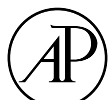
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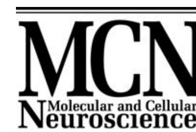

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Nogo-C is sufficient to delay nerve regeneration

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Abstract

Axonal regeneration succeeds in the peripheral but not central nervous system of adult mammals. Peripheral clearance of myelin coupled with selective CNS expression of axon growth inhibitors, such as Nogo, may account for this reparative disparity. To assess the sufficiency of Nogo for limiting axonal regeneration, we generated transgenic mice expressing Nogo-C in peripheral Schwann cells. Nogo-C includes the panisoform inhibitory Nogo-66 domain, but not a second Nogo-A-specific inhibitory domain, allowing a selective consideration of the Nogo-66 region. The *oct-6::nogo-c* transgenic mice regenerate axons less rapidly than do wild-type mice after mid-thigh sciatic nerve crush. The delayed axonal regeneration is associated with a decreased recovery rate for motor function after sciatic nerve injury. Thus, expression of the Nogo-66 domain by otherwise permissive myelinating cells is sufficient to hinder axonal reextension after trauma.
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Introduction

In a range of neurologic conditions, CNS axonal damage results in profound and persistent functional deficits. Regeneration of damaged CNS axons has been attributed both to limited expression of growth promoting proteins within axons (Skene, 1989; Neumann and Woolf, 1999; Bonilla et al., 2002) and to axon growth inhibitors in the CNS environment (David and Aguayo, 1981; Benfey and Aguayo, 1982; Bandtlow et al., 1990; Bregman et al., 1995; Fournier and Strittmatter, 2001; Bradbury et al., 2002). Nogo was identified as a CNS myelin-derived axonal regeneration inhibitor not found in peripheral myelinating Schwann cells (Chen et al., 2000; GrandPre et al., 2000; Prinjha et al., 2000; Huber et al., 2002). Antibody perturbation and receptor antagonist studies have suggested a role for Nogo in restricting CNS axon reextension (Bregman et al., 1995; GrandPre et al., 2002).

The *nogo* gene encodes three isoforms; all contain a 66-aa extracellular domain that interacts with a cell surface Nogo receptor (Fournier et al., 2001). The predominant form in oligodendrocytes, Nogo-A, also contains a domain with separate inhibitory activity (Fournier et al., 2001). The relative role of these domains and the sufficiency of Nogo for limiting axonal regeneration have not been examined by in vivo genetic methods. Other in vitro myelin-derived growth inhibitors also bind to NgR (Domeniconi et al., 2002; Liu et al., 2002; Wang et al., 2002a), but their relative role in vivo remains unclear (McKerracher et al., 1994; Mukhopadhyay et al., 1994; Bartsch et al., 1995; Schafer et al., 1996). In addition to these myelin-derived inhibitors, chondroitin sulfate proteoglycan-laden glial scars limit axonal regeneration (Davies et al., 1999; Bradbury et al., 2002). Clearly, there is a need for in vivo genetic assessment of the role of Nogo-66 in restricting axon regeneration. Here, our analysis of peripheral nerve regeneration in transgenic mice expressing Nogo-C in permissive Schwann cells demonstrates that the Nogo-66 domain is competent for blocking axon regeneration.

Results

There is little or no endogenous *nogo* expression in Schwann cells of the intact adult peripheral nervous system (GrandPre et al., 2000). After sciatic nerve transection, high stringency in situ hybridization with a panisoform specific *nogo* probe reveals little or no expression in distal nerve

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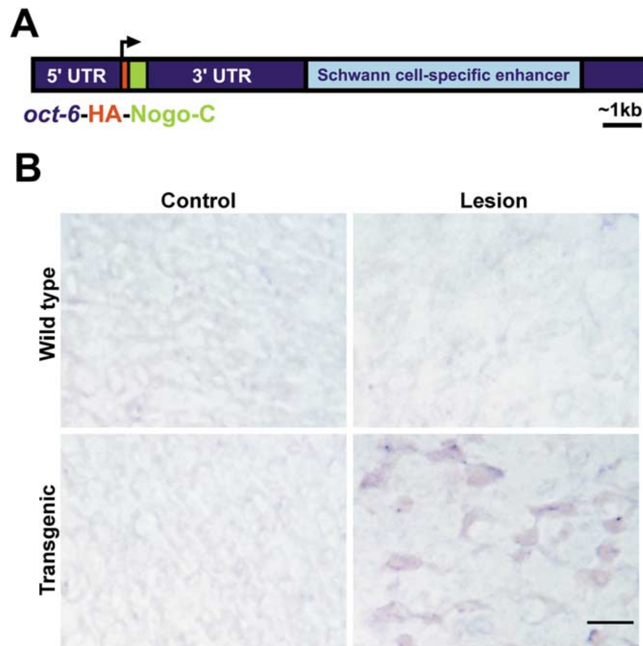


Fig. 1. Transgenic expression of *HA-nogo-c* in Schwann cells. (A) Diagram of the transgene. The *oct-6* elements, the HA epitope tag, and the rat *nogo-c* coding region are illustrated. (B) In situ hybridization for *nogo-c* in the transverse sciatic nerve sections from wild-type or *oct-6::nogo-c* (transgenic) mice. Tissue was collected from an uninjured nerve (control) and from a nerve segment 5 mm distal to a crush injury 7 days postinjury (lesion). Reaction with anti-sense probe is illustrated; the sense probe yielded no hybridization. Scale bar, 50 μm.

segments (Fig. 1B). This contrasts with strong CNS expression in oligodendrocytes detected by the same method (GrandPre et al., 2000). To express HA-tagged Nogo-C in Schwann cells we utilized promotor and enhancer elements from the *oct-6* gene (Fig. 1A). These regulatory elements are highly specific for Schwann cells and drive developmental expression followed by down-regulation in adults (Mandemakers et al., 1999). However, Wallerian degeneration after nerve injury is associated with strong reinduction of Oct-6 in Schwann cells. The predicted transgene expression pattern should allow an assessment of Nogo-C selectively in the axon regeneration process, minimizing any potential role in adult myelin or axon maintenance. We established three independent *oct-6::nogo-c* lines by pronuclear DNA injection. Expression of *nogo-c* in transgenic sciatic nerve becomes detectable in the sciatic nerve distal to a crush injury site (Fig. 1B), as shown for the endogenous Oct-6 protein (Mandemakers et al., 1999).

To detect the presence of Nogo-C protein, the epitope tag was assessed by anti-HA immunoblot and immunohistology (Fig. 2). The distal stump of a transected nerve lacks neurofilament protein due to Wallerian degeneration, but shows upregulated HA-Nogo-C of the expected molecular size, with similar expression in three transgenic lines (Fig. 2A). The selective up-regulation of HA-Nogo-C in transgenic Schwann cells distal to a nerve crush injury is also observed by immunohistologic studies (Fig. 2B). The identity of the cells expressing the HA epitope was verified by Schwann cell culture from the transgenic animals (Fig. 2C). Spindle-shaped cells in these cultures are immunoreactive for the Schwann cell marker, S100β (data not shown), and express HA-Nogo-C throughout their processes (Fig. 2C). Nogo protein has the potential to localize both to the plasma membrane as in oligodendrocytes and to the endoplasmic reticulum as in COS-7 cells (GrandPre et al., 2000; Wang et al., 2002b). High-magnification confocal images of the distal nerve segment after crush injury reveals HA-Nogo-C at the cell periphery, demonstrating relatively greater association with the Schwann cell plasma membrane as opposed to the endoplasmic reticulum (Fig. 2D). Double labeling for filamentous actin confirms that Nogo-C is not distributed uniformly throughout the cytoplasm of the transgenic Schwann cell. Thus, Nogo-C is situated so that it might influence regenerating nerves. While a quantitative comparison to endogenous Nogo levels is limited by the use of different immunologic methods, the Nogo-C level in the distal segments of injured nerves appears to be similar to or less than physiologic Nogo-A levels in CNS oligodendrocytes (Wang et al., 2002b).

Prior to assessing a role for Nogo-C in limiting axonal regeneration, we considered whether nerve fibers or their myelination might be altered in the peripheral nerves of transgenic mice due to developmental Nogo-C expression. Toluidine blue-stained semi-thin sections of *oct-6::nogo-c* sciatic nerve reveal an axon caliber, axon density, and myelin structure that is indistinguishable from that of wild-type mice (Fig. 3A). After nerve injury, the distal nerve stump undergoes a reactive process that actively clears degenerating myelin and axonal debris and is accompained by Schwann cell proliferation. The reactive changes in nerve segments distal to a sciatic nerve injury of transgenic animals are identical to those in littermate control mice (Fig. 3A and B). Thus, injury-regulated Schwann cell expression of Nogo-C does not alter peripheral nerve morphology or Wallerian degeneration.

If peripheral nerves are to be responsive to Nogo-C, then they would be expected to express some level of its receptor, NgR. Previously, we demonstrated that cultured embryonic day 13 chick dorsal root ganglion (DRG) neurons do express NgR and respond to Nogo-66 in vitro (Fournier et al., 2001). Immunohistologic localization demonstrates that mouse NgR is expressed in both motoneuron and DRG cell bodies after injury and is transported through regenerating axons past the lesion site (Fig. 4A; Wang et al., 2002b). Expression in wild-type and uninjured specimens (data not shown) was indistinguishable from that in injured transgenic animals. Thus, the NgR is situated in a fashion to receive an ectopic Nogo-66 signal from Schwann cell Nogo-C in the transgenic mice. This peripheral expression of NgR at sites without endogenous Nogo may reflect both the fact that peripheral sensory and motor neurons also have central processes where NgR may exert a physiologic role

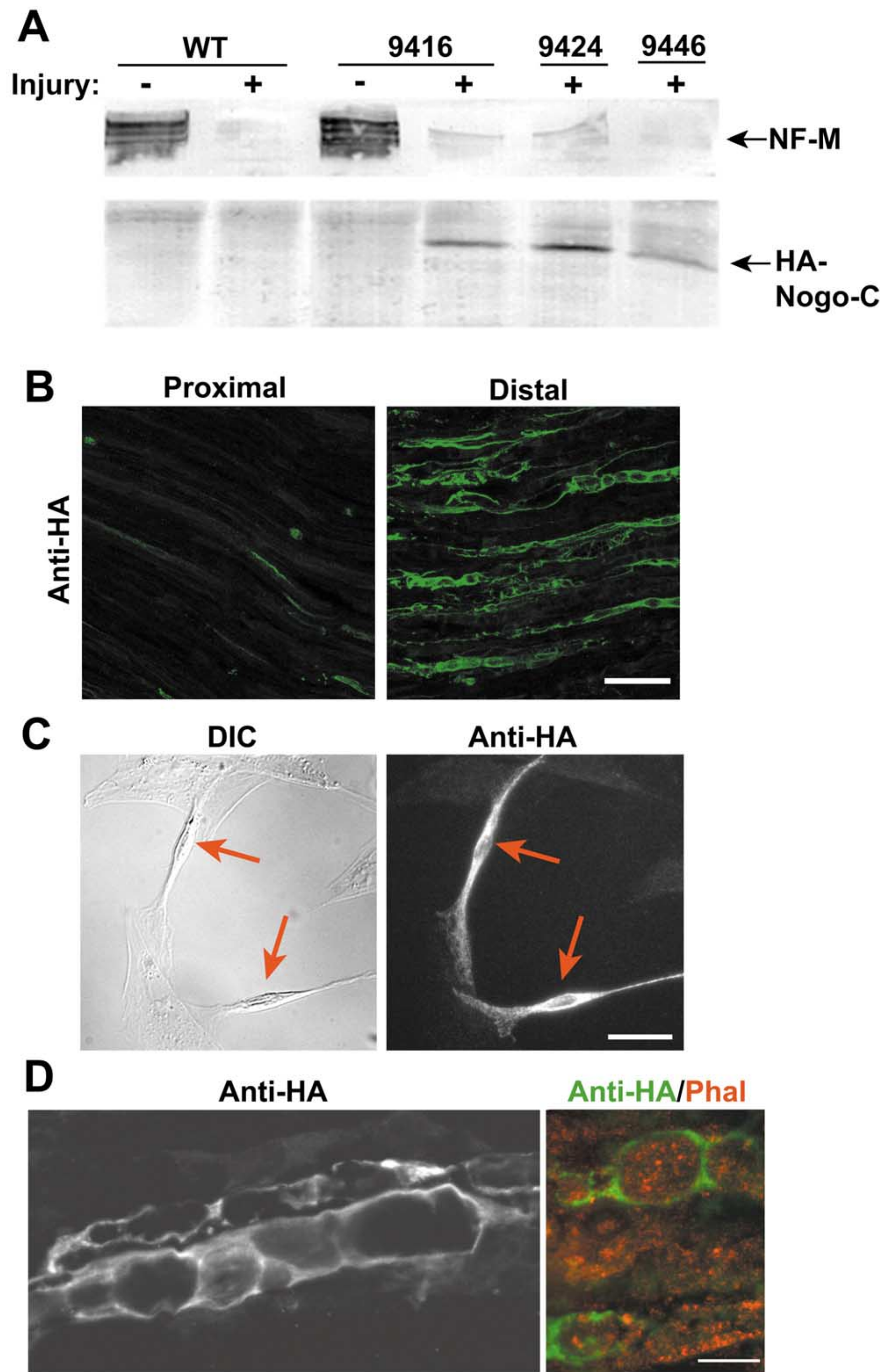


Fig. 2. Transgenic Nogo-C protein in Schwann cells. (A) Immunoblot for HA-Nogo-C in *oct-6::nogo-c* (NC) mice. Expression in uninjured sciatic nerves and in the distal segment of the nerve from three independent strains at 7 days posttransection is probed. Immunoblot for neurofilament medium chain (NF-M) demonstrates the degeneration of nerve fibers in the distal segment. (B) Anti-HA localization of transgene expression. Segments of sciatic nerve 3 mm proximal or distal to a crush injury are stained for HA-Nogo-C 4 days postinjury. Scale bar, 50 μm. (C) Localization of transgenic Nogo-C in cultured Schwann cells. Schwann cells cultured from *oct-6::nogo-c* mice are visualized in a DIC image as spindle-shaped cells (left, arrows), while fibroblasts have a flattened morphology. Anti-HA immunohistology (right) demonstrates the distribution of the transgenic protein. Scale bar, 50 μm. (D) HA-Nogo-C distribution in Schwann cells. Confocal images of the sciatic nerve 2 mm distal to a crush injury 4 days previously. On the left, the HA antigen is visualized predominantly as a rim at the plasma membrane of cells with the appearance of Schwann cells. On the right, double staining for Nogo-C (anti-HA, green) and filamentous actin (phalloidin, red) demonstrates that the cytoplasm containing actin has relatively little Nogo-C compared to the plasma membrane. Scale bar, 10 μm.

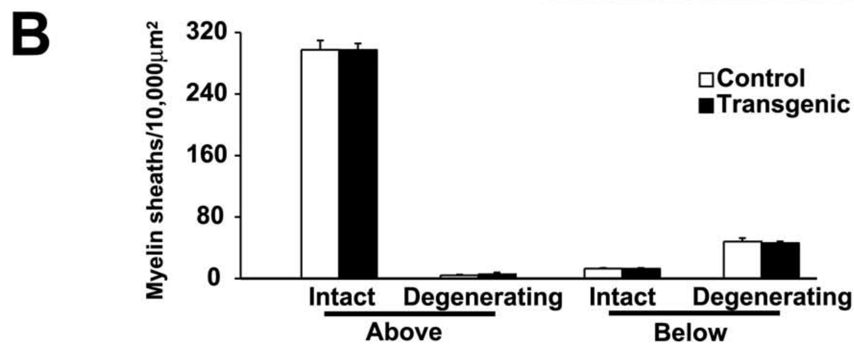
A

Wild type Transgenic

Proximal

Distal

B



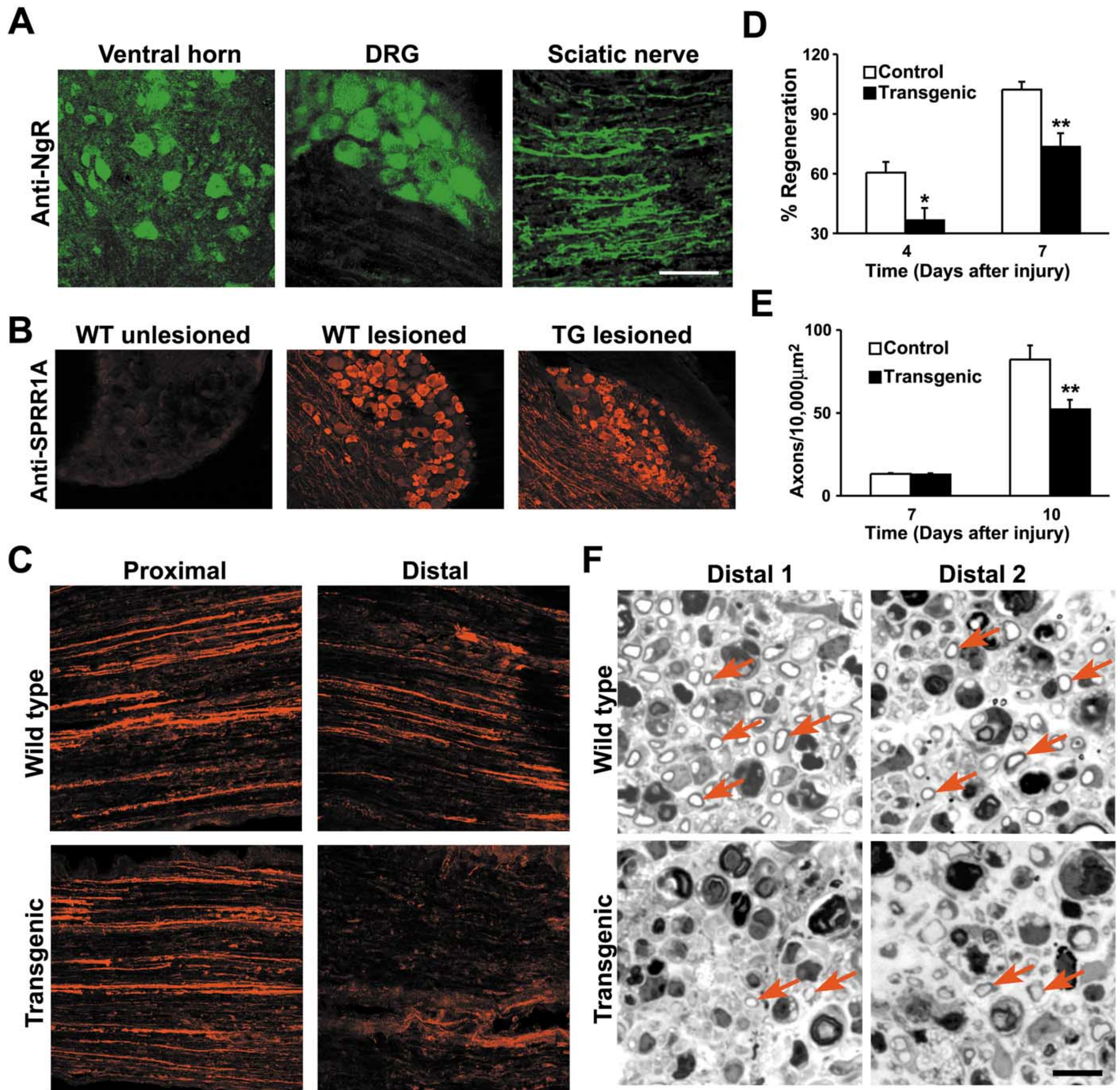


Fig. 4. Delayed axon regeneration in transgenic mice. (A) NgR expression in peripheral nerve. Anti-NgR immunohistology of lumbar ventral horn, DRG, and distal sciatic nerve (1 mm from injury), 4 days after sciatic nerve crush in a transgenic animal. (B) SPRR1A expression in L4–5 DRG with or without sciatic nerve injury in wild-type (WT) and transgenic *oct-6::nogo-c* (TG) mice. (C) Anti-SPRR1A staining of sciatic nerve 4 days postcrush at sites 2 mm proximal or 2 mm distal to the lesion. (D) Failure of axonal regeneration in transgenic mice. The number of SPRR1A-positive fibers 2 mm proximal and 2 mm distal to a crush injury were counted from sections as in C. The distal fiber count per section is normalized to the proximal fiber number for the same nerve. Proximal fiber counts were indistinguishable between groups. Mean ± SEM from six control mice and six transgenic mice, three from each of two independent lines. $*P \leq 0.01$; $**P \leq 0.0001$ compared to wild type (Student's *t* test). (E) Regenerating nerve profiles 5 mm distal to a nerve crush injury performed 10 days before in six control mice and six transgenic mice, three from each of two independent lines. Mean ± SEM. $**P \leq 0.0001$ compared to wild type (Student's *t* test). (F) Semi-thin sections of nerve 5 mm distal to a 10-day-old nerve crush. Examples of the regenerating axon profiles counted in (E) are illustrated (arrows) for two control mice and two transgenic mice from independent lines. Scale bar, 25 μm.

Fig. 3. Peripheral nerve structure and Wallerian degeneration in *oct-6::nogo-c* mice. (A) Semi-thin toluidine-blue-stained sections of uninjured sciatic nerve or the distal (5 mm) segment of sciatic nerve 7 days after crush injury. Distal segments with degenerating profiles (arrows) from two different animals of each genotype are illustrated. Scale bar, 25 μm. (B) The density of intact myelinated fibers and degenerating myelinated axon profiles is illustrated for wild-type and transgenic samples from 5 mm proximal or distal to a nerve crush injury 7 days previously. Mean ± SEM from six control mice and six transgenic mice, three from each of two independent lines.

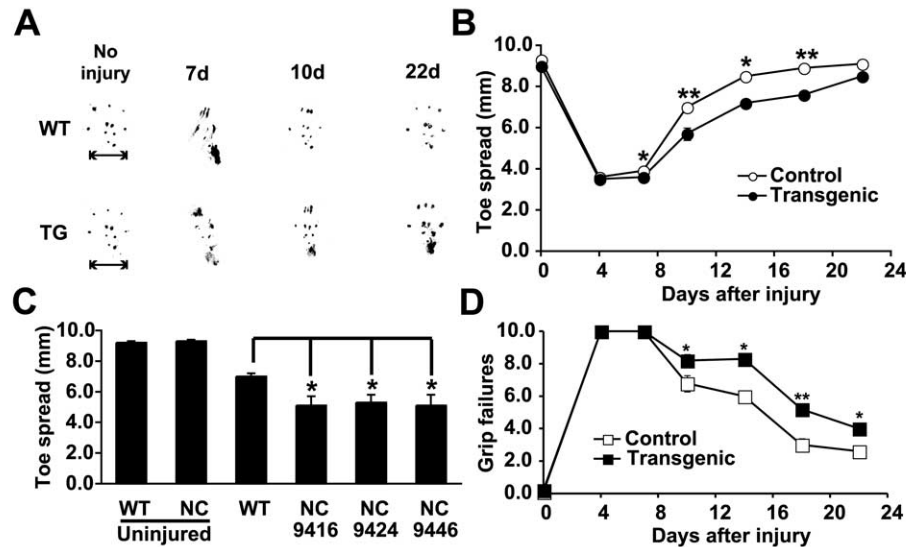


Fig. 5. Sciatic functional recovery after crush injury is delayed by Nogo-C. (A) Footprints of wild-type (WT) or *oct-6::nogo-c* (TG) mice at various times after sciatic nerve injury. In the first column, the toe spread distance is indicated. (B) Recovery of toe spread after sciatic nerve crush. Most error bars are smaller than the symbols. (C) Delayed toe spread recovery in independent *oct-6::nogo-c* lines from the 10-day-postinjury time point. (D) Grip failures after sciatic nerve crush. Most error bars are smaller than the symbols. All data are represented as means ± SEM from 12 control mice and 12 transgenic mice, 3–5 from each of three independent lines. *$P \leq 0.05$ compared to wild type; **$P \leq 0.005$ compared to wild type (Student's *t* test).

and the possibility that there is a lack of specificity in NgR protein transport within one neuron.

To assess anatomical evidence for peripheral axon regeneration in the presence of Nogo-C, we made use of SPRR1A immunohistology. We have recently identified SPRR1A as a highly specific marker for regenerating peripheral axons (Bonilla et al., 2002). There is no detectable SPRR1A expression in uninjured nerves or DRG cell bodies (Fig. 4B). After sciatic nerve crush injury, SPRR1A is strongly induced in wild-type and transgenic L4–5 DRG cell bodies (Fig. 3B) and motor neurons (data not shown and Bonilla et al., 2002). In the sciatic nerve, SPRR1A labels equally the numerous regenerating fibers proximal to the crush in wild-type and *oct-6::nogo-c* mice (Fig. 4C). Distal to the injury, many fewer SPRR1A-positive fibers are observed in the transgenic mice (Fig. 4C and D). Four days postinjury, only a third of wild-type SPRR1A-positive axons have failed to regenerate through a distance of 2 mm or greater. In contrast, nearly twice that percentage of fibers have failed to reach the same distance in the presence of Schwann cell Nogo-C. By 7 days postinjury, regeneration of SPRR1A-positive fibers in wild-type animals is nearly complete to a distance of 2 mm. However, a significant fraction of axons have still failed to reach this distance in the *oct-6::nogo-c* transgenic environment. The extent of axon regeneration in the transgenic animals at 7 days resembles that in littermate control animals at 4 days. Thus, the sciatic nerve fiber regeneration rate is slowed by a factor of 2 with this level of Nogo-C.

Anatomical evidence for nerve regeneration was confirmed by SPRR1A-independent morphometry of thin sections. At 7 and 10 days after injury, the density of regenerating fiber profiles at a distance 5 mm distal to a crush lesion was scored (Fig. 4E and F). In sections from wild-type mice, few regenerating fibers are detectable at 7 days, but a fiber density approximately 30% that of the uninjured nerve (compare Fig. 3B) is found by 10 days postcrush. The wild-type nerve regeneration time course appears slower than that observed by the SPRR1A staining method because samples were analyzed 5 mm distal, rather than 2 mm distal to the injury. In transgenic samples, there is a significant reduction in the density of regenerating axons compared to wild-type values in the distal nerve segment. Thus, two methods verify reduced axon regeneration in the transgenic *oct-6::nogo-c* mice during the subacute period after sciatic nerve crush.

Delayed axonal regeneration is expected to produce a more prolonged period of hindlimb dysfunction after sciatic nerve injury. We assessed two measurements of sciatic nerve activity in the affected hindlimb, toe spread and grip strength (Fig. 5). Both of these measures exhibit significant declines immediately after sciatic injury in control mice and then recover progressively from 7 to 22 days postinjury. In the *oct-6::nogo-c* animals, the deficit at days 4–7 is identical to that in control animals (Fig. 5B and D). However, the recovery rate is significantly delayed, with a lag period of 4–8 days from control samples. Toe spread function is reduced in three independent *oct-6::nogo-c* lines (Fig. 5C), confirming that the delayed regeneration is not secondary to insertion site mutagenesis.

Discussion

This work demonstrates that Nogo-C is capable of delaying axonal regeneration rates in vivo. Peripheral axon

reextension after injury is reduced by transgenic Nogo-C expression in Schwann cells, but regeneration is not prevented, as in the wild-type CNS. In part, this may be due to the presence of other axon growth inhibitors in the CNS, such as chondroitin sulfate proteoglycans (Davies et al., 1999; Fournier and Strittmatter, 2001; Bradbury et al., 2002), oligodendrocyte myelin glycoprotein (Wang et al., 2002a), and persistent myelin-associated glycoprotein not cleared by phagocytic cells (McKerracher et al., 1994; Mukhopadhyay et al., 1994; Bartsch et al., 1995; Schafer et al., 1996). Alternatively, the fact that transgenic Schwann cell Nogo-C is induced only after injury in the distal segment, as opposed to constitutive oligodendrocyte Nogo-A expression throughout the CNS (Huber et al., 2002; Wang et al., 2002b), may explain the observation of slowed (but not absent) regeneration. It also remains possible that transgenic Nogo-C never reaches as high a concentration as does endogenous oligodendrocyte Nogo.

As this study was being prepared for publication, a transgenic Schwann cell Nogo-A expression study was published (Pot et al., 2002) with results remarkably similar to those presented here. While that study could not distinguish between Nogo-66 and amino-Nogo effects, the current work verifies that the region of Nogo containing Nogo-66 is active in delaying peripheral nerve regeneration. A comparison of axon regeneration in the two studies is limited by the fact that different promotors driving temporally and quantitatively different levels of Nogo expression were employed. However, one might speculate that the more pronounced delay in sciatic recovery in the study of Pot et al. (2002) compared to the current study is due to the additive actions of amino-Nogo and Nogo-66. Both studies are based on the finding that endogenous *nogo* expression is low in Schwann cells. This has been verified by in situ hybridization for both intact and regenerating nerves in this study. Reports of *nogo* expression in regenerating nerves (Hunt et al., 2002) might be explained by detection of very low *nogo* levels with sensitive radioactive methods or by cross-reaction of *nogo* probes with other *rtn* mRNAs that are known to be highly similar to *nogo* but do not inhibit axonal outgrowth (GrandPre et al., 2000).

The subcellular distribution of Nogo protein has been a subject of investigation since it determines the extent to which Nogo is exposed on the surface of intact myelinating cells and can contact axons. In transfected COS cells most of the protein is intracellular and localized to endoplasmic reticulum (GrandPre et al., 2000). In myelinating oligodendrocytes, a much greater fraction of the protein is associated with the plasma membrane and can be detected at the outer and inner surfaces of compact myelin (GrandPre et al., 2000; Huber et al., 2002; Wang et al., 2002b). This is more apparent for fully mature oligodendrocytes in vivo than for differentiating oligodendrocyte lineage cells in vitro. Here, transgenic HA-Nogo-C could be detected by virtue of the carboxyl epitope tag and is expressed in a predominantly intracellular endoplasmic reticulum pattern in Schwann cell lineage cells in tissue culture. A smaller percentage of the protein appears to be associated with the plasma membrane. Sections from regenerating distal nerve stumps containing Schwann cells in the process of remyelinating regenerating axons demonstrate a greater proportion of plasma membrane staining for Nogo-C.

Transgenic Schwann cell Nogo-C and endogenous oligodendrocyte Nogo are likely to act by exposing Nogo-66 to the NgR present on peripheral and central axons (Fournier et al., 2001; Wang et al., 2002b). Thus, methods to block ligand interactions at the Nogo-66 receptor are expected to promote CNS axon regeneration (GrandPre et al., 2002).

Experimental methods

In situ hybridization

Digoxigenin-labeled a riboprobes spanning the entire *nogo-c* coding region were hybridized to tissue sections as described previously (Goshima et al., 1995; GrandPre et al., 2000). Bound probe was detected with anti-digoxigenin antibody conjugated to alkaline phosphatase using nitroblue tetrazolium plus 5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) as substrate.

Generation of transgenic mice

HA-tagged rat *nogo-c* cDNA was subcloned into the *oct-6*-derived SCE-R3 expression vector containing the 4.3-kb Schwann-cell-specific enhancer element, a generous gift from Dr. Dies Meijer at Erasmus University Rotterdam (Mandemakers et al., 1999). This construct was linearized with *Eco*RI and was microinjected into embryos to generate transgenic mice. Transgene integration was verified using PCR and 17 founders were identified. Three founders with the highest level of transgene expression were backcrossed to C57BL/6 mice. Eight- to 10-week-old Nogo-C transgenic and wild-type littermates were used for all experiments.

Schwann cell culture

Neonatal transgenic and wild-type mice were sacrificed and Schwann cells cultured as described (Peulve et al., 1994). Individual cultures were genotyped by PCR amplification of DNA from reserved tissue and pooled according to genotype before replating on four-well chamber slides (Nalge Nunc) for immunocytochemistry.

Sciatic nerve crush and transection

Mice were anesthesized by injecting 2.5% avertin ip. The sciatic nerve was exposed at the midthigh region and crushed for 30 s using Dumont No. 5 forceps cooled in a dry ice/ethanol bath (Inserra et al., 1998). The crush was re-

peated at the same site to completely sever the axons. For transection injury and immunoblot analysis, the sciatic nerve was transected with microscissors and the distal free end was folded to prevent rejoining of the ends.

Immunoblotting and histology

Seven days after surgery, distal portions of sciatic nerve from the transected or nontransected side were homogenized in RIPA buffer (1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, PBS). Thirty micrograms of sciatic nerve lysate was separated by SDS–PAGE and blotted onto PVDF. To compare levels of transgene induction at 14 days postinjury in different founders, rabbit polyclonal antibodies against HA were used at 1:1000 (Santa Cruz Biotechnology). Immunoreactivity was visualized after incubation with biotinylated secondary antibodies and avidin–AP (Vector Laboratories) using NBT/BCIP substrates.

Four or 7 days after crush, mice were perfused transcardially with PBS followed by a 4% paraformaldehyde/PBS solution. Sciatic nerves and lumbar DRGs were dissected, flash-frozen in tissue embedding medium (TBS), and cryosectioned at 15 μm. Regenerating fibers and sensory neurons were examined with anti-SPRR1A (Bonilla et al., 2002) and anti-NgR (Wang et al., 2002b) antibodies. Rabbit polyclonal anti-HA antibodies were used at 1:200. For toluidine blue staining, sciatic nerves were postfixed overnight in 2.5% glutaraldehyde, 2.5% paraformaldehyde, PBS solution, and then dehydrated and embedded in plastic for microtome sectioning.

Quantitation of degenerating and regenerating axons

Toluidine-blue-stained sections were photographed with a 40X objective and a 10,000-μm^2 area was chosen at random for analysis. Irregular and darkly stained myelin profiles 4 to 10 μm in diameter were categorized as degenerating myelin sheaths. Circular profiles containing a lightly stained area between 2 and 6 μm in diameter were categorized as regenerating fibers. Counts of intact axons, degenerating profiles, and regenerating axons were all performed on sections from at least six control mice and six transgenic mice, with three from each of two independent lines. For each mouse, 20–200 profiles were counted for each animal.

Behavioral analysis

At various times after unilateral crush injury, mice were tested for return of hindlimb function on the crushed side by measuring toe spread and the ability to grip an inverted wire screen (Contreras et al., 1995). Toe spread was assessed by painting the hind feet with black pigment ink (Clearsnap) and allowing the mice to walk freely on a blank sheet of letter-sized paper. The distance between the first and fifth digits (outer toe spread) was measured. For the grip assay, mice were placed on a wire screen mesh, which was turned over to test the ability of the mice to correctly grip the screen with their hind paws. Each mouse was tested 10 times and the number of failed trials was recorded.

Acknowledgments

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