Generation of rationally-designed nerve growth factor (NGF) variants with receptor specificity.

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Abstract

Nerve growth factor (NGF) is the prototypic member of the neurotrophin family and binds two receptors, TrkA and the 75 kDa neurotrophin receptor ($p75^{NTR}$), through which diverse and sometimes opposing effects are mediated. Using the FoldX protein design algorithm, we generated eight NGF variants with different point mutations predicted to have altered binding to TrkA or $p75^{NTR}$. Of these, the I31R NGF variant exhibited specific binding to $p75^{NTR}$. The generation of this NGF variant with selective affinity for $p75^{NTR}$ can be used to enhance understanding of neurotrophin receptor imbalance in diseases and identifies a key targetable residue for the development of small molecules to disrupt binding of NGF to TrkA with potential uses in chronic pain.

Key words: FoldX; NGF; p75 neurotrophin receptor ($p75^{NTR}$); TrkA

Introduction

Nerve growth factor (NGF) is a member of the secreted neurotrophin family that have diverse roles in development and in the adult nervous system [1]. NGF is synthesized as a 35 kDa precursor, proNGF. The NGF prodomain is important for expression, secretion and folding of the mature protein. It is cleaved intracellularly and extracellularly to generate mature NGF comprising two 14 kDa monomers [2]. ProNGF and NGF have distinct, or even opposite biological effects. Binding of NGF to *tropomyosin* receptor kinase A (TrkA) regulates cell survival, differentiation and proliferation. The best characterized signaling pathways activated through TrkA are the PI3K/Akt and Ras/ERK pathways [3]. Activation of 75 kDa neurotrophin receptor ($p75^{NTR}$) by proNGF and NGF binding initiates a diverse array of biological effects, that are heavily dependent on the cellular context, of which the best characterized are the RhoA pathway, which regulates axon growth and degeneration, the pro-survival NF-kB pathway, and the pro-apoptotic caspase cascade [3].

An imbalance of NGF and proNGF signaling is reported to contribute to Alzheimer's disease, spinal cord injury, chemoresistance of certain cancers and chronic pain [4,5]. Therapies, such as NGF gene delivery and anti-NGF monoclonal antibodies showed great promise in clinical trials for treatment of Alzheimer’s disease [6] and pain associated with osteoarthritis, respectively [7]. However, there is still a need for alternative therapies.

Here we describe the generation of NGF variants, designed using the FoldX algorithm and the available crystal structures of NGF in complex with $p75^{NTR}$ and with TrkA [8–10]. From eight NGF mutants that were predicted to have altered receptor binding, one variant (I31R) was identified to have a selective affinity for $p75^{NTR}$, highlighting I31 as a key residue that can be targeted to selectively disrupt NGF-TrkA signaling.

Experimental Procedures:

Materials

All chemicals were from Sigma-Aldrich unless otherwise indicated.

Computational design of receptor selective NGF variants

X-ray crystal structures of NGF in complex with TrkA (PDB code 2IFG) [10] and $p75^{NTR}$ (1SG1) [9] were used as structural templates for computational design of receptor selective variants using FoldX as described previously [11] (http://foldx.crg.es/).

Cell culture

HEK293T (ATCC) and RN22 Schwannoma cells (ECACC) were cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 50 units/ml penicillin and 50 µg/ml streptomycin. For recombinant protein production HEK293T cells were seeded at 5.8 x 10^4 cells/cm^2. RN22 were seeded at a density of 3 x 10^4 cells/cm^2. Rat adrenal pheochromocytoma PC12 cells (ECACC) were cultured in DMEM supplemented with heat-inactivated 5% FBS and 10% horse serum, 50 units/ml penicillin and 50 µg/ml streptomycin. PC12 cells were seeded at 3 x 10^4 cells/cm^2 in plates coated with 10 µg/ml poly-L-lysine, and left overnight before commencing treatments.

Construction of histidine-tagged NGF variants

The mutants were constructed using the megaprimer mutagenesis method which utilized mutagenic primer, reverse and forward primers and two rounds of PCR performed on a hβNGF DNA (GeneCopoeia). All PCR reactions contained DNA template, 1 µM forward and reverse primers, 5 µM dNTPs, 1U Phusion 'Hotstart' high fidelity DNA polymerase and 1 x Phusion

buffer. Following first PCR, megaprimer was separated by gel electrophoresis and purified using the QIAquick PCR Purification Kit (Qiagen) standard protocol. After the second PCR full-length NGF mutant DNA was purified. The C-terminal His-tagged NGF constructs were generated by fusion PCR which required three PCR reactions performed on a NGF variant DNA. The first PCR reaction contained the forward primer and the reverse primer with His-tag sequence. The second PCR reaction contained the reverse NGF primer and the forward tagged primer. These two PCR reaction products were combined in the third PCR reaction with the forward and reverse primers to generate the full length NGF DNA. The PCR products were then separated by gel electrophoresis and purified. WT_NGF and NGF variants were cloned into pcDNA3.1+ (Invitrogen) using the *Bam*HI and *Not*I sites. Introduction of mutations and His-tag was confirmed by DNA sequencing.

Cell transfection

HEK293T cells were transfected using JetPEI transfection reagent (Polyplus transfection reagents) with a DNA to lipid ratio of 1:2, in antibiotic-free media. Following incubation at 37 ^{o}C for 5 h the media was replaced with fresh serum free media. After 48 h the conditioned media, containing secreted NGF, was harvested, centrifuged at 775 g at 4 ^{o}C for 10 min and stored at -80 ^{o}C until purification. Expression and secretion of NGF variants was confirmed by immunoblotting and ELISA.

NGF protein purification

Secreted NGF in HEK293T culture media was digested using 0.2 U/ml furin (New England Biolabs) for 6 h at 37 oC and then concentrated using Amicon ultra 15 filters (molecular weight cut off of 10 kDa) (Millipore). The media was concentrated to 1 ml by centrifugation in Amicon filters at 3000 rpm for 40 min and diluted in wash buffer (50 mM NaH2PO4, 0.5 M NaCl, 20 mM imidazole, pH 8). The concentrated NGF was then applied to Ni-NTA agarose beads

(QIAGEN). The beads were first washed in 1 ml wash buffer followed by incubation with the NGF overnight at 4 oC with rotating. The beads were then centrifuged and washed with 4x 1 ml of wash buffer. The beads were next resuspended in elution buffer (50 mM NaH2PO4, 0.5 M NaCl and 250 mM imidazole, pH=8), rotated for 5 min and then centrifuged. The supernatant was retained. The elution was repeated a total of 3 times. NGF purification was confirmed by Western blotting and the NGF concentration quantified by ELISA.

NGF variants binding assay

The immune assay plates (Nunc) were coated with 100 µg/ml of soluble Fc-linked TrkA and p75NTR receptors (R&D Systems, #175-TK-050 and #367-NR-050/CF) in PBS overnight at room temperature. Plates were washed 3 times with PBST and blocked with 1% BSA in PBS for 1 h. Plates were then washed as before and purified NGF variants (0.625-15 ng/ml) were applied to the plates. After 2 h incubation at room temperature, the wells were washed. Bound NGF was detected by incubation with 50 ng/ml biotin-tagged anti-NGF antibody (R&D Systems) at room temperature for 2 h. After washes, streptavidin-HRP (1/400 dilution) (R&D Systems) was applied and incubated at room temperature for 20 min. Plates were washed and incubated with 3,3',5,5'-Tetramethylbenzidine (TMB) (Thermo Scientific) in the dark for 20 min. The reaction was stopped by addition of 2 M H2SO4 and the absorbance was read at 450 and 550 nm using a microplate reader (Victor x3).

Neurite outgrowth assay

PC12 cells were seeded at density 8 x 10^3 cells/cm^2 onto PLL-coated plates and treated for 7 days with 100 ng/ml NGF variant-containing media from HEK293T cells. The cells were stained with 1 g/L hematoxylin solution and phase contrast images were taken. The neurite outgrowth was then quantified, 100 cells were counted and the number of cells extending neurites was quantified.

Immunoblotting

Cells were scraped from culture flasks, washed in PBS and lysed in 20 mM HEPES pH 7.5, 350 mM NaCl, 1 mM $MgCl_2$, 0.5 mM ethylenediaminetetraacetic acid, 0.1 mM ethylene glycol tetraacetic acid, 0.1% Nonidet P-40, 0.5 mM dithiothreitol, 0.1% phenylmethylsulphonyl fluoride (PMSF), 1% aprotinin, 5 mM NaF, and 1 mM Na_3VO_4. Protein concentration was measured using Bradford reagent with bovine serum albumin (BSA) as a standard. Protein samples were denatured using Laemmli's buffer (62 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulphate (SDS), 5% β-mercaptoethanol, 4% glycerol, 1 mM PMSF, 0.01% bromophenol blue) and boiled at 95 ^{o}C for 5 min. Proteins were separated by 15% SDS-polyacrylamide gel electrophoresis and electrophoretically transferred onto nitrocellulose membranes (Whatman). Membranes were blocked for 1 h in PBS containing 0.05% Tween 20 (PBST) and 5% (w/v) non-fat dried milk. Membranes were probed with antibodies against: phospho-ERK (Cell Signalling Technologies, CST), total ERK (CST), total IκB (Millipore), Actin (Sigma) followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibody (Jackson ImmunoResearch Laboratories) (1:10,000) for 2 h at room temperature. Protein bands were visualized using chemiluminescent reagent (Millipore).

MTT assay

PC12 cells were incubated with 0.5 mg/ml of 3-(4,5-dimethythiazol2-yl)-2,5-diphenyl tetrazolium bromide (MTT) for 3 h at 37 °C. To stop the reaction and solubilize the formazan crystals 20% (w/v) SDS in 50% (v/v) dimethyl formamide was added and the absorbance measured at 550 nm by a plate-reader (Victor x 3). Cell viability was expressed as percent of viable cells relative to the empty vector (EV) control.

Statistical analysis

Statistical analysis was carried out using SPSS software. Values are expressed as ± SD of 3 separate experiments, otherwise stated. Data were analyzed using repeated-measures ANOVA followed by LSD multiple comparisons *post hoc* test. Differences were considered statistically significant at *p<0.05; **p<0.01, ***p<0.001.

Results and discussion

Design of NGF variants

Introducing small molecules to modulate neurotrophin signaling is challenging, particularly for agonistic ligands, where bivalency is desirable [12]. The development of molecules with TrkA and $p75^{NTR}$ receptor selectivity may help to elucidate a function of each receptor and would be of therapeutic relevance in neurodegenerative disorders, chemoresistance and chronic pain. Available crystal structures for NGF in complex with TrkA and $p75^{NTR}$, and for proNGF in complex with $p75^{NTR}$ [8,10,13] enables modelling and prediction of mutations that influence ligand-receptor interactions. Here, we used the FoldX protein design algorithm [14] to predict NGF variants which alter binding to its receptors. The NGF variants were prioritized based on the free energy of unfolding. Importantly, several of the NGF mutations designed by FoldX are in residues that have been previously reported to be involved in receptor interaction, confirming the capability of the design algorithm [15,16].

Of particular interest the FoldX protein design algorithm predicted an interaction site within NGF that distinguishes between TrkA and $p75^{NTR}$ binding, the I31-K32-G33 containing loop (Fig. 1). It has previously been reported that I31 and the nearby residues T29, K32, G33 in NGF are important for interaction with TrkA, while residues K32 and K34 are important for binding to $p75^{NTR}$ [10]. The G33M mutant was chosen as FoldX predicted it would specifically

bind to TrkA receptor (Fig. 1). The K32R was predicted to increase binding at both receptors, while H84Q should bind to both receptors with an affinity comparable with WT_NGF. The A98I and I31R variants would have an increased affinity for $p75^{NTR}$. The I31 residue is located in the loop I of NGF that interacts with $p75^{NTR}$ at cysteine rich domain CRD2. However, I31 has also been shown to interact directly with TrkA [17]. Therefore, it was expected that alteration of this amino acid would affect TrkA signaling and results in a selective activation of $p75^{NTR}$. The R69 residue is located in the loop IV of NGF that binds to $p75^{NTR}$. The R69D mutant was expected to have a reduced affinity for this receptor (Fig. 1). Along the residues of the N-terminal domain of NGF, the H4 residue has previously been reported to have an important role in receptor binding and the H4D mutant showed a reduced binding to TrkA [18]. In support of this, FoldX predicted that substitution of H4 residue of NGF with D or E amino acid will alter TrkA affinity. The N-terminal domain of NGF is not observed in the co-crystal structure with $p75^{NTR}$ due to conformational flexibility and thus difficult to resolve by x-ray crystallography, and as such is not thought to contribute to binding of $p75^{NTR}$ [19]. The predicted changes in free energy of binding of the NGF variant-receptor complex ($\Delta\Delta G$) and on protein stability are shown in Fig. 1D. Negative $\Delta\Delta G$ values indicate that the mutations should increase the binding affinity of NGF to its receptor.

Binding of the NGF variants to TrkA and $p75^{NTR}$

Constructs encoding His-tagged wild-type and variants proNGF were transiently expressed in HEK293T cells [2,20]. Mature NGF variants secreted to culture media were His-trap purified and then applied to immobilized TrkA and $p75^{NTR}$ to determine the binding affinity for receptors (Fig. 2).

The NGF variants H4E and H4D exhibited an increase in binding to TrkA at the lower concentrations, while at concentrations above 5 ng/ml of NGF they showed ~40% and ~60%

reduction in binding compared with WT_NGF, respectively (Fig. 2A). H4D exhibited binding affinity to $p75^{NTR}$ that was similar to WT_NGF. In contrast, H4E had a greatly reduced interaction with $p75^{NTR}$ (Fig. 2C). Contrary to FoldX predictions, the G33M and H84Q variants both showed ~50% reduction in binding to TrkA. While G33M binding to $p75^{NTR}$ was similar to that of WT_NGF, the H84Q variant showed a markedly different binding curve to $p75^{NTR}$ (Fig. 2C and D). The altered binding of H84Q to $p75^{NTR}$ was not expected, as H84 residue is not in the $p75^{NTR}$ receptor ligand interface.

The K32R and A98I variants displayed a marked increase in $p75^{NTR}$ receptor binding compared with WT_NGF (Fig. 2C and D). Both had also a higher binding to TrkA, with A98I showing only a modest increase and K32R exhibited a ~40% stronger affinity for TrkA at higher NGF concentrations (Fig. 2A and B). The R69D variant was unable to bind to either TrkA or $p75^{NTR}$ (Fig. 2B and D). This is likely due to a decrease in stability as predicted by FoldX, which could affect protein folding and activity (Fig. 1D). Notably, the I31R variant, predicted to have an opposing profile to that of G33M and be selective for $p75^{NTR}$, displayed a dramatic decrease in TrkA affinity and retained binding for $p75^{NTR}$ although it was lower in comparison with WT_NGF (Fig. 2B and D).

Biological activity of the NGF variants

The biological activity of the NGF variants was compared with that of WT_NGF. NGF can induce neurite outgrowth [21] and protect PC12 cells against thapsigargin (TG)-induced apoptosis through TrkA-dependent activation of ERK signaling [22]. The NGF variants H4D, H4E, K32R, G33M, H84Q and A98I caused an extensive neurite outgrowth after 7 days of PC12 cells incubation with 100 ng/ml NGF variant-containing media (Fig. 3A). Those NGF variants induced phosphorylation of ERK in PC12 cells to similar levels as WT_NGF (Fig.3B). The same mutants protected PC12 cells against TG-induced toxicity (Fig. 4A) and this

protective effect was lost upon inhibition of kinase TrkA receptor activity with K252a (Fig. 4B). Furthermore, the variants exhibited similar potency to WT_NGF as determined by the dose dependency of the protection, which was statistically significant only at the highest dose of 50 ng/ml (Fig. 4C and D). Thus, although those variants had different binding affinities for TrkA this did not alter their biological activity at this receptor.

The I31R and R69D NGF variants did not exhibited the biological activity at TrkA. They could not stimulate neurite outgrowth after 7 days of PC12 cells incubation with NGF variant-containing media (Fig. 3A). They were also unable to induce ERK phosphorylation and protect the PC12 cells from TG-induced cell death (Fig. 4A,B and D), consistent with previous observations that these variants do not bind to TrkA. The data suggest that I31R may possess selective activity through $p75^{NTR}$ as it was unable to bind to TrkA and activate TrkA signaling. Previously, other variants of I31 have been generated, I31A, I31M and I31V [16]. Variants I31A and I31M exhibited 30% TrkA binding, whereas I31V showed 130% stronger TrkA binding relative to WT_NGF, demonstrating the importance of this residue for NGF interaction with TrkA and the effect of amino acid substitution at this residue on TrkA interaction [23]. The activity of I31 mutants at $p75^{NTR}$ was not determined in that study.

$p75^{NTR}$ is known to activate pro-survival signaling through nuclear factor-κB (NF-κB) or pro-apoptotic signaling through the adaptor proteins NADE, NRAGE and NRIF [24]. Activation of these contrasting pathways depends on the expression levels of the receptors [25]. We tested the biological activity of I31R NGF through $p75^{NTR}$ signaling in RN22 Schwannoma cells which do not express TrkA and are known to stimulate NF-κB signaling in response to $p75^{NTR}$ activation accompanied by IκB degradation [26]. Treatment of RN22 cells with 100 ng/ml I31R NGF for 15 min, led to a higher degradation of total IκB compared with WT_NGF, which

induced a modest reduction in the level of IκB (Fig. 3E), confirming the ability of I31R to activate NF-κB.

Here we have designed and characterized a novel NGF variant that selectively binds and activates $p75^{NTR}$. This I31R variant could be used as a valuable tool to discriminate between NGF's two receptors and further determine the role of $p75^{NTR}$ signaling in different biological systems. We observed that in I31R, the replacement of the hydrophobic isoleucine with the charged arginine side chain prevents the binding of NGF to TrkA, while it still allows binding to $p75^{NTR}$. Activity towards $p75^{NTR}$ might be further improved by combining I31R mutation with another that improves binding to $p75^{NTR}$, such as K32R. I31, as well as adjacent residues, D30 and G33, are conserved in NGF across several species [16], supporting its importance in receptor binding. Furthermore, sequence alignment of NGF with the other neurotrophins show that neurotrophin-3 (NT3), which also binds TrkA, is the only member of the family to have an isoleucine at position 31, while members that bind TrkB and TrkC do not [16], suggesting that this binding site might be one of the specificity determinants in discriminating between the different Trk receptors. Currently available small molecules hypothesized to have a docking site at the loop I/IV cleft of NGF do not possess a selectivity toward blocking binding of NGF to TrkA [14]. Our data suggest that the area around I31 represents an attractive target site for small molecule intervention promising for pain management and that I31R NGF can be used to analyze $p75^{NTR}$-specific effects.

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Figure legends:

FIGURE 1. Crystal structures of NGF-TrkA and NGF-p75NTR complexes showing FoldX-predicted mutations

(A) NGF (dark blue) in complex with TrkA (light blue) (PDB code 2IFG). **(B)** NGF (blue) in complex with p75NTR (green) (PDB code 1SG1). **(C)** proNGF (blue) in complex with p75NTR (green). The prodomain of NGF was not resolved because of its flexibility (PDB code 3IJ2). NGF mutations predicted by FoldX algorithm to alter receptor activity are highlighted in red. **(D)** The predicted effects of point mutations on NGF binding affinity and protein stability.

FIGURE 2. Binding of NGF variants to TrkA and p75NTR

The purified NGF variants were incubated with immobilized Fc-TrkA **(A and B)** or Fc-p75NTR **(C and D)** and the NGF binding was determined using ELISA. The binding of individual NGF variants was expressed as a percentage of WT_NGF binding.

FIGURE 3. NGF variants biological activity downstream of TrkA

(A) PC12 cells were culture in conditioned media containing 50 ng/ml NGF variants for 7 days. Cells were stained with hematoxylin and the neurite outgrowth was then quantified. **(B)** PC12 cells were incubated for indicated times with conditioned media of NGF variant expressing HEK293T. Cell lysates were immunoblotted for phospho-ERK and total ERK.

FIGURE 4. NGF variant-mediated protection against TG-induced cell death

The 50 ng/ml NGF-containing conditioned media were applied to PC12 cells **(A)** without or **(B)** with K252a 2 h before addition of 0.25 μM TG and viability was assessed by MTT. (**C and D**) PC12 cells were treated with indicated concentrations of NGF variants in culture media followed by TG treatment and MTT assay. **(E)** RN22 cells were treated with 100 ng/ml NGF

variants and WT_NGF for 15 min and cells lysates were immunoblotted for total IκB and Actin.