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Peripheral nerve regeneration is delayed in neuropilin 2-deficient mice

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Abstract

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 than control mice. Thus, neuropilin-2 facilitates peripheral nerve axonal regeneration.

Keywords

peripheral nervous system; axons; Schwann cells; semaphorins; sciatic nerve

Introduction

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; Guttmann-Raviv et al, 2007; Staton et al, 2007). These effects of class 3 semaphorins are transduced by target cell plasma membrane receptor complexes which contain neuropilin-1 (Npn1) and/or neuropilin-2 (Npn2). Npn1 is required for semaphorin-3A (Sema3A) signaling, Npn2 for signaling by Sema3F and Sema3B, and 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 mRNAs encoding the 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).

While 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 the *Npn2* ligands, *Sema3B*, *Sema3F*, and *Sema3C*, in epineurial and perineurial cells at the injury site (Scarlato 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 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.

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), were provided by W.C. Skarnes. These mice were backcrossed to a C57BL/6J background for at least 6 generations before use in our studies.

Quantitation of *Npn1* and *Npn2* mRNAs 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 mRNAs 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 pre-cooled with liquid nitrogen. Total RNA was then isolated using Qiagen RNeasy Mini reagent kits. First strand complementary DNA (cDNA) was synthesized from 1 µg portions of total RNA using Superscript II Reverse transcriptase (Invitrogen, San Diego) and oligo (dT)₁₈ primer (Invitrogen, San Diego). Assays for mouse *Npn1*, *Npn2*, and *GAPDH* mRNAs 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 µl containing 1X TaqMan Universal PCR Master Mix (Applied Biosystems), 250 nM of each primer and 200 nM probe. The thermal cycling conditions were initial denaturation at 95°C for 10 minutes followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing at 60°C for 1 minute. Each sample was tested in duplicate. Data were normalized by dividing the copy number of the target cDNA by the copy number of *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 1-2mm distal to the sciatic notch for 20 seconds with number 4 size forceps. The crush site was marked by gently squeezing with forceps dipped in sterile powdered charcoal for 5 seconds, followed by suturing the incision.

Footprint analysis

Gait was tested pre-operatively, and again 1, 2 and 3 weeks post-operatively. Walking lanes (4" × 48") were constructed from hardboard and placed on a strip of white, ink jet-coated paper (92 brightness, Amerigo, Cincinnati). 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 blind observers for the presence or absence of 3 evidences 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 anti-serum 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 anti-rat (for MBP) or rhodamine-conjugated donkey anti-rabbit 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 postfixed 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 35 min with chicken anti-NF-L (1:300, Chemicon), washed in PBS, then incubated with biotin-conjugated donkey anti-chicken (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 0.1 M 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 40x oil objective and a Leica laser scanning

confocal 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 express markedly reduced levels 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/1000 copies GADPH mRNA) was less than 3% of that in Npn2^{+/+} mice (9.55 ± 0.33 copies/1000 copies GADPH mRNA), with approximately half-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/1000 copies GADPH mRNA) than that in Npn2^{+/+} mice (4.17 ± 0.82 copies/1000 copies GADPH mRNA).

Adult Npn2^{-/-} mice, though 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 (3 month old male Npn2^{-/-} 25.9 ± 2.5 grams, vs 29.2 ± 2.7 grams for 3 month postnatal male Npn2^{+/+} mice (mean $\pm$ SD, $n=16$ in each group, $p<0.01$). Gaits of uninjured 3 month postnatal C57BL/6J Npn2^{-/-} and Npn2^{+/+} mice, assessed by footprint analysis, did not differ significantly, nor did the uninjured Npn2^{-/-} and Npn2^{+/+} mice differ in numbers of myelinated axons in their sciatic nerves (Table 1).

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 post-crush, footprints had returned to normal in 86% of the Npn2^{+/+} mice, but in only 19% of the Npn2^{-/-} mice (Table 1). Typically, most Npn2^{-/-} mice exhibited foot-dragging and unmeasurable toe spread and/or paw length at that time-point (Figure 1C). At 3 weeks post-crush, nearly all Npn2^{-/-} and Npn2^{+/+} mice had measurable footprints and no foot-dragging (Table 1).

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 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 two weeks post-crush, myelinated axons had reappeared distal to the crush in both the Npn2^{+/+} and Npn2^{-/-} mice. At this time-point, 24% more myelinated axons were present in the sciatic nerves of the Npn2^{+/+} than the Npn2^{-/-} mice (Table 1 and Figure 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. Here, also, there were 24% more myelinated axons in the Npn2^{+/+} than the Npn2^{-/-} mice (905 ± 133 vs 727 ± 59 , mean $\pm$ SD, $n = 6$, $p < 0.02$).

Penetration of regenerating axons through the sciatic crush site is delayed in Npn2^{-/-} mice

The slower post-crush regeneration of myelinated axons and return to normal gait in Npn2^{-/-} mice than in 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 discriminate 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 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 (Figure 3).

Axonal down-regulation of Schwann cell p75^{NTR} is delayed in Npn2^{-/-} mice

Following axotomy, Schwann cells upregulate expression of p75^{NTR}. When Schwann cells re-establish 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 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 time-point, however, expression of p75^{NTR} immunoreactivity in sciatic nerve at and just distal to the crush was substantially lower in Npn2^{+/+} than Npn2^{-/-} mice (Figure 4), thus indicating more rapid re-establishment of functional contact by regenerating axons with Schwann cells at and immediately below the sciatic nerve crush site in the Npn2^{+/+} than Npn2^{-/-} mice.

Discussion

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

There are at least 3 possible mechanisms by which systemic Npn2 deficiency might slow axonal regeneration. First, class 3 semaphorins induced in 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). Since 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. Electrophysiological, axon tracing, and transmission electron microscopic studies will be required, however, 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 band 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} immunohistology (Figure 4), were not obviously different in Npn2^{+/+} vs 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) (Staton et al, 2007; Shraga-Heled et al, 2007), as well as for class 3 semaphorins, and retinal new capillary formation 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 *Sema3A* 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, since *Sema3A*, 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 et al, 2007), and knock-down of *Npn1* expression in an immortalized line of neurons ("F11 cells";) enhanced the capacity of their neurites to grow on sections prepared from *Sema3A*-expressing human neuromas (Tannemaat et al, 2007). No comparable studies of the effects of *Npn2* and its' ligands on PNS axonal regeneration have been reported, though it has been observed that homozygous disruption of the genes encoding either *Npn2* or *Sema3F* disrupts the migration of neural crest cells (Gammill et al, 2007) 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 to enhance recovery after neural injury.

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Figure 1. Footprint analysis demonstrates delayed functional recovery following sciatic nerve crush in *Npn2*^{-/-} mice

Lane A - pre-crush footprints are normal in an *Npn2*^{-/-} mouse.

Lane B - normal footprints 2 weeks post-crush in an *Npn2*^{+/+} mouse.

Lane C - right foot-drag (arrowheads) 2 weeks post-crush in an *Npn2*^{-/-} mouse. Note also the impaired toe spread on the right.

Figure 2. Regeneration of myelinated axons 5 mm distal to sciatic nerve crush is delayed in *Npn2*^{-/-} mice

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

Figure 3. NF-L immunostaining demonstrates delayed axonal penetration through the sciatic nerve crush zone in *Npn2*^{-/-} mice

NF-L+ axons are shown in an *Npn2*^{+/+} mouse at the crush site (Panel A, with upper limit of crush marked with asterisks). Panel B and Panel C are respectively 2 mm and 3.5 mm distal to the crush site in the same nerve. Note the presence of fine regenerating axons coursing through Panels A, B, and C. Panel D shows the crush site in an *Npn2*^{-/-} mouse. The upper limit of the crush is marked with horizontal asterisks, and arrows point to the termination of axons within the field. Panel E is 2 mm distal to the crush site in the same nerve. Note the paucity of NFL+ axons in this field, in comparison to the corresponding field from the *Npn2*^{+/+} mouse. Magnification and confocal microscope settings were the same for all

fields; the size bar in A indicates 30 μm . Panel F shows NF-L immunoperoxidase labeling at the top of a crush site in an $\text{Npn2}^{-/-}$ mouse. In this panel, arrowheads mark charcoal particles at the edge of the crush site while arrows indicate axonsterminating before penetrating through the crush site. The scale bar in Panel F = 10 μm .

Figure 4. Axonal down-regulation of Schwann cell p75^{NTR} distal to sciatic nerve crush is delayed in Npn2^{-/-} mice

Panel A: p75^{NTR} immunoreactivity from the sciatic nerve at the crush site in an Npn2^{+/+} mouse 1 week post-crush. This field corresponds to Panel A of Figure 3. **Panel B:** p75^{NTR} immunoreactivity 5 mm distal to the crush site in an Npn2^{+/+} mouse 1 week post-crush. This field corresponds to Panel B of Figure 3. **Panel C:** p75^{NTR} immunoreactivity at the crush site in an Npn2^{-/-} mouse 1 week post-crush. This field corresponds to Panel D of Figure 3. 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 Panels A and C. The size bar in Panel A = 10μm.

Table 1

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

<u>Percentage of mice with normal gait*</u>	<u><i>Npn2^{+/+}</i> mice</u>	<u><i>Npn2^{-/-}</i> mice</u>
	(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)
<u>Myelinated axons/distal sciatic nerve ***</u>	<u><i>Npn2^{+/+}</i> mice</u>	<u><i>Npn2^{-/-}</i> mice</u>
	(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).