

# ProNGF promotes neurite growth from a subset of NGF-dependent neurons by a p75<sup>NTR</sup>-dependent mechanism

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## SUMMARY

The somatosensory and sympathetic innervation of the vertebrate head is derived principally from the neurons of trigeminal and superior cervical ganglia (SCG), respectively. During development, the survival of both populations of neurons and the terminal growth and branching of their axons in the tissues they innervate is regulated by the supply of nerve growth factor (NGF) produced by these tissues. NGF is derived by proteolytic cleavage of a large precursor protein, proNGF, which is recognised to possess distinctive biological functions. Here, we show that proNGF promotes profuse neurite growth and branching from cultured postnatal mouse SCG neurons. In marked contrast, proNGF does not promote the growth of trigeminal neurites. Studies using compartment cultures demonstrated that proNGF acts locally on SCG neurites to promote growth. The neurite growth-promoting effect of proNGF is not observed in SCG neurons cultured from p75<sup>NTR</sup>-deficient mice, and proNGF does not phosphorylate the NGF receptor tyrosine kinase TrkA. These findings suggest that proNGF selectively promotes the growth of neurites from a subset of NGF-responsive neurons by a p75<sup>NTR</sup>-dependent mechanism during postnatal development when the axons of these neurons are ramifying within their targets *in vivo*.

**KEY WORDS:** Proneurotrophins, p75<sup>NTR</sup>, Neurite growth, Sympathetic neurons, Mouse

## INTRODUCTION

In the developing vertebrate peripheral nervous system (PNS), neurotrophins sustain the survival of particular populations of neurons and promote the growth and branching of their axons (Bibel and Barde, 2000; Huang and Reichardt, 2001; Davies, 2009). In so doing, this family of secreted proteins plays a key role in regulating neuron number and tissue innervation density. Each of the four members of the neurotrophin family of higher vertebrates, nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT3; also known as NTF3) and NT4 (also known as NTF5), is synthesised as a proneurotrophin precursor protein that is proteolytically cleaved to generate the corresponding mature neurotrophin with its extensively characterised neurotrophic functions (Seidah et al., 1996a; Seidah et al., 1996b; Lee et al., 2001). In recent years, proneurotrophins have been reported to exhibit a distinctive set of functions, including induction of neuronal apoptosis (Nykjaer et al., 2004; Teng et al., 2005; Kenchappa et al., 2006; Yano et al., 2009) and modulation of synaptic function (Pang et al., 2004; Woo et al., 2005). Whereas the effects of mature neurotrophins on neuronal survival are mediated by members of the Trk family of receptor tyrosine kinases (Huang and Reichardt, 2001) and are modulated by the common neurotrophin receptor p75<sup>NTR</sup> (also known as NGFR) (Davies et al., 1993; Lee et al., 1994b), the pro-apoptotic effects of proneurotrophins are mediated by a receptor complex composed of p75<sup>NTR</sup> and sortilin (Nykjaer et al., 2004; Nykjaer et al., 2005).

To explore further the biological functions of proNGF in the developing PNS, we studied the effects of a cleavage-resistant form of proNGF on two well-characterised populations of NGF-dependent neurons cultured at stages throughout late fetal and early postnatal development. The neurons of the trigeminal ganglion and superior cervical ganglion (SCG) provide somatosensory and sympathetic innervation, respectively, to a diversity of structures throughout the head. The timing of neurogenesis, naturally occurring neuronal death and target field innervation and the timing of NGF dependency have been described in detail for both populations of neurons (Davies, 1997; Davies, 2009; Enokido et al., 1999). Here, we find that proNGF acts locally and independently of mature NGF (mNGF) on SCG neurites, but not on trigeminal ganglion neurites, to promote profuse growth and branching at the stage in development when the axons of these neurons are branching extensively in their target tissues. This growth-promoting effect of proNGF on sympathetic neurites becomes evident in development after the growth-promoting effect of mNGF on these neurites has become established. These findings raise the possibility that proNGF, in addition to mNGF, plays a role in regulating tissue innervation, but that its effects are more selective among populations of NGF-dependent neurons than mNGF and that it acts over a more restricted period of development. We show that the effects of proNGF on neurite growth depend on the common neurotrophin receptor p75<sup>NTR</sup> and do not require receptor tyrosine kinase (Trk) activation. We speculate that the relative availability of proNGF and mNGF in particular target tissues might play a role in regulating relative innervation density by different populations of NGF-dependent neurons.

## MATERIALS AND METHODS

### Neuron culture

SCG, trigeminal and nodose ganglia were dissected from embryonic and postnatal CD1 mice. The ganglia were trypsinised, and plated at very low density (~500 neurons per dish) in poly-ornithine/laminin-coated 35 mm tissue culture dishes (Greiner, Germany) in serum-free Hams F14 medium (Davies et al., 1993) supplemented with 0.25% Albumax I (Life

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Technologies, Paisley, UK). Mouse breeding was approved by Cardiff University Ethical Review Board for Animal Research and performed under the 1986 Animal Procedures Act with personal and project licenses granted by the Home Office, UK.

Neuronal survival was estimated by counting the number of attached neurons within a 12 × 12 mm grid in the centre of each dish 2 hours after plating and again after 48 hours, and expressing the 48-hour count as a percentage of the 2-hour count. Analysis of the size and complexity of neurite arbours was carried out by labelling the neurons at the end of the experiment with the fluorescent vital dye calcein-AM (Life Technologies). For every condition in each experiment, images of at least 50 neurons were digitally acquired by fluorescence microscopy and analysed to obtain total neurite length, number of branch points and Sholl profiles (Gutierrez and Davies, 2007). Statistical analyses were performed using one-way ANOVA with Bonferroni-Holm post-hoc test. Pair-wise comparisons were made using Student's *t*-test.

To identify and analyse separately the neurite arbours of SCG and trigeminal neurons co-cultured in the same dish, these two kinds of neurons were transfected with YFP- and DsRed-expressing plasmids, respectively. To do this, freshly dissociated suspensions of each kind of neuron were electroporated using the Neon Transfection System (Life Technologies, Paisley, UK) prior to being plated together in the same culture dishes at low density.

For small interfering RNA (siRNA) experiments, dissociated suspensions of SCG neurons were electroporated with Silencer Select siRNAs (Life Technologies) directed against TrkA (also known as NTRK1) or with scrambled control siRNA. Co-transfection of YFP allowed visualisation of transfected neurons, and knock down was confirmed by immunocytochemistry (not shown).

For studying the effects of proNGF on local neurite growth, SCG or trigeminal neurons were seeded in one compartment of a two-compartment microfluidic device (Xona Microfluidics, CA, USA). The cell body compartment was supplemented with 10 ng/ml mNGF to sustain neuronal survival and the neurite compartment received either no factors, mNGF or proNGF. Calcein-AM was added to the neurite compartment 24 hours after plating to label neurites in this compartment.

Mature recombinant NGF, BDNF and CNTF were obtained from R&D Systems (MN, USA). Native murine proNGF, cleavage-resistant murine proNGF that has an R-to-G substitution at amino acid position 104 and cleavage-resistant murine proBDNF that has RR-to-AG substitutions at amino acid positions 112 and 113 were obtained from Alomone (Jerusalem, Israel). Cleavage-resistant human proNGF that has RR-to-AA substitutions at amino acids 64 and 65 and KR-to-AA substitutions at amino acids 94 and 95 and at 135 and 136 was kindly provided by GlaxoSmithKline. Native human proNT3 was obtained from Novoprotein (NJ, USA).

Where indicated, the culture medium was supplemented with the caspase inhibitor Boc-D-FMK (Calbiochem, Nottingham, UK) to prevent neuronal apoptosis in the absence of mNGF and with the broad-spectrum matrix metalloproteinase (MMP) inhibitor batimastat (Tocris Bioscience, Bristol, UK) to prevent proneurotrophin degradation.

#### Real-time QPCR (RT-QPCR)

The level of *p75<sup>NTR</sup>* mRNA was quantified relative to mRNA levels for reference genes encoding glyceraldehyde phosphate dehydrogenase (GAPDH) and succinate dehydrogenase (SDHA) by RT-QPCR. Total RNA was extracted and purified from tissue samples using the RNeasy Mini Extraction Kit (Qiagen, Manchester, UK) according to manufacturer's instructions. Each RNA sample (10 µl) was reverse transcribed for 1 hour at 45°C, with StrataScript reverse transcriptase (Agilent Technologies, Edinburgh, UK) in a 40 µl reaction volume containing the manufacturer's buffer supplemented with 5 mM deoxyribonucleotide triphosphates (dNTPs; Agilent Technologies, Edinburgh, UK) and 10 µM random hexamers (Fermentas, Sankt Leon-Rot, Germany). For each target cDNA, 2 µl aliquots of each completed reverse transcriptase reaction were amplified in a 20 µl reaction volume using Brilliant III ultrafast QPCR reagents (Agilent Technologies). PCR amplification was carried out on an Mx3000P qPCR machine (Agilent Technologies) and consisted of 45 cycles of 95°C for 12 seconds and 60°C for 35 seconds. Three separate dissections were

performed for each age. PCR primer probe sets were as follows: p75 forward, 5'-ACCAGAGGGAGAGAAACT-3'; p75 reverse, 5'-GCAG-GCTACTGTAGAGGT-3'; p75 probe, 5'-FAM-ACAGCGACAGCG-GCATCT-BHQ1-3'; GAPDH forward, 5'-GAGAAACCTGCCAAG-TATG-3'; GAPDH reverse, 5'-GGAGTTGCTGTTGAAGTC-3'; GAPDH probe, 5'-FAM-AGACAACCTGGTCCCTCAGTGT-BHQ1-3'; SDHA forward, 5'-GGAACACTCCAAAACAG-3'; SDHA reverse, 5'-CCACAGCATCAAATTCAT-3'; SDHA probe, 5'-FAM-CCTGCGGC-TTTCCTCTCT-BHQ1-3'. The relative levels of each target cDNA were determined by running a standard curve made from serial dilutions of reverse-transcribed adult mouse brain RNA (Zyagen, San Diego, USA) in every QPCR reaction.

#### Western blotting

For detection of TrkA phosphorylation in SCG neurons, the neurons were plated at high density in polyornithine/laminin-coated 96-well plates (5000 neurons per well). Neurons were incubated overnight in medium containing 25 µM Boc-D-FMK (Calbiochem) before treating them for different lengths of time with either 10 ng/ml mNGF or 10 ng/ml proNGF. After treatment, neurons were lysed in RIPA buffer and insoluble debris was removed by centrifugation. Samples were transferred to polyvinylidene difluoride membranes using the Bio-Rad TransBlot (Bio-Rad, CA, USA). Membranes were blocked with 5% bovine serum albumin in PBS containing 0.1% Tween-20. The membranes were then incubated with anti-phospho-TrkA antibody (1:1000; Cell Signaling Technology, MA, USA), anti-total TrkA antibody (1:500; Cell Signaling Technology) or anti-β-III tubulin antibody (1:10,000; R&D Systems), which were detected using a peroxidase-linked secondary antibody (Promega, WI, USA) and the ImmunoCruz Western Blotting Luminol Reagent (Santa Cruz, CA, USA).

#### Quantification of NF-κB transcriptional activity

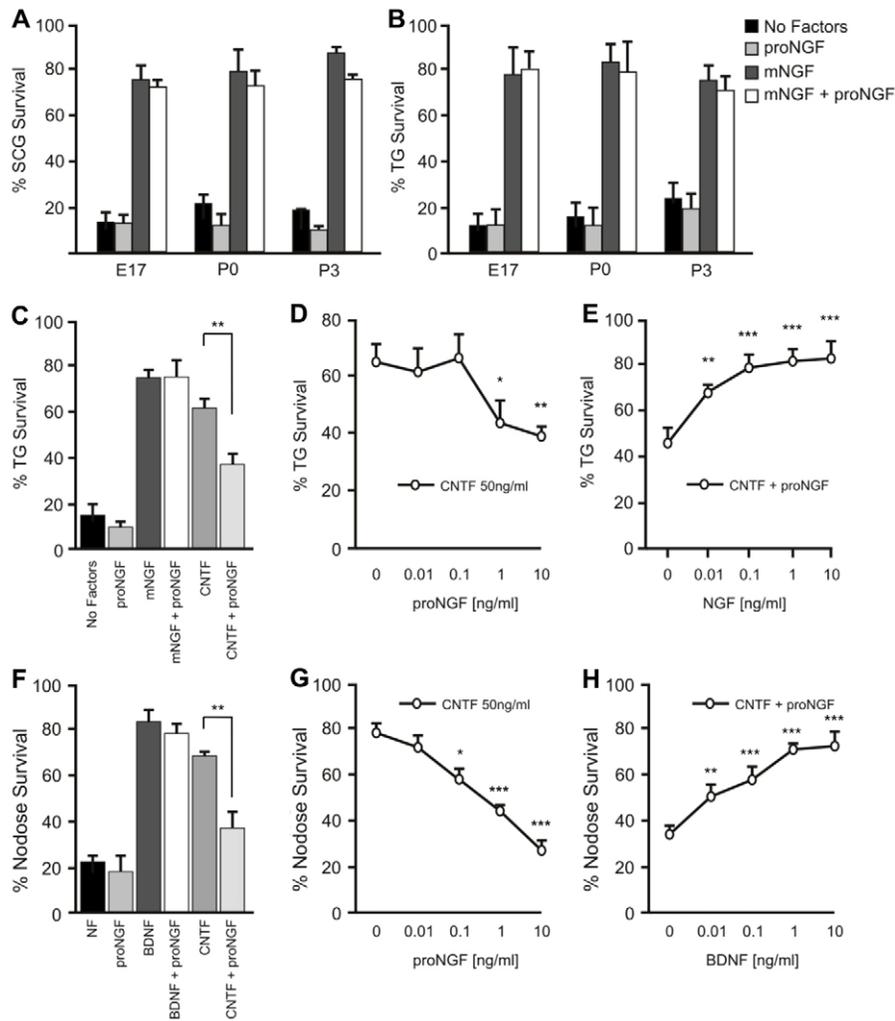
To estimate the relative level of NF-κB activation, freshly dissociated neurons were transfected with a reporter construct in which GFP expression is under the control of a series of κB regulatory elements (Gutierrez et al., 2005). Fluorescent cells were visualised using a Zeiss Axiovert 200 inverted fluorescence microscope, and the numbers of fluorescent neurons per well were counted. All imaging and quantification was performed blind. Statistical comparisons were performed by ANOVA followed by Bonferroni-Holm post-hoc test.

## RESULTS

### ProNGF does not affect the survival of SCG and trigeminal neurons supported by mNGF

We began characterising the effects of proNGF on developing SCG and trigeminal neurons by studying the effects of cleavage-resistant murine proNGF on the survival of these neurons cultured at low density at stages throughout late fetal and early postnatal development when these neurons are dependent on a supply of mNGF from their targets for survival. In control cultures (no added factors), the majority of neurons died within 48 hours of plating, and there was no significant difference in the small number of neurons surviving in these cultures and in cultures treated with proNGF. The survival of the majority of neurons was sustained by mNGF, and mNGF-promoted survival was unaffected by the presence of proNGF (Fig. 1A,B). These results show that proNGF neither exerts a survival-promoting effect itself nor interferes with the survival-promoting effects of mNGF on developing trigeminal and SCG neurons.

To ascertain whether proNGF is capable of promoting apoptosis in the absence of mNGF, we studied the effects of proNGF on the survival of newborn trigeminal neurons supported by CNTF in culture. In contrast to the insignificant effect of proNGF on the survival of trigeminal neurons maintained by mNGF, proNGF significantly reduced the survival-promoting effects of CNTF (Fig. 1C). Dose-response analysis revealed that this pro-apoptotic



**Fig. 1. proNGF does not affect the survival of SCG and trigeminal neurons supported by mNGF.** (A,B) Percentage survival of E17, P0 and P3 SCG (A) and trigeminal (B) neurons incubated for 48 hours without factors, or with 10 ng/ml proNGF, 10 ng/ml mNGF, or proNGF plus mNGF. (C) Survival of P0 trigeminal neurons incubated for 48 hours without factors, or with 10 ng/ml proNGF, 10 ng/ml mNGF, 50 ng/ml CNTF or proNGF plus either mNGF or CNTF. (D) Survival of P0 trigeminal neurons incubated for 48 hours with 50 ng/ml CNTF and a range of concentrations of proNGF. (E) Survival of P0 trigeminal neurons incubated for 48 hours with 50 ng/ml CNTF, 10 ng/ml proNGF and a range of concentrations of mNGF. (F) Survival of P0 nodose neurons incubated for 48 hours without factors, or with 10 ng/ml proNGF, 10 ng/ml BDNF, 50 ng/ml CNTF or proNGF plus either BDNF or CNTF. (G) Survival of P0 nodose neurons incubated for 48 hours with 50 ng/ml CNTF and a range of concentrations of proNGF. (H) Survival of P0 nodose neurons incubated for 48 hours with 50 ng/ml CNTF, 10 ng/ml proNGF and a range of concentrations of BDNF. The mean  $\pm$  s.e.m. for at least three separate experimental datasets is shown. \* $P < 0.05$ , \*\* $P < 0.001$ , \*\*\* $P < 0.0001$ , ANOVA with Bonferroni-Holm post-hoc test.

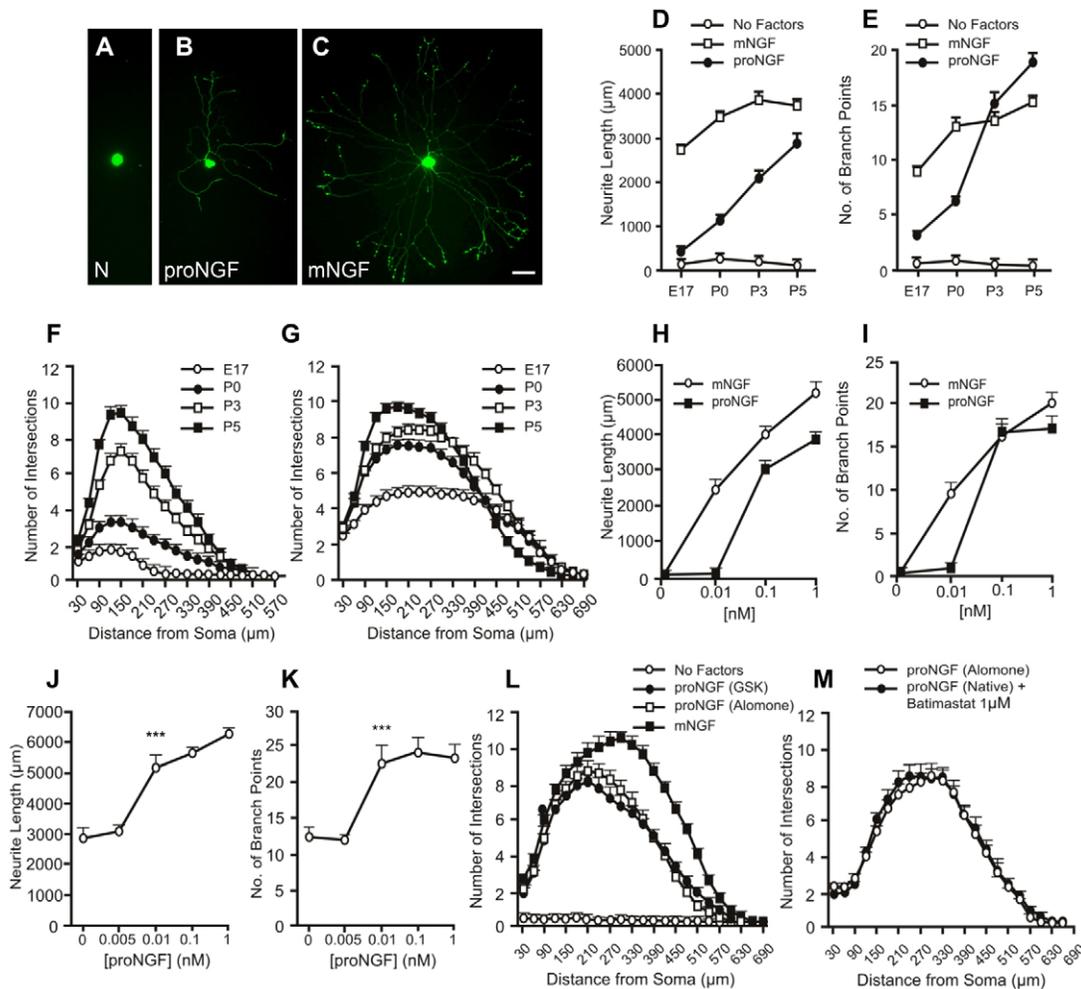
effect of proNGF on CNTF-promoted survival was significant at concentrations of 1 ng/ml and higher (Fig. 1D). Dose-response studies additionally showed that the pro-apoptotic effect of 10 ng/ml proNGF on CNTF-promoted survival was significantly impaired by as little as 0.01 ng/ml mNGF (Fig. 1E). Very similar results were obtained in studies of newborn nodose ganglion neurons supported in culture by either CNTF or the neurotrophin BDNF. Concentrations of proNGF as low as 0.1 ng/ml significantly reduced the survival of nodose neurons cultured with CNTF, and concentrations of BDNF as low as 0.01 ng/ml significantly countered the pro-apoptotic effect of proNGF (Fig. 1F-H). These results suggest that proNGF exerts a pro-apoptotic action on neurons supported with CNTF that is effectively antagonised by very low levels of mature neurotrophins.

### ProNGF promotes neurite growth and branching from postnatal SCG neurons

To investigate whether proNGF is capable of promoting neurite growth from NGF-dependent neurons independently of mNGF, SCG neurons from late fetal and postnatal mice were incubated with proNGF in medium containing the caspase inhibitor Boc-D-FMK to prevent apoptosis in the absence of mNGF. After 24 hours incubation, the neurons were labelled with the fluorescent vital dye calcein-AM and images of individual neurons were acquired for quantification of total neurite length, number of branch points

and Sholl analysis, which provides a graphic illustration of neurite length and branching in relation to distance from the cell body (Sholl, 1953). In the absence of factors (control cultures), there was negligible neurite outgrowth at all ages studied (Fig. 2). ProNGF exerted small but statistically significant increases in neurite length and branching in embryonic day (E)17 SCG cultures compared with controls (Fig. 2D,E). There were progressive and marked increases in neurite length and branching in proNGF-treated cultures between E17 and postnatal day (P)5, with overall neurite length and branch point number becoming sixfold greater at P5 than at E17. This age-related effect of proNGF on neurite growth is graphically illustrated by the Sholl analyses carried out at different ages (Fig. 2F).

To compare the effect of proNGF on sympathetic neurite growth at different ages with that of mNGF, cultures of SCG neurons were treated in parallel with mNGF at each age. Boc-D-FMK was also added to these cultures to keep the experimental conditions the same in proNGF- and mNGF-treated cultures. Unlike proNGF, mNGF was very effective at promoting neurite growth from SCG neurons at all ages studied. However, like proNGF, an age-related effect of mNGF on the extent of neurite growth was also evident, although this was less pronounced than that observed with proNGF (Fig. 2D,E,G). Direct comparison of the effects of equimolar concentrations of mNGF and proNGF on neurite growth from P5 SCG neurons revealed that mNGF was more potent and



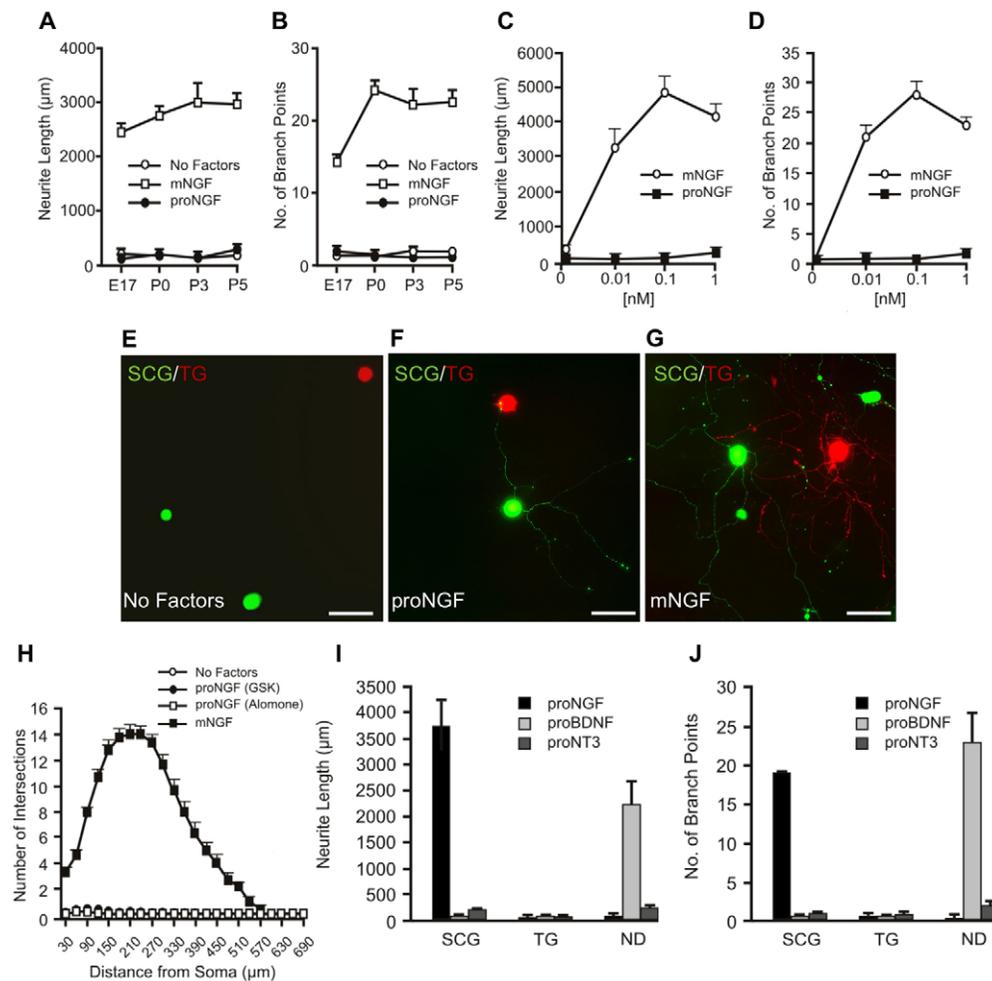
**Fig. 2. proNGF promotes neurite growth from postnatal SCG neurons.** (A-C) Images of calcein-AM-stained P3 SCG neurons cultured for 24 hours without factors (N) (A), with 10 ng/ml cleavage-resistant murine proNGF (B) or with 10 ng/ml mNGF (C). Scale bar: 50  $\mu$ m. (D,E) Neurite length and branching of E17 to P5 SCG neurons cultured for 24 hours without NGF (No Factors) or with 10 ng/ml cleavage-resistant murine proNGF or 10 ng/ml mNGF. (F,G) Sholl plots of E17 to P5 SCG neurons cultured with cleavage-resistant murine proNGF (F) or 10 ng/ml mNGF (G). (H,I) Neurite length and branching of P5 SCG neurons cultured with a range of concentrations of mNGF and cleavage-resistant murine proNGF. (J,K) Neurite length and branching of P3 SCG neurons cultured with 0.005 nM mNGF plus a range of concentrations of proNGF. (L) Sholl plot of P3 SCG neurons incubated with mNGF, cleavage-resistant human proNGF and cleavage-resistant murine proNGF. (M) Sholl plot of P3 SCG neurons incubated with cleavage-resistant murine proNGF or native murine proNGF in medium containing 1  $\mu$ M batimastat. All cultures were supplemented with 25  $\mu$ M Boc-D-FMK. The mean $\pm$ s.e.m. for at least three individual datasets is shown. \*\*\* $P$ <0.0001, ANOVA with Bonferroni-Holm post-hoc test.

efficacious than proNGF (Fig. 2H,I). At 0.01 nM, proNGF was ineffective in promoting neurite growth whereas mNGF exerted a clear effect on length and branching. At ten- and 100-fold higher molar concentrations, the effectiveness of proNGF in promoting neurite growth approached that of mNGF.

To investigate whether mNGF and proNGF in combination have additive or synergistic effects on neurite growth, P3 SCG neurons were grown with a half maximally effective concentration of mNGF for neurite growth (0.005 nM) and a range of concentrations of proNGF from those that fail to elicit neurite growth alone to those that are maximally effective in promoting neurite growth. ProNGF at a concentration that does not elicit neurite growth on its own (0.01 nM), in combination with 0.005 nM mNGF promoted neurite elongation (Fig. 2J) and branching (Fig. 2K) to a similar extent to that elicited by maximally effective concentrations of either factor alone (Fig. 2H,I). These results suggest that at low concentrations proNGF and mNGF have synergistic effects on neurite growth.

There are no additive effects on neurite growth of combining saturating concentrations of proNGF and mNGF (not shown), suggesting that essentially the same subset of SCG neurons respond to proNGF and mNGF.

To investigate whether the neurite growth-promoting action of the murine proNGF that had an R-to-G substitution at amino acid 104 used above is a peculiarity of this cleavage-resistant form of proNGF, we examined the effects of a different cleavage-resistant form of proNGF and the effects of native proNGF on neurite growth. Human proNGF that has amino acid substitutions at multiple cleavage sites (64 and 65, RR to AA; 94 and 95, KR to AA; 135 and 136, KR to AA) was just as effective as the cleavage-resistant murine proNGF in promoting neurite growth from P3 SCG neurons (Fig. 2L). Likewise, native proNGF, in cultures containing the broad spectrum MMP inhibitor batimastat to impair its degradation, had a similar effect on neurite growth from P3 SCG neurons (Fig. 2M).



**Fig. 3. Specificity of neurite growth-promoting effects of proNGF, proBDNF and proNT3.**

(A,B) Neurite length and branching of E17 to P5 trigeminal neurons cultured for 24 hours without factors or with 10 ng/ml cleavage-resistant murine proNGF or 10 ng/ml mNGF. (C,D) Neurite length and branching of P5 trigeminal neurons cultured with a range of concentrations of mNGF and cleavage-resistant murine proNGF. (E-G) YFP-transfected SCG neurons (green) and pDsRed-transfected trigeminal neurons (red) co-cultured for 24 hours either without factors (E), or with 10 ng/ml cleavage-resistant murine proNGF (F) or 10 ng/ml mNGF (G). Scale bars: 100 μm. (H) Scatter plot of P3 trigeminal neurons incubated with mNGF, cleavage-resistant murine proNGF or cleavage-resistant human proNGF. (I,J) Neurite length and branching of P3 SCG, trigeminal and nodose neurons cultured for 24 hours with 10 ng/ml cleavage-resistant murine proNGF, 10 ng/ml cleavage-resistant murine proBDNF or 10 ng/ml native human proNT3. All cultures were supplemented with 25 μM Boc-D-FMK and the cultures with native proNT3 additionally received 1 μM batimastat. The mean ± s.e.m. for at least three individual datasets are shown.

### ProNGF does not promote neurite growth from developing trigeminal neurons

In marked contrast to the pronounced neurite growth-promoting effect of proNGF on postnatal SCG neurons, murine cleavage-resistant proNGF at concentrations as high as 1 nM did not promote neurite growth from trigeminal neurons in cultures established from E17 to P5 (Fig. 3A-D). However, at each of these ages, mNGF promoted neurite growth from trigeminal neurons to a comparable extent to and with similar potency to its effect on SCG neurons. As a further stringent test of the selective effect of proNGF in promoting neurite outgrowth from different kinds of NGF-dependent neurons, SCG and trigeminal neurons were separately labelled with different fluorophores and co-cultured in medium containing proNGF. SCG neurons, labelled with YFP, exhibited profuse neurite growth, whereas trigeminal neurons, labelled with pDsRed, exhibited no neurite growth (Fig. 3F). However, when these neurons were co-cultured in medium containing mNGF, both populations of neurons exhibited profuse neurite growth (Fig. 3G). Like the cleavage-resistant proNGF, cleavage-resistant human proNGF did not elicit neurite growth from trigeminal neurons (Fig. 3H). These results confirm that proNGF has a selective effect on neurite growth from a subset of NGF-dependent neurons.

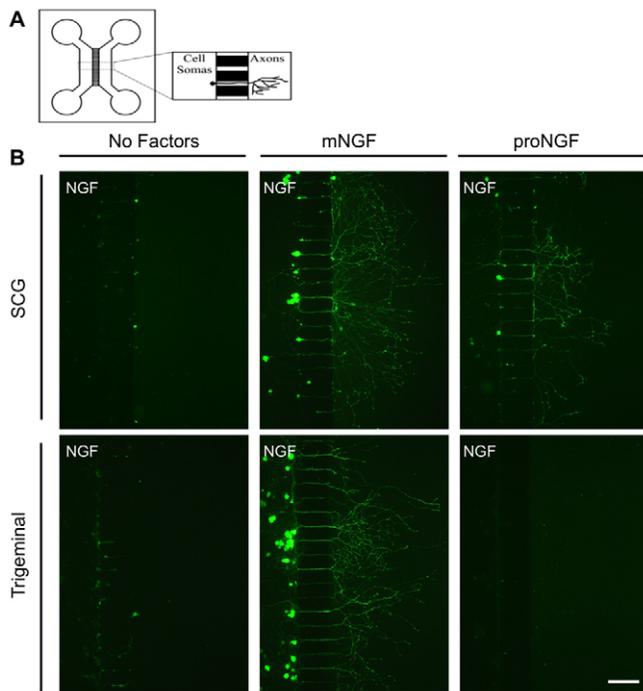
### Effects of proneurotrophins on neurite growth from different populations of neurons

To extend our investigation of the potential neurite growth-promoting action of proNGF and to determine whether other

proneurotrophins promote neurite growth from different populations of postnatal neurons, we cultured P3 SCG, trigeminal and nodose neurons with cleavage-resistant forms of either proNGF or proBDNF and native proNT3 in the presence of the MMP inhibitor batimastat (Fig. 3I-J). In contrast to proNGF, neither proBDNF nor proNT3 promoted neurite growth from SCG neurons. Trigeminal neurons were unresponsive to all three proneurotrophins, and nodose neurons displayed no response to proNGF and proNT3, but responded to proBDNF with profuse neurite growth. These studies reveal distinctive and highly specific effects of proneurotrophins on neurite growth from different populations of developing PNS neurons.

### ProNGF exerts a local growth-promoting effect on sympathetic neurites

To explore aspects of the response of SCG neurons to proNGF that might be relevant to its mode of action *in vivo*, we cultured these neurons in microfluidic devices to determine if, like mNGF, proNGF is capable of acting locally on neurites to promote growth. In these devices, the cell bodies and neurite terminals are grown in different compartments separated by a culture-medium impermeable barrier (Fig. 4A). P3 SCG neurons were seeded into one compartment of this device (the cell body compartment), which was supplemented with mNGF to maintain neuronal viability. The other compartment (the neurite compartment) received either proNGF or mNGF or no factors. After 24 hours incubation, the neurites in the neurite compartment were labelled with the



**Fig. 4. proNGF acts locally on SCG neurites to promote growth.**

(A) Schematic of a two-compartment microfluidic device. (B) Representative images of calcein-AM-labelled P3 SCG and P3 trigeminal neurons incubated for 24 hours with no NGF or with 10 ng/ml mNGF or 10 ng/ml cleavage-resistant murine proNGF in the neurite compartment. Neuronal viability was maintained by supplementing the cell body compartment with 10 ng/ml mNGF in all cases. Scale bar: 150  $\mu\text{m}$ .

fluorescent vital dye calcein-AM. Profuse neurite growth in the neurite compartment was observed when this compartment was supplemented with either mNGF or proNGF, but no neurites were observed in this compartment in the absence of NGF (Fig. 4B). When similar experiments were carried out using P3 trigeminal neurons, neurite growth in the neurite compartment was only observed when this compartment was supplemented with mNGF and not with proNGF. These observations demonstrate that proNGF acting directly on the neurites of postnatal SCG neurons can induce local increases in growth.

#### ProNGF-promoted neurite growth is mediated by $p75^{\text{NTR}}$

Because the common neurotrophin receptor  $p75^{\text{NTR}}$  has been implicated in regulating neurite growth and is a receptor for proNGF, we investigated whether it has a role in the neurite growth-promoting effects of proNGF on postnatal sympathetic neurons. We began investigating this question by quantifying the level of  $p75^{\text{NTR}}$  transcripts expressed in the SCG over the period of development when SCG neurons become responsive to proNGF (E17 to P5). We found a highly significant, threefold increase in the level of  $p75^{\text{NTR}}$  mRNA relative to the level of transcripts for the housekeeping enzymes GAPDH and SDHA over this period of development (Fig. 5A).

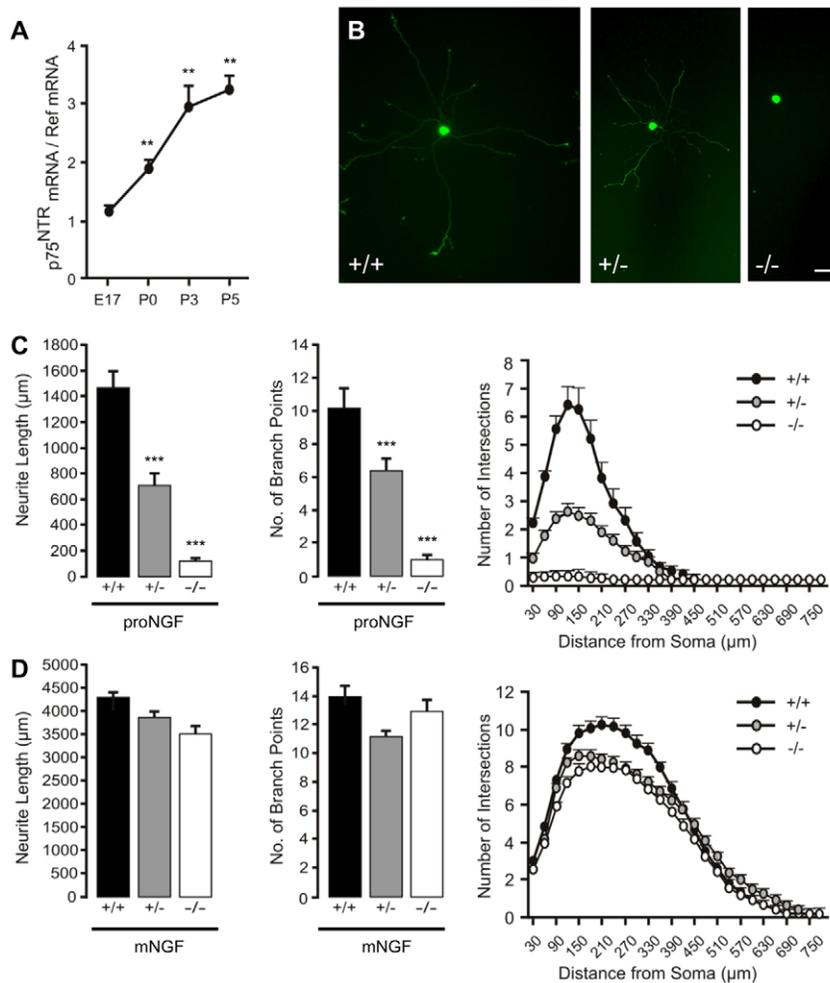
We investigated the importance of  $p75^{\text{NTR}}$  in mediating the neurite growth-promoting effects of proNGF by comparing the effects of proNGF on cultures of SCG neurons obtained from  $p75^{\text{NTR}+/+}$ ,  $p75^{\text{NTR}+/-}$  and  $p75^{\text{NTR}-/-}$  mice. For these studies,

$p75^{\text{NTR}+/-}$  mice were crossed, and separate cultures were established from the littermates at P3. The neurons were incubated in medium containing Boc-D-FMK with and without proNGF, and neurite growth was quantified in each of the three genotypes 24 hours after plating. ProNGF-promoted neurite growth was completely eliminated in neurons lacking  $p75^{\text{NTR}}$  (Fig. 5B,C). There was no significant difference in the negligible extent of neurite growth in these cultures between control and proNGF-supplemented conditions (not shown). Neurite length and branch number was reduced by about half in proNGF-supplemented cultures established from  $p75^{\text{NTR}+/-}$  mice compared with cultures established from wild-type mice. These differences in proNGF-promoted neurite growth between neurons of the three  $p75^{\text{NTR}}$  genotypes are clearly illustrated in the respective Sholl plots. In contrast to the marked effect of the  $p75^{\text{NTR}}$  genotype on proNGF-promoted neurite growth, there was little difference in the extent of mNGF-promoted neurite growth between wild-type and  $p75^{\text{NTR}}$ -deficient neurons. Although there was a small (19%), statistically significant decrease in neurite length in  $p75^{\text{NTR}}$ -deficient neurons compared with wild-type neurons ( $P < 0.01$ ), there were no significant differences in branching (Fig. 5D). These small differences in neurite growth cannot be accounted for by the known modest differences in neuronal survival between wild-type and  $p75^{\text{NTR}}$ -deficient postnatal SCG neurons (Lee et al., 1994b), because Boc-D-FMK was included in the medium to ensure similar levels of survival in all cultures (confirmed by cell counts, not shown). These results demonstrate that  $p75^{\text{NTR}}$  is crucial for proNGF-promoted neurite growth but not for mNGF-promoted neurite growth from postnatal SCG neurons. The clear gene dosage effect observed in cultures established from heterozygous mice suggests that the extent of the response to proNGF is dependent on the level of  $p75^{\text{NTR}}$  expressed.

#### ProNGF does not activate either TrkA or NF- $\kappa$ B in postnatal sympathetic neurons

Although proNGF is generally not thought to bind TrkA directly (Boutillier et al., 2008), several studies have reported TrkA activation in PC12 cells in response to treatment with cleavage-resistant proNGF (Fahnestock et al., 2004; Masoudi et al., 2009). We examined whether TrkA participates in the neurite growth-promoting actions of proNGF in postnatal SCG neurons by first ascertaining whether proNGF activates TrkA in these neurons. P5 SCG neurons were grown overnight without NGF in medium containing Boc-D-FMK before the neurons were treated with either proNGF or mNGF. Western blot analysis of cell lysates with a specific anti-phosphotyrosine TrkA antibody revealed that mNGF promoted TrkA phosphorylation within 5 minutes of exposure. By contrast, there was no indication of enhanced TrkA phosphorylation following proNGF treatment at intervals up to 60 minutes (Fig. 6A). These results suggest that mNGF, but not proNGF, activates TrkA in postnatal SCG neurons.

We also examined the participation of TrkA in the neurite growth-promoting effects of mNGF and proNGF by studying the consequences of transfecting P5 SCG neurons with siRNA directed against TrkA. Whereas neurons transfected with TrkA siRNA displayed a substantial reduction in mNGF-promoted neurite growth compared with neurons transfected with a scrambled control RNA, TrkA siRNA had no significant effect on the magnitude of proNGF-promoted neurite growth compared with control-transfected neurons (Fig. 6B). These results suggest that TrkA plays a major role in mediating the neurite growth-promoting effects of mNGF in postnatal SCG neurons, but plays no clear role in mediating the neurite growth-promoting effects of proNGF.



**Fig. 5. The ability of proNGF to promote the growth of postnatal sympathetic neurites depends on p75<sup>NTR</sup>.** (A) Level of p75<sup>NTR</sup> mRNA relative to reference mRNAs in the SCG at E17 to P5. (B) Representative photomicrographs of calcein-AM-stained SCG neurons from P3 p75<sup>NTR</sup>+/+ (+/+), p75<sup>NTR</sup>+/- (+/-) and p75<sup>NTR</sup>-/- (-/-) littermates cultured for 24 hours with 10 ng/ml proNGF in medium containing 25 μM Boc-D-FMK. Scale bar: 50 μm. (C,D) Bar charts of neurite length and branching and Sholl analysis from at least three separate data sets of cultures of these neurons cultured with either 10 ng/ml proNGF (C) or 10 ng/ml mNGF (D). The mean±s.e.m. are shown. \*\**P*<0.01; \*\*\**P*<0.0001, statistical comparison with either E17 (A) or p75<sup>NTR</sup>+/+ (C,D).

Because mNGF is known to activate the transcription factor NF-κB by a p75<sup>NTR</sup>-dependent mechanism (Carter et al., 1996), and because NF-κB has been implicated in regulating neurite growth (Gutierrez and Davies, 2011), we investigated the influence of proNGF on NF-κB transcriptional activity in SCG and trigeminal neurons. To do this, we transfected freshly dissociated neurons with a reporter construct in which GFP expression is under the control of a series of κB regulatory elements (Gutierrez et al., 2005). The neurons were then plated in Boc-D-FMK-supplemented medium with either mNGF, proNGF or with no factors. After 24 hours, quantification of the NF-κB reporter signal revealed that mNGF caused an approximately threefold increase in both SCG and trigeminal neurons but proNGF had no effect on the signal in either neurons (Fig. 6C,D). This suggests that p75-dependent proNGF-promoted neurite growth is not dependent on NF-κB signalling.

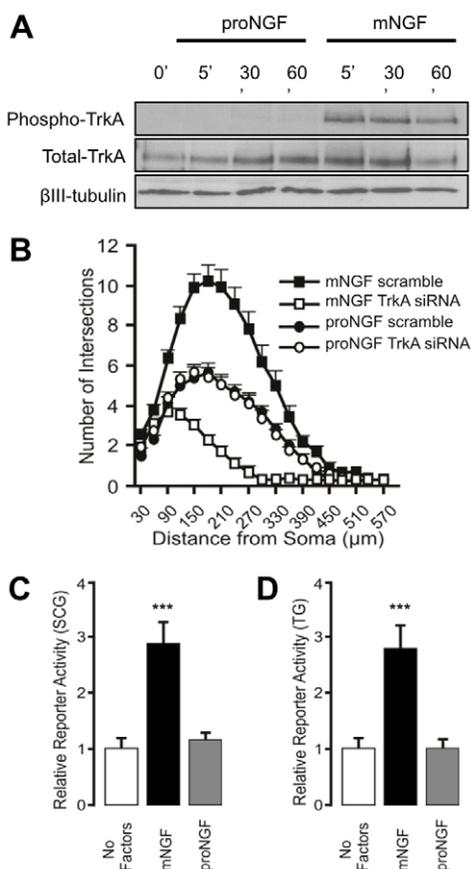
## DISCUSSION

We report a marked and surprising effect of proNGF on selective promotion of the growth and branching of the neurites of a subset of NGF-dependent neurons during the stage in development when these neurites are normally branching extensively within their targets. During the early postnatal period, proNGF promotes the growth and branching of SCG sympathetic neurites but has no effect on the growth of trigeminal sensory neurites. The ability of proNGF to promote sympathetic neurite growth begins at birth and increases markedly in magnitude during early postnatal development. By P5,

proNGF has become almost as potent and efficacious as mNGF in its capacity to promote the growth of sympathetic neurites. The capacity of mNGF to enhance the growth of sympathetic and sensory neurites appears earlier in fetal development and displays only a modest increase in magnitude throughout early postnatal development. These findings reveal a potent, highly selective, age-related effect of proNGF on neurite growth.

The correlation between the increasing level of p75<sup>NTR</sup> expression in SCG neurons and the acquisition of the neurite growth-promoting function of proNGF during postnatal development, together with our demonstration that homozygous deletion of the p75<sup>NTR</sup> gene completely eliminates the ability of proNGF to promote neurite growth, suggests that p75<sup>NTR</sup> is essential for this function of proNGF. Furthermore, our finding that the magnitude of proNGF-induced neurite growth is halved in SCG neurons obtained from heterozygous neonates suggests that the level of p75<sup>NTR</sup> expression is a crucial determinant of the magnitude of responsiveness of postnatal SCG neurons to proNGF.

Studies carried out on a variety of neurons have revealed that p75<sup>NTR</sup> can have opposing effects on neurite growth depending on the ligand and neuron. Perhaps the clearest example of p75<sup>NTR</sup> mediating enhanced neurite growth in response to mature neurotrophins in the absence of Trk signalling is observed in ciliary neurons, which express p75<sup>NTR</sup> but not Trks (Yamashita et al., 1999). Here, we observe a small statistically significant reduction in the effect of mNGF on neurite length from the SCG neurons of



**Fig. 6. proNGF-promoted neurite growth from postnatal SCG neurons does not depend on activation of either TrkA or NF- $\kappa$ B.**

(A) Representative western blot showing the levels of Tyr-490 phosphorylated TrkA following stimulation of P5 SCG neurons with either 10 ng/ml mNGF or 10 ng/ml cleavage-resistant murine proNGF for the times indicated. 0' represents unstimulated neurons. (B) Sholl plot showing neurite outgrowth from P5 SCG neurons transfected with either siRNA against TrkA or a scrambled control RNA and cultured for 24 hours with either 10 ng/ml mNGF or 10 ng/ml cleavage-resistant murine proNGF in medium containing 25  $\mu$ M Boc-D-FMK. (C,D) Bar charts of the number of fluorescent SCG (C) and trigeminal (D) neurons 24 hours after transfection with the NF- $\kappa$ B reporter. The numbers of fluorescent neurons in cultures treated with either mNGF or cleavage-resistant murine proNGF are expressed relative to the number of fluorescent neurons in untreated cultures (normalised to 1). Data obtained from three separate experiments. In each experiment, the total number of fluorescent cells for each condition was assessed in 16 wells of a standard 96-well plate. The mean $\pm$ s.e.m. is shown. \*\*\* $P$ <0.0001, ANOVA with Bonferroni-Holm post-hoc test.

$p75^{NTR-/-}$  mice compared with those of  $p75^{NTR+/+}$  mice. This is similar to the small statistically significant reduction in the survival response of  $p75^{NTR}$ -deficient neurons to mNGF compared with wild-type neurons (Davies et al., 1993; Lee et al., 1994b), and may reflect a modulatory role of  $p75^{NTR}$  on Trk signalling. By contrast, the binding of mature BDNF to  $p75^{NTR}$  causes growth cone collapse and degeneration in cultured sympathetic neurons *in vitro* and is required for axon pruning *in vivo* (Kohn et al., 1999; Singh and Miller, 2005; Singh et al., 2008). ProBDNF also causes growth cone collapse in DRG and cortical neuron cultures by a  $p75^{NTR}$ -dependent mechanism (Sun et al., 2012) and proNGF causes growth cone collapse in cultured hippocampal neurons by binding to

$p75^{NTR}$  (Deinhardt et al., 2011).  $p75^{NTR}$  also participates in a receptor complex with NgR1 (also known as RTN4R) and LINGO-1 to mediate neurite outgrowth inhibition by Nogo (also known as RTN4), myelin-associated glycoprotein and oligodendrocyte myelin glycoprotein (Wang et al., 2002; Yamashita et al., 2002; Mi et al., 2004).  $p75^{NTR}$  has also been implicated in mediating neurite growth inhibition to ephrins A and B2 and semaphorins 3A and 3F (Lim et al., 2008; Naska et al., 2010). These positive and negative influences of  $p75^{NTR}$  on neurite growth might explain why  $p75^{NTR-/-}$  mice display either enhanced or reduced axon growth from different kinds of neurons *in vivo* (Yamashita et al., 1999; Walsh et al., 1999; Bentley and Lee, 2000). An intriguing question arising from our study is why there is such a marked difference in the response of SCG and trigeminal neurons to proNGF. This cannot simply be explained by differences in the expression of  $p75^{NTR}$ , as both kinds of neurons express similar levels of  $p75^{NTR}$  mRNA at P3 and P5 (data not shown). Alternatively, it is possible that these neurons differ in the expression of a  $p75^{NTR}$  co-receptor that is required for or inhibits proNGF-promoted neurite growth or might differ in downstream signal transduction pathways that link  $p75^{NTR}$  ligation by proNGF with neurite extension.

Although it is clear that  $p75^{NTR}$  is essential for the neurite growth-promoting effects of proNGF on postnatal SCG neurons, TrkA function is apparently not required. ProNGF does not activate TrkA in P5 SCG neurons, even after 60 minutes of stimulation, whereas mNGF causes rapid and marked phosphorylation of TrkA under identical experimental conditions. Furthermore, siRNA against *TrkA* mRNA selectively reduces the neurite growth-promoting effects of mNGF, but has no effect on the capacity of proNGF to promote neurite growth. These findings contrast with certain reports of neurotrophic activities of proNGF observed in neurons and cell lines. Cleavage-resistant proNGF has been reported to promote the survival of cultured P1 mouse SCG neurons just as effectively as mNGF and to increase the percentage of process-bearing neurons in these cultures (Fahnestock et al., 2004; Masoudi et al., 2009). Although proNGF activates TrkA in the PC12 tumour cell line (Fahnestock et al., 2004; Masoudi et al., 2009), this depends on its endocytosis, cleavage by furin-like enzymes and the generation of mNGF (Boutillier et al., 2008). In addition to finding no evidence for TrkA activation by proNGF in P5 SCG neurons, we also find that proNGF does not enhance the survival of these neurons over a wide range of ages. In this regard, our findings accord more closely with *in vitro* studies of adult rat SCG neurons in which proNGF was found to promote neurite growth without enhancing neuronal survival (Al-Shawi et al., 2008). The observations of differing cellular responses to proNGF could be due to differences in the mutated proNGF used or to methodological differences employed in the various studies. For example, whereas Fahnestock et al. (Fahnestock et al., 2004) used a cleavage-resistant proNGF that has the same amino acid substitution that disrupts the tetrabasic cleavage site (-1, R to G) as one of the forms of cleavage-resistant proNGF used in our study, they cultured SCG neurons at extremely high density (20,000 neurons per 35 mm dish), and observed an increase in the proportion of process-bearing neurons as well as enhanced survival. The cleavage-resistant proNGF used by Lee et al., which was reported to promote apoptosis but not neurite growth from SCG neurons (Lee et al., 2001), has amino acid substitutions at -2, -1, 118 and 119 (RR to AA in both cases). Using this same cleavage-resistant proNGF, one that has different multiple amino acid substitutions (-73 and -72, RR to AA; -43 and -42, KKRR to KAAR; and -2 and -1, KR to AA) (Pagadala et al., 2006) and one with two other substitutions (-1 and +1 RS to AA), Masoudi found

that each proNGF enhanced the survival of SCG neurons, rather than promoting apoptosis, and increased the number of neurons with processes (Masoudi et al., 2009). However, SCG neurons were also grown at exceptionally high density in these studies. It is important to note that in our study, two different forms of cleavage-resistant proNGF and native proNGF elicited neurite growth of the same magnitude and neuronal specificity.

The broader physiological significance and developmental relevance of our intriguing and unexpected findings are unclear, but it is possible that the highly selective effects of proNGF on neurite growth during the phase of target field innervation may assist in establishing regional differences in the distribution and density of the axon terminals of different kinds of NGF-dependent neurons in various tissues. Studies using compartment cultures have demonstrated that mNGF acts locally on neurite growth and promotes only the growth of neurite branches exposed to it (Campenot, 1987). Similarly, using compartment cultures, we also show that proNGF acts locally on neurite growth. Moreover, we demonstrate that mNGF and proNGF have synergistic actions on SCG neurite growth and branching at very low concentrations. Thus, it is possible that the relative availability of mNGF and proNGF in different tissues or different cellular locations within such tissues could selectively regulate the relative innervation density by subsets of NGF-responsive neurons that have distinctive responses to mNGF and proNGF. Selective regional defects in innervation have indeed been reported in mice lacking p75<sup>NTR</sup> (Lee et al., 1992; Lee et al., 1994a). However, it is important to note that such defects in innervation might arise not only from the altered responses of p75<sup>NTR</sup>-deficient neurons to neurotrophins, but because the absence of p75<sup>NTR</sup> on pathways along which axons grow towards their targets (Yan and Johnson, 1988) may affect the presentation of neurotrophins to growing axons in p75<sup>NTR</sup> null mutant mice (Lee et al., 1994b). Although we made numerous attempts to detect the presence of proNGF in media conditioned by relevant SCG target tissues at the appropriate stage of development, we failed to obtain convincing results because limited material precluded necessary analytical steps, such as immunoprecipitation experiments, prior to western blot analysis.

SCG and trigeminal neurons innervate many of the same tissues of the head, and, within these areas, tissues or regions in which extracellular proNGF is relatively more abundant than mNGF might exhibit greater local growth and branching of SCG neurites without additional growth of trigeminal neurites. If so, this would represent a novel mechanism for regulating the density and distribution of different subsets of neurites within the tissue they innervate that is governed by regional differences in the proteolytic processing and/or secretion of precursor and mature forms of the same factor rather than on regional differences in the synthesis of different neurotrophic factors.

#### Acknowledgements

We thank Yves Alain Barde for the p75<sup>NTR</sup> mutant mice and GlaxoSmithKline for the cleavage-resistant human proNGF.

#### Funding

This work was supported by a grant from the Wellcome Trust [grant number 085984 to A.M.D.]. Deposited in PMC for immediate release.

#### Competing interests statement

The authors declare no competing financial interests.

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