NEURITES REGROWTH OF CORTICAL NEURONS BY GSK3β INHIBITION INDEPENDENTLY OF NOGO RECEPTOR 1.

Oscar Seira[1,2,3], Rosalina Gavín[1,2,3], Vanessa Gil[1,2,3], Franc Llorens[1,3], Alejandra Rangel[1,2,3], Eduardo Soriano[2,3,4] and José Antonio del Río[1,2,3,*].

1) Molecular and Cellular Neurobiotechnology, Institute for Bioengineering of Catalonia, Baldiri Reixac 15-21, E-08028, Barcelona Spain.
2) Department of Cell Biology, Faculty of Biology, University of Barcelona; Diagonal 645, E-08028 Barcelona, Spain.
3) Centro de Investigación Biomédica en Red de Enfermedades Neurodegenerativas (CIBERNED).
4) Developmental Neurobiology and Regeneration Laboratory. Institute for Research in Biomedicine (IRB Barcelona), Josep Samitier 1-5, 08028 Barcelona.

***Corresponding author:** Prof. Jose A. Del Río, Molecular and Cellular Neurobiotechnology laboratory, Institute of Bioengineering of Catalonia, Baldiri Reixac 15-21, E-08028 Barcelona, Spain. Phone: +34-93-40335923 Fax: +34-93-4020183. E-mail: jadelrio@ibec.pcb.ub.es.

Number of pages: 30
Number of figures: 5 + 5 Suppl. Figures

Abbreviations used: AT, Axon terminal; CGN, Cerebellar granule neurons; CRMP, Collapsing response mediator protein; ChABC, chondroitinase ABC; CSPG, Chondroitin sulphate proteoglycan; DAL, Days after lesion; DIV, Days *in vitro*; EGFr, Epidermal growth factor receptor; EH, Entorhino-hippocampal, EHP, Entorhino-hippocampal pathway; IGF1, Insulin-like growth factor-1; MAIs, Myelin-associated inhibitors; MAG, Myelin-associated glycoprotein; MAPs, Microtubule associated proteins; MBP, Myelin binding protein; MEM, Minimum essential medium; NgR1, Nogo Receptor 1; OMgp, Oligodendrocyte myelin glycoprotein; SEM, Standard error of the mean; TPA, phorbol ester.

Abstract

Lesioned axons do not regenerate in the adult mammalian central nervous system, owing to the overexpression of inhibitory molecules such as myelin-derived proteins or chondroitin sulphate proteoglycans. In order to overcome axon inhibition, strategies based on extrinsic and intrinsic treatments have been developed. For myelin-associated inhibition, blockage with NEP1-40, receptor bodies or IN-1 antibodies has been used. In addition, endogenous blockage of cell signalling mechanisms induced by myelin-associated proteins is a potential tool for overcoming axon inhibitory signals. We examined the participation of glycogen synthase kinase 3 (GSK3β) and ERK1/2 in axon regeneration failure in lesioned cortical neurons. We also investigated whether pharmacological blockage of GSK3β and ERK1/2 activities facilitates regeneration after myelin-directed inhibition in two models: i) cerebellar granule cells and ii) lesioned entorhino-hippocampal pathway in slice cultures, and whether the regenerative effects are mediated by Nogo Receptor 1 (NgR1). We demonstrate that, in contrast to ERK1/2 inhibition, the pharmacological treatment of GSK3β inhibition strongly facilitated regrowth of cerebellar granule neurons over myelin independently of NgR1. Lastly these regenerative effects were corroborated in the lesioned EHP in *NgR1* -/- mutant mice. These results provide new findings for the development of new assays and strategies to enhance axon regeneration in injured cortical connections.

Keywords: Nogo Receptor complex, axon inhibition, pharmacological treatment, organotypic slice cultures

Running title: GSK3β inhibition and cortical axon regeneration

Introduction

Damaged axons in the adult central nervous system do not regenerate after lesion, largely because of the presence of growth inhibitory molecules at the meningo-glial scar (Silver & Miller 2004, Rolls *et al.* 2009). Most of these molecules derive from disrupted myelin sheaths and non-neuronal cells, which proliferate in the damaged region (see (Xie & Zheng 2008, Fitch & Silver 2008) for recent reviews). Myelin-associated proteins such as Nogo-A, myelin-associated glycoprotein (MAG) and oligodendrocyte myelin glycoprotein (OMgp), as well as chondroitin sulphate proteoglycans (CSPGs) together with secreted Semaphorins or Ephrins, have been identified as the main molecular barriers to axon regeneration (Silver & Miller 2004, Rolls et al. 2009). Myelin-associated inhibitors (MAIs) act primarily through a common neuronal receptor complex which comprises NgR1, P75, TROY and Lingo1 (Park *et al.* 2005, Wang *et al.* 2002, Shao *et al.* 2005, Schweigreiter *et al.* 2004). In addition to this receptor complex, MAG also inhibits axonal regeneration through binding to other receptors (Vinson *et al.* 2001, Vyas *et al.* 2002, Venkatesh *et al.* 2005, Goh *et al.* 2008), and the receptors mediating Amino-Nogo-A region inhibition comprise several integrins (Hu & Strittmatter 2008). Moreover, the participation of the epidermal growth factor receptor (EGFr) and mitogen-activated kinase pathways (ERK1/2) in CSPG- and MAI-mediated inhibition has also been reported (Koprivica *et al.* 2005). Lastly, a new MAIs receptor has recently been described (Atwal *et al.* 2008) as well as a new NgR1 ligand (Zhang *et al.* 2009) and a CSPG receptor (Shen *et al.* 2009), increasing the complexity of the scenario.

CSPGs and myelin-based intracellular signalling are the most widely studied inhibitory mechanisms in the adult CNS. Although there is some controversy (Zhou *et al.* 2006), PKC activation and RhoA GTPase are considered convergent points in myelin- and CSPG-induced inhibition of axon regeneration (He &

Koprivica 2004, Sivasankaran *et al.* 2004, Hasegawa *et al.* 2004, Schweigreiter et al. 2004). Targets for RhoA or PKC activation by MAIs are linked to proteins which modulate polymerization/depolymerization of actin filaments, such as cofilin through LIM kinase activation (Hsieh *et al.* 2006) or microtubules, such as collapsing response mediator protein-2 (CRMP-2) (Mimura *et al.* 2006) or CRMP-4 (Alabed *et al.* 2007).

Several microtubule-associated proteins (MAPs) play relevant roles in microtubule dynamics and stabilization (Baas & Qiang 2005, Maccioni & Cambiazo 1995). Two of the most widely studied MAPs in healthy and neurodegenerative nervous systems are MAP1B (Riederer 2007, Gordon-Weeks & Fischer 2000, Gonzalez-Billault *et al.* 2004) and Tau (Johnson & Stoothoff 2004, Nunez & Fischer 1997). These MAPs are regulated at the post-translational level by serine threonine phosphorylation through kinases such as Extracellular-Related Kinase (ERK1/2), Glycogen Synthase Kinase 3β (GSK3β) and Cyclin-Dependent Kinase 5 (cdk5) (Riederer 2007, Gordon-Weeks & Fischer 2000, Gonzalez-Billault et al. 2004, Bhat *et al.* 2004, Del Rio *et al.* 2004, Gonzalez-Billault *et al.* 2005). MAIs regulation of cdk5, ERK1/2 and GSK3β is different. Cdk5 and ERK1/2 activities are regulated by MAG expression (Dashiell *et al.* 2002). However, no modification in GSK3β activity occurs in *mag -/-* mice (Franzen *et al.* 2001). At the same time, GSK3β activity has also been associated with CRMP-2 and CRMP-4 phosphorylation in neuroblastoma cells after IGF1 and TPA incubation, whereas cdk5 promotes only CRMP-2 phosphorylation (Cole *et al.* 2006). In terms of regeneration, one study reported that pharmacological blockage of GSK3β activity with lithium chloride (LiCl) or SB-415286 induces a moderate regeneration of damaged corticospinal tract (CST) axons after dorsal lesion of the rat spinal cord (Dill *et al.* 2008). Nevertheless, the number of CST regenerative axons in this study was low following inhibitor treatments, in contrast to other studies using different methodologies. However, the putative participation of NgR1 in this process has not been explored. As recently described elsewhere (Worter *et al.* 2009), the response of different neurons to a particular inhibitor should be different. In the present study, we used translational research to assess whether GSK3β and ERK1/2 are activated by myelin and MAIs, using two different models: in 2D culture of cerebellar granule neurons and in 3D organotypic slices of the entorhino-hippocampal (EH) connection, with the aim of exploring further the potential use of GSK3β and ERK1/2

inhibition in promoting axon regeneration. Our results indicate that both ERK1/2 and GSK3β are differentially activated by myelin and Nogo-66 in cultured cerebellar granule neurons and in lesioned EH co-cultures. We also found that treatment with the maleimide derivatives SB-415286 and SB-216763 inhibit activated GSK3β, thereby inducing axon regeneration in both culture models, in contrast to ERK1/2 inhibition by U0126. However, although the absence of NgR1 moderately increased neurite extension in CGN cultured over MAIs, EH co-cultures from *NgR1 -/-* did not regenerate after entorhino-hippocampal pathway (EHP) axotomy as wild-type co-cultures. More relevantly, the observed neurite extension of CGNs and EHP regeneration is not mediated by NgR1 in either culture models since CGN cultures over myelin and lesioned EH cultures from NgR1 mutant mice regenerated after pharmacological blockage of GSK3β.

Material and Methods

Animals

A total of 26 pregnant OF1 mice (Iffra Credo, Lyon, France) were used. The mating day was considered as embryonic day 0.5 (E0.5) and the day after birth was considered postnatal day 0 (P0). In addition, we used 20 pregnant *NgR* -/- mice (Zheng *et al.* 2005). Animals were used at postnatal stage P5-P7 (n = 65) for cerebellar granule neuron (CGN) cultures or P0-P1 (50 mice, 26 *NgR1* -/- and 24 wild-type) for entorhino-hippocampal cultures. In addition, 10 adult Sprague-Dawley rats were used to obtain purified myelin. All animal procedures were performed in accordance with the guidelines established by the Spanish Ministry of Science and Technology and the European Community Council Directive 86/609 EEC.

Antibodies and biochemical reagents

The following antibodies were used at a dilution of 1:1000 unless otherwise indicated. CS-56 antibody (diluted 1:200) was from Sigma (St. Louis, MO). GSK3β phospho-serine 9 (diluted 1:250), GSK3 phospho-Y279/Y216 (diluted 1:500) and totalGSK3β were obtained from Upstate (Lake Placid, NY). Akt phospho-serine 473 and ERK phospho-threonine 202/phospho-tyrosine 204 were from Cell Signaling Technology (Beverly, MA). Total ERK and total Akt antibodies were from Transduction Laboratories (Lexington, KY) and Santa Cruz Biotechnology respectively. The monoclonal antibody against Actin (diluted 1:10,000) and Myelin-basic protein (MBP) were from Chemicon (Temecula, CA). Goat-raised anti-OMgp was from R&D Systems (Minneapolis, USA). The total Tau (Tau5) was from Biosource Europe, S.A. (Nivelles, Belgium) and phosphor-serine 202-205 Tau (AT8, diluted 1:250) was a gift from Prof. Jesús Ávila (CBM-UAM, CSIC, Madrid). Phalloidin-FITC and Hoechst (Bisbenzimide) were purchased from Sigma. Alexa Fluor 488 goat anti-mouse immunoglobulin G (IgG) (H+L) and Alexa Fluor 568 goat anti-rabbit immunoglobulin G (IgG) (H+L) were from Molecular Probes (Leiden, Netherlands). The goat anti-mouse-HRP and rabbit anti-goat-HRP secondary antibodies used in Western blotting were purchased from DAKO (Glostrup, Denmark). Goat anti-rabbit-HRP was from Sigma. The ERK1/2 kinase

inhibitor U0126 was obtained from Promega (Madison, USA) and the GSK3β inhibitors SB-415286 and SB-216763 was purchased from Sigma (St. Louis, MO). In addition, a membrane-permeable isoform of the C3 transferase (TAT-C3) was used (CT04-A) (Cytoskeleton, Denver), and the NEP1-40 peptide was purchased (Alpha diagnostics, San Antonio, Texas).

In vitro experiments

CGNs from P5-P7 mouse pups (from wild-type and *NgR1* -/- litters*)* were dissociated by combined trypsinization as described previously (Niederost *et al.* 2002). Cells were placed in 24-well tissue culture dishes (Nunc, Roskilde, Denmark) on coated coverslips (see below) and grown for 24 to 48 h in DMEM medium supplemented with N2 and B27. Two procedures were used to treat cells with MAIs or myelin: i) the cell surface was coated with purified myelin essentially as described elsewhere (Fournier *et al.* 2001); and ii) acute treatments with myelin or AP-Nogo66 were carried out. Phalloidin-labeled cultures were counterstained with bisbenzimide, mounted in Fluoromount^{TM} (Vector Labs, Burlingame, CA) and analyzed with a confocal microscope (TCS SPII, Leica). Neurite length in cultures was assessed following confocal image acquisition using ImageJ software.

Determination of GSK3β and ERK1/2 activities

GSK3β and ERK1/2 kinase activity assays were carried out as described elsewhere (Sayas *et al.* 1999, Simo *et al.* 2007). Cultured cell extracts were prepared after peptide treatments. Cells were collected with a scraper and homogenized in a buffer containing 20 mM HEPES (pH 7.4) 100 mM NaCl, 100 mM NaF, 1 mM sodium orthovanadate and 5 mM EDTA. The soluble fraction was immunoprecipitated with agarose-conjugated antibodies against total GSK3β or total ERK1/2. Samples of 10 µl were incubated in a buffer containing 25 mM HEPES, pH 7.5, 1 mM DTT, 10 mM $MgCl_2$ and a specific GSK3 substrate peptide (pGSK3 peptide-2, Upstate Biotechnology Inc) at a final concentration of 0.75 mg/ml, and in the presence of γ^{32}P-ATP. After 1 hour, the reaction was stopped with 1% H_3PO_4 and the reaction products were transferred to Immobilon membranes. The difference between kinase activity in the presence or absence of the GSK3 inhibitor LiCl (20 mM) was considered to reflect GSK3β kinase activity. For

ERK1/2 activity, reactions were initiated by resuspending immunoprecipitates in assay buffer (25 mM Tris–Cl [pH 7.4], 5 mM □-glycerophosphate, 2 mM dithiothreitol, 0.1 mM Na_3VO_4, 10 mM $MgCl_2$, and 1 mM phenylmethylsulfonyl fluoride) containing 20 μM of unlabeled adenosine triphosphate, 5 μCi of γ^{32}P-ATP, and 5 μg of MBP (Sigma). Reaction mixtures were incubated at 30°C for 1 hour. ^{32}P-GSK3 peptide-2 and ^{32}P-MBP loaded membranes were dried and exposed overnight to a phosphor-imager screen (Fuji). Radioactive levels were quantified using a Fuji BAS1000 phosphor-imager and PCBAS 2.0 software (Fuji Photo Film Co. Ltd., Japan).

Entorhino-hippocampal slice co-cultures

Entorhino-hippocampal slice co-cultures from wild-type and *NgR1 -/-* mice were prepared from P0 (day of birth) or P1 mouse pups as described elsewhere (del Rio *et al.* 2002, del Rio & Soriano 2010). Animals were anaesthetized by hypothermia, their brains were aseptically removed, and the hippocampus and the entorhinal cortex were dissected out. Using a McIIwain chopper, tissue pieces were cut into single horizontal sections (300-400 μm thick) containing both the entorhinal cortex and the hippocampus and maintained in Minimum Essential Medium (MEM) supplemented with glutamine (2 mM) for 45 min at 4^o C (MEM dissecting salt solution). Selected slices were cultured using the membrane interface method (Stoppini *et al.* 1991). Slices were placed on 30-mm Ø sterile membranes (Millicell-CM, Millipore) and transferred into six-well tissue culture trays. Cultures were fed 1 ml of culture medium (50% MEM; 25% horse serum; 25% HBSS) containing 2 mM of glutamine and 0.044% $NaHCO_3$ adjusted to pH 7.3. The membrane cultures were maintained in a humidified incubator at 36º C in 5% CO_2. After 15 days *in vitro* (15 DIV), cultures were axotomized (see below).

Axotomy of the EHP in vitro, biocytin labeling, and quantification of regenerating axons

After 15 DIV, the EHP of *NgR1-/-* and wild type was axotomized by cutting the co-cultures from the rhinal fissure to the ventricular side along the entire entorhino-hippocampal (EH) interface with a tungsten knife (see del Río et al., 2002, for details). Co-cultures were allowed to grow after axotomy for times ranging from 30 min to 15 days and were then processed for biochemical or morphological studies.

Parallel cultures were treated pharmacologically with the GSK3β inhibitors SB-415286 and SB-216763 (*NgR1* -/- and wild-type), NEP1-40 or C3-transferase for 10 days and the degree of EHP regeneration was determined by biocytin labelling (wild-type cultures). Two small crystals of biocytin (Sigma) were placed at the entorhinal slice. The following day, cultures were fixed with phosphate buffered 4% paraformaldehyde and processed (Del Rio *et al.* 1997). Some traced co-cultures of *NgR1* -/- were processed for electron microscopy analysis as described (del Rio et al. 2002). For quantification, a calibrated eyepiece was used to count the number of biocytin-labelled fibres which crossed a 400-µm segment in the hippocampus located at a distance of 75-80 µm parallel to the lesion interphase of consecutive sections from each culture (40x oil immersion objective)(Mingorance *et al.* 2004, Mingorance *et al.* 2005). A Student *t*- test was used to assess statistical significance.

Results

Kinase activation in cultured CGNs.

In contrast to AP-Mock-treated cultures, the CGNs cultures incubated with AP-Nogo66 showed increased ERK1/2 and Akt activity (Fig. 1A-C). This activation was also observed after incubation with myelin and after the radioactive kinase activity assay, in which ERK1/2 activity increased 2.5-fold after 30 min (Fig. 1C), decreasing to a 2-fold increase at 1 h of incubation. In addition a concentration dependent response was observed in ERK1/2 activation against myelin (Suppl. Fig 1). In contrast to ERK1/2, GSK3β activity decreased by 40% 30 min after myelin incubation, but increased rapidly to peak at 90 min, thereafter to further decrease in a radioactive kinase activity assay (Fig. 1D). These data was also corroborated by Western blotting using antibodies against phosphorylated residues of GSK3β (Fig. 1D). Next, we explored the degree of phosphorylation of Tau (a target of GSK3β) in these conditions (Fig. 1D). Western blotting experiments showed a correlation of phospho-Tau with the time course of GSK3β activation (Fig. 1D). Thus, a parallel peak of Tau phosphorylation and GSK3β activity was observed 90 min after incubation with myelin.

Differential kinase activation in cultured CGNs after acute treatment with myelin or growing over myelin-coated substrates.

We measured the activation of ERK1/2 and GSK3β in cultured CGNs after acute treatment with myelin or after growing over myelin-coated substrates for 24 h (Fig. 2). Phospho ERK1/2 and the GSK3β-phosphoserine 9 levels (which indicate low activity of GSK3β) increased 30 min after acute myelin treatment (Fig. 2B). However, when CGNs were cultured over myelin substrates (substrate in Fig. 2B) for 24 hours no relevant activation of ERK1/2 was seen in Western blots (Fig. 2B). In contrast, there was a relevant decrease in GSK3β-phosphoserine 9 level, which indicates GSK3β activation in CGNs growing over myelin for 24 hours (Fig. 2B). This activation was corroborated by radioactive kinase assays of GSK3β activity in CGNs cultured over myelin as above (Fig. 2C). These results suggest that, in contrast to GSK3β inhibitors, ERK1/2 inhibitors are not appropriate for enhancing axon regeneration in cultured

CGNs over myelin. Indeed, when cultured over myelin, the neurite length of CGNs decreased (47.3%, Fig. 2D). GSK3β inactivation with SB-415286 (15 and 30 μM) increased neurite length by 21.3 % and 21.6 % respectively (Fig. 2D). In addition, GSK3β inactivation with SB-216763 (3 and 10 μM) increased neurite length by 18.9 % and 23.8 % respectively (Fig. 2D). In contrast, treatments with U0126 (10 μM) to block ERK1/2 did not induce significant CGNs neurite regrowth over myelin (Fig. 3D).

GSK3β inhibition enhances outgrowth of CGNs neurites independently of *NgR1* expression.

Next we explored whether the positive effects of SB-415286 CGN neurite extension are dependent on *NgR1* expression. For this, we prepared time-matched CGNs cultures from *NgR1* deficient mice (Zheng et al. 2005). CGNs growing over PD-Lysine extended their neurites in a way similar to that observed in wild-type cultured neurons (45.2 ± 4 μm *vs* 42.3 ± 3.5 μm; mean ± sem, wild-type and knockout neurons respectively) (Fig. 2E). Moreover, *NgR1* -/- CGNs growing over myelin showed reduced neurite length compared to *NgR1* -/- neurons growing over PD-Lysine (27.7 %; 30,48 ± 2.95 *vs* 42.27 ± 3.53 respectively) (Fig. 2E), but greater extension (35%) than wild-type CGNs growing over myelin-containing substrates (30,48 ± 2.95 *vs* 22,5 ± 3.3 respectively) (compare second bar of Fig. 3E and Fig. 3D histograms). However, when treated with 30 μM SB-415286 neurite growth of *NgR1* -/- cells reached neurite lengths similar to those seen growing over PD-Lysine (40,57 ± 3,47 *vs* 42.27 ± 3.53 respectively) (Fig. 2E).

Gene expression profiling analysis after EHP axotomy *in vitro*.

To evaluate the genes whose transcription was regulated after 1, 3 and 7 days after EHP axotomy (see Material and Methods for details), RNA samples were analyzed with Agilent whole genome mouse long oligonucleotide (44base) probe based microarrays. A total of 699 genes were regulated in this way, with a maximum of 407 genes regulated at 3 days after EHP axotomy, and clustering analysis showed that genes gather in 5 expression patterns (Suppl. Fig. 2). First, we were interested to test whether EHP axotomy induces relevant cell death or apoptotic pathways in axotomized EHP. However, no relevant changes in the expression of apoptotic or cell death markers were observed in axotomized EHP (Fig. 3A). As

expected, some genes which are known to be up-regulated after physical injury in neurons Clu (Wehrli *et al.* 2001), FGF-2 (Yoshimura *et al.* 2001) and IGF-2 (Gluckman *et al.* 1998) were up-regulated after 3 days of EHP axotomy. Likewise, we checked whether MAIs were up-regulated after EHP axotomy. In fact, most of the proteins included in the arrays (e.g., *MAG, MBP, MOG, MOBP* and *Plp*) were strongly up-regulated at 3 and 7 days after EHP axotomy. In contrast, *Rtn4* (Nogo-A) gene expression remained constant after EHP axotomy.

Pattern of myelin inhibitory proteins, CSPGs and correlated kinase activity in axotomized EH organotypic slice co-cultures.

To corroborate the data obtained in the microarrays study, we first determined the expression levels of myelin-associated proteins Nogo-A (*Rtn4*), OMgp and MBP after 30 min, 90 min, 3 days and 12 days following EHP axotomy at 15 DIV in wild-type slices, using the Western blot technique (Fig. 4A). As expected, OMgp and MBP protein levels increased in the lesioned EH co-culture, especially at 3 and 12 days after lesion (DAL) (Fig. 4A). On the other hand, CS-56 levels were relevant over time in wild-type EH axotomized co-cultures, especially after 10 DAL (Fig. 4B) (see also (Mingorance *et al.* 2006)).

Next, we analyzed the kinase activity in lesioned wild-type and *NgR1 -/-* EH co-cultures (Fig. 4C-D). ERK1/2 activity showed an initial increase at 30 min and 90 min after axotomy but decreased markedly at 3 and 12 DAL in wild-type and *NgR1 -/-* cultures (Fig. 4C-D). Furthermore, immunohistochemical analysis of wild-type lesioned co-cultures showed a relevant increase in phosphorylated ERK1/2 close to the lesion site when compared to unlesioned cultures, which contrasts with the unchanged pale labeling observed in projecting EH neurons in control and lesioned co-cultures (Suppl. Fig. 3A-B). This increased phospho-ERK1/2 labeling is almost absent in the EH co-culture 2 DAL (Suppl. Fig. 3C). These results suggest that in EH axotomized slice co-cultures, ERK1/2 activation is mainly associated with reactive cells on the lesion side not affecting axotomized projecting neurons. However, we cannot rule out a putative participation of neuronal ERK1/2-mediated gene expression not determined in our histological analysis in regulating neuronal factors that could be involved in regenerative responses of damaged axons

or neuronal survival. In contrast, parallel Western blotting experiments demonstrated that GSK3β activity increased steadily after EHP lesion in wild-type slices, specially 3 and 12 DAL (Fig. 4C-D). We also determined that, although less relevant than wild-type slices, a GSK3β activation also occurs in *NgR1 -/-* lesioned organotypic slice co-cultures at the same DAL (Fig 4C-D). Unfortunately our GSK3β antibodies did not recognize phosphorylated GSK3β residues in histological sections of EH co-cultures. The activation of GSK3β in *NgR1 -/-* slices suggests that other inhibitory molecules (e.g. CSPGs (Mingorance et al. 2006)(present results), or secreted Semaphorins (Montolio *et al.* 2009) also present in the lesioned organotypic slice may act on GSK3β activity during these late stages in both wild-type and in a lower level in knockout cultures likely due to the absence of the NgR1. Altogether, the present data points GSK3β as a putative target for enhancing axon regeneration after EHP lesion *in vitro*.

Repair of the lesioned EHP by blocking GSK3β activity *in vitro* in wild-type and *NgR1 -/-* co-cultures.

To further corroborate the potential of GSK3β inhibition in EHP regeneration, we treated lesioned cultures from wild-type mice with SB-415286, SB-216763; and a membrane-permeable form of C3-transferase (TAT-C3) (Tan *et al.* 2007) to block RhoA-dependent activity, and with NEP1-40 peptide, as previously described (Mingorance et al. 2006) (Fig. 5). The ensuing cultures demonstrated that acute treatment of axotomized organotypic co-cultures for 10 days with SB-415286 (30 μM) resulted in the regrowth of numerous entorhinal axons entering the hippocampus (Fig. 5D-E,F) (25.3 ± 6.4; mean ± sem). Similarly, parallel axotomized organotypic co-cultures treated for 10 days with SB-216763 (10 μM) resulted in the regrowth of entorhinal axons (Fig. 5G) (18,5 ± 4.6; mean ± sem). In contrast, in unlesioned co-cultures most of the EH axons stopped at the lesion interface and very few entered into the hippocampus (Fig. 5F-G). Regenerating axons, ending in growth cones (Fig. 5C,E), did not always grow directly towards the *stratum lacunosum moleculare/molecular layer* (slm/ml) and often grew ectopically but crossed the lesion (Fig. 5B-E). Compared with controls, treatment with NEP1-40 (100 μM) led to a significant increase in the number of regenerating biocytin-labeled axons entering the hippocampus, similar to the effect of SB-415286 and SB-216763 (Fig. 5F). However, treatment with TAT-C3 resulted in lower regeneration (14,3

± 3.2) of the EHP compared to treatment with SB-415286, SB-216763 or NEP1-40 (Fig. 5F). Next, we prepared EH co-cultures from *NgR1 -/-* mice which were axotomized after 15 DIV. Due to the results obtained for wild-type cultures, *NgR1 -/-* co-cultures were treated with 30 μM of SB-415286, 10 μM of SB-216763 or with buffer (Fig. 5H-L). After biocytin labeling, we observed that axons did not enter the hippocampus in controls (Fig. 5H, K-L), in contrast to the high numbers which did so after treatment (4.1 ± 0.5 *vs* 18.2 ± 3.1, controls *vs* SB-415286 treated cultures; 3,2 ± 0.7 *vs* 14,6 ± 2,7, controls *vs* SB-216763 treated cultures) (Fig. 5K-L). Regenerating axons displayed random trajectories in the cultures treated with both GSK3β inhibitors. Thus, SB-415286 treatment is most efficient than SB-216763 for improving axon regeneration in wild-type as well as *NgR1 -/-*, and reinforces the notion that the axonal regeneration obtained with both treatments previously in CGNs cultures is independent of *NgR1* expression.

Discussion

Involvement of intracellular kinases in axon growth and regeneration.

GSK3β plays critical roles in the regulation of neuron physiology. Highly expressed in neurons during development, GSK3β is crucial for establishing neuronal polarity and neurite branching and elongation (Garrido *et al.* 2007). For example, the participation of members of the Wnts (e.g., Wnt-7) and neurotrophins (e.g., NGF) in GSK3β-mediated axon growth has been reported (e.g., (Arevalo & Chao 2005, Zhou *et al.* 2008, Zhou *et al.* 2004)). GSK3β activity depends on the balance of tyrosine/serine phosphorylation. In addition, other intracellular kinases have been implicated in the neuritogenic process. For example, ERK1/2 activation enhances neurite outgrowth in PC12 cells (Xiao & Liu 2003), Neuro2A (Singleton *et al.* 2000) and spinal ganglion neurons (Aletsee *et al.* 2001) after several stimuli. Moreover, the activity of another kinase, PI3K, is essential for neurite elongation and maintenance, since its inhibition causes neurite retraction through GSK3β activation (Sanchez *et al.* 2001), considered a sensor of extracellular factors in neural growth (Luikart *et al.* 2008). At this respect, a recent study indicates that PTEN, a negative regulator or PIK3 kinase induce a robust regeneration of ganglion cells (Park *et al.* 2008). This data also suggests that other PTEN-regulated pathways such as GSK3β could be involved in controlling axon growth (Kim & Snider 2008). It is well described that, among others, main targets of GSK3β activity are members of the microtubule associated proteins (e.g., Tau or MAP1B) (Cohen & Frame 2001), and that the microtubule cytoskeleton stability is largely impaired after lesion (Erturk *et al.* 2007) .

To date, few studies have analyzed the role of GSK3β preventing axon regeneration. The GSK3β-mediated inhibition of axon regeneration of lesioned neurons has been studied mainly through the use of pharmacological treatments with LiCl. Indeed, LiCl treatment combined with ChABC (Yick *et al.* 2004) promotes axon regeneration of ganglion cells in the retina (Huang *et al.* 2003) and rubrospinal tract. These effects correlate with increased Bcl-2 expression and GSK3β inhibition (Yick et al. 2004, Dill et al. 2008).

Thus, a dual effect of LiCl in cell survival and axon growth is likely to be responsible for these results. Indeed, a single application of LiCl after spinal cord lesion does not promote the regeneration of rubrospinal axons (Yick et al. 2004), and the overexpression of Bcl-2 does not stimulate axon regeneration *per se* in several lesion models (Chierzi *et al.* 1999, Inoue *et al.* 2002, Sole *et al.* 2004, Chen & Tonegawa 1998). In contrast, LiCl has recently been used after spinal cord lesion (Dill et al. 2008), and the results contrast with those reported by Alabed and co-workers (Alabed et al., 2007, *Society for Neuroscience*) with respect to the inhibition of GSK3β. These differences may be attributable to direct delivery of LiCl to the lesion site, which would prevent cell death, or to activating infiltrating cells located at the lesion site to secrete growth factors or cytokines (Victoratos *et al.* 1997, Feinstein 1998), which may also improve axon regeneration (Mizusawa *et al.* 2003). LiCl is a non-specific GSK3β activity inhibitor (Pardo *et al.* 2003) and additional effects occur after its application. Due to these putative indirect effects we have used two well characterized GSK3β inhibitors instead LiCl in our experiments.

Differential kinase activation after myelin treatment and experimental axotomy *in vitro*: lessons from CGNs and EH slice co-cultures and *NgR1* mutants.

Different neurons may react differently to the same inhibitory molecule (e.g., (Worter et al. 2009). Thus, it is reasonable to analyze the effects of MAIs inhibitory activities in different models.

CGNs: We have shown that acute treatment with myelin and AP-Nogo66 activate ERK1/2 and PI3K/Akt, which correlates with GSK3β activity in cultured CGNs. The activation also correlates with the increased phosphorylation of Tau in Serine 199/202, a target of GSK3β. However, ERK1/2 activation only occurs shortly after myelin or AP-Nogo66 exposure, which was corroborated in cultured CGNs growing over myelin for prolonged periods. Indeed, the application of the specific ERK1/2 inhibitor did not enhance axon regeneration in CGNs cultured over myelin in these conditions in contrast to GSK3β inhibitors, suggesting a time window of effectiveness for ERK1/2 blockage. Recent data indicates that MAIs-induced NgR1-mediated inhibition occurs only in acute phases following lesion (Chivatakarn *et al.* 2007).

However, this finding contrasts with other studies reporting that NgR1 inhibitors (e.g., NEP1-40 peptide) are useful in chronic treatment following lesion (Mingorance et al. 2006, Li & Strittmatter 2003), and raise the question of whether the action of NEP1-40 is NgR1-specific, which would warrant further study. Our present results indicate that blockage of ERK1/2 with a specific inhibitor does not enhance neurite outgrowth of CGNs growing over myelin after 24 h, and reinforce the notion of a transient effect of NgR1 inhibiting neurite growth by MAIs (Chivatakarn et al. 2007).

On the other hand, using *NgR1 -/-* CGNs growing over myelin we observed an increase in neurite length in mutant neurons in contrast to parallel wild-type cultures, and the length of the CGNs neurites treated with the GSK3β inhibitors was similar to those seen in *NgR1 -/-* and *NgR1 +/+* neurons growing over PD-Lysine. This implies the involvement of other receptors than NgR1 participating in the activation of GSK3β by MAIs and that ERK1/2 and GSK3β could be activated by independent intracellular pathways in CGNs. At this respect a participation of EGFr in ERK1/2 activation after MAIs treatment has been described (Koprivica et al. 2005).

EH organotypic slice cultures: In EH co-cultures, a similar model to *in vivo*, we described the up-regulation of MAIs (except Nogo-A) shown using microarray and Western blotting methods. These data corroborated previous studies of our group (Mingorance et al. 2006, Mingorance et al. 2004, Mingorance et al. 2005). However, we must distinguish between early and late responses after the EHP lesion in terms of kinase activation. GSK3β remain active in lesioned cultures in all analyzed post-lesion days whereas the ERK1/2 activation is only active shortly after lesion and decreases after several days *in vitro* (especially at 3 and 12 DAL). This short ERK1/2 activation after lesion may be attributed to the activation of reactive glia in the lesioned area. (Suppl. Fig. 3). The participation of NgR1 in this ERK1/2 activation is plausible since increased NgR1 immunoreactivity in reactive astrocytes after CNS injury has been shown, and EGFr is involved in NgR1-mediated responses (Satoh *et al.* 2005, Koprivica et al. 2005). EGFr receptor is up-regulated in astrocytes after injury and promotes their transformation into reactive astrocytes. Furthermore, EGFr inhibitor PD168393 has been shown to enhance axon regeneration in the

injured optic nerve and promote recovery after spinal cord injury (Erschbamer *et al.* 2007). In these studies, EGFr inhibitors may reduce MAPK activation, decreasing glial reactivity and the delivery of inhibitory molecules impairing axon regrowth (e.g., CSPG) (Erschbamer et al. 2007). A study by Rhodes *et al*. established that the reduction in glial scar proliferation with cytosine-D-arabinofuranoside (Ara-C) temporarily increased the number of regenerating axons after CNS lesion, this effect decreasing after 18 DAL (Rhodes *et al.* 2003). However, treatment with the selective EGFr inhibitor in the study of (Erschbamer et al. 2007) was only effective shortly after lesion. It would be of interest to determine whether this treatment is also useful in delayed application after lesion in the absence of relevant ERK1/2 activation. On the other hand, our results indicate that although the development of the EHP connection in *NgR1 -/-* is normal (not shown), it does not spontaneously regenerate after lesion. This finding has also been described for the adult cortico-spinal tract *in vivo* (Zheng et al. 2005), although it regenerates after GSK3β inhibition as EH *NgR1 -/-* co-cultures (present data). Whilst some discrepancies exist (Dimou *et al.* 2006), mice bearing a single mutation in ligands or receptors did not show robust regeneration (Dimou et al. 2006), demonstrating that axon inhibition depends on different ligands/receptors and intracellular cascades. However, our results also indicate that GSK3β is activated in *NgR1 -/-* cultures and two GSK3β inhibitors are able to enhance axon regeneration after axotomy of EHP in these slices. Total GSK3β levels were similar between wild-type and *NgR1 -/-* unlesioned organotypic slice co-cultures (not shown). However, although present, GSK3β activation in *NgR1 -/-* is lower than in wild-type axotomized co-cultures. Thus, the characterization of the cellular factors responsible of GSK3β activation in *NgR1 -/-* cultures warrant further studies, but it is well known that secreted Semaphorins trigger the activation of several kinases including GSK3β (Ahmed & Eickholt 2007) (Suppl. Fig. 4). This may be of relevance in the delayed activation of GSK3β observed after EHP axotomy in the present study, since correlates with the increased expression of Semaphorins in the lesioned EHP *in vitro* (Montolio et al. 2009) (Suppl. Fig. 4). Taken together, our data indicate the need to develop combined strategies for enhancing axon regeneration and that GSK3β inhibitory treatment can be considered as a putative target to enhance cortical axon regeneration after injury.

Acknowledgements

The authors would like to thank Isabel Jiménez for technical assistance and Prof. Ramón Trullas for critical reading of the manuscript. We also thank Robin Rycroft for correcting the English manuscript and Lauro Sumoy for microarray analysis. The authors are particularly grateful to Prof. Marc Tessier-Lavigne (Genentech) for sharing the Nogo Receptor 1 mutant mice. This work was supported by grants from MICINN (BFU2009-10848), Instituto Carlos III, Generalitat de Catalunya (SGR2009-366) and the FP7/2007-2013 under grant agreement 222887 PRIORITY to J.A.D.R., and grants awarded by MICINN, Instituto Carlos III and Generalitat de Catalunya to E.S. F.LL. is a "Juan de la Cierva" investigator at the MICNN. A.R. was supported by the Programme Alban, the European Union Programme of High Level Scholarships for Latin America (E04D034892VE). VG and OS were supported by MEC.

References

Ahmed, A. and Eickholt, B. J. (2007) Intracellular kinases in semaphorin signaling. *Adv Exp Med Biol,* **600,** 24-37.

Alabed, Y. Z., Pool, M., Ong Tone, S. and Fournier, A. E. (2007) Identification of CRMP4 as a convergent regulator of axon outgrowth inhibition. *J Neurosci,* **27,** 1702-1711.

Aletsee, C., Beros, A., Mullen, L., Palacios, S., Pak, K., Dazert, S. and Ryan, A. F. (2001) Ras/MEK but not p38 signaling mediates NT-3-induced neurite extension from spiral ganglion neurons. *J Assoc Res Otolaryngol,* **2,** 377-387.

Arevalo, J. C. and Chao, M. V. (2005) Axonal growth: where neurotrophins meet Wnts. *Current opinion in cell biology,* **17,** 112-115.

Atwal, J. K., Pinkston-Gosse, J., Syken, J., Stawicki, S., Wu, Y., Shatz, C. and Tessier-Lavigne, M. (2008) PirB is a functional receptor for myelin inhibitors of axonal regeneration. *Science (New York, N.Y,* **322,** 967-970.

Baas, P. W. and Qiang, L. (2005) Neuronal microtubules: when the MAP is the roadblock. *Trends in cell biology,* **15,** 183-187.

Bhat, R. V., Budd Haeberlein, S. L. and Avila, J. (2004) Glycogen synthase kinase 3: a drug target for CNS therapies. *Journal of neurochemistry,* **89,** 1313-1317.

Cohen, P. and Frame, S. (2001) The renaissance of GSK3. *Nat Rev Mol Cell Biol,* **2,** 769-776.

Cole, A. R., Causeret, F., Yadirgi, G. et al. (2006) Distinct priming kinases contribute to differential regulation of collapsin response mediator proteins by glycogen synthase kinase-3 in vivo. *The Journal of biological chemistry,* **281,** 16591-16598.

Chen, D. F. and Tonegawa, S. (1998) Why do mature CNS neurons of mammals fail to re-establish connections following injury--functions of bcl-2. *Cell death and differentiation,* **5,** 816-822.

Chierzi, S., Strettoi, E., Cenni, M. C. and Maffei, L. (1999) Optic nerve crush: axonal responses in wild-type and bcl-2 transgenic mice. *J Neurosci,* **19,** 8367-8376.

Chivatakarn, O., Kaneko, S., He, Z., Tessier-Lavigne, M. and Giger, R. J. (2007) The Nogo-66 receptor NgR1 is required only for the acute growth cone-collapsing but not the chronic growth-inhibitory actions of myelin inhibitors. *J Neurosci,* **27,** 7117-7124.

Dashiell, S. M., Tanner, S. L., Pant, H. C. and Quarles, R. H. (2002) Myelin-associated glycoprotein modulates expression and phosphorylation of neuronal cytoskeletal elements and their associated kinases. *Journal of neurochemistry,* **81,** 1263-1272.

Del Rio, J. A., Gonzalez-Billault, C., Urena, J. M. et al. (2004) MAP1B is required for Netrin 1 signaling in neuronal migration and axonal guidance. *Curr Biol,* **14,** 840-850.

Del Rio, J. A., Heimrich, B., Borrell, V. et al. (1997) A role for Cajal-Retzius cells and reelin in the development of hippocampal connections. *Nature,* **385,** 70-74.

del Rio, J. A., Sole, M., Borrell, V., Martinez, A. and Soriano, E. (2002) Involvement of Cajal-Retzius cells in robust and layer-specific regeneration of the entorhino-hippocampal pathways. *Eur J Neurosci,* **15,** 1881-1890.

del Rio, J. A. and Soriano, E. (2010) Regenerating cortical connections in a dish: the entorhino-hippocampal organotypic slice co-culture as tool for pharmacological screening of molecules promoting axon regeneration. *Nat Protoc,* **5,** 217-226.

Dill, J., Wang, H., Zhou, F. and Li, S. (2008) Inactivation of glycogen synthase kinase 3 promotes axonal growth and recovery in the CNS. *J Neurosci,* **28,** 8914-8928.

Dimou, L., Schnell, L., Montani, L., Duncan, C., Simonen, M., Schneider, R., Liebscher, T., Gullo, M. and Schwab, M. E. (2006) Nogo-A-deficient mice reveal strain-dependent differences in axonal regeneration. *J Neurosci,* **26,** 5591-5603.

Erschbamer, M., Pernold, K. and Olson, L. (2007) Inhibiting epidermal growth factor receptor improves structural, locomotor, sensory, and bladder recovery from experimental spinal cord injury. *J Neurosci,* **27,** 6428-6435.

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

Feinstein, D. L. (1998) Potentiation of astroglial nitric oxide synthase type-2 expression by lithium chloride. *Journal of neurochemistry,* **71,** 883-886.

Fitch, M. T. and Silver, J. (2008) CNS injury, glial scars, and inflammation: Inhibitory extracellular matrices and regeneration failure. *Experimental neurology,* **209,** 294-301.

Fournier, A. E., GrandPre, T. and Strittmatter, S. M. (2001) Identification of a receptor mediating Nogo-66 inhibition of axonal regeneration. *Nature,* **409,** 341-346.

Franzen, R., Tanner, S. L., Dashiell, S. M., Rottkamp, C. A., Hammer, J. A. and Quarles, R. H. (2001) Microtubule-associated protein 1B: a neuronal binding partner for myelin-associated glycoprotein. *The Journal of cell biology,* **155,** 893-898.

Garrido, J. J., Simon, D., Varea, O. and Wandosell, F. (2007) GSK3 alpha and GSK3 beta are necessary for axon formation. *FEBS letters,* **581,** 1579-1586.

Gluckman, P. D., Guan, J., Williams, C., Scheepens, A., Zhang, R., Bennet, L. and Gunn, A. (1998) Asphyxial brain injury--the role of the IGF system. *Molecular and cellular endocrinology,* **140,** 95-99.

Goh, E. L., Kim, J. Y., Kuwako, K., Tessier-Lavigne, M., He, Z., Griffin, J. W. and Ming, G. L. (2008) Beta1-integrin mediates myelin-associated glycoprotein signaling in neuronal growth cones. *Molecular brain,* **1,** 10.

Gonzalez-Billault, C., Del Rio, J. A., Urena, J. M. et al. (2005) A role of MAP1B in Reelin-dependent neuronal migration. *Cereb Cortex,* **15,** 1134-1145.

Gonzalez-Billault, C., Jimenez-Mateos, E. M., Caceres, A., Diaz-Nido, J., Wandosell, F. and Avila, J. (2004) Microtubule-associated protein 1B function during normal development, regeneration, and pathological conditions in the nervous system. *Journal of neurobiology,* **58,** 48-59.

Gordon-Weeks, P. R. and Fischer, I. (2000) MAP1B expression and microtubule stability in growing and regenerating axons. *Microscopy research and technique,* **48,** 63-74.

Hasegawa, Y., Fujitani, M., Hata, K., Tohyama, M., Yamagishi, S. and Yamashita, T. (2004) Promotion of axon regeneration by myelin-associated glycoprotein and Nogo through divergent signals downstream of Gi/G. *J Neurosci,* **24,** 6826-6832.

He, Z. and Koprivica, V. (2004) The Nogo signaling pathway for regeneration block. *Annu Rev Neurosci,* **27,** 341-368.

Hsieh, S. H., Ferraro, G. B. and Fournier, A. E. (2006) Myelin-associated inhibitors regulate cofilin phosphorylation and neuronal inhibition through LIM kinase and Slingshot phosphatase. *J Neurosci,* **26,** 1006-1015.

Hu, F. and Strittmatter, S. M. (2008) The N-terminal domain of Nogo-A inhibits cell adhesion and axonal outgrowth by an integrin-specific mechanism. *J Neurosci,* **28,** 1262-1269.

Huang, X., Wu, D. Y., Chen, G., Manji, H. and Chen, D. F. (2003) Support of retinal ganglion cell survival and axon regeneration by lithium through a Bcl-2-dependent mechanism. *Investigative ophthalmology & visual science,* **44,** 347-354.

Inoue, T., Hosokawa, M., Morigiwa, K., Ohashi, Y. and Fukuda, Y. (2002) Bcl-2 overexpression does not enhance in vivo axonal regeneration of retinal ganglion cells after peripheral nerve transplantation in adult mice. *J Neurosci,* **22,** 4468-4477.

Johnson, G. V. and Stoothoff, W. H. (2004) Tau phosphorylation in neuronal cell function and dysfunction. *Journal of cell science,* **117,** 5721-5729.

Kim, W. Y. and Snider, W. D. (2008) Neuroscience. Overcoming inhibitions. *Science,* **322,** 869-872.

Koprivica, V., Cho, K. S., Park, J. B. et al. (2005) EGFR activation mediates inhibition of axon regeneration by myelin and chondroitin sulfate proteoglycans. *Science (New York, N.Y,* **310,** 106-110.

Li, S. and Strittmatter, S. M. (2003) Delayed systemic Nogo-66 receptor antagonist promotes recovery from spinal cord injury. *J Neurosci,* **23,** 4219-4227.

Luikart, B. W., Zhang, W., Wayman, G. A., Kwon, C. H., Westbrook, G. L. and Parada, L. F. (2008) Neurotrophin-dependent dendritic filopodial motility: a convergence on PI3K signaling. *J Neurosci,* **28,** 7006-7012.

Maccioni, R. B. and Cambiazo, V. (1995) Role of microtubule-associated proteins in the control of microtubule assembly. *Physiological reviews,* **75,** 835-864.

Mimura, F., Yamagishi, S., Arimura, N., Fujitani, M., Kubo, T., Kaibuchi, K. and Yamashita, T. (2006) Myelin-associated glycoprotein inhibits microtubule assembly by a Rho-kinase-dependent mechanism. *The Journal of biological chemistry,* **281,** 15970-15979.

Mingorance, A., Fontana, X., Sole, M. et al. (2004) Regulation of Nogo and Nogo receptor during the development of the entorhino-hippocampal pathway and after adult hippocampal lesions. *Mol Cell Neurosci,* **26,** 34-49.

Mingorance, A., Fontana, X., Soriano, E. and Del Rio, J. A. (2005) Overexpression of myelin-associated glycoprotein after axotomy of the perforant pathway. *Mol Cell Neurosci,* **29,** 471-483.

Mingorance, A., Sole, M., Muneton, V., Martinez, A., Nieto-Sampedro, M., Soriano, E. and del Rio, J. A. (2006) Regeneration of lesioned entorhino-hippocampal axons in vitro by combined degradation of inhibitory proteoglycans and blockade of Nogo-66/NgR signaling. *Faseb J,* **20,** 491-493.

Mizusawa, I., Abe, S., Kanno, K., Yabashi, A., Honda, T., Suto, M. and Hiraiwa, K. (2003) Expression of cytokines, neurotrophins, neurotrophin receptors and NOS mRNA in dorsal root ganglion of a rat tourniquet model. *Legal medicine (Tokyo, Japan),* **5 Suppl 1,** S271-274.

Montolio, M., Messeguer, J., Masip, I., Guijarro, P., Gavin, R., Antonio Del Rio, J., Messeguer, A. and Soriano, E. (2009) A semaphorin 3A inhibitor blocks axonal chemorepulsion and enhances axon regeneration. *Chem Biol,* **16,** 691-701.

Niederost, B., Oertle, T., Fritsche, J., McKinney, R. A. and Bandtlow, C. E. (2002) Nogo-A and myelin-associated glycoprotein mediate neurite growth inhibition by antagonistic regulation of RhoA and Rac1. *J Neurosci,* **22,** 10368-10376.

Nunez, J. and Fischer, I. (1997) Microtubule-associated proteins (MAPs) in the peripheral nervous system during development and regeneration. *J Mol Neurosci,* **8,** 207-222.

Pardo, R., Andreolotti, A. G., Ramos, B., Picatoste, F. and Claro, E. (2003) Opposed effects of lithium on the MEK-ERK pathway in neural cells: inhibition in astrocytes and stimulation in neurons by GSK3 independent mechanisms. *Journal of neurochemistry,* **87,** 417-426.

Park, J. B., Yiu, G., Kaneko, S., Wang, J., Chang, J. and He, Z. (2005) A TNF Receptor Family Member, TROY, Is a Coreceptor with Nogo Receptor in Mediating the Inhibitory Activity of Myelin Inhibitors. *Neuron,* **45,** 345-351.

Park, K. K., Liu, K., Hu, Y. et al. (2008) Promoting axon regeneration in the adult CNS by modulation of the PTEN/mTOR pathway. *Science,* **322,** 963-966.

Rhodes, K. E., Moon, L. D. and Fawcett, J. W. (2003) Inhibiting cell proliferation during formation of the glial scar: effects on axon regeneration in the CNS. *Neuroscience,* **120,** 41-56.

Riederer, B. M. (2007) Microtubule-associated protein 1B, a growth-associated and phosphorylated scaffold protein. *Brain research bulletin,* **71,** 541-558.

Rolls, A., Shechter, R. and Schwartz, M. (2009) The bright side of the glial scar in CNS repair. *Nature reviews,* **10,** 235-241.

Sanchez, S., Sayas, C. L., Lim, F., Diaz-Nido, J., Avila, J. and Wandosell, F. (2001) The inhibition of phosphatidylinositol-3-kinase induces neurite retraction and activates GSK3. *Journal of neurochemistry,* **78,** 468-481.

Satoh, J., Onoue, H., Arima, K. and Yamamura, T. (2005) Nogo-A and nogo receptor expression in demyelinating lesions of multiple sclerosis. *J Neuropathol Exp Neurol,* **64,** 129-138.

Sayas, C. L., Moreno-Flores, M. T., Avila, J. and Wandosell, F. (1999) The neurite retraction induced by lysophosphatidic acid increases Alzheimer's disease-like Tau phosphorylation. *J Biol Chem,* **274,** 37046-37052.

Schweigreiter, R., Walmsley, A. R., Niederost, B. et al. (2004) Versican V2 and the central inhibitory domain of Nogo-A inhibit neurite growth via p75NTR/NgR-independent pathways that converge at RhoA. *Mol Cell Neurosci,* **27,** 163-174.

Shao, Z., Browning, J. L., Lee, X. et al. (2005) TAJ/TROY, an Orphan TNF Receptor Family Member, Binds Nogo-66 Receptor 1 and Regulates Axonal Regeneration. *Neuron,* **45,** 353-359.

Shen, Y., Tenney, A. P., Busch, S. A., Horn, K. P., Cuascut, F. X., Liu, K., He, Z., Silver, J. and Flanagan, J. G. (2009) PTPsigma is a receptor for chondroitin sulfate proteoglycan, an inhibitor of neural regeneration. *Science,* **326,** 592-596.

Silver, J. and Miller, J. H. (2004) Regeneration beyond the glial scar. *Nature reviews,* **5,** 146-156.

Simo, S., Pujadas, L., Segura, M. F., La Torre, A., Del Rio, J. A., Urena, J. M., Comella, J. X. and Soriano, E. (2007) Reelin induces the detachment of postnatal subventricular zone cells and the expression of the Egr-1 through Erk1/2 activation. *Cereb Cortex,* **17,** 294-303.

Singleton, D. W., Lu, C. L., Colella, R. and Roisen, F. J. (2000) Promotion of neurite outgrowth by protein kinase inhibitors and ganglioside GM1 in neuroblastoma cells involves MAP kinase ERK1/2. *Int J Dev Neurosci,* **18,** 797-805.

Sivasankaran, R., Pei, J., Wang, K. C., Zhang, Y. P., Shields, C. B., Xu, X. M. and He, Z. (2004) PKC mediates inhibitory effects of myelin and chondroitin sulfate proteoglycans on axonal regeneration. *Nat Neurosci,* **7,** 261-268.

Sole, M., Fontana, X., Gavin, R., Soriano, E. and del Rio, J. A. (2004) Bcl-2 overexpression does not promote axonal regeneration of the entorhino-hippocampal connections in vitro after axotomy. *Brain research,* **1020,** 204-209.

Stoppini, L., Buchs, P. A. and Muller, D. (1991) A simple method for organotypic cultures of nervous tissue. *Journal of neuroscience methods,* **37,** 173-182.

Tan, E. Y., Law, J. W., Wang, C. H. and Lee, A. Y. (2007) Development of a cell transducible RhoA inhibitor TAT-C3 transferase and its encapsulation in biocompatible microspheres to promote survival and enhance regeneration of severed neurons. *Pharmaceutical research,* **24,** 2297-2308.

Venkatesh, K., Chivatakarn, O., Lee, H., Joshi, P. S., Kantor, D. B., Newman, B. A., Mage, R., Rader, C. and Giger, R. J. (2005) The Nogo-66 receptor homolog NgR2 is a sialic acid-dependent receptor selective for myelin-associated glycoprotein. *J Neurosci,* **25,** 808-822.

Victoratos, P., Yiangou, M., Avramidis, N. and Hadjipetrou, L. (1997) Regulation of cytokine gene expression by adjuvants in vivo. *Clinical and experimental immunology,* **109,** 569-578.

Vinson, M., Strijbos, P. J., Rowles, A., Facci, L., Moore, S. E., Simmons, D. L. and Walsh, F. S. (2001) Myelin-associated glycoprotein interacts with ganglioside GT1b. A mechanism for neurite outgrowth inhibition. *J Biol Chem,* **276,** 20280-20285.

Vyas, A. A., Patel, H. V., Fromholt, S. E., Heffer-Lauc, M., Vyas, K. A., Dang, J., Schachner, M. and Schnaar, R. L. (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.

Wang, K. C., Kim, J. A., Sivasankaran, R., Segal, R. and He, Z. (2002) P75 interacts with the Nogo receptor as a co-receptor for Nogo, MAG and OMgp. *Nature,* **420,** 74-78.

Wehrli, P., Charnay, Y., Vallet, P. et al. (2001) Inhibition of post-ischemic brain injury by clusterin overexpression. *Nature medicine,* **7,** 977-979.

Worter, V., Schweigreiter, R., Kinzel, B., Mueller, M., Barske, C., Bock, G., Frentzel, S. and Bandtlow, C. E. (2009) Inhibitory activity of myelin-associated glycoprotein on sensory neurons is largely independent of NgR1 and NgR2 and resides within Ig-Like domains 4 and 5. *PLoS One,* **4,** e5218.

Xiao, J. and Liu, Y. (2003) Differential roles of ERK and JNK in early and late stages of neuritogenesis: a study in a novel PC12 model system. *Journal of neurochemistry,* **86,** 1516-1523.

Xie, F. and Zheng, B. (2008) White matter inhibitors in CNS axon regeneration failure. *Experimental neurology,* **209,** 302-312.

Yick, L. W., So, K. F., Cheung, P. T. and Wu, W. T. (2004) Lithium chloride reinforces the regeneration-promoting effect of chondroitinase ABC on rubrospinal neurons after spinal cord injury. *Journal of neurotrauma,* **21,** 932-943.

Yoshimura, S., Takagi, Y., Harada, J., Teramoto, T., Thomas, S. S., Waeber, C., Bakowska, J. C., Breakefield, X. O. and Moskowitz, M. A. (2001) FGF-2 regulation of neurogenesis in adult hippocampus after brain injury. *Proceedings of the National Academy of Sciences of the United States of America,* **98,** 5874-5879.

Zhang, L., Zheng, S., Wu, H., Wu, Y., Liu, S., Fan, M. and Zhang, J. (2009) Identification of BLyS (B lymphocyte stimulator), a non-myelin-associated protein, as a functional ligand for Nogo-66 receptor. *J Neurosci,* **29,** 6348-6352.

Zheng, B., Atwal, J., Ho, C., Case, L., He, X. L., Garcia, K. C., Steward, O. and Tessier-Lavigne, M. (2005) Genetic deletion of the Nogo receptor does not reduce neurite inhibition in vitro or promote corticospinal tract regeneration in vivo. *Proc Natl Acad Sci U S A,* **102,** 1205-1210.

Zhou, F., Zhang, L., Wang, A. et al. (2008) The association of GSK3 beta with E2F1 facilitates nerve growth factor-induced neural cell differentiation. *The Journal of biological chemistry,* **283,** 14506-14515.

Zhou, F. Q., Walzer, M., Wu, Y. H., Zhou, J., Dedhar, S. and Snider, W. D. (2006) Neurotrophins support regenerative axon assembly over CSPGs by an ECM-integrin-independent mechanism. *Journal of cell science,* **119,** 2787-2796.

Zhou, F. Q., Zhou, J., Dedhar, S., Wu, Y. H. and Snider, W. D. (2004) NGF-induced axon growth is mediated by localized inactivation of GSK-3beta and functions of the microtubule plus end binding protein APC. *Neuron,* **42,** 897-912.

Figure Legends

Figure 1. ***Activation of ERK1/2, Akt and GSK3β and phosphorylation of MAPs in cultured CGNs by myelin incubation.*** **A)** Schematic procedure of the experiments. Isolation of CGNs of P5-P7 mouse pups for Western blotting and kinase activity assays after myelin or AP-Nogo66/Mock treatments. **B)** Time course activation of ERK1/2 and Akt in cultured CGNs after incubation with AP-Nogo66 or AP-Mock

containing media. The quantification of ERK1/2 and Akt-phosphorylation is shown below in the histograms. Values represent the mean ± sem of three separate experiments **C)** ERK1/2 activity in cultured CGNs measured by radioactive assay (see Material and Methods for details) after myelin treatment. The level of total ERK1/2 in autoradiographic samples is shown in the immunoblots. ** $p < 0.05$ by the Student´s *t* test **D)** Histograms showing the GSK3β activity using a radioactive method following myelin treatment. Time course phosphorylation levels of GSK3β-Ser and GSK3β-Tyr after myelin treatment. Time course phosphorylation of Tau (α-AT8) in cultured CGNs following acute myelin treatment. Levels of Actin and total Tau (α-Tau5) are presented as loading controls. The quantification of the Tau phosphorylation experiments is shown in the histograms. Values represent the mean ± sem of three separate experiments. * $p < 0.05$ by the Student´s *t* test

Figure 2. ***Differential activation of ERK1/2 and GSK3β in CGNs after acute and chronic treatment with myelin.*** **A)** Isolation of CGNs from P5-P7 mouse pups. The CGNs were cultured over PD-Lysine or PD-Lysine + myelin to perform activity assays, morphological studies, kinase activity assays and also to carry out the pharmacological treatments. **B)** Immunoblot showing the activation of ERK1/2 and GSK3β in cultured CGNs growing over PD-Lysine and treated with PBS 0.1 M or myelin (acute, left) or, alternatively growing over PD-Lysine or PD-Lysine + myelin (substrate, right). As observed, acute treatment with myelin activates ERK1/2 and increases levels of Serine 9 phosphorylated GSK3β. Cultured CGNs show no activation of ERK1/2 after 24 h in the presence of myelin. In contrast, levels of Serine 9 phosphorylated GSK3β are reduced in the same experimental conditions, indicating global activation of GSK3β. Total ERK1/2 and GSK3β are displayed as loading controls. This activation was corroborated by using a radioactive assay **(C)**. Values represented in the histogram correspond to the mean ± sem of four separate experiments. ** $p < 0.05$ by the Student´s *t* test. **D)** High power microphotographs illustrating examples of *NgR1* +/+ CGNs cultured over PD-Lysine or PD-Lysine + myelin (8 μg per 35 mm ø culture surface). CGNs growing over PD-Lysine extend long neurites (arrows) shortly after being cultured (48 h) and often display several branches (asterisk). In contrast, when CGNs are cultured over PD-Lysine +

myelin there is a great reduction in neurite extension (arrowheads). CGNs cultured over PD-Lysine + myelin partially recover neurite length after treatment with SB-415286 (30 μM) (arrows) in contrast to U0126 (10 μM) treatment (arrowheads). The quantification of the experiments is shown below in the histogram, where values represent the mean ± sem of neurite length of 40 neurons for SB-415286 (30 μM), 42 neurons in SB-415286 (15 μM) and 30 neurons for U0126 treatments in three separate experiments. In the lower histograms the effect of the treatment of SB-216763 on CGNs cultured over myelin is shown. Values represent the mean ** mean ± sem of neurite length of 40 neurons for SB-216763 (3μM) and 50 neurons for SB-216763 (10 μM). $p < 0.05$ by the Student´s *t* test. **E)** High power microphotographs illustrating examples of Phalloidin-labeled *NgR1 -/-* CGNs cultured over PD-Lysine or PD-Lysine + myelin as above. Notice that CGNs growing over PD-Lysine + myelin extend neurites (arrows) and often display several branches (asterisk) as in *NgR1 -/-* CGNs growing over PD-Lysine. CGNs cultured over PD-Lysine + myelin show high levels of neurite length recovery after treatment with GSK3β inhibitors, in contrast to *NgR1 +/+* CGNs (D). The quantification of the experiments is shown below in the histogram, where values represent the mean ± sem of neurite length of 90 neurons for each experimental group in four separate experiments. ** $p < 0.05$ by the Student´s *t* test.

Figure 3. ***Gene expression profiling analysis of apoptotic and neuronal injury genes and myelin associated genes after EHP axotomy in wild-type cultures.*** Gene expression profiling from apoptotic and neuronal injury markers **(A)** and myelin associated proteins **(B)** after 1, 3 and 7 days of EHP axotomy using mouse whole genome Agilent arrays (44k). The colour in each cell of the table represents the mean-adjusted expression level of the gene from direct and dye-swap measurements, with blue showing underexpression and yellow indicating overexpression, according to the chart. Abbreviations: Casp3-9, Caspase 3 and 9; Bcl-2, B-cell lymphoma protein 2; Bax, BCL2-associated X protein; Bad, BCL2-associated agonist of cell death; Mtap2, Microtubule-associated protein 2, Clu, Clusterin; FGF-2, Fibroblast growth factor 2; IGF-2, Insulin growth factor 2; MAG, Myelin associated glycoprotein; MBP, Myelin basic protein; MOG, Myelin Oligodendrocyte Glycoprotein; MOBP, myelin-associated

oligodendrocyte basic protein; Plp, proteolipoprotein; RTN4, Reticulon 4 (Nogo-A); MT3, metallothionein 3.

Figure 4. ***Presence of MAIs and CSPGs correlated with GSK3β and ERK1/2 kinase activation after EHP axotomy.*** **A-B)** Time course analysis of MAIs (A) and CSPGs (B) in axotomized EH slice co-cultures measured by Western blotting (left). Quantification of the immunoblotting analysis is shown on the right. Values represent the mean ± sem of four separate experiments. * $p < 0.05$ by the Student´s *t* test. A relevant increase in OMgp and MBP can be seen after lesion (A). In contrast, Nogo-A remained constant several days after lesion compared to CS-56-positive CSPGs (B). **C)** Western blotting analysis of GSK3β and ERK1/2 kinase activity in axotomized EH slices from *NgR1 +/+* (left) and *NgR1 -/-* (right) mice. As observed above, ERK1/2 decreases 3 and 12 DAL in both cases. In contrast, GSK3β activity increases at these stages compared to basal conditions. **D)** Time course of GSK3β (left) and ERK1/2 activation (right) in *NgR1 +/+* and *NgR1 -/-* after the EHP axotomy. Notice the increasing GSK3β activity over time after EHP axotomy in wild-type and *NgR1 -/-* cultures. Values represent the mean ± sem of four (*NgR1 +/+*) and three (*NgR1 -/-*) separate experiments.

Figure 5. ***Regeneration of the axotomized EHP after treatment with SB-415286 and SB-216763 in NgR1 +/+ and NgR1 -/- .*** **A)** Schematic diagram illustrating the *in vitro* axotomy model. The EHP was axotomized at 15 DIV (dashed line) with a tungsten needle; the cultures were treated with different drugs for 10 DIV and were then labeled with biocytin. **B-E)** Pattern of entorhino-hippocampal regeneration in TAT-C3 treatment (B-C) and after SB-415286 treatment (D-E). The EHP did not show a high regeneration level after TAT-C3 treatment in contrast to SB-415286. The presence of fibers ending in growth cones (arrows) in the hippocampal slice are shown in the insert boxes in C and E. **F-G)** Histograms showing the mean number of biocytin-labeled fibers which crossed a 400-μm segment in the hippocampus located 75-80 μm parallel to the transection of consecutive sections from each culture after TAT-C3, NEP-1-40, SB-415286 and SB-216763 treatments. The number of cultures in each treatment

was as follows: Control, n = 62 (41 (upper graph) + 21 (lower graph)); NEP1-40, n = 30; TAT-C3, n = 20; SB-415286, n = 23, SB-216763, n = 18. ** $p < 0.05$ by Student´s *t* test. **H)** Low power photomicrograph showing the absence of spontaneously entorhino-hippocampal regeneration in *NgR1 -/-* axotomized EHP (see Material and Methods for details). **I-J)** Pattern of entorhino-hippocampal regeneration after SB-415286 treatment in *NgR1 -/-* EH cultures. The presence of fibers ending in growth cones (arrows) in the hippocampal slice are shown in the insert box in I. **K-L)** Histograms showing the mean number of regenerating biocytin-labeled fibers in *NgR1 -/-* cultures after GSKβ inhibitors. The number of cultures in each treatment was as follows: Control, n = 43 (20 (upper graph) + 23 (lower graph)); SB-415286, n = 22. SB-216763, n = 16. Quantification was performed as above. ** $p < 0.05$ by Student´s *t* test. Abbreviations: CA1-3, hippocampal fields; DG, dentate gyrus; EC, entorhinal cortex; EHP. Entorhinal-hippocampal pathway; S, subiculum. Scale bars: B and G = 200 μm pertains to D and H; C = 100 μm pertains to E. I = 50 μm).

Supplementary Figure 1. ***Activation of ERK1/2 after different myelin treatment.*** Immunoblot showing the activation of ERK1/2 in cultured CGNs growing over PD-Lysine and treated with PBS 0.1 M, 50 μg/ml or 100 μg/ml of myelin during 30 min. Levels of Actin are presented as loading controls.

Supplementary Figure 2. ***Gene expression regulation after EHP axotomy.*** **A)** Venn diagrams showing the number of overlapping regulated genes at different times following EHP axotomy. Several genes were regulated at more than 1 time and 9 of them were regulated at all the times studied: Cds1, Edg2, Egr1, Hspa5, Insig1, Mcam, Ns5atp9, Plp, Spp1 (Complete updated gene list available on GEO database). **B)** Clustering analysis by MeV (Multiple Expression Viewer) from the regulated genes following EHP axotomy, showing the different expression patterns of regulated genes during the timing of the experiment.

Supplementary Figure 3. ERK1/2 ***activation in EH co-cultures after axotomy.*** **A)** Low power photomicrograph illustrating the pattern of expression of phospho-ERK1/2 immunoreactive elements at 1

DAL in EH co-cultures. Notice that most immunoreactive elements are close to the lesion site. **B)** High power photomicrograph of the boxed area shown in A). Notice the presence of immunoreactive cells arrows. **C)** Photomicrograph illustrating the decrease in phospho-ERK1/2 immunoreactivity 3 DAL in lesioned EH co-cultures. Abbreviations as in Figure 5. Scale bars: A = 200 μm and B-C 100 μm.

Supplementary Figure 4. Schematic diagram showing a proposed activation of GSK3β by MAIs. MAIs acting through Nogo receptor complex activate both EGFr (1, (Koprivica et al. 2005)) leading to ERK1/2 activation as well as Rho-kinase cascade leading to LIM kinase activation and F-Actin depolimerization (2, (Hsieh et al. 2006)). In addition, MAIs could activate GSK3β through Nogo receptor complex trhough PIK3/Akt (see results) as well as other unknown receptor/s. Activated GSK3β increases phosphorylated Tau leading to microtubule instability (3). Moreover, other inhibitory molecules could also activate GSK3β in parallel (4), (e.g., secreted Semaphorins (Montolio et al. 2009); see Discussion). The overall F-Actin and microtubule instability lead to a relevant neurite outgrowth inhibition in lesioned CNS neurons.

Supplementary Figure 5. Electron micrograph of a regenerating EHP axon terminal (AT) in the SLM, which established synaptic contact (arrows) with a dendritic spine. The axon (black reaction end-product) was labelled by biocytin injection in the EC of the *NgR1 -/-* organotypic slice after 10 DIV of treatment with the GSK3β inhibitor SB-415286. Abreviations: AT, axon terminal. Scale bar: A = 0.5 μm.