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Examining spinal axonal degeneration and regeneration with in vivo imaging and experimental
spinal cord injury

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of
Philosophy

in

Biomedical Sciences

by

Ariana Joy Orduna Lorenzana

Committee in charge:

Professor Binhai Zheng Chair
Professor Yishi Jin
Professor David Kleinfeld
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Professor Tony Yaksh

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University of California, San Diego

2014

DEDICATION

I dedicate this dissertation to my husband, Gleb Martovetsky, and my daughter, Mila Lorenzana Martovetsky. There is nothing in life that parallels the love I have for both of you. You are the light at the end of the tunnel. Thank you for a lifetime of inspiration.

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Chapter 3, is a preliminary study. The dissertation author was the primary investigator. Jeff Kwan assisted in node quantifications. I thank Pearl Shih, Omeed Ghassemi, and Amy Chang for their technical assistance and hours looking at and quantifying axons. Binhai Zheng supervised the project and provided advice.

Chapter 4, in part is currently being prepared for submission for publication of the material by authors: Cedric Geoffroy, Ariana Lorenzana, Jeffrey Kwan, Binhai Zheng. The dissertation author was a co-investigator and co-author of this material. The authors would like to thank Omeed Ghassemi, Daniel Creger for their technical support, behavioral analysis, and many hours quantifying.

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ABSTRACT OF THE DISSERTATION

Examining spinal axonal degeneration and regeneration with in vivo imaging and experimental spinal cord injury

by

Ariana Lorenzana

Doctor of Philosophy in Biomedical Sciences

University of California, San Diego, 2014

Professor Binhai Zheng, Chair

Spinal cord injury is a permanent, physically, and occasionally mentally, debilitating condition with no robust panacea available. Currently, there are 5.6 million Americans living with spinal cord injury, with an estimate of 10,000-12,000 new injuries per year [1]. The permanency of the functional deficits after spinal cord injury is due to the inability of central nervous system (CNS) axons to regenerate, which in turn is attributed to a multitude of neuron-intrinsic and extrinsic factors. Studies of spinal cord injury have traditionally been hampered by the difficulty of identifying regenerating axons, a myriad of confounding variables, and limited temporal and spatial resolution.

The main goal of my thesis work is to develop in vivo imaging with 2-photon microscopy to study spinal axon responses to injury in living mice, and to start to apply this experimental paradigm to the studies of axon growth regulators. I have found that in vivo imaging is a powerful tool to investigate the dynamic responses of spinal axons to axonal injury. In Chapter 2, the main and publication-ready part of my thesis, I describe the baseline axonal dynamics, including degeneration and regeneration, after laser-mediated axotomy in the mouse spinal cord. The data depict the detailed behaviors of single sensory axons over hours (acute), days (sub-acute), weeks (sub-chronic), and months (chronic). An earlier phase of axon degeneration is followed by later phases of axonal regeneration, pruning and remodeling up to 6 months after the initial axotomy. Branch points and nodes of Ranvier emerge as important determinants of axonal degeneration and regeneration following injury. It will be difficult, if not impossible, to gain such insights from conventional models of spinal cord injury. In Chapter 3, I describe efforts to apply this imaging paradigm to examine the effects of deleting the prototypical myelin associated axon growth inhibitor, Nogo. I found that Nogo deletion slows the acute degenerative process, and also (regeneration results coming). Chapter 4 describes my collaborative effort with another lab member to examine the effect of combined deletion of Nogo and PTEN, a neuron-intrinsic inhibitor of regeneration. The results provide proof of principle evidence that manipulating both intrinsic and extrinsic growth regulators may lead to further enhancement in axonal growth after injury. My involvement in this project was meant to complement my imaging studies, which used a minimal laser injury model. In Chapter 5, I provide perspective for future endeavors.

CHAPTER 1.

Introduction

Background and Significance

Spinal cord injury: Brief Historical Account

The connection between spinal cord injury and functional deficits that occur as a result of injury has been known and studied since the age of ancient Egyptians, when it was documented that this is an “ailment not to be treated” [2, 3]. Later, the Greeks and Romans endeavored to treat the disease or at least the complications, such as ulcers [4]. It was not until a more systematic analysis by Ramon y Cajal (1852-1934), who documented in his early drawings the inability of the CNS axons to regrow with the first retraction bulbs after injury, that led to the term “abortive regeneration” [5]. Even more recently, David and Aguayo demonstrated that, in fact, the CNS axons are able to grow in a permissive environment such as one provided by a peripheral nerve graft; however, once reaching back to the CNS, regeneration abruptly stops[6]. Echoing the results from an earlier study by Tello, a disciple of Cajal, this study implicated the non-permissive nature of the CNS.

Mammalian Spinal Cord

The spinal cord and the brain comprise the mammalian central nervous system. The mammalian spinal cord is a complex input and output system that is essentially a highway for signal trafficking, integration, and in part, execution. The spinal cord itself is composed of four main cell types: neurons, oligodendrocytes, astrocytes, and microglia. Neurons are the principal cell type in the nervous system and carry out the task of signal transmission. Glial cells play supporting roles. Anatomically, spinal cord can be divided into a core, butterfly-shaped

graymatter, where the cell bodies of neurons reside, and the peripheral white matter. Within the white matter, there is a complex network of ascending and descending axonal tracts, which continuously receive input from and provide output into the neurons in the spinal gray matter throughout the rostral – caudal axis of the cord. Ascending sensory tracts provide pain, temperature, pressure, stretch information from the periphery, whereas descending motor tracts, send signals out from the brain to execute behavior. Surrounding the spinal cord are three meningeal layers: the dura mater, the dural sac, and the pia matter, from most external to internal [7].

The spinal cord can further be separated into different rostro-caudal segments: the 8 cervical spinal roots, 12 thoracic , 5 lumbar, 5 sacral, and one coccygeal (in humans). Within the spinal nerves are the dorsal and ventral roots. The dorsal roots contain sensory axons and the dorsal root ganglion, which contains all of the cell bodies of the sensory afferents. The ventral roots contain the axons of the motor neurons originating in the spinal cord.

Within the spinal cord, there is further separation of white and grey matter, where the white matter is mostly comprised of myelinated axonal tracts, and the grey matter is composed of neuronal cell bodies. Within the grey matter, there is continued division into ten laminae, also known as the Rexed lamina, which further divides the spinal cord into segments where specific neuronal types are localized. For the purpose of my imaging studies, I chose to examine injury to large myelinated ascending sensory axons for two reasons: 1) This particular group of neurons is responsible for sensory input from the environment, and restoring sensation after injury are critical to preventing further injury. 2) In addition these axons are located on the dorsal aspect of the spinal cord, and thus, are the most technically feasible tract to examine with in vivo multi-photon imaging.

Pathophysiology

Spinal cord injury consists of two main stages: 1) First, there is the initial primary injury, which consists of shearing/severing and or contusion of neuronal tissue, damage to the

vasculature, and immediate, non-specific neuronal death. 2) Within hours to days, the secondary injury begins, and is a result of the body's response to the primary injury [8]. The secondary injury consists of, but is not limited to, ischemia, excitotoxicity, inflammation, continued axonal damage, free radicals and lipid peroxidation, ultimately leading to necrosis and apoptosis etc[8]. The primary and secondary injury affect the sensory and motor systems, along with autonomic function, which can often lead to autonomic dysreflexia [9].

Humans and rats share a similarity in injury formation and progression in that a fluid filled cyst forms in the spinal cord surrounded by dense gliotic tissue. This is in contrast to mice, whose immune response is different [10] and after injury the epicenter is full of cells and tissue [10-12]. However, all three have infiltration of macrophages, microglia activation, secretion of inflammatory cytokines, which in turn recruit more inflammatory cells and lymphocytes[13, 14]. While many initial studies on spinal cord injury used rats as the model organism, the laboratory mouse has seen increased utility in spinal cord injury research because of its genetic amenability [15].

In mice, the center of the lesion becomes filled with a dense fibrous tissue consisting of fibroblasts, extracellular matrix proteins, and other connective tissue within days of the injury [14, 16]. Meanwhile, astrocytes take on a reactive and proliferative phase, which is the beginning of glial scar formation. The glial scar acts as both a physical and chemical barrier to regeneration[17, 18]. The physical barrier is the condensed reactive astrocytes [19], the chemical barrier being the chondroitin sulphate proteoglycans that are secreted by the reactive astrocytes [20-22]. In addition, microglia quickly switch from an amoeboid to reactive state, and can also be seen in a second inflammatory wave months after injury [23].

After ~24 hours, the distal axonal segment undergoes Wallerian degeneration and is eventually phagocytosed by macrophages[24]. Monocytes/macrophages are recruited to the site of injury 2-3 days after the initial insult, and can remain activated for several weeks [25, 26]. At the sub acute phase (~ 2-3 weeks), the glial scar begins to stabilize, the neutrophils begin to decline, microglia and macrophages are still active and phagocytosing dead tissue. It is not until

after 6 months that an injury is considered stable and no further cell proliferation/recruitment occurs.

Differences between the CNS and PNS

The complexity of spinal cord injury is further convoluted by the fact that the CNS is averse to regeneration. The inability of axons to regenerate long distances is one defining feature of the adult mammalian CNS that differentiates it from the peripheral nervous system (PNS) [27]. This facet, unique to the CNS, has been well established. The idea that CNS axon regeneration is possible is supported by the fact that lower vertebrates such as fish and tailed amphibians *can* regenerate their CNS axons [28, 29]. In addition, embryonic CNS axons can regenerate in mammals [30]. Also, in the mammalian adult CNS, injured axons initially form growth cones that resemble those of developing axons [31]. A widely cited study in the 1980's by Aguayo and colleagues showed that CNS axons could regenerate when placed in a growth-permissive environment, such as that of a PNS graft [32-34]. This and other studies implicated a difference between the CNS and PNS environment. Scientists soon proposed the idea of extrinsic factors in the CNS environment playing a role in regeneration inhibition. The cellular/environmental differences between the CNS and PNS, however, were not elucidated until later. Some of the major differences will be described below.

Extrinsic Inhibitors

Myelin

First, one main difference between the CNS and PNS is the cellular environment, where the CNS contains oligodendrocytes while the PNS contains Schwann cells. Both are myelin-forming cells. Studies by Schwab and colleagues showed that CNS axons could grow on PNS myelin and not CNS myelin [35]. Thus, CNS myelin is inhibitory [36, 37] but PNS myelin is

growth permissive [34, 38, 39]. From these studies molecules now termed myelin associated inhibitors (MAIs) were found, with the three prototypical myelin inhibitors being, MAG [40], Omgp [41], and Nogo [42-44]. These MAIs are known to interact with Nogo-66 receptor (NgR1) to limit axonal growth. More recently, studies have shown that these three structurally unrelated ligands can also interact with other receptors, such as paired immunoglobulin-like receptor B (PirB) [45]. Typically, these MAIs are not found or are less abundant (in the case of MAG) in PNS myelin, which further supports the idea that the CNS environment is inhibitory to regeneration. A more in depth discussion of these MAIs will be reviewed in Chapter 3.

Among the myelin inhibitors, Nogo gained most attention because of evidence that antibodies to Nogo promotes long distance axon regeneration and functional recovery in rodent models of spinal cord injury. This was initially done with a monoclonal antibody IN-1, which may recognize protein species in addition to Nogo, and was later done with antibodies more specific for Nogo following the identification of the Nogo protein and gene. However, genetic studies in mice produced mixed results, indicating that targeting Nogo alone is more likely to affect the growth of uninjured axons (i.e. sprouting) than that of injured axons (i.e. frank regeneration).

In addition to these prototypical myelin associated inhibitors are netrin, semaphorin, and ephrin [46]. These newer myelin associated inhibitors were initially characterized as molecules primarily involved in attraction and repulsion, and thus guidance of developing growth cones, but have recently been associated with regeneration inhibition [47, 48]. However, their exact roles in injury-induced axonal growth remain to be fully explored.

Glial Scar and CSPGs

Unlike the PNS, the CNS forms an astrocyte derived “glial scar” after injury. Along with CNS myelin, the glial scar is also thought to be a major component of regeneration inhibition [49] Davies 1999. The glial scar presents not only a non-permissive physical barrier to regenerating axons, but also a chemical barrier, as it secretes molecules, such as chondroitin sulfate proteoglycans (CSPGs), which have been shown to inhibit regeneration [50-53]. CSPGs are

upregulated after injury, including neurocan, versican, brevican, phosphacan, aggrecan, and NG2, [54]. Several CSPG receptors have been identified, such as PTPsigma, NgR1 and NgR3 [55]. Degrading the inhibitory GAG side chains of CSPGs by Chondroitinase ABC (ChABC) has been shown to be beneficial functionally and anatomically after SCI [56]. However, glial scar also plays a protective role after spinal cord injury, such as reducing inflammation and secondary injury [16, 57]. In addition to reducing secondary injury, studies have also shown the glial scar to be growth promoting/permissive to specific subsets of neurons [58].

Growth factors and Guidance Cues

In addition to the presence of extrinsic inhibitors to regeneration, there is a lack of factors that may otherwise promote the regenerative response, such as growth factors and guidance cues. It is believed that the absence of these factors also contribute to the lack of regeneration seen in the CNS. Unlike the PNS, whose schwann cells secrete growth factors after injury, such as NGF, BDNF, IGF, CNTF, GDNF, etc, the CNS does not [59, 60]. Moreover, the PNS forms physical bridges at injury sites to help promote growth across the injury [61, 62]. In the PNS, after injury, the ability of an axon to regenerate may be limited by the absence of necessary growth cues and growth supporting molecules. Various studies have shown an increased regenerative response after SCI with the administration of guidance molecules and growth promoting factors [46, 63].

Intrinsic factors

Another difference between the CNS and PNS is the difference in their upregulation of regeneration associated genes after injury (RAGs) [64-66]. Studies by Bomze 2001 show that overexpressing RAGs increases sensory regeneration after SCI. In addition to the extrinsic barriers to regeneration are the intrinsic factors that favor the growth response.

Previously a major focus of the intrinsic pathway was on examining the conditioning effect of a peripheral nerve lesion and its growth enhancing effects on the CNS [67]. While this

approach led to the identification of various intrinsic factors that may increase an axon's regenerative capacity, none alone had as robust an effect as the conditioning lesion. For example, studies have shown that a single injection of a cAMP analogue can mimic the regeneration enhancing effects of a conditioning lesion [68-70]. While cAMP has been identified as a key molecule involved in the intrinsic pathway stimulating regeneration, its effects are not as robust as the conditioning lesion alone.

This suggests that there are other intrinsic factors that can modulate axonal regrowth after injury. Various researchers have endeavored to find other intrinsic molecules that may play a role in increasing axonal growth after injury [71], [70]. In recent years intrinsic molecules have been highlighted as important regulators of axon growth after injury. In a few cases, convincing evidence that manipulating such molecules increases CNS axon regeneration. Many transcription factors such as c-Jun [72, 73], SMAD1 [74, 75], ATF3 [76], KLF [77], STAT3 [78], others are kinases (Dlk1) [79, 80] [81], and more recently, phosphatases have implicated in playing a role in intrinsic regulation of SCI [82-86].

One such group has recently identified PTEN, a negative regulator of the mammalian target of rapamycin (mTOR) pathway, as a key player responsible for the regeneration failure after SCI [85]. After PTEN deletion, there is a dramatic increase in retinal axon regeneration after a complete crush. While the direct link between PTEN and axon regeneration has only recently been made, the association between increasing neuronal size and modifying the PTEN/mTOR pathway has been known for several years [87, 88]. Interestingly, the PTEN/mTOR pathway regulates axon regeneration likely via its effect on protein translation, which distinguishes this pathway from most other pathways identified so far. Chapter 4 extends the findings of Liu et al and Park et al by combining PTEN deletion with Nogo deletion.

Past and Current approaches to experimental and clinical SCI

Many studies have sought to improve functional recovery after spinal cord injury through a variety of approaches, some of which include: neuroprotection, providing trophic support and

guidance, minimizing inhibitory influences in the CNS environment, cell replacement, retraining through rehabilitation, and stimulating the intrinsic growth response. Each of these approaches target different aspects of spinal cord injury. First, with neuroprotection, it is understood that reducing further damage to the spinal cord will help to reduce the detrimental effects on motor and sensory function. Some of these neuroprotective strategies involve reducing secondary injury and inflammation with the use of drugs such as riluzole[89, 90], which has been shown to sodium and glutamate related secondary damage. A second approach is providing trophic support and guidance to injured cells as with the administration of neurotrophins such as NGF, BDNF, and NT-3. This strategy has shown that administration of growth factors can prevent cell loss[91] and promote axonal growth[92]. To target the inhibitory nature of the glial scar a popular approach is the administration of chondroitinase ABC (ChABC) to degrade the inhibitory chondroitin sulphate proteoglycans (CSPGs)[56]. Another approach has been to use stem cells as a way to replace lost cells and potentially bridge the gap between injured and uninjured tissue by providing a permissive and growth enhancing environment. A less invasive approach has been to induce axonal sprouting through behavioral rehabilitation and exercise[93]. A more recent approach is to target the intrinsic growth response by upregulating growth promoting pathways, or removing growth inhibiting pathways[77, 83].

For each of these approaches, while each targets a specific facet of spinal cord injury, specific mechanism of action is usually poorly understood. Therefore, the development of a more simple model to isolate specific variables is necessary to better understand not only axonal behavior after injury, but also how each approach can effectively be applied to spinal cord injury treatments.

Experimental Treatments for SCI

Though many experimental treatments are promising, most are targeted at reducing secondary injury and the complications associated with SCI. These treatments typically involve stabilization of the spinal column and minimizing the factors responsible for secondary injury,

which are, as described above, not limited to: excitotoxicity, inflammation, ischemia, free radical damage and lipid peroxidation, hypoxia, and apoptosis. Some of these experimental treatments include methylprednisolone, GM-1 ganglioside (reducing excitotoxicity and apoptosis, and increasing neurite sprouting)[94], minocycline (reduce cytokines, free radicals and matrix metalloproteinases) [95] [96]. Currently, of the treatments tested in spinal cord injury patients, only methylprednisolone treatment has resulted in a measurable improvement. The one caveat being the significant dose needed to obtain this effect is simultaneously deleterious, and recent studies have shown that the risks outweigh the benefits[97]. While MP is a treatment option, it is not the standard of care. In addition to treatments reducing secondary injury, there are treatments for complications associated with spinal cord injury. Some of these are pregabalin for neuropathic pain [98-100], zolmitriptan for sensory transmission and muscle spasticity [101].

While these studies have, to some degree, improved the quality of life for SCI patients, it is important to note that no clinical trial to date has implemented a therapeutic strategy that increases regeneration or sprouting after acute or chronic spinal cord injury. Thus the need to advance research on regeneration and sprouting is ever present and essential to the future of full functional recovery after spinal cord injury.

Tools to Investigate SCI

The need for a new experimental SCI paradigm: In vivo imaging

Reports of CNS axon regeneration often fall short of independent verification by multiple groups, which is widely accepted as the ultimate proof for the robustness of any positive findings in the scientific literature. In fact, it is not unusual that enhanced axon regeneration reported by one group could not be reproduced by an independent group [102]. Further complicating the matter is the possibility of artifactual labeling with the conventional surgical tract tracing method [103]. At least part of this dispute can be attributed to the limitations of current experimental paradigms relying on experimental spinal cord injury and conventional tracing techniques.

Developing a new experimental paradigm that takes advantage of recent technological advancement has the potential to uncover aspects of axonal degeneration and regeneration that have escaped studies with more conventional techniques.

This dissertation, in large part, has further developed a model to study responses of spinal axons to axotomy using two photon in vivo imaging. Coupled with laser ablation of single axons, in vivo imaging allowed me to follow the same axon over time before and after axotomy. Thus, I was able to visualize changes to the same axotomized axon at acute (hours), sub-acute (days), sub-chronic (weeks) and chronic (months) after axonal injury. Following the same injured axons over such time, even when only acute and sub-acute time points were examined (which constituted the majority of my study), provided a wealth of information that could not have been possible with experimental spinal cord injury. As such, I was able to make the unequivocal identification of any regenerating axons since axotomy of any single axon was visually verified. The vastly reduced complexity – and thus variability – allowed me to probe into the innate behavior of axons to axonal injury. I have started to use this imaging paradigm to assess the role of a single myelin inhibitor with minimal variables compared to experimental spinal cord injury.

In vivo imaging with two photon microscopy

Within the past decade, two-photon microscopy has become a valuable tool to examine anatomical and physiological characteristics in vivo [104, 105]. Some of its advantages over previous microscopic methods include micrometric resolution and an imaging depth up to 1 mm [106]. The ability to image to this depth is made possible for three reasons: 1) The light source used (red and infrared) undergoes less scattering than shorter (bluer) wavelengths, therefore more excitation light reaches the specimen. 2) The localized excitation not only reduces the background, but almost the entire signal collected is in focus light. 3) Scattered light is less detrimental to two-photon microscopy than it is to confocal microscopy [107, 108]. Moreover, while the total laser power for two-photon is higher, due to the use of pulsed lasers, the overall average laser power is much lower than with confocal microscopy. This contributes to

substantially less phototoxicity for biological specimens. Additionally, unlike confocal microscopes, the photo bleaching is confined to a much smaller area, which is extremely important when working with live organisms.

The first attempt to image spinal axons in live mice was achieved with wide-field epifluorescence microscopy [109]. New insights were gained from this study including the description of acute phase degeneration. Our lab has established a method to image fluorescently labeled spinal sensory axons in live mice with two-photon microscopy [110]. The use of two-photon microscopy is expected to induce less phototoxicity and has the potential to image deeper into the tissues. In addition, our method does not require intubation of the mice or any post imaging computer aided realignment to minimize movement artifact.

Impact

The research herein has immediate implications on our understanding of axonal responses to injury and provides insights that could not have been gained from experimental spinal cord injury. With my in vivo imaging data, I show that axonal responses to axonal injury in the spinal cord, including degeneration and regeneration, are much more dynamic and complex than previously thought. Growth, pruning and new growth alternate during the course of days to weeks or even months after axonal injury. There are new logics emerging from my study that govern the behaviors of spinal axons to injury, such as the presence of a branch point and nodes of Ranvier. Chapter 2, the main the publication-ready chapter of my study, describes in detail the innate behavior of axons without the confounding variables seen in experimental spinal cord injury. The work in Chapter 3 presents a proof-of principle study to demonstrate how the specific contribution of a molecule can be investigated with in vivo imaging. Chapter 4 is collaboration with another lab member where we examined the effect of combined deletion of a neuron-intrinsic and extrinsic inhibitor in spinal axon growth after CNS injury. This complements my training with in vivo imaging studies.

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Chapter 2.

In vivo imaging reveals location specific effects on axon degeneration and regeneration after laser axotomy in the mouse spinal cord

ABSTRACT

The poor axon regeneration in the mammalian central nervous system (CNS) underlies the limited functional recovery in patients of spinal cord injury. Studies of axon regeneration using animal models of spinal cord injury have been hampered by the difficulty in distinguishing regenerated axons from spared axons and the limited spatial and temporal resolution. In vivo optical imaging may overcome these limitations. Here we present detailed degeneration and regeneration profiles from live imaging of fluorescently labeled spinal dorsal column sensory axons with 2-photon microscopy following highly localized laser-mediated axotomy. Bidirectional acute axon degeneration in the form of fragmentation and retraction occurs within hours. This is followed by axon regeneration in the form of branching and elongation within days in the absence of any molecular intervention. Branch points serve as a barrier for acute axon degeneration while the location of axotomy in relevance to the branch point dramatically influences axon regeneration. In addition, nodes of Ranvier frequently adjoin where degeneration subsides and regeneration arises. Most strikingly, dynamic axon regeneration, pruning and remodeling can be observed up to 5 months following the initial axotomy, the longest time point examined. These data highlight a wider therapeutic window than previously envisioned to promote axon regeneration and CNS repair. In vivo imaging of the mouse spinal cord can reveal features of axonal responses to injury that have eluded conventional studies with experimental spinal cord injury.

BACKGROUND AND SIGNIFICANCE

The main roadblock for the failure of modern medicine to provide an effective restorative treatment for spinal cord injury is the lack of axon regeneration in the adult mammalian central nervous system (CNS). Axon regeneration occurs readily after injury in the peripheral nervous system (PNS), but generally fails in the CNS[6, 111, 112]. The lack of axon regeneration after CNS injury has been attributed to a number of factors such as a lack of neuron-intrinsic growth potential, a lack of growth promoting factors and the presence of inhibitory factors that actively block long distance re-growth in the CNS environment [37, 49, 113-117].

Studies of spinal axon regeneration have traditionally relied on animal models of spinal cord injury[118-120]. Typically, a lesion to the spinal cord is made using one of a variety of methods such as a surgical transection (partial or full), crush, contusion or compression[120]. An axonal population of interest is then visualized with immunohistochemistry and/or an axonal tracer, surgically applied or genetically encoded, on tissue samples that are collected terminally[121]. There are three inherent limitations to this approach. First, it is exceedingly difficult to distinguish regenerated axons from spared axons. As a result, spared axons are often mistakenly identified as regenerated axons [122]. Second, the same biological samples cannot be examined over time, limiting the temporal and spatial resolutions of the analyses. Third, the myriad of variables such as lesion production, complex tissue and systemic responses to injury including edema, hematoma, microglial activation, macrophage infiltration, astroglial reactivity, influx of cytokines and chemokines make it extremely challenging to isolate the effect of a single molecular intervention on axonal responses to injury [14, 123, 124].

We and others have developed methods to image fluorescently labeled spinal axons over time in live mice [125-127]. Kerschensteiner et al were the first to demonstrate the feasibility of imaging mouse spinal axons and studying their degeneration and regeneration using wide field fluorescence microscopy [125]. We developed a method to repetitively image mouse spinal axons with 2-photon microscopy without the need for intubation or image post-processing [127]. Others have further demonstrated the utility of in vivo imaging to study cellular responses to injuries in

the mouse spinal cord [78, 128-132].

We reasoned that to better understand how populations of axons behave following a complex injury such as a spinal cord injury, it is important to understand the behavior of single axons after axonal injury under conditions of minimal environmental variables. Here we present data from in vivo imaging of mouse spinal sensory axons with 2-photon microscopy at acute, sub-acute, sub-chronic and chronic phases following laser-mediated axotomy of single axons. With this minimal lesion paradigm, we found that the location of axotomy in relevance to the branch point impacts both axon degeneration and regeneration. Nodes of Ranvier also influence axonal responses to axotomy. Strikingly, regeneration, pruning and remodeling continue for up to 5 months after the initial axotomy, the longest time point examined, in the absence of any genetic or pharmacologic intervention, indicating a wider therapeutic window for promoting axon regeneration than previously envisioned. Our study revealed features of axonal responses to axotomy that have eluded conventional analysis with experimental spinal cord injury and indicate that in vivo imaging provides a valuable approach to study injury-induced responses of spinal axons.

MATERIALS AND METHODS:

Animal subjects

Adult Thy1-YFPH (YFP) line transgenic mice, ages 8-12 weeks, were used for this study [133]. The Institutional Animal Care and Use Committee approved animals in these experiments. For all surgical procedures, mice were anaesthetized with 100mg/kg ketamine (Fort Dodge Animal Health, Fort Dodge, IA) 15mg/kg xylazine (Vedco, Saint Joseph, MO) and 2.5mg/kg acepromazine (Vedco, Saint Joseph, MO) in a 0.9% NaCl solution. After the imaging session was completed, animals were allowed to recover on a heating pad and given 0.05 mg/kg buprenorphine subcutaneously every 12 hours for 5 days, sterile saline for dehydration, and Baytril. For terminal procedures, animals were anaesthetized with 10mg/kg of a 50mg/mL solution of Nembutal (Ovation Pharmaceuticals, Deerfield, IL).

Animal Surgery

Adult transgenic mice were anaesthetized 100mg/kg ketamine (Fort Dodge Animal Health, Fort Dodge, IA) 15mg/kg xylazine (Vedco, Saint Joseph, MO) and 2.5mg/kg acepromazine (Vedco, Saint Joseph, MO) in 0.9% NaCl solution. The midline of the back was shaved and sterilized with 70% ethanol. A midline dermal incision (~ 1cm in length) was made to expose the vertebral muscles. The vertebral muscles were carefully removed to expose the thoracic vertebrae at the T8-T10 level and a laminectomy at the T9 level of the spinal cord was made to expose the spinal cord as described[127].

Stabilization of the spinal column

The spinal column was stabilized with customized Narishige spinal cord clamps and head holding adaptors mounted on a steel plate as described (Davalos et al, 2008). Briefly, the clamps were placed on both the T8 and T10 vertebrae to stabilize the spinal column, while the head was placed in the head holding adaptor, and the tail was clamped so that the mouse maintained a relatively level position. A well of petroleum jelly was created around the exposed spinal cord and filled with artificial cerebrospinal fluid (ACSF) in order to accommodate the immersion lens.

In vivo imaging of ascending sensory neurons

After the mouse was secured in the stabilization device, it was then placed in a heat-controlled chamber underneath a customized FV300 Olympus microscope as described (Davalos et al, 2008). Imaging was performed with a Spectra Physics Mai-Tai laser tuned to 920 nm for two-photon excitation of YFP. In order to obtain a map of the entire imaging area an Olympus 10x .3 NA water-immersion lens was used. Figure 1D depicts the typical viewing area with a 10x objective. In this horizontal overview ascending sensory axons labeled with YFP enter the spinal cord and bifurcate into an ascending and descending branch. The blue arrows point to the bifurcation point. To examine labeled axons in finer detail, an Olympus 40x 0.8 NA water-immersion lens was used. Axons were chosen based on the visibility of the axon and proximity to

other surrounding axons. The three locations chosen for axotomy were the ascending branch, which travels up the spinal cord eventually terminating in the *nucleus gracilis* and *nucleus cuneatus*, the descending branch, which sends its projections into the grey matter, and finally the main branch -the branch that is directly attached to the DRG cell body. We chose these different locations for the laser injuries as it has been shown that different branches elicit different regenerative responses [134].

For laser axotomy, we used the point function in the Spectra Physics Mai Tai Software for the Ti:Sapphire laser, with duration of 0.2 seconds without a neutral density filter at a wavelength of 820 nm. Our average power output was 1-1.5 Watts for each laser axotomy event. This allowed us to focally sever a single labeled axon without visible damage to adjacent labeled axons.

Additionally, axons were chosen in an area that was not densely populated with other labeled axons. After locating a suitable axon, the laser was tuned down to 820 nm to perform the laser ablation at one of three sites chosen for their unique anatomic relation relative to the bifurcation point, a schematic of which can be seen in (Fig.1B). To confirm that the axons of interest were in the CNS as opposed to the PNS we stained for both myelin protein zero (P0), a PNS specific marker, and glial fibrillary acidic protein (GFAP) to delineate the boundaries of the PNS and CNS, respectively (Fig. 1C). After laser ablation, the axon was imaged for up to 5 hours to capture the acute axonal degeneration. We have determined that 2.5 hours is an appropriate time point for which to terminate the first imaging session, as the acute degenerative phase usually reaches a plateau by 2.5 hours. For re-imaging experiments, animals were anesthetized as described above, the original wound was reopened, and any tissue overgrowth was removed to expose the original imaging area. All animals included in the study had normal ambulation and did not exhibit any signs of motor dysfunction after each surgery. Animals that displayed motor deficits or any visible trauma to the spinal cord, such as bruising, after any surgery were excluded.

Image Processing

Acquisition of the images was executed with Olympus FluoView software. Z-stacks were taken every 10 μm with the 10x objective, and every 1 μm with the 40x objective throughout the entire focal area. The final images are a compression of all z-stacks to recreate the three-dimensional space in a two-dimensional image. During the acquisition phase laser power and offset were adjusted to minimize background. For post-acquisition image processing only brightness and contrast were manipulated using Adobe Photoshop. ImageJ was used for quantification of degeneration distances.

Quantification of axonal changes (degeneration and regeneration)

We used ImageJ to measure acute axonal degeneration as distance from the center of the injury location to the tip of the last contiguous piece of the axon both proximal and distal to the cell body. We measured sub-acute regeneration as any new growth from the terminal tip of the injured axon. For more chronic time points, total regeneration was measured as the sum of all growth new as of Day 0. For all acute degeneration images, we used the segmented line function and measured degeneration from the injury location to the tip of the last contiguous axonal segment. These quantifications were done in 2D from the maximum projection Z-stack. For all sub-acute and chronic time points, we measured regeneration using the simple neurite tracer plugin for Fiji, which allowed for measurement in 3D.

Statistical Analysis

One-way ANOVA was used for multiple comparisons with a Bonferroni post-test. For multiple observations between groups we used two-way ANOVA. Unpaired t-tests were used for pairwise comparisons. Results are expressed as mean values $\pm$ S.E.M. The chi-square test was used for group frequency data, and the Fisher's exact test was used to determine significance between specific groups. Linear regression (R^2 values) analysis was used to test the significance of distance from the bifurcation point and degeneration. Non-linear regression was used to test

the relationship and significance between degeneration and regeneration. A p value < 0.05 was considered to be statistically significant. All statistical analyses were done using Prism Graphpad, with the exception of the non-linear regression, which was executed with Sigmaplot software.

Immunohistochemistry

To identify the boundaries between the central nervous system and the peripheral nervous system we used a standard immunostaining protocol. Briefly, Thy1-YFPH mice were perfused transcardially with 4% paraformaldehyde in PBS. The spinal cord was then carefully dissected so as to keep all dorsal root ganglia intact. After 2 hours of post-fixation in PFA, spinal cords were transferred to 30% sucrose in PBS overnight at 4°. After the overnight cryoprotection the thoracic levels T8-10 were embedded in embedding solution (Sakura Fine Tech, Japan). Cross sections were then cut on a cryostat at 20 µm and collected on Superfrost Plus slides (Fisher Scientific). For immunostaining sections were washed in PBS, blocked for 90 minutes in 5% normal goat serum (NGS) (Vector Labs) in PBS, and then incubated overnight at 4° in 1:100 myelin protein zero (Abcam), and 1:500 glial fibrillary acidic protein (Dako). The next day sections were rinsed in PBS and incubated with secondary antibodies (1:500 goat anti-rabbit 350 and 1:500 donkey anti-chicken 546) (Invitrogen) in 5% NGS for 90 minutes. Sections were rinsed in PBS and coverslipped with Fluoromount-G (Southern Biotech). Images were captured and analyzed with an Axio Imager fluorescent microscope (Zeiss).

Animal subjects

Adult Thy1-YFPH (YFP) line transgenic mice, ages 8-12 weeks, were used for this study [133]. Animals in these experiments were approved by the Institutional Animal Care and Use Committee. For all surgical procedures, mice were anaesthetized with 100mg/kg ketamine (Fort Dodge Animal Health, Fort Dodge, IA) 15mg/kg xylazine (Vedco, Saint Joseph, MO) and 2.5mg/kg acepromazine (Vedco, Saint Joseph, MO) in a 0.9% NaCl solution. After the imaging

session was completed, animals were allowed to recover on a heating pad and given 0.05 mg/kg buprenorphine subcutaneously every 12 hours for 5 days, sterile saline for dehydration, and Baytril. For terminal procedures, animals were anaesthetized with 10mg/kg of a 50mg/mL solution of Nembutal (Ovation Pharmaceuticals, Deerfield, IL).

Animal Surgery

Adult transgenic mice were anaesthetized 100mg/kg ketamine (Fort Dodge Animal Health, Fort Dodge, IA) 15mg/kg xylazine (Vedco, Saint Joseph, MO) and 2.5mg/kg acepromazine (Vedco, Saint Joseph, MO) in 0.9% NaCl solution. The midline of the back was shaved and sterilized with 70% ethanol. A midline dermal incision (~ 1cm in length) was made to expose the vertebral muscles. The vertebral muscles were carefully removed to expose the thoracic vertebrae at the T8-T10 level and a laminectomy at the T9 level of the spinal cord was made to expose the spinal cord as described[127].

Stabilization of the spinal column

The spinal column was stabilized with customized Narishige spinal cord clamps and head holding adaptors mounted on a steel plate as described (Davalos et al, 2008). Briefly, the clamps were placed on both the T8 and T10 vertebrae to stabilize the spinal column, while the head was placed in the head holding adaptor, and the tail was clamped so that the mouse maintained a relatively level position. A well of petroleum jelly was created around the exposed spinal cord and filled with artificial cerebrospinal fluid (ACSF) in order to accommodate the immersion lens.

In vivo imaging of ascending sensory neurons

After the mouse was secured in the stabilization device, it was then placed in a heat-controlled chamber underneath a customized FV300 Olympus microscope as described (Davalos et al, 2008). Imaging was performed with a Spectra Physics Mai-Tai laser tuned to 920 nm for two-photon excitation of YFP. In order to obtain a map of the entire imaging area an Olympus 10x

.3 NA water-immersion lens was used. Figure 1a depicts the typical viewing area with a 10x objective. In this horizontal overview ascending sensory axons labeled with YFP enter the spinal cord and bifurcate into an ascending and descending branch. The yellow arrows point to the bifurcation point. To examine labeled axons in finer detail, an Olympus 40x 0.8 NA water-immersion lens was used. Axons were chosen based on the visibility of the axon and proximity to other surrounding axons. The three locations chosen for axotomy were the ascending branch, which travels up the spinal cord eventually terminating in the *nucleus gracilis* and *nucleus cuneatus*, the descending branch, which sends its projections into the grey matter, and finally the main branch -the branch that is directly attached to the DRG cell body. We chose these different locations for the laser injuries as it has been shown that different branches elicit different regenerative responses [134].

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imaging area. All animals included in the study had normal ambulation and did not exhibit any signs of motor dysfunction after each surgery. Animals that displayed motor deficits or any visible trauma to the spinal cord, such as bruising, after any surgery were excluded.

Image Processing

Acquisition of the images was executed with Olympus FluoView software. Z-stacks were taken every 10 μm with the 10x objective, and every 1 μm with the 40x objective throughout the entire focal area. The final images are a compression of all z-stacks to recreate the three-dimensional space in a two-dimensional image. During the acquisition phase laser power and offset were adjusted to minimize background. For post-acquisition image processing only brightness and contrast were manipulated using Adobe Photoshop. ImageJ/Fiji was used for quantification of degeneration/regeneration distances.

Quantification of axonal changes (degeneration and regeneration)

We used ImageJ and Fiji to measure acute axonal degeneration as distance from the center of the injury location to the tip of the last contiguous piece of the axon both proximal and distal to the cell body. We measured sub-acute regeneration as any new growth from the terminal tip of the injured axon. For more chronic time points, total regeneration was measured as the sum of all growth new as of Day 0.

Statistical Analysis

One-way ANOVA was used for multiple comparisons with a Bonferroni post-test. For Multiple observations between groups we used two-way ANOVA. Unpaired t-tests were used for pairwise comparisons. Results are expressed as mean values $\pm$ S.E.M. The chi-square test was used for group frequency data, and the Fisher's exact test was used to determine significance between specific groups. Linear regression (R^2 values) analysis was used to test the significance of distance from the bifurcation point and degeneration. A p value < 0.05 was considered to be

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RESULTS

The primary goal of our in vivo imaging study was to understand the innate behavior of single spinal axons after axonal injury under conditions of minimal environmental variables. As in our previous study [127], we used the Thy1-YFP-H transgenic line [133] in which a subset of dorsal column sensory axons and other axonal populations are strongly labeled with the yellow fluorescent protein (Fig. 1A). We focused on dorsal column sensory axons, as these are the most superficial (dotted area in Fig. 1A) and thus the most accessible for in vivo imaging. These axons have cell bodies in the dorsal root ganglions (DRGs) outside of the spinal cord (Fig. 1B). Their primary axon bifurcates, with one peripheral branch and one central branch that extends into the

spinal cord. After the central branch enters the spinal cord, it bifurcates again, with one ascending branch extending rostral towards the brain for a long distance in the dorsal column white matter (Fig. 1B) that ultimately synapses on secondary order neurons in the dorsal column nuclei of the brain stem. The other, descending branch courses down the spinal cord for a shorter distance and synapses locally within the spinal cord.

In this study, we focused our analyses on the CNS part of the sensory axons after they have entered the spinal cord. Accordingly, the branch point (or BP) below refers exclusively to the secondary branch point (blue arrow in Fig. 1B) after DRG axons have entered the spinal cord. “M” refers to the main CNS branch before the axon bifurcates in the spinal cord; “A” refers to the ascending branch after the axon bifurcates in the spinal cord; “D” refers to the descending branch (Fig. 1B). Immunoreactivity against myelin protein zero (P0, a marker for PNS myelin) indicates that the axons to be imaged are well within the CNS border (Fig. 1C).

At the beginning of each imaging session, we used epifluorescence microscopy to provide a low magnification roadmap for the region to be imaged, which illustrates the densely labeled dorsal column sensory axons in the Thy1-YFP-H line (Fig. 1D). A chosen area of interest (dotted box in Fig. 1D) was then imaged at a higher magnification with 2-photon microscopy (Fig. 2) as described previously [127, 135]. To induce axonal injury with minimal environmental variables, we used the femtosecond 2-photon laser, with the minimum laser power and duration necessary, to focally axotomize a single YFP-labeled axon without visible damage to adjacent labeled axons. To provide a convenient reference point for axotomy, we injured axons close to the branch point either before or after an axon bifurcates in the spinal cord (indicated by a red cross for A, D, M respectively in Fig. 1B). Other fluorescently labeled axons served as landmarks for in vivo imaging. Following laser axotomy, a small gap in YFP signal developed instantaneously at the site of injury (Fig. 2C). Axons were then followed for several hours, during which time there was no re-connection of the proximal and distal segments, verifying the completeness of laser axotomy. Immunoreactivity against glial fibrillary acidic protein (GFAP) indicates that there was minimal local astroglial reactivity at sites of laser axotomy (data not

shown).

The first imaging session usually lasted 4-5 hours, during which time we axotomized up to ~3 axons per mouse. Each axon was followed for ~3 hours, with images taken as often as every 5-15 minutes because axon degeneration at this acute phase is quite dynamic (see below). Imaging on a later day was achieved by re-opening the imaging window surgically 5 days (sub-acute), 5 weeks (sub-chronic), and ~5 months (chronic) after the initial imaging session (Day 0). The later imaging sessions usually lasted 2 hours or less, as they were intended to capture snapshots during axonal regeneration, which exhibit a much slower dynamic (see below). Most mice were imaged on Day 0 and Day 5; a few were additionally imaged at 5 weeks and fewer at ~5 months after the initial axotomy.

Acute axonal degeneration via fragmentation and retraction following laser axotomy

Consistent with previous reports with a mechanical pinprick lesion [125, 129], the first noticeable change to axons upon laser axotomy was the bidirectional (i.e. both anterograde and retrograde) acute axon degeneration. Figure 2 illustrates a time sequence of this acute axon degeneration following laser axotomy of an ascending branch (also see Movie 1). Prior to laser axotomy, the axon to be injured (pseudo-colored in yellow in Fig. 2A, with neighboring axon pseudo-colored in aqua) exhibited continuous and smooth morphology. Immediately after laser axotomy, there was a visible gap where the laser had severed the axon (Fig. 2C). Within minutes to hours, axonal degeneration proceeded both anterogradely and retrogradely from the site of axotomy. Degeneration occurred via a combination of blebbing/fragmentation and retraction, and subsided by ~ 2-3 hours following laser axotomy (Fig. 2C-R).

Individual axonal segments may exhibit a dominant mode of axotomy-induced acute degeneration: some degenerated primarily via axonal blebbing/fragmentation (Fig. 3A-D) while others primarily via retraction (Fig. 3E-H). Just as for ascending branches, axotomies of descending and main branches elicited qualitatively similar, bidirectional acute axon degeneration (Fig. 4). Thus, acute axon degeneration upon axotomy at any of the three injury locations (A, D,

M, as in Fig. 1B) followed a similar pattern: a combination of fragmentation and/or retraction persisted for ~1-3 hours before degeneration subsided. We noted that for ascending and descending branch axotomies, retrograde degeneration often proceeded to (Fig. 3D) or close to the branch point leaving a stub connected to the branch point (Fig. 2R, 3H, 4D), but rarely breached the branch point (see more below).

To examine the degeneration dynamics in more detail, we profiled individual degeneration events by plotting the degeneration distance over time on Day 0 for each axonal segment, clustered according to the injury location (ascending, descending or main branch axotomies) and the direction of degeneration (proximal/retrograde or distal/anterograde) (Fig. 6). For proximal/retrograde degeneration after ascending/descending branch axotomies or distal/anterograde degeneration after main branch axotomies, it was possible for degeneration to breach the branch point. An incidence of branch point breach is reflected by a line splitting into two (one solid and one dotted of the same color, Fig. 6A,C,F), reflecting the ensuing degeneration of two separate branches beyond the branch point. Overall, there was a great variability in the degeneration profiles among individual axonal segments. Some axonal segments exhibited an initial delay (red arrows, Fig. 6) while others initiated degeneration soon after axotomy (black arrows, Fig. 6). Sometimes axonal segments exhibited a steady and relatively slow speed of degeneration over an extended period of time (i.e. several hours) (blue arrows, Fig. 6), while at other times axonal segments exhibited a burst of degeneration within a short period of time as reflected by a precipitous slope (green arrows, Fig. 6). Upon closer inspection, these bursts of axon degeneration were often associated with fragmentation (see more below). Still other axonal segments exhibited a more complex degeneration profile with multiple, alternating phases of slow and fast degeneration (orange arrows, Fig. 6). Regardless, it is readily discernable from these individual degeneration profiles that for most axonal segments acute degeneration subsided within 1-3 hours.

Just as the individual degeneration profiles, group degeneration profiles (the average degeneration distance over time for each cluster or all axonal segments) followed a similar trend

for degeneration to slow down within hours after axotomy (Fig. 5A). However, the inflection point was less obvious in the group degeneration profiles likely due to the variable time points at which degeneration of individual axonal segments subsided. At 2.5 hours after axotomy average degeneration distances ranged between ~50 and ~130 μm for the six clusters, with the average degeneration distance for all axonal segments at ~89 μm . Noticeably, the six clusters displayed somewhat different degeneration dynamics (see more below).

To determine whether there was any correlation in degeneration between the proximal and distal segments, we plotted the distance of distal degeneration against that of proximal degeneration for individual axons, after normalizing against the average degeneration distance of the respective cluster (Fig. 5B). Indeed, there is a significant albeit inconspicuous correlation between the distances of distal and proximal degeneration for individual axons (Fig. 5B, linear regression: $r^2 = 0.55$, $p = 0.0001$). Thus, the wide variety of degeneration dynamics of individual axons is not solely due to the stochastic nature of acute axon degeneration, and reflects in part the intrinsic properties of a given axon that are shared by the proximal and distal or may merely be due to the stochastic nature of acute axon degeneration. A major assumption for this analysis is that active acute axon degeneration is regulated at least in part intrinsically within axons. If the extent of acute degeneration reflects some intrinsic property of the axons, we would expect to see some correlation in degeneration between the distal and proximal segments of individual axons. If, on the other hand, the extent of acute degeneration is a purely stochastic event, we expected to see no correlation between distal and proximal degeneration. Thus, the extent of acute degeneration reflects in part the intrinsic properties of the axons.

To determine whether there were any correlations between degeneration speed and degeneration mode, we divided axonal segments into three groups: those that degenerated primarily (i.e. for 80% or more of the distance degenerated) via fragmentation, via retraction, or via a combination of both (>20% but <80% of the distance degenerated in any one mode), and calculated the respective average degeneration speed for each group (Fig. 7A). Degeneration speed via fragmentation was significantly faster than degeneration via retraction (0.73 ± 0.06

$\mu\text{m}/\text{min}$ and $0.28 \pm 0.03 \mu\text{m}/\text{min}$, respectively), while degeneration via a combination of both averaged somewhere in between ($0.58 \pm 0.10 \mu\text{m}/\text{min}$). Additionally, 67% of fragmenting axons had a degeneration speed of $\geq 0.5 \mu\text{m}/\text{min}$, while only 9% of retracting axons had a degeneration speed of $\geq 0.5 \mu\text{m}/\text{min}$. Thus, fragmentation is associated with a faster speed of degeneration while retraction is associated with a slower speed of degeneration.

When measured with the distance degenerated, fragmentation was the dominant mode of acute axon degeneration (Fig. 7B), representing $\sim 80\%$ of the cumulative (or average) distance degenerated by all axonal segments. In contrast, retraction represented only $\sim 20\%$ of the cumulative (or average) distance degenerated (Fig. 7B). This was the case even when the three injury locations (main, ascending and descending branches) were considered separately (Fig. 7B). The average distance of fragmentation per axon at $85 \pm 13 \mu\text{m}$ was significantly larger than that of retraction at $21 \pm 5.8 \mu\text{m}$. $p < 0.0001$, Student's t -test, $n = 39$ axons). This trend can also be seen when the absolute distances of fragmentation and retraction were examined for each of the experimental groups (Fig. 7C). In addition, $\sim 61\%$ of axonal segments (48 out of 78) degenerated primary via fragmentation (i.e. fragmentation accounting for $\geq 80\%$ of the distance degenerated) while $\sim 27\%$ primarily via retraction and $\sim 12\%$ via both modes ($>20\%$ but $<80\%$ for any one mode).

Overall, the distal segments degenerated more via fragmentation as compared to the proximal segments. When the absolute distance was examined, distal segments degenerated significantly more via fragmentation than proximal segments after descending branch axotomies; a similar trend for the ascending branch axotomies did not reach statistical significance (Fig. 7C). On the other hand, proximal segments degenerated more via retraction after main or descending branch axotomies (Fig. 7C). When the average degeneration mode (i.e. the average of the percentage of distance degenerated by fragmentation vs. retraction for individual axonal segments) was examined, there was a consistent trend for more fragmentation in distal than proximal degeneration, and correspondingly more retraction in proximal than distal degeneration, regardless of the injury location (Fig. 7D). Together, these data indicate that there is a difference

in the relative contribution of fragmentation vs. retraction between retrograde degeneration of proximal segments and anterograde degeneration of the distal segments. This is the first of two pieces of evidence (see more below) indicating that while, unlike Wallerian degeneration, acute axon degeneration is bidirectional, there is still a level of asymmetry in the degeneration of proximal and distal segments, which implicates a cell body influence on acute axon degeneration.

Branch point is a barrier for retrograde axon degeneration

Our observation that retrograde degeneration after axotomies of ascending and descending branches often stopped at or near the branch point (see above, Fig. 2R, 3D, 3H, 4D); however, it rarely breached the branch point: only 2 total incidents of branch point breach were observed from retrograde degeneration of the proximal segment after ascending or descending branch axotomies (Fig. 6C,E). This prompted us to hypothesize that branch point serves as a barrier for acute axon degeneration. If this hypothesis is correct, we would anticipate that when axotomy is targeted relatively close to the branch point (e.g. ~100 - 150 μm as was done in the above experiments), degeneration towards the branch point might be truncated by the branch point while degeneration away from the branch point may proceed more freely. As a consequence, degeneration towards the branch point may persist for a shorter distance than degeneration away from the branch point. Indeed, in pair-wise comparison between distal and proximal degenerations of the same injury location, the average distance for proximal degeneration was significantly less than that for distal degeneration after ascending branch axotomies ($60 \pm 11 \mu\text{m}$ and $111 \pm 25 \mu\text{m}$, respectively) (Fig. 8A). There was a similar trend for descending branch axotomies but this trend did not reach statistical significance (Fig. 8A). In contrast, there was no appreciable difference between distal and proximal degeneration distances after main branch axotomies. This differential effect among the three injury locations was not a consequence of different distances between the axotomy site and the branch point because the average of this distance remained relatively constant after main, ascending and descending branch axotomies (Fig. 8B. $p = 0.89$, one-way ANOVA). These data are consistent

with the branch point serving as a barrier to retrograde acute degeneration but not anterograde degeneration.

To further test the hypothesis that branch point serves as a degeneration barrier, we shortened the distance between the site of axotomy and the branch point to ~ 50 μm , which would force degenerating axons to encounter the branch point sooner after axotomy than in our previous experiments. Following descending or ascending branch axotomies, most proximal axonal segments still degenerated up to but not beyond the branch point (exemplified in Fig. 9A-C); only in 1 of 7 cases ($\sim 14\%$) did retrograde degeneration breach the branch point.

To visualize the effect of branch points on acute degeneration, we plotted degeneration distance against the distance between the axotomy site and the branch point, including all axotomy sites that are ~ 50 - 150 μm away from the branch point (Fig. 8C-E). For distal degeneration after main branch axotomies and proximal degeneration after ascending or descending branch axotomies, the 45-degree lines in these graphs represent the point where axon degeneration reaches the branch point; any data points above the 45-degree line represent an incidence of branch point breach. After ascending or descending branch axotomies, retrograde acute degeneration of the proximal segments breached the branch point only in $\sim 11\%$ (3/27) of the cases (purple arrows, Fig. 8C,D). Interestingly, in $\sim 63\%$ (17/27) of the cases degeneration stopped within 25 μm of the branch point, with $\sim 33\%$ (9/27) stopping exactly at the branch point (Fig. 8C,D). In contrast, the corresponding anterograde degeneration of the distal segments, where there was no branch point along the degeneration path, proceeded more freely (with 15/27, or $\sim 56\%$ degenerating beyond the distance equivalent to that between the axotomy site and the branch point) and the degeneration endpoints spread out up to ~ 300 μm (Fig. 8C,D). The block of retrograde degeneration by the branch point was persistent, as any additional retrograde degeneration observed at 5 days after axotomy did not breach the branch point ($\sim 89\%$, or 24/27), as exemplified in Fig. 9. This indicates that the branch point is a barrier for retrograde axonal degeneration both at the acute and sub-acute time points.

Because acute axon degeneration is bidirectional (i.e. it occurs both anterogradely and

retrogradely), which distinguishes itself from the Wallerian degeneration that occurs only anterogradely[24, 136], we anticipated that anterograde acute axon degeneration may also be blocked by the branch point following main branch axotomy. However, this was not the case. In ~42% (8/19) of cases anterograde degeneration of the distal axonal segment breached the branch point within hours of a main branch axotomy (Fig. 8E). This frequency is indistinguishable from that of retrograde degeneration of the corresponding proximal segments proceeding beyond the equivalent distance (~42%, or 8/19), where there was no branch point along the degeneration path following main branch axotomies (Fig. 8E). Thus, while the branch point appears to block retrograde degeneration, it does not block anterograde degeneration. This observation provides a second piece of evidence that there is a cell body influence even at this early phase of axon degeneration and that not all aspects of acute axon degeneration is regulated locally within axons.

To determine whether these results were influenced by any intrinsic barrier to acute axon degeneration, we axotomized the ascending axons at ~700 μm from the branch point (which we termed Long Distance axotomy site). This would allow a more extended distance for axon to degenerate bidirectionally without encountering a branch point. Degeneration, either anterogradely or retrogradely, was observed to extend up to ~250 μm from the axotomy site but did not reach the branch point (exemplified in Fig. 10A-C, quantified in Fig. 8F), even 5 days after injury ($n = 7$). These degeneration distances were comparable to the maximal degeneration distances of ~300 μm following main, ascending and descending branch axotomies where there was no branch as a barrier (Fig. 8C-E).

Long Distance axotomies allowed us to examine the potential role of another anatomical structure on acute axon degeneration: the nodes of Ranvier. Interestingly, out of nine cases of Long Distance axotomy of an ascending branch, six degenerated retrogradely to or close to a node of Ranvier (Fig. 10B,D). Out of these seven cases where we were able to follow on Day 5, none degenerated beyond the node 5 days after axotomy (exemplified in Fig. 10C). Thus, the nodes of Ranvier also serve as a barrier of acute and subacute axon degeneration.

Branch point is an important determinant of sub acute axonal regeneration

During the first several hours after laser axotomy (i.e. in the first imaging session on Day 0), there was no significant regeneration in any of the axons imaged. Re-imaging the same axons at a later day allowed us to detect the regeneration of individual axons in response to axotomy. Five days after axotomy especially that of main branches (see more below), many proximal axonal segments exhibited new growth that could be definitively characterized as regeneration. Here we define regeneration as the growth of axons from the injured axonal tips. In practice, regeneration was only positively identified (or considered detectable) when there was at least 5-10 μm of new growth.

There were primarily two non-mutually exclusive modes of new axonal growth: elongation and branching. Elongation may proceed in the forward direction beyond the initial endpoint of acute axon degeneration (Fig. 11A-C, green arrows in panel C), or in the reverse direction following a U-turn (Fig. 10A-C). Even when the dominant mode was elongation, there were often signs of axonal branching (green arrowheads in Fig. 11C). Where there was extensive axon branching, individual branches may also exhibit significant axonal elongation (Fig. 11D-F'). In this example, regeneration originated from the terminal tip of the injured axon and bifurcated into two primary branches, which elongated while extending secondary branches. Growth cone-like structures could be seen at the tip of the newly growing axonal branches (magenta arrowheads, Fig. 11F'). In contrast to the smooth appearance of the original or the surrounding intact axons, regenerating axons took tortuous, winding paths, often in seemingly random directions. In addition, they had an irregular and often thinner axon diameter. Occasionally, an axon would branch in a manner similar to its original bifurcation pattern, extending an ascending and a descending branch in the "correct" direction (Fig. 11F,F').

Intriguingly, regeneration almost always originated from the terminal tip of the injured axon instead of as collateral sprouts close to the terminal tip. Furthermore, the terminal tip on Day 5 after main branch axotomies was often at the same location where retrograde degeneration had stopped on Day 0, indicating that for the majority of cases, we had observed the acute

degeneration process to or near its end on Day 0. Anterogradely, Wallerian degeneration had taken over by Day 5, where fragmented remnants of distal axonal segments were often observed (brown arrows in Fig. 11C,F).

The ability to precisely direct laser axotomy to specific locations allowed us to examine the effect of axotomy site on axon regeneration. Indeed, following main, ascending and descending branch axotomies, there was a significant effect of injury location in relevance to the branch point on regeneration frequency ($X^2 = 25.95$, $df = 2$, $p < 0.0001$). The frequency of axon regeneration following main branch axotomies was substantially greater than that after descending and ascending branch axotomies ($p = 0.0004$, and $p = 0.0001$, respectively, Fisher's exact) (Fig. 12A). Axotomizing the main branch elicited a regenerative response in ~89% (16/18) of the cases, while only ~13% (2/16) of ascending branch axotomies and ~11% (1/9) of descending branch axotomies elicited a detectable regenerative response. Interestingly, the only instances where there was a detectable regenerative response after an ascending or descending branch axotomy were those with retrograde degeneration breaching the branch point: 2 out of 16 ascending branch axotomies and 1 out of 9 for descending branch axotomies (example shown in Fig. 13A-C').

Among the main branch axotomies that resulted in detectable regeneration, there was a correlation between the distance of degeneration and that of regeneration. Proximal axonal segments that degenerated the most on Day 0 tend to exhibit less axon regeneration on Day 5 while those that regenerated most tend to exhibit less axon degeneration acutely after axotomy (Fig. 12B). This observation indicates that the initial degeneration may have a lasting effect on axon regeneration at a later time point, a possibility that remains to be fully investigated.

There are two plausible explanations for the low regeneration frequencies after ascending or descending branch axotomies: 1) the existence of a nearby branch point *per se* may suppress axon regeneration; 2) the remaining intact branch may actively suppress regeneration. To distinguish these two possibilities, we axotomized both the ascending and descending branches simultaneously at ~100-150 μm from the branch point (i.e. double branch axotomies).

We reasoned that if the former hypothesis were correct, regeneration would be more likely to occur only when retrograde degeneration breaches the branch point following double branch axotomies. However, if the latter hypothesis were correct, double branch axotomies would lead to a higher regeneration frequency regardless of any branch point breach; in other words, there would be no correlation between branch point breach and incidence of axon regeneration under this scenario. We found that after double branch axotomies, in ~67% (6/9) of the cases axons regenerated, approaching the regeneration frequency after main branch axotomies (Fig. 12A). When there was a branch point breach, regeneration occurred in 2 of 4 cases (~50%). When there was no branch point breach, regeneration occurred in 4 out of 5 cases (~80%) (exemplified in Fig. 13D-F'). Thus, compared to single axon axotomies of either the descending or ascending branch, double branch axotomies led to a higher regeneration frequency, where branch point breach was not associated with a higher regeneration frequency. These data support the hypothesis that following double branch axotomies, it is the loss of the remaining branch, rather than the loss of the branch point *per se*, that de-represses axon regeneration. If so, the remaining intact branch and potentially their synaptic contacts may suppress a regenerative response following the elimination of one axonal branch.

The above cases of axon regeneration were observed following axotomies relatively close to the branch point. We next asked whether moving the site of axotomy further away from the branch point would elicit a similar regenerative response. Intriguingly, when the ascending branch is axotomized ~500 - 900 μm from the branch point (average ~700 μm , referred to as Long Distance axotomies above), an intermediate regeneration frequency (~43%, or 3/7) was observed (Fig 12A, exemplified in Fig. 10A-C). In all of these cases, retrograde acute degeneration proceeded up to ~250 μm (Fig. 10D, see above), and a proximal axonal stub (~300 – 900 μm) remained beyond the branch point. These data are consistent with the possibility that the length of the remaining proximal segment of an axotomized branch influences regeneration, a possibility that remains to be formally tested in the future.

To determine whether there were any quantitative differences in regeneration among

different injury locations, we measured the maximum, total and average lengths of newly regenerating axons and the numbers of primary and secondary branches for axotomies that resulted in a regenerative phenotype (Fig. 12C,D). We did not include axotomies of a descending or ascending branch alone (other than the Long Distance ascending branch axotomies) in this analysis because these two groups led to only one incidence of axon regeneration each. Main branch axotomies led to the most maximum and total lengths of axon regeneration along with the most secondary branch numbers 5 days after axotomy (Fig. 12C,D). Double branch axotomies were associated with total axon lengths and secondary branch numbers that are intermediate between main branch axotomies and Long Distance ascending branch axotomies. Together, these data reinforced the notion that main branch axotomies elicit most axon regeneration and double branch axotomies somewhat mimic main branch axotomies in the effect on axon regeneration.

Pruning, remodeling and new growth at subchronic and chronic time points

We next asked the question whether axonal regeneration at sub acute time points could be sustained at subchronic and chronic time points or is only short lived. To examine axonal dynamics at more chronic time points, we chose to focus on the main branch axotomies as these exhibited most regenerative responses at sub acute time points (i.e. 5 days after axotomy). We chose 5 weeks after axotomy ($n = 5$) as a sub-chronic time point, and 5 months ($n = 3$) for our chronic time point. The small numbers of axons examined reflected the technical challenges for imaging at subchronic and chronic time points. Interestingly, however, while all 5 axons examined up to at least 5 weeks exhibited significant growth at 5 days after the axotomy, this new growth was consistently pruned to a significant extent at 5 weeks following axotomy (exemplified in Fig. 14 and Fig. 15A-C). Sometimes, there was modest new growth at 5 weeks in addition to the more noticeable pruning (Fig. 14C). Our results do not distinguish address whether pruning and regrowth can occur simultaneously at any given time. Nevertheless, it is striking that a significantly amount of newly grown axonal segments observed at the sub acute time point was

pruned at the subchronic time point.

Even more remarkably, for the 3 axons that we were able to re-examine at the 5-month time point, new axonal growth was observed again (Fig. 15D,D'). Varicosities along and at the terminal tip of the regenerated axons could be observed, possibly representing synaptic pre-synaptic structures (orange arrows, Fig. 15D'). The overall trend is that new axonal growth observed at 5 days after axotomy was replaced by remodeling and pruning at 5 weeks, and again by new axonal growth at 5 months (Fig. 15E). Furthermore, individual axons that exhibited a greater regenerative response at 5 days also exhibited a greater degree of pruning at 5 weeks, and where data were available, further exhibited a greater extent of new growth at 5 months (Fig. 15E). These data indicate that individual axons vary in their intrinsic abilities for axon regeneration, pruning and remodeling such that some axons are more dynamic while others are more static. This intrinsic property does not appear to be animal-specific as there was wild variability among axons from the same animal (e.g. axons #1 in pink and #2 in blue are from the same animal, Fig 15 E). Interestingly, comparing the 5 day and 5 month time points, while the overall length of new axonal growth was not significantly different, there was a switch from a branching phenotype to elongation type of growth, as the length of axon per branch was significantly increased at the chronic time point (Fig. 15F). Because of the great technical difficulty of following axons for 5 months after axotomy, the number of axons imaged at this time point was small. Nevertheless, these data illustrate the principle that axonal growth, pruning and remodeling continue weeks and even months after axotomy, implicating a wider time window for modulating axon regeneration following injury.

DISCUSSION

To definitively identify regenerating spinal axons and to study the dynamics of axonal responses to injury, we applied in vivo imaging with 2-photon microscopy on fluorescently labeled dorsal column sensory axons at acute, sub-acute and chronic phases after laser-mediated axotomy in mice. We injured a single axon among densely labeled axons and followed the same

injured axon over time: continuously within the first few hours after axotomy, then with snapshots at days, weeks and even months after the initial axotomy. In the absence of any molecular intervention, acute axon degeneration by fragmentation and/or retraction is followed by spontaneous, continuous and dynamic axon regeneration, pruning and remodeling for up to five months after axonal injury, the last time point examined. Our results provide new insights on the innate degenerative and regenerative responses of spinal sensory axons to axonal injury and highlight an extended time frame following injury when injured axons can still undergo dynamic changes in the mammalian CNS.

Acute axon degeneration subsides several hours after initial axotomy

As in previous reports with a pin prick-based lesion [78, 125, 129], we observed acute axon degeneration of spinal sensory axons following laser-mediated axotomy. Acute axon degeneration is rapid, occurring within 5 minutes of laser axotomy. It is bidirectional and proceeds at approximately the same initial speed in both directions from the site of injury (but see below). This distinguishes acute axon degeneration from Wallerian degeneration, which only proceeds distally to the site of axotomy. Interestingly, acute axon degeneration subsides ~2-3 hours after axotomy and typically stops within 100-300 μm from the initial site of axotomy. This indicates that there is an intrinsic limitation to the extent of acute axon degeneration. 5 days after axotomy, Wallerian degeneration distal to axonal injury was clearly seen in all cases, while rarely (?) did we observe significant ($> 30 \mu\text{m}$) additional (Wallerian) degeneration to the proximal segment. In the discussion below, we will simply refer to acute axon degeneration as degeneration, unless otherwise noted.

Branch point is a barrier to acute axon degeneration

Although acute axon degeneration proceeds bidirectionally, we did observe a modest but significant difference in proximal vs. distal acute axon degeneration when ablating ascending or descending branches. Because we ablated axons at ~100-150 μm away from the branch point

(i.e. the secondary branch point after DRG axons enter the spinal cord), we reasoned that this may be due to the presence of the branch point. In particular, we hypothesized that the branch point serves as a barrier for acute axon degeneration. Several pieces of evidence support this hypothesis. First, we observed that degeneration of an ablated ascending or descending axon rarely breached the branch point. In other words, after injury of either the ascending or descending branch, the other uninjured branch almost always remained intact. Out of 19 total ascending or descending axons axotomized at about 100-150 μm away from the branch point, only 2 breached the branch point. In most, the branch point remained intact even 5 days after injury. Second, when we moved the ablation site closer to the branch point, i.e. to ~ 50 μm , degeneration continued to halt by the branch point. Finally, we plotted the distance of degeneration against the distance between the branch point and the laser ablation site. We found that in almost all cases (22 out of 25) retrograde degeneration of an ascending or descending branch did not breach the branch point, while anterograde degeneration did not appear to observe any barrier. In contrast, for the main branch, degeneration did not appear to observe any barrier either retrogradely or anterogradely, and anterograde degeneration readily extended beyond the branch point (distal to injury). Together, these data support that the branch point that is still connected to the cell body serves as a barrier for acute axon degeneration, which has been described in *C. elegans* [134]. The molecular and cellular mechanisms for this remain to be identified.

Spontaneous axon regeneration and location-specific effect on regeneration

As with previous studies with pin prick lesions [125, 127, 129, 131, 132], our study provides definitive evidence that dorsal column spinal sensory axons spontaneously regenerate within days after laser mediated axotomy. This demonstrates that adult spinal sensory axons are intrinsically capable of axon regeneration. While this apparently contradicts with the prevalent view that axons in the adult CNS do not regenerate, there are three points of consideration. First, the degree of such regeneration is still limited, usually hundreds of microns days after axotomy.

Second, such axon growth in limited scale can be readily detected by in vivo imaging but would have eluded analyses with conventional models of spinal cord injury. Third, our model does not elicit a widespread secondary injury response including significant microgliosis, astrogliosis, fibrosis, infiltration of other immune cells and molecules, thus may or may not have allowed for a higher degree of axon regeneration.

Not all axons exhibited significant regeneration, however. When they do, the total length of new axonal growth ranges 98 to 603 μm at 5 days after axotomy. Strikingly, the injury location in relevance to the branch point had a strong effect on the frequency of axon regeneration. Ablating descending or ascending branch at 100-150 μm from the branch point elicited little axon regeneration (less than 20% of axons regenerated), while ablating the main branch prior to the bifurcation point elicited strong regenerative response (~90% axons regenerated). Intriguingly, in rare instances when axon regeneration was observed after laser ablation of the descending or ascending branch, this was always following a branch point breach at the acute degeneration phase. Thus, either a branch point breach per se or the axotomy of both ascending and descending branches is a prerequisite for the regenerative response after laser axotomy.

To distinguish between these two possibilities, we simultaneously ablated both the ascending and descending branches at 100-150 μm from the branch point (termed double ablation). If the branch point breach per se stimulates axon regeneration, we would expect a high correlation between branch point breach and axon regeneration following double ablation. On the other hand, if the axotomy of both the ascending and the descending branch rather than the branch point breach per se is an important variable, we would expect that there would be no strong correlation between branch point breach and axon regeneration. Indeed, even with the limited number of axons examined, it was clear that branch point breach was not correlated with a higher frequency of axon regeneration. Thus, it is likely the ablation of both the ascending and descending branches rather than the branch point breach stimulates axon regeneration following laser axotomy. We speculate that ablating both axonal branches would lead to the loss of most if not all synaptic targets of a sensory axon, which may stimulate axon regeneration; meanwhile,

when one branch remains intact, there is insufficient stimulation of axon regeneration as some synaptic targets remain. This hypothesis remains to be investigated.

Regeneration is dynamic and continuous

A most striking finding of our in vivo imaging study is that not only does an injured spinal sensory axon go through an initial regenerative phase consisting of extensive branching and elongation days after injury, but it could go through an extensive pruning, remodeling and continuous regeneration for up to 5 months after the initial axotomy. In our experimental paradigm, it appears that axons typically eliminate unnecessary branches in favor of one or a few at 5 weeks after axotomy, a process that is comparable to axon pruning during development. By 5 months after injury, this same axon again switches from pruning to another growth phase that consists of an elongation type of growth as opposed to branching. Technically, it is exceedingly difficult to follow a single axon for such a long time, hence the limited number of axons we examined at this chronic time point. However, it was clear even from the few examples that axonal responses to axotomy is dynamic: the initial growth of mixed branching/elongation at sub-acute phase (5 days) is followed by pruning at a sub-chronic phase (5 days to 5 weeks), further followed by new axon elongation at a more chronic phase (5 weeks to 5 months).

In addition to the thriving axonal dynamism, an indication of future functionality might be indicated by the primitive formations of axon indentations almost recapitulating nodes of Ranvier in the uninjured axons. Studies have shown that after axonal injury, remyelination results in regularly spaced nodes, though the intermodal distance, as compared to the uninjured axon, is significantly shorter. This provides new insights on axonal behavior after axotomy in the absence of any molecular or cellular intervention, and implies that the time window when administration of potential therapies can still harness this intrinsic growth phase is much more extended than previously thought.

Unlike previous studies, we were able to track a single axon over many months and have discovered a novel process in the regenerative response of an injured axon. Previous studies

have reported abortive sprouting, or even a more long-term regenerative response; however, no study to date has been able to track the dynamics of a single axon over various months without the complication of a larger lesion. We have shown that not only is there a robust initial phase of growth and branching, but there is also a second phase of pruning that occurs weeks after injury. Moreover, there is continues growth even at what would be clinically considered to be a chronic phase after injury, where no further change to the injury occurs.

In vivo imaging as a tool to study spinal axon responses to axonal injury

Along with recent reports using in vivo imaging to study spinal axons in response to axotomy, our study illustrates in vivo imaging as a powerful experimental approach that may provide new insights on axonal behavior after injury that have eluded conventional analysis with experimental spinal cord injury. For example, acute axon degeneration was first described with in vivo imaging [125]. In our study, we describe first time the effect of axotomy location on the degenerative and regenerative responses after single axon laser axotomy. In addition, we found that the branch point serves as a barrier for acute axon degeneration. Others have used in vivo imaging to describe blood vessel – axon interactions following injury (ref) or described a temporal stage-specific role for STAT3 in axon regeneration that would have been difficult to detect with experimental spinal cord injury [78]. Thus, in vivo imaging serves as an excellent model to study the detailed dynamics of axonal responses to injury. We speculate that the effect of any single molecular invention might be revealed using in vivo imaging more readily than experimental spinal cord injury, such as in the case of STAT3.

Our in vivo imaging paradigm allows for stable and repetitive imaging of spinal axons up to 5 months following the initial axotomy. In addition, our model produces a minimal lesion to the injured axons with limited effect on nearby tissues and cells, which allows for the study of axonal responses to axotomy in the absence of significant secondary injury responses. Furthermore, the preservation of the dura and minimal removal of vertebral lamina, along with absence of intubation allows us to examine the innate axonal responses in a minimally disturbed

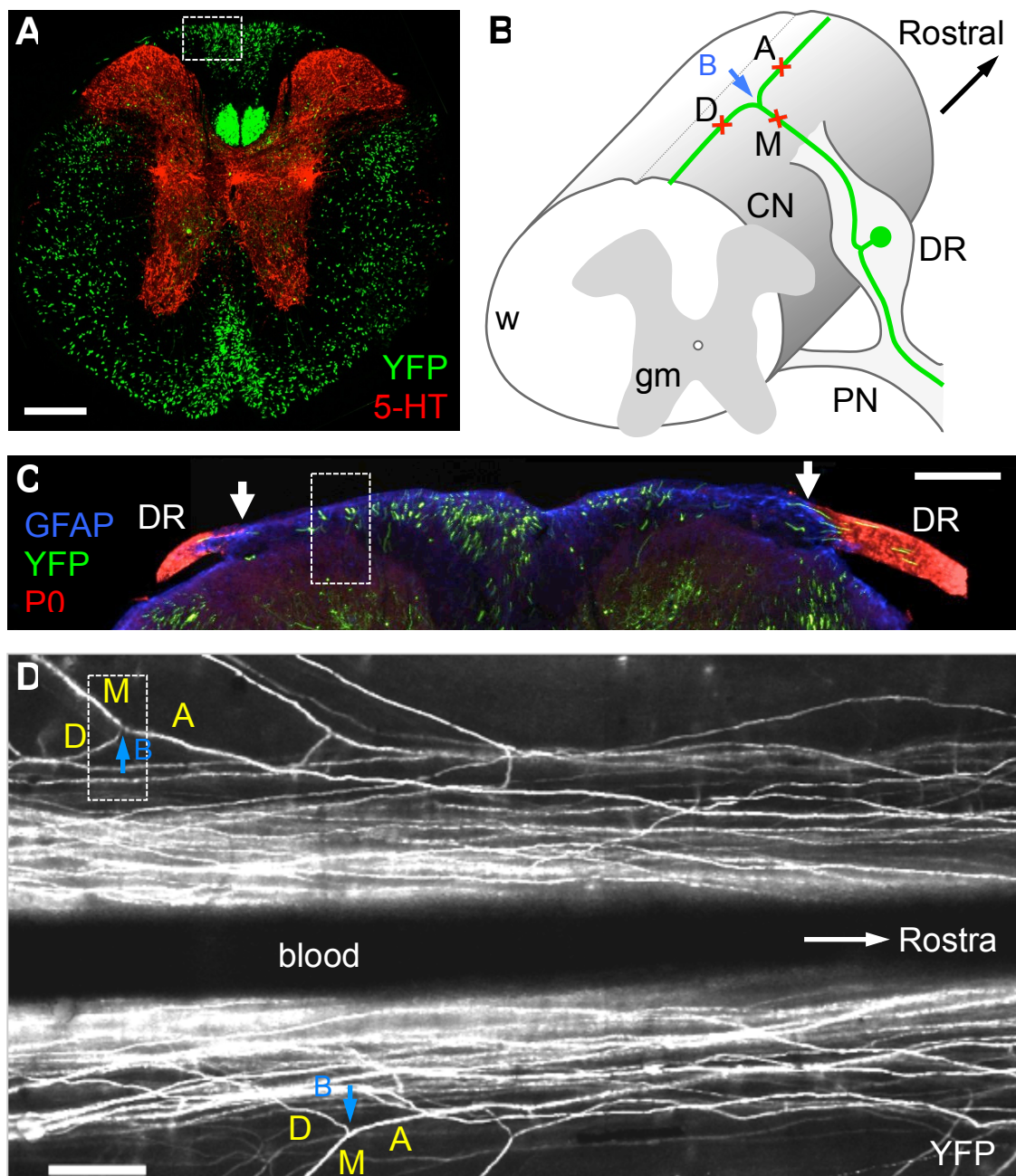
environment. The clarity at which we may describe the behavior of a single injured axon allows for precise measurements of degeneration and regeneration to be made with little ambiguity. There is no doubt when an axon has regenerated, or pruned. Future studies will improve the model by increasing the frequency of repetitive imaging, such as using a chronic window that has recently been developed [132, 137]. Additionally, this study establishes a framework by which future manipulations can be measured against. In addition, it remains a challenge how well other axonal types can be imaged in vivo due to their deeper location in the cord. Nevertheless, we anticipate that in vivo spinal axon imaging will continue to shed light on spinal axon behavior after injury with and without additional molecular and cellular intervention.

ACKNOWLEDGMENTS

Chapter 2, in part, is being submitted for publication. The dissertation author was the primary investigator and wrote the manuscript. Jae K. Lee assisted in the design and implementation of the in vivo imaging paradigm. Matt Mui assisted in data acquisition and analysis. Jeff Kwan assisted in node quantifications. I thank Pearl Shih, Omeed Ghassemi, and Amy Chang for their technical assistance and hours looking at and quantifying axons. Binhai Zheng helped write the manuscript, supervised the project and provided advice.

Figure 2.1 Illustration of the in vivo imaging model

A) A transverse section of mid thoracic spinal cord of an adult Thy1-YFP-H mouse showing the fluorescently labeled axons. Green, YFP labeled spinal mostly axons coursing along the rostral – caudal axis (perpendicular to the plane of the paper) in the white matter. Red, 5-HT (5-hydroxytryptophan) immunoreactivity to outline the gray matter. Dotted box outlines the area where the superficially located dorsal column sensory axons (to be imaged in vivo) reside. Note that many axonal populations are labeled by YFP including a subset of the corticospinal axons (the two densely labeled, medially located axonal tracts just below the dorsal column sensory axons). **B)** Schematic of sites of axotomy in relevance to the (secondary) branch point (blue arrow). Each of the branches (A, D or M) may be individually axotomized at the location shown (red cross) with a laser. **C)** The most dorsal part of a transverse Thy1-YFP-H spinal cord section stained for GFAP (blue) and P0 (red) to delineate the CNS – PNS boundaries (arrows). DR, dorsal root. Note that the imaged axons (green, YFP direct fluorescence) in the area indicated by the dotted box are well within the CNS. **D)** A lower magnification image of the dorsal spinal cord acquired with epifluorescence microscopy to serve as a road map for higher magnification two-photon imaging (dorsal view). Note many fluorescently labeled sensory axons coursing in the rostral – caudal axis. Some axons are seen to enter the main bundle while bifurcating into an ascending branch (towards the brain) and a descending branch, with two examples marked as in panel B. The boxed area is the location for time-lapse 2-photon in vivo imaging shown in Figure 2. The dark “shadow” in the middle of the cord is a blood vessel (dorsal vein). In this and all other in vivo imaging figures, rostral is to the right. A, ascending branch; D, descending branch; M, main branch; bp, branch point. DRG, dorsal root ganglion; CNS, central nervous system; PNS, peripheral nervous system; wm, white matter; gm, gray matter. Solid green circle indicates sensory neuron cell body. Scale bars = 200 μ m (A, C, D).



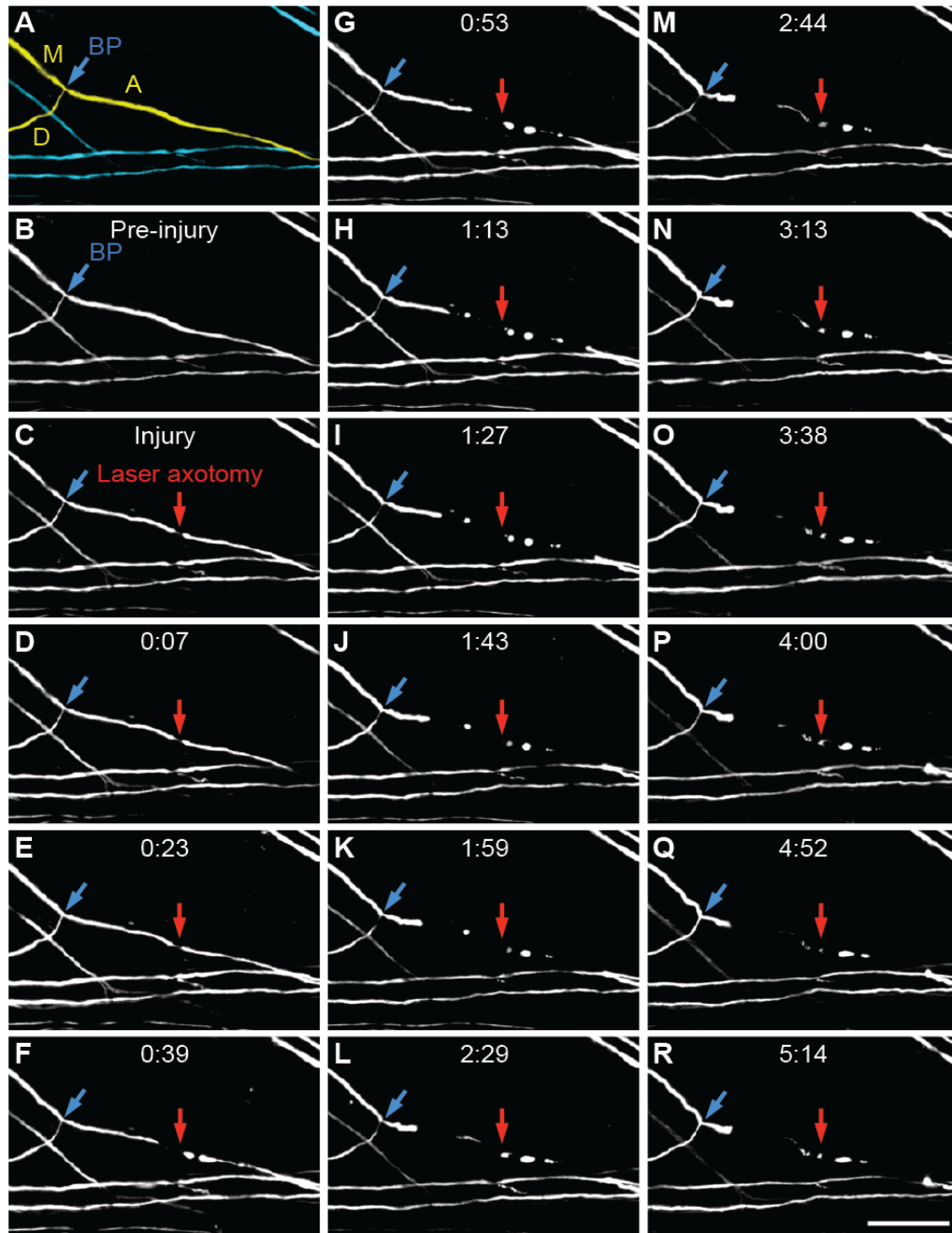


Figure 2.2 Time-lapse in vivo imaging of ascending branch degeneration after laser axotomy.

A) Artificially colored image to depict the axon to be axotomized (yellow) in relation to other axons (aqua). **B-R)** Time lapse imaging. Blebbing of the distal axonal segment was observed at 39 min after axotomy (**F**). Fragmentation was observed from 1 hr 13 min (**H**) onward for the proximal segment and from 39 min (**F**) onward for the distal segment. Axonal retraction was observed from 1 hr 13 min to 2 hr 29 min for the proximal segment (**H-L**). M, main branch; A, ascending branch; D, descending branch. For the ascending branch (**A**), distal is to the right. Times shown are in hours and minutes. "0:00" is immediately after axotomy. Panel A = pseudo-colored version of panel B. Red arrow = site of laser axotomy. Blue arrow = branch point (bp). Scale bar = 100 μ m.

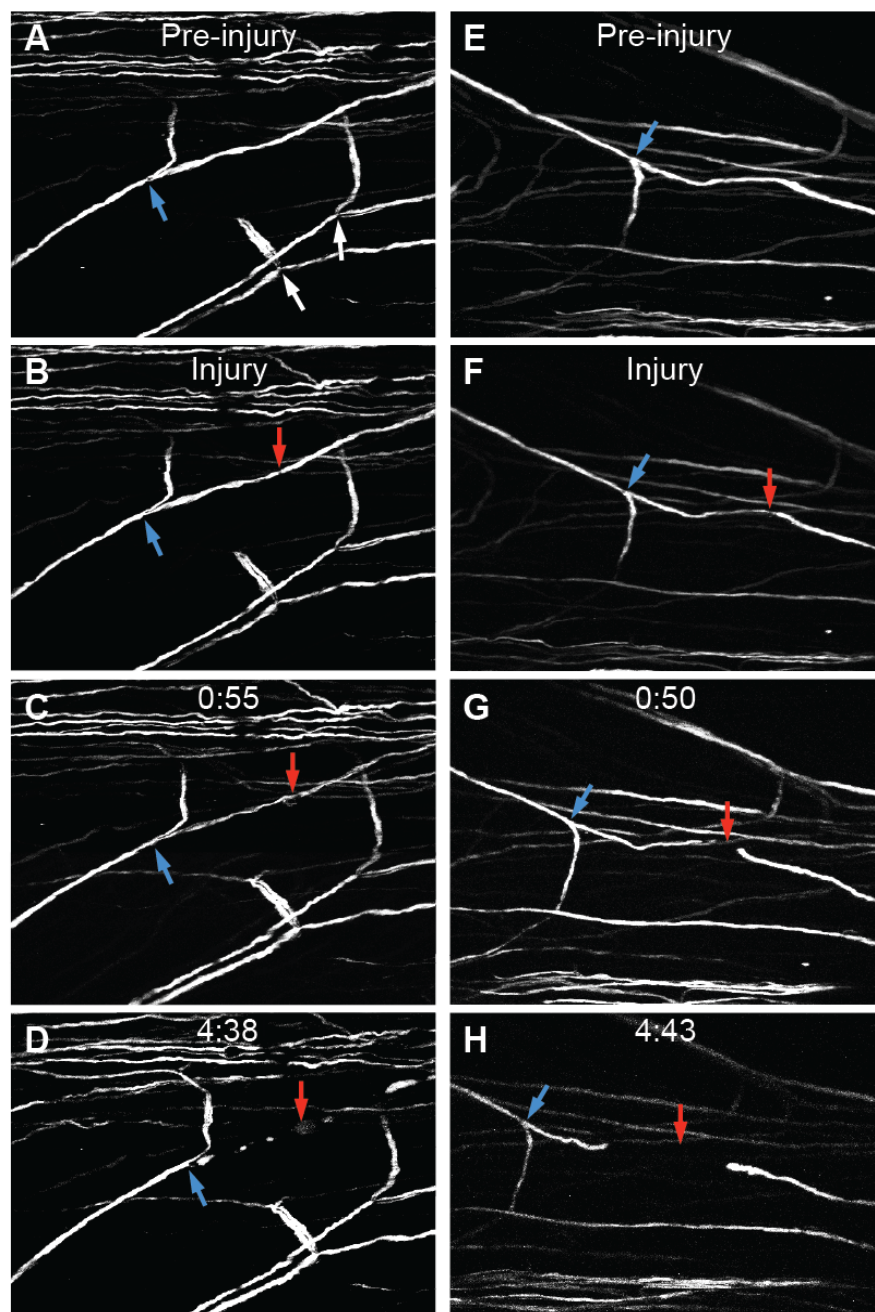


Figure 2.3 Individual axons exhibit different modes of degeneration.

A-D) An ascending branch degenerating via blebbing/fragmentation of both the proximal and distal segments. **A)** Immediately after injury. **(B)** There is a clear disconnect where the laser has severed the axon. **(C)** By one hour both the proximal and distal segments exhibited some blebbing and fragmentation. **(D)** By 5 hours substantial fragmentation of the proximal and distal segments had occurred and remnants of axons in the form of discontinuous axonal segments were visible. Note that the proximal axonal segment degenerated to the bifurcation point. **(E-H)** An ascending axon degenerating via retraction of both the proximal and distal segments. **(E)** After laser axotomy, there was a visible gap where the laser had severed the axon. **(F)** This gap expanded in the next four hours, with both the distal and proximal axonal segments degenerating via retraction. Times shown are in hours and minutes. "0:00" is

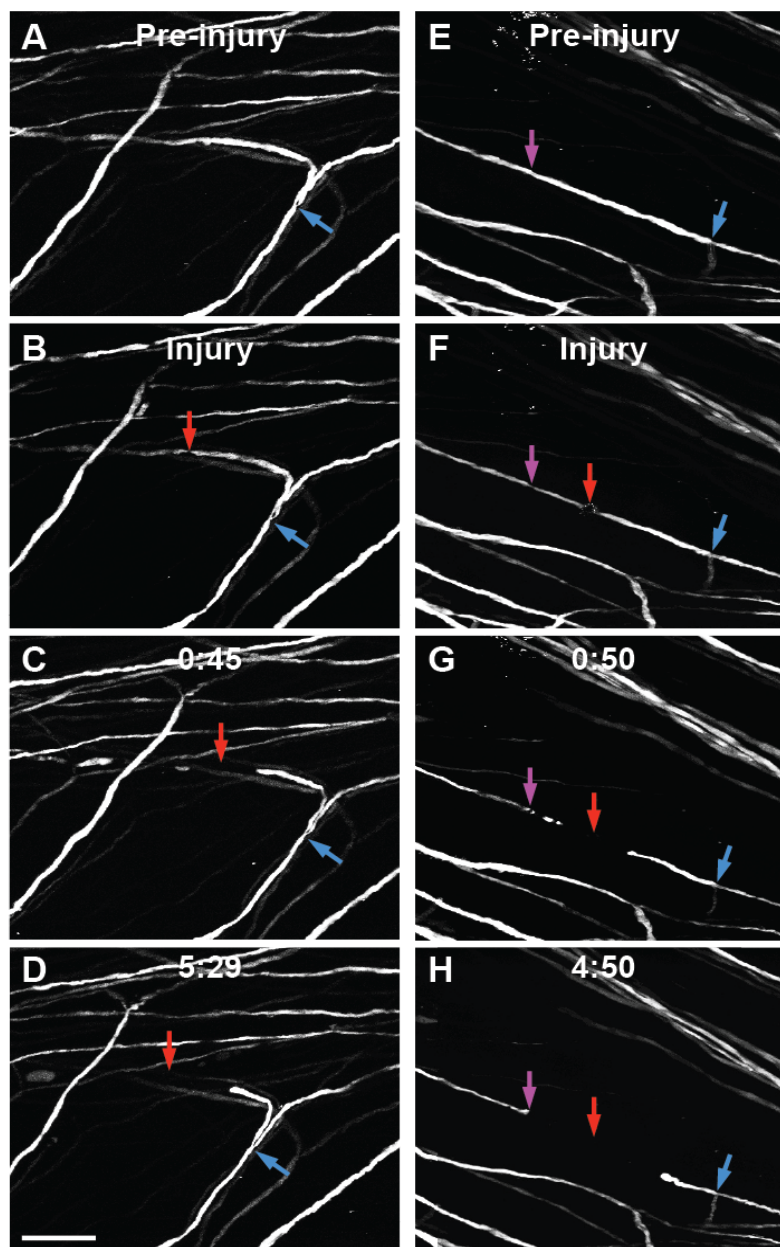


Figure 2.4 Different injury locations exhibit similar degeneration modes. **A-D)** A descending branch degenerating via both fragmentation and retraction. **A)** Intact descending axon before injury. **B)** Immediately after laser injury there is a disconnect between the proximal and distal axonal segment. **C)** By 45 minutes the distal axonal segment fragments, while the proximal axonal fragment retracts towards the bifurcation point. **D)** After 5 hours and 29 minutes, the degeneration has slowed, evident by the minimal retraction between 45 minutes and 5 hours. The fragmented distal segments have continued to further fragment. **E-J)** Main branch acute degeneration via fragmentation and retraction. **E)** Axon before injury. **F)** Immediately after injury there is a disconnect between proximal and distal segments. **G-H)** In this example, the proximal axonal segment degenerates via fragmentation while the distal axonal segment degenerates via retraction over the course of 50 minutes to almost 5 hours. Times shown are in hours and minutes. "0:00" is immediately after axotomy. Red arrow = injury site. Blue arrow = bifurcation point. Purple arrow = node of Ranvier. Scale bar = 50 μ m. For each injury location at least ten axons from a minimum of seven mice were analyzed.

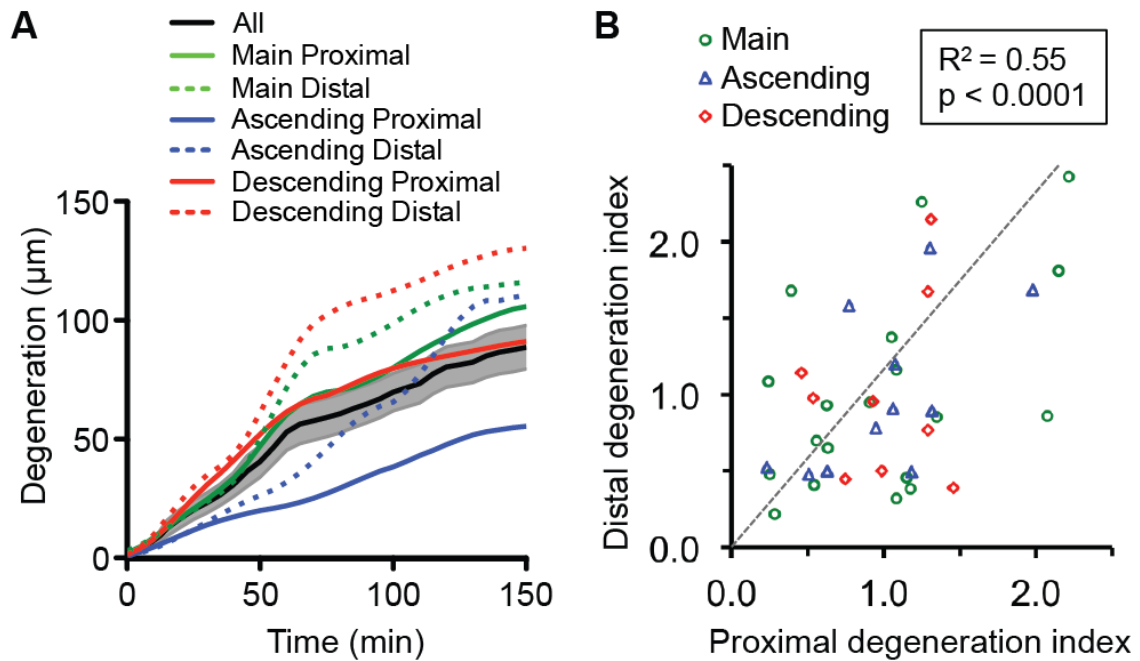
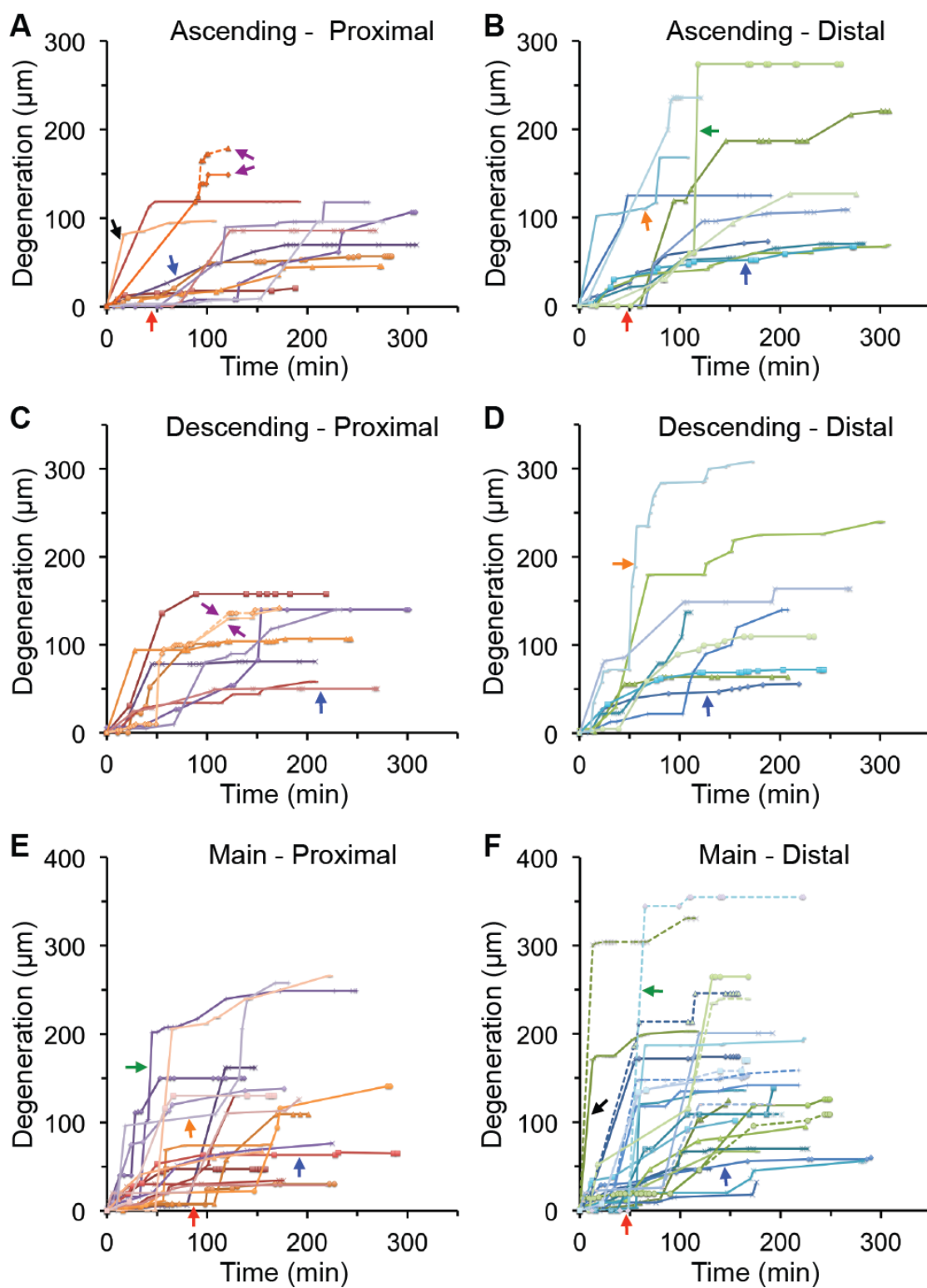


Figure 2.5 Acute axonal degeneration subsides after a few hours. A) Acute axonal degeneration is not significantly different between the different injury locations, and slows by ~2.5 hours. Animals with at least 2.5 hours were included in this graph. Data are represented as average degeneration for all animals. The black line represents the cumulative degeneration for all injury location. The grey borders represent SEM. B) The graph represents the ratio of distal degeneration to proximal degeneration for individual axons at the last time point observed on Day 0, when most axon degeneration has subsided. Correlation analysis shows that there is a significant relationship between the extent of degeneration in the distal and proximal axonal segments. $r = 0.55$, $n = 39$, and $**p = 0.0001$.

Figure 2.6 Acute axonal degeneration is governed by intrinsic axonal differences. All graphs represent the degeneration for distal and proximal axonal segments over time each axon. **E)** Main proximal branch. **F)** Main Distal Branch. **A)** Ascending Proximal Branch **B)** Ascending Distal Branch. **C)** Descending Proximal Branch. **D)** Descending Distal Branch. Degeneration and distance are represented in microns. Red arrows = axons exhibiting an initial delay in degeneration. Black arrows = degeneration initiated immediately after injury. Blue arrows = axons with slow steady speed of degeneration. Green arrows = axons displaying bursts of rapid degeneration. Orange arrows = axons with multiple oscillating phases of fast and slow degeneration. Purple arrows = degeneration beyond the bifurcation point. The split indicates breach location.



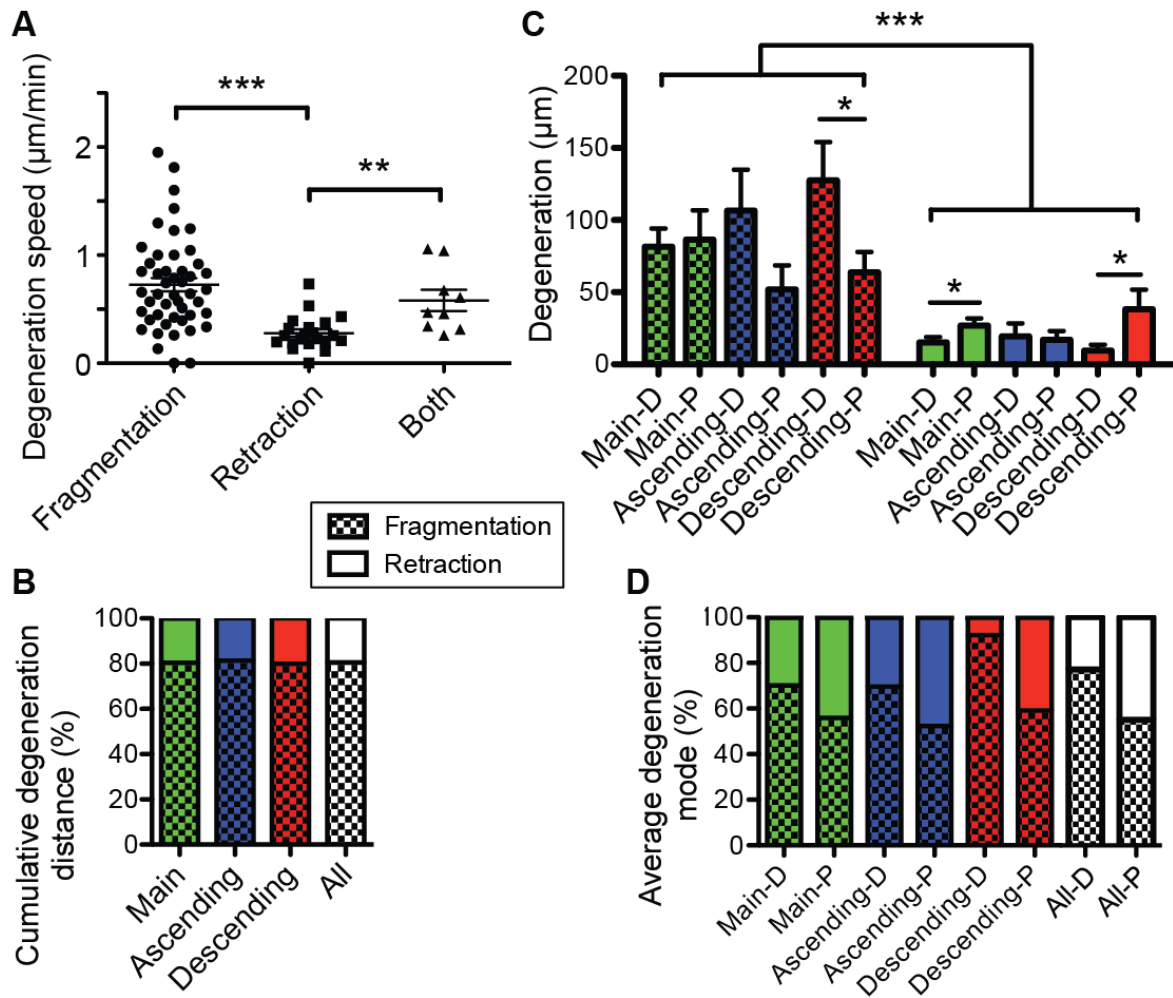


Figure 2.7 Fragmentation is the preferred mode of acute axonal degeneration

A) Acute axonal degeneration rates. Data are represented as average degeneration rates over 2.5 hours for individual axons. *** $P = 0.0001$, and ** $p = 0.002$, two-way paired t-test. **C)** Location specific differences in degeneration distance of fragmentation and retraction. Graph represents average degeneration distance by 2.5 hours. * $p < 0.05$, two-way paired t-test. For all injury locations, degeneration occurs mainly through fragmentation, *** $p = 0.0001$, two-way ANOVA. All error bars are SEM. **B)** Most of the distance degenerated in acute axon degeneration is via fragmentation. Data are represented as the cumulative distance degenerated in % after 2.5 hours. The cumulative degeneration distance, represented as percent of total degeneration distance was surprisingly similar between injury locations, in that there was a significant difference between degeneration modes, where axons degenerated greater distances via fragmentation as opposed to retraction for all ablation locations, 80.4%, 81.3%, 80% fragmentation compared to 19.6%, 18.7%, and 20% retraction for Main, Ascending, and Descending, respectively. ** $p < 0.01$, 2-way ANOVA. **D)** Average degeneration mode %. Data are represented as the percent of total distance degenerated by either fragmentation or retraction. All statistics are two-tailed paired t-test, unless otherwise stated.

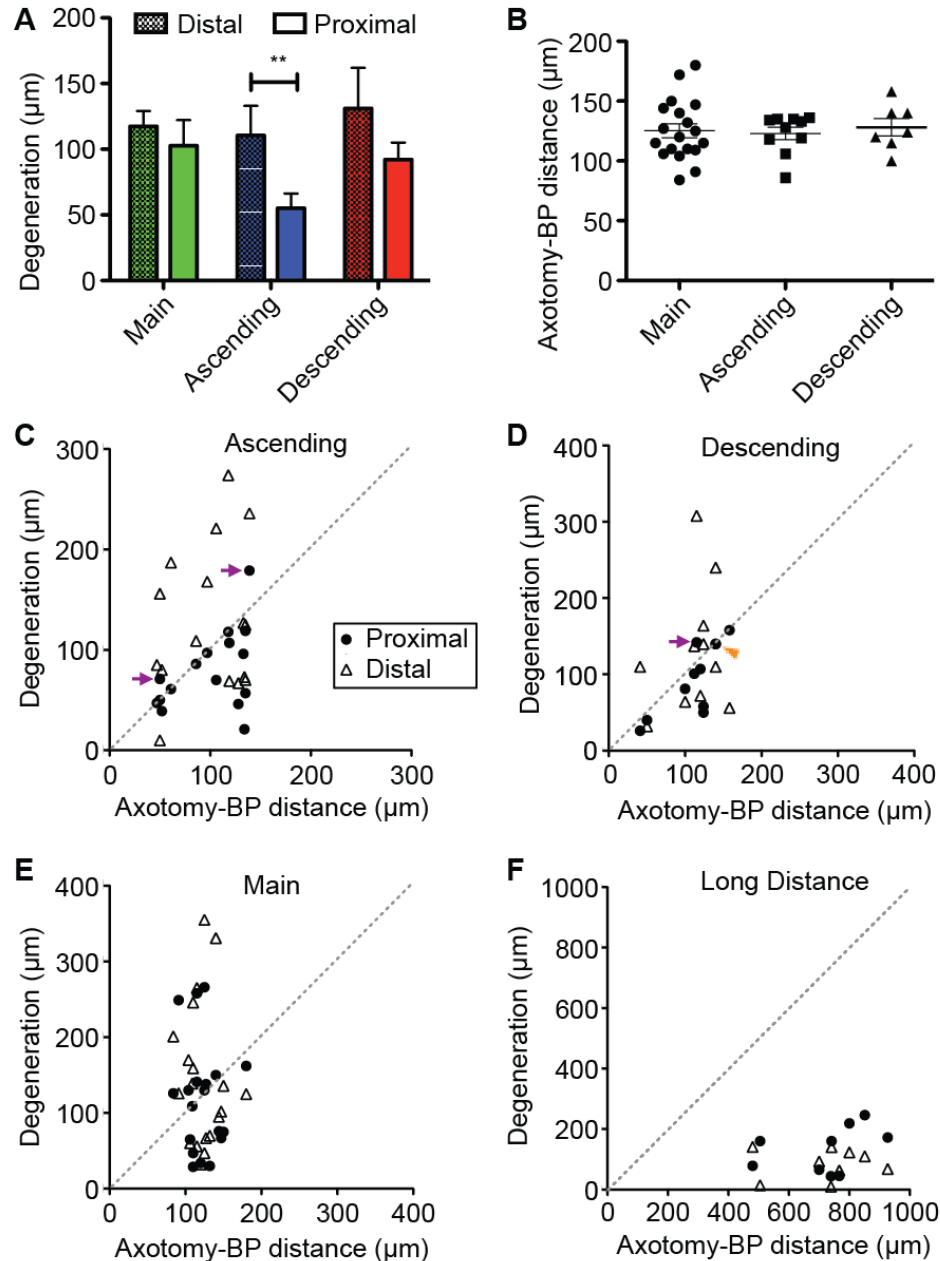


Figure 2.8 Location specific differences in acute axonal degeneration reveal a barrier to degeneration. **A)** Average acute axonal degeneration by 2.5 hours for all three injury locations. **B)** Graph represents the axotomy distance to bifurcation point for single axons. All graphs (**C-F**): distance of axon degeneration for distal and proximal axonal segments in relation to their distance from the bifurcation point after Ascending (**C**), Descending (**D**) Main branch (**E**), or long distance injury (**F**). Note the two proximal axonal segments breaching the bifurcation point for the Ascending branch ablation and one for the Descending branch ablation. Dotted grey line = bifurcation point boundary. For the main branch proximal axonal segment, this is the theoretical bifurcation point boundary. Purple triangles = proximal axonal segments that have breached the bifurcation point. Orange arrowhead indicates two data points that are superimposed on one another. Degeneration and axotomy distance are represented in microns. * $p = 0.03$, paired t-test.

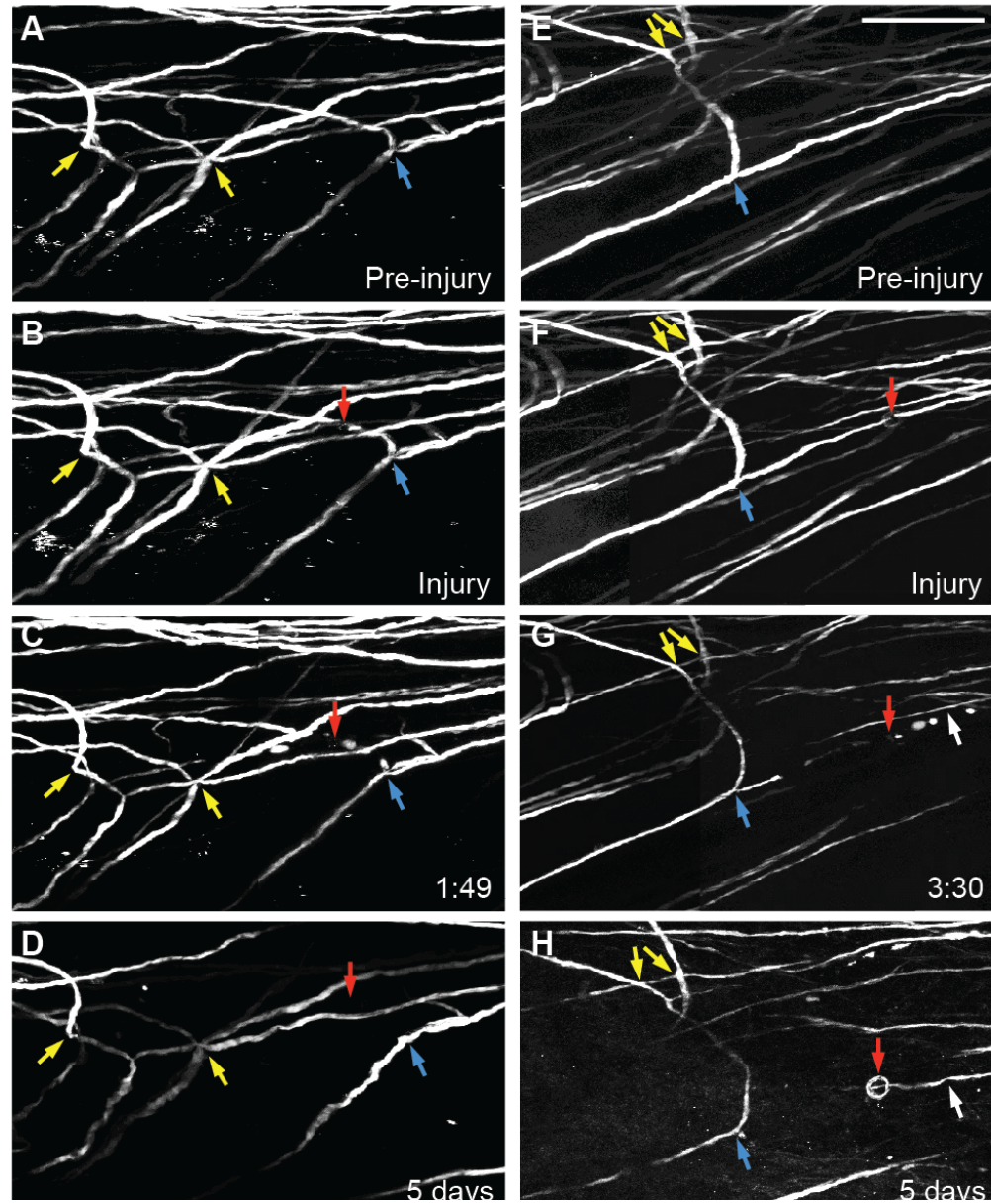


Figure 2.9 The bifurcation point is a barrier to acute and sub acute degeneration. (A-D): example of an injury for a descending branch close to bifurcation point before (A), immediately after (B) and 1:49 after injury (C). Note the disconnect between the proximal and distal axonal segment in B and the fragmentation of the distal and proximal axonal segment in C. By day 5 (D), the proximal axonal stub has degenerated to the bifurcation point with no damage or change to the descending axonal branch while the distal axonal fragments are no longer present. **E-H):** example of an ascending axon before injury. **(F)** immediately after **(G)** and 3 hours and 30 minutes after injury. Note the fragmentation of the distal segment and retraction of the proximal axonal segment. After the acute degenerative phase there is a small proximal axonal stub remaining. **(H)** By day 5 the proximal axonal stub has degenerated to the bifurcation point, again, there is no damage or change to the remaining descending or main branches. There is a small auto-fluorescent circle that is a remnant of the initial laser injury. Red arrows = injury location. Yellow arrows = landmarks that persist throughout the acute and sub acute imaging sessions. The blue arrows = bifurcation point. White arrow = uninjured axon. Scale bar = 100 μ m.

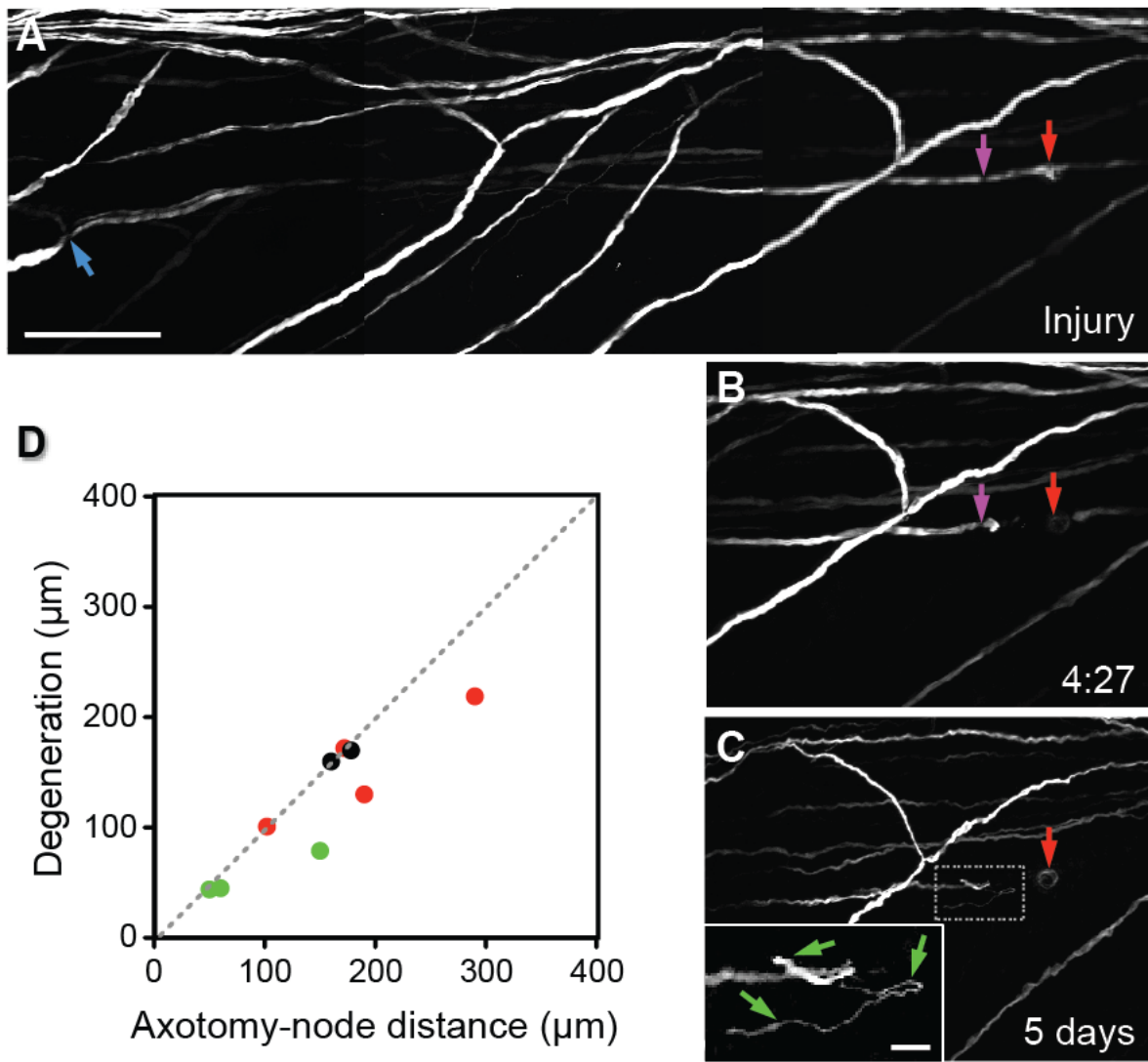


Figure 2.10 Long distance injury reveals intrinsic barrier to degeneration and point of origin for regeneration.

A-C) Long distance injury at acute and sub-acute time points. **A)** Immediately after injury and **B)** after 4 hours 27 minutes. **C)** Regeneration 5 days after injury. X-axis = distance from axotomy to the first proximal node of Ranvier. Y-axis = degeneration at the last time point for individual axons. Green arrows = new growth. Red arrows = injury location. Blue arrow = bifurcation point of injured axon. Purple arrows = node of Ranvier. Dotted grey line = location at which degeneration reaches node of Ranvier. A-C scale bar = 100 μm . Inset scale bar = 10 μm .

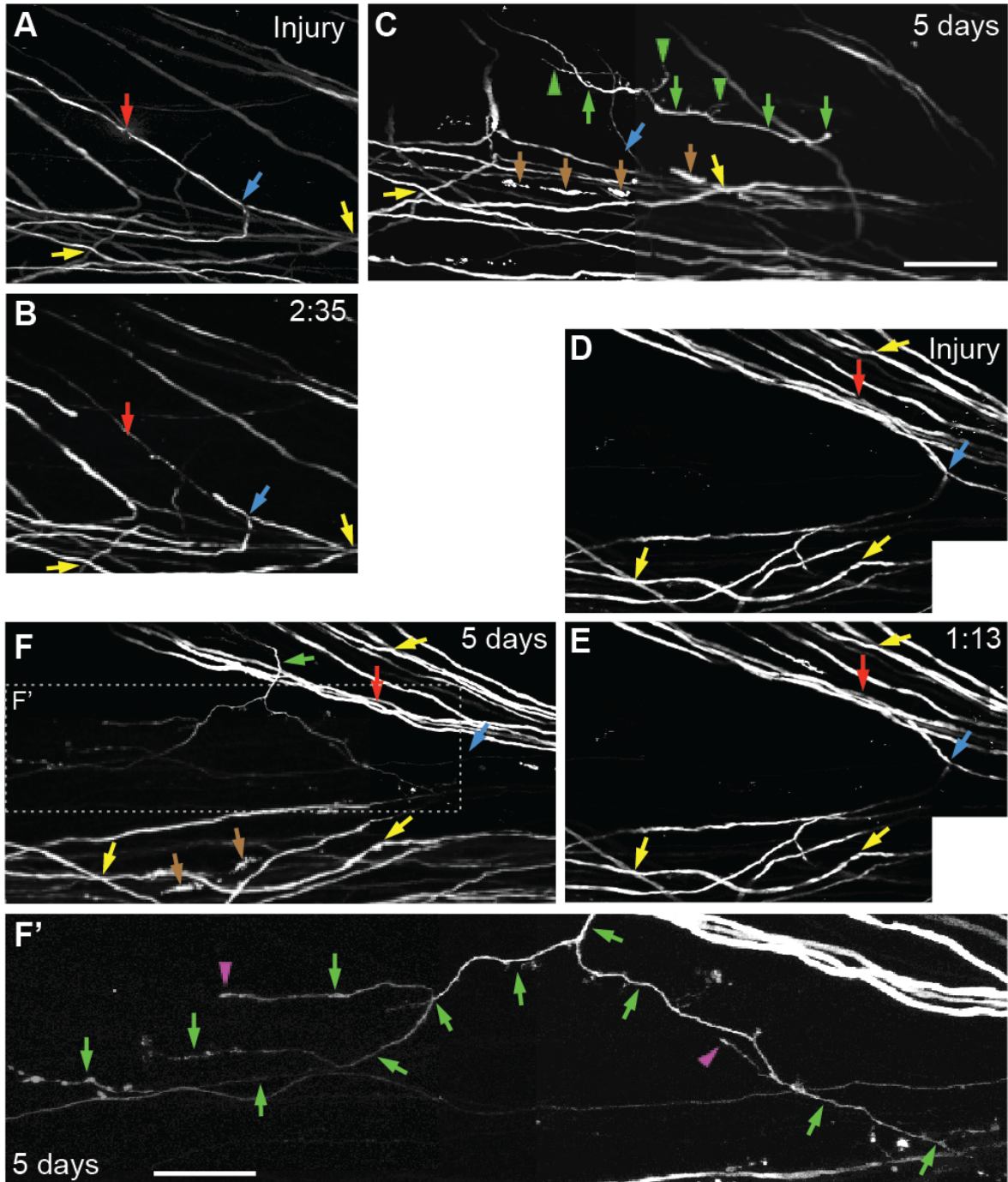


Figure 2.11 Sub-acute modes of axon regeneration: axonal extension and axonal branching. (A-C) Main branch degeneration and regeneration via axonal branching (A) immediately after injury and (B) by 2 hours and 35 minutes both the proximal and distal axonal segments have degenerated. (C) At Day 5, the main branch extends one primary regenerating axon along with other smaller regenerating branches. (D-F') Main branch injury branching phenotype. (D) Immediately after injury (E) 1 hour 13 minutes after injury (F) and at day 5. (F') High magnification image of inset in F. Scale Bar A-F = 100 μm. Scale bar C' and F' = 50 μm. Red arrows = injury location. Yellow arrows = landmarks. Green arrows = regenerating axon. Blue arrows = bifurcation point. Brown arrows = degenerating injured axon. Purple arrowheads = growth cones.

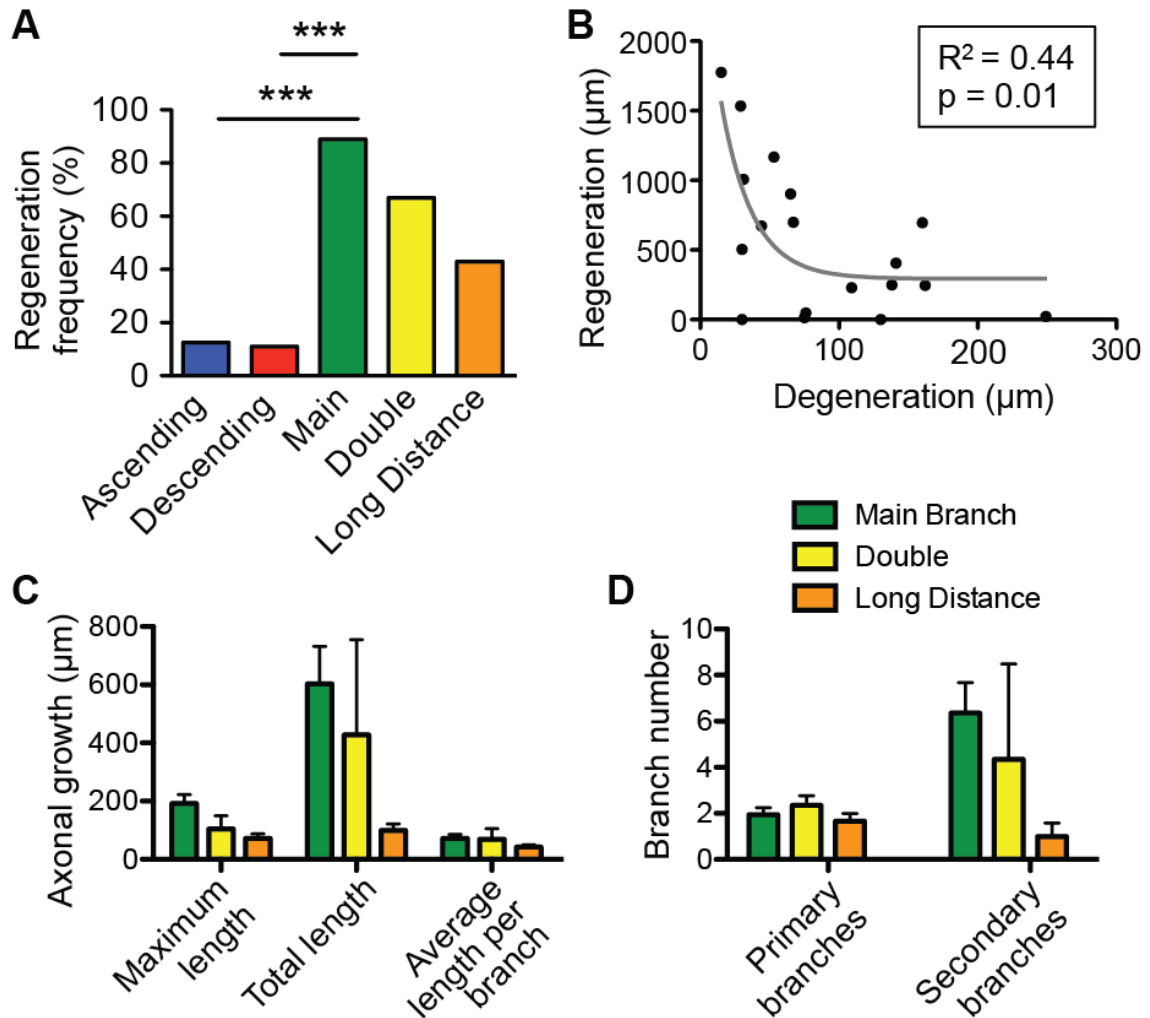


Figure 2.12 The loss of ascending and descending input elicits a sub acute regenerative response. A-C') Bifurcation point breach after descending branch injury leads to regeneration. A) Immediately after injury and B) at 3 hours C) and at day 5 . C') High magnification image of inset in C. D-F') Double branch injury leads to regenerative response. D) Immediately after injury and E) 4 hours and 21 minutes F) and at day 5. F') High magnification of inset in F. C' F' Scale bar = 50 μm . A-C, D-F scale bar = 100 μm . Red arrow points to location of injury. Yellow arrows = landmarks. Blue arrows = bifurcation point. Green arrows = new growth. Brown arrows = degenerating injured axon.

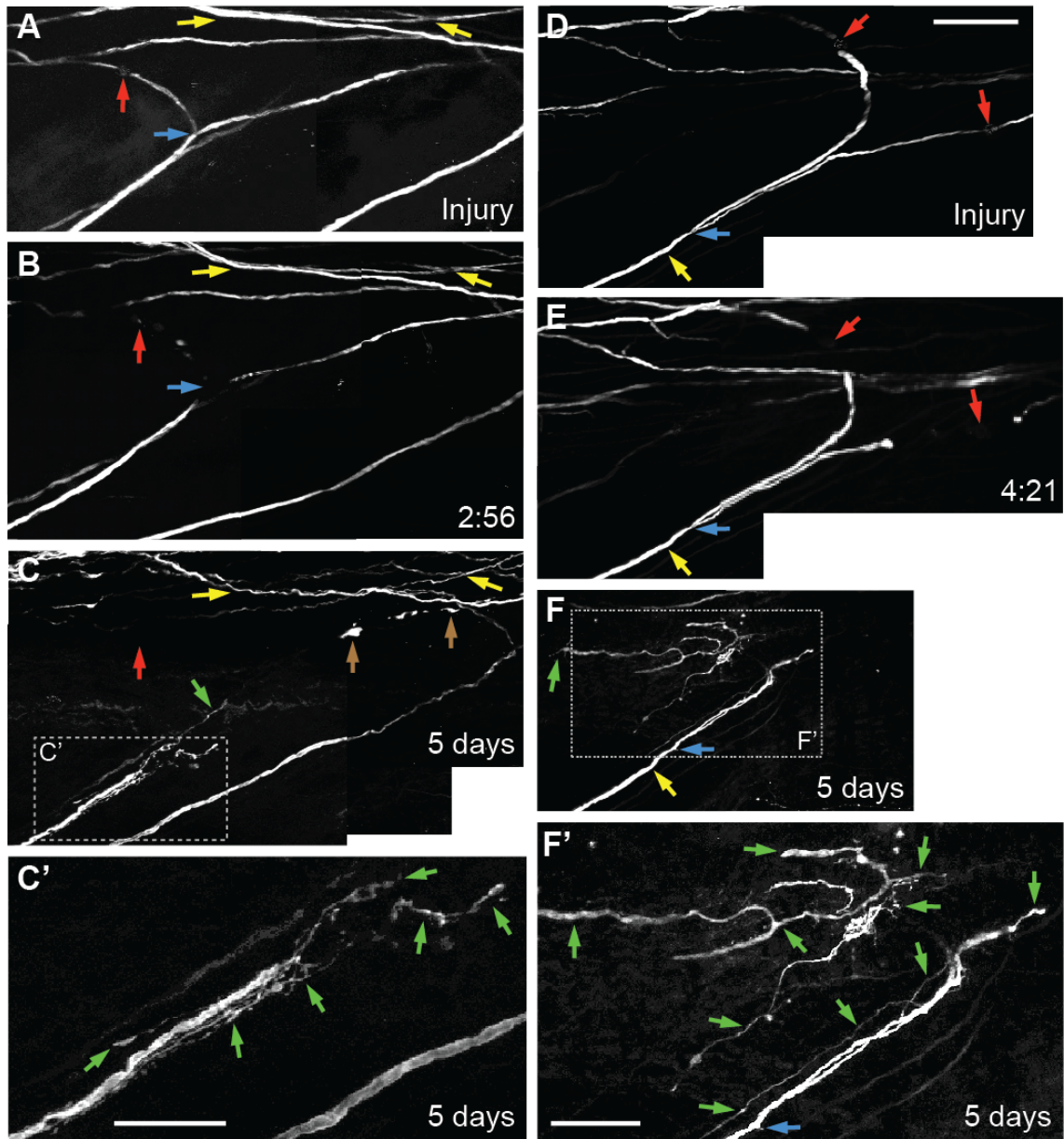


Figure 2.13 Regeneration after injury is correlated with location of injury and is inversely related to degeneration. **A)** Graph represents regeneration frequency 5 days after injury. Ablation of the main branch results in regeneration 89% of the time and is significantly different from the ascending and descending ablations, but not double or long distance ablations ($p = 0.0004$, $p = 0.0001$, $p = 0.58$, and $p = 0.53$ respectively). P values were determined using Chi-square and Fisher's exact test. **B)** Quantification of regenerative growth. **C)** Quantification of primary and secondary branching. **D)** Regeneration is inversely correlated with degeneration. The x-axis is the degeneration distance in μm at the last time point taken for each injured axon that had a corresponding day 5 time point. The y-axis represents the total regeneration in μm on day 5. There is a significant non-linear correlation with the regeneration as a function of degeneration ($f = y_0 + a \cdot \exp(-b \cdot x)$; $y_0 = 288.7$, $a = 2487.3$, $b = 0.04$). $R^2 = 0.44$, $*p = 0.01$.

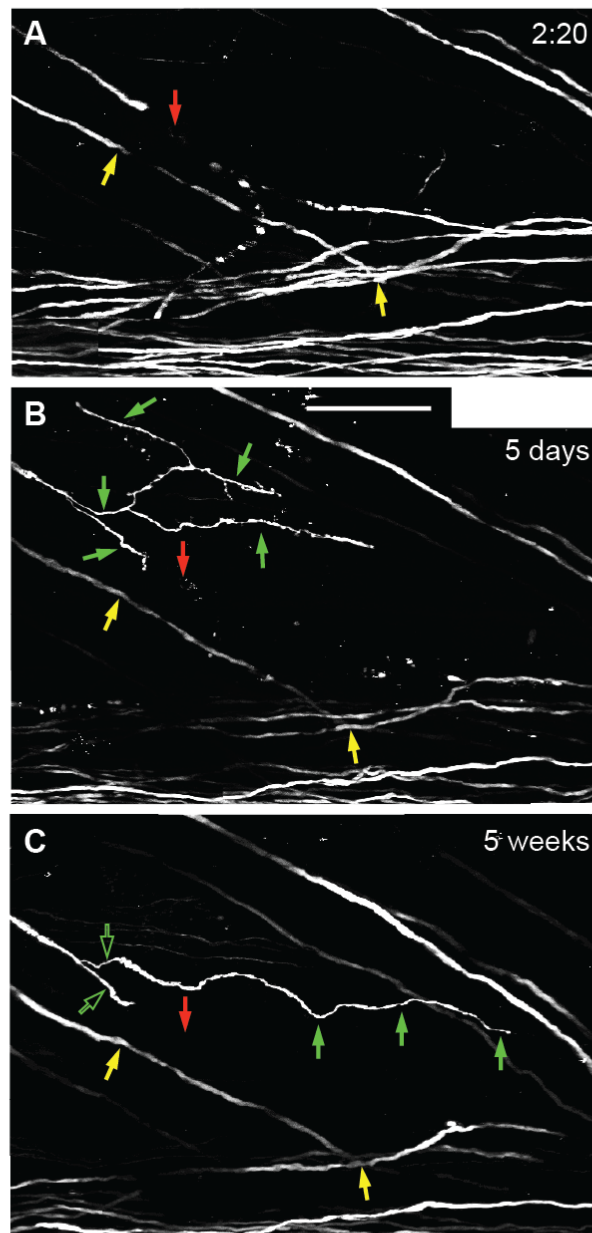


Figure 2.14 Regeneration is dynamic and continuous. **A)** Main branch 2 hours and 20 minutes after injury. **B)** Regeneration in the form of branching at day 5. **C)** Pruning and modest elongation at 5 weeks after injury. Scale bar = 100 μm. Red arrows = injury location. Yellow arrows = landmarks. Green arrows = new axonal growth. Open green arrows = sustained regeneration from day 5.

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CHAPTER 3.

Examining the specific effects of Nogo deletion on degeneration and regeneration with in vivo imaging

ABSTRACT

The effect of Nogo deletion on modulating long distance regeneration is still unclear. Studies have shown that Nogo has a significant effect on inhibiting neurite growth *in vitro*. However, when applied to mouse models of spinal cord injury, the effects of Nogo deletion have been less clear. With in vivo imaging, I was able to see specific effects of Nogo deletion on the degenerative and regenerative response after injury. First, while there were no significant differences in the total acute degeneration between Nogo mutant mice, Nogo deletion did seem to decrease the distal degenerative response. We showed that this decreased degeneration might be a result of increased retraction, which we showed (in Chapter 2) had a slower speed of degeneration. While there were no significant effects of Nogo deletion on increasing regeneration frequency there was a significant effect on increasing elongation after injury. However, this effect was not seen in the sub-acute and chronic stages. We did find that while Nogo did not significantly modify the growth over time, there did seem to be a general pattern of growth and pruning over time. This imaging paradigm revealed aspects of axonal response to injury after Nogo deletion at a temporal and spatial resolution that was not previously attainable. A better understanding of the molecular mechanisms of intrinsic and extrinsic regulation of CNS axon regeneration will greatly benefit therapeutic development for spinal cord and brain injury, stroke and various neurodegenerative disorders.

BACKGROUND AND SIGNIFICANCE

Of the three main CNS myelin inhibitors (Nogo, MAG, and OMgp), Nogo has been of particular interest since it was found to be a major protein responsible for regeneration block

through extensive studies with function blocking antibodies [15]. The Nogo gene, also known as Reticulon-4 (RTN4), encodes three major isoforms, Nogo-A, B, C, which arise from alternative promoter usage and alternative splicing [16]. All three forms share a C-terminal region with two hydrophobic regions separated by a 66-amino acid region (Nogo-66). Nogo-66 has also been shown to cause growth cone collapse and decrease neurite sprouting [138]. More specifically, Nogo -A is a transmembrane protein of 1192 amino acids that is partially encoded by exon 2 and 3. Nogo-A and B share a common N-terminal sequence encoded by exon 1. In addition, Nogo-A has a 24 amino acid sequence that facilitates binding of Nogo-66 to NgR1 (Hu et al 2005). In addition to NgR1, Nogo-66 has been shown to interact with other receptors such as PirB [45]. The other Nogo isoforms, B and C are not found naturally in myelin, can if overexpressed, delay PNS regeneration [139]. Of the three isoforms, Nogo-A is the primary form expressed in oligodendrocytes, and the main Nogo isoform recognized to be inhibitory [17]. Both Nogo-66 and the N-terminal region of Nogo-A are inhibitory to neurite growth [18].

PHYSIOLOGICAL ROLES

Nogo has well-established roles during CNS development. These include a role in neural migration, branching fasciculation, myelin formation, axon guidance [140]. During development Nogo is expressed in neural precursors in the cortex and is thought to play a role in cortical development and neuronal maturation by [141].

In the adult, Nogo is expressed highly in the hippocampus and has been shown to modulate synaptic transmission [142, 143]. In addition, neuronal Nogo-A has been shown to negatively inhibit growth cone motility [144] in the intact CNS through its regulation of the actin cytoskeleton. It is also a member of the reticulon family of proteins, and may play a role in shaping the curvature of the endoplasmic reticulum, but the relationship between this function and neurite inhibition is unknown [19].

NOGO RECEPTORS

Nogo, and its' receptors are thought to be relatively promiscuous molecules in that there is no one receptor that only binds one MAI, and there is not one MAI that only binds to one receptor. For this reason, we chose to specifically delete Nogo, as opposed to targeting its receptor. Some of the known receptors for Nogo are NgR1, NgR2, and NgR3, and Pirb [45, 145]. While the main receptor for Nogo, NgR, is not highly expressed in DRGs, other putative receptors for Nogo exist [146] [45]. For instance, studies have shown that NgR deletion does not reduce neurite growth inhibition effected by Nogo or CNS myelin [147]. Additionally, in vitro studies have demonstrated that DRG neurons can extend neurites on Nogo deficient myelin and in the presence of IN-1 inhibition is also relieved [43, 148, 149].

CNS DEGENERATION AND REGENERATION

Nogo was initially implicated in having a role in the inhibiting regeneration in the early IN-1 antibody neutralizing studies. IN-1 binds to, but is not specific to Nogo [150], and has been shown to promote corticospinal and raphespinal regeneration after spinal cord injury[151]. With regard to regeneration, three groups have found that a Nogo-A deletion can lessen the growth inhibition of myelin in vitro [7, 8, and 20]. Briefly, when neurons are cultured on Nogo-deficient myelin, there is significantly longer neurite growth as compared to wild type myelin. This inhibition release supports Nogo's role in regeneration inhibition in vitro. While there is no dispute on the in vitro role of Nogo inhibiting neurite growth, the in-vivo evidence is less clear.

Various groups have examined Nogo's role in-vivo, but the results are not concordant, as the phenotype of Nogo mutant mice from two studies show little to no regeneration, where another group reports extensive regeneration of the corticospinal tract [7, 8, and 20]. Two papers further contributed to this dispute as one reports regeneration in the optic nerve in Nogo deficient mice, and the other reports strain dependent differences in regeneration for Nogo deficient mice [21, 22]. However, the extensive spinal axon regeneration reported in one original Nogo knockout study has now been challenged by the discovery of artifactual labeling with the established tract tracing method observed in this mutant line [9]. One explanation was the differing generation of

the Nogo knockout mice, where one line was a gene trap, and lacked Nogo-A and Nogo-B, found significant regeneration. A second line only deleted Nogo-A, and found a trend for significance. And finally, the third Nogo knockout targeted all three isoforms of Nogo, Nogo A, B, C, yes did not result in increased regeneration. Another explanation was the differing genetic backgrounds, and finally a third observation was the technical aspect of labeling artifact. Due to these discrepant findings, it was thought that Nogo deletion caused compensatory up regulation of other MAIs, specifically MAG and OMgp. Our lab has previously shown that deletion of even three of the most potent myelin inhibitors is still not sufficient to alleviate the regeneration block. Therefore a reassessment of Nogo's contribution to regeneration failure in vivo is needed.

RATIONALE

In conventional spinal cord injury models, axon regeneration is defined as the growth of axons beyond an injury site. Because these models are terminal analyses, injured and uninjured axons are not readily distinguished. Thus, it remains unknown whether manipulating an extrinsic inhibitor such as Nogo modulates the growth of injured axons at a temporal and spatial scale that would have eluded analysis with conventional injury models. By circumventing the need for conventional tract tracing with two-photon microscopy and the Thy1-YFP-H transgenic and Nogo deletion mice (ND $-/-$ YFP $+$) I will assess the role of Nogo in preventing axon regeneration, independent of other major inhibitors, such as CSPGs.

MATERIALS AND METHODS:

Generation of mutant mice

The Nogo null mutants have previously been described in our lab. Briefly, the Nogo null was created by targeting the N- and C- terminal regions of the Nogo coding exons and Cre-mediated excision of this targeted sequence by loxP recombination. This mutant line was then backcrossed a minimum of 8 generations to C57BL/6 mice. This mutant was then bred to the

Thy1-YFPH (YFP) line, which was backcrossed a minimum of 8 times to C57BL/6 mice, as described in Chapter 2. The Nogo heterozygous mutants were then intercrossed to eventually obtain Nogo null homozygous and heterozygous mice used in this study, referred to as NDYFPH mice.

Animal subjects

Animals in these experiments were approved by the Institutional Animal Care and Use Committee. For all surgical procedures, mice were anaesthetized with 100mg/kg ketamine (Fort Dodge Animal Health, Fort Dodge, IA) 15mg/kg xylazine (Vedco, Saint Joseph, MO) and 2.5mg/kg acepromazine (Vedco, Saint Joseph, MO) in a 0.9% NaCl solution. After the imaging session was completed, animals were allowed to recover on a heating pad and given 0.05 mg/kg buprenorphine subcutaneously every 12 hours for 5 days, sterile saline for dehydration, and Baytril. For terminal procedures, animals were anaesthetized with 10mg/kg of a 50mg/mL solution of Nembutal (Ovation Pharmaceuticals, Deerfield, IL).

Animal Surgery

Adult transgenic mice were anaesthetized 100mg/kg ketamine (Fort Dodge Animal Health, Fort Dodge, IA) 15mg/kg xylazine (Vedco, Saint Joseph, MO) and 2.5mg/kg acepromazine (Vedco, Saint Joseph, MO) in 0.9% NaCl solution. The midline of the back was shaved and sterilized with 70% ethanol. A midline dermal incision (~ 1cm in length) was made to expose the vertebral muscles. The vertebral muscles were carefully removed to expose the thoracic vertebrae at the T8-T10 level and a laminectomy at the T9 level of the spinal cord was made to expose the spinal cord as described[127].

Stabilization of the spinal column

The spinal column was stabilized with customized Narishige spinal cord clamps and head holding adaptors mounted on a steel plate as described [127]. Briefly, the clamps were placed on

both the T8 and T10 vertebrae to stabilize the spinal column, while the head was placed in the head holding adaptor, and the tail was clamped so that the mouse maintained a relatively level position. A well of petroleum jelly was created around the exposed spinal cord and filled with artificial cerebrospinal fluid (ACSF) in order to accommodate the immersion lens.

In vivo imaging of ascending sensory neurons

After the mouse was secured in the stabilization device, it was then placed in a heat-controlled chamber underneath a customized FV300 Olympus microscope as described[127]. Imaging was performed with a Spectra Physics Mai-Tai laser tuned to 920 nm for two-photon excitation of YFP. In order to obtain a map of the entire imaging area an Olympus 10x .3 NA water-immersion lens was used. Figure 1A depicts the typical viewing area with a 10x objective. In this horizontal overview ascending sensory axons labeled with YFP enter the spinal cord and bifurcate into an ascending and descending branch. The blue arrows point to the bifurcation point. To examine labeled axons in finer detail, an Olympus 40x 0.8 NA water-immersion lens was used. Axons were chosen based on the visibility of the axon and proximity to other surrounding axons. The three locations chosen for axotomy were the ascending branch, which travels up the spinal cord eventually terminating in the *nucleus gracilis* and *nucleus cuneatus*, the descending branch, which sends its projections into the grey matter, and finally the main branch -the branch that is directly attached to the DRG cell body. We chose these different locations for the laser injuries as it has been shown that different branches elicit different regenerative responses [134].

For laser axotomy, we used the point function in the Spectra Physics Mai Tai Software for the Ti:Sapphire laser, with a duration of 0.2 seconds without a neutral density filter at a wavelength of 820 nm. Our average power output was 1-1.5 Watts for each laser axotomy event. This allowed us to focally sever a single labeled axon without visible damage to adjacent labeled axons.

Additionally, axons were chosen in an area that was not densely populated with other labeled axons. After locating a suitable axon, the laser was tuned down to 820 nm to perform the

laser ablation at one of three sites chosen for their unique anatomic relation relative to the bifurcation point, a schematic of which can be seen in (Fig.1B). As with wildtype mice there were no significant differences between the injury location distances from the bifurcation point (Fig 1C). After laser ablation, the axon was imaged for up to 5 hours to capture the acute axonal degeneration. We have determined that 2.5 hours is an appropriate time point for which to terminate the first imaging session, as the acute degenerative phase usually reaches a plateau by 2.5 hours. For re-imaging experiments, animals were anesthetized as described above, the original wound was reopened, and any tissue overgrowth was removed to expose the original imaging area. All animals included in the study had normal ambulation and did not exhibit any signs of motor dysfunction after each surgery. Animals that displayed motor deficits or any visible trauma to the spinal cord, such as bruising, after any surgery were excluded.

Image Processing

Acquisition of the images was executed with Olympus FluoView software. Z-stacks were taken every 10 μm with the 10x objective, and every 1 μm with the 40x objective throughout the entire focal area. The final images are a compression of all z-stacks to recreate the three-dimensional space in a two-dimensional image. During the acquisition phase laser power and offset were adjusted to minimize background. For post-acquisition image processing only brightness and contrast were manipulated using Adobe Photoshop. ImageJ was used for quantification of degeneration distances.

Quantification of axonal changes (degeneration and regeneration)

We used ImageJ to measure acute axonal degeneration as distance from the center of the injury location to the tip of the last contiguous piece of the axon both proximal and distal to the cell body. We measured sub-acute regeneration as any new growth from the terminal tip of the injured axon. For more chronic time points, total regeneration was measured as the sum of all growth new as of Day 0. For all acute degeneration images, we used the segmented line function

and measured degeneration from the injury location to the tip of the last contiguous axonal segment. These quantifications were done in 2D from the maximum projection Z-stack. For all sub-acute and chronic time points, we measured regeneration using the simple neurite tracer plugin for Fiji, which allowed for measurement in 3D.

Statistical Analysis

One-way ANOVA was used for multiple comparisons with a Bonferroni post-test. For multiple observations between groups we used two-way ANOVA. Unpaired t-tests were used for pairwise comparisons. Results are expressed as mean values $\pm$ S.E.M. The chi-square test was used for group frequency data, and the Fisher's exact test was used to determine significance between specific groups. Linear regression (R^2 values) analysis was used to test the significance of distance from the bifurcation point and degeneration. Non-linear regression was used to test the relationship and significance between degeneration and regeneration. A p value < 0.05 was considered to be statistically significant. All statistical analyses were done using Prism Graphpad, with the exception of the non-linear regression, which was executed with Sigmaplot software.

RESULTS

Nogo deletion slows the anterograde degenerative response.

While Nogo did not have a global effect on decreasing the degenerative response (Fig. 1 D), there seemed to be a decrease in the distal degeneration of the ascending and descending branches (Fig 3. A). Unlike wild type mice, where the distal segment degenerated freely above and below the theoretical bifurcation point barrier at acute timepoints (Fig. 2 C), the Nogo deficient mice rarely crossed this bifurcation point boundary $n = 9$ axons (Fig. 2 A). To confirm that this difference was not due to a difference in injury distance from the bifurcation point, I compared the average distances between the different injury locations. There was no significant difference between the distance of ablation to the bifurcation point between different injury

locations (Fig 1C), (Main = $132.4 \pm 4.58 \mu\text{m}$, Ascending = $132.5 \pm 17.06 \mu\text{m}$, Descending = $142.6 \pm 15.14 \mu\text{m}$). To see if there was a significant difference in the acute degeneration distance, we measured total distance degenerated at 2.5 hours between Nogo mutant and WT mice. We did find that while there were no significant differences in the degeneration of the proximal axonal segments for ascending and descending (A + D) injury locations, Nogo mutants did degenerate significantly less in the distal segment of A + D injury locations, where Nogo $-/-$ = $81.44 \pm .09 \mu\text{m}$ $n = 12$ axons, and Nogo $+/+$ = $95.64 \pm 23.06 \mu\text{m}$ $n = 20$ axons (Fig 3 A) ($p = 0.02$, ANOVA). There were no significant differences in acute axonal degeneration between any of the genotypes and Nogo $+/-$ mice.

Nogo deletion does not affect the degeneration mode or speed

To explore whether Nogo deletion decreased the degeneration speed, we measured the total percentage of axons degenerating via retraction (38.5%) as opposed to fragmentation (61.5%) in the distal axonal segment as compared to retraction (23%) and fragmentation (77%) in wildtype mice. There were no significant differences in the percentage of fragmentation (57% vs. 55%) and retraction (42% vs. 45%) in the proximal axonal segments of Nogo and wildtype mice, respectively (Fig 3. B). There were no significant differences the rate of degeneration between Nogo and wildtype mice, for fragmentation, retraction, or both modes of growth (Figure 3 C). In addition, fragmentation and retraction looked morphologically similar between Nogo and wildtype mice (Fig. 4 A-F) for Nogo mutant mice, as compared to (Fig. 4 A-H) in Chapter 2.

Nogo deletion does not increase the frequency of regeneration, but may enhance an elongating mode of growth

In addition to examining the acute differences between wildtype and Nogo mutant mice, I wanted to look at the differences at a sub-acute time point, 5 days. Interestingly, I found that Nogo deletion in heterozygous and homozygous mice did not increase the regenerative frequency for any of the injury locations. I hypothesized that Nogo deletion may increase the regenerative frequency for all injury locations as there would be less of an inhibitory environment.

Regeneration frequencies were similar within injury locations for all three genotypes (Fig 5 A) Nogo $-/-$ M= 80% A= 0% D= 0%, Nogo $+/-$ M= 100% A= 0% D= 0%, Nogo $-/-$ M= 89% A= 12% D= 11% n = a minimum of 3 axons for all injury locations.

While I did not see an increase in regeneration frequency, we did see a significant difference in total growth per branch between Nogo $-/-$ and Nogo $+/+$ mice, where Nogo $-/-$ ($120 \pm 23 \mu\text{m}$) axons grew significantly more than the Nogo $+/+$ ($72 \pm 13 \mu\text{m}$) regenerating axons (Fig 6 B, $p= 0.02$, two-tailed t-test). This only occurred for the main branch ablations, as we did not observe regeneration from ascending or descending injury locations in Nogo mutant mice.

The pattern of growth from Day 0, 5, 5 weeks, and 5 months is similar between Nogo and wildtype mice

Finally, to determine if there were any differences at chronic timepoints, I compared the regeneration between Nogo mutants and wildtype mice at 5 weeks and 5 months. I found that like wildtype mice, there seems to be a pattern of growth and pruning that is consistent with that seen in wildtype mice, where Nogo homozygous and heterozygous mutants initially have a rapid branching and growth phase at 5 days, which over the course of 5 weeks turns to a pruning phase in favor of preserving the strongest axons (Fig 6 A-D), and then from 5 weeks to 5 months, the axons that remained at 5 weeks switched to an elongation mode of growth, albeit significantly slower than the growth seen in the first few weeks after injury. This global growth pattern suggests that regenerative growth and pruning is Nogo independent and is likely regulated by a cell intrinsic growth mechanism.

DISCUSSION

While this Chapter is a preliminary investigation into the effects of Nogo deletion on degeneration and regeneration, I provide novel insight into potential roles that Nogo may play in regulating acute, sub acute, and chronic axonal remodeling after laser ablation injury. Nogo did not seem to play a role in the average distance degenerated between Nogo mutant and wildtype mice (Fig 1D); however, when injury locations were separated, the ascending and descending

distal segments seemed to degenerate less than the wildtype counterparts (Fig 3 A). This suggests that Nogo may have an active role in promoting degeneration of dying branches. The lack of a difference in the proximal segment suggests that a cell intrinsic mechanism is more likely to influence proximal degeneration.

One potential factor may be the bifurcation point. I showed that the bifurcation point was a barrier to acute degeneration in the wildtype mice. This phenomena was also present in the Nogo mutant mice, where the proximal degeneration of the ascending and descending branches stopped at or before the bifurcation point (Fig 2 A). In fact, the proposition that the bifurcation point is a barrier to degeneration was reinforced at the sub-acute time point, as the proximal segments never passed this barrier (Fig 2 B), where in the wildtype mice, there were a few instances of sub-acute breaching of the bifurcation point. Whether or not Nogo deletion has a role in this sub-acute effect remains to be tested; however, this further confirms that the bifurcation point presents some type of barrier for degeneration.

Nogo mutants also showed a similarity to wildtype mice in the types of acute degeneration patterns. There were similar frequencies of fragmentation and retraction in the proximal segments of Nogo mutant and wildtype mice (Fig 3 B). The distal segments of Nogo mutants did degenerate more via retraction (Fig 3. B). As retraction is significantly slower than fragmentation, this fits with the decreased degeneration of the distal segment in Nogo mutant mice. The specific effect of Nogo on promoting retraction as opposed to fragmentation remains to be tested. Though there were differences in the distal segment, the overall speed of degeneration via fragmentation and retraction were not significantly different between Nogo and wildtype mice (Fig 3 C), suggesting that Nogo does not affect the speed of degeneration, but rather the distribution of the degeneration pattern.

Finally, with regard to regeneration at sub acute timepoints, Nogo did not increase the frequency of regeneration suggesting that Nogo does not play a role in inhibiting the initiation of the regenerative response (Fig 5 A). However, there was a significant effect on the total length

per branch at sub-acute timepoints, where Nogo mutant mice had significantly longer branches as compared to WT mice (Fig 5 B). This novel observation suggests that Nogo deletion does play a role in the regenerative response, but can only potentiate a regenerative response and not initiate one. Also, if Nogo did have an effect on inhibiting the regenerative response, it would likely have been revealed in the ascending and descending injury locations of Nogo mutant mice, as there were no instances of a regenerative response for these injury locations in wildtype mice.

Also, like wildtype mice, there is a robust growth and branching at the sub acute timepoints, which is followed by a pruning phase at 5 weeks. It is surprising that there were no significant effects between Nogo and wildtype mice at either the 5 week or 5 month time points, as I have previously showed that Nogo affects the elongation at 5 days. This suggests that the effect of Nogo's inhibition is more limited to the initial growth response. The lack of an effect at 5 weeks implies the presence of another factor governing the pruning response. Lastly, as there is another elongation period at 5 months, I would have expected some effect of Nogo deletion; however, as there was no difference between Nogo or wildtype mice, it would imply that Nogo's inhibitory role is more restricted to the initial sub-acute regenerative response. It will be interesting to determine what factors/signaling pathways become activated or switched off at the chronic and subchronic timepoints, as these timepoints are more clinically relevant. This study indicates that there is a more universal, Nogo-independent, signaling pathway that is present at chronic and subchronic timepoints, and highlights the importance of in vivo imaging as a tool to complement experimental spinal cord injury studies.

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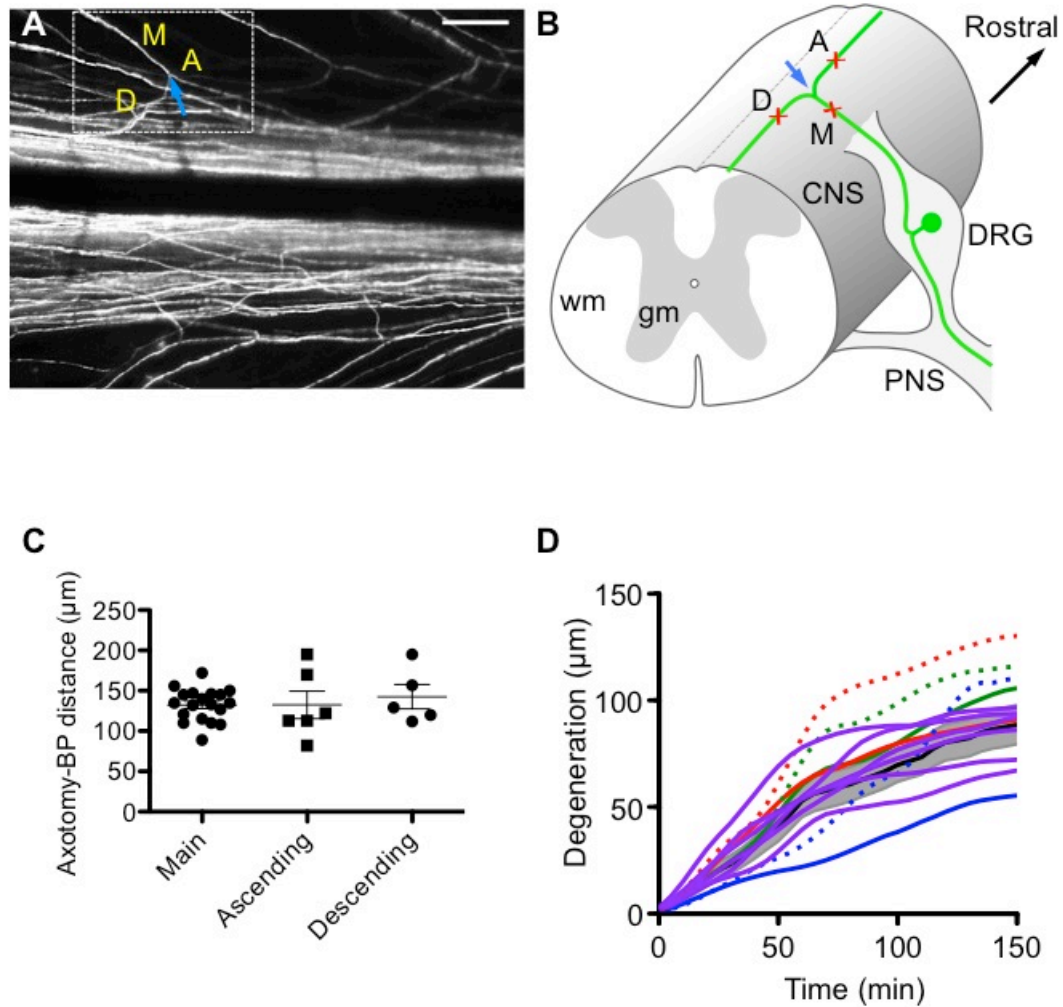


Figure 3.1 Acute axonal degeneration after laser ablation in Nogo mutant mice. A) Transverse image of the T-10 thoracic region in a Thy1YFP Nogo^{-/-} mutant mouse. Injury locations are designated by M = main, A = Ascending, D = Descending. Blue arrow points to bifurcation point. **B)** Schematic of sites of axotomy in relevance to the (secondary) branch point (blue arrow). Each of the branches (A, D or M) may be individually axotomized at the location shown (red cross) with a laser. **C)** Degeneration distance in relation the bifurcation point for Nogo null mice was not significantly different between injury locations, ANOVA $p = 0.76$. $n = 5-19$ axons per group. Nogo^{-/-}, Nogo^{+/-}, or Nogo^{+/+} mice and distance to bifurcation point for the main branch, where the average distances are $89 \pm 4.6 \mu\text{m}$, $71 \pm 11 \mu\text{m}$, and $84 \pm 5.8 \mu\text{m}$. ANOVA, $p = 0.5$. $N =$ a minimum of 8 axons in at least 4 mice. **D)** Graph of the acute axonal degeneration for all injury locations of Nogo mutant and wildtype mice. Only animals with at least 2.5 hours of degeneration were included in this graph. Degeneration distance in relation the bifurcation point for wildtype mice. $N =$ a minimum of 9 animals and 19 axons for each genotype. All degeneration data are represented as μm .

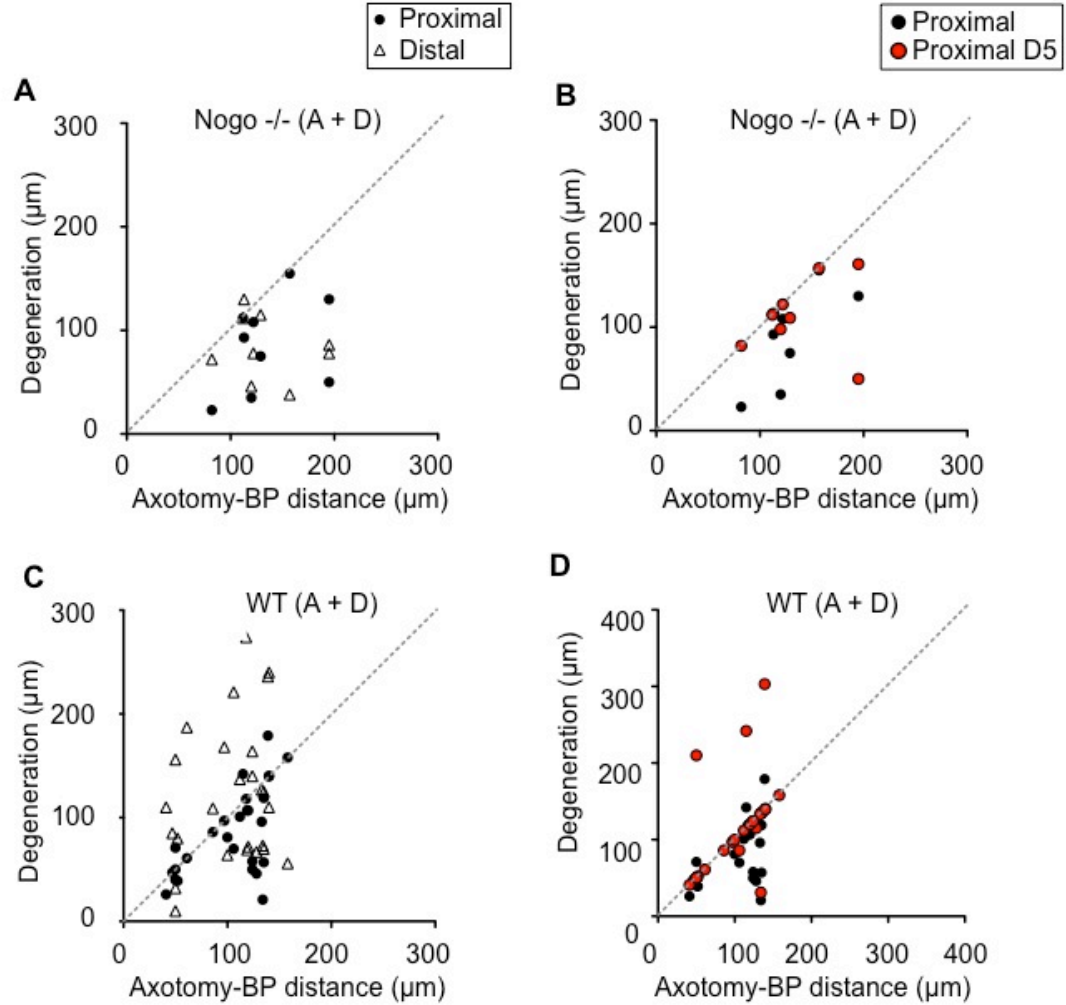


Figure 3.2 The bifurcation point is still a barrier to acute and sub-acute degeneration in Nogo mutant mice. All graphs (A-D): distance of axon degeneration for distal and proximal axonal segments in relation to their distance from the bifurcation point for the Ascending and Descending branch injury locations in Nogo^{-/-} (A and B) and wildtype (C and D) mice. A and C represent the acute time point where B and D represent sub-acute timepoints. Dotted grey line = bifurcation point boundary. Degeneration and axotomy distance are represented in micrometers.

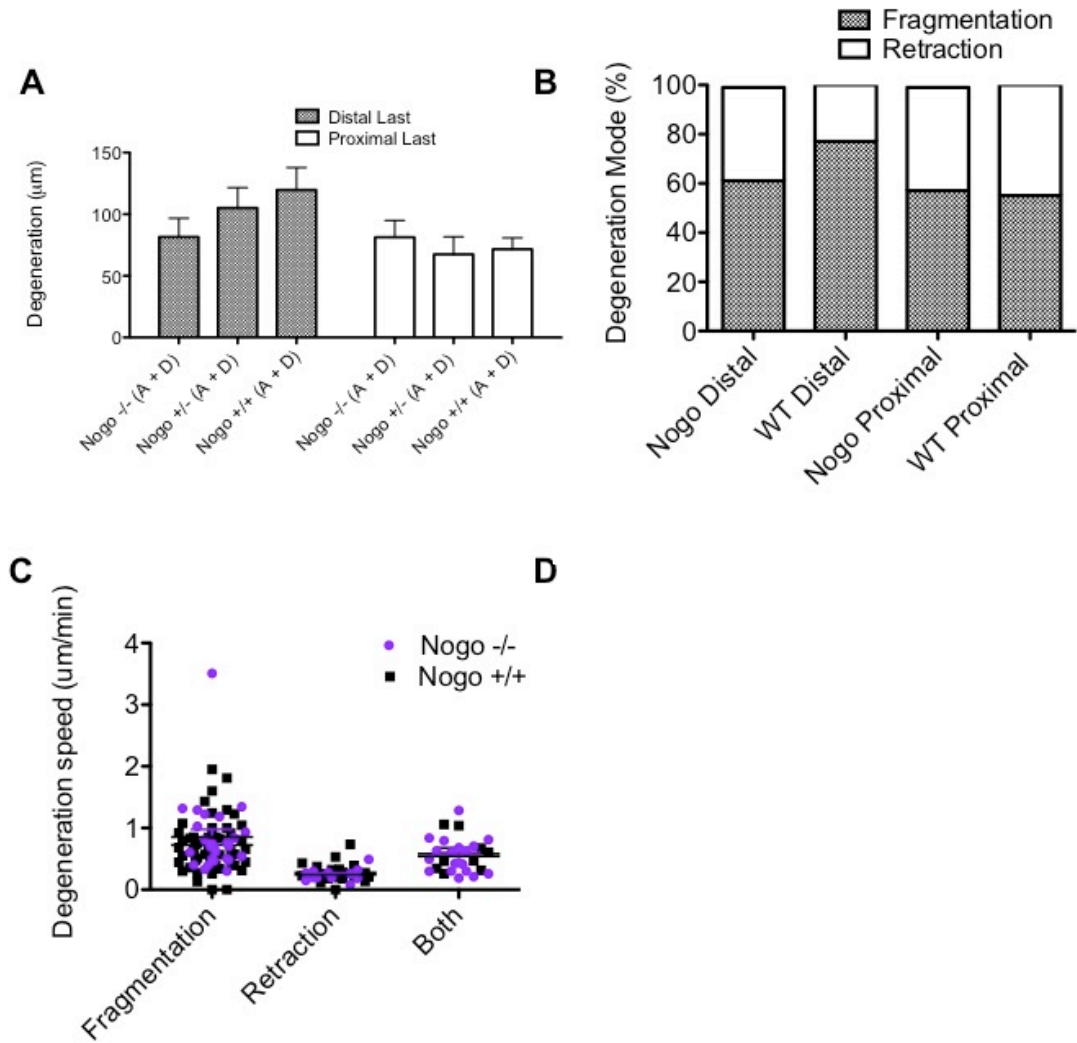


Figure 3.3 Nogo deletion may decrease the degeneration of proximal and distal axonal segments. **A)** Graph of the acute degeneration of proximal and distal axonal segments. Ascending and descending branches have been combined for all genotypes. Data are represented in μm as mean $\pm$ SEM **B)** Graph of the percentage of degeneration via either fragmentation or retraction in the distal and proximal axonal segments for Nogo -/- and WT mice. **C)** Comparison of the individual degeneration speeds between Nogo -/- and WT mice for fragmentation, retraction, and both. The y-axis represents degeneration in $\mu\text{m}/\text{minute}$.

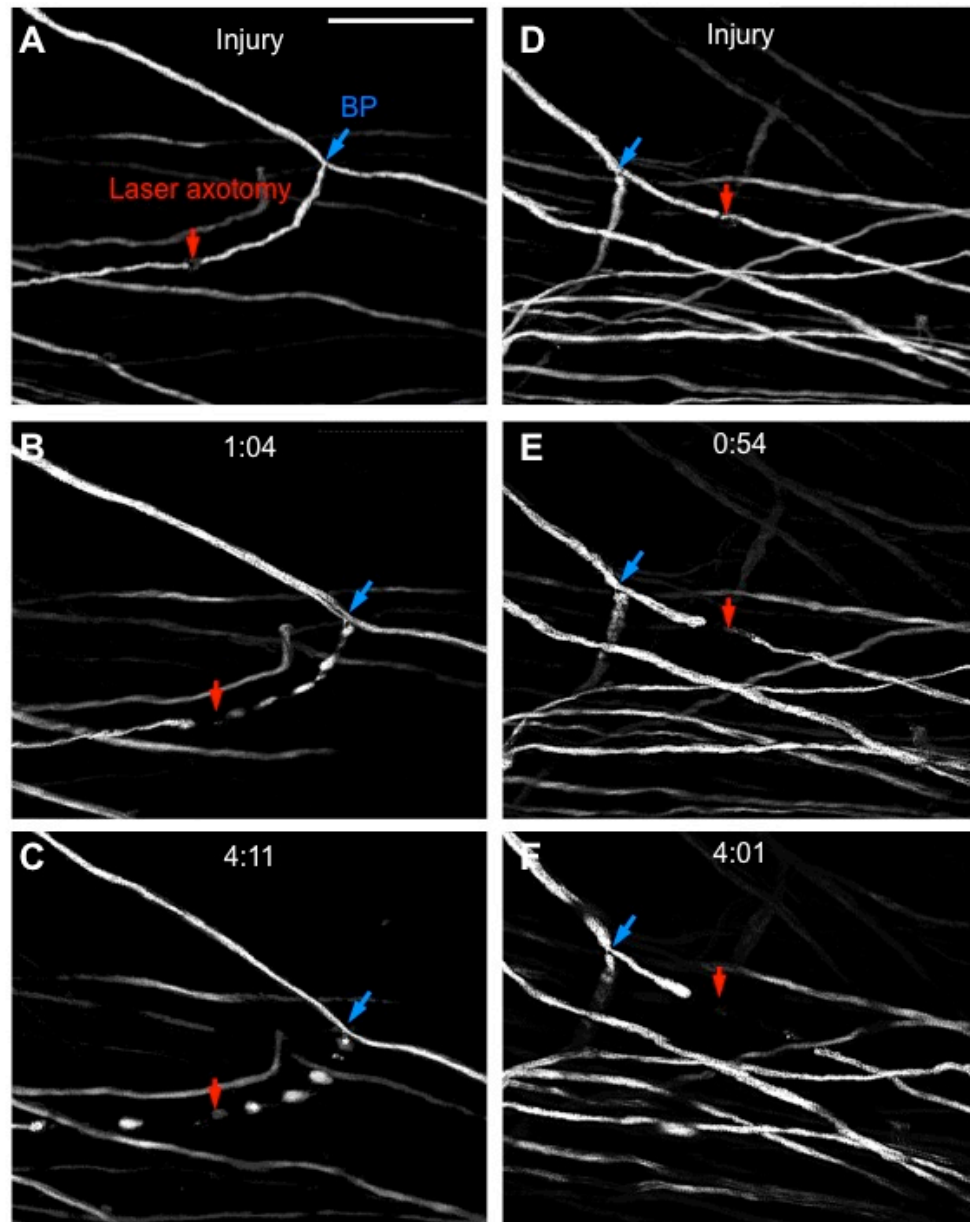


Figure 3.4 Nogo mutant mice still display fragmentation and retraction patterns of acute axonal degeneration. (A-C): Acute axonal degeneration in Nogo mutant mice via fragmentation (A) immediately after laser axotomy (B) one hour later, (C) four hours after initial injury. (D-F) Acute axonal degeneration via retraction (D) immediately after injury (E) proximal and distal retraction 54 minutes after injury, (F) and continued degeneration via retraction 4 hours after laser ablation. Blue arrow = bifurcation point. Red arrow = laser axotomy/ Scale bar = 100 μ m.

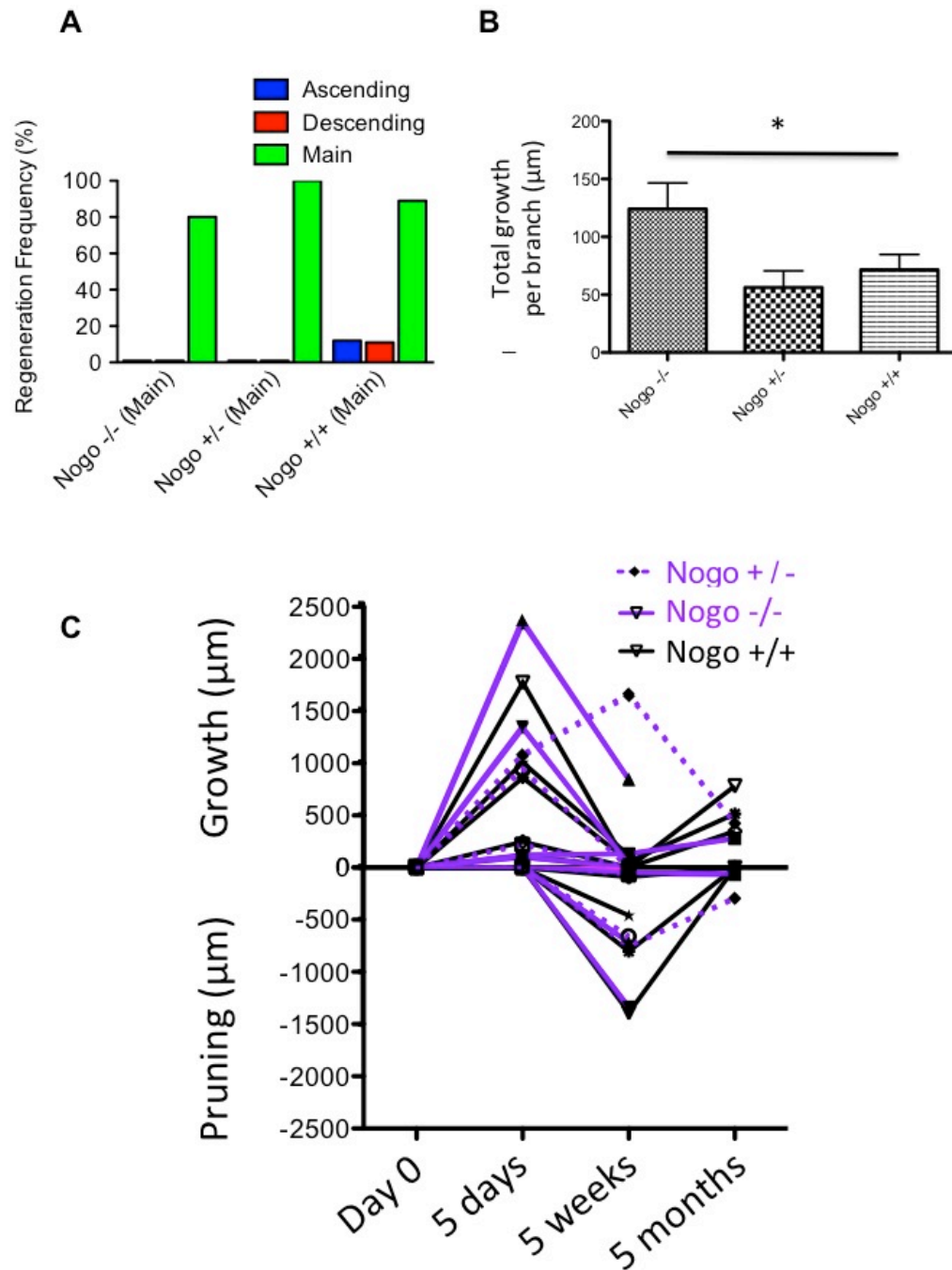


Figure 3.5 Nogo does not increase the frequency of regeneration, but may increase elongation of regenerating axons. **A)** Bar graph represents the frequency of regeneration between Nogo -/-. Nogo +/-, and Nogo +/+ mice for the three different ablation locations (Ascending, Descending, and Main). **B)** Graph represents the total regenerative growth per branch, represented in μm . Error bars = SEM, $p < 0.05$. **C)** Graph represents the growth and pruning over the four imaging timepoints. Data are represented in μm . Note the prevalence of growth during at 5 days and the prevalence of pruning at 5 weeks.

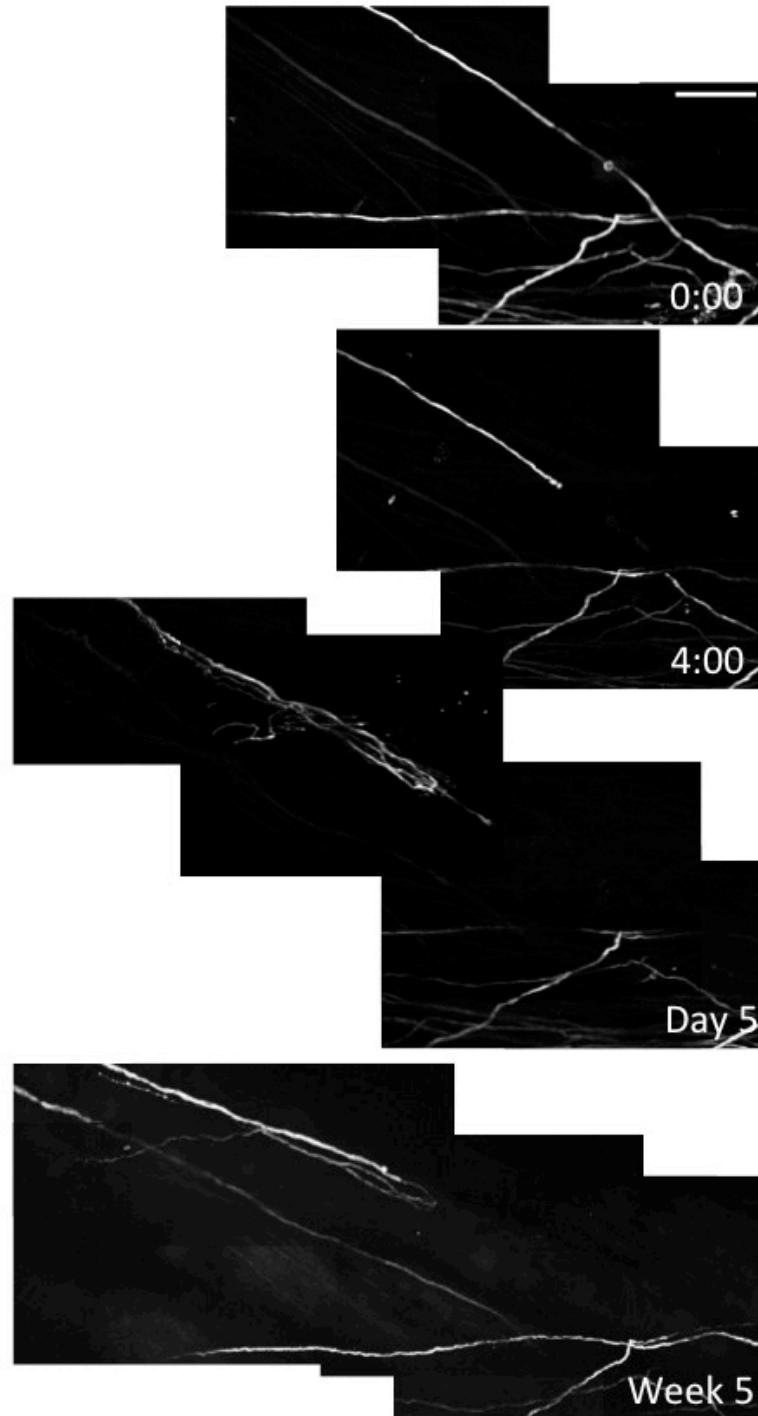


Figure 3.6 Continuous regenerative remodeling in Nogo mutant mice. **A)** Main branch ablation immediately after laser ablation and **B)** 4 hours after injury **C)** Regenerative growth at 5 Days **D)** Continued growth and pruning at 5 weeks. Scale Bar = 100 μ m. Error bars are SEM.

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Chapter 4.

Interaction of Conditional PTEN Deletion in Combination with Nogo Deletion on CNS Sprouting and Regeneration

ABSTRACT

Central nervous system axons, unlike the peripheral nervous system axons, are known to be relatively growth incompetent. This has been attributed to the developmental decline in intrinsic growth capacity and to the presence of various extrinsic inhibitory factors. Selecting the correct molecules to investigate in vivo and in vitro are challenging due to the wide diversity of candidate molecules. Here, we combine two intrinsic and extrinsic inhibitors of regeneration, PTEN and Nogo, respectively, to investigate whether increasing the growth competence of an axons in combination with reducing the inhibitory environment can lead to a more successful regenerative response after spinal cord injury. We extend the proposition that a combinatorial attack on regeneration inhibition is more efficient than a single manipulation. More specifically, we show that the adult deletion of PTEN, in combination with Nogo deletion results in significantly more regeneration than each molecule examined alone. Interestingly, we show that the adult deletion of PTEN, again in combination with Nogo, is more effective at enhancing the regenerative response than deleting PTEN at a neonatal stage. Understanding how these molecules complement each other and what potential compensatory mechanisms are upregulated after neonatal PTEN deletion will be important for future studies on experimental spinal cord injury and to the eventual implementation of effective therapeutic interventions.

INTRODUCTION

Increasing long distance axon growth after spinal cord injury is imperative to the future of successful functional recovery. In developing mammalian neurons, axons have the ability to extend long processes, prune, regrow and synapse onto the correct targets [152]. This intrinsic growth capacity declines with age [153, 154], and is thought to be one of the main reasons for CNS regeneration failure after injury. Earlier work on spinal cord injury identified extrinsic inhibitory factors as being the main barrier to regeneration [35, 38, 152]. As such, most of the work on experimental spinal cord injury aimed to alleviate the inhibitory block of these extrinsic molecules [36, 37, 114, 148, 155, 156]. These studies identified specific myelin-associated inhibitory proteins (MAIs) MAG, OMgp, Nogo, and MAG. Among the known extrinsic inhibitors, Nogo remains the one for which there is most evidence, with peptide antagonists and antibody function blocking agents, supporting its role in injury-induced axonal plasticity (but not necessarily long distance regeneration) [42, 151].

More recently, there has been a paradigm shift to target the intrinsic regenerative potential of injured neurons. It has only been in the past 1-2 decades that the intrinsic pathways have been studied. This is due, in part, to the failure of studies on extrinsic regulators to yield robust results [155-158], in addition to studies that demonstrate the upregulation of a developmental-like growth program yields regeneration in the CNS [67, 159, 160]. Previously a major focus of the intrinsic pathway was on examining the conditioning effect of a peripheral nerve lesion and its growth-enhancing effects on the CNS branch of dorsal root ganglion neurons [67, 70]. While this approach led to the identification of various intrinsic factors that may increase an axon's regenerative capacity, none alone had as robust an effect as the conditioning lesion. In recent years intrinsic molecules have been highlighted as the more promising approach to increase regenerative growth. Many are transcriptional regulators cJun [72, 73], SMAD1 [74, 75], ATF3 [76], KLF [77], STAT3 [78], SOCS3 [82], SnoN [161], second messengers (c-AMP [160]), kinases (Dlk1) [79, 80] [81], microRNAs [162], with a recent focus on the phosphatase, PTEN [82-86].

Phosphatase and tensin homolog (PTEN) is a dual phosphatase whose primary substrate is phosphatidylinositol (3,4,5)-triphosphate (PtdIns (3,4,5) P_3 or PIP $_3$) [163], and is highly conserved across species. Humans and mice differ in only one amino acid, a Ser-Thr exchange. PTEN is enriched in the brain, with preferential expression in neurons, and is localized to both the nucleus and cytoplasm [164-166]. PTEN's well known physiological roles include regulation of genomic instability and DNA repair [167-169], stem cell self-renewal [170], cellular senescence [171], muscle regeneration [172], and wound healing [173]. It is most famously known for its role in tumor suppression by negatively regulating Akt/PKB signaling pathway, which is known to regulate apoptosis, proliferation and oncogenesis [174]. Loss of the PTEN gene or mutations to PTEN is known to cause cancer, and can be linked to other disorders such as Cowden syndrome and autism [175, 176]. In mice, loss of one PTEN allele usually leads to the development of cancers and autoimmune diseases [177]. Homozygous loss of PTEN results in embryonic lethality [178]. In the CNS, PTEN has an involvement in neural development [179], cell proliferation (Cite), growth and apoptosis (cite), arborization [88], neuronal differentiation [165], synaptogenesis [180], and neurite outgrowth [181].

In the past few years, PTEN has gained momentum as being an important intrinsic regulator of CNS regeneration [84]. The first study to demonstrate this association examined how PTEN deletion increased axon regeneration after an optic nerve crush [85]. Next, this group in collaboration with our lab examined PTEN's effect on CST regeneration [86]. Another group also showed that dual knockout/knockdown of PTEN and SOCS3 has been shown to synergistically increase regeneration in optic nerve [82].

Based on continuing findings that combinatorial deletion of different pathways results in a greater regenerative response, we sought to combine conditional PTEN deletion and deletion of an extrinsic negative regulator, Nogo, to maximize the regenerative potential after injury. Studies have independently shown the effects of deleting either Nogo or PTEN on regeneration or sprouting after spinal cord injury; however, the combined effect of both of these inhibitors has yet to be explored in the CST after SCI [158, 182]. Furthermore, neuronal mTOR activity is

downregulated as development proceeds and is further downregulated after axonal injury [85, 183]. Age and injury induced decline of growth capacity may be one reason researchers have seen limited regeneration in Nogo mutants. We hypothesize that if intrinsic growth potential were increased it may make injured neurons more responsive to a reduction in myelin inhibition. Therefore combining Nogo and PTEN would be expected to have a synergistic effect on promoting axon regeneration.

MATERIALS AND METHODS

Generation of mutant mice

The Nogo null mutants have been described recently [158]. Briefly, the Nogo mutation was obtained by consecutive gene targeting disrupting the N- and C-terminal region of the Nogo coding exons, followed by Cre-mediated excision of the intervening sequence between loxP recombination sites introduced at the targeted sites [158]. The $PTEN^{flox}$ was obtained from Jackson Labs (C;129S4-*Ptentm1Hwu*/J). The single mutant mice were bred to each other to obtain heterozygous double mutants in a pure C57BL/6 (N8 minimum) background. The respective genotypes were then intercrossed among themselves to obtain $PTEN^{ff};Nogo^{-/-}$ double mutant. Scheme experiment breeds were set up by mating $PTEN^{ff};Nogo^{-/-}$ to $PTEN^{ff};Nogo^{+/-}$ so all the progeny could be used for experimental purposes. The two groups of mice were injected with AAV-Cre or AAV-GFP to obtain the four experimental groups: $PTEN^{ff};Nogo^{+/-}$ (WT mice, after GFP injection), $PTEN^{ff};Nogo^{-/-}$ (ND mice, single Nogo deletion, after GFP injection), $PTEN^{-/-};Nogo^{+/-}$ (PTEN, single PTEN deletion, after AAV-Cre), $PTEN^{-/-};Nogo^{-/-}$ (PTEN-ND, PTEN-Nogo double deletion, after AAV-Cre),

Viral production and injection

Viral vectors were obtained from Dr. Zhigang He laboratory. Viruses were produced at the Salk Institute Viral Vector Core. Viral concentrations were tittered by qPCR. AAV-Cre reached a titer of 0.5×10^{11} TU/ml and AAV-eGFP 0.7×10^{11} TU/ml. All experimental procedures were

performed in compliance with animal protocols approved by the Institutional animal care and use committee and UCSD, San Diego.

For Neonatal injections, post-natal day 1 (P1) *PTEN^{ff};Nogo^{-/-}* and *PTEN^{ff};Nogo^{+/-}* mice were cryo-anesthetized, placed on a ice-cold pad and injected with 1µl of either AAV-GFP or AAV-Cre in the right sensorimotor cortex. A modified 10µl Hamilton syringe attached to a fine glass pipette was mounted on a stereotaxic device for precise injection. After injection, mice were placed on a 37°C warming pad and covered with some home cage bedding to decrease the risk of rejection by the mother. Mice were returned to their mothers once they regained consciousness and normal color and behavior.

For young adult injections (4-6weeks old) *PTEN^{ff};Nogo^{-/-}* and *PTEN^{ff};Nogo^{+/-}* mice were anesthetized with 10% Avertin (Sigma). Female were injected with a total of 1.2µl of AAV-GFP or AAV-Cre into the right hind limb sensorimotor cortex at the following six sites: 0.1, 0.6 and 1.1 mm posterior; 1.0 and 1.4 mm lateral (in reference to bregma, 0.2 µl/site), 0.7 mm deep. These mice will be used to perform dorsal hemisection. For the pyramidotomy group, AAV-GFP or AAV-Cre were injected into the right forelimb sensorimotor cortex at the following six sites: 0.5 and 0.1 mm anterior, and 0.3 mm posterior; 1.2 and 2.2 mm lateral, 0.7 mm deep (0.2 µl/site).

Surgical procedures.

All procedures were approved by the Institutional Animals Care and Use Committee at UCSD. Spinal cord injuries, pyramidotomy and BDA tracer injection into the sensorimotor cortex were performed as described with minor modifications [158, 184]. Adult female mice were used for spinal cord injuries; adult male mice were used for pyramidotomy. In total, 162 mice of various genotypes were used in surgical experiments. Surgeries were performed 6weeks after P1 viral injections, and 4 weeks after adult injections.

Pyramidotomy: For pyramidotomy, mice were anesthetized with Avertin 10% and an incision made on the left side of the trachea. Skull base was exposed and craniotomy of the

occipital bone was performed using laminectomy forceps to expose the underlying pyramidal tract of adult male mice (Starkey et al., 2005). A micro feather scalpel was used to puncture the dura and lesion the entire left pyramidal tract. Wound was closed with 6.0 sutures and Dermabond. The pyramidotomy groups had a survival period of 4 weeks following injury.

Dorsal hemisection: For spinal cord injuries, a T8 dorsal hemisection was performed. T8 laminectomy was performed to expose the spinal cord. To achieve the dorsal hemisection, first, the dura was punctured bilaterally with a 30G needle at appropriate locations. Next, a pair of superfine iridectomy scissors was used to cut the spinal cord: the dorsal half of the cord at a depth of 0.8 mm for dorsal hemisection. Finally, a micro feather ophthalmic scalpel was used to retrace the lesion to ensure its completeness. The muscle layers were sutured with 6.0 sutures and the skin was secured with wound clips and Dermabond.

BDA tracing: Two weeks before euthanasia (2weeks post PYR, 4weeks post DH), mice received stereotaxic injection of BDA (10%, Invitrogen) to label CST axons by anterograde tracing. For the dorsal hemisection groups, BDA was injected at the same coordinates used for viral injections, six injections at 0.7 mm deep into the right sensorimotor cortex at the following coordinates: 0.1, 0.6 and 1.1 mm posterior; 1.0 and 1.4 mm lateral (in reference to bregma, 0.2 µl/site). For the pyramidotomy group, BDA was injected into the right sensorimotor cortex at the following six sites: 0.5 and 0.1 mm anterior, and 0.3 mm posterior; 1.2 and 2.2 mm lateral.

Behavioral assessment

All behavioral tests were performed by observers blind to the genotypes and quantified by different observers also blind to the genotypes. The following assays were performed:

1) Rearing test (pyramidotomy group only): this test assesses the forepaw preference during rearing behavior for forelimb function in a normal exploratory activity [184]. For forepaw

preference test, mice were placed in a new cage. Mice were videotaped from above for 15min, and, at a later date, an experimenter blinded to the animal genotype scored forepaw use for weight-support against the cage wall while rearing on its hind limbs 25 times. Mice were tested on days 2, 7, 14, 21 and 28 post-surgery. The percentage of using left only, right only or both forepaws for weight support while rearing on hind limbs was measured. Both forepaw score was subdivided into right touching the wall first or left touching the wall first using a slow motion parameter. Movements where left or right were undistinguishable were counted as both.

2) Tape removal test (pyramidotomy group only): this test was performed as described in (Starkey et al., 2005) with some modifications. Mice were habituated to the testing environment by being handled as they would during testing to decrease risks of anxiety. This consisted in scruffing the mice and touching their forepaws as it would be done to attach a sticker for the real recording session. Mice were then left for 2 minutes in the cylinder as in normal session. Pre-cut round stickers (Office Depot, 1/4" diameter) were applied in 2 trials for each forepaw, alternating left and right. A camera (Panasonic) was used to videotape mice until the sticker was removed, or for a maximum of length of 2 minutes. Mice were tested at days -1 (baseline), 2, 7, 14, 21 and 28, by the same experimenters, at the same time of the day. An observer blind to the mouse genotype later analyzed the video recordings. Scoring consisted of measuring the amount of time necessary to notice the sticker (shaking hand, bring paw to the mouth, scrub on the head) giving the sensory score, and the amount of time necessary to remove the sticker (after sensing), to obtain the motor score.

3) Ladder rung test (pyramidotomy and dorsal hemisection group): was performed as described in (Metz et al 2009) with some modifications. Briefly, mice were habituated to run on the apparatus for 5 consecutive days prior to surgeries to decrease the risk of any anxiety. A camera (Panasonic) was positioned at a slight ventral angle, so positions of all four limbs could be recorded simultaneously. Testing consisted in having the mice walk for a minimum of 6

passages in front of the camera to obtain sufficient step numbers (minimum of 30 steps). Mice were tested for a regular pattern (allowing the mice to learn the pattern during the training and to anticipate the position of the rungs), and irregular patterns, which was changed from trial to trial to prevent the mice from learning the pattern. The same irregular patterns were used for all the animals at the corresponding day of testing. Similar tests and recording were performed at days - 1 (baseline), 2, 7, 14, 21 and 28 for the pyramidotomy group, and 2, 7, 14, 21, 28, 35 and 42 for the dorsal hemisection group. All recordings were performed by the same experimenters, at the same time of the day. The video recordings were analyzed by an observer blind to the mice genotype using frame-by-frame analysis at 30 f/sec as described [185]. A foot fault scoring system provided a score from 0 (total miss step) to 6 (perfect placement) for each consecutive step for individual paws, giving the percentage of missteps as well as average score.

4) BMS (dorsal hemisection group only): For the BMS (Basso Mouse Scale, and adaptation of the widely used BBB scoring system from rat to mouse), mice were placed in an open field and observed for 5 minutes by two observers (Basso et al., 2006). Many features were noted, including ankle movements, stepping pattern, frequency, coordination, paw placement, trunk instability and tail position. The BMS score were calculated later, ranging from 0 (no movement) to 9 (normal locomotion). Mice with a BMS > 3 (some stepping) at 1 day post injury were removed from the analysis.

5) Rotarod (dorsal hemisection group only): For rotarod test, mice were placed on a rod (Ugo Basile) rotating at increasing speeds from 5 to 50 rpm in 3 minutes with constant acceleration. The latency to fall (in seconds) was averaged between two trials per session. Mice were first acclimated to the test for 2 sessions for five days the week before injury, and 1 additional session one day before injury (baseline). Mice were then tested at day 2, 7, 14, 21, 28, 35 and 42 after spinal cord injury.

Tissue processing

Tissue processing was performed as described with minor modifications (Lee et al., 2009b). Mice were given lethal dose of Fatal plus, and perfused transcardially with 4% paraformaldehyde. After removal of the brain and spinal cord, the tissue was post fixed overnight at 4C in the same fixative solution. Tissue was incubated in 30% sucrose for 3 days for cryo-protection. Brain, medulla and different segments of the spinal cord (from C7 to C3 for the pyramidotomy group, and 4mm rostral and 4mm caudal to the injury for the dorsal hemisection group, as well as a segment 4mm above injury, to ensure efficient labeling with BDA and one segment 4mm below injury, to assess the efficacy of the surgery and the lack of spared axons) were embedded in OCT compound, and snapped frozen on dry ice. Tissues were sectioned with a cryostat at 20 μ m thickness for further processing.

For CST axons, transverse sections of the medullary pyramids were processed to control for labeling efficiency and for counting BDA-labeled CST fibers (see below). For the dorsal hemisection group, floating consecutive serial sagittal sections centered at the injury site were stained for BDA and GFAP. Sections were incubated in Vectastain ABC solution (Vector Labs) overnight, washed in PBS and mounted on gelatin-coated slides. BDA was detected with TSA Plus Fluorescein/ Cyanine System (10min, room temperature, 1:200, Perkin Elmer). For the dorsal hemisection group, the injury sections were co-stained with polyclonal Rabbit anti-GFAP (overnight incubation, 1:500, Dako) after blocking in PBS-Tx-0.4% with 5% Horse Serum for 2 hours at room temperature. The next day, sections were washed and secondary anti-rabbit alexa fluor 488 (1:500, Invitrogen) was added for 1hour at room temperature. After several washes with PBS, sections were cover-slipped with Fluoromount-G (Southern Biotech). For the pyramidotomy group, transverse sections of the cervical C7 were processed similarly, but without maintaining consecutive order and without GFAP staining. However, for this group, lesion sites at the medulla were carefully examined. Transverse sections of the cervical spinal cord C7 were stained with antibody to PKC γ (Santa Cruz Biotechnology, 1:100) to further examine the completeness of the lesion (data not shown). Mice with incomplete lesion were excluded from the study.

Briefly, sections were washed in TBS followed by blocking in 5% NHS for 2 hours at room temperature. Primary antibody for PTEN (Cell signaling) (1:100) and PS6 (Cell signaling) (1:500) were incubated at 4 degrees overnight. Sections were then washed with TBS-T (.05% tween) and incubated with 1:250 donkey anti-rabbit biotinylated IgG (Jackson Immunolabs) in 2.5% NHS TBS for 2 hours at room temp. Sections were washed and incubated in ABC with 10 μ L of solution A and B for every 1mL of TBS overnight at 4 degrees. Sections were washed and mounted onto slides and TSA was added 1:200 to TBS and incubated for 10 minutes.

Quantification of axons

Images were taken with a digital camera mounted on a Zeiss epifluorescence microscope using the 10x or 20x and axon density and number quantified using ImageJ. Observers blind to the genotypes conducted all axon quantification. The ImageJ program was used as described [186]. For the pyramidotomy group, Sprouting Axon Density and Sprouting Axon Number Index were quantified. Transverse sections of the cervical C7 of the spinal cord were taken. Axon density in each side of the gray matter (denervated or not) was measure after subtraction of the background using ImageJ. The outcome measure of the sprouting density index was the ratio of contralateral and ipsilateral counts. 5 randomized sections per animal were quantified and averaged. Axons in the main CST were excluded from analysis.

To quantify the Sprouting Axon Number Index, lines were drawn through the central canal and across the dorso-ventral axis, at 50 μ m from the midline, then every 100 μ m laterally in the denervated side of the gray matter. Number of axons crossing theses lines was quantified over five sections and normalized against total axon count in medulla to obtain axon index, which is then plotted as a function of the distance from the midline.

For the dorsal hemisection group, the density of fibers rostral to injury site was analyzed quantitatively by measuring the BDA staining intensity. A series of rectangles of 100 μ m wide and covering the entire dorso-ventral spinal cord were superimposed onto sagittal sections, starting from the injury site (delimited by GFAP staining) all the way to 1.5mm rostral to the injury. After

subtraction of the background, intensity was measured in every rectangle using ImageJ; and the pixel value of each segment was normalized against the intensity at 1.5mm rostral to injury. The ratio represents the Fiber Density Index and is expressed in function of the distance to the injury.

The number of axons present in all regions containing BDA-labeled axons caudal to injury was also analyzed to assess regeneration. Segments of 100 μ m wide covering the dorso-ventral axis of the spinal cord were drawn caudal to the injury, and the total number of axons in each segment quantified. The axon numbers, normalized against the total number of axons labeled in the medulla, were averaged from 10 sagittal sections (containing or adjacent to the labeled main CST bundle) and expressed as a function of the distance from the injury site in a scheme similarly as described [148, 156] (Lee et al., 2009b; Simonen et al., 2003; Zheng et al., 2005; Zheng et al., 2003). The ratio represents the Fiber Number Index and is expressed in function of the distance to the injury. Regeneration was defined as BDA-labeled axon exiting the injury site, and entering the GFAP positive region caudal to the injury. The number of axons present within the injury site, delimited by the GFAP staining, was similarly quantified as mentioned above, and corresponds to the 0 μ m. on the graphs.

RESULTS

The primary goal of this study was to extend the findings of Liu, et. al. 2010, that PTEN deletion increased the regenerative ability of CST axons. More specifically, we sought to combine PTEN deletion and Nogo deletion and examine their combined effects after injury. We specifically wanted to examine the interplay of Nogo and PTEN deletion in sprouting and regeneration after both neonatal and adult deletion of PTEN. To examine the effects on sprouting we chose to injure the CST at the level of the medullary pyramids. To examine the effects on long distance regeneration, we chose to injure long distance descending projection neurons, the corticospinal tract (Deumens 2005) at the thoracic level of the spinal cord. Additionally, this tract is responsible for controlling voluntary movement, and improving regeneration of this tract would play a significant role in functional recovery. Thus, assessing the effectiveness of Nogo and

PTEN deletion on increasing regeneration in the CST is critical to future therapeutic interventions.

Nogo does not enhance neonatal PTEN deletion compensatory effect on sprouting

We first examined the effects of CST sprouting across the midline following pyramidotomy of mice that had PTEN deleted at P1. This descending tract originates in the sensorimotor cortex, crosses at and descends through the pyramids to the spinal cord. The CST is primarily responsible for voluntary movement. As the dorsal CST is believed to be relatively averse to sprouting and regeneration, we reasoned that any effect on sprouting would be readily detected with this model. To examine compensatory sprouting (defined here as growth from uninjured axons toward the injured side of the spinal cord), we made a unilateral lesion on the left side of the medullary pyramids and assessed sprouting from the right CST. To trace the growing axons, we injected BDA into the right motor cortex two weeks after injury, and perfused two weeks later. Figures 1 (A, C, E, G) are representative images of cervical 7 (C7) spinal cord sections stained for BDA.

First, completeness of the lesion was confirmed after a PKC- γ staining was performed on caudal cross sections. Mice that had any axons in the injured portion of the CST were excluded from the study (only a total 3 mice were excluded). We then analyzed the total intensity (sprouting axon density index) and total number of axons (sprouting axon number) crossing the midline using BDA staining. For the later, measurements were normalized to the total number of labeled axons in the medulla to control for labeling variability.

Next, and as demonstrated previously [158], we observed a slight increase in sprouting in the Nogo deleted mice (Fig. 1 C, D) compared to WT mice (Fig. 1 A, B). Indeed, ND mice showed a higher sprouting axon density index that failed to reach statistical significance (Fig. 1I, WT 0.066 ± 0.006 , ND 0.088 ± 0.013). The sprouting axon number was also higher for the ND mice at 50 and 150 μ m from the midline, but again was not statistically different from the WT (Fig. 1J, WT: 0.009 ± 0.001 , ND: 0.012 ± 0.001 at both 50 and 150 μ m). Next, we confirmed results reported in previous studies (Liu et al), as we found that deleting PTEN after birth (Fig. 1 E,F) significantly

increased contralateral sprouting after P1 deletion, as compared to WT and Nogo mutant mice (Fig. 1 A- H, quantified in I,J). More specifically, we found that the density of axons crossing midline after PTEN deletion alone (0.142 ± 0.014) was more than two times greater than wild type, and less than two times greater than Nogo mutant (Fig1. I). In addition, the total number of axons to cross the midline was significantly higher in the PTEN single deletion group, compared to Nogo and WT mice, from 50 μ m to 450 μ m away from midline (0.017 ± 0.002 at 50 μ m, Fig. 1J). However, we did not see any additive effects on sprouting when Nogo deletion was combined with PTEN deletion. This was the case for both the sprouting axon index (PTEN-ND: 0.152 ± 0.02), or the sprouting axon number index ($0.020x \pm 0.003$ at 50 μ m, Fig. 1 G-J). These results suggest that, at least for the effect on sprouting, it is more important to modulate the intrinsic pathway as opposed to the inhibitory extrinsic pathway.

Locomotor improvement in PTEN mice after pyramidotomy (P1)

Because of the important increase in sprouting in the PTEN and PTEN-ND groups, we sought to examine if these effects on sprouting were mimicked in forelimb functional recovery. We first performed the rearing test to assess the forepaw preference during rearing behavior for forelimb function in a normal exploratory activity. No differences were observed between groups for any of the quantifications performed (data not shown). We next performed the ladder rung test. Unilateral lesion increased the percentage of missteps of the injured side (Fig. 2E-F) and decreases the average score (Fig. 2G) percentage of missteps, demonstrating that this test is sensitive enough to assess functional recovery. However, no differences between groups were observed for both the percentage of missteps, and the average walking score.

Finally the tape removal test was performed to assess sensory and motor behavior. Unilateral lesion specifically increased the time mice spent to realize a sticker is present on their paw (sensory score, Fig. 2A-B) and the time for its successful removal (motor score, which measures the latency to remove the tape after sensation, Fig. 2C-D). We found that there were no significant differences for the right sensory score between the different groups. On the other

hand, there was a significant difference in the motor score, but only transient. Indeed, ND, PTEN and PTEN-ND mutants removed the tape significantly faster than WT mice at day 7-post injury (WT: 49.5sec $\pm$ 18.5, ND: 17.0sec $\pm$ 6.5, PTEN: 25.4sec $\pm$ 10.2, and PTEN-ND: 14.1sec $\pm$ 4.8). However, Nogo did not confer any additive synergistic effect on the motor score.

Improved regeneration after dorsal hemisection in single PTEN and PTEN Nogo mutant mice

While sprouting did not yield any synergistic effects of PTEN and Nogo combined deletion, we hypothesized that the effects of Nogo deletion would be more readily detected in a dorsal hemisection model, as it has been shown that myelin is one of the main growth inhibiting factors present after spinal cord injury. We wanted to test whether increasing the neurons intrinsic growth properties in a more favorable environment would lead to an increase of regenerating axons.

As described for the pyramidotomy study, we used mice that had PTEN deleted at P1, and performed a dorsal hemisection at the thoracic 8 (T8) level of the spinal cord. Mice were traced with a unilateral BDA injection to measure CST regeneration across the injury 4 weeks after injury. As with our previous studies, and others, we did not observe any regeneration in the WT or Nogo mutant mice (Fig. 3 A, A', B, B'). However, both PTEN and PTEN-ND mice presented numerous regenerating CST axons, as many BDA-labeled fibers were seen within and past the injury, delimited by the GFAP staining (Fig. 3 C-D'). Quantification of the number of regenerated axons (fiber number index) revealed a similar pattern for both the PTEN and the PTEN-ND groups in that both were significantly higher than Nogo or WT mice, up to 700 μ m caudal to the injury site (Fig. 3 B). Interestingly, the PTEN-ND mutants presented significantly more axons within the injury than the PTEN single mice (0.013 ± 0.006 for PTEN versus 0.023 ± 0.010 for PTEN-ND). Additionally, while not significant, there was a trend for the PTEN-ND double mutants to extend their axons for longer distances than the PTEN single (Fig. 4 B).

Examination of the segments rostral to the injury revealed clear visual differences.

Indeed, whereas characteristic die-back of the cut axons was noticed for the WT group, both PTEN and PTEN-ND group presented an extension of the main CST bundle very close to the injury border. Additionally, fewer retraction bulbs were noted in these 2 groups compared to the WT. we quantified this observation by measuring the BDA staining intensity rostral to the injury and obtain the fiber density index. As expected, PTEN and PTEN-ND presented a significantly higher fiber density index than the WT all the way to 0.5mm rostral to the injury (Fig. 4 A). Surprisingly, the Nogo group was in between WT and PTEN groups. However, we did not find any synergistic effects between PTEN single and PTEN-ND double mutants.

Because of the significant increase of axon regeneration in the PTEN and PTEN-ND groups, we wanted to test whether this regeneration was linked with an increase of functional recovery. Several behavioral tests were performed, including the open field test, to obtain the well-accepted BMS score, the rotarod test, and the ladder rung. Unfortunately, none of these tests demonstrated a functional recovery for any of the 4 groups tests (Fig. 5, A-D). Sensitivity and specificity of these tests could explain these negative results, but it is important to note that the axon regeneration observed in the PTEN and PTEN-ND groups, even if significant, remained moderate compare to original number of CST axons in non injured animals. This low absolute number is most likely responsible for the lack of detectable functional recovery improvement.

Adult PTEN deletion increases compensatory sprouting but additional deletion of Nogo does not enhanced sprouting

As neonatal deletion of PTEN may have resulted in a compensatory upregulation of redundant molecules, we also performed PTEN deletion in young adult mice (4 weeks old) and assessed anatomical and behavioral outcomes. As described for the P1 group, we first quantified the BDA labeling intensity in the injured and non-injured side to obtain the sprouting axon density index. As observed for the P1 study, we noticed a small, but not significant, increase in the Nogo deleted group when compared to the WT group (respectively 0.063 ± 0.006 and 0.043 ± 0.003 , Fig. 5E). More importantly, both PTEN and PTEN-ND group presented a significant increase in

the density when compared to both WT and ND single group (Fig. 5A-D). Indeed, the intensity of both PTEN deleted groups was 3 times higher than the WT group, and twice the ND group (PTEN: 0.124 ± 0.008 and PTEN-ND: 0.136 ± 0.037 , Fig. 5E). This is the first time to our knowledge that adult PTEN deletion is associated with increase in sprouting. However, co-deletion of PTEN and Nogo did not result in an increase of the sprouting density index (Fig. 5 E).

We next quantified the sprouting axon number in function of the distance from midline, and, similar to the results of neonatal study, we found that ND mice presented a slight increase in number compared to WT, albeit not significant (WT: 0.007 ± 0.002 and ND: 0.01 ± 0.002 at $50\mu\text{m}$, Fig. 6 F). Moreover, there was a significant increase in axon number index in PTEN and PTEN-ND mutants as compared to Nogo and WT mice (Fig. 6 A-D, quantified in F). Indeed, PTEN (0.016 ± 0.002 at $50\mu\text{m}$) and PTEN-ND (0.018 ± 0.003 at $50\mu\text{m}$) were significantly different from the WT and Nogo mutants, from the midline to $450\mu\text{m}$. However, as observed for the P1 study, we didn't see any additive effect in the double deleted mice. These data again suggest the importance of growth intrinsic modulation with regard to compensatory sprouting over extrinsic inhibitory factors.

Because of the massive increase of compensatory sprouting observed after PTEN deletion, we wanted to determine if there was any functional recovery in the adult mouse after injury. As described for the P1 study, we performed the rearing test, the ladder rung test and the tape removal test. We found that mice in all groups did not show behavioral further improvement for the rearing and the ladder test (data not shown). Tape removal assay did not show improvement of the sensory score for the PTEN or PTEN-ND group in comparison to the WT and ND groups (Fig 6. G), similar to what observed in the P1 groups. However, at 7days post injury, there is a trend for the PTEN and PTN ND group to recover faster then WT group, but it failed to reach statistical significance (Fig. 6H).

Adult PTEN deletion in combination with Nogo enhances the regenerative effect seen in

PTEN single mutants after dorsal hemisection.

We first looked at the axon density rostral to the T8 dorsal hemisection. Indeed, we notice a visible difference in term of staining intensity and density close to the injury site for the two groups deleted from PTEN, as observed for P1 deletion (Fig. 7A-A' and B-B'). We quantified this intensity by segments of 100µm moving rostral, all the way to 1.5mm, and we found that PTEN and PTEN-ND groups presented a significantly higher fiber density index than the WT and ND from 0.2mm all the way to 0.7mm rostral to the injury (Fig. 7 C).

We next investigated whether PTEN deletion in young adult could promote CST axonal regeneration. First, as expected, we never observed axon regeneration (axon growing through the glial scar) for the WT and ND groups (Fig. 7 A-A'). Moreover, PTEN deletion in young adult promoted significant regeneration in both PTEN and PTEN-ND groups ((Fig. 7 B-B'). Interestingly, and more importantly, quantification of the fiber number index suggested that more axons are present within the injury in PTEN-ND group compared to the PTEN group (0.036 ± 0.013 and 0.02 ± 0.007 , respectively). Additionally, this difference was even more significant at 50, 150 and 250 µm caudal to the injury. Finally, as observed for the neonatal deletion, we found a trend for the axons to travel on a longer distance caudal to the injury in the PTEN-ND mice than in the PTEN single, albeit not significant. These data clearly suggest that removing some extrinsic inhibitory molecules, here Nogo, can work synergistically with promoting adult neurons intrinsic growth capacities in order to promote axon regeneration.

Finally, we wanted to determine if this increase of regeneration was paralleled with an increase in functional recovery. As described for the P1 group, we performed the open field test (Fig 8A-B), the rotarod test (Fig. 8C) and the ladder rung test (Fig. 8D). Here again, none of these tests demonstrated a better functional recovery for the PTEN and PTEN-ND groups compared to WT and ND groups.

DISCUSSION

Results from a multitude of recent studies have shown that no single manipulation can

alleviate the regeneration block after spinal cord injury. Increasing evidence has shown that a multiple pronged approach of targeting extrinsic and intrinsic factors will be necessary to achieve long distance functional regrowth. Here we present data that show how two extrinsic and intrinsic pathways interact to increase the regenerative response after spinal cord injury. We investigate how deletion of two extrinsic and intrinsic regulators of regeneration, Nogo and PTEN, respectively, interact to synergistically increase regeneration. To be comprehensive, we analyzed their effect on both regeneration and sprouting after deletion at P1 and during adulthood. Our results demonstrate the necessity to understand the specific contribution of each molecule. Several studies from our lab and others have previously reported the limited effect of Nogo on regeneration. However, we now show a specific, significant contribution of Nogo, in combination with PTEN on promoting long distance regeneration. This study provides exciting evidence that previously understated molecules may have dramatic effects when combined with the appropriate partner.

It is believed that an age related downregulation of growth competence is partly responsible for the lack of adult CNS regeneration. Park et al further supported this by showing that mTOR pathway is downregulated in the adult mouse. Our results show that PTEN deletion in the young adult can restore the PS6 expression in cortical neurons, and therefore make these cells potentially more growth responsive to injury. We hypothesized that by reducing the inhibitory environment, through Nogo deletion, the increased neuronal growth competence from PTEN deletion would allow a greater regenerative response to be measured.

Sprouting

We show that in line with previous studies, single Nogo deletion increases slightly sprouting after pyramidotomy [158]. We also show that the PTEN single mutant increases sprouting greater than two-fold in comparison to wildtype mice. This is true for P1 PTEN deletion (as shown by Liu et al, 2010) but also for adult PTEN deletion. Indeed, adult deletion seems to make the cortical neurons more responsive to injury, by re-activating the mTOR pathway (as

demonstrated by PS6 upregulation). When we combined deletion of PTEN and Nogo, we did not observe increase of the sprouting above that of the single PTEN mutant. This is true for both the P1 and the adult injections. One explanation for the lack of increased sprouting after pyramidotomy could be the ceiling effect on sprouting reached in the PTEN deleted mice. Alternatively, the lack of additional effect could be due to the lack of a myelin associated “barrier”, as the pyramidotomy is not a true spinal cord injury. Here the sprouting occurs in a native, non-injured environment, albeit denervated of the original CST; therefore, any potential synergistic effect after injury is hidden. To address this, a different injury model, such as a cervical dorsal column lesion, would be better suited to examine sprouting in the injured CNS. Alternatively, examination of the C7 coronal sections after DH may be another solution to examining sprouting (at T3/4 it seems to be increase of sprouting in PTEN/PTEN-ND). A third possibility is that the different molecules, though acting on different pathways, eventually activate a single pathway responsible for increasing sprouting. This would suggest that multiple intrinsic and extrinsic molecules can regulate sprouting, but all converge onto one single pathway responsible for increased sprouting. This proposition is supported by studies that have shown multiple manipulations do not increase sprouting [156].

Regeneration

To extend the roles of PTEN and Nogo in regeneration after spinal cord injury, we performed dorsal hemisections on mice that had PTEN deleted at both the neonatal and adult stages. We found, like others that PTEN significantly increases regeneration for the neonatal deleted group. However, we did not find any significant synergistic effect of the added Nogo deletion. For all groups we did not see any significant functional recovery after gridwalk, ladder run, rotarod, or BMS. This is likely due to the need for a more sensitive assay to examine functional recovery after dorsal hemisection, and to the low absolute numbers of regenerating axons.

While only a small percentage of axons regenerated across the lesion, a significant

number of axons are observed rostral to the injury, with the majority stopping at the injury border. This clearly suggests that injured axons are actively growing rostral to the injury, but lack the ability to penetrate the injury. Indeed, Liu and al noted that many robustly growing axons failed to penetrate into the GFAP-negative area at the lesion site. We believe that further reducing the inhibitory nature of the injury epicenter, as with ChABC, would allow us to better examine the effects of Nogo deletion. This hypothesis is supported by two of our findings. First, we found that in PTND double mutants the axons that did cross the lesion extended significantly greater distances for the adult PTEN deletion group, with a trend for longer distances in the neonatal deletion group. We believe that the lack of significant extension in the neonatal double mutant group is likely due to an upregulation of redundant molecules after PTEN deletion. Second, in both P1 and adult deleted mice, the PTND group shows significantly more axons within the injury site than in the single PTEN mutants. This particularly shows that Nogo deletion reduces the inhibitory nature of myelin debris in and around the injury site allowing regenerative axons to extend farther than in PTEN deletion alone. However, due to other inhibitory factors, continued growth beyond the lesion is dampened. Therefore, the next obvious target would be to down regulate CSPG expression in the injury site so that the growth competent neurons (PTEN deleted) can extend past and continue their growth in a less inhibitory environment (Nogo deletion).

In summary we found that there are benefits to simultaneously manipulate intrinsic and extrinsic factors to enhancing regeneration of CST axons. It is important to note that while there was an enhanced regenerative response, it seems that removal of Nogo and PTEN is not sufficient to increase functional recovery. As mentioned above, this may be due to the low absolute numbers of axons crossing the injury. Nonetheless, we show that combined deletion of Nogo and PTEN enhance the distance of regenerating axons caudal to the lesion, which contributes to the goal of increasing long distance regeneration after spinal cord injury.

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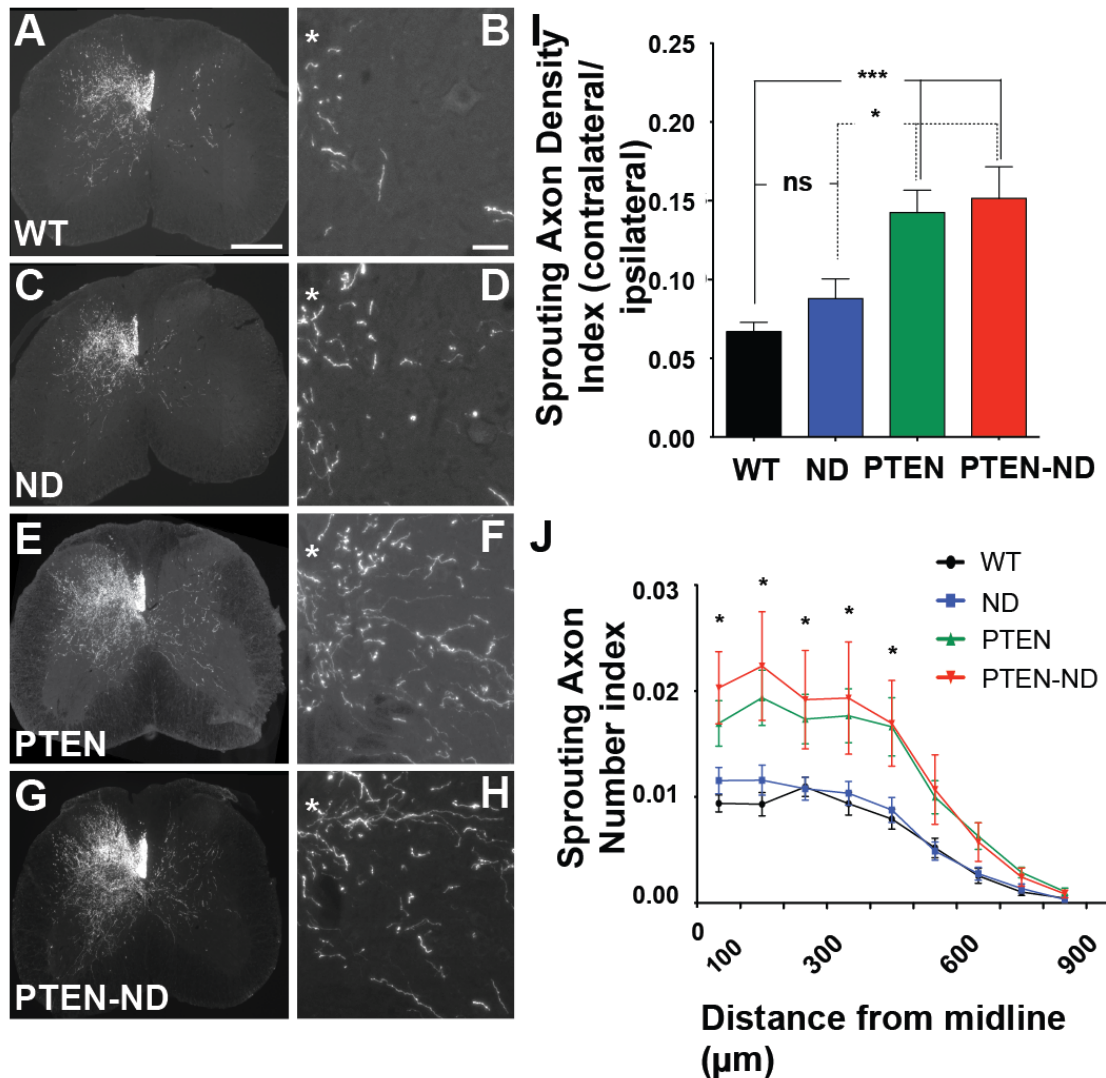


Figure 4.1 CST sprouting in mice with unilateral pyramidotomy after neonatal PTEN deletion. **A-H:** Representative images of cervical 7 (C7) spinal cord transverse sections from wild-type (A-B), Nogo Deletion (C-D), PTEN deletion (E-F) and PTEN-Nogo Deletion (G-H) mice with a left pyramidotomy. *PTEN^{loxp/loxp}-Nogo* heterozygote and *PTEN^{loxp/loxp}-Nogo* Null were injected at P1 in the right sensorimotor cortex with either AAV-GFP or AAV-Cre. Left pyramidotomy was performed 8weeks later; BDA was injected in the right sensorimotor cortex 2weeks after injury and mice perfused 2 weeks later. **I:** Quantification of Sprouting axon Density index, measuring the intensity of the BDA staining of the contralateral/ipsilateral gray matter. **J:** Sprouting Axon Number index, quantification of the number of axons crossing specific distance in the contralateral gray matter of the spinal cord, normalized against the number of axons labeled in the medulla. Adult PTEN deletion increases sprouting after pyramidotomy, but no further increase was observed in the PTEN-Nogo deleted mice. N are: WT=15, ND=13, PTEN=16, PTEN-ND=9. 5 sections per animal were quantified. *P<0.05, ***P<0.01 2-ways ANOVA followed by Bonferroni's *post hoc* test. Scale bars represent 300µm in A, C, E, G and 100µm in B, D, F, H. * indicates central canal in B, D, F, H.

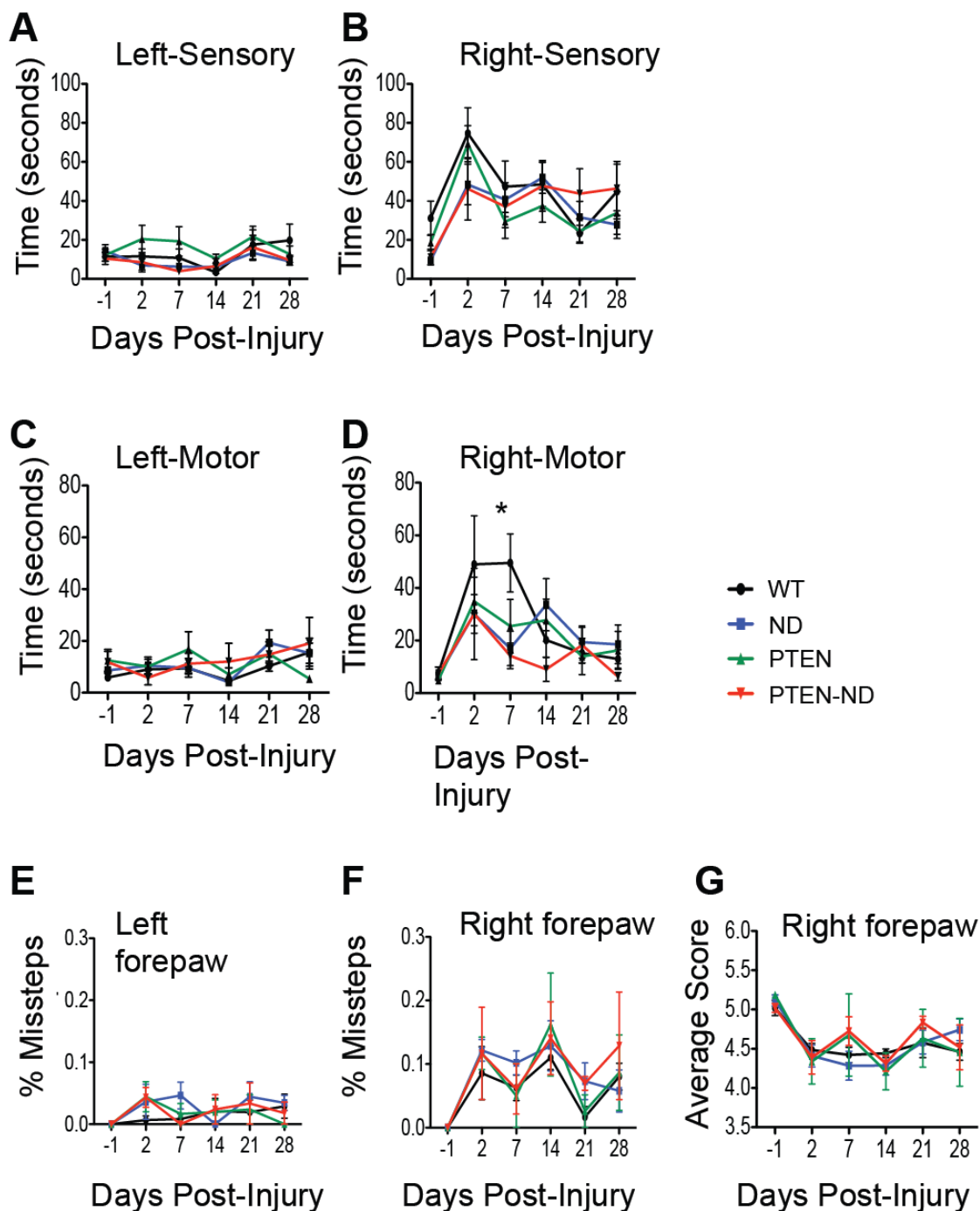


Figure 4.2 Assessment of functional recovery of forelimbs functions after unilateral pyramidotomy. A-D: Tape removal assay scoring for the time before noticing the sticker on the paws (sensory score, A-B), and time to remove the sticker after sensing it (motor score, C-D). A faster recovery was observed at 7days for ND, PTEN and PTEN-ND groups. E-G: Ladder Rung test showed an increase of the number of missteps for the injured side (right paw, F) compared to the uninjured side (left paw, E). No improvement in the % of missteps was observed during the time period of the study (F), nor did the average walking score (G). * $P < 0.05$, 2-ways ANOVA followed by Bonferroni's *post hoc* test.

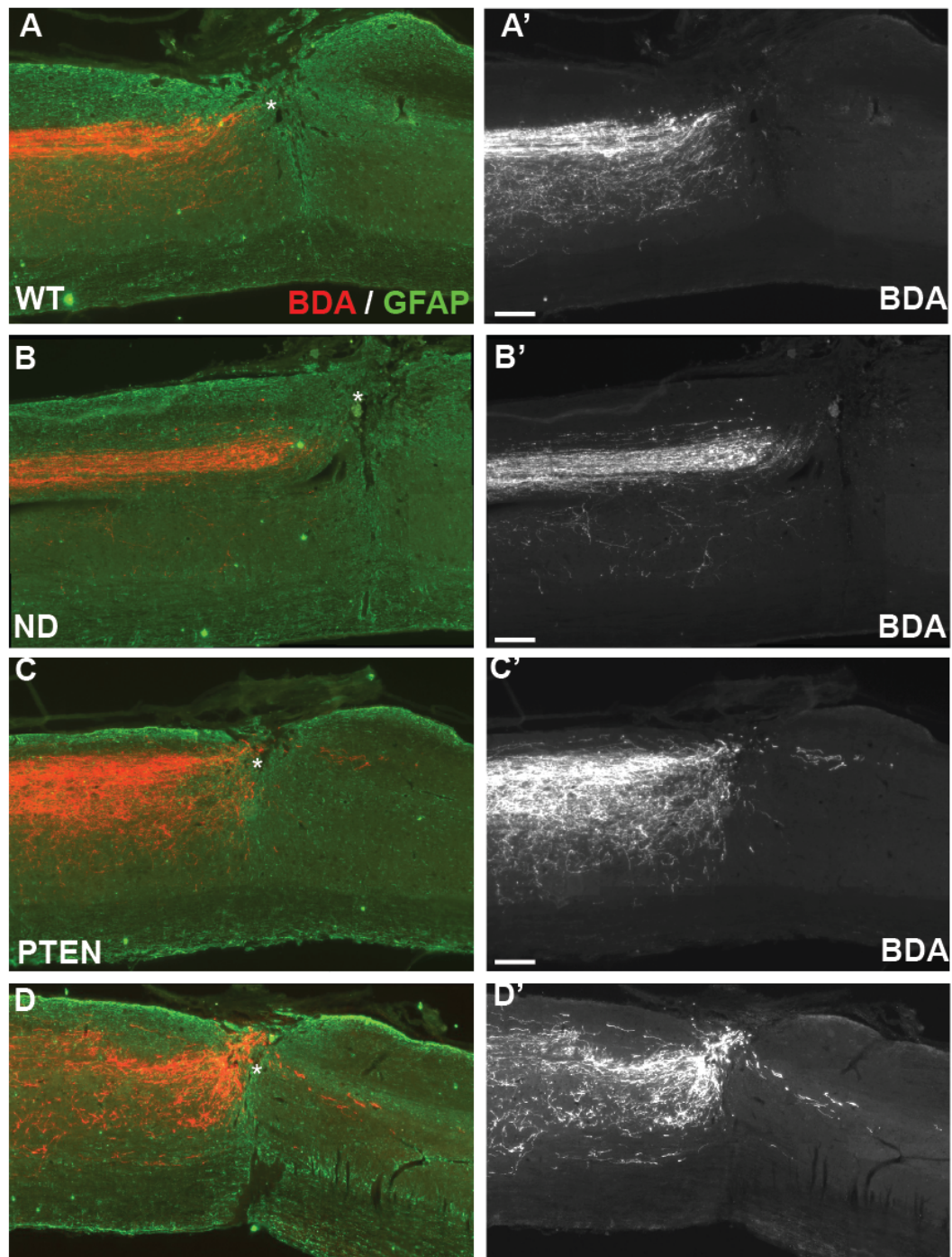


Figure 4.3 CST regeneration in neonatal PTEN deleted mice after adult T8 dorsal hemisection A-H: Representative images of spinal cord sagittal sections from wild-type (A-A'), Nogo Deletion (B-B'), PTEN deletion (C-C') and PTEN-Nogo Deletion (D-D') mice with a T8 dorsal hemisection. *PTEN*^{loxp/loxp}-Nogo heterozygote and *PTEN*^{loxp/loxp}-Nogo Null were injected at P1 in the right sensorimotor cortex with either AAV-GFP or AAV-Cre. T8 dorsal hemisection was performed 8 weeks later; BDA was injected in the right sensorimotor cortex 4 weeks after injury and mice perfused 2 weeks later. Sections were stained for BDA (red) and GFAP (green). Scale bars represent 200µm. * indicates injury in A, B, C, D.

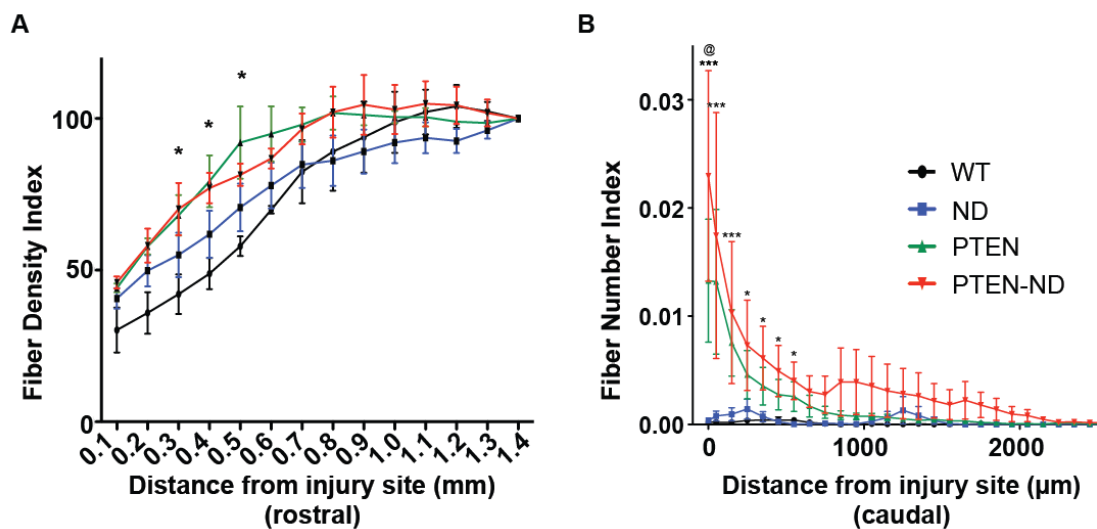


Figure 4.4 CST regeneration in neonatal PTEN deleted mice after adult T8 dorsal hemisection. **A:** Quantification of Fiber Density index, measuring the intensity of the BDA rostral to the injury site in function to the distance from the injury site. **B:** Fiber Number index, quantification of the number of axons crossing specific distance caudal to the injury site, normalized against the number of axons labeled in the medulla. Adult PTEN deletion increases CST regeneration after dorsal hemisection; PTEN-Nogo double deletion increases the distance of regeneration. N are: WT=14, ND=11, PTEN=11, PTEN-ND=9. 5 sections per animal were quantified. * $P < 0.05$, *** $P < 0.01$, between WT or ND to PTEN or PTEN-ND, @ $P < 0.05$ between PTEN-ND and PTEN. 2-ways ANOVA followed by Bonferroni's *post hoc* test.

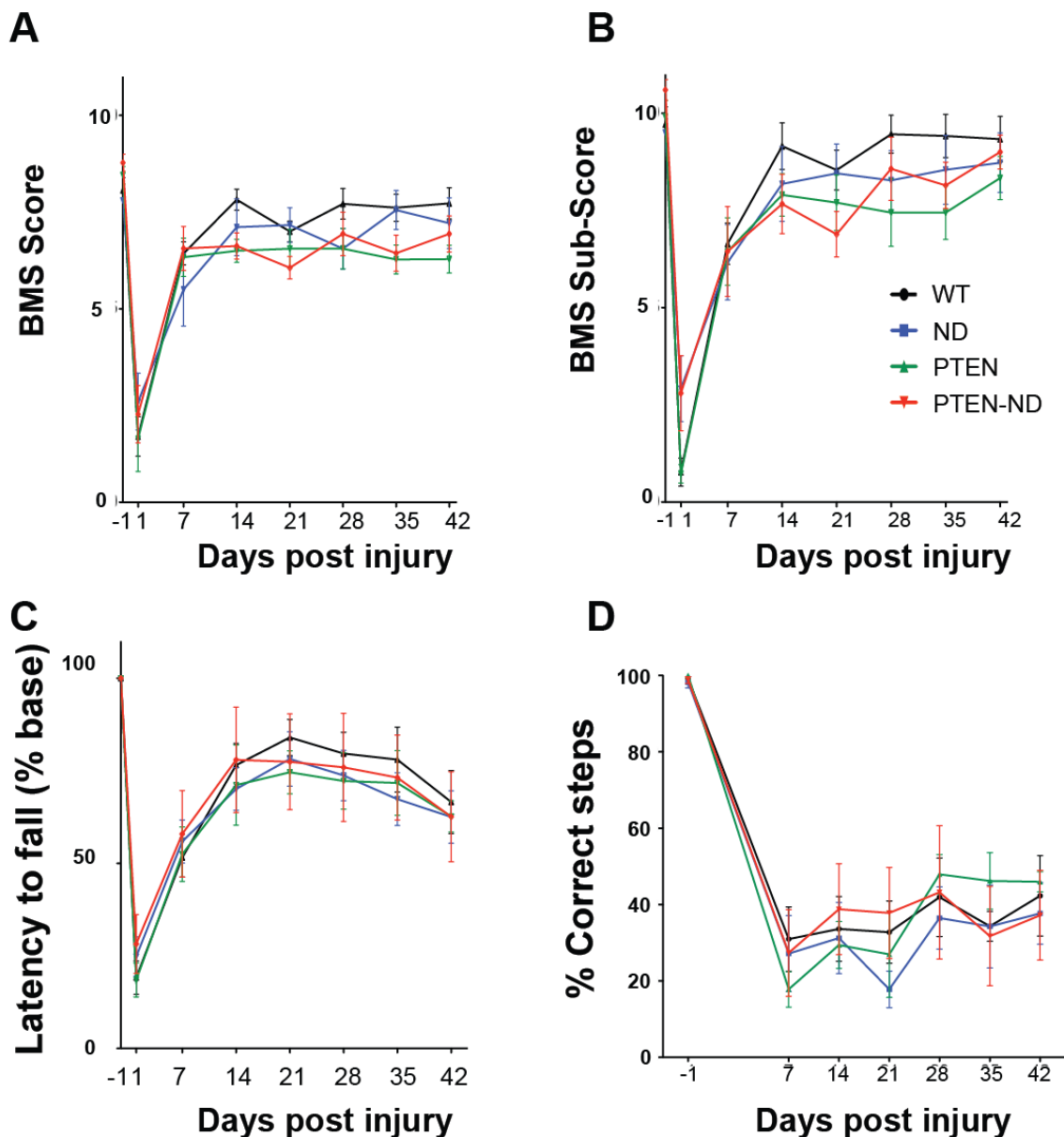
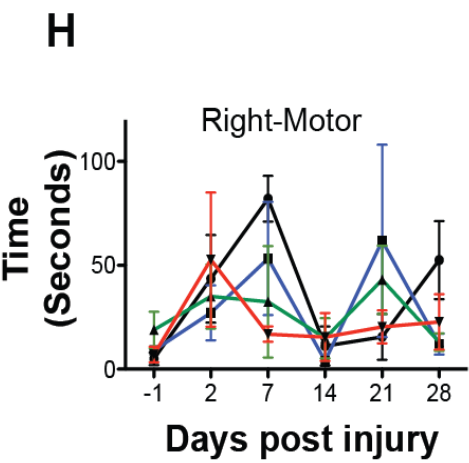
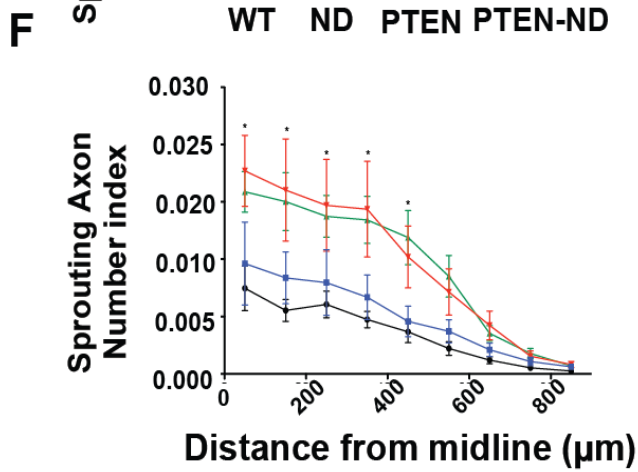
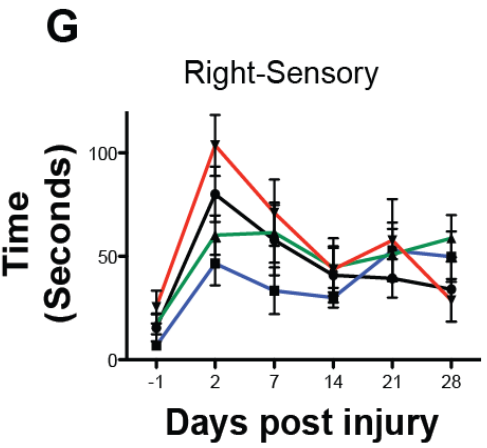
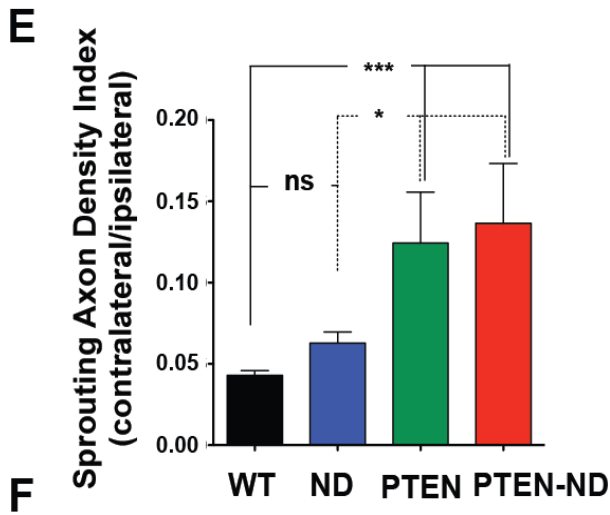
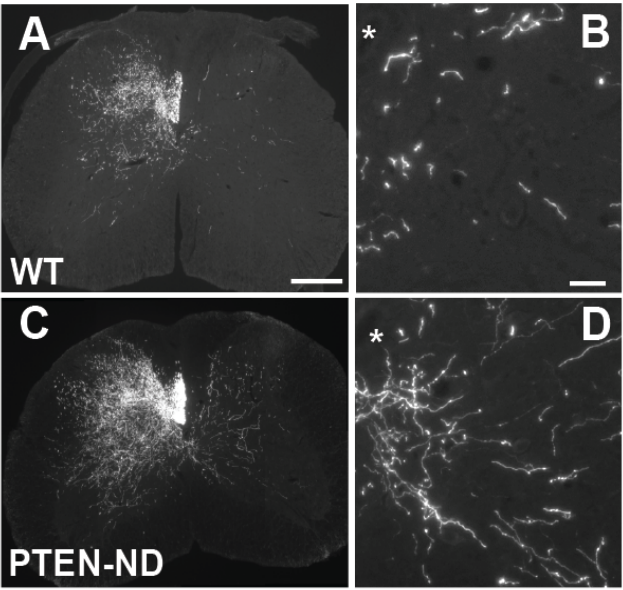


Figure 4.5 Behavioral assessment of functional recovery of hind limbs functions after T8 dorsal hemisection. A-B: open field test, giving the BMS score and the BMS sub score. **C:** rotarod (latency to fall). **D:** ladder rung. No functional improvement were observed in between the 4 groups for any of test performed up to day 42 post injury. Neonatal injection (WT=14, ND=11, PTEN=11, PTEN-ND=9)

Figure 4.6 CST sprouting in mice with unilateral pyramidotomy after adult PTEN deletion. **A-D:** Representative images of cervical 7 (C7) spinal cord transverse sections from wild-type (A-B), and PTEN-Nogo deletion (C-D) mice with a left pyramidotomy. *PTEN^{loxp/loxp}-Nogo heterozygote* and *PTEN^{loxp/loxp}-Nogo Null* were injected in the right sensorimotor cortex of young adult (4-6weeks old) with either AAV-GFP or AAV-Cre. Left pyramidotomy was performed 4 weeks later; BDA was injected in the right sensorimotor cortex 2weeks after injury and mice perfused 2 weeks later. **E:** Quantification of Sprouting axon Density index, measuring the intensity of the BDA staining of the contralateral/ipsilateral gray matter. **F:** Sprouting Axon Number index, quantification of the number of axons crossing specific distance in the contralateral gray matter of the spinal cord, normalized against the number of axons labeled in the medulla. Adult PTEN deletion increases sprouting after pyramidotomy, but no further increase was observed in the PTEN-Nogo deleted mice. **G-H:** Tape Removal assay scoring for the time before noticing the sticker on the paw (sensory score, G), and time to remove the sticker after sensing it (motor score, H). N are: WT=8, ND=10, PTEN=8. PTEN-ND=8. For E and F, 5 sections per animal were quantified. *P<0.05, 2-ways ANOVA followed by Bonferroni's *post hoc* test. Scale bars represent 300µm in A, D and 100µm in B, E. * indicates central canal in B and D.



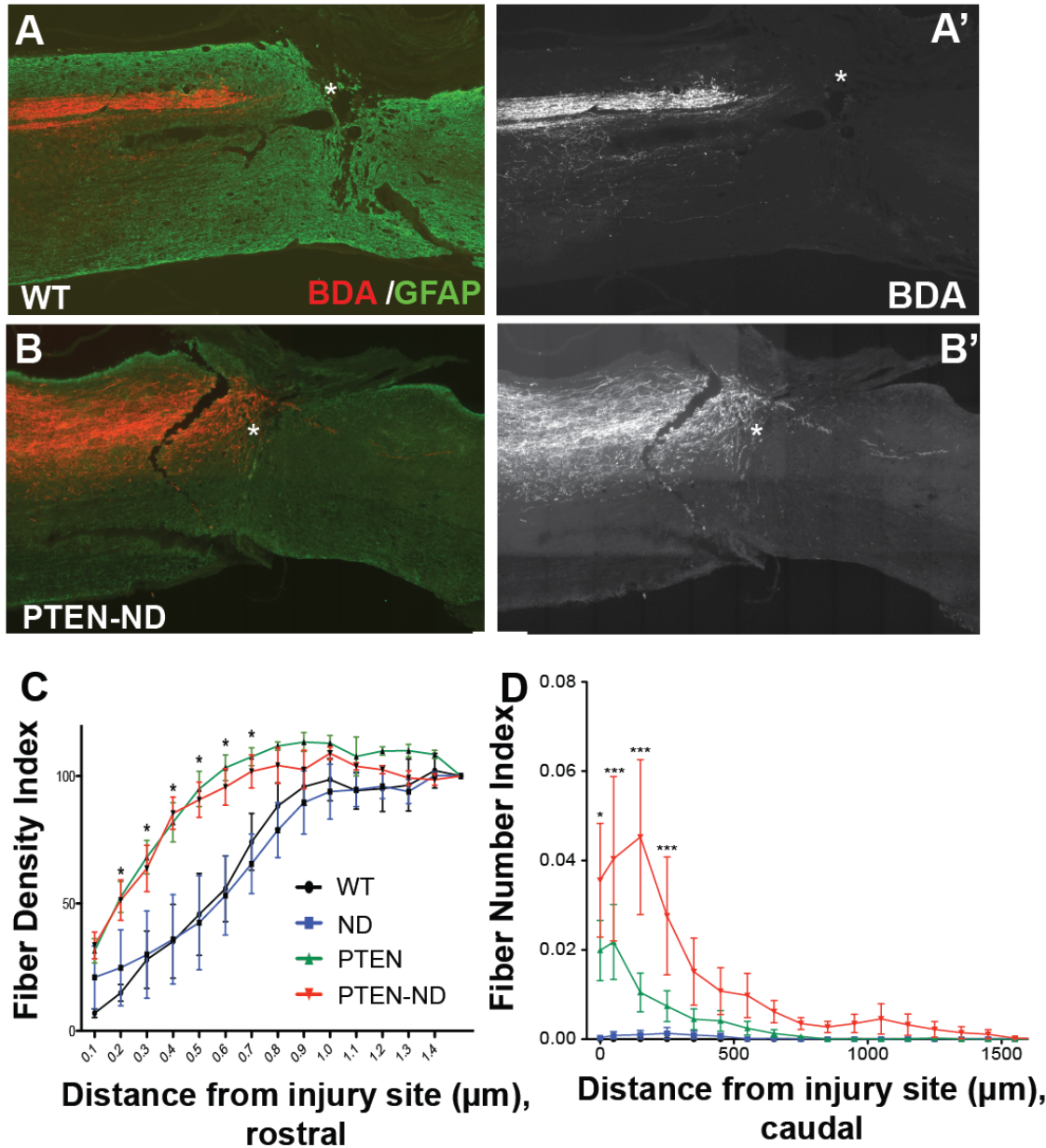


Figure 4.7 CST regeneration in adult PTEN deleted mice after adult T8 dorsal hemisection. **A-B'**: Representative images of spinal cord sagittal sections from wild-type (A-A') and PTEN-Nogo deleted (B-B') mice with a T8 dorsal hemisection. Young adult *PTEN^{loxp/loxp}-Nogo* heterozygote and *PTEN^{loxp/loxp}-Nogo Null* were injected in the right sensorimotor cortex with either AAV-GFP or AAV-Cre. T8 dorsal hemisection was performed 4 weeks later; BDA was injected in the right sensorimotor cortex 4 weeks after injury and mice perfused 2 weeks later. Sections were stained for BDA (red) and GFAP (green). **C**: Quantification of Fiber Density index, measuring the intensity of the BDA rostral to the injury site in function to the distance from the injury site. **D**: Fiber Number index, quantification of the number of axons crossing specific distance caudal to the injury site, normalized against the number of axons labeled in the medulla. Adult PTEN deletion increases CST regeneration after dorsal hemisection; PTEN-Nogo double deletion increases the number of regenerating axon and the distance of regeneration. 5 sections per animal were quantified. **P*<0.05, ****P*<0.01, between PTEN and PTEN-ND. 2-ways ANOVA followed by Bonferroni's *post hoc* test. Scale bars = 200μm. * indicates injury in A, B, C, D.

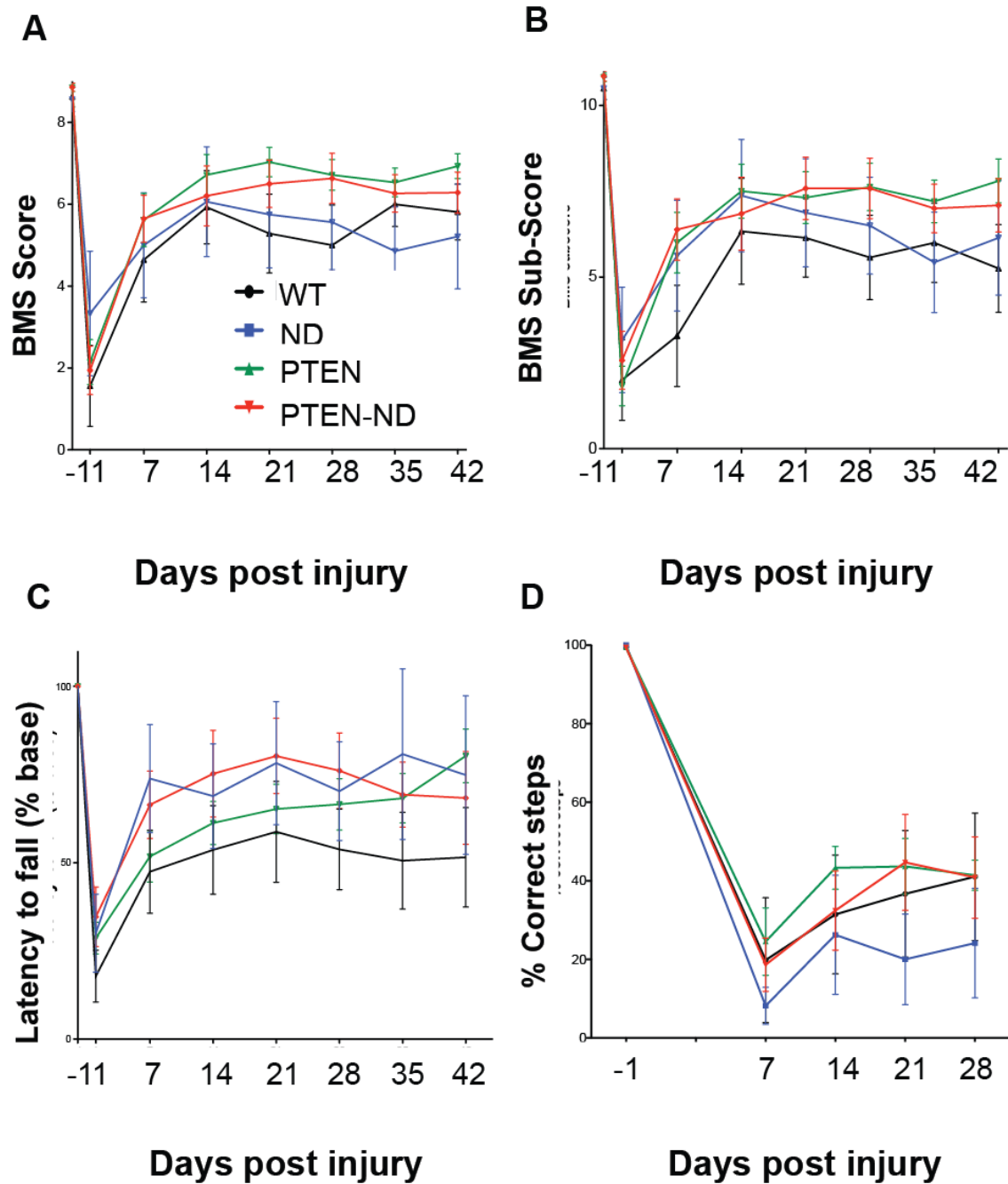


Figure 4.8 Behavioral assessment of functional recovery of hind limbs functions after T8 dorsal hemisection. A-B: open field. C: rotarod. D: ladder rung. No functional improvement were observed in between the 4 groups for any of test performed up to day 42 post injury. Adult injections (WT=14, ND=11, PTEN=11, PTEN-ND=9).

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CHAPTER 5.

General Discussion

Understanding the baseline dynamics of the axonal response to injury is important to the understanding of how different molecular interactions contribute to the degenerative and regenerative response. Many studies have shown that different molecular, pharmacologic, and/or genetic manipulation result in changes to regeneration and sprouting; however, how these specific interventions act on the injured axons are often unclear. The main body of work in this dissertation demonstrates the importance of complementing experimental spinal cord injury models and manipulations with in vivo imaging to examine the specific effects a particular manipulation can have on axon degeneration and regeneration.

Previously published imaging studies have focused mostly on the characterization of degeneration and regeneration of spinal sensory axons after mechanical lesions such as a needle or pin prick [125, 129, 131]. Many axons are severed simultaneously in such models, along with significant albeit localized secondary injury response. In our study, a single fluorescently labeled axon, among many, is visibly severed while leaving all nearby fluorescently labeled axons uncut. Thus, we characterized baseline axon degeneration and regeneration profiles in detail in such a laser axotomy model. This detailed knowledge would allow us to understand the variables that influence axonal responses to injury, which would be necessary for using the model to interrogate the effect of molecular intervention on axon regeneration in future. In addition, this would allow us to assess whether a smaller laser induced lesion would yield results qualitatively similar to a pinprick lesion.

I demonstrated that there are distinct aspects of degeneration and regeneration, and these aspects can be used to distinguish how a particular molecule, in this case Nogo, may affect the injury response. In, addition, as in vivo imaging is meant to complement experimental spinal cord injury, I demonstrated, in collaboration with other members in lab, that the results from my

imaging studies can be seen in larger injury models. This Chapter will discuss the main findings of my dissertation work, and how they relate to spinal cord injury and future studies in the field.

Fragmentation and Retraction

It is known that fragmentation and retraction are characteristic features of acute axonal degeneration. I showed that each mode had a specific speed of degeneration, where fragmentation was a more rapid form of acute degeneration and retraction progressed significantly more slowly. In addition I showed that there was a significant difference in the preference for degeneration via fragmentation or retraction between the distal and proximal axonal segments, where the distal segments fragmented significantly more than the proximal axonal segments, and the proximal axonal segments degenerated more via retraction. This is consistent with in vitro studies that show surviving proximal axonal segments preferred a retraction mode of degeneration, and that this was associated with caspase-3 and calpain activity [187]. In addition, I show that regardless of the injury location, and despite a wide variability in the degeneration profiles, all axons slow or stop their acute degeneration by ~ 3 hours after injury. Interestingly, most axons do not degenerate beyond the bifurcation point for the ascending and descending branches, and these results suggest that the bifurcation point is a barrier to acute and sub acute degeneration. Several interesting points come from these three injury locations. First, while the ascending and descending branches are essentially stopped at, or stop close to, the bifurcation point on Day 0, it is interesting that for the segments that have a stub remaining at Day 0, these degenerate by Day 5 after injury to the bifurcation point, suggesting that there may be a specific mechanism behind delayed proximal degeneration, similar to the Wallerian degeneration seen in the distal axonal segment.

Future studies to understand the mechanism behind this delayed proximal degeneration may reveal important molecules that can potentially delay degeneration after spinal cord injury, and especially progressive neurodegenerative diseases, and thus preserve or slow the loss of function. Second, while the main branch is not limited by the bifurcation point, the degeneration

from Day 0-Day 5 stays relatively stable, suggesting that, like the proximal ascending and descending branches, I have captured the beginning, middle, and end of the acute degenerative phase, and any further degeneration is a result of a delayed proximal degeneration mechanism. It will be interesting to injure a moderate distance from the bifurcation point (~200-400 μm) and see if this delayed proximal degeneration program does exist, and what the potential molecule regulating this added degeneration are, and whether or not they are concentrated at the bifurcation point (for the ascending and descending injury locations, or a node of Ranvier (for the main branch ablations). Or this may simply be due to a delayed injury response that can potentially be measured at the cell body 1-2 days after injury, in a transgenic mouse with even fewer labeled axons.

The bifurcation point is an acute and sub acute barrier to degeneration

Additionally, we are the first to demonstrate that the bifurcation point is an acute and sub acute barrier to degeneration. The bifurcation point is a unique location on the axon where there is a unique upregulation of different cytoskeletal proteins, in addition to mitochondria. The bifurcation point is also a node of Ranvier[5, 188], which further adds to the complexity of this anatomical location, in that it is also directly in contact with astrocytes and oligodendrocytes, which, after injury, may provide some growth support or mitigate the injury signal.

Location specific regeneration

When the bifurcation point is breached (in rare cases for the ascending and descending branch), this is the only time that there is a regenerative response for this injury location. This suggests three possibilities: 1) that the injury signal is stopped at the bifurcation point due to the unique composition of this location. Or 2) that the healthy axon actively inhibits the injury response, and instead, sends out a signal to maintain stability as opposed to degeneration. And 3) there is the more passive possibility that simple loss of signaling initiates the growth response. In addition, Nogo deletion did not modify this regenerative response, suggesting that it is more

important that, first, the intrinsic pathway is modified, and once the intrinsic pathway increases the growth response, the less inhibitory environment will allow the growth competent neuron to progress through. From this observation, it will be important to see if an action potential blocker, i.e. tetrodotoxin applied to each branch can elicit a growth response from the bifurcation point. Another potential experiment would be to injure one branch, allow 5 days for degeneration, and then injury the second branch. This would test both the possibility of the bifurcation point being a stabilizing factor, and also if losing both branches initiates a regenerative response.

Is pruning due to developmental or injury induced degeneration program?

My data also show that there is a specific pruning stage, at 5 weeks that is consistent in wildtype and Nogo deletion mice. This is the first report of a sub-chronic pruning response for regenerating axons, and understanding the switch from growth to pruning may be important to directing regenerative growth in the future. Previous studies have shown that there are distinct mechanisms that govern injury induced degeneration and developmental pruning, which are phenotypically similar [189]. To tease apart whether the pruning is more closely related to the injury or developmental pruning mechanism it may be interesting to look at Wlds mice in the same imaging paradigm. If the axons prune via a injury induced program, using the Wlds mice would abolish or slow this response at the 5 week time point. Thus these studies remain to be explored.

Chronic regeneration emphasizes the more dominant role of extrinsic inhibition over intrinsic inhibition of regeneration

Moreover, my data show that in the absence of any molecular, cellular, genetic, or pharmacologic intervention there is a continued growth response in the injured axon. This is also true in the Nogo mutant mice, although the total regeneration observed is not greater than the wildtype mouse. While this is true, it suggests that the extrinsic inhibition is stronger than the

intrinsic inhibitory factors, as I demonstrate that the injured axons do regenerate without intervention continuously and progressively over the course of 5-6 months. Thus,

In addition, I observed that there are stable regions of the regenerating axon that remain relatively unchanged from day 5-5 months. It will be interesting to see what type of microtubules or other proteins are present in these stable regions versus those that are pruned.

Of course, the results from these in vivo imaging studies cannot be translated directly to experimental spinal cord injury models; they provide starting points to investigate the basic mechanisms, and also bring forward new questions and potential molecular candidates to explore with a larger injury model. It also does allow an investigation of how specific manipulations contribute, as we see that with Nogo mutant mice, there is a delayed anterograde degenerative response, which could not have been captured with eSCI. In addition we see that while Nogo does not modify the frequency of regenerative response, we show that in the presence of growth competent axons, Nogo promotes the elongation of these axons. One caveat is that due to the minimal lesion there is no inhibitory myelin debris, or glial scar, thus there is minimal inhibition for my regenerating axons. However, this suggests that there may be a role for neuronal nogo in modifying the intrinsic growth response after injury. Neuronal nogo has been associated with neurite fasciculation, branching, and extension, at least in the developmental CNS[140]. This study demonstrated that deletion of neuronal Nogo led to longer neurites and less branching, which is what we see in our study. Also, Neuronal Nogo has also been shown to modify synaptic transmission[142], which may be one reason that the anterograde degenerative response is delayed.

Nodes of Ranvier

In addition to the thriving axonal dynamism, an indication of future functionality might be indicated by the primitive formations of axon indentations almost recapitulating nodes of Ranvier in the uninjured axons. Studies have shown that after axonal injury, remyelination results in regularly spaced nodes, though the intermodal distance, as compared to the uninjured axon, is

significantly shorter. Our 5-month WT image shows regularly spaced potential nodes of Ranvier every 40-50 μ m. This provides new insights on axonal behavior after axotomy in the absence of any molecular or cellular intervention, and implies that the time window when administration of potential therapies can still harness this intrinsic growth phase is much more extended than previously thought.

Unlike previous studies, we were able to track a single axon over many months and have discovered a novel process in the regenerative response of an injured axon. Previous studies have reported abortive sprouting, or even a more long-term regenerative response; however, no study to date has been able to track the dynamics of a single axon over various months. We have shown that not only is there a robust initial phase of growth and branching, but there is also a second phase of pruning that occurs weeks after injury. Moreover, there is continues growth after the pruning phase that we have shown to be close to one millimeter of growth.

The effect of Nogo on elongating neurons that are primed for growth is validated in our experimental spinal cord injury model, where we only see an additive effect of Nogo on elongation when combined with deleting PTEN, and this creating a regeneration competent neuron. As in my imaging study, Nogo did not increase the frequency or number of regenerating axons; however, Nogo did significantly increase the distance of axons that did regenerate in PTEN Nogo double knockouts. Thus it is clear from our results that it is first important to increase the intrinsic growth capacity, and then extrinsic modulators may potentiate their growth.

While I focused on the dorsal column sensory axon tract, and assessed the regeneration in this specific axonal population, it does not mean that the regeneration or degeneration phenotype observed is characteristic of all neuronal tracts. As the Nogo receptor is differentially expressed in various neuronal populations [190, 191], this may affect regeneration in various axonal populations in a different manner. That being said, it will be important to measure other axonal populations in the future.

Conclusions

While the results from my in vivo imaging studies shed new light on interesting axonal phenomena after injury, extrapolating this information to larger spinal cord injury models may lose some of the effect. This may be due to the specific localization of the injury, where a larger injury may activate significantly more pathways, and as the injury becomes larger and more complicated, the results from my studies may be diluted.

However, it does show that there is not only an increased growth response in sensory neurons after injury, it shows that there are multiple aspects of the degenerative and regenerative response both at the anatomical and temporal level that remain to be explored and characterized. This could not have been done without the in vivo imaging technique, and demonstrates the utility and importance of complementary approaches to spinal cord injury.

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