UNIVERSITY OF CALIFORNIA SAN DIEGO

Assessing the effect of neuronal LZK and DLK deletion on Axonal Sprouting after CNS Injury

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

in

Biology

by

Juliet Suen

Committee in charge:

Professor Binhai Zheng, Chair
Professor Yishi Jin, Co-Chair
Professor Yimin Zou

2020

The Thesis of Juliet Suen is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Co-chair

Chair

University of California San Diego

2020

DEDICATION

I dedicate this thesis to my incredible family, who have continuously been my support system for as long as I can remember. Despite the thousands of miles that separate us every year, they have always been my constant source of guidance and support. I truly would not be who I am or where I am today without them.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AAV: adeno-associated virus

BDA: biotinylated dextran amine

CNS: central nervous system

Cre: Cre recombinase

chABC: chondroitinase ABC

dCST: dorsal corticospinal tract

dlCST: dorsolateral corticospinal tract

DLK: dual leucine zipper-bearing kinase

GFAP: glial fibrillary acidic protein

KX: Ketamine-Xylazine

LZK: leucine zipper-bearing kinase

MAP3K13: mitogen-activated protein kinase kinase kinase 13

mTOR: mammalian target of rapamycin

OE: overexpression

PTEN: phosphatase and tensin homolog

RGC: retinal ganglion cell

SCI: spinal cord injury

LIST OF FIGURES

ACKNOWLEDGEMENTS

First and foremost, I would like to start by thanking Dr. Binhai Zheng for welcoming me into his lab and for allowing me to continue my journey in scientific research. He has provided me a space that continuously challenges me to think critically and intellectually. I appreciate his ongoing support in my goals and aspirations and after years of being under his guidance, I have learned a tremendous amount and will carry all of these lessons with me in the future.

I would also like to thank Junmi Saikia for taking me under her wing and teaching me almost everything that I know today about scientific research. With her patience and mentorship, I have been able to learn and experience what it means to be a true researcher in science. I want to thank her for her guidance over my work, experimental design, and for performing the necessary surgeries needed for my project. She has been a valuable member on my team and has continued to challenge me to critically think about scientific literature and finding.

My genuine appreciation also extends to Dr. Yishi Jin and Dr. Yimin Zou for their time and effort invested into being in my committee. I would like to thank them for being exceptional educators and for their unending commitment to science and their students.

Finally, I would like to thank the members of the Zheng Lab: Hugo Kim, Joshua Sanchez, Daniel Romaus-Sanjurjo, Sahar Allibhoy, Baredu Morka, Kristen Tsai, Noah Kim, and Carmine Chavez-Martinez for being my lab family and making every moment worth it.

Figure 6 is currently being prepared for submission for publication of the material. Suen, Juliet; Saikia, Junmi. The thesis author was the primary investigator and author of this material.

ABSTRACT OF THE THESIS

Assessing the effect of neuronal LZK and DLK deletion on Axonal Sprouting after CNS Injury

by

Juliet Suen

Master of Science in Biology

University of California San Diego 2020

Professor Binhai Zheng, Chair
Professor Yishi Jin, Co-chair

The goal of this project is to understand the roles of Leucine Zipper-bearing Kinase (LZK) and Dual Leucine Zipper Kinase (DLK), a pair of conserved mitogen-activated protein kinase kinase kinases (MAP3Ks) involved in axonal repair after central nervous system (CNS)

injury. CNS injury often leads to partial and even complete loss of function mainly due to the inability of neurons to repair themselves after injuries. This lack of regenerative capacity in the CNS can be attributed to both intrinsic and extrinsic factors. The regeneration of severed axons and the compensatory sprouting of uninjured axons are two forms of axonal repair after CNS injury. The two MAP3Ks are believed to work in concert to regulate responses after injury to axons in the mammalian CNS. Of the two homologues, DLK has been the only mammalian homologue shown to be involved in axon regeneration after peripheral or optic nerve injury. However, the role of DLK in axonal repair in the mammalian spinal cord is not known, let alone LZK. Teamed with an MD/PhD student in the lab, I pursued a project to understand the isolated roles of DLK and LZK in models of corticospinal axon and sprouting in order to better understand their individual function in axonal repair after injury. The results complement the ongoing projects in the lab for a thorough understanding of the two MAP3Ks in axonal repair in the mammalian spinal cord after injury.

INTRODUCTION

Overview of Spinal Cord Injury

According to the statistics provided by the National Spinal Cord Injury Statistical Center (NSCISC), there are 291,000 estimated number of individuals in the United States who live with spinal cord injuries (SCI) and around 18,000 new incidents each year. Vehicle accidents are the most prevalent cause of injury and such injuries often result in loss of function. With nearly 50% of all cases resulting in incomplete tetraplegia, our research focusing on the mechanisms that impact recovery are crucial to advancing our current understanding of injury responses and allow us to create new avenues for effective therapeutics.

Two types of axonal repair within the spinal cord after injury

The corticospinal tract (CST) is a white matter motor pathway responsible for voluntary and skilled motor movement (Lemon et al, 2005). Starting at the cerebral cortex, CST neurons project down through the brainstem and into the spinal cord, decussating at the level of the medullary pyramids. In rodents, the main cohort of the CST travels down the ventral portion of the dorsal column often referred to as the dorsal CST (dCST). To a much lesser extent, CST axons also travel down through the dorsal portion of the lateral column often referred to as the dorsolateral CST (dlCST). The dorsolateral CST axons project out in similar areas of gray matter and are thought to be potential sources of sparing (Steward et al.,2004).

There are two types of axonal repair that occur within the CNS: regeneration of severed axons and compensatory sprouting of uninjured axons (Figure 1). What is considered regeneration is the growth and extension of injured axons beyond the site of injury while

compensatory sprouting is the growth of uninjured axons. CNS sprouting can occur spontaneously after spinal cord injury and similar to the synapses formed in regenerated axons, sprouted axons also have the ability to form synapses. Newly formed sprouts display extensive plasticity and are naturally occurring and are likely responsible for some amount of functional recovery in human spinal cord injury patients. In animal models, the ability of CST sprouting, and regeneration can be enhanced by the deletion of neuronal phosphatase and tensin homolog (PTEN), a tumor suppressing protein that negatively regulates the mammalian target of rapamycin (mTOR) pathway (Figure 2).

Regulatory Pathways of Axon Growth:

Axon regeneration and repair is variant across animal lineages and across the mammalian nervous systems. The CNS injury and repair mechanism are influenced by both intrinsic and extrinsic factors. The peripheral nervous system (PNS) has baseline regenerative capacity, but the axons in the adult CNS lacks the capacity for self-repair (Tedeschi, 2017). As seen in Figure 3, the limited regenerative capacity seen in the adult CNS is hindered by extrinsic factors such as astrogliosis, myelin debris, and scar tissue, while the intrinsic factors seen are impacted by protein expression and injury signaling pathways (Kaplan, 2015). In addition to these inhibitory extrinsic factors seen in the CNS, there is also a lack of extrinsic growth factors and growth promoting factors. The manipulation of external factors alone is generally insufficient for regeneration of injured axons (Geoffroy, 2014).

One protein of interest, phosphatase and tensin homolog (PTEN), is a tumor suppressing protein that negatively regulates the mammalian target of rapamycin (mTOR) pathway. The

mTOR pathway is important in the cell-signaling pathways that are deregulated in cancer. The mTOR pathway is involved in the regulation of cell growth by initiating mRNA translation, controlling ribosome synthesis, metabolism, and autophagy (Guertin and Sabatini, 2007). PTEN is believed to inhibit the mTORC1 pathway in which the deletion of this PTEN promotes CNS axon regeneration (Huang, 2019). At the molecular level, we are interested in deleting PTEN as knocking out PTEN allows greater regenerative capacity in the mammalian CNS.

The mitogen-activated protein kinase (MAPK) are activated phosphorylation pathways involved in important cellular functions such as cell proliferation, differentiation, and even cell death (Morrison, 2012). The MAPK pathway contains a series of three activated protein kinases that are key to the signal transduction pathway. Each cascade is initiated by very specific cues extracellularly and leads to a sequential activation of MAPK followed by the phosphorylation of MAPK by the MAPK kinase (MAP2K) and later phosphorylated by MAP2K kinase (MAP3K) (Chang and Karin, 2001). As discussed above, DLK and LZK are MAP3K members of the MAPK pathways that are thought to be involved in neuronal and astrocytic responses to CNS injury.

The role of DLK

Nerve injury usually induces axonal regeneration, degeneration and even neuronal cell death. The mammalian DLK protein has been shown to involve both regenerative and degenerative processes, and cell death (Tedeschi & Bradke, 2013). However, the role of DLK in spinal cord axon regeneration is unclear. DLK, or dual leucine zipper kinase, has the ability to promote each of these responses—implicating the role that DLK has on the injury response

mechanism. It is believed that cytoskeletal disruption is what activates the DLK pathway and may actually result in a pro-regenerative state in response to injury (Valakh, 2015). A preconditioned injury activates the DLK pathway and improves the regenerative capacity of axons. It is concluded that DLK is essential for these responses and serves as a vital signaling mediator for sensory neurons after injury.

DLK plays a role in synaptogenesis and axonal outgrowth in *C. elegans*. In addition to its role in synapse formation and axon morphology, the Jin group found that by stabilizing mRNA, DLK-1 is also shown to be involved in sensory axon regeneration (Yan, 2009). They found that DLK-1 kinase signals via the MAPKAP kinase, MAK-2, in order to stabilize the mRNA that encodes for a bZip protein, CEBP-1. The dysregulation of this protein results in disrupted synapses and axonal morphology (Yan, 2009). It was found that the DLK-1 pathway is crucial as a regulator of mRNA stability in the formation and maintenance of synapses and plays a vital role in adult axon regeneration. Consistent with this study, it was found that the DLK-1 MAP kinase pathway is also crucial for the regeneration of motor neurons in *C. elegans* (Hammarlund, 2009). Activation of this pathway leads to improved regeneration in motor neurons while the loss of DLK-1 MAP kinase pathway reduces regeneration. Hammarlund also discovered that these proteins are involved in regulating growth cone migration. After axons are severed, neurons can extend and form a new growth cone from the stump and potentially regrow. His studies show that activation of MAP kinase cascade is required to promote the capacity of neurons to grow following axon injury. DLK-1 is critical for axon regeneration seen in the worm model, but the role of the mammalian DLK protein remained to be understood.

Watkins (2013) explored how DLK, an upstream mediator of JNK and p38 MAPK pathways, plays a role in intrinsic regulations of both neuronal regeneration and apoptosis. His studies in optic nerve showed that optic nerve crush leads to elevated levels of DLK protein firstly in the axons of retinal ganglion cells and then in their cell bodies. DLK plays an essential role in injury induced gene expression, and deletion of DLK in the retina results in protection of retinal ganglion cells (RGC) from degeneration after optic nerve injury. It is seen in that DLK is required for the pro-growth and pro apoptotic transcriptional pathways and knocking out DLK renders both pathways inactive. This can potentially explain the survival phenotype of RGCs even after injury. Despite the improved neuronal survival, however, axonal growth after injury was reduced suggesting contradictory responses through the DLK detection pathway. It has been shown that DLK protein levels are increased following axonal injury, whereas the loss of DLK seems to protect axons from Wallerian degeneration and reduce stress-induced c-Jun N-terminal kinase (JNK) signaling (Watkins, 2013).

The role of LZK

Just as DLK, LZK also signals through the MAPK cascade which orchestrates cellular responses after some external stimuli. LZK, the most structurally similar MAP3K to DLK, exhibits axon growth- promoting activities in mouse neuroblastoma cells and primary CNS neurons (Chen, 2016). The manipulation of both LZK and DLK revealed interaction and cross regulation at the protein level, suggesting the co-regulation of axonal responses from injury by both kinases. In order to determine the role of LZK in the intrinsic control of neurite growth and extension, a study was performed in mouse neuroblastoma cells—a cell line used extensively for studies of neuronal differentiation (Tremblay, 2010). LZK was shown to promote neurite growth

in vitro and the results of the study suggest that neuronal cells overexpressing LZK had a twofold increase in neurite growth as compared to those with inactive LZK (Chen, 2016).

Understanding the LZK effects of neuronal extension, another experiment was performed which tested the endogenous signaling that takes place in N2a (neuroblastoma) cells. MKK4 and JNKs were discovered to be downstream signaling molecules of LZK that positively feedback on LZK protein levels in mouse N2a cells. Chen et al. also discovered that the upregulation of LZK indeed promotes and enhances axonal growth in the neurons of the mammalian central nervous system. In order to confirm whether or not endogenous LZK has a role in axonal growth, a targeted LZK experiment was performed in which the depletion of LZK impaired the activity of JNK, axon growth and regeneration in vitro, implying an important role in axonal regeneration of the central nervous system. From these studies, two activating conditions have been identified: overexpression of LZK promotes axonal growth and potassium withdrawal which ultimately deprives the neurons of any activity. Overexpression of either LZK and DLK alone resulted in enhanced growth of axons at levels comparable to the overexpression of both LZK and DLK together. The knockout or knockdown of both kinases is shown to reduce axonal growth.

In vivo, LZK has been shown to mediate reactive astrogliosis and astrocyte scar formation (Chen, 2018). The neuronal role of LZK in spinal cord injury and repair has not been reported.

Previous Experimental Results: (DLK/LZK/PTEN triple KO vs PTEN KO)

In previous findings by Dr. Jessica Meves (a former MD/PhD student in the lab), it was seen in her results that DLK conditional knockout in wildtype background enhanced CST sprouting while the induced overexpression (iOE) of DLK indicated diminished sprouting levels as compared to wildtype. Furthermore, DLK overexpression again diminished the enhanced sprouting in a PTEN deletion background. These results are shown in Figure 4 and Figure 5. In order to better understand how these two kinases work together in injury responses, Junmi Saikia (a current MD/PhD student in the lab) performed additional experiments focusing on the double knock out of DLK and LZK in a PTEN deleted background (TKO). The results of DLK/LZK/PTEN TKO showed similar levels of sprouting compared to wildtype, which were significantly lower than PTEN deletion alone. These results are indicated in Figure 6.

LZK and DLK signaling in peripheral nerve injury

In comparison to the mammalian central nervous system, the peripheral nervous system is believed to have some innate capacity for regeneration. While PTEN deletion is used to enhance baseline regeneration in the CNS, it is not necessary in models of peripheral nerve regeneration. Thus, sciatic nerve crush models have been utilized to study the role of axonal repair in environments where baseline regenerative capacity is present.

In the peripheral nervous system, an injury-induced response signaling leads to what is known as a preconditioning injury effect. Essentially, a neuron exposed to a prior lesion appears to have significant improvement in axonal regeneration compared to neurons who have not been pre-conditioned (Neumann and Woolf, 1999). Following peripheral nerve injury, there is a local regenerative response that results in the formation of a growth cone and other various cytoskeletal changes that promote axonal repair (Bradke, 2012). There are two possible stages in its regenerative response. The first, as aforementioned, results in a fast, local alteration seen in the cytoskeleton which essentially promotes growth cone formation and outgrowth (Bradke, 2012). The second initiates a retrograde injury response that signals to activate transcription factors that ultimately turn on pro-regenerative programs (Liu, 2011). These two transcriptional processes are responsible for mediating the preconditioning effect—an effect when neurons that are pre-exposed to a lesion allows for a more robust regenerative response after injury. As seen in previous studies on *C. elegans* by Hammarlund and Yan, DLK is involved in the formation of regenerative growth cones after injury. In a study by the DiAntonio group, they found that *in vivo,* the outgrowth of injured axons is seemingly normal in the first 24 hours after injury and slowly reduced over the course of three days (Shin, 2012). Ultimately, it was found that the

regeneration to targets is severely impaired. This finding was particularly interesting in that DLK is shown to be necessary for the second phase of the regenerative response and is necessary for pro-regenerative programs that are responsible for axonal growth after injury.

In addition, DLK is also identified to be necessary for mediating the preconditioning effect of a prior injury. Because injury-signaling pathways are believed to modify transcriptional changes that ultimately support axon regeneration, Shin found that DLK is required for the induction of pro-regenerative transcriptional programs after peripheral nerve injury. Ultimately, they propose that DLK-dependency might provide some sort of selective filter for candidate regeneration genes (Shin, 2019).

DiAntonio's group provided additional evidence to support the finding that DLK is involved as a retrograde injury signal. Consistent with findings from the La Fleur studies, they found that control sensory axons regenerated beyond the site of lesion 3 days following injury (La Fleur, 1996). In addition to this finding, however, they also found that the length of regenerating axons is reduced in the absence of DLK. From previous literature, axon regeneration is understood to be promoted from a series of factors: growth cone formation, axonal extension, and the preconditioning effect (Shin, 2012). In each of these steps, they performed studies to determine which of these required DLK. They found that DLK is required for the preconditioning effect, or the accelerated regeneration that is induced by a preconditioned injury. Their results show that the preconditioning effect in WT shows a 2-fold increase in regeneration. As predicted in DLK knockouts, the preconditioning effect was reduced

significantly, suggesting that DLK is, in fact, necessary for the injury-induced acceleration of regeneration (Shin, 2012).

MATERIALS AND METHODS

Mutant Mice

Mice in a C57Bl/6 genetic background with DLK, LZK, and DLK/LZK knockout and their littermate controls were used.

For the peripheral nerve study, we generated mice with tamoxifen-inducible Cre to investigate the role of DLK, LZK, and DLK/LZK KO in tandem. In order to generate our mice, we used an $LZK^{f/f}/DLK^{f/f}$ mouse bred to a Ub-$CreER^{T2}$ transgenic mouse line (Figure 7A). We utilized tamoxifen inducible Cre to conditionally knock out LZK and DLK in the mouse.

Viral production and cortical injection

AAV-CAG-Cre and control AAV-CAG-GFP were distributed from the Boston Children's Hospital and viral concentrations were titered by qPCR. AAV-Cre reached a titer of 0.5×10^{12} TU/mL and AAV-GFP 0.7×10^{12}TU/mL. For AAV injections, 6-week-old mice were anesthetized via intraperitoneally (I.P.) injection of freshly prepared 5mg/mL Ketamine-Xylazine (KX) and were then injected with 1.2µL of either AAV-Cre or AAV-GFP into the right sensorimotor cortex. The animal model schematic is as shown in Figure 8. Microinjection needles were created by pulling from glass capillaries with a two-step needle puller. These were then attached to a 10µL Hamilton syringe and was fixed onto a stereotaxic device for injections. The right sensorimotor cortex was injected, targeting the left forelimb was injected (1.2 mm lateral and 0.5 mm anterior bregma, 1.2 mm lateral and 0.5 mm posterior bregma, 2.2 mm later and 0.0 anterior/posterior bregma). Depth of injection is 0.7 mm and rate of injection is 0.1µL per minute for 4 minutes at each injection site.

Surgical procedures for injury production and axon tracing

Unilateral pyramidotomy and biotinylated dextran amine (BDA) tracer injection into the sensorimotor cortex were performed. DLK, LZK, and DLK/LZK conditional knockout mice and wildtype mice were used in surgical experiments. All pyramidotomy surgeries were performed 2 weeks after AAV injections in young adult mice. For all surgical procedures, mice were administered with Ketamine-Xylazine (Ketamine HCl; VetOne, Xylazine; AnaSed at 100mg/10mg/kg mixture, respectively) mixture and delivered intraperitoneally (I.P.). After each surgical procedure, mice were administered 0.05 mL of Buprenex (Buprenorphine HCl; Reckitt Bencksier) and 0.5 mL of sterile saline subcutaneously. Mice were then placed in a cage and placed on top of a water jacket heating pad in the surgery room. Mice were carefully monitored until mice recovered from anesthesia. Following the surgical procedure, mice were observed twice a day for five consecutive days in case there were any post-surgical complications. All mouse husbandry and experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of California, San Diego.

Pyramidotomy

Mice were anesthetized by I.P. injection with Ketamine-Xylazine (KX) and an incision on the left side of the trachea was made. Mice toe pinch reflexes were used to confirm proper anesthetizing. The surgical area was then shaved and cleaned with alcohol wipes (Webcol). All pyramidotomy surgeries were conducted by Junmi Saikia. The skull base was exposed and blunt dissection techniques were used to expose the underlying pyramidal tract of the adult mice. A micro feather scalpel marked at 700 microns was then used to puncture the dura and lesion the entire left pyramidal tract. Skin wounds were closed with Vetbond and mice were administered

0.05 mL of Buprenex (Buprenorphine HCl; Reckitt Bencksier, 5 mg/mouse) and 0.5 mL of sterile saline subcutaneously, and allowed to recover on a pre-warmed cage.

Sciatic Nerve Crush

Surgeries are performed on 6-week-old mice. Mice were anesthetized by I.P. injection with Ketamine-Xylazine (KX). Both hind limbs were carefully shaved and cleaned off with alcohol wipes. All limbs were taped down, and the hind limbs were carefully set symmetrical to one another. A sterile surgical field was created and all instruments were sterilized by hot bead sterilization. An incision was made in the skin and blunt dissection was performed until the sciatic nerve was exposed. After isolating the sciatic nerve, the crush was made perpendicular to the nerve and was crushed using hemostatic forceps for 15 seconds. Carbon powder that had been previously sterilized by UV light was utilized to mark the crush site. After complete injury, the gluteal musculature was sutured. The skin incision was closed using 9mm reflex clips (World Precision Instruments). Enough clips were used to ensure the entire incision was closed. After surgery, the mice were administered 0.05 mL of Buprenex (Buprenorphine HCl; Reckitt Bencksier, 5 mg/mouse) and 0.5 mL of sterile saline subcutaneously. All animals were placed on a heating pad at 37°C.

For the three-day sciatic nerve crush, surgeries were performed three days following the initial injury to allow ample time for the induced preconditioning effect. All procedures were the same as the initial injury. The new crush injury was performed more proximally compared to the preconditioned lesion. All crushes were performed bilaterally.

Tamoxifen Administration

Tamoxifen was administered to mice at 8 weeks for 5 days. Tamoxifen was prepared by adding 100 mg Tamoxifen into 5 mL of sunflower oil (20mg/mL final concentration). The scintillation vial was wrapped with aluminum foil and was then heated for about 2 hours. Tamoxifen was then administered to mice using the gavage technique. The amount we administered across the five days was 0.375% of their daily body weight. Tamoxifen induced Cre-deletion of DLK and LZK was confirmed by Western blot (Figure 9).

BDA tracing

Two weeks following the pyramidotomy surgeries and two weeks prior to sacrifice, mice received stereotaxic injection of BDA (10% Invitrogen) to label CST axons by anterograde tracing. Microinjections of the same volumes, concentration, and coordinates as those used for AAV injections were performed. Mice received cortical injections of 1.2µL at 3 sites (0.4µL per site). BDA was administered using the same modified 10µL Hamilton syringe.

Tissue Processing

Mice were perfused two weeks after BDA injections. Before perfusions, mice were euthanized with a lethal dose (0.25 mL) of a pentobarbital-based solution (Fatal-Plus). Utilizing a peristaltic pump, mice were perfused transcardially with 4% paraformaldehyde. Immediately following, the brain and spinal cord were dissected and post-fixed overnight with 4% PFA. The tissues were then transferred into 30% sucrose solution in order to cryo-protect the tissue. After 3 days of tissues being post-fixed in sucrose, the brain and spinal cords were embedded in blocks (Sakura Tissue-Tek Cryomold) using OCT compound (SAKURA FINETEK USA) where they

were then frozen over dry ice. Tissue blocks were kept in the -80°C to preserve samples. For cervical spinal cord tissue, 3 millimeters of tissue were collected to sample the C5-7 region of the spinal cord. Gross anatomy was used to identify the transition from cervical to thoracic spinal cord. For medullas, 2 millimeters of tissue were harvested, using gross anatomy to identify regions of interest.

Peripheral nerve dissection

The following day after the sciatic crush, all mice were euthanized with a lethal dose (0.25 mL) of a pentobarbital-based solution (Fatal-Plus, MWI). The nerves were dissected bilaterally, placed in a 2 mL centrifuge tube, and post-fixed overnight with 4% PFA. The tissues were then transferred into 30% sucrose solution in order to cryo-protect the tissue. After 3 days of tissues being post-fixed in sucrose, the sciatic nerves were embedded into a cryomold specimen mold (Tissue-Tek, 25 mm x 20 mm x 5 mm). All samples were stored in the -80°C freezer.

Cryostat Microtome

All tissue samples were sliced using a cryostat microtome set at -20°C. The brain and spinal cord were sectioned at a thickness of 20 µm and placed in wells filled with phosphate-buffered saline (PBS). For cervical tissue, 120 sections were collected step serially; for medullas, 80 sections were collected in step serial fashion.

Direct Mounting

For the peripheral nerve study, tissues were sliced coronally at 12 µm and were directly mounted onto Gelatin-coated microscope slides (Thermo Fisher Scientific). The nerves were mounted so that the proximal end was directed at the top of the slide. All sections were stored in the -80°C freezer. On average, about 7 nerves were collected per slide.

Immunohistochemistry

In order to validate the completeness of the pyramidal lesions, the transverse sections of cervical spinal cord (C7) were stained for PKCgamma (Figure 10). To confirm proper cortical injections and BDA tracing of transverse cervical spinal cord and medullas, tissues were stained overnight for BDA using avidin/biotin complex staining kit (Vectastain ABC-Peroxidase Kit). Sections were mounted onto microscope slides (Fisherbrand) and a hydrophobic PAP pen was used to draw a barrier around the tissue for the final step of staining. The BDA signal was enhanced using 1:200 dilution of TSA Plus Fluorescein System (PerkinElmer) for 10 minutes at room temperature. After the glass slides have dried, the microscope slides were cover-slipped using Fluoromount and stored in the 4°C fridge.

For the peripheral nerve study, slide mounted immunohistochemistry was performed. All tissues were stained for SCG-10 Antibody (STMN2, Fisher Scientific), a marker for peripheral nerve regeneration. DAPI (4', 6-diamidino-2-phenylindole), a blue-fluorescent DNA stain, was also used (Figure 11). Brightfield images were also taken and shown in Figure 12.

Quantifications of axons

Images were taken with a digital camera fixed on a Zeiss epifluorescence microscope using the 10X and 20X objective lenses. Sprouting index was quantified using the software AxioVision (Zeiss) and Zen. All staining, imaging and quantifications were performed by a blinded researcher. Images of transverse cervical spinal cord (C5-7) sections were taken using the Zeiss microscope. From ten stained sections, the first five complete and undamaged sections of the cervical spinal cord were imaged and quantified. The ratio of the contralateral (denervated) and ipsilateral counts was taken as the measure for the sprouting index. In order to quantify the sprouting index number, a line was drawn through the central canal and across the dorsoventral axis. The initial line was marked at 50 µm from the midline then 100 µm laterally on the denervated side of the gray matter. The number of axons that crossed the lines were labeled with events and were averaged from the five sections. The data were then normalized against the total axon count in the medullas.

Quantification of medullas

Medulla images were taken with a digital camera fixed on a Zeiss epifluorescence microscope using the 100X objective lenses and oil. Two well-labeled sections of the medullas were imaged and quantified. Quantifications were performed on the software, Zen. Individual medulla sections were outlined using the "Contour" tool. A grid template was placed in front of the image and was used for all sections. For every full alternating square along the length of the section, axons were counted using the "Events" tool. After completing counts, numbers were inputted into a quantification template sheet to record the area of the section, number of events, and number of full boxes quantified.

Quantification for sciatic nerve

All staining, imaging and quantifications were performed by a blinded researcher. Images were taken with a digital camera fixed on a Zeiss epifluorescence microscope using the 10X objective lens. Bright field images were also taken. Images of both left and right sciatic nerve sections were taken using the Zeiss microscope. Regeneration index was quantified using the software AxioVision (Zeiss). The injury site was identified and cross sections were taken at every 100-micron mark after the injury site. At each 100 microns mark, the total number of events was recorded. Each of these values were normalized to the number of axons labeled at the preinjury mark 100 microns below the injury site as shown in Figure 13. In order to adjust for background intensity, a threshold value of 1.56 was selected that was considered signal and any signal below that were not quantified.

RESULTS

Experimental setup

My primary goal of this study was to analyze whether conditionally knocking out DLK and LZK would impact axonal sprouting after CNS injury. In particular, I wanted to determine the isolated roles of LZK and DLK in a wildtype background in order to better understand their role in injury response. A unilateral pyramidotomy injury model was used to study corticospinal tract (CST) axon sprouting. For my peripheral axon regeneration study, we used a sciatic nerve crush model.

Age- and genetic background-matched littermates (C57BL/6) were used to provide a direct comparison among different genotypes. The experimental timeline is shown in Figure 6B. We applied unilateral AAV-Cre (adeno-associated virus carrying a Cre expression construct) injection into the sensorimotor cortex of $DLK^{f/f}$ and $LZK^{f/f}$ (homozygous floxed, DLK and LZK conditional mutant) mice in order to induce DLK and LZK deletion. For the next six weeks at 2-week intervals, we performed 1) the unilateral pyramidotomy injury, 2) cortical injection of BDA to trace CST axons, 3) sacrificing the mice for terminal histological analysis. We then performed BDA immunohistochemistry stain in order to visualize traced axons. The BDA injection was injected at the same coordinates as the original AAV injection.

My first experimental cohort included LZK cKO, DLK cKO, and LZK/DLK KO. Unfortunately, some experimental mice from the DLK and LZK/DLK cohort had died due to Ketamine-Xylazine (KX) and resulted in an insignificant number of animals for DLK cKO and LZK/DLK cKO groups. These groups were excluded from the final data analysis. After cervical spinal cord sections were collected, a a PKCγ stain was used to confirm complete pyramidotomy

injuries (Figure 10).

The effect of LZK conditional knockout on corticospinal axonal sprouting after pyramidotomy injury

LZK is one of the two mammalian homologues and is of particular interest because, along with DLK, LZK regulates axon growth in vitro (Chen, 2016). In our lab, LZK has previously been studied in a PTEN deleted background and under PTEN/DLK co-deletion. Our interest in this LZK cKO project aimed to isolate the role of LZK in a wildtype background in order to better understand its function in CNS injury. At 6-weeks-old, mice were injected with either AAV-Cre or AAV-GFP into the right sensorimotor cortex. Two weeks after AAV injections, Junmi Saikia performed pyramidotomies on all mice. We identified 6 out of 7 mice that had complete pyramidotomies and proceeded with BDA staining, a stain used to label CST axons by anterograde tracing. Transverse cervical spinal cord sections were then imaged and quantified for further analysis (Figure 16). The results of LZK cKO were compared to wildtype littermates. After thorough normalization and analysis of the data, it was seen in the results that the LZK cKO cohort revealed similar sprouting levels as compared to those seen in wildtype (Figure 17). These results are important as the data suggests that knocking out LZK alone does not promote nor dampen the sprouting capacity after CNS injury. As a result of the low number of experimental mice (n=3), a follow up experiment was performed. In the second cohort that we analyzed, 7 mice were identified to have complete pyramidotomies, including both LZK cKO and their wildtype littermates. Consistent with results from the first cohort, there is no significant difference in the sprouting level between LZK cKO mice and wildtype controls (Figure 18).

Together, the results from these two cohorts of mice indicate that LZK alone does not enhance or reduce the sprouting capacity seen after injury.

The effect of DLK conditional knockout on corticospinal axonal sprouting after pyramidotomy injury

To understand the importance of DLK, the other close MAP3K homologue, in axon injury response, I also compared the effect of DLK conditional knockout to wildtype control littermates. At 6-weeks-old, mice were injected with either AAV-Cre or AAV-GFP into the right sensorimotor cortex. Two weeks after AAV injections, Junmi Saikia performed pyramidotomies on all mice. The DLK conditional knockout study was replicated and a total of 9 out of the 11 mice had complete lesions and were further analyzed. Six of the mice were DLK cKO (injected with AAV-Cre) and the other three mice were wild type (AAV-GFP). Images of the transverse cervical spine were taken and normalized against total axon counts measured in the medullas (Figure 16). The results of this suggest that sprouting may be enhanced when DLK is conditionally knocked out (Figure 20).

Towards understanding the role of DLK and LZK in peripheral nerve regeneration

In order to complement past and present studies conducted in our lab, the focus of the peripheral nerve study was to further understand the roles of DLK and LZK in the peripheral nervous system. This project was done in collaboration with a graduate student, Junmi Saikia. The peripheral nervous system has some baseline regenerative capacity. The genotype of all mice used had UBC-Cre/ERT2 for pan tissue inducible knockout. In our preliminary study, with the sciatic nerve, a total of 13 mice were analyzed. At 8-weeks, tamoxifen was administered via

oral gavage for 5 days. The amount administered over the course of five days was 0.375% of their daily body weight. Tamoxifen induced Cre-deletion of DLK and LZK was later confirmed by Western blot as seen in Figure 9. All mice received the same injury on both sides and were performed at 10-weeks-old. Carbon powder was used to mark the crush site. For the three-day sciatic nerve crush, surgeries an initial conditioning injury was performed followed by the sciatic crush injury three days after. This timeline can be seen in Figure 7B. The crush was performed proximal to the preconditioned lesion. The initial results of our experiment suggest that UBC-cre/ERT2 mice have fewer regenerating axons at around 450-750 microns away from the original injury site (Figure 21). These results suggest that deletion of DLK and LZK diminishes the regenerative capacity of axons after injury. This may support the original hypothesis that both DLK and LZK may be necessary to initiate an injury response that is seen in both the central and peripheral nervous system. Further experiments are needed to confirm these results.

DISCUSSION

From previous literature, injury to the adult central nervous system (CNS) results in permanent functional deficits largely due to lack of axonal repair. The experiments we have conducted and the results that were yielded were aimed to gain a better understanding of the roles of DLK and LZK in the axonal response to injury. We initially wanted to explore the role of DLK and LZK in both the adult central and peripheral nervous system, but in the end focused on the CNS as this is the primary interest of our lab. In addition to studying their roles independently, my project fits into the large project in the lab that aims to understand how these two kinases work together to signal injury and to mediate cellular responses. In analyzing the endogenous roles of DLK and LZK, I focused my thesis work on how deleting DLK and LZK affects corticospinal axon sprouting in an otherwise wildtype background. In this regard, my work complements other projects in the lab that examine DLK and LZK loss of function in an enhanced axon growth background (e.g. by PTEN deletion). It should be noted that all experiments during my thesis project were performed in close collaboration with Ms. Saikia in the lab.

Based on the worm and sciatic nerve studies, we initially had predicted that knocking out DLK would diminish axon sprouting capacity after injury. However, my results suggest that deleting DLK does not decrease sprouting. More animals are required to determine whether deleting DLK increases CST axon sprouting. The RGC literature indicates that DLK can trigger either a pro-growth or a pro-apoptotic response to injury.

In Dr. Jess Meves' results, she showed that induced overexpression of DLK resulted in the decreased level of axonal sprouting compared to respective PTEN deletion and

wildtype control groups, and knocking out DLK does the reverse, which was unexpected. In order to further investigate this, Junmi Saikia worked to delete both DLK and LZK, which blocked sprouting-enhancing effect of PTEN deletion and indeed the DLK/LZK/PTEN triple KO mice showed very similar levels of sprouting as wildtype controls. Thus, it appears that even in a PTEN deleted background, deleting both LZK and DLK loses the ability to trigger any type of sprouting response that occurs after injury. Together, these results suggest that there may be some amount of kinase signaling from DLK/LZK that is necessary to initiate any injury response mechanism, and particularly axonal sprouting in the context of this study. Additionally, I have also worked in collaboration with Ms. Saikia to investigate the role of LZK alone in a PTEN deleted background. The results appeared to have similar levels to those seen in wildtype backgrounds. These results, however, need to be replicated.

The results of the LZK cKO cohort showed similar levels to that of control levels, suggesting that the deletion of LZK alone is not enough to reduce or enhance levels of sprouting seen after CNS injury. This is in contrast to the DLK cKO cohort that appeared to have enhanced levels of sprouting index as compared to the control, although that result needs more animals for confirmation. Regardless, so far neuronal deletion of DLK or LZK alone does not appear to reduce CST axon sprouting. Together with the results from Ms. Saikia on DLK/LZK/PTEN triple knockout mice, it is likely that DLK and LZK have redundant roles in mediating some type of injury signals to enable a sprouting response.

In a separate experiment, our preliminary results from the peripheral nerve study suggests that knocking out DLK and LZK have less regeneration of axons at around 450-750 microns

away from the injury site. Though this is a preliminary result, this finding suggests that knocking out both kinases may actually reduce the amount of regeneration seen in the peripheral nervous system, thus providing support to previous studies that suggest there must be some level of kinase activity that is necessary to initiate an injury response. The peripheral nerve study was particularly difficult as it required a lot of troubleshooting. In particular, the identification of where the injury site remained the recurring and forefront challenge of this study. As a result, we were unable to produce repeatable results and were thus, unable to draw any significant conclusions on our study.

While we first utilized fluorescent beads to mark the site of injury, we found it incredibly challenging to identify any signal when taking images and visualizing the tissue sample under the microscope. As a result, we resorted to using carbon powder and saw better results. In the most recent cohort that we ran, I attempted to use a new method of immunohistochemistry taught to me by a Postdoctoral fellow, Dr. Daniel Romaus-Sanjurjo. This new method did not require the use of a vacuum for slide mounted immunohistochemistry and instead used a slip of Parafilm to cover all tissue sections on the slide. There were no noticeable differences using this method. Furthermore, when several tissue sections were lost during the post-processing stage, we decided to proceed with gelatin-coated slides to allow for better tissue adhesion. Though several steps were taken to improve our experiment, there still remains much to be done. For starters, while our peripheral nerve models have been in the form of 3-day crush, I believe that having future experiments that focus on complete transection of the nerve. This will allow for a clear landmark to identify the injury site. Furthermore, with our traditional method of quantifying axons, I

believe that ongoing methods of measuring and analyzing axon length would help enhance and improve the accuracy of our results.

In conclusion, the data from my CNS sprouting study suggest that conditionally knocking out LZK does not reduce nor enhance CST axon sprouting levels after injury. Furthermore, results from conditionally knocking out DLK suggest that sprouting, however, may be enhanced. In our peripheral nerve study, the results suggest that knocking out both DLK and LZK show dampened regeneration of axons around 450-750 microns away from the injury site. These results, along with other unpublished findings from the lab indicate that DLK and LZK have complex roles in regulating axonal repair in the mammalian spinal cord.

Figure 6 is currently being prepared for submission for publication of the material. Suen, Juliet; Saikia, Junmi. The thesis author was the primary investigator and author of this material.

REFERENCES

Bradke, F., Fawcett, J. & Spira, M. Assembly of a new growth cone after axotomy: the precursor to axon regeneration. *Nat Rev Neurosci* **13,** 183–193 (2012).

Chang L, Karin M. (2001). Mammalian MAP kinase signaling cascades. *Nature* **410**: 37–40.

Chen M, Geoffroy CG, Wong HN, Tress O, Nguyen MT, Holzman LB, Jin Y, Zheng B (2016) Leucine Zipper-bearing Kinase promotes axon growth in mammalian central nervous system neurons. Sci. Rep. 6: 1-16.

David S, Aguayo AJ. Axonal elongation into peripheral nervous system "bridges" after central nervous system injury in adult rats. Science. 1981; 214:931–933.

Geoffroy CG, Zheng B. Myelin-associated inhibitors in axonal growth after CNS injury. *Curr Opin Neurobiol*. 2014; 27:31-38.

Geoffroy CB, Lorenzana AO, Kwan JP, Lin K, Ghassemi O, Ma A, Xu N, Creger D, Liu K, He Z, Zheng B (2015) Effects of PTEN and Nogo Codeletion on Corticospinal Axon Sprouting and Regeneration in Mice. J. Neurosci. 35: 6413- 6428.

Guertin DA, Sabatini DM. Defining the role of mTOR in cancer. *Cancer Cell*. 2007;12(1):9-22.

Hammarlund M, Nix P, Hauth L, Jorgensen EM, Bastiani M (2009) Axon Regeneration Requires a Conserved MAP Kinase Pathway. Science. 323: 802-806.

Huang, H., Miao, L., Yang, L., Liang F., Wang, Q., Zhuang, P., Sun, Y., Hu, Y.,. AKT-dependent and -independent pathways mediate PTEN deletion-induced CNS axon regeneration. *Cell Death Dis* 10, 203 (2019).

Huebner, E. A., & Strittmatter, S. M. (2009). Axon regeneration in the peripheral and central nervous systems. *Results and problems in cell differentiation*, *48*, 339–351.

Jin Y, Zheng B (2019) Multitasking: Dual Leucine Zipper-Bearing Kinases in Neuronal Development and Stress Management. Annu Rev Cell Dev Biol. 35:501-521.

Kaplan, A., Ong Tone, S., & Fournier, A. E. (2015). Extrinsic and intrinsic regulation of axon regeneration at a crossroads. *Frontiers in molecular neuroscience*, *8*, 27. https://doi.org/10.3389/fnmol.2015.00027

Karin M, Chang L (2001) Mammalian MAP kinase signaling cascades. Nature 410: 37-40.

La Fleur M, Underwood JL, Rappolee DA, Werb Z. Basement membrane and repair of injury to peripheral nerve: defining a potential role for macrophages, matrix metalloproteinases, and tissue inhibitor of metalloproteinases 1. The Journal of experimental medicine. 1996; 184:2311–2326.

Lemon RN, Griffiths J. Comparing the function of the corticospinal system in different species: organizational differences for motor specialization?. *Muscle Nerve*. 2005;32(3):261-279.

Liu, K., Tedeschi, A., Park, K. K., & He, Z. (2011). Neuronal Intrinsic Mechanisms of Axon Regeneration. *Annual Review of Neuroscience, 34*(1), 131-152. doi:10.1146/annurev-neuro-061010-113723

National Spinal Cord Injury Statistical Center, Facts and Figures at a Glance. Birmingham, AL: University of Alabama at Birmingham, 2019.

Neumann S, Woolf CJ. Regeneration of dorsal column fibers into and beyond the lesion site following adult spinal cord injury. *Neuron*. 1999;23(1):83-91.

Shin, J. E., Ha, H., Kim, Y. K., Cho, Y., & DiAntonio, A. (2019). DLK regulates a distinctive transcriptional regeneration program after peripheral nerve injury. *Neurobiology of disease*, *127*, 178–192.

Shin, J. E., Cho, Y., Beirowski, B., Milbrandt, J., Cavalli, V., & DiAntonio, A. (2012). Dual leucine zipper kinase is required for retrograde injury signaling and axonal regeneration. *Neuron*, *74*(6), 1015–1022. https://doi.org/10.1016/j.neuron.2012.04.028

Steward, O., Zheng, B., Ho, C., Anderson, K. and Tessier-Lavigne, M. (2004), The dorsolateral corticospinal tract in mice: An alternative route for corticospinal input to caudal segments following dorsal column lesions. J. Comp. Neurol., 472: 463-477.

Tedeschi A, Bradke F. The DLK signalling pathway--a double-edged sword in neural development and regeneration. *EMBO Rep*. 2013;14(7):605-614.

Tedeschi A, Bradke F. Spatial and temporal arrangement of neuronal intrinsic and extrinsic mechanisms controlling axon regeneration. *Curr Opin Neurobiol*. 2017; 42:118-127.

Tremblay RG, Sikorska M, Sandhu JK, Lanthier P, Ribecco-Lutkiewicz M, Bani-Yaghoub M. Differentiation of mouse Neuro 2A cells into dopamine neurons. *J Neurosci Methods*. 2010;186(1):60-67.

Valakh, V., Frey, E., Babetto, E., Walker, L. J., & DiAntonio, A. (2015). Cytoskeletal disruption activates the DLK/JNK pathway, which promotes axonal regeneration and mimics preconditioning injury. *Neurobiology of disease*, *77*, 13–25. https://doi.org/10.1016/j.nbd.2015.02.014

Watkins TA, Wang B, Huntwork-Rodriguez S, Yang Z, Eastham-Anderson J, Modrusan Z, Kaminker J, Tessier-Lavigne M, Lewcock J (2013) DLK initiates a transcriptional program that couples apoptotic and regenerative responses to axonal injury. Proc. Natl. Acad. Sci. United States 110: 4039-4044.

Yan D, Wu Z, Chisholm AD, Jin Y (2009) The DLK-1 kinase promotes mRNA stability and local translation in C. elegans synapses and axon regeneration. *Cell* vol.138,5: 1005-1018.

FIGURES

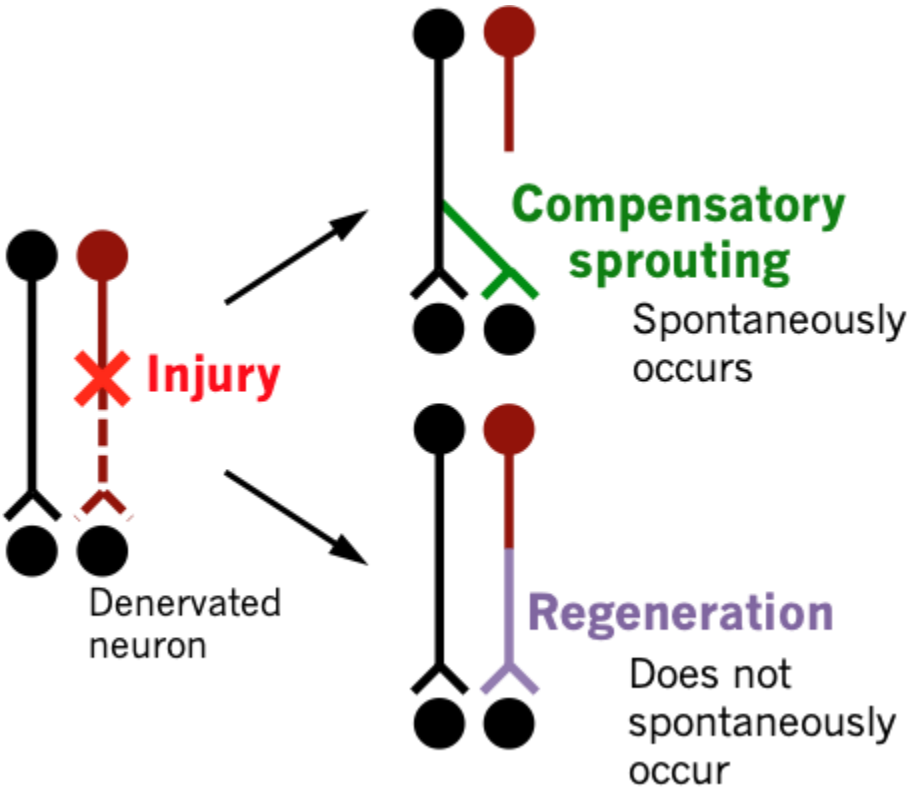


Figure 1: Diagram of two types of axonal growth after spinal cord injury.
After spinal cord injury, compensatory sprouting occurs when growth is induced from uninjured axons and forms synapses with targets from the denervated neurons. The other type of axonal growth is regeneration and occurs when growth is induced from previously injured axons. (Meves et al., 2018)

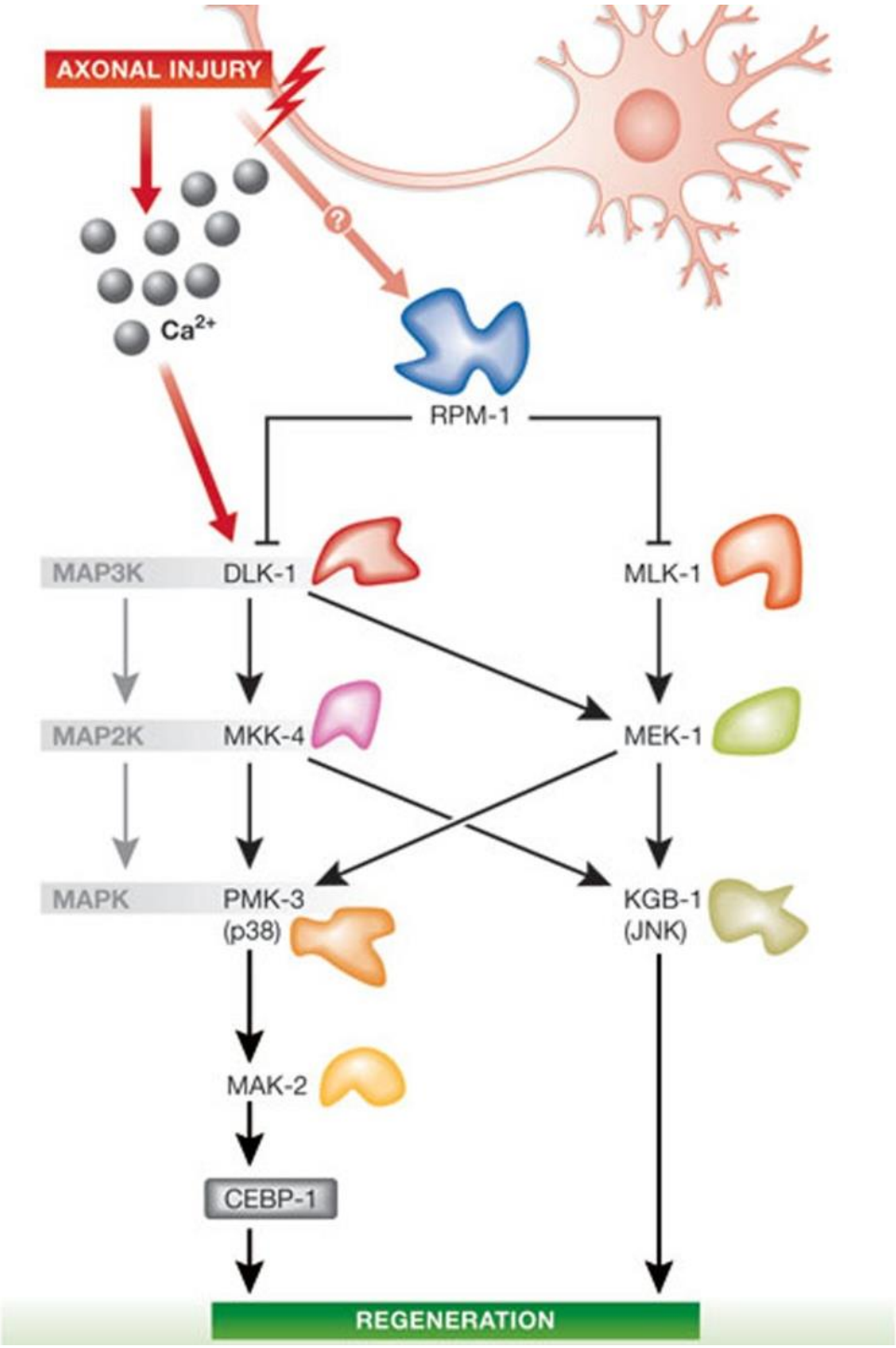


Figure 2: Illustration of MAP3K pathway (Tedeschi & Bradke, 2013).

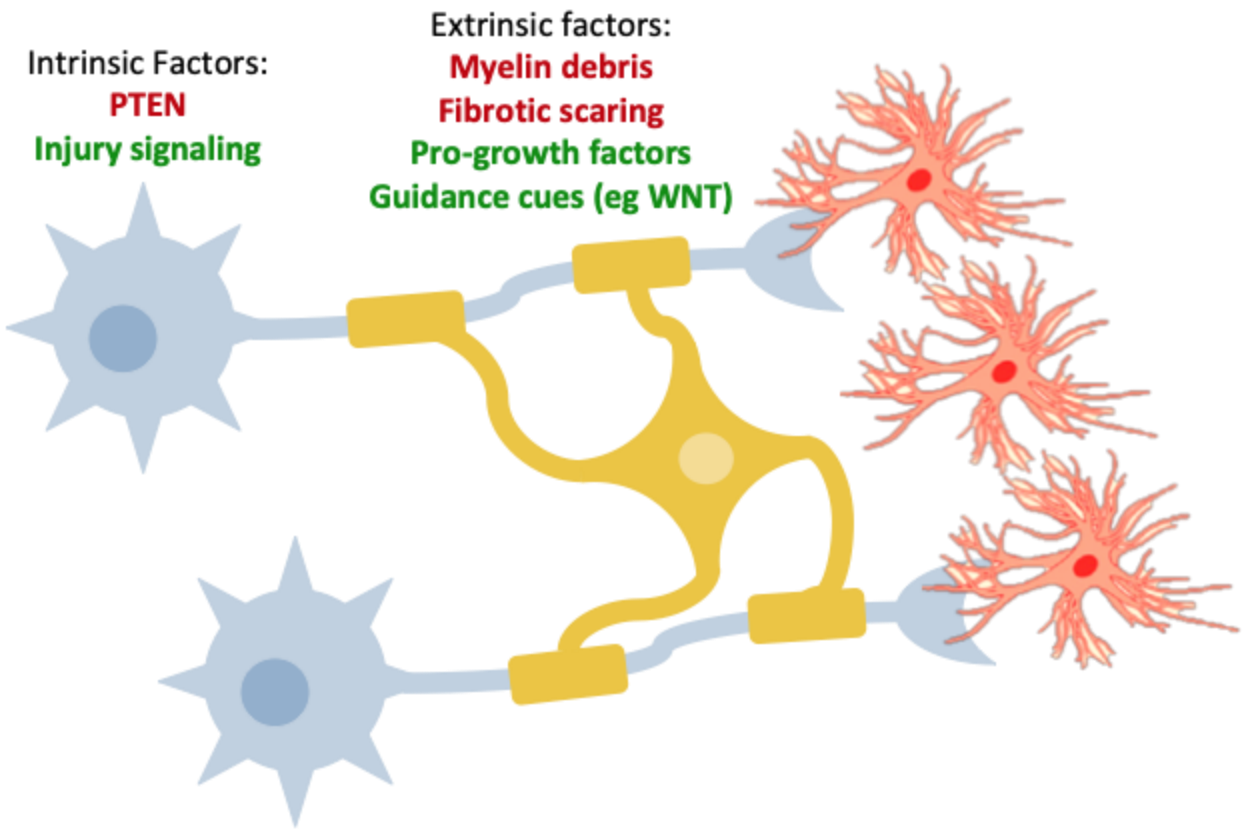
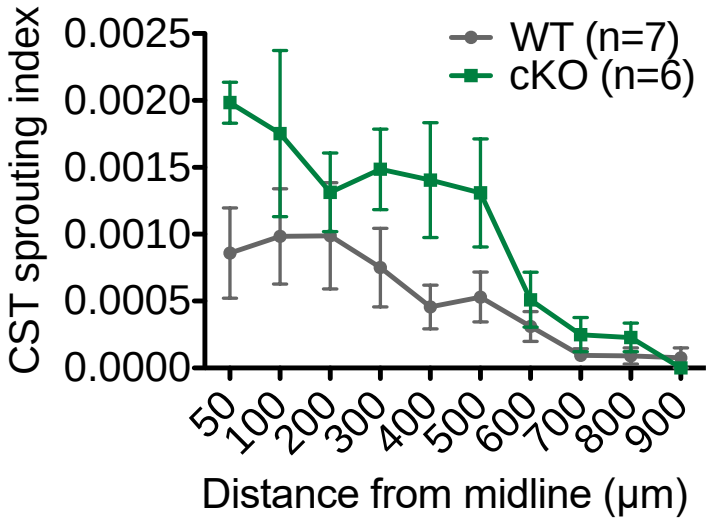


Figure 3: Diagram of intrinsic and extrinsic factors affecting axonal repair mechanisms in the mammalian central nervous system (CNS).
The regenerative capacity seen in the adult CNS is hindered by extrinsic factors such as astrogliosis, myelin debris, fibrotic scarring. Intrinsic factors may be attributed to protein expression and injury signaling pathways (Tedeschi and Bradke, 2017).

A



B

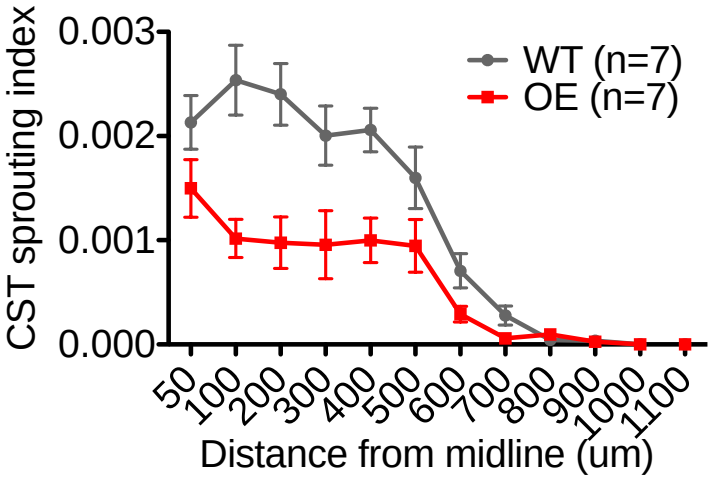


Figure 4: Graph of sprouting index of DLK KO and DLK OE in a wildtype background from previous experiments conducted by Dr. Jessica Meves.

(A) Graph of sprouting index of DLK KO and (B) DLK OE in wildtype background (Meves).

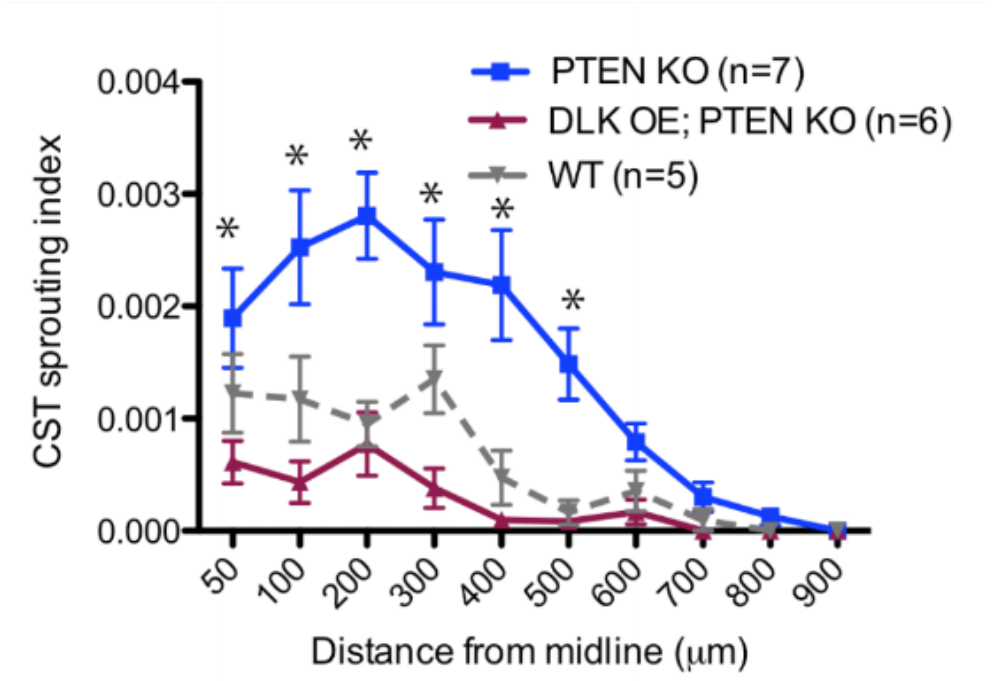


Figure 5: Graph of sprouting index of DLK KO in a PTEN deleted background in a previous experiment conducted by Dr. Jessica Meves.
Graph of sprouting index of DLK KO in PTEN deleted background (Meves).

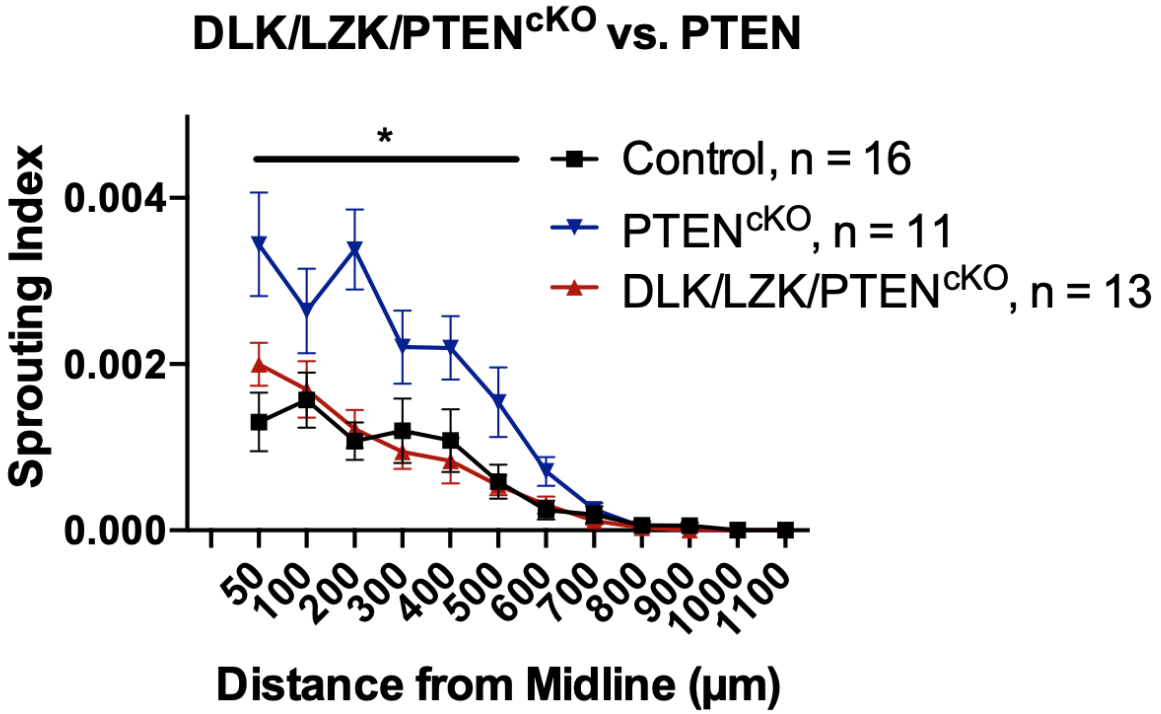
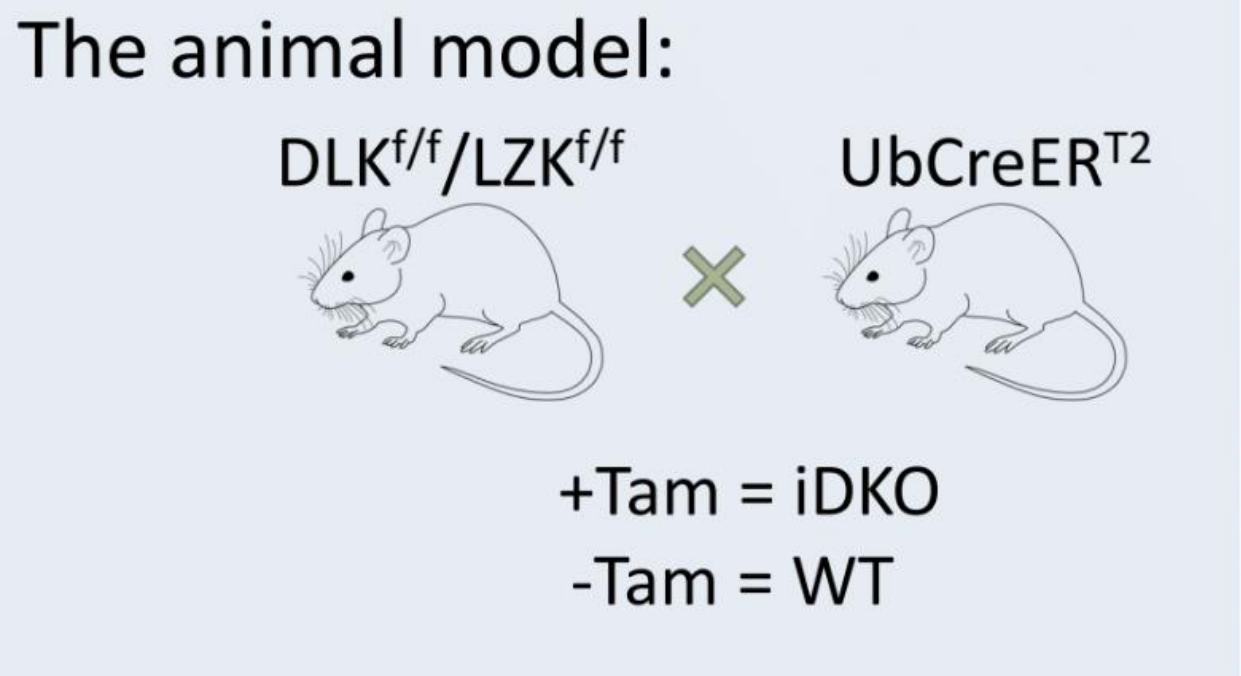


Figure 6: Graph of sprouting index of DLK/LZK/PTEN TKO and PTEN cKO taken from previous experimental findings conducted by Junmi Saikia.
Graph of sprouting index of TKO (DLK/LZK/PTEN) and PTEN cKO data taken from previous experiments (Saikia, 2019).

A



B

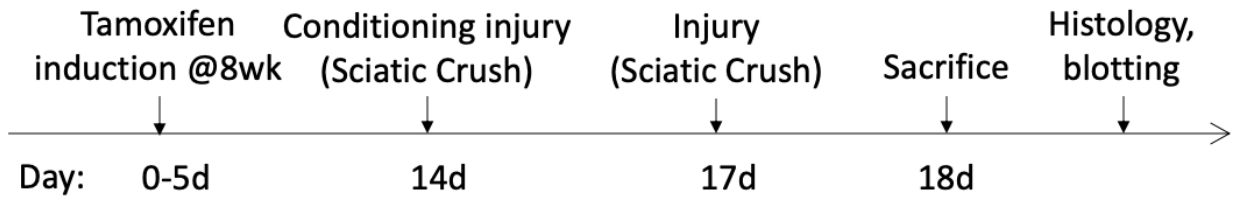
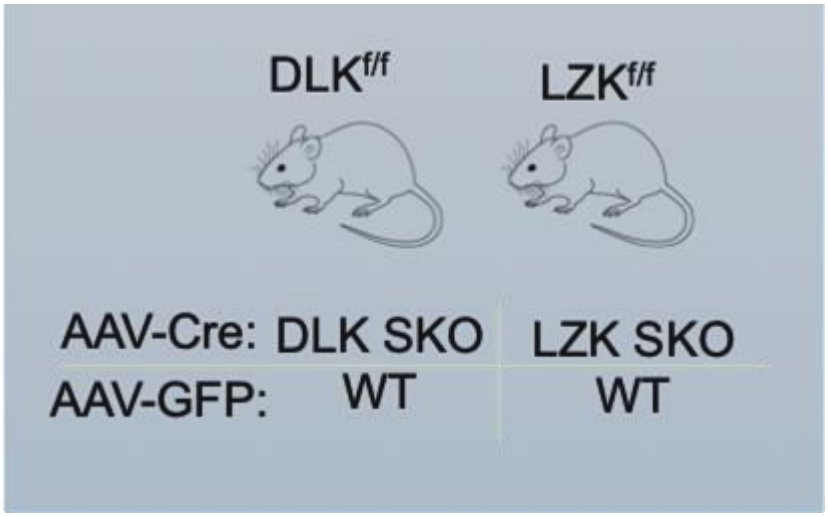


Figure 7: Breeding scheme of tamoxifen induced Cre-deletion of DLK and LZK
(A) Schematic of $LZK^{f/f}/DLK^{f/f}$ mouse bred to a Ub-$CreER^{T2}$ transgenic mouse line. (B) Schematic timeline of experiment of peripheral nerve study.

A



B

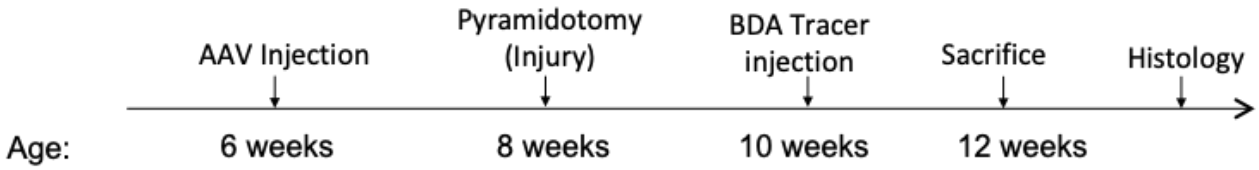


Figure 8: Schematic of animal model and experimental timeline.
(B) Animal model for experiment. (B) Schematic timeline of experiment starting with AAV injections at 6 weeks old for all mice.

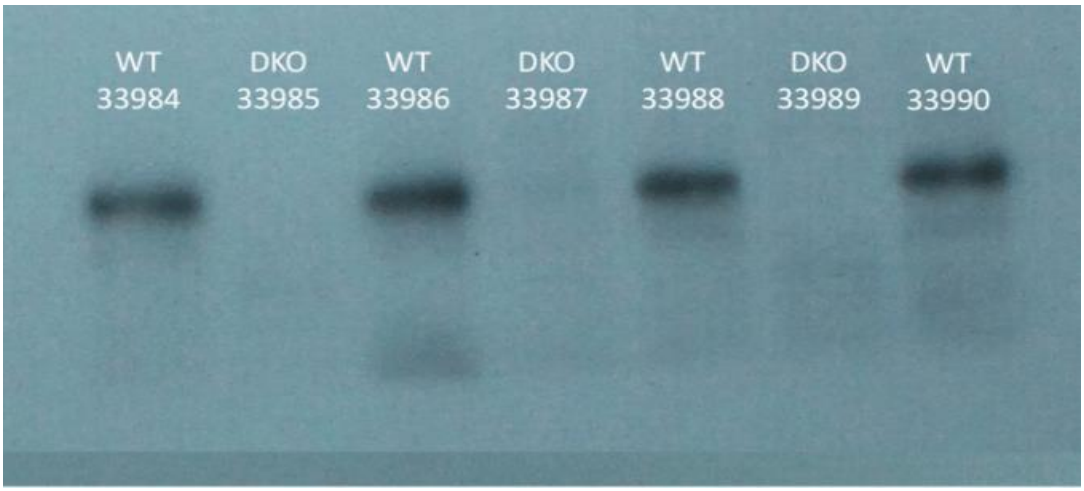


Figure 9: Western blot
In order to confirm the Tamoxifen induced Cre-deletion of DLK and LZK, a western blot was performed.

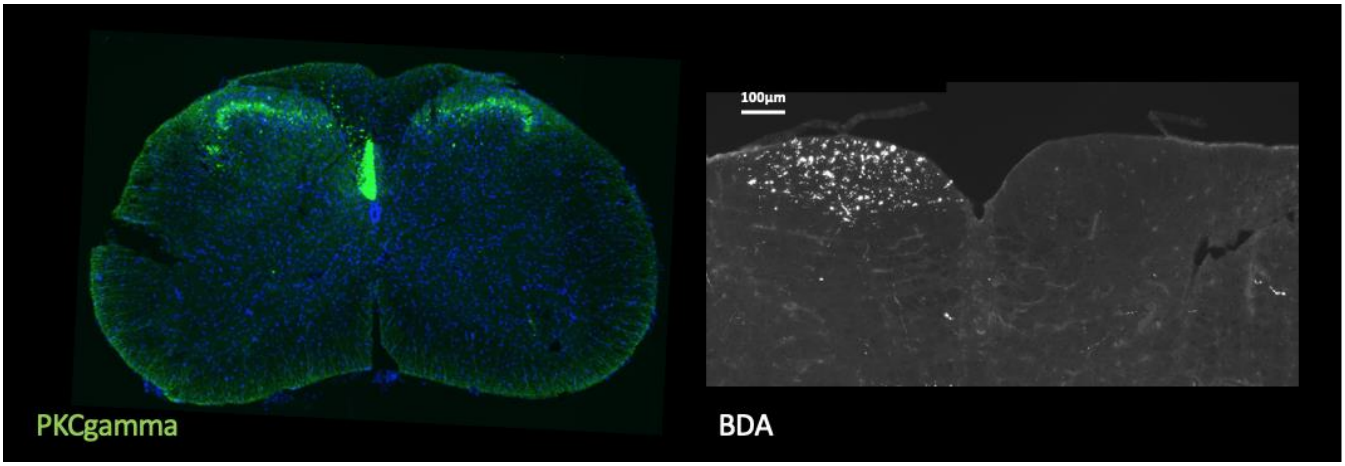


Figure 10: Validating methods using PKCgamma and BDA immunohistochemistry.
PKCgamma staining was used to validate complete pyramidotomy injuries. BDA staining was performed to stain traced axons.

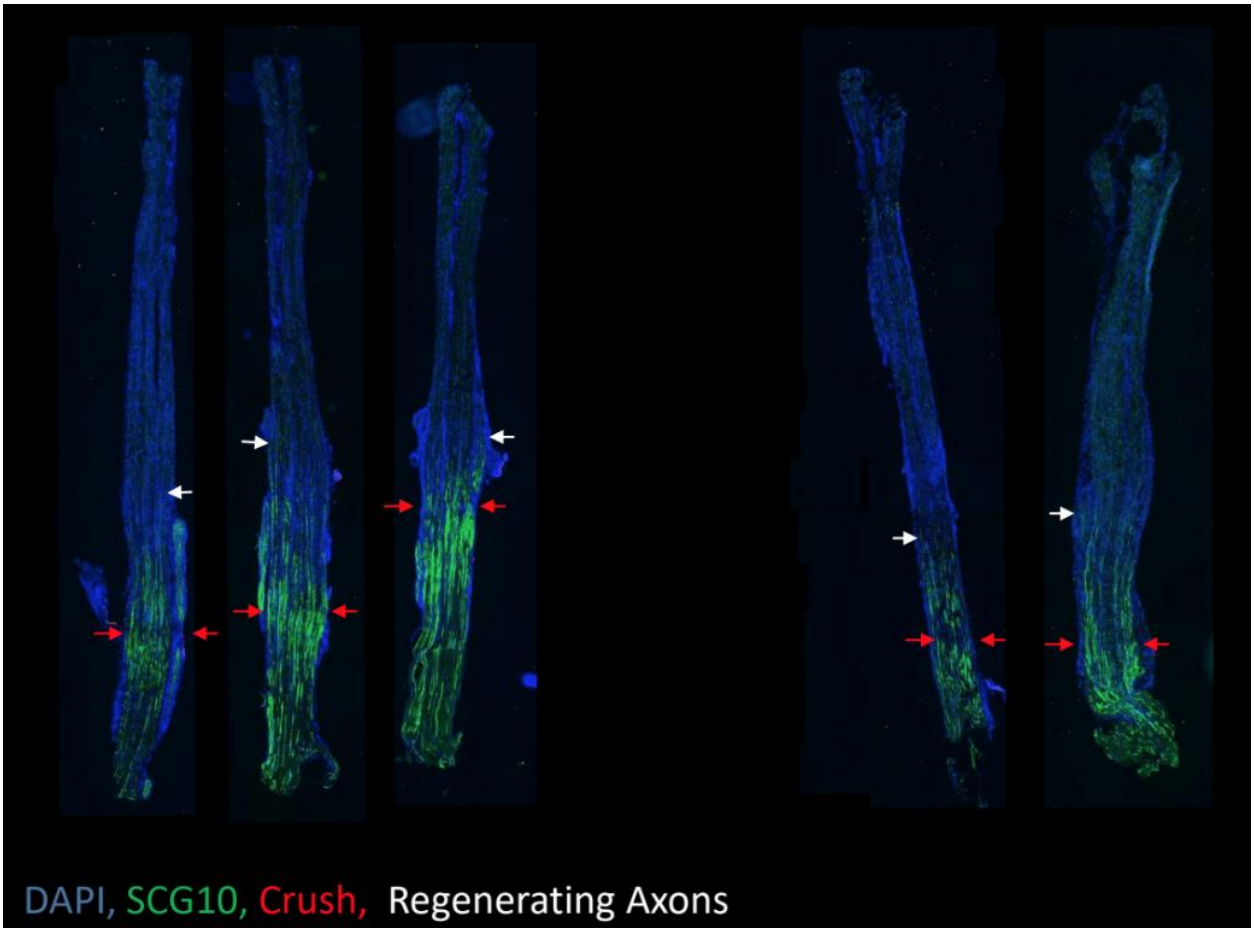


Figure 11: Images of Sciatic Nerve Crush
Wildtype (left) and UbCre+ (right) images of sciatic nerve crush. SCG10, a marker for peripheral nerve regeneration is indicated in green while the red arrows indicate the site of crush.

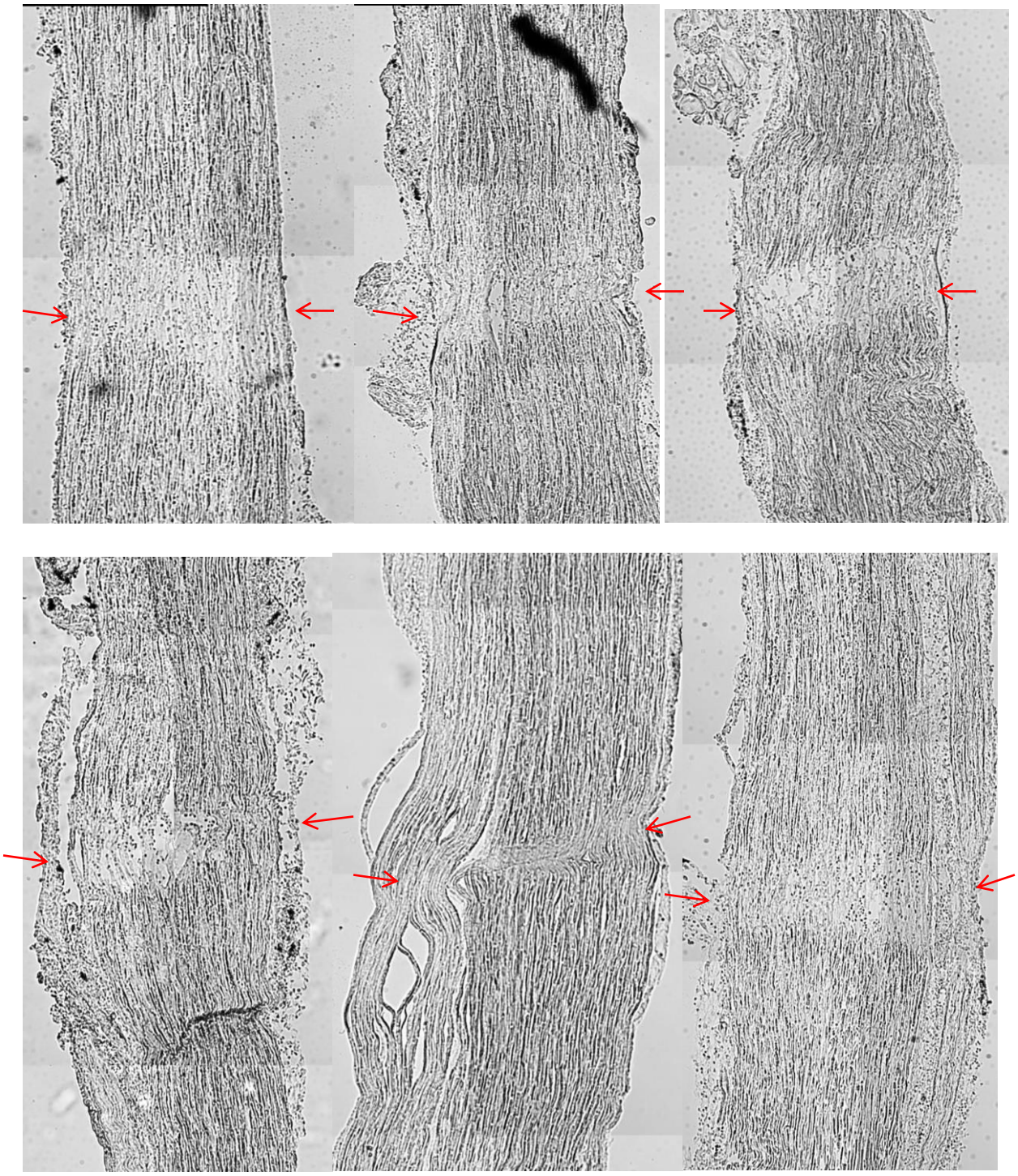


Figure 12: Brightfield Images
Brightfield images of sciatic nerve from wildtype (top) and UbCre+ (bottom) mice. The red arrows indicate the site of crush injury.

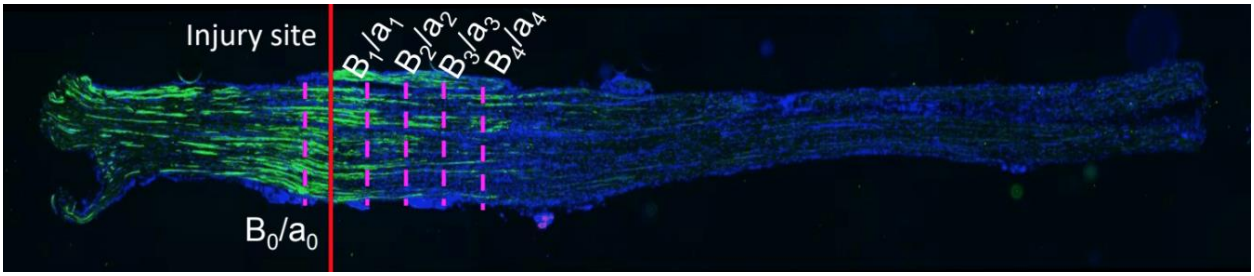


Figure 13: Quantification Method
Visualization of how quantifications were normalized for data analysis. The red line indicates the site of injury and B_0/a_0 marks 100 microns below injury site. B_x/a_x marks each 100 microns after injury. Total number of events was recorded and normalized with B_0/a_0. B indicated the number of events and a represented the cross sectional area.

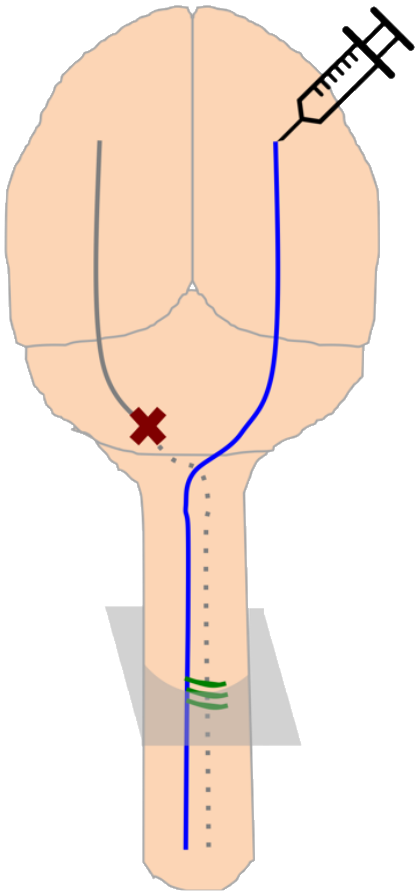


Figure 14: Illustration of a unilateral pyramidotomy model used in experiments
Red "x" marking cut site of injury performed at medullary pyramids. Blue track indicating the BDA tracing. Green lines represent sprouting across the midline.

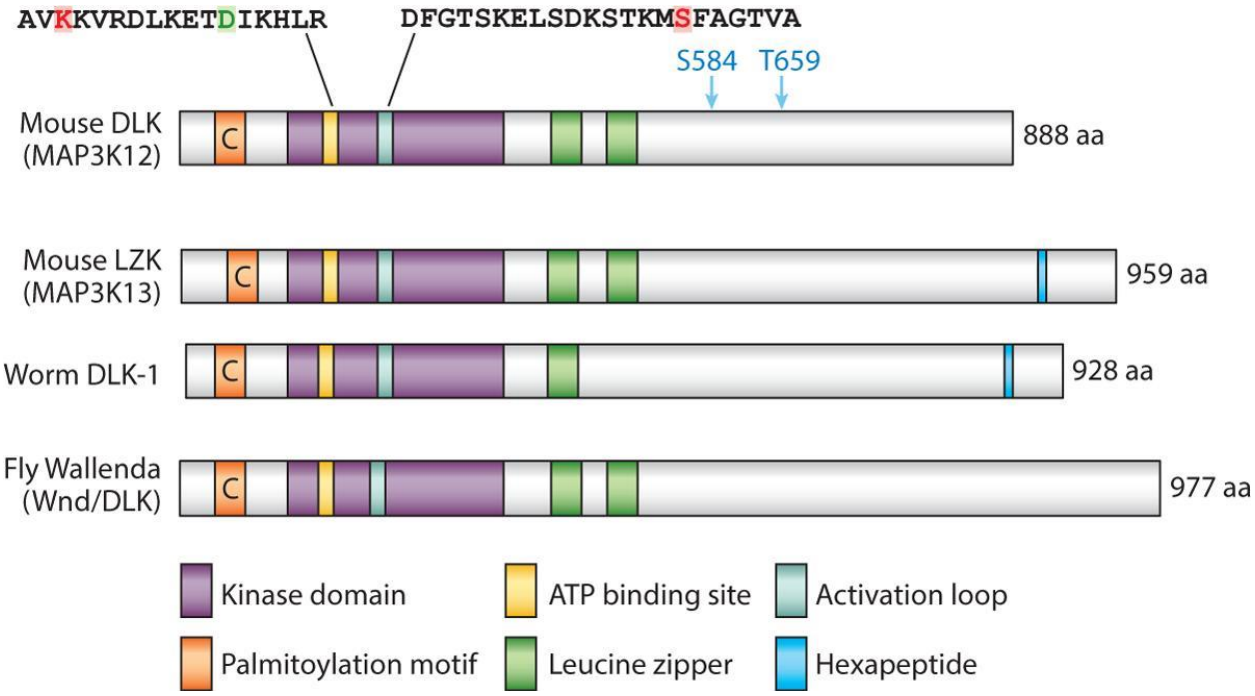
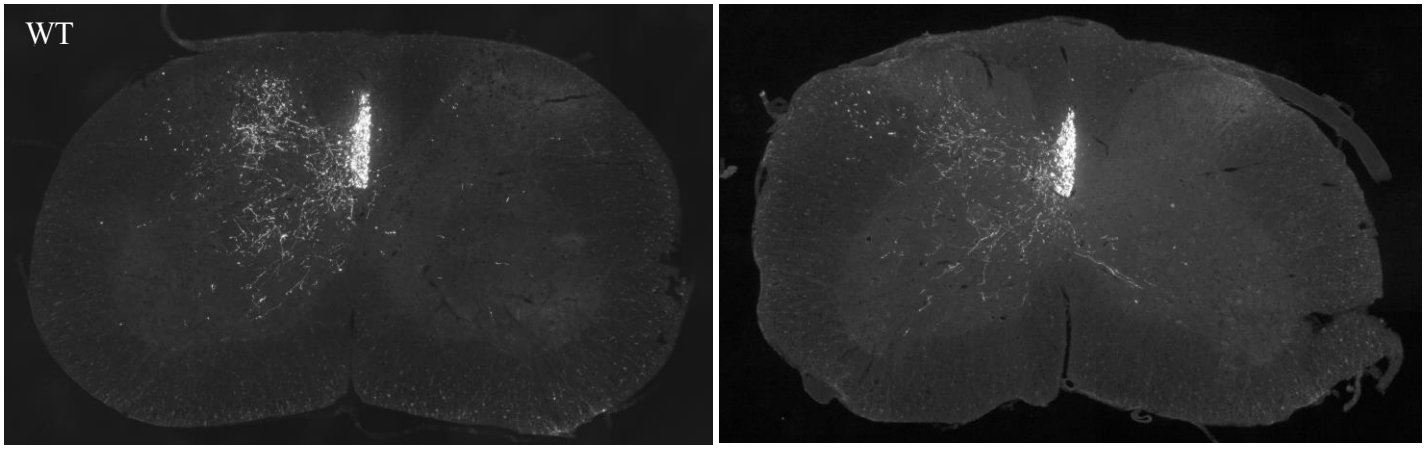
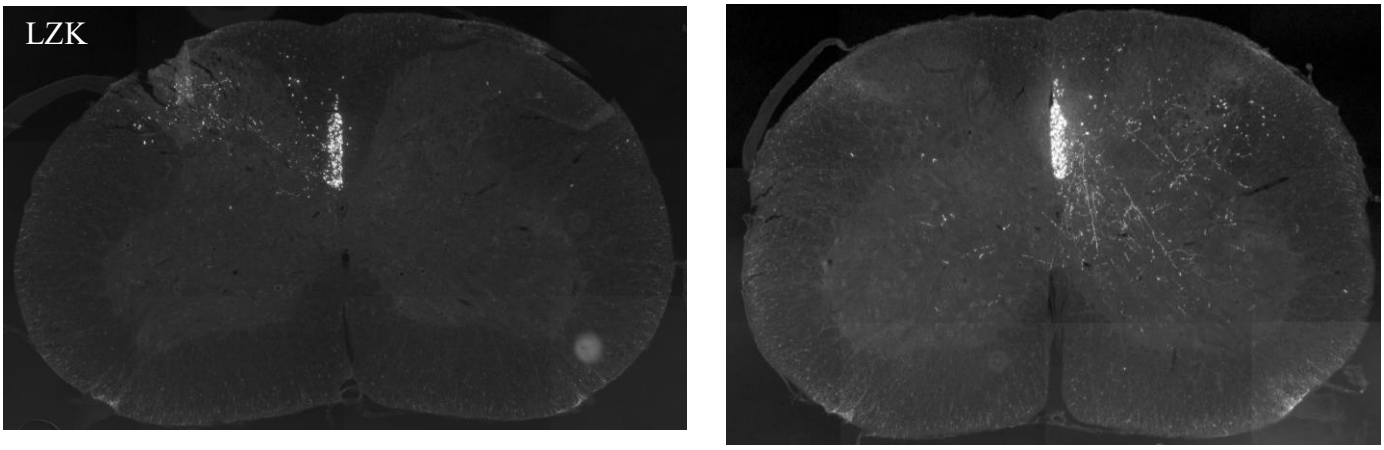


Figure 15: Schematic of experimental timeline and DLK and LZK construct.
(A) DLK-1 construct of *C. elegans* and in mammals (Jin, 2020).

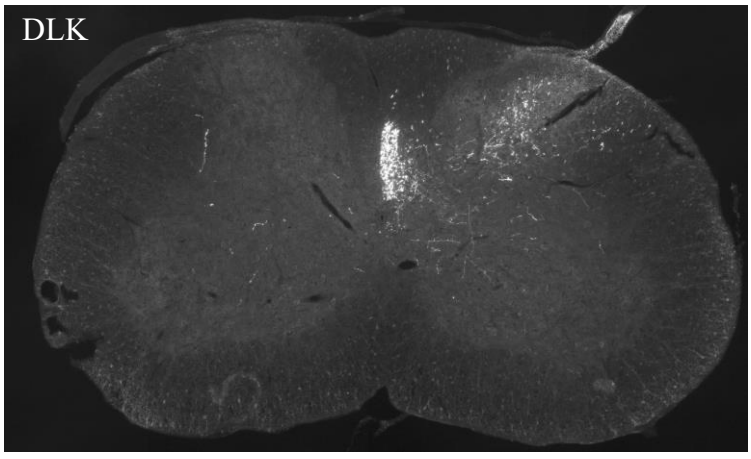
A



B



C



D

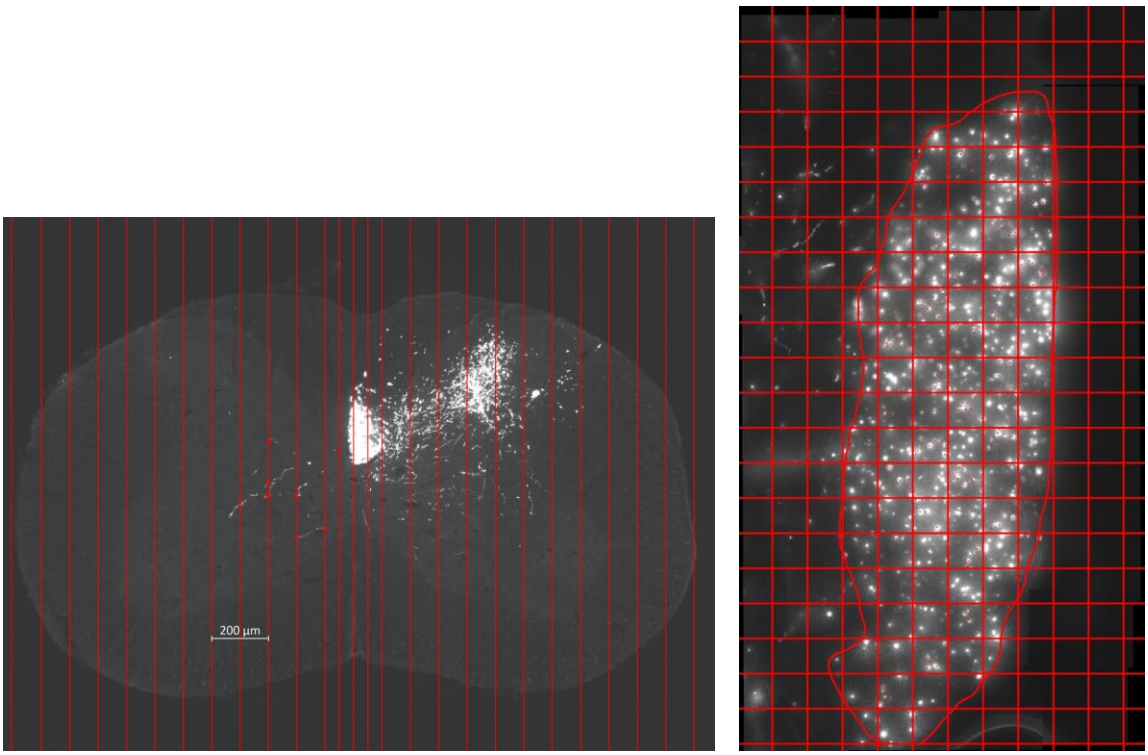


Figure 16: Images for cervical spines and medulla quantifications
Transverse cervical spines were imaged and quantified using Zen. (A) Transverse cervical spine taken from wildtype mice. (B) Transverse cervical spine taken from LZK cKO mice. (C)Transverse cervical spine taken from DLK cKO. Medulla images were taken at 20x and quantified. (D) Quantification template for cervical spine (left) and medullas (right).

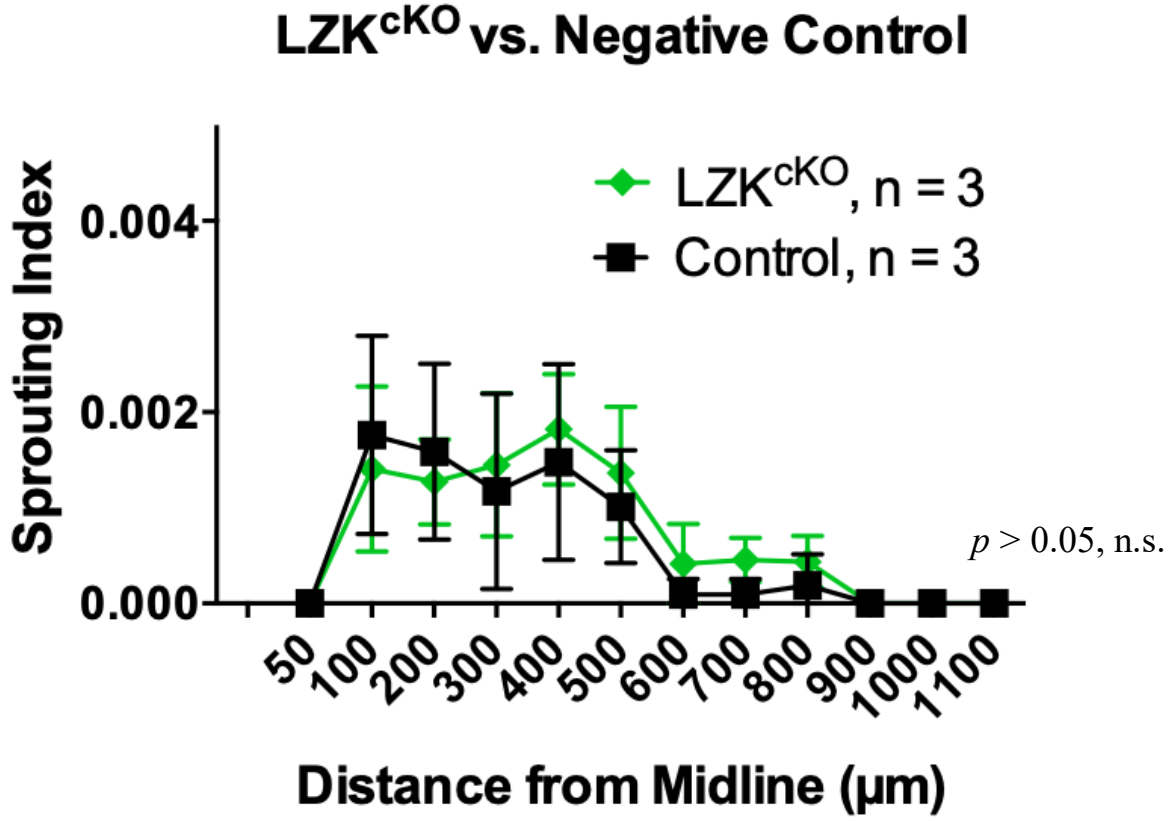


Figure 17: Graph of sprouting index of LZK cKO
Graph of sprouting index of LZK cKO and wildtype data taken from previous experiments. Wildtype mice were littermates with experimental mice.

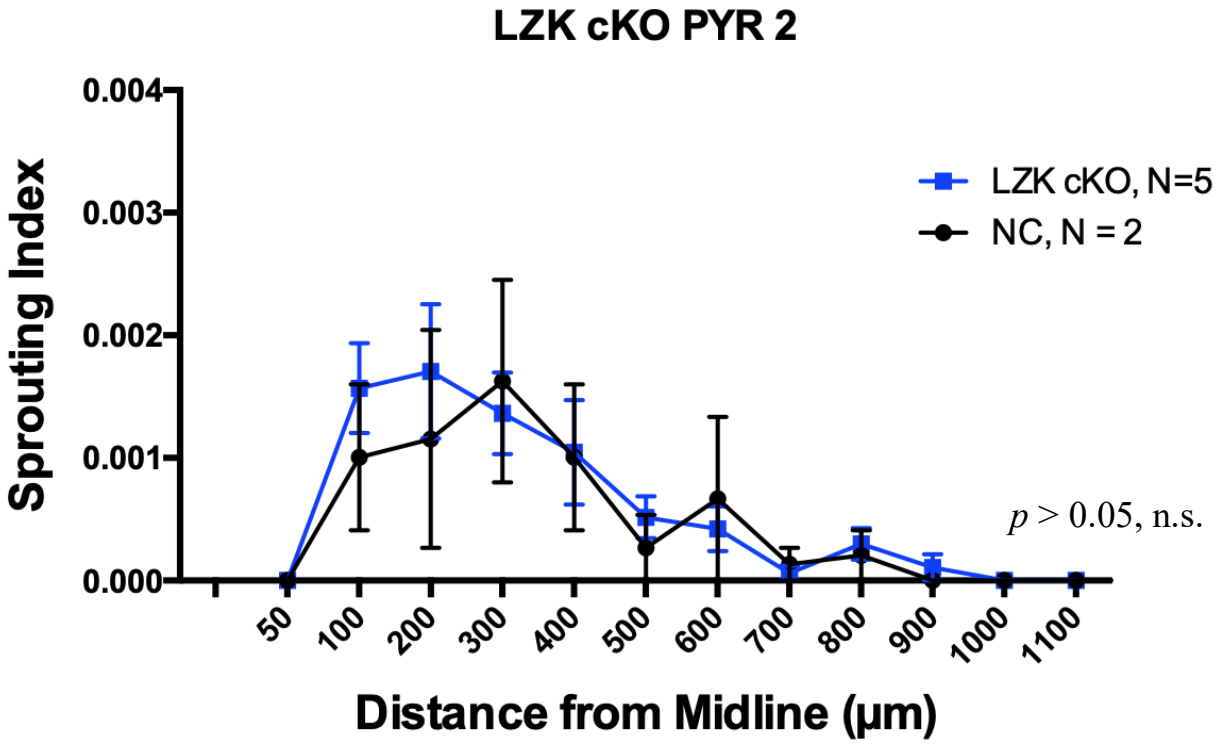


Figure 18: Graph of sprouting index of second LZK cKO cohort conducted
Graph of sprouting index of LZK cKO and wildtype data. Wildtype mice were littermates with experimental mice.

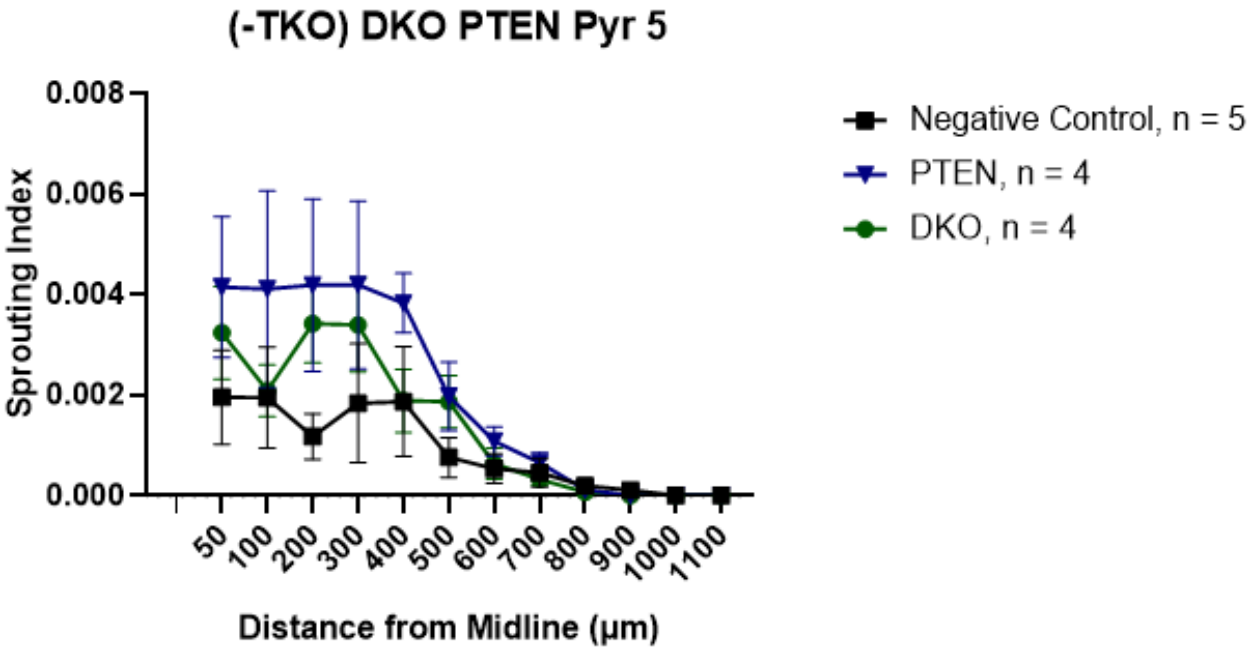


Figure 19: Graph of sprouting index of LZK/PTEN DKO and PTEN cKO from previous experiments conducted by Junmi Saikia.
Graph of sprouting index of LZK/PTEN and PTEN cKO data taken from previous experiments (Saikia, 2019).

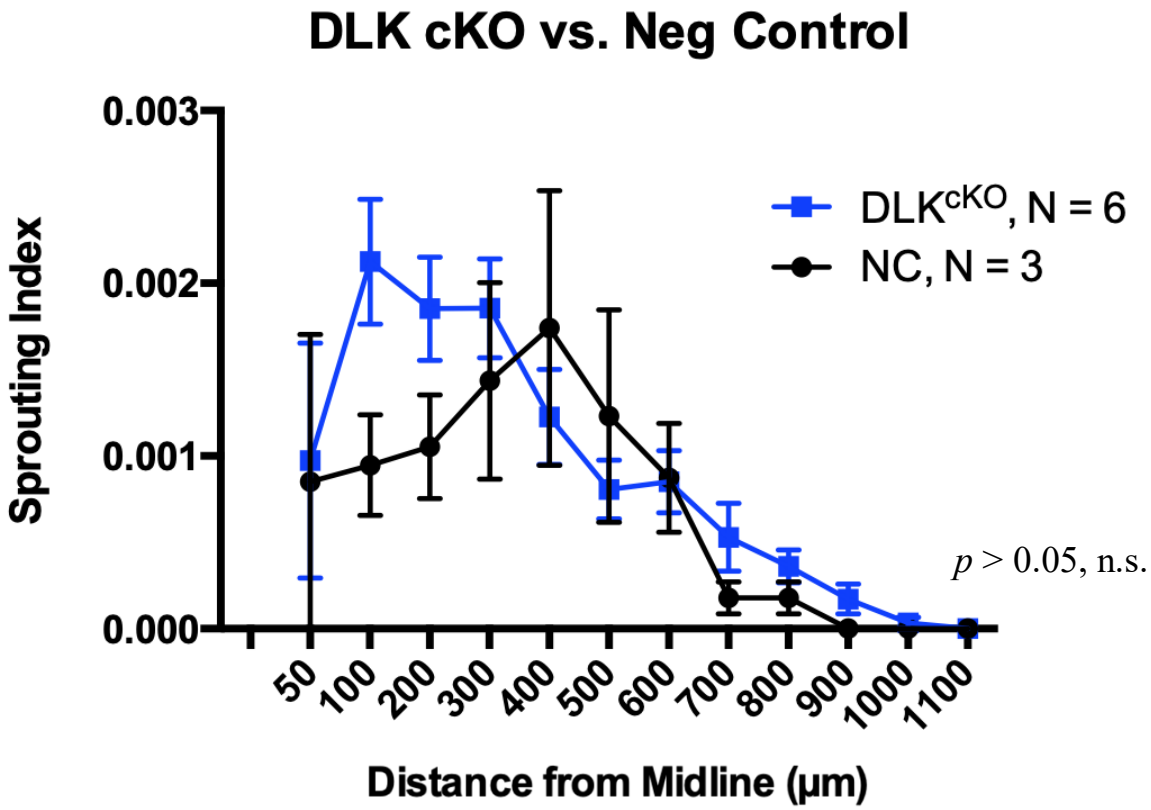


Figure 20: Graph of sprouting index of DLK cKO
Graph of sprouting index of DLK cKO and wildtype data taken from previous experiments. Wildtype mice were littermates with experimental mice.

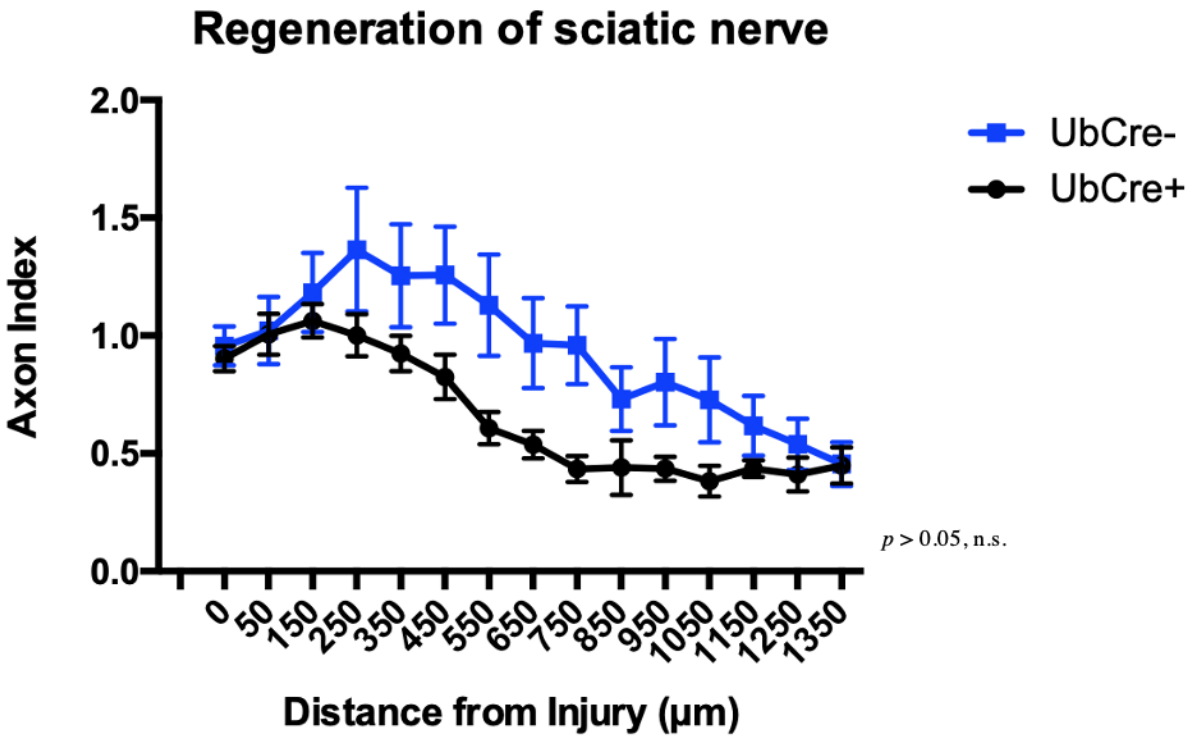


Figure 21: Graph of Regeneration of Sciatic Nerve
Preliminary data of regeneration index from peripheral nerve study.