Sputa nerve growth factor forms a preferable substitute to mouse 7S- β nerve growth factor

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The NGF (nerve growth factor) from *Naja sputatrix* has been purified by gel filtration followed by reversed-phase HPLC. The protein showed a very high ability to induce neurite formation in PC12 cells relative to the mouse NGF. Two cDNAs encoding isoforms of NGF have been cloned and an active recombinant NGF, sputa NGF, has been produced in *Escherichia coli* as a His-tagged fusion protein. Sputa NGF has been found to be non-toxic under both *in vivo* and *in vitro* conditions. The induction of neurite outgrowth by this NGF has been found to involve the high-affinity $\text{trkA-p75}^{\text{NTR}}$ complex of receptors. The pro-survival mechanism of p75^{NTR} has been mediated by the activation of nuclear factor κB gene by a corresponding down-regulation of inhibitory κB gene.

Real-time PCR and protein profiling (by surface-enhanced laser-desorption-ionization time-of-flight) have confirmed that sputa NGF up-regulates the expression of the endogenous NGF in PC12 cells. Preliminary microarray analysis has also shown that sputa NGF is capable of promoting additional beneficial effects such as the up-regulation of arginine vasopressin receptor 1A, voltage-dependent T-type calcium channel. Hence, sputa NGF forms a new and useful NGF.

Key words: *Naja sputatrix*, nerve growth factor (NGF), neurite outgrowth, PC12 cells, sputa NGF.

INTRODUCTION

In the past decade, the neurotrophin family has gathered much interest as potentially useful therapeutic agents against neurological disorders. The neurotrophin family is made up of NGF (nerve growth factor), BDNF (brain-derived growth factor), NT-3 (neurotrophin-3), NT-4 and a newly discovered NT-6. Neurotrophins are made up of small, homodimeric proteins and they use two different receptors (i) Trk (tropomyosin-related kinase) receptor tyrosine kinase and (ii) p75^{NTR} neurotrophin receptor [a member of the TNF (tumour necrosis factor) receptor superfamily]. The binding of neurotrophin to its receptors has been shown to mediate the survival, differentiation, growth or apoptosis of neurons. Both receptors have been found in the same cell and are known to act synergistically to bring about the targeted response of neurotrophins from the neurons during the development of the nervous system [1]. NGF is required for the survival of the differentiated cells. Both p75^{NTR} and TrkA receptors act synergistically to mediate the trophic effects of NGF in cells that produce them (e.g. PC12 cells). Co-expression of p75^{NTR} has also been found to enhance the biological response of cells expressing TrkA [2]. Interestingly, the TrkA receptor activates positive signals such as increased survival and growth, whereas the p75^{NTR} receptor is known to activate both positive and negative signals. Complexes of p75^{NTR} with TrkA have been found to enhance the neurotrophin-binding affinity and TrkA-associated signalling pathway [3]. Neuroprotection by NGF and activation of NF- κB (nuclear factor κB , a pro-survival transcription factor) have also been shown to be dependent on the expression of p75^{NTR} in PC12 cells [4].

NGF has been detected in various animals including snakes [5–7]. NGFs from human placental tissues and body fluids have

been documented by Goldstein et al. [8] and Lipps [7]. However, the most extensively studied NGF has been obtained from the male mouse submaxillary glands (mouse NGF; [9]). NGF from snakes, particularly from cobras, has been reported to be superior in inducing neurite outgrowth on PC12 cells when compared with NGFs derived from other types of snakes [9]. Server et al. [10] reported that NGFs from cobra, *Naja naja*, and mouse submaxillary glands could also elicit neurite outgrowth from chick embryonic dorsal root ganglion.

NGF is normally produced as a pre-pro-precursor, which is then processed to a mature protein by endopeptidases [11]. The NGF from snakes has also been produced as a pre-pro-NGF molecule with 241 amino acids, which is later processed to form a mature NGF comprising 119 amino acids. Approx. 60 % homology has been seen between the NGF from snakes and rodents. cDNAs encoding mouse, human, bovine, chick and snake NGFs have also been cloned and sequenced [5,6]. In the present study, we describe the cloning and sequencing of two new cDNAs encoding the NGF in the venom glands of a spitting cobra, *Naja sputatrix*. The expressed and refolded recombinant protein, sputa NGF, has been found to be equally active as the mouse NGF in forming neurites in PC12 cells. Functional studies have shown that the sputa NGF promotes neurite outgrowth via the TrkA and p75^{NTR} receptors. Activation of NF- κB gene showed that the pro-survival mechanism of p75^{NTR} -mediated signalling is initiated after the addition of sputa NGF. Furthermore, the endogenous NGF in the PC12 cells has been found to be up-regulated only after treatment with sputa NGF. Oligoprobe microarray analysis also showed that the sputa NGF, although acting through similar mechanisms to mouse NGF, may be capable of bringing about additional beneficial effects.

Abbreviations used: AKAP, A-kinase anchoring protein; ATF3, activating transcription factor 3; BAD, Bcl-2/Bcl-X_L-antagonist, causing cell death; CaM, calmodulin; CE, cathepsin E; CNS, central nervous system; Egr 1, early growth response 1; I κ B, inhibitory κB ; IL-12, interleukin-12; NF- κB , nuclear factor κB ; NGF, nerve growth factor; NT-3, neurotrophin-3; RT, reverse transcription; SELDI-TOF, surface-enhanced laser-desorption-ionization-time-of-flight; SHP-1, Src homology 2 domain-containing tyrosine phosphatase-1; Trk, tropomyosin-related kinase; V1a, vasopressin receptor 1A.

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The nucleotide sequence data reported have been submitted to the DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under the accession numbers AY527215 and AY527216.

MATERIALS AND METHODS

Venom, venom glands and PC12 cells

An adult snake (*N. sputatrix*) obtained from Singapore Zoological Gardens (Singapore), kept undisturbed, was milked and the venom was freeze-dried and stored at -20°C . The venom glands from the same snake were frozen in liquid nitrogen immediately after killing the animal and stored at -85°C . PC12 cells were obtained from A.T.C.C. (Manassas, VA, U.S.A.) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum and 1% antibiotics (penicillin and streptomycin; [12]) at 37°C in 5% CO_2 . The ability of the sputa NGF to elicit neurite outgrowth on PC12 cells was determined as described by Greene and Tishler [13].

Purification of native cobra NGF

Freeze-dried crude venom (50 mg) was reconstituted in 1 ml of water and subjected to gel filtration followed by reversed-phase HPLC (Vision system; Applied Biosystems, Foster City, CA, U.S.A.) using a Sephasil C18 column as described by Armugam et al. [14]. Fractions showing neurite outgrowth on PC12 cells were used for further studies.

Cloning and sequencing of cDNAs

Total RNA was isolated from the venom glands using the guanidine isothiocyanate method [15], and the integrity of the total RNA was analysed by denaturing formaldehyde agarose electrophoresis. Oligonucleotide primers were synthesized by Oswel DNA Services (Southampton, U.K.) based on the sequence at the 5'- and 3'-untranslated regions of cDNA encoding NGF in *Agkistrodon halys* Pallas [6]. Total RNA (3 μg) was reverse-transcribed and amplified by PCR as described by Armugam et al. [14].

The PCR products were cloned into pT7Blue (R) vector (Novagen, Madison, WI, U.S.A.) and transformed into *Escherichia coli* DH5 α : *supE44* Δ *lacU169* (Φ 80*lacZ* Δ m15) *hsdR17* *recA1* *endA1* *gyrA96* *thi-1* *relA1* [16]. The recombinant plasmids from the transformants were subjected to Sanger di-deoxy DNA sequencing using M13/pUC forward and reverse primers [14,17] on an automated DNA sequencer (Model 3100; Applied Biosystems).

Expression and purification of recombinant sputa NGF

The cDNA-encoding NGF was amplified using sense and anti-sense primers, 5'-cctgatccgaagatcatctgtg-3' and 5'-ctcaagcttcagggttcagtt-3' respectively, and subcloned into pQE-30 expression vector (Qiagen, Chatsworth, CA, U.S.A.) between *Bam*HI and *Hind*III sites.

The expression of the sputa NGF was induced by isopropyl β -D-thiogalactoside (1 mM) at 37°C for 3 h and the protein was purified under denaturing conditions (8 M urea) on a Ni^{2+} -nitrilotriacetate Sepharose resin (Qiagen) column. The purified fractions were analysed by Tris/Tricine SDS/10%-(w/v)-PAGE [18]. *In vitro* renaturation [19] of the denatured proteins was performed overnight at room temperature (25°C) in $1 \times$ PBS (pH 8.0), containing 4 mM GSH and 2 mM GSSG.

CD spectral analysis

CD spectra for both the native NGF and recombinant sputa NGF were recorded on a Jasco spectropolarimeter (J715) equipped with an interfaced personal computer. It was calibrated with ammonium *d*-10-camphorsulphonate. Data collected and averaged from at least five recordings were considered acceptable. Spectral

measurements were performed at appropriate concentrations using cells of 0.1 cm path-length [19].

Western-blot analysis

Western blotting was performed as described by Cher et al. [20], using either monoclonal anti-phosphotyrosine (AG10; Upstate Biological, Lake Placid, NY, U.S.A.) or polyclonal anti-TrkA and affinity-purified polyclonal anti-p75^{NTR} (Advanced Targeting Systems, San Diego, CA, U.S.A.) at 1 $\mu\text{g}/\text{ml}$ or monoclonal anti- β -actin (Sigma Chemicals, St. Louis, MO, U.S.A.) at a dilution of 1:2500. Anti- β -actin was used as a reference for quantification.

Real-time quantitative PCR

Total RNA from PC12 cells was extracted by a single-step method using TRIzol[®] reagent (Invitrogen, Carlsbad, CA, U.S.A.). Gene-specific primers for TrkA, p75^{NTR}, endogenous NGF, NF- κ B and κ B (inhibitory κ B) were designed using the PrimerExpress 2.0 software (Applied Biosystems) and their corresponding mRNA sequences. The primers were designed to yield amplicons of < 100 bp and synthesized by Alpha-DNA (Montreal, Quebec, Canada). β -Actin was used as an internal calibrator. The primer sequences were as follows: TrkA, 5'-catggagaaccacagctacttcag-3' (sense) and 5'-ctagctcccacttgagaatgatgtc-3' (antisense); p75^{NTR}, 5'-gtggtcgtggccttg-3' (sense) and 5'-gcgctgtttttttgttg-3' (antisense); rat endogenous NGF, 5'-catgttacaatcccttcaac-3' (sense) and 5'-ccaaccacacactgacactg-3' (antisense); NF- κ B, 5'-gaagagtcctttcaatggacaa-3' (sense) and 5'-tcgggaaggcagcgaata-3' (antisense); κ B, 5'-gctgcccagagtgccgat-3' (sense) and 5'-cagtcacgtagggaactcatc-3' (antisense); β -actin 5'-agggaatcgtcggtgaca-3' (sense) and 5'-gccatctcctgctcgaagtc-3' (antisense).

In the present study, a two-step RT (reverse transcription)-PCR was used. RT was performed as described in [20]. PCR mixtures contained RT products, forward and reverse primers each at 125 nM and SYBR[®] Green master mixture (Applied Biosystems). The PCR amplification was then performed for 1 cycle at 50°C for 2 min and 95°C for 10 min followed by 40 cycles with each cycle at 94°C for 15 s and 60°C for 1 min. All reactions were performed in triplicate using the ABI Prism 7000 SDS (Applied Biosystems).

Protein profiling [SELDI-TOF (surface-enhanced laser-desorption-ionization time-of-flight)]

PC12 cells showing neurite outgrowth were rinsed with 0.9% NaCl and lysed with lysis buffer (50 mM Tris/HCl, pH 7.4, 0.4 mM NaCl, 0.25 M sucrose and 1 mM EDTA, containing 8 M urea). The supernatant was fractionated on hydrophobic reverse-phase chip (H4 Protein Chip TM) using SELDI-TOF MS (Protein Biology System II) both from Ciphergen (Palo Alto, CA, U.S.A.) according to the manufacturer's instructions.

Microarray GeneChip[™] analysis

Microarray analysis was performed as described by Cher et al. [20] with the following modifications. Total RNA isolated from control and treated (mouse NGF or sputa NGF) PC12 cells were processed and hybridized to each array of the rat expression 230A GeneChip[™] Array set according to the methods described in the GeneChip[™] expression analysis package (Affymetrix, Santa Clara, CA, U.S.A.). Relative mRNA expression levels were expressed as positive or negative fold changes when compared with untreated controls based on the Microarray Suite software 5.0 (Affymetrix). All genes showing a change of 2-fold or more were included in subsequent analysis. Members in each cluster were classified according to their biological functions as described in the NetAffix database (Affymetrix).

Table 1 Neurite outgrowth in PC12 cells

Protein concentrations at 100 ng/ml were used unless otherwise stated. '+' denotes neurite outgrowth observed.

Sample	Neurite outgrowth
Mouse NGF (positive control; 100 ng/ml)	+++
<i>B. candidus</i> (250 ng/ml)	++
<i>N. sputatrix</i> (250 ng/ml)	+++++
<i>A. superbis</i> (250 ng/ml)	++
<i>P. textilis</i> (250 ng/ml)	++++
Chinese scorpion (250 ng/ml)	++
Indian scorpion (250 ng/ml)	+
Bee venom (250 ng/ml)	++
Biogel P10 gel fractions of <i>N. sputatrix</i> (Figure 1A)	
Fraction 27	++
Fraction 28	++++
Fraction 29	++
Fraction 30	++++
Fraction 31 (1 ng/ml)	+++++
Fraction 33	++
Fraction 34	Lysis
Fraction 35	Lysis
Fraction 36	Lysis
HPLC fractions (Figure 1B)	
Fraction 3	—
Fraction 4	—
Fraction 5	+
Fraction 6	++
Fraction 7	+++++
Fraction 8 (1 ng/ml)	+++++
Fraction 9	+++
Fraction 10	+
Denatured and refolded recombinant NGF	
Denatured recombinant NGF (100 ng/ml)	++
Refolded recombinant NGF (100 ng/ml)	++++

RESULTS

Bioassay of NGF on PC12 cells

Venoms from several elapids, *N. sputatrix*, *Bungarus candidus*, *Pseudonaja textilis* and *Austrelaps superbis*, as well as venoms from Chinese and Indian scorpions (*Buthus martensi* Karsch and *Mesobuthus tumulus*) and honey bee (*Apis mellifera*) were tested for neurite outgrowth in PC12 cells. β -NGF (7S β -NGF) from the mouse submaxillary gland was used as a positive control. The highest neurite outgrowth was observed for *N. sputatrix* crude venom at 250 ng/ml. Mouse NGF (7S) showed a comparable activity at 100 ng/ml (Table 1).

Identification of a NGF in *N. sputatrix* venom

The *N. sputatrix* venom was chromatographed on Biogel P10 (Figure 1A). The first peak, Peak I, consisted of high-molecular-mass proteins (14–50 kDa), whereas Peak II contained the low-molecular-mass proteins (4–10 kDa). Each fraction (100 ng/ml) was tested for its ability to promote neurite growth (Table 1). The corresponding fraction was also subjected to SDS/PAGE analysis to verify their homogeneity and to determine the molecular mass. The fraction showing the highest neurite outgrowth was purified further on HPLC (Figure 1B). One homogeneous protein peak was obtained, which showed neurite outgrowth at 1 ng/ml (Table 1). This active protein was 14 kDa in mass (Figure 1B, inset). The N-terminal amino acid sequence of this protein was found to be -ETHPVHNRGEYSV- and the protein was found to be non-lethal up to 1 μ g/g in mouse.

Figure 1 Purification of NGF

(A) Gel-filtration chromatography of crude *N. sputatrix* venom. (B) Reversed-phase chromatography of the active fraction obtained from gel filtration. Inset shows the protein (native sputa NGF fraction) analysed by SDS/PAGE. (C) Purification and refolding of recombinant NGF (sputa NGF). Lane 1, protein molecular mass standards (sizes indicated in kDa); lane 2, *E. coli* cell lysate before induction; lane 3, *E. coli* cell lysate (inclusion bodies) after 3 h induction; lane 4, purified sputa NGF; lane 5, refolded sputa NGF. (D) CD spectrum obtained from refolded recombinant sputa NGF and (E) CD spectrum of native NGF protein.

Cloning and expression of the NGF from *N. sputatrix*

The cDNA encoded for a 22-amino-acid signal peptide, a 112-amino-acid pre-pro-domain of NGF and a mature NGF protein

Figure 2 Comparison of amino acid sequences of various NGF

Sputa nsngfI and nsngfII refer to the two isoforms of NGF from *N. sputatrix*; bmNGF is from *Bungarus multicinctus* (S56212); agpngf is from *Agkistrodon halys* Pallas [6]; mousengf (K01759) and human ngf (humNGF, X52599). The pro-NGF region is underlined and the mature peptides are shown in boldface letters. Identical amino acids are shown by (*) and the proteolytic cleavage site (KR) for the mature peptide is indicated by ^^.

consisting of 117 amino acid residues. Analysis of the sequences indicated the presence of two isoforms (nsNGFI and nsNGFII) of venom NGF (Figure 2). The cDNA encoding the mature protein of nsNGFI (identical with the NGF isolated from the venom, Figure 1B) was expressed as a His₆-tagged fusion protein (sputa NGF) and analysed on an SDS/12% (w/v) polyacrylamide gel (Figure 1C, lane 3). The sputa NGF protein was purified by affinity chromatography using a Ni²⁺-nitrilotriacetate column (Figure 1C, lane 4) and was renatured in the presence of GSSG and GSH (Figure 1C, lane 5). The yield of the refolded protein was found to be approx. 3 mg/l. Both the denatured and refolded recombinant NGFs varied in their potency to induce neurite outgrowth on PC12 cells (Table 1). Both the mouse and sputa NGF were found to exhibit comparable levels of biological activity (Figures 3A–3D). The CD spectrum of the refolded recombinant sputa NGF has been found to correlate well with that of the native NGF protein (Figures 1D and 1E). Quantification of neurite outgrowth in the presence of either mouse or sputa NGF (i) with increasing concentration (Figure 3E) and (ii) with increasing duration of NGF treatment (Figure 3G) has shown that the refolded sputa NGF exhibits a comparable activity with that of

mouse NGF. The native NsNGF has also been found to induce neurite outgrowth in a dose-dependent manner but at relatively lower protein concentrations (1–10 ng/ml; Figure 3F).

Expression of TrkA and p75 receptors

Western-blot analysis of the PC12 cells treated separately with the mouse and sputa NGF for 15 min showed that the sputa NGF increased the production of the precursor gp130^{trkA} receptors when compared with the mouse NGF. However, the mature gp140^{trkA} receptor was found in equal amounts for both treatments (Figures 3H and 3I). Neurite extensions can be observed only after 12 h of treatment (optimal at 16–18 h). Under this condition, the cells showed higher concentrations of TrkA and p75^{NTR} receptors than the untreated control. These results were also confirmed by real-time PCR (Figure 3J). Figure 3(J) also shows the expression patterns of endogenous NGF, NF-κB and IκB. The expression of IκB (inhibitor of NF-κB gene) has been found to be negatively regulated. Interestingly, an increased expression of the endogenous NGF gene has been observed only after treatment with the sputa NGF (Figure 3J).

Figure 3 Neurite extension and receptor protein expression after treatment with mouse and sputa NGF

PC12 cells at 1×10^6 were plated and grown overnight. On the following day, cells were treated with either mouse or sputa NGF, at 200 ng for 16–18 h unless otherwise stated. (A, B) Neurite extensions observed after treatment with mouse NGF. (C, D) Neurites induced by sputa NGF. (E) Neurite extensions quantified for mouse (black bars) and sputa (white bars) NGF (10, 50 and 100 ng). (F) Neurite outgrowth observed for native nsNGF (stippled bars) at 1, 5 and 10 ng. (G) Time-course induction of neurite outgrowth by mouse (black bars) and sputa (white bars) NGF. (H) Activation of TrkA receptor determined by Western-blot analysis. Cells were treated for 15 min and total protein was extracted. Equal amounts of protein were separated on a SDS/7.5 % polyacrylamide gel and subsequently blotted on to nitrocellulose membrane. (i) Phosphorylated TrkA receptors (gp110 and gp140) were detected using monoclonal anti-phosphotyrosine antibody. The same membrane was stripped and probed with (ii) anti-TrkA and (iii) β -actin antibodies. Lane 1, mouse NGF-treated cells; lane 2, sputa NGF-treated cells and lane 3, untreated control cells. (I) Ratios of the phosphorylated TrkA and β -actin (internal control) levels measured by densitometry. (J) Quantitative gene analysis via SYBR Green assay. The RNAs from treated and untreated samples were used to study the expression of five genes: TrkA, p75^{NTR}, NF- κ B and I κ B.

Protein profiling (SELDI-TOF) analysis

Protein profiling identified five proteins that showed significant changes after NGF treatment (Figure 4). The analysis showed changes in expression of both growth-related (NGF precursor and neurogranin) and an apoptotic protein (BAD, Bcl-2/Bcl-X_L-antagonist, causing cell death). Cells treated with sputa NGF (Figure 4A) showed a decrease (approx. 6-fold) in an 8 kDa protein and an increase (approx. 2-fold) in an 11.4 kDa protein.

Cells treated with mouse NGF (Figure 4B) showed a down-regulation (approx. 10-fold) of a 13.9 kDa protein. Comparison between sputa NGF and mouse NGF treatments (Figure 4C) showed that sputa NGF caused an increase in the growth-associated proteins similar to AKAP (A-kinase anchoring protein, 11.4 kDa) and the endogenous NGF precursor (13.9 kDa) when compared with pro-apoptotic protein (BAD). Expression of protein at 8 kDa (possibly neurogranin) was greatly decreased (approx. 5-fold), whereas proteins at 22.3 kDa (BAD) and

Figure 4 Protein profiling analysis

Total protein extracts from cells treated with either mouse NGF or sputa NGF for 16–18 h were used. (A) Protein profiles obtained from cells treated with sputa NGF versus untreated control. (B) Protein profiles obtained from cells treated with mouse NGF versus untreated control. (C) Protein profiles obtained for sputa NGF versus mouse NGF.

67.2 kDa [SHP-1 (Src homology 2 domain-containing tyrosine phosphatase-1)] showed a marginal up and down-regulation respectively (Figure 4C).

Microarray analysis of NGF-treated PC12 cells

The global gene expression after treatment with both NGFs was studied using oligoprobe microarrays. The genes whose expression is altered by 2-fold or more were taken as significant from approx. 730 genes examined in our study. These probe sets were subjected to a self-organizing map (Data Mining Tool; Affymetrix) clustering and were grouped according to their expression patterns. The genes were also further assigned to categories according to their biological functions. Some of them are shown in Table 2. Most of these genes were found to be involved in the cell cycle and growth pathways.

DISCUSSION

Cloning and expression of a new recombinant NGF protein

A widely studied and commercially available NGF, β -NGF, is from the mouse submaxillary gland. Lipps [9] reported that the NGFs from venomous sources are of the highest potency and particularly the NGF from snakes is approx. 1000-fold more active than the commercially available mouse NGF. The crude venom (250 ng/ml) from four untested snakes when assayed for neurite

outgrowth on PC12 cells showed that the venom of *N. sputatrix*, Malayan spitting cobra, exhibited the highest activity. This was followed by the venom from an Australian elapid, *P. textilis*. Thus the NGF from *N. sputatrix* venom was purified, and its activity and properties were compared with those of mouse NGF. The molecular mass of the native sputa NGF was found to be 14 kDa, thus indicating that it is a monomer belonging to β -NGF family as suggested by Inoue et al. [21].

The cDNA encoding NGF from *Agkistrodon halys* Pallas has been reported previously [6]. In the present study, we report the cloning of cDNAs encoding two isoforms of sputa NGF from *N. sputatrix*. Each cDNA encoded a signal peptide, pre-pro-domain and the mature protein. The mature protein-coding region for *N. sputatrix* was identified from its N-terminal amino acid sequence as well as from the amino acid sequence of *Naja atra* NGF [21,22]. Comparison of sequences from the venom-derived NGFs with those of mouse NGF showed distinct variations at their N-terminal ends. The snake NGF shows higher homology (approx. 80%) with the mammalian-derived NGFs, thus suggesting similar function to the mammalian homologue. The pro-region of the cobra NGF however shows approx. 50% homology to the mammalian counterpart. Pro-NGF has long been postulated to contain a biologically active peptide that could induce phosphorylation of the tyrosine residue of the TrkA receptor protein [23]. On the other hand, secreted pro-NGF has been reported to activate cell death in brain following an injury via p75 and sortilin receptors [24], thus indicating a pathophysiological role for the pro-NGF during CNS (central nervous system) injury. In contrast, Fahnestock et al. [25] have shown that the pro-NGF is in abundance in CNS tissues where mature NGF is not detected. The researchers have demonstrated that this pro-NGF is responsible for the biological activity normally attributed by mature NGF, in the CNS tissues. The pro-NGF in snakes shows approx. 50% homology to the mammalian pro-NGF. Furthermore, a deletion of five amino acids can be observed in the mammalian pro-NGF sequence. Hence, their functions may be different. The snake pro-NGF could possibly be involved in the positioning of NGF moiety for proper proteolytic processing and activation of proper *in vitro* folding of the mature peptide [26]. The death-inducing property, if any, of the pro-NGF from snakes remains to be elucidated.

The venom-derived NGFs showed approx. 80–90% homology among them, and the two isoforms of *N. sputatrix* NGF (nsNGFI and nsNGFII) showed approx. 80% homology between them. nsNGFI and nsNGFII showed 70 and 60% homology to the mammalian NGF respectively. Whereas the mature protein region for both nsNGFI and nsNGFII showed high homology, the pro-region showed considerable variation among them. Owing to its higher homology to the mammalian homologue, nsNGFI was selected for the expression of recombinant protein in *E. coli* and termed sputa NGF. This recombinant molecule appears to have two pairs of β -sheets that are linked by three disulphide bonds. We were able to refold it in redox solution containing 2 mM GSSG and 4 mM GSH. The refolded recombinant protein showed a CD spectrum similar to that of the native protein, and it showed enhanced activity when compared with the unfolded NGF (Table 1). This refolded sputa NGF was also found to be non-toxic to PC12 cells at 1 μ g/ml. It was able to bring about enhanced neurite outgrowth in a dose-dependent manner from 100 to 500 ng/ml.

Expression of TrkA and p75 receptors

The sputa NGF has been found to elicit neurite outgrowth via the TrkA–p75^{NTR} complex. Two protein forms of TrkA that predominate in cell extracts are: (i) the 110 kDa N-glycosylated precursor form, gp110^{TrkA} and (ii) the 140 kDa fully matured form, gp140^{TrkA}.

Table 2 Classification of genes from microarray

Genes were grouped based on their biological functions. Notable changes in gene expression are shown in boldface.

Category of genes	Description	Mouse NGF (fold change)	Sputa NGF (fold change)
Cell cycle	Growth arrest and DNA-damage-inducible 45 α	3.63	3.18
	Proliferating cell nuclear antigen	−2.07	−1.74
	ret proto-oncogene	2.87	2.91
	ret proto-oncogene	4.06	4.41
	Cyclin-dependent kinase inhibitor 1A	4.99	4.26
	Growth-associated protein 43	2.27	2.10
Apoptosis	Apoptotic death agonist BID	3.36	2.85
	Bcl-2-like-1	2.22	1.77
Eicosanoid synthesis	Prostaglandins endoperoxide synthase 1	4.59	2.36
	IL-12 p35 subunit	6.19	4.72
Transcription factors	Core promoter element-binding protein	2.53	1.57
	fos-like antigen 1	8.57	3.51
	Nuclear transcriptional factor-Y- β	2.01	1.14
	ISL 1 transcriptional factor, LIM/homeodomain 1	2.75	1.74
	Global ischaemia-induced protein GII15B	−2.38	−2.07
	DNA polymerase β	2.20	1.88
	GATA-binding protein 4	1.39	2.17
	Myelin transcription factor 1-like	−2.20	−1.33
	Signal transducer and activator of transcription 3	2.04	1.66
	ATF3	2.2	−1.54
	Egr 1	15.14	12.91
Translational factors	Eukaryotic initiation factor-5	2.03	1.60
	Eukaryotic initiation factor-2B, subunit 3	2.04	1.82
Others	Meprin 1 α	2.55	2.10
	CE	11.08	8.22
	Mast cell protease 7	88.65	71.51
	Pls1a ras-related homologue A2	2.69	2.33
	Small GTP-binding protein rab5	2.20	1.79
	GTP cyclohydrolase 1	3.20	2.10
	Phospholipase D	2.41	2.55
	Kinesin family member 1B	1.45	2.01
	Procollagen, type XII, α 1	2.30	1.41
	Integrin β 1	2.31	2.39
	Insulin receptor substrate 1	2.06	1.27
	Double cortin and calcium/CaM-dependent protein kinase-like 1	2.69	1.84
	Gap junction membrane channel protein β 5	2.79	2.04
	Jun-B oncogene	2.28	2.39
	Opioid receptor-like	2.13	2.73
	Arginine V1a	5.43	20.25
	Purinergic receptor P2X, ligand-gated ion channel 2	−2.91	−1.59
	Purinergic receptor P2X, G-protein coupled 2	2.06	2.11
	Adenosine monophosphate deaminase 3	2.48	1.85
	Myelin basic protein	1.04	2.35
	Prolactin receptor	2.17	1.54
	Serum-inducible kinase	7.26	3.12
	Syntaxin	2.71	1.45
	Dynamin 1	−2.07	−1.39
	Guanosine diphosphate dissociation inhibitor 3	2.17	2.39
	Annexin 5	3.03	2.77
	Neural receptor protein-tyrosine kinase	9.19	3.16
	Moesin	2.97	2.48
	Vesicle-associated membrane protein, associated proteins B and C	1.71	2.10
	ADP-ribosylation-like 4	2.71	2.28
	Solute carrier family 1, member 3	6.28	4.99
	Solute carrier family 9, member 4	−1.44	2.23
	Syndecan 1	2.95	2.39
	Myosin IXA	2.22	1.99
	Potassium inwardly rectifying channel, subfamily J, member 6	2.20	1.95
	Protein kinase, cAMP-dependent regulatory, type I, α	2.53	2.08
	Coated vesicle membrane protein	2.00	1.89
	Regulator of G-protein signalling protein 2	2.25	1.38
	Cold-inducible RNA-binding protein	1.97	2.30
	Microtubule-associated protein 1b	2.31	1.29
	Acid phosphatase 5	2.01	1.91
	Profilin II	2.38	2.06
	Glutamate receptor, ionotropic, NMDA2C	−2.50	−1.91
	Lysosomal membrane glycoprotein 2	−1.97	−2.20
	NGF receptor-associated protein	−2.03	−1.55

Table 2 (contd.)

Category of genes	Description	Mouse NGF (fold change)	Sputa NGF (fold change)
Calcium-related pathway	Very-low-density lipoprotein receptor	2.16	1.83
	Very-low-density lipoprotein receptor	2.57	2.00
	Very-low-density lipoprotein receptor	2.22	2.10
	Calcium-binding protein p22	3.66	2.19
	S-100-related protein, clone 42C	3.36	2.71
	S-100-related protein A4	2.06	1.95
	Cadherin	-3.14	-2.33
	Calcium channel, voltage-dependent, T-type, α 1 subunit	5.54	7.21
	Protein phosphatase 3, regulatory subunit B, α isoform, type 1	2.23	1.78

[27]. PC12 cells are known to respond rapidly to NGF within minutes, with a reproducible burst of tyrosine phosphorylation of many cellular proteins and include the TrkA receptors. We observed that the ratio of gp140^{TrkA} to gp110^{TrkA} decreased on both mouse and sputa NGF treatments for the purpose of replenishing the mature gp140^{TrkA} at the cell surface as described by Jullien et al. [28]. TrkA and p75^{NTR} receptors work in concert to co-ordinate and modulate responses of neurons to NGF. A competitive study using specific receptor antibodies on the percentage of neurite outgrowth showed that sputa NGF is more sensitive to receptor inhibition compared with that of mouse NGF. Reduced TrkA activation in both PC12 cells and sympathetic neurons has been observed after the addition of p75^{NTR}-specific antibodies [29]. The researchers have also shown that any alteration in the optimal TrkA–p75^{NTR} interaction or direct activation of p75^{NTR} at the expense of TrkA, will result in an inhibition of NGF-dependent neurite outgrowth in adult sensory neurons. In the present study, inhibition by TrkA and p75^{NTR} receptor-specific antibodies caused a reduction in neurite outgrowth for both mouse and sputa NGF by 34 and 70 % for TrkA and 10 and 40 % for p75^{NTR} respectively. Thus the greater reduction in neurite formation on sputa NGF treatment implicates that the sputa NGF is more specific to TrkA and p75^{NTR}, and stimulates signalling using the TrkA–p75^{NTR} high-affinity complex as for mouse NGF. This antibody-based competitive study is in agreement with the quantitative analysis of the receptor (TrkA and p75^{NTR}) genes examined via real-time PCR. The expression of both receptors was higher with sputa NGF when compared with mouse NGF. Activation of TrkA and p75^{NTR} has been reported to induce the expression of NF- κ B, which in turn is known to cause neurite extension in PC12 cells [30]. Real-time PCR (Figure 3C) showed a down-regulation of the NF- κ B inhibitor, I κ B, in both NGF treatments, implying that NF- κ B will be made available to activate and promote neurite extension in PC12 cells. However, an increase in NF- κ B expression is observed for sputa NGF treatment compared with mouse NGF treatment and such an NGF-induced increase in NF- κ B is beneficial for the survival of neurons [31]. Similarly, the endogenous NGF level was up-regulated after treatment with sputa NGF in contrast with mouse NGF. Several studies have shown that neurotrophic factors, including NGF, constitute an important class of endogenous modulators of excitotoxicity, and are known to protect neurons against injury from several causes [32]. NGF has caused increase in glutathione levels of PC12 cells [33], and hence up-regulation (of NF- κ B and endogenous NGF genes) by sputa NGF may have potential neuroprotective roles.

Global gene and protein analysis

Microarray analysis (Table 2) showed for the first time the global changes in gene expression after treatment with either mouse NGF

or sputa NGF on PC12 cells. This study also yielded comparable data between the two NGFs. The differentiation pathway after NGF treatment is characterized by expression of immediate early genes [such as Egr 1 (early growth response 1) and serum-inducible kinase] and delayed response genes (such as Fos-like antigen, Fra-1 and prostaglandin-endoperoxide synthase 1). It was shown that Egr 1 acts via the extracellular-signal-regulated kinase pathway to produce eventually neurite outgrowth in PC12 cells [34]. Liby et al. [35] have shown that serum-inducible kinase has a role in cell proliferation. Expression of transcription factors, e.g. Fra-1, may participate in long-term neural responses [36], whereas overexpression of ATF3 (activating transcription factor 3) inhibited apoptosis [37]. Enhanced expression of transcription factors (Fra-1, ATF3 and Egr 1) may also cause subsequent increase in CE (cathepsin E) expression [38]. This explains the increase in CE observed in the present study (Table 2). CE is an aspartic protease, which has been implicated in antigen processing in the class II MHC pathway and is known to be up-regulated late in B-cell activation [39]. The concurrent up-regulation of mast cell protease 7 in the present study is in agreement with the previously reported effects of NGF on mast-cell development and differentiation [40].

Arginine V1a (vasopressin receptor 1A) was observed to be highly expressed by sputa NGF treatment when compared with mouse NGF. V1a is widely distributed and known to regulate nearly all the physiological actions of the neuropeptide, vasopressin (AVP), except antidiuresis (V2) and corticotrophin secretion (V1b; [41]). In addition to these, channel transporter genes (solute carrier family 1, member 3; SLC1A3, voltage-dependent calcium channel and purinergic P2X receptors) were found to be affected by the NGF treatment. Mouse NGF activated both the expression of SLC1A3 and P2X receptor genes, whereas sputa NGF activated the voltage-dependent calcium channel gene. The SLC1A3 gene encodes the glutamate receptor GLAST (glutamate/aspartate transporter) responsible for the removal of glutamate to terminate neurotransmission and prevent neuronal cell death [42], whereas the expression of voltage-dependent calcium channel subunit II is restricted to neuronal tissues for the maintenance of the neuronal physiology [43]. In the present study, the down-regulation of P2X receptor was observed in both NGF treatments. This proved to be beneficial as P2X is postulated to be involved in astrocyte-mediated inflammation and neurodegeneration [44]. Up-regulation of IL-12 (interleukin-12) and prostaglandin-endoperoxide synthase 1 seen in both cases also supports their beneficial roles. Prostaglandin-endoperoxide synthase 1 is believed to have a physiological role in neuronal differentiation of PC12 cells [45]. Similarly, IL-12 is known to play an important role in neuronal regeneration [46]. Hence, both NGFs showed similar activities, except for V1a expression. Arginine vasopressin that acts mainly through the V1a receptor

is known to enhance NGF secretion in vascular smooth muscles and rat brain cells [47,48].

Protein profiling and real-time PCR results confirmed that an endogenous NGF synthesis is up-regulated after treatment with sputa NGF. Hence, sputa NGF could possibly be acting through V1a receptor to enhance the endogenous NGF production, an effect that will prove to be useful under conditions that require neuronal regeneration and differentiation.

The sputa NGF is also found to enhance the production of growth-associated proteins such as AKAP. The AKAP (11.4 kDa) belongs to a family of scaffolding proteins that bind to the regulatory subunits of protein kinase A, which regulates the phosphorylation of various proteins including those involved in synaptic plasticity. AKAP induction is known to stimulate protein kinase A-dependent phosphorylation of the pro-apoptotic protein BAD (protein at 22.3 kDa), thereby inhibiting the release of cytochrome *c* from mitochondria and protecting the cells from apoptosis [49]. Although sputa NGF showed a marginal increase in BAD protein when compared with mouse NGF, with AKAP up-regulated approx. 2-fold more than BAD, the pro-apoptotic property of BAD could be suppressed. This interpretation was confirmed experimentally by measuring for caspase activity on PC12 cells treated with sputa NGF. No caspase activity could be determined in these cells.

The mechanisms by which NGF exerts its neuroprotective effects are currently unknown. However, *in vitro* studies have shown that NGF can stabilize intracellular calcium by preventing accumulation of intracellular calcium and subsequent neuronal damage [50]. The protein profiling results showed a down-regulation in neurogranin (8 kDa). Neurogranin is a neural-specific Ca^{2+} -sensitive CaM (calmodulin)-binding protein. It is believed that phosphorylated neurogranin has the potential to modulate neuronal free- Ca^{2+} and CaM levels [51]. Gene knock-out experiments leading to a total absence of neurogranin have resulted in deficiency of hippocampal synaptic plasticity and hippocampus-dependent spatial learning [51]. McNamara et al. [52] predicted that an increase in neurogranin expression would perturb Ca^{2+} -CaM signalling that may in turn impair the formation and/or maintenance of synapses. Thus a decreased level of free neurogranin detected after treatment with sputa NGF indicates that a basal level of intracellular Ca^{2+} is maintained for neuronal differentiation and function.

Protein tyrosine phosphatase (SHP-1; 67.2 kDa) was identified as a phosphotyrosine phosphatase, which negatively regulates the expression of TrkA. It was shown in sympathetic neurons that expression of SHP-1 induces apoptosis and TrkA dephosphorylation [53]. Hence, a down-regulation of SHP-1 expression is also in agreement with TrkA activation after treatment with both NGFs.

In conclusion, sputa NGF seems to exert potentially life-sustaining and neuroprotective (NF- κ B and endogenous NGF genes) effects in a manner similar to that of mouse NGF. The life-sustaining effects have been observed at both gene (microarray) and protein (SELDI-TOF) levels. Sputa NGF has also been found to increase the expression of V1a and endogenous NGF genes as well as the NGF precursor and the AKAPs. However, both neurogranin and SHP-1 proteins have been found to be down-regulated after treatment with sputa NGF. Real-time PCR analysis has revealed additional pro-survival effects of sputa NGF such as enhanced expression of NF- κ B and the endogenous NGF genes. These effects could have potential neuroprotective roles.

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