

Neu3 Sialidase-Mediated Ganglioside Conversion Is Necessary for Axon Regeneration and Is Blocked in CNS Axons

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PNS axons have a high intrinsic regenerative ability, whereas most CNS axons show little regenerative response. We show that activation of Neu3 sialidase, also known as Neuraminidase-3, causing conversion of GD1a and GT1b to GM1 ganglioside, is an essential step in regeneration occurring in PNS (sensory) but not CNS (retinal) axons in adult rat. In PNS axons, axotomy activates Neu3 sialidase, increasing the ratio of GM1/GD1a and GM1/GT1b gangliosides immediately after injury *in vitro* and *in vivo*. No change in the GM1/GD1a ratio after axotomy was observed in retinal axons (*in vitro* and *in vivo*), despite the presence of Neu3 sialidase. Externally applied sialidase converted GD1a ganglioside to GM1 and rescued axon regeneration in CNS axons and in PNS axons after Neu3 sialidase blockade. Neu3 sialidase activation in DRGs is initiated by an influx of extracellular calcium, activating P38MAPK and then Neu3 sialidase. Ganglioside conversion by Neu3 sialidase further activates the ERK pathway. In CNS axons, P38MAPK and Neu3 sialidase were not activated by axotomy.

Key words: axon regeneration; axotomy; gangliosides; Neu3 sialidase

Introduction

Mammalian CNS axons fail to regenerate after damage, whereas PNS axons can regenerate vigorously, many reconnecting with their targets. Mature CNS axons have been shown to be intrinsically poor at regenerating even in permissive environments (Sun and He, 2010; Moore and Goldberg, 2011; Wang and Jin, 2011; Chew et al., 2012; Cho and Cavalli, 2012). Successful axon regeneration begins with growth cone formation involving coordinated proteolytic, cytoskeletal, and cell surface regulatory events (Bradke and Dotti, 1999; Long and Lemmon, 2000; Erez et al., 2007; Witte et al., 2008; Afshari et al., 2009; Bradke et al., 2012) as well as activation of local protein translation as in PNS axons (Zheng et al., 2001; Verma et al., 2005). Integrins and other signaling molecules involved with control of regeneration can asso-

ciate with lipid microdomains, leading to modulation of many intracellular signaling events (Guirland et al., 2004; Kamiguchi, 2006; Guirland and Zheng, 2007; Grider et al., 2009; Ohkawa et al., 2010; Stuermer, 2010; Gao et al., 2011; Norambuena and Schwartz, 2011).

Gangliosides, complex glycosphingolipids on the plasma membrane containing one or more sialic acid residues, are key components of microdomains. The adult mammalian nervous system has four dominant types of gangliosides: GM1, GD1a, GD1b, and GT1b. Whereas embryos can develop normally without complex gangliosides, postnatally they develop demyelination, neurodegeneration, and impaired peripheral nerve regeneration (Sheikh et al., 1999; Okada et al., 2002; Kittaka et al., 2008). GM1 ganglioside has been shown to promote axon growth (Facci et al., 1984; Skaper et al., 1985) and regeneration (Yang et al., 2006; Mountney et al., 2010), but a clinical trial of GM1 treatment in spinal cord injury was not successful (Geisler et al., 2001). GD1a ganglioside, on the other hand, has been shown to be a receptor for inhibition of axon growth by MAG (Vyas et al., 2002; Zhang et al., 2011), and GT1b forms a complex with the Nogo receptor to inhibit axon growth (Williams et al., 2008). Neu3 sialidase, an enzyme that hydrolyzes polysialic acid-containing gangliosides to GM1 ganglioside (Geisler et al., 2001; Vyas et al., 2002), acts on outer sialic acid groups and has been shown to effectively increase GM1 levels (Oehler et al., 2002; Monti and Miyagi, 2012). Additionally, Neu3 has been shown to be necessary for neuronal differenti-

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ation (Da Silva et al., 2005), for maintenance of polarity after axotomy (Rodriguez et al., 2001), and for regulating invasive and migratory activity (Yamaguchi et al., 2006; Miyata et al., 2011; Tringali et al., 2012).

In this paper, we examine Neu3 sialidase action on axonal gangliosides and resultant axon regeneration, and we assess whether these mechanisms help to explain the different regeneration capacities of CNS and PNS axons. We find that after PNS axotomy, but not CNS, Neu3 sialidase converts GD1a to GM1. Pharmacological blocking of Neu3 sialidase, downregulation of Neu3 with siRNA, or depleting gangliosides all inhibit PNS regeneration. Conversely, exogenously applied sialidase increases GM1 ganglioside and promotes regeneration in CNS axons, suggesting that conversion of polysialylated gangliosides is necessary for axon regeneration.

Materials and Methods

Chemicals and reagents

All chemicals, antibodies, and reagents were purchased from Sigma-Aldrich, unless otherwise specified.

DRG cultures

Adult (3- to 4-month-old) male Sprague Dawley rats were euthanized and DRGs removed.

Explants

DRGs were trimmed of meningeal coverings, divided into segments, and plated onto coated chamber slides (Nunc) [10 μ g/ml poly-D-lysine (PDL) and 1 μ g/ml laminin]. Explants were maintained at 37°C and 7% CO₂ in DMEM (Invitrogen) with 0.11 g/L sodium pyruvate with pyridoxine, 4.5 g/L glucose, 20 ng/ml NGF, 1% insulin-transferrin-selenium, 1% penicillin-streptomycin-fungizone.

Dissociated DRGs

DRGs were dissociated with 0.2% collagenase followed by 0.1% trypsin and triturated with Pasteur pipettes of decreasing tip diameters. The suspension was centrifuged through a density gradient (15% BSA). Cells were cultured in DMEM supplemented with 10% FBS (Invitrogen), 1% penicillin-streptomycin-fungizone, 0.1% mitomycin-C, and 10 ng/ml NGF and plated on PDL (20 μ g/ml) + laminin (10 μ g/ml).

Retina explant cultures

Unilateral conditioning optic nerve crush was performed on adult male Sprague Dawley rats, 7 d before retinal dissection. Retinas were separated from underlying pigment epithelium and sclera in HBSS before mounting flat onto Petri dishes and cutting into 400- μ m-thick sections using a McIlwain tissue chopper (Vibratome). Retinal sections were plated onto plastic chamber slides coated with 300 μ g/ml PDL and 10 μ g/ml laminin, with the ganglion cell layer facing the substratum. Cultures were maintained in DMEM and Neurobasal-A (Invitrogen) in a 1:1 ratio, supplemented with 50 IU erythropoietin-A, 760 μ g/ml BSA, N2 supplement (Invitrogen), 100 μ g/ml sodium pyruvate, 4 μ g/ml of T3-T4, 2 mM glutamine (Invitrogen), 1.1 mg/ml glucose, 100 μ g/ml gentamycin (Invitrogen), and 50 ng/ml ciliary neurotrophic factor.

Axon regeneration assay

Axons were grown for 48–72 h (DRGs) or 5–7 d (retina explants) and then axotomized with a pulled glass electrode leaving the site of axotomy clearly demarcated on the plastic chamber slide. Axons were photographed immediately, 15 min, and 1 h after injury and assessed for regeneration. Images were captured on a Nikon phase-contrast ELWD 0.3 microscope equipped with a Nikon DXM 1200 digital camera using a 10 \times (10 \times /0.25) or 20 \times (20 \times /0.4) objective lens. For analysis, only cut axons that had retracted and begun to grow back were regarded as regenerating axons.

Pharmacological treatments

Inhibition of ganglioside synthesis. Axons were treated with either fumonisin B1 (3 μ M) or (\pm)-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol hydrochloride (PDMP) (20 μ M) 18 h before axotomy.

Modulation of Neu3 sialidase activity. Axons were treated with 2,3-dehydro-2-deoxy-N-acetylneuraminic acid (NeuAc2en; 800 μ M) for inhibition of Neu3 sialidase activity and/or with exogenous *Clostridium* sialidase (0.4 IU; Roche), immediately before axotomy. NeuAc2en was removed before adding *Clostridium* sialidase as NeuAc2en inhibited *Clostridium* sialidase activity.

Modulation of signaling pathways. To analyze the regulation of Neu3 sialidase activity after adult DRG axotomy, one of the following was added to the media 30 min before axotomy and left in the media for a further 60 min: U0126 (MEK-ERK inhibitor, 20 μ M, Promega), rapamycin (mTOR inhibitor, 10 nM, Calbiochem), and SB203580 (P38MAPK inhibitor, 5 μ M, Calbiochem). Anisomycin (10 μ M, Alomone Labs) was used to activate P38MAPK.

To examine the effect of absence of eCa²⁺ on Neu3 sialidase activity and axonal regeneration, cultures were incubated with normal growth media devoid of CaCl₂, 30 min before axotomy.

siRNA transfection

Dissociated DRGs were transfected with siRNA 48 h after plating. The Neu3 siRNA consists of four siRNA sequences: GCAGAGAUGCGUAC-CUCAA, CCAACAACUCUGCGAGCCU, CCAAACAAAUUCCGAG-CAG, and GGACAGGGCUUGUUCGCGU (ON-TARGETplus SMART pool rat Neu3 siRNA, 117185, Thermo Scientific). siGLO RISC-Free siRNA (Thermo Scientific) was used as a negative control and transfection indicator. Briefly, DharmaFECT 3 (Thermo Scientific) and siRNA were diluted separately in serum-free DMEM supplemented with 1% insulin-transferrin-selenium and 10 ng/ml NGF before mixing together and incubating with the DRGs (100 nM; 4 h) and then replacing with normal medium.

RNA purification and RT-PCR

Dissociated DRGs were lysed and collected as per the manufacturer's instructions (PureLink RNA Mini Kit, Invitrogen) before being homogenized (Ultra-Turrax T8 homogenizer, IKA-Werke). RNA purification was performed before reverse transcription. The purified RNA was then reverse-transcribed using the SuperScript III First-Strand Synthesis SuperMix (Invitrogen). The cDNA synthesis reaction was followed by PCR with the following primers: Neu3, forward, 5'-CAGCTGGGATAGCA-GAGGTC-3'; reverse, 5'-GAGTCCTGAAGCAAGCCAAC-3', resulting in a 209 bp fragment; Neu4, forward, 5'-CCTGACCCTAGGACGAA-CAG-3'; reverse, 5'-GATGTGCGTGGTGATCAGAG-3', resulting in a 179 bp fragment. The PCR was run at 26, 29, and 32 cycles separately and imaged using agarose gel electrophoresis.

Immunocytochemical staining of cultures

Both DRG and retina explant cultures were fixed with 4% PFA for 10 min and washed with PBS before immunostaining. To analyze Neu3 sialidase activity, we calculated the relative levels of GD1a (substrate) and GM1 (product) on individual axonal membranes rather than absolute mean values of GD1a and GM1 ganglioside. A mean of 30–60 axons per chamber (2 DRGs/chamber) was considered as $n = 1$ /experimental condition. Fixed cultures were incubated with one of the following antibodies: rabbit anti-Neu3 sialidase/PMGS (Rodriguez et al., 2001), mouse anti-GD1a ganglioside (kind gift from Prof. Ronald Schnaar, Johns Hopkins University, Baltimore, MD), mouse anti-GD1a ganglioside and mouse anti-GT1b ganglioside (Merck Millipore) or stained using recombinant cholera toxin-B subunit (CTB) conjugated to AlexaFluor-555 (Invitrogen), and mouse anti β -tubulin. To analyze the signaling cascade regulating Neu3 sialidase activity, DRG and retinal cultures were analyzed with the following primary antibodies: mouse anti-P38MAPK, rabbit anti-phospho P38MAPK, and rabbit anti-ERK and mouse anti-phospho ERK (Cell Signaling Technology). Subsequently, cultures were washed in PBS and incubated with the following fluorescent secondary antibodies:

anti-rabbit AlexaFluor-660, and anti-mouse or anti-rabbit AlexaFluor-488 (Invitrogen) before being mounted onto slides using Fluorosave (Calbiochem).

Surgeries

All surgeries were performed in accordance with the United Kingdom Animals (Scientific Procedures) Act of 1986 and United Kingdom Home Office regulations. Adult (2- to 3-month-old) male Sprague Dawley rats were used for all *in vivo* experiments. Animals were kept in 12 h light/dark exposure, and food and water was provided *ad libitum*.

Animals were anesthetized using 1–2% isoflurane in a mixture of 50% nitrous oxide and 50% oxygen for sciatic nerve surgery. The right sciatic nerve was exposed and crushed proximal to its division into tibial and common peroneal nerves with Dumont #5 forceps, crushing twice for 10 s duration each.

Optic nerve crushes were performed under ketamine:xylazine (2:1) anesthesia at a dosage of 0.1 ml/100 g. The conjunctiva of the right optic nerve was cut laterally near the cornea, and the retractor bulbar muscle was separated. The optic nerve was exposed and cleared of fascial coverings and crushed twice for 10 s duration each time.

Immunohistochemistry

Animals were euthanized with an overdose of Euthatal (sodium pentobarbital) at different time points after injury (2, 6, and 12 h) and transcardially perfused with PBS, pH 7.4, and 4% PFA, pH 7.4. A 2-cm-long segment of sciatic or optic nerve centered around the crush site (or contralateral control nerves) was removed and postfixed overnight in 4% PFA before cryoprotecting in 30% sucrose in PBS, pH 7.4. Tissue was embedded in OCT (RA Lamb) and sectioned longitudinally at 12 μ m (sciatic nerve) or at 10 μ m (optic nerve) thickness in a cryostat and mounted on slides (Superfrost Plus; VWR).

Sections were permeabilized in 0.2% saponin in PBS and blocked with 10% NGS and 2% BSA in PBS before incubating with one of the following antibodies: rabbit anti neurofilament-heavy chain (sciatic nerve) or rabbit anti- β III tubulin, mouse anti-GD1a, CTB (for GM1 ganglioside), and anti-S100 β . Sections were rinsed in PBS-Saponin before incubating with appropriate secondary antibodies, and coverslipping with Fluorosave.

Quantification of immunofluorescence

In vitro. Photographs of cut axons were captured on a Leica DM6000B epifluorescent microscope equipped with a Leica DFC 350FX digital camera (Leica) using a 40 \times objective lens (40 \times /1.00–0.50 Oil). Image acquisition using Leica Application Suite used identical exposure times and microscope settings. Quantification of images was performed using NIH ImageJ software.

In vivo. Photographs of sciatic (SN) and optic nerve (ON) sections adjacent to the crush site were captured using a Leica TCS SP2 confocal microscope (Leica) using 40 \times (40 \times /1.15 Oil CS) (for SN) or 63 \times (63 \times /1.30 Oil CS) (for ON) magnification. Image acquisition using Leica Application Suite used identical exposure times and microscope settings. Image quantification was performed using confocal software using optical sections (0.800 μ m thick) to analyze the levels of GM1 and GD1a gangliosides parallel and between axonal and Schwann cell markers. Areas of interest were randomly chosen based on areas adjacent and parallel to NF-H-positive staining from a single confocal image. Intensities were measured as ratio of CTB (for GM1 ganglioside):GD1a ganglioside normalized against background. In experiments where CTB or Neu3 sialidase intensity was measured, normalization was performed using intensity of an axonal marker, β III-tubulin. To ensure that intensity differences were not due to differences in axon size, intensity measurements were normalized for the area of individual axon. Images were processed with Adobe Photoshop CS4 and assembled in Adobe Illustrator CS4.

Statistical analysis

Data were first analyzed for normality using Shapiro-Wilk normality test (Origin version 8; Originlab). *p* values of all data were above set α levels

of 0.05 and therefore considered to be normally distributed. Results from the analyses were expressed as mean \pm SEM. Statistical analysis was performed using Prism version 5 (Graphpad). For all experiments, Student's *t* test (two-tailed), one-way ANOVA, and two-way ANOVA were applied as appropriate. Significant interactions were analyzed using paired *t* tests and Bonferroni *post hoc* tests as appropriate.

Results

Axotomy leads to an increase in the ratio of GM1/GD1a gangliosides due to Neu3 sialidase activation

As a model of the first steps in successful axon regeneration, we used regeneration of growth cones in adult DRG axons grown on PDL/laminin. These axons regenerate a new growth cone and begin to elongate within 30 min after axotomy (Chierzi et al., 2005). We investigated levels of GM1 and GD1a gangliosides on these axons and asked whether their relative levels change after axotomy and during successful neurite regeneration. Both DRG explant and dissociated cultures were fixed at 15 min or 1 h after axotomy and stained with CTB (marker for GM1 ganglioside, which we use throughout the paper to label GM1) and anti-GD1a ganglioside antibody (Fig. 1). Axotomy led to conversion of most of the GD1a to GM1, the ratio of GM1/GD1a having doubled within 15 min and returned back to uncut levels by 1 h (Fig. 1A–C, G, H–J, N). The change in the ratio was observed along the entire length of axons in our field of observation (200–300 μ m from the proximal end of cut axons). Results are expressed as a ratio between GM1 and GD1a because absolute levels in individual axons vary depending on axon size while the ratio varies little, serving as a more representative indicator of local Neu3 sialidase activity on axons. The overall ganglioside level, measured as GD1a + GM1, did not change significantly after axotomy or after other our interventions, except for inhibition of ganglioside synthesis (data not shown). Because desialylation of GD1a to produce GM1 on the cell surface is usually mediated by Neu3 sialidase, we confirmed the presence of Neu3 sialidase on DRG axons by immunostaining using an antibody described previously (Rodriguez et al., 2001) (Fig. 1V). Axons were immunolabeled without permeabilization demonstrating the presence of Neu3 sialidase on the axonal surface, which could be removed by inhibition of cholesterol synthesis with lovastatin or using methyl β cyclodextrin (data not shown), additionally confirming its presence in cholesterol-rich domains (Kopitz et al., 1996; Kalka et al., 2001; Miyagi et al., 2008a,b). To verify that conversion of GD1a to GM1 is due to the action Neu3 sialidase, we blocked its activity in DRG explant cultures using the specific sialidase inhibitor, NeuAc2en (Rodriguez et al., 2001), or in dissociated DRG cultures with siRNA for NEU3, the gene that generates Neu3 sialidase. The increase in GM1 after axotomy was greatly reduced by NeuAc2en treatment; there was only a small change in the GM1/GD1a ratio after axotomy (Fig. 1D–G). Using RNA interference for NEU3, we further confirmed that a reduction in Neu3 sialidase prevented the change in the GM1/GD1a ratio after axotomy (Fig. 1K–N; controls described below). Furthermore, as Neu3 sialidase acts to convert many gangliosides, including GD1a, GT1b, GD1b, and GQ1b to GM1, we investigated whether GT1b was also affected by axotomy. Comparable to GD1a, axotomy led to conversion of GT1b to GM1, the ratio of GM1/GT1b having almost tripled within 15 min and decreasing back near uncut levels by 1 h (Fig. 1O–Q, U). After siRNA knockdown of Neu3, the GM1/GT1b ratio was unchanged after axotomy (Fig. 1R–U). To determine whether axotomy increases GM1 conversion through Neu3 sialidase upregulation or by activation, we compared Neu3 sialidase protein levels on uncut axons and on

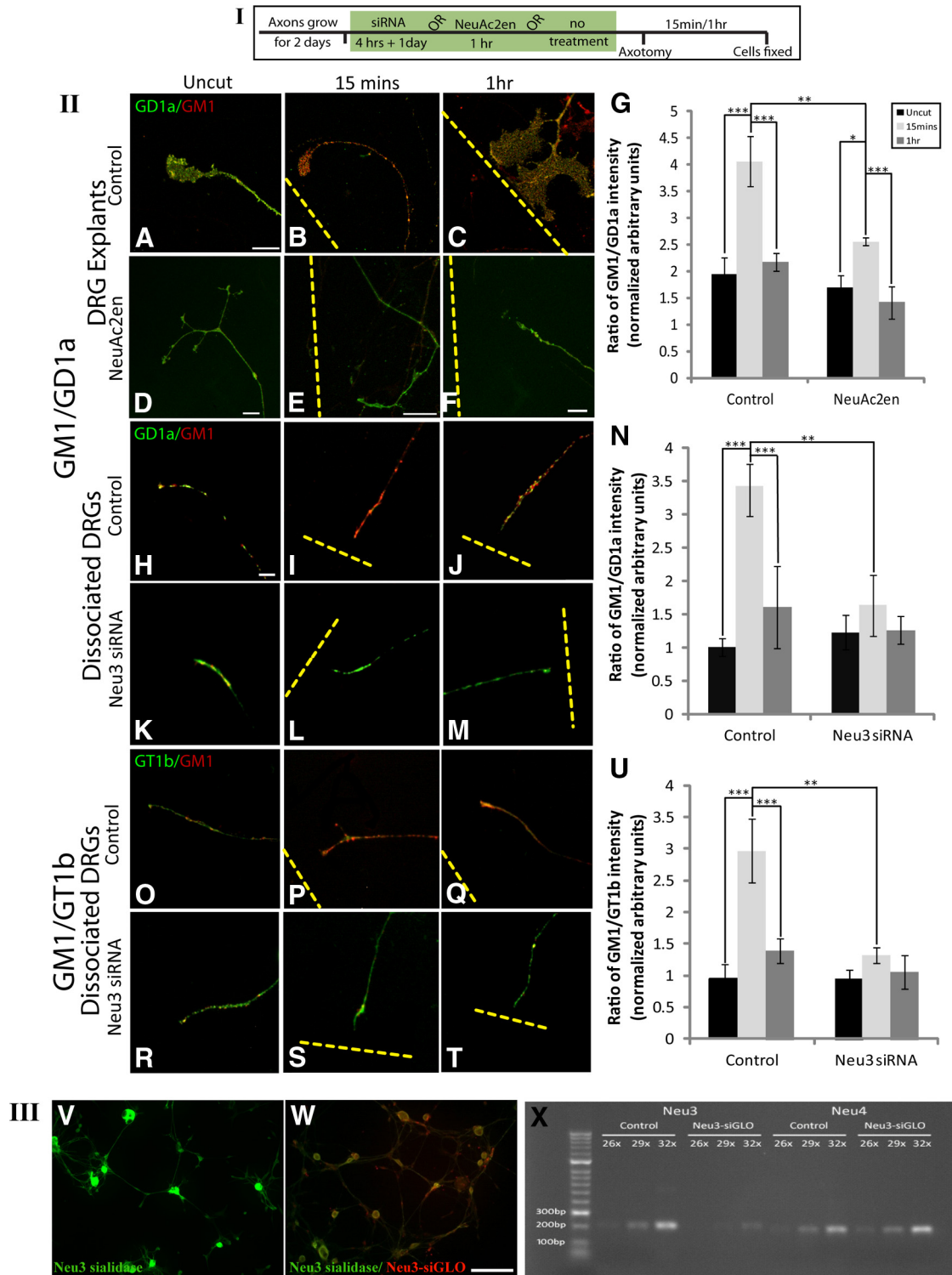


Figure 1. DRG axotomy causes a transient increase in the ratio of GM1/GD1a and GM1/GT1b gangliosides. *I*, Schematic of experimental design. *II*, Fluorescent images of adult DRG explants (*A–F*) or dissociated DRGs (*H–M*, *O–T*) with axons uncut (*A*, *D*, *H*, *K*, *O*, *R*), 15 min (*B*, *E*, *I*, *L*, *O*, *P*, *S*), or 1 h after axotomy (*C*, *F*, *J*, *M*, *Q*, *T*). DRGs are double-labeled for GD1a ganglioside (green) in *A–F*, *H–M* (or GT1b ganglioside in *O–T*) and CTB for GM1 ganglioside (red) under normal conditions (*A–C*, *H–J*, *O–Q*), after exposure to NeuAc2en (*D–F*), or after Neu3 siRNA transfection (*K–M*, *R–T*). In this and subsequent images, axotomy/nerve crush is indicated by dashed yellow lines, where applicable. GM1 and GD1a or GT1b ganglioside intensities were plotted (\pm SEM) in *G* (DRG explants, 40–50 axons/chamber, 2 DRG/chamber, $n \geq 4$ chambers) or *N*, *U* (dissociated DRGs, 2 axons/chamber, $n = 5$). *III*, Reduction of Neu3 expression with Neu3 siRNA. *V*, *W*, Fluorescent images of adult DRG neurons after immunocytochemistry for Neu3 sialidase, without siRNA transfection (*V*), or after Neu3-siGLO transfection (*W*). Neurons transfected with Neu3-siGLO (red; *W*) have reduced intensity for Neu3 sialidase staining (green). *X*, RT-PCR of siRNA-transfected adult DRG neurons, run at 26, 29, and 32 cycles. Neu3 siRNA is specific in knocking down the expression of Neu3 by ~70% and has no effect on Neu4, a closely related sialidase. Controls are siGLO-transfected-only neurons (nonfunctional and nontargeting siRNA). * $p < 0.05$ (one-way or two-way ANOVA, where appropriate). ** $p < 0.01$ (one-way or two-way ANOVA, where appropriate). *** $p < 0.001$ (one-way or two-way ANOVA, where appropriate). Scale bars: *A* for *A–F*, 25 μ m; *H* for *H–M* and *O–T*, 50 μ m; *V–W*, 250 μ m.

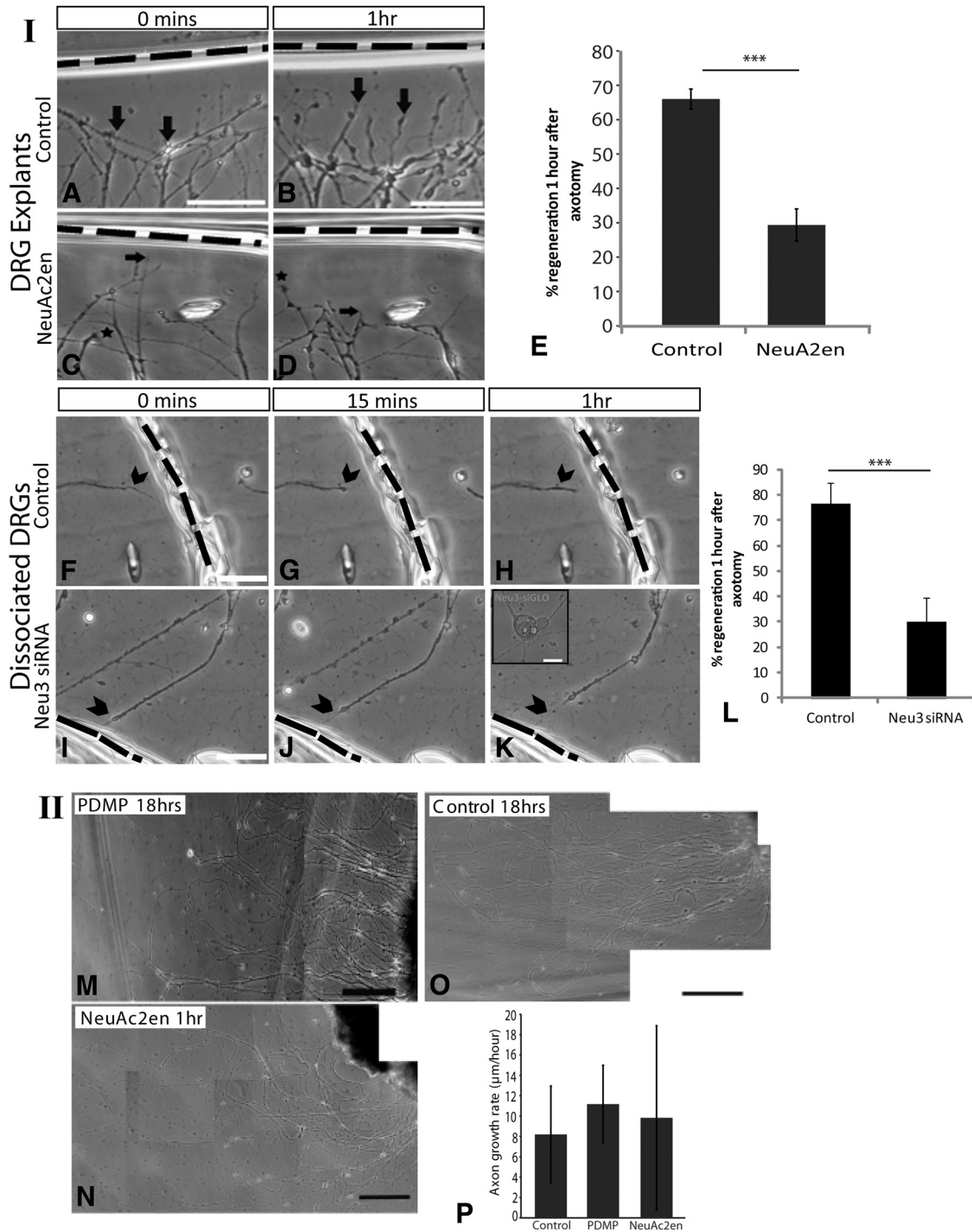


Figure 2. Reduction of Neu3 sialidase activity by the inhibitor, NeuAc2en, or by Neu3 siRNA transfection reduced regeneration after axotomy but not axon growth rate. *I*, Phase-contrast photomicrographs showing axons of DRG explants (*A–D*) or dissociated DRGs (*F–K*) immediately (*A, C, F, I*), 15 min (dissociated DRGs only; *G, J*), or 1 h after axotomy (*B, D, H, K*), under control conditions (*A, B, F–H*), and after exposure to NeuAc2en (*C, D*), or Neu3 siRNA transfection (*I–K*). Percentage regeneration 1 h after axotomy (\pm SEM) plotted in *E* ($n = 6$ DRG explants) or after Neu3 siRNA transfection in *L* (2 axons/chamber, $n = 15$ chambers, dissociated DRGs). Arrows/arrowheads indicate axons that were cut, retracted at 15 min, and regenerated at 1 h. *C, D*, \star An uncut axon that continued to grow in the presence of NeuAc2en. *K*, Inset, A merged phase-contrast and fluorescent image of the DRG cell body from *I–K* demonstrating the presence of siGLO (red) confirming the siRNA uptake in the neuron. Controls (*F–H*) were performed with siGLO transfection only, or no treatment (*A, B*). *II*, *M–O*, Phase-contrast photo-montages of adult DRG axons 18 h after exposure to PDMP (*M*), 1 h after exposure to NeuAc2en (*N*), and control (*O*). Photo-montages from similar areas were taken before addition of drugs and analyzed for growth rate in these as well as in corresponding control axons. Axon lengths were measured in drug-treated cultures and compared with corresponding control cultures to calculate the growth rate of axons (in $\mu\text{m}/\text{h}$; \pm SEM), which is plotted in *P* (each column represents a mean of 30–40 axons from $n = 3$ chambers/condition). $***p < 0.001$ (two-tailed Student's *t* test). Scale bars: *A–D*, 20 μm ; *F–K*, 100 μm ; *K*, inset, 50 μm ; *M–O*, 200 μm .

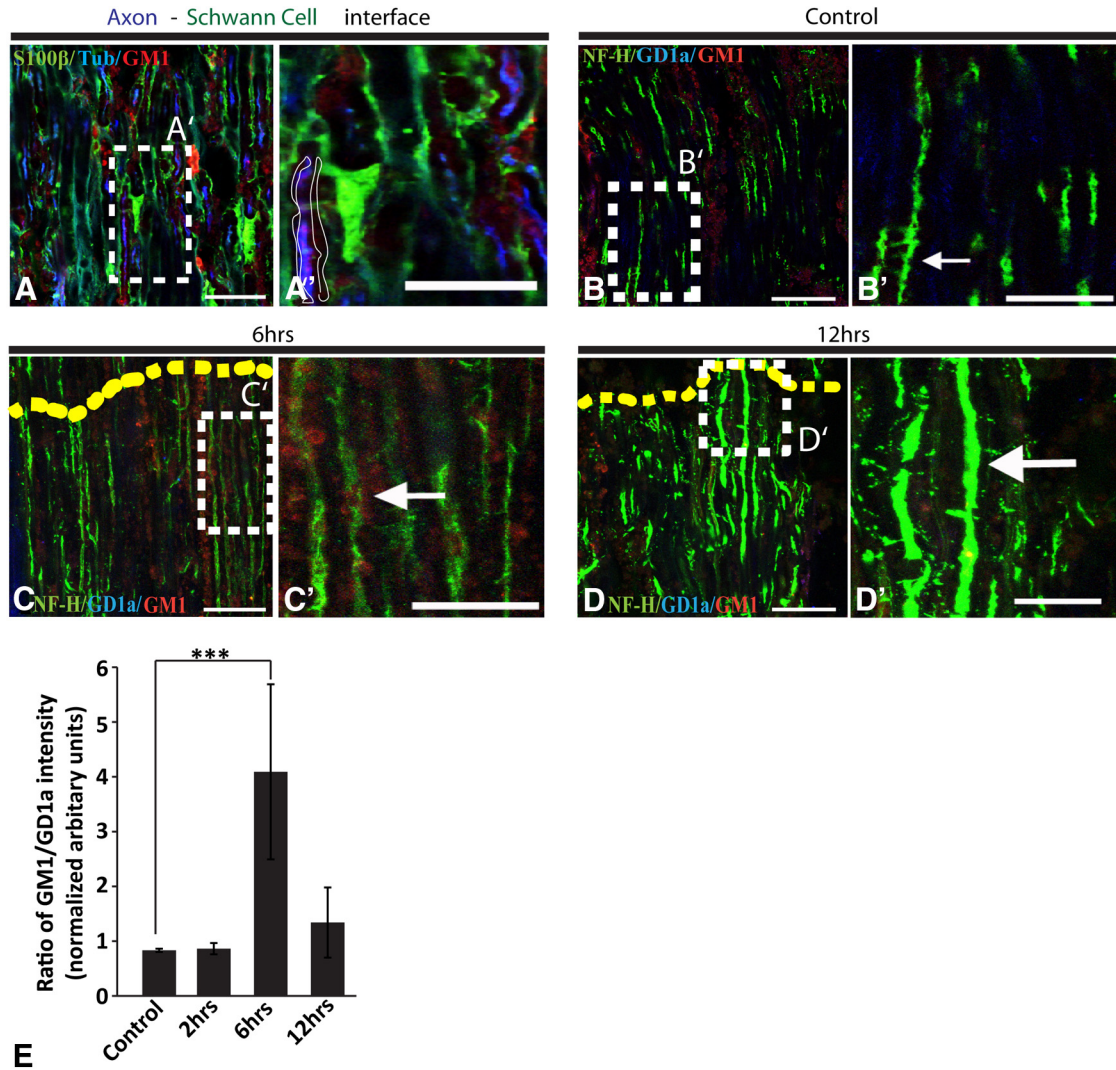


Figure 3. Neu3 sialidase is transiently activated in the sciatic nerve after crush injury. **A, A'**, Confocal micrograph of sciatic nerve immunolabeled with S100β (green), CTB (red), and βIII-tubulin (blue). Area within dotted line (**A'**) indicates representative regions parallel to axonal staining (blue) used to measure the intensities of GM1 and GD1a gangliosides. Schwann cell staining (green) performed to show its relative position to area of measurement. **B–D**, Fluorescent confocal images (longitudinal) of sciatic nerve uncrushed (**B, B'**), 6 h after crush (6 h; **C, C'**), and 12 h after crush (12 h; **D, D'**), triple-labeled for neurofilament-heavy chain (green), GD1a (blue), and GM1 gangliosides (CTB, red), with high-magnification images from dotted squares shown on right. Arrows indicate representative regions adjacent and parallel to axonal staining (NF-H-positive) used for measuring GM1 and GD1a ganglioside intensities. **E**, The ratio of GM1/GD1a ganglioside intensities (\pm SEM) over time after injury is plotted ($n = 6$ /time point). *** $p < 0.001$ (one-way ANOVA). Scale bars: **A–D**, 50 μ m; **A'–D'**, 25 μ m. Orientation of figures: top, caudal; bottom, rostral.

axons 15 min after injury by immunocytochemistry (Rodriguez et al., 2001; Da Silva et al., 2005). No significant change in Neu3 sialidase staining intensity was observed at 15 min after injury (data not shown), suggesting that an increase in Neu3 sialidase activity rather than an increase in expression or targeting to membrane was responsible for the acute rise in GM1/GD1a ratio after axotomy. These results indicate that reduction in Neu3 sialidase activity will reduce the level of desialylation of many gangliosides to GM1. However, to streamline our analyses for remaining experiments, we have chosen to assess changes in the ratio of GM1/GD1a intensity.

Knocking down the expression of Neu3 sialidase with siRNA does not affect the levels of closely related sialidases

To verify that the observed effect of knockdown of Neu3 on GM1 and GD1a and GT1b was specifically due to Neu3 sialidase, we evaluated expression levels of other related sialidases. After treatment with NEU3 siRNA, RT-PCR demonstrated a distinct reduction in Neu3 levels in dissociated DRG cultures, whereas levels of

Neu4 remained unaffected (Fig. 1X). Immunohistochemical staining for Neu3 sialidase in NEU3 siRNA-transfected cultures revealed decreases in Neu3 sialidase levels, indicating a reduction in Neu3 sialidase protein level compared with control cultures (Fig. 1V,W). As the NEU3 siRNA specifically reduced only Neu3, leaving Neu4 constant, our results suggest that changes in Neu3 sialidase activity are responsible for the conversion of GD1a (and GT1b) to GM1.

Inhibiting Neu3 sialidase activity reduces axon regeneration after axotomy

Because Neu3 sialidase activation converts more complex gangliosides to GM1 ganglioside and because GM1 has been shown to promote axonogenesis and axon growth, we hypothesized that inhibiting Neu3 sialidase’s activity might inhibit regeneration in adult DRG axons. DRG explants (or dissociated DRG cultures) were grown for 48 h on PDL-laminin, exposed to NeuAc2en for 1 h (or siRNA transfection for 4 h followed by a 1 d incubation), axotomized, and regeneration was assessed at 15 min and 1 h.

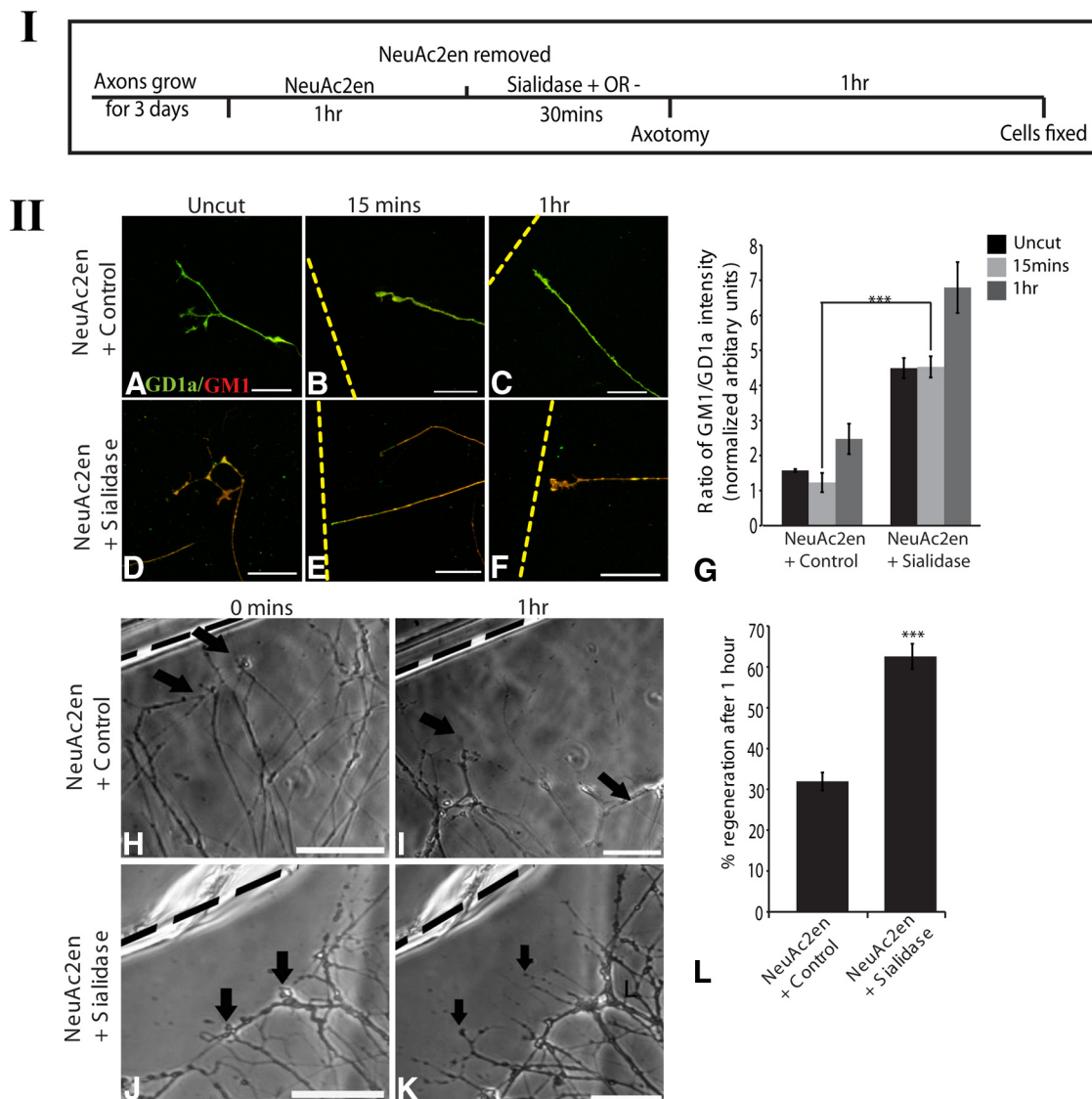


Figure 4. Exogenous sialidase rescues NeuAc2en-mediated inhibition of Neu3 sialidase activity and promotes axonal regeneration. *I*, Schematic of experimental design for sialidase rescue experiments in adult DRG axons. *II*, *A–F*, Fluorescent photomicrographs after exposure to Neu3 sialidase inhibitor (NeuAc2en) for 1 h, which was removed and followed by no treatment (NeuAc2en + control; *A–C*) or exposure to exogenous *Clostridium* sialidase (NeuAc2en + Sialidase; *D–F*) with axotomy performed 30 min later showing adult DRG axons uncut (*A, D*), 15 min (*B, E*), and 1 h after axotomy (*C, F*), double-labeled for GD1a (green) and GM1 gangliosides (CTB, red). The ratio of GM1/GD1a gangliosides (\pm SEM) was plotted in *G*, with the GM1 and GD1a ganglioside intensity ratio normalized to background ($n \geq 4$). $***p < 0.001$ (two-way ANOVA). *H–K*, Phase-contrast photomicrographs showing DRG axons immediately after axotomy (*H, J*) and 1 h after exposure to NeuAc2en and removal (NeuAc2en + control; *H, I*), or after exposure to NeuAc2en followed by exogenous *Clostridium* sialidase (NeuAc2en + Sialidase; *J, K*). Arrows indicate selected axons at axotomy and after 1 h. Percentage regeneration (\pm SEM) 1 h after axotomy plotted in *L* ($n = 6$). $***p < 0.001$ (Student's two-tailed *t* test). Scale bars: *A–F*, 50 μ m; and *H–K*, 30 μ m.

Compared with untreated cultures (Fig. 2*A, B, F–H*), NeuAc2en-treated DRG explants (Fig. 2*C, D*) and Neu3 siRNA-transfected dissociated DRGs (Fig. 2*I–K*) showed a marked reduction in regeneration (Fig. 2*E, L*). Axons were scored as regenerating if a growth cone was present at their tip and they had grown beyond their retraction point (Fig. 2*B, G*). Within dissociated cultures, retraction of axons in controls was most obvious within 15 min after axotomy, with regeneration occurring between 15 min and 1 h. Uncut axons within the same cultures continued to grow in the presence of NeuAc2en with normal growth cone morphology (Fig. 2*C, D*). Additionally, we attenuated total ganglioside synthesis using two different inhibitors, fumonisins B1 and PDMP, and found that adult DRG axon regeneration was severely impaired (data not shown). To rule out toxicity from the ganglioside inhibitors, we measured the growth rate of uncut axons in the presence of PDMP or NeuAc2en and found no difference be-

tween treated and control cultures over the same periods (Fig. 2*M–P*) with axon growth in uncut DRG cultures not affected by NeuAc2en (Fig. 2*N*). These results suggest that the GD1a to GM1 conversion mediated by rapid activation of Neu3 sialidase during the retraction period plays a significant role in the regeneration of cut DRG axons. However, high levels of GM1, or likewise reduced levels of complex gangliosides such as GD1a, are not needed for the continuing growth of mature axons.

Sciatic nerve crush leads to GD1a to GM1 conversion *in vivo*

We next investigated whether the conversion of GD1a to GM1 after axotomy occurs in the PNS *in vivo*. Sciatic nerve crushes were performed in adult rats, and the GM1/GD1a ganglioside ratio was measured immunohistochemically. To visualize changes in the vicinity of axon surfaces, we measured the intensity of GD1a and GM1 in single optical sections, selecting narrow areas of interest

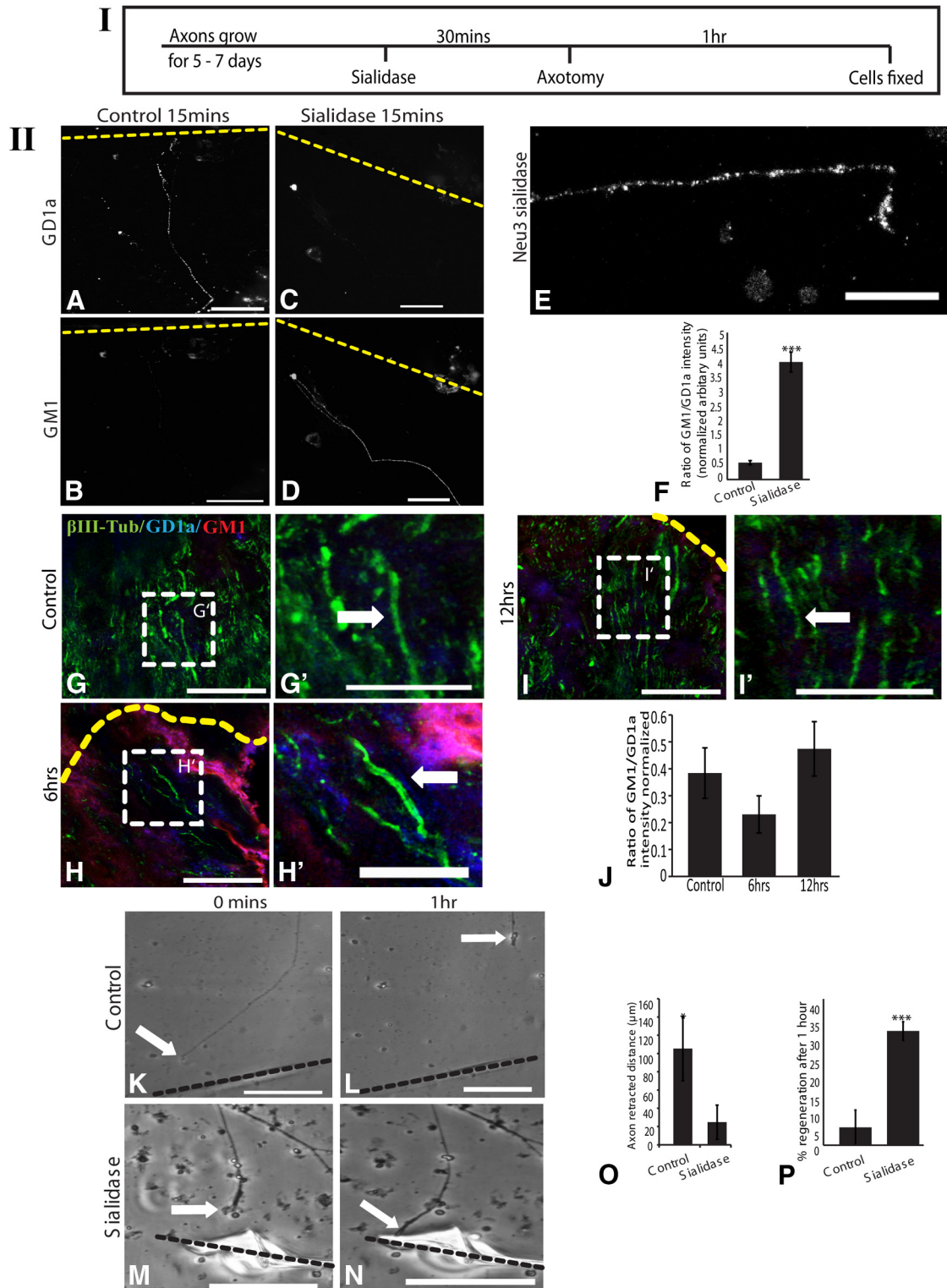


Figure 5. Neu3 sialidase remains inactivated after retinal axonal injury; however, exogenous sialidase can promote retinal axon regeneration. *I*, Schematic of timeline for retinal axon experiments. *II*, *A–D*, Fluorescent photomicrographs of adult retinal axons 15 min after axotomy. Axons are double-labeled for GD1a (*A*, *C*) and GM1 gangliosides (CTB; *B*, *D*) under normal conditions (control 15 min; *A*, *B*) or after exposure to *Clostridium sialidase* (sialidase 15 min; *C*, *D*). *E*, Fluorescent photomicrograph showing Neu3 sialidase enzyme on an adult retinal axon. The ratio of GM1/GD1a ganglioside is plotted in *F* (30–40 axons/chamber, $n \geq 6$). *G–I*, Fluorescent confocal photomicrographs (longitudinal) of optic nerve uncrushed (*G*, *G'*), 6 h (*H*, *H'*), and 12 h after crush (*I*, *I'*), triple-labeled for β III-tubulin (green), GD1a (blue), and GM1 gangliosides (CTB, red). High-magnification images (*I'*) from the dotted squares in the corresponding pictures denote representative areas used for GD1a and GM1 quantification. Arrows indicate regions parallel to axonal staining used for measuring intensities of GM1 and GD1a gangliosides (\pm SEM) parallel to axonal staining before and after optic nerve injury and normalized against background intensity levels is plotted in *J* ($n = 3$). *p* values by one-way ANOVA. *K–N*, Phase-contrast photomicrographs showing retinal axons at time of axotomy (0 min; *K*, *M*) and 1 h after axotomy (1 h; *L*, *N*), retracting under normal conditions (control; *K*, *L*), or regenerating after exposure to *Clostridium sialidase* applied 30 min before axotomy (sialidase; *M*, *N*). Plots of mean distance of axon tip retraction from site of injury measured 1 h after injury in the presence or absence of exogenous sialidase ($n \geq 6$) (*O*) and percentage of axons that regenerated past retraction position in retinal axons 1 h after injury ($n \geq 6$) (*P*) \pm SEM. *** $p < 0.001$; * $p < 0.05$ (Student's *t* test, two-tailed). Scale bars: *A–D*, 100 μ m; *E*, *G'–I'*, 25 μ m; *G–I*, *K–N*, 50 μ m.

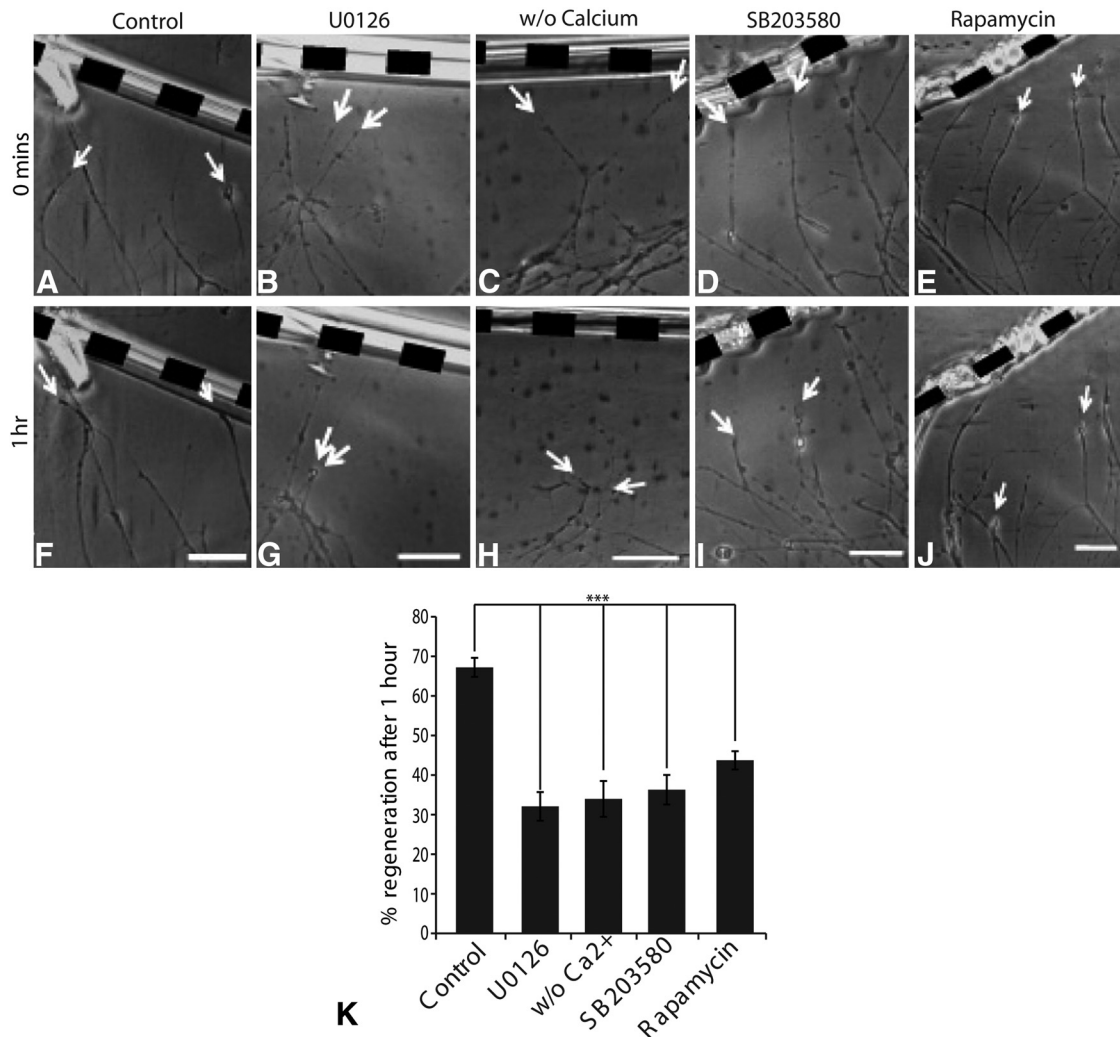


Figure 6. Axon regeneration is regulated by eCa^{2+} , P38MAPK, ERK, and mTOR signaling cascades. **A–J**, Phase-contrast photomicrographs of adult DRG axons photographed immediately after axotomy (**A–E**) and 1 h after axotomy (**F–J**), under normal conditions (control; **A, F**) and after exposure to inhibitors of ERK (U0126; **B, G**), removal of Ca^{2+} (without calcium; **C, H**), inhibitors of P38MAPK (SB203580; **D, I**), and mTOR (rapamycin; **E, J**). Inhibition of all these pathways attenuated the ability of axons to regenerate. Percentage regeneration of axons (\pm SEM) is plotted in **K** ($n \geq 5$). $***p < 0.001$ (one-way ANOVA). Scale bars, 50 μm .

parallel to axons in between S100 β -stained Schwann cells and β III-tubulin or neurofilament stained axonal profiles (Fig. 3A'). Normal adult sciatic nerve had a very low ratio of GM1/GD1a (Fig. 3B, B'). However, this ratio changed after nerve crush resulting in a fourfold increase within 6 h after crush injury, returning back to uncrushed levels by 12 h (Fig. 3B–E). Because axons and Schwann cells are in direct contact, we cannot rule out that some of the changes in sciatic nerve occurred on Schwann cells, but our *in vivo* results closely parallel changes we observed on axons *in vitro*, albeit with a longer time course.

Exogenous sialidase rescues NeuAc2en-mediated inhibition of Neu3 sialidase activity and promotes neurite regeneration

To confirm the requirement for sialidase action in axon regeneration and to control for nonspecific effects of NeuAc2en, a rescue experiment was performed in cultured DRGs. After inhibiting endogenous Neu3 sialidase activity, we treated the cultures with exogenous sialidase to convert axonal gangliosides to GM1 (NeuAc2en + sialidase). First, a control experiment demonstrated that the effects of NeuAc2en inhibition on axon regeneration persist for at least 1 h after removal. Axotomy 30 min after

removing NeuAc2en (NeuAc2en + control) showed that the inhibitory effect of NeuAc2en on Neu3 sialidase activity persisted after its removal, as indicated by a lack of increase in the GM1/GD1a ratio (Fig. 4A–C, G). The regenerative ability of axons 30 min after NeuAc2en removal remained low, similar to cultures in which the inhibitor was still present (Fig. 4H, I, L). The persistent reduction of Neu3 activity after NeuAc2en withdrawal is unexpected after removal of a soluble competitive inhibitor and suggests longer-term effects that influence the activity of Neu3 in the axonal membrane. In a previous study, this inhibitor caused changes in axonal membrane structures and the cytoskeleton that persisted after inhibitor removal (Da Silva et al., 2005).

Having confirmed the persistent blocking of Neu3 sialidase activity after NeuAc2en removal, we assessed whether exogenous *Clostridium* sialidase dissolved in the culture medium could convert GD1a to GM1. Application of sialidase increased the GM1/GD1a ratio in uncut axons to the level found after axotomy, and axotomy after sialidase treatment did not further increase GM1 levels (Fig. 4D–G). Treatment of Neu3 sialidase-inhibited cultures with sialidase restored their regenerative ability near the levels observed in normal conditions (Fig. 4J–L). These results

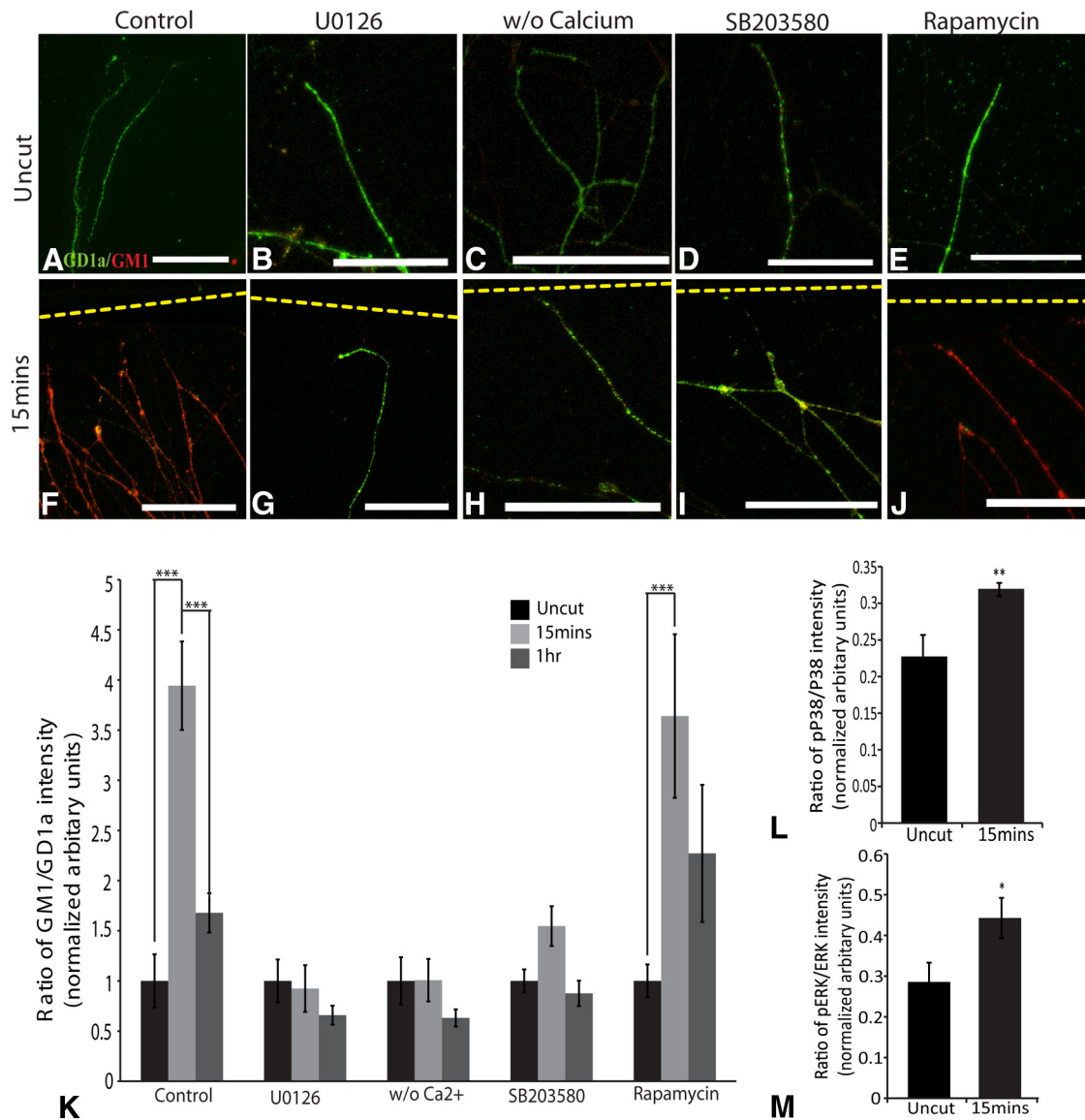


Figure 7. Neu3 sialidase activity is affected by eCa^{2+} , P38MAPK, and ERK in adult DRG axons after axotomy. **A–J**, Fluorescent photomicrographs of adult DRG axons uncut (**A–E**) and 15 min after axotomy (**F–J**), double-labeled for GD1a (green) and GM1 gangliosides (CTB, red) under normal conditions (control; **A, F**) and after exposure to inhibitors of ERK (U0126; **B, G**), removal of Ca^{2+} (without calcium; **C, H**), inhibitors of P38MAPK (SB203580; **D, I**) or mTOR (rapamycin; **E, J**). The ratio of GM1/GD1a ganglioside intensities (\pm SEM) normalized against background intensity levels is plotted in **K** (40–50 axons/chamber, 2 DRGs/chamber, $n \geq 3$ chambers). To avoid variability among uncut axons, axonal ratios of GM1/GD1a intensity levels were assigned the arbitrary value of 1 and GM1/GD1a levels at 15 min and 1 h after axotomy were normalized against corresponding uncut axons' ratio. **L, M**, The ratio of pP38MAPK/P38MAPK (**L**; uncut, $n = 4$; 15 min, $n = 6$) and pERK/ERK (**M**; uncut, $n = 4$; 15 min, $n = 4$) uncut and 15 min after injury is plotted \pm SEM. *** $p < 0.001$ (two-way ANOVA). ** $p < 0.01$ (Student's *t* test, two-tailed). * $p < 0.05$ (Student's *t* test, two-tailed). Scale bars: **A–J**, 50 μ m.

suggest that GD1a to GM1 ganglioside conversion mediated either by Neu3 sialidase or by externally applied sialidase is important for regeneration of peripheral axons.

The Neu3 sialidase-mediated transient increase in the ratio of GM1/GD1a ganglioside is not observed in retinal axons

In light of previous results, we asked whether the failure of regeneration of CNS axons might involve an inability to increase GM1 after axotomy. For these experiments, an axotomy was performed on axons extending from adult rat retinal explants. We have previously shown that only 5% of adult retinal axons regenerate after *in vitro* axotomy compared with 70% of adult DRG axons (Chierzi et al., 2005; Verma et al., 2005). The axons usually retract from the cut site and form an active retraction bulb, but fail to make a new growth cone. We found

that adult retinal axons have a lower ratio of GM1/GD1a gangliosides on their surface than DRG axons. Additionally, there was no conversion of GD1a to GM1 ganglioside 15 min after axotomy in retinal axons (Fig. 5*A, B*). To determine whether this was due to low levels of Neu3 sialidase in retinal axons, its abundance and distribution were assessed by immunofluorescence. Similar levels of staining and distribution of Neu3 sialidase to that found in DRG cultures were observed (Fig. 5*E*). To investigate whether the conversion of GD1a to GM1 can occur in these axons in the presence of active sialidase, we applied exogenous sialidase to retinal cultures. The treatment induced high levels of GM1 ganglioside on retinal axons, bringing the GM1/GD1a ratio close to that of axotomized or sialidase-treated adult DRG axons (Fig. 5*C, D, F*). These experiments suggest that Neu3 sialidase is present on adult retinal axons but is not activated by axotomy.

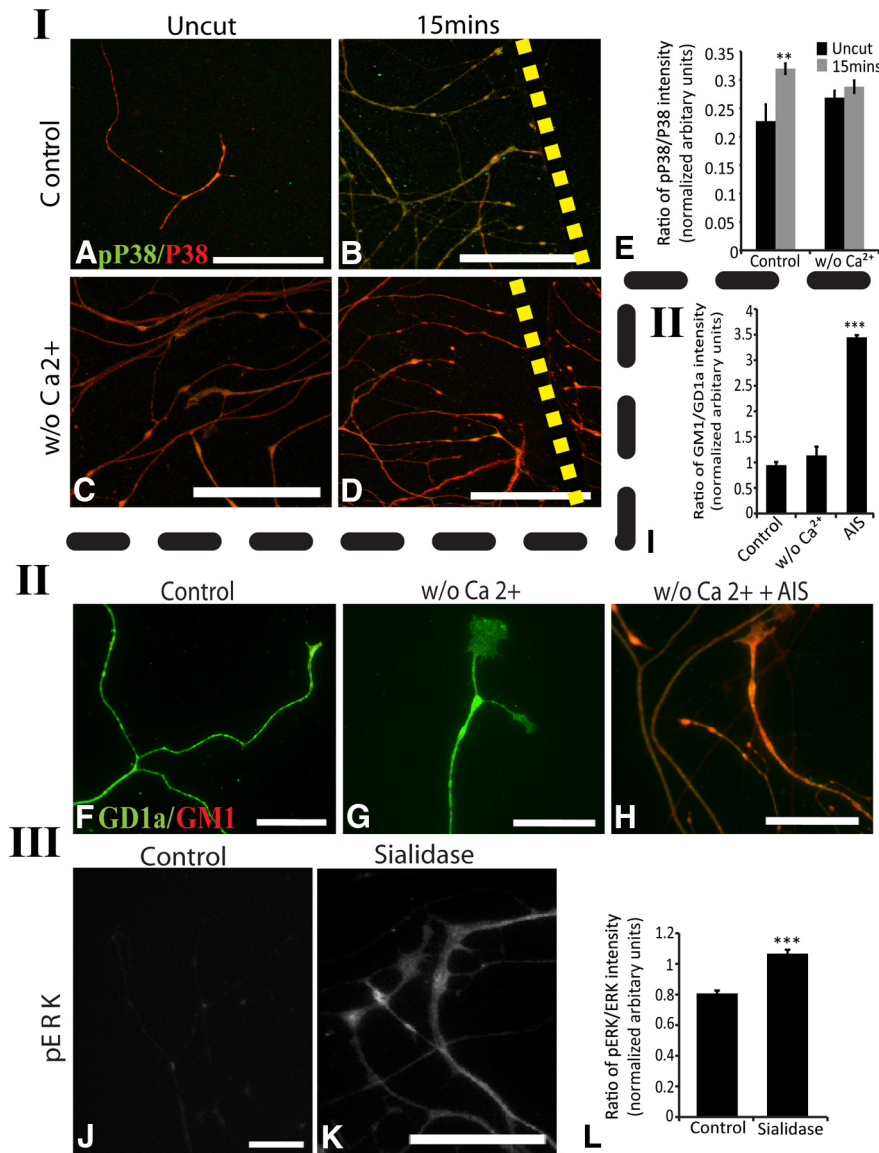


Figure 8. P38MAPK activation is the intermediary step between eCa^{2+} influx and Neu3 sialidase activation after axonal injury, whereas ERK activation is downstream of Neu3 sialidase activation. **I, A–E**, Fluorescent photomicrographs of adult DRG axons uncut (**A, C**) and 15 min after axotomy (**B, D**), double-labeled for P38MAPK (red) and phospho P38MAPK (green) under normal conditions (control; **A, B**) and after removal of eCa^{2+} (without Ca^{2+} ; **C, D**). The ratio of phospho P38MAPK/P38MAPK intensities (\pm SEM) normalized against background intensity levels is plotted in **E** (30–40 axons/chamber, 2 DRGs/chamber, $n = 4–6$ chambers). **II**, Activation of P38MAPK using anisomycin increases Neu3 sialidase activity even in the absence of eCa^{2+} . **F–H**, Fluorescent photomicrographs of uncut adult DRG axons, double-labeled for GD1a (green) and GM1 gangliosides (CTB, red) and under normal conditions (control; **F**), after removal of eCa^{2+} (without Ca^{2+} ; **G**) and after exposure to anisomycin (AIS) in the absence of eCa^{2+} (without Ca^{2+} + AIS; **H**). The ratio of GM1/GD1a ganglioside intensities (\pm SEM) normalized against background intensity levels is plotted in **I** (30–40 axons/chamber, 2 DRGs/chamber, $n = 4$ chambers). **III**, ERK activation is downstream of Neu3 sialidase activation. **J–L**, Fluorescent photomicrographs of adult uncut DRG axons, labeled for pERK under control conditions (**J**) and after exposure to exogenous sialidase (**K**). The ratio of pERK/ERK intensities (\pm SEM) normalized against background intensity levels is plotted in **L** (30–40 axons/chamber, containing 2 DRGs, $n = 6$ chambers). *** $p < 0.001$ (Student’s *t* test, two-tailed). ** $p < 0.01$ (Student’s *t* test, two-tailed). Scale bars, 50 μ m.

To determine whether conversion of gangliosides by sialidase activity occurs after damage to the CNS *in vivo*, we performed an optic nerve crush injury in adult rats and measured GM1/GD1a levels in narrow regions adjacent to the axonal marker β III-tubulin. No change in the GM1/GD1a ratio was observed in these axonal regions of the optic nerve after injury, and the ratio in undamaged nerves was similar to uncrushed sciatic nerve (Fig. 5G–I, G’–I’, J).

Treatment of retinal axons with sialidase increases their regeneration after axotomy

We next examined whether exogenous sialidase treatment enhances the ability of adult retinal axons to regenerate. After axotomy, retinal axons showed greater retraction than DRG axons, with many axons retracting out of the field of view (Fig. 5K–O). Furthermore, only 4.8% of control axons regrew 1 h after axotomy beyond their point of initial retraction, whereas 21.3% of axons exposed to sialidase regenerated past their retraction point (Fig. 5P). These results demonstrate that conversion of more complex gangliosides to GM1 mediated by sialidase is an important component of the regenerative response and that exogenous sialidase treatment can enhance regeneration in adult retinal axons *in vitro*, although not to the level seen in sensory axons.

Neu3 sialidase activity is regulated by eCa^{2+} , ERK, and P38MAPK in adult DRG axons after axotomy

The preceding experiments indicate that axotomy activates Neu3 sialidase in adult DRG axons, but not in adult retinal axons, and that Neu3 sialidase activation is necessary for a successful regenerative response. We therefore investigated the signaling events that link axotomy to Neu3 sialidase activation and then to axon regeneration. Previous work has identified three signaling pathways (ERK, P38MAPK, and mTOR) (Agthong et al., 2009, 2012; Abe et al., 2010) as well as a local Ca^{2+} influx (Mandolesi et al., 2004; Cho and Cavalli, 2012; Cho et al., 2013), which occurs in response to axotomy and whose inhibition diminishes successful DRG axon regeneration (Chierzi et al., 2005; Verma et al., 2005). We confirmed that transient inhibition of any of these pathways with standard inhibitors attenuated the ability of adult DRG axons to regenerate (Fig. 6).

Next, we asked whether blocking these signaling pathways affected the conversion of GD1a to GM1 after axotomy. Removal of eCa^{2+} , inhibition of ERK or P38MAPK prevented the axotomy-mediated increase in the GM1/GD1a ratio after axotomy, whereas blocking mTOR had no effect on the ratio (data shown in

Fig. 7 legend). These results suggest that Neu3 sialidase activation in axotomized adult DRGs is regulated by a signaling cascade involving eCa^{2+} , ERK, and P38MAPK.

Based on our results combined with knowledge of damage responses in other cell types where P38MAPK has been shown to be a stress response kinase (Coulthard et al., 2009), we hypothesized that, after axonal injury, the rapid influx of Ca^{2+} activates P38MAPK, which in turn leads to increased Neu3 sialidase activ-

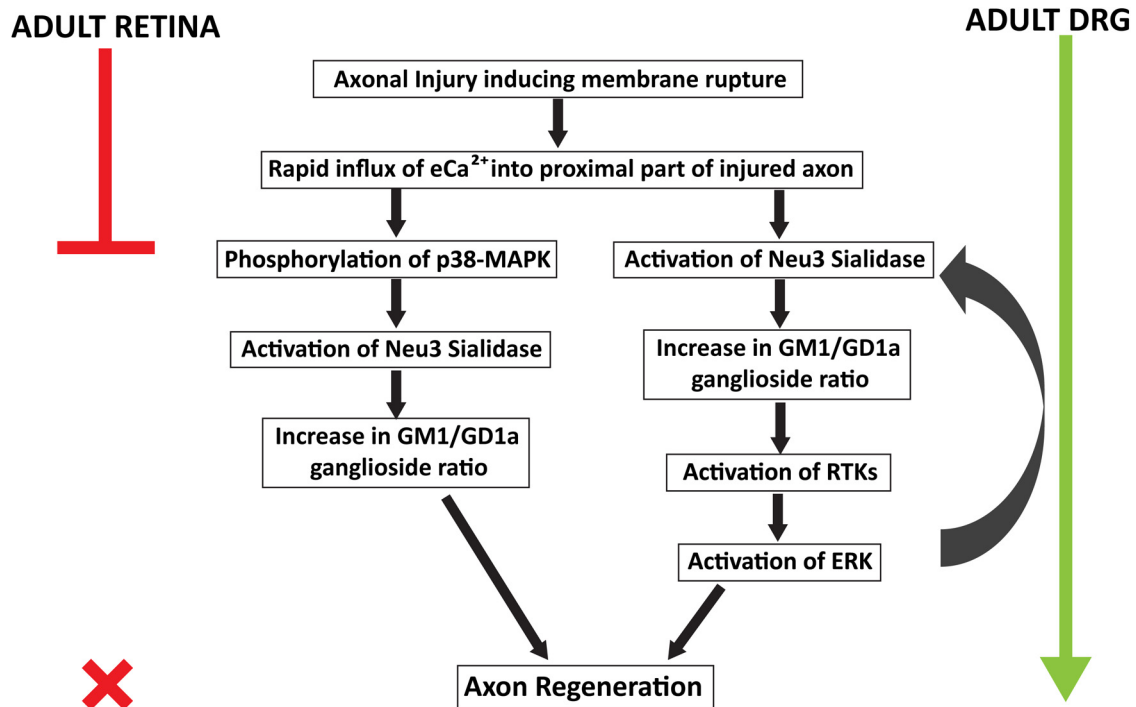


Figure 9. Diagram of proposed signaling cascade regulating Neu3 sialidase activity. Membrane rupture after axotomy leads to increased influx of eCa^{2+} into injured axons. Whereas in adult DRGs this results in either direct activation of Neu3 sialidase or an increased phosphorylation of P38MAPK resulting in increased Neu3 sialidase activation, this process is absent in adult retinal axons. Furthermore, ERK is activated downstream of Neu3 sialidase, which in turn further activates Neu3 sialidase through a positive feedback loop, a step attenuated in retinal axons, resulting in a two step block for successful Neu3 sialidase activity mediated regeneration.

ity. Based on previous work, Neu3 sialidase-mediated ganglioside conversion leads to increased signaling via the ERK pathway (Da Silva et al., 2005). ERK activation acts on axonal growth mechanisms likely as part of a positive feedback loop leading to further activation of Neu3 sialidase (see Discussion and Fig. 9). We first investigated phosphorylation of P38MAPK (pP38MAPK) 15 min after axotomy of adult DRG axons in the presence or absence of eCa^{2+} . In the absence of eCa^{2+} , the increase in the pP38MAPK/P38MAPK ratio after axotomy was significantly reduced, suggesting that P38MAPK is downstream of eCa^{2+} entry (Fig. 8A–E). We next assessed whether P38MAPK activation could activate Neu3 sialidase. P38MAPK in uncut axons in the absence of eCa^{2+} was first activated by a common P38MAPK activator, anisomycin (Katoh-Semba et al., 2009; Mei et al., 2012), before the surface levels of GM1 and GD1a were measured. Anisomycin treatment led to a mild increase in pP38MAPK/P38MAPK, and this in turn resulted in a 3.5-fold increase in the ratio of GM1/GD1a. Although anisomycin is not entirely specific to p38, coupled with the other results, the experiment suggests that Neu3 sialidase was activated by P38MAPK activation (Fig. 8F–I). Next, we examined whether ganglioside conversion can activate the ERK pathway, as demonstrated previously in embryonic neurons (Da Silva et al., 2005). To test this, we treated uncut adult DRG axons with exogenous sialidase to convert GD1a to GM1. This conversion resulted in an increased ratio of pERK/ERK (Fig. 8J–L). Together, these results support the proposed signaling events shown in Figure 9.

P38MAPK is not activated in adult retinal axons after axotomy

CNS axotomy has been shown to invoke a large calcium influx (Mandolesi et al., 2004); therefore, we hypothesized that the block in Neu3 sialidase activation in retinal axons might occur at the P38MAPK activation step. We evaluated whether the ratio of

pP38MAPK/P38MAPK changes 15 min after axotomy in retinal axons as observed in DRG axons. After axotomy, we did not observe an increase in the ratio of either pP38MAPK/P38MAPK or pERK/ERK levels (Fig. 10A–F). Therefore, we assessed whether Neu3 sialidase activation consequent on P38MAPK activation is operative in retinal axons. P38MAPK was activated using anisomycin, and we measured the GM1/GD1a ganglioside ratio. Anisomycin treatment led to a 100% increase in the levels of GM1/GD1a ganglioside (Fig. 10G–I).

Overall, our experiments indicate that, in DRG axons, Neu3 sialidase activation after axonal injury is triggered by influx of eCa^{2+} after membrane rupture, leading to activation of P38MAPK. However, in retinal axons, neither P38MAPK nor Neu3 sialidase is activated after axotomy. Active Neu3 sialidase or an externally applied sialidase changes the ratio of GM1/GD1a ganglioside, leading to ERK pathway activation and a successful regeneration response.

Discussion

Our study demonstrates that ganglioside composition on axons is important for growth cone production and that conversion of axonal gangliosides to GM1 by the axonal surface enzyme Neu3 sialidase is required for adult axon regeneration. CNS axons lack intrinsic regenerative ability, and our results strongly suggest that the lack of Neu3 sialidase activation after axotomy is a factor in poor regenerative ability. The importance of sialidase activity for CNS regeneration is supported by a previous study in which sialidase treatment after spinal cord injury promoted axon regeneration (Yang et al., 2006) and functional recovery (Mountney et al., 2010, 2013). Further work has shown that conversion of gangliosides to GM1 by Neu3 sialidase is critical during early neuronal differentiation for axonal specification (Da Silva et al., 2005; Abad-Rodriguez and Robotti, 2007). Furthermore, poor regen-

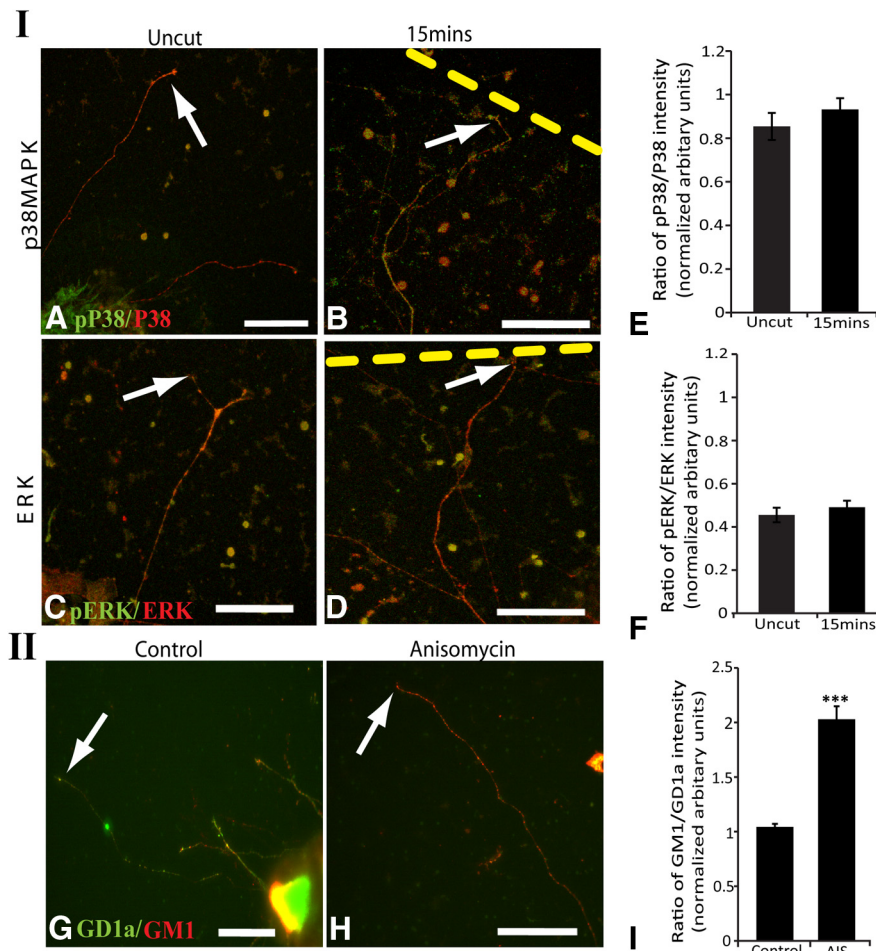


Figure 10. P38MAPK and ERK are not activated within 15 min after axotomy in retinal axons, but forced activation of P38MAPK increases Neu3 sialidase activity in retinal axons. *I, A–D*, Fluorescent photomicrographs of adult retinal axons uncut (*A, C*) and 15 min after axotomy (*B, D*), double-labeled for P38MAPK (*A, B*) or ERK (*C, D*) (red) and pP38MAPK (*A, B*) or pERK (*C, D*) (green). Ratios of pP38MAPK/P38MAPK and pERK/ERK intensities (\pm SEM) normalized against background intensity levels are plotted in *E* and *F* (30–40 axons/chamber, $n = 4$ chambers). *II, G, H*, Fluorescent photomicrographs of adult uncut retinal axons, double-labeled for GD1a (green) and GM1 gangliosides (CTB, red). Anisomycin increased the GM1/GD1a ratio (compare *H* with *G*). *I*, Ratios of GM1/GD1a ganglioside intensities (\pm SEM) normalized against background intensity levels are plotted in *I* (30–40 axons/chamber, $n \geq 7$). *** $p < 0.001$ (Student's *t* test, 2-tailed). Scale bars, 50 μ m.

eration of PNS axons has been observed in animals lacking complex gangliosides (Kittaka et al., 2008) and after treatment with antibodies to GM1 ganglioside (Lehmann et al., 2007; Lopez et al., 2010).

Regulation of Neu3 sialidase activity

Sialidases, such as Neu3, act to remove terminal sialic acid groups, converting gangliosides, such as the main CNS gangliosides GD1a, GD1b, and GT1b to GM1. After axotomy of DRG neurons, we observed a rapid increase in GM1 and a decrease in both GD1a and GT1b, which was prevented by Neu3 sialidase inhibition through pharmacological inhibition or siRNA knock-down. Preventing ganglioside conversion to GM1 inhibited regeneration in PNS axons. However, replacing Neu3 sialidase with external sialidase after blocking it rescued regeneration by inducing an increase in GM1 (and reduction in GD1a). This suggests that PNS axotomy rapidly activates Neu3 sialidase, converting major nervous system gangliosides to GM1. Within 1 h after axotomy, as axons regenerated their growth cones, GD1a and GM1 levels reverted to preaxotomy values, implying that Neu3 sialidase activation is transient. Although regulatory mechanisms

for Neu3 sialidase are unknown, the structure of Neu3 sialidase protein has been characterized, revealing potential phosphorylation sites on the C terminus (Miyagi et al., 1999). After *in vitro* axotomy, there is a transient influx of calcium and rapid activation of several signaling pathways, including P38MAPK, ERK, and PI3 kinase (Liu and Snider, 2001; Chierzi et al., 2005; Verma et al., 2005). Our results show that blockade of these pathways prevents Neu3 sialidase activation after axotomy. The first step in Neu3 sialidase activation is calcium influx into the damaged axon which we found to be necessary for GD1a to GM1 ganglioside conversion, and for activation of other signaling pathways. Calcium influx after axotomy initiates several other processes involved in regeneration, including activation of calpain and Ca^{2+} -dependent kinases (Ziv and Spira, 1997; Chierzi et al., 2005; Kamber et al., 2009). The next step we identified was activation of P38MAPK, a stress-related kinase, activated by stressors including calcium (Nozaki et al., 2001). Activation of P38MAPK by anisomycin triggered sialidase activity, confirming P38MAPK as a link between axotomy and Neu3 sialidase activation. However, we do not know whether P38MAPK binds directly to Neu3 sialidase or whether it acts via an intermediary step. Evidence demonstrates that P38MAPK activation is essential for efficient axon regeneration (Myers et al., 2003; Verma et al., 2005; Nix et al., 2011; Kato et al., 2013). Axotomy also activated ERK, which is a step in the major signaling pathway controlling axon growth (Chierzi et al., 2005; Waetzig and Herdegen, 2005). Neu3 sialidase activation and ganglioside conversion is sufficient to activate ERK, confirmed with ERK activation in axons treated with exogenous sialidase. These results suggest that P38MAPK and ERK are acting in parallel within DRG neurons downstream of the initial calcium influx arising immediately after axotomy. This is likely the result of the construction of cell surface signaling domains (rafts), of which GM1 is a major organizer (Ichikawa et al., 2009; Ohkawa et al., 2010; Singh et al., 2010; Furukawa et al., 2011). These domains contain various activators of signaling pathways, such as integrins, growth factor receptors, and other signaling molecules. In embryonic neurons, Neu3 sialidase action can affect axons by increasing TrkA signaling resulting in ERK activation (Da Silva et al., 2005).

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Neu3 sialidase activation fails in CNS axons

Although Neu3 sialidase was activated by axotomy in DRG axons, we observed no such change in adult retinal axons, despite the presence of Neu3 sialidase protein on the axons. These results suggest that there is a failure in the mechanisms that activate Neu3 after axotomy. Where might activation fail? Axotomy of CNS axons causes calcium influx as in PNS axons (Mandolesi et al., 2004), but we did not observe activation of P38MAPK after

axotomy in retinal axons. Alternatively, if P38MAPK is pharmacologically activated, retinal axons show sialidase activity with ganglioside conversion, suggesting a defect in the pathway leading to P38MAPK activation. Interestingly, axonal regeneration in P38MAPK knock-out mice was shown to be substantially lower than in wild-type mice (Kato et al., 2013). It is not clear why there is a differential regulation of P38MAPK between the two neuronal types; however, we hypothesize that the differential activity profile of RhoA in CNS and PNS axons may partially explain this difference. It has been shown to be activated by the myelin inhibitor Nogo-A (absent in PNS), which acts to suppress P38MAPK levels in CNS neurons after (MCAO) injury (Kilic et al., 2010), inhibiting regeneration. There is also evidence demonstrating that the formation of a complex between GT1b and the Nogo receptor NgR1 inhibits axon growth in cerebellar granular neurons (Williams et al., 2008). Likewise, upregulation of cytokines after injury, such as TNF- α , has also been shown to activate RhoA in cultured hippocampal neurons leading to reduced P38MAPK activation (Neumann et al., 2002). However, TNF- α has an opposite effect in DRG neurons leading to increases in after injury calcium influx and activation of P38MAPK (Pollock et al., 2002).

Actions of Neu3 sialidase

Neu3 sialidase is membrane-associated, and its action is known to be specific to membrane gangliosides (Miyagi et al., 1999). Neu3 sialidase might affect axon regeneration in several ways. In one well-established mechanism, GD1a is a coreceptor for the inhibitory molecule MAG, present on PNS- and CNS-myelinating cells (Collins et al., 1997; Vyas et al., 2002). Conversion of GD1a to GM1 therefore diminishes inhibition by MAG. Although important *in vivo*, where axons and myelinating glia are in close contact, it is not likely to be the mechanism behind the positive effect of sialidase in our *in vitro* assays as these axons were not in contact with myelinating glia, and inhibition of ganglioside synthesis blocked rather than stimulated axon regeneration. Instead, our results suggest a positive effect of GM1 on axon regeneration. However, in addition to the “growth-promoting effect” of increased GM1 levels, it is possible that the increase in regeneration was also the result of a reduction of complex gangliosides, such as GD1a or GT1b. All interventions that increased axonal GM1 increased regeneration and those that inhibited conversion of polysialylated gangliosides to GM1 blocked regeneration. This conclusion is strengthened by our results with axotomized retinal axons, which do not upregulate GM1 ganglioside and fail to regenerate unless treated with sialidase to convert surface gangliosides to GM1. Potential mechanisms have been proposed to explain how GM1 might enable growth cone regeneration, mostly based on the ability of GM1 to organize lipid-rich microdomains/rafts (Furukawa et al., 2011). Disruption of one type of raft through manipulation of flotillins affects regeneration, for example (Stuermer, 2010). Additionally, extensive literature on cancer invasion and Neu3 sialidase exists showing that Neu3 sialidase is localized to membrane ruffles in GM1-rich rafts during metastasis (Yamaguchi et al., 2006; Miyagi et al., 2008a,b; Miyata et al., 2011; Tringali et al., 2012). Metastatic transformation and growth cone advance share a requirement for membrane extension, decreased adhesiveness to substrate at the leading edge, and formation of filopodia and lamellipodia. In tumor cells, Neu3 sialidase has effects on adhesion, integrin turnover and activation, caspase activity, caveolins, and several signaling pathways (Roche et al., 1997; Miyagi et al., 2008a,b; Tringali et al., 2012). Disruption of microdomains/rafts might explain why mice lacking complex gangliosides have decreased expression of

neurotrophic factors and their receptors (Kittaka et al., 2008), and why GM1 ganglioside potentiates the activity of NGF receptor, TrkA, in addition to other neurotrophin receptors (Bähr et al., 1989; Cuello et al., 1994; Rabin and Mocchetti, 1995; Rabin et al., 2002). Additionally, it is likely that Neu3 sialidase activation may lead to its association with integrins, further leading to activation of FAK and calcium influx (Wu et al., 2007), an event important for cytoskeletal reorganization and axonal guidance (Kato et al., 2006; Miyagi et al., 2008b).

The experiments in this paper demonstrate that an increase in the ratio of growth-promoting gangliosides (GM1) to growth-inhibiting gangliosides (such as GD1a and GT1b) mediated by Neu3 sialidase play an important role in changes that lead to axon regeneration after axotomy. Much of the detail about cytoskeletal changes that occur after axotomy is known (Spira et al., 2003; Erez et al., 2007; Ertürk et al., 2007), and we are beginning to understand the process of local mRNA translation (Hanz et al., 2003; Willis et al., 2005; Vogelaar et al., 2009; Yoo et al., 2010; Jung et al., 2012). Equally important are changes on the cell surface, and our results strongly suggest that understanding these events will enable interventions that enhance the intrinsic regenerative ability of axons.

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