Soluble Nogo Receptor Down-regulates Expression of Neuronal Nogo-A to Enhance Axonal Regeneration*□

Xiangmin Peng, Zhigang Zhou, Jian Hu, David J. Fink, and Marina Mata

From the Department of Neurology, University of Michigan, and Ann Arbor Veterans Affairs Healthcare System, Ann Arbor, Michigan 48109

Nogo-A, a member of the reticulin family, is present in neurons and oligodendrocytes. Nogo-A in central nervous system (CNS) myelin prevents axonal regeneration through interaction with Nogo receptor 1, but the function of Nogo-A in neurons is less known. We found that after axonal injury, Nogo-A is increased in dorsal root ganglion (DRG) neurons unable to regenerate following a dorsal root injury or a sciatic nerve ligation-cut injury and that exposure in vitro to CNS myelin dramatically enhanced neuronal Nogo-A mRNA and protein through activation of RhoA while inhibiting neurite growth. Knocking down neuronal Nogo-A by small interfering RNA results in a marked increase of neurite outgrowth. We constructed a nonreplicating herpes simplex virus vector (QHNgSR) to express a truncated soluble fragment of Nogo receptor 1 (NgSR). NgSR released from QHNgSR prevented myelin inhibition of neurite extension by hippocampal and DRG neurons in vitro. NgSR prevents RhoA activation by myelin and decreases neuronal Nogo-A. Subcutaneous inoculation of QHNgSR to transduce DRG neurons resulted in improved regeneration of myelinated fibers in both the dorsal root and the spinal dorsal root entry zone, with concomitant improvement in sensory behavior. The results indicate that neuronal Nogo-A is an important intermediate in neurite growth dynamics and its expression is regulated by signals related to axonal injury and regeneration, that CNS myelin appears to activate signaling events that mimic axonal injury, and that NgSR released from QHNgSR may be used to improve recovery after injury.

Myelin proteins, Nogo-A, myelin-associated glycoprotein (MAG),2 and oligodendrocyte myelin glycoprotein, play important roles in the failure of axonal regeneration after central nervous system (CNS) injury (1, 2). Nogo-A is expressed in oligodendrocytes and neurons at sites of axon-myelin interactions and in synaptic regions (3–7). Recombinant Nogo-A causes growth cone collapse and limits neurite extension in vitro through two growth-inhibitory domains that function independently. Nogo66 (amino acids 1–66) is active as a monomer and is neuron-specific, although two regions within the N-terminal domain, amino-Nogo and NiGΔ20, are inhibitory to neurons and non-neuronal cells (3–5, 8).

These myelin inhibitors bind the Nogo receptor, a family of glycosylphosphatidylinositol-anchored membrane glycoproteins (NgR1–3) that are highly expressed in neurons. NgR1 binds with high affinity to Nogo66 (8), whereas MAG preferentially binds NgR2 (9). NgR1 functions as the ligand-binding domain of a multicomponent receptor complex that may include the low affinity p75 neurotrophin receptor (p75NTR) and LINGO-1 (LRR and Ig domain-containing, Nogo receptor-interacting protein) or TROY as signaling transducers of neurite growth (10–12). In addition to its function as a neurite growth inhibitor, Nogo-A has been implicated in synaptic destabilization, and NgR1 engagement alters synaptic plasticity (13, 14). Despite conflicting results, transgenic mice lacking Nogo-A, Nogo-A and -B, or Nogo-A, -B, and -C, and mice lacking NgR1 have been reported to exhibit improved axonal regeneration after spinal cord injury (15–18). Pharmacological delivery of a Nogo inhibitory peptide (NEP-(1–40)) (19, 20), NgR1 gene-related peptide (21, 22), or antibodies to Nogo-A to rodents (25–27) or primates (28) enhances axonal growth after CNS injury.

Although Nogo-A was initially isolated from CNS myelin, in situ hybridization studies have shown that Nogo-A and NgR1 are expressed in most neurons (29, 30). In DRG neurons in vitro, Nogo-A appears in the plasma membrane by surface biotinylation studies and localized in growth cones and neurite branching points (31, 32). In adult sciatic nerve, Nogo-A is seen in fiber bundles and in regenerating sprouts after nerve injury (31). Neuronal Nogo-A has been found to play a role in the maintenance of inhibitory synapses by modulating the expression of synaptic anchoring molecules (14) and modulates growth cone motility (33). In this study, we explored the role of neuronal Nogo-A in axon regeneration in the lesioned adult peripheral nervous system and CNS. We found that neuronal Nogo-A is increased following axonal injury in vivo and by exposure to CNS myelin proteins and by exposure to CNS myelin proteins in vitro. Neuronal Nogo-A is regulated by RhoA and plays a role in the inhibition of neurite outgrowth. Nogo-soluble receptor (NgSR) expressed from the HSV-based vector inhibits RhoA activation by myelin and decreases neuronal Nogo-A in vitro. NgSR expressed in DRG neurons transduced by subcutaneous inoculation resulted in enhanced regeneration of primary afferents in the dorsal root.
and into the spinal cord with improved functional outcome. These studies demonstrate that interfering with Nogo-NgR1 interactions by NgSR blocks myelin inhibitory effect and enhances regeneration while altering transcriptional regulation of Nogo-A in neurons.

**EXPERIMENTAL PROCEDURES**

**Vector Construction**

The recombinant genomic HSV-based vector expressing NgSR was constructed as follows. A rat cDNA library (prepared from rat total RNA extracted from rat spinal cord by using TRIzol reagent (Invitrogen)) was used as template. The signal region and eight leucine-rich repeat domain of rat Nogo66 receptor were amplified using the primers NgSR forward, AAA GGA TCC ATG AAG AGG GCG TCC TGA GGA, and NgSR reverse, AAT GGA TCC TTA TTA TCA AGC ACA ACC CTC TAA GTC ACT. The NgSR PCR fragment was extracted by chloroform once at room temperature, and 2 μl of the chloroform-extracted NgSR PCR fragment was ligated into EcoRV sites of the pSTBlue-1 vector using a Perfectly Blunt cloning kit (Novagen, Madison, WI). This plasmid (pNgSR) was sequenced to ensure the correct reading frame.

Sphl-Sall (AflIII) (124,485–126,412) and BglII-Smal (StyI)-BglII (XhoI) (131,934–133,520) of HSV were cloned into plasmid SP72 (BglII-SphI). This plasmid, named SAB3S, is completely deleted for both copies of ICP4. An 850-bp BglII fragment of HCMV-BamHI-poly(A) was inserted into SAB3S. Only one orientation of the plasmid was selected for NgSR cloning, in which the HCMV promoter was ligated with Smal site, and the poly(A) was inserted with Sall. The latter plasmid was named HCMV-poly(A)/SAB3S-16. The entire open reading frame of NgSR was cut from pNgSR by BamHI, purified by gel electrophoresis, and cloned into BamHI-cut dephosphated HCMV-poly(A)/SAB3S-16. The orientation of the NgSR insertion was verified by restriction digestion analysis by BglII and Sall and followed by DNA sequencing. The HCMV-poly(A)/SAB3S-16 plasmid with the correct NgSR insertion was selected.

Two copies of the ICP4 gene were completely removed and replaced with HCMV-EGFP to construct the HSV recombinant UL41E1G6. This HSV recombinant is also deleted for ICP27. 0.3 million 7B cells in a 6-well plate were infected with about 50 plaque-forming units of UL41E1G6 and incubated at 37 °C for 1 h. One μg of HCMV-NgSR-poly(A)/SAB3S-16 plasmid was transfected into 7B cells using Lipofectamine 2000 (Invitrogen). After 5 days in vitro, the viral recombinant was harvested and sonicated, and 10 μl of the solution was used to isolate white viral plaques (deleted for both copies of HCMV-EGFP). Each round of purification was confirmed by Southern blot. Expression of NgSR from the vector was confirmed by infection of primary DRG neurons using immunocytochemistry, and release of the protein was determined by Western blot. The final isolate was propagated to high titer and purified on a Nycodenz (Nycomed) gradient.

**Tissue Culture and in Vitro Experiments**

DRG were removed from P10 rats and dissociated into single cells by incubation with 0.1% collagenase and 0.025% trypsin for 1 h at 37 °C. Neurons were dissociated and plated on poly-d-lysine- (100 μg/ml) and laminin (5 μg/ml)-coated plates with defined Neurobasal Medium containing B27, Glutamax I, Albumax I, and penicillin/streptomycin, according to the manufacturer’s recommendation (Invitrogen), and supplemented with 100 ng/ml 7.0 S nerve growth factor (Sigma). Cerebral cortex from E17 rat pups was removed and dissociated into single cells by incubation with 0.25% trypsin/EDTA (Sigma) for 15 min at 37 °C. Cortical neurons were plated on poly-d-lysine-coated 6-well plates with 2 ml of defined Neurobasal media with supplements as above. Both P10 DRG and E17 cortical neurons were transfected with either QHNgSR or QHGFP for 2 h and replaced with fresh medium. The cell lysates and medium for P10 DRG and cortical neurons were collected at 48 and 24 h, respectively, for determination of NgSR by Western blot. Medium from transfected cortical neurons was used as conditioned medium for the subsequent experiments. In some experiments, P10 DRG neurons were plated on increasing concentrations of myelin and treated with C3 transferase (Cytoskeleton) and Bt2cAMP (Sigma) at the time of plating and cultured for 24 h. Cell lysates were collected for Western blot analysis and RT-PCR.

**Myelin Isolation**

CNS myelin was prepared by sucrose gradient centrifugation as described (34). The purified myelin contained three bands consistent in size with the myelin inhibitory molecules, Nogo-A, MAG, and OMgp on 12% Tris-glycine gel.

**Neurite Outgrowth Assay**

Hippocampus was dissected from P1 rats, digested with 0.25% trypsin/EDTA at 37 °C for 15 min, dissociated into single cells, and plated with defined Neurobasal medium with containing B27, Glutamax I, Albumax I, and penicillin/streptomycin. P1 hippocampal neurons and P10 DRG, prepared as described above were plated in 8-well chamber slides at 2 × 10^4 cells per well. Conditioned medium was added at plating. At 20 h after plating, the cells were fixed and immunostained with an antibody against neuron-specific βIII tubulin (TuJ1, Covance). The length of neurites was determined using Meta Imaging software (Molecular Devices). More than 100 randomly selected individual neurons were analyzed per condition in each experiment, and the experiment was repeated three times.

**siRNA Preparation and Transfection**

ON-TARGET plus SMARTpool siRNA directed against Nogo-A were provided by Dharmacon (Chicago). The siRNA sequences used for Nogo-A were as follows: sequence 1, 5′-CGAUAUCACUUCAAGAAGA-3′; sequence 2, 5′-UUAGAAGAUCUCCGACAA-3′; sequence 3, 5′-GAAUGGACAGGUGUCA-3′; sequence 4, 5′-GAAUAAGGACUCGGGGAA-1′; ON-TARGET plus SMARTpool targeting pool siRNA(Dharmacon) was used as control. For siRNA transfection, 2.5 μl of siRNA in 47.5 μl of antibiotic-free cultured medium was combined with 2 μl of DharmaFECT siRNA transfection reagent 3 (Dharmacon) in 48 μl of antibiotic-free cultured medium, and the mixture was allowed to incubate for 20
min at room temperature. DRG neurons in vitro were incubated with the siRNA transfection solution for 48 h before cell lysis or immunocytochemistry.

**RT-PCR**

Total RNA was isolated from P10 DRG using TRIzol isolation reagent (Invitrogen) according to the manufacturer’s instructions. cDNA prepared from mRNA was amplified using following primer sets: β-actin forward, 5'-CAG TTC GCC ATG GAT GAC CAT-3', and β-actin reverse, 5'-CAC CTC CAG ACC TCA GGA TCT TCA TG-3'; Nogo-A forward, 5'-GAG ACC CTG TTT GCT CCT CTG G-3', and Nogo-A reverse, 5'-AAT GAT GGG CAA AGC TGT GCT G-3'. All reactions involved initial denaturation at 94 °C for 5 min followed by 24 cycles (94 °C for 30 s, 68 °C for 2 min, and 1 cycle 68 °C for 8 min using a GeneAmp PCR 2700 (Applied Biosystems)).

**Western Blot**

L4 and L5 dorsal root ganglia were dissected and homogenized in lysis buffer containing 50 mM Tris, 10 mM NaCl, 1% Nonidet P-40, 0.02% NaN₃, protease inhibitor, and phosphatase inhibitor mixtures (Sigma) at pH 7.4. Cultured P10 DRG neurons and E17 cortical neurons were collected in the same lysis buffer after being dislodged from the culture plates with a cell scraper. Cell lysates and tissue homogenates were sonicated and centrifuged at 15,000 × g for 10 min at 4 °C. Protein bands were visualized using a GeneAmp PCR 2700 (Applied Biosystems).

**Experimental Animals and Surgical Procedures**

Female Sprague-Dawley rats weighing 200–250 g were used in all experiments. Housing conditions and experimental procedures were approved by the University of Michigan Committee on Use and Care of Animals.

**Dorsal Root Injury**—Under isoflurane anesthesia, the left lumbar dorsal roots were exposed by a laminectomy. The L4 and L5 roots were crushed twice by forceps for 15 s at 1 cm distance from the spinal cord entry. Muscle and fascia were sutured closed, and the skin was closed with autoclips. Following surgery, all animals were maintained under preoperative conditions and were eating and drinking within 3 h after surgery. Thirty min after crush, animals were inoculated in the plantar surface of left hind paw with 30 μl of vector containing

**FIGURE 1.** Nogo-A (red) colocalized with TuJ1 (green)-stained P10 DRG neurons in vitro (a) and in normal adult lumbar DRG in vivo (b) is shown. Nogo-A protein in L4 and L5 DRG is markedly increased 1 week after sciatic nerve cut and ligation (c and d). Nogo-A level was significantly increased in L4 and L5 DRG 1 week after dorsal root crush near the DREZ as detected by Western blot (d and f), n = 3; *, p < 0.05. Scale bar, a = 40 μm; b = 50 μm.
Sciatic Nerve Injury—In other animals, under isoflurane anesthesia, the sciatic nerve was cut at the level of the upper thigh and just below the sciatic notch, and the proximal and distal nerve ends were ligated to prevent axonal regeneration, or the sciatic nerve was crushed for 30 s with a Dumont number 5 forceps, prior to suturing the muscles and closing the skin with autoclips. The contralateral sciatic nerve was kept intact, and a similar cohort of animals was used as uninjured controls. At 1 and 4 weeks post-surgery, the lumbar DRG were collected from the ipsilateral and contralateral sites for protein studies.

Behavioral Analysis—Grid walk was used to evaluate proprioception. The animals were placed on a 1-meter long horizontal runway of metal grid bars elevated 30 cm from the ground with regular gaps (4 cm). The number of errors of foot placing was counted in each crossing. Three crossings were counted per animal at each time point. All animals were pretrained in the task 1 week prior to surgery. Thermal sensory perception was determined by latency to hind paw withdrawal to radiant heat using a modified Hargreave’s testing device (35). After a 30-min habituation to a glass plate maintained at 30 °C, the plantar surface of the paw was exposed to a beam of radiant heat with a 20-s cutoff time. Testing was performed three times at 5-min intervals. Behavioral measures were determined weekly for the 6-week duration of the study by an observer blinded to treatment group.

Morphometric Analysis of Dorsal Roots—Rats were perfused intracardially with 0.5% glutaraldehyde and 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.4. Dorsal roots were dissected and kept in same fixative overnight at 4 °C. Under the dissecting microscope, 1-mm segments of L4 and L5 dorsal roots proximal to the crush injury and near the dorsal root entry into the spinal cord (distal to the crush) were taken as blocks. Tissues were rinsed in 0.1 m cacodylate buffer, dehydrated, osmicated, and embedded in Polybed (Ted Pella) resin. One-micron semithin sections were cut with a Reichert Ultracut and
stained with toluidine blue. Sections of the dorsal root proximal and distal to the injury site from three animals in each group were photographed digitally, and measurements of myelinated fiber density and size were determined using Meta Imaging software (Molecular Devices).

### Immunochemistry

Rats were perfused transcardially with 0.9% NaCl followed by freshly made 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.4. The DRG, L4–L5 segment of spinal cord, and attached roots were removed, post-fixed in the same solution for 8 h, and cryoprotected with 30% sucrose in 0.1M phosphate buffer for 2 days at 4 °C. Cryostat sections of DRG (15 μm) and L4–L5 spinal cord with attached roots (30 μm) were thaw-mounted onto cold Superfrost microscope slides (Fisher), blocked with 5% normal goat serum in PBS-T, incubated with rabbit anti-Nogo-A (1:100, Santa Cruz Biotechnology), anti-NogoR (1:500, Santa Cruz Biotechnology), anti-Tuj1 (1:5000, Covance), anti-CGRP (1:500 Peninsula Laboratories), and anti-CTB (1:500, Sigma) overnight at 4 °C followed by fluorescent anti-rabbit IgG Alexa Fluor 594 or anti-mouse IgG Alexa Fluor 488 (1:2000, Molecular Probes).

### Data Analysis

 Statistical significance of the difference between vector-treated and control animals was determined by one-way analysis of variance with post hoc comparisons where appropriate, and parametric statistics, using the general linear model for repeated measures, were used to identify significant effects of treatment conditions on the behavioral measure over time. All analyses were performed using SPSS 12.0 for Windows (SPSS Inc.). Data are expressed as means ± S.E., with *p* < 0.05 considered significant.

### RESULTS

### Neuronal Nogo-A Is Increased following Axonal Injury in Vivo and by Exposure to CNS Myelin Proteins in Vitro

— We observed high levels of Nogo-A protein in most postnatal DRG neurons in vitro and in adult DRG in vivo by immunocytochemistry (Fig. 1, a and b). We did not detect Nogo-A in Schwann cells or in satellite cells in vitro or in vivo in agreement with prior published observations (36). We examined Nogo-A levels following two different axonal injuries in which regeneration is blocked as follows: a sciatic nerve cut with ligation and a dorsal root crush near the DREZ. In both cases, we observed a marked increased in Nogo-A protein levels in DRG following injury (Fig. 1, c–f). In contrast, a minor and insignificant increase in Nogo-A protein in DRG occurred after a sciatic nerve crush injury that is permissive of regeneration (supplemental Fig. 1). These results suggest that neuronal Nogo-A is regulated by axonal injury. CNS myelin proteins have been implicated in the failure of axonal regeneration after injury and in neurite growth inhibition in vitro. We isolated myelin from young adult rat brain (33) stained with toluidine blue. Sections of the dorsal root proximal and distal to the injury site from three animals in each group were photographed digitally, and measurements of myelinated fiber density and size were determined using Meta Imaging software (Molecular Devices).

### Neuronal Nogo-A in Axonal Regeneration

FIGURE 3. Knocking down neuronal Nogo-A by siRNA enhances neurite outgrowth. P10 DRG transfected with Nogo-A siRNA for 24 h showed marked reduction in Nogo-A mRNA and protein as compared with scramble siRNA (a and b). P10 DRG cultured on CNS myelin (3 μg/well) in 8-well chamber slides transduced with Nogo-A siRNA for 48 h blocks the inhibition of neurite growth by myelin (c and d). P10 DRG was stained with Tuj1 (green) (c). *, *p* < 0.05. Scale bar, 100 μm.
to be consistent within a single animal species in our experiments, and we observed a marked increase of neuronal Nogo-A mRNA and protein in DRG neurons exposed to CNS myelin in vitro (Fig. 2, a and b), a transcriptional response similar to that we found in DRG neurons after axonal injury in vivo.

Neuronal Nogo-A Is Decreased by RhoA Inhibitor, Neurotrophic Factor, and Bt2cAMP—RhoA is the major downstream inhibitory molecule mediating myelin inhibition of neurite extension through activation of the NgR1. Inhibition of RhoA and its downstream kinase ROCK have been found to enhance neurite growth (37, 38). To explore the mechanism underlying up-regulation of neuronal Nogo-A by CNS myelin, we used the cell-permeable bacterial ADP-ribosyltransferase C3, which inhibits RhoA activity by promoting Rho-GDI complex formation and decreasing binding of RhoA to the membrane (39). Addition of C3 to DRG neurons exposed to myelin prevented the increase in expression of neuronal Nogo-A mRNA and protein (Fig. 2, c and d), indicating that RhoA-dependent signaling plays a role in the transcription regulation of neuronal Nogo-A. Increasing cAMP promotes neurite outgrowth (40, 41), and cAMP-dependent kinase negatively regulates RhoA activity (42). We found that treatment of DRG neurons with Bt2cAMP prevented the increase of Nogo-A mRNA and protein in DRG neurons exposed to myelin (Fig. 2, e and f). Artemin, a member of the glia-derived neurotrophic factor family, reverses myelin inhibition of neurite growth by cAMP-PKA-cAMP-response element-binding protein-dependent pathway (43) and supports growth of DRG central and peripheral axons in vivo (44). Addition of artemin to the medium of DRG neurons plated on CNS myelin prevented the increase in Nogo-A induced by CNS myelin (Fig. 2, g and h).

Knocking Down Neuronal Nogo-A Increases Neurite Outgrowth—To directly test the role of neuronal Nogo-A in neurite growth, we next constructed siRNA to Nogo-A and tested its ability to knock down Nogo-A in DRG neurons after axonal injury in vitro. Nogo-A siRNA silenced Nogo-A mRNA and protein in DRG neuron 24 h after exposure, whereas a scrambled version had no effect (Fig. 3, a and b). The biological effects of knocking down neuronal Nogo-A is manifested by a reversal of myelin inhibitory effects on neurite outgrowth measured at 48 h (Fig. 3, c and d).

Vector QHNgsR Expresses NgSR—To study the role that Nogo-A and NgR1 interactions might play in neurite growth and downstream signaling events, we constructed a nonreplicating HSV-based vector expressing the truncated soluble Nogo receptor (NgSR), which avoids off-target effects resulting from intrathecal or systemic delivery of these proteins. A soluble form of the NgR1 ligand-binding domain has been used successfully to block CNS myelin inhibition in vitro (45). We constructed QHNgsR, a nonreplicating HSV vector containing two copies of the soluble ectodomain of the NgR1 (NgSR), corresponding to amino acids 1–310, under the control of the HCMV-IEp (Fig. 4a). Control vector QHGFP was identical to QHNgsR except that it contained the green fluorescent protein reporter in place of NgSR (Fig. 4b). Greater than 90% of cortical or DRG neurons were transduced by the vector, determined using green fluorescent protein fluorescence as reporter. We observed that the endogenous level of the NgR1 protein.
(66 kDa) was substantially higher in cortical neurons (Fig. 5a) than in DRG neurons (Fig. 5b). Infection of either cortical neurons (Fig. 5, a and c) or DRG neurons (Fig. 5, b and d) in culture resulted in expression of NgSR transgene protein with the predicted mass of 37 kDa detected by Western blot of cell lysates (Fig. 5, a and b) and release of NgSR from the transduced cells confirmed by Western blot of the culture medium (Fig. 5, c and d). DRG neurons synthesized and released higher levels of the transgene product from HSV vector than did transected cortical neurons, suggesting that DRG neurons have a greater synthetic capability. Immunocytochemistry of primary DRG neurons confirmed the expression of NgSR within DRG neurons in the transduced cultures (Fig. 5e).

**Vector-mediated NgSR Overcomes the Inhibitory Effects of Myelin on Neurite Elongation**—We tested the effect of vector-expressed NgSR on preventing myelin inhibition of neurite growth using rat DRG neurons and hippocampal neurons cultured on CNS myelin isolated from young adult rat brain (34) to be consistent within a single animal species in all our studies. Western blot of the purified myelin preparation showed the presence of the three major inhibitors OMgp, MAG, and Nogo-A proteins (Fig. 6d). We used conditioned medium from cortical neurons transected with QHNgSR to be certain that the effect was mediated by released peptide acting at the cell...
NogoSR and Neuronal Nogo-A in Axonal Regeneration

We chose to produce the conditioned medium from infected cortical neurons because they can be grown in the same culture medium but do not require nerve growth factor, thus avoiding a potential confound in the analysis of neurite growth. The amount of NgSR in the conditioned medium was confirmed by Western blot (Fig. 5c). Conditioned medium from control vector-transfected cells contained no NgSR. Tissue culture plates were coated with 1–3 μg of myelin dried onto the poly-D-lysine and overcoated with laminin. P10 DRG neurons cultured on the myelin-treated plates showed progressive inhibition of neurite extension with increasing amounts of myelin (Fig. 6, a and b). Myelin-induced inhibition of neurite extension was markedly reduced by the addition of QHNgSR-conditioned medium but not by QHGFP-conditioned medium. Similar effects were seen after the addition of conditioned medium to cultured P1 hippocampal neurons (Fig. 6c) despite the fact that hippocampal neurons were more resistant to myelin inhibition compared with DRG neurons. Exposure to myelin resulted in a 40–50% inhibition of neurite extension in hippocampal neurons compared with 80–90% inhibition of neurite extension in DRG neurons, but NgSR-conditioned medium restored ~40% of neurite extension in both DRG and hippocampal cultures. The response to NgSR is consistent with the observation that DRG and hippocampal neuron preparations express similar levels of NgR1 protein by Western blot at the time of study (data not shown), and the neurons are exposed to a similar amount of myelin proteins. The profound inhibitory effects of CNS myelin on DRG neurites when compared with hippocampal neurites suggest that DRG cells have additional receptor-coupled signaling pathways engaged by myelin-mediating neurite growth inhibitory cues at this developmental stage.

NgSR Inhibits RhoA Activation by Myelin and Decreases Neuronal

**FIGURE 7.** Transgene-mediated NgSR attenuates RhoA activity, Nogo-A and CRMP-2 (Thr(P)-555) phosphorylation in vitro. DRG neurons cultured on myelin substrate for 24 h and treated without or with conditioned medium containing NgSR. α, RhoA activity assay. DRG neurons plated on a surface coated with 8 μg/well myelin in a 24-well plate show increased RhoA activity that is attenuated by exposure to QHNgSR-conditioned medium. Myelin significantly increases Nogo-A mRNA detected by RT-PCR (b and c) and protein level detected by Western blot (d and e), an effect that was substantially blocked by NgSR-containing conditioned medium (b–e). Western blot of cell lysates demonstrates that exposure to myelin increases phosphorylation of CRMP-2 (Thr(P)-555), an effect that is markedly attenuated by exposure to QHNgSR-conditioned medium (d and g). Results are representative of three independent experiments. *, p < 0.05; **, p < 0.01. Ctrl, control.
Nogo-A—The effects of Nogo-A are mediated in part by Rho-GTPase and activation of Rho kinase, which in turn result in growth cone collapse and neurite growth inhibition (37, 46). To assess whether NgSR produced by the vector improved neurite elongation through this pathway, we examined RhoA activity in P10 DRG neurons plated on the myelin-containing substrate. Compared with DRG neurons plated in the absence of myelin, DRG neurons exposed to myelin showed a 3-fold increase in RhoA GTPase activity; this increase in RhoA activity was significantly prevented in cells exposed to NgSR-conditioned medium but was not changed by exposure to control vector-conditioned medium (Fig. 7a). Treatment of DRG neurons with NgSR produced by vector QHNgSR prevents the increase in both Nogo-A mRNA (Fig. 7, b and c) and protein (Fig. 7, d and e) induced by myelin. This is consistent with our observation that neuronal Nogo-A is transcriptionally regulated by RhoA.

Within the family of collapsin-response mediator proteins, CRMP-2 is an important Rho-kinase substrate. Phosphorylation of CRMP-2 at threonine 555 (Thr(P)-555) by Rho-kinase is involved in microtubule assembly or stability (47, 48). Exposure of DRG neurons to myelin significantly increased the amount of p-CRMP-2 (Thr(P)-555) relative to total CRMP-2, an effect that was prevented by the addition of QHNgSR-conditioned medium (Fig. 7, f and g). NgSR did not modulate the phosphorylation state of protein kinase C (Ser-657) or GSK-3β (Ser-9) induced by myelin. (data not shown).

Transfection of DRG by QHNgSR Increases Regeneration of Central Axons in Dorsal Roots—We used the model of dorsal root crush to test the biological effects of transgene-mediated NgSR in vivo. We have previously reported that nonreplicating HSV vectors similar to the one used in this study are effective in delivering transgenes to DRG in vivo (49, 50), and the transgene product is released by the central afferent terminals of transduced DRG (51). We confirmed that subcutaneous inoculation of QHNgSR resulted in the production of NgSR in DRG neurons in vivo. Morphometric analysis within the dorsal root, 4 weeks after crush, showed a marked loss of myelinated axons distal to the crush compared with the dorsal root proximal to the crush site (Fig. 8a). Animals that were injected with QHNgSR showed a significant increase in the number of regenerating myelinated axons per cross-sectional area with a concomitant increase in the total area occupied by myelinated fibers (Fig. 8a and b). The average size of myelinated fibers was not significantly affected by vector-mediated transgene expression (Fig. 8c). The morphometric changes suggest that NgSR expressed by DRG neurons promotes axonal regeneration and increases branching of myelinated fibers within the dorsal root.

Transfection of DRG by QHNgSR Increases Regeneration into the Spinal Dorsal Root Entry Zone—After dorsal root crush injury, regeneration of the central projection of the pseudounipolar DRG neuron stops at the DREZ where the change from Schwann cells to oligodendrocytes inhibits the entry of these fibers into the spinal cord. Because the DREZ contains only axons projecting from the root, the measurements reflect true regeneration of damaged fibers and not sprouting of neighboring fibers. Six weeks after injury, the L5 DREZ of control animals was virtually devoid of sensory afferent terminals, measured either by TuJ1 immunocytochemistry for the neuron-specific βIII tubulin isofrom recognizing all axons independent of size or by immunoreactivity for the CGRP that identifies mainly small afferent fiber terminals.

There was a significant increase of TuJ1-labeled axons crossing DREZ in the NgSR vector-treated animals as compared with animals treated with control vector QHGFP (Fig. 9, a–c). Vector-mediated expression of NgSR did not significantly change CGRP-labeled axons in the dorsal horn (Fig. 9, a and d), indicating that NgSR predominantly improved the regeneration of medium and large myelinated fibers. As an independent measure of dorsal root regeneration into the dorsal root entry zone, we injected CTB into the sciatic nerve 5 weeks after nerve crush and 1 week prior to sacrifice. The summed area of CTB immunostaining in the dorsal root entry zone of the injured side compared with the contralateral, intact dorsal root was substantially greater in QHNgSR-inoculated animals than in control vector-inoculated animals (Fig 10, a and b). This result further confirms that the increase in fibers observed in the DREZ in vector-inoculated animals represents regeneration of fibers from the dorsal root.
Transfection of DRG Neurons with QHNgSR Improves Functional Recovery after Dorsal Root Crush in Vivo—We confirmed that subcutaneous inoculation of QHNgSR resulted in the production of NgSR in DRG neurons in vivo (Fig. 11a). Using the grid walk test to assess proprioceptive function and feedback control of limb placement, we found that animals injected subcutaneously with QHNgSR into the ipsilateral paw 1 week after L4–L5 dorsal root crush showed a sustained and significant improvement in foot placement over 6 weeks (Fig 11b) compared with animals inoculated with the control vector QHGFP. Withdrawal of the paw from a painful thermal stimulus was not significantly different between NgSR-expressing and control vector-inoculated animals (data not shown). These results are consistent with the preferential regenerative response of myelinated fibers seen by the histological measures and suggest that expression of NgSR from vector can support sustained improvement after injury.

**DISCUSSION**

There are five principal results of this study as follows. 1) Nogo-A expression in sensory neurons is increased after axonal injury and after exposure to myelin that inhibits axonal growth. 2) Neuronal Nogo-A expression is controlled in part by the NgR1 acting through RhoA. 3) Knocking down neuronal Nogo-A leads to marked increase of neurite outgrowth. 4) Disruption of NgR1 signaling by vector-mediated
expression of NgSR overcomes neurite growth inhibition and prevents the increase in neuronal Nogo-A expression resulting from exposure to CNS myelin in vitro. 5) Subcutaneous inoculation of QHNgSR to express NgSR in DRG after nerve root injury increases regeneration of central afferents in the dorsal root and into dorsal root entry zone resulting in a significant improvement in behavioral recovery in vivo (Fig. 12).

Previous reports have documented that Nogo-A is expressed in peripheral sensory neurons (31), but this is the first report to demonstrate that acute axonal injury increases neuronal Nogo-A expression. Increases in neuronal Nogo-A mRNA or protein have been reported previously in hippocampal pyramidal neurons following entorhinal cortex injury, in cortical neurons following percussion injury, and after middle cerebral artery occlusion (52–54). In our studies, we found that the increase in neuronal Nogo-A occurs specifically after axonal injury in situations in which regeneration is inhibited, either by a physical barrier (cut and ligature of the peripheral projection of the DRG axon) or by myelin-mediated receptor interaction in the DREZ. The enhanced expression of Nogo-A in neurons in axon exposed to myelin suggests that neurons respond to these different insults by engaging similar signaling pathways.

Activation of RhoA has been described in experimental models of sciatic nerve injury (38) and after exposure to CNS myelin (37). Our studies demonstrate that RhoA signaling is also a key regulator of Nogo-A expression in sensory neurons. Inhibition of RhoA by C3 transferase or inhibition of the downstream Rho kinase promotes neurite growth in vitro and axonal regeneration after injury (37, 55) that correlated with a reduction in transcription of Nogo-A. We interpret these findings to suggest that regulation of Nogo-A expression in neurons by RhoA is closely linked to neurite dynamics. Elevation of cAMP and activation of PKA by neurotrophic factors promotes axon growth through phosphorylation and activation of cAMP-response element–binding protein (40, 41). We have previously reported that artemin, a neurotrophic factor of the GDNF family of proteins with potent neurite growth activity for sensory neurons, reverses myelin inhibition of neurite extension, increases cAMP levels, and activates PKA in DRG neurons (43). RhoA has been identified as a substrate for PKA. There are several potential mechanisms for this interaction that include direct phosphorylation of RhoA at Ser-188 (42), phosphorylation of GTPases with potent neurite growth activity for sensory neurons, reverses myelin inhibition of neurite extension, increases cAMP levels, and activates PKA in DRG neurons (43). RhoA has been identified as a substrate for PKA. There are several potential mechanisms for this interaction that include direct phosphorylation of RhoA at Ser-188 (42), phosphorylation of Gα13 protein upstream of Rho (56, 57), or phosphorylation of GTP dissociation inhibitor (58). Our data suggest that either artemin or Bt2cAMP acting through cAMP-PKA signaling may partially inhibit RhoA activity and thus negatively regulate Nogo-A expression.

Montani et al. (33) recently reported that in the unlesioned adult nervous system, neuronal Nogo-A restricts neuronal growth through negative modulation of growth cone motility. Our finding that in the presence of CNS myelin, knocking down neuronal Nogo-A in DRG results in increased neurite outgrowth and provides direct evidence that neuronal Nogo-A plays a role in the inhibition of neurite outgrowth.

Rho-GTPases are thought to be a convergent point signaling growth cone development and neurite extension. Activation of
NogoSR and Neuronal Nogo-A in Axonal Regeneration

RhoA induces growth cone collapse by phosphorylation of myosin light chain and activation of myosin II leading to a decreased F-actin (59). In accord with previous work (47, 60, 61), we found that NgSR prevented the phosphorylation of CRMP-2 at threonine 555, and our observation supports the interpretation that the effects on neurite growth are through the Rho-Rho kinase-CRMP2 pathway.

The role of Nogo-A in this process in vitro and in vivo was confirmed using the nonreplication HSV vector expressing NgSR, the soluble NgR1 ectodomain (amino acids 1–310). NgSR differs from the fusion construct (NgR1(310)-Fc) reported previously (21, 23) in that NgSR does not contain the human IgG Fc fragment that leads to the formation of high molecular weight oligomers. Nonetheless, NgSR was able to completely reverse myelin inhibition of neurite inhibition in hippocampal neurons in culture. NgSR partially reversed the myelin inhibition of neurite extension in DRG neurons that correlated with a partial reduction of RhoA activity in DRG neurons induced by myelin. These results suggest that in DRG neurons there may be additional inhibitory cues acting through Rho signaling that are independent of the myelin Nogo-A-NgR1 interaction, although the possibility that a higher dose of NgSR might provide complete reversal is not excluded.

The dorsal root entry zone provides an excellent model to directly test the effect of NgSR on nerve regeneration in vivo because of the switch from Schwann cells to oligodendroglia at the Obersteiner-Redlich zone that demarcates the entry of the axons into the DREZ. We used transfection of DRG by subcutaneous inoculation of the HSV-based vector QHNgSR to express NgSR in DRG neurons. We chose this approach because HSV-based vectors efficiently transduce DRG neurons from skin inoculation (49, 62, 63), reflecting the conserved neurotropic properties of HSV, and produce transgene products that are transported to afferent terminals of those cells in the dorsal horn of spinal cord (64, 65). We used several different measures to assess the effect of NgSR expression by QHNgSR on regeneration in vivo. There was an increased number of regenerating myelinated fibers distal to the dorsal root crush site in QHNgSR-transfected animals compared with control animals. This observation might result from axonal regeneration from a larger pool of DRG neurons or from increased branching of injured axons within the dorsal root. The former is more likely because DRG afferents to the spinal cord are largely unbranched. There was no significant increase in the diameter of the axons or the thickness of myelin per axon to support an additional trophic effect. The increased number of regenerating axons in the dorsal root prior to DREZ suggests NgSR promotes axon regeneration by a mechanism independent of CNS myelin effect. The density of all fiber types regenerating into the DREZ, as measured by βIII-tubulin, was significantly increased, whereas the difference in CGRP-immunoreactive fibers failed to reach statistical significance. The differential effect of NgSR on myelinated axons was confirmed by studies with CTB, a tracer that is preferentially taken up by large myelinated axons.

The morphological evidence of improved nerve regeneration in animals inoculated with QHNgSR was confirmed by behavioral analysis in which we found that the number of foot misplacements was reduced in animals inoculated with the NgSR-expressing vector. Foot placement represents in part the spinal reflex arc involving principally large myelinated fibers. The failure of the NgSR-expressing vector to modify withdrawal from a thermal stimulus, behavior representing another spinal reflex arc in which the afferent input is mediated by unmyelinated fibers, is consistent with the morphological evidence of improved regeneration preferentially of myelinated axons in animals inoculated with the NgSR-expressing vector.

Taken together, the results of these studies provide insight into the possibility of a link between neuronal Nogo-A in axonal injury and neurite extension. The local release of NgSR by regenerating fibers may provide a therapeutic effect, without the off-target effects of NgSR that may be observed with systemic or even intrathecal or systemic delivery of this molecule. The results of the animal experiments provide preclinical evidence for a strategy that may be particularly suitable for treatment of CNS injury.

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