University of Alberta

Rehabilitative reaching training and plasticity following spinal cord injury in the adult rat

by

Aleksandra Krajacic

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

Spring 2011
Edmonton, Alberta

Examining Committee

_____

Karim Fouad, Faculty of Rehabilitation Medicine, Centre for Neuroscience, Supervisor

_____

Keir Pearson, Department of Physiology, Centre for Neuroscience, Committee member

_____

Jaynie Yang, Dept. of Physical Therapy, Centre for Neuroscience, Committee member

_____

Simon Gosgnach, Department of Physiology, Centre for Neuroscience

_____

Gillian Muir, University of Saskatchewan, External examiner

To my mom- “Du schaffst das. Jetzt setz Dich hin und schreib!”

Abstract

Injury to the cervical spinal cord is a devastating event that results in a transient to permanent loss of sensory and motor functions following injury. Moderate recovery has been reported to occur in individuals and in animal models after spinal cord injury (SCI). One approach to promote recovery after SCI is rehabilitative training. This thesis examines the relation of reaching training with adaptive changes (i.e. plasticity) and functional recovery following SCI. In my first experiment, I investigated whether plasticity of the corticospinal tract (CST) is the cause for reaching recovery after ablation of the dorsal and lateral CST. Rats that received reaching training were significantly better in reaching than their untrained counterparts. A relesion of the CST revealed that the reaching recovery mainly depended on plasticity of the CST itself.

Since it is controversial whether training should be initiated immediately after SCI, I investigated whether a delayed initiation of reaching training after SCI is beneficial. I compared the reaching success of rats that received reaching training on day 4 post SCI with rats that received training on day 12 post SCI. I found that the reaching success in rats that either received reaching training on day 4 or 12 following SCI was similar.

Lastly, I investigated whether training efficacy is declined in chronically injured rats. Since it has been shown that the inflammatory response after SCI declines, it is questionable whether there is a relation between the inflammatory response after SCI and training efficacy. In my last experiment I injected chronically injured rats with a substance that induces a systemic inflammation. I

found that rehabilitative reaching training in chronic injured rats only resulted in an improved reaching recovery when the training was combined with the administration of the substance that induces inflammation (lipopolysaccharide).

Although there are still unanswered questions regarding the underlying mechanism for functional recovery after SCI, the results of this thesis could be used as a basic to improve future rehabilitative training strategies and therefore improve the quality of life in individuals that suffer from SCI.

Acknowledgements

I would like to thank my supervisor and mentor Dr. Karim Fouad for being such an excellent supervisor. I definitely admire his critical thinking, strong writing and editing skills that gave my manuscripts perfection. I am very grateful for all of his suggestions and the advice he gave me during my PhD.

I thank my supervisory committee Dr. Keir Pearson and Dr. Jaynie Yang for their guidance, contributions and their patience. I would also like to thank my examining committee: Dr. Gosgnach and Dr. Muir.

Thanks to Dr. Tetzlaff, who performed the pyramid lesions. I thank Dr. Pearse and Mousumi Ghosh who performed the Western blots of the delayed training study.

A big thank you to all my "little" participants who dedicated their lives towards research.

Thanks to Carol Ann Johnson and Megan Airmet for their assistance and for keeping me on track for my degree. I would like to thank Romana Vavrek, and Lisa (Siwei) Li for their conversations and support. Thanks to Caitlin Hurd and Nina Weishaupt for reading and editing my thesis and for being such great friends. I thank my good friends Kathrin Gutsche, Antje Bock, Francois Roy, Sylvia Ciurysek, Kelly Brunton, Katelyn Brown, Cheryl Pilipchuk and Lisa Scott. You've always been there for me and helped me to stay strong.

A special thanks to my parents, Dirkje and Dragutin Krajacic and my sisters, Patricia and Natascha, who encouraged and supported me in every situation over the last 5 years. I definitely appreciate all the phone calls that we had, since we are so far away from each other. I couldn't have done this without you!

I would like to thank Herb, Dianne and Jessica King for their support and many special thanks to my beloved boyfriend and soulmate Grant King, who supported and listened to me, and discussed the Hebbian theory with me. Thank you Grant for being part of team "awesome"!

Table of Contents

List of Figures

1. REHABILITATION AFTER INCOMPLETE SPINAL CORD INJURY

2. TRAINING-INDUCED PLASTICITY IN RATS WITH CERVICAL SPINAL CORD INJURY: EFFECTS AND SIDE EFFECTS

3. ADVANTAGES OF DELAYING THE ONSET OF REHABILITATIVE RACHING TRAINING IN RATS WITH IMCOMPLETE SPINAL CORD INJURY

4. COUNTERACTING THE DECLINE OF TRAINING EFFICACY IN SPINAL CORD INJURED RATS

5. INTERPRETATION OF RESULTS OF REHABILITATIVE REACHING TRAINING AND PLASTICITY FOLLOWING SPAINL CORD INJURY IN THE ADULT RAT

6. APPENDIX

LESION SIZES

Abbreviations

ABC – Avidin-biotin complex

BCA – Bicinchoninic acid

BDA – Biotinylated dextran amine

BDNF – Brain-derived neurotrophic factor

BSA – Bovine serum albumin

BSCB – Blood-spinal cord barrier

cAMP – Cyclic adenosine monophosphate

CSPG – Chondroitin sulfate proteoglycan

CST – Corticospinal tract

CNS – Central nervous system

DAB – Diaminobenzidene

DLQ- lesion – Dorso-lateral quadrant lesion

ELISA – Enzyme-linked immunosorbent assay

EMG – Electromyography

GABA – Gamma (γ)-aminobutyric acid

ICMS – Intracortical micro-stimulation

IgG – Immunoglobulin G

i.p – Intraperitoneally

LPS – Lipopolysaccharide

MAG – Myelin associated glycoprotein

MEP – Motor evoked potential

NSCISC – National Spinal Cord Injury Statistical Center

Nogo-A – A myelin associated glycoprotein

NT-3 – Neurotrophin-3

Omgp – Oligodendrocyte myelin associated glycoprotein

PDE – Phosphodiesterase

PKA – Protein kinase A

PMSF – Phenylmethylsufonyl fluoride

RST – Rubrospinal tract

RT – Room temperature

SCI – Spinal cord injury

SEM – Standard error of the mean

TBS – Tris-buffered saline

TBS-T – Tris-buffered saline containing 0.15% Tween 20

TrkB – Tropomyosin-receptor-kinase B

CHAPTER 1

REHABILITATIVE REACHING TRAINING AND PLASTICITY FOLLOWING SPINAL CORD INJURY IN THE ADULT RAT

1.1 Spinal cord injury

The spinal cord is a part of the central nervous system (CNS) that serves as an interface between the brain and the periphery and allows them to communicate with each other. The spinal cord is not only a caudal outgrowth of the brain, but rather a highly specialized structure that controls reflexes and contains neural circuitries. Injury to the mammalian spinal cord is a devastating event that, depending on the lesion severity, can result in transient or permanent loss of sensation and motor function below the injury. Spinal cord injury (SCI) affects more than 250,000 people in the United States with more than 12,000 new cases occurring annually (as reviewed in Darian-Smith, 2009; National Spinal Cord Injury Statistical Center (NSCISC)). During the last decade the NSCISC reported that most injuries occur at the cervical level of the spinal cord than in the previous decades. The cervical spinal cord contains motor neuron pools that innervate muscles of the upper extremities. Injury to the cervical spinal cord can result in loss of sensorimotor functions below the level of injury including arm and hand function. In this respect, individuals with SCI are left with invalidity which most consider as a diminishment of their quality of life. The average age of those living with SCI is 40.2 years, however individuals with SCI are considered to retain a nearly normal life expectancy. A recent survey shows that individuals suffering from quadriplegia (paralysis of all limbs and torso), rank arm function the highest in improving their quality of life (Anderson, 2004; figure 1.1). The reason for this desire is that the ability to use their arms would make these individuals more

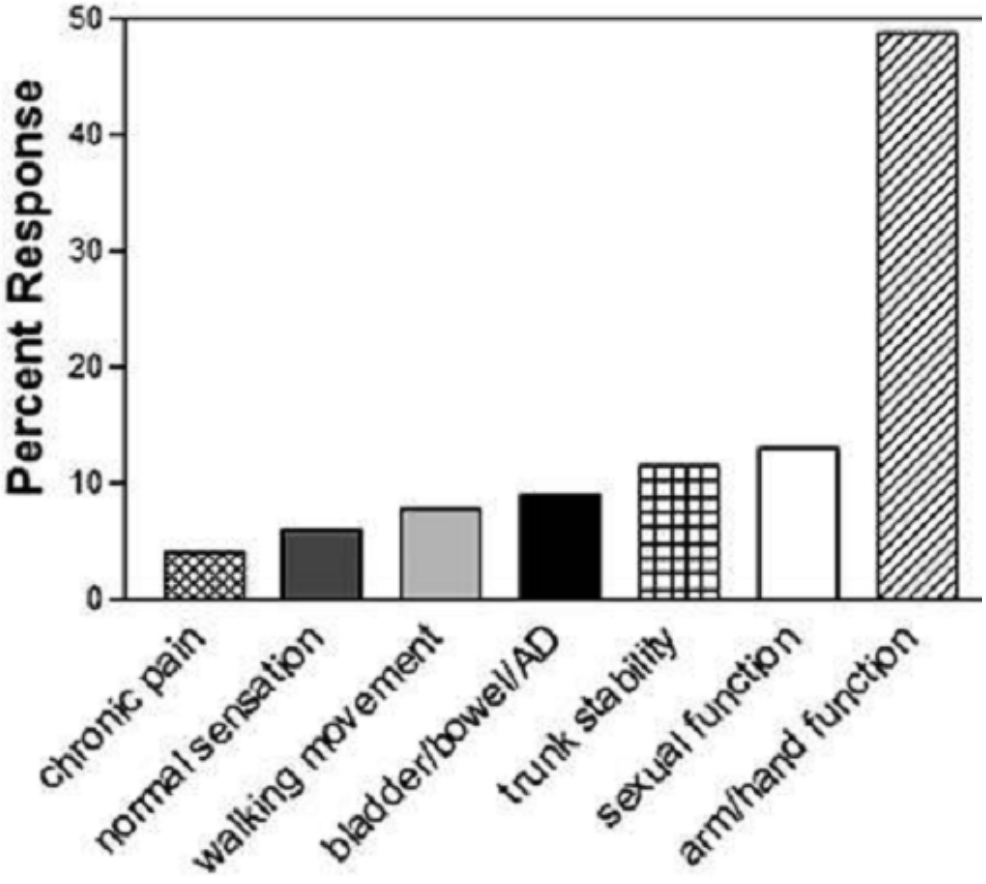


Figure 1.1. Ranking of the most desired regain of function in quadriplegics. The bar graph represents areas where functional recovery is rated the highest in quadriplegics. Percent response is the percentage of individuals ranking that function as being most important for improving their quality of life (Anderson, 2004).

independent. Thus, treatments that can diminish their disabilities regarding arm and hand functions are of high importance.

In order to design treatment strategies it is important to understand what happens after SCI on a physiological level. One major problem is that injury to the spinal cord is accompanied by several events such as haemorrhage, vascular damage and disruption of the blood-spinal cord barrier (BSCB; as reviewed in Hausmann, 2003). The disruption of the BSCB leads to an invasion of immune cells after SCI into the lesioned area. The following section gives a detailed description of the consequences after a BSCB disruption.

1.2 Disruption of the blood-spinal barrier and inflammation after SCI

The BSCB usually protects the spinal cord from the entry of potentially harmful immune cells and serum proteins, thereby maintaining the fluid microenvironment within a narrow limit (Sharma, 2005). A disruption of the BSCB facilitates immune cells such as macrophages to infiltrate the lesioned area in a matter of few days (Saville *et al.*, 2004; Jones *et al.*, 2005; Wu *et al.*, 2005; Conta and Stelzner, 2008; Donnelly and Popovich, 2008; see figure 1.2), which triggers a host defense mechanism, also known as a cellular immune response or inflammatory response. The inflammatory response following SCI is a controversial subject as it is still unclear whether the inflammatory response after SCI is beneficial or detrimental to functional recovery and repair of the CNS

(Crutcher *et al.*, 2006). So far it has a predominantly negative reputation. The reason for this is that SCI is accompanied by axonal damage, edema, neuronal cell death and invasion of immune cells such as macrophages through the disruption of the BSCB. It has been suggested that macrophages can release various factors that trigger apoptosis in neurons and glial cells (Stoll *et al.*, 2002)

Animal studies have revealed that SCI activates immune cells that reside around a spinal lesion which then mediate deteriorating processes such as glutamate-induced intracellular Ca^{2+} increase, proteolysis, formation of free radicals and nitric oxide (as reviewed in Hausmann, 2003; Hagg and Oudega, 2006). These processes lead to damage of initially spared tissue, a process that has been referred to as 'secondary injury'. For example, cytotoxic inflammatory products can cause degeneration, demyelination and dysfunction of axons that were not affected by the initial injury. Also, damage to nervous tissue triggers signal cascades that result in a self-perpetuating progression of degenerative processes and cause the development of a fluid filled cyst that persists in the chronic state (Ahn *et al.*, 2006). The cyst cavity is filled with granular debris of myelinated and unmyelinated axons, and is interspersed with macrophages. Additionally, the cyst is enclosed by activated microglia and astrocytes which play a principal role in the scarring process after SCI, which is explained explicitly in the next section.

1.3 The glial scar and inhibition of regeneration

Formation of the glial scar (gliosis) is a process that is induced by damage to the CNS. The main cellular components of the glial scar are astrocytes and microglia. Following SCI, the glial scar creates a physical barrier for regenerating axons. For example, it has been shown that the regenerative inhibition of axons, which is found in the white matter of the spinal cord, is caused by myelin associated glycoprotein (MAG), oligodendrocyte myelin glycoprotein (Omgp) and other growth inhibitors that are associated with CNS myelin (i.e., Nogo-A; Schwab and Caroni, 1988). These myelin associated growth inhibitors can decrease the chance of regeneration through certain receptors and intracellular signalling pathways in the axon. Also, several extracellular matrix proteins have been shown to influence the regenerative failure of axons. Some of these extracellular proteins include tenascin as well as certain proteoglycans, especially chondroitin sulfate proteoglycans (CSPG). The latter have been shown to have properties that inhibit axonal growth in vitro (Snow *et al.*, 1990; McKeon *et al.*, 1995) and in vivo (Fitch and Silver, 1997). Together, these mechanisms are the reason why the inhibitory environment in the mammalian CNS was ascribed to be the cause for the inability of axons to regenerate (David and Aguayo, 1981; Schwab and Thoenen, 1985; Schwab and Caroni, 1988). As a result of axonal regeneration failure within and around the spinal lesion, the overall recovery in motor and sensory functions below the spinal lesion is very limited (Schwab and Bartholdi, 1996). The increasing knowledge in discovered intra- and extracellular

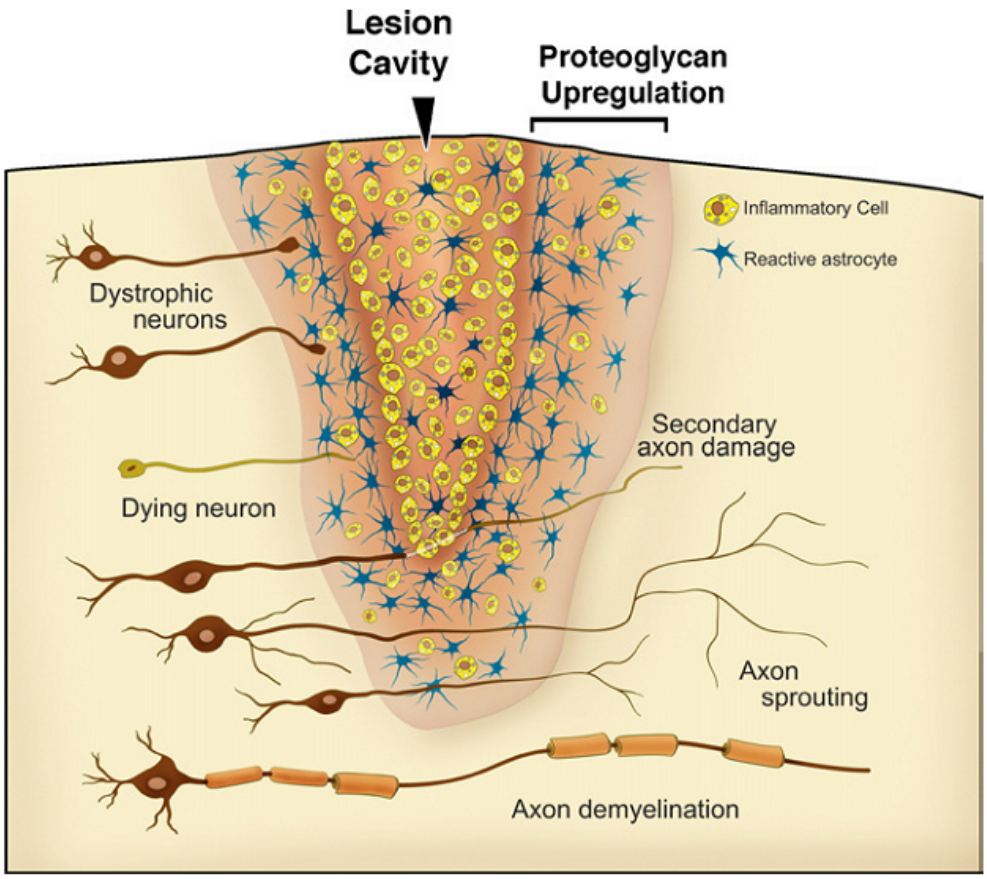


Figure 1.2. Illustration of mechanisms occurring following injury to the spinal cord. Damage to the spinal cord leaves a lesion cavity that is surrounded by a glial scar (proteoglycan up-regulation in astrocytes). Neurons that are injured die or withdraw from the lesion. Spared axons sprout and form new connections. Adapted from Fitch and Silver (2008).

inhibitory signalling pathways attract scientists to design approaches to circumvent this problem of regeneration failure and to promote treatment strategies after SCI (Hagg and Oudega, 2006). Approaches to improve axon regeneration and functional recovery include the prevention of neuronal tissue loss, promoting axonal growth of injured fibers (Hagg and Oudega, 2006), remyelination of spared fibers (Jeffery and Blakemore, 1997), implantation of cellular grafts of various types to create a favourable environment for regenerating axons (Ramon-Cueto *et al.*, 2000; Pearse *et al.*, 2004), modification of extracellular matrix proteins (Bradbury et al. 2002) and blocking the myelin associated inhibitory molecules with antibodies (Schnell and Schwab, 1993; Liebscher *et al.*, 2005). These topics however fall outside the scope of this thesis and the reader is referred to more adequate reviews regarding these subjects (Hagg and Oudega, 2006; Boulenguez and Vinay, 2009).

It is noteworthy that some recovery occurs in patients and animal models with incomplete SCI. This spontaneous recovery occurs over weeks and months after a phase of spinal shock in which the spinal tissue below the level of injury remains unexcitable (Hiersemenzel *et al.*, 2000). The underlying mechanisms of such recovery are described in the next section.

1.4 Plasticity and recovery after SCI

More studies provide evidence that the entire CNS is undergoing injury-induced adaptations, generally referred to as neuronal plasticity (Liu and Chambers, 1958; Murray and Goldberger, 1974; Basbaum and Wall, 1976; Fawcett, 2002; Fouad and Tse, 2008). This is surprising as for many decades it was believed that the mammalian CNS is rigid, hard-wired and not capable of such adaptations. It has now been suggested that adaptations of the CNS occur continuously throughout the life of an individual. This is surprising as adaptations (i.e. plasticity) of the CNS have been only reported to occur in certain developmental stages. First evidence for plasticity in such developmental stages came from experiments by Wiesel and Hubel where they showed that the CNS passes through "critical periods" in which unused neural connections are pruned during the development of the visual system of the cat (Hubel and Wiesel, 1970). This suggests that rearrangements, such as forming new connections as well as pruning, are activity-dependent, which was first postulated by the Canadian researcher Donald Hebb in 1949. He stated that only "neurons that fire together, wire together". This phenomenon of activity-dependent rearrangement/ plasticity also occurs following SCI in humans. It was shown that the cortical representation of the hands in humans rearranges following SCI (Levy *et al.*, 1990; Topka *et al.*, 1991; Bruehlmeier *et al.*, 1998; Turner *et al.*, 2003). Commonly, the cortical representation of the hand which was affected by the injury decreases and the cortical representation of the non-injured hand increases. This phenomenon might be mediated through the diminished use of the injured hand as well as the

extensive use of the hand that is not or only less affected by the injury. Animal models of SCI reveal that plasticity in neuronal circuitries occurs in the entire CNS, above and below the level of the injury. Reported mechanisms of spontaneously occurring plasticity include changes of neuronal properties, cortical map changes (Fouad et al., 2001), collateral sprouting of lesioned axons rostral to the injury (Bareyre *et al.*, 2004; Courtine *et al.*, 2008) and collateral sprouting of spared fibres caudal to the injury (Weidner *et al.*, 2001; Ballermann and Fouad, 2006). Further, SCI also disconnects motor neurons from their supraspinal inputs and thus these motor neurons no longer receive essential neuromodulators such as serotonin and norepinephrine (Bennett *et al.*, 2004). In response to the loss of these neuromodulators, the receptors on motor neurons within the spinal cord are altered (Boulenguez and Vinay, 2009; Murray *et al.*, 2010). This form of adaptation in particular has been suggested to be one potential mechanism that occurs during the development of spasticity. Neuronal plasticity not only mediates desirable adaptations following SCI but also contributes to a range of undesirable effects besides spasticity such as chronic pain (Mariano, 1992) and autonomic dysreflexia (Weaver et al., 2001). For example, investigations in the field of neuronal plasticity are used to diminish such undesirable consequences of SCI.

As functional recovery following SCI is also linked to neuronal plasticity, many laboratories are addressing this issue by investigating the underlying mechanisms, principles and limitations of adaptive changes. More research is needed however to link various forms of plasticity to functional impairments and improvements. A study that focuses on this issue reported that compensatory

movement strategies are acquired following incomplete SCI and it is thought that injury-induced plasticity might be related to compensatory movement strategies (McKenna and Whishaw, 1999).

One structure that is often damaged after cervical SCI in humans and has been shown to undergo plasticity, is the corticospinal tract (CST). The CST is a descending pathway that originates in the cortex, projects to the cervical and lumbar enlargements in the spinal cord and is involved in controlling voluntary movements of the digits. It has been reported that sensorimotor recovery after SCI can be linked to neuronal plasticity (Kanagal and Muir, 2009) (e.g. axonal sprouting) in descending systems including the CST and the rubrospinal tract (RST; Weidner *et al.*, 2001). The improved recovery occurred in parallel with increased sprouting of the CST at various levels of the CNS (see figure 1.3; Weidner *et al.*, 2001; Girgis *et al.*, 2007). Approaches that can promote plasticity after SCI are mentioned in the next section.

1.5 Approaches to promote plasticity after SCI

One approach to promote plasticity is to find strategies to circumvent the inhibitory environment and promote plasticity within and around the lesion after SCI attracted. For instance, it has been reported that following SCI in rats the application of a Nogo-A antibody (mAB IN-1), which decreases the inhibitory activity of oligodendrocytes and myelin, resulted in enhanced sprouting of RST fibers towards the grey matter (Raineteau *et al.*, 2001).

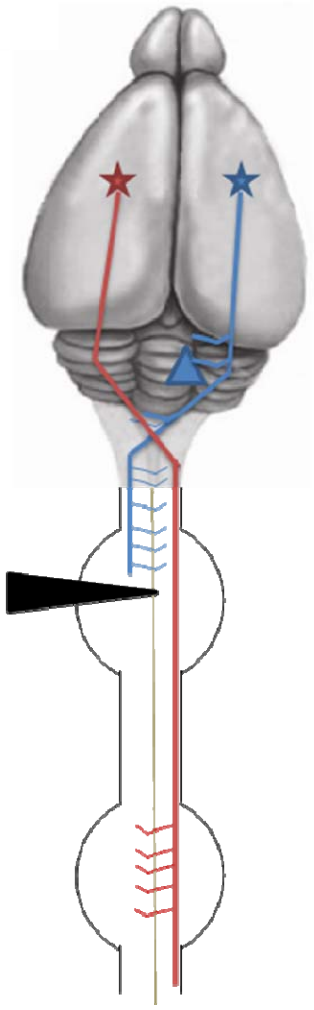


Figure 1.3. Plasticity of the CST occurs at various levels of the CNS following SCI. The projections of the cortical neurons that give rise to the CST (red and blue star), cross at the level of the medulla and project down the spinal cord (red line). If the CST is injured, sprouting of damaged and spared fibers can occur above (shown as blue horizontal lines) and below (shown as red horizontal lines) the lesion (black triangle), respectively.

Besides Nogo-A inhibition, the digestion of sugar chains of the glial scar (CSPGs) by chondroitinase ABC was also shown to reactivate plasticity (Kwok *et al.*, 2008) and promote recovery following SCI (as reviewed in Bradbury and Carter, 2010). Other approaches to promote plasticity after SCI focused on the application of neurotrophic factors such as neurotrophin 3 and brain-derived-neurotrophic factor (BDNF) because these factors are known to have neuroprotective properties. Additionally, their exogenous application was shown to minimize secondary damage after SCI (Tobias *et al.*, 2003). BDNF was reported to bind on both a high- (TrkB) and a low-affinity receptor (p75). It has been suggested that BDNF promotes neuronal survival and axonal sprouting through the activation of an adenylyl cyclase and consequently increases intracellular cyclic adenosine monophosphate (cAMP) levels (Cai *et al.*, 2001; Gao *et al.*, 2003; Gordon *et al.*, 2009). cAMP is a second messenger that has been shown to be involved, among other processes, in the process of learning and memory. Further, it has been reported that artificially increasing intraneuronal cAMP levels can promote axonal growth in the presence of myelin-associated inhibitors (Cai et al., 2001).

Another approach to promote plasticity after SCI is the administration of electrical stimulation. It has been shown that electrical stimulation of the motor cortex also increases the expression of BDNF (Carmel *et al.*, 2010; Fritsch *et al.*, 2010) and results in adaptive changes of the CST (Nitsche and Paulus, 2000). It has been suggested that increased neuronal activity can enhance cortical cAMP levels through BDNF signalling and by directly activating voltage- and Ca^{++}

dependent adenylyl cyclases (Ferrendelli *et al.*, 1980; Xia *et al.*, 1991; Reddy *et al.*, 1995). Further, an earlier study from Girgis *et al.* (2007) revealed that rats with cervical SCI receiving rehabilitative reaching training had an increase in markers for plasticity (GAP-43 expression) within the cortex. To date the most successful approach to promote plasticity and functional recovery following SCI is rehabilitative training, which is described in detail in the next section.

1.6 Rehabilitative training as a tool to promote plasticity

The history of using rehabilitative training as a tool to improve functional recovery after SCI began with studies of spinalized cats. The discovery that chronic cats with a complete transaction of the spinal cord were still able to step without the descending input from the brain (Lovely *et al.*, 1986) led to the assumption that the spinal cord contains networks and circuitries (Sherrington, 1910; Brown, 1914). With the application of treadmill training animals regained their stepping ability and were able to support their body weight within a few weeks. This suggests that the spinal cord can not only learn or adapt but also that such adaptations (plasticity) are activity-dependent. A study with individuals suffering from SCI and exposed to treadmill training showed that the training not only improved locomotor recovery but also improved the connectivity of spared corticospinal pathways (Thomas and Gorassini, 2005). Treadmill training is now probably the best established training strategy that has been successfully translated from animal studies into the clinic.

Besides treadmill training various other approaches have been addressed to mediate rehabilitative training following injury to the CNS in humans and animal models including constraint movement therapy (Taub *et al.*, 1999), running wheel training (Engesser-Cesar *et al.*, 2005), robotically assisted training (de Leon *et al.*, 2002) and reaching training (Girgis *et al.*, 2007). Since it was shown that the reaching pattern in rodents and humans is similar, reaching training in rodents with SCI has become an approach to study treatments to promote functional recovery. For example, a recent study by Girgis and colleagues (2007) showed

that rats that were exposed to rehabilitative reaching training following a cervical SCI had a significant increase in reaching recovery when compared to their untrained counterparts. This proves the power of rehabilitative training but also poses one question: "What the possible underlying mechanisms for functional improvements?". So far, one underlying mechanism that has been reported to be related to the exposure to rehabilitative training is the increased expression of brain-derived neurotrophic factor (BDNF; Ying *et al.*, 2008). The relation between training-induced BDNF-expression and the up-regulation of cAMP is interesting because cAMP seems to be involved in injury-induced mechanisms. Therefore it is important to investigate the relationship between cAMP and rehabilitative training.

One major problem that has been discussed is the optimal time point to initiate rehabilitative training following SCI. The onset of rehabilitative training in patients is usually delayed by a phase of spinal shock and other injuries that occurred in parallel with SCI. In a few hospitals however, individuals are exposed to weight-supported treadmill training that allows them to start with their rehabilitative training early after SCI (as reported in Dobkin *et al.*, 2003). Recently it has been reported that rehabilitative training in rodents (Norrie *et al.*, 2005) as well as patients is more efficient when initiated immediately or within a few days following SCI (Winchester *et al.*, 2005; 2009). This is based on findings that growth- and plasticity associated genes are up-regulated directly after SCI (Song *et al.*, 2001; Di Giovanni *et al.*, 2005). Additionally, it has been reported that BDNF levels in the grey matter of rodents peak within 24 hours after the

BSCB becomes permeable. On one hand emphasizes that training immediately following SCI could be beneficial as a state of increased plasticity in the CNS would further promote training-induced recovery. On the other hand it suggests the existence of a "window of opportunity" in which the CNS exhibits enhanced plasticity following SCI. There are speculations about the time frame of the "window of opportunity", but it appears that the more delayed the initiation of rehabilitative training, the lesser is the achieved functional recovery. In Chapter 3, the issue of early versus delayed training is described in detail.

1.7 Interaction of rehabilitative training with pharmacological interventions

Pharmacological treatments such as the administration of BDNF or NT-3 has been shown to promote axonal growth following SCI (Zhou and Shine, 2003) but do not translate into improved functional recovery. Conversely, rehabilitative training has been shown to increase plasticity and promote functional recovery in rats (Girgis *et al.*, 2007). A combination of pharmacologically enhanced axonal growth with training would be the next logical step as the enhanced sprouting could be tuned by rehabilitative training to make it more functional. One problem with this approach is that it still remains unknown when rehabilitative training should be initiated and when plasticity promoting treatments should be applied after SCI.

As mentioned earlier, it has been shown that cAMP levels decline gradually following SCI. Another, partially neglected process that also gradually declines after SCI is the injury-induced inflammatory response. Inflammation after SCI is thought to be detrimental to nervous tissue, however, it is very controversial now whether the contribution of the inflammatory response after injury to the CNS could also be beneficial. Accumulating evidence suggests that the injury-induced inflammation not only contributes to pathology but is also involved in neuro-reparative functions (Schwartz *et al.*, 1999; Schwartz and Yoles, 2006; Yin *et al.*, 2006; Beck *et al.*, 2010). For example, macrophages have been shown to also promote the regeneration of CNS axons (Gensel *et al.*, 2009). This would emphasize the role of the inflammatory response in creating a 'window of opportunity' following SCI. If this is true, then the reintroduction of an inflammatory response in a chronic model of SCI will allow for enhanced functional recovery. Evidence for is provided by a study from Chen et al. (2008) who found that a reintroduction of an inflammatory response is required for NT-3-induced axonal plasticity in chronic injured rats. This NT-3-induced plasticity however was apparent when chronic injured rats were immunosuppressed. The reintroduction of inflammation was achieved by the administration of the bacterial endotoxin Lipopolysaccharide (LPS). LPS induces inflammation systemically, leads to sickness and transient behavioural changes in animals (Dantzer, 2004) and activates intracellular signalling pathways that elicit a macrophage response similar to what has been seen after CNS injury. A study that addressed the effect of LPS after SCI shows that LPS is also involved in the expression of growth-

associated genes (Hossain-Ibrahim *et al.*, 2006). This suggests that the administration of LPS might induce plasticity and promote functional recovery. If a reintroduction of inflammation in a chronic model of SCI can promote plasticity, the application of reaching training in combination with the inflammation should result in an improved recovery after SCI. This matter will be addressed in Chapter 4 of this thesis.

1.8 Summary

SCI is an event that disconnects neurons from their target cells, which can result in a transient to permanent loss in sensorimotor functions. Following an incomplete lesion of the spinal cord spontaneous and trainig-induced recovery has been reported to occur.

The aim of this thesis was to investigate structures that are involved in reaching recovery (Chapter 2) and to promote reaching performance by the administration of rehabilitative reaching training in rats with cervical SCI. Additionally, it seemed to be a necessity to explore whether reaching training that was initiated on day 12 post-SCI could still be efficacious in promoting recovery (Chapter 3) since there is no standard when reaching training after SCI should be initiated.

Finally, if the inflammatory response after SCI plays a role in recovery, can a reinstatement of inflammation at a chronic stage after SCI enhance the reaching performance? This issue is addressed in Chapter 4 in the present thesis.

1.9 References

Ahn Y. H., Lee G. and Kang S. K. (2006). "Molecular insights of the injured lesions of rat spinal cords: Inflammation, apoptosis, and cell survival." Biochem Biophys Res Commun 348(2): 560-70.

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

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

Bareyre F. M., Kerschensteiner M., Raineteau O., Mettenleiter T. C., Weinmann O. and Schwab M. E. (2004). "The injured spinal cord spontaneously forms a new intraspinal circuit in adult rats." Nat Neurosci 7(3): 269-77.

Basbaum A. I. and Wall P. D. (1976). "Chronic changes in the response of cells in adult cat dorsal horn following partial deafferentation: the appearance of responding cells in a previously non-responsive region." Brain Res 116(2): 181-204.

Beck K. D., Nguyen H. X., Galvan M. D., Salazar D. L., Woodruff T. M. and Anderson A. J. (2010). "Quantitative analysis of cellular inflammation after traumatic spinal cord injury: evidence for a multiphasic inflammatory response in the acute to chronic environment." Brain 133(Pt 2): 433-47.

Bennett D. J., Sanelli L., Cooke C. L., Harvey P. J. and Gorassini M. A. (2004). "Spastic long-lasting reflexes in the awake rat after sacral spinal cord injury." J Neurophysiol 91(5): 2247-58.

Boulenguez P. and Vinay L. (2009). "Strategies to restore motor functions after spinal cord injury." Curr Opin Neurobiol 19(6): 587-600.

Bradbury E. J. and Carter L. M. (2010). "Manipulating the glial scar: Chondroitinase ABC as a therapy for spinal cord injury." Brain Res Bull.

Brown T. G. (1914). "On the nature of the fundamental activity of the nervous centres; together with an analysis of the conditioning of rhythmic activity in progression, and a theory of the evolution of function in the nervous system." J Physiol 48(1): 18-46.

Bruehlmeier M., Dietz V., Leenders K. L., Roelcke U., Missimer J. and Curt A. (1998). "How does the human brain deal with a spinal cord injury?" Eur J Neurosci 10(12): 3918-22.

Cai D., Qiu J., Cao Z., McAtee M., Bregman B. S. and Filbin M. T. (2001). "Neuronal cyclic AMP controls the developmental loss in ability of axons to regenerate." J Neurosci 21(13): 4731-9.

Carmel J. B., Berrol L. J., Brus-Ramer M. and Martin J. H. (2010). "Chronic electrical stimulation of the intact corticospinal system after unilateral injury restores skilled locomotor control and promotes spinal axon outgrowth." J Neurosci 30(32): 10918-26.

Chen Q., Smith G. M. and Shine H. D. (2008). "Immune activation is required for NT-3-induced axonal plasticity in chronic spinal cord injury." Exp Neurol 209(2): 497-509.

Conta A. C. and Stelzner D. J. (2008). "Immunomodulatory effect of the purine nucleoside inosine following spinal cord contusion injury in rat." Spinal Cord 46(1): 39-44.

Courtine G., Song B., Roy R. R., Zhong H., Herrmann J. E., Ao Y., Qi J., Edgerton V. R. and Sofroniew M. V. (2008). "Recovery of supraspinal control of stepping via indirect propriospinal relay connections after spinal cord injury." Nat Med 14(1): 69-74.

Crutcher K. A., Gendelman H. E., Kipnis J., Perez-Polo J. R., Perry V. H., Popovich P. G. and Weaver L. C. (2006). "Debate: "is increasing neuroinflammation beneficial for neural repair?"" J Neuroimmune Pharmacol 1(3): 195-211.

Dantzer R. (2004). "Cytokine-induced sickness behaviour: a neuroimmune response to activation of innate immunity." Eur J Pharmacol 500(1-3): 399-411.

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

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

de Leon R. D., Kubasak M. D., Phelps P. E., Timoszyk W. K., Reinkensmeyer D. J., Roy R. R. and Edgerton V. R. (2002). "Using robotics to teach the spinal cord to walk." Brain Res Brain Res Rev 40(1-3): 267-73.

Di Giovanni S., Faden A. I., Yakovlev A., Duke-Cohan J. S., Finn T., Thouin M., Knoblach S., De Biase A., Bregman B. S. and Hoffman E. P. (2005). "Neuronal plasticity after spinal cord injury: identification of a gene cluster driving neurite outgrowth." FASEB J 19(1): 153-4.

Dobkin B. H., Apple D., Barbeau H., Basso M., Behrman A., Deforge D., Ditunno J., Dudley G., Elashoff R., Fugate L., Harkema S., Saulino M. and Scott M. (2003). "Methods for a randomized trial of weight-supported treadmill training versus conventional training for walking during inpatient rehabilitation after incomplete traumatic spinal cord injury." Neurorehabil Neural Repair 17(3): 153-67.

Donnelly D. J. and Popovich P. G. (2008). "Inflammation and its role in neuroprotection, axonal regeneration and functional recovery after spinal cord injury." Exp Neurol 209(2): 378-88.

Engesser-Cesar C., Anderson A. J., Basso D. M., Edgerton V. R. and Cotman C. W. (2005). "Voluntary wheel running improves recovery from a moderate spinal cord injury." J Neurotrauma 22(1): 157-71.

Fawcett J. (2002). "Repair of spinal cord injuries: where are we, where are we going?" Spinal Cord 40(12): 615-23.

Ferrendelli J. A., Blank A. C. and Gross R. A. (1980). "Relationships between seizure activity and cyclic nucleotide levels in brain." Brain Res 200(1): 93-103.

Fitch M. T. and Silver J. (1997). "Activated macrophages and the blood-brain barrier: inflammation after CNS injury leads to increases in putative inhibitory molecules." Exp Neurol 148(2): 587-603.

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

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

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

Fritsch B., Reis J., Martinowich K., Schambra H. M., Ji Y., Cohen L. G. and Lu B. (2010). "Direct current stimulation promotes BDNF-dependent synaptic plasticity: potential implications for motor learning." Neuron 66(2): 198-204.

Gao Y., Nikulina E., Mellado W. and Filbin M. T. (2003). "Neurotrophins elevate cAMP to reach a threshold required to overcome inhibition by MAG through extracellular signal-regulated kinase-dependent inhibition of phosphodiesterase." J Neurosci 23(37): 11770-7.

Gensel J. C., Nakamura S., Guan Z., van Rooijen N., Ankeny D. P. and Popovich P. G. (2009). "Macrophages promote axon regeneration with concurrent neurotoxicity." J Neurosci 29(12): 3956-68.

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

Gordon T., Udina E., Verge V. M. and de Chaves E. I. (2009). "Brief electrical stimulation accelerates axon regeneration in the peripheral nervous system and promotes sensory axon regeneration in the central nervous system." Motor Control 13(4): 412-41.

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

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

Hiersemenzel L. P., Curt A. and Dietz V. (2000). "From spinal shock to spasticity: neuronal adaptations to a spinal cord injury." Neurology 54(8): 1574-82.

Hossain-Ibrahim M. K., Rezajooi K., MacNally J. K., Mason M. R., Lieberman A. R. and Anderson P. N. (2006). "Effects of lipopolysaccharide-induced inflammation on expression of growth-associated genes by corticospinal neurons." BMC Neurosci 7: 8.

Hubel D. H. and Wiesel T. N. (1970). "The period of susceptibility to the physiological effects of unilateral eye closure in kittens." J Physiol 206(2): 419-36.

Jeffery N. D. and Blakemore W. F. (1997). "Locomotor deficits induced by experimental spinal cord demyelination are abolished by spontaneous remyelination." Brain 120 (Pt 1): 27-37.

Jones T. B., Hart R. P. and Popovich P. G. (2005). "Molecular control of physiological and pathological T-cell recruitment after mouse spinal cord injury." J Neurosci 25(28): 6576-83.

Kanagal S. G. and Muir G. D. (2009). "Task-dependent compensation after pyramidal tract and dorsolateral spinal lesions in rats." Exp Neurol 216(1): 193-206.

Kwok J. C., Afshari F., Garcia-Alias G. and Fawcett J. W. (2008). "Proteoglycans in the central nervous system: plasticity, regeneration and their stimulation with chondroitinase ABC." Restor Neurol Neurosci 26(2-3): 131-45.

Levy W. J., Jr., Amassian V. E., Traad M. and Cadwell J. (1990). "Focal magnetic coil stimulation reveals motor cortical system reorganized in humans after traumatic quadriplegia." Brain Res 510(1): 130-4.

Liebscher T., Schnell L., Schnell D., Scholl J., Schneider R., Gullo M., Fouad K., Mir A., Rausch M., Kindler D., Hamers F. P. and Schwab M. E. (2005). "Nogo-A antibody improves regeneration and locomotion of spinal cord-injured rats." Ann Neurol 58(5): 706-19.

Liu C. N. and Chambers W. W. (1958). "Intraspinal sprouting of dorsal root axons; development of new collaterals and preterminals following partial denervation of the spinal cord in the cat." AMA Arch Neurol Psychiatry 79(1): 46-61.

Lovely R. G., Gregor R. J., Roy R. R. and Edgerton V. R. (1986). "Effects of training on the recovery of full-weight-bearing stepping in the adult spinal cat." Exp Neurol 92(2): 421-35.

Mariano A. J. (1992). "Chronic pain and spinal cord injury." Clin J Pain 8(2): 87-92.

McKenna J. E. and Whishaw I. Q. (1999). "Complete compensation in skilled reaching success with associated impairments in limb synergies, after dorsal column lesion in the rat." J Neurosci 19(5): 1885-94.

McKeon R. J., Hoke A. and Silver J. (1995). "Injury-induced proteoglycans inhibit the potential for laminin-mediated axon growth on astrocytic scars." Exp Neurol 136(1): 32-43.

Murray K. C., Nakae A., Stephens M. J., Rank M., D'Amico J., Harvey P. J., Li X., Harris R. L., Ballou E. W., Anelli R., Heckman C. J., Mashimo T., Vavrek R., Sanelli L., Gorassini M. A., Bennett D. J. and Fouad K. (2010). "Recovery of motoneuron and locomotor function after spinal cord injury depends on constitutive activity in 5-HT2C receptors." Nat Med 16(6): 694-700.

Murray M. and Goldberger M. E. (1974). "Restitution of function and collateral sprouting in the cat spinal cord: the partially hemisected animal." J Comp Neurol 158(1): 19-36.

Nitsche M. A. and Paulus W. (2000). "Excitability changes induced in the human motor cortex by weak transcranial direct current stimulation." J Physiol 527 Pt 3: 633-9.

Norrie B. A., Nevett-Duchcherer J. M. and Gorassini M. A. (2005). "Reduced functional recovery by delaying motor training after spinal cord injury." J Neurophysiol 94(1): 255-64.

Pearse D. D., Pereira F. C., Marcillo A. E., Bates M. L., Berrocal Y. A., Filbin M. T. and Bunge M. B. (2004). "cAMP and Schwann cells promote axonal growth and functional recovery after spinal cord injury." Nat Med 10(6): 610-6.

Raineteau O., Fouad K., Noth P., Thallmair M. and Schwab M. E. (2001). "Functional switch between motor tracts in the presence of the mAb IN-1 in the adult rat." Proc Natl Acad Sci U S A 98(12): 6929-34.

Ramon-Cueto A., Cordero M. I., Santos-Benito F. F. and Avila J. (2000). "Functional recovery of paraplegic rats and motor axon regeneration in their spinal cords by olfactory ensheathing glia." Neuron 25(2): 425-35.

Reddy R., Smith D., Wayman G., Wu Z., Villacres E. C. and Storm D. R. (1995). "Voltage-sensitive adenylyl cyclase activity in cultured neurons. A calcium-independent phenomenon." J Biol Chem 270(24): 14340-6.

Saville L. R., Pospisil C. H., Mawhinney L. A., Bao F., Simedrea F. C., Peters A. A., O'Connell P. J., Weaver L. C. and Dekaban G. A. (2004). "A monoclonal antibody to CD11d reduces the inflammatory infiltrate into the injured spinal cord: a potential neuroprotective treatment." J Neuroimmunol 156(1-2): 42-57.

Schnell L. and Schwab M. E. (1993). "Sprouting and regeneration of lesioned corticospinal tract fibres in the adult rat spinal cord." Eur J Neurosci 5(9): 1156-71.

Schwab M. E. and Bartholdi D. (1996). "Degeneration and regeneration of axons in the lesioned spinal cord." Physiol Rev 76(2): 319-70.

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

Schwab M. E. and Thoenen H. (1985). "Dissociated neurons regenerate into sciatic but not optic nerve explants in culture irrespective of neurotrophic factors." J Neurosci 5(9): 2415-23.

Schwartz M., Moalem G., Leibowitz-Amit R. and Cohen I. R. (1999). "Innate and adaptive immune responses can be beneficial for CNS repair." Trends Neurosci 22(7): 295-9.

Schwartz M. and Yoles E. (2006). "Immune-based therapy for spinal cord repair: autologous macrophages and beyond." J Neurotrauma 23(3-4): 360-70.

Sharma H. S. (2005). "Pathophysiology of blood-spinal cord barrier in traumatic injury and repair." Curr Pharm Des 11(11): 1353-89.

Sherrington C. S. (1910). "Flexion-reflex of the limb, crossed extension-reflex, and reflex stepping and standing." J Physiol 40(1-2): 28-121.

Snow D. M., Steindler D. A. and Silver J. (1990). "Molecular and cellular characterization of the glial roof plate of the spinal cord and optic tectum: a possible role for a proteoglycan in the development of an axon barrier." Dev Biol 138(2): 359-76.

Song G., Cechvala C., Resnick D. K., Dempsey R. J. and Rao V. L. (2001). "GeneChip analysis after acute spinal cord injury in rat." J Neurochem 79(4): 804-15.

Stoll G., Jander S. and Schroeter M. (2002). "Detrimental and beneficial effects of injury-induced inflammation and cytokine expression in the nervous system." Adv Exp Med Biol 513: 87-113.

Taub E., Uswatte G. and Pidikiti R. (1999). "Constraint-Induced Movement Therapy: a new family of techniques with broad application to physical rehabilitation--a clinical review." J Rehabil Res Dev 36(3): 237-51.

Thomas S. L. and Gorassini M. A. (2005). "Increases in corticospinal tract function by treadmill training after incomplete spinal cord injury." J Neurophysiol 94(4): 2844-55.

Tobias C. A., Shumsky J. S., Shibata M., Tuszynski M. H., Fischer I., Tessler A. and Murray M. (2003). "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 184(1): 97-113.

Topka H., Cohen L. G., Cole R. A. and Hallett M. (1991). "Reorganization of corticospinal pathways following spinal cord injury." Neurology 41(8): 1276-83.

Turner J. A., Lee J. S., Schandler S. L. and Cohen M. J. (2003). "An fMRI investigation of hand representation in paraplegic humans." Neurorehabil Neural Repair 17(1): 37-47.

Weaver L. C., Verghese P., Bruce J. C., Fehlings M. G., Krenz N. R. and Marsh D. R. (2001). "Autonomic dysreflexia and primary afferent sprouting after

clip-compression injury of the rat spinal cord." J Neurotrauma 18(10): 1107-19.

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

Winchester P., McColl R., Querry R., Foreman N., Mosby J., Tansey K. and Williamson J. (2005). "Changes in supraspinal activation patterns following robotic locomotor therapy in motor-incomplete spinal cord injury." Neurorehabil Neural Repair 19(4): 313-24.

Winchester P., Smith P., Foreman N., Mosby J. M., Pacheco F., Querry R. and Tansey K. (2009). "A prediction model for determining over ground walking speed after locomotor training in persons with motor incomplete spinal cord injury." J Spinal Cord Med 32(1): 63-71.

Wu D., Miyamoto O., Shibuya S., Okada M., Igawa H., Janjua N. A., Norimatsu H. and Itano T. (2005). "Different expression of macrophages and microglia in rat spinal cord contusion injury model at morphological and regional levels." Acta Med Okayama 59(4): 121-7.

Xia Z. G., Refsdal C. D., Merchant K. M., Dorsa D. M. and Storm D. R. (1991). "Distribution of mRNA for the calmodulin-sensitive adenylate cyclase in rat brain: expression in areas associated with learning and memory." Neuron 6(3): 431-43.

Yin Y., Henzl M. T., Lorber B., Nakazawa T., Thomas T. T., Jiang F., Langer R. and Benowitz L. I. (2006). "Oncomodulin is a macrophage-derived signal for axon regeneration in retinal ganglion cells." Nat Neurosci 9(6): 843-52.

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

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

CHAPTER 2

TRAINING-INDUCED PLASTICITY IN RATS WITH CERVICAL SPINAL CORD INJURY: EFFECTS AND SIDE EFFECTS

Adapted from Krajacic A, Weishaupt N, Girgis J, Tetzlaff W and Fouad K, (2010)

Behav Brain Res.

2.1 Preface

Previous work from the Fouad laboratory showed that rehabilitative reaching training in rats with a cervical lesion of the spinal cord resulted in a significant increase in reaching success in rats that were exposed to reaching training over those that did not (Girgis *et al.*, 2007). One major finding of the study was that in parallel with the enhanced reaching success, rats that received training also had a significant increase in the number of collateral sprouts of the corticospinal tract (CST). The spinal lesion in that particular study ablated not only the dorsal and lateral part of the CST but also parts of the rubrospinal tract (RST). This leads to the question of which descending CNS structure (i.e. CST or RST) is the major contributor for the reaching success. To answer this question we designed an experiment in which rats received a spinal lesion that targeted either the dorsal CST, the lateral CST and RST or a combination of both. Further, if the increase in collateral sprouting of the CST is the main neuronal mechanism for the recovery in reaching, a complete ablation of the CST should result in a great reduction of the reaching success. This Chapter addresses this problem by describing the ablation the dorsal and lateral part of the CST, followed by a lesion of the medullary pyramids.

2.2 Introduction

Following incomplete spinal cord injury (SCI), moderate functional recovery can be found in patients and animal models, however, axonal growth is suppressed by a variety of intrinsic and extrinsic factors (Huebner and Strittmatter, 2009). This spontaneous recovery is generally attributed to adaptive changes within spared neuronal circuitry, frequently referred to as plasticity (reviewed in Fouad and Tse, 2008). Plasticity occurs at various anatomical locations and physiological levels within the entire central nervous system, ranging from collateral sprouting to changes in neuronal properties (Fouad *et al.*, 2001; Li *et al.*, 2004; Rank *et al.*, 2007; Vavrek *et al.*, 2006; Weidner *et al.*, 2001). Thus, it remains unclear, which adaptations are involved in the recovery process, and whether some changes are actually maladaptive, resulting in adverse effects like neuropathic pain or spasticity. For the corticospinal tract (CST), for example, sprouting above and below the level of the injury has been associated with recovery (Girgis *et al.*, 2007; Vavrek *et al.*, 2006; Weidner *et al.*, 2001), and conversely, recovery of forelimb function has been linked to plasticity in various descending systems especially the CST and the rubrospinal tract (RST; Kanagal and Muir, 2009; Weidner *et al.*, 2001).

A well established approach to enhance both recovery and plasticity following injuries of the nervous system is rehabilitative training (Beekhuizen, 2005; Edgerton *et al.*, 2006). Despite its general acceptance as the current most successful treatment paradigm following SCI, there remain important and

unanswered questions regarding the efficacy of rehabilitative training, the best design, timing and its limitations for both animal models and patients. One critical issue for example, is that task-specific training in animal models has been reported to reduce the performance in other, unrelated and untrained tasks (de Leon *et al.*, 1999; Edgerton and Roy, 2009; Garcia-Alias *et al.*, 2009; Girgis *et al.*, 2007). The reduced performance in unrelated/ untrained tasks might depend on the early onset of rehabilitative training, something that, in the case of reaching, has not been observed when training was delayed by 12 days (Krajacic *et al.*, 2009). Thus, more knowledge on the interaction of plasticity and the activation of spared and rearranged networks due to rehabilitative training are necessary in order to design effective and relevant rehabilitative strategies. This knowledge will also be essential when rehabilitative training is combined with plasticity-promoting drugs and broad training might possibly interfere or mask the drug treatment (e.g., Garcia-Alias *et al.*, 2009).

In the present study we used three different lesion types, and found that the lesion location determines the effect of training success and its effects on untrained tasks. A unilateral relesion of the CST at the level of the pyramids confirms the importance of training-induced CST plasticity on the recovery, but also suggests the contribution of other descending projections.

2.3 Methods

2.3.1 Subjects

Adult female Lewis rats (Charles River, 200-250g, total n= 60) were group-housed and received water *ad libitum*. In order to motivate rats to grasp for sugar-pellets (45mg, TestDiet, Richmond, CA), measured amounts of food (10-13g/day) were presented directly after training/testing sessions allowing a constant increase in weight. Grasping movements within the home cage were reduced by feeding chow mash (rodent chow and water). To prevent effects of high glucose intake between trained and untrained rats (Plunet *et al.*, 2008), untrained rats received a comparable amount of pellets in their food mash. The study was approved by the local animal welfare committee, and complies with the guidelines of the Canadian Council for Animal Care.

2.3.2 Pre-surgery training

a) Skilled reaching: Prior to surgery rats were familiarized with a reaching task and their preferred paw was determined during the first training sessions. During training sessions, rats were placed individually into a transparent Plexiglas chamber (30x36x30cm) with a slit in the front wall as reported earlier (figure 2.1D; Girgis *et al.*, 2007). A small platform was situated on the outside of the chamber in front of the slit onto which single sucrose pellets were placed. The skilled reaching task involved the following steps: the rat had to walk to the back wall, turn around, walk to the front, reposition itself, and grasp for a presented

pellet. A successful reach was counted when a rat was able to retrieve the presented pellet and ate it. If the pellet was knocked off the shelf or if it dropped through the metal grid used as the chamber floor, a failure was scored. For evaluation, the total number of successfully grasped and eaten pellets over a 10min period was counted. Further, the success rate was defined as the percentage of successfully grasped and eaten pellets in relation to all presented pellets.

b) Horizontal ladder: Rats were placed on a 1m long runway consisting of randomly spaced (3-4cm) 1.5mm thick steel rungs, and encouraged to cross it (Fig. 2.1E). The distance between the rungs was altered between testing sessions to prevent the rats from learning a movement pattern (Bolton *et al.*, 2006). Each animal had to cross the ladder 3 times. Crossings were video-recorded and analyzed offline by counting slips and falls of the affected forelimb which were defined as errors.

2.3.3 Surgical procedure

Rats were divided into 3 groups with each group receiving a different lesion type: a unilateral lesion of the dorsal funiculus (to target the dorsal portion of the CST; n= 13, figure 2.1A), a lesion targeting the dorsal part of the lateral funiculus (targeting the majority of the lateral CST and rubrospinal tract, RST; n= 13, figure 2.1B) or a lesion of the entire dorsolateral quadrant (DLQ, n= 30, figure 2.1C) ablating both, the dorsal and lateral CST as well as the majority of the RST.

Rats were anesthetized (Hypnorm 0.16mg/kg; fentanyl citrate, 120ul per 200g body weight, Janssen Pharmaceutics, Beerse, Belgium and Midazolam 2.5mg/kg; Sabex, Boucherville, QC, Canada) and mounted into a stereotaxic frame (Kopf Instruments, Germany). The incision site was cleaned with 10% Chlorhexadine Digluconate (Sigma-Aldrich Canada LTD., Oakville, ON, Canada) and an incision to the skin above vertebrae C2-C4 was made. Then the overlying muscles above C3 were split. Without performing a laminectomy, the spinal lesions were performed between C2 and C3 ipsilateral to the preferred paw by using a custom-made tapered micro-blade. Muscle layers and skin were sutured. Following surgery rats were maintained at 37˚C and received saline and the analgesic Buprenorphine (0.03mg/kg, diluted in sterile water, Temgesic®, Schering-Plough Ltd., Hertfordshire, UK) over the next two days.

2.3.4 Rehabilitative training

Rats of each lesion type were distributed into a training group and a control group (i.e., untrained group) in such a manner that both groups contained animals with comparable post-lesion deficits. Therefore, a scoring system from 1 to 3 was used, where a score of 1 was assigned if paw and digits were partially paralyzed but the rat was able to stand on the affected forelimb. A score of 2 was given if the paw and digits were paralyzed and no plantar stepping occurred, and a score of 3 if the paw, digits and the elbow were paralyzed.

Starting on day 4 post-lesion, rats of the training-group received rehabilitative training in the reaching task (described above) for 6 weeks. One training session consisted of 10min of training per day, 6 days a week. To prevent differences in handling and acclimatization to the experimental apparatus, untrained rats were placed 6 days a week into the reaching chamber. However, the slit of the apparatus was blocked off so that untrained rats were not able to practice reaching movements. Additional subgroups (n= 4 each) with the DLQ-lesion received reaching training/no training for a period of 3 weeks only, which was followed by a pyramidotomy contralateral to the preferred paw.

2.3.5 Functional testing

Rats were tested in their ability to reach for a pellet and to cross the horizontal ladder (i.e., the untrained task) after 3 or 6 weeks of rehabilitation. Rats in the control groups were tested on the same outcome tasks at the same intervals. The performance in both tasks was assessed over 3 days as described above.

2.3.6 Pyramidotomy

Following 3 weeks of recovery a group of rats with DLQ-lesion (n=8) received a pyramidotomy to transect unilaterally all fibers (spared or lesioned) of the CST at the level of the medulla. Therefore, anaesthetized rats (Hypnorm and Midazolam as described above) were placed on their back, their skin was incised and the paratracheal tissue was gently pulled to the side in order to drill a hole

into the bone overlying the medullary pyramids. Then the dura was cut and the pyramids were exposed. The pyramid contralateral to the preferred paw was transected rostral to the decussation by using spring-scissors. Subsequently the skin was closed and the rats placed in a cage with a heating pad. When animals showed first reflex responses, post surgical care was provided as described above.

2.3.7 Intracortical Micro-Stimulation (ICMS)

Stimulation of the caudal portion of the forelimb motor area was performed in all rats to investigate the cortical area where motor evoked potentials (MEP) and potentially wrist movements could be elicited. A control group of rats (n= 4, no SCI) was added to show differences between lesioned and unlesioned animals.

After the functional testing period (see above) rats underwent the ICMS procedure, where they received buprenorphine (0.06 mg/kg; s.c.) in combination with ketamine (100 mg/kg; i.p., Sandoz Canada inc., Boucherville, QC, Canada) for anesthesia. Animals were placed into a stereotaxic frame and four electrodes with exposed tips (seven-stranded Teflon coated wire; A-M systems, Carlsborg, WA, USA) were inserted into the wrist extensors (of both forelimbs) to record MEP's as we described earlier (Krajacic *et al.*, 2009). The electromyographic (EMG) signal was amplified (Grass, Astro-Med. Inc, West Warwick, USA), digitized (5kHz, Digidata 1322A; Axon instruments, Foster city, CA, USA), filtered (30-300Hz).

The skull of the animals was exposed and a dental drill was used to make two rectangular openings over the caudal forelimb cortex of both hemispheres (1-4mm lateral and 0.5 to 3mm rostral to Bregma). The exposed cortex on each hemisphere was divided into 25 squares. The stimulation of the caudal forelimb cortex was performed by using a tungsten microelectrode (FHC, Bowdoin, ME, USA; 8-10 MΩ), which was lowered between 1.5 and 1.9mm into the sensory motor cortex. Each of the 25 squares was stimulated on both hemispheres, if vascularization allowed it. Stimulation was performed by delivering a train of cathodal pulses (n= 30, 0.25ms, 330 Hz) with a starting current of 60µA. The maximal current used in order to evoke a response in the wrist was 180µA. The wrist MEP's were recorded and evaluated in later analysis by using Axoscope software (Axoscope 9.0.1.16; Molecular Devices, Sunnyvale, CA, USA).

2.3.8 Tracing

To evaluate the amount of collateral sprouting of injured CST fibers, anterograde tracing with biotinylated dextran amine (BDA, 10%, Microprobes, Eugene, OR, USA) was performed subsequently after the ICMS procedure. At 3 different sites 1µl of the tracer was injected 1.5mm deep into the cortex contralateral to the preferred paw, over a time period of 3min by using a Hamilton syringe (Reno, NV, USA). Additionally, a subgroup of rats with a DLQ-lesion (n = 6) received Alexa Fluor-488 tracing into the forelimb cortex to investigate existing projections from the damaged CST to the reticular formation. A total of

3µl Alexa Fluor-488 (5%; 10,000MW, Molecular Probes, Eugene, Oregon, USA) was injected at 3 different sites within the window used for the ICMS procedure into the forelimb motor cortex contralateral to the spinal injury.

Following tracer injections the skin was sutured and post-surgical care was provided as described above.

2.3.9 Construction of cortical maps

The ICMS results were used to create cortical maps as described earlier (Girgis *et al.*, 2007). If stimulation of a square resulted in a MEP, the response was noted and averaged for each square in all rats within a group. As MEP recordings are more sensitive to stimulation than visible wrist movements, they were used to create the cortical maps. When MEP's could not be evoked at all, we routinely stimulated the other side of the cortex with an intact CST to confirm the appropriateness of anaesthesia depth. To illustrate differences between groups a color code was created, and for statistical analysis this code was transferred into a scale from 0-3. The white squares indicate that no wrist movement was found (0%), light grey, dark grey and black squares represent that on average wrist MEP's were found in more than 0%, 33% and 66% of the animals respectively.

2.3.10 Injection of muscimol into Red Nucleus in rats with pyramidotomy

To block transmission through the red nucleus and to confirm the injection site a mixture of muscimol (γ-aminobutyric acid agonist, Sigma-Aldrich Canada LTD., Oakville, ON, Canada) and the tracer dextran tetramethyl-rhodamine (TMR, Invitrogen, Molecular Probes, Eugene, Oregon, USA) was pressure-injected into the red nucleus in a group of rats that had received a DLQ lesion (n=8). The coordinates of the injection site were 5.7mm caudal, 1mm lateral to Bregma, and 7.6mm deep. After injection, the needle of the Hamilton syringe remained in the brain for 2min to prevent leakage and was then slowly removed. Thirty minutes later the spots where MEPs or wrist movements were elicited prior injection, were stimulated once more, to confirm whether CST connections to motor neuron pools were rerouted through the red nucleus as we described following anti-Nogo treatment (Raineteau et al. 2001). Subsequently rats were euthanized and perfused.

2.3.11 Perfusion

Perfusions were performed either following the administration of muscimol (see above) or 2 weeks after tracer injection. Rats were euthanized by a lethal dose of pentobarbital (Euthanyl, Bimeda-MTC, Animal-Health Inc., Cambridge, ON, CA). Then a transcardial perfusion was performed by using saline with 0.02g heparine/liter, followed by a 4% formalin solution with 5% sucrose. Brains and

spinal cords were dissected, post fixed overnight in 4% formalin (with 5% sucrose) and transferred for 3 days into a 30% sucrose solution for cryoprotection. Then the tissue was cut into a brainstem, a C1, and a C2-C6 section, embedded in Tissue Tek (Sakura Finetek USA, Inc., Torrance, CA, USA) and frozen in 2-methyl-butane at -60ºC. The brain stem and C1 segments were mounted for cross-sections and the C2-C6 section was mounted for horizontal sections. All tissue was cryosectioned (25µm), mounted onto slides (Fisherbrand, colorfrost microscope slides, Fisher Scientific, Ottawa, ON, Canada) and stored at -80ºC until a staining procedure was performed.

2.3.12 Immunohistochemistry

A staining procedure was performed to visualize traced axons. Frozen sections from brain stem and spinal cord tissue were attached to the slides by heating the slides for 30min at 37ºC. The sections on the slides were then rehydrated by bathing by 2x10min in Tris-buffered saline (TBS, pH 7.4), followed by two washes for 45min in TBS with 0.5% Triton X-100. Afterwards the sections were incubated overnight at 4ºC in an avidin-biotin complex solution (ABC, Elite, Vector Laboratories, Burlingame, CA, USA). Then the ABC solution was washed off (2x10min), and a diaminobenzindine (DAB, Vector kit, SK4100) reaction was performed. The reaction was halted after 5min by washing slides in distilled water. Subsequently, slides were washed (2x10min) in TBS, dehydrated in

increasing alcohol concentrations, cleared with xylene and coverslipped with Permount (Fisher Scientific).

Slides with brainstem sections containing the muscimol injection site were counterstained for 2min in 0.1% cresyl violet. The reaction was halted by washing slides in distilled water. Dehydration was performed in increasing concentrations of ethanol sections were then cleared in xylene and coverslipped with Permount.

In order to investigate whether fibers of the CST project towards the reticular formation, tissue from rats which received an injection of Alexa Fluor-488 into the forelimb motor cortex contralateral to spinal injury was stained.

Slides were dried at 38^{o}C for 1hour, then washed in 2x10min TBS followed by 2x 45min TBS-TX. Subsequently, slides were incubated in blocking solution containing 10% Normal Goat Serum (NGS, Vector Laboratories, Burlingame, CA, USA) for 2 hours before application of primary antibody (rabbit Anti-AF488, Molecular Probes, Eugene, Oregon, USA) at 1:750 overnight. On the next day, slides were again washed in 2x10min TBS and incubated overnight in secondary antibody solution containing biotinylated goat anti rabbit secondary antibody (Vector Laboratories) and NGS. Incubation with an avidin-biotin-peroxidase complex (ABC; Elite, Vector Laboratories) overnight was followed by the DAB reaction using the Vector DAB kit (SK4100, Vector Laboratories). Tissue dehydration and coverslipping was performed as described above.

2.3.13 Histology

Analysis was performed by using bright-field or phase contrast microscopy in order to evaluate: *1)* the total number of traced CST axons, *2)* the lesion size, and *3)* collateral sprouting from injured CST fibers, *4)* reconstruct CST projections to the reticular formation.

The number of traced CST axons was analyzed by counting them on one representative C1 cross-section.

Analysis of the lesion size was performed on every 4th section throughout the entire spinal cord tissue of the C2-C6 segments. Landmarks (i.e., central canal, gray-white matter interfaces) were used to reconstruct the lesion size and transfer it onto a schematic of a cross section of the cervical spinal cord.

For quantification of the number of collateral sprouts of injured CST fibers, every 4th section of the horizontally sectioned C2-C6 segments was counted. The number of collateral sprouts emanating from the injured CST by crossing an imaginary line at the gray-white matter interface and projecting into the gray matter (for more than 30µm) was counted (figure 2.1F). This number was normalized to the length (mm) of the analyzed tissue and the number of total traced axons counted on the C1 tissue.

Projections of traced CST fibers projecting from the pyramidal tract towards the reticular formation were reconstructed at -11mm from bregma using a camera lucida connected to a Leica DM LB microscope.

2.3.14 Statistics

A one way ANOVA followed by a Tukey *post hoc* test (Prism, V 4.01; Graph Pad Software Inc., La Jolla, CA, USA) was used to determine differences between groups in all outcome measures. All results and figures are presented as means ± standard error of the mean. Statistical significance was stated when *P*-values ≤ 0.05 were found and indicated by an asterisk. *P*-values ≤ 0.01 were indicated by two asterisks.

2.4 Results

2.4.1 Training-induced task-specific recovery was only found in rats with lesions involving the dorsal funiculus

Rats with the three different lesion types (figure 2.1A-C) were trained in a skilled reaching task. The quantification of the lesion volume (%) in respect to a transverse section of the spinal cord shows no statistical difference between trained and untrained rats in the three different lesion groups (figure 2.2A-D). After six weeks of rehabilitative training they were tested in the trained paradigm and the score was normalized to the baseline (pre-surgical) value. Overall untrained animals with the DLQ-lesion showed the most significant deficits whereas rats with dorsal or lateral funiculus lesion performed comparably. In line with earlier findings (Girgis *et al.*, 2007), trained animals with a DLQ-lesion (n= 9) showed a highly significant increase of 28.9% (P ≤ 0.01, figure 2.3A) in their

success rate when compared to their untrained counterparts (n= 7, 60.33% ± 4.0 and 31.4% ± 5.67 respectively). Damage to the dorsal funiculus only, also allowed a highly significant training effect (27.1%; $P \leq 0.01$). With this lesion, trained rats reached with a 104.6% ± 4.44 average success rate (n= 7) compared to 77.50% ± 7.33 (n= 6) success in the untrained group. Surprisingly, no training effect at all was found in the group that received a lesion of the lateral funiculus (trained 66.71% ± 4.52, n= 7; untrained 69.33% ± 3.75, n= 6).

Note that the reaching performances are represented as % of the baseline value. The actual success rate (in %) in the skilled reaching task after 6 weeks of rehabilitative training mirror these results. Trained rats with a DLQ-lesion performed significantly better than their untrained counterparts (41.44 ± 4.01% and 20.36 ± 3.91%, respectively, $P \leq 0.01$) and an ablation of the dorsal funiculus also resulted in a significant difference ($P \leq 0.01$) between trained (77.29 ± 5.08%) and untrained animals (54.50 ± 5.60%). In addition, the quantification of the success rates of rats that received a lateral funiculus lesion did not reveal any differences between trained (51.43 ± 5.18%) and untrained rats (47.17 ± 5.34%). Thus, in our experiment only rats with lesions to the dorsal column containing sensory fibers and the majority of the CST (dorsal column and DLQ-lesion group) showed a training-induced recovery.

2.4.2 Deficits in the untrained task are only found in rats with DLQ-lesion

Previous reports indicated that training after cervical spinal cord injury may result in impairments in an untrained task (de Leon *et al.*, 1999; Garcia-Alias *et al.*, 2009; Girgis *et al.*, 2007). Thus, it is of great importance to understand the relation between training, and the consequences of intensive rehabilitation. Comparing the different groups of the present study to each other we found that trained rats with a DLQ-lesion (2.09 ± 0.31; figure 2.3B) performed significantly worse (difference between groups 1.203; $P \leq 0.01$) when crossing a horizontal ladder than their untrained counterparts (0.89 ± 0.35; figure 2.3B). Animals with training following a dorsal funiculus lesion showed a statistically insignificant increase in the error rate (trained 1.53 ± 0.69; untrained 1.24 ± 0.44), and in rats with a lesion to the lateral funiculus there was no difference at all (trained 1.03 ± 0.28; untrained 0.95 ± 0.32; figure 2.3B). It should be noted that rats of the DLQ-lesion group showed the biggest training benefits (in the reaching task) but also showed the biggest deficits in the untrained task. Rats of the RST-lesion group (i.e. lateral funiculus group) did not show either beneficial or detrimental training effects.

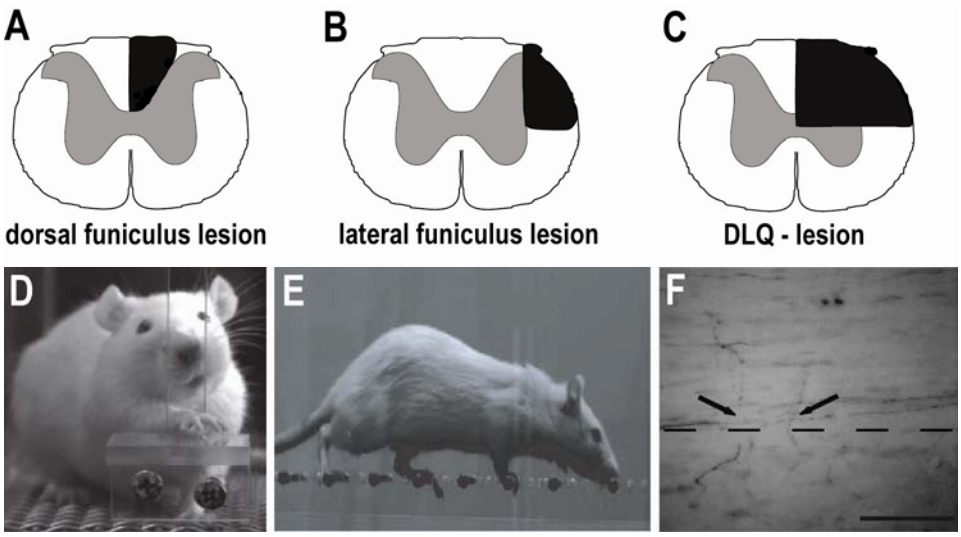


Figure 2.1. Schematic illustration of the different lesion paradigms (dorsal funiculus lesion, lateral funiculus lesion and DLQ-lesion) are shown in **A - C**. An example of a rat reaching for a pellet six weeks after a spinal lesion (**D**). A rat crossing the horizontal ladder (**E**). Microscopic photograph of a rat spinal cord section showing the labeled injured CST sending out collaterals into the gray matter (**F**). The dashed line indicates the white/gray matter interface. Arrows pointing at collateral sprouts emanating the labeled CST and projecting into the gray matter. Scale bar: 100μm.

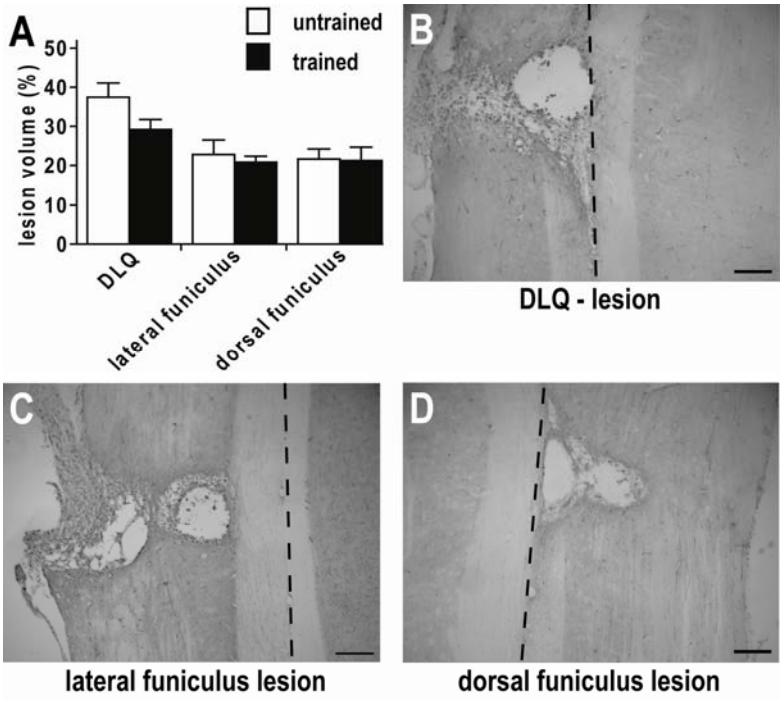


Figure 2.2. Lesion size analysis. A comparison of the spinal lesion volume between trained and untrained rats (as of transverse section) is shown in (A). There was no significant difference between the groups. Photomicrographs show horizontal sections of the spinal cord of rats with a DLQ (B), lateral funiculus (C) and a dorsal funiculus lesion (D). Dashed line represents the midline, scale bar: 200µm. Data are shown as mean, error bars show the standard error of the mean.

2.4.3 Training and collateral sprouting

To elucidate whether functional recovery in skilled reaching is related to an increase in collateral sprouting of the lesioned CST fibers towards targets in the gray matter, we examined differences in the number of collateral sprouts emanating from the injured CST rostral to the lesion between groups (figure 2.3C). The number of counted collaterals was averaged per animal and normalized to the length (in mm) of analyzed CST and the number of total labeled axons at the level of C1.

Trained animals which received a DLQ-lesion (untrained 0.0031 ± 0.0004, trained 0.0042 ± 0.0008; figure 2.3C) or a lesion of the dorsal funiculus (trained 0.0041 ± 0.0015; untrained 0.0035 ± 0.0006) showed a trend to have more collaterals, however this difference was not statistically significant. Rats with a lesion of the lateral funiculus showed no change in the number of collaterals at all (trained 0.0032 ± 0.0005; untrained 0.0034 ± 0.0003). It is noteworthy that although statistically insignificant the results mirror the training-induced success rate and the deficits in an untrained task (figure 2.3A, B).

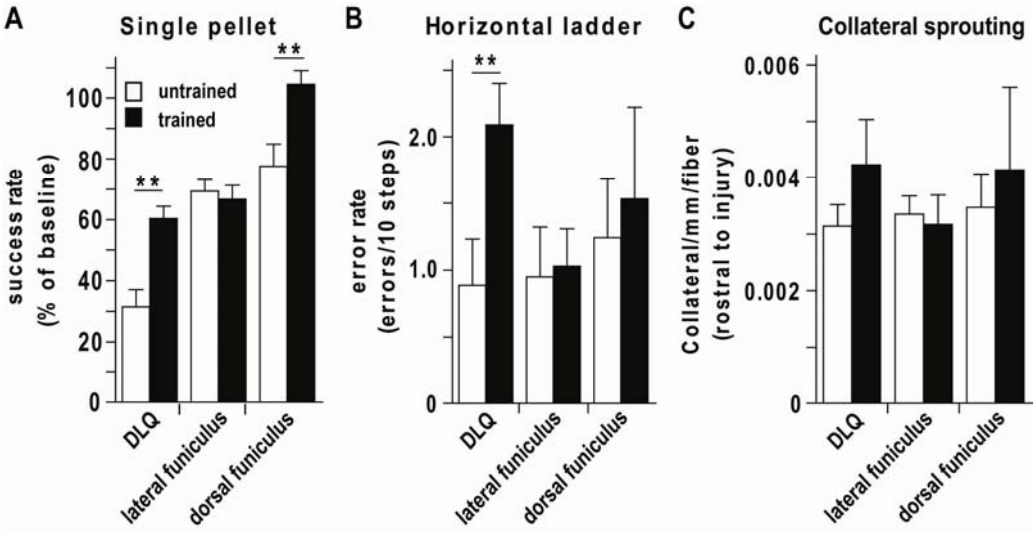


Figure 2.3. Functional testing & collateral sprouting of the injured CST rostral to the injury. The performance of rats in the skilled reaching task is shown in **A**. Trained rats of the DLQ-lesion group and of the dorsal funiculus group show a significant increase in reaching success when compared to their untrained counterparts. No training effect was found in rats with laterals funiculus lesion. **B**: The performance in the horizontal ladder task shows that trained rats with DLQ-lesion make significantly more errors than their untrained counterparts. Only statistically insignificant changes are found following dorsal and none at all after lateral funiculus lesion. **C**: The evaluation of the number of collateral sprouts emanating from the injured CST shows only statistical insignificant difference between trained and untrained rats in any of the lesion groups. Nevertheless it is noteworthy that changes reflect those found with training effect and side effect (**A, B**) **$P \leq 0.01$, error bars show standard error of the mean.

2.4.4 Training and cortical maps

Following 6 weeks of rehabilitative training in the reaching task, rats underwent intracortical microstimulation (ICMS) and recording of MEP's in wrist extensor muscles. Cortical maps of uninjured (control rats) and injured rats (contralateral to the spinal lesion) with and without training, are shown in figure 2.4A.

The results of the ICMS were transferred into a score to allow statistical comparisons. None of the training-induced changes reached significance; nevertheless, the trend follows all other results so far described. For example when the dorsal funiculus was involved in the lesion an insignificant training effect could be found (figure 2.4B; 0.48 ± 0.14 versus 0.72 ± 0.16 in the DLQ-group and 0.6 ± 0.15 versus 1.0 ± 0.22 in the rats with dorsal funiculus lesion). Lateral funiculus lesions did not result in a decline in cortical motor output (0.80 ± 0.15) when compared to the value of uninjured controls (dashed line), thus a restoration/training induced increase did not occur (0.80 ± 0.17).

Further, we observed a significant correlation (r=0.57) between the rearrangements in the cortical maps and the increase in the number of collateral sprouts from the injured CST in trained rats with a lesion of the dorsal CST (DLQ-lesion and dorsal funiculus lesion; figure 2.4C). This indicates that the amount of axonal sprouts of the CST may be involved in the rearrangements within the caudal portion of the forelimb motor cortex.

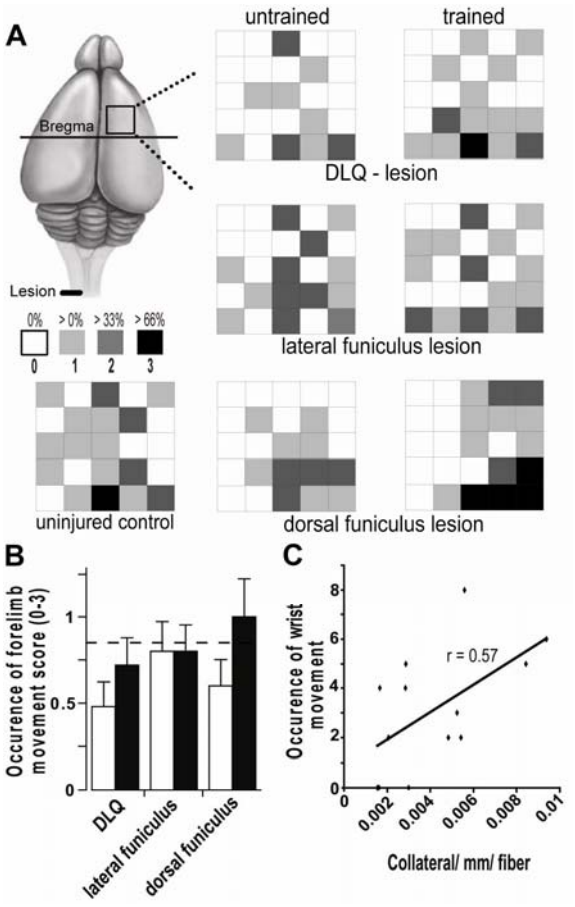


Figure 2.4. Intracortical microstimulation of the caudal forelimb area. The stimulated area contralateral to the spinal lesion was divided into 25 squares (**A**). The color code represents the responsiveness of the ICMS in wrist movements, averaged over all rats within a group. The white color represents no movement elicited (0%) and black represents wrist movement elicited in more than 66% of the animals within the group. To perform statistical analysis, the color code was transferred into values (**B**). No statistical difference was found between trained and untrained rats in either lesion group. The dashed line represents the value of uninjured controls. A significant correlation between the changes in the cortical map and the number of collateral sprouts in trained rats with a lesion of the dorsal CST (DLQ-lesion and dorsal funiculus lesion) is illustrated in **C**. White bars indicate untrained and black bars represent trained rats. Error bars show standard error of the mean.

2.4.5 Spontaneous but not training-induced recovery in reaching depends specifically on CST plasticity

Our results show that in rats with DLQ- or dorsal funiculus lesions rehabilitative training further increases plasticity and spontaneous recovery. To investigate the contribution of plasticity in spared (i.e., ventral fibers) and lesioned CST fibers (above the level of the injury) in the recovery process we unilaterally ablated the CST at the level of the pyramids following 3 weeks of spontaneous and training-induced recovery (figure 2.5A). One week following the second lesion, rats were tested again in the reaching task. The results show a highly significant decrease ($P \leq 0.01$) in the success rate in trained and untrained animals after lesion to the pyramids (trained before 84.69% ± 17.08, after 17.41% ± 5.25; untrained before 49.83% ± 9.77, after 3.36% ± 2.64; figure 2.5B). This indicates that spontaneous recovery was mainly based on CST function, leaving the relesioned rats with no grasping ability at all. Importantly, following relesion trained rats demonstrated a significant higher success rate than untrained rats, indicating that training-induced plasticity must involve other tracts than the CST ipsilateral to the spinal lesion.

Following behavioral testing and perfusion, the completeness of the lesions was confirmed in cross sections through the brainstem (figure 2.5C). Only animals with complete ablation of the pyramids are included in this study.

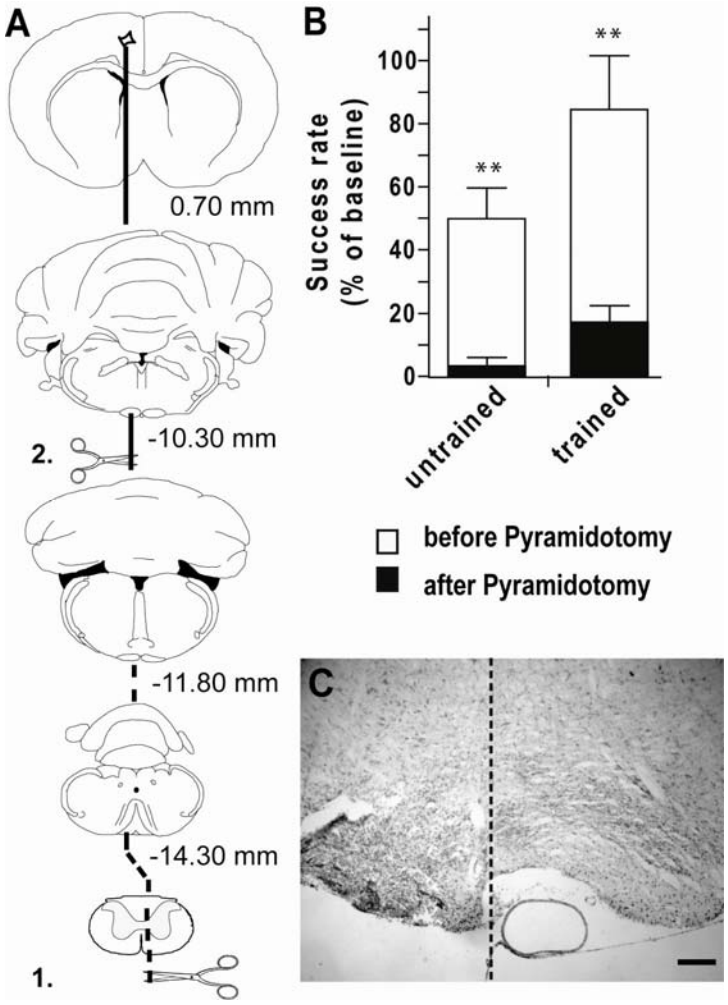


Figure 2.5. Pyramidotomy and skilled reaching performance. The lesion sequence and location is illustrated in **A**. Schematics at various levels are shown according to their location to Bregma (Paxinos, 1997). Scissors indicate the lesions of the CST at cervical level of the spinal cord (1.), and above the pyramidal decussation (2.). The bar graph in **B** shows the success rate in the skilled reaching task 3 weeks after a DLQ-lesion (before Pyramidotomy) to the cervical spinal cord of trained and untrained rats. Black bars represent the reaching success after ablation of the pyramidal tract. All values are normalized to the baseline. Picture of a cross section of the brainstem illustrating a Pyramidotomy on the left side of the brainstem in a representative animal (**C**). Bar indicates 200µm. **$P \leq 0.01$, error bars show standard error of the mean.

2.4.6 Training-induced recovery and cortical connectivity to forelimb motoneurons is not due to rerouting injured CST fibers via the red nucleus

In the last experiment we showed that training-induced recovery was not based on plasticity in the CST system solely, since functional benefits were still seen after complete CST ablation. When performing cortical micro-stimulation however, both trained and untrained rats showed that following pyramidotomy, the cortex is still indirectly connected to the periphery (i.e., to motoneurons). A likely system involved in the recovery is the RST, which was only partially ablated by our lesion. In a previous study, we demonstrated that after Nogo-A neutralization, the lesioned CST can reconnect to the periphery via the RST (Raineteau *et al.*, 2002). Using the same approach as in this previous study, we investigated whether training could induce similar plasticity. Therefore, we injected the GABA-agonist muscimol into the Red nucleus and examined changes in the connectivity of cortical spinal projections to forelimb motoneurons. This injection was performed in 3 trained animals. Following 30 minutes another round of ICMS was performed. Contrary to our experiments following Nogo-neutralization, we did not find muscimol-induced changes in cortical connectivity. All stimulated areas where a wrist MEP was noted before injection and responses with similar delays could still be observed after injection.

Although the amplitude in post-muscimol MEP's was slightly reduced, this suggest that the RST may not function as a relay for the loss of the dorsal and lateral CST (figure 2.6).

Injection locations were confirmed histologically, and only animals where the tracer (injected together with muscimol) had spread into the red nucleus (see definition by Martin, 1991) are included in the study.

In summary, these results suggest that spared RST fibers are not the major relay for disconnected CST fibers. Other descending pathways such as the reticulospinal and the vestibulospinal tract might offer a relay to the periphery and contribute to the recovery after ablation of the CST.

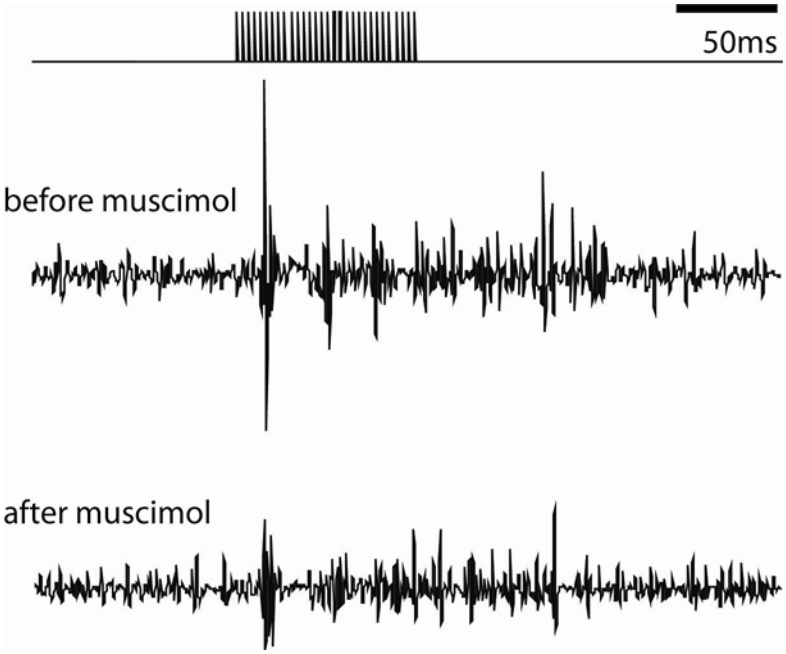


Figure 2.6. Motor evoked potentials in forelimb extensor muscles before and after muscimol injection into the Red Nucleus. Stimulation (upper trace) of the forelimb motor cortex contralateral to the lesioned paw resulted in short latencies MEPs. These could still be elicited 30 min after the administration of muscimol into the red nucleus (bottom trace).

2.4.7 The reticulospinal tract, a possible relay for injured CST fibers

To confirm that the reticulospinal tract in rats is a reasonable candidate for rerouted CST connectivity to the periphery, we investigated CST projections towards the reticular formation (figure 2.7B). As the majority of reticular fibers do not decussate, and the CST at this level of the brainstem is not decussated, we focused on CST projections crossing the midline towards the gigantocellular reticular nucleus. Considering that BDA tracing only labels around 5% of CST fibers we found a substantial amount of CST collaterals innervating both the ipsi- and contralateral reticular formation in uninjured rats. These projections could therefore be involved in rerouting CST connectivity to the periphery.

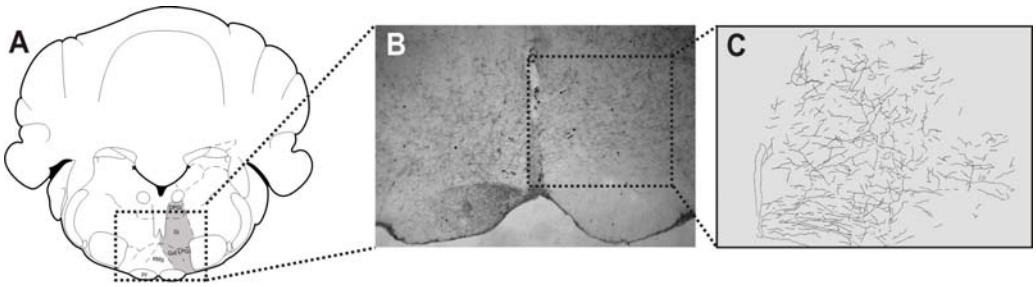


Figure 2.7. Corticospinal fibers project to the ipsi- and contralateral reticular formation in the brainstem. *Left:* Schematic cross section of the brainstem at 10.8mm posterior to bregma (modified from Paxinos, 1997). DPGi = dorsal paragigantocellular nucleus, Gi = gigantocellular reticular nucleus, GiA = gigantocellular reticular nucleus alpha, LPGi = lateral paragigantocellular nucleus, py = pyramidal tract. Gray area represents reticular formation contralateral to left pyramidal tract. *Middle:* Cross section of the brainstem at 50x magnification showing traced pyramidal tract (left) and its projections. *Right:* Camera lucida drawing depicting all contralateral projections of traced pyramidal fibers in one representative brainstem section.

2.5 Discussion

In the present study we confirmed recent findings that training rats with a cervical lesion to the dorsal column in a reaching task translates into enhanced recovery but negatively impacts a non-trained task (Garcia-Alias *et al.*, 2009; Girgis *et al.*, 2007; Krajacic *et al.*, 2009). Surprisingly, neither effects on the trained task nor on the untrained task were found following a lesion of the lateral funiculus. This raises two questions: why does not every lesion allow for training-induced benefits (and deficits on untrained tasks), and, is training success but not training per se detrimental to the performance in untrained tasks?

Although the results of training-induced changes in collaterals sprouting of injured CST axons and cortical map changes mirror the training effects (i.e., changes only occurred in lesion types where training effects were found), the numbers of collateral sprouts were not statistically significant and only allow speculations about their relation. Nevertheless, a significant correlation between collateral sprouting and cortical map changes was found, which supports the idea that cortical map changes are influenced by alternative connections of injured axons as we suggested earlier (Fouad *et al.*, 2001). Further, the present results indicate that spinal plasticity contributes to cortical map changes but the lack of clear statistical relation between recovery and map changes further suggests that recovery is likely the product of various sites of plasticity and not dependent on cortical map changes only.

A relesion at pyramid level in animals with a DLQ-lesion (that showed significant training effects) confirms the important contribution of plasticity in the CST to spontaneous recovery as reported earlier (Girgis *et al.*, 2007; Kanagal and Muir, 2009; Vavrek *et al.*, 2006; Weidner *et al.*, 2001). However, after training and lesioning the pyramidal tract, the substantial amount of remaining motor function indicates the involvement of other motor systems in the recovery. One likely candidate is the intact CST which has posited to function as a substitute after CST lesions (Kanagal and Muir, 2009). In addition, bilateral EMG responses were seen in all rats, supporting the idea that the contralateral uninjured CST could have played a crucial role in the recovery. Another likely candidate contributing to the recovery in reaching is the reticulospinal pathway, which receives substantial input from the CST (figure 2.7) and has been described to be involved in the recovery of reaching in cats and primates (Pettersson *et al.*, 2007). Unlike following anti-Nogo neutralization (Raineteau *et al.*, 2002, Raineteau *et al.*, 2001) the training-induced plasticity did not allow detouring the CST via the red nucleus, which does not necessarily exclude rubrospinal adaptations in the recovery process.

Probably the most counterintuitive result of this study is the lack of training benefits in rats with lesion of the lateral funiculus. A possible reason could be that lesion-induced deficits were too insignificant to allow for a detectable training-induced recovery. However, this is unlikely because rats with a dorsal funiculus lesion had comparable deficits.

Another reason might be the distinctive roles of the RST and CST in reaching and grasping (Kanagal and Muir, 2009) and their different level in the hierarchy of motor control. For example, damage to the majority of RST fibers might be compensated by spontaneous plasticity of the CST.

Noteworthy are also the cortical map changes that occurred only in lesion groups where the dorsal column was injured. Damage to the dorsal column but not the lateral funiculus alone allowed for noticeable (but statistically insignificant) training-induced map adaptations. Lastly the role of sensory fibers projecting in the dorsal funiculus has to be considered. These fibers might be involved in the training effect as their integrity is clearly involved in reaching success, but can be well compensated for over time (Kanagal and Muir, 2007; Whishaw *et al.*, 1998).

Another important finding of the present study is that animals that show no training-induced benefit also do not present deficits in an untrained task. This indicates that side effects are not necessarily due to an exacerbation of the injury by the training, as it has been described following stroke (DeBow *et al.*, 2003). Only when training promotes functional meaningful rewiring of "spared neuronal hardware" deficits will be found in an untrained task. This concept would be applicable also to effects found in the locomotor system where walking versus standing was trained/tested in spinal cats (de Leon *et al.*, 1999). Most important, regarding the clinical aspect of rehabilitative training, is that the present study confirms the assumption that task-specific training appears to use "spared

neuronal hardware" and recruits this new circuitry toward the trained task. Thus, untrained tasks (even if related, see Garcia-Alias *et al.*, 2009) might not benefit from the training, or even worse, recover less well. This finding leads to questions about the nature of rehabilitation following SCI, specifically, whether a broad spectrum of tasks should be considered (Musselman *et al.*, 2009), whether training of more tasks is limited by spared neuronal hardware, and to what degree training effects and side effects are reversible when the phase of injury-induced plasticity declines and leaves the CNS once more in a more rigid state.

As the muscimol injection did not yield any apparent effect in inhibiting the signal transmission, it may be argued that the experiment had been executed successfully. However, based on our earlier experience and careful examination of the injection site, we are confident that the muscimol injection has been performed precisely. The negative result following its injection implies that training-induced plasticity might not allow a rerouting via the RST, but uses different avenues to restore motor function than approaches neutralizing growth inhibitors as Nogo (Raineteau *et al.*, 2002; Raineteau *et al.*, 2001) or chondroitin sulfate proteoglycans (Garcia-Alias *et al.*, 2009). This might explain why combinatory effects using rehabilitative training and pharmacological plasticity enhancers are most successful as recently demonstrated by Garcia-Alias and colleagues (Garcia-Alias *et al.*, 2009).

In conclusion, the present study suggests that the CST plays a central role in trained recovery in a reaching task. However, various descending systems besides

the CST are involved in the training effect on recovery. Our results also indicate that although participation in activity/ training is not directly detrimental to untrained tasks, success on such tasks may disadvantage untrained behaviours and associated neuronal circuitries.

2.6 References

Beekhuizen KS. (2005). "New perspectives on improving upper extremity function after spinal cord injury." J Neurol Phys Ther 29: 157-162.

Bolton DA, Tse AD, Ballermann M, Misiaszek JE, Fouad K. (2006). "Task specific adaptations in rat locomotion: runway versus horizontal ladder." Behav Brain Res 168: 272-279.

de Leon RD, Tamaki H, Hodgson JA, Roy RR, Edgerton VR. (1999). "Hindlimb locomotor and postural training modulates glycinergic inhibition in the spinal cord of the adult spinal cat." J Neurophysiol 1999; 82: 359-369.

DeBow SB, Davies ML, Clarke HL, Colbourne F. (2003). "Constraint-induced movement therapy and rehabilitation exercises lessen motor deficits and volume of brain injury after striatal hemorrhagic stroke in rats." Stroke 34: 1021-1026.

Edgerton VR, Kim SJ, Ichiyama RM, Gerasimenko YP, Roy RR. (2006). "Rehabilitative therapies after spinal cord injury." J Neurotrauma 23: 560-570.

Edgerton VR, Roy RR. (2009). "Robotic training and spinal cord plasticity." Brain Res Bull 78: 4-12.

Fouad K, Pedersen V, Schwab ME, Brosamle C. (2001). "Cervical sprouting of corticospinal fibers after thoracic spinal cord injury accompanies shifts in evoked motor responses." Curr Biol 11: 1766-1770.

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

Garcia-Alias G, Barkhuysen S, Buckle M, Fawcett JW. (2009). "Chondroitinase ABC treatment opens a window of opportunity for task-specific rehabilitation." Nat Neurosci 12: 1145-1151.

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

Huebner EA, Strittmatter SM. (2009). "Axon regeneration in the peripheral and central nervous systems." Results Probl Cell Differ 48: 339-351.

Kanagal SG, Muir GD. (2007). "Bilateral dorsal funicular lesions alter sensorimotor behaviour in rats." Exp Neurol 205: 513-524.

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

Krajacic A, Ghosh M, Puentes R, Pearse DD, Fouad K. (2009). "Advantages of delaying the onset of rehabilitative reaching training in rats with incomplete spinal cord injury." Eur J Neurosci 29: 641-651.

Li Y, Gorassini MA, Bennett DJ. (2004). "Role of persistent sodium and calcium currents in motoneuron firing and spasticity in chronic spinal rats." J Neurophysiol 91: 767-783.

Martin JH. (1991). "Autoradiographic estimation of the extent of reversible inactivation produced by microinjection of lidocaine and muscimol in the rat." Neurosci Lett 127: 160-164.

Musselman KE, Fouad K, Misiaszek JE, Yang JF. (2009). "Training of walking skills overground and on the treadmill: case series on individuals with incomplete spinal cord injury." Phys Ther 89: 601-611.

Paxinos G. (1997). "The rat brain in stereotaxic coordinates." Academic Press, San Diego, xxxiii, [78] pp.

Pettersson LG, Alstermark B, Blagovechtchenski E, Isa T, Sasaski S. (2007). "Skilled digit movements in feline and primate--recovery after selective spinal cord lesions." Acta Physiol (Oxf) 189: 141-154.

Plunet WT, Streijger F, Lam CK, Lee JHT, Liu J, Tetzlaff W. (2008). "Dietary restriction started after spinal cord injury improves functional recovery." Exp Neurol 213: 28-35.

Raineteau O, Fouad K, Bareyre FM, Schwab ME. (2002). "Reorganization of descending motor tracts in the rat spinal cord." Eur J Neurosci 16: 1761-1771.

Raineteau O, Fouad K, Noth P, Thallmair M, Schwab ME. (2001). "Functional switch between motor tracts in the presence of the mAb IN-1 in the adult rat." Proc Natl Acad Sci U S A 98: 6929-6934.

Rank MM, Li X, Bennett DJ, Gorassini MA. (2007). "Role of endogenous release of norepinephrine in muscle spasms after chronic spinal cord injury." J Neurophysiol 97: 3166-3180.

Vavrek R, Girgis J, Tetzlaff W, Hiebert GW, Fouad K. (2006). "BDNF promotes connections of corticospinal neurons onto spared descending interneurons in spinal cord injured rats." Brain 129: 1534-1545.

Weidner N, Ner A, Salimi N, Tuszynski MH. (2001). "Spontaneous corticospinal axonal plasticity and functional recovery after adult central nervous system injury." Proc Natl Acad Sci U S A 98: 3513-3518.

Whishaw IQ, Gorny B, Sarna J. (1998). "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 93: 167-183.

CHAPTER 3

ADVANTAGES OF DELAYING THE ONSET OF REHABILITATIVE REACHING TRAINING IN RATS WITH INCOMPLETE SPINAL CORD INJURY

Adapted from Krajacic A, Ghosh M, Puentes R, Pearse DD, Fouad K (2009)

Eur J Neurosci, 2009; 29: 641-651.

3.1 Preface

In the previous Chapter (Chapter 2), I found that the functional outcome in rats following a cervical spinal cord injury depends mainly on plasticity (e.g., sprouting) of the CST. One approach to enhance sprouting of spared and lesioned axons of the CNS is rehabilitative training. Considering that individuals suffering from SCI most likely have other injuries that do not allow them to start rehabilitative training right away, it remains undetermined when rehabilitative training should be initiated following SCI. In a few SCI rehabilitation centres, rehabilitative training is initiated immediately after SCI as it was reported that an early onset of rehabilitative training with a mechanically assisted locomotion apparatus (lokomat) results in better functional outcome than if the initiation of training was delayed (Dietz, 2009). However, it remains controversial whether starting rehabilitative training immediately after SCI is beneficial or if it could also result in impairments. Findings from the field of stroke suggest that an early onset of training is not as beneficial as once believed. Researchers found that following stroke an early start of rehabilitative training may exacerbate the lesion volume in rats (DeBow *et al.*, 2003).

It has been speculated that a "window of opportunity" after SCI exists, where the CNS is most susceptible to adaptive changes. It is very important to start rehabilitative training following SCI within this window, however, it is not clear whether a small delay in the onset of training will still lead to functional recovery. If yes, does delayed rehabilitative training also result in impairments in untrained

tasks as it was demonstrated by a previous study (Girgis et al. 2007) by the Fouad laboratory? This question is addressed in the present Chapter.

3.2 Introduction

Moderate recovery of sensory and motor function following incomplete spinal cord injury (SCI) can be found in patients and animal models (Waters *et al.*, 1994; You *et al.*, 2003; Ballermann & Fouad, 2006). This recovery is likely due to various mechanisms including collateral sprouting of lesioned and spared axons, synaptic rearrangements and changes of cellular properties in spared neuronal circuitries (Bennett *et al.*, 2001; Fouad *et al.*, 2001; Weidner *et al.*, 2001; Bareyre *et al.*, 2004; Ballermann & Fouad, 2006; reviewed in Rossignol, 2006). These mechanisms are often referred to as plasticity (Fouad & Tse, 2008; Raineteau & Schwab, 2001). One approach, which has been shown to enhance plasticity and functional recovery following SCI, is intensive training. For example, it has been shown that locomotor training in animal models (Lovely *et al.*, 1986; Barbeau & Rossignol, 1987) and patients with SCI resulted in functional recovery (Dietz *et al.*, 1994; Dobkin *et al.*, 2007; Wernig *et al.* 1995) and plasticity (de Leon *et al.*, 1999; Harkema, 2001; Edgerton *et al.*, 2001; Behrman & Harkema, 2007; Babu & Namasivayam, 2008; Barriere *et al.*, 2008). The effects of training forelimb (Girgis *et al.*, 2007) or hand function following cervical SCI are less well studied (Beekhuizen & Field-Fote, 2005; Beekhuizen & Field-Fote, 2008). In contrast, following stroke, forelimb/hand rehabilitation therapies are well established. For example, in animal models it has been demonstrated that the forced use of the affected limb leads to significant plasticity and improved recovery (Taub *et al.*, 2006). This approach has been successfully translated into the clinical setting (Taub & Uswatte, 2003; Shaw *et al.*, 2005) however, until now the ideal onset of

rehabilitative training in patients and animal models is still unclear, due to possible effects on lesion size (Kozlowski *et al.*, 1996; DeBow *et al.*, 2003; Humm *et al.*, 1998) and inappropriate compensatory strategies (Alaverdashvili *et al.*, 2008). Besides avoiding such side effects, it has been shown that a 2 week delay of training increased the expression of brain-derived neurotrophic factor (BDNF) and promoted recovery when compared to a group with immediate training onset (Griesbach *et al.*, 2007). A comparable uncertainty about the ideal timing of rehabilitative training also exists in the field of SCI, where it is generally assumed that an early onset is most beneficial. This is based on the common assumption that training-induced recovery is facilitated by injury-induced plasticity, involving an activated immune system (Schwartz & Yoles, 2006; Chen *et al.*, 2008; Benowitz & Yin, 2008) and among others the up-regulation of neurotrophic factors (Jones *et al.*, 1999). Functional evidence for the benefits of an early training onset however is sparse, as only very few studies addressed this issue (Norrie *et al.*, 2005; Rupp *et al.*, 2002). In contrast to these studies, we reported earlier that an acute training onset might also have negative effects as we found task-specific recovery in a reaching task, but also impairments in an untrained task (i.e., crossing a horizontal ladder, Girgis *et al.*, 2007).

In conclusion, the present study addresses the important but frequently neglected question about the efficacy of delayed training on recovery and neuronal plasticity following an incomplete cervical SCI.

3.3 Methods

Experiments were performed using adult female Lewis rats (Charles River, 200-250g, n=20), which were group housed, kept at 12h:12h light/dark cycle, and received water *ad libitum*. In order to motivate rats to grasp for sugar-pellets (45mg, chocolate flavor, TestDiet, Richmond, CA) in the reaching task, measured amounts of food were presented only directly after the training/testing sessions. Amounts were chosen to allow a constant increase in weight during the experiment. Grasping movements within the homecage were reduced by feeding rat chow mash (rodent chow and water). To avoid any influence of the sucrose pellets used for training on recovery, untrained rats received a comparable amount of diluted sucrose pellets in their mash.

The study was approved by the local animal welfare committee, and complies with the guidelines of the Canadian Council for Animal Care.

3.3.1 Behavioral tasks

Prior to surgery all rats were familiarized with two behavioral tasks:

Single pellet reaching task: Rats were placed individually into a transparent Plexiglas chamber (30x36x30cm) with a slit in the front wall as it was reported by Whishaw *et al.* (1993). Outside of the chamber a shelf (in 2cm distance from the inside of the front wall, 3cm above the floor) with indentations was attached. The indentations in the shelf were positioned so that the rats can not reach the pellets

with their tongue and therefore were forced to grasp them with their forelimbs (McKenna & Whishaw, 1999). The floor of the chamber consisted of Plexiglas in the back half and of a metal grid in the front half to prevent rats from eating dropped pellets. Rats, like humans have a preferred paw that they use for grasping, which was determined during the first practice sessions.

A successful reach was counted when a rat was able to retrieve the presented sugar pellet, brought it to its mouth and ate it. As soon as the presented pellet was knocked off from the shelf or if it dropped through the metal grid while retrieving, the trial was scored as a failure. For evaluation the total number of successfully grasped and eaten pellets over a 10min period was counted. The success rate was calculated by counting the number of successfully grasped pellets and expressed as percentage of totally presented pellets.

Horizontal ladder walking: Rats had to cross a 1m long runway consisting of randomly spaced (3 - 4cm) 1.5mm thick steel rungs as it was described earlier (Bolton *et al.*, 2006). The distance between the rungs was altered between testing sessions to prevent the rats from learning a movement pattern. Prior to the lesion rats were familiarized to the horizontal ladder task by encouraging them to cross the ladder in order to get to their home cage, which was placed at the other end of the ladder. A maximum of five runs per rat was performed. Six weeks after lesion animals were tested in crossing the horizontal ladder. Each animal had to cross the ladder 3 times. Crossings were video-recorded and analyzed offline by counting slips and falls of the affected forelimb which were defined as errors.

3.3.2 Spinal cord injury

Rats were anesthetized (using Hypnorm 0.16mg/kg; Sabex, Canada and Midazolam 2.5mg/kg; Vetapharma, Leeds, UK, diluted in sterile water) and mounted into a stereotaxic frame (Kopf Instruments, Germany). Their head was flexed in a 90 degree angle, an incision to the skin above the level of C2-3 cervical vertebrae was performed, and the spinal cord between C2-3 was exposed without performing a laminectomy. Subsequently, the dura mater was cut and the spinal cord was lesioned using a custom made micro-blade. A unilateral dorso-lateral quadrant lesion (figure 3.1B) was performed to ablate the corticospinal tract (CST) and most of the rubrospinal tract (RST) ipsilateral to the non-preferred paw. After the lesion, muscle layers and skin were sutured. Then rats were placed

on a heating pad until they were awake and received analgesic Buprenorphine (0.03mg/kg) and 4ml of Saline over the next two days.

3.3.3 Rehabilitative training

Three days following surgery rats were divided into a trained and untrained group. In order to match the animals with comparable deficits the rats were visually inspected and the paralysis in the digits, wrist, elbow and shoulder in the injured forelimb was scored as described earlier (Girgis *et al.*, 2007). In brief, a ranking system from 1 to 3 was used, where a score of 1 was assigned if paw and digits were partially paralyzed and the rat was able to stand on the affected limb. A score of 2 was given if the paw and digits were paralyzed, and a score of 3 if the paw, digits and elbow were paralyzed. Thus, the designated trained group (n= 10) consisted of rats with comparable deficit to those in the untrained group (n= 10).

On post-operative day 12 rehabilitative training in the skilled reaching task was initiated for 6 days a week over a period of 6 weeks. The training procedure consisted of one session (10min/rat/day) in the reaching task. To avoid grasping with the unlesioned paw, during the first training sessions the unlesioned forelimb was restrained by a bracelet (Whishaw *et al.*, 1986). Untrained rats were handled on daily basis and placed into the reaching chamber to avoid any difference in performance because of animals' familiarity to the experimenter or the environment. However, no pellets were offered to reach for.

3.3.4 Assessment of functional recovery and intracortical microstimulation

Eight weeks following SCI, trained and untrained rats underwent a final testing session over three days in the single pellet reaching task. Further, animals were tested in an untrained task (horizontal ladder walking). Performances were recorded and scored as mentioned above.

Additionally, in order to evaluate whether the training-induced plasticity also influenced sensory systems (e.g. training-induced sprouting of sensory fibers which can result in an increased pain response), thermal sensitivity was tested by using a plantar heater apparatus (UGO Basile, Italy). In brief, rats were placed into a Plexiglas box and after settling down for a few minutes, an infrared beam was directed onto the animal's front palm from underneath the box. Time was recorded until rats withdrew their paws from the infrared light which generated heat sensitization. The procedure was repeated 3 times for both paws with a 2min resting interval between repetitions. Averages in the latency of the withdrawal response were calculated and compared between trained and untrained rats.

Following the testing in all paradigms, rats underwent an intracortical micro-stimulation (ICMS), which was used to determine the cortical area where wrist movements could be elicited. For this procedure animals received the analgesic Buprenorphine (0.06mg/kg; s.c.) in combination with Ketamine (100mg/kg, i.p.) for anesthesia. The head was shaved and the rats were placed into a stereotaxic frame.

In order to record motor evoked potential (MEP) of the wrist extensors 4 electrodes (2 per forelimb) with exposed tips (7 stranded Teflon-coated wire, A-M systems, Carlsborg, WA, USA), were inserted into wrist extensors as we described earlier (Raineteau *et al.*, 2002). The electromyographic (EMG) signal was amplified (Cyber-Amp 380, Axon Instruments, Forster City, CA, USA), digitized (5kHz, Digidata 1322A, Axon Instruments), filtered (30-300Hz) and used to measure the latencies and the duration of the evoked responses.

For the ICMS the skin over the skull was incised and the skull was exposed. Using a dental drill two rectangular openings were drilled into the skull overlaying the caudal forelimb motor area on both cortical hemispheres of the rat as described earlier (Girgis *et al.*, 2007). The coordinates for the opening were 1mm to 4mm lateral to bregma and extended from 0.5mm to 3mm rostral to bregma. The exposed area (of each side) was divided into 25 squares. The stimulation was performed by using a tungsten microelectrode (FHC Inc., Bowdoin, ME, USA; 8-10 MΩ; 250μm shank diameter) which was placed on the cortex over each square (subsequently), avoiding blood vessels. The electrode was then lowered from 1.5mm to 1.9mm into the sensory motor cortex. Stimulations were performed by delivering a train of cathodal pulses (n=30, 0.25msec, 330Hz) with a starting current of 60μA up to a maximum of 180μA or until a response

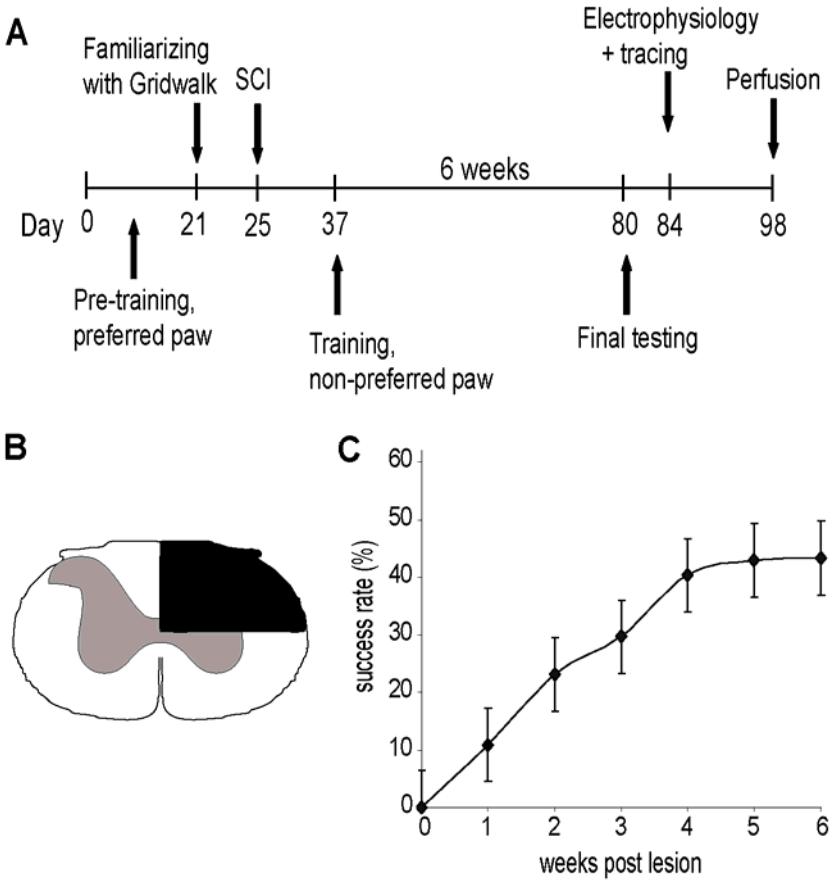


Figure 3.1. Experimental flow (**A**) and a schematic of a dorso-lateral quadrant lesion (black area, **B**) at the level of C3 vertebrae. **C**: The recovery in the success rate of skilled reaching during the training period. Trained rats reached a plateau in their performance 5 weeks post lesion.

was evoked. If a response occurred, the threshold for the response was determined by decreasing the stimulus intensity in 10μA steps until the movement response was no longer elicited. Cortical maps were created by using areas that responded to the stimulation with a MEP as we described earlier (Girgis *et al.*, 2007). Briefly, we noted for each stimulated square, in each animal whether the stimulation resulted in a MEP. The resulting sum was then divided by the number of animals within the group and expressed as a percentage. If the stimulation of a square did not give rise to a MEP or just one animal showed a response, the value for that square was calculated as the average of the adjacent squares. To illustrate differences in the cortical map between the groups the calculated percentage was used to create a 4-colour code. For statistical analysis the colour code was transferred into a scale (0-3).

3.3.5 Tracing

In order to investigate sprouting of the unlesioned CST, anterograde tracing with biotinylated dextran amine (BDA, 10%, Microprobes, Eugene, OR) of the forelimb motor cortex ipsilateral to the lesion site was performed directly following the electrophysiological assessment. BDA was pressure-injected at 1.5mm lateral and 1.5mm rostral to bregma by using a Hamilton syringe (Reno, Nevada, USA). The syringe was lowered up to 1.5mm deep into the motor cortex and 1μl BDA was injected. After injection the syringe stayed for two minutes in the cortex to minimize leakage of the tracer. This procedure was repeated twice so

that a total amount of 3µl BDA was injected. Following the injection the skin was sutured and animals received post-surgical care as described above.

In an additional group of rats (n=5) anterograde tracing with BDA of the lesioned CST was performed. These animals received the same lesion and rehabilitative training (start on day 12 following lesion) as described above.

3.3.6 Perfusion

Two weeks following tracing, all rats were euthanized and subsequently perfused through the heart with saline and paraformaldehyde. The spinal cord was extracted, post fixed in 4% formalin (with 5% sucrose) overnight, cryoprotected by storing tissue for 3 days in 30% sucrose, cut into several pieces (i.e., C1, C2-C4), embedded in Tissue Tek and frozen in 2-methyl-butane over dry ice (-60˚C). Then tissue was stored at -80˚C until further processing. For further analysis the C1 segments were mounted for cross sections, and the C2-C4 segments were mounted for horizontal sections. Both were cut in a cryostat into 25µm thick sections and mounted onto slides (Fisherbrand colorfrost microscope slides, Fisher Scientific Ltd. Ottawa, ON, Canada) and stored at -80˚C until staining procedure for BDA was performed.

3.3.7 Immunohistochemistry

Frozen C1 and C2-4 sections were dehydrated for 30min in an incubator at 37˚C. Slides were rehydrated 2 x 10min in Tris-buffered saline (TBS, pH 7.4),

followed by two 45min washes in TBS containing 0.5% Triton-X. Then slides were incubated overnight (4˚C) in avidin-biotin complex (ABC) solution (Elite, Vector Laboratories, Burlingame, CA, USA). The next day the ABC solution was washed off (2 x 10min in TBS) and Diaminobenzidene (DAB Vektor kit, SK4100, Vector Laboratories) reaction was performed for 5min. Afterwards, slides were washed in distilled water to halt the reaction, and subsequently washed twice for 10min in TBS. Sections were dehydrated in reduced alcohol concentrations, cleared with Xylene and coverslipped with Permount (Fisher Scientific Ltd. Ottawa, ON, Canada).

3.3.8 Histology

Analysis was performed comparable to our earlier studies (Vavrek *et al.*, 2006; Girgis *et al.*, 2007) using bright field or phase contrast microscopy (Leica). The total number of traced axons of the CST was counted on cross sections at the level of C1.

Further anatomical analysis focused on lesion size, which was reconstructed from C2-4 spinal cord tissue by analyzing every 4^{th} section through the dorso-ventral plane of the spinal cord by using landmarks (i.e., grey/white matter interphases and central canal). Then the surface area of any cavitations observed at the injury site was recorded and the gray/white matter damage was selected (Adobe Photoshop CS2) calculated and presented as ratio of the percentage of the lesion in a schematic cross section of the cervical spinal cord. Rats with

significant larger lesion sizes than a unilateral ablation of the dorsal and lateral CST and mostly of the RST (dorso-lateral quadrant lesion), were excluded from the study.

Further analysis of the lesion size was focusing on the lesion extent in rostro-caudal axis in order to examine training induced changes in lesion size. Therefore, for each animal the lesion extent was also measured on every fourth horizontal C2-C4 section. The greatest value was used, averaged and the result compared between groups.

Another evaluation performed on the C2-C4 spinal cord tissue was the count of CST collaterals rostral and caudal to the lesion, which was performed with light microcopy (400 x magnification). Therefore, every 4th section throughout the entire spinal cord was analyzed. The number of collateral sprouts emanating from the intact or lesioned CST by crossing an imaginary line at the white/grey matter interphase (for more than 30μm), and projecting into the grey matter was counted. Then this number was normalized to the length of analyzed tissue (in mm) per section and to the number of total labeled axons at the level of C1.

3.3.9 Preparation of protein samples and Western blot analysis

An additional group of rats was used to perform protein samples and Western blot analysis. Animals received skilled reaching training for 10 days (starting either on day 4 or 14 following SCI) or remained untrained. These subgroups of animals were deeply anesthetized at 14 or 24 days after lesion respectively. The

ipsilateral and contralateral motor cortices were extracted and immediately frozen at -80˚C in liquid nitrogen (n = 4 per treatment group).

Tissue samples were prepared and homogenized as described previously (Pearse *et al.*, 2001). Briefly, tissue samples were rapidly thawed and then homogenized with a Dounce homogenizer (30 strokes, 4°C) in 1ml of ice-cold cell lysis buffer (20mM Tris-HCl (pH 7.5), 150mM NaCl, 1mM EDTA, 1mM EGTA, 1% Triton X-100, 2.5mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 50mM NaF, 1μg/ml leupeptin, 1mM PMSF, and 1x Roche's complete protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany)). The samples were centrifuged (1000 ×g, 10min, 4°C) and the supernatants were assayed for total protein. All procedures were performed at 4°C. Protein concentrations were determined by the BCA protein assay, according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA). Protein extracts were normalized to a final concentration of 1mg/ml and boiled at 95°C for 10min in Laemmli buffer (100mM Tris, pH 6.8, 250mM β-mercaptoethanol, 4% sodium dodecyl sulfate, 0.01% bromophenol blue and 20% glycerol). A total of 20μg of protein was loaded per lane for separation by 10% SDS-polyacrylamide gel electrophoresis and transferred overnight (room temperature, 25V) onto a Nitrocellulose blotting membrane (PALL Life Sciences Corporation, BioTraceNT, Fl). Membranes were then blocked in 3% BSA (fraction V; Sigma) in TBS-T (50mM Tris-HCl, 150mM NaCl, 0.15% Tween-20, pH 8.0) and probed with different primary antibodies (rabbit polyclonal anti-pPKA (Ser-338, Abcam Inc., Cambridge, MA; rabbit polyclonal anti-PKA (Santa Cruz); rabbit polyclonal

anti-EPAC-1 (Abcam))). PKA α-cat (C-20), (Santa Cruz Biotechnology, Inc. Cat No. sc-903) is an affinity purified rabbit polyclonal IgG antibody raised against a peptide mapping at the C-terminus of the PKAα catalytic subunit of human origin. As per the manufacturer's data sheet, it shows specificity towards the PKAα catalytic subunit of mouse, rat, human, dog and mink origin by Western Blotting, with partial cross-reactivity to the β and γ subunits. The antibody was used at a dilution of 1:1000 in 1XTBST buffer containing 3% BSA, allowing detection of a single band at 38kDa, the expected molecular weight of PKAα cat (Maldonado *et al*., 1988).

PhosphoPKA beta (S338) (Abcam; Cat No. ab5816) is a rabbit polyclonal antibody to phosphoPKA beta (catalytic subunit) which was developed against a synthetic phosphopeptide derived from the carboxyl terminus of human PKA catalytic beta subunit containing serine 338 as its immunogen. The antibody is known to react with human and mouse protein and is also predicted to react to other mammalian species due to sequence homology. Partial cross-reactivity is also seen with the the β and γ subunits. The manufacturer has demonstrated that a peptide corresponding to PKA cat beta [pS338] blocks the antibody signal, verifying specificity and that phosphatase stripping eliminates the signal, verifying phospho-specificity. The antibody was used at a dilution of 1:1000, identifying a band at 42 kDa, the predicted molecular weight of phosphoPKA beta (catalytic subunit).

The Epac1 antibody (Abcam; Cat No. ab21236) is a polyclonal antibody raised in rabbit using a synthetic peptide: DHSVLTLQLPVTASVR, corresponding to amino acids 526-541 of Epac1 as the immunogen. The antibody to Epac1 was then affinity purified over immobilized antigen based chromatography. It is reported to react with Epac1 from human, mouse, rat and rabbit but does not cross react with Epac2. This antibody was used at a concentration of 1:1000, allowing detection of a single band at 99kDa, the predicted molecular weight of this protein (Sabbatini *et al*., 2008).

The anti-beta-actin antibody was used as a loading control for all our Western blot experiments. This is a monoclonal antibody produced in mouse (Sigma-Aldrich cat_no: A1978). The immunogen used to raise this antibody was a beta-cytoplasmic actin N-terminal peptide: Ac-Asp-Asp-Asp-Ile-Ala-Ala-Leu-Val-Ile-Asp-Asn-Gly-Ser-Gly-Lys, conjugated to KLH and is obtained as a purified antibody from the manufacturer that has been reported to have reactivity towards human, bovine, sheep, pig, rabbit, feline, canine, mouse, rat, guinea pig and chicken. This antibody was used at a dilution of 1:5,000 and identified a band at 42kDa, the expected molecular weight of beta-actin (Gimona *et al*., 1994).

The antibodies were diluted 1/1000 in the blocking buffer for a 2hr incubation at room temperature. Next a secondary anti-rabbit/IgG conjugated with horseradish peroxidase, diluted 1/10,000, and was used for 1hr at room temperature (Jackson ImmunoResearch Laboratories, Inc. West Grove, PA). Visualization of the proteins was accomplished by using the SuperSignal West Pico Chemiluminescent detection kit (Pierce Biotechnology, Thermo Fisher

Scientific Inc, Rockford, IL). For subsequent immunoprobing of the same membrane with multiple antibodies, the membranes were first incubated at 37°C in stripping buffer (62.5mM Tris-HCl, pH 6.7, 10mM β-mercaptoethanol, 2% (w/v) SDS) with gentle agitation for 2h followed by 3 washes with TBS-T buffer. Following the stripping of the blot, the blocking step and incubation with a different primary antibody was performed. The relative amount of immunoreactive protein in each band was determined by scanning densitometric analysis of the X-ray films (Fluor-S Multi Imager, Bio-Rad). Densitometry readings were normalized to β-actin (1:5,000, Sigma-Aldrich). Densitometric data from the injured, contralateral motor cortex was then expressed as a percentage of the unaffected, ipsilateral cortex.

3.3.10 Statistics

A Student's t-test (Prism, V 4.0, GraphPad, San Diego, CA, USA) was used to assess the effects in the sucessrate in the reaching task, the error rate in the gridwalk, of sensitivity in heat on the plantar heater, the occurrence, the delay of an EMG when stimulating the caudal forelimb motor area and the threshold to elicit a movement response. Further the t-test was used to determine statistical differences in the lesion extent in the rostro-caudal axis. Welch's correction was applied to data due to unequal variances. For the statistical analysis of the collateral sprouting and for comparing changes in the production of specific signaling intermediaries between cortices after injury a one-way ANOVA followed by a Tukey post-test (Prism, V 4.0, GraphPad, San Diego, CA, USA)

was used. All results are presented as means ± standard error of the mean. Statistical significance was stated when P-values < 0.05 were found, and indicated with one asterisk in the figures. P-values < 0.01 were considered highly significant and indicated with two asterisks.

3.4 Results

3.4.1 Delayed onset of reaching training results in task specific improvements without impairments in non-trained tasks

Previously we found that the immediate start of training following cervical SCI resulted in task-specific recovery and impairments in a non-trained task (Girgis *et al.*, 2007). The present study was focusing on the prevention of such impairments after intensive rehabilitative training in a reaching task by introducing a short delay in the training onset. The experimental flow and the timing of the training is illustrated in figure 3.1A. During the 6 weeks of rehabilitation the trained rats constantly improved in the reaching task until week 5, where they reached a plateau in their success rate (figure 3.1C). In the final testing session (at week 8 post lesion) trained rats (n=7, 3 rats were excluded due to lesion size, see material and methods) achieved a success rate of 34.3% ± 5.8 (Mean ± SEM) with their affected paw. When comparing the success rate between the last training session and the final testing a decrease in the performance was found, this could be explained by the fact that the single pellet reaching task is very sensitive to changes in the daily routine, such as filming procedure or the performance of

other tests on the same day. Untrained rats (n=10) reached only a success rate of 5.6% (± 5.6; figure. 3.2A). This represents a highly significant difference between the groups ($p = 0.006$). Thus, a delayed training onset of 12 days following incomplete SCI promotes task-specific recovery to a comparable degree, as we reported following immediate training (Girgis *et al.*, 2007). In addition, we found that the delayed training onset did not impact the non-trained task. There was no statistical difference in the errors (slips and falls) when crossing a horizontal ladder between rats that received reaching training (0.5 ± 0.1; figure 3.2B) and their untrained counterparts (1.3 ± 0.5).

Strikingly, further analysis of the horizontal ladder crossing showed that rats with cervical SCI tend to use a new strategy (i.e., stand on their wrist/lower forelimb instead of their paw) more frequently possibly to avoid slipping between the rungs. This compensatory movement was counted and set into relation to the overall steps. The results show that trained animals made significantly less ($p = 0.018$) compensatory movements (1.3 ± 0.3) than untrained rats (3.3 ± 0.7; figure 3.2C). This difference might have been influenced by the fact that trained rats also practiced stepping on rungs, by frequently walking on the grid in the front half of the reaching box. Untrained rats in contrast, spend most of their time in the back half of the reaching chamber with a smooth surface. Directly after the lesion they initially tried to reach for non- existent pellets but they discontinued this behaviour after 2 weeks. This might confound the conclusion that reaching training influenced the walking strategy on the horizontal ladder.

Although the training influenced the use of the compensatory strategy, no correlation between the success rate in the single pellet reaching task and the compensatory movement strategy was found ($r = - 0.05$).

In a last behavioural task we examined the effects of rehabilitative training on a sensory system. Therefore, rats were tested on a plantar heater, where the time to withdrawal the affected paw from a heat source was measured. No significant differences in thermal sensitivity between trained (5.9sec ± 0.5) and untrained rats (5.8sec ± 0.3) were found (figure 3.2D).

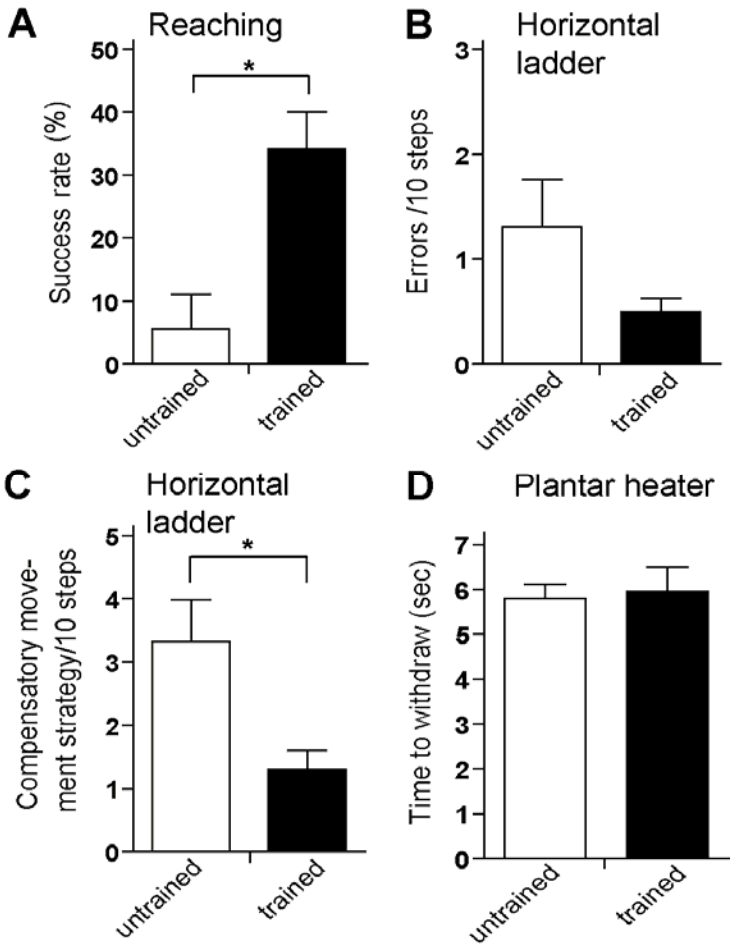


Figure 3.2. Functional testing. **A**: Success rate in the trained paradigm after 8 weeks post SCI. Trained rats were significantly better in the reaching task than their untrained counterparts. **B**: The horizontal ladder task showed no statistical difference between trained and untrained rats. **C**: Detailed analysis of the horizontal ladder crossing showed that untrained rats compensate significantly more than trained rats. **D**: Thermal sensitivity was tested using a plantar heater. No statistical difference between the groups was found. * indicates $p < 0.05$. Error bars show standard error of the mean.

3.4.2 Delayed reaching training did not influence cortical map changes

After 6 weeks of rehabilitative training and completion of motor and sensory testing, rats underwent ICMS in both hemispheres of the caudal forelimb motor area. The results of stimulating the forelimb motor cortex showed no differences in eliciting wrist extensor activity between trained and untrained rats. When transferring the color code (figure 3.3A) into a numerical code (0-3), the representation of the cortical map ipsilateral to the lesion between trained (1.0 ± 0.2) and untrained rats (1.1 ± 0.1; figure 3.3B) was not statistically different. Comparably, stimulation of the caudal forelimb motor area contralateral to the lesion did not lead to statistical differences between trained (1.0 ± 0.1) and untrained rats (0.8 ± 0.1; figure 3.3C). Thus a short delay in the onset of reaching training after incomplete SCI does not promote significant cortical rearrangements as observed following immediate onset of training (Girgis *et al.*, 2007).

Recordings from wrist extensor muscles (figure 3.4A, B) in the forelimb ipsilateral to the lesion were used to determine the delay of the motor evoked potentials (MEPs) as well as the thresholds to elicit a response. When analyzing the delay of MEPs we found that stimulation of the cortex with injured axons triggered slightly faster responses in the affected paw in trained rats (14.9ms ± 1.0), which was however not statistically different to the latency found in untrained rats (18.5ms ± 2.9; figure 3.4C). When analyzing the effects of ICMS of the hemisphere ipsilateral to the lesion we also did not find statistical difference

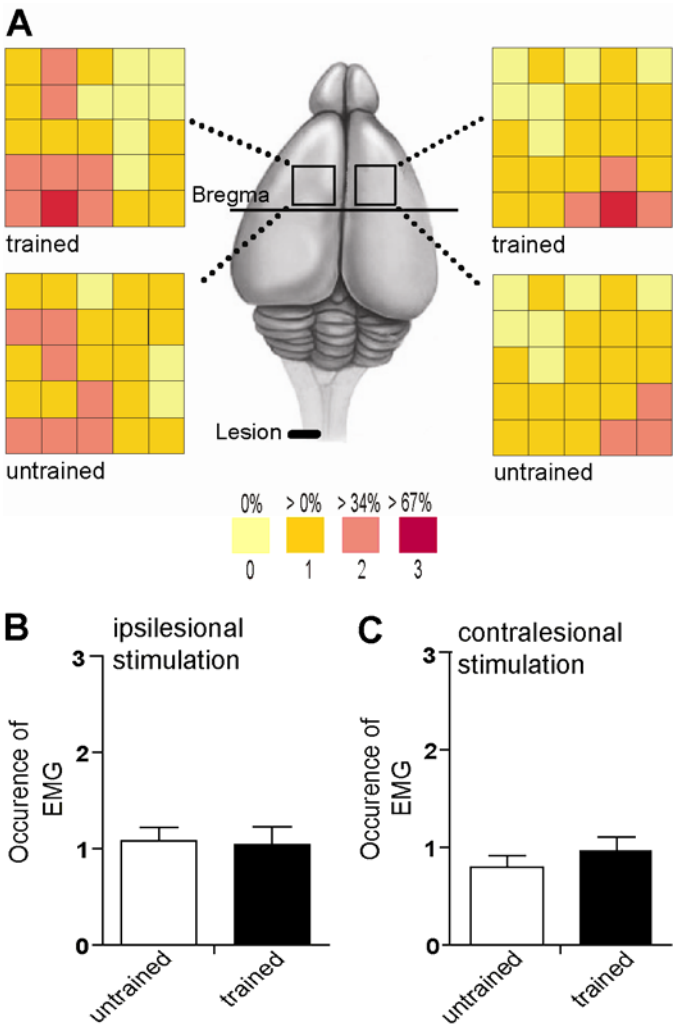


Figure 3.3. Mapping of the motor cortex using intracortical micro-stimulation. **A**: Each forelimb motor area was divided into 25 squares, which were stimulated individually. The colour-code, indicates the responsiveness of each square, averaged over all the animals, ranging from no evoked wrist movement (light yellow) to a high number of evoked wrist movement (>67%, dark red). For statistical analysis the colour-code was translated into a score (0-3). Neither ICMS of the cortex ipsilateral (**B)** nor contralateral (**C)** to the lesion showed a statistical difference in the occurrence of elicited wrist movements between trained and untrained rats. Error bars show standard error of the mean.

in the delay of the MEPs (21.2ms ± 2.91, 2 out of 5) and trained rats (21.7ms ± 3.4, 5 out of 8; figure 3.4D).

When stimulating the cortex contralateral to the lesion, the intensity (threshold) to elicit a wrist extensor MEP was comparable between untrained (64.9μA ± 7.8) and trained rats (88.6μA ± 10.6; figure 3.4E). Similarly, there was no statistical difference when stimulating the cortex ipsilateral to the lesion (53.7μA ± 4.9 in untrained and 80.4μA ± 12.5 in trained animals; figure 3.4F).

3.4.3 Trained rats have smaller lesion sizes in the rostro-caudal axis

In our lesion paradigm, the rostro-caudal extent is an important factor as the lesion is located within the cervical enlargement containing motor and interneuron pools involved in forelimb control. Thus, a larger lesion in the rostro-caudal axis extent can contribute to further functional impairments as the innervation to these neuronal populations is lost. We did not find a difference in the lesion size when the lesion was reconstructed from horizontal sections into a cross section. In trained animals the damage in relation to a cross section was 30.4% (± 3.7) and in untrained rats 31% (± 3.1; figure 3.5A). In 4 trained and 4 untrained rats the dorsal CST (see Brosamle & Schwab, 1997) and the entire RST (see Kuchler *et al.*, 2002) was completely ablated. In 5 trained and 6 untrained rats the dorsal CST was less than 30% spared and the RST in these animals was either completely ablated or less than 30% spared.

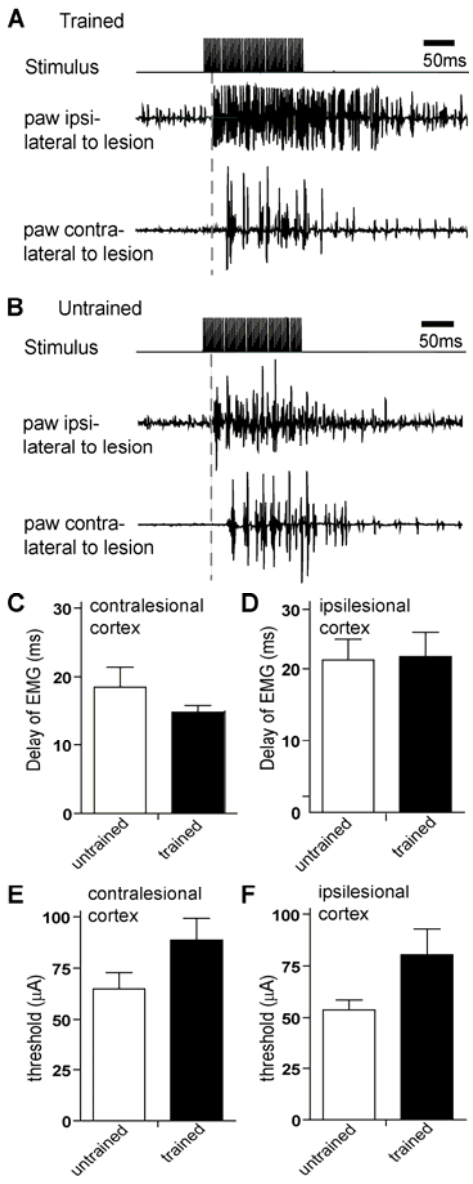


Figure 3.4. Analysis of electromyographic (EMG) activity of wrist extensors elicited by ICMS. **A**, **B:** Illustration of bilateral recordings in a trained and an untrained rat respectively. The dashed line indicates the onset of EMG activity in the paw ipsilateral to the lesion (contralateral to the stimulation). **C-D:** Neither stimulation in the hemisphere contralateral nor ipsilateral to the lesion resulted in a statistical difference between trained and untrained rats. Also no statistical difference in the current threshold to elicit an EMG response was found between groups when stimulating contralaterally (**E**) or ipsilaterally (**F**) to the lesion. Error bars show standard error of the mean.

When analyzing the lesion in the rostro-caudal extent, we found that trained rats had a significantly smaller lesion extent in the rostro-caudal axis (3.3mm ± 0.2) compared to untrained counterparts (4.6mm ± 0.5; p= 0.018; figure 3.5B-D). This difference in lesion size did not correlate with the success rate in the single pellet reaching task (r = -0.3).

3.4.4 Collateral sprouting

Here we examined whether axons of the intact dorsal CST will send out collaterals to the grey matter rostral and caudal to the injury (figure 3.6A) comparable to what we reported earlier for the injured CST (Girgis *et al.*, 2007). The number of counted collaterals emanating from the CST was averaged per animal and normalized to the length (in mm) analyzed per section and to the number of total labeled axons at the level of C1.

The results of the normalized collateral counts show no statistical difference of the unlesioned CST caudal to the lesion between trained (0.0005 ± 0.0002; figure 3.6B) and untrained rats (0.002 ± 0.0008). Quantification of collateral sprouting emanating from the CST rostral to the lesion was insignificant different between groups (0.003 ± 0.0005 in untrained and 0.002 ± 0.0004 in trained animals; figure 3.6B). In agreement with increased collateral sprouting of the lesioned CST following early rehabilitative training (Grigis *et al.*, 2007), we found a high significant difference ($F_{39} = 13.55$, $p < 0.001$) in collateral sprouting between the

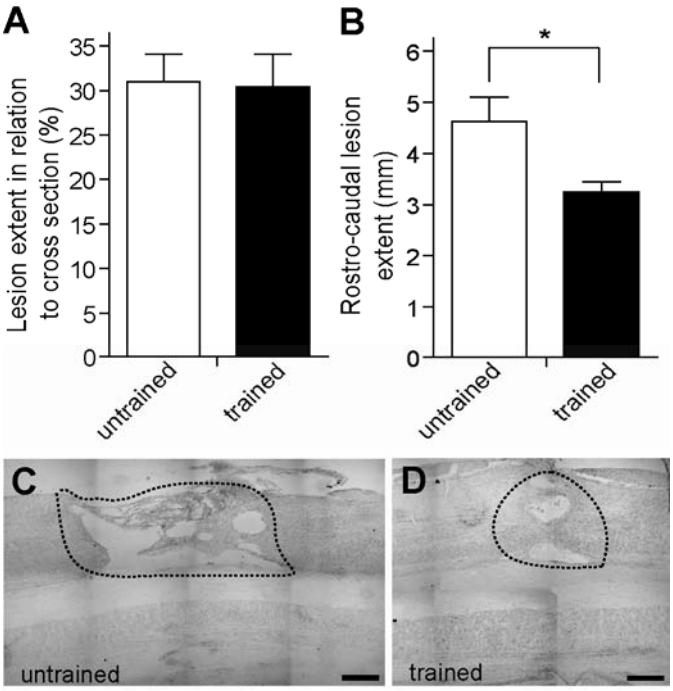


Figure 3.5. Evaluation of the lesion size. **A:** The lesion extent in relation to a cross section was not different between groups. **B:** Analysis of the lesion extent in the rostro-caudal axis of the spinal cord showed that untrained animals had a significantly larger lesion. Microscopic photographs of untrained (**C**) and trained (**D**) rats demonstrate this difference in lesion extent (dotted circle). Scale bars represent 500μm and * indicates $p < 0.05$. Error bars show standard error of the mean.

unlesioned CST (0.0025 ± 0.0004) and the lesioned CST (0.008 ± 0.0009) in rats that received delayed reaching training (figure 3.6B).

3.4.5 The timing of rehabilitative reaching training differentially affects injury-induced changes in cyclic AMP signaling intermediaries.

Following CNS injury there are marked and persistent reductions in levels of cyclic AMP in the brain and/or spinal cord (Pearse *et al.*, 2004; Atkins *et al.*, 2007), which may be indicative of reduced activity in brain areas that projected to or across the lesion. Therefore, we examined whether there were injury-induced changes in cyclic AMP signaling intermediaries within the motor cortex and if these changes could be modulated by task-specific rehabilitative training initiated either immediately or delayed after SCI. A unilateral lesion to the C2-3 cervical spinal cord produces a dramatic reduction in cyclic AMP levels in the contralateral motor cortex that is affected by the injury (Fouad *et al.*, unpublished). Although we did not observe significant, injury-induced changes in the total levels of the downstream kinase of cyclic AMP, protein kinase A (PKA; as measured by levels of catalytic subunit α; no significant group effect by ANOVA, $F_{9,30} = 1.39$, $p>0.05$; figure 3.7A) in the contralateral motor cortex when compared to the unaffected ipsilateral cortex, at both 2 and 4 weeks post-injury, a dramatic change in serine-388 phosphorylated, activated PKA, among groups was

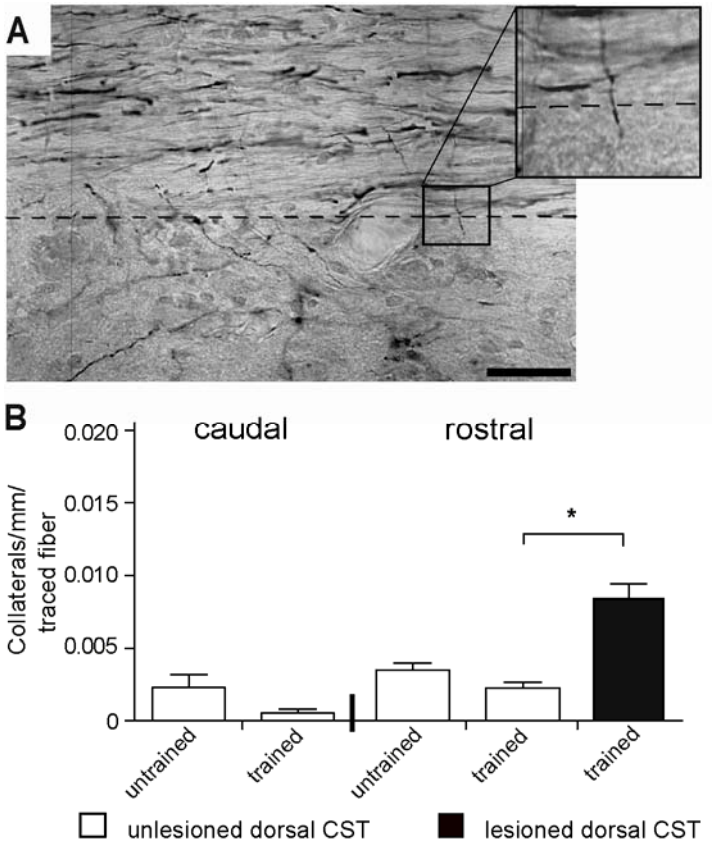


Figure 3.6. Collateral sprouting of the intact corticospinal tract (CST) at cervical level. **A:** The microscopic photograph shows that the labeled CST is sending out collaterals into the grey matter. The dashed line indicates the white/grey matter interphase. A magnification of the window in **A** shows a collateral sprout leaving the labeled tract and emanating into the grey matter. Scale bar represents 40μm. **B:** Quantification of collaterals caudal and rostral to the lesion showed only statistically insignificant differences between the groups. A comparison between the unlesioned and the lesioned dorsal CST shows a statistical increase in the number of collateral sprouts emanating from the injured CST to the grey matter. * indicates $p < 0.05$ and error bars show standard error of the mean.

found, $F_{9,30} = 17.49$, $p<0.0001$. Unilateral cervical injury induced a 54.9 ± 5.0% reduction in $pPKA^{Ser338}$ at 2 weeks ($q_{39} = 10.23$, $p<0.001$) and a further reduction to 17.9 ± 8.0% of ipsilateral levels at 4 weeks ($q_{39} = 5.67$, $p<0.01$ vs. 2 weeks; figure 3.7B) in the contralateral motor cortex as compared to the ipsilateral cortex. While the use of an acute training paradigm had no significant effect on injury-induced changes in phosphorylated PKA ($q_{39} = 1.21$ at 2 weeks, $q_{39} = 4.64$ at 4 weeks; $p>0.05$ at both timepoints), a delay in the implementation of training was able to significantly restore these levels at 4 weeks post-injury (to 48.8 ± 7.4% of the ipsilateral cortex, $q_{39} = 5.75$, $p<0.01$; figure 3.7B). Analysis of the canonical cAMP pathway, which signals through Rap-1 and EPAC-1, showed a significant change among groups in EPAC-1 levels ($F_{9,30} = 14.01$, $p<0.0001$). Following a unilateral cervical injury, a reduction in EPAC1 within the contralateral cortex to 23.2 ± 11.7% of ipsilateral levels was observed at 2 weeks, $q_{39} = 8.85$, $p<0.001$; these levels remained low through 4 weeks; Fig. 3.7C). Again, an early onset of training had no affect on these decreased levels of EPAC-1, $q_{39} = 0.04$, $p>0.05$. Unlike activation of the classic cAMP-PKA signaling cascade, however, delayed training did not restore, but rather further reduced, the production of EPAC-1 (to 11.6 ± 3.9% of ipsilateral levels, $q_{39} = 5.48$, $p<0.05$; figure 3.7C).

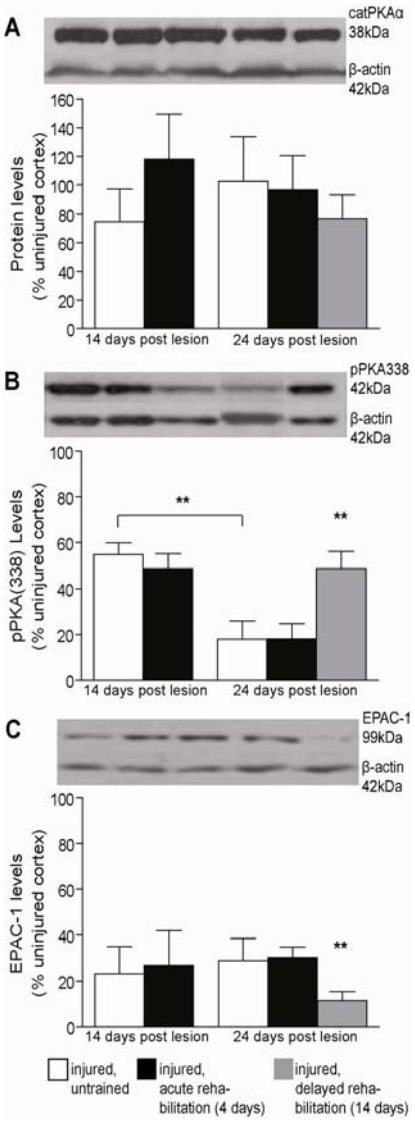


Figure 3.7. Injury-induced changes in cyclic AMP signaling intermediaries: the effect of acute and delayed rehabilitation. **A:** Total PKA levels in the contralateral motor cortex are unaffected by C2-3 spinal cord injury. **B:** Marked reductions in $pPKA^{338}$ occur following SCI in the contralateral motor cortex, decreasing further over time. Delayed, but not acute rehabilitation prevents this progressive decrease. **C:** Levels of EPAC-1 are also dramatically reduced in the contralateral motor cortex following cervical injury. Acute rehabilitation is without effect while delayed rehabilitation further reduces these levels. Data is expressed as mean ± standard error, n = 4 per group. * indicates $p < 0.05$ and ** $p < 0.01$.

3.5 Discussion

In an earlier study we showed that rehabilitative training following cervical SCI in rats starting 4 days post lesion significantly improved the recovery of the trained task (Girgis *et al.*, 2007). The present study demonstrates that a delay of 12 days before the onset of rehabilitative training still allows a comparable task-specific recovery, although here the non-preferred side was undergoing rehabilitative training. This possibly made the recovery even more challenging and could explain a decrease in post-injury performance when compared to our earlier study.

The appearance of task-specific recovery after a delayed onset of rehabilitative training is somewhat surprising as the existence of a "window of opportunity" for rehabilitative training immediately following injuries of the CNS in generally agreed upon. This is based on findings of up-regulation of growth promoting factors including BDNF within the first days following SCI (Donnelly & Popovich, 2008), and that an acute inflammatory response is a necessity for NT-3 induced sprouting following pyramidal tract lesions (Chen *et al.*, 2008). Accordingly, a delayed onset of rehabilitative training has been reported to be less effective, since the nervous system is once more in a "rigid" state. Examples following SCI include studies in primates (Barbay *et al.*, 2006), or in rats (Norrie *et al.*, 2005). However, in these studies the delay of training was significantly longer (1 and 3 months post lesion respectively) than in our study. Experiments that focused on a shorter delay, comparable to that used in the present study,

could show an exercise-induced up-regulation of BDNF only in a group of rats that was trained from 7 to 14 days but not in a group that was trained immediately to 6 days post brain injury (Griesbach *et al.*, 2007). Studies in animal models of stroke also reported that an early training onset might promote side effects such as learned bad use (Alaverdashvili *et al.*, 2008) or even the exacerbation of the injury size (DeBow *et al.*, 2003; Risedal *et al.*, 1999; Humm *et al.*, 1998). Negative effects of early training after SCI in rats were found in an earlier study from our laboratory, where immediate training (Girgis *et al.*, 2007) but not the delayed onset of rehabilitative training (current study) resulted in impairments in an untrained task. It has already been reported that task specific training does not necessarily translate into the recovery of untrained tasks (e.g., backwards and forward stepping in humans with SCI, Grasso *et al.*, 2004), and also that task specific training can affect untrained ones (e.g., standing versus walking in cats with SCI, De Leon *et al.*, 1998). While the current results could not help to unravel the mechanism causing the reduced performance of trained rats with SCI in an untrained task, it is evident that training one task has eventually consequences on the performance in untrained tasks.

When considering the onset of rehabilitative training following SCI it also has to be kept in mind that, especially in humans, an immediate training onset is clinically impractical. This is due to the frequent occurrence of multiple injuries in SCI patients, a phase of spinal shock where the transmission of spared axonal pathways is transiently blocked (Holaday & Faden, 1983; Hiersemenzel *et al.*,

2000) and reduced excitation in the spinal cord caudal to the lesion (Harvey *et al.*, 2006).

It has been shown that the excitability of spinal networks can be affected by BDNF (Jakeman *et al.*, 1998) and that the expression of BDNF is up-regulated a second time at around 10 days following SCI (Donnelly & Popovich, 2008). A signaling molecule involved in the up-regulation of BDNF is cAMP, which is important for the survival, growth and guidance of neurons and their axons (Pearse, 2004). We have previously reported that SCI reduces levels of cAMP, in those brain regions projecting to or across the lesion site (Pearse *et al.*, 2004). In the current study, we investigated whether a reaching training in the rat following cervical SCI can influence the expression of downstream effectors of cAMP such as protein kinase A (PKA) and EPAC-1. We observed that following lesion both phosphorylated PKA, and canonical, EPAC-1, were reduced in the motor cortex contralateral to the spinal injury, as compared to the unaffected, ipsilateral side. Interestingly, we only found significant restoration of PKA levels when rehabilitative training was started after a delay of 14 days, but not when performed between day 4 to 14 post-injury. This timing phenomenon for the effect of training on growth promoting factors resembles reports after brain injury (Griesbach *et al.*, 2007). In that study it has been shown that increased expression of cAMP signaling intermediaries, such as CREB, as well as improved functional recovery occurs only when exercise is started at 14 days following injury. However, training within the first week post injury did not up regulate CREB or BDNF (Griesbach *et al.*, 2007).

We also found that following a delayed training onset the stimulation of the caudal forelimb motor area did not reveal significant changes/rearrangements in cortical maps. This result is surprising but for three reasons. First, we showed in an earlier study with an immediate training onset that cortical rearrangements were significantly increased (Girgis *et al.*, 2007). Second, increased cortical PKA activation following delayed training would suggest increased plasticity of the brain and spinal cord and third, it is generally assumed that such cortical rearrangements are involved in promoting functional recovery in primates and rats after brain injury or SCI (Kaas *et al.*, 2008). However, our current results indicate that increased recovery seems not to be necessarily dependent on cortical map changes. Alternatively, important map changes might have been overseen due to a limited number of muscles (i.e., wrist extensors) and the limited cortical area that had been examined. Another reason that might have masked rehabilitative training induced cortical rearrangements is the pre-injury experience. In contrast to the study by Girgis *et al.,* (2007), the current study employed rehabilitative training on the side that has not been exposed to this task pre-injury. However, it could be argued that the cortex that was not exposed to training prior the spinal lesion (see current study) should be especially susceptible to rehabilitative training-induced plasticity, but this has not been found. Further, the percentage of recovery in the rats that had no pre-injury exposure on the lesioned side is comparable to that reported in our earlier study with pre-injury exposure. Finally, it might be that in our earlier study the occurrence of significant cortical rearrangements is related to the adverse effect found in an untrained task (horizontal ladder crossing).

Here we could show that after a delayed onset of rehabilitative training collateral sprouting of the lesioned CST is significantly increased when compared to the unlesioned CST. This result stands in line with earlier studies where sprouting of the lesioned CST was shown (Fouad *et al.*, 2001; Bareyre *et al.*, 2004; Girgis *et al.,* 2007). Unlike our finding of increased sprouting of the lesioned CST we observed that the collateral density of the unlesioned CST is unchanged. This result appears to stand in contrast to an earlier report, where sprouting of unlesioned CST fibers (Weidner *et al.*, 2001) was reported. However, it has to be kept in mind that in the present study the evaluated spared CST fibers were projecting into the lesioned side of the spinal cord (as compared to the ventral fibers on the injured side; Weidner *et al.*, 2001). We cannot exclude that enhanced sprouting of spared ventral CST fibers as reported by (Weidner *et al.*, 2001) also contributed to the training-induced recovery. Furthermore, it is likely that adaptive changes in other descending systems are involved in the training induced recovery. For example, it has to be considered that in the present study the lesion targeted the dorso-lateral quadrant of the spinal cord. Such a lesion would ablate the majority of rubrospinal tract (RST) fibers, however as this tract projects a few fibers even ventral to the midline (Kuchler *et al.*, 2002), a small percentage was likely spared. It has been shown earlier that the RST is involved to compensate following CST lesion (Alstermark et al. 1987; Perfiliev et al. 1998). But it appears that the contribution of spared RST fibers is not a necessity for functional recovery. As discussed by Alstermark *et al.* (1981), following the ablation of the CST and RST, cats showed a recovery of grasping movements.

Later studies with complete lesions of the CST and partly or complete lesions of the RST indicated that the recovery was most likely mediated by other descending tracts such as the reticulospinal pathway and propriospinal interneurons (Alstermark *et al.*, 1987; Blagovechtchenski *et al.*, 2000; Pettersson *et al.*, 2007).

In conclusion, our results demonstrate that a short delay in the onset of training in a forelimb task can significantly alter various parameters that are important for successful rehabilitative training.

3.6 References

Alaverdashvili M., Foroud A., Lim D. H. & Whishaw I. Q. (2008). " "Learned baduse" limits recovery of skilled reaching for food after forelimb motor cortex stroke in rats: A new analysis of the effect of gestures on success." Behav Brain Res 188: 281-90.

Alstermark B., Lundberg A., Norrsell U. & Sybirska E. (1981). "Integration in descending motor pathways controlling the forelimb in the cat. 9. Differential behavioural defects after spinal cord lesions interrupting defined pathways from higher centres to motoneurones." Exp Brain Res 42: 299-318.

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

Atkins C. M., Oliva A. A., Jr., Alonso O. F., Pearse D. D., Bramlett H. M. & Dietrich W. D. (2007). "Modulation of the cAMP signaling pathway after traumatic brain injury." Exp Neurol 208: 145-58.

Babu R. S. & Namasivayam A. (2008). "Recovery of bipedal locomotion in bonnet macaques after spinal cord injury: footprint analysis." Synapse 62: 432-47.

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

Barbay S., Plautz E. J., Friel K. M., Frost S. B., Dancause N., Stowe A. M. & Nudo R. J. (2006). "Behavioral and neurophysiological effects of delayed training following a small ischemic infarct in primary motor cortex of squirrel monkeys." Exp Brain Res 169: 106-16.

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

Bareyre F. M., Kerschensteiner M., Raineteau O., Mettenleiter T. C., Weinmann O. & Schwab M. E. (2004). "The injured spinal cord spontaneously forms a new intraspinal circuit in adult rats." Nat Neurosci 7: 269-77.

Barriere G., Leblond H., Provencher J. & Rossignol S. (2008). "Prominent role of the spinal central pattern generator in the recovery of locomotion after partial spinal cord injuries." J Neurosci 28: 3976-87.

Beekhuizen K. S. & Field-Fote E. C. (2005). "Massed practice versus massed practice with stimulation: effects on upper extremity function and cortical plasticity in individuals with incomplete cervical spinal cord injury." Neurorehabil Neural Repair 19: 33-45.

Beekhuizen K. S. & Field-Fote E. C. (2008). "Sensory stimulation augments the effects of massed practice training in persons with tetraplegia." Arch Phys Med Rehabil 89: 602-608.

Behrman A. L. & Harkema S. J. (2007). "Physical rehabilitation as an agent for recovery after spinal cord injury." Phys Med Rehabil Clin N Am 18: 183-202.

Bennett D. J., Li Y., Harvey P. J. & Gorassini M. (2001). "Evidence for plateau potentials in tail motoneurons of awake chronic spinal rats with spasticity." J Neurophysiol 86: 1972-82.

Benowitz L. & Yin Y. (2008). "Rewiring the injured CNS: lessons from the optic nerve." Exp Neurol 209: 389-98.

Blagovechtchenski E., Pettersson L. G., Perfiliev S., Krasnochokova E. & Lundberg A. (2000). "Control of digits via C3-C4 propriospinal neurones in cats; recovery after lesions." Neurosci Res 38: 103-7.

Bolton D. A., Tse A. D., Ballermann M., Misiaszek J. E. & Fouad K. (2006). "Task specific adaptations in rat locomotion: runway versus horizontal ladder." Behav Brain Res 168: 272-9.

Brosamle C. & Schwab M. E. (1997). "Cells of origin, course and termination patterns of the ventral, uncrossed component of the mature rat corticospinal tract." J Comp Neurol 386: 293-303.

Chen Q., Smith G. M. & Shine H. D. (2008). "Immune activation is required for NT-3-induced axonal plasticity in chronic spinal cord injury." Exp Neurol 209: 497-509.

de Leon R. D., Tamaki H., Hodgson J. A., Roy R. R. & Edgerton V. R. (1999). "Hindlimb locomotor and postural training modulates glycinergic inhibition in the spinal cord of the adult spinal cat." J Neurophysiol 82: 359-69.

DeBow S. B., Davies M. L., Clarke H. L. & Colbourne F. (2003). "Constraint-induced movement therapy and rehabilitation exercises lessen motor deficits and volume of brain injury after striatal hemorrhagic stroke in rats." Stroke 34: 1021-6.

Dietz V., Zijlstra W. & Duysens J. (1994). "Human neuronal interlimb coordination during split-belt locomotion." Exp Brain Res 101: 513-20.

Dobkin B., Barbeau H., Deforge D., Ditunno J., Elashoff R., Apple D., Basso M., Behrman A., Harkema S., Saulino M. & Scott M. (2007). "The evolution of walking-related outcomes over the first 12 weeks of rehabilitation for incomplete traumatic spinal cord injury: the multicenter randomized Spinal Cord Injury Locomotor Trial." Neurorehabil Neural Repair 21: 25-35.

Donnelly D. J. & Popovich P. G. (2008). "Inflammation and its role in neuroprotection, axonal regeneration and functional recovery after spinal cord injury." Exp Neurol 209: 378-88.

Edgerton V. R., Leon R. D., Harkema S. J., Hodgson J. A., London N., Reinkensmeyer D. J., Roy R. R., Talmadge R. J., Tillakaratne N. J., Timoszyk W. & Tobin A. (2001). "Retraining the injured spinal cord." J Physiol 533: 15-22.

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

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

Gimona M.,Vandekerckhove J., Goethals M., Herzog M., Lando Z. & Small J. V. (1994). "Beta-actin specific monoclonal antibody." Cell Motil Cytoskel 27: 108-116.

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

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

Griesbach G. S., Gomez-Pinilla F. & Hovda D. A. (2007). "Time window for voluntary exercise-induced increases in hippocampal neuroplasticity molecules after traumatic brain injury is severity dependent." J Neurotrauma 24: 1161-71.

Harkema S. J. (2001). "Neural plasticity after human spinal cord injury: application of locomotor training to the rehabilitation of walking." Neuroscientist 7: 455-68.

Harvey P. J., Li X., Li Y. & Bennett D. J. (2006). "Endogenous monoamine receptor activation is essential for enabling persistent sodium currents and repetitive firing in rat spinal motoneurons." J Neurophysiol 96: 1171-86.

Hiersemenzel L. P., Curt A. & Dietz V. (2000). "From spinal shock to spasticity: neuronal adaptations to a spinal cord injury." Neurology 54: 1574-82.

Holaday J. W. & Faden A. I. (1983). "Spinal shock and injury: experimental therapeutic approaches." Adv Shock Res 10: 95-8.

Humm J. L., Kozlowski D. A., James D. C., Gotts J. E. & Schallert T. (1998). "Use-dependent exacerbation of brain damage occurs during an early post-lesion vulnerable period." Brain Res 783: 286-92.

Jakeman L. B., Wei P., Guan Z. & Stokes B. T. (1998). "Brain-derived neurotrophic factor stimulates hindlimb stepping and sprouting of cholinergic fibers after spinal cord injury." Exp Neurol 154: 170-84.

Jones T. A., Chu C. J., Grande L. A. & Gregory A. D. (1999). "Motor skills training enhances lesion-induced structural plasticity in the motor cortex of adult rats." J Neurosci 19: 10153-63.

Kaas J. H., Qi H. X., Burish M. J., Gharbawie O. A., Onifer S. M. & Massey J. M. (2008). "Cortical and subcortical plasticity in the brains of humans, primates, and rats after damage to sensory afferents in the dorsal columns of the spinal cord." Exp Neurol 209: 407-16.

Kozlowski D. A., James D. C. & Schallert T. (1996). "Use-dependent exaggeration of neuronal injury after unilateral sensorimotor cortex lesions." J Neurosci 16: 4776-86.

Kuchler M., Fouad K., Weinmann O., Schwab M. E. & Raineteau O. (2002). "Red nucleus projections to distinct motor neuron pools in the rat spinal cord." J Comp Neurol 448: 349-59.

Lovely R. G., Gregor R. J., Roy R. R. & Edgerton V. R. (1986). "Effects of training on the recovery of full-weight-bearing stepping in the adult spinal cat." Exp Neurol 92: 421-35.

Maldonado F. & Hanks S. K. (1988). "A cDNA clone encoding human cAMP-dependent protein kinase catalytic subunit C alpha." Nucleic Acids Res 16: 8189-8190.

McKenna J. E. & Whishaw I. Q. (1999). "Complete compensation in skilled reaching success with associated impairments in limb synergies, after dorsal column lesion in the rat." J Neurosci 19: 1885-94.

Norrie B. A., Nevett-Duchcherer J. M. & Gorassini M. A. (2005). "Reduced functional recovery by delaying motor training after spinal cord injury." J Neurophysiol 94: 255-64.

Paxinos G. (1997). "The rat brain in stereotaxic coordinates." Vol. xxxiii, San Diego: Academic Press; 1997. p.78.

Pearse D. D. (2004). "Targeting intracellular signaling molecules within the neuron promote repair after spinal cord injury." Topics in Spinal Cord Injury Rehabilitation 10: 1-16.

Pearse D. D., Bushell G. & Leah J. D. (2001). "Jun, Fos and Krox in the thalamus after C-fiber stimulation: coincident-input-dependent expression, expression across somatotopic boundaries, and nucleolar translocation." Neuroscience 107: 143-59.

Pearse D. D., Pereira F. C., Marcillo A. E., Bates M. L., Berrocal Y. A., Filbin M. T. & Bunge M. B. (2004). "cAMP and Schwann cells promote axonal growth and functional recovery after spinal cord injury." Nat Med 10: 610-6.

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

Raineteau O., Fouad K., Bareyre F. M. & Schwab M. E. (2002). "Reorganization of descending motor tracts in the rat spinal cord." Eur J Neurosci 16: 1761-71.

Raineteau O. & Schwab M. E. (2001). "Plasticity of motor systems after incomplete spinal cord injury." Nat Rev Neurosci 2: 263-73.

Risedal A., Zeng J. & Johansson B. B. (1999). "Early training may exacerbate brain damage after focal brain ischemia in the rat." J Cereb Blood Flow Metab 19: 997-1003.

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

Rupp R., Eberhard S., Schreier R. & Colombo G. (2002). " [Reha-Stepper locomotion therapy in early rehabilitation of paraplegic patients]." Biomed Tech (Berl.) 47: 708-11.

Sabbatini M. E., Chen X., Ernst S. A. & Williams J. A. (2008). "Rap1 activation plays a regulatory role in pancreatic amylase secretion." J Biol Chem 283: 23884-94.

Schwartz M. & Yoles E. (2006). "Immune-based therapy for spinal cord repair: autologous macrophages and beyond." J Neurotrauma 23: 360-70.

Shaw S. E., Morris D. M., Uswatte G., McKay S., Meythaler J. M. & Taub E. (2005). "Constraint-induced movement therapy for recovery of upper-limb function following traumatic brain injury." J Rehabil Res Dev 42: 769-78.

Taub E. & Uswatte G. (2003). "Constraint-induced movement therapy: bridging from the primate laboratory to the stroke rehabilitation laboratory." J Rehabil Med 41: 34-40.

Taub E., Uswatte G., Mark V. W. & Morris D. M. (2006). "The learned nonuse phenomenon: implications for rehabilitation." Eura Medicophys 42: 241-56.

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

Waters R. L., Adkins R. H., Yakura J. S. & Sie I. (1994). "Motor and sensory recovery following incomplete paraplegia." Arch Phys Med Rehabil 75: 67-72.

Weidner N., Ner A., Salimi N. & Tuszynski M. H. (2001). "Spontaneous corticospinal axonal plasticity and functional recovery after adult central nervous system injury." Proc Nat Acad Sci USA 98: 3513-8.

Wernig A., Muller S., Nanassy A., Cagol E. (1995). "Laufband therapy based on 'rules of spinal locomotion' is effective spinal cord injured persons." Eur J Neurosci 7: 1429.

Whishaw I. Q., O'Connor W. T. & Dunnett S. B. (1986). "The contributions of motor cortex, nigrostriatal dopamine and caudate-putamen to skilled forelimb use in the rat." Brain 109: 805-43.

Whishaw I. Q., Pellis S. M., Gorny B., Kolb B. & Tetzlaff W. (1993). "Proximal and distal impairments in rat forelimb use in reaching follow unilateral pyramidal tract lesions." Behav Brain Res 56: 59-76.

You S. W., Chen B. Y., Liu H. L., Lang B., Xia J. L., Jiao X. Y. & Ju G. (2003). "Spontaneous recovery of locomotion induced by remaining fibers after spinal cord transection in adult rats." Restor Neurol Neurosci 21: 39-45.

CHAPTER 4

COUNTERACTING THE DECLINE OF TRAINING EFFICACY IN SPINAL CORD INJURED RATS

4.1 Preface

The previous Chapter described an experiment that was conducted to elucidate the importance of the onset of rehabilitative reaching training. The results show that a short delay in reaching training following cervical SCI leads to a similar success rate in reaching as it was observed after an early onset of training (Girgis *et al.*, 2007). Interestingly, measurements of a downstream factor of cyclic AMP, called protein kinase A (PKA), revealed significant differences between rats that were exposed to early (training initiated on day 4) and rats that received delayed (initiation of training on day14) reaching training.

Another important process that was not addressed in the study in Chapter 3, is that following SCI the blood brain barrier is disrupted and immune cells are capable of entering the spinal or brain tissue. This leads to a complex and not fully understood inflammatory mechanism. It is still debated whether the inflammatory response after SCI is detrimental or beneficial for recover but recently it has been reported that an inflammatory response is necessary to allow for NT-3 induced axonal growth in a chronic rat model of SCI (Chen et al. 2008). That would suggest that if the inflammatory response is involved in recovery, is it inevitable to combine rehabilitative training with inflammation? In the following Chapter, rats with a chronic spinal lesion received an injection of a bacterial endotoxin (Lipopolysaccharide, LPS) that leads to an inflammatory response; similar to what has been seen following SCI. The experiment was designed to investigate the importance of inflammation in the recovery after SCI. Because the

acute phase of inflammation is well studied and it is generally agreed that the CNS becomes more rigid and thus adaptive changes are less likely to occur with time after SCI, we investigated the combination of rehabilitative reaching training in a chronic model of SCI with systemically induced inflammation.

4.2 Introduction

Spinal cord injury (SCI) is a devastating event that is accompanied by a permanent loss of motor and sensory functions below the injury. To date the most successful approach to promote recovery following SCI is rehabilitative training. There is currently only limited information regarding the time frame when rehabilitative training is most beneficial. Evidence from stroke research suggests that there might be a "window of opportunity" in which the central nervous system (CNS) is most susceptible to training-induced changes. Only a few studies have investigated this important issue after SCI and suggested a decline in the training efficacy when the rehabilitative training was initiated at a chronic stage (Norrie *et al.*, 2005; Winchester *et al.*, 2005; 2009). There are several possible reasons for this decline in the efficacy for rehabilitative reaching training, including a deterioration in neuronal and/or muscle function (Dietz *et al.*, 1995), declining levels of the second messenger cyclic adenosine monophosphate (cAMP; Pearse *et al.*, 2004; Krajacic *et al.*, 2009), declining expression of growth-and plasticity-associated factors (Song *et al.*, 2001; Di Giovanni *et al.*, 2005) and an abatement in the injury-induced inflammatory response over time (Donnelly and Popovich, 2008).

Although it appears counterintuitive that inflammation is a possible contributor to recovery, various studies have reported that certain aspects of inflammation within the CNS are involved in neuro-reparative functions (Schwartz *et al.*, 1999; Yoles *et al.*, 2001; Yin *et al.*, 2006; Chen *et al.*, 2008; Beck *et al.*, 2010). One

approach to induce systemic inflammation is the administration of a bacterial endotoxin like lipopolysaccharide (LPS). It has been suggested that LPS activates intracellular signaling pathways that elicit a macrophage and microglial response similar to what has been seen after CNS injury (Guth *et al.*, 1994a; Guth *et al.*, 1994b; Montero-Menei *et al.*, 1994; Lazar *et al.*, 1999). LPS has been recently linked to be involved in repair mechanisms following SCI since its application to the cortex of rats with SCI up-regulated the expression of growth-associated genes (Hossain-Ibrahim *et al.*, 2006). Most importantly, Chen and colleagues (2008) found that only the administration of LPS in chronically injured rats could promote sprouting of spared CST fibers. This treatment was ineffective in the chronic stage after SCI, but sprouting of the CST could be reactivated through the administration of LPS (Chen *et al.*, 2008). Since axonal sprouting is a likely mechanism contributing to the effects of forelimb rehabilitative training in rats with SCI (Girgis *et al.*, 2007; Krajacic *et al.*, 2009), we hypothesize that similar to the study by Chen et al., (2008), also the effect of rehabilitative training in the chronic situation can be enhanced by the application of LPS. In the current study we test this hypothesis in rats with cervical spinal cord injury receiving single pellet reaching training.

4.3 Methods

Fifty-one adult female Lewis rats (Charles River, 200-250g) were group-housed and received water *ad libitum*. The study was approved by the local

animal welfare committee, and complies with the guidelines of the Canadian Council for Animal Care.

4.3.1 Experimental design

The present study was carried out in three parts.

I: Training efficacy in rats with chronic SCI

This part of the study was designed to investigate the training efficacy in chronically injured rats and to compare the reaching success to what we reported when rehabilitative training was initiated on day 12 after SCI (Krajacic *et al.*, 2009).

4.3.2 Pre-surgical training

a) Skilled reaching: Prior to SCI rats were familiarized with a single pellet reaching task and their preferred paw was determined during the first training sessions. For training, rats were placed individually into a Plexiglas chamber with a slit in the front wall as reported earlier (Girgis *et al.*, 2007; see figure 4.5A) and motivated to reach through the slit and grasp for banana-flavored sugar pellets (45mg, TestDiet, Richmond, CA) from the shelf. To promote reaching a measured amount of food (10g/day) was presented directly after training/testing sessions allowing a small but constant increase in body weight over time.

A successful reach was counted when a rat was able to retrieve the presented pellet and ate it. If the pellet was knocked off the shelf or if it dropped through the metal grid used as the chamber floor, a failure was scored. The total number of successfully grasped and eaten pellets and the attempts to retrieve the pellets over a 10min period was noted.

b) Horizontal ladder: Rats were placed on one end of a 1m long runway consisting of randomly spaced (3-4cm) 1.5mm thick steel rungs. The distance between the rungs was altered between testing sessions to prevent the rats from learning a movement pattern. Each animal had to cross the ladder 3 times. Ladder crossings were video-recorded and analyzed offline by counting slips and falls of the affected forelimb which were defined as errors.

4.3.3 Spinal cord injury

For this procedure rats were anesthetized (Hypnorm 0.16mg/kg; fentanyl citrate, 120µl per 200g body weight, Janssen Pharmaceutics, Beerse, Belgium and Midazolam 2.5mg/kg; Sabex, Boucherville, QC, Canada) and received a lesion of the dorsolateral quadrant ablating the dorsal and lateral CST as well as the majority of the rubrospinal tract (see figure 4.1B, C). Rats were mounted into a stereotaxic frame (Kopf Instruments, Germany), the surgical site was cleaned with 10% Chlorhexadine Digluconate (Sigma-Aldrich Canada LTD., Oakville, ON, Canada) and the skin above vertebrae C2-C4 was incised. Then the overlying

muscles above spinal C3 were split. A laminectomy and a spinal lesion unilaterally between C3 and C4 ipsilateral to animals preferred paw was performed using a custom-made tapered micro-blade. Muscle layers and skin were sutured. Saline and the analgesic Buprenorphine (0.03mg/kg, Temgesic®, Schering-Plough Ltd., Hertfordshire, UK) were applied to animals over the next two days.

4.3.4 Rehabilitative training

On day 4 post lesion, rats were distributed into training groups and control groups (i.e., untrained groups) so that all experimental groups contained animals with comparable post-lesion deficits (as described in Krajacic *et al.*, 2010).

Eight weeks after SCI, rats of the training group received training for 10min in the skilled reaching task 6 days a week for a total of 6 weeks. For untrained rats the slit in front of the reaching chamber was blocked off in order to prevent them from practicing grasping movements. These untrained animals received a comparable amount of pellets in the reaching chamber to avoid effects of high glucose intake between trained and untrained rats (Plunet *et al.*, 2008).

4.3.5 Final(behavioural) testing

Following 6 weeks of reaching training animals underwent a final testing in reaching and crossing a horizontal ladder (i.e., the untrained task) on 3

consecutive days. Rats were tested randomly so that control groups were tested in the same manner as trained rats. For evaluation, the success rate in reaching was defined by dividing the amount of successfully grasped and eaten pellets by the attempts needed to retrieve the pellets. Errors (slip and falls of the affected forelimb) of horizontal ladder crossings were analyzed offline from video recordings.

4.3.6 Perfusion

Following the final testing, rats were anesthetized with isoflurane (Halocarbon Products Corporation, River Edge, USA), their head was shaved and they were fixed in a stereotaxic frame. The skin was incised and a piece of skull was removed to expose the cortical forelimb area. Then the animals were euthanized by a lethal dose of pentobarbital (Euthanyl, Bimeda-MTC, Animal-Health Inc., Cambridge, ON, CA), followed by a transcardial perfusion with saline containing 0.02g heparine/L. The vascular system was flushed for 10sec, brain tissue of the caudal motor cortex of the forelimb area was harvested and snap frozen in liquid nitrogen. Then the perfusion was continued until the liver cleared. At this point the saline was replaced by a 4% formalin solution with 5% sucrose. Remaining brain and spinal cord tissue was dissected, post fixed overnight in 4% formalin (with 5% sucrose) and transferred into a 30% sucrose solution for 3 days. Cortex samples were transferred from the liquid nitrogen into a -80ºC freezer until lysed for western blots and enzyme-linked immunosorbent assays (ELISA).

Spinal tissues of all rats were cut into a C1 and a C2-C6 section, embedded in Tissue Tek (Sakura Finetek USA, Inc., Torrance, CA, USA) and frozen in 2-methyl-butane at -60ºC. The brain and C1 segments were mounted for cross sections and the C2-C6 sections were mounted for horizontal sections. All tissue was cryosectioned (25µm), mounted onto slides (Fisherbrand, Fisher Scientific, Ottawa, ON, Canada) and stored at -20ºC until a staining procedure was performed.

4.3.7 Phosphorylated PKA levels

In order to determine phorsphorylated PKA levels in chronically injured rats, cortical tissue samples were thawed and homogenized in lysis buffer (20mM Tris-HCl (pH 7.5), 150mM NaCl,1mM EDTA, 1mM EGTA, 1% Triton X-100, 1x Halt™ phosphatase inhibitor cocktail (Thermo Scientific, USA)) and 1 tablet of Roche's complete protease inhibitor cocktail (Roche Diagnostics GmbH, Germany). Then samples were centrifuged (5000g, 10min, 4ºC) and the supernatants were assayed for total protein concentration using a bicinchoninic acid (BCA) protein assay (Thermo Scientific, USA) and an xMark® spectrophotometer (Bio-Rad Laboratories, Inc., USA). A total of 10µg protein was denaturated in NuPAGE® LDS sample buffer with 1x NuPAGE® reducing agent and then loaded on each lane of 4-12 % Bis-Tris Gel (Invitrogen, USA). Proteins were separated and transferred onto nitrocellulose membranes using XCell II™ Blot Module (Invitrogen). Membranes were then blocked in 5ml of

membrane blocking solution (Invitrogen) for 1hr at room temperature (RT) on a shaker, followed by an incubation with Rabbit anti-Phospho-PKA beta (1:2000; S338, Abcam, USA), Rabbit anti-PKAα-cat (1:1000; C-20; Santa Cruz Biotechnology, USA) or mouse monoclonal anti-beta-actin (1:2500; Sigma-Aldrich, USA) at 4ºC over night. The next day, membranes were washed 3x for 5min in Tris-buffered saline containing 0.15% Tween 20 (TBS-T) and then incubated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG, 1:5000; Chemicon International, USA) or horseradish peroxidase-conjugated goat anti-mouse IgG (1:5000; Millipore, USA) for 60min at RT. Membranes were washed 3x for 5min in TBS-T then incubated with Amersham™ ECL™ Western Blotting Detection Reagents (GE Healthcare, UK) for 1min and then exposed to BioMax MR film (Kodak, USA). Membranes were stripped for 10min at RT using Restore™ Western Blot Stripping Buffer (Thermo Scientific) and then washed 5x for 10min in TBS-T between incubation with different primary antibodies.

4.3.8 Cyclic AMP ELISA

Lysis and protein assay of cortical tissue was performed as for western blotting. A total of 20µg protein containing lysate was used to measure cAMP levels using a cAMP ELISA Kit (Cell Biolabs, Inc., USA). Lysates containing protein and cAMP standards were incubated with Peroxidase cAMP tracer in a goat anti-Rabbit IgG coated 96-well plate on a shaker at RT for 2hrs. Each tested well was

washed 5x with 250µl wash buffer and then 100µl of substrate solution were added to each well. The plate was then incubated on a shaker at RT for 20min. Enzyme reaction was stopped by adding 100µl of stop solution into each well, and the plate was read at 450nm on a xMark® spectrophotometer.

4.3.9 Histological analysis

Lesion size assessment

Evaluation of the lesion size was performed on every 4th horizontal section throughout the dorso-ventral extent of spinal cord tissue of the C2-C6 segments. Reconstruction of the lesion extent into a schematic of a cervical transverse section (as shown in figure 4.1C) was made using landmarks (i.e., central canal, grey-white matter interphases). Lesion sizes were analyzed using bright-field or phase contrast microscopy (Leica) and expressed as percentage of the overall transverse section.

4.3.10 Statistical analysis

All measures were quantified with a one-way ANOVA or a one way ANOVA repeated measures (i.e., reaching success) followed by a Tukey *post hoc* test (Prism, V 4.01; Graph Pad Software Inc., La Jolla, CA, USA) in order to determine differences among groups. Significant differences in cortical cAMP and PKA levels were quantified using a Students t-test. Throughout the

manuscript results and figures are presented as means ± standard error of the mean (SEM). Statistical significances are stated when *P*-values ≤ 0.05.

II: Physiological and behavioral responses to different doses of LPS

In the second part of the study we investigated rats' recovery from various LPS concentrations and the effect on microglia/ macrophage recruitment in the spinal cord. LPS is produced in different batches, possibly resulting in different responses of the animals. Thus, we did not use doses described by others (e.g., Chen *et al.*, 2008), but performed this dose finding experiment.

LPS was derived from *Escherichia coli* endotoxin (serotype 055:B5, Sigma-Aldrich Canada, Ltd., Oakville, ON, Canada) and dissolved in sterile saline (pyrogen-free 0.9% saline, Hospira, Montreal, QC, Canada). Rats received a unilateral cervical lesion of the spinal cord and 3 weeks later an intraperitoneal (i.p.) injection of LPS of either 50, 125, 250µg/kg or saline (n=3 per group).

The injection of LPS in rodents also results in an increase in body temperature, therefore we monitored rats' overall behaviour and recovery. Body temperature (rectal measurements) was taken prior and 3 times a day up to 40 hours following LPS administration.

Rats that in this part of the study were not exposed to reaching training and received food and water *ad libitum*. The SCI was conducted as described earlier.

4.3.11 Perfusion

Rats were euthanized 2, 9 or 16 days following injection and cortex samples were extracted for western blotting. Then animals were perfused and spinal cord tissue was used for lesion size analysis and the quantification of microglia/ macrophages at various LPS concentrations (50, 125, 250μg/kg). The perfusion procedure, harvest of cortical tissue, and quantification of the lesion size was performed as described earlier.

4.3.12 Microglia/ Macrophage staining

In order to quantify the LPS induced activation of microglia/ macrophages we performed an immuno-staining of spinal tissue with the Iba-1 antibody (rabbit Anti-Iba-1 ,Wako Chemicals Inc., Richmond, VA, USA; diluted in1:1000 Triton X-100). Thus, frozen spinal tissue was mounted on slides and incubated overnight at 4ºC with the primary antibody. Slides were washed 3x 10min in TBS and incubated for 2hrs at room temperature with a secondary antibody (Donkey-anti-rabbit Alexafluor 488, Jackson Inc., Baltimore, PA, USA; diluted in 1:500 in TBS). Subsequently, the slides were washed (3x10min in TBS), then dehydrated in increasing alcohol concentrations. Finally sections were cleared in xylene and coverslipped with Permount (Fisher Scientific).

Quantification of microglia was performed by counting all stained microglia/macrophages in every 10^{th} saggital section of the spinal cord piece C2-

C6 under a fluorescent microscope (Leica). The western blotting procedure to determine cortical PKA levels, was performed as described earlier for one animal per group/dose.

III: Effect of LPS on training efficacy in a chronic model of SCI

Part three of the study focused on the effect of LPS (250μg/kg) on reaching training efficacy in a chronic model of SCI (for flow of the experiment see Fig. 4.4A). Rats were pre-trained/familiarized with the reaching apparatus, then received a unilateral spinal lesion and were allowed to recover 8 weeks in their home cages. Then, rats were assigned into different groups (Vehicle + Training, LPS + Training, LPS + No Training; n=8, 10 and 9 respectively). Rats in the training groups were trained for 6 days a week for 6 weeks and received an injection of LPS 2 days before the onset of training and another injection 2 weeks following the first (see figure 4.4A). Animals' body temperature was monitored prior and 7, 12, 20 and 30 hrs following LPS administration. Also, rats were weighed every second day throughout the experiment. Following 6 weeks of rehabilitative training all animals underwent a final testing procedure, tracing of the forelimb motor cortex and were euthanized 2 weeks after tracing.

Pre-surgical training, lesion to the spinal cord, rehabilitative training and behavioural testing were performed as in part one of the study. In addition, we evaluated the rats' grasping ability from the video recordings during the final testing period employing the following scoring system: The score '0' was given if

the animal was unable to reach through the slit of the reaching chamber. A score of '1'was given when the rat was able to reach through the slit with its affected limb but was unable to grasp a pellet. '2' was assigned if animals were able to reach through the slit but licked the pellet from the shelf. When animals were shoveling instead of grasping the presented pellets, a '3' was given. A score of '4' was given for rats which were able to grasp the pellets but dropped them in the chamber and '5' was assigned when rats showed normal grasping movements and were able to retrieve and eat pellets.

Furthermore, at the end of the training period rats were filmed exploring the walls of a Plexiglas cylinder. This test was used in order to evaluate the spontaneous usage of either paw. All wall touches of both front paws were counted during 10 rearing movements. The sum of wall touches per injured paw is expressed as percentage of the total amount of touches with both paws. Non-lesioned rats have a symmetry ratio of ~50% per paw (Schallert *et al.*, 2000).

4.3.13 Tracing

To evaluate the amount of collateral sprouts of the CST into the grey matter and sprouting of CST fibers within the grey matter rostral to the spinal lesion, anterograde tracing with biotinylated dextran amine (BDA, 10%, Microprobes, Eugene, OR, USA) was performed after the final testing period. At 3 different sites within the forelimb motor cortex contralateral to the preferred/affected paw,

1µl BDA was injected with a Hamilton syringe (Reno, NV, USA) at a depth of 1.5mm.

4.3.14 Perfusion

Rats were euthanized with pentobarbital and a transcardial perfusion was conducted as described above without collecting cortical tissue.

4.3.15 Immunohistochemistry

In order to visualize BDA-labeled axons, frozen sections of spinal cord tissue were thawed for 30min at 37ºC rehydrated by bathing in 2x10min TBS, followed by 2 washes in TBS with 0.5% Triton X-100 for 45min. Afterwards the sections were incubated overnight at 4ºC in an avidin-biotin complex solution (ABC, Elite, Vector Laboratories, Burlingame, CA, USA). Then slides were washed in TBS (2x 10min) and a diaminobenzidine (DAB, Vector kit, SK4100) reaction was performed. The reaction was halted after 5min by washing slides in distilled water. Subsequently, slides were washed (2x 10min) in TBS, dehydrated in increasing alcohol concentrations, cleared with xylene and coverslipped with Permount.

Lesion size analysis was performed as described before.

Collateral sprouting of the injured CST

Evaluation of the total number of traced CST fibers consisted of counting all BDA labeled CST axons in one C1 cross section. For quantification of collateral sprouts of traced CST fibers rostral to the spinal lesion, fibers emanating from the injured CST and projecting into the grey matter (for more than 30µm) were counted on every 4th horizontal section. This number was then normalized to the length (mm) of the tissue analyzed and the number of traced axons counted on C1 cross sections.

Densitometry of traced CST fibers in the grey matter

The density of traced CST fibers/ arborisations in the grey matter was assessed using ImageJ (National Institutes of Health, USA). Photomicrographs of the grey matter rostral to the spinal lesion on horizontal sections were taken with a Leica microscope and processed in ImageJ. For each picture the contrast and threshold were set at the same level. In laminae V-VI, a 1x1" square was placed over an area with the highest amount of fibers/ arborisations and no obvious artifacts. The analysis was performed in a double blinded manner and the results were normalized to the number of traced axons counted at the level of C1.

4.3.16 Statistical analysis

All results of this part of the study were quantified by using a one-way ANOVA or a one way ANOVA repeated measures (i.e., reaching success) followed by a Turkey *post hoc* test. A Mann Whitney-test was applied for the reaching score and the horizontal ladder. Statistical significances (*) are stated when *P*-values ≤ 0.05 and *P*-values ≤ 0.01 (**).

4.4 Results

Part I

4.4.1 Reaching training efficacy declines over time

The first part of the present study was performed to investigate the efficacy of reaching training in a chronic model (8 weeks post lesion) of SCI. We compared the training efficacy following chronic SCI (present study) with that of rats that received rehabilitative reaching training started on day12 after injury (published data from Krajacic *et al.*, 2009). A condition to allow such a comparison between studies is matching lesion sizes. The comparison of lesion sizes between rats that received reaching training 12 days after SCI in an earlier study (trained 30.42% ± 3.65; untrained 31.10% ± 3.10) showed no statistical difference to rats in the current study that were trained starting 8 weeks post SCI (trained 28.07% ± 3.17; untrained 30.19% ± 2.75; figure 4.1A).

Confirming the matching lesion sizes, we found that by the end of the experiment, the reaching success of untrained animals with chronic SCI (5.71% ± 2.01) was comparable to that of untrained rats from the earlier study (Krajacic *et al.*, 2009; training initiated on day 12 post SCI; 5.00% ± 5.00; figure 4.1D). However, when we compared the reaching success of trained rats we found that although the training procedure was similar in both studies, animals with a chronic spinal lesion were significantly less successful in reaching for pellets than rats that received reaching training starting on day 12 after SCI (Krajacic *et al.*, 2009; 32.86 % ± 3.43 and 56.07% ± 1.33 respectively). Nevertheless, under both conditions (i.e., training initiated on day 12 or after 8 weeks) the rehabilitative training significantly ($P \leq 0.01$) increased the reaching success. In summary, although training following chronic SCI significantly improves recovery in the trained task, it is less beneficial to training with an earlier onset.

4.4.2 Reaching training in chronic injured rats increases cAMP levels and PKA activity

Previously we found that reaching training initiated on day 12 after SCI can up-regulate cAMP dependent phosphorylated PKA levels in the cortex contralateral to the spinal lesion (Krajacic *et al.*, 2009). The analysis of the cortical cAMP levels of chronic injured rats in the present study also resulted in a significant ($P \leq 0.05$) increase of cortical cAMP levels in trained rats (632.6 %± 258.1) versus untrained rats (100% ± 92.87; figure 4.2A). Consequently, quantification of phosphorylated PKA in rats with chronic SCI shows that animals

that received reaching training (142.9% ± 4.88) have a significant increase ($P \leq 0.05$) in cortical phosphorylated PKA when compared to their untrained counterparts (100.3% ± 13.69; Fig. 4.2B). Please note that values of phosphorylated PKA were not normalized to the ipsilateral cortical hemisphere because it has been reported that the uninjured site is also affected by the training (Girgis *et al.*, 2007).

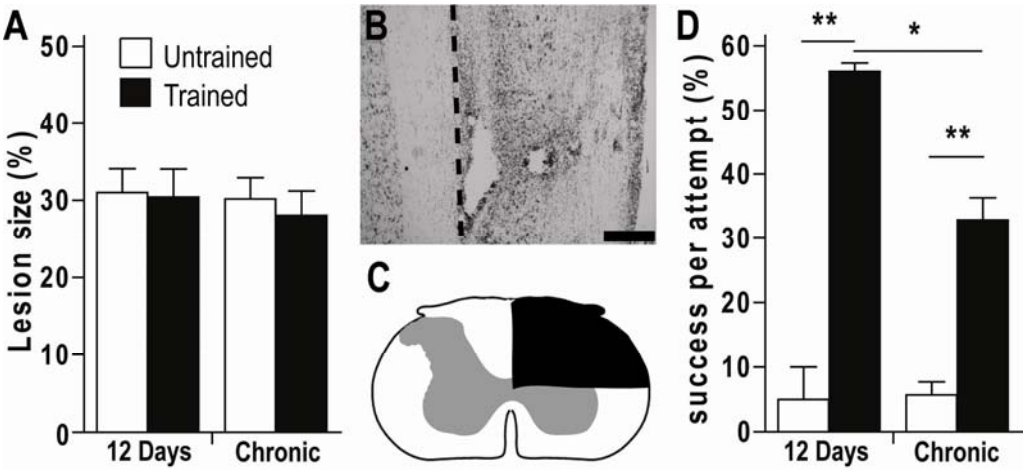


Figure 4.1. Chronically injured rats have a decreased reaching success than rats that received reaching training on day 12 after SCI. In **A** we show that the lesion size is not significantly different between rats that received reaching training on day 12 after SCI and rats that were started to be trained 8 weeks after SCI. **B**: A horizontal section of the spinal cord of a rat with a spinal lesion. The dashed line indicates the midline of the cord. The schematic in **C** shows a cross section of a spinal cord at the level of C3 with a reconstructed lesion (black area). **D**: Performance of rats where reaching training was initiated on from day 12 after SCI (data from Krajacic et al. 2009) and rats with a chronic lesion (training initiated 8 weeks post SCI) have a significantly higher success rate than their untrained counterparts. When the reaching success of rats that received training on day 12 was compared to the reaching success of rats with a chronic lesion, we found that trained rats with a chronic lesion were significantly worse at reaching. Interestingly, untrained rats of both groups (white bars) were not different in the reaching success. *indicates $P \leq 0.05$ and ** $P \leq 0.01$, error bars show standard error of the mean and scale bar is 20μm.

In summary, these results show that rehabilitative reaching training at a chronic stage (8 weeks) after SCI up-regulates cortical cAMP levels and its downstream factor PKA.

Part II & III

4.4.3 LPS increases recruitment of Microglia/ Macrophages in the spinal cord

This part of the study was designed to investigate the most suitable dose that triggers microglia/ macrophage recruitment in the spinal cord, but also allows for a fast recovery from the LPS injection. This is important so that animals would be able to be trained immediately following injection (part three of the study). Please note that only one rat per dosage and time point was used. We found that the administration of a concentration of 50µg/kg of LPS does not result in a higher number of microglia/ macrophages than the administration of saline (figure 4.3A-C). Further, the higher the concentration of LPS the more microglia/ macrophages were counted over a time course of 16 days (125µg/kg and 250µg/kg, figure 4.3C). Administration of 250µg/kg LPS 3 weeks following cervical SCI results in an increase (figure 4.3B, C) in the number of microglia in the spinal cord when compared to animals that received saline injections (figure 4.3A). Temperature measurements show for concentrations of 125µg/kg and 250µg/kg an increase in the animals body temperature within 12hrs (figure 4.3D). Body temperatures

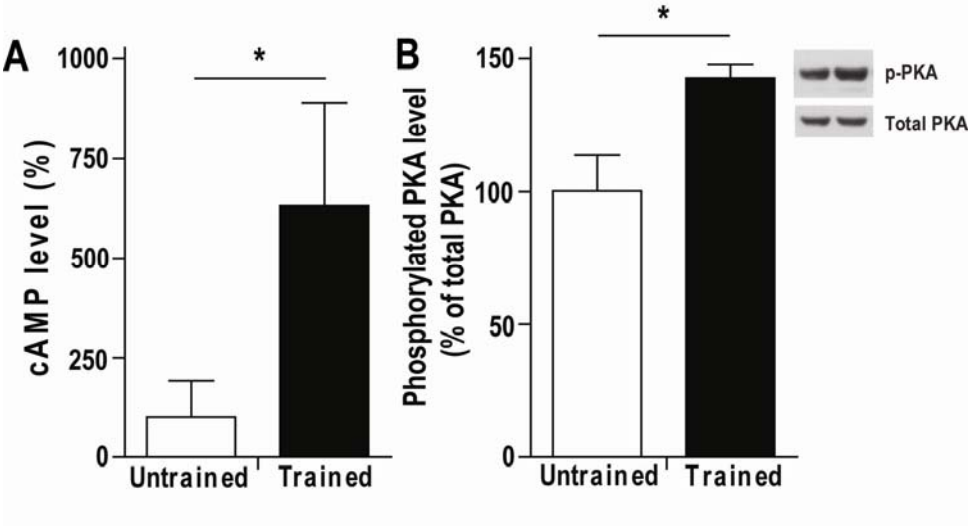


Figure 4.2. Cortical cyclic AMP and PKA-activity in chronically injured rats is increased in the trained group. Results from the ELISA procedure show that trained rats with chronic SCI have significantly increased cortical cAMP levels when compared to their untrained counterparts (**A**). **B** illustrates Western blots of cortical tissue of trained and untrained rats. A significant increase in cortical phosphorylated PKA levels was found in trained rats when compared to untrained rats. *indicates $P \leq 0.05$ and error bars show standard error of the mean.

peaked at 20hrs (38.2 ºC ± 0.22 for rats receiving 250µg/kg) after LPS administration and declined to 37.5 ºC (± 0.09, at 40hrs post injection for rats receiving 250µg/kg). Also, the results from western blots that were performed on cortical tissue harvested 16 days following LPS-injection indicate that the administration of LPS resulted in an increase of phosphorylated PKA levels in rats receiving the highest LPS dose (250µg/kg) when compared to animals receiving saline injections (figure 4.3E). Thus, a dosage of 250µg/kg LPS was chosen for subsequent experiments (part three).

In the third part of the study we investigated the interaction between LPS injection and training efficacy in rats with chronic SCI.

4.4.4 LPS induces a short term fever in chronically injured rats

First we examined the physical recovery of chronically injured rats following LPS injections. Eight weeks following SCI rats received injections of LPS and were observed for flu-like symptoms (e.g., fever). After the first LPS injection there was a rise in rats` core body temperature. The body temperature increased from 37.3ºC (± 0.09) up to 38.0 ºC (± 0.07; LPS + Training group) and stayed elevated until the last measurement (30 hours after injection, 37.5ºC ± 0.08; figure 4.4B). Surprisingly, rats that were treated with the vehicle solution also had increased temperatures for a short time period (38.2ºC ± 0.18; measured at 7hrs after first injection). Following the second LPS injection only rats receiving LPS

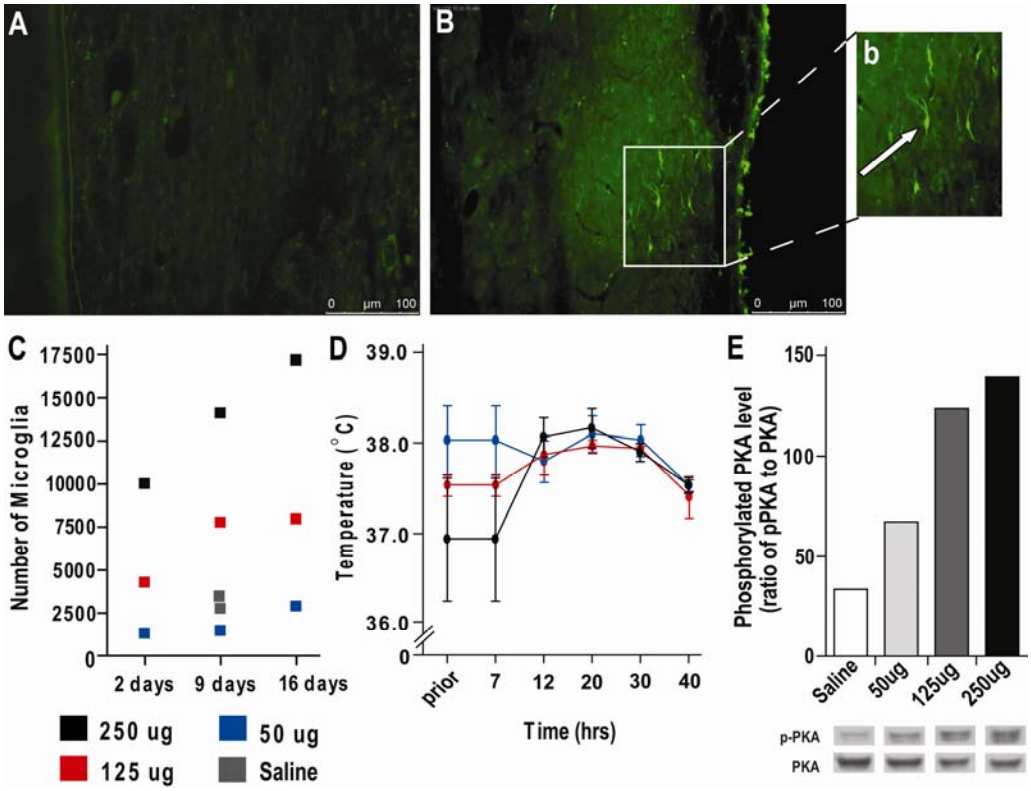


Figure 4.3. Administration of LPS triggers microglia recruitment and increases cortical PKA-activity. Photomicrographs of Iba-1 stained spinal cords from rats receiving LPS (250µg/kg; **B**) or saline (control vehicle; **A**) show that rats that received LPS had more Microglia (arrow, magnification in **b**). Evaluation of the number of Microglia in rats receiving different concentrations of LPS demonstrates that more Microglia were present in the highest LPS concentration (**C**). The amount of Microglia increases over a time course of 16 days. **D**: Body temperature of rats receiving 250µg/kg LPS increases within 12 hrs after LPS injection, peaks at 20 hrs and returns to normal levels at 40 hrs following LPS administration. **E**: Western blots of the PKA-activity in cortical tissue at 16 days following LPS injection from rats receiving saline or 50, 125 and 250µg/kg LPS show higher cortical pPKA activity in LPS treated (250µg/kg, right lanes) animals than rats that received saline injections (left lanes). Each dot/bar demonstrates a single animal. No statistical analysis was performed. Scale bar is 100µm.

had increased temperatures (38.3 ºC ± 0.2 and 38.3ºC ± 0.2; for rats in the LPS + Training and LPS + No Training group respectively) which, after 7hrs declined to values similar to those measured prior to LPS injection. To detect potential differences in weight due to the application of LPS, animals were weighed every second day. The averages of the animal weights were not different among groups over the course of the experiment (figure 4.4C).

4.4.5 Combination of LPS and reaching training improves reaching ability

In order to investigate the effects of LPS on training efficacy in a chronic model of SCI, rats were tested following 6 weeks of reaching training (figure 4.5A). A highly significant difference ($P \leq 0.01$, figure 4.5B) could be found in the reaching success rate between the LPS + Training group (9.15% ± 0.46) and Vehicle + Training group (3.04 %± 0.66). Also, the reaching success of rats in the LPS + Training group was also significantly increased ($P \leq 0.01$) when compared to the LPS + No Training group (2.63% ± 0.10). Also, when rats' grasping ability was scored (0-5, as described above), the LPS + Training group excelled (figure 4.5C). A significant difference ($P \leq 0.05$) could be found between rats in the LPS + Training group (2.40 ± 0.48) and rats in the LPS + No Training group (1.0 ± 0.52). Rats that received the vehicle solution and reaching training (1.38 ± 0.50) were not significantly different from the other groups.

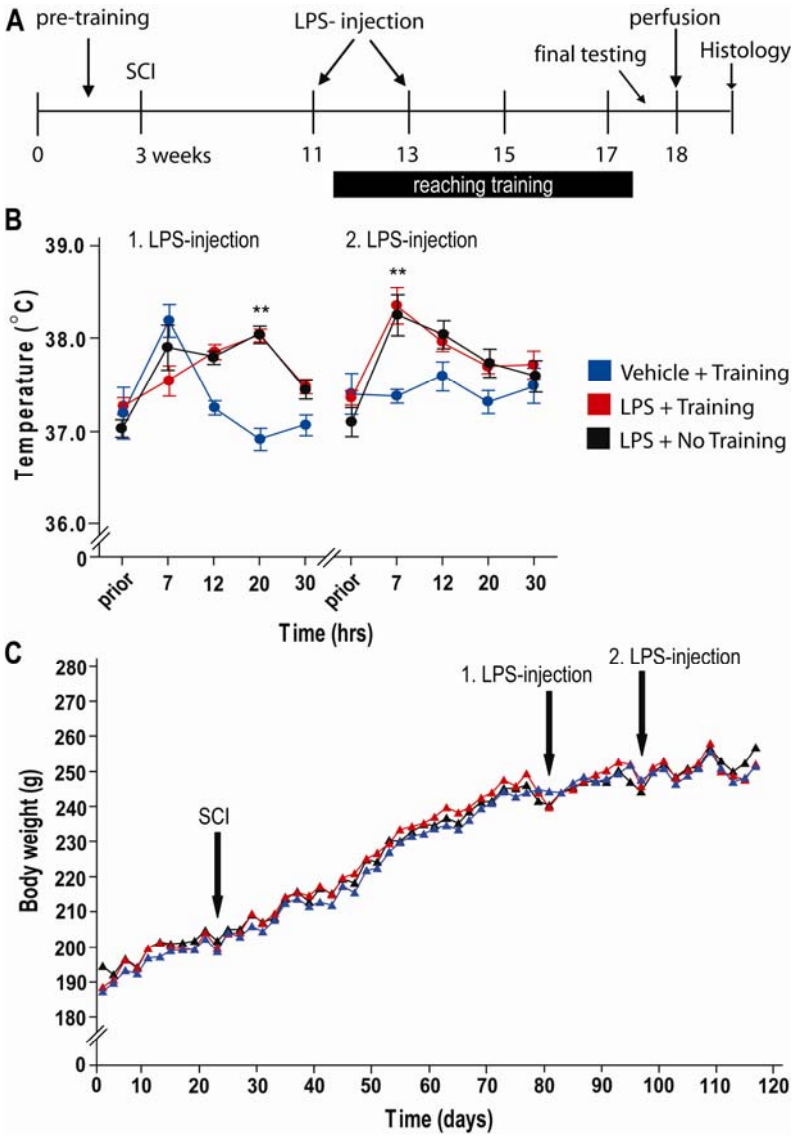


Figure 4.4. Experimental outline and physiological measurements following LPS administration in chronic injured rats. An Illustration of the flow of the experiment is shown in **A**. The temperature assessment prior and after LPS injection show that rats that were treated with LPS had a transient increase in their body temperature (**B).** The graph in **C** demonstrates that body weight measurements of rats in various groups were increasing over the time of the experiment and did not differ from between animals. **indicates $P \leq 0.01$ compared to Vehicle + Training group and error bars show standard error of the mean.

In summary, the administration of LPS allowed for a higher rehabilitative training efficacy in rats with chronic SCI.

4.4.6 Untrained tasks

Assessment of the horizontal ladder task revealed that rats that were not assigned to rehabilitative reaching training made significantly ($P \leq 0.05$) more mistakes (2.53 ± 0.41) when crossing the horizontal ladder than rats receiving reaching training in combination with LPS (1.51 ± 0.25; figure 4.5D). Animals in the Vehicle + Training group (1.92 ± 0.44) performed comparable to rats in the LPS + No Training or LPS + Training group.

Quantification of the paw usage in a cylinder (figure 4.5E) did not result in significant differences among groups. The mean percentage of wall touches with the injured paw was around 30% in each group (Vehicle + Training 30.29% ± 3.08; LPS + Training 30.72% ± 5.87; LPS + No Training 33.16 ± 4.10), indicating that rats were predominantly using their uninjured paw (figure 4.5F).

4.4.7 Lesion size is not different among groups

Evaluation of the lesion size (expressed as percentage of lesioned tissue in a transverse section of the cervical spinal cord, as illustrated in figure 4.6A) showed no significant difference among groups (Vehicle + Training 41.52% ± 3.11; LPS + Training 35.72% ± 1.88; LPS + No Training 37.92 ± 3.28; figure 4.6B). This

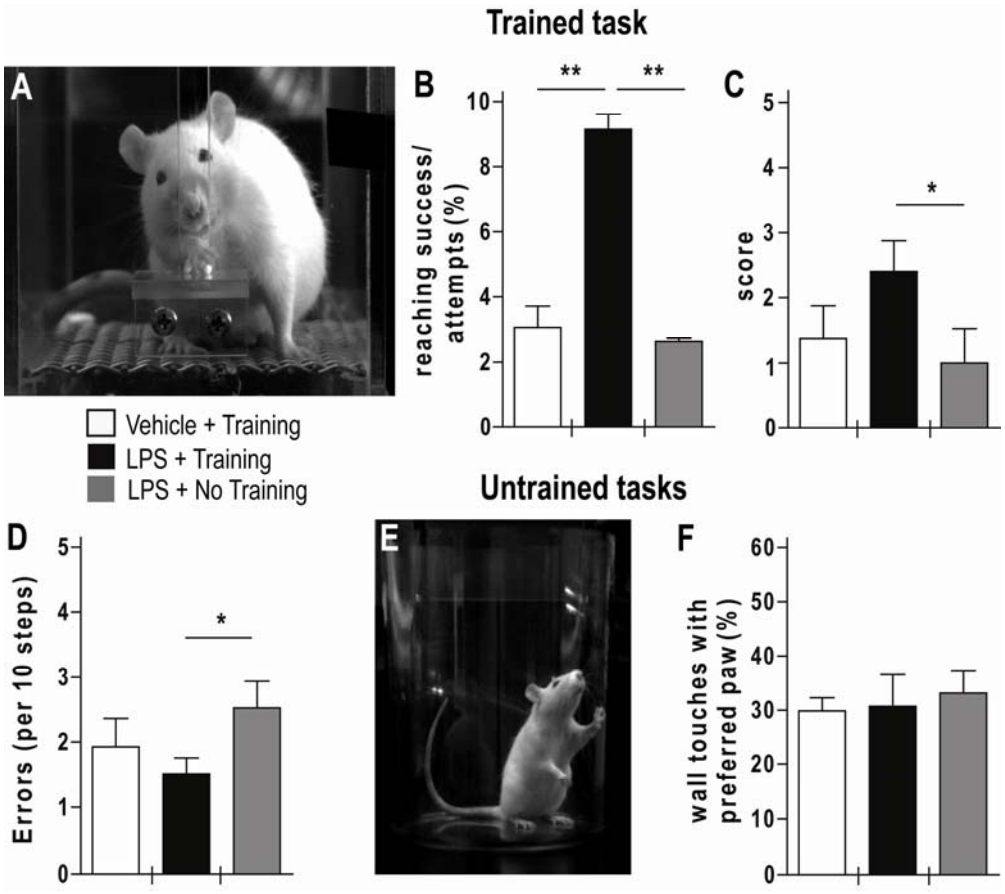


Figure 4.5. Rats that received a combination of reaching training and LPS administration show an increase in reaching success and make fewer mistakes while crossing a horizontal ladder. Rats receiving LPS and reaching training are significantly more successful in reaching for pellets (**A**) than animals receiving reaching training and saline or rats receiving LPS injections only (**B**). When the reaching was scored from 1-5, animals receiving LPS and reaching training were significantly better than rats that were treated with LPS alone (**C**). When crossing a horizontal ladder, rats receiving LPS alone made significantly more errors while crossing than rats receiving LPS in combination with reaching training (**D**). Evaluation of the cylinder task (**E)** demonstrates no difference in the amount of wall touches with the injured paw among experimental groups (**F**). *indicates $P \leq 0.05$ and ** $P \leq 0.01$, error bars show standard error of the mean.

shows that it was not a difference in lesion size between the groups, but the administration of LPS in combination with training that resulted in improved recovery. Although there is no direct comparison between experiments in Part I and III, it is noteworthy that the average lesion sizes of groups in Part III are significantly larger than those in Part I, explaining the difference in reaching success between the experiments.

4.4.8 LPS in combination with training increases CST fiber/ arborisation density

Quantification of collateral sprouts emanating from the traced CST and projecting into the grey matter rostral to the spinal lesion (photomicrograph in Fig. 4.6C) revealed no significant differences among groups (Training + Vehicle 0.008 ± 0.001; LPS + Training 0.009 ± 0.001; LPS + No Training 0.009 ± 0.001; figure 4.6D). However, when analyzing the density of BDA labeled CST fibers/ arborisations in the grey matter (laminae V-VI; see figure 4.7A-E) rostral to the spinal lesion, we found the highest density of BDA labeled CST fibers/ arborisations within the grey matter in the LPS + Training group (0.025 ± 0.005), which was significantly higher ($P \leq 0.05$) than rats receiving LPS alone (0.010 ±0.003; figure 4.7F).

In summary, only the combination of training and LPS resulted in a significant increase in sprouting within the grey matter.

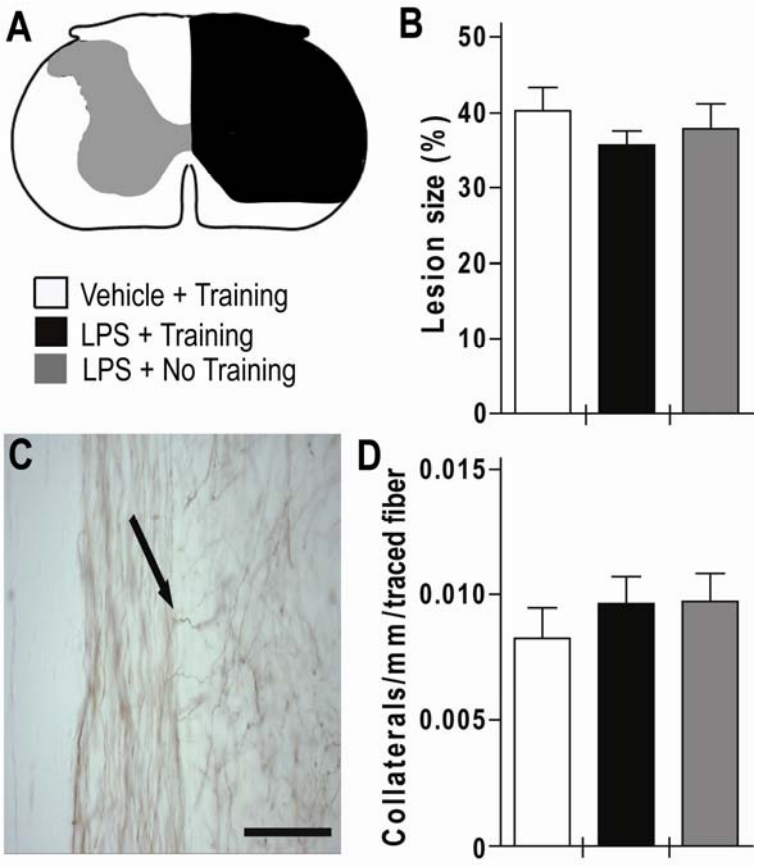


Figure 4.6. Administration of LPS did not alter lesion extent and collateral sprouting of the lesioned CST in rats with chronic SCI. A schematic illustration of the average lesion extent in a cervical cross section in chronic injured rats is shown in **A**. No difference in the lesion size was found among groups (**B**). The microscopic photograph of a rat spinal cord section illustrating the labeled injured CST sending out collaterals into the grey matter is shown in **C**. The arrow shows a collateral leaving the CST and projecting into the grey matter. Evaluation of the number of collateral sprouts projecting into the grey matter did not result in statistical differences among experimental groups (**D**). Scale bar is 200μm.

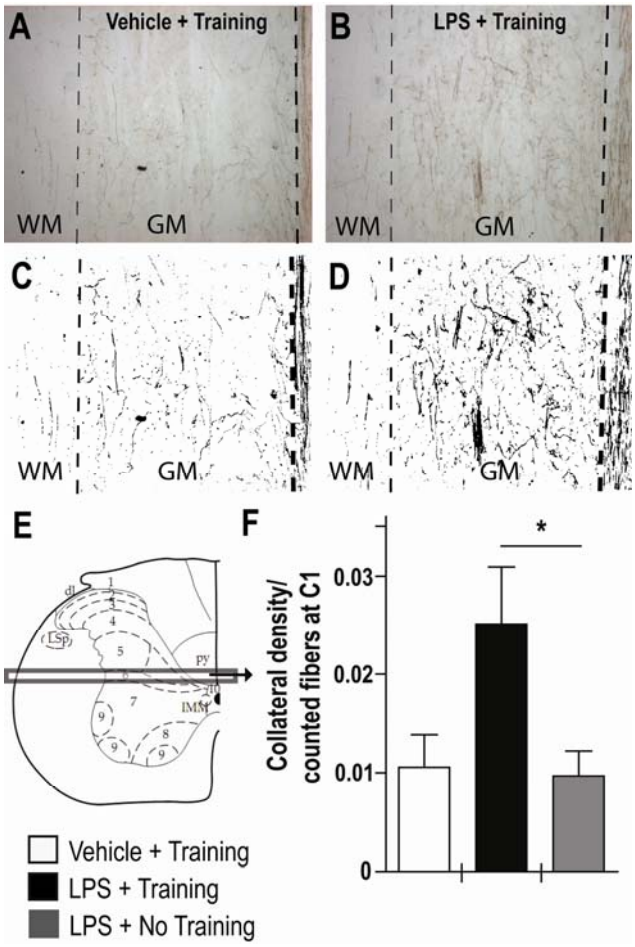


Figure 4.7. Density of arborisations of the labeled CST is increased in rats receiving combination of training and LPS. Microscopic photographs of a horizontal section of a rat spinal cord show labeled CST collaterals in the grey matter of rats receiving saline and reaching training (**A**), and rats receiving LPS and reaching training (**B**). Dashed lines indicate grey (GM)/ white matter (WM) interphase. For density evaluation images were converted into a black and white picture (**C, D**). Schematic illustration of a cross section of the cervical spinal cord shows where measurements for collateral density within the grey matter were made (**E**). Quantification of aborisation density in the grey matter in laminae V-VI shows that rats receiving LPS in combination with reaching training have a significantly higher density of arborisations in the grey matter than rats receiving LPS alone (**F**). *indicates $P \leq 0.05$ and error bars show standard error of the mean.

4.5 Discussion

In the present study we found that reaching training at a chronic stage of SCI is still efficacious in promoting recovery, however the training is less efficient compared to training that is initiated on day 12 following SCI (Krajacic *et al.*, 2009). This confirms what has been suggested following SCI (Norrie *et al.*, 2005; Winchester *et al.*, 2005; 2009) and is comparable to findings reported at a chronic stage following stroke (Salter *et al.*, 2006). Importantly, we show that the decline of training efficacy could be counteracted by the administration of LPS during the training phase.

There are various theories that could explain the nature of the decline in training efficacy following SCI. One of them involves the fact that this decline is paralleled by a post injury decline/ change in the inflammatory response (Alexander and Popovich, 2009). It is possible that this decline/ change plays a prominent role in the decline of susceptibility of the CNS to training and plasticity (i.e., in closing the window of opportunity). A possible mechanism could involve changes in microglia/ macrophages number or type (Gensel *et al.*, 2009). Microglia/ macrophages remain for a long time in the damaged spinal cord and their role in chronic SCI is still debated (as reviewed in Crutcher *et al.*, 2006). It has been shown that macrophages can promote regeneration of CNS axons (Zeev-Brann *et al.*, 1998; Gensel *et al.*, 2009), and studies from the Benowitz's laboratory report that one potential mechanism for axonal growth following CNS injury is the expression of the macrophage-derived growth factor Oncomodulin

(Yin *et al.*, 2006; Kurimoto *et al.*, 2010). Following optic nerve injury it was reported that cAMP enables Oncomodulin to bind to retinal ganglion cells (Yin *et al.*, 2006). Thus, it could be speculated that a training induced elevation of cortical cAMP levels in rats with chronic SCI (as shown in figure 4.2) may enable macrophage mediated axonal regeneration/ sprouting within the grey matter. This potential mechanism could be enhanced by increasing the number, phenotype or reactivity of microglia/ macrophages in the spinal cord, for example with the administration of LPS (figure 4.3).

Alternatively, the post injury decline in cAMP (Pearse *et al.*, 2004; Krajacic *et al.*, 2009) levels in the chronically injured animals might not be sufficiently counteracted by training alone, only with the addition of LPS (which increase cAMP dependent PKA levels, figure 4.3) the activity in cAMP dependent pathways might be increased to a functionally relevant levels.

It seems paradoxical that LPS, which is known mostly for its detrimental effects (e.g., induction of pathological alterations) after injury to the CNS (Semmler *et al.*, 2005; Vallieres *et al.*, 2006; Semmler *et al.*, 2007), can also trigger beneficial effects such as axonal sprouting. This effect was possibly triggered through the up-regulation of growth associated factors (Hossain-Ibrahim *et al.*, 2006) . Here we show that the combination of LPS and reaching training in a chronic model of SCI promotes spinal neuroplasticity that is associated with increased axonal sprouting within the grey matter, especially in laminae V-VI. These results match findings from the Shine laboratory, where following SCI

sprouting of spared CST fibers was induced by NT-3 expression, which only occurred in the acute scenario (Chen *et al.*, 2006), but not in chronically lesioned rats (Chen *et al.*, 2008). This plasticity could be reestablished by the application of LPS (Chen *et al.*, 2008).

Another interesting finding is that the combination of LPS and reaching training leads to an improved performance in the trained and in one of the untrained tasks (i.e., crossing a horizontal ladder) when compared to rats receiving LPS alone or rehabilitative training in combination with a vehicle solution. Conversely, we reported earlier that reaching training can result in impairments in other, untrained tasks (Girgis *et al.*, 2007). This however was only the case when training was initiated within 4 days after SCI (Girgis *et al.*, 2007) but not when training was initiated on day 12 post SCI (Krajacic *et al.*, 2009). Therefore, it is important that future studies address whether initiating training early after SCI might have detrimental effects that had not been detected in our earlier study (Girgis *et al.*, 2007).

Our results show that the administration of LPS leads to transient sickness behaviour, for instance fever, as it has been reported earlier (Dantzer, 2004; Rowsey *et al.*, 2006). We measured increased body temperature that returned to normal values within 30 hours following injection. Surprisingly, we observed that following the first injection rats in the Vehicle + Training group had a similar increase in temperature as LPS treated rats. This effect can possibly be explained by the fact that rats assigned to different groups were housed together. After the

first injection animals showed thermo-regulating behaviour which is generally observed in hibernating rodents but also in rat and hamster pups (Sokoloff and Blumberg, 2002). By huddling together, temperatures in a population lead to equalization of the body temperature. Following the second injection animals that received LPS recovered faster and the groups showed less thermoregulatory behaviour, likely explaining the lack of increase in body temperature in animals treated with saline.

In conclusion, the present study shows that the training efficacy after SCI is reduced in rats receiving rehabilitative reaching training at a chronic stage. Strikingly, we observed that the administration of LPS in a chronic model of SCI in combination with training resulted in significant behavioural improvements, which were likely due to the altered microglia/ macrophage response caused by the application of LPS. Nevertheless, as Gensel et al. (2009) suggested, additional work is necessary to investigate the significance of microglia/ macrophage response in order to separate the beneficial from detrimental aspect of inflammation.

4.6 References

Alexander J. K. and Popovich P. G. (2009). "Neuroinflammation in spinal cord injury: therapeutic targets for neuroprotection and regeneration." Prog Brain Res 175: 125-37.

Beck K. D., Nguyen H. X., Galvan M. D., Salazar D. L., Woodruff T. M. and Anderson A. J. (2010). "Quantitative analysis of cellular inflammation after traumatic spinal cord injury: evidence for a multiphasic inflammatory response in the acute to chronic environment." Brain 133(Pt 2): 433-47.

Chen Q., Smith G. M. and Shine H. D. (2008). "Immune activation is required for NT-3-induced axonal plasticity in chronic spinal cord injury." Exp Neurol 209(2): 497-509.

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

Crutcher K. A., Gendelman H. E., Kipnis J., Perez-Polo J. R., Perry V. H., Popovich P. G. and Weaver L. C. (2006). "Debate: "is increasing neuroinflammation beneficial for neural repair?"" J Neuroimmune Pharmacol 1(3): 195-211.

Dantzer R. (2004). "Cytokine-induced sickness behaviour: a neuroimmune response to activation of innate immunity." Eur J Pharmacol 500(1-3): 399-411.

Di Giovanni S., Faden A. I., Yakovlev A., Duke-Cohan J. S., Finn T., Thouin M., Knoblach S., De Biase A., Bregman B. S. and Hoffman E. P. (2005). "Neuronal plasticity after spinal cord injury: identification of a gene cluster driving neurite outgrowth." FASEB J 19(1): 153-4.

Dietz V., Colombo G., Jensen L. and Baumgartner L. (1995). "Locomotor capacity of spinal cord in paraplegic patients." Ann Neurol 37(5): 574-82.

Donnelly D. J. and Popovich P. G. (2008). "Inflammation and its role in neuroprotection, axonal regeneration and functional recovery after spinal cord injury." Exp Neurol 209(2): 378-88.

Gensel J. C., Nakamura S., Guan Z., van Rooijen N., Ankeny D. P. and Popovich P. G. (2009). "Macrophages promote axon regeneration with concurrent neurotoxicity." J Neurosci 29(12): 3956-68.

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

Guth L., Zhang Z., DiProspero N. A., Joubin K. and Fitch M. T. (1994a). "Spinal cord injury in the rat: treatment with bacterial lipopolysaccharide and indomethacin enhances cellular repair and locomotor function." Exp Neurol 126(1): 76-87.

Guth L., Zhang Z. and Roberts E. (1994b). "Key role for pregnenolone in combination therapy that promotes recovery after spinal cord injury." Proc Natl Acad Sci U S A 91(25): 12308-12.

Hossain-Ibrahim M. K., Rezajooi K., MacNally J. K., Mason M. R., Lieberman A. R. and Anderson P. N. (2006). "Effects of lipopolysaccharide-induced inflammation on expression of growth-associated genes by corticospinal neurons." BMC Neurosci 7: 8.

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

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

Kurimoto T., Yin Y., Omura K., Gilbert H. Y., Kim D., Cen L. P., Moko L., Kugler S. and Benowitz L. I. (2010). "Long-Distance Axon Regeneration in the Mature Optic Nerve: Contributions of Oncomodulin, cAMP, and pten Gene Deletion." J Neurosci 30(46): 15654-63.

Lazar D. A., Ellegala D. B., Avellino A. M., Dailey A. T., Andrus K. and Kliot M. (1999). "Modulation of macrophage and microglial responses to axonal injury in the peripheral and central nervous systems." Neurosurgery 45(3): 593-600.

Montero-Menei C. N., Sindji L., Pouplard-Barthelaix A., Jehan F., Denechaud L. and Darcy F. (1994). "Lipopolysaccharide intracerebral administration induces minimal inflammatory reaction in rat brain." Brain Res 653(1-2): 101-11.

Norrie B. A., Nevett-Duchcherer J. M. and Gorassini M. A. (2005). "Reduced functional recovery by delaying motor training after spinal cord injury." J Neurophysiol 94(1): 255-64.

Pearse D. D., Pereira F. C., Marcillo A. E., Bates M. L., Berrocal Y. A., Filbin M. T. and Bunge M. B. (2004). "cAMP and Schwann cells promote axonal growth and functional recovery after spinal cord injury." Nat Med 10(6): 610-6.

Plunet W. T., Streijger F., Lam C. K., Lee J. H., Liu J. and Tetzlaff W. (2008). "Dietary restriction started after spinal cord injury improves functional recovery." Exp Neurol 213(1): 28-35.

Rowsey P. J., Metzger B. L., Carlson J. and Gordon C. J. (2006). "Effects of chronic exercise conditioning on thermal responses to lipopolysaccharide and turpentine abscess in female rats." Arch Toxicol 80(2): 81-7.

Salter K., Jutai J., Hartley M., Foley N., Bhogal S., Bayona N. and Teasell R. (2006). "Impact of early vs delayed admission to rehabilitation on functional outcomes in persons with stroke." J Rehabil Med 38(2): 113-7.

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

Schwartz M., Lazarov-Spiegler O., Rapalino O., Agranov I., Velan G. and Hadani M. (1999). "Potential repair of rat spinal cord injuries using stimulated homologous macrophages." Neurosurgery 44(5): 1041-5; discussion 1045-6.

Semmler A., Frisch C., Debeir T., Ramanathan M., Okulla T., Klockgether T. and Heneka M. T. (2007). "Long-term cognitive impairment, neuronal loss and reduced cortical cholinergic innervation after recovery from sepsis in a rodent model." Exp Neurol 204(2): 733-40.

Semmler A., Okulla T., Sastre M., Dumitrescu-Ozimek L. and Heneka M. T. (2005). "Systemic inflammation induces apoptosis with variable vulnerability of different brain regions." J Chem Neuroanat 30(2-3): 144-57.

Sokoloff G. and Blumberg M. S. (2002). "Contributions of endothermy to huddling behavior in infant Norway rats (Rattus norvegicus) and Syrian golden hamsters (Mesocricetus auratus)." J Comp Psychol 116(3): 240-6.

Song G., Cechvala C., Resnick D. K., Dempsey R. J. and Rao V. L. (2001). "GeneChip analysis after acute spinal cord injury in rat." J Neurochem 79(4): 804-15.

Vallieres N., Berard J. L., David S. and Lacroix S. (2006). "Systemic injections of lipopolysaccharide accelerates myelin phagocytosis during Wallerian degeneration in the injured mouse spinal cord." Glia 53(1): 103-13.

Winchester P., McColl R., Querry R., Foreman N., Mosby J., Tansey K. and Williamson J. (2005). "Changes in supraspinal activation patterns following robotic locomotor therapy in motor-incomplete spinal cord injury." Neurorehabil Neural Repair 19(4): 313-24.

Winchester P., Smith P., Foreman N., Mosby J. M., Pacheco F., Querry R. and Tansey K. (2009). "A prediction model for determining over ground walking speed after locomotor training in persons with motor incomplete spinal cord injury." J Spinal Cord Med 32(1): 63-71.

Yin Y., Henzl M. T., Lorber B., Nakazawa T., Thomas T. T., Jiang F., Langer R. and Benowitz L. I. (2006). "Oncomodulin is a macrophage-derived signal for axon regeneration in retinal ganglion cells." Nat Neurosci 9(6): 843-52.

Yoles E., Hauben E., Palgi O., Agranov E., Gothilf A., Cohen A., Kuchroo V., Cohen I. R., Weiner H. and Schwartz M. (2001). "Protective autoimmunity is a physiological response to CNS trauma." J Neurosci 21(11): 3740-8.

Zeev-Brann A. B., Lazarov-Spiegler O., Brenner T. and Schwartz M. (1998). "Differential effects of central and peripheral nerves on macrophages and microglia." Glia 23(3): 181-90.

CHAPTER 5

INTERPRETATION OF RESULTS OF REHABILITATIVE REACHING TRAINING AND PLASTICITY FOLLOWING SPINAL CORD INJURY IN THE ADULT RAT

5.1 Training efficacy depends mainly on CST plasticity after incomplete SCI

One goal of the current thesis was to investigate the role of training-induced corticospinal tract (CST) plasticity following incomplete cervical SCI in adult rats. In Chapter 2, I investigated whether adaptations of the CST following an injury are a potential mechanism involved in spontaneous and training-induced recovery of reaching success. Chapter 2 of the current thesis is an expansion of previous work from Ms. Girgis, a former student of the Fouad laboratory. We both found differences in the reaching success between animals with different lesion types. Since animal numbers were insufficient in her study, I repeated and expanded the experiment. We both found an increase in the reaching success in rats with a lesion of the dorsal column as opposed to a lateral lesion or both. One of the major findings of the present thesis is that improvements in reaching ability following incomplete cervical SCI in rats seem to depend on the input of the CST because a relesion of the CST following a lesion of the dorsolateral quadrant of the spinal cord resulted in a significantly reduced reaching performance. This reduced performance in reaching following a relesion of the CST occurred in trained as well as in untrained animals. Therefore, we suggest that plasticity of the CST is involved in spontaneous and training-induced improvements in reaching success. Improvement after SCI in a function, such as reaching, is thought to be related to plastic changes of the CNS. Following SCI, sprouting of the CST into the grey matter of the spinal cord can be seen as one potential mechanism of

plasticity that can lead to functional recovery. Nevertheless, collateral sprouting of the dorsal CST rostral to the lesion was not significantly different among groups with different lesions. The decline of function after CST relesion could be explained by the evidence that spared, ventral CST fibers can compensate for the injured dorsal CST as it has been shown before (Weidner *et al.* 2001) or that a statistical insignificant increase in collaterals not necessarily represents a functionally meaningless increase. Another possible explanation why no difference in the number of collateral sprouts was found, is that other descending tracts, such as the rubrospinal (RST) and reticulospinal tract (RtST), likely contributed to the recovery especially after training. An analysis and quantification of sprouts emanating from the rubrospinal and reticulospinal tract has not been performed and presents a limitation to the experiment (Chapter 2).

In Chapter 2, the reaching recovery was compared among groups with different lesion types of SCI: which either ablated the dorsal CST, the lateral CST in combination with damage to parts of the RST or all above structures (DLQ-lesion). Since both the CST and RST have been shown to be involved in voluntary skilled movements such as fine digit control (Kanagal and Muir 2009), it was surprising that after an ablation of the lateral funiculus no training benefits were found. This is interesting and it could be suggested that following an ablation of the RST the CST is able to compensate for the injury to a degree that training has no more benefits.

Interestingly, if only the dorsal funiculus was ablated, almost all trained animals had greater success post-injury than what was measured before SCI. When compared to baseline measurements in reaching, which are usually between 45-65% in Lewis rats (see appendix figure 6.4), those with SCI that were exposed to reaching training for 6 weeks achieved a success rate that was on average 10-20% higher than the baseline value. On one hand this would suggest that a rather minor injury to the CST can trigger plasticity of the injured and spared CST that results in an increase in training efficacy/skill performance as it was detected in animals that received a dorso-lateral quadrant lesion followed by an ablation of the pyramids. On the other hand it elucidates that input of the CST is involved in a training-induced increase in the reaching performance after SCI.

In Chapter 2 we also found in rats that first received a spinal lesion followed by a pyramidotomy that the inactivation of the *Red Nucleus,* the origin of the RST, by the administration of Muscimol altered but did not eliminate motor evoked potentials (MEP's) that were elicited by stimulation of the caudal part of the forelimb motor cortex. Since the administration of muscimol did not eliminate MEP's, it seems plausible that other descending pathways (e.g., reticulospinal tract) might be involved in the underlying mechanisms of the reaching recovery, as it was described by (Pettersson *et al.*, 2007). This is consistent with the results of the study in Chapter 2.

Interestingly, in rats that received an injury to the dorsal part of the CST (dorsal funiculus and DLQ-group) intensive reaching training resulted in impairments in

an untrained task (i.e. crossing a horizontal ladder). This result is in parallel with findings from Girgis *et al.* (2007) and suggests that intensive rehabilitative reaching training promotes reaching performance but may also result in deficits in an untrained task. Improvements in a trained task (i.e., task-specific improvements) are generally thought to not affect the performance of other tasks. Nevertheless, I observed that only rats that received a DLQ-lesion were showing impairments when tested in an untrained task. This could mean that the decreased performance in an untrained task is specific to the DLQ-lesion model (which is also the most severe injury). Similar findings were reported in cats where step training affected standing and vice versa (de Leon *et al.*, 1999). Additionally, Kuerzi and colleagues have reported that step-training in shallow water in rats with SCI improved recovery in the trained task but was not translated into improvements in overground walking (Kuerzi *et al.*, 2010). All these studies, including the results in Chapter 2 of this thesis suggest that rehabilitative training after SCI might function as an implement to fine tune newly formed and/ spared connections to strengthen networks orchestrating the trained task. As a result of task-specific training, movement patterns that distinguish themselves from the trained pattern may not be supported and therefore a decline in the performance of non-trained movements can be observed. This suggests on one hand that training multiple tasks at the same time could result in a broader recovery but it is unclear whether training multiple tasks would limit the improvements of the separate tasks. Future experiments have to address the important issue of task-specific

training and training in multiple tasks in order to design rehabilitation strategies in the clinic.

5.2 Advantages of delaying the onset of rehabilitative reaching training after incomplete SCI in rats

In Chapter 3 we investigated whether reaching training that was initiated on day 12 post-SCI would be effective in promoting reaching success. Currently, the most successful approach to promote reaching success following incomplete SCI is rehabilitative training. Until now there has been only limited information regarding the most beneficial time point to initiate rehabilitative training after SCI in order to promote improvements in a trained task. However, results from stroke research suggest that there might be a limited "window of opportunity", in which the CNS is most susceptible to training-induced changes. In Chapter 3, possible advantages in delaying the initiation of rehabilitative reaching training were examined. From the results in Chapter 3, we found that when the onset of rehabilitative reaching training was shifted to 12 days post SCI, rats were able to improve their grasping ability similar to what has been reported following an immediate onset of rehabilitative reaching training (Girgis *et al.*, 2007). Unlike reaching training that was initiated on day 4 post SCI, the initiation of rehabilitative reaching training on day 12 post SCI did not result in impairments in an untrained task (shown in Chapter 3; Girgis *et al.*, 2007). This would suggest that delaying the onset of rehabilitative training to 12 days post lesion allows

improvements in the trained task but also allows the CNS to build and strengthen connections that are needed for an untrained task such as crossing a horizontal ladder. Since we did not perform histological analysis of spinal tissue in a subgroup of rats in order to address the issue whether trained animals had smaller lesion sizes in general, we cannot exclude the possibility that rats performed better in the trained task because their lesion sizes were smaller initially. Alternatively, it might be that early reaching training (initiated on day 4 post SCI) can promote destruction of initially spared tissue, a process known as secondary damage, and therefore lead to an exacerbation of the lesion extent. Evidence for this comes from research in the field of stroke, where immediate rehabilitative training after brain injury in rats leads to an exacerbation of the lesion volume (Humm *et al.*, 1999; Risedal *et al.*, 1999; DeBow *et al.*, 2004). Because an increase in the expression of the neuroprotective BDNF has frequently been shown to occur with exercise (e.g., Ying *et al.*, 2005), training initiated at a time point when inflammatory processes are still present, could result in a preservation of spared tissue and functional recovery. Results in Chapter 3 of this thesis confirm this idea by showing that rehabilitative reaching training initiated on day 12 following SCI is beneficial in regards to lesion extent in the rostro-caudal axis because trained animals displayed a significantly smaller lesion extent than their untrained counterparts. Conversely, others reported that forced exercise (Sandrow-Feinberg *et al.*, 2009) or voluntary exercise (Engesser-Cesar *et al.*, 2005) following SCI improved functional recovery however it did not influence the lesion size. It remains speculative whether rehabilitative training that is initiated within a few

days after SCI can actively influence the lesion extent in the rostro-caudal axis. One might suggest that a time point exists which determines whether rehabilitative training will lead to tissue destruction or preservation. Such a tipping point is most likely linked to the inflammatory response that occurs after SCI.

It is generally agreed upon that tissue preservation will result in better recovery following SCI. A common assumption is that training, which is initiated early after SCI, would be more beneficial on recovery as opposed to training that would be initiated at later stages after SCI (Brown *et al.*, 2011). In comparison to this, studies in the field of stroke show that immediate forced use of the affected limb following stroke causes damage of surviving tissue around the lesioned area which then results in an exacerbation of the lesion size (Kozlowski *et al.*, 1996). Also it remains unclear whether such metabolic responses are enhanced by early training interventions after SCI, resulting in non-task-specific impairments. Support for this was found in a study by Girgis and colleagues (2007) where rehabilitative reaching training lead to impairments in an untrained task. The underlying mechanisms for such impairment however have not been investigated. Considering that early rehabilitative training may enhance secondary damage and therefore result in impairments, one might suggest that rehabilitative training be initiated in a slightly delayed manner as it was executed in the study in Chapter 2. The onset of rehabilitative training was chosen to be the time after injury when BDNF, a neurotrophic factor, which is associated with neuroprotection, is highly expressed. It has been shown that following SCI in rats the level of BDNF within

the grey matter is up-regulated within 24 hours after breakdown of the blood-spinal cord barrier, then declines and is up-regulated again from 6-14 days after SCI (Donnelly and Popovich, 2008). Because reaching training that was initiated within 4 days after SCI resulted in impairments in an untrained task (Girgis *et al*., 2007) a short delay in the onset of reaching training after SCI (started on day 12 post-SCI) would not only coincide with the second wave of BDNF expression but also be more clinically relevant as patients with SCI are usually not able to start rehabilitative training because of other injuries that accompany the SCI.

Probably the most important finding of the experiment in Chapter 3 is that levels of the cAMP-dependent kinase PKA, especially in its activated form, are declining following SCI. This decline in PKA activity could be prevented by the initiation of rehabilitative reaching training on day 14 post-SCI but not by reaching training initiated on day 4 post-injury. There are several hypotheses that could explain the phenomenon that only delayed reaching training can enhance cortical PKA activity in rats. On one hand it might be possible that an increase in PKA activity could not have been detected as rats that were exposed to reaching training on day 4 after SCI were not motivated/able to reach and therefore train. It has been observed that rats that receive training at such a time point after SCI try to reach for pellets initially but do not continue to reach after being unsuccessful. Such behaviour disappears within a few training sessions. On the other hand one might suggest that a critical level for plasticity and functional recovery related to PKA levels following SCI exists (see Fig. 5.2.1), which means that if training is initiated early after SCI, when PKA activity is still high (above a critical level), no

change in the PKA activity can be detected. If the training is initiated at a later time point (e.g., months) following SCI, animals will exhibit a reduced use or even a non-use of the affected limb as was demonstrated in humans and monkeys (as reviewed in Taub *et al.*, 2006). Thus, the activity-induced plasticity and therefore PKA activity would be reduced (below a critical level). This suggests that PKA activity is dependent on cell activation, which can be elicited by rehabilitative training. Training that is not initiated immediately after SCI could enhance "below threshold" PKA activity (training-induced activity; see Fig. 5.2.1 dashed line) and thus extent the "window of opportunity" which could explain why we found elevated PKA activity in animals that received delayed reaching training but not rats that received training immediately after SCI.

Another important process that also declines with time following SCI is the inflammatory response. It is unclear whether the decline in the inflammatory response after SCI correlates with the decline in PKA activity following SCI. If they are correlated, a reintroduction of an inflammatory response at a chronic stage after SCI should lead to an enhanced PKA activity and therefore to improved training-efficacy. Evidence for this comes from results in Chapter 4 where we found that the administration of lipopolysaccharide (LPS) to chronic injured rats increases cortical PKA levels. This increase in cortical PKA level by the application of LPS alone was not sufficient to improve reaching success, however if LPS was combined with reaching training, a significant increase in reaching success was found. This would suggest that only the combination of training and LPS raised PKA levels above the critical level and therefore result in

a recovery in reaching success. In addition, LPS does not only increase PKA activity but has also been shown to enhance mircoglial/macrophage recruitment following its application to rodents (as reviewed in Nguyen *et al*., 2002). It remains unclear whether the administration of LPS can improve reaching recovery through PKA activity or by the enhanced recruitment of macrophages. In Chapter 4, I investigated whether the administration of LPS in rats can increase reaching success and investigated potential mechanisms that could be involved in the recovery such as PKA activity and microglia/macrophage recruitment. The main findings of the experiment in Chapter 4 are discussed below.

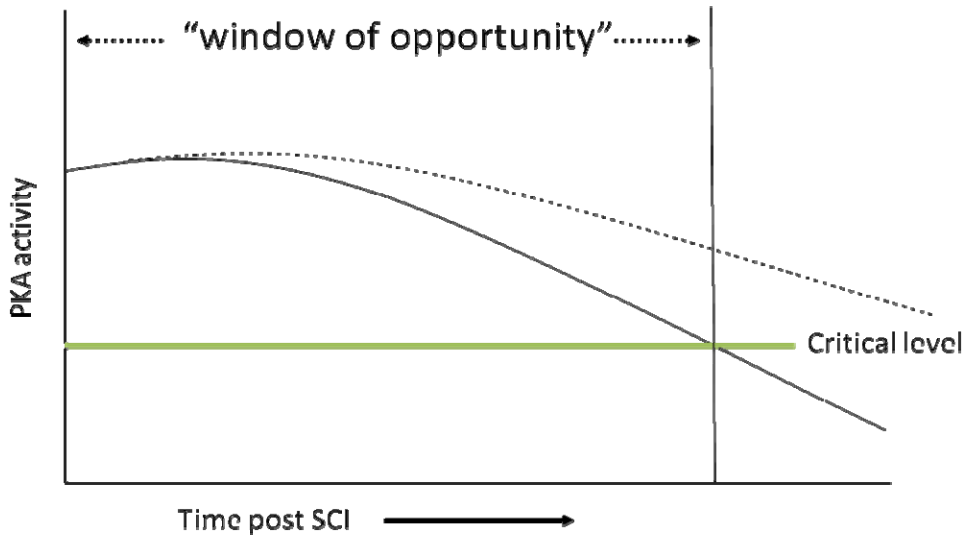


Figure 5.2.1. A schematic illustrating the hypothetical decline of PKA activity after SCI. The green line represents a critical level of PKA activity that must be surpassed in order to translate to functional improvements. If rehabilitative training is applied within a "window of opportunity", it potentially increases PKA activity and might result in improved recovery after SCI (dashed line).

5.3 Reintroduction of an inflammatory response at a chronic stage of SCI can promote training efficacy in rats

In Chapter 4, we investigated whether rehabilitative training at a chronic stage (8 weeks post SCI) is as efficacious as reaching training initiated on day 12 after SCI. Since there is only limited information regarding the most efficient onset of rehabilitative training after incomplete SCI, it was necessary to investigate this detail. The results in Chapter 4 show that rehabilitative reaching training in chronic injured rats translates into task-specific recovery but is significantly less efficacious than rehabilitative training initiated on day 12 after SCI. These results are consistent with findings from the field of stroke, where it has been shown that training efficacy also declines with time after focal brain injury (Biernaskie *et al.*, 2004). Unpublished data from the Pearse laboratory in Miami show that cAMP within the CNS declines over a time period of 7 months after SCI (Pearse et al. unpublished). A gradual reduction of cAMP levels in the developing CNS has been shown to be in parallel with an age-dependent decline in the adaptability of the nervous system (Cai et al., 2001). Therefore, we suggest that the reduced responsiveness to rehabilitative training in a chronic model of SCI is effected by reduced levels of cAMP within the CNS. This hypothesis is supported by the finding that following SCI, damaged neuronal tissue and tissue within the vicinity of the lesion have inadequate levels of the important second messenger cAMP, especially at a chronic stage after SCI. However, data in Chapter 4 in this thesis suggest that rehabilitative training in rats can elevate cAMP levels and PKA

activity when compared to untrained animals. If activation at a chronic stage after SCI is needed to increase cAMP levels and thus PKA activity (as suggested earlier), trained rats with a chronic lesion should have a significant increase in cAMP as well as activated PKA levels when compared to their untrained counterparts. This is exactly what I discovered while analyzing western blots of cortical tissue of rats with chronic SCI. Also, I found that the application of the endotoxin LPS 3 weeks following SCI increases PKA levels, which suggests a correlation between the decline in PKA activity and the inflammatory response after SCI. Nevertheless, future studies are needed to prove the relationship between the decline of PKA activity and the decline of the inflammatory response after SCI.

Taken together, the results presented in Chapter 4 lead to the conclusion that training efficacy after SCI declines over time which is in parallel with a decline in cortical cAMP levels and PKA activity. We also found that rehabilitative training at a chronic stage of SCI is still beneficial, possibly through elevated cortical cAMP and PKA levels. Nevertheless, these elevated cortical cAMP levels are most likely lower than cortical cAMP levels at an early stage after SCI. It might be possible that the combination of training and LPS administration at a chronic stage after SCI is sufficient to enhance training efficacy.

5.4 Interpretations and applications

5.4.1 *Rehabilitative reaching training and CST-plasticity*

The results in Chapter 2 show that recovery after SCI is activity-dependent as only rats that were exposed to reaching training had an increased success in retrieving pellets. This is consistent with an earlier report from the field of stroke research in which it was shown that non-use of an affected limb exacerbates motor deficits following injury, a process that is paralleled by the loss of cortical function in areas surrounding the lesion site (Nudo and Milliken, 1996). It has been reported that humans also develop injury-induced learned non-use of body parts that were affected by a stroke (reviewed in Taub et al., 2002). This strongly suggests that only the activation after injury leads to improvements, which also has been supported by Schallert *et al.* (Schallert et al. 2000) and coincides with Chapter 2, where following SCI, a relesion of the CST lead to a significant reduction in the acquired recovery in trained rats. Nevertheless, a relesion of the CST in untrained rats also reduced functional improvements which had been the result of spontaneously occurring plasticity. Thus, to know the spontaneous and naturally occurring mechanisms of plasticity and the potential effect of plasticity on functional recovery is very important for designing future rehabilitation strategies following SCI.

5.4.2 *Delayed onset of rehabilitative reaching training allows for substantial recovery in the trained task*

The most effective time frame for rehabilitative training in patients following SCI has yet to be investigated. In Chapter 3, we discuss the finding that rehabilitative reaching training in injured rats that was initiated with a short delay after SCI (12 days) results in similar reaching recovery when compared to rats that started reaching training immediately after SCI. One underlying mechanism that seemed to accompany the activity-dependent recovery is the up-regulated activity of PKA in the cortex in trained rats as compared to their untrained counterparts. The cAMP pathway has been shown to play a crucial role in learning and memory, however more extensive research is needed to identify the relation between functional recovery and cAMP-dependent plasticity.

5.4.3 *Systemically induced inflammation and recovery in chronically injured rats*

There is an ongoing debate whether the inflammatory response following SCI has beneficial effects on repair and recovery in addition to the more commonly accepted detrimental effects (as reviewed in Crutcher *et al.*, 2006). The negative reputation of inflammation is based on the fact that pro-inflammatory cytokines induce apoptosis, increase tissue damage, impair functional recovery and are responsible for neuronal and glial loss in the lesion epicentre within 4 hrs of the

injury (Tetzlaff *et al.*, 1994). They also play a role in scar formation and retrograde neuronal cell loss. Macrophages are one of the most potent components of the inflammatory response because they can trigger secondary damage to the spared neural tissue through the expression of pro-inflammatory cytokines. It has recently been reported that two types of macrophages exist, a classically activated pro-inflammatory and an alternatively activated anti-inflammatory form (Kigerl *et al.*, 2009). In addition, it was found that macrophages express growth promoting factors such as BDNF at later stages of the inflammatory response, which is generally thought to stimulate axonal growth and potentially plasticity. If different types of macrophages are expressing various pro- and anti-inflammatory cytokines throughout the injury-induced inflammatory response, it would not be advisable to completely eliminate macrophages from entering the injury site. A more appropriate approach to promote recovery is to boost the growth stimulating phase of macrophages. Administration of the macrophage stimulant zymosan was originally used to model the injurious effect of macrophages without considering beneficial effects of the drug (Fitch *et al.*, 1999; Popovich *et al.*, 2002). The administration of zymosan however results in activation of macrophages that can for example promote regeneration of retinal ganglion cells (Yin *et al.*, 2003). Further, studies from the Benowitz laboratory suggest that the expression of the macrophage-derived growth factor Oncomodulin can promote axonal growth following CNS injury (Yin *et al.*, 2006; Kurimoto *et al.*, 2010). This suggests that axonal growth following SCI could also be mediated by macrophages through the expression of Oncomodulin. One approach to activate macrophages other than by

an injury to the CNS is the administration of zymosan or LPS. Zymosan and LPS are both wall components of yeast and gram-negative bacteria respectively, which induce a systemic inflammation and activate macrophages. Their ability to promote axon regeneration however differs. LPS has been shown to be a less potent promoter of regeneration but LPS-stimulated macrophages are less toxic than zymosan-activated macrophages (Gensel *et al.*, 2009). Most importantly, Chen and colleagues found that LPS in combination with NT-3 could significantly increase sprouting of the CST over non-treated animals with a chronic SCI (Chen *et al.*, 2008). This suggests that LPS activates the "alternatively activated" macrophages, which are known to express anti-inflammatory cytokines, could enhance axonal growth. From the results in Chapter 4 however, it appears that the administration of LPS alone is insufficient to promote plasticity (e.g. axonal sprouting) and therefore functional recovery. However the combination of LPS with rehabilitative reaching training in chronically injured rats is sufficient to be translated into recovery, as it is described in my last experiment (Chapter 4).

5.5 Limitations

The aim of the experiments in the present thesis was to investigate the role of plasticity of the CST following SCI in rats. Plasticity describes adaptations of the CNS due to training and/or injury. Since plasticity following SCI occurs throughout the entire CNS, it is challenging to find difference between groups when the focus of analysis is narrowed to only one level of the CNS. For instance,

in Chapter 2 I saw a training-induced increase in the number of collateral sprouts emanating from the CST and projecting into the grey matter when compared between trained and untrained rats. However, the difference in sprouting appeared not to be significant. Interestingly, when the cortical maps of rats with different lesions were analyzed I found again a difference between trained and untrained rats in the DLQ-lesion and dorsal funiculus group. This difference between trained and untrained animals was again not significantly different even though the cortical map representation of the wrist mirrored the reaching success data. One might argue that statistical differences are not necessarily representing the true image of recovery after SCI and that the results are diluted by plasticity in other systems.

One limitation of this thesis was the insufficient representation of the origin and ablation/sparing of descending and ascending spinal tracts for the different spinal lesions in Chapter 2. Therefore, a figure to the appendix (see Appendix figure 6.1) was added to clarify which tracts were ablated/spared (figure 6.1 A) and where collateral sprouts of the dorsal CST were counted (figure 6.1 B). In the schematic the origins as well as the projection within the cervical spinal cord of descending tracts are shown. A schematic cross section as used in Chapter 2 was added to illustrate which lesion model ablated which spinal tracts.

Another major limitation of this thesis is that improvements following a dorsolateral quadrant lesion (DLQ-lesion; ablates the dorsal and lateral part of the CST as well as the majority of the RST) in the trained paradigm were

accompanied by impairments in the performance in an untrained task (Chapter 2). This result was very interesting and surprising as only this lesion in combination with reaching training in rats resulted in an impaired performance when crossing a horizontal ladder. It has to be investigated whether training that was initiated on day 4 post-SCI might have played an essential role in leading to impairments in an untrained task (e.g., exacerbation of the lesion) compared to reaching that was initiated on day 12 post-SCI which did not exhibit to be detrimental to an untrained task (as described in Chapter 3).

Another shortcoming of this thesis is that I stated that I found an increase in reaching recovery, however none of the manuscripts actually showed a recovery over time. Since only measurements of the reaching performance alone or the reaching performance normalized to the pre-surgical value is shown, one might argue that the enhanced performance in reaching is no recovery but rather an improvement in skill acquisition. It can be speculated whether the enhanced performance in reaching can be seen as recovery. There are two terminologies that describe recovery after SCI but they have very distinctive meanings. On one hand is the motor recovery, which describes improvements in motor ability following injury. On the other hand is the functional recovery, which does not describe motor or sensory improvements but rather improvements in a task such as reaching. Therefore, in the experiments that are described in the current thesis we used functional recovery. In our manuscripts we failed to show that functional recovery occurs over time after SCI and only show measurements from the endpoint (6 weeks) of rehabilitative reaching training. In order to prove that we

did find an increase in functional recovery I now added two figures to appendix (figure 6.2 and 6.3). The graph in figure 6.2 shows the recovery in reaching success of trained (black circles) and untrained rats (grey circles) that received a dorsolateral funiculus lesion. The graph in figure 6.3 represents the reaching recovery over time for animals that received LPS in combination with reaching training (black circles), rats that received LPS alone (grey circles) and rats that received training alone (white circles).

Another limitation of the current thesis is that the reaching success following 6 weeks of training is illustrated either as success rate that was shown as a percentage of the pre-lesion value (Chapter 2), as actual reaching success (Chapter 3) or as reaching success that was divided by the attempt rate (Chapter 4). The different representation of the reaching data is confusing for the reader but the reason for choosing different illustrations between the experiments is the following: with time we recognized that a normalization of the reaching success to the pre-lesion value is not necessarily the most useful representation. This is based on the fact that regained function after SCI does not necessarily show the exact pattern of the same function as it was prior to injury function. Therefore, all function that will be acquired will automatically be a compensation of the function that was seen prior to injury. The reaching recovery is consequently due to the development of a compensatory movement. Thus, in the latter Chapters the normalization to pre-lesion values was not performed as the two reaching patterns (before and after SCI) are completely different from each other. Nevertheless, in order to compare the reaching success among studies, a normalization of the

reaching success to the pre-lesion performance was added to the appendix for data shown in Chapter 3 and 4 (figure 6.5 and 6.6 respectively).

Lastly, the representation of the lesion size in Chapter 2 and 4 is not sufficient to show differences in lesion sizes among animals in one group. Therefore, schemata of lesion sizes in a cross section and ablation of the pyramids are included in the appendix.

5.6 Future directions

5.6.1 Exogenous application of cAMP and its inhibition

Results from experiments in this thesis (Chapter 3 & 4) indicate that cortical cAMP and PKA levels are closely related to functional recovery. One could suggest that if the levels of cAMP stayed elevated, the CNS would be more susceptible to adaptive changes and therefore would allow for a higher level of recovery. One study addressing this specific issue shows that exogenously applying a phosphidiesterase inhibitor to the cortex of rats after ischemia enhances cAMP signaling (MacDonald *et al.*, 2007). This results in an improved recovery in skilled reaching within ten days (MacDonald *et al.*, 2007). A likely mechanism for the improved recovery could be by an enhanced synaptic plasticity between neurons in the cortex that was triggered by cAMP.

It remains unclear whether the exogenous application of a cAMP analogue in combination with intensive rehabilitative training after SCI can enhance recovery.

One approach to test whether cAMP is essential for functional recovery following SCI would be the inhibition of cAMP or PKA. This can be achieved for instance by cortical administration of H-89 a potent cell-permeable and reversible PKA-inhibitor. If cAMP and PKA are involved in training-induced recovery after SCI, the inhibition of cAMP or PKA should reduce the training effect drastically, as suggested in Fig. 5.6.1.

5.6.2 Role of reticulospinal tract in reaching recovery after pyramiditomy

In Chapter 2, we suggested that the reaching recovery after pyramidotomy can depend on other descending pathways of the CNS such as the reticulospinal tract. The reticulospinal tract has been shown to play a major role in locomotion but it also projects to motoneuron pools that innervate shoulder muscles (McKenna *et al.*, 2000). The results in Chapter 2 indicate that the CST of uninjured animals projects towards the reticular formation, the origin of the reticulospinal tract (RtST). Certainly, more research is needed to show their relation and changes in the number of these projections between trained and untrained rats after SCI. For instance, to prove this relation, administration of muscimol to the reticular formation after an ablation of the entire CST and RST should result in a disappearance of MEP's when the forelimb motor cortex is stimulated. One limitation of the study would be that the RtST is only minimally involved in

reaching recovery and therefore no changes in the signal, which is recorded from wrist extensor muscles, might be seen.

5.6.3 *Enhancing collateral sprouting by the application of electrical stimulation in combination with reaching training*

Collateral sprouting of the CST after SCI has been closely related to functional recovery (Girgis et al. 2007). One approach to increase axonal growth is the application of electrical stimulation. It has been shown that electrical stimulation to a peripheral nerve following lesion enhances the expression of BDNF and pro-regenerative genes (Geremia *et al.*, 2007; Asensio-Pinilla *et al.*, 2009). The expression of BDNF acts most likely through the TrkB receptor and thus triggers the cAMP pathway (Gordon *et al.*, 2009). It has been shown that drugs that enhance the cAMP pathway mimic the effects that were seen following electrical stimulation (Gordon *et al.*, 2009). This would suggest that brief electrical stimulation to the caudal part of the forelimb motor cortex following SCI could possibly enhance the expression of BDNF and thus up-regulate cAMP levels, which in turn would allow for an increased plasticity of the lesioned structures (e.g. CST). Also, in Chapter 4 I showed that rehabilitative reaching training even at a chronic stage after SCI still increases cortical cAMP levels. If brief electrical stimulation to the cortex can be combined with rehabilitative training following

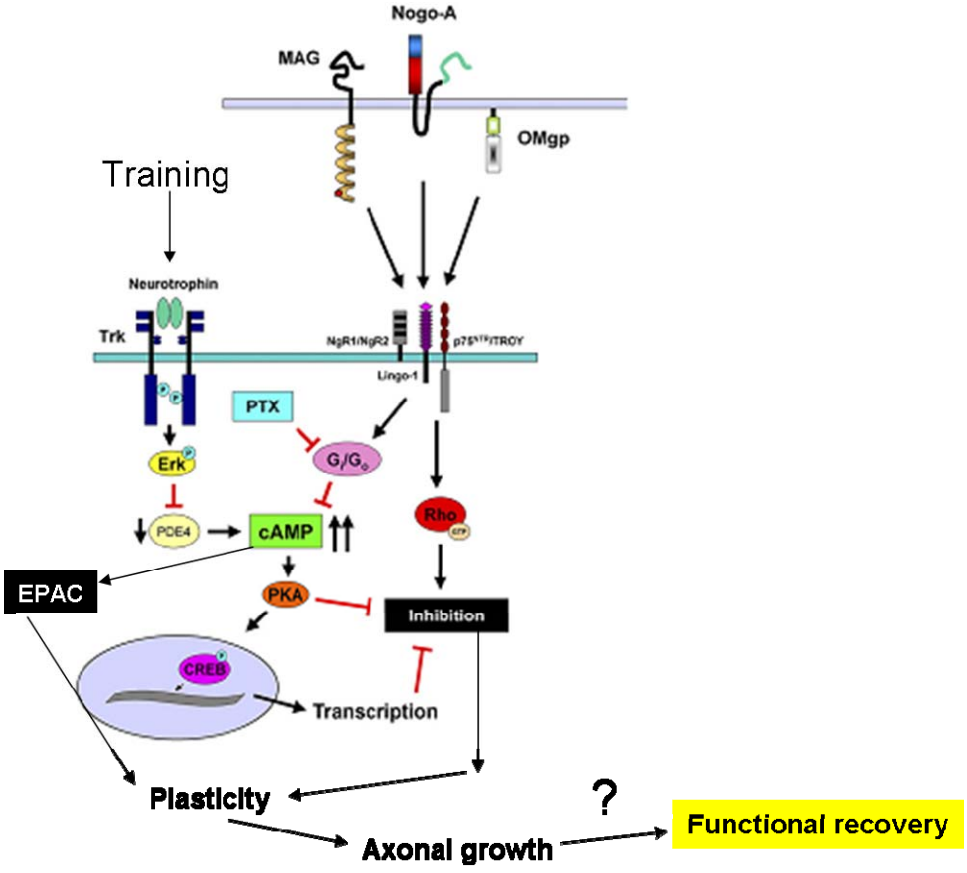


Figure 5.6.1. A schematic illustrating the role of cAMP in plasticity after SCI. The activation of a neurotrophin (e.g. BDNF) can up-regulate the expression of cAMP and promote plasticity. Adapted from Hannila and Filbin (2008) The potential role of cyclic AMP signaling in promoting axonal growth and plasticity after spinal cord injury.

SCI, cortical cAMP levels should increase and allow for a better recovery than training or stimulation alone. One limitation of such approach is that rat's performance in reaching can be decreased by exposure to stress. To successfully conduct the proposed experiment in rats, a connector must be implanted to the forelimb motor cortex. A wireless device would be preferred, however this would increase the size and weight of the connector which could influence rats posture. Thus, the weight of the connector has to be in a range so it does not affect animals' behaviour.

5.6.4 *Administration of LPS in an acute phase after SCI*

As described in Chapter 4, the reinstatement of inflammation, through the administration of LPS in combination with reaching training in a chronic model of SCI promoted plasticity and recovery in rats. Since the application of LPS in combination with reaching training in a chronic model of SCI resulted in an improvement in the reaching success, it needs to be investigated if the administration of LPS in combination with reaching training during the acute (within 4 days after SCI) or sub-acute phase (within 14 days after SCI) will also promote plasticity and enhance reaching recovery. If the hypothesis in figure 5.2.1. is correct then the administration of LPS in the acute phase after SCI would not improve the reaching ability significantly as the level of plasticity (measured by the activity of PKA) at this phase is relatively high already. Another possibility is that with the administration of LPS in the acute phase after SCI a certain level

of plasticity (and therefore PKA activity) is exceeded and most adaptations of the CNS result to be inappropriate and therefore potentially lead to impairments rather than improvements. If reaching training in combination with LPS would be initiated in a sub-acute phase after SCI an improvement in the reaching success might be seen, as both, training and LPS administration could increase PKA activity and therefore plasticity. It remains uncertain whether PKA activity, the microglia/macrophage response or an unknown factor is the underlying mechanism for enhancing training efficacy after SCI and LPS injection. Thus the suggested experiment with LPS application at different post-lesion time points presents a necessity in order to design future rehabilitation treatments.

5.6.5 *Relationship between LPS-induced inflammation and functional recovery*

In Chapter 4 we implied that reaching recovery at chronic stage of SCI in rats can be enhanced by the administration of LPS with reaching training. We suggested that the application of LPS triggers a macrophage response in the spinal cord. If the administration of LPS enables alternatively activated macrophages to express BDNF and Oncomodulin (as suggested earlier) then an experiment using genetically modified mice with a macrophage deficiency should reduce the effect of reaching recovery. Currently, there are no macrophage-deficient knockout mice available. However, it has been reported that a spontaneous macrophage-deficient mouse exists, known as op/op. This type is unable to produce any macrophage

colony-stimulating factors, which are required for macrophage differentiation (as described in Wiktor-Jedrzejczak *et al.*, 1990). A study with this type of mice would clarify whether enhanced recovery at a chronic stage of SCI is evoked by macrophages and their expression of growth factors like Oncomodulin and BDNF. One major limitation of this experiment is that the op/op mice are only spontaneous macrophage deficient and that such animals suffer from congenital osteopetrosis, a condition characterized by over-density of bone tissue. These mice tend to suffer very easily from bone fractures which would compromise their reaching performance. In addition to this it needs to be clarified whether microglia/macrophages or the cAMP pathway could be the underlying mechanism for the enhanced recovery. The proposed experiment is necessary as it is important to know the mechanism that can enhance functional recovery in order to design future rehabilitation strategies in the clinic.

5.7 Concluding remarks

The data of the present thesis suggest that probably the best approach to initiate rehabilitative reaching training following SCI is during the sub-acute phase, when the CNS is most susceptible to adaptive changes. This stems from the results from Chapter 2, 3 and 4 as after a DLQ-lesion in rats reaching training initiated at an acute phase (4 days post-SCI) results in an increase in skilled performance but also in impairments in untrained tasks. Reaching training that was initiated at a sub-acute phase after SCI (12 days post-SCI) showed a similar increase in the

reaching performance in trained animals without displaying negative effects on an untrained task. Also, reaching training that was initiated at a chronic phase of SCI (8 weeks post-SCI) still enhanced the reaching performance in trained rats over their untrained counterparts, however when the actual performance was compared between studies the reaching training seemed to be less efficacious in enhancing the reaching performance than training that was initiated at a acute or sub-acute phase after SCI. As mentioned above, parts of the experiments need to be repeated and performed in a time course experiment in order to be able to compare the reaching success between the different training onsets. In the future such a comparison represents a necessity as no standard treatment and no time line outlining an appropriate time to initiate training is currently available for patients suffering from SCI. There is a dissenting opinion among researchers and clinicians as i) the severity of SCI varies between individuals ii) individuals may or may not suffer from other injuries and iii) initiation of rehabilitative training must be adjusted according to the patient's ability. All these factors need to be considered to design a rehabilitation therapy for patients with SCI. In addition to this, if the underlying mechanism that is involved in enhancing functional and motor recovery after SCI can be determined, rehabilitative training can be even more beneficial. If parts of the inflammatory response are essential for enhancing recovery after SCI, an increase or reinstatement of certain parts of the inflammation need to be explored.

In order to design effective rehabilitation strategies, future studies are necessary to investigate the most efficient time point to start rehabilitative training after SCI and the importance of the inflammatory response following SCI.

5.8 References

Asensio-Pinilla E., Udina E., Jaramillo J. and Navarro X. (2009). "Electrical stimulation combined with exercise increase axonal regeneration after peripheral nerve injury." Exp Neurol 219(1): 258-65.

Biernaskie J., Chernenko G. and Corbett D. (2004). "Efficacy of rehabilitative experience declines with time after focal ischemic brain injury." J Neurosci 24(5): 1245-54.

Brown A. K., Woller S. A., Moreno G., Grau J. W. and Hook M. A. (2011). "Exercise therapy and recovery after SCI: evidence that shows early intervention improves recovery of function." Spinal Cord.

Cai D., Qiu J., Cao Z., McAtee M., Bregman B. S. and Filbin M. T. (2001). "Neuronal cyclic AMP controls the developmental loss in ability of axons to regenerate." J Neurosci 21(13): 4731-9.

Chen Q., Smith G. M. and Shine H. D. (2008). "Immune activation is required for NT-3-induced axonal plasticity in chronic spinal cord injury." Exp Neurol 209(2): 497-509.

Crutcher K. A., Gendelman H. E., Kipnis J., Perez-Polo J. R., Perry V. H., Popovich P. G. and Weaver L. C. (2006). "Debate: "is increasing neuroinflammation beneficial for neural repair?"" J Neuroimmune Pharmacol 1(3): 195-211.

de Leon R. D., Tamaki H., Hodgson J. A., Roy R. R. and Edgerton V. R. (1999). "Hindlimb locomotor and postural training modulates glycinergic

inhibition in the spinal cord of the adult spinal cat." J Neurophysiol 82(1): 359-69.

DeBow S. B., McKenna J. E., Kolb B. and Colbourne F. (2004). "Immediate constraint-induced movement therapy causes local hyperthermia that exacerbates cerebral cortical injury in rats." Can J Physiol Pharmacol 82(4): 231-7.

Donnelly D. J. and Popovich P. G. (2008). "Inflammation and its role in neuroprotection, axonal regeneration and functional recovery after spinal cord injury." Exp Neurol 209(2): 378-88.

Engesser-Cesar C., Anderson A. J., Basso D. M., Edgerton V. R. and Cotman C. W. (2005). "Voluntary wheel running improves recovery from a moderate spinal cord injury." J Neurotrauma 22(1): 157-71.

Fitch M. T., Doller C., Combs C. K., Landreth G. E. and Silver J. (1999). "Cellular and molecular mechanisms of glial scarring and progressive cavitation: in vivo and in vitro analysis of inflammation-induced secondary injury after CNS trauma." J Neurosci 19(19): 8182-98.

Geremia N. M., Gordon T., Brushart T. M., Al-Majed A. A. and Verge V. M. (2007). "Electrical stimulation promotes sensory neuron regeneration and growth-associated gene expression." Exp Neurol 205(2): 347-59.

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

Gordon T., Udina E., Verge V. M. and de Chaves E. I. (2009). "Brief electrical stimulation accelerates axon regeneration in the peripheral nervous system and promotes sensory axon regeneration in the central nervous system." Motor Control 13(4): 412-41.

Hannila S. S. and Filbin M. T. (2008). "The role of cyclic AMP signaling in promoting axonal regeneration after spinal cord injury." Exp Neurol 209(2): 321-32.

Humm J. L., Kozlowski D. A., Bland S. T., James D. C. and Schallert T. (1999). "Use-dependent exaggeration of brain injury: is glutamate involved?" Exp Neurol 157(2): 349-58.

Kigerl K. A., Gensel J. C., Ankeny D. P., Alexander J. K., Donnelly D. J. and Popovich P. G. (2009). "Identification of two distinct macrophage subsets with divergent effects causing either neurotoxicity or regeneration in the injured mouse spinal cord." J Neurosci 29(43): 13435-44.

Kozlowski D. A., James D. C. and Schallert T. (1996). "Use-dependent exaggeration of neuronal injury after unilateral sensorimotor cortex lesions." J Neurosci 16(15): 4776-86.

Kuerzi J., Brown E. H., Shum-Siu A., Siu A., Burke D., Morehouse J., Smith R. R. and Magnuson D. S. (2010). "Task-specificity vs. ceiling effect: step-training in shallow water after spinal cord injury." Exp Neurol 224(1): 178-87.

Kurimoto T., Yin Y., Omura K., Gilbert H. Y., Kim D., Cen L. P., Moko L., Kugler S. and Benowitz L. I. (2010). "Long-distance axon regeneration in

the mature optic nerve: contributions of oncomodulin, cAMP, and pten gene deletion." J Neurosci 30(46): 15654-63.

MacDonald E., Van der Lee H., Pocock D., Cole C., Thomas N., VandenBerg P. M., Bourtchouladze R. and Kleim J. A. (2007). "A novel phosphodiesterase type 4 inhibitor, HT-0712, enhances rehabilitation-dependent motor recovery and cortical reorganization after focal cortical ischemia." Neurorehabil Neural Repair 21(6): 486-96.

Nudo R. J. and Milliken G. W. (1996). "Reorganization of movement representations in primary motor cortex following focal ischemic infarcts in adult squirrel monkeys." J Neurophysiol 75(5): 2144-9.

Nguyen M. D., Julien J. P., Rivest S. (2002). "Innate immunity: the missing link in neuroprotection and neurodegeneration? Nat Rev Neurosci 3: 216-227.

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

Popovich P. G., Guan Z., McGaughy V., Fisher L., Hickey W. F. and Basso D. M. (2002). "The neuropathological and behavioral consequences of intraspinal microglial/macrophage activation." J Neuropathol Exp Neurol 61(7): 623-33.

Risedal A., Zeng J. and Johansson B. B. (1999). "Early training may exacerbate brain damage after focal brain ischemia in the rat." J Cereb Blood Flow Metab 19(9): 997-1003.

Sandrow-Feinberg H. R., Izzi J., Shumsky J. S., Zhukareva V. and Houle J. D. (2009). "Forced exercise as a rehabilitation strategy after unilateral cervical spinal cord contusion injury." J Neurotrauma 26(5): 721-31.

Taub E., Uswatte G. and Elbert T. (2002). "New treatments in neurorehabilitation founded on basic research." Nat Rev Neurosci 3(3): 228-36.

Taub E., Uswatte G., Mark V. W. and Morris D. M. (2006). "The learned nonuse phenomenon: implications for rehabilitation." Eura Medicophys 42(3): 241-56.

Tetzlaff W., Kobayashi N. R., Giehl K. M., Tsui B. J., Cassar S. L. and Bedard A. M. (1994). "Response of rubrospinal and corticospinal neurons to injury and neurotrophins." Prog Brain Res 103: 271-86.

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

Wiktor-Jedrzejczak W., Bartocci A., Ferrante A. W., Jr., Ahmed-Ansari A., Sell K. W., Pollard J. W. and Stanley E. R. (1990). "Total absence of colony-stimulating factor 1 in the macrophage-deficient osteopetrotic (op/op) mouse." Proc Natl Acad Sci U S A 87(12): 4828-32.

Yin Y., Cui Q., Li Y., Irwin N., Fischer D., Harvey A. R. and Benowitz L. I. (2003). "Macrophage-derived factors stimulate optic nerve regeneration." J Neurosci 23(6): 2284-93.

Yin Y., Henzl M. T., Lorber B., Nakazawa T., Thomas T. T., Jiang F., Langer R. and Benowitz L. I. (2006). "Oncomodulin is a macrophage-derived signal for axon regeneration in retinal ganglion cells." Nat Neurosci 9(6): 843-52.

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

6. Appendix

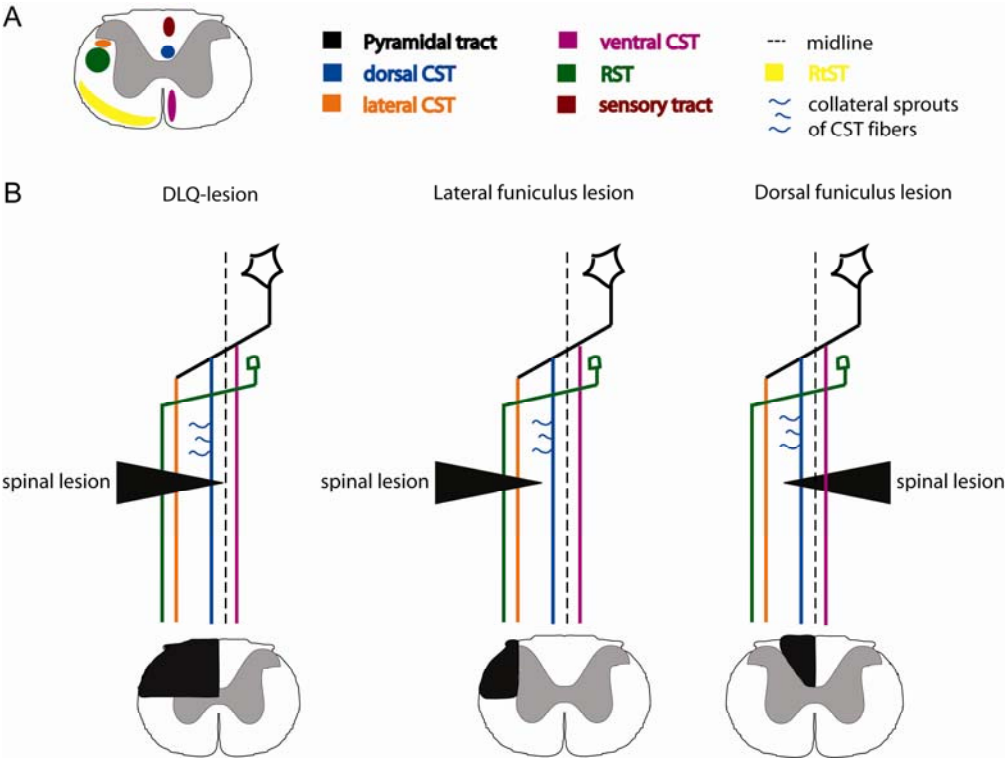


Figure 6.1. Schematic representation of the different lesion models in Chapter 2. **A** shows a schematic cross section of the spinal cord with the location of ascending (sensory tract) and descending spinal tracts. In **B** the different descending tracts that were ablated by the spinal lesion are shown. The dashed line indicates the midline. Collateral sprouts (blue horizontal lines) of the CST were counted above the spinal lesion. CST= corticospinal tract; RST= rubrospinal tract; RtST= reticulospinal tract.

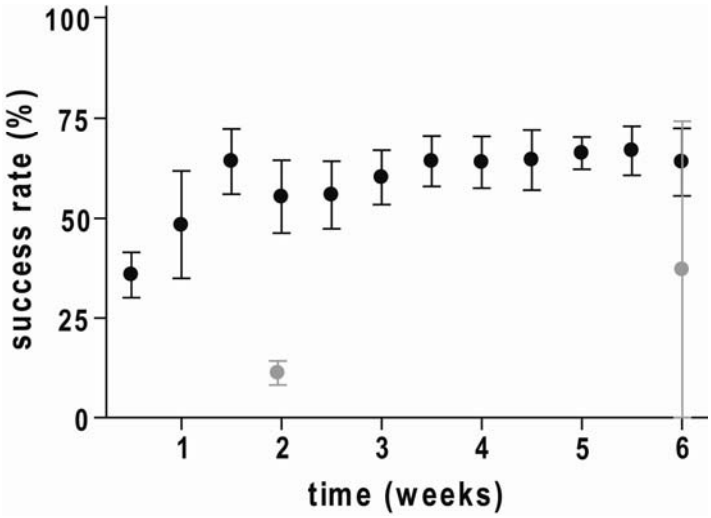


Figure 6.2. An illustration of the reaching performance of rats with a dorsal funiculus lesion over the time course of 6 weeks. Shown are reaching performances of rats that were exposed to reaching training (black circles) and rats that remained untrained (grey circles). Animals in both groups exhibit a recovery in skilled reaching over time.

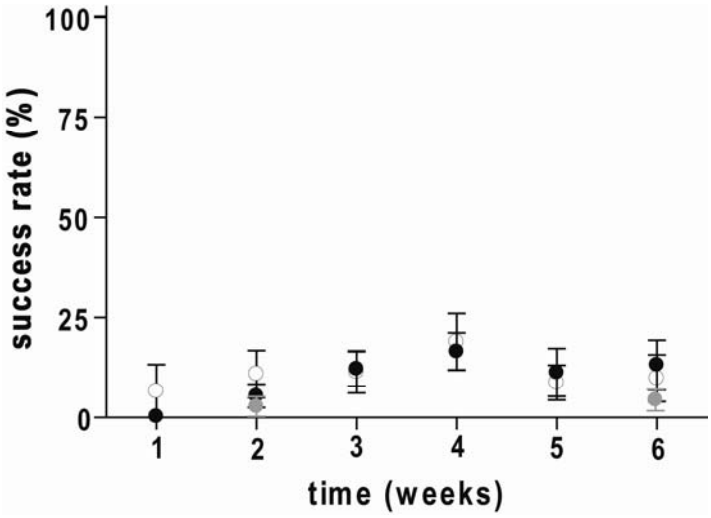


Figure 6.3. An illustration of the reaching performance of chronically injured rats that received LPS in combination with reaching training (black circles) or training alone (white circles) over the time course of 6 weeks. Untrained animals (grey circles) were tested following 2 and 6 weeks. The graph shows that trained as well as untrained rats recover their reaching ability over time.

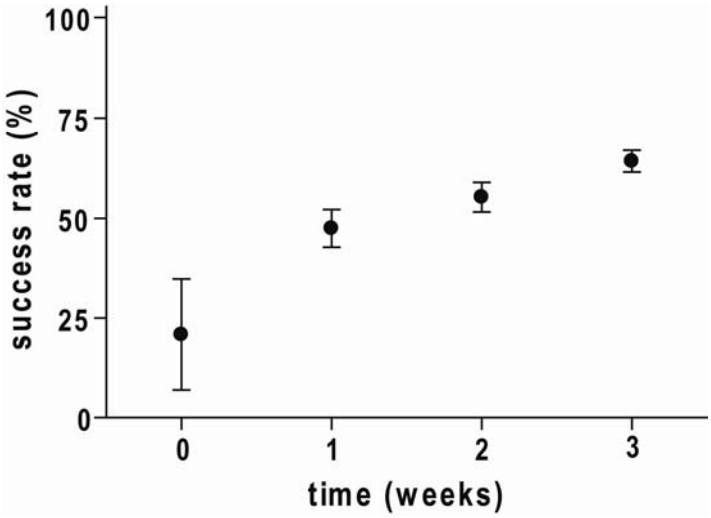


Figure 6.4. An illustration of the reaching success of rats before SCI (animals from dorsal funiculus group). Each square represents the average of 10 animals per day. Rats were familiarized with the task/ trained for about 3 weeks before SCI. The graph shows an improvement in the reaching task over the time course of 3 weeks.

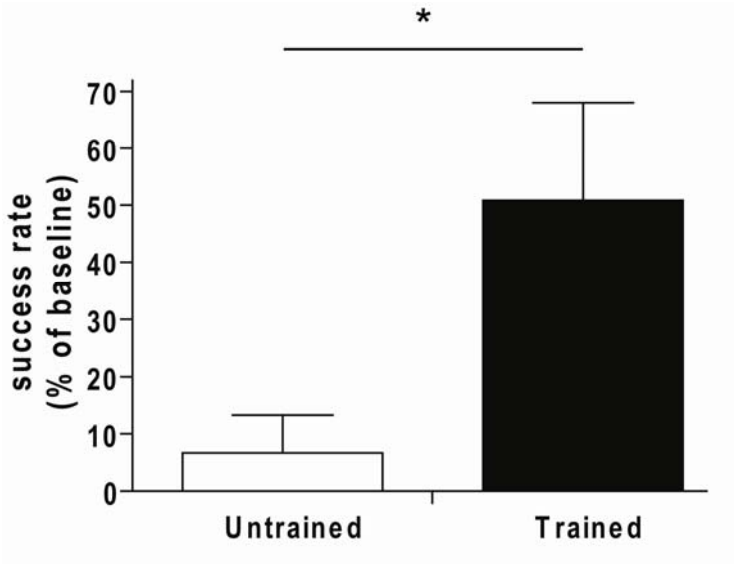


Figure 6.5. Reaching training that was initiated on day 12 post-SCI in rats still promotes recovery in reaching success. The success rate in rats is significantly ($P < 0.05$) increased in trained rats with a dorsolateral quadrant lesion that were exposed to reaching training when compared to their untrained counterparts.

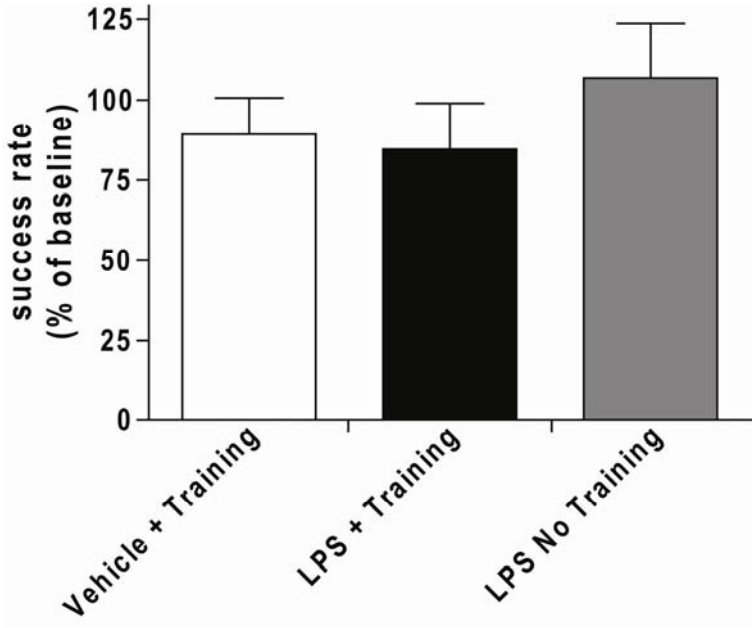


Figure 6.6. Reaching performance of rats that received either reaching training alone, LPS alone or reaching training in combination with LPS. No statistical difference was detected.

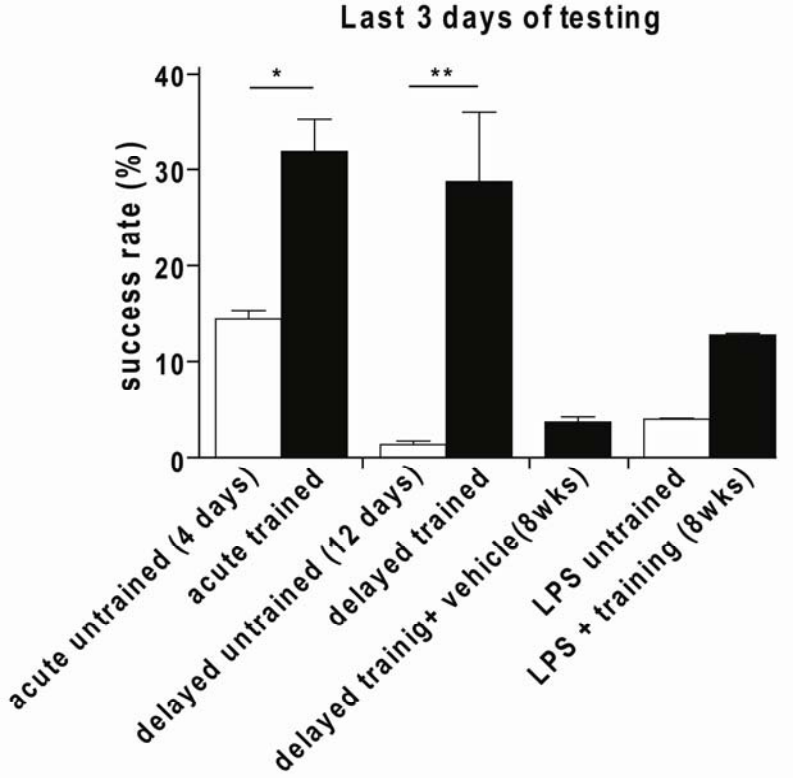
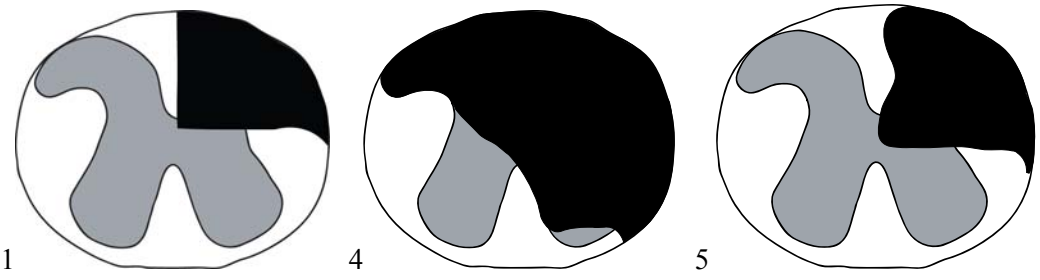


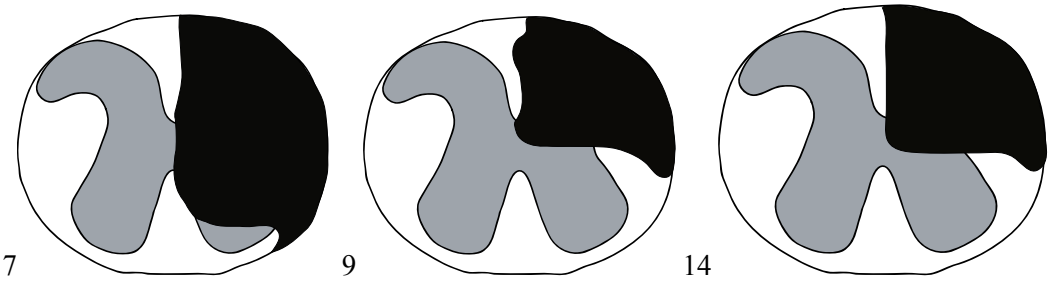
Figure 6.7. An illustration of the reaching performance of animals in different experiments. When the reaching performance was compared among groups, trained animals exhibit a better reaching performance than their untrained counterparts.

Lesion sizes:

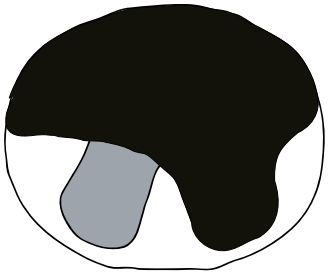
Relesion of the CST (Chapter 2):

Trained rats with DLQ-lesion



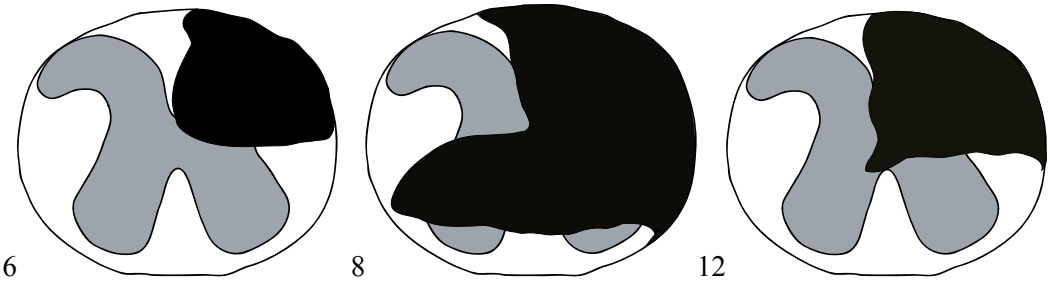


7 9 14

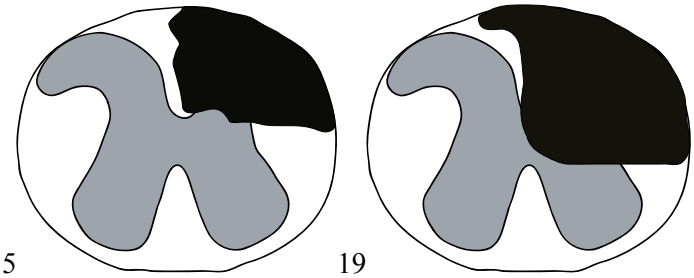


23

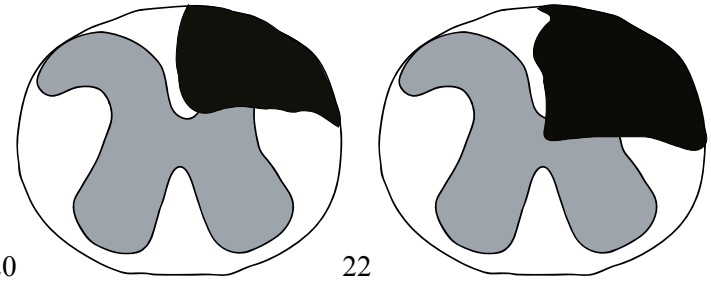
Relesion of the CST, untrained animals with DLQ-lesion (Chapter 2)



6 8 12

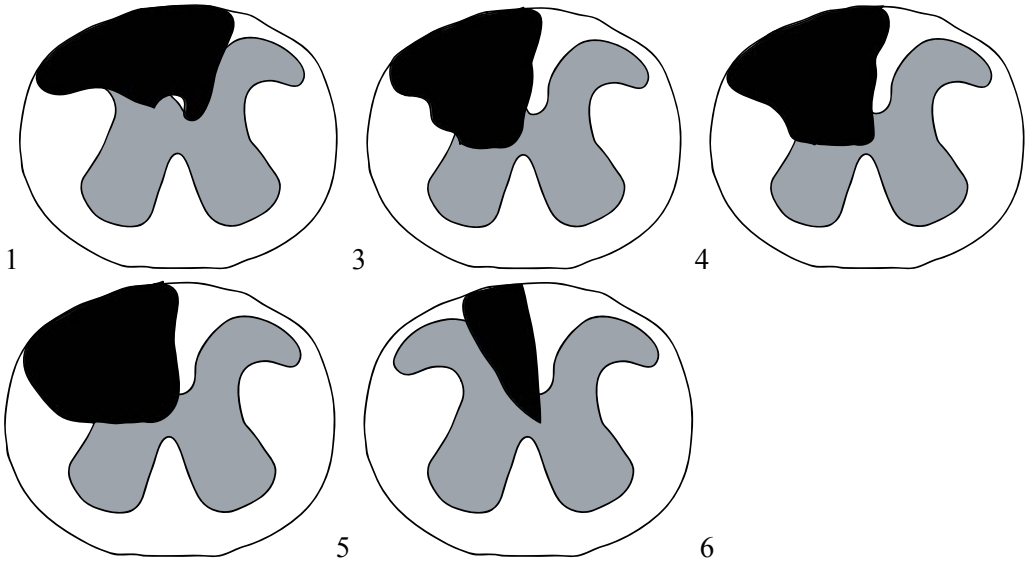


15 19

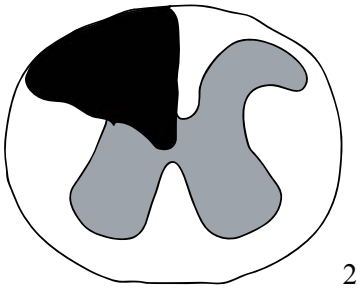


20 22

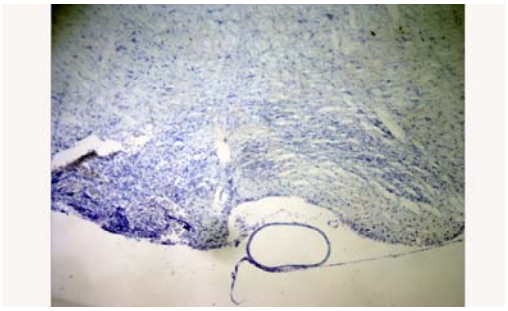
Trained rats with a dorsal funiculus lesion (Chapter 2)



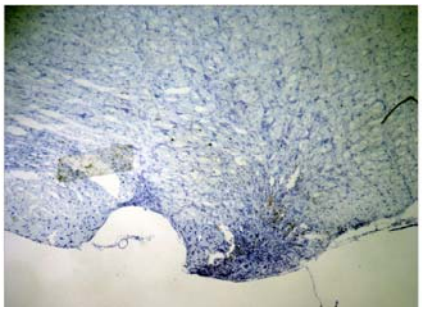
Untrained rats with dorsal funiculus lesion (Chapter 2)



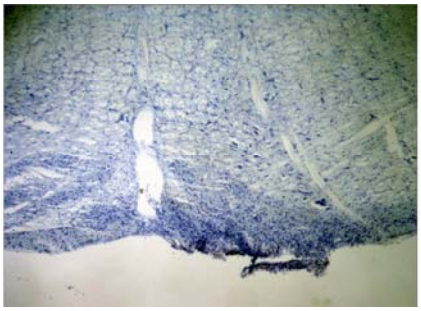
Pyramidal lesions of trained and untrained animals (Chapter 2)



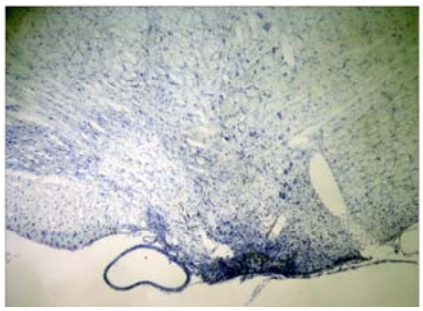
4



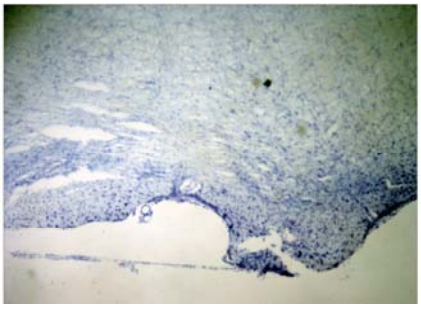
7



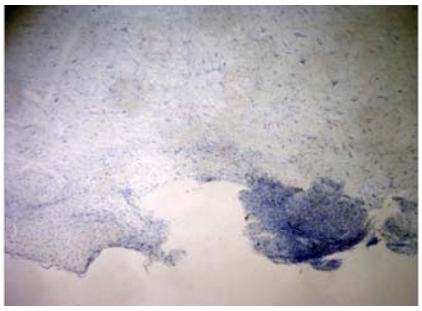
9



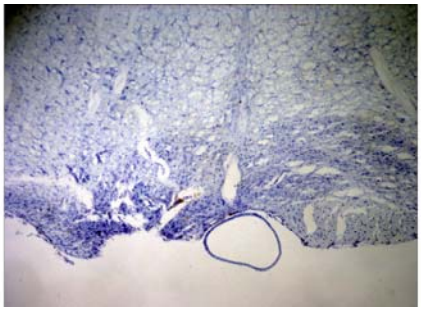
15



19

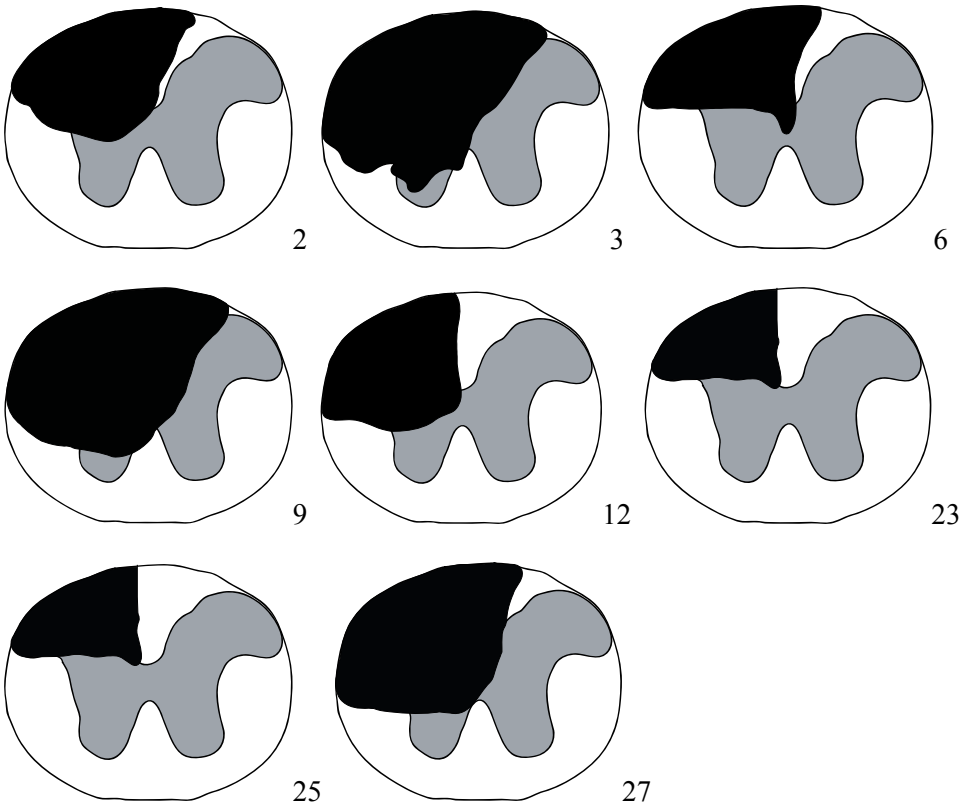


20

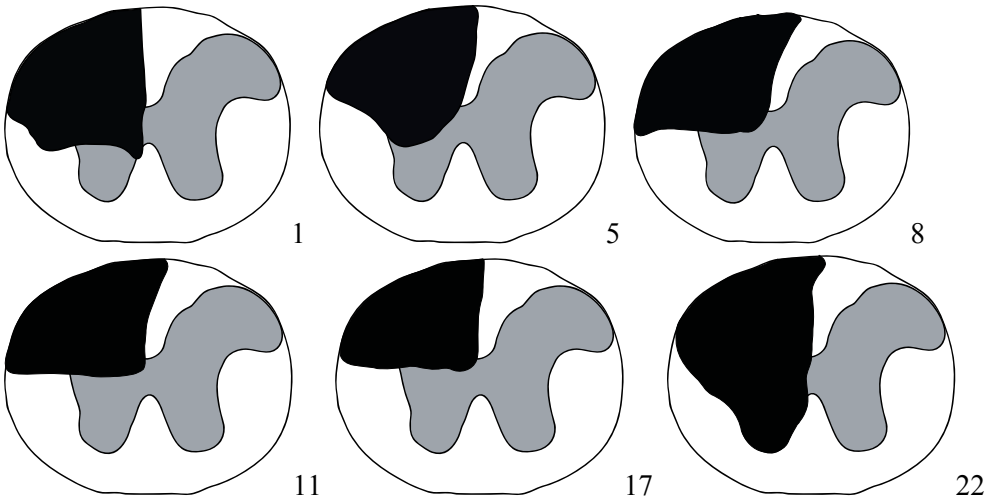


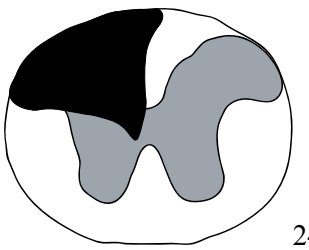
23

Delayed training study, trained rats (Chapter 3)

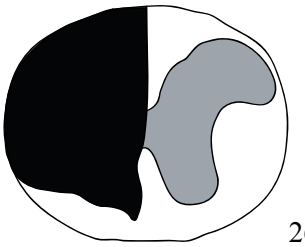


Delayed training study, untrained rats (Chapter 3)

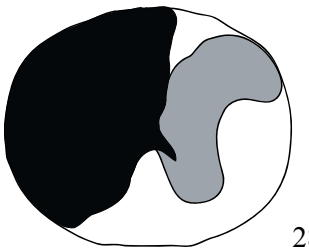




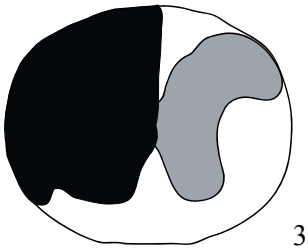
24



26

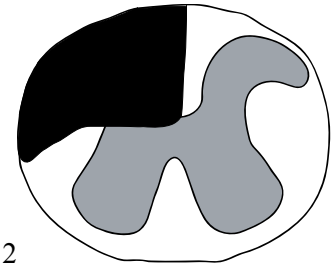


28

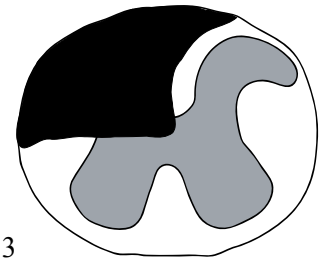


30

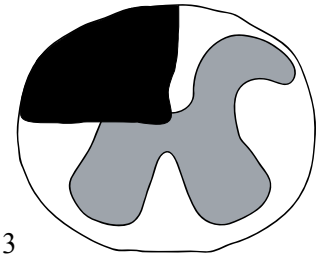
Delayed training addition, trained rats (Chapter 3)



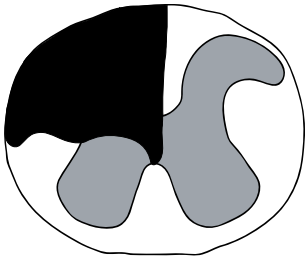
2



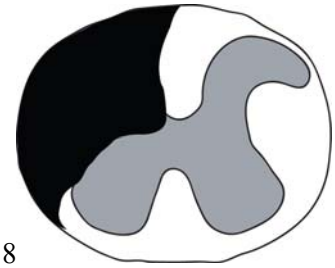
3



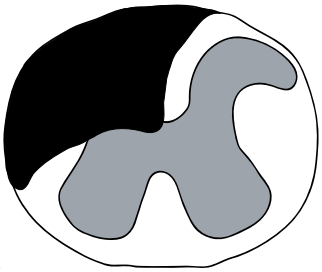
13



17

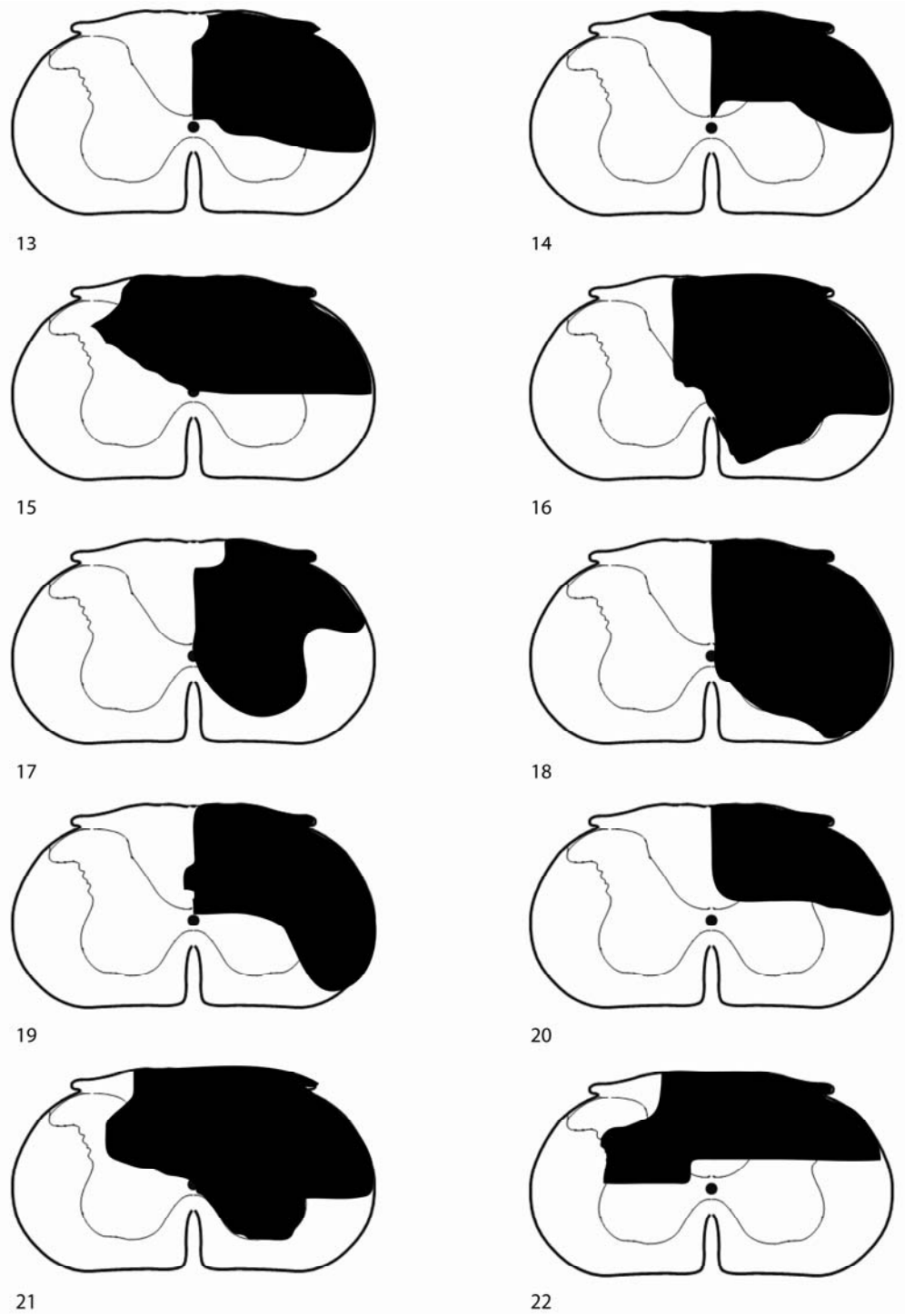


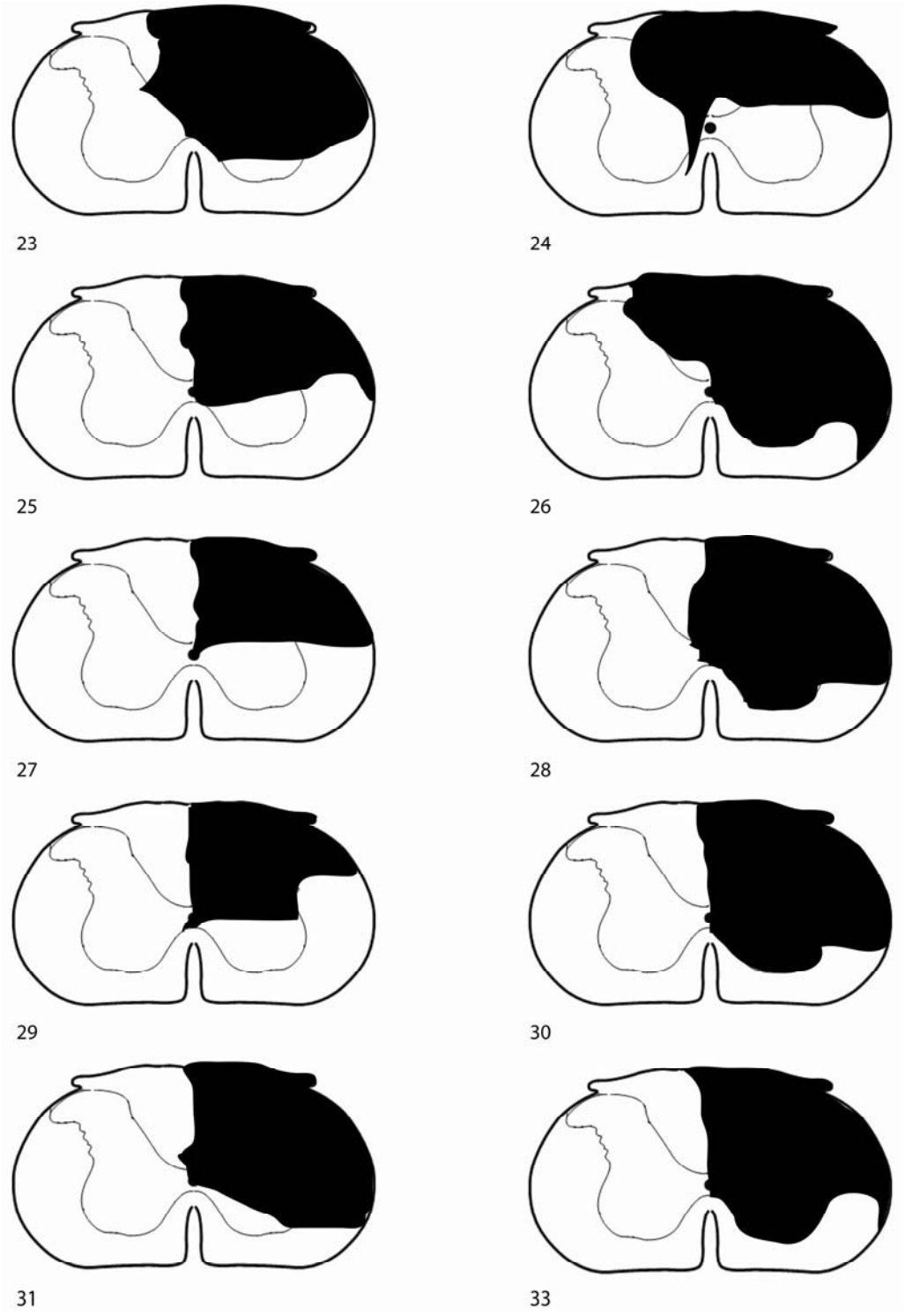
18

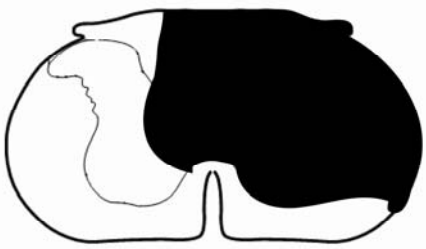


21

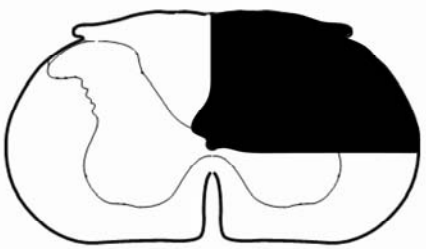
Lesion sizes LPS study (Chapter 4), trained and untrained rats (mixed)



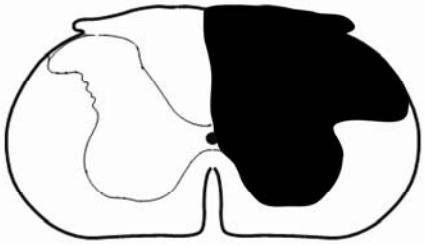

23
24
25
26
27
28
29
30
31
33



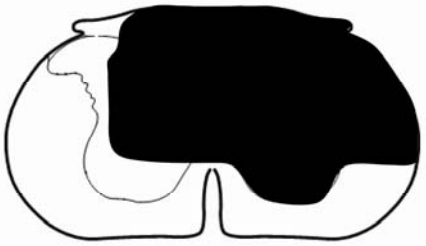
34



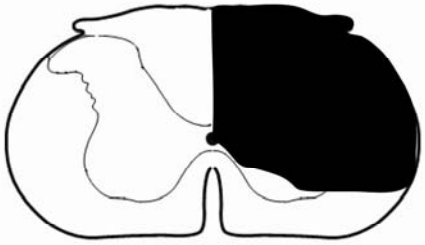
35



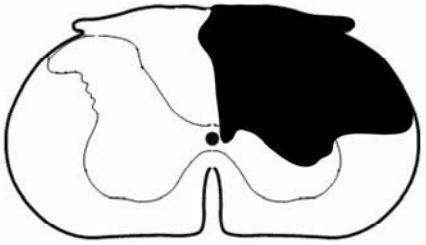
36



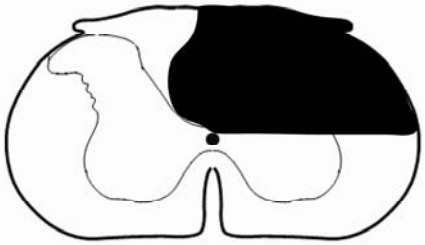
37



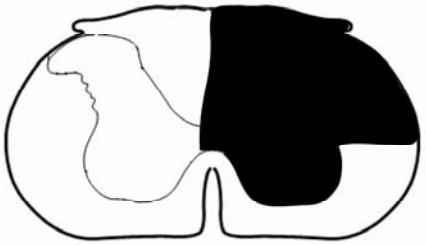
38



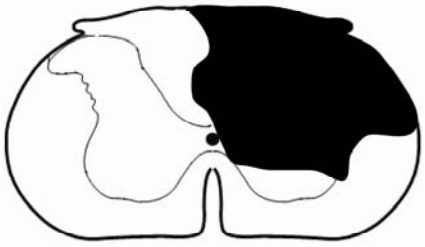
39



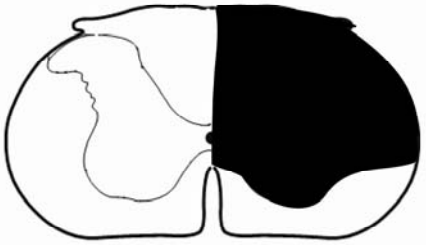
40



41



42



43