UNIVERSITY OF CALIFORNIA SAN DIEGO

Neuron-intrinsic and extrinsic control of axon sprouting after central nervous system injury

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

in

Neurosciences

by

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The Dissertation of Jessica Moore Meves is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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LIST OF ABBREVIATIONS

AAV	Adeno-associated virus
BDA	Biotinylated dextran amine
CNS	Central nervous system
Cre	Cre recombinase
CST	Corticospinal tract
CTB-555	Cholera toxin subunit B, Alexa Flour 555 conjugate
DLK	Dual leucine zipper-bearing kinase
iOE	Inducible overexpressing
LZK	leucine zipper-bearing kinase
n.s.	Not significant
NgR1	Nogo Receptor 1
OE	Overexpressing
PKCγ	Protein kinase C gamma
PTEN	Phosphatase and tensin homolog
tdT	tdTomato
WT	Wild-type

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PUBLICATIONS

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ABSTRACT OF THE DISSERTATION

Neuron-intrinsic and extrinsic control of axon sprouting
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Damage to the central nervous system (CNS) such as from brain or spinal cord injury leads to permanent functional impairment, resulting in substantial social and economic burden. Sustained functional deficits are due in large part to the limitation of axon growth after injury. Damaged axons do not regenerate after CNS injury, whereas uninjured axons show some degree of spontaneous sprouting after CNS injury. Sprouting is believed to contribute to partial (albeit often insufficient) recovery in individuals with less severe injury or stroke, and can be promoted by manipulating axon

growth regulators in model organisms, suggesting a potential approach to enhance recovery in humans. Nonetheless, our understanding of the molecular regulation of sprouting is incomplete. My dissertation work investigates the degree to which factors expressed by neurons and other CNS cell-types regulate axon sprouting and whether promotion of sprouting improves functional recovery from injury. I investigated the cell-type specific roles of a classically described myelin-associated inhibitor of axon growth, Nogo, and a novel neuron-intrinsic regulator of axon growth, DLK, in sprouting of the corticospinal tract (CST) after experimental unilateral pyramidotomy in mice. In order to evaluate whether promotion of CST sprouting correlates with improved functional recovery from injury, I evaluated and optimized behavioral tests to monitor functional recovery from pyramidotomy. I found that oligodendrocytic-specific deletion of Nogo enhances CST sprouting, showing for the first time that Nogo specifically expressed by oligodendrocytes restricts sprouting and spinal axon growth after CNS injury, supporting a longstanding but heretofore untested hypothesis. I was not able to detect a functional benefit associated with this enhanced sprouting, highlighting the challenge in harnessing structural plasticity to promote functional recovery. Additionally, I provide the first evidence for a role of DLK in sprouting and spinal axon growth after CNS injury that, to our surprise, supports a growth-reducing role for DLK in CST sprouting. These results illustrate that sprouting is an accessible form of axonal growth following CNS injury that can be modulated by manipulating neuron-intrinsic or extrinsic factors, but that building on enhanced sprouting to promote functional recovery remains a challenge.

Chapter 1. Introduction

1.1 Overview

Damage to the central nervous system (CNS) such as from brain or spinal cord injury leads to permanent functional impairment, resulting in substantial social and economic burden. Currently there are no therapeutics to promote recovery of CNS function after injury. Permanent functional deficits are due in large part to the limitation of axon growth after injury. Much research has focused on why axons of the adult mammalian CNS fail to regenerate after injury, with the hopes of elucidating a therapeutic approach that encourages the growth of damaged axons and results in functional recovery from CNS injury. Unfortunately, few if any experimental interventions have been found to promote functionally meaningful regeneration of damaged axons in vivo. Nonetheless, a second form of axon growth, the growth of uninjured axons here termed “sprouting,” occurs spontaneously after CNS injury and is believed to contribute to partial (albeit often insufficient) recovery in individuals with less severe traumatic injury or stroke. Experiments in models of CNS injury in rodents and primates have revealed that this compensatory axon sprouting can be promoted by manipulating various axon growth regulators, suggesting a potential alternative approach to promote recovery from CNS injury in humans. Nonetheless, our understanding of the molecular regulators of this important form of injury-induced axonal growth is incomplete. My dissertation work aims to investigate the degree to which manipulation of axon growth regulators modulates axon sprouting and whether promotion of this form of axon growth has the potential to improve functional recovery from injury.

1.2 Axon growth after injury

Recovery from injury to the CNS is limited in the mammalian adult. This limitation

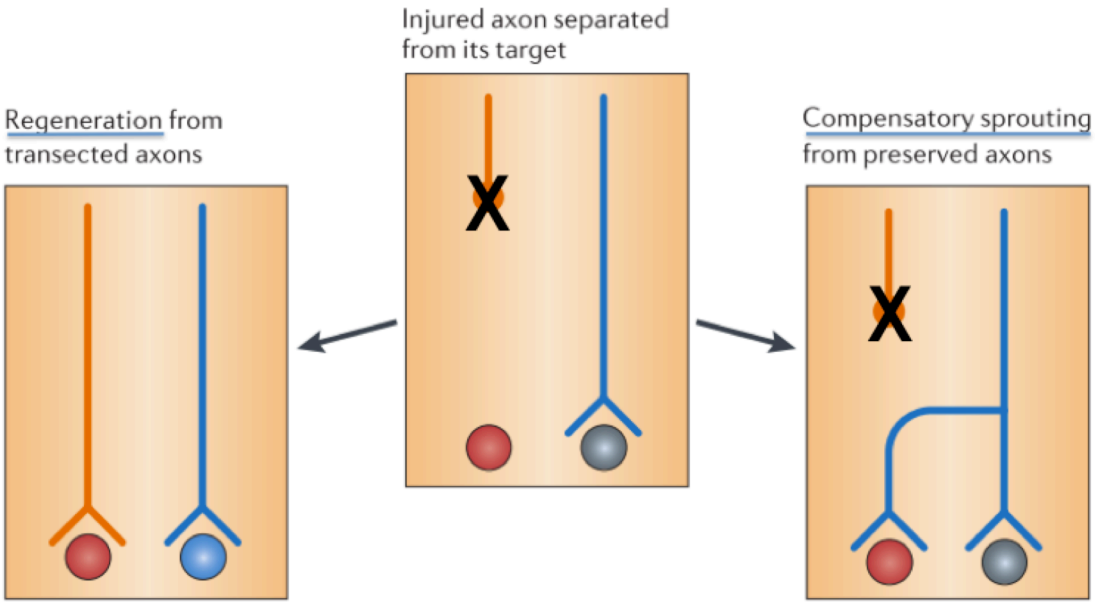

Figure 1.1 Regeneration versus compensatory sprouting: distinct forms of axon growth after injury. Neuronal connections are disrupted by CNS injury including brain or spinal cord injury (black X). Reinnervation of target neurons can occur via regeneration of damaged axons (left panel) or via compensatory axon sprouting of intact axons (right panel). (Yiu and He, 2006)

is due in large part to the failure of CNS axons to grow after injury. There are two principal types of injury-induced axonal growth: 1) regeneration, the growth from injured neurons, and 2) sprouting, the growth from uninjured neurons (Figure 1.1). Many factors are thought to play a role in restricting axon growth after injury, including the poor intrinsic axon growth ability of CNS neurons, the presence of growth inhibitory molecules in the CNS environment, and a lack of growth-promoting factors in the CNS environment (see "*1.3 Neuron-intrinsic vs. extrinsic regulators of axon sprouting*). Unfortunately, attempts to enhance functional recovery from injury by the promotion of regeneration have been mostly futile. Instead, harnessing the structural plasticity of remaining tissue by promoting axon sprouting may be a more accessible approach to improve recovery from spinal cord injury.

Axon sprouting is thought to contribute to spontaneous partial recovery after incomplete spinal cord injuries and stroke (see *"1.4 Functional recovery from CNS injury*") (Fouad et al., 2001; Weidner et al., 2001; Bradbury and McMahon, 2006; Rosenzweig et al., 2010; Ueno et al., 2012). It is believed that this innate recovery process can be promoted by targeting inhibitors of axon growth expressed by neurons and by non-neuronal cell types in the CNS, resulting in improved functional recovery from injury. Importantly, the majority of spinal cord injuries are anatomically incomplete, which is underscored by the fact that even neurologically complete injuries may be anatomically incomplete (Angeli et al., 2014). This leaves the possibility for plasticity in spared systems to mediate functional recovery (Fouad et al., 2001; Weidner et al., 2001; Bradbury and McMahon, 2006; Rosenzweig et al., 2010; Ueno et al., 2012). Furthermore, some interventions found to improve behavioral recovery from injury show

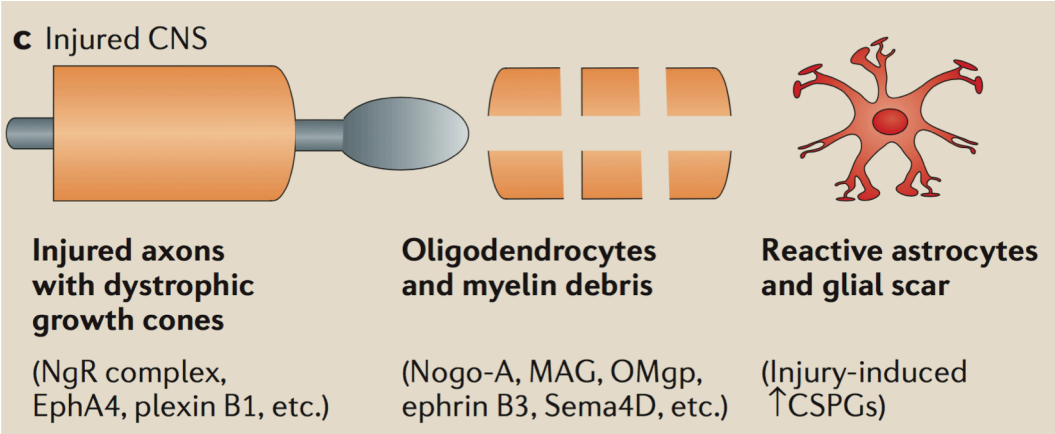

Figure 1.2 Neuron-extrinisic regulators of axon growth. Factors in the CNS environment including myelin-associated inhibitors of axon growth (Nogo-A, MAG, OMgp) and their receptor NgR; guidance molecules ephrin B3 and Sema4D and their respective receptors EphA4 and plexin B1; astrocyte-associated extracellular matrix molecules CSPGs; and neurotrophins (not shown) influence axon growth after injury. (Yiu and He, 2006)

only modest, if any, regeneration of damaged axons, but a more robust effect on the growth of undamaged axons, highlighting the potential importance of this form of axon growth in mediating functional recovery from spinal cord injury (Lee et al., 2009; Schwab and Strittmatter, 2014).

1.3 Neuron-intrinsic vs. extrinsic regulators of axon sprouting

In the efforts to promote axon growth after injury, much research has focused on targeting neuron-extrinsic regulators of axon growth present in the CNS environment (Figure 1.2) (Silver and Miller, 2004; Yiu and He, 2006). Various molecules expressed in the CNS are inhibitory to axon growth, many of which originate from mature oligodendrocytes. The prototypical myelin inhibitors Nogo, myelin-associated glycoprotein (MAG), and oligodendrocyte myelin glycoprotein (OMgp) are expressed on the surface of myelin and have been extensively characterized as inhibitors of neurite outgrowth in vitro. In vivo, genetic deletion of each of these myelin inhibitors was found to promote axon sprouting of specific axon tracts but not regeneration nor functional recovery after CNS injury (Lee et al., 2010). Interestingly, although all three of the prototypical inhibitors Nogo, MAG, and OMgp signal through common receptors, genetic deletion of Nogo but not MAG or OMgp was found to increase sprouting of the corticospinal tract (CST) after unilateral pyramidotomy (see *"1.5 Unilateral pyramidotomy model to study corticospinal axon sprouting*") (Lee et al., 2010). Surprisingly, MAG deletion was found to *decrease* CST axon sprouting after injury in this model (Lee et al., 2010), suggesting that MAG and perhaps myelin inhibitors in general have a dual role in modulating axonal sprouting after injury.

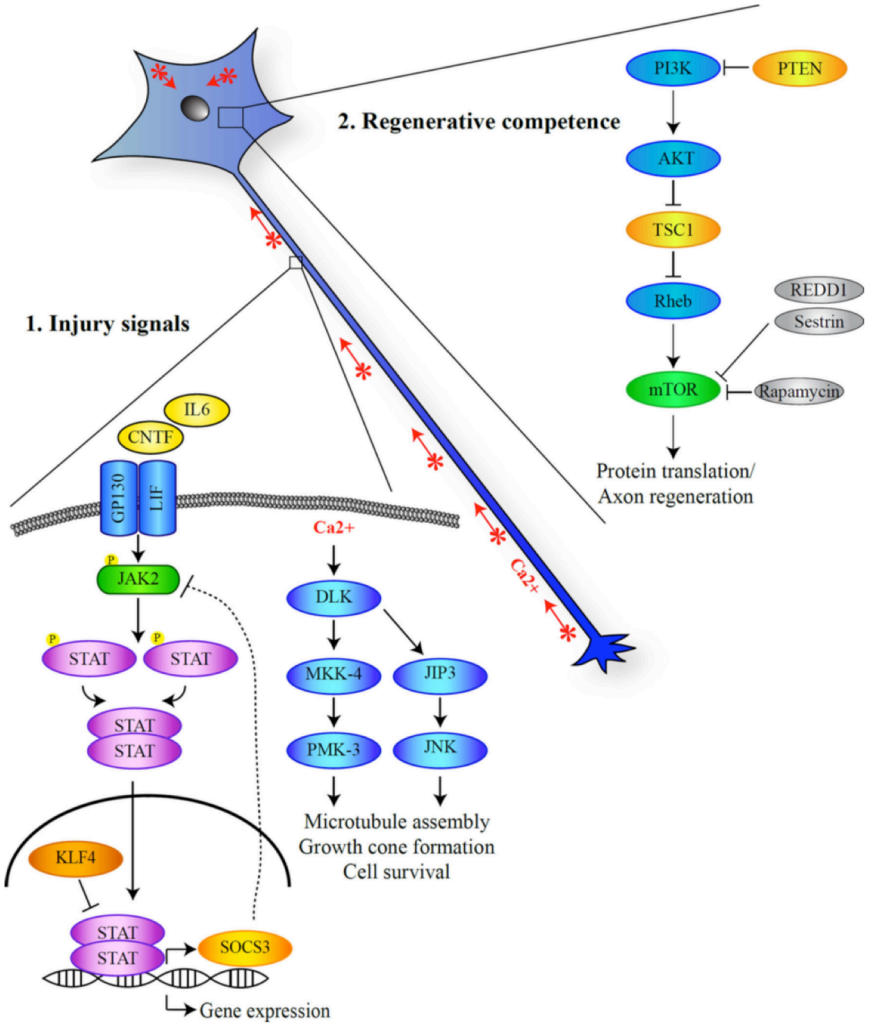

Figure 1.3 Neuron-intrinsic regulators of axon growth. The activity of various signaling pathways and factors within neurons including PTEN/mTOR, SOCS3/STAT3, KLFs, c-Myc (not shown), Doublecortin-Like Kinases (not shown), and DLK regulate axon growth after CNS injury. (Lu et al., 2014)

In addition to the prototypical myelin inhibitors, oligodendrocytes also express axon guidance molecules such as ephrins and semaphorins that may inhibit axon growth after injury. For instance, Sema6A is highly expressed by mature oligodendrocytes and inhibits neurite outgrowth from dorsal root ganglion neurons *in vitro* via its receptor PlexinA2 (Shim et al., 2012). Genetic deletion of PlexinA2 leads to increased sprouting of the intact CST on both sides of the cervical spinal cord after unilateral pyramidotomy, as well as increased functional recovery in a pellet-reaching assay (Shim et al., 2012). The extent to which various axon guidance molecules modulate spinal axon sprouting after CNS injury remains to be fully explored.

Chondroitin sulfate proteoglycans (CSPGs) present in the extracellular matrix of the glial scar have also been shown to inhibit axon growth *in vitro* and *in vivo.* The bacterial enzyme chondroitinase ABC (ChABC) degrades side chains from CSPGs, attenuating their inhibitory nature. In the pyramidotomy model, ChABC treatment has been shown to increase CST sprouting and functional recovery of paw preference for weight support during rearing (Starkey et al., 2012). In contrast to the bilateral sprouting observed after PlexinA2 deletion, ChABC treatment increased sprouting on the denervated side of the spinal cord only, suggesting distinction in the mechanisms involved.

Recently the importance of neuron-intrinsic factors in regulating axon growth after injury has been increasingly recognized by the finding of multiple intracellular signaling pathways such as PTEN/mTOR, SOCS3/STAT3, KLFs, c-Myc, and Doublecortin-Like Kinases that regulate axon regeneration and/or compensatory sprouting after CNS injury (Figure 1.3) (Moore et al., 2009; Smith et al., 2009; Liu et al.,

2010; Liu et al., 2011; Lu et al., 2014; Belin et al., 2015; Nawabi et al., 2015). Targeting PTEN, a negative regulator of the mammalian target of rapamycin (mTOR), promotes axon regeneration and sprouting in the CNS and some degree of functional recovery from experimental spinal cord injury, highlighting the potential of targeting neuron-intrinsic factors to promote recovery from CNS injury (Liu et al., 2010; Zukor et al., 2013; Lewandowski and Steward, 2014; Danilov and Steward, 2015).

The Dual Leucine zipper-bearing Kinase (DLK), a Mitogen-activated Protein Kinase Kinase Kinase (MAPKKK or MAP3K), was initially discovered for its critical role in axon degeneration and regeneration after injury in invertebrate model organisms *C. elegans* and Drosophila (Hammarlund et al., 2009; Miller et al., 2009; Yan et al., 2009; Xiong et al., 2010). Recent studies of one of its mammalian homologs, DLK, have revealed its role in neuronal death, degeneration and regeneration following peripheral nervous or optic nerve injury (Itoh et al., 2009; Shin et al., 2012; Watkins et al., 2013; Welsbie et al., 2013). However, the role of this molecule in sprouting or in spinal axon growth after experimental CNS injury has not been described.

While some factors such as PTEN regulate both axon regeneration and compensatory sprouting, other axon growth regulators such as myelin-associated inhibitors have been found to primarily regulate sprouting but not regeneration, indicating that sprouting and regeneration have both shared and distinct molecular regulation (Lee et al., 2010). A greater understanding of the differential role of axon growth regulators in these distinct forms of axon growth will become increasingly important if manipulation of these factors is to move forward in treating a diverse patient population suffering from CNS injury.

1.4 Functional recovery from CNS injury

Multiple mechanisms likely contribute to the partial recovery that spontaneously occurs after CNS injury. Recovery in spinal cord injury patients is thought to be due to both compensatory growth of uninjured axons ("sprouting") and synaptic strengthening of pre-existing circuits (Raineteau and Schwab, 2001).

Evidence that sprouting contributes to functional recovery comes from re-lesioning studies in model organisms. In these studies, partial CNS lesions that caused a behavioral impairment were followed by a recovery period during which growth of spared fibers and behavioral recovery was observed. Subsequent lesion of the spared fibers attenuated the observed functional recovery, suggesting their contribution to recovery (Weidner et al., 2001; Ueno et al., 2012). The corticospinal tract (CST), a major descending motor pathway important for voluntary movement, is often the focus of these studies due to its traceability and its contribution to motor behavior. For example, spontaneous functional recovery after dorsal column lesion to disrupt the dorsal main CST was abolished when a second lesion was performed to eliminate the ventral CST in rats (Weidner et al., 2001). Similarly, after unilateral lesion of the sensorimotor cortex, mice show spontaneous sprouting of intact CST fibers in the denervated cervical spinal cord and gradual partial spontaneous improvement in fine motor control; this functional recovery is attenuated by subsequent lesion of the uninjured CST fibers (Ueno et al., 2012). These experiments demonstrate that the spared CST, which is a source of axon sprouting, is important for behavioral recovery after partial lesion to the CST system. However, definitive demonstration that the sprouted fibers themselves mediate functional recovery remains a challenge.

Considering that compensatory sprouting of uninjured axons likely contributes to partial recovery from injury in humans and model organisms, one might wonder whether this spontaneous recovery mechanism could be harnessed to further promote recovery from injury by manipulating factors that regulate axon growth. Indeed, while many attempts have been made to encourage regeneration of damaged axons by modulating axon growth regulators, few experimental manipulations have led to robust, functionally meaningful regeneration. Nonetheless, extensive literature indicates that targeting various inhibitory molecules present in the CNS environment such as myelin-associated inhibitors and chondroitin sulfate proteoglycans may improve functional recovery in models of spinal cord injury, first shown with the IN-1 antibody (Bregman et al., 1995) and later with chondroitinase ABC (Bradbury et al., 2002). While subsequent studies raised the question of how robustly targeting these extrinsic inhibitors improves axon regeneration, a consistent theme has emerged that manipulating these extrinsic inhibitors alters the axonal sprouting response of intact axons, suggesting that enhanced sprouting may underlie the improved functional recovery (Bartus et al., 2012; Lee and Zheng, 2012).

In studies using the unilateral pyramidotomy model to study sprouting of the CST (see *"1.5 Unilateral pyramidotomy model to study corticospinal axon sprouting"),* manipulating extrinsic growth inhibitors has been found to both increase CST sprouting and functional recovery from injury, as assessed by pellet retrieval (Thallmair et al., 1998; Shim et al., 2012), sticky paper removal retrieval (Thallmair et al., 1998; Shim et al., 2012), paw preference for weight support during rearing (Starkey et al., 2012), or rope climbing (Thallmair et al., 1998). Furthermore the ability of these sprouted CST

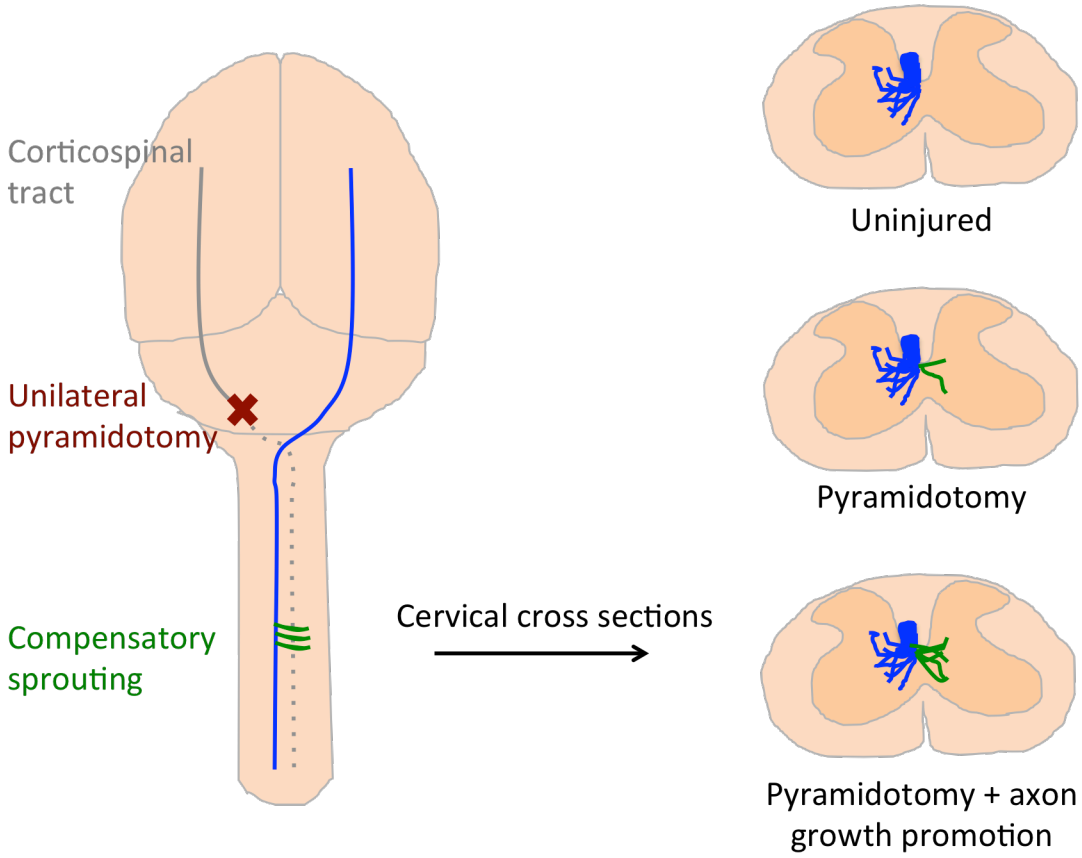

Figure 1.4 Unilateral pyramidotomy model to study compensatory sprouting of the corticospinal tract. In this CNS injury model, one side of the CST is lesioned at the level of the medulla. The unlesioned CST is traced (blue) and new axonal growth in the denervated side of the cervical spinal cord (green) is assessed.

axons to form functional synapses has been implicated by their co-localization with a variety of pre- and post-synaptic markers including vGlut1 (Starkey et al., 2012), synaptophysin, SV2, and PSD-95 (Shim et al., 2012), suggesting the possibility that these sprouted fibers mediate functional recovery.

Yet the question remains of whether the observed increase of sprouting of CST axons in the cervical spinal cord is directly responsible for functional recovery. Indeed, performance in these behavioral tests may be partially mediated or compensated for by plasticity of other tracts involved in motor functioning, such as the rubrospinal tract or the reticulospinal tract. For instance, with IN-1 antibody treatment the rubrospinal tract has been shown to innervate motor neurons denervated by bilateral CST transection (Raineteau et al., 2002). It is also possible that growth of CST axons rostral to the lesion into subcortical nuclei important for motor functioning may be involved in behavioral recovery (Z'Graggen et al., 1998).

1.5 Unilateral pyramidotomy model to study corticospinal axon sprouting

The corticospinal tract (CST) is a major descending motor pathway important for voluntary motor control. Sprouting of the CST in rodents can be readily assessed after experimental unilateral pyramidotomy (Figure 1.4). In this injury model, one side of the CST is lesioned at the level of the medulla as it travels from the cortex to the contralateral spinal cord via the pyramids of the medulla, with the lesion placed just above where the tract crosses the midline. Pyramidotomy allows for a “clean” injury to one side of the CST, as an artery separates the two pyramids in the medulla. Lesion completeness can then be assessed by staining cervical spinal cord sections for PKCγ,

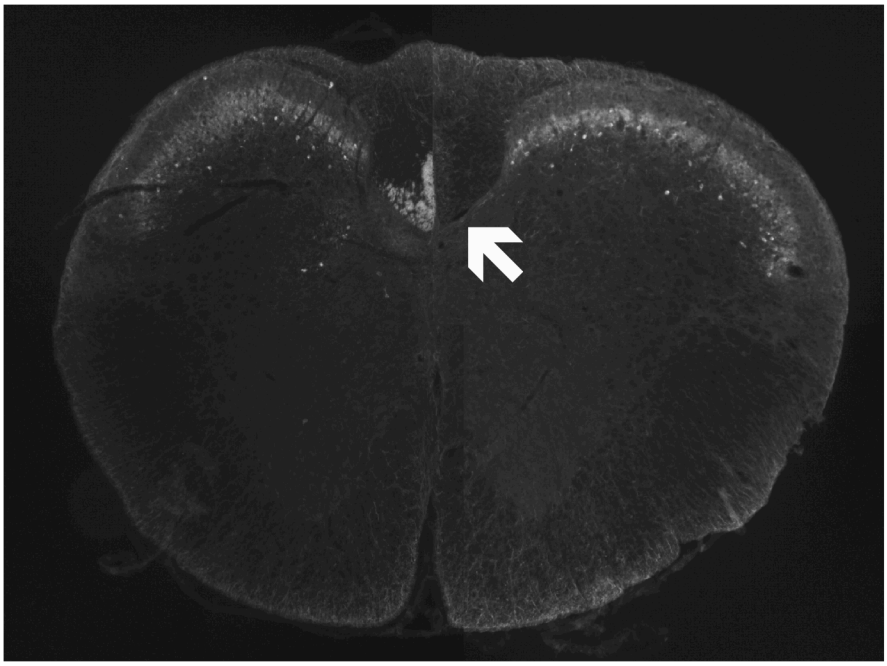

Figure 1.5 Unilateral pyramidotomy results in loss of one side of the main CST in the cervical spinal cord. PKCγ staining reveals unilateral loss of main CST axons in the dorsal columns of the cervical spinal cord (white arrow).

which specifically labels the CST within the white matter (Figure 1.5).

In order to assess compensatory sprouting of the CST after injury, the intact side of the CST is labeled via stereotactic cortical injection of neuronal tracer into the forelimb-projecting region of the sensorimotor cortex (Figure 1.6). Sprouting of fibers in the denervated side of the cervical spinal cord is then assessed. In rodents, there is a small but significant amount of spontaneous CST axon sprouting after unilateral pyramidotomy (Thallmair et al., 1998; Lee et al., 2010; Shim et al., 2012). Therefore, it is important to take into consideration this baseline level of spontaneous sprouting when assessing the effect of any molecular manipulation. Interestingly, the level of spontaneous CST sprouting appears to be much more extensive in non-human primates than in rodents after a lateral cervical injury, implicating the translational importance of CST sprouting in anatomically incomplete spinal injuries (Rosenzweig et al., 2010).

The unilateral pyramidotomy model is well equipped for assessing compensatory sprouting in the CNS for a few reasons. First, due to the anatomy of the tract, the CST can be cleanly lesioned on one side and labeled on the other, avoiding any ambiguity between regeneration versus sprouting or what population of neurons are traced. Second, while robust behavioral assays to monitor functional recovery from this injury are still under development, in theory this axonal tract is important for a quantifiable behavioral outcome (i.e., voluntary and fine motor control), and thus functional benefit associated with increased sprouting can be assessed. Lastly, the corticospinal tract is important for voluntary motor control in humans and is resistant to regeneration;

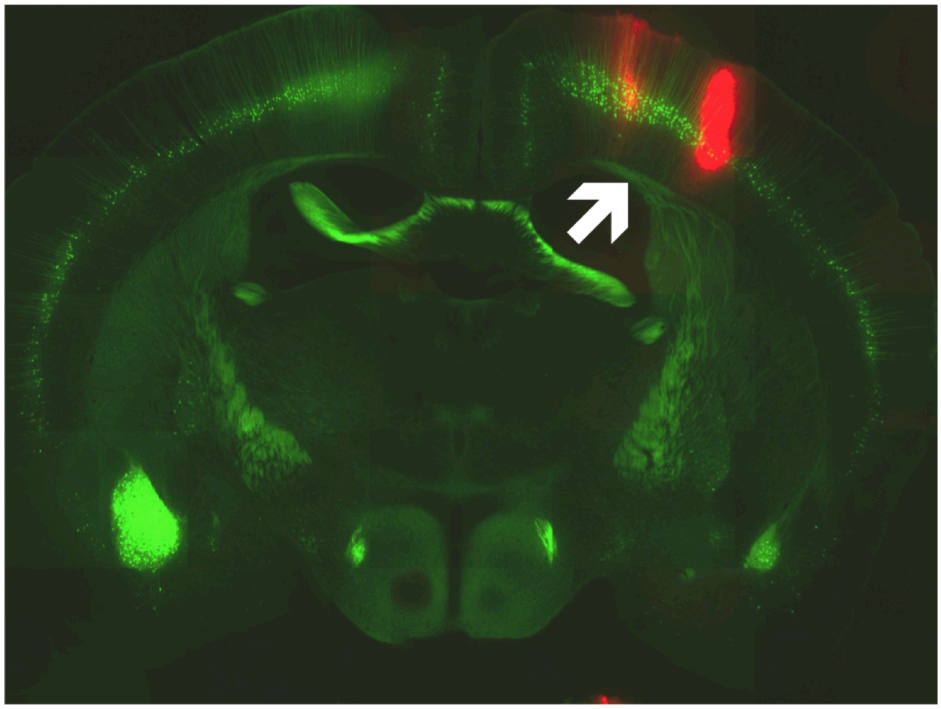

Figure 1.6 Neuronal tracer can be stereotactically injected into the mouse sensorimotor cortex to target one side of the corticospinal tract. Arrow points to injection sites of CTB-555 (red) targeting layer V neurons of the sensorimotor cortex, a subset of which are neurons of the CST. Layer V pyramidal cells are labeled in the mouse reporter line thy1-YFP-H (green).

restoring function of this tract via promoting sprouting has the potential to provide great clinical benefit to patients with brain or spinal cord injuries.

Various interventions that increase CST compensatory sprouting have been found to improve functional recovery of skilled forelimb use, suggesting that sprouted axons may play a role in recovery from injury (Figure 1.7, see also "*1.4 Functional recovery from CNS injury")* (Shim et al., 2012; Starkey et al., 2012; Lang et al., 2013; Meves and Zheng, 2014). Nonetheless, robust and objective functional assays for monitoring recovery from unilateral pyramidotomy are lacking.

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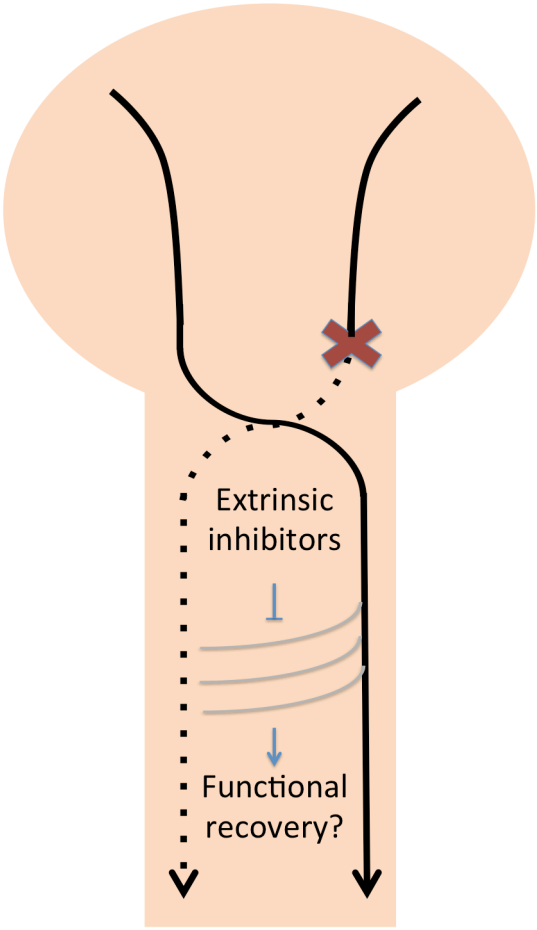

Figure 1.7 Axon growth inhibitors attenuate anatomical and functional recovery from injury. After an axonal tract in the central nervous system is lesioned (bold X), the distal segments degenerate (dotted line). Uninjured axon fibers sprout in the denervated side of the spinal cord after injury (horizontal curved lines), which is attenuated by neuron-extrinsic and intrinsic inhibitors (intrinsic not shown). This sprouting may contribute to functional recovery from spinal cord injury. Arrows denote the direction of the two descending axonal tracts (one on each side) within the spinal cord. (Meves and Zheng, 2014)

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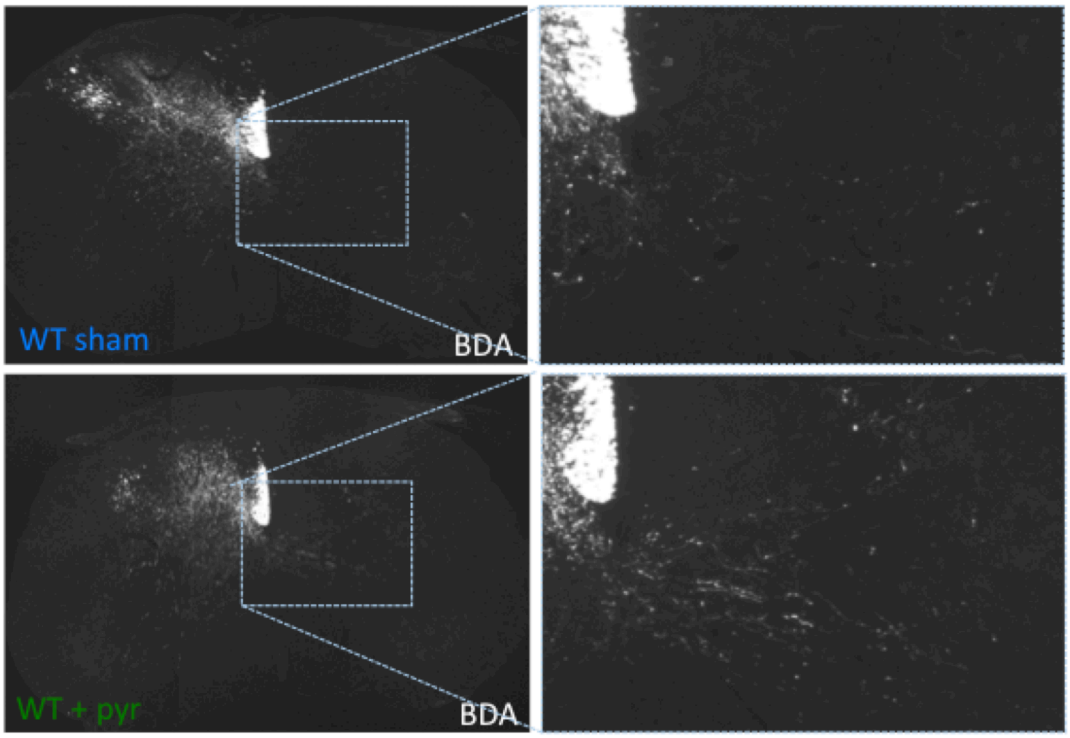
Chapter 2. Improved assessment of functional recovery after pyramidotomy in mice

2.1 Abstract

The unilateral pyramidotomy model is a valuable tool for assessing compensatory growth of uninjured corticospinal tract (CST) axons after damage to the central nervous system. In this model, one side of the CST is lesioned at the level of the medullary pyramids, which induces spontaneous growth of the contralateral intact CST axons in the denervated side of the spinal cord. Previous studies have shown that this form of axon growth can be increased by manipulating a variety of neuron-intrinsic or extrinsic axon growth regulators. While this model allows clear anatomical assessment of axon growth after injury, the use of this model in mice has been hampered by a lack of consistent and robust behavioral assessments. Here I show that a modified staircase-reaching test reveals a clear, persistent functional deficit after a unilateral pyramidotomy injury in mice. This behavioral paradigm requires minimal hands-on training of animals or subjective scoring of performance, lending itself as a valuable tool for ongoing studies that assess the functional relevance of molecular manipulations that promote compensatory axon growth.

2.2 Introduction

In contrast to damaged axons in the adult mammalian CNS that largely fail to regenerate after injury, uninjured axons spontaneously sprout after CNS injury and are thought to contribute to functional recovery (see "*1.4 Functional recovery from CNS injury*"). While the unilateral pyramidotomy injury model serves as a useful tool for assessing axon sprouting after injury, evaluating functional recovery from this injury model remains a challenge. Limitations in existing behavioral assays stem from a lack



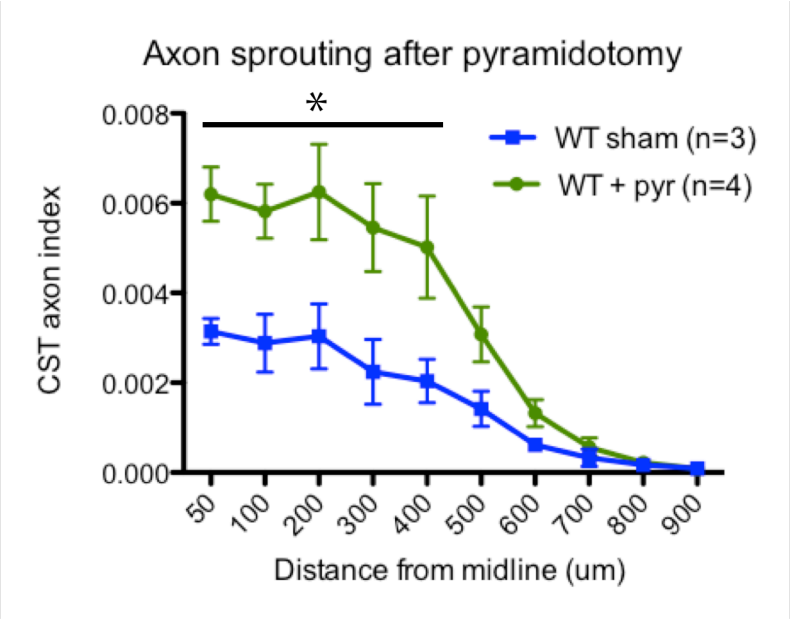


Figure 2.1 Unilateral pyramidotomy induces compensatory sprouting of the corticospinal tract in the denervated side of the spinal cord. Top panel, BDA-labeled CST axons in cervical sections from WT mice with and without unilateral pyramidotomy. Bottom panel, quantification of the number of BDA-labeled axons at specific distances from the midline in the denervated side of the spinal cord normalized to the total labeled CST axon count at the medulla in WT sham (n=3) and WT + pyr (n=4) mice. Two-way repeated measures ANOVA with Bonferroni post test, *$p < 0.05$ compared to WT sham. Error bars denote standard error of the mean (SEM).

of initial functional deficit, spontaneous functional recovery to baseline, and/or reliance on subjective behavioral measures. In order to evaluate whether manipulations that promote axon sprouting promote functional recovery, a robust behavioral test is of utmost importance. Here I describe the effect of unilateral pyramidotomy on grooming and pasta handling in mice for the first time, and describe an optimized staircase reaching assay with the potential to detect both enhanced and reduced recovery from injury.

2.3 Materials and Methods

Surgical procedures

Pyramidotomy and biotinylated dextran amine (BDA) tracer injection were performed as described previously with minor modifications (Starkey et al., 2005; Lee et al., 2010; Liu et al., 2010; Geoffroy et al., 2015). Surgeons performing the surgeries were blinded to genotype. All animals were anesthetized with 2.5% Avertin (Sigma) and incision wounds were closed using Vetbond (3M). An incision was made overlying the trachea and the pyramidal tracts were accessed at the base of the skull (Starkey et al., 2005). A Feather micro scalpel was used to lesion the entire left pyramidal tract just caudal to the foramen magnum. Cortical injection of BDA (10%, Invitrogen) was performed 2 weeks prior to sacrifice for all studies. BDA was delivered via a modified 10 µL Hamilton syringe attached to a fine glass pipette mounted on a stereotaxic device for cortical injection. Animals were anesthetized with freshly prepared 2.5% Avertin (Sigma) and a total of 1.2 µL of BDA was injected into the sensorimotor cortex at 3 sites (0.4 µL per site). The right sensorimotor cortex targeting the left forelimb was injected 0.7 mm

below the cortical surface at the following coordinates relative to Bregma: 0.5 mm anterior, 1.2 mm lateral; 0.3 mm posterior, 1.2 mm lateral; and 0.1 mm anterior, 2.2 mm lateral.

Tissue processing

Tissue processing was performed as described previously with minor modifications (Lee et al., 2009; Lee et al., 2010). Mice were administered a lethal dose of Fatal plus and perfused transcardially with 4% PFA. Brain and spinal cord were dissected out, and the tissues were post-fixed overnight at 4°C in the same fixative solution. Tissues were incubated in 30% sucrose for cryo-protection. Brain, medulla, and C1-C7 cervical spinal cord were embedded in OCT compound and frozen on dry ice. Tissues were sectioned with a cryostat at a thickness of 20 μm. For sprouting studies, transverse sections of the medullas were processed to obtain estimates of the total number of CST axons labeled to control for labeling efficiency (see below). Cervical spinal cord and medulla sections were incubated in Vectastain ABC solution (Vector Laboratories) overnight at 4°C, washed in PBS, and mounted on gelatin-coated slides. BDA was detected with TSA Plus Fluorescein System (10 min, room temperature, 1:200, PerkinElmer). Selected transverse sections of cervical spinal cord (C7) were immunostained for PKCγ (1:100, Santa Cruz Biotechnology) to examine the completeness of the lesion for each animal, as described previously (Lee et al., 2010). Mice with incomplete lesion were excluded from the study.

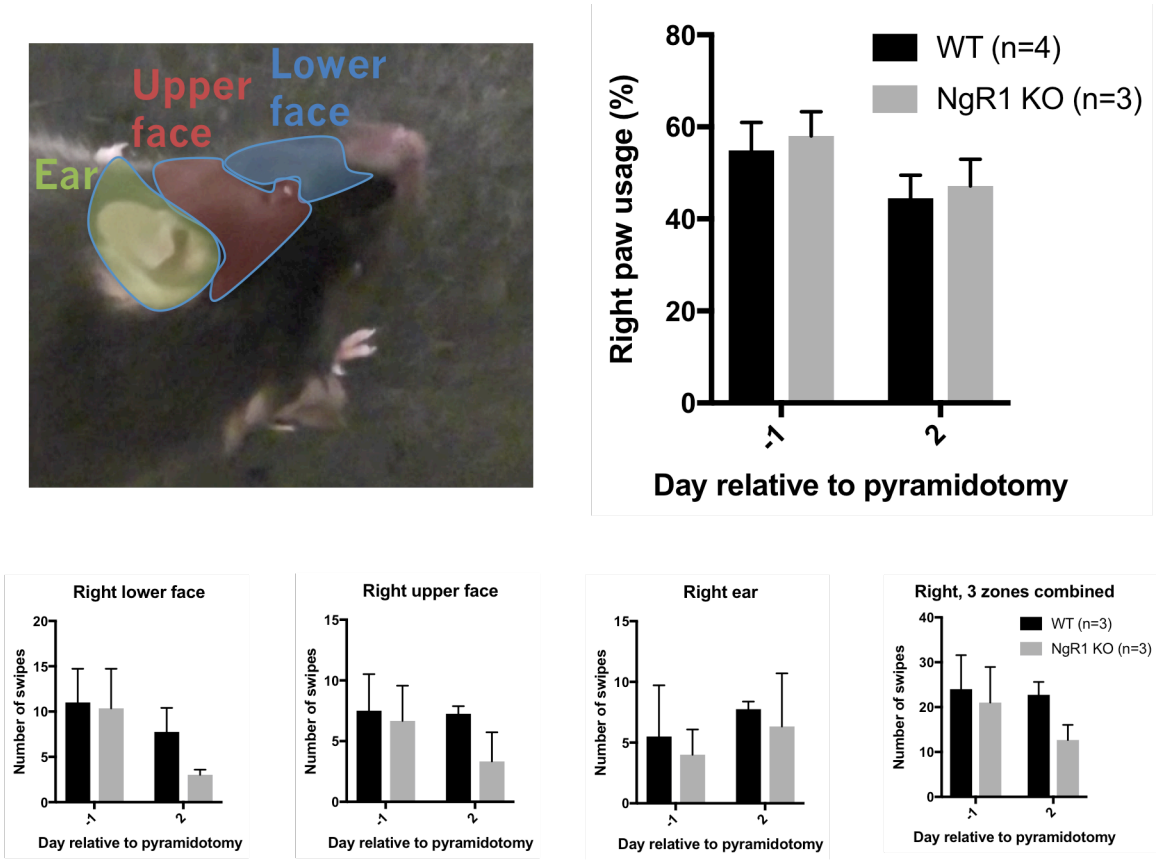


Figure 2.2 Grooming test does not reveal functional impairment from unilateral pyramidotomy. Top left, zones in which number of paw swipes with the ipsilateral paw were counted. Note that the same zones were evaluated on the right side of the head (not shown). Top right, right paw usage for grooming showed a modest trend toward reduction after pyramidotomy (two way repeated measures ANOVA, $p = 0.0794$ for main effect of time). Bottom four panels, no appreciable effect of pyramidotomy on number of swipes in any individual zone nor all three zones combined.

Quantification of sprouting index

To determine the sprouting index, transverse spinal cord sections were taken from C5-C7 cervical levels and imaged for BDA (see Tissue Processing). Lines were drawn parallel to the midline dorsoventral axis at 50 and 100 microns from the midline, then every 100 microns laterally in the denervated gray matter. The number of axons crossing each line was manually counted in five randomly chosen sections per animal by a blinded observer. Counts were averaged for each animal and normalized against the total axon count in medulla (Lee et al., 2010) to obtain the sprouting index and percent midline crossing axons, which was plotted as a function of distance from the midline.

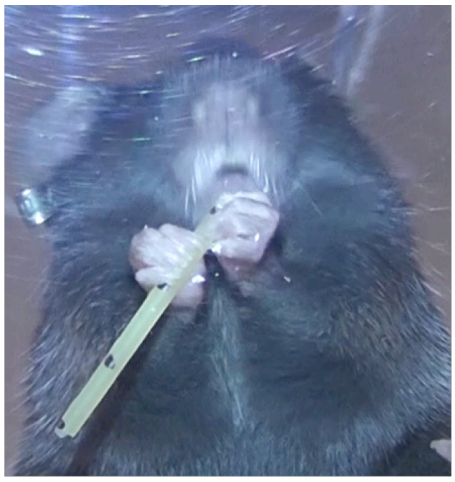
Behavior testing

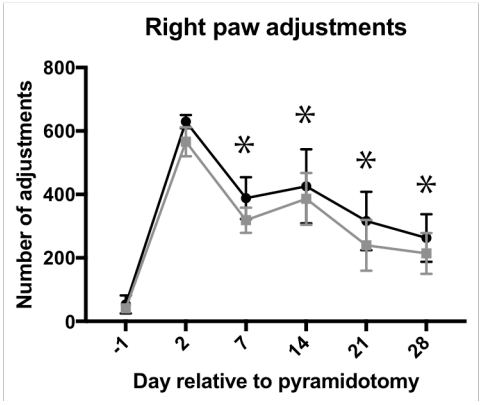
All behavioral tests were performed and quantified by observers blinded to genotype. For both staircase reaching and pasta handling tests, mice were deprived of food at approximately 5pm and tested at approximately 9am the following morning resulting in approximately 16 hours food deprivation. Animals were weighed weekly to monitor for excessive weight loss (greater than 10%).

For the grooming test, water was rubbed along the midline portion of the head of the mouse running between the eyes and the ears using a cotton swab (Bertelli and Mira, 1993; Gensel et al., 2006; Hilton et al., 2013). Mouse behavior was immediately filmed for two minutes from above. Video playback was quantified frame-by-frame to evaluate the number of times each paw entered one of three zones on the side of the head (lower face, upper face, ear) (Figure 2.2, top panel).

For pasta handling, mice were tested as described previously (Tennant et al., 2010). Briefly, mice were video-recorded while eating a 2.5 cm piece of dry capellini pasta and the number of adjustments made with each paw was quantified by watching frame-by-frame video playback. Adjustments consisted of paw release and re-contact with the pasta or any movement of the paw or digits while in contact with the pasta as described previously (Tennant et al., 2010). Before testing, mice were exposed to 3-5 pieces of pasta in the home cage for 5 days and were habituated to the testing environment for 2-3 days until eating pasta in the testing environment.

For staircase reaching, mice were pre-trained and tested as described previously with slight modifications (Baird et al., 2001; Starkey et al., 2005; Kloth et al., 2006). For habituation prior to pre-training, animals were exposed to dyed sucrose pellets (Fisher Scientific Dustless Precision Pellets 14 mg, dyed with AmeriColor Student Soft Gel Paste Food Color) in their home cages and habituated to the testing environment, experimenter, and staircase chambers (Campden Instruments) for approximately 10 minutes per day for 3 days. Staircase chambers were modified to prevent build up of displaced pellets on lower steps by removing a plastic barrier at the base of the staircase and by adding a plastic barrier between the staircase chamber and the main chamber. During pre-training, animals were placed in sucrose-pellet baited chambers for 10 minutes once daily for 4-5 days per week until performance plateau, for a total of approximately 25 sessions. For all pre-training and testing sessions, staircases were baited with 4 pellets on each step other than the top two steps to prevent usage of the tongue to retrieve pellets (unless noted otherwise). Initial pre-training sessions were baited with additional pellets on the top steps and the central platform to encourage





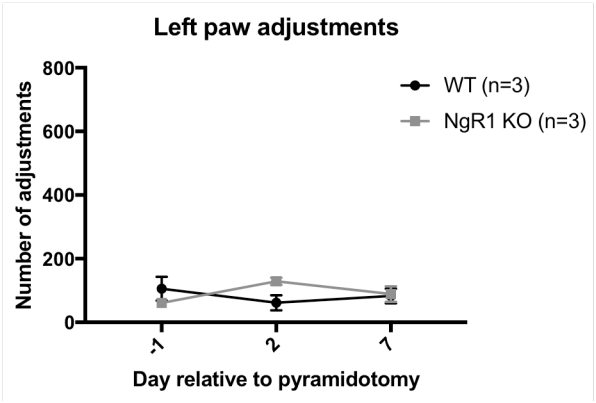


Figure 2.3 Pasta handling test reveals injury-specific effect on fine motor control after unilateral pyramidotomy. Top, photo of mouse performing the pasta-handling test. Bottom left, the number of paw adjustments made with the right paw after left pyramidotomy remains significantly elevated through day 28. Bottom right, the number of adjustments made by the left paw is unaffected. Two-way repeated measures ANOVA $p = 0.0001$ for main effect of time, * $p < 0.05$ for both groups combined vs. Day -1. Note day 2 excluded from analysis due to one WT data point missing (WT n=2 for Day 2, right paw adjustments).

exploration and pellet retrieval; however, the number of sessions conducted in this way was kept to a minimum to avoid reliance on licking pellets (i.e., as soon as mice learned to retrieve pellets, the pellets on the top 2 steps and on and the central platform were removed.) Throughout pre-training and testing, performance was monitored to ensure pellets were retrieved with forepaws and not tongues. One mouse was found to use the tongue to retrieve pellets beyond early pre-training sessions from the 3rd step and was excluded from analysis. After pre-training, animals were tested once every 7 days on day 3, 10, 17, etc. after pyramidotomy. Test sessions were 10 minutes, after which the number and location of remaining pellets was recorded to calculate pellets eaten (successful retrievals) and pellets eaten relative to pellets displaced (success rate).

Experimental design and statistical analysis

Sprouting index data were analyzed via two way repeated measures ANOVA with Bonferroni post-tests. For all statistical analyses, GraphPad Prism 5 was used with $p < 0.05$ set as the threshold for statistically significance, denoted by asterisks in graphs. All data is displayed as means with error bars denoting SEM. Specific n values for each study are listed in figures. For analysis of sprouting index, five cervical sections and two medulla sections were quantified for each animal (see Quantification of sprouting index and percent midline crossing axons for specific details*).* A mix of male and female mice in an approximately 1:1 ratio were used for all experiments.

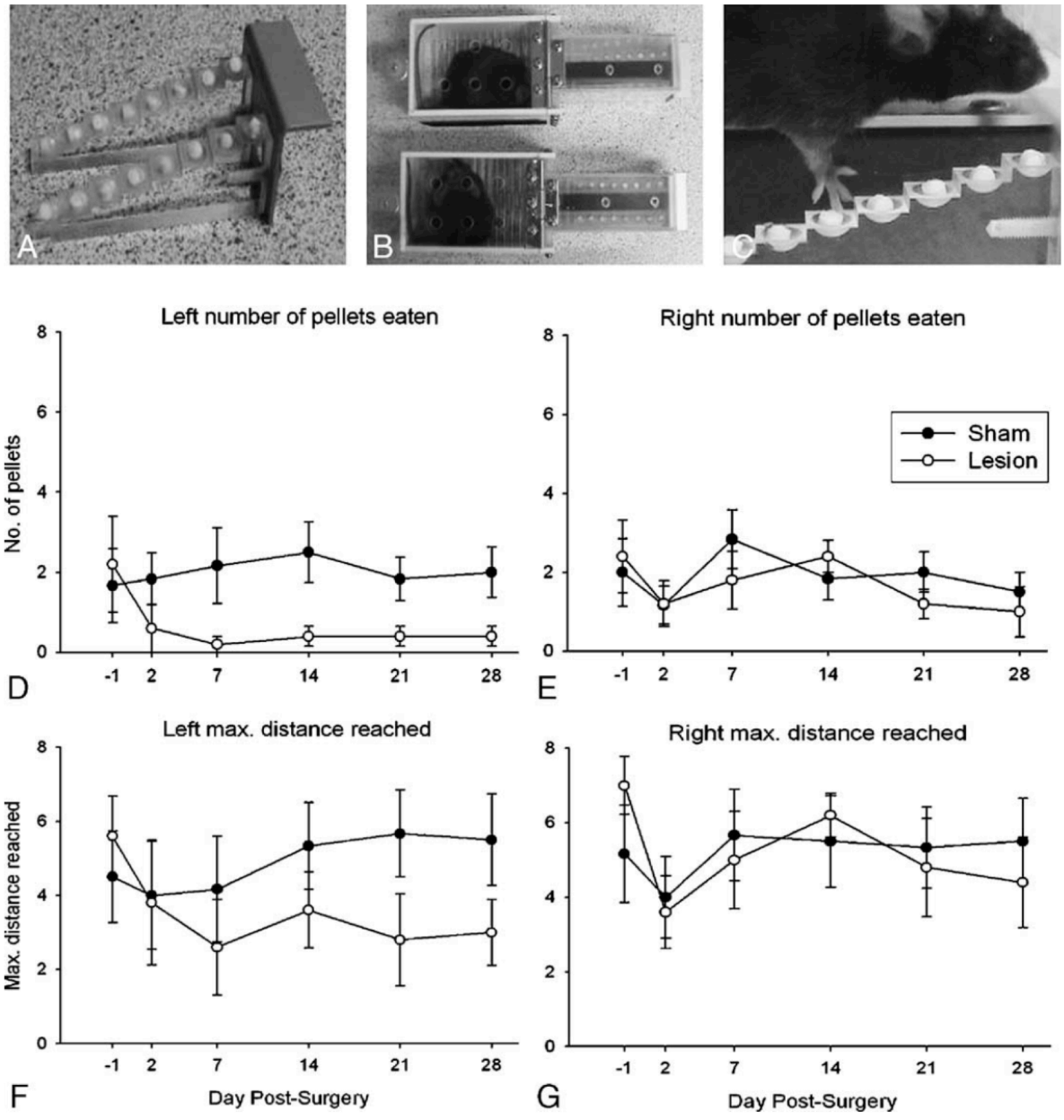


Figure 2.4 Original staircase reaching assay fails to show significant effect of pyramidotomy. (A) Staircase baited with one sucrose pellet per step. (B) Top view of chambers with mouse in main chamber, from which they can freely explore the staircase chamber to retrieve pellets. (C) Side view of mouse in staircase portion of chamber reaching to retrieve a sucrose pellet. (D-G) Original assay is not sensitive to impairments after unilateral pyramidotomy. (Starkey et al., 2005)

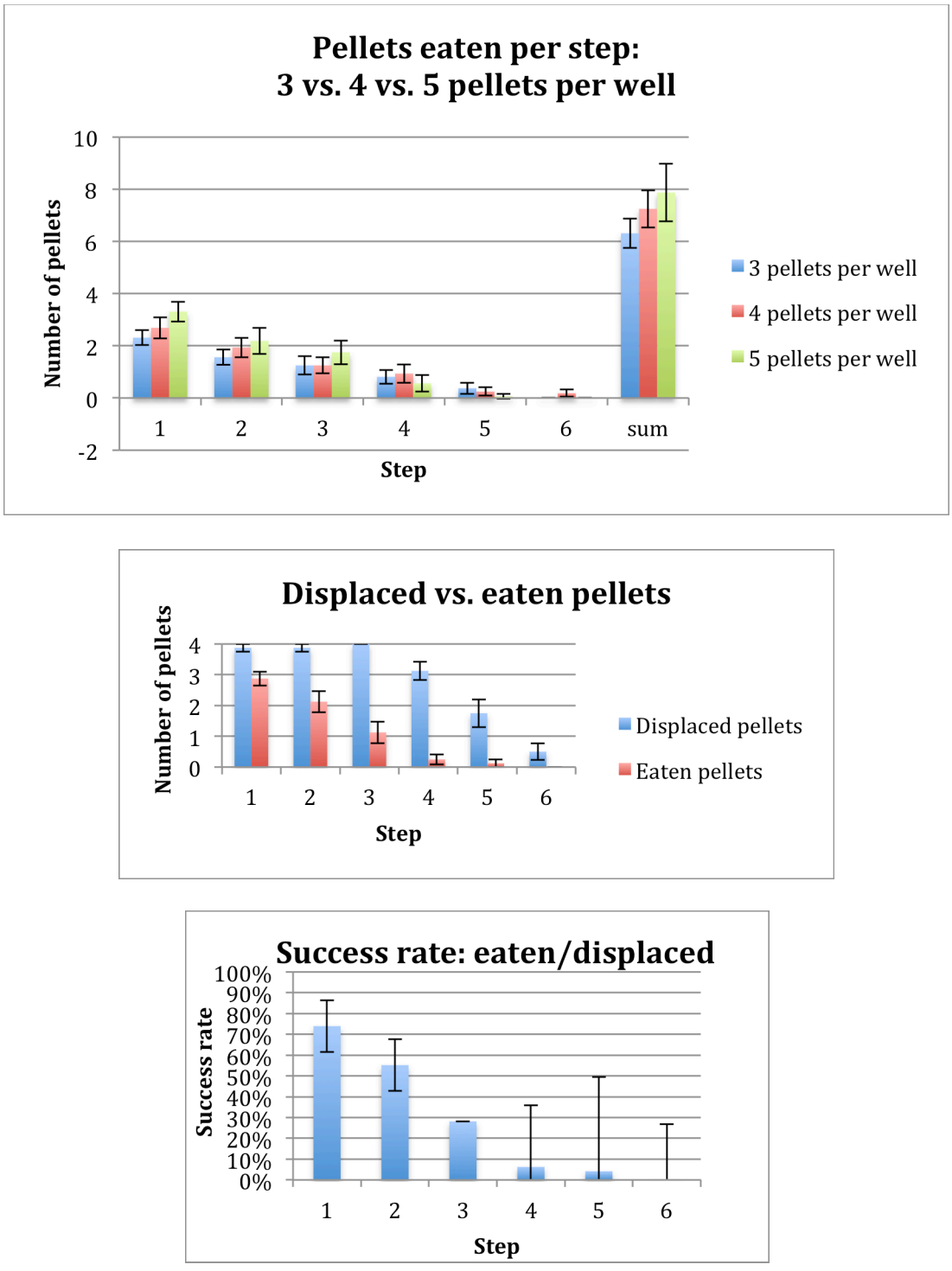
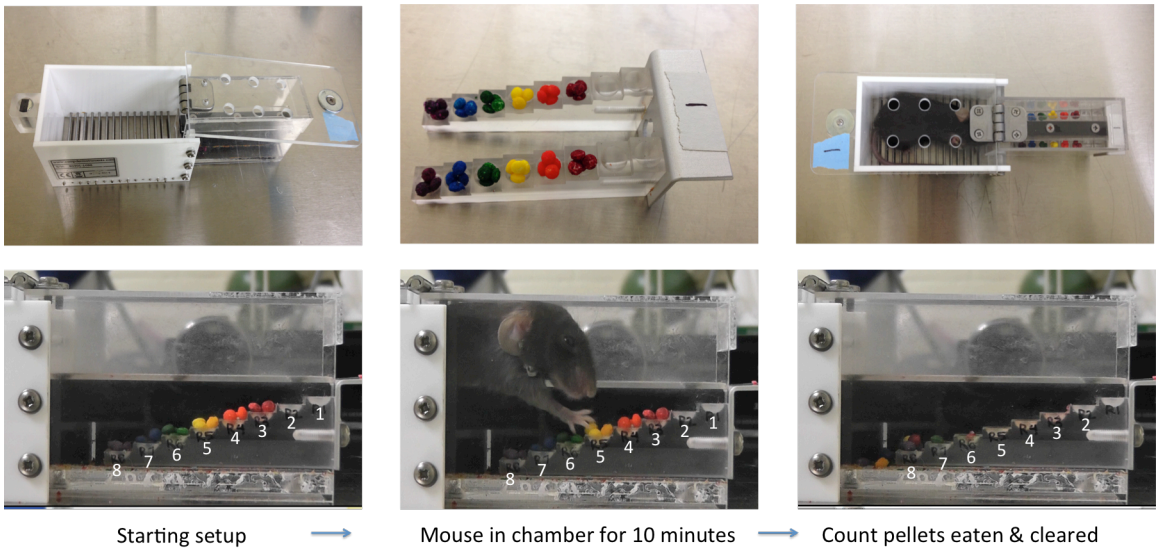


Figure 2.5 Optimization of staircase reaching assay. Top, comparison of the number of pellets eaten per step when steps are baited with 3, 4, or 5 pellets each. Middle and bottom, displaced, eaten, and success rate measures for 4 pellet setup. Error bars denote standard deviation.

2.4 Results and Discussion

Unilateral pyramidotomy was found to induce a ~2x increase in the number of CST axons in the denervated side of the spinal cord (Figure 2.1). In order to evaluate the functional deficits and subsequent recovery of fine motor control associated with this injury, mice were assessed in grooming, pasta handling, and staircase reaching tests. The grooming test did not reveal any significant functional effects of pyramidotomy in a range of measures assessed, nor any effect of deleting the Nogo receptor NgR1 (Figure 2.2). In contrast, the pasta handling test in which the number of adjustments made by each paw while eating a piece of dry pasta revealed a dramatic effect of pyramidotomy, which was still evident 28 days after injury (Figure 2.3). This effect was only seen on the affected side (right paw with unilateral pyramidotomy on the left, above the pyramidal decussation) confirming the specificity of the effect to the unilateral CST lesion. Still, no functional benefit of deleting NgR1 was evident, in contrast to evidence in the literature that NgR1 deletion promotes functional recovery from unilateral pyramidotomy (Cafferty and Strittmatter, 2006).

While the pasta-handling test revealed a functional effect of pyramidotomy, the test requires subjective scoring of performance that may vary between experimenters. In order to explore more objective measures of function, attempts were made to optimize the staircase-reaching test, which had failed to show a significant effect of pyramidotomy in the past (Figure 2.4) (Starkey et al., 2005). Two modifications were made to the test: (1) an increased number of pellets per step to improve the sensitivity of the test to detect functional deficits and (2) color-coded pellets to give measures of performance by step, displaced pellets, and success rate. Comparisons of performance



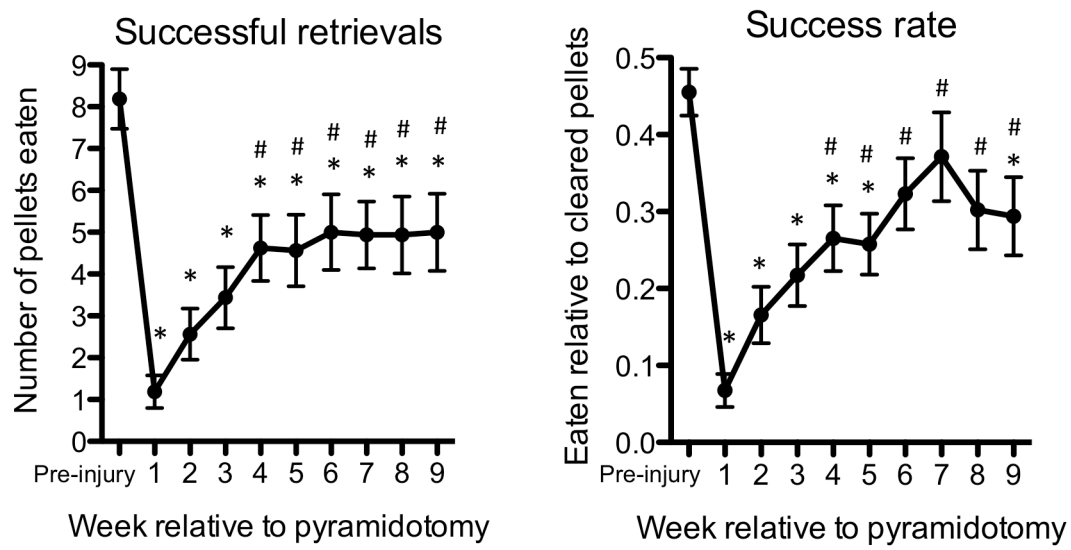


Figure 2.6 Optimized staircase-reaching test with four color-coded pellets per well reveals sustained partial deficit after unilateral pyramidotomy. Top, experimental setup in which mouse is placed in chamber for 10 minutes and allowed to retrieve pellets. Bottom, through 9 weeks after pyramidotomy, performance is significantly improved relative to week 1 but remains impaired relative to pre-injury performance. One-way repeated measures ANOVA with Bonferroni post test, *p < 0.05 compared to pre-injury, #p < 0.05 compared to week 1, n=16. Error bars denote SEM.

when steps were baited with 3, 4, or 5 pellets suggested that baiting with 4 or 5 pellets per step may increase the total number of pellets eaten relative to 3 pellets (note 1 pellet per step was used in the original evaluation of this assay for use with pyramidotomy) (Starkey et al., 2005). Additionally, when comparing baiting with 4 versus 5 pellets per step, the use of 4 pellets maintained pellet retrieval across a range of steps when compared to the use of 5 pellets (i.e., more pellets eaten from steps 4, 5, and 6 for 4 pellets vs. 5 pellets), which may increase sensitivity of the assay to impairments that preferentially affect retrieval on lower steps from which pellet retrieval is presumably more difficult. Thus, 4 pellets per step was chosen as the to be used in future experiments with the staircase reaching assay.

In addition to optimizing the number of pellets per step in the staircase-reaching test, pellets were also color-coded by step. This color-coding enabled assessment of performance by step and additional measures of the number of pellets displaced off of each step and the success rate (number of pellets eaten relative to number of pellets displaced) (Figure 2.5). Preliminary data suggested that while the top 3 steps showed a similar number of displaced pellets, retrieval of pellets from lower steps was more difficult.

This optimized staircase-reaching test revealed a sustained functional deficit through 9 weeks after pyramidotomy in both the number of pellets eaten and success rate (Figure 2.6). As such, this test can be used to evaluate whether manipulations that promote sprouting improve functional recovery from injury as well. Nonetheless, this optimized assay was able to detect partial recovery from injury as well, and could also be used to evaluate whether any manipulations *impair* recovery from pyramidotomy.

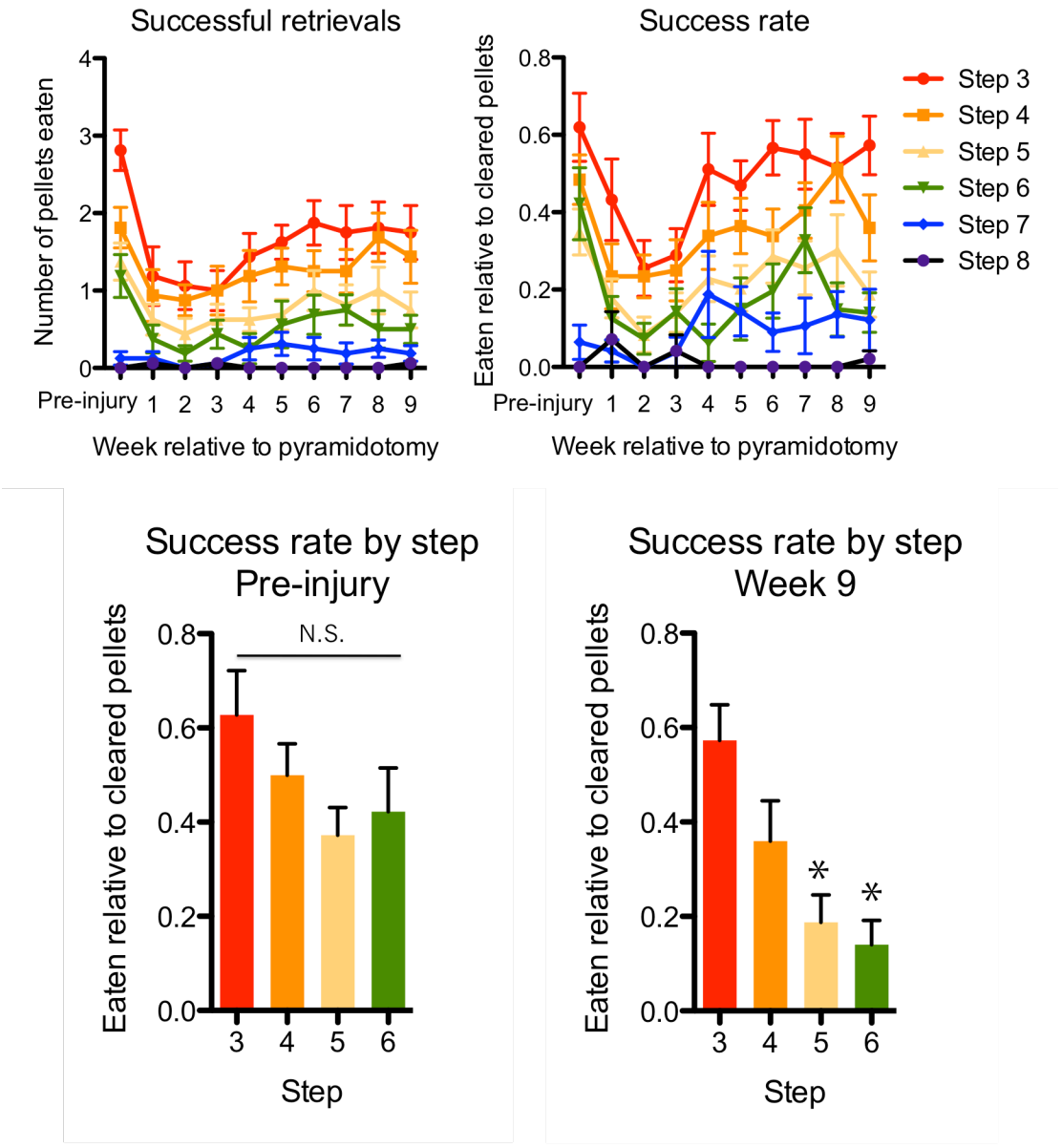


Figure 2.7 Evaluation of staircase reaching performance by step. Success rate varies across steps after injury, which may enable more sensitive detection of the effect of manipulations that promote functional recovery. One-way repeated measures ANOVA with Bonferroni post test, *p < 0.001 compared to step 3, n=15. Error bars denote SEM.

Finally, evaluation of success rate by step, enabled by color-coded pellets, revealed that while there was no significant difference in success rate between steps before injury, at 9 weeks the success rate at lower steps was significantly reduced relative to higher steps (Figure 2.7). This suggests that pyramidotomy preferentially impairs performance at lower steps, and as such evaluation of performance at individual steps may increase the sensitivity of the assay. To summarize, color-coded pellets enables assessment of function at varying difficulty levels after injury.

In line with my behavioral data comparing performance of WT and NgR1 KO mice in the grooming test and pasta-handling test, I was not able to detect improved functional recovery in NgR1 KO mice in staircase reaching (two-way repeated measures ANOVA on performance after injury, n.s.) (Figure 2.8). Upon attempting to reproduce a report in the literature of increased CST sprouting in NgR1 KO, I in collaboration with Cèdric Geoffroy did not detect enhanced CST sprouting in two independent experiments (Cafferty and Strittmatter, 2006).

To conclude, the grooming test did not reveal any functional effect of pyramidotomy, whereas the pasta-handling test and staircase-reaching test both show utility for monitoring functional recovery after pyramidotomy. While the pasta-handling test showed a striking and sustained effect of injury, the optimized staircase-reaching test provides an objective measure of performance. The increased number of pellets per well allows for detection of sustained functional deficit after pyramidotomy and color-coded pellets enable evaluation of success rate and performance by step, providing assessment of function across a range of difficulties.

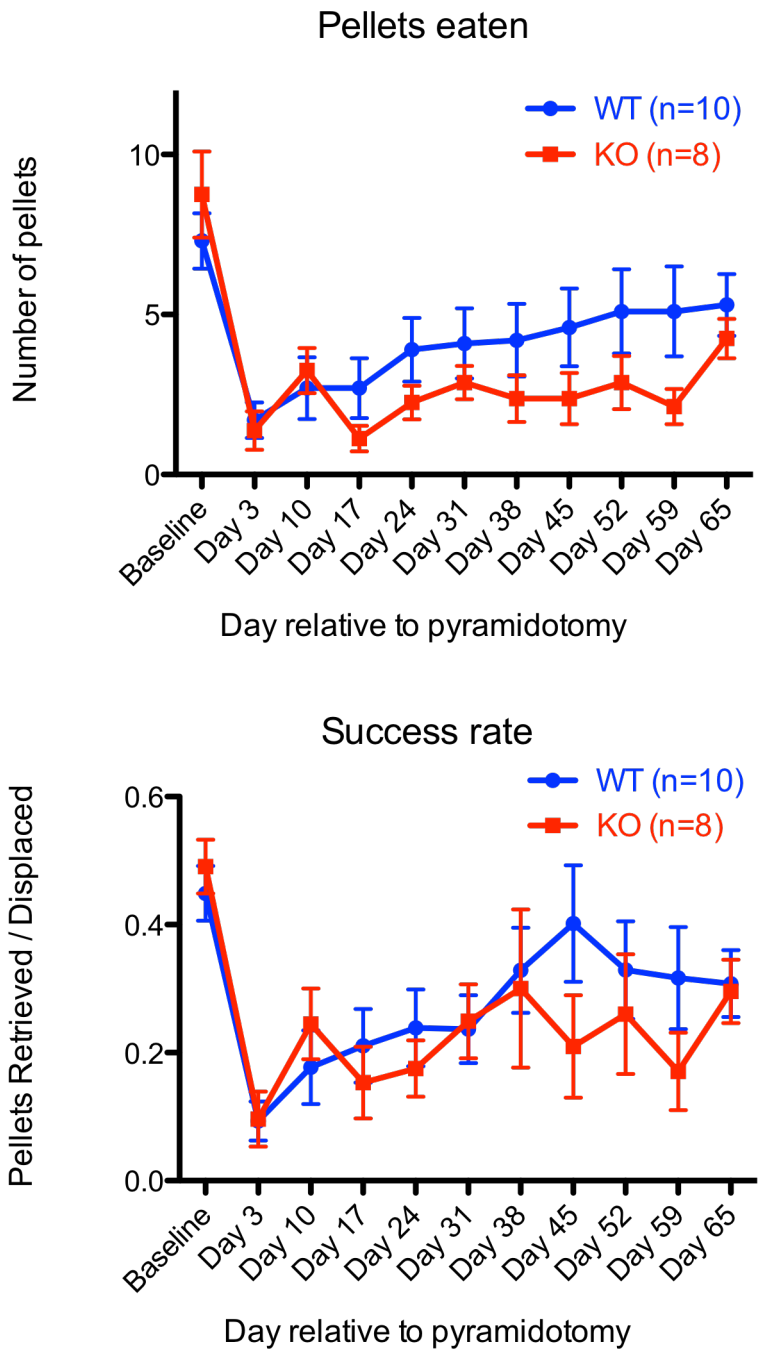


Figure 2.8 Deletion of NgR1 does not promote recovery in staircase reaching test. Top, number of pellets eaten by WT and NgR1 KO mice after pyramidotomy. Bottom, success rate of WT and NgR1 KO mice after pyramidotomy.

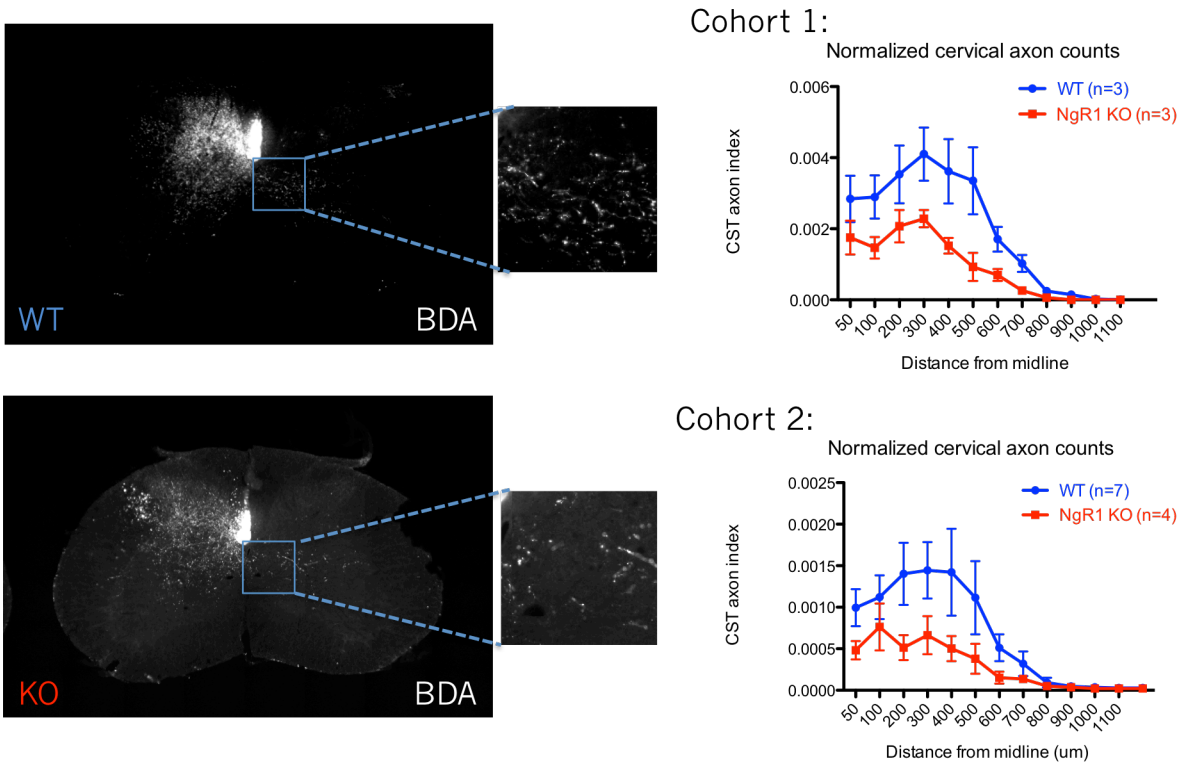


Figure 2.9 Deletion of NgR1 does not promote CST sprouting. Left, BDA-labeled CST axons in cervical sections from WT and NgR1 KO mice after unilateral pyramidotomy. Right, quantification of the number of BDA-labeled axons at specific distances from the midline in the denervated side of the spinal cord normalized to the total labeled CST axon count at the medulla in WT and NgR1 KO mice. Cohort 1, pyramidotomy at 2 months and sacrifice at 3 months. Cohort 2, pyramidotomy at 5 months and sacrifice at 8 months.

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Chapter 3. Oligodendrocytic but not neuronal Nogo restricts corticospinal axon sprouting after central nervous system injury

3.1 Abstract

Recovery from injury to the central nervous system (CNS) is limited in the mammalian adult. Nonetheless, some degree of spontaneous recovery occurs after partial CNS injury. Compensatory axonal growth from uninjured neurons, here termed sprouting, contributes to this naturally occurring recovery process and can be enhanced by molecular intervention. Extensive studies employing pharmacological and genetic manipulations have depicted a long-held hypothesis that oligodendrocyte-derived Nogo restricts axonal sprouting and functional recovery after CNS injury. However, the cell type-specific role of Nogo in oligodendrocytes or neurons in compensatory sprouting or functional recovery from CNS injury has not been reported. Here I show that inducible and cell type-specific deletion of Nogo from oligodendrocytes leads to a ~50% increase in the compensatory sprouting of corticospinal tract (CST) axons after unilateral pyramidotomy in mice of either sex, whereas deleting neuronal Nogo does not significantly affect sprouting. While sprouting axons express the synaptic marker VGLUT1, I did not detect any functional improvement in fine motor control associated with the observed increase in sprouting. These data show for the first time that Nogo specifically expressed by oligodendrocytes restricts compensatory sprouting after CNS injury, supporting a longstanding but heretofore untested hypothesis.

3.2 Significance Statement

Nogo has long been proposed to inhibit axonal growth after CNS injury through its expression in oligodendrocytes. However, its cell type-specific function has not been tested in the context of spinal axon repair or compensatory axon sprouting from

uninjured neurons, an important form of axonal growth that contributes to functional recovery. Using inducible and cell type-specific gene deletion, I provide the first evidence for a role of Nogo in oligodendrocytes but not neurons in inhibiting compensatory axon sprouting following CNS injury. My findings demonstrate the potential of targeting oligodendrocytic Nogo to promote compensatory axon sprouting, highlight the challenge of promoting functional recovery, and do not support a previously reported growth-promoting role for neuronal Nogo.

3.3 Introduction

Injury to the CNS often results in permanent functional impairment largely due to the limited ability of axons to grow after injury. There are two forms of injury-induced axonal growth: regeneration, axonal growth from injured neurons; and sprouting, compensatory axonal growth from uninjured neurons (Fig. 3.1) (Lee and Zheng, 2008; Tuszynski and Steward, 2012; Geoffroy and Zheng, 2014). In comparison to the very limited axon regeneration in the CNS, sprouting occurs spontaneously to some extent and is thought to contribute to partial recovery after brain or spinal cord injury (Fouad et al., 2001; Weidner et al., 2001; Bradbury and McMahon, 2006; Rosenzweig et al., 2010; Ueno et al., 2012). Harnessing and enhancing this innate form of structural plasticity in the CNS represents an important strategy in promoting repair and recovery after injury.

CNS myelin is considered inhibitory to axon growth due to the presence of a variety of factors expressed by oligodendrocytes (Geoffroy and Zheng, 2014; Schwab and Strittmatter, 2014). Nogo is one such protein. This membrane-bound protein binds to receptors expressed on the surface of neurons including NgR1 and PirB (Fournier et

al., 2001; Yiu and He, 2006; Atwal et al., 2008). The Nogo gene encodes three major isoforms, Nogo-A,B,C, through alternative splicing and alternative promoter usage (Chen et al., 2000; GrandPre et al., 2000). Two domains of Nogo-A, Nogo-66 and NogoΔ20, are inhibitory to neurite outgrowth in vitro and destabilize the cytoskeleton via Rho-A signaling (Fournier et al., 2001; Niederost et al., 2002; Oertle et al., 2003; Joset et al., 2010). All three Nogo isoforms contain Nogo-66, the ligand for NgR1 and PirB, while only Nogo-A contains NogoΔ20.

Function-blocking antibodies and peptides against Nogo-A increase axon growth and functional recovery in rodent and non-human primate models of spinal cord injury (Gonzenbach and Schwab, 2008; Zorner and Schwab, 2010). While pharmacological studies implicated enhanced axon regeneration as the underlying mechanism, genetic deletion of Nogo or its receptors NgR1 and PirB failed to demonstrate substantial and consistent regeneration of the corticospinal tract (CST), a commonly used model system in studying axonal repair after spinal cord injury (Kim et al., 2003; Simonen et al., 2003; Zheng et al., 2003; Kim et al., 2004; Song et al., 2004; Zheng et al., 2005; Steward et al., 2007; Ji et al., 2008; Lee et al., 2009; Nakamura et al., 2011). Nonetheless, Nogo deletion consistently resulted in increased compensatory axon sprouting after partial injury (Cafferty and Strittmatter, 2006; Lee et al., 2010).

In addition to oligodendrocytes, Nogo is also expressed by neurons. Neuronal expression of Nogo is most widespread during development, but persists in highly plastic regions of the adult CNS such as the hippocampus and cortex (Huber et al., 2002). Unexpectedly, silencing or deleting Nogo-A in retinal ganglion cells reduces axon regeneration and the expression of growth-related molecules after optic nerve crush,

while Nogo-A overexpression promotes regeneration, implicating a growth-promoting role for neuronal Nogo-A (Pernet et al., 2012; Vajda et al., 2015). This suggests the intriguing possibility that neuronal Nogo and oligodendrocytic Nogo have opposing roles, which has not been tested in the context of compensatory sprouting or spinal axon repair.

Genetic studies on Nogo in compensatory sprouting and in spinal axon repair have exclusively relied on germline Nogo deletion, leaving open the possibility that developmental compensation has limited the effect of Nogo deletion on anatomical or functional recovery from injury. Using inducible cell type-specific deletion of Nogo, here I formally test the hypothesis that Nogo expressed by oligodendrocytes limits CST axon sprouting and functional recovery from injury, while neuronal Nogo promotes CST axon sprouting. I found that oligodendrocytic-specific deletion of Nogo promoted CST sprouting but not functional recovery, while neuronal Nogo deletion had no significant effect on CST sprouting. These results provide the first evidence that oligodendrocytic but not neuronal Nogo inhibits compensatory axonal sprouting following CNS injury, and illustrate that other strategies are required to harness the functional benefits of enhanced sprouting.

3.4 Materials and Methods

Generation of Nogo conditional mice

A conditional allele of Nogo was generated in which the first two exons common to all 3 major Nogo isoforms (Nogo-A,B,C) were flanked by loxP sites (“floxed”). A targeting vector containing the floxed exons preceded by a floxed Neomycin selection

cassette (thus with a total of three loxP sites) was targeted to the endogenous Nogo locus by homologous recombination in AB2.2 mouse embryonic stem cells to generate the targeted allele, which was then transmitted through the germline. Targeted events were initially identified by Southern blot analysis. The expected loxP sites in the targeted allele were verified by PCR and sequencing analysis. The targeted mutant was bred to a universal deleter strain Actin-Cre (Lewandoski et al., 1997) to excise the Neomycin cassette via Cre-mediated partial excision, generating mice harboring the conditional allele of Nogo (Fig. 3.2*A*) as determined by PCR genotyping. Nogo conditional mutant mice were backcrossed to C57BL/6 at least 10 generations. To generate inducible oligodendrocytic-specific Nogo gene knockout mice, mice homozygous for the Nogo conditional allele ($Nogo^{f/f}$) were bred to $PLPCreER^{T}$ mice (Doerflinger et al., 2003) and backcrossed to generate $Nogo^{f/f}$ and $Nogo^{f/f}$;$PLPCreER^{T}$ breeders that were used to generate littermate $Nogo^{f/f}$ control and $Nogo^{f/f}$;$PLPCreER^{T}$ mice for sprouting studies. To generate conditional Nogo mice with gene knockout from CST neurons, conditional Nogo mice were bred to a tdTomato reporter line to generate littermate $Nogo^{+/f}$ control and $Nogo^{f/f}$ mice all heterozygous for the inducible tdTomato reporter gene (Madisen et al., 2010).

Tamoxifen administration

Animals were administered 2 mg of tamoxifen (VWR) dissolved in sunflower oil or sunflower oil vehicle via IP injection daily for 5 days beginning at 5 weeks of age for all studies. For sprouting and behavior studies, both $Nogo^{f/f}$;$PLPCreER^{T}$ mice and $Nogo^{f/f}$ (Cre-less) controls were administered tamoxifen.

Western blot

Spinal cord protein was extracted by homogenization in 20 mM Tris-HCl (pH 7.5), 50 mM NaCl, 0.5% TritonX-100, 0.25% DOC, 1% SDS, 5 mM NaF, 1 mM EDTA (pH 8.0), 1 mM PMSF, and Complete Mini (Roche). After sitting on ice for 30 min, tissue homogenate was centrifuged at 11,000g for 30 min at 4°C. The protein concentration of the supernatant was determined using the Bradford assay (Bio-Rad) and 30 µg of protein in NuPAGE LDS sample buffer (Invitrogen) was separated with SDS-PAGE, and transferred to nitrocellulose membrane (Amersham). Membranes were washed in TBS-T 4x5 minutes, blocked with 5% milk for 1 hour at room temperature, and incubated overnight at 4°C with primary antibody. The next day, membranes were washed in TBS-T 4x5 minutes and incubated in appropriate HRP-conjugated secondary antibody (1:50,000, Peirce) while shaking for 1 hour at room temperature. After washing in TBS-T 4x5 minutes, membranes were incubated in Supersignal West Dura (Peirce) chemiluminescence solution for 5 min and then exposed to film (Genesee Scientific). For loading control detection, membrane portion containing actin was cut and detected as described above. Primary antibodies used were rabbit anti-Nogo-A at 1:500 (Zheng et al., 2003) and mouse anti-actin at 1:1000 (Millipore).

Tissue processing

Tissue processing was performed as described previously with minor modifications (Lee et al., 2009; Lee et al., 2010). Mice were administered a lethal dose of Fatal plus and perfused transcardially with 4% PFA. Brain and spinal cord were dissected out, and the tissues were post-fixed overnight at 4°C in the same fixative

solution. Tissues were incubated in 30% sucrose for cryo-protection. Brain, medulla, and C1-C7 cervical spinal cord were embedded in OCT compound and frozen on dry ice. Tissues were sectioned with a cryostat at a thickness of 20 μm. For sprouting studies, transverse sections of the medullas were processed to obtain estimates of the total number of CST axons labeled to control for labeling efficiency (see below). For tissues containing BDA-labeled axons, cervical spinal cord and medulla sections were incubated in Vectastain ABC solution (Vector Laboratories) overnight at 4°C, washed in PBS, and mounted on gelatin-coated slides. BDA was detected with TSA Plus Fluorescein System (10 min, room temperature, 1:200, PerkinElmer). For tissues containing tdTomato-labeled axons, cervical spinal cord and medulla sections were processed without immunostaining for tdTomato. For immunohistochemistry, the following antibodies were used: purified polyclonal rabbit anti-Nogo-A (1:500) as described previously (Zheng et al., 2003), mouse anti-APC clone CC-1 (1:200, Millipore), rabbit anti-VGLUT1 (1:500, Abcam), biotinylated mouse anti-NeuN (1:100, Millipore), and rat anti-GFAP (1:200, Invitrogen). For sprouting studies, selected transverse sections of cervical spinal cord (C7) were immunostained for PKCγ (1:100, Santa Cruz Biotechnology) to examine the completeness of the lesion for each animal, as described previously (Lee et al., 2010). Mice with incomplete lesion were excluded from the study. A total of 1 animal was excluded based on this criteria of the genotype $Nogo^{f/f}$;$PLPCreER^{T}$.

Immunofluorescence quantifications

To quantify the intensity of Nogo-A immunofluorescence in white and gray matter

of cervical spinal cord, the mean intensity of the entire white and gray matter regions of transverse spinal cord sections were measured in AxioVision and averaged across five sections per animal. Mean intensity of the white and gray matter of germline Nogo knockout tissue was subtracted as the background for white and gray matter measurements. To quantify the number of APC^+ cells that express Nogo-A, the total number of APC^+ cells and the number of $APC^+Nogo\text{-}A^+$ cells were counted in a 335 μm x 448 μm sample region of cervical gray matter and of cervical white matter by an observer blinded to genotype. To quantify the percent of BDA^+ axons expressing VGLUT1, the total number of BDA^+ axons and the number of $BDA^+VGLUT1^+$ axons were counted in a 168 μm x 224 μm sample region of cervical spinal cord gray matter with percent averaged between 3 sections per animal by an observer blinded to genotype. To calculate the percent of $tdTomato^+$ cells expressing NeuN, APC and GFAP and the percent of each cell type expressing tdTomato, the following were counted in a 335 μm x 448 μm sample cortical region near the injection site (where overall tdTomato expression rate was roughly 75% of $DAPI^+$ cells): the total number of cells expressing tdTomato, NeuN, APC, and GFAP, and the total number of cells expressing both tdTomato and each cell type marker. Percent calculations from 3 regions were averaged for each animal.

Surgical procedures

Pyramidotomy and biotinylated dextran amine (BDA) tracer injection were performed as described previously with minor modifications (Starkey et al., 2005; Lee et al., 2010; Liu et al., 2010; Geoffroy et al., 2015). Surgeons performing the surgeries

were blinded to genotype. All animals were anesthetized with 2.5% Avertin (Sigma) and incision wounds were closed using Vetbond (3M). Pyramidotomy was performed at 2.5 months of age for anatomy studies (neuronal Nogo-A deletion, oligodendrocytic Nogo-A deletion without injury) and at approximately 4 months of age for behavior studies after completion of behavioral pre-training and baseline performance assessment. An incision was made overlying the trachea and the pyramidal tracts were accessed at the base of the skull (Starkey et al., 2005). A Feather micro scalpel was used to lesion the entire left pyramidal tract just caudal to the foramen magnum. Sham surgeries consisted of accessing the pyramidal tract in the same manner without lesioning the pyramidal tract. Cortical injection of BDA (10%, Invitrogen) was performed 2 weeks prior to sacrifice for all studies using the same injection volumes and coordinates as for AAV-injection (see Viral production and injection). Animals were sacrificed at approximately 4 months of age for anatomy studies and at approximately 8 months of age for behavior studies.

Viral production and injection

AAV-Cre was produced at the Salk Institute Viral Vector Core as described previously (Liu et al., 2010; Geoffroy et al., 2015). Viral concentration titer was determined to be 0.5 x 10^{12} TU/ml via qPCR. Virus was delivered via a modified 10 µL Hamilton syringe attached to a fine glass pipette mounted on a stereotaxic device for cortical injection. 5 week old animals were anesthetized with 2.5% Avertin (Sigma) and a total of 1.2 µL of AAV-Cre was injected into the sensorimotor cortex at 3 sites (0.4 µL per site). The right sensorimotor cortex targeting the left forelimb was injected 0.7 mm

below the cortical surface at the following coordinates relative to bregma: 0.5 mm anterior, 1.2 mm lateral; 0.3 mm posterior, 1.2 mm lateral; and 0.1 mm anterior, 2.2 mm lateral.

Quantification of sprouting index and percent midline crossing axons

To determine the sprouting index and percent midline crossing axons, transverse spinal cord sections were taken from C5-C7 cervical levels and imaged for BDA and tdTomato (see Tissue Processing). Lines were drawn parallel to the midline dorsoventral axis at 50 and 100 microns from the midline, then every 100 microns laterally in the denervated gray matter. The number of axons crossing each line was manually counted in five randomly chosen sections per animal by a blinded observer. Counts were averaged for each animal and normalized against the total labeled CST axon count in medulla (Lee et al., 2010) to obtain the sprouting index and percent midline crossing axons, which was plotted as a function of distance from the midline.

Behavioral assessment

All behavioral tests were performed and quantified by observers blinded to genotype. For both staircase reaching and pasta handling tests, mice were deprived of food at approximately 5pm and tested at approximately 9am the following morning resulting in approximately 16 hours food deprivation. Animals were weighed weekly to monitor for excessive weight loss (greater than 10%). For staircase reaching, mice were pre-trained and tested as described previously with slight modifications (Baird et al., 2001; Starkey et al., 2005; Kloth et al., 2006). For habituation prior to pre-training,

animals were exposed to dyed sucrose pellets (Fisher Scientific Dustless Precision Pellets 14 mg, dyed with AmeriColor Student Soft Gel Paste Food Color) in their home cages and habituated to the testing environment, experimenter, and staircase chambers (Campden Instruments) for approximately 10 minutes per day for 3 days. Staircase chambers were modified to prevent build up of displaced pellets on lower steps by removing a plastic barrier at the base of the staircase and by adding a plastic barrier between the staircase chamber and the main chamber. During pre-training, animals were placed in sucrose-pellet baited chambers for 10 minutes once daily for 4-5 days per week until performance plateau, for a total of approximately 25 sessions. For all pre-training and testing sessions, staircases were baited with 4 pellets on each step other than the top two steps to prevent usage of the tongue to retrieve pellets. Initial pre-training sessions were baited with additional pellets on the top steps and the central platform to encourage exploration and pellet retrieval. Throughout pre-training and testing, performance was monitored to ensure pellets were retrieved with forepaws and not tongues. After pre-training, animals were tested once every 7 days on day 3, 10, 17, etc. after pyramidotomy. Test sessions were 10 minutes, after which the number and location of remaining pellets was recorded to calculate pellets eaten (successful retrievals) and pellets eaten relative to pellets displaced (success rate).

For pasta handling, mice were tested as described previously (Tennant et al., 2010). Briefly, mice were video-recorded while eating a 2.5 cm piece of dry capellini pasta and the number of adjustments made with each paw was quantified by watching frame-by-frame video playback. Adjustments consisted of paw release and re-contact with the pasta or any movement of the paw or digits while in contact with the pasta as

described previously (Tennant et al., 2010). Mice were habituated to the testing setup for 2 sessions prior to testing. Pasta handling testing occurred 10 weeks after pyramidotomy after the conclusion of staircase testing.

Experimental design and statistical analysis

Western blot data were analyzed via one-way ANOVA. Sprouting index, percent midline crossing axons, staircase reaching, pasta handling and Nogo-A immunofluorescence data were analyzed via two way repeated measures ANOVA with Bonferroni or Fisher post-tests as indicated. VGLUT1 expression data was analyzed via unpaired t-test. For all statistical analyses, GraphPad Prism 7 was used with $p < 0.05$ set as the threshold for statistically significance, denoted by asterisks in graphs. All data is displayed as means with error bars denoting SEM. Specific n values for each study are listed in figure legends. For analysis of sprouting index and percent midline crossing axons, five cervical sections and two medulla sections were quantified for each animal (see Quantification of sprouting index and percent midline crossing axons for specific details*).* A mix of male and female mice in an approximately 1:1 ratio was used for all experiments.

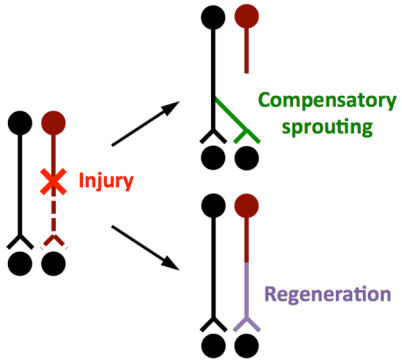

Figure 3.1 Compensatory axon sprouting is a CNS repair mechanism distinct from axon regeneration. There are two forms of injury-induced axonal growth: regeneration, axonal growth from injured neurons; and sprouting, compensatory axonal growth from uninjured neurons. After a partial CNS injury that severs axons from a subset of neurons (as exemplified by the neuron in dark red), uninjured neurons (black) may exhibit spontaneous compensatory axon sprouting into denervated regions (green), a process distinct from axon regeneration from injured neurons (purple).

3.5 Results

Establishing inducible, oligodendrocyte-specific Nogo gene knockout mice

To investigate the effects of deleting Nogo from specific cell types after development is complete, my mentor Binhai Zheng designed and generated a conditional allele of Nogo using the Cre-*loxP* site-specific recombination system (Fig. 3.2*A*). The Nogo gene encodes three major isoforms, Nogo-A, B and C, through alternative splicing and alternative promoter usage (Chen et al., 2000; GrandPre et al., 2000). Previous studies indicate that targeting one isoform, Nogo-A, can cause unwanted, substantial upregulation of another isoform, Nogo-B (Simonen et al., 2003; Vajda et al., 2015). The targeting strategy therefore was designed similarly to what was previously used to disrupt all three isoforms simultaneously (Zheng et al., 2003).

Specifically, the first two common exons for all three major Nogo isoforms were flanked by *loxP* sites. Cre-mediated excision of these two exons would lead to a frame shift for all downstream exons and was predicted to result in a null for Nogo-C and at least a severe hypomorph for Nogo-A and Nogo-B (see Methods for more details on gene targeting). Importantly, this strategy would eliminate the expression of the growth inhibitory Nogo-66 amino acid loop shared by all three Nogo isoforms (GrandPre et al., 2000), and targeting a similar region of Nogo was previously shown to result in a null or severe hypomorph for all three major Nogo isoforms (Zheng et al., 2003). Mice harboring this allele were crossed to the PLPCreER^{T} mouse line in which an inducible Cre transgene is expressed under the myelin proteolipid protein (PLP) promoter, which is highly expressed in mature oligodendrocytes (Doerflinger et al., 2003). To assess the extent of Nogo deletion using this approach, Nogo$^{f/f}$;PLPCreER^{T} mice and Nogo$^{f/f}$ (Cre-

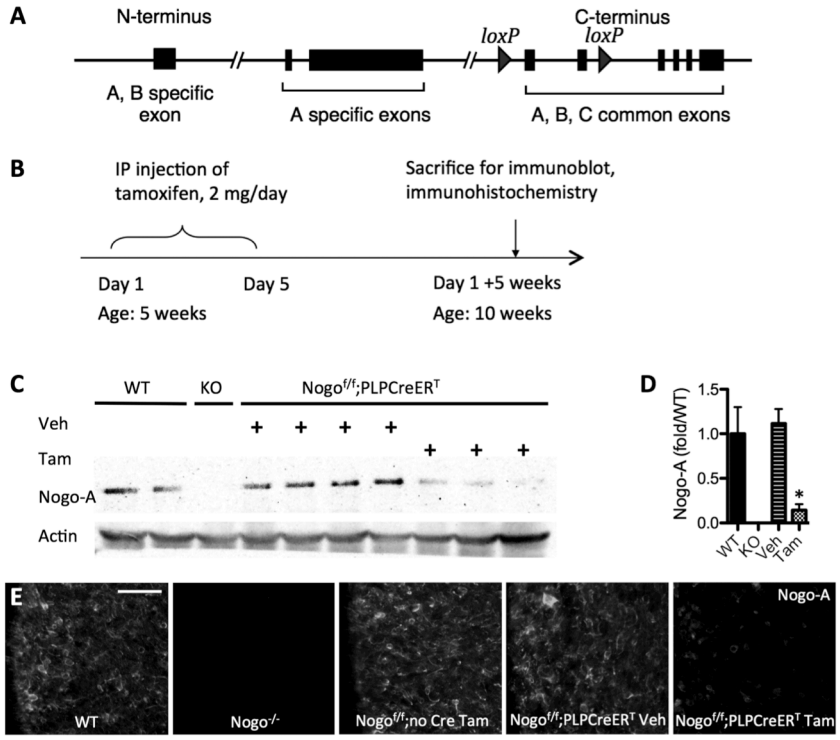

Figure 3.2 Generation and characterization of Nogo$^{f/f}$;PLPCreER^{T} mice. (A) Conditional Nogo allele in which two exons common to all Nogo isoforms is flanked by loxP sites. (B) Timeline of procedures for expression characterization. (C) Immunoblot against Nogo-A in spinal cord of WT, germline KO, control mice, and inducible KO mice. (D) Quantification of immunoblot in (C); *p<0.05 compared to WT and to Veh via ANOVA. Error bar, SEM. (E) Immunostain against Nogo-A in spinal cord white matter of WT, germline KO, control mice, and inducible KO mice. Scale bar = 50 μm.

less) controls were administered 2 mg of tamoxifen or sunflower oil vehicle via IP injection daily for 5 days beginning at 5 weeks of age. Five weeks later, mice were sacrificed for immunoblot and immunohistochemical analyses (Fig. 3.2*B*). Nogo-A protein levels in whole spinal cord of vehicle-treated Nogo$^{f/f}$;PLPCreER^{T} mice remained similar to wild-type (WT) levels, while tamoxifen administration led to an ~87% reduction in Nogo-A protein levels relative to vehicle-treated Nogo$^{f/f}$;PLPCreER^{T} mice [$F(2,6) = 9.951$, $p = 0.0124$, ANOVA, WT vs. TAM and Veh vs. TAM $p < 0.05$] (Fig. 3.2*C,D*). The incomplete loss of total Nogo-A signal is likely due to the presence of Nogo-A in other cell types including neurons and also possibly incomplete removal of Nogo-A from oligodendrocytes or myelin (see below). Likewise, immunostaining against Nogo-A in cervical spinal cord revealed reduction of Nogo-A levels in Nogo$^{f/f}$;PLPCreER^{T} mice treated with tamoxifen but not in vehicle-treated Nogo$^{f/f}$;PLPCreER^{T} mice or tamoxifen-treated Nogo$^{f/f}$ (Cre-less) control mice (Fig. 3.2*E*).

Inducible Nogo deletion from oligodendrocytes promotes corticospinal tract axon sprouting

To provide a rigorous genetic test on the long-held hypothesis that oligodendrocytic Nogo inhibits axonal sprouting, I assessed the effect of deleting all three Nogo isoforms from PLP-expressing cells in young adult mice on corticospinal tract (CST) axon sprouting after unilateral pyramidotomy. In the unilateral pyramidotomy injury model, one side of the CST is lesioned at the level of the medullary pyramids, and compensatory sprouting of CST axons into the contralateral, denervated side of the cervical spinal cord is assessed (Fig. 3.3*A*). After the same induction method described

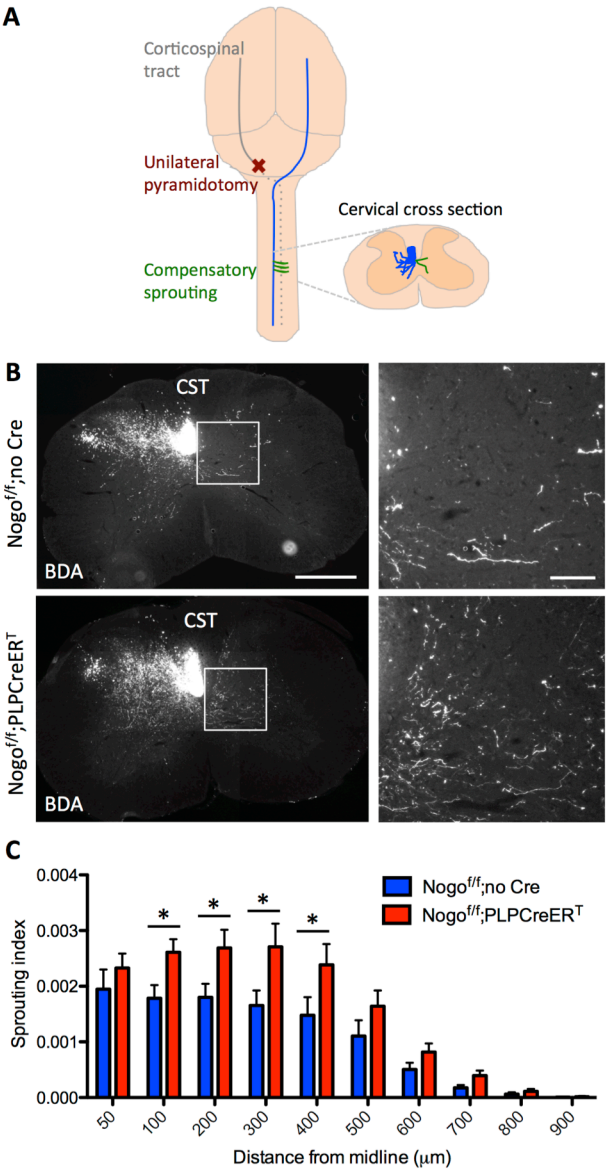

Figure 3.3 Inducible deletion of Nogo from oligodendrocytes promotes compensatory sprouting of corticospinal tract axons. (A) Drawing of unilateral pyramidotomy model. (B) BDA-labeled CST axons in cervical sections from Nogo$^{f/f}$;no Cre and Nogo$^{f/f}$;PLPCreER^{T} mice after unilateral pyramidotomy. White box depicts enlarged region shown in right panels. Scale bars = 500 μm (low mag, left), 100 μm (high mag, right). (C) Quantification of the number of BDA-labeled axons at specific distances from the midline in the denervated side of the spinal cord normalized to the total labeled CST axon count at the medulla in Nogo$^{f/f}$;no Cre (n=8) and Nogo$^{f/f}$;PLPCreER^{T} (n=12) mice. *$p < 0.05$, two-way RM ANOVA followed by Fisher's least significant difference test. Error bar, SEM.

previously in which mice were administered tamoxifen beginning at 5 weeks of age (Fig. 3.2*B*), unilateral pyramidotomy was performed at approximately 4 months of age and animals were sacrificed two weeks after cortical injection of axon tracer biotinylated dextran amine (BDA) at approximately 8 months of age. This protracted timeline was necessary to allow time for behavioral pre-training and assessment. Axon tracer was visualized in the cervical spinal cord of Nogo$^{f/f}$ control and Nogo$^{f/f}$;PLPCreER^{T} mice by ABC and TSA amplification of BDA (Fig. 3.3*B*).

Axon sprouting indices were quantified by counting the numbers of axons crossing predetermined distances from the midline in the contralateral cervical cord, normalized against the total CST axon count in the medulla as previously described (Fig. 3.3*C*) (Lee et al., 2010; Geoffroy et al., 2015). Nogo$^{f/f}$;PLPCreER^{T} mice exhibited an increase in the number of axons crossing defined distances from the midline relative to tamoxifen-treated Nogo$^{f/f}$ littermate control mice not expressing Cre [$F(9,162) = 2.11$, $p = 0.0313$, two-way repeated measures (RM) ANOVA] (Fig. 3.3*C*). This effect was largest at 300 μm from the midline, where normalized axon counts were increased by ~64% [$F(9,162) = 2.11$, $p = 0.0313$ for two-way RM ANOVA; Fisher's least significant difference post-test $p = 0.0251$ at 100 μm, 0.0163 at 200 μm, 0.0046 at 300 μm, and 0.0144 at 400 μm].

To confirm efficient deletion of Nogo-A from oligodendrocytes in these animals, cervical sections were doubly immunostained for Nogo-A and APC, a mature oligodendrocyte marker (Fig. 3.4*A*). Total Nogo-A expression was significantly reduced in both white and gray matter [$F(1,4)=49.69$, $p = 0.0021$ for two-way RM ANOVA; Bonferroni's post-test, $p < 0.0001$ for white matter and $p = 0.0123$ for gray matter] (Fig.

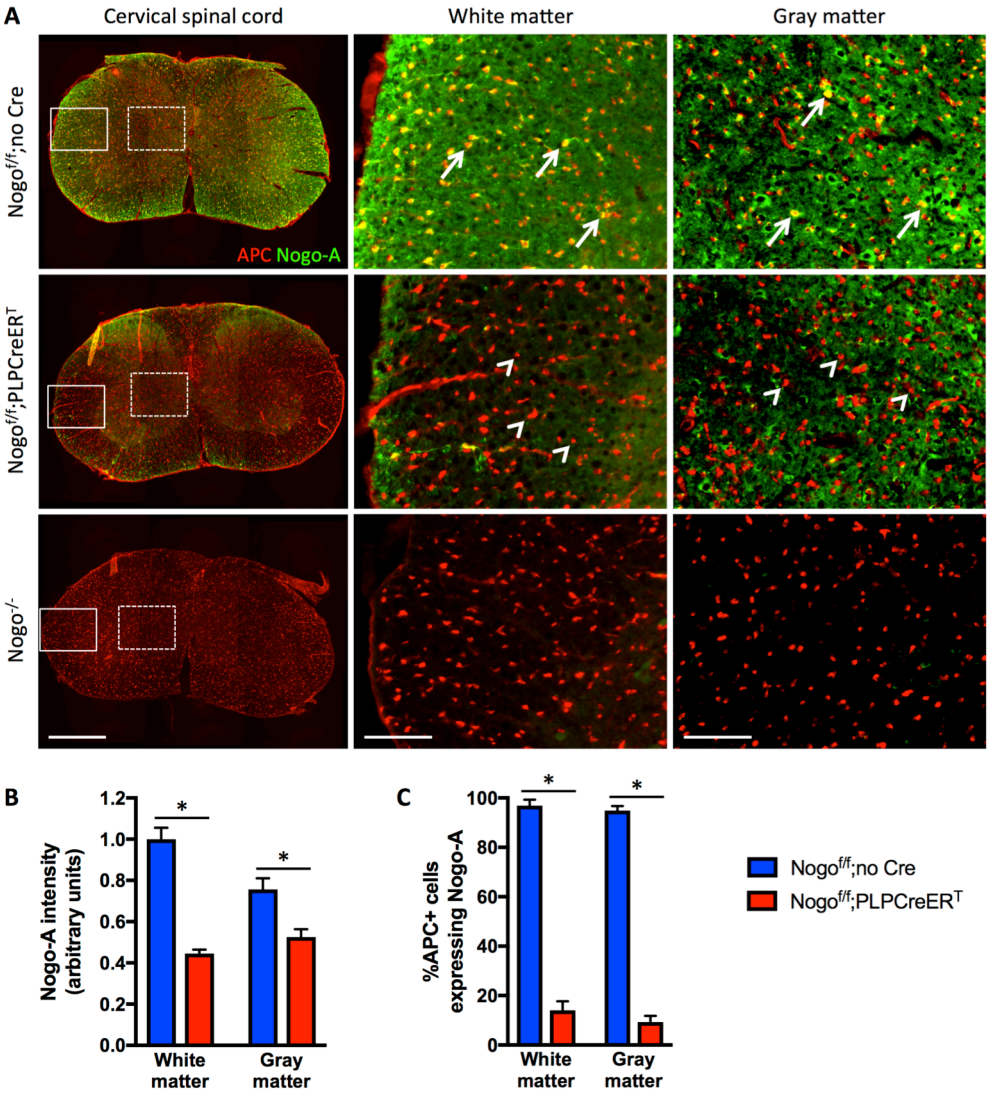

Figure 3.4 Validation of Nogo deletion from experimental animals. (A) Double immunostain of APC and Nogo-A in experimental animals from Figure 3 and germline Nogo KO (Nogo$^{-/-}$) tissue. White boxes depict regions shown in the middle panels; white dashed boxes depict regions show in the right panels. Arrows indicate APC^{+}Nogo-A^{+} cells; arrowheads indicate APC^{+} cells that are negative for Nogo-A. Note that, in the gray matter, neuronal Nogo-A is expected to remain in the oligodendrocyte-specific Nogo knockout (tamoxifen treated Nogo$^{f/f}$;PLPCreER^{T}) mice. Scale bars = 500 µm (low mag, left panels), 100 µm (high mag, middle and right panels). (B,C) Quantification of background-subtracted Nogo-A immunofluorescence (B) and percent of APC^{+} cells expressing Nogo-A (C) in Nogo$^{f/f}$;no Cre (n=3) and Nogo$^{f/f}$;PLPCreER^{T} (n=3) mice. *$p < 0.05$, two-way RM ANOVA with Bonferroni post-tests. Error bar, SEM.

3.4*B*). Since neuronal Nogo-A was expected to remain in Nogo$^{f/f}$;PLPCreER^{T} mice, the reduction of Nogo-A immunoreactivity was more noticeable in the white matter than in the gray matter (Fig. 3.4*A,B*). Furthermore, Nogo-A was below detectable levels in ~91% of gray matter APC^{+} cells in tamoxifen-treated Nogo$^{f/f}$;PLPCreER^{T} mice, confirming efficient Nogo deletion in oligodendrocytes even in the gray matter [$F(1,4)=700.8$, $p < 0.0001$ for two-way RM ANOVA; Bonferroni's post-test, $p < 0.0001$ for both white and gray matter] (Fig. 3.4*C*).

Importantly, sham lesioned Nogo$^{f/f}$;PLPCreER^{T} and tamoxifen-treated Nogo$^{f/f}$ littermate controls in which the medullary pyramids were surgically accessed but not lesioned exhibited no difference in midline crossing axons [$F(9,36) = 0.11$, $p = 0.9994$, two-way RM ANOVA] (Fig. 3.5*A,B*). Together, these data indicate that inducible, oligodendrocyte-specific Nogo deletion enhances CST sprouting across the midline in the cervical spinal cord after unilateral pyramidotomy.

To assess the potential for sprouted axons to form synapses, I examined the expression of the pre-synaptic marker VGLUT1 in conjunction with BDA tracing of CST axons in Nogo$^{f/f}$ control and Nogo$^{f/f}$;PLPCreER^{T} mice (Fig. 3.6). In both control Nogo$^{f/f}$ and Nogo$^{f/f}$;PLPCreER^{T} mice, VGLUT1 staining is evident along BDA-labeled axons, consistent with the formation of synaptic structures by sprouting axons (Fig. 3.6*A,B,C*). A substantial proportion of BDA-labeled axons expressed VGLUT1 in both genotypes, with Nogo$^{f/f}$;PLPCreER^{T} mice exhibiting a trend for a higher percentage of co-labeling than control Nogo$^{f/f}$ mice that did not reach statistical significance [~79% and ~67% respectively, $t(4) = 2.553$, $p = 0.0631$, unpaired t-test] (Fig. 3.6*D*).

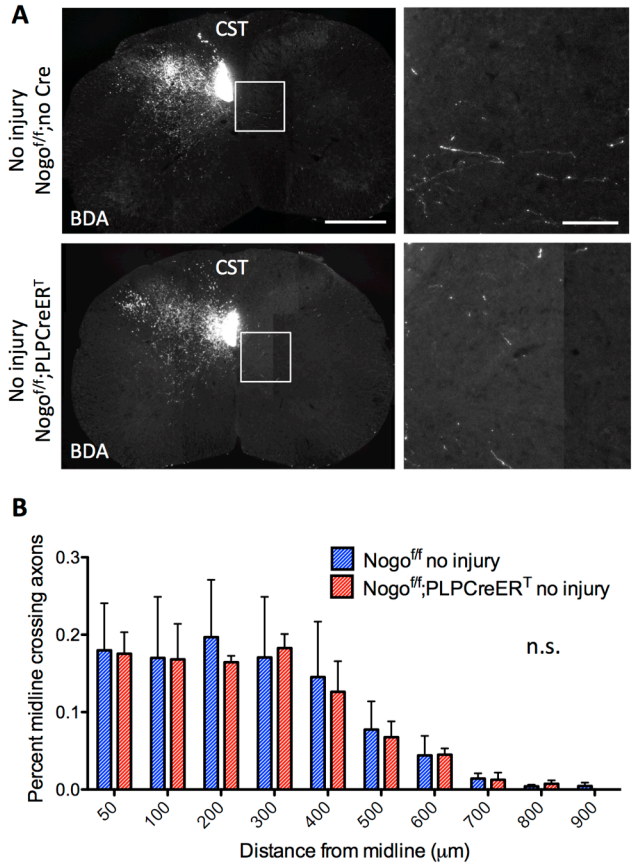

Figure 3.5 Inducible deletion of Nogo from oligodendrocytes does not affect axon counts in the absence of injury. (A) BDA-labeled CST axons in cervical sections from Nogo$^{f/f}$;no Cre and Nogo$^{f/f}$;PLPCreER^{T} mice without injury. White boxes depict enlarged regions shown in the right panels. Scale bars = 500 μm (low mag, left), 100 μm (high mag, right). (B) Quantification of the number of BDA-labeled axons at specific distances from the midline contralateral to the main labeled CST normalized to the total labeled CST axon count at the medulla in Nogo$^{f/f}$;no Cre (n=3) and Nogo$^{f/f}$;PLPCreER^{T} (n=3) mice. n.s, not significant by two-way RM ANOVA. Error bar, SEM.

Inducible Nogo deletion from oligodendrocytes does not promote functional recovery after injury

To determine whether the increase in CST sprouting was associated with improved functional recovery from injury, I tested Nogo$^{f/f}$ control and Nogo$^{f/f}$;PLPCreER^{T} mice in the staircase reaching test and the pasta handling test, two assays of fine motor control (Fig. 3.7*A,D*) (Baird et al., 2001; Tennant et al., 2010). Both groups of mice showed similar pre-injury and initial post-injury performance in staircase reaching (Fig. 3.7*B,C*). Furthermore, while this assay was able to detect significant functional impairment in both groups, Nogo$^{f/f}$ control mice showed significant recovery by 6 weeks post-injury whereas Nogo$^{f/f}$;PLPCreER^{T} mice did not (Fig. 3.7*B,C*). [Successful retrievals: two-way repeated measures ANOVA with Bonferroni post-tests on week 6 versus -1 and 0, $F(2,52) = 38.12$, $p < 0.0001$, week 6 vs. -1 $p < 0.01$ for both groups, week 6 versus 0 $p < 0.05$ for Nogo$^{f/f}$ but not Nogo$^{f/f}$;PLPCreER^{T}. Success rate: two-way repeated measures ANOVA with Bonferroni post-tests on week 6 versus -1 and 0, $F(2,52) = 30.93$, $p < 0.0001$, week 6 vs. -1 $p < 0.05$ for both groups, week 6 versus 0 $p < 0.05$ for Nogo$^{f/f}$ but not Nogo$^{f/f}$;PLPCreER^{T}.] Similarly, the affected paw showed impairment in pasta handling 10 weeks after injury as evidenced by increased adjustments relative to the intact paw with no significant difference between groups [$F(1,7) = 12.57$, $p = 0.0094$, two-way repeated measures ANOVA with Bonferroni post-tests] (Fig. 3.7*E*).

Neuronal deletion of Nogo does not significantly affect sprouting

Previous studies indicate that in the optic nerve system, neuronal Nogo-A and

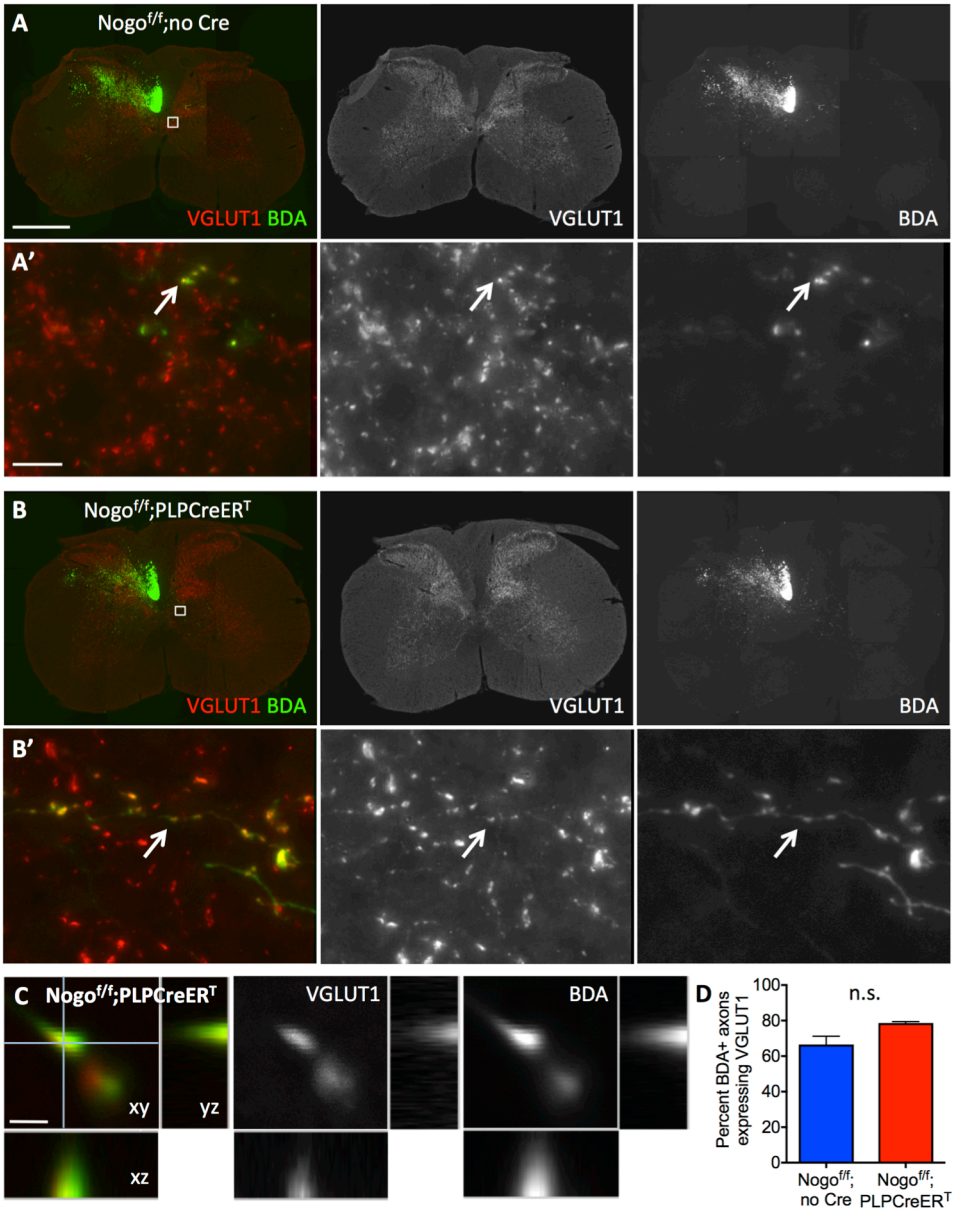

Figure 3.6 Sprouting axons form synapses. (A-B') VGLUT1 and BDA double labeling in cervical sections from Nogo$^{f/f}$;no Cre (A and A') and Nogo$^{f/f}$;PLPCreER^{T} mice (B and B') after unilateral pyramidotomy. (C) Confocal image showing colocalization of VGLUT1 and BDA. (D) Quantification of the percent of BDA^{+} axons expressing VGLUT1 in Nogo$^{f/f}$;no Cre (n=3) and Nogo$^{f/f}$;PLPCreER^{T} (n=3) mice. Small white boxes in (A) and (B) depict enlarged regions shown in (A') and (B') respectively. Arrows in (A') and (B') exemplify VGLUT1 and BDA co-labeled axons. Scale bars = 500 µm (A), 10 µm (A'), 1 µm (C). n.s, not significant by unpaired t-test.

oligodendrocytic Nogo-A have opposite roles in regulating axon growth following injury, which was proposed as a contributing factor to the less-than-expected effect of germline Nogo deletion on axonal repair after CNS injury (Pernet et al., 2012; Vajda et al., 2015). I therefore assessed whether deletion of Nogo from corticospinal neurons themselves affects sprouting. Nogo conditional mice were bred to a tdTomato reporter line to generate littermate $Nogo^{+/f}$ control and $Nogo^{f/f}$ mice both of which were also heterozygous for the inducible tdTomato reporter gene (Madisen et al., 2010). Upon AAV2-Cre injection in the motor cortex, cortical cells expressed tdTomato and concomitantly exhibited a substantial reduction of Nogo-A immunoreactivity in neurons identified through NeuN staining (~94% reduction in the percentage of $tdTomato^{+}NeuN^{+}$ cells that express Nogo-A) (Fig. 3.8*A,B*). Furthermore, based on tdTomato reporter expression near the injection sites, ~95% of AA2-Cre transduced cells were $NeuN^{+}$, ~0.3% were APC^{+}, and ~0.8% were $GFAP^{+}$; meanwhile, transduction efficiencies for cells expressing each cell type marker were found to be ~74% for NeuN, ~1.2% for APC, and ~8% for GFAP (Fig. 3.8*C,D*). TdTomato expression in cervical sections enabled tracing of CST axons only from neurons that had been infected with AAV2-Cre (Fig. 3.9*A*). There was no statistically significant difference in the degree of CST sprouting between control and neuronal Nogo-A conditional gene deletion mice (Fig. 3.9*B*) [$F(1,10) = 2.28$, $p = 0.1618$, two-way repeated measures ANOVA], indicating that neuronal Nogo is unlikely to be a significant factor, at least by itself, in regulating CST axon sprouting after injury.

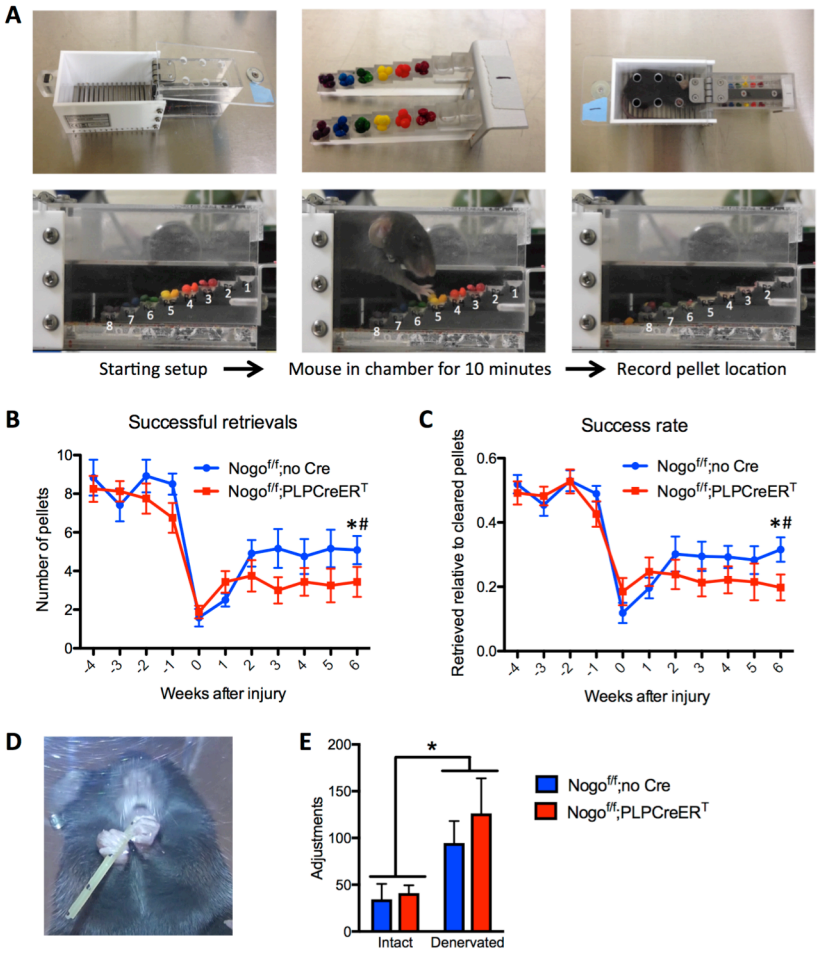

Figure 3.7 Inducible deletion of Nogo from oligodendrocytes does not promote functional recovery from injury. (A) Staircase reaching test in which the mouse is allowed to retrieve color-coded sucrose pellets from steps of varying heights over the course of 10 minutes. (B) Number of pellets retrieved using denervated paw during 10-minute sessions each week after injury. Week 6 performance is significantly reduced relative to baseline week -1 for both genotypes, whereas week 6 performance is significantly improved from week 0 for control Nogo$^{f/f}$;no Cre mice but not for Nogo$^{f/f}$;PLPCreER^{T} mice. (C) Success rate for denervated paw during 10-minute sessions each week after injury. Week 6 performance is significantly reduced relative to baseline week -1 for both genotypes, whereas week 6 performance is significantly improved from week 0 for Nogo$^{f/f}$;no Cre mice but not Nogo$^{f/f}$;PLPCreER^{T} mice. (D) Mouse performing pasta-handling test where the impaired paw exhibits more adjustments than the intact paw. (E) Number of adjustments made with intact vs. denervated paw during pasta-handling test 10 weeks after injury. For (B) and (C) two-way RM ANOVA with Bonferroni post-tests on week 6 versus -1 and 0, * $p < 0.05$ week 6 vs. 0 and 1 for Nogo$^{f/f}$;no Cre, # $p < 0.05$ week 6 vs. -1 for Nogo$^{f/f}$;PLPCreER^{T}, Nogo$^{f/f}$ n=12, Nogo$^{f/f}$;PLPCreER^{T} n=16. For (E) two-way RM ANOVA with Bonferroni post-tests $p = 0.0094$ for paw, Nogo$^{f/f}$;no Cre n=7, Nogo$^{f/f}$;PLPCreER^{T} n=5.

3.6 Discussion

In the present study I investigated the role of Nogo expressed by oligodendrocytes and by neurons in the compensatory sprouting of CST axons and functional recovery from CNS injury using inducible and cell type-specific gene deletion in mice. In doing so I tested a longstanding hypothesis that Nogo expressed by oligodendrocytes limits anatomical and functional recovery from injury. I found that Nogo expressed by oligodendrocytes limits CST sprouting, while neuronal Nogo does not significantly modulate CST sprouting. My results provide the first evidence with rigorous genetic analyses that oligodendrocytic Nogo restricts compensatory axon sprouting in the mammalian CNS after injury.

Oligodendrocytic Nogo inhibits compensatory sprouting of CST axons while neuronal Nogo has no effect

In this study, I focused exclusively on the compensatory sprouting of CST axons after a partial CNS injury. I found that deleting Nogo from oligodendrocytes using inducible $Nogo^{f/f}$;$PLPCreER^{T}$ mice resulted in a ~50% increase in the number of sprouting CST axons after pyramidotomy. This level of sprouting enhancement is comparable to — and at the upper limit of — that from previous studies from our laboratory indicating an up to ~50% increase in CST sprouting following the same pyramidotomy injury in germline Nogo knockout mice (Lee et al., 2010; Geoffroy et al., 2015). Together, these results indicate that oligodendrocytic Nogo is primarily responsible for its growth-inhibitory effect on CST sprouting. As a reference point, neuronal Pten deletion consistently leads to a ~2 fold or more increase in a similar CST

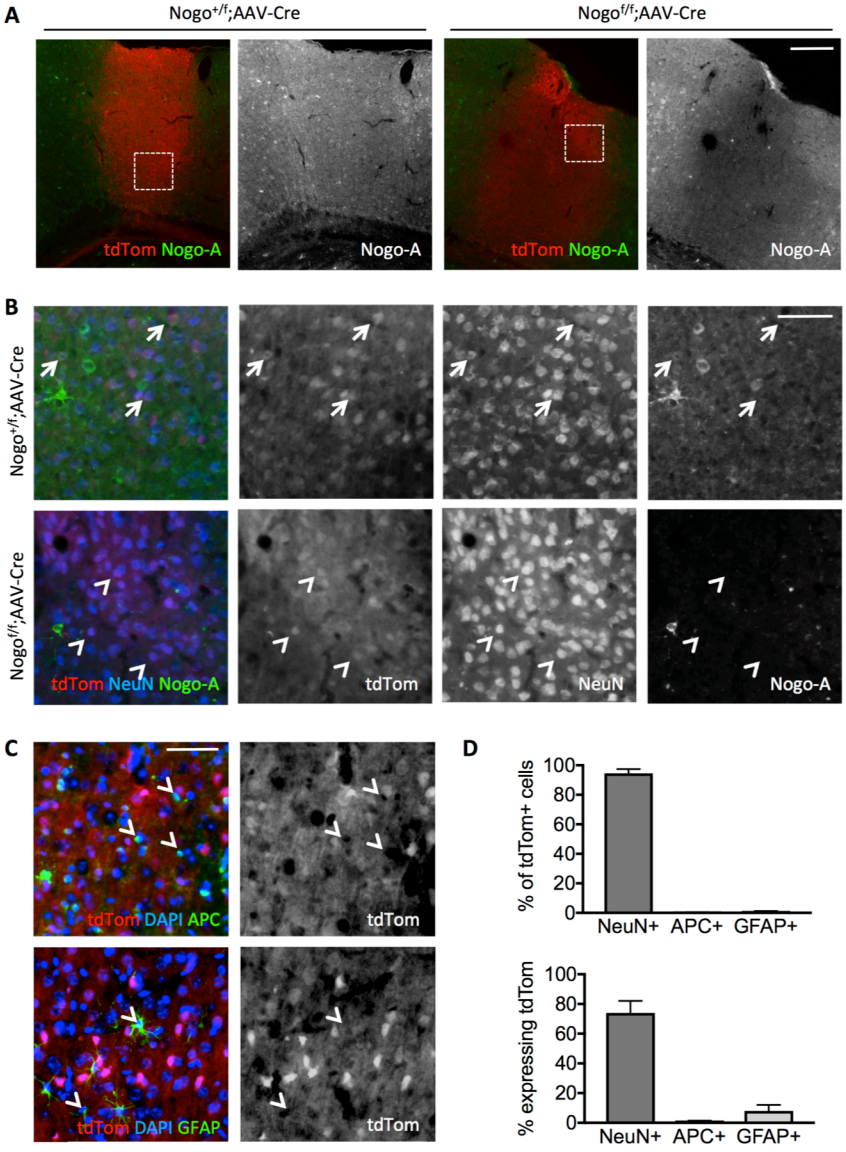

Figure 3.8 AAV2-Cre cortical injection induces tdTomato expression and deletes neuronal Nogo-A in Nogo$^{f/f}$;tdtom mice. (A) Coronal brain slices from Nogo$^{+/f}$;tdTomato ("Nogo$^{+/f}$") and Nogo$^{f/f}$;tdTomato ("Nogo$^{f/f}$") mice near cortical AAV2-Cre injection sites immunostained for Nogo-A showing induction of tdTomato reporter and deletion of Nogo-A. White boxes depict enlarged regions shown in (B). (B) High magnification images showing Nogo-A signal in tdTomato, NeuN positive cells in control Nogo$^{+/f}$ mice and loss of Nogo-A signal in Nogo$^{f/f}$ mice following AAV-Cre mediated recombination. Arrows indicate tdTomato^{+}NeuN^{+}Nogo-A^{+} cells; arrowheads indicate tdTomato^{+}NeuN^{+} cells that are negative for Nogo-A. (C) High magnification images showing relative lack of transduction of APC^{+} and GFAP^{+} cells by AAV2-Cre as evidenced by tdTomato reporter (white arrowheads). (D) Quantification of the percent of tdTomato^{+} cells expressing each of the cell type markers NeuN, APC, GFAP (top panel) and the percent of each cell type expressing tdTomato near cortical injection sites (bottom panel). Scale bars = 200 μm (A), 50 μm (B, C).

sprouting paradigm (Liu et al., 2010; Geoffroy et al., 2015). Indeed, Pten deletion led to a higher level of CST sprouting enhancement in a side-by-side comparison with germline Nogo deletion (Geoffroy et al., 2015). Thus, while I did not conduct a side-by-side comparison in the current study, it is apparent that oligodendrocytic Nogo deletion produces a more modest effect on CST sprouting than neuronal Pten deletion.

In previous studies, deleting or silencing Nogo-A in retinal ganglion cells reduced axon regeneration after optic nerve crush (Pernet et al., 2012; Vajda et al., 2015). In contrast, in the present study I did not detect a growth-reducing effect of neuronal Nogo deletion in CST sprouting (if anything, neuronal Nogo deletion may have slightly increased CST sprouting although the difference did not reach statistical significance). This was unlikely due to a floor effect of CST sprouting after pyramidotomy injury since my lab was previously able to detect a consistent level of reduction in CST sprouting in MAG knockout mice with the same injury model (Lee et al., 2010). The apparent discrepancy in the effect of neuronal Nogo deletion between our study and Vajda et al. (2015) could reflect the distinct roles of Nogo in different neuron subtypes (e.g., corticospinal neurons versus retinal ganglion cells) or in different forms of axon growth after injury (sprouting versus regeneration). On the one hand, optic nerve injury leads to substantial retinal ganglion cell death (Li et al., 1999) whereas experimental spinal cord injury does not cause significant corticospinal neuron death (Nielson et al., 2010; Nielson et al., 2011). On the other hand, our current study focused exclusively on the compensatory axon sprouting from uninjured neurons using an injury model (unilateral pyramidotomy) that is specifically designed to evaluate CST sprouting; in contrast, Vajda et al. (2015) focused on axon regeneration (albeit short distance

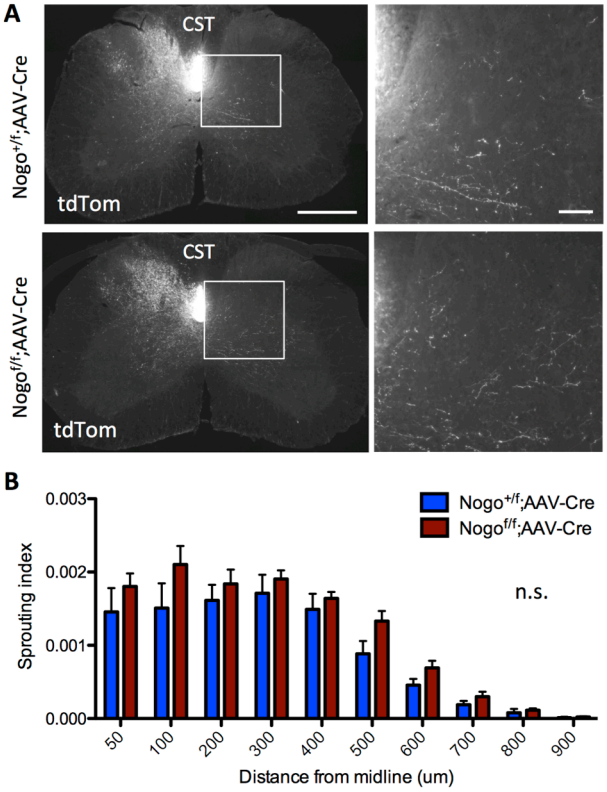

Figure 3.9 Deletion of Nogo from cortical neurons via AAV2-Cre injection does not significantly affect CST sprouting. (A) tdTomato-labeled CST axons in cervical sections from Nogo$^{+/f}$;tdTomato control and Nogo$^{f/f}$;tdTomato mice after cortical injection of AAV2-Cre and unilateral pyramidotomy. White boxes depict enlarged regions shown on the right. Scale bars = 500 μm (low mag, left), 100 μm (high mag, right). (B) Quantification of the number of tdTomato-positive axons at specific distances from the midline in the denervated side of the spinal cord normalized to the total labeled CST axon count in the medulla in Nogo$^{+/f}$ control (n=5) and Nogo$^{f/f}$ (n=7) mice. n.s, not significant by two-way RM ANOVA.

regeneration) from injured retinal ganglion neurons based on the commonly accepted definitions of regeneration and sprouting (Fig. 3.1) (Lee and Zheng, 2008; Tuszynski and Steward, 2012), even though they referred to this form of regeneration as “regenerative sprouting” (Vajda et al., 2015). Importantly, the strategy used by Vajda et al. (2015) to conditionally delete Nogo-A undesirably led to substantial Nogo-B upregulation, confounding the interpretation of their data regardless of the focus on regeneration or sprouting (Vajda et al., 2015). In comparison, our gene targeting strategy allowed us to cleanly disrupt all 3 Nogo isoforms (Nogo-A,B,C) in the current study, leaving no ambiguity in our conclusions on the cell type-specific function of Nogo.

Lack of functional benefit of deleting Nogo from oligodendrocytes

I was not able to detect a functional benefit resulting from deletion of oligodendrocytic Nogo in two independent measures of forelimb function. Similarly, germline deletion of Nogo does not improve various measures of recovery from pyramidotomy including forepaw preference during rearing, ladder rung missteps, or tape removal assays, but has been shown to increase affected paw usage in food pellet retrieval (Cafferty and Strittmatter, 2006; Lee et al., 2010; Geoffroy et al., 2015). Nonetheless, a large body of literature supports that pharmacologically or functionally blocking Nogo-A in rats and non-human primates promotes functional recovery from CNS injury including pyramidotomy, an effect that appears to be distinct from that of oligodendrocytic deletion of Nogo I evaluated here (Thallmair et al., 1998; Z'Graggen et al., 1998; Schwab and Strittmatter, 2014). In fact, in the present study control mice showed some degree of spontaneous recovery from injury, whereas oligodendrocytic

Nogo knockout mice did not. Nonetheless, sprouted axons on the denervated side in both groups expressed the pre-synaptic marker VGLUT1, suggesting an ability to form functional synapses, consistent with previous reports of VGLUT1 expression along pyramidotomy-induced sprouting axons (Maier et al., 2008; Starkey et al., 2012).

Thus, promotion of axon growth alone may not be sufficient to promote functional recovery from injury. Indeed, in certain cases increased sprouting even correlates with worse functional recovery from injury (Wang et al., 2015). Rehabilitative training could be an important factor in harnessing the enhanced sprouting for functional improvement. In the case of Nogo-A manipulations, the timing of rehabilitative training might be an additional important consideration, as differently timed rehabilitative training may either enhance or worsen functional recovery after CNS injury (Wahl et al., 2014; Chen et al., 2017). My results indicate that removal of oligodendrocytic Nogo is sufficient to promote anatomical recovery from CNS injury, but alone may not be sufficient to promote functional recovery. Whether rehabilitative training will unmask a functional benefit of deleting oligodendrocytic Nogo remains to be tested.

Concluding remarks

Here I showed that inducible, cell type-specific Nogo deletion in oligodendrocytes enhances compensatory sprouting of CST axons after a partial CNS injury. This study represents the first rigorous genetic test demonstrating that oligodendrocytic Nogo inhibits compensatory sprouting and that oligodendrocytic Nogo inhibits axonal repair in the spinal cord. The results have important implications for ongoing clinical trials testing anti-Nogo antibodies, emphasizing the potential for enhanced sprouting while

highlighting the challenge in harnessing this structural plasticity for functional recovery.

3.7 Appendix: Towards characterization of Nogo receptor homolog NgR2-deletion mice

In order to further characterize the role of myelin-associated inhibitors of axon growth in sprouting, our lab sought to evaluate CST sprouting in mice with deletion of sequence homologues of NgR1 including NgR2 and NgR3. I specifically studied CST trajectory and sprouting in NgR2 mutant mice. While sharing sequence homology with the Nogo receptor NgR1, NgR2 itself does not bind Nogo but binds with high affinity another classically described myelin-associated inhibitor of axon growth MAG (Myelin-Associated Glycoprotein) (Venkatesh et al., 2005). In a previous study, our lab made the surprising finding that that MAG deletion *decreases* CST sprouting, suggesting that MAG and perhaps myelin inhibitors in general have a dual role in modulating axonal sprouting after injury (Lee et al., 2010). Thus I hypothesized that deletion of NgR2, a receptor for MAG, would also reduce CST sprouting.

Initial experiments in NgR2 deletion mice were carried out in a “dirty” null line in which exon 2 of the NgR2 gene was deleted but a beta-galactosidase — neomycin selection cassette remained (Figure 3.10A). Surprisingly, mice homozygous for this “dirty” null allele showed no CST axons in the dorsal columns of the cervical spinal cord as evidenced by PKCγ staining, with heterozygous mice showing nearly no remaining $PKC\gamma^{+}$ CST axons (Figure 3.10B). Upon breeding out the selection cassette to generate “clean” null mice, this phenotype was lost but remained in littermates heterozygous for

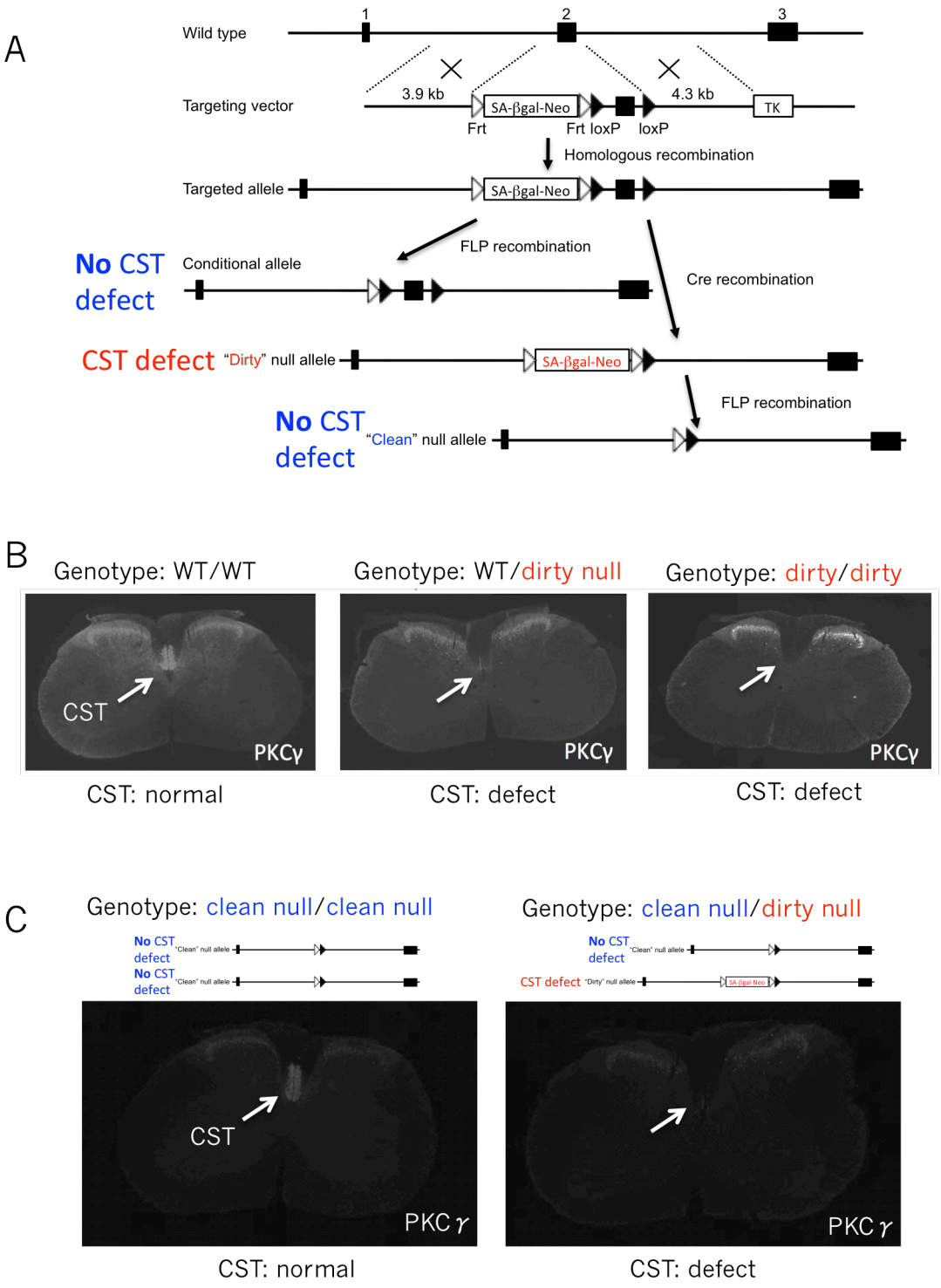

Figure 3.10 CST defect in mice with "dirty" null allele. (A) NgR2 mouse line design. (B) CST defect is present in mice heterozygous and homozygous for the "dirty" null allele. (C) CST is normal in mice homozygous for the "clean" null allele (selection cassette removed) (left), while the CST defect is present in mice with one "clean" null allele and one "dirty" null allele (right).

the “dirty” null allele (with one “clean” null allele and one “dirty”), suggesting a gain of function of the “dirty” null allele (Figure 3.10C).

Characterization of the “dirty” null mice beyond PKCγ staining of the cervical spinal cord revealed a range of brain defects including shrunken cerebral peduncles and absent medullary pyramids (Figure 3.11A), enlarged ventricles and abnormal hippocampi (Figure 3.11B), and an apparent targeting defect of the CST as evidenced by BDA tracing (Figure 3.11C). BDA-labeled CST axons were present at the level of the pons but no longer formed a tract in the trapezoid body or in the region of the medullary pyramids.

We hypothesized that the molecular cause of the phenotype could be due to a gain of function of the remaining non-targeted exon 3, but reverse transcription PCR demonstrated no expression of exon 3 in “dirty” null mice (Figure 3.11D). The molecular cause of this phenotype only present in animals with the selection cassette remains unknown.

Upon generating “clean” NgR2 null mice, preliminary CST sprouting studies were conducted. Among the limited number of animals tested, I did not observe a decrease in CST sprouting in NgR2 null mice (if anything, there could be a trend toward increased CST sprouting, but more studies are needed to be conclusive) (Figure 3.12). Results from this small cohort do not provide evidence for our hypothesis that NgR2 serves as a neuronal receptor to facilitate sprouting. Whether NgR2 deletion *increases* CST sprouting remains inconclusive and requires follow-up investigation.

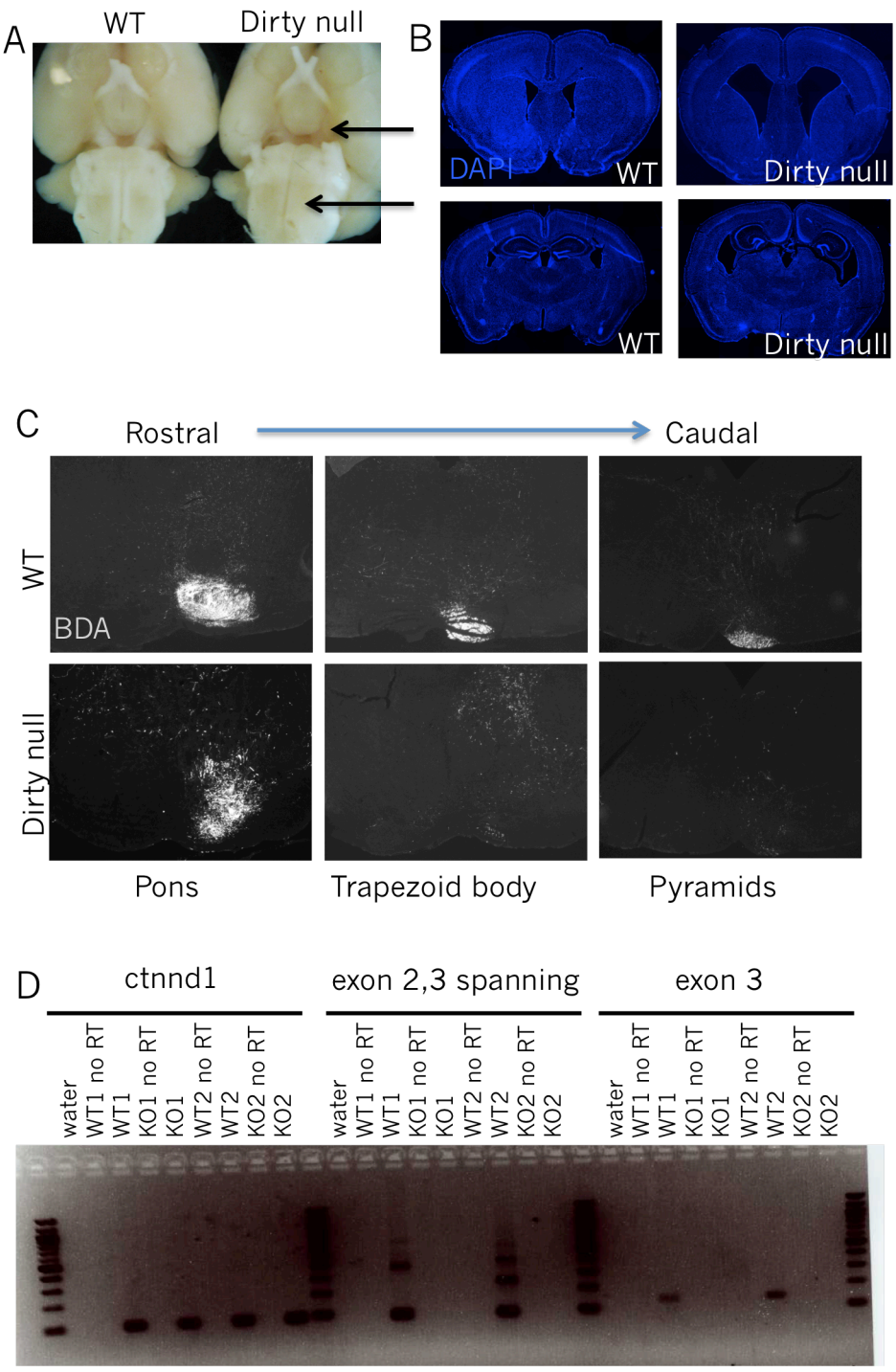

Figure 3.11 Further characterization of mice with "dirty" null allele. (A) Shrunken cerebral peduncles and absent medullary pyramids (black arrows) in "dirty" null homozygous mice. (B) Enlarged ventricles and abnormal hippocampi in "dirty" null homozygous mice. (C) BDA labeling of sensorimotor neurons reveals CST defect evident at the level of the pons through the medullary pyramids. (D) Reverse transcription PCR revelas "dirty" null mice ("KO") do not express exon 3. Ctnnd1, nearby gene serving as a positive control; "no RT" = negative control without reverse transcriptase.

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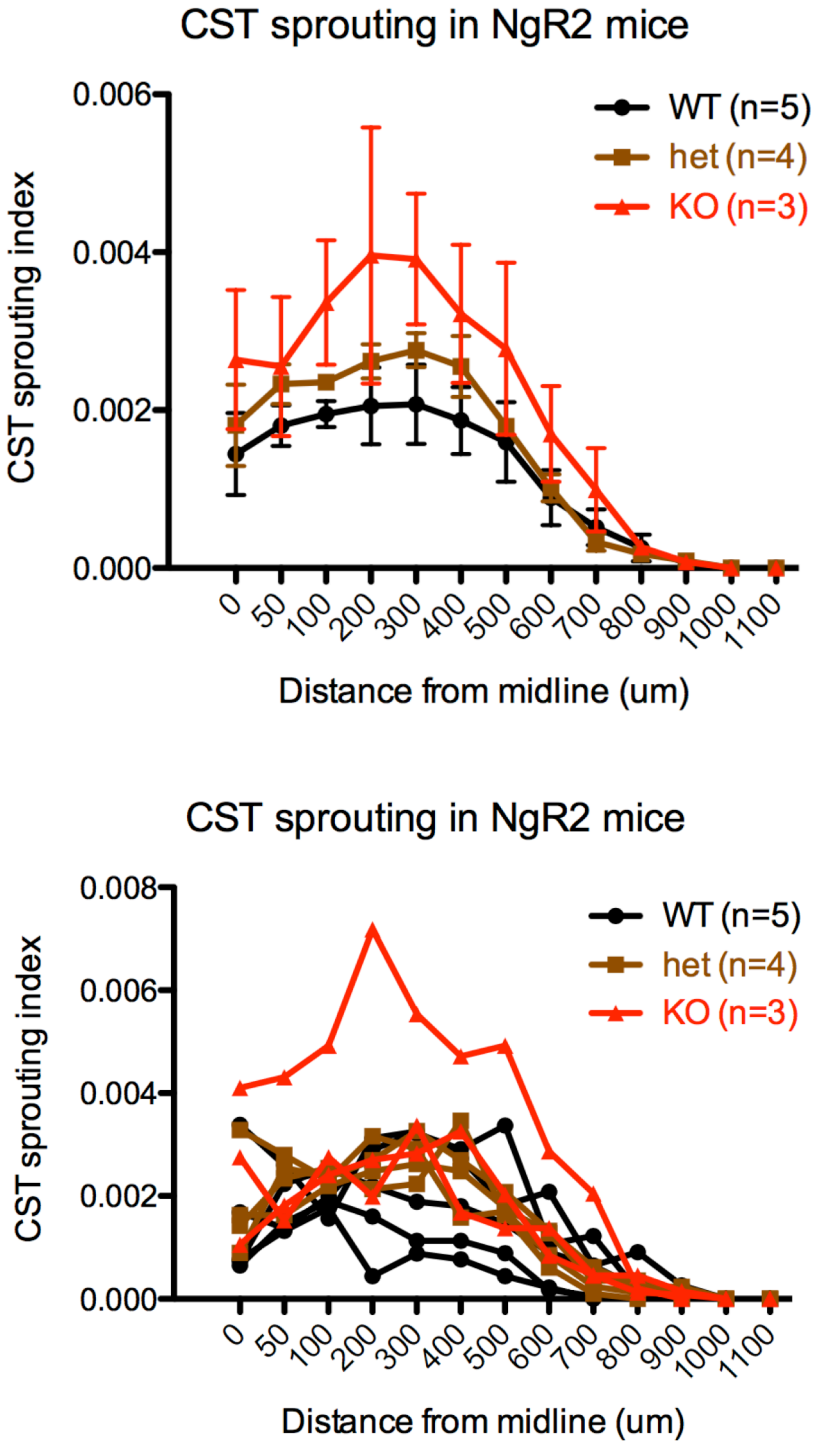

Figure 3.12 Preliminary CST sprouting data in NgR2 KO mice. Top panel, mean +/- SEM. Bottom panel, data for individual mice.

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Chapter 4. Role of mammalian dual leucine zipper-bearing kinase DLK in corticospinal axon sprouting

4.1 Abstract

Recovery from injury to the central nervous system (CNS) is limited in the mammalian adult, resulting in permanent functional impairments after CNS damage such as from traumatic injury or stroke. This is largely due to insufficiency of two forms of axon growth after injury: regeneration of damaged axons and compensatory sprouting of uninjured axons. Significant regeneration does not occur spontaneously after CNS injury in the mammalian adult. Some degree of compensatory sprouting occurs after CNS injury and contributes to the partial but incomplete spontaneous functional recovery that often occurs after less-severe CNS injury. These two forms of axon growth have both shared and distinct molecular regulation, the details of which have yet to be fully elucidated. The Dual Leucine zipper-bearing Kinase (DLK), a Mitogen-activated Protein Kinase Kinase Kinase (MAPKKK or MAP3K), was initially discovered for its critical role in axon degeneration and regeneration after injury in invertebrate model organisms *C. elegans* and Drosophila. Recent studies of one of its mammalian homologs, DLK, have revealed its role in neuronal death, degeneration and regeneration following peripheral nervous or optic nerve injury. However, the role of this molecule in compensatory sprouting or in spinal axon growth after CNS injury has not been described. Here I test the hypothesis that DLK promotes corticospinal tract (CST) axon sprouting after unilateral pyramidotomy. Using a combination of gain and loss of function analyses, results from ongoing studies suggest that DLK may in fact play a growth-reducing role in CST sprouting. Interestingly, the same reduction in CST sprouting is seen even in an enhanced growth state induced by PTEN deletion, strengthening the surprising possibility that DLK may negatively regulate CST sprouting.

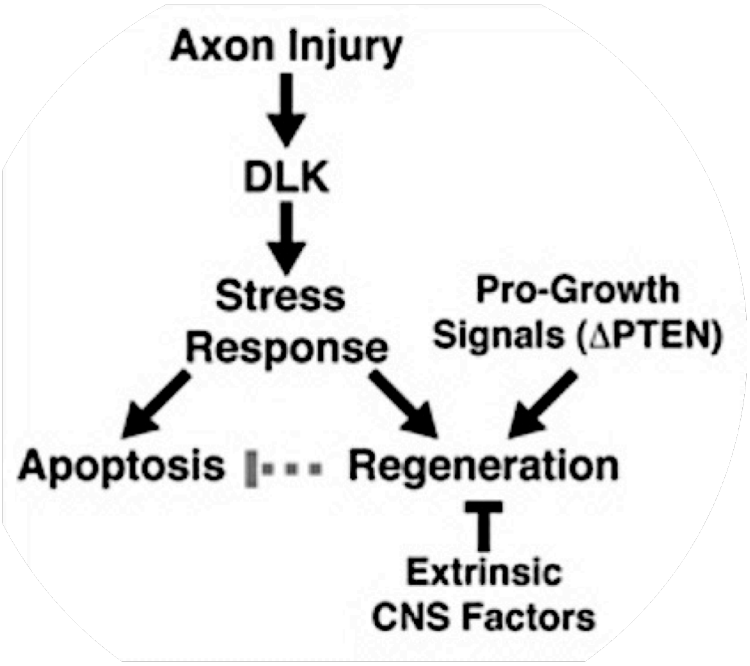


Figure 4.1 DLK is an upstream regulator of cellular responses to injury including apoptosis and regeneration. Stress response activation via DLK signaling induced by axon injury can result in apoptosis, axon regeneration, and axon degeneration (not shown). Gray dashed lines, proposed mechanism whereby DLK promotes either apoptosis or regeneration depending on the growth state of the cell, which can be influenced by deletion of the tumor suppressor gene PTEN. (Watkins et al., 2013)

4.2 Introduction

The Dual Leucine zipper-bearing Kinase-1 (DLK-1) was first identified as a regulator of presynaptic development in *C. elegans* by Dr. Yishi Jin (Nakata et al., 2005). DLK is a mitogen-activated protein kinase kinase kinase (MAPKKK or MAP3K), part of a mitogen-activated protein kinase (MAPK) signaling pathway. MAPK signaling regulates gene expression, cellular proliferation, apoptosis, and responses to external stimuli including stress, and as such may be involved in early decisions on cellular responses to injury (Figure 4.1) (Chang and Karin, 2001; Tedeschi and Bradke, 2013). After delineating the role of DLK in presynaptic development, Dr. Jin and others identified DLK as necessary for axon regeneration in multiple neuron types in *C. elegans* (Hammarlund et al., 2009; Yan et al., 2009). Furthermore, DLK overexpression promotes regeneration in *C. elegans* (Hammarlund et al., 2009; Yan et al., 2009). The Drosophila homologue of DLK, Wallenda, is also required for axon regeneration, likely as a regulator of an injury-signaling cascade (Xiong et al., 2010).

With regards to the mammalian system, there are two close sequence homologues of invertebrate DLK: DLK (also know as MAP3K12) and Leucine Zipper-bearing Kinase (LZK, also know as MAP3K13). DLK has been show to be important for peripheral regeneration of dorsal root ganglion (DRG) axons and particularly important for the pre-conditioning lesion effect where a prior injury stimulates regeneration following a second injury (Itoh et al., 2009; Shin et al., 2012). Furthermore, DLK is required for the robust axon regeneration of the optic nerve that results from PTEN deletion (Watkins et al., 2013). The mechanism underlying activation of mammalian DLK in the context of injury remains unclear but likely involves axonal cytoskeletal

destabilization (Valakh et al., 2015). With regards to the function of DLK in axon outgrowth from mammalian CNS neurons, silencing or deleting DLK reduces neurite length of primary cultured cortical neurons (Eto et al., 2010; Hirai et al., 2011). Furthermore, my lab has found that overexpression of either LZK or DLK increases maximum and total neurite lengths of cultured postnatal cerebellar granular neurons, although there is no synergistic effect of overexpressing both kinases (Chen et al., 2016). Conversely, genetically deleting DLK using a conditional mutant mouse line leads to reduced neurite growth from cerebellar granule neurons. Furthermore, DLK-deficient postnatal hippocampal neurons also exhibit reduced neurite growth in culture. Thus, manipulating DLK appears to have a widespread effect on neurite growth from different CNS neurons in culture. However, whether DLK regulates compensatory sprouting of uninjured axons after CNS injury remains unexplored.

Here I test the hypothesis that DLK positively regulates corticospinal tract (CST) axon sprouting after CNS injury by investigating the effect of deletion and overexpression of DLK in CST neurons in the unilateral pyramidotomy injury model. Contrary to our hypothesis, I found that DLK is dispensable for CST sprouting, and further that overexpression of DLK unexpectedly reduces CST sprouting. Strikingly, overexpression of DLK reduced CST sprouting even in PTEN deletion mice. My results suggest that DLK negatively regulates CST sprouting, even when neurons are primed toward a pro-growth state.

4.3 Materials and Methods

Experimental animals

DLK conditional knockout (KO) mice were generated in which exon 2 of the DLK gene is flanked by loxP sites (Figure 4.2). To test efficiency of inducible gene deletion, mice were bred to a ubiquitously expressed inducible Cre-recombinase mouse line ("Ub-Cre") and administered tamoxifen, resulting in near complete deletion of DLK expression (Ruzankina et al., 2007). For DLK cKO pyramidotomy studies, "WT" denotes DLK mice homozygous for the conditional allele ("DLK f/f") with cortical injection of AAV2-GFP control virus, while "cKO" denotes littermate DLK f/f mice with cortical injection of AAV2-Cre to delete DLK from the motor cortex. DLK overexpressing (OE) mice were generated in collaboration with Dr. Yishi Jin's lab through Applied StemCell by targeted knock-in of a DLK and tdTomato expressing construct preceded by a LoxP-stop-LoxP sequence to the Hipp11 locus, in which Cre-recombinase activity is expected to result in DLK overexpression and tdTomato expression (Figure 4.3, top panel). Upon cortical injection of AAV2-Cre, tdTomato and DLK expression is evident near cortical injection sites (Figure 4.3, bottom panel). For DLK OE pyramidotomy studies, littermates with or without the knock-in allele were injected with AAV2-Cre to generate "WT" and "OE" mice. For DLK OE pyramidotomy studies in a PTEN KO background, all mice were littermate PTEN f/f mice with or without the DLK knock-in allele, injected with AAV2-Cre to generate "PTEN KO" and "PTEN KO;DLK OE" mice or AAV2-GFP control virus to generate "WT" mice.

Tissue processing

Tissue processing was performed as described previously with minor modifications (Lee et al., 2009; Lee et al., 2010). Mice were administered a lethal dose

of Fatal plus and perfused transcardially with 4% PFA. Brain and spinal cord were dissected out, and the tissues were post-fixed overnight at 4°C in the same fixative solution. Tissues were incubated in 30% sucrose for cryo-protection. Brain, medulla, and C1-C7 cervical spinal cord were embedded in OCT compound and frozen on dry ice. Tissues were sectioned with a cryostat at a thickness of 20 μm. For sprouting studies, transverse sections of the medullas were processed to obtain estimates of the total number of CST axons labeled to control for labeling efficiency (see below). For tissues containing BDA-labeled axons, cervical spinal cord and medulla sections were incubated in Vectastain ABC solution (Vector Laboratories) overnight at 4°C, washed in PBS, and mounted on gelatin-coated slides. BDA was detected with TSA Plus Fluorescein System (10 min, room temperature, 1:200, PerkinElmer). For immunohistochemistry, the following antibodies were used: DLK (1:200, made by the Holzman lab) (Holzman et al., 1994), NeuN (1:500, Millipore), GFAP (1:500, DAKO). Neurotrace 640/660 (1:200, 20 minute incubation, ThermoFisher) was used for fluorescent Nissl staining and Thionin (0.25%, Fisher) for brightfield Nissl staining follow standard procedures. For sprouting studies, selected transverse sections of cervical spinal cord (C7) were immunostained for PKCγ (1:100, Santa Cruz Biotechnology) to examine the completeness of the lesion for each animal, as described previously (Lee et al., 2010). Mice with incomplete lesion were excluded from the study.

Surgical procedures

Pyramidotomy and biotinylated dextran amine (BDA) tracer injection were performed as described previously with minor modifications (Starkey et al., 2005; Lee et

al., 2010; Liu et al., 2010; Geoffroy et al., 2015). Surgeons performing the surgeries were blinded to genotype. All animals were anesthetized with 2.5% Avertin (Sigma) and incision wounds were closed using Vetbond (3M). Pyramidotomy was performed at 7 weeks for all studies. An incision was made overlying the trachea and the pyramidal tracts were accessed at the base of the skull (Starkey et al., 2005). A Feather micro scalpel was used to lesion the entire left pyramidal tract just caudal to the foramen magnum. Cortical injection of BDA (10%, Invitrogen) was performed 2 weeks prior to sacrifice for all studies using the same injection volumes and coordinates as for AAV-injection (see Viral production and injection). Animals were sacrificed at 11 weeks for all studies.

Viral production and injection

AAV-Cre was produced at the Salk Institute Viral Vector Core as described previously (Liu et al., 2010; Geoffroy et al., 2015). Viral concentration titer was determined to be 0.5 x 10^{12} TU/ml via qPCR. Virus was delivered via a modified 10 µL Hamilton syringe attached to a fine glass pipette mounted on a stereotaxic device for cortical injection. 5 week old animals were anesthetized with 2.5% Avertin (Sigma) and a total of 1.2 µL of AAV-Cre was injected into the sensorimotor cortex at 3 sites (0.4 µL per site). The right sensorimotor cortex targeting the left forelimb was injected 0.7 mm below the cortical surface at the following coordinates relative to Bregma: 0.5 mm anterior, 1.2 mm lateral; 0.3 mm posterior, 1.2 mm lateral; and 0.1 mm anterior, 2.2 mm lateral.

Quantification of sprouting index

To determine the sprouting index, transverse spinal cord sections were taken from C5-C7 cervical levels and imaged for BDA (see Tissue Processing). Lines were drawn parallel to the midline dorsoventral axis at 50 and 100 microns from the midline, then every 100 microns laterally in the denervated gray matter. The number of axons crossing each line was manually counted in five randomly chosen sections per animal by a blinded observer. Counts were averaged for each animal and normalized against the total axon count in medulla (Lee et al., 2010) to obtain the sprouting index and percent midline crossing axons, which was plotted as a function of distance from the midline.

Experimental design and statistical analysis

Sprouting index data were analyzed via two way repeated measures ANOVA with Bonferroni post-tests. For all statistical analyses, GraphPad Prism 5 was used with $p < 0.05$ set as the threshold for statistically significance, denoted by asterisks in graphs. All data is displayed as means with error bars denoting SEM. Specific n values for each study are listed in figures. For analysis of sprouting index, five cervical sections and two medulla sections were quantified for each animal (see Quantification of sprouting index and percent midline crossing axons for specific details*).* A mix of male and female mice in an approximately 1:1 ratio were used for all experiments.

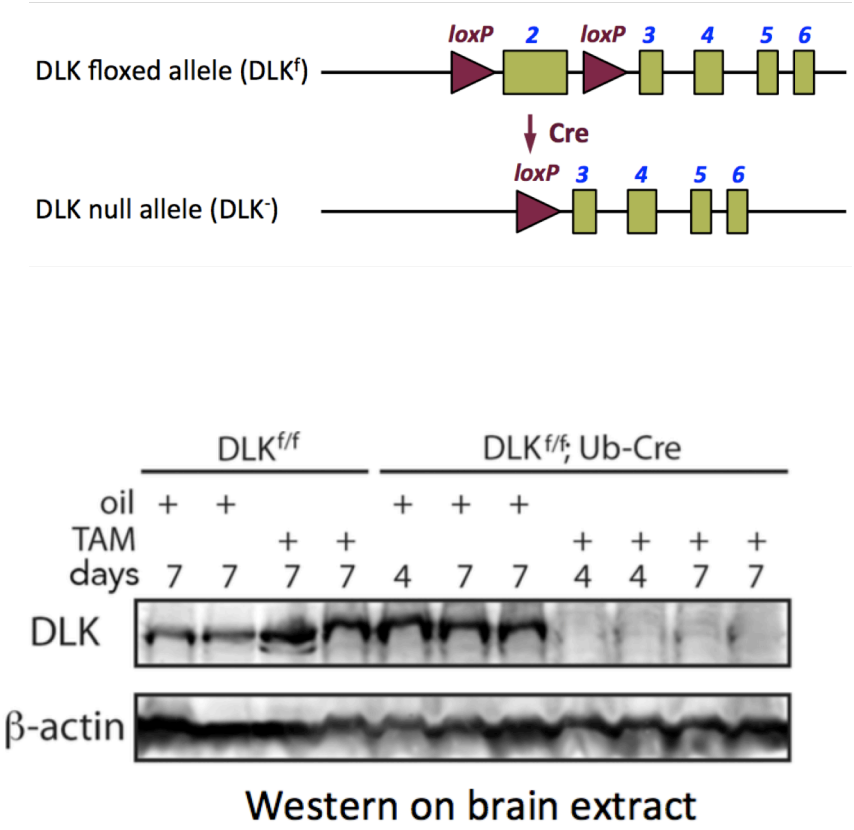


Figure 4.2 DLK conditional knockout mouse line. Top panel, targeted DLK allele in which exon 2 of the DLK gene is flanked by loxP sites. Cre recombinase activity is expected to excise exon 2 resulting in no expression of DLK**.** Bottom panel, tamoxifen administration to DLK f/f; Ub-Cre mice results in efficient deletion of DLK protein. (Western blot data generated by Amy Chen, PhD.)

4.4 Results

To test the hypothesis that DLK promotes compensatory sprouting of the CST, I tested DLK conditional knockout mice (Figure 4.2) and DLK inducible overexpressing mice (Figure 4.3) in CST sprouting after unilateral pyramidotomy. Contrary to our hypothesis, I found that deletion of DLK did not reducing sprouting; indeed there was a trend toward *enhanced* sprouting in DLK KO mice (two way repeated measures ANOVA, $p = 0.062$ for interaction between distance and genotype, $p = 0.086$ for main effect of gene) (Figure 4.4). Conversely, in both WT and PTEN KO background, DLK OE mice exhibited a significantly reduced CST sprouting index (two way repeated measures ANOVA with Bonferroni post-test, $p < 0.05$ for WT vs. cKO [Figure 4.5], $p < 0.05$ for PTEN KO vs. PTEN KO;DLK OE [Figure 4.6]), with no change in the total number of labeled main CST axons in the medulla (Figures 4.5, 4.6).

In order to assess whether the reduced number of sprouting axons per main labeled CST axon in DLK OE mice was associated with any loss of CST neurons, Nissl staining was performed to check for dropout of layer V pyramidal neurons. Nissl staining revealed an expected localized loss of neurons near cortical injection sites in both PTEN KO control and PTEN KO;DLK OE mice (Figure 4.7A,B). While Nissl staining in PTEN KO;DLK OE mice did not reveal “clean” dropout of layer V pyramidal neurons, a distinct Nissl staining pattern was evident near injection sites relative to PTEN KO controls, which overlapped with bright tdTomato expression. To further assess potential phenotypes revealed by the Nissl staining, additional cortical sections were immunostained for NeuN and GFAP to (1) investigate any potential loss of neurons and (2) to assess whether the bright tdTomato^{+} cells that expressed distinct Nissl staining in

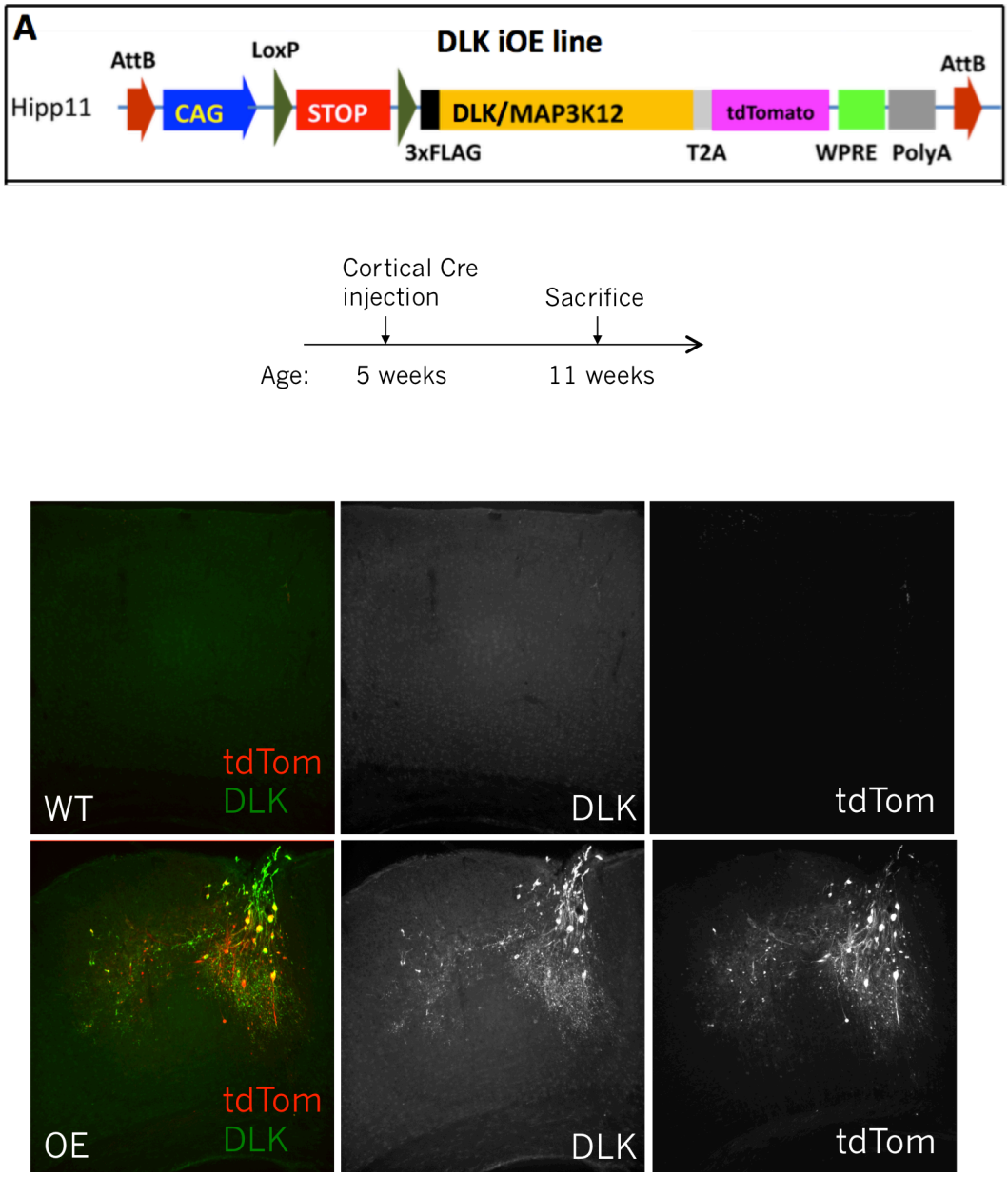


Figure 4.3 DLK inducible overexpressing mouse line. Top panel, DLK inducible overexpression construct in which Cre-recombinase activity is expected to result in overexpression of DLK and expression of tdTomato. Middle panel, experimental timeline for checking efficiency of DLK OE upon cortical injection of AAV2-Cre. Bottom panel, cortical injection of AAV2-Cre induces expression of tdTomato and overexpression of DLK.

PTEN KO;DLK OE mice (Figure 4.7A) express neuron and/or astrocyte cell-type markers (NeuN for neurons, GFAP for astrocytes). NeuN immunostaining revealed a localized decreased density of $NeuN^+$ cells near cortical injection sites in both genotypes (Figure 4.8A), with no substantial difference between PTEN KO;DLK OE tissue and PTEN KO control. A number of bright $tdTomato^+$ cells co-expressed GFAP in PTEN KO;DLK OE mice (Figure 4.8B), suggesting these may be the distinctly-Nissl staining cells (4.7B). Note that weaker tdTomato+ cells co-expressed NeuN (Figure 4.8C).

4.5 Discussion

DLK is dispensable for CST sprouting

My results show that deletion of DLK from CST neurons via cortical injection of AAV2-Cre does not reduce CST sprouting. These results are in contrast to the finding that DLK is required for regeneration of retinal ganglion cell axons after optic nerve crush injury (Watkins et al., 2013). Nonetheless, important distinctions lie in these two experimental paradigms. First, retinal ganglion cells and CST neurons show distinct responses to injury; for example, while a substantial number of retinal ganglion cells undergo apoptosis in response to injury (Li et al., 1999), the majority of CST neurons do not (Nielson et al., 2010; Nielson et al., 2011). Second, all axons are damaged in optic nerve injury and thus the axon growth assessed is of regeneration, whereas in the pyramidotomy injury model the axon growth of only uninjured neurons (i.e., sprouting) is evaluated. Thus the contrasting results of the role of DLK in axon growth after optic nerve crush and in the unilateral pyramidotomy model may be due to differences in the

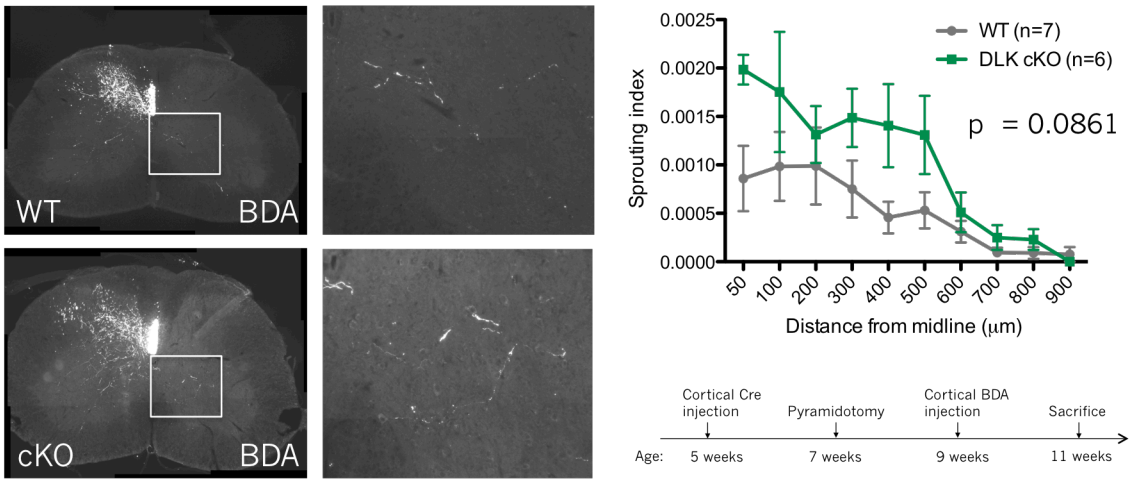
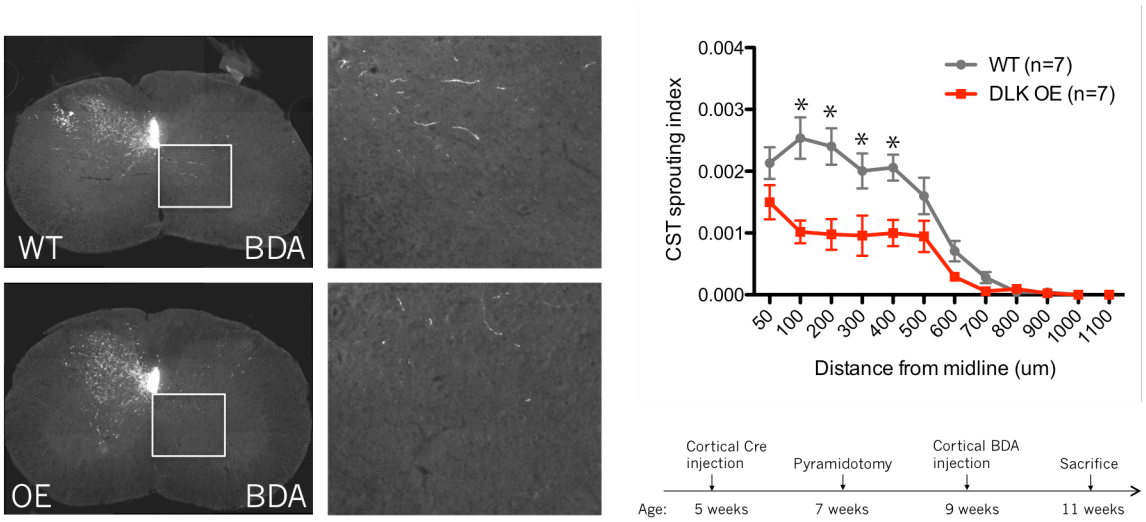


Figure 4.4 DLK is dispensable for CST sprouting. Left, BDA-labeled CST axons in cervical sections from WT and DLK cKO mice after unilateral pyramidotomy. White box depicts enlarged region shown in right panels. Upper right, quantification of the number of BDA-labeled axons at specific distances from the midline in the denervated side of the spinal cord normalized to the total labeled CST axon count at the medulla in WT and DLK cKO mice. Two-way RM ANOVA, p= 0.0861 for main effect of gene. Error bars denote SEM. Lower right, timeline of experimental procedures.

cell type assessed or differences in whether the neurons are injured or not. Finally, it cannot be ruled out that LZK (leucine zipper-bearing kinase), a homolog of DLK, may functionally compensate for loss of DLK in CST sprouting, although in the optic nerve LZK does not appear to fully compensate for the loss of DLK in supporting retinal axon regeneration (Watkins et al., 2013).

Interestingly, DLK cKO mice showed a trend toward increased CST sprouting, suggesting that endogenous DLK may negatively regulate the number of CST sprouting axons that are present at the time point investigated here (four weeks after pyramidotomy). If this were verified in future experiments, two different mechanisms may be at play: (1) DLK may prevent axon outgrowth in sprouting CST neurons or (2) DLK may contribute to the pruning phase of corticospinal collateral remodeling. Indeed, the number of sprouting CST axons is higher at two weeks than at four weeks after traumatic brain injury, and the number of main CST collaterals proximal to spinal injury is elevated at 10 days relative to three weeks (Lee et al., 2011; Lang et al., 2012). I have only assessed the number of CST midline-crossing collaterals at four weeks after pyramidotomy, which may be after pruning of collaterals has occurred. Considering that DLK can be activated by both actin and microtubule destabilization, perhaps the cytoskeletal rearrangements required for sprouting and subsequent pruning could activate DLK as well (Bounoutas et al., 2011; Valakh et al., 2015).



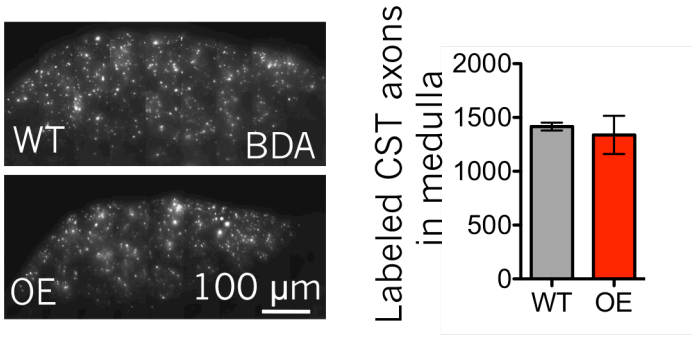


Figure 4.5 Overexpression of DLK reduces CST sprouting without significantly affecting the number of main CST axons labeled in the medulla. Upper left, BDA-labeled CST axons in cervical sections from WT and DLK OE mice after unilateral pyramidotomy. White box depicts enlarged region shown in right panels. Upper right, quantification of the number of BDA-labeled axons at specific distances from the midline in the denervated side of the spinal cord normalized to the total labeled CST axon count at the medulla in WT and DLK OE mice, with timeline of experimental procedures shown below. Bottom left, BDA-labeled CST axons in medullary pyramids of WT and DLK OE mice. Bottom right, quantification of the number of BDA-labeled axons in medullary pyramids (n=7 per genotype). * $p < 0.05$, two-way repeated measures ANOVA followed by Bonferroni post-test. Error bars denote SEM.

Overexpression of DLK reduces CST sprouting without substantial loss of CST neurons

I found that overexpression of DLK in both a WT and PTEN KO background significantly decreases CST sprouting. While we hypothesized that DLK would function in accordance with its role of promoting axon growth in optic nerve regeneration studies, important distinctions lie between these two cell-types and forms of axon growth (see "DLK is dispensable for CST sprouting"). Furthermore, beyond the well-described role of DLK in promoting Wallerian degeneration of distal axon segments after injury (Tedeschi and Bradke, 2013), recently DLK has been implicated in the axon degeneration that occurs in a model of amyotrophic lateral sclerosis (ALS) (Le Pichon et al., 2017). In this model, axon lumen area in the sciatic nerve is reduced prior to the loss of axons, an effect that is partially rescued by DLK deletion, suggesting DLK may promote axon degeneration in some contexts outside of Wallerian degeneration.

One might wonder whether the reduction in CST sprouting is correlated with a more general degenerative process, i.e., the loss of neurons or main axons of the CST. It should be noted that the CST sprouting index reflects the number of midline-crossing CST axons per labeled main CST axon at the level of the medulla, and as such reflects the degree of collateral formation and/or stability independent of the number of main CST axons that are labeled. Nonetheless, I saw no effect of genotype on the number of labeled main CST axons. Additionally, I do not see evidence of substantial loss of CST neurons. First, considering we see no difference in the number of BDA-labeled main CST axons at the level of the medulla neuronal tracer injected into the same sites as AAV2-Cre appears to be taken up by the same number of CST neurons in DLK OE and

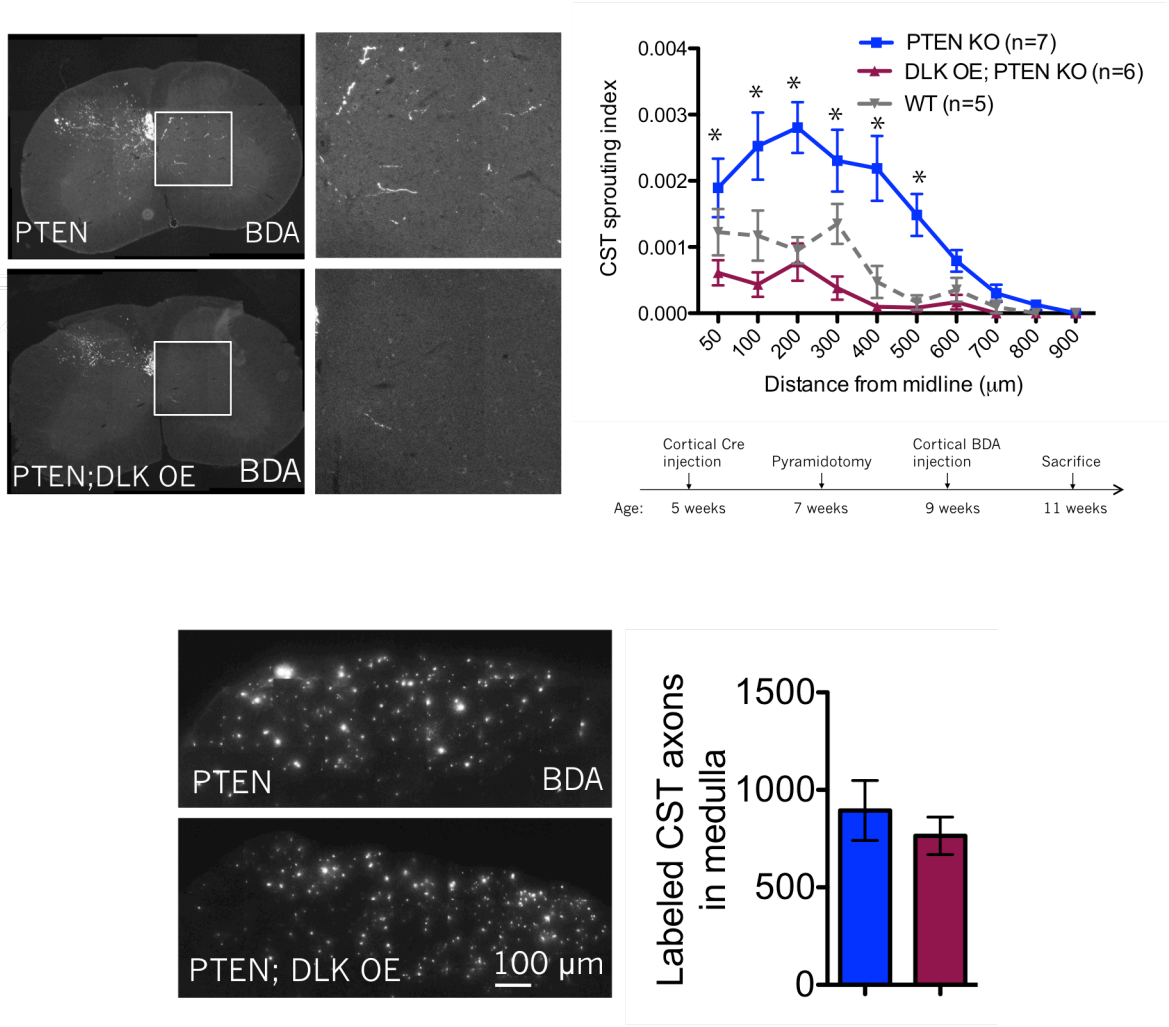


Figure 4.6 Overexpression of DLK reduces CST sprouting in PTEN KO background without significantly affecting the number of main CST axons labeled in the medulla. Left, BDA-labeled CST axons in cervical sections from PTEN KO and PTEN KO;DLK OE mice after unilateral pyramidotomy. White box depicts enlarged region shown in right panels. Upper right, quantification of the number of BDA-labeled axons at specific distances from the midline in the denervated side of the spinal cord normalized to the total labeled CST axon count at the medulla in WT, PTEN KO, and PTEN KO;DLK OE mice, with timeline of experimental procedures shown below. Bottom left, BDA-labeled CST axons in medullary pyramids of PTEN KO and PTEN KO;DLK OE mice. Bottom right, quantification of the number of BDA-labeled axons in medullary pyramids (n=7 PTEN, n=6 PTEN KO; DLK OE). * $p < 0.05$ PTEN KO vs. PTEN KO;DLK OE, two-way repeated measures ANOVA followed by Bonferroni post-test. Error bars denote SEM.

control mice, arguing against significant loss of CST neurons. Second, we see a substantial number of tdTomato+ CST axons in the medullas of DLK OE mice, which we would not expect were there to be significant CST loss (Figure 4.9). Third, NeuN staining near cortical injection sites did not reveal substantial loss of neurons, although future studies directly identifying AAV2-Cre transduced regions of the cortex in DLK OE and control mice would be required to further investigate this possibility.

The ability of DLK overexpression to reduce CST sprouting to below WT levels even in a PTEN KO background is striking. While it had been proposed that the balance between DLK degenerative versus regenerative signaling may be influenced by the growth state of the cell including PTEN deletion (Figure 4.1), my findings suggest that this is not the case for CST sprouting (Watkins et al., 2013). On the contrary, the degree to which CST sprouting is reduced in DLK OE mice relative to WT appears to be unaffected by PTEN deletion, which by itself leads to a 2-3 fold increase in CST sprouting. Future studies confirming that PTEN deletion upregulates the mTOR pathway in our DLK OE mice would be illustrative, as it remains possible that DLK overexpression interferes with PTEN pathway signaling. Regardless, when CST neurons are primed toward a pro-growth state via PTEN deletion, overexpression of DLK substantially reduces CST sprouting.

While initially performed to check for loss of layer V pyramidal neurons, Nissl staining revealed a distinct staining pattern associated with highly expressing tdTomato^{+} cells in PTEN KO;DLK OE cortical tissue that was not evident in PTEN KO control tissue. Upon further investigation, I discovered that many highly expressing tdTomato^{+} cells express the astrocyte marker GFAP. Indeed, in a separate study I found that while

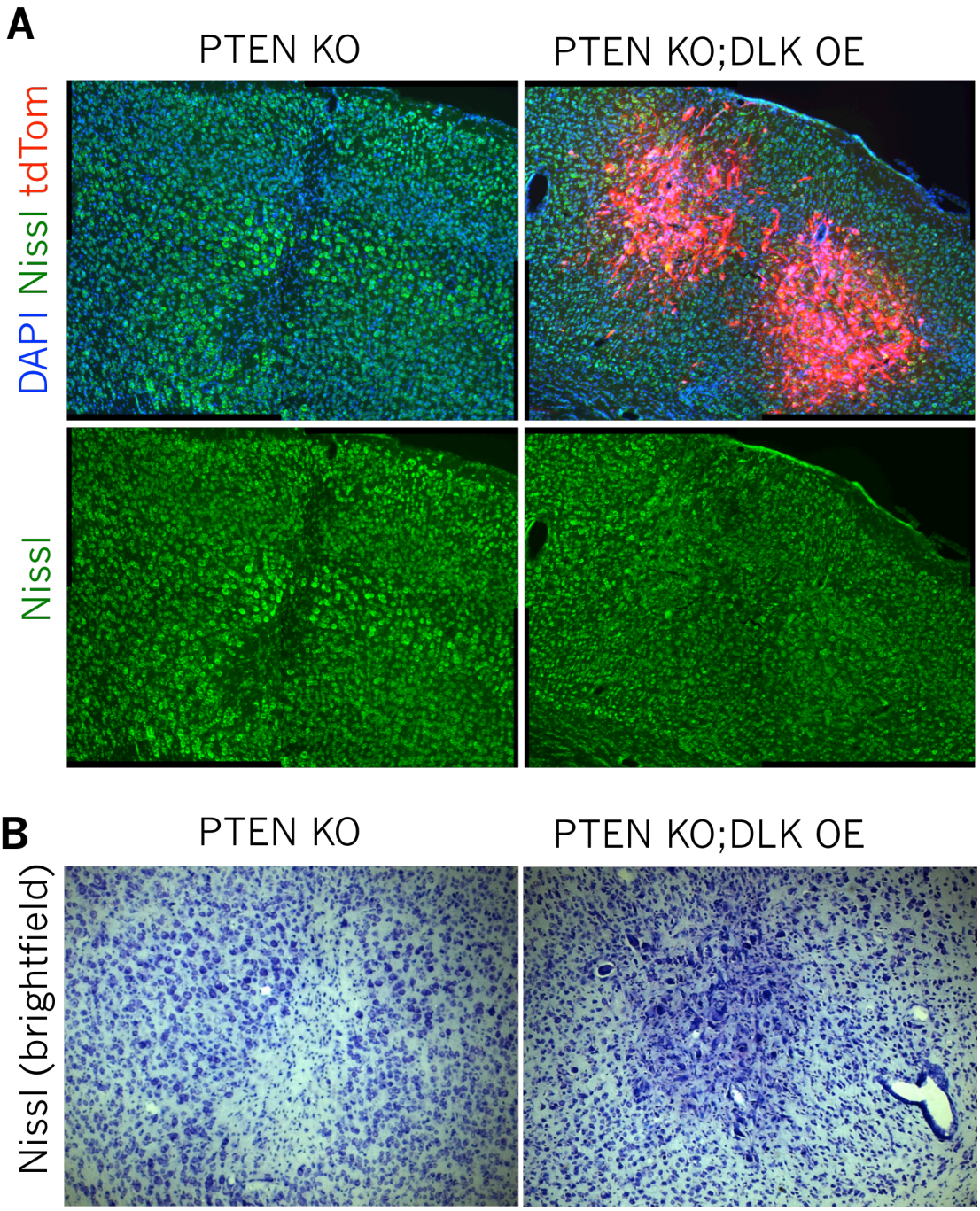


Figure 4.7 Nissl stain inconsistent with clean loss of layer V pyramidal neurons in PTEN KO;DLK OE mice. (A) Immunofluorescence images showing expected localized loss of neurons along cortical injection needle track in PTEN KO mice (left) and distinct pattern of Nissl staining in PTEN KO;DLK OE mice (right). Note that some needle tracks were evident in PTEN KO;DLK OE mice as well (not shown). (B) Higher-magnification images of brightfield Nissl staining near injection sites, with staining patterns similar to (A). Note tissue sections in (B) are distinct from tissue sections in (A).

~95% of cells transduced by motor cortex cortical injection of AAV2-Cre cells are $NeuN^+$ and only ~0.8% are $GFAP^+$, transduction efficiencies for cells expressing each cell type marker near injections sites were found to be ~74% for NeuN ~8% for GFAP (Meves et al., 2018). While the role of DLK in astrocytes is unknown, my lab recently discovered that overexpression of LZK, a homolog of DLK, induces astrogliosis (for which I am a co-author), consistent with the increased astrogliosis I see in PTEN KO;DLK OE mice relative to PTEN KO control (Chen et al., 2018).

4.6 Future Directions

A number of questions remain in our ongoing studies of the role of DLK in CST sprouting. First, is the trend toward increased CST sprouting in DLK cKO mice reproducible? If this trend is not reproduced, follow-up studies assessing CST sprouting in DLK;LZK double KO mice would inform whether any lack of phenotype in DLK cKO mice is due to functional compensation by the DLK homolog LZK. Conversely, if repeat studies in DLK cKO mice confirmed an increase in CST sprouting, whether this is due to increased outgrowth of midline-crossing CST axons or decreased later-stage pruning would be interesting to assess. Similarly, overexpression of DLK may reduce sprouting either by preventing outgrowth or by promoting pruning. Assessing sprouting at an earlier time point before significant pruning has occurred may help inform which of these underlying mechanisms is at play.

Additionally, while my Nissl and NeuN stainings in PTEN KO control vs. PTEN KO;DLK OE mice suggest that overexpression of DLK can reduce CST sprouting even without marked loss of CST neurons, whether overexpression of DLK causes loss of

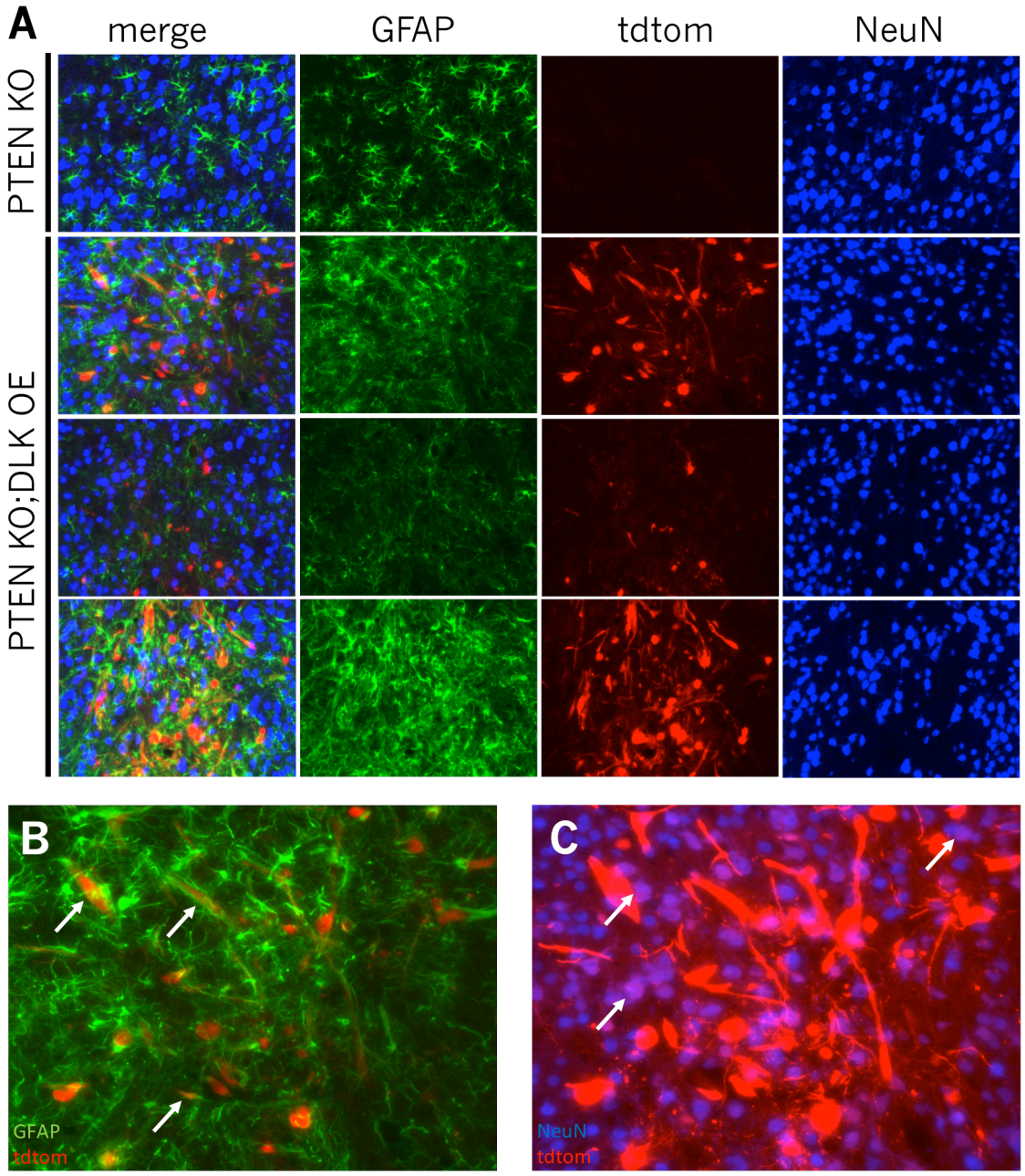
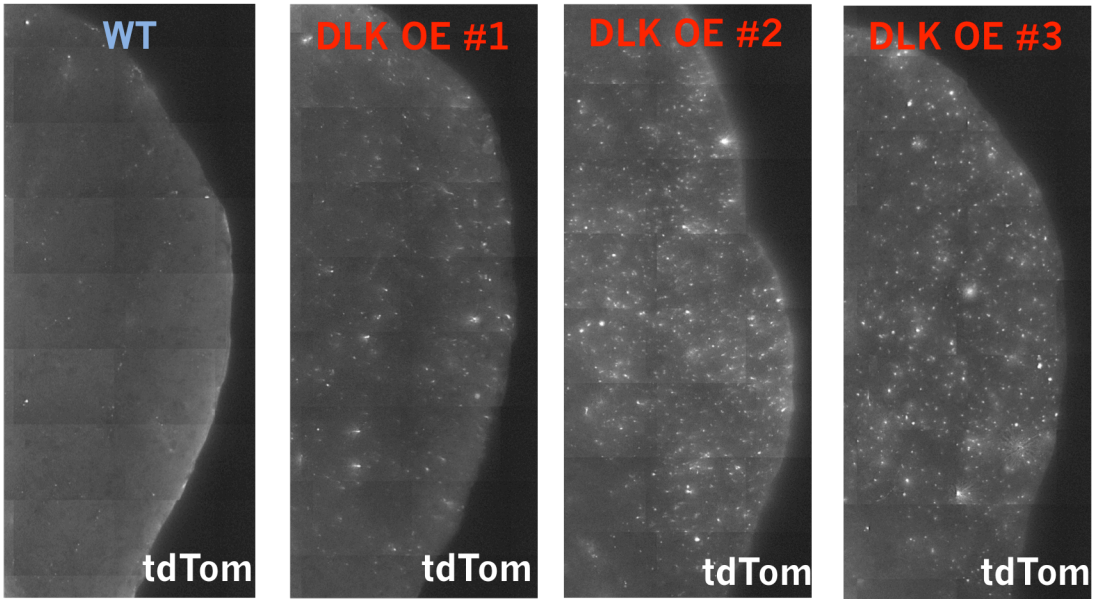


Figure 4.8 Lack of decreased NeuN$^+$ cell density and increased GFAP immunostaining in PTEN KO;DLK OE mice. (A) GFAP, tdTomato, and NeuN expression near cortical injection sites. N=3 shown for PTEN KO;DLK OE mice. (B) Enlarged overlay of GFAP and tdTomato signal. White arrows point to cells positive for both GFAP and tdTomato. (C) Enlarged overlay of NeuN and tdTomato signal. White arrows point to cells positive for both NeuN and tdTomato.

CST neurons in a WT background has not been assessed in this manner. Nonetheless, the lack of difference in the number of BDA-labeled main CST axons and the presence of tdTomato$^+$ CST axons in the medulla suggest that any effect would be minor. Still, considering the known role of DLK in apoptosis, thorough investigation of the effects of overexpression of DLK on cortical cell death both with and without CNS injury are warranted. To fully explore whether overexpression of DLK is associated with any loss of NeuN$^+$ cells (both in WT and PTEN background), future studies directly identifying AAV2-Cre transduced regions of the cortex should be undertaken in order to identify injection sites and to enable quantification of the number of NeuN$^+$ cells present. Additionally, future studies assessing early markers of cell death would rule out the possibility that cell loss was just starting to occur at the time of sacrifice. While unlikely considering the time point at which animals were sacrificed (six weeks after AAV2-Cre injection to overexpress DLK, four weeks after pyramidotomy, and two weeks after BDA injection), it cannot be ruled out that any neuronal degeneration was just beginning at the time of sacrifice and was affecting CST collaterals (including sprouting collaterals) before affecting the number of main CST axons labeled or CST neurons present. We also cannot rule out that overexpression of DLK reduces the number of midline-crossing CST axons even without injury, although ability to detect any effect may be limited by the low number of midline-crossing axons that are present without injury.

Two questions remain regarding underlying signaling at play in these experiments. First, as mentioned above, confirming that PTEN deletion upregulates the mTOR pathway in the PTEN KO;DLK OE mice (in which PTEN deletion did not enhance sprouting) would shed light on whether these cells truly are in a pro-growth



F**igure 4.9 tdTomato-expressing cells project toward the spinal cord in DLK OE mice.** tdTomato expression in axons in the pyramids of the medullas in DLK OE mice (n=3). Note WT control mice do not express tdTomato.

state or, instead, DLK overexpression is interfering with the PTEN signaling pathway. One such way to do this would be to stain for the phospho-S6 ribosomal protein, an indicator of mTOR pathway activity (Geoffroy et al., 2016). Additionally, in order to interpret the biological relevance of the phenotype we see with DLK overexpression, it will be important to assess the degree to which DLK is overexpressed and/or signaling enhanced, for example by immunohistochemistry against DLK (hindered by our present inability to detect endogenous DLK) or downstream signaling molecules and/or by RNA in situ hybridization.

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Chapter 5. Conclusions

Injury to the central nervous system often results in permanent functional impairment. Many efforts have been made to understand the biological underpinnings of this limitation in recovery, with the hopes that therapeutics may some day be developed to promote recovery in patients with brain or spinal cord injuries.

The work summarized in this dissertation strives to elucidate the degree to which a form of spontaneous recovery can be manipulated by molecular interventions. More specifically, the efforts outlined here attempt to answer whether deleting or overexpressing specific factors hypothesized to play a role in axon growth after injury influences compensatory sprouting of uninjured axons after CNS injury. Furthermore, if a manipulation is found to promote sprouting, is that associated with improved functional recovery from injury as well?

The unilateral pyramidotomy model is a robust experimental paradigm to investigate the degree to which axons of the corticospinal tract, an important tract to investigate due both to its resistance to recovery and its importance for voluntary motor control, show compensatory sprouting after CNS injury. While this injury model is a well-established tool for investigating CST axon sprouting, functional (i.e., behavioral) tests to assess recovery from this injury model are less well established, especially in the genetically amenable animal model, the laboratory mouse. Therefore, Chapter 2 of this dissertation summarizes efforts to assess and develop functional assays to test whether manipulations that promote CST sprouting are associated with functional recovery. Three assays, a grooming test, a pasta handling test, and a staircase reaching test, were evaluated and/or optimized for their ability to show sustained deficits after injury, such that they would be sensitive to detect improvements associated with molecular

manipulation of CST sprouting. Two of the three assays, the pasta-handling test and an optimized staircase-reaching test, showed sensitivity to sustained functional impairments resulting from pyramidotomy, and thus were performed in future experiments in which I saw increased CST sprouting. While not the primary goal of these efforts, both assays were also able to detect spontaneous *recovery* from pyramidotomy, expanding the utility of these tests. For example, the pasta-handling test and the staircase-reaching test have the potential to answer not only whether a manipulation might promote functional recovery, but also whether any manipulations hinder spontaneous functional recovery. Indeed, the settings in which promoting axon growth is beneficial for functional recovery remains unknown and warrants further investigation (as discussed below).

Ultimately the goal of this dissertation is to investigate the extent to which specific factors expressed by neurons and by non-neuronal cell-types influence compensatory sprouting and recovery from CNS injury. As such, a major part of this dissertation sought to test a longstanding hypothesis that had never been tested directly regarding the cell-type specific role of a classically described inhibitor of axon growth on CST sprouting (Chapter 3 of this dissertation). I showed for the first time that the protein Nogo expressed by oligodendrocytes restricts CST sprouting, while Nogo expressed by neurons of the CST is dispensable for sprouting. This is an important question regarding the biological function of Nogo, especially considering the completed and ongoing clinical trials investigating therapeutic use of Nogo antibodies to treat spinal cord injury, amyotrophic lateral sclerosis (ALS), and multiple sclerosis (ClinicalTrials.gov, NCT00406016, NCT00875446, NCT01753076, NCT01424423, NCT01435993).

Understanding the cell-type specific roles of Nogo has other important implications. Specifically, cell-type specific manipulation of Nogo in an optic nerve injury model paradoxically implicated a *pro-regenerative* role for neuronal Nogo in retinal axon regeneration, which suggests that simultaneously targeting both oligodendrocytic and neuronal Nogo would be counterproductive in promoting axonal repair in the optic nerve system (Vajda et al., 2015). In contrast, I found that genetic deletion of Nogo in corticospinal neurons does not impede CST sprouting. Thus, my finding predicts that targeting both oligodendrocytic and neuronal Nogo would not impede with the overall strategy to enhance CST sprouting in promoting repair and recovery after incomplete spinal cord injury.

The fact that my findings in CST sprouting contrast with evidence in the literature supporting an injury-induced pro axonal-growth role for neuronal Nogo in the context of optic nerve regeneration raises an interesting biological question. Is this difference due to distinctions between cell types (retinal ganglion cells versus corticospinal tract neurons) or between different forms of injury-induced axon growth (regeneration versus compensatory sprouting)? Indeed, it would be interesting to test the role of oligodendrocytic versus neuronal Nogo in CST regeneration to begin to address this question. This would also be informative for therapeutic development in order to help identify which brain or spinal cord injury patients might benefit from therapeutics targeting Nogo. My data support that targeting oligodendrocytic Nogo may enhance axon sprouting in spared pathways in patients with incomplete CNS injuries, but whether this enhanced axon growth could be extrapolated to increased regeneration in patients with anatomically complete spinal cord injuries remains unknown.

My findings also do not support that targeting oligodendrocytic Nogo is sufficient to promote functional recovery from CNS injury. In fact, while wild-type animals show significant spontaneous recovery from pyramidotomy, oligodendrocytic-Nogo deletion animals do not. Indeed, there is evidence in the literature that some manipulations that enhance sprouting and regeneration can paradoxically lead to worse functional recovery from injury, suggesting that in certain situations enhanced axon growth can in fact be maladaptive (Wang et al., 2015). Rehabilitative training may show utility in harnessing enhanced CST sprouting to promote functional recovery, a hypothesis that remains to be tested in the context of oligodendrocytic-specific deletion of Nogo.

In addition to my studies on a well-known neuron-extrinsic inhibitor of axon growth and spinal cord injury repair (Nogo), I also investigated the role of a novel neuron-intrinsic axon growth regulator that had not been tested in the context of spinal axon repair, the dual leucine zipper-bearing kinase (DLK, Chapter 4). While ongoing, these studies suggest that overexpression of DLK reduces CST sprouting. This result contrasts with evidence in the literature that DLK overexpression promotes axon regeneration in *c. elegans*; nonetheless, DLK was found to mediate both cell death and axon regeneration in the mammalian CNS (i.e., retinal ganglion cells) by loss-of-function analysis. My study is the first to report on the effect of overexpressing DLK in vivo in mammalian CNS neurons, and shows that overexpression of DLK does not promote CST sprouting.

For a more thorough understanding of the role of DLK in CST sprouting, future studies further investigating my finding of a trend toward increased CST sprouting in DLK deletion mice or additional experiments in mice with deletion of both DLK and LZK,

a homologous protein that may functionally compensate for DLK, are of high priority. Furthermore, while preliminary data supports that the effect of DLK overexpression appears to be specific to CST sprouting without affecting the number of CST neurons or main CST axons present, further investigation into these possibilities is warranted (as discussed in *"4.6 Future Directions").*

I have shown that both neuron-extrinsic and intrinsic axon growth regulators can be important targets for modulating axon sprouting after CNS injury, as explored here with investigation into the role of Nogo and DLK in CST sprouting. Important questions remain before these and related studies can move forward toward the goal of providing clinical benefit to patients with CNS injury. Definitive evidence for the exact role of compensatory sprouting in spontaneous and enhanced functional recovery from CNS injury remains a challenge. In what contexts is enhanced sprouting associated with functional recovery? What axonal tracts may be involved beyond the corticospinal tract? These questions are important to consider when determining where to target therapeutics and what types of clinical injuries are likely to see benefit from therapies targeting regulators of axon sprouting.

Much remains to be investigated regarding the roles of various regulators of axon growth in compensatory sprouting and axon regeneration after injury. We are only beginning to understand the ways in which promoting these forms of axon growth might contribute to functional recovery from injury. Nonetheless, the ability to manipulate axon sprouting by targeting neuron-intrinsic and extrinsic factors suggests it may someday be possible to promote recovery in patients with CNS injury who would otherwise suffer life-long functional impairments.

Acknowledgement

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