



## Review

# Understanding pain perception through genetic painlessness diseases: The role of NGF and proNGF

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## ABSTRACT

Nerve growth factor (NGF), by binding to TrkA and p75<sup>NTR</sup> receptors, regulates the survival and differentiation of sensory neurons during development and mediates pain transmission and perception during adulthood, by acting at different levels of the nervous system. Key to understanding the role of NGF as a pain mediator is the finding that mutations (namely, R121W, V232fs and R221W) in the *NGF* gene cause painlessness disease Hereditary Sensory and Autonomic Neuropathy type V (HSAN V). Here we shall review the consequences of these *NGF* mutations, each of which results in specific clinical signs: R221W determines congenital pain insensitivity with no overt cognitive disabilities, whereas V232fs and R121W also result in intellectual disability, thus showing similarities to HSAN IV, which is caused by mutations in TrkA, rather than to HSAN V. Comparing the cellular, biochemical and clinical findings of these mutations could help in better understanding not only the possible mechanisms underlying HSAN V, but also mechanisms of NGF signalling and roles. These mutations alter the balance between NGF and proNGF in favour of an accumulation of the latter, suggesting a possible role of proNGF as a molecule with an analgesic role. Furthermore, the neurotrophic and pronociceptive functions of NGF are split by the R221W mutation, making NGF variants based on this mutation interesting for designing therapeutic applications for many diseases. This review emphasizes the possibility of using the mutations involved in “painlessness” clinical disorders as an innovative approach to identify new proteins and pathways involved in pain transmission and perception.

*Outstanding questions:* Why do homozygous HSAN V die postnatally? What is the cause of this early postnatal lethality?

Is the development of a mouse or a human feeling less pain affecting higher cognitive and perceptual functions? What is the consequence of the HSAN V mutation on the development of joints and bones? Are the multiple fractures observed in HSAN V patients due exclusively to the carelessness consequent to not feeling pain, or also to an intrinsic frailty of their bones?

Are heterodimers of NGF<sup>WT</sup> and NGF<sup>R221W</sup> in the heterozygote state formed? And if so, what are the properties of these heterodimeric proteins?

How is the processing of proNGF<sup>R221W</sup> to NGF<sup>R221W</sup> affected by the mutation?

## 1. Introduction

Pain is a subjective experience that can vary among individuals. It alerts us about danger, thus exerting an immediate physiological role in preserving bodily integrity. The description of pain as an alarm system was first proposed by Descartes in the *Traité de l'Homme*, in which pain is represented as a line that links the stimulus – fire, in that case – to the brain. Descartes's vision of pain has been very influential on modern

pain research. He proposed that a foot coming in contact with fire excites the terminations of sensory fibers in the foot and that this information is transmitted to the brain, where an alarm is triggered in the form of the unpleasant sensation of pain [1]. In this regard, it is important to note that pain sensation is not necessarily linearly related to the nociceptive input that triggered it, neither is it solely for vital protective functions. Sensory and affective dimensions also concur to determine the final sensation [2]. Thus, pain can be considered as the result of brain

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processes elicited by peripherally encoded sensory-discriminative inputs, which are integrated with affective and emotional aspects [3].

Pain is, therefore, a highly subjective experience, as illustrated by the accepted definition given from the International Association for the Study of Pain [4] “an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage”. This general definition covers all the various aspects of pain.

Most of the studies on the interplay between pain perception, cognition and emotion are carried out on animal models of hyperalgesia [5]. In fact, this is a very practical approach, as setting up an experimental protocol based on painful stimuli is the most reasonable manner to obtain a clear readout (e.g., licking or retraction of a paw). On the other hand, creating a model based on the *decrease* of nociception is much less obvious, but can nonetheless contribute to complete our understanding of the chain of events that starts from sensing an external stimulus as harmful and ends with a complex sensation elaborated by the brain.

This task can be accomplished by taking inspiration from human diseases characterized by varying degrees of pain insensitivity. Their genetic forms collectively fall within the category of Hereditary Sensory and Autonomic Neuropathies (HSANs) [6], and can be considered unique tools to study the physiopathology of this essential sensory perception.

In this review, after describing the general characteristics of HSANs, we will summarize the clinical features of HSAN V, a rare disease linked to mutations in the Nerve Growth Factor (NGF) gene. We will clarify the nomenclature of NGF mutations, by numbering the residues from the first amino acid residue of the pre-proNGF (Fig. 1).

Then, we will discuss the main findings on the mechanisms underlying this disease, also clarifying our current understanding of the role of NGF and proNGF in pain perception, an aspect often neglected.

Finally, we shall discuss the possibility of using the painlessness disorders as a basis for the development of novel analgesic drugs.

## 2. The other side of the coin of pain: HSAN diseases

HSANs are a clinically and genetically heterogeneous group of inherited peripheral neuropathies [7]. HSANs can be transmitted either as an autosomal dominant (AD) or autosomal recessive (AR) trait. AD types present marked sensory involvement and minimal autonomic and variable motor involvement. On the other hand, AR types present either as congenital syndromes with striking sensory and autonomic nervous system abnormalities, or as autonomic disorders [8]. The classification of different forms of HSANs is based on genetic diagnosis, inheritance pattern and predominant clinical features [6,7]. Based on this, eight phenotypically diverse HSANs were identified, three of which were

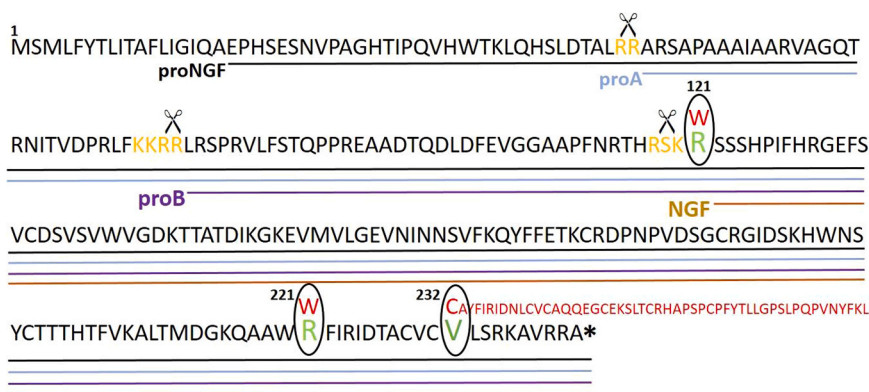
added in this past decade [6].

HSAN II and IV can be thought of as a sort of “pain blindness”, caused by impaired function of nociceptors or receptors involved in nociceptive signaling. For instance, mutations in the genes coding for key elements of peripheral sensory transmission, such as sodium ion channels (e.g.,  $Na_v1.7$ , responsible for HSAN II; [9]) which are responsible for nociceptor activation, straightforwardly result in impaired pain transmission and perception.

## 3. HSANs and the NGF system

NGF has potent pronociceptive functions, contributing to sensitization of peripheral and central sensory neurons and, potentially, driving local neuronal sprouting at the site of injury [10]. Nociceptors are particularly dependent on NGF-mediated signalling for proper development and function [11]. Indeed, the absence of NGF-induced TRPV1 sensitization in sensory neurons underlies the lack of heat hyperalgesia in the naked mole-rat [12], as well as mutations in the gene for NGF or its receptor TrkA result in congenital insensitivity to pain [13]. These diseases, named HSAN IV and V, are notable exceptions among HSANs, as they are not caused by mutations in genes directly, or exclusively, related to peripheral nociceptor function but rather in genes involved both in the proper development of sensory neurons and in the function of the adult pain transmission and perception, at different levels of the nervous system. HSAN IV is associated with mutations (mostly nonsense mutations, but also missense, small insertions and deletions, splicing variants) in the gene coding for tropomyosin receptor A (*TRKA*), the tyrosine kinase receptor for Nerve Growth Factor (NGF) [13–15], whereas HSAN V is caused by mutations in the nerve growth factor beta gene (*NGFB*) [16]. Thus, HSAN IV and V represent a peculiar pair of diseases, caused by mutations in a key neurotrophin receptor and its ligand, respectively. The partly overlapping clinical features of these diseases (please see below for details) are in line with the general finding that the NGF–TrkA signaling axis has a crucial role not only in the development but also in the adult function of the nociceptive system [17].

HSAN IV is a rare recessive congenital insensitivity to pain accompanied by anhidrosis, caused by a failure of nociceptive and sympathetic neuron development. Patients are prone to oral injuries as well as multiple accidental injuries such as falls, burns and bone and joint fractures [18,19]. Further key features of HSAN IV are a deficit in temperature sensing [20] and, notably, intellectual disability [15]. Recently, lack of TrkA signaling has been shown to also increase the susceptibility to infections, in particular to *Staphylococcus aureus*, through monocyte/macrophage-specific NGF/TrkA pathway [21], but this feature still needs to be investigated in HSAN IV patients. The hypofunctionality of TrkA is also at the basis of the lack of NGF-induced



**Fig. 1.** An overview of preproNGF with the relative positions of HSAN V-causing mutations. The mutation sites are shown in green, with the corresponding mutated amino acids in red. The missense R121W mutation (c. 0.361 C>T; [38]) occurs in the last residue of the furin cleavage motif RSKR. The R122W mutation (c. 661 C>T; [16]) changes a basic arginine to a non-polar tryptophan at position 221 in the proNGF, corresponding to amino acid 100 in the mature protein. The V232fs mutation (c. 680 C>T + 681\_682delGG; [37]) leads to the bases CGG being changed to A, replacing the C-terminal 15 amino acids of mature NGF with a different amino acid sequence (shown in red).

The scheme also shows the three different proNGF forms (proNGF, proA, proB) and mature NGF (NGF) with the corresponding sequences, marked by colored lines (black, blue, purple, gold, respectively). The scissor symbols indicate the cleavage sites (highlighted in yellow). All the

residues are numbered from the first residue of the pre-propeptide (“M” surmounted by number “1”).

thermal hyperalgesia in naked mole-rat [12], showing once again the importance of TrkA-mediated signaling in the transmission and perception of nociceptive stimuli.

HSAN V is also characterized by a severe reduction in pain perception, with subsequent propensity to multiple bodily lesions, but it is not accompanied by intellectual disability nor cognitive impairment ([22]; please see below for details).

These clinical features suggest that dysfunction of NGF [16] results in a less severe phenotype than dysfunction of its receptor, TrkA [13]. This could be due to the fact that mutated NGF is still able to bind and activate TrkA, albeit in a defective manner [23–25], whereas HSAN IV-related mutations in TrkA itself lead to a more dramatic loss of function [15]. In this regard, it is tempting to speculate that the less severe phenotype observed in HSAN V patients may also be due to the ability of NT-3 to bind TrkA, which could partly compensate for reduced activation of this receptor by mutated NGF. However, this aspect remains to be explored in detail.

To help shed light on the cellular, molecular and physiological basis of HSAN IV and V, novel transgenic mouse models have been developed [26]. In addition, these models can also be exploited to set up experimental designs which do not solely rely of measuring pain in a pain-sensing animal (i.e., a wild type mouse), but allow to create assays centered on inducing a pain-related experimental readouts in an animal which does not usually display it. In the next paragraphs, we will focus on the genetic basis of HSAN V and discuss how knock-in mice for one of the mutant forms of NGF helped in discovering new possible therapeutic targets.

#### 4. An overview of HSAN V

HSAN V is an autosomal recessive genetic disorder, whose symptoms have been described for the first time by Low, without identifying the genetic cause [27]. Later, Einarsdottir and Minde, analyzing the clinical features of a multi-generational family suffering from loss of pain perception, identified the associated gene mutation [16]. This family was located in the Norrbottnian region of Sweden and, for this reason, HSAN V is also known as “Norrbottnian congenital insensitivity to pain”. HSAN V is associated with a point mutation in exon 3 of the *NGFB* gene, located on chromosome 1p11.2-p13.2 and consists of the substitution of C to T at nucleotide 661. This turns a basic arginine (CGG) into a non-polar tryptophan (TGG) at the position corresponding to residue 221 in proNGF, corresponding to position 100 in mature NGF (R221W) (Fig. 1) [16].

The most evident clinical features of Swedish HSAN V patients are fractures of the lower legs and feet, appearing from childhood in homozygous subjects [16,22,28]. In contrast, in heterozygous subjects, fractures appear during adulthood and are less frequent than in homozygotes [29]. This correlates with the fact that, in homozygotes, pain sensations are decreased mainly at the forearms and legs, with normal pain sensation in the trunk [16], whereas these symptoms are not present in heterozygous carriers [29], even though they display a reduction in nociception. Is the increased number of fractures due to carelessness secondary to reduced pain sensitivity, or to an intrinsic frailty of bones in HSAN V patients? It has been shown that NGF-TrkA signalling by sensory nerves controls the correct vascularization and ossification of developing bone [30]. Indeed, inhibition of TrkA-mediated signalling or deletion of NGF in perichondral osteochondral precursor cells affects primary and secondary ossification, leading to decreased bone mass and length [30]. NGF-TrkA signalling is also required in adult mice for skeletal adaptation to mechanical loads [31]. Therefore, these data may help to explain the skeletal disorders observed in patients with HSAN caused by loss-of function mutations in NGF and TrkA [13,22].

In both heterozygous and homozygous HSAN V patients, cold and heat thresholds are increased [16,28,29]. Consistently, the expression of TRPV1, TRPV2 and TRPM8 (Transient receptor potential) channels, which are responsible for the detection of different thermal stimuli [32],

is reduced in skin nerve fibers of heterozygous and homozygous individuals, compared to healthy subjects, as revealed by immunohistochemistry [33]. Interestingly, despite the reduced autonomic innervation of sweat glands, revealed by immunostaining for positive-fibers to vasoactive intestinal peptide (VIP) and tyrosine hydroxylase (TH) [33], Swedish HSAN V patients present normal sweating [16]. Indeed, HSAN V do not suffer from repeated high fever episodes during childhood, unlike the recurrent hyperthermia and febrile convulsions that are the first clinical signs of HSAN IV in children [34]. Moreover, HSAN V patients do not present visceral pain, while the sensitivity to soft touch, joint position and vibration sensation are normal [16,28,29]. Notably, motor conduction velocity and amplitude of peripheral nerves are normal in both heterozygous and homozygous patients [16,29].

Sural nerve biopsy and morphometric analysis revealed that all patients show a small reduction of Aδ fibers and a severe reduction of C fibers, which carry the fast- and slow-speed components of nociceptive input, respectively [28]. This reduction was less severe in heterozygous patients, thus explaining their milder phenotype, compared to homozygotes [29]. Using in vivo corneal confocal microscopy (CCM) to quantify C-fiber loss in the cornea, Perini and colleagues showed that homozygotes present a significant afferent reduction compared to heterozygotes and control subjects. Importantly, peripheral C-fiber loss correlated negatively with subjective pain evaluation, as revealed by the results of Situational Pain Questionnaire (SPQ) [35].

At the cognitive level, mental retardation is not observed in Swedish HSAN V patients, except for one homozygous child, who presents a light attention deficit [28]. On the contrary, HSAN IV patients show various degrees of intellectual and learning disabilities, and many individuals suffer from severe attention-deficit/hyperactivity disorder (ADHD) [36].

In addition to the Swedish R221W mutation, a mutation 680 C>A + 681\_682delGG in the *NGFB* gene, replacing the COOH-terminal 15 amino acids of mature NGF with a different 43 amino acid sequence (V232fs) (Fig. 1), was found in five children from a consanguineous Arabic family [37]. All of them suffer from congenital insensitivity to pain, inability to discriminate temperatures, chronic immunodeficiency, anhidrosis, automutilation and severe injuries with Charcot joints or osteomyelitis. Mental retardation was also reported [37]. Notably, this condition, which closely approaches HSAN IV, manifests only in the sole homozygote individual known to date. Both parents and a sibling, which are heterozygous, do not show overt clinical signs, and have no detectable alterations in pain perception. NGF<sup>V232fs</sup> is not secreted when expressed in COS-7 cells [37]. It is not known whether cells from heterozygote individuals secrete only NGF<sup>WT</sup>, or whether a heterodimeric NGF<sup>WT</sup>/NGF<sup>V232fs</sup> protein can also be secreted, in a functional form which would explain the normal phenotype of heterozygotes.

Recently, a third mutation (c.0.361 C>T, p.R121W) has been identified in the *NGFB* gene. This mutation occurs in the last residue of the furin cleavage site of the proNGF precursor (Fig. 1), right ahead of the mature NGF sequence [38]. A homozygous subject, carrying this mutation, displays a similar phenotype as V232fs subjects: lack of sweating, reduced learning abilities, multiple Charcot's joints and tendency to ignore painful injuries [38]. Thus, the clinical phenotype of HSAN V caused by V232fs and R121W mutations is more similar to that of HSAN IV, indicating that these mutations have more dramatic consequences on the activation of TrkA signaling by mutated NGF than R221W mutation.

Finally, many other cases, both in Japan and Brazil, can be considered as manifestations of HSAN V, but the genotype of the patients has not been described [39,40]. Further analysis of these cases will be instrumental to deepen our understanding of the phenotypic consequences of missense mutations in NGF. Considering the importance of genetic analysis in the diagnosis of neuropathy, the allocation of these last two forms of HSANs (namely V232fs and R121W mutations) is controversial.

Therefore, it is important to consider the specific clinical features

associated with the three known mutations in the *NGFB* gene, in order not to generalize the different forms of HSAN V, and to possibly distinguish the Swedish from the other forms of HSAN V (see Table 1).

### 5. Effect of the Swedish mutation NGF<sup>R221W</sup> on processing and secretion of NGF

The clinical features of HSAN V caused by the NGF<sup>R221W</sup> mutation suggest a more complex picture than a simple loss of function.

When the mutant NGF<sup>R221W</sup> gene is transfected into PC12 cells, the transfected cells fail to differentiate [41], unlike PC12 cells transfected with NGF<sup>WT</sup> that undergo differentiation. In this assay, PC12 differentiation is expected to occur because NGF is secreted and acts in an autocrine or paracrine manner, on the same or on neighbouring cells respectively, to induce differentiation. The failure of NGF<sup>R221W</sup> to induce PC12 cell differentiation can be therefore interpreted as due to a failure of being secreted, even if a direct intracellular signalling in the secretory pathway of the PC12 cells cannot be excluded. Indeed, PC12 and COS-7 cells transfected with human NGF<sup>R221W</sup> secrete a very low amount of mature NGF in the culture medium, compared to transfection with NGF<sup>WT</sup> [41]. The defect in secretion is accompanied by a reduced processing to mature NGF<sup>R221W</sup>, an intracellular accumulation of proNGF<sup>R221W</sup> and a reduced sorting of proNGF<sup>R221W</sup> and NGF<sup>R221W</sup> into the secretory granules, where the protein would be available for proteolytic processing and secretion. The causal relationships between these facts and whether the primary defect is a processing failure or a mis-sorting of proNGF<sup>R221W</sup> are still open questions. Despite this intracellular mis-sorting in the secretory pathway, the expression of proNGF<sup>R221W</sup> appears not to induce endoplasmic reticulum (ER) stress [41]. To further investigate this aspect, NGF<sup>R221W</sup> and NGF<sup>WT</sup> were co-expressed, as it happens in heterozygous carriers of HSAN V [26]. Interestingly, co-expression of both NGF<sup>R221W</sup> and NGF<sup>WT</sup> in HEK293 cells leads to decreased NGF abundance in the cell culture medium, with respect to the cells expressing NGF<sup>WT</sup> indicating that NGF<sup>R221W</sup> interferes with the secretion of wild type NGF [26]. This result points to a possible dominant-negative role for NGF<sup>R221W</sup>, possibly through the formation of NGF<sup>WT</sup> / NGF<sup>R221W</sup> heterodimers.

These findings extend those in the report by Covaceuszach et al. [24]. Here, human proNGF<sup>R221W</sup> was expressed in *Escherichia coli*, taking advantage of this system to refold the precursor protein from inclusion bodies and then cleave it to obtain the mature form [42]. The yield of human NGF<sup>R221W</sup> was much lower than that of NGF<sup>WT</sup>, expressed in the same conditions, which led to conclude that the substitution of the positively charged arginine with tryptophan increases the hydrophobicity of the protein, thus causing the formation of aggregates [24]. In

fact, the optimal conditions required to produce large amounts of NGF<sup>R221W</sup> is still an unsolved problem. For this reason, in vivo studies were performed using the mutant protein NGF<sup>R221E</sup>, since the R221W and R221E mutants have totally superimposable TrkA binding [24] and signalling activation [23] properties, while the production yield for R221E protein was much higher than that for R221W [24].

The structural basis for the inefficient processing of pro NGF<sup>R221W</sup> is currently not understood. One possibility is that residues in the region of position R221 of mature NGF might be involved in some interaction with the pro domain [42–44] that might be disrupted, or stabilized, by the mutation, influencing the cleavage to yield mature NGF.

### 6. NGF<sup>R221W</sup>: a painless molecule with unaltered neurotrophic properties

The biological actions of NGF are triggered by binding and activation of its receptors, namely TrkA and p75<sup>NTR</sup> [45,46]. TrkA has the highest affinity for NGF and their interaction promotes NGF-mediated survival and differentiation of target neurons [47,48]. p75<sup>NTR</sup>, unlike TrkA, presents a more promiscuous binding for all neurotrophins and their precursor forms [49,50]. The effect of signaling mediated by p75<sup>NTR</sup> depends on its interacting partners: with sortilin, p75<sup>NTR</sup> causes apoptosis mediated by proNTs [51]; with LINGO-1 and Nogo-A, it is involved in myelin-dependent inhibition of axonal growth [52]; with Trks, it supports survival, axonal growth and differentiation [50].

Considering the complexity of signal transduction mediated by the binding of NGF and proNGF to their receptors, the effect of R221W mutation on these processes was initially investigated by analysis of the crystallographic structures of the complexes formed by NGF with the extracellular domains of TrkA and p75<sup>NTR</sup> [24]. Inspection of these structures show that the residue R221 of human NGF appears not to be involved in the interaction surface with TrkA [24]. On the contrary, in the human NGF-p75<sup>NTR</sup> complex, the R221W residue participates in an extensive charge complementary surface, making an electrostatic interaction with residue D75 of p75<sup>NTR</sup> [24] (Fig. 2A). As mentioned above, residue R221 is conserved in the primary sequences of NGF and other neurotrophins from all species, except for *Danio rerio* NT3. Interestingly, residue D75 is also conserved in p75<sup>NTR</sup> from most species, with the notable exception of *Danio rerio* (N75) [24] (Fig. 2B).

We anticipated from this structural analysis that the NGF<sup>R221W</sup> mutation might preserve the interaction with TrkA, but disrupt the interaction with p75<sup>NTR</sup>. Indeed, human NGF<sup>R221W</sup> has a binding affinity to TrkA very similar to that of wild-type NGF, whereas the mutation induces a significant reduction in the binding of mature human NGF<sup>R221W</sup> to p75<sup>NTR</sup> [24]. Intriguingly, the effect of the R221W mutation is only

**Table 1**

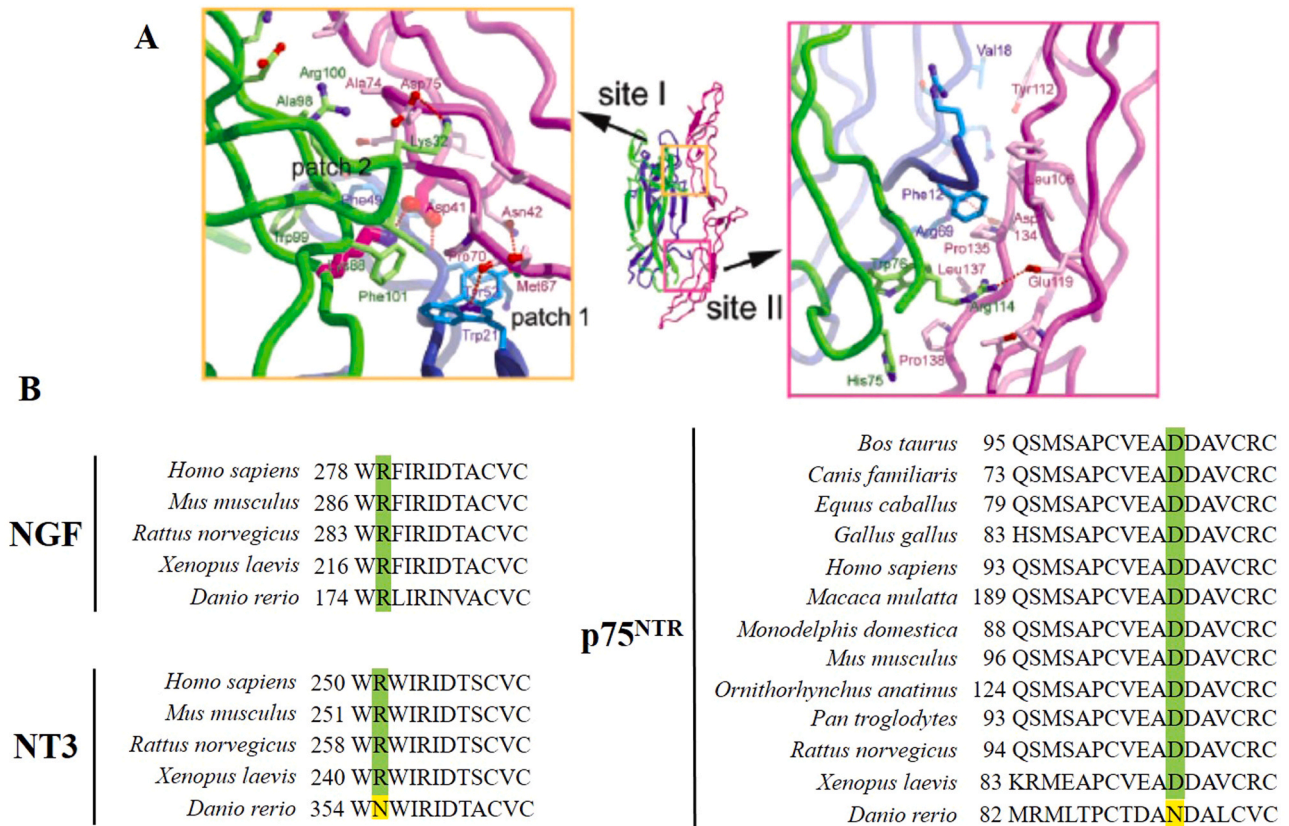
Clinical presentation of different forms HSAN V compared to HSAN IV symptoms.

Clinical features	HSAN V				HSAN IV
	R221W		V232fs	R121W	
	Heterozygous	Homozygous			
Mutation	NGF	NGF	NGF	NGF	TrkA
Manifestation of orthopedic symptoms	Adulthood	Childhood	Childhood	Childhood	Childhood
Charcot's joints	Yes	Yes	Yes	Yes	Yes
Temperature sensation	Decreased		Decreased	nr	Decreased
Sweating	Normal	Normal	No	Absent	Absent
Anhidrosis	No	No	Yes	nr	Yes
Visceral pain	Normal	Normal	Absent	nr	Absent
Touch, vibration and position	Normal	Normal	nr	nr	Normal
Motor conduction velocity	Normal	Normal	nr	nr	Normal
Nerve Biopses	Aα: normal Aδ: decreased C: decreased	Aα: normal Aδ: decreased C: strongly decreased	nr	nr	Aα: normal Aδ: strongly decreased C: decreased
Mental retardation	No	No	Yes	Yes	Yes

NGF, nerve growth factor; TrkA, tropomyosin receptor A; nr, not reported.

This table updates Capsoni, 2014





**Fig. 2.** Structural insights into the NGF-p75<sup>NTR</sup> complex. A, The crystallographic structures of human NGF (in green) complexed with p75<sup>NTR</sup> extracellular domain (in purple) (by [108]) show that the human NGF residue R221 participates in the hNGF-p75<sup>NTR</sup> interaction surface (in particular with site I), by interacting with residue D75 of p75<sup>NTR</sup> [24]. B, The residue R221 is conserved in the primary sequences of NGF and NT3 from all species, except for *D. rerio* NT3 (N221; for a more comprehensive alignment of all neurotrophins in the various species see supplementary Tables 2 and 3 of Covaceuszach et al. [24]). Notably, residue D75 is also conserved in p75<sup>NTR</sup> across species, with the notable exception of *D. rerio* (N75; [24]).

observed in the context of the mature NGF<sup>R221W</sup> protein, since the affinity of proNGF<sup>R221W</sup> to p75<sup>NTR</sup> is very similar to that of wild type proNGF.

Regarding the activation of signal transduction, NGF<sup>R221W</sup> is able to bind TrkA, but the ensuing phosphorylation at Tyr490 was reduced, correlating with a strong reduction in the activation of phospholipase C-γ (PLC-γ; [23]). Notably, TrkA signalling is essential to promote NGF-mediated survival, and this neurotrophic property is maintained by NGF<sup>R221W</sup>, which displayed an unaltered potential to support survival of dorsal root ganglion (DRG) neuron cultures [23]. Subsequently, Sung et al. [25] confirmed previous findings from our group, namely that the mutation reduces the binding to p75<sup>NTR</sup>, failing to stimulate its downstream effectors, while still being able to activate TrkA-mediated signaling pathways, with the notable exception of the PLC-γ signalling stream. PLC-γ regulates the upregulation in the expression of TRP channels in response to NGF and bradykinin [53], thus controlling the cellular reaction to algogens and pro-inflammatory molecules – which shows once more the intimate relationship between these processes [54]. The reduced ability of NGF<sup>R221W</sup> to activate PLC-γ [23,25] provided the first clear indication on a specific impairment of pain-related pathways, and prompted subsequent in vivo investigations, which showed that NGF<sup>R221W</sup> does not elicit acute thermal or mechanical hyperalgesia when administered in vivo [25,26]. On the contrary, the receptor binding and signaling capabilities necessary to induce hyperalgesia priming are retained, indeed, in presence of NGF<sup>R221W</sup>, prostaglandin E2 induces prolonged hyperalgesia (Sung K et al., 2018). In keeping with this, the ability of human NGF<sup>R221W</sup> to sensitize DRG cultures in response to bradykinin, through Substance P release, expression of bradykinin receptor 2 and activation of TRPV1, was lower

in comparison to NGF<sup>WT</sup> [26] (Fig. 3A, B). This is in line with the failure of NGF<sup>R221W</sup> application to potentiate H<sup>+</sup>-evoked responses in DRG cultures [25].

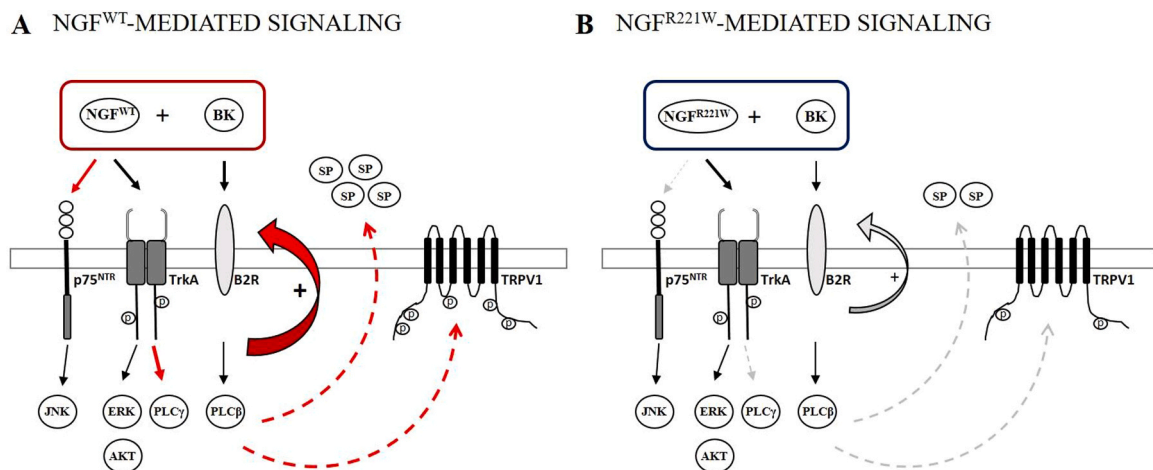
It is worth noting that the binding of NGF to p75<sup>NTR</sup> is required for the upregulation of bradykinin receptors [55]. As in DRGs treated with NGF<sup>R221W</sup>, reduced expression of bradykinin 2 receptor is observed in neurons from mice lacking p75<sup>NTR</sup> or in neurons from wild-type mice treated with a p75<sup>NTR</sup>-blocking antibody [55]. Thus, these data suggest that the differential engagement of TrkA and p75<sup>NTR</sup> receptors could explain the reduced expression of the bradykinin receptor following treatment with NGF<sup>R221W</sup>, suggesting a possible molecular mechanism for the reduction in DRG priming. In this sense, NGF<sup>R221W</sup> can be considered, from the point of view of the signalling, as a TrkA-biased agonist [24,56].

Taken together, the findings of the papers by Sung and Testa [25,26] agree in showing that NGF<sup>R221W</sup> reduces the propensity of DRGs to sensitize and to increase transmission of noxious stimuli, while maintaining an unaffected neurotrophic potency. Therefore, the R221W mutation seems to interfere with the typical switch of NGF from survival molecule during development to pain mediator in the adult.

## 7. The V232fs and R121W mutations alter the balance between NGF and proNGF

In comparison to the R221W mutation, fewer data are available on V232fs [37] and R121W [38].

COS-7 cells fail to release NGF<sup>V232fs</sup> in the culture medium, in contrast to the small amount of mature NGF<sup>R221W</sup> which could be detected in the same experimental conditions [37]. This lack of secretion



**Fig. 3.** Comparison between pain-related signaling pathways activated by NGF<sup>WT</sup> and NGF<sup>R221W</sup>. A, Treatment of DRG neurons with NGF<sup>WT</sup> for 5 days, followed by acute administration of the inflammatory peptide bradykinin (BK) (3 h), induces the activation of pain-related intracellular cascades. B, NGF<sup>R221W</sup> exhibits a reduced binding affinity to the p75<sup>NTR</sup> receptor (gray, dashed arrow) and a diminished capability to activate PLCγ (gray, dashed arrow). The same application of NGF<sup>R221W</sup> as in A, followed by acute administration of BK, leads to reduction in (i) bradykinin B2R receptor expression (gray, thin arrow), (ii) release of substance P (SP; gray, dashed arrow, also note the fewer SP particles released), and (iii) TRPV1 receptor phosphorylation (gray, dashed arrow and fewer phosphorylated sites; [26]), suggesting a possible mechanism to explain how reduced nociceptive signaling in DRG neurons might contribute to the reduced pain perception in HSAN V. Comparison between red arrows and gray arrows in panels A and B, respectively, highlights specific aspects of signaling which are affected by the R221W mutation. Black arrows indicated unimpaired signaling functions in the context of the R221W mutation.

was also observed in PC12 cells, and is responsible for their failure to differentiate [37]. Moreover, it is not known if the V232fs mutations make the protein functionally inactive.

ProNGF is cleaved predominantly by furin to generate mature NGF, but the pro domain also contains two other cleavage sites, responsible for the production of the intermediate proA and proB forms of proNGF (see Fig. 1).

The R121W mutation, recently identified by the Woods group, interferes with the full cleavage of proNGF, abolishing the formation of mature NGF, with only the proB form being secreted [38]. Interestingly, transfection with NGF<sup>R121W</sup> apparently caused a slight increase in apoptosis. However, transfection with the empty vector resulted in the highest induction of apoptosis, which led the authors to conclude that transfection per se caused apoptosis and that neither NGF<sup>WT</sup>, nor NGF<sup>R121W</sup> were pro-apoptotic [38].

However, this R121W cleavage-resistant form of proNGF involves the same amino acid of one of the experimental cleavage-resistant proNGF molecules analyzed by the Fahnestock group [57]. In this regard, they showed that proNGF activity strongly depends on the levels of its receptors, and not simply by amino acid substitutions in the proNGF cleavage region [57]. In PC12 cells, proNGF, but not NGF, switches from neurotrophic to pro-apoptotic function in response to reduction of TrkA levels [58].

On the contrary, the proNGF mutant used by the Hempstead group, is resistant to cleavage by furin (residues RR in the dibasic consensus site for furin were mutated to AA) and has a well defined pro-apoptotic action [49]. Thus, differences in the number of amino acid substitutions, purification method, addition of a tag, expression system, along with different bioassay procedures, can result in opposite biological actions of proNGF, namely neurotrophic versus apoptotic [59].

The signaling mediated by proB-NGF<sup>R121W</sup> was also analyzed, showing a significant reduction of both TrkA and MAPK phosphorylation, accompanied with a reduced capability to induce neurite outgrowth in PC12 cells [38]. The binding of proB-NGF<sup>R121W</sup> to its receptors was qualitatively assessed in Hek393T cells co-transfected with TrkA/p75<sup>NTR</sup>, showing that membrane labelling of receptors by proB-NGF<sup>R121W</sup> was higher in TrkA/p75<sup>NTR</sup>-expressing cells compared to cells transfected with TrkB/p75<sup>NTR</sup> and TrkC/p75<sup>NTR</sup> [60]. However, the binding affinities for individual receptors and the p75<sup>NTR</sup>

downstream signaling remain unknown. Shahik et al. [38] concluded that the HSAN V R121W phenotype is caused by an insufficiency of mature NGF, rather than by the effects of induced apoptosis on developing or adult nociceptors, but this conclusion does not seem to be fully substantiated. The mechanism HSAN V R121W phenotype is therefore still unclear.

## 8. The role of proNGF in pain perception

One common aspect between the R221W and R121W mutations is that they determine an unbalance in the proNGF to NGF levels. This prompts to examine the pronociceptive effects of proNGF.

ProNGF represents a significant fraction of total NGF in adult sensory neurons, spinal cord and peripheral target tissues [61–63]. In addition, high levels of TrkA, p75<sup>NTR</sup> and sortilin are found in DRG neurons [61, 64], providing a basis for a possible role of proNGF in nociception. In this regard, mechanical allodynia was evaluated in CD1 mice after intraplantar injection of human proNGF<sup>WT</sup>, at a concentration equimolar to that of NGF, that would cause a maximal hyperalgesic response. No behavioural nociceptive responses were observed in response to proNGF administration, showing that proNGF<sup>WT</sup>, unlike NGF<sup>WT</sup>, is not able to induce pain sensitization [23]. In addition, both NGF<sup>R221E</sup> and proNGF<sup>R221E</sup> displayed a reduced effectiveness in eliciting nociceptive responses in CD1 mice, similarly to proNGF<sup>WT</sup> [23]. These data, together with the recently described R121W mutation, open the field of proNGF and pain, showing that proNGF, unlike NGF, is unable to support physiological pain sensitization.

Based on these data, we postulate that the pain insensitivity manifested by HSAN V patients carrying the cleavage-resistant R121W mutation, that ultimately results in the secretion of a protease-resistant proNGF, might due to an imbalance between NGF and proNGF. Accordingly, one would expect to find higher levels of proNGF in humans or mice carrying the mutation, which would act as an endogenous analgesic, suppressing pain perception.

## 9. The humanized hNGF<sup>R221W</sup> mouse as a validated model of human HSAN V

The study of pathologies characterized by hypoalgesia is

experiencing a rising interest [5]. This can be motivated also by the fact that understanding the basis of pathological silencing of pain perception can guide the design of new therapies for effectively counteracting chronic pain, a condition that worsens the life of millions of individuals worldwide [65].

Mouse models carrying pain insensitivity mutations are instrumental for mechanistic studies of the painlessness mutations and their clinical consequences. In this regard, mice with genetic deletion of either NGF or TrkA [11,66] were the first transgenic animal models used to analyze the peripheral neuropathy related to this signalling axis.

NGF is required for the survival and differentiation of DRG neurons, and many of the NGF-dependent neurons are small-diameter TrkA-expressing peptidergic neurons involved in nociception [67]. Thus, as expected, NGF<sup>-/-</sup> mice, examined in the first weeks after birth, show a severe reduction of small neurons in DRGs and significant reduction of sensory innervations, leading to decreased pain perception [11]. Moreover, NGF<sup>-/-</sup> mice also display loss of autonomic innervation, accompanied by a severe reduction of the number of cells in Superior Cervical Ganglia (SCGs). Heterozygous NGF<sup>+/-</sup> mice reach adulthood, and present learning and memory deficits with a reduction in Choline acetyltransferase-immunoreactive neurons in the medial septum of the basal forebrain and striatum [26,68]. However, autonomic and cognitive functions are normal in patients with NGF<sup>R221W</sup>-related HSAN V, and only partially impaired in the cases of V232fs and R121W. Thus, neither homozygous nor heterozygous NGF knockout mice accurately reproduce the features of any of the HSAN V variants.

The lack of a valid model to reproduce all clinical features of HSAN V, prompted us to generate the first knock-in mouse carrying a human NGFB allele carrying the R221W mutation [26,69] (hNGFR221W/R221W mice), with no other modification to the gene.

Compared to the only three known homozygous patients who reached the adult age despite their severe nociceptive defects [16], homozygous hNGF<sup>R221W/R221W</sup> mice die within the first month of life [69]. Their early postnatal lethality could be rescued by NGF<sup>WT</sup> administration [69], reminiscent of the phenotype caused by the complete deletion of the NGF gene [11]. A reduced hNGF<sup>R221W</sup> bioavailability, due to defects in its secretion [26,41,70], represents the main explanation for the lethality of hNGF<sup>R221W/R221W</sup> mice.

Early lethality is also observed in another mouse model for HSAN V, harbouring the R221W mutation in the context of the mouse Ngf gene [71]. Histological analyses of the embryos showed no developmental defect in major organs of homozygous mNGF<sup>R221W/R221W</sup> mice [71], thus suggesting that more subtle alterations may be responsible for the embryonic lethality of these mice. It should be noted, however, that as of today, also the reasons for the early lethality of NGF<sup>-/-</sup> mice are not yet explained in detail.

On the other hand, heterozygous mice harbouring the mutant human allele, along with a wild type murine allele (hNGF<sup>R221W/m</sup>) are vital and show nociceptive impairment [26], which fully manifests in adulthood, in line with patients [29]. Their reactivity to noxious stimuli is decreased, correlating with reduced innervation of glabrous and hairy skin, along with reduced density of non-myelinated fibers. Progressive loss of the skin innervation with age was also confirmed in the HSAN V mouse model carrying the mutation in the context of murine NGF developed by Wu's group [70]. The skin content of NGF in adult NGF<sup>R221W/m</sup> mice is reduced [26]. This is in accordance to the fact that in adults NGF is required for the maintenance of skin innervation [72] and thus sensitivity to noxious stimuli. Accordingly, treatment of hNGF<sup>R221W/m</sup> mice with hNGF<sup>WT</sup> rescued their impaired responsiveness to capsaicin-induced pain [26]. However, the functionality of the remaining fibers is normal since nerve conduction speed was not different from control mice, in line with similar electrophysiological findings from heterozygous patients [28].

These behavioural, histological and electrophysiological results suggest that heterozygous humanized hNGF<sup>R221W/m</sup> mice represent a valid model for HSAN V because they display the typical features of

heterozygous patients (The summary of the main features of the HSAN V mouse model are shown in Fig. 4).

## 10. Unaltered function of NGF<sup>R221W</sup> in sensory and sympathetic neurons in vivo

Proper development of nociceptors is the prototypical example of a cellular process that is totally dependent on NGF-TrkA signaling [11, 73]. This evidence has led to automatically considering altered development of nociceptor neurons as part of HSAN IV and V clinical pictures [74]. However, the analysis of the main NGF-sensitive neuronal populations of DRG in NGF<sup>R221W/m</sup> mice showed a normal distribution in both newborns and adults [26], in line with the unaltered neurotrophic power of NGF<sup>R221W</sup> [25,26]. Thus, these findings ruled out the possibility of an effect of the mutation on DRG development in heterozygous condition. In addition, NGF also promotes differentiation and maturation of nonpeptidergic neurons, which are Ret<sup>+</sup>/TrkA<sup>-</sup> [67]. This action is maintained by NGF<sup>R221W</sup>, since nonpeptidergic neurons, stained by means of the IB4 isolectin marker, were unaffected in NGF<sup>R221W/m</sup> mice [26].

NGF is also essential for (i) sympathetic neuron survival in vivo [75] and in vitro [76]; (ii) development [11] and axonal growth of sympathetic neurons [77] and (iii) correct innervation of sympathetic targets in vivo [78]. The cellular density of SCG and peripheral sympathetic innervation of internal organs were unaltered in NGF<sup>R221W/m</sup> mice, further supporting the conclusion that NGF<sup>R221W</sup> also retains its key functions in the peripheral nervous system. In addition, a possible compensatory role could be played by NT-3 with NGF coordinate sympathetic axon growth, survival and innervation of targets [79].

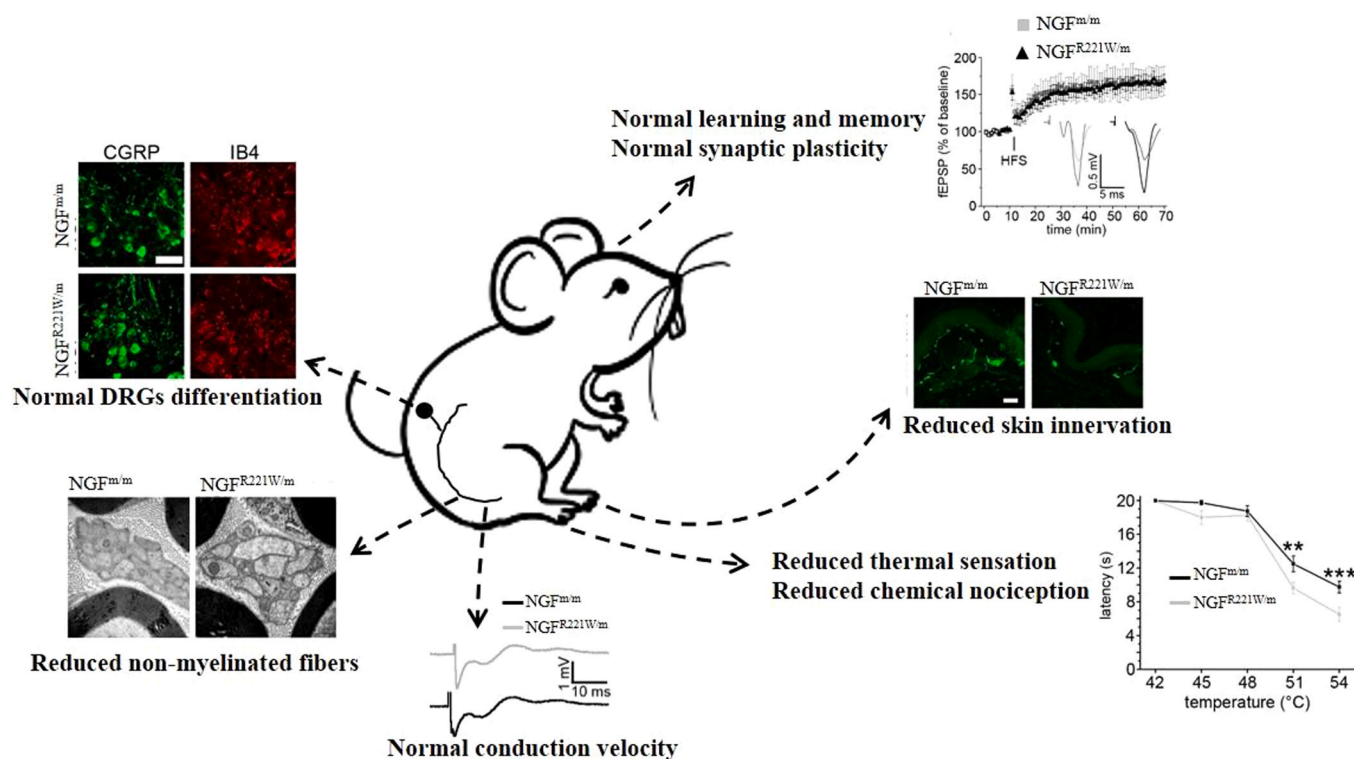
## 11. Normal cognitive performance is a hallmark of Swedish HSAN V

HSAN V, differently from HSAN IV, is not characterized by mental retardation [14,15], indicating that mutations in the ligand and in its receptor have not completely overlapping phenotypic and clinical consequences. In addition, the absence of mental retardation is a distinctive feature that distinguishes Swedish HSAN V, caused by the R221W mutation, from the other two forms of the disease, caused by the V232fs and R121W mutations [16,37,38].

NGF<sup>R221W/m</sup> mice allowed us to analyse in detail learning and memory. Despite lower brain NGF levels, behavioural performance in the Morris water maze task [26,70] (and long-term potentiation (LTP) of the Schaffer collateral pathway, were normal, in stark contrast with heterozygous NGF<sup>+/-</sup> mice [26]. This difference between pure NGF haploinsufficiency (i.e., NGF<sup>+/-</sup> mice) and heterozygosity for the R221W missense mutation emphasizes the selective loss of pain-related actions of NGF, while not affecting its neurotrophic and neuroprotective actions in the central nervous system. In keeping with this, the R221W mutation preserves the binding of NGF<sup>R221W</sup> to TrkA and the corresponding downstream signaling [23,25]. Moreover, normal function of TrkA is required for adequate maturation of basal forebrain cholinergic neurons, thus impacting cognitive functions such as attention, learning and memory [80]. Maturation and differentiation of NGF target neurons in the medial septum of the basal forebrain and striatum – which are involved in memory and cognitive processes – were also unaffected in NGF<sup>R221W/m</sup> mice, thus lending further support to a normal activation of TrkA [26].

In addition, homozygous NGF<sup>R221W/R221W</sup> mice showed unaffected cholinergic neuron density in the basal forebrain and, surprisingly, a significant increase in the striatum [69]. Similarly to NGF<sup>R221W/R221W</sup> mice, p75<sup>NTR/-</sup> mice show an increase in the number of striatal cholinergic neurons [81]. As NGF<sup>R221W/m</sup> mice, p75<sup>NTR/-</sup> mice have a milder phenotype, with reduced skin innervation but unaffected Calcitonin Gene-Related Peptide (CGRP)-immunoreactive DRG neurons, normal learning [82] and unaltered sympathetic innervation of target





**Fig. 4.** Infographic summarizing the main phenotypic features of the NGF<sup>R221W</sup> transgenic mouse model. This model reproduces the key clinical signs of HSAN V.

tissues [83]. These data, once again, lend support to an imbalance of NGF<sup>R221W</sup> signaling via TrkA over p75<sup>NTR</sup>. Thus, the R221W mutation splits the trophic and nociceptive actions of NGF, maintaining the downstream signaling of TrkA, required for cognitive functions and cholinergic maturation [80,84], but failing to engage p75<sup>NTR</sup>, which would be required to elicit nociception [85,86].

## 12. Applying “painlessness” pathologies to pain therapy and beyond

Persistent pain represents an important global health and social problem. It is often associated with many comorbidities, such as depression, having a significant negative impact on quality of life [65]. The area of chronic and inflammatory pain is, actually, a heterogeneous group of conditions triggered by various pathologies, implying different origins and mechanisms of action. Consequently, an adequate treatment should take into account the type and severity of pain status.

A well-known pain mediator is NGF that, in addition to its neurotrophic actions, has also proinflammatory and sensitizing functions [17]. Application of NGF to primary sensory neurons results in a rapid sensitization of those neurons to a variety of stimuli, including noxious heat [87], mechanical stimuli [88], or chemical stimuli [89]. Cutaneous administration of NGF to humans [90] and mice [91] leads to a rapid sensitization of cutaneous nociceptors. These rapid effects are mediated by NGF binding to TrkA expressed on mast cells, causing degranulation and release of various algogenic mediators [92]. NGF, indeed, has an important role in inflammatory pain. It is up-regulated in experimental models of inflammation, such as formalin injection and complete Freund's adjuvant administration [93], as well as in models of Rheumatoid arthritis [94]. Based on these data, the interest in targeting NGF and its downstream signaling pathways has grown, representing a promising target for pain relief therapies.

A number of strategies have been developed to investigate the role of endogenous NGF in acute and chronic pain, and the most straightforward approach has been to develop anti-NGF antibodies [95,96] or a

TrkA-IgG “scavenger” to sequester NGF, thus blocking its biological activity [97].

In addition, TrkA also represents an important target for pain therapy. MNAC13, an anti-human TrkA monoclonal antibody induces analgesia in both inflammatory and neuropathic pain models, with a long-lasting effect in the latter case [98].

Among several approaches to counteract NGF-TrkA-mediated sensitization, a special interest has been focused on selective monoclonal antibodies against NGF [99–101].

Among anti-NGF antibodies, tanezumab and fasinumab have shown efficacy in preclinical and clinical studies for the treatment of osteoarthritis (OA) [102]. However, it is important to consider that the aim of immunotherapy should be to normalize NGF levels and signaling, not their complete elimination, which would cause side effects as a result of neurotrophin deprivation of adult tissues. In this regard, in clinical trials anti-NGF antibodies showed important adverse effects, such as rapid progression osteoarthritis (OA), osteonecrosis and neurogenic arthropathy. In 2010, this led the US Food and Drug Administration (FDA) to stop the clinical studies [103], also for concerns on effects on the sympathetic system. After careful consideration, clinical trials involving tanezumab and fasinumab in OA have now been resumed [102].

Despite this effort, current treatments for acute and chronic pain have limited efficacy, which makes the need for novel analgesics a compelling issue in medicine. The lessons taken from the relationship between NGF and nociception, combined with the peculiar features of HSAN V, could be relevant to discover new targets to treat pathological pain conditions. Within this context, it will be interesting to exploit the humanized HSAN V hNGFR221W/m mouse model by inducing different forms of pain and discover which genes are differentially modulated with respect to wild type mice. This type of studies, performed in both in humans and mice unable to feel pain, could contribute to reveal novel genes, proteins and pathways involved in pain perception, thus helping in defining new treatments to alleviate altered nociception conditions, such as chronic and neuropathic pain.

In addition, the fact that NGF<sup>R221W</sup> is able to split the trophic from



the nociceptive action makes this molecule (and similar derivatives) interesting for designing therapeutic applications for many other diseases such as Alzheimer's disease and Down Syndrome, where the use of NGF as therapeutic agent has been hindered by its pro-nociceptive activity [104].

Furthermore, painlessness pathologies could be used to study the cognitive and emotional effect of hypoalgesia, along with the cellular and neurophysiological substrates of the "pain matrix", i.e., the complex of cortical and sub-cortical areas that activate when the brain generates the perception of pain [105], going beyond the common observation that many HSAN IV patients display severe cognitive problems. For instance, the clinically validated HSAN V transgenic mouse model is a platform which offers a new point of view to study how the brain constructs the "pain" perception, generates pain-related memories and produces the ensuing behavioral responses [106,107]. This has direct implications for fear- and anxiety-related disorders, such as post-traumatic stress disorder (PTSD).

Taken together, this rapidly expanding and evolving body of evidence highlights the importance of experimental models reproducing pain insensitivity diseases as a novel discovery tool to gain a deeper insight into the physiopathology of nociception and pain.

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## Conflict of interest

The authors declare no conflict of interest regarding this manuscript.

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