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Neurotrophic actions initiated by proNGF in adult sensory neurons may require peri-somatic glia to drive local cleavage to NGF

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Abstract

The nerve growth factor (NGF) precursor, proNGF, is implicated in various neuropathological states. ProNGF signals apoptosis by forming a complex with the receptors p75 and sortilin, however it can also induce neurite growth, proposed to be mediated by the receptor of mature NGF, tyrosine kinase receptor A (TrkA). The way in which these dual effects occur in adult neurons is unclear. We investigated the neurotrophic effects of proNGF on peptidergic sensory neurons isolated from adult mouse dorsal root ganglia (DRG) and found that proNGF stimulated neurite extension and branching, requiring p75, sortilin and TrkA. Neurite growth rarely occurred in sortilin-expressing neurons but was commonly observed in TrkA-positive, sortilin-negative neurons that associated closely with sortilin-positive glia. ProNGF was unable to induce local trophic effects at growth cones where sortilin-positive glia were absent. We propose that in adult sensory neurons the neurotrophic response to proNGF is mediated by NGF and TrkA, and that peri-somatic glia may participate in sortilin- and p-75 dependent cleavage of proNGF. The potential ability of local glial cells to provide a targeted supply of NGF may provide an important way to promote trophic (rather than apoptotic) outcomes under conditions where regeneration or sprouting is required.

Keywords

neurotrophin; sortilin; p75; satellite cells; dorsal root ganglion; nociceptor

Introduction

The neurotrophin family, comprising nerve growth factor (NGF) and related proteins, regulates survival, differentiation and axon growth in populations of peripheral and central neurons (Lewin and Barde, 1996; Huang and Reichardt, 2001; Ernsberger, 2009). These actions occur via receptor tyrosine kinases (Trks), and p75 that modulates tyrosine kinase receptor (Trk) receptor binding (Barbacid, 1994; Kaplan and Miller, 2000). Neurotrophins also promote axon regeneration in injured neurons, so understanding their mechanism in adult systems may suggest therapeutic targets for repair (Chen et al., 2007).

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Neurotrophins are synthesised as precursors (proneurotrophins) that are cleaved by proteases to the mature form (Seidah et al., 1996; Lee et al., 2001). Proneurotrophins are important signaling molecules in their own right, with many studies showing that proNGF binds to p75 and the Vps10p domain protein, sortilin, to induce apoptosis (Lee et al., 2001; Nykjaer et al., 2004). The theme emerging is that proNGF acts as a death-inducing ligand under pathological conditions, including Alzheimer's disease and spinal cord injury (Fahnestock et al., 2001; Harrington et al., 2004; Nykjaer et al., 2004; Volosin et al., 2006; Domeniconi et al., 2007; Jansen et al., 2007; Yune et al., 2007; Al-Shawi et al., 2008). This has led to the hypothesis that mature and proneurotrophins exert opposing effects using distinct (i.e., Trk vs. p75/sortilin) signaling pathways (Teng et al., 2010). However, there are also reports of neurotrophic proNGF actions, potentially mediated by TrkA (Fahnestock et al., 2004; Al-Shawi et al., 2008; Masoudi et al., 2009). It is not known what determines the type of action (neurotrophic vs. apoptotic) of proNGF, although this may be influenced by the ratio of TrkA to p75/sortilin expressed at the cell surface (Fahnestock et al., 2004; Al-Shawi et al., 2008; Clewes et al., 2008; Masoudi et al., 2009). It has also been reported that proNGF does not bind TrkA (Nykjaer et al., 2004; Boutilier et al., 2008), or activates this receptor more weakly than NGF (Clewes et al., 2008). To reconcile these observations, based on cell line experiments it has been proposed that TrkA-dependent neurotrophic effects of proNGF require p75-mediated endocytosis and cleavage to mature NGF (Boutilier et al., 2008). The site and mechanism of this conversion has not been identified. In order to fully integrate the available mechanistic data it is important to recognise that it has been obtained from diverse cell types, including embryonic sympathetic, sensory and central neurons, as well as cell lines; these vary in the expression of each receptor type and their physiological functions and growth behaviours, so may also differ in their mechanisms of proNGF signalling.

ProNGF is a significant form of NGF in adult sensory neurons, peripheral target tissues and the spinal cord (Reinshagen et al., 2000; Bierl et al., 2005; Arnett et al., 2007; Buttigieg et al., 2007). Many sensory neurons in dorsal root ganglia (DRG) express one or more of the receptors, TrkA, p75, and sortilin (Wright and Snider, 1995; Arnett et al., 2007), raising the possibility of complex effects induced by proNGF. Our goal is to determine the mechanism of proNGF trophic actions in mature sensory neurons, as embryonic systems are more difficult to extrapolate to injury, regeneration and pain. In this study we found that neurotrophic responses to proNGF are dependent on TrkA, p75, and sortilin. Our results support a model in which sortilin- and p75-dependent cleavage of proNGF occurs in perisomatic glia, to produce NGF that activates TrkA in the adjacent neuronal soma. The proximity and activity of glial cells may determine whether proNGF has pro-apoptotic or trophic actions in adult sensory neurons.

Materials and Methods

DRG cultures

All experiments were performed in accordance with the Code of Practice for the Care and Use of Animals for Experimental Purposes (National Health & Medical Research Council of Australia) and approved by the Animal Ethics Committees of the University of Sydney and Royal North Shore Hospital. The ARRIVE guidelines have been followed for all aspects of the study.

A total of 46 six- to eight-week old male C57BL/6J mice (Gore Hill Research Laboratories, Sydney, Australia) were used. Mice were housed in a 12h light/dark cycle under conditions controlled for temperature and humidity, and access to standard rodent chow and water *ad libitum*. Animals were deeply anaesthetised with sodium pentobarbitone (40 mg / kg i.p.) and decapitated before dissecting DRG from all spinal levels. DRGs were incubated for 1 hour in 0.15% collagenase (Worthington type I, ScimaR, Templestow, Victoria, Australia)

followed by a further 1 hour in 0.15% collagenase and 0.25% trypsin (Sigma, St Louis, MO, USA). DRGs were washed in Tyrodes solution (130 mM NaCl, 20 mM NaHCO₃, 10 mM HEPES, 3 mM KCl, 10 mM glucose, 4 mM CaCl₂, 1 mM MgCl₂, 0.5% antimycotic/antibiotic, pH 7.4 (Invitrogen, Mt Waverly, Victoria, Australia) then gently triturated using a fire-polished Pasteur pipette and centrifuged at 400×g for 5 minutes. The pellet was re-suspended in Neurobasal A medium with 2% B27 supplement, 100 U penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B, and 200 mM Glutamax I (all from Invitrogen). Dispersed cells were plated onto glass coverslips coated with poly-DL-ornithine (Sigma, St. Louis, MO, USA) and laminin (Invitrogen).

Neurotrophins were added to cultures after plating and remained in the culture medium for the duration of the experiment (approximately 20 hours). All cultures included an internal control (i.e., no added neurotrophic factor) to identify any basal neuronal growth; in contrast to embryonic neurons, survival of adult sensory neurons in culture does not require neurotrophic factors and many neurons begin to grow neurites within the first day of culture (Kalous and Keast, 2010).

For neurite growth experiments, chemical inhibitors or blocking antibodies (TrkA/Fc chimera, 4 ng/ml; anti-mouse p75 (1:400); anti-mouse sortilin (1:200) were added to the culture medium after plating, 50 minutes prior to the addition of NGF or proNGF. At the end of the experiment, neurons were fixed in 4% phosphate-buffered formaldehyde (pH 7.4) for 30 minutes.

For growth cone experiments, cultures were incubated overnight in NGF-supplemented medium; after approximately 20 hours, the medium was removed and replaced with fresh NGF-free medium for 2 hours (trophic factor “starvation”) prior to adding proNGF or NGF for a further 2 hours. This period of trophic factor deprivation was essential for initiating growth cone collapse. Growth cone collapse was measured at the same time point in all cultures, i.e. in controls this starvation period lasted for 4 hours. Neurons were fixed in pre-warmed 4 % phosphate buffered formaldehyde (pH 7.4) containing 10 % sucrose at 37°C for 30 minutes.

Western Blot analysis

In six cultures, the medium was harvested 20 h after treatment with proNGF or NGF. After centrifugation, 100 µl of medium was added with 25 µl 5X sample buffer (250 mM Tris pH 6.6, 50% glycerol, 10% SDS, 15% β-mercaptoethanol, 0.2 mg/ml bromophenol blue), boiled for 5 minutes, and run in a 15% SDS-polyacrylamide gel. Proteins in the gel were transferred to a 0.45 µm PVDF membrane, blocked with milk (5% in Tris-buffered saline with 0.2% Tween 20), and incubated with anti-NGF M20 antibodies (1:1000, 2 hours at 20–25°C, Santa Cruz Biotechnology), followed by horseradish peroxidase-conjugated anti-rabbit secondary antibodies (1:5000, 1 hour at 20–25°C, Calbiochem). Bands were visualized by enhanced chemiluminescence (ECL, Amersham) and scanned.

Immunohistochemistry

DRG cultures—Neurons were incubated for 2 hours in combinations of the following antisera: goat anti-rat CGRP antibody (1:1500, AbD serotec, Kidlington, Oxford, UK, Cat. No. 1720-9007, lot 0906), mouse monoclonal anti-β-tubulin isotype III (1:200; Sigma, Cat. No. T8660, lot 055K4771), rabbit anti-mouse p75 (1:500; Chemicon, Cat. No. AB1554, lot 24120100), goat anti-mouse sortilin (1:100; R&D Systems, Cat. No. AF2934, lot VYL01), rabbit anti-rat TrkA (1:100; Upstate Biotech, Lake Placid, NY, USA, Cat. No. 06-574, lot 22778). Phalloidin conjugated to Alexa Fluor 488 (1:40, Molecular Probes, Invitrogen) was used for growth cone collapse experiments to visualise growth cone morphology. Donkey

anti-goat Cy3 (1:1000; Jackson Immunoresearch Laboratories, West Grove, PA, USA), donkey anti-mouse Cy2 (1:400; Jackson) or donkey anti-rabbit Alexa Fluor 488 (1:2000; Molecular Probes, Eugene, OR, USA), were used to visualise markers in neurite outgrowth experiments. For growth cone collapse experiments, donkey anti-goat Cy3 and horse anti-mouse biotin (1:300; Vector Laboratories, Burlingame, CA, USA) were used, followed by AMCA-avidin D (1:100, Vector). 4',6-diamidino-2-phenylindole (DAPI, 1 µg/ml; Sigma) was used as a nuclear counterstain. We identified unhealthy or dead neurons as having irregular nuclei with condensed or peripheral chromatin. Such cells were rejected from analysis. In some figures the grey scale was inverted to allow better visualisation of neurites and growth cone structure. Minor brightness and contrast adjustments were made using Adobe Photoshop CS4 (Adobe Systems, San Jose, CA, USA) to best represent the immunostaining as seen under the microscope.

DRG sections—Male C57BL/6J mice (8 weeks old) were deeply anaesthetised with sodium pentobarbitone (40 mg/kg, i.p.) and perfused transcardially with 0.9 % saline containing 1.8 IU/ml heparin and 1 % sodium nitrite, followed by 4 % phosphate-buffered formaldehyde. DRG from cervical, thoracic, and lumbar levels were removed and post-fixed for 1 hour, then cryoprotected in 30 % sucrose in PBS overnight. DRG were cut into 12 µm sections using a cryostat and thaw-mounted onto gelatin-subbed glass slides. Slides were washed in PBS then blocked and permeabilised for 2 hours in 10 % horse serum and 0.1 % triton X-100 in PBS. DRG were incubated overnight in various combinations of goat anti-mouse sortilin antibody (1:100, as above), rabbit anti-rat CGRP (calcitonin gene-related peptide; 1:1500, Chemicon International, Cat. No. AB5920, lot 25010374), rabbit anti-bovine GFAP (glial fibrillary acid protein; 1:1000, Dako, Glostrup, Denmark, Cat. No. Z0334), and rabbit anti-mouse p75 (1:2000, as above). After washing, sections were incubated for 3 hours in donkey anti-goat Cy3 (1:1000; Jackson Immunoresearch Laboratories) and donkey anti-rabbit fluorescein isothiocyanate (FITC; 1:100; Jackson), then coverslipped in bicarbonate-buffered glycerol (pH 8.6). Sections used for confocal microscopy were coverslipped in Vectashield (Vector Laboratories).

Microscopy

For assessment of neurite growth and status of growth cones, cultured neurons were viewed and analysed under an Olympus BX51 microscope (Olympus Australia, Melbourne, Australia). Digital images were acquired using an RT Spot camera (Diagnostic Instruments, Sterling Heights, MI, USA) and Image Pro Plus 4.5 software (Media Cybernetics, Carlsbad, CA, USA). Neurite initiation was quantified as the percentage of neurons with one or more neurites longer than the diameter of the cell body. For each treatment, 200 neurons per replicate were counted. To analyse neurite elongation and branching, images of 40–50 randomly selected neurons per coverslip were acquired. Neurite length and branching were analysed using HCA-Vision image analysis software (CSIRO, North Ryde, NSW, Australia, <http://www.hca-vision.com>). For growth cone collapse experiments, 50 neurite-bearing neurons per coverslip were assessed. Growth cone collapse was quantified as the percentage of neurons with > 50% of their growth cones displaying a collapsed morphology. Laser-scanning confocal images of DRG sections and cultures were acquired using either an Olympus Fluoview FV300 system with Olympus Fluoview version 4.3 software (Olympus, Japan), or a Leica TCS SP5 system with Leica Application Suite software (Wetzlar, Germany). Excitation wavelengths of 488 nm or 543 nm were used to detect FITC/Cy2 or Cy3, respectively, employing sequential scanning. The optical section thickness was 1.5 µm.

Statistical analysis

Replicates for each experiment were obtained from separate cultures from different animals, where n equals the number of replicates performed. At least 3 replicates for each experiment

were analysed. Data are plotted as the mean $\pm$ standard error of the mean. Statistical analyses were performed using GraphPad Prism 5.0a (San Diego, CA). Paired t-tests or one-way ANOVA followed by Bonferroni *post hoc* tests on selected pairs were performed to detect differences in control versus treated groups. $P < 0.05$ was regarded as statistically significant.

Materials

Activity and properties of recombinant proNGF have been described previously (Lee et al., 2001). Purified NGF from mouse submaxillary glands was purchased from Sigma (St Louis, MO, USA; Cat. No. N6009). Recombinant rat TrkA/Fc chimera (4 ng/ml) was purchased from R&D Systems (Minneapolis, MN, USA; Cat. No. 1056-TK) and functional blocking anti-mouse p75 polyclonal antibody (1:400) from Chemicon International (Temecula, CA, USA; Cat. No. AB1554, lot 0606031680). LY294002 (50 μ M), wortmannin (10 μ M), U0126 (20 μ M), PD98059 (50 μ M), SU6656 (2 μ M), and K252a (100 nM) were all purchased from Biomol Research Laboratories (Plymouth Meeting, PA, USA). Batimastat and doxycycline were purchased from Tocris Bioscience (Ellsville, MO, USA).

Results

ProNGF has neurotrophic actions on adult peptidergic sensory neurons

We first examined the effects of NGF and recombinant furin-resistant proNGF (Lee et al., 2001) in both CGRP-positive and CGRP-negative populations of DRG neurons *in vitro*. In adult rodent DRG, almost all CGRP neurons express TrkA, and vice versa (Averill et al., 1995), and in our cultures, 35–40% of DRG neurons express CGRP (Kalous and Keast, 2010). Some neurons grew neurites in untreated control cultures (16.1 ± 1.9 % of all neurons, $n=12$), and approximately half of these were CGRP-positive. This will be referred to as “basal growth”.

NGF increased the proportion of neurons that possessed neurites (“neurite initiation”), total neurite length, maximum neurite length, and branching in the CGRP-positive population (Fig. 1A, B, D–F). Therefore, the increase in total neurite length per neuron was due to increased complexity as well as increased linear extension of the neurite field. Neurite initiation in CGRP-negative neurons was unaffected by NGF treatment (8.0 ± 1.2 % control vs. 7.9 ± 0.7 % NGF-treated, $P > 0.05$, $n = 12$). In contrast to NGF, in the CGRP-positive population proNGF did not significantly increase the proportion of neurons that possessed neurites (Fig. 1C), using physiological concentrations previously shown to have neurotrophic actions in sympathetic neurons *in vitro* (Fahnestock et al., 2004; Nykjaer et al., 2004). However, even though there was no stimulation of neurite initiation, proNGF treatment increased neurite elongation and branching in CGRP-positive neurons (Fig. 1A, D–F). The magnitude of this response was similar to NGF. ProNGF did not increase the proportion of neurons possessing neurites, strongly suggesting that the response of increased neurite elongation and branching must have occurred in neurons that had already begun to grow neurites in overnight culture (i.e. basal growth).

Around 5–10% of CGRP-negative neurons underwent neurite growth in untreated control cultures, but proNGF did not affect neurite length and branching in these neurons (total length, control 933.8 ± 9.4 μ m vs. proNGF 889.3 ± 32.6 μ m; maximum length, control 180.2 ± 8.0 μ m vs. proNGF 193.7 ± 12.2 μ m; branch points control 16.4 ± 0.3 μ m vs. proNGF 12.9 ± 0.4 μ m; all $n = 3$). ProNGF also had no effect on neurite initiation in the CGRP-negative population (control 14.6 ± 2.7 % vs. proNGF-treated 13.4 ± 3.9 %, $P > 0.05$, $n = 4$).

Western blot analysis of culture medium harvested at the end of six experiments failed to detect NGF following 20 h treatment with 10 ng/ml proNGF (Examples shown in Fig 1G,

lanes 2, 3, 7, and 9). However, NGF added to cultures at the beginning of the culture period was easily detectable in the media 20 h later (Fig. 1G, lanes 4, 5). This indicates that if large quantities of NGF had been produced from proNGF cleavage, some should still be detectable at this time. This suggests that the growth response to proNGF may occur through mechanisms other than conversion to NGF that accumulates in the culture media.

The neurotrophic action of proNGF is dependent on sortilin, p75 and TrkA

To determine the mechanism of proNGF action in adult sensory neurons we first conducted pharmacological studies to define the receptors required for neurite elongation and branching. Functional blocking antibodies against sortilin (Kim and Hempstead, 2009) or p75 (Fiorentini et al., 2002) inhibited neurite elongation (Fig. 2A, C, D) and branching (not shown) in proNGF-treated cultures but had no effect on NGF-treated or control cultures. Therefore, sortilin and p75 were required for the neurotrophic response to proNGF but not to NGF. If the proNGF response involved prior cleavage to NGF, we would expect that the typical receptor target of NGF, TrkA, was also required. Pre-treatment with the functional blocker, recombinant rat TrkA/Fc chimera, or the broad-spectrum tyrosine kinase inhibitor, K252a, significantly attenuated or blocked neurite elongation (Fig. 2A, B, E–H) and branching (not shown) in both proNGF- and NGF-treated cultures. Together, these experiments show that proNGF actions on neurite elongation and branching require all three receptors (sortilin, p75 and TrkA) and may require cleavage to NGF. Treatment with these receptor antibodies had no effect on basal growth parameters, demonstrating that our results are unlikely to be confounded by significant amounts of endogenous neurotrophic factors produced within our cultures.

ProNGF-sensitive neurons do not express sortilin, but are closely associated with sortilin-positive peri-somatic glia

To understand how all three receptors are involved in the proNGF response, we then investigated the immunohistochemical properties of neurons that responded to proNGF *in vitro*. We specifically asked if proNGF-induced neurite elongation and branching were likely to involve neuronal sortilin. As indicated above, proNGF did not stimulate growth of new neurites (neurite initiation) but did stimulate elongation and branching of neurites in the population of neurons that underwent basal growth, i.e. these neurons grew neurites without trophic factor treatment. Therefore we focused our first set of studies on this group of neurons by analysing control cultures.

In our control cultures (i.e., no NGF or proNGF treatment), we found that $21.8 \pm 1.5\%$ ($n=3$) of all tubulin-positive neurons expressed sortilin-immunoreactivity (IR). This proportion did not include a small number of sortilin-IR neurons that, based on their unhealthy appearance, were excluded from analysis. These latter neurons had rich sortilin-IR, however their nuclei were often condensed or irregular in shape (DAPI staining); in addition, they failed to grow neurites. To our surprise, very few of the healthy, sortilin-positive neurons grew neurites in overnight culture ($7.3 \pm 2.8\%$; $n=3$) (Fig. 3A–H); moreover, in these sortilin-positive neurons that did grow neurites in the absence of neurotrophin, sortilin-IR was invariably dim. In summary, only a very small population ($\sim 1\%$, i.e. 7% of 21%) of all healthy cultured neurons expressed sortilin *and* were able to grow neurites in the absence of neurotrophin. This suggests that the neurotrophic effects (neurite elongation and branching) of proNGF on peptidergic neurons in our cultures may not be mediated by neuronal sortilin.

In contrast to the poor correlation between neuronal sortilin expression and basal neurite growth, many TrkA-positive (i.e., peptidergic) neurons underwent neurite initiation in the absence of neurotrophic factors. These comprised almost one-third ($27.2 \pm 1.0\%$; $n=3$) of TrkA-positive neurons. TrkA-positive neurons co-express CGRP (Averill et al., 1995) and

CGRP neurons comprise ~35–40% of all neurons in our cultures, so this population represents ~10% of all neurons.

During the course of this careful inspection of sortilin localisation in our cultures, we also noticed that many (but not all) of the glial cells expressed bright sortilin-IR, especially in their peri-nuclear cytoplasm. Sortilin-positive glia had a characteristic distribution, being located very close to the neuronal soma or proximal neurites but rarely near neurite terminals (Fig. 3A–D, I–L; 4A–H). Sortilin-positive glial cells were commonly associated with neurons that were sortilin-negative but TrkA- and p75-positive, and that had grown neurites overnight (Fig. 4A–H). That is, in TrkA-positive neurons there was a strong correlation between the presence of peri-somatic sortilin-positive glia and neurite initiation. The majority ($75.3 \pm 1.8\%$, $n=3$) of TrkA-positive somata that *had* initiated neurites in control cultures were closely associated with sortilin-positive glia, whereas only $35.2 \pm 2.2\%$ ($n=3$) of TrkA-neurons that had *not* grown neurites were associated with these cells. We also noted that many sortilin-positive glia showed low to moderate levels of p75-IR (Fig. 4I–L); this staining was granular and considerably less intense than p75-IR associated with neurons. Together, these observations support that neuronal sortilin is not involved prominently in the neurotrophic response to proNGF. However, these results suggest peri-somatic glia as alternative potential targets for sortilin-dependent signaling in our cultures.

Peri-somatic glia express proNGF receptors *in vivo*

In fixed cryosections of adult mouse DRG, cytoplasmic sortilin-IR was observed in many DRG neurons that were mainly of small and medium size (Fig. 5A–C). We did not quantify these neurons because immunostaining intensity varied widely between neurons and while there were many positively-stained neurons, for others it was difficult to determine with confidence whether or not they should have been regarded as positive. However, a previous study reported that approximately 70% of all neurons in mouse lumbar DRG express sortilin (Arnett et al., 2007). By performing dual-labeling for sortilin and CGRP, a marker for NGF-sensitive peptidergic neurons, we examined the potential for proNGF signalling in this population *in vivo*. Sortilin-IR was expressed by around half of the peptidergic neurons and, in turn, around half of all CGRP-IR neurons expressed sortilin (Fig. 5A–C). In addition, the majority of neuronal profiles, including those without cytoplasmic sortilin labelling, were bordered by a “halo” of punctate sortilin-IR. Dual-labelling with an antibody against the glial marker, GFAP, showed that these “halos” were satellite glial cells (Fig. 5D–F). Therefore, peri-somatic glia may also be important sites of sortilin signaling *in vivo*.

Matrix metalloproteinase activity may be required for the neurotrophic actions of proNGF

ProNGF can be cleaved by intracellular furins (Seidah et al., 1996), or by the secreted and transmembrane proteases plasmin and matrix metalloproteinases (MMPs) (Lee et al., 2001). Many DRG neurons and satellite glial cells express MMPs and tissue plasminogen activator *in vivo* (Yamanaka et al., 2004, 2005; Kawasaki et al., 2008; Huang et al., 2011). Since we used furin-resistant proNGF, we hypothesised that secreted/transmembrane proteases expressed by peri-somatic glial cells may have participated in cleavage of proNGF in our cultures, potentially providing a local supply of NGF to somata of adjacent TrkA-expressing neurons. We therefore tested if MMP activity in our cultures was required for the neurotrophic actions of proNGF (10 ng/ml) and showed that the broad-spectrum MMP inhibitor batimastat (5 μ M) abolished proNGF effect on total neurite length (control: 2673 ± 705 ; proNGF: 4258 ± 633 ; proNGF + batimastat: 2845 ± 325 μ m; $n=3$). The tetracycline antibiotic, doxycycline (10 μ M), inhibits MMPs but also affects many other signalling pathways and did not attenuate the proNGF-induced neurite growth (4423 ± 465 μ m; $n=3$).

ProNGF does not have neurotrophic actions at sites distant from peri-somatic glia

It is not feasible to completely remove glia from these short-term cultures, so to further investigate the role of peri-somatic glia in the neurotrophic action of proNGF, we took advantage of the observation that in our cultures, glia typically cluster around neuronal somata, but rarely near distal neurites or growth cones. Growth cone morphology is modulated by local NGF signalling (Connolly et al., 1987; Campenot, 1994). We predicted that if the neurotrophic actions of proNGF depend on local cleavage to NGF by peri-somatic glia, proNGF may be unable to modulate growth cone morphology in our cultures. To test this, we compared the actions of proNGF and NGF on growth cone morphology using a growth cone collapse assay. A neurite was judged to have a collapsed growth cone if the tip was tapered and lacked filopodia, and intact if lamellipodia and filopodia were present.

Cultures were incubated overnight in NGF-supplemented (1 ng/ml) medium to stimulate neurite outgrowth and growth cone elaboration (Fig. 6A). Under these conditions, approximately half of all neurite-bearing neurons displayed growth cones with a collapsed morphology on at least 50% of their neurites (Fig. 6E); a similar state was observed after treatment with 10 ng/ml NGF (not shown). After overnight incubation, the NGF-supplemented medium was replaced with fresh NGF-free medium for two hours, during which time the proportion of neurons with collapsed growth cones increased from approximately 50% to 80% (Fig. 6B,E). Mechanical forces during replacement of the medium did not contribute to growth cone collapse, as removing the overnight medium and replacing it back immediately did not change the proportion of neurons with collapsed growth cones ($47.9\% \pm 1.6$ no medium removal vs. $47.8\% \pm 2.9$ mechanical control, $n = 3$).

After incubation in NGF-free medium, cultures were then treated with either proNGF or NGF to compare their ability to restore collapsed growth cones. Treatment with NGF (10 ng/ml) for 2 hours induced growth cone formation, reversing the proportion of neurons with collapsed growth cones from approximately 80% to 50% (Fig. 6C,E). This action of NGF was dependent on TrkA signalling, as pretreatment with TrkA/Fc chimera prior to the addition of NGF blocked the reversal of growth cone collapse ($51.4\% \pm 4.5$ NGF vs. $84.8\% \pm 0.4$ TrkA/Fc chimera plus NGF). In contrast, treatment with proNGF (1 or 10 ng/ml) did not alter the proportion of neurons with collapsed growth cones (Fig. 6D,E), indicating that proNGF was unable to stimulate growth cone formation.

The neurotrophic actions of proNGF and NGF are dependent on similar downstream signalling mechanisms

Our results support a model of sortilin- and p75-dependent glial cleavage of proNGF to NGF, which then acts on nearby TrkA-positive neurons to cause neurite growth. This model predicts that neurite elongation and branching initiated by proNGF and NGF would utilise the same downstream neuronal signaling mechanisms. Therefore, we examined enzyme pathways that typically mediate the Trk A-dependent neurotrophic actions of NGF. We blocked the phosphatidylinositol-3 (PI3)-kinase pathway in NGF- and proNGF-treated cultures using the inhibitors LY294002 and wortmannin, and blocked the upstream kinase Src using SU6656. SU6656 blocked neurite length (Fig. 7A–C) and branching (not shown) in both NGF- and proNGF-treated cultures, without affecting neurite growth in control cultures. LY294002 and wortmannin reduced total (Fig. 7A, D, E) and maximum neurite length in NGF- and proNGF-treated cultures, but only wortmannin reduced branching (not shown). The reduction in branching in the presence of wortmannin but not LY294002 suggests an involvement of PI 4-kinase, which is inhibited only by the former (Meyers and Cantley, 1997).

NGF also activates mitogen-activated protein kinase (MAPK) that promotes neurite growth (Riccio et al., 1999; Markus et al., 2002; Paveliev et al., 2007; Tucker et al., 2008). In our experiments, two different inhibitors of the MAPK MEK, PD98059 and U0126, did not affect neurite growth in control or NGF-treated cultures (Fig. 7A, F, G), although these concentrations used are effective in blocking MEK in adult rodent DRG cultures (Purves-Tyson and Keast, 2004). In contrast, U0126 but not PD98059, slightly reduced proNGF-induced branching (not shown). This might be explained by inhibitory actions of U0126 on p70 S6 kinase, downstream of PI 3-kinase (Fukazawa and Uehara, 2000). These results show that the neurotrophic response to proNGF requires initiation of signaling cascades typically activated by trkA.

Discussion

Whereas many studies have investigated the role of proNGF as a death-inducing ligand, its potential to drive neurotrophic events is less well understood, especially in adult neuronal systems. We used growth assays, pharmacological and immunocytochemical approaches to investigate the site and mechanism of proNGF-induced neurite growth in sensory neurons of adult mice. Here, NGF has been strongly implicated in diverse responses to injury, including axon regeneration (Segal, 2003; Chen et al., 2007) and, in the cases of nociceptors, sensitisation (Pezet and McMahon, 2006).

We showed that in isolated mature sensory neurons, proNGF has neurotrophic actions that require sortilin, TrkA and p75. These three receptors are expressed in partially overlapping populations of adult DRG neurons, as shown in the present study and by Arnett et al. (2007). The major finding of our study is that the neurotrophic action of proNGF required sortilin but the locus of growth did not correlate spatially with neuronal sortilin expression. Instead, we identified that many glial cells were sortilin-immunoreactive and commonly associated with the soma and proximal neurites of those neurons that demonstrated basal growth (i.e., grew processes overnight in the absence of added neurotrophins). Therefore our results suggest that glial sortilin mediates these neurotrophic effects of proNGF. This was further supported by our growth cone collapse studies that highlighted the distinct actions of NGF and proNGF – i.e., the poor response to proNGF in this assay correlates well with the distant location of sortilin-positive glia in relation to growth cones. The model of proNGF action we propose based on our results involves sortilin- and p75-dependent conversion of proNGF to NGF at the level of peri-somatic glia (satellite cells), providing a local source of NGF to the adjacent neuronal soma. Therefore the activity of satellite glia may have significant implications for whether a neuron undergoes a regenerative or apoptotic event in the presence of proNGF. Peri-somatic glial expression of p75 is upregulated by injury (Zhou et al., 1996; Hu and McLachlan, 2003; Obata et al., 2006), which could also potentially influence the participation of sortilin in cleavage of proNGF.

A critical observation made early in our study was that in contrast to NGF, proNGF was unable to stimulate a robust neurite initiation response. By revealing this distinguishing feature of proNGF signalling we were able to infer that the neurotrophic action (elongation and branching of neurites) of proNGF quantified here had occurred in neurons that were already undergoing growth in the absence of added neurotrophins (basal growth). By carefully assessing this population of neurons, we were able to directly correlate the neurotrophin action of proNGF with neurons having closely associated sortilin-positive glia. We did not specifically quantify p75-positive glia in our cultures, however many showed low to moderate levels of granular expression, and previous studies in sensory ganglia have reported that glial expression of p75 is upregulated by injury (Zhou et al., 1996; Hu and McLachlan, 2003; Obata et al., 2006). While not revealed by our study, our proposed model does not exclude an effect of proNGF on neurite initiation. However, we could only assess

trophic factor-induced neurite initiation in neurons that had *not* already grown neurites (i.e. did not contribute to basal growth). Only a very small proportion of these were both TrkA-positive and associated with sortilin-positive peri-somatic glia, i.e. the cellular machinery to elicit a growth response through our proposed glial mechanism. In contrast, neurons with the appropriate characteristics to be targeted by locally cleaved proNGF were the same population that underwent basal growth of neurites, so any potential for additional growth of this type could not be assessed.

We used furin-resistant proNGF (Lee et al., 2001) to reduce the possibility of intracellular cleavage and conversion to NGF to potentially be released into the culture medium. Our Western blotting analysis failed to detect NGF in media from proNGF-treated DRG cultures. Moreover, if high levels of NGF were produced and secreted into the medium, proNGF should have mimicked all of the actions of NGF. However, there was little effect of proNGF on neurite initiation or growth cone formation, indicating that significant levels of NGF were not released into the culture medium. Rather, our model predicted that production of NGF from proNGF occurred in a highly localised manner, such that only the soma adjacent to sortilin- and p75-expressing glia were able to be stimulated by newly produced NGF. It is possible that this locally produced NGF is secreted in a very targeted manner so is difficult to measure in culture media using conventional Western blotting, so alternative approaches will be needed to probe this potential mechanism further.

The model suggested by our results may resolve some of the previous complexities of the literature that identified an ability of proNGF to stimulate TrkA phosphorylation and MAPK activity (Fahnestock et al., 2004; Masoudi et al., 2009) but having a low affinity for TrkA (Nykjaer et al., 2004) (contrasting with its high affinity for p75 and sortilin to form a pro-apoptotic signaling complex). Such discrepancies may have partly arisen from the diverse experimental systems studied but, irrespective, these different types of experimental outcomes have delayed the broad acceptance of proNGF as a genuine neurotrophic molecule. To at least partly resolve this, Boutilier et al. (2008) provided evidence that the neurotrophic actions of proNGF are indirect and involve conversion to NGF, which may be subsequently secreted to activate TrkA. They showed in PC12 cells (i.e., cultures that do not include glial cells) that activation of TrkA and downstream Erk and Akt required endocytosis and intracellular cleavage of proNGF by the protease furin. In addition, TrkA activation by proNGF has a delayed time course compared with NGF, and evidence from immunoprecipitation experiments indicates that proNGF does not bind to TrkA. Our results from adult ganglion cultures extend this model by proposing that a site of cleavage may be the population of glial cells close to neuronal somata and proximal neurites.

It is not known exactly how sortilin and p75 are involved in the trophic response, e.g., whether they are required for internalisation of proNGF or if they bind proNGF for extracellular presentation to a local source of secreted MMPs (see below). Given the technical challenges involved in separately manipulating glial and neuronal function in this unavoidably mixed short-term culture system, new approaches will be required to ascertain exactly how glial are involved. Nevertheless, highlighting this potential target of neurotrophic factor signalling provides a new way to investigate the function of proNGF in injury or disease states. This complements the report that showed mature pig oligodendrocytes can cleave proNGF (Althaus and Kloppner, 2006). While our results are consistent with glial involvement in proNGF actions, we also initially considered neurons as a site of sortilin-dependent proNGF cleavage. However, very few of the sortilin-positive neurons undergo basal growth during the culture period so do not closely match the properties of proNGF-responsive neurons.

We were able to prevent the neurotrophic response to proNGF by pretreatment with the broad spectrum MMP inhibitor, batimastat. Therefore, it is possible that glial-derived MMPs are involved with proNGF cleavage in our cultures (although the lack of action of doxycycline suggests additional contributing mechanisms). This contrasts with PC12 cells where proNGF cleavage depends on endocytosis and intracellular cleavage of proNGF by furins, and in cerebellar granule cells proBDNF conversion is mediated by a combination of intracellular furins and MMPs (Boutillier et al., 2008). MMPs are widely implicated in tissue remodelling and neural plasticity after injury (Yong et al., 2001; Rivera et al., 2010). *In vivo*, the activity of plasmin and MMPs is tightly regulated by tissue plasminogen activator and tissue inhibitors of MMPs (TIMPs), respectively. These are expressed in DRG satellite glia, where they are regulated by injury (Yamanaka et al., 2004, 2005; Kozai et al., 2007; Huang et al., 2011). It would be of great interest to explore further the large family of MMPs and their endogenous regulators in the context of proNGF cleavage, both *in vitro* and *in vivo*.

The properties of our culture system, focused on peptidergic TrkA-expressing sensory neurons, allowed us to specifically visualise the proNGF neurotrophic response on neurite length and branching in peptidergic neurons undergoing basal growth (neurite initiation). However, sortilin was also expressed by some non-peptidergic DRG neurons and their associated glia, so proNGF may also have TrkA-independent actions, such as promoting cell death. While we did not specifically investigate degenerative events in our cultures, we noted that in our control cultures some neurons had the appearance of unhealthy or dying neurons and that these neurons typically expressed high levels of sortilin (data not shown). Arnett et al. (2007) also noted that sortilin-positive DRG neurons were lost after sciatic nerve injury and proposed that these were more susceptible to injury-induced death. We did not see any reduction in the proportion of neurons initiating neurites after proNGF treatment, which one would have expected if proNGF had a significant negative effect on neuronal health in this early period. However, investigating a potential action of proNGF on neuronal death is an interesting question that would be best examined after longer treatments; our culture protocol (short-term cultures, low neuronal density) was designed to optimise analysis of neurites.

In conclusion, proNGF is able to stimulate neurotrophic responses in adult sensory neurons and we propose that these responses are mediated by NGF produced locally by perisomatic glia in a sortilin-, p75- and MMP-dependent manner. Further understanding of the signalling events underlying the neurotrophic response and how these components are regulated *in vivo* may provide new targets for enhancing regeneration.

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Abbreviations

CGRP	calcitonin gene-related peptide
DAPI	4', 6-diamidino-2-phenylindole
DRG	dorsal root ganglion or ganglia

FITC	fluorescein isothiocyanate
PI	phosphatidyl inositol
GFAP	glial fibrillary acid protein
-IR	immunoreactivity or immunoreactive
MAPK	mitogen-activated protein kinase
MMP	matrix metalloproteinase
NGF	nerve growth factor
PBS	phosphate-buffered saline
TrkA	tyrosine kinase receptor A

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Figure 1.

Effects of NGF and proNGF on neurite initiation, elongation and branching in mature DRG neurons *in vitro*. **A**, Representative images of CGRP-positive neurons in control, NGF- and proNGF-treated cultures, showing inverted images of β -tubulin immunofluorescence. Scale bar, 150 μ m. **B–F**, Quantification of neurite initiation (**B**, **C**), total neurite length (**D**), maximum neurite length (**E**), and number of branch points (**F**) in CGRP-positive neurons *in vitro*. In **B** and **D–F**, NGF and proNGF treatments were each 10 ng/ml. NGF increased neurite initiation, elongation, and branching. ProNGF increased neurite elongation and branching but not initiation (* $P < 0.05$, ** $P < 0.01$ compared to control, all $n = 5$ except **C**, $n = 10$). **G**, Western blot to identify NGF in culture media harvested at the end of three experiments (exp 1–3) from separate mice. This showed no detectable NGF in control media (20h incubation, no neurotrophic factor added; lanes 1, 6, 8) or in cultures treated for 20h with 10 ng/ml proNGF (lanes 2, 3, 7, 9). NGF added to cultures at the beginning of the incubation was still detectable 20 h later (lane 4, 10 ng/ml; lane 5, 50 ng/ml).

Figure 2.

Effect of sortilin, p75 and TrkA receptor blockade on neurite elongation responses to proNGF and NGF (each 10 ng/ml) in mature CGRP-positive DRG neurons *in vitro*. **A, B**, Representative inverted images of neurons, using β -tubulin immunoreactivity to demonstrate their neurite field. Scale bar, 150 μ m. Neurons treated with proNGF in the presence of sortilin- or p75 blocking antibodies, TrkA /Fc chimera or K252a (**A**). Neurons treated with NGF (**B**) in the presence of TrkA /Fc chimera or K252a. **C–H**, Quantitative analyses of the effect of receptor or tyrosine kinase signaling blockade on neurite growth responses to proNGF (**C, E, G**) or NGF (**D, F, H**), expressed as total neurite length (μ m).

Parallel measurements on the longest neurite (“maximum neurite length”) and neurite branching (“branch points”) from the same cultures are not shown. Anti-sortilin (1:200) and anti-p75 (1:400) blocked neurite elongation in proNGF- (**C**) but not NGF-treated (**D**) cultures. (** $P < 0.01$, *** $P < 0.001$ compared to proNGF, all $n = 4$). Neither antibody affected neurite growth in control cultures. Both TrkA/Fc chimera (100 ng/ml) and K252a (100 μ M) attenuated or blocked neurite elongation in both proNGF- (**E**, **G**) and NGF-treated (**F**, **H**) cultures (** $P < 0.01$, *** $P < 0.001$ compared to the appropriate neurotrophin-treated control, all $n = 3$). Neither TrkA/Fc chimera nor K252a affected neurite length in control cultures.

Figure 3.

Sortilin expression in DRG neurons and glia cultured in the absence of neurotrophic factor.

A–H, The majority of sortilin-immunoreactive neurons failed to grow neurites after overnight culture (arrow in **A**; **E**). Conversely, neurons with neurites invariably lacked sortilin in their cell bodies (**A**, **I**); note that the dim signal seen in these neurons is due to autofluorescence that is common in adult sensory neurons (i.e., it is present even if primary or secondary antibodies are omitted from the processing). Sortilin-immunoreactivity was observed in many satellite glial cells in culture (arrowheads in **A**, **I**). These glial cells tended to cluster around neuronal cell bodies (see DAPI staining in **C**, **K**), and were closely associated with somata of neurons that had grown neurites. Scale bar, 75 μm (**A–D**) or 20 μm (**E–L**).

Figure 4.

Sortilin and neurotrophic factor expression in DRG neurons and glia cultured in the absence of neurotrophic factor. Many neurons that underwent basal neurite growth overnight expressed TrkA (A–D) and p75 (E–H). Their somata were typically associated with sortilin-positive glia (arrowheads in A, E). I–L, Glia had variable p75-immunoreactivity, but this was usually granular and paler than neuronal staining. Scale bar, 20 μ m (A–H) or 14 μ m (I–L).

Figure 5. Co-localisation of sortilin with CGRP and GFAP in cryosections of adult DRG. **A–C**, Image pairs of sortilin- and CGRP-immunoreactivity, showing that some neurons express both molecules (arrows). In addition, a “halo” of sortilin-IR puncta bordered the profiles of many neurons (arrowheads). These profiles co-localised with GFAP (**D–F**, arrows), identifying them as satellite glial cells. Scale bar, 25 μ m.

Figure 6.

Effect of NGF and proNGF on growth cone morphology in mature CGRP-positive DRG neurons *in vitro*. **A–D**, Representative images of growth cone morphology in neurons grown in the presence of NGF (**A**), after a period of NGF starvation (**B**), and after exposure to NGF (**C**) or proNGF (**D**) following starvation. Scale bar, 20 μ m. **E**, Quantitative analysis of reversal of growth cone collapse by NGF and proNGF in NGF-starved cultures (** $P < 0.01$, $n = 4$).

Figure 7.

Effect of Src, PI 3-kinase and MAPK inhibitors on neurite elongation responses to NGF (10 ng/ml) and proNGF (10 ng/ml) in mature CGRP-positive DRG neurons *in vitro*. **A**, Representative images of neurons in control and treated cultures; β -tubulin immunofluorescence; SU6656, Src kinase inhibitor; LY294002, PI 3-kinase inhibitor; PD98059, MAPK inhibitor (2 μ M). Scale bar, 150 μ m. **B–G**, Quantitative analyses of the effects of inhibitors on total neurite length in proNGF- and NGF-treated cultures. Parallel measurements on maximum neurite length and neurite branching from the same cultures are not shown. **B, C**, SU6656 (2 μ M) blocked neurite elongation in NGF and proNGF-treated

cultures (** $P < 0.01$, *** $P < 0.001$ compared to the appropriate neurotrophin-treated control, $n = 3$). SU6656 had no effect in control cultures. **D, E**, The PI 3-kinase inhibitors LY294002 (50 μM) and wortmannin (10 μM) reduced neurite elongation in NGF- and proNGF-treated cultures (* $P < 0.05$) compared to the appropriate neurotrophin-treated control, all $n = 4$). **F, G**, Neither of the MAPK inhibitors, PD98059 (50 μM) or U0126 (20 μM), significantly affected total neurite length in neurotrophin-treated or control cultures (all $n = 4$).