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Neuronal responses to myelin are mediated by rho kinase

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Abstract

CNS myelin inhibits axon growth due to the expression of several growth-inhibitory proteins, including myelin-associated glycoprotein, oligodendrocyte myelin glycoprotein and Nogo. Myelin-associated inhibitory proteins activate rho GTPase in responsive neurons. Rho kinase (ROCK) has been implicated as a critical rho effector in this pathway due to the ability of the pharmacological inhibitor Y-27632 to circumvent myelin-dependent inhibition. Y-27632, however, inhibits the activity of additional kinases. Using three independent approaches, we provide direct evidence that ROCKII is activated in response to the myelin-associated inhibitor Nogo. We demonstrate that Nogo treatment enhances ROCKII translocation to the cellular membrane in PC12 cells and enhances ROCKII kinase activity towards an *in vitro* substrate. In addition, Nogo treatment enhances phosphorylation of myosin light chain II, a known ROCK substrate. Further, we demonstrate that primary dorsal root ganglia neurons can be rendered insensitive to the inhibitory effects of myelin via infection with dominant negative ROCK. Together these data provide direct evidence for a rho-ROCK-myosin light chain-II signaling cascade in response to myelin-associated inhibitors.

Keywords: CNS regeneration, myelin inhibition, protein kinase C-related kinase, rho kinase.

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Neurons in the adult mammalian CNS do not spontaneously regenerate after injury due to the combination of an inhibitory environment and a lack of positive cues. CNS myelin contains several growth-inhibitory proteins, including myelin-associated glycoprotein (McKerracher *et al*. 1994; Mukhopadhyay *et al*. 1994), oligodendrocyte myelin glycoprotein (Kottis *et al*. 2002; Wang *et al*. 2002b) and Nogo (Chen *et al*. 2000; GrandPre *et al*. 2000; Prinjha *et al*. 2000). These myelin-associated inhibitors (MAIs) exert their effects via a common, tripartite neuronal receptor complex consisting of Nogo receptor (NgR) (Fournier *et al*. 2001), $p75^{NTR}$ (Wang *et al*. 2002a; Wong *et al*. 2002) and Lingo1 (Mi *et al*. 2004).

Intracellularly, rho GTPase plays a critical role in transducing MAI signals to the actin cytoskeleton. Rho GTPase is activated in response to MAIs (Dergham *et al*. 2002; Niederost *et al*. 2002; Fournier *et al*. 2003) and blockade of rho function with C3, a rho-specific ADP ribosyltransferase, partially circumvents myelin-dependent inhibition (Jin and Strittmatter 1997; Lehmann *et al*. 1999; Dergham *et al*. 2002). This pathway has been particularly well studied in response to Nogo-66, a potent inhibitory component of full-length Nogo-A. Many effector proteins downstream of rho modulate actin cytoskeletal dynamics and are reasonable candidates for the transduction of myelin-associated signals, including citron kinase, mDia, rho kinase (ROCK) and protein kinase N (Bishop and Hall 2000). ROCK has been the favored effector candidate due to the ability of the drug Y-27632, a ROCK antagonist, to promote neuronal outgrowth on MAI substrates *in vitro* and *in vivo* (Dergham *et al*. 2002; Niederost *et al*. 2002; Fournier *et al*. 2003). ROCK is a serine/threonine kinase with two isoforms (p160ROCK/ROCK-I/ROCKβ and ROCKII/ROKα). ROCKS are ubiquitously expressed in rat tissue (Riento and Ridley 2003) where they phosphorylate multiple downstream substrates (Riento and Ridley 2003). It is reasonable to speculate that ROCK mediates myelin-dependent growth cone collapse by virtue of its ability to regulate myosin light chain (MLC)

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Abbreviations used: AP, alkaline phosphatase; DNROCK, dominant negative rho kinase; DRG, dorsal root ganglia; E, embryonic day; MAI, myelin-associated inhibitor; MLC, myosin light chain; NGF, nerve growth factor; NgR, Nogo receptor; PRK, protein kinase C-related kinase; ROCK, rho kinase.

phosphorylation and consequently actomyosin dynamics within the growth cone (Amano *et al*. 1996; Kimura *et al*. 1996; Dent and Gertler 2003). However, to date there are no reports directly demonstrating that ROCK is activated in response to MAIs. This is a key issue in the rational design of antagonists to promote axon outgrowth following CNS injury.

The importance of this question is highlighted by several observations. First, treatment with Y-27632 promotes neurite outgrowth on permissive substrates in addition to inhibitory substrates bringing into question the supposition that ROCK is directly activated by MAIs (Fournier *et al*. 2003). Second, Y-27632 is an ATP-competitive inhibitor which inhibits a number of kinases. The majority of studies published to date have used Y-27632 at a concentration of 10 μM. At this concentration Y-27632 also inhibits protein kinase C-related kinase (PRK)2/PKNγ. PRK2 is a serine/threonine kinase which binds to rho and regulates cytoskeletal dynamics affecting actin stress fiber formation (Vincent and Settleman 1997) and cell–cell adhesions (Calautti *et al*. 2002). Other studies have used Y-27632 at concentrations up to 50 μM *in vitro*, a concentration at which other kinases are inhibited (Davies *et al*. 2000). This is also relevant to *in vivo* studies examining the effects of Y-27632 in spinal cord injury models where it is difficult to accurately estimate the active concentration of the drug (Dergham *et al*. 2002; Fournier *et al*. 2003). Finally, the notion that myosin II, the presumptive target of ROCK in this pathway, provides the critical activity for retrograde actin flow and neurite retraction has recently been challenged (Brown and Bridgman 2003).

To resolve these ambiguities, we set out to determine whether ROCKII is directly activated by MAI signals. Using two independent approaches, a membrane translocation assay and an *in vitro* phosphorylation assay, we find that ROCKII is directly activated in response to Nogo stimulation. In contrast PRK2, although expressed in many types of neurons, is not activated. Further, we demonstrate a ROCK-dependent increase in MLCII phosphorylation in response to MAIs. We find that dominant negative ROCK (DNROCK), a more specific blocking reagent than Y-27632, blocks myelin-dependent growth cone collapse and neurite outgrowth inhibition. Together, these data provide a direct demonstration of a critical rho-ROCK-MLCII signaling cascade in response to MAIs.

Materials and methods

Plasmid construction and recombinant HSV preparation

To construct pHSVmycDNROCK, a dominant negative ROCKII fragment was amplified by PCR from pMalC2-Rho-kinase/RB/PH(TT) (Amano *et al*. 1998) (a gift from Dr Kozo Kaibuchi, Nagoya University) using a 5′ primer containing a c-Myc epitope sequence. The dominant negative construct encompasses the rho binding/pleckstrin homology (RB/PH) domain (residues 941-1388) of *bos taurus* rho kinase (ROCKII). Site directed mutagenesis of Asn1036 and Lys1037 to threonine prevents binding of this domain to GTPγS-Rho. ROCKI and ROCKII share 60% amino acid identity in the RB/PH domain (comparison in mouse), raising the possibility that this construct may also antagonize the kinase domain of ROCKI. The PCR product was ligated into the *Bam*HI and *Eco*RI site of pHSVprPUC (provided by Dr Rachel Neve, Harvard Medical School). The resulting plasmid was transfected into 2-2 Vero cells which were superinfected with 5*dl* 1.2 HSV helper virus 1 day later. Recombinant virus was amplified through three passages and stored at − 80°C as previously described (Neve *et al*. 1997).

Preparation of myelin and recombinant proteins

To purify alkaline phosphatase (AP)-conjugated Nogo-66 or AP, conditioned medium was collected from HEK293A cells stably transfected with pcDNA3.1AP-Nogo66-His (generously provided by Dr Stephen Strittmatter, Yale University) or HEK293T cells transiently transfected with pcDNA-AP-His. Secreted protein was purified by Ni^{2+} affinity chromatography (Nakamura *et al*. 1998). For all cellular treatments AP-Nogo66-His (8 nM) or AP (8 nM) was pre-aggregated with 100 ng/mL anti-human AP (Niederost *et al*. 2002). Glutathione *S*-transferase or glutathione *S*-transferase-Nogo-66 was expressed in *Escherichia coli* and purified on glutathione-Sepharose (Amersham Pharmacia Biosciences, Baie D'Urfe, Quebec, Canada) as previously described (GrandPre *et al*. 2000). Glutathione *S*-transferase-Nogo-66 was added at a final concentration of 50 nM. Myelin extracts were prepared from the bovine brain as previously described (Igarashi *et al*. 1993). After clarification of the myelin extract by centrifugation at 400 000 *g*, the detergent was removed by dialysing with phosphate-buffered saline.

RT-PCR

Total RNA was prepared using an RNeasy kit (Qiagen, Mississauga, Ontario, Canada). cDNA libraries were made with a Thermoscript Reverse Transcriptase kit (Invitrogen, Burlington, Ontario, Canada). Primers for PCR detection were designed to span an intron boundary: ROCKII primers, 5′-AGATCAGTGCAGCGGCTATT-3′ and 5′-GAATTTGGCTCTCTTCAGC-3′; PRK2 primers, 5′-CTCCATGGTACAGCTCAGCA-3′ and 5′-CTTGTTCGACTGCGACGG-3′.

Immunofluorescence

Cerebellar neurons were dissected from post-natal day 8 rat brain, dissociated with trypsin and mechanical trituration and cultured on poly-L-lysine-coated substrates for 24 h in Sato medium (Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 1% penicillin/streptomycin, 1% N2 supplement, 400 ng/mL tri-iodothyronine and 400 ng/mL tetraiodothyronine). Cultures were fixed with 4% paraformaldehyde, permeabilized in 0.2% Triton X-100, and incubated with an anti-ROCKII antibody generated with a peptide corresponding to residues 567-718 of rat ROCKII (BD Bioscience, Mississauga, Ontario, Canada) or an anti-PRK2 antibody generated with a peptide from the amino terminus of human PRK2 (Cell Signaling, Beverly, MA, USA), followed by a CY2-conjugated secondary antibody.

In vitro kinase assay

An S6 Kinase Assay kit (Upstate Biotechnology, Waltham, MA, USA) was used to measure the kinase activity of ROCKII and PRK2

in vitro. PC12 cells were transfected for 24 h with pCDNA3-flag-PRK2 (a gift from Dr Jeff Settleman, Harvard Medical School) or pCAG-myc-mROCKII (a gift from Dr Shuh Narumiya, Kyoto University) using Lipofectamine 2000 (Invitrogen). The cells were then differentiated with 50 ng/mL nerve growth factor (NGF) (Upstate Biotechnology) in serum-free media for 24 h [Roswell Park Memorial Institute 1640, 5% bovine serum albumin, 1% penicillin-streptomycin]. Cells were treated with pre-clustered AP or AP-Nogo-66 for 10 min, washed twice with ice-cold phosphate-buffered saline and lysed in 50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% (v/v) Triton X-100, 1 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride and Cømplete protease inhibitors (Roche Diagnostics, Laval, PQ, Canada). The supernatant fractions containing 2 mg of protein were pre-cleared with protein A/G-agarose (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 20 min at 4°C and then ROCKII and PRK2 were immunoprecipitated with myc-agarose or flag-agarose antibody at 4°C for 2 h (Sigma Chemical Co., Oakville, Ontario, Canada). After washing three times with 50 mM Tris (pH 7.4) and 150 mM NaCl, the immunoprecipitates were analysed for *in vitro* kinase activity as per the manufacturer's instructions. As a control, ROCKII and PRK2 kinase activity was blocked with 10 μM Y-27632 (Calbiochem, San Diego, CA, USA). For the PRK2 assay, the PKC inhibitor, which is included in the kit's assay mixture, was replaced with assay dilution buffer. In parallel with the *in vitro* kinase assay, ROCKII and PRK2 were immunoprecipitated from 1 mg of transfected cell lysates and analysed by western blotting to ensure equal immunoprecipitation of the relevant kinase for each treatment. After subtraction of background from each of the samples, the kinase activity was normalized relative to total ROCKII or PRK2 levels detected by western blotting. The AP-Nogo-66 treatment values were then normalized to AP controls and expressed as fold change.

To assess the non-specific *in vitro* kinase activity from the immunoprecipitation, *in vitro* kinase activity associated with myc-agarose or flag-agarose immunoprecipitations from 2 mg of mock-transfected cell lysates was analysed. The amounts of incorporated phosphate from mock immunoprecipitation and from control treated samples were compared. Myc-agarose and flag-agarose immunoprecipitates from mock-transfected cells account for 1.5 and 30.9% of the control ROCKII and PRK2 kinase activity, respectively. This background is not subtracted from the presented results.

PC12 cell fractionation

PC12 cells were grown to subconfluence on collagen-coated plates and then differentiated for 24 h with 50 ng/mL NGF (Upstate Biotechnology). After stimulation with Nogo-66 or the appropriate control ligand, cells were washed twice in ice-cold Tris-buffered saline and harvested in lysis buffer A (20 mM HEPES, pH 7.3, 150 mM NaCl) with 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride and Cømplete protease inhibitors. Cells were sonicated on ice, after which they were centrifuged for 10 min at 800 *g* to remove any unlysed cells. Supernatants were then centrifuged at 100 000 *g* for 30 min. Supernatants containing the cytosolic fraction were removed and the membrane fraction-containing pellets were washed twice with lysis buffer A and resuspended in lysis buffer (Tris-buffered saline containing 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate). Protein concentrations were determined with a detergent-compatible protein assay (Bio-Rad, Mississauga, Ontario, Canada). ROCKII or PRK2 protein content in the membrane fraction was analysed by separating the lysates by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on 4–15% gradient gels, transferring to polyvinylidene difluoride membranes (Bio-Rad) and probing with anti-ROCKII antibody. Membrane fractions were analysed with anti-p75 antibody (generously provided by Dr Phil Barker, McGill University) as a control for equal protein loading. Bands were analysed by densitometry using IMAGEJ analysis software.

Growth cone collapse and neurite outgrowth assays

For growth cone collapse experiments, embryonic day (E)13 chick dorsal root ganglia (DRG) explants were cultured in DRG media (F-12 medium, 10% fetal bovine serum, 1% penicillin/streptomycin, 1% L-glutamine, 50 ng/mL NGF) on poly-L-lysine- and laminin-coated substrates for 18 h. For viral infections, recombinant viral preparations were added to the media 1 h after plating. After 18 h, cultures were treated and fixed with 4% paraformaldehyde/20% sucrose/phosphate-buffered saline and double stained with rhodamine-phalloidin (Molecular Probes, Eugene, OR, USA) and anti-myc antibody to assess the mycDNROCK infection efficiency. For the HA1077 experiments, cultures were treated with 10 μM of HA1077 (Calbiochem) for 30 min prior to myelin treatment.

For neurite outgrowth assays, Nogo or myelin was dried down on poly-L-lysine-coated substrates. Substrates were washed and coated with 10 μg/mL laminin for 1 h. To examine the effects of HA1077, dissociated E13 chick DRG neurons were grown in the presence or absence of 10 μM HA1077 for 4–6 h, fixed and stained with rhodamine phalloidin. For HSVDNROCK experiments, dissociated E13 chick DRG neurons were grown in the presence of virus for 24 h and double stained with anti-βIII tubulin and anti-myc antibody. For shorter term outgrowth experiments examining the effects of HA1077, potent inhibition could be obtained with approximately 100 ng of myelin substrate. For 24-h experiments with the DNROCK virus potent inhibition was achieved with 400 ng of myelin. Neurite outgrowth lengths per cell were assessed using IMAGEJ, a public domain JAVA image-processing program (http://rsb.info.nih.gov/ij/) as previously described (Fournier *et al*. 2003).

Myosin light chain phosphorylation

PC12 cells were differentiated for 24 h with 50 ng/mL NGF and treated with pre-clustered AP-Nogo-66. Lysates were analysed by western blot using anti-phospho-MLC (Santa Cruz Biotechnology) and anti-MLC (Sigma Aldrich, Oakville, ON, USA) antibodies. Bands were analysed by densitometry using IMAGEJ analysis software. Phospho-MLC levels were normalized to total MLC levels for each lysate.

Results

HA1077 enhances neurite outgrowth on laminin and myelin substrates

Previous studies have shown that Y-27632 attenuates myelin-dependent neurite outgrowth inhibition (Fournier *et al*.

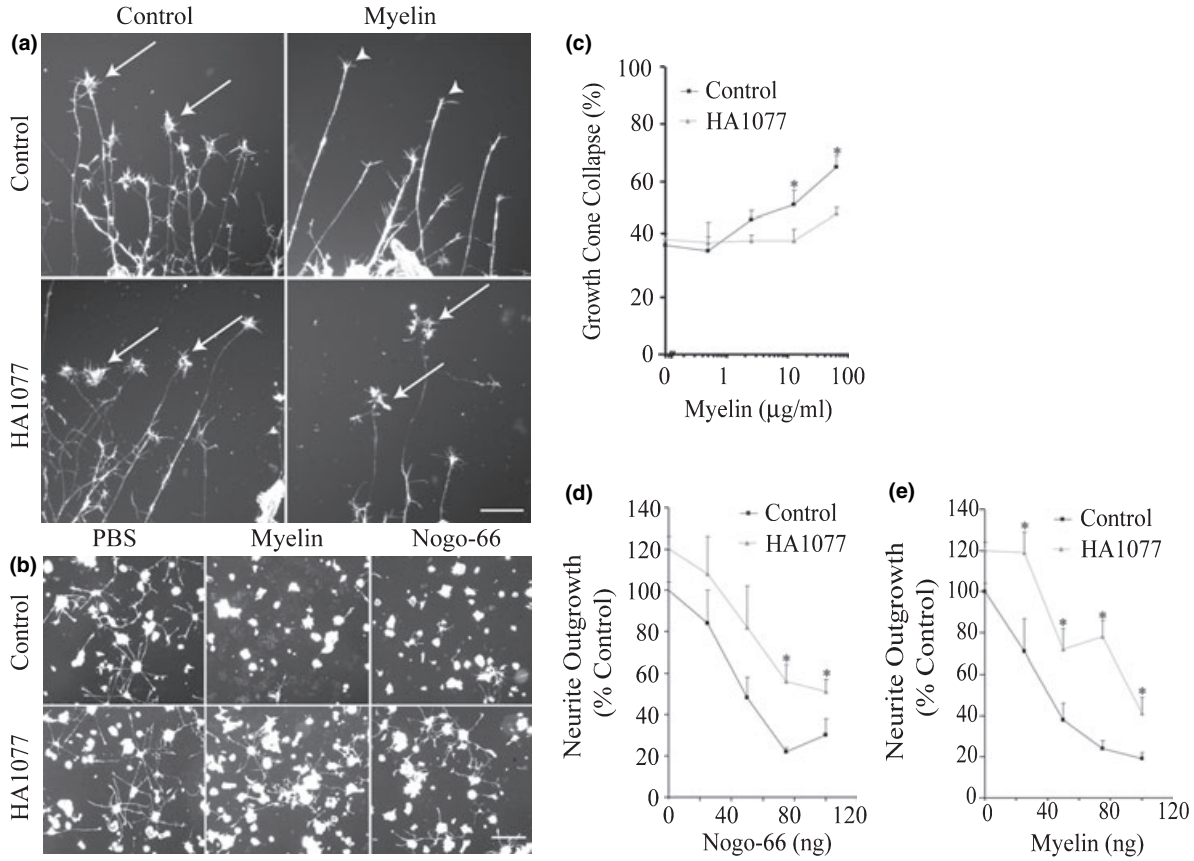

Fig. 1 HA1077 protects neurons from myelin-induced growth cone collapse and neurite outgrowth inhibition. (a) Embryonic day 13 chick dorsal root ganglia (DRG) explants were treated for 1 h with soluble myelin following a 30-min pre-treatment with phosphate-buffered saline (PBS) (control) or 10 μM HA1077. Explants were stained with rhodamine-phalloidin. Arrows, spread growth cones; arrowheads, collapsed growth cones. Scale bar, 40 μm. (b) Dissociated chick DRG neurons were cultured on control (PBS), myelin or Nogo-66 substrates in the presence or absence of 10 μM HA1077 and stained with rhodamine-phalloidin. Scale bar, 100 μm. Quantification of growth cone collapse (c) or neurite outgrowth per cell (d and e). Outgrowth is expressed as a percentage of control (control growth on 0 ng Nogo-66) ± SEM. Determinations are from four separate experiments each performed in duplicate. *$p < 0.05$ compared with controls by Student's *t*-test.

2003). Similar to Y-27632, HA1077 antagonizes ROCK and PRK (Davies *et al*. 2000); however, it may have distinct activity towards other targets. To address the functional specificity of this drug, we analysed the effects of HA1077 on myelin-dependent neuronal responses. We first assessed the effect of HA1077 on myelin-dependent growth cone collapse. DRG explants from E13 chick were pre-treated with 10 μM HA1077 prior to a 1-h exposure to myelin. DRG explants treated with HA1077 were significantly less sensitive to the growth cone collapse activity of myelin (Figs 1a and c). We also assessed the effect of HA1077 on the neurite outgrowth-inhibitory properties of myelin and Nogo-66. Dissociated E13 chick DRG neurons were cultured for 4–6 h on inhibitory substrates in the presence or absence of 10 μM HA1077. HA1077-treated DRG neurons grew significantly better on myelin and Nogo substrates (Figs 1b, d and e). These results, together with previous data (Fournier *et al*. 2003), demonstrate that two different pharmacological inhibitors with common target proteins, ROCK and PRK, both promote outgrowth on myelin substrates. These data suggest that either ROCK or PRK may mediate MAI signaling. However, a confounder of these results with HA1077, and of previous results with Y-27632 treatment, is that these agents also promote outgrowth on permissive substrates. Therefore, a more direct demonstration of the regulated activity of ROCK and PRK is required to fully implicate these proteins in MAI signaling.

Rho kinase II and protein kinase C-related kinase 2 are expressed in PC12 cells and in the rat nervous system

To begin to assess the relative contribution of ROCK and PRK to MAI signaling we analysed their expression in neural tissue by RT-PCR and immunofluorescence. mRNA for ROCKII and PRK2 was present in rat brain, cerebellum, DRG and in PC12 cells (Fig. 2a). To analyse the distribution of ROCKII and PRK2 in primary neurons, dissociated post-

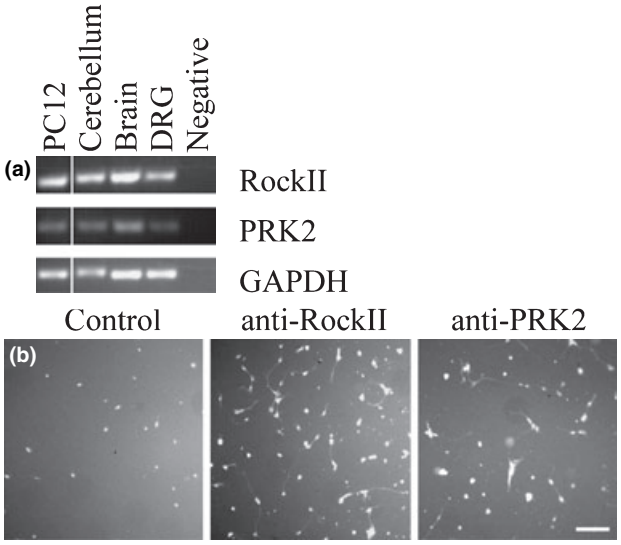

Fig. 2 Expression of rho kinase (ROCK)II and protein kinase C-related kinase (PRK)2 in PC12 cells and rat neural tissue. (a) mRNA detection of ROCKII and PRK2 in various tissues by RT-PCR. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is detected as a positive control. For the negative control, a parallel RT-PCR reaction was run on brain lysates in the absence of reverse transcriptase. (b) ROCKII and PRK2 immunostaining of post-natal day 8 rat cerebellar neurons demonstrating that both proteins are expressed in neuronal cell bodies and neurites. The control culture is stained with Cy-2-conjugated secondary antibody only. Scale bar, 100 μm. DRG, dorsal root ganglia.

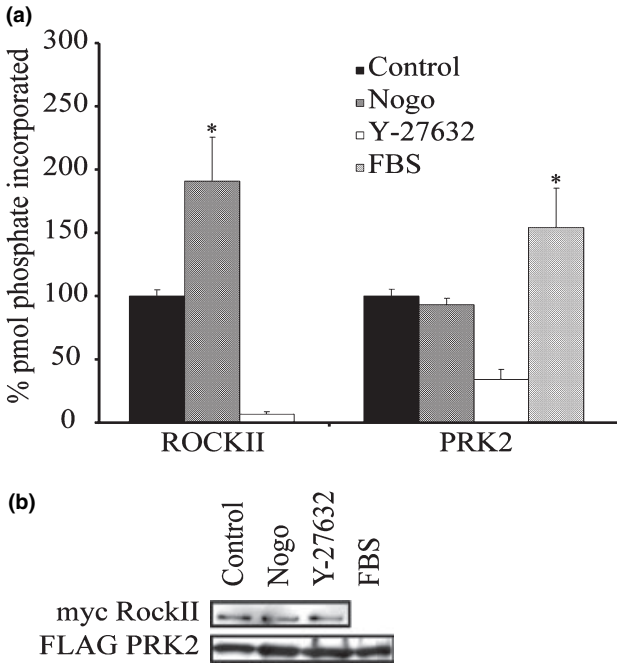

Fig. 3 Nogo-66 activates rho kinase (ROCK)II kinase activity. (a) Kinase activity assay demonstrates the ability of Nogo-66 to enhance ROCKII- but not protein kinase C-related kinase (PRK)2-dependent incorporation of ^{32}P-labeled-ATP into S6 substrate peptide. Y-27632 blocks the *in vitro* kinase activity of ROCKII and PRK2. (b) Immunoprecipitation of myc-ROCKII and flag-PRK2 from 30% of the lysates used for the *in vitro* kinase assay demonstrates that equal amounts of kinase were immunoprecipitated for each treatment. Determinations are from three independent experiments each performed in duplicate. Error bars represent the SEM. *$p < 0.05$. FBS, fetal bovine serum.

natal day 8 rat cerebellar neurons were immunostained with anti-ROCKII and anti-PRK2 antibodies. ROCKII and PRK2 were present in both cell bodies and neurites of dissociated cerebellar neurons (Fig. 2b).

Nogo-66 modulates the kinase activity of rho kinase II but not of protein kinase C-related kinase 2

Binding to active rhoA enhances the kinase activity of both ROCKII and PRK2 (Leung *et al*. 1995; Ishizaki *et al*. 1996; Matsui *et al*. 1996). To directly assess the activity of ROCKII and PRK2 in the MAI pathway, we assessed their kinase activities towards S6 kinase substrate *in vitro* (Borisoff *et al*. 2003) following treatment with Nogo-66, a potent inhibitory fragment of Nogo-A (GrandPre *et al*. 2000). Nogo-66 was chosen as a potent soluble ligand which contributes significantly to the inhibitory activity of MAIs (GrandPre *et al*. 2000). Evidence to date suggests that the intracellular signaling cascades engaged by Nogo-66 binding to NgR are similar to those of other MAIs. PC12 cells transfected with myc-ROCKII or flag-PRK2 were treated for 10 min with AP or AP-Nogo-66. ROCKII and PRK2 were immunoprecipitated from cell lysates using anti-myc-agarose or anti-flag-agarose, respectively, and tested for their ability to phosphorylate the S6 substrate using [γ-^{32}P] ATP. Equal immunoprecipitations for control and treated samples were verified by western blot analysis (Fig. 3b). Kinase activity was normalized to control (AP stimulation) for each experiment. Treatment of PC12 cells with AP-Nogo-66 for 10 min caused a 91% increase in ROCKII kinase activity (Fig. 3a). Treatment with Y-27632 blocked the kinase activity of ROCKII by 96% as anticipated. PRK2 was also able to phosphorylate the S6 kinase substrate *in vitro* (Zhu *et al*. 2004), although less efficiently than ROCKII (data not shown). Treatment of PC12 cells with fetal bovine serum enhanced PRK2 kinase activity, presumably due to lysophosphatidic acid-dependent stimulation of rho (Kranenburg *et al*. 1999) (Fig. 3a); however, Nogo treatment had no direct effect on PRK2 kinase activity (Fig. 3a). PRK2 kinase activity was also inhibited by treatment with Y-27632. Together, these data demonstrate that Nogo-66 stimulates the kinase activity of ROCKII but not PRK2.

Nogo-66 induces rho kinase II translocation to the membrane in PC12 cells

To verify that Nogo can activate ROCKII in an independent assay, we examined the ability of Nogo-66 to stimulate

ROCKII translocation to the cell membrane. ROCKII has been previously shown to translocate to the membrane in cultured cells transfected with constitutively active rhoAV14 (Leung *et al*. 1995). Differentiated PC12 cells were stimulated with Nogo-66 for 0.5, 15 and 60 min. Membrane fractions were isolated and analysed for ROCKII content by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and western blotting. Nogo-66 treatment increased the translocation of ROCKII from the cytosol to the membrane by 30 s and ROCKII was retained at the membrane at 60 min (Figs 4a and b). Control glutathione *S*-transferase treatment had no effect on ROCKII translocation (data not shown). These results demonstrate that Nogo-66 treatment enhanced the recruitment of ROCKII to the membrane providing independent evidence that ROCKII is activated by Nogo.

Nogo-66 mediates phosphorylation of myosin light chain

To examine potential downstream targets of ROCKII in the MAI signaling cascade, we examined the effect of Nogo-treatment on MLCII phosphorylation. MLCII is a substrate for ROCKII phosphorylation, which can regulate actomyosin dynamics. Differentiated PC12 cells were stimulated with AP-Nogo-66 and lysates were analysed by western blotting with anti-phospho-MLCII antibody. MLCII phosphorylation was rapidly (30 s) increased following Nogo-66 stimulation and this was maintained at 30 min (Figs 5a and b). The ability of Y-27632 to block the Nogo-dependent increase in MLCII phosphorylation was also assessed. Pre-treatment of cells with 10 μM Y-27632 prior to Nogo stimulation completely blocked the Nogo-dependent increase in MLCII phosphorylation (Figs 5a and b).

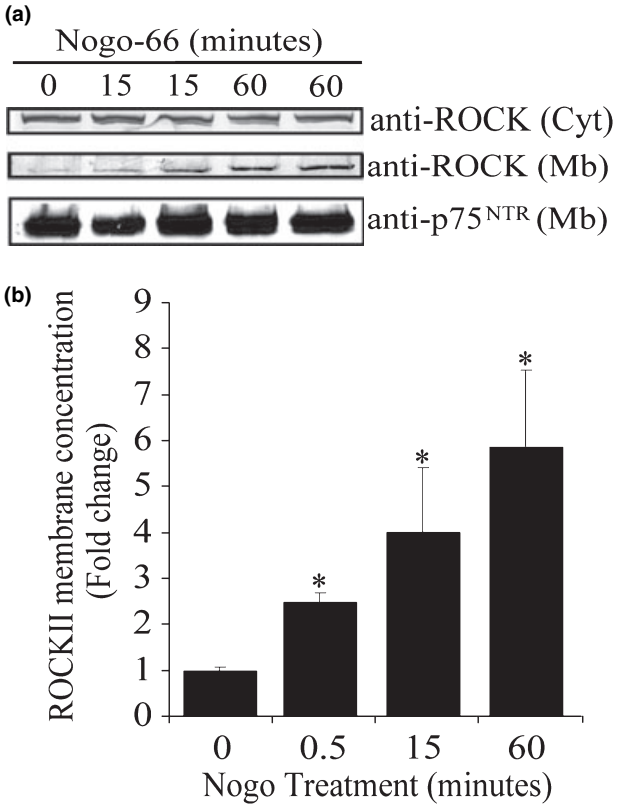

Fig. 4 Nogo-66 induces rho kinase (ROCK)II translocation to the membrane in PC12 cells. (a) PC12 cells were treated with glutathione *S*-transferase-Nogo-66 for 0, 0.5, 15 or 60 min. Crude membrane fractions (Mb) were prepared and analysed for ROCKII content by western blotting. Membrane lysates were analysed for $p75^{NTR}$ content to control for equal membrane protein loading. ROCK levels in the cytosolic fraction (Cyt) were also assessed. (b) ROCKII levels in the membrane fraction of PC12 cells were analysed by densitometry. ROCKII levels were normalized to p75 levels and expressed as a percentage of ROCKII levels in non-stimulated PC12 lysates (0) for each experiment. $^*p < 0.05$.

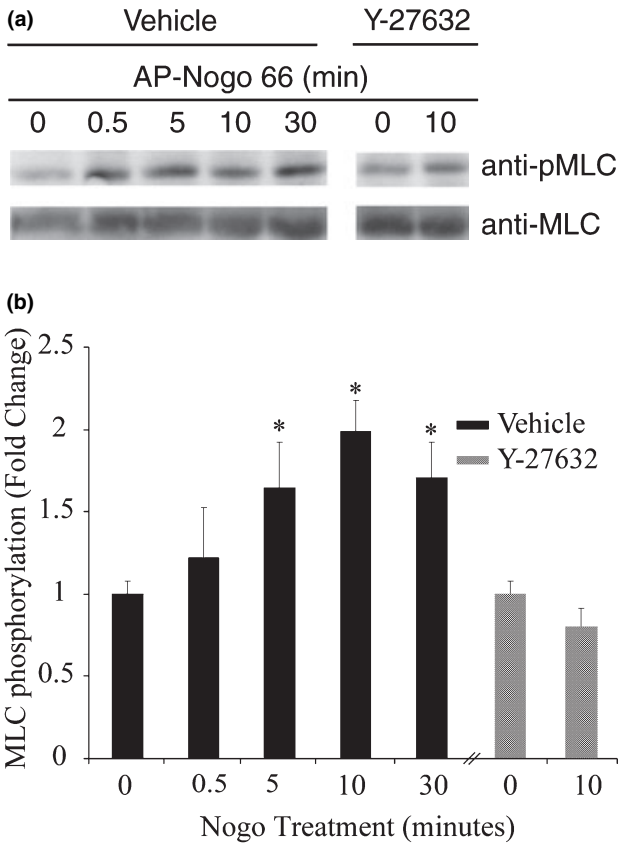

Fig. 5 Nogo-66 increases phosphorylation of myosin light chain (MLC)II. (a) PC12 cells were treated with Nogo-66 and lysates were analysed by western blotting with anti-phospho-MLC or anti-MLC antibody. Cells were pre-treated for 30 min with 10 μM Y-27632 or vehicle control. (b) Phosphorylated MLC (pMLC) in cell lysates was quantified by densitometry. Values were normalized to untreated control values for each experiment. In the case of Y-27632 pre-treatment, values following Nogo stimulation were normalized to the Y-27632-pre-treated condition. Values are for at least three independent experiments. Error bars represent the SEM; $^*p < 0.05$. AP, alkaline phosphatase.

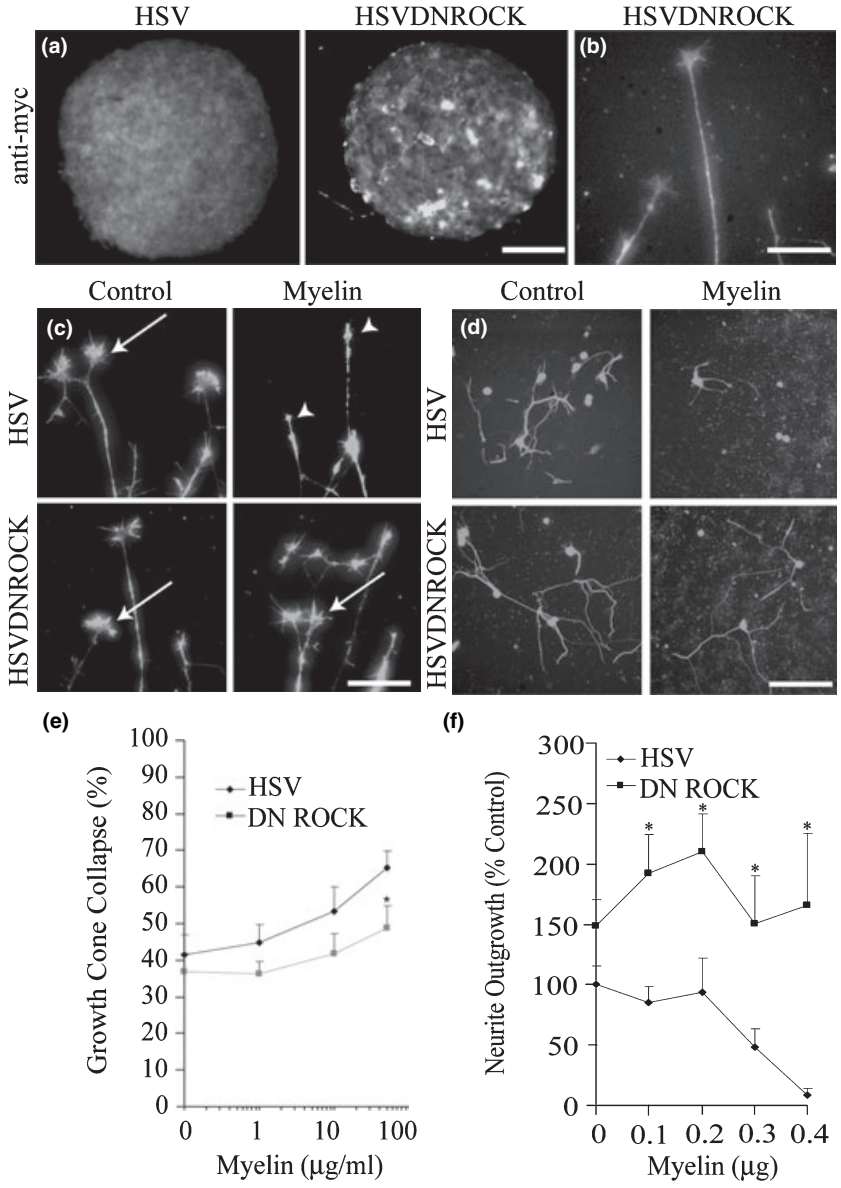

Fig. 6 Dominant negative (DN) rho kinase (ROCK)II rescues neurons from myelin-dependent inhibition. (a) Low magnification views of HSV- or HSVDNROCK-infected explants immunostained with anti-myc antibody demonstrates the efficiency of viral infection. Scale bar, 100 μm. (b) High magnification view of an HSVDNROCK-infected growth cone stained with anti-myc antibody demonstrates that the virus is transported into dorsal root ganglia (DRG) neurites and growth cones. Scale bar, 40 μm. (c) HSV or HSVDNROCK-infected embryonic day (E)13 DRG explants were treated with myelin for 1 h and stained with rhodamine phalloidin. Arrows, spread growth cones; arrowheads, collapsed growth cones. Scale bar, 40 μm. (d) HSV- or HSVDNROCK-infected dissociated E13 DRG neurons were cultured on myelin and stained with anti-βIII tubulin antibody. Scale bar, 100 μm. Quantification of myelin-dependent DRG growth cone collapse (e) and neurite outgrowth/cell on myelin substrate (f). Neurite outgrowth lengths were normalized to control (HSV outgrowth on 0 μg myelin) for each experiment. Determinations are from at least three separate experiments each performed in duplicate. Error bars represent the SEM; $*p < 0.05$.

Dominant negative rho kinase II protects neurons from myelin inhibition

To directly assess the effect of ROCK antagonism on neuronal responses to myelin, we examined DRG growth cone collapse and neurite outgrowth following neuronal infection with DNROCK. E13 DRG explants were infected with mycDNROCK recombinant viral preparations for 24 h prior to myelin stimulation and assessment of growth cone collapse. The efficiency of viral infection was verified by immunofluorescence for myc (Fig. 6a). We further verified that recombinant mycDNROCK protein was efficiently transported into DRG neurites and growth cones (Fig. 6b). Infection of neurons with DNROCK significantly decreased the percentage of collapsed growth cones in response to myelin (Figs 6c and e).

To analyse the effects of DNROCK on neurite outgrowth inhibition, dissociated E13 chick DRG neurons were infected with DNROCK or control HSV recombinant viral preparations and tested for their ability to grow on myelin substrates. DRG neurite outgrowth was significantly improved on inhibitory myelin substrates in neurons infected with DNROCK compared with control HSV preparations (Figs 6d and f). Taken together, these results demonstrate that antagonism of ROCK is sufficient to enhance neuronal outgrowth on permissive and inhibitory substrates.

Discussion

Previous data have implicated ROCK proteins in the MAI signaling cascade. By *in vitro* phosphorylation and mem-

brane translocation assays we provide direct evidence that ROCK is activated in response to Nogo-66 stimulation. Further, we find that the ROCKII effector MLCII is phosphorylated in response to stimulation with Nogo-66. We are unable to detect Nogo-dependent activation of PRK2, a kinase which is equally inhibited by many small molecule ROCK inhibitors such as Y-27632 and HA1077. Functional studies further demonstrate that blockade of ROCK activity with a previously characterized DNROCKII construct circumvents myelin-dependent growth cone collapse and neurite outgrowth inhibition.

Targeting intracellular signaling substrates has proven to be an effective approach to antagonize the inhibitory environment at the CNS lesion site (Dergham *et al*. 2002; Fournier *et al*. 2003). This approach may be more advantageous than targeting inhibitory ligands or their neuronal receptors for several reasons. The efficacy of blocking individual inhibitors is limited by the presence of multiple ligands in CNS myelin (He and Koprivica 2004). Blockade of individual ligands with function-blocking antibodies has met with some success (Fouad *et al*. 2001). However, gene knockout studies of individual inhibitory ligands have either proven ineffective in CNS injury models (Bartsch *et al*. 1995) or difficult to interpret due to different phenotypes in different strains and age-dependent responses (Kim *et al*. 2003; Simonen *et al*. 2003; Zheng *et al*. 2003). Antagonism of NgR, a common binding partner for all three MAIs, has also yielded promising effects in a variety of injury models (GrandPre *et al*. 2002; Lee *et al*. 2004; Li *et al*. 2004a,b), although positive effects on regeneration in NgR knockout mice are restricted to select neuronal populations (Kim *et al*. 2004; Zheng *et al*. 2005). Further, other members of the NgR family (NgR2 and NgR3) have now been identified (Pignot *et al*. 2003) which may contribute to the transduction of MAI signals (Venkatesh *et al*. 2005). Similarly, regeneration in $p75^{NTR}$ null mice has been disappointing (Song *et al*. 2004) and this may be partially attributed to heterogeneity in coreceptor requirements based on neuronal cell types (Park *et al*. 2005; Shao *et al*. 2005). A promising aspect of experiments utilizing small molecule inhibitors such as Y-27632 has been their ability to antagonize multiple inhibitory influences including MAIs (Dergham *et al*. 2002; Fournier *et al*. 2003) and components of the glial scar (Borisoff *et al*. 2003). However, the effects of these drugs on multiple physiological processes in many cell types (Riento and Ridley 2003) highlight the need to better understand the intracellular signaling substrates with the aim of developing more specific and potent antagonists.

The direct demonstration of Nogo-regulated ROCKII activity in membrane translocation and *in vitro* kinase assays strongly implicates ROCKII as a critical target in this pathway. The ability of a DNROCKII reagent to circumvent myelin inhibition further suggests that it plays a critical role in myelin-dependent inhibition. However, a ROCKI contribution to the functional effects of MAIs cannot be ruled out due to the potential ability of the DNROCK construct to also antagonize ROCKI (Amano *et al*. 1998; Kobayashi *et al*. 2004). DNROCK is a truncated protein lacking its kinase domain and containing a double mutation that prevents its binding to rho (Amano *et al*. 1997). It is thought to exert its dominant negative effect by binding to and inhibiting the kinase domain of endogenous wild-type ROCK. The ability of DNROCK to inhibit wild-type ROCK without binding to rho suggests that the effects seen on growth cone collapse and neurite outgrowth inhibition are mediated by effectors downstream of ROCK. The ability of DNROCK to promote neurite outgrowth on control substrates is consistent with the neurite outgrowth-promoting activity of pharmacological antagonists suggesting that basal ROCK activation also modulates neurite outgrowth under permissive conditions. It is also intriguing that DNROCK is a more efficient antagonist of myelin-dependent outgrowth inhibition than HA1077. While it is possible that 10 μM HA1077 does not block ROCKII activity as efficiently as DNROCK, it is also possible that DNROCK may sequester additional target substrates that are critical for myelin-dependent inhibition. The inability of HA1077 or DNROCK to completely circumvent myelin inhibition also raises the possibility that additional rho effectors may be involved in this process.

While ROCKII has many substrates (Riento and Ridley 2003), in neuronal cells the rho-ROCK-actomyosin pathway leads to growth cone collapse and neurite retraction and inhibition of this pathway promotes neurite outgrowth (Amano *et al*. 1998). ROCK's ability to drive actomyosin contractility is largely attributed to its ability to regulate MLCII by both direct phosphorylation and phosphorylation and inhibition of MLC phosphatase (Fujita *et al*. 2001). The mechanism by which MLCII promotes neurite outgrowth is not completely understood. While it is clear that blockade of actin retrograde flow with general myosin inhibitors such as 2,3-butanedione monoxime correlates with growth at the leading edge of *Aplysia* neurons (Cheung *et al*. 2002), inactivation of myosin isoforms via microchromophore-assisted laser inactivation suggests that myosin 1C not IIB may be the critical isoform for mediating retrograde F-actin flow (Diefenbach *et al*. 2002). Irrespective of the mechanism, functional data for the critical role of myosin II in neurite outgrowth continue to mount. In one study examining the mechanism of neurite retraction, inhibition of ROCK with Y-27632 or of myosin II with bebbistatin blocked axon retraction following an *in vitro* cut. This type of effect correlates well with the protective effects on growth-inhibitory substrates (Gallo 2004). Together, these data encourage further study of the basic cellular mechanisms of growth cone collapse and neurite retraction in the context of inhibitory influences.

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