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In vitro receptor binding properties of a "painless" NGF mutein, linked to hereditary sensory autonomic neuropathy type V

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ABSTRACT

Nerve Growth Factor (NGF) signalling is mediated by the TrkA and p75NTR receptors. Besides its neurotrophic and survival activities, NGF displays a potent pro-nociceptive activity. Recently, a missense point mutation was found in the NGFB gene (C661T, leading to the aminoacid substitution R100W) of individuals affected by a form of hereditary loss of pain perception (hereditary sensory and autonomic neuropathy type V, HSAN V). In order to gain insights into the functional consequences of the HSAN V NGF mutation, two sets of hNGFR100 mutants were expressed in *Escherichia coli* and purified, as mature NGF or proNGF, for *in vitro* receptor binding studies. Here, we show by Surface Plasmon Resonance analysis that the R100 mutation selectively disrupts binding of hNGF to p75NTR receptor, to an extent which depends on the substituting residue at position 100, while the affinity of hNGFR100 mutants for TrkA receptor is not affected. As for unprocessed hproNGF, the binding of the R100 variants to p75NTR receptor shows only a limited impairment, showing that the impact of the R100 mutation on p75NTR receptor binding is greater in the context of mature, processed hNGF. These results provide a basis for elucidating the mechanisms underlying the clinical manifestations of HSAN V patients, and provide a basis for the development of "painless" hNGF molecules with therapeutic potential.

Introduction

Nerve Growth Factor (NGF) [1] is the prototype member of the structurally related neurotrophin family [2–4], that regulate many functions of neuronal cells. NGF exerts its effects in responsive cells by interacting with either one or both of two cell surface receptors: the tyrosine kinase TrkA and p75NTR [5–7]. Among its numerous actions in the central and peripheral nervous system, NGF is involved in pain transduction mechanisms and plays a key role in many persistent pain states, regulating both the neuronal and the inflammatory component of pain (reviewed in [8,9]). Specifically, tissue damage or inflammation increase NGF expression and secretion. NGF, in turn, directly activates through TrkA/p75NTRs the sensory nerve cells that recognize painful stimuli (nociceptors), sensitizing and regulating the expression of ion channels, such as the capsaicin receptor TRPV1, conveying nociceptive signals. On the inflammatory side, NGF recruits inflammatory cells and stimulates the release of chemicals (*e.g.* substance P, CGRP, histamine), that increase the sensitivity of nociceptors to painful stimuli [8,9]. The pro-nociceptive activity of NGF has been demonstrated in humans, in the course of clinical trials, limiting severely the therapeutical applications of NGF [10].

The physiological relevance of the NGF system as a crucial regulator of pain has been highlighted by genetic evidence in humans. Rare forms of congenital insensitivity to pain (human sensory and autonomic neuropathy type IV and V, HSAN IV and HSAN V) are caused by mutations in the *NTRK1* gene, coding for the NGF receptor, TrkA [11], and the *NGFB* gene [12], respectively. HSAN IV NTRK1 mutations abolish or reduce TrkA responsiveness to NGF [11]. The recently described single nucleotide missense mutation in the *NGFB* gene, found in HSAN V patients, results in the aminoacid R to W substitution at position 100 of mature NGF protein [12] and its impact on NGF functions is unclear [13]. Homozygous cases for this mutation show impaired temperature sensation and an

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almost complete loss of deep pain perception, leading to joint destruction and multiple painless fractures, while heterozygous members of the family showed a milder pain insensitivity [13]. Importantly, HSAN V patients, unlike HSAN IV patients, show no mental retardation nor other neurological and cognitive deficits [14], suggesting that neuro-developmental effects on NGF target sensory neurons are probably minor in HSAN V. We deduced that these genetic data could, in principle, provide a basis for the design of a "painless" NGF variant of therapeutic interest [15].

In this paper, in order to gain insights into the functional consequences of the HSAN V NGF mutation, a series of hNGFR100 mutants were expressed in *Escherichia coli* and the *in vitro* receptor binding studies, and the *in vitro* receptor binding profile of hNGFR100 and hproNGF variants was characterized.

Methods

hNGF muteins expression and purification. Wild type hNGF was expressed in *E. coli* and purified as described before [16], according to a procedure slightly modified from Rattenholl et al. [17] and Paoletti et al. [18]. hNGF muteins were generated by site-specific mutagenesis according to Stratagene's protocol (Stratagene, La Jolla, CA, USA) and, after DNA sequencing, were cloned as hproNGF precursors (short form [18]) in prokaryotic expression vector pETM11 for expression in *E. coli*. The corresponding proteins were expressed in *E. coli* inclusion bodies, re-naturated by means of the pulsed refolding protocol [17], cleaved by controlled proteolysis with trypsin (enzyme:substrate ratio of 1:250) to yield mature hNGF and, after addition of protease inhibitors, purified by size exclusion and ionic exchange chromatography [19] and quantified by Lowry assay (Bio-Rad, Hercules, CA, USA). Unprocessed hproNGF muteins were prepared using the same protocol, just omitting the proteolytical cleavage step. Protein purity was assessed using capillary electrophoresis (Bioanalyzer; Agilent Technologies, Wilmington, DE).

Surface Plasmon Resonance. Surface Plasmon Resonance (SPR) measurements were performed by a BIACore 2000 instrument (BIACore AB, Uppsala, Sweden). The extracellular domains of TrkA and p75NTR, expressed in the form camel immunoadhesins [20] and purified from baculovirus-infected insect cells, were immobilized on a CM5 sensor chip by cross-linking the amine groups according to the manufacturer's instructions, obtaining SPR signals, after completion of the chip regeneration cycle, of 8900 resonance units (RU) and of 4300 RU, respectively. The binding kinetics were determined by injection in PBS buffer with addition of 0.005% v/v Surfactant P20 of hNGF or hproNGF muteins (in the 4–500 nM concentration range) at a flow rate of 30 μl/min. Data were analyzed using the BIA evaluation 3.0 package (GE Healthcare) to yield the apparent equilibrium constant K_D (defined as the k_a/k_d ratio).

Results

Structural insights into the R100W HSAN V mutation in NGFB protein

Examination of the crystallographic structures of hNGF complexes with p75NTR [21] and TrkA [22,23] extracellular domains suggests that the R100W HSAN V mutation in *NGFB* gene is likely to have a grater impact on the hNGF-p75 than on the hNGF–TrkA interaction (Fig. 1).

Indeed, as assessed by the protein interfaces, surfaces and assemblies service PISA at the European Bioinformatic Institute [24], hNGF residue R100 is not directly involved in the interface between hNGF and TrkA (Fig. 1A). However, considering that in both crystallographic structures of the hNGF–TrkA complexes [22,23] the residue R100 is close to the C-terminus of the extracellular portion of TrkA receptor and that the C-terminal 58 aminoacids linker, connecting the last Ig-like domain to the transmembrane region of TrkA receptor, is structurally not defined, we cannot exclude that hNGF residue R100 may interact with a portion of such a linker region of TrkA.

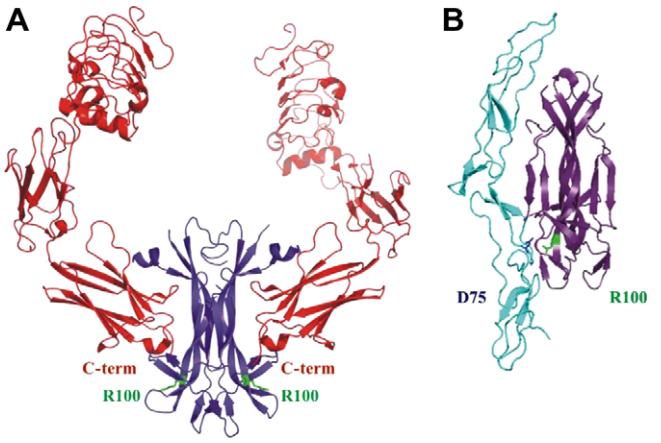

Fig. 1. Structural insights into the R100W HSAN V mutation in NGFB protein. The crystallographic structures of hNGF (in blue) complexed with TrkA (A) and with p75NTR (B) extracellular domains show that hNGF residue R100 (in green) is not directly involved in the interface between hNGF and TrkA (A), while (B) it participates in the hNGF–p75NTR interaction surface. Cartoon representations created with *Pymol* (http://www.pymol.org).

On the other hand, in the hNGF–p75NTR complex residue R100 of hNGF participates in an extensive charge complementary surface (Fig 1B). In particular, residue R100 is engaged in an electrostatic interaction (3.43 Å) with residue D75 of p75NTR and 89% of its solvent-accessible surface area is shown to be buried upon complex formation with p75NTR (Supplementary Table 1). Considering that hNGFR100 solvation energy gain upon formation of the hNGF–p75NTR interface (Δ^iG) is one of the lowest (−1.33) among all hNGF residues involved in the interface with p75NTR (Supplementary Table 1), R100 is likely to give a strong positive contribution to the binding affinity of the hNGF–p75NTR complex. The non-conservative mutation R100W is very likely to disrupt this electrostatic interaction and to negatively affect the corresponding Δ^iG, compromising the stability of the hNGF–p75NTR complex. Residue R100 is conserved in the primary sequences of NGF and other neurotrophins from all species (Supplementary Table 2), with the only exception of *Danio rerio* NT3 (N100). Interestingly, residue D75 is also conserved in p75NTR from most species, with the notable exception of *Danio rerio* (N75) (Supplementary Table 3).

Finally, residue R100 might be part of the surface patch of mature hNGF that interacts with its prodomain of hNGF, since it is structurally close in space to residue W21, known to be involved in such an interaction in the proNGF molecule [19] (Supplementary Fig. 1). This could alter the overall folding of proNGF, particularly in its compact form [18].

Design, expression and purification of hNGF muteins

To characterize the effects of the mutation of hNGF residue R100 *in vitro* and *in vivo*, two sets of hNGFR100 mutants were designed (Supplementary Table 4). In the first set, residue R100 was substituted with different amino acids, in the context of wild type hNGF. The second set of mutants contained, in addition to the R100 mutations, a second substitution (P61S), which replaces the proline residue at position 61 of hNGF with Ser residue present in mouse NGF. The hNGFP61S "tagged" molecules are equally bioactive as hNGF [16] and are selectively detectable against wild type hNGF, with a specific monoclonal antibody [16].

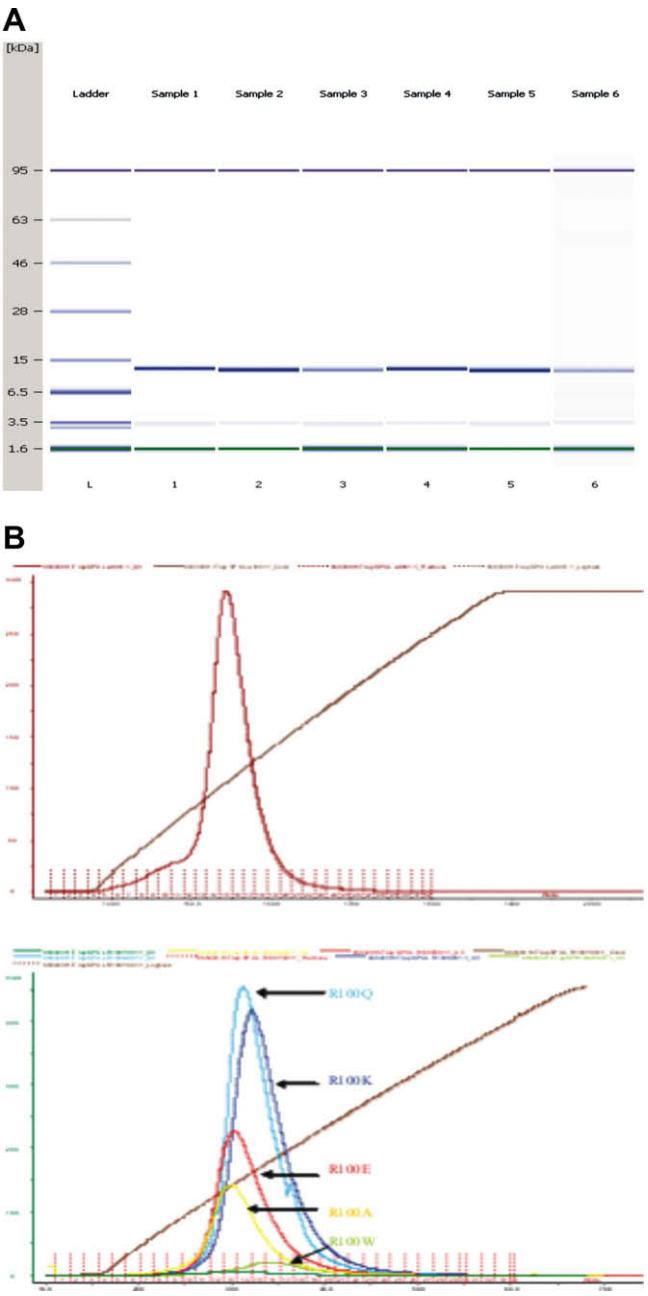

Fig. 2. Purification of hNGF muteins. (A) Purity of hNGF muteins after refolding. Sample 1: hNGF, Sample 2: hNGFR100E, Sample 3: hNGFR100W, Sample 4: hNGFP61S, Sample 5: hNGFP61S/R100E, Sample 6: hNGFP61S/R100W. (B) Upper panel: Elution profile from Hitrap SP Sepharose XL of hproNGF WT after pulsed refolding: the area below the curve is proportional to the total amount of purified protein. Bottom panel: Comparison of elution profiles from Hitrap SP.

The two sets of hNGF mutants were expressed in *E. coli* as hproNGF precursor proteins, refolded from inclusion bodies, cleaved by controlled proteolysis to yield mature hNGF and purified [19,25] to 99% purity (Fig. 2A and B). While all mutants yielded comparable amounts of protein in inclusion bodies, they gave distinct yields after refolding and purification, which was, respectively, maximum for wild type hNGF, hNGFR100K and hNGFR100Q, intermediate for hNGF-R100A and hNGFR100E and very low for the genetic mutant hNGFR100W (or for mutants with hydrophobic residues R100Y, R100I, R100L and R100V) (Fig. 2B and Supplementary Table 5). The very low yields of the R100W mutants are due to a reduced refolding efficiency and not to a lower cleavage efficiency after refolding (not shown). Indeed, the substitution of the positively charged R100 with an hydrophobic residue is likely to increase the overall hydrophobicity of the molecule, inducing folding intermediates to aggregate and subtracting protein to the refolding process.

Binding affinity of hNGF and hproNGF muteins for purified TrkA and p75NTR receptors

Binding of hNGF mutants to recombinant, purified forms of extracellular domains of TrkA and p75NTR receptors, was studied by Surface Plasmon Resonance (SPR). As shown in previous studies [16], hNGFP61S and wild type hNGF bound TrkA (dissociation constants (K_D) ~ 1.38 and 0.94 nM, respectively) and p75NTR (K_D ~ 2.25 and 1.53 nM, respectively) with identical affinities (Fig. 3 and Supplementary Table 6). On the other hand, the hNGF muteins hNGFR100E and hNGFP61S/R100E, while showing an affinity for TrkA identical to that of hNGF or hNGFP61S (K_D ~ 1.44 and 1.35 nM, respectively, for hNGFR100E and hNGFP61S/R100E), proved to have a significantly lower affinity for p75NTR (K_D ~ 125 and 200 nM, respectively) (Fig. 3 and Table 1). The mutants hNGFR100W and hNGFP61S/R100W showed qualitatively the same selective disruption of p75NTR binding as the R100E mutants, although a quantitative determination of the binding affinity was not possible, due to their poor expression yields (Supplementary Table 5). All remaining hNGFR100 and hNGFP61S/R100 muteins showed an unchanged affinity for TrkA and variable but consistently lower affinities for p75NTR (Supplementary Table 6).

The precursor, unprocessed form of hNGF (hproNGF) is endowed with a biological activity distinct from that of mature hNGF [26], due to a distinct receptor binding profile. It was therefore, of interest to investigate the impact of the R100 mutations on TrkA and p75NTR receptor binding, in the context of unprocessed proNGF. A SPR analysis was carried out using hproNGF and hproNGFP61S mutated in position R100. The data obtained show that the mutation R100 does not affect at all binding to the TrkA receptor (Table 1; K_D = 20.6 nM for hproNGFR100E vs 17.8 nM hproNGF and K_D = 20.4 nM for hproNGFP61S/R100E vs 22.6 nM hproNGFP61S). Contrary to what we found for hNGF and hNGFP61S mutants, the SPR analysis showed that the binding to p75NTR of unprocessed proNGF R100 mutants was not greatly affected, revealing only a twofold decrease in affinity (Table 1; K_D = 52.5 nM for hproNGFR100E vs 19.7 nM hproNGF and K_D = 49 nM for hproNGFP61S/R100E vs 18.9 nM hproNGFP61S).

Thus, *in vitro*, the major impact of the R100 mutation (notably with the R100W and R100E substitutions) is on hNGF binding of the p75NTR, which is largely abolished, while the same mutation, in the context of unprocessed proNGF, has a much lower effect on p75NTR binding.

Discussion

In this paper, we characterized the *in vitro* receptor binding properties of recombinant NGF variants, carrying mutations at position R100, inspired by a human genetic mutation causing reduced sensitivity to pain in HSAN V patients [12].

HSANs are a heterogeneous group (I–V) of peripheral neuropathies characterized by sensory and autonomic dysfunctions, involving at least eight different genetic loci (with six identified genes) [27]. A recent study of a large multi-generational Swedish family, suffering from the rare HSAN V form, has led to the identification of a mutation in the *NGFB* gene (exon 3, nt C661T) [12]. This mutation changes a basic arginine (CGG) to a non-polar tryptophan (TGG) at a position corresponding to residue R100 in mature NGF [12].

HSAN V patients suffer from loss of pain perception but show no mental retardation and have most neurological functions intact [14], showing that the mutation dissociates the developmental ef-

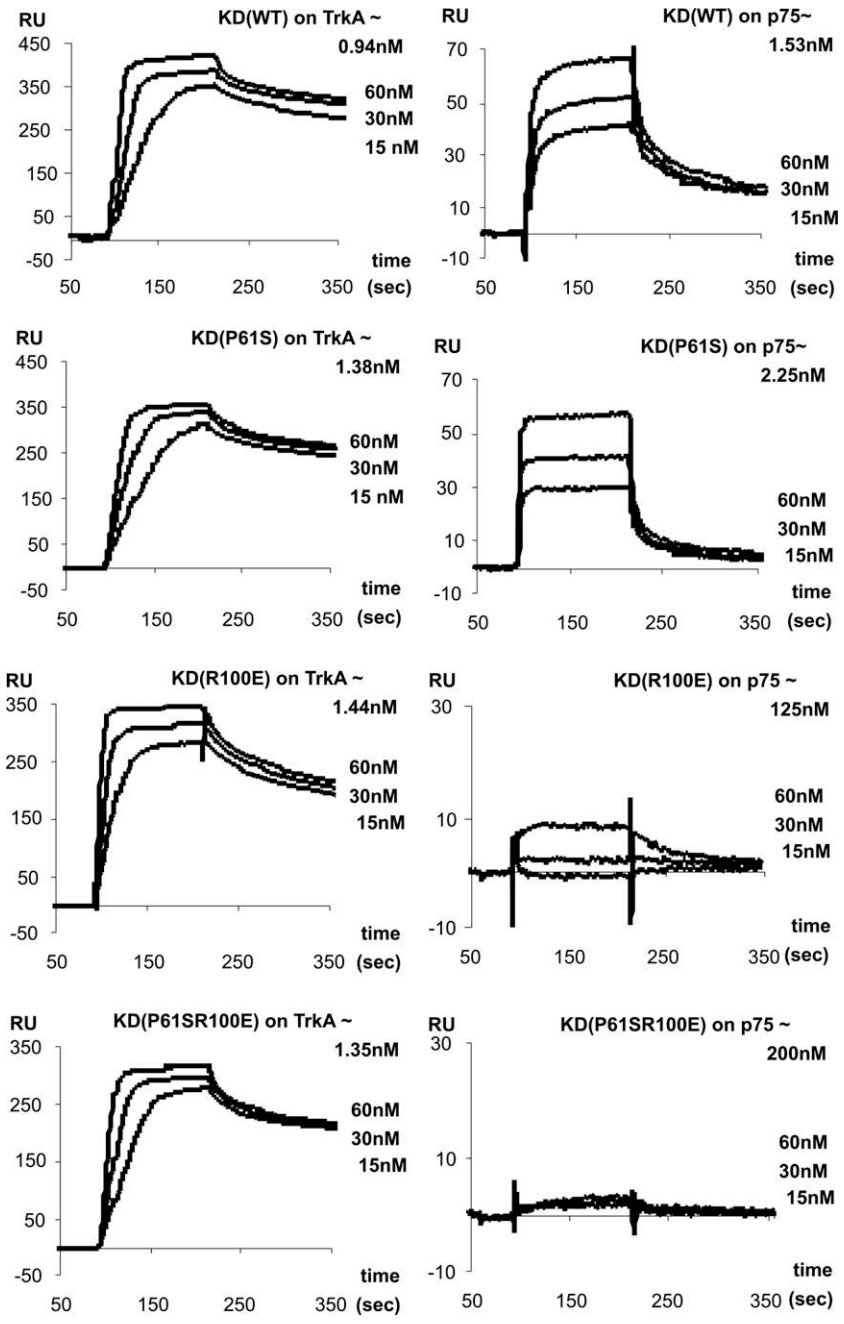

Fig. 3. Binding affinity of hNGF muteins for purified TrkA and p75NTR receptors. Binding curves of 15 and 60 nM hNGF, hNGFP61S, hNGFR100E and hNGFP61S/R100E to immobilized TrKA (8400 RU) and p75NTR (4300 RU) are shown. K_D values (calculated from the full range of curves in the 15–640 nM range) are indicated.

fects of NGF from its effects as a master regulator of pain responses. The mechanisms whereby the mutant NGFR100W exerts its effects in HSAN V remain to be investigated, as no data are available on the receptor binding properties and the downstream signalling activated by the mutant hproNGF and hNGFR100 mutants.

This study provides clear-cut evidence that the main impact of the R100 mutation is on the binding of NGF with the p75NTR receptor, greatly reducing this interaction, at least under these *in vitro,* well controlled conditions. Interestingly, the R100 mutation has only a minor impact on p75NTR binding, when expressed in the context of the unprocessed hproNGF R100 protein.

On the other hand, TrkA binding affinity is unchanged. It remains to be seen whether, in face of an unchanged overall binding affinity, more subtle changes in the downstream TrkA signalling are induced by the R100 mutants.

These data help explaining why neuro-developmental effects are minor in HSAN V [14,27,28]. Thus, the molecular explanation for the HSAN V NGFR100W mutation would lie in an alteration of the separate signalling streams normally activated by NGF through its TrkA and p75NTR receptors. NGFR100 proteins would maintain the neurotrophic signalling stream unchanged, for which signalling through TrkA plays a predominant role [29], while showing a reduced signalling involved in nociceptor sensitization. While the final answer will come from future experiments, made possible by the recombinant proteins characterized in this paper, the predicted failure of NGFR100 proteins to activate and sensitize nociceptors deserves some comment. Thus, although the general consensus is that the inflammatory component of pain is mediated by TrkA, expressed on mast cells [30], a growing body of evidence suggest that p75NTR signalling provides a significant contribution

Table 1
SPR analysis. Summary of the derived kinetic and equilibrium binding constants of hproNGF and hNGF and their muteins in position 100 towards TrkA and p75 receptors.

	K_a (M^{-1} s^{-1})	K_d (s^{-1})	K_D (M)	KA (M^{-1})
TrkA				
hproNGF	6.63×10^4	1.18×10^{-3}	17.8	5.62×10^7
hproNGF R100E	4.21×10^4	0.86×10^{-3}	20.6	4.86×10^7
hNGF	1.23×10^6	1.16×10^{-3}	0.94	1.06×10^9
hNGFR100E	1.34×10^6	1.93×10^{-3}	1.44	6.9×10^8
hproNGF P61S	3.69×10^4	8.35×10^{-4}	22.6	4.42×10^7
hproNGFP61S/R100E	4.1×10^4	8.35×10^{-4}	20.4	4.91×10^7
hNGFP61S	0.79×10^6	1.09×10^{-3}	1.38	7.26×10^8
hNGFP61S/R100E	1.07×10^6	1.45×10^{-3}	1.35	7.41×10^8
	k_a	k_d	K_D	KA
p75NTR				
hproNGF	2.39×10^5	4.72×10^{-3}	19.7	5.07×10^7
hproNGF R100E	4.18×10^4	2.2×10^{-3}	52.5	1.9×10^7
hNGF	3.05×10^6	4.68×10^{-3}	1.53	6.52×10^8
hNGFR100E	4.51×10^4	5.62×10^{-3}	125	8.02×10^6
hproNGFP61S	1.96×10^5	3.7×10^{-3}	18.9	5.3×10^7
hproNGFP61S/R100E	5.92×10^4	2.9×10^{-3}	49	2.04×10^7
hNGFP61S	3.92×10^6	8.83×10^{-3}	2.25	4.44×10^8
hNGFP61S/R100E	5.43×10^4	10.9×10^{-3}	200	4.98×10^6

to pain transmission and sensitization (reviewed by Nicol and Vasko [9]). Besides the fact that a number of p75NTR-mediated signalling pathways activated by NGF, independent of TrkA activity, are known to mediate peripheral sensitization (depicted in Supplementary Fig. 2), p75NTR has been associated with NGF-induced excitability of nociceptors in culture [31], with pain states in which bradykinin is an important mediator [32]. Moreover, p75NTR functional block has been shown to suppresses injury-induced neuropathic pain [33] and the hyperalgesia arising from complete Freund's Adjuvant-induced inflammation or with an intraplantar injection of NGF [34].

In light of the unchanged TrkA binding of NGFR100, the limited neurodevelopmental loss of sensory Aδ and C fibers observed in HSAN V patients [12] would be determined by lower levels of NGFR100 protein, resulting from its impaired processing and accumulation as proNGF (possibly involving an altered intramolecular interaction of the R100W residue with the pro domain [13] (see Supplementary Fig. 1), rather than by a neurotrophic signalling defect. In this respect, how the mutation impacts the processing of proNGF and how proNGF regulates pain transmission signals represent questions for future investigations.

Conclusions

In this scenario our work provides clear-cut evidence that the main impact of the R100 mutation is on the binding of NGF with the p75NTR receptor, completely abolishing this interaction and its functional outcome, blocking the contribution of signalling through p75NTR to nociceptive actions of NGF.

The hNGF muteins described in this paper could provide a basis for designing "painless" NGF variant molecules, tailored for therapeutic applications in AD [15], circumventing the most serious hurdle that have limited such applications.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.11.146.

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