UNIVERSITY OF CALIFORNIA, SAN DIEGO

Corticospinal Tract Axons Regenerate after Spinal Cord Injury: Growth from the Injured Tip of Axon

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

in

Biology

by

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The thesis of Arthi Suresh Rao is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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University of California, San Diego

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Dedication

I would like to dedicate this to my parents and younger brother, who have always supported and encouraged all of my pursuits.

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ABSTRACT OF THE THESIS

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by

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Master of Science in Biology

University of California, San Diego, 2017

Professor Mark Tuszynski, Chair

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Neural stem cells or neural progenitor cells have been extensively studied in spinal cord injury (SCI) rat models to attempt to reconnect injured circuits to restore function. Attempts have shown significant success, and further studies have revealed corticospinal tract (CST) axon growth into the neural stem cell graft. The purpose of this study is to understand whether CST axons undergo regeneration, regenerative sprouting, or sprouting into a neural stem cell graft. The animals were grafted with E14 spinal neural progenitor cells in a C4 dorsal column lesion for this study. The rat models were then injected with a small volume and low concentration of a tracer, AAV8-CAG-rCOMET or biotinylated dextran amine, in the motor cortex of the brain to label a few individual axons in order to visualize their growth pathway. Through the visualization on the confocal microscope, the injured axons of the CST are shown to regenerate into the graft cells. There was minimal regenerative sprouting and no sprouting seen with this model. Thus, CST axons regenerate into a neural stem cell graft.

CHAPTER 1

INTRODUCTION

Spinal Cord Injury Statistics

Approximately, 17,000 new cases of Spinal Cord Injuries (SCIs) develop every year in the United States[1]. SCIs can be caused by a variety of situations and can present different symptoms ranging in severity depending on the injury. Causes of injury are most commonly automobile accidents, falls, violence, sports, alcohol/drug abuse, and other diseases[2]. These injuries are more prevalent in males than females[3]. The lifespan for those with spinal cord injuries have also seen no significant increase since 1973[4]. Due to the varying range in injury, healthcare costs can be anywhere from $1,578,274 for a twenty-five-year-old with an ASIA D injury to $4,724,181 for a twenty-five-year old with a cervical ASIA A-C injury[5]. Due to the increase in technology and research, people are working towards giving those with SCIs longer and healthier lives than previously expected.

Symptoms of SCI

Typically, the local neurons and axon tracts in the spinal cord are demyelinated or cut at the point of injury. Many of these axons in the spinal cord have cell bodies in the brain. These axons typically form a relay between motor and sensory neurons to the brain. Severing or destroying these nerves in the spinal cord can lead to varying severities of motor and sensory deficits to the regions beneath the site of injury. This primary injury is caused by the mechanical stress of impact. There is a secondary injury response which leads to an expansion of the injury cavity for weeks after the initial injury. This includes but is not limited to paralysis, decreased mobility, spasms, loss of bladder and bowel control, pain, amongst other symptoms[6]. The paralysis that can result from these injuries can be classified as paraplegia or tetraplegia. It has been noted that patients of Spinal Cord

Injuries tend to show signs of depression or anxiety due to the changes and stresses that an SCI imparts on lifestyle[7].

Injury Classification

Clinically, these injuries are classified on the American Spinal Injury Association (ASIA) scale which is a modified version of the previous Frankel scale, and are also measured on a motor scale. There are 5 levels of clinical injury classification. ASIA A, or complete, implies a lack of motor and sensory function below the point of injury, specifically in sacral segments 4 and 5. ASIA B, sensory incomplete, is used to classify injuries that preserves sensory function at the S4 and S5 region, but there is not much preservation of motor function to the areas beneath the injury. ASIA C and D correspond to varying levels of motor incomplete injuries, where muscles have varying degrees of function at the area below the injury. ASIA C has more severe motor deficit compared to ASIA D. ASIA E is used to describe someone with an injury but no current deficit[8].

Neuron Development

The main cell type affected with this injury are neurons in the spinal cord. Neurons develop through neurogenesis, where progenitor cells from the ectoderm form neuroblasts and glioblasts in the embryo. These cells are mitotic, but then differentiate into neuronal and glial subtypes respectively[9]. Neuroblasts undergo asymmetric cell division through Notch and Numb signaling producing two daughter cells: one neuroblast and one ganglion mother cell(GMC)[10,11]. The differentiating GMC then divides further to produce neurons and glia which can no longer undergo mitotic division[12]. The fate or temporal identity of the progeny are determined through the transcription factor expression in the progenitor cells[13]. The neurons and neuroblasts migrate throughout the brain and if peripherally

produced from the neural crest travel throughout the body to find their specific place[14]. The differentiation of these neuroblasts occurs through specific signaling dependent on their path and signaling glia[14].

Next axons work their way to their targets through axon guidance. The growth cone at the end of the axon follows intrinsic and extrinsic cues to find its synaptic partner in the body. Examples of cues include netrins, slit, semaphorins, Wnts, and ephrins[15,16]. Intrinsically, elevated levels of cAMP, cGMP, Ca^{2+}, amongst others impact the way the axons respond to the extrinsic cues[17]. An example of this can be seen with netrins, which can be both repulsive or attractive for growth, depending on the intrinsic factors present in the cell[17]. The growth cone responds to this combination of cues, which are either secreted or membrane bound, moving towards the promoting factors and shying away from the inhibitory ones, to eventually find its target. Through the same cues, these axons further undergo synaptic pruning to ensure the appropriate targets are connected to[18].

The Axon Regeneration Problem

After development, these axons remain synapsed to their specific targets with minimal changes through the course of their lives. Neurons are fixed. Unlike other cells in the body these cells cannot replace themselves once they die. It is important to recognize that axons from the peripheral nervous system, and those from the central nervous system have a difference in their ability to regenerate if injured. When PNS neurons are cut, they can undergo complete regeneration, typically restoring full function; CNS axons, on the other hand, are incapable of regenerating[19]. Due to this property of CNS axons, corticospinal tract (CST) axons, the main type of neurons observed in this study, can produce some detrimental side effects when damaged.

Inhibitors of Regeneration: Extrinsic Factors

There are a variety of factors that influence these neurons to respond differently to damage. Compared to the PNS, the CNS axons face mostly inhibitory signaling, both extrinsic and intrinsic, for regeneration. Extrinsic inhibitors arise from myelin and the glial scar that is produced after damage[20]. Interestingly, factors involved in axon guidance can produce an inhibitory effect in adult CNS axons[21]. Primarily, the myelin produced by oligodendrocytes for CNS axons releases a variety inhibitors compared to the PNS myelin and Schwann cells. Three isoforms of Nogo are expressed in oligodentrocytes and play a crucial role in axon growth inhibition after damage, specifically Nogo-A, which contains a unique N-terminus[21,22]. Nogo's extracellular domain has been seen to associate with the endoplasmic reticulum and contribute to the collapse of growth cone formation in DRGs and neurite outgrowth[22,23]. The lack of Nogo in Schwann Cells contributes to the ability of the PNS to continue to regenerate and restore initial function. Myelin associated glycoprotein (Mag) further influences axon regrowth. Though found both in the CNS and PNS, it exhibits a more inhibitory effect, and is in fact a promoter of growth during the early stages of axon growth, specifically DRG growth up to a few days postnatal[20,24]. Oligodendrocyte myelin protein (OMgp), is not restricted to expression in only oligodendrocytes as it is also found in neurons as it also is restrictive of regrowth in the CNS[25].

From the glial scar arise further inhibitory factors to growth. Mainly, chondroitin sulfate proteoglycans (CSPGs) are released by activated glia, and are also seen during development to inhibit axonal growth in certain regions[26]. CSPGs have been noted to be present surrounding the lesion or injury cavity, promoted by the glial cells that form the

scar tissue[27]. Beyond this, there are other factors present in the extracellular matrix which include many axon guidance cues like ephrins, slit, and semaphorin 3A, which though they initially contribute to axon growth and pathfinding, also have inhibitory effects on adult CNS axons[28,29,30]. For example, in an injured CNS, Wnt signaling is noted to increase and has been shown to be an inhibitor of CST axon regeneration[16].

Inhibitors of Regeneration: Intrinsic Factors

There are intrinsic factors within the neurons which influence the lack in the ability of axons to regenerate. The internal growth program of the axon shuts down post-development. Specifically, there are a variety of pathways known to inhibit axon regeneration. cAMP, for example is typically seen at elevated levels during development, however at lower levels, the regenerative capacity seems to decrease though the exact mechanism of influence by cAMP is unknown[31]. Another example of an intrinsic inhibitor is the PTEN/mTOR pathway. The presence of PTEN inhibits multiple downstream effectors, including mTOR, which promote cell growth and regeneration[32]. There are still other pathways that are being studied that contribute to the inability of the CNS to regenerate after injury.

Current Research

Currently, there is a growing field of research studying potential therapeutics for SCIs. This includes three main categories of study: neural protection, remyelination, and regeneration.

Neuroprotection

Within spinal cord injuries, after the primary injury or mechanical stress, there is a secondary injury. Beyond the initial severing of axons in the cord, the secondary injury is

a term used to describe further pathophysiological pathways that lead to further degradation of tissue surrounding the injury site[33]. Neuroinflammatory responses, apoptosis, and a variety of other pathways contribute significantly to the secondary degradation, and serve as targets for potential studies[34]. Amongst many currently in study, the lipid peroxidation plays an active role in secondary degradation, and as seen in clinical trials, methylprednisolone and naloxone were pharmacological therapeutics used to target this peroxidation pathway which demonstrated some promise in neuroprotection[35]. Another current study shows the use of minocycline hydrochloride which shows an increase in functional recovery alongside a reduction in the secondary degradation in rat SCI models[36].

Remyelination

With certain SCIs, not all axons are severed and have lost complete connectivity, but many lose their myelination. Another targeted field of study to improve function after SCI is to promote remyelination of these injured axons. Promoting oligodendrocyte myelination in the CNS, is currently an important study for Multiple Sclerosis patients, that can also aid with SCI[37]. Further, T-cells, mainly T_{reg}, are thought to play an important role in both remyelination as well as oligodendrocyte differentiation according to a recent 2017 study[38]. There are many more studies underway to address remyelination in diseases of the CNS which focusing on oligodendrocyte progenitor cell transplantation, as well as the intracellular signaling methods associated with myelination[39].

Regeneration

Studies further attempt to promote regeneration of injured or damaged axons in SCI to regain lost function. There are currently two main ways to attempt to promote

regeneration in injured axons of the CNS: one is to transplant cells to fill in the lesion cavity, and the other is to manipulate pre-existing factors.

The primary purpose of cell transplantation studies is to provide a more promoting environment and permissible substrate for axon regrowth after injury. Stem cells, fibroblasts, and scaffolds are common substrates placed in the lesion cavity to bridge the gap between the lesioned tissue and the severed connectivity of the host.

Primarily, the usage of neural stem cells for this purpose is two-fold. As these cells mimic developmental stages, these cells are ideal due to their ability to successfully differentiate into necessary neuronal subtypes *in vitro,* which have high intrinsic growth and connectivity capacity that could restore functions for spinal cord injury[40,41]. Embryonic stem cells show differentiation potential into neuron progenitor cells which have the capability to differentiate further into the neuronal subtypes necessary for motor function recovery in injured rats[42,43]. Specifically, embryonic stem cells have been derived into neural stem cells or neural progenitor cells to utilize as potential grafting cells[44]. Further, stem cells differentiate into oligodendrocytes to promote myelination of the injury site, and further produce motor benefits[45]. Neural subtype derived from embryonic stem cells grafted into the injury site, and oligodendrocyte progenitor cells further support myelination and regeneration[46]. As shown through the Lu et. al study, a neural stem cell graft promisingly survives when grafted into the lesion cavity, showing dense and robust axonal growth for long distances with attempts at connectivity with the host[47]. Neural progenitor cell transplants have shown to contribute to the plasticity and functional recovery in traumatic cord injuries in rat models[48]. These stem cell models are currently

being optimized to ensure promotion of host growth capability, myelination, and graft outgrowth and connectivity to host cells.

Cultured Schwann cells *in vitro* have been optimized to transplant in rat spinal cords to promote regeneration, and even further have been carried into clinical trials[49]. These cells have shown to successfully survive and promote both regeneration and myelination. Further the use of modified fibroblasts serve as a growth promoting substrate in SCIs. Fibroblasts modified to express higher levels of BDNF showed increased distances of axonal growth as well as increased forelimb functional recovery[50]. Transplantation of BDNF and NT-3 producing fibroblasts promote regenerative pathways overall[51]. Bone marrow stromal cells have further been studied as a potential therapeutic as these cells have been seen to increase axonal growth when grafted into a contusion injury[52].

Through the use of various biomaterials, scaffolds are also a vastly researched area for therapeutics. A freeze-dried agarose scaffold was shown to integrate into a graft providing individual channels to linearize axonal growth[53]. This study saw increased benefits combined with BDNF[53]. Further, utilization of a polymer scaffold incorporated with guiding neural stem cells have further shown functional recovery[54].

Gene therapies study the effect of the introduction of additional neurotrophins or degradation of other inhibitors after injury in order to promote cell growth in the injured CNS. As studied by Lu et al the combination of cAMP and NT-3 administration promotes significant regeneration of dorsal column sensory axons[55]. Others currently under study include growth factors like FGF and neuropoetic cytokines like LIF and CNTF[56].

Degradation or inhibition of extrinsic inhibitors of CNS regeneration have shown some promising levels of regeneration. Peptide blockers of Nogo receptors or antibody

administration reduces the inhibitory effects of growth and result in robust axonal regeneration and compensatory sprouting[57]. Further, co-inhibition of PTEN and SOCS3 pathway is studied as a potential regeneration promoting pathway for injured CNS axons, as PTEN typically inhibits mTOR, a gene which promotes regeneration and growth[58]. As seen with mouse CST axons, downregulation of PTEN promoted regeneration and connectivity[59]. Usage of chondroitinase ABC has shown to reduce the inhibitor activity of CSPGs to further enhance functional recovery[60,61]. Though all show promise to aid in relieving functional deficits, more study is under way to optimize these therapies. Ideally, combination therapies of cell transplantation and gene therapies can prove to be more effective.

My Research

Regeneration and Properties

As set forth by Tuszynski and Steward, there are three main terms to define axon growth after injury. Though sometimes used to describe general growth of a cell, it is important to distinguish that the term regeneration is used to describe the growth from the injured axon tip of a transected axon[62]. Though commonly main tract axons tend to grow linearly, regeneration indicates non-linear, arborous, and varicose growth[55]. Further, the axon can continue to grow proximal off the shaft of an injured axon, known as regenerative sprouting[62]. Finally, axons are thought to undergo compensatory sprouting where uninjured axons attempt to form connectivity[62]. Many axons, have been suggested to not grow further. As they undergo axonal retraction, the end of the axon swells due to the accumulation of organelles[63]. As the axon unsuccessfully attempts to form a growth cone and faces further inhibition, a retraction bulb may result[16,63].

CST Axons

Corticospinal tract (CST) axons support voluntary motor function, and are considered to be one the most important axon tracts in humans. Damage to this tract leads to the severe paralysis, a common symptom of spinal cord injury. Many studies as expressed above focus more specifically on promoting CST axonal regeneration as well as myelination. As studied by Zheng et al, the neuronal Nogo receptor was deleted in hopes of reducing myelin associated inhibitors (MAI) inhibition and shown not to have increased CST axonal regeneration[64]. However, in contrast, a study conducted in 2003 supported CST axon sprouting when the Nogo receptor was subject to a peptide antagonist[65]. Further, mice without Nogo A/B showed sprouting tendencies of the CST[66]. Chondroitinase ABC further has the power to encourage CST regeneration below the lesion[60]. Further, deletion of PTEN, upregulates mTOR which has shown to promote CST axon regeneration[59]. Others studies mainly show the ability of axons from the CST to undergo sprouting after a treatment attempt for SCI, which typically remains in the white matter, not the lesion site[55,63,67].

A pivotal study of cell transplantation conducted by Lu et al, showed promising survival of neural stem cells or progenitor cells derived from embryonic spinal cord, embryonic stem cells, and human induced pluripotent stem cell derived neural stem cells (iPSC-NSCs) in a C5 rat hemisection model in the host[47,70]. Further, these grafted cells showed robust axonal outgrowth both high in density and distance suggesting attempts at connectivity with host axons[47,70]. From here, the Kadoya et al study results set the framework for this study. When given a neural progenitor cell graft, the CST axons were

shown to regenerate indicating that NPCs provide a promoting substrate for regrowth[68]. Most axons regenerated in the rostral portion of the graft[68].

Hypothesis

Based on this previous research, our goal is to be able to visualize the growth of individual axons into a neural stem cell graft. We hypothesize that CST axons undergo regeneration, regenerative sprouting, and sprouting into grafted neural stem cells. As the Kadoya et al paper labeled a large number of axons, it is difficult to clearly distinguish each individual axon's pathway to regrowth after an injury. The ideal strategy for this study is to reduce the number of axons traced with either AAV8-CAG-rCOMET or BDA. Through a three-fold method: reduction of tracer injection sites, injection volume, and concentration of tracer—we attempt to reduce the number of axons labeled in order to visualize individual axon growth into the graft. The visualization technique used was the z-stack function on an Olympus confocal Microscope.

CHAPTER 2: MY EXPERIMENT

Abstract

Neural stem cells or neural progenitor cells have been extensively studied in spinal cord injury (SCI) rat models to attempt to reconnect injured circuits to restore function. Attempts have shown significant success, and further studies have revealed corticospinal tract (CST) axon growth into the neural stem cell graft. The purpose of this study is to understand whether CST axons undergo regeneration, regenerative sprouting, or sprouting into a neural stem cell graft. The animals were grafted with E14 spinal neural progenitor cells in a C4 dorsal column lesion for this study. The rat models were then injected with a small volume and concentration of a tracer, AAV8-CAG-rCOMET or BDA, in the motor cortex of the brain to label a few individual axons in order to visualize their growth pathway. Through the visualization on the confocal microscope, the injured axons of the CST are shown to regenerate into the graft cells. There was minimal regenerative sprouting and no sprouting seen with this model. Thus, CST axons regenerate into a neural stem cell graft.

Introduction

Typically, SCIs cause damage to local neurons as well as white matter tracts in the spinal cord, including the most important tract, the corticospinal tract. CST is specifically important in the voluntary circuits and fine motor skill[69]. As part of the CNS, CST axons face a wide range of both intrinsic and extrinsic inhibitory cues, making it difficult to regenerate[21]. Currently many studies are underway to attempt to promote CST axon regeneration, which if successful can aid in reversing the motor deficits and paralysis seen in SCI.

To clarify, the definition of regeneration is more specific than commonly used. As described by Tuszynski and Steward, regeneration is the growth from the injured tip of a transected axon[62]. Regenerative sprouting is growth of the proximal shaft of the injured axon[62]. Further, compensatory sprouting is the growth from a spared axon[62]. As shown by Kadoya et al, injury is necessary for sprouting to occur[68].

Multiple studies have been conducted to counteract the inhibition of myelin associated inhibitors, Nogo, MAG, and OMgp, and promote regeneration of the CST. Zheng et al showed that the deletion of the Neuronal Nogo Receptor showed no increase in CST axon regeneration, in contrast to other studies which either knocked out Nogo or used a receptor antagonist which resulted CST regeneration[64,65,66]. Further, to study intrinsic growth capabilities, PTEN gene knockdown has further indicated an upregulation of mTOR and promotion of intrinsic growth of axons in the CNS[59]. However, most CST growth is around the injury site and a few axons penetrate the injury site through spared glial processes.

Based on the Lu et al studies in 2012 and 2014, neural stem cells or progenitor cell derived from embryonic stem cells, embryonic stem cells, and induced pluripotent stem cell derived neural stem cells proved a useful cell transplantation therapeutic[47,70]. The grafted cells survived and grew into the host to form connections with host axons. From here, the Kadoya et al study showed promising levels of host axonal regeneration and regenerative sprouting into grafted neural stem cells[68].

Based on these prior studies, our objective is to be able to visualize individual CST axonal growth into grafted tissues. We hypothesize that these axons undergo regeneration, regenerative sprouting, and sprouting into a neural stem cell graft. To study this, our main strategy is to have sparse labeling of the main tract axons using tracers like AAV8-CAG-rCOMET or BDA at low volume, concentration, and fewer points of injection in the motor cortex. Our model is the C4 wire knife dorsal column lesion with a E14 spinal cord derived neural progenitor cell transplant.

Materials and Methods

Strategy

In order to properly study the individual axon growth into the graft, the ideal approach would be to label a very few axons using tracers such as AAV8-CAG-rCOMET, which is the Codon Optimized Membrane Embedded tdTomato, or biotinylated dextran amine (BDA). As it is a variant of RFP, AAV8-CAG-rCOMET will be also be referred to as RFP throughout the paper. BDA is a common anterograde tracer used for studies like this. RFP uses a viral delivery system to label the membrane of the injected cell.

Previous studies used an 18-point injection per hemisphere delivery system. In order to achieve individual axon labeling we chose a 2-point injection method. Further, the volume was reduced from the typical 4-5 μL per hemisphere to 1 μL per hemisphere. Finally, the dosage was reduced from 10^{13} genome copies/mL, the original virus titer, to somewhere between 10^{11}-10^{12}. For BDA, the concentration was decreased from 10% to a concentration between .5% -1%.

Preliminary Research

In order to address the strategy, some preliminary experiments were conducted to determine the appropriate virus dosage or BDA concentration.

AAV8-CAG-rCOMET- Dosage Study

A dosage study was conducted to determine the appropriate titer of AAV8-CAG-rCOMET. Female F344 rats were used. The rats were tested for an appropriate dilution of the virus in order to get sparse labeling of the axons. The virus was tested at three separate dilutions 10^{12}, 3 x 10^{11}, and 10^{11}. Each rat was cortically injected at 2 points per hemisphere to label axons in the main CST. The injection coordinate on the rostral-caudal axis was

2mm caudal to the bregma, and on the medial-lateral axis, 1.9mm, 3mm, -1.9mm, and -3mm. Three animals were injected with the lowest titer, two with the middle titer, and two with the highest titer. The animals were then perfused 4 weeks post graft, and the tissue was processed as described below.

BDA Dosage Study

Three male rats were injected at 2 points per hemisphere. A total of 2 μL were used per animal with 0.5μL per point. The coordinates were 2mm caudal to the bregma, and on the medial-lateral axis, 1.9mm, 2.9mm, -1.9mm, and -2.9mm. Two animals were tested with 1% BDA, and the other was tested with 0.1%. The animals were perfused 4 weeks post graft and tissue was processed.

Experimental Research

E14 Spinal Cord Derived Neural Progenitor Cell Harvesting

As adapted from the Kadoya et al study, E14 spinal cord derived neural progenitor cells were harvested as follows. GFP-expressing transgenic rats were used. E14 spinal cords were removed and subject to .05% trypsin to digest for dissociation[68]. These cells were then re-suspended in PBS prior to grafting in the injury site.

A total of 24 Female Fischer 344 rats were introduced to a C4 dorsal column lesion to transect the dorsal CST main tract through a wire knife lesion. After 4 days, the rats were grafted with freshly harvested E14 spinal cord derived neural progenitor cells. Some animals were grafted on the same day as the lesion. Each animal was injected in the lesion region with approximately 200,000-500,000 cells in 1-2μL of volume. A week later, the animals were injected cortically in the at the following coordinates: on the rostral caudal axis, the animals were injected at 1.5mm caudal to the bregma, and on the medial lateral

axis, they were injected at 1.8mm, -1.8mm, 3mm, and -3mm in relation to the bregma. Some animals were injected with the AAV8-CAG-rCOMET: 4 were injected at $3x10^{11}$ genome copies/mL, 3 were injected at 10^{11} genome copies/mL, and 4 were injected with 1.75×10^{11} genome copies/mL. Further, five animals total were injected with 1% BDA. The remaining 8 were subject to RFP and BDA co-injections of $5x10^{10}$ RFP and .5% BDA. The animals were perfused 4 weeks post-graft.

Tissue Processing

Perfusion

The rats were deeply anesthetized and transcardially perfused with the following materials: 4% paraformaldehyde (PFA) and perfusion saline. The rats were opened and pumped with cold saline solution (100ml-200ml/rat), followed by cold 4% PFA (300mL/rat). The cords were then collected from the animals and post-fixed in the 4%PFA solution. They were switched within 4 hours to sucrose for cryoprotection for a minimum of 48 hours before sectioning in the cryostat.

Sectioning

The cords were then dissected. For the preliminary study animals, the cords were cut into a 2mm block at the cervical C3, Thoracic T6-7, and Lumbar L3-4. The cords were then cut at the coronal plane at a thickness of 30-35 μm using the cryostat. They were collected in tissue collection solution (TCS) and stored at $4^{o}C$. For the animals that received a neural progenitor cell graft, the cord was cut into a 1 cm block, which contained the lesion and graft in the center of the block, and cut on the sagittal plane on the cryostat at a thickness of 30 μm.

Immunohistochemistry

RFP labeling

To visualize RFP labeled CST axons, the sections were immunolabeled with antibodies against RFP. The sections were washed in TBS before undergoing a graded methanol treatment to enhance immunolabeling. The sections were then blocked in TBS containing 5% donkey or goat serum and .25% Triton-X100 for an hour at room temperature before being placed in this block serum with primary antibodies against RFP (Rockland, 1:1000, rabbit), GFP at (Aves, 1:1000, chicken), and NeuN at (Millipore, 1:1000, mouse) overnight at 4 degrees. The sections were then rinsed the next day in TBS with 3% donkey or goat serum (animal serum must remain consistent throughout protocol) and .25% Triton-X100, before being placed in the same solution with secondary antibodies at room temperature for 2.5 hours, ThermoFischer Alexa Fluors (488, 568, and 647nm, 1:833).

BDA RFP Co-Labeling

When labeled together, the sections were treated as above for RFP labeling, and included an additional BDA step during the incubation with other primaries. The BDA axons were labeled using Streptavidin Alexa Fluor at 647nm (1:250), specifically when co-injected with RFP.

BDA

For BDA only sections, a variation of the RFP protocol was used. BDA was labeled with Streptavidin Alexa Fluor 568nm at 1:250 during the primary incubation step with GFP (Aves, 1:1000, chicken) and NeuN (Millipore, 1:1000, mouse) which were then labeled with the secondary antibodies ThermoFischer Alexa Fluor 488nm and 647nm

respectively. The sections were then washed, mounted on slides, and coverslipped with Fluormount G (Southern Biotechnology Associates, Inc., Birmingham, AL).

Visualization

Microscopy

Images were taken on the Olympus Confocal Microscope. Images were captured using the z-stack function on the 20x objective with oil. Additional images were captured using the Keyence BZ –X700 (Keyence, Woodcliff Lake, NJ) at 20x. The images were taken using the z stack function as well as the xy stitch. From here the images were merged and stitched on the Keyence Analysis Software. Through the use of Adobe Photoshop CS6 (Adobe Systems, San Jose, CA) captured images were adjusted for both brightness and exposure for better visualization.

Quantification Criteria

In order to quantify the number of axons that fall under each specification, imaged sections were primarily categorized by definitions set forth in the Tuszynski and Steward review[62]. Axons were classified as direct regeneration if a main tract axon was seen parallel to the other main tract CST and directly penetrated from the white matter into the graft cells. Regenerative sprouting posed a bigger challenge, however, through our best judgement, we decided a branch off of a main tract CST which specifically penetrated the graft host interface, could be considered an axon undergoing regenerative sprouting. Sprouting was judged based on whether any intact axons from ventral or lateral CST branched into the graft.

Results

Preliminary Research

Strategy

As part of the strategy expressed previously, three main changes occurred to control the number of axons labeled. Typical cortical injections are conducted in an 18 point per hemisphere manner. After reducing the number of points of injection to 2 points per hemisphere, the volume was also significantly reduced. With typical 18 point injections, the volume is 4-5uL per hemisphere. In this case, the volume was reduced to 1uL per hemisphere.

Typical titer of the virus used for labeling axons is 10^{13}. From the preliminary dosage study, as seen in Figure 1, the aim of this was to label a few axons so as to be able to trace single axons into the graft. It was determined that a concentration between $3x10^{11}$ (Figure 1B) and 10^{11} (Figure 1C) of the RFP virus would be best to trace a minimal number axons, as opposed to the hundreds of axons labeled in the highest concentration condition (Figure 1A). The optimal number of axons were typically traced with $3x10^{11}$ or $1.75x10^{11}$ of the RFP. Similarly, a similar dosage study conducted for BDA titer suggested a percentage of .5%-1% would provide the appropriate labeling of axons.

Looking at the sagittal sections of the cord provides a clearer view of the axons, indicating in fact how few axons are labeled for this study. Again, the labeling using the 10^{13} titer (Figure 1D) strongly labels many main tract axons, making it difficult to specifically trace the path of one single axon. For the RFP and BDA co-injections, the typical number of axons labeled are shown in Figure 1E. The individual axons are much

more distinguishable from one another at 5 x 10^{10} RFP and .5% BDA compared to the tracing in Figure 1D.

Figure 2 depicts a typical graft with this model. The main tract CST axons are labeled with RFP and are located rostral to the graft. There is a prominent graft host interface, and the main area of focus for this study will be the rostral interface where the CST axons encounter the graft. An intact axon was found to innervate the gray matter through sprouting behavior and further innervated the gray matter (Figure 3). The axon was used as comparison for the many branching behaviors of the experimental axons.

Experimental Research

Regeneration

As expressed before by Tuszynski and Steward, regeneration is the term used to describe the growth from the injured tip of a severed axon[62]. As seen in Figure 4, axons from the injured end of a main tract CST axon directly penetrate the graft. Once in the graft, these axons display a variety of described characteristics of regenerative axons like branching and curving. As previously shown, regenerative axons no longer show a linearity in growth as shown by the parent intact CST axons[55]. During regeneration. these axons undergo curves and turns, and follow a more tumultuous path. Figure 4A &4B depict a main tract axon as it encounters the graft host interface. Though the origin is not seen on this plane, it can be considered a main tract axon as it is starts parallel to other labeled main tract axons. It grows for a distance before characteristically turning almost ninety degrees. Another example seen with Figure 4C & 4D depict another CST axon from the main tract in the white matter as it penetrates the graft. This axon travels for a distance into the graft before depicting branching and terminal-like structures.

As seen with Figure 5A-B two main tract CST axons penetrate the graft host interface from the white matter into the graft and display these prominent branches. The epitome of regeneration is Figure 5C-D depicting a linear main tract axon, running parallel to other main tract axons, as it penetrates the graft and further depicts a branch point. Other main tract axons are observed to form retraction bulbs prior to the graft host interface. CST main tract axons from the white matter into a graft further show vibrant branching abilities potentially mimicking the ability of CST axons to branch into the gray matter (Figure 5E-F).

Regenerative Sprouting

Axonal growth from the proximal shaft of an injured axon was seen in two potential examples of regenerative sprouting. These axons have a main tract parent axon which form branch points which further penetrate the graft. Figures 6A-B depicts a potential example of both regeneration and regenerative sprouting. At the primary branch point, it is unclear which branch is in fact the parent axon, however both penetrate the graft tissue. Figures 6C-D depict an axon which branches at two points. One makes it into the graft potentially classifying it as an example of regenerative sprouting. However, the remainder of the axon is not seen in this plane of section.

Sprouting

There were no forms of sprouting observed within this model. The expectation is to see sprouting from the dorsal CST which terminates in the gray matter, or lateral or ventral CST which remain intact. The axons must be touching the graft in an injured model to observe any sprouting. As seen in Figure 7, the ventral CST is proximal to the graft, however, there is no sprouting or penetration of the graft from these intact axons.

Primate Models

Dr. Ephron Rosenzwieg provided primate samples. A human neural stem cell graft in a C7 hemisection primate model and the main tract CST was labeled with BDA. The subject survived for 7 months post-grafting. The primate model also indicated examples of regeneration (Figure 8A-F).

Quantification

There are many limitations to this study, as will be discussed in the discussion section. However, based on our selection criteria, we were able to visualize 32 total axons that depicted growth into the graft. About 30 of these were examples of regeneration, and two were potential examples of regenerative sprouting.

Figures

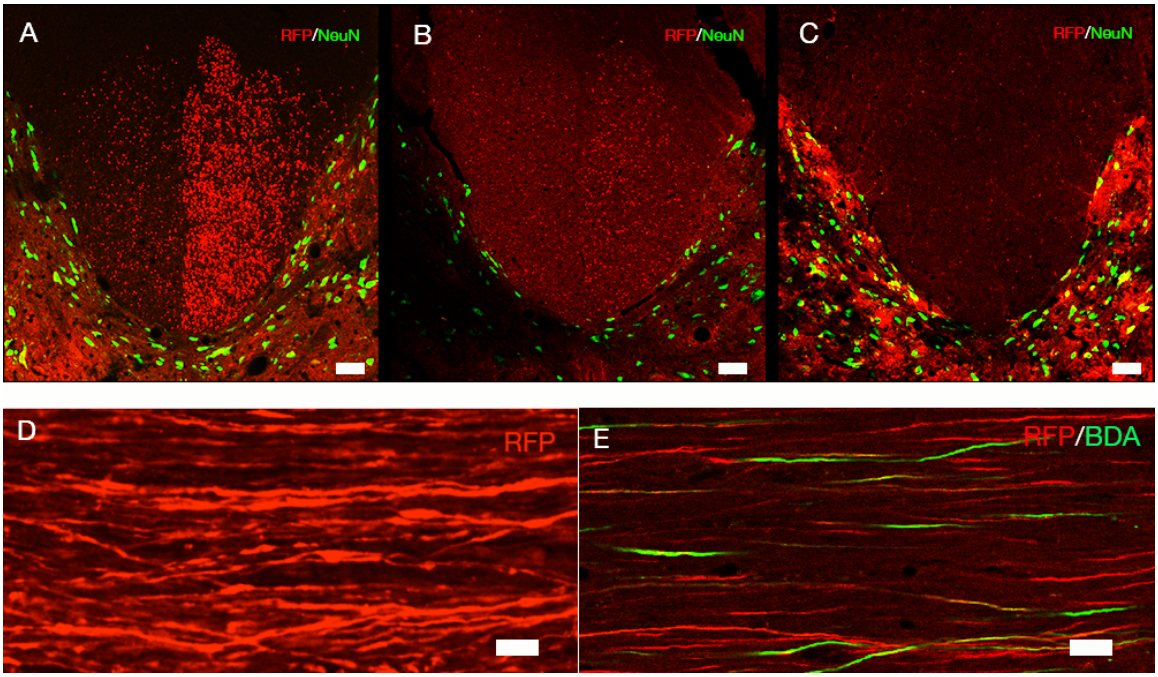

Figure 1. Dosage Study Results These images show the number of CST axons labeled based on different dosage treatments of AAV8-CAG-rCOMET injected in the motor cortex. **(A)** shows the labeling of the dorsal column of a C3 cross section of 30 µm thickness. The axons were labeled for RFP and the section was co-labeled with NeuN to indicate gray matter cells. This animal was originally treated with 10^{12} titer of AAV8-CAG-rCOMET. Scale bar represents 50 µm. **(B)** shows a cross section at C3 of the dorsal column where the CST was labeled with $3x10^{11}$ of RFP, and the section was also stained with NeuN. Scale bar represents 50 µm. **(C)** shows a cross section at C3 with labeling for RFP at a concentration of 10^{11}, and the section was counterstained for NeuN. Scale bar represents 50 µm. **(D)** shows a sagittal section of 30 µm in the C2-C3 region of the rat spinal cord treated with 10^{13}, the original virus titer. Scale bar represents 10 µm. **(E)** shows a sagittal section of an animal co-injected with $5x\ 10^{5}$ RFP and .5% BDA. Scale bar represents 10 µm.

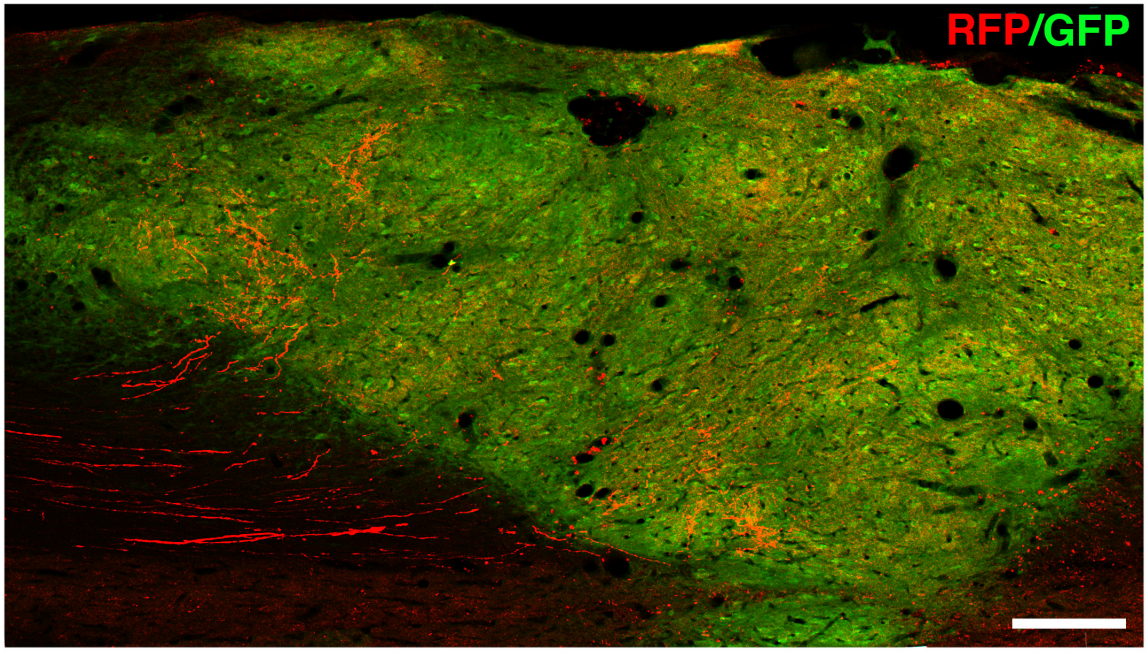

Figure 2. Typical Graft Size This image shows a typical graft size of the spinal cord derived neural progenitor cells which express GFP. The CST axons run rostral to caudal, and the area of interest for this study is the rostral graft-host interface. The scale bar represents 200 µm.

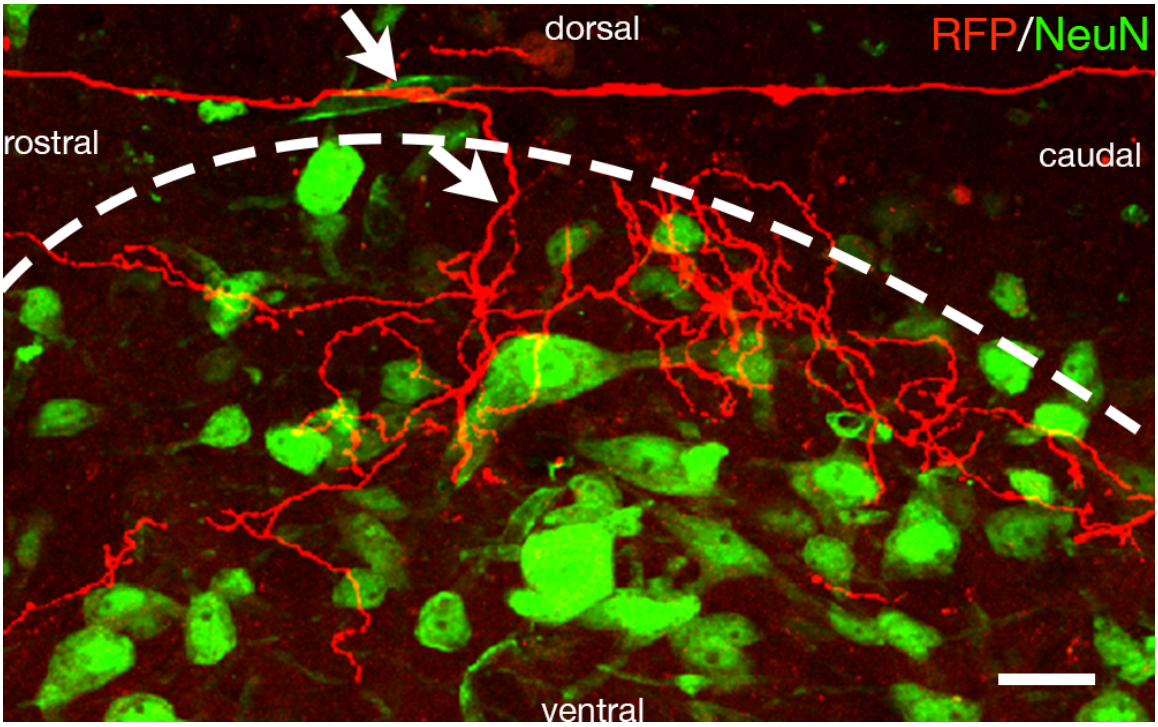

Figure 3. Intact CST This image shows an intact CST axon from the main tract sprouting into and innervating the gray matter. This section was counterstained with NeuN to label the nuclei of neurons in the gray matter. Scale bar represents 50 μm.

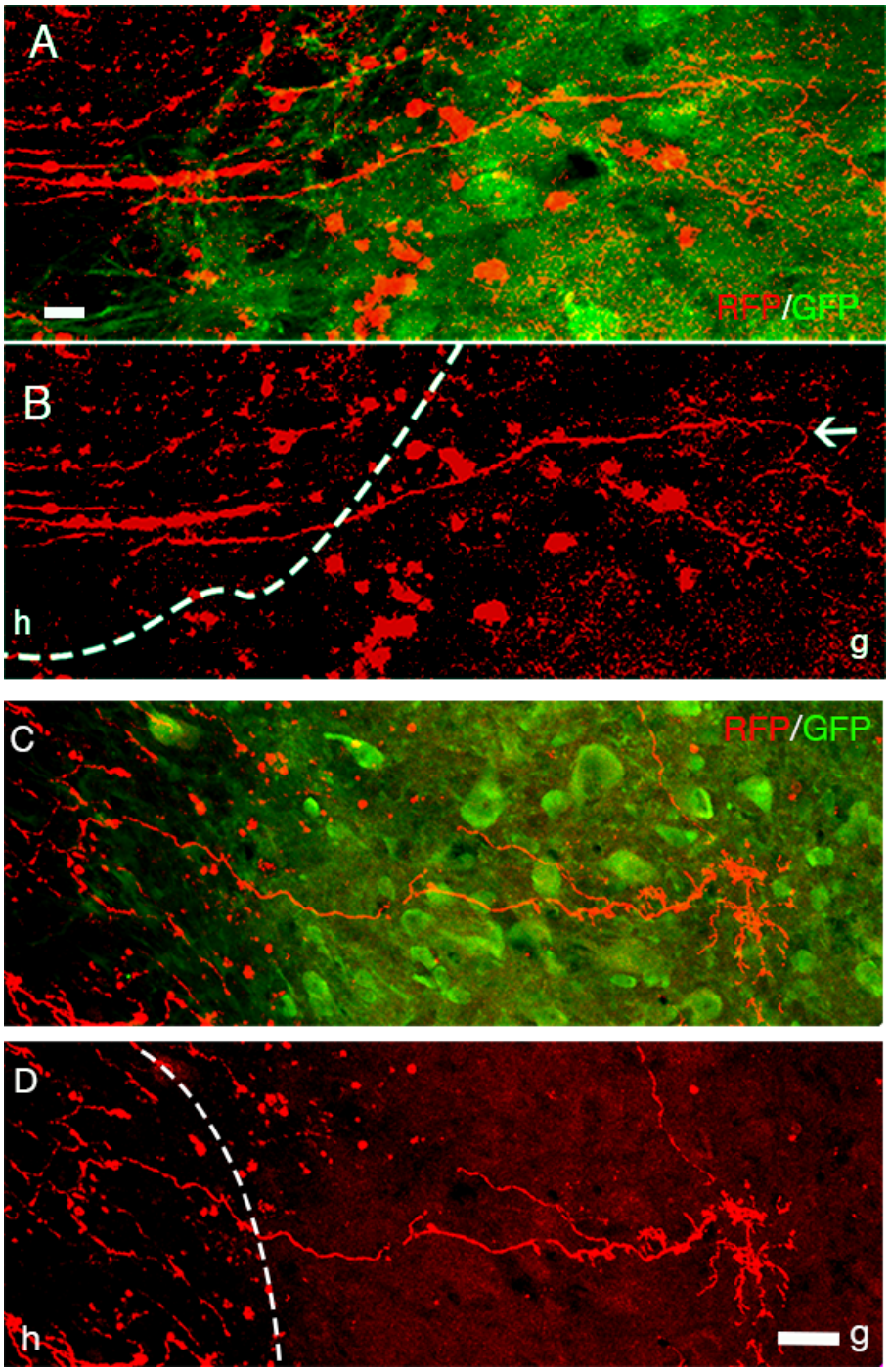

Figure 4. Examples of True Regeneration These images depict examples of true regeneration. **(A)** shows a main tract CST axon as it penetrates the graft labeled with GFP. **(B)** shows just the RFP channel, with the host (h) and graft (g) interface indicated. The arrow points to the curvature at the end of the axon in this plane. The scale bar represents 20 µm. **(C)** shows another example of direct regeneration. **(D)** depicts the RFP channel only with the interface indicated. Scale bar represents 60 µm.

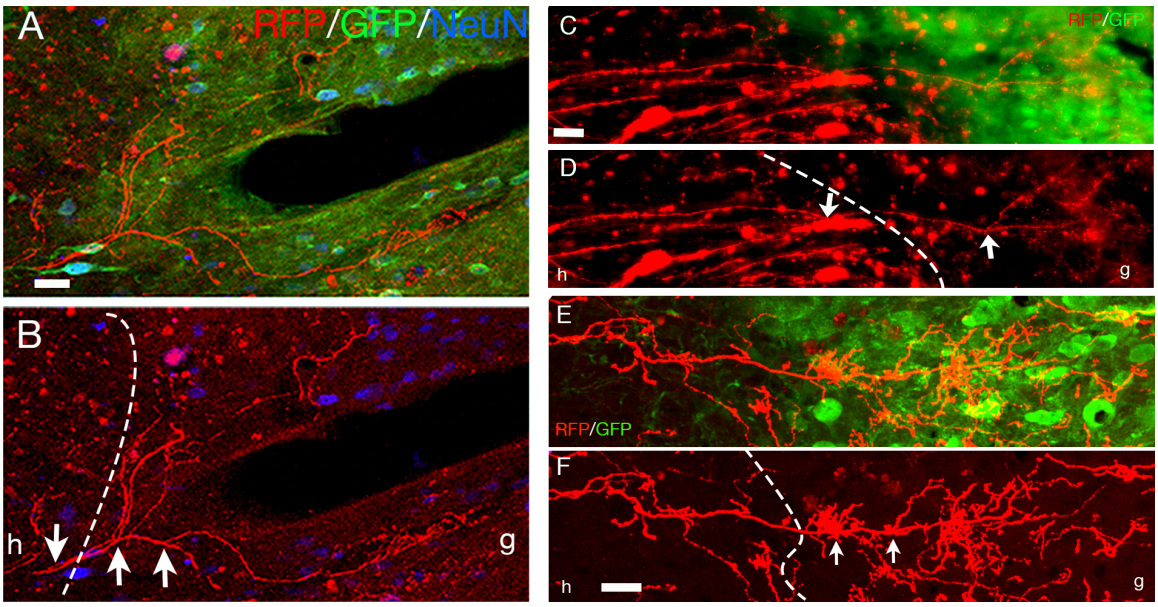

Figure 5. Additional Examples of Regeneration. **(A)** shows two main tract axons following a similar path into the graft labeled with GFP. The section is counterstained with NeuN which is a neuronal marker that labels neurons in the graft. **(B)** shows only the red and blue channels. The axon branches once in the graft. Scale bar represents 40 µm. **(C)** shows an additional example of regeneration. The main tract axon grows from the white matter main tract and penetrates the graft. **(D)** shows only the RFP labeling. The left arrow indicates the continuity of the same main tract axon, as other RFP may hinder the visibility of the axon. The scale bar is 20 µm. **(E)** shows an example of a main tract axon with robust branching into the graft. The scale bar represents 20 µm.

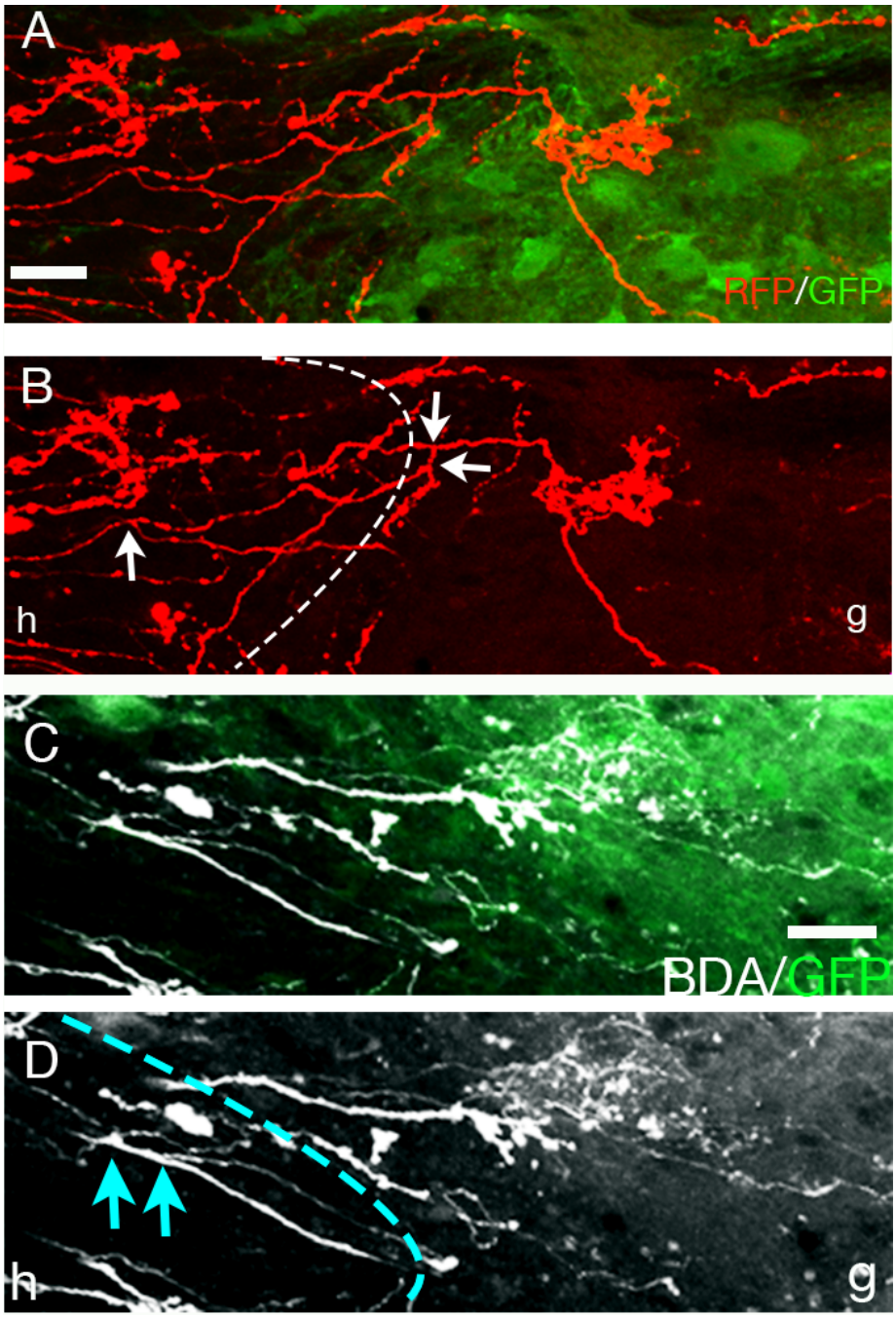

Figure 6. Examples of Regenerative Sprouting (A) shows an example of a main tract axon entering the graft. The scare bar represents 20 µm. **(B)** shows only the axon, without the GFP labeling. At the primary arrow, the axon can be seen to branch, growing proximal off the main shaft. The axon then seems to continue into the graft at multiple branch points both from the cut end and a branch point. **(C)** shows a main tract axon labeled with BDA entering the graft labeled for GFP. The scale bar represents 20 µm. **(D)** shows an axon labeled with BDA branching at two points, one of which enters the graft.

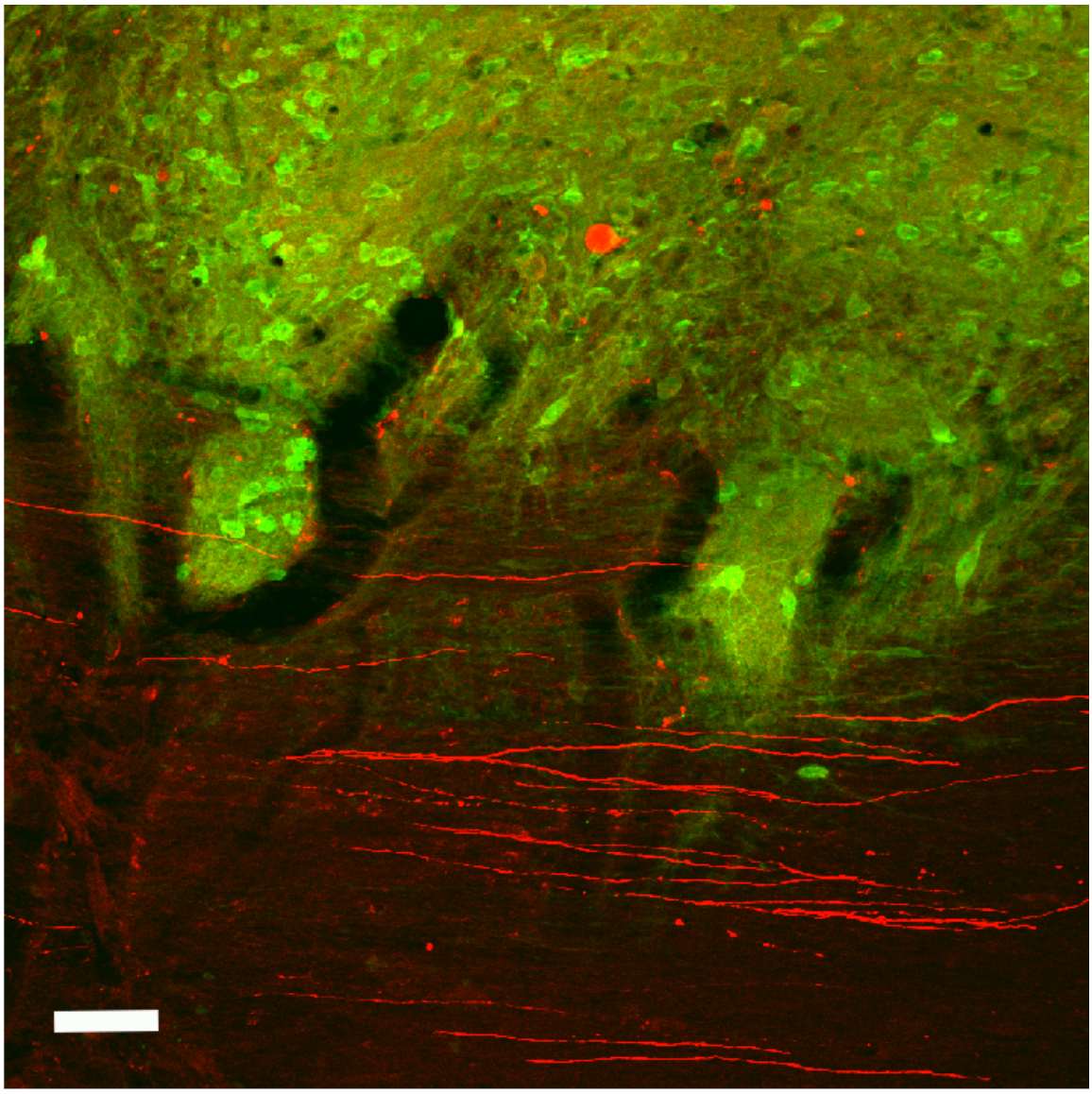

Figure 7. Ventral CST. This image depicts the ventral CST as it encounters the graft. The scale bar represents 64 μm.

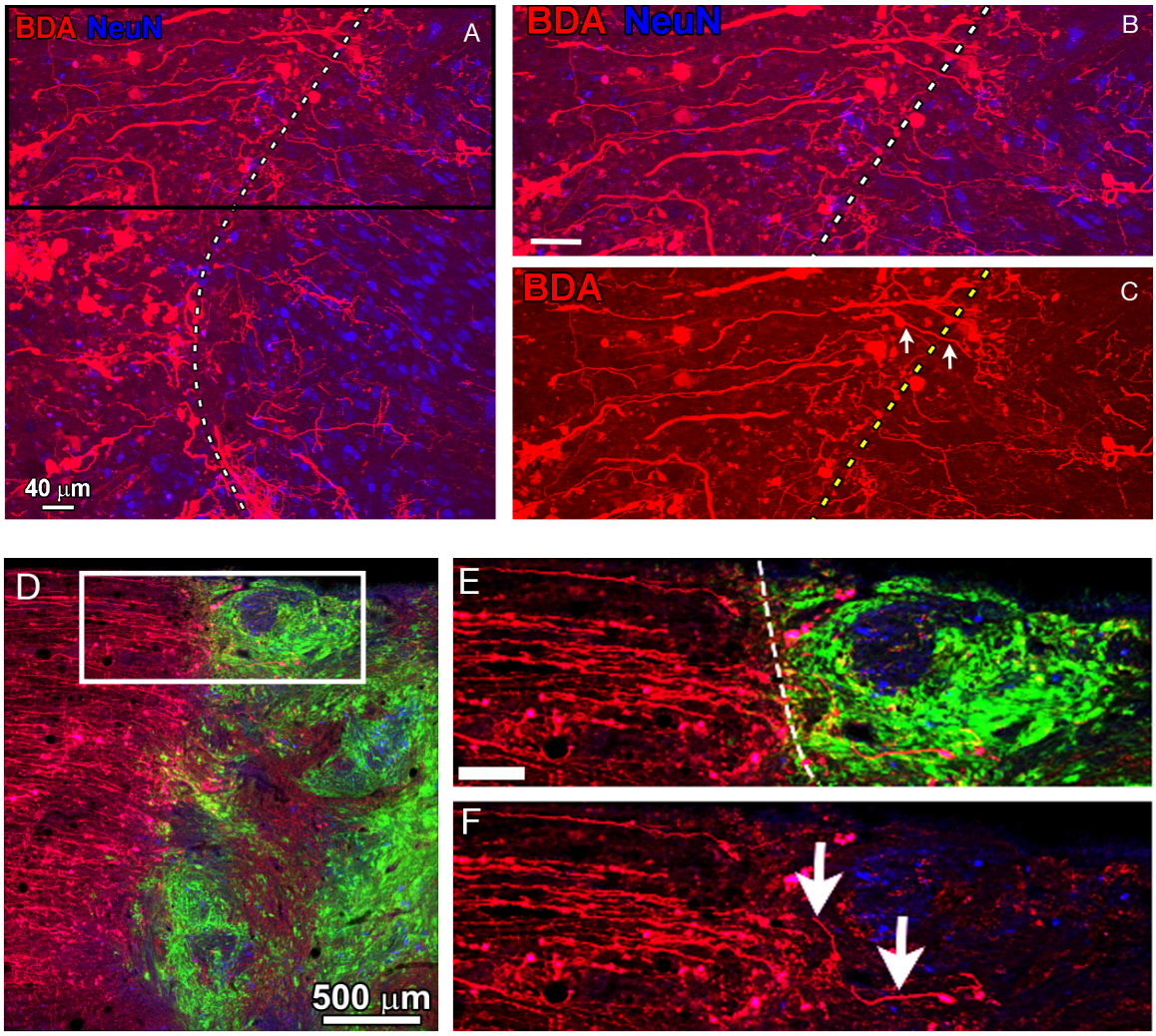

Figure 8. Examples of Regeneration in Primate Models. All images were obtained from Dr. Ephron Rosenzweig. **(A)** shows BDA labeling of the CST in the cervical region. The boundary represents the graft host interface, and the blue labeling is a NeuN counterstain. **(B)** and **(C)** show a specific region of A. The arrows indicate a specific axon crossing the graft host interface. The scale bar represents 40 μm. **(D)** shows a different primate where the graft again is labeled with GFP antibodies, and the main tract is labeled for BDA. **(E)** and **(F)** show a region of D where an axon can be seen crossing the main tract. The scale bar represents 200 μm.

Discussion

Previous studies show that though main tract CST axons can in fact regenerate; axons have shown to sprout or regeneratively sprout around the lesion but seldom penetrate into the lesion site[55,63,67]. The results from this study support the idea that in the face of injury, given a neural stem cell substrate, host CST axons do in fact have a regenerative potential. Specifically, within three described concepts for growth post injury, a majority of the axons studied seemed to display direct regeneration, where the axons grow directly from the cut end of the injured axons. This suggests that the active formation of the growth cone and successful actin polymerization to aid in the axon's growth into the graft. Further, we found evidence to support that there was some potential for regenerative sprouting that occurred with this model. There were no forms of sprouting observed.

In the Kadoya et al study, the concept of regenerative sprouting was observed when a graft was introduced above the main tract and the tract was cut slightly caudal to the site of the graft[68]. In our model, this was also studied, and a few axons supported the idea that regenerative sprouting can occur. However, regenerative sprouting is difficult to particularly study and find, so the actual prevalence of this form of growth could potentially be higher than we actually observed. The difficulty arises again with our specific method limitations where not all axons remain in the same plane. Ideally, any 3D visualization technique could perhaps provide better input on whether it is in fact regenerative sprouting that occurs.

From here, after observing that a majority of growing axons undergo, it is important to understand what in fact causes this growth potential. What cues or target cells in the graft help to promote this CST axon regeneration? Ideally, we would want to enrich the

graft with this cell type or specific extrinsic cues to upregulate the growth of CST after injury. As observed, very few axons actually grow into the graft post-injury, and the vast majority of injured CST axons displayed retraction bulbs. Using the graft's cues, we would hope to increase both the density of axons that undergo regeneration, and the distances that these axons are capable of regenerating. Above all, we would hope to understand these targets, and their attraction to regeneration to understand and encourage connectivity from these regenerating axons.

Method Limitations

Studying individual axons can be tricky, as they do not necessarily remain in the same plane in the spinal cord. The ideal method to really study axon regeneration would be to visualize the individual axons in a three-dimensional view. This would allow complete tracing of the individual axon, and would better help us observe the phenomenon of sprouting, regeneration, or regenerative sprouting. To address this, two separate methods were tested to obtain this 3D view of the axons.

Tissue Clearing

Primarily, tissue clearing was attempted. Though the clearing itself was successful, the native fluorescence of the RFP being weak combined with the harsh clearing conditions provided minimal to no visual clarity through a microscope. From here, the next steps that can be tried are antibody staining of the cleared cord.

3D Reconstruction

Further, 3D reconstruction using serial sections was attempted using the software Volocity. After many attempts of taking images from the Keyence, to utilizing Image J to align the individual images, and further using Volocity to render the images, this didn't

produce the result we wanted to see. Unfortunately, despite our attempts to label a low density of axons, the number of axons labeled were still too many, making it difficult to track the path of an individual axon. At this point, it is best to attempt to further reduce the number of axons labeled to help us really study individual axons.

Microscopy

Finally, within our own methods, there were some limitations. Primarily, the section thickness itself is 30μm. Within that, the axon doesn't necessarily travel within the same section. Due to this, it is difficult to completely trace an axon from its origin into the graft. Many times, the axon will be present in the graft indicating some sort of growth, but the true origin is unknown making it difficult to truly decipher which axon grew, and whether it underwent regeneration, regenerative sprouting, or sprouting itself.

Again, with these limitations it is difficult to successfully interpret the results as strictly one form of growth over the other. With these method limitations, quantification was a difficult process. With limited 3D capacity of our visualization technique, it is difficult to determine with complete certainty whether an image is an example of regeneration or regenerative sprouting. Further, with more than one axon per section, classification of each individual axon posed great difficulty. Above all, axons didn't remain in the plane of the section, making it difficult to truly determine the type of growth. However, with the results set forth from previous studies, the results from this study truly support that CST axons undergo true regeneration.

CHAPTER 3:

CONCLUSION

As observed with this study, CST axons do in fact regenerate into a neural stem cell graft, suggesting that these can serve as potential targets or growth promoting cues for regeneration. Even with the methodical limitations present, we were still able to observe significant true regeneration and growth primarily from the cut end of an injured CST axon. Further, the number of axons that seemed to have grown into the graft are quite limited. To ensure that the cell therapeutics work, it is important to understand the promoters of both direct regeneration and regenerative sprouting to further enrich the graft. Ideally, we would hope to promote growth of these axons to increase in both distance and density. Further, it would be important to focus on the innervation capabilities of these axons within the graft to promote connectivity to graft cells. The hope with this method is to set an appropriate relay to other host systems that may have been severed due to injury.

Spinal cord injuries can be severe, and ideally, we would want to reduce and eventually obliterate the negative symptoms of loss in motor and sensory function and paralysis caused by this injury. There are currently a variety of therapeutic options being studied that work to reduce the severity of these symptoms as done through neural protection, remyelination, and regeneration. Within regeneration studies, a growing field are cell transplantation studies where stem cells are placed in the lesion cavity to act as a substrate for regeneration and connectivity. Understanding the internal capacity of an axon's ability to respond is important to study and clearly understand. The regeneration ability will provide information regarding the direction research must take to further promote this in more axons and for higher distances. The results from this research support the concept of regeneration and can serve as a foundational basis for future studies in spinal cord injuries and other neurodegenerative diseases.

CHAPTER 4:

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