B-RAF kinase drives developmental axon growth and promotes axon regeneration in the injured mature CNS

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Citation

Published Version
doi:10.1084/jem.20131780

Accessed
June 19, 2017 8:12:31 AM EDT

Citable Link
http://nrs.harvard.edu/urn-3:HUL.InstRepos:13454857

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Axon growth is essential for the establishment of a functional nervous system as well as for the restoration of neuronal connectivity after injury or disease. It has long been hypothesized that re-activation of developmental growth mechanisms might help to achieve axon regeneration in the injured adult nervous system (Filbin, 2006). The role of MAP kinases in axon growth signaling has been much studied and discussed (Markus et al., 2002; Hanz and Fainzilber, 2006; Agthong et al., 2009; Hollis et al., 2009). However, depending on the model systems used, the outcomes have been controversial or even contradictory (Pernet et al., 2005; Sapieha et al., 2006; Hollis et al., 2009). We have shown that RAF–MEK signaling robustly promotes axon growth in primary sensory neurons in vitro (Markus et al., 2002). In vivo, conditional gene targeting studies have shown that RAF signaling is necessary for developing sensory neurons to arborize in their target fields in the skin (Zhong et al., 2007). However, it remains unknown whether RAF signaling is sufficient to enable axon growth in vivo or whether concomitant activation of other signaling pathways is necessary to drive long-range axon projections. Furthermore, it is unclear whether this pathway can promote axon growth in neuronal populations beyond the sensory neurons and the extent to which it can be harnessed to promote regeneration in the injured central nervous system (CNS). To address these questions, we have used conditional B-RAF gain-of-function mouse models to show that cell-intrinsic RAF signaling is a crucial pathway promoting developmental and regenerative axon growth in the peripheral and central nervous systems.

Activation of intrinsic growth programs that promote developmental axon growth may also facilitate axon regeneration in injured adult neurons. Here, we demonstrate that conditional activation of B-RAF kinase alone in mouse embryonic neurons is sufficient to drive the growth of long-range peripheral sensory axon projections in vivo in the absence of upstream neurotrophin signaling. We further show that activated B-RAF signaling enables robust regenerative growth of sensory axons into the spinal cord after a dorsal root crush as well as substantial axon regrowth in the crush-lesioned optic nerve. Finally, the combination of B-RAF gain–of–function and PTEN loss–of–function promotes optic nerve axon extension beyond what would be predicted for a simple additive effect. We conclude that cell-intrinsic RAF signaling is a crucial pathway promoting developmental and regenerative axon growth in the peripheral and central nervous systems.
activation of intraneuronal RAF–MEK signaling is sufficient to promote robust axon growth in developing and regenerating neurons in the peripheral nervous system and CNS.

RESULTS
Activation of B-RAF signaling alone is sufficient to promote sensory axon extension during early development
In vivo, the neurotrophin nerve growth factor (NGF) signals through its receptor kinase TrkA to promote developmental extension of dorsal root ganglion (DRG) nociceptive TrkA+ peripheral axons into the epidermis. To examine whether RAF signaling alone is sufficient to promote long-range axon extension of TrkA-positive neurons, we set out to selectively activate RAF kinase signaling in these neurons in a TrkA-null background.

To this end, we first conditionally activated RAF signaling in a WT background using a genetically modified loxP-STOP-loxP-BrafV600E (LSL-kaBraf) knock-in mouse line (Mercer et al., 2005), in which a kinase-activated B-RAF (kaB-RAF) mutant is expressed from the endogenous B-RAF locus upon Cre recombination. We next bred LSL-kaBraf mice with a neuronal

Figure 1. Conditional expression of kaB-RAF specifically activates MAP kinase signaling in the nervous system. (A) E18.5 mouse embryos expressing kaB-RAF develop grossly normally, except for substantial hydrocephalus. (B) DRG size is unaffected by kaB-RAF expression. (C) Representative Western blots show increased levels of pMEK1/2 and pERK1/2 in the E12.5 neocortex and spinal cord. (D) Quantitation of Western blots as in C. Amounts are normalized to corresponding βIII-tubulin levels. n = 4 repeats with three animals per group for each bar. Student’s t test: *, P < 0.05; **, P < 0.01. (E–G) Analysis of the interaction of kaB-RAF with other signaling pathways in the E12.5 nervous system. Representative Western blots are from three independent experiments, each with two or three E12.5 embryos for each genotype. (G) B-RAF protein levels are not increased in E12.5 DRG and spinal cord of LSL-kaBraf:nesCre embryos and control littermates. Molecular mass is indicated in kilodaltons. (H) Representative images of nerve endings in trunk skin of E13.5 embryos. Bars: (B) 200 µm; (H) 40 µm. (I) The number of branches per nerve trunk was quantitated, blinded to the genotypes, for a defined skin section by counting the total number of branch points observed along each major nerve trunk entering the skin (Zhong et al., 2007). n = 3 for each group. (D and I) Error bars indicate SEM.
Figure 2. Expression of kaB-RAF substantially rescues sensory afferent growth in the absence of TrkA/NGF signaling. (A, left) Normal sensory cutaneous innervation at E16.5. (middle) Sensory cutaneous innervation is lost in embryos lacking the NGF receptor TrkA. (right) Expression of kaB-RAF restores cutaneous innervation. Arrowheads label the blue β-gal–positive (presumptive TrkA+) sensory trajectories. (B) Visualization of axon growth patterns after tissue clearing. The thoracic somatosensory innervation driven by kaB-RAF in a TrkA−/− embryo (bottom; compare with middle for TrkA−/− alone) is similar to that seen in a control TrkAWT/− littermate (top). White arrowheads indicate the normal pathways of peripheral axons extending from thoracic DRGs. Red arrowheads indicate sensory projections rescued by kaB-RAF in the TrkA−/− background. (C) Expression of kaB-RAF substantially rescues trigeminal TrkA− afferent growth in the absence of TrkA/NGF signaling. Presumptive TrkA+ trigeminal axon projections (top) are lost in TrkA-deficient mice (middle) and are rescued by kaB-RAF (bottom). Ga, great auricular nerve; Go, greater occipital nerve; Mn, mandibular branch; Mx, maxillary branch; Op, ophthalmical branch. Images show littermates and are representative of three embryos per genotype. Bars: (A) 2 mm; (B and C) 1 mm.

nestin promoter–driven Cre deleter (nes-Cre; Tronche et al., 1999). In DRG neurons, nes-Cre–mediated recombination occurs as early as embryonic day (E) 11.5 (Galabova-Kovacs et al., 2008). Embryos heterozygously expressing kaB-RAF progressively developed macrocephaly from E13.5 onwards (Fig. 1 A) but appeared otherwise normal, including normally sized DRGs (Fig. 1 B). The known RAF effectors were activated in neuronal tissues expressing Cre recombinase, as indicated by elevated phospho-MEK1/2 (pMEK1/2) and pERK1/2 in the neocortex and spinal cord of E12.5 _LSL-kaBraf:nes-Cre_ mice (Fig. 1, C and D). Note that compared with MEK1/2, ERK1/2 activation appears minor in the kaB-RAF–expressing DRGs; this is because of relatively high levels of pERK1/2 in the DRG at baseline. B-RAF activation did not affect mTOR phosphorylation (Fig. 1 E). Levels of pAKT, pS6K, and pGSK3β were not changed significantly in the DRG of _LSL-kaBraf:nes-Cre_ mice (Fig. 1 F), indicating minimal cross talk between the MAP kinase and PI3-kinase–AKT pathways. Because the expression of kaB-RAF is under the control of endogenous _Braf_ promoter, the expression level of B-RAF protein is not changed in
the DRG and spinal cord at E12.5 (Fig. 1 G). At E13.5, the branching pattern of sensory nerves in the skin was not changed by kaB-RAF expression (Fig. 1, H and I).

**B-RAF activation rescues nociceptor axon extension in embryos lacking TrkA**

To test whether kaB-RAF is sufficient to drive nociceptor axon growth in the absence of TrkA signaling, we next mated the LSL-kaBraf:nes-Cre line with available TrkAtauLacZ and Bax<sup>−/−</sup> lines to generate LSL-kaBraf:TrkA<sup>tauLacZ/tauLacZ</sup>:Bax<sup>−/−</sup>:nes-Cre mice. In TrkA<sup>tauLacZ</sup> mice, the WT TrkA gene is replaced by a <i>tauLacZ</i> expression cassette, such that the axonal morphology of putative TrkA<sup>+</sup> neurons can be visualized by β-gal staining (Moqrich et al., 2004). Because TrkA expression is absent in homozygous TrkA<sup>tauLacZ/tauLacZ</sup> mice, we refer to the "TrkA<sup>tauLacZ/tauLacZ</sup>" as "TrkA<sup>−/−</sup>" in the text below. Removal of the Bax gene blocks apoptosis in embryonic DRG neurons, rescuing them from cell death that is otherwise observed in the absence of TrkA signaling. The Bax<sup>−/−</sup> background thus allows for the molecular dissection of signaling pathways that specifically affect axon growth (Knudson et al., 1995; Lentz et al., 1999; Patel et al., 2000; Markus et al., 2002; Kuruvilla et al., 2004; Moqrich et al., 2004). In TrkA<sup>−/−</sup>:Bax<sup>−/−</sup> mice, DRG neurons survive, but sensory afferent innervation in the skin is completely abolished (Fig. 2, A and B; Patel et al., 2000). Compared with control littersmates (which include LSL-kaBraf:TrkA<sup>−/−</sup>:Bax<sup>−/−</sup>, nes-Cre:TrkA<sup>−/−</sup>:Bax<sup>−/−</sup>, and TrkA<sup>−/−</sup>:Bax<sup>−/−</sup> genotypes), in which we detected no LacZ-positive fibers in the skin at E16.5, expression of kaB-RAF in TrkA<sup>−/−</sup>:Bax<sup>−/−</sup> DRG and trigeminal neurons substantially restored cutaneous sensory axon projections (Fig. 2, A–C). The morphologies of the radial thoracic trajectories derived from spinal DRGs as well as those growing from trigeminal ganglia induced by kaB-RAF in the TrkA<sup>−/−</sup>:Bax<sup>−/−</sup> background were grossly similar to those seen in control mice. Epidermal innervation of the footpad was partially rescued by kaB-RAF (Fig. 3, A and B). Thus, sustained B-RAF kinase activity can, to a large extent, substitute for TrkA-mediated axon growth signaling in presumptive TrkA<sup>+</sup> sensory neurons.

**B-RAF-mediated axon growth indirectly rescues calcitonin gene-related peptide (CGRP) expression in TrkA<sup>−/−</sup>:Bax<sup>−/−</sup> neurons**

CGRP expression in the peptidergic subset of nociceptive DRG neurons is induced by skin-derived factors and therefore indicates that sensory axon peripheral innervation into the skin is complete (Hall et al., 1997, 2001; Patel et al., 2000; Xu and Hall, 2007). In TrkA<sup>−/−</sup>:Bax<sup>−/−</sup> mice, DRG neurons and their centrally projecting axons are devoid of CGRP because of the lack of cutaneous innervation as previously described (Patel et al., 2000), whereas CGRP expression in the spinal motor neurons remains unaffected (Fig. 4 A, middle).
Specifically, nociceptive TrkA+ fibers terminate in the superficial laminae I and II of the dorsal horn, and proprioceptive parvalbumin-positive afferents project to intermediate laminae or to the ventral spinal cord.

In B-RAF gain-of-function mice, we observed excessive growth of both nociceptive and proprioceptive afferents (Fig. 5). Nociceptive axons normally restricted to superficial dorsal horn extended ectopically into deeper layers of dorsal spinal cord, and many axons aberrantly crossed the midline (Fig. 5 A). This kaB-RAF–driven overgrowth was substantially rescued by concomitant elimination of MEK1/2, the canonical downstream kinases of RAF (Fig. 5 C), suggesting that the effect of kaB-RAF expressed from the endogenous Braf locus depends strictly on canonical signaling.

In WT mice, different subpopulations of sensory neurons project from the DRG to highly specific targets in the spinal cord. Specifically, nociceptive TrkA+ fibers terminate in the superficial laminae I and II of the dorsal horn, and proprioceptive parvalbumin-positive afferents project to intermediate laminae or to the ventral spinal cord.

In WT mice, the central proprioceptive afferents enter the cord medially at tightly circumscribed dorsal root entry zones (DREZs; Fig. 5 B, left). kaB-RAF expression caused the proprioceptive sensory axons to enter the spinal cord all across its surface and to aberrantly terminate some branches in the superficial dorsal laminae (Fig. 5 B, right). Proprioceptive axons in the DREZs normally are subject to repulsive guidance from Semaphorin 6C/D (Sema6) expressed in the spinal cord, acting on PlexinA1 on the sensory axons (Yoshida et al., 2006). kaB-RAF expression did not detectably alter the protein (Fig. 5 D) or transcript levels (RNAseq; not depicted).

kaB-RAF causes overgrowth of nociceptive and proprioceptive afferent fibers in the spinal cord

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phenotype suggested that reactivation of the B-RAF pathway in injured adult neurons might be exploited to promote regeneration.

of these factors in E12.5 DRG and spinal cord. The overgrowth phenotype for both nociceptive and proprioceptive afferents was observed at all levels of the spinal cord (Fig. 5, E–J). This
Figure 6. Activation of B-RAF signaling in mature DRG neurons elevates their growth competency. (A, top) Schematic of the brn3a-CreERT2 construct used to generate the brn3a-CreERT2 deleter mouse line. (bottom) A cross section of the spinal cord of a 10-wk-old Rosa26-lacZ:brn3a-CreERT2 mouse treated with tamoxifen. Blue LacZ staining indicates CreERT2-mediated recombination in the DRG neurons. (B) Representative DRGs from adult LSL-kaBraf:brn3a-CreERT2:brn3a-CreERT2 mice without (top left) and with (bottom left) tamoxifen treatment. TdTom expression indicates recombination in DRG neurons. Cells were counterstained with Draq5 (Dq5) to label nuclei. (C) ATF3 is induced by preconditioning lesion. Blue shows nuclear stain Draq5. (D) Representative images of adult DRG neurons derived from intact brn3a-CreERT2:TdTom (left), LSL-kaBraf:brn3aCreERT2:TdTom
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As expected, in T2 One-way ANOVA with post-hoc Tukey’s HSD test: *, P < 0.01; **, P < 0.005.

(middle), and WT preconditioning lesioned mice (right) after 24 h in vitro. TdT om expression (Fig. 6 B). ATF3, a

kaB-RAF enables regeneration of injured adult DRG central axons across the DREZ

To test whether activation of B-RAF kinase signaling can promote axon regeneration of injured mature CNS neurons, we used an optic nerve regeneration model (Fig. 8, A and B; Park et al., 2008; Benowitz and Yin, 2010). 8–12-wk-old LSL-kaBraf: Bax−/− mice and Bax−/− controls were injected intravitreally with AAV2-Cre to induce kaB-RAF expression in retinal ganglion cells (RGCs) and then subjected to optic nerve crush. The Bax−/− background was used to minimize apoptotic death of retinal ganglion neurons triggered by optic nerve injury, which may amount to 80% at 2 wk after optic nerve crush (Li et al., 2000). 2 wk after the injury, we observed robust regenerative axon growth up to 3 mm past the lesion site in the kaB-RAF–expressing optic nerve (Fig. 8 C), consistent with previous observations that survival alone is not sufficient to promote growth of adult RGC axons (Goldberg et al., 2002).

Combined deletion of the canonical RAF effector kinases MEK1 and MEK2 substantially suppressed the regenerative axon growth caused by kaB-RAF (Fig. 8 E), indicating that kaB-RAF drives axon growth through the canonical MEK effectors. Whereas the length of axon extension induced by

Figure 7. Activation of B-RAF signaling enables crushed sensory axons to regenerate into adult spinal cord. (A–D) Confocal views of regenerating dorsal root axons in whole mounts (A and B) or transverse sections (C and D) 2 wk after root crush. Axons were labeled by AAV-GFP injected into C6 and C7 DRGs at the time of the crush injury. (A and C) Control mice (lrn3a-CreER)2; (B and D) Mice expressing activated B-RAF in DRGs (LSL-kaBraf:lrn3a-CreER)2. Dashed yellow lines indicate the DREZ, dashed gray lines indicate the border between gray and white matter, and arrowheads indicate the extent of axon growth across the DREZ (B) and into gray matter (D). DH, dorsal horn; DR, dorsal root; PNS, peripheral nervous system; SC, spinal cord. n = 2 DRGs from each of three animals per genotype. Bars, 200 µm.
B-RAF gain-of-function is comparable with the maximal axon growth reported in PTEN deletion mice (Fig. 8 F; Park et al., 2008), in a direct comparison, we found up to a 3.9-fold higher density of regenerating axons in the LSL-kaBraf;Bax<sup>−/−</sup> mice 1.5 mm distal to the crush site than is seen in the crushed Pten<sup>−/−</sup>:Bax<sup>−/−</sup> optic nerve (Fig. 8, D, F, and G).
DISCUSSION

An understanding of the mechanisms that drive axon growth is important, both to decipher how connectivity develops in the nervous system and to develop therapeutic strategies for nervous system repair after injury or disease. We show that the RAF–MEK axis plays a key role in axon growth signaling. Activation of B-RAF in neurons is sufficient to drive sensory axon growth in the embryo, to enable adult sensory axons to regenerate across the DREZ and further into the spinal cord, and to induce robust regeneration of adult retinal axons in the injured optic nerve. Both developmental DRG axon overgrowth in the spinal cord and mature RGC axon regeneration in the optic nerve were abrogated by concomitant ablation of MEK1 and MEK2. We thus establish classical cell-autonomous RAF–MEK signaling as a fundamental driver of axon growth. We should note that this pathway seems to be selective to axon growth signaling because we have never observed that B-RAF activation supports neuronal survival (unpublished data).

In vitro work has long suggested a potential role for RAF–MEK signaling in axon growth. Previous in vivo data, however, have been scarce and controversial. In the retina, for example, pharmaceutical inhibition of MEK–ERK signaling abrogated optic nerve regeneration supported by FGF2 (Sapieha et al., 2006). Two putative intracellular activators of RAF signaling, BAG1 and Mst3b, have been shown to promote regenerative axon growth in the optic nerve (Planchamp et al., 2008; Lorber et al., 2009), but the expression of a constitutively active MEK1 did not drive any regeneration in the optic nerve (Pernet et al., 2005). Others have concluded that ERK activity promotes RGC axon regeneration via an indirect mechanism dependent on glial cells (Müller et al., 2009). Although it is likely that multiple mechanisms, direct as well as indirect, will contribute to axon regeneration in the inhibitory environment of the CNS, the current cacophonic state of the field is likely caused by the mainly indirect approaches of incomplete penetration that have been taken by various laboratories. When using small molecule inhibitors or transient viral overexpression of interfering or activating constructs, it is difficult to accurately titrate the dose for the entire duration of an experiment. We believe that we have applied a stringent approach toward activation of RAF signaling in RGCs, and our data argue strongly for a direct positive effect of RAF–MEK signaling on axon growth and regeneration of RGCs, as well as in DRG neurons. Possible downstream mechanisms beyond the MEK kinases remain speculative at this point. Stabilization of microtubules improves axon regeneration in a spinal cord injury (SCI) model through both neuron-intrinsic and -extrinsic mechanisms (Hellal et al., 2011), and it is likely that activation of RAF–MEK signaling will directly affect microtubule stability in injured axons via its effects on microtubule-regulating enzymes such as HDAC6 (Williams et al., 2013). Furthermore, B-RAF has been shown to directly interact with tubulin (Bonfiglio et al., 2011). Activation of B-RAF signaling is also likely to trigger the expression of axon growth–enhancing gene sets in injured neurons. The elucidation of exact mechanisms awaits further study.
The developmental phenotypes we observed in the B-RAF gain-of-function embryos were generally complementary to those previously observed in B-RAF/C-RAF loss-of-function mice (Zhong et al., 2007). In contrast to nociceptors’ peripheral projections, the development of their central projections does not depend on NGF/TrkA signaling (Patel et al., 2000; Harrison et al., 2004; Zhong et al., 2007). Notably, we found that activation of B-RAF resulted in overgrowth of both proprioceptive and nociceptive axons in the spinal cord, whereas the expression of two known repulsive signaling molecules in the dorsal cord and DRG; Semaphorin and PlexinA1, remained unaltered. Thus, kaB-RAF appears to activate a normally quiescent axon growth signaling pathway in the central nervous system that seems to be unaffected by repulsive guidance cues.

The importance of this effect, the lack of response to repulsive or inhibitory cues, becomes clear in the context of regeneration of central sensory branches after dorsal root crush injury. Sensory axons expressing kaB-RAF robustly regenerated into the DREZ and spinal cord.

The regeneration failure of DRG axons after dorsal root avulsion injuries has been variously attributed to the lack of intrinsic growth capacity, to extrinsic growth barriers such as glia-associated growth inhibitors at the DREZ, and to premature synaptic differentiation (Han et al., 2012; Smith et al., 2012). Application of neurotrophic factors acting via tyrosine kinase receptors has shown substantially enhanced regeneration (Ramer et al., 2000; Wang et al., 2008; Harvey et al., 2010), even functional recovery with the systemic administration of artemin (Wang et al., 2008), although these results await independent replication. Future studies will test whether a combination of RAF activation with trophic growth factors can further enhance axon regeneration and reinnervation of presumptive targets in the spinal cord.

Compared with spinal cord lesions, the optic nerve’s simple structure allows for clear evaluation of both lesion and regeneration. In recent years, the optic nerve model has revealed several intracellular signaling pathways that can drive CNS axon regeneration, most prominently the PI3-kinase–mTOR and the JAK–STAT pathways, engaged by growth factor tyrosine kinase receptors and cytokines (Park et al., 2008; Smith et al., 2009; Buchser et al., 2012; Leibinger et al., 2013; Pernet et al., 2013). Combined deletion of endogenous inhibitors of these two pathways enhanced regeneration above the level reported for deletion of either gene alone (Sun et al., 2011). Activation of PI3-kinase–mTOR via PTEN deletion also enhanced regenerative growth in an SCI model (Liu et al., 2010), Activation of PI3-kinase–mTOR via PTEN deletion also enhances optic nerve axon regeneration even more strongly than would be expected for a simple additive effect.

The extent of regeneration achieved by direct genetic activation of specific intracellular signaling pathways, including B-RAF; DLK, PI3-kinase–mTOR, KLF, and JAK–STAT pathways (Smith et al., 2009; Yan et al., 2009; Park et al., 2010; Blackmore et al., 2012; Shin et al., 2012; Lang et al., 2013), compares favorably with what has been reported for application of growth factors such as NGE, BDNF, GDNF, and CNTF (Lykissas et al., 2007; Zhang et al., 2009; Allen et al., 2013; Leibinger et al., 2013). Growth factors as promoters of regeneration are hobbled by two major issues. First, the signaling machinery that enables growth factors to drive axon growth in the developing nervous system is not expressed at sufficient levels in the adult nervous system (Hollis et al., 2009). Indeed, growth inhibitory signaling molecules such as the SOCS family or phosphatases are up-regulated upon maturation (Lu et al., 2002; Smith et al., 2009; Park et al., 2010; Gatto et al., 2013). Therefore, the most promising studies using growth factors have combined them with genetic intervention to up-regulate growth factor receptors or down-regulate their intrinsic inhibitors (Hollis et al., 2009; Sun et al., 2011). The second issue is that of undesirable side effects, especially that of neuropathic pain caused by neurotrophin administration (Obata et al., 2006; Jankowski and Koerber, 2009). Development of “painless” neurotrophins (Capsoni et al., 2011) may improve the usefulness of this family of growth factors in the context of regeneration. Future combined activation of several growth signaling pathways with blockade of growth inhibitory pathways may lead to realistic treatment options for patients with loss of vision, sensation, or locomotion.

MATERIALS AND METHODS

Mouse models. Mouse breeding and genotyping were performed as described previously (Mercer et al., 2005; Zhong et al., 2007). The animal protocol was approved by the Institutional Animal Care and Use Committee at Weill Cornell Medical College, and experiments were conducted in accordance with the National Institutes of Health Guide for the Use and Care of Laboratory Animals. The LSL-kaBraf mouse line was provided by C.A. Pritchard (University of California, San Francisco, CA; Moqrich et al., 2004). Nestin-Cre deleter and Bax-null mice were generated in R. Klein’s laboratory (Max Planck Institute of Neurobiology, Martinsried, Germany; Tronche et al., 1999) and S.J. Korsmeyer’s laboratory (Dana-Farber Cancer Institute, Boston, MA; Knudson et al., 1995), respectively. The brn3a-CreER2 deleter mouse line was generated by J. Zhong in W.D. Smider’s laboratory (University of North Carolina at Chapel Hill, Chapel Hill, NC). All mice were on a mixed 129Sv and C57BL/6 background. We used littermates as controls throughout.

Generation of the brn3a-CreER2 deleter mouse line. Brn3a is a POU domain transcription factor that is selectively expressed in DRG neurons. Using a brn3a promoter construct (Eng et al., 2001), we generated a brn3a-CreER2 deleter mouse line using the pronuclear injection technique (Fig. 6 A).

Western blotting and immunohistochemical staining. Western blotting and immunohistochemical staining were performed as described previously (Zhong et al., 2007). An equal amount of total protein was loaded in each lane. The antibodies used were as follows: TrkA, Brn3a, and PlexinA1 antibodies were provided by L. Reichardt, E. Turner (University of California, San Diego, La Jolla, CA), and T. Jessell (Columbia University, New York, NY), respectively. Antibodies against MEK1/2 (9122), pMEK1/2 (9121), ERK1/2 (9102), pERK1/2 (9101), pAKT (9271 and 9275), phospho-p70S6K (811)
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Surgeons and all other personnel performing experi-
ments and analyses were blinded as to genotypes. 10–14-wk-old mice were
abolished and plated on laminin-coated coverslips. Cells were then
cultured in serum-free media supplemented with 1× N2 (Invitrogen) and
1% BSA. FdU, NGF, or AV2-Cre (Vector Laboratories) were added as
described previously (Markus et al., 2002; Zhong et al., 2007).

Dorsal root crush. Surgeons and all other personnel performing experi-
mints and analyses were blinded as to genotypes. 10–14-wk-old mice were
jected s.c. with tamoxifen (5 μg/10 g body weight) for a consecutive 5 d. After
another 2 d, i.e., 1 wk from the first tamoxifen injection, crush injury
of the C5, C6, C7, and C8 dorsal roots was performed using a fine forceps
(Dumont 8) as described previously (Di Maio et al., 2011). AV2-GFP
(∼1010 GC, 7004-Vector Laboratories) was then injected to C6 and C7 DRGs.
2 wk after the crush, mice were perfused, and axon regeneration through the
C6 and C7 dorsal roots was analyzed in a thin dorsal slice preparation of whole
spinal cord or in cryostat sections.

Sciatic nerve crush. Mice were injected with tamoxifen (2 μg/10 g body
weight) for a consecutive 5 d, followed by 5 d of rest. Unilateral sciatic nerve
 crush was then performed as described previously (Zhong et al., 1999). Adult
DRGs were collected and cultured as described previously (Zou et al., 2009).
Images were taken using a Carl Zeiss LSM710 NLO confocal microscope. Axon
length was quantified as described previously (Zou et al., 2009).

Optic nerve crush. Surgeons and all other personnel performing experi-
mints and analyses were blinded as to genotypes. The crush-regeneration and
axon counting protocol is adapted from Park et al. (2008). Whole-mount
optic nerves were treated with FocusClear (CelExper Lobars) and scanned
using an LSM710 NLO multiphoton confocal microscope. OD was det-
mined with the Carl Zeiss ZEN2009 software.

We would like to thank Louis Reichardt for the TrkαCreER mice and Trkα antibody,
Catherine Pritchard for the LSL-koBraf mice, Eric Turner for the Brn3a promoter, and
Thomas Jessell for the Plexin1A antibody. Larry Benowitz provided experimental
advice on the optic nerve injury model. Rajiv Ratan, David Ginty, William D. Snider,
and Annette Markus are acknowledged for insightful discussion and suggestions.
This work was supported by startup funds from the Burke Foundation as well as
Whitehall Foundation research grant 2010-08-61, grants 1R01EY022409 and
3R01EY022409-01S1 from the National Eye Institute (NEI), and grant ZBI-1102-1
from the Christopher & Dana Reeve Foundation to J. Zhong. Z. He is supported by
grants SR1EY12516 and EY021342 from NEI. Y.-J. Son is supported by grant
1R01NS079631 from the National Institute of Neurological Disorders and Stroke
and grants from Shriners Hospitals for Children and the Muscular Dystrophy
Association. H. Zou is supported by grants from the National Institutes of Health
(1R01NS073596) and the Irma T. Hirschl/Monique Well-Caulier Foundation.
K.J. O’Donovan is a Goldsmith fellow.

The authors declare no competing financial interests.

Submitted: 24 August 2013
Accepted: 18 March 2014

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