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An expression analysis of the mammalian dual leucine zipper-bearing kinase (DLK) after axonal injury

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

in

Biology

by

Jeffrey Kwan

Committee in charge:

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

2011

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University of California, San Diego

2011

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List of Abbreviations

cDNA	complementary deoxyribonucleic acid
CNS	central nervous system
CST	corticospinal tract
Ct	cycle threshold
DLK	dual leucine zipper-bearing kinase
DRG	dorsal root ganglion
Het.	heterozygous
IHC	immunohistochemistry
JNK	c-Jun N-terminal kinase
KO	knockout
MAPK	mitogen-activated protein kinase
MAPKK	mitogen-activated protein kinase kinase
MAPKKK	mitogen-activated protein kinase kinase kinase
MAP3K12	mitogen-activated protein kinase kinase kinase 12
MFC	microfluidic chamber
mRNA	messenger ribonucleic acid
PNS	peripheral nervous system
P0	postnatal day 0
qPCR	quantitative real time polymerase chain reaction
SC	spinal cord

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ABSTRACT OF THE THESIS

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by

Jeffrey Kwan

Master of Science in Biology

University of California, San Diego, 2011

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

The peripheral nervous system (PNS) and the central nervous system (CNS) vary in their ability for axon regrowth after trauma resulting in limited or lack of functional recovery in the CNS. Studies in *C. elegans* and *Drosophila* have shown that an evolutionarily conserved mitogen-activated protein kinase kinase kinase (MAPKKK), DLK, is necessary for axon growth cone formation and axon regeneration. Whether its mammalian ortholog plays a similar role remains unknown. We assessed the expression of murine DLK after *in vitro* axotomy and after sciatic nerve crush and spinal dorsal

hemisection injury in vivo. We did not observe any change in the DLK mRNA expression level in cell bodies *in vivo* or *in vitro* after axotomy of dorsal root ganglion (DRG) neurons. Following spinal cord injury, DLK mRNA levels exhibited a significant 4-fold decrease 3 and 5 days post injury, which was accompanied by a significant decrease of DLK protein at the injury site. Finally, DLK mRNA levels in the sciatic nerve after PNS injury decreased initially (i.e. one day post injury), but increased 2 fold compared to controls 5 days post injury. These data are consistent with the hypothesis that DLK may regulate mammalian axon regeneration.

I. Introduction

Spinal cord injury currently affects ~265,000 Americans with ~12,000 newly documented cases per year (National Spinal Cord Injury Statistical Center, 2011). This debilitating condition can cost patients millions of dollars in treatments, severely decrease the life expectancy of patients, and decrease quality of life depending on location and severity of spinal cord injury. Damage to the central nervous system (CNS) leads to partial or complete loss of signals sent to and from the peripheral nervous system (PNS) which, in turn, impairs or eliminates the communication between the brain and body. The PNS distributes nerves throughout the entire body with many nerves in close proximity to the surface of the skin leading to higher susceptibility to injury from everyday life, whereas the CNS is encased in a bony skull and vertebral column in addition to cerebrospinal fluid to absorb external shocks for extra protection (Afshari et al., 2009). A possible evolutionary explanation for the lack of CNS regeneration could stem from the fact that in higher organisms, a complex functional organization would be disturbed from an increased number of unwanted sprouting of axons or miswirings of CNS circuitry after injury to the CNS (Afshari et. al., 2009). Permanent disability can occur after CNS injury due to the limited regenerative ability of the CNS, while the PNS often times is able to recover from injuries. Severed axons displaying retraction dystrophic terminal bulbs were originally thought incapable of regrowth (Ramon y Cajal, 1928). It was later shown that when severed axons were introduced to a permissive growth environment supplied by a peripheral nerve graft, the ability for injured axons to regrow were restored (David and Aguayo, 1981). The inhibitory growth environment of

the CNS differs from the permissive growth environment of the peripheral nervous system (PNS) and partially explains the failure of axon regeneration in the mature CNS.

Following the axotomy of a healthy axon, the segment distal to the cell body undergoes the process of Wallerian degeneration where axon and myelin sheaths are actively degraded. The proximal segment reseals the plasmalemma of the axon using Ca^{2+} as a signaling molecule (Yoo et. al, 2003). Over time, damaged axons proximal to the injury site tend to form a retraction bulb and die back from the lesion site instead of maintaining a growth cone, which is necessary in order to elongate the axon and re-establish contact with the denervated area (Hill et. al., 2001). The injured axons initially do attempt to form sprouts and regrow, but these attempts tend to be abortive and are mostly ephemeral in the CNS due to the short time period for regrowth (Bregman et. al., 1989).

The failure of axon regeneration in the mature CNS lies in the differences in regenerative ability of the PNS and the CNS. In addition to their role in the myelination of PNS axons, Schwann cells release cytokines leading to the recruitment of macrophages to phagocytose myelin debris (Shamash et. al., 2002). In turn, the release of cytokines like interleukin-1 from macrophages may lead to an increase in nerve growth factor expressed in an injured nerve (Lindholm et. al., 1987). Along with many growth factors, regeneration associated molecules such as transcription factor c-Jun and growth-associated protein 43 are activated after injury in the PNS (Raivich et. al., 2004; Skene J.H., 1989). A combination of the rapid degradation of myelin debris along with secreted growth factors and the lack of inhibitory factors such as chondroitin sulfate

proteoglycans, myelin inhibitors and a glial scar, which are present in the CNS, can contribute to the permissive growth environment of the PNS (Afshari et. al., 2009).

In the CNS, oligodendrocytes are responsible for myelinating axons and their role in clearing myelin debris is minimal compared to Schwann cells (Ludwin, S.K., 1990). The rapid removal of myelin debris in the PNS is not seen in the CNS due to the decreased phagocytic activity of microglia; however, when macrophages from the PNS were introduced to a degenerating optic nerve, there was not increased CNS myelin phagocytosis (Kuhlmann et. al., 2002).

Another contributing factor to the lack of axon regeneration in the adult CNS is the age dependent decline in regenerative ability. Studies with purkinje cells and retinal ganglion cells *in vitro* have shown that as time progresses, neurons lose their intrinsic ability to regenerate axons after axotomy, perhaps due to the process of synaptogenesis and dendrite remodeling turning off the genetic program responsible for axonal elongation seen in the early phase of neuronal development (Dusart et. al., 1997; Goldberg et. al., 2002; Chen et. al., 1995). This phenomenon is also seen *in vivo* where neonates are capable of some spontaneous regeneration whereas regeneration is limited in the mature CNS (Schwab and Bartholdi, 1996; Jaervea et. al., 2011).

At the molecular level, factors influencing, CNS axon regeneration can be divided into extrinsic and intrinsic factors in relation to neurons within the CNS. Extrinsic factors include molecules associated with the glial scar, myelin associated inhibitors, factors secreted by macrophages and other repulsive/attractive cues such as *Wnt*s. Formation of the glial scar can lead to a physical as well as chemical barrier by blocking sprouting axons attempting to cross the injury site while also upregulating growth

inhibitory molecules (Stichel and Muller, 1998). Myelin-associated inhibitors include Nogo, myelin-associated glycoprotein (MAG), and oligodendrocyte myelin glycoprotein (OMgp). Inhibition of Nogo using neutralizing antibodies was previously shown to increase corticospinal tract (CST) regeneration after experimental spinal cord injury, but it was later shown that genetically deleting Nogo and all of its isoforms is insufficient for CST regeneration after injury (Kim, J.E. et. al., 2003; Simonen et. al., 2003; Zheng et. al., 2003; Steward et. al., 2007; Cafferty et. al., 2007; Lee et. al, 2009). The microglia and inflammatory response had previously been thought to lead to the active destruction of neuronal cells in neurodegenerative diseases with the release of reactive oxygen species causing neurotoxicity (Block et. al., 2007). However, evidence has shown that microglia/macrophages are necessary for secretion of growth factors such as brain-derived neurotrophic factor and removing debris and that there is a direct relationship between inflammation and improved locomotor function after spinal cord injury (Bouhy et. al., 2006; Hashimoto et. al., 2005). Recent studies indicate that different types of macrophages may mediate opposite (beneficial vs. detrimental) effects after spinal cord injury (Kigerl et. al., 2009). The expression of repulsive *Wnt* molecules may inhibit axon regeneration after CNS injury. Administration of a function-blocking antibody to *Ryk*, a *Wnt* receptor, leads to reduced CST retraction and/or increased sprouting of CST collaterals past the injury site (Liu, Y. et. al., 2008). There are many proposed extrinsic factors involved in CNS regeneration, yet it is still unknown which ones are most important for CNS regeneration.

Intrinsic factors that are involved in axon regeneration in the CNS involve many pathways, one of them being the mammalian target of rapamycin (mTOR) signaling

pathway. Phosphatase and tensin homolog (PTEN), an upstream negative regulator of mTOR, was originally studied for its importance in tumor suppression, and later was shown to negatively regulate neural progenitor cells *in vivo* (Li et. al., 1997; Ross et. al., 2001). Genetic deletion of PTEN led to robust regeneration of retinal ganglion axons past an optic nerve crush lesion (Park et. al., 2008). Rapamycin, a potent inhibitor of mTOR, was able to eliminate regrowth of axotomized retinal ganglion cells, confirming that the effect of PTEN deletion was through mTOR (Park et. al, 2008). It was later shown that, strikingly, deletion of PTEN also led to successful regrowth of CST axons past a spinal cord lesion *in vivo* (Liu, K. et. al., 2010).

In addition to the mTOR pathway, calcium and cyclic adenosine monophosphate (cAMP) have been shown to increase regeneration in the CNS. *In vitro* studies have shown that Ca^{2+} influx into the damaged axon signals the sealing off of the plasma membrane in addition to containment of membrane vesicles to be available for regrowth of the damaged axon (Xie et. al., 1991). Furthermore, *in vitro* experiments have shown that Ca^{2+} stimulates the turning of growth cones, suggesting that Ca^{2+}may be involved as a potential molecule for axon guidance (Zheng, J.Q., 2000). Conditioning lesion experiments take advantage of the fact that the PNS has the ability to regenerate and a lesion to the PNS elicits a robust genetic program of regeneration. Thus, after an initial lesion to the peripheral branch of the dorsal root ganglion (DRG) is performed, a subsequent lesion to the CNS branch of the DRG neurons is followed by some, albeit still modest, degree of regenerative responses. Shortly after the peripheral lesion, increased levels of cAMP are seen in the corresponding DRG neurons, partly underlying the increased regenerative response of DRG neurons (Qiu et. al, 2002). Increased levels of

cAMP led to an increase in arginase I and interleukin-6, which have been shown to overcome the growth-inhibiting effects of myelin (Hannila and Filbin, 2008). After axotomy in *C. elegans*, an increase in Ca^{2+} and cAMP levels is important for axon regeneration. Most relevant to this project, the DLK-1 mitogen-activated protein kinase (MAPK) cascade has recently been shown to play a critical role in axonal regeneration in the *C. elegans*. (Yan et. al., 2009; Hammarlund et. al., 2009; Ghosh-Roy et. al., 2010).

The MAPK pathways play important roles via activation of a phosphorylation cascade in a variety of biological processes including embryogenesis, cell differentiation, cell proliferation, and programmed cell death (Pearson et. al., 2001). The functions of these serine/threonine-specific protein kinases form a family of protein kinases that have been evolutionarily conserved from yeast all the way to humans (Johnson and Lapadat, 2002). There are several MAPK subfamilies that exist downstream of the MAPK cascade. Namely, they are the extracellular signal-regulated kinases (ERK1 and ERK2), c-Jun NH_2-terminal kinases (JNK1, JNK2 and JNK3), and p38 enzymes (p38α, p38β, p38γ and p38δ) (Johnson and Lapadat, 2002). ERK is generally activated by growth factors and hormones, while JNK and p38 are predominantly activated by stress stimuli (Lewis et. al., 1998; Kyriakis and Avruch, 2001). These MAP kinases are activated by phosphorylation on threonine and tyrosine by mitogen-activated protein kinase kinase (MAPKK), which are, in turn, activated via phosphorylation of serine and threonine by mitogen-activated protein kinase kinase kinase (MAPKKK) (Yang et. al., 2003). MAP kinases phosphorylate and activate downstream protein kinases such as MAPK-activated protein kinase, MAPK-interacting kinase, and mitogen- and stress-activated protein kinase families, which, in turn, regulate gene expression via phosphorylation of histones

and transcriptional regulatory proteins (Lewis et. al., 1998; Kyriakis and Avruch, 2001). There are many MAPK pathways, but one MAPKKK molecule in particular is of interest because of its capability of activating downstream molecules required for axon regeneration; this molecule is dual leucine zipper-bearing kinase 1 (DLK-1).

DLK-1 was discovered to be involved in axon regeneration independently in two different studies. First, using a β-spectrin mutant (*unc-70) C. elegans* with fragile GABAnergic motor axons, which break during locomotion and constantly attempt to reestablish connections with the dorsal cord, an RNAi screen was performed in order to isolate mutants with decreased axon regrowth. RNAi targeting DLK-1 gave the strongest phenotype for lack of axon regrowth (Hammarlund et. al., 2009). Loss and gain of function studies of DLK-1 in the *unc-70* mutants demonstrated that DLK-1 is necessary for growth cone formation and morphology during axon regeneration but not for axonal development (Hammarlund et. al., 2009). In a separate study by a different group, after laser axotomy in PLM mechanosensory neurons, *dlk-1* loss of function mutants were unable to regenerate, whereby overexpression of DLK-1 in these same neurons led to increased regeneration after laser axotomy (Yan et. al., 2009). DLK-1 signals through a MAPKAP, MAK-2, to activate a basic leucine zipper (bZip) transcription factor CEBP-1 (the CCAAT/enhancer-binding protein) after laser axotomy to mediate axon regeneration (Yan et. al., 2009). Recent data indicate that Downstream of DLK-1 in *C. elegans*, PMK-3 (p38) and KGB-1 (JNK) MAPK pathways must be activated in synchrony in order for axon regeneration (Nix et. al., 2011). From these findings, it has been suggested that DLK and the coordinated activation of MAPKs are necessary for growth cone formation

and migration, and thus axon regeneration in *C. elegans* (Hammarlund et. al.; 2009, Yan et. al, 2009; Nix et. al., 2011).

The DLK MAPK pathway has also been shown to be necessary for axon regeneration after crush injury in *Drosophila*. Wild-type *Drosophila* motoneurons show significantly more regeneration after crush injury compared to RNAi knockdown of Wallenda, a *Drosophila* ortholog to *C. elegans* DLK-1 (Xiong et. al., 2010). In addition to a role in axon regeneration, Wallenda has also been shown to regulate Wallerian degeneration as shown by the slowed Wallerian degeneration in olfactory receptor neurons in Wallenda loss of function mutants (Miller et al., 2009). The downstream cascade after injury in *Drosophila* leads to *wallenda* eventually activating Jun N-terminal Kinase (JNK), a MAP kinase, which in turn leads to Wallerian degeneration and axon regrowth (Miller et. al., 2009).

DLK-1 in *C. elegans* is degraded by an E3 ubiquitin ligase, regulator of presynaptic morphology (*rpm-1)* (Nakata et. al., 2005). DLK-1 MAPKKK is present in presynaptic regions while its MAPKK, MKK-4, is mainly in the cytoplasm (Fig. 1). Downstream of the linear MAPK pathway lies PMK-3, a p38 MAPK, which was mainly present in the nucleus and cytoplasm (Nakata et. al., 2005). In *C. elegans rpm-1* loss of function mutants, an increased level of DLK-1 is present along with disorganized presynaptic cytoarchitecture and uncoordinated locomotion (Nakata et al., 2005). The lack of *RPM-1* causes axons to overshoot their targets, but does not affect their overall migration to these targets (Jin et al., 2008). These results coincide with DLK-1 being involved in axon migration and growth cone formation. In a *highwire* (*Drosophila* ortholog of *rpm-1*) mutant, synaptic overgrowth was seen in motor terminals exhibiting

excessive branching (Wan et al., 2000). Although embryonic mice missing Phr1 (a mouse ortholog of *Highwire and RPM-1*) showed an increase in excessive sprouting of nerve terminals beyond their respective targets like *highwire* and *rpm-1* mutants, a complete loss of function knockout results in postnatal lethality due to lack of the phrenic nerve innervating the diaphragm (Burgess et al., 2004, Bloom et al., 2007).

DLK was discovered originally in mouse in a screen looking for developmentally regulated protein kinases in the kidney, but high levels of DLK mRNA transcripts were also developmentally expressed in the brain (Holzman et. al., 1994). In adult mice, DLK mRNA was found most abundantly in the CNS, more specifically, in the hippocampal neurons and in cerebellar purkinje cells (Blouin et. al., 1996). Two groups have shown that DLK localization, specifically in the neocortex, was located in axons that have originated from pyramidal neurons. (Hirai et al., 2006; Eto et. al., 2010). The subcellular locations of DLK in neurons include plasma membrane and cytosol of synaptic terminals where it associated most strongly with microtubules (Mata et. al., 1996; Hirai et. al, 2005). Both cytosolic and plasma membrane-associated DLK can exist in a dephosphorylated state, but interestingly, the phosphorylated state was found only in cytosolic fractions (Mata et. al., 1996). Activation of DLK has been suggested to be due to dimerization, particularly, of the leucine zipper region and deletion of this region prevents stimulation of the JNK pathway (Nihalani et. al., 2000). Another mechanism behind activation of DLK may be attributed to tyrosine phosphorylation upon cell stimulation in the presence of a tyrosine phosphatase inhibitor (Daviau et. al., 2009).

DLK was revealed to be part of a new subfamily of mixed-lineage kinases (MLK) due to similarity with serine/threonine kinases, but being a hybrid between the

microtubule-associated protein kinase kinase kinase and the fibroblast growth factor receptor families (Holzman et. al. 1994). DLK activated MKK7, a MAPKK, *in vitro* via phosphorylation, but was unable to activate MKK4 in a similar manner in transfected COS cells (Merritt et. al, 1999). In addition to all MLKs regulating JNK through MKKs, DLK has also been shown to activate the p38 MAPK pathway (Gallo and Johnson, 2002). The human DLK, also termed zipper leucine protein kinase (ZPK), MAPK-upstream kinase (MUK) and mitogen-activated protein kinase kinase kinase 12 (MAP3K12), is most similarly related to the leucine zipper-bearing kinase (LZK) found in mouse sharing 86% identity in catalytic domain and leucine zipper domain similarity (Sakuma et. al., 1997).

The downstream JNK that DLK activates is particularly important because it has been shown to increase phosphorylation of microtubule associated protein 1 B (MAP1B) by regulation of MKK-4 and MKK-7 (Hirai et. al., 2006). After knockout of JNK1 and JNK2 genes in mice, regulation of brain development was significantly hindered due to the failure of neural tube closure along with an increase in cell death in various regions of the brain leading to inviable fetuses (Sabapathy et. al., 1999). Efficient Wallerian degeneration of axons in both flies and mammals requires DLK and its downstream effector, JNK; disrupting this pathway delayed axon fragmentation after injury (Miller et. al., 2009). In addition to *in vivo* developmental studies showing the effect of DLK and JNK on axon formation, DLK and JNK have been shown to function in a common pathway supporting axon formation via RNA interference rescue experiments *in vitro* (Hirai et. al., 2011). Knockdown of DLK decreased phosphorylation of JNK and its substrate, MAP1B, a molecule involved in axonal elongation. This suggests that

phosphorylation of MAP1B is the mechanism by which the DLK pathway regulates axonal growth (Eto et al., 2010).

DLK-1 in *C. elegans* and wallenda in *Drosophila* are intrinsic molecules related to regeneration in many ways, i.e. growth cone formation, axon migration, Wallerian degeneration. But in mice, targeted deletion of DLK leads to perinatal death and overexpression of DLK *in vitro*, inhibits cell growth (Hirai et. al, 2006; Bergeron et. al., 1997). Mouse neonatal DRG explants from DLK gene-trap have shown limited neurite growth *in vitro* compared to wild-type littermates (Itoh et. al., 2009). The E3 ubiquitin ligase ortholog of RPM-1, *Phr1*, does not increase DLK after deletion, suggesting that DLK may be regulated differently in mammals. The effects of DLK and the molecules that it phosphorylates in mice are not completely known, but in order to assess the effects of DLK after spinal cord injury, it is important to first determine its baseline expression before and after injury before artificially manipulating it.

The specific goals of my project are: (1) assess changes in *Map3k12* (i.e. DLK) mRNA in neonatal DRG cell bodies after axonal injury *in vitro*, (2) assess changes in *Map3k12* mRNA over time after PNS and CNS injury *in vivo*, and (3) assess changes in DLK protein over time after CNS injury *in vivo*.

First, we hypothesized that axotomy of neonate DRG axons *in vitro* will cause upregulation of *Map3k12* mRNA in DRG cell bodies, which may contribute to DRG axon regeneration. In addition to assessing *Map3k12* mRNA in DRG cell bodies, we tested the possibility that there could be *Map3k12* mRNA present in the axons.

We hypothesized that the PNS axons regenerate due to an increased level of DLK after injury and the CNS could not regenerate due to a decrease of DLK after injury. If

this hypothesis is correct, we would anticipate that *Map3k12* mRNA would increase in the L3-L5 DRG cell bodies following a crush injury to the sciatic nerve. This study represents a first step toward the test of the hypothesis that DLK/MAP3K12 is evolutionarily conserved amongst *C. elegans*, *Drosophila* and mammals in promoting axonal regeneration.

II. Materials and Methods

DRG Cell Culture (performed as described in Lee, J.K. et. al., 2010)

Postnatal day 0 (P0) mice (C57BL/6) were decapitated, and DRGs extracted and preserved in F12+2%B27 (Invitrogen, Carlsbad, CA) until further manipulation. Tissue was washed with Hank's Balanced Salt Solution (Invitrogen, Carlsbad, CA) without calcium and magnesium, and incubated with collagenase/dispase (Roche Applied Science, Indianapolis, IN) for 40 min at 37°C followed by 15 min of trypsin treatment at 37°C. After removal of the enzymes, tissue was triturated using fire- polished glass pipettes in a trypsin inhibitor medium. After centrifugation, DRG neurons were plated onto appropriate substrates.

Preparation of Microfluidic Chamber (MFC) Cultures

Preparation of the devices was performed using the manufacturer recommendations (Xona Microfluidics, Temecula, CA) with some differences. Sterile glass slides were coated with poly-ornithine (1mg/m; Sigma-Aldrich, St. Louis, MO) over night by plating 350µl onto the slides and a coverslip to allow even distribution of the poly-ornithine on the slide. After three washes with water, slides were dried for several hours. Next, sterile MFCs were mounted onto the slides and carefully checked for proper sealing. 150µl of laminin (10 µg/ml; Invitrogen, Carlsbad, CA) was added to the bottom left well of the cell body compartment (Fig. 2a); 15 min later, 150µl of laminin was added to the other side. Special care was taken to make sure that the laminin would diffuse towards the microgrooves (where the axons grow through later in the process).

Axon compartments on the right (Fig. 2b) were then filled with 120µl of laminin on both sides, and the whole device was placed into a sterile petri dish and incubated overnight at 37°C. The next day, laminin was removed and washed with neuron medium (Neurobasal-A medium, 10% fetal bovine serum, 2%B27, L-glutamine, antibiotic, and lacking brain-derived neurotrophic factor [BDNF]). The apparatus could then be processed for cell plating.

Cell Plating in MFCs

For each chamber, 6µl of neuron medium (with BDNF at 10ng/ml, Sigma-Aldrich, St. Louis, MO) containing 50,000 cells were added in one side of the cell body compartment, and 4µl of medium on the other side, to minimize fluid movement and increase cell adherence. 4µl of neuron medium enriched with BDNF (20ng/ml) were added in the two wells of the axon compartment (Fig. 2b). Petri dishes were sealed with parafilm and placed in incubator for 4hr. Next, 150µl of neuron medium (containing BDNF) and 120µl of BDNF enriched-neuron medium were added to the cell body compartment and axon compartment, respectively. Cells were fed every other day, with half of the medium changed. Neurons survived up to 21days.

MFC Axotomy

Axotomy was performed by using a 200µl pipette and performing 3 up/down movements using the medium already present in the wells with the tip facing toward the channel. In order to ensure complete axotomy, direct aspiration using a vacuum tip in the axonal channel was performed with the tip toward the channel. Most medium from the

opposing well was aspirated through the axonal channel separating the two wells. The force of the vacuum was sufficient enough to sever the axons, but the high fluidic resistance of the microgrooves allowed the cell body compartment to not be disturbed (Fig. 3a,b). 120 µl of BDNF enriched-neuron medium was added in each well.

Surgery and Care of Animals

All analyzed animals were female with a C57BL/6 background that were approximately 6-8 weeks of age. There were 2 different types of independent injuries performed: T-10 dorsal hemisection (1) and sciatic nerve crush (2). Each injury required mice to be anesthetized with a mixture of Ketamine, Xylazine, and Acepromazine given via intraperitoneal injection (Ketamine HCl; Fort Dodge Animal Health, Fort Dodge, IA/ Xylazine HCl; VEDCO, INC., St. Joseph, MO/ Acepromazine Maleate; VEDCO, INC., St. Joseph, MO in a 100mg:15mg:2.5mg/ kg ratio, respectively).

For dorsal hemisection and crush, back hair was first shaved off and swabbed with 70% isopropyl alcohol. A midline incision was made over the thoracic vertebrae, the paravertebral muscles were separated from the vertebral column and retracted, and a laminectomy was performed at T-10. A dorsal hemisection was performed using microscissors pre-measured to .8mm in order to cut the dorsal spinal cord and ensuring the ventral part of spinal cord was not injured in the process. After hemostasis was achieved, the muscle layers and skin were sutured and the skin was stapled.

For sciatic nerve crush, the dorsal portion of both thighs were shaved and swabbed with 70% isopropyl alcohol. A vertical incision was made in the skin over the quadriceps. The quadriceps were separated and located the sciatic nerve with a blunt

curette and injuries were performed in bisection from the hip to the knee. The sciatic nerve was crushed with No. 5 forceps. After hemostasis was achieved, the skin was sutured.

All animals were given Buprenex (Buprenorphine HCl; Reckitt Benckisser Healthcare (UK) Ltd., Hull, England; .1mg/kg), Baytril (2.27% Enroflaxacin; Bayer Healthcare LLC, Animal Health Division, Shawnee Mission, Kansas) and 1mL of saline immediately after surgery given subcutaneously. All mice were given Buprenex, Baytril, and saline twice a day until sacrifice. Dorsal hemisection mice had their bladders expressed twice a day by applying manual abdominal pressure.

RNA isolation and Quantitative Real-Time PCR (qPCR)

Animals were euthanized at specified post-injury times by anesthetic overdose with Nembutal Sodium (Pentobarbital 50mg/mL; MWI Veterinary Supplies, Meridian, ID) and motor cortex, spinal cord 2mm rostral and caudal to the injury, and DRGs corresponding to the injury site were dissected (Rigaud et. al., 2008). RNA extraction on tissue was performed using TRIzol (Invitrogen; Carlsbad, CA), and treated with DNase I (New England Bio Labs; Ipswich, MA). *In vitro* DRG RNA isolation was performed using the MagMAX-96 kit (Ambion; Austin, TX). The MagMAX-96 kit was used for its ability to purify low amounts of RNA by employing a nano-magnetic bead system that binds RNA, which can then be pulled down by a magnet for subsequent washes. Sample concentrations were verified using the NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE). Reverse transcription of 400ng of total RNA from *in vivo* experiments and 35ng of total RNA from *in vitro* MFC were performed using the

SuperScript III First-Strand Synthesis Kit (Invitrogen, Carlsbad, CA) and qScript cDNA Synthesis Kit (Quanta BioSciences, Gaithersburg, MD), respectively. Thermal cycling conditions were as prescribed by the manufacturer.

PCR of cDNA of *in vitro* cell bodies and axons cultured in microfluidic chambers to confirm MAP3K12 presence was performed using primers coding for beta-actin (*Actb*), to ensure RNA presence, calcium/calmodulin-dependent protein kinase II alpha (*Camk2a*), to ensure no contaminating RNA from the cell body compartment had penetrated the axon compartment, and MAP3K12, to detect our gene of interest (primers: *Actb*, 5'-AGC-CAT-GTA-CGT-AGC-CAT-CC-3' and 5'-CTC-TCA-GCT-GTG-GTG-GTG-AA-3'; *Camk2a*, 5'-GCC-CGG-GAG-TAT-TAC-AGT-GA-3' and 5'-GGG-TTG-ATG-GTC-AGC-ATC-TT-3' as described (Taylor et. al., 2005) and MAGIC, [intron spanning primer coding for *Map3k12*], 5'-GTG-GTG-CTA-TGG-GAA-CTA-CT-3' and 5'TGT-AGA-GAG-CAC-ATC-AGC-GGA-3'.)

Quantitative real time polymerase chain reaction (qPCR) was performed using PerfeCTa SYBR Green FastMix for iQ (Quanta BioSciences, Gaithersburg, MD) using fast 3-step cycling conditions (Thermal cycling parameters were initiated with 95°C for 30 sec, followed by 40 cycles of 95°C for 5□sec, 62°C for 15□sec, and 68°C for 10□sec) with data acquisition at each 68°C cycle. The melting curve was performed to ensure only one product was being amplified during qPCR (Melting curve parameters were initiated with 55°C for 1 min, followed by 81 cycles of 0.5°C increments for 10 sec starting at 55°C until 95°C was reached with data acquisition occurring at the end of each increment cycle). qPCR amplification and detection was conducted in Bio-Rad iQ5 Real-Time PCR Detection System (Bio-Rad, Hercules, CA). Many unique primers were made for

qPCR to detect MAP3K12, the primer pair with the lowest Ct value was chosen (primer: QPCR MAP CED 3, 5'-CGC-CCC-ACA-GCA-AAA-GGC-CA-3' and 5'-TGC-CAC-GCC-GAC-TCC-TTC-CA-3'). The relative amount of expressed RNA was calculated by comparison with the expression of a housekeeping gene, 60S ribosomal protein L19 (*Rpl19*) (primers: *Rpl19*, 5'-GCC-AAT-GCC-AAC-TCC-GCT-CAG-3' and 5'-CGC-TTT-CGT-GCT-TCC-TTG-GTC-TTA-3')

Western Blots

Cortex and spinal cord protein were extracted by sonication in homogenization buffer containing 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, Indianapolis, IN). After sitting on ice for 30min, tissue homogenate was centrifuged at 11,000*g* for 30 min at 4°C and pellet discarded. The protein concentration of the supernatant was determined using the Lowry assay (Bio-Rad, Hercules, CA) and 20 μg of protein in 5x NuPAGE LDS sample buffer (Invitrogen, Carlsbad, CA) was separated with 7.5% SDS-PAGE, and transferred to nitrocellulose membrane (Amersham, Little Chalfont, Buckinghamshire, UK) for 110 minutes. Membranes were washed in tris-buffered saline in .1% tween-20 (TBS-T) for 4 × 5 min, blocked with 5% milk/bovine serum albumin (BSA) depending on antibody for 1 hr at room temperature, and incubated overnight at 4°C with a primary antibody in blocking solution for loading control antibodies. Anti-DLK primary antibody was incubated at 4°C overnight and then 3 hours at RT before being washed with TBS-T. Blot was incubated in original blocking solution with conjugated secondary antibody (1:1000-1:5,000, depending on antibody,

Thermo Scientific, Rockford, IL) for 1 hr at room temperature. After washing in TBS-T, membranes were incubated in Supersignal West Dura and Supersignal West Femto chemiluminescence solution (Thermo Scientific, Rockford, IL) for 5 min and then exposed to film (Genesee Scientific, San Diego, CA) or exposed on the Fluorchem Q imaging system (Protein Simple, Santa Clara, CA). Primary antibodies used: DLK rabbit antiserum generated against a fusion protein of Glutathione S-transferase - C-terminal 223 amino acid as described (Holzman et al, 1994) diluted to 50% concentration in 100% glycerol and used at 1:2000 dilution; mouse anti-α-Tubulin at 1:5000 (Sigma-Aldrich, St. Louis, MO).

Quantification

Expression of DLK protein compared relative to α-tubulin was determined using the FluorChem Q imaging software (Protein Simple, Santa Clara, CA). A pre-determined area was drawn around each protein band to detect band intensity and the software subtracted background automatically.

Expression of MAP3K12 and RPL-19 RNA was determined using the iQ5 optical system software (Bio-Rad, Hercules, CA). The software calculated Ct values and melting curve data automatically. The $2^{-\Delta\Delta Ct}$ method was performed to analyze qPCR results.

Statistics were performed on samples that had n=3 with Prism using one-way ANOVA followed with Tukey's multiple comparison test (Graphpad Software, La Jolla, CA). Statistics for *in vitro* experiments with n=1 but performed in triplicate during qPCR had standard deviation (stdev) for fold change calculated using the formula, $=(\ln 2)(\text{stdev}_{\Delta\Delta Ct})(2^{-\Delta\Delta Ct})$; (Bookout and Mangelsdorf, 2003)

III. RESULTS

In vitro expression of *Map3k12* mRNA in neonatal DRG neurons after axotomy

To examine the change in *Map3k12* mRNA expression after axotomy in MFCs, injury was performed in 2 hour increments up to 8 hours, and RNA was extracted from the cell compartment. After qPCR, no significant change in *Map3k12* mRNA was seen in the DRG neurons compared to the non-injured DRG neurons (Fig. 4). To determine if DLK mRNA was present in the axons, P0 DRG cell bodies were grown in MFC culture; axons that extended into the specialized axon compartment were axotomized and mRNA was isolated. Reverse Transcriptase PCR results confirmed that mRNA was successfully purified as seen by detection of *Actb* in the axon compartment and no contaminating cell bodies were present in the axon compartment as seen by the absence of *Camk2a* in the axon compartment (Fig. 5) PCR results showed no *Map3k12* mRNA presence in the axon compartment, but was abundant within the cell body compartment as expected (Fig. 5).

In vivo expression of *Map3k12* mRNA after CNS and PNS injury in adult mice

To assess changes in *Map3k12* levels in the PNS, we crushed the sciatic nerve and harvested mRNA 1, 3, and 5 days after injury. We found that after sciatic nerve crush, a significant 4-fold decrease in *Map3k12* mRNA levels in the sciatic nerve was detected by qPCR 1 day after sciatic nerve crush. Following the initial decrease in *Map3k12* mRNA levels at day 1, there was a steady and significant increase in *Map3k12* mRNA resulting in a 2-fold increase on day 5 post injury compared to control (Fig. 6). A similar trend at

day 1 post injury was seen in the L3-L5 DRG neurons after sciatic nerve crush, though changes in *Map3k12* mRNA levels were not significant (Fig. 7)

To assess changes in *Map3k12* mRNA levels after CNS injury, we performed a dorsal hemisection at T-10 in 7-week old mice and harvested mRNA 1, 3, and 5 days from the T10 spinal cord and T10-L5 DRG neurons. Interestingly, we observed a significant 4-fold decrease 3 and 5 days post injury in *Map3k12* mRNA at the injury site (Fig. 8). A trend for decrease in *Map3k12* mRNA in T10-L5 DRG neurons after dorsal hemisection just failed to reach statistical significance one day post injury (Fig. 9). The same trend, however, was observed for DRG neurons and sciatic nerves after sciatic nerve crush. Finally, no significant changes in *Map3k12* mRNA in the motor cortex were observed after dorsal hemisection (Fig. 10).

Determining antibody specificity for DLK protein

Several commercial antibodies of DLK were tested without success (results not shown). The DLK antibody used in this study was a gift of Dr. Holzman from University of Pennsylvania (Holzman et. al., 1994). Before further use of this antibody, the specificity of the DLK antibody for western blots was tested. We confirmed that the antibody detected a specific band for DLK at a size of 130 kDa. As a control, embryonic day 13 knockout brains did not present a band at 130 kDa, whereas littermate heterozygote brains and wild-type P0 mouse brain presented the expected band (E13 tissue courtesy of DiAntonio laboratory) (Fig. 11). Similarly, a 130 kDa was detected in P0 mouse spinal cord and adult wild-type spinal cord extracts, but not in spinal cord extract from adult DLK gene-trap mutant mice (tissue courtesy of Itoh Laboratory) (Fig.

11). Confirmation of similar protein loading was indicated with a nonspecific band (Fig. 11). Therefore, DLK antibody could be used with confidence for further experiments.

In vivo expression of DLK protein after CNS injury in adult mice

Dorsal hemisection spinal cord injury was chosen as the experimental spinal cord injury model because it enabled us to target the corticospinal tract involved in fine motor control as well as targeting the dorsal column sensory pathways. We saw a significant decrease in DLK protein levels at the injury site (T-10) one day post injury after dorsal hemisection and no further significant changes in DLK protein levels at day 3 or day 5 after dorsal hemisection suggesting a steady state trend of no change after one day post injury (Fig. 12). These results are consistent with our hypothesis that DLK protein decreases after injury, which may partially contribute to the limited axon regeneration after CNS injury. In addition to examining DLK protein levels at the spinal cord where the tissue is comprised of interneurons, spinal motoneurons, glial cells and, importantly, axonal components of injured cells, we wanted to determine if there were changes within the cell bodies of injured axons, starting with the corticospinal axons. In order to do this we harvested tissue from the motor cortex, which contains a majority of the cell bodies from the main corticospinal tract axons injured after dorsal hemisection. One day following a T-10 dorsal hemisection, there was a trend for a decrease in DLK protein levels in the motor cortex but this trend did not reach statistical significance (Fig. 13).

IV. Discussion

The CNS, generally, is thought to be incapable of regrowth and self-repair as compared to the PNS, which intrinsically has the ability for growth and repair after injury. While there have been many attempts to manipulate extrinsic factors in the CNS that are involved in inhibiting regrowth after injury such as manipulating myelin inhibitors or macrophages, there have been inconsistent findings (Kim, J.E. et. al., 2003; Hashimoto et. al., 2005; Bouhy et. al. 2006; Block et. al. 2007; Lee, J.K. et. al., 2009). Due to the conflicting data and lack of robust regeneration after manipulating these extrinsic factors, eliminating inhibitory extrinsic factors is unlikely to be sufficient to increase regeneration and manipulating neuron-intrinsic molecules may be required to obtain substantial regenerative growth in the CNS. Recent studies have shown promising data indicating that manipulation of neuron-intrinsic molecules such as PTEN or cAMP can lead to increased regrowth in the CNS *in vivo* (Hannila and Filbin, 2008; Liu et. al., 2010). An intrinsic molecule shown to be critical for axon regeneration, DLK-1, was independently discovered in 2009 by two different groups studying *C. elegans* (Hammarlund et. al., 2009; Yan et. al, 2009). In addition to its role in regeneration in *C. elegans,* DLK-1 has been shown to play a role in regeneration in axon regeneration in *Drosophila*, axon degeneration in both *Drosophila* and mammals, and mammalian neurite growth *in vitro* (Itoh, A. et. al., 2009; Miller et. al., 2009; Xiong et. al., 2010). Due to the impact of DLK-1 on the robust regeneration seen in *C. elegans*, we wanted to know if DLK plays a role in mammalian CNS axon regeneration after spinal cord injury. Towards this end, we aimed first to establish its expression in the adult mouse nervous

system before and after injury. Specifically, we wanted to determine if DLK expression changes at the level of mRNA or protein after either CNS or PNS injury and sought to understand the expression of the protein and mRNA after injury in different locations of the mouse *in vivo*.

Knowing the expression of DLK before and after injury in mice without viral or genetic manipulation is essential to establishing a baseline for future experimental manipulations. Additionally, it is important to know if it is more of DLK expression or kinase activity of DLK that could be responsible for any effect on CNS regeneration. Assuming that increased expression of mRNA leads to an increased amount of translated protein, we examined both mRNA and protein to determine at what stage DLK may be regulated.

For our *in vitro* cell culture experiments, we hypothesized that *Map3k12* mRNA levels would increase after injury leading to more MAP3K12 protein being synthesized and thus contributing to the *in vitro* regrowth of axons. The results of these experiments indicate that there is no significant change in mRNA levels of *Map3k12* from neonatal DRG neurons even though they readily extended axons through microgrooves in MFCs after axotomy. This was surprising to us because neonatal DRG neurons have higher capability for regrowth and survival *in vitro* as seen by their ability to regrow axons after injury compared to adult DRG neurons. A possible caveat in interpreting this result was that the actual number of DRG neurons that had their axons cut was far less than the total number of DRG neurons in the cell body compartment of the MFC, leading to potentially underestimating a change in *Map3k12* mRNA levels in axotomized neurons. In other words, a change in *Map3k12* mRNA levels may have missed the detection amongst such

a large population of cells that were not axotomized. Explanations for lack of change in *Map3k12* can be due to the proposed time course for the experiment not being long enough and upregulation of *Map3k12* occurring at a later time as well as neonatal mice having already high levels of *Map3k12* as seen by their intrinsic ability for regrowth. Future experiments should test for longer duration after axotomy as well as using adult DRG neurons.

Another possibility is that DLK expression is changed at the protein level but not at the mRNA level. In order to detect changes in expression of DLK/MAP3K12, retrograde labeling of cell bodies that have successfully extended axons through microgrooves can be accomplished by addition of tracer dye into the axon well. After axotomy, neurons can be fixed at different time points and stained for DLK to detect protein level changes, comparing cells labeled as injured and cells not labeled as non-axotomized controls. Finally, there is a possibility that there is a change in the activity of the DLK protein rather than a change in mRNA or protein expression levels after *in vitro* axotomy. In order to address this possibility, it would be interesting to assess the phosphorylation states of the downstream molecules in the MAPK pathway (p38 and JNK) using the same tracer experimental design proposed above.

Interestingly, for our *in vivo* experiments, *Map3k12* mRNA levels increased in the sciatic nerve after peripheral injury, but remained unchanged in the corresponding DRG neurons 5 days post injury. This is puzzling because axoplasmic transport of mRNA and local translation of protein in the axons means that an increase of mRNA would be present in the cell body first because mRNA is synthesized in the nucleus of the cell body, which is not what was seen. In the future, a gene such as *Gap43* that is upregulated

in the cell body should be used as a positive control to ensure that lack of changes seen in the DRG neurons after injury are not due to technical errors (Mason et. al., 2002).

However, one should note that the source of mRNA in the sciatic nerve is not restricted to the axonal compartments of sensory and motor neurons, but also the glial cells, fibroblasts and other cell types present in the sciatic nerve. Further analysis is required to definitively interrogate this question. We were unable to see any *Map3k12* mRNA in a pure axon population in MFCs after 40 cycles suggesting that there is no local translation of *Map3k12* in axons that are not freshly injured which, however, may exhibit a different expression profile from what is seen in axons that are freshly injured. Our experimental testing for axonal mRNA differed from a published paper also utilizing the MFC as we did not include a pre-amplification step of mRNA (Taylor et. al., 2009). Therefore, there is a possibility that an extremely low amount of *Map3k12* mRNA is present in the axons, but was not detectable with the technique used in the present study. It is also important to note that qPCR data was initially normalized against a housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which was originally thought to be unchanged after injury. However, because of data obtained (not shown), and after further reading of the literature, these data were removed, as it had been suggested that common housekeeping genes such as GAPDH and Beta-actin change after injury and can therefore not be used as reference (Fan et. al, 2001; Bustin, S.A., 2002; Wan, G. et. al., 2010; Nelissen et. al., 2010). Instead, the ribosomal protein L (RPL) family was a more favorable reference gene and was thus used in our study (Wan, G. et. al., 2010; Nelissen et. al., 2010). Optimally the best reference gene for detecting changes

in *Map3k12* mRNA in axons would be a gene solely expressed in axons and/or cell bodies, but is unaffected after injury.

Interestingly, after dorsal hemisection, a similar trend in decrease of DLK/MAP3K12 protein expression in the motor cortex was seen whereas *Map3k12* mRNA levels in the motor cortex were unchanged. However, DLK protein in the spinal cord after dorsal hemisection continued to stay decreased 5 days post injury. Additionally, the lack of significant change that was seen at the DRG neurons may be due to the fact that the roots of the DRG neurons collected from T10-L5 were above the T10 injury level and were actually uninjured. Determining changes in expression of DLK in the sciatic nerve and DRG neurons were not performed due to low levels of protein extracted, however, a protein array could be used as an alternative way to determine expression changes due to its sensitivity. Nevertheless, the decrease in *Map3k12* mRNA and DLK protein at the injury level after dorsal hemisection may explain the lack of regeneration seen in the CNS. However, aside from injured axons, there are many different cell types present after spinal cord injury that may skew DLK protein expression leading to complexities in DLK expression change that occur at the injury area. Future experiments should include immunohistochemistry (IHC) to visualize colocalization of DLK and axon markers after injury allowing for a more definitive conclusion. In addition to IHC, checking DLK activity is not possible due to lack of commercial antibodies, but phosphorylated downstream antibodies (as mentioned above) are commercially available.

Especially tantalizing is our observation of an increase in *DLK* mRNA in the sciatic nerve after PNS injury, but a decrease of DLK protein and mRNA in the spinal

cord after CNS injury. Future experiments should focus on the specific cell types in which DLK expression of activity levels may change after axonal injury by IHC or *in situ* hybridization. This would be followed by further manipulation to investigate whether manipulating this signaling pathway would provide a meaningful approach to stimulate axonal regeneration in order to achieve improved functional recovery after spinal cord injury.

Appendix

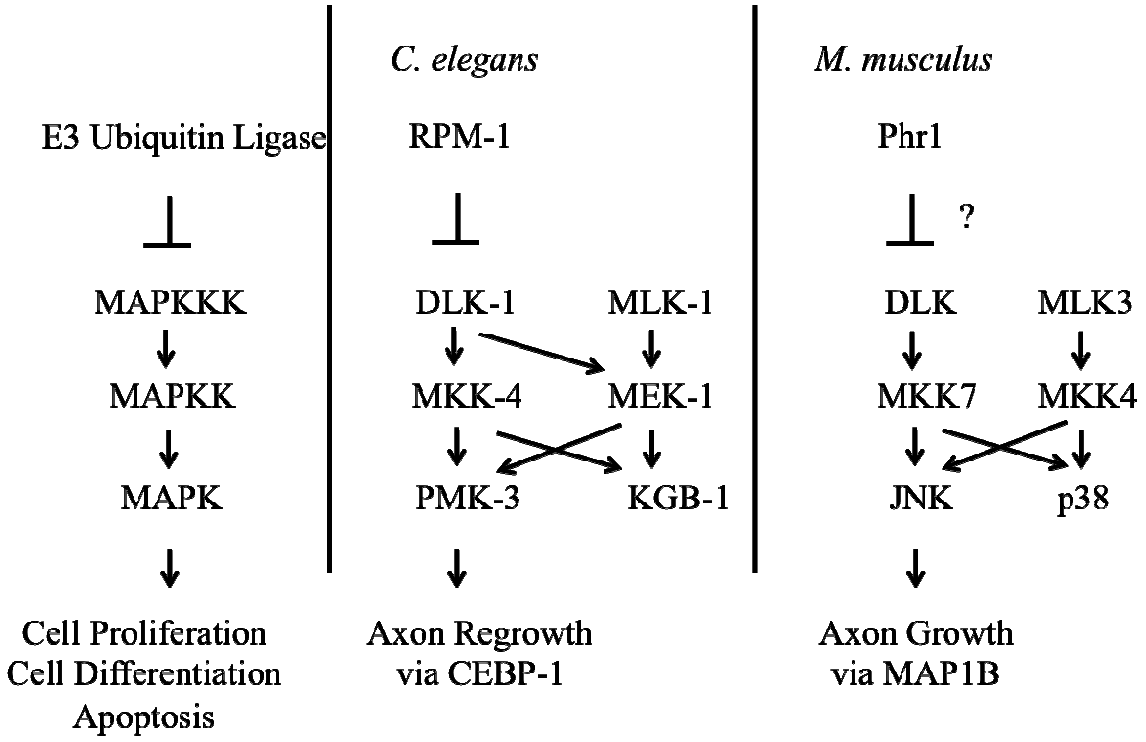


Figure 1. Mitogen Activated Protein Kinase Kinase Kinase pathway
An evolutionarily conserved pathway containing orthologs found in worms and mice with the focus on dual leucine zipper-bearing kinase and its downstream molecules.

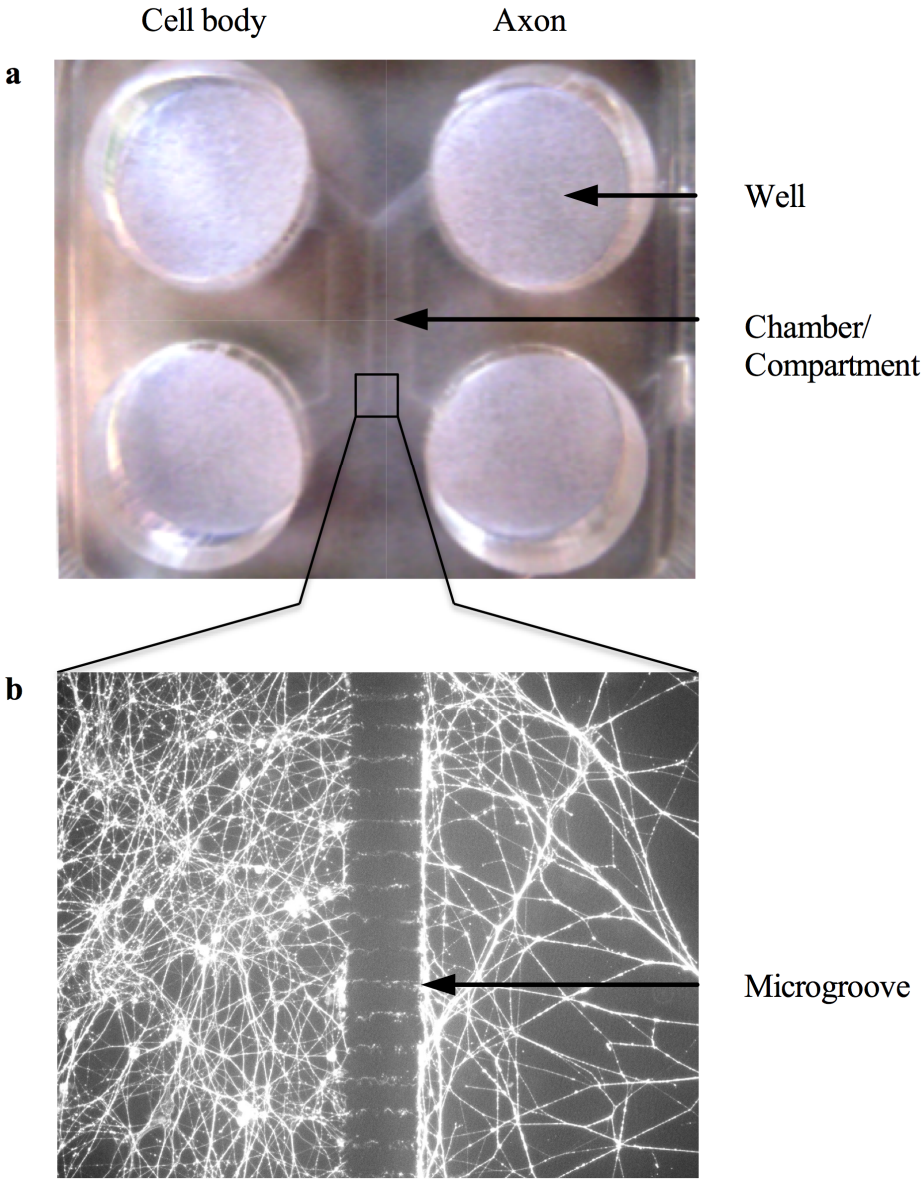


Figure 2. Isolation of axons for axotomy in microfluidic chambers.
(a) DRG neurons are cultured *in vitro* in the cell body compartment (left) and project axons through microgrooves to end up in an isolated compartment exclusively comprised of axons (right). (b) P0 DRG neurons stained against beta III tubulin.

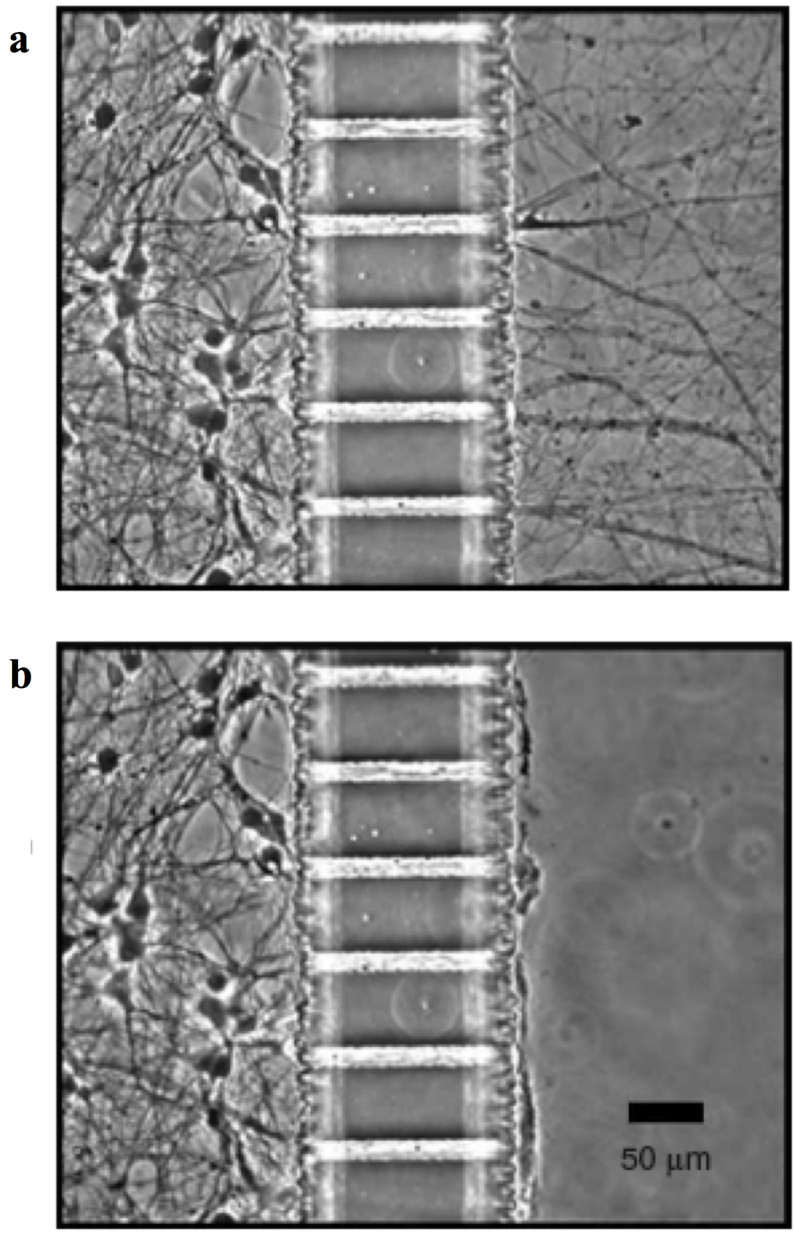


Figure 3. Overview of axotomy procedure in the microfluidic device.
(a) The cell bodies are on the left and the isolated axons are on the right. (b) The cell bodies are undisturbed, while the axon compartment shows complete severing of axons after axotomy with a vacuum tip. Reprinted by permission from NPG and Macmillan Publishers LTD.: [Nature Protocols] (Park, J.W. et. al., 2006).

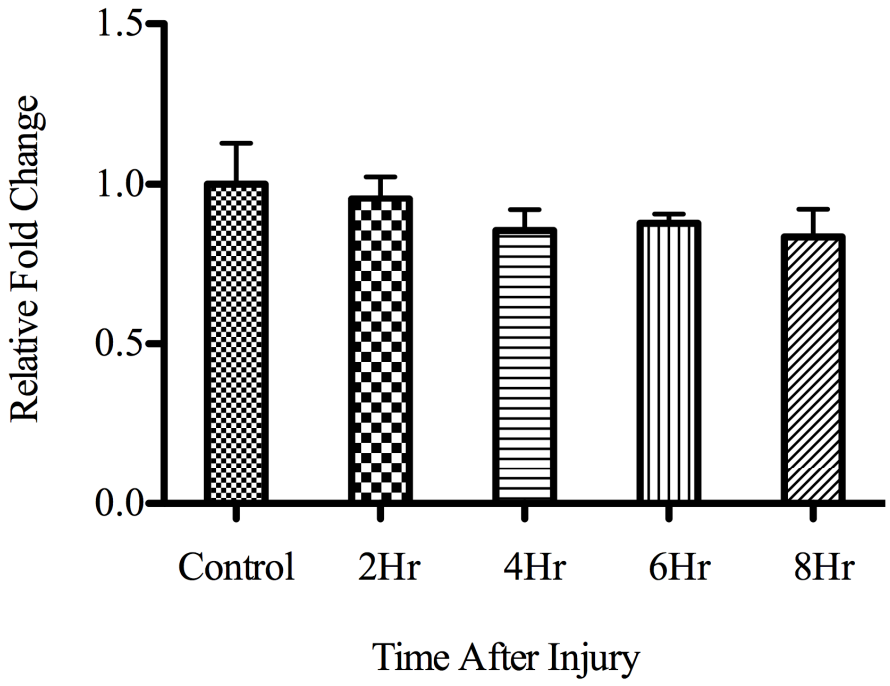


Figure 4. Expression of *Map3k12* mRNA in P0 DRG neurons after axotomy in MFC. There are no significant changes in *Map3k12* mRNA after axotomy in P0 DRG neurons. Relative *Map3k12* levels were determined by comparison to the reference gene *Rpl19* using the $2^{-\Delta\Delta Ct}$ method. The columns are means ± standard deviations of 1 experiment performed in triplicate. (P>0.05; One-way ANOVA with Tukey's Multiple Comparison Test).

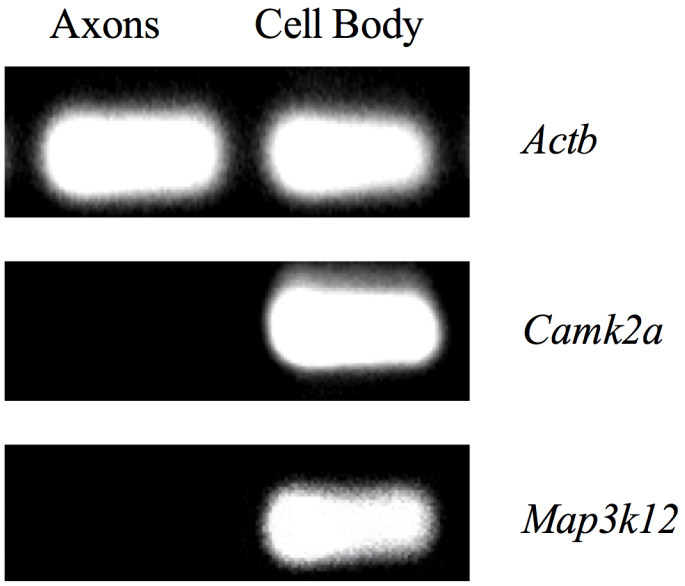


Figure 5. No detectable *Map3k12* mRNA in axon compartment of MFC.
P0 DRG neurons were cultured 12 days *in vitro* in MFC. PCR was performed for 40 cycles with no visually detectable levels of Map3k12.

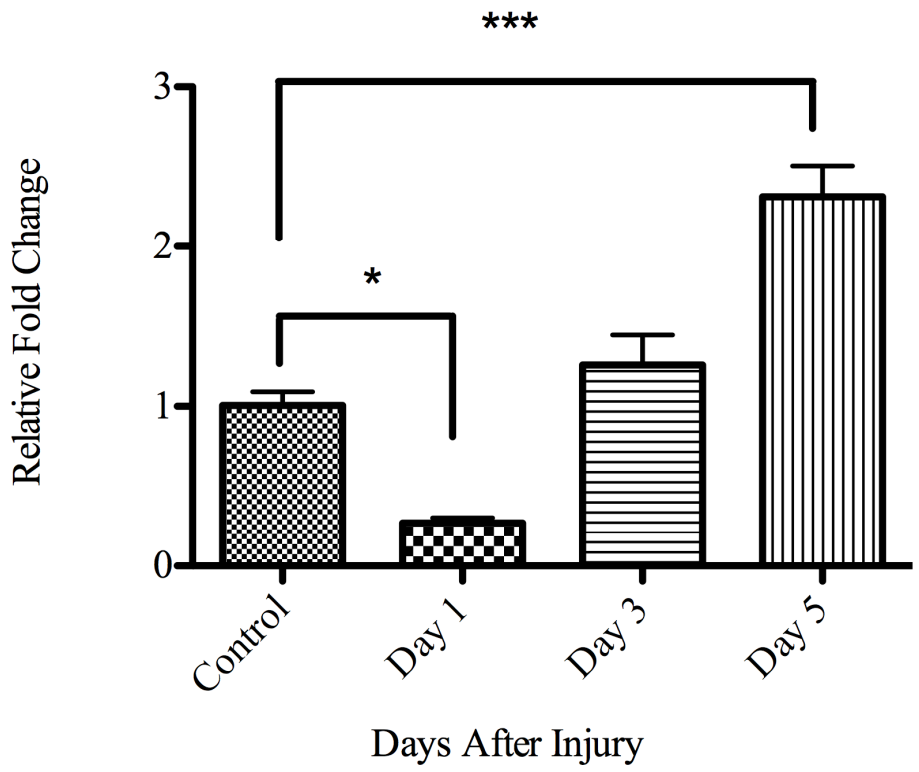


Figure 6. Expression of *Map3k12* mRNA in sciatic nerve after sciatic nerve crush.
A significant decrease in *Map3k12* levels was detected 1 day post injury; by day 5 post injury, a significant increase in *Map3k12* levels compared to control levels was detected. Relative *Map3k12* levels were determined by comparison to the reference gene *Rpl19* using the $2^{-\Delta\Delta Ct}$ method. The columns are means ± SEM with 3 samples each performed in triplicate. (*: P<0.05 and ***: P<0.0005; One-way ANOVA with Tukey's Multiple Comparison Test).

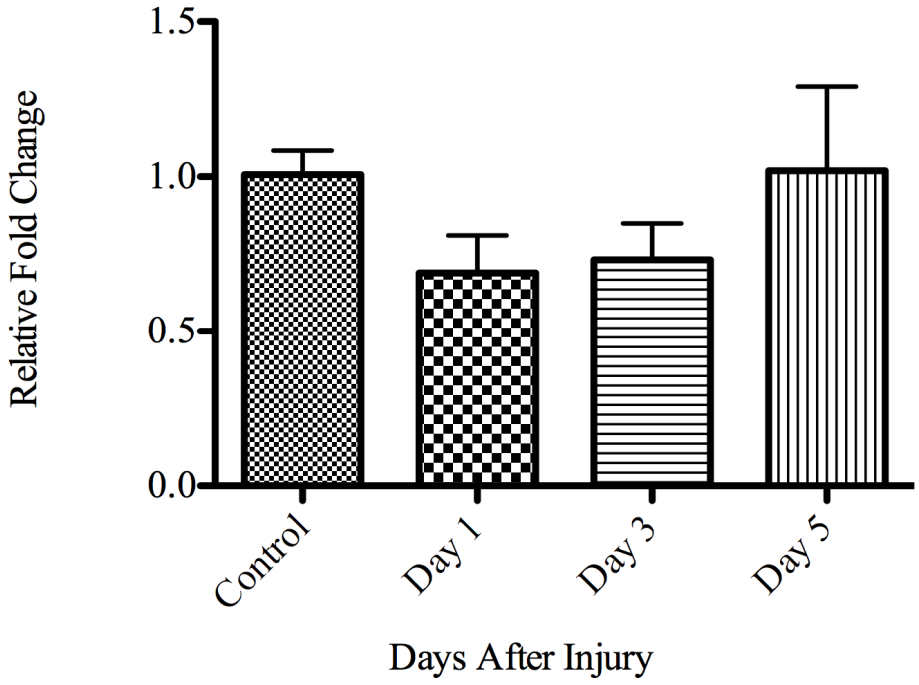


Figure 7. Expression of *Map3k12* mRNA in L3-L5 DRG neurons after sciatic nerve crush.
A trend in decrease was seen in the DRG neurons as the sciatic nerve after sciatic nerve crush; however, there was no significant change after injury. Relative *Map3k12* levels were determined by comparison to the reference gene *Rpl19* using the $2^{-\Delta\Delta Ct}$ method. The columns are means ± SEM with 3 samples each performed in triplicate. (P>0.05; One-way ANOVA with Tukey's Multiple Comparison Test).

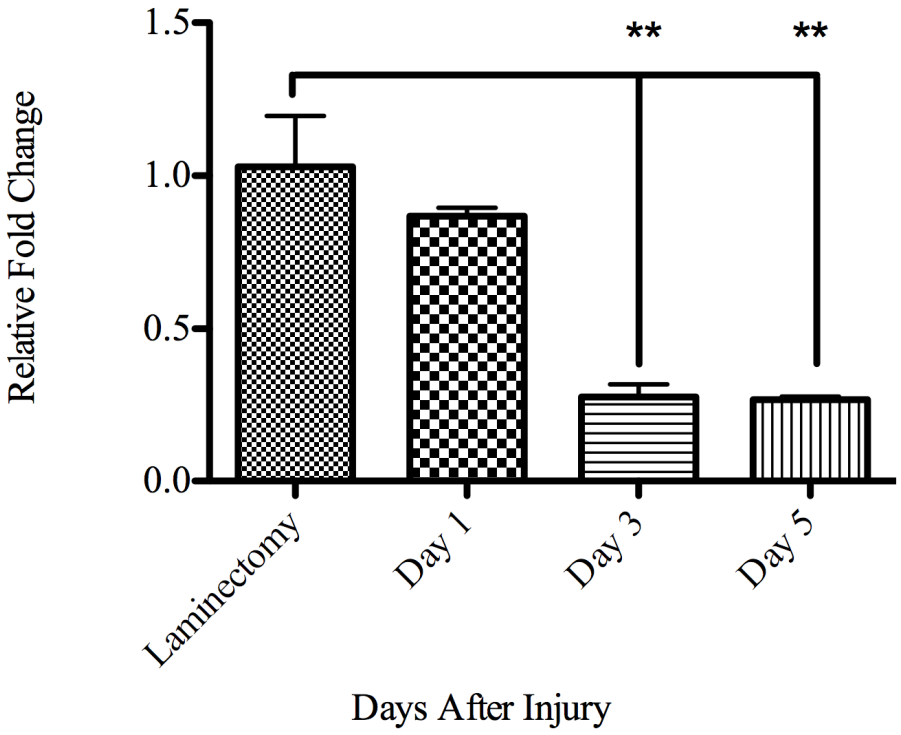


Figure 8. Expression of *Map3k12* mRNA at T10 spinal cord after dorsal hemisection. There was a significant decrease in *Map3k12* mRNA at day 3 and day 5 after dorsal hemisection. Relative *Map3k12* levels were determined by comparison to the reference gene *Rpl19* using the $2^{-\Delta\Delta Ct}$ method. The columns are means ± SEM with 3 samples each performed in triplicate. ($P<0.005$; One-way ANOVA with Tukey's Multiple Comparison Test).

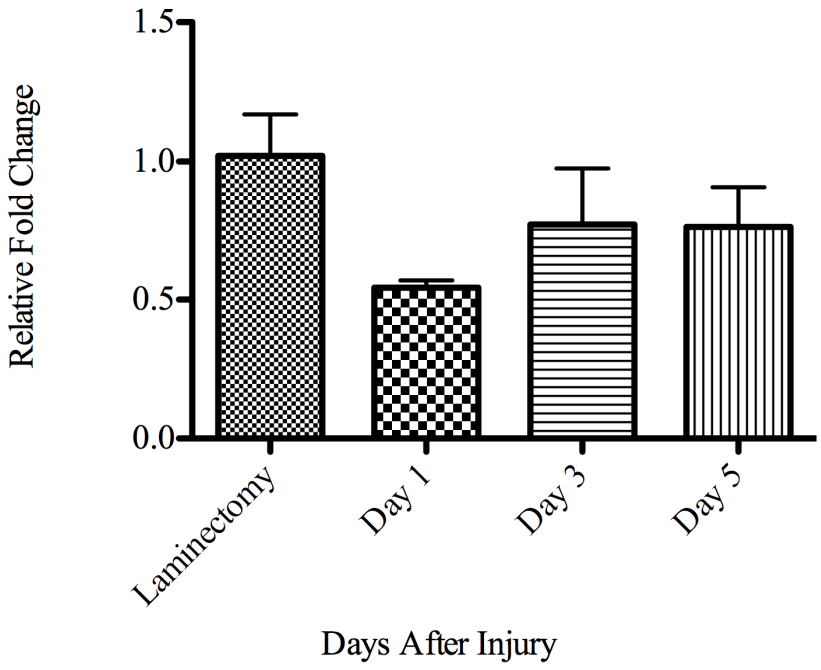


Figure 9. Expression of *Map3k12* mRNA in T10-L5 DRG neurons after dorsal hemisection.
There was a trend for a decrease in *Map3k12* levels 1 day post injury but this trend did not reach statistical significance in T10-L5 DRG neurons after dorsal hemisection. Relative *Map3k12* levels were determined by comparison to the reference gene *Rpl19* using the $2^{-\Delta\Delta Ct}$ method. The columns are means ± SEM with 3 samples each performed in triplicate. ($P>0.05$; One-way ANOVA with Tukey's Multiple Comparison Test).

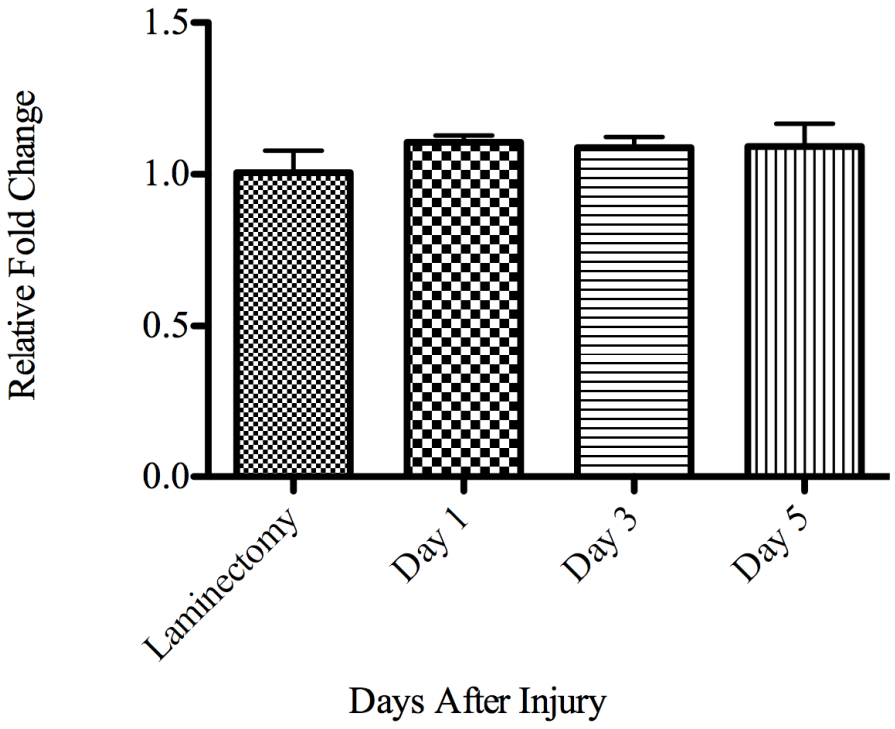


Figure 10. Expression of *Map3k12* mRNA in the motor cortex after dorsal hemisection. No significant change was seen in *Map3k12* mRNA levels in the motor cortex after dorsal hemisection. Relative *Map3k12* levels were determined by comparison to the reference gene *Rpl19* using the $2^{-\Delta\Delta Ct}$ method. The columns are means ± SEM with 3 samples each performed in triplicate. (P>0.05; One-way ANOVA with Tukey's Multiple Comparison Test).

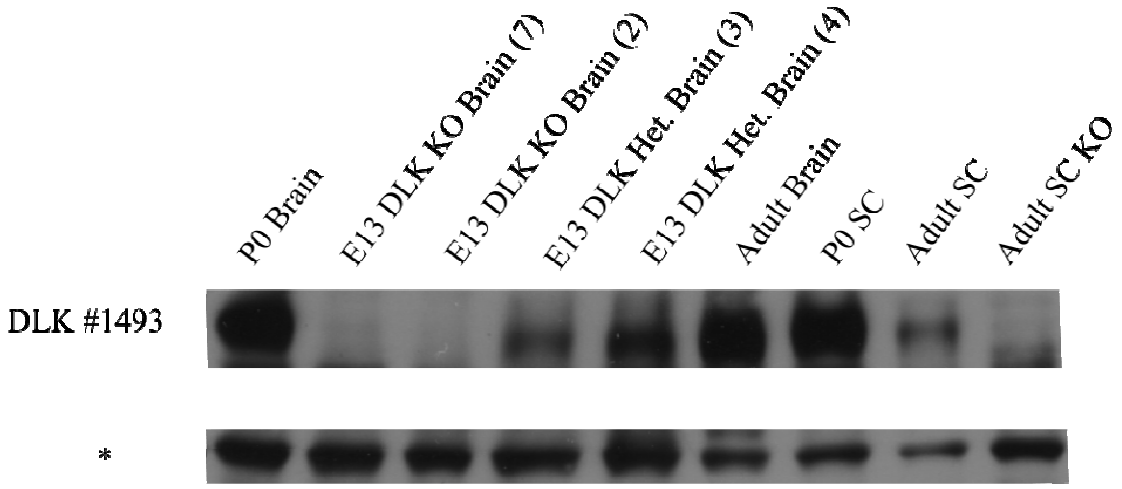


Figure 11. DLK antibody specificity in brain and spinal cord.
DLK Rabbit antiserum #1493 (Holzman, et. al., 1994) was confirmed to be specific by comparing 3 separate negative controls (E13 DLK KO Brain (2,7) (DiAntonio et. al., 2009) and Adult SC KO (Itoh, T. et. al., 2009)). * indicates a nonspecific bands serving as a loading control.

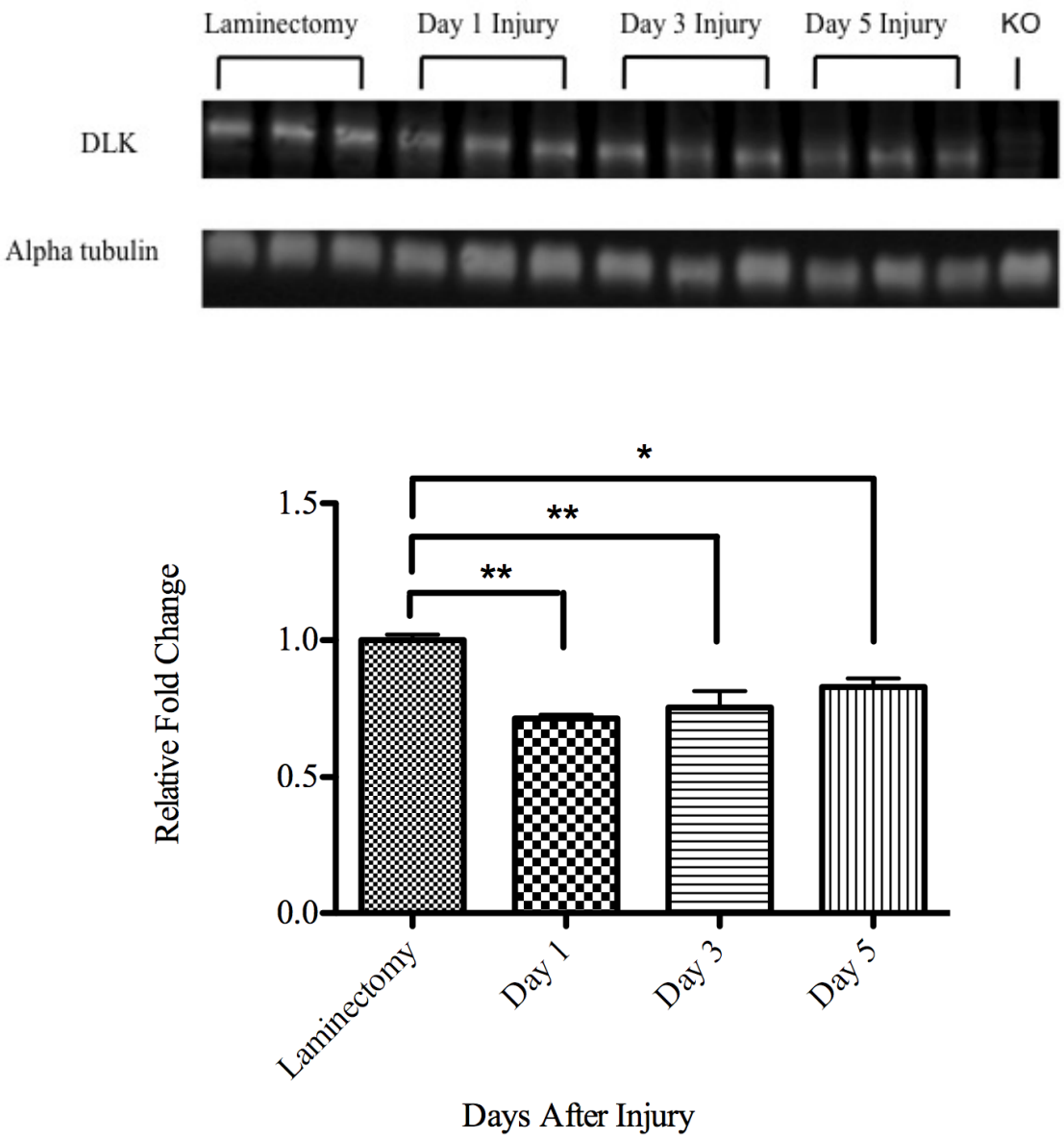


Figure 12. Expression of DLK protein in spinal cord after dorsal hemisection. There was a significant decrease in protein levels of DLK in the spinal cord after dorsal hemisection that remained decreased 5 days post injury. Relative ratios of DLK were determined using alpha tubulin as a reference gene. The columns are means ± SEM with 3 samples each performed in triplicate. (**: $P<0.005$ and *: $P<0.05$; One-way ANOVA with Tukey's Multiple Comparison Test).

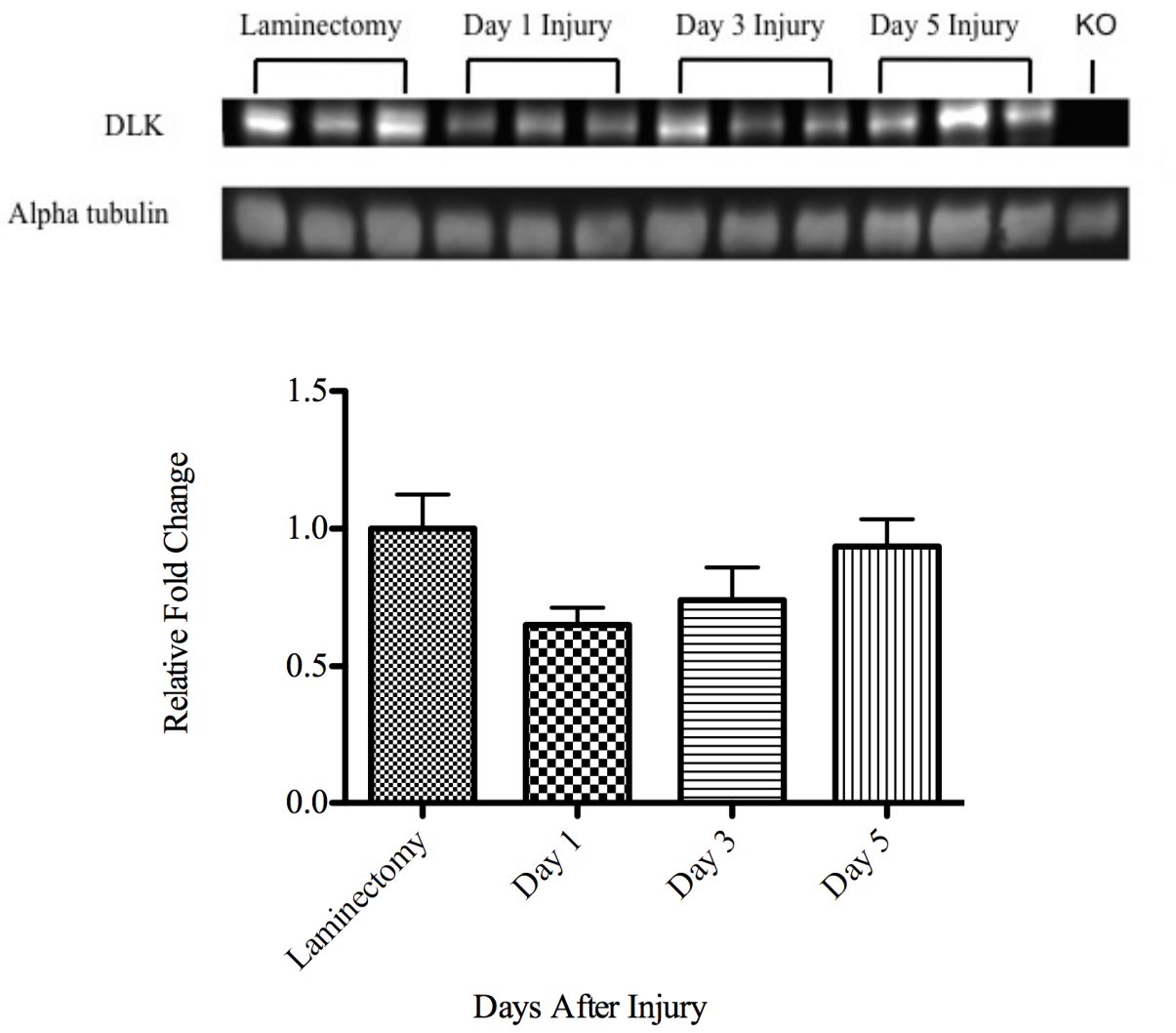


Figure 13. Expression of DLK protein in motor cortex after dorsal hemisection.
There was a trend in decrease of DLK protein in the motor cortex one day post injury, but did not reach statistical significance. Relative ratios of DLK were determined using alpha tubulin as a reference gene. The columns are means ± SEM with 3 samples each performed in triplicate. ($P>0.05$; One-way ANOVA with Tukey's Multiple Comparison Test).

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