University of Alberta

MODELING INCOMPLETE CERVICAL SPINAL CORD INJURY IN RATS TO EXPLORE MECHANISMS OF REHABILITATIVE TRAINING

by

Caitlin Hurd

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

Master of Science

Centre for Neuroscience

Fall 2013

Edmonton, Alberta

ABSTRACT

Although limited functional recovery is observed following spinal cord injury (SCI), the most successful approach to promote recovery to date has been rehabilitative training. However, the effects of training are not stunning. With a thorough understanding of the intracellular mechanisms involved in training-induced recovery as well as a re-evaluation of the animal models used, it may be possible to enhance training efficacy following SCI. This thesis describes a study in which the cyclic AMP/protein kinase A intracellular signaling pathway was inhibited throughout rehabilitative reaching training in a rat model of incomplete cervical SCI to characterize its role in training-induced recovery. Additionally, a re-evaluation of a single pellet skilled reaching test is described in which an animal model of rehabilitative training and reaching recovery is analyzed. These data contribute to our understanding of rehabilitative training in animal models of SCI and may lead to greater improvements in this treatment approach.

ACKNOWLEDGEMENTS

I would like to thank the members of my supervisory committee, Dr. Declan Ali and Dr. Dave Bennett, for helpful suggestions and collaboration. Thank you to Megan Airmet for all of the administrative assistance throughout the course of my degree. I would like to sincerely thank R. Vavrek, S. Li, A. Krajacic, N. Weishaupt, A. Mason, Z. May and D. Wei for being such great colleagues and friends. Thank you for all of the laughs, conversations and assistance you have provided. I would like to thank my family and friends for their love and encouragement throughout the course of my thesis work. Thank you in particular to my parents, who have always been incredibly supportive while reminding me to “remember who you are”. Finally, I would like to thank my supervisor Dr. Karim Fouad for his endless support and encouragement. Thank you for all of the advice and reassurance and for always having an open door.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

- aCSF: artificial cerebrospinal fluid
- ANOVA: analysis of variance
- ASIA: American Spinal Injury Association
- BBB: Basso, Beattie and Bresnahan locomotor scale
- BDNF: brain-derived neurotrophic factor
- BL: baseline
- cAMP: cyclic adenosine monophosphate
- cAMPS-Rp: PKA inhibitor
- CE3F4: Epac inhibitor
- chABC: chondroitinase ABC
- CNS: central nervous system
- CPG: central pattern generator
- CREB: calcium response element binding
- CSPG: chondroitin sulfate proteoglycan
- CST: corticospinal tract
- CV: cresyl violet
- db-cAMP: cAMP analog
- DLQ: dorsolateral quadrant
- Epac: exchange protein activated by cAMP
- GAP-43: growth-associated protein 43
- GTPase: Enzyme that can bind and hydrolyze guanine triphosphate

- H89: PKA inhibitor
- IN-1: Inhibitory protein 1, (described by Caroni and Schwab 1988)
- KT 5720: PKA inhibitor
- MAG: myelin-associated glycoprotein
- NGF: nerve growth factor
- NT-3: neurotrophin-3
- Nogo-A: Neurite outgrowth inhibitor
- OMgP: oligodendrocyte myelin glycoprotein
- PDE: Phosphodiesterase
- PKA: protein kinase A
- pPKA: phosphorylated PKA
- PNS: peripheral nervous system
- RST: rubrospinal tract
- SCI: spinal cord injury
- SDS: sodium dodecyl sulfate
- SEM: standard error of the mean
- TBS: tris-buffered saline
- TBS-T: tris-buffered saline with 0.15% tween

CHAPTER I

INTRODUCTION TO SPINAL CORD INJURY

1.1 SPINAL CORD INJURY: THE FACTS AND FIGURES

Spinal cord injury (SCI) is a devastating event that can lead to permanent motor and sensory impairments below the level of injury. Injury at higher levels of the spinal cord (i.e., cervical) will result in tetraplegia, motor as well as sensory deficits in upper and lower limbs. Injuries below the cervical levels of the spinal cord may result in paraplegia, motor and sensory loss in the lower limbs and trunk (Fig 1.1). There are currently an estimated 270, 000 people living with SCI in the United States and approximately 12, 000 new cases each year (National SCI Statistical Centre, 2012). Because SCI occurs most frequently in young adults and only slightly decreases a person's life expectancy, individuals with SCI often live for quite some time with motor and sensory impairments as well as secondary health complications such as pain, spasticity (as reviewed by Hsieh et al., 2008) and pressure ulcers (Richardson and Meyer, 1981). These health effects drastically decrease a person's quality of life (Tate et al., 2002). Recovery of motor function, especially hand function, is a top priority among individuals with cervical SCI. Other factors that reduce the quality of life of those with SCI include reductions in bladder and sexual function (Anderson, 2004; Simpson et al, 2012). Finally, the effects of SCI can be costly for health care systems, with estimated lifetime costs of 1 to 4.5 million per person with SCI, depending on the severity and location of injury (National SCI Statistical Centre, 2012). Considering all these factors as well as the fact that there are currently no effective treatments to repair the injured spinal cord, investigation into potential treatments for SCI is of great importance.

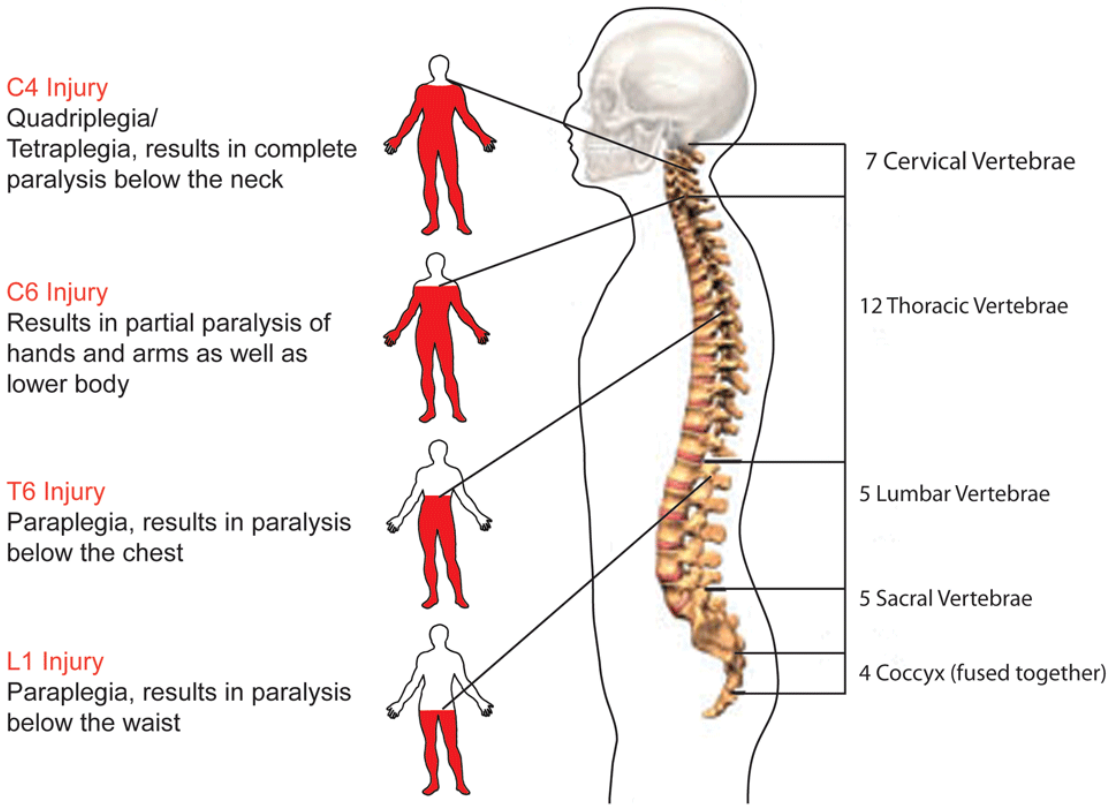

Figure 1.1

Spinal cord segments and injuries.

The degree of motor and sensory impairment following SCI depends on the extent and location of injury to the spinal cord. For example, tetraplegia occurs when there is injury to the spinal cord in the cervical area. This may cause loss of feeling and/or movement in the arms, chest, stomach area and legs. Paraplegia may occur when there is injury to the spinal cord in the thoracic, lumbar, or sacral area. This may cause loss of feeling and/or movement in the chest, stomach area, and legs. Figure adapted from Spinal Injuries Ireland (2013).

1.2 EARLY RESEARCH IN SPINAL CORD INJURY

In the early stages of neuroscience research it was unclear whether the central nervous system (CNS) is capable of regeneration. The first description of neurite growth cones was reported by Santiago Cajal (1928). Later, axons in the peripheral nervous system (PNS) were shown to successfully regrow at rates of over 3mm/day following transection (Danielsen et al., 1986), however, a similar degree of regeneration had still not been observed for axons of the CNS. It became increasingly clear that one of the differences between the CNS and PNS that contributes to this disparity in the potential for regeneration is the inhibitory environment that exists in the CNS. This was investigated in the early 1900's, when Santiago Cajal reported collapse of growth cones after only a few millimeters of growth (1928). Cajal's work was expanded upon in the 1980s when axons from the PNS were shown to be unable to regenerate onto CNS glia (Schwab and Caroni, 1988) and when CNS axons were shown to grow for approximately 30 millimeters into peripheral nerve grafts (David and Aguayo, 1981) but not within the CNS (Schwab and Thoenen, 1985). These results clearly demonstrated that a difference in the environment between the CNS and the PNS is a major determinant of the potential for axonal regeneration, however it remained unclear whether the difference was due to greater inhibition or suboptimal amounts of growth-promoting factors in the CNS.

1.3 ENVIRONMENT OF THE CNS: INHIBITORY OR LACK OF TROPHIC SUPPORT?

In his initial studies on neurite growth (1928), Santiago Cajal speculated that a lack of growth-promoting (i.e., trophic) factors was the main reason for a lack of regeneration in the CNS. One of the first of these trophic factors to be described was nerve growth factor (NGF; Cohen and Levi-Montalcini, 1954; 1956), which was found to be necessary for survival and differentiation of specific populations of neurons (Thoenen and Barde, 1980). NGF was also found to stimulate the rate of neurite outgrowth and influence the directionality of growth cone movement (Gundersen and Barret, 1980; Sofroniew et al., 2001). Although these studies demonstrated the importance of trophic factors, namely NGF, for growth and development of neurons, the degree to which they played a role in neurite growth in the CNS versus the PNS was unclear. The possible difference in inhibitory factors in the CNS versus the PNS also remained unclear. This led to an important study by Schwab and Thoenen (1985) who used an *in vitro* neuronal culture with NGF to determine the relative importance of NGF and inhibitory factors for neurite growth. They used explants of optic (from CNS) and sciatic (from PNS) nerves as "bridges" for cultured neurons and found no axons grew into the optic nerve explants whereas many axons were found in the sciatic nerve explants. This study demonstrated the extremely poor conditions for neurite outgrowth in the CNS that could not be overcome by the strong growth-promoting effects of NGF and prompted further research aimed at characterizing the nature of inhibitory factors in the CNS.

One of the major differences between the CNS and the PNS is the different types of glial cells that are present; myelin is composed of Schwann cells in the PNS but oligodendrocytes in the CNS. This fact, combined with the observation that not only living but also dead CNS fibers were avoided by growing neurons in culture (Schwab and Thoenen, 1985), led to the hypothesis that it is not a soluble inhibitory factor in the CNS, but rather a glial membrane component that is responsible for inhibiting regeneration. Studies by Martin Schwab and colleagues to test this hypothesis elegantly demonstrated that cell adhesion and axonal elongation are prevented by membrane-bound components of white matter, particularly oligodendrocytes (Caroni and Schwab; 1988a; Savio and Schwab, 1989). These myelin-associated inhibitory proteins include Nogo-A, myelin-associated glycoprotein (MAG) and oligodendrocyte myelin glycoprotein (OMgP) and have been shown to inhibit axon growth (Caroni and Schwab, 1988a; Filbin, 2003; Mukhopadhyay et al, 1994) by triggering the collapse of growth cones (Kottis et al, 2002). Further, neutralization of these myelin-associated proteins, for example by administering the monoclonal antibody for Nogo-A, IN-1 (Caroni and Schwab, 1988b), was shown to enhance axon collateral growth and regenerative sprouting (Schnell and Schwab, 1990; as reviewed by Zorner and Schwab, 2010).

In addition to myelin-associated proteins, proteins called proteoglycans further inhibit neurite outgrowth in the CNS. Chondroitin sulfate proteoglycans (CSPGs) are a major component of the extracellular matrix in the CNS and have been shown to be important for cell migration, maturation, differentiation and

survival throughout development (Oohira et al, 2000). In the grey matter of the spinal cord, the perineuronal net is comprised of proteoglycans, a very condensed matrix that surrounds cell bodies and proximal dendrites of neurons (Celio and Blumcke, 1994; Murakami and Ohtsuka, 2003). Following CNS injury, CSPGs are up-regulated by macrophages, astrocytes and oligodendrocyte progenitor cells and are inhibitory to axonal regeneration (Jones et al, 2002; Lemons et al, 1999; McKeon et al, 1995). The bacterial enzyme chondroitinase ABC (chABC) breaks down CSPGs and application of chABC following SCI has been shown to enhance neurite growth and result in functional recovery (Bradbury et al, 2002; Fouad et al, 2005; Garcia-Alias et al, 2008).

In addition to components in the CNS environment that actively inhibit growth of axons, there are also differences in inflammatory cell recruitment and function between the PNS and CNS that contribute to differences in the capacity for neurite growth. One environmental factor in the PNS that facilitates regeneration following injury is the ability of non-neuronal cells in the PNS, such as macrophages, to quickly remove debris (including the inhibitory myelin debris) from the site of injury. This clearance occurs earlier in the PNS than in the CNS (George and Griffin, 1994; Perry et al., 1987). The slow process of removing debris following injury in the CNS contributes to the proliferation of astrocytes (Bologa et al., 1985) as they invade sites of CNS injury in a process called reactive gliosis or glial scarring. The formation of this glial scar is extremely inhibitory to axonal regeneration (Davies et al., 1997; McKeon et al., 1991), particularly because astrocytes produce CSPGs. The accumulation of astrocytes,

and therefore CSPGs, following SCI results in a physical and chemical barrier to axonal growth.

From these results it is clear that the inhibitory environment in the CNS following injury plays a major role in limiting neurite growth. The application of antibodies to neutralize myelin-associated inhibitory proteins as well as the use of chABC to degrade CSPGs have yielded promising results to date. Interestingly, although these treatments were developed with the intention to promote regeneration of the injured spinal cord, these treatments function primarily via the promotion of plasticity. Plasticity is the ability of the nervous system to change or adapt and is often used to describe changes in the nervous system throughout development as well as following injury. These adaptations have been reported on a number of levels including synaptic rearrangements or alterations in synaptic strength (Lu and Chow, 1999), collateral sprouting of injured or spared axons (Fouad et al., 2001; Vavrek et al., 2006; Weidner et al., 2001) as well as changes on a larger scale such as reorganization of motor maps in the cortex (Girgis et al., 2007; Nudo et al., 1996). Administrating chABC and IN-1 following SCI, for example, was found to promote plasticity in the form of axonal sprouting in the spinal cord (Barritt et al., 2006; Bregman et al., 1995) and resulted in functional recovery. With observations of plasticity such as these, the field of SCI began to shift to include the investigation of plasticity-promoting effects rather than just the original, less effective, attempts to promote regeneration of injured axons in the spinal cord.

1.4 REDUCED CAPACITY FOR REGENERATION OF ADULT INJURED NEURONS

Another factor to take into account in efforts to repair the injured spinal cord is that injured axons in the adult CNS have a reduced intrinsic capacity for neurite outgrowth. For this reason, both the growth permissiveness of the environment as well as the natural ability of an axon to regenerate must be considered. One striking example of the reduced intrinsic capacity for regeneration among injured adult neurons involved grafting neural stem cells derived from rat as well as human embryos into the site of a complete spinal transection in rats. Lu et al., (2012) found that, despite the inhibitory environment in the injured adult spinal cord, embryonic stem cells were able to extend over very long distances. These embryonic cells formed functional relays across the site of SCI thereby facilitating functional recovery. It is clear from these results that adult neurons have a reduced capacity for regeneration yet immature embryonic CNS neurons are capable of remarkable axonal growth, even in the inhibitory environment of the adult spinal cord that includes myelin inhibitors and CSPGs.

There are a number of intrinsic cellular factors that change with development and likely influence the reduced capacity for neurite growth of adult axons after injury. These factors include regeneration-associated genes, which are up-regulated immediately following SCI but decrease over time (Di Giovanni et al., 2005; Song et al., 2001). Some of these genes code for proteins such as growth-associated protein 43 (GAP-43), microtubule-associated protein and

myelin oligodendrocyte protein. Various intracellular signaling pathways such as those involving cyclic AMP (cAMP), ERK, Akt and the Jak-STAT3 pathway are also differentially regulated throughout development and are thought to be involved in the reduced potential for regeneration and plasticity following SCI.

A number of treatment approaches have been applied to address the reduced growth capacity of adult neurons in the injured CNS. One approach is through the administration of neurotrophins, such as neurotrophin-3 (NT-3) and brain-derived neurotrophic factor (BDNF), which have been shown to promote neuronal survival, synaptic plasticity and axonal growth (Bregman et al., 1997; Hiebert et al., 2002; Namiki et al., 2000; Poo, 2001; Zhou et al., 2003). Neurotrophins, particularly BDNF, are thought to promote axonal sprouting by increasing cAMP levels (Cheng et al., 2011; Gallo et al., 2002; Gao et al., 2003; Gordon et al., 2009). The use of neurotrophins has resulted in promising results to date in terms of the promotion of plasticity following SCI. For example, local expression of NT-3 via a viral vector promoted axonal sprouting of uninjured corticospinal tract (CST) fibers, which were found to cross the midline and re-innervate the denervated side of the spinal cord (Zhou et al., 2003). Also, the administration of BDNF to the motor cortex was shown to increase collateral sprouting of the CST as well as the number of contacts made onto propriospinal interneurons rostral to a thoracic SCI (Vavrek et al., 2006). Interestingly, enhancing the production and secretion of BDNF can be achieved by increased neuronal activity (Lu et al., 2005; Udina et al., 2011). Some promising ways to enhance neuronal activity, and thereby increase the activity-dependent growth-

promoting actions of BDNF, include electrical stimulation (Fritsch et al., 2010) and rehabilitative training (Weishaupt et al., 2013, as revieved by Weishaupt et al., 2012).

Electrical stimulation of the motor cortex, for example, has been shown to increase the expression of BDNF (Fritsch et al., 2010), and promote plasticity in the form of collateral sprouting of the CST (Nitsche and Paulus, 2000). Electrical stimulation has been shown to enhance the regenerative ability the CST and result in functional recovery following SCI (Carmel et al., 2010). Similar to the mechanisms behind improved recovery with neurotrophin treatment, it has been suggested that elevating neuronal activity with electrical stimulation or rehabilitative training enhances cortical cAMP levels via BDNF signaling and by directly activating voltage- and Ca^{++} dependent- adenylyl cyclases (Gao et al., 2003; Gordon et al., 2009; Xia et al., 1991). Both electrical stimulation and rehabilitative training have been shown to promote neurite growth (Brus-Ramer et al., 2007; Udina et al., 2008). Girgis et al. (2007) also found that rehabilitative reaching training following SCI increased markers for plasticity, namely GAP-43 expression, in the cortex of rats. Similarly, exercise has been reported to restore injury-induced decreases in spinal BDNF levels caudal to injury (Ying et al., 2005). Approaches such as neurotrophin treatment, electrical stimulation and rehabilitative training all promote plasticity in the injured spinal cord in an activity-dependent manner. Promoting activity-dependent plasticity through rehabilitative training is particularly interesting because it is a safe and effective way to promote functional recovery following SCI in animal models and humans.

Training following injury may be similar to the development of the nervous system where activity strengthens connections and neuronal inactivity leads to pruning of unused connections (reviewed in Murakami et al., 1992).

Although rehabilitative training and electrical stimulation are known to enhance recovery from SCI in an activity-dependent manner, the intracellular mechanisms responsible for functional recovery with rehabilitative training remain unclear. One cellular pathway downstream of BDNF that likely plays a role in plasticity-promoting effects of rehabilitative training is the cAMP, cAMP-dependent protein kinase A (PKA) pathway. Levels of cAMP have been shown to decline with age as animals mature and this decline in cAMP has been associated with a drop in axonal growth capacity (Cai et al., 2001). Relatively low levels of cAMP have been identified as a reason for the inability of neurons in the adult CNS to regenerate within inhibitory white matter and artificially increasing cAMP enhances the growth and plasticity of neurons in the presence of myelin-associated inhibitors (Cai et al., 2001). Krajacic et al. (2009) have reported that cAMP levels in the motor cortex and the subsequent activation of PKA decline within a few days of SCI. Interestingly, the decline in cAMP level was found to correspond with the decline in post-injury rehabilitative reaching success. This is consistent with other reports (MacDonald et al., 2007) that following stroke, the application of a phosphodiesterase inhibitor, which essentially maintains elevated cAMP levels by inhibiting cAMP degradation, improved motor recovery. These results suggest an important role for the cAMP pathway in plasticity and subsequent motor recovery following CNS injury, however, a causal relationship

between the decline in cortical cAMP level and rehabilitative training effectiveness has yet to be established. This relationship will be addressed in Chapter 2 of this thesis.

1.5 REHABILITATIVE TRAINING TO PROMOTE RECOVERY FOLLOWING SCI

To date, despite a lack of a thorough understanding of the intracellular mechanisms responsible, rehabilitative training is one of the safest and most effective approaches to repair the injured spinal cord. The field of rehabilitative training following SCI gained momentum in the late 1980s following studies of the central pattern generators (CPGs) in the lumbar section of the spinal cord. A spinal CPG is a network of neurons that functions to orchestrate locomotion by producing rhythmic, patterned outputs. Early studies of spinal cord CPGs were often performed in cats, where locomotor patterns were observed in paralyzed hindlimbs following complete spinal transections (Forssberg et al., 1980a; Forssberg et al., 1980b; Grillner and Zangger, 1979). Interestingly, the locomotor ability of spinally transected cats was shown to improve with treadmill training (Barbeau and Rossignol, 1987; Lovely et al., 1986). This demonstrated that spinal CPGs could be trained and spurred the study of rehabilitative training following SCI in humans. Some of the first reports of improved functional recovery with rehabilitative training in humans were demonstrated with the use of treadmill training. Humans with injuries at the thoracic level of the spinal cord were shown

to produce coordinated stepping movements following weight-supported treadmill training (Dietz et al., 1994; Wernig and Muller, 1992). These studies also demonstrated that, in addition to producing stepping movements on a treadmill, rehabilitative treadmill training also promoted walking ability on a stationary surface.

The early studies of rehabilitative training were performed following thoracic SCI, however, over time there has been an increase in the frequency of cervical SCIs in humans (National Spinal Cord Injury Statistical Centre; 2012). This increase necessitated a more thorough study of the effects of training following cervical injuries. The use of cervical injury models was further propagated by studies that suggested that reaching, unlike rhythmic movements such as locomotion, is volitional not only orchestrated by spinal networks. The mounting evidence that hand/paw function involves CST function (Lawrence and Kuypers, 1968; Schwartzman, 1978), a descending motor tract with clinical relevance and a convenient anatomical location for analysis, further encouraged the study of cervical lesions. Finally, the use of cervical models is favourable for rehabilitative training studies because unlike thoracic lesions after which animals self-train in their cage by walking, the voluntary, in-cage use of injured forepaws can be reduced after injury. All of these factors contributed to the expansion of animal models to study rehabilitative training from a focus on thoracic SCI to include lesions at the cervical level.

1.6 BEHAVIOURAL TESTS FOR CERVICAL SCI

A number of behavioural tests have been developed for rehabilitative training of the forelimb in animal models of cervical SCI. Rats are often used in these studies because their experimental use is ethically less controversial than larger animals such as primates. For example rats can be trained to reach for food or sugar pellets, they use a movement sequence of reaching that is similar to that of humans (Whishaw et al., 1993) and the factors that inhibit neurite growth in the injured adult spinal cord in rats are quite similar to those of humans (Josephson et al., 2001). Some common tests for forelimb motor control include tray reaching (Whishaw et al., 1986), the Montoya staircase task (Montoya et al., 1991) and single pellet skilled reaching (Whishaw et al., 1993; Whishaw and Pellis, 1990). These behavioural tests have enabled the evaluation of potential treatments following cervical SCI by providing a way to quantify functional recovery. One important factor to consider when testing SCI with these behavioural measures is the combination of the model of injury and the behavioural test that is used. The combination of these factors is important to optimize the potential to observe treatment effects. An injury should induce significant functional deficits, however, it is important that lesions are not so severe that no recovery can be observed. It is essential that appropriate functional tests to cover deficits of different severities be paired with appropriate lesion models.

Rehabilitative training studies using various behavioural tests have demonstrated many (primarily beneficial) functional and anatomical effects of training following SCI. Given the effectiveness of rehabilitative training to date, it

is interesting that the mechanisms of recovery are still not well understood. Although rehabilitative training appears to function through the activation of cAMP, perhaps via BDNF (Ying et al., 2005; Ying et al., 2008), a causal mechanistic relationship has not yet been established. Establishing this relationship will be important in order to enhance rehabilitative training approaches in the future, particularly at chronic time points when training efficacy is reduced.

1.7 CONCLUDING REMARKS

SCI is a devastating event and, although progress has been made in terms of understanding the pathological processes following injury, many questions still remain unanswered in terms of potential treatments. This thesis is comprised of two projects that were performed to enhance our understanding of rehabilitative training as a treatment for incomplete cervical spinal cord injury. The first project, as described in Chapter 2, was aimed at understanding mechanisms by which rehabilitative training improves motor recovery, and focuses on the cAMP/PKA signaling pathway. The second project, described in Chapter 3, was performed to evaluate the lesion extent and location that is most appropriate to use with single pellet skilled reaching tests for the rat. The importance of appropriate models of SCI to test potential treatments is discussed. The implications of the results presented in Chapters 2 and 3 as well as future directions and conclusions are discussed in detail in Chapter 4.

1.8 REFERENCES

Anderson, K. D., 2004. Targeting recovery: priorities of the spinal cord-injured population. Journal of neurotrauma 21**,** 1371-1383.

Barbeau, H., and Rossignol, S., 1987. Recovery of locomotion after chronic spinalization in the adult cat. Brain research 412**,** 84-95.

Barritt, A. W., Davies, M., Marchand, F., Hartley, R., Grist, J., Yip, P., McMahon, S. B., and Bradbury, E. J., 2006. Chondroitinase ABC promotes sprouting of intact and injured spinal systems after spinal cord injury. The Journal of neuroscience : the official journal of the Society for Neuroscience 26**,** 10856-10867.

Bologa, L., Deugnier, M. A., Joubert, R., and Bisconte, J. C., 1985. Myelin basic protein stimulates the proliferation of astrocytes: possible explanation for multiple sclerosis plaque formation. Brain research 346**,** 199-203.

Bradbury, E. J., Moon, L. D., Popat, R. J., King, V. R., Bennett, G. S., Patel, P. N., Fawcett, J. W., and McMahon, S. B., 2002. Chondroitinase ABC promotes functional recovery after spinal cord injury. Nature 416**,** 636-640.

Bregman, B. S., Kunkel-Bagden, E., Schnell, L., Dai, H. N., Gao, D., and Schwab, M. E., 1995. Recovery from spinal cord injury mediated by antibodies to neurite growth inhibitors. Nature 378**,** 498-501.

Bregman, B. S., McAtee, M., Dai, H. N., and Kuhn, P. L., 1997. Neurotrophic factors increase axonal growth after spinal cord injury and transplantation in the adult rat. Experimental neurology 148**,** 475-494.

Brus-Ramer, M., Carmel, J. B., Chakrabarty, S., and Martin, J. H., 2007. Electrical stimulation of spared corticospinal axons augments connections with ipsilateral spinal motor circuits after injury. The Journal of neuroscience : the official journal of the Society for Neuroscience 27**,** 13793-13801.

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. The Journal of neuroscience : the official journal of the Society for Neuroscience 21**,** 4731-4739.

Cajal, S. R., 1928. Degeneration & regeneration of the nervous system (Vol. 1). Oxford University Press, Humphrey Milford.

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. The Journal of neuroscience : the official journal of the Society for Neuroscience 30**,** 10918-10926.

Caroni, P., and Schwab, M. E., 1988a. Two membrane protein fractions from rat central myelin with inhibitory properties for neurite growth and fibroblast spreading. The Journal of cell biology 106**,** 1281-1288.

Caroni, P., and Schwab, M. E., 1988b. Antibody against myelin-associated inhibitor of neurite growth neutralizes nonpermissive substrate properties of CNS white matter. Neuron 1**,** 85-96.

Celio, M. R., and Blumcke, I., 1994. Perineuronal nets--a specialized form of extracellular matrix in the adult nervous system. Brain research. Brain research reviews 19**,** 128-145.

Cheng, P. L., Song, A. H., Wong, Y. H., Wang, S., Zhang, X., and Poo, M. M., 2011. Self-amplifying autocrine actions of BDNF in axon development. Proceedings of the National Academy of Sciences of the United States of America 108**,** 18430-18435.

Cohen, S., and Levi-Montalcini, R., 1956. A Nerve Growth-Stimulating Factor Isolated from Snake Venom. Proceedings of the National Academy of Sciences of the United States of America 42**,** 571-574.

Cohen, S., Levi-Montalcini, R., and Hamburger, V., 1954. A Nerve Growth-Stimulating Factor Isolated from Sarcom as 37 and 180. Proceedings of the National Academy of Sciences of the United States of America 40**,** 1014-1018.

Danielsen, N., Lundborg, G., and Frizell, M., 1986. Nerve repair and axonal transport: outgrowth delay and regeneration rate after transection and repair of rabbit hypoglossal nerve. Brain research 376**,** 125-132.

David, S., and Aguayo, A. J., 1981. Axonal elongation into peripheral nervous system "bridges" after central nervous system injury in adult rats. Science 214**,** 931-933.

Davies, S. J., Fitch, M. T., Memberg, S. P., Hall, A. K., Raisman, G., and Silver, J., 1997. Regeneration of adult axons in white matter tracts of the central nervous system. Nature 390**,** 680-683.

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 journal : official publication of the Federation of American Societies for Experimental Biology 19**,** 153-154.

Dietz, V., Colombo, G., and Jensen, L., 1994. Locomotor activity in spinal man. Lancet 344**,** 1260-1263.

Filbin, M. T., 2003. Myelin-associated inhibitors of axonal regeneration in the adult mammalian CNS. Nature reviews. Neuroscience 4**,** 703-713.

Forssberg, H., Grillner, S., and Halbertsma, J., 1980a. The locomotion of the low spinal cat. I. Coordination within a hindlimb. Acta physiologica Scandinavica 108**,** 269-281.

Forssberg, H., Grillner, S., Halbertsma, J., and Rossignol, S., 1980b. The locomotion of the low spinal cat. II. Interlimb coordination. Acta physiologica Scandinavica 108**,** 283-295.

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. Current biology : CB 11**,** 1766-1770.

Fouad, K., Schnell, L., Bunge, M. B., Schwab, M. E., Liebscher, T., and Pearse, D. D., 2005. Combining Schwann cell bridges and olfactory-ensheathing glia grafts with chondroitinase promotes locomotor recovery after complete transection of the spinal cord. The Journal of neuroscience : the official journal of the Society for Neuroscience 25**,** 1169-1178.

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**,** 198-204.

Gallo, G., Ernst, A. F., McLoon, S. C., and Letourneau, P. C., 2002. Transient PKA activity is required for initiation but not maintenance of BDNF-mediated protection from nitric oxide-induced growth-cone collapse. The Journal of neuroscience : the official journal of the Society for Neuroscience 22**,** 5016-5023.

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. The Journal of neuroscience : the official journal of the Society for Neuroscience 23**,** 11770-11777.

Garcia-Alias, G., Lin, R., Akrimi, S. F., Story, D., Bradbury, E. J., and Fawcett, J. W., 2008. Therapeutic time window for the application of chondroitinase ABC after spinal cord injury. Experimental neurology 210**,** 331-338.

George, R., and Griffin, J. W., 1994. Delayed macrophage responses and myelin clearance during Wallerian degeneration in the central nervous system: the dorsal radiculotomy model. Experimental neurology 129**,** 225-236.

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 : a journal of neurology 130**,** 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**,** 412-441.

Grillner, S., and Zangger, P., 1979. On the central generation of locomotion in the low spinal cat. Experimental brain research. Experimentelle Hirnforschung. Experimentation cerebrale 34**,** 241-261.

Gundersen, R. W., and Barrett, J. N., 1980. Characterization of the turning response of dorsal root neurites toward nerve growth factor. The Journal of cell biology 87**,** 546-554.

Hiebert, G. W., Khodarahmi, K., McGraw, J., Steeves, J. D., and Tetzlaff, W., 2002. Brain-derived neurotrophic factor applied to the motor cortex promotes sprouting of corticospinal fibers but not regeneration into a peripheral nerve transplant. Journal of neuroscience research 69**,** 160-168.

Hsieh, J. T., Wolfe, D. L., Miller, W. C., and Curt, A., 2008. Spasticity outcome measures in spinal cord injury: psychometric properties and clinical utility. Spinal cord 46**,** 86-95.

Jones, L. L., Yamaguchi, Y., Stallcup, W. B., and Tuszynski, M. H., 2002. NG2 is a major chondroitin sulfate proteoglycan produced after spinal cord injury and is expressed by macrophages and oligodendrocyte progenitors. The Journal of neuroscience : the official journal of the Society for Neuroscience 22**,** 2792-2803.

Josephson, A., Widenfalk, J., Widmer, H. W., Olson, L., and Spenger, C., 2001. NOGO mRNA expression in adult and fetal human and rat nervous tissue and in weight drop injury. Experimental neurology 169**,** 319-328.

Kottis, V., Thibault, P., Mikol, D., Xiao, Z. C., Zhang, R., Dergham, P., and Braun, P. E., 2002. Oligodendrocyte-myelin glycoprotein (OMgp) is an inhibitor of neurite outgrowth. Journal of neurochemistry 82**,** 1566-1569.

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. The European journal of neuroscience 29**,** 641-651.

Lawrence, D. G., and Kuypers, H. G., 1968. The functional organization of the motor system in the monkey. I. The effects of bilateral pyramidal lesions. Brain : a journal of neurology 91**,** 1-14.

Lemons, M. L., Howland, D. R., and Anderson, D. K., 1999. Chondroitin sulfate proteoglycan immunoreactivity increases following spinal cord injury and transplantation. Experimental neurology 160**,** 51-65.

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. Experimental neurology 92**,** 421-435.

Lu, B., and Chow, A., 1999. Neurotrophins and hippocampal synaptic transmission and plasticity. Journal of neuroscience research 58**,** 76-87.

Lu, B., Pang, P. T., and Woo, N. H., 2005. The yin and yang of neurotrophin action. Nature reviews. Neuroscience 6**,** 603-614.

Lu, P., Wang, Y., Graham, L., McHale, K., Gao, M., Wu, D., Brock, J., Blesch, A., Rosenzweig, E. S., Havton, L. A., Zheng, B., Conner, J. M., Marsala, M., and Tuszynski, M. H., 2012. Long-distance growth and connectivity of neural stem cells after severe spinal cord injury. Cell 150**,** 1264-1273.

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. Neurorehabilitation and neural repair 21**,** 486-496.

McKeon, R. J., Hoke, A., and Silver, J., 1995. Injury-induced proteoglycans inhibit the potential for laminin-mediated axon growth on astrocytic scars. Experimental neurology 136**,** 32-43.

McKeon, R. J., Schreiber, R. C., Rudge, J. S., and Silver, J., 1991. Reduction of neurite outgrowth in a model of glial scarring following CNS injury is correlated with the expression of inhibitory molecules on reactive astrocytes. The Journal of neuroscience : the official journal of the Society for Neuroscience 11**,** 3398-3411.

Montoya, C. P., Campbell-Hope, L. J., Pemberton, K. D., and Dunnett, S. B., 1991. The "staircase test": a measure of independent forelimb reaching and grasping abilities in rats. Journal of neuroscience methods 36**,** 219-228.

Mukhopadhyay, G., Doherty, P., Walsh, F. S., Crocker, P. R., and Filbin, M. T., 1994. A novel role for myelin-associated glycoprotein as an inhibitor of axonal regeneration. Neuron 13**,** 757-767.

Murakami, F., Song, W. J., and Katsumaru, H., 1992. Plasticity of neuronal connections in developing brains of mammals. Neuroscience research 15**,** 235-253.

Murakami, T., and Ohtsuka, A., 2003. Perisynaptic barrier of proteoglycans in the mature brain and spinal cord. Archives of histology and cytology 66**,** 195-207.

Namiki, J., Kojima, A., and Tator, C. H., 2000. Effect of brain-derived neurotrophic factor, nerve growth factor, and neurotrophin-3 on functional recovery and regeneration after spinal cord injury in adult rats. Journal of neurotrauma 17**,** 1219-1231.

National Spinal Cord Injury Statistical Centre, 2012. Facts and Figures at a Glance. https://www.nscisc.uab.edu/reports.aspx (November 2012, date last accessed).

Nitsche, M. A., and Paulus, W., 2000. Excitability changes induced in the human motor cortex by weak transcranial direct current stimulation. The Journal of physiology 527 Pt 3**,** 633-639.

Nudo, R. J., Wise, B. M., SiFuentes, F., and Milliken, G. W., 1996. Neural substrates for the effects of rehabilitative training on motor recovery after ischemic infarct. Science 272**,** 1791-1794.

Oohira, A., Matsui, F., Tokita, Y., Yamauchi, S., and Aono, S., 2000. Molecular interactions of neural chondroitin sulfate proteoglycans in the brain development. Archives of biochemistry and biophysics 374**,** 24-34.

Perry, V. H., Brown, M. C., and Gordon, S., 1987. The macrophage response to central and peripheral nerve injury. A possible role for macrophages in regeneration. The Journal of experimental medicine 165**,** 1218-1223.

Poo, M. M., 2001. Neurotrophins as synaptic modulators. Nature reviews. Neuroscience 2**,** 24-32.

Richardson, R. R., and Meyer, P. R., Jr., 1981. Prevalence and incidence of pressure sores in acute spinal cord injuries. Paraplegia 19**,** 235-247.

Savio, T., and Schwab, M. E., 1989. Rat CNS white matter, but not gray matter, is nonpermissive for neuronal cell adhesion and fiber outgrowth. The Journal of neuroscience : the official journal of the Society for Neuroscience 9**,** 1126-1133.

Schnell, L., and Schwab, M. E., 1990. Axonal regeneration in the rat spinal cord produced by an antibody against myelin-associated neurite growth inhibitors. Nature 343**,** 269-272.

Schwab, M. E., and Caroni, P., 1988. Oligodendrocytes and CNS myelin are nonpermissive substrates for neurite growth and fibroblast spreading in vitro. The Journal of neuroscience : the official journal of the Society for Neuroscience 8**,** 2381-2393.

Schwab, M. E., and Thoenen, H., 1985. Dissociated neurons regenerate into sciatic but not optic nerve explants in culture irrespective of neurotrophic factors. The Journal of neuroscience : the official journal of the Society for Neuroscience 5**,** 2415-2423.

Schwartzman, R. J., 1978. A behavioral analysis of complete unilateral section of the pyramidal tract at the medullary level in Macaca mulatta. Annals of neurology 4**,** 234-244.

Simpson, L. A., Eng, J. J., Hsieh, J. T., and Wolfe, D. L., 2012. The health and life priorities of individuals with spinal cord injury: a systematic review. Journal of neurotrauma 29**,** 1548-1555.

Sofroniew, M. V., Howe, C. L., and Mobley, W. C., 2001. Nerve growth factor signaling, neuroprotection, and neural repair. Annual review of neuroscience 24**,** 1217-1281.

Song, G., Cechvala, C., Resnick, D. K., Dempsey, R. J., and Rao, V. L., 2001. GeneChip analysis after acute spinal cord injury in rat. Journal of neurochemistry 79**,** 804-815.

Spinal Cord Injuries Ireland, 2013. What is Spinal Cord Injury? https://www.spinalinjuries.ie (May 2013, date last accessed).

Tate, D. G., Kalpakjian, C. Z., and Forchheimer, M. B., 2002. Quality of life issues in individuals with spinal cord injury. Archives of physical medicine and rehabilitation 83**,** S18-25.

Thoenen, H., and Barde, Y. A., 1980. Physiology of nerve growth factor. Physiological reviews 60**,** 1284-1335.

Udina, E., Cobianchi, S., Allodi, I., and Navarro, X., 2011. Effects of activity-dependent strategies on regeneration and plasticity after peripheral nerve injuries. Annals of anatomy = Anatomischer Anzeiger : official organ of the Anatomische Gesellschaft 193**,** 347-353.

Udina, E., Furey, M., Busch, S., Silver, J., Gordon, T., and Fouad, K., 2008. Electrical stimulation of intact peripheral sensory axons in rats promotes outgrowth of their central projections. Experimental neurology 210**,** 238-247.

Vavrek, R., Girgis, J., Tetzlaff, W., Hiebert, G. W., and Fouad, K., 2006. BDNF promotes connections of corticospinal neurons onto spared descending interneurons in spinal cord injured rats. Brain : a journal of neurology 129**,** 1534-1545.

Weidner, N., Ner, A., Salimi, N., and Tuszynski, M. H., 2001. Spontaneous corticospinal axonal plasticity and functional recovery after adult central

nervous system injury. Proceedings of the National Academy of Sciences of the United States of America 98**,** 3513-3518.

Weishaupt, N., Blesch, A., and Fouad, K., 2012. BDNF: the career of a multifaceted neurotrophin in spinal cord injury. Experimental neurology 238**,** 254-264.

Weishaupt, N., Li, S., Di Pardo, A., Sipione, S., and Fouad, K., 2013. Synergistic effects of BDNF and rehabilitative training on recovery after cervical spinal cord injury. Behavioural brain research 239**,** 31-42.

Wernig, A., and Muller, S., 1992. Laufband locomotion with body weight support improved walking in persons with severe spinal cord injuries. Paraplegia 30**,** 229-238.

Whishaw, I. Q., O'Connor, W. T., and Dunnett, S. B., 1986. The contributions of motor cortex, nigrostriatal dopamine and caudate-putamen to skilled forelimb use in the rat. Brain : a journal of neurology 109 (Pt 5)**,** 805-843.

Whishaw, I. Q., and Pellis, S. M., 1990. The structure of skilled forelimb reaching in the rat: a proximally driven movement with a single distal rotatory component. Behavioural brain research 41**,** 49-59.

Whishaw, I. Q., Pellis, S. M., Gorny, B., Kolb, B., and Tetzlaff, W., 1993. Proximal and distal impairments in rat forelimb use in reaching follow unilateral pyramidal tract lesions. Behavioural brain research 56**,** 59-76.

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**,** 431-443.

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. Experimental neurology 193**,** 411-419.

Ying, Z., Roy, R. R., Zhong, H., Zdunowski, S., Edgerton, V. R., 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., Baumgartner, B. J., Hill-Felberg, S. J., McGowen, L. R., and Shine, H. D., 2003. Neurotrophin-3 expressed in situ induces axonal plasticity in the adult injured spinal cord. The Journal of neuroscience : the official journal of the Society for Neuroscience 23**,** 1424-1431.

Zorner, B., and Schwab, M. E., 2010. Anti-Nogo on the go: from animal models to a clinical trial. Annals of the New York Academy of Sciences 1198 Suppl 1**,** E22-34.

CHAPTER II

INHIBITION OF PKA TO INTERFERE WITH REHABILITATIVE TRAINING-INDUCED REACHING RECOVERY FOLLOWING SCI

2.1 INTRODUCTION

Only moderate recovery is observed following complete SCI because injured axons of the adult mammalian CNS are unable to regenerate. Although there are no effective treatment approaches to completely repair the injured spinal cord to date, the use of rehabilitative training has been shown to improve functional recovery. Rehabilitative training likely facilitates motor recovery by enhancing plasticity, the ability of the nervous system to adapt. Plasticity in the central nervous system has been observed on a number of scales, from small scale, synaptic plasticity including changes in density or trafficking of receptors (Opazo et al., 2010), to intermediate and larger scales, which include neurite outgrowth and reorganization of motor maps of limb or digit representation in the cortex, respectively. The use of rehabilitative training following SCI has been shown to enhance plasticity on each of these scales, most notably through intermediate and large-scale plasticity. In terms of neurite outgrowth, collateral sprouting of the CST (a tract necessary for fine motor control of the digits; Schwartzman, 1978), for example, has been related to functional recovery following SCI (Fouad et al., 2001). This likely occurs through the formation of a relay onto interneurons that bridge an incomplete injury site through connections to neurons below the injury (Bareyre et al., 2004; Vavrek et al., 2006). Following incomplete SCI, rehabilitative training has been shown to enhance plasticity in the form of sprouting of CST fibers, which corresponded to improved functional recovery (Girgis et al., 2007). Training-induced plasticity on a larger scale following stroke

and SCI has been shown to involve enhanced representation of trained limbs or digits in the motor cortex of animals (Girgis et al., 2007; Nudo et al., 1996).

Rehabilitative training following SCI likely promotes plastic changes such as collateral sprouting and reorganization of motor maps through activity-dependent mechanisms. For example, training has been shown to enhance activity-dependent intracellular signaling cascades that are associated with plasticity and the expression of growth-promoting factors including growth-associated protein 43 (GAP-43; Girgis et al, 2007) and brain-derived neurotrophic factor (BDNF; Gomez-Pinilla et al, 2002). Although it is clear that rehabilitative training increases these growth-promoting factors, the intracellular signaling cascades responsible for plasticity and functional recovery with rehabilitative training have yet to be clearly established. In order to optimize and enhance rehabilitative training following SCI, it will be important to understand the mechanisms behind training-induced functional recovery.

One mechanism by which rehabilitative training likely promotes recovery following SCI is through signaling cascades involving the secondary messengers cyclic AMP (cAMP) and cAMP-dependent protein kinase A (PKA). cAMP has been known to play a role in neuronal development, growth and survival (Cui and So, 2004; Merz et al., 2011) as well as synaptic plasticity and learning through activity-dependent mechanisms (Ferguson & Storm, 2004). Low levels of cAMP in adult CNS neurons have been identified as a reason for the inability of neurons to regenerate because artificially increasing cAMP was shown to promote neurite outgrowth on a substrate that is normally inhibitory to growth (Cai et al., 1999;

2001). Interestingly, cortical cAMP levels, and subsequent activation of PKA, decrease following SCI (Krajacic et al., 2009; Pearse et al., 2004) and this decrease parallels the observed decrease in training efficacy over time following SCI (Norrie et al., 2005; Winchester et al., 2009). More recent evidence for the potential involvement of cAMP in promoting training-induced plasticity following CNS injury came from MacDonald et al., (2007). They reported that administration of a phosphodiesterase 4 inhibitor, to prevent degradation of cAMP, enhanced rehabilitation-dependent motor recovery and cortical reorganization following a focal ischemic infarct. This study implicates cAMP in motor recovery following CNS injury and warrants further investigation, particularly as cAMP levels pertain to recovery from other CNS injuries such as SCI. The contribution of various downstream effectors of cAMP that may be responsible for the observed motor recovery, such as PKA or the exchange protein activated by cAMP (Epac; Murray et al., 2009) also warrant further investigation.

Although a causal link has not yet been established, cAMP/PKA signaling is a promising avenue to study the mechanisms behind the observed beneficial effects of rehabilitative training following SCI. In order to determine if cortical cAMP signaling and subsequent PKA activation is a critical component of training-induced recovery following incomplete, cervical SCI, we inhibited PKA to interfere with the efficacy of motor training. We hypothesized that administering cAMPS-Rp, a cell-permeable, competitive inhibitor of PKA, would inhibit the training-induced functional recovery following SCI, thereby

demonstrating the necessity for active, phosphorylated PKA (pPKA) for motor recovery with a plasticity-promoting treatment such as training.

2.2 MATERIALS AND METHODS

2.2.1 Animals and Experimental Groups

All experimental procedures were approved by the University of Alberta Animal Care and Use Committee and complied with the guidelines of the Canadian Council for Animal Care. A total of 23 female Lewis rats (Charles River Laboratories, Canada) weighing 180 g – 200 g were group housed on a 12h:12h light/ dark cycle. Eight animals received rehabilitative reaching training and an osmotic mini pump (Alzet, Cupertino, CA, USA) containing saline that was continually infused to the forelimb motor cortex corresponding to their preferred paw. Eight animals received rehabilitative training and a cAMPS-Rp (Tocris, Bristol, UK)-filled osmotic mini pump and 7 rats received a cAMPS-Rp-filled pump and no rehabilitative training. Animals were fed *ad libitum* except for the days preceding single pellet reaching sessions when food pellets were reduced to approximately 8 g/ rat to motivate rats to reach. Weights were closely monitored to ensure the rats maintained a stable weight over time. An experimental timeline is presented in figure 2.1.

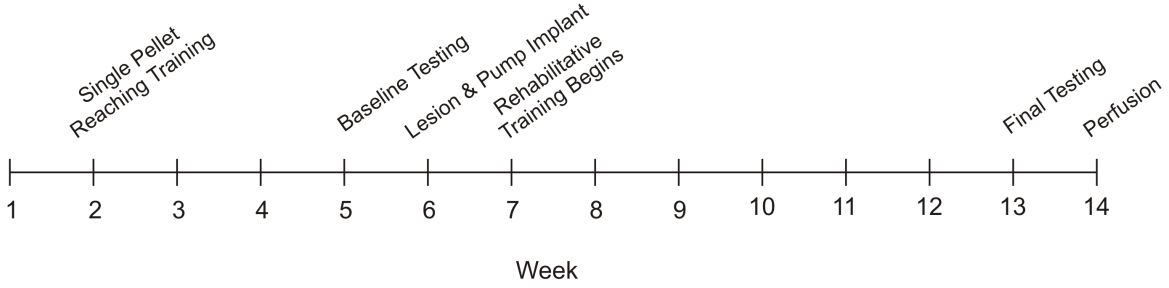

Figure 2.1

Experimental timeline.

All animals were trained on the skilled reaching task and baseline testing was performed before incomplete cervical (C4) SCI and osmotic pump implant surgery. Following a 1 week recovery period, trained animals began rehabilitative training on the skilled reaching task for 6 weeks. Final testing was performed before tissue extraction and perfusion.

2.2.2 Single Pellet Skilled Reaching

Rats were trained, as previously described (McKenna & Whishaw, 1999), on a single-pellet skilled-reaching task. Rats were individually placed in a Plexiglas chamber that was 45 cm in height, 12.5 cm in width and 38.5 cm in length. To ensure that any dropped pellets could not be retrieved, the entire chamber was raised 3 cm from the ground and the floor of the front half of the chamber was made of metal grating. Rats were trained to reach through a 1 cm wide opening with their dominant forepaw to retrieve sucrose pellets (45 mg; Research Diets, New Brunswick, NJ, USA) from a Plexiglass shelf. Sucrose pellets were placed into an indentation on the shelf 2 cm away from the front wall above the grid floor, a distance far enough that rats are forced to grasp the pellet with one forelimb rather than retrieve the pellet with their tongue. Baseline testing was performed on 3 consecutive days to assess the rats' pre-operative reaching ability following five weeks of training. Baseline reaching scores were analyzed by counting the total number of pellets retrieved and eaten divided by the number of total reaching attempts. Following SCI, rats were left for one week to recover before rehabilitative training was initiated. Rehabilitative training was performed for 6 weeks (10 minutes/day, 5 days/week) before final testing. Final testing of all rats, trained and untrained, consisted of 3 consecutive days of testing and was quantified by counting the total number of pellets retrieved and eaten divided by the number of total reaching attempts.

2.2.3 Spinal Cord Injury and Pump Implant

Rats underwent a unilateral spinal lesion at the cervical level (C4) on the side of their preferred paw, as established during reaching training. Animals were anesthetized by gas anesthesia (4% isoflurane for induction, 1.5-2% for maintenance). The surgical area was shaved and disinfected with 10% chlorhexadine digluconate (Sigma–Aldrich Canada Ltd., Oakville, ON, Canada) and the animal was mounted into a stereotactic frame (Kopf Instruments, Tujunga, CA, USA). Throughout the surgery, body temperature was maintained at 37 °C with a heating blanket. Following a skin incision and dissection of muscle, the spinal cord under C4 was exposed with a partial (1/2 a segment) laminectomy. To make the lesion, a custom-made microblade was lowered 1 mm into the spinal cord at the midline then moved laterally to create a unilateral dorsolateral quadrant (DLQ) lesion. The lesion ablated the dorsal funiculus containing the dorsal CST and a large percentage of the rubrospinal tract (RST) projecting in the lateral funiculus. Following the lesion, the muscles over the injury site were sutured and the skin was closed with surgical clips.

Osmotic mini pumps were implanted at the time of the lesion surgery. The pumps were filled with either saline or cAMPS-Rp (3.536 μM solution in saline) and were implanted subcutaneously in the back of the rats. The pump was connected with silicone tubing to a brain infusion kit (Alzet, Cupertino, CA, USA), which was glued with Loctite instant adhesive cyanoacrylate gel (Cedarlane, Burlington, ON, Canada) to the rats' skull at +1.5 mm rostral and +1.5 mm lateral from bregma, at a depth of 1.5 mm, for infusion into the forelimb motor cortex

corresponding to the preferred paw. Osmotic mini pumps were replaced under isoflurane anesthesia every two weeks for a period of six weeks. Post-operative hydration was ensured by s.c. injection of 4 ml of saline and pain was managed by s.c. injections of buprenorphine (0.05 mg/kg, Temgesic, Schering-Plough, QC, Canada). Animals were kept on a heating blanket until fully awake.

2.2.4 Perfusion and Tissue Collection

At 7 weeks post-injury rats were anesthetized with isoflurane and an approximately 3 x 3 mm piece of cortical tissue from both the right and left forelimb motor cortex was extracted and immediately frozen in liquid nitrogen. Rats were euthanized with pentobarbital (3.2ml/kg; Euthanyl, Biomeda-MTC, Cambridge, ON, Canada) and transcardially perfused with saline followed by a 4% formalin solution containing 5% sucrose. The spinal cords were dissected and post-fixed in 4% formalin solution for 24 hours before cryoprotection in 30% sucrose for 3 days. Spinal cords were mounted onto filter paper using Tissue Tek (Sakura Finetek, Torrance, CA, USA) and frozen in methylbutane over dry ice at -45 ºC. Tissue was stored at -80 ºC before being sectioned on a cryostat at -20 ºC at a thickness of 25 µm.

2.2.5 Western Blot

Cortical tissue was analyzed for PKA content using western blot procedures.

Tissue samples were thawed and homogenized with pestles on ice in 300 µl of ice-cold cell lysis buffer [in mM: Tris–HCl (pH 7.5), 20; NaCl, 150; EDTA, 1; EGTA, 1; 1% Triton X-100; 1 tablet of Roche's complete protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany)]. The samples were centrifuged (2000 x g, 10 min, 4 °C) and the supernatants were assayed for total protein concentration. 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, USA). Protein extracts were heated at 70 °C for 10 min in NuPAGE LDS sample buffer [100 mm Tris, pH 6.8, 250 mm β-mercaptoethanol, 4% sodium dodecyl sulfate (SDS), 0.01% bromophenol blue and 20% glycerol] and NuPAGE reducing agent. A total of 20 µg of protein was loaded per lane for separation by 10% SDS–polyacrylamide gel electrophoresis for 1.5 hours (room temperature, 140 V) and transferred for 1 hour (room temperature, 25 V) onto a nitrocellulose blotting membrane (PALL Life Sciences, BioTraceNT, FL, USA). Membranes were then blocked in Membrane Blocking Solution (BSA; fraction V; Sigma) and probed with primary antibodies (i.e., polyclonal anti-PKA and polyclonal anti-pPKA). PKA-cat (C-20; Santa Cruz Biotechnology, CA, USA) is an affinity-purified rabbit polyclonal IgG antibody raised against a peptide mapping at the C-terminus of the PKAa 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 with the b- and c-subunits. The antibody was used at a dilution of 1:1000 in Membrane

Blocking Solution allowing detection of a single band at 38 kDa, the expected molecular weight of PKAa-cat (Maldonado & Hanks, 1988). PhosphoPKA beta (S338; Abcam, MA, USA) 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 was used at a dilution of 1:2000, identifying a band at 42 kDa, the predicted molecular weight of pPKA beta (catalytic subunit).

Following membrane blocking and application of primary antibody, the membrane was washed with TBS-T (0.15% Tween-20, pH 8.0) 3 times at 5 minutes per wash and the the membrane was then incubated for 1 hour at room temperature in a secondary anti-rabbit IgG conjugated with horseradish peroxidase, diluted 1:5000 (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Visualization of the proteins was accomplished by using the SuperSignal West Pico Chemiluminescent detection kit (Pierce Biotechnology, Thermo Fisher Scientific, Rockford, IL, USA). For subsequent immunoprobing of the same membrane with multiple antibodies, the membranes were first incubated on the shaker at room temperature for 15 minutes in membrane stripping buffer [62.5 mm Tris–HCl, pH 6.7, 10 mm b-mercaptoethanol, 2% (w ⁄ v) SDS] followed by three, 10 minute washes with TBS-T. Following stripping of the blot, the blocking step and incubation with a different primary antibody were performed. The relative amount of immunoreactive protein in each band was determined by densitometric analysis of the X-ray films (ImageJ Software, NIH).

Densitometry readings were expressed as the amount of pPKA/total PKA.

2.2.6 Histology

Horizontal sections of the cervical spinal cord were cut at a thickness of 25 µm on a cryostat and stored at -80 ºC before being stained with cresyl violet (CV). For CV staining slides were dried for 30 minutes at 37 ºC to adhere tissue to the slide. Tissue was rehydrated in TBS, placed in 0.1% CV for 3 minutes, dipped in dH_2O to remove excess CV and then serially dehydrated in EtOH (50%, 75%, 99%) for 1 minute each. Following dehydration, tissue was cleared in xylene for 1 minute and then coverslipped with Permount (Fisher Scientific, Unionville, Canada).

2.2.7 Lesion Size Analysis

CV-stained spinal cord sections were assessed for lesion size using light microscopy. Every fourth horizontal section was analyzed at 10x magnification under bright field and phase contrast settings. The maximal extent of the injury was determined using landmarks such as the relative amount of grey versus white matter as well as the central canal and mapped onto a schematic of the cervical spinal cord. The size and shape of the lesion was reconstructed using ImageJ (NIH Software) and the lesioned area was calculated as a percent of the total cross sectional area.

2.2.8 Statistical Analysis

We compared the lesion sizes, baseline and final reaching success and cortical PKA levels between experimental groups using one-way ANOVAs. A student's t-test was used to compare the level of cortical pPKA between saline and cAMPS-Rp treated groups, regardless of training. Statistical analyses were performed using Graph Pad Prism software (LaJolla, CA, USA). All data are presented as mean ± SEM and rounded to two decimal places. A p value ≤ 0.05 was considered significant (*).

2.3 RESULTS

2.3.1 Lesion Size Analysis

The DLQ lesion used in this study severed the CST and RST to some extent in all animals. The minimal, median and maximal lesions included in this study are represented in figure 2.2A. Lesion sizes in each of the 3 experimental groups were compared to ensure that any subsequent results were not due to differences in lesion size between groups. Overall, there was no significant difference in lesion size between the experimental groups ($p = 0.73$; Fig. 2.2B). The saline and rehabilitative training group had an average lesioned area of 29.6% ± 3.0, 32.8% ± 4.2 for the cAMPS-Rp and rehabilitative training group and 34.1% ± 5.3 for the cAMPS-Rp and no rehabilitative training group.

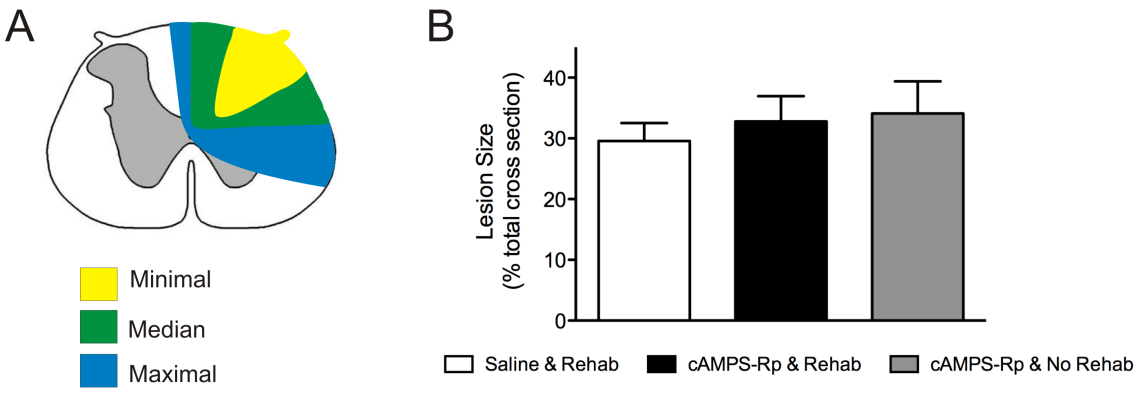

Figure 2.2

Lesion size analysis.

A) The minimal, median and maximal lesions of all rats are represented on a spinal cord cross-section schematic in yellow, green and blue, respectively. B) The average lesion size, expressed as the lesioned area as a percent of the total cross-sectional area, is shown for each experimental group (Saline & Rehabilitative Training n = 8, cAMPS-Rp & Rehabilitative Training n = 8, cAMPS-Rp & No Rehabilitative Training n = 7). Error bars represent SEM.

2.3.2 Single Pellet Reaching

Single pellet reaching was performed to test fine motor control of the forelimb following SCI. Baseline reaching scores were recorded before injury and were analyzed to confirm that there was no difference between experimental groups. This ensures that any differences between the experimental groups observed following injury are not the result of differences in baseline reaching performance. There was no difference in baseline reaching success between experimental groups ($p = 0.99$; Saline and rehabilitative training, 38.3% ± 6.2, cAMPS-Rp and rehabilitative training, 37.7% ± 6.4, cAMPS-Rp and no rehabilitative training, 38.0% ± 7.3; Fig. 2.3A). Final single pellet success was calculated as the best score achieved for each rat over the final 3 days of testing and reported as a percent of their baseline reaching score.

There was no significant difference in final reaching success between any of the three experimental groups ($p = 0.20$; saline and rehabilitative training, 14.8% ± 10.1, cAMPS-Rp and rehabilitative training, 53.5% ± 16.2, cAMPS-Rp and no rehabilitative training, 38.5% ± 18.6; Fig.2.3B). However, the cAMPS-Rp and rehabilitative training group showed greater functional recovery over the course of rehabilitative training (Fig.2.3D, $p = 0.01$) than saline-treated animals (Fig.2.3C, $p = 0.06$). This result is contrary to our central hypothesis that cAMPS-Rp-treated rats would have lower single pellet success than saline-treated rats following SCI.

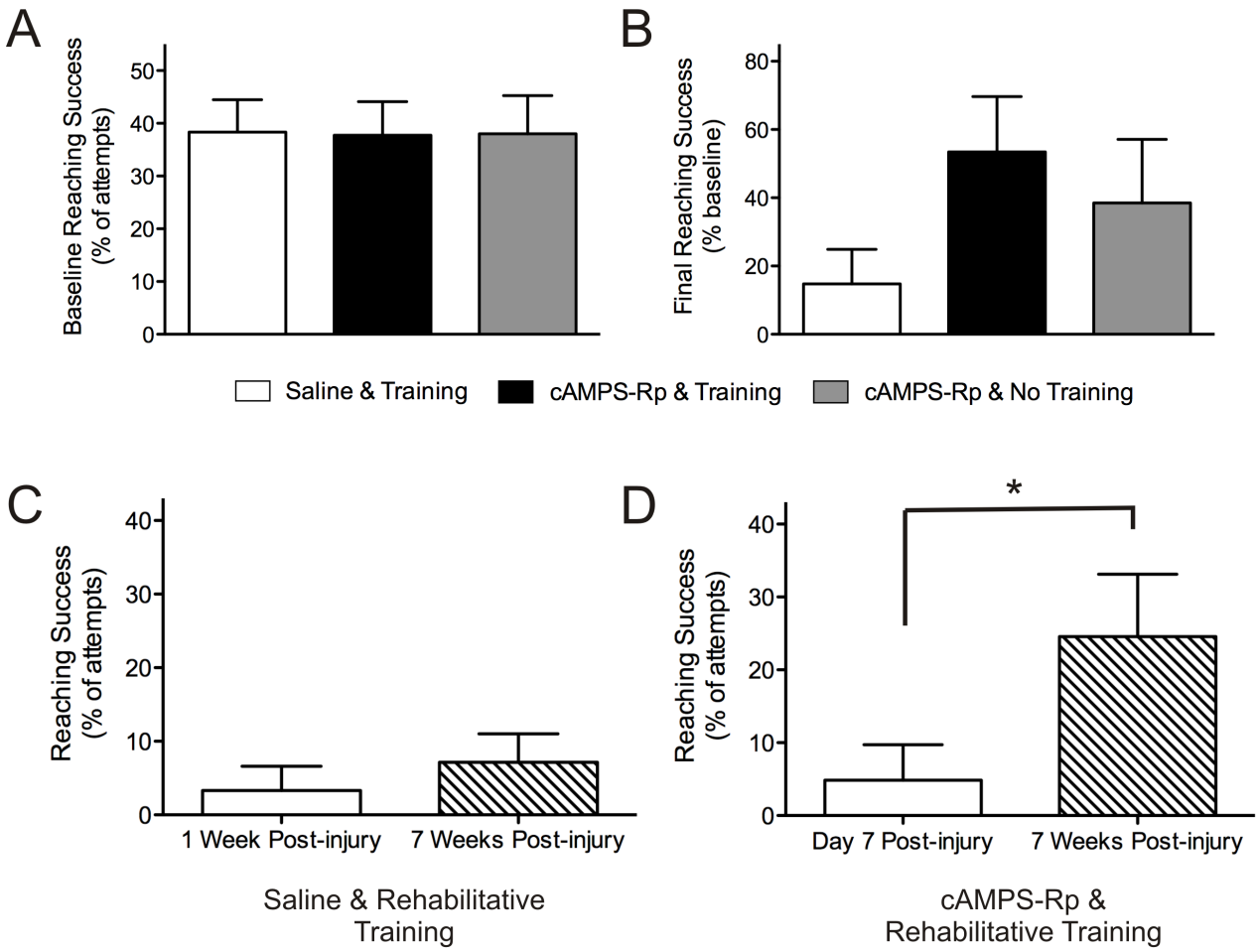

Figure 2.3

Single pellet reaching results.

A) There was no difference in baseline reaching success, expressed as the total number of pellets retrieved and eaten divided by the number of total reaching attempts, between the experimental groups. B) Final reaching success, reported as a percent of baseline reaching success, is shown for each of the experimental groups, $p = 0.20$. C) Reaching success, expressed as a percent of the number of reaching attempts was not different 1 week post-spinal cord injury compared to the end of the rehabilitative training period (7 weeks post-injury) for the saline-treated group. D) Reaching success significantly improved over the course of rehabilitative training in the cAMPS-Rp-treated group, $p = 0.01$. Error bars represent SEM.

2.3.3 Western Blot

A western blot analysis for pPKA was performed on cortical tissue to ensure that cAMPS-Rp administration did, in fact, decrease pPKA levels in the forelimb motor cortex. Contrary to what was expected, we did not see a significant difference in activated PKA levels between experimental groups ($p = 0.93$; saline and rehabilitative training, 0.70 ± 0.1, cAMPS-Rp and rehabilitative training, 0.72 ± 0.1, cAMPS-Rp and no rehabilitative training, 0.65 ± 0.1; expressed as pPKA/PKA; Fig. 2.4). We then grouped all cAMPS-Rp-treated rats, regardless of whether or not they had rehabilitative training, and compared their cortical phosphorylated PKA levels to saline-treated rats. Again, there was no difference between these two groups ($p = 0.93$, data not shown).

2.4 DISCUSSION

This study was conducted to demonstrate the role of PKA signaling in plasticity following SCI by inhibiting PKA levels in the motor cortex throughout post-injury rehabilitative reaching training. Our results did not reveal a statistically significant effect of PKA inhibition on rehabilitative training, however, there was a trend towards higher reaching success among rats treated with a PKA inhibitor as compared to those treated with saline. This trend is the opposite of what was hypothesized and presents an interesting opportunity for further investigation. To interpret these results and expand on them with future studies, it is important to address some experimental considerations including

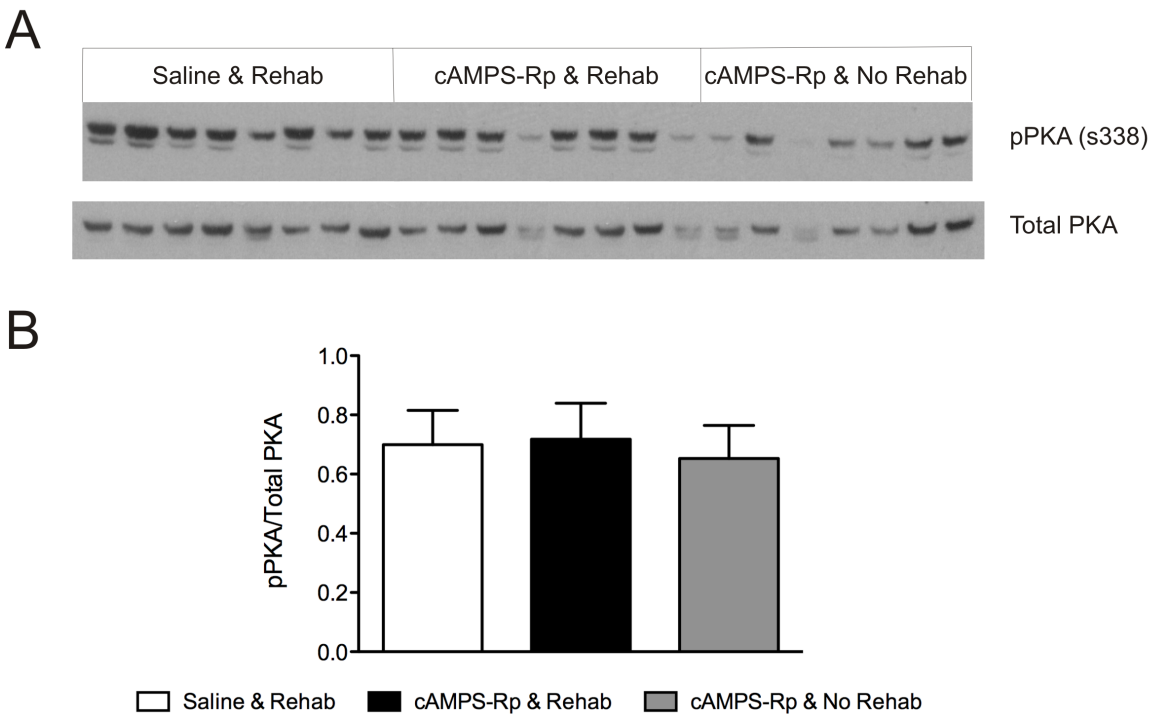

Figure 2.4

Western blot results.

A) Expression of pPKA (top) and total PKA in motor cortices corresponding to the rats' preferred paw was evaluated by western blot and are shown for all animals (n = 23). B) Quantification of pPKA/total PKA demonstrated no difference between experimental groups. Error bars represent SEM.

possible explanations for observations that were opposite of what had been hypothesized. Some factors that must be considered are the inconclusive western blot results as well as downstream effectors of cAMP signaling that may play a more prominent role in rehabilitation-induced motor recovery than was initially anticipated.

2.4.1 Western Blot Considerations

Perhaps the most important experimental consideration in this study is the inconclusive western blot results that were obtained. A western blot analysis of cortical tissue was performed to ensure that the PKA inhibitor, cAMPS-Rp, was actually functioning to inhibit active, or phosphorylated, PKA (pPKA) in the motor cortex. Because no differences in the level of phosphorylated PKA were found between saline and cAMPS-Rp-treated rats (Fig. 2.4A), we cannot be sure that the skilled reaching observations were a result of decreased cortical PKA. These unexpected results are likely the result of issues with our western blot protocols, which will be discussed below. Without proof that the drug decreased the level of pPKA, we cannot confidently state that behavioural trends were caused by PKA inhibition.

It is interesting to note that we have observed two distinct bands of protein in the pPKA blots when performing western blot analysis of the cortical cAMPS-Rp and saline-treated tissue (Fig. 2.4A). Notably, inconsistent results are observed depending on how these bands are quantified (i.e., whether both bands or only the

largest one are analyzed). The two bands are a concern because they may be the result of nonspecific binding of the antibody to different isoforms of PKA (Olsen and Uhler, 1989; Shuntoh et al., 1992) or perhaps different phosphorylation sites (Steinberg et al., 1993). This antibody has been reported to cross-react with the b- and c-subunits of PKA, however, the manufacturer has demonstrated that a peptide corresponding to PKA-cat beta (pS338) blocks the antibody signal, thereby verifying specificity. The manufacturer has also reported that phosphatase stripping eliminates the signal, thereby verifying phospho-specificity of the antibody. We are in the process of testing alternative pPKA antibodies in an attempt to identify an antibody with improved phospho-specificity and limited cross-reaction with the various PKA subunits.

Another factor that is important to consider when optimizing our western blot procedure is the tissue extraction process. Many studies citing the effects of PKA inhibitors have been conducted *in vitro* and are often performed in cell cultures. Freezing and lysis of cells from culture differs from protocols to remove cortical tissue from our *in vivo* model of PKA inhibition. For example, in this study, the animals were under isoflurane anesthesia at the time of cortical tissue extraction, which may alter PKA activity (Tanaka et al., 2007). When optimizing our western blot protocol, other tissue extraction methods, such as decapitation as well as alternative anesthetics, must be explored. Also, the addition of phosphatase inhibitors to the lysis buffer will be an important part of improving our tissue extraction and sample preparation process.

2.4.2 Power Analysis

The skilled reaching data from this study suggests that a behavioural effect of cAMPS-Rp administration may be present, although opposite of the effect that was hypothesized. With a relatively small sample size (n = 23), it may be possible that there was simply insufficient power to detect a statistically significant result. Statistical power can be defined as the probability that a null hypothesis will be rejected given that it is false. In other words, was our experimental design sensitive enough to have been able to detect a significant effect of cAMPS-Rp on skilled reaching if it was truly producing a behavioural effect? We performed a post hoc power analysis using G*Power software (Faul et al, 2007) and found that our power for this study is estimated at 0.32. This suggests that we had a 32% chance of statistically detecting a difference between the experimental groups if indeed there was a real difference. This power is quite low compared to the standard 0.8 or 0.9 that is usually accepted in experimental design. In order to increase our power, the current study should be repeated to increase the total sample size and decrease the observed variability. A plot of power as a function of sample size is shown in figure 2.5 to illustrate to effect of increasing sample size on power level for this experiment.

2.4.3 Intracellular Signaling Cascades

We used cAMPS-Rp to inhibit PKA in the motor cortex of rats with incomplete cervical SCI. To our knowledge this particular inhibitor has not yet

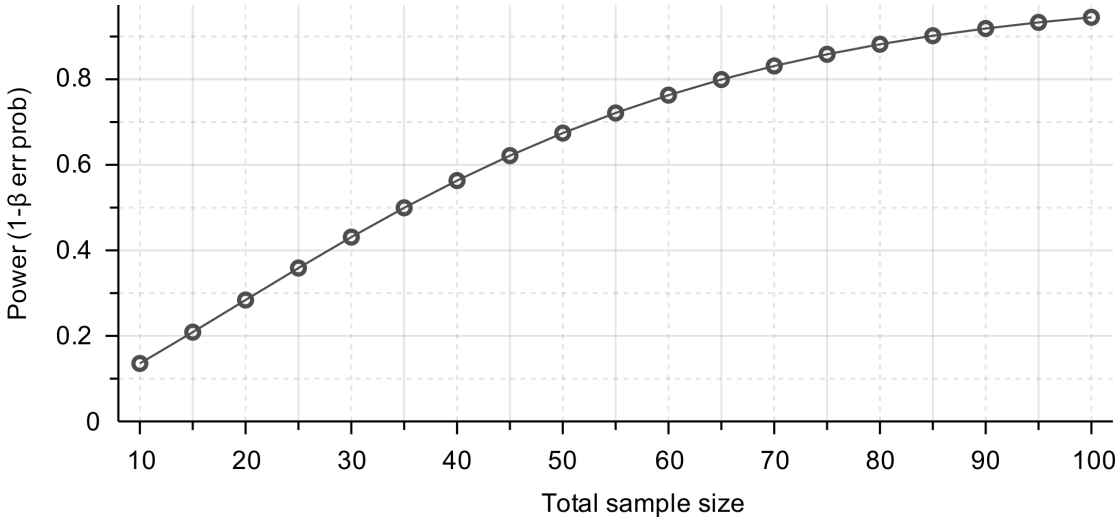

Figure 2.5

Statistical power as a function of total sample size.

A plot of the estimated statistical power (as predicted using G*Power software) that would be expected for any given total sample size in this study. It is clear that to acquire traditional levels of power (0.8-0.9), the total sample size would have to be greater than 65 animals.

been used *in vivo* but has been shown to decrease transient calcium currents in dendritic spines and inhibit synaptic plasticity *in vitro* (Fu et al., 2008; Hoogland & Saggau, 2004). Some alternative PKA inhibitors that are often used include H89 and KT 5720, however, these inhibitors bind to a different site on PKA and affect other signaling cascades, resulting in widespread nonspecific effects (as reviewed by Murray, 2008). By using cAMPS-Rp, a membrane-permeable, competitive inhibitor of PKA which functions by inhibiting the cAMP binding site, we attempted to better understand the role of PKA in post-injury rehabilitative training.

In contrast to what was reported by MacDonald et al., (2007), who found that administering a phosphodiesterase (PDE) inhibitor to increase cAMP levels resulted in improved motor performance following a cortical lesion, we found that inhibiting PKA seemed to somewhat improve motor recovery following SCI. This disparity can possibly be explained by a few factors including a negative feedback mechanism when PKA is inhibited as well as effects of downstream targets of cAMP other than PKA.

A feedback mechanism has been reported (Sunahara et al., 1996) where active PKA (i.e., phosphorylated PKA; pPKA) activates PDEs, which degrade cAMP to AMP. Ultimately, this feedback results in decreased cAMP with increases in pPKA. In the current study, inhibiting PKA may have reduced this feedback so fewer PDEs were activated and therefore the level of cAMP increased. If this is the case, the increased cAMP would either compete with the cAMPS-Rp for binding sites on PKA (importantly, the PKA inhibitor is less

effective with increased cAMP) or the excessive cAMP may have a more profound effect on alternative downstream targets of cAMP such as the exchange protein activated by cAMP (Epac; Murray et al., 2009) or cyclic nucleotide-gated ion channels (as reviewed by Kaupp and Seifert, 2002).

Cyclic nucleotide-gated ion channels are nonselective cation channels that have been characterized mainly in the retinal photoreceptors, so their potential role in cortically-driven motor recovery following SCI remains unclear and therefore speculative at best. In contrast, the role of Epac in neurite growth has become increasingly clear in recent years. Epac is a guanine nucleotide exchange factor that is directly activated by cAMP and can signal independently of PKA (Bos, 2003). Based in *in vitro* data, Murray et al. (2009) have proposed that Epac is activated when cAMP levels are high and this results in neurite outgrowth. In contrast, when cAMP levels are low Epac is not activated and instead, PKA mediates retraction of neurites. This may be in line with previous studies looking at the role of cAMP in neurite growth and motor function. For example, a decline in neuronal cAMP level with age has been associated with the simultaneous decline in capacity for axonal growth (Cai et al., 2001). Similarly, enhancing cAMP with an analog, db-cAMP, has been shown to stimulate neurite growth (Neumann et al., 2002). These studies may have observed increased growth and recovery by PKA, as is often assumed and discussed, however, as Murray suggests (2009), these promising results may have instead resulted from increased activation of Epac.

In light of these considerations, the improved motor recovery with PDE inhibitor treatment as previously reported in the field of stroke (MacDonald et al., 2007) can be critically evaluated. For instance, because PDE inhibitor treatment prevents the degradation of cAMP, rather than more specific downstream effectors such as PKA or Epac, it is not possible to determine the specific signaling cascades responsible for mediating the reported recovery. It will be necessary to repeat these studies in the future with more, and likely combinations of, inhibitors to include factors such as PKA and Epac.

2.4.4 Conclusions and Future Directions

Although our observations were opposite of what was hypothesized, our behavioural results emphasize the potential for some interesting avenues of study. There are some technical considerations that must be resolved before further experiments can be completed. Given these initial results, there remain a number of factors to analyze in order to better understand the intracellular signaling mechanisms behind functional recovery with rehabilitative training. This may include the use of inhibitors for other targets of cAMP, such as Epac, as well as an investigation into downstream effects of the cAMP pathway, including calcium response element binding (CREB) protein, a transcription factor activated by both calcium and cAMP with a role in neuronal plasticity. Following a thorough analysis of the factors that may be involved, it will be important to test manipulations of these factors in an attempt to enhance the beneficial effects of

rehabilitative training. Ultimately, there is still much to be learned with respect to the intracellular mechanisms involved in improved functional recovery with rehabilitative training following SCI.

2.5 REFERENCES

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. Nature neuroscience 7**,** 269-277.

Bos, J. L., 2003. Epac: a new cAMP target and new avenues in cAMP research. Nature reviews. Molecular cell biology 4**,** 733-738.

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. The Journal of neuroscience : the official journal of the Society for Neuroscience 21**,** 4731-4739.

Cai, D., Shen, Y., De Bellard, M., Tang, S., and Filbin, M. T., 1999. Prior exposure to neurotrophins blocks inhibition of axonal regeneration by MAG and myelin via a cAMP-dependent mechanism. Neuron 22**,** 89-101.

Cui, Q., and So, K. F., 2004. Involvement of cAMP in neuronal survival and axonal regeneration. Anatomical science international 79**,** 209-212.

Faul, F., Erdfelder, E., Lang, A. G., and Buchner, A., 2007. G*Power 3: a flexible statistical power analysis program for the social, behavioral, and biomedical sciences. Behavior research methods 39**,** 175-191.

Ferguson, G. D., and Storm, D. R., 2004. Why calcium-stimulated adenylyl cyclases? Physiology 19**,** 271-276.

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. Current biology : CB 11**,** 1766-1770.

Fu, Y., Han, J., Ishola, T., Scerbo, M., Adwanikar, H., Ramsey, C., and Neugebauer, V., 2008. PKA and ERK, but not PKC, in the amygdala contribute to pain-related synaptic plasticity and behavior. Molecular pain 4**,** 26.

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 : a journal of neurology 130**,** 2993-3003.

Gomez-Pinilla, F., Ying, Z., Roy, R. R., Molteni, R., and Edgerton, V. R., 2002. Voluntary exercise induces a BDNF-mediated mechanism that promotes neuroplasticity. Journal of neurophysiology 88**,** 2187-2195.

Hoogland, T. M., and Saggau, P., 2004. Facilitation of L-type Ca2+ channels in dendritic spines by activation of beta2 adrenergic receptors. The Journal of neuroscience : the official journal of the Society for Neuroscience 24**,** 8416-8427.

Kaupp, U. B., and Seifert, R., 2002. Cyclic nucleotide-gated ion channels. Physiological reviews 82**,** 769-824.

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. The European journal of neuroscience 29**,** 641-651.

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. Neurorehabilitation and neural repair 21**,** 486-496.

Maldonado, F., and Hanks, S. K., 1988. A cDNA clone encoding human cAMP-dependent protein kinase catalytic subunit C alpha. Nucleic acids research 16**,** 8189-8190.

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. The Journal of neuroscience : the official journal of the Society for Neuroscience 19**,** 1885-1894.

Merz, K., Herold, S., and Lie, D. C., 2011. CREB in adult neurogenesis--master and partner in the development of adult-born neurons? The European journal of neuroscience 33**,** 1078-1086.

Murray, A. J., 2008. Pharmacological PKA inhibition: all may not be what it seems. Science signaling 1**,** re4.

Murray, A. J., Tucker, S. J., and Shewan, D. A., 2009. cAMP-dependent axon guidance is distinctly regulated by Epac and protein kinase A. The Journal of neuroscience : the official journal of the Society for Neuroscience 29**,** 15434-15444.

Neumann, S., Bradke, F., Tessier-Lavigne, M., and Basbaum, A. I., 2002. Regeneration of sensory axons within the injured spinal cord induced by intraganglionic cAMP elevation. Neuron 34**,** 885-893.

Norrie, B. A., Nevett-Duchcherer, J. M., and Gorassini, M. A., 2005. Reduced functional recovery by delaying motor training after spinal cord injury. Journal of neurophysiology 94**,** 255-264.

Nudo, R. J., Wise, B. M., SiFuentes, F., and Milliken, G. W., 1996. Neural substrates for the effects of rehabilitative training on motor recovery after ischemic infarct. Science 272**,** 1791-1794.

Olsen, S. R., and Uhler, M. D., 1989. Affinity purification of the C alpha and C beta isoforms of the catalytic subunit of cAMP-dependent protein kinase. The Journal of biological chemistry 264**,** 18662-18666.

Opazo, P., Labrecque, S., Tigaret, C. M., Frouin, A., Wiseman, P. W., De Koninck, P., and Choquet, D., 2010. CaMKII triggers the diffusional trapping of surface AMPARs through phosphorylation of stargazin. Neuron 67**,** 239-252.

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. Nature medicine 10**,** 610-616.

Schwartzman, R. J., 1978. A behavioral analysis of complete unilateral section of the pyramidal tract at the medullary level in Macaca mulatta. Annals of neurology 4**,** 234-244.

Shuntoh, H., Sakamoto, N., Matsuyama, S., Saitoh, M., and Tanaka, C., 1992. Molecular structure of the C beta catalytic subunit of rat cAMP-dependent protein kinase and differential expression of C alpha and C beta isoforms in rat tissues and cultured cells. Biochimica et biophysica acta 1131**,** 175-180.

Steinberg, R. A., Cauthron, R. D., Symcox, M. M., and Shuntoh, H., 1993. Autoactivation of catalytic (C alpha) subunit of cyclic AMP-dependent protein kinase by phosphorylation of threonine 197. Molecular and cellular biology 13**,** 2332-2341.

Sunahara, R. K., Dessauer, C. W., and Gilman, A. G., 1996. Complexity and diversity of mammalian adenylyl cyclases. Annual review of pharmacology and toxicology 36**,** 461-480.

Tanaka, K., Kawano, T., Nakamura, A., Nazari, H., Kawahito, S., Oshita, S., Takahashi, A., and Nakaya, Y., 2007. Isoflurane activates sarcolemmal adenosine triphosphate-sensitive potassium channels in vascular smooth muscle cells: a role for protein kinase A. Anesthesiology 106**,** 984-991.

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. The journal of spinal cord medicine 32**,** 63-71.

CHAPTER III

ANATOMICAL CORRELATES OF RECOVERY IN SINGLE PELLET REACHING IN SPINAL CORD INJURED RATS

Adapted from C. Hurd, N. Weishaupt, K. Fouad, Exp Neurol, (2013)

3.1 PREFACE

The previous chapter describes a study in which rats received an incomplete cervical (C4) SCI and recovery was evaluated using a single pellet skilled reaching task. Although there is a trend towards differences in reaching recovery between the experimental groups, statistical significance was not reached. Interestingly, a power analysis of the data in Chapter 2 revealed that a total sample size of greater than 65 animals would likely be required to acquire traditional levels of power (0.8-0.9).

To increase the power and reliability of the single pellet reaching test it will be important to re-evaluate our model of SCI by identifying the factors that contribute to the extremely high variability in reaching success. There are many possible reasons for this variability including the lesion, motivation and alternative reaching strategies following injury. For example, is the lesion severity appropriate to assess recovery in single pellet skilled reaching? This chapter describes an analysis of our animal model of incomplete cervical SCI in order to optimize the potential to observe treatment effects by reducing variability in success on the single pellet skilled reaching task.

3.2 INTRODUCTION

Modeling spinal cord injury (SCI) in animals to test potential treatments is challenging, partly due to the variables involved, including different lesion levels, severities and causes of injury (e.g., contusion, compression, section). A number

of combinations of these variables have been applied, driven by different treatment questions ranging from neuroprotection to axonal regeneration. Much of the early research in the field of SCI focused on lesions at the thoracic level using locomotor recovery as functional readout (Barbeau and Rossignol, 1987; Behrmann et al., 1992; Little et al., 1988). Because of its clinical relevance and convenient neuroanatomical access (relatively easy to trace and examine because it descends in the dorsal funiculus), the corticospinal tract (CST) was the preferred descending system involved in many of these studies (Eidelberg and Yu, 1981; Liebscher et al., 2005; Metz et al., 1998; Muir and Whishaw, 1999). However, it became clear (at least in rodents) that it is not lesions to the CST, but to the reticulospinal tract, projecting in the ventrolateral funiculus, that determine the degree of locomotor deficits (Loy et al., 2002; Schucht et al., 2002; Steeves and Jordan, 1980). Another challenge in the interpretation of locomotor recovery is that locomotion is orchestrated by so called spinal central pattern generating networks. Thus, locomotor patterns can be initiated without direct descending input (Barbeau and Rossignol, 1987; Lovely et al., 1986), making the interpretation of treatment effects even more difficult.

A shift in the field of SCI research towards cervical lesions occurred as a result of a number of factors. These factors included a strong base of knowledge that hand/paw function involves CST function (Lawrence and Kuypers, 1968; Schwartzman, 1978), an increase in the frequency of incomplete, cervical SCIs in humans (National Spinal Cord Injury Statistical Centre; 2012), and the assumption that reaching movements are volitional, and directly orchestrated by

the brain and not only by spinal networks. The majority of these studies involved rats, which can be trained to grasp for food pellets in tests such as a single pellet skilled reaching task (Whishaw et al., 1993; Whishaw and Pellis, 1990), tray reaching (Whishaw et al., 1986) or the Montoya staircase test (Montoya et al., 1991). Studies using these reaching tests showed that damage to both the lateral and dorsal funiculus, housing the rubrospinal tract (RST) and CST respectively, results in permanent functional deficits in reaching recovery (Anderson et al., 2007; Morris et al., 2011; Whishaw et al., 1998). Cervical SCI models have quickly evolved over the past few years to overcome apparent limitations of thoracic lesions. However, some of the challenges introduced above remain, especially the search for the ideal combination of efficient functional readout and lesion type. The current study attempts to address this issue by describing the reaching recovery of rats with various lesion severities to highlight the ideal lesion to be used to test plasticity-promoting treatments with single pellet skilled reaching.

To be useful for demonstrating the benefits of regeneration and/or plasticity-promoting treatments, an animal model of SCI must fulfill various requirements. First, considering the currently limited treatment options for SCI and the fact that effective treatments will likely consist of combinatory approaches, moderate treatment effects should be detectable. By assessing the effects of rehabilitative training we use one of the currently most effective treatments for promoting recovery following SCI, thereby providing a good estimation of how sensitive a current testing approach needs to be. Second, the

injury has to induce significant functional deficits, but should not be so severe that a floor effect is observed. A floor effect describes the effect when data cannot be recorded lower than a designated value (e.g. success of 0% in the single pellet test). This can result in an accumulation of animals with success rates of 0 although they still might vary in their reaching abilities. It also has to be kept in mind that plasticity-inducing treatments require a certain amount of tissue sparing. Lastly, the variability in spontaneous functional recovery should be small because the margin between spontaneous recovery, treatment effects and pre-lesion motor function are often very small.

To define an optimal cervical lesion model for testing effects of regeneration and/or plasticity-promoting treatments (including rehabilitative training), we investigated the functional deficits caused by unilateral lesions, ranging from a minimal dorsolateral lesion to a complete lateral hemisection. To also investigate the effect of lesion location (with respect to projection of descending tracts) we distributed the lesions into categories and studied spontaneous recovery as well as the effect of rehabilitative reaching training (as a potent plasticity-promoting treatment) to identify the most appropriate lesion type to use when testing treatments following SCI.

3.3 MATERIALS AND METHODS

3.3.1 Animals and Experimental Groups

All experimental procedures were approved by the University of Alberta Animal

Care and Use Committee and complied with the guidelines of the Canadian Council for Animal Care. The rats in this study were derived from control groups in earlier studies from our laboratory. A total of 139 female Lewis rats (Charles River Laboratories, Canada) weighing 180 g – 200 g were included in this study. Rats were group housed on a 12h :12h light/ dark cycle and received a cervical (C4) lesion with the intention to create a dorsolateral quadrant (DLQ) lesion. 6 Fisher rats (Charles River Laboratories, Canada), also control rats from a previous study, received a lateral hemisection at C4. All animals were fed ad libitum except for the day preceding single pellet reaching sessions when food pellets were reduced to approximately 8 g/ rat to motivate rats to reach. Weights were closely monitored to ensure stable body weight over time.

3.3.2 Spinal Cord Injury

Rats received a spinal cord injury on the side of their preferred paw, as established during reaching training. Animals were anesthetized by a subcutaneous (s.c.) injection of Fentanyl (0.2 mg/ kg, Hypnorm, Janssen Pharmaceutics, Beerse, Belgium) mixed with Midazolam (4 mg/ kg, Versed, Sabex, Boucherville, QC, Canada). The surgical area was shaved and disinfected. Throughout the surgery, body temperature was maintained with a heating blanket. The spinal cord between C4 and C5 was exposed without laminectomy. To make the DLQ lesions, a custom-made blade was lowered 1 mm into the spinal cord at the midline then moved laterally. For hemisection lesions, the blade was lowered through the entire

spinal cord at the midline and moved laterally. Finally, muscle layers were sutured and the skin was closed with staples. Post-operative hydration was ensured by s.c. injection of 4 ml saline and pain was managed by s.c. injections of buprenorphine (0.05 mg/kg, Temgesic, Schering-Plough, QC, Canada). Animals were kept on a heating blanket until fully awake.

3.3.3 Single Pellet Reaching

Rats were individually placed in a Plexiglas chamber as previously described (Girgis et al., 2007) and were trained to reach through an opening to retrieve sucrose pellets (45 mg) placed into an indentation on a shelf 2 cm away from the front wall above an elevated grid floor (Fig. 3.1A). This distance forces rats to grasp with one forelimb through the opening. All rats were trained on the single pellet task for four weeks before SCI and some rats (n = 90) underwent rehabilitative training for 6 weeks beginning one week post-injury (p.i.). For baseline and final scores in the reaching task, the total number of reaching attempts, as well as the number of successful grasps, was recorded on three consecutive days. The day with the highest success rate during baseline and final testing was used. Overall reaching recovery success rate is calculated as final reaching success as a percent of the baseline reaching success. The proportion of animals with final reaching success rates of zero was taken into account by dividing the number of rats in each lesion category with zero success by the total number of rats in the category. Since rats have been known to use compensatory

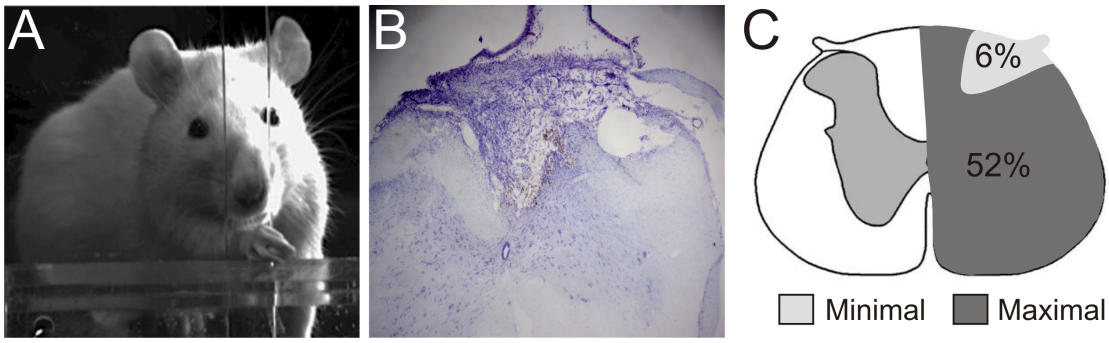

Figure 3.1

Single pellet testing and lesion reconstruction.

A) Rats were trained and tested on a single pellet skilled reaching task which required successful retrievals of sucrose pellets from a shelf outside a custom Plexiglass reaching box. B) A cresyl violet stained cervical (C4) cross section of the spinal cord demonstrating a DLQ lesion. C) Schematic reconstructions of minimal (light grey area), and maximal (dark grey) lesion extents.

movements after injury (Metz et al., 2005; Whishaw et al., 1991), which may artificially increase reaching scores, a simple qualitative analysis of the reaching pattern was conducted using high-speed video recordings of baseline and final reaching periods. We also analyzed at what lesion severity the rats begin to scoop or drag the pellet to their mouth rather than actually grasping it from the shelf. This compensation is reported for each lesion category (established as described below) as the number of rats that scoop or drag the sugar pellets to their mouth as a percent of the total number of rats in each category.

3.3.4 Perfusion and Tissue Collection

Following 7 weeks of recovery after SCI, rats were euthanized with an overdose of pentobarbital (Euthanyl, Biomeda-MTC, Cambridge, ON, Canada) and transcardially perfused with saline followed by a 4% formalin solution containing 5% sucrose. The spinal cords were dissected and postfixed in formalin solution for 24 hours before cryoprotection in 30% sucrose for three days. Spinal cords were mounted onto filter paper using Tissue Tek (Sakura Finetek, Torrance, CA, USA), frozen in methylbutane at -45 ºC and stored at -80 ºC.

3.3.5 Histology

Cervical spinal cords including the lesion site were cut at 25 µm on a cryostat. For cresyl violet staining, tissue was dried for 30 min at 37 ºC so that it adhered to the

slide. Tissue was then rehydrated in TBS, placed in 0.1% cresyl violet for 3 min, dipped in dH_2O 3 times to remove excess cresyl violet and then serially dehydrated in EtOH (50%, 75%, 99%) for 1 min each. Following dehydration, tissue was cleared in xylene for 1 minute and coverslipped with Permount (Fisher Scientific, Ottawa, ON, Canada).

3.3.6 Lesion Size Quantification

Cresyl violet-stained spinal cord sections were assessed for lesion size using light microscopy (Fig. 3.1B). Each lesion was reconstructed on a schematic of a C4 spinal cord cross section (Fig. 3.1C) based on a stereotaxic atlas (Paxinos and Watson, 1998) and the amount of lesioned area was calculated as a percent of the total cross sectional area in ImageJ (NIH, Bethesda Maryland, USA). Using ImageJ, the lesion was analyzed further to specify the extent of injury to particular spinal tracts such as the corticospinal tract (CST) and the rubrospinal tract (RST) using reported projection sites (Kuchler et al, 2002). Although DLQ or hemisection lesions were intended, the extent of lesion varied and rats were therefore grouped more precisely into specific lesion categories based on size and location. These categories include 'minimal' (n=8) lesions where the dorsal CST and the RST were spared and there was minimal damage to the dorsal horn, 'small' (n=51) lesions which had incomplete, unilateral damage to the dorsal CST and the RST, DLQ (n=41) lesions which included the dorsal funiculus containing the dorsal CST on one side of the spinal cord and a large percentage of the RST projecting in the lateral funiculus, 'large' (n=39) lesions which extended more

ventrally and included all RST projections on one side of the spinal cord, leaving mainly reticulo- and vestibulospinal tract fibers intact, and finally, 'hemisection' (n=6) lesions which unilaterally ablate the entire half of the spinal cord, the maximal lesion extent that was studied. Figure 3.1C depicts schematics of the minimal and most extensive lesions included in the analysis.

3.3.7 Statistics

Statistical analyses included Kendall's tau tests for all correlations (SPSS, IBM, Armonk New York, USA). This test was chosen because the data was not normally distributed (see floor effect in introduction). The correlation was considered significant when the correlation coefficient value was higher than 0.70. The reference values to make this decision were based on values reported by Munro (2005). GraphPad Prism (GraphPad Software, La Jolla California, USA) was used for all other statistical analyses. Two-way ANOVAs were performed to analyze the effects of training and lesion categories on reaching success as well as attempt rates. Bonferroni multiple comparison post-hoc tests were performed on reaching success rates and attempt rates of trained versus untrained rats in each lesion category. Chi squared tests were performed on zero reaching success data as well as the analysis of rats that compensate in the reaching task by scooping. All data are presented as mean ± SEM and rounded to two decimal places. A p value < 0.05 or < 0.01 was considered significant and a p value < 0.001 was considered highly significant.

3.4 RESULTS

3.4.1 Correlating Lesion Size with Reaching Success

The severity of a SCI is frequently described in two ways, either as the percentage of lesioned tissue or as spared white matter in a spinal cross section. In contrast to thoracic injuries, both gray and white matter damage at cervical levels greatly influence functional recovery so we measured the total area of lesioned tissue in this study. The results ranged from 6% to 50% of the total spinal cross section in trained animals and 8% to 52% in untrained animals. There was no statistical difference in the average lesion size between the trained and untrained group. When plotting lesion size versus success rate in the single pellet reaching (Fig. 3.2A), it was evident that there is considerable variability (especially between rats with lesion sizes of about 15% to 40% of total cross sectional area) and there were animals with 0 success rates throughout the lesion spectrum. This variability was reflected in small correlation coefficients, -0.25 and -0.16 for untrained and trained rats, respectively.

A possible reason for high variability in reaching success is that the extent of injury to gray matter or particular descending spinal tracts projecting in specific spinal areas rather than total percent lesioned area may influence this relation. Therefore, we analyzed the relationship between reaching success and gray matter damage, CST damage (main projection in the dorsal funiculus only; Fig. 3.2B) and RST (dorsolateral funiculus) ablation Fig. 3.2C. Gray matter damage did not correlate with reaching recovery (data not shown). Because the CST plays a major

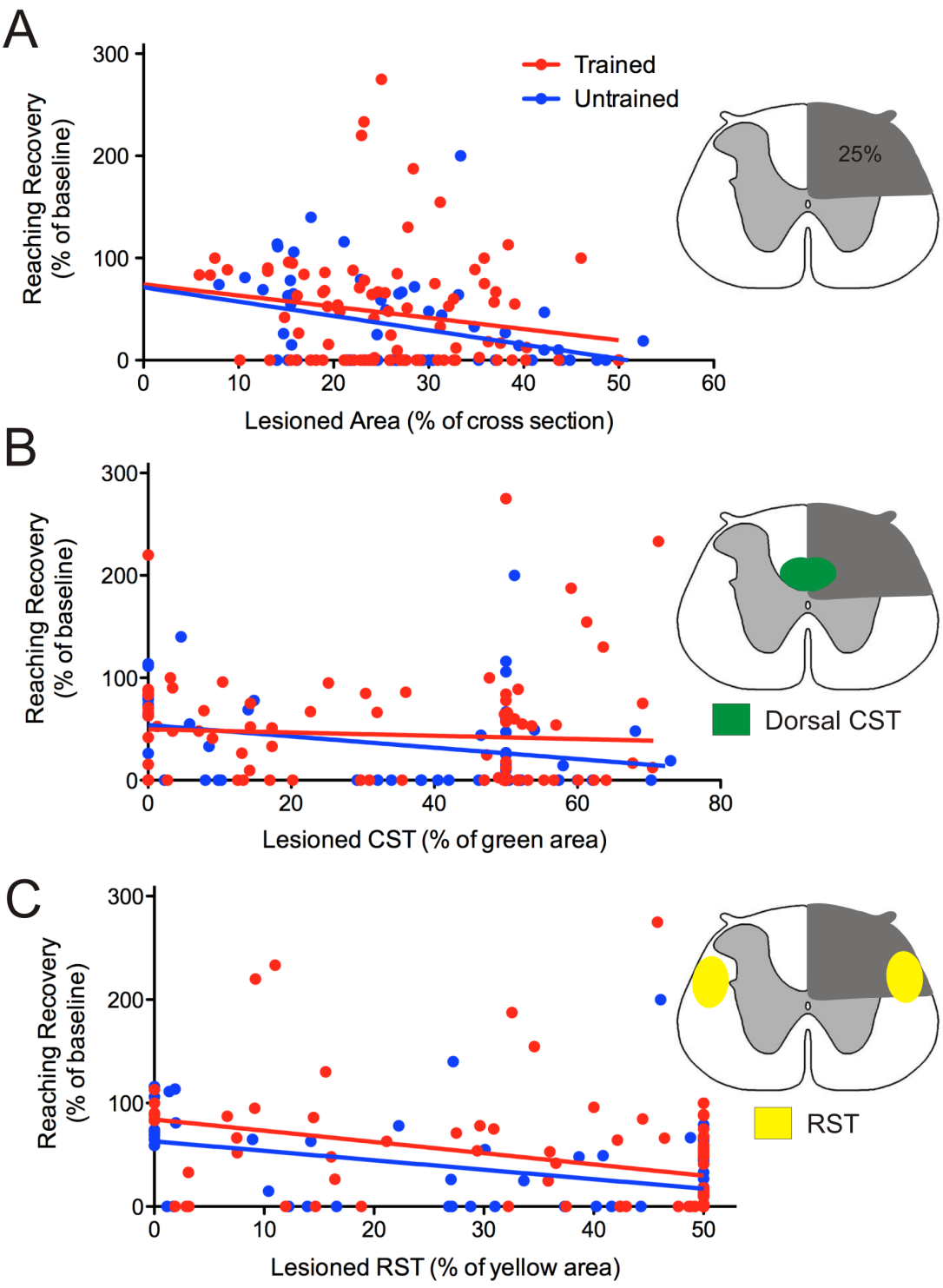

Figure 3.2

Correlations of lesion and reaching success.

Figure 3.2

Correlations of lesion and reaching success.

A) Total lesioned area was quantified using lesion reconstructions and was not found to be correlated with final reaching success for untrained (blue) and trained (red) rats (Kendall's tau = -0.25 and -0.16, respectively) Cross section schematic (right) depicts the area quantified for the mean lesion size, the DLQ lesion (grey). B) Reaching success was not found to be correlated with extent of CST injury for untrained (blue) and trained (red) rats (Kendall's tau = -0.16 and -0.10, respectively). CST injury is calculated as the injured portion of the CST as a percent of the total (bilateral) dorsal CST projection, depicted in the cross section schematic (right). C) Reaching success was not correlated with extent of damage to the RST for untrained (blue) and trained (red) rats (Kendall's tau = -0.33 and -0.31, respectively). Cross section schematic (right) demonstrates the quantification for the most common, DLQ lesion where the RST damage is expressed as a percent of the total, bilateral RST projection.

role in successful single pellet reaching, we hypothesized that the extent of CST injury would be negatively correlated with final reaching scores. In other words, greater injury to the CST would result in greater reaching impairments and therefore, a lower success rate. This hypothesis was not confirmed for untrained nor trained rats. Recovery was not significantly correlated with the extent of CST injury for untrained or trained rats (Kendall's tau = -0.16 and -0.10 respectively). The extent of damage to the RST was also not significantly correlated with final reaching scores for untrained (Kendall's tau = -0.33) or trained (Kendall's tau = -0.31) groups.

3.4.2 Effects of Lesion Severity, Location and Training

Because there was no correlation between reaching success and lesion size, we explored whether there was a relationship between lesion and functional outcome when the extent of injury to the CST and RST were both taken into account. All animals were sorted into 5 lesion categories based on lesion location (Fig. 3.3A, as described in Materials and Methods). The reaching recovery of Lewis rats was comparable to Fisher rats with similar lesion severities, so the inclusion of both strains of rats was considered admissible. We found a statistically significant effect of lesion category on reaching recovery irrespective of training (Fig. 3.3A; two-way ANOVA, $p < 0.05$). This confirms the importance of lesion size and location on post-injury reaching recovery. More importantly, there was no effect of training found across all lesion categories with a two-way ANOVA analysis

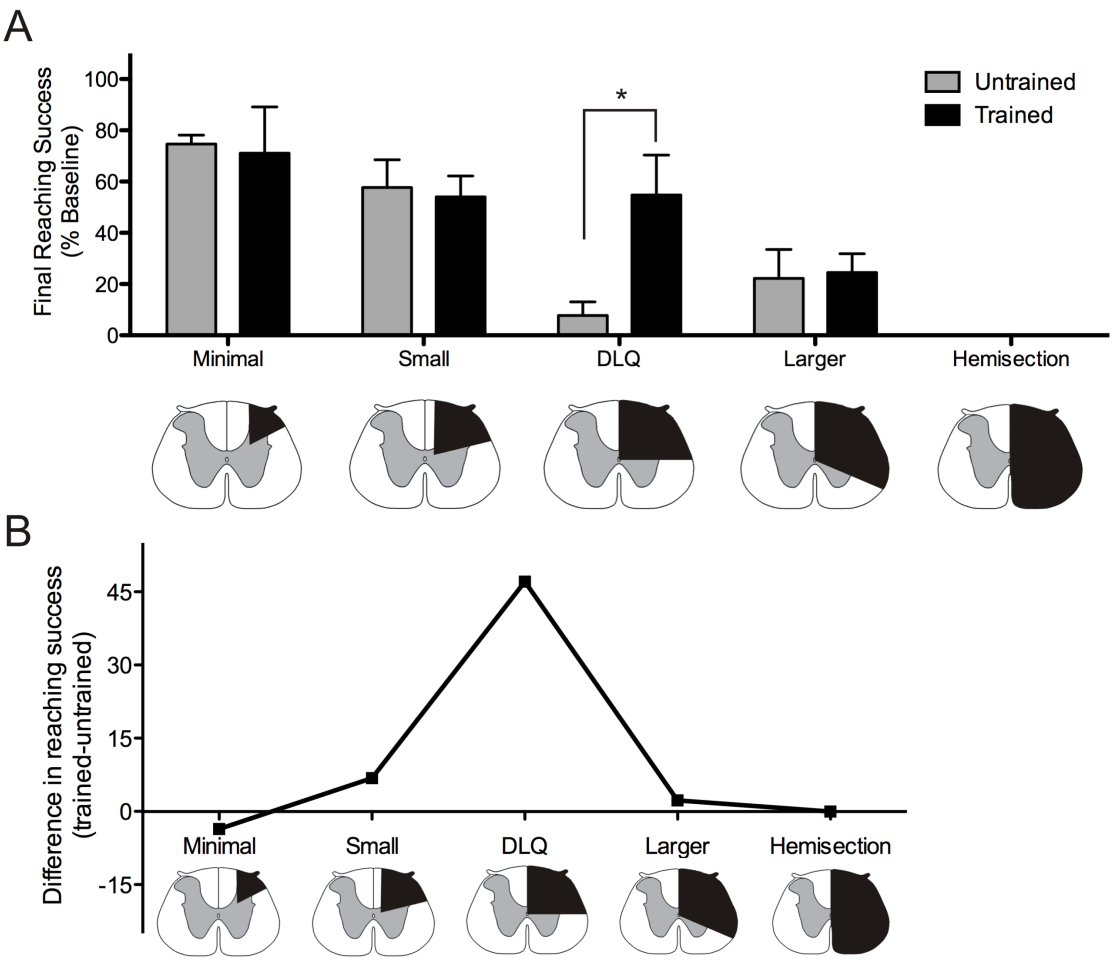

Figure 3.3

Reaching success of trained and untrained animals in each lesion category.

A) Average single pellet reaching success for untrained (grey) and trained (black) rats for each lesion category, as depicted in schematics below graph. There was a significant main effect of lesion category ($p < 0.05$) and there was a significant difference in average success rates between untrained and trained rats in the DLQ lesion category ($p < 0.05$). B) Difference in reaching success between trained and untrained rats at each lesion category, expressed as the average reaching success of trained rats minus the average reaching success of untrained rats. (* indicates $p < 0.05$)

($p > 0.05$). This can likely be explained by a ceiling effect, animals with a small lesion spontaneously recovered close to baseline success rates and further improvement in their reaching performance with rehabilitative training could not be detected. In addition, animals with severe lesions might have been subjected to a floor effect. They may have been responsive to training, but not to a degree quantifiable with single pellet reaching, and thus remained completely unable to successfully grasp food pellets. Reaching success of untrained animals in the 'minimal' lesion category was comparable to average success rates of trained animals (74.7% ± 3.5 and 71.1% ± 18.0 respectively). Average success rates were also comparable in the 'small' lesion category (untrained animals, 57.7% ± 10.9 and trained animals, 54.0% ± 8.2). The biggest difference was in the 'DLQ' lesion category where untrained animals had a much lower average reaching success than trained animals (7.7% ± 5.3 and 54.8% ± 15.5 respectively). The 'larger' and 'hemisection' categories showed very little or no difference in reaching success between untrained and trained animals ('larger' group, 24.2% ± 7.3 and 36.6% ± 11.2 respectively and average reaching success of 0 ± 0, both untrained and trained groups in 'hemisection' category). It is evident from these data that animals in the 'DLQ' lesion category, which showed significant reaching deficits but were still able to reach, significantly benefit from rehabilitative training. Unlike post-hoc comparisons for all other lesion categories, a Bonferroni multiple comparison post-hoc test revealed a significant effect of training in the 'DLQ' lesion category ($p < 0.05$, Fig.3.3A). These results are summarized in figure 3.3B, which shows the difference in reaching success for each lesion

category, expressed as the average reaching success of trained rats minus average reaching success of untrained rats.

3.4.3 Compensatory Strategy

The finding that lesion size did not correlate with the reaching success rate could possibly be explained by the implementation of different (compensatory) reaching strategies with increasing lesion severity. The use of compensatory movements has been characterized following CNS injuries (Alaverdashvili and Whishaw, 2010; Gharbawie et al., 2005; Starkey et al., 2011), and could allow for the maintenance of success rates over a wide range of lesion severities. To investigate this possibility we analyzed the use of different reaching strategies with increasing lesion sizes. A strategy that significantly increased in parallel to increases in lesion size was that pellets were not actually grasped, but scooped or dragged towards the mouth (Fig. 3.4A). We found that for untrained animals, 0% of rats in the 'minimal', 0% of rats in the 'small', 60% of rats in the 'DLQ' and 89% of rats in the 'larger' lesion category applied this strategy (Fig. 3.4B; left). In trained animals, 0% of rats in the 'minimal', 20% of rats in the 'small', 36% of rats in the 'DLQ' and 100% of rats in the 'larger' lesion category applied this strategy (Fig. 3.4B; right). Since animals with hemisections often could not reach through the opening of the reaching box, they could not be analyzed for scooping movements (Fig. 3.4B). Overall, a significant percent of untrained and trained rats employed this dragging or scooping movement with increasing lesion extent ($p = 0.006$ and

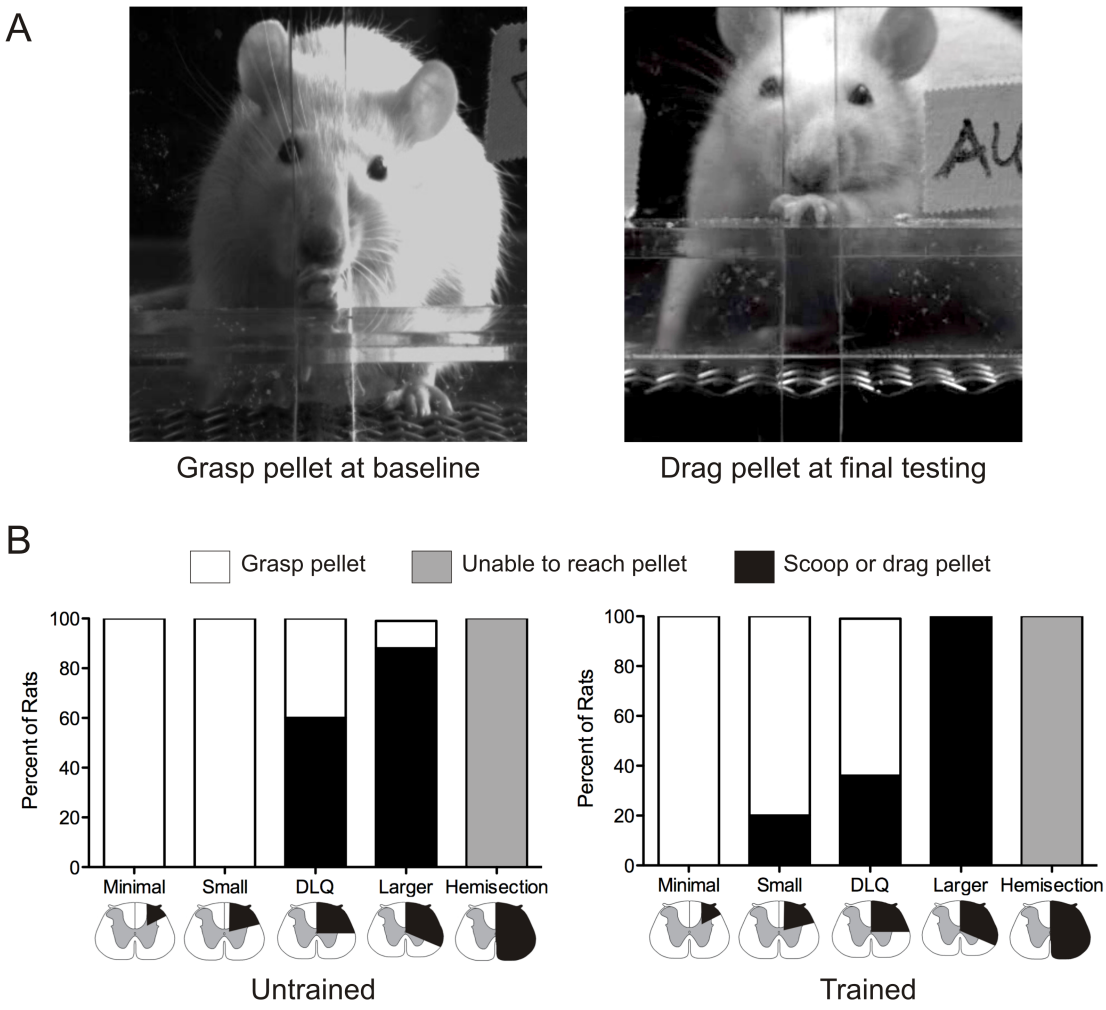

Figure 3.4

Utilization of compensatory reaching strategies.

A) Images of a representative rat reaching at baseline (left) and using a dragging strategy while reaching at 7 weeks post-injury (right). B) Scooping or dragging strategies increased significantly with increasing lesion size for untrained ($p = 0.006$) and trained ($p = 0.036$) rats. The percent of rats in each lesion category that use a scooping strategy is represented in black and the percentage of rats that reach as they did at baseline recording is represented in white. Animals in the hemisection categories (grey bars) were unable to reach through the opening in the box and could, therefore, not be analyzed for compensatory movements.

p = 0.036, respectively). For statistical reasons (i.e., Chi squared calculations are only valid when all expected values are greater than 1 and no more than 20% of the expected values are less than 5) this analysis was only performed on, the 'small', 'DLQ' and 'larger' lesion categories.

3.4.4 Zero Scores and Attempt Rates

Reaching success rates of 0 were found throughout the lesion spectrum, which reduced the strength of our statistical analysis. A reaching success of 0 provides very little information about a rat's reaching ability so it is important to identify the source of these 0 success rates. The main question is whether rats with a success rate of 0 truly cannot reach or simply attempt to reach less often and are therefore less likely to be successful. Assigning a 0 reaching success rate to rats with attempt rates of 0 may artificially decrease reaching correlations.

To better understand the reason for reaching success rates of 0 and take attempt rates into account, we analyzed the reaching success and attempt rates of the lesion categories during final testing (Fig. 3.5). Chi squared analyses of untrained and trained rats showed highly significant effects of lesion category on the proportion of success rates of 0 ($p = 0.0008$ and $p = 0.0079$, respectively). In the 'minimal' lesion category, very few rats had reaching success rates of zero (0% of untrained and 2% of trained). A comparable percent of untrained and trained rats in the 'small' lesion category had reaching success rates of 0 (22% and 25% respectively). The biggest difference was seen in the 'DLQ' group, where a greater proportion of untrained rats than trained rats had success rates of zero

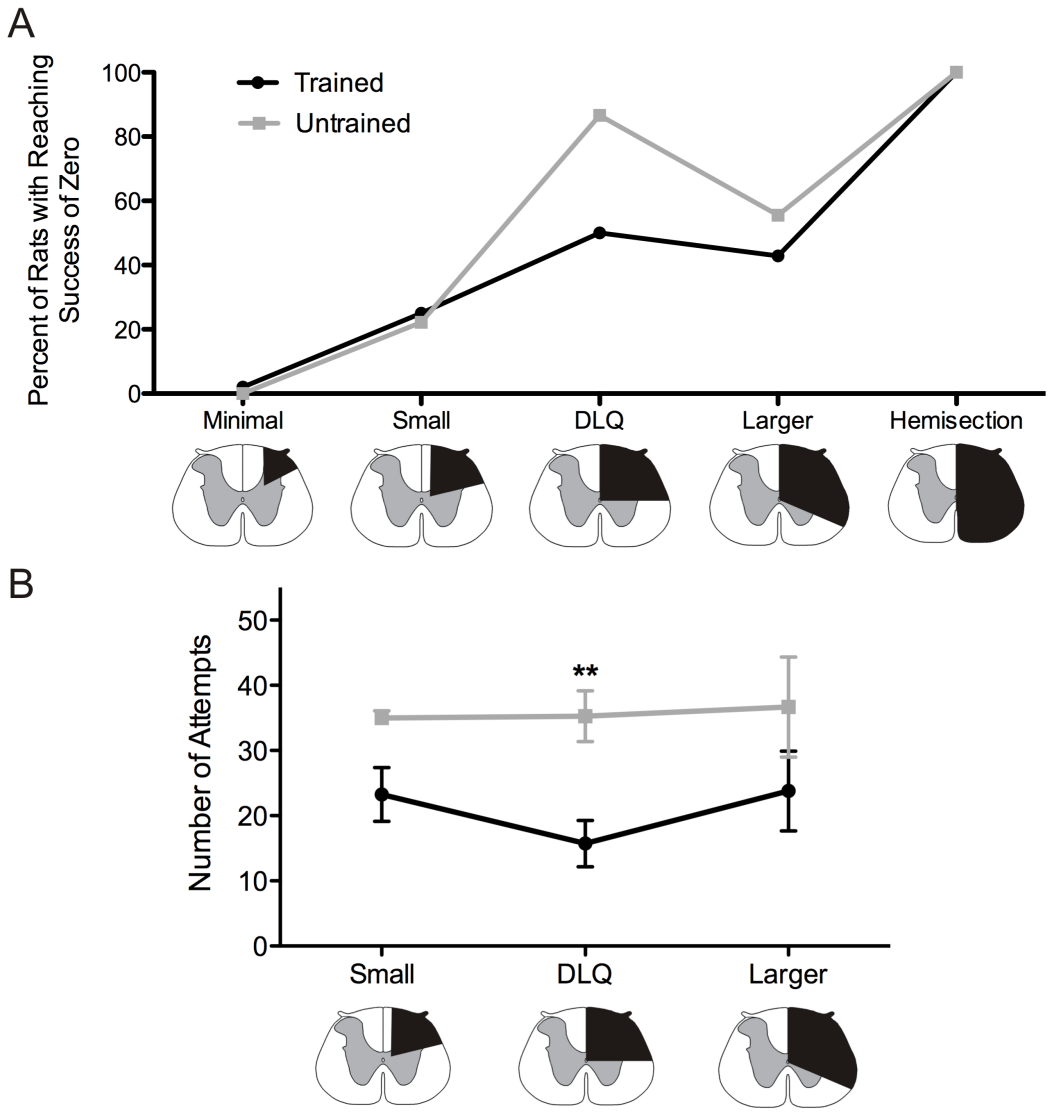

Figure 3.5

Success rates of zero and attempt rates.

A) The percent of rats with reaching success rates of zero are plotted for each lesion category in both untrained (grey line) and trained (black line) groups. There were significantly more zero success rates with increasing lesion size for untrained ($p = 0.0008$) and trained ($p = 0.0079$) rats. B) The greatest number of reaching attempts out of the final three testing days was used to calculate average attempt rates for each lesion category and are shown for untrained (grey line) and trained (black line) groups. In the DLQ lesion category, untrained animals had significantly more attempts than trained animals ($p < 0.01$). Overall, there was a highly significant training effect on reaching attempts ($p < 0.001$). (** indicates $p < 0.01$)

(87% and 50% respectively). In the 'larger' lesion category more untrained than trained rats had reaching success rates of zero (56% and 43% respectively). All hemisected rats had final reaching success rates of 0. To understand whether these zeros result from a true inability to reach or a lack of motivation to reach, we analyzed the number of reaching attempts at the final testing period for the animals with success rates of zero in each lesion category (Fig. 3.5B). The highest attempt rate out of the three days in the final testing period was used. Because there were no untrained rats with a reaching success of zero in the 'minimal' lesion category and animals in the 'hemisection' category were often unable to reach through the slot in the reaching box, the attempt rates of the 'minimal' and 'hemisection' lesion categories could not be analyzed further.

There was a highly significant effect of reaching attempts between untrained and trained rats, i.e., untrained rats had a greater number of attempts ($p < 0.001$, two-way ANOVA). In the 'small' lesion category, untrained rats with success rates of 0 reached, on average, 35 ± 1.1 times, whereas trained rats reached an average of 23 ± 4.1 times. The biggest difference was seen in the 'DLQ' lesion category where untrained rats reached significantly more than trained rats (35 ± 3.9 and 15 ± 3.5 respectively, $p < 0.01$, Fig. 3.5B). Finally, untrained rats with 'larger' lesions reached more often on average than trained rats with comparable lesions (37 ± 7.7 and 24 ± 6.1 respectively).

These data clearly demonstrate that untrained rats with reaching success rates of zero, particularly those in the 'DLQ' category, attempted to reach more, albeit less

successfully than trained rats. The fact that there was a greater proportion of animals with 0 reaching success in the untrained group was likely due to their inability to successfully grasp food pellets. Although the reason for this inability remains unclear, our data suggest that training promotes compensatory movement strategies.

3.5 DISCUSSION

3.5.1 The "Optimal" Lesion Model

Although SCI research has substantially increased our basic understanding of the processes limiting the self-repair of the injured CNS, effective treatments to promote repair are still missing. This could be due in part to limitations in animal models, where functional recovery is a key outcome measure. Devising sensitive functional tests and scores according to lesion location and severity is important to ensure that the potential to observe meaningful treatment effects is maximized. It is possible that some treatments have been dismissed prematurely because functional recovery could not be detected. Recently there has been increasing interest in rat models of incomplete cervical SCI, which allow for the testing of treatment effects on hand/paw function. This led to the challenge of matching lesion type and severity to appropriate functional outcome measures. Our study investigated the optimal lesion extent and location of a cervical SCI in rats to assess the recovery of single pellet reaching, a task involving CST function. Our results indicate that the optimal lesion to increase the effectiveness of testing

plasticity-promoting treatments (e.g., training) was a dorsolateral quadrant lesion, ablating the dorsal funiculus and part of the lateral funiculus. Considering such a specific lesion is needed to observe treatment effects in addition to the inherent variability in lesion size, it becomes clear that only robust treatment effects can be detected with this test. Nevertheless, this might be advantageous for successful translation into the clinic (Kwon et al., 2011; Reier et al., 2012).

3.5.2 Motor Tracts and Lesion Deficits

The question of what spinal lesion size and location at thoracic level would provide an optimal window to test locomotor recovery has been explored previously (Loy et al., 2002; Schucht et al., 2002). These studies showed that evaluations of locomotor recovery are not necessarily linearly correlated with lesion size. Further, the location of the injury is important in predicting functional outcome because even a small amount of spared tissue in the ventral and ventrolateral funiculus allowed for substantial recovery, but comparable sparing in the dorsal funiculus did not. These findings differ from those of the current study following cervical injuries and the evaluation of reaching abilities. For example, lesion sizes beyond the DLQ resulted in large, sustained impairment in reaching ability. Because ventral lesions were not examined in the current study, no comparison can be made to analyze whether a similar lesion size of the ventrolateral funiculus would allow for the recovery of reaching.

The effects of the extent and location of lesion at the cervical level have been previously investigated, providing evidence for the role of particular descending spinal tracts for motor recovery. It is clear from studies investigating lesions to specific tracts or graded injuries (Dunham et al., 2010; Gensel et al., 2006; Kanagal and Muir, 2007; Morris et al., 2011) that the CST and RST are important for recovery of reaching following SCI. More specific lesions of the CST at the level of the pyramids showed that the CST was found to be important for skilled reaching performance (Whishaw et al., 1998) however, pathways in the dorsolateral funiculus (e.g., the RST) may compensate for CST loss. This is consistent with our finding that reaching ability significantly declines with greater injury to the lateral funiculus (Fig. 3.3A).

The observation that deficits from RST lesions cannot be overcome by training suggests that the contribution of the RST to reaching is more significant than that of the CST in rats or that, in combination with a RST lesion, considerable damage was done to the reticulospinal tract. Extensive damage to these tracts would intensify the lesion effect because the CST lesion could not be compensated for.

An important factor to consider is that to investigate plasticity of descending tracts, lesions were applied using a blade, which spared most of the ventral gray matter. Contusive injuries (that inherently cause greater spread of injury in gray matter) would likely result in a different functional outcome, because gray matter at the cervical level of the spinal cord contains motor neurons that directly innervate forelimb muscles.

3.5.3 Zero Success Rates

One of the most striking observations when looking at the reaching data is the number of rats with reaching success rates of 0, which may result from an inability to reach successfully or, although rats are food-restricted prior to reaching sessions, from a lack of motivation to reach or a learned nonuse (Erickson et al., 2007). Our results show that the 0 reaching successes in the untrained groups, especially in the DLQ lesion category, are due to an inability to reach successfully. Importantly, this suggests that the decreased occurrence of success rates of 0 in trained rats is actually based on improved motor abilities with rehabilitative training, possibly through compensatory movement strategies.

3.5.4 Outcome Measures and Compensation in Single Pellet Reaching

Single pellet reaching can be quantified in various ways. These include the absolute number of pellet retrievals (i.e. success without recording attempt rates; Schrimsher and Reier, 1993), the success rate achieved when a standard number of pellets are presented (e.g., 20, Erickson et al., 2007; Farr and Whishaw, 2002) or success achieved within a particular amount of time (as was used in the current study). These various ways to characterize reaching success have different benefits and disadvantages. For example, measuring success as successful retrievals out of number of attempts better accounts for motivational issues, rather than an analysis that looks at just the number of pellets retrieved and therefore may be more affected by stress.

Another way to analyze reaching recovery is to use movement scores, scoring different portions of reaching and movement pattern, which provides a qualitative analysis of reaching. Such a score offers the obvious advantage that information is gained about the reaching strategy, however, the discrimination between compensatory changes and injury-induced deficits is difficult. Further, qualitative analyses add much more time to an already labor-intensive testing paradigm. This is an issue because testing drugs or potential treatments needs to be performed in many animals and therefore relatively fast, efficient analyses are often preferable. Lastly, compensation generally has a negative connotation, however, recovery of function in a clinical setting, even by the adaptation of compensatory strategies, is of importance. Since restoration of original function following SCI is currently not achievable, the promotion of plasticity and the subsequent compensation appears desirable.

Compensation in single pellet reaching following stroke has been discussed in detail in the literature (Alaverdashvili et al., 2008; Alaverdashvili and Whishaw, 2010; Gharbawie et al., 2005; Metz et al., 2005). To compensate for impairments after injury, changes in body (Farr and Whishaw, 2002) and head (Gharbawie et al., 2005) posture were reported. It is likely that recovery in reaching success in our study is based on some form of compensation that may include a mechanism such as scooping (Fig. 3.4). Earlier reports show that lesioned rats recover by using compensatory mechanisms (Gharbawie and Whishaw, 2006, Metz et al., 2005), which are likely perfected by training. Neuronal plasticity may be seen as a neuronal correlate of functional

compensation. In other words, training and any form of motor learning will engage and rewire neuronal circuitry in an alternate fashion (Girgis et al., 2007; Nudo et al., 1996), which might or might not result in observable compensatory movement strategies (Ballerman and Fouad, 2006). An analysis of compensation was performed in this study to provide more insight into the reason for the lack of correlation observed in our data. It is evident that compensation strategies become most prevalent with more extensive lesions (Fig. 3.4B). This suggests that a lack of linear decline in reaching success with increasing lesion size may be due to compensation; the new strategy maintains success scores at a higher level with increased lesion severity.

3.5.5 Variability

Variability in success rates is an issue that can be found between animals but more importantly between testing days in the same animal. Variability between animals with similar lesions may result from different compensatory strategies as discussed above, different suppliers or different batches of animals (O'Bryant et al., 2011). The variability in individual animals can only be explained by other factors including stress, appetite, time of day or year (O'Bryant et al., 2011), day of the week (Schubring-Giese et al., 2007), estrus cycle (Allred and Jones, 2004) as well as the trainer (e.g. differences in experience of the trainer; Schubring-Giese et al., 2007). To control for some of these variables we trained and tested food restricted rats in a secluded room with

the radio on for background noise. Rats were trained by the same person at the same time each day and no stressful procedures involving other animals in the room were performed before testing. By controlling for as many of these conditions as possible we limited variability between training days in the rats, however, this did not completely reduce the variability, which can be seen as an inherent weakness of the test.

3.5.6 Floor and Ceiling Effects

To maximize the potential for statistically significant outcomes following treatments, a relatively large difference in performance between untreated and treated rats is needed. Considering that the baseline success rate for uninjured rats in single pellet reaching, depending on the strain of rat, can be as low as 30-40%, this window is already limited (Fig. 3.6). An experimentally valuable lesion should significantly decrease the animals' functional abilities from the baseline level to avoid ceiling effects, but ideally should not lead to a floor effect (i.e., reaching scores of 0). If animals reach a success rate of 0 and a treatment does not improve function, it is unclear whether the treatment was ineffective or the task was too challenging.

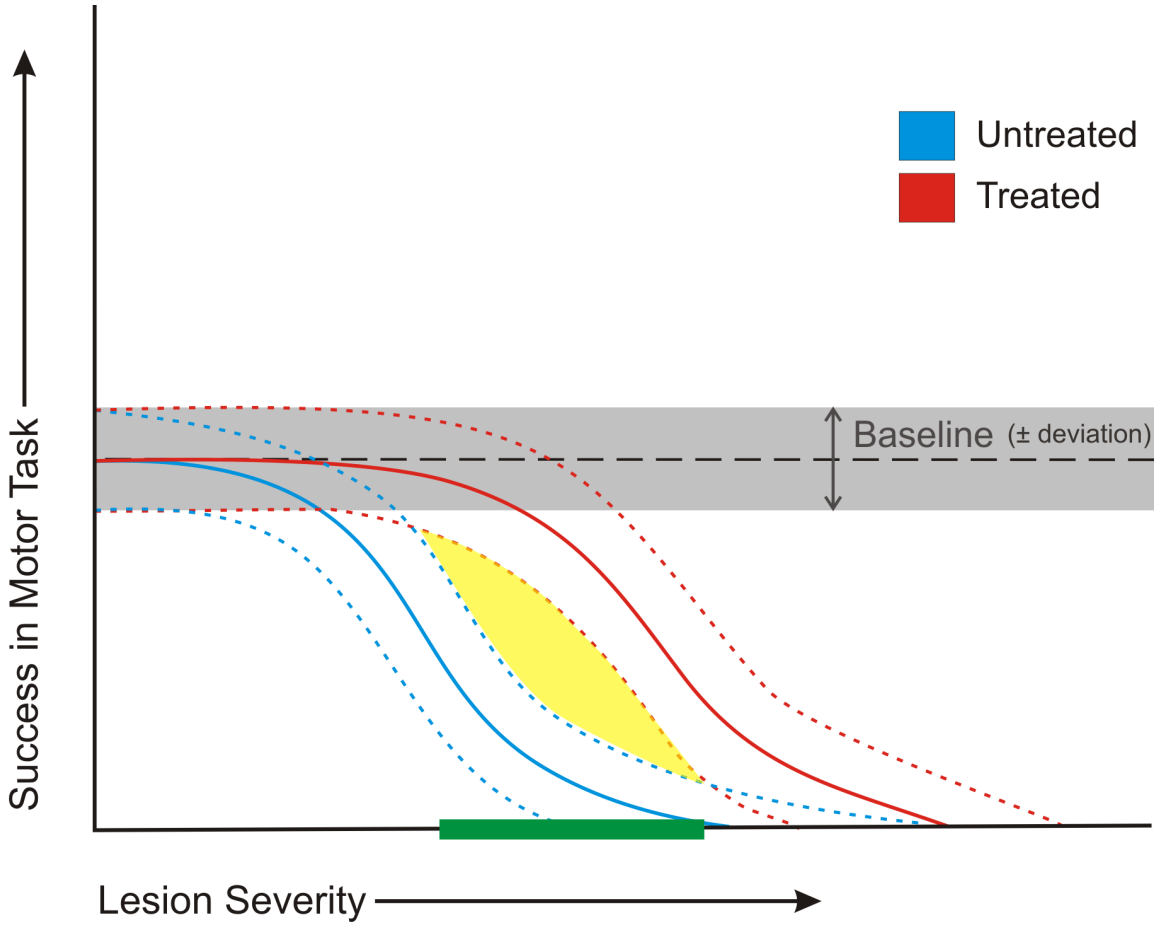

Figure 3.6

Schematic of the potential to observe motor recovery.

The average baseline motor score and standard error of the mean are represented by the black dashed line and light grey, rectangular area, respectively. The y axis refers to success in a motor task with greater success at the top and lower success rates towards the bottom. The x axis represents lesion severity, with smaller lesions on the left and extremely severe lesions on the right. Motor success and deviation with respect to lesion severity for untreated animals are represented by the solid blue line and dashed blue lines, respectively. The solid and dashed red lines represent success and deviation, respectively, with a treatment. The yellow area is the region in which a treatment effect would be observed and therefore, may correspond to an 'ideal' lesion size (green).

3.5.7 Conclusion

To test functional recovery using the single pellet reaching task in spinal cord injured rats, a lesion like the DLQ lesion should be used. This will maximize the limited potential to observe spontaneous and treatment-promoted reaching recovery. However, following the analysis of the relation between recovery and lesion size of 145 rats, we have come to the conclusion that due to high variability, the large number of "non performers", a very small range of lesion size that allows for the detection of treatment effects, and the labor-intensive nature of the test, the single pellet reaching task is not well suited to quantify effects of currently available treatments for cervical SCI. This test may be viewed instead as a stringent behavioural test that may be useful for predicting robust effects and therefore the translational success of potential treatments for cervical SCI.

3.6 REFERENCES

Alaverdashvili, M., Moon, S. K., Beckman, C. D., Virag, A., and Whishaw, I. Q., 2008. Acute but not chronic differences in skilled reaching for food following motor cortex devascularization vs. photothrombotic stroke in the rat. Neuroscience 157**,** 297-308.

Alaverdashvili, M., and Whishaw, I. Q., 2010. Compensation aids skilled reaching in aging and in recovery from forelimb motor cortex stroke in the rat. Neuroscience 167**,** 21-30.

Allred, R. P., and Jones, T. A., 2004. Unilateral ischemic sensorimotor cortical damage in female rats: forelimb behavioral effects and dendritic structural plasticity in the contralateral homotopic cortex. Experimental neurology 190**,** 433-445.

Anderson, K. D., Gunawan, A., and Steward, O., 2007. Spinal pathways involved in the control of forelimb motor function in rats. Experimental neurology 206**,** 318-331.

Ballermann, M., and Fouad, K., 2006. Spontaneous locomotor recovery in spinal cord injured rats is accompanied by anatomical plasticity of reticulospinal fibers. The European journal of neuroscience 23**,** 1988-1996.

Barbeau, H., and Rossignol, S., 1987. Recovery of locomotion after chronic spinalization in the adult cat. Brain research 412**,** 84-95.

Behrmann, D. L., Bresnahan, J. C., Beattie, M. S., and Shah, B. R., 1992. Spinal cord injury produced by consistent mechanical displacement of the cord in rats: behavioral and histologic analysis. Journal of neurotrauma 9**,** 197-217.

Dunham, K. A., Siriphorn, A., Chompoopong, S., and Floyd, C. L., 2010. Characterization of a graded cervical hemicontusion spinal cord injury model in adult male rats. Journal of neurotrauma 27**,** 2091-2106.

Eidelberg, E., and Yu, J., 1981. Effects of corticospinal lesions upon treadmill locomotion by cats. Experimental brain research. Experimentelle Hirnforschung. Experimentation cerebrale 43**,** 101-103.

Erickson, C. A., Gharbawie, O. A., and Whishaw, I. Q., 2007. Attempt-dependent decrease in skilled reaching characterizes the acute postsurgical period following a forelimb motor cortex lesion: an experimental demonstration of learned nonuse in the rat. Behavioural brain research 179**,** 208-218.

Farr, T. D., and Whishaw, I. Q., 2002. Quantitative and Qualitative Impairments in Skilled Reaching in the Mouse (Mus musculus) After a Focal Motor Cortex Stroke. Stroke 33**,** 1869-1875.

Gensel, J. C., Tovar, C. A., Hamers, F. P., Deibert, R. J., Beattie, M. S., and Bresnahan, J. C., 2006. Behavioral and histological characterization of unilateral cervical spinal cord contusion injury in rats. Journal of neurotrauma 23**,** 36-54.

Gharbawie, O. A., Gonzalez, C. L., and Whishaw, I. Q., 2005. Skilled reaching impairments from the lateral frontal cortex component of middle cerebral artery stroke: a qualitative and quantitative comparison to focal motor cortex lesions in rats. Behavioural brain research 156**,** 125-137.

Gharbawie, O. A., and Whishaw, I. Q., 2006. Parallel stages of learning and recovery of skilled reaching after motor cortex stroke: "oppositions" organize normal and compensatory movements. Behavioural brain research 175**,** 249-262.

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 : a journal of neurology 130**,** 2993-3003.

Kanagal, S. G., and Muir, G. D., 2007. Bilateral dorsal funicular lesions alter sensorimotor behaviour in rats. Experimental neurology 205**,** 513-524.

Kuchler, M., Fouad, K., Weinmann, O., Schwab, M. E., and Raineteau, O., 2002. Red nucleus projections to distinct motor neuron pools in the rat spinal cord. The Journal of comparative neurology 448**,** 349-359.

Kwon, B. K., Okon, E. B., Tsai, E., Beattie, M. S., Bresnahan, J. C., Magnuson, D. K., Reier, P. J., McTigue, D. M., Popovich, P. G., Blight, A. R., Oudega, M., Guest, J. D., Weaver, L. C., Fehlings, M. G., and Tetzlaff, W., 2011. A grading system to evaluate objectively the strength of pre-clinical data of acute neuroprotective therapies for clinical translation in spinal cord injury. Journal of neurotrauma 28, 1525-1543.

Lawrence, D. G., and Kuypers, H. G., 1968. The functional organization of the motor system in the monkey. I. The effects of bilateral pyramidal lesions. Brain : a journal of neurology 91**,** 1-14.

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. Annals of neurology 58**,** 706-719.

Little, J. W., Harris, R. M., and Sohlberg, R. C., 1988. Locomotor recovery following subtotal spinal cord lesions in a rat model. Neuroscience letters 87**,** 189-194.

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. Experimental neurology 92**,** 421-435.

Loy, D. N., Talbott, J. F., Onifer, S. M., Mills, M. D., Burke, D. A., Dennison, J. B., Fajardo, L. C., Magnuson, D. S. K., and Whittemore, S. R., 2002. Both Dorsal and Ventral Spinal Cord Pathways Contribute to Overground Locomotion in the Adult Rat. Experimental neurology 177**,** 575-580.

Metz, G. A., Antonow-Schlorke, I., and Witte, O. W., 2005. Motor improvements after focal cortical ischemia in adult rats are mediated by compensatory mechanisms. Behavioural brain research 162**,** 71-82.

Metz, G. A., Dietz, V., Schwab, M. E., and van de Meent, H., 1998. The effects of unilateral pyramidal tract section on hindlimb motor performance in the rat. Behavioural brain research 96**,** 37-46.

Montoya, C. P., Campbell-Hope, L. J., Pemberton, K. D., and Dunnett, S. B., 1991. The "staircase test": a measure of independent forelimb reaching and grasping abilities in rats. Journal of neuroscience methods 36**,** 219-228.

Morris, R., Tosolini, A. P., Goldstein, J. D., and Whishaw, I. Q., 2011. Impaired arpeggio movement in skilled reaching by rubrospinal tract lesions in the rat: a behavioral/anatomical fractionation. Journal of neurotrauma 28**,** 2439-2451.

Muir, G. D., and Whishaw, I. Q., 1999. Complete locomotor recovery following corticospinal tract lesions: measurement of ground reaction forces during overground locomotion in rats. Behavioural brain research 103**,** 45-53.

Munro, B. H., 2005. Statistical Methods for Health Care Research, fifth ed. Lippincott Williams & Wilkins, Philadelphia.

National Spinal Cord Injury Statistical Centre, 2012. Facts and Figures at a Glance. https://www.nscisc.uab.edu/reports.aspx (November 2012, date last accessed).

Nudo, R. J., Wise, B. M., SiFuentes, F., and Milliken, G. W., 1996. Neural substrates for the effects of rehabilitative training on motor recovery after ischemic infarct. Science 272**,** 1791-1794.

O'Bryant, A. J., Allred, R. P., Maldonado, M. A., Cormack, L. K., and Jones, T. A., 2011. Breeder and batch-dependent variability in the acquisition and performance of a motor skill in adult Long-Evans rats. Behavioural brain research 224**,** 112-120.

Paxinos, G., and Watson, C., 1998. The Rat Brain in Stereotaxic Coordinates. Academic Press: New York.

Reier, P. J., Lane, M. A., Hall, E. D., Teng, Y. D., and Howland, D. R., 2012. Translational spinal cord injury research: preclinical guidelines and challenges. Handbook of clinical neurology / edited by P.J. Vinken and G.W. Bruyn 109, 411-433.

Schrimsher, G. W., and Reier, P. J., 1993. Forelimb motor performance following dorsal column, dorsolateral funiculi, or ventrolateral funiculi lesions of the cervical spinal cord in the rat. Experimental neurology 120**,** 264-276.

Schubring-Giese M, Molina-Luna K, Hertler B, Buitrago MM, Hanley DF, Luft AR. Single pellet reaching task in rat: a retrospective analysis of confounding factors. 2007 Neuroscience Meeting Planner Online; 2007 [Program No. 206.5]

Schucht, P., Raineteau, O., Schwab, M. E., and Fouad, K., 2002. Anatomical Correlates of Locomotor Recovery Following Dorsal and Ventral Lesions of the Rat Spinal Cord. Experimental neurology 176**,** 143-153.

Schwartzman, R. J., 1978. A behavioral analysis of complete unilateral section of the pyramidal tract at the medullary level in Macaca mulatta. Annals of neurology 4, 234-244.

Starkey, M. L., Bleul, C., Maier, I. C., and Schwab, M. E., 2011. Rehabilitative training following unilateral pyramidotomy in adult rats improves forelimb function in a non-task-specific way. Experimental neurology 232**,** 81-89.

Steeves, J. D., and Jordan, L. M., 1980. Localization of a descending pathway in the spinal cord which is necessary for controlled treadmill locomotion. Neuroscience letters 20**,** 283-288.

Whishaw, I. Q., Gorny, B., and 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. Behavioural brain research 93**,** 167-183.

Whishaw, I. Q., O'Connor, W. T., and Dunnett, S. B., 1986. The contributions of motor cortex, nigrostriatal dopamine and caudate-putamen to skilled forelimb use in the rat. Brain : a journal of neurology 109 (Pt 5)**,** 805-843.

Whishaw, I. Q., and Pellis, S. M., 1990. The structure of skilled forelimb reaching in the rat: a proximally driven movement with a single distal rotatory component. Behavioural brain research 41**,** 49-59.

Whishaw, I. Q., Pellis, S. M., Gorny, B., Kolb, B., and Tetzlaff, W., 1993. Proximal and distal impairments in rat forelimb use in reaching follow unilateral pyramidal tract lesions. Behavioural brain research 56**,** 59-76.

Whishaw, I. Q., Pellis, S. M., Gorny, B. P., and Pellis, V. C., 1991. The impairments in reaching and the movements of compensation in rats with motor cortex lesions: an endpoint, videorecording, and movement notation analysis. Behavioural brain research 42**,** 77-91.

CHAPTER IV

GENERAL DISCUSSION OF RESULTS AND FUTURE DIRECTIONS

4.1 SUMMARY OF RESULTS

The previous chapters describe two studies that were conducted to enhance our understanding of rehabilitative training following incomplete, cervical SCI in rats. The first study (Chapter 2) focused on the mechanisms of rehabilitative training-induced plasticity and requires further discussion of issues with western blot protocols, which are addressed below. This study also demonstrated the difficulties associated with pharmacologically manipulating signaling pathways when using *in vivo* models of SCI. A similar approach to promote recovery following SCI that was recently tested in a clinical trial is discussed below. The second study (Chapter 3) analyzed the effect of various lesion sizes on recovery of reaching ability and training efficacy in a skilled reaching task. The results of this study emphasize the critical importance of matching appropriate behavioural tests to lesion severity. General considerations for choosing or establishing animal models of SCI to assess functional recovery are included below.

4.2 WESTERN BLOT METHODOLOGICAL CONSIDERATIONS

Chapter 2 describes a study in which we applied a PKA inhibitor to the forelimb motor cortex of rats throughout rehabilitative training. The intent of the study was to better understand the intracellular signaling cascades responsible for training-induced motor recovery following SCI. We showed that rats with the PKA inhibitor had greater reaching success rates than saline-treated animals,

opposite of what we had hypothesized. In order to confirm that this behavioural trend was due to the drug treatment, we performed a western blot analysis with tissue of the motor cortices of rats to ensure that the cortex of rats treated with the PKA inhibitor had lower levels of active PKA. Active PKA is phosphorylated (pPKA) and is quantified as pPKA normalized to the total level of PKA in the tissue sample. We did not observe a difference in cortical pPKA level between PKA inhibitor-treated and saline-treated groups (Fig.2.4). This result may reflect one of two possibilities, either the drug did not decrease cortical pPKA levels or we were unable to detect a difference with our current western blot protocol.

The western blot procedure used in our laboratory was adapted based on a protocol from a previous collaborator that had been shown to successfully detect levels of PKA and pPKA in the motor cortex (Krajacic et al., 2009). When no difference in pPKA level was observed between PKA inhibitor-treated rats and saline treated rats (Chapter 2), we began to re-evaluate this western blot protocol and have had difficulty producing consistent results. In order to evaluate and optimize the protocol, we have recently performed western blots of *in vitro* sacral spinal cord tissue. This *in vitro* preparation is particularly useful for two reasons; first it eliminates the process of harvesting *in vivo* tissue under anaesthesia, a component that might introduce errors. Second, the *in vitro* model allows for electrophysiological recording of motoneron activity (by recording from ventral roots of the spinal cord) at the time of drug administration and shows a clear change in activity of the treated tissue unlike the *in vivo* model, in which observing a physiological effect of the drug is more complicated. In the *in vitro*

preparation we have used, the spinal cord was bathed in artificial cerebrospinal fluid (aCSF) and forskolin, a molecule that increases adenylyl cyclase activity, thereby increasing the level of pPKA (Hurley, 1999; Seamon and Daly, 1986). By using this *in vitro* preparation, increases in spontaneous activity in motoneurons of the spinal cord can be recorded following forskolin application. The sacral spinal cord is then frozen in liquid nitrogen, lysed and subsequently analyzed with western blot procedures to determine if greater levels of pPKA can be detected in forskolin-treated spinal cords as compared to control spinal cords that are bathed in aCSF without forskolin. These experiments are ongoing and include the use of alternative antibodies, as discussed in Chapter 2, and will be necessary to optimize the sensitivity of our western blot protocol so that future drug treatments *in vivo* can be confidently evaluated.

4.3 ALTERNATIVE SIGNALING CASCADES INCLUDING RHO, ROCK AND EPAC

The results from Chapter 2 are interesting because, contrary to what was hypothesized, PKA inhibition seemed to promote greater reaching recovery following SCI as compared to a saline control group. As discussed in Chapter 2, these findings may have been the result of activation of other downstream targets of cAMP, such as Epac (Murray, 2009). Another likely possibility is that inhibiting PKA enhanced a negative feedback effect that resulted in reduced PDE activity and therefore, higher levels of cAMP than would have been predicted

(Sunahara et al., 1996). Our results emphasize the importance of a systematic evaluation of the signaling cascades involved in training-induced plasticity. In the future it will be necessary to repeat our behavioural findings and use pharmacological blockade and activation of additional components of the cAMP/PKA pathway to better understand the intracellular mechanisms involved. For example, it will be necessary to perform a similar experiment using an Epac inhibitor, such as CE3F4 (Courilleau et al., 2012), given the evidence that it may be Epac and not necessarily PKA that is responsible for neurite outgrowth (Murray et al., 2009). To enhance the effects of PKA and Epac inhibitors *in vivo*, it will also be important to administer them in combination with PDE inhibitors. This would allow for better control of cAMP levels, which could be dramatically increased with PDE inhibitors, and therefore result in greater activation of PKA or Epac and likely more striking behavioural effects.

Pharmacologically inhibiting one component of an intracellular signaling cascade to enhance functional recovery in an *in vivo* model of SCI is not a novel approach. The ambiguous and sometimes unexpected results in such an experimental manipulation have been reported previously. One classic example is the development of Cethrin, a Rho antagonist. Rho is a guanine triphosphatase (GTPase) that activates ROCK kinase. The Rho-ROCK pathway is one of the primary regulators of actin dynamics in cells and is therefore responsible for axon growth and guidance. When the Rho-ROCK pathway is activated, which can occur by signaling from CSPGs and membrane-bound myelin inhibitors following SCI, growth cone collapse is triggered (Gopalakrishnan et al., 2008; Yamashita

and Tohyama, 2003). So it follows that antagonists of Rho (e.g., BA-210, trademarked Cethrin) and ROCK (e.g., Y-27632) would inhibit growth cone collapse and therefore may enhance functional recovery following SCI. Early *in vitro* assays demonstrated that Rho and ROCK inhibitors enhanced axon growth in the presence of the inhibitory CSPGs from glial scar tissue (Monnier et al., 2003). *In vivo* studies demonstrated similar results including promotion of axonal regeneration in an optic nerve injury model (Lehmann et al., 1999), and axonal sprouting and motor recovery in rodent models of SCI (Dergham et al., 2002; Dubreuil et al., 2003; Hara et al., 2000). These promising results were eventually tested in humans in a phase I/IIa clinical trial to evaluate safety and tolerability of the Rho antagonist Cethrin. Individuals with complete injury (defined as American Spinal Injury Association [ASIA] A) at the cervical or thoracic level of the spinal cord were included in the study and all participants received Cethrin treatment (doses ranged from 0.3 mg to 9 mg). There was very little motor recovery over one year for those with thoracic SCI, however, those with cervical SCI demonstrated more substantial recovery, particularly those who received an intermediate concentration of the drug (3 mg; Fehlings et al., 2011; Fig. 4.1). It is important to note that this trial did not include a non-Cethrin-treated (i.e., vehicle) control group. This is important because individuals were treated within 72 hours of SCI so some spontaneous motor recovery would be expected over time but all the observed motor recovery was attributed to Cethrin treatments because of the lack of a control. Interestingly, clinical trials for Rho antagonists have since stopped (ClinicalTrials.gov, 2010) supposedly as a result of limited funding.

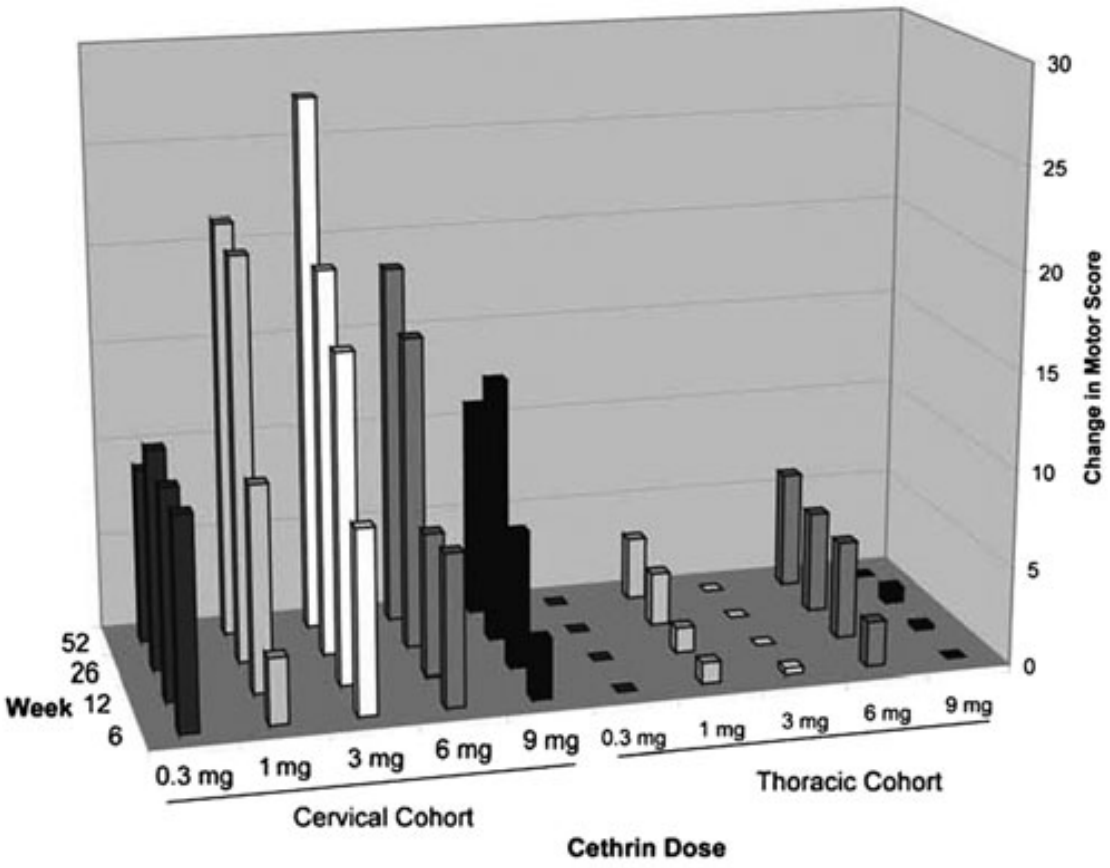

Figure 4.1

Changes in motor score over time for all Cethrin treatment doses.

The cervical cohort of patients and the thoracic cohort of patients were followed for 52 weeks. The mean change in ASIA motor scores is shown for each dose group at each time point. Ten key muscle groups, five in the arm and five in the leg, were tested bilaterally, and given a score of 0-5 each, resulting in a possible total score of 100. Figure adapted from Fehlings et al., 2011.

It is clear that the study and manipulation of intracellular signaling using *in vivo* models of SCI is extremely complex. These are highly regulated signaling cascades given their importance in growth and development. For this reason as well as potential interactions with other pathways, they remain difficult to manipulate in an *in vivo* model. For example, the Rho-ROCK and cAMP-PKA pathways are related through the inhibition of RhoA by PKA, which results in neurite formation (Dong et al., 1998). By inhibiting the components of either pathway, the other is certainly affected. Even though the aim of the study described in Chapter 2 of this thesis was not to identify a new potential treatment for SCI, but merely to better characterize the pathways involved in motor recovery with training, it is important to keep these considerations in mind. Given the reported difficulties in the study of these cascades, the systematic targeting of multiple components may allow for elucidation of the mechanisms involved in training-induced motor recovery following SCI in the future.

4.4 ANIMAL MODELS OF SCI

The results from Chapter 3, the study of an incomplete cervical lesion model and recovery in the single pellet skilled reaching task, identified the "ideal" lesion size to study plasticity-promoting treatment effects on reaching recovery following incomplete injury. This calls attention to some of the factors and limitations that must be taken into account in the design of appropriate animal models to test potential treatments for SCI such as the extent of injury to particular descending motor tracts and compensatory behavioural strategies. This

study demonstrated the surprisingly limited "window of opportunity" in lesion size that allows for detecting treatment-induced reaching recovery. These results can be considered a prompt to generate discussions leading to greater refinements and more critical analyses of behavioral outcome measures of potential treatments for SCI in an attempt to translate them to the clinic.

The use of animal models has enabled much progress in the field of SCI over the past few decades. Unfortunately, there are still no effective treatments to repair the injured spinal cord, likely because promising treatment approaches are often not able to be reproduced and are rarely able to be translated to the clinic. For this reason, studies like the one described in Chapter 3 of this thesis may be increasingly important for re-evaluation of laboratory models of SCI. The re-evaluation of animal models should include factors such as the way lesions are modeled in the laboratory (e.g., incision or contusion) and differences in projection and contribution of various motor tracts, factors that can influence the degree of functional recovery with a potential treatment as well as how relevant it may be for humans with SCI.

In terms of type of lesion, it is important to note that the majority of human SCIs are due to mechanical forces and result in contusion injuries (Bunge et al., 1993). These injuries often span more than one spinal segment and are particularly detrimental to gray matter due to spreading ischemia (Norenburg et al., 2004). Additionally, in contrast to incision lesions for example, contusion injuries tend to trigger a greater inflammatory response and may result in secondary processes such as hemorrhage, cavitation and demyelination

(Siegenthaler et al., 2007; Tator, 2006). For these reasons, it is important to evaluate the type of lesion that will be used for a given study, a decision that is often based on the focus of the experiment. For example, if promoting axonal regeneration is the objective of a study, a complete incision lesion is ideal in order to most accurately examine regenerated axons. In contrast, if potential neuroprotective treatments were to be evaluated, a contusion injury would be preferable. It is important to note, however, that studying contusion injuries at the cervical level of the spinal cord is particularly difficult. This is because the large extent of injury spread in gray matter can substantially damage motoneuron pools and therefore results in detrimental effects on reaching recovery. Also, compared to more controlled incision injuries, contusion injuries often spread across the midline of the spinal cord. This is particularly difficult for cervical models of SCI because of animal wellbeing and welfare issues.

To translate potential treatments to the clinic, a survey of professionals in the field of SCI found that the majority of respondents felt that the contusion model is the most clinically relevant model of SCI but that a therapy should be shown to be effective in different injury models and different injury severities before being translated into clinical trials (Kwon et al., 2010). In the case of the study of mechanisms of training-induced functional recovery (Chapter 2), our aim was to evaluate the effect of a PKA inhibitor and training in three groups with similar lesion locations and severity so an incomplete, controlled lesion was necessary. If these studies were to result in a potential treatment in the future, it

would be necessary to evaluate the effectiveness of the treatment in a more clinically relevant lesion model such as a contusion injury.

Another factor to consider in animal models of SCI is the difference in projection of particular motor tracts between animals, particularly rodents, and humans. In addition, the differences in contribution to motor function of these tracts between species should also be taken into account. This point is especially important for potential treatments that target the CST. The CST in primates and humans is essential for voluntary motor control (Lawrence and Kuypers, 1968), whereas in rodents, the CST plays a more minor role, contributing mostly to fine motor control (Whishaw et al., 1998). Anatomically, there is a much greater proportion of the cortex that forms the CST in humans compared to rats and the CST axons project in the lateral columns of the spinal cord in humans and nonhuman primates, whereas they project in the dorsal column in rats. There are also differences in the termination of CST axons between the rat and human spinal cords. As shown in figure 4.2 (adapted from Courtine et al., 2007), the CST in rats projects mainly to interneurons, whereas many of the CST axons in humans and nonhuman primates synapse directly onto motoneurons (Lemon et al., 2004). This is interesting when discussing animal models because the number of direct connections between the cortex and motoneurons strongly correlates with the level of manual dexterity, as reported in nonhuman primates (Lemon et al., 2004). An additional consideration for the projection of the CST is that the CST in nonhuman primates decussates extensively across the spinal cord midline (Rosenzweig et al., 2009) and likely contributes to spontaneous motor recovery

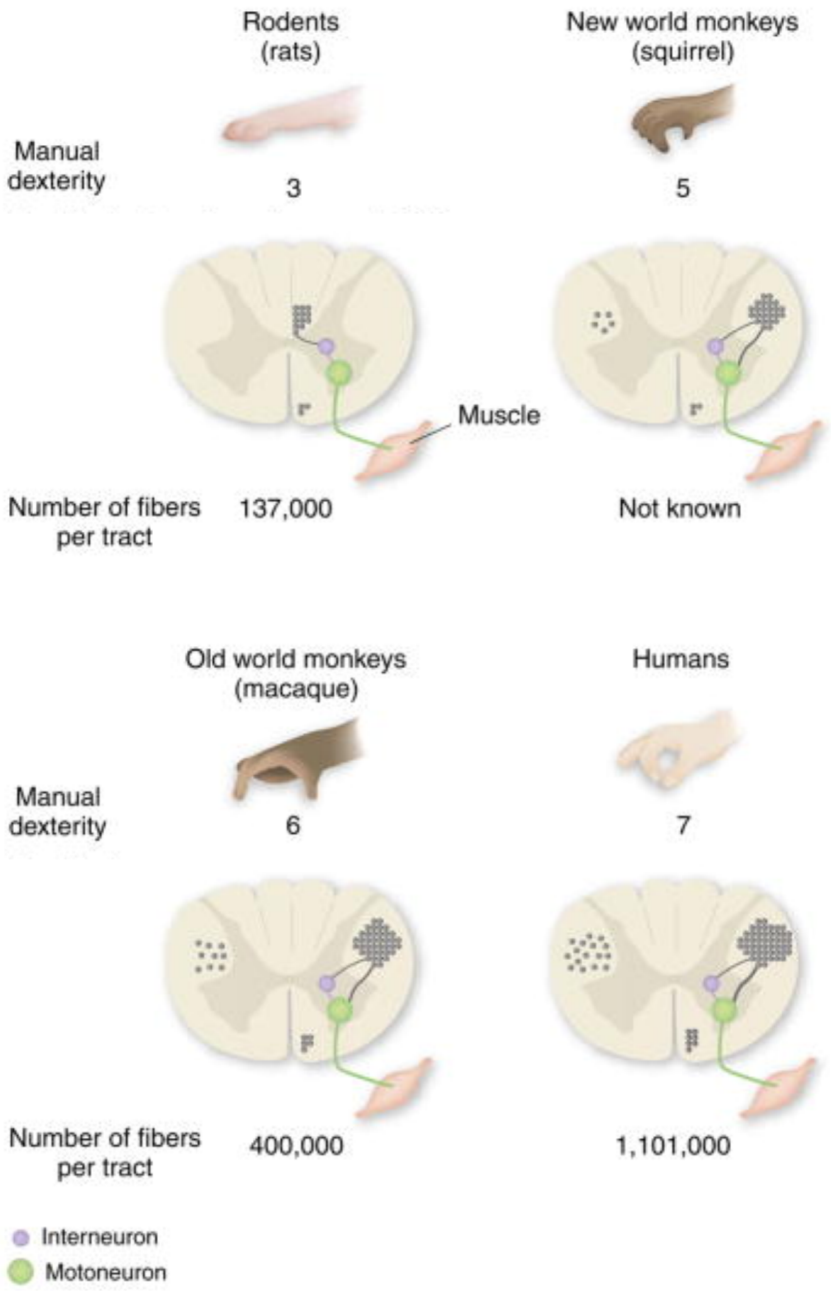

Figure 4.2

Relationship between the development of the corticospinal tract and the emergence of fine motor control abilities.

Figure 4.2

Relationship between the development of the corticospinal tract and the emergence of fine motor control abilities.

In rodents, there are no direct connections between corticospinal neurons and the cervical motoneurons that innervate forelimb muscles—interneurons relay cortical input to motor neurons. In the evolution of the corticospinal tract in nonhuman primates and humans, direct corticospinal connections with motoneurons have emerged, together with an increase in the size and number of the corticospinal fibers. Furthermore, most of the corticospinal tract fibers in rodents travel in the dorsal columns. In contrast, the primate corticospinal tract is mostly located in the lateral columns, and a significant proportion of corticospinal fibers (10–20%) descend ipsilaterally. Development of the corticospinal tract correlates with the improvement in the index of dexterity, particularly in the ability to perform finger-thumb precision grip. Figure adapted from Courtine et al., 2007.

observed after incomplete SCI in humans (Rosenzweig et al., 2010). These anatomical differences, therefore, warrant the testing of potential treatments for SCI in larger, nonhuman primate models following promising results in rodents.

Finally, it is important to note that therapies for SCI must ultimately result in functional improvements that overcome or compensate for deficits induced by the lesion. For this reason, it is important to effectively assess and evaluate functional recovery in animal models of SCI so it is important to evaluate behavioural outcome measures in the laboratory, as demonstrated in the study described in Chapter 3. We found that the single pellet skilled reaching task may be considered a good, stringent test for recovery with plasticity-promoting treatments because there was a specific lesion severity that enabled the evaluation of plasticity-promoting (i.e., training) treatment. Alternatively, the single pellet reaching task may also be considered a test that is too stringent to test a wide range of lesion sizes. This is similar to what has been reported for a common test to evaluate locomotor recovery, the BBB scale (Basso et al., 1995). Schucht et al., (2002) evaluated this scale and found that rats clustered at two scores, indicating that it is a non-linear analysis which can lead to data bias and ceiling effects. Additionally, it is important to consider what constitutes "clinically meaningful efficacy" in animal models of SCI. Kwon et al (2010) found that the majority of professionals in the field of SCI thought that a treatment that resulted in plantar weight-supported stepping and interlimb coordination, compared to dragging of hindlimbs and only uncoordinated steps in control animals, constituted clinical meaningful recovery in a rodent model of thoracic SCI.

In terms of the single pellet task, determining what constitutes "clinically meaningful efficacy" is more difficult. As demonstrated in figure 3.6, the baseline success rates on this task can be quite low (approximately 40%) so recovery to baseline after a treatment, although impressive, may not be considered improvement enough to constitute clinically meaningful recovery. Because of the variability in the single pellet reaching and the use of compensation, the evaluation of treatment efficacy becomes even more complex. For these reasons, a more important indication of treatment efficacy on cervical injury models should include consistent, reproducible effects of a treatment. Overall, it is clear that behavioural tests must be optimized and standardized wherever possible and that "clinically meaningful outcomes" are clearly defined in order to effectively and reliably evaluate potential treatments for SCI.

4.5 PUBLICATION, REPLICATION AND TRANSLATION

One of the reasons that translation of potential treatments for SCI to the clinic has yet to be successful is that, common to any field of science, there exists a publication bias in the field of SCI research. The publication of negative results, even when research methods are sound, can be quite difficult. This issue in the field of SCI was addressed in a survey by Kwon et al. (2010), who found that 84% of respondents felt that scientists are reluctant to publish negative results. Additionally, 81% of respondents felt that high-impact journals tend to reject submitted papers that describe negative results. This publication bias exists in

basic research but also within clinical trials. For example, the results of all the clinical trials at a particular medical center were recently analyzed based on impact, time to and rate of publication (Sune et al., 2013). It was found that, although the impact of positive and negative clinical trial results were the same, the rate of publication was higher and the time to publication was shorter for positive results than negative clinical trial results.

In addition to more frequent publication of negative results, replication of positive results is an important step in the process to translate a promising treatment in the laboratory to the clinical setting. Individuals in the SCI community overwhelmingly agree that independent (i.e., different laboratory) replication of promising results is important (Kwon et al., 2010). The National Institutes of Health (NIH) in the United States has addressed this concern by funding the replication of certain promising potential treatments for SCI to confirm their reported success in independent laboratories. These treatments have included the Nogo-66 receptor antagonist, minocycline, erythropoetin and a combinatory treatment including lipopolysaccharide, pregnenolone and indomethacin (Pinzon et al, 2008a; 2008b; Popovich et al., 2012; Steward et al, 2008) and have generally yielded results that are less encouraging than initially reported.

Establishing appropriate animal models and replication of promising results are important to demonstrate robust treatment effects in the laboratory setting before translating a potential treatment to the clinic. This is important primarily for human safety, but also because of the large costs and time associated

with clinical trials (Fawcett et al, 2007; Courtine et al, 2007; Steeves et al, 2007; Tuszynski et al., 2007). Also, a number of variables are controlled for in the laboratory, however this is not possible in clinical trials (e.g., differences in age, injury level and severity, motivation to participate in demanding treatments such as rehabilitative training, secondary complications of injury, time before treatment can be administered). All of these factors contribute to greater variability in human trials and therefore, a large number of human subjects are needed in order to effectively evaluate a treatment in the clinic. For these reasons, establishing appropriate animal models to test potential treatments in the laboratory is of critical importance.

4.6 CONCLUSIONS

Over the past few decades, the field of SCI research has made significant advances in our understanding of the pathological mechanisms that accompany this type of injury. These findings form the framework for the development of potential treatments in order to improve the quality of life of those affected by SCI. Although translation of potential treatments deemed promising in the laboratory has proven difficult so far, a thorough evaluation of our animal models of injury and behavioural recovery is a step in the right direction. This re-evaluation is especially important for potential treatments for SCI but can also be useful, as demonstrated in Chapter 3 of this thesis, for the evaluation of treatments that are currently used in the clinic (e.g., rehabilitative training).

The studies described in this thesis contribute to our understanding of rehabilitative training following incomplete, cervical SCI. Elucidating the mechanisms involved and the appropriate models to test potential treatments can enhance rehabilitative training on its own as well as in combination with other potential treatments. Given that rehabilitative training is the most effective treatment to repair the injured spinal cord to date, it will be beneficial to gain a more complete understanding of this potent treatment for SCI. This understanding should include a re-evaluation of the animal models that can be used to evaluate training, as well as other potential approaches to promote functional recovery following SCI.

4.7 REFERENCES

Basso, D. M., Beattie, M. S., and Bresnahan, J. C., 1995. A sensitive and reliable locomotor rating scale for open field testing in rats. Journal of neurotrauma 12**,** 1-21.

Bunge, R. P., Puckett, W. R., Becerra, J. L., Marcillo, A., and Quencer, R. M., 1993. Observations on the pathology of human spinal cord injury. A review and classification of 22 new cases with details from a case of chronic cord compression with extensive focal demyelination. Advances in neurology 59**,** 75-89.

ClinicalTrials.gov, 2010. Safety and efficicy of Cethrin in adult subjects with acute cervical spinal cord injury. http://clinicaltrials.gov/show/NCT00610337 (March 2013, date last accessed).

Courilleau, D., Bisserier, M., Jullian, J. C., Lucas, A., Bouyssou, P., Fischmeister, R., Blondeau, J. P., and Lezoualc'h, F., 2012. Identification of a tetrahydroquinoline analog as a pharmacological inhibitor of the cAMP-binding protein Epac. The Journal of biological chemistry 287**,** 44192-44202.

Courtine, G., Bunge, M. B., Fawcett, J. W., Grossman, R. G., Kaas, J. H., Lemon, R., Maier, I., Martin, J., Nudo, R. J., Ramon-Cueto, A., Rouiller, E. M., Schnell, L., Wannier, T., Schwab, M. E., and Edgerton, V. R., 2007. Can experiments in nonhuman primates expedite the translation of treatments for spinal cord injury in humans? Nature medicine 13**,** 561-566.

Dergham, P., Ellezam, B., Essagian, C., Avedissian, H., Lubell, W. D., and McKerracher, L., 2002. Rho signaling pathway targeted to promote spinal cord repair. The Journal of neuroscience : the official journal of the Society for Neuroscience 22**,** 6570-6577.

Dong, J. M., Leung, T., Manser, E., and Lim, L., 1998. cAMP-induced morphological changes are counteracted by the activated RhoA small GTPase and the Rho kinase ROKalpha. The Journal of biological chemistry 273**,** 22554-22562.

Dubreuil, C. I., Winton, M. J., and McKerracher, L., 2003. Rho activation patterns after spinal cord injury and the role of activated Rho in apoptosis in the central nervous system. The Journal of cell biology 162**,** 233-243.

Fawcett, J. W., Curt, A., Steeves, J. D., Coleman, W. P., Tuszynski, M. H., Lammertse, D., Bartlett, P. F., Blight, A. R., Dietz, V., Ditunno, J., Dobkin, B. H., Havton, L. A., Ellaway, P. H., Fehlings, M. G., Privat, A., Grossman, R., Guest, J. D., Kleitman, N., Nakamura, M., Gaviria, M., and

Short, D., 2007. Guidelines for the conduct of clinical trials for spinal cord injury as developed by the ICCP panel: spontaneous recovery after spinal cord injury and statistical power needed for therapeutic clinical trials. Spinal cord 45**,** 190-205.

Fehlings, M. G., Theodore, N., Harrop, J., Maurais, G., Kuntz, C., Shaffrey, C. I., Kwon, B. K., Chapman, J., Yee, A., Tighe, A., and McKerracher, L., 2011. A phase I/IIa clinical trial of a recombinant Rho protein antagonist in acute spinal cord injury. Journal of neurotrauma 28**,** 787-796.

Gopalakrishnan, S. M., Teusch, N., Imhof, C., Bakker, M. H., Schurdak, M., Burns, D. J., and Warrior, U., 2008. Role of Rho kinase pathway in chondroitin sulfate proteoglycan-mediated inhibition of neurite outgrowth in PC12 cells. Journal of neuroscience research 86**,** 2214-2226.

Hara, M., Takayasu, M., Watanabe, K., Noda, A., Takagi, T., Suzuki, Y., and Yoshida, J., 2000. Protein kinase inhibition by fasudil hydrochloride promotes neurological recovery after spinal cord injury in rats. Journal of neurosurgery 93**,** 94-101.

Hurley, J. H., 1999. Structure, mechanism, and regulation of mammalian adenylyl cyclase. The Journal of biological chemistry 274**,** 7599-7602.

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. The European journal of neuroscience 29**,** 641-651.

Kwon, B. K., Hillyer, J., and Tetzlaff, W., 2010. Translational research in spinal cord injury: a survey of opinion from the SCI community. Journal of neurotrauma 27**,** 21-33.

Lawrence, D. G., and Kuypers, H. G., 1968. The functional organization of the motor system in the monkey. I. The effects of bilateral pyramidal lesions. Brain : a journal of neurology 91**,** 1-14.

Lehmann, M., Fournier, A., Selles-Navarro, I., Dergham, P., Sebok, A., Leclerc, N., Tigyi, G., and McKerracher, L., 1999. Inactivation of Rho signaling pathway promotes CNS axon regeneration. The Journal of neuroscience : the official journal of the Society for Neuroscience 19**,** 7537-7547.

Lemon, R. N., Kirkwood, P. A., Maier, M. A., Nakajima, K., and Nathan, P., 2004. Direct and indirect pathways for corticospinal control of upper limb motoneurons in the primate. Progress in brain research 143**,** 263-279.

Monnier, P. P., Sierra, A., Schwab, J. M., Henke-Fahle, S., and Mueller, B. K., 2003. The Rho/ROCK pathway mediates neurite growth-inhibitory activity associated with the chondroitin sulfate proteoglycans of the CNS glial scar. Molecular and cellular neurosciences 22**,** 319-330.

Murray, A. J., Tucker, S. J., and Shewan, D. A., 2009. cAMP-dependent axon guidance is distinctly regulated by Epac and protein kinase A. The Journal of neuroscience : the official journal of the Society for Neuroscience 29**,** 15434-15444.

Norenberg, M. D., Smith, J., and Marcillo, A., 2004. The pathology of human spinal cord injury: defining the problems. Journal of neurotrauma 21**,** 429-440.

Pinzon, A., Marcillo, A., Pabon, D., Bramlett, H. M., Bunge, M. B., and Dietrich, W. D., 2008a. A re-assessment of erythropoietin as a neuroprotective agent following rat spinal cord compression or contusion injury. Experimental neurology 213**,** 129-136.

Pinzon, A., Marcillo, A., Quintana, A., Stamler, S., Bunge, M. B., Bramlett, H. M., and Dietrich, W. D., 2008b. A re-assessment of minocycline as a neuroprotective agent in a rat spinal cord contusion model. Brain research 1243**,** 146-151.

Popovich, P. G., Tovar, C. A., Wei, P., Fisher, L., Jakeman, L. B., and Basso, D. M., 2012. A reassessment of a classic neuroprotective combination therapy for spinal cord injured rats: LPS/pregnenolone/indomethacin. Experimental neurology 233**,** 677-685.

Rosenzweig, E. S., Brock, J. H., Culbertson, M. D., Lu, P., Moseanko, R., Edgerton, V. R., Havton, L. A., and Tuszynski, M. H., 2009. Extensive spinal decussation and bilateral termination of cervical corticospinal projections in rhesus monkeys. The Journal of comparative neurology 513**,** 151-163.

Rosenzweig, E. S., Courtine, G., Jindrich, D. L., Brock, J. H., Ferguson, A. R., Strand, S. C., Nout, Y. S., Roy, R. R., Miller, D. M., Beattie, M. S., Havton, L. A., Bresnahan, J. C., Edgerton, V. R., and Tuszynski, M. H., 2010. Extensive spontaneous plasticity of corticospinal projections after primate spinal cord injury. Nature neuroscience 13**,** 1505-1510.

Schucht, P., Raineteau, O., Schwab, M. E., and Fouad, K., 2002. Anatomical correlates of locomotor recovery following dorsal and ventral lesions of the rat spinal cord. Experimental neurology 176**,** 143-153.

Seamon, K. B., and Daly, J. W., 1986. Forskolin: its biological and chemical properties. Advances in cyclic nucleotide and protein phosphorylation research 20**,** 1-150.

Siegenthaler, M. M., Tu, M. K., and Keirstead, H. S., 2007. The extent of myelin pathology differs following contusion and transection spinal cord injury. Journal of neurotrauma 24**,** 1631-1646.

Steeves, J. D., Lammertse, D., Curt, A., Fawcett, J. W., Tuszynski, M. H., Ditunno, J. F., Ellaway, P. H., Fehlings, M. G., Guest, J. D., Kleitman, N., Bartlett, P. F., Blight, A. R., Dietz, V., Dobkin, B. H., Grossman, R., Short, D., Nakamura, M., Coleman, W. P., Gaviria, M., and Privat, A., 2007. Guidelines for the conduct of clinical trials for spinal cord injury (SCI) as developed by the ICCP panel: clinical trial outcome measures. Spinal cord 45**,** 206-221.

Steward, O., Sharp, K., Yee, K. M., and Hofstadter, M., 2008. A re-assessment of the effects of a Nogo-66 receptor antagonist on regenerative growth of axons and locomotor recovery after spinal cord injury in mice. Experimental neurology 209**,** 446-468.

Sunahara, R. K., Dessauer, C. W., and Gilman, A. G., 1996. Complexity and diversity of mammalian adenylyl cyclases. Annual review of pharmacology and toxicology 36**,** 461-480.

Sune, P., Sune, J. M., and Montoro, J. B., 2013. Positive outcomes influence the rate and time to publication, but not the impact factor of publications of clinical trial results. PloS one 8**,** e54583.

Tator, C. H., 2006. Review of treatment trials in human spinal cord injury: issues, difficulties, and recommendations. Neurosurgery 59**,** 957-982; discussion 982-957.

Tuszynski, M. H., Steeves, J. D., Fawcett, J. W., Lammertse, D., Kalichman, M., Rask, C., Curt, A., Ditunno, J. F., Fehlings, M. G., Guest, J. D., Ellaway, P. H., Kleitman, N., Bartlett, P. F., Blight, A. R., Dietz, V., Dobkin, B. H., Grossman, R., and Privat, A., 2007. Guidelines for the conduct of clinical trials for spinal cord injury as developed by the ICCP Panel: clinical trial inclusion/exclusion criteria and ethics. Spinal cord 45**,** 222-231.

Whishaw, I. Q., Gorny, B., and 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. Behavioural brain research 93**,** 167-183.

Yamashita, T., and Tohyama, M., 2003. The p75 receptor acts as a displacement factor that releases Rho from Rho-GDI. Nature neuroscience 6**,** 461-467.