP:

−18.75 ± 4.12 %, BDNF/NT-3: −4.05 ± 6.67 %). Animals that received both neurotrophins and training reached the best scores at final assessment and therefore showed a tendency to recover better than untrained control and BDNF-only treated animals (BDNF/NT-3 plus training: −2.14 ± 4.83 %). Taken together, although group differences were modest, combined neurotrophin treatment had a greater effect on recovery in this untrained task than reaching training. Also, the expression of NT-3 in the reticular formation seemed to be of greater importance for motor control on the horizontal ladder than for single pellet reaching.

2.4.5. Cylinder test

In contrast to the two previously mentioned motor tasks, the cylinder test measures spontaneous, voluntary paw usage. Additionally, the motor control necessary for wall exploration and limited weight support are not directly related to the motor skills measured in the two previously mentioned motor tasks. Although animals that received both neurotrophins and training showed a tendency to use their ipsilateral paw more often than other groups (−1.64 ± 2.96% difference to baseline), there were no statistical differences due to high variability in paw usage scores (GFP/GFP: −7.64 ± 4.07 %, plus training: −5.37 ± 1.60 %, BDNF/NT-3: −5.71 ± 2.18 %, BDNF/GFP: −3.87 ± 4.43 %, plus training: −6.43 ± 3.01 % difference to baseline; Fig. 2.4.C).

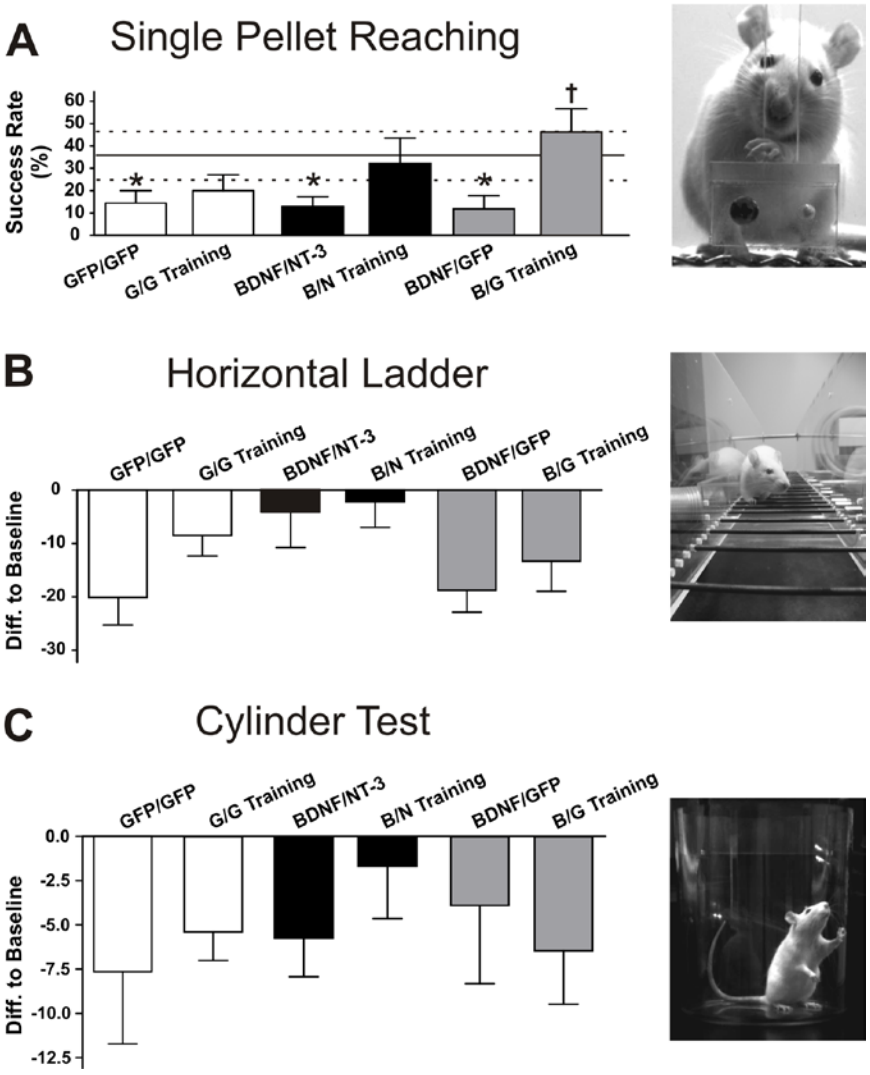


Figure 2.4. Recovery of motor function.

(A) Following a 6 week recovery period, untrained animals performed significantly poorer in reaching than average baseline (solid horizontal line), regardless of neurotrophin or control treatment. The small effect of training on GFP treated animals elevated their reaching score to a success level not significantly different from baseline. However, neurotrophin treated animals benefited the most from training, with trained BDNF/GFP treated animals recovering reaching ability to a degree even higher than baseline. These animals performed significantly better than untrained BDNF/GFP treated animals. Animals receiving both neurotrophins and training recovered close to baseline values. Solid line marks average baseline score, dashed lines mark standard deviation for baseline values. Asterisks indicate $p < 0.05$

compared to baseline, † indicates $p < 0.05$ compared to BDNF/GFP). **(B)** On the horizontal ladder, reaching training had a modest beneficial effect on performance regardless of neurotrophin or control treatment. The best recovery was measured in trained animals treated with BDNF and NT-3, which recovered horizontal ladder walking close to baseline levels. Also, untrained BDNF/NT-3 animals tended to perform better when compared to other untrained animals. All group differences are statistically insignificant. **(C)** In the cylinder test, no significant group differences were found, partly due to high variability in scores within the groups. However, treatment with both neurotrophins and training was modestly effective in promoting spontaneous use of the injured forelimb. Error bars represent SEM.

2.4.6. CST collateral projection in the brainstem

Tracing of reticular neurons revealed that the majority of traced reticulospinal fibers project in the ipsilateral white matter in the spinal cord. Therefore, we sought to encourage CST axons, which are still uncrossed at the pyramidal level, to connect to reticular neurons in the contralateral brainstem, which mainly project ipsilateral to CST injury (Fig. 2.1.B). One indication for an increase in connections between pyramidal axon collaterals and the targeted reticular neurons would therefore be an increase in the number of pyramidal CST fibers that cross the brainstem midline (Fig. 2.5.A). In fact, the amount of midline-crossing CST collaterals was found to remain relatively stable among the groups when normalized to the number of traced axons (range from BDNF/NT-3: 3.47 ± 0.8 up to BDNF/GFP: 8.35 ± 3.07, Fig. 2.5.B). Therefore, we could find no evidence for the hypothesized rewiring between CST collaterals and the contralateral reticular formation, nor did we observe a reaction of CST collateral growth to the chemo-attractant NT-3 expressed in the contralateral reticular formation.

2.4.7. CST collateral projection directly above the injury

BDNF exerts many of its stimulating effects by initiating protein synthesis in the cell body, therefore increased collateral sprouting may be expected on any level along the axon. We chose to investigate the amount of CST collaterals projecting into the grey matter directly above the injury, a well-

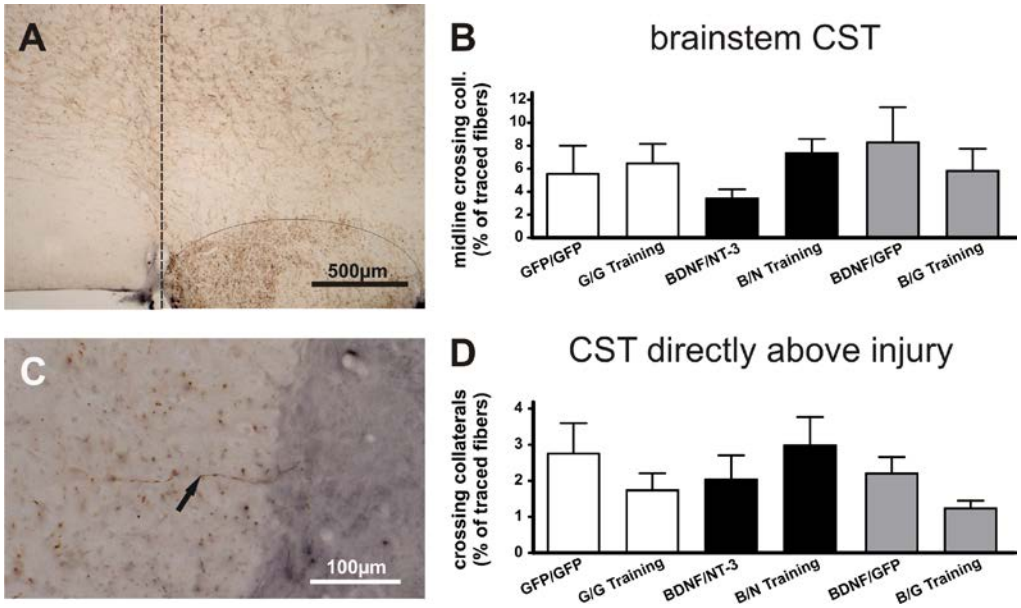


Figure 2.5. Collateral sprouting of the injured CST in the brainstem and immediately rostral to the injury.

(A) The ventral-middle part of a DAB stained brainstem cross section. Traced CST collaterals emerging from the right pyramid (circled) were counted when crossing the midline (dashed line) toward the contralateral reticular formation. **(B)** The number of midline-crossing CST fibers, after normalization to tracing efficacy, is similar among all groups. Thus, neither neurotrophin treatment nor training increased CST collateral sprouting across the brainstem midline. **(C)** A collateral (arrow) of the injured dorsal CST is crossing into the grey matter immediately above the level of injury. **(D)** Although the normalized number of CST collaterals projecting across the grey-white matter interface at this level fluctuates a little across treatment groups, there is no overall effect of any treatment condition on the amount of CST collateral sprouting. Error bars represent SEM.

known location for increased collateral growth following this kind of injury (Fig. 2.1.B; Fouad *et al.*, 2010, Girgis *et al.*, 2007). Similar to our findings at brainstem level, no group differences were evident in the normalized number of dorsal CST collaterals entering the grey matter (range from BDNF/GFP plus training: 1.28 ± 0.21 up to BDNF/NT-3 plus training: 3.0 ± 0.79; Fig. 2.5.C, D). In summary, neither training nor BDNF treatment had a stimulating effect on CST collateral growth immediately rostral to the injury.

2.4.8. RtST collateral projection below the injury

If the RtST could act as a detour for CST signals, there might be an increased "demand" for connections to neurons below the lesion level. We therefore quantified the number of RtST collaterals that cross into the grey matter at level C5 on the injured side of the spinal cord (Fig. 2.6.A). The resulting normalized values were comparable among all groups (range from BDNF/NT-3 plus training: 15.95 ± 2.29 up to BDNF/GFP plus training: 25.47 ± 4.34; Fig. 2.6.B). Thus, at this location we did not find evidence for anatomical re-arrangements that would indicate that the reticulospinal tract might take over targets denervated from CST input.

2.4.9. Side effects of neurotrophin treatment

To discover a potential change in the wiring of sensory/pain networks following neurotrophin treatment, we tested some of our animals in the planter heater apparatus before injury and at the end of the recovery period.

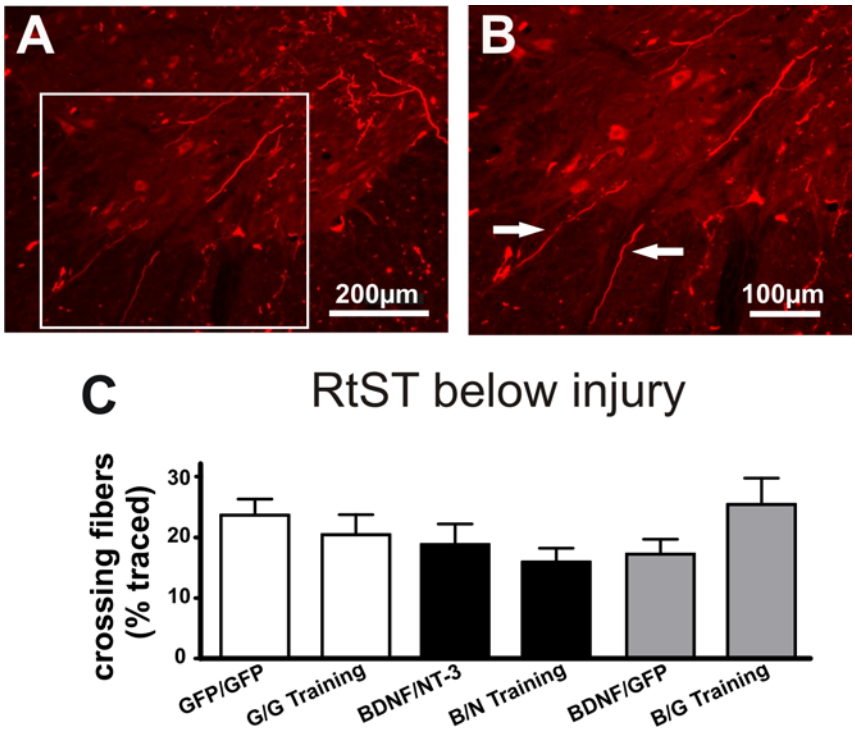


Figure 2.6. RtST collateral sprouting below injury level.

(A) Part of the left ventral white matter and the left ventral horn (ipsilateral to lesion and to the traced reticular formation) in a cross section below the lesion level. Traced RtST fibers in the ventral white matter send collateral branches into the grey matter. White rectangle is magnified in **(B)**. White arrows point to RtST collaterals crossing the grey-white matter interface. **(C)** We quantified the number of these collaterals to investigate whether neurotrophin treatment and/or training increases RtST projections into the grey matter below the lesion. We found that after normalization to the number of traced fibers, the number of RtST collaterals crossing into grey matter on the lesioned side did not change in response to any treatment. Error bars represent SEM.

No statistical differences in the change of response time to the heat stimulus could be found among the combined treated, BDNF-only or GFP-only control groups (-0.24 ± 1.73 s, -1.88 ± 0.95 s, -0.86 ± 1.06 s, respectively; Fig. 2.7.). This indicates that neurotrophin treatment did not have an obvious effect on behaviour related to sensory pathways.

2.5. Discussion

In the present study, we investigate the effectiveness of AAV-delivered neurotrophins and rehabilitative training to promote rewiring of the injured CST following a unilateral cervical spinal cord injury. Using targeted expression of BDNF and NT-3 in combination with training, we find that while BDNF and training may promote performance in a trained task (single pellet reaching), this improved recovery may not translate to an untrained task (walking across a horizontal ladder). Most importantly, there is a strong interaction between BDNF treatment and rehabilitative training which suggests that training is necessary for translating BDNF effects into functional recovery. NT-3 treatment did not produce a similar effect on motor performance in the trained or the untrained condition. These behavioural findings were not reflected in anatomical re-arrangements at the levels analyzed, which suggests an underlying mechanism independent of collateral CST or RtST sprouting, potentially including synaptic plasticity or changes in neuronal excitability (Darian-Smith, 2009, Murray *et al.*, 2010).

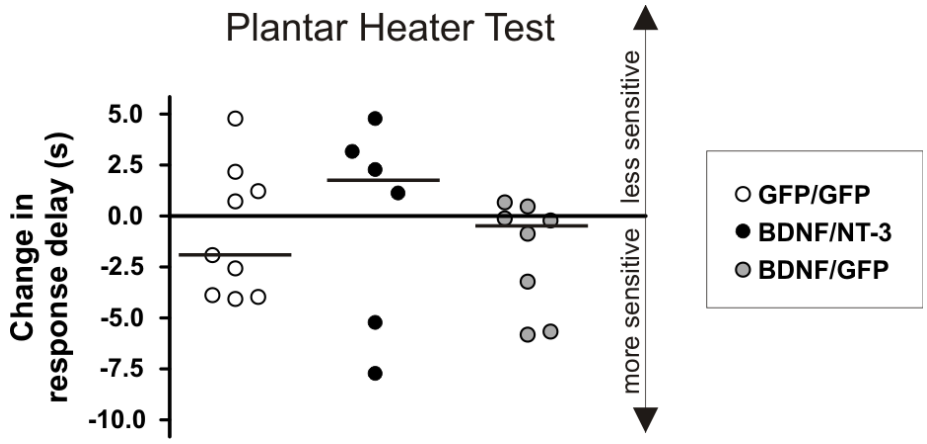


Figure 2.7. Side effects of neurotrophin treatment.

As BDNF is a known modulator of pain pathways, we tested animals for development of hypersensitivity in the plantar heater apparatus. It was evident that neither cortical BDNF treatment nor NT-3 expression in the brainstem lead to changes in sensitivity to a heat stimulus. Horizontal lines mark median.

Adeno-associated viral (AAV) vectors of serotype 2, which preferentially transfect neurons, are frequently used to express a protein of choice over a time period of several weeks in a defined location (Blits *et al.,* 2010, Burger *et al.*, 2004, Passini et al., 2004, Bartlett *et al.*, 1998, Lin *et al.*, 2011). Plasmids similar to those used in our AAV-2 have successfully been used in AAV-2s in other studies (Lu *et al.*, 2012). Their advantages over alternative methods such as the use of osmotic mini-pumps, which need frequent re-loading, are more invasive and may cause a substantial inflammatory response, make viral vectors a more elegant and straightforward method for long-term substance delivery. One limitation of our study is that protein levels of the neurotrophins expressed following AAV-2 injection in the brain and brainstem are not known. Klein et al. measured levels of AAV-2 expressed neurotrophins and GFP in rat brains using ELISA and immunolabeling, thereby providing a general idea of the spread of transfection and the expression efficiency using this method (Klein *et al.*, 2002). In contrast to our AAV-2s however, in which BDNF and NT-3 were driven by a CMV (cytomegalovirus) promoter, Klein et al. used a CBA (cytomegalovirus/chicken beta-actin) promoter to express GDNF or NGF. Measuring meaningful BDNF levels constitutes a different challenge altogether, as BDNF protein inside the cell is not pharmacologically active until it is released into the extracellular space in an activity-dependent manner (Goodman *et al.*, 1996, Griesbeck *et al.*, 1999). To our knowledge it is also unclear what concentrations of BDNF or NT-3 would have to be reached

in the tissue to be functionally relevant in the context of our study. Because the vector constructs we used were not tagged with a marker and reliable immune-histochemical detection of the expressed neurotrophins themselves proved extremely challenging, we confirmed the ability of our AAV-2s to express BDNF or NT-3 *in vitro*. Together with histologic confirmation of GFP expression in brainstem cells around the injection site as well as the observed effect of BDNF treatment on weight gain *in vivo*, we can interpret the collected behavioural and histological data with the confidence that the encoded neurotrophins were expressed in the tissue following AAV-2 injection.

The most compelling effect of neurotrophin treatment on motor function was achieved when BDNF was combined with rehabilitative training. Importantly, comparisons among the groups reveal that this is not an additive effect but rather it seems that training is necessary for a BDNF treatment effect to emerge at all. One reason why training has such an impact on the emergence of BDNF effects might be the fact that any plastic changes in the nervous system rely heavily on activity for development, maintenance and fine-tuning of new synaptic connections. This has been demonstrated in visual deprivation studies early in life where activity-dependent, competitive changes were observed in cortical connectivity (Wiesel and Hubel, 1965). It is well known that training following spinal cord injury can have a major impact on recovery-promoting plasticity (Lovely *et al.*, 1986, Barbeau and Rossignol, 1987, Vaynman and Gomez-Pinilla, 2005). A potentiating

relationship between drug treatment and training has been reported for chondroitinase ABC where a minor effect of drug treatment by itself was raised substantially when chondroitinase administration was combined with training (Garcia-Alias *et al.*, 2009). Also, there is a substantial body of evidence indicating that training-induced activity in the nervous system can up-regulate neurotrophin expression and that training-induced expression of BDNF specifically is pivotal for recovery after spinal cord injury (Griesbach *et al.*, 2004, Vaynman and Gomez-Pinilla, 2005, Ying *et al.*, 2005, Ying *et al.*, 2008). In our experiment, a training-induced up-regulation of endogenous BDNF in addition to the vector-mediated expression might increase total BDNF levels to a threshold concentration that results in functionally meaningful plasticity. Additionally, the synergistic effect of training and BDNF might be attributable to a training-induced release of BDNF from virus-transfected cortical neurons. The neurotrophin, once translated, is released via a regulated, activity dependent pathway (Goodman *et al.*, 1996, Griesbeck *et al.*, 1999). BDNF signals through membrane receptors and thus can only exert its effects extracellularly. Making BDNF available for extracellular signaling by increasing its release might well explain why training "unmasks" an effect of BDNF on a functional level. Curiously however, the functional improvement was less striking for animals treated with both BDNF and NT-3.

Taken together, these considerations regarding rehabilitative training lead to the conclusion that a successful treatment for SCI will most likely be a combination of several components, given that rehabilitative training is

essential to promote functional relevance of other treatments.

Yet, using complex tests like the single pellet reaching task to assess training benefits in animals is not straightforward. Contrary to previous reports, we did not find a marked beneficial effect of training by itself in the control condition (GFP/GFP: 14.38 ± 5.21%, plus training: 20.00 ± 7.20%; Girgis *et al.*, 2007, Krajacic *et al.*, 2009, Krajacic *et al.*, 2010). This might be due to an insufficient training intensity (25 pellets 5 times a week for 5 weeks instead of 10 min 6 days a week for 6 weeks), but could also be attributed to a diluting effect by self-training of the rats in their home cage. In contrast to beneficial training effects that our group and others reported previously, rats in this study received solid food pellets instead of mashed food and were also given a few sunflower seeds daily for enrichment. This self-training through eating behaviour might have diluted the effect of skilled reaching rehabilitative training similar to what has been reported for treadmill training (Fouad *et al.*, 2000, Caudle *et al.*, 2011). The potential for a training effect to arise also relies heavily on the lesion severity, which might have been slightly too severe in the present study to allow for significant reaching recovery (unpublished observations). Another factor that plays a role in reaching success is motivation, especially following a lesion that makes it extremely hard for animals to be successful in reaching initially after the injury. We tried to maintain motivation of unsuccessful animals by rewarding legitimate attempts and did not notice a decline in attempt rate as a measure of motivation in any group during training or at final testing.

Returning to our results regarding training and treatment interaction, it is not surprising that the addition of training to neurotrophin treatment has the greatest benefits in the trained reaching task, as opposed to untrained tasks, as similar results have been reported for spinalized cats trained to locomote (Boyce *et al.*, 2007). The horizontal ladder is also designed to assess fine motor control of the forelimb, including wrist and paw, but it is important to consider that walking over rungs is still a fundamentally different motor skill than reaching for pellets. As ladder walking is related to locomotor activity, the reticulospinal tract, which is mainly involved in controlling locomotion and posture, probably plays a greater role in this task than in the single pellet reaching task. The fact that the additional expression of NT-3 in the reticular formation is somewhat beneficial in the ladder task may therefore be explained by a plasticity-promoting effect on the reticulospinal tract.

A forelimb motor control test not heavily relying on fine motor control of the paw is the cylinder task, where we did not find effects of neurotrophin treatment or training. In general, the results of this study highlight the perceived impossibility to find "the" most beneficial treatment in a complex system such as the injured nervous system. More specifically, our findings emphasize that employing neurotrophins *in vivo* might not be straight forward, and point out the need to improve predictability of neurotrophin treatment before it can be translated into clinical use in the future.

In order to achieve an understanding of these complex processes, we

need to investigate which mechanisms are mediating neurotrophin effects and their interactions with training. Because our motivation for the treatment tested here was to promote rewiring of the injured CST via the reticulospinal tract, we looked for histological evidence of newly induced collateral growth.

Quantification of CST collaterals crossing the midline in the brainstem and growing toward the contralateral reticular formation revealed no differences among the treatment groups. One possible explanation for this finding might be an insufficient gradient of NT-3. It is unclear what magnitude an NT-3 gradient would have to be in order to elicit long distance tropism from CST collaterals on the contralateral side of the brainstem. Also, a shortcoming of using viral vectors is that the amount of produced protein and the resulting tissue concentration is hard to predict and control and will probably vary at any given time in any given animal. Also, it is important to take into consideration that contradicting reports for effects of NT-3 exist, ranging from chemo-attractive properties with increased CST sprouting (Zhou *et al.*, 2003), the lack of an effect on sprouting (Vavrek *et al.*, 2006) to a reduction in CST sprouting (Hagg *et al.*, 2005).

Although our approach of quantifying midline-crossing collaterals in the brainstem did not provide evidence for the desired rewiring, this does not exclusively prove that there are no re-arrangements between the CST and the RtST. For example, we found that control animals already had an abundance of pyramidal collaterals projecting across the midline. These

already established connections might merely be strengthened, or primary collaterals might branch and create secondary collaterals within the vicinity of the reticular formation to increase connectivity. The fact that we could not detect an increase in midline-crossing pyramidal fibers might reflect that, economically speaking, there might not have been a need for an increase in newly growing fibers to mediate increased connectivity or signaling. To clarify this issue, an electrophysiological experiment could be added in the future to investigate the strength of descending input from the motor cortex and the reticular formation.

As an alternative to rewiring via the reticular formation, the injured CST can theoretically build new meaningful connections with any spared descending structure, such as interneurons. Contrary to what has been reported in studies delivering BDNF with osmotic mini-pumps, we did not find increased sprouting in BDNF treated animals directly above the injury (Hiebert *et al.*, 2002, Vavrek *et al.*, 2006). Of notice, the BDNF-stimulated CST sprouting reported in these two studies was seen following spinal lesions of different size and location (thoracic level) compared to the cervical dorsolateral quadrant lesion used here. Also, Vavrek *et al.* (2006) did not observe functional recovery as a result of sprouting. One alternative explanation for the lack of BDNF-induced sprouting of the dorsal CST in the present study might be that the extracellular concentration of BDNF in the motor cortex needed to elicit such a growth response was not reached. Similar circumstances might explain the lack of group differences in the

number of RtST collaterals entering the grey matter directly below the lesion. However, increased RtST sprouting has been reported to occur following a thoracic spinal lesion, and has been suggested to be involved in functional recovery (Ballermann and Fouad, 2006). Therefore, the lack of such sprouting together with the stable number of CST collaterals targeting the reticular formation in the brainstem suggests that the behavioural effects in this study are probably not mediated by new connections formed by the CST or RtST. A caveat to this conclusion is the fact that we did not analyze sprouting of the CST or RtST along the full length of their projections. Specifically, we cannot exclude a potential sprouting response of the ventral CST, whose ability to mediate functional recovery has been reported (Weidner *et al.*, 2001).

Alternatively to the hypothesized rewiring, what other mechanisms might mediate the observed significant benefits of BDNF treatment for motor recovery? First, changes on a synaptic level might contribute to these functional effects. BDNF's role in short-term and long-term synaptic plasticity is very well established (Lu *et al.*, 2004, Bramham and Messaoudi, 2005, Luikart and Parada, 2006, Minichiello, 2009). Second, BDNF is known to be a potent neuronal excitant (Kafitz *et al.*, 1999, Blum *et al.*, 2002). Its excitatory actions have been investigated in the brain, where it is known to promote epileptic activity (Scharfman, 2005), as well as in the spinal cord, where it can contribute to central sensitization and spasticity (Boudes and Menigoz, 2009, Boulenguez *et al.*, 2010). Therefore, some of the functional effects seen

in this study might be mediated by an increase in overall activity in motor systems affected by increased availability of BDNF signaling.

2.6. Conclusion

The present study emphasizes the challenge to predict responses of the injured nervous system to neurotrophin treatment. Most importantly, we found that training can interact powerfully with neurotrophin signaling to a level where functional effects of neurotrophin treatment emerge only in combination with training. On the basis that viral vector delivery of neurotrophins proved successful, our results suggest that interactions between neurotrophins and training are essential in promoting functional recovery, yet they are far from straight forward. Although we report promising leads, more work needs to be done to understand the mechanism behind the observed effects and interactions to optimize the use of neurotrophins and realize their potential to become a valuable tool for repairing the injured spinal cord.

2.7. References

Alto LT, Havton LA, Conner JM, Hollis Ii ER, Blesch A, Tuszynski MH. Chemotropic guidance facilitates axonal regeneration and synapse formation after spinal cord injury. Nat. Neurosci. 2009;12(9):1106-13.

Baker SN. The primate reticulospinal tract, hand function and functional recovery. J. Physiol. 2011;589(Pt 23):5603-12.

Ballermann M, Fouad K. Spontaneous locomotor recovery in spinal cord injured rats is accompanied by anatomical plasticity of reticulospinal fibers. Eur. J. Neurosci. 2006;23(8):1988-96.

Barbeau H, Rossignol S. Recovery of locomotion after chronic spinalization in the adult cat. Brain Res. 1987;412(1):84-95.

Bareyre FM, Kerschensteiner M, Raineteau O, Mettenleiter TC, Weinmann O, Schwab ME. The injured spinal cord spontaneously forms a new intraspinal circuit in adult rats. Nat. Neurosci. 2004;7(3):269-77.

Barritt AW, Davies M, Marchand F, Hartley R, Grist J, Yip P, et al. Chondroitinase ABC promotes sprouting of intact and injured spinal systems after spinal cord injury. J. Neurosci. 2006;26(42):10856-67.

Bartlett JS, Samulski RJ, McCown TJ. Selective and rapid uptake of adeno-associated virus type 2 in brain. Hum. Gene Ther. 1998;9(8):1181-6.

Blits B, Derks S, Twisk J, Ehlert E, Prins J, Verhaagen J. Adeno-associated viral vector (AAV)-mediated gene transfer in the red nucleus of the adult rat brain: comparative analysis of the transduction properties of seven AAV serotypes and lentiviral vectors. J. Neurosci. Methods. 2010;185(2):257-63.

Blum R, Kafitz KW, Konnerth A. Neurotrophin-evoked depolarization requires the sodium channel Na(V)1.9. Nature. 2002;419(6908):687-93.

Bolton DA, Tse AD, Ballermann M, Misiaszek JE, Fouad K. Task specific adaptations in rat locomotion: runway versus horizontal ladder. Behav. Brain Res. 2006;168(2):272-9.

Boudes M, Menigoz A. Non-neuronal BDNF, a key player in development of central sensitization and neuropathic pain. J. Physiol. 2009;587(Pt 10):2111-2.

Boulenguez P, Liabeuf S, Bos R, Bras H, Jean-Xavier C, Brocard C, et al. Down-regulation of the potassium-chloride cotransporter KCC2 contributes to spasticity after spinal cord injury. Nat. Med. 2010;16(3):302-7.

Boyce VS, Tumolo M, Fischer I, Murray M, Lemay MA. Neurotrophic factors promote and enhance locomotor recovery in untrained spinalized cats. J. Neurophysiol. 2007;98(4):1988-96.

Bramham CR, Messaoudi E. BDNF function in adult synaptic plasticity: the synaptic consolidation hypothesis. Prog. Neurobiol.

2005;76(2):99-125.

Burger C, Gorbatyuk OS, Velardo MJ, Peden CS, Williams P, Zolotukhin S, et al. Recombinant AAV viral vectors pseudotyped with viral capsids from serotypes 1, 2, and 5 display differential efficiency and cell tropism after delivery to different regions of the central nervous system. Mol. Ther. 2004;10(2):302-17.

Caudle KL, Brown EH, Shum-Siu A, Burke DA, Magnuson TS, Voor MJ, et al. Hindlimb immobilization in a wheelchair alters functional recovery following contusive spinal cord injury in the adult rat. Neurorehabil. Neural Repair. 2011;25(8):729-39.

Cote MP, Azzam GA, Lemay MA, Zhukareva V, Houle JD. Activity-dependent increase in neurotrophic factors is associated with an enhanced modulation of spinal reflexes after spinal cord injury. J. Neurotrauma. 2011;28(2):299-309.

Courtine G, Song B, Roy RR, Zhong H, Herrmann JE, Ao Y, et al. Recovery of supraspinal control of stepping via indirect propriospinal relay connections after spinal cord injury. Nat. Med. 2008;14(1):69-74.

Darian-Smith C. Synaptic plasticity, neurogenesis, and functional recovery after spinal cord injury. Neuroscientist. 2009;15(2):149-65.

Fouad K, Metz GA, Merkler D, Dietz V, Schwab ME. Treadmill training in incomplete spinal cord injured rats. Behav. Brain Res. 2000;115(1):107-13.

Fouad K, Pedersen V, Schwab ME, Brosamle C. Cervical sprouting of

corticospinal fibers after thoracic spinal cord injury accompanies shifts in evoked motor responses. Curr. Biol. 2001;11(22):1766-70.

Fouad K, Tetzlaff W. Rehabilitative training and plasticity following spinal cord injury. Exp. Neurol. 2012;235(1):91-9.

Fouad K, Tse A. Adaptive changes in the injured spinal cord and their role in promoting functional recovery. Neurol Res. 2008;30(1):17-27.

Fouad K, Vavrek R, Cho S. A TrkB antibody agonist promotes plasticity following cervical spinal cord injury in adult rats. J. Neurotrauma. 2010, in press.

Garcia-Alias G, Barkhuysen S, Buckle M, Fawcett JW. Chondroitinase ABC treatment opens a window of opportunity for task-specific rehabilitation. Nat. Neurosci. 2009;12(9):1145-51.

Girgis J, Merrett D, Kirkland S, Metz GA, Verge V, Fouad K. Reaching training in rats with spinal cord injury promotes plasticity and task specific recovery. Brain. 2007;130(Pt 11):2993-3003.

Goodman LJ, Valverde J, Lim F, Geschwind MD, Federoff HJ, Geller AI, et al. Regulated release and polarized localization of brain-derived neurotrophic factor in hippocampal neurons. Mol. Cell Neurosci. 1996;7(3):222-38.

Griesbach GS, Hovda DA, Molteni R, Wu A, Gomez-Pinilla F. Voluntary exercise following traumatic brain injury: brain-derived neurotrophic factor upregulation and recovery of function. Neuroscience. 2004;125(1):129-39.

Griesbeck O, Canossa M, Campana G, Gartner A, Hoener MC, Nawa H, et al. Are there differences between the secretion characteristics of NGF and BDNF? Implications for the modulatory role of neurotrophins in activity-dependent neuronal plasticity. Microsc. Res. Tech. 1999;45(4-5):262-75.

Hagg T, Baker KA, Emsley JG, Tetzlaff W. Prolonged local neurotrophin-3 infusion reduces ipsilateral collateral sprouting of spared corticospinal axons in adult rats. Neuroscience. 2005;130(4):875-87.

Hiebert GW, Khodarahmi K, McGraw J, Steeves JD, Tetzlaff W. Brain-derived neurotrophic factor applied to the motor cortex promotes sprouting of corticospinal fibers but not regeneration into a peripheral nerve transplant. J. Neurosci. Res. 2002;69(2):160-8.

Kafitz KW, Rose CR, Thoenen H, Konnerth A. Neurotrophin-evoked rapid excitation through TrkB receptors. Nature. 1999;401(6756):918-21.

Kato M, Murakami S, Yasuda K, Hirayama H. Disruption of fore- and hindlimb coordination during overground locomotion in cats with bilateral serial hemisection of the spinal cord. Neurosci. Res. 1984;2(1-2):27-47.

Klein RL, Hamby ME, Sonntag CF, Millard WJ, King MA, Meyer EM. Measurements of vector-derived neurotrophic factor and green fluorescent protein levels in the brain. Methods. 2002;28(2):286-

92.

Krajacic A, Ghosh M, Puentes R, Pearse DD, Fouad K. Advantages of delaying the onset of rehabilitative reaching training in rats with incomplete spinal cord injury. Eur. J. Neurosci. 2009;29(3):641-51.

Krajacic A, Weishaupt N, Girgis J, Tetzlaff W, Fouad K. Training-induced plasticity in rats with cervical spinal cord injury: effects and side effects. Behav. Brain Res. 2010;214(2):323-31.

Lin LH, Dragon DN, Jin J, Talman WT. Targeting neurons of rat nucleus tractus solitarii with the gene transfer vector adeno-associated virus type 2 to up-regulate neuronal nitric oxide synthase. Cell. Mol. Neurobiol. 2011;31(6):847-59.

Lovely RG, Gregor RJ, Roy RR, Edgerton VR. Effects of training on the recovery of full-weight-bearing stepping in the adult spinal cat. Exp. Neurol. 1986;92(2):421-35.

Lu B. Acute and long-term synaptic modulation by neurotrophins. Prog. Brain Res. 2004;146:137-50.

Lu P, Blesch A, Graham L, Wang Y, Samara R, Banos K, et al. Motor axonal regeneration after partial and complete spinal cord transection. J. Neurosci. 2012;32(24):8208-18.

Lu Y, Ji Y, Ganesan S, Schloesser R, Martinowich K, Sun M, et al. TrkB as a potential synaptic and behavioral tag. J Neurosci. 2011;31(33):11762-71.

Luikart BW, Parada LF. Receptor tyrosine kinase B-mediated excitatory

synaptogenesis. Prog. Brain Res. 2006;157:15-24.

Minichiello L. TrkB signalling pathways in LTP and learning. Nat. Rev. Neurosci. 2009;10(12):850-60.

Murray KC, Nakae A, Stephens MJ, Rank M, D'Amico J, Harvey PJ, et al. Recovery of motoneuron and locomotor function after spinal cord injury depends on constitutive activity in 5-HT2C receptors. Nat. Med. 2010;16(6):694-700.

Onifer SM, Smith GM, Fouad K. Plasticity after spinal cord injury: relevance to recovery and approaches to facilitate it. Neurotherapeutics. 2011;8(2):283-93.

Passini MA, Watson DJ, Wolfe JH. Gene delivery to the mouse brain with adeno-associated virus. Methods Mol. Biol. 2004;246:225-36.

Pettersson LG, Alstermark B, Blagovechtchenski E, Isa T, Sasaski S. Skilled digit movements in feline and primate--recovery after selective spinal cord lesions. Acta Physiol. (Oxf). 2007;189(2):141-54.

Rosas-Vargas H, Martinez-Ezquerro JD, Bienvenu T. Brain-derived Neurotrophic Factor, Food Intake Regulation, and Obesity. Arch. Med. Res. 2011; 42(6):482-94.

Rossignol S, Frigon A, Barriere G, Martinez M, Barthelemy D, Bouyer L, et al. Chapter 16--spinal plasticity in the recovery of locomotion. Prog. Brain Res. 2011;188:229-41.

Schaal SM, Kitay BM, Cho KS, Lo TP, Jr., Barakat DJ, Marcillo AE, et al. Schwann cell transplantation improves reticulospinal axon growth

and forelimb strength after severe cervical spinal cord contusion. Cell Transplant. 2007;16(3):207-28.

Schallert T, Fleming SM, Leasure JL, Tillerson JL, Bland ST. CNS plasticity and assessment of forelimb sensorimotor outcome in unilateral rat models of stroke, cortical ablation, parkinsonism and spinal cord injury. Neuropharmacology. 2000;39(5):777-87.

Scharfman HE. Brain-derived neurotrophic factor and epilepsy--a missing link? Epilepsy Curr. 2005;5(3):83-8.

Taylor L, Jones L, Tuszynski MH, Blesch A. Neurotrophin-3 gradients established by lentiviral gene delivery promote short-distance axonal bridging beyond cellular grafts in the injured spinal cord. J. Neurosci. 2006;26(38):9713-21.

Vavrek R, Girgis J, Tetzlaff W, Hiebert GW, Fouad K. BDNF promotes connections of corticospinal neurons onto spared descending interneurons in spinal cord injured rats. Brain. 2006;129(Pt 6):1534-45.

Vavrek R, Pearse DD, Fouad K. Neuronal populations capable of regeneration following a combined treatment in rats with spinal cord transection. J. Neurotrauma. 2007;24(10):1667-73.

Vaynman S, Gomez-Pinilla F. License to run: exercise impacts functional plasticity in the intact and injured central nervous system by using neurotrophins. Neurorehabil. Neural Repair. 2005;19(4):283-95.

Waldron HA, Gwyn DG. Descending nerve tracts in the spinal cord of the

rat. I. Fibers from the midbrain. J. Comp. Neurol. 1969;137(2):143-53.

Weidner N, Ner A, Salimi N, Tuszynski MH. Spontaneous corticospinal axonal plasticity and functional recovery after adult central nervous system injury. Proc. Natl. Acad. Sci. USA. 2001;98(6):3513-8.

Wiesel TN, Hubel DH. Extent of recovery from the effects of visual deprivation in kittens. J. Neurophysiol. 1965;28(6):1060-72.

Ying Z, Roy RR, Edgerton VR, Gomez-Pinilla F. Exercise restores levels of neurotrophins and synaptic plasticity following spinal cord injury. Exp. Neurol. 2005;193(2):411-9.

Ying Z, Roy RR, Zhong H, Zdunowski S, Edgerton VR, Gomez-Pinilla F. BDNF-exercise interactions in the recovery of symmetrical stepping after a cervical hemisection in rats. Neuroscience. 2008;155(4):1070-8.

Zemlan FP, Behbehani MM, Beckstead RM. Ascending and descending projections from nucleus reticularis magnocellularis and nucleus reticularis gigantocellularis: an autoradiographic and horseradish peroxidase study in the rat. Brain Res. 1984;292(2):207-20.

Zhou L, Baumgartner BJ, Hill-Felberg SJ, McGowen LR, Shine HD. Neurotrophin-3 expressed in situ induces axonal plasticity in the adult injured spinal cord. J. Neurosci. 2003;23(4):1424-31.

Zhou L, Shine HD. Neurotrophic factors expressed in both cortex and

spinal cord induce axonal plasticity after spinal cord injury. J. Neurosci. Res. 2003;74(2):221-6.

CHAPTER 3

Vector-induced NT-3 expression promotes collateral growth of injured corticospinal tract axons in a rat model of spinal cord injury

N. Weishaupt, A. L. O. Mason, C. Hurd, Z. May, D. C. Zmyslowski, K. Fouad

In preparation for submission to Experimental Neurology

3.1. Preface

NT-3's chemo-attractive and growth-promoting effects on injured axons in the vicinity of a spinal lesion are well established (Schnell *et al.*, 1994, Taylor *et al.*, 2006). NT-3 has also successfully been employed to promote sprouting from the spared CST far caudal to a unilateral pyramidotomy (Zhou *et al.*, 2003). Nevertheless, expression of NT-3 in the reticular formation in chapter 2 did not influence collateral growth from the cervically injured CST across the brainstem midline towards the source of NT-3. A potential explanation for the failure of NT-3 to act on contralateral CST axons within the brainstem may be the lack of local inflammation and immune activation, as suggested by Chen and colleagues (Chen *et al.*, 2008). Yet, if we are to use NT-3 as a tool to promote novel connections between the injured CST and relay neurons, the neurotrophin will have to be effective in grey matter regions rostral to the SCI. In such regions, where neither injury-induced nor degeneration-associated inflammatory processes prevail, NT-3 has so far failed to induce sprouting from the injured CST (chapter 2; Vavrek *et al.*, 2006). Following the lead that inflammation may be essential for a growth-promoting effect of NT-3 on axons to occur, the question whether systemic immune activation can facilitate such an effect of NT-3 rostral to an SCI is addressed next.

3.2. Introduction

The corticospinal tract (CST), which is important for voluntary control and skilled use of the extremities (Brochier and Umilta, 2007, Lemon, 2008), typically does not regenerate when severed by a spinal cord injury (SCI). However, the tract has been shown to grow new collaterals rostral to an injury (Fouad *et al.*, 2001, Bareyre *et al.*, 2004), a process that is facilitated by rehabilitative training (Girgis *et al.*, 2007). Such collateral sprouting can result in new connections with neurons spared by the injury (Bareyre *et al.*, 2004), which may allow the motor signal to reach denervated areas below the injury via a detour circuit (Courtine *et al.*, 2008, van den Brand *et al.*, 2012).

While several interventions are known to increase CST collateral sprouting (Hiebert *et al.*, 2002, Vavrek *et al.*, 2006, Girgis *et al.*, 2007), tools to direct any growing collaterals to new targets by chemo-attraction are more difficult to find. One example is the neurotrophin-3 (NT-3), which in sensory neurons attracts axon growth even to inappropriate targets (Alto *et al.*, 2009). CST responsiveness to NT-3 has been demonstrated by increased sprouting at a spinal lesion site (Schnell *et al.*, 1994), and by increased collateral growth of spared fibers across the spinal cord midline towards NT-3 expressing motoneurons caudal to a unilateral pyramidotomy (Zhou *et al.*, 2003). In contrast, we previously failed to promote midline-crossing CST collateral growth when NT-3 was expressed in the reticular formation remote from a

cervical SCI (Weishaupt *et al.*, 2013). Likewise, intrathecal delivery of NT-3 rostral to a SCI failed to promote sprouting of the injured CST (Vavrek *et al.*, 2006), and even a reduction of CST collateral growth in response to NT-3 delivery has been reported (Hagg *et al.*, 2005). Recent evidence indicates that these inconsistencies in the responsiveness of the CST to NT-3 may be explained by the necessity of an inflammatory environment. Shine and colleagues found that NT-3-induced midline-crossing CST collateral growth far caudal to a pyramidotomy only in the acute phase after injury, or following lipopolysaccharide- (LPS-) induced re-activation of immune cells associated with Wallerian degeneration (WD) in the chronic phase (Chen *et al.*, 2006, Chen *et al.*, 2008). This is consistent with the notion that NT-3 seems ineffective when administered in regions far rostral to an SCI where injury-induced inflammation and immune activation are limited (Schnell *et al.*, 1999), and where grey matter is largely devoid of degenerating collaterals from injured axons (Fleming *et al.*, 2006, McKay *et al.*, 2007, Wang *et al.*, 2009).

Based on the suggested necessity of immune activation for NT-3 effects on CST sprouting to occur, we hypothesized that LPS-induced immune activation would be able to promote collateral growth from various motor tracts into regions with high NT-3 concentration rostral to an SCI. To test this hypothesis, we investigated the collateral growth response from the left and right CST as well as the right RST to systemic LPS administration and NT-3 expression in the right cervical grey matter, rostral from a severe thoracic

SCI. While the RST remained unresponsive under all experimental conditions, our findings indicate that CST sprouting rostral to an injury can be promoted by NT-3 into areas that lack degenerative processes, but might strongly depend on the availability and/or the concentration of NT-3.

3.3. Materials and methods

3.3.1. Animals and experimental groups

31 female Lewis rats (Charles River Laboratories, Wilmington, MA, USA) weighing 200 – 210 g received an incomplete SCI at level T8 and were randomly assigned to 1 of 4 experimental groups. Group GFP (green fluorescent protein): Injection of adeno-associated viral vectors (AAV) of serotype 2 encoding GFP in the cervical cord and intraperitoneal (i.p.) saline injections (n = 7); Group NT-3: AAV-2 encoding NT-3 injection in the cervical cord and i.p. saline injections (n = 8); Group GFP/LPS: Injection of AAV-2 encoding GFP in the cervical cord and i.p. LPS injections (n = 8); Group NT-3/LPS: AAV-2 encoding NT-3 injection in the cervical cord and i.p. LPS injections (n = 8). The experimental flow is demonstrated in Fig. 3.1.A. Ten additional rats served as unlesioned controls for behavioural analysis. All animals were group housed and kept on a 12 h: 12 h light/ dark cycle. All procedures involving animals were approved by the Health Sciences Animal Care and Use Committee of the University of Alberta.

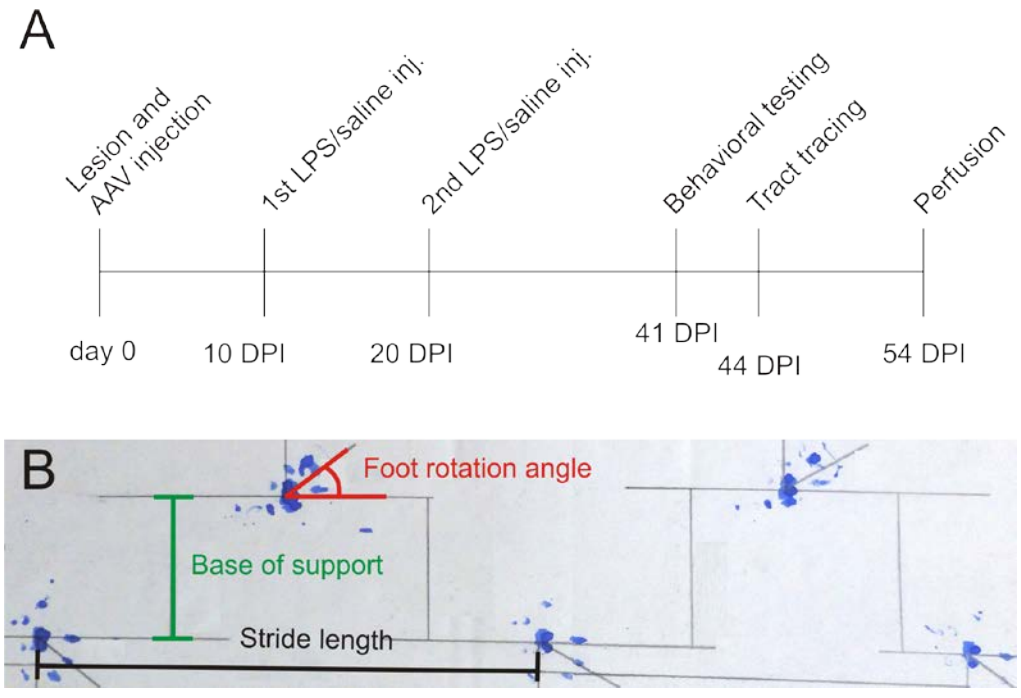


Figure 3.1. Experimental timeline and footprint gait analysis.

(A) Timeline demonstrates sequence of events. DPI = days post injury/AAV injection. **(B)** In a series of footprints, stride length (black line), base of support (green line) and foot rotation angle (marked in red) were measured for analysis of gait.

3.3.2. Spinal cord injury

Spinal lesion surgeries and AAV injections were performed under ketamine-xylazine anesthesia (ketamine 60mg/kg, Sandoz, Boucherville, QC, Canada; xylazine 7.5 mg/ kg, Rompun, Bayer Inc., Toronto, ON, Canada). Body temperature was maintained at 37 °C with a heating blanket throughout the surgery. The spinal cord was exposed at level T8 and a severe incomplete SCI was induced with a custom-made microblade. Muscle layers were sutured and the skin was closed with staples.

3.3.3. Injection of AAV vectors

In order to deliver AAV vectors encoding either NT-3 or GFP (Vector Laboratories, Chapel Hill, NC, USA) in the right cervical grey matter, the right hemicord between vertebrae C4 and C5 was exposed while animals were still under anesthesia from the lesion surgery. After puncturing the dura, a glass electrode filled with AAV-2 containing solution was lowered into the right hemicord to a depth of 1 mm (to target the intermediate grey matter) and 1 µl of AAV containing solution (NT-3-AAV-2: 1.6 x 10e13 particles/ ml; GFP-AAV-2: 2.5 x 10e12 particles/ ml) was pressure injected at pulses of 15 msec duration at 15 - 25 psi. Muscle layers were sutured and the skin closed with staples. Post-operative hydration was restored by s.c. injection of 4 ml saline, and pain was managed by s.c. injections of buprenorphine (Temgesic, Schering-Plough, Kirkland, QC, Canada, 0.05 mg/kg). Animals were kept on a

heating blanket until fully awake.

3.3.4. Injection of LPS or saline

LPS can differ in its potency to induce immune activation between batches. From pilot experiments in unlesioned animals, a dose of LPS was selected that would produce transient signs of sickness (such as puffy hair coat, hunched back, increased porphyrin staining, segregation from cage mates, decreased spontaneous activity) for at least one day post injection, and that would raise body temperature about 1 °C. Animals in the LPS treatment groups received i.p. injections of LPS on day 10 (400 µg/kg) and day 20 (350 µg/kg) post injury, while animals in the two remaining groups received an equal volume of saline i.p. All animals were briefly anaesthetized with isoflurane for i.p. injections and baseline temperature measurement using a rectal probe. Thereafter, rectal temperature was measured while awake at 5 hrs, 10 hrs, 25 hrs, 34 hrs and 58 hrs post injection and animals were closely monitored for signs of sickness.

3.3.5. Footprint gait analysis

At the end of the 6 week recovery period, footprint gait analysis was performed for all injured and 10 uninjured rats (de Medinaceli *et al.*, 1982, Teunissen *et al.*, 2001, Klein *et al.*, 2009). The rats' hindpaws were dipped in standard non-toxic ink, and the rats were encouraged to walk along a 4' x 4.5" Plexiglass runway covered with white paper. This was repeated until 3 clean

runs were recorded on paper. Three measurements were analyzed (Fig. 3.1.B). (1) Stride length: The distance between two subsequent prints of the same hindpaw. The difference between the stride lengths of the left and right hindpaws was taken as a measure of symmetry. (2) Base of support: The distance between the left and right paw prints at 90° to the forward stepping direction. (3) Foot rotation angle: The angle between the centre of the footpad and the third digit.

3.3.6. Plantar heater test

Following 6 weeks of recovery all rats underwent thermal sensitivity testing in the plantar heater apparatus (Ugo Basile, Comerio, Italy). Ten additional animals served as uninjured controls. A radiant heat source, with infrared light intensity set to 50, was shone underneath each hindpaw and the time in seconds taken for the animal to withdraw its paw was measured. If the rat did not withdraw its hindpaw 30 s after the start of a trial, stimulus delivery was stopped. The left and right hindpaws were tested separately 3 times with a 2 min break between trials.

3.3.7. Tracing

After final behavioural testing, animals were anesthetized with isoflurane and their heads secured in a stereotaxic frame (Kopf Instruments, Tujunga, CA, USA) in order to inject tracers into both hindlimb motor cortices (1.6 – 3.3 mm caudal and 2 – 3 mm lateral to bregma, at 1.5 mm depth) as well as

into the left red nucleus (5.7 mm caudal, 0.9 mm lateral to bregma and 7.5 mm beneath the dura). The right hindlimb motor cortex was injected with 1 µl Alexa Fluor 488 (10 %, Molecular Probes, Eugene, OR, USA) at 3 locations using a Hamilton syringe. The left hindlimb motor cortex was injected in the same manner with biotinylated dextran amine (10 % BDA 10.000 MW, Molecular Probes). Neurons of the left red nucleus and their axons were traced by pressure injection (pulses of 15 msec duration and 15 – 25 psi) of 1 µl Tetramethylrhodamine (10 % TMR, Molecular Probes). The wound was closed with stitches and post-surgical care was the same as described in 3.3.3.

3.3.8. Perfusion and tissue collection

Two weeks following tracer injection, animals were euthanized with an overdose of pentobarbital (Euthanyl, Biomeda-MTC, Cambridge, ON, Canada) and then perfused with saline followed by 2 % paraformaldehyde (PFA) solution containing 0.2 % *p*-benzoquinone (PBQ, Sigma-Aldrich). Brains and spinal cords were dissected, post-fixed in PFA/PBQ solution on ice for 2 hrs and subsequently transferred into a cryoprotective 30 % sucrose solution for 2 – 4 days. Tissue blocks were mounted on filter paper using Tissue Tek (Sakura Finetek, Torrance, CA, USA), frozen in methylbutane at -50 °C and cryo-sectioned at 25 µm (cross sections: C1 and lumbar region; horizontal sections: cervical enlargement and lesion area at T8).

3.3.9. NT-3 staining procedure

To visualize viral vector-mediated expression of NT-3 in the cervical enlargement, horizontal sections were dehydrated at 37 °C for 1 hr, then rehydrated 2 x 10 min in TBS, rinsed in 0.6% H_2O_2 on shaker for 15 min and washed again. Sections were blocked in 5% normal donkey serum (NDS) in 0.25 % TX in TBS for 1 hr and incubated in goat-anti-NT-3 antibody (GT 15000, Neuromics, Edina, MN, USA; 1:100 in 0.25 % TBS-TX/1 % NDS) for two nights at room temperature (RT). On day 3, slides were washed 3 x 10 min in 0.25 % TBS-TX/1% NDS, incubated in donkey-anti-goat FITC antibody (diluted 1:50 in 0.25 % TBS-TX/1% NDS, # 705-095-003 Jackson Immuno Research Laboratories, West Grove, PA, USA) for 2 hr at RT, washed 3 x 10 min in TBS and coverslipped with Permount (Fisher Scientific).

3.3.10. CST staining procedure

The tracers for the right (BDA) and left (AF 488) CST were developed with DAB and NovaRED, respectively, in a double staining procedure. To secure attachment of sections to slides, slides were dehydrated at 37 °C for 1 hr. Following 2 x 10 min TBS and 2 x 45 min TBS-TX, slides were incubated with Vectakit ABC solution (Vector Laboratories) at RT for 2 hrs. After 3 x 10 min TBS, the DAB reaction (Vector Laboratories) was performed and halted in H_2O. Slides were washed 3 x 10 min in TBS, blocked for 1 hr at RT in NGS and then incubated in anti-AF 488 antibody (rabbit IgG, Molecular Probes,

Eugene, OR, USA) at 4 °C overnight. Following 3 x 10 min TBS, slides were incubated in Vecta Kit goat-anti-rabbit antibody (Vector Laboratories) overnight at 4 °C. After 3 x 10 min TBS, slides were incubated in ABC solution for 2 hrs at RT, followed by 3 x 10 min TBS and the NovaRED (Vector Laboratories, SK-4800) reaction, which was halted in H_2O. After 3 x 10 min TBS, slides were dehydrated in increasing concentrations of ethanol, 2 x 2 min xylene and coverslipped using Permount.

3.3.11. Lesion reconstruction

Lesion extent was analyzed in horizontal sections including the lesion epicenter at spinal level T8. This analysis was performed under fluorescent light (Leica Microsystems) to judge the integrity of each section. Using landmarks such as the grey white matter borders and the central canal, lesions were reconstructed onto a schematic of a cross section. Lesion of motor tracts was verified by examining sections caudal to the lesion for spared traced fibers.

3.3.12. Quantification of traced axons

Cross sections of the spinal cord at level C1 were either coverslipped and examined under fluorescent light to detect TMR traced RST axons, or developed with DAB and NovaRED and examined with a light microscope to detect axons of the right and left dorsal CST, respectively. Pictures covering the area of projection of each of these tracts were taken at 400 x

magnification and merged into collages using Corel Draw. Using an overlying grid, the number of traced fibers was then counted in these collages for each of the tracts and subsequently used for normalization of collateral counts as described below.

3.3.13. Quantification of collaterals from right CST

In horizontal sections of the cervical enlargement, BDA-traced fibers of the right CST were developed with DAB. CST fibers that left the main projection of the tract in the dorsal funiculus and crossed the white-grey matter interface to project in the adjacent grey matter were counted in two slides per animal (sections throughout the entire dorso-ventral extent of the cord were cut in staggered fashion on 8 slides). Only those areas of a section that contained DAB-stained CST axons projecting in the dorsal white matter were analyzed. Collateral numbers were normalized to the distance analyzed as well as to the number of traced fibers at level C1 for each animal. Because the area of GFP/NT-3 expression was consistently located within the caudal half of horizontal sections, the rostral and caudal halves of each section were separately screened for collaterals. To detect a potential effect of NT-3/GFP-expressing AAV on collateral growth, all final values are expressed as the difference between the caudal half (region of NT-3/GFP expression) and the rostral half (no NT-3/GFP expression) of the cervical enlargement.

3.3.14. Quantification of collaterals from left CST

In horizontal sections of the cervical enlargement, fibers of the left CST traced with Alexa Flour 488 were developed with Vector NovaRED substrate kit (SK-4800). Using a Leica light microscope, NovaRED stained CST collaterals that project across the midline within the grey matter were counted in two slides per animal. Normalization was done as for right CST collaterals.

3.3.15. Quantification of collaterals from right RST

Horizontal sections of the cervical enlargement were coverslipped and examined under fluorescent light. TMR-traced axon collaterals that left the main projection of the tract in the lateral white matter and crossed over into the adjacent grey matter were counted and normalized using the same protocol as for CST collaterals.

3.3.16. Statistical analyses

All data sets were tested for fitting a Gaussian distribution. Comparisons of temperatures between LPS and saline injected animals were analyzed using two-way ANOVA across time points. Lesion size was compared among all four groups using one-way ANOVA and Bonferroni's Multiple Comparison Test. The same test was used to compare behavioural outcomes among the uninjured and all four injured treatment groups. Histological results of axon and collateral counts were analyzed using a Kruskal-Wallis test followed by

Dunns post-test. All data are presented as mean ± SEM, which is outlined in scatter plots. A p value ≤ 0.05 was considered significant.

3.4. Results

3.4.1. Body temperature development following LPS injections

Before LPS or saline were administered for the first time at day 10 post injury, temperatures among the 31 rats varied from 36.3 °C to 37.8 °C. Animals injected with LPS (n = 16, 400 µg/kg) showed sickness behaviour over the course of about 24 hrs after the first injection, however a significant increase in body temperature of LPS versus saline injected animals was only evident at 34 hrs post injection (LPS: 37.42 °C ± 0.12 °C, saline: 36.9 °C ± 0.13 °C, $p < 0.05$, Fig. 3.2.). Overall, body temperature of saline injected animals increased similarly to that of LPS injected animals (Fig. 3.2.). We speculate that this equalization of body temperature may have been facilitated by the animals resting and sleeping closely huddled together, as animals receiving LPS or saline were housed together. Baseline body temperatures before the second administration of LPS or saline at day 20 post injury varied similarly to what was observed 10 days prior. To avoid potential equalization of body temperature by group housing, animals were housed only with cage mates of the same treatment group over the following 58 hrs. A significant difference in temperature rise between the LPS (350 µg/kg) and saline injected groups was first evident at 5 hrs after this second

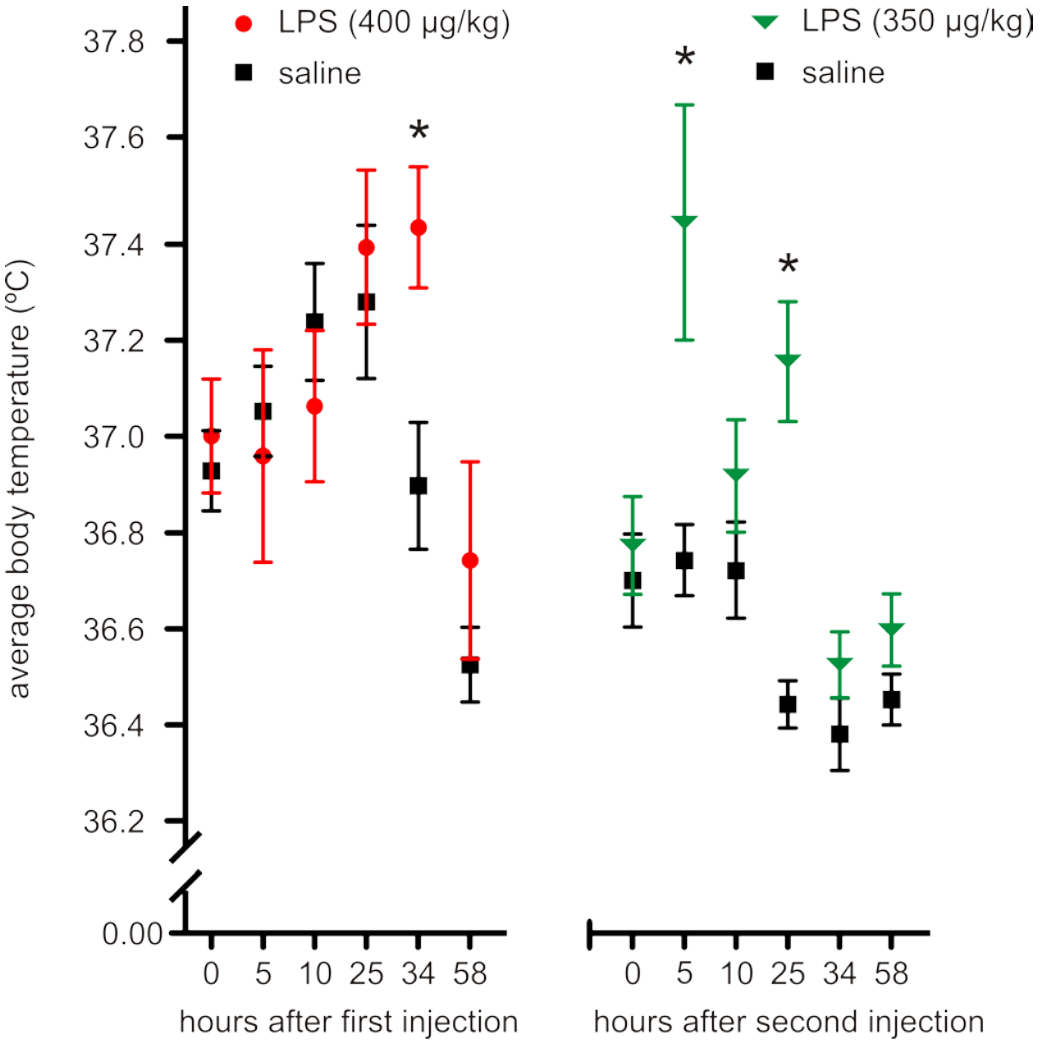


Figure 3.2. Body temperature following LPS administration.

Body temperature following LPS administration. To confirm an immune-activating effect of LPS administration, body temperature was closely monitored following each LPS injection. Body temperature of LPS injected animals (red circles) was only significantly elevated compared to saline injected animals (black squares) at 34 hrs after the first injection. Following the second injection of LPS or saline, when animals were group housed according to treatment, LPS injected animals (green triangles) showed a significant increase in body temperature at 5 hrs and 25 hrs post injection compared with saline injected animals (black squares). Error bars show SEM, asterisk indicates $p < 0.05$.

injection (LPS: 37.43 °C ± 0.24 °C, saline: 36.75 °C ± 0.07 °C, $p < 0.05$). Such an early response is consistent with a second exposure to the same endotoxin.

Sickness behaviour after this second LPS injection was less pronounced than after the initial injection. Another peak of core temperature rise was recorded at 25 hrs post injection (LPS: 37.15 °C ± 0.12 °C, saline: 36.46 °C ± 0.05 °C, $p < 0.05$). Interestingly, body temperature of saline injected animals fell to a low of about 0.25° below the pre-injection baseline at 25 hrs post injection, which was maintained at 34 hrs and 58 hrs post injection. LPS injected animals returned to a similar low at 34 hrs and 58 hrs post injection.

3.4.2. Footprint analysis

To assess whether NT-3 and/or LPS treatment affect hindlimb motor function after a severe incomplete thoracic SCI, we analyzed the animals' gait using the traditional footprint technique (de Medinaceli *et al.*, 1982, Teunissen *et al.*, 2001, Klein *et al.*, 2009). A measure of stride length symmetry was derived by calculating the difference between right and left stride lengths, which was on average 2.76 mm ± 0.59 mm in uninjured animals. SCI resulted in a higher asymmetry (GFP: 7.0 mm ± 1.89 mm, Fig. 3.3.A), which was reduced to values closer to the uninjured condition in the GFP/LPS group (NT-3: 6.75 mm ± 1.22; GFP/LPS: 3.21 mm ± 0.72 mm, NT-3/LPS: 5.63 mm ± 1.0 mm, Fig. 3.3.A). Injured animals also walked with a significantly wider base of support (GFP: 35.53 mm ± 1.52 mm) compared to uninjured counterparts (25.10 mm ± 1.03 mm, $p < 0.01$, ANOVA), yet no

differences among the treatment groups were evident (Fig. 3.3.B). Foot rotation for the right hindlimb (GFP: 30.87° ± 2.73°) was likewise increased as a result of SCI (uninjured: 18.6° ± 1.06°, $p < 0.05$, ANOVA). Whereas neither LPS nor NT-3 by itself decreased right hindlimb foot rotation, the combined NT-3/LPS treatment was able to reduce rotation angle to a value not significantly different from the uninjured condition (NT-3/LPS: 27.25° ± 4.30°, $p > 0.05$, ANOVA; Fig. 3.3.C).

3.4.3. Thermal sensitivity test

Increasing plasticity in the CNS always comes at the risk of producing undesired side effects, such as a potential increase in sensory fiber connectivity, which has been associated with hypersensitivity and pain (Deumens *et al.*, 2008). To assess whether sensitivity of the hindpaws was altered by NT-3 and/or LPS treatment, the delay to withdrawal from an infrared heat stimulus to the footpad was measured. Withdrawal delays among the four treatment groups and the left and right hindlimbs were similar (Fig. 3.3.D). Yet, all lesioned animals reacted significantly earlier to the heat stimulus compared to uninjured control animals, indicating lesion-induced hypersensitivity that was unaltered by NT-3 and/or LPS treatment (injured left: 10.55 s ± 0.36 s; uninjured left: 14.64 s ± 0.74 s; $p < 0.0001$, t-test; injured right: 9.67 s ± 0.43 s; uninjured right: 14.70 s ± 0.84 s; $p < 0.0001$, t-test; Fig. 3.3.D).

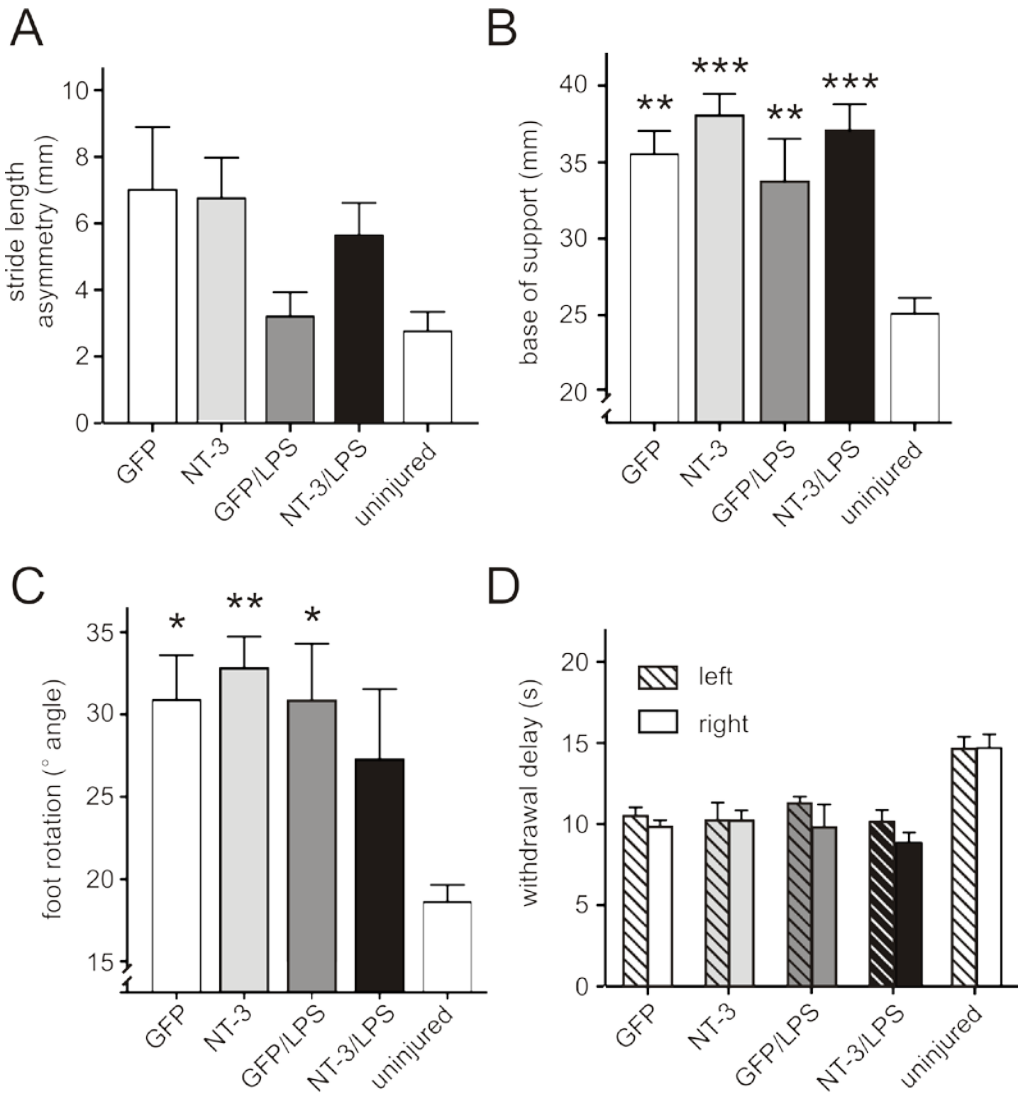


Figure 3.3. Footprint gait analysis.

(A) Spinal cord injury tended to facilitate asymmetry between stride lengths of the left and right hindlimbs, which was completely abated in the GFP/LPS group. **(B)** A significant increase in the base of support as a result of spinal cord injury (ANOVA) was not affected by NT-3 and/ or LPS treatment. **(C)** An injury-induced increase in foot rotation of the right hindlimb ($p < 0.05$, ANOVA) was reduced by combined NT-3/LPS treatment to a value not significantly different from the uninjured condition. **(D)** An injury-induced increase in hindpaw thermal sensitivity was not affected by any of the treatments. Error bars show SEM, asterisk indicates $p < 0.05$, double asterisks indicate $p < 0.01$, triple asterisks indicate $p < 0.001$.

3.4.4. Lesion analysis

The aim for this experiment was to create an incomplete SCI with injury to the right RST and both dorsal CSTs (Fig. 3.4.A). Lesion reconstruction (Fig. 3.4.B) revealed that the left dorsal CST was partly or completely injured in 14 animals, while the majority of the right dorsal CST projection was injured in 23 animals. All animals were included in the analyses as sparing is not a major concern for the design of this study. RST projections were consistently lesioned completely, with evident sparing in only one animal of the GFP/LPS group. Parts of the ventral and ventrolateral white matter, where the reticulospinal tract (Waldron and Gwyn, 1969, Zemlan *et al.*, 1984) and vestibulospinal tract project (Waldron and Gwyn, 1969, Bankoul and Neuhuber, 1992), were spared in the majority of animals (22 out of 31 animals; Fig. 3.4.A). Because sparing of these motor tracts was found equally across all experimental groups, group differences in behavioural outcomes can confidently be attributed to effects of intervention rather than to lesion variability. An approximation of the average lesion extent is outlined in Fig. 3.4.C. Overall, lesion size expressed as percent lesioned area out of the total cross sectional area varied from 14.92 % to 60.46 %. Lesion size distribution across the treatment groups was not significantly different (GFP: 32.76 % ± 3.72 %, GFP/LPS: 40.36 % ± 5.05 %, NT-3: 49.98 % ± 5.08 %, NT-3/LPS: 42.01 % ± 2.70 %, Fig. 3.4.D). It is worth noting that the LPS-induced re-activation of inflammatory processes did not result in a significant increase of

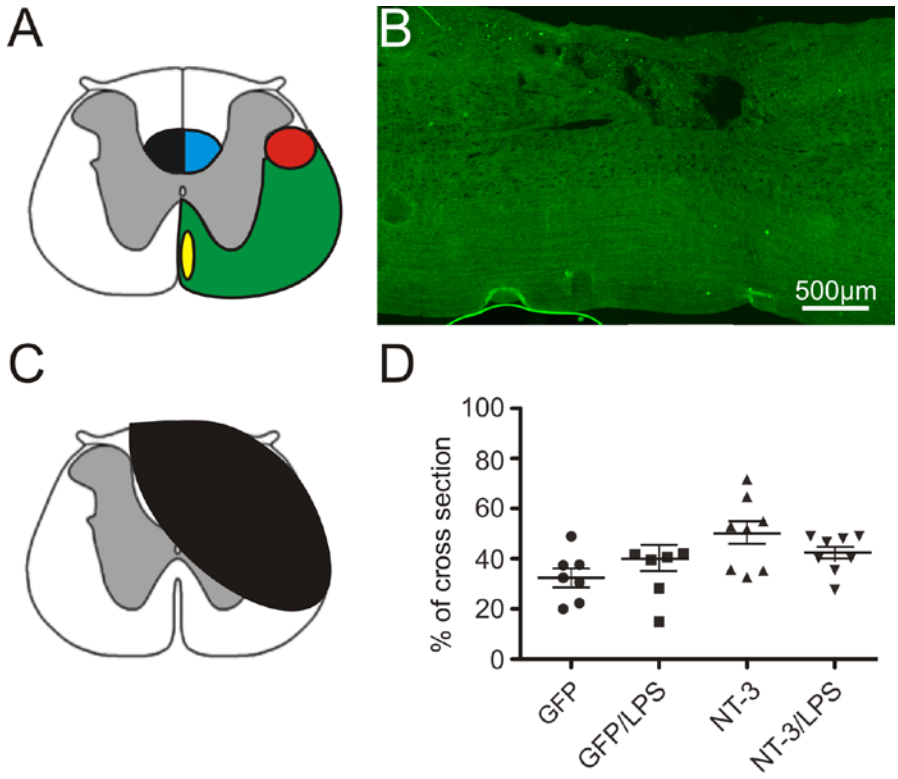


Figure 3.4. Lesion reconstruction.

(A) Schematic cross section of the spinal cord at level T8 shows projections of important motor tracts. Black: left dorsal CST; Blue: right dorsal CST; Red: right rubrospinal tract; Green: reticulospinal tract in the right white matter; Yellow: right vestibulospinal tract. **(B)** A representative lesion is shown in a horizontal section of the thoracic spinal cord. **(C)** An approximation of the average lesion extent across all animals is represented by the black area in a schematic cross section of the spinal cord at level T8. **(D)** Quantification of lesioned area expressed as percentage of complete cross sectional area reveals no difference in lesion size across all experimental groups. Lines represent mean ± SEM.

lesion extent.

3.4.5. Vector-mediated GFP and NT-3 expression

AAV-induced GFP expression was confirmed in coverslipped horizontal sections under fluorescent light. The majority of GFP signal was visible in spinal laminae IV-VI. In most cases, GFP expression covered the medio-lateral extent of the right grey matter and had also spread into the ventral part of the right dorsal funiculus (Fig. 3.5.A). Longitudinal spread of GFP signal was consistently confined to the caudal half of horizontal sections of the cervical enlargement. AAV-induced expression of NT-3 was confirmed by NT-3 immunostaining in a sample of 8 animals (Fig. 3.5.B).

3.4.6. Tracing efficacy

Tracing of layer V neurons in the left motor cortex with BDA was successful in 28 animals. The number of traced fibers was not significantly different among the four experimental groups at C1 (GFP: 866.7 ± 208, GFP/LPS: 819.7 ± 177.2, NT-3: 1053 ± 113, NT-3/LPS: 1453 ± 192.5), and was used for normalization purposes.

Injection of AF 488 into the right motor cortex resulted in tracing of the left CST in 26 animals. It is worth noting that significantly more fibers were traced in the NT-3/LPS group at C1 (Kruskal-Wallis and Dunns test; GFP: 848.7 ± 108.7, $p < 0.05$, GFP/LPS: 1020 ± 256.8, NT-3: 728.8 ± 226.7, $p < 0.01$, NT-3/LPS: 1959 ± 197.7, Fig. 3.6.A). This difference in tracing efficacy

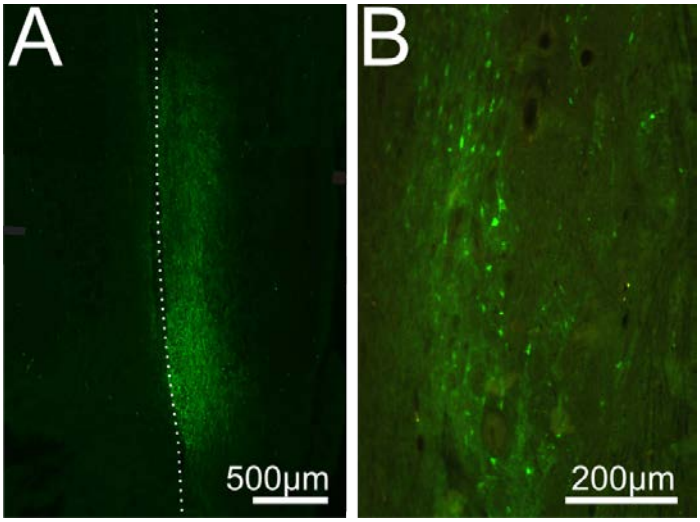


Figure 3.5. AAV-induced protein expression.

(A) Green GFP signal is visible in the right dorsal white matter and the adjacent right grey matter in a horizontal section of the cervical spinal cord from an animal injected with GFP-expressing AAV. GFP fluorescence was frequently observed to spread in the rostro-caudal direction, but was mostly confined to the caudal half of the section. **(B)** NT-3 immunostaining confirms successful expression of NT-3 in cells within the right grey matter of the cervical spinal cord in a horizontal section from an animal injected with NT-3-expressing AAV.

is accounted for by normalizing all collateral counts for the left CST to the number of traced fibers for each individual animal.

Injection of TMR into the left red nucleus resulted in tracing of RST fibers in the right white matter in 26 animals. The number of traced fibers was not significantly different among the four experimental groups at C1 (GFP: 143.6 ± 46.3, GFP/LPS: 282 ± 85.96, NT-3: 200.3 ± 71.05, NT-3/LPS: 181 ± 74.11), but was nevertheless used for normalization purposes to yield the most accurate value for collateral sprouting.

3.4.7. Quantification of right CST collaterals

To detect an effect of AAV-induced NT-3 or GFP expression on collateral growth, the difference in collateral numbers between the NT-3 and GFP expressing caudal half of each section and the rostral half of each section (where no NT-3 or GFP expression was evident) was used for statistical comparisons. The normalized values for collaterals crossing into the grey matter in the GFP group indicate that the right CST innervates the rostral and caudal segments of the cervical enlargement almost equally with an average of 0.51 ± 0.33 more collaterals found in the caudal half (Fig. 3.6.B). One outlier with a high count of 2.12 collaterals more in the caudal half accounted for most of the variability in this group. While LPS administration by itself did not change the number of CST collaterals crossing into the grey matter in the caudal versus the rostral part of the enlargement (GFP/LPS: 0.59 ± 0.12), the expression of NT-3 significantly increased the number of such CST

collaterals in the area of NT-3 expression (Kruskal-Wallis and Dunns, NT-3: 2.06 ± 0.55, vs. GFP $p < 0.05$, Fig. 3.6.B). The addition of LPS did not increase this count further, but instead seemed to dampen the effect of NT-3 (NT-3/LPS: 1.25 ± 0.49). A two-way ANOVA revealed a significant overall effect of NT-3 treatment ($p < 0.05$). Although normalization of collateral counts to the number of traced fibers was considered necessary (tracing efficacy was not equal across all groups), analyses were also performed without any normalization to the number of traced collaterals, with no difference in statistical significance of the results.

3.4.8. Quantification of left CST collaterals

As a chemo-attractant, NT-3 can potentially be used to promote growth of axonal collaterals into an area with high NT-3 concentration. In order to reach the intermediate layers of the right grey matter where NT-3 was expressed, collaterals emanating from the left CST (labeled with NovaRed) have to cross the spinal cord midline. Because no such collaterals were observed to cross into the grey matter from the right dorsal funiculus, we counted NovaRed labeled collaterals that crossed the midline within the grey matter. In the GFP group, such collaterals were equally found in the rostral (devoid of NT-3 or GFP expression) and caudal half (region of NT-3 or GFP expression) of the right cervical enlargement, with 0.14 ± 0.10 midline-crossing collaterals more in the caudal half (Fig. 3.6.C). Overall, midline-crossing collaterals were sparse, which is in line with previous reports for

the injured rodent spinal cord (Brus-Ramer *et al.*, 2007). In fact, no midline-crossing collaterals were counted within the caudal half of the enlargement in 6 animals across all experimental groups. A comparison of normalized values among all groups revealed no significant increase of midline-crossing collaterals under any condition (GFP/LPS: 0.64 ± 0.50, NT-3: 0.28 ± 0.27, NT-3/LPS: 0.34 ± 0.21). To exclude a potential effect of injury on the growth response of left CST axons, the number of midline-crossing collaterals was compared between animals where this CST was mostly lesioned and those animals where it was mostly spared (data not shown). No trends toward an effect of injury were evident in any experimental group.

3.4.9. Quantification of RST collaterals

Quantification of RST collaterals entering the grey matter in the cervical enlargement revealed that the RST preferentially innervates more rostral segments of the enlargement, irrespective of NT-3 treatment within the caudal segments. Thus, the average difference between caudal and rostral segments was 1.73 ± 0.95 fewer collaterals in caudal segments for the GFP group (Fig. 3.6.D). Combined LPS and NT-3 treatment was not able to reverse this relationship in favour of the NT-3 treated caudal segments (GFP/LPS: -0.68 ± 1.86, NT-3: -0.94 ± 0.47, NT-3/LPS: -1.51 ± 0.50, Fig. 3.6.D). Overall, differences in RST collaterals entering the grey matter were non-significant among the 4 experimental groups.

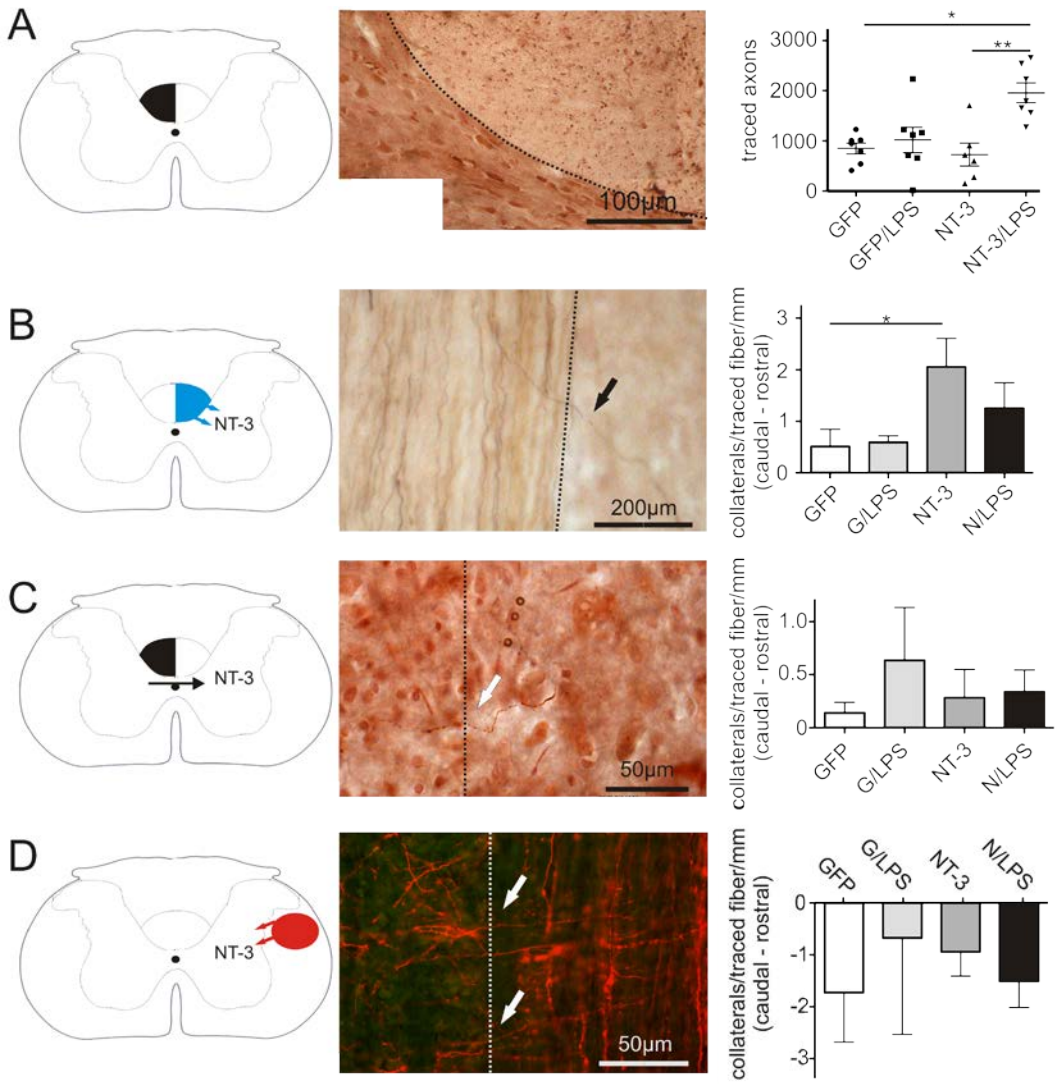


Figure 3.6. Quantification of traced axons and collateral sprouting in the cervical spinal cord.

(A) Quantification of AF488 traced axons within the left dorsal CST (visualized with NovaRED) at spinal level C1 reveals a significantly higher number of traced axons in the NT-3/LPS combined treatment group (Kruskal-Wallis and Dunns test, single asterisk marks $p < 0.05$, double asterisks mark $p < 0.01$). Dashed line represents the grey matter/ white matter border, lines in dot plot represent mean ± SEM. **(B)** BDA traced collaterals emanating from the right dorsal CST and entering the right grey matter (marked by arrow), where NT-3 or GFP was expressed, were quantified in horizontal sections. Dashed line represents the grey matter/ white matter border.

NT-3 significantly increased the number of collaterals in the vicinity of its expression compared to the GFP group (Kruskal-Wallis and Dunns test, asterisk marks $p < 0.05$). **(C)** Collaterals from axons within the left dorsal CST have to cross the midline to reach the region of NT-3 (or GFP) expression. Dashed line represent the midline separating the left and right half of the cervical spinal cord. Quantification of midline-crossing collaterals (marked by arrow) reveals no significant differences across the experimental groups. **(D)** Quantification of RST axon collaterals entering the grey matter from the lateral white matter (marked by arrows) reveals no significant group differences. Dashed line represents the grey matter/white matter border. Error bars show SEM.

3.5. Discussion

The present findings indicate that, contrary to previous reports (Hagg et al. 2005; Vavrek *et al.*, 2006, Weishaupt *et al.*, 2013), NT-3 is able to promote CST outgrowth in spinal cord regions several segments rostral to an injury site. The main difference from previously reported failures of NT-3 to induce such growth is that in this study we offer a source of NT-3 in the direct vicinity of the projection of the ipsilateral dorsal CST. This proximity ensures easy access for the axon to a high concentration of the neurotrophin. The fact that there is no discernible gap in NT-3 availability between the CST axon and the NT-3 source might be pivotal as such gaps have been demonstrated to impede an effect of NT-3 on regenerating sensory axons (Taylor *et al.*, 2006). This may also explain why the left dorsal CST failed to respond to NT-3 expression in the present study, as the distance between the left dorsal funiculus and NT-3 expression in the right grey matter might have been too long to provide a sufficient concentration of NT-3. Nevertheless, NT-3 expression in ventral horn motoneurons has been reported to elicit midline-crossing collateral growth of the spared contralateral CST in areas of Wallerian Degeneration (WD; (Zhou *et al.*, 2003). This indicates that certain factors in the microenvironment of WD, which are lacking where we administered NT-3 in the present study, may play a crucial role in overcoming the impediment of neurotrophin signaling across long distances. Alternatively, Zhou and colleagues might have induced a higher

concentration of NT-3 by vector-mediated expression than what was reached in the present study. Unfortunately, it is still unclear what concentration of NT-3 is necessary to affect CST axon growth. For example, Hagg and colleagues infused human recombinant NT-3 into the spinal grey matter at 3 and 10 μg/day, but neither concentration was able to induce midline-crossing CST growth (Hagg *et al.*, 2005). We are left to conclude that in the present study, the left dorsal CST did not reach the "NT-3-rich" right grey matter because of an insufficient concentration of NT-3 in the vicinity of the left dorsal funiculus. It remains to be seen whether a gradient of NT-3 may be able to promote CST collateral growth over longer distances and across the midline at this location (Taylor *et al.*, 2006).

Potential differences in the intrinsic growth state between the left and right dorsal CST are also worth considering. The loss of target innervation for any injured neuron can favour collateral growth in an effort to connect to new targets. Thus, thoracically injured CST axons have been reported to spontaneously grow collaterals that innervate the cervical grey matter (Fouad *et al.*, 2001, Bareyre *et al.*, 2004). Since the left and the right CST were both injured across animals in the present study, loss of axonal targets seems an unlikely explanation for the lack of response from the left dorsal CST to NT-3. In addition, there was no difference in midline-crossing collateral numbers between animals with injury to the left dorsal CST and animals with intact left CST.

The finding that NT-3 fails to promote midline-crossing CST collateral growth multiple segments rostral to an SCI requires further explanation, as midline-crossing CST growth is readily elicited caudal to an injury (Zhou *et al.*, 2003). Shine and colleagues have provided evidence that midline-crossing CST plasticity induced by NT-3 only occurs when processes of WD are active (Chen *et al.*, 2006, Chen *et al.*, 2008). Whatever factors act together with NT-3 to elicit this growth response can even be re-introduced at a chronic stage of injury when WD is re-activated by systemic administration of LPS (Chen *et al.*, 2008). So far, CD4+ T-cells have been identified as the essential cellular component for mediating this effect (Chen *et al.*, 2008). As WD is largely absent in cervical grey matter regions after a SCI at level T8 (Schnell *et al.*, 1999, Fleming *et al.*, 2006, McKay *et al.*, 2007, Wang *et al.*, 2009), we investigated whether administration of LPS might stimulate an immune response necessary to promote a growth response to NT-3 across the midline. Our results indicate that LPS-induced immune activation is insufficient to promote either growth of the RST, or CST collateral growth across the midline, or increased collateral growth of the ipsilateral CST. We are confident that the LPS stimulus was strong enough to stimulate immune activation since we observed sickness behaviour and increases in body temperature following two consecutive injections, whereas Chen et al. were able to mount a sufficient stimulus with a single dose of LPS that did not result in significant temperature elevation (Chen *et al.*, 2008). While this may point towards WD as a necessary prerequisite for the release of factors

essential to induce NT-3 promoted midline-crossing CST growth, *in vitro* evidence offers an interesting alternative explanation. Cell culture experiments indicate that microglial activation by LPS is impeded by NT-3 when microglia are pre-exposed to NT-3 (Tzeng and Huang, 2003, Tzeng *et al.*, 2005). Such NT-3 pre-treatment resulted in decreased production of a number of pro-inflammatory signaling molecules such as NO, TNF-alpha and IL-1-beta in response to LPS stimulation. Consistent with this, Chen and colleagues successfully re-activated immune cells by an injection of LPS seven days prior to NT-3 expression with viral vectors. In the present study, the first stimulation with LPS was performed 10 days after NT-3-expressing AAVs were injected. According to reports that characterize AAV-mediated neurotrophin expression in the nervous system, transfected cells would have started to produce NT-3 by day 10 (Bartlett *et al.*, 1998, Blits *et al.*, 2010). Therefore, we cannot exclude that NT-3 dampened full microglia activation by LPS, leading to an immune response insufficient to interact with NT-3 in order to promote midline-crossing CST growth.

Contrary to the CST, a growth response from the RST could not be elicited under any experimental conditions tested here. Because expression of GFP and NT-3 was more consistently located at the medial border of the grey matter than close to the lateral white matter where the RST projects, a gap in NT-3 availability might also have occurred here. A lack of TrkC receptor on RST neurons is unlikely, as neuroprotective and regeneration-promoting effects of NT-3 on RST axons have been described (Novikova *et al.*,

2000, Tobias *et al.*, 2003), and red nucleus neurons have been shown to express TrkC mRNA (King *et al.*, 1999, Tetzlaff et al. 1992). Nevertheless, a significant sprouting response of the RST to NT-3 has, to our knowledge, not yet been reported in the literature. It is therefore currently uncertain if and under what conditions NT-3 might be able to induce collateral sprouting from thoracically injured RST axons.

One unexpected finding of the present study is the significantly higher number of AF 488 traced CST fibers in the left dorsal CST in the combined treatment group compared to all other experimental groups. CST tracing of all animals was performed by the same surgeon, who was blinded to treatment group. In addition, surgeries were not performed in order of experimental group identity. Although we cannot exclude an effect of combined NT-3/LPS treatment on tracer uptake at this point, we deem this explanation improbable as neither NT-3 nor LPS treatment by itself tended to increase the number of traced CST fibers at cervical level C1.

In conclusion, we have shown that NT-3-induced collateral sprouting from CST axons rostral to their injury is possible even in an environment where no noteworthy active degenerative processes take place. However, the sprouting response of CST fibers appears limited by NT-3 concentrations, and this limitation is not overcome by LPS-induced inflammation.

3.6. References

Alto LT, Havton LA, Conner JM, Hollis ER, 2nd, Blesch A, Tuszynski MH. Chemotropic guidance facilitates axonal regeneration and synapse formation after spinal cord injury. Nat. Neurosci. 2009;12(9):1106-13.

Bankoul S, Neuhuber WL. A direct projection from the medial vestibular nucleus to the cervical spinal dorsal horn of the rat, as demonstrated by anterograde and retrograde tracing. Anat. Embryol. (Berl). 1992;185(1):77-85.

Bareyre FM, Kerschensteiner M, Raineteau O, Mettenleiter TC, Weinmann O, Schwab ME. The injured spinal cord spontaneously forms a new intraspinal circuit in adult rats. Nat. Neurosci. 2004;7(3):269-77.

Bartlett JS, Samulski RJ, McCown TJ. Selective and rapid uptake of adeno-associated virus type 2 in brain. Hum. Gene Ther. 1998;9(8):1181-6.

Blits B, Derks S, Twisk J, Ehlert E, Prins J, Verhaagen J. Adeno-associated viral vector (AAV)-mediated gene transfer in the red nucleus of the adult rat brain: comparative analysis of the transduction properties of seven AAV serotypes and lentiviral vectors. J. Neurosci. Methods. 2010;185(2):257-63.

Brochier T, Umilta MA. Cortical control of grasp in non-human primates. Curr. Opin. Neurobiol. 2007;17(6):637-43.

Brus-Ramer M, Carmel JB, Chakrabarty S, Martin JH. Electrical stimulation of spared corticospinal axons augments connections with ipsilateral spinal motor circuits after injury. J. Neurosci. 2007;27(50):13793-801.

Chen Q, Smith GM, Shine HD. Immune activation is required for NT-3-induced axonal plasticity in chronic spinal cord injury. Exp. Neurol. 2008;209(2):497-509.

Chen Q, Smith GM, Shine HD. Immune activation is required for NT-3-induced axonal plasticity in chronic spinal cord injury. Exp. Neurol. 2008;209(2):497-509.

Chen Q, Zhou L, Shine HD. Expression of neurotrophin-3 promotes axonal plasticity in the acute but not chronic injured spinal cord. J. Neurotrauma. 2006;23(8):1254-60.

Courtine G, Song B, Roy RR, Zhong H, Herrmann JE, Ao Y, et al. Recovery of supraspinal control of stepping via indirect propriospinal relay connections after spinal cord injury. Nat. Med. 2008;14(1):69-74.

de Medinaceli L, Freed WJ, Wyatt RJ. An index of the functional condition of rat sciatic nerve based on measurements made from walking tracks. Exp. Neurol. 1982;77(3):634-43.

Deumens R, Joosten EA, Waxman SG, Hains BC. Locomotor dysfunction and pain: the scylla and charybdis of fiber sprouting after spinal cord injury. Mol. Neurobiol. 2008;37(1):52-63.

Fleming JC, Norenberg MD, Ramsay DA, Dekaban GA, Marcillo AE, Saenz

AD, et al. The cellular inflammatory response in human spinal cords after injury. Brain. 2006;129(Pt 12):3249-69.

Fouad K, Pedersen V, Schwab ME, Brosamle C. Cervical sprouting of corticospinal fibers after thoracic spinal cord injury accompanies shifts in evoked motor responses. Curr. Biol. 2001;11(22):1766-70.

Girgis J, Merrett D, Kirkland S, Metz GA, Verge V, Fouad K. Reaching training in rats with spinal cord injury promotes plasticity and task specific recovery. Brain. 2007;130(Pt 11):2993-3003.

Hagg T, Baker KA, Emsley JG, Tetzlaff W. Prolonged local neurotrophin-3 infusion reduces ipsilateral collateral sprouting of spared corticospinal axons in adult rats. Neuroscience. 2005;130(4):875-87.

Hiebert GW, Khodarahmi K, McGraw J, Steeves JD, Tetzlaff W. Brain-derived neurotrophic factor applied to the motor cortex promotes sprouting of corticospinal fibers but not regeneration into a peripheral nerve transplant. J. Neurosci. Res. 2002;69(2):160-8.

King VR, Michael GJ, Joshi RK, Priestley JV. trkA, trkB, and trkC messenger RNA expression by bulbospinal cells of the rat. Neuroscience. 1999;92(3):935-44.

Klein A, Wessolleck J, Papazoglou A, Metz GA, Nikkhah G. Walking pattern analysis after unilateral 6-OHDA lesion and transplantation of foetal dopaminergic progenitor cells in rats. Behav. Brain Res. 2009;199(2):317-25.

Lemon RN. Descending pathways in motor control. Annu. Rev. Neurosci. 2008;31:195-218.

McKay SM, Brooks DJ, Hu P, McLachlan EM. Distinct types of microglial activation in white and grey matter of rat lumbosacral cord after mid-thoracic spinal transection. J. Neuropathol. Exp. Neurol. 2007;66(8):698-710.

Novikova LN, Novikov LN, Kellerth JO. Survival effects of BDNF and NT-3 on axotomized rubrospinal neurons depend on the temporal pattern of neurotrophin administration. Eur. J. Neurosci. 2000;12(2):776-80.

Schnell L, Fearn S, Klassen H, Schwab ME, Perry VH. Acute inflammatory responses to mechanical lesions in the CNS: differences between brain and spinal cord. Eur. J. Neurosci. 1999;11(10):3648-58.

Schnell L, Schneider R, Kolbeck R, Barde YA, Schwab ME. Neurotrophin-3 enhances sprouting of corticospinal tract during development and after adult spinal cord lesion. Nature. 1994;367(6459):170-3.

Taylor L, Jones L, Tuszynski MH, Blesch A. Neurotrophin-3 gradients established by lentiviral gene delivery promote short-distance axonal bridging beyond cellular grafts in the injured spinal cord. J. Neurosci. 2006;26(38):9713-21.

Teunissen CE, Steinbusch HW, Angevaren M, Appels M, de Bruijn C, Prickaerts J, et al. Behavioural correlates of striatal glial fibrillary acidic protein in the 3-nitropropionic acid rat model: disturbed

walking pattern and spatial orientation. Neuroscience. 2001;105(1):153-67.

Tobias CA, Shumsky JS, Shibata M, Tuszynski MH, Fischer I, Tessler A, et al. Delayed grafting of BDNF and NT-3 producing fibroblasts into the injured spinal cord stimulates sprouting, partially rescues axotomized red nucleus neurons from loss and atrophy, and provides limited regeneration. Exp. Neurol. 2003;184(1):97-113.

Tzeng SF, Huang HY. Downregulation of inducible nitric oxide synthetase by neurotrophin-3 in microglia. J. Cell. Biochem. 2003;90(2):227-33.

Tzeng SF, Huang HY, Lee TI, Jwo JK. Inhibition of lipopolysaccharide-induced microglial activation by preexposure to neurotrophin-3. J. Neurosci. Res. 2005;81(5):666-76.

van den Brand R, Heutschi J, Barraud Q, DiGiovanna J, Bartholdi K, Huerlimann M, et al. Restoring voluntary control of locomotion after paralyzing spinal cord injury. Science. 2012;336(6085):1182-5.

Vavrek R, Girgis J, Tetzlaff W, Hiebert GW, Fouad K. BDNF promotes connections of corticospinal neurons onto spared descending interneurons in spinal cord injured rats. Brain. 2006;129(Pt 6):1534-45.

Waldron HA, Gwyn DG. Descending nerve tracts in the spinal cord of the rat. I. Fibers from the midbrain. J. Comp. Neurol. 1969;137(2):143-

53.

Wang L, Hu B, Wong WM, Lu P, Wu W, Xu XM. Glial and axonal responses in areas of Wallerian degeneration of the corticospinal and dorsal ascending tracts after spinal cord dorsal funiculotomy. Neuropathology. 2009;29(3):230-41.

Weishaupt N, Li S, Di Pardo A, Sipione S, Fouad K. Synergistic effects of BDNF and rehabilitative training on recovery after cervical spinal cord injury. Behav. Brain Res. 2013;239:31-42.

Zemlan FP, Behbehani MM, Beckstead RM. Ascending and descending projections from nucleus reticularis magnocellularis and nucleus reticularis gigantocellularis: an autoradiographic and horseradish peroxidase study in the rat. Brain Res. 1984;292(2):207-20.

Zhou L, Baumgartner BJ, Hill-Felberg SJ, McGowen LR, Shine HD. Neurotrophin-3 expressed in situ induces axonal plasticity in the adult injured spinal cord. J. Neurosci. 2003;23(4):1424-31.

CHAPTER 4

Reticulospinal plasticity after cervical spinal cord injury in the rat involves withdrawal of projections below the injury

Adapted from N. Weishaupt, C. Hurd, D. Z. Wei, K. Fouad (2013)

Experimental Neurology

4.1. Preface

The reticulospinal tract (RtST) has been suggested to contribute to residual motor function and to recovery of motor performance of the forelimb following injury to the CST (Alstermark *et al.*, 1987, Pettersson *et al.*, 2007, Krajacic *et al.*, 2010). While results from chapter 2 do not further elucidate the role of the RtST in functional recovery, they do indicate that the observed treatment effect on reaching performance was not accompanied by treatment-induced changes in the RtST projection pattern directly below the lesion. The question whether RtST plasticity might have occurred at different spinal levels remained unanswered. Because the experiment in chapter 2 did not include an unlesioned control group, it is also unclear whether the injury itself might have induced changes in the RtST projection pattern, which may have mediated a limited degree of spontaneous recovery irrespective of experimental group. The following experiment was designed to address these questions by investigating the projection pattern of RtST collaterals across multiple spinal segments in injured compared to uninjured animals.

4.2. Introduction

Cervical spinal cord injury (SCI) results in the disruption of descending pathways important for motor control of the extremities. One such pathway, which has been the focus of intense research efforts to reorganize the injured spinal cord (Oudega and Perez, 2012), is the corticospinal tract (CST), the

main pathway to contribute to fine motor control of the distal arm and hand in humans (Schieber, 2007). In rats, the injured CST has repeatedly been reported to sprout at cervical level in response to thoracic SCI, thereby building novel connections with lesion-bridging relay neurons that may then transmit the signal to denervated neurons below the injury (Fouad *et al.*, 2001, Bareyre *et al.*, 2004). In contrast, CST sprouting rostral to a cervical lesion was only reported when rehabilitative training was added during the recovery period (Girgis *et al.*, 2007). Yet, recent investigations have led to the conclusion that corticospinal plasticity alone might not suffice to mediate the observed degree of recovery following training in forelimb motor tasks (Krajacic *et al.*, 2010).

A tract well suited to contribute to the recovery of reaching in rats is the rubrospinal tract (RST; Kanagal and Muir, 2009, Morris *et al.*, 2011). Interestingly, lesions that involve both the CST and the RST still allow substantial spontaneous and training-induced recovery (Krajacic *et al.*, 2010). As a consequence, the reticulospinal tract (RtST), which projects mainly in the ventrolateral funiculus (Waldron and Gwyn, 1969, Zemlan *et al.*, 1984, Martin *et al.*, 1985), has gained attention as another potential contributor to the recovery of forelimb function after CST lesions in cats and primates (Pettersson *et al.*, 2007). Reticular neurons receive cortical input and have been shown to project to spinal motoneurons that control a variety of upper extremity muscles (Davidson and Buford, 2006, Riddle *et al.*, 2009). RtST axons commonly regenerate more readily than CST axons (Xu *et al.*, 1995,

Vavrek *et al.*, 2007) and exhibit the ability to sprout and rewire following SCI (Ballermann and Fouad, 2006). In light of these findings and because the RtST projects within a large portion of the ventral and ventrolateral white matter (Waldron and Gwyn, 1969, Zemlan *et al.*, 1984, Martin *et al.*, 1985), and is therefore mostly spared by dorsolateral lesions, the RtST might prove useful in efforts to improve motor control of the upper limb following ablation of the dorsal CST. To promote new connections between the CST and RtST in the brainstem in the present study, we injected adeno-associated viral vectors (AAV) expressing brain-derived neurotrophic factor (BDNF; to promote collateral growth) and neurotrophin-3 (NT-3; to serve as a chemo-attractant to direct CST sprouting towards the reticular formation). GFP-expressing vectors were used as a control. Viral vector-mediated neurotrophin treatment did not promote observable plasticity or recovery. Yet, limited spontaneous recovery irrespective of AAV treatment was evident, which has been suggested to involve RtST function (Krajacic *et al.*, 2010), (Pettersson *et al.*, 2007). Whether this potential contribution of the RtST to recovery also includes re-organization of the tract's projection pattern is currently unknown. To investigate this, RtST projection patterns were analyzed above and below the C4 injury level using 3 different outcome measures. AAV-treated, injured animals were pooled and compared to AAV-treated, uninjured controls to examine solely the effect of injury on RtST plasticity.

4.3. Materials and methods

4.3.1. Animals and experimental groups

A total of 26 female Lewis rats (Charles River Laboratories, Canada) weighing 180 g – 200 g were group housed at a 12h :12 h light/ dark cycle. Twenty-one animals received a unilateral incomplete cervical SCI. In an effort to promote plasticity, these animals received the following injections of adeno-associated viral vectors (AAV) of serotype 2: AAV expressing brain-derived neurotrophic factor (BDNF) in the motor cortex and neurotrophin-3 (NT-3) in the reticular formation (n = 7), AAV expressing the non-pharmacologically active green-fluorescent protein (GFP) in the motor cortex and NT-3 in the reticular formation (n = 7) or GFP-expressing AAV into both locations (n = 7). Five control animals remained unlesioned but received BDNF-expressing AAV into the motor cortex and NT-3 expressing AAV into the reticular formation to provide a close control condition for detecting an injury-induced effect. Three animals remained unlesioned and did not receive AAV injections. Some animals had to be excluded from individual histological analyses because data could not be obtained from a sufficient number of sections meeting the necessary quality standards (e.g., tears or folding of tissue, which would produce significant artifacts in densitometry measures). The number of animals included in each analysis is indicated in brackets in the respective results sections (4.4.). Animals were fed ad libitum except for the day preceding single pellet reaching sessions when food pellets were

reduced to 8 g/ rat. Weights were closely monitored to assure stable body weight over time. All procedures involving animals were approved by the Health Sciences Animal Care and Use Committee at the University of Alberta.

4.3.2. Spinal cord injury and AAV injection

Animals in the injured groups received a dorsolateral quadrant spinal lesion unilateral to their preferred paw (as established during reaching training). Rats were anesthetized by a subcutaneous injection of Fentanyl (0.2 mg/ kg, Hypnorm, Janssen Pharmaceutics, Beerse, Belgium) mixed with Midazolam (4 mg/ kg, Versed, Sabex, Boucherville, QC, Canada). The surgical area was shaved and disinfected and the animal mounted into a stereotactic frame (Kopf Instruments, Tujunga, CA, USA). Throughout the surgery, body temperature was maintained at 37 °C with a heating blanket. Following a skin incision and dissection of muscle, the spinal cord between C3 and C4 was exposed with a laminectomy of half a vertebral segment (C3). A custom-made microblade was lowered 1 mm into the spinal cord at the midline, then moved lateral. Muscle layers were sutured and the skin was closed with staples.

In injured as well as in 5 uninjured animals, the brain was accessed via two drill holes to allow injection of AAV vectors. The forelimb motor cortex contralateral to lesion was targeted at coordinates 1.5 mm anterior and 1.5 mm lateral to bregma, 1.5 mm below the dura. The gigantocellular nucleus of the reticular formation ipsilateral to lesion was targeted at 2.8 mm

caudal and 0.8 mm lateral to bregma, at a depth of 9.2 mm. One µl of the respective AAV in solution was slowly pressure injected into each location at 13-25 psi using a custom-made glass electrode connected to a picospritzer. The incision was then closed with stitches. Post-operative hydration was ensured by s.c. injection of 4 ml saline and pain was managed by s.c. injections of buprenorphine (0.05 mg/kg, Temgesic, Schering-Plough, QC, Canada). Animals were kept on a heating blanket until fully awake.

4.3.3. Single pellet reaching

Before lesion surgery, all animals assigned to injured groups were trained to reach through a slot (1.5 cm wide) in a Plexiglas box (15 x 36 x 30 cm) to grasp sugar pellets offered to them in a small indentation on a tray (pellet 2 cm away from front wall at a height of 3 cm above the elevated grid floor). In addition, the rats were taught to go back to the other end of the box before the next pellet was offered. Success rates per session were calculated as the percentage of pellets successfully grasped and eaten out of 20 pellets offered. Starting at day 7 post injury (p.i.), all animals were tested in reaching twice per week. Performance scores before the injury, at the beginning of reaching testing p.i. and at the end of the recovery period were determined by selecting the best performance for each animal out of 3 consecutive sessions.

4.3.4. Horizontal ladder

Rats were videotaped while crossing an elevated horizontal ladder with rungs (1.5 mm in diameter) randomly spaced between 2 cm and 5 cm apart (Bolton *et al.*, 2006). Before lesion surgery, animals were familiarized with the task and then 3 ladder crossings were videotaped with a JVC digital camera and analyzed frame-by-frame on a computer screen for baseline scores. Final testing was done the same way at the end of the recovery period. An error made by the preferred forepaw was defined as a fall or a deep slip with either the rat losing balance or the paw dropping underneath the rung level up to the point of the carpal/tarsal joint. Error rate was calculated by averaging the score of 3 ladder crossings and expressed as percentage of erroneous steps made by the preferred/injured paw out of the total number of steps taken to cross the ladder.

4.3.5. Cylinder test

At the end of the recovery period, rats were filmed with a JVC digital camera as they explored the walls of a clear Plexiglas cylinder (24 cm high, 19 cm inner diameter; (Schallert *et al.*, 2000). Spontaneous usage of either forepaw was analyzed frame-by-frame on a computer screen by counting how many times either paw touches the wall of the cylinder during ten rearings. The sum of paw touches was used to calculate to what percentage the ipsilateral paw was engaged in exploration.

4.3.6. Tracing

After a recovery time of 6 weeks, the forelimb motor cortex contralateral to the spinal lesion was injected at 3 locations with 1 µl of Alexa Fluor 488 (10 %, Molecular Probes, Eugene, OR, USA) at a depth of 1.5 mm under isoflurane anesthesia. Neurons in the gigantocellular division of the reticular formation in the brainstem were traced by injecting 1 µl of Fluororuby (10 %, Molecular Probes, Eugene, OR, USA) at coordinates 2.8 mm posterior and 0.8 mm lateral to lambda, 9.2 mm below the dura, on the side ipsilateral to the spinal lesion. Fluororuby administration was performed by pressure injection with a picospritzer at pulses of 15 msec duration at 13 – 25 psi. The wound was closed with stitches and post-operative care was as described in section 4.3.2. Unlesioned control animals were traced in the same manner.

4.3.7. Perfusion and tissue collection

Two weeks following tracer injection, animals were euthanized by an overdose of pentobarbital (Euthanyl, Biomeda-MTC, Cambridge, ON, Canada) and then perfused with saline followed by 4 % paraformaldehyde containing 5 % sucrose. Brains and spinal cords were dissected, kept in formalin solution for 24 hours and subsequently transferred into a 30 % sucrose solution for 2 to 4 days. Pieces of tissue to be cut on a cryostat were mounted on filter paper using Tissue Tek (Sakura Finetek, Torrance, CA, USA), and were frozen in Methylbutane at -50 °C. Tissue was stored at -80 °C until

sectioned. The cervical spinal cord was cross-sectioned at 25 µm at spinal level C1 to C3, at lesion level (C4) and immediately below the lesion at C5. The brainstem was sectioned at 35 µm thickness to allow efficient data collection and analysis along a long rostro-caudal extent.

4.3.8. Cresyl violet staining and lesion assessment

Slides with sections of the spinal lesion were dehydrated at 37 ºC for 1 hr to ensure attachment to the slides. They were then rehydrated 3 x 10 min in TBS, followed by incubation in 0.1 % Cresyl Violet for 3 min. Excess stain was removed by 5 dips in ddH_2O. Tissue was serially dehydrated in ascending concentrations of ethanol for 1 min each, cleared 1 min in Xylene and then coverslipped using Permount (Fisher Scientific, Ottawa, ON, Canada). Maximal lesion extent was reconstructed onto a schematic cross section by examining cresyl violet stained cross sections spanning the full length of the lesion using light microscopy (Leica Microsystems, Concord, ON, Canada; Fig. 4.2.A). Lesion size was quantified using Image J and is expressed as lesioned area as a percent of the full cross sectional area of the schematic.

4.3.9. Immunohistochemistry

Brainstem sections as well as C2 and C3 spinal cord cross sections were stained with DAB against AF 488 (CST tracer) for assessment of CST collateral sprouting. Slides were dehydrated at 37 ºC for 1 hr prior to washing 2 x 10 min in TBS. After two additional washes in TBS-TX, slides

were incubated with blocking solution containing 10 % NGS for 2 hrs at room temperature. Then, anti-AF 488 antibody (rabbit IgG, Molecular Probes, Eugene, OR, USA) was applied at 1:750 in TBS and slides were incubated at 4 °C overnight. The next day, slides were washed 2 x 10 min in TBS and the Vecta Kit goat-anti-rabbit antibody (Vector Laboratories, Burlington, ON, Canada) was applied according to kit instructions and incubated at 4 °C overnight. On the third day, slides were washed 2 x 10 min in TBS before Vectastain ABC solution was applied according manufacturer's instructions for an incubation of 2 hrs at room temperature. Following 3 x 10 min washes in TBS, DAB (Vector Laboratories) was applied on each slide and the reaction was stopped in distilled water as soon as the desired colour intensity was reached. Slides were washed 3 x 10 min in TBS, dehydrated in ascending concentrations of alcohol (2 min each), immersed 2 x 2 min in Xylene and coverslipped using Permount (Fisher Scientific, Ottawa, ON, Canada).

4.3.10. Quantification of tracing

For normalization purposes, traced CST and RtST axons were counted in the spinal cord. This allows the quantification of tracing efficacy of fibers descending in the spinal cord and allows us to take into account the well-documented tracing variability. CST axons were counted in a picture collage of one DAB stained brainstem and one C1 cross section per animal at 400 x magnification with a Leica microscope. Quantification of traced reticulospinal

axons on both sides of the spinal cord was performed in picture collages of C1 and C5 cross sections taken under fluorescent light at 100 x.

4.3.11. Quantification of CST collaterals in the brainstem and the spinal cord

The combined BDNF and NT-3 treatment was aimed at promoting corticospinal connections with the reticular formation at the pyramidal level. Because the RtST is mostly spared by the lesion and because more than half of reticular neurons project ipsilaterally in the spinal cord, we expressed NT-3 in the ipsilateral reticular formation. Thus, traced CST collaterals (in the yet uncrossed pyramidal tract contralateral to injury) attracted by NT-3 need to cross the brainstem midline in order to reach the NT-3-rich target area. To quantify these collaterals, all traced CST collaterals crossing the ventral portion of the midline (along a 850 µm vertical line) were counted in 10 DAB stained brainstem sections at intervals of 315 µm for each animal. The sum of counted fibers was then normalized to the amount of traced fibers in the pyramids and final numbers are expressed as percentage of fibers traced.

In 10 DAB-stained sections each at level C2 and C3, collaterals emerging from the dorsal CST and crossing into the adjacent grey matter were counted at intervals of about 500 µm. The sum of crossing collaterals was then normalized to the amount of traced CST fibers at C1 for each animal.

4.3.12. Quantification of reticulospinal collaterals

The number of traced RtST collaterals crossing the grey-white matter interface was counted in 10 spinal cross sections per animal at level C1 as well as level C5, and in 5 cross sections per animal each at level C2 and C3 (covering a total distance of about 1 mm or 500 µm, respectively, per level analyzed; Fig. 4.1.A, B). The different number of sampled sections arises because larger blocks of tissue could be cross-sectioned for C1 and C5. To avoid counting the same fiber twice, sections were sampled at regular intervals. The sum of counted collaterals was then normalized to the number of traced fibers on the respective side of the spinal cord either rostral or caudal to the injury. Thus, collateral numbers at C1 to C3 were normalized to traced axons at C1, and collaterals below the lesion were normalized to axon counts at C5. This takes into account that reticulospinal fibers might have been lesioned. The resulting value allows for a more accurate comparison with the unlesioned condition below the lesion level. RtST collaterals crossing the spinal cord midline within the grey matter, previously described in the cat (Peterson *et al.*, 1975), were quantified similarly (Fig. 4.1.G). The sum of midline-crossing fibers counted in 10 cross sections per animal and level was normalized to the sum of traced fibers of both sides at the corresponding spinal level (levels above injury were normalized to C1, values below the injury were normalized to C5 tracing counts).

4.3.13. Densitometry of reticulospinal fibers within the grey matter

To quantify reticulospinal projections in the grey matter, we measured the density of fluorescent tracer signal in 3 areas of interest on each side at spinal level C1, C2, C3 and C5. Microscopic images were taken at 100 x, converted into an 8-bit black and white image and then identically thresholded in Image J (Fig. 4.1.C, D). The threshold setting was chosen to amplify the signal in a way that axons faintly visible in the fluorescent image would still be detected in the black and white image, while at the same time limiting background and artifacts to a minimum. Laminae VII / IX and VIII were deemed areas of interest as reticulospinal projections are known to terminate in these areas in the cervical cord (Zemlan *et al.*, 1984), Fig. 4.1.E, F). Lamina X in close vicinity to the central canal was of interest as an additional measure to support the counting of midline-crossing fibers (Fig. 4.1.E, F). In Image J, a frame in the shape of a circle of identical dimension for all analyses was placed over each lamina of interest. Signal density was noted for each region ipsi- as well as contralateral to the traced side of the spinal cord in 8 cross sections per animal at level C1 and C5, and in 4 sections per animal at level C2 and C3 (covering a total distance of about 800 µm or 400 µm, respectively, per level analyzed). This number of sampled sections arises from the need to analyze sections at intervals to avoid measuring one projection fiber multiple times, combined with the need for excellent tissue quality to minimize artifacts in the densitometry analysis. Density values

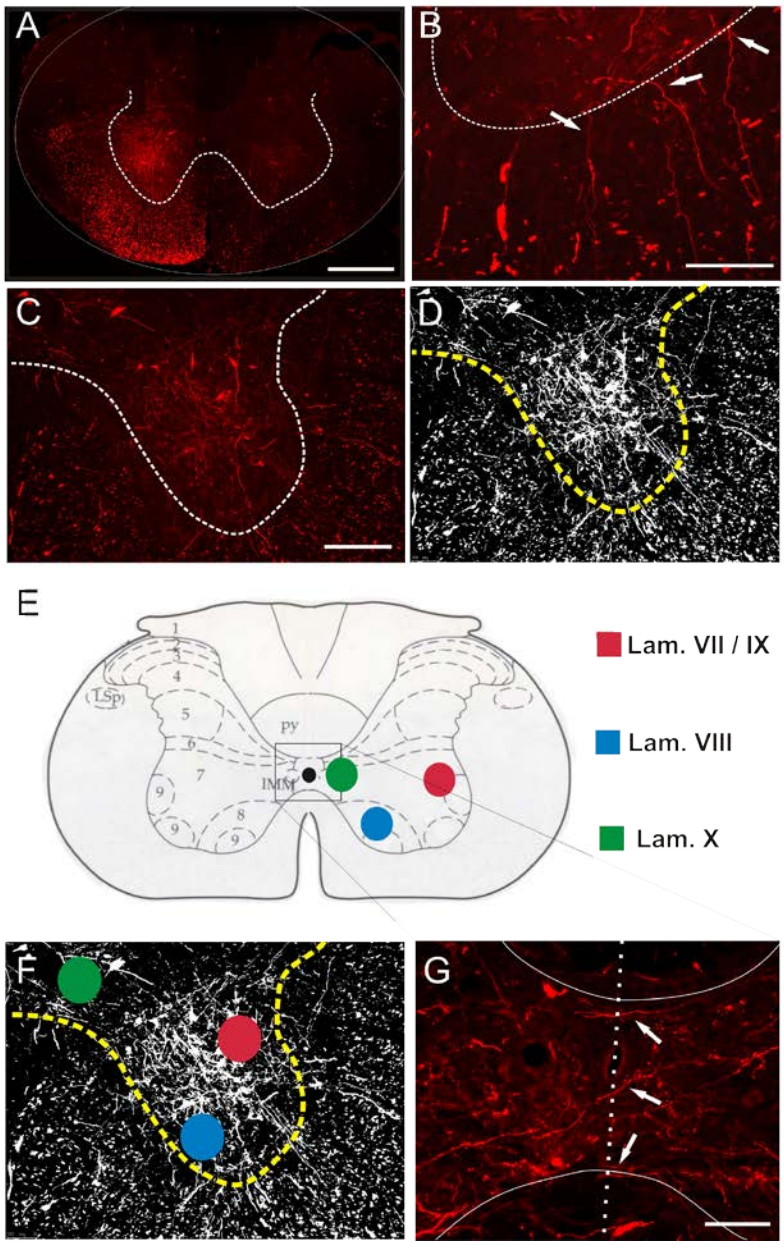


Figure 4.1. Methods of analysis for RtST projections.

Traced RtST projections at level C1 in a representative cross section demonstrate the strong ipsilateral (here left) projection of the tract (**A**, scale bar = 500 μm). Dashed line indicates the white-grey matter interface along which collaterals entering the grey matter were counted. White arrows in a magnified cross sectional area including the ventral horn and ventral white matter mark RtST collaterals entering the grey matter (**B**, scale bar = 100 μm). Pictures of fluorescent RtST

tracing signal in the ventral horn and intermediate laminae were taken for densitometry (**C**, scale bar = 200 μm). These raw pictures were then converted into 8 bit images and identically thresholded (**D**, scale bar as in C) before density of the white signal was measured. Densitometry was performed in 3 laminae on each side of the spinal cord grey matter (E, adapted from Paxinos and Watson, 1998). Colour coded circles indicate areas of density measurement in a schematic and a histological cross section (**E, F,** scale bar as in C). RtST collaterals crossing a virtual spinal cord midline through the central canal were counted within the grey matter (**G**, scale bar = 100 μm). Arrows point to crossing collaterals, dashed line indicates midline of spinal section, solid line marks the grey-white matter interface.

were divided by the number of traced fibers at C1 for all sections above injury and at C5 for below the injury. Next, the average of these normalized values per lamina was calculated for each animal. The resulting values for all 3 laminae were finally summed and express the overall density in arbitrary units for each side of the cord.

4.3.14. Statistical analyses

Comparisons between treatment groups were performed using one-way ANOVA. Unpaired student's t-tests were used to compare the lesioned versus the unlesioned condition as well as single pellet reaching recovery. All data are presented as Mean ± SEM. A p value ≤ 0.05 was considered significant (*). A p value < 0.01 was marked (**) and a p value < 0.0001 was marked (***).

4.4. Results

4.4.1. Lesion size and lesioned pathways

Reconstruction of lesion extents in a series of cross sections at spinal level C4 revealed various degrees of damage to the dorsal and lateral portions of the CST as well as to the RST projection among the 21 injured animals (Fig. 4.2.A). All lesions included ablation of sensory pathways in the dorsal white matter, which contributes to deficits in performance in skilled motor tasks. Projection of the ipsilesional RtST was affected to various extents by spreading of the injury into ventrolateral white matter. Even in the animal

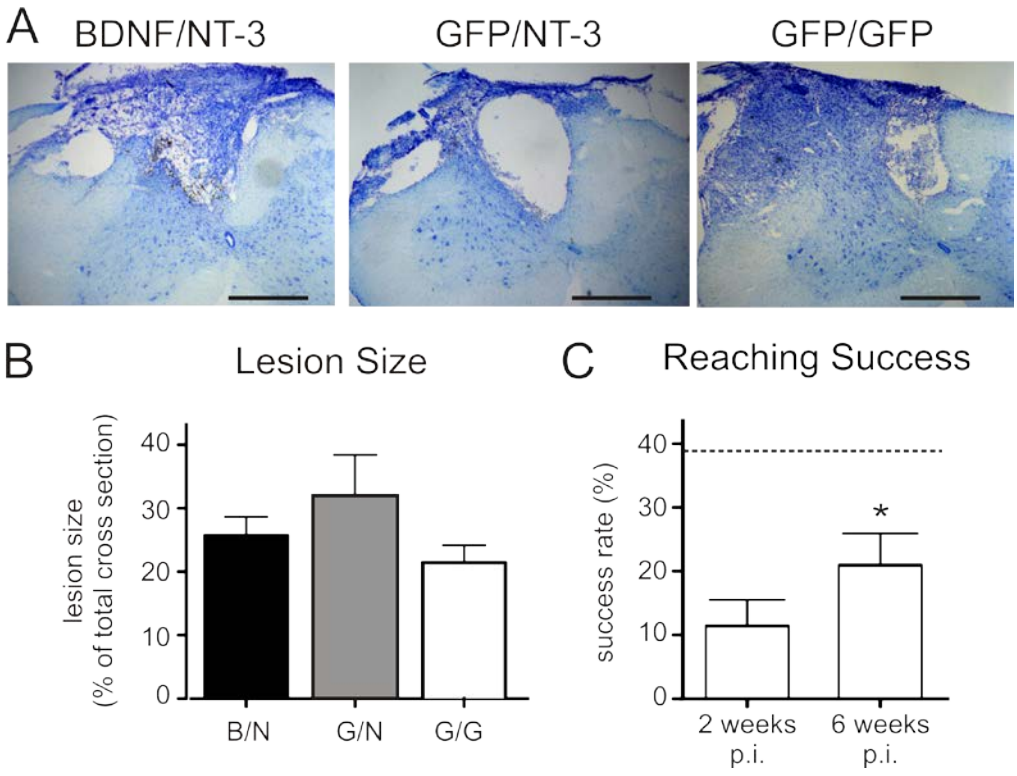


Figure 4.2. Lesion size and functional recovery.

Cresyl-violet stained cross sections of averaged-size lesions demonstrate that average lesion size is similar among the experimental groups (**A**, scale bar = 400μm). Lesion size measured as percent lesioned tissue out of the full cross section is not significantly different across experimental groups **(B)**. B/N: BDNF-expressing AAV to motor cortex, NT-3 expressing AAV to brainstem; G/N: GFP-expressing AAV to motor cortex, NT-3 expressing AAV to brainstem; G/G: GFP expressing AAV into both injection locations. Comparison of the best reaching performance out of 3 consecutive sessions at week 2 post injury (p.i.) versus at the end of the recovery period (week 6 p.i.) of all animals reveals a significant improvement in reaching success **(C)**. Dashed line indicates the average success rate of all animals at baseline. Error bars indicate Standard Error of the Mean, asterisk marks $p < 0.05$.

with the fewest spared traced RtST fibers, 349 traced axons were found projecting within the ipsilesional white matter at C5. Considering that only a fraction of fibers are traced, this represents a substantial number. Thus, no animals were excluded. All measures of RtST projections at level C5 were normalized to the number of spared RtST axons to minimize an effect of lesion size on these values. To ensure that any subsequent analyses of neurotrophin effects are not influenced by differences in lesion size among the various virus-treated groups, we analyzed the lesion sizes of each experimental group and found that they were not significantly different (Fig. 4.2.B).

4.4.2. Motor performance

Single pellet reaching is a demanding task for rats with injured CST and RST fibers. Reaching testing starting at day seven post injury (p.i.) revealed a substantial deficit across all injured animals with an average success rate of 11.43 % ± 4.085 % compared to 39.29 % ± 2.519 % at baseline (n = 21; Fig. 4.2.C). Rats suffering from dorso-lateral quadrant lesions do not usually recover fully without rehabilitative training, yet a limited but significant improvement in reaching success was achieved by week 6 p.i. (20.95 % ± 4.96 %, n = 21; p = 0.048; Fig. 4.2.C). No difference in motor performance was evident in any task at this final time point among the AAV injected groups (Fig. 4.3.)

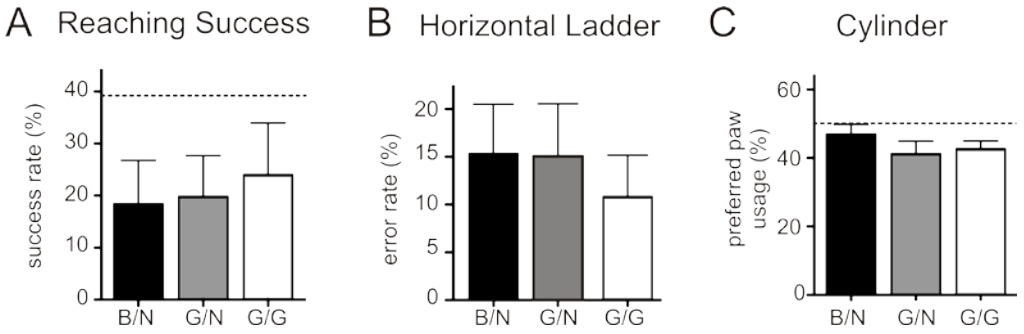


Figure 4.3. Motor performance across experimental groups.

No difference among the groups was evident in the successful reaching for sugar pellets (A, dashed line indicates average baseline performance) or in errors made while crossing a horizontal ladder (B). Spontaneous usage of the preferred forelimb in the cylinder shows scores close to baseline in all experimental groups (C). Dashed line indicates baseline score of 50 % for the preferred paw. Error bars indicate Standard Error of the Mean. B/N = BDNF to cortex, NT-3 to brainstem; G/N = GFP to cortex, NT-3 to brainstem; G/G = GFP to cortex and to brainstem.

4.4.3. Collateral projection of the injured CST above the lesion

Functional recovery following incomplete cervical SCI has frequently been associated with an increase in collateral sprouts emanating from the injured dorsal CST and entering the grey matter rostral to the lesion site. No differences in such collateral sprouting were evident among the neurotrophin-treated groups or between injured and uninjured control animals (injured n = 20, uninjured n = 4; Fig. 4.4.A). To test whether vector-mediated neurotrophin expression led to an increase in CST collaterals at the level of the pyramids (contralateral to injury) projecting towards the reticular formation (ipsilateral to injury), we counted midline-crossing CST collaterals within the brainstem and found no effect of AAV injection (Fig. 4.4.B). Therefore, the observed spontaneous recovery in reaching was not accompanied by increased collateral sprouting of the injured CST at the level of the brainstem or in the spinal cord at level C2.

4.4.4. RtST plasticity above and below the lesion

Because there was no effect of vector-mediated neurotrophin treatment on any of the investigated behavioural tests or anatomical plasticity readouts (including all RtST plasticity analyses reported from here on, see Fig. 4.4.C-E for representative results from level C2), pooling all AAV-injected, spinal cord lesioned animals was considered admissible. To investigate a potential effect of injury alone on RtST plasticity, 5 uninjured control animals were injected

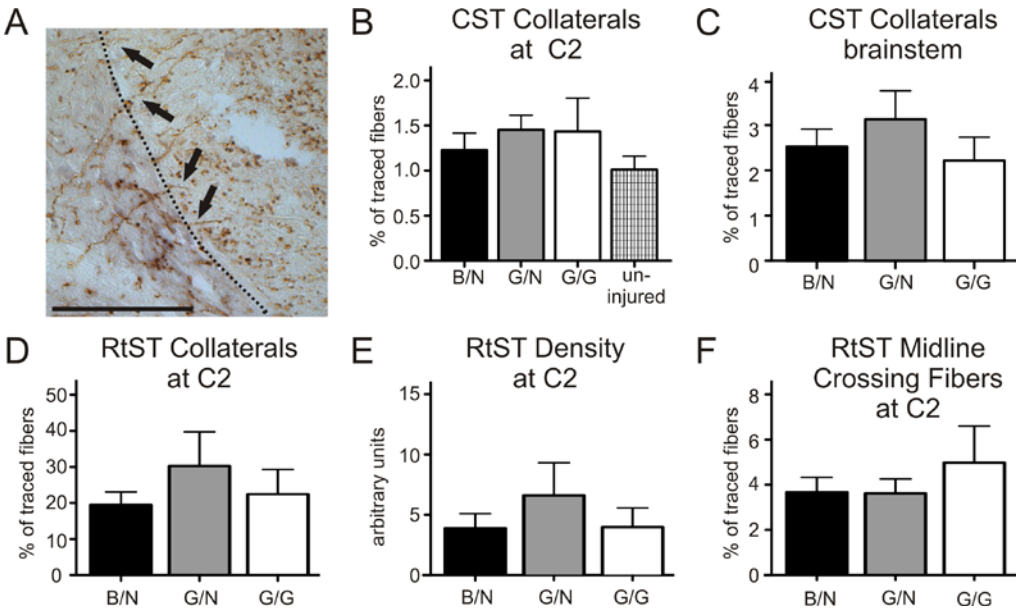


Figure 4.4. CST and RtST plasticity following neurotrophin treatment.

CST collaterals entering the grey matter (indicated by arrows) were counted in cross sections at spinal level C2 (**A**, scale bar = 100 μm). Neither the number of injured CST collaterals entering the grey matter rostral to injury at level C2 **(B)**, nor the number of midline-crossing (contralateral) pyramidal collaterals projecting to the (ipsilateral) reticular formation in the brainstem showed an effect of neurotrophin treatment **(C)**. Likewise, neither the number of contralateral RtST collaterals entering the grey matter at C2 **(D)**, nor the density of RtST fibers in the contralateral grey matter at this level **(E)**, nor the number of RtST fibers crossing the midline **(F)** reveal any differences among the experimental groups. Similarly, no changes were observed in any RtST analysis at other segments of the cord (not shown). Error bars indicate Standard Error of the Mean. B/N = BDNF to cortex, NT-3 to brainstem; G/N = GFP to cortex, NT-3 to brainstem; G/G = GFP to cortex and to brainstem.

with the same AAV-2 constructs (expressing BDNF and NT-3, respectively; see section 4.3.1.) as injured animals to provide the best control condition possible. This uninjured control group is referred to from this point forward in text and figures. Three additional uninjured control animals were not injected with AAV-2, and were not found to be significantly different from AAV-injected counterparts in measures of RtST projections (except for having more midline-crossing fibers at level C5, data not shown). Reticulospinal projections were analyzed using 3 different outcome measures at spinal levels C1 to C3 above the lesion, and at level C5 below the lesion. Collaterals entering the grey matter were counted on each side of the cord, density of traced RtST fibers was measured in 3 laminae on each side of the grey matter, and finally midline-crossing RtST fibers within the grey matter were quantified.

RtST projections at C1: No injury-induced changes in RtST collateral count or density were seen at C1 ipsilateral to injury or across the midline (Fig. 4.5.A, C; Fig. 4.7. A, B). However, on the contralateral side, slightly fewer collaterals were seen crossing into the grey matter (45.13 % ± 5.34 % for injured n = 20 vs. 71.2 % ± 12.02 % for uninjured n = 4), and density within the grey matter decreased significantly in injured animals compared to uninjured controls (4.63 ± 0.63 for injured n = 21 vs. 10.26 ± 4.31 for uninjured n = 5; $p = 0.025$; Fig. 4.5.B; Fig. 4.7.A, B).

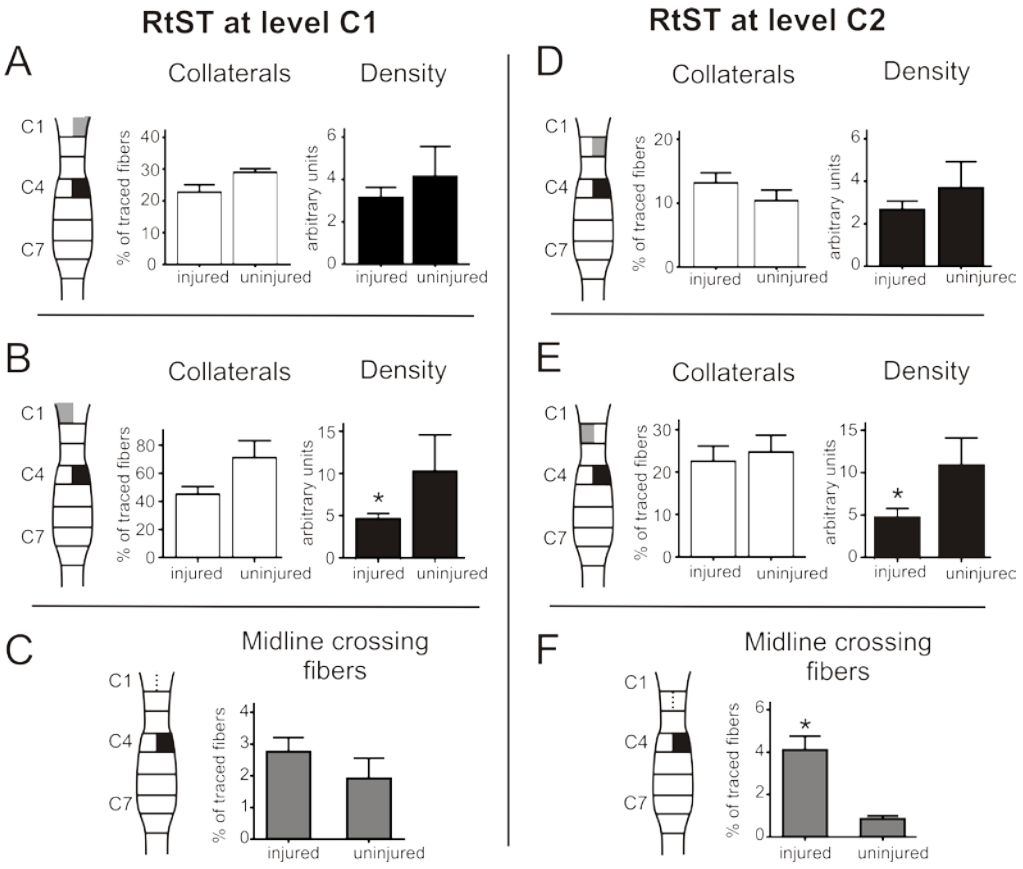


Figure 4.5. Injury-induced RtST plasticity at level C1 and C2.

Areas of analysis are indicated in the schematics of the spinal cord, either in grey **(A,B,D,E)** or with a dashed line **(C,F)**. At level C1, no changes with injury were detected in RtST projections ipsilateral to the lesion **(A)** or in the number of midline crossings **(C)**. Contralateral to the lesioned side, a significant drop in RtST fiber density was evident in injured animals **(B)**. At level C2, no changes were detected in RtST projections ipsilateral to the lesion in injured compared to uninjured animals **(D)**. In the contralateral grey matter, density decreased significantly with injury **(E)**, while the number of midline- crossing fibers increased **(F)**. All values are normalized to the number of traced RtST fibers at level C1. Error bars indicate Standard Error of the Mean, asterisks indicate $p < 0.05$.

RtST projections at C2: Similar to C1, no significant changes were detected in RtST projection patterns ipsilateral to injury (Fig. 4.5. D; Fig. 4.7.A, B). In the contralateral grey matter, RtST fiber density dropped significantly with injury (4.72 ± 1.05 for injured n = 20 vs. 10.88 ± 3.22 for uninjured n = 5; p = 0.027; Fig. 4.5.E; Fig. 4.7.A), whereas an increase was observed in midline-crossing RtST fibers (4.11 % ± 0.65 % for injured n = 19 vs. 0.85 % ± 0.15 % for uninjured n = 5; p = 0.02; Fig. 4.5.F; Fig. 4.7.C).

RtST projections at C3: The region directly rostral to the injured segment of the spinal cord did not show many changes in RtST projection patterns as an effect of injury (Fig. 4.6.A, B; Fig. 4.7.A, B). There was a significant increase in collaterals entering the contralateral grey matter (11.22 % ± 1.55 % for injured n = 19 vs. 21.54 % ± 4.49 % for uninjured n = 5; p = 0.012; Fig. 3.6.B; Fig. 4.7.B) and a relatively small but significant increase in midline-crossing fibers (1.80 % ± 0.24 % for injured n = 19 vs. 0.77 % ± 0.16 % for uninjured n = 5; p = 0.041; Fig. 4.6.C; Fig. 4.7.C).

RtST projections at C5: The segment directly below the spinal cord lesion displayed the greatest re-arrangements in RtST projections, both in degree and location. Significant decreases were measured in injured animals for collaterals entering the grey matter on both sides (ipsil.: 18.5 % ± 1.88 % for injured n = 21 vs. 47.83 % ± 9.15 % for uninjured n = 5; p < 0.0001; Fig. 4.6.D; Fig. 4.7.B; contral.: 15.75 % ± 1.14 % for injured n= 19 vs. 53.10 % ± 10.73 % for uninjured n = 5; p < 0.0001; Fig. 4.6.E; Fig.4.7.B), as well as for density of RtST fibers in the ipsilateral grey matter (1.57 ± 0.22 for injured n = 19 vs.

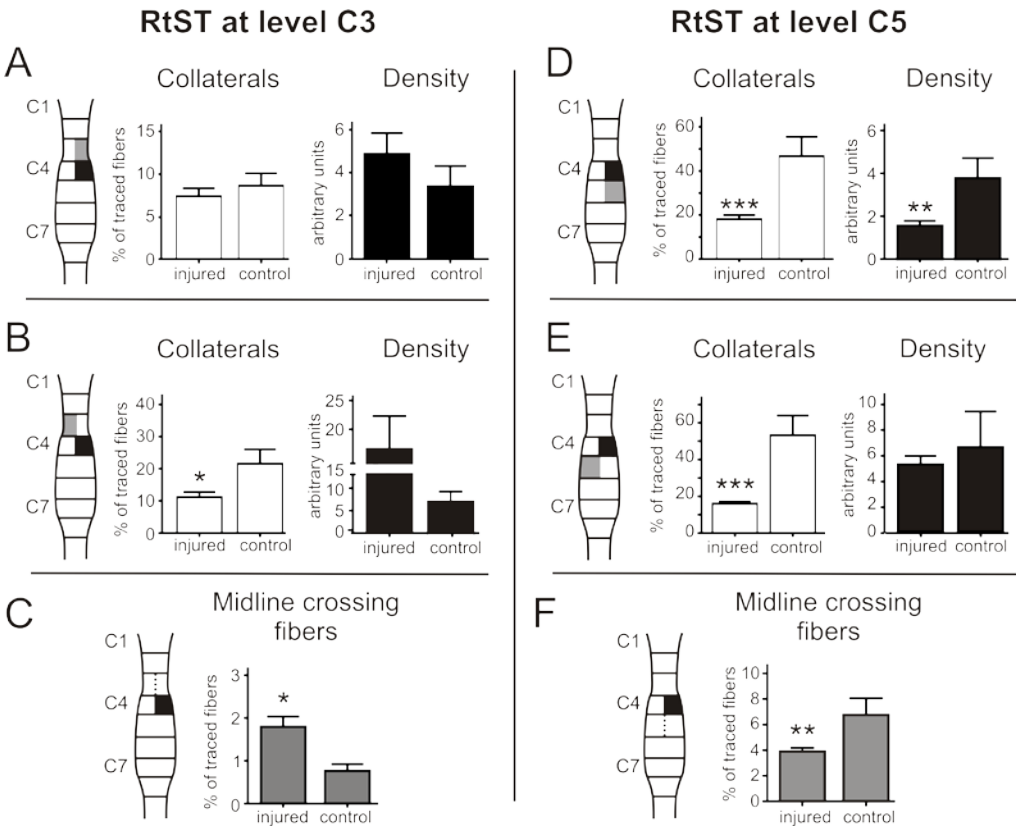


Figure 4.6. Injury-induced RtST plasticity at level C3 and C5.

Areas of analysis are indicated in the schematics of the spinal cord, either in grey **(A, B, D,E)** or with a dashed line **(C,F)**. At level C3, injury-induced changes in RtST density or number of collaterals entering the grey matter were not observed for either side of the cord except for collateral numbers on the contralateral side **(A, B)**. A small but significant increase in midline-crossing RtST fibers was evident **(C)**. All values are normalized to the number of traced RtST fibers at level C1. Ipsilateral to injury at C5, both the number of RtST collaterals entering grey matter and the density of RtST fibers within the grey matter were significantly decreased in injured animals compared to uninjured controls **(D)**. On the opposite side of the cord, only the drop in collaterals in the injured condition reached significance **(E)**. Also, the amount of midline-crossing RtST fibers at C5 was lower after injury compared to intact controls **(F)**. All values are normalized to the number of traced RtST fibers at C5. Error bars indicate Standard Error of the Mean, single asterisk indicates $p < 0.05$, double asterisks indicates $p < 0.01$, triple asterisks indicates $p < 0.0001$.

3.79 ± 0.93 for uninjured n = 5; p = 0.0018; Fig. 4.6.D; Fig. 4.7.A). Numbers of midline-crossing fibers reversed in comparison to above injury levels and dropped significantly in injured animals compared to intact controls (3.91 % ± 0.28 % for injured n = 20 vs. 6.75 % ± 1.32 % for uninjured n = 5; p = 0.0023; Fig. 4.6.F; Fig. 4.7.C). To exclude the possibility that these surprisingly strong results were influenced by different efficacy of RtST tracing in injured versus uninjured animals, we compared the numbers of traced fibers between the two groups. The number of traced axons proved to be similar between the groups (results not shown).

In summary, although some loss of RtST fibers in the grey matter and an increase in midline-crossing fibers occur rostral to injury, the majority of changes in RtST projection are seen directly below the injured spinal segment (Fig. 4.7.). Contrary to the frequently reported increase in axonal collaterals with injury, this cervical injury induces a marked reduction in all RtST parameters measured below the injury. Loss of traced fibers within the tract at C5 due to the severity of the upstream lesion is controlled for by normalization of all values measured below lesion to the number of traced fibers at level C5.

To characterize these changes below the injury further, we looked at the RtST signal density in laminae VII / IX, VIII and X individually (Fig. 4.8.). Ipsilateral to injury, RtST fiber density dropped significantly in laminae VIII, where collaterals from the ventral white matter were observed to enter the grey matter frequently (p = 0.0003; injured n = 18, uninjured n = 5; Fig. 4.8.A).

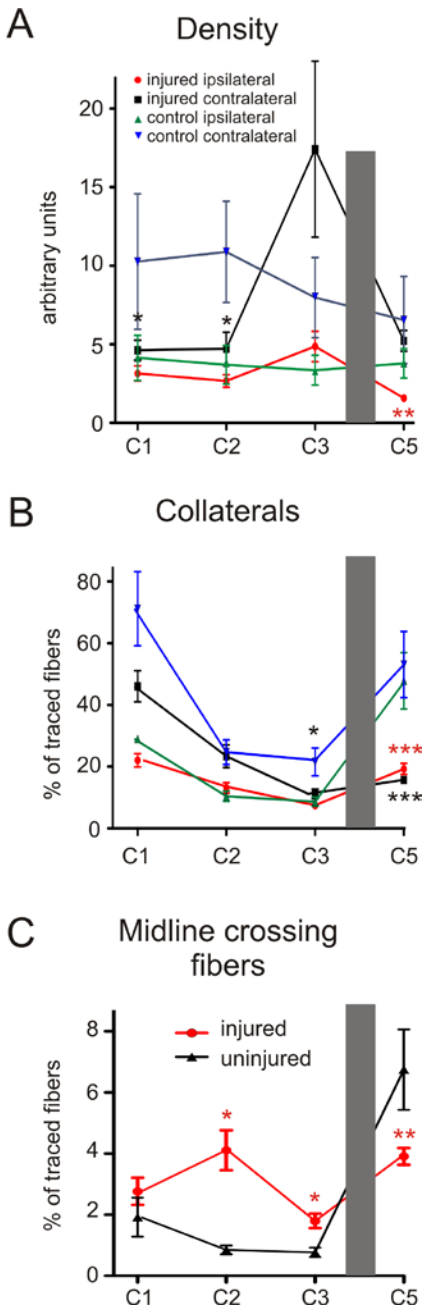


Figure 4.7. Overview of injury-induced changes in RtST projections at all levels analyzed.

Comparisons were made between the injured and uninjured condition on the respective side of the cord. Grey bar represents lesioned segment. Error bars indicate Standard Error of the Mean, single asterisk indicates $p < 0.05$, double asterisks indicates $p < 0.01$, triple asterisks indicates $p < 0.0001$.

No significant change was detected for lamina VII / IX, where dense networks of RtST fiber clusters were commonly found. A significant decline in RtST fiber density in ipsilateral lamina X, adjacent to the midline, is consistent with a decrease in midline-crossing fibers at this level (p = 0.0027; injured n = 18, uninjured n = 5; Fig. 4.6.F; Fig. 4.8.A). On the contralateral side of the spinal cord, only minor decreases in density were found across the laminae (injured n = 19, uninjured n = 5; Fig. 4.8.B).

4.5. Discussion

In order to investigate anatomical responses of the RtST to SCI, we characterized its projections across five different spinal levels using 3 different outcome measures. Injured animals were pooled together from 3 groups that were injected with AAV expressing neurotrophins in an effort to promote plasticity of the CST and RtST. Such pooling was considered admissible as no effect of vector-mediated neurotrophin treatment was recorded in any of the behavioural or histological analyses. We did not pursue quantification of neurotrophin expression because such analysis is unreliable in perfused tissue and because the concentration necessary to promote sprouting has not yet been defined. Furthermore, the lack of effect of this neurotrophin treatment is in line with previously reported data (Weishaupt *et al.*, 2013). To provide the best possible uninjured control condition for investigating injury-induced RtST re-organization, 5 uninjured

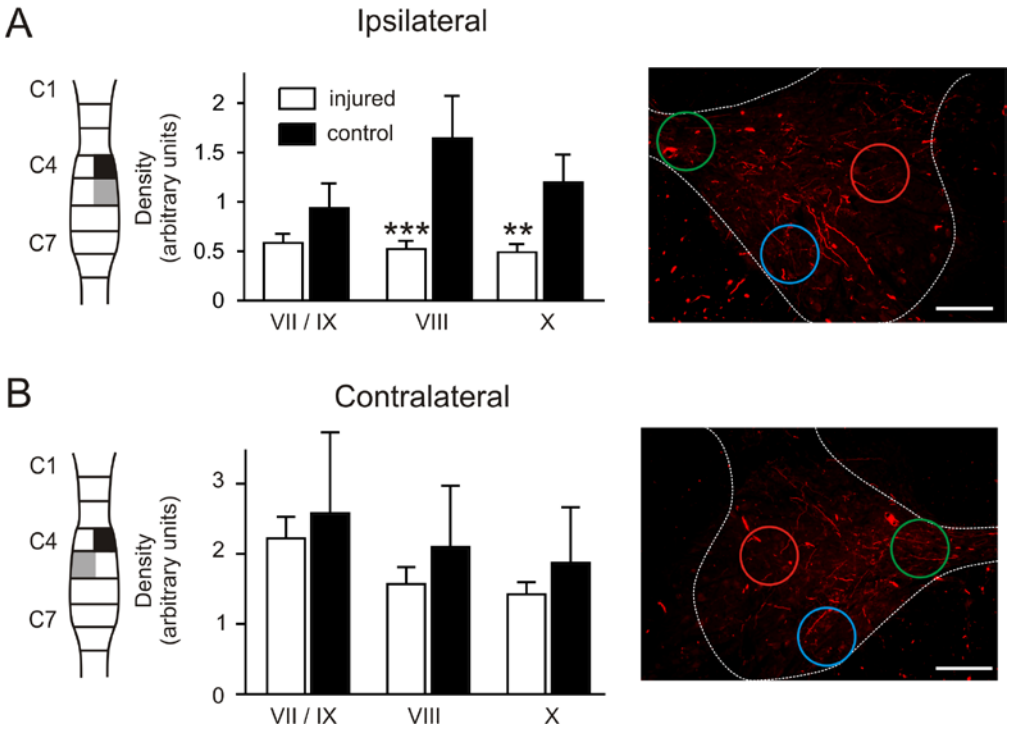


Figure 4.8. Changes in RtST fiber density in different laminae at level C5.

Significant decreases in density with injury were detected in laminae VIII and X ipsilateral to the lesion **(A)**. The 3 areas in which density was measured are indicated by colour-coded circles (red: Lam. VII / IX, blue: Lam. VIII, green: Lam. X, scale bar = 200 µm) in a representative picture of the ipsilateral grey matter used for densitometry. Densities were similar across all laminae in the contralateral grey matter and no effect of the SCI on RtST fiber density was evident **(B)**. The areas in which density was measured on the contralateral side are indicated by colour-coded circles as in A in a representative picture used for densitometry (scale bar = 200 µm). All values are normalized to the number of traced RtST fibers at level C5. Error bars indicate Standard Error of the Mean, double asterisks indicates $p < 0.01$, triple asterisks indicates $p < 0.0001$.

animals received AAV injections of vectors expressing both neurotrophins. This is to ensure that differences between injured and uninjured animals demonstrate an effect of injury. The RtST projection pattern analyzed in 3 naïve, uninjured animals was similar to that of AAV-injected uninjured controls.

Compensatory sprouting in various pathways including the RtST, has frequently been associated with functional recovery following SCI (Weidner et al., 2001, Raineteau *et al.*, 2002, Bareyre et al., 2004, Ballermann and Fouad, 2006, Courtine *et al.*, 2008, Rosenzweig *et al.*, 2010). Considering the potential role of the RtST in recovery it is therefore somewhat counterintuitive that we do not find evidence for similar anatomical RtST plasticity. We instead demonstrate a significant decline in RtST projections below the SCI, consistent across 3 different outcome measures. To our knowledge, such a significant loss of axonal collaterals has not yet been described in the literature. For these reasons, we critically discuss all values at level C5 to assess explanations for and against these results reflecting any potential artifacts.

First, we consider the number of traced fibers at C5 as a potential factor contributing to the high difference in values between the groups. Although the normalization procedure is identical for all animals, a significant difference in the number of traced fibers between the injured and uninjured animal groups might still influence the overall outcome. Comparing the raw numbers of traced fibers, we find that the number of

traced fibers at C5 is similar between the two groups, excluding this factor from potentially contributing to the statistical differences. The RtST is known for its diversity in fiber origin, projection and transmitter content. In the cat, distinct reticular neuron populations have been defined to project preferably to rostral cervical segments versus more caudal segments such as C4 and below (Peterson *et al.*, 1975). It is therefore theoretically possible that in a subset of experimental animals, a different population of reticulospinal fibers was traced, partly accounting for the loss of projection within the grey matter. We consider this possibility as highly unlikely, based on the results of cervical levels C1 to C3. At these levels, no such discrepancy in reticulospinal projection between the experimental groups is evident.

Second, we consider the power of our analysis. At level C5, the sample size per group is at least 19 animals for all analyses of the injured condition and exactly 5 animals for the uninjured condition. The finding that RtST projections are reduced at this level is reflected in four out of five data sets (the difference in grey matter density on the contralateral side did not reach significance), and thus is a very robust finding across the different outcome measures.

Third, we consider potential explanations for the differences at level C5 with regards to data collected at the other spinal levels across groups. In uninjured animals, more collaterals project in the grey matter at level C5 than at more rostral levels. These projections are reduced following injury to a level that is similar to the innervation of levels rostral to the injury. While

reticulospinal branching within the grey matter has been characterized in the cat (Peterson *et al.*, 1975), the amount of RtST axonal collaterals crossing the white-grey matter interface has to our knowledge not yet been reported in detail for rodents. We thus have to rely on the measurements taken here.

Fourth, we found a few fluorescent cell bodies in the grey matter of each of the analyzed spinal cord sections. This is likely the result of the TMR tracer also retrogradely labeling neurons. This introduced a small error, consistent in both groups and at every level of analysis. However, a significant change in RtST projections was found only at C5. It is highly unlikely that this change is due to an artifact at this level.

After having consolidated our main findings with regards to potential experimental errors, we would now like to consider potential explanations for the loss of RtST projections below the injury. Decreases in RtST fiber density are more prominent within laminae of the ipsilateral spinal cord side, an observation that potentially points towards a causal difference in pathological processes between the ipsilateral spared RtST and the contralateral intact projection.

First, it is conceivable that Wallerian Degeneration of axons severed by the lesion may spread into adjacent regions of the white matter (Hausmann, 2003, Hagg and Oudega, 2006). We would therefore expect a greater impact of this secondary damage on the side ipsilateral to the SCI, while the contralateral white matter may still be largely devoid of degenerative and inflammatory processes (Wang *et al.*, 2009, Weishaupt *et*

al., 2010). We may hypothesize that inflammatory processes adjacent to surviving fibers may somehow negatively regulate their growth, leading to reduction in fibers specifically on the ipsilesional side. One caveat is that secondary damage and subsequent degeneration of fibers could lead to a decrease in the number of viable RtST axons projecting in the white matter at level C5. We controlled for this potential degeneration by normalizing all values to the number of traced RtST fibers at the level analyzed.

Second, remodeling processes within the grey matter, as well as partial denervation of neurons that the RtST connected with may account for a decrease in RtST innervation. Denervated neurons that are significantly less active due to deprivation of CST and RST input may affect synaptic pruning and die-back of reticulospinal fibers, in analogy to what is known about activity-dependent plasticity in the visual system (Hubel and Wiesel, 1963a/b, Wiesel and Hubel, 1963). Because some reticulospinal fibers cross the midline to innervate the contralateral spinal cord, any reduction in axonal collaterals on the ipsilateral side (Peterson *et al.*, 1975) might have an effect on the activity of cells on the contralateral side. If reduced target cell activity were confirmed to be a major contributor to the loss of reticulospinal fibers, the key to further improvement of recovery may lie in the stimulation of input-deprived target cells to rescue remaining synapses.

Third, we have to take into account the differential effect of pruning of excitatory versus inhibitory reticulospinal inputs to grey matter cells (Du Beau *et al.*, 2012). It is known that sub-populations of reticulospinal fibers

contain inhibitory transmitters. A selective pruning of inhibitory inputs may overall increase the activity of denervated neurons and may thereby potentially contribute to a reorganization of the spinal cord below the injury that is overall beneficial with regards to motor recovery.

Taken together, the most significant injury-induced changes in RtST projection found in this study indicate a loss of projections in the grey matter especially below the lesion. However, reorganization of spinal RtST networks may not be limited to the remodeling of RtST fibers observed here. Other plastic changes in RtST connections not detectable by gross fiber anatomy may still have occurred in response to injury. The same principle applies to the CST. Although increased CST sprouting rostral to the injury was not detected in this study, we cannot exclude that the CST played a role in mediating the observed recovery because we did not re-lesion the tract. Such re-lesion studies have provided mixed results (Weidner *et al.*, 2001, Krajacic *et al.*, 2010). It is important to keep in mind that CST sprouting may have developed at different locations (Weidner *et al.*, 2001), and plasticity other than axonal sprouting may still have occurred (e.g., collateral branching, synaptic plasticity, neuronal excitability; Oudega and Perez, 2012). The finding that spontaneous recovery was not accompanied by an increase in collaterals for either the injured dorsal CST or the spared RtST in this study raises the question of what the underlying mechanisms of the observed recovery may be. Unraveling these mechanisms warrants further study of different levels of plasticity, such as synaptic plasticity and excitability. Also,

the question arises of how axonal sprouting and other anatomical measures of plasticity relate to functional effects (Whishaw *et al.*, 2008, Oudega and Perez, 2012, Zaaimi *et al.*, 2012). Making inferences on meaningful plasticity from collateral counts alone may not be feasible without further supportive evidence, such as results from re-lesions or electrophysiology.

4.6. References

Alstermark B, Lundberg A, Pettersson LG, Tantisira B, Walkowska M. Motor recovery after serial spinal cord lesions of defined descending pathways in cats. Neurosci. Res. 1987;5(1):68-73.

Ballermann M, Fouad K. Spontaneous locomotor recovery in spinal cord injured rats is accompanied by anatomical plasticity of reticulospinal fibers. Eur. J. Neurosci. 2006;23(8):1988-96.

Bareyre FM, Kerschensteiner M, Raineteau O, Mettenleiter TC, Weinmann O, Schwab ME. The injured spinal cord spontaneously forms a new intraspinal circuit in adult rats. Nat. Neurosci. 2004;7(3):269-77.

Bolton DA, Tse AD, Ballermann M, Misiaszek JE, Fouad K. Task specific adaptations in rat locomotion: runway versus horizontal ladder. Behav. Brain Res. 2006;168(2):272-9.

Courtine G, Song B, Roy RR, Zhong H, Herrmann JE, Ao Y, et al. Recovery of supraspinal control of stepping via indirect propriospinal relay connections after spinal cord injury. Nat. Med. 2008;14(1):69-74.

Davidson AG, Buford JA. Bilateral actions of the reticulospinal tract on arm and shoulder muscles in the monkey: stimulus triggered averaging. Exp. Brain Res. 2006;173(1):25-39.

Du Beau A, Shakya Shrestha S, Bannatyne BA, Jalicy SM, Linnen S, Maxwell DJ. Neurotransmitter phenotypes of descending systems in the rat lumbar spinal cord. Neuroscience. 2012;227C:67-79.

Fouad K, Pedersen V, Schwab ME, Brosamle C. Cervical sprouting of corticospinal fibers after thoracic spinal cord injury accompanies shifts in evoked motor responses. Curr. Biol. 2001;11(22):1766-70.

Girgis J, Merrett D, Kirkland S, Metz GA, Verge V, Fouad K. Reaching training in rats with spinal cord injury promotes plasticity and task specific recovery. Brain. 2007;130(Pt 11):2993-3003.

Hagg T, Oudega M. Degenerative and spontaneous regenerative processes after spinal cord injury. J. Neurotrauma. 2006;23(3-4):264-80.

Hausmann ON. Post-traumatic inflammation following spinal cord injury. Spinal Cord. 2003;41(7):369-78.

Hubel DH, Wiesel TN. Receptive Fields of Cells in Striate Cortex of Very Young, Visually Inexperienced Kittens. J. Neurophysiol. 1963;26:994-1002.

Kanagal SG, Muir GD. Task-dependent compensation after pyramidal tract and dorsolateral spinal lesions in rats. Exp. Neurol. 2009;216(1):193-206.

Krajacic A, Weishaupt N, Girgis J, Tetzlaff W, Fouad K. Training-induced plasticity in rats with cervical spinal cord injury: effects and side effects. Behav. Brain Res. 2010;214(2):323-31.

Martin GF, Vertes RP, Waltzer R. Spinal projections of the gigantocellular reticular formation in the rat. Evidence for projections from different areas to laminae I and II and lamina IX. Exp. Brain Res. 1985;58(1):154-62.

Morris R, Tosolini AP, Goldstein JD, Whishaw IQ. Impaired arpeggio movement in skilled reaching by rubrospinal tract lesions in the rat: a behavioral/anatomical fractionation. J. Neurotrauma. 2011;28(12):2439-51.

Oudega M, Perez MA. Corticospinal Reorganization after Spinal Cord Injury. J. Physiol. 2012; 590 (Pt 16):3647-63.

Peterson BW, Maunz RA, Pitts NG, Mackel RG. Patterns of projection and braching of reticulospinal neurons. Exp. Brain Res. 1975;23(4):333-51.

Pettersson LG, Alstermark B, Blagovechtchenski E, Isa T, Sasaski S. Skilled digit movements in feline and primate--recovery after selective spinal cord lesions. Acta. Physiol. (Oxf). 2007;189(2):141-54.

Raineteau O, Fouad K, Bareyre FM, Schwab ME. Reorganization of descending motor tracts in the rat spinal cord. Eur. J. Neurosci. 2002;16(9):1761-71.

Riddle CN, Edgley SA, Baker SN. Direct and indirect connections with upper limb motoneurons from the primate reticulospinal tract. J. Neurosci. 2009;29(15):4993-9.

Rosenzweig ES, Courtine G, Jindrich DL, Brock JH, Ferguson AR, Strand SC, et al. Extensive spontaneous plasticity of corticospinal projections after primate spinal cord injury. Nat. Neurosci. 2010;13(12):1505-10.

Schallert T, Fleming SM, Leasure JL, Tillerson JL, Bland ST. CNS plasticity and assessment of forelimb sensorimotor outcome in unilateral rat

models of stroke, cortical ablation, parkinsonism and spinal cord injury. Neuropharmacology. 2000;39(5):777-87.

Schieber MH. Chapter 2 Comparative anatomy and physiology of the corticospinal system. Handb. Clin. Neurol. 2007;82:15-37.

Vavrek R, Pearse DD, Fouad K. Neuronal populations capable of regeneration following a combined treatment in rats with spinal cord transection. J. Neurotrauma. 2007;24(10):1667-73.

Waldron HA, Gwyn DG. Descending nerve tracts in the spinal cord of the rat. I. Fibers from the midbrain. J. Comp. Neurol. 1969;137(2):143-53.

Wang L, Hu B, Wong WM, Lu P, Wu W, Xu XM. Glial and axonal responses in areas of Wallerian degeneration of the corticospinal and dorsal ascending tracts after spinal cord dorsal funiculotomy. Neuropathology. 2009;29(3):230-41.

Weidner N, Ner A, Salimi N, Tuszynski MH. Spontaneous corticospinal axonal plasticity and functional recovery after adult central nervous system injury. Proc. Natl. Acad Sci. USA. 2001;98(6):3513-8.

Weishaupt N, Li S, Di Pardo A, Sipione S, Fouad K. Synergistic effects of BDNF and rehabilitative training on recovery after cervical spinal cord injury. Behav. Brain Res. 2013;239:31-42.

Weishaupt N, Silasi G, Colbourne F, Fouad K. Secondary damage in the spinal cord after motor cortex injury in rats. J. Neurotrauma.

2010;27(8):1387-97.

Whishaw IQ, Alaverdashvili M, Kolb B. The problem of relating plasticity and skilled reaching after motor cortex stroke in the rat. Behav. Brain Res. 2008;192(1):124-36.

Wiesel TN, Hubel DH. Effects of Visual Deprivation on Morphology and Physiology of Cells in the Cats Lateral Geniculate Body. J. Neurophysiol. 1963a;26:978-93.

Wiesel TN, Hubel DH. Single-Cell Responses in Striate Cortex of Kittens Deprived of Vision in One Eye. J. Neurophysiol. 1963b;26:1003-17.

Xu XM, Guenard V, Kleitman N, Aebischer P, Bunge MB. A combination of BDNF and NT-3 promotes supraspinal axonal regeneration into Schwann cell grafts in adult rat thoracic spinal cord. Exp. Neurol. 1995;134(2):261-72.

Zaaimi B, Edgley SA, Soteropoulos DS, Baker SN. Changes in descending motor pathway connectivity after corticospinal tract lesion in macaque monkey. Brain. 2012;135(Pt 7):2277-89.

Zemlan FP, Behbehani MM, Beckstead RM. Ascending and descending projections from nucleus reticularis magnocellularis and nucleus reticularis gigantocellularis: an autoradiographic and horseradish peroxidase study in the rat. Brain Res. 1984;292(2):207-20.

Zemlan FP, Kow LM, Pfaff DW. Analgesia after lesions of nucleus reticularis magnocellularis: differential effect on supraspinal versus spinal pain reflexes. Pain. 1984;18(3):221-37.

CHAPTER 5

Training following unilateral cervical spinal cord injury in rats affects the contralesional forelimb

Adapted from N. Weishaupt, R. Vavrek, K. Fouad (2013)

Neuroscience Letters

5.1. Preface

Once feasible strategies to rewire the injured CST are at hand, they will most certainly be combined with rehabilitative training in the clinic. Yet, although rehabilitative training is often a daily routine for individuals with SCI, mechanisms and limitations of training are still not fully understood. One limitation of task-specific training is demonstrated in chapter 2. A significant improvement in single pellet reaching performance with cortical BDNF treatment and reaching training did not translate in better motor performance on the horizontal ladder or in more symmetrical limb use in the cylinder test. Other reports indicate that task-specific training may even come at the expense of performance in non-trained tasks (De Leon *et al.*, 1998, Girgis *et al.*, 2007, Garcia-Alias *et al.*, 2009). Developing training strategies therefore seems similar to making investment decisions, as available neuronal networks appear to be committed solely to the trained task. Unfortunately, promoting recovery of ipsilesional skilled forelimb function in incomplete spinal cord injured rats beyond the limited spontaneous recovery observed in chapter 4 is generally difficult to achieve, especially across various tasks (chapter 2). Therefore, a training investment in the performance of the less affected forelimb, which may have more intact neuronal networks available for motor control, may be worth considering. The following experiment was designed to tackle the question whether the less affected forelimb may benefit from training, and whether training

involving both forelimbs will impact the performance level of the less affected forelimb due to competitive use of neuronal circuits.

5.2. Introduction

Currently, rehabilitative training is the most widely used and likely the most effective strategy to improve motor recovery in individuals with spinal cord injury (SCI; Scholtes *et al.*, 2012). Yet, many important details as to how the effectiveness of training protocols can be maximized remain poorly studied. While the limited translation of improvements after task-specific training into untrained tasks in animal models of SCI as well as in affected humans has received increasing attention (Grasso *et al.*, 2004, Smith *et al.*, 2006, Magnuson *et al.*, 2009), recent reports from animal models suggest that task-specific training may even negatively influence performance in untrained tasks (Garcia-Alias *et al.*, 2009). For example, cats trained to walk on a treadmill did not reach a similar performance level as control animals in a standing test, whereas cats trained to stand performed considerably poorer at walking (De Leon *et al.*, 1998). Further evidence that task-specific training may adversely impact performance in untrained tasks comes from rat models of incomplete cervical SCI (Girgis *et al.*, 2007). Here, cervically injured rats trained in a reaching task performed worse at walking across a horizontal ladder than untrained animals.

The observation that training-induced improvements in task-specific motor recovery often come at the cost of performance in untrained tasks suggests that training induces a task-specific rewiring of neural circuits in the central nervous system. If training leads to the commitment of neural networks to produce a certain functional outcome, the circuitry that remains available for untrained tasks may be limited. Based on this concept, how much should training be focused on the circuitry primarily affected by an incomplete injury? This question should be considered in a bigger picture than just the affected limb(s). The idea that neuronal circuitry not directly involved in the primary injury can be affected by an injury to the central nervous system has recently been described (Starkey *et al.*, 2012).

After incomplete SCI, it is conceivable that spared circuitry may mediate compensatory movements to increase the overall functional versatility in every-day life. Considering the emerging evidence for negative aspects of task-specific rewiring after SCI, a critical view on a rehabilitation concept focused solely on the affected extremity may be warranted for individuals with incomplete cervical SCI. Here, we address the question whether training involving the contralesional forelimb (CF) may be beneficial in overall performance and how training restricted to the primarily affected forelimb (ipsilesional forelimb = IF) following unilateral incomplete SCI can impact performance of the CF. This will contribute to defining interactions between training and spared neuronal circuitry on a behavioural level. Knowledge of these interactions will be crucial for designing an optimized

training regime to achieve maximal motor function in the clinical setting, taking into account both extremities.

5.3. Materials and methods

5.3.1. Animals and experimental groups

47 female Lewis rats weighing 180-200g were group housed and kept at a 12 h: 12 h light/ dark cycle. They were fed ad libitum except for the day preceding a reaching session when food was reduced to 10 g / rat for all rats across all experimental groups. Weights were closely monitored to ensure weight stability over time. Forty-eight hours after lesion surgery, forelimb deficits on the side of the lesion were subjectively categorized upon close observation of overground walking (light: paw placement deficits subtle; moderate: obvious paw placement deficits, involving the wrist joint; severe: forelimb placement deficits involving the forearm and affecting walking). Based on this score, animals were assigned to 3 groups to ensure equal distribution of deficit severities. One group (n = 16) received post-injury (p.i.) training in single pellet reaching (SP group), a second group (n = 16) received p.i. training in the horizontal ladder task (HL group) and a third group (n = 15) received training in both tasks (double task group DT). The study includes two identically designed experiments with 24 and 23 animals each to allow for adequate training intensity/duration (see design and timeline in

Fig. 5.1.A). All experimental procedures were approved by the University of Alberta Health Science Animal Care and Use Committee.

5.3.2. Spinal cord injury

All animals received a cervical dorsal quadrant spinal lesion unilateral to their preferred paw (as established during pre-injury reaching training). Once rats were anesthetized with isoflurane, their body temperature was maintained at 37 °C with a heating blanket throughout the surgery. After exposure of the spinal cord between C3 and C4, a custom-made microblade was lowered 1 mm into the spinal cord at the midline and then moved laterally to create a unilateral dorsal quadrant lesion. Finally, muscle layers were sutured and the skin was closed with staples. Post-operatively, hydration was restored by saline injections and pain was managed by s.c. injections of buprenorphine (0.05 mg/kg, Temgesic, Schering-Plough, Kirkland, QC, Canada). Animals were kept on a heating blanket until fully awake.

5.3.3. Single pellet reaching

Before lesion surgery, all animals were trained to reach through a slot (1.5 cm wide) in a Plexiglass box (15 x 36 x 30 cm) to grasp sugar pellets offered to them in a small indentation on a tray (pellet 2 cm away from front wall at a height of 3 cm above the elevated grid floor). In addition, the rats were taught to go back to the other end of the box before the next pellet was offered.

Success rates per session were calculated as the number of pellets successfully grasped and eaten during a 10 minute period. Starting on day seven after surgery, animals in groups SP and DT received one 10 minute session of reaching training (consisting on average of 25.5 reaching attempts) 5 days per week for 5 weeks. At the same time, animals in the HL group were offered an equal number of pellets on the floor of the reaching box while the slot was closed with tape. Before final assessment of all rats at the end of the recovery period, HL animals were reintroduced to the task in one session that was not included in scoring. Baseline and final testing consisted of 3 consecutive sessions and the best performance for each animal was taken for statistical analysis.

5.3.4. Horizontal ladder

Rats were encouraged to cross an elevated ladder with rungs (1.5 mm in diameter) randomly spaced between 2 cm and 5 cm apart (Bolton *et al.*, 2006). An error was defined as a fall or a deep slip with either the rat losing balance or the paw dropping underneath the rung level at least to the point of the carpal joint. The success rate for each forelimb was calculated by averaging the score of 3 runs and is expressed in percentage of correct steps out of the total number of steps taken to cross the ladder. Before lesion surgery, animals were familiarized with the task and then 3 ladder crossings in each direction were videotaped with a JVC digital camera and analyzed frame-by-frame (30 Hz) on a computer screen for baseline scores. Final

testing was done similarly at the end of the recovery period. Post-injury training of DT and HL groups consisted of 5 ladder runs in each direction per day (consisting on average of 73 steps), 5 days a week for 5 weeks starting on day 7 after injury. At the same time, SP rats were put in a Plexiglass runway with solid flooring to provide a control condition.

5.3.5. Perfusion and tissue collection

At the end of the recovery period, animals were euthanized with an overdose of pentobarbital (Euthanyl, Biomeda-MTC, Cambridge, ON, Canada) and perfused with saline followed by 4 % paraformaldehyde containing 5 % sucrose. Dissected spinal cords were kept in formalin solution for 24 hours and subsequently transferred into a cryoprotective 30 % sucrose solution for two to four days. Pieces of tissue to be cut on a cryostat were mounted on filter paper using Tissue Tek (Sakura Finetek, Torrance, CA, USA) and frozen in methylbutane at -50 °C. Tissue was stored at -80 °C until sectioned.

5.3.6. Lesion assessment

Lesions were reconstructed by examining horizontal sections spanning the full dorsal-ventral extent of the lesion and were then outlined in a schematic cross section. From these reconstructions, the lesion area was measured using Image J and is expressed as the percentage of the injured half of the cross section. Animals with a lesion size of 80 % or more were excluded from

all analyses. Animals with lesion sizes < 15% were probed for reaching deficits following injury and excluded if deficits were negligible.

5.3.7. Statistical analyses

Because lesions in the second experiment were overall significantly larger than in the first experiment (based on a students t-test), which was especially reflected in the lesser ability of rats in the second experiment to reach for sugar pellets, all behavioural scores underwent the following normalization processes: First, individual final reaching success scores and ladder walking success rates were deducted from the individual baseline scores in the respective test for each rat to gain the difference from baseline (p.i. score – baseline score = individual score). Then, the average performance score for all rats within the first and the second experiment, respectively, was determined for each test. All scores stated in the text and shown in graphs represent the individual rat's difference in score from the average of the respective experiment (individual score – average of test and experiment = final score). This normalization allowed for a focus on training group differences rather than on lesion size differences between the two otherwise identical experiments.

All comparisons among the 3 different training groups were made using one-way ANOVA. Statistical significance was awarded if Bonferroni's Post Test resulted in a p value of 0.05 or less. All data is presented as mean ± SEM.

5.4. Results

5.4.1. Lesion assessment

Even a relatively small variation in lesion size can have a great impact on functional recovery and on the ability to achieve training effects. Therefore, 4 animals were excluded from all analyses, two of them based on the predetermined exclusion criterion of a lesion size of more than 80 % of the injured half of the spinal cross section. Two more animals (with the smallest lesion extents) were excluded on the basis that they did not show any obvious deficits in the reaching task after injury. When the two identical experiments (to increase statistical power, see section 5.3.1.) were combined, the resulting group sizes for behavioural results were n = 14 for the SP group, n = 14 for the DT group and n = 15 for the HL group. Without these four excluded animals, lesion sizes in the first experiment ranged from 10.14 % to 32.63 % with an average of 19.33 %, and were overall significantly less severe than lesions in the second experiment, which ranged from 13.27 % to 38.77 % with an average of 23.68 % (p = 0.034; Fig. 5.1.B, C). We also detected distinct variations between the two experiments in functional recovery, especially in the reaching paradigm, which are consistent with our observations of lesion size. Because of this difference between the experiments, obvious also within the experimental groups (Fig. 5.1.D), we decided to pool results into a single analysis. All functional

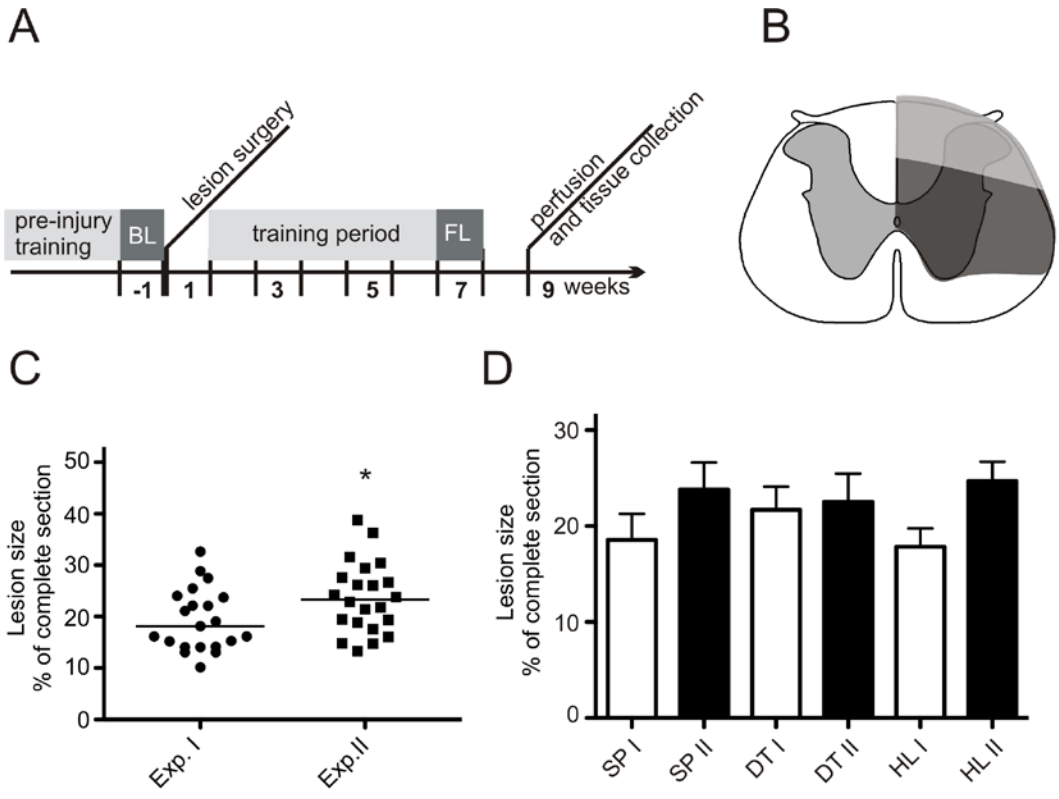


Figure 5.1. Experimental flow and lesion analysis.

(A) Once rats learned how to perform the single pellet reaching task, they were assessed in this task as well as on the horizontal ladder to yield pre-injury baseline levels (BL). Rehabilitative training, specific to training group, started at day 7 following a cervical dorsal quadrant lesion. Animals were trained for 5 weeks, followed by final testing (FL) and perfusion. **(B)** Smallest (light grey) and largest (dark grey) lesion extent are indicated in a schematic spinal cord cross section. **(C)** Overall, lesion sizes (as a percentage of the complete spinal cross section) in the second experiment were significantly larger than lesion sizes in the first experiment (p = 0.034, horizontal lines mark group median). **(D)** Lesion sizes do not significantly differ among training groups (white bars = experiment I, black bars = experiment II). Therefore, we can confidently attribute group differences in recovery to the training regime. SP = single pellet, DT = double training, HL = horizontal ladder. Error bars indicate Standard Error of the Mean, asterisk indicates $p < 0.05$.

performance scores were thus normalized as described in section 5.3.7. Importantly, lesion sizes and the degree to which cortico-, rubro- and reticulospinal tracts were lesioned were comparable among the 3 different training groups so that any differences in functional performance can confidently be attributed to an effect of training rather than lesion variability. To minimize effects of lesion severity, one animal with slight damage to the contralateral dorsal white matter, affecting sensory projections only, was excluded from horizontal ladder analysis of the non-preferred paw (resulting in a group size of 14 for the DT group).

5.4.2. Recovery of single pellet reaching

Single pellet reaching is a demanding task for animals with a unilateral cervical dorsal-quadrant lesion. In the final assessment, only 3 out of 14 animals (21.4 %) and 4 out of 15 animals (26.7 %) were able to successfully grasp the pellet at all in the SP and HL groups, respectively, whereas almost half of the animals in the DT group managed to grasp and eat the pellet (40 %). This indicates that in this experiment single pellet training by itself did not increase the rats' ability to grasp post injury, an unexpected result, but that the added horizontal ladder training was somewhat beneficial for promoting reaching performance. A look at the normalized success rates of all animals, taking into account the degree of grasping performance, does not reveal any statistical differences among the groups, certainly in part due to the high variability (SP = -3.04 ± 6.08, n = 14; DT = 7.44 ± 4.91, n = 15; HL

= -4.61 ± 6.10, n = 15; Fig. 5.2.A). However, a trend for the DT group to perform better than the other two groups is apparent.

5.4.3. Recovery of horizontal ladder walking

Rungs of the horizontal ladder have to be targeted correctly and grasped in a way that supports the animal's weight during stepping to achieve successful, non-erratic walking across the ladder. In this task, deficits of the IF were observed to differing degrees in most animals at final assessment, whereas in some cases in each training group success scores actually reached values above baseline level. This variability prevents the trend for the DT animals' better performance to reach significance (Fig. 5.2.B).

Recovery of motor control for the IF as measured on the horizontal ladder mirrors exactly the results in the single pellet reaching paradigm (Fig. 5.2.A). Normalized performance scores on the ladder similarly suggest a lack of training effect in the HL group and a slight but not significant beneficial effect of double task training on the recovery of the IF. Error rates for the IF across all groups in the first experiment were on average very similar to what has been reported from our laboratory for untrained animals with similar lesions (Krajacic *et al.*, 2010) .

In contrast to this, scores for the CF reveal a significant effect of ladder training only versus added reaching training of the IF (Fig. 5.2.C). It has to be kept in mind here that although the lesion is limited to one side of the spinal cord, the performance of the CF will also be affected by the lesion. For the CF,

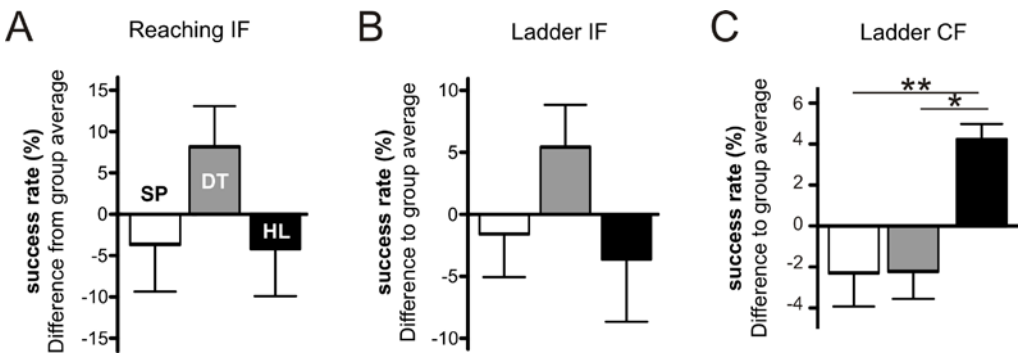


Figure 5.2. Motor performance at final assessment.

White bars = single pellet training only (SP), grey bars = both single pellet and horizontal ladder training (DT), black bars = horizontal ladder training only (HL). **(A)** In the reaching task, there is no significant difference in performance among the training groups. Yet, animals that received training in both tasks tend to benefit to a non-significant degree. **(B)** On the horizontal ladder, results for the ipsilesional forelimb (IF) are comparable to success in the reaching task. Although differences do not reach statistical significance, double trained animals once more tend to perform better. **(C)** The contralesional forelimb (CF) benefits significantly from horizontal ladder only training. Of notice, when single pellet reaching training is added to ladder training, the beneficial effect disappears. Error bars indicate Standard Error of the Mean, single asterisk indicates $p < 0.05$, double asterisks indicate $p < 0.01$.

training restricted to horizontal ladder walking significantly increased the success rate (HL = 3.89 ± 0.80, n = 15) compared to animals that were not trained on the ladder at all (SP = -2.35 ± 1.73, n = 14; $p < 0.01$). This result confirms that ladder training at the intensity used here is sufficient to enhance performance in this specific task at least for the CF. However, a clear interference of added single pellet training with the training effect in the HL group becomes evident (HL = 3.89 ± 0.80, n = 15; DT = -1.82 ± 1.405, n= 14; $p < 0.05$). Thus, the addition of reaching training restricted to the IF seems to prevent the CF from achieving the same level of performance that was reached after ladder training alone.

5.5. Discussion

As animal models and clinical observations reveal previously undefined interactions between trained and untrained movement patterns (Grasso *et al.*, 2004, Girgis *et al.*, 2007, Garcia-Alias *et al.*, 2009), rehabilitative training after SCI might not be as straight forward a process as once thought. Some of these recent reports suggests that training may result in the full commitment of the available neuronal circuitry to the trained task only (Grasso *et al.*, 2004, Girgis *et al.*, 2007), leading to the question of what and paradigms to invest in to optimize functional recovery for every-day life situations (Musselman *et al.*, 2009). Whereas the issue of task-specificity versus more generalized training has received some attention (Garcia-Alias *et al.*, 2009, Musselman *et*

al., 2009), it is currently unknown how much focus on the primarily affected extremity is beneficial to maximize overall functional versatility after incomplete SCI.

In the current study, we address the question whether concentrating rehabilitative training on the ipsilesional extremity following incomplete SCI is beneficial when motor performance of both extremities is taken into account. Our results indicate that the contralesional forelimb (CF) profits more from training when tested in the trained task than the ipsilesional forelimb (IF), similar to what has been reported after a TrkB antibody treatment (Fouad *et al.*, 2010). This holds true even if the IF receives more specific training, such as single pellet reaching training in the present study. These findings suggest that in order for training effects to occur, a certain amount of circuitry needs to be spared. Comparison of lesion characteristics and reaching training scores in a meta-analysis of a large number of animals indicate that the 'threshold lesion' for IF training effects to emerge may be a lesion slightly smaller than the one used in this study, with complete ablation of the dorsal CST and slight damage to the rubrospinal projection (Hurd et al., 2013). This also becomes evident when comparing the limited performance of rats in this study with pyramidotomized, similarly trained rats (Starkey *et al.*, 2011).

If a critical amount of neuronal circuitry needs to be spared to mediate a training effect on recovery, the question arises of how the rewiring of this spared circuitry changes if a different form of training is added. The

present study allows us to examine the addition of a more general motor training to a specific, fine motor training (looking at reaching and ladder walking with the IF), as well as to investigate the effect of added IF training on performance of the CF. While the IF tends to benefit to a small extent from combined training in the single pellet and the horizontal ladder in the present study, added IF-specific training significantly interferes with training improvements on the horizontal ladder for the CF. A potential explanation for the different outcomes for the IF and CF following combined training may be that a significant effect of single task training is necessary to detect any interference from added training involving the respective CF. The interference of added IF-restricted training to general ladder training with motor control of the CF suggests a competition for neuronal circuitries between the two tasks. Because the IF has limited access to descending motor control after the injury, IF-specific training seems to recruit networks involved in motor control of the CF. Therefore, less spared circuitry is available to mediate sophisticated CF performance on the ladder when IF-specific training is added to ladder training. Unfortunately, the added IF training proves to be a poor investment in the study at hand as no significant benefit results from the added training either in performance on the ladder, or in performance in the reaching task. By comparing animals that received only training on the ladder to those that received combined training on the ladder and in reaching (overall more training), we can draw additional conclusions about the plasticity underlying the differences in recovery. For

the CF, more training did not lead to more functional rewiring (because DT animals are not as good at ladder walking as HL animals) but rather, to different rewiring. This is in line with the concept of training-specific rewiring in a situation where the supply of neuronal circuitry to be engaged in plasticity is limited.

5.6. Conclusion

Training-specific rewiring in combination with a limited supply of neuronal circuitry available for rewiring emphasizes the need to make informed decisions when designing rehabilitative training protocols. In the case of incomplete cervical spinal cord injury, the potential task-specific functional improvements of the contralesional extremity should be carefully weighed against the potential of ipsilesional functional recovery in terms of extent and relevance in every-day life situations. In a broader context, the potential of functional compensation mediated by spared circuitry should be carefully weighed against the potential of damaged circuitry to mediate training-induced recovery.

5.7. References

Bolton DA, Tse AD, Ballermann M, Misiaszek JE, Fouad K. Task specific adaptations in rat locomotion: runway versus horizontal ladder. Behav. Brain Res. 2006;168(2):272-9.

De Leon RD, Hodgson JA, Roy RR, Edgerton VR. Full weight-bearing hindlimb standing following stand training in the adult spinal cat. J. Neurophysiol. 1998;80(1):83-91.

Fouad K, Vavrek R, Cho S. A TrkB antibody agonist promotes plasticity following cervical spinal cord injury in adult rats. J. Neurotrauma. 2010, in press.

Garcia-Alias G, Barkhuysen S, Buckle M, Fawcett JW. Chondroitinase ABC treatment opens a window of opportunity for task-specific rehabilitation. Nat. Neurosci. 2009;12(9):1145-51.

Girgis J, Merrett D, Kirkland S, Metz GA, Verge V, Fouad K. Reaching training in rats with spinal cord injury promotes plasticity and task specific recovery. Brain. 2007;130(Pt 11):2993-3003.

Grasso R, Ivanenko YP, Zago M, Molinari M, Scivoletto G, Lacquaniti F. Recovery of forward stepping in spinal cord injured patients does not transfer to untrained backward stepping. Exp. Brain Res. 2004;157(3):377-82.

Hurd C, Weishaupt N, Fouad K. Anatomical correlates of recovery in single

pellet reaching in spinal cord injured rats. Exp. Neurol. Epub ahead of print.

Krajacic A, Weishaupt N, Girgis J, Tetzlaff W, Fouad K. Training-induced plasticity in rats with cervical spinal cord injury: Effects and side effects. Behav. Brain Res. 2010;214(2):323-31.

Magnuson DS, Smith RR, Brown EH, Enzmann G, Angeli C, Quesada PM, et al. Swimming as a model of task-specific locomotor retraining after spinal cord injury in the rat. Neurorehabil. Neural Repair. 2009;23(6):535-45.

Musselman KE, Fouad K, Misiaszek JE, Yang JF. Training of walking skills overground and on the treadmill: case series on individuals with incomplete spinal cord injury. Phys. Ther. 2009;89(6):601-11.

Scholtes F, Brook G, Martin D. Spinal cord injury and its treatment: current management and experimental perspectives. Adv. Tech. Stand. Neurosurg. 2012;38:29-56.

Smith RR, Shum-Siu A, Baltzley R, Bunger M, Baldini A, Burke DA, et al. Effects of swimming on functional recovery after incomplete spinal cord injury in rats. J. Neurotrauma. 2006;23(6):908-19.

Starkey ML, Bleul C, Maier IC, Schwab ME. Rehabilitative training following unilateral pyramidotomy in adult rats improves forelimb function in a non-task-specific way. Exp. Neurol. 2011;232(1):81-9.

Starkey ML, Bleul C, Zorner B, Lindau NT, Mueggler T, Rudin M, et al. Back seat driving: hindlimb corticospinal neurons assume forelimb

control following ischaemic stroke. Brain. 2012;135(Pt 11):3265-81.

CHAPTER 6

Critical discussion of findings

6.1. Challenges of using the neurotrophins NT-3 and BDNF in efforts to rewire the injured corticospinal tract

The literature on NT-3 and BDNF is vast and our knowledge of the neurotrophins keeps growing with new articles being published continuously (Fig. 6.1.). Both neurotrophins have been characterized extensively *in vitro* as well as *in vivo* (reviewed in Vaynman and Gomez-Pinilla, 2005, in Lu *et al.*, 2008, and in Weishaupt *et al.*, 2012) and the cellular signaling pathways they trigger by binding to their respective high and low affinity receptors are known in detail (reviewed in Kaplan and Miller, 2000, and in Minichiello, 2009). Nevertheless, using these neurotrophins as promoters of plasticity for rewiring the injured corticospinal tract, as attempted in the work reported here, is cutting-edge research. If neurotrophins are characterized so well, why is there still so much uncertainty and unpredictability in regards to the effect of BDNF and NT-3 in the injured nervous system? For instance, although treating cortical neurons with BDNF has been repeatedly reported to facilitate CST sprouting, this result was not reproduced in chapter 2. Further, NT-3 has shown remarkable chemo-attractive and growth-promoting effects on CST and other axons (Schnell *et al.*, 1994, Zhou *et al.*, 2003, Alto *et al.*, 2009). Yet these actions of NT-3 were likewise not reliably reproduced in the literature (Hagg *et al.*, 2005, Vavrek *et al.*, 2006) or in chapter 2 and 3. One reason for the inconsistencies in results across all *in vivo* experiments certainly is the sheer complexity of the nervous system.

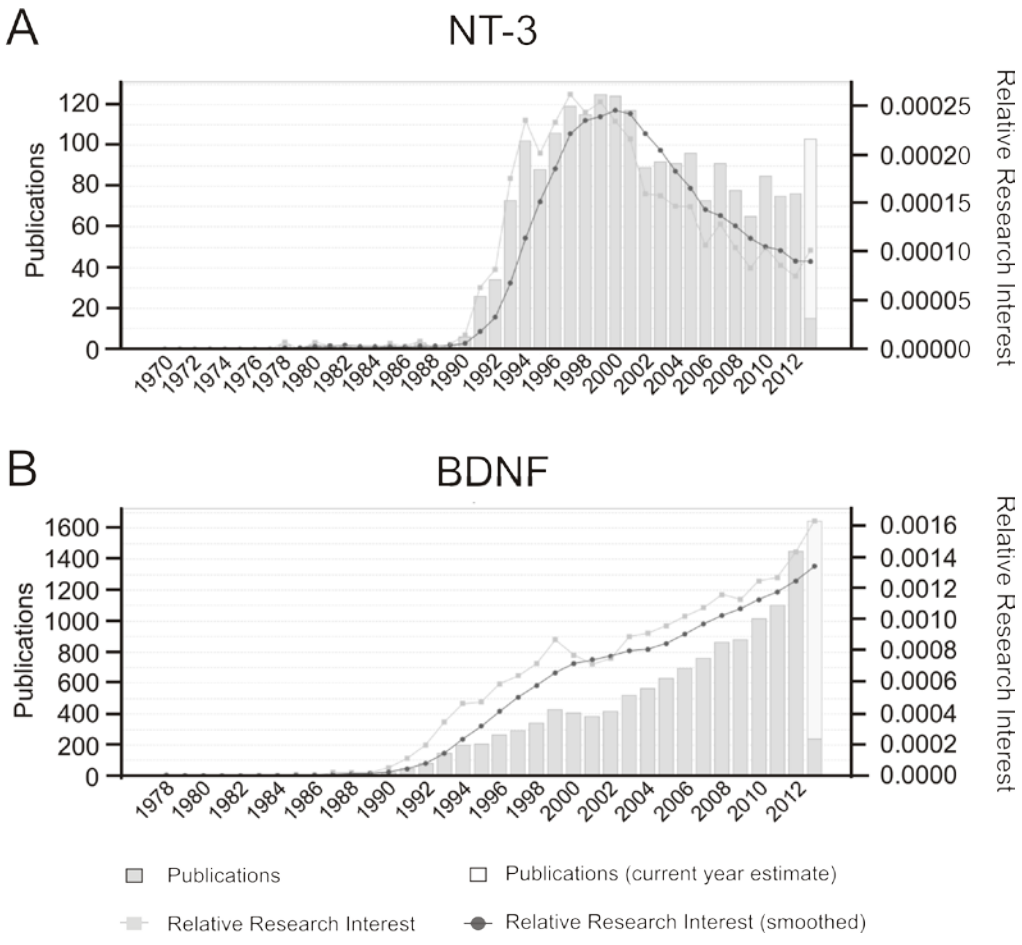


Figure 6.1. Number of annual publications on NT-3 (1970-2013) and on BDNF (1978-2013, adapted from www.gopubmed.com).

Thus, the difficulty to reproduce reported effects of neurotrophins or to translate 'known' neurotrophin actions into slightly different contexts can often stem from seemingly negligible changes in techniques or experimental design. For example, the method of neurotrophin delivery (infusion versus viral vector systems) and the location of neurotrophin delivery/ expression (into the lesion cavity, above or below an SCI) might make the difference between failure and success of treatment. This emphasizes that there is still a lot of unknown territory for understanding how neurotrophins work *in vivo.* The findings in this thesis, achievements and failures of neurotrophin treatment alike, lead to new insights on how we can optimize the use of neurotrophins to improve their reliability and predictability in a system as complex as the CNS.

6.1.1. Optimizing the use of BDNF

BDNF is a multifaceted effector in both the healthy developing (reviewed in Cohen-Cory *et al.*, 2010, and in Park and Poo, 2013) and the mature nervous system (reviewed in Poo, 2001, in Lu *et al.*, 2008, and in Rosas-Vargas *et al.*, 2011), and it seems to be ubiquitous throughout the CNS (Hofer *et al.*, 1990, Yan *et al.*, 1997, Dreyfus *et al.*, 1999). Yet, in states of injury, enhanced signaling activity through BDNF's high-affinity receptor TrkB is beneficial, for example to promote axonal plasticity and regeneration (Kobayashi *et al.*, 1997, Ye and Houle, 1997, Hiebert *et al.*, 2002, Vavrek *et al.*, 2006), or to

change cell intrinsic signaling from an atrophic and degenerative program to a pro-survival and growth-promoting program (reviewed in Kaplan and Miller, 2000, Geremia *et al.*, 2010). What concentration of BDNF is needed to facilitate pro-survival and growth promoting effects in any given context is not exactly known and probably depends on a myriad of factors, not least of which is the density of TrkB and p75 receptors on the surface of the receiving cell (Kwon *et al.*, 2004, reviewed in Lu *et al.*, 2005, Hollis *et al.*, 2009). Two reports of successful promotion of CST sprouting in the cervical enlargement following BDNF infusion into the motor cortex provide an idea of what dose is sufficient to elicit CST sprouting (Hiebert *et al.*, 2002, Vavrek *et al.*, 2006). Both studies infused BDNF into the motor cortex at 0.5 μg/hr over 2 weeks via an osmotic minipump. Using AAV-2 induced BDNF expression in the motor cortex in chapter 2 did not result in similar plasticity. Although BDNF expression in chapter 2 remains unquantified and may well be lower than tissue concentrations of BDNF achieved with infusion of the protein, the neurotrophin was expressed over the full length of the 6-week recovery period in chapter 2. Despite no observable increase in CST sprouting, the addition of rehabilitative training translated BDNF expression into a behavioural effect. This result is suggestive of two concepts, which are important to consider when using BDNF: First, plasticity that mediates recovery may occur on levels other than anatomical growth. BDNF is well known to have excitatory effects predominantly through its high affinity receptor TrkB, which may manifest independent of the promotion of

collateral outgrowth and potentially at a lower concentration BDNF. Second, the result is suggestive of training and neuronal activity somehow influencing BDNF/TrkB signaling.

If training can unmask or substantiate effects of BDNF, then the signaling intensity of BDNF may be amenable to control by training. This becomes important when considering some of the undesired effects that BDNF can have when it is overexpressed. BDNF's role in the development of seizures, hyperreflexia and neuropathic pain is well established (Scharfman, 1997, Zhu and Roper, 2001, reviewed in Pezet *et al.*, 2002, Boyce *et al.*, 2012, Lu *et al.*, 2012). These potential adverse effects of BDNF limit the quantity of the neurotrophin that can be administered safely in experiments and potential future therapeutic use. Hence, an easily controllable up-regulator of BDNF signaling intensity, such as training, is extremely valuable. For a factor like BDNF, the difference between a harmful concentration (Boyce *et al.*, 2012, Lu *et al.*, 2012) and a concentration sufficient to produce a desired effect (Boyce *et al.*, 2012) may be minute. What constitutes a harmful, a sufficient and an insufficient concentration (chapter 1) of BDNF will also likely vary with the tissue environment (healthy versus injured, spinal cord versus brain), making dose finding for BDNF a delicate balancing act. To fully explore the potential of BDNF for nervous system repair, it will be essential to have control over BDNF's local availability and signaling intensity in a timely manner. While training may be one such control mechanism, at least

for increasing BDNF signaling, future endeavors into how BDNF signaling can be tightly controlled are warranted.

6.1.2. Optimizing the use of NT-3

While some reports of NT-3's actions on plasticity in the CNS are remarkable (Schnell *et al.*, 1994, Zhou *et al.*, 2003, Alto *et al.*, 2009), one may also discover studies reporting a lack of an effect of NT-3 when looking thoroughly through the literature (Hagg *et al.*, 2005, Vavrek *et al.*, 2006). Furthermore, the incidence of NT-3 not producing a growth-promoting effect in experimental settings may potentially be grossly underestimated due to the difficulty to publish negative results in scientific journals. Dissecting the conditions under which NT-3 is reported to remain ineffective is important as a means to deduce a potential explanation for why, for example, NT-3 expression is insufficient to promote midline-crossing CST collateral growth in the brainstem, as found in chapter 2. It seems that in order for NT-3 to affect CST axons at a distance, such as across the midline (chapter 2 and 3), other supportive factors have to be at play. While Chen and colleagues narrowed down these so-far elusive factors to being somehow associated with the presence of CD4+ T-cells (Chen *et al.*, 2008), more work is needed to actually identify the process that can promote NT-3-induced CST collateral growth across the spinal cord midline, and potentially even across the

brainstem midline in the future. The systematic study of a potential dose-response relationship may already be a good start to investigate this problem.

Several mechanisms of how immune activation may promote NT-3 induced midline-crossing growth of CST axon collaterals are imaginable. For example, growth factors released by activated immune cells may influence a CST axon's intrinsic growth state (reviewed in Schwartz and Yoles, 2006, Kigerl *et al.*, 2009) and thus make it more receptive to (even a low concentration of) NT-3. Additionally, it is also conceivable that factors released by immune cells may affect the efficiency of NT-3 signaling by influencing TrkC/p75 receptor expression on axons, similar to LPS up-regulating TrkC expression on microglia (Elkabes *et al.*, 1998). Furthermore, it is possible that immune-associated signaling factors may affect the expression and/or the release of NT-3 in virus-transfected cells. Further work is needed to elucidate the so-far speculative mechanisms by which immune activation can facilitate an effect of NT-3 on midline-crossing CST growth (Chen *et al.*, 2008). Until that is achieved, it is worth investigating how else we can improve the results of NT-3 expression in areas where no noteworthy degenerative processes take place in grey matter as a means to overcome the limitations of NT-3 observed in chapter 2 and chapter 3.

A working hypothesis that future experiments may explore is that NT-3's chemo-attractive and growth-promoting abilities may facilitate midline-crossing CST growth if a gradient of NT-3 is created, starting at a close vicinity to CST axons and leading to the desired target area. Creating a

neurotrophin gradient is not a new concept. It has been successfully employed to allow axons inside a lesion-filling graft to leave a growth-permissive graft environment and to enter (axons from grafted cells) or to re-enter (regenerating host axons) the host spinal cord (Taylor *et al.*, 2006, Bonner *et al.*, 2010). It is plausible that a similar strategy may be able to overcome a potential gap in NT-3 availability to CST axons in the hemicord contralateral to NT-3 expression.

6.1.3. Optimizing neurotrophin delivery with viral vectors

In the previous chapters, AAV of serotype 2 was used to force the expression of either BDNF, NT-3 or the pharmacologically inactive control substance green fluorescent protein (GFP) for a number of reasons. First, sites of neurotrophin delivery were the intact spinal cord (chapter 3), the motor cortex (chapter 2) and the reticular formation (chapter 2). These are altogether areas not already disrupted by the SCI, so a relatively non-invasive technique of neurotrophin delivery with minimal tissue damage was preferable, such as injection via a thin glass-electrode. In addition, the gigantocellular division of the reticular formation is targeted at 8.6 mm beneath the dura (past the cerebellum), making implantation of an infusion cannula unfeasible. Cannula fixation to the skull could cause extensive nervous tissue damage since the brain may still move within the confines of the skull. Second, animals included in this research underwent more than 5

weeks of neurotrophin treatment (taking into account a 1 week lag phase for AAV-mediated neurotrophin expression and a 6 week recovery period). Osmotic minipumps, which maximally deliver over a time of up to 14 days, would have had to be implanted, exchanged twice and then removed in a total of four surgeries to cover the period of treatment desired. In contrast, viral vector mediated expression of neurotrophins over the full treatment period was achieved with just one injection at the time of SCI surgery and was thus minimally invasive and therefore preferred with regard to animal welfare. An important consideration for potential future translational use of viral vectors is the kinetics of protein expression with viral vectors, which generally entails a lag phase of a few days (in which cell transfection takes place), a long phase of high protein expression (up to several years in monkeys and dogs with AAVs; Niemeyer *et al.*, 2009, Nathwani *et al.*, 2010), followed by a decline in expression mostly due to silencing of the promoter (Papadakis *et al.*, 2004).

Certain features of a viral vector system can have great impact on the success of vector-mediated neurotrophin expression in the experimental settings described in previous chapters. To start with, it is essential that the vector can transfect cells of the CNS. A broad variety of different viral vector constructs is available for use in the CNS today, mostly based on lenti- and adeno-associated viruses (AAV; reviewed in Jakobsson and Lundberg, 2006, in Gray *et al.*, 2010, and in Lentz *et al.*, 2012). In addition, AAVs display divergent serotypes, which refers to the composition of proteins that act as

receptor-ligands on the virus capsid (Wang *et al.*, 2003, Burger *et al.*, 2004, reviewed in Van Vliet *et al.*, 2008). These capsid proteins may determine how the virus interacts with host cells, shaping in particular the affinity of the virus' serotype for transfection of specific kinds of host cells. For our purposes, a viral vector was needed that efficiently transfects cells of the nervous system, preferentially post-mitotic neurons, and that elicits as small an immune reaction in the host as possible, while at the same time being non-pathogenic to humans and experimental animals. AAVs are best suited to fulfill this profile and have been extensively used to transfect neurons and glia (reviewed in McCown, 2011, and in Lentz *et al.*, 2012). To achieve vector-mediated expression of BDNF in the motor cortex and NT-3 in the brainstem and spinal cord, we chose AAV of serotype 2 because it is frequently used for transfection of CNS cells experimentally (reviewed in Burger *et al.*, 2005), in clinical trials (McPhee *et al.*, 2006, Mittermeyer *et al.*, 2012, www.clinicaltrials.gov), and has been successfully employed by our collaborators (Hollis *et al.,* 2010, Lu *et al.*, 2012). Recent evidence suggests that AAV of serotype 1 and 5 may be more efficient than AAV-2 at transducing corticospinal neurons (Hutson *et al.*, 2012). If CST transduction specifically is desired, these serotypes may therefore be employed in favour of AAV-2. However, for our purposes it was essential that BDNF be made available to TrkB receptor bearing corticospinal neuron somata (Lu *et al.*, 2001), therefore transduction of corticospinal neurons themselves was not necessarily required.

An additional point of consideration is the construction of the DNA plasmid that the virus carries. This includes choosing a promoter sequence that is highly active in transduced cells, as well as choosing the genetic code for the desired transgene, which ideally allows easy detection of vector-induced gene expression. The plasmid used in this work contained one of the most effective and most universally active promoters available, the cytomegalovirus (CMV) promoter, which had been successfully employed previously by our collaborators (Hollis *et al.,* 2010, Lu *et al.,* 2012). The CMV promoter is known to be differentially effective at stimulating transcriptional activity in different neuronal populations, therefore its use needs to be carefully considered when the goal is to promote gene expression in specific neurons. For our purposes, however, expression and release of neurotrophin in a defined anatomical area was more important than the origin of the neurotrophin, as we were not manipulating intracellular pathways but rather increasing the concentration of an extracellular signaling molecule. Following the CMV promoter region, our AAV-2 plasmid contained the genetic code for GFP, human BDNF or NT-3, respectively. Because similar plasmids had previously been successfully used in experiments by the Tuszynski group (Hollis *et al.,* 2010, Lu *et al.,* 2012) and because there is wide homology between the human and rat neurotrophin gene sequences, we concluded that human BDNF and NT-3 are similarly effective in the rat as rat-derived BDNF and NT-3. Coding for human BDNF and human NT-3 in a viral vector has the advantage that virus-induced expression of BDNF/NT-3 is easily

distinguishable from BDNF/NT-3 derived from the rat genome. This helped confirm the functionality of our AAV constructs *in vitro* (chapter 2) and *in vivo* (unpublished data), since any evidence of human BDNF mRNA or human NT-3 mRNA was proof of successful AAV gene delivery and expression. Alternatively, co-expression of a reporter gene, such as GFP, may also allow detection of transgene expression in the tissue. Unfortunately, testing the GFP reporter in our NT-3 expressing AAV-2 produced underwhelming results. More sophisticated reporter gene constructs will be advantageous to determine successful transgene expression, particularly in experiments that yield negative results.

Apart from detecting successful gene expression, achieving the right rate of transgene expression is a major concern as well, particularly when expressing BDNF. A concentration of released BDNF that is too high may have devastating adverse effects such as hyperreflexia, seizure activity and increased pain (Scharfman, 1997, Zhu and Roper, 2001, Pezet *et al.*, 2002, Boyce *et al.*, 2012, Lu *et al.*, 2012). Regulatable viral vector technology was still in its infancy at the time when these experiments were planned (reviewed in Vilaboa and Voellmy, 2006, and in Stieger *et al.*, 2009), making it impossible to stop expression of neurotrophins in case undesired effects arose. Therefore, while trying to avoid the emergence of adverse effects of BDNF, one may end up using vector doses that create a concentration of BDNF insufficient to promote the desired plasticity. Fortunately, there are factors other than the dose of viral vector systems that may influence BDNF

or NT-3 availability and/ or signaling efficiency, such as neuronal activity for BDNF (reviewed in Vaynman and Gomez-Pinilla, 2005, Beaumont *et al.*, 2008, Nagappan *et al.*, 2009, Cote *et al.*, 2011) and immune activation for NT-3 (Chen *et al.*, 2008). In addition, construction of viral vectors that may be turned on or off by controllable triggers has been pioneered (Chtarto *et al.*, 2003, Georgievska *et al.*, 2004, Bockstael *et al.*, 2008). A prominent technique for regulating transgene expression is to employ so-called Tet-Off or Tet-On systems. Here, the presence of the antibiotic tetracycline or one of its derivatives acts as an off- or on- switch, respectively, for promoter activity (Sprengel and Hasan, 2007). A viral vector delivery system that can be adjusted at any time will not only solve some of the dosing problems we are dealing with to make neurotrophins work safely for our purposes, it will also open exciting new ways of conducting research. For example, whether the recovery-mediating effect of BDNF combined with training, as seen in chapter 2, is indeed due to an increase in excitability rather than due to changes at anatomical level will be easy to test with adjustable vectors. If increased excitability is the mechanism underlying the behavioural effect, switching BDNF off will be expected to make the effect disappear in a timely manner (Ankeny *et al.*, 2001). If anatomical plasticity, for example newly grown connections between cells, was the predominant mechanism underlying recovery, then one would expect that switching BDNF expression off would not result in any major changes in the outcome measures, at least not in a timely manner. I suspect, however, that such an experiment might

not deliver results as conclusive as theoretically possible. It is plausible that the CNS may have responded to BDNF by both anatomical plasticity (undetected by us so far), and an excitatory component in chapter 2. Still, advancing viral vector technology towards adjustable transgene expression is met with optimistic enthusiasm for the new opportunities it provides, both for fine-tuning dose of transgene expression and for studying the acute impact of switching neurotrophin expression on or off.

6.2. The role of reticulospinal plasticity after cervical SCI

The experiments described in chapters 2 and 4 include investigating the anatomical correlates of a potential involvement of the RtST in plastic changes after injury. I hypothesized that if the RtST is somehow involved in mediating functional improvement after cervical incomplete SCI, it will likely undergo adaptive anatomical changes, such as increased collateral sprouting. This hypothesis was not supported by the experimental findings in chapters 2 and 4. This stands in contrast to what has been demonstrated to occur spontaneously for the injured CST above (Fouad *et al.*, 2001), and for the spared RtST below a thoracic injury (Ballermann and Fouad, 2006). In the lesion models used in this thesis, the ipsilesional RtST was at least partly spared. It therefore seems likely that an increased RtST innervation would occur to compensate for lost CST innervation ipsilesional below the injury. Instead, we observed a remarkable loss of RtST projections within the grey

matter at the spinal level immediately below the injury in chapter 4. While this result was as robust in magnitude and across outcome measures as it was unexpected, more work needs to be done before we can reject the hypothesis that the RtST is involved in compensation after CST denervation. Future studies may look more closely at RtST projections to spinal levels further caudal than C5. The complexity of the spinal cord would certainly allow for increased RtST innervation of lower segments (e.g. C7) to have an impact on more rostral spinal levels (e.g. C5) via intersegmental interneuronal connections. What complicates investigating the RtST is that its projections are substantially more complex than the CST's in that any one of its axons can innervate multiple segments along the full length of the spinal cord (reviewed in Matsuyama *et al.*, 2004a). Further, the tract is comprised of excitatory as well as inhibitory fibers (Du Beau *et al.*, 2012), which originate from neurons within various brainstem regions across the vast reticular formation (de Boer-van Huizen and ten Donkelaar, 1999, Matsuyama *et al.*, 2004a/b). RtST axon collaterals are known to terminate in a diverse fashion, both contacting motor neurons and various interneurons (Matsuyama *et al.*, 2004a/b, Szokol and Perreault, 2009). For all these reasons, it may in the end not be overly surprising that our understanding of how the RtST is impacted by incomplete cervical injury, and what its potential role is in mediating functional recovery, is still very limited. If an overall increase in RtST collateral sprouting will fail to be detected in future experiments, it might be useful to dissect RtST plasticity using other methods.

This could potentially include differentiating between changes in projection pattern of excitatory and inhibitory fibers, or employing electrophysiological methods. Electrophysiology may ultimately determine whether partially denervated areas below an incomplete injury may receive increased RtST input mediated either by alternate neuronal routes or by functional changes within the existing projection pattern.

Although future work may elucidate the role of the RtST in functional recovery after cervical lesions, it is still curious that in the previous chapters, significant functional improvements were in no case accompanied by increased collateral innervation of grey matter, neither by the injured CST, nor by the spared RtST. This leads us to the question of how meaningful the commonly analyzed collateral sprouting is for mediating improvements in motor performance.

6.3. The hunt for recovery-mediating plasticity

In the experiments described in chapters 2 and 4, an increase in collateral sprouting either from the injured CST or the mostly spared RtST did not accompany the observed treatment-induced (chapter 2) or spontaneous (chapter 4) recovery. Naturally, we cannot exclude that sprouting occurred at spinal levels that were not analyzed in these experiments, or the involvement of other spared tracts such as the spared ventral portion of the ipsilateral CST (Weidner *et al.*, 2001), or the spared contralateral CST (Ghosh *et al.*, 2009).

Yet, especially the lack of injured CST sprouting similar to what has been previously reported to accompany training-induced recovery after the same lesion (Girgis *et al.*, 2007, Krajacic *et al.*, 2010) is somewhat puzzling. That collateral sprouting is not increased, although functional improvements are measurable, raises some doubts about the meaningfulness of increased collateral sprouting when it is observed. In support of sprouting contributing to functional recovery are reports from re-lesions studies (Weidner *et al.*, 2001, Bareyre *et al.*, 2004). Cervical CST sprouting following thoracic SCI has also been linked to cortical map changes (Fouad *et al.*, 2001). Some authors find that collateral sprouting correlates with the degree or time course of observed functional recovery (Ballermann and Fouad, 2006, Girgis *et al.*, 2007, Krajacic *et al.*, 2010). However, we cannot make any inferences about causal relationships from correlations alone. In theory, an increase in collateral sprouting may just be a meaningless side effect, as in many studies we simply do not know whether these collaterals make functional connections, and whether these connections are meaningful for behavioural recovery. We do know that re-organizations between descending motor tracts and intrinsic spinal networks can result in an astonishing level of recovery even when all projections of descending motor tracts are ablated following two staggered hemisections (Courtine *et al.*, 2008). However, how many new connections from descending motor tracts for example to lesion-bridging interneurons are necessary to produce a detectable effect on recovery after SCI has yet to be established.

It appears the study of plasticity following spinal cord injury may profit greatly from other methods of analyses being routinely added to histological analyses of sprouting. This becomes particularly relevant when the experimental condition fails to produce a detectable increase in grey matter innervation by important motor tracts, as observed in chapters 2 and 4 for the CST and the RtST. Based on these experimental results, and on the notion that plasticity may occur on any physiological level, including molecular, cellular, and anatomical levels, it seems that examining plasticity by collateral counts only represents a somewhat reductionist approach. Therefore, I would like to advocate a more holistic examination of plasticity for any experiments aimed at assessing plastic changes following SCI. On a molecular level, neuronal adaptations can potentially be detected by measuring expression of plasticity-related proteins (e.g., growth-associated protein GAP-43, immediate early genes such as cfos), by assessing markers for the activation of cellular pathways involved in growth and plasticity (ERK, MAPK, PKA, GSK-3, Rho, PKC), by examining the expression of cell surface receptors, as well as by analyzing the differential regulation of gene expression. On a micro-anatomical level, such approaches may include measuring changes in the abundance of transmitter phenotypes in the area of interest (e.g., 5-Hydroxy-tryptamine (5-HT) for serotonergic neurons, glutamic acid decarboxylase (GAD) for gamma-aminobutyric acid- (GABA)-ergic neurons), and quantification of synaptic connections between certain axons and neurons, possibly by double-labeling immunohistochemistry (Lu

et al., 2012). Also, following the synaptic network of such connections by employing trans-synaptic tracers or by using retrograde (for lesion-bridging neurons) together with anterograde tracers (to label the motor tract in question) may shed light on how many relevant connections are actually formed (Vavrek *et al.*, 2006). However, considering the complexity of the CNS, what is relevant may well include larger, more complex circuits in addition to direct contacts between for example a CST axon and a lesion-bridging neuron. One way of investigating plastic changes that span across multiple-neuron networks is electrophysiology. By analyzing responses below the lesion (either from within the spinal cord or from muscle) to stimulation of areas of interest above the lesion, such as the motor cortex, one may be able to shed light on plasticity on a network level. On the other hand, electrophysiology may also prove useful for detecting changes in neuronal excitability, which may underlie the effect of BDNF and training reported in chapter 2.

A multifaceted search for plastic changes will enhance the chances of identifying the mechanisms that underlie recovery, as findings from previous chapters strongly indicate that these mechanisms are insufficiently addressed by analyzing collateral sprouting alone. Understanding how a treatment (chapter 2), or the injury itself (chapter 4), affect plasticity to mediate recovery will be crucial to optimize treatment strategies and to maximize the potential of meaningful plasticity in future endeavors to repair the injured spinal cord.

6.4. How useful is the single pellet reaching task for training and functional assessment of rats with cervical SCI?

The study of motor behaviour has produced an array of well-established motor tests that are widely used for rat or mice models of SCI, stroke, traumatic brain injury and for models of neurological disorders (reviewed in Muir and Webb, 2000, in Kleim *et al.*, 2007, and in Onifer *et al.*, 2007). Some of these tests are more suited to assess locomotion and hindlimb function (e.g., the BBB score (Basso *et al.*, 1995), kinematic (Fouad *et al.*, 2010) and gait pattern analysis (de Medinaceli *et al.*, 1982, Klein *et al.*, 2009), grid walk (Bolton *et al.*, 2006), while others are primarily designed to assess fine motor control of the rat's forepaw [e.g., single pellet reaching (Whishaw *et al.*, 1991), Montoya staircase test (Montoya *et al.*, 1991), pasta reaching task (Ballermann *et al.*, 2000), and tray reaching task (Whishaw *et al.*, 1986)]. Among the paradigms designed to measure forepaw function, the single pellet reaching test is the most sensitive and most sophisticated method to detect changes in reaching and grasping behaviour (Morris *et al.*, 2011, Klein and Dunnett, 2012). In fact, the task is difficult to perform even for uninjured rats, which have to be trained intensively to learn the task properly. To allow detection of significant recovery in reaching, it is crucial that baseline success rate be as high as possible. If baseline performance is low, then the likelihood of reaching a "ceiling" in recovery is high. This will prevent the detection of any treatment effect because untreated control animals already reach

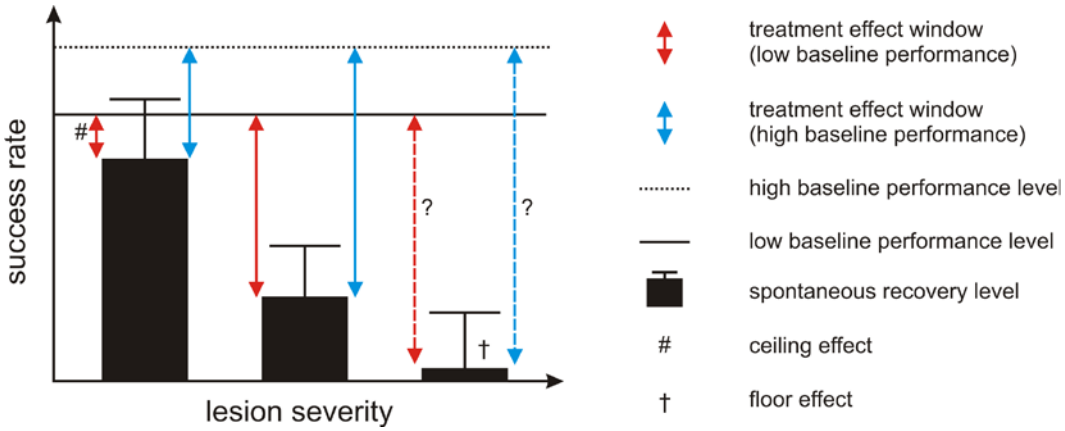


Figure 6.2. Schematic illustrating the size of treatment effect windows based on baseline performance and spontaneous recovery in the single pellet reaching task.

Left bar: The treatment effect window is increased by higher baseline performance, which can reduce the risk of reaching a ceiling effect (marked by #). Middle bar: An ideal lesion severity results in moderate spontaneous recovery that leaves room for a wide treatment window. Right bar: Severe lesions generally lead to high deficits that result in no or minimal spontaneous recovery. Although the treatment effect window can theoretically reach a maximum width in models of severe SCI, deficits of this severity cannot be overcome by currently available treatments, resulting in a floor effect (marked by cross).

maximal (baseline) performance (6.2.). While in the literature, average baseline pellet reaching success rates of (sometimes far) more than 50 % are reported (Whishaw *et al.*, 2003, Morris *et al.*, 2011), the rats used in the experiments reported here typically performed around 40 % average success. This seems consistent across experiments performed with Lewis rats in our laboratory. We find that other rat strains excel in pellet reaching compared to Lewis rats. A group of Fisher rats, for example, reached an average of 78 % success rate at baseline (unpublished observation), while a group of Long Evans rats achieved a success rate of 53 % on average (unpublished observations). A curious finding in chapter 2 is that rats recovering from SCI and receiving BDNF treatment and training seem to perform better in the reaching task than they did before the injury and BDNF treatment (training intensity was similar before and after surgery). Unless the treatment combination had extreme general performance-enhancing effects, which can be tested in uninjured animals that were not part of the experiment in chapter 2, this finding is possibly an artifact produced by the low average baseline performance. The question as to why baseline performance was relatively low compared to performance at the end of the experiment leads us to another disadvantage of this test: test results are sensitive to the motivation and stress level of the animals. This becomes strikingly obvious when plotting an individual rat's performance over the course of the experiment. The day-to-day performance variation for an individual animal may be so high that potential differences among experimental groups

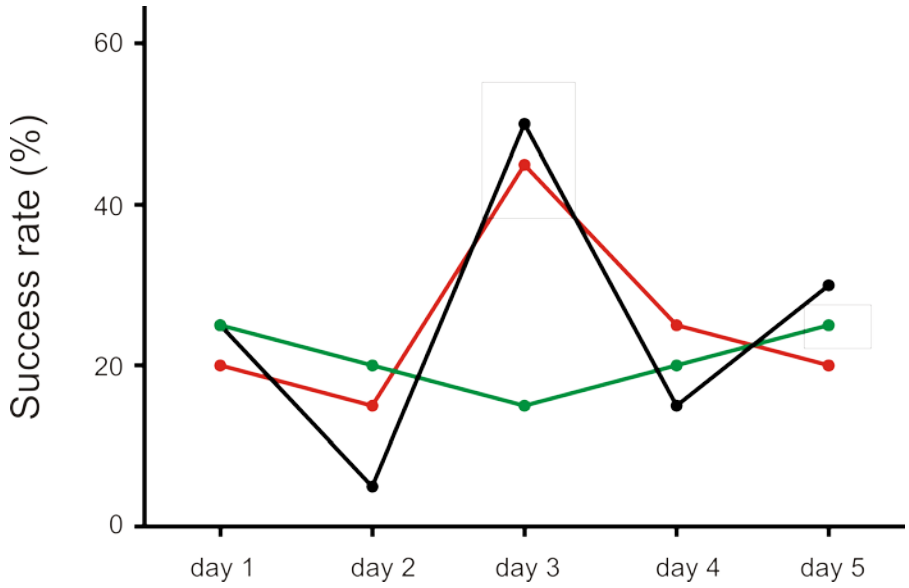


Fig. 6.3. Variability in reaching performance for 3 representative rats over the last 5 days of pre-injury training.

The black, red and green data points represent the score for each of 3 representative rats that were part of the experiments described in chapter 2. The highest score during the last 3 sessions of baseline training (here day 3, 4 and 5; high score marked by rectangles) was chosen to represent the respective rat's ability to grasp at baseline.

become difficult to detect statistically over time (Fig. 6.3.). We also noticed that any activity within the animal room can have an impact on overall performance in single pellet reaching (unpublished observation). For example, rats usually drop in performance when they are tested the day after surgeries were performed on a different group of rats housed in the same room. In order to minimize the influence of this variation on statistics, I decided to use the best value out of 3 consecutive sessions (for baseline as well as final assessment) instead of the average score of 3 sessions. I consider this feasible as the best score represents well what each animal is capable of doing. Nevertheless, one has to be aware that performance in single pellet reaching is prone to be affected by any changes in routine, environment or food deprivation.

Apart from trying to achieve high baseline performance, and from minimizing motivational/stress-related variability, it is also important to choose a lesion model that produces a severe enough deficit in reaching ability. If spontaneous recovery is close to baseline performance, then the ceiling effect will only allow for small treatment effects to be detectable (Fig. 6.2.). However, a too severe lesion will reduce the potential for a fair amount of recovery entirely, resulting in a prolonged inability to successfully grasp a pellet ("floor effect"), which often cannot be overcome by treatment (Fig. 6.2.). Indeed, a substantial number of animals with lesions of the dorsolateral quadrant (chapter 2, 4, 5) do not recover the ability to perform the reaching task successfully at all, resulting in a score of zero. Zeros can not only be a

statistician's nightmare, they also do not reveal any information on how well these animals actually do, besides the fact that they are not able to eat the pellet. Some zeros are scored because the animal is not able to advance its forearm through the slot of the pellet box, which is common during the initial testing sessions early after the SCI (earliest time point for testing in my experiments was day 7 post-injury). In contrast, some zeros are scored because the animal fails to target the pellet correctly, while forearm advance, supination of the wrist and the finger arpeggio during the grasping movement look fairly good. This may be due to a lack of 'concentration', franticness, or indeed to the inability to physically direct the paw accurately to the pellet. Another category of zeros consists of successful reaches, followed by successful grasps, ending in a premature release of the pellet because the rat does not realize that the pellet was grasped. This may be due to the non-avoidable ablation of the dorsal column sensory pathways in this lesion model, which substantially reduces sensory input from the forepaw to the brain. In rare cases, animals reach and grasp well but fail to release the pellet out of its grasp, maybe due to a high flexor tone similar to what is observed in spastic patients. Thus, success rates of zero do not allow further characterization of differences in motor performance of animals that cannot reach the threshold for successful grasping. While this "floor effect" may be a disadvantage of the test, as it reduces the resolution of the single pellet reaching test in low performing animals, it may on the other hand simply be regarded as evidence for the impotence of the tested treatment. This is an

especially valid argument when it comes to translational efforts, where robust experimental treatment effects are desired to increase the likelihood of treatment efficacy in clinical trials.

As a fair portion of rats usually fall into the zero success category following a dorsolateral quadrant lesion, we routinely record a number of reaching trials with a high-speed video camera for each rat. Frame-by-frame playback of these clips allows qualitative analysis of the movement components (Whishaw *et al.*, 1991), which gives a more detailed picture of motor control for unsuccessful rats than the reaching success rate itself. In contrast, qualitative analysis often decreases resolution of motor performance for rats that are highly successful in grasping. Rats with high success rates usually either max out or reach the exact same level in qualitative scoring. Therefore, instead of resulting in a cloud of normally distributed values in a dot plot, qualitative scores in successful reachers often converge on one single line. Furthermore, qualitative analysis is based on comparison of an individual animal's reaching movement components after the recovery period with the same animal's movements at baseline. Therefore, animals that develop a successful compensatory reaching and grasping strategy during recovery from SCI are not awarded a high score in qualitative analysis, while at the same time they may achieve outstanding success rates in pellet grasping.

In summary, the single pellet reaching task poses a number of challenges for accurately measuring recovery of motor control of the forearm

and forepaw across various lesion severities. Given the inevitable variability of lesion extent, and the labor-intensiveness of functional assessment using this reaching task, single pellet reaching is in my opinion not well suited to detect treatment effects in rat models of cervical SCI. Potential alternative paradigms include the Montoya staircase test and the horizontal ladder test, which have draw-backs as well. The ladder test for example relies on locomotor-like activity involving all four limbs, as well as on balance, which makes it difficult to judge forelimb motor control by itself accurately. Hope is high that a better way of testing forearm and forepaw motor function after cervical SCI will be developed. Instead of choosing the lesion severity for the test in an effort to avoid ceiling and floor effects, I think the field would profit from an array of tests that together provide high resolution scoring across the full span of forelimb motor abilities that can be expected following diverse cervical injury severities. A starting point may be the introduction of kinematic analysis for skilled reaching movements.

6.5. Optimizing rehabilitative training after SCI

Rehabilitative training is the only therapy for individuals with SCI that is considered effective and safe without major controversy. However, the systematic study of some important aspects of training, such as the optimal onset of training, its intensity and what training paradigms are most beneficial, is still in its infancy. Recent training studies in animal models of

SCI have hinted at side effects of training and the need to explore strategies for optimization. For example, task-specific training may lead to significant improvements in the trained task, as seen in chapter 2, but often does not translate into untrained tasks (chapter 2, Grasso *et al.*, 2004, Smith *et al.*, 2006, Magnuson *et al.*, 2009). Of notice, the experiments in chapter 2 and 5 did not yield an effect of single pellet reaching training alone on reaching recovery at all. This stands in contrast to what has been previously achieved in our laboratory (Girgis *et al.*, 2007, Krajacic *et al.*, 2010). The only difference between those earlier reports and the experiments described in the previous chapters is that rats in my studies were from a different supplier and were trained only 5 days a week instead of 6. This begs the question as to whether small differences in training intensity or minute genomic variations (the rat strain was unchanged) can have a significant impact on the potential to benefit from training. Colleagues of mine are currently tackling this question with an experiment in which the effects of different training intensities on performance in different motor paradigms are tested.

When motor performance of the forearm and forepaw is assessed with either the single pellet reaching test or on the horizontal ladder in rat models of cervical SCI, the overall outcome score does not reflect the degree to which these animals return to original limb function. In fact, a lot of these animals seem to develop compensatory strategies to reach the same goal. For example, instead of grasping the pellet from above, animals with an incomplete cervical SCI can sometimes be observed to grasp the pellet with a

side-ways scooping motion. Although the original movement component of pronation of the forearm and wrist is not restored, the new strategy is just as successful. Similarly, compensatory stepping is often observed in injured animals when they cross the horizontal ladder. Instead of correctly grasping the rung by wrapping the fingers around the rung and supporting body weight with the palm, compensatory steps typically result in an overshoot of the paw, with the wrist or the lower forearm coming to rest on the rung, and the fingers hanging loosely in the space between rungs. Surprisingly, this kind of stepping can result in a balanced, uninterrupted, error-free (if errors are counted as slips and falls) crossing of the ladder. In conclusion, a portion of the recovery reported for the injured limb in chapters 2 and 4 is due to compensatory movement strategy, and not due to what in the medical community is referred to as "restitutio ad integrum". Studying how the CNS may re-establish communication among neuronal networks disrupted by injury, we can now expect with certainty that a restoration of neuronal pathways identical to the pre-injury state will never occur. How then can we expect functional plasticity, or rewiring, to restore movement control in a way that matches pre-injury conditions exactly? It would be somewhat ignorant to suggest that currently available treatments to promote plasticity should be judged by how accurately they can restore pre-injury movement control. As long as we cannot 'cure' individuals with SCI, compensation should in my opinion find broader acceptance as a therapeutic goal.

If successful compensation is considered a valid goal of recovery, then a holistic view of the affected individual or the spinally injured rat can take into account compensatory strategies beyond the use of the primarily affected forearm (PAF). While maximizing the performance of the PAF is of course important, it may be of equal value to maximize the performance of the less affected forearm (LAF). Patients who lose the use of their dominant arm temporarily, for instance due to a bone fracture, typically feel extremely helpless, as the non-dominant extremity is often not skilled enough to compensate. While the control over the PAF in this scenario is clearly limited, the potential of enhancing skill of the LAF is immense. Training the LAF can result in a level of skill that can not only compensate for lost function of the PAF, the LAF may now be able to contribute better to two-handed tasks. When this notion is applied to the case of cervical SCI, every day life tasks may ultimately be best accomplished not only by maximizing the control over the PAF, which is likely limited and may plateau early, but by maximizing the performance of the LAF as well. The findings in chapter 5 corroborate this concept in that task-specific training for all limbs (horizontal ladder) resulted in significant training effects for the LAF but not for the PAF, while task-specific training of the PAF alone (single pellet reaching) did not result in improved performance in this task. These results confirm that the potential to improve function and skill is naturally higher in the LAF, as more neuronal pathways are available for motor control of this limb following incomplete SCI. What is more interesting, however, is the finding that as these two task-

specific training paradigms are combined, neither limb profits. While added training for the PAF (reaching plus ladder) did not advance its pellet reaching performance, the training effect previously recorded for the LAF (ladder) disappeared when the PAF was trained in reaching at the same time. In other words, while training efforts for improving function of the PAF did not result in improvement, these same efforts interfered with the LAF reaching its training potential. I speculate that the reason for the interference effect may lie in the limited availability of intact neuronal networks spared by the injury, which can be committed to specific movement programs. If some networks are being committed during reaching training of the PAF (although not resulting in measurable functional recovery), there may be less neuronal networks available to mediate motor control of the LAF during ladder training than when ladder crossing is the only paradigm trained. In other words, spared descending fibers contralateral to an injury may be rewired to communicate with ipsilesional motorneurons via commissural connections (in response to single pellet training), thereby reducing the potential of these spared fibers to exert control over contralesional motorneurons (observed as a failure to reach high performance levels with the LAF in ladder walking when reaching is trained). How can these findings affect rehabilitative training in the clinic? While rehabilitative training will naturally have to be focused on the PAF in the early phases of recovery, when the potential for improvement is relatively high, I think it may be worth shifting emphasis onto the LAF once the PAF reached a plateau in performance. It is commonly

reported that recovery of SCI is gradual in the first months after the trauma and then reaches a plateau from whereon further significant improvements are rarely achieved (Burns *et al.*, 2012). Moreover, the interference of training the PAF on potential skill development for the LAF should be kept in mind when first assessing an SCI and the degree of recovery that can reasonably be expected for the PAF. The findings in chapter 5 also shed a different light on constraint-induced movement therapy (CIMT), which has been explored in animal models of stroke (reviewed in Janssen *et al.*, 2013), and which is clinically practiced for individuals suffering from stroke (reviewed in Sirtori *et al.*, 2009). Any potential use of CIMT for individuals with SCI should be carefully assessed based on the caveats raised in chapter 5.

6.6. Limitations of experiments

Each of the experiments described in the previous chapters has its unique limitations, which are covered in the respective chapter's discussion. I would like to take this opportunity to raise a few general limitations that many of the experiments described here have in common.

First, the number of animals one can reasonably start these experiments with is limited (24 maximal if training is part of the experiment). One reason for the limitation is the time and labor intensive one-on-one training that is required if single pellet reaching testing or any post-injury

training protocol is part of the experiment. Another limitation is posed by the time-intensive surgeries that these experiments all require. If surgeries spanned over the course of a week (instead of 2-3 days for 24 animals), keeping post-injury interventions and procedures consistent with regards to the time point after injury would be challenging. Another factor that limits the number of animals one can include in each of these experiments is the high level of early post-injury care that these animals require. The highest standards of care to ensure animal welfare and experimental success require considerable time commitment and careful organization over the course of the days and nights following surgeries. To address this limitation, we pooled results from two identical experiments in chapter 2 and 5, respectively. This way, the number of animals included in statistics can be raised substantially. The problem with this strategy, however, emerges when results from the two experiments are compared before pooling is done. Repeating an experiment always introduces variation, which is evident in chapter 5 where the overall lesion size was significantly different between the two "identical" experiments, calling for normalization before data could be pooled.

Second, all experiments except for the one described in chapter 3 do not include an uninjured control group. The reason for not having uninjured animals in studies involving training is again due to the time investment that training requires. Thus, the decision as to what control conditions shall be included in any one experiment is usually made in favour of injured control groups that lack a treatment component. When treatment combinations are

tested, as is increasingly common in the field, controls need to be in place for the effect of one treatment component only. Therefore, experiments described here that test combinatorial treatments (chapter 2, 3 and 5) do not include an uninjured control group. Nevertheless, we could probably learn a lot from how a given treatment may affect the intact animal. Especially for the BDNF/training combination in chapter 2, and for the double training group in chapter 5, it would be interesting to see whether these interventions have effects on uninjured rats.

A third limitation that all these experiments have in common is that histology was the only method used to analyze plasticity. Unfortunately, to achieve high quality tissue processing for histology, perfusion is necessary following euthanasia of the animal. Thus, harvesting samples for protein analyses (e.g., western blot or ELISA), would only be possible in the anesthetized animal, which is not considered optimal, or in a subset of animals that is not used for histology. Likewise, quality electrophysiological assessment, a terminal procedure, is not always achievable in traced animals. The procedure would rely on stimulation of an area that was previously injected with a tracer substance, which may affect physiological properties of these neurons. Forced to decide between these methods of analysis, we opted to give histology priority over these other assessments for various reasons. First, increased collateral sprouting has frequently been reported in response to SCI, and it was therefore reasonable to hypothesize that changes in sprouting may occur. Second, the experiment described in chapter 2 was

designed to test the hypothesis that targeted rewiring for the injured CST may be promoted with the help of neurotrophins and training. The treatment was specifically designed to promote the growth of new collaterals, making histology the obvious choice for analysis. It would nevertheless be valuable to include other methods of analysis when investigating plasticity, as discussed in detail in section 6.3. To perform these analyses properly, either different sets of animals or refinements in the methodology (e.g., tissue harvesting techniques) will be needed.

6.7. Future directions

As is common in scientific research, the findings reported in this thesis lead to an array of new questions. Even in cases where the working hypothesis was not fully supported (chapter 2, 3 and 4), the positive results suggest interesting new hypotheses. I will here briefly summarize what direction the experimental findings from each chapter may point to for future endeavors to investigate and promote plasticity after SCI.

6.7.1. Chapter 2: Synergistic effects of BDNF and rehabilitative training on recovery after cervical spinal cord injury

The main question that arises from this study is what the mechanism behind the treatment-induced recovery may be. Candidate mechanisms include an increased excitability of BDNF treated neurons, which may facilitate signaling

through spared pathways, changes in synaptic plasticity, such as increases in transmission efficacy, as well as anatomical rewiring at locations that were not included in our analysis. Future experiments may investigate the excitability of cortical motor neurons using electrophysiological methods. For example, a decrease in the cortical stimulus intensity required to activate reticular neurons, or needed to evoke a muscle response as measured by electromyogram (EMG), would be evidence for increased excitability of the neuronal network involved.

The advance of viral vector technology offers the exciting new prospect of being able to switch protein expression off. Switching off BDNF expression in these animals may provide evidence as to whether the behaviour is mediated by changes in anatomical plasticity or is rather mainly due to temporary effects of BDNF signaling on cell excitability and activity.

Another finding in chapter 2 that warrants further investigation is that only the combination of BDNF and training resulted in improved recovery, an effect that was more than additive. A thorough investigation of the relationship between neuronal activity and BDNF release/ signaling is called for to address this interesting finding. Modeling this combined treatment in organotypic slice cultures, where synaptic regions are more readily available for detailed molecular analysis, may be one way to examine this *in vitro*. Neuronal activity can for instance be elicited by current injection (Fritsch *et al.*, 2010), or application of excitatory transmitters (Pooler *et al.*,

2013, Yamagata *et al.*, 2013). *In vivo*, protein analysis of the treated brain region may also contribute to shed light on the relationship between BDNF and activity. Apart from rehabilitative training, *in vivo* neuronal activity can be elicited experimentally by electrical stimulation (English *et al.*, 2007, Carmel *et al.*, 2010, Huie *et al.*, 2012).

6.7.2. Chapter 3: Vector-induced NT-3 expression adjacent to injured corticospinal axons promotes collateral growth in a rat model of spinal cord injury

Keeping in mind that NT-3 concentrations were not measured in this experiment, the question arises of how one can promote collateral growth across a distance into an "NT-3 rich" target region that is devoid of degenerative processes. Two straight-forward approaches to achieve such growth immediately come to mind: increasing the concentration of NT-3 in the target area and/or creating a gradient of NT-3 by multiple AAV injections to guide collaterals from their origin all the way into the target area. Gradients of NT-3 have proved successful in promoting the entry or re-entry of fibers from cell grafts into host tissue (Taylor *et al.*, 2006). A different way of tackling the problem is to make motor tract axons more sensitive to NT-3 signaling, for example by increasing TrkC receptor expression. Up-regulation of TrkB receptors for example has been achieved previously by viral vector transduction of cortical motoneurons, and was accompanied by an enhanced

responsiveness of these transduced cells to BDNF (Hollis *et al.*, 2009). Further progress in the identification of essential immune factors that may positively influence the cross-talk between NT-3 and more distant axons, such as indicated by Shine and colleagues' work (Chen *et al*., 2008), may also lead to elegant solutions for overcoming the hindrances of long distance signaling of NT-3. It will be interesting to see whether those factors can be used safely, and through what mechanism they are able to promote midline-crossing CST growth in response to NT-3 expression.

6.7.3. Chapter 4: Reticulospinal plasticity after cervical spinal cord injury in the rat involves withdrawal of projections below the injury

In order to characterize the role of the RtST in recovery of hand/paw function after incomplete SCI, a considerable amount of work still needs to be done. Unfortunately, the experiment in chapter 4 is not able to contribute much to the question of the functional role of the RtST, as positive changes in plasiticity, which would have suggested the tract's involvement in recovery, were not evident. Electrophysiological experiments may be well suited to investigate the functional role of the RtST after injury. It is conceivable that by stimulating the reticular formation and recording forearm EMG, changes in connectivity between reticular neurons and spinal motoneurons will be detectable. In order to investigate the connectivity between the motor cortex and the reticular formation, cortical stimulation and recording from reticular

cells may be useful. However, the fact that the reticular formation is such a diverse and vast brainstem region may prove to be a major hindrance to this approach. Alternatively, the role of the reticular formation may be elucidated by experimentally blocking its input to spinal motoneurons and examining the resulting deficits. When dealing with a more defined motor tract such as the CST, it is relatively easy to lesion the tract at a level rostral to SCI (e.g., by pyramidotomy), where the tract is well defined and can be lesioned completely with little damage to neighboring structures (Zhou *et al.*, 2003, Hagg *et al.*, 2005, Krajacic *et al.*, 2010). Unfortunately, the RtST projects in a wide area of the spinal white matter and therefore cannot be lesioned in a comparable manner. Another strategy to abolish input from a neuronal population to spinal motoneurons is to block neuronal activity. For example, injection of the $GABA_A$-agonist Muscimol into the red nucleus successfully silences most of the neurons in the region (Krajacic *et al.*, 2010). Unfortunately, an injection of a GABA agonist into the reticular formation could be risky, as neuronal centres for the control of vital bodily functions, such as respiration and heart function, are located in the vicinity of the reticular formation. At the same time, RtST neurons reside within a wide anatomical area, which makes silencing the majority of these neurons via muscimol injection difficult. While it seems extremely challenging to abolish RtST function experimentally at this point in time, in the future optogenetics techniques may provide safer and more elegant ways to silence defined populations of neurons.

Apart from elucidating the role of the RtST in recovery, future endeavors will likely identify further plastic changes in the RtST, may it be on a molecular or cellular level, or on spinal levels not analyzed in chapter 4. In pursuit of the mechanism behind the collateral withdrawal observed in chapter 4, evidence for inflammatory activity at this spinal level would be valuable. However, such inflammation, which may contribute to or indeed cause the RtST collateral withdrawal, may only be detectable at an earlier time point during the recovery phase. Linking collateral withdrawal to the time course and location of secondary injury processes would certainly substantiate the core finding in chapter 4.

6.7.4. Chapter 5: Training following unilateral cervical spinal cord injury in rats affects the contralesional forelimb

The finding in chapter 5 that adding a training paradigm can negatively affect performance in another training paradigm raises interesting concepts about the way neuronal networks are involved in motor learning and perhaps committed to certain motor programs. The mechanistic hypothesis of limited neuronal network availability coupled with task-specific commitment of available networks has been explained in detail earlier in this chapter (section 6.5). This hypothesis is not only fit to explain the phenomenon observed in chapter 5, but also similar phenomona in previous reports (De Leon *et al.*, 1998, Girgis *et al.*, 2007, Garcia-Alias *et al.*, 2009). It can be tested

by double training experiments with animals recovering from an incomplete cervical SCI. For example, Group A could be trained in task A until a significant training effect is achieved. Group B could be trained in task B until a significant training effect is achieved. The hypothesis predicts that due to an equal amount of neuronal networks being available for use in all animals, group C trained in tasks A and B will not reach the same level of performance in either task as group A or group B. One limitation that would need to be overcome is that ideally, both tasks should be comparable in the motor skill level required as well as in their sensitivity to detect changes in motor performance. If the hypothesis is confirmed, the question follows how similar tasks have to be in order to have a synergistic or antagonistic effect on each other if trained in parallel. Training intensity and onset after injury may also influence the commitment of available neuronal networks if the hypothesis is supported, and can easily be studied in follow-up experiments.

6.8. Summary and conclusion

This thesis is a contribution to the exploration of how CNS plasticity can mediate recovery in hand/forepaw function after incomplete cervical SCI. Important achievements were made using BDNF, rehabilitative training and NT-3 to stimulate plasticity on multiple levels, while challenges and caveats were identified as well. The results point towards a promising union between BDNF and rehabilitative training to promote recovery and to exceed the

limits of rehabilitative training by itself. The data also indicate that NT-3 is effective at promoting CST collateral growth in healthy tissue regions rostral to an SCI, where it can be used to promote rewiring of CST fibers with interneurons in future endeavors. However, since measured improvements in motor performance were in no case accompanied by increased plasticity of the CST or RtST, the present data suggest a limited functional relevance of collateral sprouting. It therefore seems likely that other correlates of plasticity, such as adaptations on a synaptic and cellular level, are potentially responsible for the improved motor function observed in these experiments. Further research is warranted to elucidate mechanisms behind the recovery observed in the previous chapters, including the role of the RtST in mediating recovery.

A closer look at how task-specific training impacts functional recovery of both forelimbs following a unilateral cervical SCI indicates that training effects for the contralesional paw are eliminated when intensive training of the ipsilesional paw is added. This finding spurs interesting concepts of how training affects plasticity of spared neuronal networks, which, if further confirmed, may have widespread implications for how we can optimize rehabilitative training for individuals with incomplete SCI.

6.9. References

Alto LT, Havton LA, Conner JM, Hollis ER, 2nd, Blesch A, Tuszynski MH. Chemotropic guidance facilitates axonal regeneration and synapse formation after spinal cord injury. Nat. Neurosci. 2009;12(9):1106-13.

Ankeny DP, McTigue DM, Guan Z, Yan Q, Kinstler O, Stokes BT, et al. Pegylated brain-derived neurotrophic factor shows improved distribution into the spinal cord and stimulates locomotor activity and morphological changes after injury. Exp. Neurol. 2001;170(1):85-100.

Ballermann M, Fouad K. Spontaneous locomotor recovery in spinal cord injured rats is accompanied by anatomical plasticity of reticulospinal fibers. Eur. J. Neurosci. 2006;23(8):1988-96.

Ballermann M, Tompkins G, Whishaw IQ. Skilled forelimb reaching for pasta guided by tactile input in the rat as measured by accuracy, spatial adjustments, and force. Behav. Brain Res. 2000;109(1):49-57.

Bareyre FM, Kerschensteiner M, Raineteau O, Mettenleiter TC, Weinmann O, Schwab ME. The injured spinal cord spontaneously forms a new intraspinal circuit in adult rats. Nat. Neurosci. 2004;7(3):269-77.

Basso DM, Beattie MS, Bresnahan JC. A sensitive and reliable locomotor rating scale for open field testing in rats. J. Neurotrauma.

1995;12(1):1-21.

Beaumont E, Kaloustian S, Rousseau G, Cormery B. Training improves the electrophysiological properties of lumbar neurons and locomotion after thoracic spinal cord injury in rats. Neurosci. Res. 2008;62(3):147-54.

Bockstael O, Chtarto A, Wakkinen J, Yang X, Melas C, Levivier M, et al. Differential transgene expression profiles in rat brain, using rAAV2/1 vectors with tetracycline-inducible and cytomegalovirus promoters. Hum. Gene Ther. 2008;19(11):1293-305.

Bolton DA, Tse AD, Ballermann M, Misiaszek JE, Fouad K. Task specific adaptations in rat locomotion: runway versus horizontal ladder. Behav. Brain Res. 2006;168(2):272-9.

Bonner JF, Blesch A, Neuhuber B, Fischer I. Promoting directional axon growth from neural progenitors grafted into the injured spinal cord. J. Neurosci. Res. 2010;88(6):1182-92.

Boyce VS, Park J, Gage FH, Mendell LM. Differential effects of brain-derived neurotrophic factor and neurotrophin-3 on hindlimb function in paraplegic rats. Eur. J. Neurosci. 2012;35(2):221-32.

Burger C, Gorbatyuk OS, Velardo MJ, Peden CS, Williams P, Zolotukhin S, et al. Recombinant AAV viral vectors pseudotyped with viral capsids from serotypes 1, 2, and 5 display differential efficiency and cell tropism after delivery to different regions of the central nervous system. Mol. Ther. 2004;10(2):302-17.

Burger C, Nash K, Mandel RJ. Recombinant adeno-associated viral vectors in the nervous system. Hum. Gene Ther. 2005;16(7):781-91.

Burns AS, Marino RJ, Flanders AE, Flett H. Clinical diagnosis and prognosis following spinal cord injury. Handb. Clin. Neurol. 2012;109:47-62.

Carmel JB, Berrol LJ, Brus-Ramer M, Martin JH. Chronic electrical stimulation of the intact corticospinal system after unilateral injury restores skilled locomotor control and promotes spinal axon outgrowth. J. Neurosci. 2010;30(32):10918-26.

Chen Q, Smith GM, Shine HD. Immune activation is required for NT-3-induced axonal plasticity in chronic spinal cord injury. Exp. Neurol. 2008;209(2):497-509.

Chtarto A, Bender HU, Hanemann CO, Kemp T, Lehtonen E, Levivier M, et al. Tetracycline-inducible transgene expression mediated by a single AAV vector. Gene Ther. 2003;10(1):84-94.

Cohen-Cory S, Kidane AH, Shirkey NJ, Marshak S. Brain-derived neurotrophic factor and the development of structural neuronal connectivity. Dev. Neurobiol. 2010;70(5):271-88.

Cote MP, Azzam GA, Lemay MA, Zhukareva V, Houle JD. Activity-dependent increase in neurotrophic factors is associated with an enhanced modulation of spinal reflexes after spinal cord injury. J. Neurotrauma. 2011;28(2):299-309.

Courtine G, Song B, Roy RR, Zhong H, Herrmann JE, Ao Y, et al. Recovery of supraspinal control of stepping via indirect propriospinal relay

connections after spinal cord injury. Nat. Med. 2008;14(1):69-74.

de Boer-van Huizen RT, ten Donkelaar JH. Early development of descending supraspinal pathways: a tracing study in fixed and isolated rat embryos. Anat. Embryol. (Berl). 1999;199(6):539-47.

De Leon RD, Hodgson JA, Roy RR, Edgerton VR. Full weight-bearing hindlimb standing following stand training in the adult spinal cat. J. Neurophysiol. 1998;80(1):83-91.

De Medinaceli L, Freed WJ, Wyatt RJ. An index of the functional condition of rat sciatic nerve based on measurements made from walking tracks. Exp. Neurol. 1982;77(3):634-43.

Dreyfus CF, Dai X, Lercher LD, Racey BR, Friedman WJ, Black IB. Expression of neurotrophins in the adult spinal cord in vivo. J. Neurosci. Res. 1999;56(1):1-7.

Du Beau A, Shakya Shrestha S, Bannatyne BA, Jalicy SM, Linnen S, Maxwell DJ. Neurotransmitter phenotypes of descending systems in the rat lumbar spinal cord. Neuroscience. 2012;227:67-79.

Elkabes S, Peng L, Black IB. Lipopolysaccharide differentially regulates microglial trk receptor and neurotrophin expression. J. Neurosci. Res. 1998;54(1):117-22.

English AW, Schwartz G, Meador W, Sabatier MJ, Mulligan A. Electrical stimulation promotes peripheral axon regeneration by enhanced neuronal neurotrophin signaling. Dev. Neurobiol. 2007;67(2):158-72.

Fouad K, Pedersen V, Schwab ME, Brosamle C. Cervical sprouting of . corticospinal fibers after thoracic spinal cord injury accompanies shifts in evoked motor responses. Curr. Biol. 2001;11(22):1766-70.

Fouad K, Rank MM, Vavrek R, Murray KC, Sanelli L, Bennett DJ. Locomotion after spinal cord injury depends on constitutive activity in serotonin receptors. J. Neurophysiol. 2010;104(6):2975-84.

Fritsch B, Reis J, Martinowich K, Schambra HM, Ji Y, Cohen LG, et al. Direct current stimulation promotes BDNF-dependent synaptic plasticity: potential implications for motor learning. Neuron. 2010;66(2):198-204.

Garcia-Alias G, Barkhuysen S, Buckle M, Fawcett JW. Chondroitinase ABC treatment opens a window of opportunity for task-specific rehabilitation. Nat. Neurosci. 2009;12(9):1145-51.

Georgievska B, Jakobsson J, Persson E, Ericson C, Kirik D, Lundberg C. Regulated delivery of glial cell line-derived neurotrophic factor into rat striatum, using a tetracycline-dependent lentiviral vector. Hum. Gene Ther. 2004;15(10):934-44.

Geremia NM, Pettersson LM, Hasmatali JC, Hryciw T, Danielsen N, Schreyer DJ, et al. Endogenous BDNF regulates induction of intrinsic neuronal growth programs in injured sensory neurons. Exp. Neurol. 2010;223(1):128-42.

Ghosh A, Sydekum E, Haiss F, Peduzzi S, Zorner B, Schneider R, et al.

Functional and anatomical reorganization of the sensory-motor cortex after incomplete spinal cord injury in adult rats. J. Neurosci. 2009;29(39):12210-9.

Girgis J, Merrett D, Kirkland S, Metz GA, Verge V, Fouad K. Reaching training in rats with spinal cord injury promotes plasticity and task specific recovery. Brain. 2007;130(Pt 11):2993-3003.

Grasso R, Ivanenko YP, Zago M, Molinari M, Scivoletto G, Lacquaniti F. Recovery of forward stepping in spinal cord injured patients does not transfer to untrained backward stepping. Exp. Brain Res. 2004;157(3):377-82.

Gray SJ, Woodard KT, Samulski RJ. Viral vectors and delivery strategies for CNS gene therapy. Ther. Deliv. 2010;1(4):517-34.

Hagg T, Baker KA, Emsley JG, Tetzlaff W. Prolonged local neurotrophin-3 infusion reduces ipsilateral collateral sprouting of spared corticospinal axons in adult rats. Neuroscience. 2005;130(4):875-87.

Hiebert GW, Khodarahmi K, McGraw J, Steeves JD, Tetzlaff W. Brain-derived neurotrophic factor applied to the motor cortex promotes sprouting of corticospinal fibers but not regeneration into a peripheral nerve transplant. J. Neurosci. Res. 2002;69(2):160-8.

Hofer M, Pagliusi SR, Hohn A, Leibrock J, Barde YA. Regional distribution of brain-derived neurotrophic factor mRNA in the adult mouse brain. EMBO J. 1990;9(8):2459-64.

Hollis ER, 2nd, Jamshidi P, Low K, Blesch A, Tuszynski MH. Induction of corticospinal regeneration by lentiviral trkB-induced Erk activation. Proc. Natl. Acad. Sci. USA. 2009;106(17):7215-20.

Hollis ER, Jamshidi P, Lorenzana AO, Lee JK, Gray SJ, Samulski RJ, Zheng B, Tuszynski MH. Transient demyelination increases the efficiancy of retrograde AAV transduction. Mol. Ther. 2010;18(8):1496-500.

Huie JR, Garraway SM, Baumbauer KM, Hoy KC, Jr., Beas BS, Montgomery KS, et al. Brain-derived neurotrophic factor promotes adaptive plasticity within the spinal cord and mediates the beneficial effects of controllable stimulation. Neuroscience. 2012;200:74-90.

Hutson TH, Verhaagen J, Yanez-Munoz RJ, Moon LD. Corticospinal tract transduction: a comparison of seven adeno-associated viral vector serotypes and a non-integrating lentiviral vector. Gene Ther. 2012;19(1):49-60.

Jakobsson J, Lundberg C. Lentiviral vectors for use in the central nervous system. Mol. Ther. 2006;13(3):484-93.

Janssen H, Speare S, Spratt NJ, Sena ES, Ada L, Hannan AJ, et al. Exploring the efficacy of constraint in animal models of stroke: meta-analysis and systematic review of the current evidence. Neurorehabil. Neural Repair. 2013;27(1):3-12.

Kaplan DR, Miller FD. Neurotrophin signal transduction in the nervous system. Curr. Opin. Neurobiol. 2000;10(3):381-91.

Kigerl KA, Gensel JC, Ankeny DP, Alexander JK, Donnelly DJ, Popovich PG.

Identification of two distinct macrophage subsets with divergent effects causing either neurotoxicity or regeneration in the injured mouse spinal cord. J. Neurosci. 2009;29(43):13435-44.

Kleim JA, Boychuk JA, Adkins DL. Rat models of upper extremity impairment in stroke. ILAR J. 2007;48(4):374-84.

Klein A, Dunnett SB. Analysis of skilled forelimb movement in rats: the single pellet reaching test and staircase test. Curr. Protoc. Neurosci. 2012;Chapter 8:Unit8 28.

Klein A, Wessolleck J, Papazoglou A, Metz GA, Nikkhah G. Walking pattern analysis after unilateral 6-OHDA lesion and transplantation of foetal dopaminergic progenitor cells in rats. Behav. Brain Res. 2009;199(2):317-25.

Kobayashi NR, Fan DP, Giehl KM, Bedard AM, Wiegand SJ, Tetzlaff W. BDNF and NT-4/5 prevent atrophy of rat rubrospinal neurons after cervical axotomy, stimulate GAP-43 and Talpha1-tubulin mRNA expression, and promote axonal regeneration. J. Neurosci. 1997;17(24):9583-95.

Krajacic A, Weishaupt N, Girgis J, Tetzlaff W, Fouad K. Training-induced plasticity in rats with cervical spinal cord injury: effects and side effects. Behav. Brain Res. 2010;214(2):323-31.

Kwon BK, Liu J, Oschipok L, Teh J, Liu ZW, Tetzlaff W. Rubrospinal neurons fail to respond to brain-derived neurotrophic factor applied to the spinal cord injury site 2 months after cervical

axotomy. Exp. Neurol. 2004;189(1):45-57.

Lentz TB, Gray SJ, Samulski RJ. Viral vectors for gene delivery to the central nervous system. Neurobiol. Dis. 2012;48(2):179-88.

Lu B, Pang PT, Woo NH. The yin and yang of neurotrophin action. Nat. Rev. Neurosci. 2005;6(8):603-14.

Lu P, Blesch A, Graham L, Wang Y, Samara R, Banos K, et al. Motor axonal regeneration after partial and complete spinal cord transection. J. Neurosci. 2012;32(24):8208-18.

Lu P, Blesch A, Tuszynski MH. Neurotrophism without neurotropism: BDNF promotes survival but not growth of lesioned corticospinal neurons. J. Comp. Neurol. 2001;436(4):456-70.

Lu P, Wang Y, Graham L, McHale K, Gao M, Wu D, et al. Long-distance growth and connectivity of neural stem cells after severe spinal cord injury. Cell. 2012;150(6):1264-73.

Lu Y, Christian K, Lu B. BDNF: a key regulator for protein synthesis-dependent LTP and long-term memory? Neurobiol. Learn. Mem. 2008;89(3):312-23.

Magnuson DS, Smith RR, Brown EH, Enzmann G, Angeli C, Quesada PM, et al. Swimming as a model of task-specific locomotor retraining after spinal cord injury in the rat. Neurorehabil. Neural Repair. 2009;23(6):535-45.

Matsuyama K, Mori F, Nakajima K, Drew T, Aoki M, Mori S. Locomotor role of the corticoreticular-reticulospinal-spinal interneuronal system.

Prog. Brain Res. 2004a;143:239-49.

Matsuyama K, Nakajima K, Mori F, Aoki M, Mori S. Lumbar commissural interneurons with reticulospinal inputs in the cat: morphology and discharge patterns during fictive locomotion. J. Comp. Neurol. 2004b;474(4):546-61.

McCown TJ. Adeno-Associated Virus (AAV) Vectors in the CNS. Curr. Gene Ther. 2011;11(3):181-8.

McPhee SW, Janson CG, Li C, Samulski RJ, Camp AS, Francis J, et al. Immune responses to AAV in a phase I study for Canavan disease. J. Gene Med. 2006;8(5):577-88.

Minichiello L. TrkB signalling pathways in LTP and learning. Nat. Rev. Neurosci. 2009;10(12):850-60.

Mittermeyer G, Christine CW, Rosenbluth KH, Baker SL, Starr P, Larson P, et al. Long-term evaluation of a phase 1 study of AADC gene therapy for Parkinson's disease. Hum. Gene Ther. 2012;23(4):377-81.

Montoya CP, Campbell-Hope LJ, Pemberton KD, Dunnett SB. The "staircase test": a measure of independent forelimb reaching and grasping abilities in rats. J. Neurosci. Methods. 1991;36(2-3):219-28.

Morris R, Tosolini AP, Goldstein JD, Whishaw IQ. Impaired arpeggio movement in skilled reaching by rubrospinal tract lesions in the rat: a behavioral/anatomical fractionation. J. Neurotrauma.

2011;28(12):2439-51.

Muir GD, Webb AA. Mini-review: assessment of behavioural recovery following spinal cord injury in rats. Eur. J. Neurosci. 2000;12(9):3079-86.

Nagappan G, Zaitsev E, Senatorov VV, Jr., Yang J, Hempstead BL, Lu B. Control of extracellular cleavage of ProBDNF by high frequency neuronal activity. Proc. Natl. Acad. Sci. USA. 2009;106(4):1267-72.

Nathwani AC, Rosales C, McIntosh J, Rastegarlari G, Nathwani D, Raj D, Nawathe S, Waddington SN, Bronson R, Jackson S, Donahue RE, High KA, Mingozzi F, Ng CY, Zhou J, Spence Y, McCarville MB, Valentine M, Allay J, Coleman J, Sleep S, Gray JT, Nienhuis AW, Davidoff AM. Long-term safety and efficacy following systemic administration of a self-complementary AAV vector encoding human FIX pseudotyped with serotype 5 and 8 capsid proteins. Mol. Ther. 2011;19(5):876-85.

Niemeyer GP, Herzog RW, Mount J, Arruda VR, Tillson DM, Hathcock J, van Ginkel FW, High KA, Lothrop CD Jr. Long-term correction of inhibitor-prone hemophilia B dogs treated with liver-directed AAV2-mediated factor IX gene therapy. Blood 2009;113(4):797-806.

Onifer SM, Rabchevsky AG, Scheff SW. Rat models of traumatic spinal cord injury to assess motor recovery. ILAR J. 2007;48(4):385-95.

Papadakis ED, Nicklin SA, Baker AH, White SJ. Promoters and control elements: designing expression cassettes for gene therapy. Curr. Gene Ther. 2004;4(1):89-113.

Park H, Poo MM. Neurotrophin regulation of neural circuit development and function. Nat. Rev. Neurosci. 2013;14(1):7-23.

Pezet S, Malcangio M, McMahon SB. BDNF: a neuromodulator in nociceptive pathways? Brain Res. Brain Res. Rev. 2002;40(1-3):240-9.

Poo MM. Neurotrophins as synaptic modulators. Nat. Rev. Neurosci. 2001;2(1):24-32.

Pooler AM, Phillips EC, Lau DH, Noble W, Hanger DP. Physiological release of endogenous tau is stimulated by neuronal activity. EMBO Rep. 2013. Epub ahead of print.

Rosas-Vargas H, Martinez-Ezquerro JD, Bienvenu T. Brain-derived neurotrophic factor, food intake regulation, and obesity. Arch. Med. Res. 2011;42(6):482-94.

Scharfman HE. Hyperexcitability in combined entorhinal/hippocampal slices of adult rat after exposure to brain-derived neurotrophic factor. J. Neurophysiol. 1997;78(2):1082-95.

Schnell L, Schneider R, Kolbeck R, Barde YA, Schwab ME. Neurotrophin-3 enhances sprouting of corticospinal tract during development and after adult spinal cord lesion. Nature. 1994;367(6459):170-3.

Schwartz M, Yoles E. Immune-based therapy for spinal cord repair: autologous macrophages and beyond. J. Neurotrauma. 2006;23(3-4):360-70.

Sirtori V, Corbetta D, Moja L, Gatti R. Constraint-induced movement

therapy for upper extremities in stroke patients. Cochrane Database Syst. Rev. 2009(4):CD004433.

Smith RR, Shum-Siu A, Baltzley R, Bunger M, Baldini A, Burke DA, et al. Effects of swimming on functional recovery after incomplete spinal cord injury in rats. J. Neurotrauma. 2006;23(6):908-19.

Sprengel R, Hasan MT. Tetracycline-controlled genetic switches. Handb. Exp. Pharmacol. 2007;(178):49-72.

Stieger K, Belbellaa B, Le Guiner C, Moullier P, Rolling F. In vivo gene regulation using tetracycline-regulatable systems. Adv. Drug Deliv. Rev. 2009;61(7-8):527-41.

Szokol K, Perreault MC. Imaging synaptically mediated responses produced by brainstem inputs onto identified spinal neurons in the neonatal mouse. J. Neurosci Methods. 2009;180(1):1-8.

Taylor L, Jones L, Tuszynski MH, Blesch A. Neurotrophin-3 gradients established by lentiviral gene delivery promote short-distance axonal bridging beyond cellular grafts in the injured spinal cord. J. Neurosci. 2006;26(38):9713-21.

Van Vliet KM, Blouin V, Brument N, Agbandje-McKenna M, Snyder RO. The role of the adeno-associated virus capsid in gene transfer. Methods Mol. Biol. 2008;437:51-91.

Vavrek R, Girgis J, Tetzlaff W, Hiebert GW, Fouad K. BDNF promotes connections of corticospinal neurons onto spared descending interneurons in spinal cord injured rats. Brain. 2006;129(Pt

6):1534-45.

Vaynman S, Gomez-Pinilla F. License to run: exercise impacts functional plasticity in the intact and injured central nervous system by using neurotrophins. Neurorehabil. Neural Repair. 2005;19(4):283-95.

Vilaboa N, Voellmy R. Regulatable gene expression systems for gene therapy. Curr. Gene Ther. 2006;6(4):421-38.

Wang C, Wang CM, Clark KR, Sferra TJ. Recombinant AAV serotype 1 transduction efficiency and tropism in the murine brain. Gene Ther. 2003;10(17):1528-34.

Weidner N, Ner A, Salimi N, Tuszynski MH. Spontaneous corticospinal axonal plasticity and functional recovery after adult central nervous system injury. Proc. Natl. Acad. Sci. USA. 2001;98(6):3513-8.

Weishaupt N, Blesch A, Fouad K. BDNF: the career of a multifaceted neurotrophin in spinal cord injury. Exp. Neurol. 2012;238(2):254-64.

Whishaw IQ, Gorny B, Foroud A, Kleim JA. Long-Evans and Sprague-Dawley rats have similar skilled reaching success and limb representations in motor cortex but different movements: some cautionary insights into the selection of rat strains for neurobiological motor research. Behav. Brain Res. 2003;145(1-2):221-32.

Whishaw IQ, O'Connor WT, Dunnett SB. The contributions of motor cortex,

nigrostriatal dopamine and caudate-putamen to skilled forelimb use in the rat. Brain. 1986;109 (Pt 5):805-43.

Whishaw IQ, Pellis SM, Gorny BP, Pellis VC. The impairments in reaching and the movements of compensation in rats with motor cortex lesions: an endpoint, videorecording, and movement notation analysis. Behav. Brain Res. 1991;42(1):77-91.

Yamagata Y, Kaneko K, Kase D, Ishihara H, Nairn AC, Obata K, et al. Regulation of ERK1/2 mitogen-activated protein kinase by NMDA-receptor-induced seizure activity in cortical slices. Brain Res. 2013. Epub ahead of print.

Yan Q, Rosenfeld RD, Matheson CR, Hawkins N, Lopez OT, Bennett L, et al. Expression of brain-derived neurotrophic factor protein in the adult rat central nervous system. Neuroscience. 1997;78(2):431-48.

Ye JH, Houle JD. Treatment of the chronically injured spinal cord with neurotrophic factors can promote axonal regeneration from supraspinal neurons. Exp. Neurol. 1997;143(1):70-81.

Zhou L, Baumgartner BJ, Hill-Felberg SJ, McGowen LR, Shine HD. Neurotrophin-3 expressed in situ induces axonal plasticity in the adult injured spinal cord. J. Neurosci. 2003;23(4):1424-31.

Zhu WJ, Roper SN. Brain-derived neurotrophic factor enhances fast excitatory synaptic transmission in human epileptic dentate gyrus. Ann. Neurol. 2001;50(2):188-94.