Supporting Information

Hur et al. 10.1073/pnas.1011258108

SI Materials and Methods

Materials. Chondroitin sulfate proteoglycans (CSPGs) were obtained from Millipore. Myelin extracts were kindly provided by Dr. Shuxin Li (University of Texas Southwestern Medical Center, Dallas, TX). NGF was obtained from Harlan Bioproducts for Science, laminin from Invitrogen, and blebbistatin and 6-bromoindirubin-3′-acetoxime from Calbiochem. Aggrecan, polylysine, and anti-β-tubulin antibodies were from Sigma-Aldrich. Anti-nonmuscle myosin IIA (NMIIA) antibodies were kindly provided by Robert Adelstein (National Institutes of Health, Bethesda, MD), and anti-NMIIB antibodies were from Covance. All fluorescence-tagged secondary antibodies were provided by Molecular Probes. NMIIA, NMIIB (ON-TARGETplus SMART POOL), and control siRNAs were obtained from Thermo Scientific Dharmacon.

Cell Culture and Substrate Coating. Dorsal root ganglion (DRG) neurons were isolated from embryonic day 14–15 embryos or from 8–12 wk adult mice, and cerebellar granule neurons were prepared from postnatal day 4–8 mice, according to the Institutional Animal Care and Use Committee regulations. Dissection and culture conditions are described in detail elsewhere (1, 2). To study axon growth on permissive substrates, glass coverslips were coated with a mixture of poly-D-lysine (100 μg/mL) and laminin (5 or 10 μg/mL) for 2–4 h. For preparing inhibitory substrates, glass coverslips were coated with poly-D-lysine (100 μg/mL) for 1 h, washed with PBS, and then coated with a mixture of laminin and 10 μg/mL CSPGs (or 50 μg/mL aggrecan) for 2–4 h. Polylysine was coated under all conditions to support neuronal attachment. For adult DRG neurons, 10 μg/mL of laminin was used. For embryonic DRG neurons, 5 μg/mL laminin was used unless indicated otherwise because at this concentration growth cones of embryonic DRG neurons were larger and thus enabled detailed analysis of the cytoskeletal proteins in the growth cone.

Microfabrication and Substrate Coating of the Two-Compartment Chamber. A microfluidic two-compartment chamber was prepared as previously described (3). Polylysine, laminin, and CSPGs were used at a concentration of 100 μg/mL, 10 μg/mL, and 10 μg/mL, respectively. To create permissive substrates for both the somal and the axonal compartments in the two-compartment chamber experiments (as in Fig. 6*D*, *Top*), the chamber was uniformly coated with a mixture of polylysine (100 μg/mL) plus laminin (10 μg/mL). To create a boundary for the permissive–inhibitory environment, after the uniform coating step with a mixture of polylysine and laminin, the axonal compartment was additionally coated with CSPGs. The boundary between the permissive and the inhibitory terrains was ensured through careful deposition. CSPGs were introduced into the axonal chamber very slowly and carefully using a micropipette. The hydrostatic pressure difference between the somal and axonal chambers produces a fluid flow that prevents the diffusion of CSPGs into the microchannels. DRG neurons were loaded into the somal side of devices and grown for 2–4 d before blebbistatin was treated. Control and blebbistatin-treated neurons were examined at the same time to compare axon growth.

RNA Interference. For knocking-down experiments, neurons were transfected with siRNAs against NMIIA (siNMIIA) and/or NMIIB (siNMIIB) together with Venus, a variant of YFP, to identify transfected neurons. Transfection was performed according to the Amaxa protocols for mouse neurons. Briefly, dissociated adult DRG neurons were spun down to remove the supernatant completely and resuspended in 100 μL of Amaxa electroporation buffer with 2 μg of Venus and 2 μg of siRNA. For control, 2 μg of control siRNA (Thermo Scientific Dharmacon) was used. Suspended cells were then transferred to a 2.0-mm cuvette and electroporated with an Amaxa Nucleofector apparatus. After electroporation, cells were immediately transferred to the desired volume of prewarmed culture medium and plated on culture dishes coated with polylysine (100 μg/mL) plus laminin (10 μg/mL). After neurons fully attached to the substrates (3–5 h), medium was replaced to remove the remnant transfection buffer. Transfected neurons were replated at 4 d after transfection on glass coverslips coated with permissive or inhibitory substrates, as indicated.

Immunostaining and Fluorescence Microscopy. For NMII staining, neurons were simultaneously fixed and detergent-extracted using 0.15% glutaraldehyde, 0.2% TX-100, and 4% paraformaldehyde to remove soluble proteins. To stain growth cone MTs, neurons were fixed with prewarmed 4% paraformaldehyde containing 0.25% glutaraldehyde at 37 °C for 15 min. For all other staining, neurons were fixed with 4% paraformaldehyde at room temperature for 20–30 min. To visualize and quantify F-actin content in the growth cone, growth cones were stained with Alexa-594 phalloidin as previously described (4). All coverslips in any one experiment were fixed and processed together. For collection of fluorescence images, all images for one experiment were acquired in a single session. Parameters of time interval and gain setting on the digital camera were adjusted so the brightest areas did not reach saturation, and the same gain and time interval was applied to capture all images of any particular staining. An average intensity of phalloidin staining after background subtraction was determined for the entire growth cone area. Neurons were viewed with an inverted light microscope (Zeiss Axiovert 200, Carl Zeiss MicroImaging) equipped with epifluorescence optics. Images were captured with a CCD camera controlled by the Axiovision software. A 5×, a 10×, or a 20× objective (0.45 N.A.) was used to record whole neurons, and a 100× (1.3 N.A.) oil objective was used for high-resolution imaging of growth cone cytoskeletal structures.

Time-Lapse Video Microscopy. For time-lapse analysis of axon growth, DRG neurons from embryonic day 14 embryos or conditioning lesioned mice (7 d after conditioning lesion) were plated on permissive or inhibitory substrates, as indicated. Neurons growing on permissive substrates were allowed to attach for 5–7 h before the time-lapse capture began. For neurons growing on aggrecan, imaging was initiated after overnight culture to ensure the inhibitory effect of aggrecan. For neurons growing in the two-compartment chambers, recording was initiated when axons entered the microchannels, which was typically 2–4 d after plating the neurons in the somal side. Live cell imaging was performed in an environmental chamber (37 °C, 5% CO_2) mounted onto the stage of a motorized inverted microscope (Zeiss Axiovert 200M) equipped with a CCD camera. Digital images were automatically recorded using the AxioVision software (Carl Zeiss MicroImaging).

1. Vyas AA, et al. (2002) Gangliosides are functional nerve cell ligands for myelin-associated glycoprotein (MAG), an inhibitor of nerve regeneration. *Proc Natl Acad Sci USA* 99:8412–8417.

2. Zhou FQ, et al. (2006) NGF-induced axon growth is mediated by localized inactivation of GSK-3beta and functions of the microtubule plus end binding protein APC. *J Cell Sci* 119:2787–2796.

3. Yang IH, Siddique R, Hosmane S, Thakor N, Höke A (2009) Compartmentalized microfluidic culture platform to study mechanism of paclitaxel-induced axonal degeneration. *Exp Neurol* 218:124–128.

4. Roche FK, Marsick BM, Letourneau PC (2009) Protein synthesis in distal axons is not required for growth cone responses to guidance cues. *J Neurosci* 29:638–652.

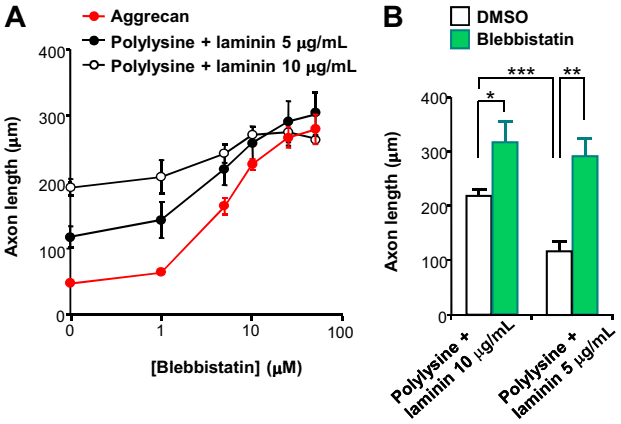


Fig. S1. Axon growth-promoting effect of blebbistatin on different substrates. (*A*) Embryonic DRG neurons were cultured on permissive (polylysine plus 5 or 10 μg/mL laminin, as indicated) or inhibitory substrates (aggrecan) and were treated with varying concentrations of blebbistatin or DMSO as a vehicle control. Neurons growing on different substrates were all fixed at the same time (at 12–14 h after plating) to measure axon length. (*B*) Embryonic DRG neurons were cultured on polylysine plus 5 or 10 μg/mL laminin, as indicated, and were treated with blebbistatin or DMSO as a vehicle control, as indicated. Neurons were all fixed at the same time (at 12–14 h after plating), and axon growth was assessed. **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

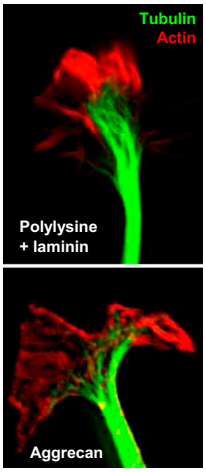


Fig. S2. Blockade of Rho kinase (ROCK) activity has little effect on growth cone cytoskeletal structures. Embryonic DRG neurons growing on permissive (polylysine plus laminin) or inhibitory substrates (aggrecan) were treated with Y27632 and were stained with phalloidin and anti-tubulin antibodies. Representative images are shown.

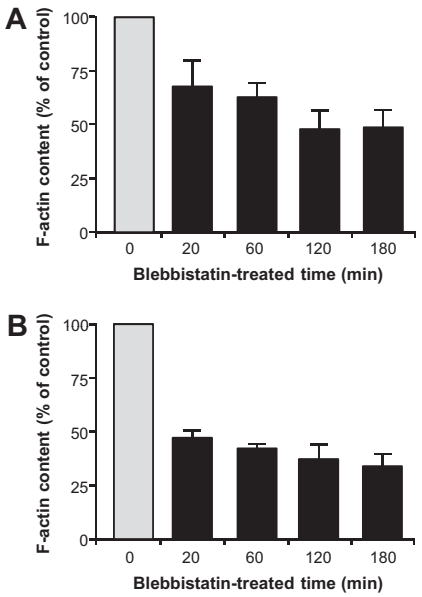


Fig. S3. Blebbistatin reduces F-actin content in the growth cone on permissive and inhibitory substrates. Embryonic DRG neurons cultured on polylysine plus laminin (*A*) or aggrecan (*B*) were treated with DMSO (0 min) or blebbistatin, as indicated. Neurons were stained with phalloidin, and F-actin content in the growth cone was quantified as described in *SI Materials and Methods*.

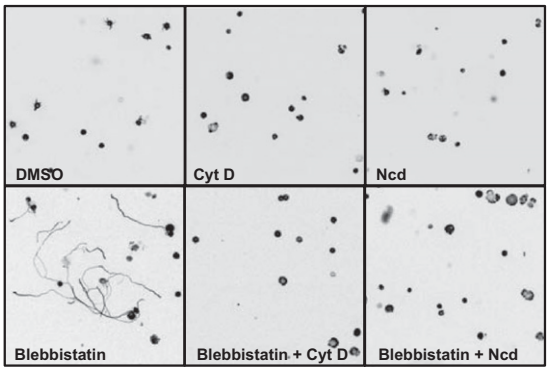


Fig. S4. Initiation of axon growth over CSPGs induced by NMII inhibition requires actin and MT dynamics. Adult DRG neurons from conditioning lesioned mice were plated on CSPGs and were treated with cytochalasin D (Cyt D) (100 nM), nocodazole (Ncd) (50 nM), or DMSO as a vehicle control in the presence or absence of blebbistatin as indicated. Note that initiation of axons was completely prevented by application of cytochalasin D or nododazole.

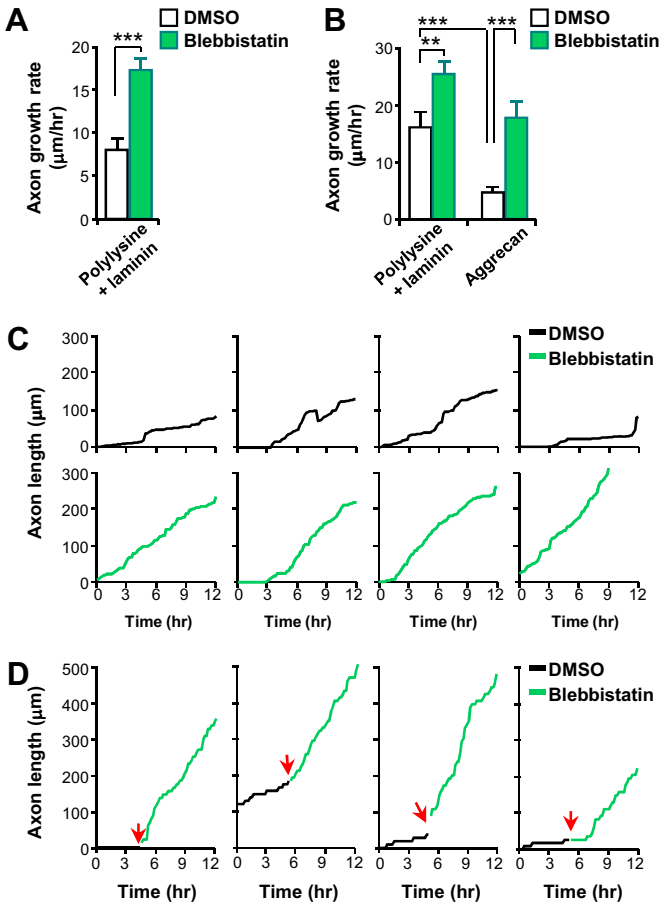


Fig. S5. Inhibition of NMII activity accelerates axon growth rate. (*A* and *C*) Embryonic DRG neurons were cultured on polylysine plus laminin in the presence or the absence of blebbistatin, as indicated, and axon growth was monitored by time-lapse microscopy. Average axon growth rate obtained from 12 h of recording is presented in *A*, and the growths of representative axons are presented in *C*. (*B* and *D*) Adult DRG neurons from conditioning lesioned mice were cultured on permissive or inhibitory substrates in the presence or absence of blebbistatin, as indicated, and axon growth was monitored by time-lapse microscopy. Average axon growth rate obtained from 12 h of recording is presented in *B*, and the growth of representative axons over aggrecan before and after blebbistatin treatment is presented in *D*. The red arrows indicate the time points when blebbistatin was added. Note the immediate acceleration of axon growth upon the addition of blebbistatin. $^{**}P < 0.01$; $^{***}P < 0.001$.

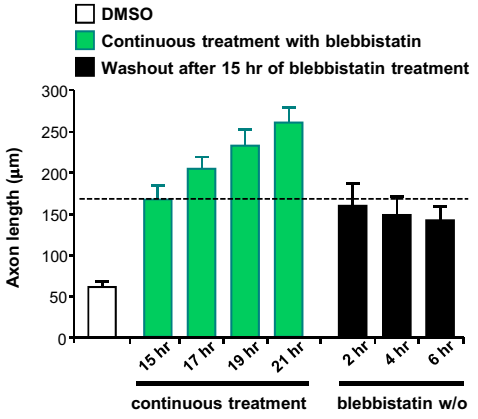


Fig. S6. Axon growth-promoting effect of blebbistatin is reversible. Adult DRG neurons from conditioning lesioned mice were cultured on CSPGs and were treated with blebbistatin (green and black bars) or DMSO as a control (white bar). After 15 h of treatment, blebbistatin was washed out and were treated with DMSO for the indicated period (black bars). Note that the axons stopped growing after blebbistatin washout (black bars), as opposed to the actively growing axons that were continuously treated with blebbistatin (green bars).

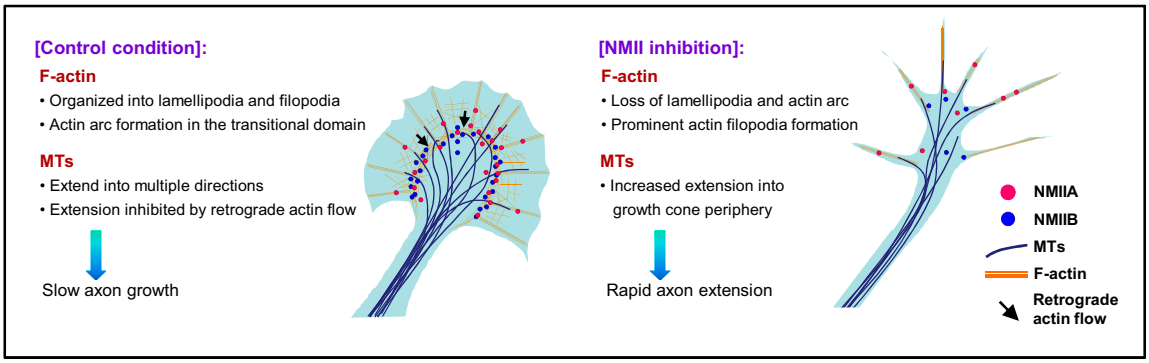
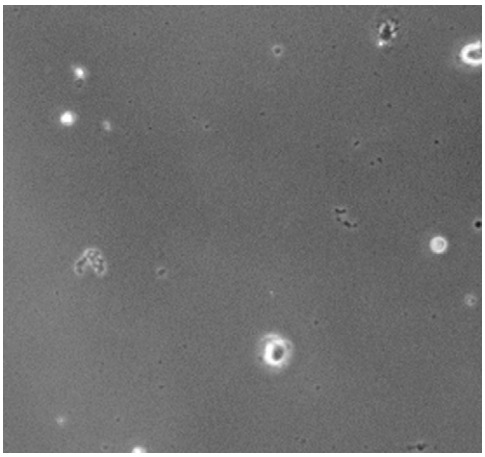
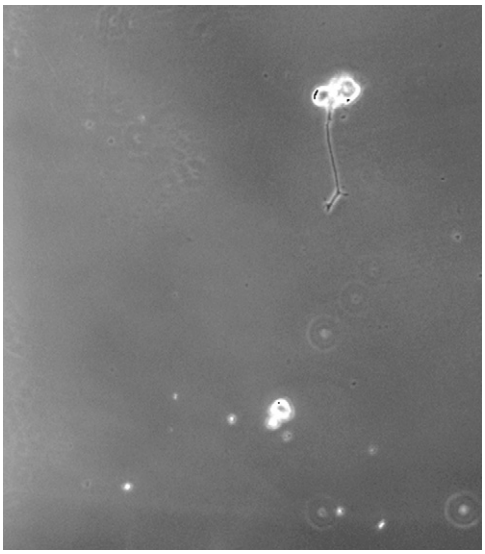


Fig. S7. Suggested model for how inhibition of NMII promotes axon growth over inhibitory substrates. In control growth cones, actin filaments, in particular the actin arc structures, actively inhibit MT extension via NMII-driven retrograde flow. NMII inhibition induces loss of lamellipodial areas, depletion of actin arc structures in the transitional zone, and reduction in total F-actin content in the growth cone. By contrast, actin bundles in the growth cone periphery become more prominent. Together with the reorganization of F-actin, marked extension of MTs into the growth cone periphery occurs, and MTs eventually form tight bundles. The interplay between F-actin and MTs is required for the acceleration of axon growth over inhibitory substrates.



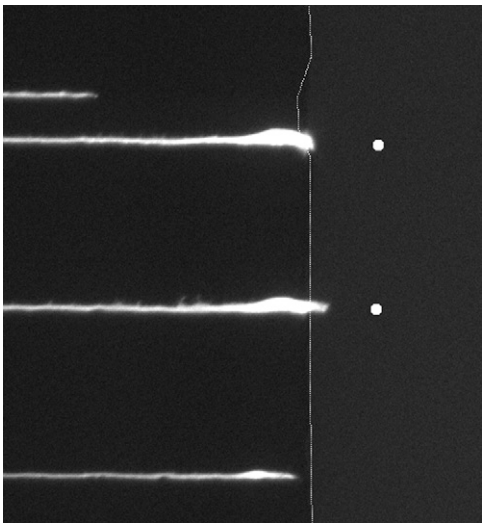
Movie S1. NMII inhibition by blebbistatin triggers marked acceleration of axon growth from adult DRG neurons on aggrecan. Adult DRG neurons from conditioning lesioned mice were plated on aggrecan, and axon growth was monitored by time-lapse microscopy. Recording was started at 15 h after plating and pictures were taken every 10 min. After blebbistatin treatment ("+ Blebbistatin"), recording was reinitiated and pictures were taken at 10-min intervals. Note that neurons cultured on aggrecan had very short or no axons even after overnight culture, but started to grow axons robustly upon exposure to blebbistatin.

Movie S1



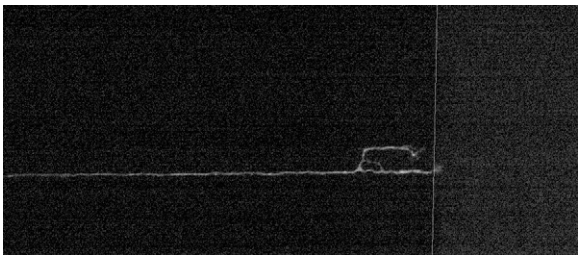
Movie S2. NMII inhibition by blebbistatin triggers marked acceleration of axon growth from adult DRG neurons on aggrecan. Adult DRG neurons from conditioning lesioned mice were plated on aggrecan, and axon growth was monitored by time-lapse microscopy. Recording was started at 15 h after plating and pictures were taken every 10 min. After blebbistatin treatment ("+ Blebbistatin"), recording was reinitiated and pictures were taken at 10-min intervals. Note that neurons cultured on aggrecan had very short or no axons even after overnight culture, but started to grow axons robustly upon exposure to blebbistatin.

Movie S2



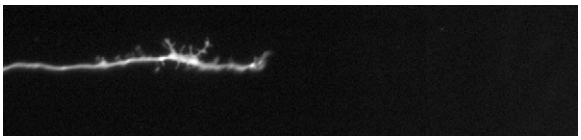
Movie S3. Axons facing the permissive–inhibitory boundary in the two-compartment chamber system do not grow into the CSPG-coated axonal compartment and form dystrophic end bulbs. Adult DRG neurons were plated in the somal compartment and allowed to grow axons in the two-compartment chamber system. Axons entering the microchannels grew inside the channels toward the axonal compartment (right). When the axons encountered the permissive–inhibitory border (dotted line), axons in the microchannels failed to enter the axonal compartment and generated retraction bulb-like swellings at the border. Note that the dystrophic growth cones retract back into the channels (white circles) after exploring the CSPG-coated axonal compartment.

Movie S3



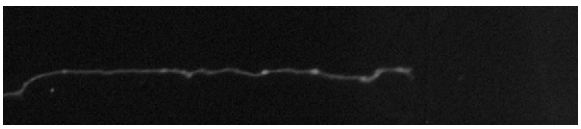
Movie S4. Time-lapse recording of a representative axon facing the permissive–inhibitory boundary in the two-compartment chamber system. Adult DRG neurons were plated in the somal compartment and allowed to grow axons in the two-compartment chamber system. Once the axons entered the microchannels, they grew inside the channels toward the axonal compartment (right). Shown is an axon growing along the permissive–inhibitory border (dotted line) as it exits the microchannel. Note that this axon grows back into a microchannel, which is coated with permissive substrates, to avoid the CSPG-coated inhibitory terrain.

Movie S4



Movie S5. Local administration of blebbistatin to the axonal compartment enables growth cones to cross the permissive–inhibitory boundary. Adult DRG neurons were plated in the somal compartment and allowed to grow axons in the two-compartment chamber system. Axons entering the microchannels grew inside the channels toward the axonal compartment (right). Blebbistatin was locally applied to the axonal compartment. Note that axons grow into the CSPG-coated axonal compartment without pausing at the permissive–inhibitory border (dotted line).

Movie S5



Movie S6. Local administration of blebbistatin to the axonal compartment enables growth cones to cross the permissive–inhibitory boundary. Adult DRG neurons were plated in the somal compartment and allowed to grow axons in the two-compartment chamber system. Axons entering the microchannels grew inside the channels toward the axonal compartment (right). Blebbistatin was locally applied to the axonal compartment. Note that axons grow into the CSPG-coated axonal compartment without pausing at the permissive–inhibitory border (solid line).

Movie S6