Combined Gene Therapy and Rehabilitative Approaches to Repair the Corticospinal Tract Following Injury

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COMBINED GENE THERAPY AND REHABILITATIVE APPROACHES TO REPAIR THE CORTICOSPINAL TRACT FOLLOWING INJURY

By

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ABSTRACT
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Spinal cord injury (SCI) is a condition that has plagued humans for centuries, and still there is no effective treatment to restore neuronal deficits caused by trauma. Unlike the peripheral system, the mature central nervous system is highly limited in intrinsic regenerative capacity. The signaling from the brain to the spinal cord and through the corticospinal tract (CST), important in fine motor control, through the gray matter and out to the muscles through motoneurons is highly specialized to each muscle signal. Within the gray matter the cells representing extensor and flexor muscles are positioned close together, making individual cell to cell connections imperative for appropriate activation of one muscle over the other. The work presented here studies the CST in axon growth studies following injury, utilizing a known growth-promoting gene therapy (KLF6) and novel rehabilitation to in an attempt to enhance sprouting and functional recovery of the tract.

Using the unilateral pyramidotomy model the CST is left transected on one side, with the other side intact. A cortical injection into the motor cortex representing the CST on the intact side is injected with a tracer virus or a tracer virus + KLF6 to trace and treat the intact CST and measure axon sprouting into the denervated tissue. The initial study found that the intact CST will only sprout in the presence of an injury response. Also, KLF6 will only induce axon sprouting in the CST in the presence of injury. Next by probing the time course over which KLF6 induces axon sprouting it became clear that at 4 weeks post injury significant sprouting into the denervated cord existed.

In a following study the 4-week time point was used to bring in task-based rehabilitation. Animals received cortical injections of tracer or tracer + KLF6 and pyramidotomies and 4 weeks later the rehabilitation group began task-based pellet retrieval and general walking rehabilitation for 10 weeks. Each week all animals were assessed for pellet retrieval and wheel walking behavior. At the end of the experiment the axon sprouting in the cervical cord was measured in all animals. Neither KLF6 nor rehabilitation effected behavioral measures, but KLF6 continued to promote sprouting as seen in the previous experiments. A preliminary study of an intensive forced-use rehabilitation, constraint-induced movement therapy (CIMT) was use on animals that had received unilateral pyramidotomies. This experiment sought to intensively rehabilitate the affected limb following injury but found no behavioral or axon sprouting differences between treated and control groups. Collectively, this work attempted to improve axon growth following injury. Ultimately, it identified basic principles concerning sprouting, the injury requirement for sprouting, and the time course of sprouting, all of which are critical information for further studies involving timed rehabilitative intervention.

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AAV	Adeno-associated virus
BDNF	brain derived neurotrophic factor
Celsr3	cadherin epidermal growth factor laminin G seven-pass G-type receptor 3
ChABC	chondroitinase ABC
CIMT	constraint-induced movement therapy
CNTF	ciliary neurotrophic factor
CNS	central nervous system
CPG	central pattern generator
CST	corticospinal tract
CSPG	chondroitin sulfate proteoglycan
DNA	deoxyribonucleic acid
DRG	dorsal root ganglion
EGFP	enhanced green fluorescent protein
Emx1	empty spiracles homeobox 1
GAG	glycosaminoglycan
IGF	insulin growth factor
KLF-4/6/7	Krüppel-like factor 4/6/7
KO	knockout
luc	luciferase
MAG	myelin associated growth protein
NPC	neural progenitor cells
NT-3	neurotrophin-3
OMG	oligodendrocyte myelin glycoprotein
P#	postnatal day #
PBS	phosphate buffered solution
PFA	paraformaldehyde
PNS	peripheral nervous system
PTEN	phosphatase and tensin homolog
PKC	protein kinase C
RAG	regeneration associated gene
RARß2	retinoic acid receptor ß2
RGC	retinal ganglion cell
RNA	ribonucleic acid
RNA-seq	ribonucleic acid sequencing
mRNA	messenger ribonucleic acid
PTPσ	receptor-type tyrosine-protein phosphatase sigma
SEM	standard error of the mean
SCI	spinal cord injury
TBI	traumatic brain injury
TF	transcription factor

Chapter I

INTRODUCTION

Part 1: General Introduction

Prevalence and Cause of Spinal Cord Injury in Human Populations

Spinal cord injury (SCI) is a condition that affects 250,000-500,00 individuals globally each year. SCI is a devastating diagnosis because there are currently no effective therapeutic interventions to treat neurological damage in spinal injury. The World Health Organization reports that transportation accidents, falls, and acts of violence are the top three causes of SCI (WHO, 2013). The severity and extent of injuries are directly dependent on the location, size, depth of damage as well as other physiological systems that are affected by the trauma. Injuries that occur more rostral result in more significant deficits affecting the entire body as compared to more caudal SCIs.

Historically speaking the survival rate for SCI patients in the first 24-72 hours following injury was low, such that spinal injury was almost considered a death sentence. The evolution of modern medicine has increased the life expectancy for individuals living with spinal injuries (Stover and Fine, 1987; Frankel et al., 1998). The National Spinal Cord Injury Statistical Center currently reports that individuals who suffer from a severe injury in their 20's and survive the first 24 hours will live for an average of 45.7 years post injury, as compared to 59.6 years for healthy age matched controls (NSCISC, 2018). These data are representative of individuals who are paraplegic. The life expectancy for individuals with more severe injuries decreases with increased injury severity. While injured patients have a life expectancy that is shorter than the general population, the gap

between these two groups continues to grow smaller as medical advances increase the life expectancy for SCI patients (DeVivo et al., 1999; DeVivo, 2012). Here the case can be made that the SCI community is placing an extensive monetary burden on the economy due to their extensive care needs. There is also a moral burden placed on the scientific community to find effective treatments to support this population so that they are capable of leading more productive lives post-injury.

In the initial phase of SCI, multiple systems of the body are damaged and require immediate medical attention for survival. At this stage, a decompression surgery to the spinal cord may take place, but treatment of the spinal cord may be delayed as other body systems are prioritized. Frequent interventions include stabilization of blood pressure to support the cardiovascular system, and the respiratory system may require intubation and the use of a ventilator to regulate breathing (Rogers and Todd, 2016). These systems may be prioritized to improve chances of survival. The primary goal of decompression surgery is to remove any damaged structures that may be putting pressure on the spinal cord such as vertebrae and secondarily to stabilize the spinal cord. This will improve blood flow, relieve pressure, and physically stabilize the cord (Lee et al., 2018; Sewell et al., 2018).

While decompression surgery has been used in SCI dating back to the early 1900's, its effects on long term neurological outcomes are generally positive, but are dependent on many factors including site of injury and timing of surgery with respect to initial injury (Papadopoulos et al., 2002; Wilson et al., 2020). Retrospective studies on humans have mixed findings on the effects of decompression surgeries, although studies with shortest injury-surgery intervals (≤ 8hrs postinjury) as opposed to long intervals of

~48hrs tend to report the best neurological outcomes and thus leave it as the main standard of care following SCI (Fehlings et al., 2012; Li et al., 2014; Wilson et al., 2017).

While it is not practically possible for some SCI patients to receive initial decompression surgeries in the immediate hours following injury, the data supports pursuing these procedures as soon as possible for these patients. Unfortunately for the SCI population the initial phase of treatment is just the beginning of lifelong medical hurdles involving the spinal cord and other physiological systems. The long-term concerns for individuals with SCI include secondary complications such as urological, respiratory and skin infections (Sweis and Biller, 2017; Wahman et al., 2019). These infections are often reoccurring and fatal for this population, further illustrating the need for a cure for SCI to treat and restore the damage caused by spinal injury.

Anatomy and Function of the Spinal Cord

Together the spinal cord and brain make up the central nervous system (CNS). The spinal cord is an important site of communication for the trunk and limbs, where descending motor commands and ascending sensory signals are relayed out to the body and sensorimotor cortex, respectively. In humans the spinal cord is approximately 45cm long and 6.5-13mm wide (Frostell et al., 2016). It is encased in protective vertebrae that simultaneously allow for bending and twisting movement while supporting the spinal cord (Li et al., 2009). Together the spinal cord and vertebrae compose the spinal column. The projections within the spinal cord are usually myelinated for fast neuronal communication, but a small percentage are composed of more slowly conducting unmyelinated fibers (Rivot et al., 1980). The spinal cord is highly organized

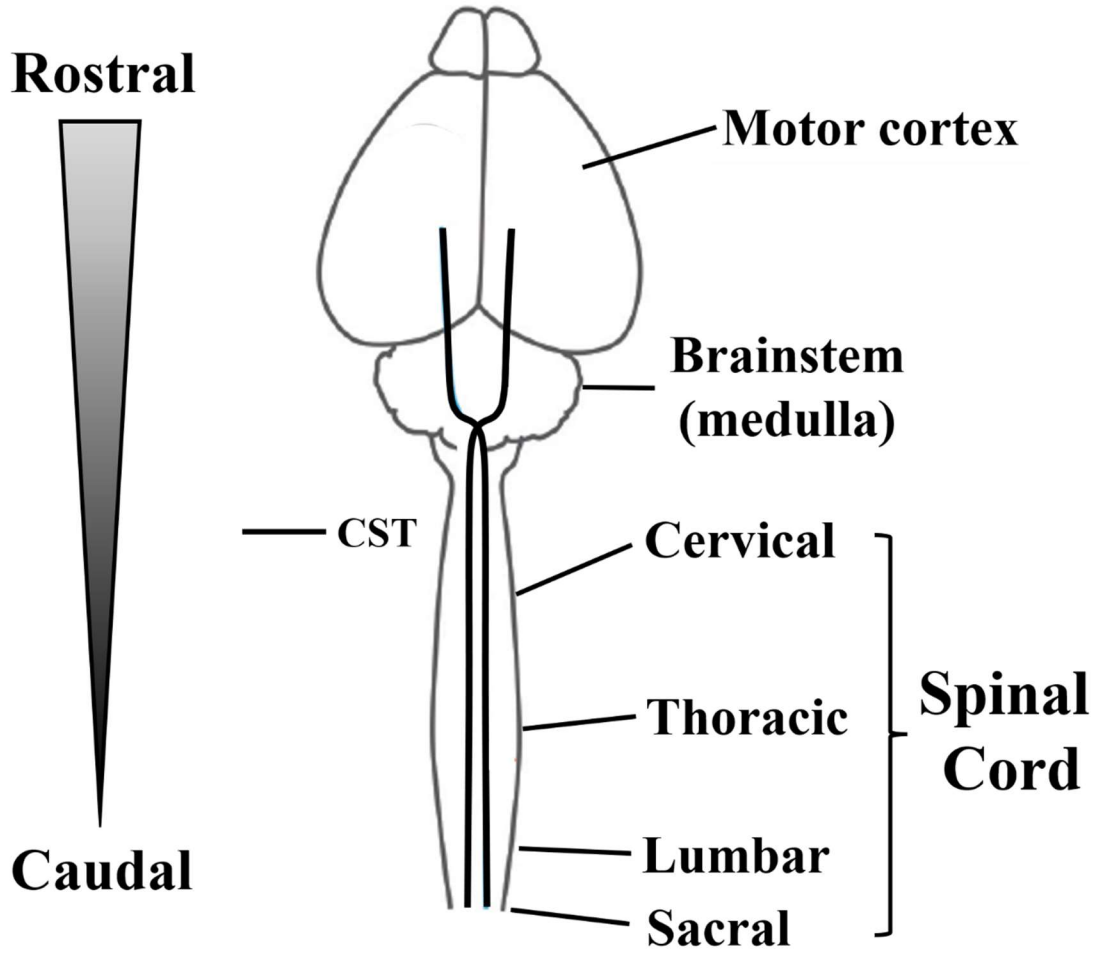


Figure 1.1 Schematic of brain and spinal cord. The brain and spinal cord are arranged in a rostral to caudal orientation. The spinal cord is broadly divided into four sections (cervical, thoracic, lumbar and sacral) which innervate the body at the corresponding topographic levels. The corticospinal tract projects from the motor cortex through the brainstem and down to the sacral region of the spinal cord.

topographically in the transverse plane and segmentally in the coronal plane to facilitate diverse functions.

The rostral to caudal regions of the spinal cord are divided into four main sections: cervical, thoracic, lumbar, and sacral (Fig. 1.1). Each level of spinal cord is responsible for innervating different regions of the body, each section approximately innervating the portion of the body that it topographically covers (Jenny and Inukai, 1983; Hanson et al., 2008). As part of the CNS the spinal cord relays information and connects to the peripheral nervous system (PNS) through spinal nerves that bifurcate as they approach the cord and separate into dorsal and ventral roots. These roots connect directly to the spinal cord.

The dorsal root ganglion (DRG) contains a group of cell bodies that will send sensory information from the PNS to the spinal cord and ultimately the brain (Ahimsadasan and Kumar, 2018). The spinal cord contains the cell bodies that will send motor signals out to the spinal nerve via the ventral root to innervate muscle and tissue innervation (O'Donovan and Landmesser, 1987). The mature PNS has much higher intrinsic regenerative ability than the CNS, so therapeutically targeting the ventral root at a site of SCI is one approach to restoring motor control that has been lost to injury (Lin et al., 2014). Functionally the human spinal cord is divided into 31 pairs of spinal segments in the human spinal cord and 34 in the mouse. The spinal nerves are distributed rostrally to caudally with 8 cervical, 12 thoracic, 5 lumbar, 5 sacral and 1 coccygeal nerves in the human. The rodent system is highly conserved with 8 cervical, 13 thoracic, 7 lumbar, 4 sacral and 2 coccygeal spinal nerves (Sengul and Watson, 2012). Because each nerve

innervates a very specific set of muscles and organs it is very difficult to reproduce or regenerate the original pathways following an injury.

For example, the cervical region of the spinal cord innervates the diaphragm, the head and neck muscles, and controls function of the forelimb. To dissect out the fine function of the cervical spinal nerve in the forelimb, it is essential to consider all of the muscles that function in this portion of the body. Opposing muscles such as biceps brachii and triceps brachii, forearm extensors and forearm flexors, and the many muscles that control the digits of the forepaw are all controlled by spinal nerves in the same region. These nerves are physically located between C4-T2, a span of approximately 6mm in the mouse (Bácskai et al., 2013). The balance of control of these muscles relies in part on the somatosensory feedback loop of information coming in through the dorsal root ganglion but is gated through motor neurons that are located in the cervical spinal cord and send their signals out through the ventral roots. Major descending tracts such as the corticospinal tract display a high degree of specificity to relay signals through a series of interneurons within the cord. The specificity of the signaling must be exact so that the correct motor neurons activate the biceps and not the triceps so that a person picks up a cup of coffee instead of pushing it down (Watson et al., 2009).

In the case of cervical SCI the balance of signal to the flexors and extensors is no longer appropriately regulated which can either lead to no input to the motor neurons and therefore no modulatory signal out from the cord, or more likely, an inappropriate signal. Here the injured condition is sufficient to drive either the flexor or the extensor and potentially give the patient intermittent spasms (Mayo et al., 2017). This often occurs in a muscle such as the biceps where the biceps receive constant motoneuron firing which

draws the arms up to the patient's body and can become quite painful. Interventions such as electrical stimulation, medications and physical therapy practices such as splinting work to reduce these effects over time (Dimitrijevic et al., 1986). Innervation of muscles from the spinal cord via spinal nerves is highly specified and likely one reason that SCI has proven so challenging to treat.

One way to visualize the topographic organization of the spinal cord is with a transverse section (Fig. 1.2). The white myelinated tracts run along the circumference of the spinal cord carrying information, with gray matter running along the inner core. At the very center is the central canal, which allows for cerebrospinal fluid to flow through the cord. The white matter tracts carry sensory or motor signals between the body and the brain. The gray matter is divided into three general regions; the dorsal, lateral and ventral. The dorsal horns are broadly responsible for receiving sensory information, and ventral horns generally hold the motoneurons which are responsible for sending out motor signals to the muscles and periphery (Purves et al., 2001; Hochman, 2007).

The gray matter is mainly composed of cell bodies, axons, synapses and glial cells. Within the gray matter there are local interneuron circuits that act as feedback loops and can be used to modulate the signals received or sent out by the dorsal and ventral horns (Fig. 1.2). The shape of the spinal cord changes as it descends from the brain to the most caudal point with enlargements in the cervical and lumbar regions corresponding to dense innervation in those areas (Purves et al., 2001). Also within a region such as the cervical spinal region the white:gray matter pattern changes at different levels which reflects the changing path and thickness of myelinated tracts that are running through that region, but also the number, sizes and density of the cell bodies in the gray matter of that

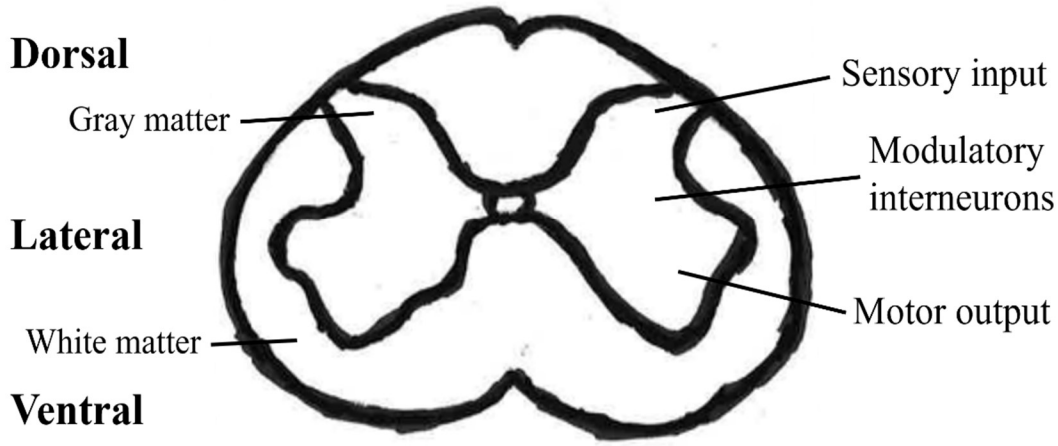


Figure 1.2 Transverse cervical spinal cord slice schematic. Left side of schematic depicts the orientation of a transverse section from dorsal to ventral and identifies the internal gray matter and the outer white matter column that surrounds the gray matter. The central canal is the circle dividing the gray matter into two parts along the midline. Labels on the right side identify the general function of cells found in the gray matter as sensory input in the dorsal horns, modulatory interneurons in the lateral zones and motor output in the ventral horns.

region. The pattern of cellular expression and cytoarchitecture of the gray matter has been highly studied in the mouse and is indexed into 10 layers, or laminae that are arranged dorsal-ventral at each spinal level (Watson et al., 2009). The cells in dorsal laminae are typically more involved in receiving and transmitting sensory signals from the DRG whereas the more ventral laminae contain interneurons and motoneurons that are involved in processing motor signals (Sengul and Watson, 2012).

The CST is one classically studied tract that originates in the cortex and extends to the sacral region of the spinal cord (Fig. 1.1). Functionally the CST is the major descending tract that is involved in fine motor control of the forelimb and is a critical path to study for regeneration and repair following SCI in an animal model (Iwaniuk and Whishaw, 2000). It is clinically relevant to patients who have suffered a cervical level SCI as rehabilitation of this pathway allows individuals to regain or relearn skills that were lost due to injury (Jang, 2014). In both primates and rodents the CST originates in the somatosensory/motor cortex and projects through the midbrain and into the brainstem where it decussates in the medullary pyramids and projects into the spinal cord (Van Wittenberghe and Peterson, 2020).

A recent study conducted in transgenic mice analyzed the relative distribution of premotor interneurons associated with both flexors and extensors, along the coronal plane rostral to caudal in the spinal cord. In the rostral-caudal orientation they found topographic coverage with higher cervical levels C2-3 innervating the shoulder and neck, at level C5-6 innervating the elbow and wrist, and at thoracic levels trunk control muscles were represented. Interestingly, premotor interneurons for flexor and extensor pairs were consistently found to be positioned close to each other within a transverse plane at the

corresponding spinal segment (Wang et al., 2017). This indicates that control of each distinct muscle must be tightly coordinated because the spatial resolution is low. Descending tracts that signal through premotor interneurons, such as the CST, must show a high degree of specificity in the cells that they make contact with in order to innervate the appropriate pathway. If the CST were to make inappropriate contacts, an extensor pathway as opposed to a flexor, for example, could be activated.

Development of the Corticospinal Tract

Early development is a critical period for the CST as this is when this tract experiences the most plasticity. After maturity the capacity for growth and plasticity is greatly reduced. In both rodent and primate nervous systems the CST is fully extended by approximately postnatal day 14 (P14) in the rodent or a late *in utero* timepoint in the primate (Terashima, 1995; ten Donkelaar, 2000). During development the immature CST branches hyperextensively in the brain, reaching as far as the visual cortex (Eyre, 2007). As the tract matures the nonfunctional excessive collaterals die off, and the main tract exits the cortical plate, enters the intermediate zone and extends rostrally to the internal capsule and midbrain (Watson et al., 2009). The CST then projects through the brainstem and medulla before entering the dorsal spinal cord where the axons begin to sprout and branch laterally into the cord in addition to continuing growth down the cord in a caudal direction (Canty and Murphy, 2008). The collateral branching and growth cone bifurcation that leads to expansive sprouting within the gray matter of the spinal cord occurs in spurts. These bursts of growth happen after the descending axons reach appropriate levels within the cord (Schreyer and Jones, 1982). These sprouts create numerous putative synaptic contacts which are then pared back to only maintain

functional sites that correlate with behavioral motor circuits (Dent et al., 2011; Lang et al., 2012; Tedeschi and Bradke, 2017). This selective maintenance of synapses is a combined effort of information coming from the developing tract, and information coming from the poorly coordinated, but physically active animal.

The development of the CST is highly specialized on a molecular level. When CST axons are in an extension phase early in development, the axon extends over long distances and the leading edge of the axon is characterized by a specialized zone known as the growth cone. Growth cones are a functionally active site containing F-actin terminating in filipodia. These structures respond to environmental cues in one of three ways: attraction, repulsion or passive (Geraldo and Gordon-Weeks, 2009; Kahn and Baas, 2016). The function of the growth cone and its response to the environment is essential for proper CST guidance to the spinal cord and innervation within the cord.

Pioneer axons are specialized structures that lead the CST from the motor cortex through the midbrain, hindbrain and along the spinal column, until they terminate at the appropriate spinal level and the remaining axons follow in tightly fasiculated bundles (Gorgels, 1991). The bundles stay bound until entering the spinal cord where they will ultimately sprout as independent axons (Canty and Murphy, 2008). As the axon elongates the growth cone is driven by constant attractive, repulsive and passive interactions with the CNS substrate. These external cues when added with internal transcriptional cues provide a molecular view of the dynamic environment that ultimately determines the path that the fully developed CST will travel (Kolodkin, 1996; Hu and Strittmatter, 2004; Polleux et al., 2007).

External cues come in the form of secreted proteins such as semaphorin 3A (Sema3A) which acts as a repellent and Sema3C, an attractant. The chemical gradient created by the diffusion of these two proteins likely functions to attract the developing CST out of the cortical plate and into the intermediate zone to begin descending to deeper brain structures en route to the spinal cord (Bagnard et al., 1998). Different transcription factors (TF) are important at specific points of development. One such factor, fasciculation and elongation protein zeta 1 (Fezl), has been found to be essential for the fate specification of CST neurons in the developing motor cortex, and when it is knocked out there is marked absence of pyramidal cells in layer V of the neocortex (Molyneaux et al., 2005). The CST uses external signals cues such as Semaphorins and internal signals like Fezl to establish its tract from the developing neocortex to the spinal cord. The expression pattern of these guidance molecules is essential for proper localization of this extensively projecting tract (Ten Donkelaar et al., 2004).

Maturation of the CST is concomitant with physical experience. As the animal begins to learn how to move its limbs, even in the most basic of modalities, the tract integrates that information to finalize collateral growth (Martin et al., 2007). The developmental switch from growth cone to presynaptic bouton is dynamic and occurs over a period of time which corresponds to the transition through which the cell downregulates growth cone f-actin cytoskeletal elements and upregulates synaptic vesicle related proteins (Burry, 1991). After the CST has fully developed the neurons switch to a cell maintenance state which is resistant to growth and regeneration (Donatelle, 1977). Importantly, the dynamic formation of synapses and presynaptic terminals allows even

the mature CST to undergo limited experience-dependent plasticity at the level of the synapse.

Pathway and function of the Corticospinal Tract

The developmental time course of the CST is variable depending on the specific animal species, but generally in the rodent the leading axons enter the spinal cord near the time of birth and reach the lumbar region by P5 (Canty and Murphy, 2008). In a study that tracked the ultrastructural development of the CST in the mouse at C7 and L4 from P0 to P28 they found that unmyelinated axons made up a large portion of the developing CST. Overall, there was a large decrease in unmyelinated axons between P14 and P28 that corresponded with an increase in myelinated axons, indicating that as the system matured and made final synaptic contacts the unmyelinated portion of axons decreased. This corresponds with the paring back of collaterals that are not functionally required. Interestingly between P14 and P28 some of the unmyelinated axons became myelinated, specifically in the cervical region which then gave them permanence (Hsu et al., 2006). This developmental information provides insight into how the mature CST has formed and highlights potential points of plasticity; collateralization and synaptogenesis.

In the adult mammalian system the CST originates in layer V of the sensorimotor cortex and projects ipsilaterally through the internal capsule and to the medulla oblongata. Once the tract reaches the brainstem approximately 90% of the CST fibers decussate to the contralateral side where they will descend through the brain stem and into the spinal cord through the dorsal horn (Welniarz et al., 2017). The remaining uncrossed axons will project ipsilaterally into the ventral funiculus as the ventral component of the CST. The main or dorsal CST projects from the cervical through the

lumbar regions, with axon fibers leaving the main tract to innervate the cord at the appropriate level.

When CST fibers exit the descending tract there is some debate as to where exactly they land or are targeted. The main action of the excitatory CST axons is to activate motoneurons that lie in the ventral lamina 7-9, but many studies have shown that the action of CST input is indirect and works by signaling through interneurons as opposed to direct inputs onto motoneurons (Welniarz et al., 2017). Recent work has indicated that the CST may even have a role in modulating sensory information by activating interneuron networks in dorsal laminae II/III.

Specifically one study looked at a population of excitatory interneurons that normally function to detect light touch, but interestingly when knocked out animals displayed a deficit on the ladder walking task (Bourane et al., 2015). This is the first study that implicates that proprioceptive inputs modulate CST-dependent behaviors such as fine motor ladder walking. Another study aimed to identify neuronal populations of cells within the spinal cord at the level of the hindlimb by using RNA sequencing. Here researchers collected total RNA, created profiles of cell populations, and were able to identify 43 unique populations at the hindlimb level of the cord. Next they attempted to link individual clusters of neurons to locomotor activity and were able to further classify at least 9 clusters that were responsive during rotarod behavioral activity (Sathyamurthy et al., 2018). The cell clusters that were transcriptionally active during locomotor behavior reached from the dorsal horn, to the intermediate lamina and then to the ventral region where motor neurons are expected to reside. This work supports the study from Bourane *et al* 2015 implicating proprioceptive-receiving interneurons as playing a role in

stepping behavior. Clearly an integration of signals within the interneuron networks allows for cross talk between tracts that are classically thought of as acting in isolation (Levine et al., 2012). As scientists continue to push forward on deciphering the complex signaling between the diverse interneuron populations, the CST will still likely be treated as a fine motor tract. Moving forward it will be important to keep in mind that the system is more complex and integrated than is currently understood (Moreno-López et al., 2016).

The synaptic mapping of the CST historically has focused on signaling that leads to motor neurons. The work in the nonhuman primate has shown both the presence of monosynaptic and polysynaptic connections between the CST and motoneurons (Galea and Darian-Smith, 1995; Isa, 2012). An interesting study using retrograde transsynaptic rabies virus injected into individual muscles in the rhesus monkey to label monosynaptic connections identified a subset of these cells in the motor cortex that is present in only certain higher primates and humans. This study further classifies the monosynaptic CST-motoneuron connections as controlling the muscles of the digits and possibly the elbow and shoulder (Rathelot and Strick, 2009). This work further supports previous studies that show the higher the dexterity of a species of monkey, the higher the direct input from the CST to the motoneurons (Bortoff and Strick, 1993; Nakajima et al., 2000). In contrast data from rodent studies have shown that CST axons terminate within the intermediate lamina 5-7, and only occasionally fall within the motoneuron-containing region of laminae 7, but even then fail to make direct connections to motoneurons (Liang et al., 1991; Yang and Lemon, 2003). The species-specific differences in the ability to monosynaptically connect the CST to the motoneuron may support a larger picture argument for the role of the CST as a driving force in evolution of fine motor capabilities.

Animal Models of Corticospinal Tract Injuries

As previously mentioned the CST originates in the motor cortex and projects through the brainstem, where it decussates in the medulla, and continues running contralaterally down the spinal cord (Brösamle and Schwab, 1997). The path of projection is similar in both rodents and primates until innervation of the spinal cord. In the rodent the majority of CST runs along the dorsal column, whereas in the higher primate/human the projections are spread more evenly between lateral and ventral projections. Knowledge of the projections of the tract allow for the design of injuries to the CST that can specifically target the tract at levels as high as the medulla, or from lower cervical to lumbar regions in the spinal cord.

When a human suffers a SCI it is typically a very traumatic event both at the site of the spinal cord due to a strong inflammatory response, a potentially compromised respiratory system, and the results from loss of descending/ascending signaling at and below the site of injury. This is in addition to other parts of the body that may have been injured at the time of the accident requiring immediate stabilization for survival (Sekhon and Fehlings, 2001; Kwon et al., 2004). Human SCI is such a traumatic event that it can be difficult to model in animal systems. There are multiple options when deciding on a model of SCI; they range from recreating the traumatic injury state that the human would experience to specifically isolating and injuring a portion of the spinal cord while limiting damage to the rest body.

From the aspect of axon growth each model is very different. Each injury model creates a specific neurological condition to attempt for neurorehabilitation and recovery. The properties of neuronal regeneration from injured cells versus collateral sprouting

from uninjured or spared tracts are one difference to consider when choosing between the different models.

Contusion-In this model the exposed cord is submitted to blunt force trauma for a short period of time, or may be injured using an electromagnetic impactor (Gruner, 1992; Bhalala et al., 2013). The nature of these injuries is quite severe, causing immediate widespread damage to the spinal cord, but also engaging the immune response which will lead to secondary damage to the neuronal tissue. The force of the injury is proportional to the behavioral deficit in animals that have recovered (Jakeman et al., 2000). When traced the lesions are generally bilateral (but can be precisely targeted to one side), extensive when compared to other models such as transection and pyramidotomy and can have increased variability likely due to challenges with procedural replication.

The contusion model can decrease or ablate the output of the phrenic motor neurons if the injury is given at cervical level C4 or higher which can limit the ability of the diaphragm to function sufficiently to maintain respiration. While this is a real-life problem for humans who suffer a high cervical injury, a unilateral contusion model at C4/C5 has been developed that allows for sufficient innate function of the diaphragm such that the animal can survive without supportive ventilation (Nicaise et al., 2012). These injuries are complicated to treat in the mouse because the animal suffers from the same secondary effects that a human would in a comparable scenario including inflammation, potential loss of bladder control and post-injury pain and infection (Krishna et al., 2013).

The ability to study axon growth in the contusion model is dependent on the presence and quantity of spared axons. Given the extensive damage in this injury, it is a

potential model for use in studies where the goal is to induce regeneration from injured axons (Zarei-Kheirabadi et al., 2020).

Compression- In the compression model the spinal cord is exposed but instead of a brief impact as in the contusion model, it is compacted for a longer period of time. The methodology of compression originally involved physical clips to the spinal cord, where the force exerted on the cord and duration of time were controlled (Rivlin et al., 1978). Current technologies tend towards using a manual crush model wherein the spinal cord is exposed and physically crushed using forceps, a model similar to the impact-driven contusion, but may provide higher reproducibility and increased specificity (Plemel et al., 2008). The forceps are generally calibrated with a spacer so that when they are closed a specific space remains and the cord is crushed for to a specific diameter and for a set time. This model would invoke the same physiological systems as above but may prove to be even more traumatic than the contusion model as the entire cord is damaged. For rehabilitative studies in the compression/crush model relies on regeneration of damaged axons across the injury site because this is a near complete injury with very few surviving axons (Zukor et al., 2013).

Transection-Transection models seek to injure targeted tracts or regions of the spinal cord. There are two main types of transection models. The first type takes place in the spinal cord. The cord is exposed, as with the models above, and either a vibrating knife or a wire knife is used to create a targeted partial lesion within the spinal cord (Lu et al., 2003; Wang et al., 2018). This injury will elicit the immune response but is generally less invasive than the contusion or compression models and does not leave the animal in such a compromised state for infections and bladder control. Studies of axon growth in

transection models will depend on the severity of the model. In the case where the transection completely removes CST bilaterally a stem cell graft can be put in place, so in this case the study is looking at regeneration of injured axons into the graft (Lu et al., 2012). Ideally growth through the graft and into the denervated tissue could be achieved, but that technology is still being developed.

The second type of transection is the injury to a spinal cord tract outside of the spinal cord. The pyramidotomy is an example of this type of transection. The unilateral pyramidotomy is a transection of the CST as it runs through the medullary pyramids in the brainstem (Kathe et al., 2014). The benefit to this injury is that it is highly tract specific and that the localized immune response is in the brainstem, but the regeneration analysis will take place in the spinal cord, so the immune effect will not have an impact on the recovery and axon growth process. Because of the relative ease of access to the tract in the medulla there is a relatively low risk of infection or other recovery complications. In this injury only one side of the CST has been injured, leaving the other intact. This allows for sprouting of the intact CST into the denervated tissue (Blackmore et al., 2012; Jayaprakash et al., 2016).

Part II: Molecular Mechanisms of Regeneration

Extrinsic mechanisms inhibiting axon regeneration

Extrinsic mechanisms are processes that act to block axonal regeneration and originate from outside of the injured neurons. These processes can broadly be divided into three categories: myelin, immune factors and scar-associated. Extrinsic signaling is

present immediately following the injury and persists for the life of the individual. This signaling originates from CNS myelin, macrophages and glial cells. It originates at both the injury site and systemic responses. After spinal injury locally sourced and bone-marrow derived macrophages as well as glial cells will become activated and infiltrate the injury locale (Kong and Gao, 2017). The nature of this signaling is so widespread and long lasting that a better understanding of how these mechanisms interact with neuron intrinsic signaling is critical for effective treatment of SCI.

The mature myelin in the CNS expresses many factors that are inhibitory to axon growth including Nogo-A, myelin associated growth protein (MAG) and oligodendrocyte myelin glycoprotein (OMG) (Vogelaar, 2016). Nogo-A expressed in oligodendrocytes functions by binding to Nogo receptors on axons to block regeneration. The Nogo-A signaling pathway has been experimentally manipulated to demonstrate its role in limiting neuronal regeneration (Fouad et al., 2004). *In vitro* studies have applied exogenous Nogo-A to neuronal cultures to show that this protein inhibits neuronal growth. *In vivo* studies have used an antibody that binds to Nogo-A, effectively inhibiting Nogo-meditated signaling. These studies have demonstrated that by blocking this signaling pathway in an injury model there is increased regeneration and improved behavioral function (Schweigreiter and Bandtlow, 2006). This Nogo inhibition has been further studied in the context of SCI and rehabilitation. Here the combination of treatments showed modest improvement on behavior, but significantly increased CST sprouting and synapses onto motoneurons (Chen et al., 2017).

Additionally, in the mature uninjured spinal cord a class of proteins called ephrins is upregulated (Goldshmit et al., 2006). Ephrins bind to EphA and EphB receptor

signaling families. Neurons and sprouting axons are growth aversive to ephrins and ephrin receptors (Giger et al., 2010). Furthermore, multiple ephrin receptors have been shown to be upregulated following SCI (Willson et al., 2003; Fabes et al., 2006). This indicates that spinal injury pushes the mature spinal cord to an even more growth aversive state as compared to that of newly sprouting axons.

External injury cues activate the immune system and drive macrophages, specifically microglia, to the injury site. Once there, they release inflammatory cytokines such as tumor necrosis factor alpha (TNFα), interleukin 12 (IL-12) and interleukin 1 beta (IL-1ß) (Profyris et al., 2004). These proinflammatory cytokines can amplify the damage to injured oligodendrocytes and neurons, specifically in the case of TNFα. This cytokine is known to be involved in apoptotic pathways through receptor-mediated signaling, and knockout of the main TNFα receptor has been shown to decrease inflammation and spinal cord trauma following injury (Genovese et al., 2008). Targeted depletion of inflammatory macrophages following SCI has proven to increase axon regeneration (Popovich et al., 1999). Administration of a TNFα antagonist 1hr following thoracic SCI in rats resulted in increased motor function two weeks post injury and decreased levels of TNFα, tumor necrosis factor receptor 1 (TNFR1) and TNFR2 (Chen et al., 2011). This study suggests that antagonism of the proinflammatory pathway is sufficient to improve recovery following SCI in a behaving animal. Macrophages are not the only cell type known to migrate in response to SCI, glial cells also play an important and dynamic role in this process.

Following SCI activated glial cells migrate to the injury site to create a physical and chemical scar that works to block regeneration. (Yuan and He, 2013). The

consequences of this protective scar are varied as the effect of the reactive astroctyes is proportional to the intensity of the injury. In the most severe injuries the resulting astrogliosis prevents neuroregeneration by proliferation of astrocytes at the site of injury. In less severe injury conditions the astrocytic response will not result in a permanent change to the environment (Sofroniew, 2009). In these cases the reactive astrocytes may be beneficial to the damaged tissue by acting in a variety of ways such as repairing the blood brain barrier or supporting local synaptogenesis (Barres, 2008). Okada *et al* 2006 suggests that immediately following injury reactive astrocytes may act to repair and support damaged tissue, but over a chronic period the persistence of these cells in the injury site can become inhibitory to neuronal function (Okada et al., 2006). In the severe injury case the astrocytic response is termed the glial scar because over time the factors released by the glial cells as well as the migration of glial cells to the injury site will create a barrier. This scar is seen in human clinical patients as well as rodent models. Physiologically this scar naturally functions to protect the uninjured tissue from the toxic, inflammatory injury environment. It is important to better understand the signaling mechanisms within the scar so that they can be therapeutically targeted to reverse the formation of this regeneration barrier.

Chondroitin sulfate proteoglycans (CSPGs) are one of the most abundant chemical components of the glial scar. They are extracellular matrix components and inhibit neuronal outgrowth. CSPGs are composed of a main protein with a chondroitin sulfate side chain. Many different CSPGs are upregulated following injury including brevican, neurocan, phosphacan, versican and neuron-glial antigen protein. Together these chains form glycosaminoglycan (GAG) chains to globally act against neuronal

growth (Yuan and He, 2013; Harlow and Macklin, 2014). Chondroitinase ABC (ChABC) is an enzyme that can specifically digest the GAG side chain and has proven to be an effective treatment for managing the extrinsic-derived glial scar and can lead to behavioral improvement following SCI (Huang et al., 2006; Yiu and He, 2006; Mironets et al., 2016). While extrinsic factors create strong physical barriers to neuronal growth following SCI, intrinsic factors are an entirely different set of challenges to regeneration that occur in parallel to the extrinsic counterparts.

Intrinsic factors of SCI

Broadly speaking, intrinsic factors can be categorized into three parts: trophic factors, transcription factor (TF) expression and cell growth/survival pathways. The CST has the highest capacity for growth early in development. One approach to overcoming the intrinsic barriers to axon regeneration is to try to get the neuron look or act like a developing neuron experimentally. One way of achieving this is to express proteins that are developmentally downregulated or to turn off pathways that signal maturity (Liu et al., 2011).

Trophic factors such as brain derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), insulin growth factor (IGF), and neurotrophin-3 (NT-3) are involved in many processes including the growth and development of neurons. Expression of these factors has all shown success at regeneration in various CNS injury models (Bregman et al., 1997; Leaver et al., 2006; Hodgetts and Harvey, 2017). Recently, the combinatorial treatment of BDNF and NT-3 in a chronic model of SCI was found to promote motor recovery up to four months following injury (Martiñón et al., 2016). Trophic factors are a logical strategy for treatment of injured neurons as they mediate not

only axonal growth and synaptogenesis but also cell survival (Kelamangalath and Smith, 2013). These factors are so promising that they are currently being used in human clinical trials (Keefe et al., 2017).

Transcription factors (TF) regulate the expression for many genes, and the study of TF expression as a therapeutic intervention could prove to be more impactful than just a single gene treatment. Evidence of developmentally timed control of gene expression has historically been studied manually on a target-by-target basis using protein or mRNA quantification studies. Proteins critical to CST function such as growth associated protein 43 (GAP-43) and protein kinase C (PKC) were first studied using in situ hybridization and electron microscopy, but these are labor intensive experiments that are only capable of testing very few proteins or gene targets at a time (Mahalik et al., 1992; Miki, 1996). Blackmore *et al.* 2012 identified a TF, Krüppel-like factor 7 (KLF7), that was known to be upregulated in the PNS and RGC in response to injury and applied it to the CST. They found that overexpression of KLF7 induced neurite outgrowth in cultured neurons and induced sprouting in the adult injured CST (Blackmore et al., 2012).

The goal of this work is to identify TFs that are developmentally regulated and then to determine if they are involved in regeneration, or regeneration-associated genes (RAGs). In the CNS this allows us to look for proteins that are involved in axon growth and guidance pathways that are downregulated over time as neurons mature. These proteins and pathways can then be targeted to be reactivated following SCI to promote axon growth. Venkatesh *et al*. 2018 has developed a pipeline that integrates sequencing data with bioinformatics programming of chromatin accessibility to predict TF pro-growth networks in the CST (Venkatesh et al., 2018).

An emerging idea in the field of SCI is that cells must be treated with multiple TFs to induce the strongest growth following injury. This approach can either use multiple genes that work on the same pathway to increase the function of that pathway, or as will be described in greater detail later on, use genes that work on different pathways but are predicted to work synergistically to promote regeneration (Venkatesh and Blackmore, 2017). One capability of bioinformatics sequencing platforms is to footprint or capture the locations on DNA where specific TF can bind, thus predicting the genes that may be regulated by any given factor (Boyle et al., 2011). If you layer on this information with chromatin accessibility at a given age, you could predict the ability of a TF to facilitate transcription of a specific gene.

Cell growth and survival pathways are critical targets for inducing regeneration following SCI. The mechanistic target of rapamycin (mTOR), WNT/ßCatenin, and ERK pathways have been targets to promote regeneration, but must be carefully targeted as many of the signaling intermediates crossover between pathways (Afshari et al., 2009). Regeneration in the CNS, specifically in the CST, is inherently difficult to study, so scientists first look to successful interventions in similar systems. One such system for neuronal regeneration are the retinal ganglion cells (RGC), which retain some regeneration capacity into adulthood and have been well studied in goldfish, zebrafish and more recently in the rodent (Becker and Becker, 2007; Berry et al., 2008; Fleisch et al., 2011) Protein phosphatase and tensin homolog (PTEN) is a negative regulator of mTOR. Knockout of PTEN in RGCs was found to promote axon growth following optic nerve crush. This initial study pharmacologically validated that PTEN was working through the mTOR pathway, a cell survival path, and laid the groundwork for future

mTOR regeneration studies (Park et al., 2008). PTEN is a tumor suppressor that was identified in the late 1990's to be commonly mutated in cancer, allowing for uncontrolled cell growth (Shi et al., 2012). This made it an obvious candidate to study in neuronal regeneration.

In 2010 Liu *et al* manipulated the mTOR growth-promoting signaling pathway by knocking out PTEN in neonatal animals. They then performed a unilateral pyramidotomy in adults, and PTEN knockouts showed increased CST sprouting compared to controls (Liu et al., 2010). This study illustrates that there are specific signaling pathways that can be targeted to direct the cell back into a developmentally young growth-like state. Geoffrey *et al* built upon this work to determine if the PTEN/mTOR pathway had an effect in aged animals. Increased age in animals decreases regenerative capacities, but studies in adult animals are critical for their translational properties. This study initiated PTEN knockdown at different ages, ranging from P1 to 12-18months, and found that all groups showed axonal growth to the injury site. As expected, increased aged of onset of PTEN knockout was directly correlated with decreased ability of the CNS to regenerate following a dorsal hemisection. Approximately half of the P1 deletion group showed regeneration caudal to the injury, as compared to 0% of animals in the 12-18month deletion group (Geoffroy et al., 2016). This study demonstrates that barriers to regeneration are multifaceted and while a treatment such as PTEN knockout may be sufficient to induce growth in young cortical neurons, it does not have the same effect when age is layered on. Even in the youngest age group, only half of the animals showed regeneration caudal to the injury.

When assessing PTEN as a potential translational therapy, in the "best" condition possible with treatment at a very young age there is still much room for improvement, and in the "more likely" condition of an adult onset of treatment there is no effect. PTEN is often seen within the field as a high standard for regenerative therapy, but alone would not likely be clinically relevant. This allows for the possibility of combinations of therapies that target multiple systems, both intrinsic and extrinsic, to maximize the regenerative capacity in a translational manner (Griffin and Bradke, 2020). Increasing our understanding of the intrinsic mechanisms that limit neuronal regeneration can lead to novel therapies to overcome these barriers to effectively treating SCI.

Plasticity following SCI

Plasticity is the ability of the nervous system to respond to a stimulus. It is a feature of the nervous system that allows for essential functions such as learning and memory and to recover following injury (Shaw et al., 1994; Sasmita et al., 2018). There are several levels of plasticity in the spinal cord following SCI. Plasticity can be in the form of new axonal sprouts in response to SCI or traumatic brain injury (TBI), the growth of new synapses, or even the change in strength of existing synapses. The end result of plasticity is the ability of the nervous system to functionally respond to the environment and to mediate the negative effects of injury (Ramon y Cajal, 1928). The developing nervous system has high levels of intrinsic plasticity, but that drops with maturity. The ability to capture and enhance plasticity in the adult system is a therapeutic focus in conditions such as brain injury and SCI.

In the case of SCI the goal of plasticity is to induce axonal sprouting into the newly denervated tissue, with the end goal of recovery of function lost in the injury

(Courtine and Sofroniew, 2019). In the spinal cord low levels of spontaneous recovery have been reported, but these levels are not sufficient to drive full recovery from injury (Blesch and Tuszynski, 2009; Rosenzweig et al., 2010). There are two main approaches to obtaining a therapeutic level of axonal growth, either directing axons to grow through the injury site or around it. In spared injuries, where the axon growth is around the injury site, the pattern of regeneration is actually a complete reorganization of the spinal circuit (Bareyre et al., 2004; Courtine et al., 2008). In this case spared neurons are now sending out axons to make new interneuron circuits that will ideally compensate for the injured and missing circuitry. This is in opposition to inducing growth of injured axons to grow and reestablish appropriate contacts and normal circuitry (Courtine and Sofroniew, 2019). Both cases are difficult as intrinsic regenerative capacity is low in the mature CNS. Many treatments including IN-1 antibody, gene therapy, stimulation, the receptor-type tyrosine-protein phosphatase sigma (PTPσ) CSPG inhibitor, and activity-dependent plasticity have been found that increase axonal sprouting following SCI (Powers et al., 2012; Jayaprakash et al., 2016; Ohtake et al., 2016; Quraishe et al., 2018; Sasmita et al., 2018). Increasing axonal sprouting following SCI is essential to recovering gross function after injury. Once the sprouts are in place a synaptic level response can refine cell specific connectivity to restore behavioral activity.

CSPGs are found in the glial scar and are inhibitory to neuronal growth and blocking the action of CSPGs is a therapeutic approach to creating a growth-permissive glial scar. They act on neurons in part by signaling through PTPσ. CSPG signaling through this receptor initiates the rhodopsin (Rho)/rho-associated protein kinase (RACK) pathway which collapses growth cones and leads to neurite retraction and apoptosis

(Ohtake et al., 2016). Lang *et al* 2015 created a PTPσ peptide that bound to PTPσ receptor (RPTPσ) and blocked CSPG binding. This tool applied *in vivo* over an extended time course allowed them to see that blocking RPTPσ CSPG signaling naturally leads to the innervation of serotonergic axons through the injury site and promoted functional recovery of locomotor function. In the control intact RPTPσ CSPG signaling destabilization of growth cones led to the lack of regeneration and no change in function (Lang et al., 2015). These data are in line with a 2009 study that used RPTPσ knockout mice with a CST SCI injury that found that the knockout animals showed significant growth following injury as compared to the controls (Fry et al., 2009). By blocking one element of the CSPG inhibitory pathway not only is the environment growth-permissive, but it promotes functional growth.

Plasticity on the level of the individual synapse dictates the exact circuit that a regenerating axon will interact with. The synapse, composed of the presynaptic zone, synaptic cleft, and post synaptic density is specifically designed to send and receive neurotransmitter signals that are the basis of communication between two cells. These communications are one important component of the developing nervous system, but they are also critical for the regenerating neuron following injury (Griesbach and Hovda, 2015). Plasticity can define the ability of a cell to make a new synapse with a cell or to prune one away, or on a micro level plasticity can change the frequency of neurotransmitter release, proximity of pre and postsynaptic membranes and expression of proteins and receptors in the synaptic cleft (Hübener and Bonhoeffer, 2014). Critical periods are times in early development characterized by high plasticity. During these periods neurons are sending out projections and developing their ultimate trajectory

(Lillard and Erisir, 2011). This is especially true of neurons that project from the motor cortex through the brainstem and down into the spinal cord where they make their synaptic connections such as the CST.

Changes in synapse morphology during neuroplasticity are classically studied in learning and memory, as an adaptation to addiction, and more recently in response to injury or insult such as respiratory motor control or spinal cord injury (Malenka and Nicoll, 1999; Mitchell and Johnson, 2003). The mechanisms through which neurons make new synaptic partners, send out new processes, and regenerate injured compartments is similar to the initial mechanisms that induce growth: appropriate intrinsic programming and extrinsic signaling (Castaldi et al., 2020). Synaptic plasticity is generally an activity dependent process that is known to be modulated by many different proteins including BDNF, N-methyl-D-aspartate receptor, ion channels, cAMP response element-binding protein (CREB), cyclic adenosine monophosphate (cAMP), and calcium calmodulin-dependent protein kinase (CAMK) among many other kinases and structural proteins found at the synapse (Carlezon et al., 2005; Pittenger and Duman, 2008; Lerch and Buchser, 2017; Debanne et al., 2019).

Short term plasticity is mediated by synaptic function or through immediate translation via local mRNAs located in or near synaptic structures. Short term plasticity can lead to increased docking of synaptic vesicles, resulting in increased postsynaptic responses. Local mRNAs can be found in and around presynaptic boutons and are a site for fast translation of proteins involved in synaptic signaling. (McClung and Nestler, 2008; Terenzio et al., 2018). If the activity is prolonged, the nucleus responds by altering transcription and trafficking of appropriate proteins and mRNAs to the synapse to

maintain the changes in strength and connectivity over a long time period (Pittenger and Duman, 2008). One such protein is BDNF, which has been implicated in neuroplasticity in human clinical studies as well as rodent models (Rojas Vega et al., 2008; Grande et al., 2010). Overall this is a process that involves the integration of many signaling pathways and the timed response by the cell and other cells in that population to induce a functional, measurable change in cellular activity (Shaw et al., 1994). In the CNS where the intrinsic capacity for neuroplasticity is low, modalities such as gene therapies, pharmacological interventions and stimulation paradigms are used to enhance plasticity of these cells and circuits (Cramer et al., 2011; Kusiak and Selzer, 2013; Jack et al., 2018).

One study took advantage of the anatomy of the CST and spinal cord anatomy to examine multiple levels of neuroplasticity following SCI. Here, Jayaprakash *et al* expressed a light-sensitive rhodopsin channel (ChR2) either with SRY-box transcription factor 11 (Sox11), a known CNS RAG, or with control virus. After several weeks of expression time the Sox11-induced neuroplasticity was assessed at the level of the behaving animal and then terminally in the spinal cord at the level of functional connectivity using optogenetics. Throughout the expression time the Sox11 animals showed no difference in a behavioral CST-mediated task, pellet retrieval, but using optogenetics they found that Sox11 did induce functional synaptic connections (Wang et al., 2015; Jayaprakash et al., 2016). This study found that Sox11 expression in the CST was sufficient to generate functional synaptic connections in the cervical spinal cord, an indication of RAG-mediated neuroplasticity even in the absence of a behavioral phenotype.

The nature of spinal injury requires plasticity on the gross scale of axon regeneration as well as the refined level of synapse specific connectivity to overcome the functional deficits seen in SCI. The low level of intrinsic capacity for neuroplasticity seen in the mature CNS is likely a means of allowing an individual to adapt to the environment. This is usually thought of as the ability to learn both didactic and motor skills (Dayan and Cohen, 2011). While the plasticity described here is in the direction of regaining lost function and rewiring lost circuits, neuroplasticity can be maladaptive for the individual's survival in the environment. One example of maladaptive plasticity is neuropathic pain due to cellular hyperexcitability following injury (Brown and Weaver, 2012). While this provides a cautionary tale to consider the possible negative effects of neuroplastic therapeutics, the potential for benefit as seen in the PTPσ blocking peptide study is huge in terms of recovery for SCI patients.

Part III: *In vivo* therapeutic approaches

Current strategies to treat SCI in human and animal models

SCI has proven difficult to treat in humans, but the increasing longevity of individuals living with spinal cord injuries demonstrates a clear need for effective treatments for these individuals. Rodent models of SCI can be used to study specific neuronal pathways and injury types. Given that the rodent CNS is developmentally and anatomically similar to that of the human, these animal models are also an important first step in testing therapies to support neuronal regeneration following SCI. Current strategies including gene therapy, rehabilitation, stem cell transplants, external milieu treatments and stimulation paradigms are all being actively studied in the context of SCI.

Gene therapy to treat SCI

The initial therapeutic approaches in rodent models have attempted to address the previously mentioned deficits in the mature CNS: extrinsic and intrinsic factors, and decreased plasticity. One of the most logical approaches has been gene therapy. The question is basic: can we supply neurons with a gene that will change it to a growth promoting state, and can we induce axonal growth following SCI and induce functional recovery? To answer this question techniques initially developed in the 1970's packaged genes of interest into viruses and then precisely delivered them to the cell bodies in the motor cortex that constitute the CST and project to the spinal cord (Friedmann, 1992; Osten et al., 2007).

Can forced expression of one gene in the motor cortex lead to neuronal outgrowth in the spinal cord? One approach to this question is to look to the PNS where regeneration is much greater than the CNS and to identify genes that promote regeneration in this system. Research in nonmammalian systems has extensively studied axonal transport and regeneration following PNS injuries. In these studies a targeted injury, conditioned lesion, to the PNS results in increased trafficking of proteins and mitochondria to the injury site which facilitates recovery and regeneration (Perry and Wilson, 1981; Zhang et al., 2004; Mar et al., 2014).

The DRG is a unique ganglion in that the cell bodies reside in a ganglion in the PNS just outside of the spinal cord and have pseudouniopolar projections with one into the PNS and the other into the CNS. In the DRG conditioning lesion the peripheral nerve branch is first injured, followed by a lesion to the CNS branch which then results in regeneration that would not normally be seen in the CNS (McQuarrie et al., 1977;

Oblinger and Lasek, 1984). Here the PNS lesion acts to prime the CNS branch of the DRG neuron for regeneration. It effectively increases the intrinsic growth capacity of the cell (Hoffman, 2010). Axon transport and protein expression studies of these lesions have found that the PNS lesion-induced regeneration program upregulates cytoskeletal neurofilaments as well as genes known to be involved in regeneration such as interleukin 6 (IL-6), signal transducer and activator of transcription 3 (STAT3), and GAP-43 (Oblinger et al., 1989; Dubový et al., 2019). Another way to use the uniquely positioned DRG system is to look for genes that are known to induce growth in the mature PNS and translate that to CNS studies.

One such factor, retinoic acid receptor ß2 (RARß2), has previously been shown to promote neuron regeneration and functional behavior when expressed in DRGs (Corcoran et al., 2000; Wong et al., 2006). RARß2 is involved in cell growth and differentiation and is downregulated in aged mice. Corcoran *et al* 2002 asked if supplying this single gene to *in vitro* CNS cultures was sufficient to induce neurite outgrowth. They found that viral expression of RARß2 led to neurite outgrowth in their CNS *in vitro* explant study (Corcoran et al., 2002). The next step was to supply RARß2 as a gene therapy to *in vivo* models. Yip *et al* 2006 virally injected RARß2 into layer V of the motor cortex, corresponding to the cell bodies for the CST. They then performed a dorsal column crush at C4, ablating the descending CST. They examined RARß2-mediated CST axon growth to and around the injury site whereas the control animals showed expected Wallerian degeneration-type retraction bulbs from the injury site, and near zero levels of growth to and around the lesion site. While the RARß2-mediated growth effect was minimal, the treated animals did show a modest improvement in sensory and locomotor

tasks (Yip et al., 2006). Taken together this RARß2 work demonstrates that gene therapy is a viable treatment approach that has proven effective in *in vitro* and *in vivo* models and suggests that it may be effective as a translational approach to humans.

RARß2 was identified as a putative candidate from previous work and then screened for CNS injury and growth studies. While this proved to be successful in the case of RARß2, the process of identifying proregenerative genes in the PNS and studying them one by one in the CNS is both time and resource consuming. The next step towards quickly identifying potential RAG or proregenerative genes was to look for types or classes of genes that might have been identified in other fields as regulators of cell cycle or cellular division. In many ways asking an adult neuron in the CNS to regenerate is similar to the way that cancer takes over cells and causes them to divide or grow when they otherwise would not. The field of cancer research has been using screens to identify genes involved in cancer pathology since the late 1990's (Steck et al., 1997; Ali et al., 1999). The field of neuroregenerative research utilized these findings and started testing known oncogenic genes such as PTEN, a component of the mTOR signaling pathway involved in growth, in therapies for SCI (Musatov et al., 2004; Gutilla and Steward, 2016; Salmena, 2016). By borrowing from the field of oncology, neuroscience advanced gene therapy with a more directed approach for identifying potential targets.

Over time high content screens were developed to screen for the sufficiency of a single gene to induce neurite outgrowth in an *in vitro* growth assay of dissociated neonatal cortical neurons. One of the most logical classes of genes to use as a therapy is a TF. Transcription factors are responsible for regulating the transcription of many different genes, so by overexpressing a single TF one can effectually upregulate many

distinct genes (Libermann and Zerbini, 2006). This high content screen can be used to identify both growth promoters such as doublecortin, yes-associated protein 1, retinoblastoma-associated protein1 and Krüppel-like Factor 6 (KLF6) and growth inhibitors such as ephexin, aldolase a, paired-like homeodomain and hematopoietically-expressed homeobox protein (Blackmore et al., 2010, 2012; Simpson et al., 2015; Wang et al., 2015).

An emerging tool to identify likely successful candidates for gene therapy is bioinformatics. Bioinformatics is a way to use computer simulations to interpret large *in vitro* and *in vivo* data sets such as epigenetic, chromatin accessibility or gene profiling (Weng et al., 2016; Palmisano and Di Giovanni, 2018). One application of this work is the ability to use *in silico* approaches to explore transcriptional changes that may be driving phenotypes seen in the lab.

An example of transcriptional profiling is seen in Fig. 1.3 with overexpression of KLF6. KLF6 has been shown to increase axon sprouting following injury, but the mechanism behind that was unclear. RNA-seq was used to capture a snapshot of the transcriptome in cortical cells where KLF6 was overexpressed. Computer generated analysis of the genes that were upregulated in treated cells allowed the experimenters to identify distinct clusters or nodes of genes based on function, and they found that KLF6 turns on genes that are responsible for cell growth (Wang et al., 2018). This is consistent with the growth phenotype that was reported in the same 2018 paper, and is confirmed here in Chapter III. In the case of KLF6 the RNA-seq data informed future research because while the upregulated genes fit into functional categories matching what would be expected of a growth phenotype, there was a clear lack of genes involved in synapse

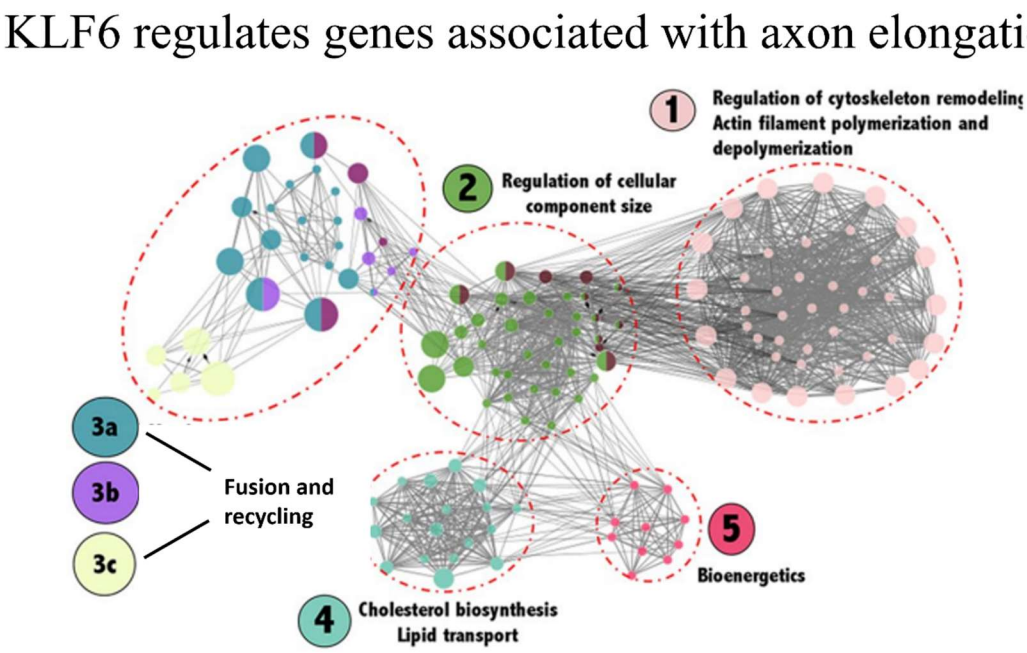


Figure 1.3 RNA-Seq analysis identifies functions of genes upregulated by KLF6 overexpression. Gene ontology analysis identifies several clusters of genes involved in functions important for axon elongation including 1-cytoskeleton remodeling, 2-regulation of size of cellular components, 3-fusion and recycling of component parts, 4-cholesterol biosynthesis and lipid transport and 5-bioenergetics.

Adapted from Wang *et al* 2018, Scientific reports.

formation and maturation. For KLF6 the field is now able to design a better informed experiment, likely one where multiple genes are included to complement each other's functions. This is one clear example where the bioinformatics backed up the benchwork, and the findings are informing future studies.

Venkatesh *et al* used epigenetic profiling to determine if chromatin surrounding the promotor region of RAGs was accessible at different developmental timepoints in CNS neurons. They found that accessibility of known RAGs decreased with time, which could potentially lead to the decreased intrinsic capacity for growth in neurons as they age (Venkatesh et al., 2016). This is one application of bioinformatics, but the possibilities to use massive computing power for *in silico* examination of TF binding sites and binding partners while taking into consideration the genes that they transcribe can predict not only single TFs, but also TFs that may be used in combination to synergistically promote regeneration (Venkatesh and Blackmore, 2017; Wang et al., 2018).

A major benefit to this approach to identification of potential therapeutic targets is the ability to use publicly available data sets and apply lab specific analysis or controls to answer a specific scientific question (Jin et al., 2014). This can be used to identify genes that are developmentally regulated or change expression with injury (Wen et al., 2016). Because the basis of bioinformatics is computer-oriented work, data sharing and program design sharing is much easier than with traditional benchwork. This facilitates the collaborative process and allow scientists from different fields to work together more easily.

Rehabilitation to treat SCI

Rehabilitation has been the gold standard to facilitate recovery following conditions such as stroke, traumatic brain injury and SCI in human patients, but it does not generally lead to significant functional gains. Because the neuronal damage caused by spinal injuries cannot directly be treated in human subjects, the current rehabilitative approaches use work around approaches to avoid the deficient pathways (Nash, 2005; Morawietz and Moffat, 2013; Behrman et al., 2017). One functional example of this is teaching patients to roll over in bed using their upper limbs to propel themselves, because they no longer have control over their lower body. Currently these work arounds are the best functional interventions for human SCI patients (Behrman and Harkema, 2007). Within the field of rehabilitation many of the human studies come from stroke patients rather than spinal cord injuries because current treatments lead to greater functional rehabilitation in the stroke population. As therapeutic interventions for SCI improve in efficacy rehabilitation studies will follow.

With the human SCI population living longer life spans and reintegrating into the workforce, there is a demand for actual gain of function interventions for these individuals. The studies to address the role of rehabilitation as a means of rerouting circuitry versus reestablishing disrupted pathways are ongoing in animal models. Importantly, the rehabilitation described here refers to purposefully placed movements as opposed to a simple locomotor motion. Best studied in cats and lower vertebrate animals such as lamprey, central pattern generators in the spinal cord can lead to a stereotypical locomotion activity even in the absence of descending cortical input (Mcclellan, 1989; Whelan, 1996; Mullins et al., 2011). This is distinctly separate from the rehabilitation

described here wherein descending input is modulated to interact with existing or regenerated circuitry in the spinal cord, resulting in purposeful movement. Purposeful movement requires an animal to adapt the behavioral response to the environmental conditions, whereas stereotypical movement is a completely repetitive motion.

There are two general approaches to rehabilitation: task specific and gross or generalized rehabilitation. The theory behind task specific rehabilitation is that after injury if you specifically train an individual for the task that you are more likely to improve performance on that task (Rensink et al., 2009; Starkey et al., 2011). In a 2012 study Arya *et al* found that in human stroke patients meaningful task-specific training in upper limb rehabilitation as opposed to a standard training group led to clinically relevant improvements in recovery (Arya et al., 2012). This study, along with many others, has laid the groundwork for targeted rehabilitation as a translational approach to use in animal models to assess functional regeneration in neuronal injury models (Szturm et al., 2008; Hubbard et al., 2009). Rehabilitation therapies in rodents can be combined with therapies that are already known to induce axon regeneration such as chondroitinase treatment or even activating the inflammatory macrophages to further improve behavioral gains (Wang et al., 2011; Torres-Espín et al., 2018a)

One study by Guillermo *et al* 2009 combined a known treatment of an extrinsic barrier to growth, chondroitinase ABC, with behavioral rehabilitation to look for functional improvements following a cervical SCI. Rats were treated with or without chondroitinase ABC and either task-specific or general locomotion rehabilitation. This group found that only the chondroitinase + skilled reaching rehabilitation group showed an improvement on a skilled reaching task. Interestingly, when challenged on a general

locomotion task, the skilled reaching group performed worse than the animals that had received general locomotion rehabilitation. The chondroitinase + general locomotion group performed worse on the skilled reaching task than animals that received no treatment at all (García-Alías et al., 2009). These results support the hypothesis that task-specific rehabilitation is necessary to improve the function of specific muscles and specific actions: generalized rehabilitation by an overall increase in activity does not improve all behavioral outputs.

Another study by Girgis *et al* 2007 looked at CST-dependent forelimb reaching rehabilitation in the rat following cervical SCI over a 6 week time period. They then assessed functional recovery, expression of known RAGs GAP-43 and BDNF, and the cortical mapping of the wrist joint. Importantly, this study assessed rehabilitation on three different physiological levels: behavior, gene expression, and cortical representation of joint function. These findings once again support the hypothesis that task-specific training is essential for task-specific improvement. Animals trained on a forelimb reaching task performed better on a reaching task over time, but actually performed worse on a generalized walking task. They found that the animals that underwent task-specific rehabilitation expressed a significantly higher level of GAP-43 in the motor cortex, correlating increased RAG expression with increased function following injury. And finally, this study was able to use electrophysiology to assess the region of the motor cortex that was committed to wrist function- the cortical representation of the injured limb. They found that in the trained animals there was an increased representation of cortical area, which correlated with increased collateral sprouting in the spinal cord (Girgis et al., 2007). Altogether this paper demonstrated that rehabilitation alone is

sufficient to induce expression of RAGs, increase collateral sprouting and cortical mapping, and to improve behavior in a task-specific manner. This work builds the case that physical rehabilitation may work synergistically with other regenerative interventions to create an optimized treatment.

Many research groups focus on the end products of regeneration as behavior, ignoring the circuitry and reorganization of cortical area that underlies the long-term plasticity. One method of studying the sensorimotor cortex is to observe the reorganization and representation of anatomical components following injury and rehabilitation (Raineteau and Schwab, 2001; Girgis et al., 2007; Asante and Martin, 2013; Li and Hollis II, 2017; Mohammed and Hollis, 2018). Another approach is to electrophysiologically manipulate the sensorimotor cortex as a means of inducing regeneration and functional recovery, potentially in the context of a rehabilitation paradigm. Yang *et al* 2019 used a paired cortical and spinal cord electrical stimulation in rats that had received cervical SCI. They found that after 10 days of stimulation the stimulation group performed consistently better on a CST-dependent walking task (Carmel and Martin, 2014; Yang et al., 2019). This study is one of many that has demonstrated that cortical, spinal, or epidural stimulation, or any combination of the three can induce functional improvements (Minassian et al., 2004; Gerasimenko et al., 2008; Krajacic et al., 2010; Martin, 2016). This opens the field to a whole new approach of inducing growth and regeneration: stimulation. The potential to use stimulation in conjunction with gene therapy and rehabilitation creates an endless number of ways to combat SCI.

Constraint-Induced Movement Therapy

One specific form of rehabilitation is constraint-induced movement therapy (CIMT) wherein the uninjured or unaffected limb is restrained so that the individual is forced to use the affected limb. Initially identified in 1940 by Edward Taub as a mechanism to improve limb function following unilateral lesion of the pyramidal tract in the monkey, this therapy is currently used as a rehabilitative approach in several human conditions including stroke, traumatic brain injury and SCI (Tower, 1940; Wolf et al., 2002; Yu et al., 2017). Historically CIMT has only been utilized in patients with the greatest potential for recovery, but it has proven so effective in this population that it is now being employed in individuals with more severe injuries. The theory behind CIMT is that by physically restraining the unaffected limb in either a sling or a cast, the affected limb will be forced to engage with the environment without any compensation from the unaffected side (Uswatte and Taub, 2013).

In the rodent model of cervical spinal cord injury CIMT is employed by casting the unaffected limb, effectively forcing the animal to use the injured forepaw. Here casting requires the animal to use the affected limb 24 hours a day, creating a highly intensive rehabilitative therapy environment. A CIMT study that used a stroke model to look at recovery of the forelimb function found that starting therapy 1day post injury led to behavioral recovery of skilled reaching and ladder walking task whereas a 17 day delay in initiation of therapy did not recover either task (Ishida et al., 2015). In a study that used CIMT in a CST pyramidotomy model they found that after 3 weeks of forced use animals had fully recovered behavioral ladder walking task and had increased axon

sprouting in the cervical spinal cord (Maier et al., 2008). Together these studies show promise for the use of CIMT as a rehabilitative tool for CST injuries.

Stem Cell Therapy

Stem cell therapy is a current research topic for many conditions, including SCI, TBI and degenerative neurological disorders (Kim and de Vellis, 2009). Many basic science and clinical labs are actively pursuing the ideal stem cell treatment for SCI (Doulames and Plant, 2016). Stem cells are an attractive target because at early stages in development cells are less and less fate determined, and are capable of maturing into different cell type (Kim and de Vellis, 2009). As an embryo develops cells become more fate determined, if we can experimentally capture cells that are pluripotent, developmentally young, or revert them back to their nascent stages then they could potentially be experimentally turned into neurons (Sylvester and Longaker, 2004; Gazdic et al., 2018). This would allow us to inject neural stem/progenitor cell grafts into the lesion sites created by spinal cord injuries, potentially decreasing many of the negative secondary effects of injury such as scarring (Ronaghi et al., 2009; Mothe and Tator, 2013). These neurons could also potentially act as a bridge to synaptically connect the neurons above and below the injury site in a sort of relay.

Stem cell research dates back to the 1980's. Initially the studies used stem cell grafts in an attempt to extend the critical period of the CST. This work found that by placing fetal spinal cord tissue in the lesion site they extended the critical period of high plasticity (Bregman et al., 1989). The spinal cord tissue transplant contained neural progenitor cells (NPC), cells that were able to differentiate into neurons or glia. In 2012 Lu *et al* published a method for collecting cells from the neural tube of embryonic day

E14 rat embryos and then exposing them to a cocktail of growth factors, leading them to differentiate into neurons, astrocytes and oligodendrocytes. These cells were grafted into adult rats with severe SCI and over a period of time appeared to integrate into the host tissue, sending projections caudally down the cord (Lu et al., 2012). This study provides proof of concept evidence for host-graft interactions and marginal neuronal differentiation from NPCs but leaves room for much improvement before the results are clinically or behaviorally relevant.

For this stem cell-mediated recovery to take place the stem cells have to meet many requirements. The pluripotent cells must differentiate into mainly neurons and supportive oligodendrocytes (Coutts and Keirstead, 2008). The stem cell graft must survive and integrate into host tissue. The presynaptic injured cells must send out new collaterals that will synaptically integrate into the graft. The graft must send out collaterals into the tissue caudal to the injury site (Lu et al., 2014). And at some point the appropriate circuit needs to be engaged from the cortex, through the graft and out to the muscle, leading to a behavioral response. These are the basic requirements for the stem cell approach to work in a translationally meaningful way. This initially seems like many barriers to success in the field of stem cell therapy, but work is currently underway on all fronts. A 2019 publication from the Tuszynski lab indicated that NPC cells transplanted into a host injury site successfully differentiated into sensory and motor interneurons, demonstrating that the grafted cells are maturing as expected (Kumamaru et al., 2019). This group has also shown evidence that the CST can regenerate into NPC grafts and make synaptic contacts with the graft (Kadoya et al., 2016). This work shows that the barriers to effective stem cell grafts in the CST can be overcome.

Part IV: Behavioral Testing of CST Function

The main functions of the CST in the forelimb include limb placement and grasping or fine dexterity in both the rodent and primate. Both functions require high levels of precision for accurate movements (Welniarz et al., 2017). Multiple neuroanatomical tracts underlie these behaviors including CST, rubrospinal tract, reticulospinal tract and propriospinal relay. Each tract has a specific role in locomotion, thus it is imperative to consider the contribution of individual tracts that work together to allow for a complex animal behavior (Watson and Harrison, 2012; Lu et al., 2015). Neuroplasticity from spared fibers or other intact descending tracts can lead to partial recovery following a spinal injury. The corticospinal, propriospinal and rubrospinal tracts can compensate for each other in an injury condition (García-Alías et al., 2015; Isa et al., 2019). Many of the mechanisms explained above are designed to engage intact neurons in the recovery process, whereas the assays listed below function as readouts of behavioral function.

To further study the role of the CST in humans, work by Lemon *et al.* 1995 used transcranial magnetic stimulation in healthy controls. They found that the tract regulates several aspects of hand control including the physical positioning of the hand and fingers in space as well as the command of muscles involved in grasping an object to pick it up (Lemon et al., 1995). Han *et al.* 2015 studied the role of the CST in skilled forelimb function by creating a genetic knockout of the CST, the cadherin epidermal growth factor laminin G seven-pass G-type receptor 3/empty spiracles homeobox 1 (*Celsr3/Emx1)* mouse. They found that behaviorally these animals showed decreased ability to manipulate food pellets that correlated with decreased numbers of motoneurons and

evoked muscle responses in the biceps brachii. Here they showed that the animals were capable of using the forelimb but demonstrated extensive deficits. The *Celsr3/Emx1* mice have increased rubrospinal projections through the upregulation of calretinin-positive propriospinal projections, and increased numbers of serotonin and tyrosine hydroxylase-containing fibers in the spinal cord, which point to compensatory rubrospinal tract signaling. The next step was to experimentally injure the rubrospinal tract in the *Celsr3/Emx1* animal, which led to significant deficits in fine forelimb function that did not recover over time (Han et al., 2015). This study points to the CST and rubrospinal tracts as mutually important for fine forelimb function. Here the overlapping functional innervation may allow for a redundancy in the system that could allow for important behaviors such as fine forelimb control to be maintained even in the case of injury.

The reticulospinal system has long been accepted as a gross motor system, important for positioning and control of entire limbs during locomotion as opposed to the fine movements previously mentioned (Drew et al., 1986; Perreault et al., 1994; Prentice and Drew, 2001; Davidson and Buford, 2006). Recent work in the monkey has shown evidence that the reticulospinal tract has monosynaptic connections on motoneurons that innervate muscles of the hand, potentially implicating a role in fine motor function similar to the CST and rubrospinal tracts (Riddle et al., 2009).

The propriospinal relay is composed of interneurons that receive information from descending pathways and then propagate signals to locomotor circuits (Laliberte et al., 2019). These relays can be short or long, communicating within one spinal segment ipsilaterally, or can be long, travelling several segments and spanning contralaterally (Miller et al., 1973; Macaya et al., 2012). The main function of these circuits is to convey

locomotion information throughout the spinal cord, often as part of the central pattern generator (CPG) (Gosgnach et al., 2017). These circuits are generally involved in coordination of locomotion; L-R alternation, flexor/extensor activity and forelimb/hindlimb activity (Quinlan and Kiehn, 2007; Frigon, 2017; Pocratsky et al., 2017). Recent experiments have shown that propriospinal circuitry is highly plastic and may facilitate recovery from SCI via indirect relay connections to circumvent the injury site (Courtine et al., 2008; Flynn et al., 2011). Many tests are used to assess fine motor function of the forelimb, but it is important to take into consideration the pathways involved in each test as a means of determining the most appropriate assay for a given experiment.

Walking-The CST is known for its role in limb placement during walking behavior, and as such variations on walking behavior are consistently used as measures of function. The horizontal ladder is frequently used as a measure of forelimb function. In this test the rodent walks across a horizontal ladder and the paw placement of the affected limb on each rung is scored as being a “correct” or “incorrect” step. Initially the rungs are regularly spaced, but most ladders have the option of removing certain rungs to make an irregularly spaced ladder. The irregularly spaced ladder is thought to be more of a challenge to the CST and rubrospinal tracts than the regularly spaced rungs because it involves a planned paw placement instead of a CPG-dependent motion (Metz and Whishaw, 2002; Webb and Muir, 2003). The goal of this test is to assess the forelimb function of one paw at each step that the animal takes across the ladder, and has proven to be an effective tool to assess limb function following injury (Soblosky et al., 2001; Gensel et al., 2006; Cummings et al., 2007; Wang et al., 2015). One potential concern is

if there is a gross error in shifting of weight or placement of hindlimbs that could be recorded as an incorrect forelimb step. Even given this potential drawback this task has been widely reported as a measure of behavioral function in cervical SCI and has validity within the field (Carmel et al., 2010; Pawar et al., 2015; Liu et al., 2016; Wang et al., 2018).

The irregularly spaced runged wheel is a version of the irregularly spaced horizontal ladder (Kramer, in review). The goals of the wheel are similar to that of the irregularly spaced horizontal ladder, with the added abilities of controlling the speed of the animal as it walks on the wheel and confining it to a tight window for video recording. In addition the "incorrect" steps here have been broken down into "heel", "toe" and "misses". This enables the experimenter to categorize exactly what type of mistakes the animals make on the ladder and to track group-wide changes in those categories over time as the animals recover or undergo treatments. This would allow for a more precise visualization of how animals are behaving after injury and treatment and even potentially to tease out any compensatory stepping mechanisms due to treatment.

The grid walk assay is another measure of forelimb placement, similar to the ladders. The main difference between the grid walk and the ladder tasks is that in the grid walk the animals are able to move around in whatever direction they choose (Onifer et al., 2005). The goal is to count correct versus slips or misplaced steps. Here the actual measure of the fine motor pathways can be easily obscured because this is a test where the whole animal is moving, and because it is a test that is scored underneath the grid it can be difficult for the experimenter to separate out gross versus fine motor placement.

A study by Schucht *et al* 2002 looked at the difference between ventral and dorsal lesions of the spinal cord and how each led to in recovery of grid walk and open field stepping behavior. They found that animals with ventral injuries had a close correlation between injury severity and gridwalk performance. This was not true of the dorsal-injured animals. From this they concluded that the descending tracts on the dorsal side are essential for fine motor control involved in the gridwalk assay. (Schucht et al., 2002). This work indicates that the grid walk may be an assay that is worth revisiting in the context of CST-dependent injuries (Starkey et al., 2005).

The CatWalk is a proprietary setup of equipment and software from Noldus technology. In this task animals walk along a walkway and the computer tracks the animal's pawprints and then is able to give readouts on many different parameters including stride length, paw contact area, and paw drag (Hamers et al., 2001, 2006). The CatWalk is often used to describe motor coordination and forelimb function. Given that the animals are tracked walking along a flat surface, this task is inherently driven by CPGs and rhythmic motion, as found with the propriospinal tract and the gross motor function in the reticulospinal tract (Koopmans et al., 2006). The measurements captured by this software are not overly dependent on the fine motor system and is likely not the most sensitive tool for assessing the cervical corticospinal tract.

Grasping/Manipulating-The central dogma of the CST is that it is involved in directing fine motor behavior, specifically of the forelimb. Commonly used manual manipulation behavioral assessments include Montoya staircase test, single pellet retrieval and the Capellini Handling Test. The Montoya staircase test was designed to study goal-directed forelimb grasping behavior bilaterally in food restricted rats. This test requires a specially

designed chamber with two ladders that hold food pellets. This test allows a physically unrestricted animal to enter a narrow chamber and grasp for food pellets, using one forepaw to retrieve the pellets for that respective side (Montoya et al., 1991). The readout on this test is pellets retrieved which reflects success rate and pellets displaced, which represents the pellets that the animal attempted to reach but missed. The staircase task is sensitive enough to detect a pyramidotomy injury effect and allow for monitoring of recovery over time (Wang et al., 2011). Also, due to food deprivation and palatability of the food pellets in the staircase the animals stay engaged in the experiment even over an extended period of time (Starkey et al., 2005). The overwhelming benefit to this test is pellets are displaced and retrieved using an isolated limb, allowing for distinction between the affected and unaffected limbs. In terms of descending control this task is highly fine motor and CST dependent because gross motor and postural control are removed from the action by the design of the apparatus. When used as a form of rehabilitation the staircase task has been found to be an effective task-based rehabilitation that selectively increases remodeling of the corticospinal pathway as opposed to other tracts projecting from the brainstem (Okabe et al., 2017a).

Single pellet retrieval has a similar goal to the staircase task in that the animals are trained to retrieve a pellet with either one forepaw or the other based on the location of the pellet (Chen et al., 2014; Jayaprakash et al., 2016). This is also a task that takes many days to train and requires specialized equipment and the ultimate measurement is success rate of pellets retrieved versus pellets dropped or missed. Similar to the staircase this is a more clear measurement of fine motor function as the behavior is only dependent

on the movement of the specific forelimb and forepaw, therefore it should have minimal contributions from propriospinal and reticulospinal tracts.

Rodents use their forepaws to manipulate food as they eat it. This observation led to the development of the Capellini Test. In this test the animal is video recorded while it consumes a piece of capellini pasta (Tennant et al., 2010). The animal is able to use both paws to manipulate the pasta which could dilute the observed effect in unilateral injuries. This is a test that can be carried out through multiple trials without food restriction and over long periods of time. This too is a test where only the forelimbs and forepaws are involved, engaging the fine motor CST and rubrospinal tracts such that the gross motor system and reticulospinal pathways should have minimal contribution to this behavior (Khaing et al., 2013).

Summary-There are many ways to capture forelimb and forepaw function. The different tests each put the animal in slightly different environmental conditions and allow the experimenter to collect very specific information. To best study the function of the CST following an injury it is imperative to choose the task(s) that will isolate the function of the tract and provide a clear readout of the function of interest over time. This is especially important as the rubrospinal tract has overlapping functions with the CST and the reticulospinal and propriospinal tracts can inadvertently be involved in behavioral design. From the work above it should be clear that no one tract works in isolation and that the fully functional spinal cord is dependent on the interaction of many inputs and important crosstalk.

Part V: Preface to Experimental Work

Spinal cord injury is an inherently translational topic, and many of the behavioral strategies that are used in animal models are derived from clinical standards. Modern medicine does not have a cure for SCI, and most treatments are minimally effective for the individuals suffering the lifelong effects of injury. To this end the practice of physical rehabilitation is currently a gold standard for SCI. It is an intervention for patients at all levels of function and is thought to be an essential therapy to allow individuals to retain and possibly gain physical functions as the time from injury increases (Behrman and Harkema, 2007; Gómara-Toldrà et al., 2014). As a minimally invasive intervention it can easily be added to other therapeutic regimens. The work presented here utilized two distinct forms of rehabilitation in an attempt to improve function following injury.

The overall goal of this dissertation was to improve behavioral function following SCI in rodent models by inducing functional axon sprouting and innervation. The approach was to create a synergistic effect of enhanced axonal growth and function in SCI using gene therapy and rehabilitation in a pyramidotomy model. The pyramidotomy model was a key component to these studies as it is an incomplete injury, leaving the descending fibers from the opposite tract fully intact. It has recently been appreciated that humans with incomplete, even if severe, injuries have a greater capacity to regain function following SCI than previously thought (Thomas and Gorassini, 2005; Tan et al., 2012; Morawietz and Moffat, 2013).

The gene therapy model employed was a single gene approach that has previously been shown by the lab to increase axon sprouting, but this group did not find an effect on

behavior (Wang et al., 2018). Previous studies in CST injuries have shown rehabilitative training to improve behavioral function and even axon sprouting (Girgis et al., 2007; García-Alías et al., 2009; Krajacic et al., 2010). To date the intersection of gene therapy in neurons and behavioral rehabilitation has not been studied.

One key factor in these studies was the design of a physical rehabilitation regime that promoted behavioral recovery in the mouse pyramidotomy model. Multiple strategies were used including CIMT and task-based rehabilitation. The CIMT employed here was a chronic 4 week forced use generalized intervention. This first attempt did not work, potentially for a combination of technical and scientific reasons. The task-based design was 5 days/week of rehabilitative training, but each animal only received approximately 1.5hrs of training a day in total. They were not considered to be training while in the home cage, whereas the CIMT animals were. Even though these two paradigms appear to be at opposite ends of the training spectrum in terms of intensity, duration and animal's motivation to participate, they both ended in negative results.

While the overall goal of behavioral recovery was not achieved, many incremental gains for field of SCI were made. The implications for this work and a detailed discussion of the results are found in Chapter V. Studies such as these are important to push forward science, particularly in fields such as SCI where it is increasingly clear that multifaceted treatment approaches are the most likely candidates for success.

Chapter II

METHODS

Surgical Methods and Tissue Analysis

Animals

Experiments used male and female mice aged 10-16 weeks at the start of experiments, obtained from Jackson Labs (C57BL/6J #000664) or bred in-house (Ai9 RCL-tdT Jackson #007905 on C57Bl6 background, receiving no Cre). Mice were group housed under a 12:12 light:dark cycle (6am on time) with experiments carried out during the light phase. To ensure that animals were acting under food motivated conditions, all animals in necessary experiments were maintained on food deprivation 7 days/week. They were fed daily following behavioral testing and maintained at 80% of initial body weight, both during the training and testing phases. All animals had unlimited access to food starting 2 days prior to surgery and extending to 7 days post operation. All experiments were approved of by the Institutional Animal Care and Use Safety Committee at Marquette University and in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Viral delivery to cortical neurons

Cortical neurons were transduced using intracerebral microinjection as seen in Fig. 2.1 (Blackmore et al., 2012; Wang et al., 2018, 2015). Mice were anesthetized IP with ketamine/xylazine cocktail (100mg/kg, 10mg/kg). Animals received cortical injections of either control AAV9-luc-EGFP:saline 1:3 or AAV9-luc-EGFP:AAV8 KLF6 1:3 delivered into the sensorimotor cortex through a pulled glass pipette fitted onto a 10µl

Hamilton microsyringe (Chapter III). Mice received two injections of 0.5µl, for a total of 1µl injected, to target the corticospinal neurons (bregma AP 0.0 ML 1.3 DV 0.5, AP .5 ML 1.3 DV 0.5). Each 0.5µL injection was delivered at a rate of 0.05µl/min using a Stoelting QSI pump (#53311). Following each injection the pipette was left in place for 1 minute to minimize the back flow of viral solution. Viruses were designed in house and generated from the University of North Carolina Vector Core as previously described (Wang et al., 2018). Animals in the Constraint induced movement therapy study received AAV9-luc-EGFP:saline 1:3 tracer virus delivered to the motor cortex as described above (Chapter IV).

Pyramidotomy transection of CST

Animals received unilateral pyramidotomy at the level of medullary pyramid to fully transect the CST of the tract that was not virally labeled as diagramed in Fig. 2.1 (Kathe et al., 2014; Wang et al., 2018, 2015). Briefly, animals were anesthetized as described above and depth of anesthesia was maintained throughout surgery. The animals were immobilized, ventral side facing up and head pulled back so the neck and throat extended. Using asceptic technique an incision was made spanning the throat and the adipose tissue between the neck and the trachea was separated at the midline to expose the trachea. With great care the trachea was displaced for short periods of time to expose the ventral component of the brainstem below. A #11 scalpel blade was used to transect the dura covering the ventral aspect of the medulla. At regular intervals the trachea was replaced to allow for regular breathing, if the trachea was displaced for several seconds too long the animal would die. Once the dura covering the medullary pyramids was displaced and the cerebrospinal fluid absorbed, Vannas scissors (FST 15002-08) were

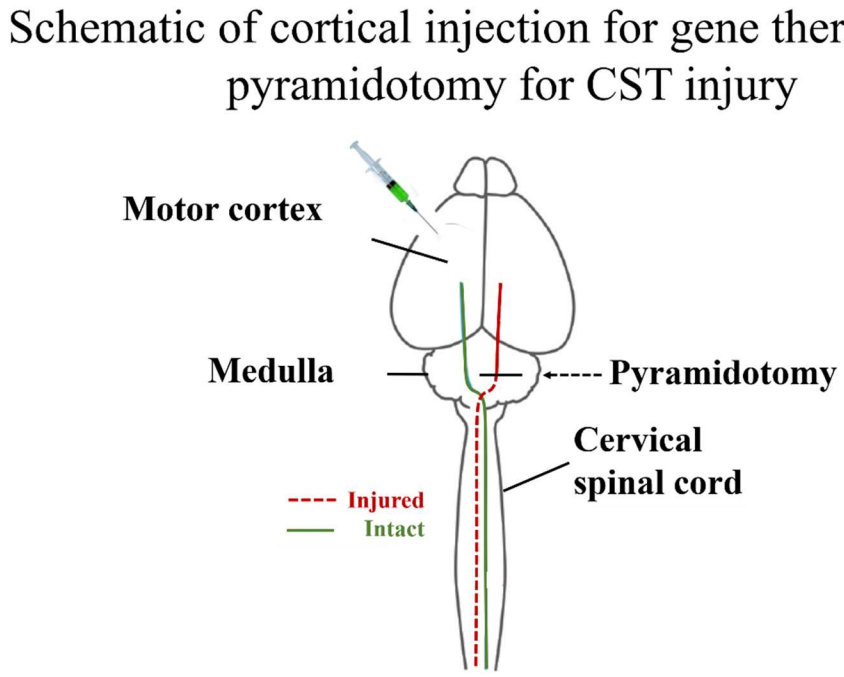


Figure 2.1 Cortical injection and pyramidotomy surgery schematic. Diagram of mouse brain and spinal cord. Green and red lines trace CST as it originates in motor cortex and projects through brainstem (medulla) and down into the spinal cord. Cortical injection takes place in the motor cortex where the gene therapy is delivered via microinjection of viral vectors. Tracer virus AAV-luc-EGFP (included in all groups) is taken up by cells and expressed by CST sprouts in the spinal cord (solid green line). In this design this labeled descending tract is left intact (solid line) to sprout into the injured cord. Unilateral pyramidotomy transects the CST on the ventral side of the medulla, leaving the descending tract to die off (dotted red line).

used to cut through the medial portion of the medullary fibers at a dept of approximately 0.5mm. Care was taken to avoid contact with the midline vein, if this vein was punctured the result was massive bleeding and generally death. The injury site was then manually traced with a 30g syringe tip to ensure complete transection of the CST (Starkey et al., 2005; Kathe et al., 2014).

Histology

Animals were euthanized using CO_2 and subjected to transcardiac perfusion with phosphate buffered solution (PBS) and 4% paraformaldehyde (PFA) in PBS. The brain and spinal column were postfixed in 4% PFA for 3-5 hours, followed by a fine dissection of brain, medulla and cervical spinal cord (a 6mm segment spanning C1-C6), which were fixed overnight in 4% PFA in 4°C and stored in PBS. Tissue was embedded in gelatin and fixed in 4% PFA overnight before being sliced to 100µm thick sections (Leica VT1200) and stored in PBS with 0.02% w/v NaAzide Fig. 2.3A. For protein kinase C gamma (PKCγ) immunohistochemistry, 100µm sections from C2-3 spinal cord were blocked in a 10% normal goat serum (NGS), 3% Triton X-100 in PBS solution for 1hr at room temperature (RT), incubated overnight at 4°C with PKCγ rabbit antibody (Abcam 4145, 1:500) in 1% NGS, 0.3% Trition X-100 in PBS, rinsed, incubated with goat anti-rabbit 546 secondary antibody (ThermoFisher A-11035 1:500) in 1% NGS, 0.03% Trition X-100 in PBS for 2hrs at RT with 4′,6-diamidino-2-phenylindole (DAPI) at a concentration of 1:2000, and then mounted on slides and imaged on an Olympus IX81 microscope at 20x Fig 2.2. Success of pyramidotomy was evaluated by measuring the intensity of PKCγ signal on the injured side as compared to the intact side in a transverse spinal cord slice taken from C3 (Fig. 2.2,2.3A). Any animal that had more than 20% of

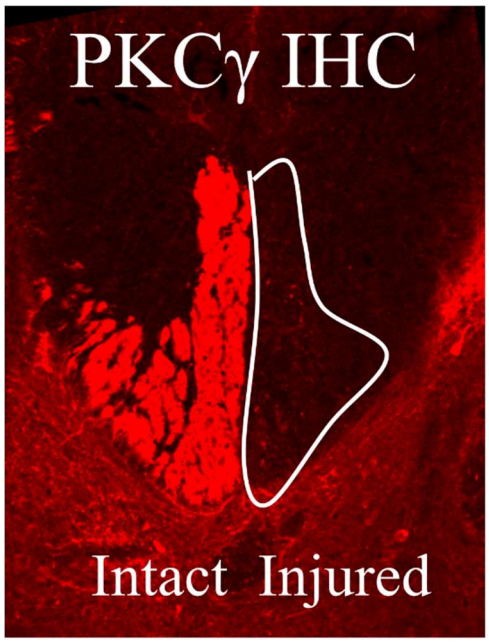


Figure 2.2 PKCγ immunohistochemistry visualized on CST dorsal columns. Pyramidotomy success is calculated by measuring the intensity of the PKCγ signal in the injured CST and dividing it by the intensity on the intact side. If an animal had > 20% signal on the injured CST the pyramidotomy surgery was considered incomplete and the animal was removed from analysis.

signal on the injured side was considered incomplete and not included in any behavioral or anatomical analyses (Table 3.2,4.2, Blackmore et al., 2012; Wang et al., 2018).

Quantification of CST sprouting and calculation of fiber index

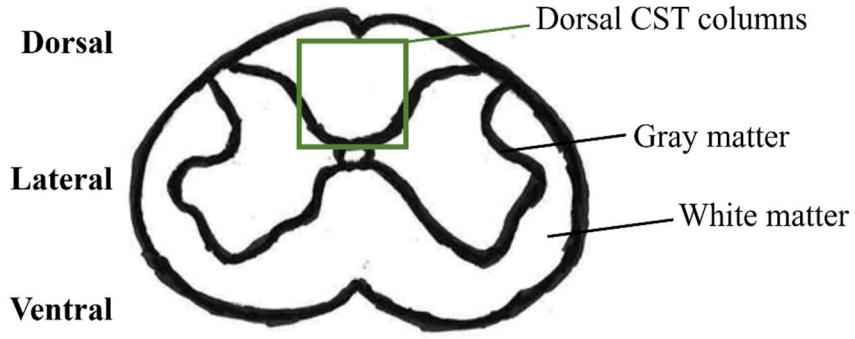
Three transverse cervical spinal cord sections taken at C1/C2, C3/C4 and C5/C6 from each animal were imaged at 20x magnification on the Olympus IX81 microscope Fig. 2.3C. The number of EGFP+ axons at 200 and 400μm distance lateral to the midline was counted (Wang et al., 2015). The medullary pyramids were imaged on the Olympus IX81 confocal microscope at 60x magnification and EGFP+ axons were counted for each animal. A minimum of 1,000 labelled CST neurons in the medulla was required for inclusion criteria (Chapter III, Table 3.2), 800 for inclusion in Chapter IV, Table 4.2. The fiber index was calculated by dividing the spinal cord axon count by the total number of EGFP+ axons quantified in transverse sections of medullary pyramid (Table 3.1, 4.1) (Blackmore et al., 2012; Wang et al., 2015, 2018).

Behavioral Rehabilitation Methods and Testing

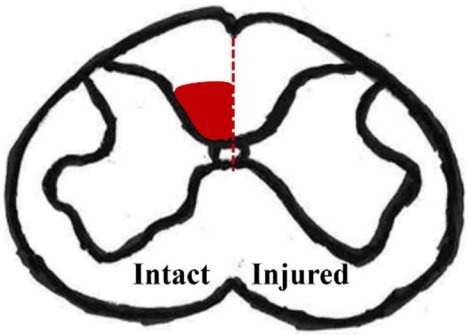
Assessment of skilled motor grasping

To assess forelimb dexterity animals were trained on the mouse staircase (Lafayette Instruments #80301) to retrieve 20mg high fat high cholesterol (21% w/v, 2.1% w/v) dustless precision pellets custom designed by BioServ Fig. 2.4F (Montoya et al., 1991; Jayaprakash et al., 2016). Staircases were modified by adding a glass barrier at the edge of the platform to prevent the animals from reaching pellets that had fallen to the floor from the stairs. To reduce neophobia animals were habituated to food pellets in home cage for 2 days preceding food deprivation. On each pretraining day animals were placed on the staircase for 30 minutes with all eight steps of the staircase loaded with 2 pellets

A. Organization of transverse spinal cord slice



B. Schematic of PKCγ IHC after pyramidotomy



C. Schematic of CST sprouting after pyramidotomy

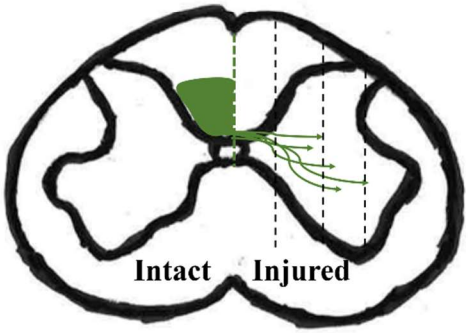


Figure 2.3 Transverse cervical spinal cord section histology schematic. A. Schematic organization of spinal cord. B. Indication of PKCγ immunohistochemistry following pyramidotomy. PKCγ is only expressed on the intact side, as visualized using antibodies. C. Schematic of sprouting from sprouting by intact CST into denervated/injured side. Intact motor cortex receives viral gene therapy containing AAV-luc-EGFP to trace sprouted axons in cervical spinal cord. Axon sprouting is quantified at each of three dotted vertical lines at 200, 400 and 600µm.

each. Both sides of the staircase were loaded with pellets, with 16 pellets per side. Only the data from the affected side are reported here. At the end of each session the number of pellets retrieved, and pellets displaced were recorded for each side of the staircase. The criterion for inclusion in experiments was a pellet retrieval of ≥ 8 pellets by the end of the training period which lasted 15-18 days. Animals that met the criterion were included in surgical experiments and further testing, animals that did not meet the criterion were dropped from the study (Table 3.2,4.2). For postinjury testing all animals were tested on the staircase 2x/week and the average for that week was used as the score for that animal for that time point (Pagnussat et al., 2009; Fouad et al., 2010). Data represent postsurgical pellet retrieval normalized to presurgical training retrieval for each animal.

Assessment of skilled fine motor limb placement

To test skilled forelimb placement we utilized a custom-built apparatus consisting of a wheel (circumference 50.26cm) of irregularly spaced rungs (spaced 0.9-1.8cm), designed as a modified form of horizontal ladder-crossing (Metz and Whishaw, 2009; Wang et al., 2015). In this task animals walked in place atop the wheel, which was rotated at a constant speed (1.26 cm/s) and the task was videorecorded with the camera placed to capture the motion of the affected limb (Fig. 2.4A). Due to the position of the camera only the affected forelimb/forepaw could be visually tracked and scored. The animal walked for three full revolutions of the wheel, with the final two rotations scored for walking behavior. Animals were habituated to the wheel in three 5-minute sessions before the first recorded time point. Animals were tested before surgery, and then weekly starting 2 weeks following pyramidotomy. Ladder testing was scored by a blinded viewer watching the videos in slow motion (Windows Media Player 10). A correct step was

classified as the paw centered on the rung with the digits closing over the rung Fig. 2.4B. Any weight bearing step in which the toes or heel were placed on the rung, or in which the foot missed the rung completely, was classified as incorrect (Fig. 2.4C-E). The number of errors at each postsurgical time point is represented as a percentage of the total steps in that session.

Task-based rehabilitation of CST forelimb behavior

AAV-Control and AAV-KLF6 animals were divided into untrained and rehabilitation trained groups following injury, and the training group received behavioral training as detailed above starting at 4 weeks post injury. The rehabilitation trained group received staircase training 5 days/week with 2 days counted for weekly testing. The trained group also received 4 days a week of ladder walking training on a grate with evenly spaced horizontal bars spaced 1.25cm apart (Fig. 2.4G). Each time an animal crossed the ladder it entered into a holding chamber and received a 20mg high fat pellet and after 30 seconds the gate was opened and the animal crossed back across the ladder to enter into the opposite holding chamber and receive another food pellet. Animals crossed the ladder 60 times each training day. Rehabilitation training continued for 10 weeks, until 14 weeks post injury, when the experiment was terminated, and tissue was collected.

Constraint-Induced Movement Therapy Casting

Immediately following cortical injection animals in the CIMT group were casted while still under surgical anesthesia. The unaffected limb was held across the animal's sternum while 2.5cm wide strips of plaster of Paris were wrapped 4-6 times around the animal's upper body (Müller et al., 2008; Zhao et al., 2009). Care was taken to allow for free range of motion of the affected limb Fig. 2.4H. Once the casting compound set any rough edges

were trimmed with scissors and softened using a moistened cotton swab. To minimize animals chewing on casts a 1.25cm wide piece of metal mesh long enough to encircle the cast and it was fastened into place with Loctite® Super Glue Gel. The integrity of the casts was checked daily throughout the 4-week period. Occasionally an animal was found with the restrained limb removed from the cast. In this case the animal was lightly anesthetized with ketamine/xylazine and a new cast was put in place (Maier et al., 2008). In several cases the metal mesh had to be reattached over the course of the 4-week CIMT rehabilitation period. This was achieved with minor restraint of the animal during which a new mesh outer cover was put in place over the plaster cast. At the end of CIMT for cast removal the animals were lightly anesthetized using low dose ketamine xylazine cocktail. The metal mesh was first removed and then using copious amounts of water the plaster cast was slowly removed. If it was found that the animal had damaged the skin underneath the cast, that area was treated with a topical antibiotic ointment as needed. To prevent hypothermia following cast removal the animals were placed in cages on top of heating pads until the fur had fully dried, the animals regained consciousness and were moving normally within the cage. Due to extended limb restraint, CIMT animals typically took 1-2 days to reestablish normal coordinated ambulation within the cage.

Task-based rehabilitation of CST forelimb behavior in CIMT animals

Animals in the CIMT group received additional rehabilitation in the form of grid walking. These animals were placed in a box on top of a grate with horizontal bars spaced 1.25cm apart for 1 hour a day 7 days/week for the 4-week duration of CIMT. During this supplemental rehabilitation period the animals were forced to use the affected limb to grasp the bars on the grate to ambulate. Multiple animals from one CIMT cage

were placed in the rehabilitation box at the same time. We found that by placing them together as a social group they were more physically active, thus increasing the level of self-motivated rehabilitation on the grate apparatus. The control animals were not exposed to the rehabilitation apparatus.

S*tatistics*

Axon Sprouting Analysis

Injury-dependence and rehabilitation axon sprouting experiments were assessed for significance using 2-way ANOVAs with Tukey's post-hoc testing at 200 and 400µm (GraphPad). The axon sprouting time course was analyzed using Student's one tailed t-test for each time point at each 200 and 400µm (Excel), Chapter III. Fiber index was analyzed with a 2-way ANOVA at 200, 400 and 600µm, also found to be not significant ($p>0.05$) in GraphPad, Chapter IV.

Behavioral Analyses

To determine if AAV-KLF6 plus rehabilitation training impacted behavior 3-way ANOVAs (SPSS) were run for each the staircase and the modified ladder walking tasks, Chapter III. Correlation between Fiber Index and PKCγ signal and Fiber Index and Terminal staircase performance was assessed using a Pearson's Correlation test (SPSS), Chapter III. To analyze ladder walking behavior in rehabilitation stepping-based statistics were assessed individually for each category (%correct, %heel, %toe and %miss) with the use of a 2-way RM ANOVA GraphPad, Chapter III and Chapter IV. The test for correlation between fiber index and terminal stepping behavior in CIMT rehabilitation was assessed using a Pearson's Correlation ($p>0.05$) in SPSS, Chapter IV. $p<0.05$ in any statistical test was considered significant.

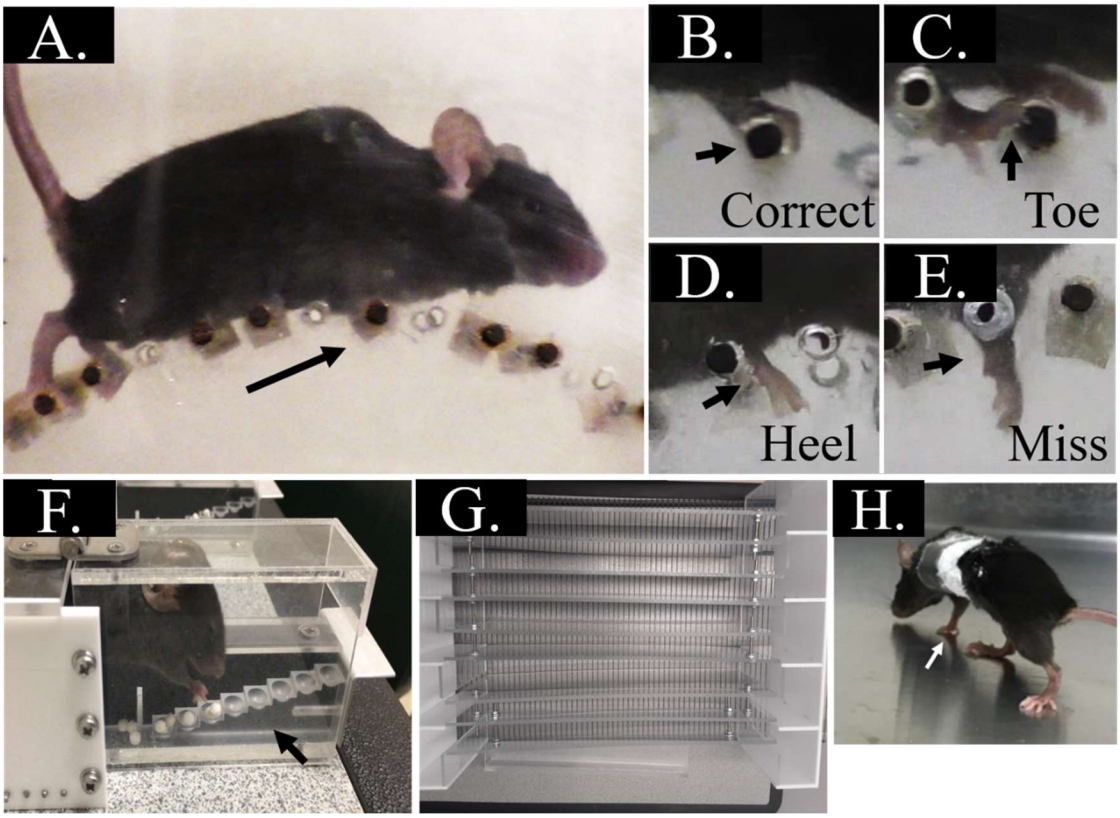


Figure 2.4 Visual of behavioral rehabilitation and testing tasks. (A) Wheel ladder testing. (B-E) Example images of step types on wheel (B) correct step, (C) incorrect toe step, (D) incorrect heel step, (E) incorrect miss step. (F) Montoya staircase grasping task for assessment and rehabilitation training. (G) Rehabilitation walking grate. (H) Casted animal, unaffected limb restrained and affected limb retains full range of motion. Plaster of Paris cast covered by metal mesh encircles the forelimb region of the animal.

Chapter III

Role of transcription factor-induced axon sprouting in corticospinal tract injury.

Abstract

Axons in the corticospinal tract (CST) display a limited capacity for compensatory sprouting after partial spinal injuries, potentially limiting functional recovery. Forced expression of a developmentally expressed transcription factor, Krüppel-like factor 6 (KLF6), enhances axon sprouting by adult CST neurons. Here, using a pyramidotomy model of injury in adult mice, we confirm KLF6's pro-sprouting properties in spared corticospinal tract neurons and show that this effect depends on an injury stimulus. In addition, we probed the time course of KLF6-triggered sprouting of CST axons and demonstrate a significant enhancement of growth within four weeks of treatment. Finally, we tested whether KLF6-induced sprouting was accompanied by improvements in forelimb function, either singly or when combined with intensive rehabilitation. We found that regardless of rehabilitative training, and despite robust cross-midline sprouting by corticospinal tract axons, treatment with KLF6 produced no significant improvement in forelimb function on either a modified ladder-crossing task or a pellet-retrieval task. These data clarify important details of KLF6's pro-growth properties and indicate that additional interventions or further optimization will be needed to translate this improvement in axon growth into functional gains.

Introduction

Spinal cord injury (SCI) results in devastating and permanent disruption of motor, sensory, and autonomic functions (Jensen et al., 2005; Sezer et al., 2015). It is increasingly appreciated that many spinal injuries, even those that present clinically as complete paralysis, are anatomically incomplete and leave a variable number of spared axons that span the injury (Wyndaele and Wyndaele, 2006; Holtz et al., 2017). Although normally insufficient for effective neural communication, this residual substrate can be coaxed to partially restore volitional control below the level of injury (Bareyre et al., 2004; Carmel and Martin, 2014; Shulga et al., 2016). One of the most effective means to improve function after spinal injury is physical rehabilitation (Dietz and Schwab, 2017; Loy et al., 2018; Torres-Espín et al., 2018a). Gains remain partial, however, which may reflect fundamental constraints imposed by the limited information that can be carried by residual axons (García-Alías et al., 2009; Onifer et al., 2011; Starkey et al., 2011). In principle, this constraint could be relieved either by increasing the number of axons that traverse the injury, or by enhancing the amount of terminal sprouting and synapse formation that each spared axon initiates in distal tissue (Girgis et al., 2007; Wang et al., 2011; Loy and Bareyre, 2019). In general the latter goal, that of improving sprouting by spared fibers, has proven more attainable than promoting long distance regeneration across injury sites (Martin, 2016; Fawcett, 2020). Thus, a promising near-term strategy to improve functional recovery from spinal injury may be to apply physical rehabilitation in conditions with elevated anatomical sprouting by spared axons (Lynskey et al., 2008; Fouad and Tetzlaff, 2012; Ishikawa et al., 2015; Griffin et al., 2020).

One promising approach to enhance axon sprouting is through forced re-expression of neuronal genes that initially participate in developmental axon growth (Blackmore et al., 2010, 2012; Wang et al., 2015; Venkatesh and Blackmore, 2017; Mahar and Cavalli, 2018). Two members of the KLF family of transcription factors (TF), KLF6 and KLF7, have demonstrated roles in developmental axon growth, are downregulated during maturation, and promote enhanced axon growth when ectopically expressed in the adult injured CNS (Laub et al., 2001; Matsumoto et al., 2006; Moore et al., 2009; Blackmore et al., 2012; Wang et al., 2018). Specifically, viral expression of KLF6 promotes regeneration and sprouting by corticospinal tract (CST) neurons, important mediators of fine motor control (Martin, 2016; Welniarz et al., 2017; Wang et al., 2018). KLF6's pro-sprouting abilities make it a likely candidate for combinatorial studies with rehabilitation, but additional information is needed to inform the optimal design of these studies. First, it is unknown whether KLF6 promotes axon growth in the absence of CNS injury, which is critical information to predict the potential for off-target growth in uninjured systems. Second, the precise timing of growth triggered by KLF6, which would inform the decision of when to initiate training aimed to sculpt new connections, remains unclear. Clarifying these properties is an important prerequisite to optimally integrate KLF6 treatment with rehabilitation following SCI.

Here we used viral delivery of KLF6 to adult mice subjected to unilateral pyramidotomy, a model of sprouting by intact CST neurons, to explore the injury dependence and time course of KLF6-triggered growth (Starkey et al., 2005, 2011; Jayaprakash et al., 2016). Our data indicate that KLF6's pro-growth effects require injury and indicate that significant KLF6-triggered growth occurs within four weeks of

treatment. Based on these data we initiated experiments in adult mice that combined KLF6 treatment with delayed rehabilitation that began four weeks after pyramidotomy. We found, however, that neither KLF6 nor delayed rehabilitation, either singly or in combination, was sufficient to improve forelimb function on a paw placement or pellet retrieval task. Combined, these data lay critical groundwork for optimal deployment of KLF6 as pro-sprouting tool, while testing and ruling out a specific paradigm of combined KLF6 and training in the corticospinal tract.

Results

Forced KLF6 expression in the corticospinal tract induces sprouting in the presence of pyramidotomy. We showed previously that following unilateral pyramidotomy injury, forced expression of KLF6 enhances cross-midline sprouting of intact CST axons in the cervical spinal cord (Wang et al., 2018). It is unknown, however, whether KLF6 expression alone is sufficient to trigger sprouting, or whether the injury stimulus is also essential. Indeed, other pro-sprouting interventions including PTEN / Suppressor of cytokine signaling 3 (SOCS3) knockout, NT-3 application, and blockade of myelin inhibitory signaling all appear to require injury to trigger axon growth (Zhou et al., 2003; Cafferty et al., 2010; Liu et al., 2010; Jin et al., 2015). To probe KLF6's dependence on injury for its pro-sprouting effects, animals were cortically injected with AAV-KLF6 and AAV-EGFP tracer or with tracer alone (AAV-Control) and then subjected to unilateral pyramidotomy or left uninjured (Fig. 3.1,A). Twelve weeks later the cervical spinal cord

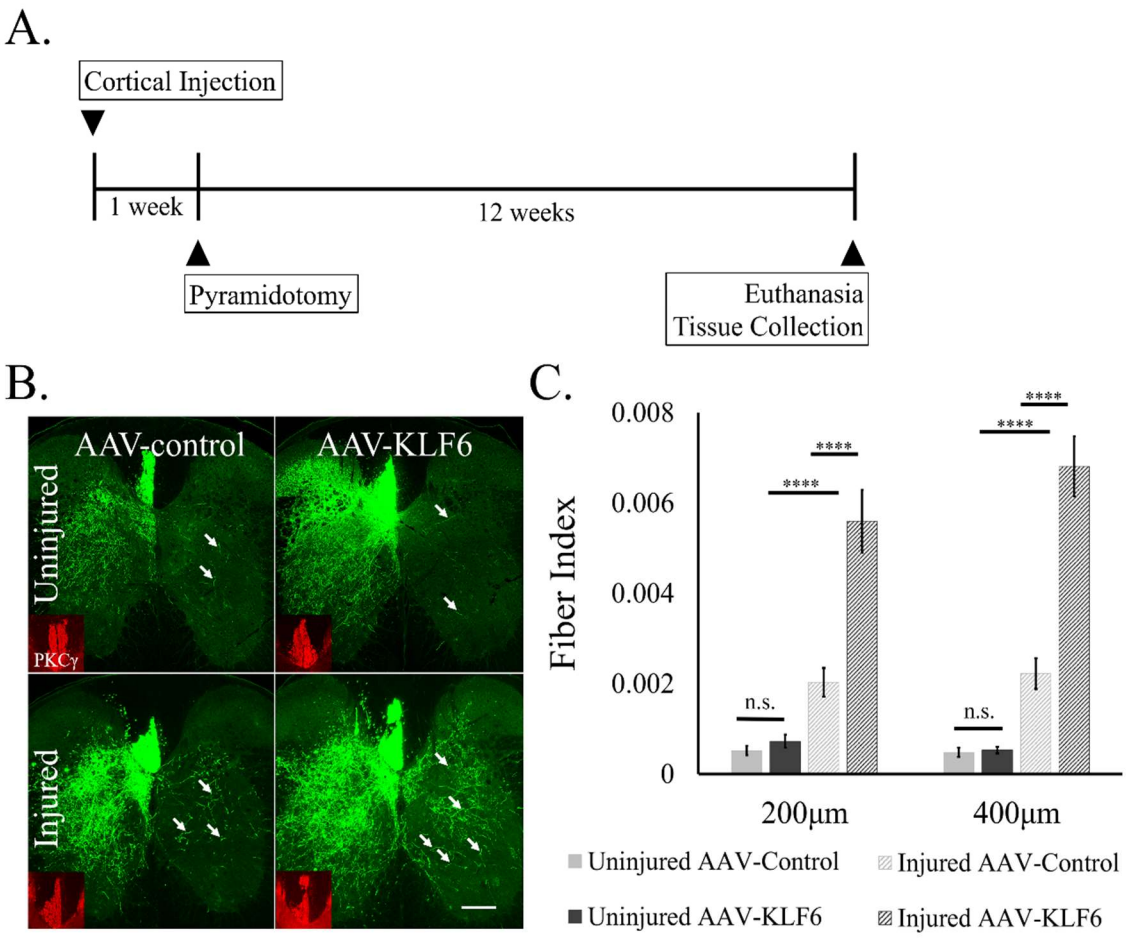


Figure 3.1. KLF6 requires injury to induce axon sprouting. Animals received cortical injections of AAV-KLF6 or AAV-Control with AAV-EGFP tracer and then received unilateral pyramidotomy or were left uninjured. Twelve weeks later tissue was collected and analyzed for CST axon sprouting. (A) Schematic of experimental design (B) Transverse sections of C3 spinal cord with CST axons labeled by EGFP (green). Increased sprouting is seen in AAV-KLF6 treated animals that received pyramidotomy (white arrows). Unilateral ablation of the CST in animals that received pyramidotomy was confirmed by PKCγ signal in the dorsal columns (red, insets). (C) CST growth quantified by axons crossing virtual lines 200 and 400µm from the midline normalized to the total number of EGFP-labeled CST axons in the medullary pyramids (fiber index). AAV-KLF6 had no significant effect on axon sprouting in uninjured animals, but significantly increased cross-midline sprouting in injured animals (**p<.0001, 2-way ANOVA with Tukey's post-hoc testing). N=8 (Uninjured AAV-Control), N=7 (Uninjured AAV-KLF6), N=8 (Injured AAV-Control), N=9 (Injured AAV-KLF6). Error bars show ±SEM. Scale bar is 200µm.**

tract was quantified and normalized to the total axons detected in the medullary pyramid, creating the fiber index (Blackmore et al., 2012; Geoffroy et al., 2015; Meves et al., 2018). Only animals that met inclusion criteria for injury status as assessed by PKCγ immunohistochemistry and effective CST label as assessed by axon counts in the medulla were included in analysis (see Fig. 2.2, Table 3.2).

Similar to previous reports, animals that received control virus and no injury showed very few CST axons in cervical spinal tissue contralateral to the labeled CST tract cord (Fig 3.1B,C) (Starkey et al., 2012; Jin et al., 2015). In AAV-Control animals, unilateral pyramidotomy triggered an approximately 4-fold increase in cross-midline growth by CST axons, indicating a level of spontaneous growth in response to injury (****$p<0.0001$, 2-way ANOVA with Tukey's post-hoc testing, Fig. 3.1C, Fiber Index values in Table 3.1). Treatment of injured animals with AAV-KLF6 further increased this injury-triggered growth about 2-fold, to a level significantly above AAV-Control (****$p<0.0001$, 2-way ANOVA with Tukey's post-hoc testing). In contrast, animals that received AAV-KLF6 but no injury showed very few CST sprouts in contralateral cord, and were indistinguishable from uninjured AAV-Control ($p>0.05$, 2-way ANOVA, Fig. 3.1C Fiber Index values in Table 3.1). Cervical spinal cord sprouting was not correlated with degree of injury completeness as measured by PKCγ immunohistochemistry at 200 and 400μm ($p>0.05$ Pearson's Correlation, Fig. 3.2A,B). This indicates that any remaining CST on the denervated side does not interfere with sprouting from the intact tract. Taken together these data confirm the pro-sprouting activity by KLF6 and reveal that the pyramidotomy injury is an essential triggering event.

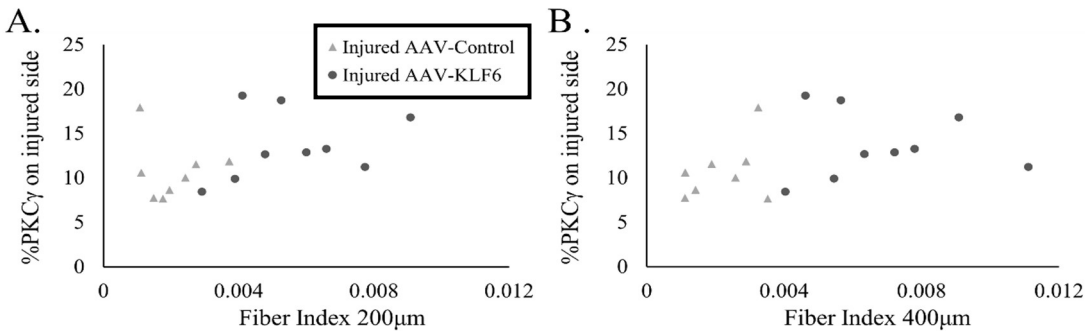


Figure 3.2. Axon sprouting in control and KLF6 treated groups does not correlate with remaining PKCγ signal on the injured side. In animals that received injections of either (A) AAV-KLF6 or AAV-Control and unilateral pyramidotomy the fiber index was plotted against the %PKCγ signal on the injured side of the CST at 200µm and (B) 400µm.

K LF6 increases sprouting at 4 weeks post pyramidotomy. We next sought to establish the time to onset of KLF6's pro-sprouting effects in injured animals. Prior studies of both spontaneous and treatment-stimulated CST growth indicated that cross-midline sprouting may occur within several weeks of injury (Fouad et al., 2001; Weidner et al., 2001; Bareyre et al., 2004). To determine if KLF6-stimulated growth follows a similar timeline, animals received a cortical injection of AAV9-KLF6 or AAV-Control. All animals then received unilateral pyramidotomy and were assigned to either 2, 4 or 8 week post-injury survival groups (Fig. 3.3A). Only animals that met inclusion criteria of effective CST label and complete injury as assessed by PKCγ were included in analyses (Fig. 3.3B insets, see Table 3.2). As before, at the designated time points animals were perfused, spinal cords sectioned and imaged, and cross-midline CST sprouting quantified as a normalized fiber index. At two weeks post-injury, KLF6-treated animals showed only a non-significant trend toward enhanced cross-midline sprouting compared to control animals. By 4 weeks, however, KLF6-treated animals showed significantly higher ime point run at 200 and 400μm, Fig. 3.3C,D). Growth at 8 weeks in the KLF6 group was unexpectedly variable but averaged near the 4 week values and showed a strong trend toward elevated growth compared to control animals ($p=0.053$ at 200μm, Student's one tailed t-test at each time point run at 200 and 400μm, Fig. 3.3C). Taken together, these data show that KLF6 stimulates cross-midline sprouting of CST axons within 4 weeks of unilateral pyramidotomy.

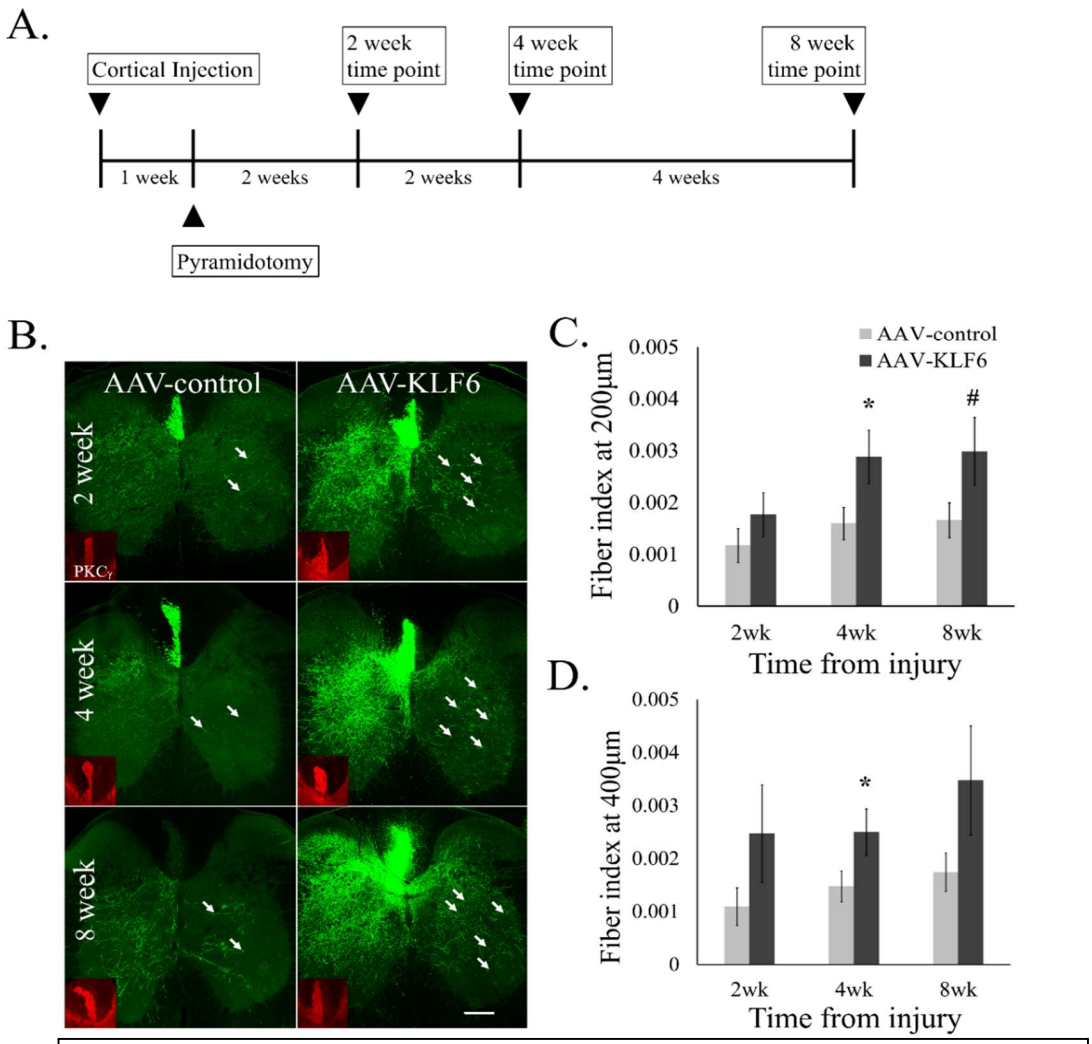


Figure 3.3 KLF6 promotes axon sprouting within 4 weeks of injury. (A) Experimental overview; animals received cortical injection of either AAV-Control or AAV-KLF6 followed by unilateral pyramidotomy and analysis of CST sprouting at 2, 4 or 8 weeks post injury. (B) CST axon sprouting in spinal axons (green) crossing virtual lines 200 and 400µm from the normalized to the total number of EGFP+ CST axons in the medullary pyramids (fiber index). AAV-KLF6 animals have increased CST sprouts across the midline over time (white arrows). Unilateral pyramidotomy was confirmed by unilateral reduction of PKCγ signal in the dorsal columns (red, insets). (C,D) CST axon sprouting quantified at 200µm (C) and 400µm (D). By four weeks post-injury axon growth in KLF6-treated animals significantly exceeded control (*p<0.05 # p=0.053, Student's one tailed t-test for each time point at 200 and 400µm). N= 5 (control 2 weeks), N=5 (control 4 weeks), N=7 (control 8 weeks), N=5 (KLF6 2 weeks), N=6 (KLF6 4 weeks), N=9 (KLF7 8 weeks). Error bars show ±SEM. Scale bar is 200µm.

KLF6 and delayed rehabilitation are insufficient to promote forelimb recovery after pyramidotomy injury. Although KLF6 promotes cross-midline CST sprouting in animals challenged by pyramidotomy, prior findings using a horizontal ladder task showed no improvement in limb placement (Wang et al., 2018). To look more carefully for possible behavioral benefits from KLF6 treatment, here we employed two alternative tests Fig. 3.4A. The first was a variant of the horizontal ladder task, in which the animal is placed atop a large runged wheel that rotates beneath them (Fig. 3.4D). This allows the experimenter to control the speed of the wheel, in contrast to the variable speed of crossing allowed by the standard horizontal ladder task (Metz and Whishaw, 2002; Starkey et al., 2011) . In addition, the animal remains in a confined frame, simplifying video analysis. The second task was the staircase pellet retrieval task in which animals are challenged to reach downward at increasing distances to retrieve high fat food pellets (Fig. 3.4B, Montoya et al., 1991; Starkey et al., 2005). All animals were pre-exposed to the ladder walking task for three 5-minute sessions. On the staircase the animals were pre-trained on the task until able to retrieve at least 8 pellets on the left side, 15-18 sessions/days (see Table 3.2 for pre-training exclusion). Animals then received left cortical injection of AAV-Control tracer or AAV-KLF6 and one week later received unilateral pyramidotomies on the right side (see Fig. 2.1). Prior to injury animals averaged 70% correct steps in the ladder task and 10.25 pellets retrieved on the left side.

As expected, injury decreased performance on both tasks, with mice stepping correctly only about 50% of the time on the ladder and retrieving 55% of the pellets that they retrieved preinjury. The injury-induced impairment of behavior was maintained over

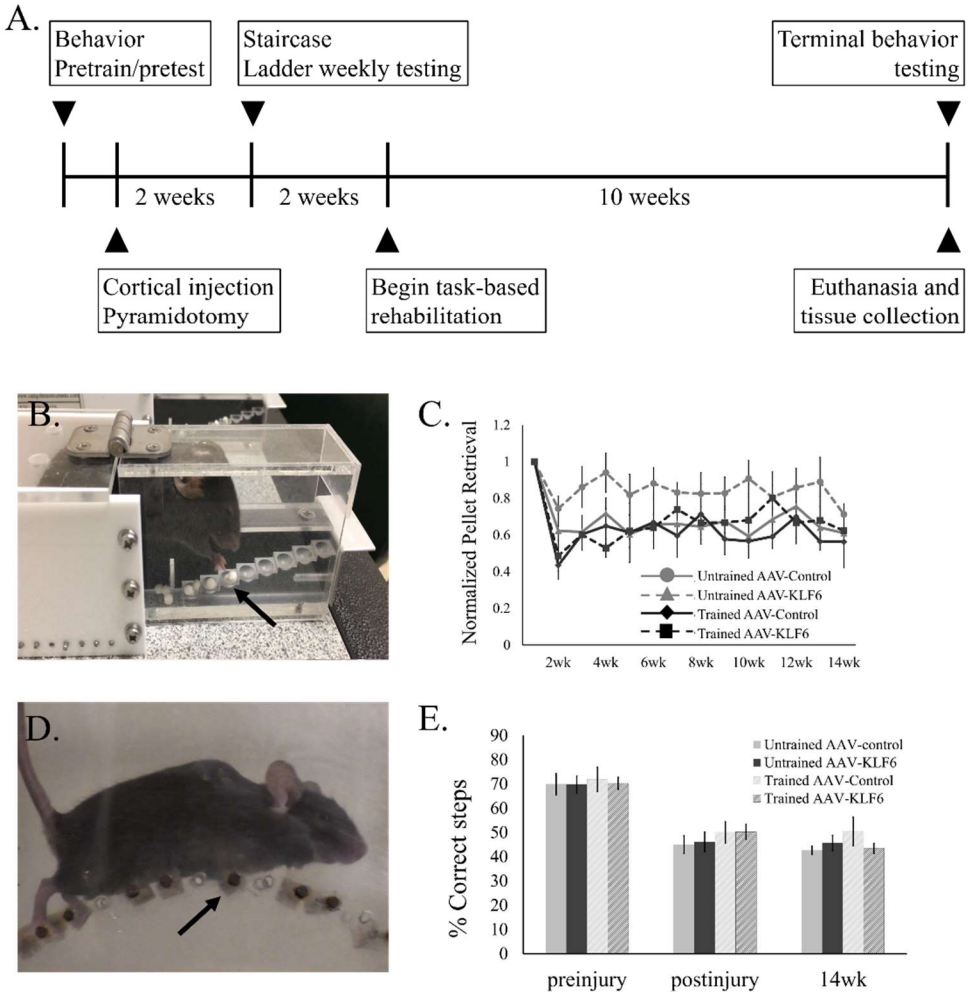


Figure 3.4 KLF6 and delayed rehabilitative training produce no improvements in forelimb function after pyramidotomy injury. (A) Experimental overview; animals were pretrained on the staircase and modified ladder tasks, treated with AAV-Control or AAV-KLF6 and then received pyramidotomy. Weekly behavioral testing began 2 weeks post-injury and half of the animals received rehabilitation starting 4 weeks post-injury. At 14 weeks post-injury tissue was analyzed for CST sprouting. (B) Staircase pellet retrieval task, where animal reaches downward to grasp food pellets (black arrow), which becomes progressively more difficult to retrieve as the stairs deepen. (C) Quantification of pellets retrieved shows an injury-triggered reduction followed by a prolonged deficit that did not differ between KLF6 or rehabilitation=retreated groups ($p>0.05$ 3-way ANOVA). (D) Modified ladder walking assay, animals walk atop a wheel with irregularly spaced rungs (black arrow) that rotates at a constant speed. (E) Quantification of correctly placed foot steps show an injury-induced reduction in performance that persisted for the duration of the experiment, and which did not differ significantly between any groups ($p>0.05$ 3way RM ANOVA). N=7 (Untrained AAV-Control), N=7 (Untrained AAV-KLF6), N=6 (Trained AAV-Control), N=10 (Trained AAV-KLF6). Error bars show ±SEM.

the course of the experiment for both groups. (Fig. 3.4C,E). The breakdown of incorrect steps as heel, toe or completely missed placements (where the forelimb completely misses the rung) is visually depicted (Fig. 3.5A-D) and graphically represented (Fig. 3.5E-G). While there was a significant increase in heel stepping following pyramidotomy in all groups, there was no difference between the groups in amounts of heel steps and this significant increase was maintained over the long time course (****$p<0.0001$ 2-way RM ANOVA Fig. 3.5E). There were no changes detected in toe stepping or complete misses ($p>0.05$ 2-way RM ANOVA Fig. 3.5F,G). Interestingly, quantification of axon growth in the cervical spinal cord at the end of the experiment confirmed significant elevation of cross-midline sprouting in AAV-KLF6 treated animals Fig. 3.6A ($p<0.05$ at each 200 and 400µm 2-way ANOVA with Tukey's post-hoc testing, Fig. 3.6B $p<0.001$ at 200µm and $p<0.05$ at 400µm, 2-way ANOVA Tukey's post-hoc testing, Fig. 3.6C). Thus, consistent with prior findings, KLF6 enhances CST axon growth but yields no behavioral improvements, even here where the behavior is designed to require finer components of forelimb function with the modified ladder task and staircase pellet retrieval.

A possible explanation for the lack of behavioral improvements is that the KLF6-stimulated growth from CST axons is not optimally targeted within the spinal cord. In humans and rodents alike, physical rehabilitation has been shown to improve behavioral function after spinal injury, presumably by reinforcing adaptive circuitry (Girgis et al., 2007; García-Alías et al., 2009; Musselman et al., 2009). We therefore asked whether KLF6 treatment can improve forelimb function in the context of exposure to daily rehabilitative tasks. As previously described, animals were pre-trained on pellet retrieval

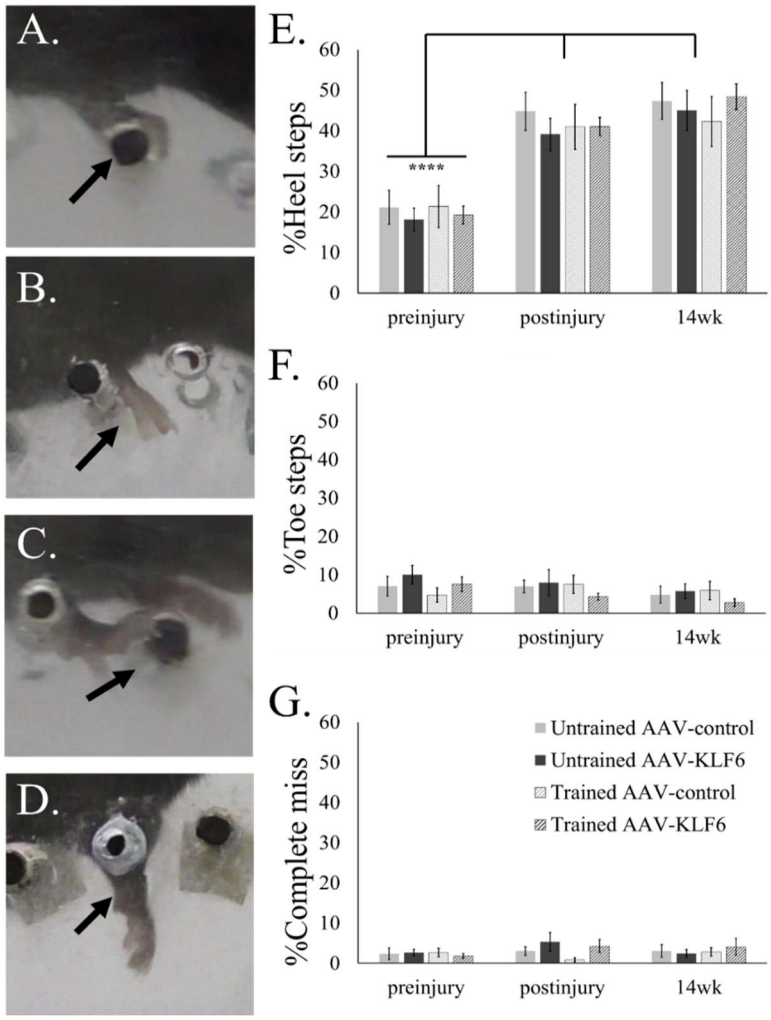


Figure 3.5 KLF6 and delayed rehabilitative training groups show increased heel steps following pyramidotomy injury. (A) Correct step (black arrow), (B) Heel step (black arrow), (C) Toe step (D) Complete miss, forelimb misses rung and falls down between two steps (black arrow), (E) Quantification of heel steps show that in all treatment groups heel steps increase after injury at a level that is maintained and do not differ between groups (**$p<0.0001$ 2-way RM ANOVA). (F) Quantification of toe steps show that there was no change in %toe steps due to injury treatment or training group ($p>0.05$ 2-way RM ANOVA). (G) Quantification complete miss steps demonstrate that there is no change in missed steps due to injury or between any groups ($p<0.05$ 2-way RM ANOVA). N=7 (Untrained AAV-Control), N=7 (Untrained AAV-KLF6), N=6 (Trained AAV-Control), N=10 (Trained AAV-KLF6). Error bars show ± SEM.**

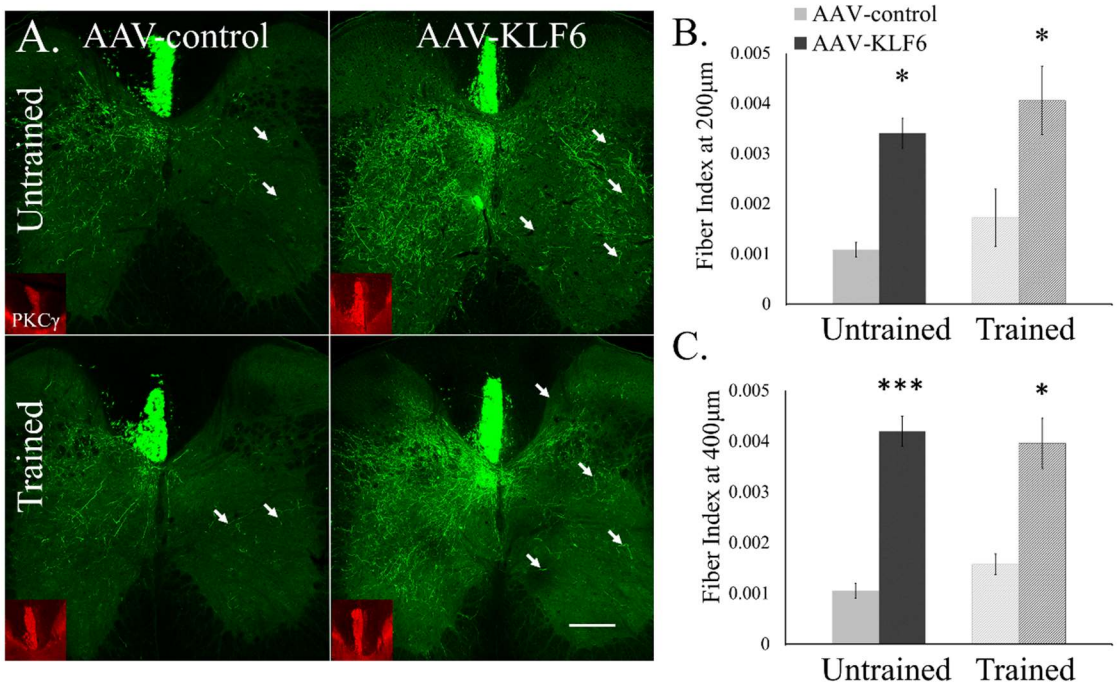


Figure 3.6 KLF6, but not delayed rehabilitative training, induces CST sprouting. (A) Transverse C3 cervical spinal cord showing CST axons sprouting across the midline (EGFP, green). AAV-KLF6 animals showed increased CST sprouting in both the untrained and trained groups (white arrows). Unilateral ablation of the CST was confirmed by reduction of PKCγ signal in the dorsal columns (red, insets). (B,C) Quantification of CST axon growth, showing that KLF6 significantly increased CST axon growth in both trained and untrained animals, whereas rehabilitation had no significant effect on axon growth in any conditions (*p<0.001 Untrained 400µm, *p<0.05 Untrained and trained 200µm, Trained 400µm groups 2-way ANOVA with Tukey's post-hoc). N=7 (Untrained AAV-Control), N=6 (Untrained AAV-KLF6), N=7 (Trained AAV-Control), N=10 (Trained AAV-KLF6). Error bars show ±SEM. Scale bar is 200µm.**

and the modified ladder task, and then received cortical injection of AAV-Control or AAV-KLF6 and unilateral pyramidotomy. Animals then started rehabilitative training at four weeks post-injury, when KLF6-stimulated growth has entered the contralateral spinal cord (Fig. 3.6A,B). Training occurred five days per week, and consisted of one-30 minute session of staircase per day and a separate session of ladder walking rehabilitation

wherein animals crossed a 60cm long horizontal ladder 60x 4 days/week and on the fifth day the animals were recorded for scoring on the ladder walking task (Fig. 3.4A, see methods). Deficits in forelimb function, however, proved insensitive to this training paradigm. Neither pellet retrieval nor foot placement improved over the 10 week period of training, leaving trained animals with deficits similar to untrained animals ($p>0.05$, 3-way ANOVA, Fig. 3.4C,E). Similarly, no differences in performance emerged between AAV-Control and AAV-KLF6 treated animals ($p>0.05$ 3-way ANOVA). Thus, task specific training, initiated 4 weeks post-injury, was insufficient to evoke behavioral improvements in KLF6-treated animals.

We assessed CST axon sprouting in animals treated with KLF6 and/or rehabilitation training (Fig. 3.6A). As previously, cross-midline CST growth was quantified in transverse sections of cervical spinal, normalized to total labeled CST axons detected in the medullary pyramids, while PKCγ immunohistochemistry confirmed injury completeness (Fig. 3.6A insets, see Table 3.2). KLF6 treated animals, in both untrained and trained groups showed increased axon sprouting compared to AAV-Control at 200 and 400μm ($p<0.05$ 2-way ANOVA with Tukey's post-hoc testing, Fig. 3.6B,C). In contrast, rehabilitation training had no significant effect on CST axon growth in either

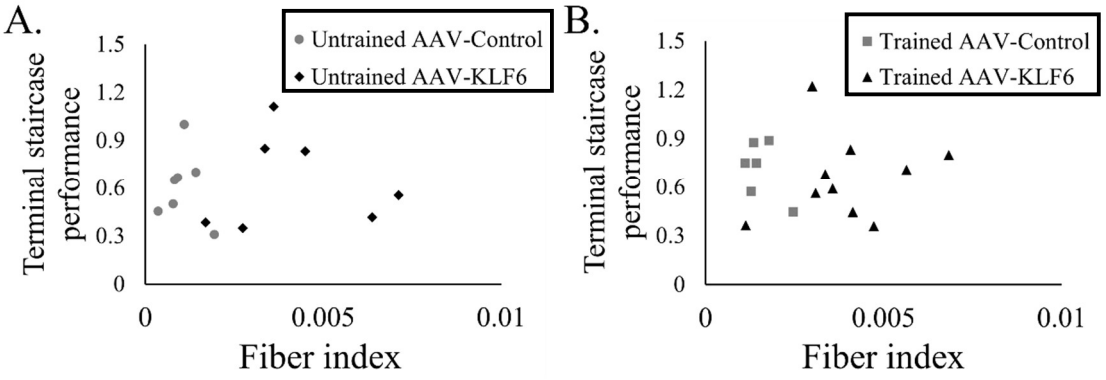


Figure 3.7 Terminal ladder walking behavior does not correlate with CST sprouting in the spinal cord in KLF6 treated or rehabilitation trained groups. (A) Untrained AAV-Control and AAV-KLF6 scatterplot of fiber index vs staircase performance show that there is no correlation between the two factors ($p>0.05$ Pearson's Correlation), (B) Rehabilitation trained AAV-Control and AAV-KLF6 scatterplot of fiber index vs staircase performance show that there is no correlation between the two factors ($p>0.05$ Pearson's Correlation). N=7 (Untrained AAV-Control), N=7 (Untrained AAV-KLF6), N=6 (Trained AAV-Control), N=10 (Trained AAV-KLF6).

AAV-Control or AAV-KLF6 groups ($p>0.05$, 2-way ANOVA). Finally we asked if sprouting in the spinal cord was correlated with terminal staircase performance and found that in each of the four experimental groups there was no correlation between behavior and the fiber index ($p>0.05$ Pearson's Correlation Fig. 3.7A,B). Taken together, these data confirm that forced expression of KLF6 produces CST sprouting that is stable for at least 14 weeks after treatment, but which is neither increased nor decreased by the applied rehabilitation paradigm.

Discussion

Our findings substantiate KLF6's ability to enhance axon sprouting and clarify important details of its evoked growth response. It is notable that across multiple experiments *in vivo,* we found forced expression of KLF6 to produce significant elevation of CST axon growth, providing robust replication of the initial report (Wang et al. 2018). Moreover, the current findings demonstrate that KLF6's effects require an injury-induced stimulus and demonstrate that four weeks of axon growth results in significantly increased sprouts in the spinal cord. Contrary to our expectations, however, axon growth stimulated by KLF6 was not accompanied by recovery of forelimb function, even in animals that received intensive task-based rehabilitation. A breakdown of post-injury behavior (Fig. 3.5E-G) did not reveal differences in compensatory mechanisms in the rehabilitation groups.

The injury requirement and timing of KLF6-triggered growth are generally in line with prior findings. For example, knockout of negative regulators of axon growth such as PTEN, SOCS3, or extrinsic myelin-associated proteins (MAG, OMgp and Nogo-A), have

all been shown to promote CST axon growth, but only in the presence of injury (Cafferty et al., 2010; Liu et al., 2010; Jin et al., 2015). Similarly, application of NT-3 to the spinal cord elevates CST sprouting in injured, but not uninjured, spinal cord (Zhou et al., 2003). Regarding the timing of growth, prior work indicates that spontaneous sprouting of CST axons after spinal injury is minimal 10 days after injury but well established by 4 weeks (Lang et al., 2012). Our current findings show KLF6-induced sprouting to follow a similar time course (Fig. 3.3), suggesting that KLF6 does not accelerate the onset of sprouting but rather increases the rate of growth once initiated.

Several non-exclusive possibilities may explain why KLF6 acted to reliably promote axon growth but did not lead to behavioral recovery. One possibility is that KLF6-induced sprouting, although consistently elevated above control, may still fall below a threshold needed to produce detectable improvement on the forelimb tasks employed here. For example, knockout of PTEN has been shown to promote axon growth in many cell types, including CST, but in a pyramidotomy model similar to the one employed here did not produce improvements in forelimb placement (Liu et al., 2010; Geoffroy et al., 2015; Ohtake et al., 2015). Interestingly, however, dual knockout of PTEN and SOCS produced a synergistic elevation of CST axon growth above the level of PTEN alone, which unlike PTEN alone was accompanied by improvements in foot placement after pyramidotomy injury (Jin et al., 2015). This indicates that increased CST sprouting correlates with improved fine forelimb function. These findings hint that KLF6-treated animals might similarly benefit from further gains in total CST growth after injury. Notably, we very recently found that the sprouting effect of KLF6 is significantly enhanced by co-expression of a synergizing factor, nuclear receptor

subfamily 5 group A member 2 (NR5A2), and future work will explore whether this elevated growth is accompanied by behavioral gains (Venkatesh et al., 2020).

A second possibility is that expression of KLF6 itself may have limited the functional output of treated CST axons. Support for this possibility comes from our recent transcriptional analysis and functional classification of genes that respond to forced KLF6 expression in neurons (Wang et al. 2018). In this study, KLF6 expression resulted in upregulation of genes that were enriched in pro-growth functions such as cytoskeletal remodeling and bioenergetics. Interestingly, however, genes downregulated by KLF6 were enriched in proteins involved in synaptic functions such as glutamatergic transmission and neurotransmitter release (Wang et al., 2018). These data raise the interesting possibility that prolonged expression of KLF6 may favor the extension of axons but interfere with proper synaptic function. Although speculative, this hypothesis would motivate experiments that deploy KLF6 in a controlled manner after injury, allowing silencing of expression and possible release from KLF6's synaptic interference once the new axon growth is in place.

Finally, it is also notable that KLF6 treatment had no detectable effect on behavior even in the presence of intensive rehabilitation. This may reflect in part the specific timing of rehabilitation in our studies, which was delayed four weeks after injury. This time was selected to potentially capitalize on the new KLF6-stimulated growth, established as present by four weeks (Fig. 3.3). Prior work, however, indicates that compared to rehabilitation initiated within seven to twelve days of injury, delayed rehabilitation yields less functional recovery (Norrie et al., 2005; Scivoletto et al., 2005; Krajacic et al., 2009; Starkey et al., 2011). It is possible that a shorter time delay would

prove more beneficial. Alternatively, to make rehabilitation more effective when applied in the chronic injury state, additional treatments may need to be provided to reopen the window for beneficial plasticity. For example, one study paired delayed rehabilitation with lipopolysaccharide (LPS)-mediated induction of an immune response. Notably, this combinatorial approach resulted in improved performance on a pellet retrieval task even when initiated eight weeks after injury (Torres-Espín et al., 2018a). Another study found that pairing chondroitinase treatment with rehabilitative training led to recovery of staircase behavior even when initiated 4 weeks after injury. This suggests that chondroitinase treatment can act to reopen the window for effective rehabilitation (Wang et al., 2011). Similarly, application of chondroitinase and L1 adhesion molecule three weeks after spinal contusion yielded long term behavioral gains that were accompanied by increased cholingergic and glutamateric positive terminals in newly sprouted axons (Lee et al., 2012). These studies raise the possibility that supplying LPS, chondroitinase, or other pro-plasticity treatments in conjunction with KLF6 may improve the functional contribution of newly grown axons in the chronic injury state. In conclusion, although KLF6-induced sprouting by CST axons did not result in behavioral improvements in the specific paradigm employed here, it remains possible that by further boosting growth, temporally controlling KLF6's expression, and/or supplying co-treatments to re-open plasticity in spinal circuits, CST sprouting triggered by KLF6 could be rendered more beneficial to behavioral improvements.

Table 3.1. Fiber Index Values				
Experiment	**Experimental group**	**Distance (µm)**	**Fiber index**	**SEM**
Figure 1-Injury dependence	Uninjured, AAV-control	200	0.00052	0.00010
	Uninjured, AAV-KLF6	200	0.00073	0.00014
	Injured, AAV-control	200	0.00204	0.00032
	Injured, AAV-KLF6	200	0.00574	0.0007
	Uninjured, AAV-control	400	0.00048	0.00010
	Uninjured, AAV-KLF6	400	0.00053	0.00007
	Injured, AAV-control	400	0.00222	0.00034
	Injured, AAV-KLF6	400	0.00666	0.00066
Figure 2-Timecourse sprouting	AAV-control 2wk	200	0.00117	0.00033
	AAV-KLF6 2wk	200	0.00177	0.00043
	AAV-control 4wk	200	0.00160	0.00031
	AAV-KLF6 4wk	200	0.00288	0.00052
	AAV-control 8wk	200	0.00166	0.00034
	AAV-KLF6 8wk	200	0.00298	0.00066
	AAV-control 2wk	400	0.00109	0.00036
	AAV-KLF6 2wk	400	0.00247	0.00092
	AAV-control 4wk	400	0.00147	0.00029
	AAV-KLF6 4wk	400	0.00250	0.00044
	AAV-control 8wk	400	0.00174	0.00037
	AAV-KLF6 8wk	400	0.00347	0.00103
Figure 3-KLF6 treatment and rehabitation training	Untrained AAV-control	200	0.00107	0.00010
	Untrained AAV-KLF6	200	0.00340	0.00030
	Trained AAV-control	200	0.00204	0.00067
	Trained AAV-KLF6	200	0.00406	0.00068
	Untrained AAV-control	400	0.00105	0.00019
	Untrained AAV-KLF6	400	0.00420	0.00074
	Trained AAV-control	400	0.00238	0.00086
	Trained AAV-KLF6	400	0.00396	0.00049

Table 3.1 Fiber Index Values for animal groups in each figure of Chapter III. Data are expressed as average fiber index values for each experimental group and SEM at each 200 and 400µm distance from the midline.

Table 3.2 Animal inclusion table for experiments in Chapter III. Specific exclusion criteria illustrate why animals were removed from each experiment, leading from the starting N for each group to the final N included in data figures.

Table 3.2 Animal Inclusion

			Excluded-behavior		Died-surgery		Excluded-histology			
Experiment	**Experimental group**	**Starting N**	**Failed to train**	**Other**	**Cortical Injection**	**Pyramidotomy**	**PKCγ**	**Medulla count**	**Final N**
Figure 1 - Injury dependence	Uninjured, AAV-contro	8	N/A	N/A	0	0	0	0	8
	Uninjured, AAV-KLF6	7	N/A	N/A	0	0	0	0	7
	Injured, AAV-control	11	N/A	N/A	1	2	0	0	8
	Injured, AAV-KLF6	11	N/A	N/A	0	1	1	0	9
Figure 2- Timecourse sprouting	AAV-control 2wk	12	N/A	N/A	0	2	1	4	5
	AAV-KLF6 2wk	11	N/A	N/A	0	3	0	2	6
	AAV-control 4wk	11	N/A	N/A	0	1	1	2	7
	AAV-KLF6 4wk	11	N/A	N/A	0	1	1	0	9
	AAV-control 8 week	13	N/A	N/A	0	4	3	1	5
	AAV-KLF6 8wk	15	N/A	N/A	0	4	4	0	7
Figure 3-KLF6 and rehabilitation tra	Untrained AAV-contro	20	5	0	1	5	2	0	7
	Untrained AAV-KLF6	20	5	0	0	5	3	1	6
	Trained AAV-control	20	5	1	0	5	1	1	7
	Trained AAV-KLF6	20	4	0	0	4	2	0	10

Chapter IV

Modulation of Corticospinal Tract Sprouting by Constraint-Induced Movement Therapy

Abstract

Mature axons in the corticospinal tract (CST) have a low intrinsic capacity for regeneration. Yet, this tract is often targeted in human patients following cervical spinal cord injuries that cause damage to these axons. Many rehabilitative therapies have been used to increase the plasticity of the CST, with varying degrees of success with regards to axon sprouting and behavioral gains. Here we employ constraint-induced movement therapy (CIMT) as a means of intensive, forced rehabilitative intervention in animals with a unilateral pyramidotomy. We used chronic CIMT period of 4 weeks and then probed the forelimb stepping behavior of treated and control animals for a further 3 weeks. While the CIMT group was restrained to use of only the affected limb in the rehabilitation period, and thus required to use this limb more than unrestrained animals, we did not find any behavioral or sprouting differences between the treatment or control groups.

Introduction

Injury and insult to the mature CNS is often highly disruptive as this system retains a low capacity for regeneration and repair. Stroke, traumatic brain injury, and spinal cord injury (SCI) are all conditions that generally have lasting effects including loss of motor control or paralysis of regions of the body caudal to the injury (Jensen et al., 2005; Rogers and Todd, 2016). This functional deficit is the result of neuroprotective mechanisms that cause injured neurons to die back and an inflammatory response that creates an inhibitory environment that is prohibitive to neuronal regeneration (Sun and He, 2010; Mahar and Cavalli, 2018). One approach to treat SCI is to target spared axons as opposed to regenerating axons for growth by engaging the individual in intense rehabilitative therapies to promote plasticity within the injured system (Raineteau and Schwab, 2001; Loy and Bareyre, 2019).

Constraint-induced movement therapy (CIMT) is a rehabilitative technique that has historically been used in stroke conditions that affect motor control (Taub et al., 1993; Ishida et al., 2015). It is a rehabilitation-intensive practice that requires animals to constantly engage the affected limb for normal movement in the home cage as well as during additional interventions. CIMT has recently been applied to SCI as an effective intervention for rehabilitation of motor deficits. CIMT is of particular interest to applications of the corticospinal tract (CST). The CST is the main descending motor tract that controls skilled forelimb behavior and is often disrupted by stroke and SCI, making it a strong candidate for rehabilitative studies (Higo, 2014; Okabe et al., 2017b). The intact CST has also shown some capacity for sprouting of spared fibers specifically in the

presence of injury, providing further evidence that it is a candidate for rehabilitation (Weidner et al., 2001; Hilton et al., 2016, Kramer *et al* under review).

One key component of rehabilitation is timing: the onset of training after injury and duration of treatment are critical factors to the success of the intervention (Jang, 2014; Burns et al., 2017). A study in rats used CIMT to rehabilitate the CST by placing a cast on the unaffected limb immediately following unilateral pyramidotomy and continued the rehabilitation for either one or three weeks. This work found that animals that were forced to use the affected limb had a full recovery of the ladder walking task after 3 weeks of CIMT and had higher levels of sprouting in the 3 week forced use group (Maier et al., 2008). This study in combination with others from the stroke literature that specifically look to CIMT in its ability to lead to skilled forelimb-specific motor gains provide further evidence that the CST is a prime candidate for CIMT rehabilitative studies (Ishida et al., 2011; Okabe et al., 2018).

Here we use CIMT in animals that have received a unilateral pyramidotomy of the CST. One week after the pyramidotomy all animals received a cortical injection of AAV-luc-EGFP into the uninjured CST motor cortex to trace the intact tract and sprouting fibers in the cervical spinal cord. At the time of cortical injection the CIMT group received casts to restrict the unaffected limb that were left in place for 4 weeks. During this time the CIMT animals were forced to use their affected forelimbs to maneuver around the home cage and the daily rehabilitation device. At the end of the rehabilitation period the casts were removed and all of the animals were tested for ladder walking behavior over a three week period. Interestingly we found that although the CIMT animals went through intensive rehabilitation for 4 weeks, they did not show an

improvement in ladder walking or an increase in axon sprouting in the cervical spinal cord.

Results

CIMT rehabilitation does not improve forelimb stepping behavior. CIMT has widely been used to treat unilateral stroke and more recently applied as an intervention to improve function of impaired limbs following unilateral spinal cord injury (Maier et al., 2008; Bani-Ahmed, 2019). Here we placed plaster casts on the unaffected upper limb of animals in the CIMT group to restrain that forepaw, leaving the affected limb with a full range of motion from the shoulder joint all of the way down to the digits (Fig. 4.1B). This then required those animals to use the affected limb for locomotion in the cage and during rehabilitation training. Intensive forced rehabilitation occurred over a 4-week period, beginning one week post injury (Fig. 4.1A) During this time Control animals were unrestricted and not subjected to supplementary rehabilitation training. After four weeks of CIMT rehabilitation we removed the casts and measured behavior via performance on an irregularly spaced runged wheel for a period of 3 weeks. The wheel is considered irregularly spaced because the rungs can be spaced immediately adjacent as in a regularly spaced wheel or variably spaced (Fig. 4.2A) This variable spacing requires higher precision in limb placement, which further engages the CST (Metz and Whishaw, 2002; Kathe et al., 2014). Control and CIMT groups did not differ in percent correctly placed steps at any time point or show any recovery over time from injury ($p>0.05$ 2-way RM ANOVA Fig. 4.2B,F). This same pattern was repeated with heel steps (Fig. 4.2C,G), toe steps (Fig. 4.2D,H) and missed steps (Fig. 4.2E,I, $p>0.05$ 2-way RM ANOVA for

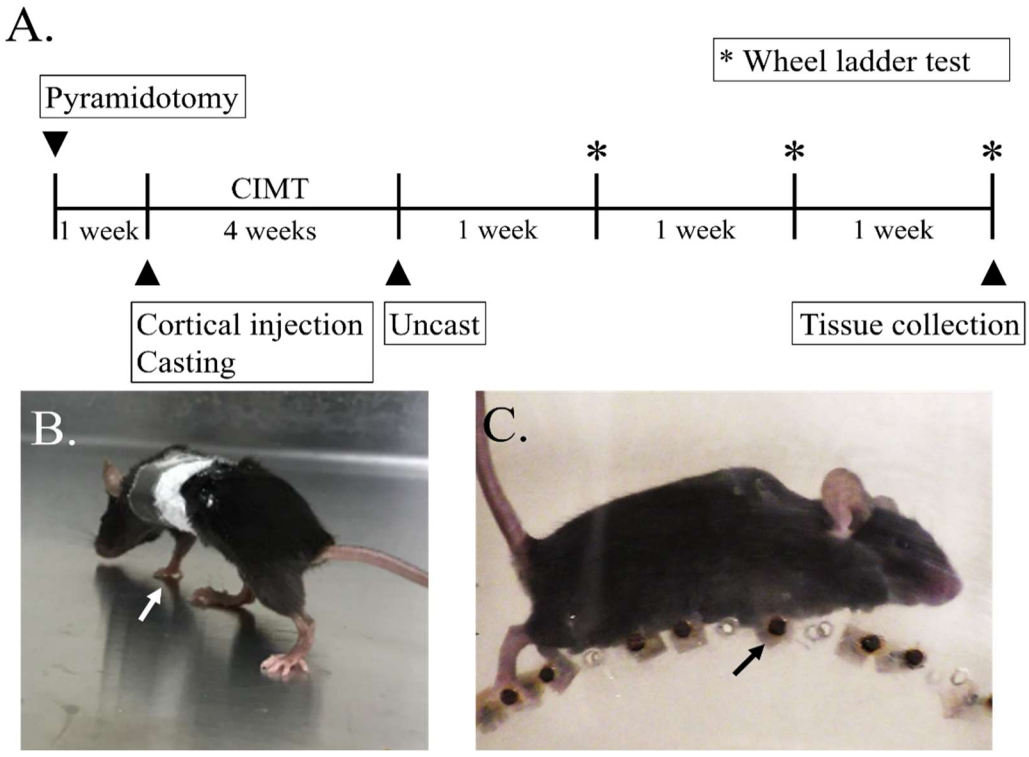


Figure 4.1 Experimental overview for Constraint-induced movement therapy. (A) Experimental timeline including CIMT-on (casting) and -off (uncast) (B) Example of casted animal, affected limb is required for mobility and manipulation (white arrow) (C) Wheel ladder task, animal has forelimb placed correctly on a rung (black arrow).

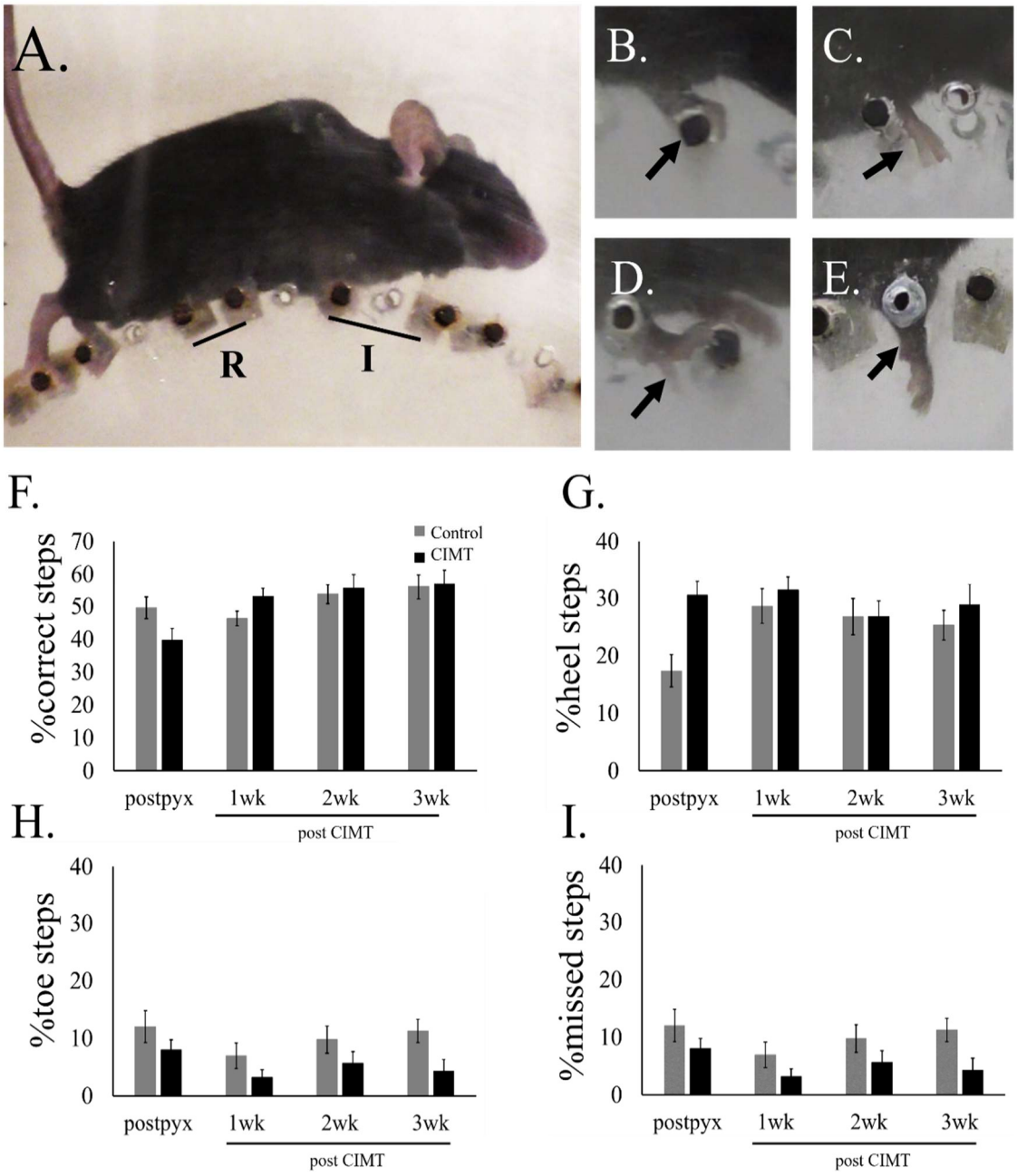


Figure 4.2 CIMT rehabilitation does not lead to differences in any measure of the wheel ladder test. (A) Example image of animal on irregularly spaced wheel "R" shows rungs that are regularly spaced, "I" shows rungs with variable spacing. (B) Correctly placed forepaw, (C) Heel step, (D) Toe step, (E) Missed step where the forepaw has fallen between two rungs, (F) %correct steps post-injury, at 1, 2 and 3 weeks post CIMT show no difference between Control or CIMT or change over time ($p>0.05$, 2-way RM ANOVA), (G) %heel steps show no difference between groups or over post CIMT time ($p>0.05$, 2-way RM ANOVA), (H) %toe steps show no difference between groups or over post CIMT time ($p>0.05$, 2-way RM ANOVA), (I) %missed steps show no difference between groups or over post CIMT time ($p>0.05$, 2-way RM ANOVA) Control N=9, CIMT N=7. Error bars ±SEM.

each step type). These data rule out any compensatory stepping behavior adapted by animals in the CIMT forced rehabilitation group.

Constraint-induced movement therapy does not lead to increased axon sprouting. Previous work has shown that forced use of the affected limb via casting has led to increased neuronal plasticity in the form of axonal sprouting and synaptic formation (Maier et al., 2008; Zhao et al., 2009). To ask if CIMT rehabilitation changed the pattern of axon innervation from the CST into the denervated cord we generated a fiber index. The fiber index is a measurement of axon sprouting in the cervical spinal cord normalized for variability of success in cortical injection(Fig. 2.3C, Liu et al., 2010; Blackmore et al., 2012). An analysis of axon sprouting in animals that had undergone CIMT and controls revealed that there was no significant difference between control and CIMT groups at 200, 400 or 600μm distance measured ($p>0.05$ 2-way ANOVA, Fig. 4.3A,B, Table 4.1).

There is no correlation between stepping behavior and sprouting in CIMT trained animals. We next set to ask whether CST sprouting positively correlated with forelimb function. Given the high levels of variability in fiber index we wanted to probe the possibility if there was a relationship between sprouting and stepping behavior. To do this we plotted terminal stepping behavior (percent correct steps) from the third week post CIMT vs the sprouting fiber index at 400μm. In both the CIMT and control conditions the Pearson's correlation was found to be non-significant ($p>0.05$ Pearson's correlation, Fig. 4.4). This indicates that CST sprouting and forelimb stepping behavior act independently of each other in this experiment, and that one is not predictive of the other.

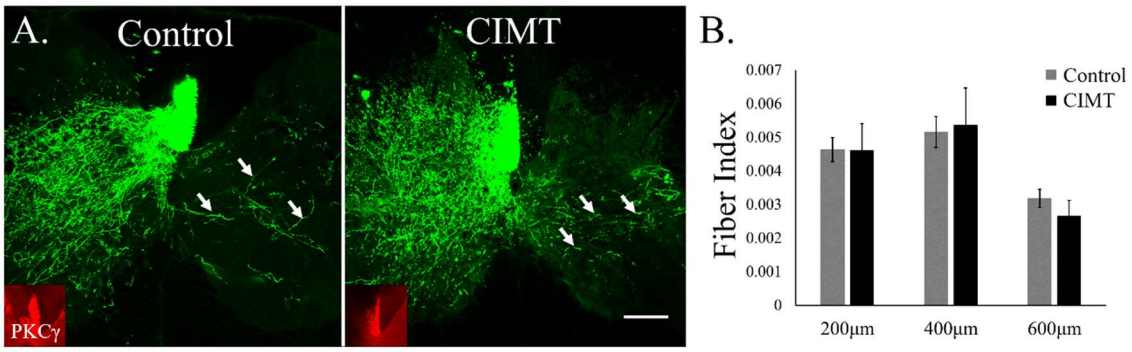


Figure 4.3 Four weeks of CIMT does not lead to a detectable increase in sprouting 8 weeks post injury. (A) Representative images of C3 transverse spinal cord slices CST axon sprouts labeled with EGFP (white arrows) PKCγ IHC confirms injury completeness (red, inset) Scale bar is 200µm (B) Quantification of axon sprouting in cervical spinal cord indicates that there is no difference in sprouting between CIMT rehabilitation and control groups (p>.05 2-way ANOVA) Control N=9, CIMT N=7. Error bars ± SEM.

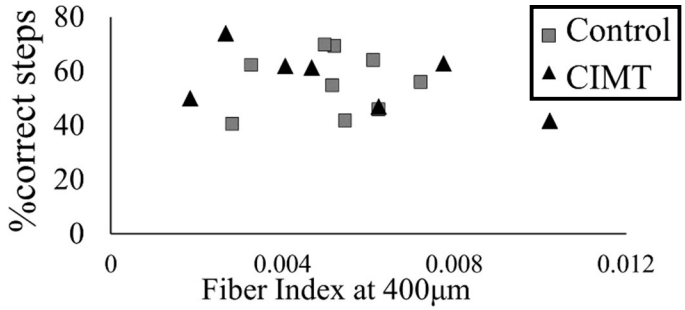


Figure 4.4 Fiber index and terminal wheel walking behavior are not correlated in the Control or CIMT groups. (p>0.05 Pearson's Correlation) Control N=9, CIMT N=7

Discussion

Here we followed a 4-week CIMT protocol that was initiated at 7 days following unilateral CST injury in adult mice. We found that this form of intense forced rehabilitation does not impact behavior in the 3 weeks immediately following the CIMT protocol. CIMT animals did not show any differences in success rate on the irregularly spaced walking wheel, nor did they show any differences in the types of errors that they made, which could have been indicative of compensatory stepping strategies. Finally, there was no difference in the fiber index of these animals, indicating that the rehabilitation did not lead to differences in axon sprouting from the uninjured tract into the denervated side of the cord.

These results were surprising given that a similar study in the CST found full recovery of stepping behavior and increased axon sprouting after three weeks of CIMT protocol (Maier et al., 2008). Other previous studies in stroke models have shown that short term CIMT lasting for a variable period from several days to several weeks maintains lasting behavioral and axon growth effects (DeBow et al., 2003; Ishida et al., 2015). Specifically, one study that used a CIMT model that started 7 days following stroke in the M1 cortex region and continued for three weeks and found a behavioral improvement in a ladder walking task that was accompanied by increases in synaptic markers in the spinal cords of the CIMT group (Zhao et al., 2009).

While CIMT has proven effective in human patients as well as animal models of TBI and stroke, it was not effective in our hands as a rehabilitation model for pyramidotomy of the CST. The time course of this experiment was such that animals received CIMT in weeks 2-5 post injury and were tested for ladder walking behavior on

post injury weeks 6, 7 and 8. In a study published by Lang *et al* 2012 CST remodeling after injury was classified into three time periods, with the later time periods being collateral formation and maturation. The difference between collateral formation at 3-4 weeks and maturation at 12 weeks is the finalization of contacts onto interneuron populations (Lang et al., 2012). In the initial growth phase the CST sends out more collaterals than are necessary, and in an experience-dependent plasticity is able to prune back those collaterals to retain only functional connections.

It is possible that this experiment captured an artificially high number of collaterals that would ultimately not be maintained if the animal had been permitted to survive for 4 more weeks because the axon sprouting from the intact cord had not yet fully pruned back to the mature state. To the point of the fiber index, it is also relevant to point out that the cortical injection was not as successful as in Chapter III (the surgeries were not as optimized) and inclusion criteria for medulla counts were lowered to 800 instead of 1,000 for Chapter IV. This would inherently drive up the fiber index, but not necessarily make it harder to tell the difference between groups.

Another possibility is that the nature of CIMT and forced generalized rehabilitation does not drive behaviors that are specific enough to the CST. This is in contrast to task-based rehabilitation which utilizes goal-based tasks that are completely centered around a specific skill such as manual dexterity or forelimb placement in a stepping task (Krajacic et al., 2010; Fouad and Tetzlaff, 2012). Research using Chondroitinase ABC in a CST injury model to promote spinal cord plasticity also studied the effect of a generalized rehabilitation versus a task-specific rehab and found that only animals that received task-specific training improved on a manual dexterity task over

time (García-Alías et al., 2009). For this reason animals in the CIMT group received "enhanced rehabilitation" by daily exposure to walking on a metal grate, but this was a self-motivated task and may not have engaged the same processes as walking on the irregularly spaced ladder.

A final, technical difference between the Zhao 2009 paper and the work presented here is that the paper is the difference between animal models. The 2009 paper used rats whereas this work used mice. It is possible that the burden of the plaster cast in mice makes it much harder to for them to ambulate normally. If this is the case then rehabilitation will be even more difficult for mice than it would for rats. A recent paper used a modified CIMT model in rats wherein the unaffected limb was restrained for only 2 hours per day using a flexible bandage (Gao et al., 2020). Importantly these animals are under less stress because they are not continuously immobilized, but the animals need to be well handled in order to accommodate this daily restraint without anesthesia. This level of handling would be very difficult to achieve in the mouse, providing further evidence that CIMT or modified CIMT may not be the best approach for this model.

Table 4.1 Fiber Index Values				
Experiment	**Experimental group**	**Distance (μm)**	**Fiber index**	**SEM**
Chapter III Animals	Control	200	0.00464	0.00036
	Control	400	0.00516	0.00046
	Control	600	0.00319	0.00027
	CIMT	200	0.00461	0.00080
	CIMT	400	0.00535	0.00111
	CIMT	600	0.00265	0.00047

Table 4.1 Average fiber index values ± SEM for each experimental group in Chapter III.

Table 4.2 Animal Inclusion Table. Details of points of exclusion in surgery and including histological criteria.

Table 4.2 Animal Inclusion			Died		Excluded		
Experiment	**Experimental group**	**Starting N**	**Pyramidotomy**	**Cortical injection**	**PKCγ**	**Medulla count**	**Final N**
Chapter III Animals	Control	22	6	1	2	4	9
	CIMT	17	6	0	1	3	7

Chapter V

DISCUSSION

Summary

The studies described in this dissertation provide evidence that CST sprouting in the cervical spinal cord is dependent on multiple factors including injury and time to axon growth. Also, axon sprouting behavior is differentially affected by the presence of gene therapy and/or rehabilitative behavior. Studies such as these are directly translational as the life expectancy for an individual who sustains an SCI has increased over time, and modern medicine has yet to find a therapeutic intervention to cure the neuronal damage caused by these injuries (Center, 2019). The CST, a tract involved in fine motor control, is of particular importance to human patients that have sustained cervical level injuries which generally damage this tract and impacts the many functions of the hands and arms.

The CST runs bilaterally down the cord with both main dorsal tracts positioned abutting each other. In the case of incomplete lesions this tract is a good candidate for regeneration studies (Watson et al., 2009). The first study presented here looked at the nature of sprouting from the intact CST in the pyramidotomy model by probing if an injury signal is necessary for growth (Chapter III). The next question was to ask over what time course KLF6 gene-induced axon sprouting into the denervated cord would take place. The second part of that study looked at task-based rehabilitation and gene therapy as employed individually or in combination. The goal of this study was to determine if KLF6 and task-based rehabilitation could work synergistically to support behavior and axon sprouting. Interestingly intensive task-based rehabilitation did not affect behavior or

axon sprouting, whereas KLF6 gene treatment had a consistent effect on axon sprouting but not behavior (Chapter III). This indicates that while KLF6 is sufficient for axon growth, it may not provide high enough levels of sprouting to properly recreate the developing innervation map interneuron circuits. This would limit the ability of KLF6 to impact behavioral improvement following injury.

The second study was a pure rehabilitation-based design. All animals received unilateral pyramidotomies, but only half of them engaged in rehabilitation. Instead of a task-based rehabilitation design, here the animals were in forced rehabilitation (Chapter IV). Constraint-induced movement therapy (CIMT) used here required the placement of a cast to immobilize the unaffected limb, forcing the animal to use the affected limb. This form of rehabilitation requires the animal to use the affected limb for all ambulation within the home cage as well as normal behaviors including manipulation of food pellets and grooming. In addition, we enhanced this forced rehabilitation by placing the animals on a parallel bar grate each day to reinforce the behavior of rung walking by the affected limb, creating an intense level of rehabilitation. CIMT lasted for 4 weeks in these animals, after which casts were removed and their performance on the walking wheel was recorded for three weeks. For reasons explained below we expected that CIMT would improve ladder walking behavior and axon sprouting, but we found that forced rehabilitation alone was not sufficient to impact either of those parameters. The following sections discuss these findings in the framework of SCI and how they may contribute to future work in the field.

1-Promise for regeneration from CST for human populations.

Emerging work from human populations is demonstrating that the majority of spinal cord injuries are incomplete (Ferro et al., 2017; Christiansen and Perez, 2018). Even injuries that have previously been classified as complete based on behavioral measures are actually incomplete in that they have remaining intact descending fibers which hold promise for therapeutic intervention (Kakulas, 1988; Heald et al., 2017). Studies of human patients have shown that canonical rehabilitation in the form of physical therapy and newer therapies such as electrical stimulation have led to rearrangement of remaining circuits, allowing for patients to regain control of muscles after periods of paralysis (Taccola et al., 2018). Together this shows the promise that the spinal cord, and specifically the CST holds for regeneration in human patients, all pointing to the importance of work in animal models.

One benefit of studying the CST in the animal model is the pyramidotomy injury. The ability to unilaterally isolate the tract by severing it at the level of the brainstem moves the effects of inflammation or scarring from the site of regeneration in the spinal cord to the site of the injury (Kathe et al., 2014). This allows for a simplistic approach to studying axon regeneration because confounding inhibitory actions of the glial scar are removed (Lee and Lee, 2013). While this simplicity is a benefit in the animal model, it is important to note that the human SCI will always be more complex. The pyramidotomy model does provide an important starting point to understand the fundamental roles of therapeutics in axon regeneration following injury (Whishaw et al., 1993; Starkey et al., 2005).

CST injuries in mouse models have shown that spontaneous plasticity coming from spared axons is capable of partial recovery of forelimb function by 6 weeks after injury (Hilton et al., 2016). Another study by Weidner *et al* investigated the spontaneous sprouting of the spinal cord by first lesioning the dorsal CST followed 5 weeks later by a lesion of the ventral CST, which abolished the functional gains acquired between the two lesions. This demonstrated that the functional recovery was CST dependent (Weidner et al., 2001). This study highlights the ability for the different components (dorsal, ventral) to sprout in response to ablation of the other, ultimately pointing to a higher level of regulation at the motor cortex. Here we show that AAV-Control injected animals display injury-dependent CST sprouting in a pyramidotomy model. It is interesting and unappreciated how uninjured axons can sense an injury signal and the cell body can respond with protein upregulation to promote growth, especially when the uninjured neurons are spatially distinct from the injured tract (Chapter III). Also studied in Chapter III is the ability of a gene therapy to increase sprouting over a relatively short time course (4 weeks). Gene therapy is an intervention that is slowly becoming available to human populations, and further studies of gene-neuron interactions that can overcome injuries will better inform future clinicians. The abundance of evidence that the CST is capable of spontaneous reorganization and sprouting in response to injury and its ability to respond to genetic intervention in the lab creates a greater platform to argue for the continued study of the CST in regenerative medicine.

2-CST is more than JUST a fine motor tract.

Long thought to be a fine motor tract, the CST has recently been implicated in wider roles of the spinal cord. Emerging evidence has found that this tract has a broader reach, with previously unappreciated roles in integration of proprioceptive signals and processing of somatosensory information. When the dorsal CST exits the column it normally sends axons into the intermediate laminae where it synapses onto interneurons. A recent study found that the CST may have a role in modulating proprioceptive information by activating interneuron networks in laminae II/III. When a population of excitatory cells in this region were knocked out, the animals had a deficit on the ladder walking task (Bourane et al., 2015). Another study classified 43 unique neuron populations within the hindlimb region of the spinal cord based on RNA-seq data and found that 9 of them were active during locomotive behavior. Interestingly these cell clusters reached from the dorsal to ventral horns, further implicating interneurons that receive proprioceptive inputs as being involved in stepping behavior (Sathyamurthy et al., 2018). These studies work together to support the idea that no pathway is truly independent, and that the nature of signaling through interneurons logically leads to the integration of systems such as the proprioceptive and the corticospinal.

To restore function the CST needs to properly synapse onto interneurons that will ultimately make contact with motoneurons. Additionally, the neurons that have made these new sprouts need to know when to fire. This is likely a dynamic learning process for CST-driven circuit that is modified by the proprioceptive inputs. As the newly sprouted axons make contacts, and the animal is behaving the receptive information will be fed back into the spinal cord. Given that between CST inputs and motoneuron outputs

there are a series of converging signals from different cell types at the level of interneurons, it is likely that proprioceptive and other sensory input received by the dorsal horn will modulate the signal in the motoneuron. In injury the CST inputs are lost and eventually replaced by axon sprouts from a different cell, but the remaining modulatory signaling in the pathway should still be in place. This would allow for the CST to reintegrate into a system that was developed around this cortical input, potentially allowing for optimal reintegration.

At the level of the cell firing and controlling contacts on both sides of the spinal cord, when a cell fires it is an all or none decision at the level of the axon hillock. If the cell fires, as myelinated fibers all of the axon sprouts should also fire. Because the CST signaling to the motoneuron is not direct and the motoneuron receives converging signals, modulation of the cortical input is always going to take place. It makes sense that modulation of the CST signal to the motoneuron will decide if a firing axon results in motor output. This is likely not only in injury models where uninjured CST is sprouting to an injured side of the spinal cord, but also in the intact cord where pools of premotor interneurons involved in opposing flexor and extensor actions are found together in a transverse plane (Wang et al., 2017). In the intact case it is likely that the same neuron sends axon terminals to multiple types of premotor interneurons, but that modulatory signaling governs ultimate motor output. The next logical step for this research is to look at the interneuron network between the CST to motoneurons and to characterize the other converging inputs that are modulating the cortical input.

3-Effective rehabilitation is critical for the human population.

Physical rehabilitation is a mainstay of treatment for any individual who has suffered a neurotraumatic event such as TBI, stroke and SCI, (Lifshutz and Colohan, 2004). Rehabilitation alone has rarely been able to overcome the devastating effects of SCI, and studies in human patients rarely report meaningful gains of function (El Tecle et al., 2018). This puts the burden of research on the academic community. There are two larger philosophies of rehabilitation: general/global rehab and task-specific rehabilitation. With general/global rehabilitation the theory is that by engaging all of the muscles the individual will regain the functions normally performed by those muscles. Task-specific rehabilitation is a goal-oriented specialized rehab design that uses repetition to reinforce a specific behavior (Bayona et al., 2005). There are conflicting studies as to the efficacy of each of these forms of rehabilitative training. One study had two groups of animals following unilateral pyramidotomy; one group was trained on single pellet grasping and the other on the ladder task. At the end of the study both groups were tested on single pellet, ladder walking and a novel staircase task. They found that the animals that received the more generalized ladder walking task fully recovered their own task and performed better on the novel staircase task, even through that task is arguably closer in function to the single pellet retrieval (Starkey et al., 2011). In contrast a study that looked at task-specific paw reaching or general in cage locomotor rehabilitation following a CST injury found that the animals that trained in the paw reaching preformed better in the staircase task (García-Alías et al., 2009). Clearly this is an area of research that needs more clarification, and perhaps the distinction of task-specific and generalized locomotor

are not as different as scientists would like to believe, and slight nuances can lead to very different results.

In Chapter III we attempted to provide both task-specific training with the Montoya staircase and a generalized training by having the animals cross the grate to retrieve pellets. The fact that after 10 weeks of rehabilitation we didn't see any improvement in the AAV-KLF6 group in either the skilled or the generalized stepping task was a surprise given our experimental design. One group was able to show in a stroke model that the combination of environmental enrichment plus reaching behavior led to the greatest recovery, but the reaching task was in the form of a single-pellet retrieval that engaged the rats for approximately 6hrs/day (Jeffers and Corbett, 2018). To put this in context, this is 12x longer than the Chapter III daily staircase task. To achieve this level of rehabilitation in the rodent options such as the automated single pellet retrieval robot need to be employed so that the animals can naturally retrieve the pellets as they would eat them on their endogenous nocturnal cycle (Fenrich et al., 2015; Torres-Espín et al., 2018b).

An alternative to automated rehabilitation interventions are combined intervention systems. Emerging evidence is pointing to electrical stimulation as a means of engaging spared axons or even activating regions below the level of injury (Hamid and Hayek, 2008). Electrical stimulation in combination with treadmill walking has been shown to help facilitate coordinated movement in humans who have been unable to ambulate in such a way for an extended period of time (Field-Fote and Tepavac, 2002). Additionally, one of the task-based studies explained above showed that Chondroitinase ABC treatment is able to extend the window for the efficacy of rehabilitative treatment

(García-Alías et al., 2009). A recent study has shown that application of LPS to induce a mild inflammatory response 8 weeks post injury was sufficient to reopen the rehabilitation window (Torres-Espín et al., 2018a). Studies such as these provide evidence that maybe rehabilitation needs to be rethought. More effective forms of rehabilitation might not mean working longer, with additive experiments, but working smarter and integrating multiple concurrent systems to make the most gains in the shortest amount of time, which is more practical to the human condition.

4-Use of gene therapy as a combinatorial design, a molecular "on" and "off" approach.

Here we employed the use of a single gene, KLF6 to promote CST axon sprouting. As previously published, KLF6 was able to induce axon sprouting (Wang et al., 2018). Here we found that sprouting across the midline was injury-dependent and that occurs within 4 weeks of injury. In the Wang *et al* 2018 paper KLF6 did not have an effect on horizontal ladder behavior. Here we added physical rehabilitation to KLF6 gene therapy with the idea that motor activity would guide the growing axon collaterals towards the appropriate interneurons for ultimate signaling on the correct motoneurons. The idea that single or even double gene therapy can induce neuronal or neurite growth has been tested over time and several genes have been identified as regulators of growth in mature neurons including PTEN, Nogo, Sox11, KLF7 and KLF6 (Liu et al., 2010; Blackmore et al., 2012; Geoffroy et al., 2015; Wang et al., 2018).

Conceptually if the goal is to get a spared axon to sprout and "take over" newly denervated tissue, there are many steps in this process. The axon(s) needs to know that it has to start sprouting, and it needs to know where spatially to do that. Next within the

gray matter the axon needs to know in general where to go and then to send out exploratory potential synaptic contacts from there. If these new contacts are reinforced by a proprioceptive or sensory motor signals then the appropriate contacts will mature and the excessive inappropriate collaterals will prune back, leaving a finalized highly reorganized network. That is a lot of requirements that are now being put on gene therapy. This leads to the idea that a combined gene, or even combined molecular treatment approach may be the best means of achieving appropriate innervation of newly growing axons following injury. One way to approach this multifactorial experiment would be to maintain temporal regulation of gene expression. This would allow for genes to be expressed as they are functionally needed to promote axon elongation, synapse formation and integration/strengthening. It would also allow the experimenter to turn off genes such as KLF6 which may prove detrimental to the maturation of sprouted axons. This concept of temporal regulation of gene expression could be expanded to temporal regulation of all aspects of rehabilitation including gene therapy, stimulation, behavior etc.

Wang *et al* used RNA-seq to study the genes that are upregulated when KLF6 is overexpressed. Unsurprisingly they fall into classes including regulation of actin filament polymerization, cholesterol biosynthesis and bioenergetics, all of which are components critical for axon growth (Wang et al., 2018). One example of the complexity of axonal growth is in the final stage of CST development: synaptogenesis. This is a dynamic process with three broad steps: 1-extensive branching of the CST to generate many putative synaptic contacts, 2-experience-dependent plasticity to facilitate the decision of which synapses will be maintained, 3-the pruning back of collaterals from contacts that

are not in the mature CST pathway. Each of these steps likely requires activation of different genes (Venkatesh and Blackmore, 2017). Potentially this process could be achieved in part by application of trophic factors as well as multiple gene therapies that are precisely timed to optimize the steps of sprouting and regeneration. The one gene approach has begun the process of axon growth, but combinatorial therapies hold a much greater potential for maximizing the effects of regeneration.

Conclusion

The circuitry that connects the brain to the arm or the hand is highly complex. Not only is there a connection between the CNS and the PNS, but the relay that originates in layer V of the motor cortex and becomes the CST is also well defined. Although this has been a known medical conundrum for centuries, there is still no therapeutic that can reverse the damage from an SCI. Here we report that a known gene therapy, KLF6, will only induce sprouting in the presence of an injury signal. This is pertinent in that it means that KLF6 gene therapy likely does not lead to aberrant neurite growth. Also, by probing the time course over which KLF6 sprouts, significant sprouting was found at 4 weeks post injury. This information is relevant to the design of combinatorial studies that may involve conditional expression of other genes, trophic factors or the use of rehabilitative therapy.

This work also looked a complex rehabilitation design wherein the animals were either control or KLF6 treated, all received pyramidotomies and those in the rehabilitation group received both a task-based pellet retrieval and a generalized rung walking rehabilitation for 10 weeks. By the end of this time the rehabilitation trained animals performed no differently from the controls, but KLF6 did persist in promoting axon sprouting. The next step is to reevaluate the methodology and timing of rehabilitation. Over time rehabilitation has proven an effective tool in treating neuronal trauma, but it is constantly revamped. The data presented here provide more information about how and when to time rehabilitative interventions surrounding axonal sprouting, and multiple rehabilitation designs that could be reworked in order to produces positive results.

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