Nogo-A expressed in Schwann cells impairs axonal regeneration after peripheral nerve injury

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Nogo-A in peripheral nerve Schwann cells, we show that axonal regeneration and functional recovery are impaired after a sciatic nerve crush. Nogo-A thus overrides the growth-permissive and -promoting effects of the lesioned peripheral nerve, demonstrating its in vivo potency as an inhibitor of axonal regeneration.

Introduction
Lesioned central axons successfully regenerate over long distances into peripheral nerves transplanted into the brain or spinal cord (CNS), whereas cultured peripheral axons grown under optimal conditions strictly refuse to invade explants of adult CNS tissue, in particular CNS white matter (Schwab and Thoenen, 1985). This observation suggested for the first time the presence of specific neurite growth-inhibitory factors in the adult CNS (Schwab and Thoenen, 1985; Caroni and Schwab, 1988). Various bioassays pointed to a high molecular weight membrane protein (NI-250, IN-1 antigen) later purified and identified as Nogo-A (Caroni and Schwab, 1988; Chen et al., 2000). Subsequently, several proteins and proteoglycans with neurite growth-inhibitory activity were identified in CNS myelin, including myelin-associated glycoprotein and chondroitin sulfate proteoglycans (Qiu et al., 2000).

To investigate in vivo the inhibitory characteristics of Nogo-A, we generated transgenic mice expressing the rat nogo A gene under the inducible control of the Schwann cell–specific P0 promoter (unpublished data). P0 is the major structural protein of peripheral myelin, and previous studies demonstrated the usefulness and specificity of the P0 promoter for transgene expression in Schwann cells (Messing et al., 1992).

Results and discussion
Transgenic mice were generated in which a myc-tagged nogo A gene was expressed postnatally using the inducible rtTA2 system (Urlinger et al., 2000) under the control of a fusion promoter (P0Cx). The P0Cx fusion promoter is composed of Schwann cell–specific regulatory elements of the 1.1-kb rat P0 promoter and of the human connexin 32 gene (Cx32; Abel et al., 1999). The 1.1-kb P0 promoter alone was often used, but transgene expression was inconsistent (Feltri et al., 1999). For high expression in mice, a Schwann cell–specific intron from the Cx32 gene was added after the P0 promoter. By inducing the expression of nogo A only after birth, we could avoid possible effects of Nogo-A protein on the development of the PNS. Transgenic lines were made that express the rtTA2 or the responder, a bidirectional tetO promoter (pBI-3 construct; Baron et al., 1995) directing nogo A and lacZ transcription (Fig. 1 A). rtTA2 is a second generation rtTA system, which requires low doses of doxycycline for activation and has almost no background activity (Urlinger et al., 2000). To test the pBI-3–nogo A, we transfected it into HeLa cells stably transfected with a tTA construct, which activates transcription from tetO promoters in the absence of doxycycline (Gossen and Bujard, 1992). The cells expressed...
both β-galactosidase and nogo A when doxycycline was omitted from the medium (Fig. 1 B).

Several founder mouse lines were obtained from pBI-3-nogo A and P0Cx-rtTA2 constructs and the strongest expressing lines were used. Single transgenic mice were mated to produce the Nogo-A-expressing double transgenic mice. The system was not leaky because X-Gal staining was only found in sciatic nerves of doxycycline-treated double transgenic mice. Nogo-A expression was induced at birth by feeding the mothers with doxycycline. The efficiency and specificity of the promoter was assessed in three ways. (1) X-Gal staining of the sciatic nerve showed large numbers of labeled cells (Fig. 2 A). No labeling was present in the spinal cord (unpublished data). Immunohistochemistry for (2) Nogo-A and (3) myc showed Nogo-A– and myc-positive cells and myelin structures in sciatic nerves exclusively of the double transgenic animals under doxycycline. Nogo-A, colocalized with the specific marker of Schwann cells S-100, was found in the Schwann cell bodies and in the outer and inner loops of the myelin, similar to that described for Nogo-A in oligodendrocytes and in myelin of the CNS (Fig. 2 C) (Huber et al., 2002). Two Nogo-A transgenic mouse lines, Tg11 and Tg16, were selected and compared using the X-Gal staining; the mice of the Tg11 line showed stronger staining than those from Tg16 (Fig. 2 A). At least 20% of the Schwann cells in line Tg16 and >45% of the Schwann cells in line Tg11 expressed a high level of the transgene (Fig. 2, B and C). The overall level of expression of the transgene in the sciatic nerve (Tg11) was about one third that of endogenous Nogo-A in the optic nerve (CNS) of the same animals (unpublished data).

Modifying the components of peripheral myelin could disturb the myelin structure (Siconolfi and Seeds, 2001). At the light and electron microscopic level, the Schwann cells, axons, and myelin of Nogo-A–expressing peripheral nerves appeared normal (Fig. 3, A–C). The transgenic mice developed normally and their gait was normal, as indicated by footprint analysis (overlapping footprint patterns for control and transgenic mice). Motor coordination and balance were tested on the rotarod (Fig. 3 D) at 10 wk of age and no deficits were recorded in transgenic mice. The animals were also indistinguishable from control littermates (single transgenics for P0-rtTA or nogo A/lacZ only or wild types), using the narrow beam test (unpublished data).

To test the influence of Nogo-A expression on axonal regeneration in the PNS, freeze-crush lesions of the sciatic nerve on 8–12-wk-old mice were performed. All motor (and presumably sensory) axons were lesioned, as indicated by the failure of Fluorogold tracer to retrogradely label motoneurons when applied 0–2 d after the crush (n = 12; unpublished data). Neurological recovery was analyzed by the sciatic functional index (SFI)* (de Medinaceli et al., 1982), which characterizes hindlimb use and foot and toe positions, and by the toe pinch reflex (Siconolfi and Seeds, 2001), a simple sensory motor reflex (Fig. 4). The number of successfully regenerating motoneuron axons was then determined by a retrograde tracing and a direct immunostaining of the axons distal to the lesion (Sagot et al., 1998). For all behavioral tasks and operations, double transgenic mice (Tg) and control littermates were used.

*Abbreviations used in this paper: PFA, paraformaldehyde; SFI, sciatic functional index.

Figure 1. Regulated expression system for the nogo A transgene, and in vitro expression of β-galactosidase and nogo A–myc. (A) Strategy used to obtain Schwann cell–specific doxycycline-regulated transgene expression. Transgenic mice carrying the rtTA gene driven by the rat 1.1-kb P0 promoter fused to 320 bp of the connexin 32 fragment were crossed with mice carrying the bidirectional tetO promoter inducing nogo A–myc and lacZ transcription when doxycycline is added. (B) TetO-nogo A/LacZ transfected into tTA HeLa cells (expression is activated by removal of doxycycline in the tTA system) showed no X-Gal, myc, or Nogo-A staining when doxycycline was in the medium (+Dox). When doxycycline was withdrawn (−Dox), the cells showed expression of X-Gal, myc, and Nogo-A. Bar, 25 μm.
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having no transgene or a single transgene (P0Cx-rtTA2 or nogo AllacZ only), were used. All animals received doxycycline from birth until the end of the experiments.

The SFI evaluates crucial aspects of locomotion involving recovery of hindlimb sensory and motor function. Nonlesioned animals have an SFI of zero (±10). Both transgenic

Figure 2. In vivo expression of the transgene in the sciatic nerve. (A) X-Gal staining of a sciatic nerve whole mount of a 3-mo-old double transgenic mouse of Tg16 results in blue-labeled Schwann cell nuclei (nuclear localization signal preceding lacZ). Cross sections of 7-wk-old mouse sciatic nerves show more X-Gal staining in the Schwann cells of Tg11 than of Tg16. (B) Colocalization of myc with Nogo-A protein. At low magnification, cross sections of P25 sciatic nerves of control mice (Ctrl) show weak, mostly axonal (insert, thin arrow) staining of Nogo-A and no myc staining. In the transgenic animal (Tg11) the Nogo-A level is higher and Nogo-A is colocalized with myc (merged). The insert shows Nogo-A in axons and additionally in the outer myelin loops (arrowheads). The immunological signals for Nogo-A were stronger than those for the myc tag in this study. (C) Colocalization of myc with the Schwann cell marker S-100 and of myc with Nogo-A. In double immunolabeled sections of Tg11, S-100/myc (left) and Nogo-A/myc (right) are colocalized in the Schwann cell bodies (arrows), as well as in the outer (closed arrowheads) and inner (open arrowhead) loops of myelin, similar to the localization of Nogo-A in oligodendrocytes and CNS myelin (Huber et al., 2002). Bars: (A and B) 60 μm; (C) 20 μm.

Figure 3. Nogo-A transgenic animals have a normal sciatic nerve histology and normal motor behavior before lesion. (A) Semi-thin sections of sciatic nerve from a 7-wk-old control (Ctrl) mouse and an age-matched Nogo-A transgenic (Tg11) stained with toluidine blue show normally myelinated large and small caliber axons. Schwann cells (arrows) are present and normal. (B) Electron micrographs show no detectable cytoplasmic abnormalities in myelinating and nonmyelinating Schwann cells in Ctrl and Tg11. (C) No differences were seen for the myelin spacing of Ctrl and Tg11 Schwann cells. (D) Behavioral assessment. 10-wk-old mice (Ctrl, Tg11, and Tg16) were tested on a rotating rod (rotarod) on two consecutive days. No differences were found in the latency to fall off from the rotarod, neither in the acceleration test (left) nor in the fatigue test (right). The means (±SEM) of control (Ctrl; n = 23), line 11 (Tg11; n = 7), and line 16 mice (Tg16; n = 13) are presented. Bars: (A) 12 μm; (B) 1.2 μm; (C) 0.25 μm.
Two independent Nogo-A transgenic mouse lines show a decrease in behavioral recovery after a sciatic nerve crush as compared with controls. (A) Recovery of locomotor function after sciatic nerve crush determined by the SFI. Nonlesioned animals have an SFI of zero. 28 d after the lesion, functional recovery was poor and the deficit long lasting in Tg11, delayed and incomplete in Tg16, and complete in the control mice. Means (±SEM) of control (Ctrl; n = 10), line 16 (Tg16; n = 5), and line 11 (Tg11; n = 3) animals are shown. *, two-tailed Mann Whitney test, P < 0.05 compared with controls. (B) Recovery of the toe pinch reflex is delayed in Tg11 and Tg16. Histogram shows the time in days after sciatic nerve crush at which initial toe pinch reflex was elicited for toe 3, toe 4, and toe 5. Mice from both lines show a delay in recovery, which is more pronounced in Tg11 than Tg16. The means (±SEM) of control (Ctrl; n = 12), line 11 (Tg11; n = 4), and line 16 (Tg16; n = 5) animals are presented. *, two-tailed Mann Whitney test, P < 0.05 compared with control. (C) Table of the distribution of animals that showed toe pinch reflex response 30 d after the operation.

and control mice showed normal SFI values before the lesion. Sciatic nerve crush produced a massive disability manifested by a negative SFI that returned to zero as the sciatic axons regrew. Control mice recovered with a typical time course of 2–3 wk (Fig. 4 A), in line with published data (Chen and Bisby, 1993). For the two Nogo-A transgenic lines, recovery was significantly delayed. Tg11 mice showed an overall poor recovery with large deficits persisting 30 d after the lesion. SFI recovery of Tg16 mice was better than of Tg11, but still significantly slower and less complete than in the control mice (Fig. 4 A).

In the toe pinch reflex, before sciatic nerve crush, all mice showed equal sensitivity; a pinch of toe 3, 4, or 5 (tested separately) reliably induced a rapid retraction of the leg (digits 1 and 2 are innervated by the saphenous nerve and were therefore not tested). After the crush, the response was totally abolished in all animals. The time taken for the injured hindlimb to show any degree of a response to the stimulus was noted. Digit function recovery occurred in a medial to lateral direction in all mice (Fig. 4 B). The recovery was monitored over 30 d, and in many of the transgenic mice the sensitivity of the last two digits did not appear. The toe pinch reflex values from those animals were extrapolated from the recovery curve. The percentage of animals responding 30 d after the lesion was calculated (Fig. 4 C). For the digit 4 of control animals, 91% showed sensitivity (75% for digit 5) compared with 80% for Tg16 (60% for digit 5) and 25% for Tg11 (25% for digit 5) (Fig. 4 C). Thus, the Nogo-A transgenic mice showed a significant delay in the recovery of the toe pinch reflex.

Spinal motoneurons were retrogradely labeled with Fluorogold 7, 14, and 28 d after the crush from a site 7 mm distal to the lesion (Fig. 5, A and C). Fluorogold was chosen as a marker because it is a long-lasting and nondiffusible tracer that undergoes rapid retrograde axonal transport. Nonlesioned transgenic mice had the same number of motoneurons as wild-type mice. A time course with wild-type mice was established to determine the time at which the transgenic animals were to be traced after the lesion. By 5 d, no motoneuron axons had reached the injection site. Between 7 and 14 d, a gradual increase in the number of retrogradely labeled motoneurons was seen and the peak number of motoneurons was reached 17 d after the lesion. Therefore, 7 and 14 d after the lesion were chosen to analyze the motor axonal regeneration, and a later time point (28 d) to analyze axon numbers at a time when functional recovery in control animals was complete. 7 and 14 d after a sciatic nerve lesion, a significantly lower number of motor axons had grown 7 mm past the lesion in transgenic animals compared with control animals (Fig. 5 B), indicating a slower regeneration of the motoneuron axons. 28 d after the lesion, only a small difference was noted in both transgenic groups compared with the control group, indicating that almost all the axons had reached the injection site. The discrepancy between this last result and the behavioral data (Fig. 4) could be due to the fact that 28 d after the lesion, most of the motoneuron axons of the transgenic mice had reached the injection site 7 mm distal of the lesion but not the muscle target (~15 mm away from the lesion), or not the appropriate target. Correct locomotion is also dependent on regeneration and correct targeting of sensory axons, which were not studied yet.

Finally, regenerating axons in the sciatic nerve were directly assessed using GAP-43 and neurofilament (SMI-32) markers. GAP-43 stains mainly growing axons. SMI-32 recognizes nonphosphorylated neurofilaments, described as being more abundant than the phosphorylated form in regenerating axons of the lesioned sciatic nerve (Pestronk et al., 1990; Tsuda et al., 2000). 7 d after the lesion, the labeled axons were counted on cross sections 4 mm distal to the lesion on randomly photographed sections. In contrast to intact nerves, most of these regenerating fibers were thin, fine cali-
Figure 5. Retrograde tracing and regenerating axons. (A) 7, 14, and 28 d after a sciatic nerve crush, the nerve was cut 7 mm distal to the lesion and crystals of the retrograde tracer Fluorogold were applied to the cut nerve. (B) 7 and 14 d after the lesion, the number of retrogradely labeled motoneurons is significantly smaller in the nerves of control animals compared with control nerves of the transgenic lines (Tg11 and Tg16) compared with control of the retrogradely labeled motoneurons. (C) Cross sections (top) were photographed at 630× and all the GAP-43–labeled axons were counted. Histograms (bottom) show the ratio of the number of GAP-43–labeled axons to the number of nonphosphorylated neurofilament marker SMI-32. Cross sections through the ventral horn of the spinal cord show a smaller number of labeled motoneurons in Tg16 versus control (Ctrl) animals 14 d after the lesion. (D) Growing axons were directly labeled 4 mm distal to the lesion using antibodies against GAP-43 and the nonphosphorylated neurofilament marker SMI-32. The means (±SEM) are represented. * = unpaired two-tailed t test, P < 0.05 compared with control. Bars: (C) 25 μm; (D) 13 μm.

Materials and methods

Plasmids for construction of the transgenic mice

For the generation of rtTA2-expressing mice, a 1.1-kb P0 rat promoter followed by a 350-bp portion of the human connexin 32 gene (30 bp of the promoter, exon 1B, intron 1, and 20 bp of exon 2) was placed upstream of the full-length rtTA2 gene. A β-globin transcription termination signal was added to the 3’ end. To generate the tet-O–nogo A–lacZ construct, a bidirectional tetO promoter, consisting of a human cytomegalovirus minimal promoter linked to the tet operator sequences was used. A 3.5-kb DNA fragment encoding nogo A with myc and Xpress tags at the COOH terminus was placed downstream of the tetO promoter in pBl-3 (Baron et al., 1995) (Fig. 1).
AI. Restriction fragments containing each transgene were isolated from vector sequences and prepared for microinjection into fertilized oocytes.

Controlling the doxycycline-regulatable expression of nogo A in HeLa cells

HeLa cells, stably expressing the TTA construct, were transfected with pBl-3-nogoA construct using FuGENE 6 transfection reagent (Roche). 3 μg/ml doxycycline solution was added to the culture medium for 24 h. The cells were fixed for 15 min in 4% paraformaldehyde (PFA) and immunostained for myc and Nogo-A, and the activity of β-galactosidase was assessed. For β-galactosidase staining, the cells were incubated for 1 h at 37°C in a solution containing 1 mg/ml X-Gal (Roche), 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 0.1% Triton X-100, and 2 mM MgCl2 in PBS. For immunofluorescence, the cells were permeabilized with 0.1% Triton X-100 in PBS and blocked with 10% FCS. Mouse anti-myc antibodies (clone 9E10; Sigma-Aldrich) were incubated simultaneously with the rabbit anti–Nogo-A antiserum 472 (Chen et al., 2000) for 30 min at RT. Rabbit antibodies were visualized by anti–rabbit FITC-conjugated secondary antibody, and mouse antibodies by anti-mouse TRITC-conjugated secondary antibody (Jackson Immunoresearch Laboratories).

Mouse breeding and genotyping

All transgenic lines were created using the hybrid strain B6D2F1. For both constructs, founder lines were crossed for two to six generations into the inbred strain C57BL/6. Double transgenic mice were obtained from crosses between mice heterozygous for each transgene. The line with the strongest expression of the transactivator (Tg[POC-xrtTA2] 693 Zbz) and of two independent reporter lines (Tg[Nogo-LacZ] 728 and 732 Zbz) were crossed, yielding the double transgenic lines Tg11 and Tg16. The genotypes of the mice were determined by PCR analysis of genomic DNA isolated from mouse tails. Primers 5’-CCGGGCGACAGCGCTTACG-3’ and 5’-CCGAACTCACATGTCTAGACTGG-3’ were used to amplify a 600-bp fragment from the TTA2 construct, and primers 5’-CTCTGGT- CATCTGAGCCTGTG-3’ (first exon of nogo A) and 5’-ACAGCTGCTAC-TACGTGACCTG-3’ (second exon of nogo A) to amplify a 548-bp fragment from the cDNA for the tetO-nogo A construct. Transgene expression was induced at birth by replacing normal drinking water with 5% sucrose containing doxycycline (2 mg/ml). The mice were kept under doxycycline until the end of the experiments.

Whole mount sciotic nerve preparation and immunofluorescence microscopy

Sciotic nerves of control and nogo A transgenic animals were dissected and fixed by immersion in 4% PFA for 20 min on ice. For β-galactosidase staining, the whole sciotic nerve was incubated overnight at 37°C in X-Gal solution. After staining and photography, the tissue was washed in PBS, postfixed for 1 h in 4% PFA, and then frozen in the same block. Cryostat sections (20 μm) were cut transversally 4 mm distal to the lesion. For immunofluorescence, the sections from wild-type and transgenic animals were processed on the same slide by permeabilization with 0.1% Triton X-100, and then frozen in the same block. Cryostat serial sections (20 μm) were viewed under fluorescence illumination, and Fluorogold-labeled motoneurons, identified by size, shape, and location in the ventral horn, were counted on every section.

Regenerating axons. 7 d after surgery, sciatic nerves were removed, fixed as described above, and cut transversally 4 mm distal to the lesion. After permeabilization with ethanol/acetic acid (95:5; 15 min), the sections (15 μm) were stained with a rabbit antiserum against GAP-43 (Chemicon) or with a mouse neurofilament antibody, SMI-32 (Steinberger Monoclonals Inc.). Areas of 10 μm2 were randomly photographed from the two or three fascicles of each nerve at 630×, and all labeled axons were counted by two different, blinded observers.

References


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