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Preferential Regeneration of Hindlimb Corticospinal Axons into a Neural Progenitor Cell Graft After Cervical Spinal Cord Injury

A thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Biology

by

Daniel Petrovich Kulinich

Committee in charge:

Professor Mark H. Tuszynski, Chair
Professor Stefan Leutgeb, Co-Chair
Professor Nick Spitzer

2018

The thesis of Daniel Petrovich Kulinich is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-Chair

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Chair

University of California San Diego

2018

DEDICATION

I would like to dedicate this to my parents for supporting me through my academic pursuits. I would also like to dedicate this to my supervisors: Dr. Paul Lu, and Dr. Mark Tuszynski, for mentoring, teaching, and giving me an opportunity to carry out such fascinating research with them.

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ABSTRACT OF THE THESIS

Preferential Regeneration of Hindlimb Corticospinal Axons into a Neural Progenitor Cell Graft After Cervical Spinal Cord Injury

by

Daniel Petrovich Kulinich

Master of Science in Biology

University of California San Diego, 2018

Professor Mark Tuszynski, Chair

Professor Stefan Leutgeb, Co-Chair

An estimated 300,000 people suffer from spinal cord injury (SCI) in the US, costing about $9.7 billion annually (French et al., 2007). Quadriplegic patients, comprising nearly 60% of SCI cases (National Spinal Cord Injury Statistics Center), list the loss of motor control, especially hand function, as the primary impediment to their daily lives. To overcome this loss, it is necessary for the corticospinal tract (CST) – the main tract controlling voluntary motor movement – to regenerate and reform functional synapses. One method to reverse such dysfunctions is to use neural progenitor cell (NPC) grafts which recapitulate lost neural tissue and promote axon regeneration, ultimately serving as a relay for neural signals.

Previously, our lab demonstrated functional recovery and robust CST regeneration into rat embryonic day 14 (E14) NPC grafts placed into cervical and thoracic SCI sites (Kadoya et al., 2016). CST regeneration into a cervical graft has the potential to restore voluntary motor function, especially hand function, for quadriplegic patients. However, whether forelimb, hindlimb or both CST populations regenerate into a cervically placed NPC graft, is unknown. Because both populations are axotomized, we hypothesized that both forelimb and hindlimb projecting CST axons would regenerate into a mid-cervically placed NPC graft. Here, we used a Cre-mediated intersectional viral approach to selectively label either forelimb or hindlimb projecting CST axons. All rats were then subjected to a C3 dorsal column lesion and received an E14 spinal cord-derived NPC graft. Surprisingly, we found preferential regeneration of hindlimb CST axons into the C3 NPC grafts, with sparse forelimb axon regeneration. This creates a functional mismatch: hypothetically, to achieve optimal functional recovery, CST axons controlling the forelimbs instead of the hindlimbs would regenerate into the lesion to form functional synapses. An examination

of mechanisms underlying this selective regeneration is necessary, and will contribute to optimizing corticospinal regeneration after SCI.

CHAPTER 1: SPINAL CORD INJURY

1.1: INTRODUCTION

SCI Epidemiology

Spinal cord injury (SCI) affects 15-40 people per million, resulting in mostly irreversible functional deficits and large financial burdens (Rowland et al., 2008). The level of injury determines the extent of the functional deficit since the original projection neurons are severed. Thus, motor synapses below, and sensory synapses above the lesion become dysfunctional. SCI is largely irreversible due to the inability of the central nervous system (CNS) to regenerate after injury (Tuszynski & Steward, 2012; Cregg et al., 2014). After incomplete spinal cord lesions, intact neurons can sprout new axonal branches resulting in partial recovery of various autonomic, sensory and motor functions (Weidner et al., 2001; Curt et al., 2008; Collyer et al., 2014). Nevertheless, extensive regeneration – growth from the axotomized tip (Tuszynski & Steward, 2012) – and subsequent recovery of original motor function is the overarching goal of spinal cord injury research (Rowland et al., 2008; McDonald & Becker, 2012). The lack of functional regeneration is important to overcome, especially for quadriplegic patients who lose hand function, comprising nearly 60% of SCI injury cases (National SCI Statistics Center). To develop therapies that help promote neural regeneration and functional reconstitution, much work has focused upon elucidating the complex mechanisms associated with axonal growth failure after injury.

Spinal Cord Injury Mechanisms

Regenerative failure is attributed to a several injury mechanisms including: (1) glial scar formation (Qui et al., 2000; Fitch & Silver 2008; Cregg et al., 2014), (2) inhibitory extracellular and myelin associated molecules (Huang et al., 1999; Mckerracher and Winton 2003; Fitch & Silver 2008), (3) lack of a growth permissive cellular substrate for regenerating axons to attach and grow in the injury site (Maxwell, 1997; Schwab, 2002), (4) lack of neurotrophic stimulation (Mitsui et al., 2005; Lu and Tuszynski, 2008) and (5) loss of an intrinsic regenerative program (Liu et al., 2011; Blesch et al., 2011; Apara & Goldberg, 2014; Bei & He, 2016).

Primary vs Secondary Injury Phases

Primary SCI involves mechanical force from a trauma that injures the neural parenchyma and vertebra. Secondary SCI refers to the multiple phases and both intracellular and extracellular mechanisms of injury following primary injury. Both immediate and early acute phase (0 - 48 hours) result in cell death through several mechanisms that induce inflammation, apoptosis, and necrosis of surrounding tissue. The subacute phase (2 days - 2 weeks) is characterized by hypertrophy of astrocytic glia at the exposed or injured spinal cord; this mechanism is hypothesized to protect the intact spinal cord from further damage (Fitch & Silver 2008; Anderson et al., 2016). The accumulated astrocytes grow by increasing production of glial fibrillary acidic protein (GFAP) and secrete chondroitin sulfate proteoglycans (CSPGs); both factors serve as physical and chemical barriers to regenerating axons (Bradbury et al., 2002; Lang et al., 2015). More so, oligodendrocytes – cells in the CNS that produce myelin, lipid layer sheath's that

increase speed of action potential propagation – contain a class of transmembrane receptor molecules (e.g. Nogo's) that likewise restrict axons from regenerating (Hunt et al., 2002, Schwab, 2014). The intermediate phase (2 weeks – 6 months) is characterized by the continued glial scar development and compensatory sprouting of intact axons that help partially regain basic motor and sensory functions. Finally, during the chronic phase (> 6 months), the glial scar stabilizes and cyst formation within the lesion cavity is observed (Rowland et al., 2008).

Therapies Targeting Intrinsic and Extrinsic Inhibitors of Regeneration

Preventing or reversing injury mechanisms is the focus of many current SCI therapies (Cadotte & Fehlings 2010; McDonald and Becker, 2012). Yet, effective clinical therapies for regeneration, and reconstitution of original synapses and functions have not yet been developed (Rowland et al., 2008; Tuszynski & Steward 2012). A possible reason is that current therapies focus on individual components in context of much more multifaceted injury mechanisms. Extracellular inhibitor target therapies including: antibodies against Nogos (Schwab, 2004) and enzymes used to degrade CSPGs, are limited in their ability to promote regeneration into or past the lesion cavity. Similarly, targeting intracellular factors such as phosphatase and tensin homolog (PTEN), a mammalian target of rapamycin (mTOR) inhibitor and tumor suppressor, render similar results (Liu et al., 2010). Upon further analysis, most of the functional recovery is due to rostral-to-injury sprouting, as opposed to true regeneration of the axotomized tip (Hunt et al., 2002; Fouad et al., 2004). Since the amount of true regeneration from such individual approaches has been minimal, utilizing a combination of therapies, which include embedding a growth

permissive substrate into the lesion cavity, is preferable (Tuszynski & Lu 2008; Blesch & Tuszynski, 2009)

Therapies to Fill the Spinal Cord Lesion Cavity

Apart from the inhibitory signaling mechanisms of myelin and glial scar, injury to the CNS results in a cavity that is not permissive for cell survival or regeneration (Maxwell, 1997; Schwab, 2002). Unlike oligodendrocytes in the CNS, myelin-producing Schwann cells of the peripheral nervous system (PNS) secrete a growth permissive matrix to promote regeneration of the peripheral nerve (Schwab et al., 1996). Therefore, most SCI combinatorial therapies utilize placing molecular, cellular or biosynthetic bridges in the lesion cavity (Tuszynski and Lu 2008, Fehlings and Vawada, 2011). Previous CNS regenerative approaches included: fibroblasts (Tuszynski et al., 1994), mesenchymal stem cell therapy (Lu et al., 2005), oligodendrocyte progenitor cells (Keirstead et al., 2005), peripheral nerve grafts (David & Aguayo, 1981), as well as biomaterial scaffolds (Stokols & Tuszynski, 2006; Kim et al., 2014); while showing promising results, these methods often fail to produce true long-distance regeneration and significant functional recovery (Tetzlaff et al., 2011; Tuszynski & Steward, 2012; Mothe & Tator, 2018). Intuitively, grafts not homologous to intact neural spinal cord tissue may attribute to the limits of such therapies. One solution is to utilize embryonically derived neural progenitor cells (NPC), which can multiply and differentiate into neurons and glia (Suda 1987; Karimi-Abdolrezaee et al., 2006). Developing embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs) derived NPC transplantation is a promising next step forward; rodent, primate and even human studies using NPC grafts have shown promising recovery

(Iwanami et al., 2005; Fehlings, Vawda, 2011; Lu et al., 2014, 2017; Vroemen et al., 2003; Tsuji et al 2010)

Stem Cell Review

Embryonic stem cells (ESCs) self-renew and eventually differentiate into somatic tissue of the adult organism (Murry and Keller, 2008). Depending on the developmental stage, stem cells become more restricted for the type of tissue they can form. While more differentiated than embryonic stem cells, NPCs are still self-renewing and neurally multipotent – can form most neural tissue cells (neurons and glia, excluding microglia) (Gage, 2000; Walker et al., 2016). NPCs form early neurons that inherently grow, make connections, and extend axons (Han et al., 2002). Thus, besides a growth-permissive environment for regenerating host axons, NPC grafts can form neuronal relays between the lesioned projection axons and their respective caudal-to-lesion denervated spinal cord neurons (Bonner and Steward, 2015; Lu et al., 2012, 2014).

Methods for Neural Progenitor Cell Generation

Obtaining NPC's for grafts can be achieved through different methods: directly harvesting NPCs from embryonic or fetal CNS tissues, differentiating cultured ESCs towards a neural fate (Reubinoff et al., 2000), or deriving NPCs from de-differentiated adult somatic cells into induced pluripotent stem cells (iPSCs), made by introduction of reprogramming molecules into terminally differentiated adult cell types (Takahashi & Yamanaka, 2006). Directly harvesting NPC from embryonic or fetal CNS tissues is a consistent and simple method for generating NPC grafts. While there are ethical concerns

with this method moving into human clinical trials, such trials have been performed for spinal cord injury (Wirth et al., 2016; Manley et al., 2017), Parkinson's disease, and Huntington's disease. iPSC derived NPC transplantation provides a more clinically relevant method, yet is currently lacking in safety, since prevailing protocols often result in tumor formation (Erceg et al., 2009).

Neural Progenitor Cell Grafts for Spinal Cord Injury

To be viable solutions for spinal cord injury, NPC grafts need to achieve the following: (1) cell survivability upon transplantation, (2) successful integration into the lesion cavity, (3) differentiate into functional neurons and glia without (4) developing intra-vertebral pressure from extensive proliferation (Lu et al., 2008). Furthermore, to reconstitute original function, NPC grafts need to help overcome the (1) inhibitory glial barrier, (2) provide a regeneration permissive environment to the lesioned axons, and (3) establish functional relays between host axons (Bonner and Steward 2015; Lu et al., 2003, 2008, 2012).

For a long time, NPC grafts failed to survive in a fully transected spinal cord model, the most rigorous model to establish regenerative ability (Tuszynski and Steward, 2012). Recently, embryonic rat and human NPCs combined with a structurally supportive fibrin matrix, nine growth factors, and one anti-cell death agent, were grafted into fully transected, sub-acutely injured rodent model. Upon examination, the graft successfully survived, promoted extensive host and graft axon regeneration and growth, remyelinated injured axons, and formed functional neuronal relays (Lu et al., 2012). In a subsequent study, the number of growth factors in a supportive fibrin matrix was reduced to a more

clinically practical number of four, while retaining extensive NPC survival and fill of the spinal cord lesion (Robinson & Lu, 2017).

Although general host regeneration was observed (Lu et al., 2012), there are many ascending and descending tracts within the spinal cord that could result in regaining of motor function (Fillini and Schwab, 2015), and are known to have a differential regenerative ability (He et al., 2011). For SCI patients, fine voluntary motor function loss is often the main impediment to daily life, especially for quadriplegic patients, whose hand function is impaired (Nathan et al 1994; Rowland et al 2008). Therefore, the regenerative ability of the corticospinal tract (CST) -- main tract responsible for voluntary motor movement-- is important to consider.

Corticospinal Tract Development and Regeneration

CST neurons originate in layer V of the cortex and project most of their axons contralaterally to innervate mainly cervical and lumbar spinal cord to control forelimb and hindlimb muscles, respectively (Kamiyama et al., 2015). If a SCI severs either or both CST sides, motor innervation to motor neurons below the injury will no longer be functional, resulting in the loss of fine voluntary motor function. While different spinal cord tracts have differential regenerative ability, the CST is largely refractory and retractive after injury (He et al, 2011). However, CST neurons do have the ability to sprout rostral to injury, more-so with different pro-regenerative strategies. Rat fibroblasts, genetically modified to secrete neurotrophin-3 (NT-3) increased both intact and injured CST fibers sprouting into the intact gray matter, but no significant change in penetration of the fibroblast graft compared to control (Grill et al., 1997). Targeting the mTOR suppressor, PTEN, primarily

increased CST sprouting but resulted in very little regeneration through lesion site (Liu et al., 2010). Similarly, targeting Nogo (Schwab 2004, Zheng 2005) or degrading CSPGs (Wang et al., 2011; Starkey et al., 2012), increase regenerative pathways that primarily lead to compensatory sprouting and not true regeneration.

Although, in the presence of NPCs within a fibrin matrix graft and growth factors (Lu et al., 2014; Robinson & Lu, 2017) placed into a SCI lesion site, severed CST neurons were able to extensively regenerate, and form functional synaptic relays between graft neurons and denervated neurons caudal to injury (Kadoya et al., 2016). In this study, Kadoya et al., showed that contact between the CST and NPC graft was necessary for regeneration; providing a possible regenerative mechanism mediated via cell adhesion pathways. Further studies must be carried out in to better understand this mechanism.

CHAPTER 2: EXPERIMENTAL DESIGN, METHODS AND RESULTS

2.1: EXPERIMENTAL DESIGN

Hypothesis

Although Kadoya et al., observed extensive CST regeneration into an NPC graft placed in a SCI lesion, detailed analysis showed that not all CST axons regenerated into the NPC graft. In this experiment, we hypothesized that both forelimb and hindlimb innervating CSTs have equal regenerative capacity in the presence of a NPC graft (Lu et al., 2014; Robinson & Lu, 2017), since both systems are axotomized thus exposing all axotomized tips to the same environment.

Experiment

Regenerative ability between forelimb and hindlimb CST populations becomes consequential when it comes to treating cervical lesions. Specifically, to achieve reconstitution of forelimb function, regeneration and functional relay formation should occur between forelimb CSTs and caudal-to-injury cervical gray matter that contain motor neurons controlling forelimb muscles. To test this, we injured the rat dorsal column, which contains the rat's main CST tract, and engrafted NPC with fibrin matrix and four growth factor cocktail (Lu et al., 2014; Robinson & Lu, 2014), assessing corticospinal regeneration using an inter-sectional viral approach to specifically trace forelimb or hindlimb CST. Our results show preferential regeneration of hindlimb CSTs in a cervical NPC graft.

2.2: METHODS

Animals

Adult female Fischer 344 rats were used in this study (100-200g, n=14). The National Institutes of Health guidelines for laboratory animal care and safety were followed. The Institutional Animal Care and Use Committee of the Department of Veterans Affairs (VA) San Diego Healthcare System approved all animal surgeries under the protocol used. Animals had free access to food and water throughout the study and health checks were performed daily. All surgeries were performed under deep anesthesia using a combination (2 mL/kg) of ketamine (25 mg/mL), xylazine (1.3 g/mL) and acepromazine (0.25 mg/mL). Animals were given post-operative injections of lactated ringers solution, banamine, and ampicillin for three days following surgery (3ml/day).

NPC Preparation

Embryonic day 14 (E14) spinal cords from transgenic Fischer 344-Tg (EGFP) rats, ubiquitously expressing GFP under the ubiquitin C promotor, provided donor tissue used for grafting in this experiment (Rat Resource and Research Center, University of Missouri, Columbia, MO). E14 spinal cords were dissected and dissociated using the same methods followed in Lu et al., 2014. Dissociated E14 cells were resuspended at a concentration of 250,000 cells/µL in a fibrin matrix (25 mg/mL fibrinogen and 25 mg/mL thrombin Sigma-Aldrich) containing a four growth factor cocktail consisting of brain-derived neurotrophic factor (BDNF), basic-fibroblastic growth factor (bFGF), vascular endothelial growth factor

(VEGF) and MDL28170, a cell death inhibitor, to support graft survival (Robinson and Lu, 2017).

Virus Injection, Lesion and Transplantation Surgeries

Two groups of adult female Fischer rats (n=7 each) had Cre dependent AAVDJ-FLEX-ArchT-tdTomato virus ($1x10^{12}$ transducing units/mL, Salk Institute, La Jolla, CA) injected into either forelimb or hindlimb motor cortex (Figure 1). Immediately after the first injection, AAV9-CamKII-Cre virus ($1.2X10^{13}$ transducing units/mL) was injected into the C6 (forelimb group, n=7) or L3 (hindlimb group, n=7) (Figure 1) of the spinal cord. Specifically, 2.1 µl virus was injected in 7 sites per primary forelimb motor hemispheres (0.300µL/site); coordinates followed: anterior-posterior(mm)/medial-lateral(mm) (0.27/±0.25; 0.17/±0.25, ±0.35; 0.07/±0.25, ±0.35; -0.03/±0.25, ±0.35) for left (+mm) and right (-mm) hemispheres, respectfully. Coordinates for 8 hindlimb injections sites followed: anterior-posterior(mm)/medial-lateral(mm) (0.02/±0.27, ±0.37; -0.08/±0.17,±0.27; -0.18/±0.19, ±0.29; -0.28/±0.19, ±0.29) for left (+mm) and right (-mm) hemispheres, respectfully. All injections were done dorsal/ventrally at 1.2mm. For spinal cord injection, 1 µL of AAV-Cre was injected into 4 points per hemisphere at following coordination: medial-lateral (mm)/dorsal-ventral (mm) (0.8/1; 0.8/0.5; 1.2/1; 1.2/0.5).

After a period of 3 (forelimb group) or 4 weeks (hindlimb group) post-viral injection, a bilateral dorsal column (DC) wire-knife lesion was performed to transect the main dorsal column CST tract. Specifically, after preforming a laminectomy at C3, the wire-knife was stereotaxically positioned 0.6mm left of the spinal cord central axis. Next, the knife was lowered by 1.2 mm from the dorsal surface into spinal cord and the knife tip

was extruded out to form a 2.25 mm-wide wire knife hook. The whole knife was then stereotaxically raised up toward the dorsal surface. To ensure the complete lesion of the axons above the knife hook, a glass pipet was used to carefully depress the white matter until the dorsal column white matter was completely transected. Finally, the wire-knife was lowered back to the original 1.2mm depth, the tip was retracted, and the knife was removed out of the spinal cord. This procedure bilaterally axotomized the afferent dorsal column sensory axons as well as the efferent dorsal corticospinal axons, ~98% of total CST axons (Weidner et al., 1999).

Four days post bilateral dorsal column lesion, both groups received 1.5µL microinjections of the NPC cell suspension (250,000 cells/µL in a fibrinogen/thrombin matrix containing 4-factor cocktail) into the C3 lesion cavity through a pulled glass micropipette using a PicoSpritzer II (General Valve Inc., Fairfield, New Jersey). All rats were perfused with 4% PFA and CNS tissue was harvested 6 weeks post-grafting.

Histology and Immunohistochemistry

All spinal cords were blocked 3 mm caudal and 3 mm rostral to graft site and sectioned into 30µm sagittal sections on a cryostat. Sections were stored in a 24 well plate in TCS solution. 1-in-6 series, free-floating sections were incubated for 3 days at 4C with primary antibodies from chicken against GFP (at 1:1000 to label grafted cells), from rabbit against RFP (at 1:1000 to label CST axons), or from mouse against Cre (1:1000 to label cell bodies, Figure 2a). Sections were then incubated in Alexa 488 (anti-chicken, 1:500) and 594 (anti-rabbit, 1:500) conjugated donkey secondary antibodies for one day at 4C. Confocal and Keyence BZ-X700 microscopy was performed to analyze CST regeneration.

Cortical sections were cut at 40 µm coronal sections on the cryostat and were similarly labeled for RFP and NeuN (anti-mouse, 1:250) (Figure 2b).

Quantification

Images of CST-labeled medio-sagittal sections were captured using Z-Stack and XY Stitch function on the Keyence BZ-X700 (Keyence, Woodcliff Lake, NJ) at ×200 magnification. The raw images were merged and stitched using Keyence Analysis Software (Keyence, Woodcliff Lake, NJ). Average CST axon density was calculated by dividing sum-total thresholded pixel RFP area over sum-total graft pixel area for forelimb (n=7) and hindlimb (n=7) animals, using ImageJ software (National Institute of Health).

2.3 RESULTS

Cre-recombinase injections, retrograde transport and activation of Cre-dependent Td-Tomato for forelimb and hindlimb CST

We used an intersectional viral approach to specifically label either forelimb or hindlimb CST neurons. Cre-dependent AAVDJ-FLEX-ArchT-tdTomato virus was injected either in the forelimb or hindlimb motor cortices; subsequently, AAV9-Cre was injected into C6 or L3 spinal cord which would be up-taken by the CST axon terminals and retrogradely transported to the cell body to forelimb and hindlimb CSTs, respectively (Wang et al., 2017). A sagittal section of the C6 spinal cord gray matter shows successful Cre-virus transduction (Figure 2a). A high-magnification overlay image shows successful activation of the Cre-dependent td-Tomato virus in Layer V of the motor cortex, as evidenced by the co-label of the RFP and NeuN – adult neuronal marker (Figure 2b). Transverse sections labeled for RFP at level C1 of the spinal cord, show successful labeling of the main CST at dorsal column (Figure 2c,d). Hindlimb CST is more concentrated towards the medial ventral dorsal column and identification is further evident with the relatively low amount of gray matter innervation (Figure 2c). Forelimb CST is more dispersed throughout the ventral dorsal column, and heavily innervates the C1 gray matter (Figure 2d).

Reduced regeneration of forelimb CST into C3 NPC graft.

Cre-dependent, anterograde AAVDJ-FLEX-ArchT-tdTomato virus was injected into rat bilateral forelimb motor cortices followed by AAV9-Cre injection into C6 spinal

cord; this allowed for visualization and analysis forelimb CST axon regeneration into GFP labeled, E14 derived, NPC graft placed in lesioned C3 rat spinal cord (Figure 3a-d). Low-magnification overlay image of a sagittal spinal cord section (Figure 3a), and without the GFP channel (Figure 3b). Host-graft interface is located at the left edge of the green fluorescent NPC graft (Figure 3a) and is marked by the dashed white lines (Figures 3b,d). The inset on Figure 3a outlines the magnified area represented in Figures 3c,d. The uninterrupted interface indicates successful NPC engraftment into the lesion site, which is a pre-requirement for CST regeneration (Kadoya et al. 2016). Following a rostral (left)-caudal (right) orientation (Figure 3), RFP labeled forelimb CST axons (RFP) approach and interface with the graft; however, higher magnification confocal images show sparse regeneration of CST axons through the host-graft interface (Figure 3d).

Robust regeneration of hindlimb CST into C3 NPC graft.

Injection of AAVDJ-FLEX-ArchT-tdTomato into rat bilateral hindlimb motor cortex, followed by AAV9-Cre injection into L3 segment, allowed for visualization and analysis of hindlimb CST regeneration into GFP labeled, E14 derived NPC graft in lesioned C3 segment of the rat spinal cord (Figure 4a-d). As in Figure 3, rostral host-graft cell interface is located at the left edge of the green fluorescent NPC graft (Figure 4a) and is marked by the dashed white lines (Figures 4b,d). The uninterrupted interface shows successful NPC engraftment into the lesion site required for CST regeneration. High-magnification confocal images indicate extensive host-graft interface penetration of the regenerating hindlimb CST axons into NPC graft (Figure 4d).

Preferential hindlimb CST regeneration into cervical NPC

Figures 5a,c are high magnification overlay images showing representative images of forelimb (Figure 5a) and hindlimb (Figure 5c) CST regeneration into GFP labeled graft, with respective RFP-only images in Figures 5b,d. White dashed lines represent rostral-edge interface between host and graft tissue (Figures 5b,d). We then quantified regeneration of RFP labeled host forelimb and hindlimb CST regeneration into the E14 NPC graft (Figure 5e). For both groups, regeneration was quantified by calculating average CST axon density by dividing sum-total threshold RFP area over sum-total graft area for forelimb (n=7) and hindlimb (n=7) animals, using ImageJ software. Results indicated significantly more hindlimb CST regeneration into a cervically placed E14 NPC graft than forelimb CST (Figure 5e). These results indicate significantly greater hindlimb than forelimb CST regeneration into NPC graft placed into cervical SCI site.

2.4: FIGURES

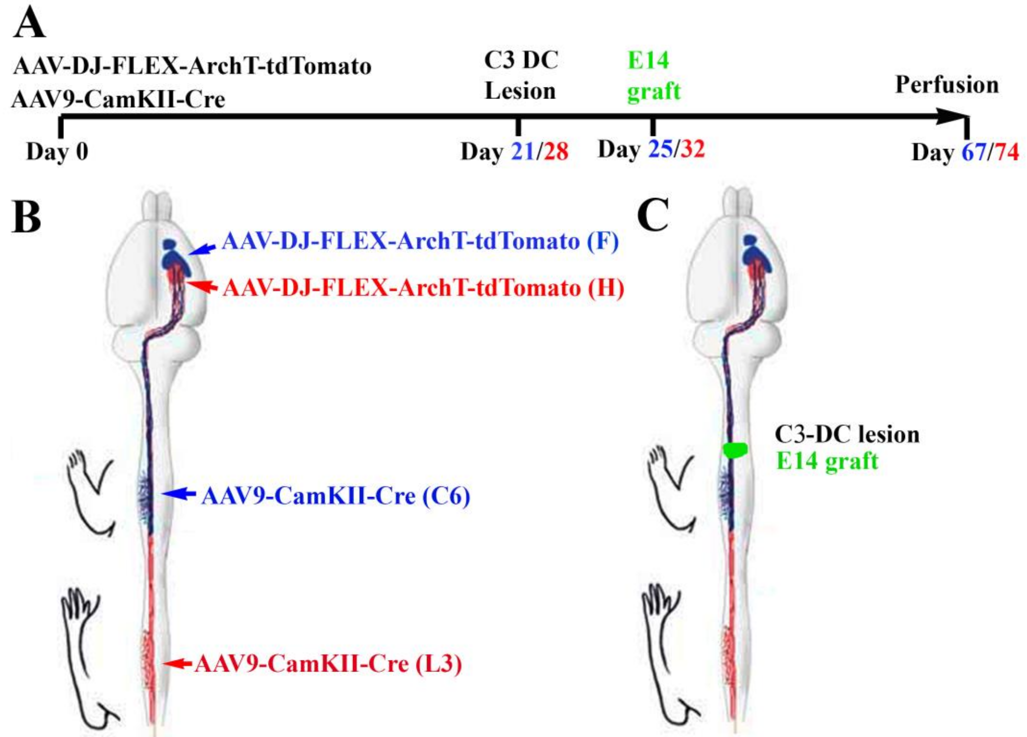

Figure 1: Experimental time course and schematic diagram of experimental design. **(A)** Schematic time-course representing order and intervals between each experimental step. **(B)** Color coded diagram representing locations of virus injections for both, forelimb or hindlimb groups (two separate groups depicted on one schematic). Red color corresponds to hindlimb CST (H-CST) axons, blue color corresponds to forelimb (F-CST) axons. **(C)** Color coded diagram representing location of the dorsal column wire-knife lesion and subsequent engraftment of E14 derived NPCs (green) for both forelimb, and hindlimb groups.

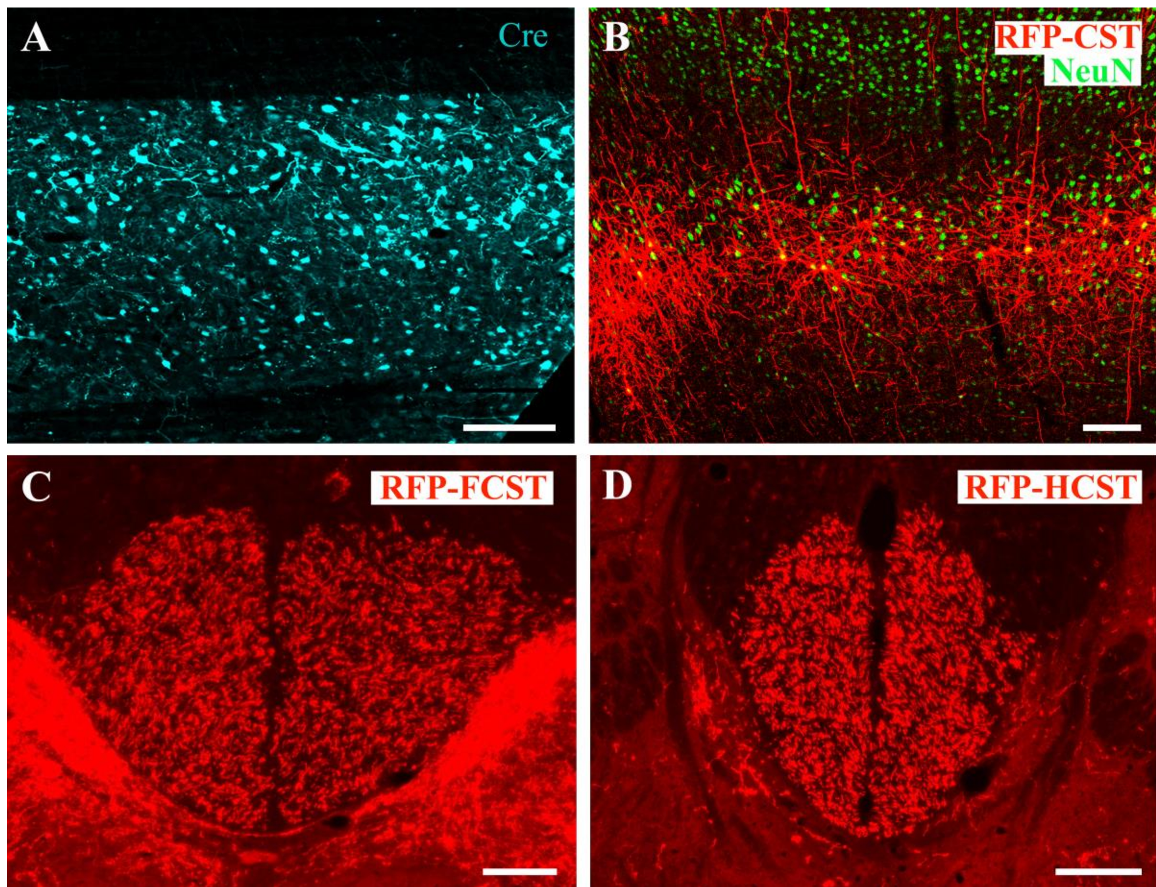

Figure 2: Cre-recombinase injections, retrograde transportation, and activation of TD-tomato expression for forelimb or hindlimb CST.

(A) Cre expression (cyan) in a sagittal section view after AAV-Cre injection into C6 spinal cord. Scale bar: 0.5 mm **(B)** Cortical TD-tomato expression (RFP) (red) in CST neurons after AAV-Cre injection (C6). NeuN labels for neurons. Scale bar: 120µm. **(C)** Hindlimb CST axonal labeling at C1 (RFP). Scale bar: 100 µm **(D)** Forelimb CST axonal labeling at C1 (RFP). Scale bar: 100 µm

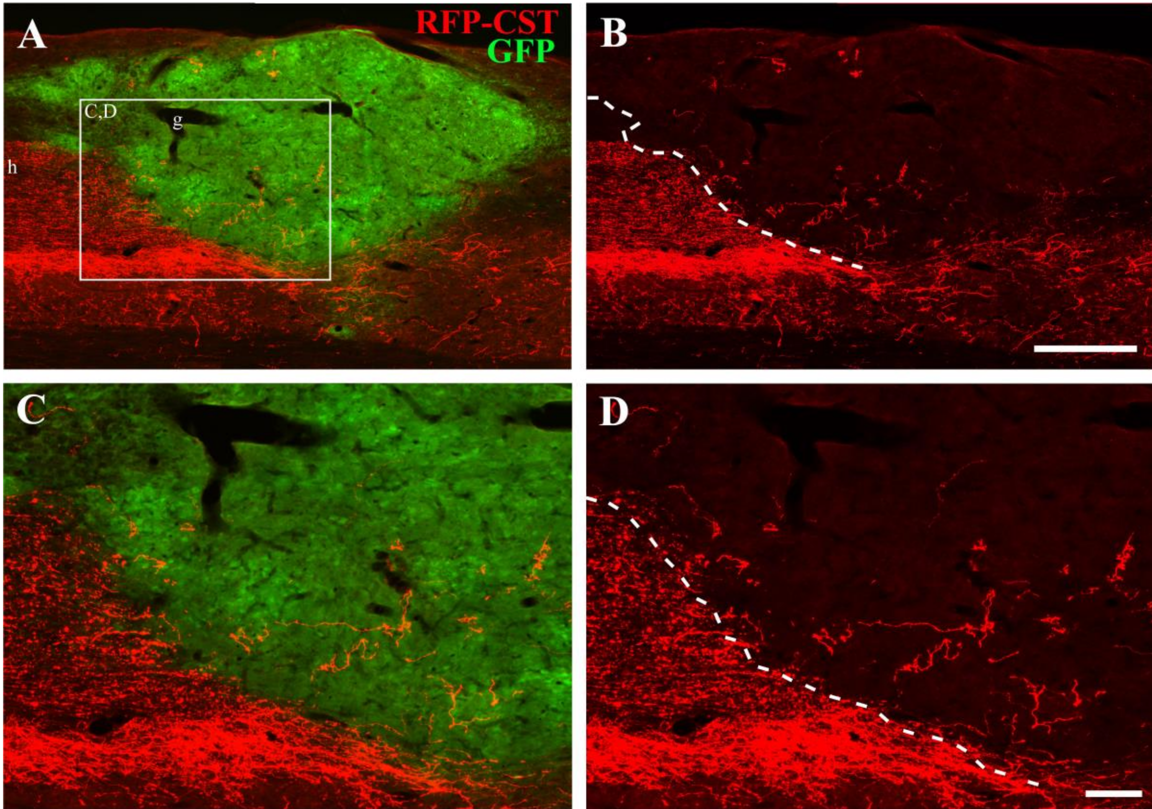

Figure 3: Poor regeneration of forelimb CST into C3 NPC graft. (A-B) A low-magnification sagittal view of dorsal column forelimb CST axons (RFP) penetrating the host (h)-graft (g) interface with sparse regeneration (6 weeks post SCI) into GFP labeled NPC graft in the C3 lesion site. Scale bar: 0.5 mm. Dashed lines indicate host-graft interface, throughout. Rostral (left)- caudal (right) orientation, throughout. **(C-D)** High-magnification view of boxed area in (A), containing a combination of overlaid images of forelimb CST axons (RFP) approaching NPC graft (GFP). Scale bars: 120 µm.

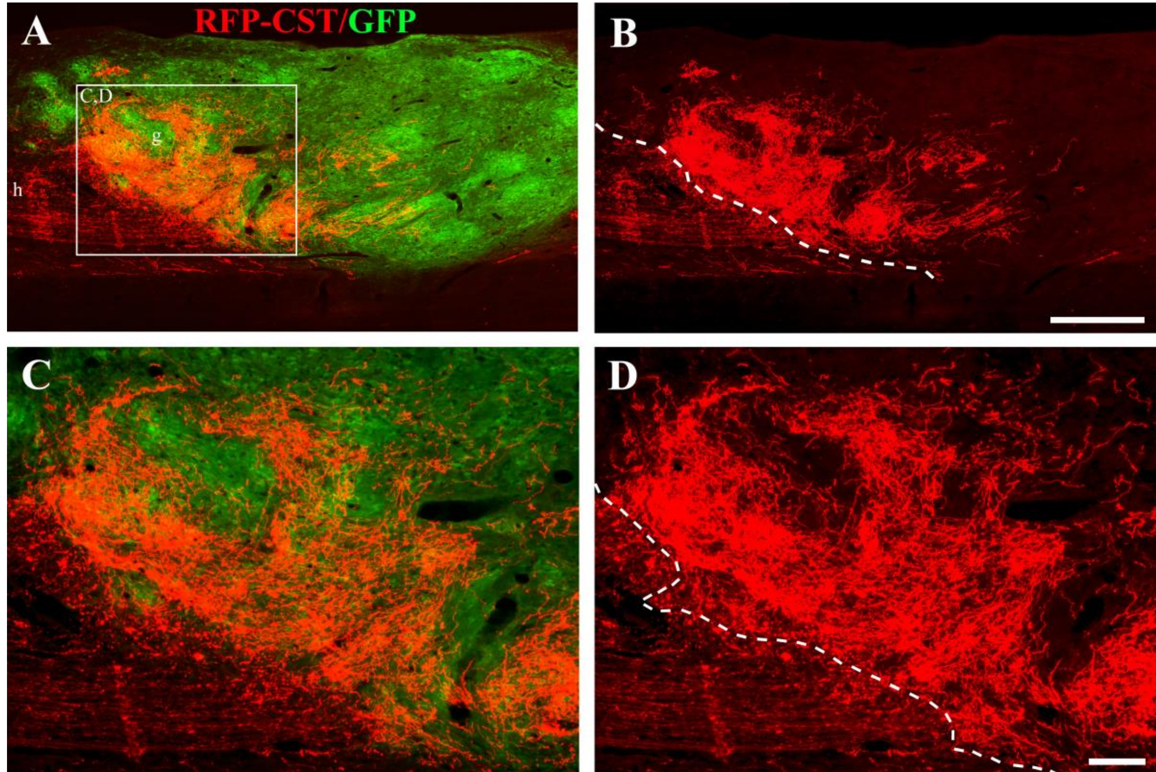

Figure 4: Robust regeneration of hindlimb CST into C3 NPC graft. (A-B) A low-magnification sagittal view of dorsal column hindlimb CST axons (RFP) penetrating the host (h)-graft (g) interface with extensive regeneration (6 weeks post SCI) into GFP labeled NPC graft in the C3 lesion site. Scale bar: 0.5 mm. Dashed lines indicate host-graft interface, throughout. Rostral (left)- caudal (right) orientation, throughout. **(C-D)** High-magnification view of outlined area in (A), containing a combination of overlaid images of hindlimb CST axons (RFP) approaching NPC graft (GFP). Scale bars: 120 µm.

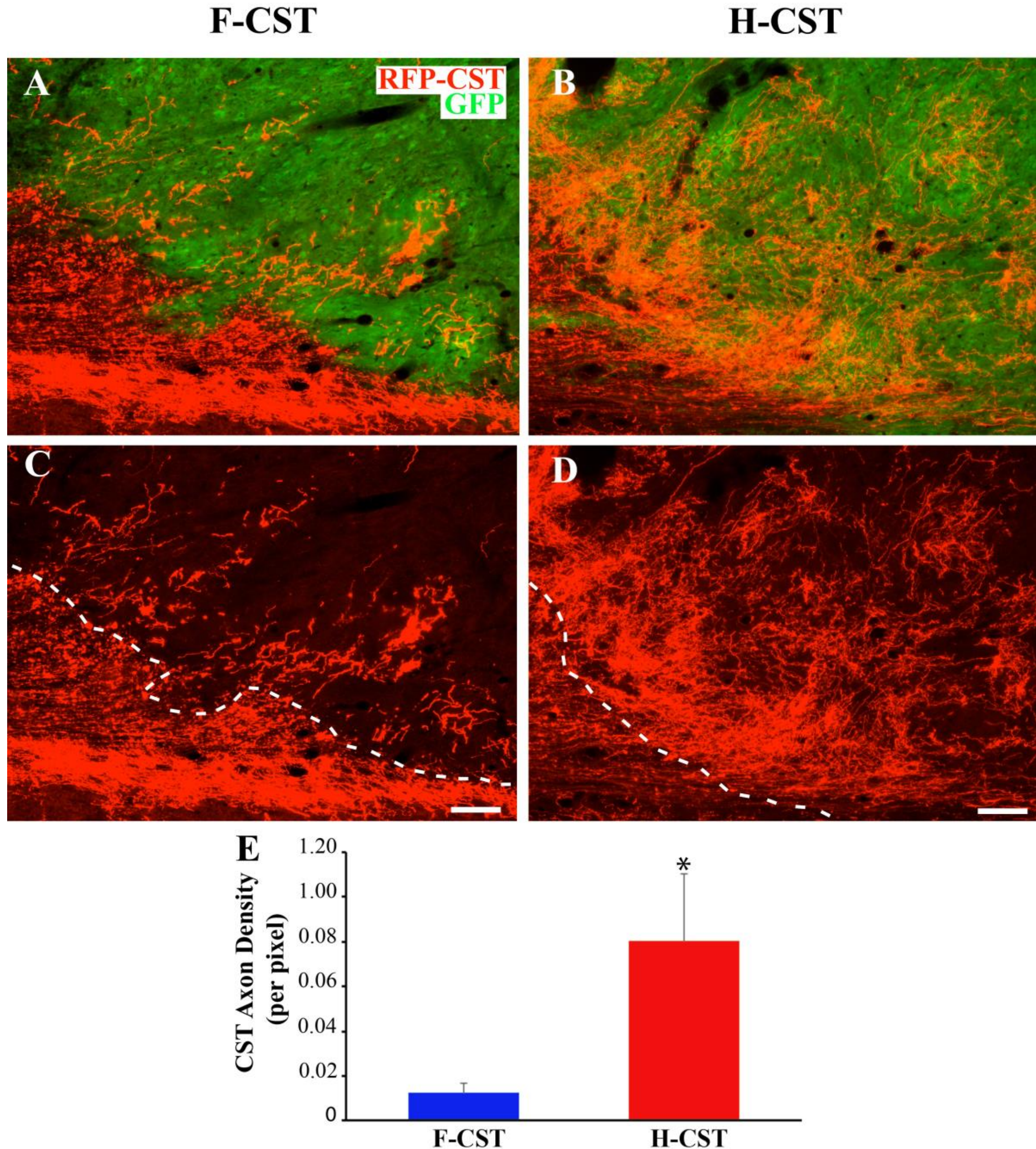

Figure 5: Preferential hindlimb CST regeneration into cervical NPC (A, C) High-magnification overlay images representing RFP labeled forelimb CST (F-CST) axons approaching GFP labeled NPC graft. Scale bar: 120 µm, in all images. Rostral (left)-caudal (right) orientation, in all images. Dashed lines indicate host (h)-graft(g) interface, in all images. **(B, D)** High-magnification overlay images representing RFP labeled hindlimb CST (H-CST) axons approaching and regenerating into GFP labeled NPC graft. **(E)** Average CST axon density calculated by dividing sum-total threshold RFP area over sum-total graft area for forelimb (n=7) and hindlimb (n=7) animals, using ImageJ software. Average regeneration of H-CST is significantly greater than F-CST (*p=0.0334, Student T test).

CHAPTER 3: DISCUSSION AND CONCLUSIONS

3.1: DISCUSSION

Recent studies have shown an extensive regenerative ability and functional relay formation of axotomized CST axons (both primate and rodent) in the presence of an NPC graft. Here, we determine that hindlimb CST axons regenerate into an NPC graft far more extensively than forelimb CST axons.

To obtain a precise characterization of the regenerative events, it was essential to use an intersectional viral approach (Wang et al., 2017). The Cre-dependent AAV-DJ-FLEX-ArchT-tdTomato reporter virus injected into either the forelimb or hindlimb motor cortex area, thus transfecting and integrating into the surrounding motor neuron cell bodies. Despite this reporter gene's integration into the host DNA, it was not expressed until Cre-recombinase interacted and activated the transfected reporter gene. Subsequent axon-terminal uptake and retrograde transport the Cre virus, at either C6 (forelimb) or L3 (hindlimb) segments, specifically labeled the neurons that were transfected by the tdTomato virus and projected their axons into either cervical or lumbar spinal cord. This protocol insured precise differentiation between the two groups of neurons. Some issues included non-specific labeling due to imprecise injection techniques or spread of virus through the cerebrospinal fluid (CSF). Therefore, extreme care and precision were taken when performing the viral injections to avoid obscuring results. The expression of tdTomato seemed specific for CST neurons and axons for most samples. Tissue with substantial nonspecific labeling, occasionally observed, were omitted from analysis.

Our results successfully confirm preliminary studies that used a less specific, anterograde virus injected separately into rostral forelimb and caudal hindlimb area in the

motor cortex, and therefore reject our original hypothesis of equal regenerative-ability between forelimb and hindlimb CST axons. Forelimb axons approach but do not significantly penetrate the host-graft interface. To control for possible confounding factors that could have led to the quantitative and qualitative differences, two main parameters needed to hold: 1) the NPC graft entirely filled the lesion cavity and formed a continuous border with the host cells, 2) the dorsal column CST was completely transected. Such conditions are necessary to allow for CST regeneration to take place (Kadoya et al. 2016); all samples presented and used to calculate quantitative significance demonstrated such requirements. Therefore, the sparsity of regeneration in the forelimb animals cannot be explained by varying graft environments.

A few possible mechanisms could explain the differential regeneration between hindlimb and forelimb CSTs.

(1) forelimb CST neurons have presumably greater branches in the brain and brainstem compared to hindlimb CSTs (Conner et al., unpublished). Specifically, about 55% of the forelimb CST branches innervate rostral to the cervical spinal cord. Therefore, after cervical CST axotomization, the regenerative cellular material has a lower probability of reaching the injured axon tip; this should result in the upstream axonal collaterals to grow after injury. Such hypothesis can be tested by quantifying CST sprouting and comparing morphological changes after injury for both groups.

(2) Another possibility -- from the assumption that forelimb CSTs have more branches -- is since the injury is cutting a small percent of the total cellular volume, the forelimb neurons are not recognizing the injury and do not increase regenerative signal.

This will be tested by comparing RNA sequencing data prior and post-injury for both forelimb and hindlimb CST neurons.

(3) Finally, the NPC grafts are derived from embryonic spinal cords. Due to the technical limitations for harvesting these spinal cord cells, it may be that we preferentially obtained cervical NPCs. Thus, the regenerating cervical CST neurons interact with the environment that recapitulates development, where the cervically projecting CSTs would naturally cease to grow. Such hypothesis will be tested by grafting cervical and lumbar embryonic spinal cord cells separately, through more diligent surgical procedures.

The preferential regeneration of hindlimb CST into a cervical SCI site filled with NPC graft poses a mismatched pattern of regeneration. Understanding and manipulating regenerative mechanisms may overcome this mismatched regeneration shown in our experiment. To get matched functional recovery, CST axons controlling the forelimbs at and below the cervical lesion must regenerate and innervate the cervical gray matter. Future studies will investigate different strategies to (1) increase forelimb CST regeneration or (2) re-train hindlimb CST to adapt control of forelimb.

Increasing forelimb CST regeneration can be done by manipulating the quiescent intrinsic cellular regenerative state. This requires developing clinically relevant strategies that will manipulate the forelimb CST system to increase its regenerative ability in the presence of NPC graft. Possible targets include suppressing PTEN and SOCS3 activity, shown to increase regeneration in CST neurons (Liu et al., 2010; Sun et al., 2011; Zukor et al., 2013), or upregulating Kruppel-like Factor (KLF) transcription factor family -- regulators of the regenerative ability in developing CST neurons (Blackmore et al., 2013).

A promising and clinically relevant method for implementing genetic modification is through antisense oligonucleotides (ASO). ASOs are short DNA sequences which can be chemically modified to suppress or alter translation of target mRNAs (Schoch & Miller, 2017). ASO technology is clinically approved and used to treat Amyotrophic Lateral Sclerosis (ALS) (Schoch & Miller, 2017). Such technology can use utilized to modify neuronal regenerative state by inhibiting targets such as PTEN and SOCS3 or increasing KLF transcription (Liang et al., 2016, 2017). Development of such therapies for clinical use is still required.

Beside genetic manipulation, rehabilitation will be critical for improving motor function after injury (Larson & Dension, 2013; Gomara-Toldra et al., 2014). A priori, physical therapy can help form matched connections between CST neurons and their respective spinal cord targets. Although, if forelimb regeneration cannot be improved, and mismatched connections between hindlimb CST neurons and cervical gray matter persist, physical therapy could still be used to retrain function of the hindlimb controlling neurons, thus allowing for reconstitution of hand function. The mechanism behind rehabilitation is that training and activity influence new circuit formation observed during development, particularly by activity-dependent stabilization of new connections and pruning of weak connections (Monfils et al., 2005; Adkins et al., 2006; Wang et al., 2016). Further research on the effectiveness of physical therapy in the presence of stem cell grafts in spinal cord injury is required.

3.2: CONCLUSION

Utilizing a combination of Cre virus and a Cre-dependent reporter gene, allowed us to precisely distinguish between forelimb and hindlimb corticospinal neurons. Here, it has been shown that hindlimb corticospinal neurons regenerate, to a significantly greater extent than forelimb corticospinal neurons, into an NPC graft located in upper cervical lesion site. This result is contrary to our original hypothesis that expected both CST systems to regenerate equally. Future work will attempt to determine the mechanism behind the difference and explore the applicability of stem cell therapy in efforts to reestablish voluntary motor control in spinal cord injury patients.

WORKS CITED

Adkins, DeAnna L., Jeffery Boychuk, Michael S. Remple, and Jeffrey A. Kleim. "Motor Training Induces Experience-Specific Patterns of Plasticity across Motor Cortex and Spinal Cord." *Journal of Applied Physiology (Bethesda, Md.: 1985)* 101, no. 6 (December 2006): 1776–82. https://doi.org/10.1152/japplphysiol.00515.2006.

Apara, Akintomide, and Jeffrey L. Goldberg. "Molecular Mechanisms of the Suppression of Axon Regeneration by KLF Transcription Factors." *Neural Regeneration Research* 9, no. 15 (August 1, 2014): 1418–21. https://doi.org/10.4103/1673-5374.139454.

Anderson, Mark A., Joshua E. Burda, Yilong Ren, Yan Ao, Timothy M. O'Shea, Riki Kawaguchi, Giovanni Coppola, Baljit S. Khakh, Timothy J. Deming, and Michael V. Sofroniew. "Astrocyte Scar Formation Aids CNS Axon Regeneration." *Nature* 532, no. 7598 (April 14, 2016): 195–200. https://doi.org/10.1038/nature17623.

Becker, D., and 3rd JW McDonald. "Approaches to Repairing the Damaged Spinal Cord: Overview." *Handbook of Clinical Neurology* 109 (2012): 445–61. https://doi.org/10.1016/B978-0-444-52137-8.00028-0.

Blackmore, Murray G., Zimei Wang, Jessica K. Lerch, Dario Motti, Yi Ping Zhang, Christopher B. Shields, Jae K. Lee, Jeffrey L. Goldberg, Vance P. Lemmon, and John L. Bixby. "Krüppel-like Factor 7 Engineered for Transcriptional Activation Promotes Axon Regeneration in the Adult Corticospinal Tract." *Proceedings of the National Academy of Sciences of the United States of America* 109, no. 19 (May 8, 2012): 7517–22. https://doi.org/10.1073/pnas.1120684109.

Blesch, Armin, Paul Lu, Shingo Tsukada, Laura Taylor Alto, Kasper Roet, Giovanni Coppola, Dan Geschwind, and Mark H. Tuszynski. "Conditioning Lesions before or after Spinal Cord Injury Recruit Broad Genetic Mechanisms That Sustain Axonal Regeneration: Superiority to Camp-Mediated Effects." *Experimental Neurology* 235, no. 1 (May 2012): 162–73. https://doi.org/10.1016/j.expneurol.2011.12.037.

Blesch, Armin, and Mark H. Tuszynski. "Spinal Cord Injury: Plasticity, Regeneration and the Challenge of Translational Drug Development." *Trends in Neurosciences* 32, no. 1 (January 2009): 41–47. https://doi.org/10.1016/j.tins.2008.09.008.

Bonner, Joseph F., and Oswald Steward. "Repair of Spinal Cord Injury with Neuronal Relays: From Fetal Grafts to Neural Stem Cells." *Brain Research* 1619 (September 4, 2015): 115–23. https://doi.org/10.1016/j.brainres.2015.01.006.

Bradbury, Elizabeth J., Lawrence D. F. Moon, Reena J. Popat, Von R. King, Gavin S. Bennett, Preena N. Patel, James W. Fawcett, and Stephen B. McMahon. "Chondroitinase ABC Promotes Functional Recovery after Spinal Cord Injury." *Nature* 416 (April 11, 2002): 636.

Cadotte, David W., and Michael G. Fehlings. "Spinal Cord Injury: A Systematic Review of Current Treatment Options." *Clinical Orthopaedics and Related Research* 469, no. 3 (March 2011): 732–41. https://doi.org/10.1007/s11999-010-1674-0.

"Cellular Delivery of Neurotrophin-3 Promotes Corticospinal Axonal Growth and Partial Functional Recovery after Spinal Cord Injury | Journal of Neuroscience." Accessed May 24, 2018. http://www.jneurosci.org/content/17/14/5560.

Collyer, E., A. Catenaccio, D. Lemaitre, P. Diaz, V. Valenzuela, F. Bronfman, and F. A. Court. "Sprouting of Axonal Collaterals after Spinal Cord Injury Is Prevented by Delayed Axonal Degeneration." *Experimental Neurology* 261 (November 2014): 451–61. https://doi.org/10.1016/j.expneurol.2014.07.014.

Cregg, Jared M., Marc A. DePaul, Angela R. Filous, Bradley T. Lang, Amanda Tran, and Jerry Silver. "Functional Regeneration beyond the Glial Scar." *Experimental Neurology* 253 (March 2014): 197–207. https://doi.org/10.1016/j.expneurol.2013.12.024.

Curt, Armin, Hubertus J. A. Van Hedel, Daniel Klaus, Volker Dietz, and EM-SCI Study Group. "Recovery from a Spinal Cord Injury: Significance of Compensation, Neural Plasticity, and Repair." *Journal of Neurotrauma* 25, no. 6 (June 2008): 677–85. https://doi.org/10.1089/neu.2007.0468.

David, S., and A. J. Aguayo. "Axonal Elongation into Peripheral Nervous System 'Bridges' after Central Nervous System Injury in Adult Rats." *Science (New York, N.Y.)* 214, no. 4523 (November 20, 1981): 931–33.

Edward D. Wirth, III, Catherine A. Priest, Jane S. Lebkowski, Nathan C. Manley, Kevin P. Nishimoto, and Ross Okamura. Pluripotent stem cell-derived oligodendrocyte progenitor cells for the treatment of spinal cord injury. United States US20160331786A1, filed May 16, 2016, and issued November 17, 2016. https://patents.google.com/patent/US20160331786A1/en.

Erceg, Slaven, Mohammad Ronaghi, and Miodrag Stojković. "Human Embryonic Stem Cell Differentiation Toward Regional Specific Neural Precursors." *Stem Cells (Dayton, Ohio)* 27, no. 1 (January 2009): 78–87. https://doi.org/10.1634/stemcells.2008-0543.

Fehlings, Michael G., and Reaz Vawda. "Cellular Treatments for Spinal Cord Injury: The Time Is Right for Clinical Trials." *Neurotherapeutics: The Journal of the American Society for Experimental NeuroTherapeutics* 8, no. 4 (October 2011): 704–20. https://doi.org/10.1007/s13311-011-0076-7.

Fitch, Michael T., and Jerry Silver. "CNS Injury, Glial Scars, and Inflammation: Inhibitory Extracellular Matrices and Regeneration Failure." *Experimental Neurology* 209, no. 2 (February 2008): 294–301. https://doi.org/10.1016/j.expneurol.2007.05.014.

Filli, Linard, and Martin E. Schwab. "Structural and Functional Reorganization of Propriospinal Connections Promotes Functional Recovery after Spinal Cord Injury." *Neural Regeneration Research* 10, no. 4 (April 2015): 509–13. https://doi.org/10.4103/1673-5374.155425.

Fouad, K., I. Klusman, and M. E. Schwab. "Regenerating Corticospinal Fibers in the Marmoset (Callitrix Jacchus) after Spinal Cord Lesion and Treatment with the Anti-Nogo-A Antibody IN-1." *European Journal of Neuroscience* 20, no. 9 (November 1, 2004): 2479–82. https://doi.org/10.1111/j.1460-9568.2004.03716.x.

Gage, Fred H. "Mammalian Neural Stem Cells." *Science* 287, no. 5457 (February 25, 2000): 1433–38. https://doi.org/10.1126/science.287.5457.1433.

Gómara-Toldrà, Natàlia, Martha Sliwinski, and Marcel P. Dijkers. "Physical Therapy after Spinal Cord Injury: A Systematic Review of Treatments Focused on Participation." *The Journal of Spinal Cord Medicine* 37, no. 4 (July 2014): 371–79. https://doi.org/10.1179/2045772314Y.0000000194.

Hunt, D., R. S. Coffin, and P. N. Anderson. "The Nogo Receptor, Its Ligands and Axonal Regeneration in the Spinal Cord: A Review." *Journal of Neurocytology* 32, no. 1 (January 1, 2003): 105–105. https://doi.org/10.1023/A:1027336500881.

Han, Steve S. W., Diana Y. Kang, Tahmina Mujtaba, Mahendra S. Rao, and Itzhak Fischer. "Grafted Lineage-Restricted Precursors Differentiate Exclusively into Neurons in the Adult Spinal Cord." *Experimental Neurology* 177, no. 2 (October 1, 2002): 360–75. https://doi.org/10.1006/exnr.2002.7995.

Huang, D.W, L McKerracher, P.E Braun, and S David. "A Therapeutic Vaccine Approach to Stimulate Axon Regeneration in the Adult Mammalian Spinal Cord." *Neuron* 24, no. 3 (November 1, 1999): 639–47. https://doi.org/10.1016/S0896-6273(00)81118-6.

Iwanami, A., S. Kaneko, M. Nakamura, Y. Kanemura, H. Mori, S. Kobayashi, M. Yamasaki, et al. "Transplantation of Human Neural Stem Cells for Spinal Cord Injury in Primates." *Journal of Neuroscience Research* 80, no. 2 (April 15, 2005): 182–90. https://doi.org/10.1002/jnr.20436.

Kadoya, Ken, Paul Lu, Kenny Nguyen, Corinne Lee-Kubli, Hiromi Kumamaru, Lin Yao, Joshua Knackert, et al. "Spinal Cord Reconstitution with Homologous Neural Grafts Enables Robust Corticospinal Regeneration." *Nature Medicine* 22, no. 5 (May 2016): 479–87. https://doi.org/10.1038/nm.4066.

Kamiyama, Tsutomu, Hiroshi Kameda, Naoyuki Murabe, Satoshi Fukuda, Noboru Yoshioka, Hiroaki Mizukami, Keiya Ozawa, and Masaki Sakurai. "Corticospinal Tract Development and Spinal Cord Innervation Differ between Cervical and Lumbar Targets." *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience* 35, no. 3 (January 21, 2015): 1181–91. https://doi.org/10.1523/JNEUROSCI.2842-13.2015.

Karimi-Abdolrezaee, Soheila, Eftekhar Eftekharpour, Jian Wang, Cindi M. Morshead, and Michael G. Fehlings. "Delayed Transplantation of Adult Neural Precursor Cells Promotes Remyelination and Functional Neurological Recovery after Spinal Cord Injury." *Journal of Neuroscience* 26, no. 13 (March 29, 2006): 3377–89. https://doi.org/10.1523/JNEUROSCI.4184-05.2006.

Keirstead, Hans S., Gabriel Nistor, Giovanna Bernal, Minodora Totoiu, Frank Cloutier, Kelly Sharp, and Oswald Steward. "Human Embryonic Stem Cell-Derived Oligodendrocyte Progenitor Cell Transplants Remyelinate and Restore Locomotion after Spinal Cord Injury." *Journal of Neuroscience* 25, no. 19 (May 11, 2005): 4694–4705. https://doi.org/10.1523/JNEUROSCI.0311-05.2005.

Kim, Moonhang, So Ra Park, and Byung Hyune Choi. "Biomaterial Scaffolds Used for the Regeneration of Spinal Cord Injury (SCI)." *Histology and Histopathology* 29, no. 11 (November 2014): 1395–1408. https://doi.org/10.14670/HH-29.1395.

Lang, Bradley T., Jared M. Cregg, Marc A. DePaul, Amanda P. Tran, Kui Xu, Scott M. Dyck, Kathryn M. Madalena. "Modulation of the Proteoglycan Receptor PTPσ Promotes Recovery after Spinal Cord Injury." *Nature* 518, no. 7539 (February 19, 2015): 404–8. https://doi.org/10.1038/nature13974.

Larson, Cathy A., and Paula M. Dension. "Effectiveness of Intense, Activity-Based Physical Therapy for Individuals with Spinal Cord Injury in Promoting Motor and Sensory Recovery: Is Olfactory Mucosa Autograft a Factor?" *The Journal of Spinal Cord Medicine* 36, no. 1 (January 2013): 44–57. https://doi.org/10.1179/2045772312Y.0000000026.

Liang, Xue-hai, Hong Sun, Wen Shen, Shiyu Wang, Joyee Yao, Michael T. Migawa, Huynh-Hoa Bui, et al. "Antisense Oligonucleotides Targeting Translation Inhibitory Elements in 5′ UTRs Can Selectively Increase Protein Levels." *Nucleic Acids Research* 45, no. 16 (September 19, 2017): 9528–46. https://doi.org/10.1093/nar/gkx632.

Liang, Xue-Hai, Wen Shen, Hong Sun, Michael T. Migawa, Timothy A. Vickers, and Stanley T. Crooke. "Translation Efficiency of MRNAs Is Increased by Antisense Oligonucleotides Targeting Upstream Open Reading Frames." *Nature Biotechnology* 34, no. 8 (2016): 875–80. https://doi.org/10.1038/nbt.3589.

Liu, Kai, Andrea Tedeschi, Kevin Kyungsuk Park, and Zhigang He. "Neuronal Intrinsic Mechanisms of Axon Regeneration." *Annual Review of Neuroscience* 34, no. 1 (June 21, 2011): 131–52. https://doi.org/10.1146/annurev-neuro-061010-113723.

Liu, Kai, Yi Lu, Jae K. Lee, Ramsey Samara, Rafer Willenberg, Ilse Sears-Kraxberger, Andrea Tedeschi, et al. "PTEN Deletion Enhances the Regenerative Ability of Adult Corticospinal Neurons." *Nature Neuroscience* 13, no. 9 (September 2010): 1075–81. https://doi.org/10.1038/nn.2603.

Lu, Paul, Lori Graham, Yaozhi Wang, Di Wu, and Mark Tuszynski. "Promotion of Survival and Differentiation of Neural Stem Cells with Fibrin and Growth Factor Cocktails after Severe Spinal Cord Injury." *JoVE (Journal of Visualized Experiments)*, no. 89 (July 27, 2014): e50641–e50641. https://doi.org/10.3791/50641.

Lu, P., L. L. Jones, and M. H. Tuszynski. "BDNF-Expressing Marrow Stromal Cells Support Extensive Axonal Growth at Sites of Spinal Cord Injury." *Experimental Neurology* 191, no. 2 (February 2005): 344–60. https://doi.org/10.1016/j.expneurol.2004.09.018.

Lu, P., L. L. Jones, E. Y. Snyder, and M. H. Tuszynski. "Neural Stem Cells Constitutively Secrete Neurotrophic Factors and Promote Extensive Host Axonal Growth after Spinal Cord Injury." *Experimental Neurology* 181, no. 2 (June 2003): 115–29. https://doi.org/10.1016/S0014-4886(03)00037-2.

Lu, P., and M. H. Tuszynski. "Growth Factors and Combinatorial Therapies for CNS Regeneration." *Experimental Neurology* 209, no. 2 (February 2008): 313–20. https://doi.org/10.1016/j.expneurol.2007.08.004.

Lu, Paul, Yaozhi Wang, Lori Graham, Karla McHale, Mingyong Gao, Di Wu, John Brock, et al. "Long-Distance Growth and Connectivity of Neural Stem Cells after Severe Spinal Cord Injury." *Cell* 150, no. 6 (September 14, 2012): 1264–73. https://doi.org/10.1016/j.cell.2012.08.020.

Manley, Nathan C., Catherine A. Priest, Jerrod Denham, Edward D. Wirth, and Jane S. Lebkowski. "Human Embryonic Stem Cell-Derived Oligodendrocyte Progenitor Cells: Preclinical Efficacy and Safety in Cervical Spinal Cord Injury." *STEM CELLS Translational Medicine* 6, no. 10 (n.d.): 1917–29. https://doi.org/10.1002/sctm.17-0065.

McDonald, John W., and Cristina Sadowsky. "Spinal Cord Injury." *The Lancet* 360, no. 9348 (December 7, 2002): 1884. https://doi.org/10.1016/S0140-6736(02)11746-6.

McKerracher, Lisa, and Matthew Winton. "Targeting Rho to Stimulate Repair After Spinal Cord Injury." *Topics in Spinal Cord Injury Rehabilitation* 8, no. 4 (January 1, 2003): 69–75. https://doi.org/10.1310/5NA7-MBGU-Q2TU-N41Q.

Mitsui, T., I. Fischer, J. S. Shumsky, and M. Murray. “Transplants of Fibroblasts Expressing BDNF and NT-3 Promote Recovery of Bladder and Hindlimb Function Following Spinal Contusion Injury in Rats.” *Experimental Neurology* 194, no. 2 (August 2005): 410–31. https://doi.org/10.1016/j.expneurol.2005.02.022.

Monfils, Marie-H., Erik J. Plautz, and Jeffrey A. Kleim. “In Search of the Motor Engram: Motor Map Plasticity as a Mechanism for Encoding Motor Experience.” *The Neuroscientist: A Review Journal Bringing Neurobiology, Neurology and Psychiatry* 11, no. 5 (October 2005): 471–83. https://doi.org/10.1177/1073858405278015.

Goodman, Corey S., and Marc Tessier-Lavigne. “Molecular Mechanisms of Axon Guidance and Target Recognition.” Accessed May 23, 2018. http://oxfordindex.oup.com/view/10.1093/acprof:oso/9780195111668.003.0004, //oxfordindex.oup.com/view/10.1093/acprof:oso/9780195111668.003.0004.

Mothe, Andrea J., and Charles H. Tator. “Advances in Stem Cell Therapy for Spinal Cord Injury.” *The Journal of Clinical Investigation* 122, no. 11 (November 1, 2012): 3824–34. https://doi.org/10.1172/JCI64124.

Murry, Charles E., and Gordon Keller. “Differentiation of Embryonic Stem Cells to Clinically Relevant Populations: Lessons from Embryonic Development.” *Cell* 132, no. 4 (February 22, 2008): 661–80. https://doi.org/10.1016/j.cell.2008.02.008.

Nathan, P. W. “Effects on Movement of Surgical Incisions into the Human Spinal Cord.” *Brain* 117, no. 2 (April 1, 1994): 337–46. https://doi.org/10.1093/brain/117.2.337.

:National Spinal Cord Injury Statistical Center, Facts and Figures at a Glance. Birmingham, AL: University of Alabama at Birmingham, 2018.

Qiu, Jin, Dongming Cai, and Marie T. Filbin. “Glial Inhibition of Nerve Regeneration in the Mature Mammalian CNS.” *Glia* 29, no. 2 (January 15, 2000): 166–74. https://doi.org/10.1002/(SICI)1098-1136(20000115)29:2<166::AID-GLIA10>3.0.CO;2-G.

Reubinoff, Benjamin E., Martin F. Pera, Chui-Yee Fong, Alan Trounson, and Ariff Bongso. “Embryonic Stem Cell Lines from Human Blastocysts: Somatic Differentiation in Vitro.” *Nature Biotechnology* 18, no. 4 (April 2000): 399–404. https://doi.org/10.1038/74447.

Rothermel, Markus, Daniela Brunert, Christine Zabawa, Marta Díaz-Quesada, and Matt Wachowiak. “Transgene Expression in Target-Defined Neuron Populations Mediated by Retrograde Infection with Adeno-Associated Viral Vectors.” *Journal of Neuroscience* 33, no. 38 (September 18, 2013): 15195–206. https://doi.org/10.1523/JNEUROSCI.1618-13.2013.

Rowland, James W., Gregory W. J. Hawryluk, Brian Kwon, and Michael G. Fehlings. "Current Status of Acute Spinal Cord Injury Pathophysiology and Emerging Therapies: Promise on the Horizon." *Neurosurgical Focus* 25, no. 5 (2008): E2. https://doi.org/10.3171/FOC.2008.25.11.E2.

Schoch, Kathleen M., and Timothy M. Miller. "Antisense Oligonucleotides: Translation from Mouse Models to Human Neurodegenerative Diseases." *Neuron* 94, no. 6 (June 21, 2017): 1056–70. https://doi.org/10.1016/j.neuron.2017.04.010.

Schwab, Martin E. "Nogo and Axon Regeneration." *Current Opinion in Neurobiology* 14, no. 1 (February 1, 2004): 118–24. https://doi.org/10.1016/j.conb.2004.01.004.

Schwab, Martin E. "Repairing the Injured Spinal Cord." *Science* 295 (February 1, 2002): 1029–31. https://doi.org/10.1126/science.1067840.

Schwab, M. E., and D. Bartholdi. "Degeneration and Regeneration of Axons in the Lesioned Spinal Cord." *Physiological Reviews* 76, no. 2 (April 1996): 319–70. https://doi.org/10.1152/physrev.1996.76.2.319.

Schwab, Martin E., and Stephen M. Strittmatter. "Nogo Limits Neural Plasticity and Recovery from Injury." *Current Opinion in Neurobiology* 27 (August 2014): 53–60. https://doi.org/10.1016/j.conb.2014.02.011.

Sekhon, L. H., and M. G. Fehlings. "Epidemiology, Demographics, and Pathophysiology of Acute Spinal Cord Injury." *Spine* 26, no. 24 Suppl (December 15, 2001): S2-12.

"Spinal Cord Injury (SCI) Facts and Figures at a Glance." *The Journal of Spinal Cord Medicine* 39, no. 3 (May 3, 2016): 370–71. https://doi.org/10.1080/10790268.2016.1177348.

Starkey, Michelle L., Katalin Bartus, Andrew W. Barritt, and Elizabeth J. Bradbury. "Chondroitinase ABC Promotes Compensatory Sprouting of the Intact Corticospinal Tract and Recovery of Forelimb Function Following Unilateral Pyramidotomy in Adult Mice." *European Journal of Neuroscience* 36, no. 12 (December 1, 2012): 3665–78. https://doi.org/10.1111/ejn.12017.

Takahashi, Kazutoshi, and Shinya Yamanaka. "Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors." *Cell* 126, no. 4 (August 25, 2006): 663–76. https://doi.org/10.1016/j.cell.2006.07.024.

Tatagiba, M., C. Brösamle, and M. E. Schwab. "Regeneration of Injured Axons in the Adult Mammalian Central Nervous System." *Neurosurgery* 40, no. 3 (March 1997): 541–46; discussion 546-547.

Tetzlaff, Wolfram, Elena B. Okon, Soheila Karimi-Abdolrezaee, Caitlin E. Hill, Joseph S. Sparling, Jason R. Plemel, Ward T. Plunet, et al. "A Systematic Review of Cellular Transplantation Therapies for Spinal Cord Injury." *Journal of Neurotrauma* 28, no. 8 (August 2011): 1611–82. https://doi.org/10.1089/neu.2009.1177.

Tsuji, Osahiko, Kyoko Miura, Yohei Okada, Kanehiro Fujiyoshi, Masahiko Mukaino, Narihito Nagoshi, Kazuya Kitamura, et al. "Therapeutic Potential of Appropriately Evaluated Safe-Induced Pluripotent Stem Cells for Spinal Cord Injury." *Proceedings of the National Academy of Sciences* 107, no. 28 (July 13, 2010): 12704–9. https://doi.org/10.1073/pnas.0910106107.

Tuszynski, Mark H., Daniel A. Peterson, Jasodhara Ray, Andrew Baird, Yasushi Nakahara, and Fred H. Gages. "Fibroblasts Genetically Modified to Produce Nerve Growth Factor Induce Robust Neuritic Ingrowth after Grafting to the Spinal Cord." *Experimental Neurology* 126, no. 1 (March 1, 1994): 1–14. https://doi.org/10.1006/exnr.1994.1037.

Tuszynski, Mark H., and Oswald Steward. "Concepts and Methods for the Study of Axonal Regeneration in the CNS." *Neuron* 74, no. 5 (June 7, 2012): 777–91. https://doi.org/10.1016/j.neuron.2012.05.006.

Tuszynski, Mark H., and Paul Lu. "13 - AXONAL PLASTICITY AND REGENERATION IN THE INJURED SPINAL CORD." In *CNS Regeneration (Second Edition)*, 319–35. San Diego: Academic Press, 2008. https://doi.org/10.1016/B978-012373994-0.50015-4.

Vroemen, Maurice, Ludwig Aigner, Juergen Winkler, and Norbert Weidner. "Adult Neural Progenitor Cell Grafts Survive after Acute Spinal Cord Injury and Integrate along Axonal Pathways." *European Journal of Neuroscience* 18, no. 4 (August 1, 2003): 743–51. https://doi.org/10.1046/j.1460-9568.2003.02804.x.

Wang, Difei, Ronaldo M. Ichiyama, Rongrong Zhao, Melissa R. Andrews, and James W. Fawcett. "Chondroitinase Combined with Rehabilitation Promotes Recovery of Forelimb Function in Rats with Chronic Spinal Cord Injury." *Journal of Neuroscience* 31, no. 25 (June 22, 2011): 9332–44. https://doi.org/10.1523/JNEUROSCI.0983-11.2011.

Wang, Ling, James M. Conner, Alan H. Nagahara, and Mark H. Tuszynski. "Rehabilitation Drives Enhancement of Neuronal Structure in Functionally Relevant Neuronal Subsets." *Proceedings of the National Academy of Sciences of the United States of America* 113, no. 10 (March 8, 2016): 2750–55. https://doi.org/10.1073/pnas.1514682113.

Wang, Xuhua, Yuanyuan Liu, Xinjian Li, Zicong Zhang, Hengfu Yang, Yu Zhang, Philip R. Williams, et al. "Deconstruction of Corticospinal Circuits for Goal-Directed Motor Skills." *Cell* 171, no. 2 (October 5, 2017): 440-455.e14. https://doi.org/10.1016/j.cell.2017.08.014.

Weidner, Norbert, Arvin Ner, Nima Salimi, and Mark H. Tuszynski. "Spontaneous Corticospinal Axonal Plasticity and Functional Recovery after Adult Central Nervous System Injury." *Proceedings of the National Academy of Sciences of the United States of America* 98, no. 6 (March 13, 2001): 3513–18. https://doi.org/10.1073/pnas.051626798.

Young, Kaylene, Tara Walker, and Jeffrey Huang. "Neural Stem and Progenitor Cells in Nervous System Function and Therapy." *Stem Cells International* 2016 (August 4, 2016): 1–2. https://doi.org/10.1155/2016/1890568.

Zheng, Binhai, Jasvinder Atwal, Carole Ho, Lauren Case, Xiao-lin He, K. Christopher Garcia, Oswald Steward, and Marc Tessier-Lavigne. "Genetic Deletion of the Nogo Receptor Does Not Reduce Neurite Inhibition in Vitro or Promote Corticospinal Tract Regeneration in Vivo." *Proceedings of the National Academy of Sciences of the United States of America* 102, no. 4 (January 25, 2005): 1205–10. https://doi.org/10.1073/pnas.0409026102

Zukor, Katherine, Stephane Belin, Chen Wang, Nadia Keelan, Xuhua Wang, and Zhigang He. "Short Hairpin RNA against PTEN Enhances Regenerative Growth of Corticospinal Tract Axons after Spinal Cord Injury." *Journal of Neuroscience* 33, no. 39 (September 25, 2013): 15350–61. https://doi.org/10.1523/JNEUROSCI.2510-13.2013