Neurotrophic factor synergy is required for neuronal survival and disinhibited axon regeneration after CNS injury

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The therapeutic effects of individual neurotrophic factors (NTF) have proved disappointing in clinical trials for neuronal repair and axon regeneration. Here, we demonstrate NTF synergistic neuronal responses after a combination of basic fibroblast growth factor, neurotrophin-3 and brain derived growth factor delivered to the somata of retinal ganglion cells promoted greater survival and axon growth than did the sum of the effects of each NTF alone. Triple and not single NTF treatments potentiated regulated intramembraneous proteolysis of p75NTR, and ectodomain shedding of Nogo receptor, correlated with a 30% decrease in activation of Rho-A, a key signalling molecule in the axon growth inhibitory cascade. Thus, combinatorial NTF administration synergistically enhanced neuronal survival, disinhibited axon growth and promoted axon regeneration through the hostile CNS environment without the intervention of scar tissue at the lesion site.

Keywords: Neurotrophic factors; regulated intramembrane proteolysis; optic nerve regeneration; p75NTR; CNS

Abbreviations: BDNF = brain derived neurotrophic factor; FGF2 = fibroblast growth factor; NT-3 = neurotrophin-3; NgR = Nogo receptor

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Introduction

Multiple diffusible neurotrophic factors (NTF) acting together promote axon outgrowth during maturation of the nervous system (Huang and Reichardt, 2001), whilst single recombinant NTF delivered into the injured adult spinal cord (Zhou et al., 2003) stimulate only limited axonal regeneration, and growth beyond the wound is rarely seen (Tuszynski et al., 1996; Ramer et al., 2002; Lu et al., 2004). Moreover, difficulties in NTF delivery and vagaries of their CNS pharmacokinetics have meant that the therapeutic potential of NTF for repair of damaged and degenerating CNS neurons has not been realized in clinical trials (Dawbarn and Allen, 2003). The efficacy of these strategies might be limited if successful CNS axon regeneration requires not only the administration of appropriate combinations of NTF (Lu et al., 2004), but also the blockade of endogenous axon growth inhibitory molecules (Hunt et al., 2002; Sandvig et al., 2004) and a neural anti-apoptotic therapy (Cho et al., 2005). Like other CNS neurons, adult retinal ganglion cells (RGC) normally fail to regenerate their axons after injury and ultimately apoptose. Paradoxically, however, transected RGC axons regenerate in vivo through the putative inhibitory environment of the scar and distal optic nerve stump and chiasm after either intravitreal peripheral nerve implantation (Berry et al., 1996, 1998) or lens injury (Leon et al., 2000; Fischer et al., 2001; Yin et al., 2003; Lorber et al., 2002, 2004, 2005). The peripheral nerve implant probably releases multiple NTF into the vitreous body of the eye promoting RGC survival and axon regeneration (Lorber et al., 2005). Peripheral nerve implantation also modulates the secretion and activity of matrix metalloproteases (MMP) by RGC axons and the surrounding optic nerve glia, which also secrete inhibitors of MMP (TIMP), leading to the dissolution of the incipient scar matrix, and unimpeded RGC axon regeneration through the inhibitory environment of the wound and beyond (Ahmed et al., 2005a).

CNS myelin contains many axon growth inhibitory molecules, including myelin-associated glycoprotein (MAG)
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(McKerracher et al., 1994; Mukhopadhyay et al., 1994; Liu et al., 2002), oligodendrocyte myelin glycoprotein (OMgp) (Wang et al., 2002b), chondroitin sulphate proteoglycans (CSPG) (Niederost et al., 1999) and Nogo (Chen et al., 2000; Prinjha et al., 2000; Fournier et al., 2001) that induce growth cone collapse by binding to the Nogo receptor (NgR) which associates with LINGO-1 and signalling receptors, including the low-affinity neurotrophin receptor p75NTR (Wong et al., 2002; Wang et al., 2002a; Mi et al., 2004), and TROY (Shao et al., 2005; Park et al., 2005). This interaction transduces inhibitory signals by activating downstream Rho-A, leading to sequential ROCK/LIM kinase/cofilin-mediated actin filament depolymerization and growth cone collapse (Wong et al., 2002; Wang et al., 2002a; Mi et al., 2004).

In other scenarios, tumour necrosis factor-α converting enzyme (TACE)-mediated cleavage of the extracellular domain (ECD) is a pre-requisite for regulated intramembranous proteolysis (RIP) of p75NTR, an event which triggers secondary cleavage within the transmembrane domain by a presenilin-containing protease complex (γ-secretase), so that a 55 kDa extracellular domain (p75ECD) and a 32 kDa cytoplasmic transitional fragment (p75CTF) are generated, and from the latter the 25 kDa intracellular domain (p75ICD) is cleaved (Jung et al., 2003; Kanning et al., 2003; Landman and Kim, 2004; Weskamp et al., 2004). Although the levels of p75ECD produced by RIP are tightly controlled by rapid removal via proteosomal degradation, p75ECD can traffic to the nucleus and activate apoptosis via the transcription factor NFκB (Kanning et al., 2003). The main function of RIP may be the disassembly of p75NTR and removal of the transmembrane domain, abolishing interaction with Trk receptors (Landman and Kim, 2004).

Whilst Domeniconi et al. (2005) suggest that RIP is required for MAG-induced inhibitory signalling, our recent work suggests that RIP of p75NTR acts to block inhibitory signalling, thereby disinhibiting CNS axon regeneration through inhibitory environments (Z. Ahmed, E. L. Suggate, E. R. Brown, R. G. Dent, S. J. Armstrong, L. B. Barrett, M. Berry and A. Logan, unpublished data). TACE, an α-secretase, also cleaves the ectodomain (ECD) of NgR, which prevents its association to p75NTR and has the potential to competitively bind all CNS myelin-derived inhibitory ligands, thus further antagonizing NgR signalling (Walsmsley et al., 2004, 2005).

Here we define the differential effects of NTF combinations and single applications on neuronal signal transducing pathways for survival and axon regeneration. For example, we have established that a combination of genes encoding neurotrophin-3 (NT-3), basic fibroblast growth factor (FGF2) and brain derived neurotrophic factor (BDNF), when delivered to an optic nerve lesion site (Berry et al., 1999), promotes significant and sustained survival of RGC after uptake, retrograde axonal transport and transcription of NTF (Berry et al., 2001b). In this paper, we show that NT-3, FGF2 and BDNF proteins, delivered to RGC somata by intravitreally implanted NTF-transfected fibroblasts, have synergistic, not additive effects on both RGC survival and axon regeneration so that, in combination, the sum of their influences is greater than would be predicted from their individual activities. In vitro, combinations of NTF, and not individual NTF, potentiate RGC TACE and γ-secretase activity triggering NgR shedding and RIP of p75NTR, suppress Rho activation and promote RGC neurite/axon regeneration by rendering growth cones insensitive to environmental axon growth inhibitory ligands.

Methods
Experimental design

In vivo experiments. Two groups of female adult Fischer (200–250 g) rats were used at 20 and 50 days after unilateral optic nerve transection and ipsilateral intravitreal implantation of control and ntf-transfected fibroblasts to study: (i), RGC survival and axon regeneration after NTF backfilling with lysinated rhodamine dextran (LRD) from the distal optic nerve stump; and (ii), axon regeneration and axon sprouting at the site of optic nerve transection by GAP-43, and GFAP/laminin immunostaining, respectively.

In vitro experiments. RGC were cultured in the presence of CNS myelin and treated with NTF. After 48 h cultures were either: (i) immunostained for βIII tubulin and positive RGC counted and the length of their neurites measured; or (ii) subjected to cell lysis, protein extraction and either (a) Western blotting to determine levels of TACE, γ-secretase (presenilin-1, PS1), p75NTR, p75ECD, p75CTF, p75ICD, NgR and TROY by densitometry, or (b) a Rho-A activation assay to determine total Rho-A and Rho-GTP.

FGF2-, NT-3-, and BDNF-transfected fibroblasts

Fibroblasts were prepared from Fischer 344 rats, engineered with either fgf2, nt-3 or bdnf (from Jaso Ray and Fred Gage, The Salk Institute, CA, USA) and characterized in detail elsewhere (Kawaja et al., 1992; Ray et al., 1995; Senut et al., 1995; Shults et al., 2000). Fibroblasts were grown to confluence in 75 cm flasks in Dulbecco’s modified Eagle’s medium with 1.0 g/l D-glucose, 10% foetal bovine serum, 2 mg/ml fungizone, 50 μg/ml gentamicin sulphate, 400 μg/ml G418, 1 mM t-glutamate (all from Invitrogen, Paisley, UK). After reaching confluence, cells were dissociated by removing the medium, rinsed in Dulbecco’s phosphate-buffered saline (PBS) with 1.0 g/l D-glucose and 2% normal rat serum, and treated with trypsin EDTA solution (226 mg/l EDTA tetrasodium, 1.0 g/l D-glucose, 400 mg/l potassium chloride, 8 g/l sodium chloride, 500 mg/l trypsin, 580 mg/l sodium bicarbonate) for 5 min. Cells were pelleted by centrifugation and resuspended at a final concentration of 400 000 cells/μl in PBS containing 0.6% glucose and implanted into the vitreous body as described below.

Intravitreal implantation of NTF transfected fibroblasts

The vitreous body was accessed through the sclera and a pellet, containing 2.0 × 10^6 fibroblasts in a polymerized fibrin clot, was inserted through the incision and retained in the vitreal cavity with Sterison (Allen and Hanbury, London, UK). Rats received implants of either single NTF releasing fibroblast (FGF2, NT-3 or BDNF) or implants containing the same total number of cells made...
up from equal numbers of each of the FGF2/NT-3, FGF2/BDNF or FGF2/NT-3/BDNF secreting fibroblasts. Control groups received implants of either (i) fibrin clot without a cell pellet (sham); (ii) fibrin clot with untransfected fibroblasts, or (iii) fibrin clot with heat-killed untransfected fibroblasts. All implants were made immediately after optic nerve lesioning.

**Optic nerve injury**

Groups of 8–12 rats were anaesthetized by intraperitoneal injection of a physiological saline solution containing ketamine (40 mg/kg), acepromazine (1.2 mg/kg) and xylazine (8 mg/kg) (Janssen Pharmaceutical, Oxford, UK). The optic nerve was accessed intraorbitally after opening the dural sheath as described elsewhere (Berry et al., 1996, 1999). In the experiments described here, optic nerves were all unequivocally severed under direct observation 2-mm distal to the lamina cribrosa without damaging the central retinal artery. Animals recovered from surgery with little or no morbidity and were maintained in accordance with a project licence issued and approved through the UK Home Office.

**RGC axon regeneration and scarring in the optic nerve**

At 20 and 50 days post transection (dpt), rats were anaesthetized with a lethal dose of pentobarbital, followed by transcardiac perfusion with PBS for 1 min and 4% paraformaldehyde in 0.1 M PBS, pH 7.2 for 5 min. After perfusion, both optic nerves were dissected, dehydrated through a graded alcohol series, embedded in polyester wax (Berry et al., 1996) and stored at 4°C. Longitudinal sections of the optic nerve, 7 μm thick, were cut using a cooled chuck, floated onto a 1% gelatin solution on slides and air dried.

Immunohistochemical staining was performed according to techniques described in Berry et al., 1996. Axonal growth in all lesions was visualized using an antibody to growth-associated protein (GAP-43, 1 : 500, Zymed, CA, USA) and the high molecular weight, phosphorylated neurofilament protein (RT97, 1 : 200, Serotec, Oxford, UK). Scarring was monitored using rabbit anti-glial fibrillary acidic protein (GFAP, 1 : 500, DAKO, Cambridge, UK) to detect activated astrocytes and anti-laminin (Sigma, Poole, UK) to detect the basement membrane. Antibodies were diluted in PBS containing 1% (w/v) bovine serum albumin (Sigma), applied to sections and incubated overnight at 4°C. Slides were washed twice in PBS, and then incubated for 1 h with either FITC-labelled or TRITC-labelled anti-IgG secondary antibodies (Promega, Southampton, UK). Tissue sections were evaluated by immunofluorescence microscopy using a Zeiss epifluorescent microscope (Zeiss, Hertfordshire, UK).

**RGC backfilling with lysinated rhodamine dextran (LRD)**

LRD (1 μl, 20% w/v) was injected into the distal optic nerve segment, 2 mm from the lesion, at 20 and 50 dpt to retrogradely fill and label RGC that had survived and regenerated axons at least 2 mm distal to the lesion. After 48 h, animals were killed, perfusion-fixed and their retinæe whole-mounted onto slides. LRD-labelled RGC were counted under fluorescence microscopy as previously described (Berry et al., 1996, 1999, 2001b). The counts were a measure of the number of RGC that had survived and regenerated their axons at least 2 mm into the distal optic nerve segment.

**Preparation of CNS myelin protein extract**

Adult rat CNS myelin extract was prepared as described earlier (Ahmed et al., 2005b). Briefly, adult Sprague–Dawley rat brains were homogenized in 0.32 M sucrose, 1 mM EDTA, pH 7.0 at 4°C, the suspension centrifuged at 800 g for 10 min and the supernatant collected. The cell pellet was diluted to the original volume in 0.32 M sucrose, 1 mM EDTA, pH 7.0 and centrifuged, supernatant collected and the combined supernatants centrifuged at 13 000 g for 20 min. After removal of the supernatant, the pellet was resuspended in 0.9 M sucrose, distributed equally amongst a set of tubes, carefully overlayed with 1–2 ml of 0.32 M sucrose and centrifuged at 20 000 g for 60 min. The white material at the interface of the two sucrose layers was collected, dispersed in 0.32 M sucrose, and centrifuged at 13 000 g for 25 min. The white pellet was collected, diluted in water, left on ice for 30 min before centrifuging at 20 000 g for 25 min. The final white pellet was resuspended in a small volume of water and freeze dried overnight. The protein content of the myelin was determined using the Pierce BCA assay (BioRad, Hercules, CA, USA). The inhibitory molecules present in these extracts have been determined previously by Western blotting as MAG, Nogo, CSPG and OMgp (Ahmed et al., 2005b).

**Adult retinal cultures**

Adult female rats (6–8 week old) were killed by cervical dislocation and retinae removed by dissection. Retinal cells were dissociated using a papain system according to the manufacturer’s protocol (Worthington Biochem, NJ, USA). Dissociated retinal cells (125 × 10⁶), containing RGC, were cultured on glass coverslips precoated with 100 μg/ml poly-d-lysine (Sigma, Dorset, UK) and 20 μg/ml merosin (Chemicon, Harrow, UK) in 4-well tissue culture plates (Nunc, UK) containing B27 supplement, l-glutamine and gentamicin (all from Invitrogen) for 4 days at 37°C in a humidified 5% CO₂ atmosphere.

**Treatment of retinal cultures with NTF**

Adult retinal cultures were grown in triplicate, as described above, in the presence of an inhibitory CNS myelin extract (10 μg/ml), containing Nogo-A, MAG, OMgp and CSPG (Ahmed et al., 2005b), and treated with either single doses of NTF [NT-3 (50 ng/ml); FGF2 (10 ng/ml) and BDNF (50 ng/ml)] (all purchased from Peprotech, London, UK); or a combined NTF cocktail of NT-3/FGF2/BDNF at the same concentration to stimulate RGC survival and neurite outgrowth. Cells were treated for 48 h before harvesting for Western blotting and subsequent densitometry, as described below.

For immunocytochemistry, cells were fixed in 4% paraformaldehyde, rinsed in several changes of PBS, permeabilized in 0.1% Triton X-100, and stained using monoclonal anti-BIII tubulin (Sigma) followed by incubation with Texas red-labelled goat anti-mouse IgG secondary antibody (Promega), mounted in Fluorsave (Merck, Nottingham, UK), and viewed under a Zeiss epifluorescence microscope as described elsewhere (Ahmed et al., 2005b).

**Protein extraction and Western blotting**

To determine levels of p75NTR, TACE, PS1 (γ-secretase), NgR and TROY in treated RGC cultures by Western blotting, 3.75 × 10⁶ cells (n = 3) were lysed in 50 μl ice-cold lysis buffer 20 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 1% NP-40 and a
cocktail of protease inhibitors (Sigma), and immunoblotted as described previously (Ahmed et al., 2005b). p75NTR, p75ECD, p75NTF, and p75ICD were detected using polyclonal rabbit anti-p75NTR (Promega, OR, USA, 1:500 dilution) (Ahmed et al., 2005b). TACE was detected using polyclonal rabbit anti-TACE antibody (Ab) (Chemicon, 1:500 dilution). PS1 was detected using a polyclonal chicken anti-PS1 (Cambridge Bioscience, Cambridge, UK, 1:200 dilution). The levels of NgR and TROY in Western blots were detected using polyclonal goat anti-human NgR Ab (1:100) and polyclonal rabbit anti-human TROY (1:500, both from Santa Cruz Biotechnology, CA, USA). To determine the levels of the p55ECD and NgR_ECD shed into the culture medium, the RGC culture supernatant was collected, concentrated using microconcentrators (Microcon YM3, Millipore, Bedford, MA, USA) and 40 μg of total protein run on Western blots as described above. Levels of detected proteins were quantified by densitometry as described below.

**Rho activation assay**

GTP-bound Rho was detected in cell lysates (n = 6) using a Rho activation assay kit (Upstate Biotechnology, Milton Keynes, UK) following the manufacturer’s instructions. The assay kit also measured total Rho. Levels of detected proteins were quantified by densitometry as described below.

**Densitometry**

Western blots were digitally scanned, keeping all scanning parameters the same, using Adobe Photoshop, and the integrated density of each band was quantified using the gel plotting macros in ScionImage, version 4 (Scion Corporation, MD, USA).

**Neurite outgrowth measurement**

The mean number of surviving RGC, RGC with neurites and mean RGC neurite length was quantified by splitting each coverslip into 9 quadrants and randomly capturing images of βIII-tubulin immunostained RGC from each quadrant using a Zeiss Axioplan epifluorescent microscope. Axiovision image analysis software (Zeiss, Hertfordshire, UK) was then used to measure neurite length and to count the number of RGC in each coverslip using the built-in macros (n = 9 coverslips/condition), as previously described (Ahmed et al., 2005b).

**Statistical analysis**

Results from total counts of LRD-labelled RGC, Western blot densitometry, RGC neurite outgrowth and survival measurements were analysed for significance using ANOVA and Bonferroni’s post hoc test using GraphPad Prism, version 4 (GraphPad Software, Inc., San Diego, CA, USA). Data were presented as means ± SEM.

**Results**

**RGC neurite outgrowth was promoted by combined NTF treatment in the presence of CNS myelin**

As expected, we found that RGC cultured in the presence of CNS myelin grew no neurites in the absence of NTF [Fig. 1A and F(i) and (iii)], but treatment with either FGF2 (Fig. 1B), NT-3 (Fig. 1C) or BDNF (Fig. 1D) singly, promoted some RGC survival [Fig. 1F(iii)] and a small number grew neurites [Fig. 1F(i) and (ii)]. By contrast, treatment with combined FGF2/NT-3/BDNF (Fig. 1E), synergistically increased the number of RGC with neurites [Fig. 1F(i)], and potentiated both mean neurite length [Fig. 1F(ii)] and RGC survival [Fig. 1F(iii)] compared to both controls (P < 0.0001), and to each of the single NTF treatments (P < 0.001).

**RGC axons regenerated after intravitreal implantation of transfected fibroblasts expressing NTF and scarring was inhibited**

The optic nerve was exposed through a supraorbital approach, the meninges incised and the nerve crushed within the pial sheath with forceps for 10 s, 2 mm behind the eye (Berry et al., 1996, 1999). On releasing the forceps, complete severance of the optic nerve was established if the crush appeared as a translucent band from which optic nerve tissue was excluded. Care was taken not to damage the central retinal artery which runs medially in the optic nerve sheath.

We determined if RGC survival and axon growth were synergistically enhanced in vivo after intravitreal implantation of either single or combined NTF-producing F12 fibroblasts 20 dpt. In the no fibroblast—(not shown) and heat-killed F12 fibroblast—(Fig. 2A) implant controls, the proximal optic nerve contained small numbers of RT97+/GAP-43+ axons, and none grew through the optic nerve lesion. After intravitreal implantation of live untransfected fibroblasts, there were more axons in the proximal optic nerve and a few regenerated into the distal optic nerve (Fig. 2B), confirming that fibroblasts secrete endogenous NTF, which sustain some RGC survival and axonal growth (Acheson et al., 1991). A similar response occurred in rats intravitreally implanted with fibroblasts transfected with either fgf2, nt-3 or bdnf genes singly (Fig. 2C–E, respectively). When we transplanted the same total number of fibroblasts used to deliver single ntf, but 1 out of 2 were transfected with fgf2 and 1 out of 2 with nt-3 (Fig. 2F), or 1 out of 2 transfected with fgf2 and 1 out of 2 with bdnf (Fig. 2G), modest improvements in GAP-43+ RGC axons were observed compared to single ntf delivery. However, when we transplanted the same total number of fibroblasts used to deliver single ntf, of which 1 out of 3 were transfected with fgf2, 1 out of 3 with nt-3 and 1 out of 3 with bdnf, this combined intravitreal ntf therapy promoted the regeneration of large numbers of GAP-43+ RGC axons through the lesion (Fig. 2H and inset; Fig. 3A and inset) at least 5 mm beyond the lesion site (Fig. 3B and inset) into the distal optic nerve segment, where the inhibitory environments of both the scar and CNS myelin-rich distal optic nerve segment would be expected to block regeneration (Hunt et al., 2002; Sandvig et al., 2004).

At 20 dpt, reactive GFAP+ astrocytes in the control non-regenerating optic nerve accumulated in a vascularized area surrounding a laminin-rich accessory glia limitans which...
Fig. 1 RGC neurite outgrowth in response to NTF treatment when cells were all grown in the presence of inhibitory CNS myelin extract. Little or no βIII-tubulin stained RGC neurite outgrowth was observed in (A) control untreated, (B) FGF2, (C) NT-3, and (D) BDNF-treated retinal cultures. However, combined FGF2/NT-3/BDNF treatment (E) produced significant and synergistic neurite outgrowth, leading to increased numbers of RGC with neurites [F(i)], longer mean neurite length [F(ii)], and enhanced RGC survival [F(iii)] compared to single NTF treatments. Scale bars = 20 μm. ***P < 0.0001. n = 3 per condition, 4 independent experiments.
defined the wound margins and enclosed a matrix core at the centre of the lesion (Fig. 3C). In the regenerating optic nerve of rats with intravitreal implants of combined fgf2/nt-3/bdnf-transfected fibroblasts, neovascularization occurred at the site of the wound and, whilst there were many reactive astrocytes surrounding the lesion, they did not associate to form a glia limitans and no scar formed (Fig. 3D). Laminin immunostaining associated with the basement membrane of blood vessels in the proximal and distal optic nerve stumps also demonstrated that wound-related angiogenesis was similar in magnitude and distribution in both the regenerating and non-regenerating optic nerve.

Intravitreal implantation of combined NTF-transfected fibroblasts-enhanced RGC survival and axon regeneration

To quantify RGC survival and axon regeneration, RGC somata were retrogradely labelled with LRD after optic nerve injection, 2 mm distal to the lesion, so that the number of RGC that had survived and regenerated at least 2 mm past the lesion site and into the distal optic nerve stump could be counted. In controls and in rats receiving intravitreal fibroblasts expressing either FGF2 alone, NT-3 alone or BDNF alone, less than 10 LRD+ RGC survived and regenerated axons into the distal optic nerve stump (Fig. 4A), whilst there were greater numbers of LRD+ RGC in rats implanted with intravitreal fibroblasts that expressed either the FGF2/NT-3, or FGF2/BDNF combinations. There were significantly greater numbers of LRD+ RGC in the eyes of rats implanted with intravitreal fibroblasts that expressed the FGF2/NT-3/BDNF triple combinations ($P < 0.0001$), demonstrating that, when NTF treatments were combined, many RGC axons had regenerated at least 2 mm into the distal optic nerve segment (Fig. 4A and B). Moreover, the number of LRD+ RGC was greater than the sum of individual LRD+ RGC surviving and regenerating after delivery of each NTF alone, emphasizing that combinatorial delivery of NTF in vivo has a synergistic effect on RGC survival and axon regeneration. The number of RGC that survived and regenerated at least 2 mm distal to the lesion site with FGF2/NT-3/BDNF combination represented approximately 1.25% of the total number of RGC in intact control retinae. At 50 dpt, the number of LRD+ RGC in the retinae of the combined NTF-treatment group had reduced <4% of that seen at 20 dpt ($P < 0.0001$) (Fig. 5A), with the numbers of RGC axons regenerating in the optic nerve also correspondingly reduced (compare Fig. 5B with Fig. 2F).

Fig. 2 RGC axon regeneration after treatment with fgf2-, and/or nt-3- and/or bdnf-transfected fibroblasts. Longitudinal sections through the optic nerve lesion site showing regenerating RGC axons 20 dpt after implantation of: (A) heat-killed untransfected fibroblasts; (B) untransfected fibroblasts; (C) fgf2-transfected fibroblasts; (D) nt-3-transfected fibroblasts; (E) bdnf-transfected fibroblasts, (F) fgf2/nt-3-transfected fibroblasts; (G) fgf2/bdnf-transfected fibroblasts and (H) combined fgf2/nt-3/bdnf-transfected fibroblasts. Inset shows high-power magnification of regenerating axons in the boxed region in (F). Tissue sections were double-stained for GAP-43 (green) and RT97 (red). Scale bars A–F = 20 μm, inset = 40 μm.

* = lesion site; # = distal optic nerve segment.
Enhanced RGC neurite outgrowth promoted by combined NTF treatment correlated with upregulation of TACE-mediated RIP of p75NTR, NgRECD shedding and suppressed Rho activation, in the presence of CNS myelin

When retinal cultures were lysed and extracted proteins analysed by Western blotting, significantly lower levels of intact p75NTR were observed in retinal cell lysates from combined recombinant FGF2/NT-3/BDNF-treated cultures than in any other group (P < 0.001) [Fig. 6A and B(ii)] which correlated with the appearance of increased levels of p75ICD recovered from the culture medium and p75ECD fragments detected in cell lysates [Fig. 6A and B(iii)]. In combined FGF2/NT-3/BDNF-treated cultures, fragmentation of NgR but not of TROY, was observed in cell lysates, while NgRECD was detected in culture media (Fig. 6A). When cell lysates were assayed for RhoA activation, the levels of Rho-GTP were significantly lower in the combined FGF2/NT-3/BDNF group than in any other group (P < 0.001) [Fig. 6A and B(iii)], while total Rho levels remained unchanged (Fig. 6A).

The observed fragmentation pattern of p75NTR and NgR suggests that cultured RGC produce and secrete (TACE) leading to NgRECD and p75ECD cleavage and γ-secretase activation to generate p75ICD (Kanning et al., 2003; Weskamp et al., 2004). Indeed, there was an up-regulation of both inactive and active TACE in
retinal cultures stimulated with NTF singly, compared to untreated control cultures \( (P < 0.0001) \) [Fig. 6A and B(iv)]. Furthermore, in response to combined FGF2/NT-3/BDNF treatment, the upregulation of both inactive and active TACE was significantly potentiated \( (P < 0.001) \) [Fig. 6A and B(iv)] co-incident with increased levels of the major component of the \( \gamma \)-secretase enzyme, PS1 (Fig. 6A) and significantly increased levels of \( \beta \)-secretase in culture media [Fig. 6B(ii)] compared to RGC receiving single NTF.

**Discussion**

We report that combined and not single intravitreal NTF therapy effectively induces RGC survival and axon regeneration within the injured optic nerve. Five key observations were made in the experiments described here: (i) NTF elicited a regenerative response of RGC axons in the optic nerve when administered intravitreally to RGC cell bodies; (ii) the RGC axon regenerative response in the optic nerve was directional, following appropriate guidance cues in spite of a milieu which is growth inhibitory and hostile to regeneration with no evidence of entrapment of axons within the wound margins; (iii) the combination of FGF2/NT-3/BDNF blocked the formation of a glial scar (mature by 8 dpt); (iv) compared to either individual or double NTF treatments, the triple combination of FGF2/NT-3/BDNF promoted greater RGC survival and axon regeneration after optic nerve injury than would be predicted by their additive activity; and (v) the combination of FGF2/NT-3/BDNF induced the production of TACE and \( \gamma \)-secretase by RGC in vitro, which led to shedding of \( \beta \)-secretase, RIP of \( \beta \)-secretase and the disinhibition of axon growth on inhibitory CNS myelin substrates by attenuating inhibitory receptor signalling, evidenced by the reduced conversion of Rho-GDP to Rho-GTP.

NTF were delivered directly to RGC somata by intravitreal transplantation of F12 fibroblasts genetically engineered to express FGF2, NT-3 and BDNF genes in contrast to other studies in which recombinant NTF are delivered to the site of CNS tract transection. Our somata delivery was designed to circumvent ‘NTF sink’ formation at the site of injury which entraps growing axons in the lesion and arrests functional regeneration. Sustained NTF delivery may account for the successful RGC axon regeneration observed over 20 dpt, but these regenerative effects were lost by 50 dpt. The complexity and the dynamics of in vivo protein delivery by cells transduced with cDNA make it difficult to know the exact combination of NTF that stimulated axon regeneration. The amounts of protein produced are likely to change according to the behaviour of cells after transfection/implantation which may affect the levels of NTF produced by the transfected cells. Cessation of regeneration over the period between 20 and 50 dpt demonstrated that optimal NTF delivery remains a significant hurdle in establishing robust, prolonged axon regeneration, and that alternative delivery strategies including gene therapy need evaluating (Berry *et al*., 2001a). In a previous study, we explored the use of gene activated matrices to deliver FGF2/NT-3/BDNF to RGC somata (Berry *et al*., 2001b).
In this case, the pinocytotic activity of regenerating axons was explored so that multiple NTF-encoding DNA were retrogradely transported from matrices implanted into optic nerve injury sites. The observed sustained effect of such gene therapy on RGC survival extended beyond 100 dpt after delivery of genes encoding the same three NTF as used here, suggests that this might be an attractive NTF delivery strategy that could prolong the therapeutic effect.

Under normal conditions, a dense collagen and CSPG-rich scar forms in the optic nerve lesion by 8 dpt but is not formed in the regenerating optic nerve (Berry *et al*., 1996, 1999). We have previously demonstrated in the same model that, after multiple NTF supply by intravitreal peripheral nerve implantation, the failure of scarring correlates with the production and secretion of MMP and TIMP by reactive optic nerve glia and plasminogen activator (PA) by RGC, leading to the dissolution of the incipient scar (Ahmed *et al*., 2005a). The absence of scarring ensures the unobstructed passage of axons through the lesion in the absence of scar-derived inhibitory ligands, including CSPG, ephrins and semaphorins from the lesion, all of which participate in the same inhibitory signalling cascade as the myelin-derived ligands (Hunt *et al*., 2002; Sandvig *et al*., 2004), thereby augmenting disinhibition of NTF-stimulated RGC axons.

The findings of our study suggest that more than one factor is required for effective RGC survival and axon regeneration and this probably explains why multiple NTF-secreting intravitreal sciatic nerve implants (Berry *et al*., 1996, 1999), injured lens (Fischer *et al*., 2001; Lorber *et al*., 2002) and intravitreal macrophages promote RGC axon growth (Yin *et al*., 2003). In some cases the regenerating RGC axons traversed the lesion, invaded the distal optic nerve and in some instances invaded the contralateral optic tract and the superior colliculus (Fischer *et al*., 2001). In our triple NTF paradigm the individual contribution of NTF was 1 out of 3 of that delivered singly. Thus, our measure of synergism is underestimated. Nonetheless, the combination of FGF2/NT-3/BDNF not only promoted RGC survival and axonal/neurite outgrowth but also abrogated NgR and p75NTR shedding. Soluble NgRECD binds all CNS myelin-derived ligands (Walmsley *et al*., 2004; 2005) and p75ECD has the potential to bind to either NgR or full length p75NTR. The ensuing competitive interaction of these complexes with the intact NgR/p75NTR inhibitory receptor complexes blocks inhibitory signalling, thereby disinhibiting RGC axon growth in the optic nerve. Accordingly, the interactions between TrkB and TrkC receptors and their co-receptor p75NTR and FGF1-4 with their heparan sulphate proteoglycan co-receptors and the signal transduction pathways they activate, deserve closer investigation. In addition to FGF2, NT-3 and BDNF, there are numerous other NTF, growth stimulatory and differentiation factors that alter the natural course of neuron survival, growth and axon regeneration in the CNS which also require further investigation in a combinatorial paradigm.

However, the exact mechanism of NTF-induced synergism on RGC survival is not known. Each NTF acts through a different intracellular signalling pathway after receptor binding, i.e. NT-3 to high affinity TrkC with optional participation of p75NTR (Huang and Reichardt, 2001), BDNF to both the TrkB and p75NTR (Lee *et al*., 2001) and FGF2 to a separate class of high affinity tyrosine kinase receptors (FGFR1) and low affinity heparan sulphate proteoglycans on the cell surface and in the extracellular matrix (Bottcher and Niehrs, 2005). Accordingly, it is likely that the many adaptor proteins and signalling pathways activated by each factor individually serve
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Fig. 6 RGC neurite outgrowth in retinal cultures stimulated by NTF in the presence of CNS myelin extract was correlated with the extent of \( p75^{\text{NTR}} \) and NgR fragmentation and reduced levels of activated Rho-A. (A) and (B) Western blotting and blot densitometry, respectively, show that there was significant fragmentation of \( p75^{\text{NTR}} \) and NgR, but not of TROY, only after combined triple FGF2/NT-3/BDNF treatment of RGC (A) and [(B)(i)], leading to the appearance of cleaved \( p75_{\text{ECD}} \) and \( \text{NgR}_{\text{ECD}} \) in the culture media and \( p75_{\text{ICD}} \) in cell lysates (A) and [(B)(ii)]. Fragmentation of \( p75^{\text{NTR}} \) and NgR was associated with a significant reduction in Rho-GTP levels without affecting total Rho levels (A) and [(B)(iii)]. Combined FGF2/NT-3/BDNF treatment also significantly enhanced levels of TACE and PS1 activation (A) and [(B)(iv)], enzymes that cleave \( p75_{\text{ECD}} \) and initiate RIP of \( p75^{\text{NTR}} \) and release \( p75_{\text{ICD}} \). *** \( P < 0.0001 \), ** \( P < 0.001 \).
to elicit a survival response when given in combination that is greater than the sum of effects obtained with each factor alone. It seems likely that, since signal pathways for BDNF, NT-3 and FGF2 share PLC-γ, Ras/MAPK and PI3K/Akt activation (Huang and Reichardt, 2001; Bottcher and Niehrs, 2005), there are opportunities for convergence at these key nodal points leading to synergistic signalling interactions. Another explanation for synergism might be the upregulation of supplementary fibroblast-derived NTF under the influence of the products of the transfected genes. However, this seems unlikely since the synergistic effects of these genes when delivered in combination to RGC from a matrix depot (Berry et al., 2001b) were comparable to those seen here with transfected fibroblasts. NTF-induced attenuation of inhibitory receptor signalling may enhance NTF synergism. TACE-mediated shedding of NgR\textsubscript{ECD} and activation of the RIP cascade induced only by the triple NTF combination leads to paralysis of p75NTR, which attenuates inhibitory signalling by myelin-related inhibitory ligands, leading in turn to a reduced conversion of Rho-GDP to Rho-GTP, thereby preserving growth cone integrity. Optimization of neuron survival and axon regeneration will require a systematic evaluation of the optimal method and composition for NTF delivery.

The results reported by Domeniconi et al. (2005) directly oppose the results we observed in our experiments. Using an in vitro model, they report that p75\textsuperscript{NT}R fragmentation is required to elicit the inhibitory effects of MAG, while our results demonstrate that p75\textsuperscript{NT}R fragmentation enhances CNS axon regeneration/neurite outgrowth in the presence of inhibitory CNS myelin. We believe that our experiments utilize physiologically relevant models, directly related to the phenomena of CNS injury, using adult primary neuronal (retinal) cultures with CNS myelin extracts added directly into the media rather than demonstrating an effect with MAG Fc expressing CHO cells on immature CNS neurons. Our CNS myelin extract contains MAG and other potent inhibitory molecules such as Nogo-A, MAG, OMgp and CSPG (Ahmed et al., 2005b). Importantly, we also include NTF in our culture media which potentiates RIPs inducing disinhibition and promotion of RGC axon/neurite outgrowth, confirming NTF-induced RIP in regenerating neurons.

In summary, our findings confirm our proposition that appropriate NTF combinations are required to promote RGC survival and regeneration in the optic nerve by: (i) replacing the NTF deficit required for RGC survival; (ii) mobilizing structural protein synthesis essential for RGC axon growth; (iii) stimulating the production and activation of TACE and γ-secretase, which paralyses inhibitory signalling by the initiation of shedding of NgR\textsubscript{ECD} and RIP of p75\textsuperscript{NT}R; (iv) attenuating the conversion of Rho-GDP to Rho-GTP, thereby reducing growth cone collapse initiated by most, if not all, inhibitory ligands; (v) stimulating the production and release of MMP/PA by glia, which block scar-production and abrogate scar derived inhibitory ligands; and (vi) circumventing ‘NTF sink’ formation at the site of axon transection by delivery to neuronal somata, thus promoting directional axon regeneration towards centrifugal targets. Our results further show that the effects of NTF are synergistic on both cell survival and axon regeneration when delivered in combination. The results presented here show that the integrated response to multiple ligands can be significantly different from the response predicted from their single activities. If confirmed in a clinical setting, then the appropriate combination of NTF delivered to injured CNS neurons could offer a pharmacological strategy for promoting their survival and disinhibited axon growth after trauma, thereby preserving neuronal numbers and promoting axon regeneration without the intervention of scar tissue.
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