Augmenting Activity Dependent Plasticity for the Repair of Spinal Cord Injury

by

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Thesis Abstract

This thesis investigates how to bolster activity dependent neuronal plasticity in conjunction with rehabilitative motor therapy to increase recovery following spinal cord injury in rats. My work focuses primarily on the injured corticospinal tract (CST) and how boosting intracellular signalling pathways related to activity dependent plasticity can lead to an increase in motor recovery.

In chapter 2, I follow up on previous work from the Fouad laboratory that demonstrated benefits of electrical stimulation on the motor forelimb cortex prior to spinal cord injury. This experiment demonstrated an increase in axonal collateralization in the rat CST. My experiment explores the effects of a single session of stimulation immediately after SCI in rats and how it can increase the benefits of functional rehabilitative therapy.

Chapter 3 serves as an introduction to the cAMP signalling pathway, which is the focus of the experiments in the following two chapters. This chapter introduces the role of cAMP and its downstream effectors, protein kinase A (PKA) and exchange protein activated by cAMP (EPAC), in neurite outgrowth.

Following a laboratory rotation in the laboratory of Dr. Sipione, where I learned to plate primary cortical neurons, I brought the protocol back to the Fouad laboratory where I tested the effects of EPAC and PKA agonists on neurite outgrowth *in vitro*. Chapter 4 describes the effects of these agonists and demonstrates an exciting novel complementary effect of simultaneous application of both agonists on neurite outgrowth. This complementary finding led us to follow up on previous Fouad laboratory results,

where PKA inhibition led to an increase in functional recovery after SCI, and test these effects *in vivo*.

Chapter 5 describes my *in vivo* experiment utilising both EPAC and PKA agonists together administered to the motor forelimb cortex innervating the injured CST alongside motor rehabilitative therapy. Following SCI, animals had osmotic mini-pumps containing either saline, an EPAC agonist, or both an EPAC agonist and a PKA agonist installed with the cannula in the moto cortex for localized drug delivery. Despite previous findings, administration of both agonists did not result in an increase in functional recovery after training, nor did it increase spinal plasticity of the CST.

A comprehensive understanding of how the cAMP pathway affects recovery after SCI and how it could be bolstered to increase the effects of rehabilitative motor therapy in the clinic is imperative for future SCI research. Whilst my final *in vivo* experiment using both agonists yielded negative results, the findings from this thesis build a strong foundation for further exploration into the signalling cascade behind activity dependent plasticity and how it can be optimized for improved recovery.

Thesis Preface

This thesis is an original work by Nicholas J. Batty. The research project, of which this thesis is a part, received research ethics approval from the University of Alberta Research Ethics Board, Project Name "Repairing the Injured Spinal Cord", No. AUP00000254, Expiration date June 17, 2022.

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I was responsible for the majority of data collection and analysis, as well as the manuscript composition. Romana Vavrek-LeCerf and Pamela Raposo provided technical assistance and helped with data collection. Dr. Karim Fouad was the supervisory author, and assisted with manuscript composition, experimental design, and surgical procedures.

Since Chapter 2 and 3 of this thesis are published as independent papers, chapter 4 and 5 will follow suit in their formatting. As such chapters 2 through 5 will all follow the same format as if they were all published papers. Other chapters in this thesis will allow for use of first-person pronouns and commentary.

Dedication

This thesis is dedicated to all of the laboratory animals who gave their lives for the research contained within this document. Their contributions to both this work and science as a whole cannot be ignored or forgotten, and I'd like to take this time to thank them for their sacrifice and acknowledge the importance of animal work in science. Scientific progress would not be as far along as it is without the involvement of research animals, but these animals should never be taken for granted. Every single animal involved in this study served as crucial a role to this work as the author themselves.

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I would like to take this opportunity to thank my supervisor, Dr. Karim Fouad. He invested five years of his life and laboratory funds in me, and I am sure that it could not have been easy all of the time. Karim has shown me what a true passion for science looks like, a passion that I hope I can emulate to its full potential one day. His guidance has made me the man and scientist that I am today, and for that I will always be grateful.

Secondly, my supervisory committee: Dr. Simonetta Sipione and Dr. David Bennett, are as deserving of accolades as my supervisor. The two of them have helped to instruct and guide me throughout these five years, having invested a significant amount of their own time. I would like to particularly thank Dr. Simonetta Sipione for allowing me to do a rotation in her laboratory to help expand my technical expertise and knowledge. It was truly a delight working with her and her students. Though I would not have made it this far were it not for the passion of Dr. David Bennett and his enthusiasm for science; whether it be his own work, journal club, or one of my committee meetings, he always shows the most genuine interest and engagement. His engagement during committee meetings was a constant reassurance that I was on the right track.

I also owe a world of debt to the phenomenal assistance of Romana Vavrek-LeCerf and Pamela Raposo. The aid of these two, day in and day out, in all laboratory manners is without compare, and without it, the journey to get this far would have been much more difficult. A special thanks to my fellow graduate students for sharing this journey with me. Graduate school is far from easy for anyone, having others along for the ride makes it a much more enjoyable trip. Thank you to Dr. Keith Fenrich and Dr. Abel

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To my friends and family, who I could always count on for love and support throughout these past five years, thank you. My parents, Barbara and Timothy Batty, and my siblings, Kaitlyn and Andrew Batty, have stuck by through a wild rollercoaster of a journey and were there for every step of the way. My Nanna, Kira Ian, has been one of my biggest fans and supporters and I deeply appreciate her eagerness to read my work. To my loving partner, Morganne Held, I would never have gotten nearly this far without you and your support. This is as much of an achievement for every one of you as it is for me.

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Abbreviations

AAV	Adeno-Associated Virus
AC	Adenylate Cyclase
AMP	Adenosine Monophosphate
ASIA	American Spinal Injury Association
BDNF	Brain Derived Neurotrophic Factor
C	Cervical
cAMP	Cyclic Adenosine Monophosphate
Cdk5	Cyclin-dependent Kinase 5
CNS	Central Nervous System
CREB	cAMP Response Element Binding protein
CSPG	Chrondroitin Sulphate Proteoglycans
CST	Corticospinal Tract
dCST	Dorsal Corticospinal Tract
DLQ	Dorsal Lateral Quadrant
DREADDs	Designer Receptors Exclusively Activated by Designer Drugs
DRG	Dorsal Root Ganglion
ECM	Extra-Cellular Matrix
eIF5A	Eukaryotic Translation Initiation Factor 5A-1
EMG	Electromyography
EPAC	Exchange Protein Activated by cAMP
ERK	Extracellular Signal-Regulated Kinase
ES	Electrical Stimulation
GDNF	Glial Derived Neurotrophic Factor
GFAP	Glial Fibrillary Acidic Protein
GTP	Guanosine Triphosphate
HARBS	High Affinity Rolipram Binding Site
HBSS	Hank's Balanced Salt Solution
L	Lumbar
LARBS	Low Affinity Rolipram Binding Site

LTP	Long Term Potentiation
MAG	Myelin Associated Glycoprotein
MEP	Motor Evoked Potential
mTOR	Mechanistic Target of Rapamycin
NGF	Nerve Growth Factor
NGS	Normal Goat Serum
NT-3	Neurotrophin-3
OMGp	Oligodendrocyte Myelin Glycoprotein
PBS	Phosphate Buffered Saline
pCREB	Phosphorylated cAMP Response Element Binding protein
PDE4	Phosphodiesterase 4
PKA	Protein Kinase A
PNS	Peripheral Nervous System
pPKA	Phosphorylated Protein Kinase A
PTEN	Phosphatase and Tensin Homolog
RG	Retinal ganglion
RhoA	Ras Homolog Family Member A
RST	Rubrospinal Tract
S	Sacral
SCI	Spinal Cord Injury
siRNA	Small Interfering Ribonucleic Acid
SLPI	Secretory Leukocyte Protease Inhibitor
SPG	Single Pellet Grasping
T	Thoracic
TBS	Tris-Buffered Saline
TMS	Transcranial Magnetic Stimulation
Trk	Tyrosine Receptor Kinase
VEGF	Vascular Endothelial Growth Factor
VF	Von Frey

Chapter 1

Introduction to Spinal Cord Injury and Repair

1.1 Spinal Cord Injury

Spinal cord injury (SCI) is a devastating condition that has lifelong impacts on the individual with the injury, as well as their family and friends. In Canada alone, it is estimated that there are more than 86000 people living with some form of spinal cord injury, with over half of all injuries being attributed to traumatic causes such as a vehicular collision. The cost of SCI to an individual extends beyond the injury itself, as the economic burden of SCI leaves individuals with an estimated lifetime cost of two million dollars (Praxis Spinal Cord Institute, 2021). With treatments yielding substantial functional recovery still out of reach, research into mechanisms for repair of the injured spinal cord is a necessity for many individuals living with SCI today.

This thesis aims to contribute to the overall investigation into potential means to increase functional outcomes after spinal cord injury by combining treatments with rehabilitative motor therapy to complement the plasticity promoting effects of motor therapy.

1.2 Pathophysiology of Spinal Cord Injury

More than just simply a set of wires connecting the brain to the rest of the bodily systems, the spinal cord is in fact a complicated framework of nerves that contains processing centres, central pattern generators, and motoneurons. As such injury to the spinal cord often results in a profound loss of motor and sensory function to areas below the site of injury. The loss of function varies depending on the injury location and how complete the injury is. Patients with SCI typically experience symptoms such as loss of

sensory and motor function (Kirshblum et al., 2011), pain (Yezierski, 2009), spasticity (Elbasiouny et al., 2010), autonomic dysreflexia (Baguley, 2008), bladder and bowel dysfunction (Craggs et al., 2006), and deficits in sexual function (as it pertains to males: (Brown et al., 2006); and females: (Sipski and Arenas, 2006)). The extent of sensory and motor loss depends heavily on the size and site of injury, with more severe injuries being associated with further detriment (Kirshblum et al., 2011). In the same regard, thoracic injuries may result in paraplegia in the legs, and cervical injuries can result in tetraplegia affecting both arms and legs (Kirshblum et al., 2011). The American Spinal Injury Association (ASIA) established criteria for assessing spinal cord injury and the extent of the loss of function. According to these guidelines, SCI is classified on a scale ranging from functionally complete, to motor complete/sensory incomplete, to motor incomplete (Kirshblum et al., 2011). Functionally complete SCI results in a complete loss of both sensory and motor function below the injury, while incomplete injuries will have varying degrees of motor and sensory function present depending on lesion severity (Kirshblum et al., 2011). It is important to note that functionally complete SCI is not the same as anatomically complete. It is uncommon for SCI to result in a complete ablation of tissue, as spared white matter tracts around the edges of the cord tend to be spared from the initial damage (Kakulas, 1999, 1984). Using ASIA's classification system, according to the American Nation SCI Database, incomplete tetraplegia (i.e. cervical injuries) are the most prevalent type of SCI ("Spinal Cord Injury (SCI) Facts and Figures at a Glance," 2016). The focus of my research and the work contained in this thesis will focus on incomplete cervical SCI, as this is the most prevalent injury type to occur.

The initial trauma is only the beginning and is referred to as primary damage. Following this there is also secondary damage which is the result of additional damaging processes that were initiated due to the primary damage (Reviewed in: Ahuja et al., 2017; Oyinbo, 2011; Tator and Fehlings, 1991).

1.2.1 Secondary Damage

Secondary damage in SCI is the delayed progressive injury of spinal tissue surrounding the primary damage as a result of a complicated signalling cascade that follows the injury. Secondary damage is caused by further cell death and apoptosis around the injury site (Beattie et al., 2000; Crowe et al., 1997; Emery et al., 1998; Liu et al., 1997), and the demyelination (Blight, 1985; Bunge et al., 1993) and degeneration (Crowe et al., 1997; Kerschensteiner et al., 2005) of injured and nearby spared axons. There is also a major vascular impact of secondary damage, as damage to the nearby blood vessels results in a lack of blood flow to the damaged area (Ahuja et al., 2017; Balentine, 1978; Tator and Fehlings, 1991). The resulting ischemia further compounds the damage as it is associated with the production of free oxygen radicals that contribute to cellular damage (Demopoulos et al., 1980). Furthermore, the contents of neurons when released en masse by damage is toxic to nearby neurons. Intracellular glutamate, when released, causes a hyperactivation of nearby cells that leads to further cell death (Li and Stys, 2000; Park et al., 2004). In addition to further cell death surrounding the injury site, further barriers to regeneration are discussed below.

1.2.2 Inflammation

In addition to further cell death after SCI, immediately following the injury a state of neuroinflammation is initiated that will typically persist as a chronic state of systemic inflammation. This inflammatory state post SCI is remarkably complex and has traditionally been associated with an array of negative outcomes and comorbidities connected with the injury. While inflammation is originally an immune response that targets invading foreign objects, chronic inflammation has been associated with a number of health concerns including depression, ongoing tissue damage, increased susceptibility to infection, and increased risk of cardiovascular disease (reviewed in: Bloom et al., 2020). Injury to the spinal cord results in a distinct inflammatory response that is unique to the cord: injury results in an infiltration of inflammatory cells such as neutrophils and macrophages (Schnell et al., 1999) that has previously been associated with further tissue damage (Fleming et al., 2006). The time course of this inflammatory invasion has been well-characterized in humans (Fleming et al., 2006) and rats (Popovich et al., 1997). It was believed that targeting this inflammation would reduce the overall injury size and improve the prognosis for patients (Gris et al., 2004; Hall, 1992). Recently however there has been an interesting change in the dynamic of inflammation research that suggests that inflammation may play a beneficial role post SCI. While this will not be the focus of this thesis, interested readers can refer to reviews discussing the duality of inflammation (Bethea, 2000; Donnelly and Popovich, 2008).

1.2.3 Glial and Fibrotic Scarring

Injury to the spinal cord activates resident astrocytes and pericytes, and alongside the pro-inflammatory cells discussed above, invading peripheral cells such as fibroblasts (Goritz et al., 2011; Soderblom et al., 2013) and Schwann cells (Beattie et al., 1997; Bruce et al., 2000; Buss et al., 2006) are recruited to form a scar surrounding the injury. A collection of deposited fibrotic cellular components such as extracellular matrices (ECM), form what is known as the fibrotic scar. Activated glial cells such as astrocytes form a thick scar surrounding the lesion, which is called the glial scar. In addition to the physical barrier that the scarring poses, a number of the components of the fibrotic scar such as CSPGs have been shown to be inhibitory to axon growth (reviewed in Morgenstern et al., 2002). More information about glial and fibrotic scarring as well as treatments that have targeted it can be found in reviews such as that by Orr and Gensel (2018).

1.2.4 Neurotrophin Downregulation

Following injury in the central nervous system, there is a lack of growth promoting factors that are present following injury in the peripheral nervous system. Blood vessels that are damaged by the injury are unable to grow back due to a decrease in vascular endothelial growth factor (VEGF) (Herrera et al., 2009), further contributing to the previously discussed ischemia following SCI. A decrease in neurotrophins such as BDNF (Gomez-Pinilla et al., 2012; Ying et al., 2008, 2005) and NT-3 (Ying et al., 2003) have also been demonstrated to occur following SCI. Neurotrophins such as BDNF and NT-3 are directly responsible for stimulating growth promoting pathways such as the

cAMP signalling pathway. Researchers have also found that there are declines in specific cell-signalling pathways that have been demonstrated to be crucial for plasticity. The mammalian target of rapamycin (MTOR) signalling pathway, shown to play a role in neuronal survival and axon growth (Swiech et al., 2008; Takei and Nawa, 2014), declines following SCI (Liu et al., 2010). This may be due in part to an increase in PTEN levels post SCI (Liu et al., 2010), as the Rho/PTEN pathway directly inhibits MTOR and its growth promoting abilities. Another important signalling molecule, cAMP, decreases following injury (Cai et al., 2001; Dougherty et al., 2000; Fenrich and Gordon, 2004; Widenfalk et al., 2001), resulting in a decline in the growth potential of neurons. This results in a decline in the signalling cascade through its downstream effectors, the exchange protein activated by cAMP (EPAC) and protein kinase A (PKA). cAMP and its downstream effectors are further introduced in chapter 3 of this thesis, as they are the focus of the later chapters.

1.2.5 Inhibitory Environment and Myelin Associated Inhibitors

The CNS is also a very inhibitory environment for neurons to grow in, having an array of inhibitory factors present that slow neuronal regeneration and growth cone development. It had been first postulated as early as 1982 (Berry) that myelin was somehow responsible for this inhibitory environment. This was eventually proven to be true (Savio and Schwab, 1989; Schwab and Bartholdi, 1996) and it later became known that there are three major myelin associated inhibitors: Nogo, Myelin-Associated Glycoprotein (MAG), and Oligodendrocyte Myelin Glycoprotein (OMgp).

The discovery of Nogo, the most well-known and studied myelin associated inhibitor, stemmed from the antigen for the monoclonal antibody IN-1 which Caroni and Schwab (1988) used to halt the inhibition of neurite outgrowth. With the injection of this antibody allowing for previously impossible growth, it became apparent that the antigen for this antibody must be the factor leading to the inhibition. The actual myelin associated antigen for this antibody, however, proved difficult to isolate until 2000, when it was purified by three separate labs and named Nogo (Chen et al., 2000; GrandPré et al., 2000; Prinjha et al., 2000). The gene responsible for Nogo actually encodes for three isoforms of Nogo: Nogo-A, Nogo-B, and Nogo-C. All three Nogo isoforms have a shared active site in the form of a 66-amino acid loop, termed Nogo-66, which contributes to the inhibitory function of Nogo (GrandPré et al., 2000). Nogo-A possesses an additional functional domain in the form of an N-terminus region, termed amino Nogo. As this is found exclusively in Nogo-A, this isoform has the highest degree of inhibitory function (Fournier et al., 2001; GrandPré et al., 2000). Nogo-A is also the only one that expresses strong inhibitory function in the CNS (GrandPré et al., 2000; Huber et al., 2002).

Myelin-Associated Glycoprotein was the first myelin associated inhibitor to be discovered chronologically in 1972, despite all of the previously mentioned work with the IN-1 antibody and Nogo. MAG was shown to inhibit neurite outgrowth in culture (McKerracher et al., 1994; Mukhopadhyay et al., 1994). Interestingly, however, this was not its only major function, as it soon became apparent that MAG acted to inhibit growth in older neurons but functioned as a growth promoting factor in young neurons (Mukhopadhyay et al., 1994). The last inhibitor, Oligodendrocyte Myelin Glycoprotein was identified long before it was known to act as an inhibitor of neurite outgrowth but

only in recent years it has been reisolated and found to cause growth cone collapse and inhibit growth like the other two major myelin associated inhibitors (Wang et al., 2002a, 2002b).

Additionally, each appears to have their own separate roles in development. OMgp for example, although identified earlier, was not originally discovered as specifically a myelin associated inhibitor. Rather, it was originally found as a peanut-agglutinin-binding protein (Mikol and Stefansson, 1988) and only later shown to be inhibitory to neuron growth. Through the few roles we do know for it however, it is evident that its inhibitory effects appear to be a side effect of its original function in development. MAG, as mentioned previously, has a well-known second function promoting growth in developing organisms. It only changes over to having an inhibitory role once the nervous system has largely developed not long after birth.

1.3 Plasticity in the Central Nervous System

The adult mammalian CNS was traditionally thought to be an unchanging network that was incapable of rearrangement or repair. This idea has been challenged by scientists over the last few decades by demonstrating that the mature CNS is, in fact, capable of rearrangement and adaptation. There has been substantial work demonstrating cortical remapping in individuals following extended fine motor training (Schwenkreis et al., 2007), and CNS injury (Fouad et al., 2001; reviewed in Wittenberg, 2010). The spinal cord is also capable of plastic changes, such as rerouting through spared tissue to restore functional connections following SCI (Courtine et al., 2008; May et al., 2017). Changes

observed under the umbrella term “plasticity” occur simultaneously throughout the CNS at different physiological and anatomical levels (Reviewed in Kusiak and Selzer, 2013; von Bernhardi et al., 2017). For the majority of the work in this thesis, plasticity will be analyzed by measuring collateral sprouting, which is the growth of collateral fibres off of the main axon shaft of injured or spared neurons (Fouad et al., 2001). It is important to differentiate plasticity, the ability of the CNS to adapt and rewire, from regeneration, which is the regrowth from the severed end of a severed axon (Cafferty et al., 2008). Regeneration as a target of recovery after spinal cord injury has yielded minimal results, and as such much of the field of SCI repair has moved towards utilizing plasticity to rewire post SCI (Fouad et al., 2015).

1.3.1 Activity Dependent Plasticity

Activity dependent plasticity is, as the name implies, plasticity that develops as a result of activity in the neuron (reviewed in Bertrand and Cazalets, 2013; Dunlop, 2008; Mueller, 2007; Tahayori and Koceja, 2012). Much of the research into activity dependent is focused on the physical activity and motor systems aspect but there has also been work into mental exercise as a means to improve plasticity (Moseley, 2007). Activity dependent plasticity has been demonstrated to work via increasing levels of important neurotrophins like VEGF, NT-3, and BDNF (Extensively reviewed in: Cotman et al., 2007; Dishman et al., 2006; Vaynman and Gomez-Pinilla, 2005). These same neurotrophins were discussed above as being key neurotrophins shown to decrease after injury, demonstrating a significant role of neuronal activity in increasing plasticity. The pathways that these neurotrophins act on will be discussed further in chapter 3. For the

time being, the important aspect to note is that neuronal activity has been shown to increase sprouting after SCI, and as such is a crucial component of recovery.

1.4 Rehabilitative Motor Therapy

Rehabilitative motor therapy is currently the safest and most reliable therapy we have to promote functional recovery following traumatic SCI. Motor rehabilitative training has been demonstrated to improve functional outcomes in a number of animal models (Barbeau and Rossignol, 1987; Battistuzzo et al., 2012; García-Alías et al., 2009; Girgis et al., 2007; Starkey et al., 2014; Tashiro et al., 2015; Torres-Espín et al., 2018c, 2018b) and improve neurological outcomes in human patients in the clinic as well (Beekhuizen and Field-Fote, 2005; Harkema, 2001; Kowalczewski et al., 2011). Motor training has been demonstrated to increase plasticity by increasing levels of the neurotrophin BDNF and intracellular signalling molecule cAMP (Vaynman and Gomez-Pinilla, 2005), both discussed earlier for their important role in neurite outgrowth. Whilst increasing plasticity is beneficial, it must be directed towards relevant connections to promote recovery. As a result it is crucial to apply rehabilitative therapy during periods of increased plastic potential so as to ensure it is being directed properly (Torres-Espín et al., 2018a). Carefully controlled neuronal activity stabilizes the important adaptive changes made by the CNS so as to ensure these connections are maintained (Stoneham et al., 2010). In the same regard, controlled neuronal activity allows researchers to prevent maladaptive plastic changes such as spasticity or pain (Hou et al., 2014; Tashiro et al., 2015). Further information on the use of rehabilitative therapy in animal models of SCI can be found in the review by Torres-Espín et al. (2018a).

1.4.1 Single Pellet Grasping

One of the most desired functions to have returned to cervical SCI patients is hand function (Anderson, 2004; Hanson and Franklin, 1976; Snoek et al., 2004), and so my work in this thesis will specifically concentrate on rehabilitative motor therapies focused on fine motor control of the injured forelimb. My work will use the single pellet grasping (SPG) model of rehabilitative motor therapy first established by Dr. Whishaw to test motor function after cortical lesions (Whishaw et al., 1986). In the original task, animals are housed in a cage with vertical metal bars, and a food tray is outside of their cage on the other side of the bars. The animals are expected to reach through the bars to obtain and place the food in their mouths. There is a mesh floor to ensure that any food that is not held on to properly by the animal will fall through and be lost, requiring another reaching attempt through the bars to obtain a new piece of food. This was later adapted to having animals reach through a thin window in a specialized enclosure with a single food pellet on a shelf on the other side of the window, and tested in a dorsal column spinal cord injury rat model (McKenna and Whishaw, 1999). A modified version of this behavioural test has also been used in humans with Parkinson's disease, demonstrating homology to rat deficits in the same model type (Whishaw et al., 2002). Previous work in the Fouad laboratory has demonstrated the use of the SPG rehabilitative training model as a means to restore functional recovery after SCI (Girgis et al., 2007; Torres-Espín et al., 2018c). Further work from our laboratory has demonstrated that treatment on its own, such as BDNF overexpression in the motor forelimb cortex, does not result in functional recovery (Vavrek et al., 2006). However, treatment in conjunction with rehabilitative motor therapy demonstrated a profound improvement in motor function (Weishaupt et

al., 2013). This suggests that rehabilitative motor training is a crucial component of the recovery process to enhance plasticity in the spinal cord following SCI.

Where the SPG training allows for scoring based on success rate of retrieved and consumed pellets (McKenna and Whishaw, 1999), there is also the potential for a further analysis of the reaching motion of the animal. First described in 1993 (Whishaw et al.), and later adapted and modernized to the single pellet grasping task (Metz and Whishaw, 2000), an analysis to assess the quality of reaching motion allows for experimenters to have a better understanding of the deficits in each animal. In this thesis this is performed using high speed (120 frames per second) video recordings, and each recorded reaching movement is assessed based on the scoring system outlined in Table 1 and further described by Metz and Whishaw (2000).

1.4.2 SPG Dispensers and Training Intensity

While rehabilitative motor therapy is the safest and most reliable treatment in the clinic, there are still many unanswered questions about how to translate it best to practical recovery. The intensity of training has been shown to be important in human locomotor models (Leech et al., 2016; Wessels et al., 2010), and demonstrated to be necessary for significant recovery in rodent stroke models (Bell et al., 2015; MacLellan et al., 2011). As a result, the work in this thesis uses a high intensity variant of the SPG task established in (Torres-Espín et al., 2018c) using two sided training enclosures with automated pellet presenters to increase the speed at which pellets can be presented. This is established to result in a higher intensity training compared to manual SPG training, with more consistent results between experimenters (Torres-Espín et al., 2018c).

Components	Behaviour scored	Rating
1. Orient	Head oriented to target	0, 0.5, 1
	Sniffing	0, 0.5, 1
2. Limb lift	Body weight shift to back	0, 0.5, 1
	Hindlimbs aligned with body	0, 0.5, 1
	Limb moves forward	0, 0.5, 1
	Digits on midline	0, 0.5, 1
3. Digits close	Palm supinated, semi-in	0, 0.5, 1
	Digits semiflexed	0, 0.5, 1
4. Aim	Elbow comes in	0, 0.5, 1
	Palm in midline	0, 0.5, 1
5. Advance	Elbow in	0, 0.5, 1
	Limb forward	0, 0.5, 1
	Limb directed to target	0, 0.5, 1
	Head and upper body raised	0, 0.5, 1
	Body weight shift front	0, 0.5, 1
	Body weight shift lateral	0, 0.5, 1
6. Digits open	Digits open	0, 0.5, 1
	Discrete limb movement	0, 0.5, 1
	Not fully pronated	0, 0.5, 1
7. Pronation	Elbow out	0, 0.5, 1
	Palm down in arpeggio	0, 0.5, 1
8. Grasp	Arm still	0, 0.5, 1
	Digits close	0, 0.5, 1
	Hand lifts	0, 0.5, 1
9. Supination I	Elbow in	0, 0.5, 1
	Palm medially before leaving slot	0, 0.5, 1
	Palm turned 90°	0, 0.5, 1
10. Supination II	Head points down	0, 0.5, 1
	Body horizontally	0, 0.5, 1
	Palm straight up	0, 0.5, 1
	Distal limb movement	0, 0.5, 1
11. Release	Open digits	0, 0.5, 1
	Puts food in mouth	0, 0.5, 1
	Head and upper body lowered	0, 0.5, 1
	Raises other paw	0, 0.5, 1

Table 1 – Scoring of 11 distinct components in the single pellet grasping task, adapted from (Metz and Whishaw, 2000)

"The 11 movement components finally included the following movement characteristics […]. (1) Orient, the head is oriented towards the target and the snout is inserted through the slot to sniff. (2) Limb lift, the mass of body weight is shifted from the reaching

forelimb to the back, and the forepaw is lifted from the floor. The hindlimbs are aligned with the body, indicating a normal base of support. (3) Digits close, the palm is partially supinated and approaches the midline of the body, the digits are semiflexed. (4) Aim, the elbow is adducted to the body midline while the digits remain positioned on the body midline. (5) Advance, the forelimb moves forward. While the body weight shifts to the front, the head and the upper body are raised to allow the forelimb to advance into the slot. This movement is accompanied by a moderate lateral body movement towards the reaching limb. (6) Digits open, the digits are opened with accompanying discrete limb movement, the palm is not fully pronated. (7) Pronation, the elbow abducts pronating the paw over the target in an arpeggio movement. (8) Grasp, the arm remains still, while the digits close and the paw is extended and raised. (9) Supination I, the paw is supinated by 90° so that it can be withdrawn through the slot. (10) Supination II, the paw is supinated so that the palm faces the mouth, and the body is in a horizontal position. (11) Release, as the rat sits back, the food pellet is released into the mouth by opening the digits. In parallel, the head and the upper body are lowered and the other paw is raised to support the preferred forelimb." (Metz and Whishaw, 2000)

Recently there has been an established connection between rehabilitative motor therapy intensity and corticospinal tract plasticity following SCI (Fenrich et al., 2021), validating my use of higher intensity training in all of my animal groups in this thesis.

1.5 The Corticospinal Tract

As discussed above, this thesis will focus on incomplete cervical spinal cord injury and hand function, as it is one of the most desired functions to have returned in patients with cervical SCI (Anderson, 2004; Hanson and Franklin, 1976; Snoek et al., 2004). It has been demonstrated across humans, primates, and rats that there is a defined forelimb area in the motor cortex that sends connections down through the corticospinal tract (CST) in spinal white matter to innervate and control forelimb movements (Asanuma and Rosen, 1972; Kleim et al., 1998; Rasmussen and Penfield, 1947). As such, this thesis will focus on encouraging plasticity in the CST, due to the role it plays in forelimb motor control.

As functional motor recovery is the overall final goal, it is crucial to ensure that the target pathway controls the motor outcome desired. It has been demonstrated as early as 1968 that the CST at the cervical level is a key component of the execution of fine digit and hand motions in primates (Lawrence and Kuypers, 1968). While in rats the CST is suggested to be involved in the single pellet reaching task in dorsally lesioned rats (McKenna and Whishaw, 1999), and described as contributing to motor forelimb function, rats are capable of fine motor control of their forelimb in cases of select CST ablation (Kanagal and Muir, 2009, 2008; Whishaw et al., 1998). This is due to the rubrospinal tract (RST) demonstrating a remarkable ability to compensate for a lack of

CST input (Raineteau et al., 2001; Whishaw et al., 1998). Nevertheless, it is evident that in rats and humans alike, the CST plays a crucial role in the fine motor control of the upper extremity, which is the targeted motor outcome of this work.

The corticospinal tract originates from a number of cortical motor and motor associated areas, with the exact wiring depending on the species (Lemon and Griffiths, 2005). However, for the purpose of this thesis the primary focus will be on the fibers that project from the primary motor cortex, as these play a key role in voluntary hand function, and this is where the initiation of forelimb reaching will occur. Corticospinal fibres descend through the internal capsule to the medullary pyramids, where most fibres cross over the other side of the spinal cord before descending through white matter tracts to their final innervation targets (Canty and Murphy, 2008; Lemon, 2008; Lemon and Griffiths, 2005; Oudega and Perez, 2012).

While the corticospinal tract is largely considered a motor tract, it does however have a number of other functions including: descending control of afferent inputs (e.g. nociception), gating of spinal reflexes, inhibition of motoneurons, autonomic control, LTP of spinal circuits, and trophic functions during development (Lemon, 2008; Oudega and Perez, 2012). For the purposes of this thesis however, the motor connections will be the primary focus.

1.5.1 The CST in Humans and Rats

There are some key differences between the human and the rodent corticospinal tracts that should be acknowledged and considered whilst reading this work. There are

anatomical location differences between the rodent and human CST, as well as potential compensation from other tracts to improve motor function in rats. There are also direct cortico-motoneuronal connections in human and primate CST that are not present in other mammalian CSTs, including rats.

The human corticospinal tract projects primarily through the lateral funiculus of the spinal cord, whereas the rat CST primarily projects through the ventral component of the dorsal funiculus (Lemon, 2008; Oudega and Perez, 2012). The location of the major component the human CST better overlaps with rodent RST projections (Raineteau et al., 2001). These projections from the red nucleus, when spared, are actually shown to compensate for the loss of CST fibers in motor forelimb control in rats (Raineteau et al., 2001; Whishaw et al., 1998). This pathway is believed to not be able to compensate for the lost CST in humans, probably due to the RST being underdeveloped (Nathan and Smith, 1982; Yang et al., 2011). While the RST will not be directly analysed or studied in this work, it is important to acknowledge its existence given its distinct compensatory role in motor function (as suggested in: Raineteau et al., 2001; Whishaw et al., 1998). As it is designed to be injured in the injury model used in these experiments, it is not expected to confound any rehabilitative therapy results by compensating for lost CST fibers in the following experiments.

In addition to the aforementioned anatomical differences, there are also connectivity differences between the rat and human CST. In humans there are what are described as cortico-motoneuronal connections, meaning the CST neuron is directly synapsing on the relevant motor neuron for the muscle it innervates (Lemon, 2008). While the function of these connections is not entirely understood, it is thought that these

serve to improve the dexterity of the associated motor functions in primates and humans (Lemon, 2008). It has been demonstrated that there are no cortico-motoneuronal connections in other species, including rodents, and instead spinal interneurons and brainstem pathways relay cortical input onto relevant motoneurons (Lemon, 2008). It is possible there may not be a functional difference in terms of the motor outputs intended, but the connectivity differences are something to keep in mind moving forward.

1.5.2 Dorsolateral Quadrant Transection injury in Rats as a Model of Incomplete Human SCI

In order to selectively injure the rat CST, which is primarily contained in the dorsal columns of the spinal cord, a dorsolateral quadrant transection injury model will be used in all *in vivo* experiments in this thesis. This involves the use of a custom-made surgical blade to transect from the center of the spinal cord, down approximately 1 mm deep to the level of the central canal, and across to the outer edge of the cord on the intended side of injury. The intent of this model is to cut a quarter of the spinal cord: either the left or right dorsal half depending on dominant paw. This ablates the ipsilateral CST and RST which, when done at level C4, serves as a model of incomplete cervical SCI. It has previously been demonstrated that injury at this level results in impairments to the fine motor control of forelimb digits (McKenna et al., 2000).

Transection models of SCI in rats when performed correctly are useful for anatomical experiments analyzing the effects of a treatment on regeneration and plasticity (reviewed in: Rosenzweig and McDonald, 2004). However, it is important to note that this is not in line with the most common injury type in human SCI, as contusion based

injuries are most prevalent ("Spinal Cord Injury (SCI) Facts and Figures at a Glance," 2016). In fact, most researchers use contusion models of SCI in their experiments (Sharif-Alhoseini et al., 2017), as the contusion model is ideal for focusing on the pathology post SCI and testing neuroprotective effects of treatment (Rosenzweig and McDonald, 2004). With this in mind however, it was felt that an anatomical plasticity approach fit best with the projects included in this manuscript and so a transection model was chosen. Future work on this project should move towards more clinically relevant injury models following the work described in this thesis.

A key factor in determining an animal model of injury is ensuring that the pathology of the injury matches well with human injury pathology. As discussed above, there is a complicated cascade of events that follow SCI in humans, including secondary damage and invading inflammatory cells. It is important to ensure that a similar response occurs in the animal model used. In terms of models of SCI, the two most commonly used are rats and mice. Rats and mice, however, have different injury pathologies and extent of recovery (Kjell and Olson, 2016). Mice have shown to exhibit cellular proliferation at the injury site following SCI to retain the structural integrity of the spinal cord (Goritz et al., 2011; Ma et al., 2001). This also results in a lack of fluid filled cysts that form, a phenomenon that does occur in humans and rats (Metz et al., 2000). Mouse models of SCI have even shown regeneration through the site of injury (Inman and Steward, 2003). Humans exhibit none of these natural regenerative mechanisms seen in mice, and neither do rats, making a rat model of SCI much more translatable to human SCI.

In addition to the pathophysiological overlap between rats and humans, the corticospinal tract has also been demonstrated to be capable of plastic changes, making it an ideal target for enhancing plasticity after injury. Studies have shown that following severance of the dorsal corticospinal tract in rats, without any intervention, collaterals sprouted from the CST into the grey matter (Weidner et al., 2001), demonstrating that there is a low level of plasticity already present in the CST that is separate from regeneration. Further plastic changes have been noted following SCI, including injured and uninjured fibres forming collateral branches that sprout from the parent axon that can restore functional connectivity by reconnecting with target cells or by rewiring with spared circuitry (Bareyre et al., 2004; Vavrek et al., 2006; Weidner et al., 2001). These collateral sprouts have been directly linked to functional changes in descending motor pathways, as demonstrated by the CST sprouting after thoracic (Fouad et al., 2001) and cervical (Weidner et al., 2001) injury. This plasticity is shown further in models using overlapping hemisections, which was done in a study by Courtine et al. (2008), where it was demonstrated that the CST is capable of profound rearrangement. Two overlapping hemisections on opposite sides were performed, resulting in a complete severance of descending input below the second hemisection. The authors demonstrated propriospinal interneurons were acting as relays and bypassing the injury site, resulting in an improvement in locomotion (Courtine et al., 2008).

Overall, the C4 DLQ injury as a model of incomplete cervical SCI presents us with a model that matches the pathophysiological profile of human SCI, is capable of substantial plastic changes certainly ripe for enhancement, and presents a clear connection between the motor outcome desired and the descending tract being targeted.

While it may not match the most common form of injury in humans, it does provide a model that allows for investigation into the plasticity promoting effects of treatments in a more reproducible manner. The plastic potential and the capability of that plasticity to be increased in the CST make the CST an ideal target for improving functional motor recovery.

1.6 References

Ahuja, C.S., Nori, S., Tetreault, L., Wilson, J., Kwon, B., Harrop, J., Choi, D., Fehlings, M.G., 2017. Traumatic Spinal Cord Injury—Repair and Regeneration. Neurosurgery 80, S9–S22. https://doi.org/10.1093/neuros/nyw080

Anderson, K.D., 2004. Targeting Recovery: Priorities of the Spinal Cord-Injured Population. J. Neurotrauma 21, 1371–1383. https://doi.org/10.1089/neu.2004.21.1371

Asanuma, H., Rosen, I., 1972. Topographical organization of cortical efferent zones projecting to distal forelimb muscles in the monkey. Exp. Brain Res. 14, 243–256. https://doi.org/10.1007/BF00816161

Baguley, I., 2008. Autonomic Complications Following Central Nervous System Injury. Semin. Neurol. 28, 716–725. https://doi.org/10.1055/s-0028-1105971

Balentine, J.D., 1978. Pathology of experimental spinal cord trauma. I. The necrotic lesion as a function of vascular injury. Lab. Investig. J. Tech. Methods Pathol. 39, 236–253.

Barbeau, H., Rossignol, S., 1987. Recovery of locomotion after chronic spinalization in the adult cat. Brain Res. 412, 84–95. https://doi.org/10.1016/0006-8993(87)91442-9

Bareyre, F.M., Kerschensteiner, M., Raineteau, O., Mettenleiter, T.C., Weinmann, O., Schwab, M.E., 2004. The injured spinal cord spontaneously forms a new intraspinal circuit in adult rats. Nat. Neurosci. 7, 269–277. https://doi.org/10.1038/nn1195

Battistuzzo, C.R., Callister, R.J., Callister, R., Galea, M.P., 2012. A Systematic Review of Exercise Training To Promote Locomotor Recovery in Animal Models of Spinal Cord Injury. J. Neurotrauma 29, 1600–1613. https://doi.org/10.1089/neu.2011.2199

Beattie, M.S., Bresnahan, J.C., Komon, J., Tovar, C.A., Van Meter, M., Anderson, D.K., Faden, A.I., Hsu, C.Y., Noble, L.J., Salzman, S., Young, W., 1997. Endogenous Repair after Spinal Cord Contusion Injuries in the Rat. Exp. Neurol. 148, 453–463. https://doi.org/10.1006/exnr.1997.6695

Beattie, M.S., Farooqui, A.A., Bresnahan, J.C., 2000. Review of Current Evidence for Apoptosis After Spinal Cord Injury. J. Neurotrauma 17, 915–925. https://doi.org/10.1089/neu.2000.17.915

Beekhuizen, K.S., Field-Fote, E.C., 2005. Massed Practice versus Massed Practice with Stimulation: Effects on Upper Extremity Function and Cortical Plasticity in Individuals with Incomplete Cervical Spinal Cord Injury. Neurorehabil. Neural Repair 19, 33–45. https://doi.org/10.1177/1545968305274517

Bell, J.A., Wolke, M.L., Ortez, R.C., Jones, T.A., Kerr, A.L., 2015. Training Intensity Affects Motor Rehabilitation Efficacy Following Unilateral Ischemic Insult of the Sensorimotor Cortex in C57BL/6 Mice. Neurorehabil. Neural Repair 29, 590–598. https://doi.org/10.1177/1545968314553031

Berry, M., 1982. Post-injury myelin-breakdown products inhibit axonal growth: an hypothesis to explain the failure of axonal regeneration in the mammalian central nervous system. Bibl. Anat. 1–11.

Bertrand, S., Cazalets, J.-R., 2013. Activity-dependent Synaptic Plasticity and Metaplasticity in Spinal Motor Networks. Curr. Pharm. Des. 19, 4498–4508. https://doi.org/10.2174/1381612811319240014

Bethea, J.R., 2000. Spinal cord injury-induced inflammation: A dual-edged sword, in: Progress in Brain Research. Elsevier, pp. 33–42. https://doi.org/10.1016/S0079-6123(00)28005-9

Blight, A.R., 1985. Delayed Demyelination and Macrophage Invasion: A Candidate for Secondary Cell Damage in Spinal Cord Injury. Cent. Nerv. Syst. Trauma 2, 299–315. https://doi.org/10.1089/cns.1985.2.299

Bloom, O., Herman, P.E., Spungen, A.M., 2020. Systemic inflammation in traumatic spinal cord injury. Exp. Neurol. 325, 113143. https://doi.org/10.1016/j.expneurol.2019.113143

Brown, D.J., Hill, S.T., Baker, H.W.G., 2006. Male fertility and sexual function after spinal cord injury, in: Progress in Brain Research. Elsevier, pp. 427–439. https://doi.org/10.1016/S0079-6123(05)52029-6

Bruce, J.H., Norenberg, M.D., Kraydieh, S., Puckett, W., Marcillo, A., Dietrich, D., 2000. Schwannosis: Role of Gliosis and Proteoglycan in Human Spinal Cord Injury. J. Neurotrauma 17, 781–788. https://doi.org/10.1089/neu.2000.17.781

Bunge, R.P., Puckett, W.R., Becerra, J.L., Marcillo, A., 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. Adv. Neurol. 59, 75–89.

Buss, A., Pech, K., Kakulas, B.A., Martin, D., Schoenen, J., Noth, J., Brook, G.A., 2006. Growth-modulating molecules are associated with invading Schwann cells and not astrocytes in human traumatic spinal cord injury. Brain 130, 940–953. https://doi.org/10.1093/brain/awl374

Cafferty, W.B.J., McGee, A.W., Strittmatter, S.M., 2008. Axonal growth therapeutics: regeneration or sprouting or plasticity? Trends Neurosci. 31, 215–220. https://doi.org/10.1016/j.tins.2008.02.004

Cai, D., Qiu, J., Cao, Z., McAtee, M., Bregman, B.S., Filbin, M.T., 2001. Neuronal Cyclic AMP Controls the Developmental Loss in Ability of Axons to Regenerate. J. Neurosci. 21, 4731–4739. https://doi.org/10.1523/JNEUROSCI.21-13-04731.2001

Canty, A.J., Murphy, M., 2008. Molecular mechanisms of axon guidance in the developing corticospinal tract. Prog. Neurobiol. 85, 214–235. https://doi.org/10.1016/j.pneurobio.2008.02.001

Caroni, P., Schwab, M.E., 1988. Antibody against myelin associated inhibitor of neurite growth neutralizes nonpermissive substrate properties of CNS white matter. Neuron 1, 85–96. https://doi.org/10.1016/0896-6273(88)90212-7

Chen, M.S., Huber, A.B., van der Haar, M.E., Frank, M., Schnell, L., Spillmann, A.A., Christ, F., Schwab, M.E., 2000. Nogo-A is a myelin-associated neurite outgrowth inhibitor and an antigen for monoclonal antibody IN-1. Nature 403, 434–439. https://doi.org/10.1038/35000219

Cotman, C.W., Berchtold, N.C., Christie, L.-A., 2007. Exercise builds brain health: key roles of growth factor cascades and inflammation. Trends Neurosci. 30, 464–472. https://doi.org/10.1016/j.tins.2007.06.011

Courtine, G., Song, B., Roy, R.R., Zhong, H., Herrmann, J.E., Ao, Y., Qi, J., Edgerton, V.R., Sofroniew, M.V., 2008. Recovery of supraspinal control of stepping via indirect propriospinal relay connections after spinal cord injury. Nat. Med. 14, 69–74. https://doi.org/10.1038/nm1682

Craggs, M.D., Balasubramaniam, A.V., Chung, E.A.L., Emmanuel, A.V., 2006. Aberrant reflexes and function of the pelvic organs following spinal cord injury in man. Auton. Neurosci. 126–127, 355–370. https://doi.org/10.1016/j.autneu.2006.03.010

Crowe, M.J., Bresnahan, J.C., Shuman, S.L., Masters, J.N., Beattie, M.S., 1997. Apoptosis and delayed degeneration after spinal cord injury in rats and monkeys. Nat. Med. 3, 73–76. https://doi.org/10.1038/nm0197-73

Demopoulos, H.B., Flamm, E.S., Pietronigro, D.D., Seligman, M.L., 1980. The free radical pathology and the microcirculation in the major central nervous system disorders. Acta Physiol. Scand. Suppl. 492, 91–119.

Dishman, R.K., Berthoud, H.-R., Booth, F.W., Cotman, C.W., Edgerton, V.R., Fleshner, M.R., Gandevia, S.C., Gomez-Pinilla, F., Greenwood, B.N., Hillman, C.H., Kramer, A.F., Levin, B.E., Moran, T.H., Russo-Neustadt, A.A., Salamone, J.D., Van Hoomissen, J.D., Wade, C.E., York, D.A., Zigmond, M.J., 2006. Neurobiology of Exercise*. Obesity 14, 345–356. https://doi.org/10.1038/oby.2006.46

Donnelly, D.J., Popovich, P.G., 2008. Inflammation and its role in neuroprotection, axonal regeneration and functional recovery after spinal cord injury. Exp. Neurol. 209, 378–388. https://doi.org/10.1016/j.expneurol.2007.06.009

Dougherty, K.D., Dreyfus, C.F., Black, I.B., 2000. Brain-Derived Neurotrophic Factor in Astrocytes, Oligodendrocytes, and Microglia/Macrophages after Spinal Cord Injury. Neurobiol. Dis. 7, 574–585. https://doi.org/10.1006/nbdi.2000.0318

Dunlop, S.A., 2008. Activity-dependent plasticity: implications for recovery after spinal cord injury. Trends Neurosci. 31, 410–418. https://doi.org/10.1016/j.tins.2008.05.004

Elbasiouny, S.M., Moroz, D., Bakr, M.M., Mushahwar, V.K., 2010. Management of Spasticity After Spinal Cord Injury: Current Techniques and Future Directions. Neurorehabil. Neural Repair 24, 23–33. https://doi.org/10.1177/1545968309343213

Emery, E., Aldana, P., Bunge, M.B., Puckett, W., Srinivasan, A., Keane, R.W., Bethea, J., Levi, A.D.O., 1998. Apoptosis after traumatic human spinal cord injury. J. Neurosurg. 89, 911–920. https://doi.org/10.3171/jns.1998.89.6.0911

Fenrich, K., Gordon, T., 2004. Canadian Association of Neuroscience Review: Axonal Regeneration in the Peripheral and Central Nervous Systems – Current Issues and Advances. Can. J. Neurol. Sci. J. Can. Sci. Neurol. 31, 142–156. https://doi.org/10.1017/S0317167100053798

Fenrich, K.K., Hallworth, B.W., Vavrek, R., Raposo, P.J.F., Misiaszek, J.E., Bennett, D.J., Fouad, K., Torres-Espin, A., 2021. Self-directed rehabilitation training intensity thresholds for efficient recovery of skilled forelimb function in rats with

cervical spinal cord injury. Exp. Neurol. 339, 113543. https://doi.org/10.1016/j.expneurol.2020.113543

Fleming, J.C., Norenberg, M.D., Ramsay, D.A., Dekaban, G.A., Marcillo, A.E., Saenz, A.D., Pasquale-Styles, M., Dietrich, W.D., Weaver, L.C., 2006. The cellular inflammatory response in human spinal cords after injury. Brain 129, 3249–3269. https://doi.org/10.1093/brain/awl296

Fouad, K., Forero, J., Hurd, C., 2015. A Simple Analogy for Nervous System Plasticity After Injury. Exerc. Sport Sci. Rev. 43, 100–106. https://doi.org/10.1249/JES.0000000000000040

Fouad, K., Pedersen, V., Schwab, M.E., Brösamle, C., 2001. Cervical sprouting of corticospinal fibers after thoracic spinal cord injury accompanies shifts in evoked motor responses. Curr. Biol. 11, 1766–1770. https://doi.org/10.1016/S0960-9822(01)00535-8

Fournier, A.E., GrandPre, T., Strittmatter, S.M., 2001. Identification of a receptor mediating Nogo-66 inhibition of axonal regeneration. Nature 409, 341–346. https://doi.org/10.1038/35053072

García-Alías, G., Barkhuysen, S., Buckle, M., Fawcett, J.W., 2009. Chondroitinase ABC treatment opens a window of opportunity for task-specific rehabilitation. Nat. Neurosci. 12, 1145–1151. https://doi.org/10.1038/nn.2377

Girgis, J., Merrett, D., Kirkland, S., Metz, G.A.S., Verge, V., Fouad, K., 2007. Reaching training in rats with spinal cord injury promotes plasticity and task specific recovery. Brain 130, 2993–3003. https://doi.org/10.1093/brain/awm245

Gomez-Pinilla, F., Ying, Z., Zhuang, Y., 2012. Brain and Spinal Cord Interaction: Protective Effects of Exercise Prior to Spinal Cord Injury. PLoS ONE 7, e32298. https://doi.org/10.1371/journal.pone.0032298

Goritz, C., Dias, D.O., Tomilin, N., Barbacid, M., Shupliakov, O., Frisen, J., 2011. A Pericyte Origin of Spinal Cord Scar Tissue. Science 333, 238–242. https://doi.org/10.1126/science.1203165

GrandPré, T., Nakamura, F., Vartanian, T., Strittmatter, S.M., 2000. Identification of the Nogo inhibitor of axon regeneration as a Reticulon protein. Nature 403, 439–444. https://doi.org/10.1038/35000226

Gris, D., Marsh, D.R., Oatway, M.A., Chen, Y., Hamilton, E.F., Dekaban, G.A., Weaver, L.C., 2004. Transient Blockade of the CD11d/CD18 Integrin Reduces Secondary Damage after Spinal Cord Injury, Improving Sensory, Autonomic, and Motor Function. J. Neurosci. 24, 4043–4051. https://doi.org/10.1523/JNEUROSCI.5343-03.2004

Hall, E.D., 1992. The neuroprotective pharmacology of methylprednisolone. J. Neurosurg. 76, 13–22. https://doi.org/10.3171/jns.1992.76.1.0013

Hanson, R.W., Franklin, M.R., 1976. Sexual loss in relation to other functional losses for spinal cord injured males. Arch. Phys. Med. Rehabil. 57, 291–293.

Harkema, S.J., 2001. Neural Plasticity after Human Spinal Cord Injury: Application of Locomotor Training to the Rehabilitation of Walking. The Neuroscientist 7, 455–468. https://doi.org/10.1177/107385840100700514

Herrera, J.J., Nesic, O., Narayana, P.A., 2009. Reduced Vascular Endothelial Growth Factor Expression in Contusive Spinal Cord Injury. J. Neurotrauma 26, 995–1003. https://doi.org/10.1089/neu.2008.0779

Hou, J., Nelson, R., Nissim, N., Parmer, R., Thompson, F.J., Bose, P., 2014. Effect of Combined Treadmill Training and Magnetic Stimulation on Spasticity and Gait Impairments after Cervical Spinal Cord Injury. J. Neurotrauma 31, 1088–1106. https://doi.org/10.1089/neu.2013.3096

Huber, A.B., Weinmann, O., Brösamle, C., Oertle, T., Schwab, M.E., 2002. Patterns of Nogo mRNA and Protein Expression in the Developing and Adult Rat and After CNS Lesions. J. Neurosci. 22, 3553–3567. https://doi.org/10.1523/JNEUROSCI.22-09-03553.2002

Inman, D.M., Steward, O., 2003. Ascending sensory, but not other long-tract axons, regenerate into the connective tissue matrix that forms at the site of a spinal cord injury in mice. J. Comp. Neurol. 462, 431–449. https://doi.org/10.1002/cne.10768

Kakulas, B.A., 1999. A review of the neuropathology of human spinal cord injury with emphasis on special features. J. Spinal Cord Med. 22, 119–124. https://doi.org/10.1080/10790268.1999.11719557

Kakulas, B.A., 1984. Pathology of spinal injuries. Cent. Nerv. Syst. Trauma J. Am. Paralys. Assoc. 1, 117–129. https://doi.org/10.1089/cns.1984.1.117

Kanagal, S.G., Muir, G.D., 2009. Task-dependent compensation after pyramidal tract and dorsolateral spinal lesions in rats. Exp. Neurol. 216, 193–206. https://doi.org/10.1016/j.expneurol.2008.11.028

Kanagal, S.G., Muir, G.D., 2008. Effects of combined dorsolateral and dorsal funicular lesions on sensorimotor behaviour in rats. Exp. Neurol. 214, 229–239. https://doi.org/10.1016/j.expneurol.2008.08.004

Kerschensteiner, M., Schwab, M.E., Lichtman, J.W., Misgeld, T., 2005. In vivo imaging of axonal degeneration and regeneration in the injured spinal cord. Nat. Med. 11, 572–577. https://doi.org/10.1038/nm1229

Kirshblum, S.C., Burns, S.P., Biering-Sorensen, F., Donovan, W., Graves, D.E., Jha, A., Johansen, M., Jones, L., Krassioukov, A., Mulcahey, M.J., Schmidt-Read, M., Waring, W., 2011. International standards for neurological classification of spinal cord injury (Revised 2011). J. Spinal Cord Med. 34, 535–546. https://doi.org/10.1179/204577211X13207446293695

Kjell, J., Olson, L., 2016. Rat models of spinal cord injury: from pathology to potential therapies. Dis. Model. Mech. 9, 1125–1137. https://doi.org/10.1242/dmm.025833

Kleim, J.A., Barbay, S., Nudo, R.J., 1998. Functional Reorganization of the Rat Motor Cortex Following Motor Skill Learning. J. Neurophysiol. 80, 3321–3325. https://doi.org/10.1152/jn.1998.80.6.3321

Kowalczewski, J., Ravid, E., Prochazka, A., 2011. Fully-automated test of upper-extremity function, in: 2011 Annual International Conference of the IEEE Engineering in Medicine and Biology Society. Presented at the 2011 33rd Annual International Conference of the IEEE Engineering in Medicine and Biology Society, IEEE, Boston, MA, pp. 7332–7335. https://doi.org/10.1109/IEMBS.2011.6091710

Kusiak, A.N., Selzer, M.E., 2013. Neuroplasticity in the spinal cord, in: Handbook of Clinical Neurology. Elsevier, pp. 23–42. https://doi.org/10.1016/B978-0-444-52901-5.00003-4

Lawrence, D.G., Kuypers, H.G.J.M., 1968. THE FUNCTIONAL ORGANIZATION OF THE MOTOR SYSTEM IN THE MONKEY: II. THE EFFECTS OF LESIONS OF THE DESCENDING BRAIN-STEM PATHWAYS. Brain 91, 15–36. https://doi.org/10.1093/brain/91.1.15

Leech, K.A., Kinnaird, C.R., Holleran, C.L., Kahn, J., Hornby, T.G., 2016. Effects of Locomotor Exercise Intensity on Gait Performance in Individuals With Incomplete Spinal Cord Injury. Phys. Ther. 96, 1919–1929. https://doi.org/10.2522/ptj.20150646

Lemon, R.N., 2008. Descending Pathways in Motor Control. Annu. Rev. Neurosci. 31, 195–218. https://doi.org/10.1146/annurev.neuro.31.060407.125547

Lemon, R.N., Griffiths, J., 2005. Comparing the function of the corticospinal system in different species: Organizational differences for motor specialization? Muscle Nerve 32, 261–279. https://doi.org/10.1002/mus.20333

Li, S., Stys, P.K., 2000. Mechanisms of Ionotropic Glutamate Receptor-Mediated Excitotoxicity in Isolated Spinal Cord White Matter. J. Neurosci. 20, 1190–1198. https://doi.org/10.1523/JNEUROSCI.20-03-01190.2000

Liu, K., Lu, Y., Lee, J.K., Samara, R., Willenberg, R., Sears-Kraxberger, I., Tedeschi, A., Park, K.K., Jin, D., Cai, B., Xu, B., Connolly, L., Steward, O., Zheng, B., He, Z., 2010. PTEN deletion enhances the regenerative ability of adult corticospinal neurons. Nat. Neurosci. 13, 1075–1081. https://doi.org/10.1038/nn.2603

Liu, X.Z., Xu, X.M., Hu, R., Du, C., Zhang, S.X., McDonald, J.W., Dong, H.X., Wu, Y.J., Fan, G.S., Jacquin, M.F., Hsu, C.Y., Choi, D.W., 1997. Neuronal and Glial Apoptosis after Traumatic Spinal Cord Injury. J. Neurosci. 17, 5395–5406. https://doi.org/10.1523/JNEUROSCI.17-14-05395.1997

Ma, M., Basso, D.M., Walters, P., Stokes, B.T., Jakeman, L.B., 2001. Behavioral and Histological Outcomes Following Graded Spinal Cord Contusion Injury in the C57Bl/6 Mouse. Exp. Neurol. 169, 239–254. https://doi.org/10.1006/exnr.2001.7679

MacLellan, C.L., Keough, M.B., Granter-Button, S., Chernenko, G.A., Butt, S., Corbett, D., 2011. A Critical Threshold of Rehabilitation Involving Brain-Derived Neurotrophic Factor Is Required for Poststroke Recovery. Neurorehabil. Neural Repair 25, 740–748. https://doi.org/10.1177/1545968311407517

May, Z., Fenrich, K.K., Dahlby, J., Batty, N.J., Torres-Espín, A., Fouad, K., 2017. Following Spinal Cord Injury Transected Reticulospinal Tract Axons Develop New Collateral Inputs to Spinal Interneurons in Parallel with Locomotor Recovery. Neural Plast. 2017, 1–15. https://doi.org/10.1155/2017/1932875

McKenna, J.E., Prusky, G.T., Whishaw, I.Q., 2000. Cervical motoneuron topography reflects the proximodistal organization of muscles and movements of the rat forelimb: a retrograde carbocyanine dye analysis. J. Comp. Neurol. 419, 286–296. https://doi.org/10.1002/(sici)1096-9861(20000410)419:3<286::aid-cne2>3.0.co;2-3

McKenna, J.E., Whishaw, I.Q., 1999. Complete Compensation in Skilled Reaching Success with Associated Impairments in Limb Synergies, after Dorsal Column

Lesion in the Rat. J. Neurosci. 19, 1885–1894. https://doi.org/10.1523/JNEUROSCI.19-05-01885.1999

McKerracher, L., David, S., Jackson, D.L., Kottis, V., Dunn, R.J., Braun, P.E., 1994. Identification of myelin-associated glycoprotein as a major myelin-derived inhibitor of neurite growth. Neuron 13, 805–811. https://doi.org/10.1016/0896-6273(94)90247-X

Metz, G.A.S., Curt, A., van de MEENT, H., Klusman, I., Schwab, M.E., Dietz, V., 2000. Validation of the Weight-Drop Contusion Model in Rats: A Comparative Study of Human Spinal Cord Injury. J. Neurotrauma 17, 1–17. https://doi.org/10.1089/neu.2000.17.1

Metz, G.A.S., Whishaw, I.Q., 2000. Skilled reaching an action pattern: stability in rat (Rattus norvegicus) grasping movements as a function of changing food pellet size. Behav. Brain Res. 116, 111–122. https://doi.org/10.1016/S0166-4328(00)00245-X

Mikol, D.D., Stefansson, K., 1988. A phosphatidylinositol-linked peanut agglutinin-binding glycoprotein in central nervous system myelin and on oligodendrocytes. J. Cell Biol. 106, 1273–1279. https://doi.org/10.1083/jcb.106.4.1273

Morgenstern, D.A., Asher, R.A., Fawcett, J.W., 2002. Chapter 22 Chondroitin sulphate proteoglycans in the CNS injury response, in: Progress in Brain Research. Elsevier, pp. 313–332. https://doi.org/10.1016/S0079-6123(02)37024-9

Moseley, L.G., 2007. Using visual illusion to reduce at-level neuropathic pain in paraplegia. Pain 130, 294–298. https://doi.org/10.1016/j.pain.2007.01.007

Mueller, P.J., 2007. EXERCISE TRAINING AND SYMPATHETIC NERVOUS SYSTEM ACTIVITY: EVIDENCE FOR PHYSICAL ACTIVITY DEPENDENT NEURAL PLASTICITY: Exercise training and the SNS. Clin. Exp. Pharmacol. Physiol. 34, 377–384. https://doi.org/10.1111/j.1440-1681.2007.04590.x

Mukhopadhyay, G., Doherty, P., Walsh, F.S., Crocker, P.R., Filbin, M.T., 1994. A novel role for myelin-associated glycoprotein as an inhibitor of axonal regeneration. Neuron 13, 757–767. https://doi.org/10.1016/0896-6273(94)90042-6

Nathan, P.W., Smith, M.C., 1982. THE RUBROSPINAL AND CENTRAL TEGMENTAL TRACTS IN MAN. Brain 105, 223–269. https://doi.org/10.1093/brain/105.2.223

Orr, M.B., Gensel, J.C., 2018. Spinal Cord Injury Scarring and Inflammation: Therapies Targeting Glial and Inflammatory Responses. Neurotherapeutics 15, 541–553. https://doi.org/10.1007/s13311-018-0631-6

Oudega, M., Perez, M.A., 2012. Corticospinal reorganization after spinal cord injury: Corticospinal tract and SCI. J. Physiol. 590, 3647–3663. https://doi.org/10.1113/jphysiol.2012.233189

Oyinbo, C.A., 2011. Secondary injury mechanisms in traumatic spinal cord injury: a nugget of this multiply cascade. Acta Neurobiol. Exp. (Warsz.) 71, 281–299.

Park, E., Velumian, A.A., Fehlings, M.G., 2004. The Role of Excitotoxicity in Secondary Mechanisms of Spinal Cord Injury: A Review with an Emphasis on the Implications for White Matter Degeneration. J. Neurotrauma 21, 754–774. https://doi.org/10.1089/0897715041269641

Popovich, P.G., Wei, P., Stokes, B.T., 1997. Cellular inflammatory response after spinal cord injury in Sprague-Dawley and Lewis rats. J. Comp. Neurol. 377, 443–464. https://doi.org/10.1002/(sici)1096-9861(19970120)377:3<443::aid-cne10>3.0.co;2-s

Praxis Spinal Cord Institute, 2021. Rick Hansen Spinal Cord Injury Registry - A Look at Traumatic Spinal Cord Injury in Canada in 2019.

Prinjha, R., Moore, S.E., Vinson, M., Blake, S., Morrow, R., Christie, G., Michalovich, D., Simmons, D.L., Walsh, F.S., 2000. Inhibitor of neurite outgrowth in humans. Nature 403, 383–384. https://doi.org/10.1038/35000287

Raineteau, O., Fouad, K., Noth, P., Thallmair, M., Schwab, M.E., 2001. Functional switch between motor tracts in the presence of the mAb IN-1 in the adult rat. Proc. Natl. Acad. Sci. 98, 6929–6934. https://doi.org/10.1073/pnas.111165498

Rasmussen, T., Penfield, W., 1947. The human sensorimotor cortex as studied by electrical stimulation. Fed. Proc. 6, 184.

Rosenzweig, E.S., McDonald, J.W., 2004. Rodent models for treatment of spinal cord injury: research trends and progress toward useful repair: Curr. Opin. Neurol. 17, 121–131. https://doi.org/10.1097/00019052-200404000-00007

Savio, T., Schwab, M., 1989. Rat CNS white matter, but not gray matter, is nonpermissive for neuronal cell adhesion and fiber outgrowth. J. Neurosci. 9, 1126–1133. https://doi.org/10.1523/JNEUROSCI.09-04-01126.1989

Schnell, L., Fearn, S., Klassen, H., Schwab, M.E., Perry, V.H., 1999. Acute inflammatory responses to mechanical lesions in the CNS: differences between brain and spinal

cord: Inflammatory responses in brain and spinal cord. Eur. J. Neurosci. 11, 3648–3658. https://doi.org/10.1046/j.1460-9568.1999.00792.x

Schwab, M.E., Bartholdi, D., 1996. Degeneration and regeneration of axons in the lesioned spinal cord. Physiol. Rev. 76, 319–370. https://doi.org/10.1152/physrev.1996.76.2.319

Schwenkreis, P., El Tom, S., Ragert, P., Pleger, B., Tegenthoff, M., Dinse, H.R., 2007. Assessment of sensorimotor cortical representation asymmetries and motor skills in violin players: Sensorimotor asymmetries in violin players. Eur. J. Neurosci. 26, 3291–3302. https://doi.org/10.1111/j.1460-9568.2007.05894.x

Sharif-Alhoseini, M., Khormali, M., Rezaei, M., Safdarian, M., Hajighadery, A., Khalatbari, M.M., Safdarian, M., Meknatkhah, S., Rezvan, M., Chalangari, M., Derakhshan, P., Rahimi-Movaghar, V., 2017. Animal models of spinal cord injury: a systematic review. Spinal Cord 55, 714–721. https://doi.org/10.1038/sc.2016.187

Sipski, M.L., Arenas, A., 2006. Female sexual function after spinal cord injury, in: Progress in Brain Research. Elsevier, pp. 441–447. https://doi.org/10.1016/S0079-6123(05)52030-2

Snoek, G.J., IJzerman, M.J., Hermens, H.J., Maxwell, D., Biering-Sorensen, F., 2004. Survey of the needs of patients with spinal cord injury: impact and priority for improvement in hand function in tetraplegics. Spinal Cord 42, 526–532. https://doi.org/10.1038/sj.sc.3101638

Soderblom, C., Luo, X., Blumenthal, E., Bray, E., Lyapichev, K., Ramos, J., Krishnan, V., Lai-Hsu, C., Park, K.K., Tsoulfas, P., Lee, J.K., 2013. Perivascular Fibroblasts

Form the Fibrotic Scar after Contusive Spinal Cord Injury. J. Neurosci. 33, 13882–13887. https://doi.org/10.1523/JNEUROSCI.2524-13.2013

Spinal Cord Injury (SCI) Facts and Figures at a Glance, 2016. . J. Spinal Cord Med. 39, 370–371. https://doi.org/10.1080/10790268.2016.1177348

Starkey, M.L., Bleul, C., Kasper, H., Mosberger, A.C., Zörner, B., Giger, S., Gullo, M., Buschmann, F., Schwab, M.E., 2014. High-Impact, Self-Motivated Training Within an Enriched Environment With Single Animal Tracking Dose-Dependently Promotes Motor Skill Acquisition and Functional Recovery. Neurorehabil. Neural Repair 28, 594–605. https://doi.org/10.1177/1545968314520721

Stoneham, E.T., Sanders, E.M., Sanyal, M., Dumas, T.C., 2010. Rules of Engagement: Factors That Regulate Activity-Dependent Synaptic Plasticity During Neural Network Development. Biol. Bull. 219, 81–99. https://doi.org/10.1086/BBLv219n2p81

Swiech, L., Perycz, M., Malik, A., Jaworski, J., 2008. Role of mTOR in physiology and pathology of the nervous system. Biochim. Biophys. Acta BBA - Proteins Proteomics 1784, 116–132. https://doi.org/10.1016/j.bbapap.2007.08.015

Tahayori, B., Koceja, D.M., 2012. Activity-Dependent Plasticity of Spinal Circuits in the Developing and Mature Spinal Cord. Neural Plast. 2012, 1–12. https://doi.org/10.1155/2012/964843

Takei, N., Nawa, H., 2014. mTOR signaling and its roles in normal and abnormal brain development. Front. Mol. Neurosci. 7. https://doi.org/10.3389/fnmol.2014.00028

Tashiro, S., Shinozaki, M., Mukaino, M., Renault-Mihara, F., Toyama, Y., Liu, M., Nakamura, M., Okano, H., 2015. BDNF Induced by Treadmill Training Contributes to the Suppression of Spasticity and Allodynia After Spinal Cord Injury via Upregulation of KCC2. Neurorehabil. Neural Repair 29, 677–689. https://doi.org/10.1177/1545968314562110

Tator, C.H., Fehlings, M.G., 1991. Review of the secondary injury theory of acute spinal cord trauma with emphasis on vascular mechanisms. J. Neurosurg. 75, 15–26. https://doi.org/10.3171/jns.1991.75.1.0015

Torres-Espín, A., Beaudry, E., Fenrich, K., Fouad, K., 2018a. Rehabilitative Training in Animal Models of Spinal Cord Injury. J. Neurotrauma 35, 1970–1985. https://doi.org/10.1089/neu.2018.5906

Torres-Espín, A., Forero, J., Fenrich, K.K., Lucas-Osma, A.M., Krajacic, A., Schmidt, E., Vavrek, R., Raposo, P., Bennett, D.J., Popovich, P.G., Fouad, K., 2018b. Eliciting inflammation enables successful rehabilitative training in chronic spinal cord injury. Brain 141, 1946–1962. https://doi.org/10.1093/brain/awy128

Torres-Espín, A., Forero, J., Schmidt, E.K.A., Fouad, K., Fenrich, K.K., 2018c. A motorized pellet dispenser to deliver high intensity training of the single pellet reaching and grasping task in rats. Behav. Brain Res. 336, 67–76. https://doi.org/10.1016/j.bbr.2017.08.033

Vavrek, R., Girgis, J., Tetzlaff, W., Hiebert, G.W., Fouad, K., 2006. BDNF promotes connections of corticospinal neurons onto spared descending interneurons in spinal cord injured rats. Brain 129, 1534–1545. https://doi.org/10.1093/brain/awl087

Vaynman, S., Gomez-Pinilla, F., 2005. License to Run: Exercise Impacts Functional Plasticity in the Intact and Injured Central Nervous System by Using Neurotrophins. Neurorehabil. Neural Repair 19, 283–295. https://doi.org/10.1177/1545968305280753

von Bernhardi, R., Bernhardi, L.E., Eugenín, J., 2017. What Is Neural Plasticity?, in: von Bernhardi, R., Eugenín, J., Muller, K.J. (Eds.), The Plastic Brain. Springer International Publishing, Cham, pp. 1–15. https://doi.org/10.1007/978-3-319-62817-2_1

Wang, K.C., Kim, J.A., Sivasankaran, R., Segal, R., He, Z., 2002a. p75 interacts with the Nogo receptor as a co-receptor for Nogo, MAG and OMgp. Nature 420, 74–78. https://doi.org/10.1038/nature01176

Wang, K.C., Koprivica, V., Kim, J.A., Sivasankaran, R., Guo, Y., Neve, R.L., He, Z., 2002b. Oligodendrocyte-myelin glycoprotein is a Nogo receptor ligand that inhibits neurite outgrowth. Nature 417, 941–944. https://doi.org/10.1038/nature00867

Weidner, N., Ner, A., Salimi, N., Tuszynski, M.H., 2001. Spontaneous corticospinal axonal plasticity and functional recovery after adult central nervous system injury. Proc. Natl. Acad. Sci. 98, 3513–3518. https://doi.org/10.1073/pnas.051626798

Weishaupt, N., Li, S., Di Pardo, A., Sipione, S., Fouad, K., 2013. Synergistic effects of BDNF and rehabilitative training on recovery after cervical spinal cord injury. Behav. Brain Res. 239, 31–42. https://doi.org/10.1016/j.bbr.2012.10.047

Wessels, M., Lucas, C., Eriks, I., de Groot, S., 2010. Body weight-supported gait training for restoration of walking in people with an incomplete spinal cord injury : a

systematic review. J. Rehabil. Med. 42, 513–519. https://doi.org/10.2340/16501977-0525

Whishaw, I.Q., Gorny, B., Sarna, J., 1998. Paw and limb use in skilled and spontaneous reaching after pyramidal tract, red nucleus and combined lesions in the rat: behavioral and anatomical dissociations. Behav. Brain Res. 93, 167–183. https://doi.org/10.1016/S0166-4328(97)00152-6

Whishaw, I.Q., O'Connor, W.T., Dunnett, S.B., 1986. THE CONTRIBUTIONS OF MOTOR CORTEX, NIGROSTRIATAL DOPAMINE AND CAUDATE-PUTAMEN TO SKILLED FORELIMB USE IN THE RAT. Brain 109, 805–843. https://doi.org/10.1093/brain/109.5.805

Whishaw, I.Q., Pellis, S.M., Gorny, B., Kolb, B., Tetzlaff, W., 1993. Proximal and distal impairments in rat forelimb use in reaching follow unilateral pyramidal tract lesions. Behav. Brain Res. 56, 59–76. https://doi.org/10.1016/0166-4328(93)90022-I

Whishaw, I.Q., Suchowersky, O., Davis, L., Sarna, J., Metz, G.A., Pellis, S.M., 2002. Impairment of pronation, supination, and body co-ordination in reach-to-grasp tasks in human Parkinson's disease (PD) reveals homology to deficits in animal models. Behav. Brain Res. 133, 165–176. https://doi.org/10.1016/S0166-4328(01)00479-X

Widenfalk, J., Lundströmer, K., Jubran, M., Brené, S., Olson, L., 2001. Neurotrophic Factors and Receptors in the Immature and Adult Spinal Cord after Mechanical Injury or Kainic Acid. J. Neurosci. 21, 3457–3475. https://doi.org/10.1523/JNEUROSCI.21-10-03457.2001

Wittenberg, G.F., 2010. Experience, cortical remapping, and recovery in brain disease. Neurobiol. Dis. 37, 252–258. https://doi.org/10.1016/j.nbd.2009.09.007

Yang, H.-S., Kwon, H.G., Hong, J.H., Hong, C.P., Jang, S.H., 2011. The rubrospinal tract in the human brain: Diffusion tensor imaging study. Neurosci. Lett. 504, 45–48. https://doi.org/10.1016/j.neulet.2011.08.054

Yezierski, R.P., 2009. Spinal cord injury pain: spinal and supraspinal mechanisms. J. Rehabil. Res. Dev. 46, 95–107.

Ying, Z., Roy, R.R., Edgerton, V.R., Gómez-Pinilla, F., 2005. Exercise restores levels of neurotrophins and synaptic plasticity following spinal cord injury. Exp. Neurol. 193, 411–419. https://doi.org/10.1016/j.expneurol.2005.01.015

Ying, Z., Roy, R.R., Edgerton, V.R., Gómez-Pinilla, F., 2003. Voluntary exercise increases neurotrophin-3 and its receptor TrkC in the spinal cord. Brain Res. 987, 93–99. https://doi.org/10.1016/S0006-8993(03)03258-X

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, 1070–1078. https://doi.org/10.1016/j.neuroscience.2008.06.057

Chapter 2

Single-Session Cortical Electrical Stimulation Enhances the Efficacy of Rehabilitative Motor Training after Spinal Cord Injury in Rats

Preface

Chapter 2 of this thesis presents my first experiment while working in the Fouad laboratory. This experiment was designed to follow up on a previous study that demonstrated the plasticity promoting effects of electrical stimulation before spinal cord injury (Jack et al., 2017). Whilst this study yielded interesting results, a further step towards clinical relevance was necessary by stimulating after SCI. Published at a similar time as the study from Jack et al. was a study from the laboratory of Dr. Armin Blesch that demonstrated that the benefits of a single session of electrical stimulation did not differ from prolonged or repeated stimulation sessions *in vitro* (Goganau et al., 2017). With this data in mind, my experiment was designed to test the same stimulation paradigm used in the study by Jack et al. to demonstrate that a single stimulation session after SCI would result in functional recovery and increased plasticity in the injured CST.

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To support FAIR data and open science principles, all data for this experiment has been published on the Open Data Commons for Spinal Cord Injury (odc-sci.org):

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2.1 Introduction

Axonal regeneration is limited within the adult central nervous system (CNS: brain and spinal cord). It is assumed that this limited repair is due to the growth inhibitory environment of the CNS (Schwab and Bartholdi, 1996) and the lack of growth promoting factors. After spinal cord injury (SCI) this lack of regrowth and the limited functional recovery has been approached from many sides, including attempts to block the action of growth inhibitors in order to regenerate the damaged neurites through the injury site (Gonzenbach and Schwab, 2008; Lee et al., 2004; Massey et al., 2006; McKerracher et al., 1994). An alternative means to elicit repair and recovery is through increasing plasticity in both the brain and spinal cord, as opposed to focusing on regeneration (Fouad et al., 2015). The goal of increasing plasticity in the injured CNS is to remodel circuitry using the remaining tissue. This can occur concomitantly at different anatomical and physiological levels (Kusiak and Selzer, 2013; von Bernhardi et al., 2017). One such anatomical change is collateral sprouting of both injured and spared neurons (Dell'Anno and Strittmatter, 2017).

An approach to increase both regenerative growth at the injured axon tip as well as neuroplasticity, is electrical stimulation (ES). ES has been used after nerve injury to facilitate regeneration speed and distance (Gordon, 2016a, 2016b; Willand et al., 2016), and has been translated to the clinical setting following surgery (Chan et al., 2016). Furthermore, substantial work in SCI models suggests that continuous electrical stimulation of the cortex innervating the spared side of the spinal cord leads to an increase in sprouting of its projections (Carmel et al., 2013, 2010; Carmel and Martin, 2014). While many studies have shown the growth promoting effects of repeated

electrical stimulation in spared CNS neurons over a period of days (Brus-Ramer et al., 2007; Carmel et al., 2014, 2013, 2010), single sessions of stimulation to injured spinal tracts have only recently been explored. For example, we have shown that similar to the peripheral nervous system (Brushart et al., 2002), stimulation of sensory fibers increases their ability to grow *in vitro* (Goganau et al., 2017) and *in vivo* (Udina et al., 2008). Our group has recently demonstrated that similar stimulation could also promote plasticity in injured descending tracts (Jack et al., 2017). However, as stimulation of a lesioned spinal tract is a difficult approach, this study rather applied a "conditioning" stimulation before a SCI was introduced. Therefore, the goal of the current study was to move a step further towards a clinical relevant stimulation approach, by stimulating after, and not before a cervical SCI, and by adding rehabilitative motor training. Here we perform reaching training, a motor function that is affected by the lesion. Such training is standard in individuals with cervical spinal cord injury and it has been shown multiple times in animal models to be essential in translating plasticity promoting treatments (like ES) into functional recovery (García-Alías et al., 2009; Torres-Espín et al., 2018a; Weishaupt et al., 2013).

Here we examine the plasticity promoting effects of a single session of electrical stimulation of the motor cortex innervating the injured side of the spinal cord immediately following a unilateral SCI in rats in conjunction with rehabilitative motor training.

2.2 Methods and Materials

2.2.1 Animals

Female adult Lewis rats (Charles River Laboratories, Canada) weighing 180-220g were used for all experiments. Rats were housed 5 per cage with a 12-hour light/dark cycle. Water and food were provided *ad libitum* apart from during the training period where, to encourage reaching behaviour in the rats, each cage was given 90% of its normal daily food intake (12g/rat, 60g/cage). Total number of animals used in this experiment was 27 animals, though only 20 remained part of the final experiment (n=11 in the stimulated group, n=9 in control) due to exclusion criteria (see statistical analysis). The experiment was run in 3 batches of animals, with each batch having animals randomized into control or stimulated groups so that each batch had an equal number of animals per group. Experiments were approved by the Health Sciences Animal Care and Use Committee of the University of Alberta.

2.2.2 Single Pellet Grasping Training

Prior to receiving a SCI, rats were pre-trained in the single pellet grasping (SPG) task (Whishaw et al., 1986) to ensure they are sufficiently able to complete the task, so as to be able to continue post-injury. Animals were acclimated to the SPG chamber and their dominant paw was determined by the experimenter while training the rats to reach for sugar pellets (45mg, Bio-serv) (Alaverdashvili, 2008; Alaverdashvili and Whishaw, 2013; McKenna and Whishaw, 1999). The training chamber is set up with semi-automated pellet dispensers attached to both sides as described previously (Torres-Espín et al., 2018b). The rats were trained to go from one side to the other reaching for pellets.

Rats were trained 5 days a week for ten minutes a day. Once their success rate reached a plateau at approximately 4 weeks of training, animals underwent SCI surgery and were given a week for recovery. Following this recovery period, the animals were reintroduced to the SPG task for 10 minutes a day, 5 days a week for rehabilitative motor therapy. All animals received training as it is a standard in the clinic. No limit of pellets was imposed during the 10 minutes of training.

Rats were assessed weekly following injury by videotaping every fifth training session and scoring each pellet grasping attempt as follows: 'Attempts' were defined as the number of times the animal reached for a pellet no matter the outcome, and 'Success rate' was defined as the percentage of attempts in which the animal was able to reach and move the pellet to their mouth and eat it, though this allows for compensatory means to obtain the pellet (Hurd et al., 2013; May et al., 2015; Torres-Espín et al., 2018a).

After 5 weeks of rehabilitative reaching training, a different assessment was performed introducing a gap between the animal and the pellet. This gap creates a barrier for animals that compensate for their inability to grasp the pellet by dragging it towards them. By having a gap in which a dragged pellet can fall into, the rats are forced to retrieve the pellet over the gap to succeed. This alteration to the SPG task differentiates between animals that compensate by scooping as opposed to animals that show recovery by firmly grasping and holding on to the pellet (Torres-Espín et al., 2018b).

2.2.3 Single Pellet Grasping Movement Analysis

At the end of the SPG training the reaching movement pattern of for each animal was analyzed as described by Metz and Whishaw (2000). High speed video recordings (120 fps) were taken of each animal successfully reaching for 3 pellets. The performance in each trial was broken up into 11 components (Metz and Whishaw, 2000) and scored based on the presence of that movement and how it was performed, scores are: 0 – absent movement, 0.5 – movement is abnormal, 1 – movement is normal. These three successes were individually scored and averaged for each animal. For animals that were unable to perform successful reaches by the end of training, their best 3 attempts were taken.

2.2.4 Spinal Cord Injuries

Each animal received a unilateral dorsal-lateral quadrant (DLQ) spinal cord transection injury at cervical vertebrae level 4 on the side of the individual animal's dominant paw as we described earlier (Girgis et al., 2007; Krajacic et al., 2009). DLQ injuries target the dorsal corticospinal tract and the majority of the rubrospinal tract, as well as ascending fibers. For lesion surgeries animals were anesthetized via intraperitoneal injection of ketamine/xylazine (70 mg/kg ketamine; 7 mg/kg xylazine) and placed in a stereotactic frame. At the onset of surgery, rats were shaved and disinfected with 2% chlorhexidine digluconate (Sigma-Aldrich Canada ltd., Oakville, ON, Canada) and ethanol. Eyes were lubricated during surgery with Alcon Systane Ointment (Novartis Pharmaceuticals Canada Inc., Dorval, QC, Canada). The skin and muscles were opened to perform a laminectomy. An opening in the dura was made and a thin custom-made blade was used to perform the injury to a depth of 1 mm. The muscle

over the spinal cord was sutured using 5-0 vicryl sutures and the skin overtop was closed using surgical staples. This injury affects the animal's fine motor control ability in their distal forelimb. Procedures were performed on a heating blanket set to 37°C to prevent hypothermia. Post procedure, animals were administered 3 mL of saline and buprenorphine (0.3 mg/kg) for hydration and pain control respectively. Animals were placed in heated recovery cages with ad libitum food and water until they demonstrated a level of wakefulness deemed sufficient to be returned to their home cages.

2.2.5 Electrical Stimulation

Prior to SCI, rats had their heads shaved and disinfected with 2% chlorhexidine digluconate (Sigma-Aldrich Canada ltd., Oakville, ON, Canada) and ethanol. A dental drill was used to create a 1.5 mm square window over the motor forelimb cortex (1 mm to 2.5 mm rostral, 1 mm to 2.5 mm lateral to bregma) contralateral to the side of the dominant paw. Two insulated tungsten electrodes (5 μm diameter, 0.1 MΩ impedance; World Precision Instruments Inc., Sarasota, FL, USA) were inserted approximately 1.5 mm deep and 1 mm apart into the motor forelimb cortex (Brus-Ramer et al., 2007; Carmel et al., 2014; Girgis et al., 2007). Placement of electrodes was confirmed by sending a single train of pulses through the electrodes prior to SCI and evoking a motor response in the forelimb. As part of this process, motor threshold was recorded by adjusting the amplitude of the stimulation until a forelimb movement was evoked in response to stimulation using a Master-8 stimulator (A.M.P.I., Jerusalem, Israel) and an isolation unit (A.M.P.I., Jerusalem, Israel), and the stimulation amplitude was recorded. Motor threshold for the animals ranged from 0.6 mA and 1.3 mA.

Once motor threshold was obtained, the SCI was performed, and stimulation was turned on immediately thereafter. Animals received 30 minutes of electrical stimulation at 1.1 x motor threshold in the form of a train of 30 biphasic pulses with a 0.2 ms pulse width at 333 hz every 0.5 seconds. Upon completion of the stimulation, the skin over the craniotomy was sutured and given buprenorphine and saline as per the spinal cord injury protocol above. Based on our previous study (Jack et al., 2017) showing no difference between sham stimulation animals and spinal cord injury animals, control animals did not have electrodes inserted into the cortex.

2.2.6 Tracing of the CST

At the end of the five weeks of SPG training animals underwent tracing of the injured CST. Surgeries were conducted under isoflurane anesthesia, 5% for induction and 3% for maintenance Using the same coordinates as for the electrical stimulation, a 5 µL Hamilton syringe was used to inject 1 µL of biotin dextran amine (BDA; 10%, Life Technologies, Grand Island, New York, USA) tracer into three locations 1.5 mm deep (layer V of the cortex). General surgical and post operative procedures were performed as stated above.

2.2.7 Perfusion and Histological Sectioning

Two weeks following CST tracing, rats were euthanized using 960mg/kg Euthanyl (pentobarbital sodium injection, Bimeda-MTC Animal Health Inc., Cambridge, ON, Canada). This was followed by a transcardial perfusion using warm (37°C) saline with heparin and cold 4% formalin solution with 5% sucrose. The spinal cord and brain

from each animal were extracted and post fixed in a 4% formalin solution for two hours at room temperature and then placed in a 30% sucrose solution for 5 days at 4°C for cryoprotection. The spinal cord tissue and brain were cut into segments and mounted on filter paper using Tissue Tek O.C.T (Sakura Finetek, CA, USA) after which they were frozen using methylbutane and dry ice at -45°C, and stored at -80°C.

Tissue was sectioned on a cryostat at -20°C at 25 µm and mounted onto coated microscope slides in a staggered fashion. Staggering the tissue over 4 slide sets creates 4 separately stainable sets of tissue, with each section being 100 µm from the next. Tissue at the lesion site was sectioned in transverse sections to allow for analysis of the lesion size and for collateral CST fibers above and below the injury site. Slides were stored at -20°C until staining procedures were performed.

2.2.8 Lesion size analysis

Tissue sections were stained using cresyl violet. For this, slides were dehydrated for 1 hour at 37°C followed by rehydration in tris-buffered saline (TBS) twice for 10 minutes. Slides were then placed in a 0.5% cresyl violet solution for 3 minutes, dipped in distilled water to remove excess stain, and serially dehydrated in increasing alcohol concentrations. Finally, slides were cleared in xylene and coverslipped with Permount (Fisher Scientific, Ottawa, ON, Canada).

Cresyl violet stained tissue was analyzed using bright field microscopy in addition to phase contrast to assess the size of the DLQ lesion. At 10x magnification, landmarks

were identified to determine the location and extent of the injured tissue. Based on these landmarks, lesion locations and size were translated onto a schematic cross-sectional representation of the spinal cord. Each lesion was then calculated as a percentage of the total area of the spinal cord and as a percentage of the dorsal CST (dCST) using ImageJ software (Schneider et al., 2012).

2.2.9 Immunohistochemistry

To visualize BDA traced CST fibers, immunohistochemical staining was performed. For this, slides were dehydrated at 37°C for 1 hour and then rehydrated twice in TBS for 10 minutes. Slides were then incubated with 10% normal goat serum (NGS) in TBS with triton-X (0.5%; TBS-TX). Slides were then incubated overnight with streptavidin conjugated to Alexa Fluor 488 (Cedarlane, Burlington, ON, Canada) in 1% NGS in TBS-TX (0.5%). Slides were then washed twice in TBS-TX (0.5%) for 10 minutes each, and then twice in TBS for ten minutes each. Slides were coverslipped with Fluoromount G (Cedarlane) and sealed around the edges of the coverslip with nail polish.

2.2.10 Analysis of regeneration at the lesion site

To quantify the effects of stimulation on the reparative growth of the injured axon tips, tissue up to 500 μm above and 100 μm below the injury was analyzed and the number of traced fibers were counted at every 100 μm (Fig. 2.3A). For each animal, tissue sections starting from the lesion site were imaged and quantification of traced fibers in the CST was performed at each slice. Since the anatomical distance between each slice was known due to the cutting thickness and the staggering of slices on slide

sets, the number of fibers at each distance from the lesion site was determined. These counts were normalized to the number of fibers traced at the C1 level in each animal.

2.2.11 Collateral Fiber Density Heatmapping

Density-based maps of collateral sprouting of the injured CST were extracted as previously described (Torres-Espín et al., 2018a). Briefly, tissue 100 µm above the lesion site was imaged using confocal microscopy to visualize the BDA tracing. For each animal, 5 tissue sections were imaged in 10 step Z-stacks which were collapsed into a maximum projection image. A customized ImageJ macro was used to extract the coordinate location of each stained collateral fiber in the grey matter pixel by pixel relative to the CST.

During this process the coordinates were recorded relative to key structures (i.e., the CST and the border of the dorsal and ventral horn) to account for changes in section shape from slice to slice during the analysis process. Using a custom made script written in R (R Core Team, 2018), these coordinates for all animals within a group were overlaid and kernel density-based heatmaps were generated using the kde2d function in the MASS R package for visualization. To quantify the traced fibers density and the differences between groups the collateral fibers where count in 75x75 micron bins and the CST count normalized between animals. This was done by dividing each bin count by the total count of CST axons traced in the dorsal CST area for each section. This generated a CST index that we used for statistical comparisons. Using this CST index, ANOVA tests were run in the 75x75 bins and translated to an additional heatmap showing the adjusted p value (by Tukey method) at each area of the spinal cord (Fig. 2.4I). The 75x75 micron bins, are not

perfectly squared due to the axes having different scales as a result of how the graph was generated in R. In addition to calculating and comparing the CST index (graphed using ggplot2, points scattered using geom_jitter), the number of bins that show an increase in collateral fibers between groups, both significant and non-significant were grouped and analyzed a Fisher's exact test in GraphPad Prism 7 (GraphPad Software) (Fig. 2.4M).

2.2.12 Statistical Analyses

Following the initial pre-training phase of the experiment, animals were randomized into either the stimulated or control groups. From this point onward the experiment was conducted blinded. Statistical analyses for all behavioural assessments and lesion size analysis were conducted using GraphPad Prism 7 (GraphPad Software). Normality was assessed using D'Agostino-Pearson omnibus test. Lesion size data was analyzed using unpaired t-tests, and data from SPG testing and regeneration measures were analyzed using repeated measures two-way ANOVA. Statistical analyses of the collateral density heatmaps in the form of two-way ANOVAs were performed in R studio as indicated above. Non-parametric Mann-Whitney test was used for the CST lesion percentage analysis due to non-normality.

Exclusion criteria include animals that did not engage with the task prior to injury (attempts >5 each session prior to injury) or those that did not train after injury (attempts >5 each session), and animals that had too severe of a deficit (total lesion size >30%) and thus were apathetic towards the SPG task post injury. Exclusion criteria required the removal of seven animals: five for lesion size and two for training apathy. Upon removal

of these animals, the final group numbers were n=11 for the stimulated group and n=9 for the control group.

2.3 Results

2.3.1 Single-Session Cortical Electrical Stimulation Enhances Training Efficacy Following Spinal Cord Injury

Reaching and grasping ability of the rats was assessed weekly post-SCI, where performance was scored as the success rate (in percentage). Pre-injury, both groups performed at very similar baseline success levels ($p=0.64$), with the stimulated animals at 53.17% (±13.13%) and the control animals at 49.92% (±17.61%) (Fig. 2.1B). Post-injury all rats declined in success as expected and, over the course of the rehabilitative motor therapy, showed improvements up to the final week of training. At the end of five weeks of training, success scores of the animals began to plateau and approach baseline levels (stimulated: 54.04 ±12.12%; $p=0.86$ paired t-test compared to baseline; control: 39.45 ±25.32%, $p=0.25$ paired t-test compared to baseline). Repeated measures ANOVA was used to analyze the functional data over time. A significant group effect was observed ($p=0.02$, RM two-way ANOVA) demonstrating that the stimulated group showed an increase in functional recovery overall compared to the control animals, although without significant group x time interaction.

Following injuries, individuals as well as rats develop and use compensatory approaches to succeed affected tasks. One of the compensatory means by which rats are able to obtain food pellets after injury is through scooping the pellets towards them as

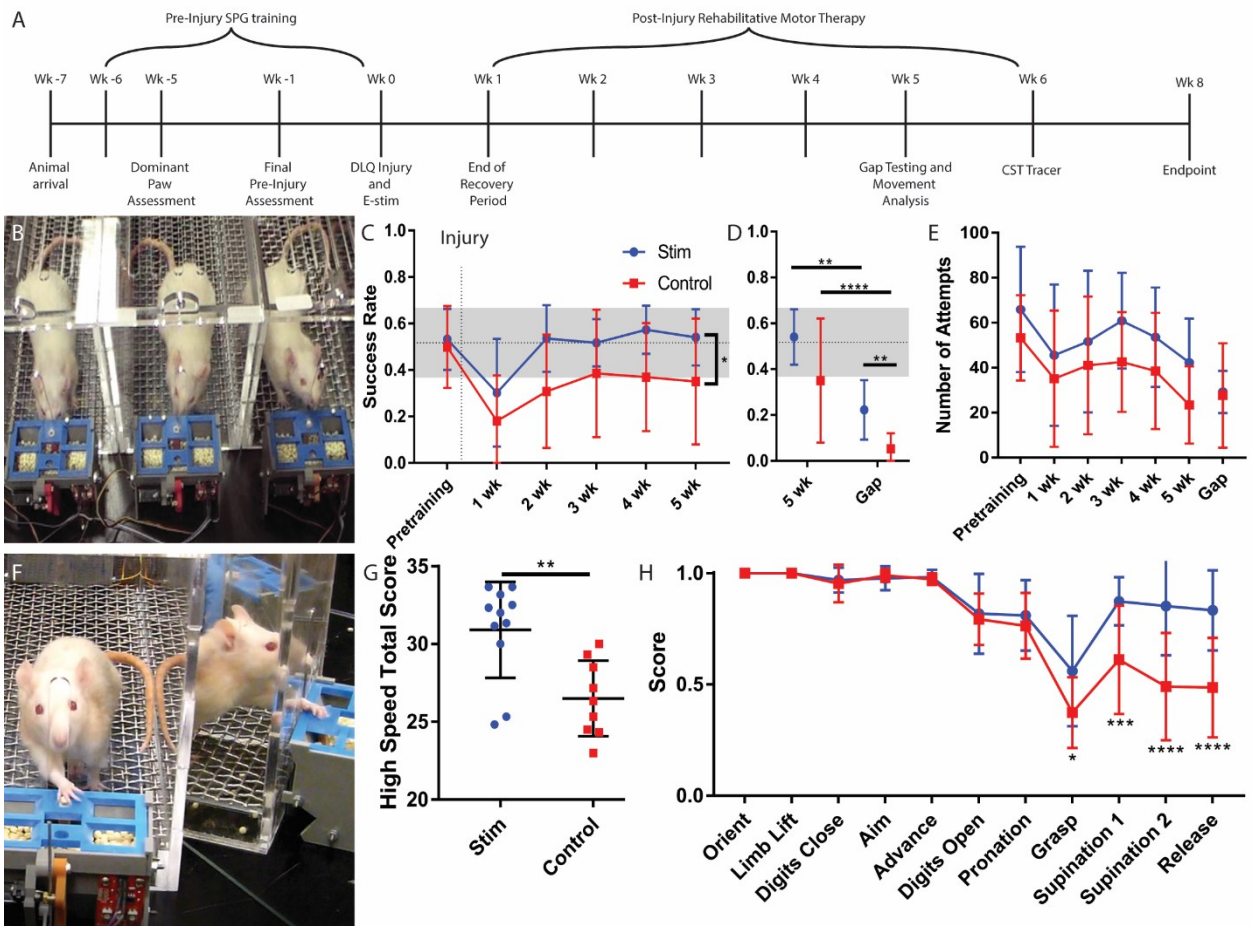


Figure 2.1 - Functional Testing Timeline and Single Pellet Grasping Analysis

(A) A timeline of the experiment demonstrating when functional testing and surgical procedures occurred. (B) A rat grasps for a pellet in the standard SPG task used for rehabilitative motor training. The stimulated group shows increased success approaching preinjury levels (baseline ± SD denoted in grey) (C) and a significantly increased success rate in the Gap testing task relative to controls (D), neither of which are the result of a difference in training intensity (E). Further investigation into the specific components of the reaching motion using high speed cameras and mirrors to see the sides of the animals (F) shows a significant difference in the overall score (G), which when broken down (H) shows that the stimulated animals have a higher score in the components of the task associated with distal digit control. SPG: single pellet grasping, CST: corticospinal tract, DLQ: dorsolateral quadrant, E-stim: electrical stimulation.

opposed to actually grasping and lifting the pellet (McKenna and Whishaw, 1999). One way to test the effects of scooping on their success score is to introduce a gap in the shelf where the pellet is presented. This will cause the pellet to fall when it is pulled toward the animal (Torres-Espín et al., 2018a) and be counted as failure. When rats were exposed to the SPG gap, the success levels declined for both groups compared to their score on the standard SPG task at 5 weeks (stimulated: $p<0.0001$, paired t-test; control: $p=0.004$, paired t-test) (Fig. 2.1C). However, the stimulated animals showed a significantly higher success rate with the gap compared to controls (22.27 ±12.96% in stimulated animals vs. 5.29 ±6.73% in controls; $p=0.049$ RM two-way ANOVA). This suggests that in the control rats recovery in the success rate was based on compensation only. In contrast, the stimulated animals showed restoration of function in grasping and lifting the pellet as per the original task as demonstrated by the significantly higher score relative to controls in the gap testing.

In order to measure the reaching strategies, the qualitative components of the reaching motion were analyzed (Metz and Whishaw, 2000). Comparing the overall scores out of 35 between the stimulated and the control groups (Fig. 2.1F), the stimulated animals showed a significantly higher overall score (30.91 ±0.93 in stimulated animals vs. 26.50 ±0.81 in control animals; $p=0.003$). When the score was broken down into the 11 individual aspects of the reaching motion (Fig. 2.1G), the stimulated animals showed significant improvement in the latter 4 components of the task: grasping (0.56 ±0.25 in stimulated vs. 0. 37 ±0.16 in control; $p=0.04$), supination 1 (0.87 ±0.11 in stimulated vs. 0.61 ±0.24 in control; $p=0.0007$), supination 2 (0.85 ±0.22 in stimulated vs. 0.49 ±0.24 in control; $p<0.0001$), and release (0.83 ±0.18 in stimulated vs. 0.49 ±0.22 in control;

$p<0.0001$). These final 4 components of the task are the components most heavily reliant on digit and fine motor control, and therefore the most affected by the DLQ injury. This indicates that the animals in the stimulated group recovered their fine digit control ability beyond recovery that occurred from rehabilitative motor therapy alone as seen in the control group.

As differences in spinal lesion sizes can make a big difference in training efficacy and recovery (Hurd et al., 2013), we compared the lesions between the groups. There was no significant difference in overall lesion size between the two groups when expressed as a percentage of the total area of the spinal cord ($p=0.88$). The average lesion size for the stimulated group was 19.95% (±6.89%) and for the control group was 20.40% (±6.11%) (Fig. 2.2B).

Comparing the two groups in terms of the extent of CST damage relative to the total area of the dorsal CST (dCST) on the intended side of lesion also showed no significant difference between the two control and stimulated groups ($p=0.37$; Mann-Whitney test). The average dCST lesion size for the stimulated and control groups were 80.93% (±26.41%) and 64.03% (±36.9%) respectively (Fig. 2.2C). In summary, lesion sizes were consistent between the two groups and within the desired size range to allow for effective rehabilitative training (Hurd et al., 2013). Consequently, differences in the outcome measures are likely not due to differences in the lesions.

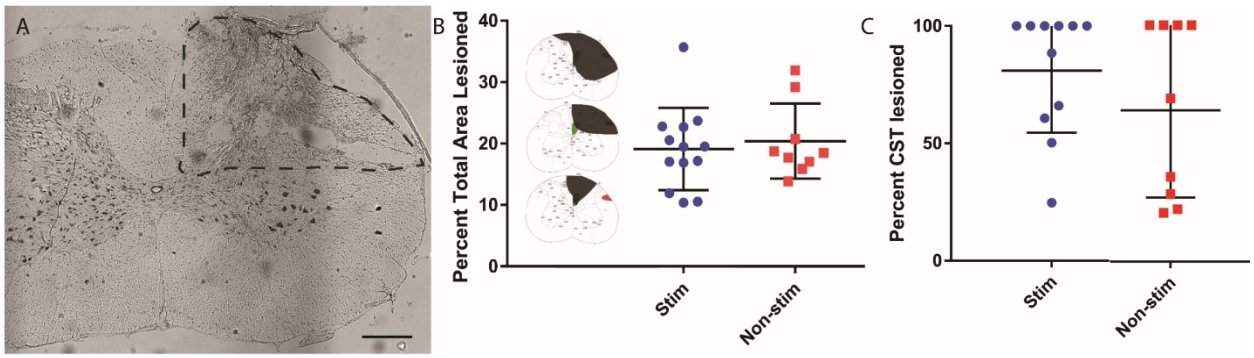


Figure 2.2 – Lesion Analysis

(A) Representative image of piece of tissue at the lesion site, stained with cresyl violet, lesion delineated by the dashed line. Scale bar is 200 μm. (B) Lesion size between groups is consistent, with a similar mean size and variation. Example lesions on the left hand side show approximations of lesions at 10, 20, and 30% total area injured. (C) Area of the corticospinal tract damaged by the lesion is similar between the stimulated and control groups.

2.3.2 Forelimb Functional Recovery is Associated with CST Collateralization but Not Regeneration

Quantifying the number of traced dCST fibers at set distances from the lesion was performed to determine whether electrical stimulation enhanced regenerative growth into and beyond the lesion or affected axonal withdraw from the lesion site. As shown in figure 2, a DLQ lesion generally do not result in wide spread cavitation enabling a fairly precise determination of the lesion epicentre. There was however no statistical difference between the two groups. Both control and stimulated animals have a similar ratio of fibers (relative to the number of fibers counted at the C1 level) at each rostral or caudal distance from the lesion measured (Fig. 2.3D) ($p= 0.75$, RM two-way ANOVA, group effect) and no interaction effect was seen ($p=0.9651$, RM two-way ANOVA). The two animals with partial sparing of the dCST were not considered in the analysis.

There was however a significant distance effect on the CST fiber count i.e., there were fewer fibres closer to the lesion in all animals ($p<0.0001$, RM two-way ANOVA). This fits with the idea of axonal withdraw following SCI that has been heavily documented in the literature (Cajal and May, 1991; Profyris et al., 2004).

To determine the adaptive changes in the injured CST occurring alongside the functional differences described above, sprouting off into the grey matter was analysed as an indicator of CST plasticity (Bareyre et al., 2004; Lindau et al., 2014; Mitchell et al., 2016) (Fig. 2.4). Immediately rostral to the lesion at vertebral level C4 there is a visible difference between the stimulated and control groups in terms of axon density and distribution (Fig. 2.4 F-G). The stimulated group shows a greater distribution of collaterals that extend further into the grey matter compared to controls. Further rostral

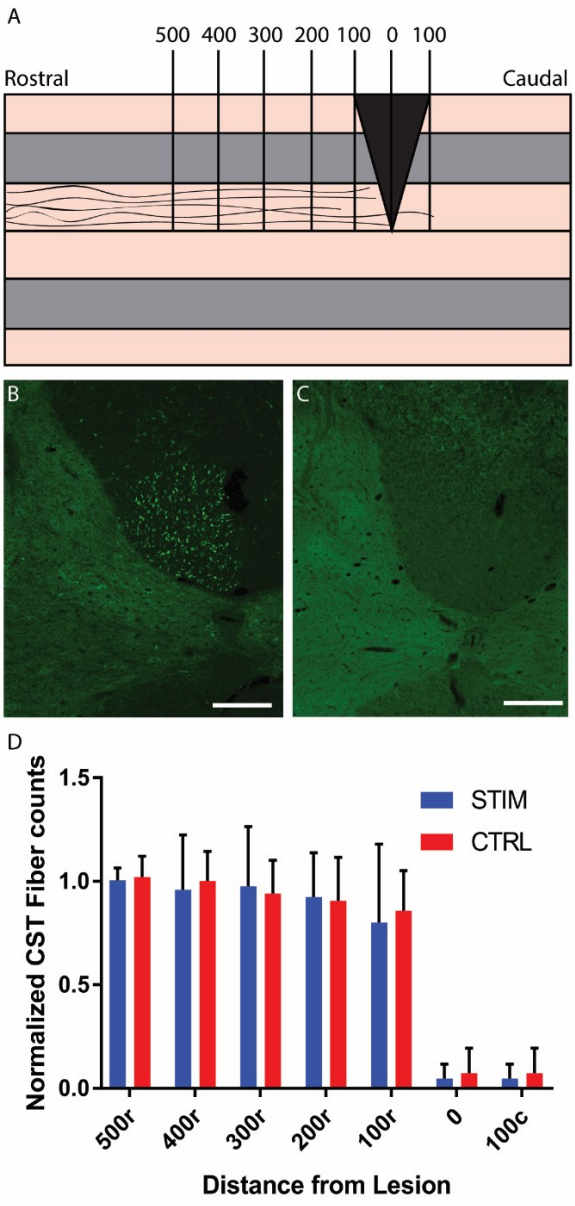


Figure 2.3 – Analysis of Severed Axon Regeneration

Schematic of the spinal cord, lesion, and the points at which axon counts were conducted (distances are in μm) (A). Example image of traced CST fibers above the lesion site are shown in (B) and from below the lesion in (C). Fibers at each level were normalized by dividing the number by the number of fibers counted at the C1 level and then graphed in (D). There are no significant differences between the groups and the fibers seen through the lesion site are only apparent in animals where lesion analysis showed sparing of the CST, and are therefore not regenerated fibers. Scale bars are 125 μm.

from the lesion site, at approximately C2-C3, both groups show similar distribution of collaterals (Fig. 2.4 D-E).

Using the CST index (normalized CST counts, see methods), statistical analyses were performed in 75-micron squared bins based on an overlay of the spinal cord grey matter, demonstrating areas of the spinal cord where a significant difference between the control and stimulated groups occurred (Fig. 2.4H). Mapping the statistical data to show which group has a higher CST index in each bin demonstrates that the stimulated group consistently had a larger CST index (Fig. 2.4I). To further confirm that the stimulated group had higher CST index values throughout the spinal cord, comparison of the CST index values in all bins (Fig. 2.4J) showed the stimulated group having a higher CST index. The CST index comparison between groups was further analyzed by taking the CST indices for each group from sections that were significantly higher in control animals (Fig. 2.4K) or in stimulated animals (Fig. 2.4L). The difference in scale of these two graphs further exemplifies that the differences in areas that were better innervated by the stimulated group were much greater (Fig. 2.4L) than the differences in areas favouring the control group (Fig. 2.4K).

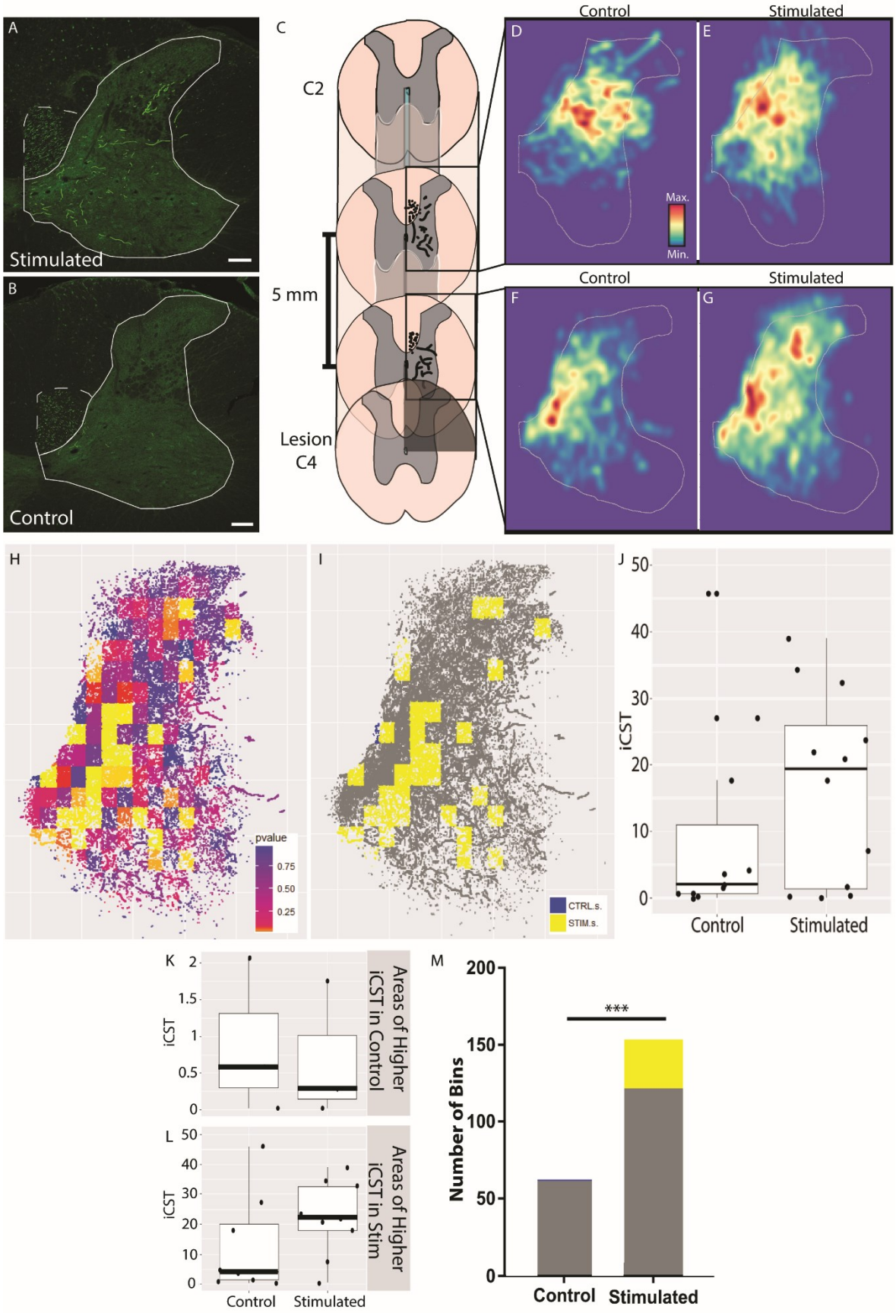
Group statistics to compare the number of bins showing higher CST index for each group were performed as an additional means to quantify these differences. Comparing bins favouring (both significantly and non-significantly) control animals to those favouring stimulated animals showed a clear difference in the two samples ($p<0.0001$; Fisher's exact test) (Fig. 2.4M). This further demonstrates the plasticity promoting effect of electrical stimulation on the injured CST.

Given the effects of electrical stimulation on the functional recovery of the animals and on the extent of CST collateralization, further analysis was performed to connect the two outcome measures. Correlational analysis of the SPG data and high-speed analysis scores were run against the CST index scores in all areas of the spinal cord grey matter and the CST index scores in the significant regions (from Fig. 2.4I). Whilst linear regression analysis showed no significant correlational link between the standard SPG scores with either CST index measures (Fig. 2.5 A&C), there was a positive correlation between the high-speed scores and the CST index, both in all bins of the heatmaps and especially the bins showing significant changes (Fig. 2.5 B&D).

2.4 Discussion

We have shown that a single session of stimulation of the motor cortex innervating the ablated CST, immediately after SCI, can promote CST plasticity and enhance the efficacy of rehabilitative training performed over weeks thereafter. This effect is correlated with an increase in the number and density of corticospinal collateral axons found in the intermediate laminae of the spinal cord grey matter, but not with CST axonal elongation across the injury site. These results suggest the recovery gained by the ES treatment are not based on axonal regeneration, but rather associated with anatomical plasticity of the CST.

Following traumatic injury, the CNS is capable of limited adaptive changes in remaining neuronal circuitry (Fouad and Tetzlaff, 2012). To enhance this plasticity by increasing neurite outgrowth in the injured CNS requires overcoming the two major


A
Stimulated
B
Control
C
C2
5 mm
Lesion
C4
Control
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0.75
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Areas of Higher iCST in Stim
Control
Stimulated
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200
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100
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0
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Control
Stimulated

Figure 2.4 – Heatmaps of Collateral Density from the CST

BDA tracing of the CST in both stimulated (A) and control animals (B) was analyzed 1 and 5 mm above the lesion site as seen in (C) the schematic of where in the spinal cord relative to the injury site that analysis occurred. Collateral density 5 mm rostral to the lesion site showed no difference between the two groups (D and E). Only collateral density 1 mm above the lesion site showed a marked change between groups (F and G) and thus was further analyzed and quantified. Collateral density was normalized based on the number of traced fibers in each animal. This normalized value creates the CST index. Fiber distribution maps were recreated with weighting based on their CST index and then statistical analyses were calculated in bins (H). Statistically significant differences in collateral density between groups are highlighted in a different colour denoting the group showing the increase (I). CST index quantification showing the overall CST index between groups (J) shows a marked increase in the stimulated group compared to control. Comparing the CST index between groups in areas where control animals have a higher CST index (K) shows a smaller difference than the comparison between groups in areas where stimulated animals have a higher CST index (L). Population statistics comparing the number of bins with a higher CST index in each group, both significant (coloured) and non-significant (grey), are shown in (M) and demonstrate a significant difference between the two groups ($p > 0.0001$; Fisher's exact test). Scale bars are 125 μm.

naturally occurring hurdles blocking repair following injury: a lack of activation in growth promoting signalling pathways, and the inhibitory environment of the CNS that hinders neurite outgrowth (Chaudhry and Filbin, 2007). Electrical stimulation is a well-established means to promote neurite growth of injured nerves in the peripheral nervous system (Chan et al., 2016; Gordon, 2016a, 2016b; Willand et al., 2016), and after CNS injury (Brus-Ramer et al., 2007; Carmel et al., 2014, 2013, 2010; Chang et al., 2015).

The mechanisms by which electrical stimulation engages intracellular signalling pathways and ultimately promotes plasticity is still not well understood. We know however that activity in neurons is capable of increasing cAMP levels and these cAMP levels can be also be bolstered through electrical stimulation (Brushart et al., 2002; Geremia et al., 2007; Udina et al., 2008). These intracellular levels of cAMP may play a decisive role in neurite growth. For example it has been shown that after the nervous system develops cAMP levels drop, which is associated with the decline or limited neuronal growth ability (Cai et al., 2001; Pearse et al., 2004; Chaudhry and Filbin, 2007; Park et al., 2008). A second potential mechanism includes that ES changes signalling of growth-promoting developmental signaling pathway. This was reported for ES of uninjured CST axons, and in the use of chronic ES, which upregulated mTOR and Jak/Stat signaling (Zareen et al., 2018).

A final crucial step to enhanced recovery is that ES induced plasticity was likely directed into meaningful directions through rehabilitative motor training. Training alone certainly does promote recovery following SCI in individuals as well as animal models (Behrman et al., 2017; Girgis et al., 2007; Krajacic et al., 2009; Zewdie et al., 2015) and based on findings during development, activity is a key in fine tuning and the

maintenance of new neuronal connections (Reviewed in Andreae and Burrone, 2014). We did not test the effect of training alone in the current experiment because a large body of literature has described these effects (Girgis et al., 2007; van den Brand et al., 2012; Weidner et al., 2001; Ying et al., 2005), and because previous work has demonstrated the important role of training in orchestrating pharmaceutically induced plasticity into functional recovery (Gonzenbach et al., 2010; Torres-Espín et al., 2018a; Weishaupt et al., 2013). Together with the fact that individuals with SCI will generally receive rehabilitative motor training to various degrees these findings make rehabilitative training a standard in all our experimental groups.

Our paradigm of a single session of motor cortex stimulation post injury shows a significant increase in the degree of reaching and grasping recovery (Fig. 2.1). This is even more evident when success rates are distilled down by impairing compensatory approaches (i.e., by introducing a gap in the SPG testing device), showing a significant difference between the ES and control animals ($p=0.049$). When broken down into the components of the reaching task to eliminate any effects on the success rate from compensational mechanisms, it was found that there was a significant increase in the overall reaching ability of animals that underwent ES of their motor forelimb cortex. This was seen specifically in the components of the task that involve a greater degree of motor control of the digits and wrist (Fig. 2.1G): grasping, supination, and release, which a DLQ injury at cervical level 4 greatly hinders (McKenna et al., 2000).

In studying collateralization of the CST after injury, it is important that the effects of tissue sparing are considered. Since the potential for collateralization is greatly increased after injury, spared fibres could potentially result in a decrease in collateral

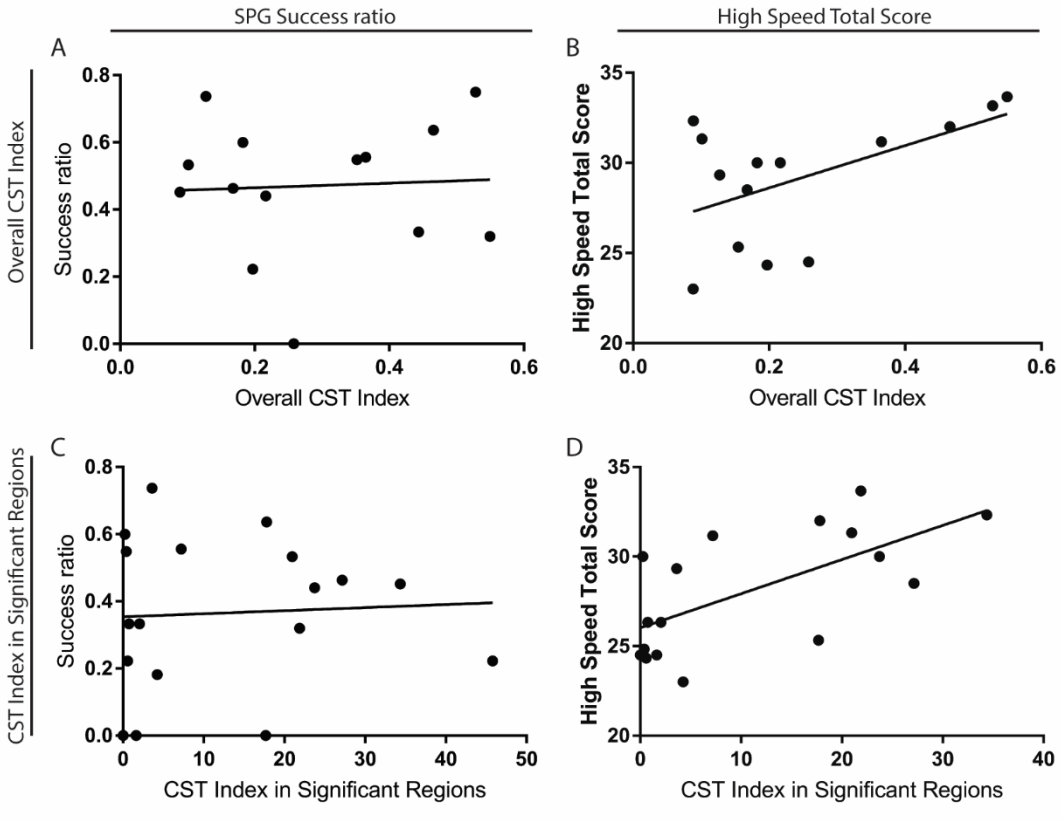

Figure 2.5 – Correlation Between Training Data and CST Index

The relationship between the CST index and the success rates of the animals are graphed comparing both major SPG measures used (standard success rate and high-speed movement analysis scoring) against overall CST index and the CST index in significant regions (see Fig. 2.4I). (A) Overall CST index and SPG success ratio at the end of the 5 weeks of training show no correlation between the two (R^2=0.0029, p=0.8545). (B) Comparing the high-speed data to the overall CST index shows a positive correlation between the two, demonstrating that the higher CST index of an animal is more likely to coincide with higher score in our movement analysis (R^2=0.2887, p=0.0475). Comparing the CST index from significant regions to the SPG success ratio (C) show a similar relationship as the overall CST index to SPG success ratio (R^2=0.0032, p=0.8246). (D) Comparing high speed score to the CST index in significant regions shows a positive correlation between the two (R^2=0.4203, p=0.0049), similar to the high-speed data compared to the overall CST index.

fibre formation in either group when present. To check for these possible effects, a comparison of the extent of collateralization (CST index) was compared within groups between animals that exhibited sparing against those that had no tissue sparing. Once the CST index was normalized to the amount of spared tissue, it was found that in both the control and stimulated groups, tissue sparing showed no effect on the amount of collateral formation (data not shown). Therefore, the amount of spared tissue present in this study does not present as a confounding variable in this body of work.

Analysis of CST fiber regeneration through the injury side yielded minimal results, showing no regenerative growth of CST fibers through the lesion as well as no significant difference between groups (Fig. 2.3). The functional recovery found in this study was correlated with a greater extent of corticospinal collateralization into the grey matter on the side of injury (Fig. 2.5B and D) indicating that there is a link between these functional changes and the plasticity exhibited by the CST. It is important to note however that although these correlations are definitely present in both cases due to the significant p-values (Fig. 2.5B: $p=0.0475$, Fig. 2.5D: $p=0.0049$), these correlations do not represent the multifaceted process leading to functional recovery. The link between these aspects of post SCI recovery, however present it may be, for this study is simply a straight forward readout for plasticity. It represents just one piece of the larger process that leads to functional recovery, as seen in the coefficient of determination (Fig. 2.5B: $R^2=0.2887$, Fig. 2.5D: $R^2=0.4203$).

Previous studies have demonstrated that after SCI, functional recovery has been correlated with collateral fibers forming novel connections with propriospinal interneurons located largely in laminae VII and VIII (Bareyre et al., 2004; Vavrek, 2006).

It is likely that the functional recovery exhibited by the rats in the stimulated group is in part due to these novel connections. Additional support for the idea that sprouting was involved in the observed recovery comes from various studies reporting sprouting in similar gray matter areas that was linked to recovery (Lindau et al., 2014; Mitchell et al., 2016; Torres-Espín et al., 2018a).

It is important to note that ipsilateral dCST sprouting is likely not the only mechanism contributing to recovery. Spared fibers for example may play a major role as they show a prominent response to SCI. The CST does not solely project through the dorsal funiculus, as has been the focus of this study, but also has projections that extend through the ventral and lateral regions of the spinal cord which have been shown to increase sprouting following lesion to the dorsal CST (Weidner et al., 2001). Furthermore, although neither tracing nor ES of the uninjured CST was not performed in this study, previous work has shown that after injury, plastic changes in the uninjured side of the spinal cord and fibers crossing to the injured side can play a role in functional recovery (Brus-Ramer et al., 2007; Carmel et al., 2014, 2013, 2010).

Beyond sprouting and larger scale physiological changes of neurons, there are a number of other changes occurring in the CNS that coincide with neuronal activity (e.g, neuronal properties or synaptic strength, (von Bernhardi et al., 2017)) that can also be causing some of the recovery seen in this study. As such the data presented in this study should be considered amongst the larger framework of changes brought on by neuronal activity after SCI.

2.4.1 Technical Challenges and Translational Considerations

Electrical stimulation of cell bodies giving rise to lesioned spinal tracts is challenging for many reasons. Firstly, it is not known in how far these cells are acutely excitable directly following an injury. Secondly, how is a reliable stimulation intensity determined, considering the tract is disconnected from its targets. Some of these questions could be addressed in the future using imaging approaches (e.g., calcium imaging) in parallel to stimulation. Nevertheless the positive results in this study and the relative simplicity of cortical stimulation makes electrical cortical stimulation a promising and feasible clinical target. For example transcranial magnetic stimulation (TMS), offers itself for such an approach though achieving the frequencies used in this or other stimulation studies would pose a challenge (Awad et al., 2015). The majority of transcranial magnetic stimulation (TMS) studies that utilize 'high frequency stimulation' stimulate at frequencies no higher than 25hz (Klomjai et al., 2015; Rossi et al., 2009; Speer et al., 2000) and a transcranial electrical stimulation paradigm might have to be considered (Bestmann and Walsh, 2017; Fertonani and Miniussi, 2017).

2.5 Conclusion

Data from this study suggests that similar to lesioned axons in the periphery and to sensory fibres in the spinal cord a single session of electrical stimulation is able to increase neurite growth in an injured descending white matter tract i.e., the CST.

Considering the importance of the CST following SCI, cortical stimulation offers itself as a potential strategy to bolster the effects of rehabilitative motor training. The possibility

to use a single session of electrical stimulation is paramount as it allows for a reduction in the amount of treatment time and costs required for such a procedure.

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2.7 References

Alaverdashvili, M., 2008. “Learned baduse” limits recovery of skilled reaching for food after forelimb motor cortex stroke in rats: A new analysis of the effect of gestures on success. Behav. Brain Res. 188, 281–290. https://doi.org/10.1016/j.bbr.2007.11.007

Alaverdashvili, M., Whishaw, I.Q., 2013. A behavioral method for identifying recovery and compensation: Hand use in a preclinical stroke model using the single pellet reaching task. Neurosci. Biobehav. Rev. 37, 950–967. https://doi.org/10.1016/j.neubiorev.2013.03.026

Andreae, L.C., Burrone, J., 2014. The role of neuronal activity and transmitter release on synapse formation. Curr. Opin. Neurobiol. 27, 47–52. https://doi.org/10.1016/j.conb.2014.02.008

Awad, B.I., Carmody, M.A., Steinmetz, M.P., 2015. Potential Role of Growth Factors in the Management of Spinal Cord Injury. World Neurosurg. 83, 120–131. https://doi.org/10.1016/j.wneu.2013.01.042

Bareyre, F.M., Kerschensteiner, M., Raineteau, O., Mettenleiter, T.C., Weinmann, O., Schwab, M.E., 2004. The injured spinal cord spontaneously forms a new intraspinal circuit in adult rats. Nat. Neurosci. 7, 269–277. https://doi.org/10.1038/nn1195

Behrman, A.L., Ardolino, E.M., Harkema, S.J., 2017. Activity-Based Therapy: From Basic Science to Clinical Application for Recovery After Spinal Cord Injury. J. Neurol. Phys. Ther. 41, S39–S45. https://doi.org/10.1097/NPT.0000000000000184

Bestmann, S., Walsh, V., 2017. Transcranial electrical stimulation. Curr. Biol. 27, R1258–R1262. https://doi.org/10.1016/j.cub.2017.11.001

Brushart, T.M., Hoffman, P.N., Royall, R.M., Murinson, B.B., Witzel, C., Gordon, T., 2002. Electrical Stimulation Promotes Motoneuron Regeneration without Increasing Its Speed or Conditioning the Neuron. J. Neurosci. 22, 6631–6638.

Brus-Ramer, M., Carmel, J.B., Chakrabarty, S., Martin, J.H., 2007. Electrical Stimulation of Spared Corticospinal Axons Augments Connections with Ipsilateral Spinal Motor Circuits after Injury. J. Neurosci. 27, 13793–13801. https://doi.org/10.1523/JNEUROSCI.3489-07.2007

Cai, D., Qiu, J., Cao, Z., McAtee, M., Bregman, B.S., Filbin, M.T., 2001. Neuronal Cyclic AMP Controls the Developmental Loss in Ability of Axons to Regenerate. J. Neurosci. 21, 4731–4739.

Cajal, S.R. y, May, R.M., 1991. Cajal's Degeneration and Regeneration of the Nervous System. Oxford University Press. https://doi.org/10.1093/acprof:oso/9780195065169.001.0001

Carmel, J.B., Berrol, L.J., Brus-Ramer, M., Martin, J.H., 2010. Chronic Electrical Stimulation of the Intact Corticospinal System after Unilateral Injury Restores Skilled Locomotor Control and Promotes Spinal Axon Outgrowth. J. Neurosci. 30, 10918–10926. https://doi.org/10.1523/JNEUROSCI.1435-10.2010

Carmel, J.B., Kimura, H., Berrol, L.J., Martin, J.H., 2013. Motor cortex electrical stimulation promotes axon outgrowth to brain stem and spinal targets that control the forelimb impaired by unilateral corticospinal injury. Eur. J. Neurosci. 37, 1090–1102. https://doi.org/10.1111/ejn.12119

Carmel, J.B., Kimura, H., Martin, J.H., 2014. Electrical Stimulation of Motor Cortex in the Uninjured Hemisphere after Chronic Unilateral Injury Promotes Recovery of Skilled Locomotion through Ipsilateral Control. J. Neurosci. 34, 462–466. https://doi.org/10.1523/JNEUROSCI.3315-13.2014

Carmel, J.B., Martin, J.H., 2014. Motor cortex electrical stimulation augments sprouting of the corticospinal tract and promotes recovery of motor function. Front. Integr. Neurosci. 8. https://doi.org/10.3389/fnint.2014.00051

Chan, K.M., Curran, M.W.T., Gordon, T., 2016. The use of brief post-surgical low frequency electrical stimulation to enhance nerve regeneration in clinical practice: Use of brief post-surgical low frequency electrical stimulation. J. Physiol. 594, 3553–3559. https://doi.org/10.1113/JP270892

Chang, W.H., Kim, H., Sun, W., Kim, J.Y., Shin, Y.-I., Kim, Y.-H., 2015. Effects of extradural cortical stimulation on motor recovery in a rat model of subacute stroke. Restor. Neurol. Neurosci. 33, 589–596. https://doi.org/10.3233/RNN-140445

Chaudhry, N., Filbin, M.T., 2007. Myelin-associated inhibitory signaling and strategies to overcome inhibition. J. Cereb. Blood Flow Metab. 27, 1096–1107. https://doi.org/10.1038/sj.jcbfm.9600407

Dell'Anno, M.T., Strittmatter, S.M., 2017. Rewiring the spinal cord: Direct and indirect strategies. Neurosci. Lett. 652, 25–34. https://doi.org/10.1016/j.neulet.2016.12.002

Fertonani, A., Miniussi, C., 2017. Transcranial Electrical Stimulation: What We Know and Do Not Know About Mechanisms. The Neuroscientist 23, 109–123. https://doi.org/10.1177/1073858416631966

Fouad, K., Forero, J., Hurd, C., 2015. A Simple Analogy for Nervous System Plasticity After Injury: Exerc. Sport Sci. Rev. 43, 100–106. https://doi.org/10.1249/JES.0000000000000040

Fouad, K., Tetzlaff, W., 2012. Rehabilitative training and plasticity following spinal cord injury. Exp. Neurol. 235, 91–99. https://doi.org/10.1016/j.expneurol.2011.02.009

García-Alías, G., Barkhuysen, S., Buckle, M., Fawcett, J.W., 2009. Chondroitinase ABC treatment opens a window of opportunity for task-specific rehabilitation. Nat. Neurosci. 12, 1145–1151. https://doi.org/10.1038/nn.2377

Geremia, N.M., Gordon, T., Brushart, T.M., Al-Majed, A.A., Verge, V.M.K., 2007. Electrical stimulation promotes sensory neuron regeneration and growth-associated gene expression. Exp. Neurol. 205, 347–359. https://doi.org/10.1016/j.expneurol.2007.01.040

Girgis, J., Merrett, D., Kirkland, S., Metz, G.A.S., Verge, V., Fouad, K., 2007. Reaching training in rats with spinal cord injury promotes plasticity and task specific recovery. Brain 130, 2993–3003. https://doi.org/10.1093/brain/awm245

Goganau, I., Sandner, B., Weidner, N., Fouad, K., Blesch, A., 2017. Depolarization and electrical stimulation enhance in vitro and in vivo sensory axon growth after spinal cord injury. Exp. Neurol. https://doi.org/10.1016/j.expneurol.2017.11.011

Gonzenbach, R.R., Gasser, P., Zörner, B., Hochreutener, E., Dietz, V., Schwab, M.E., 2010. Nogo-A antibodies and training reduce muscle spasms in spinal cord-injured rats. Ann. Neurol. 68, 48–57. https://doi.org/10.1002/ana.22009

Gonzenbach, R.R., Schwab, M.E., 2008. Disinhibition of neurite growth to repair the injured adult CNS: Focusing on Nogo. Cell. Mol. Life Sci. 65, 161–176. https://doi.org/10.1007/s00018-007-7170-3

Gordon, T., 2016a. Electrical Stimulation to Enhance Axon Regeneration After Peripheral Nerve Injuries in Animal Models and Humans. Neurotherapeutics 13, 295–310. https://doi.org/10.1007/s13311-015-0415-1

Gordon, T., 2016b. Nerve regeneration in the peripheral and central nervous systems: Editorial. J. Physiol. 594, 3517–3520. https://doi.org/10.1113/JP270898

Hurd, C., Weishaupt, N., Fouad, K., 2013. Anatomical correlates of recovery in single pellet reaching in spinal cord injured rats. Exp. Neurol. 247, 605–614. https://doi.org/10.1016/j.expneurol.2013.02.013

Jack, A.S., Hurd, C., Forero, J., Nataraj, A., Fenrich, K., Blesch, A., Fouad, K., 2017. Cortical electrical stimulation in female rats with a cervical spinal cord injury to promote axonal outgrowth. J. Neurosci. Res. https://doi.org/10.1002/jnr.24209

Klomjai, W., Katz, R., Lackmy-Vallée, A., 2015. Basic principles of transcranial magnetic stimulation (TMS) and repetitive TMS (rTMS). Ann. Phys. Rehabil. Med. 58, 208–213. https://doi.org/10.1016/j.rehab.2015.05.005

Krajacic, A., Ghosh, M., Puentes, R., Pearse, D.D., Fouad, K., 2009. Advantages of delaying the onset of rehabilitative reaching training in rats with incomplete

spinal cord injury. Eur. J. Neurosci. 29, 641–651. https://doi.org/10.1111/j.1460-9568.2008.06600.x

Kusiak, A.N., Selzer, M.E., 2013. Neuroplasticity in the spinal cord, in: Handbook of Clinical Neurology. Elsevier, pp. 23–42. https://doi.org/10.1016/B978-0-444-52901-5.00003-4

Lee, J.-K., Kim, J.-E., Sivula, M., Strittmatter, S., 2004. Nogo Receptor Antagonism Promotes Stroke Recovery by Enhancing Axonal Plasticity. J. Neurosci. 24, 6209–6217. https://doi.org/10.1523/JNEUROSCI.1643-04.2004

Lindau, N.T., Bänninger, B.J., Gullo, M., Good, N.A., Bachmann, L.C., Starkey, M.L., Schwab, M.E., 2014. Rewiring of the corticospinal tract in the adult rat after unilateral stroke and anti-Nogo-A therapy. Brain 137, 739–756. https://doi.org/10.1093/brain/awt336

Massey, J.M., Hubscher, C.H., Wagoner, M.R., Decker, J.A., Amps, J., Silver, J., Onifer, S.M., 2006. Chondroitinase ABC Digestion of the Perineuronal Net Promotes Functional Collateral Sprouting in the Cuneate Nucleus after Cervical Spinal Cord Injury. J. Neurosci. 26, 4406–4414. https://doi.org/10.1523/JNEUROSCI.5467-05.2006

May, Z., Fouad, K., Shum-Siu, A., Magnuson, D.S.K., 2015. Challenges of animal models in SCI research: Effects of pre-injury task-specific training in adult rats before lesion. Behav. Brain Res. 291, 26–35. https://doi.org/10.1016/j.bbr.2015.04.058

McKenna, J.E., Prusky, G.T., Whishaw, I.Q., 2000. Cervical motoneuron topography reflects the proximodistal organization of muscles and movements of the rat

forelimb: A retrograde carbocyanine dye analysis. J. Comp. Neurol. 419, 286–296. https://doi.org/10.1002/(SICI)1096-9861(20000410)419:3<286::AID-CNE2>3.0.CO;2-3

McKenna, J.E., Whishaw, I.Q., 1999. Complete Compensation in Skilled Reaching Success with Associated Impairments in Limb Synergies, after Dorsal Column Lesion in the Rat. J. Neurosci. 19, 1885–1894. https://doi.org/10.1523/JNEUROSCI.19-05-01885.1999

McKerracher, L., David, S., Jackson, D.L., Kottis, V., Dunn, R.J., Braun, P.E., 1994. Identification of myelin-associated glycoprotein as a major myelin-derived inhibitor of neurite growth. Neuron 13, 805–811. https://doi.org/10.1016/0896-6273(94)90247-X

Metz, G.A.S., Whishaw, I.Q., 2000. Skilled reaching an action pattern: stability in rat (Rattus norvegicus) grasping movements as a function of changing food pellet size. Behav. Brain Res. 116, 111–122. https://doi.org/10.1016/S0166-4328(00)00245-X

Mitchell, E.J., McCallum, S., Dewar, D., Maxwell, D.J., 2016. Corticospinal and Reticulospinal Contacts on Cervical Commissural and Long Descending Propriospinal Neurons in the Adult Rat Spinal Cord; Evidence for Powerful Reticulospinal Connections. PLOS ONE 11, e0152094. https://doi.org/10.1371/journal.pone.0152094

Profyris, C., Cheema, S.S., Zang, D., Azari, M.F., Boyle, K., Petratos, S., 2004. Degenerative and regenerative mechanisms governing spinal cord injury. Neurobiol. Dis. 15, 415–436. https://doi.org/10.1016/j.nbd.2003.11.015

R Core Team, 2018. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.

Rossi, S., Hallett, M., Rossini, P.M., Pascual-Leone, A., 2009. Safety, ethical considerations, and application guidelines for the use of transcranial magnetic stimulation in clinical practice and research. Clin. Neurophysiol. 120, 2008–2039. https://doi.org/10.1016/j.clinph.2009.08.016

Schneider, C.A., Rasband, W.S., Eliceiri, K.W., 2012. NIH Image to ImageJ: 25 years of image analysis. Nat. Methods 9, 671.

Schwab, M.E., Bartholdi, D., 1996. Degeneration and Regeneration of Axons in the Lesioned Spinal Cord. Physiol. Rev. 76, 319–370.

Speer, A.M., Kimbrell, T.A., Wassermann, E.M., D. Repella, J., Willis, M.W., Herscovitch, P., Post, R.M., 2000. Opposite effects of high and low frequency rTMS on regional brain activity in depressed patients. Biol. Psychiatry 48, 1133–1141. https://doi.org/10.1016/S0006-3223(00)01065-9

Torres-Espín, A., Forero, J., Fenrich, K.K., Lucas-Osma, A.M., Krajacic, A., Schmidt, E., Vavrek, R., Raposo, P., Bennett, D.J., Popovich, P.G., Fouad, K., 2018a. Eliciting inflammation enables successful rehabilitative training in chronic spinal cord injury. Brain 141, 1946–1962. https://doi.org/10.1093/brain/awy128

Torres-Espín, A., Forero, J., Schmidt, E.K.A., Fouad, K., Fenrich, K.K., 2018b. A motorized pellet dispenser to deliver high intensity training of the single pellet reaching and grasping task in rats. Behav. Brain Res. 336, 67–76. https://doi.org/10.1016/j.bbr.2017.08.033

Udina, E., Furey, M., Busch, S., Silver, J., Gordon, T., Fouad, K., 2008. Electrical stimulation of intact peripheral sensory axons in rats promotes outgrowth of their central projections. Exp. Neurol. 210, 238–247. https://doi.org/10.1016/j.expneurol.2007.11.007

van den Brand, R., Heutschi, J., Barraud, Q., DiGiovanna, J., Bartholdi, K., Huerlimann, M., Friedli, L., Vollenweider, I., Moraud, E.M., Duis, S., Dominici, N., Micera, S., Musienko, P., Courtine, G., 2012. Restoring Voluntary Control of Locomotion after Paralyzing Spinal Cord Injury. Science 336, 1182–1185. https://doi.org/10.1126/science.1217416

Vavrek, R., 2006. BDNF promotes connections of corticospinal neurons onto spared descending interneurons in spinal cord injured rats. Brain 129, 1534–1545. https://doi.org/10.1093/brain/awl087

von Bernhardi, R., Bernhardi, L.E., Eugenín, J., 2017. What Is Neural Plasticity?, in: von Bernhardi, R., Eugenín, J., Muller, K.J. (Eds.), The Plastic Brain. Springer International Publishing, Cham, pp. 1–15. https://doi.org/10.1007/978-3-319-62817-2_1

Weidner, N., Ner, A., Salimi, N., Tuszynski, M.H., 2001. Spontaneous corticospinal axonal plasticity and functional recovery after adult central nervous system injury. Proc. Natl. Acad. Sci. 98, 3513–3518. https://doi.org/10.1073/pnas.051626798

Weishaupt, N., Li, S., Di Pardo, A., Sipione, S., Fouad, K., 2013. Synergistic effects of BDNF and rehabilitative training on recovery after cervical spinal cord injury. Behav. Brain Res. 239, 31–42. https://doi.org/10.1016/j.bbr.2012.10.047

Whishaw, I.Q., O'Connor, W.T., Dunnett, S.B., 1986. THE CONTRIBUTIONS OF MOTOR CORTEX, NIGROSTRIATAL DOPAMINE AND CAUDATE-PUTAMEN TO SKILLED FORELIMB USE IN THE RAT. Brain 109, 805–843. https://doi.org/10.1093/brain/109.5.805

Willand, M.P., Nguyen, M.-A., Borschel, G.H., Gordon, T., 2016. Electrical Stimulation to Promote Peripheral Nerve Regeneration. Neurorehabil. Neural Repair 30, 490–496. https://doi.org/10.1177/1545968315604399

Ying, Z., Roy, R.R., Edgerton, V.R., Gómez-Pinilla, F., 2005. Exercise restores levels of neurotrophins and synaptic plasticity following spinal cord injury. Exp. Neurol. 193, 411–419. https://doi.org/10.1016/j.expneurol.2005.01.015

Zareen, N., Dodson, S., Armada, K., Awad, R., Sultana, N., Hara, E., Alexander, H., Martin, J.H., 2018. Stimulation-dependent remodeling of the corticospinal tract requires reactivation of growth-promoting developmental signaling pathways. Exp. Neurol. 307, 133–144. https://doi.org/10.1016/j.expneurol.2018.05.004

Zewdie, E.T., Roy, F.D., Yang, J.F., Gorassini, M.A., 2015. Facilitation of descending excitatory and spinal inhibitory networks from training of endurance and precision walking in participants with incomplete spinal cord injury, in: Progress in Brain Research. Elsevier, pp. 127–155. https://doi.org/10.1016/bs.pbr.2014.12.005

Chapter 3

The Role of cAMP and its Downstream Targets in Neurite Growth in the Adult Nervous System

Preface

As part of my undergraduate studies, I built upon a review paper for a class and turned it into the basis of my graduate work. This was technically one of the first things I did in the Fouad laboratory, having been published before the previous chapter was. It does however serve as a secondary introduction here to the downstream effects of stimulation and how it relates to activity dependent plasticity. This chapter is meant to introduce cAMP and its downstream effectors and tie the stimulation study to the following chapters that focus on EPAC and PKA, the downstream effectors of cAMP.

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3.1 Introduction

Ever since David and Aguayo (David and Aguayo, 1981) first demonstrated that injured central nervous system (CNS) axons can grow into a peripheral nerve graft, a major question has been: why do CNS axons not regenerate within the injured CNS? Three streams of research tackled this question. One stream examined whether the lack of CNS regeneration was due to an inhibitory environment. This research later identified inhibitory factors for neurite outgrowth including myelin associated inhibitors (Schwab and Bartholdi, 1996), and chondroitin sulfate proteoglycans (CSPGs) in the glial scar and the perineuronal net (Silver and Silver, 2014; Tom et al., 2004). The second stream examined whether reduced CNS regeneration was due to a lack of neurotrophic support resulting in the neurons not having the ability to regrow, as proposed by Aguayo himself (David and Aguayo, 1981). The third stream examined the question of whether age related changes might influence the decline in neuronal growth abilities (Cai et al., 2001; Park et al., 2008). These streams of research led to three main treatment strategies. Some laboratories attempted to neutralize neurite growth inhibitors (e.g., MAG (McKerracher et al., 1994) or Nogo (Craveiro et al., 2013; Schneider et al., 2015); , and CSPGs (Massey et al., 2006; Morgenstern et al., 2002)), while others augmented neurotrophic support by adding growth factors like brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), or glial cell-derived neurotrophic factor (GDNF), to the injured spinal cord (Bregman et al., 1998, 1997; Gao et al., 2003; Kwon et al., 2002; Plunet et al., 2002) (Figure 3.1). Alternatively, some groups attempted to modulate intracellular signalling pathways with the idea to recreate conditions of the developing CNS in order to allow injured neurons to regrow (Cai et al., 2002, 2001; Dergham et al., 2002; Gao et al., 2003;

Park et al., 2008). As a result of all of this research, it is now well accepted that a combination of all of three factors (growth inhibitors, in sufficient of growth promoting factors and developmental changes) are responsible for the limited neurite outgrowth and repair after a spinal cord injury (SCI) in adult mammals.

With many intrinsic and extrinsic factors converging to prevent regeneration (growth at the tip of lesioned axons) and plasticity (e.g., growth of new collaterals) after SCI, there is a major effort to identify whether there is a common cell signalling pathway responsible for inhibiting neurite growth. This effort has resulted in the knowledge that both the intrinsic and extrinsic factors inhibiting axonal growth after SCI seem to converge on a number of signalling pathways associated with cAMP, calcium/calmodulin, Ras homolog gene family member A (RhoA), and the mechanistic target of rapamycin (mTOR). A number of recent reviews have focused on roles of RhoA (Dergham et al., 2002; Monnier et al., 2003) and mTOR (Lu et al., 2014; Park et al., 2008) in axon growth and regeneration; this review will focus on cAMP and its downstream effectors. Specifically, we will discuss the relationship between cAMP and neurite outgrowth in the adult CNS, recently identified cAMP cell signalling pathways linked to axonal growth after SCI, and possible treatment strategies aimed to activate relevant cAMP dependent pathways.

3.2 The Role of cAMP in Neurite Growth

In the embryonic CNS, cAMP levels are high and are linked to the extensive axonal growth that occurs during development (Cai et al., 2001). It has been shown that

not long after birth, cAMP levels in dorsal root ganglia (DRG) neurons rapidly decline in parallel with the reduced regenerative capacity of their CNS axons (Cai et al., 2001; Chaudhry and Filbin, 2007). Similarly, blocking protein kinase A (PKA), a downstream effector of cAMP, using the PKA antagonists KT5720 and Rp-cAMPS in cultured rat DRG and retinal ganglion (RG) neurons resulted in reduced neurite outgrowth (Cai et al., 2001). This was done using both young CNS neurons (before postnatal day 4), where the PKA antagonists effectively stopped myelin's growth promoting effects, and in adult CNS neurons (P5 and older), where the inhibitory effects of the myelin-associated glycoprotein MAG, a neurite outgrowth inhibitor, were counteracted (Cai et al., 2001). They observed similar results in adult raphespinal neurons given their decrease in neurite growth when harvested from older animals (Cai et al., 2001). Collectively, these data suggest that cAMP is a key signalling molecule for switching the role of myelin-associated inhibitors from growth promoting in the developing nervous system to growth inhibiting in the mature nervous system.

3.3 Neurotrophins and cAMP

Neurotrophins are important signalling molecules for cell survival and neurite outgrowth. They can promote axon growth even in adult neurons exposed to myelin-associated inhibitors. For example, cerebellar and DRG neurons plated with the neurotrophins BDNF or neurite growth factor (NGF) prior to exposure to MAG and/or myelin, have increased neurite outgrowth compared to neurons without neurotrophins (Cai et al., 1999). Dougherty et al. (Dougherty et al., 2000) further explored neurotrophin expression in the spinal cord and found that in the adult spinal cord neurotrophins are

expressed at low basal levels but transiently increase near SCI sites. However, this transient expression of neurotrophins in the spinal cord does not occur to the same extent as in the peripheral nervous system (PNS) following injury. This discrepancy could be part of the reason for increased axonal growth in the PNS compared to the CNS (Dougherty et al., 2000; Fenrich and Gordon, 2004; Widenfalk et al., 2001).

The mechanisms of neurotrophin-induced priming on neurite outgrowth have also been examined by inhibiting tropomyosin receptor kinases (Trk; one of the receptors known to be activated by neurotrophins; Figure 3.1). Inhibiting Trk leads to a reduction of the growth promoting effects of neurotrophins, suggesting that that their growth promoting signalling occurs via Trk receptors, namely Trk A for NGF and Trk B for BDNF (Gao et al., 2003). Trk A and B signalling leads to the activation of the extracellular signal-regulated kinase (ERK) pathway, which inhibits phosphodiesterases (PDE, see upper portion of Figure 3.1). ERK inhibition of PDE was shown by priming cerebellar neurons with BDNF followed by the application of MAPK/ERK kinase inhibitors, to reverse the effect of BDNF (Gao et al., 2003). MAPK/ERK kinase inhibition reduced the inhibition of PDE, subsequently reducing cAMP levels and demonstrating that BDNF induced cAMP upregulation is ERK dependent. Most relevant to the regulation of cAMP levels is the inhibition of PDE4, which directly hydrolyzes cAMP. The blockade of the PDE inhibitor ERK shows that exposure to neurotrophins increases cAMP levels by reducing the rate of cAMP breakdown, leading to increased neurite outgrowth and regeneration (Gao et al., 2003). The findings that neurotrophins activate ERK and increase cAMP levels, supports previous results showing that cerebellar and DRG neurons plated with BDNF, NGF, or GDNF reduce MAG inhibition

(Cai et al., 1999). Collectively, when neurotrophin activation of ERK is considered, these findings show that neurotrophins increase cAMP.

Cai et al. (1999) also demonstrated in cerebellar and DRG cell culture models that neurotrophins need to be added prior to MAG exposure to be effective in promoting neurite outgrowth, as simultaneous treatment was not able to counteract MAG inhibition. These findings suggest that PDE4 must be inactivated and cAMP levels allowed to rise prior to MAG exposure to allow neurite outgrowth in adult neurons. Furthermore, injecting the injured spinal cord with neurotrophins and injecting a cAMP analog into the L4 DRG to precondition the soma prior to a cervical dorsal column transection results in a much larger increase in regeneration than either treatment alone (Lu et al., 2004). This finding suggests that neurotrophins affect neurite growth by increasing cAMP levels and other pathways. However, in this study the combination of neurotrophins and db-cAMP did not result in enhanced functional recovery in tasks such as tape-removal (Hernandez and Schallert, 1988), horizontal ladder (Kunkel-Bagden, 1993), or rope tasks (Kim et al., 2001), which indicates that an additional treatment component is needed to translate neurite growth into functional recovery (e.g., rehabilitative training (García-Alías et al., 2009; Krajacic et al., 2010)).

Given the importance of neurotrophins as growth factors during development and their ability to promote axon growth in adult CNS neurons, numerous studies have examined the effects of neurotrophins as potential therapeutic targets for promoting neurite outgrowth after SCI (for reviews see: (Awad et al., 2015; Harvey et al., 2015; Weishaupt et al., 2012)). From these studies it can be concluded that despite the abilities of neurotrophins to promote neurite outgrowth and their transient increase after injury,

the CNS still does not overcome growth inhibition after injury and is unable to repair itself. A likely reason for this persistent lack of axon growth is that the injury induced up regulation of neurotrophins is insufficient and not sustained long enough to enable axonal regeneration (Gordon and Tetzlaff, 2015).

Understanding why pro-regenerative mechanisms that are normally activated after CNS injury, such as neurotrophic or cAMP signalling, are not sufficient to overcome growth inhibition could open the door to novel treatment strategies to boost these pathways in the future.

3.4 Elevating cAMP Levels to Promote Neurite Outgrowth

With the knowledge that elevated cAMP levels could promote neurite outgrowth in the inhibitory environment of the injured adult mammalian CNS, it was a logical next step to target cAMP and other parts of its signalling pathway to promote regeneration. Neumann and colleagues (Neumann et al., 2002) demonstrated the pro-regenerative effect of high cAMP levels by injecting a cAMP analog (db-cAMP) in DRGs *in vivo*, thus activating downstream effectors including PKA, and CREB (see Figure 3.1) and promoting regeneration of the central branch in the spinal cord. Similarly, Qiu et al. (Qiu et al., 2002) found that a lesion to the peripheral branch of DRG cells via a conditioning lesion (Neumann and Woolf, 1999) tripled cAMP levels and increased regeneration of subsequently lesioned sensory fibers in the spinal cord. These experiments led to the findings that there are two phases of regeneration. The first phase is dependent upon PKA (i.e., transcription independent phase; see Figure 3.1), extends up to a week, and was

found through the inhibition of PKA prior to the end of this time period using KT5720 to halt regeneration (Qiu et al., 2002). The subsequent phase is PKA independent (i.e., transcription dependent phase; see Figure 3.1) and was detected by the lack of effect of the PKA inhibitor KT5720 at 7 days post-injury when cAMP levels had returned to pre-injury levels. The cAMP levels returning to pre-injury levels were confirmed via cAMP staining during both phases showing greatly increased cAMP levels in the first, PKA dependent, phase and normal levels after a week. It was also shown that blocking PKA early in the PKA dependent stage, while simultaneously increasing cAMP halts both phases, suggesting a role for PKA in triggering the subsequent phase (Qiu et al., 2002). Based on the findings that increasing neuronal cAMP levels promotes neurite outgrowth, but is insufficient to promote axon growth through a spinal lesion, Pearse et al (Pearse et al., 2004) grafted Schwann cells into a thoracic contusion site in addition to increasing cAMP levels in an attempt to overcome this hurdle. The cAMP signalling pathway was activated by administration of dibutyryl cAMP, a cAMP analog, or rolipram, a PDE inhibitor (see figure 3.1). In both cases, activation of cAMP signalling promoted the ability of axons to grow into and beyond a Schwann cell graft (Pearse et al., 2004).

3.5 Raising cAMP Using Electrical Stimulation

The importance of cAMP in the regulation of neurite outgrowth and myelin-associated inhibition is illustrated even further by the finding that cAMP levels drop in the spinal cord, brainstem, and cortex following SCI (Pearse et al., 2004), consistent with lack of axonal regeneration. A possible reason for the post injury decline in cAMP is the disuse of affected limbs and the associated inactivity of neurons within the involved

circuitry. This is supported by the finding that directly following unilateral SCI in rats there is a decrease in cAMP levels in the motor cortex contralateral to the lesion leading to a significant decrease in phosphorylated PKA (pPKA) despite no effect on overall PKA levels (Krajacic et al., 2009). Furthermore, rehabilitative motor training was able to restore the levels of pPKA at 4 weeks post injury (Krajacic et al., 2009). Reduced cAMP levels due to disuse can also explain the success of constrained induced movement therapy in promoting motor recovery after stroke, which is enforcing the activation (and thus the increase in cAMP) of disused circuitry (David M et al., 1997; Taub and Wolf, 1997).

An alternative method to increase the activity of neuronal networks besides training is electrical stimulation. Electrical stimulation is a well-established approach to enhance regeneration of injured peripheral axons (Gordon, 2016a, 2016b; Willand et al., 2016) and has been translated to the clinical setting, for example in individuals undergoing carpal tunnel release surgery (Chan et al., 2016). It has also been shown that following unilateral SCI, electrical stimulation of the motor cortex innervating the uninjured side promotes neurite outgrowth of the stimulated spared fibers (Carmel et al., 2013, 2010; Carmel and Martin, 2014). One proposed mechanism for the stimulation enhanced growth capacity of injured and spared axons is that electrical stimulation increases cAMP (Brushart et al., 2002; Geremia et al., 2007). With this in mind, Udina and colleagues (Udina et al., 2008) tested whether electrical stimulation of peripheral nerve could promote neurite outgrowth of DRG neurons *in vitro*. For these experiments, sciatic nerves of rats were stimulated for 1 hour, before collecting the DRGs either 1 or 7 days later to measure neurite outgrowth. The outgrowth from the 1 and 7 day group was

then compared to the conditioning lesion group. These experiments showed that electrical stimulation 7 days prior to DRG extraction increased neurite outgrowth four times compared to both the control and 1 day group. These *in vitro* findings provided the basis for testing if electrical stimulation could increase cAMP *in vivo* (Udina et al., 2008). For these experiments, rats received a pre-conditioning lesion followed by a dorsal column thoracic SCI, or a dorsal column thoracic SCI followed by electrical stimulation of peripheral nerves innervating the hindlimbs (Udina et al., 2008). In this setting electrical stimulation increased axon growth of the lesioned central branch of DRGs, though not to the same extent as in a conditioning lesion group. It is noteworthy that both the conditioning lesion group and the electrical stimulation group showed increased intracellular cAMP to a similar level (Udina et al., 2008). This suggests that, conditioning lesions involve other signalling pathways working in tandem with the cAMP pathway.

In conclusion, increasing neuronal cAMP levels following SCI is a promising avenue to enable/promote neurite outgrowth of injured and spared nerve cells and thus to enhance neuro-reparative processes as regeneration and plasticity. Possible treatment approaches to enhance cAMP levels range from pharmacology to electrical stimulation or rehabilitative training (Chan et al., 2016; David M et al., 1997; Dietz and Fouad, 2014; García-Alías et al., 2009; Gordon, 2016a; Krajacic et al., 2010, 2009; Taub and Wolf, 1997; Willand et al., 2016).

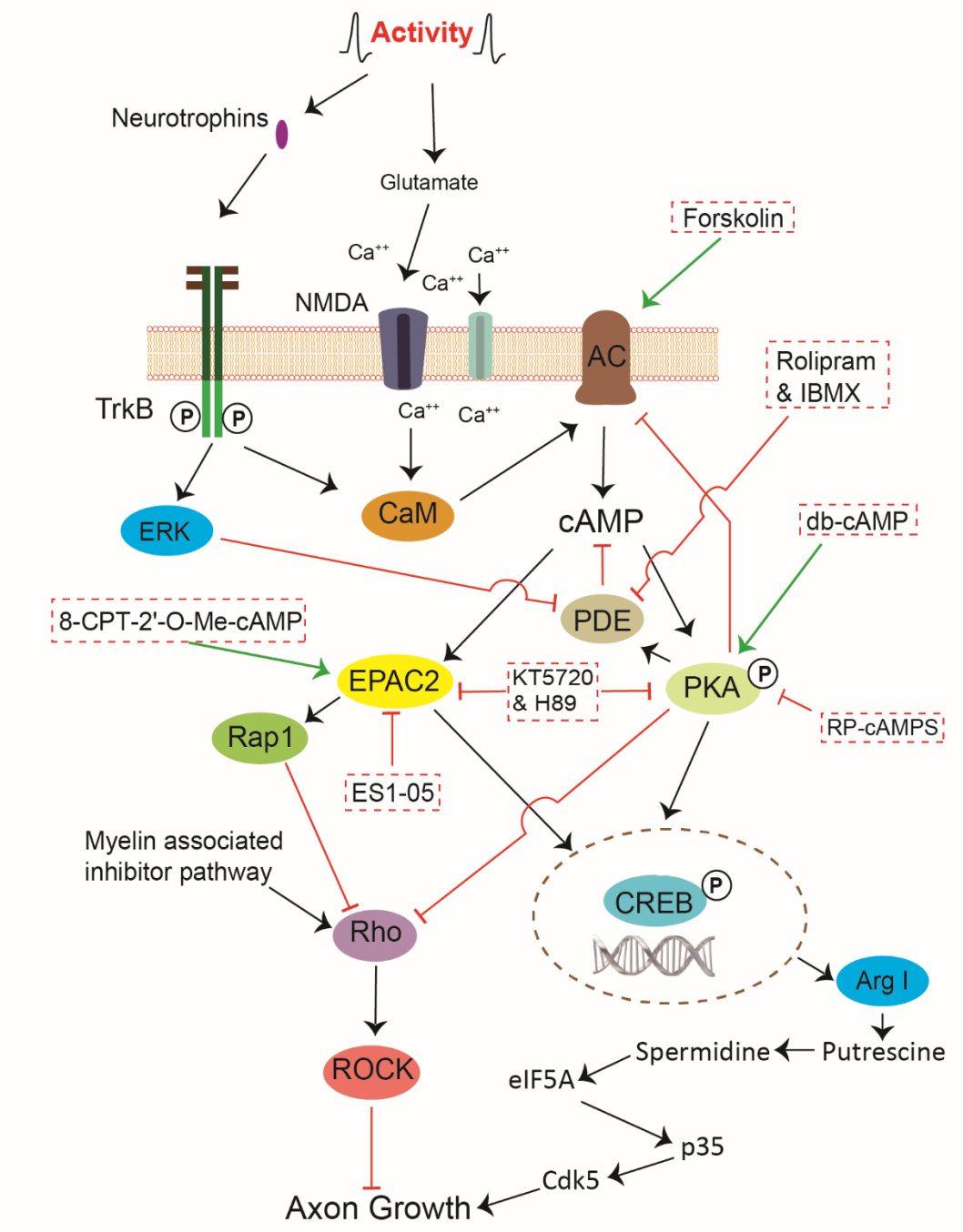

Figure 3.1 – The cAMP Pathway and Axon Growth

Intracellular cAMP levels can be increased by various means including via neurotrophins, which results in a decreased rate of hydrolytic cAMP breakdown through ERK activation, inhibiting PDE. cAMP levels can also be increased via activity in the neuron, e.g. by electrical stimulation, or agonist application. cAMP directly activates PKA and EPAC. PKA is regulated via a feedback look in that phosphorylated PKA activates PDE,

causing cAMP breakdown and therefore a decrease in further PKA activation. Activated PKA directly inhibits RhoA, which is part of PKA's actions in the transcription independent phase and leads to CREB phosphorylation. This has been associated with increased Arginase I transcription, resulting in enhanced growth promoting polyamine production. CREB phosphorylation and Arginase I transcription are known as the transcription dependent phase of neuronal growth. The drugs used in experiments to either activate or inhibit the activity of effectors along the path are highlighted in dashed boxes. AC: Adenylate cyclase, ERK: Extracellular signal-regulated kinase, CaM: Calmodulin, cAMP: cyclic AMP, PDE: Phosphodiesterase, PKA: Protein Kinase A, EPAC2: Exchange protein activated by cAMP, Rap-1: Ras-proximate-1, Rho: Ras homolog family member, CREB: cAMP response element-binding protein, Arg I: Arginase I, eIF5a: Eukaryotic translation initiation factor 5A-1, Rock: Rho associated protein kinase, Cdk5: Cyclin-dependent kinase 5.

3.6 cAMP Signalling

High neuronal cAMP levels are linked to increased neurite outgrowth in both permissive and inhibitory environments, but the mechanisms by which cAMP increases neuronal growth have yet to be fully understood. A well-known downstream effector of cAMP is PKA (Figure 3.1). Various studies have shown that blocking PKA using KT5720 eliminates the growth promoting effects of increased cAMP levels (Cai et al., 2002, 2001, 1999; Gao et al., 2003; Ming et al., 1997; Qiu et al., 2002) (Figure 3.1). Qiu et al. (Qiu et al., 2002), showed that there are two phases of regeneration, the PKA dependent phase (<7d post-injury) and the PKA independent phase (starting at 7d post-injury). During the PKA dependent phase, PKA is leading to the activation of cAMP-response element binding protein (CREB) (Chaudhry and Filbin, 2007), which in turn leads to the PKA independent phase and transcription of the Arginase I gene (Figure 3.1). This proposed signalling pathway is supported by findings that Arginase I is up-regulated following administration of db-cAMP or BDNF in cerebellar neurons (Cai et al., 2002), and a corresponding increase in growth promoting polyamine synthesis was found, indicating that Arginase I is a downstream effector of cAMP. Therefore, the transcription dependent phase (i.e., PKA independent) of neuronal regrowth that occurs once cAMP levels have returned to normal could be due to conversion of cAMP to AMP and the downstream activation of CREB and subsequently Arginase. Recent studies have established that Arginase I synthesis of polyamines leads to an increase in putrescine, which is then converted to spermidine (He et al., 2016). Spermidine in turn activates the eukaryotic translation initiation factor 5A-1 (eIF5A), leading to increased translation of p35, which in turn activates cyclin-dependent kinase 5 (Cdk5). Thus, it has been

proposed that Cdk5 is responsible for the growth promoting actions of this pathway and activity dependent dendrite development in general and therefore the transcription dependent phase of regeneration (He et al., 2016; Liang et al., 2015; Ye et al., 2012).

Besides activating PKA, cAMP has also been implicated in other neurite growth promoting pathways. For example, Hannila et al. (Hannila et al., 2013) showed that secretory leukocyte protease inhibitor (SLPI) is upregulated in a cAMP dependent manner and boosting its activity increases the reversal of MAG inhibition in both P1 cortical neurons and P5 DRGs. Additionally, an upregulation of Interleukin-6 (Cao et al., 2006) and Metallothionein-I/II (Siddiq et al., 2015) following conditioning lesions has been discovered and artificial upregulation of these factors also increase reversal of MAG inhibition, though it has been suggested that these factors are independent to the PKA pathway (Cao et al., 2006; Siddiq et al., 2015).

The role of PKA in controlling neurite growth becomes even more complicated once the transcription independent phase of neuronal growth is examined in detail. It has been reported that between days 1 and 7 following artificial cAMP elevation, mature DRG neurons can grow on both MAG and myelin (Qiu et al., 2002). To prove that enhanced axon growth was due to elevated cAMP levels, and under the assumption that PKA was the only downstream effector of cAMP responsible for axon growth, the PKA inhibitor KT5720 was added to cultured DRG neurons on MAG expressing CHO cells. In this scenario the inhibition of PKA led to a decrease in the growth promoting effects of cAMP (Qiu et al., 2002) (Figure 3.1). Additionally, PKA is also suggested to play a role in guidance of regenerating axons. Using spinal neurons of young *Xenopus*, Ming et al. (Ming et al., 1997) showed growth cone attraction to netrin-1 in their base state, but when

a cAMP competitor or the PKA inhibitor KT5720 was used, this turned into a repulsive response, with netrin-1 repelling growth cones (Ming et al., 1997). Together these results indicate that the PKA dependent mechanisms underlying regeneration and axon guidance are potentially related. Additionally it is believed that PKA directly inhibits RhoA, a key signalling component for translating the growth inhibitory effect of both myelin associated inhibitors (Chaudhry and Filbin, 2007; Domeniconi et al., 2002) and CSPGs (Dergham et al., 2002; Monnier et al., 2003). Therefore, the transcription independent phase is highly dependent upon the actions of PKA both as an axonal guidance promoter and as a direct blocker of inhibition.

Despite the detailed knowledge gained on PKA and neurite outgrowth, there is a possible confounding factor regarding many studies involving PKA inhibition. The vast majority of studies done prior to 2008 used KT5720 and/or H89 to inhibit PKA in order to elucidate its role in cAMP signalling and axonal regeneration. In a seminal study by Murray in 2008 (Murray, 2008) both KT5720 and H89 were shown to have widespread effects inhibiting other protein kinases including the exchange protein activated by cAMP (EPAC), a signalling molecule downstream of cAMP, often at lower concentrations than that needed to block PKA (Figure 3.1). This insight to unspecific mechanisms of action of PKA inhibiting drugs raised doubts about the conclusion that PKA is the key component in the cAMP mediated axon growth. The wider inhibition brought on by KT5720, however, does not necessarily refute the role of PKA in triggering the transcription dependent phase. Importantly, the knowledge that PKA may not be responsible for all functions previously attributed to it opens the door to the targeting of other cAMP dependent pathways to promote neurite outgrowth after CNS injury.

3.7 Exchange Protein Activated by cAMP

EPAC is activated by cAMP independently of PKA (Figure 3.1) and likely a key factor in cAMP induced neurite outgrowth, as well as many other aspects of cAMP signalling in other areas of the body that had been previously thought to be the result of PKA activation (Cheng et al., 2008). Two isoforms have been described, EPAC1 and EPAC2, with varying expression levels and tissue distributions throughout development (Murray and Shewan, 2008). EPAC1 is highly expressed in embryonic nerve cells while EPAC2 is expressed mostly in developed nervous tissue (Murray and Shewan, 2008) and therefore the focus of most EPAC studies. Despite their different expression patterns, the specific roles of each isoform in the normal nervous system has yet to be determined as they seem functionally very similar (Murray and Shewan, 2008). Murray and Shewan (Murray and Shewan, 2008) highlighted the importance of EPAC2 in axon growth using a combination of cAMP analogs and an siRNA knock down protocol to specifically inhibit EPAC1 and EPAC2 activity. In these experiments, EPAC1 and EPAC2 knock down resulted in reduced neurite outgrowth in DRG cells cultured on a permissive substrate. The addition of a cAMP analog, which would have activated PKA did not improve neurite outgrowth. In the same study, it was illustrated in a growth cone turning assay that culturing DRG neurons along a gradient of the PDE inhibitor rolipram resulted in growth cones turning towards the higher concentration of the drug (Murray and Shewan, 2008). Additionally, Enserink et al (Enserink et al., 2002) showed that a specific

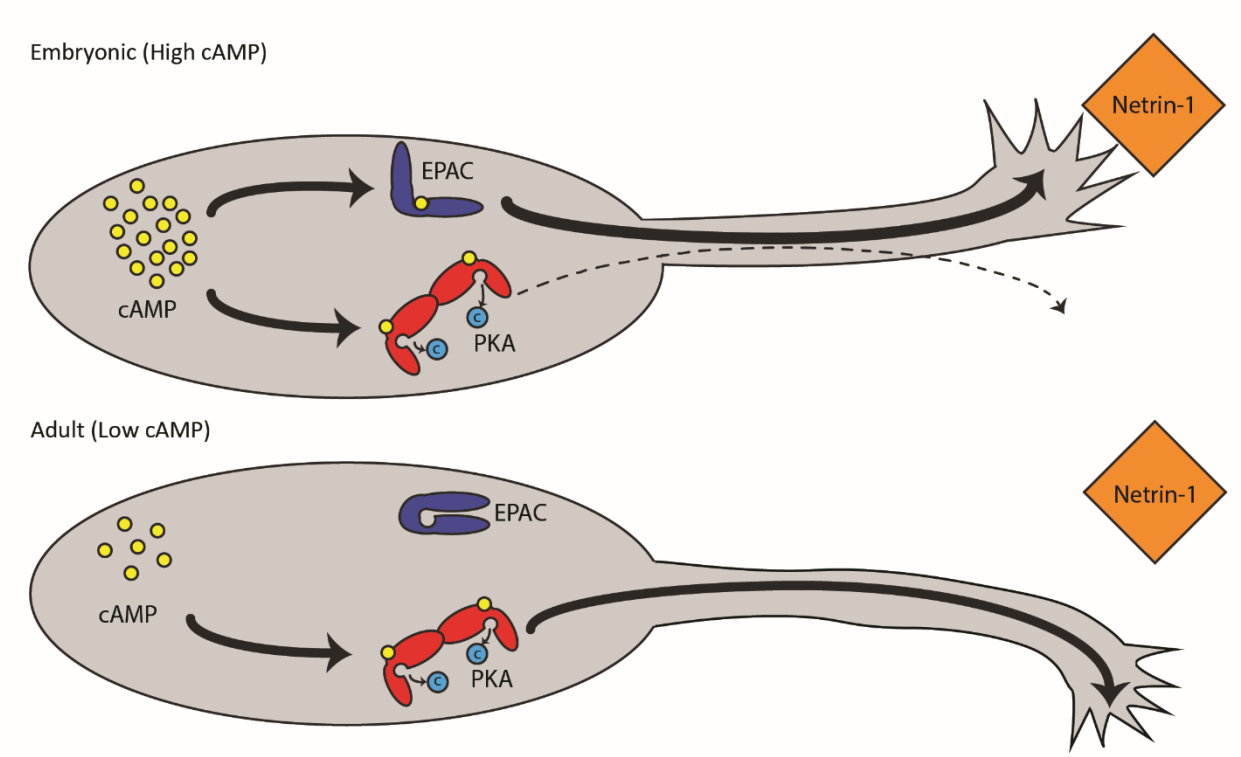

Figure 3.2 – Model for How Differing Levels of cAMP Leads to an Altered Activation of EPAC and PKA

High levels of cAMP, typically found in embryonic cells or when cAMP is artificially raised leads to activation of both EPAC and PKA, though the attraction properties of EPAC predominate resulting in growth cone attraction to Netrin-1 and MAG (Netrin-1 being shown in the image, though MAG acts in the same manner). At low levels of cAMP, as seen in adult neurons, only PKA is activated resulting in the catalytic subunits of PKA separating from the regulatory subunits, causing downstream actions and leading to growth cone repulsion to Netrin-1 and MAG.

EPAC agonist, 8-CPT-2′-O-Me-cAMP, enhanced neurite outgrowth both in young and adult neurons when they were grown on a permissive laminin substrate. Similar results were seen when a growth cone turning assay was performed using the EPAC agonist 8-CPT-2'-Me-cAMP, showing growth cone turning towards higher agonist concentrations. These results lead to new interpretations of previous work to elucidate the role of PKA in growth cone guidance that used non-specific PKA inhibitors (Ming et al., 1997). Whether PKA has no role in growth cone guidance, has a different role than previously thought, or that both PKA and EPAC2 work in tandem remains to be determined.

Murray and Shewan went on to analyze growth cone chemotaxis in relation to EPAC2 and PKA activity in the presence of netrin-1 and MAG (Murray et al., 2009). They found that growth cones from embryonic neurons, which have high levels of cAMP, were attracted to netrin-1 and MAG and, which was mediated by EPAC2. Conversely, growth cones of adult neurons, which have low cAMP levels, were repelled by netrin-1 and MAG; an effect shown to be mediated by PKA (Murray et al., 2009) (Figure 3.2). These findings were confirmed using siRNA knock down in adult neurons as well as a fluorescence resonance energy transfer (FRET) imaging protocol that they established to measure protein activity. They found that netrin-1, an axon guidance protein, and MAG activated EPAC2 in embryonic cells, but activated PKA in postnatal and adult cells (Murray et al., 2009). This information also sheds light on the potential reason why MAG switches from growth promoting to growth inhibiting after development as its growth cone guidance properties are similar in function and mechanism to that of netrin-1. Furthermore, differences in EPAC2 and PKA activation in embryonic versus adult neurons could be almost entirely cAMP dependent (Peace and

Shewan, 2011). Importantly, PKA is activated at much lower cAMP levels than EPAC2, which leads to growth cone repulsion from environmental stimuli. Conversely, increased cAMP levels lead to both PKA and EPAC2 activation, with the growth cone attractive properties outweighing the repulsive ones, leading to an overall attraction towards the stimuli (Peace and Shewan, 2011).

Recently, our group has added more pieces to the ever-expanding puzzle of EPAC2 and PKA signalling in adult neurons (Wei et al., 2016). Given the previously documented increase in cortical pPKA in SCI rats receiving rehabilitative training (in a task heavily relying on the corticospinal tract; CST), we hypothesized that pharmacological blockade of cortical PKA using Rp-cAMPS would reduce training induced CST plasticity and recovery. Contrary to our hypothesis, blocking PKA activity led to a significant increase in functional recovery in a forelimb reaching task in two separate experiments (Wei et al., 2016). We also investigated the effects of PKA inhibition on CST plasticity by tracing the injured CST and found a marked increase in CST collateral sprouting in rats treated with Rp-cAMPS and rehabilitative training compared to control groups. Subsequent *in vitro* experiments using DRG cells indicated that Rp-cAMPS treatment increased neurite outgrowth which was contrary to our expectations and the findings of Cai et al. (Cai et al., 2001) which showed Rp-cAMPs treatment reduced neurite outgrowth in newborn DRG cells with high cAMP on myelin.

One of the most important aspects of these findings relative to the bigger picture of PKA, CREB, and EPAC2, is the finding that PKA blockade did not alter CREB phosphorylation/activation in an *in vitro* experiment. For this experiment, a striatal cell line was treated with forskolin, a compound that up-regulates the activity of adenylate

cyclase to increase cAMP levels in the cell and subsequently CREB activation (Figure 3.1). It was found that CREB activation was not blocked when PKA was inhibited using Rp-cAMPS. Interestingly, Rp-cAMPS blocked CREB activation when IBMX, a PDE inhibitor (Figure 3.1), was used to increase cAMP. These results suggest that high levels of cAMP play a major role in inducing CREB phosphorylation when PKA activity is halted and are consistent with the findings of Peace and Shewan (Peace and Shewan, 2011). This points towards the importance of the EPAC pathway in CREB phosphorylation.

Following our findings suggesting the importance of the EPAC pathway in neurite outgrowth we investigated its involvement in cAMP mediated activation of CREB and axon sprouting. For this, DRG neurons growing on laminin were treated with either the PKA inhibitor Rp-cAMPS, the EPAC2 inhibitor ES1-05, or both, and the effects on neurite outgrowth and CREB phosphorylation were examined *in vitro*. Consistent with the *in vivo* results, PKA inhibition alone increased neurite outgrowth. However, EPAC2 inhibition alone did not reduce neurite outgrowth, and only when both inhibitors were applied was neurite outgrowth reduced. Looking at pCREB levels in similar experimental groups, Rp-cAMPS treatment groups showed increased pCREB, congruent with the augmented neurite outgrowth, but EPAC2 inhibition resulted in a small decrease in pCREB levels, though not to the same extent as blockade of both PKA and EPAC2 together. These findings suggest that PKA and EPAC2 both act through CREB, and can actually compensate for the loss of the other. The results showing that inhibition of PKA did not reduce pCREB whereas EPAC2 inhibition did, suggests that

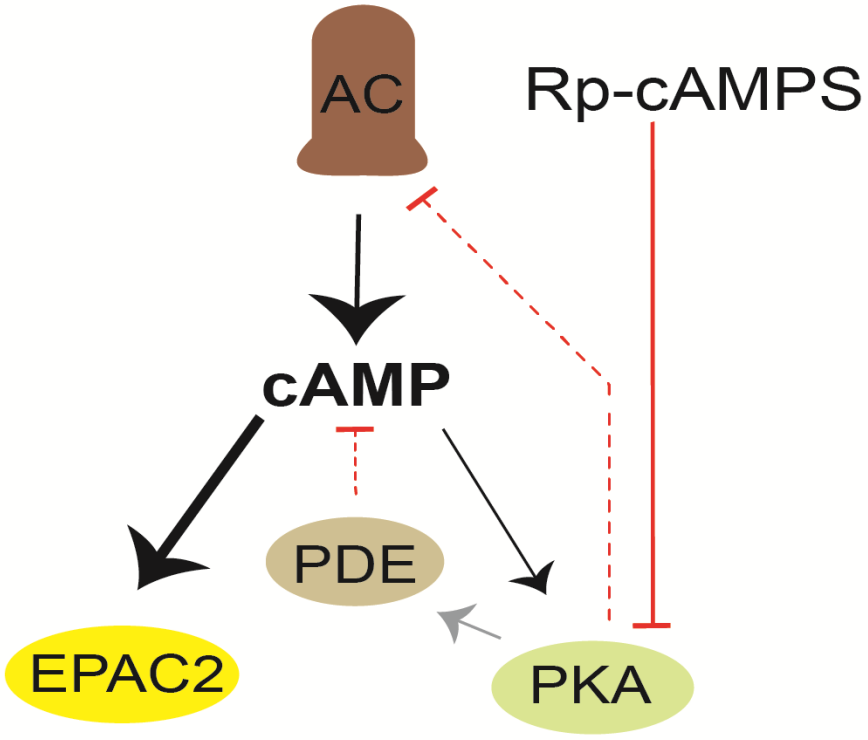

Figure 3.3 – Model of the Compensation by EPAC2 Following PKA Inhibition by Rp-cAMPs Treatment

AC: Adenylate Cyclase, cAMP: Cyclic AMP, PKA: Protein Kinase A, EPAC: Exchange protein activated by cAMP, PDE: Phosphodiesterase.

EPAC2 is more effective in phosphorylating CREB. Therefore, the findings that PKA blockade using Rp-cAMPS (a competitive inhibitor of cAMP binding to PKA) led to increased functional recovery in rats is potentially because it allowed EPAC2 full access to the cAMP pool. Collectively, the results suggest that the two signalling molecules work in concert, compensating for the loss of the other as required (Wei et al., 2016) (Figure 3.3).

With evidence that EPAC2 is important for both neurite outgrowth and growth cone guidance, two functions previously attributed exclusively to PKA, cAMP dependent neurite growth may not be as dependent on PKA as previously thought. In fact, the transcription independent phase of growth seems likely due to both the PKA and EPAC pathways using cAMP. In light of these new findings, one potential mechanism for cAMP mediated axon growth is that EPAC2 is activated in a cAMP rich environment in the first week post-injury, resulting in the short-term growth enhancement seen until cAMP levels returned to baseline. At this point, the transcription dependent phase has started and polyamines begin to encourage neurite outgrowth. Additionally, the findings from Wei et al. (Wei et al., 2016) suggest that the transcription dependent phase is also due to both PKA and EPAC2 activating CREB and its downstream effectors, and that EPAC2 appears to be the more effective of the two routes, potentially opening the door to new treatment options that target EPAC2 pathway.

In addition to its role in promoting sprouting of injured adult axons, EPAC2 may play a role in myelination after SCI. In a study by Boomkamp et al. (Boomkamp et al., 2014), it was demonstrated that at low doses rolipram enhances axonal myelination in spinal cord cultures made from dissociated embryonic spinal cords that and plated with

neurosphere derived astrocytes. Rolipram blocks PDE4, which hydrolyzes cAMP and is therefore often used to study and promote neurite outgrowth (Boomkamp et al., 2014; Mackenzie and Houslay, 2000) (Figure 3.1). It was found that the reason rolipram only enhances myelination at low doses is because rolipram preferentially inhibits the high affinity rolipram binding-site (HARBS) of PDE4. Interestingly, higher concentrations of rolipram led to the inhibition of the low affinity rolipram binding-site (LARBS) and inhibited myelination. To test the mechanism by which myelination was inhibited Rp-cAMPS was applied. Inhibiting PKA led to an increase in myelination, despite high rolipram concentrations, suggesting that PKA plays a role in the myelination blocking actions of LARBS. Remarkably, activating HARBS with low doses of rolipram while blocking PKA did not reduce myelination, showing that PKA is not the only effector downstream of these binding sites. It was proposed that HARBS actions must use a different cAMP effector, i.e., EPAC. To test this idea, myelinating spinal cord cultures were treated with the EPAC2 agonist, Me-cAMP, which enhanced myelination levels as expected. Therefore, since a PKA antagonist and an EPAC2 agonist have similar effects on myelination after SCI, this data indicates that PKA and EPAC have opposing functions in myelination. Importantly, this study provides another potential s treatment target for SCI through preferential HARBS binding. More broadly, these findings are consistent with the opposing functions of PKA and EPAC2 in growth cone guidance, and suggest that therapies that specifically stimulate EPAC2 signalling could be highly beneficial for improving both axon growth and remyelination after SCI.

3.8 Conclusion

Numerous studies suggest that increasing cAMP levels can increase neurite outgrowth and could be effective as both short and long-term treatment option for SCI. However, due to cAMP being an important and ubiquitous signalling molecule throughout the body, direct activation of cAMP would likely cause numerous adverse side effects. Thus, the understanding of how to specifically activate downstream targets is an important step in the development of clinically relevant treatments. As more accurate and selective drugs are discovered, we will be able to learn more about the processes involved in neuronal regeneration and refine our knowledge of how neurite outgrowth occurs. Up to this point, most studies examining the role of cAMP in axon regeneration focused on PKA dependent pathways. Advances in the field have greatly altered that perception and now the emerging ideas regarding the roles of EPAC sheds light on a potentially new pathway with a crucial role in regeneration and plasticity. With more specific targets for promoting plasticity and the dawn of a second cAMP dependent pathway, Murray et al. (Murray et al., 2009) suggested that a targeted activation of EPAC might be necessary to see significant improvements in functional recovery from CNS injury and limiting possible side effects that may occur from a broader cAMP boosting treatment. Further advancements in our understanding of cAMP signalling will continue to illuminate the potential functions and components of cAMP signalling that can be targeted to promote neurite growth and recovery following CNS injuries.

3.9 References

Awad, B.I., Carmody, M.A., Steinmetz, M.P., 2015. Potential Role of Growth Factors in the Management of Spinal Cord Injury. World Neurosurg. 83, 120–131. https://doi.org/10.1016/j.wneu.2013.01.042

Boomkamp, S.D., McGrath, M.A., Houslay, M.D., Barnett, S.C., 2014. Epac and the high affinity rolipram binding conformer of PDE4 modulate neurite outgrowth and myelination using an *in vitro* spinal cord injury model: Rho/ROCK and cAMP effects on SCI cultures. Br. J. Pharmacol. 171, 2385–2398. https://doi.org/10.1111/bph.12588

Bregman, B.S., Broude, E., McAtee, M., Kelley, M.S., 1998. Transplants and Neurotrophic Factors Prevent Atrophy of Mature CNS Neurons after Spinal Cord Injury. Exp. Neurol. 149, 13–27. https://doi.org/10.1006/exnr.1997.6669

Bregman, B.S., McAtee, M., Dai, H.N., Kuhn, P.L., 1997. Neurotrophic Factors Increase Axonal Growth after Spinal Cord Injury and Transplantation in the Adult Rat. Exp. Neurol. 148, 475–494. https://doi.org/10.1006/exnr.1997.6705

Brushart, T.M., Hoffman, P.N., Royall, R.M., Murinson, B.B., Witzel, C., Gordon, T., 2002. Electrical Stimulation Promotes Motoneuron Regeneration without Increasing Its Speed or Conditioning the Neuron. J. Neurosci. 22, 6631–6638.

Cai, D., Deng, K., Mellado, W., Lee, J., Ratan, R.R., Filbin, M.T., 2002. Arginase I and Polyamines Act Downstream from Cyclic AMP in Overcoming Inhibition of Axonal Growth MAG and Myelin In Vitro. Neuron 35, 711–719.

Cai, D., Qiu, J., Cao, Z., McAtee, M., Bregman, B.S., Filbin, M.T., 2001. Neuronal Cyclic AMP Controls the Developmental Loss in Ability of Axons to Regenerate. J. Neurosci. 21, 4731–4739.

Cai, D., Shen, Y., De Bellard, M., Tang, S., 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.

Cao, Z., Gao, Y., Bryson, J.B., Hou, J., Chaudhry, N., Siddiq, M.M., Martinez, J., Spencer, T., Carmel, J.B., Hart, R.P., Filbin, M.T., 2006. The Cytokine Interleukin-6 Is Sufficient But Not Necessary to Mimic the Peripheral Conditioning Lesion Effect on Axonal Growth. J. Neurosci. 26, 5565–5573. https://doi.org/10.1523/JNEUROSCI.0815-06.2006

Carmel, J.B., Berrol, L.J., Brus-Ramer, M., Martin, J.H., 2010. Chronic Electrical Stimulation of the Intact Corticospinal System after Unilateral Injury Restores Skilled Locomotor Control and Promotes Spinal Axon Outgrowth. J. Neurosci. 30, 10918–10926. https://doi.org/10.1523/JNEUROSCI.1435-10.2010

Carmel, J.B., Kimura, H., Berrol, L.J., Martin, J.H., 2013. Motor cortex electrical stimulation promotes axon outgrowth to brain stem and spinal targets that control the forelimb impaired by unilateral corticospinal injury. Eur. J. Neurosci. 37, 1090–1102. https://doi.org/10.1111/ejn.12119

Carmel, J.B., Martin, J.H., 2014. Motor cortex electrical stimulation augments sprouting of the corticospinal tract and promotes recovery of motor function. Front. Integr. Neurosci. 8. https://doi.org/10.3389/fnint.2014.00051

Chan, K.M., Curran, M.W.T., Gordon, T., 2016. The use of brief post-surgical low frequency electrical stimulation to enhance nerve regeneration in clinical practice: Use of brief post-surgical low frequency electrical stimulation. J. Physiol. 594, 3553–3559. https://doi.org/10.1113/JP270892

Chaudhry, N., Filbin, M.T., 2007. Myelin-associated inhibitory signaling and strategies to overcome inhibition. J. Cereb. Blood Flow Metab. 27, 1096–1107. https://doi.org/10.1038/sj.jcbfm.9600407

Cheng, X., Ji, Z., Tsalkova, T., Mei, F., 2008. Epac and PKA: a tale of two intracellular cAMP receptors. Acta Biochim. Biophys. Sin. 40, 651–662. https://doi.org/10.1111/j.1745-7270.2008.00438.x

Craveiro, L.M., Weinmann, O., Roschitzki, B., Gonzenbach, R.R., Zörner, B., Montani, L., Yee, B.K., Feldon, J., Willi, R., Schwab, M.E., 2013. Infusion of anti-Nogo-A antibodies in adult rats increases growth and synapse related proteins in the absence of behavioral alterations. Exp. Neurol. 250, 52–68. https://doi.org/10.1016/j.expneurol.2013.09.015

David M, M., Jean E, C., Stephanie C, D., Rama D, P., Edward Taub, 1997. Constraint-induced movement therapy for moter recovery after stroke. NeuroRehabilitation 29–43. https://doi.org/10.3233/NRE-1997-9104

David, S., Aguayo, A.J., 1981. Axonal Elongation into Peripheral Nervous System "Bridges" after Central Nervous System Injury in Adult Rats. Science 214, 931–933.

Dergham, P., Ellezam, B., Essagian, C., Avedissian, H., Lubell, W.D., McKerracher, L., 2002. Rho Signaling Pathway Targeted to Promote Spinal Cord Repair. J. Neurosci. 22, 6570–6577.

Dietz, V., Fouad, K., 2014. Restoration of sensorimotor functions after spinal cord injury. Brain 137, 654–667. https://doi.org/10.1093/brain/awt262

Domeniconi, M., Cao, Z., Spencer, T., Sivasankaran, R., Wang, K.C., Nikulina, E., Kimura, N., Cai, H., Deng, K., Gao, Y., He, Z., Filbin, M.T., 2002. Myelin-Associated Glycoprotein Interacts with the Nogo66 Receptor to Inhibit Neurite Outgrowth. Neuron 35, 283–290.

Dougherty, K.D., Dreyfus, C.F., Black, I.B., 2000. Brain-Derived Neurotrophic Factor in Astrocytes, Oligodendrocytes, and Microglia/Macrophages after Spinal Cord Injury. Neurobiol. Dis. 7, 574–585. https://doi.org/10.1006/nbdi.2000.0318

Enserink, J.M., Christensen, A.E., de Rooij, J., van Triest, M., Schwede, F., Genieser, H.G., Døskeland, S.O., Blank, J.L., Bos, J.L., 2002. A novel Epac-specific cAMP analogue demonstrates independent regulation of Rap1 and ERK. Nat. Cell Biol. 4, 901–906. https://doi.org/10.1038/ncb874

Fenrich, K., Gordon, T., 2004. Canadian Association of Neuroscience Review: Axonal Regeneration in the Peripheral and Central Nervous Systems – Current Issues and Advances. Can. J. Neurol. Sci. 31, 142–156. https://doi.org/10.1017/S0317167100053798

Gao, Y., Nikulina, E., Mellado, W., Filbin, M.T., 2003. Neurotrophins Elevate cAMP to Reach a Threshold Required to Overcome Inhibition by MAG through

Extracellular Signal-Regulated Kinase-Dependent Inhibition of Phosphodiesterase. J. Neurosci. 23, 11770–11777.

García-Alías, G., Barkhuysen, S., Buckle, M., Fawcett, J.W., 2009. Chondroitinase ABC treatment opens a window of opportunity for task-specific rehabilitation. Nat. Neurosci. 12, 1145–1151. https://doi.org/10.1038/nn.2377

Geremia, N.M., Gordon, T., Brushart, T.M., Al-Majed, A.A., Verge, V.M.K., 2007. Electrical stimulation promotes sensory neuron regeneration and growth-associated gene expression. Exp. Neurol. 205, 347–359. https://doi.org/10.1016/j.expneurol.2007.01.040

Gordon, T., 2016a. Electrical Stimulation to Enhance Axon Regeneration After Peripheral Nerve Injuries in Animal Models and Humans. Neurotherapeutics 13, 295–310. https://doi.org/10.1007/s13311-015-0415-1

Gordon, T., 2016b. Nerve regeneration in the peripheral and central nervous systems: Editorial. J. Physiol. 594, 3517–3520. https://doi.org/10.1113/JP270898

Gordon, T., Tetzlaff, W., 2015. Regeneration-associated genes decline in chronically injured rat sciatic motoneurons. Eur. J. Neurosci. 42, 2783–2791. https://doi.org/10.1111/ejn.13070

Hannila, S.S., Siddiq, M.M., Carmel, J.B., Hou, J., Chaudhry, N., Bradley, P.M.J., Hilaire, M., Richman, E.L., Hart, R.P., Filbin, M.T., 2013. Secretory Leukocyte Protease Inhibitor Reverses Inhibition by CNS Myelin, Promotes Regeneration in the Optic Nerve, and Suppresses Expression of the Transforming Growth Factor-Signaling Protein Smad2. J. Neurosci. 33, 5138–5151. https://doi.org/10.1523/JNEUROSCI.5321-12.2013

Harvey, A.R., Lovett, S.J., Majda, B.T., Yoon, J.H., Wheeler, L.P.G., Hodgetts, S.I., 2015. Neurotrophic factors for spinal cord repair: Which, where, how and when to apply, and for what period of time? Brain Res. 1619, 36–71. https://doi.org/10.1016/j.brainres.2014.10.049

He, H., Deng, K., Siddiq, M.M., Pyie, A., Mellado, W., Hannila, S.S., Filbin, M.T., 2016. Cyclic AMP and Polyamines Overcome Inhibition by Myelin-Associated Glycoprotein through eIF5A-Mediated Increases in p35 Expression and Activation of Cdk5. J. Neurosci. 36, 3079–3091. https://doi.org/10.1523/JNEUROSCI.4012-15.2016

Hernandez, T.D., Schallert, T., 1988. Seizures and recovery from experimental brain damage. Exp. Neurol. 102, 318–324. https://doi.org/10.1016/0014-4886(88)90226-9

Kim, D., Schallert, T., Yi Liu, Browarak, T., Nayeri, N., Tessler, A., Fischer, I., Murray, M., 2001. Transplantation of Genetically Modified Fibroblasts Expressing BDNF in Adult Rats with a Subtotal Hemisection Improves Specific Motor and Sensory Functions. Neurorehabil. Neural Repair 15, 141–150. https://doi.org/10.1177/154596830101500207

Krajacic, A., Ghosh, M., Puentes, R., Pearse, D.D., Fouad, K., 2009. Advantages of delaying the onset of rehabilitative reaching training in rats with incomplete spinal cord injury. Eur. J. Neurosci. 29, 641–651. https://doi.org/10.1111/j.1460-9568.2008.06600.x

Krajacic, A., Weishaupt, N., Girgis, J., Tetzlaff, W., Fouad, K., 2010. Training-induced plasticity in rats with cervical spinal cord injury: Effects and side effects. Behav. Brain Res. 214, 323–331. https://doi.org/10.1016/j.bbr.2010.05.053

Kunkel-Bagden, E., 1993. Methods to Assess the Development and Recovery of Locomotor Function after Spinal Cord Injury in Rats. Exp. Neurol. 119, 153–164. https://doi.org/10.1006/exnr.1993.1017

Kwon, B.K., Liu, J., Messerer, C., Kobayashi, N.R., McGraw, J., Oschipok, L., Tetzlaff, W., 2002. Survival and regeneration of rubrospinal neurons 1 year after spinal cord injury. Proc. Natl. Acad. Sci. 99, 3246–3251. https://doi.org/10.1073/pnas.052308899

Liang, Z., Ye, T., Zhou, X., Lai, K.-O., Fu, A.K.Y., Ip, N.Y., 2015. Cdk5 Regulates Activity-Dependent Gene Expression and Dendrite Development. J. Neurosci. 35, 15127–15134. https://doi.org/10.1523/JNEUROSCI.1443-15.2015

Lu, P., Yang, H., Jones, L.L., Filbin, M.T., Tuszynski, M.H., 2004. Combinatorial Therapy with Neurotrophins and cAMP Promotes Axonal Regeneration beyond Sites of Spinal Cord Injury. J. Neurosci. 24, 6402–6409. https://doi.org/10.1523/JNEUROSCI.1492-04.2004

Lu, Y., Belin, S., He, Z., 2014. Signaling regulations of neuronal regenerative ability. Curr. Opin. Neurobiol. 27, 135–142. https://doi.org/10.1016/j.conb.2014.03.007

Mackenzie, S.J., Houslay, M.D., 2000. Action of rolipram on specific PDE4 cAMP phosphodiesterase isoforms and on the phosphorylation of cAMP-response-element-binding protein (CREB) and p38 mitogen-activated protein (MAP)

kinase in U937 monocytic cells. Biochem. J. 347, 571–578. https://doi.org/10.1042/bj3470571

Massey, J.M., Hubscher, C.H., Wagoner, M.R., Decker, J.A., Amps, J., Silver, J., Onifer, S.M., 2006. Chondroitinase ABC Digestion of the Perineuronal Net Promotes Functional Collateral Sprouting in the Cuneate Nucleus after Cervical Spinal Cord Injury. J. Neurosci. 26, 4406–4414. https://doi.org/10.1523/JNEUROSCI.5467-05.2006

McKerracher, L., David, S., Jackson, D.L., Kottis, V., Dunn, R.J., Braun, P.E., 1994. Identification of myelin-associated glycoprotein as a major myelin-derived inhibitor of neurite growth. Neuron 13, 805–811. https://doi.org/10.1016/0896-6273(94)90247-X

Ming, G., Song, H., Berninger, B., Holt, C.E., Tessier-Lavigne, M., Poo, M., 1997. cAMP-Dependent Growth Cone Guidance by Netrin-1. Neuron 19, 1225–1235.

Monnier, P.P., Sierra, A., Schwab, J.M., Henke-Fahle, S., Mueller, B.K., 2003. The Rho/ROCK pathway mediates neurite growth-inhibitory activity associated with the chondroitin sulfate proteoglycans of the CNS glial scar. Mol. Cell. Neurosci. 22, 319–330. https://doi.org/10.1016/S1044-7431(02)00035-0

Morgenstern, D.A., Asher, R.A., Fawcett, J.W., 2002. Chondroitin sulphate proteoglycans in the CNS injury response. Prog. Brain Res. 137, 313–332.

Murray, A.J., 2008. Pharmacological PKA Inhibition: All May Not Be What It Seems. Sci. Signal. 1, re4–re4. https://doi.org/10.1126/scisignal.122re4

Murray, A.J., Shewan, D.A., 2008. Epac mediates cyclic AMP-dependent axon growth, guidance and regeneration. Mol. Cell. Neurosci. 38, 578–588. https://doi.org/10.1016/j.mcn.2008.05.006

Murray, A.J., Tucker, S.J., Shewan, D.A., 2009. cAMP-Dependent Axon Guidance Is Distinctly Regulated by Epac and Protein Kinase A. J. Neurosci. 29, 15434–15444. https://doi.org/10.1523/JNEUROSCI.3071-09.2009

Neumann, S., Bradke, F., Tessier-Lavigne, M., Basbaum, A.I., 2002. Regeneration of Sensory Axons within the Injured Spinal Cord Induced by Intraganglionic cAMP Elevation. Neuron 34, 885–893.

Neumann, S., Woolf, C.J., 1999. Regeneration of Dorsal Column Fibers into and beyond the Lesion Site following Adult Spinal Cord Injury. Neuron 23, 83–91. https://doi.org/10.1016/S0896-6273(00)80755-2

Park, K.K., Liu, K., Hu, Y., Smith, P.D., Wang, C., Cai, B., Xu, B., Connolly, L., Kramvis, I., Sahin, M., He, Z., 2008. Promoting Axon Regeneration in the Adult CNS by Modulation of the PTEN/mTOR Pathway. Science 322, 963–966. https://doi.org/10.1126/science.1161566

Peace, A.G., Shewan, D.A., 2011. New perspectives in cyclic AMP-mediated axon growth and guidance: The emerging epoch of Epac. Brain Res. Bull. 84, 280–288. https://doi.org/10.1016/j.brainresbull.2010.09.002

Pearse, D.D., Pereira, F.C., Marcillo, A.E., Bates, M.L., Berrocal, Y.A., Filbin, M.T., Bunge, M.B., 2004. cAMP and Schwann cells promote axonal growth and functional recovery after spinal cord injury. Nat. Med. 10, 610–616. https://doi.org/10.1038/nm1056

Plunet, W., Kwon, B.K., Tetzlaff, W., 2002. Promoting axonal regeneration in the central nervous system by enhancing the cell body response to axotomy. J. Neurosci. Res. 68, 1–6. https://doi.org/10.1002/jnr.10176

Qiu, J., Cai, D., Dai, H., McAtee, M., Hoffman, P.N., Bregman, B.S., Filbin, M.T., 2002. Spinal Axon Regeneration Induced by Elevation of Cyclic AMP. Neuron 34, 895–903.

Schneider, M.P., Schwab, M.E., Kessler, T.M., 2015. Anti-Nogo-A antibody: a treatment option for neurogenic lower urinary tract dysfunction? BJU Int. 115, 16–17. https://doi.org/10.1111/bju.13007

Schwab, M.E., Bartholdi, D., 1996. Degeneration and Regeneration of Axons in the Lesioned Spinal Cord. Physiol. Rev. 76, 319–370.

Siddiq, M.M., Hannila, S.S., Carmel, J.B., Bryson, J.B., Hou, J., Nikulina, E., Willis, M.R., Mellado, W., Richman, E.L., Hilaire, M., Hart, R.P., Filbin, M.T., 2015. Metallothionein-I/II Promotes Axonal Regeneration in the Central Nervous System. J. Biol. Chem. 290, 16343–16356. https://doi.org/10.1074/jbc.M114.630574

Silver, D.J., Silver, J., 2014. Contributions of chondroitin sulfate proteoglycans to neurodevelopment, injury, and cancer. Curr. Opin. Neurobiol. 27, 171–178. https://doi.org/10.1016/j.conb.2014.03.016

Taub, E., Wolf, S.L., 1997. Constraint Induced Movement Techniques To Facilitate Upper Extremity Use in Stroke Patients. Top. Stroke Rehabil. 3, 38–61. https://doi.org/10.1080/10749357.1997.11754128

Tom, V.J., Steinmetz, M.P., Miller, J.H., Doller, C.M., Silver, J., 2004. Studies on the Development and Behavior of the Dystrophic Growth Cone, the Hallmark of Regeneration Failure, in an In Vitro Model of the Glial Scar and after Spinal Cord Injury. J. Neurosci. 24, 6531–6539. https://doi.org/10.1523/JNEUROSCI.0994-04.2004

Udina, E., Furey, M., Busch, S., Silver, J., Gordon, T., Fouad, K., 2008. Electrical stimulation of intact peripheral sensory axons in rats promotes outgrowth of their central projections. Exp. Neurol. 210, 238–247. https://doi.org/10.1016/j.expneurol.2007.11.007

Wei, D., Hurd, C., Galleguillos, D., Singh, J., Fenrich, K.K., Webber, C., Sipione, S., Foaud, K., 2016. Inhibiting Cortical Protein Kinase A in Spinal Cord Injured Rats Enhances Efficacy of Rehabilitative Training. Exp. Neurol. 283, 365–374. https://doi.org/10.1016/j.expneurol.2016.07.001

Weishaupt, N., Blesch, A., Fouad, K., 2012. BDNF: The career of a multifaceted neurotrophin in spinal cord injury. Exp. Neurol. 238, 254–264. https://doi.org/10.1016/j.expneurol.2012.09.001

Widenfalk, J., Lundströmer, K., Jubran, M., Brené, S., Olson, L., 2001. Neurotrophic Factors and Receptors in the Immature and Adult Spinal Cord after Mechanical Injury or Kainic Acid. J. Neurosci. 21, 3457–3475.

Willand, M.P., Nguyen, M.-A., Borschel, G.H., Gordon, T., 2016. Electrical Stimulation to Promote Peripheral Nerve Regeneration. Neurorehabil. Neural Repair 30, 490–496. https://doi.org/10.1177/1545968315604399

Ye, T., Fu, A.K.Y., Ip, N.Y., 2012. Cyclin-Dependent Kinase 5 in Axon Growth and Regeneration, in: International Review of Neurobiology. Elsevier, pp. 91–115.

Chapter 4

Simultaneous activation of the cAMP downstream effectors PKA and EPAC Bolsters Neurite Outgrowth

Preface

Following the results presented in Chapter 2, I originally intended to follow up with an experiment utilizing Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) to selectively activate target neurons *in vivo* over an extended period using the selective agonist for these receptors, clozapine-N-oxide. Viral transfection would have allowed for targeted expression of these receptors in the primary motor cortex so that the same area stimulated in Chapter 2 with intracranial electrodes could be stimulated with an intraperitoneal agonist injection. In order to use these *in vivo*, I needed confirmation that I could get these to work in my hands and so I spent a semester doing a laboratory rotation with Dr. Sipione where I learned to plate primary neuronal cell cultures. The aim was to transfect cell cultures with viruses expressing the desired DREADDs and prove they could be activated with the agonist. Despite many attempts and two separate batches of DREADDs ordered, I was unable to successfully transfect DREADD receptors into cortical neurons as so the use of DREADDs *in vivo* was put on hold. The DREADDs infection experiments are documented in Appendix I.

Following my time in the laboratory of Dr. Sipione, I established a cell culture protocol in the Fouad Laboratory. We then wanted to investigate downstream effectors of cAMP in light of the paradoxical increases in functional recovery with PKA inhibition from the study by Wei et al. (2016) where PKA antagonist application to the motor cortex resulted in increased plasticity. I developed a protocol for measuring neurite growth and designed the following proof-of-concept experiment to guide future *in vivo* studies and explore potential mechanisms of cAMP mediated neurite growth.

4.1 Introduction

The adult mammalian central nervous system (CNS) has long been known to have a reduced capacity for growth and repair after injury compared to the peripheral nervous system (PNS). Over the last few decades, a vast amount of research has gone into the mechanisms of this reduction, covering a multitude of different signalling pathways. One proposed mechanism of this lack of regenerative potential is a natural attenuation in the signalling pathways involving cyclic AMP (cAMP) in the injured neurons (Cai et al., 2001; Dougherty et al., 2000; Fenrich and Gordon, 2004; Widenfalk et al., 2001).

cAMP is a ubiquitous secondary messenger molecule that is found throughout the body and in many different cell types including neurons. Its role in neurite growth has largely been driven by a focus on its downstream effector, protein kinase A (PKA), either through inhibition of PKA (Cai et al., 2001), elevation of cAMP levels via drug analogues (Neumann et al., 2002), or conditioning lesions (Neumann and Woolf, 1999; Qiu et al., 2002). In more recent years it has become apparent that there is in fact a second important downstream effector of cAMP that plays a similar role to PKA, the exchange protein activated by cAMP (EPAC) (Cheng et al., 2008; Murray and Shewan, 2008; Peace and Shewan, 2011; Quilliam et al., 2002). Research into the role of EPAC has suggested that some of the roles previously attributed to PKA are performed by EPAC, and EPAC may be more effective at neuronal growth promotion than PKA but requires higher cAMP concentrations to be activated (Cheng et al., 2008; Peace and Shewan, 2011).

Research focusing on the role of EPAC in CNS repair (or its lack thereof) has demonstrated a remarkable potential for EPAC targeting as a means to increase the length

of neurites *in vitro* (Enserink et al., 2002; Murray et al., 2009; Murray and Shewan, 2008). Furthermore, studies have also recently shown EPAC as a means to increase neurite extension following spinal cord injury (SCI) in *ex vivo* rat models (Guijarro-Belmar et al., 2019).

Studies investigating the potential for PKA induced neurite outgrowth to increase functional recovery after SCI (Wei et al., 2016) found that inhibition of PKA resulted in an increase in functional recovery and corticospinal tract plasticity, contradictory to older, established results (Cai et al., 2001). This study showed that PKA antagonism did not negatively impact CREB phosphorylation, and further examination suggests the role of EPAC is increased in the absence of PKA. This is hypothesized to be the result of cAMP moving towards EPAC in the absence of available PKA, something that has been demonstrated in HEK293 cells when PKA is inhibited via PKA-inhibitor proteins (Hoy et al., 2020).

The goal of the current study is to compare the effects of EPAC and PKA activation and antagonism *in vitro* to elucidate differences in the role and mechanisms underlying the growth promoting effects of these two pathways. Here we treat neurons with a combination of EPAC and PKA agonists and antagonists to compare their effects on neurite outgrowth. Finally we attempt to use siRNA to knockdown known GTPases downstream of EPAC, Rap1a and Rac1 (Birukova et al., 2010), to demonstrate that EPAC is working on downstream effectors beyond the established CREB phosphorylation/protein transduction pathway that PKA and EPAC have been reported to act on (Delghandi et al., 2005; Yang, 2018).

4.2 Materials and Methods

4.2.1 Materials

Reagents used for cell cultures include: Poly-L-lysine (Sigma-Aldrich, P5899), Neurobasal-A based growth media (Neurobasal-A: Gibco, 10888-022; B27 supplement, Gibco, 27504-044), papain (Worthington, No. 3119-26.4U/mgP), DNA (Sigma, DN-25), cytosine arabinoside (Sigma, 147-94-4), HBSS (Gibco, 14175-095).

Pharmacological reagents include: ESI-05 (Sigma-Aldrich, SML1907-5MG), 8-CPT-2`-Me-cAMP (Tocris, Cat. 1645), Rp-cAMPs (Tocris, Cat. 1337), 6-BnZ-cAMP (Tocris, Cat. 5255)

siRNA treatments were purchased from Dharmacon, Inc. and include: ON-TARGETplus Rat Rac1 siRNA SMARTpool (363875), ON-TARGETplus Rat Rap1a siRNA SMARTpool (295347), and DharmaFECT transfection reagent.

Immunocytochemistry antibodies used were anti-microtubule-associated protein 2 (Millipore, AB5622) anti-glial fibrillary acidic protein clone GA5 (Millipore, MAB360), anti-Rac1 (Abcam, ab33186) anti-Rap1a (Abcam, ab197673), and DAPI (Thermofisher, D1306).

4.2.2 Animals

P0-P1 Sprague-Dawley rat pups (Charles River Laboratories, Canada) were used for all experiments. For each cell culture prep, pregnant dams were housed with a 12-h light/dark cycle. Water and food were provided ad libitum. Once the litter was born, pups

were removed from their mother and processed for cell cultures. Total number of pups used in this experiment was 27 over 6 culture preparations.

4.2.3 Cell Culture Plating

Cell cultures were plated in 24-well plates on poly-L-lysine coated glass coverslips. Coverslips were sterilized and treated with poly-L-lysine for 24 hours prior to cell plating.

Cortical tissue was extracted from rat pups via decapitation and opening of the scalp from the magnum foramen to the middle of the eyes. The brain was extracted and placed in a dissection buffer. At this time, the meninges were removed from the brain, the olfactory bulb and cerebellum were removed, and the cortex was separated from midbrain structures. Isolated cortices were placed in a 50 mL falcon tube with neurobasal-A based growth media until all tissue was extracted.

Tissue was then transferred to a clean falcon tube and the media removed. Cortices were re-suspended in a papain solution for 10 minutes before DNase was added for an additional 5 minutes. Tissue was centrifuged at 200g for 1 minute and the supernatant removed. Tissue was repeatedly triturated in growth media using a fire-polished Pasteur pipette. Supernatant containing single cells was collected into a separate tube and then passed through a 40 μm mesh. Cells were then counted using trypan blue dye and a hemocytometer to determine the number of cells per millilitre of media for optimal plating density.

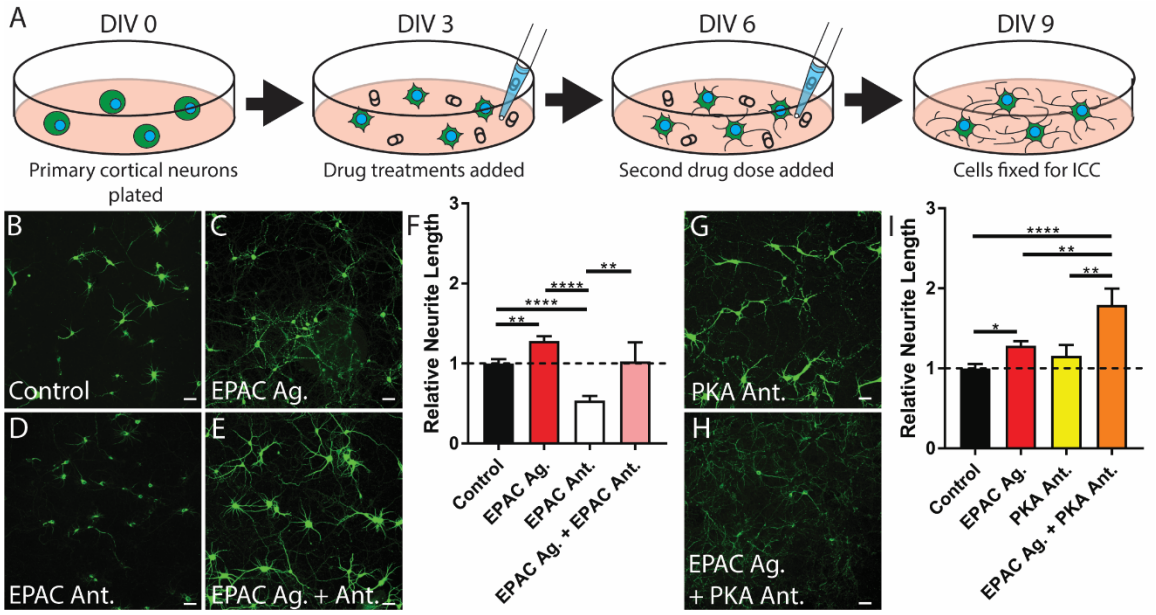

Figure 4.1 – EPAC Activation and PKA Antagonism Increase Neurite Extension *in vitro*

(A) A schematic demonstrating on which day in vitro (DIV) various treatments were added to the cells. Untreated control cells (n=27) (B) are compared to cells that received the EPAC agonist, 8-CPT-2`-Me-cAMP (n=24) (C), the EPAC antagonist, ESI-05 (n=17) (D), and cells that received both drugs (n=6) (E). Neurite lengths for these groups are quantified in (F). Effects of EPAC activation are compared to the effects of PKA antagonism, comparing untreated controls (n=27) to Epac activation (n=24), PKA antagonism, Rp-cAMPs (n=10) (G), and a group receiving both EPAC activation and PKA antagonism (n=15) (H). This data is quantified and compared in (I) showing a marked increase in extension when EPAC activation is applied alongside PKA antagonism. Error bars show standard error. Scale bar represents 50 µm. P-value asterisks: *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$, ****: $p \leq 0.0001$.

Cells were plated at $1.5x10^5$ cells per well with 1 mL of growth media. Media was completely removed and replaced the following day. On day 2 after plating, cytosine arabinoside was added, and media was fully changed on day 3. Following this, media was changed every 3 days with half of the replaced media being new fresh media and half being old media passed through a steriflip (Millipore) apparatus.

4.2.4 Drug Treatments

Drug treatments were added directly to the media in the plates following media changes on day *in vitro* (DIV) 3 and DIV 6 (Fig. 4.1A). Drugs used in this experiment include: EPAC agonist 8-CPT-2`-Me-cAMP (10 µM), EPAC antagonist ESI-05 (1 µM), PKA antagonist Rp-cAMPs (10 µM), PKA agonist 6-BnZ-cAMP (100 µM). Group n values are all greater than 10 with exception of the 8-CPT and ESI-05 group (n=6) and the Rp-cAMPs and 6-BnZ-cAMP group (n=3), individual n values listed in figure legends.

4.2.5 siRNA Treatments

siRNA treatments were initially added to cell cultures following the provided protocol on DIV 1 following full media change to allow 48 hours for protein knock down prior to drug treatments starting on DIV 3. A second siRNA treatment was added following media change on DIV 3 to ensure continued knock down while drug treatments were present (Fig. 4.4A).

siRNA was added alongside DharmaFECT transfection agent to increase chances of successful transfection. Group n values for siRNA analyses are all greater than 4, with individual values listed in figure legends.

4.2.6 Cell Fixing and Immunocytochemistry

To visualize neurons and GTPases, immunocytochemical staining was performed. On DIV 9, cultures were washed twice with 37° $HBSS^{++}$ and then fixed with cold 4% paraformalin (Sigma-Aldrich, 30525-89-4) solution for 15 minutes. Cells were then permeabilized in PBS_{TX} (0.1% solution) for five minutes and washed four times with PBS. Cultures were then incubated with 4% normal goat serum (NGS; Vector, S1000) in PBS for one hour at room temperature before being incubated with primary antibodies in 4% NGS in PBS for another hour at room temperature. Cells were washed three times with PBS and then incubated for an hour with secondary bodies in the same 4% NGS solution. Cells were washed with 1:5000 DAPI in PBS for five minutes followed by two more washes of PBS. Cover slips were mounted onto slides using prolong gold (Thermofisher).

4.2.7 Confocal Imaging and Analyses

Slides with mounted coverslips were imaged using confocal microscopy for import into ImageJ. Cultures showing greater than 10% astrocyte population based on GFAP staining were not used for final analyses. Total number of cell cultures used for

analysis in this study was 209 individual primary cortical cultures, n values per group can be found in figure legends.

Analysis of neurite length and branch density was performed using ImageJ (Rueden et al., 2017) plugin NeurphologyJ (Ho et al., 2011). Branch density was calculated by dividing the number of neurite end points measured by the number of cell body attachment points (i.e. the number of primary neurites extending from the soma).

4.2.8 Statistical Analyses

Statistical analyses for all assessments were conducted using GraphPad Prism version 7 (GraphPad Software). Normality was assessed using D'Agostino-Pearson omnibus test. Neurite length data was analyzed using multiple comparisons one-way ANOVA, and data from branch density measures were analyzed using unpaired t-tests.

4.3 Results

4.3.1 EPAC Activation Increases Neurite Outgrowth and PKA Inhibition Benefits Neurite Extension

The Fouad laboratory have previously argued that PKA inhibition may lead to an increase in neurite outgrowth by pushing cAMP towards EPAC (Wei et al., 2016). We expand upon this in the current study by treating primary cortical neurons with the EPAC agonist 8-CPT-2`-Me-cAMP (8-CPT) and comparing average neurite length to that of untreated control cells. Cultures show that treatment with the EPAC agonist resulted in neurites with a 1.28-fold (± 0.29, $p = 0.006$) increase in length (Fig. 4.1C and F). This

change was confirmed to be due to the effects of EPAC using the selective EPAC antagonist, ESI-05, which completely negated the effect of the agonist 8-CPT (Fig. 4.1E; 1.02 ± 0.53, $p = 0.998$, Fig. 4.1F). On its own, ESI-05 (without the agonists present, Fig. 4.1D) reduced relative neurite length to 0.54 (± 0.24, $p < 0.0001$, Fig. 4.1F).

In contrast to previous *in vivo* results (Wei et al., 2016), when primary cortical cultures were treated with the PKA antagonist Rp-cAMPs, only a statistically insignificant increase in neurite length was found (1.16 ± 0.36, $p = 0.670$, Fig. 4.1G and I). Interestingly, Rp-cAMPs treatment alongside 8-CPT led to a profound increase in neurite length, resulting in a 1.79 fold increase in length (± 0.54, $p < 0.0001$ versus controls, Fig. 4.1H and I). This increase in length is larger than both 8-CPT treated cells (1.28 ± 0.29, $p = 0.0028$), and the expected additive effect of the 8-CPT and Rp-cAMPs combined.

These results indicate that EPAC activation increases the growth capacity of neurons, and despite not directly increasing neurite outgrowth on its own, PKA appears to compliment EPAC to enhance neurite growth.

4.3.2 PKA Activation and Antagonism have Similar Effects on Neurite Extension

Given our seemingly paradoxical results that PKA antagonism leads to an increase in neurite length *in vivo* (Wei et al., 2016) and the suggestion that PKA inhibition benefits neurite outgrowth seen in the current study, we compared the effects of PKA activation and antagonism in neuron cultures. Application of the PKA agonist, 6-BnZ-cAMP, to primary cortical cells (Fig. 4.2B) showed a non-significant trend towards

an increase in neurite length (1.24 fold increase over controls ± 0.46; $p = 0.2491$, Fig. 4.2E). It is worth noting that this result is not statistically different from cells treated with the PKA antagonist, Rp-cAMPs ($p = 0.949$), despite eliciting entirely opposite effects on PKA (Fig. 4.2C).

4.3.3 Simultaneous Activation of EPAC and PKA Results in Increases in Neurite Length

Given the complementary effect of combining EPAC activation and PKA antagonism, the cause of this unexplained growth warranted investigation. This led us to test the effects of activating both EPAC and PKA in the same culture. Simultaneous activation of EPAC and PKA resulted in two very evident changes: firstly, a 2.02-fold increase in neurite length (± 0.72) over controls ($p < 0.0001$), and second, a striking increase in neurite diameter and a visual change neuron morphology (Fig. 4.2D, best representative image). The increase in neurite length seen in the dual agonist group (2.02 relative neurite length) is far more than the expected additive effects of EPAC and PKA activation individually (1.28 and 1.24 relative neurite length, respectively). The dual agonist group differed from the individual EPAC and PKA activation groups as well, each with the same level of significance ($p < 0.0001$) (Fig. 4.2F).

4.3.4 Dual Agonism and EPAC-Focused Treatment Have Similar Effects on Neurite Extension Despite Visual Differences

EPAC activation with either PKA antagonism (EPAC-focused group, Fig. 4.1H) and PKA activation (dual agonist group, Fig. 4.2D) results in pronounced increases in

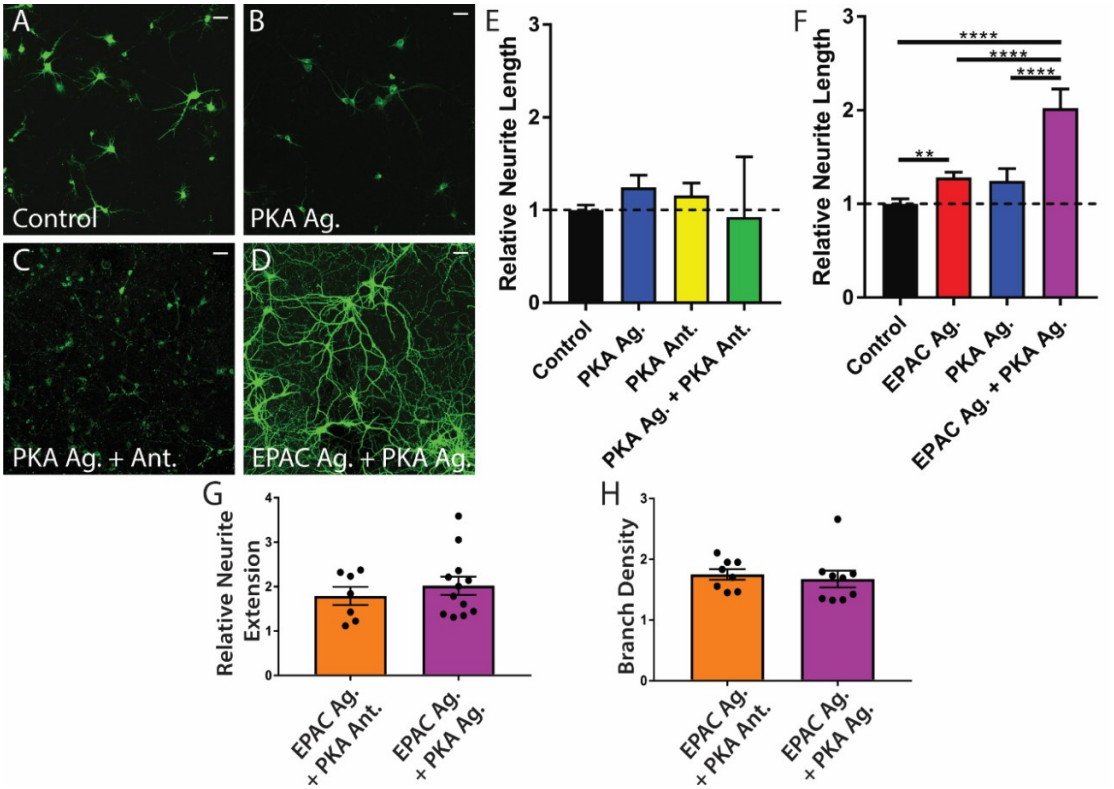

Figure 4.2 – Effects of PKA Activation on Neurite Extension

To better understand how PKA plays a role in neurite extension compared to EPAC, a PKA agonist, 6-BnZ-cAMP, was used to compared activation and antagonism of PKA. Untreated control cells (n=27) (A) are compared to 6-BnZ-cAMP treated cells (n=12) (B), Rp-cAMPs treated cells (n=10), and cells that were treated with both agonist and antagonist as a control measure (n=3) (C). Differences between these groups are quantified in (E). To further examine the effects of PKA activation in conjunction with EPAC, untreated cells (n=27) were compared to EPAC agonist treated cells (n=24), PKA agonist treated cells (n=12), and cells treated with both agonists (n=15) (D, best representative image). Quantification of the differences between groups (F) demonstrates that dual activation results in a marked increase in neurite extension. Comparing the relative neurite extension between the cells that received both agonists as shown in (D) and cells that received the EPAC agonist and PKA Antagonist (figure 1), reveal that both groups have increased neurite extension to a similar degree (G) though it is possible that the extension seen is due to fasciculation of multiple neurites. Branch density calculation demonstrates that both groups have a similar number of branches formed (H). Error bars show standard error. Scale bars represent 50 µm.

neurite length compared to untreated controls, but show no difference in length when compared to each other (Fig. 4.2G, $p = 0.4761$). Despite this, these two groups exhibit clear visual differences in other aspects of cellular morphology.

To determine if these treatments affected the number of branches formed along neurites, the branch density was also calculated by dividing the number of neurite end points measured by the number of soma attachment points. The higher this number is, the more branches are formed along the neurites. Determination of branch density (Fig. 4.2H) however showed no significant differences between the EPAC-focused group (1.75 ± 0.247) and the dual agonist group (1.68 ± 0.418; $p>0.9999$). Due to the high density of these cultures, accurate determination of neurite thickness and branch angles were unable to be determined as it is impossible to ascertain whether it is due to changes in neurite morphology or the fasciculation of overlapping neurites that result in these changes. Visually comparing the dual agonist group (Fig 4.2D) to the EPAC focused group (Fig. 4.1H) demonstrates what appears to be a higher cell density and thicker neurites in the dual agonist group and other potential changes in morphology that would warrant further experimentation.

These findings suggest that while both the EPAC-focused treatment and dual agonist treatment promote the outgrowth of neurites, there is a key difference with activating both cAMP effectors (i.e., PKA and EPAC) that potentially results in thicker (or more bundled), potentially more developed neurites (Wu et al., 2015), as opposed to simply increasing in length.

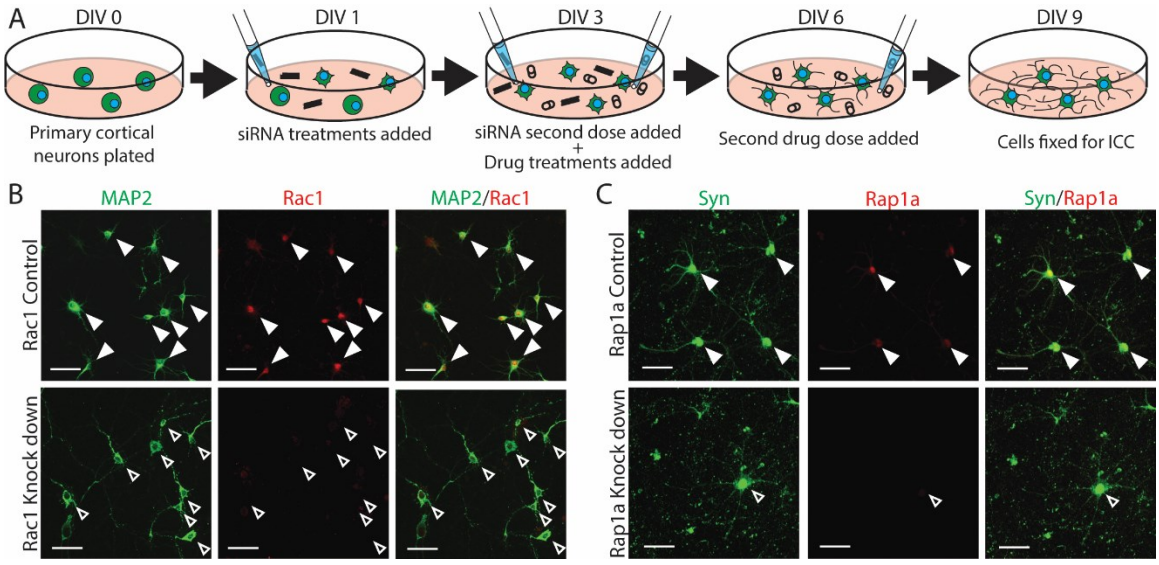

Figure 4.3 – Confirmation of siRNA Efficacy

(A) Schematic demonstrating the timeline of siRNA and drug treatments on primary cortical cultures used for this experiment. Confirmation of knockdown of Rac1 protein using immunocytochemistry (B) is demonstrated by the co-localization of Rac1 staining (denoted with filled arrows) with MAP2 staining in the Rac1 control cells in the top row, and the lack of Rac1 staining in the bottom row (hollow arrows). Confirmation of Rap1a knockdown (C) is demonstrated via positive staining of Rap1a (filled arrows) alongside synaptophysin staining in the Rap1a control cells (top row) as opposed to the lack of Rap1a staining (hollow arrows) in the siRNA treated cells in the bottom row. Scale bars represent 50 µm.

4.3.5 Use of siRNA to Differentiate EPAC and PKA Mechanisms of Action

4.3.5.1 Confirmation of siRNA Efficacy

To better differentiate the mechanisms of action of EPAC and PKA, we attempted to take a further look at known downstream GTPases of EPAC, (i.e., Rac1 and Rap1; (Birukova et al., 2010)), to determine how these directly impact the growth promoting effects of EPAC and PKA. It is also important to verify where overlap of these pathways may occur so as to better understand how we may target these pathways with future treatments. Knockdown of Rac1 and Rap1a using siRNA in conjunction with the EPAC-focused treatment or the dual agonist treatment would help elucidate where along these pathways PKA and EPAC activation differ to better understand their roles. Prior to this knockdown however, confirmation of the efficacy of siRNA is required.

Successful knockdown of Rac1 and Rap1a was confirmed via immunocytochemistry. Comparison of cells treated with Rac1 siRNA and untreated controls was performed under confocal microscopy by comparing colocalization of staining of MAP2 (for neurons) and Rac1 staining. Untreated controls demonstrated clear expression of Rac1 whereas cells that were treated with siRNA for Rac1 showed no expression (Fig. 4.3B). The process was repeated for Rap1a siRNA treated cells by staining for Rap1a. Untreated controls showed clear Rap1a expression whilst cells treated with Rap1a siRNA did not (Fig. 4.3C). This demonstrates that the siRNA knockdown of these GTPases was successful and could be used alongside pharmacological activation to better differentiate between PKA and EPAC activation.

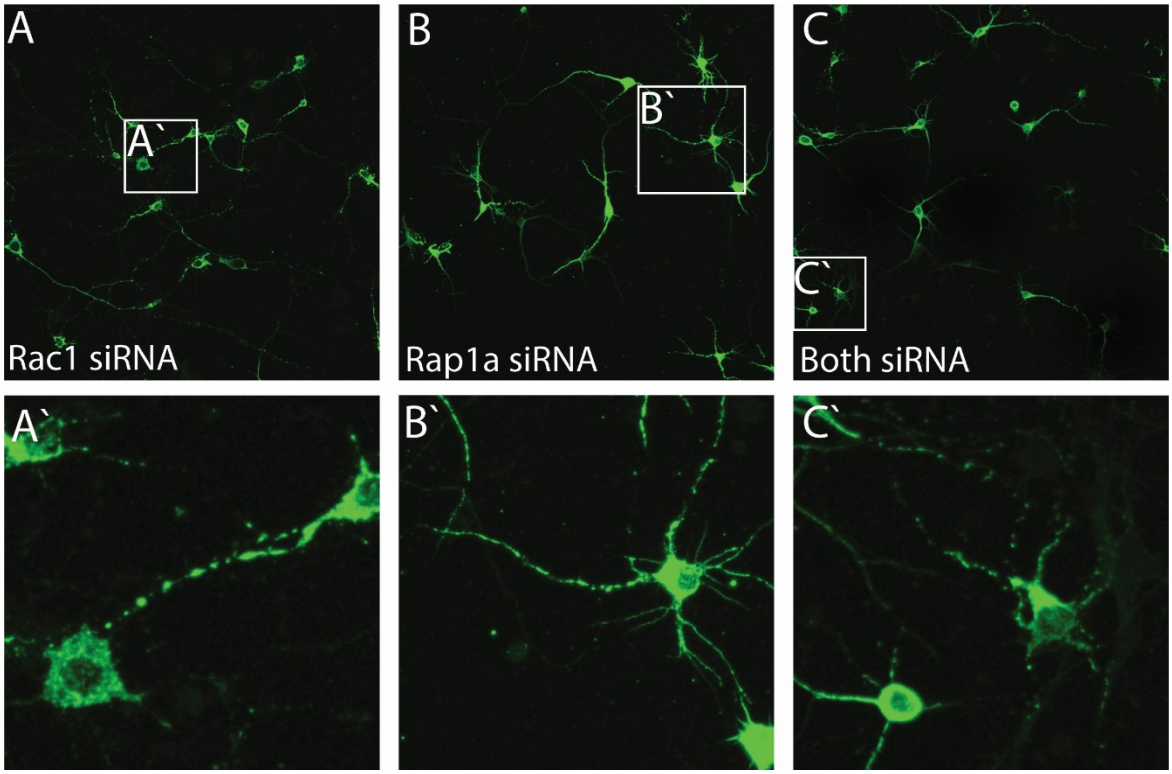

Figure 4.4 – siRNA Treatment to Knockdown Downstream GTPases Effected Neuron Health Resulting in Beading

Example images of each siRNA treatment targeting either Rac1, Rap1a, or both siRNA together. As highlighted by each example image in the insets, siRNA treatment resulted in extensive neurite beading in all siRNA treatment cases. Due to the effect on neurite health and extensive toxicity, further analysis of neurite length was unable to be performed.

4.3.5.2 siRNA Knockdown Resulted in Toxicity to Cells

Despite confirmation of knockdown of the intended GTPases via immunocytochemistry, use of the siRNA as a means to target downstream components of the cAMP signalling cascade and measure the impact on neurite extension resulted in neurite beading and a reduction in overall neuronal health. Due of extensive toxicity, neuronal measurements could not reliably be performed in these experiments. Future studies will be required to optimize siRNA design, dose, and delivery methods to minimize toxicity and reliably knockdown the intended GTPase targets to determine their effect on neurite extension.

4.4 Discussion

In this study we have demonstrated that simultaneous activation of cAMP's downstream effectors PKA and EPAC results in a profound increase in neurite outgrowth that extends beyond the changes seen from activation of either one. This effect may be due to the actions of multiple pathways beyond the established CREB phosphorylation pathway (Delghandi et al., 2005; Yang, 2018), including but not limited to the Rac1 and Rap1a GTPases, with EPAC and PKA each playing separate but complementary roles. This complementary effect of EPAC activation and PKA activation has not been previously reported in cortical neurons, though it has been hypothesized to occur (Cheng et al., 2008) and a synergistic effect benefitting neurite length has been demonstrated in a PC12 cell line (Christensen et al., 2003). Presently, many studies target either EPAC or PKA (cf: Guijarro-Belmar et al., 2019; Murray and Shewan, 2008), but our data suggests that the ideal treatment may involve targeting of both effectors in order to increase

neurite growth through the cAMP pathway. Potential applications of this complementary effect *in vivo* should be further validated and tested to determine optimal dosing, route of administration, and whether the cell needs to be treated at the cell body or along the neurites.

Following injury to the adult CNS, there is a limited capacity for growth and repair, partly due to the changes of intrinsic properties of the neurons themselves (Cai et al., 2001; Dougherty et al., 2000; Fenrich and Gordon, 2004; Widenfalk et al., 2001), as well as the post-injury decline in cAMP that has been previously described (Krajacic et al., 2009; Pearse et al., 2004). Compared to the PNS, there is a marked decrease in cAMP levels and signalling in the post-developmental CNS (Gao et al., 2003). One of the means by which the PNS is better able to repair itself following injury is the transient local upregulation of neurotrophins around the site of injury (Dougherty et al., 2000), which signal through TRK receptors to increase cAMP levels (Gao et al., 2003). This effect is much less prevalent in the CNS, hindering its overall potential to overcome the growth inhibitory environment present (Dougherty et al., 2000; Fenrich and Gordon, 2004; Widenfalk et al., 2001). As such, targeting the downstream effectors of cAMP serves as a viable pharmacological means to increase recovery and has been the focus of much research in recent years (cf: Batty et al., 2017; Murray et al., 2009; Murray and Shewan, 2008; Neumann et al., 2002; Pearse et al., 2004; Qiu et al., 2002; Wei et al., 2016).

While studies using targeted activation of cAMP or its downstream effectors is already underway *in vivo* (Guijarro-Belmar et al., 2019; Neumann et al., 2002; Pearse et al., 2004; Qiu et al., 2002; Wei et al., 2016), many of the mechanisms of action have thus far remained elusive. Recent work has shed new light on the role of PKA in neurite

outgrowth and suggests that EPAC may play a prominent role alongside its better understood counterpart, PKA (Cheng et al., 2008; Enserink et al., 2002; Murray, 2008; Murray and Shewan, 2008; Wei et al., 2016). It has been suggested that PKA antagonism results in an increase in neurite outgrowth by allowing any intracellular cAMP to be used by EPAC, which requires a higher concentration of cAMP than PKA to be activated (Cheng et al., 2008; Peace and Shewan, 2011).

The current study supports our previous findings that PKA inhibition increases neurite growth (Wei et al., 2016), despite earlier work suggesting otherwise (Cai et al., 2001; Ming et al., 1997). This discrepancy could be due, in part, to the PKA inhibitors that have commonly been used in the past, such as KT5720 and H89, which have been shown in recent years to not be nearly as specific for PKA as first thought, and in fact inhibit EPAC as well (Murray, 2008). Based on previous results that PKA inhibition elevates neurite growth (Wei et al., 2016) and on the notion that PKA inhibition sends cAMP over to EPAC, we tested an EPAC-focused treatment, in which PKA inhibition was combined with EPAC activation, and hypothesized a resulting ceiling effect due to saturation of the EPAC pathway. Instead, this EPAC-focused treatment resulted in increases in neurite length beyond the expected additive values of PKA inhibition or EPAC activation alone (Fig. 4.1I). However, it is important to consider the possibility that the lack of a ceiling effect could have been merely due to insufficient activation of the pathway at the dose used. Regardless, these results warranted further investigation into its mechanism of action and so we compared the effects of PKA activation and antagonism on neurite outgrowth. Interestingly, PKA activation alone was indistinguishable from PKA antagonism (Fig. 4.2E).

One potential cause for the effects of PKA agonism and antagonism being indistinguishable from each other in terms of neurite outgrowth may be due to the effects of pharmacological competition. Since both the agonist and antagonist of PKA used are cAMP analogs they would bind to the same binding site as cAMP, outcompeting cAMP. As such the lack of available binding sites on PKA for cAMP, may be sending cAMP towards EPAC, as proposed by Wei et al. (2016). Its possible that the small effect being seen in both the PKA agonist and antagonist groups is caused by a small amount of EPAC activation that would occur with the endogenous cAMP being unable to bind to PKA.

When a PKA agonist was combined with an EPAC agonist, there is a similar increase in neurite length as was demonstrated in the EPAC-focused treatment. This supports the idea that the PKA agonist and antagonist used are both serving to push cAMP toward EPAC due to competitive binding. This increased activation of EPAC from both the EPAC agonist and endogenous cAMP is the most likely reason for the similar increases in neurite length seen in both treatments. With that in mind, there did appear to be a visual difference between the two groups that warranted further investigation but could not be accurately quantified in this experiment due to the high density of cultures (such as in Fig. 2D) and resulting fasciculation making individual neurite measures impossible, and even likely influencing the neurite extension measurements performed. It is worth noting that in the dual agonist treated cells there appears to be a higher density of cell bodies (suggesting a potential effect on cell survival) and thicker neurites (or more bundled neurites due to fasciculation and increased cell density). This suggests benefits of simultaneous PKA and EPAC activation

that could not be quantified here but should be followed up with in future experiments. While in this experiment, the primary effect on neurite outgrowth is most likely due to the actions of EPAC, it is probable that the apparent visual differences present between the dual agonist group and the EPAC-focused group may be due to the PKA activation in the dual agonist group.

Following our finding that PKA and EPAC activation have a complementary effect on neuronal growth, we attempted to used siRNA to inhibit GTPases that have been implicated in the downstream effects of EPAC, Rac1 (Thies and Davenport, 2003) and Rap1a (Wu et al., 2015). The goal was to highlight that these signalling pathways produce the most profound effects in concert and that EPAC works through a more complex pathway than previously established. Unfortunately use of the siRNA resulted in a toxic effect on the neurons (as seen in the beading in figure 4) and so reliable measurements could not be obtained as the impact on neuron health would have a major impact on their growth. Future experiments will need to be performed to optimize the siRNA design, dose, and delivery to minimize toxicity and ensure optimal conditions for the neurons.

Based on the literature (Thies and Davenport, 2003), it was hypothesized that use of a Rac1 siRNA would result in a significant decline in neurite extension when combined with an EPAC agonist and compared to EPAC agonist treated control cells. Given the implications of Rap1a in neuronal differentiation (Wu et al., 2015), it was expected that there would be a lesser impact of Rap1a knockdown on the effects of EPAC activation compared to Rac1 knockdown. The effects of Rap1a knockdown were expected to be more evident when combined with EPAC agonist and PKA agonist

together; PKA has been shown to phosphorylate Rap1a (Bokoch, 1993) and have an impact on its downstream effects (Hu et al., 1999). It was expected that cells receiving the dual agonist treatment with Rap1a knocked down would demonstrate a lesser increase in neurite length and have a morphology like the EPAC agonist treated control cells as opposed to the fuller appearing dual agonist treated cells. This hypothesis was based on the fact that there would still be the growth promoting effects of EPAC through CREB phosphorylation and Rac1, but the effects of Rap1a on neuronal polarization would not occur, effectively eliminating a major contribution of PKA in this instance. Following optimization of the siRNA treatment as discussed above, testing of these hypotheses should follow soon after.

Despite the findings of this study, there are careful considerations that must be made prior to the use of EPAC and PKA agonists *in vivo*. It has been previously suggested (Murray et al., 2009) that targeted application of these pharmacological agents to the relevant structures and cell types must first be considered. Given the ubiquity of cAMP and its functions in the body, widespread upregulation of cAMP in non-targeted bodily systems is likely to cause undesirable effects in other areas. The targeted activation of downstream effectors allows for a more reliable activation of these relevant growth factors, and perhaps even suggests that the ideal target(s) lie further downstream of PKA and EPAC.

4.4.1 Technical Challenges and Translational Considerations

As previous findings have reported that EPAC is directly involved in neuronal polarization (Munoz-Llancao et al., 2015; Wu et al., 2015), it would be interesting to see

how combined EPAC and PKA agonist treatments may effect neuronal polarization. Due to technical limitations, polarization analysis in the current study was unable to be performed. However given the implication of Rap1a in neuronal polarization (Wu et al., 2015) and the fact that PKA has been shown to phosphorylate Rap1a (Bokoch, 1993), it would be reasonable to expect that the dual agonist treatment is likely causing an increase in neuronal polarization, resulting in some of the morphological differences observed. As such a full cellular polarization analysis would benefit the findings of this study immensely.

As was previously discussed, a higher density of cells resulted in neuronal fasciculation and eliminated the ability to measure certain aspects of cellular morphology reliably. A lower density of cells in future repetitions or short term cultures to allow for more reliable individual cell analysis would substantiate the findings discussed above.

The use of immunocytochemistry to check for protein knockdown of the targeted GTPases may have been sufficient for this initial proof of concept study, however future experiments aiming to repeat and improve upon these findings should confirm siRNA knockdown through PCR analysis..

As was mentioned above, *in vivo* applications of this complementary effect should be further tested to determine appropriate dosing, how it should be administered, and whether the treatment needs to be targeted at the cell body or along the ends of neurites.

4.5 Conclusion

Data from this study demonstrates a complementary effect on neuronal growth in primary cortical neurons when agonists for both PKA and EPAC are administered simultaneously. This supports previous hypotheses from the literature. Our research suggests that simultaneous activation of EPAC and PKA may be a viable treatment *in vivo* following CNS injuries, such as SCI, given the previously unreported effect seen when both are upregulated.

Considering how widespread cAMP is found in the body, targeted activation of its downstream effects in focused locations stands out as the most viable option for *in vivo* experimentation to avoid potential off target effects.

4.6 Acknowledgements

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4.7 References

Batty, N.J., Fenrich, K.K., Fouad, K., 2017. The role of cAMP and its downstream targets in neurite growth in the adult nervous system. Neurosci. Lett. 652, 56–63. https://doi.org/10.1016/j.neulet.2016.12.033

Birukova, A.A., Burdette, D., Moldobaeva, N., Xing, J., Fu, P., Birukov, K.G., 2010. Rac GTPase is a hub for protein kinase A and Epac signaling in endothelial barrier protection by cAMP. Microvasc. Res. 79, 128–138. https://doi.org/10.1016/j.mvr.2009.11.007

Bokoch, G.M., 1993. Biology of the Rap proteins, members of the ras superfamily of GTP-binding proteins. Biochem. J. 289, 17–24. https://doi.org/10.1042/bj2890017

Cai, D., Qiu, J., Cao, Z., McAtee, M., Bregman, B.S., Filbin, M.T., 2001. Neuronal Cyclic AMP Controls the Developmental Loss in Ability of Axons to Regenerate. J. Neurosci. 21, 4731–4739.

Cheng, X., Ji, Z., Tsalkova, T., Mei, F., 2008. Epac and PKA: a tale of two intracellular cAMP receptors. Acta Biochim. Biophys. Sin. 40, 651–662. https://doi.org/10.1111/j.1745-7270.2008.00438.x

Christensen, A.E., Selheim, F., de Rooij, J., Dremier, S., Schwede, F., Dao, K.K., Martinez, A., Maenhaut, C., Bos, J.L., Genieser, H.-G., Døskeland, S.O., 2003. cAMP Analog Mapping of Epac1 and cAMP Kinase: DISCRIMINATING ANALOGS DEMONSTRATE THAT Epac AND cAMP KINASE ACT SYNERGISTICALLY TO PROMOTE PC-12 CELL NEURITE EXTENSION. J. Biol. Chem. 278, 35394–35402. https://doi.org/10.1074/jbc.M302179200

Delghandi, M.P., Johannessen, M., Moens, U., 2005. The cAMP signalling pathway activates CREB through PKA, p38 and MSK1 in NIH 3T3 cells. Cell. Signal. 17, 1343–1351. https://doi.org/10.1016/j.cellsig.2005.02.003

Dougherty, K.D., Dreyfus, C.F., Black, I.B., 2000. Brain-Derived Neurotrophic Factor in Astrocytes, Oligodendrocytes, and Microglia/Macrophages after Spinal Cord Injury. Neurobiol. Dis. 7, 574–585. https://doi.org/10.1006/nbdi.2000.0318

Enserink, J.M., Christensen, A.E., de Rooij, J., van Triest, M., Schwede, F., Genieser, H.G., Døskeland, S.O., Blank, J.L., Bos, J.L., 2002. A novel Epac-specific cAMP analogue demonstrates independent regulation of Rap1 and ERK. Nat. Cell Biol. 4, 901–906. https://doi.org/10.1038/ncb874

Fenrich, K., Gordon, T., 2004. Canadian Association of Neuroscience Review: Axonal Regeneration in the Peripheral and Central Nervous Systems – Current Issues and Advances. Can. J. Neurol. Sci. 31, 142–156. https://doi.org/10.1017/S0317167100053798

Gao, Y., Nikulina, E., Mellado, W., Filbin, M.T., 2003. Neurotrophins Elevate cAMP to Reach a Threshold Required to Overcome Inhibition by MAG through Extracellular Signal-Regulated Kinase-Dependent Inhibition of Phosphodiesterase. J. Neurosci. 23, 11770–11777.

Guijarro-Belmar, A., Viskontas, M., Wei, Y., Bo, X., Shewan, D., Huang, W., 2019. Epac2 Elevation Reverses Inhibition by Chondroitin Sulfate Proteoglycans *In Vitro* and Transforms Postlesion Inhibitory Environment to Promote Axonal Outgrowth in an *Ex Vivo* Model of Spinal Cord Injury. J. Neurosci. 39, 8330–8346. https://doi.org/10.1523/JNEUROSCI.0374-19.2019

Ho, S.-Y., Chao, C.-Y., Huang, H.-L., Chiu, T.-W., Charoenkwan, P., Hwang, E., 2011. NeurphologyJ: An automatic neuronal morphology quantification method and its application in pharmacological discovery. BMC Bioinformatics 12, 230. https://doi.org/10.1186/1471-2105-12-230

Hoy, J.J., Salinas Parra, N., Park, J., Kuhn, S., Iglesias-Bartolome, R., 2020. Protein kinase A inhibitor proteins (PKIs) divert GPCR-Gαs-cAMP signaling toward EPAC and ERK activation and are involved in tumor growth. FASEB J. 34, 13900–13917. https://doi.org/10.1096/fj.202001515R

Hu, C.-D., Kariya, K., Okada, T., Qi, X., Song, C., Kataoka, T., 1999. Effect of Phosphorylation on Activities of Rap1A to Interact with Raf-1 and to Suppress Ras-dependent Raf-1 Activation. J. Biol. Chem. 274, 48–51. https://doi.org/10.1074/jbc.274.1.48

Krajacic, A., Ghosh, M., Puentes, R., Pearse, D.D., Fouad, K., 2009. Advantages of delaying the onset of rehabilitative reaching training in rats with incomplete spinal cord injury. Eur. J. Neurosci. 29, 641–651. https://doi.org/10.1111/j.1460-9568.2008.06600.x

Ming, G., Song, H., Berninger, B., Holt, C.E., Tessier-Lavigne, M., Poo, M., 1997. cAMP-Dependent Growth Cone Guidance by Netrin-1. Neuron 19, 1225–1235.

Munoz-Llancao, P., Henriquez, D.R., Wilson, C., Bodaleo, F., Boddeke, E.W., Lezoualc'h, F., Schmidt, M., Gonzalez-Billault, C., 2015. Exchange Protein Directly Activated by cAMP (EPAC) Regulates Neuronal Polarization through Rap1B. J. Neurosci. 35, 11315–11329. https://doi.org/10.1523/JNEUROSCI.3645-14.2015

Murray, A.J., 2008. Pharmacological PKA Inhibition: All May Not Be What It Seems. Sci. Signal. 1, re4–re4. https://doi.org/10.1126/scisignal.122re4

Murray, A.J., Shewan, D.A., 2008. Epac mediates cyclic AMP-dependent axon growth, guidance and regeneration. Mol. Cell. Neurosci. 38, 578–588. https://doi.org/10.1016/j.mcn.2008.05.006

Murray, A.J., Tucker, S.J., Shewan, D.A., 2009. cAMP-Dependent Axon Guidance Is Distinctly Regulated by Epac and Protein Kinase A. J. Neurosci. 29, 15434–15444. https://doi.org/10.1523/JNEUROSCI.3071-09.2009

Neumann, S., Bradke, F., Tessier-Lavigne, M., Basbaum, A.I., 2002. Regeneration of Sensory Axons within the Injured Spinal Cord Induced by Intraganglionic cAMP Elevation. Neuron 34, 885–893.

Neumann, S., Woolf, C.J., 1999. Regeneration of Dorsal Column Fibers into and beyond the Lesion Site following Adult Spinal Cord Injury. Neuron 23, 83–91. https://doi.org/10.1016/S0896-6273(00)80755-2

Peace, A.G., Shewan, D.A., 2011. New perspectives in cyclic AMP-mediated axon growth and guidance: The emerging epoch of Epac. Brain Res. Bull. 84, 280–288. https://doi.org/10.1016/j.brainresbull.2010.09.002

Pearse, D.D., Pereira, F.C., Marcillo, A.E., Bates, M.L., Berrocal, Y.A., Filbin, M.T., Bunge, M.B., 2004. cAMP and Schwann cells promote axonal growth and functional recovery after spinal cord injury. Nat. Med. 10, 610–616. https://doi.org/10.1038/nm1056

Qiu, J., Cai, D., Dai, H., McAtee, M., Hoffman, P.N., Bregman, B.S., Filbin, M.T., 2002. Spinal Axon Regeneration Induced by Elevation of Cyclic AMP. Neuron 34, 895–903.

Quilliam, L.A., Rebhun, J.F., Castro, A.F., 2002. A growing family of guanine nucleotide exchange factors is responsible for activation of ras-family GTPases, in: Progress in Nucleic Acid Research and Molecular Biology. Elsevier, pp. 391–444. https://doi.org/10.1016/S0079-6603(02)71047-7

Rueden, C.T., Schindelin, J., Hiner, M.C., DeZonia, B.E., Walter, A.E., Arena, E.T., Eliceiri, K.W., 2017. ImageJ2: ImageJ for the next generation of scientific image data. BMC Bioinformatics 18, 529. https://doi.org/10.1186/s12859-017-1934-z

Thies, E., Davenport, R.W., 2003. Independent roles of Rho-GTPases in growth cone and axonal behavior. J. Neurobiol. 54, 358–369. https://doi.org/10.1002/neu.10135

Wei, D., Hurd, C., Galleguillos, D., Singh, J., Fenrich, K.K., Webber, C.A., Sipione, S., Fouad, K., 2016. Inhibiting cortical protein kinase A in spinal cord injured rats enhances efficacy of rehabilitative training. Exp. Neurol. 283, 365–374. https://doi.org/10.1016/j.expneurol.2016.07.001

Widenfalk, J., Lundströmer, K., Jubran, M., Brené, S., Olson, L., 2001. Neurotrophic Factors and Receptors in the Immature and Adult Spinal Cord after Mechanical Injury or Kainic Acid. J. Neurosci. 21, 3457–3475.

Wu, Y., Zhou, J., Li, Y., Zhou, Y., Cui, Y., Yang, G., Hong, Y., 2015. Rap1A Regulates Osteoblastic Differentiation via the ERK and p38 Mediated Signaling. PLOS ONE 10, e0143777. https://doi.org/10.1371/journal.pone.0143777

Yang, L., 2018. Neuronal cAMP/PKA Signaling and Energy Homeostasis, in: Wu, Q., Zheng, R. (Eds.), Neural Regulation of Metabolism, Advances in Experimental Medicine and Biology. Springer Singapore, Singapore, pp. 31–48. https://doi.org/10.1007/978-981-13-1286-1_3

Chapter 5

Cortical Infusion of PKA and EPAC Agonists and Rehabilitative Motor Training After Cervical Spinal Cord Injury in Female Rats

Preface

Despite the limitations of the findings presented in chapter 4, the *in vitro* results spurned a desire to test the potential for this effect to be used *in vivo* to boost plasticity with rehabilitative motor therapy. Following up on previous studies from the Fouad laboratory targeting similar pathways at the same anatomical target (Vavrek, 2006; Wei et al., 2016), I designed an experiment aiming to infuse the motor forelimb cortex innervating the SCI affected limb with PKA and EPAC agonists to increase plasticity and promote functional recovery. As previous experiments using the planned methodology had demonstrated brain damage as a result of the cannula, I needed to find ways to minimize damage to cortical tissue. I discussed my project with Dr. Molly Shoichet from the University of Toronto. She presented data that suggested small molecules (such as drugs) are able to diffuse into cortical tissue as seen in her work with hydrogel drug delivery (Tuladhar et al., 2020), and helped me formulate dosing for this experiment. In order to minimize cortical damage, the cannula for the brain infusion kits were shortened to only extend 0.5 mm deep into cortical tissue. Due to the fact that the agonists would be applied directly into the cortex, it was decided to use doses similar to those used in vitro, and as such it was calculated so that the same mass of drug was applied to the cortex per day as was performed in vitro in Chapter 4.

To support FAIR data and open science principles, all data for this experiment has been published on the Open Data Commons for Spinal Cord Injury (odc-sci.org):

Batty N. J., Vavrek R., Raposo P., Fouad K. (2021) Behaviour and histopathology after simultaneous cortical infusion of PKA and EPAC agonists and rehabilitative forelimb motor training after incomplete cervical spinal cord injury in female rats. Open Data Commons for Spinal Cord Injury. ODC-SCI:556 http://dx.doi.org/10.34945/F56C7W

5.1 Introduction

Following injury to the central nervous system (CNS: brain and spinal cord) there is limited capacity for axonal regeneration. This lack of regeneration has been studied in depth and has been attributed to limited internal growth capabilities (David and Aguayo, 1981) and the inhibitory growth environment surrounding the CNS (Schwab and Bartholdi, 1996). Following spinal cord injury (SCI), attempts to increase axonal regeneration have focused on blocking growth inhibitors to increase regeneration through the site of injury (Gonzenbach and Schwab, 2008; Lee et al., 2004; Massey et al., 2006; McKerracher et al., 1994). While this has been met with limited success, an alternate approach to bring about repair and functional recovery is through increasing plasticity in the brain and spinal cord (reviewed in: Fouad et al., 2015). The main goal of targeting plasticity over regeneration in the injured CNS is to use the circuitry within surviving tissue to remodel and allow for circumvention of the injury as opposed to direct repair. Part of why plasticity focused treatments show such promise is that changes in plasticity occur simultaneously throughout the CNS at different physiological and anatomical levels (Kusiak and Selzer, 2013; von Bernhardi et al., 2017). Additionally, neuronal plasticity is a naturally occurring repair mechanism that needs encouraging and direction, as opposed to regeneration which requires more forceful interventions. In this study, the focus on plasticity is of the collateral sprouting of both injured and spared neurons (Dell'Anno and Strittmatter, 2017).

As previously mentioned, there is a lack of regenerative potential in the adult CNS following an injury. This is due in part to a further decline in cyclic AMP (cAMP) in injured neurons (Cai et al., 2001; Dougherty et al., 2000; Fenrich and Gordon, 2004;

Widenfalk et al., 2001). This decline from baseline levels makes the cAMP signalling cascade an ideal target for increasing regenerative potential after SCI, as we know that when cAMP levels are elevated the neurons are capable of growth despite the inhibitory environment. Targeting this pathway would be restoring the natural growth potential of these cells as opposed to attempting to "teach an old neuron a new trick". cAMP is a ubiquitous signalling molecule found throughout the body, in many physiological systems, and in many different cell types including neurons. It primarily acts through two downstream effectors: Protein Kinase A (PKA), discovered in 1968, and the Exchange Protein Activation by cAMP (EPAC), discovered 30 years later. Much of the research on cAMP signalling has been suggested to be through PKA, as this downstream effector is more thoroughly studied than EPAC. However recent work has demonstrated a potential confound in many of these studies (Murray, 2008), and studies into EPAC suggest many of PKA's previously attributed roles are actually due to EPAC (review: Batty et al., 2017). EPAC activation has been demonstrated to be a noteworthy target for increasing neurite length *in vitro* (Enserink et al., 2002; Murray et al., 2009; Murray and Shewan, 2008). Targeting EPAC has also been shown to increase neurite extension in an *ex vivo* model of rat spinal cord injury (SCI) (Guijarro-Belmar et al., 2019).

In experiments examining the effects of PKA on neurite outgrowth and functional recovery after SCI (Wei et al., 2016) we found that blocking PKA led to an increase in functional recovery and neurite outgrowth in the corticospinal tract (CST), contrary to the findings of previous studies (Cai et al., 2001). The study went further to show that inhibition of PKA did not reduce levels of CREB phosphorylation, and went on to indicate that in the absence of PKA, EPAC has a more prominent role in cAMP

signalling. It is believed to be due to cAMP being more readily available to EPAC in the absence of available PKA, which has been shown *in vitro* (Hoy et al., 2020).

Whilst following up on our seemingly paradoxical finding that cortical Rp-cAMPS infusion led to increases in functional recovery and CST plasticity after SCI (Wei et al., 2016), we have demonstrated a complementary effect on neurite growth of simultaneous application of PKA and EPAC agonists *in vitro* (as demonstrated in Chapter 4). Given our previous experience targeting the cAMP signalling cascade using slow cortical infusion (Vavrek, 2006; Wei et al., 2016), we use this same methodology to test the complementary effect of PKA and EPAC agonists *in vivo,* in adult rats with a cervical SCI.

Rehabilitative motor training is standard in the clinic with individuals with cervical spinal cord injury and has repeatedly been demonstrated in animal models to be crucial, alongside plasticity promoting treatments, to eliciting functional recovery (García-Alías et al., 2009; Torres-Espín et al., 2018b; Weishaupt et al., 2013), and as such all groups received reaching training, a skilled motor function that is directly affected by our lesion model. This experiment examines the plasticity promoting effects of EPAC and PKA agonist application directly to the motor cortex innervating the injured side of the spinal cord immediately following a unilateral SCI in rats in conjunction with rehabilitative motor training.

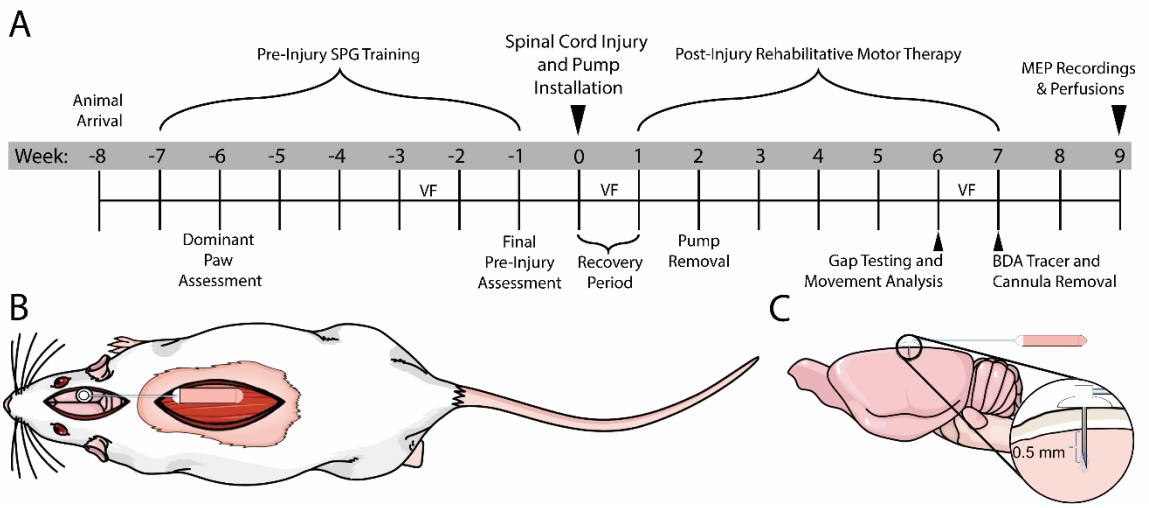

Figure 5.1 – Timeline and Experimental Schema

(A) A timeline demonstrating the time course of the experiment, when injury occurred, and the timing of all tests described in the study. VF denotes time points where Von Frey testing was performed. (B) A schematic of a rat with an Alzet osmotic mini-pump demonstrating the pump location atop the muscle over the animal's back, the silicone tubing going underneath the skin, and the brain infusion cannula adhered to the surface of the skull. The cannula from the brain infusion kit is cut so that it only extends 0.5 mm into the cortical tissue after the base is glued to the skull (C).

5.2 Methods and Materials

5.2.1 Animals

Female adult Lewis rats (Charles River Laboratories, Canada) weighing 180-220g were used. Rats were housed 5 per cage with a 12-hour light/dark cycle. Water and food were provided *ad libitum* apart from during the training period where, to encourage reaching behaviour in the rats, each cage was given 90% of its normal daily food intake (11g/rat, 55g/cage).

Total number of animals used in this experiment was 52 animals, though only 34 remained part of the final experiment (n=8 in the Dual agonist group, n=7 in the EPAC agonist group, n=19 in control) due to predetermined exclusion criteria (see statistical analysis). The experiment was run in 2 cohorts of animals, with each cohort having animals randomized into control or agonist groups. Experiments were approved by the Health Sciences Animal Care and Use Committee of the University of Alberta.

5.2.2 Single Pellet Grasping Training

Prior to receiving a SCI, rats were pre-trained in the single pellet grasping (SPG) task (Whishaw et al., 1986) to ensure they are sufficiently able to complete the task, and to be able to continue post-injury. Animals were acclimated to the SPG chamber and their dominant paw was determined by the experimenter while training the rats to reach for food pellets (45mg, Bio-serv) (Alaverdashvili, 2008; Alaverdashvili and Whishaw, 2013; McKenna and Whishaw, 1999). The training chamber is set up with semi-automated pellet dispensers attached to both sides as described previously (Torres-Espín et al., 2018c). The rats were trained to go from one side to the other reaching for pellets. Rats

were trained 5 days a week for ten minutes a day. Once their success rate reached a plateau at approximately 4 weeks of training, animals underwent SCI surgery and were given a week for recovery. Following this recovery period, the animals were reintroduced to the SPG task for 10 minutes a day, 5 days a week for rehabilitative motor therapy. All animals received training as it is a standard in the clinic. No limit of pellets was imposed during the 10 minutes of training.

Rats were assessed weekly following injury by videotaping every fifth training session and scoring each pellet grasping attempt as follows: 'Attempts' were defined as the number of times the animal reached for a pellet no matter the outcome, and 'Success rate' was defined as the percentage of attempts in which the animal was able to reach and move the pellet to their mouth and eat it, though this allows for compensatory means to obtain the pellet (Hurd et al., 2013; May et al., 2015; Torres-Espín et al., 2018b).

After 6 weeks of reaching training, a different assessment was performed introducing a gap between the animal and the pellet. This gap creates a barrier for animals that compensate for their inability to grasp the pellet by dragging it towards them. By having a gap in which a dragged pellet can fall into, the rats are forced to retrieve the pellet over the gap to succeed. This alteration to the SPG task differentiates between animals that compensate by scooping as opposed to animals that show recovery by firmly grasping and holding on to the pellet (Torres-Espín et al., 2018c).

5.2.3 Single Pellet Grasping Movement Analysis

At the end of the SPG training the reaching movement pattern for each animal was analyzed as described by Metz and Whishaw (2000). High speed video recordings (120 fps) were taken of each animal successfully reaching for 3 pellets. The performance in each trial was broken up into 11 components (Metz and Whishaw, 2000) and scored based on the presence of that movement and how it was performed, scores are: 0 – absent movement, 0.5 – movement is abnormal, 1 – movement is normal. These three successes were individually scored and averaged for each animal. For animals that were unable to perform successful reaches by the end of training, their best 3 attempts were taken.

5.2.4 Von Frey

Animals were acclimated to the Von Frey testing chamber (IITC Life Science, CA, USA) prior to any tactile testing. This was done by placing each animal in the testing chamber for 15 minutes on a day prior to testing. To test the tactile sensitivity of both forepaws, an electronic Von Frey device with a rigid tip on the probe was used to gradually apply increasing pressure to the paw until a marked nociceptive response was exhibited. A Nociceptive response is whenever the animal would deliberately retract their paw or demonstrate clear signs of discomfort. The maximum force (in grams) was recorded from the electronic Von Frey device. Each paw is tested a minimum of three times per testing session with a minimum five minutes between assessments, and an average of these values per animal is used for statistical analysis.

5.2.5 Spinal Cord Injuries and Pump Installation

Each animal received a unilateral dorsal-lateral quadrant (DLQ) spinal cord transection injury at cervical level 4 on the side of the individual animal's dominant paw as described previously (Girgis et al., 2007; Krajacic et al., 2009). DLQ injuries target the dorsal corticospinal tract and the majority of the rubrospinal tract, as well as ascending fibers. For lesion surgeries animals were anesthetized using gaseous isofluorane (5% for induction, 3% for maintenance) and placed in a stereotactic frame. At the onset of surgery, the scalp and back of the rats were shaved and disinfected with 2% chlorhexidine digluconate (Sigma-Aldrich Canada ltd., Oakville, ON, Canada) and ethanol. Eyes were lubricated during surgery with Alcon Systane Ointment (Novartis Pharmaceuticals Canada Inc., Dorval, QC, Canada). The skin and muscles over the shoulder blades were opened to perform a laminectomy. An opening in the dura was made and a thin custom-made blade was used to perform the injury to a depth of 1 mm. The muscle over the spinal cord was sutured using 5-0 vicryl sutures and the skin overtop was closed using surgical staples. This injury affects the animal's fine motor control ability in their distal forelimb. Procedures were performed on a heating blanket set to 37°C to prevent hypothermia. Post procedure, animals were administered 3 mL of saline and buprenorphine (0.3 mg/kg) for hydration and pain control respectively. Animals were placed in heated recovery cages with ad libitum food and water until they demonstrated a level of wakefulness deemed sufficient to be returned to their home cages.

During the same surgical procedure as the lesion, animals were randomly divided between experimental groups. Animals received either saline vehicle, EPAC agonist, or

both EPAC and PKA agonists delivered to the forelimb motor cortex contralateral to the spinal cord injury.

The drug application to the forelimb motor cortex was performed as previously described (Hiebert et al., 2002). A hole was drilled at the coordinates 1.5 mm lateral and 1.5 mm rostral to bregma. An Alzet brain infusion kit (Durect Corporation, CA, USA) cannula was inserted 0.5 mm deep into the motor cortex. Cannula was adhered to the surface of the skull around the hole using Loctite instant adhesive cyanoacrylate gel (Cedarlane, Burlington, ON, Canada). The cannula was connected to a 14 day Alzet Osmotic mini-pump (0.5 µL/hour) that was placed subcutaneously on the animal's back. The pumps were filled the day prior under sterile conditions and primed in saline overnight at 37° before insertion into animals. The pumps were filled with either a 300 µM EPAC Agonist solution (8-CPT-2`-Me-cAMP in PBS), a a 300 µM EPAC Agonist and a 300 µM PKA Agonist solution (8-CPT-2`-Me-cAMP and 6-BnZ-cAMP in PBS) or saline vehicle. Pumps were removed three weeks later under isoflurane anaesthesia.

5.2.6 Tracing of the CST

At the end of the five weeks of SPG training animals underwent tracing of the injured CST. Surgeries were conducted under isoflurane anesthesia, 5% for induction and 3% for maintenance. Using the same coordinates as for the cannula installation, a 1 mm^2 window was opened using a surgical drill and a 5 µL Hamilton syringe was used to inject 1 µL of biotin dextran amine (BDA; 10%, Life Technologies, Grand Island, New York, USA) tracer into three locations 1.5 mm deep (layer V of the cortex). General surgical and post operative procedures were performed as stated above.

5.2.7 Motor Evoked Potential Recordings

Two-weeks following CST tracing, motor evoked potentials (MEP) were recorded in the wrist muscle of the affected forelimb. Rats were anaesthetized (xylazine 7mg/kg subcutaneous, ketamine 75 mg/kg intraperitoneal) and placed in a stereotactic frame where an incision was made through the scalp to expose the skull. A surgical drill was used to open a window over the motor forelimb cortex area (0.5-1.7 mm rostral, 1-3 mm lateral to bregma) of each hemisphere. Stimuli were delivered via two tungsten electrodes (5 μm diameter, 0.1 MΩ impedance; World Precision Instruments Inc., Sarasota, FL, USA) mounted in the stereotaxic arm and placed transdurally 1 mm apart, and 1.5 mm deep (Brus-Ramer et al., 2007; Carmel et al., 2014; Girgis et al., 2007). For recordings, a custom made electrode was placed into the first proximal third of the wrist extensor muscles, and a second electrode was placed in the distal third of the extensors. A final wire was placed subcutaneously on the back of the animal to act as ground. Recorded activity was amplified (Grass P5 series a.c. pre-amplifier), passed through a low pass filter, and digitized at 2000 Hz (Axon instrument Digidata 1322A, 16-bit) using AxoScope software (molecular devices). Stimuli were delivered using a Master-8, (A.M.P.I.) and an isolation unit (isoflex, A.M.P.I.), in the form of thirty pulse trains (0.2ms pulse width) at 333 Hz. The threshold of the muscle response was determined by stimulating the cortex innervating the uninjured forelimb at progressively increasing stimulus intensities until a motor response was evoked. Then the cortex innervating the affected forelimb was stimulated at 1.1x the determined threshold with minimum 30 second intervals between stimuli. Recordings were analyzed for latency between stimulus

artifact and initial response using AxoScope software (molecular devices). Animals were immediately euthanized for perfusion following MEP recordings.

5.2.8 Perfusion and Histological Sectioning

Following electrophysiological recordings, rats were euthanized using 960mg/kg Euthanyl (pentobarbital sodium injection, Bimeda-MTC Animal Health Inc., Cambridge, ON, Canada). This was followed by a transcardial perfusion using warm (37°C) saline with heparin, and cold 4% formalin solution with 5% sucrose. The spinal cord and brain from each animal were extracted and post fixed in a 4% formalin solution for two hours at room temperature and then placed in a 30% sucrose solution for 5 days at 4°C for cryoprotection. The spinal cord tissue and brain were cut into segments and mounted on filter paper using Tissue Tek O.C.T (Sakura Finetek, CA, USA) after which they were frozen using methylbutane and dry ice at -45°C, and stored at -80°C.

Tissue was sectioned on a cryostat at -20°C at 25 µm and mounted onto coated microscope slides in a staggered fashion. Staggering the tissue over 4 slide sets creates 4 separately stainable sets of tissue, with each section being 100 µm from the next. Tissue at the lesion site was sectioned in transverse sections to allow for analysis of the lesion size and for collateral CST fibers above and below the injury site. Slides were stored at -20°C until staining procedures were performed.

5.2.9 Lesion Size Analysis

Tissue sections were stained using cresyl violet. For this, slides were dehydrated for 1 hour at 37°C followed by rehydration in tris-buffered saline (TBS) twice for 10

minutes. Slides were then placed in a 0.5% cresyl violet solution for 3 minutes, dipped in distilled water to remove excess stain, and serially dehydrated in increasing alcohol concentrations. Finally, slides were cleared in xylene and coverslipped with Permount (Fisher Scientific, Ottawa, ON, Canada).

Cresyl violet stained tissue was analyzed using bright field microscopy in addition to phase contrast to assess the size of the DLQ lesion. At 10x magnification, landmarks were identified to determine the location and extent of the injured tissue. Based on these landmarks, lesion locations and size were translated onto a schematic cross-sectional representation of the spinal cord. Each lesion was then calculated as a percentage of the total area of the spinal cord and as a percentage of the dorsal CST area (dCST) using ImageJ software (Schneider et al., 2012).

5.2.10 Immunohistochemistry

To visualize BDA traced CST fibers, immunohistochemical staining was performed. For this, slides were dehydrated at 37°C for 1 hour and then rehydrated twice in TBS for 10 minutes. Slides were then incubated with 10% normal goat serum (NGS) in TBS with triton-X (0.5%; TBS-TX). Slides were then incubated overnight with streptavidin conjugated to Alexa Fluor 488 (Cedarlane, Burlington, ON, Canada) in 1% NGS in TBS-TX (0.5%). Slides were then washed twice in TBS-TX (0.5%) for 10 minutes each, and then twice in TBS for ten minutes each. Slides were coverslipped with Fluoromount G (Cedarlane) and sealed around the edges of the coverslip with nail polish.

5.2.11 Collateral Fiber Density Heatmapping

Density-based maps of collateral sprouting of the injured CST were extracted as previously described (Torres-Espín et al., 2018b). Briefly, tissue 100 μm above the lesion site was imaged using confocal microscopy to visualize the BDA tracing. For each animal, 5 tissue sections were imaged in 10 step Z-stacks which were collapsed into a maximum projection image. A customized ImageJ macro was used to extract the coordinate location of each pixel in a stained fiber within the grey matter (corresponding to a collateral fiber off the CST). Using a custom made script written in R (R Core Team, 2018), the coordinates for all animals within a group were overlaid and kernel density-based heatmaps were generated using the kde2d function in the MASS R package for visualization. To quantify the traced fibers density and the differences between groups the collateral fibers were counted in 75x75 micron bins and the count normalized between animals. This was done by dividing each bin count by the total count of CST axons traced in the dorsal CST area at C1 for each animal. This generated a CST index that we used for statistical comparisons that essentially normalizes the weight behind each collateral fiber depending on how successful tracing was in that animal. Using this CST index, ANOVA tests were run in the 75x75 bins and translated to an additional heatmap showing the adjusted p value (by Tukey method) at each area of the spinal cord. In addition to calculating and comparing the CST index, the number of bins that show an increase in collateral fibers between groups, both significant and non-significant were grouped and analyzed a Fisher's exact test in GraphPad Prism 7 (GraphPad Software). As this analysis compares only two groups, this was repeated to compare each group combination.

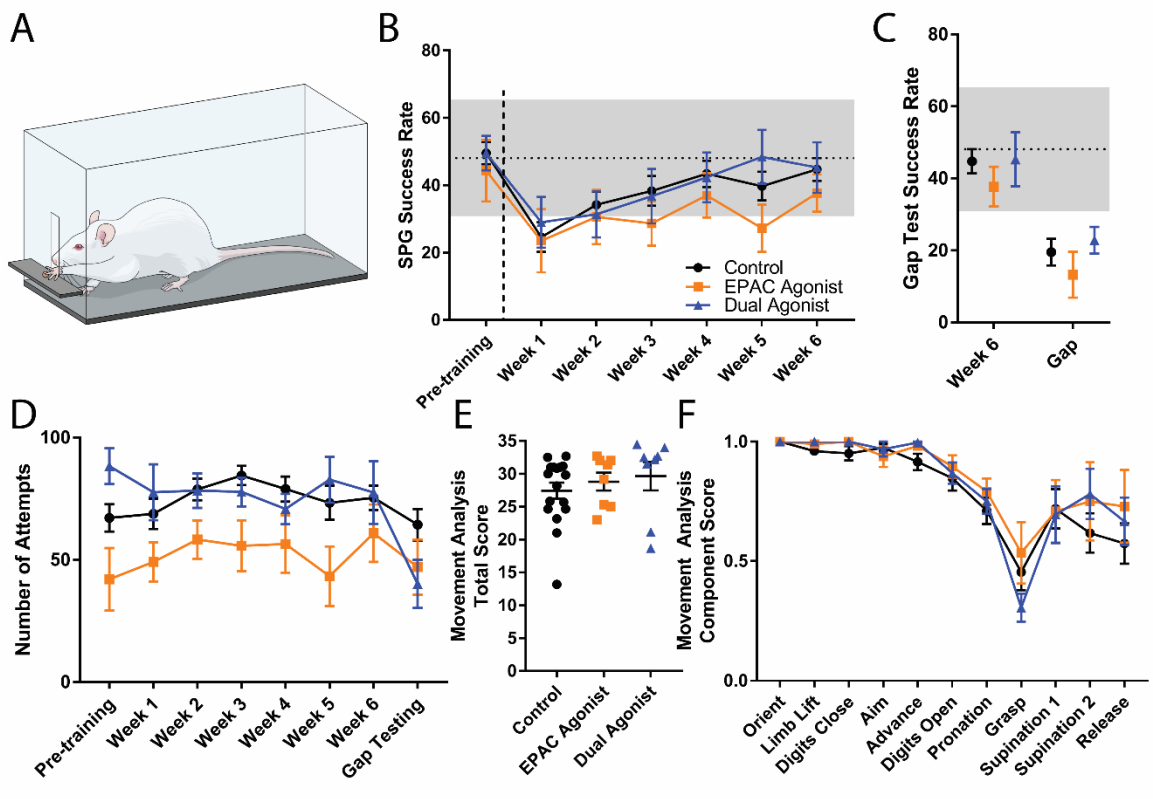

Figure 5.2 – Single Pellet Grasping

A rat grasps for a pellet in the standard SPG task used for rehabilitative motor training (A). Average scores from all three groups show increased success approaching preinjury levels (baseline ± SD denoted in grey) (B) and a significant decline in success rate in the Gap testing task (C). Training intensity between groups as seen in terms of attempt rate during training sessions was not significantly different between groups (D). Further investigation into the specific components of the reaching motion using high speed cameras and mirrors to see the sides of the animals shows no significant difference in the overall score (E), which when broken down (F) shows that whilst all animals demonstrated a decline in score in the components of the reaching task most associated with fine motor control, as that is what is directly impacted by the C4 DLQ model of injury used in this study. Error bars show SEM.

5.2.12 Statistical Analyses

Following the initial pre-training phase of the experiment, animals were randomized into control, EPAC agonist, or Dual agonist groups. Statistical analyses for all behavioural assessments and lesion size analysis were conducted using GraphPad Prism 7 (GraphPad Software). Normality was assessed using D'Agostino-Pearson omnibus test. Lesion size data was analyzed using unpaired t-tests, and data from SPG testing and regeneration measures were analyzed using repeated measures two-way ANOVA. Statistical analyses for the collateral density heatmaps in the form of two-way ANOVAs were performed in R studio as indicated above. Non-parametric Mann-Whitney test was used for the CST lesion percentage analysis due to non-normality.

Exclusion criteria include animals that did not engage with the task prior to injury (attempts >5 each session prior to injury) or those that did not train after injury (attempts >5 each session), and animals that had too severe of a deficit (total lesion size >30%) and thus were apathetic towards the SPG task post injury. Exclusion criteria required the removal of eighteen animals in total from this experiment, 9 were removed to due lesion size and 10 were due to training apathy (with one animal falling into both categories). Upon removal of these animals, the final group numbers were n=8 in the Dual agonist group, n=7 in the EPAC agonist group, n=19 in the control group. For the MEP analysis, all recordings were analyzed regardless of exclusion criteria due to low n.

5.3 Results

5.3.1 SPG Outcomes and Lesion Analysis

Reaching and grasping ability was assessed weekly following injury. Performance was evaluated primarily based on the percentage of successful pellet retrievals in relation to reaching attempts. All three groups show similar pre-injury baseline scores ($p=0.77$), as the control group had an average baseline success rate of 49.59% (±14.31%), the EPAC agonist group scored 44.33% (±24.24%), and the Dual agonist group scoring 49.55% (±14.44%) (Fig. 5.2B). Following SCI, all rats demonstrated an expected decline associated with the C4 DLQ injury. As the rats underwent rehabilitative motor therapy, all groups showed signs of recovery that increased as training progressed, beginning to plateau around week 4 and continuing until week 6 as they neared baseline scores (Control: 44.74 ±14.39%; $p=0.2008$ paired t-test compared to baseline; EPAC Agonist: 37.66 ± 14.56%, $p=0.4955$ paired t-test compared to baseline; Dual Agonist: 45.30 ± 21.23%, $p=0.6043$ paired t-test compared to baseline). Overall, the groups showed no functional difference in their SPG scores ($p=0.47$, Two-way ANOVA Treatment effect).

One of the prominent means by which rats compensate to obtain the pellets in the SPG task is by scooping the pellets towards them in a sweeping motion instead of grasping the pellet to bring it to their mouths (McKenna and Whishaw, 1999). We have previously established a means by which to introduce a gap into the SPG dispenser (Torres-Espín et al., 2018a) that will cause the pellet to fall if it is swept toward the animal as opposed to lifted. When the animals were tested using the SPG gap dispensers, the success rates for all groups declined as expected compared to their final week SPG scores, though there was no significant difference between the groups once again

suggesting that all groups have similar amounts of compensation in their actions (Fig. 5.2C).

As a further means to differentiate reaching and grasping ability, the qualitative components of the task were analyzed (Metz and Whishaw, 2000). The animals demonstrated scores in line with what has become expected given the injury type, where the most effected components are grasping and handling the pellets. That being said, all groups show similar scores when adding the 35 subcomponents together and similar score trends across the major 11 component categories (Fig. 5.2 E & F). Further demonstrating a lack of functional effect across treatment groups.

Previous work has highlighted the importance of comparable lesion size in experiments such as this, as small differences can make a large functional difference in recovery (Hurd et al., 2013). To account for this, we analyzed the spinal lesions of all the animals and found no significant difference in total lesion area across groups ($p=0.25$, One-way ANOVA; Fig. 5.3B), and in the dorsal CST (dCST) lesion area across groups ($p=0.89$, One-way ANOVA; Fig. 5.3C). The average total lesion area damage for the control group was 15.24% (±4.90%), for the EPAC agonist group was 12.40% (±1.67%), and for the Dual agonist group was 12.86% (±5.245%). The average dCST lesion size for the Control, EPAC agonist, and Dual agonist groups were 81.36% (±21.9%), 79.46% (±16.43%), and 78.55% (±24.27%), respectively. Overall, lesion sizes were consistent between groups and would not act as a confounding variable between groups for the other outcome measures in this study.

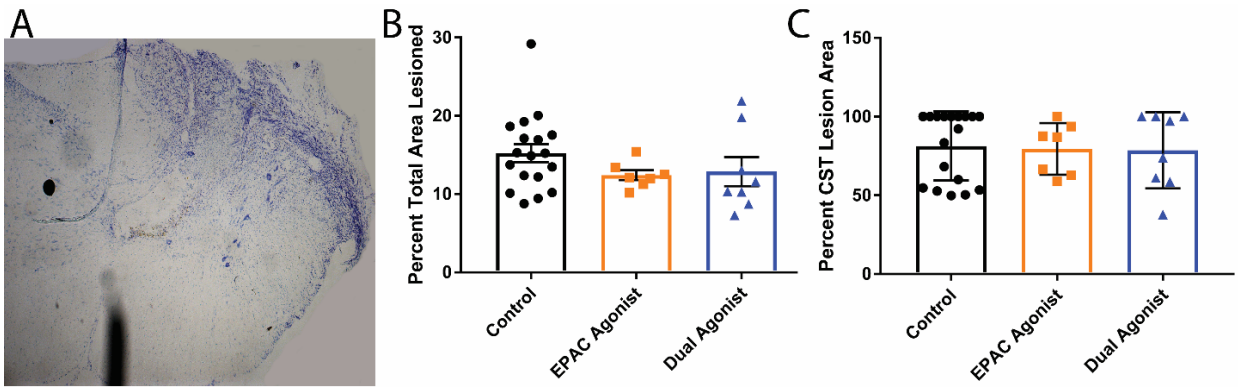

Figure 5.3 – Lesion Analysis

(A) Image of an example lesion epicenter tissue slice, stained with cresyl violet. (B) Lesion size between groups is consistent, with a similar mean size and variation. (C) Total area of the corticospinal tract damaged by the lesion (as a percentage of the whole CST) is similar across all groups. Error bars show SEM.

5.3.2 Von Frey Testing Suggests Sensory Recovery with Dual Agonist Treatment

While functional motor ability shows no differences between treatment groups and controls, analysis of tactile sensitivity was performed to assess potential sensory changes. Mechanical sensitivity was assessed using an electronic Von Frey machine with a rigid tipped probe. As expected, the SCI results in a significant reduction in sensitivity in the injured paw and an increased sensitivity in the contralesional paw (Post-injury: control $p<0.0001$, EPAC Agonist $p=0.0259$, Dual Agonist $p=0.0115$). Sensitivity was again assessed following the 6 weeks of rehabilitative motor therapy and drug treatments. The control and EPAC agonist groups both continued to show reduced sensitivity in the injured paw and increased sensitivity in the uninjured paw, however the Dual Agonist group showed no significant difference between forepaws (Post-training: control $p=0.0161$, EPAC Agonist $p=0.0688$, Dual Agonist $p=0.6220$; Fig. 5.4A.

5.3.3 MEP Latency Comparisons

As plasticity is a varied, multilevel process, it is possible that there are electrophysiological changes as a result of treatment, that did not present itself in the functional outcomes. MEP recordings were analyzed for changes in latency that may suggest changes such as increases in myelination or neuronal excitability, or a change in the number of synapses between the stimulated motor cortex and the affected forelimb. Only the control and Dual agonist groups had MEP responses recorded and analyzed ($n=4$ for control, $n=7$ for dual agonist). For analysis of the MEPs, latency of the response after the stimulus was averaged across all sweeps per animal and this data was used for the statistical comparisons. Comparison of the average latency between groups

demonstrated that there was no significant difference between groups (p=0.073, Mann-Whitney Test) with control animals having an average latency of 10.73 ± 1.618 ms and the Dual agonist group having an average latency of 14.29 ± 2.925 ms. Overall, there were no differences in MEP delay between the control and Dual Agonist treated animals, suggesting no anatomical changes occurred that affect electrophysiological responses (Fig. 5.4B). Amplitude of these recordings was unable to be compared due to the variability between animals. As the stage of anaesthesia can have a major impact on cortical excitability (Kawaguchi and Furuya, 2004) and having a consistent plane of anaesthesia between animals at the time of recording is impossible, amplitude analysis would fail to provide any meaningful observation.

5.3.4 Collateralization Increased in EPAC Treatment Group Over Dual Agonist Group

To examine the effects of Dual agonist and EPAC agonist treatments on CST plasticity, collateral sprouting from the CST into the grey matter of the spinal cord was analyzed (Bareyre et al., 2004; Lindau et al., 2014; Mitchell et al., 2016). This was done by creating density based heatmaps of collateral CST sprouts in the spinal grey matter (Fig. 5.5 A-C). Immediately rostral to the lesion we see distinct differences between the control, EPAC agonist, and Dual Agonist groups. The control group shows the expected distribution of collaterals with the majority remaining a short distance from the injured CST axons. The EPAC agonist group shows a deeper distribution of collaterals into the grey matter, and the Dual agonist group exhibits traces of collaterals deep into the dorsal horn (i.e. in laminae 1-4) (Fig. 5.5 D-F).

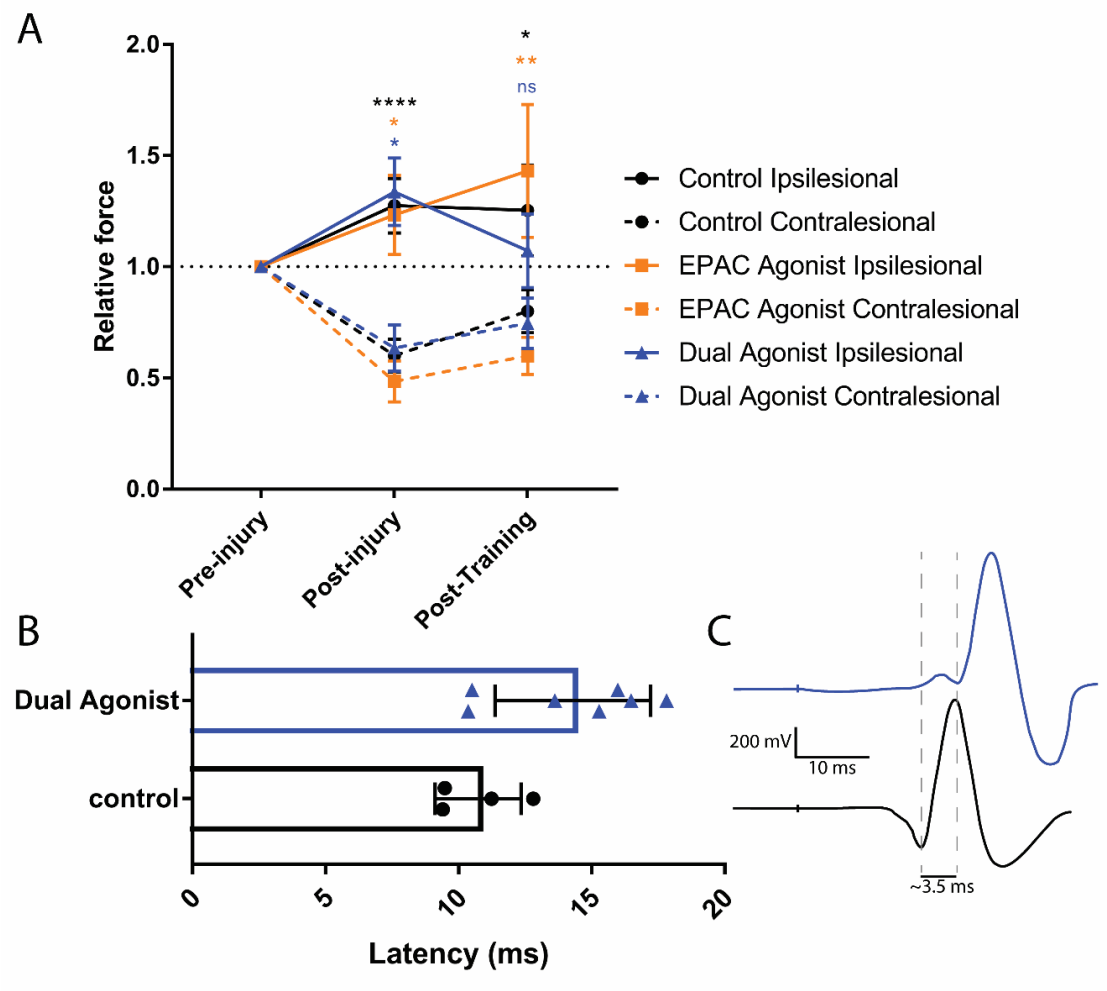

Figure 5.4 – Von Frey Data and MEP Recordings

Mechanical thresholds of both the injured and uninjured paws of the animals were tested using an electronic Von Frey apparatus at three time points throughout the experiment (A). All groups show the expected loss of sensitivity in injured paws immediately following SCI as well as the compensatory increase in sensitivity in the uninjured paw. Only in the Dual Agonist group were the injured and uninjured paws not significantly different from each other after training, suggesting further recovery in this treatment group. Motor Evoked Potential recordings were performed to look for electrophysiological changes not detected by functional tests. Quantifying the collected data (B) shows no statistical significant difference in MEP latency between groups. (C) Example MEP traces from a Dual agonist animal (blue) and a control animal (black) show the non-significant difference in latency between the two groups. Error bars show SEM.

In order to quantify and compare these heatmaps, a CST index was created by normalizing the relative impact of each collateral fiber to the number of traced CST fibres counted at C1 for that respective animal. Each group was compared to each of the other two groups separately, and statistical heatmaps for each of the comparable permutations were made. This data was then used for statistical analyses in 75-micron squared bins overlaid onto the spinal grey matter. Two heatmaps are then constructed using the statistical analyses from each bin, one that shows the magnitude of difference in the form of statistical significance values (i.e. p value) (Fig. 5A, 5G, 5M), and another that shows the direction of the change (i.e. which group has more collaterals in that bin) (Fig. 5B, 5H, 5N). Population statistics were run using the number of bins in favour of each group and whether or not they were significant. Additionally, overall CST index values (Fig. 5.6 C, I, O) and CST index values in significant bins (Fig. 5.6 D, E, J, K, P, Q) were extracted and graphed for each comparison.

Comparing the control group to the EPAC Agonist group or the Dual Agonist group reveals little of significance. When comparing the EPAC Agonist group to the Dual Agonist group, however, there is a marked increase in the number of bins showing that the EPAC Agonist group has more collaterals present ($p<0.0001$, Fisher's exact test, Fig. 5.6R). It is important to note however that a quick visual check would reveal that the majority of these bins are not far from the CST (Fig. 5.6N), indicating that there are more 'short collaterals' in the EPAC Agonist group than the Dual Agonist.

5.4 Discussion

In this study we follow up on the previous chapter's *in vitro* findings that dual PKA and EPAC agonism results in a complementary increase in neurite length. Our goal was to take this complementary effect *in vivo* and explore its effects on functional recovery and spinal anatomy after SCI. As our previous *in vivo* experiments targeting the same pathway (Vavrek, 2006; Wei et al., 2016) used slow cortical infusion, we repeated the process here but with a shorter cannula depth to limit damage to the cortex seen previously (Wei et al., 2016). No signs of cortical damage were found in any of the groups in the current experiment. Contrary to our hypothesis, our study showed no significant differences in both functional recovery and/or anatomical changes (in terms of CST plasticity) between treatment groups, with few exceptions. The study does however demonstrate the promising potential of a treatment effect seen in the increase in collaterals found in laminae 1-4 in the dual agonist group, and the differences seen in the Von Frey Analysis.

Injury to the adult mammalian CNS does not have a natural means for repair, partly due to changes to the neurons themselves brought on by age (Cai et al., 2001; Dougherty et al., 2000; Fenrich and Gordon, 2004; Widenfalk et al., 2001) and the injury itself (Krajacic et al., 2009; Pearse et al., 2004). A key difference between the CNS and the PNS is the ability for the peripheral nervous system to repair itself following injury, and one of the key factors leading to this difference is the temporary upregulation of neurotrophins at the injury site (Dougherty et al., 2000) leading to an increase in cAMP levels (Gao et al., 2003). As such, treatment aiming to increase cAMP levels or the actions of cAMP downstream effectors, would serve to increase the ability of the CNS to

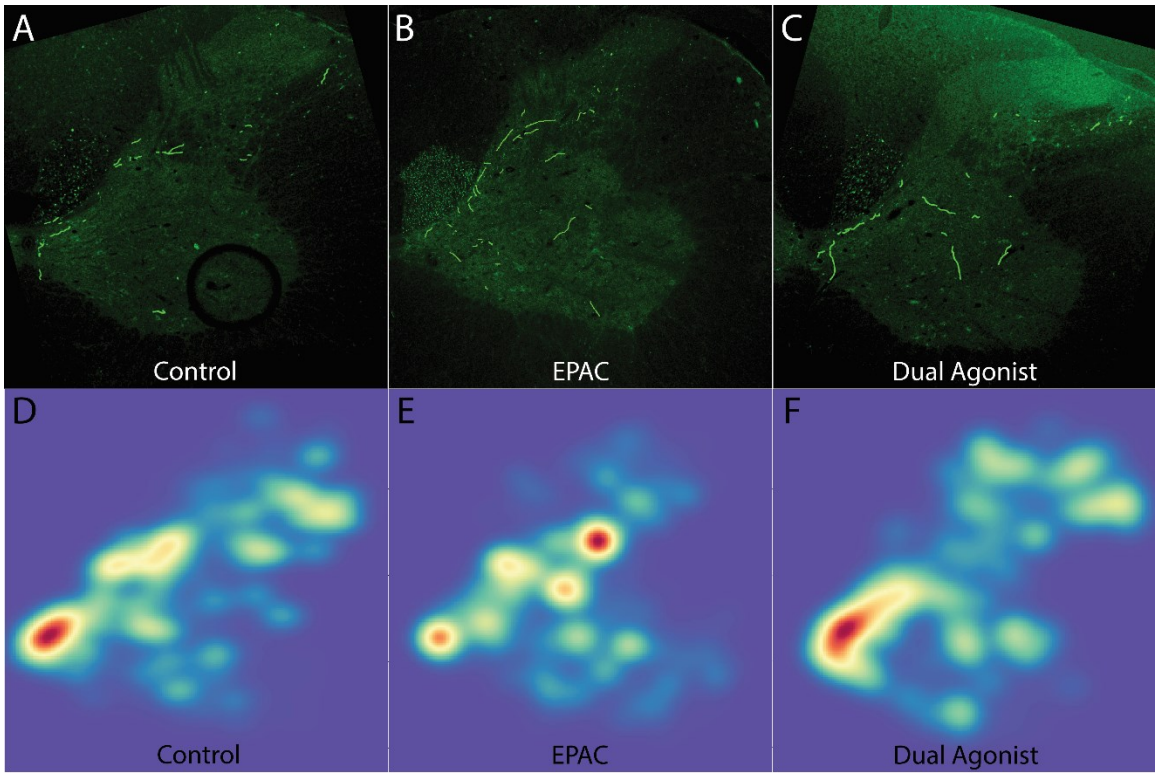

Figure 5.5 – Heatmaps of Collateral Density from the CST

BDA tracing of the CST in control animals (A), EPAC agonist animals (B), and Dual Agonist animals (C) was analyzed immediately rostral to the lesion site and used to generate density based heatmaps of collaterals sprouting off of the CST (D, E, F).

repair itself. The idea of the present study is to combine rehabilitative motor therapy with treatments to increase cAMP downstream effector signalling, essentially promoting one of the pathways that the motor training will act on to increase plasticity in the neuron (i.e. activity dependent plasticity). Work connecting neuronal stimulation to cAMP signalling (Brushart et al., 2002; Geremia et al., 2007; Udina et al., 2008) has already established a connection between activity in a neuron and the cAMP signalling pathway for its growth promoting effects. Motor training is another way to stimulate relevant motor neurons, and as such would also increase cAMP signalling. Further increasing this signalling pathway in conjunction with motor training is hypothesized to result in an increase in collateral sprouting based (on what was seen *in vitro* (Chapter 4) and functional motor recovery.

As discussed previously, rehabilitative motor therapy plays a proven role in enhancing and directing plasticity after SCI in conjunction with other therapies (Batty et al., 2020; Gonzenbach et al., 2010; Torres-Espín et al., 2018b; Weishaupt et al., 2013). This, in combination with the fact that human SCI patients will normally receive some form of rehabilitative motor therapy related to their injury is what makes motor therapy a constant between all groups in our study.

Our experiment to increase activation of cAMP downstream effectors, PKA and EPAC, through sustained cortical injection of pharmaceutical agonists did not show a significant difference in the extent of functional recovery in the SPG task compared to both controls and an EPAC agonist group (Fig. 5.2B). It is possible that what is occurring in the SPG testing data is a ceiling effect in their functional recovery as success rates approach pre-injury levels leaving no room for further recovery. In order to determine if

this is the case a more advanced test of the animal's reaching ability is required to further differentiate the groups.

In an attempt to determine if this was potentially due to a ceiling effect, and to determine the extent of compensation in the animals' recovery, a gap was introduced into the SPG pellet dispenser to ensure animals only received a pellet when they actually lifted it over the gap. If a significant difference between the groups in this test were to be a present, it would demonstrate that the lack of difference in the SPG success rate is due to a ceiling effect. The gap testing demonstrated similar results as the SPG success rate (Fig. 5.2C) in that no difference was seen when a gap was introduced, as all groups declined to similar levels, suggesting similar levels of compensation and no ceiling effect in the original SPG data. Movement analysis using high speed cameras yielded similar findings (Fig.5.2 E & F). It is believed that lacking a ceiling effect in the original SPG data, and no difference in the movement analysis, that overall there was no difference in functional recovery between treatment and control groups.

As the in vitro experiment that preceded the current experiment demonstrated that the dual agonist treatment yielded a marked increase in neurite extension (chapter 4 of this thesis), it warrants investigation here into the collaterals that formed off of the CST to determine if there was an increase in collateralization and collateral extension as hypothesized. Collaterals were analyzed by generating heatmaps to analyze CST sprouting immediately above the lesion site as per previous findings (Batty et al., 2020). The density based heatmaps for the individual groups (Fig. 5.5 D-F) show an average of where the collaterals from each group grew towards. It is worth pointing out that despite a lack of statistical significance favouring the dual agonist group in the statistical

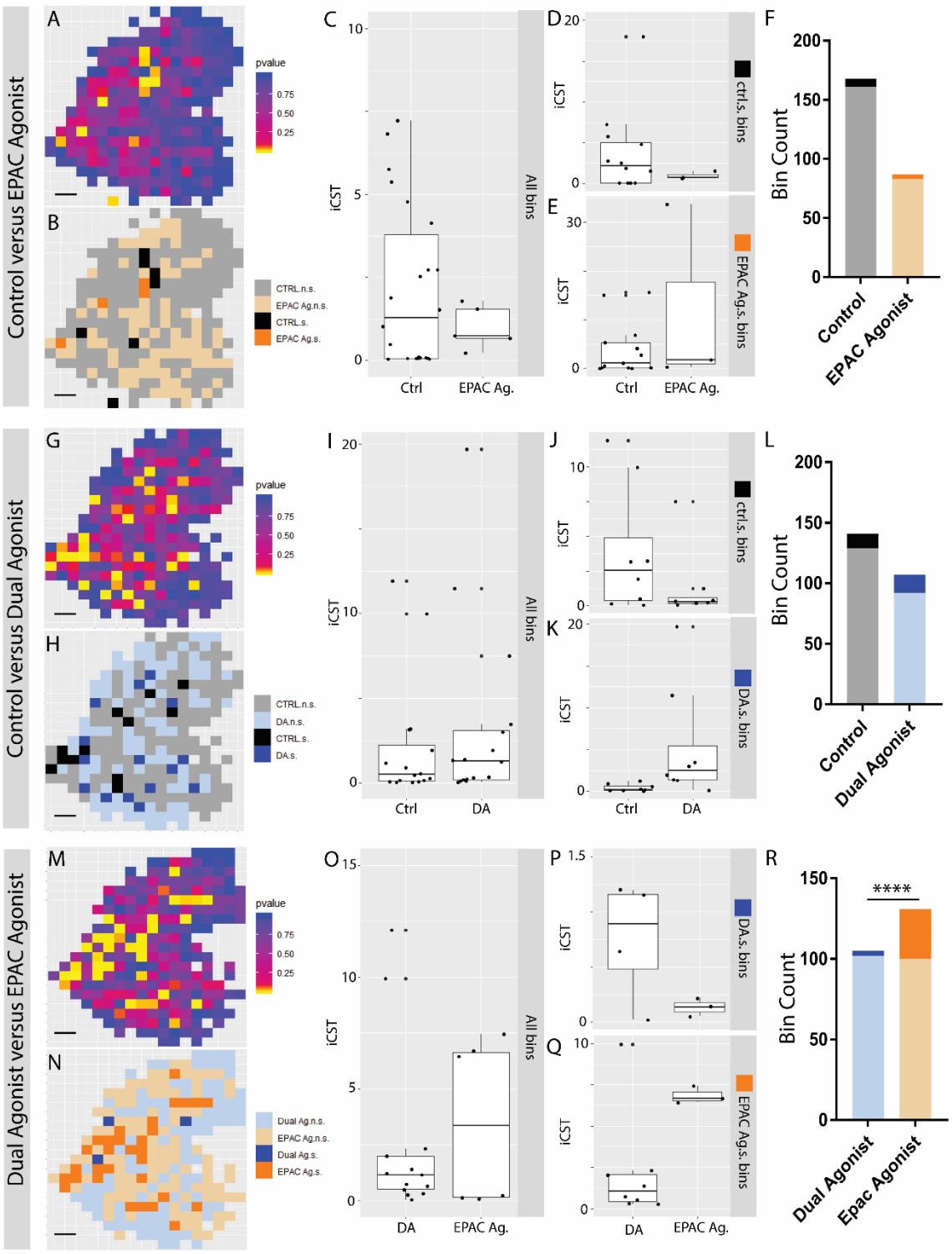

Figure 5.6 – Collateral Heatmap Statistical Analyses

Further analyses were performed by comparing heatmaps from individual groups to each other, resulting in three separate analyses. Collateral density was normalized based on the

number of traced fibers in each animal. This normalized value creates the CST index (CST index). Fiber distribution maps were recreated with weighting based on their CST index and then statistical analyses were calculated in bins (A, G, M). Statistically significant differences in collateral density between groups are highlighted in a different colour denoting the group showing the increase (B, H, N). CST index quantification showing the overall CST index between groups (C, I, O) and the CST index in statistically significant bins (organized by group that bin is significantly in favour of) (D, E, J, K, P, Q). Population statistics comparing the number of bins with a higher CST index in each group, both significant and non-significant, are shown in (F, L, R). Scale bars are 150 μm. Error bars show SEM.

heatmaps (Fig. 5.6), the dual agonist group has a larger proportion of collateral fibers deeper in the dorsal horn (Fig. 5.5F), i.e. towards laminae 1-4. It is interesting to see collaterals sprouting to this area, as this is often associated with pain and touch signalling (Reviewed in: Stachowski and Dougherty, 2021). The EPAC agonist treated animals did have a larger number of bins with statistically significant increases in collateralization compared to the dual agonist group (Fig. 5.6R), but the heatmap showing which group has more collaterals per bin (Fig. 5.6N) clearly shows that the majority of these EPAC favouring bins are proximal to the CST, suggesting more short fibres sprouted versus longer ones. Increasing the group size to increase the power of the statistics involved may demonstrate significant collateralization in the dorsal horn in the dual agonist group. This also fits with the Von Frey data, as the Dual agonist group was the only group to no longer show a difference between ipsilesional and contralesional paws in terms of nociceptive threshold. The collaterals that extended towards laminae 1-4 may be related to the recovery seen in the Von Frey, as cells in this area have been associated with pain, itch, and touch signalling (Stachowski and Dougherty, 2021).

Despite limited results, the current study does not disprove or contradict previous studies, but rather leaves room for improvement, repetition, and provides some interesting findings and questions to kickstart further studies.

5.4.1 Technical Challenges and Translational Considerations

Application of pharmaceutical agents through sustained diffusion into cortical tissue has a number of potential challenges. In our study it is important to note that while the osmotic mini-pumps were being removed, connection to the cannula was confirmed

for many of the animals, but a number were unable to be confirmed and even suspected to have come disconnected. As a result it is possible in a number of cases that our drug treatment was not even pumping into the motor cortex as intended, thus having no effect, though no link between these animals and animals were non-responders was found. Additionally, the only confirmation for the efficacy of the pump at dispelling its contents is to check the volume remaining after they are removed. No significant amount of fluid was found in any of the pumps post removal, but it does not confirm it penetrated to the intended cortical layer though literature suggests that small molecules are capable of penetrating up to 6mm into brain tissue (Tuladhar et al., 2020).

The lack of effect in this study raises a few other questions regarding this experiment. Previous work in the Fouad laboratory has use sustained cortical infusion to target neurotrophin and cAMP pathways with success (Vavrek, 2006; Wei et al., 2016), though it does raise a question of if it is the most appropriate choice for the present pharmacological agents. Other researchers working on targeting EPAC for functional recovery after SCI have targeted the injury site with the same EPAC agonist used in the present study, demonstrating a benefit beyond the cell body of this pathway. It would be worthwhile to determine the optimal location to apply PKA and EPAC agonists to the neuron, either at the soma as done in the present study, or at the site of injury. An *in vitro* experiment utilizing microfluidic chambers separating neuronal soma from their axons and allowing targeted treatment with EPAC and PKA agonists would be ideal to determine if there is a difference depending on the site of treatment with these agonists. It may suggest that it would be more beneficial to target the injury site with dual PKA and EPAC agonists as opposed to the cell body. Furthermore, it may be worthwhile to

consider an alternate means to administer the treatment to the tissue, for example a great deal of work has gone into hydrogel based drug delivery that allows for the infusion of a hydrogel with a drug allowing for only one open surgical site to apply treatment that penetrates tissue (Tuladhar et al., 2020).

Another factor to consider when it comes to the application of these pharmacological agents is the dose itself. While the dose was determined based off of *in vitro* data, and determined as such because of the direct application to cortical neurons being more similar to the *in vitro* experiment and therefore bypassing first pass metabolism, it is possible that the doses used may still be an order of magnitude too low. That being said, experiments prior to the one described here have been performed attempting higher doses of the EPAC and PKA agonists used via direct cortical injection have demonstrated convulsive behaviour in the animals receiving the agonists. As a result, pharmacological manipulation of EPAC and PKA may not be viable due to these factors. Future experiments targeting the cAMP pathway and its downstream effectors PKA and EPAC may benefit from cell autonomous genetic activation of EPAC and PKA through use of a floxed viral vector and cre tagged corticospinal neurons. This would not only remove any side effects of the agonist application, but allow for a much more specific upregulation of these effectors in the cells of interest.

When comparing the present study to our previous experiment aiming to bolster activity dependent signalling through electrical stimulation (Batty et al., 2020), the effects of stimulation augment plasticity and functional motor recovery to a stronger extent than presented in the current study. This is likely because there are a wide array of growth promoting pathways that are upregulated by neuronal activity (and stimulation) such as

the mTOR/PTEN and Jak/Stat signalling cascades (Zareen et al., 2018). The cAMP signalling pathway that is the focus of this study is only one key player amongst a myriad of other important signalling pathways that are elevated by activity. The differences between the present study and stimulation experiments support the use of combinatorial therapies targeting multiple important signalling cascades alongside cAMP signalling upregulation (such as in Lu et al., 2004).

Lastly, this study does suffer from relatively small group sizes after exclusion criteria were applied. While the study presents findings that suggest a treatment effect as seen in the collateral sprouting and the Von Frey analysis, the small remaining group sizes does limit the weight behind the findings and statistical significance would likely arise from repetitions to bolster the group sizes in the study.

5.5 Conclusion

This study follows up on *in vitro* findings reporting a complementary increase in neuron growth when PKA and EPAC agonists are applied, by applying these same agonists to injured corticospinal neurons *in vivo*. While there is no significant difference between groups in terms of functional motor recovery, there are some promising results demonstrated in the dual agonist treated animals. The von frey data suggesting the dual agonist treated animals demonstrate a return to normal nociceptive thresholds and the collaterals that extend into laminae 1-4 hint at a treatment effect that may become apparent with further repetition and larger group sizes.

5.6 Acknowledgements

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5.7 References

Alaverdashvili, M., 2008. “Learned baduse” limits recovery of skilled reaching for food after forelimb motor cortex stroke in rats: A new analysis of the effect of gestures on success. Behav. Brain Res. 188, 281–290. https://doi.org/10.1016/j.bbr.2007.11.007

Alaverdashvili, M., Whishaw, I.Q., 2013. A behavioral method for identifying recovery and compensation: Hand use in a preclinical stroke model using the single pellet reaching task. Neurosci. Biobehav. Rev. 37, 950–967. https://doi.org/10.1016/j.neubiorev.2013.03.026

Bareyre, F.M., Kerschensteiner, M., Raineteau, O., Mettenleiter, T.C., Weinmann, O., Schwab, M.E., 2004. The injured spinal cord spontaneously forms a new intraspinal circuit in adult rats. Nat. Neurosci. 7, 269–277. https://doi.org/10.1038/nn1195

Batty, N.J., Fenrich, K.K., Fouad, K., 2017. The role of cAMP and its downstream targets in neurite growth in the adult nervous system. Neurosci. Lett. 652, 56–63. https://doi.org/10.1016/j.neulet.2016.12.033

Batty, N.J., Torres-Espín, A., Vavrek, R., Raposo, P., Fouad, K., 2020. Single-session cortical electrical stimulation enhances the efficacy of rehabilitative motor training after spinal cord injury in rats. Exp. Neurol. 324, 113136. https://doi.org/10.1016/j.expneurol.2019.113136

Brushart, T.M., Hoffman, P.N., Royall, R.M., Murinson, B.B., Witzel, C., Gordon, T., 2002. Electrical Stimulation Promotes Motoneuron Regeneration without Increasing Its Speed or Conditioning the Neuron. J. Neurosci. 22, 6631–6638.

Brus-Ramer, M., Carmel, J.B., Chakrabarty, S., Martin, J.H., 2007. Electrical Stimulation of Spared Corticospinal Axons Augments Connections with Ipsilateral Spinal Motor Circuits after Injury. J. Neurosci. 27, 13793–13801. https://doi.org/10.1523/JNEUROSCI.3489-07.2007

Cai, D., Qiu, J., Cao, Z., McAtee, M., Bregman, B.S., Filbin, M.T., 2001. Neuronal Cyclic AMP Controls the Developmental Loss in Ability of Axons to Regenerate. J. Neurosci. 21, 4731–4739.

Carmel, J.B., Kimura, H., Martin, J.H., 2014. Electrical Stimulation of Motor Cortex in the Uninjured Hemisphere after Chronic Unilateral Injury Promotes Recovery of Skilled Locomotion through Ipsilateral Control. J. Neurosci. 34, 462–466. https://doi.org/10.1523/JNEUROSCI.3315-13.2014

David, S., Aguayo, A., 1981. Axonal elongation into peripheral nervous system "bridges" after central nervous system injury in adult rats. Science 214, 931–933. https://doi.org/10.1126/science.6171034

Dell'Anno, M.T., Strittmatter, S.M., 2017. Rewiring the spinal cord: Direct and indirect strategies. Neurosci. Lett. 652, 25–34. https://doi.org/10.1016/j.neulet.2016.12.002

Dougherty, K.D., Dreyfus, C.F., Black, I.B., 2000. Brain-Derived Neurotrophic Factor in Astrocytes, Oligodendrocytes, and Microglia/Macrophages after Spinal Cord Injury. Neurobiol. Dis. 7, 574–585. https://doi.org/10.1006/nbdi.2000.0318

Enserink, J.M., Christensen, A.E., de Rooij, J., van Triest, M., Schwede, F., Genieser, H.G., Døskeland, S.O., Blank, J.L., Bos, J.L., 2002. A novel Epac-specific cAMP

analogue demonstrates independent regulation of Rap1 and ERK. Nat. Cell Biol. 4, 901–906. https://doi.org/10.1038/ncb874

Fenrich, K., Gordon, T., 2004. Canadian Association of Neuroscience Review: Axonal Regeneration in the Peripheral and Central Nervous Systems – Current Issues and Advances. Can. J. Neurol. Sci. 31, 142–156. https://doi.org/10.1017/S0317167100053798

Fouad, K., Forero, J., Hurd, C., 2015. A Simple Analogy for Nervous System Plasticity After Injury: Exerc. Sport Sci. Rev. 43, 100–106. https://doi.org/10.1249/JES.0000000000000040

Gao, Y., Nikulina, E., Mellado, W., Filbin, M.T., 2003. Neurotrophins Elevate cAMP to Reach a Threshold Required to Overcome Inhibition by MAG through Extracellular Signal-Regulated Kinase-Dependent Inhibition of Phosphodiesterase. J. Neurosci. 23, 11770–11777. https://doi.org/10.1523/JNEUROSCI.23-37-11770.2003

García-Alías, G., Barkhuysen, S., Buckle, M., Fawcett, J.W., 2009. Chondroitinase ABC treatment opens a window of opportunity for task-specific rehabilitation. Nat. Neurosci. 12, 1145–1151. https://doi.org/10.1038/nn.2377

Geremia, N.M., Gordon, T., Brushart, T.M., Al-Majed, A.A., Verge, V.M.K., 2007. Electrical stimulation promotes sensory neuron regeneration and growth-associated gene expression. Exp. Neurol. 205, 347–359. https://doi.org/10.1016/j.expneurol.2007.01.040

Girgis, J., Merrett, D., Kirkland, S., Metz, G.A.S., Verge, V., Fouad, K., 2007. Reaching training in rats with spinal cord injury promotes plasticity and task specific recovery. Brain 130, 2993–3003. https://doi.org/10.1093/brain/awm245

Gonzenbach, R.R., Gasser, P., Zörner, B., Hochreutener, E., Dietz, V., Schwab, M.E., 2010. Nogo-A antibodies and training reduce muscle spasms in spinal cord-injured rats. Ann. Neurol. 68, 48–57. https://doi.org/10.1002/ana.22009

Gonzenbach, R.R., Schwab, M.E., 2008. Disinhibition of neurite growth to repair the injured adult CNS: Focusing on Nogo. Cell. Mol. Life Sci. 65, 161–176. https://doi.org/10.1007/s00018-007-7170-3

Guijarro-Belmar, A., Viskontas, M., Wei, Y., Bo, X., Shewan, D., Huang, W., 2019. Epac2 Elevation Reverses Inhibition by Chondroitin Sulfate Proteoglycans *In Vitro* and Transforms Postlesion Inhibitory Environment to Promote Axonal Outgrowth in an *Ex Vivo* Model of Spinal Cord Injury. J. Neurosci. 39, 8330–8346. https://doi.org/10.1523/JNEUROSCI.0374-19.2019

Hiebert, G.W., Khodarahmi, K., McGraw, J., Steeves, J.D., 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. J. Neurosci. Res. 69, 160–168. https://doi.org/10.1002/jnr.10275

Hoy, J.J., Salinas Parra, N., Park, J., Kuhn, S., Iglesias-Bartolome, R., 2020. Protein kinase A inhibitor proteins (PKIs) divert GPCR-Gαs-cAMP signaling toward EPAC and ERK activation and are involved in tumor growth. FASEB J. 34, 13900–13917. https://doi.org/10.1096/fj.202001515R

Hurd, C., Weishaupt, N., Fouad, K., 2013. Anatomical correlates of recovery in single pellet reaching in spinal cord injured rats. Exp. Neurol. 247, 605–614. https://doi.org/10.1016/j.expneurol.2013.02.013

Kawaguchi, M., Furuya, H., 2004. Intraoperative spinal cord monitoring of motor function with myogenic motor evoked potentials: a consideration in anesthesia. J. Anesth. 18, 18–28. https://doi.org/10.1007/s00540-003-0201-9

Krajacic, A., Ghosh, M., Puentes, R., Pearse, D.D., Fouad, K., 2009. Advantages of delaying the onset of rehabilitative reaching training in rats with incomplete spinal cord injury. Eur. J. Neurosci. 29, 641–651. https://doi.org/10.1111/j.1460-9568.2008.06600.x

Kusiak, A.N., Selzer, M.E., 2013. Neuroplasticity in the spinal cord, in: Handbook of Clinical Neurology. Elsevier, pp. 23–42. https://doi.org/10.1016/B978-0-444-52901-5.00003-4

Lee, J.-K., Kim, J.-E., Sivula, M., Strittmatter, S., 2004. Nogo Receptor Antagonism Promotes Stroke Recovery by Enhancing Axonal Plasticity. J. Neurosci. 24, 6209–6217. https://doi.org/10.1523/JNEUROSCI.1643-04.2004

Lindau, N.T., Bänninger, B.J., Gullo, M., Good, N.A., Bachmann, L.C., Starkey, M.L., Schwab, M.E., 2014. Rewiring of the corticospinal tract in the adult rat after unilateral stroke and anti-Nogo-A therapy. Brain 137, 739–756. https://doi.org/10.1093/brain/awt336

Lu, P., Yang, H., Jones, L.L., Filbin, M.T., Tuszynski, M.H., 2004. Combinatorial Therapy with Neurotrophins and cAMP Promotes Axonal Regeneration beyond

Sites of Spinal Cord Injury. J. Neurosci. 24, 6402–6409. https://doi.org/10.1523/JNEUROSCI.1492-04.2004

Massey, J.M., Hubscher, C.H., Wagoner, M.R., Decker, J.A., Amps, J., Silver, J., Onifer, S.M., 2006. Chondroitinase ABC Digestion of the Perineuronal Net Promotes Functional Collateral Sprouting in the Cuneate Nucleus after Cervical Spinal Cord Injury. J. Neurosci. 26, 4406–4414. https://doi.org/10.1523/JNEUROSCI.5467-05.2006

May, Z., Fouad, K., Shum-Siu, A., Magnuson, D.S.K., 2015. Challenges of animal models in SCI research: Effects of pre-injury task-specific training in adult rats before lesion. Behav. Brain Res. 291, 26–35. https://doi.org/10.1016/j.bbr.2015.04.058

McKenna, J.E., Whishaw, I.Q., 1999. Complete Compensation in Skilled Reaching Success with Associated Impairments in Limb Synergies, after Dorsal Column Lesion in the Rat. J. Neurosci. 19, 1885–1894. https://doi.org/10.1523/JNEUROSCI.19-05-01885.1999

McKerracher, L., David, S., Jackson, D.L., Kottis, V., Dunn, R.J., Braun, P.E., 1994. Identification of myelin-associated glycoprotein as a major myelin-derived inhibitor of neurite growth. Neuron 13, 805–811. https://doi.org/10.1016/0896-6273(94)90247-X

Metz, G.A.S., Whishaw, I.Q., 2000. Skilled reaching an action pattern: stability in rat (Rattus norvegicus) grasping movements as a function of changing food pellet size. Behav. Brain Res. 116, 111–122. https://doi.org/10.1016/S0166-4328(00)00245-X

Mitchell, E.J., McCallum, S., Dewar, D., Maxwell, D.J., 2016. Corticospinal and Reticulospinal Contacts on Cervical Commissural and Long Descending Propriospinal Neurons in the Adult Rat Spinal Cord; Evidence for Powerful Reticulospinal Connections. PLOS ONE 11, e0152094. https://doi.org/10.1371/journal.pone.0152094

Murray, A.J., 2008. Pharmacological PKA Inhibition: All May Not Be What It Seems. Sci. Signal. 1, re4–re4. https://doi.org/10.1126/scisignal.122re4

Murray, A.J., Shewan, D.A., 2008. Epac mediates cyclic AMP-dependent axon growth, guidance and regeneration. Mol. Cell. Neurosci. 38, 578–588. https://doi.org/10.1016/j.mcn.2008.05.006

Murray, A.J., Tucker, S.J., Shewan, D.A., 2009. cAMP-Dependent Axon Guidance Is Distinctly Regulated by Epac and Protein Kinase A. J. Neurosci. 29, 15434–15444. https://doi.org/10.1523/JNEUROSCI.3071-09.2009

Pearse, D.D., Pereira, F.C., Marcillo, A.E., Bates, M.L., Berrocal, Y.A., Filbin, M.T., Bunge, M.B., 2004. cAMP and Schwann cells promote axonal growth and functional recovery after spinal cord injury. Nat. Med. 10, 610–616. https://doi.org/10.1038/nm1056

R Core Team, 2018. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.

Schneider, C.A., Rasband, W.S., Eliceiri, K.W., 2012. NIH Image to ImageJ: 25 years of image analysis. Nat. Methods 9, 671.

Schwab, M.E., Bartholdi, D., 1996. Degeneration and Regeneration of Axons in the Lesioned Spinal Cord. Physiol. Rev. 76, 319–370.

Torres-Espín, A., Beaudry, E., Fenrich, K., Fouad, K., 2018a. Rehabilitative Training in Animal Models of Spinal Cord Injury. J. Neurotrauma 35, 1970–1985. https://doi.org/10.1089/neu.2018.5906

Torres-Espín, A., Forero, J., Fenrich, K.K., Lucas-Osma, A.M., Krajacic, A., Schmidt, E., Vavrek, R., Raposo, P., Bennett, D.J., Popovich, P.G., Fouad, K., 2018b. Eliciting inflammation enables successful rehabilitative training in chronic spinal cord injury. Brain 141, 1946–1962. https://doi.org/10.1093/brain/awy128

Torres-Espín, A., Forero, J., Schmidt, E.K.A., Fouad, K., Fenrich, K.K., 2018c. A motorized pellet dispenser to deliver high intensity training of the single pellet reaching and grasping task in rats. Behav. Brain Res. 336, 67–76. https://doi.org/10.1016/j.bbr.2017.08.033

Tuladhar, A., Obermeyer, J.M., Payne, S.L., Siu, R.C.W., Zand, S., Morshead, C.M., Shoichet, M.S., 2020. Injectable hydrogel enables local and sustained co-delivery to the brain: Two clinically approved biomolecules, cyclosporine and erythropoietin, accelerate functional recovery in rat model of stroke. Biomaterials 235, 119794. https://doi.org/10.1016/j.biomaterials.2020.119794

Udina, E., Furey, M., Busch, S., Silver, J., Gordon, T., Fouad, K., 2008. Electrical stimulation of intact peripheral sensory axons in rats promotes outgrowth of their central projections. Exp. Neurol. 210, 238–247. https://doi.org/10.1016/j.expneurol.2007.11.007

Vavrek, R., 2006. BDNF promotes connections of corticospinal neurons onto spared descending interneurons in spinal cord injured rats. Brain 129, 1534–1545. https://doi.org/10.1093/brain/awl087

von Bernhardi, R., Bernhardi, L.E., Eugenín, J., 2017. What Is Neural Plasticity?, in: von Bernhardi, R., Eugenín, J., Muller, K.J. (Eds.), The Plastic Brain. Springer International Publishing, Cham, pp. 1–15. https://doi.org/10.1007/978-3-319-62817-2_1

Wei, D., Hurd, C., Galleguillos, D., Singh, J., Fenrich, K.K., Webber, C.A., Sipione, S., Fouad, K., 2016. Inhibiting cortical protein kinase A in spinal cord injured rats enhances efficacy of rehabilitative training. Exp. Neurol. 283, 365–374. https://doi.org/10.1016/j.expneurol.2016.07.001

Weishaupt, N., Li, S., Di Pardo, A., Sipione, S., Fouad, K., 2013. Synergistic effects of BDNF and rehabilitative training on recovery after cervical spinal cord injury. Behav. Brain Res. 239, 31–42. https://doi.org/10.1016/j.bbr.2012.10.047

Whishaw, I.Q., O'Connor, W.T., Dunnett, S.B., 1986. THE CONTRIBUTIONS OF MOTOR CORTEX, NIGROSTRIATAL DOPAMINE AND CAUDATE-PUTAMEN TO SKILLED FORELIMB USE IN THE RAT. Brain 109, 805–843. https://doi.org/10.1093/brain/109.5.805

Widenfalk, J., Lundströmer, K., Jubran, M., Brené, S., Olson, L., 2001. Neurotrophic Factors and Receptors in the Immature and Adult Spinal Cord after Mechanical Injury or Kainic Acid. J. Neurosci. 21, 3457–3475.

Zareen, N., Dodson, S., Armada, K., Awad, R., Sultana, N., Hara, E., Alexander, H., Martin, J.H., 2018. Stimulation-dependent remodeling of the corticospinal tract requires reactivation of growth-promoting developmental signaling pathways. Exp. Neurol. 307, 133–144. https://doi.org/10.1016/j.expneurol.2018.05.004

Chapter 6

Critical Discussion of the Work

6.1 Discussion of Chapter 2: Single-Session Cortical Electrical Stimulation Enhances the Efficacy of Rehabilitative Motor Training after Spinal Cord Injury in Rats

6.1.1 Summary of Results and Conclusion

Chapter 2 examined the effects that a single session of intracortical electrical stimulation has on injured CST cell bodies after SCI. Through this experiment I demonstrated that a single session of electrical stimulation post injury is sufficient to increase the functional recovery of injured rats following rehabilitative motor therapy, and that it increases plasticity of the CST above the site of injury. This introduces the idea that, much like the peripheral nervous system, we can electrically stimulate injured CNS fibers to encourage plastic changes, and only a single session is needed to produce significant effects. As of writing, this experiment has only been done using intracortical stimulation, but the data presented do suggest that a single session of stimulation post SCI may be a viable surgical intervention option.

6.1.2 Limitations and Future Directions

This experiment showed significant results in terms of improving functional recovery after SCI, but it does have limitations that need to be acknowledged. First and foremost was the lack of a group that did not undergo rehabilitative motor therapy. While it is undoubtedly true that rehabilitative motor therapy is the treatment of choice in the clinic with human patients, therefore justifying the use of training in all groups in this experiment, it does nevertheless limit the interpretation of how the stimulation is improving plasticity. Including in this experiment a group that received stimulation

without rehabilitative therapy would have allowed for comparisons against both the stimulated group that underwent motor training and the trained unstimulated control to fully analyze the impacts of training and stimulation separately. This would thus allow for more insight into how stimulation would be boosting activity dependent plasticity as opposed to simply encouraging plasticity on its own. Comparing a trained non-stimulated group to the trained and stimulated animals may also reveal if there are changes in cortical plasticity as a result of the stimulation that would impact the animal's motivation to train, thus influencing their final functional outcomes. Although one could look at the results from the previous stimulation study from the Fouad lab (Jack et al., 2018) for the effects of stimulation without training, it is important to remember that the timings of the stimulation differ between this previous study and my own, and furthermore there was no functional motor therapy in the Jack et al. study with which to compare results. As such, a future repetition including an untrained group that receives stimulation after injury would provide more concrete evidence for the effects seen in my experiment.

Another additional control to be considered in a potential repeat of this experiment is the inclusion of a prolonged stimulation group. Specifically, one that received stimulation daily over a week, as in the study by Goganau et al. (2018), which was key in the design of my experiment. This would allow for a better understanding of the impact of a single session of stimulation, because if it were to result in the same extent of recovery, it would further solidify the conclusion that a single session of stimulation is as effective as prolonged stimulation, as concluded by Goganau et al (2018). Although my experiment provided convincing evidence for the benefits of a

single session of stimulation, it does not rule out alternative stimulation paradigms that may prove more effective.

Furthermore, the study presented in chapter 2 only examined the plastic changes of the injured CST, and did not trace the contralateral CST, or other components of the CST in the ventral and lateral regions of the spinal cord. As was brought up in chapter 2, these other components of the CST have been shown to increase sprouting after lesion to the dorsal CST (Weidner et al., 2001). The contralesional CST has also been shown to undergo plastic changes and have fibers that cross over to the other side which can play a role in functional recovery (Brus-Ramer et al., 2007; Carmel et al., 2014, 2013, 2010). The study presented in Chapter 2, having not analyzed these other aspects of CST plasticity, missed a valuable opportunity.

Another aspect of this experiment that follow up studies should consider is the potential for the tungsten electrodes and the current applied to cause damage or inflammation to the cortex during the stimulation. It is possible that the relatively high current used may cause pH changes in the surrounding area, damaging tissue and resulting in microglial changes that could heavily impact plasticity. An analysis of the microglial profile the area surrounding the site of stimulation in future repetitions would allow experimenters to determine how much of a role inflammation may play in the effects seen in this experiment.

While the notion of a single session of stimulation being sufficient to improve recovery may make an invasive treatment seem more viable as opposed to treatments requiring multiple surgeries, it is still far from ideal. Future steps with cortical stimulation to improve recovery after SCI should start to explore means of stimulation that are less

invasive. A logical follow-up study would include repeating my experiment with the addition of a group of animals receiving transdural stimulation, using ball electrodes, to compare how the delivery of stimulation may change its effect. Assuming transdural stimulation is able to deliver similar results to the intended target without stimulating extraneous brain regions, a move to even less invasive means such as transcranial magnetic stimulation (TMS) would be the following step to take this notion of boosting activity in injured CNS neurons in order to improve their plastic potential.

From a research perspective to further explore the mechanisms of activity dependent plasticity, repeating the stimulation with different tools such as optogenetics or Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) would explore alternate means to elicit neuronal activity and improve recovery. These tools would allow for a less invasive means to perform prolonged stimulation paradigms and compare the effects of these to a single stimulation session to further the findings of my study.

6.2 Discussion of Chapter 4: Simultaneous Activation of the cAMP Downstream Effectors PKA and EPAC Bolsters Neurite Outgrowth

6.2.1 Summary of Results and Conclusion

Chapter 4 explored the effects of EPAC activation alongside PKA agonism or antagonism, demonstrating remarkable complementary effects on neurite outgrowth in both cases. However, cells receiving EPAC and PKA agonists together resulted in an increase in cell density and apparent neurite diameter compared to those that received the

PKA antagonist with the EPAC agonist. A complimentary effect from dual agonism of EPAC and PKA is novel to cortical neurons but synergy from dual agonism has been demonstrated in PC12 cells (Christensen et al., 2003). While there are many limitations to this study, as will be discussed below, it does suggest that application of agonists for both downstream effectors of cAMP could serve as a means to further promote neurite extension and plasticity in the cell following SCI. As such, the effects seen *in vitro* were motivation for me to test the effects *in vivo*, as was done in chapter 5 of this thesis.

6.2.2 Limitations and Future Directions

This study demonstrated significant complementary effect (and is supported by the literature in PC12 cells (Christensen et al., 2003)), but there were some clear limitations. This study relied primarily on image analysis for its findings with little advanced analysis techniques to validate the visual differences between groups. While the findings are arguably convincing, analyzing pCREB protein levels via Western blots (as was done by Wei et al. (2016)) at the very least would validate the visual differences between groups and further the conclusions of this study. Future iterations of this study aiming to further explore the mechanism of cAMP signalling should include the additional analysis of regeneration associated genes (RAGs) such as GAP-43 and Tα1-tubulin (as done in Kobayashi et al., 1997; Kwon et al., 2002), as this would further confirm growth findings by demonstrating increases in RAGs.

Other morphological analyses would also serve to expand the scope of this study. For instance, the use of live imaging to show the growth rate of neurites after application of the agonists over 24 hours, or up to a week, would explore more of the mechanism of

how the dual agonist treatment works, differentiating between an increase in growth rate and final growth amount. Lower density cell cultures so that neurite diameter and branching angles could be reliably analyzed to determine further morphological changes would be ideal. Additionally, although my study discussed the idea that cellular polarization is likely to be increased in dual agonism, it did not have a metric for polarization. A repetition of this experiment with either a MAP2-tau antibody or the inclusion of an NF200 stain to differentiate between dendrites and axons would allow for a concrete polarization analysis, resulting in a more well-rounded conclusion.

In my study, the siRNA were confirmed to be effective at knocking down their target proteins via immunocytochemistry, which is arguably not the ideal confirmation of siRNA knockdown. A RT-PCR to confirm the absence of the target mRNA would offer a quantitative and thus more appropriate approach. As the siRNA impacted the health of the neuron cultures, future experiments will need to be done to optimize the delivery of these agents to ensure they can be effective at knocking down their target GTPases whilst not compromising health of the cells.

6.3 Discussion of Chapter 5: Cortical Infusion of PKA and EPAC Agonists and Rehabilitative Motor Training After Cervical Spinal Cord Injury in Female Rats

6.3.1 Summary of Results and Conclusion

Chapter 5 followed up on my novel findings from chapter 4 and applied the PKA and EPAC agonists that resulted in elevated growth *in vitro* to the motor forelimb cortex of rats with SCI in hopes to improve CST plasticity and functional recovery when

combined with motor therapy. While this study was unable to confirm my hypothesis that this treatment would result in increases in plasticity of the CST and in motor recovery, it does provide direction for future experimentation to fine tune the methodology before conducting further experiments testing dual agonism *in vivo*.

6.3.2 Limitations and Future Directions

The study presented in chapter 5 followed established methodology to target cell bodies presented in previous studies from the Fouad laboratory (Vavrek et al., 2006; Wei et al., 2016) and colleagues (Hiebert et al., 2002), but there were a number of limitations that should be addressed in future repetitions of this experiment. As iterated in chapter 5, not all of the pumps could be confirmed to be connected at the time of removal, introducing the possibility that either some of the pumps were disconnected for an extended period or they simply came loose during removal surgery. As the silicone tubing is connected to the cannula without adhesive, it is possible that it could have come off while the animals were going about their daily activities. Some means to secure the tubing to the cannula would remove future doubt over how well they remained connected.

The MEP analysis in this experiment was arguably one of its weakest components, as there were a series of limitations that need to be addressed. First, additional controls would have allowed for a much more thorough analysis of individual animal responses. In this experiment, the unaffected cortex was stimulated to determine motor threshold in the unaffected limb, but MEP recordings in the unaffected limb for each animal were not recorded. Recording the unaffected limb would have allowed for a

direct comparison to the injured limb in terms of latency, as well as possibly amplitude analysis within animals. Second, amplitude is heavily impacted by anaesthesia (Kawaguchi and Furuya, 2004), and it is impossible to ensure and confirm a consistent plane of anaesthesia between animals at the time of recording, rendering amplitude comparison largely without meaning. As these recordings were performed using differential EMG, the distance between electrodes in the forelimb would also impact amplitude, and inter-animal consistency was not checked in this experiment. Overall, the lack of uninjured control traces, as well as recording instability, significantly limit what the MEP data presented in this experiment can tell us.

Drug doses used in this chapter were based on those used in chapter 4; this experiment utilized direct application of the drugs to the neuron, eliminating the need for dose compensation due to metabolism and distribution. While this rationalization has merit, it does not remove the need for proper testing beforehand in order to ensure that the doses used were appropriate. Testing the effect of various concentrations of EPAC agonist against various concentrations of PKA agonist would allow for a more detailed understanding of their plasticity promoting interactions. While this would be fairly complicated and resource intensive, future iterations of this experiment should first and foremost find a dose that results in the optimal increase in CST plasticity.

Neither my experiment presented in chapter 5, nor the *in vitro* background for this study in chapter 4, addressed the question of target site *in vivo*. The *in vitro* experiment applies the drugs through the growth media so that the cells are bathed in the treatment and there is no specificity for soma or axon; the *in vivo* experiment, as previously mentioned, follows up on previous work in the Fouad laboratory (Hiebert et al., 2002;

Vavrek et al., 2006; Wei et al., 2016), and therefore the agonists are only applied to the soma of injured cells. This is despite evidence that EPAC has chemoattractant properties (Murray et al., 2009), prompting other researchers investigating its potential use after SCI to apply their EPAC agonists at the lesion (Guijarro-Belmar et al., 2019).

Therefore, the first step I would propose to move this experiment forward would be a return to *in vitro* to test how targeted application of the EPAC and PKA agonists at the soma or to the axons influences outgrowth. This can be done using microfluidic chambers to allow for plating of neurons on half of the device, and as they grow, tunnels between chambers would encourage axon growth to the other side of the device. Due to the way fluid physics behaves in tiny spaces such as in these devices, the two sides of the chamber are fluidically isolated from each other so long as the total volume of that side is slightly lower than the other (Park et al., 2009). The addition of a drug to one side (soma or axon) of the device results in the drug being unable to diffuse to the other side. This approach would allow for targeted application of EPAC and PKA agonists at the soma, axon, or combinations thereof. The results of this study would heavily influence future *in vivo* attempts.

Once the target site and drug dose are confirmed, future researchers may also want to consider other means of drug administration. One such alternative would be an injectable, drug infused hydrogel that would slowly release the agonists into and through the tissue (Tuladhar et al., 2020). Unlike the cannula, the hydrogel would likely not need to be surgically removed, necessitating fewer surgeries, and there would be less concern regarding discontinuous or premature cessation of drug administration. Overall, the goal

should be to find an easier and more reliable way to deliver the EPAC and PKA agonists to the target site.

6.4 Overall Summary and Conclusion

This thesis presented a series of experiments and literature reviews to introduce spinal cord injury and the idea of augmenting activity dependent plasticity to improve recovery after SCI. The first experiment presented in chapter 2 demonstrated that it is possible to stimulate injured CST fibers to encourage plasticity and motor recovery in conjunction with rehabilitative motor therapy with a single session of stimulation. Chapter 3 presented a literature review to connect activity dependent plasticity to cAMP and its downstream effectors EPAC and PKA, which were the targets of the rest of the thesis. Following the introduction of the cAMP pathway, chapter 4 represented a proof-of-concept study using a cell culture protocol I developed with the help of the laboratory of Dr. Sipione. In this experiment, I presented data that showed that activating both PKA and EPAC simultaneously created a complementary effect that increased neuronal growth potential, something not previously demonstrated in cortical neurons but supported by findings in other cells (Christensen et al., 2003). Based on this finding, an *in vivo* experiment was performed and presented in chapter 5, where I applied the same agonists used *in vitro* to the motor forelimb cortex of animals with incomplete SCI in conjunction with rehabilitative motor therapy. This experiment did not present significant findings to suggest that the treatment had any effect compared to saline animals or animals that received only the EPAC agonist. This is believed to be due to methodology confounds such as low group size, rather than a lack of effect of the agonists applied, as there are

some promising results presented. The experiment did present opportunities to improve upon the study and further this project, by ensuring due diligence is being done.

This thesis serves as a contribution to the ever-expanding exploration of the utilization and augmentation of plasticity in the central nervous system to improve motor recovery after incomplete spinal cord injury. Significant contributions to science were made including the presentation of data suggesting that a single session of electrical stimulation is effective at promoting motor recovery after SCI. The results of the *in vitro* work included in this thesis suggests the potential for a combined application of PKA and EPAC agonists to increase neurite outgrowth and, hopefully one day, CNS plasticity after injury. Further research is required before the work presented in this thesis would be able to move towards being a clinically viable treatment. This includes developing less invasive stimulation parameters and delivery, repeating *in vitro* experiments for validation, and fine-tuning application of EPAC and PKA agonists to injured CST neurons *in vivo*. While the cAMP pathway is but one of many pathways upregulated by activity in neurons, the investigation into its effects on CNS plasticity could be a key piece in the puzzle that is repairing the injured spinal cord.

6.5 References

Brus-Ramer, M., Carmel, J.B., Chakrabarty, S., Martin, J.H., 2007. Electrical Stimulation of Spared Corticospinal Axons Augments Connections with Ipsilateral Spinal Motor Circuits after Injury. J. Neurosci. 27, 13793–13801. https://doi.org/10.1523/JNEUROSCI.3489-07.2007

Carmel, J.B., Berrol, L.J., Brus-Ramer, M., Martin, J.H., 2010. Chronic Electrical Stimulation of the Intact Corticospinal System after Unilateral Injury Restores Skilled Locomotor Control and Promotes Spinal Axon Outgrowth. J. Neurosci. 30, 10918–10926. https://doi.org/10.1523/JNEUROSCI.1435-10.2010

Carmel, J.B., Kimura, H., Berrol, L.J., Martin, J.H., 2013. Motor cortex electrical stimulation promotes axon outgrowth to brain stem and spinal targets that control the forelimb impaired by unilateral corticospinal injury. Eur. J. Neurosci. 37, 1090–1102. https://doi.org/10.1111/ejn.12119

Carmel, J.B., Kimura, H., Martin, J.H., 2014. Electrical Stimulation of Motor Cortex in the Uninjured Hemisphere after Chronic Unilateral Injury Promotes Recovery of Skilled Locomotion through Ipsilateral Control. J. Neurosci. 34, 462–466. https://doi.org/10.1523/JNEUROSCI.3315-13.2014

Christensen, A.E., Selheim, F., de Rooij, J., Dremier, S., Schwede, F., Dao, K.K., Martinez, A., Maenhaut, C., Bos, J.L., Genieser, H.-G., Døskeland, S.O., 2003. cAMP Analog Mapping of Epac1 and cAMP Kinase. J. Biol. Chem. 278, 35394–35402. https://doi.org/10.1074/jbc.M302179200

Goganau, I., Sandner, B., Weidner, N., Fouad, K., Blesch, A., 2018. Depolarization and electrical stimulation enhance in vitro and in vivo sensory axon growth after

spinal cord injury. Exp. Neurol. 300, 247–258. https://doi.org/10.1016/j.expneurol.2017.11.011

Guijarro-Belmar, A., Viskontas, M., Wei, Y., Bo, X., Shewan, D., Huang, W., 2019. Epac2 Elevation Reverses Inhibition by Chondroitin Sulfate Proteoglycans *In Vitro* and Transforms Postlesion Inhibitory Environment to Promote Axonal Outgrowth in an *Ex Vivo* Model of Spinal Cord Injury. J. Neurosci. 39, 8330–8346. https://doi.org/10.1523/JNEUROSCI.0374-19.2019

Hiebert, G.W., Khodarahmi, K., McGraw, J., Steeves, J.D., 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. J. Neurosci. Res. 69, 160–168. https://doi.org/10.1002/jnr.10275

Jack, A.S., Hurd, C., Forero, J., Nataraj, A., Fenrich, K., Blesch, A., Fouad, K., 2018. Cortical electrical stimulation in female rats with a cervical spinal cord injury to promote axonal outgrowth. J. Neurosci. Res. 96, 852–862. https://doi.org/10.1002/jnr.24209

Kawaguchi, M., Furuya, H., 2004. Intraoperative spinal cord monitoring of motor function with myogenic motor evoked potentials: a consideration in anesthesia. J. Anesth. 18, 18–28. https://doi.org/10.1007/s00540-003-0201-9

Kobayashi, N.R., Fan, D.-P., Giehl, K.M., Bedard, A.M., Wiegand, S.J., Tetzlaff, W., 1997. BDNF and NT-4/5 Prevent Atrophy of Rat Rubrospinal Neurons after Cervical Axotomy, Stimulate GAP-43 and Tα1-Tubulin mRNA Expression, and Promote Axonal Regeneration. J. Neurosci. 17, 9583–9595. https://doi.org/10.1523/JNEUROSCI.17-24-09583.1997

Kwon, B.K., Liu, J., Messerer, C., Kobayashi, N.R., McGraw, J., Oschipok, L., Tetzlaff, W., 2002. Survival and regeneration of rubrospinal neurons 1 year after spinal cord injury. Proc. Natl. Acad. Sci. 99, 3246–3251. https://doi.org/10.1073/pnas.052308899

Murray, A.J., Tucker, S.J., Shewan, D.A., 2009. cAMP-Dependent Axon Guidance Is Distinctly Regulated by Epac and Protein Kinase A. J. Neurosci. 29, 15434–15444. https://doi.org/10.1523/JNEUROSCI.3071-09.2009

Park, J., Koito, H., Li, J., Han, A., 2009. Microfluidic compartmentalized co-culture platform for CNS axon myelination research. Biomed. Microdevices 11, 1145–1153. https://doi.org/10.1007/s10544-009-9331-7

Tuladhar, A., Obermeyer, J.M., Payne, S.L., Siu, R.C.W., Zand, S., Morshead, C.M., Shoichet, M.S., 2020. Injectable hydrogel enables local and sustained co-delivery to the brain: Two clinically approved biomolecules, cyclosporine and erythropoietin, accelerate functional recovery in rat model of stroke. Biomaterials 235, 119794. https://doi.org/10.1016/j.biomaterials.2020.119794

Vavrek, R., Girgis, J., Tetzlaff, W., Hiebert, G.W., Fouad, K., 2006. BDNF promotes connections of corticospinal neurons onto spared descending interneurons in spinal cord injured rats. Brain 129, 1534–1545. https://doi.org/10.1093/brain/awl087

Wei, D., Hurd, C., Galleguillos, D., Singh, J., Fenrich, K.K., Webber, C.A., Sipione, S., Fouad, K., 2016. Inhibiting cortical protein kinase A in spinal cord injured rats enhances efficacy of rehabilitative training. Exp. Neurol. 283, 365–374. https://doi.org/10.1016/j.expneurol.2016.07.001

Weidner, N., Ner, A., Salimi, N., Tuszynski, M.H., 2001. Spontaneous corticospinal axonal plasticity and functional recovery after adult central nervous system injury. Proc. Natl. Acad. Sci. 98, 3513–3518. https://doi.org/10.1073/pnas.051626798

Complete Bibliography

Ahuja, C.S., Nori, S., Tetreault, L., Wilson, J., Kwon, B., Harrop, J., Choi, D., Fehlings, M.G., 2017. Traumatic Spinal Cord Injury—Repair and Regeneration. Neurosurgery 80, S9–S22. https://doi.org/10.1093/neuros/nyw080

Alaverdashvili, M., 2008. "Learned baduse" limits recovery of skilled reaching for food after forelimb motor cortex stroke in rats: A new analysis of the effect of gestures on success. Behavioural Brain Research 188, 281–290. https://doi.org/10.1016/j.bbr.2007.11.007

Alaverdashvili, M., Whishaw, I.Q., 2013. A behavioral method for identifying recovery and compensation: Hand use in a preclinical stroke model using the single pellet reaching task. Neuroscience & Biobehavioral Reviews 37, 950–967. https://doi.org/10.1016/j.neubiorev.2013.03.026

Anderson, K.D., 2004. Targeting Recovery: Priorities of the Spinal Cord-Injured Population. Journal of Neurotrauma 21, 1371–1383. https://doi.org/10.1089/neu.2004.21.1371

Andreae, L.C., Burrone, J., 2014. The role of neuronal activity and transmitter release on synapse formation. Current Opinion in Neurobiology 27, 47–52. https://doi.org/10.1016/j.conb.2014.02.008

Asanuma, H., Rosen, I., 1972. Topographical organization of cortical efferent zones projecting to distal forelimb muscles in the monkey. Exp Brain Res 14, 243–256. https://doi.org/10.1007/BF00816161

Awad, B.I., Carmody, M.A., Steinmetz, M.P., 2015. Potential Role of Growth Factors in the Management of Spinal Cord Injury. World Neurosurgery 83, 120–131. https://doi.org/10.1016/j.wneu.2013.01.042

Baguley, I., 2008. Autonomic Complications Following Central Nervous System Injury. Semin Neurol 28, 716–725. https://doi.org/10.1055/s-0028-1105971

Balentine, J.D., 1978. Pathology of experimental spinal cord trauma. I. The necrotic lesion as a function of vascular injury. Lab Invest 39, 236–253.

Barbeau, H., Rossignol, S., 1987. Recovery of locomotion after chronic spinalization in the adult cat. Brain Research 412, 84–95. https://doi.org/10.1016/0006-8993(87)91442-9

Bareyre, F.M., Kerschensteiner, M., Raineteau, O., Mettenleiter, T.C., Weinmann, O., Schwab, M.E., 2004. The injured spinal cord spontaneously forms a new intraspinal circuit in adult rats. Nat Neurosci 7, 269–277. https://doi.org/10.1038/nn1195

Battistuzzo, C.R., Callister, R.J., Callister, R., Galea, M.P., 2012. A Systematic Review of Exercise Training To Promote Locomotor Recovery in Animal Models of

Spinal Cord Injury. Journal of Neurotrauma 29, 1600–1613. https://doi.org/10.1089/neu.2011.2199

Batty, N.J., Fenrich, K.K., Fouad, K., 2017. The role of cAMP and its downstream targets in neurite growth in the adult nervous system. Neuroscience Letters 652, 56–63. https://doi.org/10.1016/j.neulet.2016.12.033

Batty, N.J., Torres-Espín, A., Vavrek, R., Raposo, P., Fouad, K., 2020. Single-session cortical electrical stimulation enhances the efficacy of rehabilitative motor training after spinal cord injury in rats. Experimental Neurology 324, 113136. https://doi.org/10.1016/j.expneurol.2019.113136

Beattie, M.S., Bresnahan, J.C., Komon, J., Tovar, C.A., Van Meter, M., Anderson, D.K., Faden, A.I., Hsu, C.Y., Noble, L.J., Salzman, S., Young, W., 1997. Endogenous Repair after Spinal Cord Contusion Injuries in the Rat. Experimental Neurology 148, 453–463. https://doi.org/10.1006/exnr.1997.6695

Beattie, M.S., Farooqui, A.A., Bresnahan, J.C., 2000. Review of Current Evidence for Apoptosis After Spinal Cord Injury. Journal of Neurotrauma 17, 915–925. https://doi.org/10.1089/neu.2000.17.915

Beekhuizen, K.S., Field-Fote, E.C., 2005. Massed Practice versus Massed Practice with Stimulation: Effects on Upper Extremity Function and Cortical Plasticity in Individuals with Incomplete Cervical Spinal Cord Injury. Neurorehabil Neural Repair 19, 33–45. https://doi.org/10.1177/1545968305274517

Behrman, A.L., Ardolino, E.M., Harkema, S.J., 2017. Activity-Based Therapy: From Basic Science to Clinical Application for Recovery After Spinal Cord Injury. Journal of Neurologic Physical Therapy 41, S39–S45. https://doi.org/10.1097/NPT.0000000000000184

Bell, J.A., Wolke, M.L., Ortez, R.C., Jones, T.A., Kerr, A.L., 2015. Training Intensity Affects Motor Rehabilitation Efficacy Following Unilateral Ischemic Insult of the Sensorimotor Cortex in C57BL/6 Mice. Neurorehabil Neural Repair 29, 590–598. https://doi.org/10.1177/1545968314553031

Berry, M., 1982. Post-injury myelin-breakdown products inhibit axonal growth: an hypothesis to explain the failure of axonal regeneration in the mammalian central nervous system. Bibl Anat 1–11.

Bertrand, S., Cazalets, J.-R., 2013. Activity-dependent Synaptic Plasticity and Metaplasticity in Spinal Motor Networks. CPD 19, 4498–4508. https://doi.org/10.2174/1381612811319240014

Bestmann, S., Walsh, V., 2017. Transcranial electrical stimulation. Current Biology 27, R1258–R1262. https://doi.org/10.1016/j.cub.2017.11.001

Bethea, J.R., 2000. Spinal cord injury-induced inflammation: A dual-edged sword, in: Progress in Brain Research. Elsevier, pp. 33–42. https://doi.org/10.1016/S0079-6123(00)28005-9

Birukova, A.A., Burdette, D., Moldobaeva, N., Xing, J., Fu, P., Birukov, K.G., 2010. Rac GTPase is a hub for protein kinase A and Epac signaling in endothelial barrier protection by cAMP. Microvascular Research 79, 128–138. https://doi.org/10.1016/j.mvr.2009.11.007

Blight, A.R., 1985. Delayed Demyelination and Macrophage Invasion: A Candidate for Secondary Cell Damage in Spinal Cord Injury. Central Nervous System Trauma 2, 299–315. https://doi.org/10.1089/cns.1985.2.299

Bloom, O., Herman, P.E., Spungen, A.M., 2020. Systemic inflammation in traumatic spinal cord injury. Experimental Neurology 325, 113143. https://doi.org/10.1016/j.expneurol.2019.113143

Bokoch, G.M., 1993. Biology of the Rap proteins, members of the ras superfamily of GTP-binding proteins. Biochemical Journal 289, 17–24. https://doi.org/10.1042/bj2890017

Boomkamp, S.D., McGrath, M.A., Houslay, M.D., Barnett, S.C., 2014. Epac and the high affinity rolipram binding conformer of PDE4 modulate neurite outgrowth and myelination using an *in vitro* spinal cord injury model: Rho/ROCK and cAMP effects on SCI cultures. Br J Pharmacol 171, 2385–2398. https://doi.org/10.1111/bph.12588

Bregman, B.S., Broude, E., McAtee, M., Kelley, M.S., 1998. Transplants and Neurotrophic Factors Prevent Atrophy of Mature CNS Neurons after Spinal Cord Injury. Experimental Neurology 149, 13–27. https://doi.org/10.1006/exnr.1997.6669

Bregman, B.S., McAtee, M., Dai, H.N., Kuhn, P.L., 1997. Neurotrophic Factors Increase Axonal Growth after Spinal Cord Injury and Transplantation in the Adult Rat. Experimental Neurology 148, 475–494. https://doi.org/10.1006/exnr.1997.6705

Brown, D.J., Hill, S.T., Baker, H.W.G., 2006. Male fertility and sexual function after spinal cord injury, in: Progress in Brain Research. Elsevier, pp. 427–439. https://doi.org/10.1016/S0079-6123(05)52029-6

Bruce, J.H., Norenberg, M.D., Kraydieh, S., Puckett, W., Marcillo, A., Dietrich, D., 2000. Schwannosis: Role of Gliosis and Proteoglycan in Human Spinal Cord Injury. Journal of Neurotrauma 17, 781–788. https://doi.org/10.1089/neu.2000.17.781

Brushart, T.M., Hoffman, P.N., Royall, R.M., Murinson, B.B., Witzel, C., Gordon, T., 2002. Electrical Stimulation Promotes Motoneuron Regeneration without Increasing Its Speed or Conditioning the Neuron. J. Neurosci. 22, 6631–6638. https://doi.org/10.1523/JNEUROSCI.22-15-06631.2002

Brus-Ramer, M., Carmel, J.B., Chakrabarty, S., Martin, J.H., 2007. Electrical Stimulation of Spared Corticospinal Axons Augments Connections with Ipsilateral Spinal

Motor Circuits after Injury. Journal of Neuroscience 27, 13793–13801. https://doi.org/10.1523/JNEUROSCI.3489-07.2007

Bunge, R.P., Puckett, W.R., Becerra, J.L., Marcillo, A., 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. Adv Neurol 59, 75–89.

Buss, A., Pech, K., Kakulas, B.A., Martin, D., Schoenen, J., Noth, J., Brook, G.A., 2006. Growth-modulating molecules are associated with invading Schwann cells and not astrocytes in human traumatic spinal cord injury. Brain 130, 940–953. https://doi.org/10.1093/brain/awl374

Cafferty, W.B.J., McGee, A.W., Strittmatter, S.M., 2008. Axonal growth therapeutics: regeneration or sprouting or plasticity? Trends in Neurosciences 31, 215–220. https://doi.org/10.1016/j.tins.2008.02.004

Cai, D., Deng, K., Mellado, W., Lee, J., Ratan, R.R., Filbin, M.T., 2002. Arginase I and Polyamines Act Downstream from Cyclic AMP in Overcoming Inhibition of Axonal Growth MAG and Myelin In Vitro. Neuron 35, 711–719. https://doi.org/10.1016/S0896-6273(02)00826-7

Cai, D., Qiu, J., Cao, Z., McAtee, M., Bregman, B.S., Filbin, M.T., 2001. Neuronal Cyclic AMP Controls the Developmental Loss in Ability of Axons to Regenerate. J. Neurosci. 21, 4731–4739. https://doi.org/10.1523/JNEUROSCI.21-13-04731.2001

Cai, D., Shen, Y., De Bellard, M., Tang, S., 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. https://doi.org/10.1016/S0896-6273(00)80681-9

Cajal, S.R. y, May, R.M., 1991. Cajal's Degeneration and Regeneration of the Nervous System. Oxford University Press. https://doi.org/10.1093/acprof:oso/9780195065169.001.0001

Canty, A.J., Murphy, M., 2008. Molecular mechanisms of axon guidance in the developing corticospinal tract. Progress in Neurobiology 85, 214–235. https://doi.org/10.1016/j.pneurobio.2008.02.001

Cao, Z., Gao, Y., Bryson, J.B., Hou, J., Chaudhry, N., Siddiq, M.M., Martinez, J., Spencer, T., Carmel, J.B., Hart, R.P., Filbin, M.T., 2006. The Cytokine Interleukin-6 Is Sufficient But Not Necessary to Mimic the Peripheral Conditioning Lesion Effect on Axonal Growth. Journal of Neuroscience 26, 5565–5573. https://doi.org/10.1523/JNEUROSCI.0815-06.2006

Carmel, J.B., Berrol, L.J., Brus-Ramer, M., Martin, J.H., 2010. Chronic Electrical Stimulation of the Intact Corticospinal System after Unilateral Injury Restores Skilled Locomotor Control and Promotes Spinal Axon Outgrowth. Journal of

Neuroscience 30, 10918–10926. https://doi.org/10.1523/JNEUROSCI.1435-10.2010

Carmel, J.B., Kimura, H., Berrol, L.J., Martin, J.H., 2013. Motor cortex electrical stimulation promotes axon outgrowth to brain stem and spinal targets that control the forelimb impaired by unilateral corticospinal injury. Eur J Neurosci 37, 1090–1102. https://doi.org/10.1111/ejn.12119

Carmel, J.B., Kimura, H., Martin, J.H., 2014. Electrical Stimulation of Motor Cortex in the Uninjured Hemisphere after Chronic Unilateral Injury Promotes Recovery of Skilled Locomotion through Ipsilateral Control. Journal of Neuroscience 34, 462–466. https://doi.org/10.1523/JNEUROSCI.3315-13.2014

Carmel, J.B., Martin, J.H., 2014. Motor cortex electrical stimulation augments sprouting of the corticospinal tract and promotes recovery of motor function. Front. Integr. Neurosci. 8. https://doi.org/10.3389/fnint.2014.00051

Caroni, P., Schwab, M.E., 1988. Antibody against myelin associated inhibitor of neurite growth neutralizes nonpermissive substrate properties of CNS white matter. Neuron 1, 85–96. https://doi.org/10.1016/0896-6273(88)90212-7

Chan, K.M., Curran, M.W.T., Gordon, T., 2016. The use of brief post-surgical low frequency electrical stimulation to enhance nerve regeneration in clinical practice: Use of brief post-surgical low frequency electrical stimulation. J Physiol 594, 3553–3559. https://doi.org/10.1113/JP270892

Chang, W.H., Kim, H., Sun, W., Kim, J.Y., Shin, Y.-I., Kim, Y.-H., 2015. Effects of extradural cortical stimulation on motor recovery in a rat model of subacute stroke. RNN 33, 589–596. https://doi.org/10.3233/RNN-140445

Chaudhry, N., Filbin, M.T., 2007. Myelin—Associated Inhibitory Signaling and Strategies to Overcome Inhibition. J Cereb Blood Flow Metab 27, 1096–1107. https://doi.org/10.1038/sj.jcbfm.9600407

Chen, M.S., Huber, A.B., van der Haar, M.E., Frank, M., Schnell, L., Spillmann, A.A., Christ, F., Schwab, M.E., 2000. Nogo-A is a myelin-associated neurite outgrowth inhibitor and an antigen for monoclonal antibody IN-1. Nature 403, 434–439. https://doi.org/10.1038/35000219

Cheng, X., Ji, Z., Tsalkova, T., Mei, F., 2008. Epac and PKA: a tale of two intracellular cAMP receptors. Acta Biochimica et Biophysica Sinica 40, 651–662. https://doi.org/10.1111/j.1745-7270.2008.00438.x

Christensen, A.E., Selheim, F., de Rooij, J., Dremier, S., Schwede, F., Dao, K.K., Martinez, A., Maenhaut, C., Bos, J.L., Genieser, H.-G., Døskeland, S.O., 2003. cAMP Analog Mapping of Epac1 and cAMP Kinase. Journal of Biological Chemistry 278, 35394–35402. https://doi.org/10.1074/jbc.M302179200

Cotman, C.W., Berchtold, N.C., Christie, L.-A., 2007. Exercise builds brain health: key roles of growth factor cascades and inflammation. Trends in Neurosciences 30, 464–472. https://doi.org/10.1016/j.tins.2007.06.011

Courtine, G., Song, B., Roy, R.R., Zhong, H., Herrmann, J.E., Ao, Y., Qi, J., Edgerton, V.R., Sofroniew, M.V., 2008. Recovery of supraspinal control of stepping via indirect propriospinal relay connections after spinal cord injury. Nat Med 14, 69–74. https://doi.org/10.1038/nm1682

Craggs, M.D., Balasubramaniam, A.V., Chung, E.A.L., Emmanuel, A.V., 2006. Aberrant reflexes and function of the pelvic organs following spinal cord injury in man. Autonomic Neuroscience 126–127, 355–370. https://doi.org/10.1016/j.autneu.2006.03.010

Craveiro, L.M., Weinmann, O., Roschitzki, B., Gonzenbach, R.R., Zörner, B., Montani, L., Yee, B.K., Feldon, J., Willi, R., Schwab, M.E., 2013. Infusion of anti-Nogo-A antibodies in adult rats increases growth and synapse related proteins in the absence of behavioral alterations. Experimental Neurology 250, 52–68. https://doi.org/10.1016/j.expneurol.2013.09.015

Crowe, M.J., Bresnahan, J.C., Shuman, S.L., Masters, J.N., Beattie, M.S., 1997. Apoptosis and delayed degeneration after spinal cord injury in rats and monkeys. Nat Med 3, 73–76. https://doi.org/10.1038/nm0197-73

David, S., Aguayo, A., 1981. Axonal elongation into peripheral nervous system "bridges" after central nervous system injury in adult rats. Science 214, 931–933. https://doi.org/10.1126/science.6171034

Delghandi, M.P., Johannessen, M., Moens, U., 2005. The cAMP signalling pathway activates CREB through PKA, p38 and MSK1 in NIH 3T3 cells. Cellular Signalling 17, 1343–1351. https://doi.org/10.1016/j.cellsig.2005.02.003

Dell'Anno, M.T., Strittmatter, S.M., 2017. Rewiring the spinal cord: Direct and indirect strategies. Neuroscience Letters 652, 25–34. https://doi.org/10.1016/j.neulet.2016.12.002

Demopoulos, H.B., Flamm, E.S., Pietronigro, D.D., Seligman, M.L., 1980. The free radical pathology and the microcirculation in the major central nervous system disorders. Acta Physiol Scand Suppl 492, 91–119.

Dergham, P., Ellezam, B., Essagian, C., Avedissian, H., Lubell, W.D., McKerracher, L., 2002. Rho Signaling Pathway Targeted to Promote Spinal Cord Repair. J. Neurosci. 22, 6570–6577. https://doi.org/10.1523/JNEUROSCI.22-15-06570.2002

Dietz, V., Fouad, K., 2014. Restoration of sensorimotor functions after spinal cord injury. Brain 137, 654–667. https://doi.org/10.1093/brain/awt262

Dishman, R.K., Berthoud, H.-R., Booth, F.W., Cotman, C.W., Edgerton, V.R., Fleshner, M.R., Gandevia, S.C., Gomez-Pinilla, F., Greenwood, B.N., Hillman, C.H.,

Kramer, A.F., Levin, B.E., Moran, T.H., Russo-Neustadt, A.A., Salamone, J.D., Van Hoomissen, J.D., Wade, C.E., York, D.A., Zigmond, M.J., 2006. Neurobiology of Exercise*. Obesity 14, 345–356. https://doi.org/10.1038/oby.2006.46

Domeniconi, M., Cao, Z., Spencer, T., Sivasankaran, R., Wang, K.C., Nikulina, E., Kimura, N., Cai, H., Deng, K., Gao, Y., He, Z., Filbin, M.T., 2002. Myelin-Associated Glycoprotein Interacts with the Nogo66 Receptor to Inhibit Neurite Outgrowth. Neuron 35, 283–290. https://doi.org/10.1016/S0896-6273(02)00770-5

Donnelly, D.J., Popovich, P.G., 2008. Inflammation and its role in neuroprotection, axonal regeneration and functional recovery after spinal cord injury. Experimental Neurology 209, 378–388. https://doi.org/10.1016/j.expneurol.2007.06.009

Dougherty, K.D., Dreyfus, C.F., Black, I.B., 2000. Brain-Derived Neurotrophic Factor in Astrocytes, Oligodendrocytes, and Microglia/Macrophages after Spinal Cord Injury. Neurobiology of Disease 7, 574–585. https://doi.org/10.1006/nbdi.2000.0318

Dunlop, S.A., 2008. Activity-dependent plasticity: implications for recovery after spinal cord injury. Trends in Neurosciences 31, 410–418. https://doi.org/10.1016/j.tins.2008.05.004

Elbasiouny, S.M., Moroz, D., Bakr, M.M., Mushahwar, V.K., 2010. Management of Spasticity After Spinal Cord Injury: Current Techniques and Future Directions. Neurorehabil Neural Repair 24, 23–33. https://doi.org/10.1177/1545968309343213

Emery, E., Aldana, P., Bunge, M.B., Puckett, W., Srinivasan, A., Keane, R.W., Bethea, J., Levi, A.D.O., 1998. Apoptosis after traumatic human spinal cord injury. Journal of Neurosurgery 89, 911–920. https://doi.org/10.3171/jns.1998.89.6.0911

Enserink, J.M., Christensen, A.E., de Rooij, J., van Triest, M., Schwede, F., Genieser, H.G., Døskeland, S.O., Blank, J.L., Bos, J.L., 2002. A novel Epac-specific cAMP analogue demonstrates independent regulation of Rap1 and ERK. Nat Cell Biol 4, 901–906. https://doi.org/10.1038/ncb874

Fenrich, K., Gordon, T., 2004. Canadian Association of Neuroscience Review: Axonal Regeneration in the Peripheral and Central Nervous Systems – Current Issues and Advances. Can. j. neurol. sci. 31, 142–156. https://doi.org/10.1017/S0317167100053798

Fenrich, K.K., Hallworth, B.W., Vavrek, R., Raposo, P.J.F., Misiaszek, J.E., Bennett, D.J., Fouad, K., Torres-Espin, A., 2021. Self-directed rehabilitation training intensity thresholds for efficient recovery of skilled forelimb function in rats with cervical spinal cord injury. Experimental Neurology 339, 113543. https://doi.org/10.1016/j.expneurol.2020.113543

Fertonani, A., Miniussi, C., 2017. Transcranial Electrical Stimulation: What We Know and Do Not Know About Mechanisms. Neuroscientist 23, 109–123. https://doi.org/10.1177/1073858416631966

Fleming, J.C., Norenberg, M.D., Ramsay, D.A., Dekaban, G.A., Marcillo, A.E., Saenz, A.D., Pasquale-Styles, M., Dietrich, W.D., Weaver, L.C., 2006. The cellular inflammatory response in human spinal cords after injury. Brain 129, 3249–3269. https://doi.org/10.1093/brain/awl296

Fouad, K., Forero, J., Hurd, C., 2015. A Simple Analogy for Nervous System Plasticity After Injury. Exercise and Sport Sciences Reviews 43, 100–106. https://doi.org/10.1249/JES.0000000000000040

Fouad, K., Pedersen, V., Schwab, M.E., Brösamle, C., 2001. Cervical sprouting of corticospinal fibers after thoracic spinal cord injury accompanies shifts in evoked motor responses. Current Biology 11, 1766–1770. https://doi.org/10.1016/S0960-9822(01)00535-8

Fouad, K., Tetzlaff, W., 2012. Rehabilitative training and plasticity following spinal cord injury. Experimental Neurology 235, 91–99. https://doi.org/10.1016/j.expneurol.2011.02.009

Fournier, A.E., GrandPre, T., Strittmatter, S.M., 2001. Identification of a receptor mediating Nogo-66 inhibition of axonal regeneration. Nature 409, 341–346. https://doi.org/10.1038/35053072

Gao, Y., Nikulina, E., Mellado, W., Filbin, M.T., 2003. Neurotrophins Elevate cAMP to Reach a Threshold Required to Overcome Inhibition by MAG through Extracellular Signal-Regulated Kinase-Dependent Inhibition of Phosphodiesterase. J. Neurosci. 23, 11770–11777. https://doi.org/10.1523/JNEUROSCI.23-37-11770.2003

García-Alías, G., Barkhuysen, S., Buckle, M., Fawcett, J.W., 2009. Chondroitinase ABC treatment opens a window of opportunity for task-specific rehabilitation. Nat Neurosci 12, 1145–1151. https://doi.org/10.1038/nn.2377

Geremia, N.M., Gordon, T., Brushart, T.M., Al-Majed, A.A., Verge, V.M.K., 2007. Electrical stimulation promotes sensory neuron regeneration and growth-associated gene expression. Experimental Neurology 205, 347–359. https://doi.org/10.1016/j.expneurol.2007.01.040

Girgis, J., Merrett, D., Kirkland, S., Metz, G.A.S., Verge, V., Fouad, K., 2007. Reaching training in rats with spinal cord injury promotes plasticity and task specific recovery. Brain 130, 2993–3003. https://doi.org/10.1093/brain/awm245

Goganau, I., Sandner, B., Weidner, N., Fouad, K., Blesch, A., 2018. Depolarization and electrical stimulation enhance in vitro and in vivo sensory axon growth after spinal cord injury. Experimental Neurology 300, 247–258. https://doi.org/10.1016/j.expneurol.2017.11.011

Gomez-Pinilla, F., Ying, Z., Zhuang, Y., 2012. Brain and Spinal Cord Interaction: Protective Effects of Exercise Prior to Spinal Cord Injury. PLoS ONE 7, e32298. https://doi.org/10.1371/journal.pone.0032298

Gonzenbach, R.R., Gasser, P., Zörner, B., Hochreutener, E., Dietz, V., Schwab, M.E., 2010. Nogo-A antibodies and training reduce muscle spasms in spinal cord-injured rats. Ann Neurol. 68, 48–57. https://doi.org/10.1002/ana.22009

Gonzenbach, R.R., Schwab, M.E., 2008. Disinhibition of neurite growth to repair the injured adult CNS: Focusing on Nogo. Cell. Mol. Life Sci. 65, 161–176. https://doi.org/10.1007/s00018-007-7170-3

Gordon, T., 2016a. Electrical Stimulation to Enhance Axon Regeneration After Peripheral Nerve Injuries in Animal Models and Humans. Neurotherapeutics 13, 295–310. https://doi.org/10.1007/s13311-015-0415-1

Gordon, T., 2016b. Nerve regeneration in the peripheral and central nervous systems: Editorial. J Physiol 594, 3517–3520. https://doi.org/10.1113/JP270898

Gordon, T., 2016c. Nerve regeneration in the peripheral and central nervous systems: Editorial. J Physiol 594, 3517–3520. https://doi.org/10.1113/JP270898

Gordon, T., Tetzlaff, W., 2015. Regeneration-associated genes decline in chronically injured rat sciatic motoneurons. Eur J Neurosci 42, 2783–2791. https://doi.org/10.1111/ejn.13070

Goritz, C., Dias, D.O., Tomilin, N., Barbacid, M., Shupliakov, O., Frisen, J., 2011. A Pericyte Origin of Spinal Cord Scar Tissue. Science 333, 238–242. https://doi.org/10.1126/science.1203165

GrandPré, T., Nakamura, F., Vartanian, T., Strittmatter, S.M., 2000. Identification of the Nogo inhibitor of axon regeneration as a Reticulon protein. Nature 403, 439–444. https://doi.org/10.1038/35000226

Gris, D., Marsh, D.R., Oatway, M.A., Chen, Y., Hamilton, E.F., Dekaban, G.A., Weaver, L.C., 2004. Transient Blockade of the CD11d/CD18 Integrin Reduces Secondary Damage after Spinal Cord Injury, Improving Sensory, Autonomic, and Motor Function. Journal of Neuroscience 24, 4043–4051. https://doi.org/10.1523/JNEUROSCI.5343-03.2004

Guijarro-Belmar, A., Viskontas, M., Wei, Y., Bo, X., Shewan, D., Huang, W., 2019. Epac2 Elevation Reverses Inhibition by Chondroitin Sulfate Proteoglycans *In Vitro* and Transforms Postlesion Inhibitory Environment to Promote Axonal Outgrowth in an *Ex Vivo* Model of Spinal Cord Injury. J. Neurosci. 39, 8330–8346. https://doi.org/10.1523/JNEUROSCI.0374-19.2019

Hall, E.D., 1992. The neuroprotective pharmacology of methylprednisolone. Journal of Neurosurgery 76, 13–22. https://doi.org/10.3171/jns.1992.76.1.0013

Hannila, S.S., Siddiq, M.M., Carmel, J.B., Hou, J., Chaudhry, N., Bradley, P.M.J., Hilaire, M., Richman, E.L., Hart, R.P., Filbin, M.T., 2013. Secretory Leukocyte Protease Inhibitor Reverses Inhibition by CNS Myelin, Promotes Regeneration in the Optic Nerve, and Suppresses Expression of the Transforming Growth Factor-Signaling Protein Smad2. Journal of Neuroscience 33, 5138–5151. https://doi.org/10.1523/JNEUROSCI.5321-12.2013

Hanson, R.W., Franklin, M.R., 1976. Sexual loss in relation to other functional losses for spinal cord injured males. Arch Phys Med Rehabil 57, 291–293.

Harkema, S.J., 2001. Neural Plasticity after Human Spinal Cord Injury: Application of Locomotor Training to the Rehabilitation of Walking. Neuroscientist 7, 455–468. https://doi.org/10.1177/107385840100700514

Harvey, A.R., Lovett, S.J., Majda, B.T., Yoon, J.H., Wheeler, L.P.G., Hodgetts, S.I., 2015. Neurotrophic factors for spinal cord repair: Which, where, how and when to apply, and for what period of time? Brain Research 1619, 36–71. https://doi.org/10.1016/j.brainres.2014.10.049

He, H., Deng, K., Siddiq, M.M., Pyie, A., Mellado, W., Hannila, S.S., Filbin, M.T., 2016. Cyclic AMP and Polyamines Overcome Inhibition by Myelin-Associated Glycoprotein through eIF5A-Mediated Increases in p35 Expression and Activation of Cdk5. Journal of Neuroscience 36, 3079–3091. https://doi.org/10.1523/JNEUROSCI.4012-15.2016

Hernandez, T.D., Schallert, T., 1988. Seizures and recovery from experimental brain damage. Experimental Neurology 102, 318–324. https://doi.org/10.1016/0014-4886(88)90226-9

Herrera, J.J., Nesic, O., Narayana, P.A., 2009. Reduced Vascular Endothelial Growth Factor Expression in Contusive Spinal Cord Injury. Journal of Neurotrauma 26, 995–1003. https://doi.org/10.1089/neu.2008.0779

Hiebert, G.W., Khodarahmi, K., McGraw, J., Steeves, J.D., 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. J. Neurosci. Res. 69, 160–168. https://doi.org/10.1002/jnr.10275

Ho, S.-Y., Chao, C.-Y., Huang, H.-L., Chiu, T.-W., Charoenkwan, P., Hwang, E., 2011. NeurphologyJ: An automatic neuronal morphology quantification method and its application in pharmacological discovery. BMC Bioinformatics 12, 230. https://doi.org/10.1186/1471-2105-12-230

Hou, J., Nelson, R., Nissim, N., Parmer, R., Thompson, F.J., Bose, P., 2014. Effect of Combined Treadmill Training and Magnetic Stimulation on Spasticity and Gait Impairments after Cervical Spinal Cord Injury. Journal of Neurotrauma 31, 1088–1106. https://doi.org/10.1089/neu.2013.3096

Hoy, J.J., Salinas Parra, N., Park, J., Kuhn, S., Iglesias-Bartolome, R., 2020. Protein kinase A inhibitor proteins (PKIs) divert GPCR-Gαs-cAMP signaling toward EPAC and ERK activation and are involved in tumor growth. FASEB j. 34, 13900–13917. https://doi.org/10.1096/fj.202001515R

Hu, C.-D., Kariya, K., Okada, T., Qi, X., Song, C., Kataoka, T., 1999. Effect of Phosphorylation on Activities of Rap1A to Interact with Raf-1 and to Suppress Ras-dependent Raf-1 Activation. Journal of Biological Chemistry 274, 48–51. https://doi.org/10.1074/jbc.274.1.48

Huber, A.B., Weinmann, O., Brösamle, C., Oertle, T., Schwab, M.E., 2002. Patterns of Nogo mRNA and Protein Expression in the Developing and Adult Rat and After CNS Lesions. J. Neurosci. 22, 3553–3567. https://doi.org/10.1523/JNEUROSCI.22-09-03553.2002

Hurd, C., Weishaupt, N., Fouad, K., 2013. Anatomical correlates of recovery in single pellet reaching in spinal cord injured rats. Experimental Neurology 247, 605–614. https://doi.org/10.1016/j.expneurol.2013.02.013

Inman, D.M., Steward, O., 2003. Ascending sensory, but not other long-tract axons, regenerate into the connective tissue matrix that forms at the site of a spinal cord injury in mice. J. Comp. Neurol. 462, 431–449. https://doi.org/10.1002/cne.10768

Jack, A.S., Hurd, C., Forero, J., Nataraj, A., Fenrich, K., Blesch, A., Fouad, K., 2018. Cortical electrical stimulation in female rats with a cervical spinal cord injury to promote axonal outgrowth. J Neuro Res 96, 852–862. https://doi.org/10.1002/jnr.24209

Kakulas, B.A., 1999. A review of the neuropathology of human spinal cord injury with emphasis on special features. J Spinal Cord Med 22, 119–124. https://doi.org/10.1080/10790268.1999.11719557

Kakulas, B.A., 1984. Pathology of spinal injuries. Cent Nerv Syst Trauma 1, 117–129. https://doi.org/10.1089/cns.1984.1.117

Kanagal, S.G., Muir, G.D., 2009. Task-dependent compensation after pyramidal tract and dorsolateral spinal lesions in rats. Experimental Neurology 216, 193–206. https://doi.org/10.1016/j.expneurol.2008.11.028

Kanagal, S.G., Muir, G.D., 2008. Effects of combined dorsolateral and dorsal funicular lesions on sensorimotor behaviour in rats. Experimental Neurology 214, 229–239. https://doi.org/10.1016/j.expneurol.2008.08.004

Kawaguchi, M., Furuya, H., 2004. Intraoperative spinal cord monitoring of motor function with myogenic motor evoked potentials: a consideration in anesthesia. Journal of Anesthesia 18, 18–28. https://doi.org/10.1007/s00540-003-0201-9

Kerschensteiner, M., Schwab, M.E., Lichtman, J.W., Misgeld, T., 2005. In vivo imaging of axonal degeneration and regeneration in the injured spinal cord. Nat Med 11, 572–577. https://doi.org/10.1038/nm1229

Kim, D., Schallert, T., Yi Liu, Browarak, T., Nayeri, N., Tessler, A., Fischer, I., Murray, M., 2001. Transplantation of Genetically Modified Fibroblasts Expressing BDNF in Adult Rats with a Subtotal Hemisection Improves Specific Motor and Sensory Functions. Neurorehabil Neural Repair 15, 141–150. https://doi.org/10.1177/154596830101500207

Kirshblum, S.C., Burns, S.P., Biering-Sorensen, F., Donovan, W., Graves, D.E., Jha, A., Johansen, M., Jones, L., Krassioukov, A., Mulcahey, M.J., Schmidt-Read, M., Waring, W., 2011. International standards for neurological classification of spinal cord injury (Revised 2011). The Journal of Spinal Cord Medicine 34, 535–546. https://doi.org/10.1179/204577211X13207446293695

Kjell, J., Olson, L., 2016. Rat models of spinal cord injury: from pathology to potential therapies. Disease Models & Mechanisms 9, 1125–1137. https://doi.org/10.1242/dmm.025833

Kleim, J.A., Barbay, S., Nudo, R.J., 1998. Functional Reorganization of the Rat Motor Cortex Following Motor Skill Learning. Journal of Neurophysiology 80, 3321–3325. https://doi.org/10.1152/jn.1998.80.6.3321

Klomjai, W., Katz, R., Lackmy-Vallée, A., 2015. Basic principles of transcranial magnetic stimulation (TMS) and repetitive TMS (rTMS). Annals of Physical and Rehabilitation Medicine 58, 208–213. https://doi.org/10.1016/j.rehab.2015.05.005

Kobayashi, N.R., Fan, D.-P., Giehl, K.M., Bedard, A.M., Wiegand, S.J., Tetzlaff, W., 1997. BDNF and NT-4/5 Prevent Atrophy of Rat Rubrospinal Neurons after Cervical Axotomy, Stimulate GAP-43 and Tα1-Tubulin mRNA Expression, and Promote Axonal Regeneration. J. Neurosci. 17, 9583–9595. https://doi.org/10.1523/JNEUROSCI.17-24-09583.1997

Kowalczewski, J., Ravid, E., Prochazka, A., 2011. Fully-automated test of upper-extremity function, in: 2011 Annual International Conference of the IEEE Engineering in Medicine and Biology Society. Presented at the 2011 33rd Annual International Conference of the IEEE Engineering in Medicine and Biology Society, IEEE, Boston, MA, pp. 7332–7335. https://doi.org/10.1109/IEMBS.2011.6091710

Krajacic, A., Ghosh, M., Puentes, R., Pearse, D.D., Fouad, K., 2009. Advantages of delaying the onset of rehabilitative reaching training in rats with incomplete spinal cord injury. European Journal of Neuroscience 29, 641–651. https://doi.org/10.1111/j.1460-9568.2008.06600.x

Krajacic, A., Weishaupt, N., Girgis, J., Tetzlaff, W., Fouad, K., 2010. Training-induced plasticity in rats with cervical spinal cord injury: Effects and side effects. Behavioural Brain Research 214, 323–331. https://doi.org/10.1016/j.bbr.2010.05.053

Kunkel-Bagden, E., Dai, H.-N., Bregman, B.S., 1993. Methods to Assess the Development and Recovery of Locomotor Function after Spinal Cord Injury in Rats. Experimental Neurology 119, 153–164. https://doi.org/10.1006/exnr.1993.1017

Kusiak, A.N., Selzer, M.E., 2013. Neuroplasticity in the spinal cord, in: Handbook of Clinical Neurology. Elsevier, pp. 23–42. https://doi.org/10.1016/B978-0-444-52901-5.00003-4

Kwon, B.K., Liu, J., Messerer, C., Kobayashi, N.R., McGraw, J., Oschipok, L., Tetzlaff, W., 2002a. Survival and regeneration of rubrospinal neurons 1 year after spinal cord injury. Proceedings of the National Academy of Sciences 99, 3246–3251. https://doi.org/10.1073/pnas.052308899

Kwon, B.K., Liu, J., Messerer, C., Kobayashi, N.R., McGraw, J., Oschipok, L., Tetzlaff, W., 2002b. Survival and regeneration of rubrospinal neurons 1 year after spinal cord injury. Proceedings of the National Academy of Sciences 99, 3246–3251. https://doi.org/10.1073/pnas.052308899

Lawrence, D.G., Kuypers, H.G.J.M., 1968. THE FUNCTIONAL ORGANIZATION OF THE MOTOR SYSTEM IN THE MONKEY: II. THE EFFECTS OF LESIONS OF THE DESCENDING BRAIN-STEM PATHWAYS. Brain 91, 15–36. https://doi.org/10.1093/brain/91.1.15

Lee, J.-K., 2004. Nogo Receptor Antagonism Promotes Stroke Recovery by Enhancing Axonal Plasticity. Journal of Neuroscience 24, 6209–6217. https://doi.org/10.1523/JNEUROSCI.1643-04.2004

Leech, K.A., Kinnaird, C.R., Holleran, C.L., Kahn, J., Hornby, T.G., 2016. Effects of Locomotor Exercise Intensity on Gait Performance in Individuals With Incomplete Spinal Cord Injury. Physical Therapy 96, 1919–1929. https://doi.org/10.2522/ptj.20150646

Lemon, R.N., 2008. Descending Pathways in Motor Control. Annu. Rev. Neurosci. 31, 195–218. https://doi.org/10.1146/annurev.neuro.31.060407.125547

Lemon, R.N., Griffiths, J., 2005. Comparing the function of the corticospinal system in different species: Organizational differences for motor specialization? Muscle Nerve 32, 261–279. https://doi.org/10.1002/mus.20333

Li, S., Stys, P.K., 2000. Mechanisms of Ionotropic Glutamate Receptor-Mediated Excitotoxicity in Isolated Spinal Cord White Matter. J. Neurosci. 20, 1190–1198. https://doi.org/10.1523/JNEUROSCI.20-03-01190.2000

Liang, Z., Ye, T., Zhou, X., Lai, K.-O., Fu, A.K.Y., Ip, N.Y., 2015. Cdk5 Regulates Activity-Dependent Gene Expression and Dendrite Development. Journal of Neuroscience 35, 15127–15134. https://doi.org/10.1523/JNEUROSCI.1443-15.2015

Lindau, N.T., Bänninger, B.J., Gullo, M., Good, N.A., Bachmann, L.C., Starkey, M.L., Schwab, M.E., 2014. Rewiring of the corticospinal tract in the adult rat after unilateral stroke and anti-Nogo-A therapy. Brain 137, 739–756. https://doi.org/10.1093/brain/awt336

Liu, K., Lu, Y., Lee, J.K., Samara, R., Willenberg, R., Sears-Kraxberger, I., Tedeschi, A., Park, K.K., Jin, D., Cai, B., Xu, B., Connolly, L., Steward, O., Zheng, B., He, Z., 2010. PTEN deletion enhances the regenerative ability of adult corticospinal neurons. Nat Neurosci 13, 1075–1081. https://doi.org/10.1038/nn.2603

Liu, X.Z., Xu, X.M., Hu, R., Du, C., Zhang, S.X., McDonald, J.W., Dong, H.X., Wu, Y.J., Fan, G.S., Jacquin, M.F., Hsu, C.Y., Choi, D.W., 1997. Neuronal and Glial Apoptosis after Traumatic Spinal Cord Injury. J. Neurosci. 17, 5395–5406. https://doi.org/10.1523/JNEUROSCI.17-14-05395.1997

Lu, P., Yang, H., Jones, L.L., Filbin, M.T., Tuszynski, M.H., 2004. Combinatorial Therapy with Neurotrophins and cAMP Promotes Axonal Regeneration beyond Sites of Spinal Cord Injury. Journal of Neuroscience 24, 6402–6409. https://doi.org/10.1523/JNEUROSCI.1492-04.2004

Lu, Y., Belin, S., He, Z., 2014. Signaling regulations of neuronal regenerative ability. Current Opinion in Neurobiology 27, 135–142. https://doi.org/10.1016/j.conb.2014.03.007

Ma, M., Basso, D.M., Walters, P., Stokes, B.T., Jakeman, L.B., 2001. Behavioral and Histological Outcomes Following Graded Spinal Cord Contusion Injury in the C57Bl/6 Mouse. Experimental Neurology 169, 239–254. https://doi.org/10.1006/exnr.2001.7679

Mackenzie, S.J., Houslay, M.D., 2000. Action of rolipram on specific PDE4 cAMP phosphodiesterase isoforms and on the phosphorylation of cAMP-response-element-binding protein (CREB) and p38 mitogen-activated protein (MAP) kinase in U937 monocytic cells. Biochemical Journal 347, 571–578. https://doi.org/10.1042/bj3470571

MacLellan, C.L., Keough, M.B., Granter-Button, S., Chernenko, G.A., Butt, S., Corbett, D., 2011. A Critical Threshold of Rehabilitation Involving Brain-Derived Neurotrophic Factor Is Required for Poststroke Recovery. Neurorehabil Neural Repair 25, 740–748. https://doi.org/10.1177/1545968311407517

Massey, J.M., Hubscher, C.H., Wagoner, M.R., Decker, J.A., Amps, J., Silver, J., Onifer, S.M., 2006. Chondroitinase ABC Digestion of the Perineuronal Net Promotes Functional Collateral Sprouting in the Cuneate Nucleus after Cervical Spinal Cord Injury. Journal of Neuroscience 26, 4406–4414. https://doi.org/10.1523/JNEUROSCI.5467-05.2006

May, Z., Fenrich, K.K., Dahlby, J., Batty, N.J., Torres-Espín, A., Fouad, K., 2017. Following Spinal Cord Injury Transected Reticulospinal Tract Axons Develop

New Collateral Inputs to Spinal Interneurons in Parallel with Locomotor Recovery. Neural Plasticity 2017, 1–15. https://doi.org/10.1155/2017/1932875

May, Z., Fouad, K., Shum-Siu, A., Magnuson, D.S.K., 2015. Challenges of animal models in SCI research: Effects of pre-injury task-specific training in adult rats before lesion. Behavioural Brain Research 291, 26–35. https://doi.org/10.1016/j.bbr.2015.04.058

McKenna, J.E., Prusky, G.T., Whishaw, I.Q., 2000. Cervical motoneuron topography reflects the proximodistal organization of muscles and movements of the rat forelimb: a retrograde carbocyanine dye analysis. J Comp Neurol 419, 286–296. https://doi.org/10.1002/(sici)1096-9861(20000410)419:3<286::aid-cne2>3.0.co;2-3

McKenna, J.E., Whishaw, I.Q., 1999. Complete Compensation in Skilled Reaching Success with Associated Impairments in Limb Synergies, after Dorsal Column Lesion in the Rat. J. Neurosci. 19, 1885–1894. https://doi.org/10.1523/JNEUROSCI.19-05-01885.1999

McKerracher, L., David, S., Jackson, D.L., Kottis, V., Dunn, R.J., Braun, P.E., 1994. Identification of myelin-associated glycoprotein as a major myelin-derived inhibitor of neurite growth. Neuron 13, 805–811. https://doi.org/10.1016/0896-6273(94)90247-X

Metz, G.A.S., Curt, A., van de MEENT, H., Klusman, I., Schwab, M.E., Dietz, V., 2000. Validation of the Weight-Drop Contusion Model in Rats: A Comparative Study of Human Spinal Cord Injury. Journal of Neurotrauma 17, 1–17. https://doi.org/10.1089/neu.2000.17.1

Metz, G.A.S., Whishaw, I.Q., 2000. Skilled reaching an action pattern: stability in rat (Rattus norvegicus) grasping movements as a function of changing food pellet size. Behavioural Brain Research 116, 111–122. https://doi.org/10.1016/S0166-4328(00)00245-X

Mikol, D.D., Stefansson, K., 1988. A phosphatidylinositol-linked peanut agglutinin-binding glycoprotein in central nervous system myelin and on oligodendrocytes. The Journal of Cell Biology 106, 1273–1279. https://doi.org/10.1083/jcb.106.4.1273

Ming, G., Song, H., Berninger, B., Holt, C.E., Tessier-Lavigne, M., Poo, M., 1997. cAMP-Dependent Growth Cone Guidance by Netrin-1. Neuron 19, 1225–1235. https://doi.org/10.1016/S0896-6273(00)80414-6

Mitchell, E.J., McCallum, S., Dewar, D., Maxwell, D.J., 2016. Corticospinal and Reticulospinal Contacts on Cervical Commissural and Long Descending Propriospinal Neurons in the Adult Rat Spinal Cord; Evidence for Powerful Reticulospinal Connections. PLoS ONE 11, e0152094. https://doi.org/10.1371/journal.pone.0152094

Monnier, P.P., Sierra, A., Schwab, J.M., Henke-Fahle, S., 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 Neuroscience 22, 319–330. https://doi.org/10.1016/S1044-7431(02)00035-0

Morgenstern, D.A., Asher, R.A., Fawcett, J.W., 2002. Chapter 22 Chondroitin sulphate proteoglycans in the CNS injury response, in: Progress in Brain Research. Elsevier, pp. 313–332. https://doi.org/10.1016/S0079-6123(02)37024-9

Morris, D.M., Crago, J.E., DeLuca, S.C., Pidikiti, R.D., Taub, E., 1997. Constraint-induced movement therapy for motor recovery after stroke. NRE 9, 29–43. https://doi.org/10.3233/NRE-1997-9104

Moseley, L.G., 2007. Using visual illusion to reduce at-level neuropathic pain in paraplegia. Pain 130, 294–298. https://doi.org/10.1016/j.pain.2007.01.007

Mueller, P.J., 2007. EXERCISE TRAINING AND SYMPATHETIC NERVOUS SYSTEM ACTIVITY: EVIDENCE FOR PHYSICAL ACTIVITY DEPENDENT NEURAL PLASTICITY: Exercise training and the SNS. Clinical and Experimental Pharmacology and Physiology 34, 377–384. https://doi.org/10.1111/j.1440-1681.2007.04590.x

Mukhopadhyay, G., Doherty, P., Walsh, F.S., Crocker, P.R., Filbin, M.T., 1994. A novel role for myelin-associated glycoprotein as an inhibitor of axonal regeneration. Neuron 13, 757–767. https://doi.org/10.1016/0896-6273(94)90042-6

Munoz-Llancao, P., Henriquez, D.R., Wilson, C., Bodaleo, F., Boddeke, E.W., Lezoualc'h, F., Schmidt, M., Gonzalez-Billault, C., 2015. Exchange Protein Directly Activated by cAMP (EPAC) Regulates Neuronal Polarization through Rap1B. Journal of Neuroscience 35, 11315–11329. https://doi.org/10.1523/JNEUROSCI.3645-14.2015

Murray, A.J., 2008. Pharmacological PKA Inhibition: All May Not Be What It Seems. Science Signaling 1, re4–re4. https://doi.org/10.1126/scisignal.122re4

Murray, A.J., Shewan, D.A., 2008. Epac mediates cyclic AMP-dependent axon growth, guidance and regeneration. Molecular and Cellular Neuroscience 38, 578–588. https://doi.org/10.1016/j.mcn.2008.05.006

Murray, A.J., Tucker, S.J., Shewan, D.A., 2009. cAMP-Dependent Axon Guidance Is Distinctly Regulated by Epac and Protein Kinase A. Journal of Neuroscience 29, 15434–15444. https://doi.org/10.1523/JNEUROSCI.3071-09.2009

Nathan, P.W., Smith, M.C., 1982. THE RUBROSPINAL AND CENTRAL TEGMENTAL TRACTS IN MAN. Brain 105, 223–269. https://doi.org/10.1093/brain/105.2.223

Neumann, S., Bradke, F., Tessier-Lavigne, M., Basbaum, A.I., 2002. Regeneration of Sensory Axons within the Injured Spinal Cord Induced by Intraganglionic cAMP Elevation. Neuron 34, 885–893. https://doi.org/10.1016/S0896-6273(02)00702-X

Neumann, S., Woolf, C.J., 1999. Regeneration of Dorsal Column Fibers into and beyond the Lesion Site following Adult Spinal Cord Injury. Neuron 23, 83–91. https://doi.org/10.1016/S0896-6273(00)80755-2

Orr, M.B., Gensel, J.C., 2018. Spinal Cord Injury Scarring and Inflammation: Therapies Targeting Glial and Inflammatory Responses. Neurotherapeutics 15, 541–553. https://doi.org/10.1007/s13311-018-0631-6

Oudega, M., Perez, M.A., 2012. Corticospinal reorganization after spinal cord injury: Corticospinal tract and SCI. The Journal of Physiology 590, 3647–3663. https://doi.org/10.1113/jphysiol.2012.233189

Oyinbo, C.A., 2011. Secondary injury mechanisms in traumatic spinal cord injury: a nugget of this multiply cascade. Acta Neurobiol Exp (Wars) 71, 281–299.

Park, E., Velumian, A.A., Fehlings, M.G., 2004. The Role of Excitotoxicity in Secondary Mechanisms of Spinal Cord Injury: A Review with an Emphasis on the Implications for White Matter Degeneration. Journal of Neurotrauma 21, 754–774. https://doi.org/10.1089/0897715041269641

Park, J., Koito, H., Li, J., Han, A., 2009. Microfluidic compartmentalized co-culture platform for CNS axon myelination research. Biomed Microdevices 11, 1145–1153. https://doi.org/10.1007/s10544-009-9331-7

Park, K.K., Liu, K., Hu, Y., Smith, P.D., Wang, C., Cai, B., Xu, B., Connolly, L., Kramvis, I., Sahin, M., He, Z., 2008. Promoting Axon Regeneration in the Adult CNS by Modulation of the PTEN/mTOR Pathway. Science 322, 963–966. https://doi.org/10.1126/science.1161566

Peace, A.G., Shewan, D.A., 2011. New perspectives in cyclic AMP-mediated axon growth and guidance: The emerging epoch of Epac. Brain Research Bulletin 84, 280–288. https://doi.org/10.1016/j.brainresbull.2010.09.002

Pearse, D.D., Pereira, F.C., Marcillo, A.E., Bates, M.L., Berrocal, Y.A., Filbin, M.T., Bunge, M.B., 2004. cAMP and Schwann cells promote axonal growth and functional recovery after spinal cord injury. Nat Med 10, 610–616. https://doi.org/10.1038/nm1056

Plunet, W., Kwon, B.K., Tetzlaff, W., 2002. Promoting axonal regeneration in the central nervous system by enhancing the cell body response to axotomy. J. Neurosci. Res. 68, 1–6. https://doi.org/10.1002/jnr.10176

Popovich, P.G., Wei, P., Stokes, B.T., 1997. Cellular inflammatory response after spinal cord injury in Sprague-Dawley and Lewis rats. J Comp Neurol 377, 443–464. https://doi.org/10.1002/(sici)1096-9861(19970120)377:3<443::aid-cne10>3.0.co;2-s

Praxis Spinal Cord Institute, 2021. Rick Hansen Spinal Cord Injury Registry - A Look at Traumatic Spinal Cord Injury in Canada in 2019.

Prinjha, R., Moore, S.E., Vinson, M., Blake, S., Morrow, R., Christie, G., Michalovich, D., Simmons, D.L., Walsh, F.S., 2000. Inhibitor of neurite outgrowth in humans. Nature 403, 383–384. https://doi.org/10.1038/35000287

Profyris, C., Cheema, S.S., Zang, D., Azari, M.F., Boyle, K., Petratos, S., 2004. Degenerative and regenerative mechanisms governing spinal cord injury. Neurobiology of Disease 15, 415–436. https://doi.org/10.1016/j.nbd.2003.11.015

Qiu, J., Cai, D., Dai, H., McAtee, M., Hoffman, P.N., Bregman, B.S., Filbin, M.T., 2002. Spinal Axon Regeneration Induced by Elevation of Cyclic AMP. Neuron 34, 895–903. https://doi.org/10.1016/S0896-6273(02)00730-4

Quilliam, L.A., Rebhun, J.F., Castro, A.F., 2002. A growing family of guanine nucleotide exchange factors is responsible for activation of ras-family GTPases, in: Progress in Nucleic Acid Research and Molecular Biology. Elsevier, pp. 391–444. https://doi.org/10.1016/S0079-6603(02)71047-7

R Core Team, 2018. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.

Raineteau, O., Fouad, K., Noth, P., Thallmair, M., Schwab, M.E., 2001. Functional switch between motor tracts in the presence of the mAb IN-1 in the adult rat. Proceedings of the National Academy of Sciences 98, 6929–6934. https://doi.org/10.1073/pnas.111165498

Rasmussen, T., Penfield, W., 1947. The human sensorimotor cortex as studied by electrical stimulation. Fed Proc 6, 184.

Rosenzweig, E.S., McDonald, J.W., 2004. Rodent models for treatment of spinal cord injury: research trends and progress toward useful repair: Current Opinion in Neurology 17, 121–131. https://doi.org/10.1097/00019052-200404000-00007

Rossi, S., Hallett, M., Rossini, P.M., Pascual-Leone, A., 2009. Safety, ethical considerations, and application guidelines for the use of transcranial magnetic stimulation in clinical practice and research. Clinical Neurophysiology 120, 2008–2039. https://doi.org/10.1016/j.clinph.2009.08.016

Rueden, C.T., Schindelin, J., Hiner, M.C., DeZonia, B.E., Walter, A.E., Arena, E.T., Eliceiri, K.W., 2017. ImageJ2: ImageJ for the next generation of scientific image data. BMC Bioinformatics 18, 529. https://doi.org/10.1186/s12859-017-1934-z

Savio, T., Schwab, M., 1989. Rat CNS white matter, but not gray matter, is nonpermissive for neuronal cell adhesion and fiber outgrowth. J. Neurosci. 9, 1126–1133. https://doi.org/10.1523/JNEUROSCI.09-04-01126.1989

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J.-Y., White, D.J.,

Hartenstein, V., Eliceiri, K., Tomancak, P., Cardona, A., 2012. Fiji: an open-source platform for biological-image analysis. Nat Methods 9, 676–682. https://doi.org/10.1038/nmeth.2019

Schneider, C.A., Rasband, W.S., Eliceiri, K.W., 2012. NIH Image to ImageJ: 25 years of image analysis. Nat Methods 9, 671–675. https://doi.org/10.1038/nmeth.2089

Schneider, M.P., Schwab, M.E., Kessler, T.M., 2015. Anti-Nogo-A antibody: a treatment option for neurogenic lower urinary tract dysfunction?: Comment. BJU Int 115, 16–17. https://doi.org/10.1111/bju.13007

Schnell, L., Fearn, S., Klassen, H., Schwab, M.E., Perry, V.H., 1999. Acute inflammatory responses to mechanical lesions in the CNS: differences between brain and spinal cord: Inflammatory responses in brain and spinal cord. European Journal of Neuroscience 11, 3648–3658. https://doi.org/10.1046/j.1460-9568.1999.00792.x

Schwab, M.E., Bartholdi, D., 1996. Degeneration and regeneration of axons in the lesioned spinal cord. Physiological Reviews 76, 319–370. https://doi.org/10.1152/physrev.1996.76.2.319

Schwenkreis, P., El Tom, S., Ragert, P., Pleger, B., Tegenthoff, M., Dinse, H.R., 2007. Assessment of sensorimotor cortical representation asymmetries and motor skills in violin players: Sensorimotor asymmetries in violin players. European Journal of Neuroscience 26, 3291–3302. https://doi.org/10.1111/j.1460-9568.2007.05894.x

Sharif-Alhoseini, M., Khormali, M., Rezaei, M., Safdarian, M., Hajighadery, A., Khalatbari, M.M., Safdarian, M., Meknatkhah, S., Rezvan, M., Chalangari, M., Derakhshan, P., Rahimi-Movaghar, V., 2017. Animal models of spinal cord injury: a systematic review. Spinal Cord 55, 714–721. https://doi.org/10.1038/sc.2016.187

Siddiq, M.M., Hannila, S.S., Carmel, J.B., Bryson, J.B., Hou, J., Nikulina, E., Willis, M.R., Mellado, W., Richman, E.L., Hilaire, M., Hart, R.P., Filbin, M.T., 2015. Metallothionein-I/II Promotes Axonal Regeneration in the Central Nervous System. Journal of Biological Chemistry 290, 16343–16356. https://doi.org/10.1074/jbc.M114.630574

Silver, D.J., Silver, J., 2014. Contributions of chondroitin sulfate proteoglycans to neurodevelopment, injury, and cancer. Current Opinion in Neurobiology 27, 171–178. https://doi.org/10.1016/j.conb.2014.03.016

Sipski, M.L., Arenas, A., 2006. Female sexual function after spinal cord injury, in: Progress in Brain Research. Elsevier, pp. 441–447. https://doi.org/10.1016/S0079-6123(05)52030-2

Snoek, G.J., IJzerman, M.J., Hermens, H.J., Maxwell, D., Biering-Sorensen, F., 2004. Survey of the needs of patients with spinal cord injury: impact and priority for

improvement in hand function in tetraplegics. Spinal Cord 42, 526–532. https://doi.org/10.1038/sj.sc.3101638

Soderblom, C., Luo, X., Blumenthal, E., Bray, E., Lyapichev, K., Ramos, J., Krishnan, V., Lai-Hsu, C., Park, K.K., Tsoulfas, P., Lee, J.K., 2013. Perivascular Fibroblasts Form the Fibrotic Scar after Contusive Spinal Cord Injury. Journal of Neuroscience 33, 13882–13887. https://doi.org/10.1523/JNEUROSCI.2524-13.2013

Speer, A.M., Kimbrell, T.A., Wassermann, E.M., D. Repella, J., Willis, M.W., Herscovitch, P., Post, R.M., 2000. Opposite effects of high and low frequency rTMS on regional brain activity in depressed patients. Biological Psychiatry 48, 1133–1141. https://doi.org/10.1016/S0006-3223(00)01065-9

Spinal Cord Injury (SCI) Facts and Figures at a Glance, 2016. . The Journal of Spinal Cord Medicine 39, 370–371. https://doi.org/10.1080/10790268.2016.1177348

Starkey, M.L., Bleul, C., Kasper, H., Mosberger, A.C., Zörner, B., Giger, S., Gullo, M., Buschmann, F., Schwab, M.E., 2014. High-Impact, Self-Motivated Training Within an Enriched Environment With Single Animal Tracking Dose-Dependently Promotes Motor Skill Acquisition and Functional Recovery. Neurorehabil Neural Repair 28, 594–605. https://doi.org/10.1177/1545968314520721

Stoneham, E.T., Sanders, E.M., Sanyal, M., Dumas, T.C., 2010. Rules of Engagement: Factors That Regulate Activity-Dependent Synaptic Plasticity During Neural Network Development. The Biological Bulletin 219, 81–99. https://doi.org/10.1086/BBLv219n2p81

Swiech, L., Perycz, M., Malik, A., Jaworski, J., 2008. Role of mTOR in physiology and pathology of the nervous system. Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics 1784, 116–132. https://doi.org/10.1016/j.bbapap.2007.08.015

Tahayori, B., Koceja, D.M., 2012. Activity-Dependent Plasticity of Spinal Circuits in the Developing and Mature Spinal Cord. Neural Plasticity 2012, 1–12. https://doi.org/10.1155/2012/964843

Takei, N., Nawa, H., 2014. mTOR signaling and its roles in normal and abnormal brain development. Front. Mol. Neurosci. 7. https://doi.org/10.3389/fnmol.2014.00028

Tashiro, S., Shinozaki, M., Mukaino, M., Renault-Mihara, F., Toyama, Y., Liu, M., Nakamura, M., Okano, H., 2015. BDNF Induced by Treadmill Training Contributes to the Suppression of Spasticity and Allodynia After Spinal Cord Injury via Upregulation of KCC2. Neurorehabil Neural Repair 29, 677–689. https://doi.org/10.1177/1545968314562110

Tator, C.H., Fehlings, M.G., 1991. Review of the secondary injury theory of acute spinal cord trauma with emphasis on vascular mechanisms. Journal of Neurosurgery 75, 15–26. https://doi.org/10.3171/jns.1991.75.1.0015

Taub, E., Wolf, S.L., 1997. Constraint Induced Movement Techniques To Facilitate Upper Extremity Use in Stroke Patients. Topics in Stroke Rehabilitation 3, 38–61. https://doi.org/10.1080/10749357.1997.11754128

Thies, E., Davenport, R.W., 2003. Independent roles of Rho-GTPases in growth cone and axonal behavior. J. Neurobiol. 54, 358–369. https://doi.org/10.1002/neu.10135

Tom, V.J., Steinmetz, M.P., Miller, J.H., Doller, C.M., Silver, J., 2004. Studies on the Development and Behavior of the Dystrophic Growth Cone, the Hallmark of Regeneration Failure, in an In Vitro Model of the Glial Scar and after Spinal Cord Injury. Journal of Neuroscience 24, 6531–6539. https://doi.org/10.1523/JNEUROSCI.0994-04.2004

Torres-Espín, A., Beaudry, E., Fenrich, K., Fouad, K., 2018a. Rehabilitative Training in Animal Models of Spinal Cord Injury. Journal of Neurotrauma 35, 1970–1985. https://doi.org/10.1089/neu.2018.5906

Torres-Espín, A., Forero, J., Fenrich, K.K., Lucas-Osma, A.M., Krajacic, A., Schmidt, E., Vavrek, R., Raposo, P., Bennett, D.J., Popovich, P.G., Fouad, K., 2018b. Eliciting inflammation enables successful rehabilitative training in chronic spinal cord injury. Brain 141, 1946–1962. https://doi.org/10.1093/brain/awy128

Torres-Espín, A., Forero, J., Schmidt, E.K.A., Fouad, K., Fenrich, K.K., 2018c. A motorized pellet dispenser to deliver high intensity training of the single pellet reaching and grasping task in rats. Behavioural Brain Research 336, 67–76. https://doi.org/10.1016/j.bbr.2017.08.033

Tuladhar, A., Obermeyer, J.M., Payne, S.L., Siu, R.C.W., Zand, S., Morshead, C.M., Shoichet, M.S., 2020. Injectable hydrogel enables local and sustained co-delivery to the brain: Two clinically approved biomolecules, cyclosporine and erythropoietin, accelerate functional recovery in rat model of stroke. Biomaterials 235, 119794. https://doi.org/10.1016/j.biomaterials.2020.119794

Udina, E., Furey, M., Busch, S., Silver, J., Gordon, T., Fouad, K., 2008. Electrical stimulation of intact peripheral sensory axons in rats promotes outgrowth of their central projections. Experimental Neurology 210, 238–247. https://doi.org/10.1016/j.expneurol.2007.11.007

van den Brand, R., Heutschi, J., Barraud, Q., DiGiovanna, J., Bartholdi, K., Huerlimann, M., Friedli, L., Vollenweider, I., Moraud, E.M., Duis, S., Dominici, N., Micera, S., Musienko, P., Courtine, G., 2012. Restoring Voluntary Control of Locomotion after Paralyzing Spinal Cord Injury. Science 336, 1182–1185. https://doi.org/10.1126/science.1217416

Vavrek, R., Girgis, J., Tetzlaff, W., Hiebert, G.W., Fouad, K., 2006. BDNF promotes connections of corticospinal neurons onto spared descending interneurons in spinal cord injured rats. Brain 129, 1534–1545. https://doi.org/10.1093/brain/awl087

Vaynman, S., Gomez-Pinilla, F., 2005. License to Run: Exercise Impacts Functional Plasticity in the Intact and Injured Central Nervous System by Using Neurotrophins. Neurorehabil Neural Repair 19, 283–295. https://doi.org/10.1177/1545968305280753

von Bernhardi, R., Bernhardi, L.E., Eugenín, J., 2017. What Is Neural Plasticity?, in: von Bernhardi, R., Eugenín, J., Muller, K.J. (Eds.), The Plastic Brain, Advances in Experimental Medicine and Biology. Springer International Publishing, Cham, pp. 1–15. https://doi.org/10.1007/978-3-319-62817-2_1

Wang, K.C., Kim, J.A., Sivasankaran, R., Segal, R., He, Z., 2002a. p75 interacts with the Nogo receptor as a co-receptor for Nogo, MAG and OMgp. Nature 420, 74–78. https://doi.org/10.1038/nature01176

Wang, K.C., Koprivica, V., Kim, J.A., Sivasankaran, R., Guo, Y., Neve, R.L., He, Z., 2002b. Oligodendrocyte-myelin glycoprotein is a Nogo receptor ligand that inhibits neurite outgrowth. Nature 417, 941–944. https://doi.org/10.1038/nature00867

Wei, D., Hurd, C., Galleguillos, D., Singh, J., Fenrich, K.K., Webber, C.A., Sipione, S., Fouad, K., 2016. Inhibiting cortical protein kinase A in spinal cord injured rats enhances efficacy of rehabilitative training. Experimental Neurology 283, 365–374. https://doi.org/10.1016/j.expneurol.2016.07.001

Weidner, N., Ner, A., Salimi, N., Tuszynski, M.H., 2001. Spontaneous corticospinal axonal plasticity and functional recovery after adult central nervous system injury. Proceedings of the National Academy of Sciences 98, 3513–3518. https://doi.org/10.1073/pnas.051626798

Weishaupt, N., Blesch, A., Fouad, K., 2012. BDNF: The career of a multifaceted neurotrophin in spinal cord injury. Experimental Neurology 238, 254–264. https://doi.org/10.1016/j.expneurol.2012.09.001

Weishaupt, N., Li, S., Di Pardo, A., Sipione, S., Fouad, K., 2013. Synergistic effects of BDNF and rehabilitative training on recovery after cervical spinal cord injury. Behavioural Brain Research 239, 31–42. https://doi.org/10.1016/j.bbr.2012.10.047

Wessels, M., Lucas, C., Eriks, I., de Groot, S., 2010. Body weight-supported gait training for restoration of walking in people with an incomplete spinal cord injury : a systematic review. J Rehabil Med 42, 513–519. https://doi.org/10.2340/16501977-0525

Whishaw, I.Q., Gorny, B., Sarna, J., 1998. Paw and limb use in skilled and spontaneous reaching after pyramidal tract, red nucleus and combined lesions in the rat: behavioral and anatomical dissociations. Behavioural Brain Research 93, 167–183. https://doi.org/10.1016/S0166-4328(97)00152-6

Whishaw, I.Q., O'Connor, W.T., Dunnett, S.B., 1986. THE CONTRIBUTIONS OF MOTOR CORTEX, NIGROSTRIATAL DOPAMINE AND CAUDATE-PUTAMEN TO SKILLED FORELIMB USE IN THE RAT. Brain 109, 805–843. https://doi.org/10.1093/brain/109.5.805

Whishaw, I.Q., Pellis, S.M., Gorny, B., Kolb, B., Tetzlaff, W., 1993. Proximal and distal impairments in rat forelimb use in reaching follow unilateral pyramidal tract lesions. Behavioural Brain Research 56, 59–76. https://doi.org/10.1016/0166-4328(93)90022-I

Whishaw, I.Q., Suchowersky, O., Davis, L., Sarna, J., Metz, G.A., Pellis, S.M., 2002. Impairment of pronation, supination, and body co-ordination in reach-to-grasp tasks in human Parkinson's disease (PD) reveals homology to deficits in animal models. Behavioural Brain Research 133, 165–176. https://doi.org/10.1016/S0166-4328(01)00479-X

Widenfalk, J., Lundströmer, K., Jubran, M., Brené, S., Olson, L., 2001. Neurotrophic Factors and Receptors in the Immature and Adult Spinal Cord after Mechanical Injury or Kainic Acid. J. Neurosci. 21, 3457–3475. https://doi.org/10.1523/JNEUROSCI.21-10-03457.2001

Willand, M.P., Nguyen, M.-A., Borschel, G.H., Gordon, T., 2016. Electrical Stimulation to Promote Peripheral Nerve Regeneration. Neurorehabil Neural Repair 30, 490–496. https://doi.org/10.1177/1545968315604399

Wittenberg, G.F., 2010. Experience, cortical remapping, and recovery in brain disease. Neurobiology of Disease 37, 252–258. https://doi.org/10.1016/j.nbd.2009.09.007

Wu, Y., Zhou, J., Li, Y., Zhou, Y., Cui, Y., Yang, G., Hong, Y., 2015. Rap1A Regulates Osteoblastic Differentiation via the ERK and p38 Mediated Signaling. PLoS ONE 10, e0143777. https://doi.org/10.1371/journal.pone.0143777

Yang, H.-S., Kwon, H.G., Hong, J.H., Hong, C.P., Jang, S.H., 2011. The rubrospinal tract in the human brain: Diffusion tensor imaging study. Neuroscience Letters 504, 45–48. https://doi.org/10.1016/j.neulet.2011.08.054

Yang, L., 2018. Neuronal cAMP/PKA Signaling and Energy Homeostasis, in: Wu, Q., Zheng, R. (Eds.), Neural Regulation of Metabolism, Advances in Experimental Medicine and Biology. Springer Singapore, Singapore, pp. 31–48. https://doi.org/10.1007/978-981-13-1286-1_3

Ye, T., Fu, A.K.Y., Ip, N.Y., 2012. Cyclin-Dependent Kinase 5 in Axon Growth and Regeneration, in: International Review of Neurobiology. Elsevier, pp. 91–115. https://doi.org/10.1016/B978-0-12-398309-1.00006-8

Yezierski, R.P., 2009. Spinal cord injury pain: spinal and supraspinal mechanisms. J Rehabil Res Dev 46, 95–107.

Ying, Z., Roy, R.R., Edgerton, V.R., Gómez-Pinilla, F., 2005. Exercise restores levels of neurotrophins and synaptic plasticity following spinal cord injury. Experimental Neurology 193, 411–419. https://doi.org/10.1016/j.expneurol.2005.01.015

Ying, Z., Roy, R.R., Edgerton, V.R., Gómez-Pinilla, F., 2003. Voluntary exercise increases neurotrophin-3 and its receptor TrkC in the spinal cord. Brain Research 987, 93–99. https://doi.org/10.1016/S0006-8993(03)03258-X

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, 1070–1078. https://doi.org/10.1016/j.neuroscience.2008.06.057

Zareen, N., Dodson, S., Armada, K., Awad, R., Sultana, N., Hara, E., Alexander, H., Martin, J.H., 2018. Stimulation-dependent remodeling of the corticospinal tract requires reactivation of growth-promoting developmental signaling pathways. Experimental Neurology 307, 133–144. https://doi.org/10.1016/j.expneurol.2018.05.004

Zewdie, E.T., Roy, F.D., Yang, J.F., Gorassini, M.A., 2015. Facilitation of descending excitatory and spinal inhibitory networks from training of endurance and precision walking in participants with incomplete spinal cord injury, in: Progress in Brain Research. Elsevier, pp. 127–155. https://doi.org/10.1016/bs.pbr.2014.12.005

Appendix I

Augmentation of Activity Dependent Cellular Pathways Driving Neuronal Plasticity in Rats Using DREADDs

Preface

The work contained in this appendix was conducted during a laboratory rotation course as part of the requirements of the doctoral degree program. The following project was done in the laboratory of Dr. Simonetta Sipione, where I learned how to plate primary cortical neuron cultures under the supervision of (then graduate student) Dr. Luis Carlos Morales.

The goal of this project was to confirm the ability to infect cortical neurons with adeno-associated viruses (AAV) containing Designer Receptors Exclusively Activated by Designer Drugs (DREADDs). Following successful infection, DREADDs activation testing was planned to follow. With confirmation of the ability to infect and activate DREADDs in primary cortical neurons, the goal was to then follow up on the stimulation study presented in chapter 2 with an *in vivo* study utilizing DREADDs that can stimulate neurons when activated by CNO, and DREADDs that directly upregulate cAMP when activated. These two vectors in conjunction with rehabilitative motor therapy would have allowed for a direct comparison of increased neuronal activity to selective cAMP upregulation to better understand the role of the cAMP pathway in plasticity and functional motor recovery.

The following appendix documents the initial attempts to infect primary cortical neurons with AAVs containing DREADDs. This paper was written and presented as part of the aforementioned laboratory rotation course that this project was originally a part of and is included for the sake of documentation and to present the originally planned project.

A1.1 Introduction

Activity based neurite growth has been the target of rehabilitative therapy based treatments for spinal cord injury and other CNS diseases for many years. Rehabilitative training is considered the most successful treatment in promoting and directing plasticity to restore meaningful connections. Despite the usefulness of this treatment, knowledge regarding its mechanisms is limited. It is widely known that rehab training must be done along with additional treatments such as drugs to optimize plasticity. In order for this to be done in an effective manner, knowledge of the molecular pathways mediating rehabilitative training (such as cAMP activated PKA and EPAC pathways) induced plasticity is required before we can target these pathways.

Over the past couple of decades the effects of cAMP in relation to neurite extension in the central nervous system has been examined in detail. It has been shown that cAMP levels are naturally high in pre- and neonatal central nervous systems, corresponding with extensive axonal growth present during the development of the nervous system. These levels rapidly decline shortly after birth to low basal levels corresponding to an inability of central nervous system neurons to sprout (Cai et al., 2001). It has been shown that increasing cAMP levels in CNS neurons either via injection of a cAMP analog (Neumann et al., 2002) or via a conditioning lesion (Neumann and Woolf, 1999; Qiu et al., 2002) increases neuronal growth.

Similar effects as those seen in conditioning lesions on increasing cAMP levels have been seen using electrical stimulation of both the injured cortex (Brushart et al., 2002; Geremia et al., 2007; Gordon, 2016; Willand et al., 2016) and uninjured cortex (Carmel et al., 2013, 2010; Carmel and Martin, 2014). This increase in cAMP levels and

associated neurite growth has been linked to rehabilitative therapies and viewed as a major part of activity based neurite growth.

With cAMP having been conclusively linked to neurite growth, its downstream effectors were examined. Previous evidence has shown that cAMP increases lead to PKA activation, CREB phosphorylation, which eventually leads to a cascade of polyamines being synthesized and neuronal growth occurring (Batty et al., 2016; Cai et al., 2001, 1999; Gao et al., 2003; Ming et al., 1997; Qiu et al., 2002). A potentially large confounding variable in studies looking into the effects of cAMP based PKA activation is that many of them used inhibitors KT5720 and H89 which were recently shown to not be selective for PKA, but rather inhibited a number of secondary messengers (Murray, 2008). From this it has been shown that there is another protein, the exchange protein activated by cAMP (EPAC), that plays an important role in this activity based neurite growth pathway (Peace and Shewan, 2011). EPAC has been shown to work alongside PKA and performing the same tasks, though to a stronger extent and requiring a higher concentration of cAMP to activate. Work from our group has illustrated the effects of PKA inhibition leading to increases in neurite sprouting (Wei et al., 2016), further highlighting the suggested effects of EPAC (Batty et al., 2016; Murray et al., 2009, 2009; Peace and Shewan, 2011).

In order to optimize rehabilitative therapy in spinal cord injury treatment, the mechanisms of activity based neurite growth need to be further examined and their potential for augmentation considered. High frequency (333hz) electrical stimulation of the motor forelimb cortex prior to spinal cord injury has been shown to lead to an increase in neurite sprouting (Jack et al., 2018), which warranted follow up in a more

clinically relevant model. High frequency stimulation directly following spinal cord injury in rats in combination with rehabilitative motor therapy demonstrated increases in functional recovery and plasticity in the CST (Batty et al., 2020). Future experiments with the intent to move to lower frequencies and potentially delayed onset of stimulation should follow. Electrical stimulation has two major limitations when it comes to its role in increasing cAMP however. We are currently unable to stimulate for extended periods of time or even in multiple sessions without invasive surgery, and electrical stimulation activates all neurons around the implanted electrodes whether they be excitatory or inhibitory. As a result a better technique to increase activity in the motor forelimb cortex is desired.

With electrical stimulation being limited both in terms of selectively activating excitatory neurons and being unable to be used over extended periods of time, a new model must be used. Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) are a relatively new tool for selectively infecting groups of neurons and activating them with an exogenous ligand (Lee et al., 2014; Roth, 2016; Urban and Roth, 2015). DREADDs are genetic inserts in adeno-associated virus (AAV) particles that when inserted into cells via infection with the viral particle, cause the cell to produce the receptor encoded in the genetic insert based on the promotor used. As such there are excitatory DREADDs which are based on the human muscarinic receptor hM3Dq which causes burst firing in the cells, there are inhibitory DREADDs (hM4Di) which cause neuronal silencing when activated, and there are Gs coupled DREADDs that lead to an increase in cAMP levels following activation (Roth, 2016). All DREADDs are designed to have no basal level of activity until turned on by the exogenous ligand Clozapine-N-

oxide which is otherwise inert to the body, resulting in targeted activation of the desired receptors.

As an alternative model to increase activity in the motor forelimb cortex, infection of cortical neurons with excitatory (Gq) or cAMP increasing (Gs) DREADDs with a CamKIIa promoter would allow us to selectively activate or increase cAMP in excitatory glutamatergic neurons for prolonged periods of time. This would allow us to only turn on neurons that increase activity in the motor pathways as well as activate them for extended periods of time. DREADDs have been previously shown to increase axonal regeneration in the retinal ganglion (Lim et al., 2016) as well as in peripheral neurons (Jaiswal and English, 2017). As such we hope to move to an *in vivo* model where infection of neurons in the motor forelimb cortex with either excitatory or cAMP increasing DREADDs coupled with rehabilitative therapy would lead to increased axonal sprouting.

In order to move to this new *in vivo* model, we need a pilot project to test the effects of the DREADDs to confirm that they can infect primary cortical neurons, as well as they perform the intended function leading to increased sprouting. The goal of this project is to infect rat primary cortical neurons with an excitatory DREADD, a cAMP increasing DREADD, as well as an inert control vector, to demonstrate that when activated with CNO we can demonstrate increased depolarization in neurons with the Gq DREADD, and increased cAMP in both the Gq and Gs infected cells. In order to demonstrate this, measures for proving these effects must also be tested. This study will examine the potential of staining neurons for cFOS, an immediate early gene shown to be upregulated following neuronal activity (Herrera and Robertson, 1996) as well as the potential of performing a Western Blot to analyze levels of neurofilament 200, an

intermediate filament associated with axonal integrity and growth (Perrot and Eyer, 2009), and MAP2, a microtubule associated protein linked to dendritic extension (Diez-Guerra and Avila, 1995), as a quick measure of dendritic arborisation. Following successful infection with DREADDs, cAMP levels will be indirectly measured through western blot looking at levels of pCREB, a downstream effector of cAMP. Measuring pCREB has been used by our group previously to show increase cAMP effects as directly measuring cAMP through ELISA has been unreliable in the past (Wei et al., 2016).

A1.2 Materials and Methods

A1.2.1 Primary Neuronal Cultures

Neonatal rats (P0-P1) were decapitated and the heads were placed in a dish. Isolated cortices were placed in growth medium consisting of Neurobasal-A (Gibco, 10888-022), B-27 Supplement (Invitrogen, 17504-044), GlutaMAX x-100 (Gibco, 35050-061), and pen-strep (Hyclone, SV30010). Cortices were transferred to a 50 mL polysterene falcon tube and digested with papain (Worthington, 3120-20.7U/mgP) and DNase followed by centrifugation. Digested tissue was resuspended in plating media consisting of DMEM (Hyclone, SH30081.01), L-Glutamine (Gibco, 25030), and FBS (Sigma, F1051), and triturated with flame polished Pasteur pipettes. The solution was allowed to settle and supernatant containing individual cells was removed from remaining tissue and collected into a separate falcon tube. Remaining tissue was resuspended and trituration was repeated twice and supernatant collected. Supernatant containing individual cells was filtered via a 40 μM MESH into a separate falcon tube to remove any undigested tissue. Filtrate was then taken to a hemacytometer for counting.

Cells were plated either in 24 well plates on Poly-L-Lysine (Sigma, P4707) coated coverslips at $1.5x10^5$ cells per well, or in either 35 mm dishes or 6 well plates coated with poly-L-lysine at $7.2x10^5$ cells per plate where indicated.

A1.2.2 Immunocytochemistry

Neurons were fixed in 4% paraformaldehyde in PBS for 15 min. Fixed neurons were permeabilized with 0.1% Triton X-100 (Sigma, X100) in phosphate buffered saline (PBS) for 5 min followed by two washes in PBS for ten minutes each. Coverslips were blocked using 4% Normal Donkey Serum (sigma, D9663) in PBS for 1 h at room temperature followed by treatment with primary antibodies diluted in 4% NDS in PBS for 1 h at room temperature. Primary antibodies used include: rabbit anti-MAP2 (1:200, Millipore, Ab5622); mouse anti-MAP2 (1:250, Sternberger, Ab28032); rabbit anti-GFAP (1:500, DAKO, 20334); mouse anti-GFAP (1:500, Chemicon, mAb3580); mouse anti-cFOS (1:250, Millipore, MBE329); rabbit anti-cFOS (1:1000, Thermofisher, MA1-21190). Coverslips were washed three times in PBS and then treated for 1 h at room temperature with secondary antibodies made in the same solution. Secondary antibodies used include: donkey anti-mouse AF488 (1:500, Invitrogen, A21202); donkey anti-mouse AF594 (1:500, Invitrogen, A21203); donkey anti-rabbit AF488 (1:500, Invitrogen, A21206); donkey anti-rabbit AF594 (1:500, Invitrogen, A21207). Cells were then incubated for 5 min in a 1:5000 DAPI (Sigma, DI306) in PBS solution, and then washed twice in PBS. Coverslips were mounted onto microscope slides.

A1.2.3 Potassium Chloride and NMDA cell activation

In order to have a positive control for cFOS staining, KCl and NMDA were used to depolarize neurons for 1 h prior to fixation and staining. KCl (Fisher, BP366) and NMDA (Sigma, M3262) were added to the media of the well/plate at indicated concentrations.

A1.2.4 Cell lysates and protein quantification

Cells were lysed with NP-40 buffer containing NP-40 (Abcam, ab142227), EDTA, EGTA, MG132 (Sigma, SML1135), NEM (Sigma, E3876), phosSTOP (Roche, 04906837002), and water, and sonicated. 1:10 dilutions of cell lysates were used for protein quantification. Samples were incubated with Pierce BCA protein assay reagent A (Thermofisher scientific, 23223) and Pierce BCA protein assay reagent B (Thermofisher scientific, 23224) for 30 minutes at 37°C. Protein concentration was measured with a plate reader.

A1.2.5 Western Blot

Quantified lysates were mixed with lysis buffer and sample buffer according to calculations from a pre-made spreadsheet acquired from Luis Carlos Morales, taking into consideration the desired mass of protein to add in each well, a maximum volume of 55 µL, and the required 20% sample buffer. Samples were denatured at 60°C for 10 m. Samples were loaded into acrylamide gels (gradient concentration 4%-20%) alongside either HiMark protein ladder (Invitrogen, LC5699) or Dual colour protein ladder (Biorad,

161-0374). Gels were run at 80mV until the lower edge of the samples reached the running gel and then were run at 110mV in running buffer. Following running of the samples through acrylamide gel, the gel was equilibrated in transfer buffer (containing 0.05% SDS where large proteins are involved) for 30 minutes. Additionally sponges, filter paper, and PVDF membrane for gel transfer (activated in methanol) were equilibrated alongside it. Membrane transfer was set up overnight to run at 50 mA at 4°C.

A1.2.6 REVERT Stain

Following transfer, membrane is rinsed in Milli-Q water. To measure protein levels and normalize readings to total protein loaded in each lane, membrane was incubated in 5 mL of REVERT stain (Li-Cor, 926-11010) for five minutes, followed by two rinses in REVERT wash solution. Membrane was imaged using Li-Cor Odyssey infrared imager. Stain was removed via 5 minute incubation in reversal solution, followed by rinsing in water, and reactivating the membrane in methanol.

A1.2.7 Immunoblotting

Membrane was blocked using 5% BSA in TBS for 1 h on a shaker. Primary antibodies were then diluted in 5% TBS-T and added to membrane for 2 h at room temperature on a shaker. Primary antibodies used include: mouse anti-MAP2 (1:1000, Sternberger, Ab28032); rabbit anti-NF200 (1:1000, Sigma, N4142), mouse anti-alpha tubulin (1:1000, Abcam, Ab7291). Membrane was then washed three times quickly in TBS-T, then three times for ten minutes each. Secondary antibodies were diluted in the

same solution as primaries and added to the membrane for 1 h at room temperature. Secondary antibodies used include: donkey anti-mouse AF680 (1:10000, Li-Cor, 926-32212); donkey anti-rabbit AF800 (1:10000, Li-Cor, 926-32211). Membrane was then washed three times quickly in TBS-T, three times in TBS-T for ten minutes each, three times in TBS to remove any tween that may cause background, and twice in TBS for ten minutes each. Membrane was then scanned using Li-Cor Odyssey infrared imager.

A1.2.8 Membrane Stripping

Membrane is soaked at 60°C for 30 minutes in stripping buffer (Li-Cor, LIC-928-40028), washed twice in TBS for ten minutes, and placed in UV crosslinker for ten minutes to bleach any remaining fluorescence signal. Membrane can then be re-stained using the above immunoblotting protocol.

A1.2.9 Cell Treatment for neurite outgrowth

Cells were treated with either 50μM bGM1 (ENZO, ALX-302-001-M010), 50 μM DANA (Sigma, D9050), 25 μM ESI-05 (Biolog, M092), or 100 μM 8-CPT-2Me-cAMP (Tocris, 1645) by addition of the correct volume to the cell media starting at DIV 3 and continuing until the cells were lysed using NP-40 buffer.

A1.2.10 DREADDs and viral infection

The three DREADDs used were all serotype 2 and were as follows:
pAAV-CaMKII-mCherry

- Control vector
- C = $7.5x10^{12}$ vg/mL

pAAV-CaMKII-HA-rM3D(Gs)-IRES-mCitrine

- Increases cAMP levels in the cell without causing cellular depolarization when CNO added
- C = $1.5x10^{12}$ vg/mL

pAAV-CaMKII-HA-hM3D(Gq)-IRES-mCitrine

- Causes cellular depolarization when CNO added
- C = $2.5x10^{12}$ vg/mL

DREADDs were a gift from Bryan Roth and AAV particles were purchased from Duke University Viral Vector Core.

Two different modality of infection were tested. *Overnight infection was performed w*hen neuronal cultures reach DIV 8, viral particles were slow thawed on ice from -80°C. Multiplicity of Infection (MOI) was determined for each well and using the following formula, the amount of virus needed in each well/dilution was calculated:

$$Number\ of\ cells\ in\ well * Desired\ MOI = Total\ number\ of\ plaque\ forming\ units\ (PFU)$$

then $\frac{Total\ PFU\ needed}{\frac{PFU}{mL} of\ virus} = total\ volume\ of\ virus\ needed.$

In instances where the desired MOI required volumes smaller than 0.5 µL, serial dilutions were made. Proper virus doses were added to each well/plate as needed and virus-infected cells were incubated overnight in a 37°C incubator. Media was completely replaced the following day to remove excess viral particles.

A1.2.11

Spin-infection was performed at the time of primary neuronal cultures (i.e. DIV 0). Multiplicity of Infection (MOI) was determined for each well and using the following formula, the amount of virus needed in each well/dilution was calculated:

$Number\ of\ cells * Desired\ MOI = Total\ PFU$ then $\frac{Total\ PFU\ needed}{\frac{PFU}{mL} of\ virus} =$

$total\ volume\ of\ virus\ needed.$

Proper virus doses were added to each well/plate as needed and virus-infected cells were spun at 2500 RPM for 90 minutes at 22°C, and then incubated overnight in a 37°C incubator. Media was completely replaced the following day to remove excess viral particles.

A1.2.12 SH-SY5Y Cell plating

SH-SY5Y human neuroblastoma cells were grown in 45% MEM (Gibco, 10370-021), 45% F12, and 10% FBS.

A1.2.13 Real-time PCR

Messenger RNA was extracted from cell lysates (lysed using RLT buffer from RNeasy mini kit) using the manufacturer's instruction (Qiagen, 74104). 500 ng of extracted mRNA is added to nuclease free 0.2 mL tube alongside 1 µL of oligo(dT)12-18, I µL dNTP mix (Invitrogen, 10297018), and Milli-Q water to fill the remaining volume up to 11 µL. Mixture was heated to 65°C for five minutes, quickly chilled on ice,

and centrifuged. 4 µL of 5x first strand buffer and 2 µL of DTT was added followed by incubation for two minutes at 42°C. 1 µL of superscript II RT was added to the mixture. Solution was then incubated at 42°C for fifty minutes and the reaction inactivated by heating to 70°C for fifteen minutes. cDNA was aliquoted and diluted such that each sample had five serial dilutions including: 1:5, 1:25, 1:125, 1:625, 1:3125. Dilutions were mixed with 7.5 µL of SYBR Green master mix (Applied Biosystems, 4385610), 0.375 µL of left and right primers as well as 1.75 µL of water. Primers used were: Human fw: gccagtcactgtatccagca and rv: gaggccagcaacagattctc; Rat fw: caaagctggtgcctacgatt and rv: gggttgatggtcagcatctt. Following PCR, amplicons were run though a 3% agarose gel electrophoresis and visualized by staining with ethidium bromide. The gel was imaged and amplicon size confirmed based on comparison with a DNA ladder.

A1.3 Results

A1.3.1 Analysis of neuronal activity by cFOS staining, and Live Calcium Imaging

The ability to detect neuronal activation upon neuron infection with DREADDs was initially tested by cFOS staining. In order to test the efficacy of staining for cFOS *in vitro*, a positive control for cellular activation must be used to demonstrate elevated levels of cFOS following activation as compared to baseline levels. Potassium Chloride (KCl) has been used as a means to depolarize primary neurons in a variety of concentrations ranging from 20 mM to 90 mM (Kato et al., 2013; Lemmens et al., 2001; Lin et al., 2011; Macías et al., 2001). As such we decided to use 50 mM KCl as our positive control. Cells were exposed to 50 mM KCl for 1 h at 37°C, following which they were fixed and stained for MAP2 using mouse anti-MAP2 and rabbit anti-cFOS (a gift from Dr. Bradley

Kerr) primary antibodies. I was not able to detect any increase in cFOS immunostaining compared to not stimulated cells.

NMDA has been shown to lead to cellular depolarization at 10 µM (Krogh and Thayer, 2016). Therefore, both NMDA and KCl were used to treat primary cortical neurons (DIV 10). After incubation with 10 µM NMDA I was able to confirm neuronal activation by cFOS staining (Fig. 1)

A1.3.2 NF200 and MAP2 protein levels to measure neurite outgrowth in vitro.

Since the ultimate goal of our studies will be to test whether neuronal activation by DREADDs increases plasticity and axonal sprouting, we set to determine the extent to which we could detect neurite outgrowth *in vitro*. In order to measure the degree of dendritic arborisation in primary cortical neurons, we investigated the potential of using a western blot to have a quicker means of quantifying the extent of arborisation than tracing neurites. In doing so we looked at NF200, one of the main neurofilament proteins which are shown to play a pivotal role in axon outgrowth and structure of the neuron (Laser-Azogui et al., 2015; Lowery et al., 2015), and MAP2, a microtubule associated protein that has been used as a measure of s neuronal arborisation previously (Stefano et al., 2006).

Neuronal cultures at DIV 1, 3, 6, 8, 10, and 13 were lysed and MAP2 and NF200 levels were measured by immunoblotting. MAP2 and NF200 protein levels were normalized to total protein levels, as the latter appeared more constant than alpha tubulin levels as loading control across samples (Fig. 2). Both MAP2 and NF200 levels showed

an upward trend during neuronal cultures, reflecting neurite and dendritic growth as cultures mature in vitro, as expected, with the large isoform of MAP2 coming to a rapid plateau after DIV 6. The small isoform of MAP2 and NF200 both show progressive increases over time. These data indicate that immunoblotting for NF200 and the small isoform of MAP2 could potentially be used to estimate neurite outgrowth.

To test the potential that NF200 and the small isoform of MAP2 can be used as a of neurite outgrowth by immunoblotting, I treated neuronal cultures with various compounds that have been shown to either increase or block neurite outgrowth to see if immunoblotting would show the corresponding expected changes. GM1, a ganglioside shown to play a role in neuronal differentiation and neuritogenesis (El Sayed et al., 1991; Rodriguez et al., 2001; Wu et al., 2016); and DANA, a sialidase inhibitor that was shown to decrease axonal growth specifically (El Sayed et al., 1991; Rodriguez et al., 2001) were the first compounds tested. GM1 and DANA were added to the media of primary cortical neurons starting on DIV 3 at a final concentration of 50 μM. Treated and untreated control neurons were lysed and collected at DIV 1, 6, 9, and 13. There was a clear increase in NF200 and MAP2 as neurons developed in cultures. Statistical analysis was unable to be performed as there was only one data point per group. However, compared to the untreated group, GM1 showed a slight decrease in both NF200 and MAP2 levels, whereas DANA increased MAP2 and had little effect on NF200 (Fig. 3). All results for MAP2 for this experiment are for the large isoform only as the small isoform of MAP2 was not detected in this immunoblot.

Next, we tested the effects of the EPAC agonist 8-CPT-2-Me-cAMP and the antagonist ESI-05. The EPAC agonist has been previously shown to increase neurite

sprouting (Batty et al., 2016; Enserink et al., 2002; Wei et al., 2016) and was expected to increase NF200 and MAP2 levels to reflect this. ESI-05 has been shown to reduce neurite growth (Batty et al., 2016; Wei et al., 2016) and was expected to reduce NF200 and MAP2 levels as a result. Treatments were added on DIV 3 and then refreshed with each medium change.

The untreated group showed consistently increasing NF200 and MAP2 levels, save the final plate having a decrease in both proteins, suggesting a possible issue with this plate in particular rather than the decrease being normal. Statistical analysis was again unable to be performed due to lack of repetition in the experiment. The EPAC antagonist ESI-05 showed a strong trend towards a reduction of levels of both MAP2 and NF200 proteins (Fig. 4). The EPAC agonist also seemed to have a partial inhibitory effect on MAP2 and NF200 expression, contrary to what was expected.

A1.3.3 Infection of neuronal cultures with DREADDs

Next, we tested whether our DREADD viruses were able to infect neurons in culture. Primary cortical neurons were infected overnight on DIV 8. Based off of previous literature (Liu et al., 2014) each DREADD was used at MOIs of 0, 1, 10, 100, 400, and 1000 as an initial test to determine optimal infection rate. Infection rate was determined using the fluorescent marker encoded in each viral vector, as well as by immunocytochemistry for the Human influenza hemagglutinin (HA)-DREADD chimeric protein as a secondary confirmation. Even at an MOI of 1000, none of the three vectors showed any evidence of infection despite clear health and viability of neuronal cultures (Fig. 5-7).

A1.3.4 Infection of SH-SY5Y with DREADD viruses

Following the unsuccessful infection of primary cortical neurons with any of the three viral vectors, we tested whether the same viruses could infect SH-SY5Y cells, which are known to express CamKIIa (Kato et al., 2013) and could be infected by AAV2 viral vectors (Charbel Issa et al., 2013). Both undifferentiated SH-SY5Y cells and cells differentiated towards a neuronal phenotype with 10 μM retinoic acid (Encinas et al., 2002) were infected with an MOI of 0, 400, or 1000 of one of the three DREADDs. Cells were visualized under fluorescent microscope for expression of the fluorescent markers on each of the viral vectors but none was seen (data not shown).

A1.3.5 Spin Infection of Primary Neurons at DIV 0

Since our protocol for AAV infection of primary cortical neurons was unsuccessful, we aimed to test an alternate protocol suggested by the literature. It has been shown that at the time of neuronal plating, spinning the cells with the AAV particles at 2500 RPM for 90 minutes should successful infect the neurons (Liu et al., 2014). Thus, we attempted to infect cells with the spin protocol using all three viruses at MOIs of 0, 100, 400, 1000, and 5000.

Spinning the neurons for 90 minutes at room temperature resulted in a marked increase in cell death. Immunocytochemistry was still performed at DIV 10 on the very few live cells though imaging under fluorescent microscope showed no evidence of HA tag or fluorescent marker expression (data not shown), suggesting, once again, failure of

the viruses to infect. Results were confirmed by immunoblotting with anti-HA antibodies (Fig. 8), which was able to reveal HA expression in a control sample previously generated in the laboratory, but not in infected samples.

A1.3.6 CamKIIa Expression in Neurons and SH-SY5Y cells

Since none of our attempts were successful in infecting either rat cortical neurons or SH-SY5Y cells with the DREADDs, we sought to confirm that the CamKIIa promoter that drives expression of the receptors from the viral vectors would be active in our cell models. Thus, we performed rtPCR of endogenous CamKIIa expression. rtPCR showed that rat cortical neurons expressed high levels of CamKIIa as seen through the PCR amplification plot (Fig. 9a) and the melt curve showing the expected melt temperature for the amplicon (Fig. 9b). SH-SY5Y cells also expressed CamKIIa in both undifferentiated and differentiated conditions (data not shown).

Real-time PCR data were confirmed by running the samples through an agarose gel to confirm the size of the amplicons as being the size expected given our designed primers. The agarose gel shows high levels of expression in rat cortical neurons and the rat cortex used as a control. Undifferentiated SH-SY5Y cells show a low level of CamKIIa expression while differentiated SH-SY5Y cells show greater levels of expression (Fig. 9c).

Altogether, these data suggest that our primary neurons and SH-SY5Y would be able to express DREADDs under control of the CamKIIa promoter. Therefore, failure to

detect any DREADD protein in our experiments must be attributed to defective viral preparations unable to infect cells.

A1.3.7 High MOI infection

As a last attempt, we tried to infect neurons using a much higher concentration of viruses than previously used. One well of cells was infected with the Gq DREADD at an MOI of 1,000,000, and another well was infected with the mCherry control viral vector at an MOI of 5,000,000, both using the original overnight infection protocol. Immunocytochemistry for the HA tag showed no evidence of HA tag expression in the Gq infected cells (data not shown) however about 35% of cells infected with the control vector exhibited a low level of mCherry expression (Fig. 10). These data suggest that only a very small number of viral particle in our virus preparation was infective, and confirmed that primary cortical neurons could be infected and could express DREADDs provided that the quality of the viral preparation is high (high MOI).

A1.4 Discussion

In this study, we initially set out to determine and use a set of measures for measuring the effects of an activating and cAMP increasing DREADD on rat primary cortical neurons *in vitro* as a proof of concept prior to an *in vivo* application of these viral vectors in a spinal cord injury model.

We first determine whether cFOS expression could be used as a surrogate measure of neuronal activation in cells expressing DREADDs. Action potentials in a

neuron causes an upregulation of cFOS mRNA and the associated protein and so its use as a marker of neuronal activity has been well established (Murphy et al., 1991; Sagar et al., 1988). When KCl was used to depolarize neurons, we were not able to see a corresponding increase in cFOS, contrary to other reports in literature (Geusz et al., 1997; Herrera et al., 1993). We next used NMDA to activate neurons in culture. While at higher concentrations NMDA was ineffective in increasing cFOS levels, or even toxic, at 10 μM concentration NMDA triggered clear cFOS expression that we were able to detect by immunocytochemistry. Therefore, in future studies where a positive control for neuronal activation is required, NMDA will be used. Further tests of cFOS staining will still be necessary to confirm its validity, as well as adding further analysis of the staining such as thresholding.

The main purpose of using DREADDs in our future studies is to increase neurite growth, either through sprouting or regeneration. Current means to measure sprouting or neurite extension require tracing processes to measure their length which can be very time consuming and potentially inaccurate. To confirm these DREADDs function as intended we hoped to measure the level of proteins associated with neurites as a quicker quantifiable measure of the extent of arborisation as compared to neurite tracing. First we had to establish baseline readings of MAP2 and neurofilament in neurons at different stages of development. To do so we lysed cells at specified time points as a culture developed. MAP2 is reported to have multiple isoforms categorized into either high molecular weight MAP2 or low molecular weight MAP2. The larger isoform of MAP2 is located in neuronal cell bodies and dendrites whereas the small isoform is located in every neuronal compartment (Sánchez, 2000). The large isoforms of MAP2 reached a

plateau past DIV 6, whereas the small isoform showed a clear increase over time that could serve as a valid measure of neurite extension. Neurofilament levels reflected via measurement of NF200 levels showed a progressive increase illustrating that neurofilament levels clearly rise as neurite extension progresses. To further test the potential of measuring protein levels to measure neurite growth we applied a variety of drugs that have been shown to effect neurite growth and looked to see if protein levels reflected the expected changes. Our first treatment with ganglioside GM1 and DANA showed that GM1 caused a slight decrease in levels of both proteins as has been previously reported in the literature (El Sayed et al., 1991), whereas DANA unexpectedly raised MAP2 and did not have much of an effect on NF200 in this one test. The second treatment focusing on EPAC used an agonist, 8-CPT-2'-Me-cAMP, and an antagonist, ESI-05. This experiment showed a clear indication that antagonism of EPAC leads to a decrease in levels of both proteins as was expected, though further trials will need to be done to confirm this in a significant manner. Increasing EPAC levels via the EPAC agonist surprisingly showed no increase in protein levels which was contrary to our previous results (Wei et al., 2016). Further trials need to be done in order to confirm these results, as well as verify the use of protein levels as a measure of neurite extension via comparison with neurite tracing methods. It is important to note that the small isoform of MAP2 was remarkably undetected via immunoblotting in both of our treatments which did not allow us to check the effects of any of our treatments on its expression level. The reason for its lack of appearance is unknown and needs to be investigated further.

Lastly, the infection with the DREADDs themselves were unsuccessful in the vast majority of our experiments. Serotype 2 adeno associated viruses are, to the best of our

knowledge as well as is indicated in the literature (Fu et al., 2003; Hocquemiller et al., 2016), to be optimal for infecting central nervous system neurons. Additionally, the use of a CamKIIa promoter driven construct in glutamatergic cortical neurons is valid as excitatory neurons are reported to express CamKIIa (Hoerndli et al., 2015; Hudmon and Schulman, 2002). Our first protocol involved bath application of the neurons in viral particles by adding them at varying multiplicities of infection so as to determine the optimal concentration of virus to use. Much to our surprise there was no evidence of successful infection measured through both immunocytochemistry for the HA tag, and by looking for the fluorescence tag on each viral vector. Our next attempt to infect a different cell line, namely SH-SY5Y cells, reported to be able to be infected by serotype 2 AAVs (Charbel Issa et al., 2013) via the same protocol also was unsuccessful. Next we tested an alternate protocol found in the literature reported to be successful at infecting cells with AAVs that involved spinning the cells and viruses together, though was unsuccessful in our hands. At this point we had reason to believe that our difficulties infecting neurons were not due to our protocols or cells but that the viral particles themselves were dead, invalid, or non-functional. Though to confirm this we proved that the lack of evidence of infection was not due to a lack of transcription within the cell due to a poor choice in promoter. We confirmed this via rtPCR of both rat primary cortical neurons and SH-SY5Y cells and found that both express CamKIIa and therefore should be able to transcribe the gene encoded in the viral vector. With all of this information we firmly believe that the DREADDs used in this experiment are indeed dead, invalid, or non-functional.

At the request of Duke Viral Vector Core, the providers of the viral vectors, we attempted to infect our cells with an extremely high multiplicity of infection of DREADDs as they mentioned the potential for the viral particles to only be 0.1 – 0.01% functional. At their request we infected two wells, one with an MOI of 1000000 of the Gs DREADD and one with an MOI of 5000000 of the mCherry control vector. The Gs DREADD showed no signs of infection though to our surprise the mCherry vector showed a low level of fluorescence indicating there was a very small percentage of the viral particles that were indeed functional, though in our opinion not enough to still be considered useful.

Despite the infection seen at high MOI with the mCherry control vector, the viral particles are speculated to be the cause of the overall lack of infectivity. This may be due to the difficulty in producing viral particles with plasmid inserts greater than 5 kB, as has been reported by Duke Viral Vector Core. They report that genetic inserts above 5 kB result in an unreliable titer and less functionality of the viral particles. An alternate cause of the lack of activity in the viral particles may have been due to the manner of shipping. The DREADDs were shipped on a Friday without a label indicating dry ice and storage temperatures, and upon arrival had very little dry ice remaining. The inactivation of viral particles due to thawing may not be a valid theory however as AAV particles have been reported to be very stable and only exhibit small reductions in infectivity following freeze-thaw cycles (Howard and Harvey, 2017).

A1. 5 Conclusion

In conclusion, our study yielded two measures to test the efficacy of these viral vectors that show promise in their validity, though more testing of both protocols is required to ensure they are capable of accurately measuring the variables necessary, accurately reflecting the effects of the constructs. Our experiments also yielded no *in vitro* DREADD infected neuron model as intended though we firmly argue that this is by no fault of our own and is due to the viral particles themselves, therefore in order to continue this experiment new constructs will need to be obtained.

A1.6 Appendix Figures

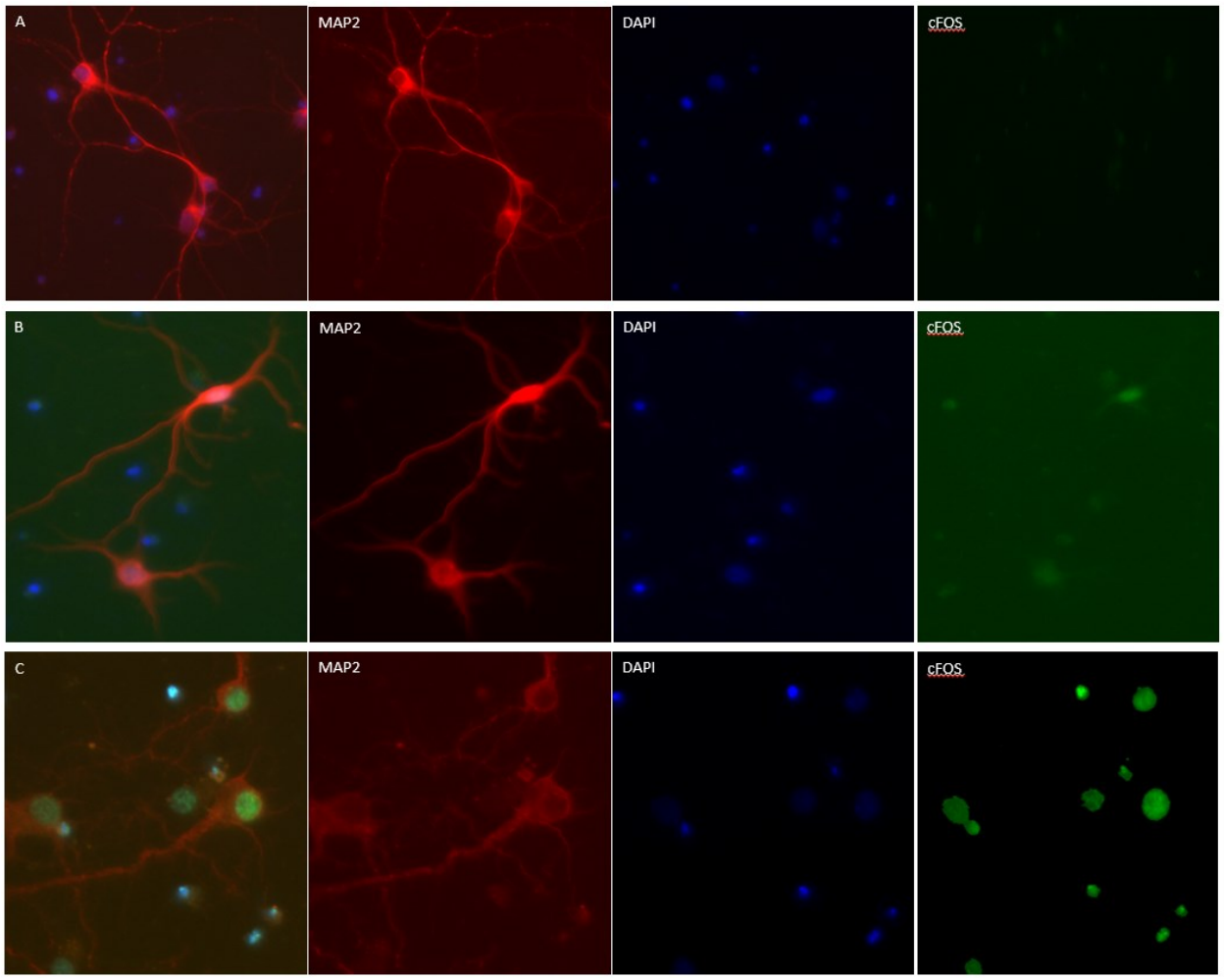

Figure A1.1 – Second Attempt at cFOS staining using two different compounds to activate cells

Staining performed with rabbit anti-MAP2 in red, mouse anti-cFOS in green, and nuclei stained with DAPI in blue. Exposure set so that negative control shows no cFOS fluorescence to account for basal constitutive activation of cFOS, these exposure parameters were used in subsequent images in the same experiment so only levels above baseline appear. **(A)** Negative control group without KCl addition showing no cFOS staining. **(B)** 25 mM KCl group showing slightly elevated levels of cFOS as seen through minor increase in green fluorescence. **(C)** 10 µM NMDA group showing elevated cFOS levels co-localized with DAPI staining in the nuclei.

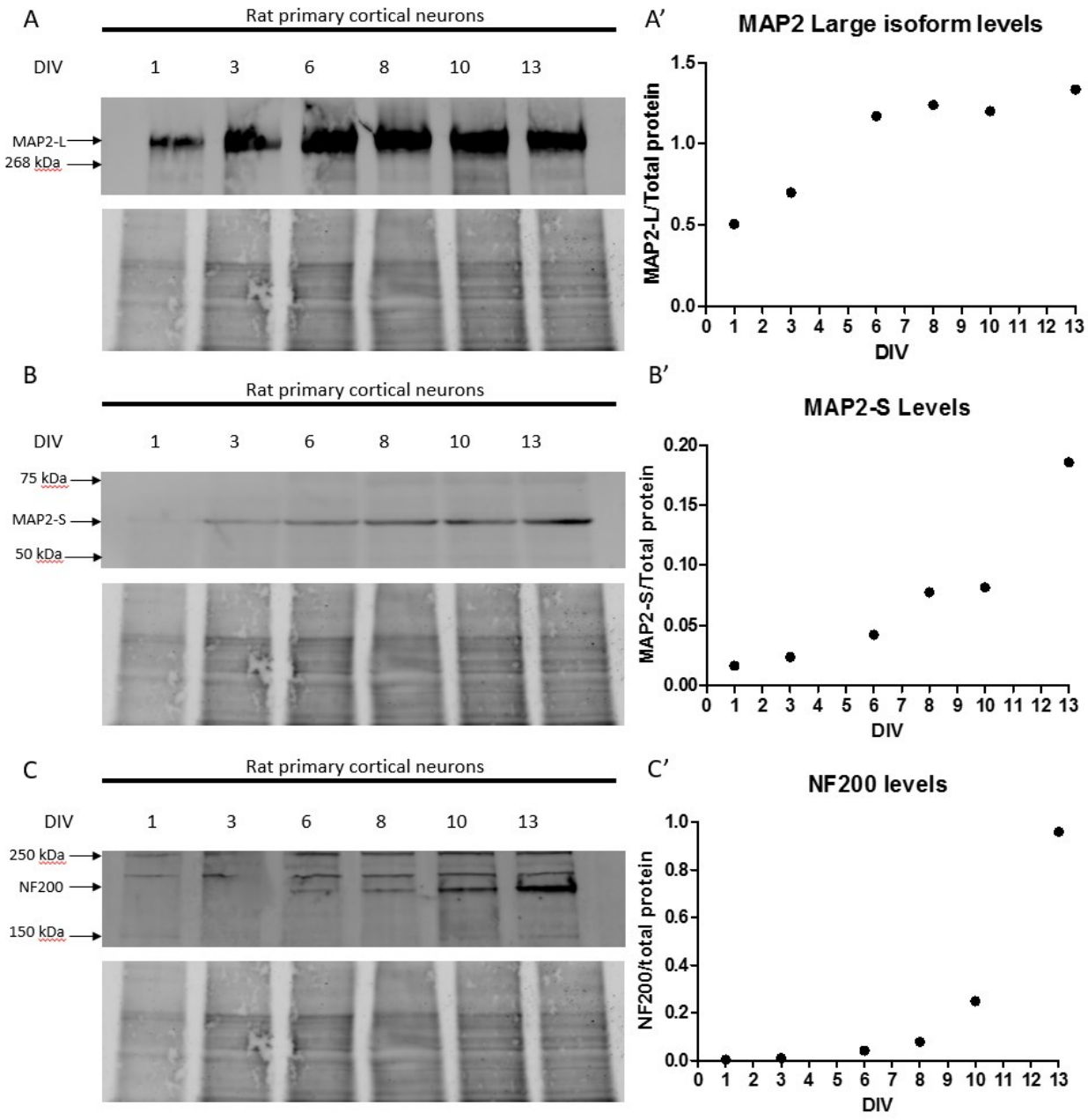

Figure A1.2 – NF200 and MAP2 protein levels progressively increase during neuronal culture *in vitro*

(A) The large isoform of MAP2 with REVERT staining below, showing the area used to normalize the fluorescence values, alongside **(A')** showing the quantified values of the fluorescence normalized to total protein levels. **(B)** Bands from the small isoform of MAP2 with REVERT below. **(B')** quantified fluorescence values normalized to total

protein values. **(C)** Bands from NF200 above the REVERT stain and **(C')** shows quantified values of NF200 fluorescence normalized to total protein levels.

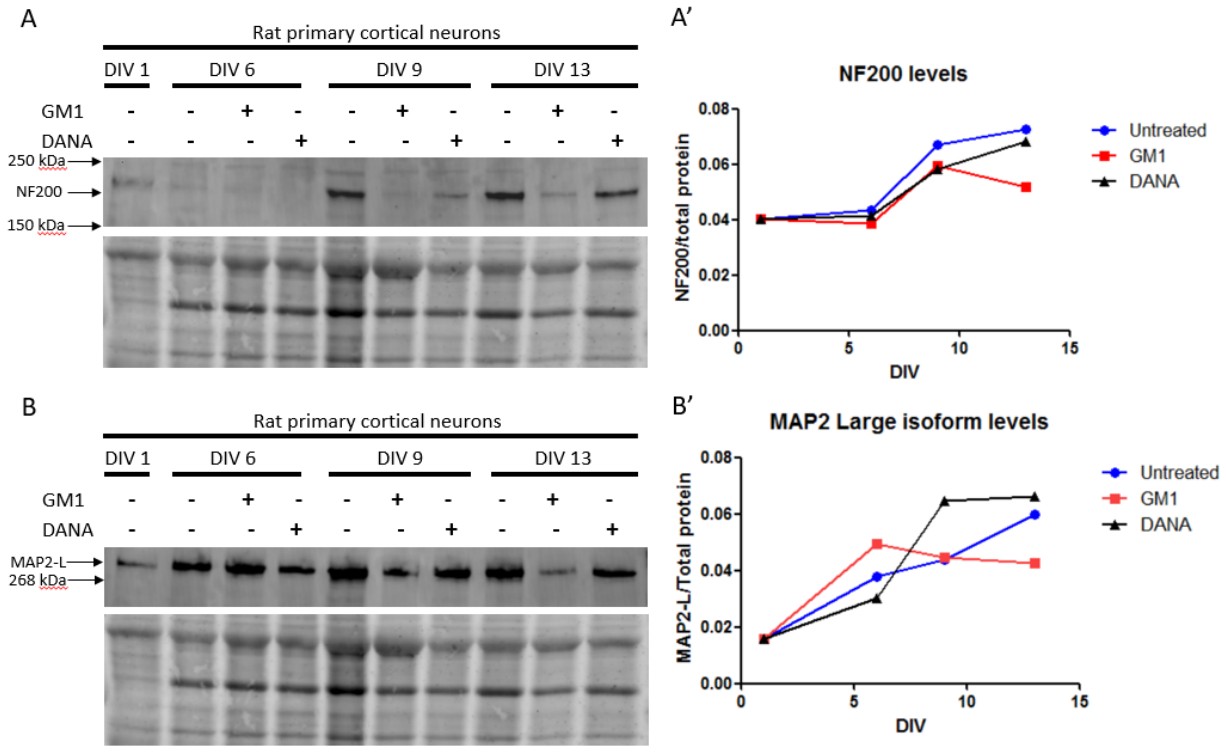

Figure A1.3 – Effects of GM1 and DANA on NF200 and MAP2 protein levels

(A) shows the Bands from NF200 above the REVERT stain, highlighting the area used to normalize the fluorescence values, alongside **(A')** showing the quantified values of the fluorescence normalized to total protein levels compared between untreated, GM1, and DANA groups. **(B)** Bands from the large isoform of MAP2 with REVERT below. **(B')** quantified fluorescence values normalized to total protein values compared between groups. The small isoform of MAP2 was not detected in this immunoblot.

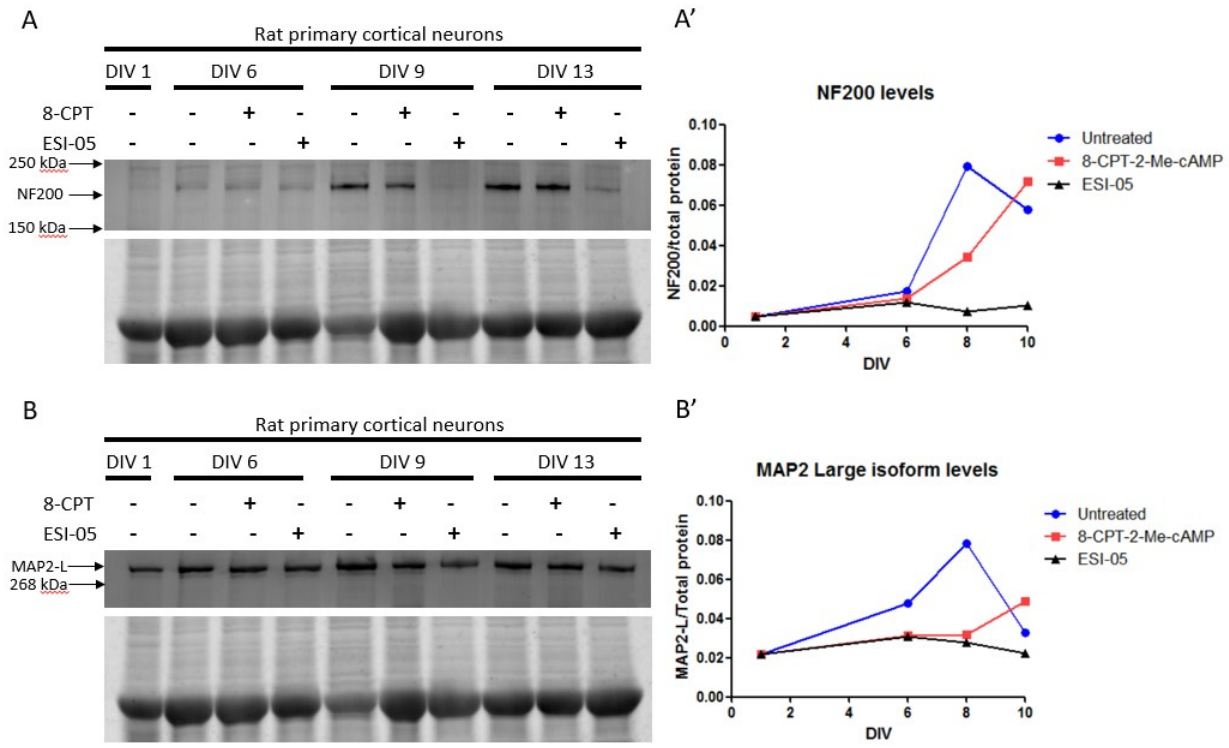

Figure A1.4 – Effects of 8-CPT-2`-Me-cAMP and ESI-05 on NF200 and MAP2 protein levels

(A) shows the Bands from NF200 above the REVERT stain, highlighting the area used to normalize the fluorescence values, alongside **(A')** showing the quantified values of the fluorescence normalized to total protein levels compared between untreated, 8-CPT-2-Me-cAMP, and ESI-05 groups. **(B)** Bands from the large isoform of MAP2 with REVERT below. **(B')** quantified fluorescence values normalized to total protein values compared between groups. The small isoform of MAP2 was not detected in this immunoblot.

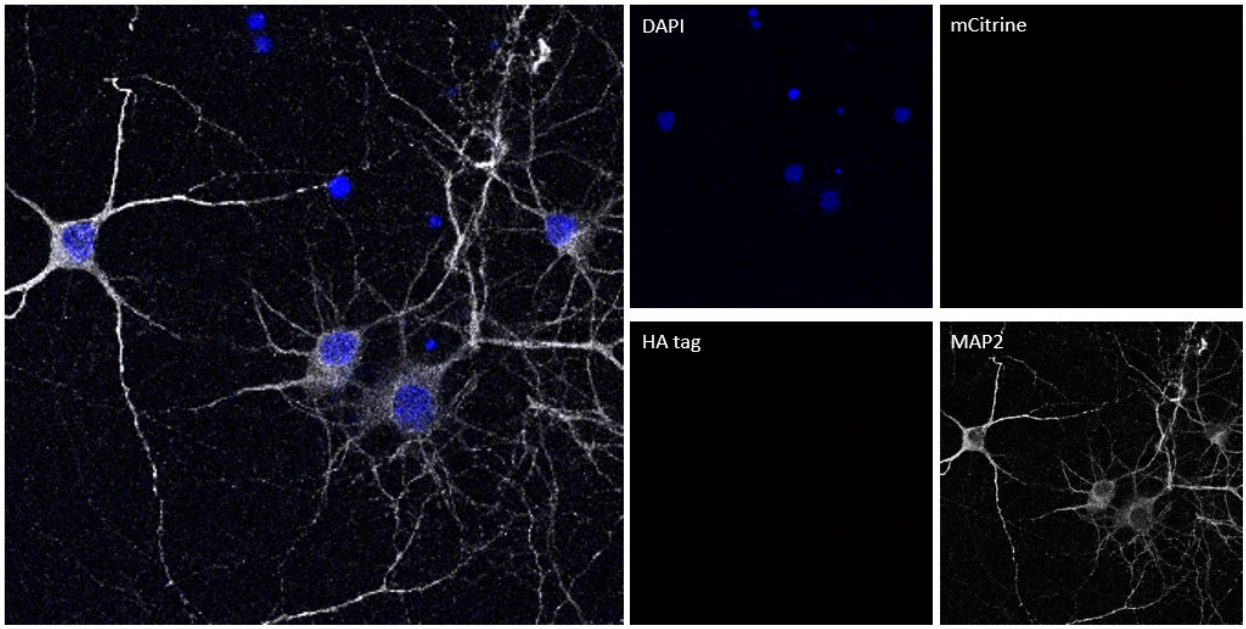

Figure A1.5 – Confocal image showing unsuccessful infection with Gs viral vector at an MOI of 1000

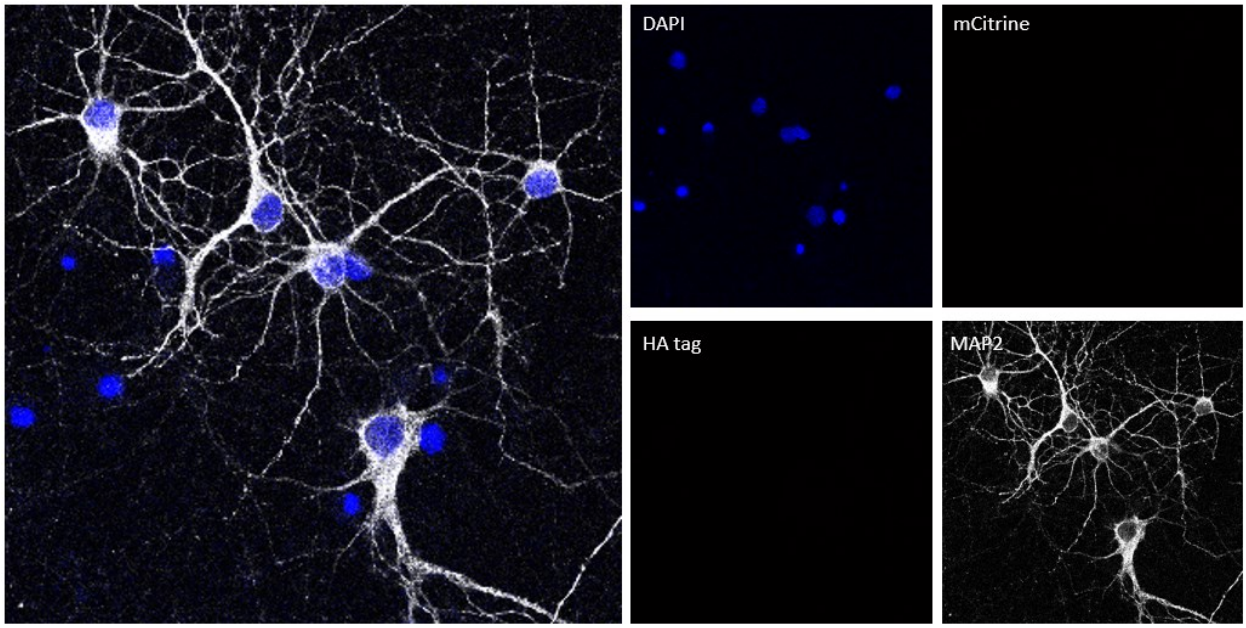

Figure A1.6 – Confocal image showing unsuccessful infection with Gq viral vector at an MOI of 1000

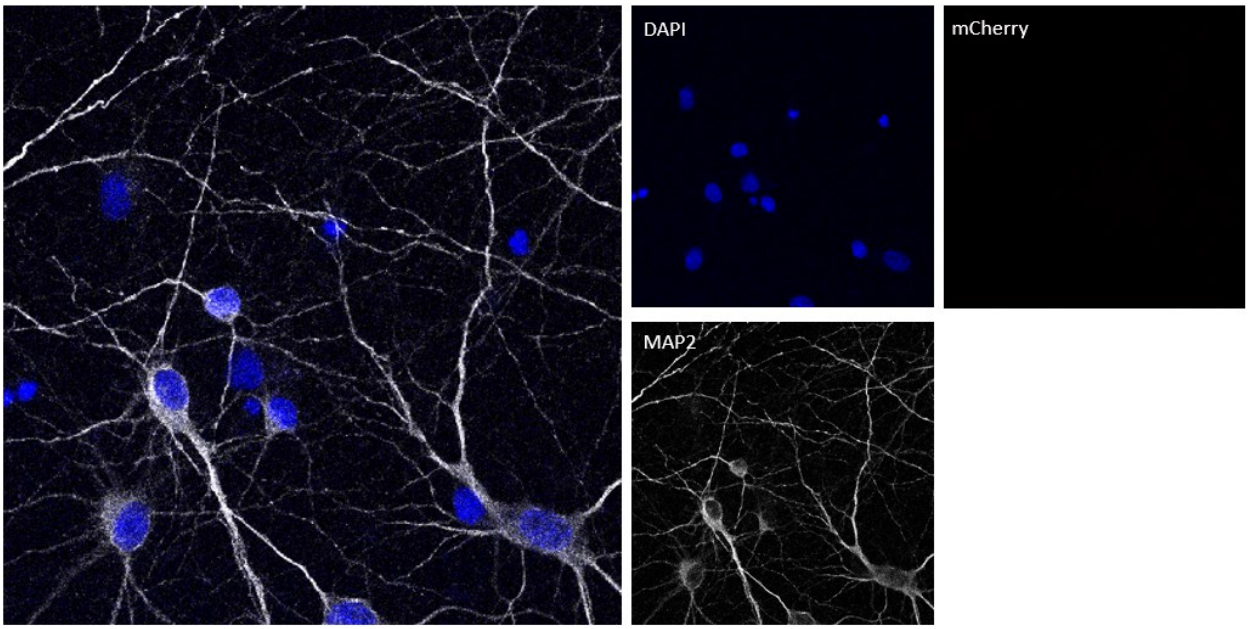

Figure A1.7 – Confocal image showing unsuccessful infection with mCherry control vector at an MOI of 1000

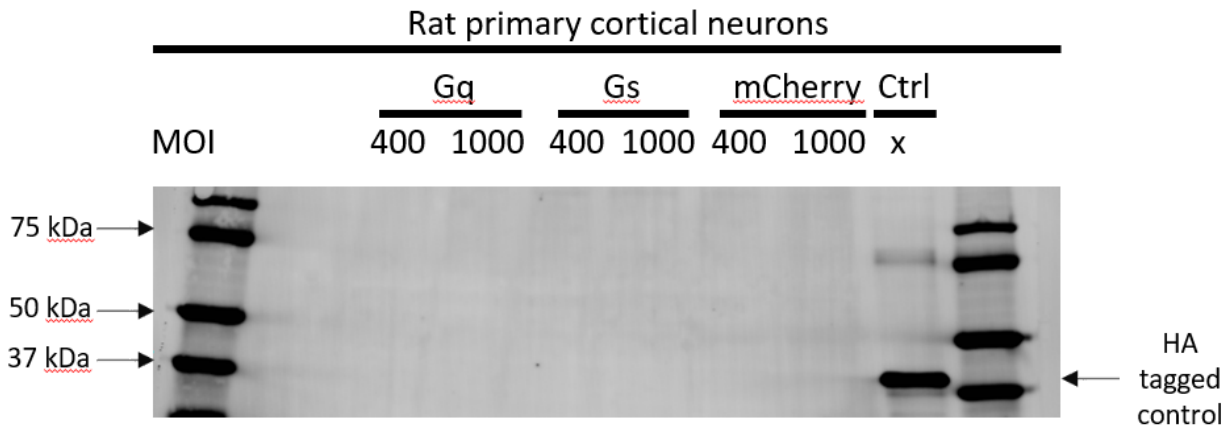

Figure A1.8 – Immunoblot showing lack of HA tag expression in cortical neurons infected with DREADDs alongside a previously generated HA tagged protein

Positive control protein runs at 40 kDa as shown. Calculated molecular weight of the DREADD receptors tagged with HA is 77 kDa. There was no protein evident in the expected area of the Western Blot in any of the infection groups.

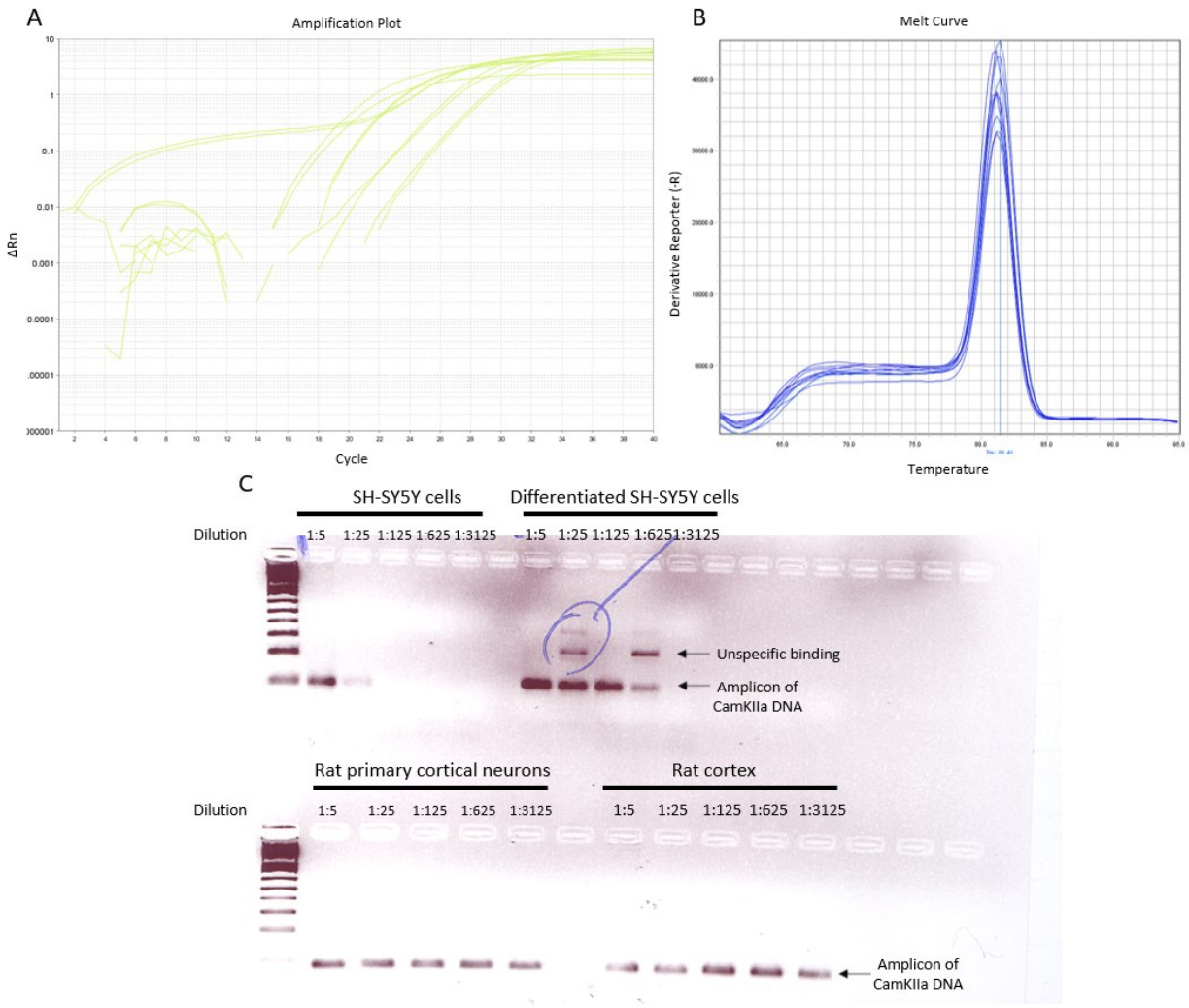

Figure A1.9 – rtPCR confirmation of CamKIIa expression in rat cortical neurons and SH-SY5Y cells

(A) shows the amplification plot from the rtPCR for the cortical neurons, plot clearly shows an increase in arbitrary fluorescent units above baseline levels of 0.01, confirming amplification of desired cDNA. **(B)** Melt curve from rtPCR of rat cortical neurons, confirming size of the amplicons through their consistency in melting point. **(C)** Agarose gel of amplicons post-PCR to confirm size of amplicons produced.

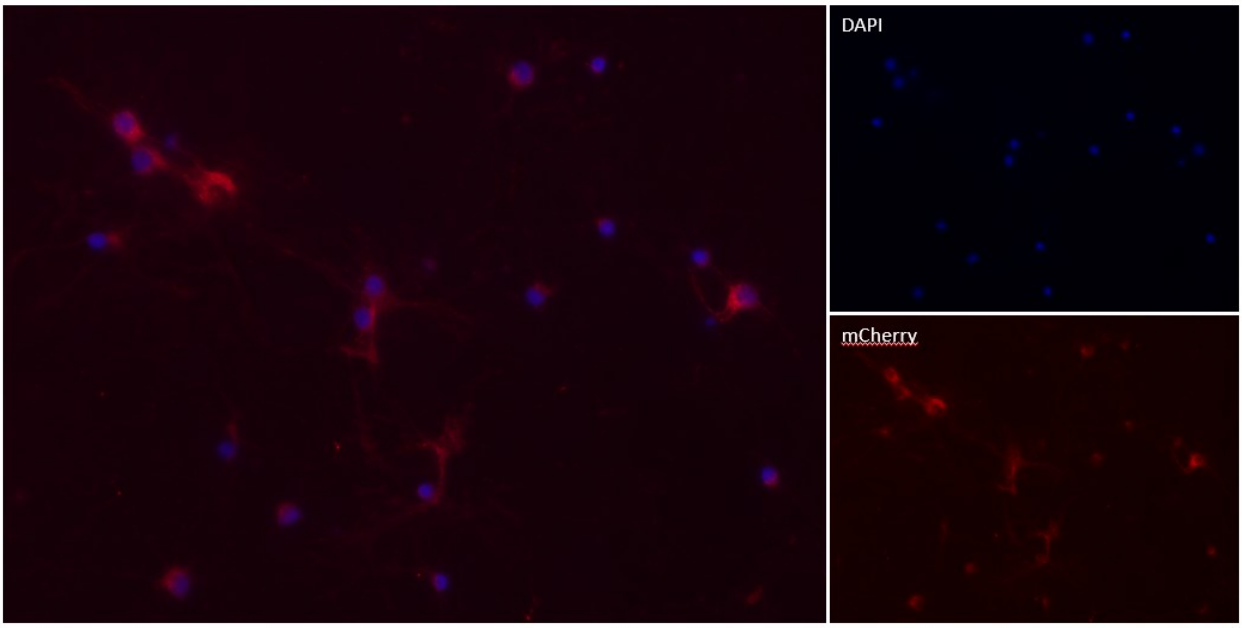

Figure A1.10 – Image from fluorescent microscope showing neurons infected with mCherry vector at MOI of 5000000

Nuclei are stained in blue and mCherry is shown in red. Neurons were not stained with anything aside from DAPI.

A1.7 Appendix References

Batty, N.J., Fenrich, K.K., Fouad, K., 2016. The role of cAMP and its downstream targets in neurite growth in the adult nervous system. Neurosci. Lett. https://doi.org/10.1016/j.neulet.2016.12.033

Batty, N.J., Torres-Espín, A., Vavrek, R., Raposo, P., Fouad, K., 2020. Single-session cortical electrical stimulation enhances the efficacy of rehabilitative motor training after spinal cord injury in rats. Exp. Neurol. 324, 113136. https://doi.org/10.1016/j.expneurol.2019.113136

Brushart, T.M., Hoffman, P.N., Royall, R.M., Murinson, B.B., Witzel, C., Gordon, T., 2002. Electrical Stimulation Promotes Motoneuron Regeneration without Increasing Its Speed or Conditioning the Neuron. J. Neurosci. 22, 6631–6638.

Cai, D., Qiu, J., Cao, Z., McAtee, M., Bregman, B.S., Filbin, M.T., 2001. Neuronal Cyclic AMP Controls the Developmental Loss in Ability of Axons to Regenerate. J. Neurosci. 21, 4731–4739.

Cai, D., Shen, Y., De Bellard, M., Tang, S., 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.

Carmel, J.B., Berrol, L.J., Brus-Ramer, M., Martin, J.H., 2010. Chronic Electrical Stimulation of the Intact Corticospinal System after Unilateral Injury Restores Skilled Locomotor Control and Promotes Spinal Axon Outgrowth. J. Neurosci. 30, 10918–10926. https://doi.org/10.1523/JNEUROSCI.1435-10.2010

Carmel, J.B., Kimura, H., Berrol, L.J., Martin, J.H., 2013. Motor cortex electrical stimulation promotes axon outgrowth to brain stem and spinal targets that control

the forelimb impaired by unilateral corticospinal injury. Eur. J. Neurosci. 37, 1090–1102. https://doi.org/10.1111/ejn.12119

Carmel, J.B., Martin, J.H., 2014. Motor cortex electrical stimulation augments sprouting of the corticospinal tract and promotes recovery of motor function. Front. Integr. Neurosci. 8. https://doi.org/10.3389/fnint.2014.00051

Charbel Issa, P., De Silva, S.R., Lipinski, D.M., Singh, M.S., Mouravlev, A., You, Q., Barnard, A.R., Hankins, M.W., During, M.J., MacLaren, R.E., 2013. Assessment of Tropism and Effectiveness of New Primate-Derived Hybrid Recombinant AAV Serotypes in the Mouse and Primate Retina. PLoS ONE 8, e60361. https://doi.org/10.1371/journal.pone.0060361

Diez-Guerra, F.J., Avila, J., 1995. An Increase in Phosphorylation of Microtubule-associated Protein 2 Accompanies Dendrite Extension During the Differentiation of Cultured Hippocampal Neurones. Eur. J. Biochem. 227, 68–77. https://doi.org/10.1111/j.1432-1033.1995.tb20360.x

El Sayed, H., Agudelo, J.D., Pearl, D.K., Yates, A.J., 1991. Effects of GM1 and 2-Deoxy-2,3-dehydro-N-acetylneuraminic acid (NeuAc2en) on neuroblastoma (Neuro 2a) and human glioma cells (U1242 MG). J. Neurooncol. 11, 199–205. https://doi.org/10.1007/BF00165527

Encinas, M., Iglesias, M., Liu, Y., Wang, H., Muhaisen, A., Ceña, V., Gallego, C., Comella, J.X., 2002. Sequential Treatment of SH-SY5Y Cells with Retinoic Acid and Brain-Derived Neurotrophic Factor Gives Rise to Fully Differentiated, Neurotrophic Factor-Dependent, Human Neuron-Like Cells. J. Neurochem. 75, 991–1003. https://doi.org/10.1046/j.1471-4159.2000.0750991.x

Enserink, J.M., Christensen, A.E., de Rooij, J., van Triest, M., Schwede, F., Genieser, H.G., Døskeland, S.O., Blank, J.L., Bos, J.L., 2002. A novel Epac-specific cAMP analogue demonstrates independent regulation of Rap1 and ERK. Nat. Cell Biol. 4, 901–906. https://doi.org/10.1038/ncb874

Fu, H., Muenzer, J., Samulski, R.J., Breese, G., Sifford, J., Zeng, X., McCarty, D.M., 2003. Self-complementary adeno-associated virus serotype 2 vector: global distribution and broad dispersion of AAV-mediated transgene expression in mouse brain. Mol. Ther. 8, 911–917. https://doi.org/10.1016/j.ymthe.2003.08.021

Gao, Y., Nikulina, E., Mellado, W., Filbin, M.T., 2003. Neurotrophins Elevate cAMP to Reach a Threshold Required to Overcome Inhibition by MAG through Extracellular Signal-Regulated Kinase-Dependent Inhibition of Phosphodiesterase. J. Neurosci. 23, 11770–11777.

Geremia, N.M., Gordon, T., Brushart, T.M., Al-Majed, A.A., Verge, V.M.K., 2007. Electrical stimulation promotes sensory neuron regeneration and growth-associated gene expression. Exp. Neurol. 205, 347–359. https://doi.org/10.1016/j.expneurol.2007.01.040

Geusz, M.E., Fletcher, C., Block, G.D., Straume, M., Copeland, N.G., Jenkins, N.A., Kay, S.A., Day, R.N., 1997. Long-term monitoring of circadian rhythms in c-fos gene expression from suprachiasmatic nucleus cultures. Curr. Biol. 7, 758–766. https://doi.org/10.1016/S0960-9822(06)00334-4

Gordon, T., 2016. Electrical Stimulation to Enhance Axon Regeneration After Peripheral Nerve Injuries in Animal Models and Humans. Neurotherapeutics 13, 295–310. https://doi.org/10.1007/s13311-015-0415-1

Herrera, D., Robertson, H., 1996. Activation of cFOS in the brain. Prog. Neurobiol. 50, 83–107. https://doi.org/10.1016/S0301-0082(96)00021-4

Herrera, D.G., Maysinger, D., Goiny, M., 1993. Induction of c-FOS immunoreactivity in the hippocampus following potassium stimulation. Neuroscience 52, 237–244. https://doi.org/10.1016/0306-4522(93)90152-6

Hocquemiller, M., Giersch, L., Audrain, M., Parker, S., Cartier, N., 2016. Adeno-Associated Virus-Based Gene Therapy for CNS Diseases. Hum. Gene Ther. 27, 478–496. https://doi.org/10.1089/hum.2016.087

Hoerndli, F.J., Wang, R., Mellem, J.E., Kallarackal, A., Brockie, P.J., Thacker, C., Madsen, D.M., Maricq, A.V., 2015. Neuronal Activity and CaMKII Regulate Kinesin-Mediated Transport of Synaptic AMPARs. Neuron 86, 457–474. https://doi.org/10.1016/j.neuron.2015.03.011

Howard, D.B., Harvey, B.K., 2017. Assaying the Stability and Inactivation of AAV Serotype 1 Vectors. Hum. Gene Ther. Methods 28, 39–48. https://doi.org/10.1089/hgtb.2016.180

Hudmon, A., Schulman, H., 2002. Neuronal Ca^{2+} /Calmodulin-Dependent Protein Kinase II: The Role of Structure and Autoregulation in Cellular Function. Annu. Rev. Biochem. 71, 473–510. https://doi.org/10.1146/annurev.biochem.71.110601.135410

Jack, A.S., Hurd, C., Forero, J., Nataraj, A., Fenrich, K., Blesch, A., Fouad, K., 2018. Cortical electrical stimulation in female rats with a cervical spinal cord injury to promote axonal outgrowth. J. Neurosci. Res. 96, 852–862. https://doi.org/10.1002/jnr.24209

Jaiswal, P.B., English, A.W., 2017. Chemogenetic enhancement of functional recovery after a sciatic nerve injury. Eur. J. Neurosci. https://doi.org/10.1111/ejn.13550

Kato, K., Iwamoto, T., Kida, S., 2013. Interactions between αCaMKII and calmodulin in living cells: conformational changes arising from CaM -dependent and -independent relationships. Mol. Brain 6, 37. https://doi.org/10.1186/1756-6606-6-37

Krogh, K.A., Thayer, S.A., 2016. Calcium Imaging to Study NMDA Receptor-mediated Cellular Responses, in: Popescu, G.K. (Ed.), Ionotropic Glutamate Receptor Technologies. Springer New York, New York, NY, pp. 221–239. https://doi.org/10.1007/978-1-4939-2812-5_15

Laser-Azogui, A., Kornreich, M., Malka-Gibor, E., Beck, R., 2015. Neurofilament assembly and function during neuronal development. Curr. Opin. Cell Biol. 32, 92–101. https://doi.org/10.1016/j.ceb.2015.01.003

Lee, H.-M., Giguere, P.M., Roth, B.L., 2014. DREADDs: novel tools for drug discovery and development. Drug Discov. Today 19, 469–473. https://doi.org/10.1016/j.drudis.2013.10.018

Lemmens, R., Larsson, O., Berggren, P.-O., Islam, Md.S., 2001. Ca^{2+} -induced Ca^{2+} Release from the Endoplasmic Reticulum Amplifies the Ca^{2+} Signal Mediated by Activation of Voltage-gated L-type Ca^{2+} Channels in Pancreatic β-Cells. J. Biol. Chem. 276, 9971–9977. https://doi.org/10.1074/jbc.M009463200

Lim, J.-H.A., Stafford, B.K., Nguyen, P.L., Lien, B.V., Wang, C., Zukor, K., He, Z., Huberman, A.D., 2016. Neural activity promotes long-distance, target-specific

regeneration of adult retinal axons. Nat. Neurosci. 19, 1073–1084. https://doi.org/10.1038/nn.4340

Lin, Q., Wei, W., Coelho, C.M., Li, X., Baker-Andresen, D., Dudley, K., Ratnu, V.S., Boskovic, Z., Kobor, M.S., Sun, Y.E., Bredy, T.W., 2011. The brain-specific microRNA miR-128b regulates the formation of fear-extinction memory. Nat. Neurosci. 14, 1115–1117. https://doi.org/10.1038/nn.2891

Liu, Y., Kim, Y.J., Ji, M., Fang, J., Siriwon, N., Zhang, L.I., Wang, P., 2014. Enhancing gene delivery of adeno-associated viruses by cell-permeable peptides. Mol. Ther. - Methods Clin. Dev. 1, 12. https://doi.org/10.1038/mtm.2013.12

Lowery, J., Kuczmarski, E.R., Herrmann, H., Goldman, R.D., 2015. Intermediate Filaments Play a Pivotal Role in Regulating Cell Architecture and Function. J. Biol. Chem. 290, 17145–17153. https://doi.org/10.1074/jbc.R115.640359

Macías, W., Carlson, R., Rajadhyaksha, A., Barczak, A., Konradi, C., 2001. Potassium chloride depolarization mediates CREB phosphorylation in striatal neurons in an NMDA receptor-dependent manner. Brain Res. 890, 222–232. https://doi.org/10.1016/S0006-8993(00)03163-2

Ming, G., Song, H., Berninger, B., Holt, C.E., Tessier-Lavigne, M., Poo, M., 1997. cAMP-Dependent Growth Cone Guidance by Netrin-1. Neuron 19, 1225–1235.

Murphy, T.H., Worley, P.F., Nakabeppu, Y., Christy, B., Gastel, J., Baraban, J.M., 1991. Synaptic Regulation of Immediate Early Gene Expression in Primary Cultures of Cortical Neurons. J. Neurochem. 57, 1862–1872. https://doi.org/10.1111/j.1471-4159.1991.tb06396.x

Murray, A.J., 2008. Pharmacological PKA Inhibition: All May Not Be What It Seems. Sci. Signal. 1, re4–re4. https://doi.org/10.1126/scisignal.122re4

Murray, A.J., Tucker, S.J., Shewan, D.A., 2009. cAMP-Dependent Axon Guidance Is Distinctly Regulated by Epac and Protein Kinase A. J. Neurosci. 29, 15434–15444. https://doi.org/10.1523/JNEUROSCI.3071-09.2009

Neumann, S., Bradke, F., Tessier-Lavigne, M., Basbaum, A.I., 2002. Regeneration of Sensory Axons within the Injured Spinal Cord Induced by Intraganglionic cAMP Elevation. Neuron 34, 885–893.

Neumann, S., Woolf, C.J., 1999. Regeneration of Dorsal Column Fibers into and beyond the Lesion Site following Adult Spinal Cord Injury. Neuron 23, 83–91. https://doi.org/10.1016/S0896-6273(00)80755-2

Peace, A.G., Shewan, D.A., 2011. New perspectives in cyclic AMP-mediated axon growth and guidance: The emerging epoch of Epac. Brain Res. Bull. 84, 280–288. https://doi.org/10.1016/j.brainresbull.2010.09.002

Perrot, R., Eyer, J., 2009. Neuronal intermediate filaments and neurodegenerative disorders. Brain Res. Bull. 80, 282–295. https://doi.org/10.1016/j.brainresbull.2009.06.004

Qiu, J., Cai, D., Dai, H., McAtee, M., Hoffman, P.N., Bregman, B.S., Filbin, M.T., 2002. Spinal Axon Regeneration Induced by Elevation of Cyclic AMP. Neuron 34, 895–903.

Rodriguez, J.A., Piddini, E., Hasegawa, T., Miyagi, T., Dotti, C.G., 2001. Plasma Membrane Ganglioside Sialidase Regulates Axonal Growth and Regeneration in Hippocampal Neurons in Culture. J. Neurosci. 21, 8387.

Roth, B.L., 2016. DREADDs for Neuroscientists. Neuron 89, 683–694. https://doi.org/10.1016/j.neuron.2016.01.040

Sagar, S., Sharp, F., Curran, T., 1988. Expression of c-fos protein in brain: metabolic mapping at the cellular level. Science 240, 1328–1331. https://doi.org/10.1126/science.3131879

Sánchez, C., 2000. Phosphorylation of microtubule-associated protein 2 (MAP2) and its relevance for the regulation of the neuronal cytoskeleton function. Prog. Neurobiol. 61, 133–168. https://doi.org/10.1016/S0301-0082(99)00046-5

Stefano, G.D., Casoli, T., Fattoretti, P., Balietti, M., Grossi, Y., Bertoni-Freddari, C., Giorgetti, B., 2006. Level and Distribution of Microtubule- Associated Protein-2 (MAP2) as an Index of Dendritic Structural Dynamics. Rejuvenation Res. 9, 94–98. https://doi.org/10.1089/rej.2006.9.94

Urban, D.J., Roth, B.L., 2015. DREADDs (Designer Receptors Exclusively Activated by Designer Drugs): Chemogenetic Tools with Therapeutic Utility. Annu. Rev. Pharmacol. Toxicol. 55, 399–417. https://doi.org/10.1146/annurev-pharmtox-010814-124803

Wei, D., Hurd, C., Galleguillos, D., Singh, J., Fenrich, K.K., Webber, C., Sipione, S., Foaud, K., 2016. Inhibiting Cortical Protein Kinase A in Spinal Cord Injured Rats Enhances Efficacy of Rehabilitative Training. Exp. Neurol. 283, 365–374. https://doi.org/10.1016/j.expneurol.2016.07.001

Willand, M.P., Nguyen, M.-A., Borschel, G.H., Gordon, T., 2016. Electrical Stimulation to Promote Peripheral Nerve Regeneration. Neurorehabil. Neural Repair 30, 490–496. https://doi.org/10.1177/1545968315604399

Wu, G., Lu, Z.-H., André, S., Gabius, H.-J., Ledeen, R.W., 2016. Functional interplay between ganglioside GM1 and cross-linking galectin-1 induces axon-like neuritogenesis via integrin-based signaling and TRPC5-dependent Ca^{2+} influx. J. Neurochem. 136, 550–563. https://doi.org/10.1111/jnc.13418