Progress in Brain Research, Vol. 146
ISSN 0079-6123

CHAPTER 7

ProNGF: a neurotrophic or an apoptotic molecule?

Margaret Fahnestock*, Guanhua Yu and Michael D. Coughlin

Department of Psychiatry and Behavioral Neurosciences, McMaster University, Hamilton, ON, Canada

Abstract: Nerve growth factor (NGF) acts on various classes of central and peripheral neurons to promote cell survival, stimulate neurite outgrowth and modulate differentiation. NGF is synthesized as a precursor, proNGF, which undergoes processing to generate mature NGF. It has been assumed, based on studies in the mouse submandibular gland, that NGF in vivo is largely mature NGF, and that mature NGF accounts for the molecule's biological activity. However, recently we have shown that proNGF is abundant in central nervous system tissues whereas mature NGF is undetectable, suggesting that proNGF may have a function distinct from its role as a precursor. A recent report that proNGF has apoptotic activity contrasts with other data demonstrating that proNGF has neurotrophic activity. This chapter will review the structure and processing of NGF and what is known about the biological activity of proNGF. Possible reasons for the discrepancies in recent reports are discussed.

Keywords: neurotrophin; precursor; TrkA; $p75^{NTR}$; neurite outgrowth; survival

Introduction

In the half a century since the discovery of nerve growth factor (NGF) by Rita Levi-Montalcini and Viktor Hamburger (1953), this molecule has never ceased to be full of surprises. From the initial finding that NGF is produced in snake venom and in inexplicably high quantities in the male mouse submandibular gland (Cohen, 1959, 1960) to the most recent reports discussed at NGF2002, the field has seen the unfolding story of NGF filled with twists and turns, and above all, controversy. The present state of affairs with respect to the NGF precursor, proNGF, is no exception.

Biosynthesis of NGF

The structure, biosynthesis, and biological activity of NGF have been studied extensively in the mouse submandibular gland because of its extraordinarily high concentration in this tissue (Cohen, 1960; Levi-Montalcini and Angeletti, 1968; Fahnestock, 1991). NGF is encoded by a single gene that is over 45 kilobases in length (Ullrich et al., 1983). Two separate promoters and a total of four exons are alternatively spliced to yield two major and two minor transcripts with the coding sequence at the 3′ end in exon four (Edwards et al., 1986; Selby et al., 1987; Racke et al., 1996). NGF protein is translated from the two major alternatively spliced transcripts to produce 34 and 27 kD prepro species, with translation initiation sites at amino acid positions −187 and −121, respectively (Fig. 1). Removal of the signal sequence in the endoplasmic reticulum reduces these translation products to proNGF species of 32 and 25kD (Darling et al., 1983; Ullrich et al., 1983; Edwards et al., 1986, 1988b; Selby et al., 1987).

ProNGF contains three potential glycosylation sites, two in the prosegment and one in the mature sequence (Fig. 1). It has been demonstrated that a 43kD form of NGF expressed by transfected BSC40 cells is N-glycosylated in the prosegment, whereas the glycosylation site in the mature sequence does not

*Correspondence to: M. Fahnestock, Department of Psychiatry and Behavioral Neurosciences, McMaster University, 1200 Main Street West, Hamilton, ON L8N 3Z5, Canada.
Tel.: +1-905-525-9140 (Ext. 23344); Fax: +1-905-522-8804;
E-mail: fahnest@mcmaster.ca

DOI: 10.1016/S0079-6123(03)46007-X

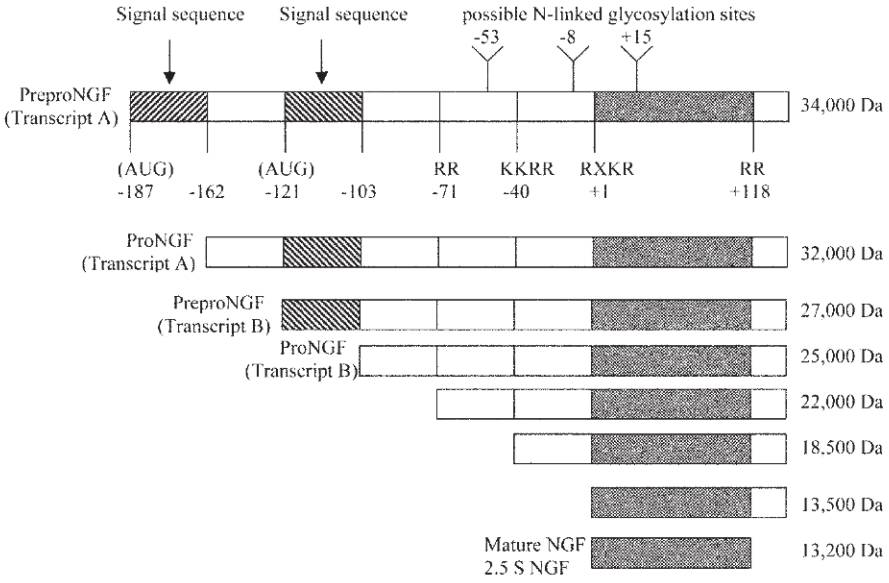

Fig. 1. Likely intermediates in NGF biosynthesis. Translation initiation sites at −187 and −121 are marked by vertical lines, as are potential cleavage sites at −71, −40 and +1. 'Y' represents potential glycosylation sites.

appear to be used (Seidah et al., 1996), except in a very minor proportion of molecules (Murphy et al., 1989). N-glycosylation of proNGF contributes to efficient protein expression (Suter et al., 1991) and may be important for exit of proNGF from the endoplasmic reticulum (Seidah et al., 1996). High-molecular-weight glycosylated NGF precursors, mostly of molecular weight 53 kD, have been identified both in vitro and in vivo (Edwards et al., 1988b; Lakshmanan et al., 1988; Bresnahan et al., 1990; Reinshagen et al., 2000; Yiangou et al., 2002). However, many tissues contain the unglycosylated proNGF (Chen et al., 1997; Fahnestock et al., 2001).

ProNGF undergoes further posttranslational processing at both amino- and carboxyl-terminal ends to generate the mature, biologically active product of 13.2 kD (Darling et al., 1983). Several intermediate sizes of NGF precursors are detectable in mouse and rat tissues, including a 27–29 kD species (Darling et al., 1983; Dicou et al., 1986) probably resulting from translation of the shorter transcript B identified by Edwards et al. (1986); a stable intermediate of 22–24 kD (Berger and Shooter, 1977; Dicou, 1992; Chen et al., 1997); and an 18–19 kD processing intermediate (Darling et al., 1983; Dicou, 1989) (Fig. 1). The functions of the precursors and their intermediates are poorly understood.

Carboxyl-terminal processing of murine NGF occurs between a pair of arginine residues, whereas processing of intermediate and amino-terminal sites of NGF occurs at four-residue basic sites. Furin, and to a lesser extent other prohormone convertases, are able to process proNGF at its amino-terminus to produce the mature form of NGF, also known as βNGF (Bresnahan et al., 1990; Seidah et al., 1996). The kallikrein γNGF, which is found in a complex with betaNGF in the mouse submandibular gland, has been shown to process proNGF at both amino- and carboxyl-termini to produce intermediate and mature forms of NGF; following processing of the C-terminus of NGF by γNGF, the γNGF remains bound to βNGF at the carboxyl-terminal arginine (Greene et al., 1968; Mobley et al., 1976; Edwards et al., 1988a; Dicou, 1989; Jongstra-Bilen et al., 1989). It is not clear whether carboxyl-terminal processing occurs in species or tissues other than the mouse submandibular gland. Recently, both plasmin and matrix metalloprotease-7 (MMP-7) have also been shown to process the NGF precursor (Lee et al., 2001). Plasmin cleavage of

proNGF produces the mature form, whereas MMP-7 cleavage results in a 17 kD intermediate that may be the same as the 18–19 kD intermediate identified previously (Darling et al., 1983; Dicou, 1989).

Secretion of proNGF

For many years, the literature has accepted that NGF biosynthesis in the mouse submandibular gland is representative of NGF biosynthesis in other species and tissues. Human NGF is highly homologous to murine NGF, and many of the same proteolytic cleavage sites and glycosylation consensus sites in the mouse sequence are conserved in human NGF (Ullrich et al., 1983). Neither NGF precursors nor intermediates have been reported in saliva, suggesting that processing occurs prior to secretion in the submandibular gland. Cell expression studies also support the model that NGF is largely processed into the mature form prior to secretion (Seidah et al., 1996). However, secretion of unprocessed proneurotrophins does occur from transfected mammalian cells, including neurons (Mowla et al., 1999), and from baculovirus-infected insect cells (Fig. 2). ProNGF, not NGF, is secreted from prostate cells, spermatids, and hair follicles (Chen et al., 1997; Delsite and Djakiew, 1999; Yardley et al., 2000). Primary sympathetic neurons reportedly secrete proNGF too (Smith et al., 2002). Here we show (Fig. 2) that primary rat cortical astrocytes also secrete proNGF. Therefore, in tissues other than the submandibular gland, NGF is likely secreted as proNGF, or at least as a mixture of proNGF and NGF.

ProNGF in tissues

Enzyme-linked immunosorbent assays (ELISA) for NGF are widely used and were thought to measure the mature 13.2 kD NGF protein in most tissues. It is now known that NGF in mouse, rat and human brain exists as 32 kD proNGF, with little or no mature NGF present (Fahnestock et al., 2001), suggesting that what was previously measured as NGF was actually proNGF. This is in contrast to the mouse submandibular gland, where large amounts of

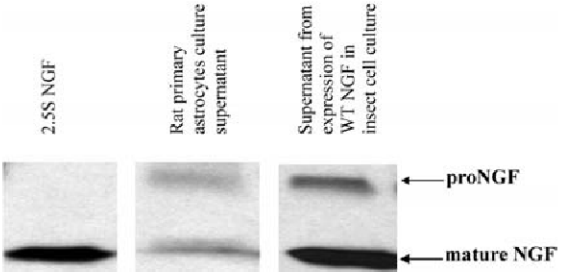

Fig. 2. ProNGF and NGF are secreted into conditioned medium of rat cortical astrocytes and baculovirus-infected insect cells in culture. Left: 2.5S NGF was isolated from mouse submandibular gland as previously described (Mobley et al., 1997; Petrides and Shooter, 1986). Middle: Single cells were isolated from cerebral cortex of newborn (0–24 h) Wistar rats as described in Hertz et al. (1989). After 14 days in culture, cells were shaken for 16 h at 200 rpm to remove oligodendrocytes. The purity of the astrocytes thus obtained was greater than 98% as estimated by indirect immunocytochemistry using antibody to glial fibrillary acidic protein. Cells were switched to serum-free DMEM. Medium was harvested after 24 h and concentrated 75-fold before analysis by Western blotting. Right: Recombinant wild-type NGF baculovirus was used to infect Sf9 insect cells in Sf-900 II serum-free medium (Invitrogen Life Technologies, Burlington, ON, Canada) as described in Van der Zee et al. (1995). Medium was harvested after three days. Western blotting was carried out as described in Fahnestock et al. (2001).

mature NGF are detectable, with lesser amounts of proNGF also found. The presence of proNGF and absence of mature NGF in central nervous system tissue is not due to rapid and selective degradation of NGF compared to proNGF, or to technical considerations such as antibody specificity, lack of sensitivity of the assay, or loss of NGF during tissue preparation. Others have noted that NGF is found as a precursor in tissues such as thyroid gland, retina, prostate, hair follicle, skin, colon, and dorsal root ganglia (Dicou et al., 1986; Chakrabarti et al., 1990; Delsite and Djakiew, 1999; Reinshagen et al., 2000; Yardley et al., 2000; Yiangou et al., 2002). Many of these tissues contain no detectable mature NGF.

It could be argued that mature NGF is taken up and transported so rapidly following secretion that it is not detectable by immunological assays, whereas proNGF is more long-lived. Basal forebrain cholinergic neurons projecting to cortex and hippocampus obtain NGF by retrograde transport from these

targets and can efficiently internalize and transport NGF. In the Alzheimer's diseased brain, however, these cholinergic neurons degenerate and can no longer transport NGF as efficiently, leading to an increase in untransported NGF in the target tissue. If the mature form of NGF were the biologically active form, one would expect mature NGF to accumulate in the Alzheimer's brain. The NGF that accumulates in cortex and hippocampus is proNGF, with no mature NGF detectable (Fahnestock et al., 2001). This argues against the role of proNGF solely as a precursor to the biologically active, mature NGF, and suggests that proNGF is the form that is active in brain.

Physical characteristics of proNGF

ProNGF most likely is a dimer in solution. Rattenholl et al. (2001a) used a series of experiments including analytical ultracentrifugation, glutaraldehyde crosslinking followed by SDS-PAGE, and gel filtration to show that recombinant human proNGF exists as a dimer. We mutated a single amino acid at the mouse proNGF cleavage site (arginine to glycine at amino acid –1) to produce a cleavage-resistant recombinant proNGF, which we call proNGF (R-1G) (Yu et al., 2002; Fahnestock et al., submitted). We used gel filtration in the presence of 4 M urea to remove any associated proteins (mature NGF dissociates at 8M urea), and, in accordance with the data of Rattenholl et al. (2001a,b), we find that recombinant proNGF (R-1G) migrates as a dimer (Fig. 3). It has, furthermore, been suggested that proNGF dimers may associate into higher order structures for binding to p75 neurotrophin receptor ($p75^{NTR}$) (Ibanez, 2002).

NGF is a basic molecule, but proNGF is less basic. Isoelectric focusing of mature NGF gives an isoelectric point of 9.3, whereas proNGF (R-1G) has an isoelectric point of 8.1 (Fig. 4).

Biological activity of proNGF

That the NGF precursor has little or no biological activity has been widely assumed. Edwards et al. (1988a) demonstrated that the neurite outgrowth activity of proNGF on chick dorsal root ganglion

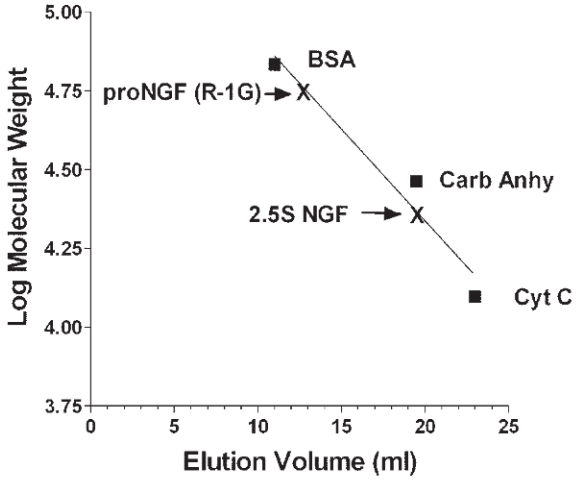

Fig. 3. ProNGF is a dimer in solution. Insect cell conditioned medium expressing a cleavage-resistant proNGF [proNGF (R-1G)] was concentrated forty-fold and chromatographed on a Sephadex G-75 column (Amersham Biosciences, Baie d'Urfé, Quebec) in 25 mM sodium phosphate pH 5.6–0.1 M NaCl–4 M urea. The column was pre-calibrated with bovine serum albumin (BSA, $M_r = 68{,}000$), carbonic anhydrase (Carb Anhyd, $M_r = 29{,}000$), and cytochrome C (Cyt C, $M_r = 12{,}500$). The calculated M_r of 2.5S NGF on this column is approximately 24,000. The calculated M_r of proNGF (R-1G) is approximately 60,000.

(DRG) neurons increased 10- to 20-fold after processing to mature NGF with trypsin. The role of the pro segment was thought to be to aid in folding of mature NGF (Suter et al., 1991; Rattenholl et al., 2001a). In contrast, a number of other investigators over the years have demonstrated that either the full-length proNGF or intermediate forms exhibit activity. Saboori and Young (1986) demonstrated that the 32 kD proNGF molecule isolated from mouse salivary gland promotes nerve growth prior to processing by γNGF. Reduced forms of the 53 kD NGF precursor bound on nitrocellulose membranes promoted neurite outgrowth and survival activity of PC12 cells (Lakshmanan et al., 1989). Suter et al. (1991) showed that a proNGF with a deletion including the processing site for conversion to mature NGF exhibited 50% of wild type neurite outgrowth activity on PC12 cells. Ibanez et al. (1992) constructed and expressed partially processed NGF precursors that exhibited neurite outgrowth and survival activity. The biological activity of full-length proNGF isolated from rat round spermatids was

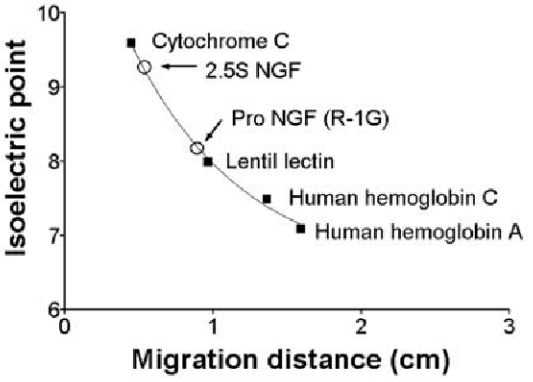

Fig. 4. Isoelectric focusing of NGF and proNGF. Isoelectric focusing in pH 3–10 gels and staining of protein standards (Bio-Rad Laboratories, Mississauga, ON, Canada) were carried out according to the manufacturer's instructions. Affinity-purified, cleavage-resistant proNGF [proNGF (R-1G)] from insect cell conditioned medium and 2.5S NGF purified from mouse submandibular glands (Mobley et al., 1976; Petrides and Shooter, 1986) were visualized using an antibody to NGF (Cedarlane, Hornby, ON) that recognizes both 2.5S NGF and proNGF (Fahnestock et al., 2001), and ECL (Amersham Biosciences). Isoelectric points of proteins used for standard curve: Cytochrome C, pI 9.6; Lentil lectin, pI 8.0; Human hemoglobin C, pI 7.5; Human hemoglobin A, pI 7.1. Calculated isoelectric point of 2.5S NGF is 9.3, and that of proNGF (R-1G) is approximately 8.1.

demonstrated in neurite outgrowth activity on PC12 cells and survival activity on Sertoli cells, whereas the 22 kD NGF intermediate exhibited less robust bioactivity (Chen et al., 1997). Using survival of DRG neurons as an assay, Rattenholl et al. (2001a) demonstrated that recombinant proNGF was equally as active as NGF. Most recently, our laboratory showed that a cleavage-resistant proNGF exhibits neurite outgrowth activity on both mouse sympathetic cervical ganglion (SCG) neurons and PC12 cells. The specific activity of proNGF in stimulating neurite outgrowth (based on the EC_{50}) is approximately five-fold less than that of mature NGF. In addition, this proNGF promotes survival of SCG neurons in culture and exhibits TrkA receptor binding and activation activity slightly less than to that of mature NGF (Yu et al., 2002; Fahnestock et al., submitted). These studies suggest that at least some forms of proNGF may indeed be neurotrophic.

In contrast, Lee et al. (2001) has recently demonstrated that a cleavage-resistant form of proNGF promotes apoptosis in primary SCG neurons and smooth muscle cells. So is proNGF apoptotic or neurotrophic?

ProNGF receptor binding

The answer may lie with the NGF receptors. NGF binds to two types of transmembrane receptors, a receptor tyrosine kinase, TrkA (Barbacid, 1995), and $p75^{NTR}$, a member of the tumor necrosis factor (TNF) receptor family (Friedman and Greene, 1999). TrkA mediates survival and neurite outgrowth by signaling through the PI3 kinase-Akt and Ras-MAP kinase pathways (Kaplan and Miller, 2000). When both receptors are present, $p75^{NTR}$ increases NGF binding affinity to TrkA, increases ligand discrimination, and enhances neurite outgrowth and survival by activating NF-kB and Rho signaling (Davies et al., 1993; Mahadeo et al., 1994; Bibel et al., 1999; Yamashita et al., 1999; Hamanoue et al., 1999). When $p75^{NTR}$ is present in the absence of TrkA, however, it can mediate cell death by signaling through the ceramide, p53, and c-Jun N-terminal kinase (JNK) pathways (Aloyz et al., 1998; Friedman, 2000; Brann et al., 2002). It follows, then, that a ligand that binds only to $p75^{NTR}$ is apoptotic, even in the presence of TrkA.

Mature NGF binds to TrkA with high affinity ($K_d \sim 10^{-11}$ M), whereas it binds to $p75^{NTR}$ with low affinity ($K_d \sim 10^{-9}$ M). Lee et al. (2001) demonstrated that their cleavage-resistant proNGF was able to bind to the low-affinity $p75^{NTR}$ receptor with enhanced affinity (10^{-10} M) but could not bind with high affinity to TrkA. Our cleavage-resistant proNGF, in contrast, binds to TrkA in cross-linking studies and activates TrkA phosphorylation at only slightly higher concentrations than NGF, suggesting it retains high-affinity binding to TrkA (Yu et al., 2002; Fahnestock et al., submitted). As expected of a molecule that binds TrkA, our proNGF exhibits neurotrophic activity.

Supporting the apoptotic model of proNGF, the amino-terminus of mature NGF has been implicated in binding to TrkA (Kahle et al., 1992; Ibanez et al., 1993; Woo et al., 1995; Kullander et al., 1997; Wiesmann et al., 1999), suggesting that unprocessed proNGF retaining an extended amino-terminus

should be sterically hindered from binding to this receptor. Supporting the neurotrophic model, NGF loop regions have been implicated in the interaction between NGF and TrkA (Ibanez, 1995, 1998; Kullander et al., 1997) and in NGF binding to $p75^{NTR}$ (Ibanez et al., 1992; Ryden and Ibanez, 1997). The loop regions might be expected to retain receptor-binding activity even in the presence of an amino-terminal pro extension. Circular dichroism suggests that the structure of mature NGF is largely maintained in the presence of the pro segment (Rattenholl et al., 2001a).

Differences between recombinant, cleavage-resistant proNGFs

Our cleavage-resistant proNGF differs from that of Lee et al. (2001) in several key structural features and in the expression and purification systems used. First, our proNGF contains a single amino acid substitution, a R-to-G substitution, at the -1 position. This substitution was designed to perturb the molecule as little as possible by substituting a small amino acid, glycine, for an arginine residue that forms part of the tetrabasic cleavage site. ProNGF from Lee et al. (2001) contains four separate amino acid substitutions: K-R to A-A at residues −1 and −2, and R-R to A-A at residues 118–119. The double alanine substitution at residues −1 and −2 was designed, like our single substitution, to eliminate the proNGF cleavage site. The double alanine substitution at residues 118–119 was designed to eliminate a carboxyl-terminal cleavage site that could release a polyhistidine tag. Our proNGF does not carry this polyhistidine tag and therefore we have no need for the carboxyl-terminal mutations. It is possible that the additional amino acid substitutions in the proNGF from Lee et al. (2001) and the fact that they are alanines rather than glycines, may unfold the molecule slightly more than a single substitution or change the structure from the native form. There is some precedent for polyhistidine tags disrupting protein structure. There is some precedent for polyhistidine tags disrupting protein structure. Polyhistidine tags have been shown to interfere with protein refolding and stabilization, and with receptor-ligand binding and biological activity (Ramage et al., 2002; Ledent et al., 1997; Lawrence et al., 2001). This may be particularly true if the amino- and carboxyl-terminal ends interact. The pro segment of NGF has been shown to be important for proper folding of the molecule (Suter et al., 1991; Rattenholl et al., 2001a,b), whereas the carboxyl-terminus is important for stability and biological activity (Drinkwater et al., 1993; Kruttgen et al., 1997). Furthermore, the carboxyl-terminus of NT-3 is a key domain for interactions of NT-3 with $p75^{NTR}$ (Urfer et al., 1994), suggesting this might also be true for NGF. Lee et al. (2001) have demonstrated no adverse effects of their polyhistidine tag on mature NGF expression or function. However, it is possible that a carboxyl-terminal polyhistidine tag might change both inter- and intramolecular interactions of proNGF in the presence of the pro domain.

It is interesting that the nickel columns commonly used to purify histidine-tagged proteins can cause oxidation and promote proteolysis by contaminating metalloproteases (Ramage et al., 2002). ProNGF from Lee et al. (2001) is extremely susceptible to cleavage by a variety of proteases including plasmin and MMP-7, a matrix metalloprotease, whereas native proNGF is also stable in tissue, as shown by its detection in a variety of sources including postmortem human brain (Fahnestock et al., 2001). It is possible that the multiple amino acid substitutions, the polyhistidine tag or the nickel column purification promote destabilization of the proNGF from Lee et al. (2001), whereas our single amino acid substitution and lack of polyhistidine tag may promote a more stable structure.

Finally, there are differences in expression systems used to produce the proNGF proteins. Lee et al. (2001) expressed proNGF in a mammalian expression system using 293 cells, whereas our proNGF is expressed in a baculovirus/insect cell system. The reasons we use baculovirus are that expressed proteins are produced in large amounts in serum-free medium for easy purification, and there are no endogenous neurotrophic factors produced by insect cells. Although 293 cells produce extremely low amounts of endogenous trophic factors, it has been demonstrated that neurotrophin subunits can freely recombine with each other (Treanor et al., 1995), which could produce artifacts. On the other hand, it has been suggested that there may be a chaperone

present in insect cell supernatants that could interact with proNGF to produce artifactual biological activity. However, we partially purified proNGF by size exclusion chromatography in the presence of urea, and this eight-fold purified material exhibits similar neurite outgrowth activity as unpurified material (Yu et al., 2002; Fahnestock et al., submitted). A contaminating molecule would have to be the same molecular weight as proNGF and stay bound to it during size exclusion chromatography in the presence of urea to be a candidate, and so this alternative is unlikely.

Is endogenous proNGF neurotrophic or apoptotic?

The cleavage-resistant proNGF molecules discussed above are mutated recombinant proteins. The characteristics and physiological role of endogenous proNGF are still a puzzle. Mature NGF is effective in low amounts at promoting cell survival and neurite outgrowth via TrkA, yet the amounts required for activation of $p75^{NTR}$ cell death pathways are at least an order of magnitude greater (Friedman, 2000). ProNGF, if its in vivo role is apoptotic, is a high-affinity ligand for $p75^{NTR}$ that could promote cell death via this receptor at much lower doses than mature NGF. This suggests that the balance between cell survival and cell death could depend upon the relative amounts of proNGF and mature NGF in tissues (Ibanez, 2002). Yet, as we have seen, most tissues examined, with the exception of the mouse submandibular gland, contain primarily, if not exclusively, proNGF.

Cleavage-resistant proNGF is neurotrophic in vitro, as we have shown. If endogenous proNGF is similarly neurotrophic, then what is its role relative to that of mature NGF? We propose that proNGF is responsible for the normal neurotrophic activity in most tissues, but that injury increases the processing of proNGF to NGF and thereby provides a rapid and local supply of the more neurotrophically active mature NGF. In support of this hypothesis, both sciatic nerve transection and kainic acid-induced seizures upregulate prohormone convertases that can process neurotrophin precursors (Meyer et al., 1996; Marcinkiewicz et al., 1999).

Much work needs to be done to clarify the differences between the biological characteristics and activities of proNGF and NGF. It is not clear whether proNGF and NGF are both internalized by cells or whether they are internalized at the same rate. It is not known whether they are both retrogradely transported at the same rate, or if NGF-induced signal transduction pathways are activated similarly by proNGF. Although several studies have reported that proNGF has neurotrophic activity similar to that of mature NGF (Chen et al., 1997; Rattenholl et al., 2001a), we and others have found its activity to be anywhere from two to twenty-fold less potent than that of mature NGF (Edwards et al., 1988a; Suter et al., 1991; Yu et al., 2002; Fahnestock et al., submitted). Quantification of proNGF is problematic because of the lack of a pure standard, and therefore these numbers must be interpreted with some caution. The use of modified proNGF molecules facilitates purification of intact proNGF but also confounds interpretation of results. Purification of native, full-length proNGF to homogeneity and its further characterization is an important next step in the study of this surprising and controversial molecule.

Summary

The nature of the activity exhibited by full-length, native proNGF is still controversial. It is not clear which, if either, of the mutated proNGF molecules now under study are representative of the proNGF found in vivo. Structural and bioactivity comparisons of native proNGF with both noncleavable proNGFs, and comparisons of the elements that distinguish our proNGF from the proNGF constructed by Lee et al. (2001), will answer some of these questions. Further work will be required, for example, on retrograde transport and on regulation following injury, to elucidate the physiological role of proNGF and the function of proNGF processing in vivo.

Acknowledgments

We thank Sean Coughlin for technical contributions, and Dr. Jack Diamond for helpful discussions. This

work was supported by grants from the Ontario Neurotrauma Foundation to M. Fahnestock and G. Yu, and by an award from the Scottish Rite Charitable Foundation of Canada to M. Fahnestock. M. Fahnestock's participation at the NGF2002 meeting was made possible by a grant from the Canadian Institutes for Health Research to R. Racine and M. Fahnestock.

Abbreviations

DRG	dorsal root ganglion
ELISA	enzyme-linked immunosorbent assay
JNK	c-Jun N-terminal kinase
MMP-7	matrix metalloprotease-7
NGF	nerve growth factor
$p75^{NTR}$	p75 neurotrophin receptor
SCG	sympathetic cervical ganglion
SDS–PAGE	sodium dodecyl sulfate- polyacrylamide gel electrophoresis
TNF	tumor necrosis factor

References

Aloyz, R.S., Bamji, S.X., Pozniak, C.D., Toma, J.G., Atwal, J., Kaplan, D.R. and Miller, F.D. (1998) p53 is essential for developmental neuron death as regulated by the TrkA and p75 neurotrophin receptors. J. Cell Biol., 143: 1691–1703.

Barbacid, M. (1995) Neurotrophic factors and their receptors. Curr. Opin. Cell Biol., 7: 148–155.

Berger, E.A. and Shooter, E.M. (1977) Evidence for pro-beta-nerve growth factor, a biosynthetic precursor to beta-nerve growth factor. Proc. Natl. Acad. Sci. USA, 74: 3647–3651.

Bibel, M., Hoppe, E. and Barde, Y.A. (1999) Biochemical and functional interactions between the neurotrophin receptors trk and p75NTR. EMBO J, 18: 616–622.

Brann, A.B., Tcherpakov, M., Williams, I.M., Futerman, A.H. and Fainzilber, M. (2002) Nerve growth factor-induced p75-mediated death of cultured hippocampal neurons is age-dependent and transduced through ceramide generated by neutral sphingomyelinase. J. Biol. Chem., 277: 9812–9818.

Bresnahan, P.A., Leduc, R., Thomas, L., Thorner, J., Gibson, H.L., Brake, A.J., Barr, P.J. and Thomas, G. (1990) Human *fur* gene encodes a yeast KEX2-like endoprotease that cleaves pro-β-NGF in vivo. J. Cell Biol., 111: 2851–2859.

Chakrabarti, S., Sima, A.A., Lee, J., Brachet, P. and Dicou, E. (1990) Nerve growth factor (NGF), proNGF and NGF receptor-like immunoreactivity in BB rat retina. Brain Res., 523: 11–15.

Chen, Y., Dicou, E. and Djakiew, D. (1997) Characterization of nerve growth factor precursor expression in rat spermatids and the trophic effects of nerve growth factor in the maintenance of Sertoli cell viability. Mol. Cell. Endocrinol, 127: 129–136.

Cohen, S. (1959) Purification and metabolic effects of a nerve growth-promoting protein from snake venom. J. Biol. Chem., 234: 1129–1137.

Cohen, S. (1960) Purification of a nerve-growth promoting protein from the mouse salivary gland and its neuro-cytotoxic antiserum. Proc. Natl. Acad. Sci. USA, 46: 302–311.

Darling, T.L., Petrides, P.E., Beguin, P., Frey, P., Shooter, E.M., Selby, M. and Rutter, W.J. (1993) The biosynthesis and processing of proteins in the mouse 7S nerve growth factor complex. Cold Spring Harb. Symp. Quant. Biol., 48 Pt 1: 427–434.

Davies, A.M., Lee, K.F. and Jaenisch, R. (1993) p75-deficient trigeminal sensory neurons have an altered response to NGF but not to other neurotrophins. Neuron, 11: 565–574.

Delsite, R. and Djakiew, D. (1999) Characterization of nerve growth factor precursor protein expression by human prostate stromal cells: a role in selective neurotrophin stimulation of prostate epithelial cell growth. Prostate, 41: 39–48.

Dicou, E. (1989) Interaction of antibodies to synthetic peptides of proNGF with in vitro synthesized NGF precursors. FEBS Lett., 255: 215–218.

Dicou, E. (1992) Nerve growth factor precursors in the rat thyroid and hippocampus. Mol. Brain Res., 14: 136–138.

Dicou, E., Lee, J. and Brachet, P. (1986) Synthesis of nerve growth factor mRNA and precursor protein in the thyroid and parathyroid glands of the rat. Proc. Natl. Acad. Sci. USA, 83: 7084–7088.

Drinkwater, C.C., Barker, P.A., Suter, U. and Shooter, E.M. (1993) The carboxyl terminus of nerve growth factor is required for biological activity. J. Biol. Chem., 268: 23202–23207.

Edwards, R.H., Selby, M.J. and Rutter, W.J. (1986) Differential RNA splicing predicts two distinct nerve growth factor precursors. Nature, 319: 784–787.

Edwards, R.H., Selby, M.J., Garcia, P.D. and Rutter, W.J. (1988a) Processing of the native nerve growth factor precursor to form biologically active nerve growth factor. J. Biol. Chem., 263: 6810–6815.

Edwards, R.H., Selby, M.J., Mobley, W.C., Weinrich, S.L., Hruby, D.E. and Rutter, W.J. (1988b) Processing and secretion of nerve growth factor: Expression in mammalian cells with a vaccinia virus vector. Mol. Cell. Biol., 8: 2456–2464.

Fahnestock, M. (1991) Structure and biosynthesis of nerve growth factor. Curr. Top. Microbiol. Immunol., 165: 1–26.

Fahnestock, M., Michalski, B., Xu, B. and Coughlin, M.D. (2001) The precursor pro-nerve growth factor is the predominant form of nerve growth factor in brain and is increased in Alzheimer's disease. Mol. Cell. Neurosci., 18: 210–220.

Fahnestock, M., Yu, G., Michalski, B., Mathew, S., Colquhoun, A., Ross, G.M., and Coughlin, M.D. The nerve growth factor precursor proNGF exhibits neurotrophic activity qulatatively similar to that of mature nerve growth factor. Submitted.

Friedman, W.J. (2000) Neurotrophins induce death of hippocampal neurons via the p75 receptor. J. Neurosci., 20: 6340–6346.

Friedman, W.J. and Greene, L.A. (1999) Neurotrophin signaling via Trks and p75. Exp. Cell Res., 253: 131–142.

Greene, L.A., Shooter, E.M. and Varon, S. (1968) Enzymatic activities of mouse nerve growth factor and its subunits. Proc. Natl. Acad. Sci. USA, 60: 1383–1388.

Hamanoue, M., Middleton, G., Wyatt, S., Jaffray, E., Hay, R.T. and Davies, A.M. (1999) p75-mediated NF-kappaB activation enhances the survival response of developing sensory neurons to nerve growth factor. Mol. Cell. Neurosci., 14: 28–40.

Hertz, L., Juurlink, B.H.J., Hertz, E., Fosmark, H. and Schousboe, A. (1989). Preparation of primary cultures of mouse (rat) astrocytes. In: A. Shahar (Ed.), A Dissection and Tissue Culture Manual of the Nervous System. Alan R. Liss, Inc, New York, pp. 105–108, Chapter 21.

Ibanez, C.F. (1995) Neurotrophic factors: from structure-function studies to designing effective therapeutics. Trends Biotechnol., 13: 217–227.

Ibanez, C.F. (1998) Emerging themes in structural biology of neurotrophic factors. Trends Neurosci., 21: 438–444.

Ibanez, C.F. (2002) Jekyll-Hyde neurotrophins: the story of proNGF. Trends Neurosci., 25: 284–286.

Ibanez, C.F., Ebendal, T., Barbany, G., Murray-Rust, J., Blundell, T.L. and Persson, H. (1992) Disruption of the low affinity receptor-binding site in NGF allows neuronal survival and differentiation by binding to the trk gene product. Cell, 69: 329–341.

Ibanez, C.F., Ilag, L.L., Murray-Rust, J. and Persson, H. (1993) An extended surface of binding to Trk tyrosine kinase receptors in NGF and BDNF allows the engineering of a multifunctional pan-neurotrophin. EMBO J., 12: 2281–2293.

Jongstra-Bilen, J., Coblentz, L. and Shooter, E.M. (1989) The in vitro processing of the NGF precursors by the gamma-subunit of the 7S NGF complex. Mol. Brain Res., 5: 159–169.

Kahle, P., Burton, L.E., Schmelzer, C.H. and Hertel, C. (1992) The amino terminus of nerve growth factor is involved in the interaction with the receptor tyrosine kinase p140trkA. J. Biol. Chem., 267: 22707–22710.

Kaplan, D.R. and Miller, F.D. (2000) Neurotrophin signal transduction in the nervous system. Curr. Opin. Neurobiol., 10: 381–391.

Krutten, A., Heymach, J.V., Jr., Kahle, P.J. and Shooter, E.M. (1997) The role of the nerve growth factor carboxyl terminus in receptor binding and conformational stability. J. Biol. Chem., 272: 29222–29228.

Kullander, K., Kaplan, D. and Ebendal, T. (1997) Two restricted sites on the surface of the nerve growth factor molecule independently determine specific TrkA receptor binding and activation. J. Biol. Chem., 272: 9300–9307.

Lakshmanan, J., Burns, C. and Smith, R.A. (1988) Molecular forms of nerve growth factor in mouse submaxillary glands. Biochem. Biophys. Res. Commun., 152: 1008–1014.

Lakshmanan, J., Beattie, G.M., Hayek, A., Burns, C. and Fisher, D.A. (1989) Biological actions of 53 kDa nerve growth factor as studied by a blot and culture technique. Neurosci Lett., 99: 263–267.

Lawrence, D., et al. (2001) Differential hepatocyte toxicity of recombinant Apo2L/TRAIL versions. Nat. Med., 7: 383–385.

Ledent, P., Duez, C., Vanhove, M., Lejeune, A., Fonze, E., Charlier, P., Rhazi-Filali, F., Thamm, I., Guillaume, G., Samyn, B., Devreese, B., Van Beeumen, J., Lamotte-Brasseur, J., Frere, J.M. (1997) Unexpected influence of a C-terminal-fused His-tag on the processing of an enzyme and on the kinetic and folding parameters. FEBS Lett., 413: 194–196.

Lee, R., Kermani, P., Teng, K.K. and Hempstead, B.L. (2001) Regulation of cell survival by secreted proneurotrophins. Science, 294: 1945–1948.

Levi-Montalcini, R. and Hamburger, V. (1953) A diffusible agent of mouse sarcoma producing hyperplasia of sympathetic ganglia and hyperneurotization of viscera in the chick embryo. J. Exp. Zool., 123: 233–288.

Levi-Montalcini, R. and Angeletti, P.U. (1968) Nerve growth factor. Physiol. Rev., 48: 534–569.

Mahadeo, D., Kaplan, L., Chao, M.V. and Hempstead, B.L. (1994) High affinity nerve growth factor binding displays a faster rate of association than p140trk binding. Implications for multi-subunit polypeptide receptors. J. Biol. Chem., 269: 6884–6891.

Marcinkiewicz, M., Marcinkiewicz, J., Chen, A., Leclaire, F., Chretien, M. and Richardson, P. (1999) Nerve growth factor and proprotein convertases furin and PC7 in transected sciatic nerves and in nerve segments cultured in conditioned media: their presence in Schwann cells, macrophages, and smooth muscle cells. J. Comp. Neurol., 403: 471–485.

Meyer, A., Chretien, P., Massicotte, G., Sargent, C., Chretien, M. and Marcinkiewicz, M. (1996) Kainic acid increases the expression of the prohormone convertases furin and PC1 in the mouse hippocampus. Brain Res., 732: 121–132.

Mobley, W.C., Schenker, A. and Shooter, E.M. (1976) Characterization and isolation of proteolytically modified nerve growth factor. Biochemistry, 15: 5543–5551.

Mowla, S.J., Pareek, S., Farhadi, H.F., Petrecca, K., Fawcett, J.P., Seidah, N.G., Morris, S.J., Sossin, W.S. and Murphy, R.A. (1999) Differential sorting of nerve growth factor and brain-derived neurotrophic factor in hippocampal neurons. J. Neurosci., 19: 2069–2080.

Murphy, R.A., Chlumecky, V., Smillie, L.B., Carpenter, M., Nattriss, M., Anderson, J.K., Rhodes, J.A., Barker, P.A., Siminoski, K., Campenot, R.B., et al. (1989) Isolation and characterization of a glycosylated form of beta nerve growth factor in mouse submandibular glands. J. Biol. Chem., 264: 12502–12509.

Petrides, P.E. and Shooter, E.M. (1986) Rapid isolation of the 7S-nerve growth factor complex and its subunits from murine submaxillary glands and saliva. J. Neurochem., 46: 721–725.

Racke, M.M., Mason, P.J., Johnson, M.P., Brankamp, R.G. and Linnik, M.D. (1996) Demonstration of a second pharmacologically active promoter region in the NGF gene that induces transcription at exon 3. Mol. Brain Res., 41: 192–199.

Ramage, P., Hemmig, R., Mathis, B., Cowan-Jacob, S.W., Rondeau, J.M., Kallen, J., Blommers, M.J.J., Zurini, M. and Rüdisser, S. (2002) Snags with tags: Some observations made with $(His)_6$-tagged proteins. Life Sci. News, 11: 1–4. (Also available at Amersham Biosciences website, http://www.lsn-online.com).

Rattenholl, A., Lilie, H., Grossmann, A., Stern, A., Schwarz, E. and Rudolph, R. (2001a) The pro-sequence facilitates folding of human nerve growth factor from Escherichia coli inclusion bodies. Eur. J. Biochem., 268: 3296–3303.

Rattenholl, A., Ruoppolo, M., Flagiello, A., Monti, M., Vinci, F., Marino, G., Lilie, H., Schwarz, E. and Rudolph, R. (2001b) Pro-sequence assisted folding and disulfide bond formation of human nerve growth factor. J. Mol. Biol., 305: 523–533.

Reinshagen, M., Geerling, I., Eysselein, V.E., Adler, G., Huff, K.R., Moore, G.P. and Lakshmanan, J. (2000) Commercial recombinant human beta-nerve growth factor and adult rat dorsal root ganglia contain an identical molecular species of nerve growth factor prohormone. J. Neurochem., 74: 2127–2133.

Ryden, M. and Ibanez, C.F. (1997) A second determinant of binding to the p75 neurotrophin receptor revealed by alanine-scanning mutagenesis of a conserved loop in nerve growth factor. J. Biol. Chem., 272: 33085–33091.

Saboori, A.M. and Young, M. (1986) Nerve growth factor: biosynthetic products of the mouse salivary glands. Characterization of stable high molecular weight and 32,000-dalton nerve growth factors. Biochemistry, 25: 5565–5571.

Seidah, N.G., Benjannet, S., Pareek, S., Savaria, D., Hamelin, J., Goulet, B., Laliberté, J., Lazure, C., Chrétien, M., Murphy, R.A. (1996) Cellular processing of the nerve growth factor precursor by the mammalian pro-protein convertases. Biochem. J., 314: 951–960.

Selby, M.J., Edwards, R.H., Sharp, F. and Rutter, W.J. (1987) Mouse nerve growth factor gene: Structure and expression. Mol. Cell Biol., 7: 3057–3064.

Smith, P.G., Pedchenko, T., Krizsan-Agbas, D. and Hasan, W. (2002) Sympathetic postganglionic neurons synthesize and secrete pro-nerve growth factor protein in culture. Soc. Neurosci. Abst., 332.3.

Suter, U., Heymach, J.V., Jr. and Shooter, E.M. (1991) Two conserved domains in the NGF propeptide are necessary and sufficient for the biosynthesis of correctly processed and biologically active NGF. EMBO J., 10: 2395–2400.

Treanor, J.J., Schmelzer, C., Knusel, B., Winslow, J.W., Shelton, D.L., Hefti, F., Nikolics, K. and Burton, L.E. (1995) Heterodimeric neurotrophins induce phosphorylation of Trk receptors and promote neuronal differentiation in PC12 cells. J. Biol. Chem., 270: 23104–23110.

Ullrich, A., Gray, A., Berman, C. and Dull, T.J. (1983) Human beta-nerve growth factor gene sequence highly homologous to that of mouse. Nature, 303: 821–825.

Urfer, R., Tsoulfas, P., Soppet, D., Escandon, E., Parada, L.F. and Presta, L.G. (1994) The binding epitopes of neurotrophin-3 to its receptors trKC and gp75 and the design of a multifunctional human neurotrophin. EMBO J., 13: 5896–5909.

Van der Zee, C.E.E.M., Rashid, K., Le, K., Moore, K.A., Stanisz, J., Diamond, J., Racine, R.J. and Fahnestock, M. (1995) Intraventricular administration of antibodies to nerve growth factor retards kindling and blocks mossy fiber sprouting in adult rats. J. Neurosci., 15: 5316–5323.

Wiesmann, C., Ultsch, M.H., Bass, S.H. and de Vos, A.M. (1999) Crystal structure of nerve growth factor in complex with the ligand-binding domain of the TrkA receptor. Nature, 401: 184–188.

Woo, S.B., Timm, D.E. and Neet, K.E. (1995) Alteration of NH2-terminal residues of nerve growth factor affects activity and Trk binding without affecting stability or conformation. J. Biol. Chem., 270: 6278–6285.

Yamashita, T., Tucker, K.L. and Barde, Y.A. (1999) Neurotrophin binding to the p75 receptor modulates Rho activity and axonal outgrowth. Neuron, 24: 585–593.

Yardley, G., Relf, B., Lakshmanan, J., Reinshagen, M. and Moore, GP. (2000) Expression of nerve growth factor mRNA and its translation products in the anagen hair follicle. Exp. Dermatol., 9: 283–289.

Yiangou, Y., Facer, P., Sinicropi, D.V., Boucher, T.J., Bennett, D.L., McMahon, S.B. and Anand, P. (2002) Molecular forms of NGF in human and rat neuropathic tissues: decreased NGF precursor-like immunoreactivity in human diabetic skin. J. Peripher. Nerv. Syst., 7: 190–197.

Yu, G., Michalski, B., Mathew, S., Coughlin, M.D. and Fahnestock, M. The nerve growth factor precursor exhibits biological activity similar to that of mature NGF. Soc. Neurosci. Abstr., 332.2.