Neu3 sialidase-mediated ganglioside conversion is necessary for axon regeneration and is blocked in CNS axons

Abbreviated title: Neu3 sialidase is required for axon regeneration (48 Characters)

Sunil Kappagantula[1], Melissa R. Andrews[1,2], Menghon Cheah[1], José Abad-Rodriguez[3], Carlos G. Dotti[4], James W. Fawcett[1*]

1 –John van Geest Centre for Brain Repair, University of Cambridge, Forvie site, Robinson way, Cambridge, CB2 0PY, UK

2 –School of Medicine, University of St Andrews, Medical and Biological Sciences Building, North Haugh, St Andrews, KY16 9TF, UK

3 - Membrane Biology and Axonal Repair Laboratory. Hospital Nacional de Parapléjicos (SESCAM), Finca La Peraleda s/n, E-45071 Toledo, Spain

4 – Centro de Biología Molecular Severo Ochoa (CSIC-UAM), Campus Cantoblanco, Nicolás Cabrera 1, 28049, Madrid, Spain

***Corresponding author:** James W. Fawcett, John van Geest Centre for Brain Repair, University of Cambridge, Forvie Site, Robinson Way, Cambridge, CB2 0PY, United Kingdom.

Tel: +441223 331188, fax +441223 331174, email: jf108@cam.ac.uk

Number of pages: 53

Number of figures: 10

Number of words: Abstract (160 words), Introduction (499 words), Discussion (1500 words)

Conflict of Interest: James Fawcett is a paid advisor to Acorda Therapeutics, Novartis, and Covidien.

Acknowledgements: The authors would like to thank Prof. Ronald L Schnaar (Johns Hopkins University) for generously providing anti-GD1a ganglioside antibody and Mr. David Story and Mr. Marc Smith (Cambridge University) for technical assistance. The work was funded by grants from the Medical Research Council, the Christopher and Dana Reeve Foundation, the John and Lucille van Geest Foundation, the Henry Smith Charity, the Commonwealth and Overseas scholarships and the Hinduja Cambridge Trust.

SUMMARY

PNS axons have a high intrinsic regenerative ability while 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 PNS axons after Neu3 sialidase blockade. Neu3 sialidase activation in DRGs is initiated by an influx of extracellular calcium, activating P38MAPK 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.

INTRODUCTION

Mammalian central nervous system (CNS) axons fail to regenerate after damage while peripheral nervous system (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 (Chew et al., 2012; Cho and Cavalli, 2012; Moore and Goldberg, 2011; Sun and He, 2010; Wang and Jin, 2011). Successful axon regeneration begins with growth cone formation involving coordinated proteolytic, cytoskeletal and cell surface regulatory events (Afshari et al., 2009; Bradke and Dotti, 1999; Erez et al., 2007; Long and Lemmon, 2000; Witte et al., 2008) as well as activation of local protein translation as in PNS axons (Verma et al., 2005; Zheng et al., 2001). Integrins and other signalling molecules involved with control of regeneration can associate with lipid microdomains, leading to the modulation of many intracellular signalling events (Gao et al., 2011; Grider et al., 2009; Guirland et al., 2004; Guirland and Zheng, 2007; Kamiguchi, 2006; Norambuena and Schwartz, 2011; Stuermer, 2010).

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. While embryos can develop normally without complex gangliosides, postnatally they develop demyelination, neurodegeneration, and impaired peripheral nerve regeneration (Kittaka et al., 2008; Okada et al., 2002; Sheikh et al., 1999). GM1 ganglioside has been shown to promote axon growth (Facci et al., 1984; Skaper et al., 1985) and regeneration (Mountney et al., 2010; Yang et al., 2006), but resulted in an unsuccessful clinical trial of GM1 in spinal cord injury (Geisler et al., 2001). GD1a ganglioside on the other hand has been shown to be

a receptor for inhibition of axon growth by myelin-associated glycoprotein (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 which 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 (Monti and Miyagi, 2012; Oehler et al., 2002). Additionally, Neu3 has been shown to be necessary for neuronal differentiation (Da Silva et al., 2005), for maintenance of polarity after axotomy (Rodriguez et al., 2001), and for regulating invasive and migratory activity (Tringali et al., 2012; Miyata et al., 2011; Yamaguchi et al., 2006).

In this paper, we examine Neu3 sialidase action on axonal gangliosides and resultant axon regeneration, and 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 polysialyated gangliosides is necessary for axon regeneration.

Materials & Methods

Chemicals and reagents

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

Dorsal root ganglion cultures

Adult (3–4 mo old) female Sprague-Dawley rats were killed and DRGs removed. **Explants:** DRGs were trimmed of meningeal coverings and divided into segments and plated onto coated chamber slides (Nunc) (10µg/ml PDL and 1µg/ml laminin). Explants were maintained at 37^0C and 7%CO_2 in DMEM (Invitrogen) with 0.11g/L Sodium pyruvate with pyrodixine, 4.5g/L glucose, 20ng/ml NGF, 1:100 Insulin-Transferin-Selenium, 1:100 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 10ng/mL NGF and plated on PDL (20µg/mL) + laminin (10µg/mL).

Retina explant cultures

Unilateral conditioning optic nerve crush was performed on adult female 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 400µm thick sections using a McIiwain tissue chopper (Vibratome, Gorsham Surrey, UK). Retinal sections were plated onto plastic chamber slides coated with 300µg/ml PDL and 10µg/ml laminin, with the ganglion cell layer facing the substratum. Cultures were maintained in DMEM and Neurobasal–A (Invitrogen) in 1:1 ratio, supplemented with 50 IU erythropoietin-A, 760µg/ml BSA, N2 supplement (Gibco), 100µg/ml Sodium pyruvate, 4µg/ml of T3-T4, 2mM glutamine (Invitrogen), 1.1mg/ml glucose, 100 µg/ml gentamycin (Invitrogen) and 50ng/ml ciliary neurotrophic factor.

Axon regeneration assay

Axons were grown for 48 – 72h (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, 15min and 1hr 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 10x (10X/0.25) or 20X (20X/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 (3μM) or PDMP (20μM) 18h before axotomy.

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

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

To examine the effect of absence of eCa^{2+} on Neu3 sialidase activity and axonal regeneration, cultures were incubated with normal growth media devoid of $CaCl_2$, 30mins before axotomy.

siRNA transfection

Dissociated DRGs were transfected with siRNA 48h after plating. The Neu3 siRNA consists of four siRNA sequences: GCAGAGAUGCGUACCUCAA, CCAACAACUCUGCGAGCCU, CCAAACAAAUUCCGAGCAG 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-Transferin-Selenium and 10ng/mL NGF before mixing together and incubating with the DRGs (100nM; 4h) before replacing with normal medium.

RNA purification and RT-PCR

Dissociated DRGs were lysed and collected as per 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'-CAGCTGGGATAGCAGAGGTC-3', reverse: 5'-GAGTCCTGAAGCAAGCCAAC-3', resulting in a 209bp fragment; NEU4, forward: 5'-CCTGACCCTAGGACGAACAG-3', reverse: 5'-GATGTGCGTGGTGATCAGAG-3', resulting in a 179bp 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 membrane rather than

absolute mean values of GD1a and GM1 ganglioside. 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 of Prof Ronald Schnaar, Johns Hopkins Univ), mouse anti-GD1a ganglioside and mouse anti-GT1b ganglioside (Merck Millipore, UK) or stained using recombinant CTB conjugated to Alexa fluor 555 (Invitrogen, UK), and mouse anti β3-tubulin. To analyze the signalling cascade regulating Neu3 sialidase activity, DRG and retinal cultures were analysed with the following primary antibodies: mouse anti-P38MAPK, rabbit anti-phospho P38MAPK, rabbit anti-ERK and mouse anti-phospho ERK (Cell Signalling Technology, USA). Subsequently, cultures were washed in PBS and incubated with the following fluorescent secondary antibodies: anti-rabbit Alexa fluor 660, and anti-mouse or anti-rabbit Alexa fluor 488 (Invitrogen, UK) 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 UK Home Office regulations. Adult (2-3 mo old) female Sprague-Dawley rats were used for all *in vivo* experiments. Animals were kept in 12h light/dark exposure and food and water was provided *ad libitum.*

Animals were anesthetized using 1 – 2% isofluorane 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 10s duration each.

Optic nerve crushes were performed under ketamine:xylazine (2:1) anesthesia at a dosage of 0.1ml/100g. 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 10s duration each time.

Immunohistochemistry

Animals were sacrificed with an overdose of Euthatal (sodium pentobarbitol) at different time points after injury (2, 6, and 12h) and transcardially perfused with PBS (pH 7.4) and 4% PFA (pH 7.4). A 2cm 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, UK) and sectioned longitudinally at 12µm (sciatic nerve) or at 10µm (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-βIII tubulin, mouse anti-GD1a, CTB (for GM1 ganglioside) and anti-S100β. 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, Germany) using a 40x objective lens (40X/1.00-0.50 Oil). Image acquisition using Leica Application Suite utilized identical exposure times and microscope settings. Quantification of images was performed using NIH Image – J 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, Germany) using 40x (40X/1.15 Oil CS) (for SN) or 63x (63X/1.30 Oil CS) (for ON) magnification. Image acquisition

using Leica Application Suite utilized identical exposure times and microscope settings. Image quantification was performed using confocal software utilizing optical sections (0.800µm 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, βIII-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 was first analyzed for normality using Shapiro-Wilk normality test (Origin Ver. 8; Originlab Corporation). P values of all data were above set alpha levels of 0.05 and therefore considered to be normally distributed. Results from the analyses were expressed as mean ± SEM. Statistical analysis was performed using Prism ver. 5 (Graphpad, USA). 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 dorsal root ganglion (DRG) axons grown on poly-D-lysine (PDL)/laminin. These axons regenerate a new growth cone and begin to elongate within 30 mins 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 15mins or 1h after axotomy and stained with Cholera toxin-b subunit (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 15mins 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 (200-300µm from the proximal end of cut axons). Results are expressed as a ratio between GM1 and GD1a since 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). Since 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 (Kalka et al., 2001; Kopitz et al., 1996; Miyagi et al., 2008). 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 which 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-F, G). Using RNA interference for NEU3, we further confirmed that a reduction in Neu3 sialidase prevented the change in the GM1/GD1a ratio post-axotomy (Fig. 1K-M, 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 15mins and decreasing back near uncut levels by 1h (Fig. 1O-Q, U). After siRNA knockdown of Neu3, the GM1/GT1b ratio was unchanged after axotomy (Fig. 1R-T, U). To determine whether axotomy increases GM1 conversion through Neu3 sialidase up-regulation or by activation, we compared Neu3 sialidase protein levels on uncut axons and on axons 15 mins after injury by immunocytochemistry (Da Silva et al., 2005; Rodriguez et al., 2001). No significant change in Neu3 sialidase staining intensity was observed at 15 min following 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 desialytion 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

In order 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 while 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 to 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.

Preventing GM1 up-regulation thus sustaining levels of complex gangliosides inhibits axon regeneration after axotomy

Since Neu3 sialidase activation generates GM1 ganglioside and because it 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 48h on PDL-laminin, exposed to NeuAc2en for 1h (or siRNA transfection for 4h followed by a 1d incubation), axotomized, and regeneration was assessed at 15 mins and 1 h. Compared to untreated cultures (Fig. 2A-B, F-H), NeuAc2en-treated DRG explants (Fig. 2C-D) and Neu3 siRNA-transfected dissociated DRGs (Fig. 2I-K)

showed a marked reduction in regeneration (Fig. 2E, L). Axons were scored as regenerating if a growth cone was present at their tip and they had grown beyond their retraction point (Fig. 2B, G). Within dissociated cultures, retraction of axons in controls was most obvious within 15min post-axotomy, with regeneration occurring between 15min and 1h. Uncut axons within the same cultures continued to grow in the presence of NeuAc2en with normal growth cone morphology (Fig. 2C-D). Additionally we attenuated total ganglioside synthesis using two different inhibitors, fumonisinB1 and (±)-threo-1-Phenyl-2-decanoylamino-3-morpholino-1-propanol hydrochloride (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 between treated and control cultures over the same periods (Fig. 2M, N) with axon growth in uncut DRG cultures not affected by NeuAc2en (Fig. 2N). 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. In order to visualise changes in the vicinity of axon surfaces, we measured the intensity of GD1a and GM1 in single optical sections, selecting narrow areas of interest parallel to axons in between S100β-stained Schwann cells and βIII-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 4-fold increase within 6h after crush injury, returning back to uncrushed levels by 12h (Fig. 3B-D, 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 non-specific 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 1h after removal. Axotomy 30 mins 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 30mins after NeuAc2en removal remained low, similar to cultures in which the inhibitor was still present (Fig. 4H-I,L).

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-F,G). Treatment of Neu3 sialidase-inhibited cultures with

sialidase restored their regenerative ability near to the levels observed in normal conditions (Fig. 4H-K, L). These results 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 if 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 15mins after axotomy in retinal axons (Fig. 5A-B). To determine if 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 was observed (Fig. 5E). 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. 5C-D, F). These experiments suggest that Neu3 sialidase is present on adult retinal axons but is not activated by axotomy.

To determine if 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 ßIII-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-N, O). Furthermore, only 4.8% of control axons regrew 1h post-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 signalling events that link axotomy to Neu3 sialidase activation and then to axon regeneration. Previous work has identified three signalling pathways (ERK, P38MAPK and mammalian target of rapamycin or

mTOR) (Abe et al., 2010; Agthong et al., 2009; Agthong et al., 2012) as well as a local Ca^{2+} influx (Mandolesi et al., 2004; Cho and Cavalli, 2012; Cho et al., 2013) which are activated/occur 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 signalling 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 signalling 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 activity. Based on previous work, Neu3 sialidase-mediated ganglioside conversion leads to increased signalling 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. 10). We first investigated phosphorylation of P38MAPK (pP38MAPK) 15mins 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 signalling events shown in Figure 10.

P38MAPK is not activated in adult retinal axons post-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 15mins 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. 9A-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. 9G-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 following 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 post-axotomy is a factor in this poor regenerative ability. The importance of sialidase activity for CNS regeneration is supported by a previous study in which treatment of the injured spinal cord with sialidase promoted axon regeneration (Yang et al., 2006) and functional recovery (Mountney et al., 2010; Mountney et al., 2013). Further work has shown that conversion of gangliosides to GM1 by Neu3 sialidase is critical during early neuronal differentiation for specification of axons (Abad-Rodriguez and Robotti, 2007; Da Silva et al., 2005). Furthermore, poor regeneration of PNS axons has been observed in animals that lack complex gangliosides (Kittaka et al., 2008), and following treatment with antibodies to GM1 ganglioside (Lehmann et al., 2007; Lopez et al., 2010).

Regulation of Neu3 sialidase *activity*

The action of sialidases such as Neu3 is to remove terminal sialic acid groups, converting gangliosides such as GD1a and GT1b to GM1. Following 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 knockdown. Preventing conversion of axonal gangliosides to GM1 led to failed 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 the major nervous system gangliosides to GM1. Within 1h after axotomy, as axons regenerated their growth cones, GD1a and GM1 levels reverted to pre-axotomy 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 signalling pathways, including P38MAPK, ERK and PI3 kinase (Chierzi et al., 2005; Liu and Snider 2001; Verma et al., 2005). Our results show that blockade of these pathways prevents Neu3 sialidase activation post-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 signalling pathways. Calcium influx after axotomy initiates several other processes involved in regeneration, including activation of calpain and Ca^{2+}-dependent kinases (Chierzi et al., 2005; Kamber et al., 2009; Ziv and Spira, 1997). 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 yet know if P38MAPK binds directly to Neu3 sialidase or if it acts via an intermediary step. P38MAPK activation has been shown to be essential for efficient axon regeneration (Kato et al., 2013; Myers et al., 2003; Nix et al., 2011; Verma et al., 2005). Axotomy also activated ERK, which is a step in the major signalling pathway controlling axon growth (Chierzi et al., 2005; Waetzig and Herdegen, 2005). Neu3 sialidase activation and ganglioside conversion is sufficient to activate ERK, confirmed when ERK was activated in axons treated with exogenous sialidase. These results suggest that P38MAPK and ERK are acting in-parallel within DRG neurons both downstream of the initial calcium influx that arises immediately post-axotomy. This is likely due to the construction of cell surface signalling domains (rafts), of which GM1 is a major organizer. These domains contain various activators of signalling pathways such as integrins, growth factor receptors and other signalling molecules. In embryonic neurons, Neu3 sialidase action can affect axons by increasing TrkA signalling resulting in ERK activation (Da Silva et al., 2005).

Neu3 sialidase *activation fails in CNS axons*

While Neu3 sialidase was activated by axotomy in DRG axons, we observed no such change in adult retinal axons, despite the presence of Neu3 sialidase. These results suggest there is a failure in the mechanisms required to 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 that leads to P38MAPK activation in retinal axons. Interestingly, axonal regeneration in a P38MAPK knockout mouse 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 leads to a lack of axon growth in cerebellar granular neurons (Williams, et al., 2008). Likewise, upregulation of cytokines following injury, such as tumor necrosis factor-alpha (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 post-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 co-receptor 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. While important *in vivo*, where axons and myelinating glia are in close contact, it is not likely the mechanism behind the positive effect of sialidase in our *in vitro* assays as these axons were not in contact with Schwann cells or oligodendrocytes, and inhibition of ganglioside synthesis blocked rather than stimulated axon regeneration. Instead, our results suggest a positive effect of GM1 on axon regeneration although we equally realize that this effect could be

due to 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 up-regulate 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. 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 (Miyagi et al., 2008; Miyata et al., 2011; Tringali et al., 2012; Yamaguchi et al., 2006). Metastatic transformation and growth cone advance share a requirement for membrane extension, decreased adhesiveness to substrate at the leading edge, and formation of fillipodia and lamellipodia. In tumor cells, Neu3 sialidase has effects on adhesion, integrin turnover and activation, caspase activity, caveolins and several signalling pathways (Miyagi et al., 2008; Roche et al., 2007). Disruption of microdomains/rafts might explain why mice lacking complex gangliosides have a marked decrease in expression levels 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 (Bahr et al., 1989; Cuello et al., 1994; Rabin et al., 2002; Rabin and Mocchetti, 1995). 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., 2008).

The experiments in this paper demonstrate that an increase in the ratio of growth-promoting gangliosides (GM1) to growth-inhibiting gangliosides (GD1a and others including GT1b) mediated by Neu3 sialidase play an important part in changes that lead to axon regeneration after axotomy. Much of the detail about cytoskeletal changes that occur after axotomy is known (Erez et al., 2007; Erturk et al., 2007; Spira et al., 2003) and we are beginning to understand the process of local mRNA translation (Hanz et al., 2003; Vogelaar et al., 2009; Willis et al., 2005). 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.

References

Abad-Rodriguez J, Robotti A (2007) Regulation of axonal development by plasma membrane gangliosides. J Neurochem 103:47-55.

Abe N, Borson SH, Gambello MJ, Wang F, Cavalli V (2010) Mammalian target of rapamycin (mTOR) activation increases axonal growth capacity of injured peripheral nerves. J Biol Chem 285:28034-28043.

Afshari FT, Kappagantula S, Fawcett JW (2009) Extrinsic and intrinsic factors controlling axonal regeneration after spinal cord injury. Expert Rev Mol Med 11:e37. doi: 10.1017/S1462399409001288.

Agthong, S, Kaewsema A, Chentanez V (2012) Inhibition of P38 MAPK reduces loss of primary sensory neurons after nerve transection. Neurol Res 34:714-720.

Agthong S, Koonam J, Kaewsema A, Chentanez V (2009) Inhibition of MAPK ERK impairs axonal regeneration without an effect on neuronal loss after nerve injury. Neurol Res 31:1068-1074.

Bahr M, Vanselow J, Thanos S (1989) Ability of adult rat ganglion cells to regrow axons in vitro can be influenced by fibroblast growth factor and gangliosides. Neurosci Lett 96:197-201.

Bradke F, Dotti CG (1999) The role of local actin instability in axon formation. Science 283:1931-1934.

Chew DJ, Fawcett JW, Andrews MR (2012) The challenges of long-distance axon regeneration in the injured CNS. Prog Brain Res 201:253-294.

Chierzi S, Ratto GM, Verma P, Fawcett JW (2005) The ability of axons to regenerate their growth cones depends on axonal type and age, and is regulated by calcium, cAMP and ERK. Eur J Neurosci 21:2051-2062.

Cho Y, Cavalli V (2012) HDAC5 is a novel injury-regulated tubulin deacetylase controlling axon regeneration. EMBO J 31:3063-3078.

Cho Y, Sloutsky R, Naegle KM, Cavalli V (2013) Injury-induced HDAC5 nuclear export is essential for axon regeneration. J Neurosci 155:894-908.

Collins BE, Yang LJ, Mukhopadhyay G, Filbin MT, Kiso M, Hasegawa A, Schnaar RL (1997) Sialic acid specificity of myelin-associated glycoprotein binding. J Biol Chem 272:1248-1255.

Coulthard LR, White DE, Jones DL, McDermott MF, Burchill SA (2009) P38(MAPK): stress responses from molecular mechanisms to therapeutics. Trends Mol Med 15:369-379.

Cuello AC, Garofalo L, Liberini P, Maysinger D (1994) Cooperative effects of gangliosides on trophic factor-induced neuronal cell recovery and synaptogenesis: studies in rodents and subhuman primates. Prog Brain Res 101:337-355.

Da Silva JS, Hasegawa T, Miyagi T, Dotti CG, Abad-Rodriguez J (2005) Asymmetric membrane ganglioside sialidase activity specifies axonal fate. Nat Neurosci 8:606-615.

Erez H, Malkinson G, Prager-Khoutorsky M, De Zeeuw CI, Hoogenraad CC, Spira ME (2007) Formation of microtubule-based traps controls the sorting and concentration of vesicles to restricted sites of regenerating neurons after axotomy. J Cell Biol 176:497-507.

Erturk A, Hellal F, Enes J, Bradke F (2007) Disorganized microtubules underlie the formation of retraction bulbs and the failure of axonal regeneration. J Neurosci 27:9169-9180.

Facci L, Leon A, Toffano G, Sonnino S, Ghidoni R, Tettamanti G (1984) Promotion of neuritogenesis in mouse neuroblastoma cells by exogenous gangliosides. Relationship between the effect and the cell association of ganglioside GM1. J Neurochem 42:299-305.

Gao X, Lowry PR, Zhou X, Depry C, Wei Z, Wong GW, Zhang J (2011) PI3K/Akt signaling requires spatial compartmentalization in plasma membrane microdomains. Proc Natl Acad Sci U S A 108:14509-14514.

Geisler FH, Coleman WP, Grieco G, Poonian D (2001) The Sygen multicenter acute spinal cord injury study. Spine 26:S87-S98.

Grider MH, Park D, Spencer DM, Shine HD (2009) Lipid raft-targeted Akt promotes axonal branching and growth cone expansion via mTOR and Rac1, respectively. J Neurosci Res 87:3033-3042.

Guirland C, Suzuki S, Kojima M, Lu B, Zheng JQ (2004) Lipid rafts mediate chemotropic guidance of nerve growth cones. Neuron 42:51-62.

Guirland C, Zheng JQ (2007) Membrane lipid rafts and their role in axon guidance. Adv Exp Med Biol 621:144-155.

Hanz S, Perlson E, Willis D, Zheng, JQ, Massarwa, R, Huerta JJ, Koltzenburg M, Kohler M, van-Minnen J, Twiss JL, Fainzilber M (2003) Axoplasmic importins enable retrograde injury signaling in lesioned nerve. Neuron 40:1095-1104.

Kalka D, von Reitzenstein C, Kopitz J, Cantz M (2001) The plasma membrane ganglioside sialidase cofractionates with markers of lipid rafts. Biochem Biophys Res Commun 283:989-993.

Kamber D, Erez H, Spira ME (2009) Local calcium-dependent mechanisms determine whether a cut axonal end assembles a retarded endbulb or competent growth cone. Exp Neurol 219:112-25

Kamiguchi H (2006) The region-specific activities of lipid rafts during axon growth and guidance. J Neurochem 98:330-335.

Kato K, Shiga K, Yamaguchi K, Hata K, Kobayashi T, Miyazaki K, Saijo S, Miyagi T (2006) Plasma-membrane-associated sialidase (Neu3) differentially regulates integrin-mediated cell proliferation through laminin- and fibronectin-derived signalling. Biochem J 394:647-656.

Kato N, Matsumoto M, Kogawa M, Atkins GJ, Findlay DM, Fujikawa T, Oda H, Ogata M (2013) Critical role of P38 MAPK for regeneration of the sciatic nerve following crush injury in vivo. J Neuroinflammation 10:1. doi: 10.1186/1742-2094-10-1.

Katoh-Semba R, Kaneko R, Kitajima S, Tsuzuki M, Ichisaka S, Hata Y, Yamada H, Miyazaki N, Takahashi Y, Kato K (2009) Activation of P38 mitogen-activated protein kinase is required for in vivo brain-derived neurotrophic factor production in the rat hippocampus. Neuroscience 163:352-361.

Kilic E, ElAli A, Kilic U, Guo Z, Ugur M, Uslu U, Bassetti CL, Schwab ME, Hermann DM (2010) Role of Nogo-A in neuronal survival in the reperfused ischemic brain. J Cereb Blood Flow Metab 30:969-984.

Kittaka D, Itoh M, Ohmi Y, Kondo Y, Fukumoto S, Urano T, Tajima O, Furukawa K (2008) Impaired hypoglossal nerve regeneration in mutant mice lacking complex gangliosides: down-regulation of neurotrophic factors and receptors as possible mechanisms. Glycobiology 18:509-516.

Kopitz J, von Reitzenstein C, Sinz K, Cantz M (1996) Selective ganglioside desialylation in the plasma membrane of human neuroblastoma cells. Glycobiology 6:367-376.

Lehmann HC, Lopez PH, Zhang G, Ngyuen T, Zhang J, Kieseier BC, Mori S, Sheikh KA (2007) Passive immunization with anti-ganglioside antibodies directly inhibits axon regeneration in an animal model. J Neurosci 27:27-34.

Liu RY, Snider WD (2001) Different signaling pathways mediate regenerative versus developmental sensory axon growth. J Neurosci 21:RC164 (1-5).

Long KE, Lemmon V (2000) Dynamic regulation of cell adhesion molecules during axon outgrowth. J Neurobiol 44:230-245.

Lopez PH, Zhang G, Zhang J, Lehmann HC, Griffin JW, Schnaar RL, Sheikh KA (2010) Passive transfer of IgG anti-GM1 antibodies impairs peripheral nerve repair. J Neurosci 30:9533-9541.

Mandolesi G, Madeddu F, Bozzi Y, Maffei L, Ratto GM (2004) Acute physiological response of mammalian central neurons to axotomy: ionic regulation and electrical activity. FASEB J 18:1934-1936.

Mei S, Gu H, Ward A, Yang X, Guo H, He K, Liu Z, Cao W (2012) P38 mitogen-activated kinase (MAPK) promotes cholesterol ester formation accumulation in macrophages through inhibition of macroautophagy. J Biol Chem 15:11761-11768.

Miyagi T, Wada T, Iwamatsu A, Hata K, Yoshikawa Y, Tokuyama S, Sawada M (1999) Molecular cloning and characterization of a plasma membrane-associated sialidase specific for gangliosides. J Biol Chem 274:5004-5011.

Miyagi T, Wada T, Yamaguchi K (2008) Roles of plasma membrane-associated sialidase Neu3 in human cancers. Biochim Biophys Acta 1780:532-537.

Miyagi T, Wada T, Yamaguchi K, Hata K, Shiozaki K (2008) Plasma membrane-associated sialidase as a crucial regulator of transmembrane signalling. J Biochem 144:279-285.

Miyata M, Kambe M, Tajima O, Moriya S, Sawaki H, Hotta H, Kondo Y, Narimatsu H, Miyagi T, Furukawa K, Furukawa K (2011) Membrane sialidase Neu3 is highly expressed in human melanoma cells promoting cell growth with minimal changes in the composition of gangliosides. Cancer Sci 102:2139-2149.

Monti E, Miyagi T (2012) Structure and Function of Mammalian Sialidases. Top Curr Chem In press.

Moore DL, Goldberg JL (2011) Multiple transcription factor families regulate axon growth and regeneration. Dev Neurobiol 71:1186-1211.

Mountney A, Zahner MR, Lorenzini I, Oudega M, Schramm LP, Schnaar RL (2010) Sialidase enhances recovery from spinal cord contusion injury. Proc Natl Acad Sci U S A 107:11561-11566.

Mountney A, Zahner MR, Sturgill ER, Riley CJ, Aston JW, Oudega M, Schramm LP, Hurtado A, Schnaar RL (2013) Sialidase, chondroitinase ABC, and combination therapy after spinal cord contusion injury. J Neurotrauma 30:181-190.

Myers RR, Sekiguchi Y, Kikuchi S, Scott B, Medicherla S, Protter A, Campana WM (2003) Inhibition of P38 MAP kinase activity enhances axonal regeneration. Exp Neurol 184:606-614.

Neumann H, Schweigreiter R, Yamashita T, Rosenkranz K, Wekerle H, Barde YA (2002) Tumor necrosis factor inhibits neurite outgrowth and branching of hippocampal neurons by a rho-dependent mechanism. J Neurosci 22:854-862.

Nix P, Hisamoto N, Matsumoto K, Bastiani M (2011) Axon regeneration requires coordinate activation of P38 and JNK MAPK pathways. Proc Natl Acad Sci U S A 108:10738-10743.

Norambuena A, Schwartz MA (2011) Effects of integrin-mediated cell adhesion on plasma membrane lipid raft components and signaling. Mol Biol Cell 22:3456-3464.

Nozaki K, Nishimura M, Hashimoto N (2001) Mitogen-activated protein kinases and cerebral ischemia. Mol Neurobiol 23:1-19.

Oehler C, Kopitz J, Cantz M (2002) Substrate specificity and inhibitor studies of a membrane-bound ganglioside sialidase isolated from human brain tissue. Biol Chem 383:1735-1742.

Okada M, Itoh M, Haraguchi M, Okajima T, Inoue M, Ohishi H, Matsuda Y, Iwamoto T, Kawano T, Fukumoto S, Miyazaki H, Furukawa K, Aizawa S, Furukawa K (2002) b-series ganglioside deficiency exhibits no definite changes in the neurogenesis and the sensitivity to Fas-mediated apoptosis but impairs regeneration of the the lesioned hypoglossal nerve. J Biol Chem 277:1633-1636.

Pollock J, McFarlane SM, Connell MC, Zehavi U, Vandenabeele P, MacEwan DJ, Scott RH (2002) TNF-alpha receptors simultaneously activate Ca2+ mobilisation and stress kinases in cultured sensory neurones. Neuropharmacology 42:93-106.

Rabin SJ, Bachis A, Mocchetti I (2002) Gangliosides activate Trk receptors by inducing the release of neurotrophins. J Biol Chem 277:49466-49472.

Rabin SJ, Mocchetti I (1995) GM1 ganglioside activates the high-affinity nerve growth factor receptor trkA. J Neurochem 65:347-354.

Roche PH, Figarella-Branger D, Daniel L, Bianco N, Pellet W, Pellissier JF (1997) Expression of cell adhesion molecules in normal nerves, chronic axonal neuropathies and Schwann cell tumors. J Neurol Sci 151:127-133.

Rodriguez JA, Piddini E, Hasegawa T, Miyagi T, Dotti CG (2001) Plasma membrane ganglioside sialidase regulates axonal growth and regeneration in hippocampal neurons in culture. J Neurosci 21:8387-8395.

Sheikh KA, Sun J, Liu Y, Kawai H, Crawford TO, Proia RL, Griffin JW, Schnaar R L (1999) Mice lacking complex gangliosides develop Wallerian degeneration and myelination defects. Proc Natl Acad Sci U S A 96:7532-7537.

Skaper SD, Katoh-Semba R, Varon S (1985) GM1 ganglioside accelerates neurite outgrowth from primary peripheral and central neurons under selected culture conditions. Brain Res 355:19-26.

Spira ME, Oren R, Dormann A, Gitler D (2003) Critical calpain-dependent ultrastructural alterations underlie the transformation of an axonal segment into a growth cone after axotomy of cultured Aplysia neurons. J Comp Neurol 457:293-312.

Stuermer CA (2010) The reggie/flotillin connection to growth. Trends Cell Biol 20:6-13.

Sun F, He Z (2010) Neuronal intrinsic barriers for axon regeneration in the adult CNS. Curr Opin Neurobiol 20:510-518.

Tringali C, Lupo B, Silvestri I, Papini N, Anastasia L, Tettamanti G, Venerando B (2012) The plasma membrane sialidase Neu3 regulates the malignancy of renal carcinoma cells by controlling β1 integrin internalization and recycling. J Biol Chem 287:42835-42845.

Verma P, Chierzi S, Codd AM, Campbell DS, Meyer RL, Holt CE, Fawcett JW (2005) Axonal protein synthesis and degradation are necessary for efficient growth cone regeneration. J Neurosci 25:331-342.

Vogelaar CF, Gervasi NM, Gumy LF, Story DJ, Raha-Chowdhury R, Leung KM, Holt CE, Fawcett JW (2009) Axonal mRNAs: characterisation and role in the growth and regeneration of dorsal root ganglion axons and growth cones. Mol Cell Neurosci 42:102-115.

Vyas AA, Patel HV, Fromholt SE, Heffer-Lauc M, Vyas KA, Dang J, Schachner M, Schnaar RL (2002) Gangliosides are functional nerve cell ligands for myelin-associated glycoprotein (MAG), an inhibitor of nerve regeneration. Proc Natl Acad Sci U S A 99:8412-8417.

Waetzig V, Herdegen T (2005) MEKK1 controls neurite regrowth after experimental injury by balancing ERK1/2 and JNK2 signaling. Mol Cell Neurosci 30:67-78.

Wang Z, Jin Y (2011) Genetic dissection of axon regeneration. Curr Opin Neurobiol 21:189-196.

Williams G, Wood A, Williams EJ, Gao Y, Mercado ML, Katz A, Joseph-McCarthy D, Bates B, Ling HP, Aulabaugh A, Zaccardi J, Xie Y, Pangalos MN, Walsh FS, Doherty P (2008) Ganglioside inhibition of neurite outgrowth requires Nogo receptor function: identification of interaction sites and development of novel antagonists. J Biol Chem 283:16641-16652.

Willis D, Li KW, Zheng JQ, Chang JH, Smit A, Kelly T, Merianda TT, Sylvester J, van Minnen J, Twiss JL (2005) Differential transport and local translation of cytoskeletal, injury-response, and neurodegeneration protein mRNAs in axons. J Neurosci 25:778-791.

Witte H, Neukirchen D, Bradke F (2008) Microtubule stabilization specifies initial neuronal polarization. J Cell Biol 180:619-632.

Wu G, Lu ZH, Obukhov AG, Nowycky MC, Ledeen RW (2007) Induction of calcium influx through TRPC5 channels by cross-linking of GM1 ganglioside associated with alpha5beta1 integrin initiates neurite outgrowth. J Neurosci 27:7447-7458.

Yamaguchi K, Hata K, Wada T, Moriya S, Miyagi T (2006) Epidermal growth factor-induced mobilization of a ganglioside-specific sialidase (Neu3) to membrane ruffles. Biochem Biophys Res Commun 346:484-490.

Yang LJ, Lorenzini I, Vajn K, Mountney A, Schramm LP, Schnaar RL (2006) Sialidase enhances spinal axon outgrowth in vivo. Proc Natl Acad Sci U S A 103:11057-11062.

Zhang G, Lehmann HC, Manoharan S, Hashmi M, Shim S, Ming GL, Schnaar RL, Lopez PH, Bogdanova N, Sheikh KA (2011) Anti-ganglioside antibody-mediated activation of RhoA induces inhibition of neurite outgrowth. J Neurosci 31:1664-1675.

Zheng JQ, Kelly TK, Chang B, Ryazantsev S, Rajasekaran AK, Martin KC, Twiss JL (2001) A functional role for intra-axonal protein synthesis during axonal regeneration from adult sensory neurons. J Neurosci 21:9291-9303.

Ziv NE, Spira ME (1997) Localized and transient elevations of intracellular Ca2+ induce the dedifferentiation of axonal segments into growth cones. J Neurosci 17:3568-3579.

Figure Legends:

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 (**D - I**) or dissociated DRGs (**K - P, R - W**) with axons uncut (**D, G, K, N, R, U**), 15mins (**E, H, L, O, S, V**) or 1h after axotomy (**F, I, M, P, T, W**). DRGs are double-labelled for GD1a ganglioside (**green**) in **D – I**, **K – P** (or GT1b ganglioside in **R – W**) and Cholera toxin B subunit (CTB) – for GM1 ganglioside (**red**) under normal conditions (**D - F, K - M, R - T**), after exposure to NeuAc2en (**G - I**), or after Neu3 siRNA transfection (**N - P, U - W**). 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 (± SEM) in **J** (DRG explants, 40 – 50 axons/chamber, 2 DRG/chamber, n≥4 chambers) or **Q and X** (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 approximately 70%, and has no effect

on Neu4, a closely related sialidase. Controls are siGLO-transfected only neurons (non-functional and non-targeting siRNA). *P<0.05, **P<0.01, ***P<0.001 calculated with one-way or two-way ANOVA, where appropriate. Bars: 250µm (**V-W**), 25µm (**A-F**), 50µm (**H-M, O-T**).

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 mins (dissociated DRGs only; **G, J**) or 1h 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**). Percent regeneration 1h after axotomy (± SEM) plotted in **E** (n=6 DRG explants) or following Neu3 siRNA transfection or **L** (2 axons/chamber, n=15 chambers, dissociated DRGs). Arrows/arrowheads indicate axons that were cut, retracted at 15min and regenerated at 1h. The★**(C,D)** indicates an uncut axon which continued to grow in the presence of NeuAc2en. The inset image (**K**) shows 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 18h after exposure to PDMP (**M**), 1h 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 µm/h with ± SEM) which is plotted in **P** (each

column represents a mean of 30-40 axons from n=3 chambers/condition). ***P<0.001 calculated using two-tailed Student's t-test. Bars: 20μm **(A - D)**, 100μm **(F - K)**, 50μm **(inset in K)**, 200μm **(M - O)**.

Figure 3: Neu3 sialidase is transiently activated in the sciatic nerve after crush injury.

(A, A') Confocal micrograph of sciatic nerve immunolabelled with S100β (**green**), CTB (**red**) and ßIII-tubulin (**blue**). Area within dotted line (**A'**) denotes 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'**), 6h after crush (**6hrs; C, C'**) and 12h after crush (**12hrs; D, D'**), triple labelled 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 gangliosides intensities. **(E)** The ratio of GM1/GD1a ganglioside intensities (± SEM) over time post-injury is plotted (n=6/time point). ***P<0.001, **P<0.01 calculated using one-way ANOVA. Bars: 50μm (**A-D**), 25μm (**A'-D'**); orientation of figures: top = caudal, bottom = rostral.

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 1h, 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 30mins later showing adult DRG axons uncut (**A, D**), 15mins (**B, E**) and 1h after axotomy (**C, F**), double labelled for GD1a (**green**) and GM1 gangliosides (CTB, **red**). The ratio of GM1/GD1a gangliosides (± SEM) was plotted in **G**, with the GM1 & GD1a ganglioside intensities ratio normalized to background (n≥4). **P<0.01 and ***P<0.001 calculated with 2-way ANOVA. **(H-K)** Phase contrast photomicrographs showing DRG axons immediately after axotomy (**H, J**) and 1h 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. Percent regeneration (± SEM) 1h post-axotomy plotted in **L** (n=6). ***P<0.001 calculated using student's two-tailed t-test. Bars: 50µm (**A-F**) and 30µm (**H-K**).

Figure 5: Neu3 sialidase remains inactivated after retina 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 15mins after axotomy. Axons are double labelled for GD1a (**A, C**) and GM1 gangliosides (CTB - **B, D**) under normal conditions (**control 15mins; A-B**) or after exposure to *Clostridium* sialidase (**sialidase 15mins; 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≥6). **(G-I)** Fluorescent confocal photomicrographs (longitudinal) of optic nerve uncrushed (**G, G'**), 6h (**H, H'**) and 12h after crush (**I, I'**), triple labelled for ßIII-tubulin (**green**), GD1a (**blue**) and GM1 gangliosides (CTB, **red**). High magnification images (**'**) 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. The ratio of GM1/GD1a gangliosides (± 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 (**0mins; K, M**) and 1h post-axotomy (**1hr; L, N**), retracting under normal conditions (**control; K, L**), or regenerating after exposure to *Clostridium* sialidase applied 30mins pre-axotomy (**sialidase; M, N**). Plots of mean distance of axon tip retraction from site of injury measured 1h post-injury in the presence or absence of exogenous sialidase (n≥6) (**O**) and percentage of axons that regenerated past retraction position in retinal axons 1h after injury (n≥6) (**P**) with ± SEM. P values (***P<0.001, *p<0.05) by student's t-test (2-tailed). Bars: 100µm (**A-D)**; 25µm (**E, G'-I'**); 50µm (**K-N, G-I**).

Figure 6: Axon regeneration is regulated by eCa^{2+}, P38MAPK, ERK and mTOR signalling cascades. (**A-J**) Phase contrast photomicrographs of adult DRG axons photographed immediately after axotomy (**A-E**) and 1h post-axotomy (**F-J**), under normal conditions (**control; A, F**) and after exposure to inhibitors of ERK (**U0126; B, G**), removal of Ca^{2+} (**w/o 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. Percent regeneration of axons (±SEM) is plotted in **K** (n≥5). P values (***P<0.001) were calculated with one-way ANOVA. Bars: 50µm.

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 15mins after axotomy (**F-J**), double labelled 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+} (**w/o calcium; C, H**), inhibitors of P38MAPK (**SB203580; D, I**) or mTOR (**rapamycin; E, J**). The ratio of GM1/GD1a ganglioside intensities (± SEM) normalized against background intensity levels is plotted in **K** (40 – 50 axons/chamber, 2 DRGs/chamber, n≥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 15mins and 1h post-axotomy were normalized against corresponding uncut axons' ratio. (**L-M**) The ratio of pP38MAPK/P38MAPK (**L**; uncut, n=4; 15mins, n=6) and pERK/ERK (**M**; uncut, n=4; 15mins, n=4) uncut and 15mins after injury is plotted ± SEM. ***P<0.001, **P<0.01, *P<0.05 calculated with two-way ANOVA (**K**) and student's t-test (two-tailed) (**L-M**). Bars: 50µm (**A-J**).

Figure 8: P38MAPK activation is the intermediary step between eCa^{2+} influx and Neu3 sialidase activation after axonal injury, while ERK activation is downstream of Neu3 sialidase activation.

I (A-E): Fluorescent photomicrographs of adult DRG axons uncut (**A, C**) and 15mins after axotomy (**B, D**), double labelled for P38MAPK (**red**) & phospho P38MAPK (**green**) under normal conditions (**control; A-B**) and after removal of eCa^{2+} (**w/o Ca^{2+}; C-D**). The ratio of phospho P38MAPK/P38MAPK intensities (± 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

labelled for GD1a (**green**) and GM1 gangliosides (CTB, **red**) & under normal conditions (**control; F**), after removal of eCa^{2+} (**w/o Ca^{2+}; G**) and after exposure to anisomycin (AIS) in the absence of eCa^{2+} (**w/o Ca^{2+} + AIS**; **H**). The ratio of GM1/GD1a ganglioside intensities (± 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, labelled for pERK under control conditions (**J**) and after exposure to exogenous sialidase (**K**). The ratio of pERK/ERK intensities (± SEM) normalized against background intensity levels is plotted in **L** (30 – 40 axons/chamber, containing 2 DRGs, n=6 chambers). ***P<0.001, **P<0.01 calculated with student's t-test (two-tailed). Bars: 50µm.

Figure 9: P38MAPK and ERK are not activated within 15mins 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 15mins after axotomy (**B, D**), double labelled for P38MAPK (**A-B**) or ERK (**C-D**) (**red**) & pP38MAPK (**A-B**) or pERK (**C-D**) (**green**). Ratios of pP38MAPK/P38MAPK and pERK/ERK intensities (± 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-labelled 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 (± SEM) normalized against background intensity levels are plotted in **I** (30 – 40 axons/chamber, n≥7). ***P<0.001, student's t-test (2-tailed). Bars: 50µm.

Figure 10: Diagram of proposed signalling cascade regulating Neu3 sialidase activity

Membrane rupture post-axotomy leads to increased influx of eCa^{2+} into injured axons. While 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 2-step block for successful Neu3 sialidase activity mediated regeneration.

Figure 1

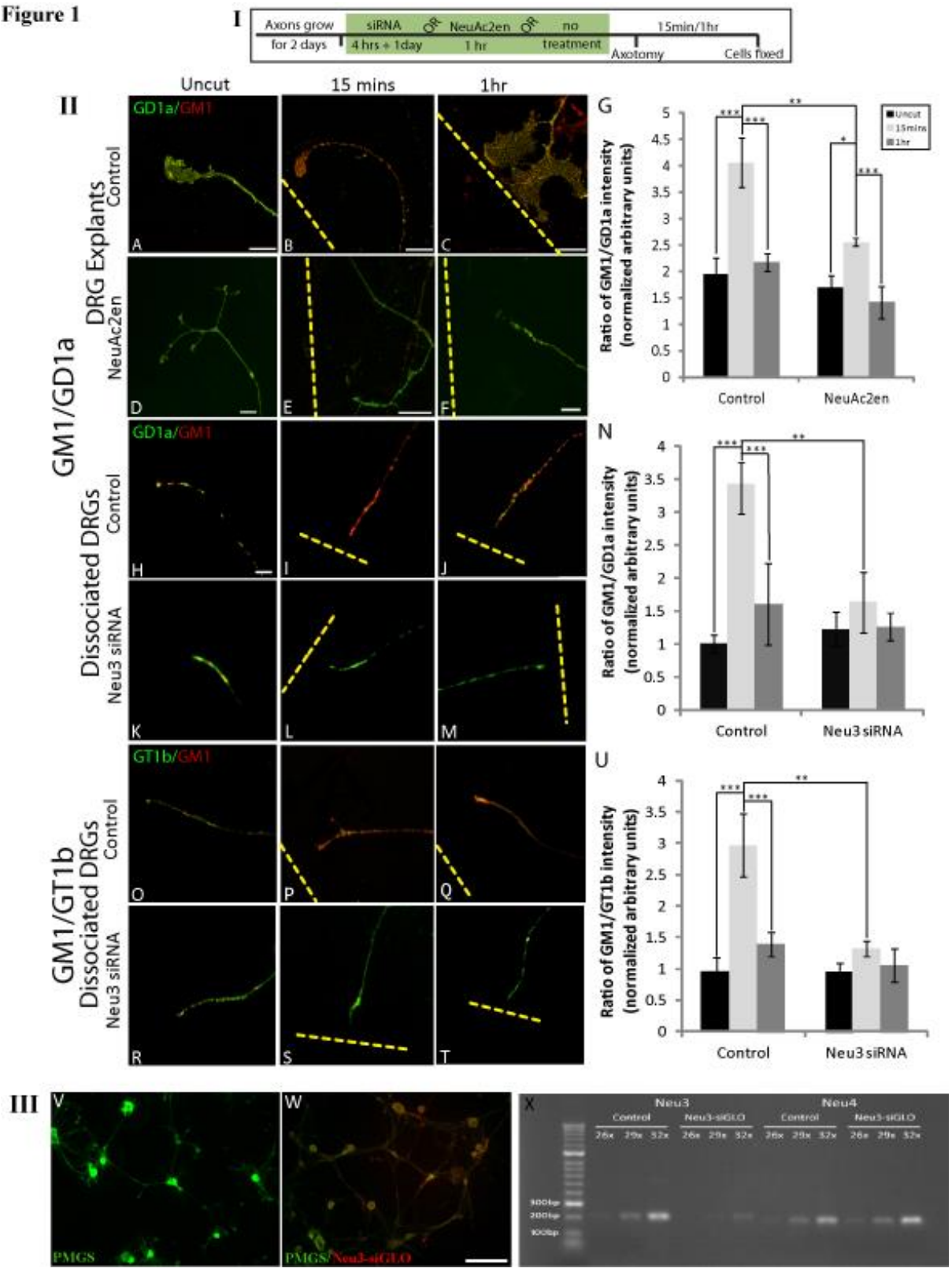

I
Axons grow for 2 days
siRNA
4 hrs + 1day
NeuAc2en
1 hr
no treatment
15min/1hr
Axotomy
Cells fixed
II
Uncut
15 mins
1hr
GM1/GD1a
DRG Explants
Control
NeuAc2en
Dissociated DRGs
Control
Neu3 siRNA
GD1a/GM1
GM1/GT1b
GT1b/GM1
A
B
C
D
E
F
H
I
J
K
L
M
O
P
Q
R
S
T
G
N
U
Ratio of GM1/GD1a intensity (normalized arbitrary units)
Ratio of GM1/GT1b intensity (normalized arbitrary units)
Uncut
15mins
1hr
Control
NeuAc2en
Neu3 siRNA
III
V
W
X
PMGS
PMGS/Neu3-siGLO
Neu3
Neu4
Control
Neu3-siGLO
26x
29x
32x
300bp
200bp
100bp

Figure 2

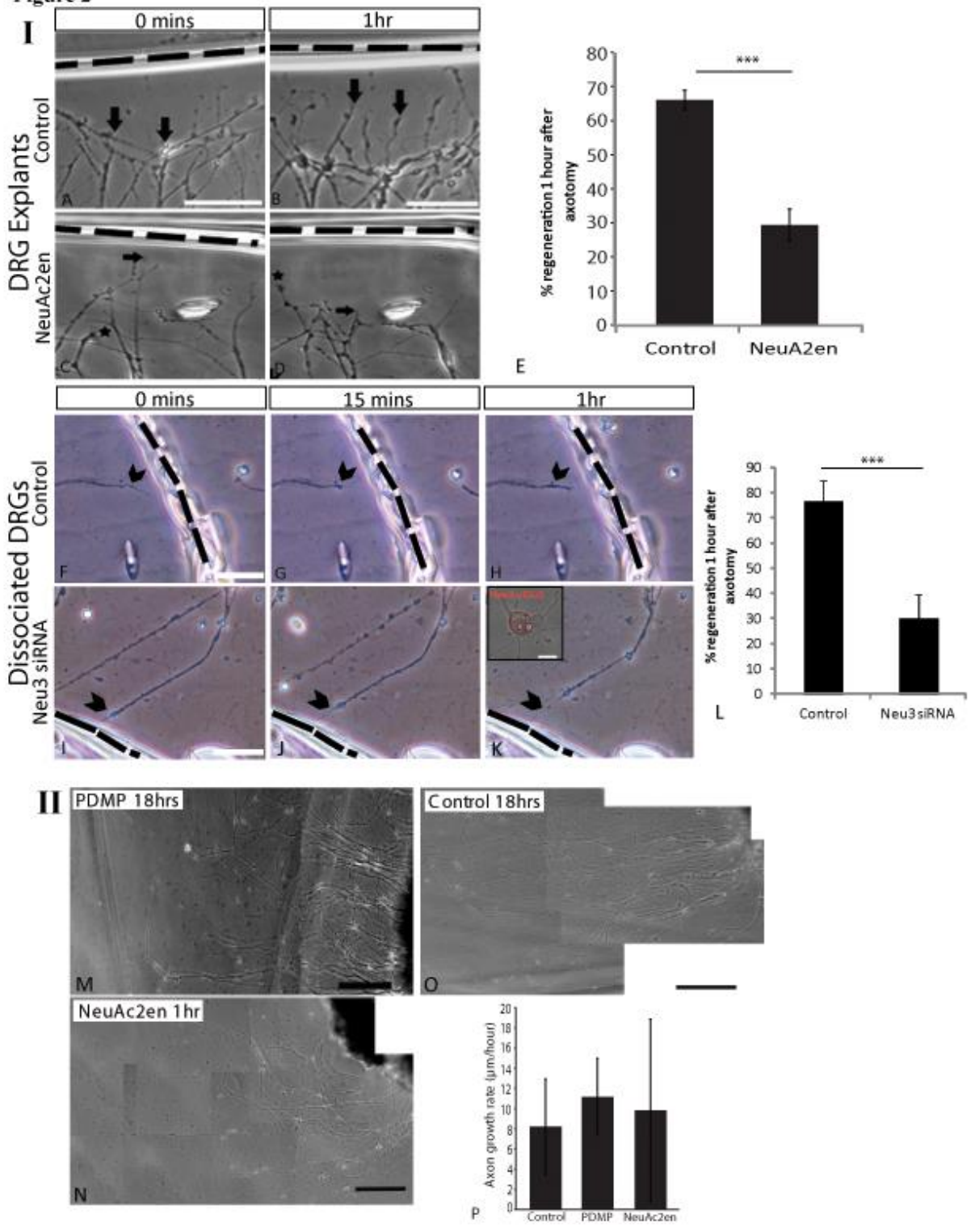

I
0 mins
1hr
DRG Explants
Control
NeuAc2en
A
B
C
D
% regeneration 1 hour after axotomy
***
Control
NeuA2en
E
0 mins
15 mins
1hr
Dissociated DRGs
Control
Neu3 siRNA
F
G
H
J
K
L
Control
Neu3siRNA
II
PDMP 18hrs
Control 18hrs
NeuAc2en 1hr
M
N
O
P
Axon growth rate (µm/hour)
Control
PDMP
NeuAc2en

Figure 3

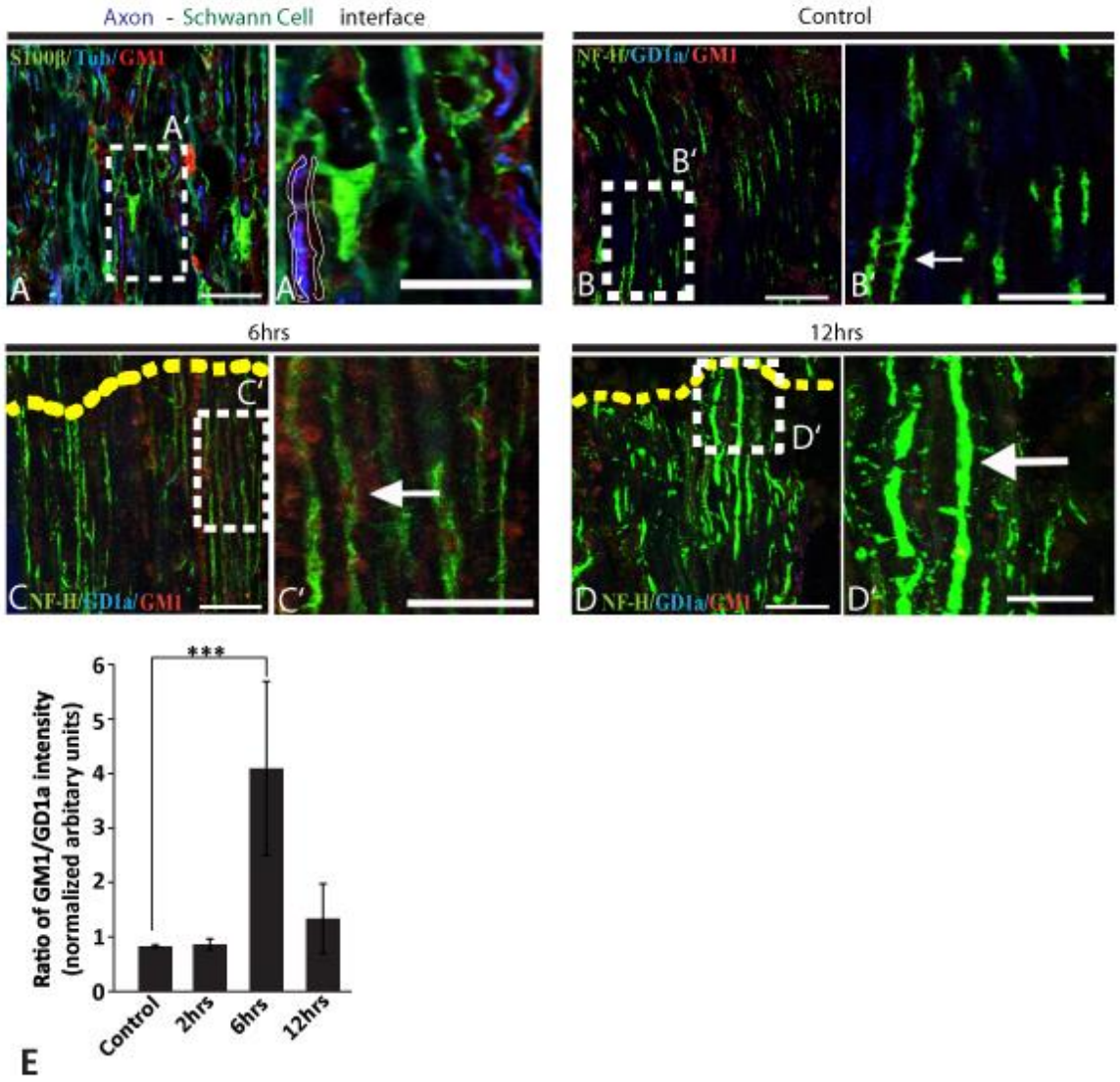

Axon - Schwann Cell interface
Control
S100β/Tuj/GM1
NF-H/GD1a/GM1
A
A'
B
B'
6hrs
12hrs
C
C'
D
D'
NF-H/GD1a/GM1
Ratio of GM1/GD1a intensity (normalized arbitary units)
***
0
1
2
3
4
5
6
Control
2hrs
6hrs
12hrs
E

Figure 4

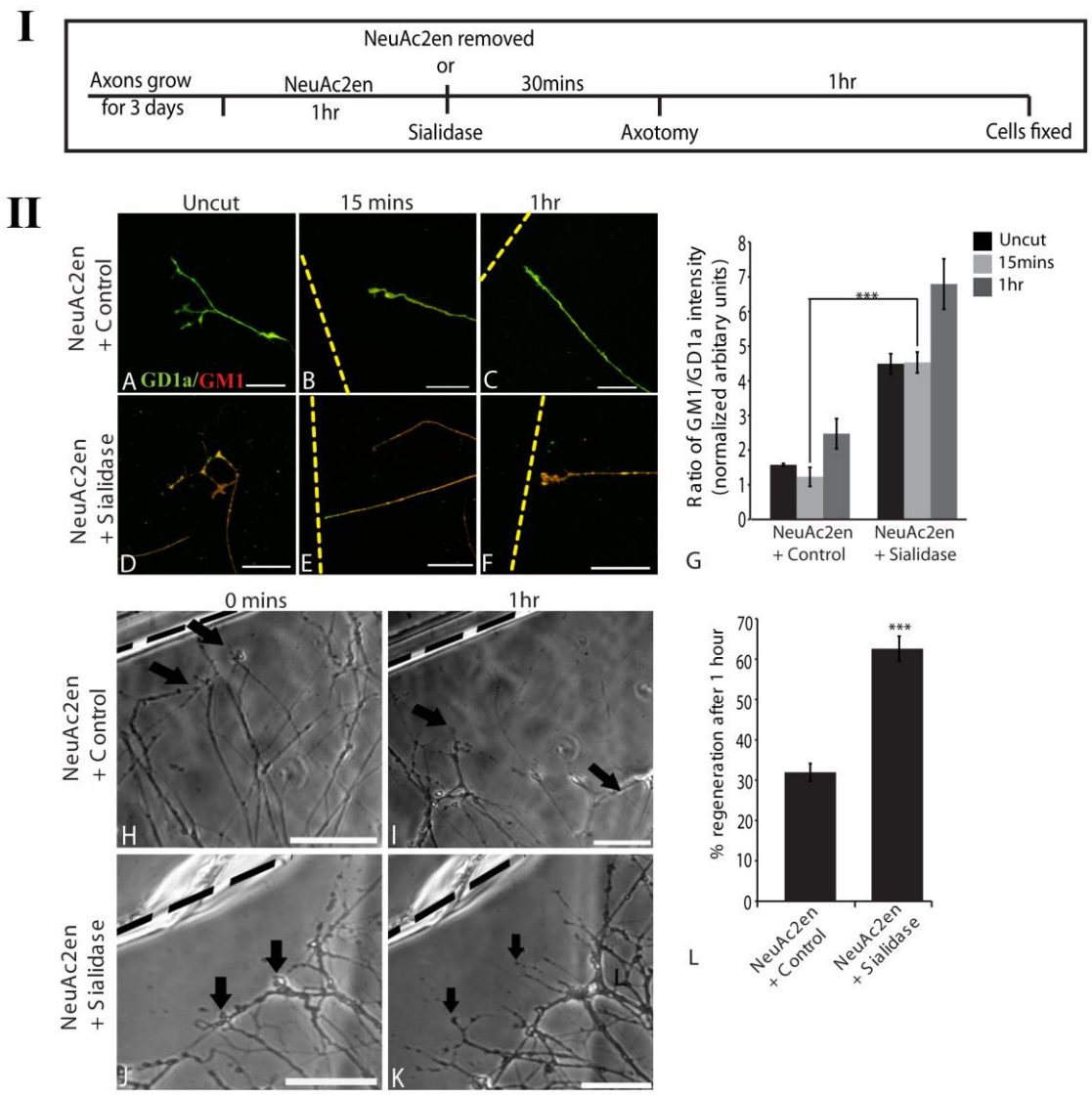

I
NeuAc2en removed
or
Axons grow
for 3 days
NeuAc2en
1hr
Sialidase
30mins
Axotomy
1hr
Cells fixed
II
Uncut
15 mins
1hr
NeuAc2en
+ Control
NeuAc2en
+ Sialidase
A GD1a/GM1
B
C
D
E
F
Ratio of GM1/GD1a intensity
(normalized arbitary units)
Uncut
15mins
1hr
***
NeuAc2en
+ Control
NeuAc2en
+ Sialidase
G
0 mins
1hr
H
I
J
K
L
% regeneration after 1 hour
***
NeuAc2en
+ Control
NeuAc2en
+ Sialidase

Figure 5

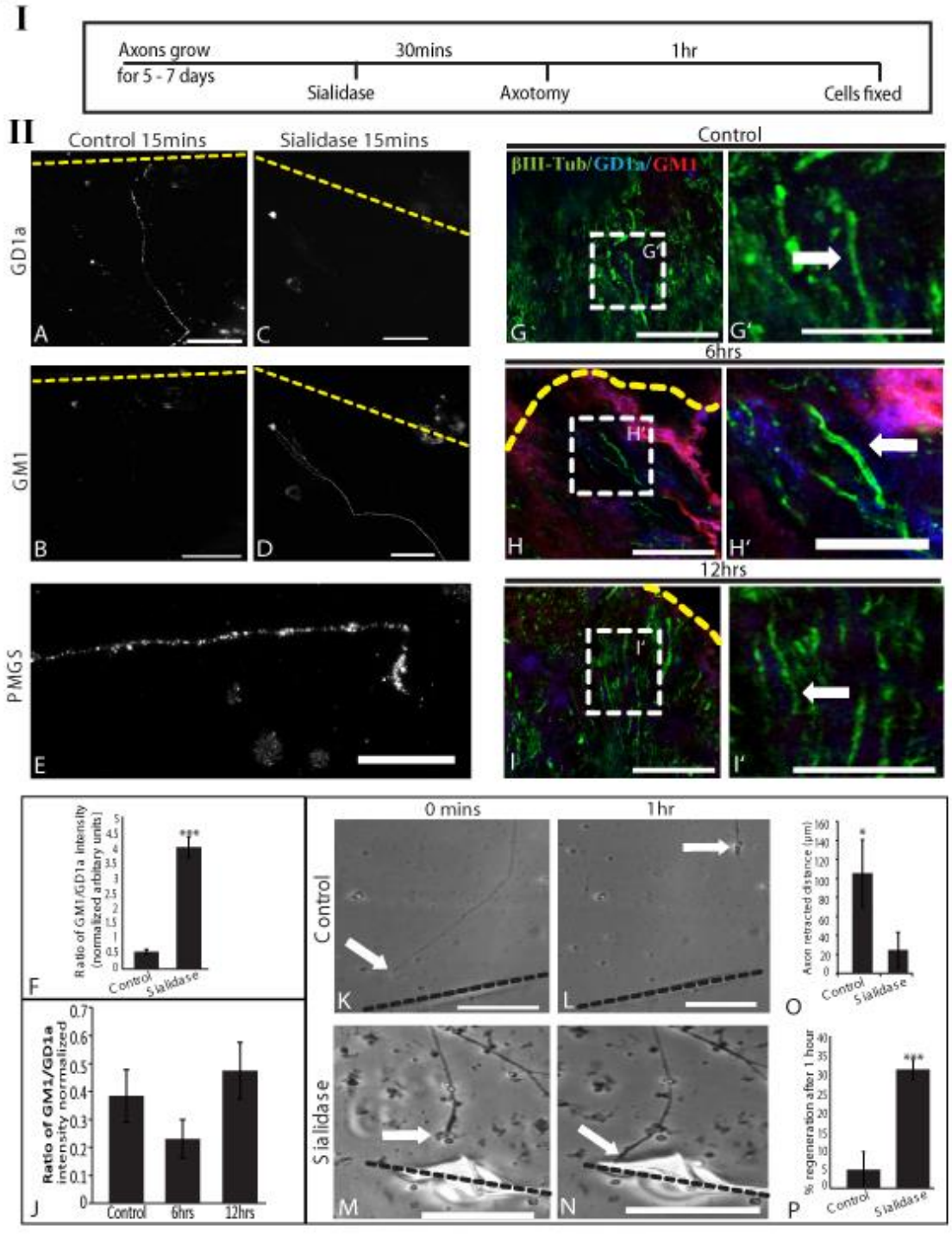

I
Axons grow for 5 - 7 days
30mins
1hr
Sialidase
Axotomy
Cells fixed
II
Control 15mins
Sialidase 15mins
Control
GD1a
GM1
PMGS
βIII-Tub/GD1a/GM1
6hrs
12hrs
A
B
C
D
E
G
G'
H
H'
I
I'
F
Ratio of GM1/GD1a intensity (normalized arbitary units)
Control
Sialidase
J
Ratio of GM1/GD1a intensity normalized
Control
6hrs
12hrs
0 mins
1hr
Control
Sialidase
K
L
M
N
O
Axon retracted distance (μm)
P
% regeneration after 1 hour

Figure 6

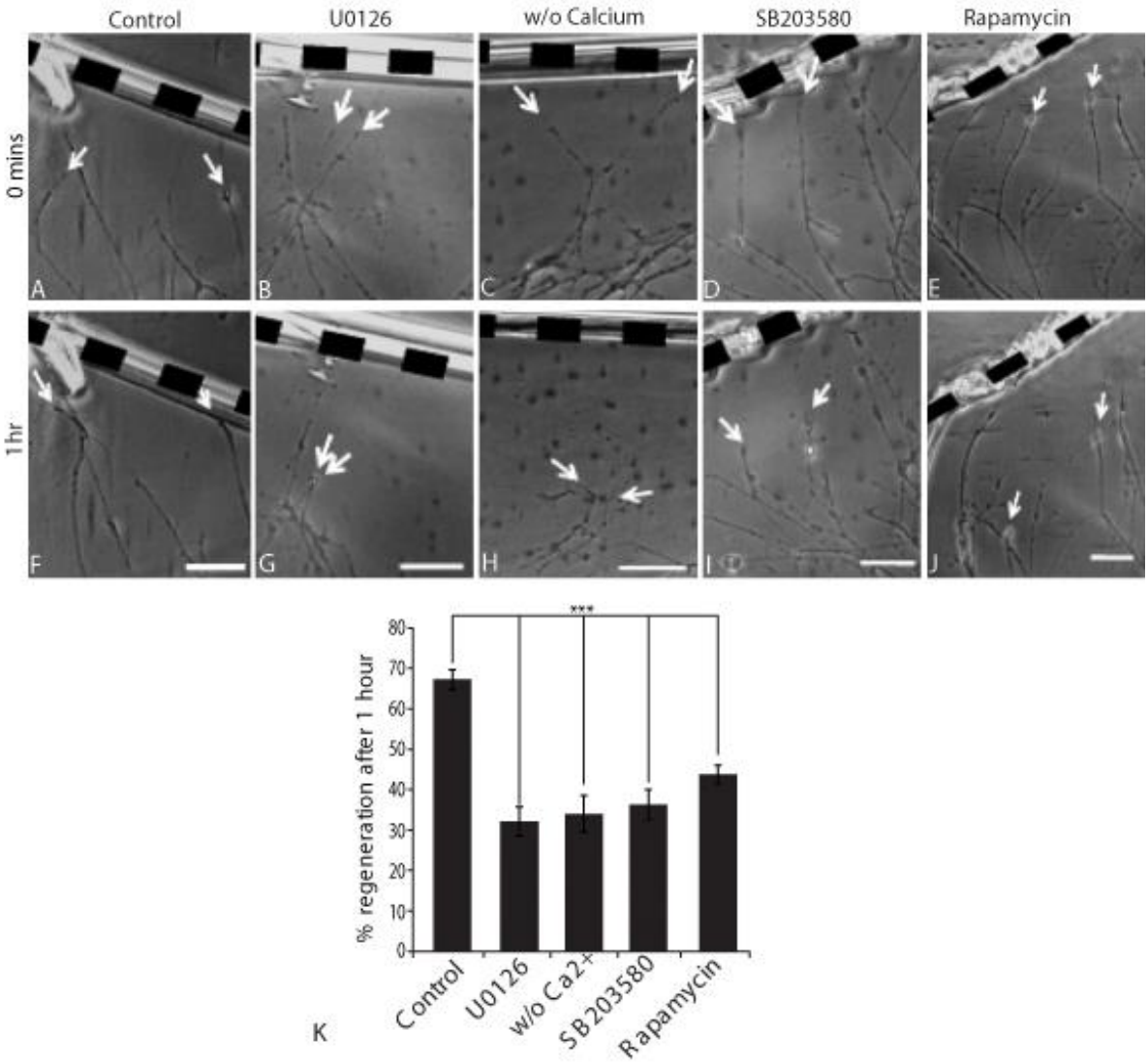
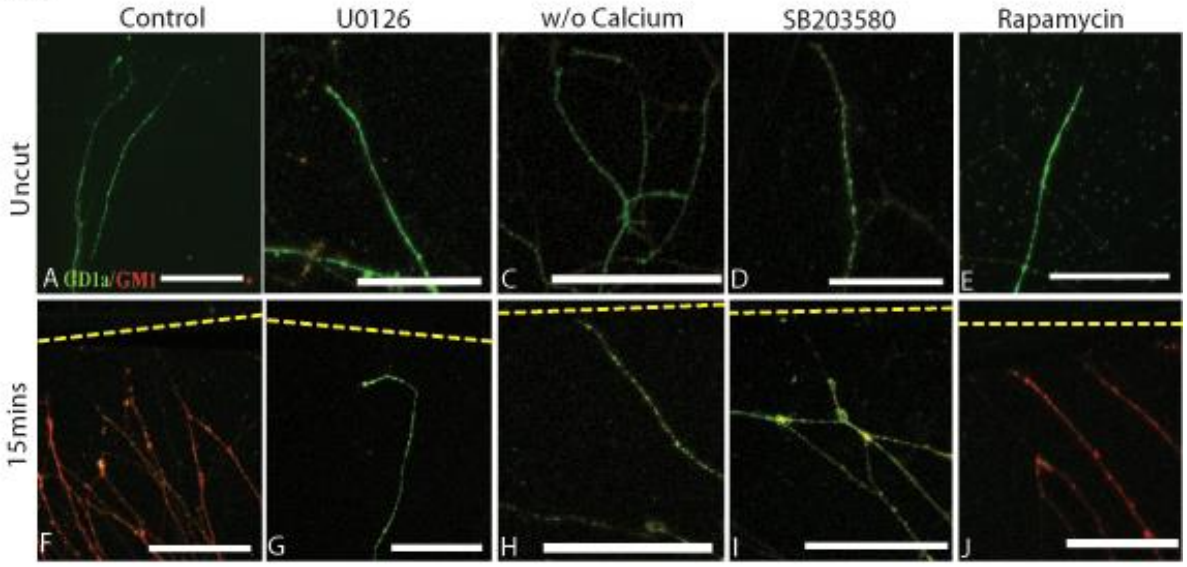
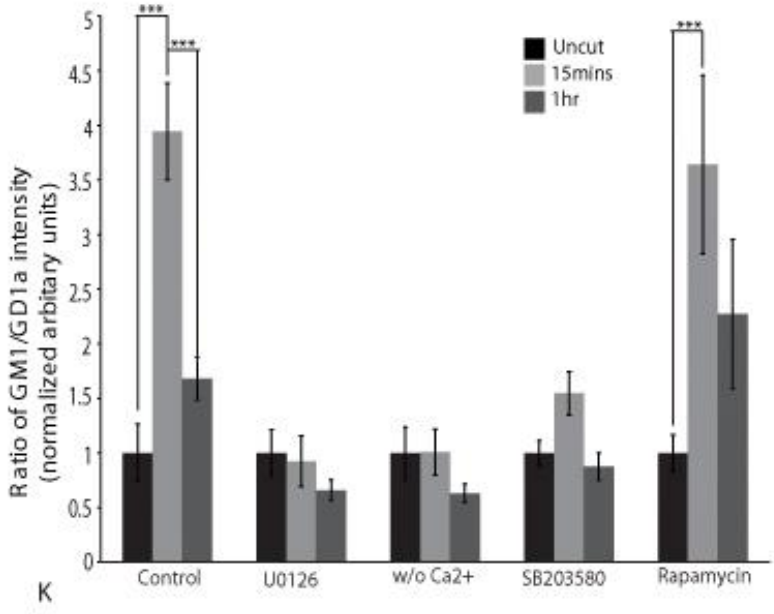
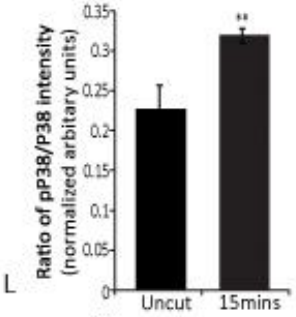

Control
U0126
w/o Calcium
SB203580
Rapamycin
0 mins
1hr
A
B
C
D
E
F
G
H
I
J
K
% regeneration after 1 hour
***
Control
U0126
w/o Ca2+
SB203580
Rapamycin

Figure 7


Control
U0126
w/o Calcium
SB203580
Rapamycin
Uncut
15mins
A GD1a/GM1
C
D
E
F
G
H
I
J


Uncut
15mins
1hr
Ratio of GM1/GD1a intensity
(normalized arbitary units)
Control
U0126
w/o Ca2+
SB203580
Rapamycin
K


Ratio of pP38/P38 intensity
(normalized arbitary units)
Uncut
15mins
L

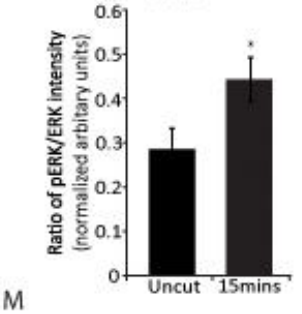

Ratio of pERK/ERK intensity
(normalized arbitary units)
Uncut
15mins
M

Figure 8

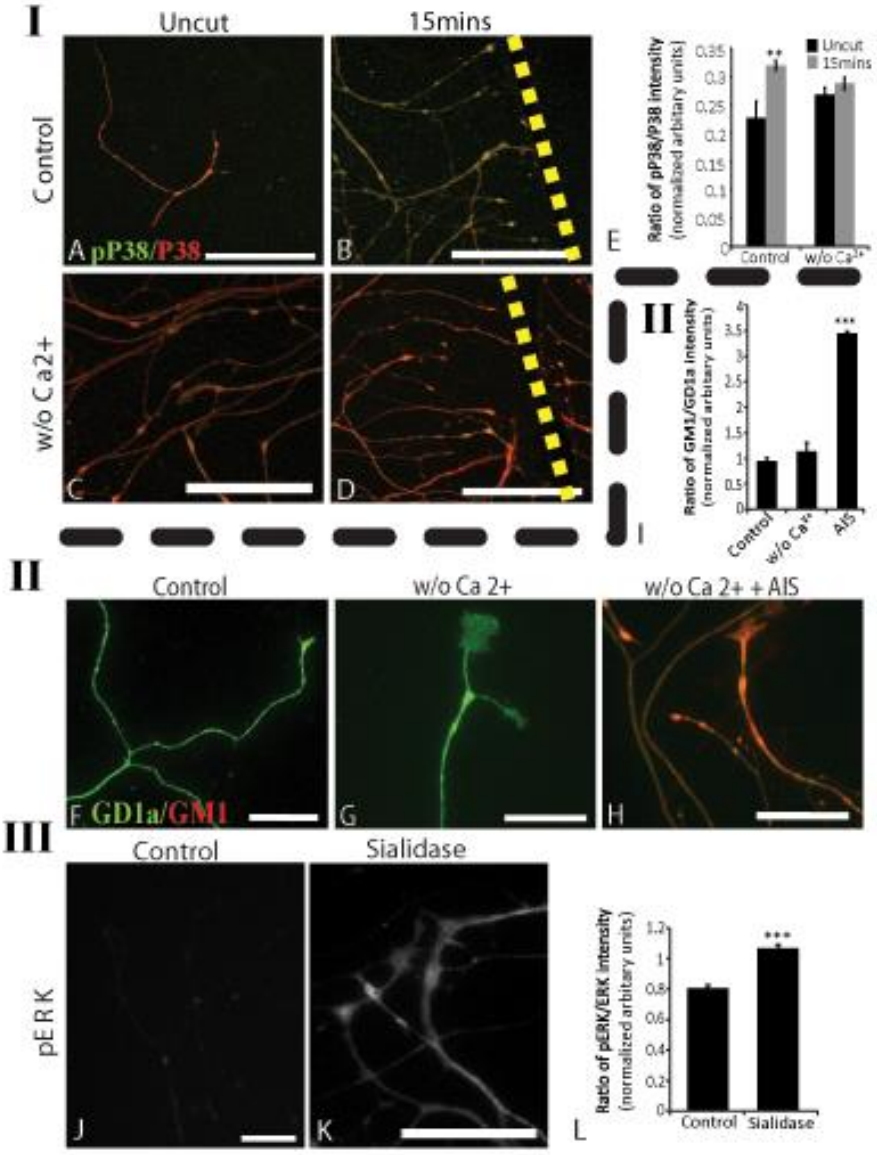

I
Uncut
15mins
Control
w/o Ca2+
A pP38/P38
B
C
D
E
Ratio of pP38/P38 intensity
(normalized arbitary units)
Uncut
15mins
Control
w/o Ca2+
II
Ratio of GM1/GD1a intensity
(normalized arbitary units)
Control
w/o Ca2+
AIS
I
II
Control
w/o Ca 2+
w/o Ca 2+ + AIS
F GD1a/GM1
G
H
III
Control
Sialidase
pERK
J
K
L
Ratio of pERK/ERK intensity
(normalized arbitary units)
Control
Sialidase

Figure 9

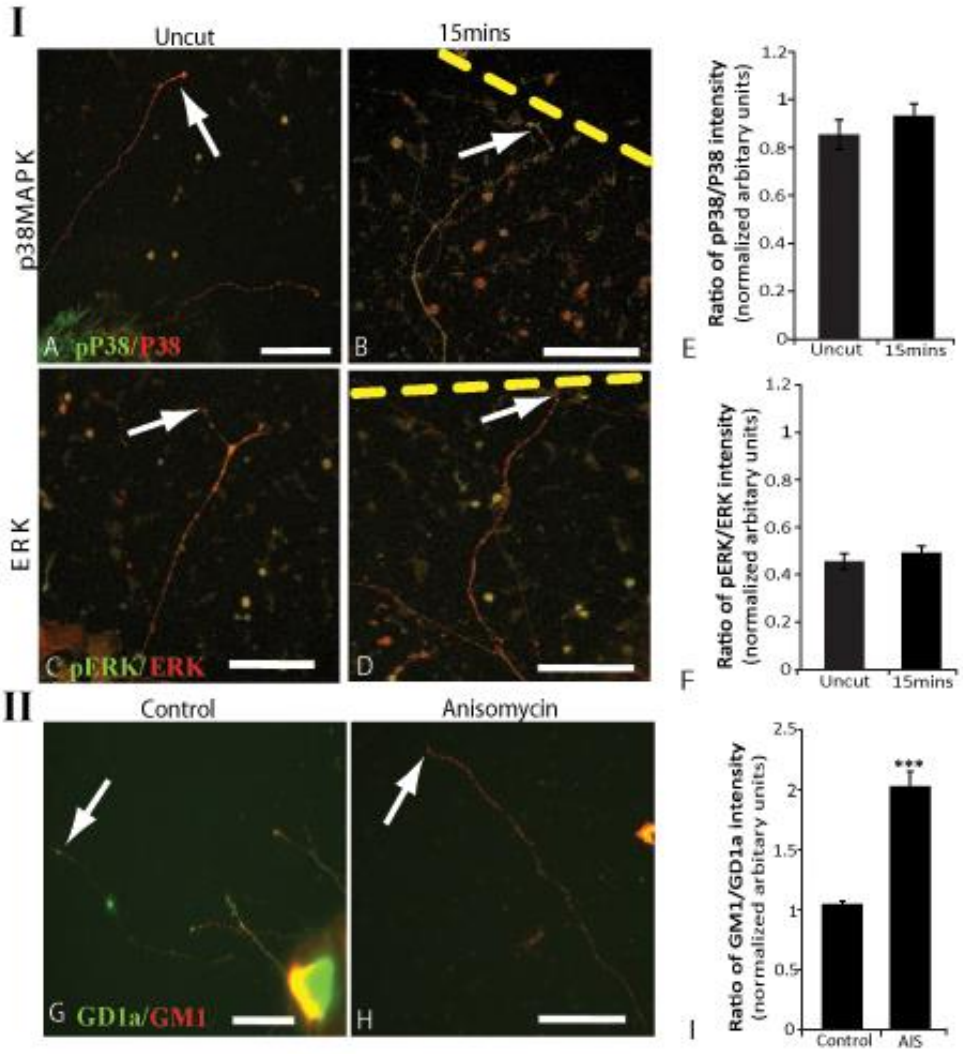
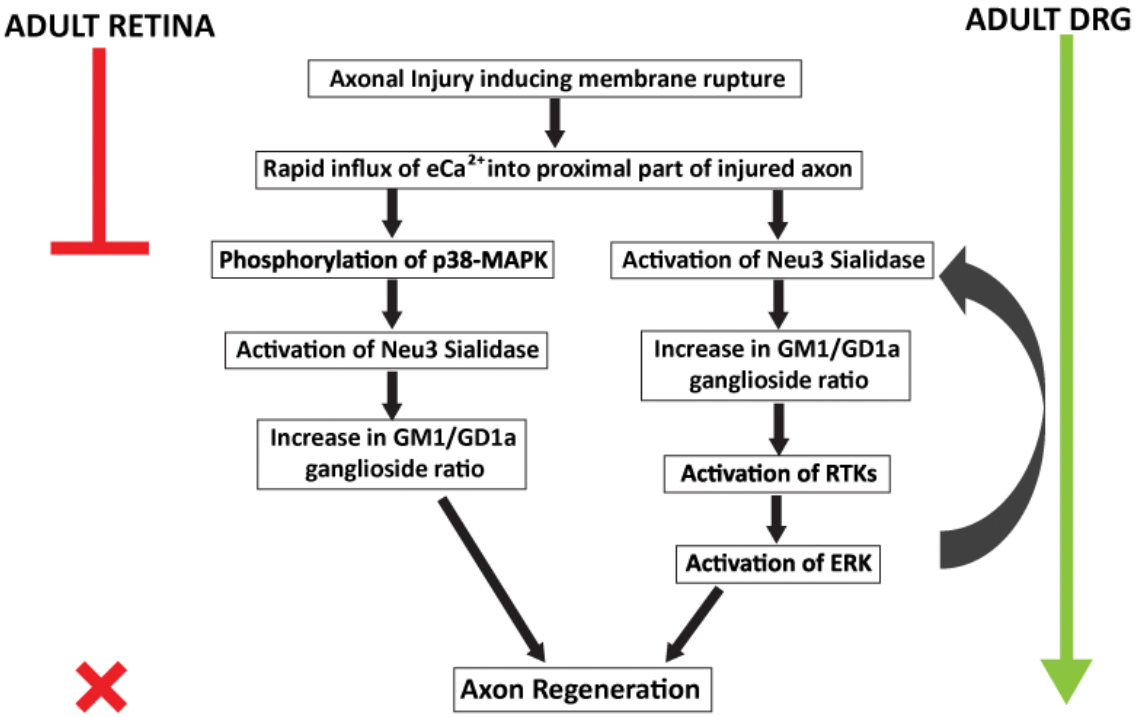

I
Uncut
15mins
p38MAPK
A pP38/P38
B
C pERK/ERK
D
ERK
II
Control
Anisomycin
G GD1a/GM1
H
E
Ratio of pP38/P38 intensity (normalized arbitary units)
Uncut
15mins
F
Ratio of pERK/ERK intensity (normalized arbitary units)
Uncut
15mins
I
Ratio of GM1/GD1a intensity (normalized arbitary units)
***
Control
AIS

Figure 10


ADULT RETINA
ADULT DRG
Axonal Injury inducing membrane rupture
Rapid influx of eCa2+ into proximal part of injured axon
Phosphorylation of p38-MAPK
Activation of Neu3 Sialidase
Activation of Neu3 Sialidase
Increase in GM1/GD1a ganglioside ratio
Increase in GM1/GD1a ganglioside ratio
Activation of RTKs
Activation of ERK
Axon Regeneration