

Peripheral Nerve Regeneration Is Delayed in Neuropilin 2–Deficient Mice

Peter Bannerman,¹ Jahan Ara,² Ashleigh Hahn,³ Lindy Hong,¹ Erica McCauley,¹ Katie Friesen,¹ and David Pleasure^{1*}

¹Institute for Pediatric Regenerative Medicine, UC Davis School of Medicine, Sacramento, California

²Department of Pediatrics, Drexel University College of Medicine, Philadelphia, Pennsylvania

³Wyeth Pharmaceuticals, Radnor, Pennsylvania

Peripheral nerve transection or crush induces expression of class 3 semaphorins by epineurial and perineurial cells at the injury site and of the neuropilins neuropilin-1 and neuropilin-2 by Schwann and perineurial cells in the nerve segment distal to the injury. Neuropilin-dependent class 3 semaphorin signaling guides axons during neural development, but the significance of this signaling system for regeneration of adult peripheral nerves is not known. To test the hypothesis that neuropilin-2 facilitates peripheral-nerve axonal regeneration, we crushed sciatic nerves of adult neuropilin-2-deficient and littermate control mice. Axonal regeneration through the crush site and into the distal nerve segment, repression by the regenerating axons of Schwann cell p75 neurotrophin receptor expression, remyelination of the regenerating axons, and recovery of normal gait were all significantly slower in the neuropilin-2-deficient mice than in the control mice. Thus, neuropilin-2 facilitates peripheral-nerve axonal regeneration. © 2008 Wiley-Liss, Inc.

Key words: peripheral nervous system; axons; Schwann cells; semaphorins; sciatic nerve

Class 3 semaphorin (Semaphorin Nomenclature Committee, 1999) gradients guide axons in the developing nervous system (Messersmith et al., 1995; Bagnard et al., 2000; Chen et al., 2000; Giger et al., 2000; Spassky et al., 2002; Walz et al., 2002; Huber et al., 2005) and also control the migration of oligodendroglial progenitor cells (Spassky et al., 2002; Cohen et al., 2005) and the assembly of endothelial cells into blood vessels (Serini et al., 2003; Guttman-Raviv et al., 2007; Staton et al., 2007). These effects of class 3 semaphorins are transduced by target-cell plasma membrane receptor complexes that contain neuropilin-1 (Npn1) and/or neuropilin-2 (Npn2). Npn1 is required for signaling by semaphorin-3A (Sema3A) and Npn2 for signaling by Sema3F and Sema3B; both Npn1 and Npn2 participate in Sema3C signaling (Kolodkin et al., 1997; Kitsukawa et al., 1997; de Castro et al., 1999; Raper, 2000; Giger et al., 2000; Zou et al., 2000; Gu et al., 2002; Pond et al., 2002; Staton et al., 2007). Mice constitutively deficient in Npn1 or Npn2 exhibit developmental abnormalities

in axonal targeting and fasciculation (Chen et al., 2000; Giger et al., 2000; Cloutier et al., 2002; Kawasaki et al., 2002; Walz et al., 2002).

Transection or contusion of the adult spinal cord induces expression of mRNA encoding class 3 semaphorins in fibroblastic/meningeal cells in the scar at the trauma site (Pasterkamp et al., 1999; De Winter et al., 2002). CNS axonal regrowth after trauma is enhanced by treatment with a Sema3A inhibitor (Kaneko et al., 2006), suggesting that in the CNS, Sema3A, signaling via axonal Npn1, limits axonal regeneration through the scar. Sema3A/Npn1 signaling can enhance as well as inhibit functional recovery after axotomy by suppressing aberrant axonal sprouting and directing regenerating axons along normal patterns of distribution (Tang et al., 2007).

Although neuropilin-mediated class 3 semaphorin signaling guides axonal development in the peripheral nervous system (PNS) as well as in the CNS, the role of this signaling pathway in modulating adult PNS axonal regeneration is unknown. It has been established, however, that axotomy induces expression of Npn2 in the perikarya of adult spinal cord motor neurons proximal to the injury (Lindholm et al., 2004) and in Schwann and perineurial cells distal to the injury and of Npn2 ligands Sema3B, Sema3F, and Sema3C in epineurial and perineurial cells at the injury site (Scarlatto et al., 2003; Ara et al., 2004). Also arguing for a role of Npn2 in PNS regeneration, antibodies directed against extracellular domains of Npn2 block assembly by cultured Schwann cells into longitudinal arrays (Ara et al., 2005); in vivo, such Schwann cell arrays (bands of Bungner) enhance axonal extension into and through nerve segments that

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*Correspondence to: David Pleasure, MD, Departments of Neurology and Pediatrics, UC Davis School of Medicine, c/o Shriners Hospital, 2425 Stockton Blvd., Sacramento, CA 95817.

E-mail: david.pleasure@ucdmc.ucdavis.edu

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have undergone Wallerian degeneration (Tetzlaff, 1982; Son and Thompson, 1995; Nguyen et al., 2002; Chen et al., 2005). To test the hypothesis that Npn2 facilitates axonal regeneration in the PNS, we compared rates of axonal regeneration following a sciatic nerve crush injury in constitutively Npn2-deficient (Npn2^{-/-}) and littermate control (Npn2^{+/+}) mice.

MATERIALS AND METHODS

Animals

Founders for our colony of Npn2^{-/-} mice, which had been mutagenized by insertion of a secretory trap vector in an intron, thus interrupting Npn2 cDNA at nucleotide 2069 (Skarnes et al., 1995; Chen et al., 2000; Mitchell et al., 2001), were provided by W. C. Skarnes. These mice were backcrossed to a C57BL/6J background for at least six generations before use in our studies.

Quantitation of Npn1 and Npn2 mRNA in Npn2^{-/-} and Npn2^{+/+} Mice by Real-Time RT/PCR

To verify deletion of Npn2 in the Npn2^{-/-} mice and to determine whether there was a compensatory increase in Npn2 in these mice, we compared expression of mRNA encoding Npn1 and Npn2 in the Npn2^{+/+} and Npn2^{-/-} mice. Tail snips from 1 week postnatal Npn2^{+/+} and Npn2^{-/-} mice were powdered in a mortar precooled with liquid nitrogen. Total RNA was then isolated using Qiagen RNeasy Mini reagent kits. First-strand complementary DNA (cDNA) was synthesized from 1- μ g portions of total RNA using Superscript II Reverse transcriptase (Invitrogen, San Diego, CA) and oligo (dT)₁₈ primer (Invitrogen, San Diego, CA). Assays for mouse Npn1, Npn2, and GAPDH mRNA were performed using an ABI PRISM 7000 sequence detection system (Applied Biosystems). The TaqMan probes were designed using Primer Express 1.5 software (Applied Biosystems). Quantitative PCR was performed in a total reaction volume of 25 μ L containing 1 \times TaqMan Universal PCR Master Mix (Applied Biosystems), 250 nM of each primer, and a 200-nM probe. The thermal cycling conditions were initial denaturation at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 sec and annealing at 60°C for 1 min. Each sample was tested in duplicate. Data were normalized by dividing the copy number of the target cDNA by the copy number of the GAPDH cDNA in each sample.

Sciatic Nerve Crush

Three month postnatal male Npn2^{-/-} and Npn2^{+/+} C57BL/6 mice were anesthetized with xylazine and ketamine. An incision was made through the skin and the upper region of the right gluteal muscle to expose the sciatic nerve, which was then crushed 1–2 mm distal from the sciatic notch for 20 sec with size 4 forceps. The crush site was marked by gently squeezing with forceps dipped in sterile powdered charcoal for 5 sec, followed by suturing of the incision.

Footprint Analysis

Gait was tested preoperatively and again 1, 2, and 3 weeks postoperatively. Walking lanes (4" \times 48") were con-

structed from hardboard and placed on a strip of white, coated inkjet paper (92 brightness, Amerigo, Cincinnati, OH). After a practice walk in the lane, both hind footpads of each mouse were painted with Carter's Neat-Flo stamp pad ink using a Q-tip cotton applicator, and the mouse was again allowed to walk. The traces were graded by blinded observers for the presence or absence of 3 types of evidence of denervation: inability to lift the foot entirely from the surface (foot-dragging), inability to spread the toes, and lack of measurable print length (e.g., see Fig. 2C) over five consecutive right-sided footprints (Inserra et al., 1998).

Immunohistology

Crushed and intact sciatic nerves excised from the Npn2^{-/-} and Npn2^{+/+} mice were fixed in 2% paraformaldehyde/phosphate-buffered saline (PBS) for 30 min. The nerves were then washed in PBS, dehydrated through ascending ethanol, cleared in xylene, and infiltrated with Paraplast wax. Wax-embedded sections (7 μ m) were collected on Superfrost/Plus slides (Fisher Scientific) and melted at 56°C for 30 min. Sections were deparaffinized in xylene, then rehydrated through descending ethanols. For indirect immunofluorescence studies, sections were overlaid with a blocking solution [minimum essential medium containing 15 mM HEPES buffer, 10% (v/v) fetal calf serum, and 0.02% (w/v) sodium azide] for 30 min, then incubated overnight at 4°C with chicken antiserum to NF-L (diluted 1:300; Chemicon) and either rabbit anti-p75^{NTR} (1:400; Chemicon) or a monoclonal myelin basic protein (MBP) antibody (hybridoma supernatant, neat; Hickey et al., 1983), then incubated with biotin-conjugated donkey anti-chicken immunoglobulins (1:500; Jackson ImmunoResearch) and either rhodamine-conjugated donkey antirat (for MBP) or rhodamine-conjugated donkey antirabbit immunoglobulins [for p75 neurotrophin receptor (p75^{NTR})]. Both rhodamine conjugates were used at 1:500 for 25 min. The biotinylated secondary label was detected using fluorescein-conjugated streptavidin (1:500; Jackson ImmunoResearch) for 20 min. The sections were postfixated with -20°C methanol prior to mounting in Vectashield (Vector Labs). For immunoperoxidase studies, endogenous peroxidase activity was inhibited by incubating the sections with methanolic hydrogen peroxide [3% (v/v)]. Following brief washes in deionized water and PBS, sections were blocked as above and incubated for 35 min with chicken anti-NF-L (1:300, Chemicon), washed in PBS, then incubated with biotin-conjugated donkey antichicken (Jackson ImmunoResearch). The biotinylated secondary label was detected using peroxidase-conjugated streptavidin (Jackson ImmunoResearch) diluted 1:200 in azide-free blocking solution for 20 min. Immunoperoxidase labeling was detected using diaminobenzidine in 0.1M TRIS containing 0.05% Triton-X100 (v/v) for 8 min. Sections were counterstained with hematoxylin, dehydrated, and mounted in Cytosol 60 (Stephens Scientific).

Counts of myelinated axons in complete sciatic and posterior tibial nerve cross sections were performed by a blinded observer. Adobe Photoshop images were assembled from stacks of ten 0.5- μ m confocal slices of sciatic and posterior tibial nerve cross sections immunostained for MBP and NF-L, using a 40 \times oil objective and a Leica laser-scanning confocal

TABLE I. Functional recovery and regeneration of myelinated axons following sciatic nerve crush are delayed in *Npn2*^{-/-} mice

Percentage of mice with normal gait*	<i>Npn2</i> ^{+/+} mice	<i>Npn2</i> ^{-/-} mice
	(number of mice in parentheses)	
A) 1 week post-crush	0% (0/15)	0% (0/14)
B) 2 weeks post-crush	86% (19/22)	19% (3/16)**
C) 3 weeks post crush	89% (8/9)	92% (12/13)
Myelinated axons/distal sciatic nerve***	<i>Npn2</i> ^{+/+} mice	<i>Npn2</i> ^{-/-} mice
	(n = 6 mice in each group)	
non-crushed	3250 ± 450	3105 ± 538
2 weeks post-crush	1609 ± 163	1297 ± 75****

*Normal gait = absence of foot drag, ability to spread the toes, and measurable print length (Inserra et al, 1998).

**At 2 weeks post-crush, the number of *Npn2*^{-/-} mice with normal gait significantly less than the number of *Npn2*^{+/+} mice with normal gait ($p < 0.001$, chi square test).

***All myelinated axons were counted 2 weeks post-crush in sciatic nerve cross-sections 5mm below the crush, and at the same level in non-crushed sciatic nerves (mean ± SD, n = 6 in each group).

****At 2 weeks post-crush, numbers of myelinated axons in *Npn2*^{-/-} distal sciatic nerves below the crush were significantly less than the number of myelinated axons in the *Npn2*^{+/+} distal sciatic nerves below the crush ($p < 0.002$, 2-tailed t-test).

microscope. Only those myelinated axons in which a continuous ring of MBP immunoreactivity was visualized surrounding a central NF-L+ axon were counted.

RESULTS

Adult *Npn2*^{-/-} Mice Have Markedly Reduced Expression of *Npn2* mRNA and Show No Compensatory Increase in *Npn1* mRNA

Quantitative RT-PCR demonstrated that the abundance of *Npn2* mRNA in *Npn2*^{-/-} mice (0.25 ± 0.02 copies/1,000 copies GADPH mRNA) was less than 3% of that in *Npn2*^{+/+} mice (9.55 ± 0.33 copies/1,000 copies GADPH mRNA), with approximately half the normal abundance of *Npn2* mRNA in *Npn2*^{+/-} heterozygotes (data not shown). The abundance of *Npn1* mRNA was not significantly different in *Npn2*^{-/-} mice (4.08 ± 0.14 copies/1,000 copies GADPH mRNA) than in *Npn2*^{+/+} mice (4.17 ± 0.82 copies/1000 copies GADPH mRNA).

Adult *Npn2*^{-/-} Mice, Although Slightly Smaller Than Adult *Npn2*^{+/+} Mice, Have a Normal Gait and Normal Numbers of Myelinated Axons in Sciatic Nerve

C57BL/6J *Npn2*^{-/-} mice were viable and fertile but were slightly smaller than wild-type C57BL/6J mice [25.9 ± 2.5 g for 3-month-old male *Npn2*^{-/-} mice vs. 29.2 ± 2.7 g for 3-month postnatal male *Npn2*^{+/+} mice (mean ± SD), $n = 16$ in each group, $P < 0.01$]. Gaits of uninjured 3-month postnatal C57BL/6J *Npn2*^{-/-} and *Npn2*^{+/+} mice, assessed by footprint

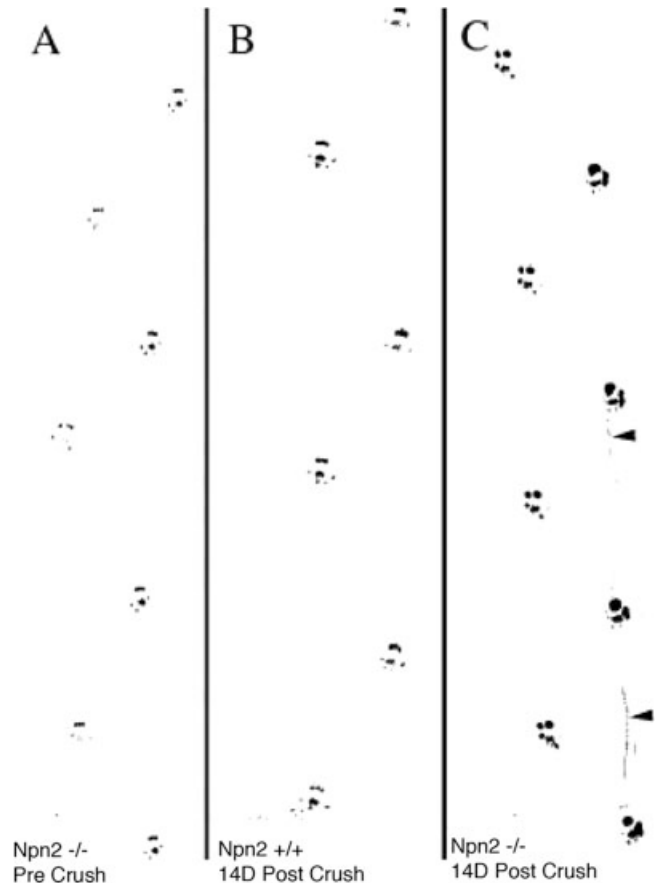


Fig. 1. Footprint analysis demonstrating delayed functional recovery following sciatic nerve crush in *Npn2*^{-/-} mice. **A:** P-crush footprints are normal in an *Npn2*^{-/-} mouse. **B:** Normal footprints 2 weeks postcrush in an *Npn2*^{+/+} mouse. **C:** Right foot-drag (arrowheads) 2 weeks postcrush in an *Npn2*^{-/-} mouse. Also note the impaired toe spread on the right.

analysis, did not differ significantly, nor did the uninjured *Npn2*^{-/-} and *Npn2*^{+/+} mice differ in numbers of myelinated axons in their sciatic nerves (Table I).

Delays in Neurological Recovery and Regeneration of Myelinated Axons after Sciatic Nerve Crush Injury in Adult *Npn2*^{-/-} Mice

One week after sciatic nerve crush, footprint analysis showed that all *Npn2*^{-/-} and *Npn2*^{+/+} mice had gait abnormalities. By 2 weeks postcrush, footprints had returned to normal in 86% of the *Npn2*^{+/+} mice but only in 19% of the *Npn2*^{-/-} mice (Table I). Typically, most *Npn2*^{-/-} mice exhibited foot-dragging and unmeasurable toe spread and/or paw length at that point (Fig. 1C). Three weeks postcrush, nearly all *Npn2*^{-/-} and *Npn2*^{+/+} mice had measurable footprints and no foot-dragging (Table I).

To determine whether the retarded recovery of normal gait in the *Npn2*^{-/-} mice was caused by a delay in regeneration of myelinated axons distal to the sciatic

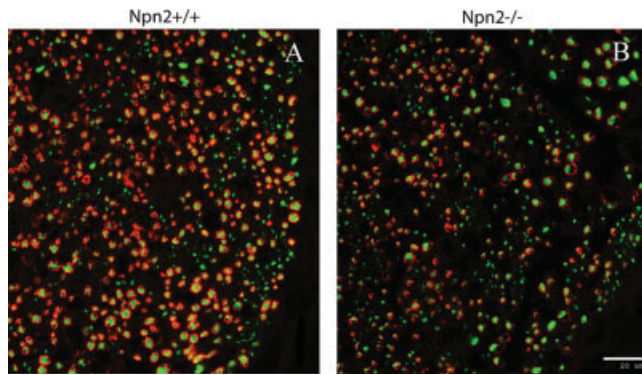


Fig. 2. Regeneration of myelinated axons 5 mm distal to sciatic nerve crush is delayed in $Npn2^{-/-}$ mice. **A, B:** Dual-wavelength indirect immunofluorescence images of tibial nerves 2 weeks post-sciatic nerve crush in an $Npn2^{+/+}$ mouse and an $Npn2^{-/-}$ mouse [red, myelin basic protein (MBP); green, neurofilament-light (NF-L)]. Note that there are more well-myelinated fibers in the tibial nerve of the $Npn2^{+/+}$ mouse than in that of the $Npn2^{-/-}$ mouse. The g ratio (axon:total fiber diameter; Lee et al., 2007) was significantly lower ($P < 0.01$, two-tailed Student *t* test) in the $Npn2^{+/+}$ nerve (**A**; mean \pm SD, 0.45 ± 0.09 ; $n = 127$) than in the $Npn2^{-/-}$ nerve (**B**; mean \pm SD, 0.57 ± 0.11 ; $n = 79$). Magnification and confocal microscope settings were the same for both fields. Scale bar = 20 μ m.

nerve crush, myelinated axons were visualized by indirect immunofluorescence microscopy, using primary antibodies against MBP and NF-L. One week following the crush, no intact myelinated axons were immunohistologically demonstrable in sciatic nerves below the crush site in either $Npn2^{+/+}$ or $Npn2^{-/-}$ mice (data not shown). By 2 weeks postcrush, myelinated axons had reappeared distal to the crush in both the $Npn2^{+/+}$ and the $Npn2^{-/-}$ mice. At this time, the sciatic nerves of the $Npn2^{+/+}$ mice had 24% more myelinated axons than did the $Npn2^{-/-}$ mice (Table I and Fig. 2). To learn whether $Npn2$ deficiency caused redistribution of axons between sciatic nerve branches, we examined tibial nerves 5 mm distal to the sciatic nerve crush in the same 2-week post-sciatic nerve crush $Npn2^{+/+}$ and $Npn2^{-/-}$ mice. In this case, too, there were 24% more myelinated axons in the $Npn2^{+/+}$ mice than in the $Npn2^{-/-}$ mice [905 ± 133 vs. 727 ± 59 (mean \pm SD), $n = 6$, $P < 0.02$].

Penetration of Regenerating Axons through Sciatic Crush Site Is Delayed in $Npn2^{-/-}$ Mice

The slower postcrush regeneration of myelinated axons and return to normal gait in $Npn2^{-/-}$ mice compared with their littermate controls could have resulted from a delay in either axonal penetration into the distal nerve segment or in remyelination of these regenerating axons. To distinguish between these possibilities, we visualized regeneration of NF-L+ axons 7 days following sciatic nerve crush in longitudinal sections of $Npn2^{+/+}$ and $Npn2^{-/-}$ sciatic nerves. In $Npn2^{+/+}$ mice, axons had extended through the crush site and

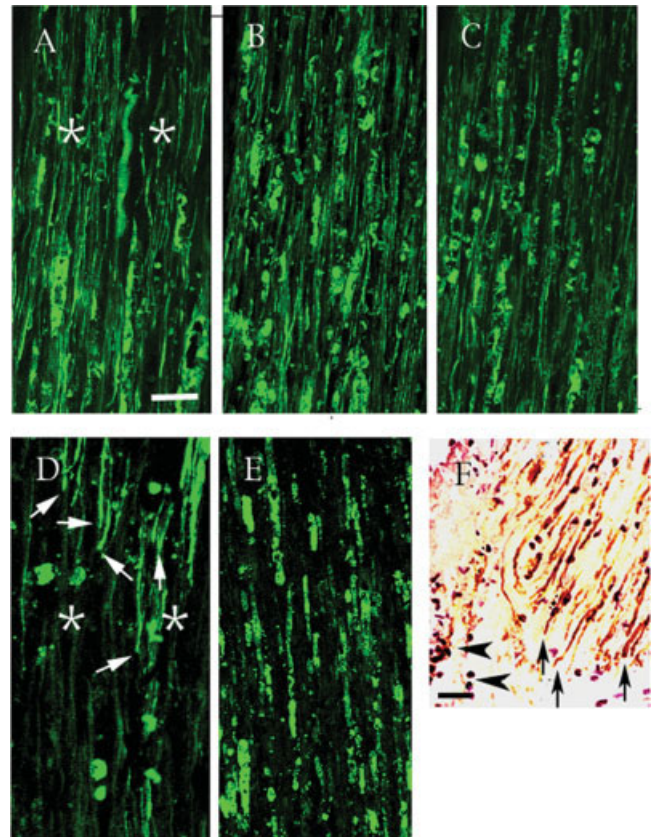


Fig. 3. NF-L immunostaining demonstrates delayed axonal penetration through the sciatic nerve crush zone in $Npn2^{-/-}$ mice. **A:** NF-L+ axons are shown in an $Npn2^{+/+}$ mouse at the crush site (upper limit of crush marked with asterisks). Scale bar = 30 μ m. **B, C:** Distances 2 and 3.5 mm, respectively, distal to the crush site in the same nerve. Note the presence of fine regenerating axons coursing through **A–C**. **D:** Crush site in an $Npn2^{-/-}$ mouse (upper limit of the crush marked with horizontal asterisks, arrows point to termination of axons within the field). **E:** Distance 2 mm distal to the crush site in the same nerve. Note the paucity of NFL+ axons in this field compared with in the corresponding field from the $Npn2^{+/+}$ mouse. Magnification and confocal microscope settings were the same for all fields. **F:** NF-L immunoperoxidase labeling at the top of a crush site in an $Npn2^{-/-}$ mouse (arrowheads mark charcoal particles at the edge of the crush site; arrows indicate axons terminating before penetrating through the crush site). Scale bar = 10 μ m.

several millimeters into the distal nerve segment in each of four 3-month-old 7-day post-sciatic nerve crush $Npn2^{+/+}$ male mice. In contrast, axons rarely penetrated through the crush site in any of the four 3-month-old $Npn2^{-/-}$ mice we examined (Fig. 3).

Axonal Down-Regulation of Schwann Cell p75^{NTR} Is Delayed in $Npn2^{-/-}$ Mice

Following axotomy, Schwann cells up-regulate expression of p75^{NTR}. When Schwann cells reestablish contact with axons, p75^{NTR} rapidly falls to undetectable levels (Taniuchi et al., 1986; Sobue et al., 1988; Scherer and Salzer, 2001; Zhou and Li, 2007). The suppression

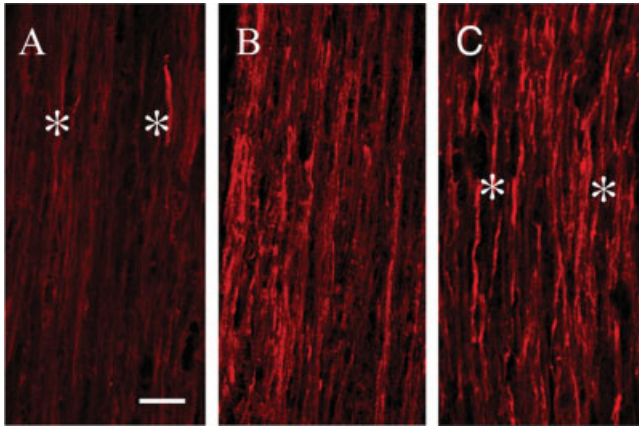


Fig. 4. Axonal down-regulation of Schwann cell p75^{NTR} distal to sciatic nerve crush is delayed in Npn2^{-/-} mice. **A:** p75^{NTR} Immunoreactivity from the sciatic nerve at the crush site in an Npn2^{+/+} mouse 1 week postcrush. This field corresponds to Figure 3A. **B:** p75^{NTR} Immunoreactivity 5 mm distal to the crush site in an Npn2^{+/+} mouse 1 week postcrush. This field corresponds to Figure 3B. **C:** p75^{NTR} Immunoreactivity at the crush site in an Npn2^{-/-} mouse 1 week postcrush. This field corresponds to Figure 3D. Note that p75^{NTR} immunoreactivity is diminished at and immediately below the crush site in the Npn2^{+/+} mouse but not in the Npn2^{-/-} mouse. The upper limits of the crush sites in the Npn2^{+/+} and Npn2^{-/-} nerves are indicated with asterisks in **A** and **C**. Scale bar = 10 μ m for **A**.

of Schwann cell p75^{NTR} can therefore be used as an index of reestablishment of functional communication between regenerating axons and Schwann cells. One week post-sciatic nerve crush, Schwann cell-immunoreactive p75^{NTR} was substantially elevated in the tibial nerve distal to the sciatic crush site of both Npn2^{-/-} and Npn2^{+/+} mice (data not shown). At this point, however, expression of p75^{NTR} immunoreactivity in sciatic nerves at and just distal to the crush was substantially lower in Npn2^{+/+} mice than in Npn2^{-/-} mice (Fig. 4), indicating more rapid reestablishment of functional contact by regenerating axons with Schwann cells at and immediately below the sciatic nerve crush site in the Npn2^{+/+} mice than in the Npn2^{-/-} mice.

DISCUSSION

We have shown that axonal regeneration through a sciatic nerve crush site, suppression of Schwann cell p75^{NTR} by regenerating axons, axonal remyelination, and recovery of normal gait are delayed in constitutive Npn2^{-/-} mice compared with in their Npn2^{+/+} littermate controls. These results support the hypothesis that Npn2 facilitates PNS axonal regeneration.

There are at least three possible mechanisms by which systemic Npn2 deficiency might slow axonal regeneration. First, class 3 semaphorins induced in the epineurium and perineurium at the sciatic nerve crush site (Scarlato et al., 2003; Ara et al., 2004) may help to guide regenerating Npn2^{+/+} axons (Lindholm et al.,

2004), but not regenerating Npn2^{-/-} axons, into the distal nerve segment and suppress aberrant axonal regeneration and traumatic neuroma formation at the nerve injury site (Nguyen et al., 2002; Tyner et al., 2007). Because sciatic nerve crush would not be expected to disrupt the continuity of the connective tissue elements that guide axonal regeneration (Nguyen et al., 2002), we would anticipate that such aberrant axonal regeneration would not be a major phenomenon post-nerve crush, even in Npn2^{-/-} mice. However, electrophysiological, axon tracing, and transmission electron microscopic studies will be required to definitively evaluate this possibility.

Second, based on Npn2 antibody blocking studies that showed that Npn2 facilitates aggregation of Schwann cells that are not in contact with axons into longitudinal arrays (Ara et al., 2005), Schwann cell Npn2 deficiency may diminish bands of Bungner formation and/or Schwann cell support for axonal regeneration at and below the site of nerve axotomy (Tetzlaff, 1982; Son and Thompson, 1995; Wanner and Wood, 2002). Schwann cell orientation in nerve segments distal to the crush injury, visualized by p75^{NTR} immunohistochemistry (Fig. 4), did not obviously differ in Npn2^{+/+} and Npn2^{-/-} mice, but this observation does not rule out a more subtle deficiency in the trophic function of bands of Bungner in Npn2^{-/-} mice.

Third, Npn2 is a receptor for splice forms of vascular endothelial growth factor (VEGF) (Shraga-Heled et al., 2007; Staton et al., 2007), as well as for class 3 semaphorins, and new capillary formation in the retina in response to ischemia has been shown to be suppressed in Npn2-deficient mice (Shen et al., 2004). Hence, it is possible that endothelial cell Npn2 deficiency diminishes the prominent intraneural neovascularization that normally takes place early after a mouse sciatic nerve crush injury (Pola et al., 2004) and thereby retards axonal regeneration.

Currently available evidence supports the concept that Semaphorin 3A induced at sites of CNS injury obstructs regeneration of Npn1-expressing CNS axons (Pasterkamp et al., 1999; De Winter et al., 2002; Kaneko et al., 2006). This phenomenon is not limited to the CNS because Semaphorin 3A, expressed either endogenously by lens epithelial cells or transfected into adult corneas, also repulsed the Npn1-expressing axons of trigeminal sensory ganglion neurons (Tanelian et al., 1997; Lwigale and Bronner-Fraser, 2007), and knockdown of Npn1 expression in an immortalized line of neurons (F11 cells) enhanced the capacity of their neurites to grow on sections prepared from Semaphorin 3A-expressing human neuroomas (Tannemaat et al., 2007). No comparable studies of the effects of Npn2 and its ligands on PNS axonal regeneration have been reported, although it has been observed that homozygous disruption of the genes encoding either Npn2 or Semaphorin 3F disrupts the migration of neural crest cells (Gammill et al., 2006) and the development of peripheral and first, third, and fourth cranial nerves (Chen et al., 2000; Giger et al., 2000; Cloutier

et al., 2002, 2004; Walz et al., 2002, 2007). Further analysis of the mechanisms by which Npn2 facilitates axonal regeneration is likely to be best accomplished by cell type-specific deletion of Npn2 or Npn2 ligands. Such studies may suggest novel approaches for enhancing recovery after neural injury.

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REFERENCES

- Ara J, Bannerman P, Hahn A, Ramirez S, Pleasure D. 2004. Modulation of sciatic nerve expression of class 3 semaphorins by nerve injury. *Neurochem Res* 29:1153–1159.
- Ara J, Bannerman P, Shaheen F, Pleasure DE. 2005. Schwann cell-autonomous role of neuropilin-2. *J Neurosci Res* 79:468–475.
- Bagnard D, Thomasset N, Lohrum M, Puschel AW, Bolz J. 2000. Spatial distributions of guidance molecules regulate chemorepulsion and chemoattraction of growth cones. *J Neurosci* 20:1030–1035.
- Chen H, Bagri A, Zupicich JA, Zou Y, Stoeckli E, Pleasure SJ, Lowenstein DH, Skarnes WC, Chedotal A, Tessier-Lavigne M. 2000. Neuropilin-2 regulates the development of select cranial and sensory nerves and hippocampal mossy fiber projections. *Neuron* 25:43–56.
- Chen YY, McDonald D, Cheng C, Magnowski B, Durand J, Zochodne DW. 2005. Axon and Schwann cell partnership during nerve regrowth. *J Neuropathol Exp Neurol* 64:613–622.
- Cloutier JF, Sahay A, Chang EC, Tessier-Lavigne M, Dulac C, Kolodkin AL, Ginty DD. 2004. Differential requirements for semaphorin 3F and Slit-1 in axonal targeting, fasciculation, and segregation of olfactory sensory neuron projections. *J Neurosci* 24:9087–9096.
- Cloutier JF, Giger RJ, Koentges G, Dulac C, Kolodkin AL, Ginty DD. 2002. Neuropilin-2 mediates axonal fasciculation, zonal segregation, but not axonal convergence, of primary accessory olfactory neurons. *Neuron* 33:877–892.
- Cohen RI, Rottkamp DM, Maric D, Barker JL, Hudson LD. 2005. A role for semaphorins and neuropilins in oligodendrocyte guidance. *J Neurochem* 85:1262–1278.
- de Castro F, Hu L, Drabkin H, Sotelo C, Chedotal A. 1999. Chemoattraction and chemorepulsion of olfactory bulb axons by different secreted semaphorins. *J Neurosci* 19:4428–4436.
- De Winter F, Oudega M, Lankhorst AJ, Hamers FP, Blits B, Ruitenberg MJ, Pasterkamp RJ, Gispens WH, Verhaagen J. 2002. Injury-induced class 3 semaphorin expression in the rat spinal cord. *Exp Neurol* 175:61–75.
- Gammill LS, Gonzalez C, Bronner-Fraser M. 2006. Neuropilin 2/semaphorin 3F signaling is essential for cranial neural crest migration and trigeminal ganglion condensation. *J Neurobiol* 67:47–56.
- Giger RJ, Cloutier J-F, Sahay A, Prinjha RK, Levensgood DV, Moore SE, Pickering S, Simmons D, Rastan S, Walsh FS, Kolodkin AL, Ginty DD, Geppert M. 2000. Neuropilin-2 is required in vivo for selective axon guidance responses to secreted semaphorins. *Neuron* 25:29–41.
- Gu C, Limberg BJ, Whitaker GB, Perman B, Leahy DJ, Rosenbaum JS, Ginty DD, Kolodkin AL. 2002. Characterization of neuropilin-1 structural features that confer binding to semaphorin 3A and vascular endothelial growth factor 165. *J Biol Chem* 277:18069–18076.
- Guttmann-Raviv N, Shraga-H, Varshavsky A, Guimaraes-Sternberg C, Kessler O, Neufeld G. 2007. Semaphorin-3A and semaphorin-3F work together to repel endothelial cells and to inhibit their survival by induction of apoptosis. *J Biol Chem* 282:26294–26305.
- Hickey WF, Lee V, Trojanowski JQ, McMillan LJ, McKearn TJ, Gontas J, Gontas NK. 1983. Immunohistochemical application of monoclonal antibodies against myelin basic protein and neurofilament triple protein subunits. advantages over antisera and technical limitations. *J Histochem Cytochem* 31:1126–1135.
- Huber AB, Kania A, Tran TS, Gu C, De Marco Garcia N, Lieberam I, Johnson D, Jessell TM, Ginty DD, Kolodkin AL. 2005. Distinct roles for secreted semaphorin signaling in spinal motor axon guidance. *Neuron* 48:949–964.
- Insera MM, Bloch DA, Terris DJ. 1998. Functional indices for sciatic, peroneal, and posterior tibial nerve lesions in the mouse. *Microsurgery* 18:119–124.
- Kaneko S, Iwanami A, Nakamura M, Kishino A, Kikuchi K, Shibata S, Okano HJ, Ikegami T, Moriya A, Konishi O, Nakayama C, Kumagai K, Kimura T, Sato Y, Goshima Y, Taniguchi M, Ito M, He Z, Toyama Y, Okano H. 2006. A selective sema3A inhibitor enhances regenerative responses and functional recovery of the injured spinal cord. *Nat Med* 12:1380–1389.
- Kawasaki T, Bekku Y, Suto F, Kitsukawa T, Taniguchi M, Nagatsu I, Nagatsu T, Itoh K, Yagi T, Fujisawa H. 2002. Requirement of neuropilin 1-mediated Sema3A signals in patterning of the sympathetic nervous system. *Development* 129:671–680.
- Kitsukawa T, Shimizu M, Sanbo M, Hirata T, Taniguchi M, Bekku Y, Yagi T, Fujisawa H. 1997. Neuropilin-2, a novel member of the neuropilin family, is a high affinity receptor for the semaphorins Sema E and Sema IV but not Sema III. *Neuron* 19:995–1005.
- Kolodkin AL, Levensgood DV, Rowe EG, Tai YT, Giger RJ, Ginty DD. 1997. Neuropilin is a semaphorin III receptor. *Cell* 90:753–762.
- Lee JM, Tos P, Raimondo S, Fornaro M, Papalia I, Geuna S, Giacobini-Robecchi MG. 2007. Lack of topographic specificity in nerve fiber regeneration of rat forelimb mixed nerves. *Neuroscience* 144:985–990.
- Lindholm T, Skold MK, Suneson A, Carlstedt T, Cullheim S, Risling M. 2004. Semaphorin and neuropilin expression in motoneurons after intraspinal motoneuron axotomy. *NeuroReport* 15:649–654.
- Lwigale PY, Bronner-Fraser M. 2007. Lens-derived semaphorin3A regulates sensory innervation of the cornea. *Dev Biol* 306:750–759.
- Messersmith EK, Leonardo ED, Shatz CJ, Tessier-Lavigne M, Goodman CS, Kolodkin AL. 1995. Semaphorin III can function as a selective chemorepellent to pattern sensory projections in the spinal cord. *Neuron* 14:949–959.
- Mitchell KJ, Pinson KI, Kelly OG, Brennan J, Zupicich J, Scherz P, Leighton PA, Goodrich LV, Lu X, Avery BJ, Tate P, Dill K, Panglilan E, Wakenight P, Tessier-Lavigne M, Skarnes WC. 2001. Functional analysis of secreted and transmembrane proteins critical to mouse development. *Nat Genet* 28:241–249.
- Nguyen QT, Sanes JR, Lichtman JW. 2002. Pre-existing pathways promote precise projection patterns. *Nature Neurosci* 5:861–867.
- Pasterkamp RJ, Giger RJ, Ruitenberg M-J, Holtmaat AJGD, De Wit J, De Winter F, Verhaagen J. 1999. Expression of the gene encoding the chemorepellent semaphorin III is induced in the fibroblast component of neural scar tissue formed following injuries of adult but not neonatal CNS. *Mol Cell Neurosci* 13:143–166.
- Pola R, Arahamian TR, Bosch-Marce M, Curry C, Gaetani E, Flex A, Smith RC, Isner JM, Losordo DW. 2004. Age-dependent VEGF expression and intraneural neovascularization during regeneration of peripheral nerves. *Neurobiol Aging* 25:1361–1368.
- Pond A, Roche FK, Letourneau PC. 2002. Temporal regulation of neuropilin-1 expression and sensitivity to semaphorin 3A in NGF- and NT3-responsive chick sensory neurons. *J Neurobiol* 51:43–53.
- Raper JA. 2000. Semaphorins and their receptors in vertebrates and invertebrates. *Curr Opin Neurobiol* 10:88–94.
- Scarlatto M, Ara J, Bannerman P, Scherer S, Pleasure D. 2003. Induction of neuropilins-1 and -2 and their ligands, Sema3A, Sema3F, and

- VEGF, during Wallerian degeneration in the peripheral nervous system. *Exp Neurol* 183:489–498.
- Scherer SS, Salzer JL. 2001. Axon-Schwann cell interactions during peripheral nerve degeneration and regeneration. In: Jessen JR, Richardson WD, editors. *Glial cell development*. Oxford, UK: Oxford University Press. p 299–330.
- Semaphorin Nomenclature Committee. 1999. Unified nomenclature for the semaphorins/collapsins. *Cell* 97:551–552.
- Serini G, Valdembrì D, Zanivan S, Mortera G, Burkhardt C, Caccavari F, Zammataro L, Primo L, Tamagnone L, Logan M, Tessier-Lavigne M, Taniguchi M, Puschel AW, Bussolino F. 2003. Class 3 semaphorins control vascular morphogenesis by inhibiting integrin function. *Nature* 424:391–397 (erratum p 974).
- Shen JK, Samul R, Zimmer J, Liu H, Liang X, Hackett S, Campochiaro PA. 2004. Deficiency of neuropilin 2 suppresses VEGF-induced retinal neovascularization. *Mol Med* 10:12–18.
- Shraga-Heled N, Kessler O, Prahst C, Kroll J, Augustin H, Neufeld G. 2007. Neuropilin-1 and neuropilin-2 enhance VEGF₁₂₁ stimulated signal transduction by the VEGFR-2 receptor. *FASEB J* 21:915–926.
- Skarnes WC, Moss e, Hurtley SM, Beddington RS. 1995. Capturing genes encoding membrane and secreted proteins important for mouse development. *Proc Natl Acad Sci U S A* 92:6592–6596.
- Sobue G, Yasuda T, Mitsuma T, Ross AH, Pleasure D. 1988. Expression of nerve growth factor receptor in human peripheral neuropathies. *Ann Neurol* 24:64–72.
- Son Y-J, Thompson WJ. 1995. Schwann cell processes guide regeneration of peripheral axons. *Neuron* 14:125–132.
- Spassky N, de Castro F, Le Bras B, Heydon K, Queraud-LeSaux F, Bloch-Gallego E, Chedoctal A, Zalc B, Thomas JL. 2002. Directional guidance of oligodendroglial migration by class 3 semaphorins and netrin-1. *J Neurosci* 22:5992–6004.
- Staton CA, Kumar I, Reed MWR, Brown NJ. 2007. Neuropilins in physiological and pathological angiogenesis. *J Pathol* 212:237–248.
- Tanelian DL, Barry MA, Johnston SA, Le T, Smith GM. 1997. Semaphorin III can repulse and inhibit adult sensory afferents in vivo. *Nat Med* 3:1398–1401.
- Taniuchi M, Clark HB, Johnson EM Jr. 1986. Induction of nerve growth factor receptor in Schwann cells after axotomy. *Proc Natl Acad Sci U S A* 83:4094–4098.
- Tang XQ, Heron P, Mashburn C, Smith GM. 2007. Targeting sensory axon regeneration in adult spinal cord. *J Neurosci* 27:6068–6078.
- Tannemaat MR, Korecka J, Ehlert EME, Mason MRJ, van Duinen SG, Boer GJ, Malessy MJA, Verhaagen J. 2007. Human neuroma contains increased levels of semaphorin 3A, which surrounds nerve fibers and reduces neurite extension in vitro. *J Neurosci* 27:14260–14264.
- Tetzlaff W. 1982. Tight junction contact events and temporary gap junctions in the sciatic nerve fibres of the chicken during Wallerian degeneration and subsequent regeneration. *J Neurocytol* 11:839–858.
- Tyner TR, Parks N, Faria S, Simons M, Stapp B, Curtis B, Sian K, Yamaguchi KT. 2007. Effects of collagen nerve guide on neuroma formation and neuropathic pain in a rat model. *Am J Surg* 193:e1–e6.
- Walz A, Rodriguez I, Mombaerts P. 2002. Aberrant sensory innervation of the olfactory bulb in neuropilin-2 mutant mice. *J Neurosci* 22:4025–4035.
- Walz A, Feinstein P, Khan M, Mombaerts P. 2007. Axonal wiring of guanylate cyclase-D-expressing olfactory neurons is dependent on neuropilin 2 and semaphorin 3F. *Development* 134:4063–4072.
- Wanner IB, Wood PM. 2002. N-cadherin mediates axon-aligned process growth and cell-cell interaction in rat Schwann cells. *J Neurosci* 22:4066–4079.
- Zhou X-F, Li H-Y. 2007. Roles of glial p75NTR in axonal regeneration. *J Neurosci Res* 85:1601–1605.
- Zou Y, Stoeckli E, Chen H, Tessier-Lavigne M. 2000. Squeezing axons out of the gray matter: a role for slit and semaphorin proteins from midline and ventral spinal cord. *Cell* 102:363–375.