

# Schwann cell-derived factor-induced modulation of the NgR/p75<sup>NTR</sup>/EGFR axis disinhibits axon growth through CNS myelin *in vivo* and *in vitro*

Zubair Ahmed, Ellen L. Suggate, Elspeth R. Brown, Russell G. Dent, Stephanie J. Armstrong, Lee B. Barrett, Martin Berry and Ann Logan

Molecular Neuroscience Group, Division of Medical Sciences, University of Birmingham, Birmingham, UK

Correspondence to: Prof Ann Logan, Molecular Neuroscience Group, Division of Medical Sciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK  
E-mail: a.logan@bham.ac.uk

**When associated with the Nogo receptor (NgR), the transmembrane receptor p75<sup>NTR</sup> signals growth cone collapse. Arrest of CNS axon growth *in vivo* is mediated by CNS myelin-derived inhibitory ligands through either an unknown pathway after NgR- and Ca<sup>2+</sup>-dependent activation of the epidermal growth factor receptor (EGFR), and/or sequential Rho-A/ROCK/LIM-kinase/cofilin phosphorylation leading to actin depolymerization. Paradoxically, rat retinal ganglion cell (RGC) axons regenerate through the CNS myelin-rich transected optic nerve after intravitreal sciatic nerve grafting without inhibitory ligand neutralization. Here, we show that optic nerve regeneration *in vivo* correlates with Schwann cell-derived factor-induced cleavage of NgR and Nogo-A, and inactivation of p75<sup>NTR</sup> signalling by the induction of regulated intramembranous proteolysis (RIP) and the release of both extracellular (p75<sup>ECD</sup>) and intracellular (p75<sup>ICD</sup>) domains. Hence, Schwann cell-derived factors compromise inhibitory signalling by (i) antagonizing ligand/NgR binding with metalloproteinase-cleaved Nogo-A peptides; (ii) RIP of p75<sup>NTR</sup>; (iii) competitively blocking NgR/p75<sup>NTR</sup> clustering with soluble p75<sup>ECD</sup>; and (iv) consequent reduced downstream EGFR phosphorylation and suppression of Rho-A activation. Moreover, in RGC cultures, exogenous tumour necrosis- $\alpha$  converting enzyme (TACE) initiates RIP of p75<sup>NTR</sup>, reduces EGFR phosphorylation, suppresses activation of Rho-A, cleaves the ECD from both NgR and TROY, and disinhibits neurotrophic factor (NTF) stimulated RGC neurite outgrowth in the presence of CNS myelin. Soluble NgR<sup>ECD</sup> binds all CNS myelin-derived ligands and thus has the potential to act as an inhibitory signalling antagonist, but the role of TROY and its shed ectodomain in growth cone mobility is unknown. siRNA knockdown of p75<sup>NTR</sup> also inactivates Rho-A and disinhibits NTF-stimulated RGC neurite outgrowth in cultures with added CNS myelin. In all the above experimental paradigms, Schwann cell-derived factor/NTF-induced attenuation of NgR/p75<sup>NTR</sup> signalling suppresses EGFR activation, thereby potentiating axon growth disinhibition.**

**Keywords:** regulated intramembranous proteolysis; optic nerve regeneration; RGC axon growth disinhibition; metalloproteinases

**Abbreviations:** BDNF = brain-derived neurotrophic factor; CNTF = ciliary neurotrophic factor; CSPG = chondroitin sulphate proteoglycans; EGFR = epidermal growth factor receptor; MAG = myelin-associated glycoprotein; MMP = matrix metalloproteinases; NRM; non-regenerating model; NTF = neurotrophic factor; NgR = Nogo receptor; PN = peripheral nerve; RGC = retinal ganglion cell; Rho-GDI = Rho-GDP dissociation inhibitor; RIP = regulated intramembranous proteolysis; RM = regenerating model; TACE = tumour necrosis- $\alpha$  converting enzyme; TIMP = tissue inhibitors of MMP.

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## Introduction

One reason why transected central nervous system (CNS) axons fail to regenerate is probably because myelin/oligodendrocyte growth inhibitory ligands bind to Nogo receptor (NgR) and form a complex with Lingo-1

and either the pan neurotrophin receptor p75<sup>NTR</sup> (Wang *et al.*, 2002; Wong *et al.*, 2002; Mi *et al.*, 2004) or other signalling co-receptors, like TROY (Park *et al.*, 2005; Shao *et al.*, 2005) located on axon growth cones. Receptor binding induces Rho-A signalling of growth cone collapse (Barde, 1989; Barrett *et al.*, 1998; Mukai *et al.*, 2000) and an NgR- and Ca<sup>2+</sup>-dependent mechanism that activates epidermal growth factor receptor (EGFR) mediated axon growth inhibition through an unknown pathway (Koprivica *et al.*, 2005). Since NgR binds a plethora of inhibitory ligands, single ligand knockout of, for example, Nogo-A fails to enhance robust CNS axon regeneration (Simonen *et al.*, 2003; Zheng *et al.*, 2003). On the other hand, manipulation of Rho-A with C3 transferase and Rho kinase inhibitors blocks downstream inhibitory pathway activation, stimulates actin polymerization and allows growth cones to advance unimpeded (Dergham *et al.*, 2002; Winton *et al.*, 2002; Borisoff *et al.*, 2003; Fournier *et al.*, 2003; Ramer *et al.*, 2004).

Retinal ganglion cells (RGC) constitutively express NgR and/or p75<sup>NTR</sup> and/or TROY, and the distal stump and scar of the transected optic nerve contains a full compliment of all known inhibitory ligands (Chierzi and Fawcett, 2001). Paradoxically, however, after intravitreal sciatic nerve grafting (Berry *et al.*, 1996, 1999) and lens injury (Leon *et al.*, 2000; Fischer *et al.*, 2001; Lorber *et al.*, 2002; Yin *et al.*, 2003), transected RGC axons in the rat and mouse do regenerate through the inhibitory ligand-rich environments of both the incipient scar and distal myelinated optic nerve and chiasm. In the former model, we have shown that regenerating RGC axons secrete matrix metalloproteinases (MMP) and down-regulate the expression of tissue inhibitors of MMP (TIMP) in optic nerve glia, which probably attenuates scarring in the lesion (Ahmed *et al.*, 2005a).

As CNS axons are generally incapable of regeneration, we investigated why intravitreal peripheral nerve (PN) implants in adult rats stimulated the regeneration of severed RGC axons though the inhibitory ligand-rich distal optic nerve (Berry *et al.*, 1996, 1999). We hypothesized that Schwann cell-derived factors, supplied through an intravitreal PN implant (Berry *et al.*, 1996, 1999), modulate NgR, p75<sup>NTR</sup> and TROY on RGC axon growth cones, thereby abrogating inhibitory ligand binding and signalling. In this study, we compared the integrity of Nogo-A, NgR, p75<sup>NTR</sup> and TROY in retina and optic nerve in our regenerating (RM) and non-regenerating (NRM) *in vivo* rat optic nerve crush models. In the RM, whilst there was elevated expression of all three receptors post-injury, they were also cleaved with associated attenuation of Rho-A signalling, and, additionally, Nogo-A was fragmented, possibly explaining why axons regenerate in the transected optic nerve after intravitreal PN grafting. After Schwann cell-derived factor/neurotrophic factor (NTF) stimulation *in vivo* and *in vitro*, respectively, levels of active tumour necrosis factor- $\alpha$  converting enzyme (TACE, which has  $\alpha$ -secretase activity) were elevated in RGC regenerating their axons. TACE initiates regulated intramembranous proteolysis (RIP) of p75<sup>NTR</sup>, a process in

which the 55 kDa extracellular domain (p75<sup>ECD</sup>) is first cleaved from p75<sup>NTR</sup> by  $\alpha$ -secretase, leaving a 32 kDa cytoplasmic transitional product p75<sup>CTF</sup> from which the 25 kDa intracellular domain (p75<sup>ICD</sup>) is secondarily cleaved by a  $\gamma$ -secretase (Logan *et al.*, 2006). Complete removal of p75<sup>NTR</sup>, by siRNA-mediated mRNA knockdown *in vitro* (Ahmed *et al.*, 2005b), significantly enhances disinhibition of neurite outgrowth of ciliary neurotrophic factor (CNTF) stimulated RGC in the presence of inhibitory CNS myelin extracts. Paralysis of p75<sup>NTR</sup> signalling by siRNA suppresses Rho-A activation, favouring actin polymerization in growth cones, thereby promoting CNTF-stimulated neurite growth in the presence of CNS myelin. We also show that EGFR-mediated axon growth inhibition (Koprivica *et al.*, 2005) is reduced in RM retinae and optic nerves by reduced phosphorylation of EGFR, probably resulting from suppressed NgR-mediated Ca<sup>2+</sup> signalling.

## Methods

### Animals and treatment groups

All animal procedures were licensed by the UK Home Office. Adult, female 200–250 g Fischer rats were anaesthetized with Hypnorm/Hypnovel anaesthesia (Janssen Pharmaceuticals, Oxford, UK). The experimental groups comprised RM in which the optic nerve was crushed (ONC) intraorbitally and a freshly teased segment of the sciatic nerve immediately transplanted intravitreally (intravitreal PN graft) and held in place with Sterispon gelatine sponge (Johnson and Johnson, UK), and NRM in which the optic nerve was crushed (ONC) without intravitreal sciatic nerve implantation. Five additional groups separately controlled for (i) baseline parameters, using untreated intact rats, that is, 0 day controls; (ii) effects of an intravitreal sciatic nerve implantation without ONC, that is, PN no ONC; (iii) intravitreal inflammation and effects of scleral/vitreous injury without ONC, by intravitreal Sterispon implantation alone; (iv) intravitreal inflammation and effects of scleral/vitreous injury with ONC, by intravitreal Sterispon implantation and ONC; and (v) presence of non-proteinaceous sciatic nerve-derived growth factors, by intravitreal implantation of freeze/thawed sciatic nerve to denature all proteins and kill all Schwann and other sciatic nerve cells. None of the animals developed cataracts, confirming that the lens had not been injured during surgery. The optic nerve was exposed through a supraorbital approach and crushed using forceps as described previously (Berry *et al.*, 1996, 1999). Optic nerves and retinae were processed for RNase protection assay, western blotting and immunohistochemistry at 6, 8 and 20 days post-injury (dpi), as described below.

### Preparation of CNS myelin extract

A CNS myelin extract was prepared as described by us elsewhere. The extract has been shown to contain Nogo-A, oligodendrocyte myelin glycoprotein (OMgp), myelin-associated glycoprotein (MAG) and chondroitin sulphate proteoglycans (CSPG) (Ahmed *et al.*, 2005b).

### Ribonuclease protection assay

A 457-bp fragment (coding region) of the rat p75<sup>NTR</sup> cDNA (from Dr M. Knipper, Tuebingen Hearing Research Centre, Tuebingen,

Germany) cloned into the *Pst*I/*Sac*II site of pBluescript KS<sup>+</sup> was used to detect p75<sup>NTR</sup> mRNA. Cyclophilin (peptidyl-propyl *cis-trans*-isomerase) cDNA, a housekeeping gene, was used as a reference DNA (from Dr J. Douglass, Oregon Health Sciences University, Portland, Oregon, USA), utilizing a 295-bp fragment (coding region) of the rat cyclophilin cDNA cloned into the *Pst*I/*Bam*HI sites of pSP65.

Total RNA was prepared from the retinae of groups of three animals per time-point using RNA-B (Biogenesis, Southampton, UK) and RNase protection assay was performed according to the method described elsewhere (Smith *et al.*, 2001). Autoradiographs were digitally scanned and intensities of bands corresponding to p75<sup>NTR</sup> and its cleaved fragments were quantified using NIH Image software (NIH, USA). For control animals, the mean intensity was given a value of 1.0 and the relative fold-change in lesioned animals was calculated. mRNA levels were internally standardized by quantifying cyclophilin mRNA levels. Data were examined statistically by one-way analysis of variance (ANOVA) with results judged as significant at  $P < 0.05$ .

### Tissue preparation and immunohistochemistry

Groups of at least three rats were killed by anaesthetic overdose and perfusion fixed with 4% paraformaldehyde, and their retinae and optic nerves were excised, cryoprotected, immersed in OCT (Miles Inc., CA, USA) and frozen in liquid nitrogen. Longitudinal cryostat sections, 10  $\mu$ m thick, were cut (Bright Instrument Co. Ltd., Cambridgeshire, UK) at 4°C, collected onto Vectabond coated slides (Vector Laboratories, Cambridgeshire, UK), air dried and processed for immunohistochemistry as described previously (Lorber *et al.*, 2002). Cultured retinal cells on glass coverslips were fixed in 4% paraformaldehyde and also processed as described previously (Lorber *et al.*, 2002).

### In situ hybridization

Longitudinal cryostat retinal sections, 10  $\mu$ m thick, were collected onto charged slides (Vector Laboratories), air dried and processed for *in situ* hybridization using a TSA Biotin System (NEN Life Sciences Inc., Boston, MA) as described previously (Lagord *et al.*, 2002). Oligonucleotide probes used for p75<sup>NTR</sup> (Radeke *et al.*, 1987; Suzuki *et al.*, 1998) and TACE (Black *et al.*, 1997) were designed from published sequences.

### Protein extraction and western blotting

At 0, 6, 8 and 20 dpi, three rats in each treatment group were killed, both retinae and optic nerves were dissected, and proteins were extracted and processed for western blotting as described previously (Winton *et al.*, 2002). The experiment was repeated three times. To determine levels of p75<sup>NTR</sup> in siRNA-treated RGC cultures by western blotting, 375  $\times$  10<sup>3</sup> cells/siRNA treatment ( $n = 6$ ) were lysed and blotted as described previously (Ahmed *et al.*, 2005b).

### Affinity precipitation of Rho-GTP

GTP-bound Rho was assayed from tissues and cell lysates using a Rho activation assay kit (Upstate Biotechnology, Milton Keynes, UK) following the manufacturer's instructions as described elsewhere (Dubreuil *et al.*, 2003).

### In situ Rho-GTP pull-down assay

*In situ* localization of Rho-GTP was performed according to a modified earlier published method (Dubreuil *et al.*, 2003). Briefly, 10  $\mu$ m thick frozen sections of retinae were post-fixed in 4% paraformaldehyde and incubated with either glutathione-S-transferase (GST)-RBD or GST alone (both from Upstate Biotechnology) overnight at 4°C. Sections were then washed 3 $\times$  in phosphate-buffered saline (PBS), blocked in 3% bovine serum albumin for 1 h at room temperature and incubated with an anti-GST antibody (New England Biolabs, Hertfordshire, UK) and  $\beta$ III-tubulin antibody (Sigma) overnight at 4°C. Sections were then washed in PBS, incubated with either FITC or Texas red secondary antibodies (Molecular Probes), mounted in Fluorsave (Calbiochem) and viewed under an epi-fluorescent microscope (Zeiss, Hertfordshire, UK).

### Adult retinal cultures

Adult rats (6–8-weeks old) were killed by cervical dislocation, and the retinae were removed by dissection and dissociated using a papain system according to the manufacturer's protocol (Worthington Biochem, New Jersey, USA). Cultures of 125  $\times$  10<sup>3</sup> dissociated retinal cells, containing RGC, were grown on glass coverslips pre-coated with 100  $\mu$ g/ml poly-D-lysine (Sigma, Dorset, UK) and 20  $\mu$ g/ml merosin (Chemicon, Harrow, UK) in 4-well tissue culture plates (Nunc, UK) in supplemented Neurobasal-A (Invitrogen) medium for 4 days at 37°C in a humidified 5% CO<sub>2</sub> atmosphere.

### Treatment of retinal cultures with NTF, TACE and modulators of enzyme activity

Experimental adult retinal cultures were grown on glass coverslips in triplicate as described above and subjected to treatment with either 25 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma) to stimulate TACE production (Kanning *et al.*, 2003; Weskamp *et al.*, 2004), or a combined cocktail of recognized Schwann cell-derived NTF, consisting of CNTF (10 ng/ml), neurotrophin-3 (NT-3) (50 ng/ml), fibroblast growth factor 2 (FGF-2) (10 ng/ml) and brain-derived neurotrophic factor (BDNF) (50 ng/ml) (all from Peprotech, London, UK), to stimulate TACE production. Retinal cultures were also treated with either recombinant TACE enzyme (10 ng/ml) (R & D Systems, UK) to cleave p75<sup>ECD</sup>, active human TIMP3 enzyme (5  $\mu$ g/ml, Chemicon) to inhibit TACE activity (Karan *et al.*, 2003; Black *et al.*, 2004), or  $\gamma$ -secretase inhibitor (30  $\mu$ M) (S2188, Sigma) to block p75<sup>ICD</sup> cleavage. Cells were treated for 48 h before harvesting for western blotting and subsequent quantitative assessment of proteins by densitometry as described below. Retinal cultures in the presence or absence of myelin were either (i) untreated; or (ii) treated with (a) combined NTF; (b) combined NTF with a TACE inhibitor, TIMP3; (c) TACE; (d) combined NTF and TACE; (e) combined NTF with a TACE activator, PMA; (f) combined NTF with a  $\gamma$ -secretase inhibitor (S2188); or (g) combined NTF with TACE and a  $\gamma$ -secretase inhibitor (S2188).

### siRNA preparation and transfection

To design target-specific siRNA duplexes, five siRNA sequences directed against the open reading frame of rat p75<sup>NTR</sup> mRNA, NCBI accession number NM012610 (Ahmed *et al.*, 2005b), were selected using criteria set out elsewhere (Elbashir *et al.*, 2001).

Oligonucleotide templates and control scrambled sequences were chemically synthesized (Alta Biosciences, University of Birmingham, UK) and siRNA sequences were constructed using the Silencer siRNA Construction kit (Ambion, Texas, USA).

RGC were transfected with siRNA using Oligofectamine reagent (Invitrogen) in 4-well tissue culture plates (Nunc) according to the manufacturer's instructions (Invitrogen). After 5 h of transfection, supplemented Neurobasal-A medium was added and incubated for a further 72 h in the presence/absence of CNS myelin and CNTF before either cell lysis or immunocytochemistry as described above.

## Antibodies

RGC and their neurites/axons were recognized using monoclonal  $\beta$ III-tubulin antibody at 1:100 (Sigma, Poole, UK). Polyclonal growth-associated protein 43 (GAP-43) antibody was used at 1:400 for both western blots and immunohistochemistry to detect regenerating axons. Polyclonal anti-p75<sup>NTR</sup> (Promega, Southampton, UK), was used at 1:500 for western blots and immunohistochemistry/cytochemistry, which recognizes intact p75<sup>NTR</sup>, p75<sup>ECD</sup>, p75<sup>CTF</sup> and p25<sup>ICD</sup>. Goat anti-human NgR (1:100), TROY (1:500) and phosphorylated EGFR (all from Santa Cruz, CA, USA) were used in western blots at 1:400. Monoclonal anti-EGFR antibody (Novocastra, Newcastle upon Tyne, UK) was used to detect total EGFR in western blots at 1:400. Rabbit polyclonal TACE (Neomarkers, CA, USA) was used at 1:500 dilution for both western blotting and immunohistochemistry. Chicken anti-presenilin-1 (PS1, Cambridge Bioscience, Cambridge, MA, USA) was used at 1:200 in western blots.

## Densitometry

Western blots were scanned digitally in Adobe Photoshop keeping scanning parameters the same for all blots to provide a densitometric value for bands of interest. TIFF files were then analysed in ScionImage (version 4.0.2, Scion Corp, Maryland, CA, USA) using the built-in gel-plotting macros. The integrated density of each band in each lane was calculated for three separate blots from three independent experiments.

## Quantification of neurite outgrowth and RGC survival

The mean number of RGC with neurites and mean neurite length were quantified by splitting each coverslip into nine quadrants and randomly capturing images of  $\beta$ III-tubulin immunostained RGC from each quadrant using a Zeiss Axioplan epi-fluorescent microscope. Axiovision image analysis software (Zeiss, Hertfordshire, UK) was then used to measure neurite length and count the number of RGC in each coverslip as described previously (Lorber *et al.*, 2002) using the built-in macros ( $n = 9$  coverslips/condition).

## Statistical analysis

The significance of differences between sample means were calculated using GraphPad Prism (GraphPad Software Inc., Version 4.0, CA, San Diego, USA) by one-way ANOVA followed by *post hoc* testing with Dunnett's method.

## Results

### *In vivo* optic nerve regeneration Upregulation of GAP-43 correlates with RGC axon regeneration

We have confirmed the original observation of Berry *et al.* (1996, 1999) that up to 10% of RGC axons regenerate in the transected optic nerve 20 days after intravitreal sciatic nerve (PN) implantation (Fig. 1A). PN-induced axon regeneration was correlated with increased GAP-43 titres in the optic nerve (Fig. 1B and C). Although there was little or no GAP-43 in 0 day intact control RGC axons, after intravitreal PN implantation without optic nerve crush (PN no ONC), optic nerve GAP-43 levels rose slightly over time, but expression was less than that seen after ONC either without (NRM) or with (RM) intravitreal PN implantation (Fig. 1B and C). Compared with GAP-43 levels seen in the optic nerve from both 0 day intact and PN no ONC controls, GAP-43 levels were increased by 6 dpi in NRM and RM optic nerves (RM > NRM,  $P < 0.0001$ ). Thereafter, GAP-43 levels fell rapidly in the optic nerves from the NRM, whereas, in the RM, they increased further at 8 dpi, and these raised levels were maintained up to 20 dpi.

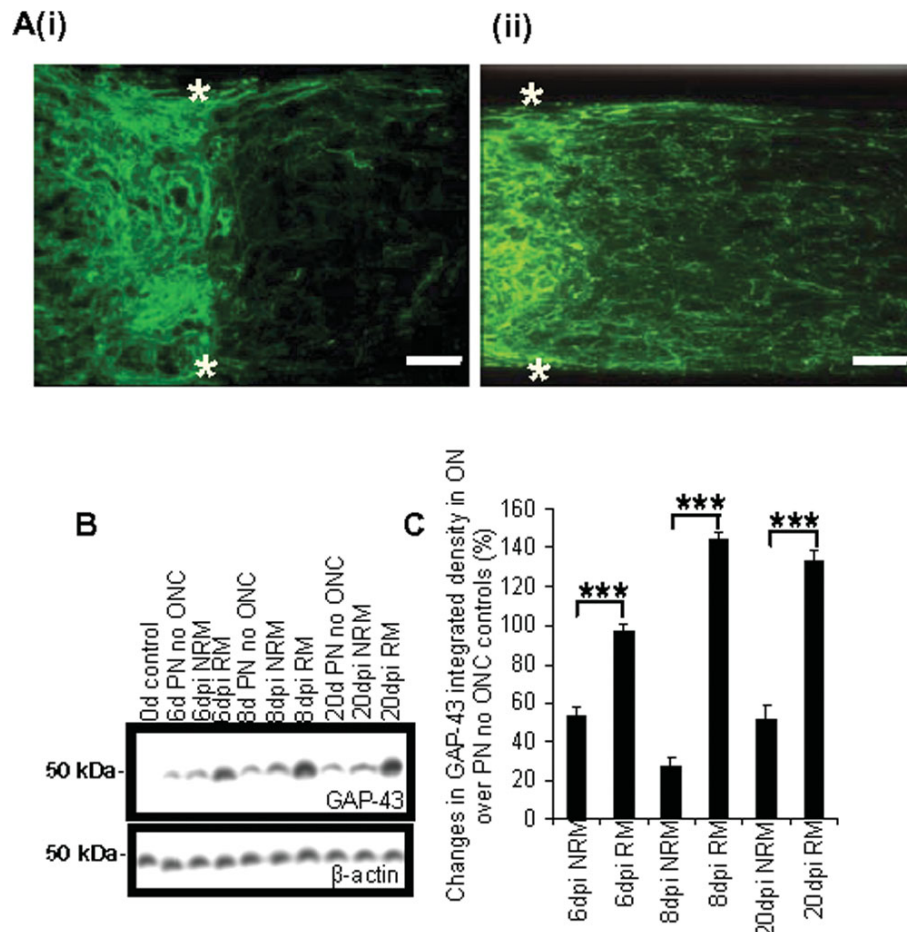
### Retinal p75<sup>NTR</sup> expression

The cellular localization of p75<sup>NTR</sup> in the adult rat retina is controversial. For example, in adult rats, p75<sup>NTR</sup> protein is found in Müller cell processes (Hu *et al.*, 1998, 1999), while mRNA is expressed in RGC (Carmignoto *et al.*, 1991). However, we observed expression of p75<sup>NTR</sup> protein (Fig. 2A) and mRNA (Fig. 2B and C) in a sub-population of ~30% of normal and axon transected adult rat RGC. Lower levels of p75<sup>NTR</sup> protein were also observed in the inner plexiform layer, probably associated with RGC dendritic fields, and occasional cells in the inner nuclear layer (INL) containing horizontal, amacrine and bipolar neurons (Fig. 2A). The levels of p75<sup>NTR</sup> mRNA rose 2-fold in the first 8 dpi in extracts of whole RM and NRM retinæ and a further 2-fold in extracts of whole RM retinæ by 20 dpi, compared with intact 0 day controls (Fig. 2D and E).

### Modulation of inhibitory receptors in RM retina

In RM and NRM retinæ, NgR and TROY levels rose post-injury (Fig. 3A). There was an incrementing appearance of cleaved TROY fragments in RM compared with NRM retinæ (Fig. 3A). Titres of full-length p75<sup>NTR</sup> and p75<sup>ECD</sup> increased in extracts of NRM retinæ by 6 dpi compared with 0 day controls, and remained elevated until 20 dpi (Fig. 3A and B), an observation that correlated with the enhanced levels of retinal p75<sup>NTR</sup> mRNA (Fig. 2D and E). By contrast, despite rising levels of p75<sup>NTR</sup> mRNA, full-length p75<sup>NTR</sup> disappeared in RM retinæ as titres of p75<sup>ECD</sup>, p75<sup>CTF</sup> and p75<sup>ICD</sup> increased proportionally (Fig. 3A and B), suggesting that p75<sup>ECD</sup> and p75<sup>ICD</sup> were generated by post-translational





**Fig. 1** GAP-43<sup>+</sup> axons (**A**) in the optic nerve of the (i) NRM and (ii) RM at 20 dpi. GAP-43<sup>+</sup> axons regenerate through the lesion and distal optic nerve segment in the RM, but not NRM (asterisks indicate lesion site, scale bar = 200  $\mu$ m). Representative western blot (**B**) and (**C**) densitometric data of GAP-43 from pooled optic nerve protein in intact 0 day controls, intravitreal PN implant alone (PN no ONC) controls, and from RM and NRM, at 6, 8 and 20 dpi ( $n = 6$ /experiment, three independent experiments).

cleavage of p75<sup>NTR</sup> and p75<sup>CTF</sup>, respectively. Post-injury fragmentation of RM p75<sup>NTR</sup> was also correlated with a failure of Rho-GDP to convert to Rho-GTP, although total Rho-A levels remained constant. At 8 dpi, for example, Rho-GTP was greatly reduced at 8 and 20 dpi in RM compared with NRM retinæ and RGC, respectively (Fig. 3A and C–E). Limited proteolytic fragmentation of p75<sup>NTR</sup> occurred by 6 days in intact controls that received an intravitreal PN implant (PN no ONC 5% > 0 day control, not shown). The disappearance of full-length p75<sup>NTR</sup> was correlated with reduced phosphorylation of EGFR at 8 and 20 dpi, without changes in total EGFR (Fig. 3A).

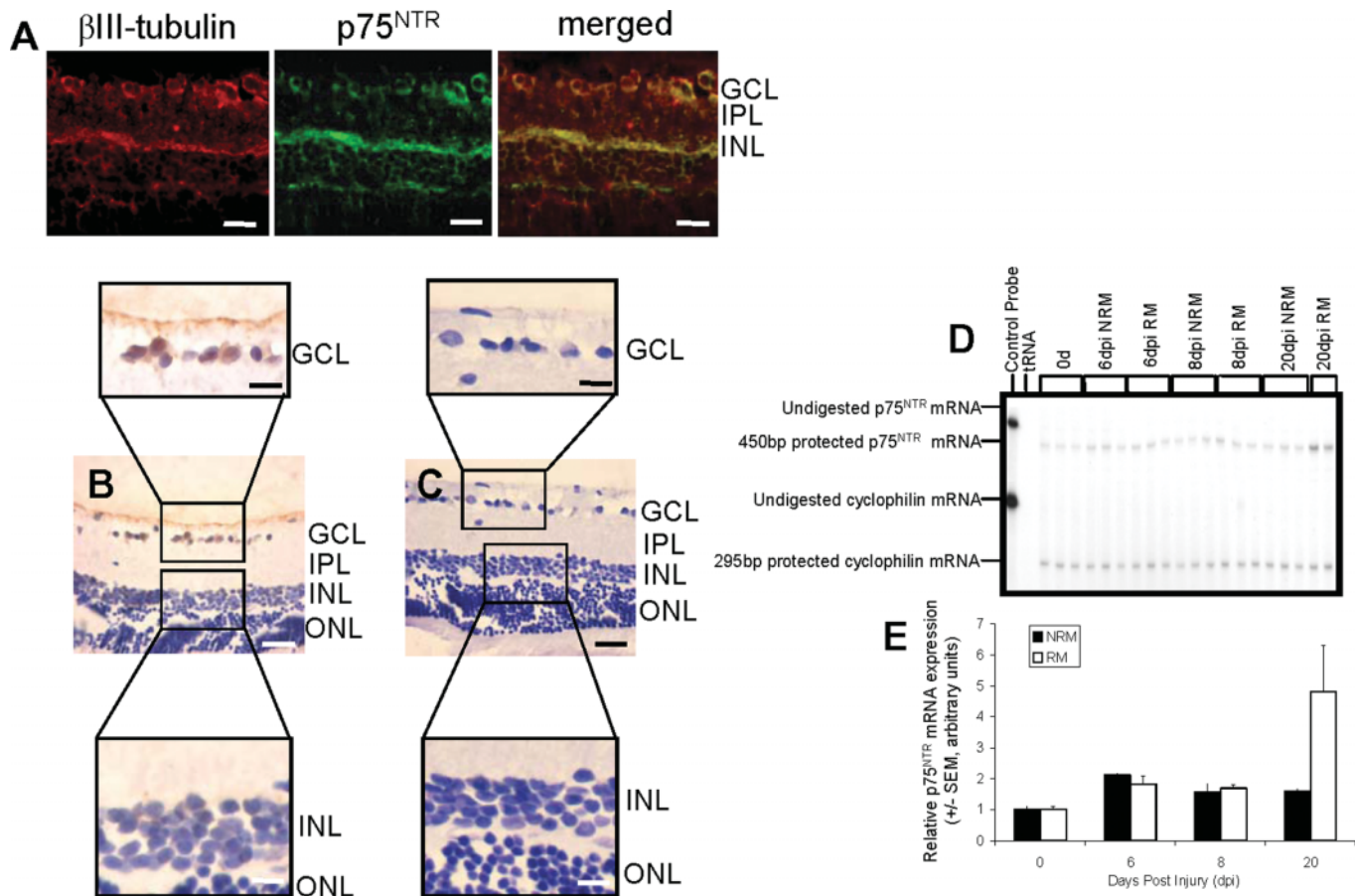
#### Modulation of inhibitory receptors in the RM optic nerve

p75<sup>NTR</sup> mRNA was neither expressed in RM nor NRM optic nerves (Fig. 4A), although at all dpi there were high titres of total p75<sup>NTR</sup> protein compared with intact 0 day controls. Increasing levels of p75<sup>ECD</sup> and p75<sup>ICD</sup> were detected over time in both RM and NRM optic nerves, with higher titres in

the RM (Fig. 4B), which correlated with local cleavage after synthesis of p75<sup>NTR</sup> in RGC somata and anterograde axonal transport into the optic nerve. Rapid cleavage of p75<sup>CTF</sup> may account for the presence of p75<sup>ICD</sup> and absence of p75<sup>CTF</sup> in the optic nerve post-injury. There was also some post-injury proteolysis of TROY, with the appearance of higher levels of a 42-kDa cleaved fragment at 8 and 20 dpi in RM than in NRM optic nerves (Fig. 4B). As in the RM retinæ, disappearance of full-length p75<sup>NTR</sup> was correlated with reduced phosphorylation of EGFR at 8 and 20 dpi, without changes in total EGFR (Fig. 4B).

#### Fragmentation of Nogo-A in RM retina and optic nerve

There was limited cleavage of Nogo-A in RM retinæ (data not shown) but, in the optic nerves, full-length Nogo-A disappeared by 8 dpi, coincident with the appearance of multiple cleaved Nogo-A fragments (Fig. 4B). Elsewhere we have shown that MMP (distinct from TACE) secreted by RGC growth cones/optic nerve glia probably degrade ON Nogo-A (Ahmed *et al.*, 2005a) (see Fig. 2C).



**Fig. 2** Representative double immunolocalization ( $n = 3$ ) of  $\beta$ III-tubulin and p75<sup>NTR</sup> (Promega antibody, directed against p75<sup>ICD</sup>) in the GCL of an intact 0 day control (**A**). *In situ* hybridization also localizes p75<sup>NTR</sup> mRNA to RGC in the GCL (**B**) (**C**, sense control). (Scale bar in **A**, **B** and **C** = 50  $\mu$ m, scale bar in all inserts = 20  $\mu$ m; GCL = ganglion cell layer; IPL = inner plexiform layer; INL = inner nuclear layer; ONL = outer nuclear layer). Representative RNase protection assay of retinal p75<sup>NTR</sup> of intact 0 day control, and RM and NRM retinæ at 6, 8 and 20 dpi (**D**) and (**E**) quantification of p75<sup>NTR</sup> mRNA to show changes in intact 0 day controls, and RM versus NRM retinæ at 6, 8, and 20 dpi.

## TACE activity in vivo and in vitro

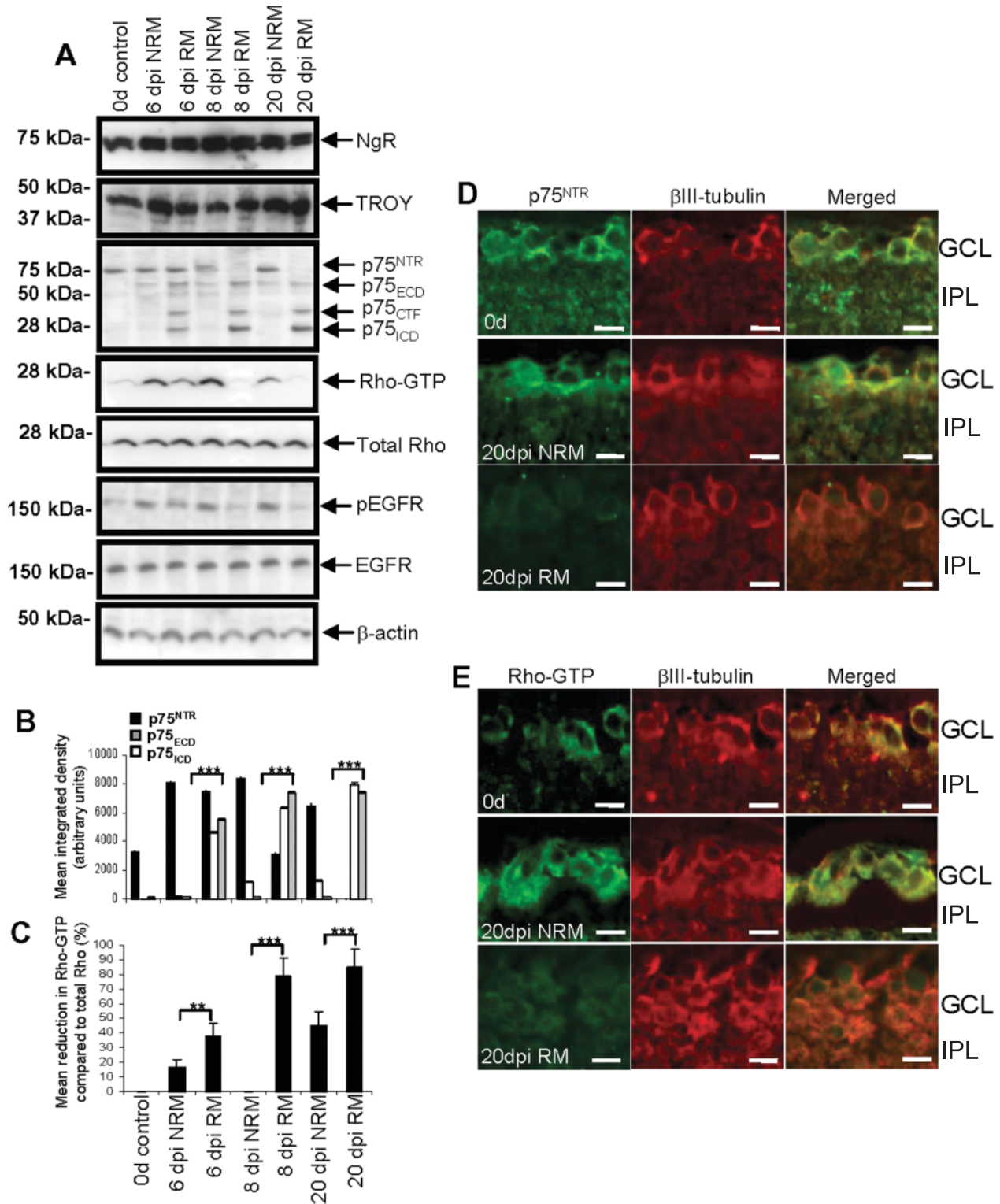
### TACE induces RIP of p75<sup>NTR</sup> in vivo

At all dpi, there was an upregulation and activation of both pro- and active-TACE, and higher levels of presenilin-1 (PS1), a component of  $\gamma$ -secretase, in RM but not NRM retinæ (Fig. 5A and C). At 20 dpi, there were higher levels of TACE immunoreactivity in RM compared with NRM RGC (Fig. 5B) and occasional GAP-43+ axons in the optic nerve (not shown). Levels of pro- and active-TACE rose post-injury in both RM and NRM lesioned optic nerves with the active form predominating in the former (Fig. 5C). There was a small increase in TACE and GAP-43 in RGC of PN no ONC control, with a modest proportional enhancement of p75<sup>NTR</sup> fragmentation, but there was no change in levels of downstream Rho-GTP (not shown). All other controls detailed in the Methods section showed no modulation of these proteins, confirming that inflammatory/macrophage cell-derived factors did not contribute to receptor shedding in RM optic nerves and retinæ (not shown).

### TACE-mediated disinhibition of RGC neurite outgrowth in vitro

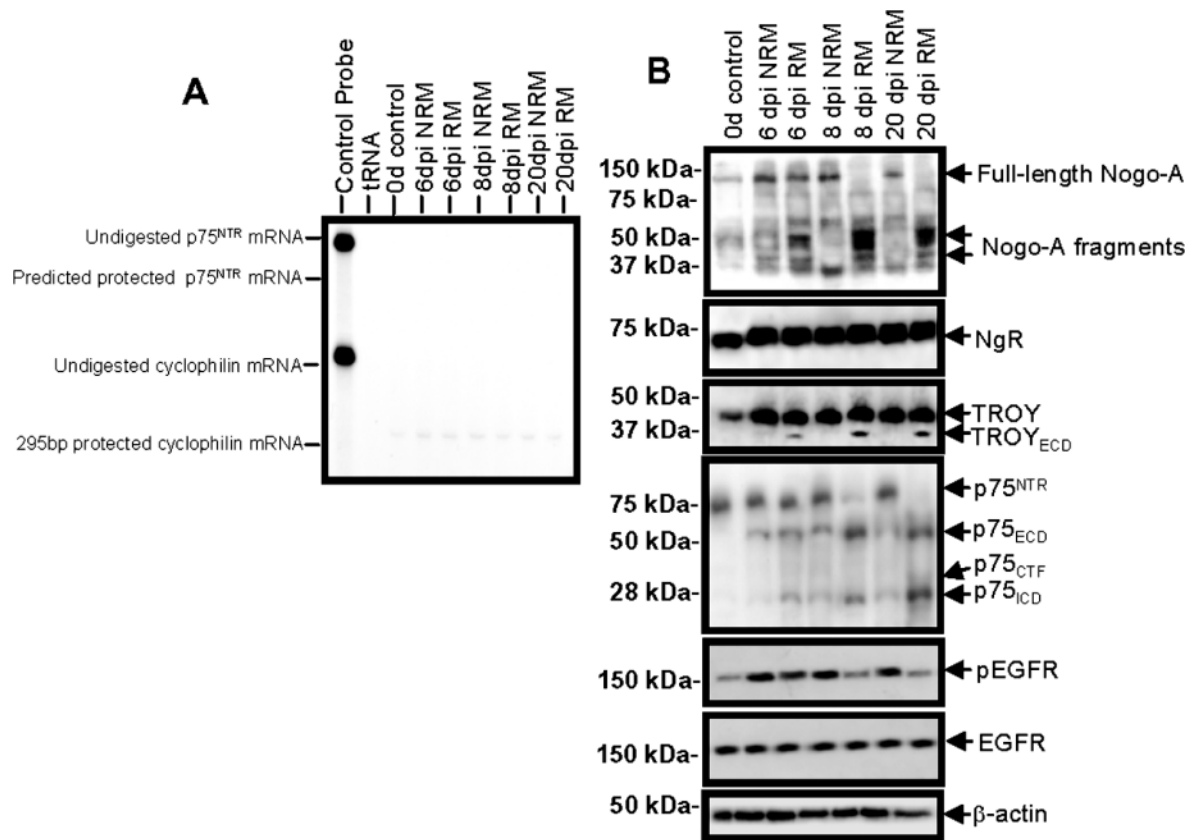
After addition of a combination of pre-optimized concentrations of known Schwann cell-secreted neurotrophic factors, CNTF, BDNF, NT-3 and FGF-2, to adult rat retinal cultures, levels of both active (\*) and pro forms (arrow) of TACE were significantly increased; the former enhanced by 43% over that seen after TACE activator (PMA) treatment ( $P < 0.0001$ ) (Fig. 6A). Addition of the TACE inhibitor TIMP3 (Karan *et al.*, 2003; Black, 2004) abolished active-TACE production in retinal cultures.

We then tested whether the addition of exogenous TACE to adult rat retinal cultures disinhibited RGC neurite outgrowth in the presence of a pre-determined inhibitory concentration of CNS myelin extract by initiating RIP of p75<sup>NTR</sup>, TROY<sup>ECD</sup>/NgR<sup>ECD</sup> shedding and decremating both phosphorylation of EGFR and Rho activation. Treatment with exogenous TACE alone, or with the NTF combination, completely cleaved p75<sup>NTR</sup> into p75<sup>ECD</sup>, p75<sup>CTF</sup> and



**Fig. 3** Proteolytic processing of NgR, p75<sup>NTR</sup> and activation of Rho-A and EGFR in the RM versus NRM retinæ. **(A)** Representative western blots and immunoprecipitation ( $n = 3$ /experiment, three independent experiments) to show levels of NgR, TROY, p75<sup>NTR</sup>, p75<sup>ECD</sup>, p75<sup>CTF</sup>, p75<sup>ICD</sup>, Rho-GTP, total Rho, phosphorylated and total EGFR, and β-actin within samples of pooled retinal protein from 0 day intact controls, and RM and NRM at 6, 8, and 20 dpi. Densitometry of western blots **(B)** to show relative levels of p75<sup>NTR</sup>, p75<sup>ECD</sup> and p75<sup>ICD</sup> levels ( $n = 3$ , three independent experiments;  $**P < 0.001$ ;  $***P < 0.0001$ ). **(C)** Mean changes in Rho-GTP compared with total Rho in intact 0 day controls, and in RM and NRM at 6, 8 and 20 dpi ( $n = 3$ /experiment, three independent experiments;  $**P < 0.001$ ;  $***P < 0.0001$ ). **(D)** Representative double immunohistochemistry of p75<sup>NTR</sup> (green) and βIII-tubulin (red) in retinæ from intact 0 day controls, and from RM and NRM retinæ at 20 dpi. **(E)** Representative *in situ* Rho-GTP (green) and βIII-tubulin (red) in intact 0 day controls, and RM and NRM retinæ at 20 dpi. (For **D** and **E**, scale bar = 10 μm; GCL = ganglion cell layer, IPL = inner plexiform layer).





**Fig. 4** p75<sup>NTR</sup> mRNA and proteolytic processing of p75<sup>NTR</sup>, Nogo-A, NgR and TROY in RM and NRM optic nerves. **(A)** Representative RNAse protection assay ( $n = 3$ ) of p75<sup>NTR</sup> in optic nerves of intact 0 d controls, and of RM and NRM at 6, 8, and 20 dpi. **(B)** Representative western blots ( $n = 3$ ) of Nogo-A, NgR, TROY, p75<sup>NTR</sup>, p75<sub>ECD</sub>, p75<sub>CTF</sub>, p75<sub>ICD</sub>, phosphorylated and total EGFR, and  $\beta$ -actin taken from optic nerves of intact 0 day controls, and from RM and NRM at 6, 8 and 20 dpi.

p75<sub>ICD</sub>, blocked Rho activation, reduced phosphorylation of EGFR (Fig. 6B and C) and enhanced NTF-stimulated RGC neurite outgrowth in the presence of CNS myelin extract (Fig. 7A–D). The numbers of RGC with neurites (Fig. 7B) and mean neurite lengths (Fig. 7C) were increased, although RGC survival was not affected (Fig. 6D). After the addition of the TACE activator, PMA, NTF-stimulated RIP of p75<sup>NTR</sup> in retinal cultures was further potentiated (Fig. 6B and C), and this correlated with the suppression of Rho activation and reduced EGFR phosphorylation. The TACE inhibitor, TIMP3, blocked NTF-induced RIP of p75<sup>NTR</sup> and NgR fragmentation, but did not affect EGFR phosphorylation (Fig. 6B and C). Levels of Rho-GTP and NgR were restored to normal (Fig. 6B and C), and RGC neurite outgrowth was inhibited in the presence of CNS myelin extracts (Fig. 7A–D). The treatment of cultures with NTF and a  $\gamma$ -secretase inhibitor (S2188) elicited p75<sub>ECD</sub> shedding but neither proteolysis of p75<sub>CTF</sub> nor reduced phosphorylation of EGFR and Rho-A activation occurred allowing CNS myelin to inhibit RGC neurite outgrowth (Fig. 6B and C; Fig. 7B and C). Addition of TACE to the NTF/S2188 treatment promoted p75<sup>NTR</sup> fragmentation and p75<sub>ECD</sub> shedding into the medium without generating p75<sub>ICD</sub>, and reduced both phosphorylation of EGFR and Rho activation, leading to RGC neurite

outgrowth equivalent to that seen in the absence of CNS myelin (Figs 6B and C, and 7A and C). Whenever p75<sup>NTR</sup> was cleaved, most p75<sub>ECD</sub> was shed into the medium, although some was retained in the cell fraction probably bound to as-yet-unspecified sites in the plasmalemma.

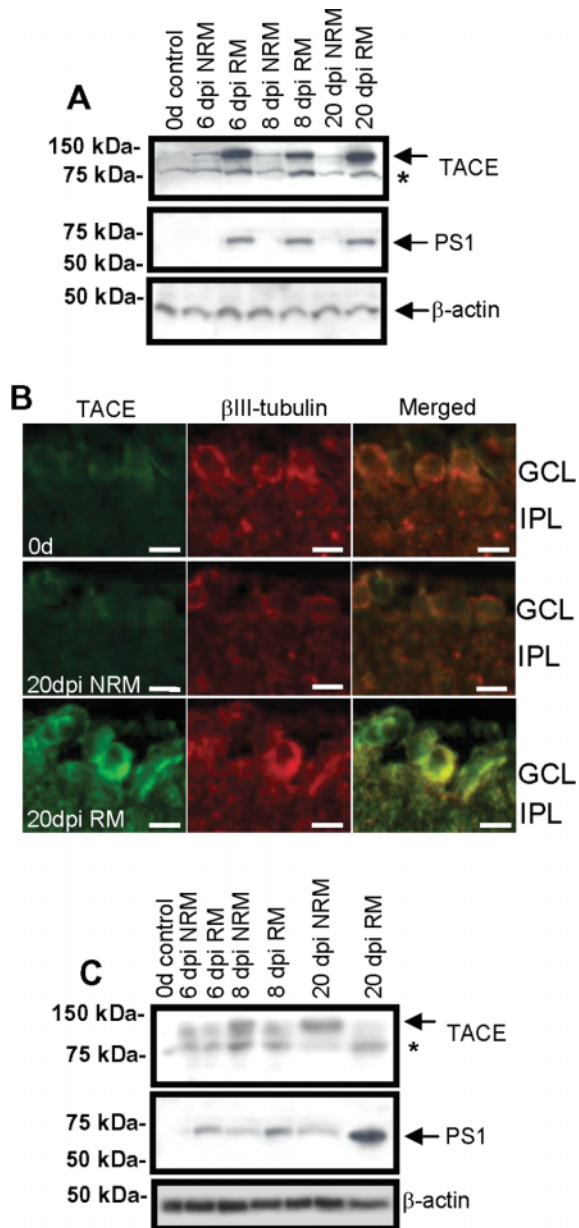
#### Nogo-A fragmentation *in vitro*

TACE did not cleave Nogo-A (Fig. 6B, lane 4) but, when NTF were added to the medium, fragmentation of Nogo-A occurred that was not blocked by the TACE inhibitor TIMP3, indicating that proteolysis was mediated by MMP other than TACE (Fig. 6B, lane 3).

#### siRNA knockdown of p75<sup>NTR</sup> disinhibits CNTF-stimulated RGC neurite outgrowth

In retinal cultures, 100 ng/ml of non-Trk-dependent CNTF, a potent RGC neurotrophic factor known to be expressed by Schwann cells (Stockli *et al.*, 1989; Meyer *et al.*, 1992; Watabe *et al.*, 1995), promoted optimal RGC neurite outgrowth. Inhibition of CNTF-mediated neurite outgrowth was optimal in 10  $\mu$ g/ml of rat CNS myelin extract that contained significant amounts of Nogo-A, OMgp, MAG and CSPG (Ahmed *et al.*, 2005b). In all control retinal cultures, full-length





**Fig. 5** Upregulation of TACE and PSI in retinae and optic nerves in the RM. **(A)** Re-probed representative western blot ( $n = 3$ ) of the same retinal samples shown in Fig. 4 of pro- (arrow) and active-TACE (\*), PS1 and  $\beta$ -actin taken from intact 0 day controls, and from RM and NRM at 6, 8 and 20 dpi. **(B)** Representative double immunolocalization ( $n = 3$ ) of TACE (green) with  $\beta$ III-tubulin (red) in the RGC layer of the retinae of intact 0 day controls, and of retinae from RM and NRM at 20 dpi (scale bar =  $10\mu\text{m}$ ; GCL = ganglion cell layer, IPL = inner plexiform layer). **(C)** Western blot of pro- (arrow) and active-TACE (\*), PS1 and  $\beta$ -actin in optic nerve samples from intact 0 day controls, and from RM and NRM at 6, 8 and 20 dpi, taken from the same optic nerve samples used in Fig. 3A.

$p75^{\text{NTR}}$  and Rho-GTP were detected (Fig. 8A). After delivery of sequences 1–5 of  $p75^{\text{NTR}}$  siRNA, sequence 2 caused 100% knockdown of  $p75^{\text{NTR}}$ , whilst sequences 1 and 3 induced 70–80% knockdown of  $p75^{\text{NTR}}$  (Fig. 8A), as assessed by

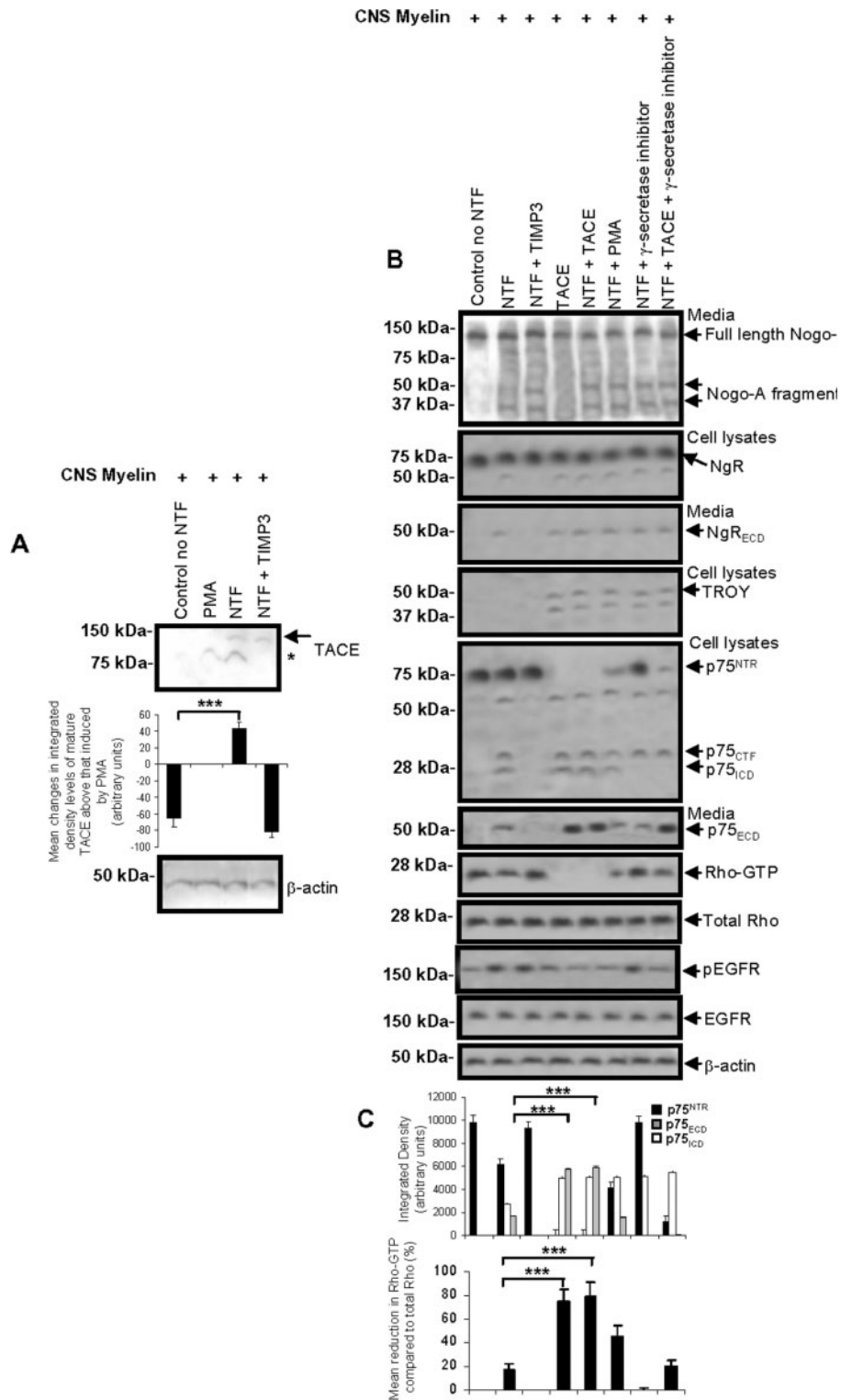
densitometry and when compared with  $p75^{\text{NTR}}$  levels in untransfected cells in the presence of CNS myelin. Rho-GTP, but not total Rho, was absent after treatment with sequences 1, 2 and 3 (Fig. 8A and B). Scrambled control sequence 2 modulated neither  $p75^{\text{NTR}}$  nor Rho-GTP (Fig. 8B).

$p75^{\text{NTR}}$  knockdown and associated suppression of Rho-A activation with sequence 2 were both correlated with a 5-fold increase in the mean number of RGC with neurites [Fig. 8C(i)] and the mean neurite length [Fig. 8C(ii)] in the presence of CNS myelin extract and CNTF, compared with that seen with CNTF alone. Moreover, both down-regulation of  $p75^{\text{NTR}}$  and the subsequent failure in Rho-A activation were correlated with a significant increase in RGC survival after 4 days in culture [Fig. 8C(iii)]. Sequence 2 enhanced RGC neurite outgrowth  $\sim 2$ -fold above that observed in CNTF-stimulated RGC cultured without inhibitory CNS myelin ligands. There was no  $p75^{\text{NTR}}$  immunostaining of RGC somata and neurites transfected with sequence 2, whilst control untransfected RGC and RGC transfected with the corresponding scrambled sequence were  $p75^{\text{NTR}}$  immunostained (Fig. 8D).

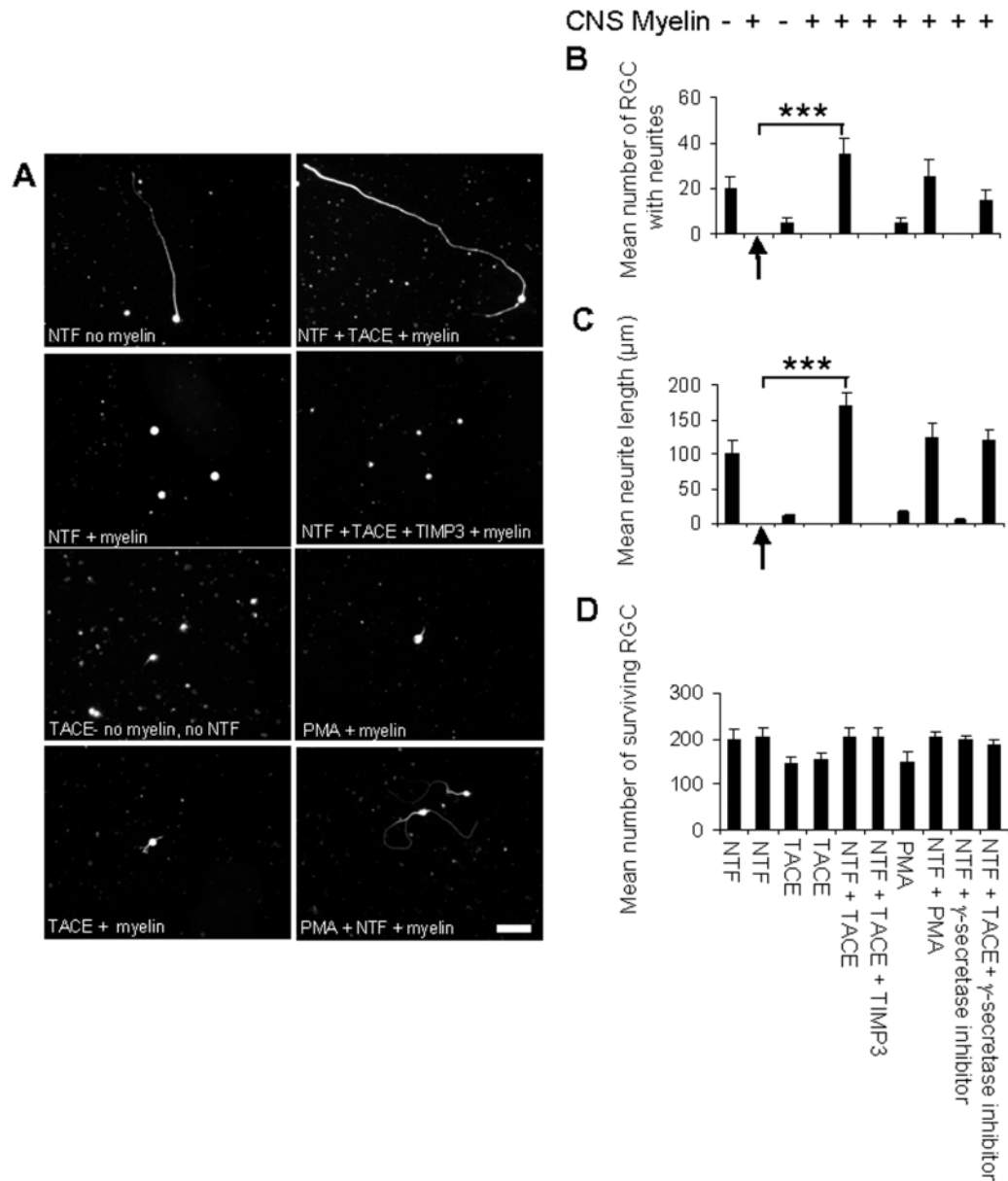
## Discussion

### Neurotrophic activity of intravitreal peripheral nerve implants

Sciatic nerve grafts, containing Schwann cells, implanted either intravitreally or onto the cut end of the optic nerve, support robust RGC axon regeneration (Berry *et al.*, 1996, 1998, 1999). Moreover, after killing all Schwann cells in the sciatic nerve implants using a freeze-thawing cycle, the number of regenerating RGC axons is greatly reduced, confirming that Schwann cells secrete axogenic molecules. Identified Schwann cell-derived NTF include nerve growth factor (NGF) (Assouline *et al.*, 1987; Bandtlow *et al.*, 1987; Matsuoka *et al.*, 1991; Weidner *et al.*, 1999), BDNF (Acheson *et al.*, 1991; Meyer *et al.*, 1992; Funakoshi *et al.*, 1993), NT-3 (Funakoshi *et al.*, 1993; Cai *et al.*, 1998; Meier *et al.*, 1999), neurotrophin-4/5 (NT-4/5; NT-4) (Ming *et al.*, 1999), FGF-2 (Grothe *et al.*, 2001) and CNTF (Stockli *et al.*, 1989; Meyer *et al.*, 1992; Watabe *et al.*, 1995). Logan *et al.* (2006) show that at least three of these NTF in combination (BDNF, FGF-2 and NT-3) synergistically promote RGC axon growth in the severed optic nerve after intravitreal delivery. Since Schwann cells remain viable in intravitreal grafts (Berry *et al.*, 1996), their potential to secrete NTF is conserved for the duration of our *in vivo* experiments. We also have evidence that treatment of cultured RGC with PN-conditioned medium significantly enhances RGC neurite outgrowth and that mAb228 (a blocker of gp130, a component of CNTF and LIF receptors), reverses this neurotogenic effect, demonstrating that a diffusible factor is released from Schwann cells that promotes RGC neurite outgrowth (observations by Lorber *et al.* in our laboratory). The NTF used for our *in vitro* studies were chosen as known components of the Schwann cell NTF repertoire.



**Fig. 6** NTF activate TACE *in vitro*, while addition of TACE to RGC cultures fragments Nogo-A, p75<sup>NTR</sup>, NgR and TROY reduces phosphorylated EGFR and blocks Rho-A activation. Representative western blot and corresponding densitometry (**A**) ( $n = 3$ ) of pro- (arrow) and active-TACE (\*) in control cultures grown in the presence of myelin extract without NTF and from cultures grown in the presence of PMA, NTF or NTF plus TIMP3 (\*\* $P < 0.0001$ ). β-actin acted as a loading control in the same western blot. Representative western blot/immunoprecipitation (**B**) of Nogo-A, NgR, TROY, p75<sup>NTR</sup>, Rho-GTP, and phosphorylated and total EGFR and (**C**) corresponding densitometry ( $n = 3$ , \*\* $P < 0.0001$ ) of p75<sup>NTR</sup>, p75<sup>ICD</sup> from cell lysates and p75<sup>ECD</sup> from supernatant of dissociated retinal cultures grown with and without CNS myelin extracts in the presence of (lane 2) NTF; (lane 3) NTF plus the TACE inhibitor, TIMP3; (lane 4) active-TACE; (lane 5) NTF plus active TACE; (lane 6) NTF plus the TACE activator, PMA; (lane 7) NTF plus γ-secretase inhibitor (S1288); and (lane 8) NTF plus TACE plus the γ-secretase inhibitor, S1288. β-actin acted as a loading control.



**Fig. 7** Addition of TACE enhances NTF RGC neurite outgrowth in the presence of inhibitory CNS myelin extract. Representative immunocytochemistry for  $\beta$ III-tubulin in retinal cultures to demonstrate RGC neurite outgrowth in the presence and absence of NTF, TACE, CNS myelin extract and PMA (A). (B) Quantification of the mean number of RGC with neurites; (C) mean neurite lengths; and (D) mean number of surviving RGC after treatment with NTF with no added CNS myelin extract, NTF plus CNS myelin extract, TACE with no CNS myelin extract, TACE plus CNS myelin extract, NTF plus TACE plus CNS myelin extract, NTF plus TACE plus TIMP3 plus CNS myelin extract (\*\* $P < 0.0001$ ; scale bar = 50  $\mu$ m). NTF = CNTF/BDNF/NT-3/FGF-2. Note: in this experiment myelin was added at titres that completely suppress NTF-stimulated neurite outgrowth (arrow).

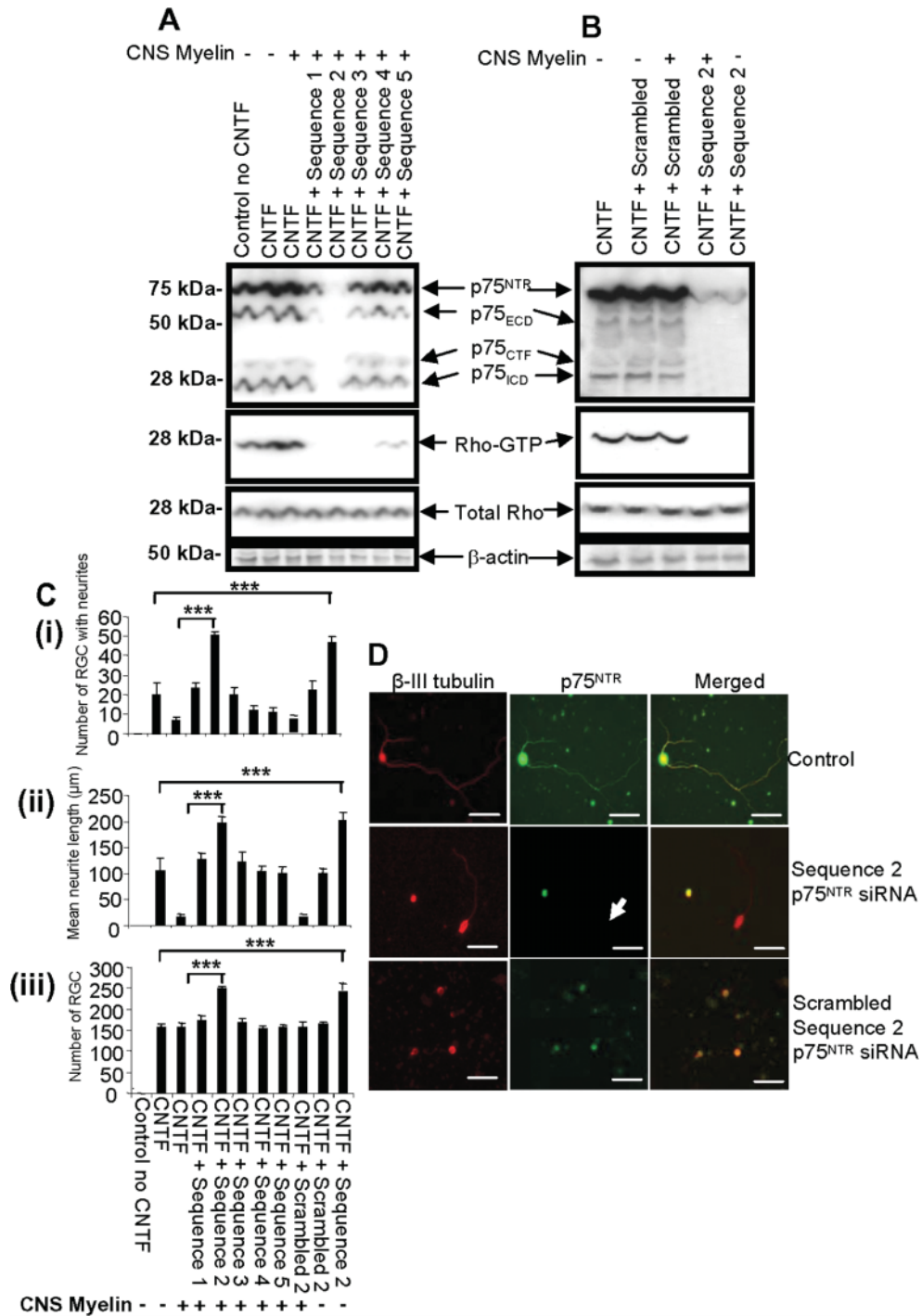
The axiogenic efficacy of intravitreal PN grafting is confirmed in this series of experiments, with enhanced numbers of GAP-43+ RGC axons visible in optic nerve sections and increased levels of GAP-43 protein measured in optic nerve extracts after ONC in the RM. Like Fischer *et al.* (2001) and Leon *et al.* (2002) (who used the same injury model, but promoted regeneration via macrophage/lens-derived factors), we also observe GAP-43 expression in control animals without optic nerve transection but with intravitreal PN implants (PN no ONC controls). These observations suggest that

Schwann cell-derived factors from the PN implant promote a state of growth readiness in RGC.

### Paradoxical regeneration of RGC axons in the inhibitory environment of the optic nerve

In this study, we investigate the paradox that Schwann cell-derived factors stimulate RGC axons to regenerate through the inhibitory myelin-rich distal segment of the transected





**Fig. 8** siRNA-mediated knockdown of p75<sup>NTR</sup> blocks Rho-A activation and promotes RGC neurite outgrowth in the presence of CNS myelin extract. **(A)** Representative western blot/immunoprecipitation ( $n = 3$ ) of p75<sup>NTR</sup>, p75<sup>ECD</sup>, p75<sup>ICD</sup>, Rho-GTP, total Rho and β-actin after siRNA-mediated silencing of p75<sup>NTR</sup> from control retinal cultures without CNS myelin extract, and cultures grown in the presence of CNTF without CNS myelin, and CNTF plus sequences 1–5 of p75<sup>NTR</sup> siRNA separately in the presence of CNS myelin extract. **(B)** Western blot/immunoprecipitation ( $n = 3$ ) of p75<sup>NTR</sup>, p75<sup>ECD</sup>, p75<sup>ICD</sup>, Rho-GTP, total Rho and β-actin after siRNA-mediated silencing of p75<sup>NTR</sup> from control cultures without CNS myelin extract; grown in the presence of CNTF alone; CNTF plus either scrambled sequence 2, or sequence 2 of p75<sup>NTR</sup> siRNA without CNS myelin extract; and either scrambled sequence 2 or sequence 2 of p75<sup>NTR</sup> siRNA with CNS myelin extract. **(C)** Quantification of (i) mean number of RGC with neurites, (ii) mean RGC neurite length, and (iii) mean number of RGC in control retinal cultures, and in retinal cultures treated with and without CNTF with no added CNS myelin, and CNTF with sequence 2, and scrambled sequence 2 of p75<sup>NTR</sup> siRNA without myelin extract, and CNTF with sequences 1–5 of p75<sup>NTR</sup> siRNA in the presence of CNS myelin extract ( $***P < 0.0001$ ). **(D)** Double immunocytochemistry to show βIII-tubulin<sup>+</sup> RGC (red) co-stained with p75<sup>NTR</sup> (green) in control retinal cultures (CNTF without CNS myelin extract), and retinal cultures treated with sequence 2, and scrambled sequence 2 of p75<sup>NTR</sup> siRNA with CNS myelin extract (scale bars = 50 μm).

optic nerve, and show that optic nerve regeneration correlates with RIP of p75<sup>NTR</sup>, NgR and TROY ectodomain shedding, suppression of EGFR phosphorylation and blockade of CNS myelin-induced Rho-A activation. RIP of p75<sup>NTR</sup> also occurs *in vitro* after the addition of exogenous TACE, which disinhibits NTF-stimulated RGC neurite outgrowth in the presence of inhibitory CNS myelin. Significant disinhibition of NTF-stimulated RGC neurite outgrowth in the presence of inhibitory CNS myelin is promoted by siRNA-mediated ablation of p75<sup>NTR</sup>. The paradox may, therefore, be explained if Schwann cell-derived factors induce  $\alpha$ - and  $\gamma$ -secretase activity in RGC that cleave p75<sup>NTR</sup> in RGC somata and growth cones, whilst additional MMP released from RGC axons and their surrounding glia in the optic nerve cleave Nogo-A (see also Ahmed *et al.*, 2005a), paralyzing inhibitory signalling through the p75<sup>NTR</sup>/NgR/Lingo-1 complex. The suppression of EGFR activation is probably effected after RIP of p75<sup>NTR</sup> and p75<sup>NTR</sup>/TROY/NgR<sub>ECD</sub> shedding and consequent attenuation of Ca<sup>2+</sup> influx. Growth cone collapse is thus suppressed by inactivation of both EGFR and Rho-A signalling, and RGC axon regeneration is disinhibited through the optic nerve. Schwann cell-derived factors stimulate axon regeneration in RM optic nerves and induce fragmentation of Nogo-A (and possibly other myelin-derived inhibitors) through increased MMP and decreased TIMP activity in the optic nerve, which also accounts for the absence of scarring in these animals (Berry *et al.*, 1996, 1999; Ahmed *et al.*, 2005a; Logan *et al.*, 2006). Not all RGC express p75<sup>NTR</sup>, although most express TROY, both *in vivo* and *in vitro*. The *in vitro* observation that ~20% RGC grow neurites in the presence of the CNTF/BDNF/NT-3/FGF-2 combination suggests that p75<sup>NTR</sup> and TROY are differentially active in RGC and that other factors determine RGC axon regenerative potential.

### Schwann cell-derived factor-induced RIP of p75<sup>NTR</sup>

In the optic nerve, significant amounts of p75<sup>NTR</sup> protein are present, although p75<sup>NTR</sup> mRNA is not detected, suggesting that the latter is manufactured in RGC somata and anterogradely transported in their axons along the optic nerve. In both RM retinae and optic nerves, the levels of p75<sub>ECD</sub>, p75<sub>CTF</sub> and p75<sub>ICD</sub> are inversely proportional to those of full-length p75<sup>NTR</sup>, supporting the conclusion that these fragments are products of p75<sup>NTR</sup> RIP. p75<sup>NTR</sup> cleavage correlates with enhanced production and activation of  $\alpha$ - (TACE) and  $\gamma$ -secretase (PS1), both of which are required for RIP of p75<sup>NTR</sup> (Jung *et al.*, 2003; Kanning *et al.*, 2003). In support of our *in vivo* observations of both ectodomain shedding and RIP of inhibitory receptors, RGC upregulated levels of pro- and active-TACE *in vitro* in response to combined CNTF/BDNF/NT-3/FGF-2 treatment. No p75<sub>ECD</sub> shedding occurs in response to either single NGF and BDNF administration, or after pro-NTF binding (Jung *et al.*, 2003; Kanning *et al.*, 2003) but, in our hands, stimulation with

combined CNTF/BDNF/NT-3/FGF-2 upregulated RGC TACE production *in vitro*, leading to RIP of p75<sup>NTR</sup>. We have already reported that treatments with other combinations of NTF produce synergistic RGC survival and axonal growth together with RIP of p75<sup>NTR</sup> (Logan *et al.*, 2006).

Astrocytes and endothelial cells constitutively express TACE in the intact CNS (Goddard *et al.*, 2001), and our results localize pro- and active-TACE to RGC and optic nerve glia. Numerous integral membrane proteins (Peschon *et al.*, 1998; Mullberg *et al.*, 2000) are proteolytically cleaved near their membrane insertion to liberate soluble ECD into the pericellular space (Ehlers and Riordan, 1991; Hooper *et al.*, 1997; Baumann and Frank, 2002). The shed soluble p75<sub>ECD</sub> may act as a p75<sup>NTR</sup> signalling antagonist by competing with full-length p75<sup>NTR</sup> and/or TROY for NgR/Lingo-1 binding, thereby potentiating the disinhibition of axon regeneration already promoted by RIP-mediated paralysis of p75<sup>NTR</sup> inhibitory transduction.

p75<sub>ICD</sub> has several potential downstream targets, including NF- $\kappa$ B that mediates gene transcription (Kanning *et al.*, 2003) regulated neuronal apoptosis (Barrett, 2000; Yamashita *et al.*, 2002). Many proteins with nuclear functions interact with the p75<sub>ICD</sub> (Roux and Barker, 2002), including the p75<sup>NTR</sup>-associated cell death executor (NADE), which signals survival/apoptosis in the presence of NTF (Mukai *et al.*, 2000). The activation of apoptosis may account for the 80–90% RGC death observed after optic nerve transection, even after intravitreal PN implantation (Berry *et al.*, 1996, 1999). In developing neurons of p75<sub>ICD</sub> over-expressing transgenic mice, p75<sub>ICD</sub> induces extensive neuronal apoptosis (Majdan *et al.*, 1997), and pro-apoptotic signalling of p75<sub>ICD</sub> is also observed in cultured cells (Coulson *et al.*, 2000; Roux and Barker, 2002). However, in the RM, PN-derived NTF do not enhance apoptosis, since significantly greater RGC survival is observed in RM compared with NRM (Berry *et al.*, 1996, 1999). In this study, the same protective effect of NTF on RGC survival was observed *in vitro*, even in the presence of TACE, suggesting that unidentified NTF-activated factors may limit p75<sub>ICD</sub>-mediated apoptosis.

### Suppression of Rho-A activation

Suppressed activation of Rho-A occurs after RIP of p75<sup>NTR</sup>. The release of Rho-GDP from Rho-GDP dissociation inhibitor (Rho-GDI) allows the GDP-bound form to be activated by guanine nucleotide exchange factors (GEF) probably by direct interaction of Rho-GDI with p75<sup>NTR</sup>, an event that liberates Rho-GDP for conversion into its active form (Yamashita *et al.*, 2002; Yamashita and Tohyama, 2003). It is probable that RIP of p75<sup>NTR</sup> perturbs the interaction of Rho-GDI with p75<sup>NTR</sup>, thereby preventing Rho-A activation through a failure of Rho-GDP/Rho-GDI dissociation. Accordingly, NTF may stimulate RGC axon regeneration after intravitreal sciatic nerve implantation by promoting actin polymerization in RGC growth cones after paralysis

of p75<sup>NTR</sup> inhibitory signalling (Amano *et al.*, 2000; Tang, 2003).

Our results conflict with those of a recent *in vitro* study that suggests that the induction of RIP of p75<sup>NTR</sup> is a prerequisite for inhibitory Rho-A signalling in immature cerebellar granule cells co-cultured with MAG Fc expressing CHO cells (Domeniconi *et al.*, 2005). In contrast, we show both *in vivo* and in primary adult retinal cultures that p75<sup>NTR</sup> fragmentation disinhibits CNS axon regeneration/neurite outgrowth in the presence of inhibitory CNS myelin. Using the same *in vivo* adult rat optic nerve injury model used here, Logan *et al.* (2006) intravitreally implanted engineered fibroblasts expressing single and combined NTF genes and demonstrated that RIP of p75<sup>NTR</sup> correlate with suppressed Rho activation and enhanced RGC axon regeneration. Furthermore, *in vitro* combinations of NTF proteins promote RGC neurite outgrowth in the presence of inhibitory CNS myelin, which also correlates with both RIP of p75<sup>NTR</sup> and reduced Rho activation (Logan *et al.*, 2006). In the current study, we show that Schwann cell-derived factors stimulate the secretion and secretion of TACE (known to activate RIP of p75<sup>NTR</sup>), and the exogenous addition of TACE to RGC cultures both stimulates RIP of p75<sup>NTR</sup> and reduces Rho activation, enhancing RGC neurite outgrowth in the presence of inhibitory CNS myelin. Blocking EGFR phosphorylation disinhibits axon growth mediated by CNS myelin and CSPG (Koprivica *et al.*, 2005). In our *in vivo* and *in vitro* experiments, EGFR phosphorylation is also blocked after RIP of p75<sup>NTR</sup>, disinhibiting RGC axon regeneration/neurite outgrowth in the presence of CNS myelin. In all of the experiments where we have demonstrated that RIP of p75<sup>NTR</sup> is associated with NTF-enhanced axon/neurite outgrowth, we have utilized physiological *in vivo* and *in vitro* models relevant to the phenomena of adult CNS injury that have a number of key differences to the Domeniconi *et al.* (2005) *in vitro* paradigms, including (i) *in vivo* activation of p75<sup>NTR</sup> RIP in NTF-stimulated adult CNS neurons; (ii) induction of adult neurite growth inhibition in cultured primary adult neurons by adding CNS myelin extracts [containing Nogo-A, MAG, OMgp and CSPG (Ahmed *et al.*, 2005b)] directly to media, rather than by the exclusive *in vitro* use of MAG Fc expressing CHO cells immature CNS neurons; and (iii) addition of NTF that potentiates RIP of p75<sup>NTR</sup> in cultured adult RGC; and (iv) reciprocal co-ordination of p75<sup>NTR</sup> and its fragments (p75<sup>ECD</sup> and p75<sup>ICD</sup>) (Logan *et al.*, 2006).

### Schwann cell-derived factor/NTF-induced TROY/NGR/EGFR modulation

TROY, a close relative of p75<sup>NTR</sup>, signals via Lingo-1 and NGR through the Rho-A pathway in the absence of p75<sup>NTR</sup> (Park *et al.*, 2005; Shao *et al.*, 2005). There is limited fragmentation of TROY at 8 and 20 dpi in both RM and NRM retinæ and optic nerves, possibly mediated by shedding mechanisms similar to RIP, with increased levels of the shed fragment

in the RM compared with the NRM. However, as knockdown of p75<sup>NTR</sup> abolishes Rho-GTP in the presence of significant levels of intact TROY, the myelin-derived factors do not seem to be actively signalling inhibition of RGC axon regeneration through TROY-activated Rho-GTP. Hence the role of TROY in RGC remains an enigma.

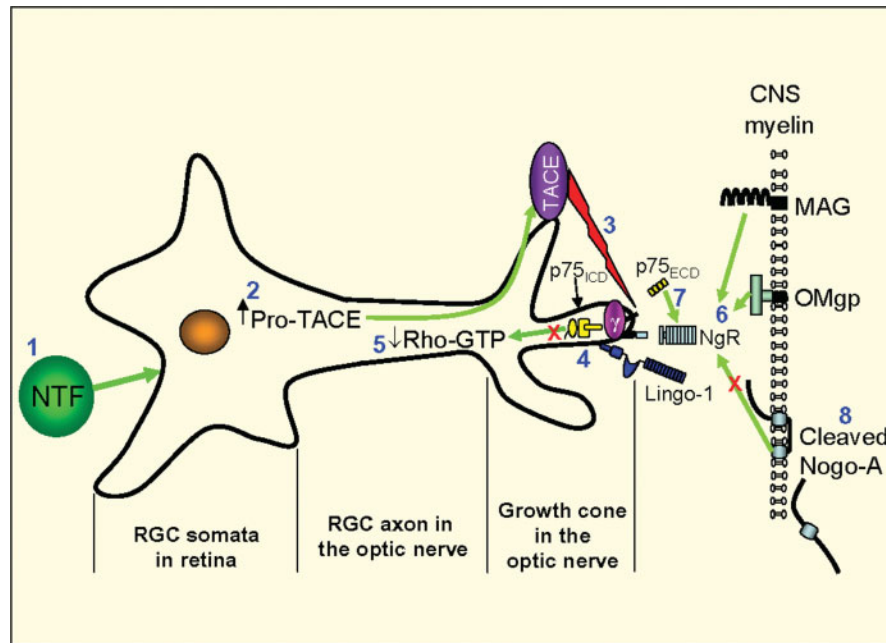
Consistent with other reports (reviewed by Hunt *et al.*, 2002 and Sandvig *et al.*, 2004), we confirm that although levels of NGR rise post-injury levels of full-length NGR are little modulated during RGC axon regeneration after intravitreal sciatic nerve implantation, but NGR<sub>ECD</sub> is shed after TACE administration, when the shed fragment may act as a dominant-negative signalling antagonist by binding all CNS myelin-derived ligands (Walmsley *et al.*, 2004, 2005).

The addition of TACE to NTF-stimulated cultured RGC activates RIP of p75<sup>NTR</sup> and NGR<sub>ECD</sub> shedding, blocks Rho-A activation, reduces EGFR phosphorylation and enhances NTF-stimulated RGC neurite outgrowth in the presence of inhibitory CNS myelin. CNS myelin inhibition is also mediated by NGR-dependent activation of EGFR (Koprivica *et al.*, 2005). Dysfunction of the NGR/p75<sup>NTR</sup> complex after RIP and NGR<sub>ECD</sub> shedding attenuates EGFR phosphorylation and potentiates disinhibition of RGC axon growth. TACE-initiated RIP of p75<sup>NTR</sup> in the absence of NTF does not promote significant neurite outgrowth of RGC cultured with CNS myelin, suggesting that priming neurons to 'growth readiness' together with the removal/neutralization of inhibitory molecules is required to maximize neurite/axon outgrowth, possibly explaining the minimal axon growth seen by others when disinhibition strategies are used without concomitant NTF treatment (Fischer *et al.*, 2004; Steinmetz *et al.*, 2005). In PN no ONC controls, increased GAP-43 expression correlates with a corresponding increase in TACE production and RIP of p75<sup>NTR</sup>. However, other optic nerve lesioned controls without intravitreal PN implantation show no modulation of either p75<sup>NTR</sup> or TACE, ruling out the possibility that inflammatory cell-derived factors (Yin *et al.*, 2003) contribute significantly to receptor shedding.

### siRNA knockdown of p75<sup>NTR</sup> mRNA

Since the processed forms of p75<sup>NTR</sup>, including p75<sup>ICD</sup>, potentially retain signalling activity *in vivo* (Kanning *et al.*, 2003), we investigated whether the complete removal of p75<sup>NTR</sup> by siRNA *in vitro* enhanced CNTF-stimulated neurite outgrowth of RGC grown with CNS myelin. Levels of p75<sup>NTR</sup> protein, and downstream Rho-A activation, are completely suppressed by p75<sup>NTR</sup> siRNA (sequence 2) in retinal cultures containing CNS myelin ligands. After p75<sup>NTR</sup> silencing, the neurite outgrowth seen in response to CNTF in the presence of CNS myelin is significantly enhanced above that seen with CNTF in the absence of myelin. Disinhibited CNTF-stimulated neurite outgrowth rose 2-fold after siRNA treatment, suggesting that the potency of CNTF is normally held in check by





**Fig. 9** Proposed mechanism of disinhibition of RGC axon growth by NTF-stimulated RIP of  $p75^{\text{NTR}}$  and MMP secretion. (1) NTF released from Schwann cells in intravitreal PN grafts stimulate the production of pro-TACE in RGC somata; (2) pro-TACE is anterogradely transported in RGC axons to growth cones in the optic nerve; (3) active TACE ( $\alpha$ -secretase) cleaves  $p75^{\text{ECD}}$  from  $p75^{\text{NTR}}$  leaving  $p75^{\text{CTF}}$  in the cytosol; (4)  $\gamma$ -secretase cleaves  $p75^{\text{ICD}}$  from  $p75^{\text{CTF}}$ ; and (5) cytosolic  $p75^{\text{ICD}}$  somehow blocks activation of Rho-GDP. Disinhibition is potentiated by (6) shedding of the  $\text{NgR}^{\text{ECD}}$  that acts as a soluble dominant-negative inhibitory signalling antagonist binding all myelin-derived ligands; (7) competition for  $\text{NgR}$  binding between shed soluble  $p75^{\text{ECD}}$  and full-length  $75^{\text{NTR}}$ ; and (8) blocking of inhibitory signalling by multiple soluble Nogo-A fragments, probably produced by MMP secreted from RGC axon growth cones/optic nerve glia. A role for TROY in disinhibition is yet to be determined. In our RM, disinhibition is potentiated by suppressed phosphorylation of EGFR secondary to  $\text{NgR}/p75^{\text{NTR}}$  inactivation and reduced  $\text{Ca}^{2+}$  influx.

other non-myelin-derived inhibitory ligands signalling through the Rho-A pathway, which might include CSPG, ephrins, tenascins and semaphorins (Hunt *et al.*, 2002; Sandvig *et al.*, 2004). Removal of  $p75^{\text{NTR}}$  by siRNA significantly promotes RGC survival *in vitro*, probably because  $p75^{\text{ICD}}$  apoptotic signalling is prevented. In the absence of a functional  $\text{NgR}/p75^{\text{NTR}}$  complex,  $p75^{\text{ECD}}$  no longer acts as a  $p75^{\text{NTR}}$  competitive signalling antagonist, although inactivation of EGFR might have been predicted, leading to enhanced disinhibition, since EGFR-mediated inhibition is  $\text{NgR}$ -dependent (Koprivica *et al.*, 2005).

In support of our *in vitro* observations using siRNA, transgenic mice over-expressing NGF grow significant numbers of sympathetic axons within myelinated portions of the maturing and adult brain, and their growth responsiveness to NGF is enhanced if  $p75^{\text{NTR}}$  is simultaneously knocked out (Walsh *et al.*, 1999). This suggests that NTF-stimulated growth of CNS axons into myelin-rich areas is facilitated by the same NTF-induced RIP of  $p75^{\text{NTR}}$  observed by us in the present experiments.

## Summary

We provide evidence for a novel model of Schwann cell factor-/NTF-stimulated optic nerve regeneration that proposes that RIP of  $p75^{\text{NTR}}$  and  $p75^{\text{NTR}}/\text{TROY}/\text{NgR}^{\text{ECD}}$  shedding blocks inhibitory signalling through suppressed Rho-A

activation and reduced EGFR phosphorylation in RGC, promoting actin polymerization in axonal growth cones and disinhibiting regeneration through the inhibitory environment of the optic nerve. Disinhibition is further potentiated by (i) fragmentation of Nogo-A, (ii) shedding of  $\text{NgR}^{\text{ECD}}$ , which acts as a dominant-negative signalling antagonist by binding all myelin-derived ligands, and (iii) competition between  $p75^{\text{ECD}}$  and full-length  $p75^{\text{NTR}}$  for  $\text{NgR}$  binding (Fig. 9). The resulting dysfunction of the  $\text{NgR}/p75^{\text{NTR}}$  complex inactivates EGFR, disinhibiting CNS axon growth further, since CNS myelin/CSPG inhibition mediated by EGFR requires upstream  $\text{NgR}$  activation and  $\text{Ca}^{2+}$ -influx.

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