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Regulation of peripheral nerve regeneration by the mTOR pathway

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REGULATION OF PERIPHERAL NERVE REGENERATION BY THE MTOR PATHWAY

by

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ABSTRACT OF THE DISSERTATION

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While neurons in the central nervous system (CNS) have limited capacity for regrowth after damage, neurons in the peripheral nervous system (PNS) have a robust ability to regenerate their axons following injury. Successful regeneration depends upon both extrinsic cues in the environment and the activation of intrinsic mechanisms to promote regrowth. A number of inhibitory molecules in the CNS environment that prevent axonal regrowth have been identified, but less is known regarding the signaling mechanisms that regulate regenerative ability in PNS neurons. Here, we explored multiple components of injury signaling in the PNS, including the retrograde transport of local axonal injury signals, enhancement of axonal growth capacity in the cell body, and the response of Schwann cells that myelinate the damaged axon.

We first addressed how axonal injury triggers enhancement of axonal growth capacity in PNS neurons. The lack of regenerative ability of CNS neurons has been linked to downregulation of the mammalian target of rapamycin (mTOR) pathway. We find that PNS dorsal root ganglia neurons (DRGs) activate mTOR following damage, and that this activity contributes to enhance axonal growth capacity following injury. Furthermore, upregulation of mTOR activity by deletion of tuberous sclerosis

complex 2 (TSC2) in DRGs is sufficient to enhance axonal growth capacity *in vitro* and *in vivo*. We identified GAP-43 as a downstream target of this pathway, which may contribute to enhance regenerative ability. However, while genetic upregulation of mTOR activity in sensory neurons facilitates axonal regrowth, it also leads to a number of developmental and functional defects, including aberrant target innervation. Thus, while manipulation of the mTOR activity could stimulate nerve regeneration in the PNS, fine control of mTOR activity may be required for proper target innervation and functional recovery.

mTOR activation in the damaged neuron is likely to represent one of several signaling events that mediate nerve regeneration. We thus also explored other aspects of peripheral nerve injury signaling, including the retrograde transport of local injury signals by axonal vesicles, and the response of myelinating Schwann cells to axonal damage. Our results indicate that several classes of signaling pathways occurring both in axons and Schwann cells cooperate to generate a robust regenerative response. A better understanding of the signaling pathways leading to increased regenerative growth ability of PNS neurons may guide new strategies to enhance nerve regeneration in the CNS.

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ABSTRACT

Although neurons within the peripheral nervous system (PNS) have a remarkable ability to repair themselves after injury, neurons within the central nervous system (CNS) do not spontaneously regenerate. This problem has remained recalcitrant despite a century of research on the reaction of axons to injury. The balance between inhibitory cues present in the environment and the intrinsic growth capacity of the injured neuron determines the extent of axonal regeneration following injury. The cell body of an injured neuron must receive accurate and timely information about the site and extent of axonal damage in order to increase its intrinsic growth capacity and successfully regenerate. One of the mechanisms contributing to this process is retrograde transport of injury signals. For example, molecules activated at the injury site convey information to the cell body leading to the expression of regeneration-associated genes and increased growth capacity of the neuron. Here we discuss recent studies that have begun to dissect the injury-signaling pathways involved in stimulating the intrinsic growth capacity of injured neurons.

INTRODUCTION

The extremely polarized morphology of neurons (i.e. axon length extending for up to 1 m) poses challenging problems for intracellular-signaling pathways. Information about distant injury, for example, has to be communicated to the cell body to initiate a proper regenerative response. Research on nerve regeneration has classically focused on identifying the inhibitory factors present in the environment, which include the glial scar and molecules such as Nogo and myelin-associated glycoprotein [1]. We know much less about the mechanisms that activate the intrinsic growth capacity of neurons following injury. Upon embryonic to adult transition, the intrinsic neuronal growth activity is repressed to allow for proper synaptic development. Injury to adult peripheral neurons, but not to central nervous system (CNS) neurons, reactivates the intrinsic growth capacity and allows regeneration to occur. Primary sensory neurons with cell bodies in the dorsal root ganglion (DRG) provide a useful model system to study the mechanisms that regulate regeneration. DRG neurons are pseudobipolar neurons and possess two axonal branches: a peripheral axon that regenerates when injured and a centrally projecting axon that does not regenerate following injury. Remarkably, injury to the peripheral branch before injury to the central branch promotes regeneration of central axons [2] [3]. This phenomenon is referred to as the 'conditioning lesion' paradigm (Figure 1) and indicates that retrograde injury signals travel from the peripheral injury site back to the cell body to increase the intrinsic growth capacity of the neuron. An increased intrinsic growth state as a result of a preconditioning lesion may enable centrally injured axons to regenerate. A series of elegant studies in the early 1990s in the mollusk Aplysia californica provided evidence for the existence of

multiple injury signals functioning in a temporal sequence [4]: injury-induced discharge of axonal potentials, interruption of the normal supply of retrogradely transported target-derived factors (also called negative injury signals) and retrograde injury signals traveling from the injury site back to the cell body (also called positive injury signals) (Figure 2).

The retrograde transport of injury signals is one of the essential cellular mechanisms leading to regeneration. Coordination between several injury-signaling pathways is necessary to regulate the appropriate genes to promote neuronal survival and increase the intrinsic growth state of injured neurons. In this thesis, we depart from the traditional focus on inhibitory factors in the non-regenerating CNS, and focus on signaling mechanisms leading to the enhanced intrinsic growth capacity of peripheral neurons following injury.

Injury signaling in the axon

Positive injury signals

The positive injury signals identified thus far cover a broad array of functionally distinct proteins that include members of the mitogen-activated protein kinase family (MAPK), cytokines, and their downstream transcription factors, as well as locally translated importin, a main regulator of nuclear import and export. Axonal transport of several kinases was initially suggested to play a role in relaying information from the nerve terminal to the cell body [5]. It is now known that axonal injury induces local activation and retrograde transport of several MAPKs, including Erk [6] [7], the c-Jun N-terminal kinase (JNK) [8] [9], and the protein kinase G [10]. These studies strongly suggest that activation of kinases, in particular JNK and Erk and their interaction with the

dynein/dynactin retrograde molecular motors is required for regeneration [6] [8]. Indeed, deletion of JNK2 or JNK3 leads to defects in axonal outgrowth in response to dissociation [8]. Transport of such injury signal is complicated by the fact that many kinases including JNK and Erk are activated by reversible phosphorylation and without proper protection this signal may not persist [11].

A key question then is how to prevent deactivation of the signal during the long journey to the cell body. One elegant solution is to protect the signal with scaffolding proteins. For example, it has been recently shown that the intermediate filament vimentin interacts with phosphorylated Erk1 to protect it from dephosphorylation by calcium-dependent steric hindrance [12]. Another mechanism proposed to protect dephosphorylation is storage within intraluminal vesicles of multivesicular bodies [13]. Indeed, kinases such as JNK can hitchhike on axonal vesicles [8] and intraluminal vesicles are not always destined to lysosomes for degradation; they can also fuse back with the limiting membrane of late endosomes [14]. This process is hijacked by several toxins and viruses to reach the cell body and could similarly be exploited by signaling proteins. Combined with a protection mechanism against phosphatases during transport, activation and retrograde transport of MAPKs might play an important role in regeneration. The upstream signaling cascade leading to MAPK activation in the axon remains yet to be established.

In addition to MAPK, axonal injury activates several transcription factors through the local release of cytokines. These include the gp130 cytokines leukemia inhibitory factor (LIF), interleukein-6 (IL-6), and ciliary neurotrophic factor (CNTF). LIF and IL-6 are required for the increased growth state of DRG neurons following peripheral injury

through activation of downstream genes such as GAP43 [15] [16], although Cao et al. [17] reported that IL-6 knockout animals do not show defects in nerve regeneration. Upregulation of IL-6 in DRG cell bodies themselves following injury [17] [18] [19] raises the possibility of paracrine or autocrine action of IL-6, which may amplify a cytokine-induced retrograde signal. The gp130 cytokines signal through a common receptor, gp130, and the JAK-STAT pathway, which leads to STAT3 phosphorylation and translocation into the nucleus [20]. Although retrograde transport of locally activated STAT3 has been suggested [21] [22], *in vitro* studies using compartmentalized cultures suggest a signaling endosome model in which the gp130/JAK complex is endocytosed and retrogradely transported to activate STAT3 in the cell body [23]. Interestingly, STAT3 activation through the Jak2-signaling pathway occurs in DRG neurons cell body after peripheral, but not central, lesion [24] [25], strongly supporting a role for STAT3 in neuronal regeneration. Although STAT3 signaling promotes axonal regrowth, in vitro studies showed that suppressor of cytokine signaling (SOCS3) inhibits STAT3 [26] and SOCS3 levels are increased by peripheral injury [27]. Although the influence of endogenous SOCS3 on axonal growth in peripheral neurons may be limited, SOCS3 may contribute to the lack of regeneration in CNS neurons [26]. Indeed, deletion of SOCS3 in retinal ganglion cells promotes regeneration [28]. The pathways leading to STAT3 activation are partially understood but the downstream targets of the cytokine-STAT3 signaling remain to be clearly defined.

Work over the past ten years has confirmed that axons have the capacity to locally synthesize proteins [29]. Axonal mRNA translation plays a role in axonal growth during development [29]and mature neurons use axonal mRNA translation to transfer injury

signals to the nucleus of injured neurons. Following peripheral nerve injury, de novo synthesis of importin-beta [7]and vimentin [6] leads to the formation of an importin-activated Erk–vimentin complex that recruits the retrograde motor dynein, linking the nuclear import machinery to retrograde injury signaling. Since a surprisingly large population of mRNAs localizes to sensory axons [30], future studies will reveal the possible role for other de novo synthesized proteins in injury signaling.

The positive injury signals identified so far share one common theme: microtubule-dependent retrograde transport. In chapter 4, we examine the localization and transport properties of axonal vesicles that contain Sunday Driver, a scaffolding protein that links active JNK molecules with axonal transport [8]. Future studies may identify new molecules involved in injury signaling and elucidate how these signals interact with the axonal transport machinery. It is tempting to speculate that the combination of several positive injury signals might serve as an indicator of the extent and nature of damage.

Negative injury signals

Although loss of negative cues represents another important mechanism to sense injury, surprisingly little is known about this type of signaling. Once a neuron is connected with its target, target-derived signals must repress the intrinsic neuronal growth activity to allow for proper synaptic development. This repression has to be relieved to allow regeneration to occur. Although neurotrophins represent the ideal candidates, evidence for their role as negative signals following injury have not yet been established.

One recently identified negative injury signal is the TGF beta/SMAD2/SMAD3 pathway (personal communication with Zhigang He). SMAD2 is downregulated following peripheral nerve injury, indicating that SMAD2-dependent gene transcription may restrict the axonal growth ability in healthy neurons and injury may relieve this inhibition. Whether SMAD2/SMAD3 contributes to the decreased regenerative ability of adult CNS neurons remains to be determined. The transcription factor ATF-2 is also rapidly suppressed in neurons following injury [31]. Similarly to SMAD2, ATF2-dependent gene transcription may repress neuronal growth capacity. Future studies are needed to explore the role of negative injury signals in axonal regeneration.

Electrical activity

Recent data suggest an important role of neural activity in regeneration. The transection of axons initiates a large depolarizing voltage discharge that travels back to the soma and triggers vigorous spiking activity and sustained depolarization [32]. This extensive electrical activity produces a strong calcium influx in both the axon and the soma. Propagation of this response requires the activation of voltage-gated sodium channels and is necessary for regeneration, since axotomy in the presence of tetrodotoxin reduces the regenerative process [32]. Calcium influx is also necessary for regeneration in vitro and is likely to act through protein kinases such as ERK or PKA [33]. In vivo studies showed that electrical stimulation accelerates motor [34] and sensory [35] axon outgrowth and increases intracellular cAMP levels in DRG neurons as effectively as the conditioning lesion [35]. However, electrical stimulation did not recapitulate all characteristics of axonal outgrowth, indicating that cAMP alone is not sufficient to

trigger a complete regenerative response [35]. In marked contrast, electrical stimulation of CNS axons does not promote regeneration, even when provided a permissive growth environment through a peripheral nerve graft [36]. Electrical activity thus may play an important role as an early injury signal in the peripheral nervous system (PNS), but might be insufficient to initiate regeneration of CNS neurons.

Signaling mechanisms in CNS axons

Induction of retrograde injury signals has so far been demonstrated in peripheral neurons. Recent studies in CNS neurons of the retina have unravelled the existence of parallel mechanisms between CNS and PNS neurons and demonstrated that the growth capacity of CNS neurons can be enhanced. While retinal ganglion cells (RGCs) normally fail to regenerate their injured axons, lens injury activates macrophages and stimulates regeneration of RGCs [37]in a process that resemble the conditioning injury in DRG neurons. Soluble factors released by activated macrophages, such as oncomodulin, are sufficient to promote RGC regeneration through a Ca^{2+}/calmodulin-dependent pathway [37]. While oncomodulin promotes neurite outgrowth in cultured central and peripheral neurons [38], its role in sensory nerve regeneration has yet to be explored *in vivo*. Lens injury also induces upregulation of CNTF in retinal astrocytes, a process that is independent of macrophages, and leads to STAT3 activation in RGCs [39] [40]. The cytokine-mediated activation of STAT3 is a central injury signaling mechanism in PNS neurons, suggesting another possible parallel between the responses of CNS and PNS neurons to injury.

A separate study in RGCs have shown that downregulation of an intrinsic growth pathway is linked to their poor regenerative ability. RGC neurons show a developmental decline in mTOR activity, which correlates with their decreased growth capacity [41]. Furthermore, axotomy to RGCs leads to a further downregulation of mTOR activity [41]. Remarkably, activation of mTOR by genetic deletion of the upstream negative regulators PTEN or TSC1 promotes regeneration in these normally non-regenerating central neurons [41]. In chapter 2, we examine whether the mTOR pathway is activated in peripheral neurons following injury, and whether this pathway contributes to enhance axonal growth capacity.

Similarly, transcriptional repression is linked to the downregulation of intrinsic growth capacity in RGCs. RGCs show a developmental increase in the expression of Kruppel-like factor-4 (KLF4), a transcriptional repressor, which is correlated with the decline in growth ability [42]. Deletion of KLF4 in adult RGCs promotes regeneration [42]. However, the KLF-4 target genes that regulate growth ability have not yet been identified.

To elucidate molecular factors responsible for the poor regenerative capacity of the CNS neurons, it will be important to determine whether CNS neurons lack the ability to activate or transport injury signals, are unable to relieve the growth inhibition brought about during their maturation or are less responsive to cytokines and other injury induced stimuli.

Somatic injury signaling

Role of cAMP

Elevation of cAMP levels in the soma following axonal injury to peripheral neurons contributes to the initiation of axonal regrowth [43]. cAMP not only increases the growth capacity of injured neurons but also partly relieves CNS myelin inhibition. The increase in cAMP levels appears to be transient and initiates a series of signaling pathways involving PKA [44]. The effects of cAMP are transcription dependent [45] and require the transcription factor cAMP response element binding protein (CREB) [46]. Interestingly, CREB mRNA is present in developing axons and CREB translation and retrograde transport is triggered by the nerve growth factor (NGF) [47]. Whether CREB translation plays a role in injury signaling in adult neurons remains to be determined. Downstream targets of cAMP signaling include Arginase1, which mediates synthesis of polyamines [48], neuropeptide Y, CREM (cAMP response element modulator), VGF (NGF-inducible growth factor), and IL-6 [17] [19]. Some of these genes were also identified in studies comparing the pattern of gene expression at different times after sciatic nerve transection [27] [49], revealing a temporal hierarchy of gene activation following injury. Although cAMP analogs fail to activate the intrinsic growth state of RGCs [50] they potentiate the effect of lens injury [40], indicating that multiple pathways act in parallel to stimulate RGCs regeneration. Although a direct link between retrograde signaling in axons and elevation of cAMP in cell body of injured neurons is still lacking, these results strongly suggest that the intrinsic growth capacity of the CNS neurons can be enhanced under appropriate conditions.

Transcription factors

Initiation of a regeneration program requires that retrograde signals from the injury site alter transcription of multiple genes [45]. Members of the immediate-early genes family, including c-Jun and JunD [51] [52], as well as members of the constitutive transcription factors CREB, STAT3, SOX11, and ATF3 [24] [46] [53] [54] are elevated and in some cases also activated in DRG cell bodies after peripheral injury. The activation of c-Jun in the cell body is essential for the initiation of transcriptional changes required for successful axonal regeneration. Some of the identified c-Jun-dependent genes include integrin a7b1, CD44, and galanin [55]. Deletion of c-Jun in the nervous system, while causing little effect on axonal growth during development, leads to a marked defect in regeneration upon nerve transection [55]. The importance of c-Jun for regeneration also comes from the observation that c-Jun activation in DRGs is drastically greater following peripheral versus central branch axotomy [56] and c-Jun activation persists until successful target reinnervation has been achieved [57] [58]. The time course of c-Jun induction depends on the distance between the injury site and the cell body [57], suggesting that JNK activation in the axon may lead to c-Jun expression in the cell body [8] [9]. Similarly to c-Jun, ATF3, and STAT3 are induced in DRG neurons after peripheral, but not central injury [24] [59]. Overexpression of ATF-3 in cultured neurons enhances neurite outgrowth [59]and transgenic expression of ATF3 can partially recapitulate a conditioning injury [60]. Conditional gene disruption of STAT3 indicates that this gene may contribute to the survival of motor neurons after peripheral nerve lesion through activation of motor neuron survival factors such as Reg-2 and Bcl-xl [61], but a direct role on nerve regeneration per se has not been demonstrated. In vitro studies show that another transcription factor Sox11 is expressed at high levels in developing and

regenerating sensory neurons and regulates neurite outgrowth and cell survival [62]. Although the identity of the genes activated by injury is being unravelled, the overall sequence and coordination of transcriptional events that initiate and sustain a regeneration program awaits further studies.

Neurotrophic factors

Neurotrophic signaling is mostly known to play a role in neuronal survival during development. The function of neurotrophins has been recently extended to other aspects of neuronal function, including regeneration [63]. Upregulation of the glial-derived neurotrophic factor GDNF and one of its receptors GFRa1 in injured nerves suggest that GDNF provides neurotrophic support for injured DRG neurons [64]. GDNF delivery directly to DRG cell bodies facilitates the conditioning injury induced growth promoting effect [65]. Although GDNF and GFRa1 are retrogradely transported in peripheral axons [66], a role of GDNF in injury signaling has not yet been examined. Fibroblast growth factor-2 (FGF-2) is another neurotrophic factor contributing to nerve regeneration [67]. FGF-2 is upregulated following injury both at the lesion site and in the cell bodies of peripheral nerves and transgenic mice overexpressing FGF2 show a greater increase in the number of regenerating axons after sciatic nerve transection [68]. The presence of neurotrophin signaling in injured nerve emphasizes the signaling crosstalk that is required to promote neuronal survival and regeneration.

Support from Schwann cells

Extrinsic factors in the environment interact with axonal injury signals for successful regeneration. In the peripheral nervous system, Schwann cells play an essential role in the maintenance of axon health and integrity in addition to providing insulation for fast saltatory conduction of action potentials. In healthy nerves, the relationship between axons and their myelinating Schwann cells is maintained by contact-mediated reciprocal signaling [69]. Disruption of Schwann-cell-axon contact, such as in the case of injury, activates a series of molecular and morphological changes in Schwann cells [70]. These include dedifferentiation and demyelination, transfer of ribosomes to axons, phagocytosis of axonal and myelin debris, synthesis of growth factors and extracellular matrix proteins, and proliferation [70] [71]. Such Schwann-cell mediated events play a critical role in the response of axons to injury and are discussed in more detail in Chapter 5.

Conclusions

A single signaling pathway is unlikely to fully mediate nerve regeneration. Several classes of injury signals may coexist to ensure precise information on the nature of the damage and its distance from the cell body (Figure 3). It is tempting to speculate that the difference in time between the arrival at the soma of the back propagating axonal depolarization—the first injury signal, and the later arrival of a positive injury signal might serve as an indicator of the distance of the injury site from the cell body. Computational modeling analyses speculate that two signals-a rapid signal carried by action potentials, and a slow signal carried by dynein-based transport-is sufficient to estimate the distance between the injury site and the cell body [72]. However, a clear link

between the arrival of injury signals and specific gene activation is still missing. Ultimately, a direct comparison between injury-signaling mechanisms in CNS and PNS neurons might shed light on the poor regenerative capacity of CNS neurons. This knowledge will be essential to our understanding and ultimately treatment of many debilitating CNS disorders, since in addition to traumatic axonal damage resulting from spinal cord injury or stroke, axonal damage can also occur in many neurodegenerative diseases in which axonal pathologies interrupt the cell body/synapse connection.

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Figure 1. Conditioning injury paradigm

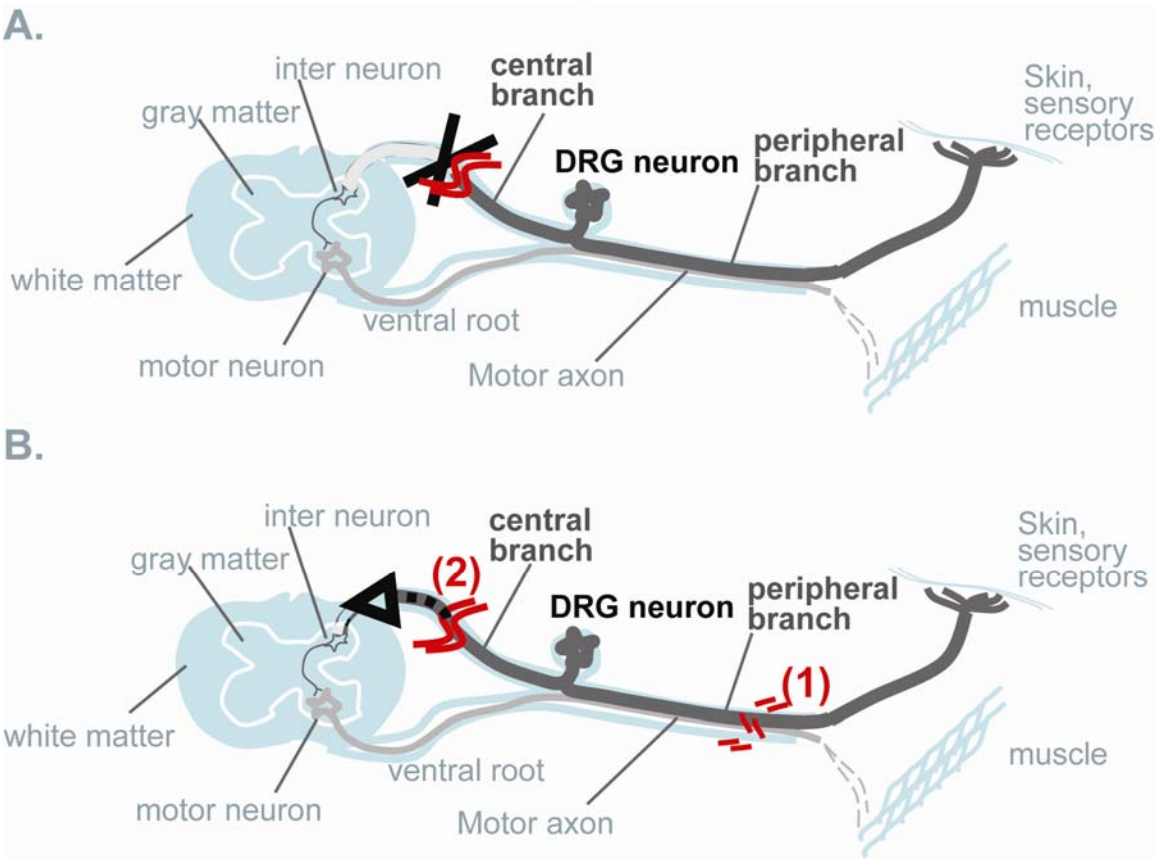

Figure 1. Conditioning injury paradigm. Primary sensory neurons within dorsal root ganglia (DRG) are particularly useful to study axonal regeneration. DRG neurons are unique in having two axonal branches; a long sensory CNS branch ascends the dorsal column in the spinal cord and a second branch projects through a peripheral nerve. Sensory axons in the adult spinal cord do not regenerate after injury (a), while peripheral injury results in a robust regenerative response. Regeneration of the central branch can be greatly enhanced by a prior injury to the peripheral branch, referred to as a 'conditioning injury' (b). The conditioning injury suggests that distinct signaling mechanisms regulate responses to central versus peripheral injury in DRG neurons and may contribute to their different abilities to axonal regrowth.

Figure 2. Injury signaling mechanisms

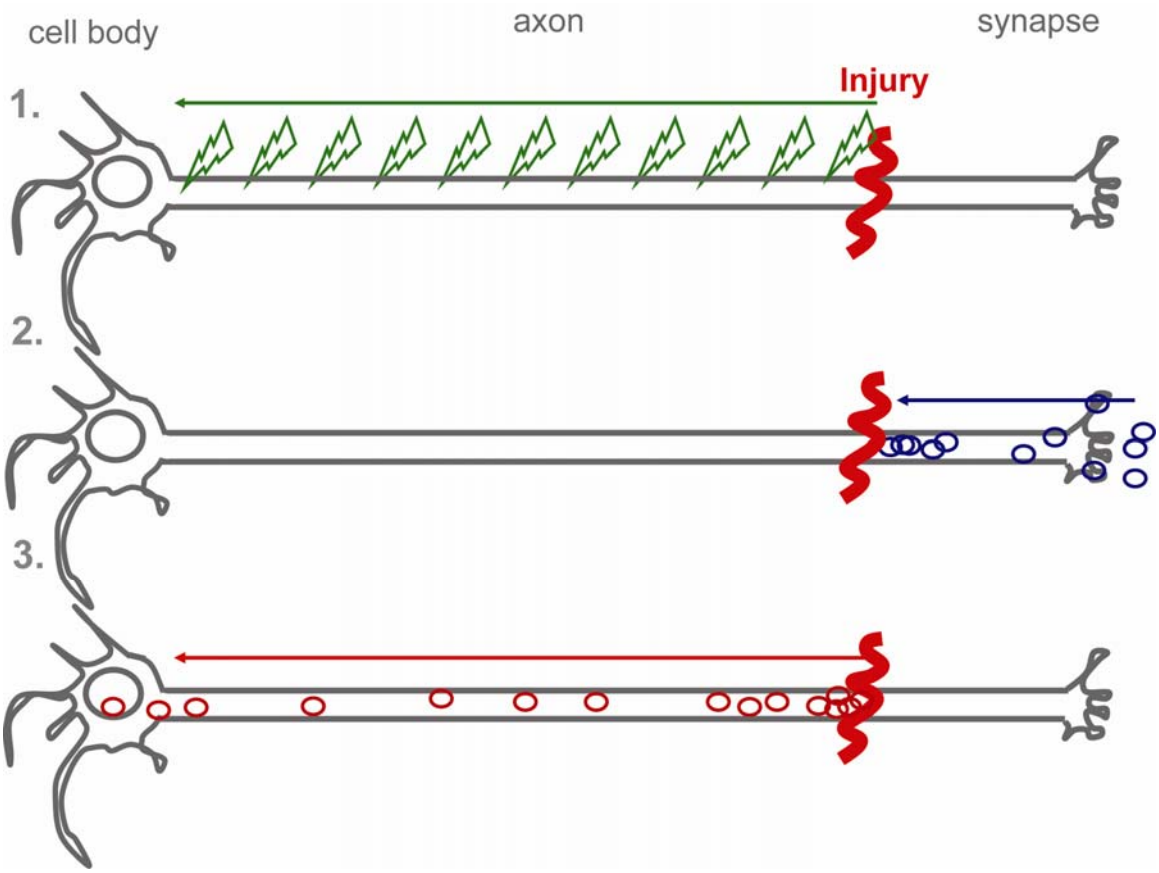

Figure 2. Injury signaling mechanisms. The cell body of injured neurons must receive accurate and timely information on the site and extent of axonal damage in order to orchestrate an appropriate response leading to successful regeneration. Pioneering work from the laboratories of Ambron and Walters have led to the notion that three distinct signaling mechanisms may act in complementary and synergistic roles: (1) injury-induced discharge of axonal potentials, (2) interruption of the normal supply of retrogradely transported trophic factors or negative regulators of neuronal growth from the target, and (3) retrograde transport of activated proteins emanating at the injury site, termed positive injury signals.

Figure 3. Activation of the intrinsic growth capacity by peripheral injury

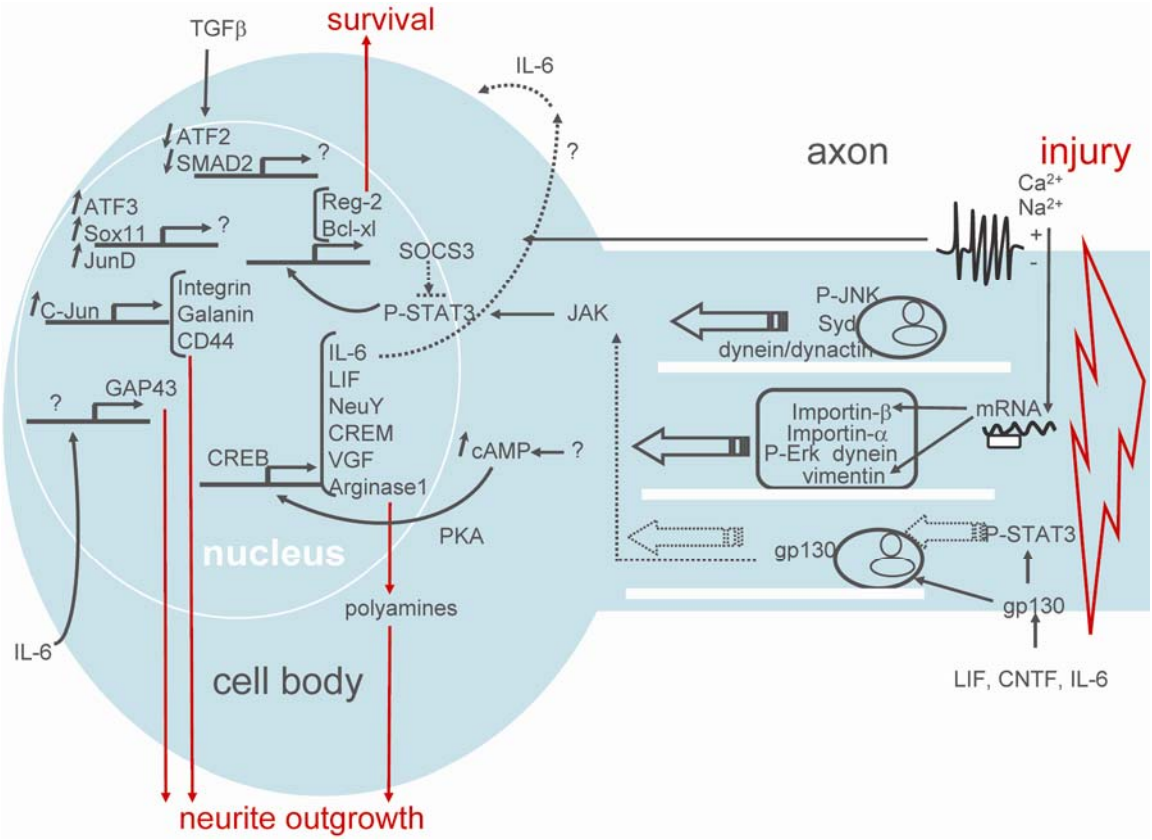

Figure 3. Activation of the intrinsic growth capacity by peripheral injury. Nerve injury triggers multiple signaling events in the axon, including membrane depolarization, JNK activation, mRNA translation, and cytokine-mediated STAT3 activation. These events lead to the microtubule-based retrograde transport of signaling molecules back to the cell body (shown by plain arrows). When these signaling molecules reach the cell body, they mediate the expression of a number of transcription factors that regulate the expression of genes involved in cell survival and neurite outgrowth. These downstream targets also include some components of the injury signal, such as IL-6 and LIF, which may amplify the injury signal via positive feedback.

REFERENCES

1. Schwab ME (2004) Nogo and axon regeneration. Curr Opin Neurobiol 14: 118-124.

2. Richardson PM, Issa VM (1984) Peripheral injury enhances central regeneration of primary sensory neurones. Nature 309: 791-793.

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

4. Ambron RT, Walters ET (1996) Priming events and retrograde injury signals. A new perspective on the cellular and molecular biology of nerve regeneration. Mol Neurobiol 13: 61-79.

5. Johanson SO, Crouch MF, Hendry IA (1995) Retrograde axonal transport of signal transduction proteins in rat sciatic nerve. Brain Res 690: 55-63.

6. Perlson E, Hanz S, Ben-Yaakov K, Segal-Ruder Y, Seger R, et al. (2005) Vimentin-dependent spatial translocation of an activated MAP kinase in injured nerve. Neuron 45: 715-726.

7. Hanz S, Perlson E, Willis D, Zheng JQ, Massarwa R, et al. (2003) Axoplasmic importins enable retrograde injury signaling in lesioned nerve. Neuron 40: 1095-1104.

8. Cavalli V, Kujala P, Klumperman J, Goldstein LS (2005) Sunday Driver links axonal transport to damage signaling. J Cell Biol 168: 775-787.

9. Lindwall C, Kanje M (2005) Retrograde axonal transport of JNK signaling molecules influence injury induced nuclear changes in p-c-Jun and ATF3 in adult rat sensory neurons. Mol Cell Neurosci 29: 269-282.

10. Sung YJ, Chiu DT, Ambron RT (2006) Activation and retrograde transport of protein kinase G in rat nociceptive neurons after nerve injury and inflammation. Neuroscience 141: 697-709.

11. Michaelevski I, Medzihradszky KF, Lynn A, Burlingame AL, Fainzilber M (2009) Axonal transport proteomics reveals mobilization of translation machinery to the lesion site in injured sciatic nerve. Mol Cell Proteomics.

12. Perlson E, Michaelevski I, Kowalsman N, Ben-Yaakov K, Shaked M, et al. (2006) Vimentin binding to phosphorylated Erk sterically hinders enzymatic dephosphorylation of the kinase. J Mol Biol 364: 938-944.

13. Weible MW, 2nd, Hendry IA (2004) What is the importance of multivesicular bodies in retrograde axonal transport in vivo? J Neurobiol 58: 230-243.

14. van der Goot FG, Gruenberg J (2006) Intra-endosomal membrane traffic. Trends Cell Biol 16: 514-521.

15. Cafferty WB, Gardiner NJ, Gavazzi I, Powell J, McMahon SB, et al. (2001) Leukemia inhibitory factor determines the growth status of injured adult sensory neurons. J Neurosci 21: 7161-7170.

16. Cafferty WB, Gardiner NJ, Das P, Qiu J, McMahon SB, et al. (2004) Conditioning injury-induced spinal axon regeneration fails in interleukin-6 knock-out mice. J Neurosci 24: 4432-4443.

17. Cao Z, Gao Y, Bryson JB, Hou J, Chaudhry N, et al. (2006) The cytokine interleukin-6 is sufficient but not necessary to mimic the peripheral conditioning lesion effect on axonal growth. J Neurosci 26: 5565-5573.

18. Murphy PG, Grondin J, Altares M, Richardson PM (1995) Induction of interleukin-6 in axotomized sensory neurons. J Neurosci 15: 5130-5138.

19. Wu D, Zhang Y, Bo X, Huang W, Xiao F, et al. (2007) Actions of neuropoietic cytokines and cyclic AMP in regenerative conditioning of rat primary sensory neurons. Exp Neurol 204: 66-76.

20. Heinrich PC, Behrmann I, Haan S, Hermanns HM, Muller-Newen G, et al. (2003) Principles of interleukin (IL)-6-type cytokine signalling and its regulation. Biochem J 374: 1-20.

21. Sheu JY, Kulhanek DJ, Eckenstein FP (2000) Differential patterns of ERK and STAT3 phosphorylation after sciatic nerve transection in the rat. Exp Neurol 166: 392-402.

22. Lee N, Neitzel KL, Devlin BK, MacLennan AJ (2004) STAT3 phosphorylation in injured axons before sensory and motor neuron nuclei: potential role for STAT3 as a retrograde signaling transcription factor. J Comp Neurol 474: 535-545.

23. O'Brien JJ, Nathanson NM (2007) Retrograde activation of STAT3 by leukemia inhibitory factor in sympathetic neurons. J Neurochem 103: 288-302.

24. Schwaiger FW, Hager G, Schmitt AB, Horvat A, Streif R, et al. (2000) Peripheral but not central axotomy induces changes in Janus kinases (JAK) and signal transducers and activators of transcription (STAT). Eur J Neurosci 12: 1165-1176.

25. Qiu J, Cafferty WB, McMahon SB, Thompson SW (2005) Conditioning injury-induced spinal axon regeneration requires signal transducer and activator of transcription 3 activation. J Neurosci 25: 1645-1653.

26. Miao T, Wu D, Zhang Y, Bo X, Subang MC, et al. (2006) Suppressor of cytokine signaling-3 suppresses the ability of activated signal transducer and activator of

transcription-3 to stimulate neurite growth in rat primary sensory neurons. J Neurosci 26: 9512-9519.

27. Nilsson A, Moller K, Dahlin L, Lundborg G, Kanje M (2005) Early changes in gene expression in the dorsal root ganglia after transection of the sciatic nerve; effects of amphiregulin and PAI-1 on regeneration. Brain Res Mol Brain Res 136: 65-74.

28. Smith PD, Sun F, Park KK, Cai B, Wang C, et al. (2009) SOCS3 deletion promotes optic nerve regeneration in vivo. Neuron 64: 617-623.

29. Twiss JL, van Minnen J (2006) New insights into neuronal regeneration: the role of axonal protein synthesis in pathfinding and axonal extension. J Neurotrauma 23: 295-308.

30. Willis D, Li KW, Zheng JQ, Chang JH, Smit A, et al. (2005) Differential transport and local translation of cytoskeletal, injury-response, and neurodegeneration protein mRNAs in axons. J Neurosci 25: 778-791.

31. Martin-Villalba A, Winter C, Brecht S, Buschmann T, Zimmermann M, et al. (1998) Rapid and long-lasting suppression of the ATF-2 transcription factor is a common response to neuronal injury. Brain Res Mol Brain Res 62: 158-166.

32. Mandolesi G, Madeddu F, Bozzi Y, Maffei L, Ratto GM (2004) Acute physiological response of mammalian central neurons to axotomy: ionic regulation and electrical activity. FASEB J 18: 1934-1936.

33. Chierzi S, Ratto GM, Verma P, Fawcett JW (2005) The ability of axons to regenerate their growth cones depends on axonal type and age, and is regulated by calcium, cAMP and ERK. Eur J Neurosci 21: 2051-2062.

34. Al-Majed AA, Tam SL, Gordon T (2004) Electrical stimulation accelerates and enhances expression of regeneration-associated genes in regenerating rat femoral motoneurons. Cell Mol Neurobiol 24: 379-402.

35. Udina E, Furey M, Busch S, Silver J, Gordon T, et al. (2008) Electrical stimulation of intact peripheral sensory axons in rats promotes outgrowth of their central projections. Exp Neurol 210: 238-247.

36. Harvey PJ, Grochmal J, Tetzlaff W, Gordon T, Bennett DJ (2005) An investigation into the potential for activity-dependent regeneration of the rubrospinal tract after spinal cord injury. Eur J Neurosci 22: 3025-3035.

37. Yin Y, Cui Q, Li Y, Irwin N, Fischer D, et al. (2003) Macrophage-derived factors stimulate optic nerve regeneration. J Neurosci 23: 2284-2293.

38. Yin Y, Henzl MT, Lorber B, Nakazawa T, Thomas TT, et al. (2006) Oncomodulin is a macrophage-derived signal for axon regeneration in retinal ganglion cells. Nat Neurosci 9: 843-852.

39. Muller A, Hauk TG, Fischer D (2007) Astrocyte-derived CNTF switches mature RGCs to a regenerative state following inflammatory stimulation. Brain 130: 3308-3320.

40. Park K, Luo JM, Hisheh S, Harvey AR, Cui Q (2004) Cellular mechanisms associated with spontaneous and ciliary neurotrophic factor-cAMP-induced survival and axonal regeneration of adult retinal ganglion cells. J Neurosci 24: 10806-10815.

41. Park KK, Liu K, Hu Y, Smith PD, Wang C, et al. (2008) Promoting axon regeneration in the adult CNS by modulation of the PTEN/mTOR pathway. Science 322: 963-966.

42. Moore DL, Blackmore MG, Hu Y, Kaestner KH, Bixby JL, et al. (2009) KLF family members regulate intrinsic axon regeneration ability. Science 326: 298-301.

43. Hannila SS, Filbin MT (2008) The role of cyclic AMP signaling in promoting axonal regeneration after spinal cord injury. Exp Neurol 209: 321-332.

44. Qiu J, Cai D, Dai H, McAtee M, Hoffman PN, et al. (2002) Spinal axon regeneration induced by elevation of cyclic AMP. Neuron 34: 895-903.

45. Smith DS, Skene JH (1997) A transcription-dependent switch controls competence of adult neurons for distinct modes of axon growth. J Neurosci 17: 646-658.

46. Gao Y, Deng K, Hou J, Bryson JB, Barco A, et al. (2004) Activated CREB is sufficient to overcome inhibitors in myelin and promote spinal axon regeneration in vivo. Neuron 44: 609-621.

47. Cox LJ, Hengst U, Gurskaya NG, Lukyanov KA, Jaffrey SR (2008) Intra-axonal translation and retrograde trafficking of CREB promotes neuronal survival. Nat Cell Biol 10: 149-159.

48. Cai D, Deng K, Mellado W, Lee J, Ratan RR, et al. (2002) Arginase I and polyamines act downstream from cyclic AMP in overcoming inhibition of axonal growth MAG and myelin in vitro. Neuron 35: 711-719.

49. Costigan M, Befort K, Karchewski L, Griffin RS, D'Urso D, et al. (2002) Replicate high-density rat genome oligonucleotide microarrays reveal hundreds of regulated genes in the dorsal root ganglion after peripheral nerve injury. BMC Neurosci 3: 16.

50. Goldberg JL, Klassen MP, Hua Y, Barres BA (2002) Amacrine-signaled loss of intrinsic axon growth ability by retinal ganglion cells. Science 296: 1860-1864.

51. Leah JD, Herdegen T, Bravo R (1991) Selective expression of Jun proteins following axotomy and axonal transport block in peripheral nerves in the rat: evidence for a role in the regeneration process. Brain Res 566: 198-207.

52. Jenkins R, Hunt SP (1991) Long-term increase in the levels of c-jun mRNA and jun protein-like immunoreactivity in motor and sensory neurons following axon damage. Neurosci Lett 129: 107-110.

53. Tanabe K, Bonilla I, Winkles JA, Strittmatter SM (2003) Fibroblast growth factor-inducible-14 is induced in axotomized neurons and promotes neurite outgrowth. J Neurosci 23: 9675-9686.

54. Tsujino H, Kondo E, Fukuoka T, Dai Y, Tokunaga A, et al. (2000) Activating transcription factor 3 (ATF3) induction by axotomy in sensory and motoneurons: A novel neuronal marker of nerve injury. Mol Cell Neurosci 15: 170-182.

55. Raivich G, Bohatschek M, Da Costa C, Iwata O, Galiano M, et al. (2004) The AP-1 transcription factor c-Jun is required for efficient axonal regeneration. Neuron 43: 57-67.

56. Broude E, McAtee M, Kelley MS, Bregman BS (1997) c-Jun expression in adult rat dorsal root ganglion neurons: differential response after central or peripheral axotomy. Exp Neurol 148: 367-377.

57. Kenney AM, Kocsis JD (1998) Peripheral axotomy induces long-term c-Jun amino-terminal kinase-1 activation and activator protein-1 binding activity by c-Jun and junD in adult rat dorsal root ganglia In vivo. J Neurosci 18: 1318-1328.

58. Herdegen T, Claret FX, Kallunki T, Martin-Villalba A, Winter C, et al. (1998) Lasting N-terminal phosphorylation of c-Jun and activation of c-Jun N-terminal kinases after neuronal injury. J Neurosci 18: 5124-5135.

59. Seijffers R, Allchorne AJ, Woolf CJ (2006) The transcription factor ATF-3 promotes neurite outgrowth. Mol Cell Neurosci 32: 143-154.

60. Seijffers R, Mills CD, Woolf CJ (2007) ATF3 increases the intrinsic growth state of DRG neurons to enhance peripheral nerve regeneration. J Neurosci 27: 7911-7920.

61. Schweizer U, Gunnersen J, Karch C, Wiese S, Holtmann B, et al. (2002) Conditional gene ablation of Stat3 reveals differential signaling requirements for survival of motoneurons during development and after nerve injury in the adult. J Cell Biol 156: 287-297.

62. Jankowski MP, Cornuet PK, McIlwrath S, Koerber HR, Albers KM (2006) SRY-box containing gene 11 (Sox11) transcription factor is required for neuron survival and neurite growth. Neuroscience 143: 501-514.

63. Cui Q (2006) Actions of neurotrophic factors and their signaling pathways in neuronal survival and axonal regeneration. Mol Neurobiol 33: 155-179.

64. Hoke A, Cheng C, Zochodne DW (2000) Expression of glial cell line-derived neurotrophic factor family of growth factors in peripheral nerve injury in rats. Neuroreport 11: 1651-1654.

65. Mills CD, Allchorne AJ, Griffin RS, Woolf CJ, Costigan M (2007) GDNF selectively promotes regeneration of injury-primed sensory neurons in the lesioned spinal cord. Mol Cell Neurosci 36: 185-194.

66. Coulpier M, Ibanez CF (2004) Retrograde propagation of GDNF-mediated signals in sympathetic neurons. Mol Cell Neurosci 27: 132-139.

67. Grothe C, Nikkhah G (2001) The role of basic fibroblast growth factor in peripheral nerve regeneration. Anat Embryol (Berl) 204: 171-177.

68. Jungnickel J, Haase K, Konitzer J, Timmer M, Grothe C (2006) Faster nerve regeneration after sciatic nerve injury in mice over-expressing basic fibroblast growth factor. J Neurobiol 66: 940-948.

69. Salzer JL, Brophy PJ, Peles E (2008) Molecular domains of myelinated axons in the peripheral nervous system. Glia 56: 1532-1540.

70. Jessen KR, Mirsky R (2008) Negative regulation of myelination: relevance for development, injury, and demyelinating disease. Glia 56: 1552-1565.

71. Court FA, Hendriks WT, Macgillavry HD, Alvarez J, van Minnen J (2008) Schwann cell to axon transfer of ribosomes: toward a novel understanding of the role of glia in the nervous system. J Neurosci 28: 11024-11029.

72. Kam N, Pilpel Y, Fainzilber M (2009) Can molecular motors drive distance measurements in injured neurons? PLoS Comput Biol 5: e1000477.

Chapter 2: mTOR activation increases axonal growth capacity of injured peripheral nerves

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ABSTRACT

Unlike neurons in the central nervous system (CNS), injured neurons in the peripheral nervous system (PNS) can regenerate their axons and re-innervate their targets. The lack of regenerative ability of CNS neurons has been linked to downregulation of the mammalian target of rapamycin (mTOR) pathway. We report here that PNS dorsal root ganglia neurons (DRGs) activate mTOR following damage, and that this activity enhances axonal growth capacity. Furthermore, genetic upregulation of mTOR activity by deletion of tuberous sclerosis complex 2 (TSC2) in DRGs is sufficient to enhance axonal growth capacity *in vitro* and *in vivo*. We further report that mTOR activation increases the expression of the growth-associated protein GAP-43, suggesting that mTOR-dependent protein synthesis contributes to enhance regeneration of injured peripheral neurons.

INTRODUCTION

While neurons in the central nervous system (CNS) have limited capacity for regrowth after damage, neurons in the peripheral nervous system (PNS) have a robust ability to regenerate their axons following injury. However, functional recovery of injured peripheral nerves often remains suboptimal, especially in cases of damage to a significant length of a peripheral nerve. Successful regeneration depends upon both extrinsic cues in the environment and the activation of intrinsic mechanisms to promote re-growth. The glial environment of the adult CNS includes inhibitory factors that prevent axon regrowth [1,2,3]. Components of the glial scar, which forms after CNS injury, act as additional barriers to axon regeneration [4]. Furthermore, CNS neurons display a decreased intrinsic capacity to regenerate, as removal of extracellular inhibitory cues is not sufficient to promote successful regeneration [5,6,7,8]. Remarkably, genetic deletion of all three major myelin inhibitors, Nogo, OMgp, and MAG does not promote CNS regeneration [8].

Injured peripheral neurons benefit from the absence of inhibitory signals in their environment, and in addition activate intracellular signaling pathways that enable axonal regrowth. The 'conditioning injury' paradigm observed in dorsal root ganglia neurons (DRGs) provides evidence for the existence of such intracellular signaling pathways induced by injury, which enhance axonal growth capacity [9,10,11]. Injury to the sciatic nerve several days prior to dissection allows affected DRG neurons to extend more elongated, rapidly growing axons in culture compared to DRG neurons not subjected to a conditioning injury [10]. This suggests that the robust response of peripheral axons to

injury is not merely a 'default' state, but results from activation of injury signals to increase axonal growth capacity.

Injury signals elicited both locally at the injury site and in the DRG cell bodies increase the intrinsic growth capacity. Members of the MAPK family including JNK [12,13], protein kinase G [14], and Erk1/Erk2 [15,16] are activated in injured axons and retrogradely transported to the cell body, where they activate downstream effectors required for regeneration. In DRGs cell bodies, transcription factors including c-Jun [17,18], CREB [19], STAT3 [20] and ATF3 [21] are activated and initiate transcriptional changes that contribute to regeneration. Despite the growing number of molecules identified that play a role in regeneration, no single signaling pathway or transcription factor alone has been shown to be sufficient for complete regeneration in the PNS, suggesting that multiple pathways work in concert to maximize axonal growth capacity.

Recent evidence implicates the control of protein synthesis by the mTOR pathway in the ability of neurons to regenerate. In non-neuronal cells, the mTOR pathway plays a critical role in the regulation of cellular growth, proliferation, and survival during development via the regulation of protein synthesis [22,23]. Upstream regulators of mTOR include Akt and the TSC1/TSC2 complex, which sense the level of growth factors, nutrients, ATP, and reactive oxygen species to either inhibit or activate mTOR [24] (Figure 1). Activation of mTOR leads to the downstream phosphorylation of S6 ribosomal protein and 4EBP-1 to initiate protein translation [24] (Figure 1). The developmental decline in mTOR activity observed in retinal ganglion cells correlates with their decreased growth capacity [25]. Moreover, axotomy to retinal ganglion cells leads to a further downregulation of mTOR activity [25]. Activation of mTOR by

deletion of the upstream negative regulators PTEN or TSC1 promotes regeneration of retinal ganglion cells, suggesting that mTOR activity is sufficient to increase growth capacity in normally non-regenerating central neurons [25]. While inactivation of mTOR activity is associated with decreased growth ability in the CNS, it is not yet known whether this pathway is activated in the PNS after injury and whether it contributes to increased axonal growth capacity.

In this study, we report that the mTOR pathway is activated in DRG neurons after injury. Inhibition of mTOR activity by rapamycin partially blocks the conditioning injury effect in DRGs, suggesting that mTOR activity contributes to the enhancement of axonal growth capacity upon injury. Furthermore, deletion of TSC2, a negative regulator of mTOR activity, leads to increased basal level of mTOR activity, which is sufficient to mimic the conditioning injury-effect and enhance regeneration *in vitro* and *in vivo.* We further report that mTOR activation increases the expression of the growth-associated protein GAP-43, suggesting that mTOR-dependent increased protein synthesis contributes to enhance regeneration of injured peripheral neurons.

RESULTS

The mTOR pathway is activated in DRGs cell bodies following injury- To test whether peripheral neurons upregulate the mTOR pathway following injury, we induced sciatic nerve injury by ligation, then dissected and analyzed L4 and L5 DRGs one to four days later. Phosphorylation of ribosomal S6 protein (S6), a downstream effector of mTOR, was used as a marker for mTOR activity. One day following sciatic nerve ligation, we observed a ~2-fold increase in the level of phosphorylated S6 protein in DRGs cell bodies from the ligated nerve compared with those from the contralateral unligated nerve (Figure 2A, 2B). By four days following ligation, S6 phosphorylation levels returned to basal, revealing the transient nature of the mTOR pathway activation (Figure 2B). To confirm that mTOR activation is the source of S6 phosphorylation following injury, we tested whether S6 phosphorylation in DRG cell bodies upon sciatic nerve ligation can be blocked by rapamycin, a potent inhibitor of mTOR. DRG cell bodies of mice injected intraperitoneally with rapamycin prior to sciatic nerve ligation showed a lower level of S6 phosphorylation compared to those of vehicle-treated ligated mice, suggesting that mTOR activity contributes to S6 phosphorylation after injury (Figure 2C, 2D). Interestingly, a minor component of S6 phosphorylation following injury was resistant to rapamycin, suggesting that in addition to mTOR activity, there may be mTOR-independent modes of S6 phosphorylation after injury (Figure 2D).

In addition to the increase in S6 phosphorylation levels, we also observed an increase in the total level of S6 protein itself in DRG cell bodies one day following sciatic nerve injury (Figure 2A, 2C, 2E). Inhibition of mTOR activity by rapamycin blocked the

increase in the total level of S6 protein after injury, suggesting that the mTOR pathway may regulate the translation of S6 itself (Figure 2E).

mTOR contributes to enhance axonal growth capacity following injury- Inactivation of the mTOR pathway following injury was shown to mediate the decreased growth capacity of retinal ganglion cells [25]. To determine whether increased mTOR activity after injury contributes to the enhanced axonal growth capacity of DRGs, we used the conditioning injury paradigm, in which prior injury causes a transition in neuronal morphology (or 'growth pattern') from compact, branched arbors to elongated, more sparsely branched axons [10]. We tested whether the presence of rapamycin, a potent inhibitor of mTOR activity during the conditioning injury blocks the increase in axonal outgrowth. To measure the extent of axon outgrowth, we calculated the distance between the cell body and the tip of the longest axon ('radial projection length'). DRGs cultured in the presence of NGF from mice injected intraperitoneally with vehicle as a control showed the expected ~2 fold increase in mean radial projection length upon a conditioning injury (Figure 3A, 3B). In contrast, intraperitoneal injection of rapamycin partially blocked the enhanced radial projection of axons upon a conditioning injury (Figure 3A, 3B, 3C). These results suggest that the mTOR pathway contributes at least in part to increase axonal growth capacity following injury.

Deletion of TSC2 in DRGs leads to elevated mTOR activity- To test whether mTOR activation is sufficient to increase axonal growth capacity, we genetically increased basal level of mTOR activity by deleting TSC2, a negative regulator of mTOR activity, in DRGs. *Tsc2*$^{flox/flox}$ mice [26] were crossed to mice expressing cre under the control of the advillin promoter, which is expressed almost exclusively in peripheral

sensory neurons [27]. *Tsc2*$^{flox/flox;}$ *Advillin*$^{Cre/+}$ mice are thereafter referred to as TSC2KO. *Tsc2*$^{flox/flox}$; *Advillin*$^{+/+}$ littermates are referred to as control. Deletion of TSC2 was confirmed by analyzing protein expression in DRGs cell bodies and brain extracts by western blot (Figure 4A). As TSC2 is a negative regulator of mTOR activity, we predicted that TSC2 deletion would upregulate mTOR activity. As expected, deletion of TSC2 in DRGs led to enhanced basal level of S6 phosphorylation, specifically in DRGs (Figure 4A and 4B). We measured a ~2 fold increase in basal S6 phosphorylation levels in DRGs cell bodies isolated from TSC2KO mice compared to control littermates, a level similar to the increase in S6 phosphorylation levels we observed in DRGs cell bodies one day after injury in wild-type mice (Figure 4B).

DRGs lacking TSC2 display enhanced axonal outgrowth in vitro and in vivo- To test whether increased mTOR activity is sufficient to increase axonal growth capacity in DRGs in the absence of a conditioning injury, we cultured DRGs from TSC2KO and control mice in the presence of NGF and assessed axonal outgrowth. TSC2KO DRGs grew more elongated, less arborized axons compared to those of control DRGs, mimicking the morphology of DRGs subjected to a conditioning injury (Figure 4C, 4D, 4E). The radial projection length of TSC2KO DRG axon was greater than that of control littermate and similar to that of control littermate DRGs subjected to a conditioning injury (Figure 4C, 4D). Conditioning injury to TSC2KO neurons did not significantly increase radial projection length compared to the uninjured condition, suggesting that even without the presence of a conditioning injury, TSC2KO DRGs have reached their maximal growth capacity in this assay (Figure 4C, 4D). The presence of the cre recombinase is not sufficient to enhance axonal growth capacity, as wild-type;

Advillin$^{Cre/+}$ mice did not show significant difference with wild-type; *Advillin*$^{+/+}$ littermate controls (Figure 4F).

To test whether TSC2 deletion facilitates axonal regeneration *in vivo,* we crushed the sciatic nerve in TSC2KO mice and control littermates and assessed the level of regeneration past the crush site 12 or 24 hours later. To visualize re-growth of damaged axons, longitudinal sciatic nerve sections were stained for the growth-associated protein GAP-43. The reported concentration of GAP-43 in axonal growth cone [28]together with its strong expression in regenerating axons [29] makes it an ideal marker to track nerve regeneration *in vivo* [30]. The length of GAP-43-expressing axons past the crush site was markedly increased in TSC2KO sciatic nerve compared to that of control littermates (Figure 5A, 5B). We quantified this increase by normalizing GAP-43 fluorescence intensity to that at the crush site to control for the increased GAP-43 expression level observed from TSC2KO neurons (Figure 6B, 6C). We calculated a regeneration index by measuring the distance away from the crush site in which the average GAP-43 intensity is half that observed at the crush site. The regeneration index was significantly higher in TSC2KO sciatic nerves compared to control nerves for both the 12 and 24 hours timepoints (Figure 5C). Thus, this result suggests that enhanced mTOR activity is sufficient to facilitate axon regeneration *in vivo.*

mTOR activity regulates GAP-43 expression following injury- GAP-43 is a crucial component of axonal outgrowth in developing and regenerating neurons [29,31,32]. The enhanced growth capacity of neurons lacking TSC2 may thus result from an enhanced expression of GAP-43. To directly test whether mTOR activity regulates GAP-43 expression following injury, we analyzed GAP-43 levels in nerve portions

proximal and distal to an axotomy site and in contralateral non-injured sciatic nerve by western blot. 24 hours after injury, GAP-43 protein level increased in the sciatic nerve proximal to the injury site in both TSC2KO and control and mice (Figure. 6B, 6C). Accumulation of GAP-43 in the proximal nerve stump is consistent with its role in promoting axonal outgrowth. However, both the basal and injury-induced levels of GAP-43 in the sciatic nerve were significantly higher in TSC2KO mice compared to controls (Figure 6B, 6C). Cultured DRGs also show a higher GAP-43 expression at the cell body and the tip of growing axons (Figure 6A). In contrast to the local increase of GAP-43 expression in sciatic nerve, injury did not increase GAP-43 levels in the DRGs cell bodies of TSC2KO mice or controls. However, the basal level of GAP-43 expression was enhanced in TSC2KO DRGs as observed in the sciatic nerve (Figure 6D, 6E).

To confirm that mTOR activity is required for the increase in GAP-43 expression after injury, we tested whether inhibition of mTOR by rapamycin reduces GAP-43 protein levels in injured and uninjured sciatic nerve. Intraperitoneal injection of rapamycin one hour prior to injury partially blocked the increase in GAP-43 expression in the proximal injured nerve stump (Figure 6F, 6G). Together, these results suggest that mTOR activity regulates GAP-43 expression in peripheral nerves following injury.

To determine whether the effect of TSC2 deletion on GAP-43 expression is specific to GAP-43 or is due to a global upregulation of protein translation, we determined whether the levels of other proteins are also increased in TSC2 KO DRGs. We examined the levels of peripherin and β–actin, whose translation after injury has been implicated in axon regeneration [16,33]. The basal level of peripherin and β–actin in uninjured sciatic nerve were not significantly different between control and TSC2 KO

mice (Figure 7A, 7B). Furthermore, the elevated level of these proteins in injured sciatic nerve were also unaffected by TSC2 deletion (Figure 7C, 7D). The expression levels of these proteins were also not significantly altered in DRGs cell bodies of TSC2 KO mice compared to control (Figure 7C, 7D). Thus, mTOR may be regulating the expression level of a specific subset of proteins that includes GAP-43.

DISCUSSION

The extent of axonal regeneration not only depends on the presence or absence of inhibitory cues in the environment, but also on the intrinsic growth capacity of the damaged neurons. Research on nerve regeneration has largely focused on identifying the inhibitory molecules in the CNS environment that act as barriers to regeneration. Recent evidence suggests that in addition to differences in their environments, CNS and PNS neurons differ in their intrinsic ability to regrow their axons after injury.

Work in retinal ganglion cells has implicated the mTOR pathway in regulating the growth capacity of neurons. Axotomy to retinal ganglion cells markedly reduces mTOR activity while genetic activation of mTOR activity by PTEN or TSC1 deletion is sufficient to boost the regenerative ability in these normally non-regenerating neurons [25]. We found that in contrast to retinal ganglion cells, DRG neurons in the PNS activate the mTOR pathway in response to injury. Activation of the mTOR pathway in DRGs implies that the ability of PNS neurons to regenerate is not solely due to the lack of inhibitory molecules in the environment, but also to an active intracellular mechanism that enhances growth capacity. Indeed, we find that rapamycin blocks the enhancement in axonal outgrowth following a conditioning injury, suggesting that mTOR activity contributes to boost the regenerative potential after damage. The observation that mTOR activation is transient and returns to basal level 3 to 4 days following injury suggests that prolonged mTOR activity may further enhance PNS regeneration.

Similarly to what was observed in CNS neurons, we found that upregulation of the mTOR pathway is sufficient to enhance axonal growth capacity in PNS neurons. Genetic ablation of TSC2, a negative regulator of mTOR activity, led to enhanced axonal

outgrowth and regenerative capacity in DRGs. TSC2KO DRGs grew faster, more elongated and sparsely branched axons in culture, similarly to wild-type DRGs subjected to a conditioning injury [10]. Thus, increased mTOR activity is sufficient to enhance axonal growth potential even in the absence of a conditioning injury. We also observed that injured TSC2KO DRGs grew further past the injury site *in vivo* compared to control DRGs. Based on our observation that TSC2 deletion primes DRGs to grow long axons *in vitro* in the absence of a conditioning injury, TSC2KO DRGs may also be primed to respond to injury *in vivo* and initiate axon regrowth earlier than control DRGs. Alternatively, TSC2 deletion may affect injury-induced retraction that occurs prior to axon elongation. Clearly, a more detailed analysis will determine the precise mechanism regulating regenerative growth in the absence of TSC2. Nevertheless, the observation that TSC2 deletion can facilitate the regrowth of crushed peripheral axons *in vivo* supports the notion that regeneration of injured axons in the PNS can be further enhanced.

The major downstream targets of mTOR are components of the translation machinery, including those that regulate the recruitment of ribosomes to mRNA. Protein synthesis plays a critical role in both injury signaling [16,34,35,36] and the formation of new growth cones during regeneration [37]. Several proteins translated in peripheral neurons after injury, including vimentin and importin β, have been shown to be critical for regeneration [16]. However, whether their expression is regulated by mTOR activity has not been explored. Our present study identified GAP-43 as a downstream target of mTOR activity. TSC2KO neurons showed enhanced GAP-43 levels both in naïve and injury-induced conditions. In addition, rapamycin treatment partially blocked the increase in GAP-43 protein levels after injury, showing that the mTOR pathway regulates

GAP-43 expression. As GAP-43 plays a key role in axon sprouting and outgrowth in regenerating axons [31,32] [38], [39], it is likely that the enhancement of axon growth capacity by the mTOR pathway is at least in part due to the regulation of GAP-43 expression. However, overexpression of GAP-43 alone is not sufficient to fully stimulate regrowth of axons in the central branch of DRGs into the spinal cord [11]. Thus, the mTOR pathway likely regulates the translation of a number of proteins in addition to GAP-43 to maximize the axonal growth capacity.

Our work indicates that PNS neurons turn on the mTOR pathway following injury, while CNS neurons do not [25]. It will therefore be important in future studies to identify the upstream regulators of mTOR that are specifically activated by PNS injury to better understand the poor regenerative ability of CNS neurons. In non-neuronal cells, a number of upstream regulators of mTOR have been identified, and include the serine/threonine protein kinase Akt and the TSC1-TSC2 complex. Growth factors, nutrients and insulin among other factors activate PI-3Kinase, which leads to the phosphorylation and activation of Akt. Active Akt in turn phosphorylates and inhibits TSC2 activity leading to mTOR activation via Rheb GTPase [40,41,42,43,44] (Figure 1). Surprisingly, we did not detect Akt phosphorylation in DRGs cell bodies 24 hr after injury at time points at which S6 is already phosphorylated (Figure 8A). A recent study reported a decrease, rather than an increase in Akt phosphorylation levels in DRGs three days following sciatic nerve injury [45]. However, as chronic activation of mTOR can downregulate Akt activation via a feedback mechanism, a more detailed time-course study of Akt activation should be performed to address whether mTOR activation after injury occurs through Akt [46].

Interestingly, we found that TSC2KO DRGs, which have an increased basal level of S6 phosphorylation, are able to further increase S6 phosphorylation levels upon sciatic nerve injury even in the absence of TSC2 (Figure 8B). Thus, inactivation of TSC2 by Akt may not fully account for mTOR activation in DRGs cell bodies after injury, and other pathways independent of TSC2 may converge to activate mTOR. One TSC2-independent mode of mTOR regulation occurs through phosphatidic acid [47] (Figure 8C). Phosphatidic acid production is mediated by phospholipase D1, which is activated by the small GTPase Cdc42 [48]. Interestingly, Cdc42 mRNA is upregulated in DRGs following axotomy, and Cdc42 overexpression induces enhanced axonal outgrowth [49]. Another possible TSC2-independent regulator of mTOR activity is the JAK-STAT–SOCS3 signaling pathway (Figure 8D). SOCS3 is a negative regulator of JAK-STAT signaling which suppresses regeneration in retinal ganglion cells [50]. Genetic deletion of SOCS3 in retinal ganglion cells is sufficient to activate the mTOR pathway and promotes regeneration [50]. In DRGs, injury induces activation of JAK-STAT signaling through the cytokine CNTF and its receptor gp130 [51], suggesting that CNTF-mediated JAK-STAT signaling may interact with the mTOR pathway after peripheral nerve injury.

Enhancement of axonal regeneration in both CNS and PNS neurons by activation of the mTOR pathway presents an exciting therapeutic target for facilitating recovery from nerve injury. However, it remains unclear whether upregulation of mTOR activity can promote successful target re-innervation in addition to facilitation of axonal regrowth. Interestingly, we observe that activation of mTOR in injured DRGs is transient, returning to basal levels after four days following injury (Figure 2A, 2B). This finding led us to question whether prolonged activation of mTOR in peripheral neurons could have

deleterious effects on target innervation and functional recovery. In Chapter 4, we examine the consequences of persistent mTOR activity in peripheral nerve development and function to speculate whether upregulation of mTOR activity can offer a safe therapeutic approach to repair injured peripheral neurons.

MATERIALS AND METHODS

Antibodies and reagents- The following antibodies were used: anti-tuberin/TSC2 (Santa Cruz Biotechnology, C-Term), anti-phosphorylated S6 ribosomal protein (Cell Signaling, Serine 240/244), anti-S6 ribosomal (Cell Signaling), anti- α–tubulin (Sigma), anti-SMI-31(Sternberger Monoclonals Incorporated), anti-beta actin (Sigma), anti-peripherin (Millipore), anti-GAP-43 (Abcam), anti-tau (Synaptic Systems), and anti-GAP-43 (Chemicon) when used with anti-tau.

Animals- For experiments involving wild-type animals, C57B6 6 to 9 month of age females from Harlan were used. *Tsc2*$^{flox/flox}$ animals were previously described [26]and *Advillin*$^{Cre/Cre}$ mice were used to drive expression of cre in sensory neurons, based on the previous characterization of the advillin-hPLAP reporter mouse [27,52]. To generate *Tsc2*$^{flox/flox}$; *Advillin*$^{Cre/+}$ conditional knockout mice, we crossed *Tsc2*$^{flox/flox}$ females to *Advillin*$^{Cre/Cre}$ males to generate *Tsc2*$^{flox/+}$*; Advillin*$^{Cre/+}$ animals. Then, *Tsc2*$^{flox/flox}$*Advillin*$^{+/+}$ females were crossed to *Tsc2*$^{flox/+}$*; Advillin*$^{Cre/+}$ males to generate *Tsc2*$^{flox/flox}$; *Advillin*$^{Cre/+}$ conditional knockout animals and *Tsc2*$^{flox/flox}$; *Advillin*$^{+/+}$ littermate control animals. Genotype was confirmed by tail PCR at weaning age. 4-8 weeks old animals and sex- matched littermate controls were used for all experiments.

Surgical procedures and drug treatment- All surgical procedures were approved by the Washington University in St. Louis, School of Medicine, Animal Studies Committee. Sciatic nerve injury experiments were performed as described previously ([12])**.** Briefly, the right sciatic nerves of mice were ligated, axotomized, or crushed unilaterally at the midpoint, and mice were sacrificed at the indicated time after surgery. For biochemistry on DRGs cell bodies, L4, L5, and L6 DRGs were dissected from both the injured side

and the contralateral uninjured side for control. For biochemistry on sciatic nerve, equal lengths (5mm) of the proximal and distal parts were homogenized. DRGs and nerves were homogenized in lysis buffer (20mM Tris-HCl pH 7.5, 150mM NaCl, 1mM Na_2EDTA, 1mM EGTA, 1% Triton, 2.5mM sodium pyrophosphate, 1mM β-glycerophosphate, 1mM Na_3VO_4, 1μg/ml leupeptin) with phosphatase inhibitor cocktail 1 and 2 (Invitrogen). Equal protein amounts were loaded and analyzed by SDS-PAGE and western blot.

Rapamycin was delivered by intraperitoneal injection at 5mg/kg body weight. Rapamycin was dissolved in 200 μl DMEM from a 10 mg/ml stock solution in DMSO. An equivalent volume of DMSO was dissolved into 200 μl DMEM for vehicle control. Intraperitoneal injection was performed 1 hr before sciatic nerve ligation, and repeated two days following ligation. Animals were sacrificed and DRGs and nerves were dissected one or four days following ligation.

Primary DRGs culture and immunofluorescence- L4, L5, and L6 DRGs were dissected and dissociated in 0.7 mg/ml Liberase Blendzyme 3 (Roche), 600 μg/ml DNAse, 10 mg/ml BSA, in DMEM AIR (DMEM/F12 with 10mM Glucose, and 1% Pen/Strep) at 37ºC for 15 minutes, followed by trypsinization with 0.25% trypsin in DMEM AIR at 37°C for 15 minutes. DRGs were then triturated and placed in culture media (DMEM, 10% FBS, 1% pen/strep) with 50ng/ml NGF (Invitrogen) on poly-D-lysine-coated plates.

After 20-24 hrs, DRGs were fixed in 4% paraformaldehyde and 4% sucrose in PBS for 10 minutes. For immunofluorescence staining, cells were permeabilized and blocked in 10% Goat Serum, 0.1% Triton-X in PBS for 15 minutes. Staining was performed with the indicated primary antibodies for 30 minutes at room temperature, and

with Alexa-conjugated secondary antibodies for 20 minutes. Images were acquired with Nikon Eclipse TE2000-E inverted microscope and analyzed using Nikon NIS Elements Advanced Research 2.30 Imaging Software. To quantify levels of axonal outgrowth, we measured the distance between the cell body and the tip of the longest axon and annotated this measurement as the radial projection length. For each condition, radial projection lengths of all cells imaged were measured, and both means and histograms were used for comparison across conditions.

Immunohistochemistry- Crushed sciatic nerves were dissected and fixed for 1-2 hours in 4% PFA in PBS, then cryoprotected overnight in 20% sucrose. Posterior hindpaw skin sections were fixed in 15% picric acid, 2% paraformaldehyde in PBS for 3-4 hours, and cryoprotected in 30% sucrose for 48 hours. Nerves and skin were embedded in OCT (Tissue-Tek) and frozen in dry-ice-cooled methanol. Serial 10-µm (nerve) or 30-µm (skin) cryostat sections were cut and mounted onto coated slides (Fisher Scientific). Sections were permeabilized and blocked with 10% goat serum, 0.1% Triton X-100 in PBS for 30 min. Primary staining with the indicated antibodies was performed in the blocking solution overnight at 4ºC. Staining with Alexa-conjugated secondary antibody was performed for 1 to 3 hours. Sciatic nerve images were acquired with Nikon Eclipse TE2000-E inverted epifluorescence microscope and analyzed using Metamorph 6.2.

ACKNOWLEDGEMENTS

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Figure 1. Major components of the mTOR pathway

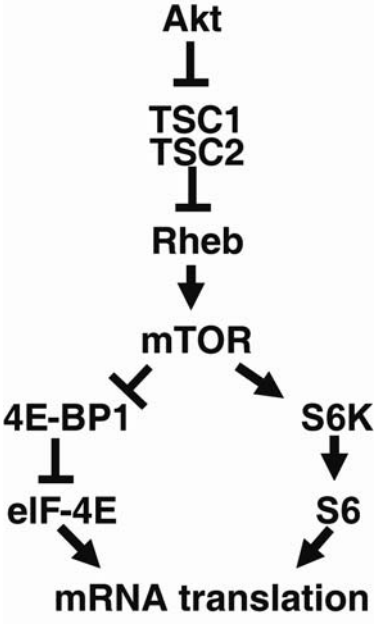

Figure 2. The mTOR pathway is upregulated in DRG cell bodies following sciatic nerve injury

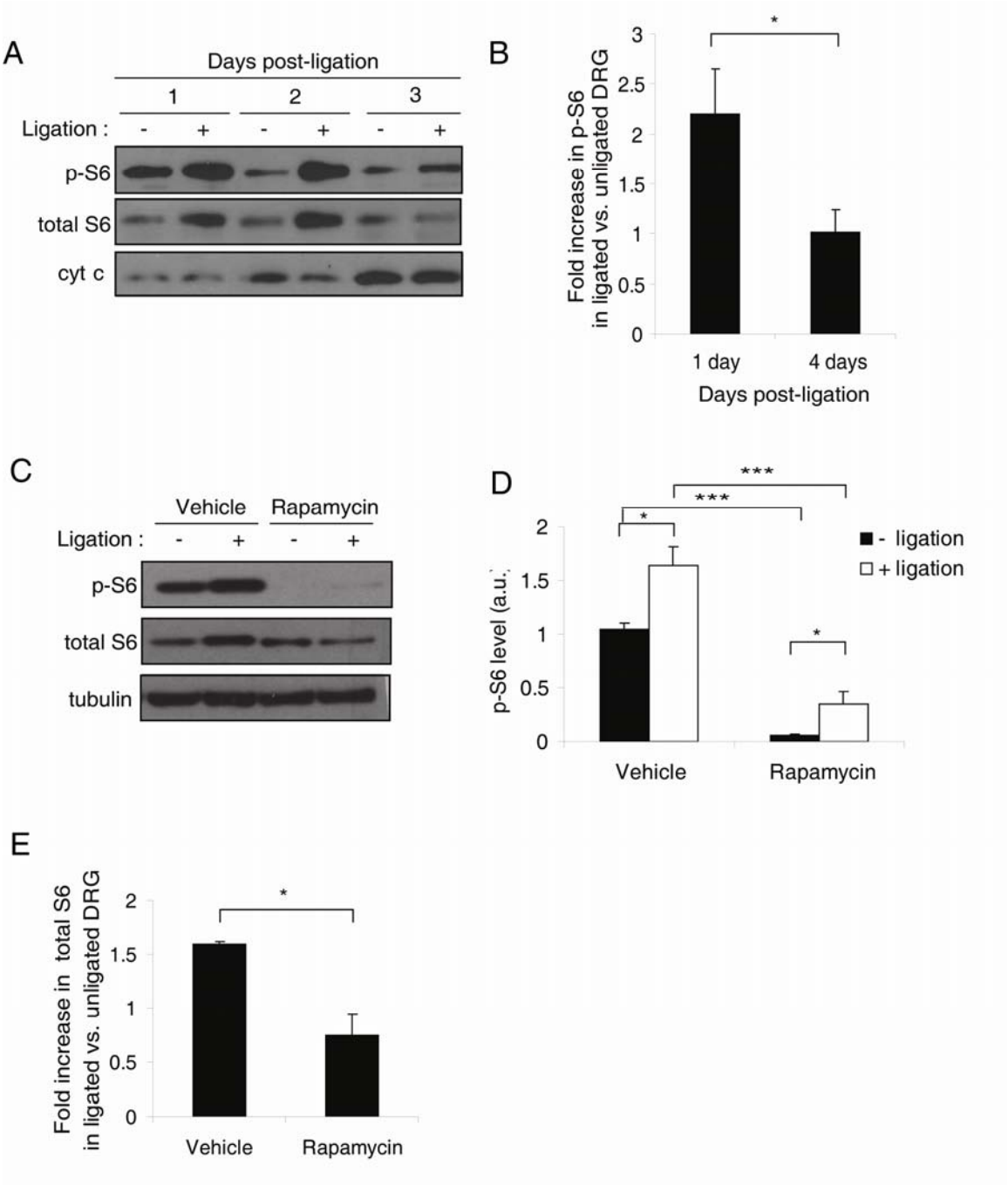

Figure 2. The mTOR pathway is upregulated in DRG cell bodies following sciatic nerve injury (A) Injury to the sciatic nerve of wild-type mice was induced by ligation and S6 phosphorylation levels were assessed in L4, L5, L6 DRG cell bodies at the indicated time points. DRG cell bodies display increased S6 phosphorylation levels one to two days following ligation. L4, L5, L6 DRG lysates from one mouse were pooled for each time point. Cytochrome c was used as loading control. (B) Quantification of fold change in S6 phosphorylation levels between DRG cell bodies from injured and un-injured nerves shows 2 fold increase in S6 phosphorylation one day after ligation which reached basal level 4 days following ligation. S6 phosphorylation levels were normalized to loading control. At least 4 mice were tested for each time point. Data are mean +/- SEM. *$p<0.05$ (Student's *t*-test). (C) Wild-type mice were treated with rapamycin or DMSO as a vehicle control by intraperitoneal injection and then subjected to sciatic nerve ligation to provoke injury one hour later. L4, L5 and L6 DRGs were dissected 24 hours later and S6 phosphorylation levels were assessed by western blot. L4, L5, L6 DRG lysates from one mouse were pooled for each time point. Basal and injury-induced levels of S6 phosphorylation are significantly reduced by rapamycin treatment. Tubulin was used as loading control. (D) Quantification of S6 phosphorylation levels from (A) shows a major component of S6 phosphorylation after injury is mTOR-dependent. S6 phosphorylation levels were normalized to loading control. 4 mice were tested for each condition. Data are mean +/- SEM. *$p<0.05$, ***$p<0.001$ (Student's *t*-test). (E) Quantification of total S6 protein levels of DRGs from (A) shows increase in total S6 levels following injury is mTOR-dependent. Total S6 levels were normalized to loading

control. 4 mice were tested for each condition. Data are mean +/- SEM.

*$p<0.05$(Student's *t*-test).

Figure 3. The mTOR pathway contributes to enhance axonal growth capacity after peripheral nerve injury

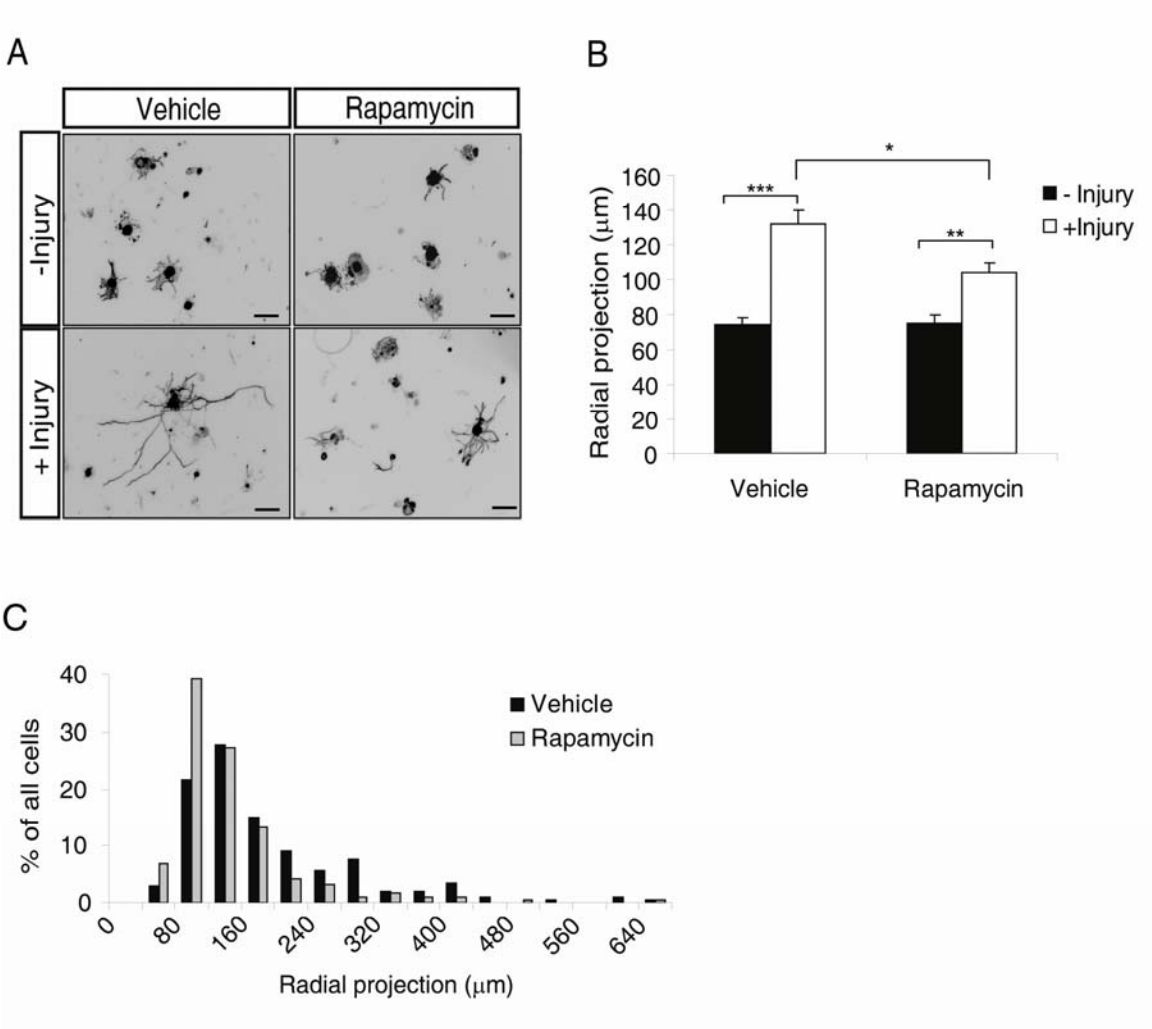

Figure 3. The mTOR pathway contributes to enhance axonal growth capacity after peripheral nerve injury. (A) Wild-type mice were treated with rapamycin or DMSO as a vehicle control by intraperitoneal injection and then subjected to sciatic nerve ligation to provoke injury one hour later. L4, L5 and L6 DRGs were dissected and cultured 4 days later. DRG cultured from injured sciatic nerve showed enhanced axonal outgrowth at 24 hours in culture. This effect was partially blocked by rapamycin. Axons were stained with α-SMI-31 antibody. (B) Quantification of radial projection length of naïve and injury-conditioned DRGs cultured from vehicle or rapamycin treated mice show rapamycin partially blocks conditioning injury paradigm. n=4 mice per condition; 100-350 neurons were analyzed per set of DRGs. Data are mean +/-SEM. *$p<0.05$, **$p<0.01$, ***$p<0.001$ (Student's *t*-test). (C) Histogram of radial projection length of injury-conditioned DRGs from one representative experiment shows shift in distribution upon rapamycin-treatment. (A) Bar=100μm.

Figure 4. TSC2KO DRGs display enhanced axonal outgrowth *in vitro*.

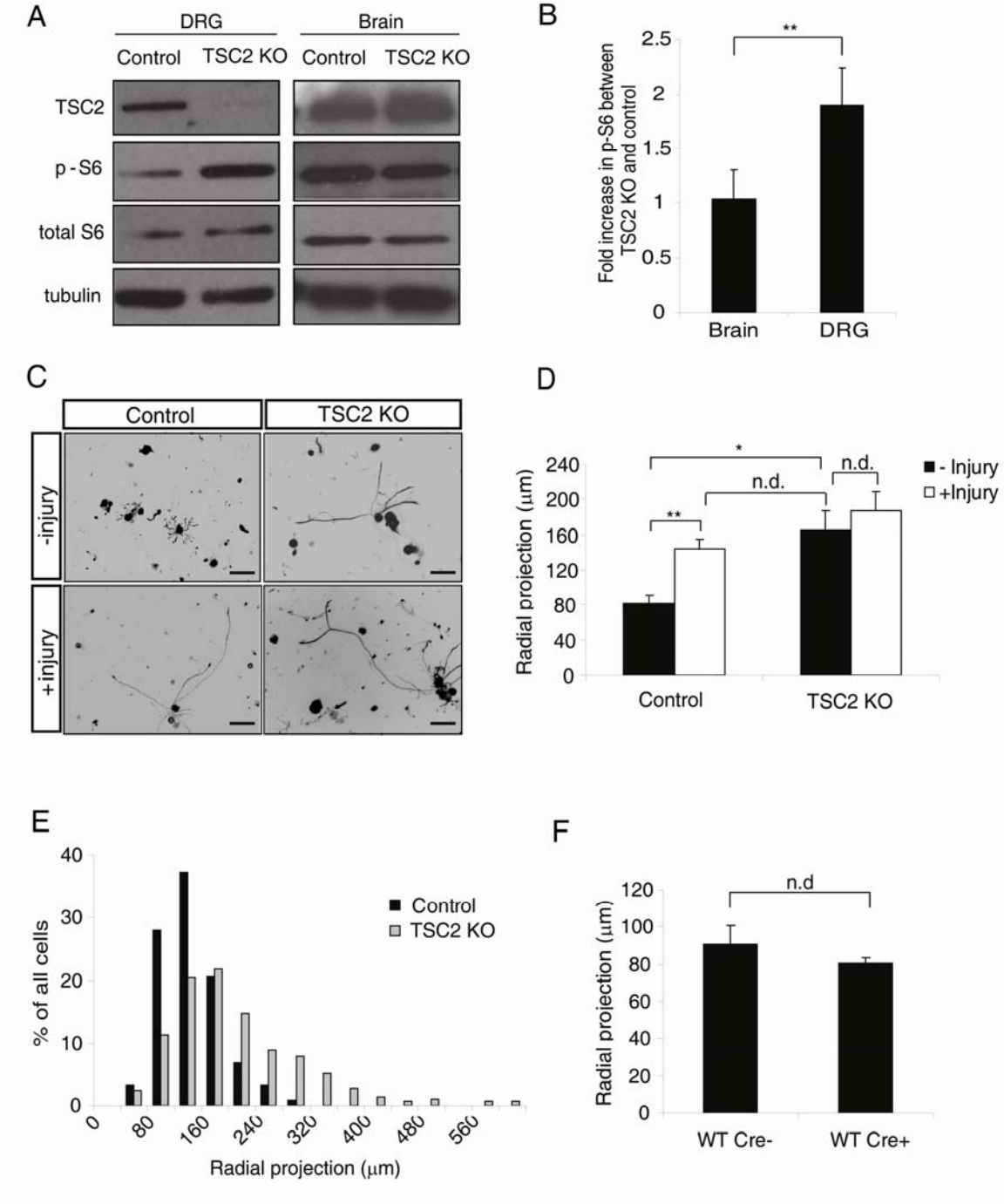

Figure 4. TSC2KO DRGs display enhanced axonal outgrowth *in vitro*. (A) Western blot of DRG cell bodies and brain lysates from TSC2KO and control animals show that TSC2 protein level are dramatically reduced in DRGs but not in brain. As expected, S6 phosphorylation level is increased in TSC2KO compared to control animals. Representative western blot is shown (B) Quantification of (A); n=5 mice per genotype, Data are mean +/- SEM. **p<0.01(Student's *t*-test). (C). Cultured TSC2KO DRGs show enhanced axonal outgrowth in the absence of a conditioning injury. Injury to the sciatic nerve 4 days prior to dissociation does not further increase axonal outgrowth in TSC2KO DRGs. Axons were stained with α-SMI-31 antibody. (D) Quantification of radial projection of naïve and injury-conditioned DRGs cultured from TSC2KO mice and controls. n=3 mice per genotype; 60-370 neurons were analyzed per set of DRGs. Data are mean +/- SEM. n.d.=no statistically significant difference, *p<0.05, **p<0.01 (Student's *t*-test). (E) Histogram of radial projection lengths of naive TSC2KO and control DRGs in culture. Data shown is from one representative experiment. (F) There was no significant difference in radial projection length between DRGs cultured from wild-type; *Advillin*$^{Cre/+}$ mice and those cultured from wild-type; *Advillin*$^{+/+}$ mice. n=3 mice per genotype. 100 to 200 neurons were measured per genotype. Data are mean +/- SEM. n.d.=no statistically significant difference (Student's *t*-test). (C) Bar=100μm.

Figure 5. TSC2KO neurons display enhanced regeneration *in vivo*.

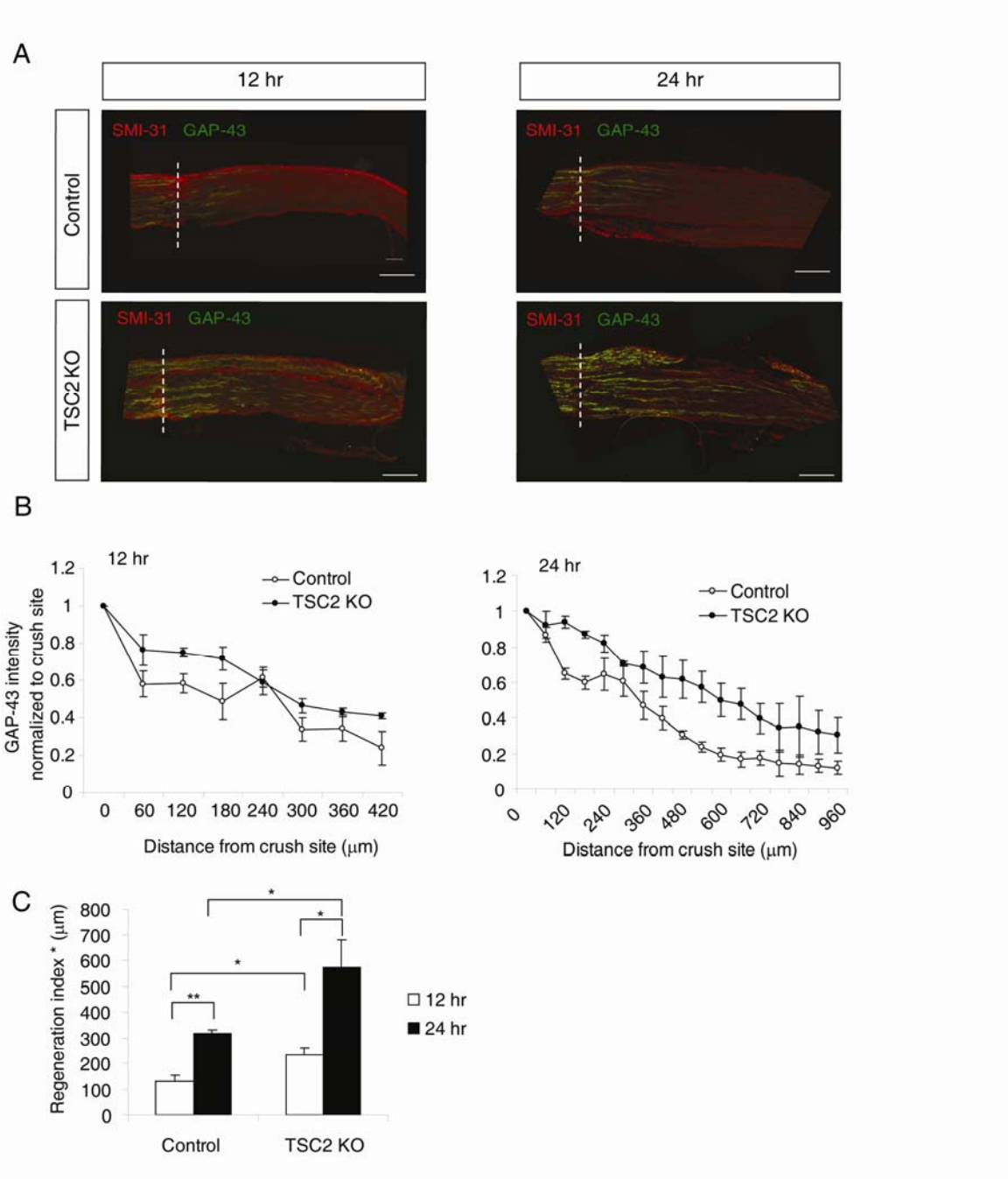

Figure 5. TSC2KO neurons display enhanced regeneration *in vivo.* TSC2KO and control mice were subject to a sciatic nerve crush, and regeneration of crushed axons was assessed 12 or 24 hours later. (A) Longitudinal section of sciatic nerve dissected 12 or 24 hours after crush reveals increased length of GAP-43 positive axons past the crush site in TSC2KO mice compared to control mice for both time points. Dashed line indicates crush site. (B) Average GAP-43 intensity at various distances distal to crush site 12 and 24 hours after crush reveal regenerating axons grew longer distances in TSC2KO mice compared to controls. GAP-43 intensity values were normalized to that at the crush site to control for the increased GAP-43 expression level observed from TSC2KO neurons. Data are mean +/- SEM. n=3 mice per genotype, 3 to 5 longitudinal sections were analyzed per mouse for both time points (Student's *t*-test). (C) Regeneration index was measured as the distance away from the crush site in which the average GAP-43 intensity is half that observed at the crush site. TSC2KO mice show a higher regeneration index compared to controls at both 12 and 24 hour timepoints.. *$p<0.05$, **$p<0.01$ (Student's *t*-test). (A) Bar=200μm.

Figure 6. The mTOR pathway regulates GAP-43 expression.

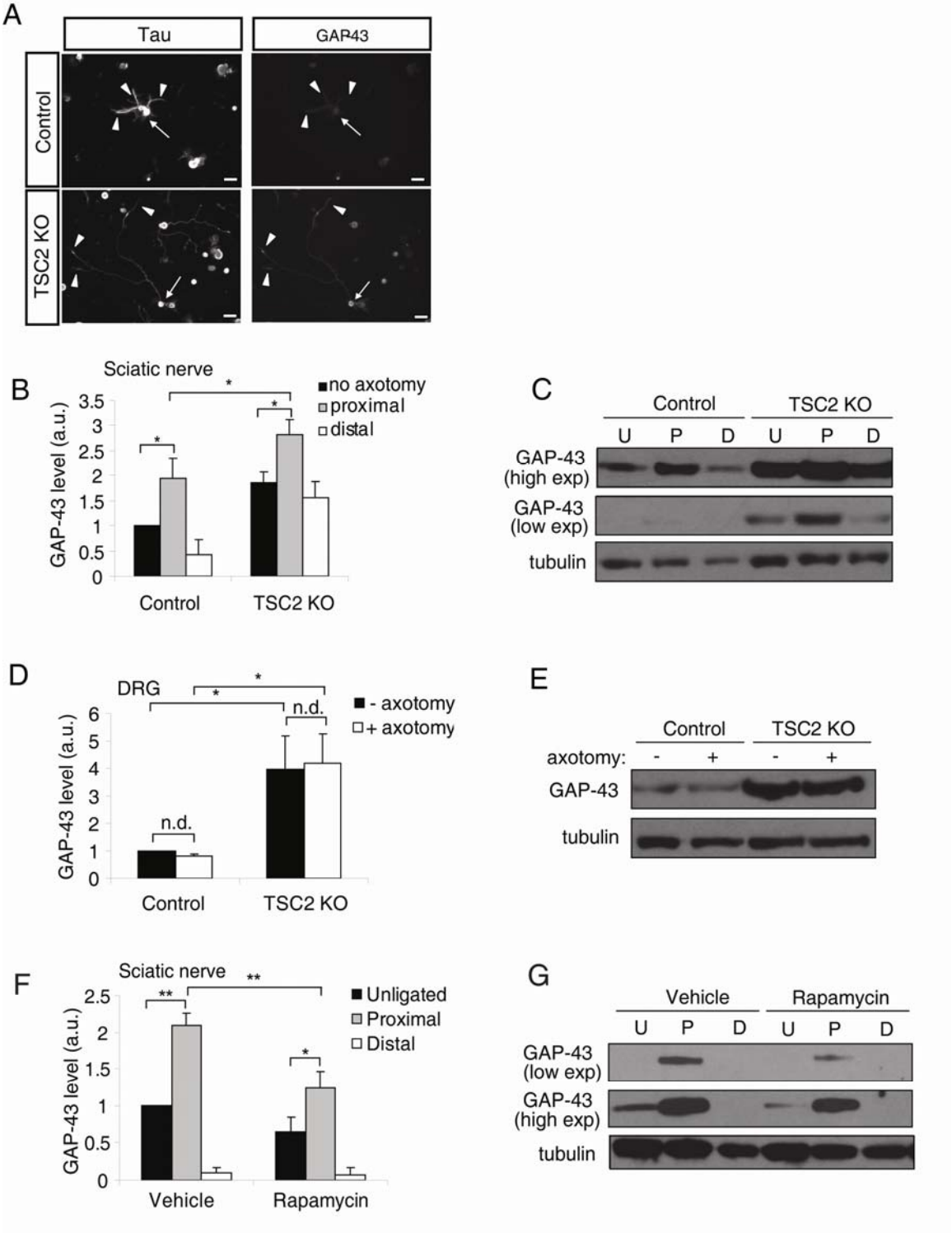

Figure 6. The mTOR pathway regulates GAP-43 expression. (A) Naïve TSC2KO DRGs show enhanced cell body and axonal GAP-43 expression in culture. Arrows indicate cell body, arrowheads indicate axonal tips. (B) Injury to the sciatic nerve of TSC2KO and control mice was induced by axotomy and GAP-43 levels in nerve portions proximal and distal to the axotomy site and in contralateral non-injured sciatic nerve were analyzed by western blot 24 hours later. Control mice show an increase in GAP-43 expression in sciatic nerve proximal to the injury site. Both the basal level and injury-induced level of GAP-43 expression is enhanced in TSC2KO mice. GAP-43 levels were normalized to loading control (tubulin). Data are mean +/- SEM. n=3 mice per genotype, *$p<0.05$ (Student's *t*-test). (C) Representative western blot from one experiment (one mouse per genotype) from (B). (D) As in (B), but DRG cell bodies were analyzed. GAP-43 levels were increased in DRG cell bodies of TSC2KO mice compared to control. Axotomy did not increase GAP-43 levels in DRG cell bodies in either genotype. n=3 mice per genotype, **$p<0.01$ (Student's *t*-test). (E) Representative western blot from one experiment (one mouse per genotype) from (C). (F) Rapamycin blocks increase in GAP-43 expression after injury. Wild-type mice were injected intraperitonealy with rapamycin or DMSO vehicle control, then sciatic nerve injury was induced by ligation. Four days following ligation, unligated nerve and ligated nerve distal and proximal to the ligation site were analyzed for GAP-43 expression. Data are mean +/- SEM. n=3 mice per condition. *$p<0.05$, *$p<0.01$ (Student's *t*-test). (G) Representative western blot from one experiment from (F). (C) Bar=100μm.

Figure 7. TSC2 deletion in DRG does not affect peripherin or β-actin expression.

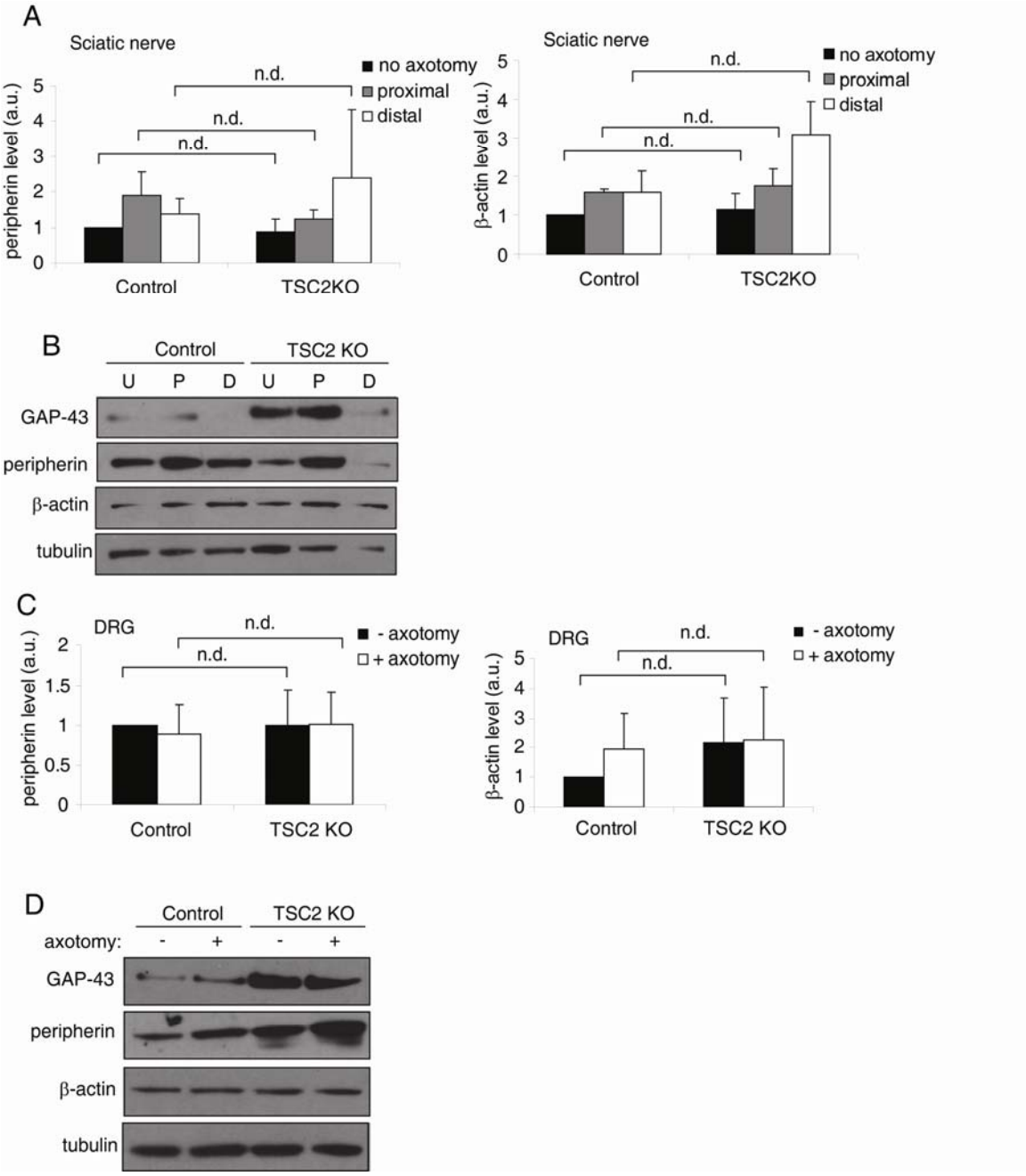

Figure 7. TSC2 deletion in DRG does not affect peripherin or β-actin expression. (A) Injury to the sciatic nerve of TSC2KO and control mice was induced by axotomy. peripherin and β-actin levels in nerve portions proximal and distal to the axotomy site and in contralateral non-injured sciatic nerve were analyzed by western blot 24 hours later. No significant difference in basal or injury-induced levels of peripherin or β-actin between TSC2KO and control mice was observed. Peripherin and β-actin levels were normalized to loading control (tubulin). Data are mean +/- SEM. n=3 mice per genotype. n.d.=no statistically significant difference (Student's *t*-test). (B) Representative western blot from one experiment (one mouse per genotype) from (A). (C) As in (A), but DRG cell bodies were analyzed. Peripherin and β-actin levels in DRG were not significantly different between TSC2KO and control. Data are mean +/- SEM. n=3 mice per genotype. n.d.=no statistically significant difference (Student's *t*-test). (D) Representative western blot from one experiment (one mouse per genotype) from (C).

Figure 8. mTOR activation after peripheral nerve injury may be Akt/TSC2-independent.

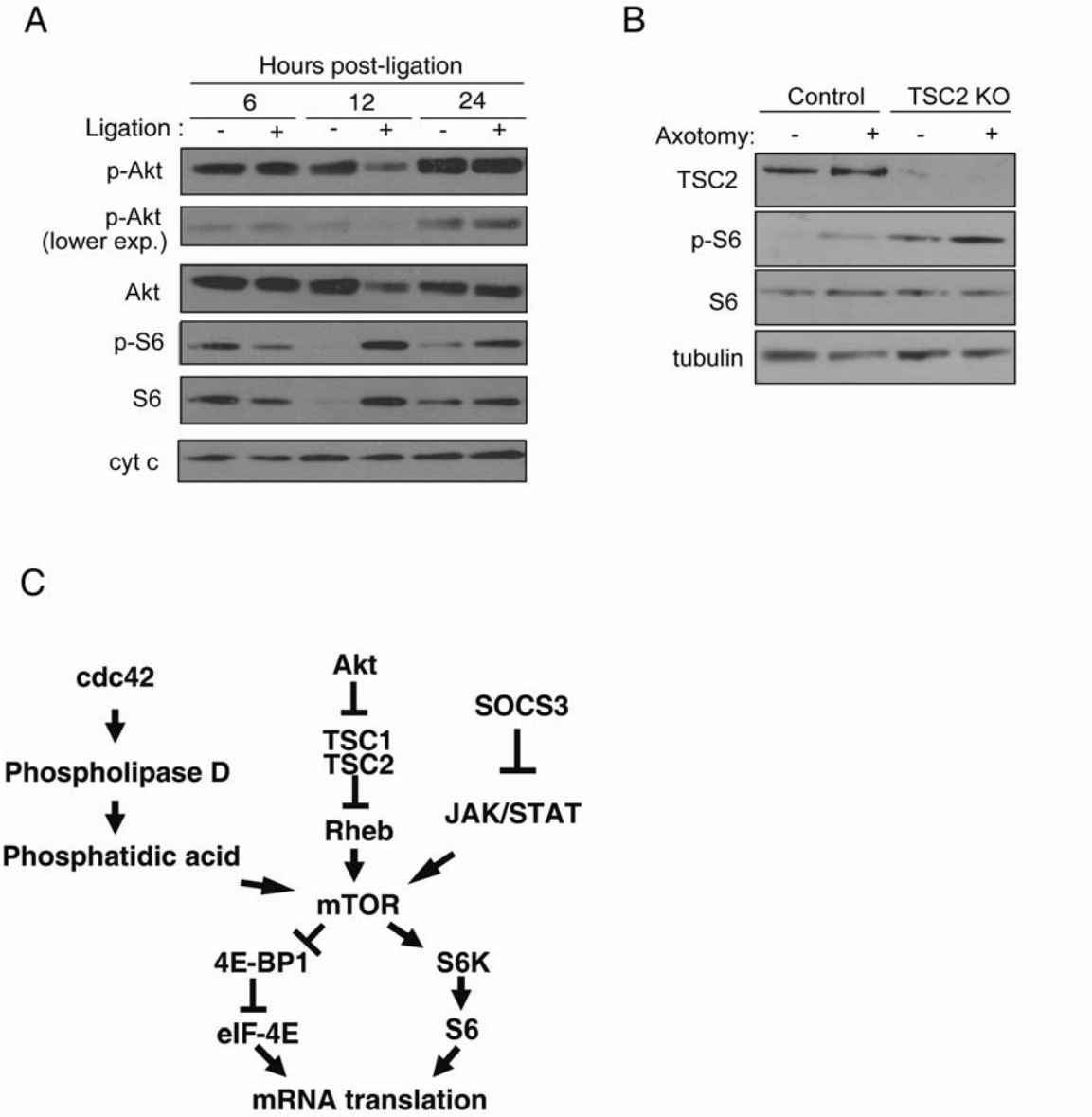

Figure 8. mTOR activation after peripheral nerve injury may be Akt/TSC2-independent.

(A) Injury to the sciatic nerve of wild-type mice was induced by ligation and phosphorylation of Akt (S473) and S6 was assessed in L4, L5, L6 DRG cell bodies at the indicated time points. While S6 phosphorylation was observed at 12 and 24 hour time points, Akt phosphorylation was not detected. Cytochrome c was used as loading control. (B) TSC2 KO DRGs cell bodies further upregulate S6 phosphorylation after sciatic nerve axotomy. DRGs were collected 24 hours after sciatic nerve axotomy and analyzed by western blot with the indicated antibodies. Tubulin was used as loading control. (C) Potential Akt/TSC2-independent modes of mTOR activation.

REFERENCES

1. Case LC, Tessier-Lavigne M (2005) Regeneration of the adult central nervous system. Curr Biol 15: R749-753.

2. Filbin MT (2003) Myelin-associated inhibitors of axonal regeneration in the adult mammalian CNS. Nat Rev Neurosci 4: 703-713.

3. Yiu G, He Z (2006) Glial inhibition of CNS axon regeneration. Nat Rev Neurosci 7: 617-627.

4. Shen Y, Tenney AP, Busch SA, Horn KP, Cuascut FX, et al. (2009) PTPsigma is a receptor for chondroitin sulfate proteoglycan, an inhibitor of neural regeneration. Science 326: 592-596.

5. Zheng B, Ho C, Li S, Keirstead H, Steward O, et al. (2003) Lack of enhanced spinal regeneration in Nogo-deficient mice. Neuron 38: 213-224.

6. Zheng B, Atwal J, Ho C, Case L, He XL, et al. (2005) Genetic deletion of the Nogo receptor does not reduce neurite inhibition in vitro or promote corticospinal tract regeneration in vivo. Proc Natl Acad Sci U S A 102: 1205-1210.

7. Woolf CJ (2003) No Nogo: now where to go? Neuron 38: 153-156.

8. Lee JK, Geoffroy CG, Chan AF, Tolentino KE, Crawford MJ, et al. (2010) Assessing spinal axon regeneration and sprouting in Nogo-, MAG-, and OMgp-deficient mice. Neuron 66: 663-670.

9. Richardson PM, Issa VM (1984) Peripheral injury enhances central regeneration of primary sensory neurones. Nature 309: 791-793.

10. Smith DS, Skene JH (1997) A transcription-dependent switch controls competence of adult neurons for distinct modes of axon growth. J Neurosci 17: 646-658.

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

12. Cavalli V, Kujala P, Klumperman J, Goldstein LS (2005) Sunday Driver links axonal transport to damage signaling. J Cell Biol 168: 775-787.

13. Lindwall C, Kanje M (2005) Retrograde axonal transport of JNK signaling molecules influence injury induced nuclear changes in p-c-Jun and ATF3 in adult rat sensory neurons. Mol Cell Neurosci 29: 269-282.

14. Sung YJ, Chiu DT, Ambron RT (2006) Activation and retrograde transport of protein kinase G in rat nociceptive neurons after nerve injury and inflammation. Neuroscience 141: 697-709.

15. Hanz S, Perlson E, Willis D, Zheng JQ, Massarwa R, et al. (2003) Axoplasmic importins enable retrograde injury signaling in lesioned nerve. Neuron 40: 1095-1104.

16. Perlson E, Hanz S, Ben-Yaakov K, Segal-Ruder Y, Seger R, et al. (2005) Vimentin-dependent spatial translocation of an activated MAP kinase in injured nerve. Neuron 45: 715-726.

17. Jenkins R, Hunt SP (1991) Long-term increase in the levels of c-jun mRNA and jun protein-like immunoreactivity in motor and sensory neurons following axon damage. Neurosci Lett 129: 107-110.

18. Broude E, McAtee M, Kelley MS, Bregman BS (1997) c-Jun expression in adult rat dorsal root ganglion neurons: differential response after central or peripheral axotomy. Exp Neurol 148: 367-377.

19. White DM, Walker S, Brenneman DE, Gozes I (2000) CREB contributes to the increased neurite outgrowth of sensory neurons induced by vasoactive intestinal polypeptide and activity-dependent neurotrophic factor. Brain Res 868: 31-38.

20. O'Brien JJ, Nathanson NM (2007) Retrograde activation of STAT3 by leukemia inhibitory factor in sympathetic neurons. J Neurochem 103: 288-302.

21. Tsujino H, Kondo E, Fukuoka T, Dai Y, Tokunaga A, et al. (2000) Activating transcription factor 3 (ATF3) induction by axotomy in sensory and motoneurons: A novel neuronal marker of nerve injury. Mol Cell Neurosci 15: 170-182.

22. Martin DE, Hall MN (2005) The expanding TOR signaling network. Curr Opin Cell Biol 17: 158-166.

23. Sarbassov DD, Ali SM, Sabatini DM (2005) Growing roles for the mTOR pathway. Curr Opin Cell Biol 17: 596-603.

24. Hay N, Sonenberg N (2004) Upstream and downstream of mTOR. Genes Dev 18: 1926-1945.

25. Park KK, Liu K, Hu Y, Smith PD, Wang C, et al. (2008) Promoting axon regeneration in the adult CNS by modulation of the PTEN/mTOR pathway. Science 322: 963-966.

26. Hernandez O, Way S, McKenna J, 3rd, Gambello MJ (2007) Generation of a conditional disruption of the Tsc2 gene. Genesis 45: 101-106.

27. Hasegawa H, Abbott S, Han BX, Qi Y, Wang F (2007) Analyzing somatosensory axon projections with the sensory neuron-specific Advillin gene. J Neurosci 27: 14404-14414.

28. Goslin K, Schreyer DJ, Skene JH, Banker G (1988) Development of neuronal polarity: GAP-43 distinguishes axonal from dendritic growth cones. Nature 336: 672-674.

29. Fu SY, Gordon T (1997) The cellular and molecular basis of peripheral nerve regeneration. Mol Neurobiol 14: 67-116.

30. Ackermann PW, Ahmed M, Kreicbergs A (2002) Early nerve regeneration after achilles tendon rupture--a prerequisite for healing? A study in the rat. J Orthop Res 20: 849-856.

31. Aigner L, Caroni P (1995) Absence of persistent spreading, branching, and adhesion in GAP-43-depleted growth cones. J Cell Biol 128: 647-660.

32. Caroni P (1997) Intrinsic neuronal determinants that promote axonal sprouting and elongation. Bioessays 19: 767-775.

33. Vogelaar CF, Gervasi NM, Gumy LF, Story DJ, Raha-Chowdhury R, et al. (2009) Axonal mRNAs: characterisation and role in the growth and regeneration of dorsal root ganglion axons and growth cones. Mol Cell Neurosci 42: 102-115.

34. Willis DE, Twiss JL (2006) The evolving roles of axonally synthesized proteins in regeneration. Curr Opin Neurobiol 16: 111-118.

35. Wang W, van Niekerk E, Willis DE, Twiss JL (2007) RNA transport and localized protein synthesis in neurological disorders and neural repair. Dev Neurobiol 67: 1166-1182.

36. Michaelevski I, Medzihradszky KF, Lynn A, Burlingame AL, Fainzilber M (2009) Axonal transport proteomics reveals mobilization of translation machinery to the lesion site in injured sciatic nerve. Mol Cell Proteomics.

37. Verma P, Chierzi S, Codd AM, Campbell DS, Meyer RL, et al. (2005) Axonal protein synthesis and degradation are necessary for efficient growth cone regeneration. J Neurosci 25: 331-342.

38. Aigner L, Arber S, Kapfhammer JP, Laux T, Schneider C, et al. (1995) Overexpression of the neural growth-associated protein GAP-43 induces nerve sprouting in the adult nervous system of transgenic mice. Cell 83: 269-278.

39. Kusik BW, Hammond DR, Udvadia AJ Transcriptional regulatory regions of gap43 needed in developing and regenerating retinal ganglion cells. Dev Dyn 239: 482-495.

40. Inoki K, Li Y, Xu T, Guan KL (2003) Rheb GTPase is a direct target of TSC2 GAP activity and regulates mTOR signaling. Genes Dev 17: 1829-1834.

41. Zhang Y, Gao X, Saucedo LJ, Ru B, Edgar BA, et al. (2003) Rheb is a direct target of the tuberous sclerosis tumour suppressor proteins. Nat Cell Biol 5: 578-581.

42. Manning BD, Tee AR, Logsdon MN, Blenis J, Cantley LC (2002) Identification of the tuberous sclerosis complex-2 tumor suppressor gene product tuberin as a target of the phosphoinositide 3-kinase/akt pathway. Mol Cell 10: 151-162.

43. Tee AR, Fingar DC, Manning BD, Kwiatkowski DJ, Cantley LC, et al. (2002) Tuberous sclerosis complex-1 and -2 gene products function together to inhibit mammalian target of rapamycin (mTOR)-mediated downstream signaling. Proc Natl Acad Sci U S A 99: 13571-13576.

44. Inoki K, Li Y, Zhu T, Wu J, Guan KL (2002) TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. Nat Cell Biol 4: 648-657.

45. Christie KJ, Webber CA, Martinez JA, Singh B, Zochodne DW (2010) PTEN inhibition to facilitate intrinsic regenerative outgrowth of adult peripheral axons. J Neurosci 30: 9306-9315.

46. Huang J, Manning BD (2009) A complex interplay between Akt, TSC2 and the two mTOR complexes. Biochem Soc Trans 37: 217-222.

47. Fang Y, Vilella-Bach M, Bachmann R, Flanigan A, Chen J (2001) Phosphatidic acid-mediated mitogenic activation of mTOR signaling. Science 294: 1942-1945.

48. Fang Y, Park IH, Wu AL, Du G, Huang P, et al. (2003) PLD1 regulates mTOR signaling and mediates Cdc42 activation of S6K1. Curr Biol 13: 2037-2044.

49. Tanabe K, Tachibana T, Yamashita T, Che YH, Yoneda Y, et al. (2000) The small GTP-binding protein TC10 promotes nerve elongation in neuronal cells, and its expression is induced during nerve regeneration in rats. J Neurosci 20: 4138-4144.

50. Smith PD, Sun F, Park KK, Cai B, Wang C, et al. (2009) SOCS3 deletion promotes optic nerve regeneration in vivo. Neuron 64: 617-623.

51. Schwaiger FW, Hager G, Schmitt AB, Horvat A, Streif R, et al. (2000) Peripheral but not central axotomy induces changes in Janus kinases (JAK) and signal transducers and activators of transcription (STAT). Eur J Neurosci 12: 1165-1176.

52. Zhou X, Wang L, Hasegawa H, Amin P, Han BX, et al. (2010) Deletion of PIK3C3/Vps34 in sensory neurons causes rapid neurodegeneration by disrupting the endosomal but not the autophagic pathway. Proc Natl Acad Sci U S A 107: 9424-9429.

Chapter 3: Persistent mTOR activity in peripheral neurons leads to developmental and functional defects

Part of this work appears in the manuscript:

Abe N, Borson SH, Gambello MJ, Wang F, Cavalli V (2010) mTOR activation increases axonal growth capacity of injured peripheral nerves. J Biol Chem. In press.

Contributions: Experiments in figure 4 were performed by Judy Golden.

ABSTRACT

Enhancement of axonal regeneration in both CNS and PNS neurons by activation of the mTOR pathway presents an exciting therapeutic target for facilitating recovery from nerve injury. However, it remains unclear whether upregulation of mTOR activity can promote successful target re-innervation and functional recovery in addition to axonal regrowth. Here, we examined the consequence of prolonged mTOR activity by conditional deletion of TSC2 in dorsal root ganglia (DRGs). Deletion of TSC2 in DRGs *in vivo* resulted in major developmental and functional defects, including increased mortality, small size, early death, sensory impairments, movement abnormalities, and aberrant target innervation and axon morphology. These developmental and functional defects suggest that prolonged elevation of mTOR activity may also interfere with axon targeting and functional recovery of regenerating axons. Thus, while manipulation of the mTOR activity could provide new strategies to stimulate nerve regeneration in the PNS, fine control of mTOR activity may be required for proper target innervation and functional recovery.

INTRODUCTION

While much research in nerve regeneration has focused on identifying extrinsic or intrinsic factors that accelerate growth, little is known regarding the consequences of prolonged potentiation of growth pathways on target re-innervation and functional recovery. As neurotrophin-mediated growth pathways are intimately linked to axon guidance [1,2], it is possible that over-activation of axon growth could interfere with axon navigation and target innervation. Genetic activation of the mTOR pathway in PNS and CNS neurons facilitates axon regrowth, raising the exciting possibility that this pathway may be targeted for therapy following nerve injury [3,4]. However, whether persistent activation of mTOR in peripheral neurons has deleterious effects on target innervation or function has not yet been explored. Given the growing evidence for the role of mTOR in multiple aspects of neuronal development, it is possible that hyperactivation of mTOR activity in peripheral neurons could disrupt axon targeting and function.

Persistent mTOR activity in the central nervous system contributes to many of the pathologies of Tuberous Sclerosis (TSC) [5,6]. TSC is an autosomal dominant disorder associated with hamartoma formation resulting from inactivating mutations in either *TSC1* or its interacting gene *TSC2* [7,8]. TSC2 is a GTPase-activating protein for Rheb-GTPase, which is an activator of mTOR [9]. TSC1 does not have any known functional domains but binds to and prevents the degradation of TSC2 [10]. While TSC can affect multiple organs including skin, kidney, heart, lung, and eye, the most significant patient morbidity is attributed to its effects on the brain [11]. Common neurological symptoms of TSC patients include epilepsy, autism, and cognitive disabilities and are associated

with developmental malformations of the cerebral cortex called cortical tubers [12,13,14]. Cortical tubers consist of giant cells, which are large ovoid cells unique to TSC patients and extend short, thickened process of unclear identity [15]. Consistent with a role of the TSC1-TSC2 complex in inhibiting mTOR activity, giant cells display an elevated level of S6 phosphorylation indicative of hyperactive mTOR signaling [15,16]. Neuron-specific deletion of *TSC1* in mice results in many of the features and pathologies of tuberous sclerosis, including spontaneous seizure activity, the presence of enlarged, aberrant neurons characteristic of those in TSC cortical tubers, and hyperactive mTOR signaling [5]. Rapamycin, an mTOR inhibitor, can reverse many of these abnormalities in this TSC mouse model, indicating that hyperactive mTOR signaling contributes to TSC pathologies [6].

The TSC1 and TSC2 tuberous sclerosis complex plays a critical role in axon specification of hippocampal neurons through its regulation of mTOR activity [17]. Specification of neuronal processes as axon or dendrite is essential for proper neuronal function and information flow. *In vitro* studies of hippocampal neurons show that TSC2 phosphorylation, and downstream mTOR signaling is restricted to the axon [17]. Deletion of TSC1 or TSC2 results in the formation of multiple axons, suggesting that spatial regulation of mTOR activity essential for axon specification and neuronal polarity [17]. Rapamycin treatment reduces the number of TSC1 or TSC2-deficient neurons with multiple axons, suggesting that the TSC1-TSC2 complex regulates axon specification through its modulation of mTOR activity [17].

Recent studies in retinal ganglion neurons have identified a role of TSC1-TSC2 and mTOR in axon guidance [18]. TSC2 heterozygote mice show aberrant projections of

retinal ganglion neurons into the dorsal lateral geniculate nucleus (dLGN) [18]. TSC2 deficient neurons in culture are insensitive to ephrin-mediated growth cone collapse, suggesting that TSC2 acts downstream of ephrin signaling which is critical for the formation of the topographic map in the dLGN [18]. Furthermore, ephrin-mediated growth cone collapse involves inhibition of protein translation by TSC2-mediated mTOR inactivation [18]. Thus, fine modulation of mTOR activity by axon guidance cues may play an instructive role in axonal targeting.

In chapter 2, we report that genetic upregulation of mTOR activity in dorsal root ganglia (DRG) facilitates axonal regrowth following injury. Given the therapeutic potential of mTOR activation in nerve injury, it is important to test the consequence of persistent mTOR activity on target innervation and function. Here, we extend the work described in chapter 2 by further characterization of the phenotype of $TSC2^{flox/flox}$; $Advillin^{Cre/+}$ conditional knockout mice (TSC2KO), in which deletion of TSC2 in DRGs results in hyperactive mTOR signaling (Chapter 2, Figure 4A, 4B). We find that TSC2 deletion in DRGs results in increased mortality, smaller body size, abnormal posture, ataxia, and early death. Furthermore, TSC2 knockout DRGs display larger soma, abnormal morphology, altered target innervation, and functional defects. Thus, while manipulation of the mTOR activity could provide new strategies to stimulate nerve regeneration in the PNS, fine control of mTOR activity may be required for proper target innervation and functional recovery.

RESULTS

TSC2KO mice have increased mortality and are smaller in size- To generate *TSC2$^{flox/flox}$; Advillin$^{Cre/+}$* mice, we bred *TSC2$^{flox/flox}$; Advillin$^{+/+}$* females to *TSC2$^{flox/+}$; Advillin$^{Cre/+}$* mice. Because female mice show leaky expression of Cre recombinase in the germline (personal communication with Fan Wang), we used male mice to pass down the Advillin^{Cre} knock-in allele. For the male in this breeding scheme, we used *TSC2$^{flox/+}$; Advillin$^{Cre/+}$* mice rather than *TSC2$^{flox/flox}$; Advillin$^{Cre/+}$* mice (TSC2KO) as TSC2KO male mice were unable to breed. Only 12% of the pups born from this breeding were TSC2KO, which is about half of the expected Mendelian percentage of 25% (Figure 1A). The remaining three genotypes were born at equal ratios (Figure 1A). Thus, TSC2 deletion in DRGs may be interfering with development *in utero*.

Of the TSC2KO mice that are born, many are smaller in size compared to control siblings (Figure 1B, 1C). Over the first few months following birth, these mice become progressively ataxic, develop tremors, and die around 2.5 ~ 3 months of age. The cause of death is not yet determined. *TSC2$^{+/+}$;Advillin$^{Cre/+}$* mice display a wild-type phenotype, confirming that these abnormalities are due to deletion of TSC2 rather than the expression of the Cre recombinase.

TSC2-deficient DRGs have axon targeting defects and abnormal morphology- To assess whether constitutive mTOR activity in DRGs alters axonal target innervation, we examined sensory nerve innervation of hindlimb glabrous footpad skin in adult TSC2KO and control littermates. TSC2 deletion led to a significant loss of epidermal innervation (Figure. 2A, 2B). We found no gross effects of TSC2 deletion on neuronal survival or the number of axons in the peripheral branch of L5 DRG (Figure 3A-3C),

suggesting that the loss of innervation is due to axon targeting defects rather than degeneration or neuronal loss. Furthermore, of the sensory nerves that innervated the epidermis, a large proportion displayed excessive branching at the axon terminal (Figure 2A, 2C). Not only was the proportion of endings with excessive branching higher in TSC2KO mice, the absolute number of these endings was also greater (Figure 2D). The presence of the cre recombinase does not alter skin innvervation, as wild-type; *Advillin*$^{Cre/+}$ mice did not show significant difference from wild-type; *Advillin*$^{+/+}$ littermate controls (Figure 2A-D). These results suggest that TSC2 deletion in peripheral neurons leads to abnormal target innervation and axonal branching. As dorsal root ganglion neurons also project into the spinal cord, it is also possible that targeting of these centrally-projecting axons is also disrupted by TSC2 deletion.

TSC2 deletion and increased mTOR-mediated protein synthesis has been shown to increase cell size in hippocampal neurons as well as non-neuronal cells. We measured soma size in dissociated DRG cultures from TSC2KO mice and found that TSC2 deletion results in larger cell size in DRGs (Figure 2E). Thus, mTOR-mediated regulation of cell size may be conserved across cell types.

TSC2KO mice have defects in sensory nerve function- To determine whether TSC2 deletion in DRGs results in impaired function in addition to axonal targeting and morphological defects, we performed a panel of behavioral tests that measure sensory nerve function. These experiments were performed by Judy Golden in Rob Gereau's laboratory. TSC2KO mice display lowered sensitivity to thermal and mechanical stimuli, suggesting that sensory function is compromised (Figure 4A). The response of TSC2KO mice to acetone, a cold stimulus, was not statistically altered (Figure 4A).

We also tracked motor behavior of TSC2KO and control mice in an open-field test. In the span of one hour measured, TSC2KO mice spent significantly less time moving, and traveled shorter distances compared to control (Figure 4B). Their movements also appear abnormal, as they walk in a "waddling" pattern with a hunched back (Figure 4C). They also display a limb clasping phenotype in response to the tail suspension test, a sign of impaired sensory-motor circuitry. Furthermore, mutant mice perform poorly on the rotarod test compared to controls, indicative of impaired motor coordination (Figure 4D). As the conditional deletion occurs in sensory neurons rather than motor neurons [19], we speculate that these motor defects in TSC2KO mice are a result of poor proprioception. In addition to defects in peripheral axons, there could also be defects in the projection of sensory axons back into the spinal cord for connection with the local motor circuit or the central nervous system. However, it will be critical to perform lineage tracing analysis of Cre expression pattern using ROSA gene trap mouse [20], to ensure that the deletion of TSC2 occurs exclusively in DRG neurons.

DISCUSSION

Deletion of TSC2 in DRGs *in vivo* resulted in marked developmental and functional defects, including increased mortality, small size, early death, sensory impairments, movement abnormalities, and aberrant axon innervation and morphology. As precise innervation of axons to their target is critical for the function of the nervous system, we speculate that axon targeting defects in TSC2KO mice contribute to the impairment of sensory function. Preliminary experiments show that the conduction velocity of sensory nerves is normal in TSC2KO mice, supporting the idea that the functional impairments are linked to innervation and morphological defects (with Bob Baloh).

In the central nervous system, Ephrins mediate axon guidance in retinal ganglion neurons by modulation of TSC2 and mTOR activity in growth cones [18]. Our finding that TSC2 deletion interferes with axonal targeting in DRGs suggest that the mTOR pathway also plays a role in the guidance of peripheral sensory axons. While we cannot exclude the possibility that these abnormalities are due to mTOR-independent effectors of TSC2, it is likely that constitutive activation of mTOR contributes to axon innervation defects given the importance of protein synthesis and degradation in growth cone dynamics[21,22,23]. Axonal guidance molecules involved in targeting of sensory axons, including Netrin-1, NGF, and Semaphorin3A mediate growth cone collapse or extension through local protein translation [24,25]. It is not yet known whether local protein translation by these axon guidance cues requires mTOR activity. It will be interesting to examine whether axon guidance molecules in the central and peripheral nervous systems

converge upon mTOR activity to regulate the directionality of growth cones during development.

Unlike c-Jun activation which persists in the DRG cell body until regeneration is complete, the increase in mTOR activity following peripheral nerve injury is a transient response, returning to basal levels four days following injury. Because prolonged mTOR activity results in axon targeting defects, it is tempting to speculate that following the boost of axonal growth by robust activation of mTOR upon injury, the level of mTOR activity is reduced to allow the axon to be sensitive to extracellular guidance cues for proper targeting. If fine modulation of mTOR activity is required for the response of axons to local guidance cues during development, it is likely that this pathway also plays an instructive role in target re-innervation following axonal damage. Thus, while targeting the mTOR pathway to increase the speed and extent of recovery of both PNS and CNS neurons may represent an attractive clinical strategy, it will be important to control the duration and level of mTOR activity to allow for proper re-innervation of targets and functional recovery.

MATERIALS AND METHODS

Antibodies and reagents- The following antibodies were used: anti-SMI-31, anti-SMI-32 (Sternberger Monoclonals Incorporated), anti-Caspase-3 (Millipore), and anti-GAP-43 (Abcam). For TUNEL staining, *in situ* cell death detection kit (Roche) was used according to product protocol.

DRG dissociation for size analysis- L4, L5, and L6 DRGs were dissected and dissociated in 0.7 mg/ml Liberase Blendzyme 3 (Roche), 600 µg/ml DNAse, 10 mg/ml BSA, in DMEM AIR (DMEM/F12 with 10mM Glucose, and 1% Pen/Strep) at 37ºC for 15 minutes, followed by trypsinization with 0.25% trypsin in DMEM AIR at 37°C for 15 minutes. DRGs were then triturated and placed in culture media (DMEM, 10% FBS, 1% pen/strep) on poly-D-lysine-coated plates. DIC images were acquired with Nikon Eclipse TE2000-E inverted microscope and analyzed using Nikon NIS Elements Advanced Research 2.30 Imaging Software.

Immunohistochemistry- Sciatic nerves and L4 DRGs were dissected and fixed for 1-2 hours in 4% PFA in PBS, then cryoprotected overnight in 20% sucrose. Posterior hindpaw skin sections were fixed in 15% picric acid, 2% paraformaldehyde in PBS for 3-4 hours, and cryoprotected in 30% sucrose for 48 hours. Nerves and skin were embedded in OCT (Tissue-Tek) and frozen in dry-ice-cooled methanol. Serial 10-µm (nerve and DRG) or 30-µm (skin) cryostat sections were cut and mounted onto coated slides (Fisher Scientific). Sections were permeabilized and blocked with 10% goat serum, 0.1% Triton X-100 in PBS for 30 min. Primary staining with the indicated antibodies was performed in the blocking solution overnight at 4ºC. Staining with Alexa-conjugated secondary antibody was performed for 1 to 3 hours. Sciatic nerve images were acquired

with Nikon Eclipse TE2000-E inverted epifluorescence microscope and analyzed using Metamorph 6.2. Fluorescent images from hindpaw skin sections were acquired using Olympus 500 confocal microscope. Confocal images were acquired at 1 μm intervals and all images from one 30 μm stack were compressed into one image using Metamorph 6.2

Behavioral assays

The Hargreaves, von Frey, acetone withdrawal, open-field, and rotarod tests were performed by Judy Golden as described previously [26].

Figure 1. TSC2KO mice have increased mortality and smaller body size

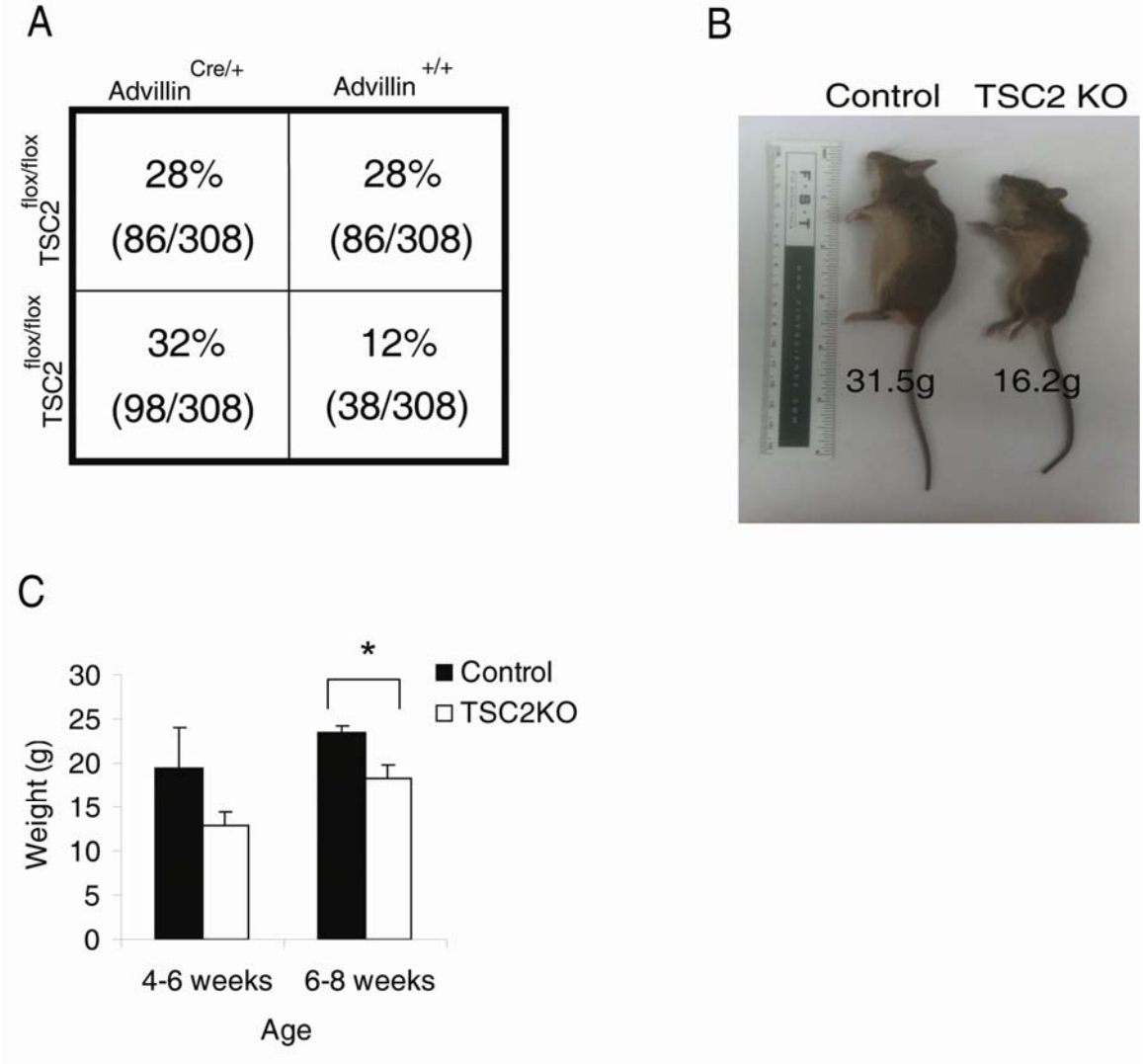

Figure 1. TSC2KO mice have increased mortality and smaller body size

(A) Percentage of each genotype born from *TSC2*$^{flox/flox}$*; Advillin*$^{+/+}$ and *TSC2*$^{flox/+}$*; Advillin*$^{Cre/+}$ breeding pair. Fewer TSC2KO pups were born than Mendelian ratio. (B) TSC2KO mice are smaller in size. Sample TSC2KO and control sibling pair. (C) Quantification of average weight of each genotype shows TSC2KO mice weigh less than controls. n=4 mice per age and genotype. *$p<0.05$ (Student's *t*-test).

Figure 2. TSC2 deletion leads to abnormal target innervation and axon morphology.

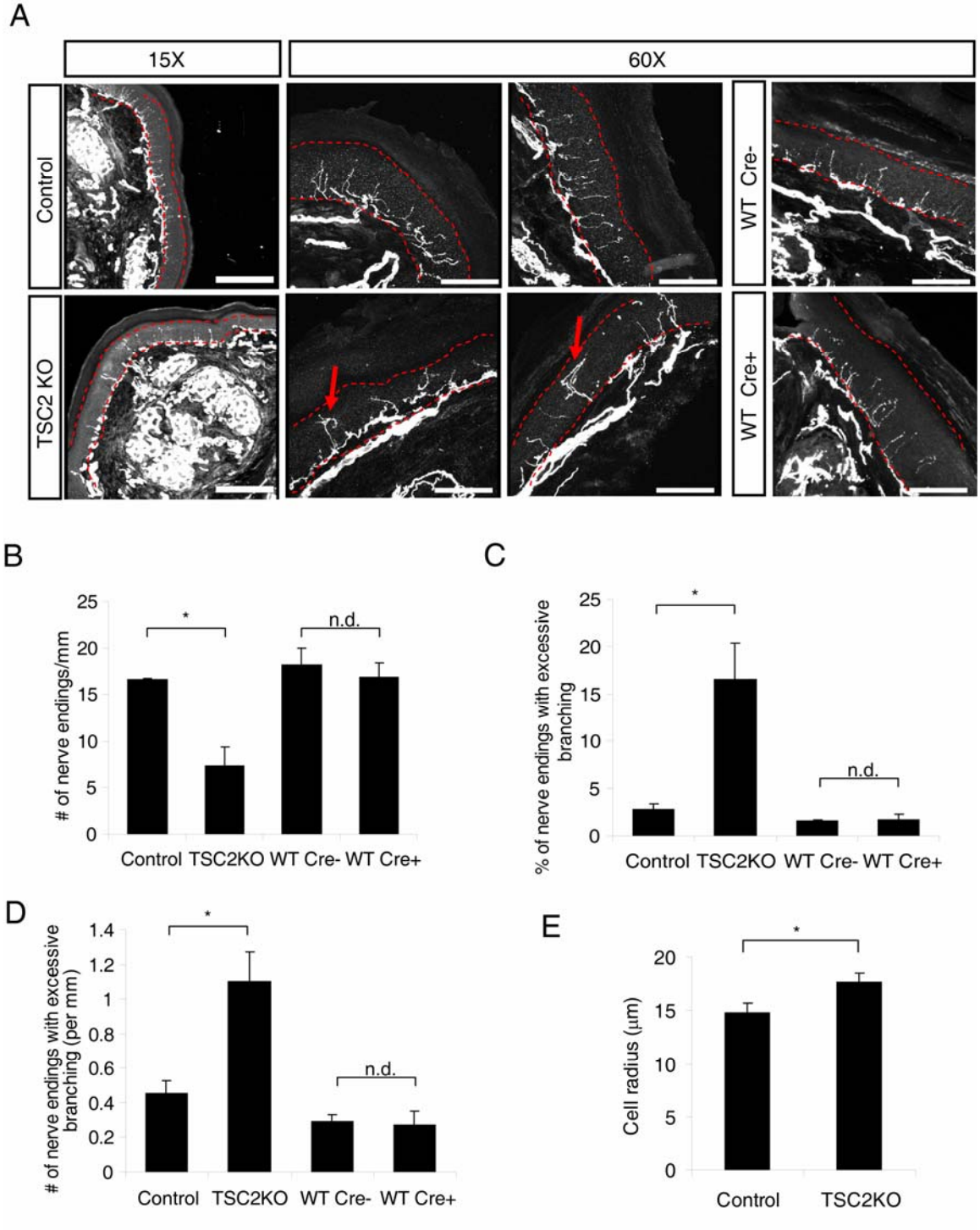

Figure 2. TSC2 deletion leads to abnormal target innervation and axon morphology. (A) Posterior hindpaw glaborous skin sections were stained with α-GAP-43 to visualize axonal endings innervating skin. Red dashed line indicate epidermal layer. Low magnification (15X) confocal images reveal fewer axons penetrating epidermal layer in TSC2KO mice compared to control. High magnification (60X) confocal images reveal enrichment of endings with excessive branching (arrow) and sharp turning (arrowhead) compared to controls. The presence of cre recombinase does not contribute to the innervation defects, as wild-type; *Advillin*$^{Cre/+}$ mice did not show significant difference from wild-type; *Advillin*$^{+/+}$ littermate or control. (B) Quantification of innervation density reveals loss of skin innervation in TSC2KO mice. Data are mean +/-SEM. n=3 mice per genotype, 17-56 sections were analyzed per mouse (Student's *t*-test). Quantification of percentage (C) and number (D) of nerve endings with excessive branching (axons with more than 2 branches at tip, or those that turned 90 degrees were counted). (E) Measurement radii of L4, L5 and L6 DRG cell bodies dissociated from TSC2KO or control mice reveal larger cell size in TSC2KO mice. n=3 animals per genotype, 70-375 cells were analyzed per animal. *$p<0.05$, n.d.=no statistically significant difference (Student's *t*-test).

Figure 3. TSC2 deletion does not lead to axon degeneration or apoptosis.

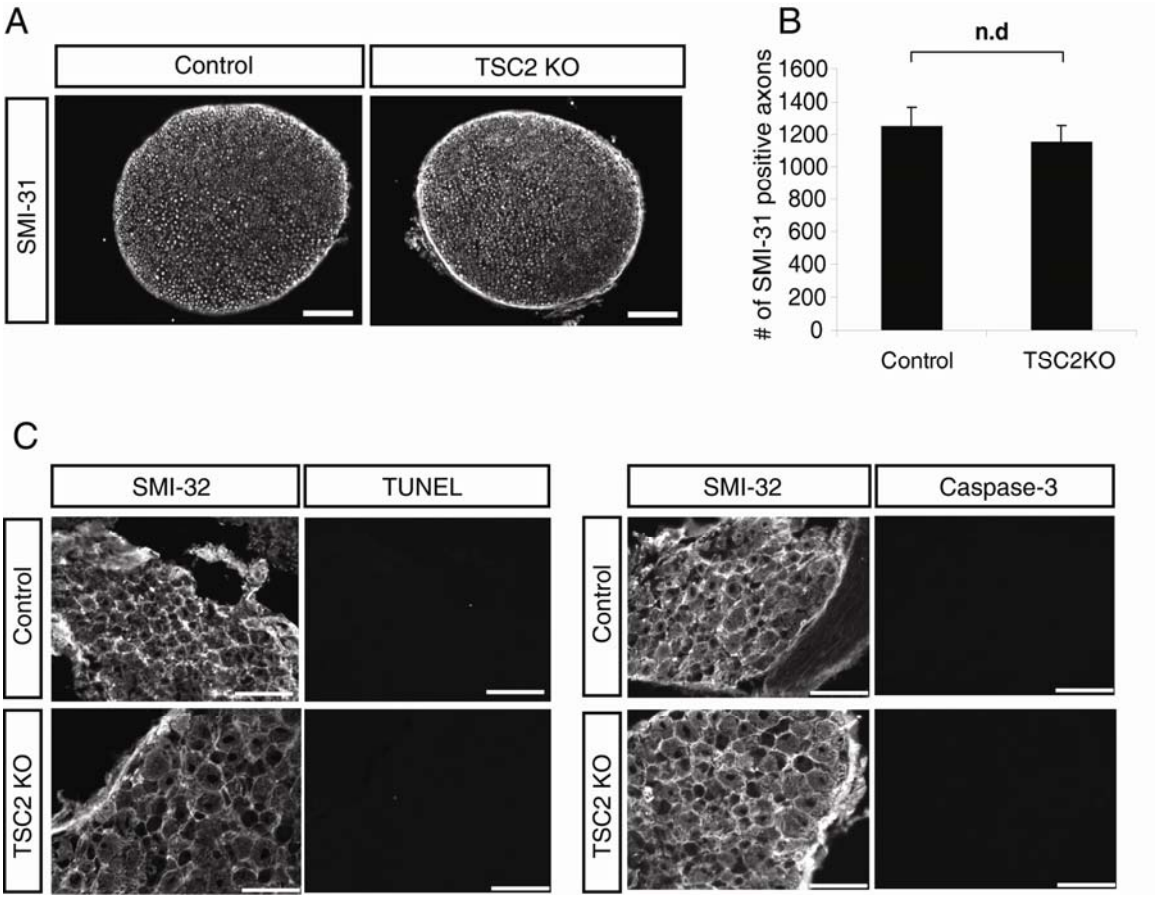

Figure 3. TSC2 deletion does not lead to axon degeneration or apoptosis. (A) Cross section of the peripheral branch of L5 DRG stained with the axonal marker SMI-31 revealed no significant difference in axon number between TSC2 KO and controls. (B) Quantification of (A). n=3 mice per genotype, 2 sections analyzed per mouse (Student's *t*-test). (C) Cross-section of L4 DRGs of 2 month-old adult TSC2KO and control mice were stained for neuronal cell body marker SMI-32 and the apoptotic markers TUNEL or Caspase-3. Neither control nor TSC2KO DRGs were positive for TUNEL or Caspase-3. (A) and (C) Bar=100μm.

Figure 4. TSC2KO mice have behavioral abnormalities indicative of impaired sensory function.

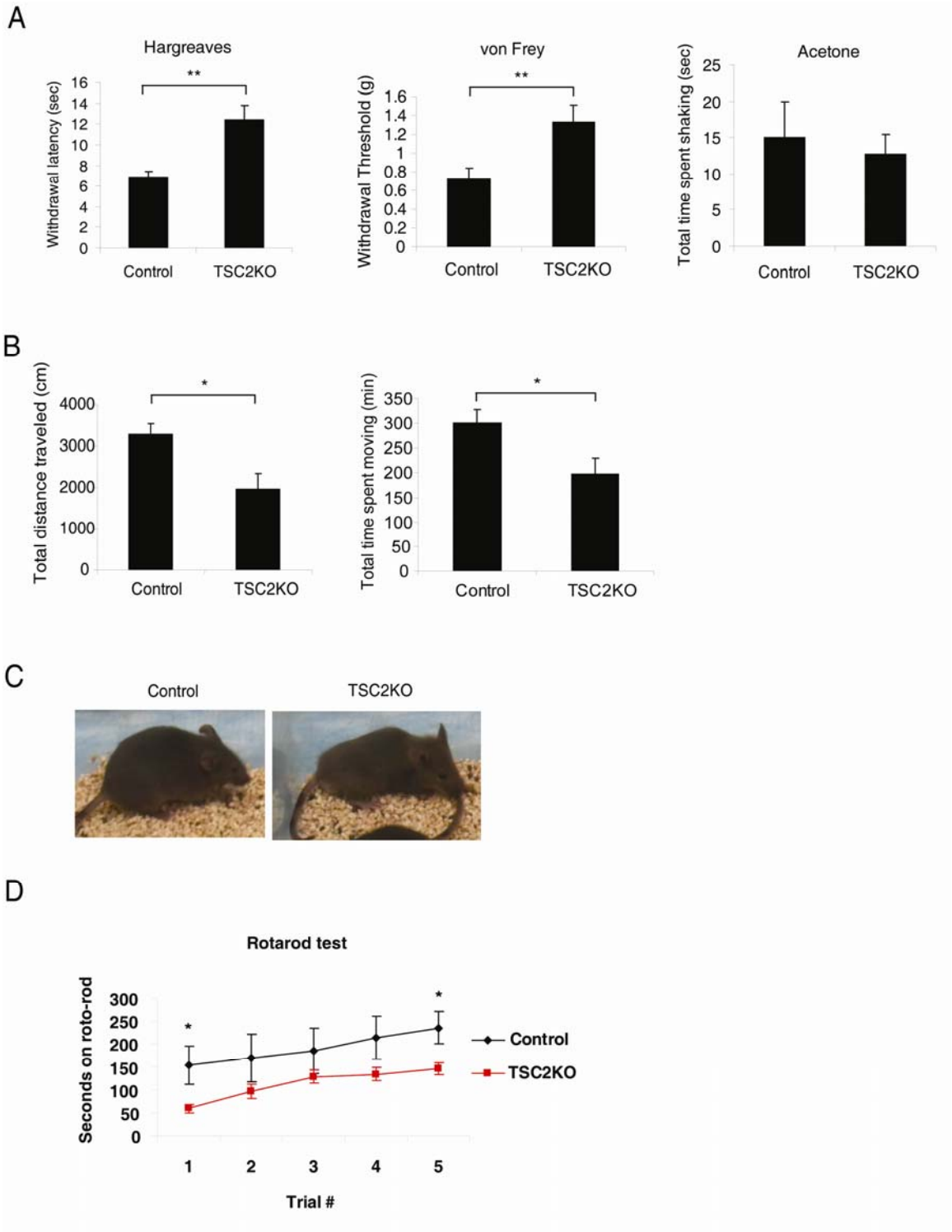

Figure 4. TSC2KO mice have behavioral abnormalities indicative of impaired sensory function. (A) TSC2KO and control mice were subjected to the Hargraves, von Frey, and acetone tests for sensitivity to heat, mechanical stimuli, and cold respectively. Sensitivity to heat and mechanical stimuli are significantly impaired in TSC2KO animals . Data are mean +/-SEM. **p<0.01 (Student's *t*-test). n=6 mice per genotype. (B) TSC2KO and control mice were subjected to the open field test for one hour, and monitored for time spent moving and distance traveled to assess motor activity. TSC2KO animals spent significantly less time moving and traveled fewer distances than controls. Data are mean +/-SEM. *p<0.05 (Student's *t*-test). n=5 mice per genotype. (C) TSC2KO animals have postural abnormalities. (D) TSC2KO animals show poor performance on the rotarod test compared to controls. Data are mean +/-SEM. *p<0.05 (Student's *t*-test). n=6 mice per genotype.

REFERENCES

1. Gillespie LN (2003) Regulation of axonal growth and guidance by the neurotrophin family of neurotrophic factors. Clin Exp Pharmacol Physiol 30: 724-733.

2. Tuttle R, O'Leary DD (1998) Neurotrophins rapidly modulate growth cone response to the axon guidance molecule, collapsin-1. Mol Cell Neurosci 11: 1-8.

3. Abe N, Borson SH, Gambello MJ, Wang F, Cavalli V (2010) mTOR activation increases axonal growth capacity of injured peripheral nerves. J Biol Chem.

4. Park KK, Liu K, Hu Y, Smith PD, Wang C, et al. (2008) Promoting axon regeneration in the adult CNS by modulation of the PTEN/mTOR pathway. Science 322: 963-966.

5. Meikle L, Talos DM, Onda H, Pollizzi K, Rotenberg A, et al. (2007) A mouse model of tuberous sclerosis: neuronal loss of Tsc1 causes dysplastic and ectopic neurons, reduced myelination, seizure activity, and limited survival. J Neurosci 27: 5546-5558.

6. Meikle L, Pollizzi K, Egnor A, Kramvis I, Lane H, et al. (2008) Response of a neuronal model of tuberous sclerosis to mammalian target of rapamycin (mTOR) inhibitors: effects on mTORC1 and Akt signaling lead to improved survival and function. J Neurosci 28: 5422-5432.

7. van Slegtenhorst M, de Hoogt R, Hermans C, Nellist M, Janssen B, et al. (1997) Identification of the tuberous sclerosis gene TSC1 on chromosome 9q34. Science 277: 805-808.

8. (1993) Identification and characterization of the tuberous sclerosis gene on chromosome 16. Cell 75: 1305-1315.

9. Tee AR, Manning BD, Roux PP, Cantley LC, Blenis J (2003) Tuberous sclerosis complex gene products, Tuberin and Hamartin, control mTOR signaling by acting as a GTPase-activating protein complex toward Rheb. Curr Biol 13: 1259-1268.

10. Chong-Kopera H, Inoki K, Li Y, Zhu T, Garcia-Gonzalo FR, et al. (2006) TSC1 stabilizes TSC2 by inhibiting the interaction between TSC2 and the HERC1 ubiquitin ligase. J Biol Chem 281: 8313-8316.

11. Sparagana SP, Roach ES (2000) Tuberous sclerosis complex. Curr Opin Neurol 13: 115-119.

12. de Vries P, Humphrey A, McCartney D, Prather P, Bolton P, et al. (2005) Consensus clinical guidelines for the assessment of cognitive and behavioural problems in Tuberous Sclerosis. Eur Child Adolesc Psychiatry 14: 183-190.

13. Thiele EA (2004) Managing epilepsy in tuberous sclerosis complex. J Child Neurol 19: 680-686.

14. Wiznitzer M (2004) Autism and tuberous sclerosis. J Child Neurol 19: 675-679.

15. Crino PB (2004) Molecular pathogenesis of tuber formation in tuberous sclerosis complex. J Child Neurol 19: 716-725.

16. Orlova KA, Crino PB (2010) The tuberous sclerosis complex. Ann N Y Acad Sci 1184: 87-105.

17. Choi YJ, Di Nardo A, Kramvis I, Meikle L, Kwiatkowski DJ, et al. (2008) Tuberous sclerosis complex proteins control axon formation. Genes Dev 22: 2485-2495.

18. Nie D, Di Nardo A, Han JM, Baharanyi H, Kramvis I, et al. (2010) Tsc2-Rheb signaling regulates EphA-mediated axon guidance. Nat Neurosci 13: 163-172.

19. Zhou X, Wang L, Hasegawa H, Amin P, Han BX, et al. (2010) Deletion of PIK3C3/Vps34 in sensory neurons causes rapid neurodegeneration by disrupting the endosomal but not the autophagic pathway. Proc Natl Acad Sci U S A 107: 9424-9429.

20. Luche H, Weber O, Nageswara Rao T, Blum C, Fehling HJ (2007) Faithful activation of an extra-bright red fluorescent protein in "knock-in" Cre-reporter mice ideally suited for lineage tracing studies. Eur J Immunol 37: 43-53.

21. Brittis PA, Lu Q, Flanagan JG (2002) Axonal protein synthesis provides a mechanism for localized regulation at an intermediate target. Cell 110: 223-235.

22. Leung KM, van Horck FP, Lin AC, Allison R, Standart N, et al. (2006) Asymmetrical beta-actin mRNA translation in growth cones mediates attractive turning to netrin-1. Nat Neurosci 9: 1247-1256.

23. Yao J, Sasaki Y, Wen Z, Bassell GJ, Zheng JQ (2006) An essential role for beta-actin mRNA localization and translation in Ca2+-dependent growth cone guidance. Nat Neurosci 9: 1265-1273.

24. Hengst U, Deglincerti A, Kim HJ, Jeon NL, Jaffrey SR (2009) Axonal elongation triggered by stimulus-induced local translation of a polarity complex protein. Nat Cell Biol 11: 1024-1030.

25. Wu KY, Hengst U, Cox LJ, Macosko EZ, Jeromin A, et al. (2005) Local translation of RhoA regulates growth cone collapse. Nature 436: 1020-1024.

26. Golden JP, Hoshi M, Nassar MA, Enomoto H, Wood JN, et al. (2010) RET signaling is required for survival and normal function of nonpeptidergic nociceptors. J Neurosci 30: 3983-3994.

Chapter 4: Sunday Driver interacts with two distinct types of axonal vesicles

This work appears as part of the manuscript:

Abe N, Almenar-Queralt A, Lillo C, Shen Z, Lozach J, et al. (2009) Sunday driver interacts with two distinct classes of axonal organelles. J Biol Chem 284: 34628-34639.

Author contributions: V.C. performed the experiments in Figures 1, 2B, 3A and 3B.

ABSTRACT

Intracellular trafficking pathways play a critical role in the communication between the neuronal cell body and distant synaptic terminals for survival, function, injury signaling, and repair. The motor-binding protein Sunday Driver (syd) has been previously shown to link vesicular axonal transport to nerve injury signaling. Electron microscopy and mass-spectrometry analyses of immunopurified syd vesicles have revealed two classes of syd-associated vesicles of distinct morphology and protein composition. Gene Ontology analyses of each vesicle protein content have revealed their unique identity and indicated that one class of syd vesicles belongs to the endocytic pathway, whereas another may belong to an anterogradely transported vesicle pool. To validate these findings, we examined the transport and localization of components of syd vesicles within axons of mouse sciatic nerve. Together, our results lead us to propose that endocytic syd vesicles function in part to carry injury signals back to the cell body, whereas anterograde syd vesicles may play a role in axonal outgrowth and guidance.

INTRODUCTION

The cell body of an injured neuron must receive accurate and timely information about the site and extent of axonal damage in order to initiate molecular changes, including the enhancement of intrinsic growth capacity and the initiation of the regeneration program. The extreme length of axons poses a challenge for such signals to arrive at the cell body. One mechanism contributing to this process in peripheral neurons is microtubule-based retrograde transport of locally-activated injury signals. Sciatic nerve injury experiments in rats and mice have identified several signaling molecules that are retrogradely transported to the cell body upon injury. For example, mitogen-activated protein kinases (MAPKs) Erk1 and Erk2 are phosphorylated in sciatic nerve axoplasm following axonal injury and are targeted for retrograde transport upon binding to vimentin and the beta-importin/dynein complex [1] [2]. c-Jun N-terminal kinase (JNK) is also activated in axons upon injury and is retrogradely transported to the cell body [3] [4]. Retrograde transport of JNK leads to JNK and c-Jun activation in the cell body [4].

Activation of JNK and c-Jun in the cell body is essential for the initiation of transcriptional changes required for axonal regeneration. JNK and c-Jun activation persists following injury until successful target re-innervation has been achieved [5] [6]. Deletion of JNK2, JNK3, or c-Jun in the nervous system, while causing little effect on axonal growth during development, leads to a marked defect in regeneration upon injury [7] [8]. Furthermore, c-Jun activation in DRGs is drastically greater following peripheral branch axotomy compared to that after central branch axotomy, suggesting that successful activation of c-Jun contributes to regenerative capacity [9]. The mechanism

by which activated JNK in peripheral axons is retrogradely transported to the cell body to initiate c-Jun activation remains unclear.

One candidate regulator of JNK transport following nerve injury is Sunday Driver (syd). Syd was originally identified in a screen for axonal transport mutants in *Drosophila* [10]. Also known as mammalian JNK interacting protein 3 (JIP3) or JSAP1, syd is part of a family of JNK interacting proteins including JIP1, JIP2, and JIP4 [11] [12] [13]. In the sciatic nerve, syd and JNK3 form a complex on axonal vesicles and interact with kinesin-1 for anterograde transport and the dynactin complex for retrograde transport [3]. Following sciatic nerve injury, JNK is locally activated and activated JNK and syd are transported mainly retrogradely. Injury induces an enhanced interaction between syd and dynactin, promoting the retrograde transport of activated JNK and syd [3]. These observations suggest that syd provides a link between axonal damage signals and axonal transport. In addition, syd-dependent vesiclar transport may be critical for axon growth and regeneration as syd deletion in the central nervous system results in axonal outgrowth defects [14] [15].

To better understand syd function in axonal transport and injury signaling, Valeria Cavalli and Larry Goldstein developed a purification strategy to isolate syd vesicles from mouse brain cortices [16]. Electron microscopy analysis of immunoisolated syd vesicles revealed the presence of two classes of organelles of distinct morphology: large, endosomal-like vesicles, and smaller-sized vesicles [16] (Figure 1). Immuno-EM analysis from ligated sciatic nerve revealed that the smaller size syd vesicles move mainly anterogradely, while the larger size syd vesicles move retrogradely [3].

In addition, mass spectrometry revealed that the two types of syd vesicles have distinct molecular compositions. Proteins found in the smaller syd vesicles include synaptic vesicle proteins (vacuolar ATPase, Rab5, AP50, synaptoporin), trafficking-related proteins (synaptotagmin VII, SNAP29), signaling proteins (sprouty, Minkk1 kinase, casein kinase), cell adhesion proteins (neogenin, neurocan), cytoskeletal proteins, and ribosomal proteins [16]. Among the proteins identified in the larger syd vesicles were molecular motors (dynein, kinesin heavy chain, Myosin Va), trafficking-related proteins (Rab15, Rab18, RabGDI), endosomal proteins (VAMP3, Syntaxin13, Rab5, Rab7, Rab11, dynamin, AP180, neurobeachin, AP2, amphiphysin, clathrin), ubiquitin-related proteins (ubiquitin carboxyl-terminal hydrolase 5, Phr1, Cullin-associated NEDD8-dissociated protein 1), signaling proteins (adenylate cyclase, phosphoinositide kinases), transporters, channels, and cytoskeletal proteins. [16].

Electron microscopy images combined with mass-spectrometry analyses of these two distinct classes of syd vesicles indicate that the large vesicles belong to the endocytic pathway, while the smaller vesicles belong to an anterogradely transported vesicle pool. To validate these findings, we examined the transport and localization of components of these distinct syd vesicle classes within axons of mouse sciatic nerve. Together, our results lead us to propose that endocytic syd vesicles function in part to carry signals back to the cell body, whereas anterograde syd vesicles may play a role in axonal outgrowth and guidance.

RESULTS

In vivo validation of components of syd vesicles -Previous biochemical analyses revealed the isolation of two populations of syd vesicles with distinct protein composition and morphology, and immuno-EM analyses showed that in mouse sciatic nerve, anterogradely moving syd-associated vesicles are mostly small vesicles/tubules and retrogradely transported syd vesicles are larger, often multivesicular organelles [3]. Small syd vesicles may belong to the anterograde pathway, whereas large syd vesicles may belong mainly to the retrograde pathway. To test this possibility, we used sciatic nerve ligation experiments to assess *in vivo* the transport properties of several markers identified in each type of syd vesicle. Mouse sciatic nerves were subjected to ligations, and nerve portions proximal or distal to the ligation site were analyzed by immunofluorescence microscopy and Western blotting. Proteins moving in the fast anterograde axonal transport pathway generally accumulate on the proximal side of a ligation, while proteins moving in the retrograde pathways generally accumulate on the distal side. Slow moving or non-axonal proteins remain unchanged.

Immunofluorescence of longitudinal sections of a ligated nerve revealed accumulation of syd on both sides of the ligature (Figure 2A), as reported previously [3]. Proteins identified in the small syd vesicle population, SNAP29 and Synaptotagmin VII, accumulated mostly proximal to the ligation site, indicative of anterograde axonal transport, as predicted (Figure 2A, 2B). In contrast, proteins identified on the large syd vesicle population, such as VAMP3/cellubrevin and syntaxin 13, accumulated on both proximal and distal sides, indicative of bidirectional transport (Figure 2B, 2D). Our results indicate that morphologically and biochemically distinct syd vesicles display

distinct axonal transport properties. Small syd vesicles primarily travel in the anterograde direction, and syd-endosomes may travel bidirectionally, consistent with the presence of both kinesin and dynein-dynactin on these vesicles, and with previous observations that early/recycling endosomes travel bidirectionally along axons of culture neurons [17,18]. Together, these findings suggest that the two distinct pools of syd vesicles may play different roles in axonal growth, maintenance, or repair.

In vivo co-localization of syd with endosomes—Previous immuno-EM analyses in sciatic nerve indicated that syd localizes to multivesicular organelles on the distal side of a ligation [3], but these experiments did not demonstrate whether these organelles belong to the endocytic pathway. To address whether syd plays a role in endosomal trafficking along axons *in vivo*, we labeled the endocytic pathway in peripheral nerves and examined syd co-localization with labeled endosomes by fluorescence microscopy. We labeled the endocytic pathway in sensory neurons by subcutaneous injection of the endocytic tracer Texas Red dextran in the mouse rear leg footpad. Nerves are able to take up tracers at the sensory terminals and transport them retrogradely along axonal tracts to the cell body in the dorsal root ganglia. We performed a sciatic nerve ligation concomitant with dye injection to increase the number of labeled structures accumulating distal to the ligation site. The sciatic nerve was analyzed 24 hours after injection. Distal to the ligation site, axons labeled with Texas Red dextran contained syd (Figure 3A). In contrast, proximal to the ligation site, no dextran was detected, but syd accumulated, as expected (Figure 2A, 3A) [3].

When examined at higher magnification (Figure 3B) or by confocal microscopy (Figure 3C) followed by deconvolution, Texas Red dextran-positive structures partially

co-localized with syd within single axons. Structures positive for syd and Texas Red dextran also partially co-localized with the endosomal marker VAMP3 [19] (Figure 3D), which we found associated with large syd vesicles. These results indicate that syd resides at least in part on axonal endosomes. However, the exact nature of the labeled endosomes awaits further investigation at the EM level. Together, our findings further our previous observations and show that syd associates with two distinct axonal vesicle populations, thereby shedding light on the function of syd in both development and regeneration.

DISCUSSION

Our data suggest that syd vesicles represent a population of bidirectional early/recycling endosomes that utilize kinesin-1 and dynein-dynactin for transport along the axon. Movement of endosomes in axons was thought to be exclusively retrograde [19], but other studies have shown bidirectional movement of recycling endosomes [20]and late endosomes/lysosomes [21]. More recently, live imaging experiments in cultured neurons revealed that the anterograde transport of endosomes mediates targeting of the adhesion molecule L1/Cam to the axon [18]. L1/Cam is present in large syd vesicles [16], suggesting that a syd-dependent recruitment of kinesin to endosomes may mediate L1/Cam transport to the axon. syd may represent a regulatory switch for motor proteins of opposing direction that controls trafficking of endocytic vesicles along the axon because the binding sites for kinesin and dynactin are mutually exclusive [22].

At the ligation site, syd-endosomes may contribute to local membrane trafficking events including exo- and endocytosis. Indeed, several studies have shown that exo- and endocytosis are required for plasma membrane repair in epithelial cells [23] [24]. Although the endocytic pathway in epithelial cells is well characterized, the molecular machinery mediating endosomal trafficking in neurons is still poorly understood. Nonetheless, the emerging picture of endosomal trafficking in neurons suggests that different endosomal pools may be involved in the regulation of distinct signaling pathways and polarized distribution of guidance and adhesion molecules [25].

The retrograde transport of endosome/multivesicular bodies (MVBs) is believed to represent the organelle that carries neurotrophic factors in axons. Although the brain-derived neurotrophic factor TrkB receptor was identified on syd vesicles [16], our data do

not allow us to establish whether syd mediates the retrograde transport of the classical neurotrophin-signaling endosome. Furthermore, the role of MVBs in retrograde signaling endosomes in axons has been recently challenged [26], and MVBs may instead represent a population of organelles that arises upon injury in axons. Indeed, the retrograde transport of MVBs may play a role in injury signaling. Storage of signaling molecules within intralumenal vesicles of multivesicular bodies may prevent their deactivation during the long journey from the axon back to the cell body [27]. Intralumenal vesicles are not always destined for lysosomal degradation; they can also fuse back with the limiting membrane of late endosomes [28]. This process is hijacked by several toxins and viruses to reach the cell body and could similarly be exploited by signaling proteins [28]. For example, storage of phosphorylated proteins within intralumenal vesicles may allow effective long range signaling in neurons and may play an important role in nerve regeneration.

The precise nature and function of the small syd vesicles await further studies. However, the presence of adhesion and cytoskeletal regulatory proteins as well as ribosomal proteins [16], together with the observation that small syd vesicles mainly travel in the anterograde pathway [3] lead us to propose that small syd vesicles may play a role in neurite outgrowth and guidance. This hypothesis is supported by the axonal growth defects observed in syd knock-out animals [14,15]. Another link between syd and axonal growth and guidance comes from *C. elegans* studies. UNC-14, a protein required for axonal elongation and guidance, interacts with syd/UNC-16 [29]. If small syd vesicles play a role in axonal outgrowth during development, they may serve a similar role during nerve repair and regeneration. Synaptotagmin VII, a member of the

synaptotagmin family of Ca^{2+}-binding proteins, was identified in small syd vesicles. Synaptotagmin VII mediates exocytosis of lysosomes, a process important for the repair of plasma membrane wounds [24] and for neurite outgrowth [30].

In summary, we have uncovered the molecular anatomy of two distinct classes of syd-associated vesicles. Our studies point to a role for endocytic syd vesicles in the transport of signals along the axon and in the recycling of synaptic vesicles. In addition, syd may play a role in axonal growth and guidance through its interaction with another class of small anterograde vesicles. The identification of syd vesicle protein composition should contribute to define the mechanisms regulating axonal growth, guidance, and repair.

MATERIALS AND METHODS

Antibodies and Reagents- syd antibodies were previously described [10]. We used the following antibodies: anti-tubulin DM1A (Sigma), anti-amyloid precursor protein (Chemicon), anti-syntaxin 13, anti-SNAP29, anti-synaptotagmin VII, and anti-cellubrevin/VAMP3 (Synaptic Systems), Alexa Fluor-labeled secondary anti-mouse and anti-rabbit antibodies (Invitrogen). Texas Red dextran 3000 MW was purchased from Invitrogen.

Sciatic Nerve Ligation- Sciatic nerve ligation experiments were performed as described previously [3]. Briefly, the sciatic nerves of mice were ligated unilaterally at the midpoint, and mice were sacrificed at the indicated time after surgery. To avoid contamination of proximal and distal parts, two ligations were placed 1 mm apart. Sciatic nerves were dissected 6 hours following ligation. For biochemistry, equal lengths of the proximal and distal parts were homogenized in sample buffer, and equal protein amounts were loaded and analyzed by SDS-PAGE and Western blotting. The tubulin Western blot serves as loading control. For *in vivo* labeling of the endocytic pathway, two ligations were placed 1 mm apart, the nerve was sectioned in between the two knots, and 5 µl of 20 mg/ml Texas Red dextran 3000 MW was injected in the rear leg footpad. All surgeries were performed using adult female C57/bl6 mice and anesthetized with isofluorane. The sciatic nerve was dissected 24 hours following ligation and dextran injection. All procedures were approved by the Washington University in St. Louis, School of Medicine, Animal Studies Committee.

Immunofluorescence- Sciatic nerves were dissected and postfixed 2 h in 4% paraformaldehyde in PBS. Nerves were incubated overnight in 20% sucrose, embedded in Tissue-Tek OCT medium, and frozen in dry ice-cooled methanol. Serial 10-μm cryostat sections were cut and mounted onto coated slides (Fisher Scientific). Sections were permeabilized and blocked with 10% goat serum, 0.1% Triton X-100 in PBS, or 5% fish skin gelatin, 0.3% Triton X-100 in PBS for 30 min. Sections were incubated with the indicated primary antibodies overnight at 4 °C and with Alexa Fluor-conjugated secondary antibodies for 3 h. For low resolution images, sections were observed with a ×20 objective on a Nikon TE2000. For high resolution images, sections were observed with a ×100 objective on an Olympus FV500 confocal microscope or a Nikon Optigrid and deconvolved using Metamorph software.

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Figure 1. syd interacts with two types of vesicles of distinct size and morphology.

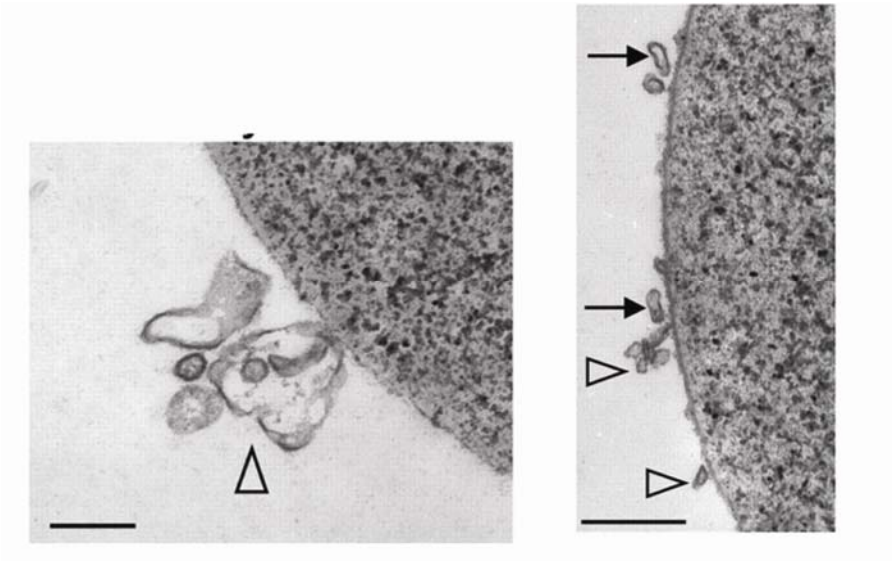

Figure 1. syd interacts with two types of vesicles of distinct size and morphology. Electron microscopy analyses of immunoisolated syd vesicles reveal larger (left) and smaller (right) vesicle types. Both single membrane vesicles (arrowheads) and tubules (arrows) were found.

Figure 2. Axonal transport of syd vesicle components.

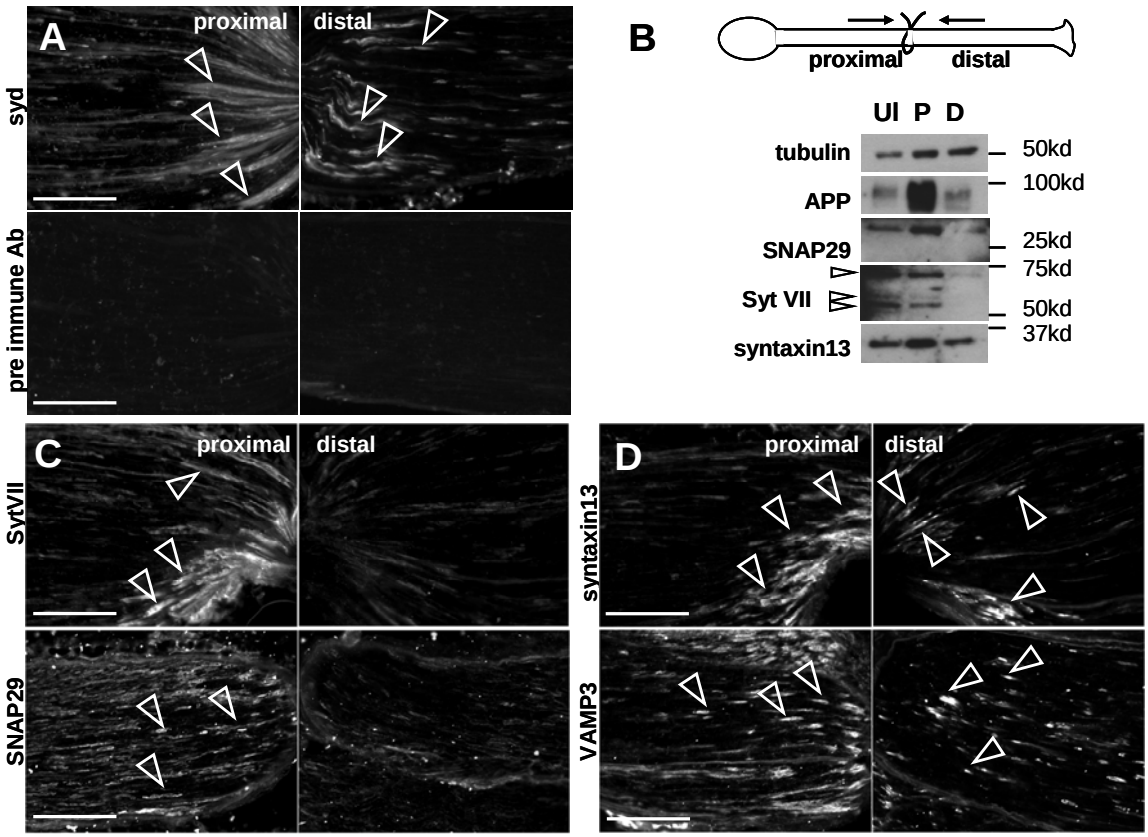

Figure 2. Axonal transport of syd vesicle components. Sciatic nerves were ligated unilaterally at the midpoint and processed for immunofluorescence microscopy or SDS-PAGE and Western blot analysis 6 hours later. (A) syd accumulates on both the proximal and the distal side of the ligation site, as expected. *Ab*, antibody. (B) Ligated and contralateral unligated sciatic nerves were dissected and extracts were analyzed by Western blot with the indicated antibodies. SNAP29 and synaptotagmin VII (SytVII), two proteins identified on the small syd vesicles, accumulated mostly on the proximal side, indicative of anterograde transport. Low levels are also detected to some extent on the distal sides, similarly to amyloid precursor protein (APP), a well established anterograde marker. The synaptotagmin antibody recognizes several isoforms, as indicated. Syntaxin 13, identified on the large syd vesicles, was detected on both the proximal and the distal sides, indicating that these proteins are transported in both anterograde and retrograde directions. Tubulin is used as a loading control. Ul, unligated; P, proximal; D, distal. C and D, SNAP29 and synaptotagmin VII are found mostly on the proximal side (C), and syntaxin 13 and VAMP3 are found on both sides (D), similarly to syd. In (A), (C), and (D), bar = 100 μm.

Figure 3. syd localization with *in vivo* labeled endosomes.

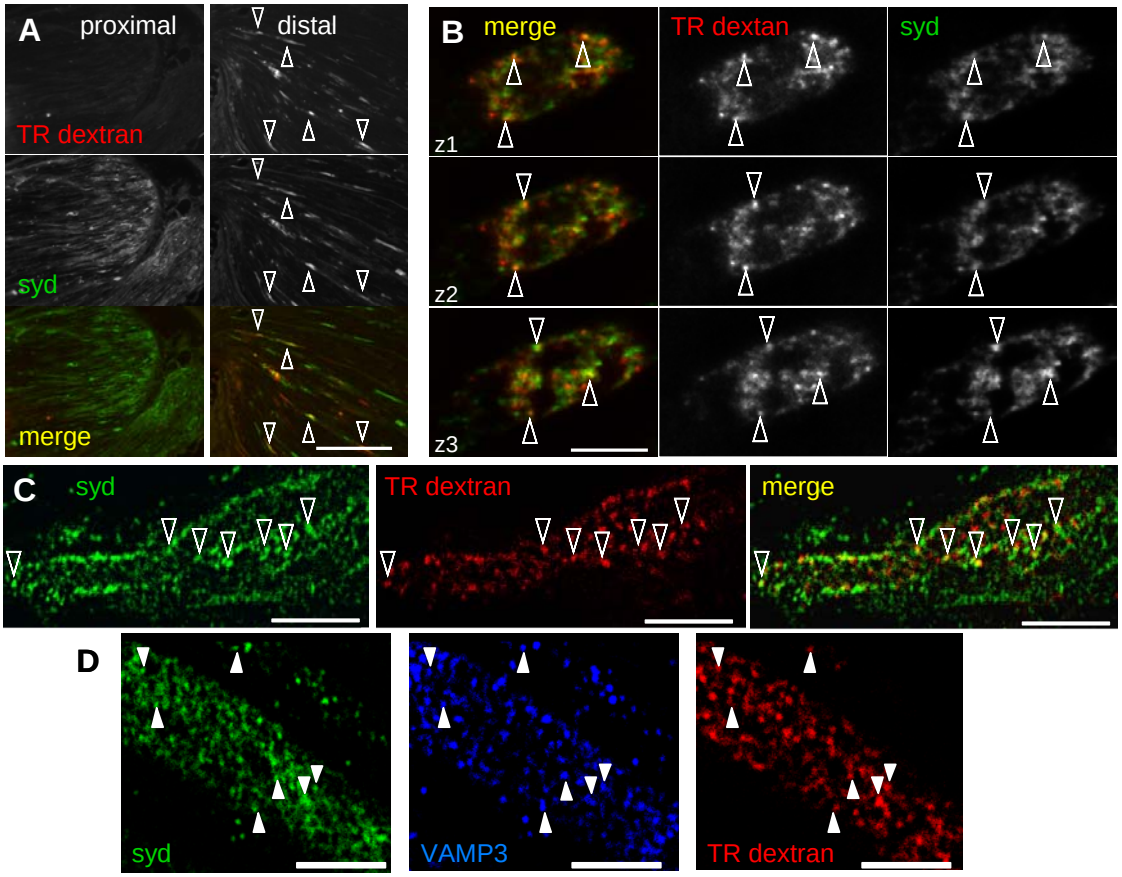

Figure 3. syd localization with *in vivo* labeled endosomes. The endocytic pathway within sensory neurons was labeled by subcutaneous injection of the tracer Texas Red dextran in the mouse rear leg footpad. A sciatic nerve ligation concomitant with dye injection was performed to increase the number of labeled structures accumulating distal to the ligation site. The sciatic nerve was dissected 24 h after injection, fixed, and embedded in cryomold. Longitudinal sections were analyzed by immunofluorescence. (A) Low magnification images showed syd accumulation in Texas Red dextran (TR dextran)-positive axons (arrowheads). (B) and (C), Nikon Optigrid structured illumination microscopy (B) or confocal microscopy (C) followed by deconvolution showed that Texas Red dextran puncta partially co-localize with syd. Three consecutive sections in the *z* plane are shown in (B) (D) Triple immunostaining showed that syd-dextran-positive structures also contained the endosomal protein VAMP3/cellubrevin, further supporting the notion that syd resides at least in part on axonal endosomes. (A) bar = 100 μm; (B), (C), (D)= 5 μm.

REFERENCES

1. Perlson E, Hanz S, Ben-Yaakov K, Segal-Ruder Y, Seger R, et al. (2005) Vimentin-dependent spatial translocation of an activated MAP kinase in injured nerve. Neuron 45: 715-726.

2. Hanz S, Perlson E, Willis D, Zheng JQ, Massarwa R, et al. (2003) Axoplasmic importins enable retrograde injury signaling in lesioned nerve. Neuron 40: 1095-1104.

3. Cavalli V, Kujala P, Klumperman J, Goldstein LS (2005) Sunday Driver links axonal transport to damage signaling. J Cell Biol 168: 775-787.

4. Lindwall C, Kanje M (2005) Retrograde axonal transport of JNK signaling molecules influence injury induced nuclear changes in p-c-Jun and ATF3 in adult rat sensory neurons. Mol Cell Neurosci 29: 269-282.

5. Herdegen T, Claret FX, Kallunki T, Martin-Villalba A, Winter C, et al. (1998) Lasting N-terminal phosphorylation of c-Jun and activation of c-Jun N-terminal kinases after neuronal injury. J Neurosci 18: 5124-5135.

6. Kenney AM, Kocsis JD (1998) Peripheral axotomy induces long-term c-Jun amino-terminal kinase-1 activation and activator protein-1 binding activity by c-Jun and junD in adult rat dorsal root ganglia In vivo. J Neurosci 18: 1318-1328.

7. Raivich G, Bohatschek M, Da Costa C, Iwata O, Galiano M, et al. (2004) The AP-1 transcription factor c-Jun is required for efficient axonal regeneration. Neuron 43: 57-67.

8. Barnat M, Enslen H, Propst F, Davis RJ, Soares S, et al. (2010) Distinct roles of c-Jun N-terminal kinase isoforms in neurite initiation and elongation during axonal regeneration. J Neurosci 30: 7804-7816.

9. Broude E, McAtee M, Kelley MS, Bregman BS (1997) c-Jun expression in adult rat dorsal root ganglion neurons: differential response after central or peripheral axotomy. Exp Neurol 148: 367-377.

10. Bowman AB, Kamal A, Ritchings BW, Philp AV, McGrail M, et al. (2000) Kinesin-dependent axonal transport is mediated by the sunday driver (SYD) protein. Cell 103: 583-594.

11. Ito M, Yoshioka K, Akechi M, Yamashita S, Takamatsu N, et al. (1999) JSAP1, a novel jun N-terminal protein kinase (JNK)-binding protein that functions as a Scaffold factor in the JNK signaling pathway. Mol Cell Biol 19: 7539-7548.

12. Kelkar N, Gupta S, Dickens M, Davis RJ (2000) Interaction of a mitogen-activated protein kinase signaling module with the neuronal protein JIP3. Mol Cell Biol 20: 1030-1043.

13. Kelkar N, Standen CL, Davis RJ (2005) Role of the JIP4 scaffold protein in the regulation of mitogen-activated protein kinase signaling pathways. Mol Cell Biol 25: 2733-2743.

14. Kelkar N, Delmotte MH, Weston CR, Barrett T, Sheppard BJ, et al. (2003) Morphogenesis of the telencephalic commissure requires scaffold protein JNK-interacting protein 3 (JIP3). Proc Natl Acad Sci U S A 100: 9843-9848.

15. Ha HY, Cho IH, Lee KW, Song JY, Kim KS, et al. (2005) The axon guidance defect of the telencephalic commissures of the JSAP1-deficient brain was partially rescued by the transgenic expression of JIP1. Dev Biol 277: 184-199.

16. Abe N, Almenar-Queralt A, Lillo C, Shen Z, Lozach J, et al. (2009) Sunday driver interacts with two distinct classes of axonal organelles. J Biol Chem 284: 34628-34639.

17. Prekeris R, Foletti DL, Scheller RH (1999) Dynamics of tubulovesicular recycling endosomes in hippocampal neurons. J Neurosci 19: 10324-10337.

18. Yap CC, Wisco D, Kujala P, Lasiecka ZM, Cannon JT, et al. (2008) The somatodendritic endosomal regulator NEEP21 facilitates axonal targeting of L1/NgCAM. J Cell Biol 180: 827-842.

19. McMahon HT, Ushkaryov YA, Edelmann L, Link E, Binz T, et al. (1993) Cellubrevin is a ubiquitous tetanus-toxin substrate homologous to a putative synaptic vesicle fusion protein. Nature 364: 346-349.

20. Foletti DL, Prekeris R, Scheller RH (1999) Generation and maintenance of neuronal polarity: mechanisms of transport and targeting. Neuron 23: 641-644.

21. Lalli G, Schiavo G (2002) Analysis of retrograde transport in motor neurons reveals common endocytic carriers for tetanus toxin and neurotrophin receptor p75NTR. J Cell Biol 156: 233-239.

22. Montagnac G, Sibarita JB, Loubery S, Daviet L, Romao M, et al. (2009) ARF6 Interacts with JIP4 to control a motor switch mechanism regulating endosome traffic in cytokinesis. Curr Biol 19: 184-195.

23. Idone V, Tam C, Goss JW, Toomre D, Pypaert M, et al. (2008) Repair of injured plasma membrane by rapid Ca2+-dependent endocytosis. J Cell Biol 180: 905-914.

24. Reddy A, Caler EV, Andrews NW (2001) Plasma membrane repair is mediated by Ca(2+)-regulated exocytosis of lysosomes. Cell 106: 157-169.

25. Salinas S, Bilsland LG, Schiavo G (2008) Molecular landmarks along the axonal route: axonal transport in health and disease. Curr Opin Cell Biol 20: 445-453.

26. Altick AL, Baryshnikova LM, Vu TQ, von Bartheld CS (2009) Quantitative analysis of multivesicular bodies (MVBs) in the hypoglossal nerve: evidence that neurotrophic factors do not use MVBs for retrograde axonal transport. J Comp Neurol 514: 641-657.

27. Weible MW, 2nd, Hendry IA (2004) What is the importance of multivesicular bodies in retrograde axonal transport in vivo? J Neurobiol 58: 230-243.

28. van der Goot FG, Gruenberg J (2006) Intra-endosomal membrane traffic. Trends Cell Biol 16: 514-521.

29. Ogura K, Shirakawa M, Barnes TM, Hekimi S, Ohshima Y (1997) The UNC-14 protein required for axonal elongation and guidance in Caenorhabditis elegans interacts with the serine/threonine kinase UNC-51. Genes Dev 11: 1801-1811.

30. Arantes RM, Andrews NW (2006) A role for synaptotagmin VII-regulated exocytosis of lysosomes in neurite outgrowth from primary sympathetic neurons. J Neurosci 26: 4630-4637.

Chapter 5: Sciatic nerve injury induces mTOR activation in Schwann cells

Contributions: Semithin sectioning and staining was performed by Howard Wynder at the Developmental Biology Histology Core

ABSTRACT

The response of Schwann cells to peripheral nerve injury play an integral role in nerve repair by creating a permissive environment for regeneration. While the morphological and functional responses that occur in Schwann cells following peripheral nerve injury have been characterized, the signaling pathways that mediate these events are not well known. We find that components of the mTOR pathway, Akt and S6 ribosomal protein are rapidly phosphorylated in Schwann cells within minutes to hours following sciatic nerve injury. Injury-induced S6 phosphorylation is abolished by rapamycin, indicating that mTOR is active following damage. Akt phosphorylation is concentrated at the paranode and spreads asymmetrically down the length of the node. In contrast, S6 phosphorylation occurs in the perinuclear space. Mechanical trauma to purified Schwann cells *in vitro* is not sufficient to activate Akt or S6 phosphorylation, suggesting that reciprocal signaling between axons and Schwann cells is important for the activation of this pathway.

INTRODUCTION

Successful regeneration depends upon not only intrinsic injury signals, but also on extrinsic factors in the environment. In the peripheral nervous system, Schwann cells act not only as insulators for fast, salutatory conduction of action potentials, but also play an essential role in the maintenance of axon health and integrity. In healthy nerves, the relationship between axons and their myelinating Schwann cells is maintained by contact-mediated reciprocal signaling [1]. Following injury, Schwann cells undergo a series of molecular and morphological changes upon disruption of contact with the axons that they myelinate [2]. These Schwann-cell mediated events play a critical role in the response of axons to injury.

One remarkable feature of Schwann cells is their ability to reverse differentiation. Within two days following nerve injury, Schwann cells undergo de-differentiation, or reversion from a differentiated myelinated state to an undifferentiated, immature state [3]. Dedifferentiation occurs through downregulation of myelination genes, including P_0, myelin basic protein (MBP), myelin associated glycoprotein (MAG), and periaxin, [4,5,6,7]. Concurrently, genes that are active in immature Schwann cells prior to myelination, such as L1, neural cell adhesion molecule (NCAM), p75 low affinity neurotrophin receptor (p75NTR) and glial fibrillary acidic protein (GFAP), become upregulated [8]. Following nerve damage, demyelination of injured axons creates a permissive environment for axonal regeneration [9,10]. Consistently, Schwann cell-specific deletion of *c-Jun*, a gene essential to drive dedifferentiation, results in a delay in myelin sheath degradation after nerve injury, as well as a significant loss of regenerative ability and functional recovery[11,12].

Following demyelination, degradation of myelin and axonal debris occurs through phagocytosis initially by Schwann cells, then later by macrophages that are recruited to the injury site [13,14,15,16]. Phagocytosis of axon and myelin debris is critical for degeneration of the nerve distal to the injury site, as well as for the regeneration of proximal axons. Myelin-associated proteins such as MAGs act as a potent negative regulators of axonal outgrowth. [17]. Thus, the removal of such inhibitory molecules by phagocytosis is essential to create a permissive environment for regeneration [18]. Indeed, the inability of the central nervous system to efficiently remove myelin debris following injury is linked to loss of regenerative ability [19].

Loss of axonal contact after injury also triggers Schwann cells to proliferate. As Schwann cells divide, they form linear arrays called bands of Bungner along the empty basement membrane of the endoneurial tube [20,21]. These bands of Schwann cells provide a platform for the growth of new axons into the distal end of the injury. Injury-induced proliferation of Schwann cells is mediated by a multitude of mitogenic factors released by macrophages, axons, and Schwann cells themselves [22].

Schwann cells are also a major source of growth factors that support the survival and growth of axons following injury. These include the neurotrophic factors NGF, BDNF, GDNF, as well as cytokines tumor necrosis factor (TNFα) and leukemia inhibitory factor (LIF) [23,24,25,26,27]. Such growth factors influence regeneration by not only enhancing survival and outgrowth of neurons, but also by regulating Schwann cell differentiation and remyelination of newly formed axons [28]. Furthermore, Schwann cells synthesize and provide surface cell adhesion molecules (CAMs), such as N-CAM, Ng-CAM/L1, N-cadherin, as well as basement membrane proteins such as

laminin, collagen, and fibronectin [22]. These molecules mediate adhesion between axons, axons and Schwann cells, and axons and the basal lamina, and thus provide substrates for the regrowth of new axons [22].

While the molecular and morphological changes that occur in Schwann cells following peripheral nerve injury have been documented, the signaling pathways that initiate these events are not well known. Furthermore, most studies have focused on events that occur days to weeks following damage, rather than early responses. Here, we sought to determine molecular pathways that become activated in Schwann cells as an immediate response to nerve injury. We find that Akt and S6 ribosomal protein, components of the mTOR pathway, are rapidly phosphorylated in Schwann cells within minutes to hours following sciatic nerve injury. Rapamycin potently blocks injury-induced S6 phosphorylation in Schwann cells, indicative of active mTOR following damage. We find that Akt phosphorylation is most robust at the paranode and spreads asymmetrically down the length of the node, while S6 phosphorylation occurs in the perinuclear region. Mechanical injury to Schwann cells *in vitro* is not sufficient to activate Akt or S6 phosphorylation, suggesting that reciprocal signaling between axons and Schwann cells is important for the activation of this pathway. Future work may shed light on the functional relevance of the mTOR pathway in Schwann cells in their response to nerve damage.

RESULTS

Akt and S6 ribosomal protein, components of the mTOR pathway, are rapidly phosphorylated in Schwann cells following injury- To identify signaling events that occur locally in the sciatic nerve following injury, we dissected the sciatic nerve 5mm proximal and distal to the ligation site at various time points after injury. Western blotting of unligated and ligated sciatic nerve extract revealed phosphorylation of Akt at Serine 473 as early as 15 minutes after ligation (Figure 1A, 1B). Akt phosphorylation levels peaked at 1 hour following injury, and returned to basal levels 9 hours later (Figure 1A, 1B). We also observed phosphorylation of S6 ribosomal protein at Serine 240/244, whose upstream kinase S6 kinase is activated by mTOR (Figure 1A, 1C). S6 phosphorylation was also observed within 15 minutes after ligation, but unlike Akt phosphorylation, steadily increased and persisted for at least 10 hours following injury (Figure 1A, 1C). Immunostaining of longitudinal sciatic nerve sections using phospho-Akt and phospho-S6 antibodies revealed that phosphorylation of these proteins occurs both proximal and distal to the ligation site (Figure 1D).

Akt and S6 ribosomal proteins are upstream and downstream components of the mTOR pathway, respectively. Injury-induced phosphorylation of S6 was completely abolished by intraperitoneal injection of the mTOR inhibitor rapamycin, indicating that mTOR activity is responsible for this phosphorylation event (Figure 1E). Intraperitoneal injection of the PI-3-kinase inhibitor LY294002 failed to inhibit the injury-induced phosphorylation of Akt. Thus, we have yet to determine whether Akt activation in the sciatic nerve following injury contributes to mTOR activity or to mTOR-independent pathways.

To determine whether injury-induced phosphorylation of Akt and S6 occur in axons or in surrounding Schwann cells, we performed immunohistochemical analyses on ligated sciatic nerve cross-sections. Phosphorylation of Akt in the unligated nerve occurs mostly in axons (Figure 2). However, in ligated sciatic nerve cross-sections, the majority of phospho-Akt signals overlap with the s100 Schwann cell marker, indicating that the increase in Akt phosphorylation levels after injury occurs in Schwann cells (Figure 2). Similarly, we found a marked increase in S6 phosphorylation levels in Schwann cells in ligated sciatic nerve cross sections (Figure 2). These results indicate that sciatic nerve injury induces phosphorylation of Akt and S6 in Schwann cells after injury.

Subcellular localization of Akt and S6 phosphorylation- To examine the subcellular localization of injury-induced Akt and S6 phosphorylation within Schwann cells, we performed immunohistochemical analyses on teased sciatic nerve preparations in which individual axons and their myelinating Schwann cells are laid flat on a two-dimensional surface. At low magnification, we observed that Akt phosphorylation is most concentrated near nodal regions and spreads unidirectionally down the internode away from the ligation site (Figure 3A, 3B). As contact-mediated reciprocal signaling between axons and Schwann cells occurs at the paranode [29] [1], we suspected that Akt phosphorylation may be concentrated at these sites. High-magnification confocal images of teased nerve sections stained for p-Akt and Caspr, a paranodal marker [29], confirmed that Akt phosphorylation occurs at the paranode on both sides of the node (Figure 3B). As observed in the low magnification images, the spread of Akt phosphorylation down the internode was detected only on one side of the node (Figure 3B, 3D). In marked contrast, phosphorylated S6 ribosomal protein is concentrated near the perinuclear

regions, consistent with the role of this protein in recruitment of ribosomes to mRNA (Figure 3C).

Mechanical injury to Schwann cells in vitro is not sufficient to activate Akt and S6 ribosomal protein- To better understand how the mechanism by which nerve injury activates Akt and S6 in Schwann cells, we tested whether mechanical injury to Schwann cells is sufficient to trigger phosphorylation of these proteins. We cultured Schwann cells from rat postnatal sciatic nerves and crushed them with a flathead instrument to induce mechanical injury. Mechanical injury to purified Schwann cells *in vitro* was not sufficient to activate Akt or S6 ribosomal protein, indicating that that reciprocal signaling between axons and Schwann cells may be required for the activation of this pathway (Figure 4).

mTOR activity is not required for demyelination- To explore whether activation of the mTOR pathway in Schwann cells have a functional role in their response to injury, we tested whether inhibition of mTOR activity by rapamycin could block demyelination of damaged axons. We chose to examine demyelination as a potential function of the mTOR pathway, as this process is one of the earlier events that occur in Schwann cells after damage and correlates temporally with the observed mTOR activation. We injected mice with either DMSO or rapamycin one hour prior to sciatic nerve ligation, dissected the sciatic nerve 48 hours later, and performed toluidine blue staining on semithin sections to visualize myelin sheaths. The degree of disorganization and unwinding of myelin sheaths was not markedly different between DMSO and rapamycin-treated ligated nerves, suggesting that mTOR activity in Schwann cells is dispensable for demyelination

after injury (Figure 5). Further studies will be required to determine the function of mTOR activity in Schwann cells in their response to damage.

DISCUSSION

While the morphological and functional responses of Schwann cells to peripheral nerve injury has been observed, little is known about the molecular pathways that mediate these processes. We find that the mTOR pathway becomes activated in Schwann cells as early as 15 minutes following sciatic nerve injury. We observed robust phosphorylation of S6 ribosomal protein which can be blocked by the mTOR inhibitor, rapamycin. Akt, one of the upstream regulators of mTOR activity, is also rapidly phosphorylated and activated in Schwann cells after injury. However, we have yet to determine whether Akt activity in Schwann cells contributes to mTOR activation, or whether it acts on other downstream targets independent of the mTOR pathway. We attempted to test this by pharmacological inhibition of Akt activation, but intraperitoneal injection of PI3-kinase inhibitor LY294002 did not successfully inhibit Akt activation. Other PI3-kinase inhibitors such as Wortmannin, or direct application of the drug into the nerve may be more effective in inhibiting kinase activity *in vivo*. As we observed in injured DRG neurons, it is possible that mTOR activation is occurring through Akt-independent mechanisms. Furthermore, the distinct subcellular localization of Akt and S6 phosphorylation suggests that activity of these proteins may have independent functions.

The concentration of p-Akt near the paranode in injured nerves indicate that reciprocal signaling between axons and Schwann cells may play an integral role in the activation of this protein. We found that mechanical trauma to purified Schwann cells *in vitro* is not sufficient to activate Akt or S6 phosphorylation, consistent with the idea that the intricate morphological and biochemical interaction between axons and Schwann

cells is critical for these signaling events. It would be interesting to test whether axotomy of myelinated axons in Schwann cell-DRG co-cultures could induce activation of Akt or S6. Our *in vivo* analyses of the subcellular localization of Akt phosphorylation revealed that the spread of Akt phosphorylation down the internode occur unidirectionally away from the injury site. In our teased nerve preparations, we could observe only a few nodes in a given nerve fiber. Preparations of longer nerve fibers may allow us to track the pattern of Akt phosphorylation in multiple nodes along a given fiber, which could provide better insight on the mechanism and relevance of the asymmetric nature of this signal.

To determine the functional relevance of mTOR activation in Schwann cells in their response to injury, we tested whether mTOR is required for demyelination of damaged axons. Rapamycin failed to block disorganization and unwinding of myelin sheaths, indicating that mTOR may not contribute to demyelination. As demyelination is tightly coupled with dedifferentiation, we speculate that dedifferentiation also does not require mTOR activity. Due to caveats of pharmacological experiments, genetic ablation of mTOR activity in Schwann cells will be necessary to confirm that this pathway is dispensable for dedifferentiation and demyelination after injury.

One potential function of the mTOR pathway in Schwann cells after peripheral nerve injury is phagocytosis of myelin and axonal debris. Akt has been implicated to mediate phagocytosis in macrophages through its activation of the mTOR pathway. Overexpression of constitutively-active Akt in macrophages *in vitro* increases efficiency of phagocytosis, while inhibition of mTOR by rapamycin blocks this effect. [30]. Furthermore, recent reports have implicated the mTOR pathway in the regulation of actin

dynamics through cdc42 and rac1 [31]. As remodeling of the actin cytoskeleton by cdc42 and rac1 is essential for phagocytosis [32], it is possible that mTOR activity in Schwann cells contributes to this process. To test this possibility, electron microscopic analyses will be required to visualize phagocytosis of debris by Schwann cells in the injured nerve *in vivo.* Phagocytosis could also be assayed *in vitro* using cultured Schwann cells; however, we would first need to identify signals or ligands that could activate phagocytosis in Schwann cells *in vitro*.

mTOR activation in Schwann cells may also contribute to protein synthesis. After sciatic nerve injury, Schwann cells synthesize and release a number of proteins that support survival and regeneration, including growth factors and extracellular matrix proteins [22,23,24,25,26,27]. Given the role of mTOR in ribosomal recruitment and protein translation, [33], it is possible that mTOR activity contributes to protein synthesis after injury. It would be interesting to test whether inhibition of mTOR activity by pharmacology or genetics could inhibit this process. As many of these proteins are soluble, immunodetection by ELISA may be required to assess changes in the levels of these Schwann-cell synthesized molecules.

It is possible that Akt activity after nerve injury has a distinct function independent of mTOR regulation. The concentration of p-Akt in paranodes in the injured nerve resembles the localization of p-ErbB2 [34]. Phosphorylation of ErbB2 is necessary and sufficient for demyelination after peripheral nerve injury [35]. Additionally, myelination of axons during development is regulated by reciprocal signaling between axons and Schwann cells, and requires ErbB2 and Akt activity [36]. *In vitro* studies show that Akt phosphorylation occurs downstream of ErbB2 activity [37]. As the

timecourse of ErbB2 phosphorylation after injury correlates with the timecourse of Akt phosphorylation, it is possible that Akt phosphorylation acts downstream of ErbB2 activation in Schwann cells after injury. As rapamycin does not block demyelination, ErbB2 and Akt may be regulating demyelination through other downstream effectors. Interestingly, ErbB2 phosphorylation occurs distal, but not proximal to the ligation site, while Akt phosphorylation occurs on both sides. Thus, it is possible that Akt activation has distinct roles on the two sides of the injury.

We speculate that cross-talk between axons and its myelinating Schwann cells is essential both for injury signaling and in the regenerative response. Understanding the molecular pathways that mediate Schwann cell response to injury, the mechanism by which they are activated, and their functional relevance could shed light on how environmental cues cooperate with intracellular growth pathways for successful regeneration.

MATERIALS AND METHODS

Antibodies and reagents- The following antibodies were used: anti-phosphorylated Akt (S473) (Cell Signaling), anti-Akt (Cell signaling), anti-phosphorylated S6 ribosomal protein (Cell Signaling, Serine 240/244), anti-S6 ribosomal protein (Cell Signaling), anti-α–tubulin (Sigma), anti-SMI-31(Sternberger Monoclonals Incorporated), anti-s100 (Sigma), anti-Caspr monoclonal (gift from Elior Peles).

Surgical procedures and drug treatment- All surgical procedures were approved by the Washington University in St. Louis, School of Medicine, Animal Studies Committee. Adult female Sprague-Dawley rats were used for experiments involving immunohistochemistry with anti-Caspr antibody. Adult female C57B6 mice were used for all other experiments. Sciatic nerve injury experiments were performed as described previously [38]**.** Briefly, the sciatic nerves of mice were ligated at mid-thigh at two points 1 mm apart. Mice were sacrificed indicated time after surgery and unligated and ligated nerves 5mm proximal and distal to the ligation site were dissected. For biochemical analyses, nerves were homogenized in lysis buffer (20mM Tris-HCl pH 7.5, 150mM NaCl, 1mM Na_2EDTA, 1mM EGTA, 1% Triton, 2.5mM sodium pyrophosphate, 1mM β-glycerophosphate, 1mM Na_3VO_4, 1μg/ml leupeptin) with phosphatase inhibitor cocktail 1 and 2 (Invitrogen). Equal protein amounts were loaded and analyzed by SDS-PAGE and western blot.

Rapamycin was delivered by intraperitoneal injection at 5mg/kg body weight. Rapamycin was dissolved in 200 μl DMEM from a 10 mg/ml stock solution in DMSO. An equivalent volume of DMSO was dissolved into 200 μl DMEM for vehicle control.

Intraperitoneal injection was performed 1 hr before sciatic nerve ligation. Animals were sacrificed and nerves were dissected 48 hours following ligation.

Schwann cell culture and immunofluorescence-. For Schwann cell cultures, sciatic nerves were dissected from P3 Sprague-Dawley rats and incubated in 0.1% collagenase in DMEM at 37º C for 30 minutes. Cells were collected by centrifugation at 800g for 10 minutes, washed, and resuspended in D-media (DMEM, 10% FBS, 2mM glutamine, 1% Pen/Strep) onto laminin-coated tissue culture flasks. After two passages, Schwann cells were purified by complement-killing of fibroblasts with anti-Thy1.1 antibody (Serotec). Purified Schwann cells were plated onto laminin-coated plastic dishes and maintained in D-media. For immunofluorescence staining, cells were fixed in 4% PFA/PBS for 30 minutes, then permeabilized and blocked in 10% Goat Serum, 0.1% Triton-X in PBS for 15 minutes. Staining was performed with the indicated primary antibodies for 30 minutes at room temperature, and with Alexa-conjugated secondary antibodies for 20 minutes. Images were acquired with Nikon Eclipse TE2000-E inverted microscope and analyzed using Nikon NIS Elements Advanced Research 2.30 Imaging Software.

Immunohistochemistry- For preparation of frozen sections, ligated and unligated sciatic nerves were dissected and fixed for 1-2 hours in 4% PFA in PBS, cryoprotected overnight in 20% sucrose, and embedded in OCT (Tissue-Tek) and frozen in dry-ice-cooled methanol. 10-µm serial cryostat sections were cut and mounted onto coated slides (Fisher Scientific). For teased nerve preparations, ligated and unligated sciatic nerves were dissected, fixed for 10 minutes in 4% PFA in PBS, and washed with PBS. After the perineurium was removed, nerves were manually teased apart in PBS with forceps, then transfered onto coated slides with a drop of PBS. Nerves were dried overnight at room

temperature and frozen at -80ºC until used for immunostaining. Frozen cryostat sections and teased nerve fibers were permeabilized and blocked with 10% goat serum, 0.1% Triton X-100 in PBS for 30 min. Primary staining with the indicated antibodies was performed in the blocking solution overnight at 4ºC. Staining with Alexa-conjugated secondary antibody was performed for 1 to 3 hours. Images were acquired with Nikon Eclipse TE2000-E inverted epifluorescence microscope or Olympus 500 confocal microscope and analyzed using Metamorph 6.2.

Semithin sections and toluidine blue staining- Fixation of nerve, embedment, sectioning, and toluidine blue staining were performed by Howard Wynder at the Developmental Biology Histology Core. Nerves were fixed in 4% PFA/2.5% gluteraldehyde/0.1M cacodylate buffer overnight at 4ºC, followed by wash with 0.1M cacodylate buffer. Secondary fixation in 1% Osmium Tetroxide was performed for 1 hour at room temperature, followed by wash with 0.1M cacodylate wash. Nerves were then dehydrated in 50% ethanol for 15 minutes, 70% ethanol for 30 miutes, 95% ethanol for 45 minutes, then 100% ethanol for 1 hour. Following dehydration, nerves were incubated in propylene oxide for 30 minutes, then in a 1:1 mixture of propylene oxide and Epon overnight at room temperature. The next day, nerves were incubated in freshly prepared Epon for several hours, then placed in another batch of fresh Epon for embedment at 65ºC overnight. Nerves were sliced into .5 to 1μm semithin sections using Leica Ultramicrotome and stained with toluidine blue.

Figure 1. Akt and S6 ribosomal protein are phosphorylated in sciatic nerve following injury.

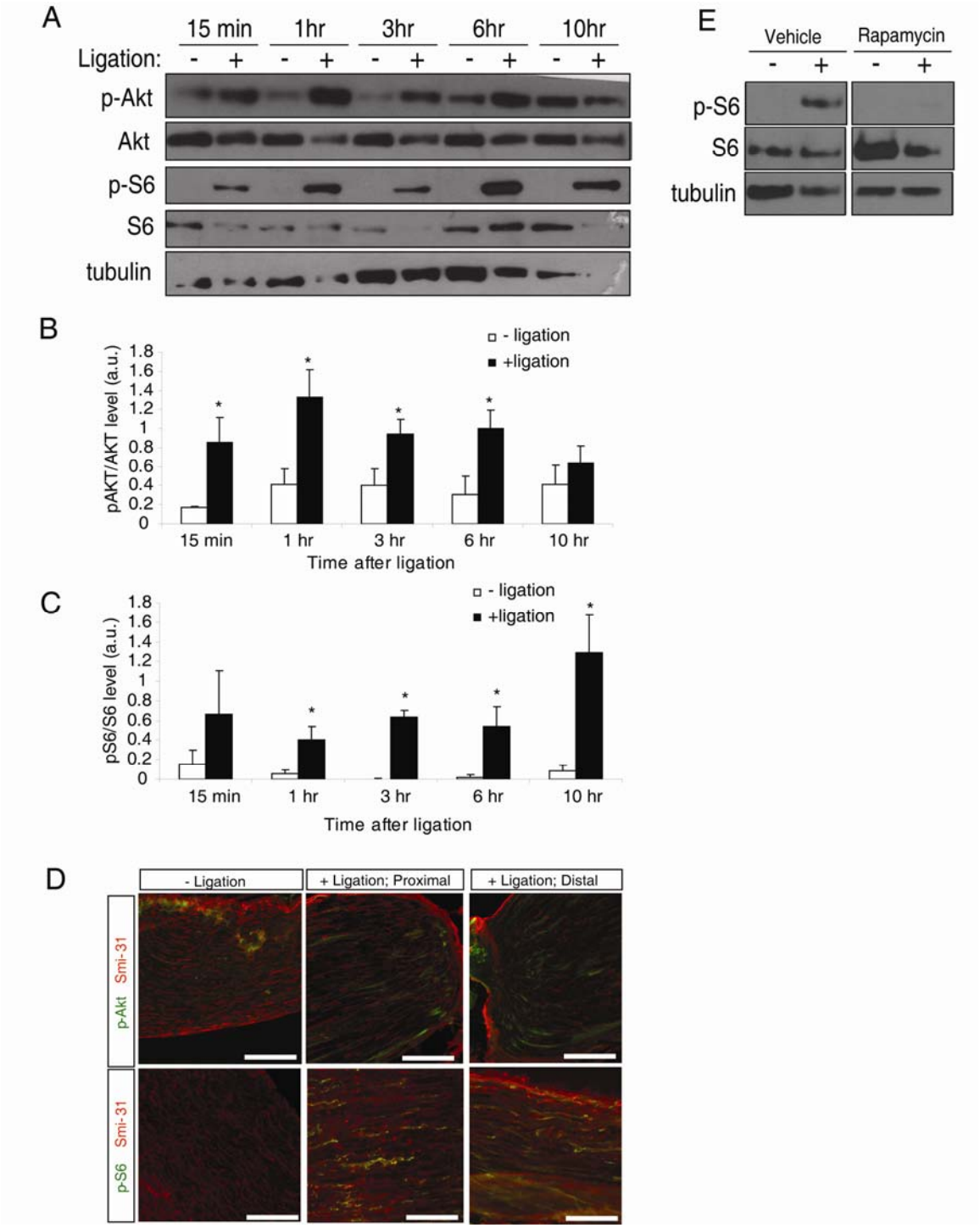

Figure 1. Akt and S6 ribosomal protein are phosphorylated in sciatic nerve following injury. (A) Unligated and ligated sciatic nerves 5mm distal and proximal to the ligation site were dissected at the indicated time points following ligation, and Akt and S6 phosphorylation levels were assessed by western blot. One mouse was used per time point. Akt and S6 phosphorylation were detected as early as 15 minutes following ligation. Quantification of Akt phosphorylation (B) and S6 phosphorylation (C) were based on 3 independent experiments. * $p<0.05$, data are mean +/- SEM (Student's *t*-test). (D) Immunohistochemistry on longitudinal sections of unligated nerve and ligated nerve 6 hours following ligation reveal Akt and S6 phosphorylation occur on both proximal and distal sides of the ligation. Bar=50μm. (E) Mice were injected intraperitoneally with rapamycin or DMSO vehicle control one hour prior to sciatic nerve ligation, and sciatic nerves were dissected 6 hours following ligation. Rapamycin treatment abolishes S6 phosphorylation in the ligated sciatic nerve.

Figure 2. Injury-induced phosphorylation of Akt and S6 ribosomal protein occurs in Schwann cells.

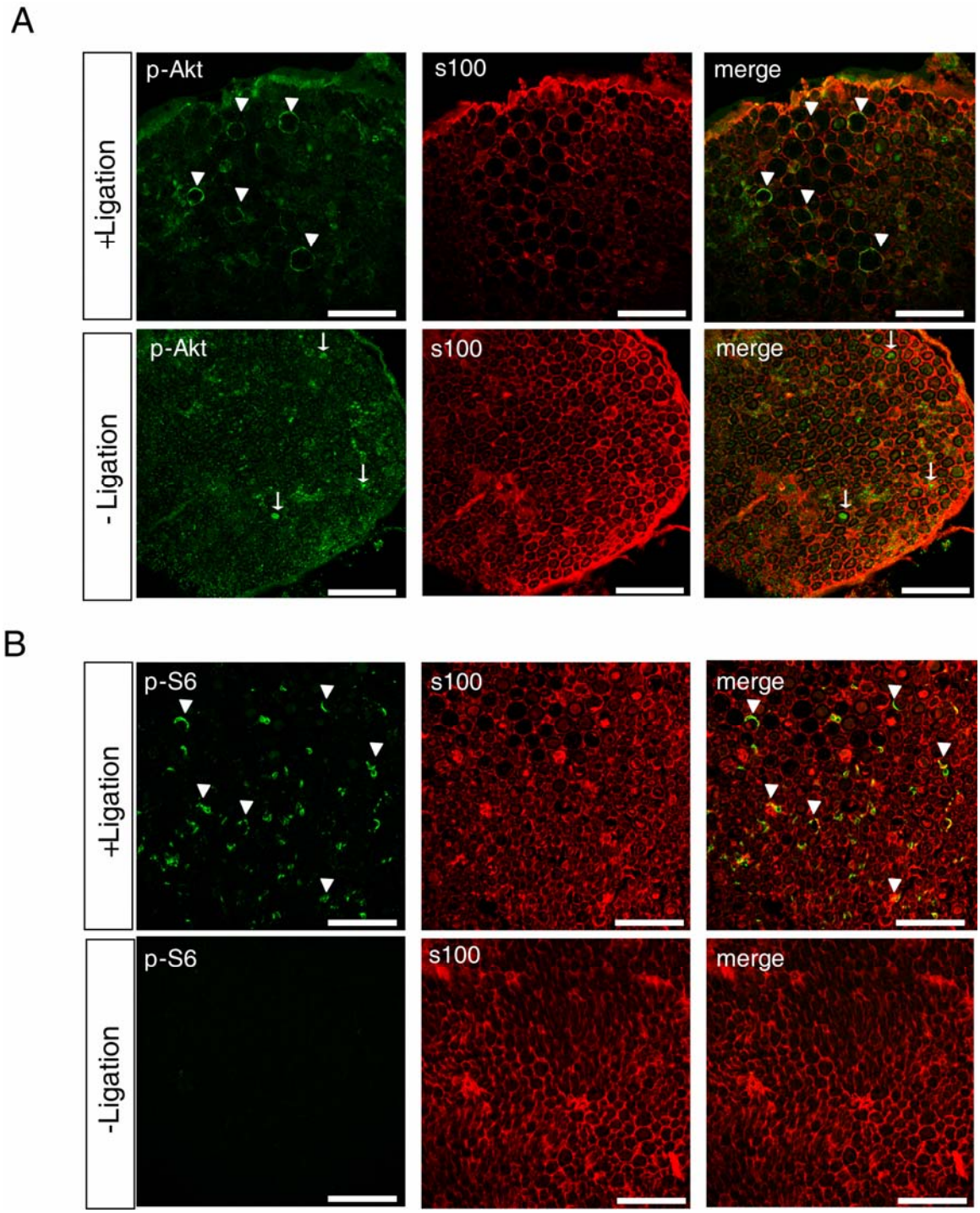

Figure 2. Injury-induced phosphorylation of Akt and S6 ribosomal protein occurs in Schwann cells. Cross sections of unligated nerve or ligated nerve 2mm distal to the ligation site were stained with s100 Schwann cell marker and (A)p-Akt or (B)p-S6 antibody. Nerves were dissected 6 hours following ligation. (A) Basal Akt phosphorylation is detected in axons, (arrows), but injury-induced phosphorylation of Akt occur predominantly in Schwann cells (arrowheads). (B) S6 phosphorylation in the ligated nerve occurs in Schwann cells (arrowheads). Bar=100μm.

Figure 3. Subcellular localization of Akt and S6 phosphorylation.

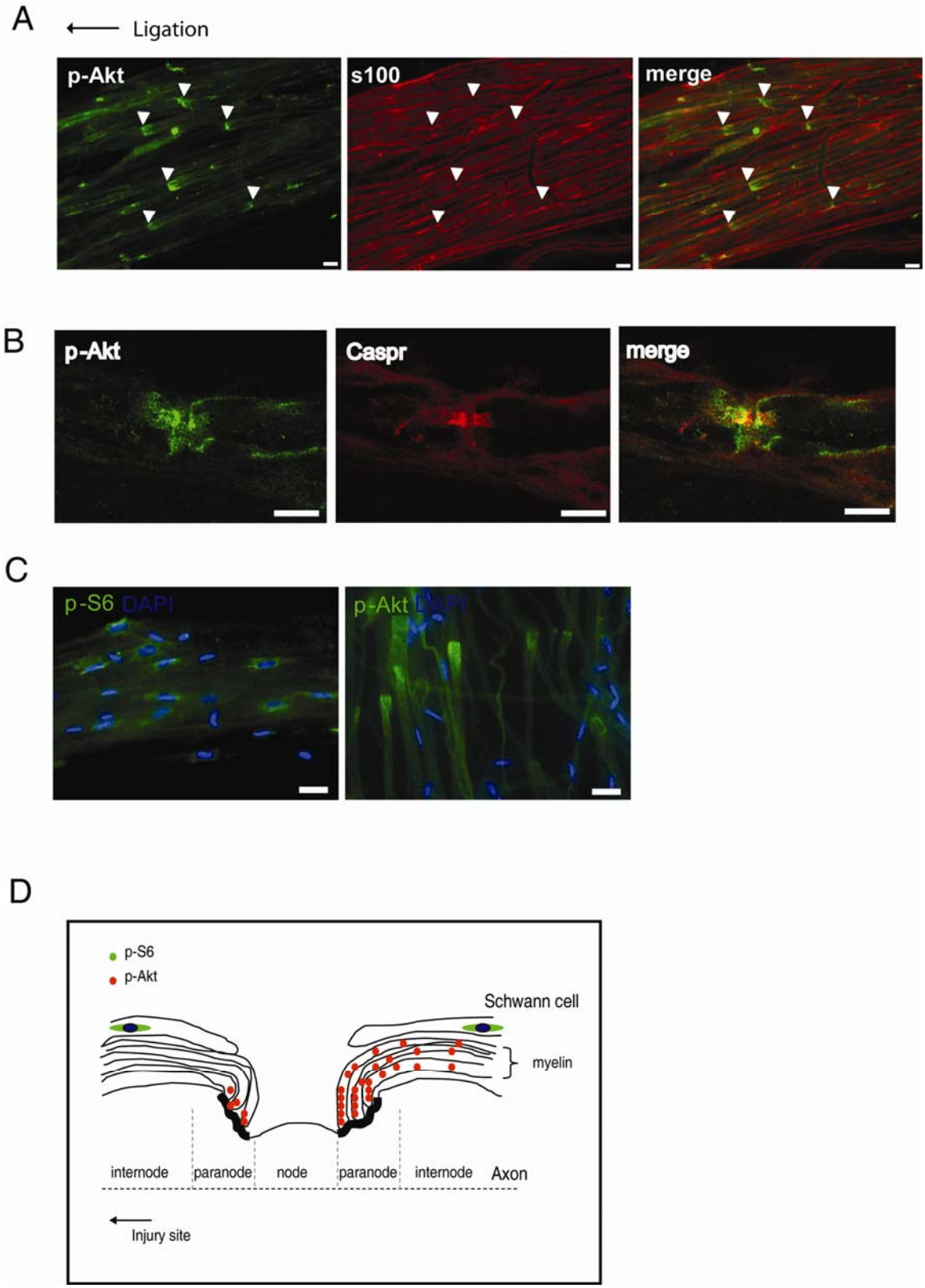

Figure 3. Subcellular localization of Akt and S6 phosphorylation. Teased nerve preparations from ligated nerve distal to the ligation site were stained with the indicated antibodies. Sciatic nerves were dissected 6 hours following ligation. (A) Akt phosphorylation occurs near nodal regions and spreads asymmetrically down the internode away from the ligation site. (B) p-Akt signal overlaps with paranodal marker Caspr, indicating that Akt phosphorylation occurs at the paranode on both sides of the node. Spread of the p-Akt signal down the internode occurs only on one side. (C) S6 phosphorylation occurs in the perinuclear region surrounding that DAPI stain. (D) Schematic of pattern of Akt and S6 phosphorylation in Schwann cells following injury. Each axonal internode is myelinated by one Schwann cell. Akt phosphorylation occurs at the paranode on both side of the node, but the spread of this signal down the internode occurs unidirectionally away from the ligation site. This pattern occurs both proximal and distal to the ligation. (A), (B), (C) Bar=10μm

Figure 4. Mechanical injury to Schwann cells *in vitro* is not sufficient to induce Akt and S6 phosphorylation.

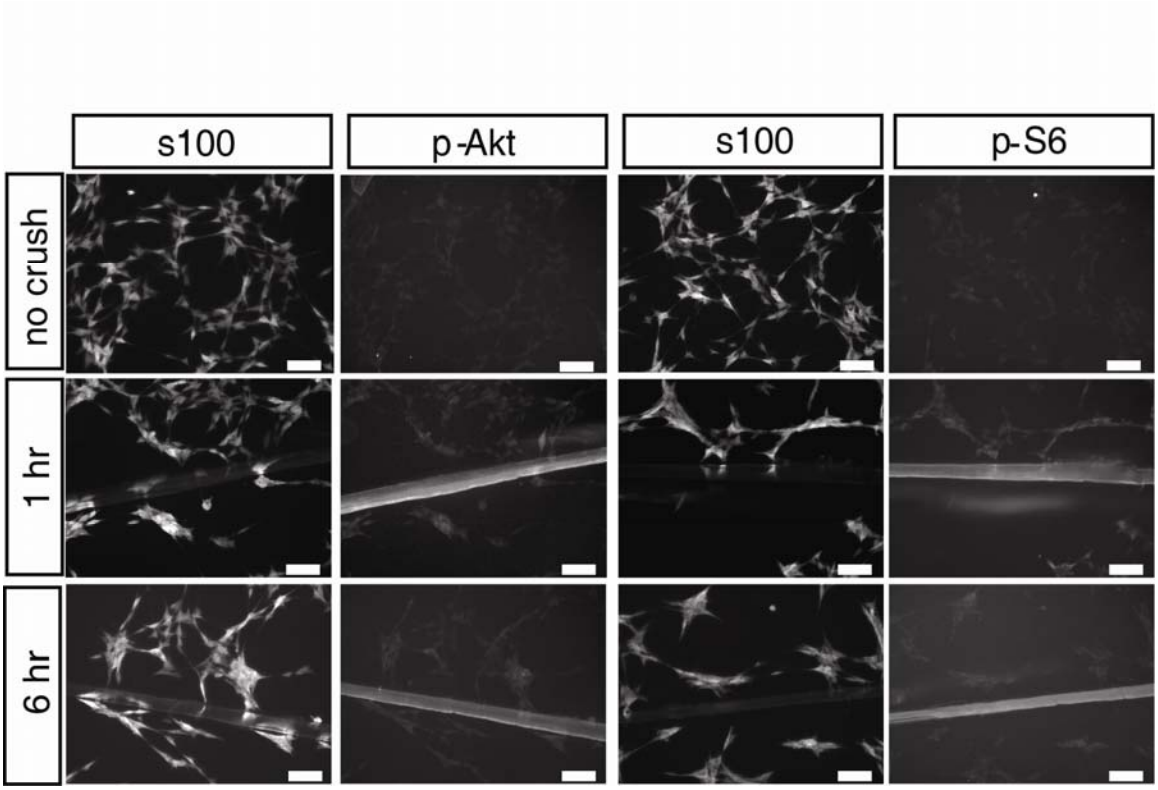

Figure 4. Mechanical injury to Schwann cells *in vitro* is not sufficient to induce Akt and S6 phosphorylation. Schwann cells from P3 rat sciatic nerves were plated and crushed *in vitro* and fixed 1 or 6 hours later. No significant increase in Akt or S6 phosphorylation was detected in crushed Schwann cells. Bar=50μm.

Figure 5. mTOR activity is not necessary for demyelination following injury.

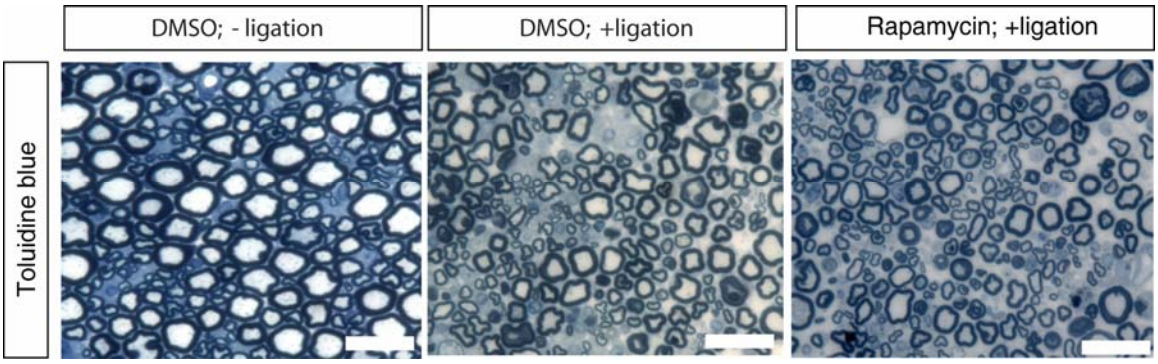

Figure 5. mTOR activity is not necessary for demyelination following injury. Mice were injected intraperitoneally with DMSO vehicle control or rapamycin one hour prior to sciatic nerve axotomy. Unligated nerve and ligated nerve ~2mm distal to the ligation site were dissected 48 hours following ligation. Toluidine blue staining of semithin sections reveal no significant difference in demyelination between DMSO and rapamycin treated ligated nerves.

Bar=50μm.

REFERENCES

1. Salzer JL, Brophy PJ, Peles E (2008) Molecular domains of myelinated axons in the peripheral nervous system. Glia 56: 1532-1540.

2. Jessen KR, Mirsky R (2008) Negative regulation of myelination: relevance for development, injury, and demyelinating disease. Glia 56: 1552-1565.

3. LeBlanc AC, Poduslo JF (1990) Axonal modulation of myelin gene expression in the peripheral nerve. J Neurosci Res 26: 317-326.

4. D'Antonio M, Michalovich D, Paterson M, Droggiti A, Woodhoo A, et al. (2006) Gene profiling and bioinformatic analysis of Schwann cell embryonic development and myelination. Glia 53: 501-515.

5. Nagarajan R, Svaren J, Le N, Araki T, Watson M, et al. (2001) EGR2 mutations in inherited neuropathies dominant-negatively inhibit myelin gene expression. Neuron 30: 355-368.

6. Nagarajan R, Le N, Mahoney H, Araki T, Milbrandt J (2002) Deciphering peripheral nerve myelination by using Schwann cell expression profiling. Proc Natl Acad Sci U S A 99: 8998-9003.

7. Verheijen MH, Chrast R, Burrola P, Lemke G (2003) Local regulation of fat metabolism in peripheral nerves. Genes Dev 17: 2450-2464.

8. Scarlato M, Xu T, Bannerman P, Beesley J, Reddy UR, et al. (2001) Axon-Schwann cell interactions regulate the expression of fibroblast growth factor-5 (FGF-5). J Neurosci Res 66: 16-22.

9. Fruttiger M, Montag D, Schachner M, Martini R (1995) Crucial role for the myelin-associated glycoprotein in the maintenance of axon-myelin integrity. Eur J Neurosci 7: 511-515.

10. Brown MC, Lunn ER, Perry VH (1992) Consequences of slow Wallerian degeneration for regenerating motor and sensory axons. J Neurobiol 23: 521-536.

11. Parkinson DB, Bhaskaran A, Arthur-Farraj P, Noon LA, Woodhoo A, et al. (2008) c-Jun is a negative regulator of myelination. J Cell Biol 181: 625-637.

12. Arthur-Farraj P, Bhaskaran A, Parkinson DB, Turmaine M, Feltri ML, et al. (2007) The transcription factor c-jun controls Schwann cell demyelination and dedifferentiation after peripheral nerve injury. J Neuron Glia Biol 3.

13. Reichert F, Saada A, Rotshenker S (1994) Peripheral nerve injury induces Schwann cells to express two macrophage phenotypes: phagocytosis and the galactose-specific lectin MAC-2. J Neurosci 14: 3231-3245.

14. Fernandez-Valle C, Bunge RP, Bunge MB (1995) Schwann cells degrade myelin and proliferate in the absence of macrophages: evidence from in vitro studies of Wallerian degeneration. J Neurocytol 24: 667-679.

15. Dailey AT, Avellino AM, Benthem L, Silver J, Kliot M (1998) Complement depletion reduces macrophage infiltration and activation during Wallerian degeneration and axonal regeneration. J Neurosci 18: 6713-6722.

16. da Costa CC, van der Laan LJ, Dijkstra CD, Bruck W (1997) The role of the mouse macrophage scavenger receptor in myelin phagocytosis. Eur J Neurosci 9: 2650-2657.

17. Tang S, Shen YJ, DeBellard ME, Mukhopadhyay G, Salzer JL, et al. (1997) Myelin-associated glycoprotein interacts with neurons via a sialic acid binding site at ARG118 and a distinct neurite inhibition site. J Cell Biol 138: 1355-1366.

18. Mears S, Schachner M, Brushart TM (2003) Antibodies to myelin-associated glycoprotein accelerate preferential motor reinnervation. J Peripher Nerv Syst 8: 91-99.

19. Filbin MT (2003) Myelin-associated inhibitors of axonal regeneration in the adult mammalian CNS. Nat Rev Neurosci 4: 703-713.

20. Thomas PK (1964) Changes in the Endoneurial Sheaths of Peripheral Myelinated Nerve Fibres during Wallerian Degeneration. J Anat 98: 175-182.

21. Bunge RP (1987) Tissue culture observations relevant to the study of axon-Schwann cell interactions during peripheral nerve development and repair. J Exp Biol 132: 21-34.

22. Fu SY, Gordon T (1997) The cellular and molecular basis of peripheral nerve regeneration. Mol Neurobiol 14: 67-116.

23. Boyd JG, Gordon T (2003) Glial cell line-derived neurotrophic factor and brain-derived neurotrophic factor sustain the axonal regeneration of chronically axotomized motoneurons in vivo. Exp Neurol 183: 610-619.

24. Boyd JG, Gordon T (2003) Neurotrophic factors and their receptors in axonal regeneration and functional recovery after peripheral nerve injury. Mol Neurobiol 27: 277-324.

25. Curtis R, Scherer SS, Somogyi R, Adryan KM, Ip NY, et al. (1994) Retrograde axonal transport of LIF is increased by peripheral nerve injury: correlation with increased LIF expression in distal nerve. Neuron 12: 191-204.

26. Meyer M, Matsuoka I, Wetmore C, Olson L, Thoenen H (1992) Enhanced synthesis of brain-derived neurotrophic factor in the lesioned peripheral nerve: different

mechanisms are responsible for the regulation of BDNF and NGF mRNA. J Cell Biol 119: 45-54.

27. Shamash S, Reichert F, Rotshenker S (2002) The cytokine network of Wallerian degeneration: tumor necrosis factor-alpha, interleukin-1alpha, and interleukin-1beta. J Neurosci 22: 3052-3060.

28. Chen ZL, Yu WM, Strickland S (2007) Peripheral regeneration. Annu Rev Neurosci 30: 209-233.

29. Einheber S, Zanazzi G, Ching W, Scherer S, Milner TA, et al. (1997) The axonal membrane protein Caspr, a homologue of neurexin IV, is a component of the septate-like paranodal junctions that assemble during myelination. J Cell Biol 139: 1495-1506.

30. Ganesan LP, Wei G, Pengal RA, Moldovan L, Moldovan N, et al. (2004) The serine/threonine kinase Akt Promotes Fc gamma receptor-mediated phagocytosis in murine macrophages through the activation of p70S6 kinase. J Biol Chem 279: 54416-54425.

31. Larson Y, Liu J, Stevens PD, Li X, Li J, et al. (2010) Tuberous sclerosis complex 2 (TSC2) regulates cell migration and polarity through activation of CDC42 and RAC1. J Biol Chem.

32. Massol P, Montcourrier P, Guillemot JC, Chavrier P (1998) Fc receptor-mediated phagocytosis requires CDC42 and Rac1. EMBO J 17: 6219-6229.

33. Hay N, Sonenberg N (2004) Upstream and downstream of mTOR. Genes Dev 18: 1926-1945.

34. Guertin AD, Zhang DP, Mak KS, Alberta JA, Kim HA (2005) Microanatomy of axon/glial signaling during Wallerian degeneration. J Neurosci 25: 3478-3487.

35. Zanazzi G, Einheber S, Westreich R, Hannocks MJ, Bedell-Hogan D, et al. (2001) Glial growth factor/neuregulin inhibits Schwann cell myelination and induces demyelination. J Cell Biol 152: 1289-1299.

36. Cotter L, Ozcelik M, Jacob C, Pereira JA, Locher V, et al. (2010) Dlg1-PTEN interaction regulates myelin thickness to prevent damaging peripheral nerve overmyelination. Science 328: 1415-1418.

37. Monje PV, Athauda G, Wood PM (2008) Protein kinase A-mediated gating of neuregulin-dependent ErbB2-ErbB3 activation underlies the synergistic action of cAMP on Schwann cell proliferation. J Biol Chem 283: 34087-34100.

38. Cavalli V, Kujala P, Klumperman J, Goldstein LS (2005) Sunday Driver links axonal transport to damage signaling. J Cell Biol 168: 775-787.

Chapter 6: Conclusions and future directions

Injury to the central nervous system (CNS) results in permanent loss of neuronal function due to the inability of injured axons to regenerate. In contrast, neurons in the peripheral nervous system (PNS) can extend new axons and reinnervate their targets following injury. Successful regeneration depends upon a balance between extrinsic factors in the environment and the intrinsic growth capacity of the injured neuron. The absence of glial-derived inhibitory cues in the PNS environment has been strongly linked to the regenerative ability of PNS neurons. However, much less is known about the intrinsic signaling pathways activated after PNS injury to promote axonal regrowth.

One potential regulator of growth capacity in injured neurons is the mTOR pathway. In non-neuronal cells, this pathway is active during development and is essential for cellular growth, proliferation, and survival [1] [2]. In the CNS, downregulation of mTOR activity following injury is linked to their lack of regenerative ability [3]. In contrast to CNS neurons, we found that dorsal root ganglia (DRG) in the PNS activate the mTOR pathway upon axonal damage, and that mTOR activation is both necessary and sufficient to enhance axonal growth capacity. Furthermore, we found that mTOR activation increases the expression of the growth-associated protein GAP-43, suggesting that mTOR-dependent protein synthesis contributes to enhance regeneration of injured peripheral neurons. However, overexpression of GAP-43 alone is not sufficient to fully stimulate regrowth of central branch axons to the level of a preconditioning injury, suggesting that the mTOR pathway regulates the translation of additional proteins to maximize axonal growth capacity [4]. Quantitative mass spectrometry analyses between wild-type and TSC2-deficient DRGs may reveal other proteins whose expression is regulated by mTOR.

Injury-induced activation of the mTOR pathway in PNS neurons demonstrate that the ability of PNS neurons to regenerate is not merely due to the lack of inhibitory cues in the environment, but also due to the active initiation of intracellular mechanisms that enhance growth capacity. Downregulation of this pathway by injured CNS neurons underscores the link between the mTOR pathway and intrinsic growth ability. To better understand the differences between CNS and PNS neurons in their ability to activate mTOR after injury, it will be important to identify the upstream regulators of mTOR that are activated by PNS injury. Our data suggest that activation of mTOR in injured DRGs occurs through Akt- and TSC2-independent mechanisms. Other candidate upstream regulators of mTOR include phosphatidic acid, and the JAK-STAT signaling pathway. [5] [6].

Activation of the mTOR pathway presents a promising therapeutic strategy to facilitate regeneration of both CNS and PNS neurons. However, we found that TSC2 deletion and persistent mTOR activation in DRGs leads to major developmental and functional defects, including increased mortality, small size, early death, sensory impairments, and aberrant target innervation and axon morphology. Though we have not yet tested whether re-innervation of injured axons is affected by TSC2 deletion, these developmental defects suggest that prolonged elevation of mTOR activity may also interfere with axon targeting and functional recovery of regenerating axons. Thus, while manipulation of mTOR activity could stimulate regrowth of injured axons, fine control of mTOR activity may be required for proper target innervation and functional recovery. Alternatively, understanding the precise mechanism by which mTOR regulates growth

versus axon targeting and morphology may offer more specific strategies on enhancing axonal regrowth without interfering with innervation.

For the injured neuron to activate molecular changes in the cell body such as the enhancement of intrinsic growth capacity or the activation of regeneration-associated genes, the cell body must first receive accurate information about the site and extent of axonal damage. Microtubule-based retrograde transport of locally activated injury signals has been proposed to contribute to this process. For example, JNK scaffolding protein Sunday driver (syd) has been shown to link vesicular axonal transport to injury signaling [7]. In naive nerves, syd binds to both kinesin for anterograde transport and dynactin for retrograde transport. Following sciatic nerve injury, JNK is locally activated and activated JNK and syd are transported mainly retrogradely [7]. The retrograde transport of JNK after injury is mediated by an enhanced interaction between syd and the dynactin complex [7].

To better understand syd function in axonal transport and injury signaling, syd vesicles were immunoisolated and analyzed by electron microscopy and mass-spectrometry. These studies have revealed two classes of syd-associated vesicles of distinct morphology and protein composition, and suggested that one class of syd vesicles belongs to the endocytic pathway, while another may belong to an anterogradely transported vesicle pool. Here, we validated these findings by examining the localization and transport of syd vesicle components within the sciatic nerve. Our results lead us to propose that the endocytic syd vesicles function to carry injury signals back to the cell body, whereas anterograde syd vesicles may play a role in axonal outgrowth and guidance. Genetic ablation of syd in DRGs will be necessary to test whether syd-

dependent axonal transport is essential for peripheral nerve regeneration. Furthermore, removing the expression of proteins that specifically interact with a specific class of syd vesicles may elucidate the distinct role of these two types of axonal organelles.

In addition to the activation of intracellular injury signaling pathways, regrowth of peripheral neurons is guided by Schwann cells in the PNS environment that create a permissive environment for axon regeneration. We found that components of the mTOR pathway, Akt and S6 ribosomal protein, are rapidly phosphorylated in Schwann cells within minutes to hours following sciatic nerve injury. Injury-induced S6 phosphorylation in Schwann cells was abolished by rapamycin, indicating that mTOR is active following damage. Mechanical trauma to purified Schwann cells *in vitro* was not sufficient to activate Akt or S6 phosphorylation, suggesting that reciprocal signaling between axons and Schwann cells is important for the activation of this pathway.

While the morphological and functional responses that occur in Schwann cells following peripheral nerve injury have been described, the signaling pathways that mediate these events are not well known. Thus, it will be valuable to determine the function of mTOR activation in Schwann cells in the context of nerve injury. In the injured peripheral nerve Schwann cells perform a number of tasks, including de-myelination, phagocytosis, proliferation, and synthesis and release of growth factors and extracellular matrix proteins. An *in vitro* myelinating Schwann cell-DRG co-culture system may offer assays to rigorously test the necessity of the mTOR pathway in each of these processes. Furthermore, it is unknown whether these Schwann-cell mediated events are necessary for nerve regeneration itself. If mTOR activation is required for any of these events, genetic ablation of mTOR components in Schwann cells could provide a

valuable tool to test the necessity of Schwann cell processes in peripheral nerve regeneration. It is likely that that cross-talk between axons and its myelinating Schwann cells is essential both for injury signaling and the regenerative response.

In this thesis, we explored multiple components of peripheral nerve injury signaling: transport of local injury signals by axonal vesicles, enhancement of intrinsic growth capacity in the cell body, and response of Schwann cells that myelinate the damaged axon. We found that the mTOR pathway is activated in both DRGs and Schwann cells after injury, and that in DRGs, this pathway plays an essential role in the enhancement of axonal growth capacity. The mTOR pathway is unlikely to be the only signal that mediates nerve regeneration; several classes of signaling pathways may act in concert to orchestrate a robust regenerative response. In fact, recent proteomic studies estimate that over 900 phosphoproteins and 4500 transcripts are involved in the injury response [8]. Additionally, it is likely that extrinsic cues derived from Schwann cells also cooperate with intracellular growth pathways for optimal regrowth. Identification of multiple components and signaling pathways that regulate nerve regeneration in both neurons and Schwann cells may unravel new therapeutic targets to promote PNS and CNS recovery from injury.

REFERENCES

1. Martin DE, Hall MN (2005) The expanding TOR signaling network. Curr Opin Cell Biol 17: 158-166.

2. Sarbassov DD, Ali SM, Sabatini DM (2005) Growing roles for the mTOR pathway. Curr Opin Cell Biol 17: 596-603.

3. Park KK, Liu K, Hu Y, Smith PD, Wang C, et al. (2008) Promoting axon regeneration in the adult CNS by modulation of the PTEN/mTOR pathway. Science 322: 963-966.

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

5. Fang Y, Vilella-Bach M, Bachmann R, Flanigan A, Chen J (2001) Phosphatidic acid-mediated mitogenic activation of mTOR signaling. Science 294: 1942-1945.

6. Smith PD, Sun F, Park KK, Cai B, Wang C, et al. (2009) SOCS3 deletion promotes optic nerve regeneration in vivo. Neuron 64: 617-623.

7. Cavalli V, Kujala P, Klumperman J, Goldstein LS (2005) Sunday Driver links axonal transport to damage signaling. J Cell Biol 168: 775-787.

8. Michaelevski I, Segal-Ruder Y, Rozenbaum M, Medzihradszky KF, Shalem O, et al. (2010) Signaling to transcription networks in the neuronal retrograde injury response. Sci Signal 3: ra53.