

Accepted Manuscript

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PII: S1043-6618(18)31280-5
DOI: <https://doi.org/10.1016/j.phrs.2018.10.028>
Reference: YPHRS 4046

To appear in: *Pharmacological Research*

Received date: 29 August 2018
Revised date: 28 October 2018
Accepted date: 28 October 2018

Please cite this article as: Cattaneo A, Capsoni S, Painless Nerve Growth Factor: a TrkA biased agonist mediating a broad neuroprotection via its actions on microglia cells, *Pharmacological Research* (2018), <https://doi.org/10.1016/j.phrs.2018.10.028>

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**Painless Nerve Growth Factor: a TrkA biased agonist mediating a broad neuroprotection
via its actions on microglia cells**

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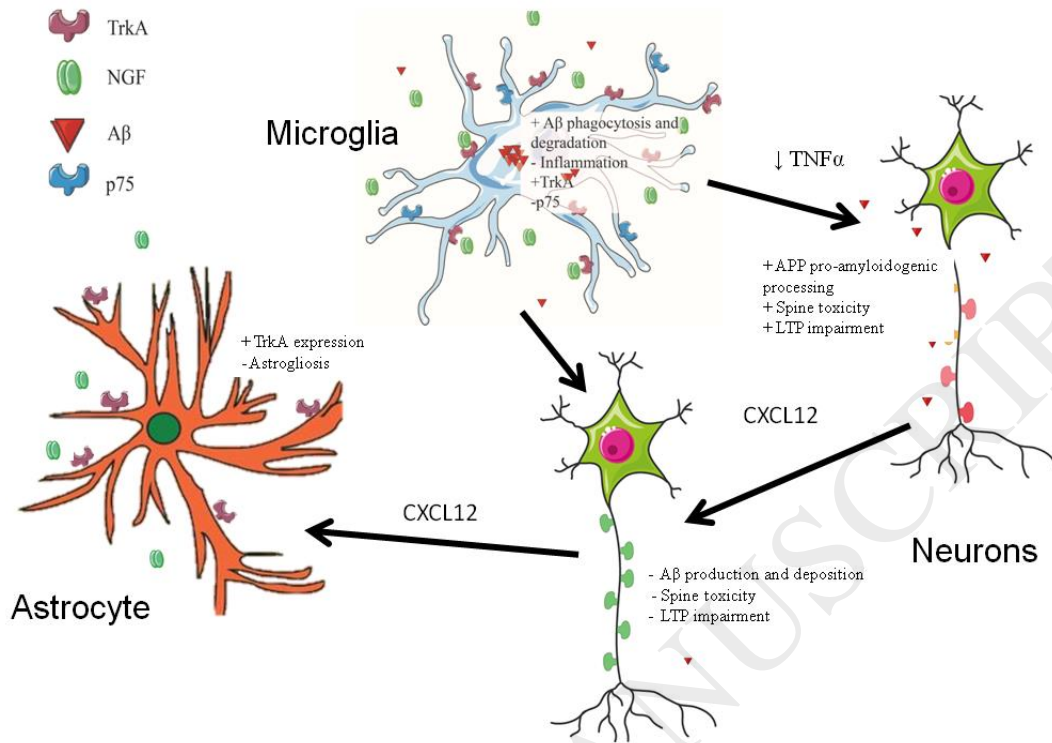
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Graphical abstract



Abstract

Nerve Growth Factor (NGF) is a therapeutic candidate for Alzheimer's disease, based on its well known actions on basal forebrain cholinergic neurons. However, because of its pro-nociceptive activity, in current clinical trials NGF has to be administered intraparenchymally into the brain by neurosurgery via cell or gene therapy approaches. To prevent the NGF pain-inducing collateral effects, thus avoiding the necessity for local brain injection, we developed painless NGF (hNGFp), based on the human genetic disease Hereditary Sensory and Autonomic Neuropathy type V (HSAN V). hNGFp has similar neurotrophic activity as wild type human NGF, but its pain sensitizing activity is tenfold lower. Pharmacologically, hNGFp is a biased receptor agonist of NGF TrkA receptor. The results of recent studies shed new light on the neuroprotective mechanism by hNGFp and are highly relevant for the planning of NGF-based clinical trials. The intraparenchymal delivery of hNGFp, as used in clinical trials, was simulated in the 5xFAD mouse model and found to be inefficient in reducing A β plaque load. On the contrary, the same dose of hNGFp administered intranasally, which was rather widely biodistributed in the brain and did not induce pain sensitization, blocked APP processing into amyloid and restored synaptic plasticity and memory in this aggressive neurodegeneration model. This potent and broad neuroprotection by hNGFp was found to be mediated by hNGFp actions on glial cells. hNGFp increases inflammatory proteins such as the soluble TNF α receptor II and the chemokine CXCL12. Independent work has shown that NGF has a potent anti-inflammatory action on microglia and steers them towards a neuroprotective phenotype. These studies demonstrate that microglia cells are a new target cell of NGF in the brain and have therapeutic significance: i) they establish that the neuroprotective actions of hNGFp relies on a widespread exposure of the brain, ii) they identify a new anti-neurodegenerative pathway,

linking hNGFp to inflammatory chemokines and cytokines via microglia, a common target for new therapeutic opportunities for neurodegenerative diseases, iii) they extend the neuroprotective potential of hNGFp beyond its classical cholinergic target, thereby widening the range of neurological diseases for which this neurotrophic factor might be used therapeutically, iv) they help interpreting the results of current NGF clinical trials in AD and the design of future trials with this new potent therapeutic candidate.

Introduction

Alzheimer disease (AD) is the most common form of dementia in elderly people, affecting approximately 50 million people worldwide. Treatments to slow, stop, or reverse the course of the disease do not exist and constitute a great medical need. Pharmacological approaches designed to modify APP processing and amyloid depositions have substantial mechanistic appeal for slowing disease progression (Vellas et al., 2007). However, the results obtained from more than a decade of clinical trials with amyloid-depleting drugs have been disappointing (Gold, 2017), calling for trials in which amyloid-modifying treatment is started in pre-symptomatic patients or those at a very early stage. There remains a great unfulfilled need to identify therapies with the potential to slow disease progression and ameliorate cognitive function in AD. Neuroprotective strategies are an important approach to be pursued. In this article we shall discuss the rationale for NGF based neuroprotective strategies, in light of new findings that demonstrate brain microglia cells as a potent target of NGF neuroprotective actions in the brain and of work that has described and extensively characterized a new variant of NGF (painless NGF), optimized for its therapeutic uses in the brain and other systems.

In this paper, we shall summarize the main findings of two recent studies (Capsoni et al 2017; Rizzi et al 2018) that address the important question of which cell types are engaged by hNGFp in the brain, with significant implications towards the development of therapeutic strategies for clinical applications of hNGF and of painless hNGFp.

NGF and AD: new twists to an old connection

In Alzheimer's Disease (AD) patients basal forebrain cholinergic neurons (BFCNs) are

vulnerable and their degeneration contributes to cognitive decline (Bartus et al, 1982; Whitehouse et al, 1982). BFCNs rely on the neurotrophin Nerve Growth Factor (NGF) (Levi-Montalcini, 1952; Levi-Montalcini, 1987) for their maintenance and survival (Hefti et al, 1993; Mufson et al, 1999). For this reason, given its robust trophic effects on BFCNs, the clinical use of NGF as a therapeutic agent for AD has been proposed long time ago (Mobley et al. 1989; Olson et al. 1993; Hefti 1994) and is being actively pursued (Mandel, 2010). Work in the past decade has extended the rationale for NGF as a therapeutic candidate for AD beyond its well established neurotrophic actions on BFCNs, based on the demonstration of a broad anti-amyloidogenic action of NGF (Mufson et al, 1999; Capsoni and Cattaneo, 2006; Capsoni et al, 2011a; Cattaneo and Calissano, 2012). Thus, independent lines of evidence point to an imbalance in the ratio between the levels of proNGF and of mature NGF as an upstream driver of neurodegeneration (Capsoni et al, 2010; Iulita and Cuello, 2014; Tiveron et al, 2013; Fasulo et al, 2017), uncovering a vicious cycle whereby high proNGF levels activate amyloidogenic processing of APP and high levels of A β inhibit the processing of proNGF to mature NGF. Consistently, the levels of proNGF are suggested as a putative biomarker for early AD (Counts et al, 2016). The cellular basis of this mechanism mediated by proNGF/NGF imbalance is still debated and poorly understood, and is unlikely to involve exclusively the BFCNs, the known neuronal target of NGF in the brain. Recent work discussed in this article points to astroglial cells as a new target cell for NGF in the brain, that can be exploited for neuroprotection. Thus, re-establishing the homeostasis of the NGF proNGF/NGF system represents a valuable and strongly motivated strategy to combat early neurodegeneration and to provide neuroprotection in AD and other neurodegenerative conditions.

Current NGF clinical trials in AD: limitations caused by essential features of NGF biology

Unfortunately, key features of the biology of NGF have limited the extent to which the therapeutic properties of NGF could be evaluated and have highlighted the difficulties that hamper this therapeutic approach (Cattaneo et al 2008 ; Cattaneo and Calissano 2012).

A first limit is linked to the poor biodistribution of NGF to the brain after systemic delivery and its inability to cross the blood-brain barrier (Poduslo and Curran, 1996). A second limit consists in the fact that, in addition to being a neurotrophic factor, NGF also has the intrinsic property of being one of the key molecules for mediating inflammatory pain and neuropathic pain in the PNS (Lewin and Mendell 1993; Lewin et al 1993; Anand et al 1996; Chuang et al 2001; Watanabe et al 2008). Clinical trials to investigate NGF as a treatment for diabetic polyneuropathy and peripheral neuropathies in HIV were discontinued, after reports of a potent dose-dependent hyperalgesia, such as back pain, injection site hyperalgesia, severe myalgia (Apfel et al 1999; Apfel et al 2000; Apfel 2002; Petty et al 1994). Therefore, the adverse effect of significant pain caused by systemically delivered NGF has severely limited its therapeutic use in treating neurodegenerative disorders. In a pilot trial in three AD patients, delivery via the ventricular system even at low doses resulted in pain and the trial was discontinued (Eriksdotter-Jonhagen et al 1998). Accordingly, NGF testing in human AD had to rely on a long-term delivery method that achieves central delivery and restricted distribution only to regions of degenerating basal forebrain cholinergic neurons. Thus, current NGF clinical trials in AD are based on the neurosurgical injection of NGF-secreting cells or of viral vectors carrying the NGF gene (Tuszynski et al, 2005; Mandel, 2010; Eriksdotter-Jonhagen et al, 2012), close to the nucleus basalis of Meynert (NBM), where the known target neurons are located. These clinical trials report a positive effect on BFCNs and on cognition, which warrants further consideration

of the NGF therapeutic approach, but the outcome of the NGF treatment on AD hallmarks, such as amyloid pathology, were not described in treated patients (Tuszynski et al, 2005; Mandel, 2010; Eriksdotter-Jonhagen et al, 2012; Ferreira et al, 2015; Karami et al, 2015; Tuszynski et al 2015; Rafii et al 2014).

The highly invasive neurosurgical procedure involved in the currently pursued forms of NGF therapy is unlikely to become generally applicable to the large numbers of patients affected by dementia.

Painless NGF (hNGFp)

To fully exploit the therapeutic potential of NGF, and avoid the need for invasive delivery approaches, we sought to reduce the pain-inducing side effects of NGF. To this aim, we developed painless NGF (hNGFp), a double mutant form of NGF (hNGFP61S/R100E) inspired by the human genetic disease Hereditary Sensory Autonomic Neuropathy type V (Einarsdottir et al, 2004).

Recently, patients in a Swedish family suffering from congenital chronic loss of pain leading to bone fractures, joint destruction, self-mutilation were shown to harbor a homozygous missense mutation in NGF (Einarsdottir et al 2004), causing a substitution of tryptophan (W) for a conserved arginine (R) at position 100 of mature NGF (corresponding to position 211 in the proNGF form). Unlike HSAN type IV (Indo 2014), which results from mutations in TrkA gene, HSAN V patients have no mental retardation and normal cognitive functions (De Andrade et al., 2008) , suggesting that the mutant NGF may retain its trophic functions in the CNS. We therefore reasoned that the protein NGF R100W might teach us how to dissect the trophic from the nociceptive actions of NGF (Covaceuszach et al 2010; Capsoni et al 2011b) and provide the

molecular basis to develop a drug based on NGF, devoid of the pitfalls of NGF itself. Due to the fact that the R100W protein is poorly expressed, and thus cannot be produced in a sufficient amount to perform extensive in vivo studies, we performed an extensive characterization of R100 mutant NGF proteins (Covaceuszach et al., 2010; Capsoni et al., 2011, 2012). The lead candidate selected for further development (Covaceuszach et al, 2010; Capsoni et al, 2012; Malerba et al, 2015) was the double mutant human NGFP61SR100E, that we named painless NGF or hNGFp. hNGFp harbors the mutation R100E, to reduce the pro-nociceptive activity of NGF (Covaceuszach et al, 2010; Capsoni et al, 2012; Malerba et al, 2015) and a second tagging mutation (P61S), allowing its specific immunodetection against endogenous NGF.

From the pharmacological point of view, hNGFp is a TrkA biased agonist, in that it effectively binds and activates TrkA, with an identical binding affinity to that of hNGF, while its binding affinity to p75NTR is strongly reduced (Covaceuszach et al, 2010; Capsoni et al, 2012). The latter result correlates with hNGFp being ineffective in activating signaling cascades downstream of p75NTR. hNGFp maintains the ability to activate TrkA and activates most TrkA downstream signaling events (Erk1/2, Akt), but is unable to fully induce phosphorylation of PLC- γ in different cell types including DRG neurons (Capsoni et al 2011; Severini et al 2017). The critical function of NGF in being retrogradely transported is preserved, in sensory neurons, by hNGFp (Sung et al 2018).

hNGFp has identical neurotrophic potency as wild type (WT) human NGF (hNGF), in a number of different assays, but a tenfold lower pain sensitizing activity (Capsoni et al, 2012; Malerba et al, 2015; Capsoni et al 2017) in different acute hyperalgesia pain assays, including mechanical or thermal hyperalgesia after intraplantar injection and orofacial pain after nasal delivery. hNGFp also shows a reduced pain sensitization activity than hNGF in chronic sensitization assays. Thus,

contrary to hNGF, DRG neurons exposed to hNGFp become less sensitive to respond to nociceptive stimuli such as bradykinin, by eliciting phosphorylation of Transient Receptor Potential Vanilloid 1 (TRPV1) or Substance P release (Severini et al 2017).

These findings provide evidence that hNGFp uncouples trophic activities from nociceptive functions, both induced by wild type hNGF. hNGFp has therefore a broader therapeutic window than hNGF, with respect to its pain inducing activity.

These properties make hNGFp an ideal therapeutic candidate for a neuroprotective approach to neurodegeneration, devoid of the pitfalls by human NGF. The bias of hNGFp to signal through TrkA, while failing to effectively engage p75NTR adds to the neurotrophic and neuroprotective properties of this innovative molecule. For these reasons, hNGFp is currently undergoing preclinical Investigational New Drug Application (IND) enabling studies, including manufacturing under Good Manufacturing Practice (GMP) conditions, towards its clinical testing in man for AD and other dementias.

Lack of anti neurodegenerative neuroprotection by intraparenchymal hNGFp locally delivered to the BFCNs

Previous studies had shown that the intranasal delivery of hNGFp to APPxPS1 mice induces a dramatic reduction of the amyloid plaque load, accompanied by a rescue of the learning and memory deficits (Capsoni et al 2012). It is of paramount interest to ascertain whether the intranasal delivery was key to the successful neuroprotection observed in that model. The effectiveness of the nasal route of delivery was therefore compared to that of the local delivery of hNGFp close to the cell bodies of the BFCNs. Thus, Capsoni et al (2017) studied the efficacy of hNGFp in counteracting neurodegeneration and behavioral deficits in the aggressive AD

mouse model 5xFAD (Oakley et al, 2006), starting the treatment at an age when the pathology is already evident, a situation that would be similar to a human clinical trial in AD patients. In this model, the amyloid neurodegeneration is diffused throughout the cortex, and in particular, in pyramidal cortical neurons that do not express NGF TrkA receptors. It was of interest to address the question whether the local intraparenchymal delivery of NGF to the NBM, mimicking the intraparenchymal delivery route current tested in NGF clinical trials, effectively decreases A β plaque load. Very significantly, it was found that hNGFp locally infused close to the NBM of 5xFAD mice, despite inducing cholinergic sprouting in the ipsilateral NBM, determined no decrease of the A β plaque load in the cerebral cortex (Fig 1 A-B-C). This is similar to what was found after an intraparenchymal administration for three months of NGF in non-human primate brains, with A β deposition found to be the same as that in age matched controls (Tuszynski et al, 1998). Thus, the intraparenchymal delivery of hNGFp to NBM is not sufficient to obtain an anti-amyloidogenic effect, despite a clear action on cholinergic neurons, suggesting that a more widespread distribution of hNGFp in the brain might be required to reduce the number of A β plaques.

Intranasal delivery of hNGFp is widely distributed throughout the brain and determines a strong neuroprotection

To investigate whether a broader exposure of the brain to hNGFp might be more efficient to reduce A β deposition, hNGFp was delivered intranasally, a non invasive way to introduce neurotrophins to the brain at pharmacologically and therapeutically relevant doses (Capsoni et al, 2002b; De Rosa et al, 2005; Capsoni et al, 2009; Capsoni et al, 2012; Thorne and Frey, 2001; Lochhead and Thorne, 2012; Capsoni et al 2002a; Malerba et al, 2011). A biodistribution study

of hNGFp after intranasal delivery, exploiting an ELISA protocol which selectively detects the P61S tagging mutation (Covaceuszach et al, 2009; Malerba et al, 2015) showed that hNGFp widely distributed to brain areas significantly affected by the neuropathology, such as the cerebral cortex and hippocampus, while its systemic levels are below the detection threshold. Thus, the intranasal administration, together with the use of hNGFp, allows delivering higher concentrations of this neurotrophic factor to maximize a broad exposure of the brain, while reducing the potentially painful consequences of systemic build-up. Very surprisingly, it was found that, after intranasal delivery, unlike what was found with local delivery to BFCNs, hNGFp determines a robust decrease of the number of amyloid plaques in many brain areas, as well as a general reduction of soluble and insoluble A β 42 and A β 40, and of A β oligomers (A β Os) (Fig 1 D-E-F). This reduced plaque load after intranasal hNGFp administration is due, at least in part, to a reduction of the pro-amyloidogenic APP processing linked to a lower expression of PS1, BACE and nicastrin after hNGFp delivery. The robust decrease of A β production after intranasal delivery of hNGFp to 5xFAD mice correlates functionally with a full rescue of synaptic plasticity deficits in the entorhinal cortex as well as of learning and memory deficits in different behavioral paradigms.

To test the specificity of hNGFp action, increasing doses of brain-derived neurotrophic factor (BDNF) were intranasally administered to 5xFAD mice, but even a 100-fold higher dose of BDNF was found not to determine rescue of A β plaque load nor of behavioral deficits, despite a robust sprouting of cholinergic innervation in the NBM, indicating that BDNF reached the brain targets (Capsoni et al., 2017). The superior properties of hNGFp over those of BDNF, in this model, is highly significant, since the latter neurotrophin is being considered as a therapeutic candidate for the treatment of AD and dementias (Nagahara and Tuszynski, 2011).

It was therefore concluded that hNGFp has potent anti-amyloidogenic actions that are not in common with another neuroprotective neurotrophin and that the intranasal strategy to deliver hNGFp is much more potent than its local administration in determining a robust decrease of A β deposition. Thus, a more widespread action of hNGFp in the brain is required to achieve a pharmacological effective neuroprotection.

Modulation of microglia and astrocytes by hNGFp

What is the cellular basis of the neuroprotective actions of hNGFp in 5xFAD mice? In these mice, A β is produced mainly by cortical pyramidal neurons, which do not express TrkA receptors. Hence, those neurons are not the first targets of the robust anti-amyloidogenic action of hNGFp. We considered unlikely that activation of BFCNs by hNGFp is commensurable to the potent anti-amyloidogenic response observed, for several reasons: because i) the 5xFAD mice do not have a cholinergic deficit, ii) the local delivery of hNGFp directly to the BFCNs proved totally ineffective in phenotypic rescue, iii) the intranasal delivery of high doses of BDNF was totally ineffective, despite evidence for BDNF reaching cholinergic neurons and fibers in the NBM. Thus, the robust neuroprotective action of hNGFp is not mediated by BFCNs, which respond to both NGF and BDNF.

Thus, we postulated that microglia and/or astrocytes might be the cells mediating hNGFp actions. Both cells are suggested target cells for NGF (De Simone et al, 2007; Cragolini et al, 2009; Cragolini et al, 2012). In cortical microglia of WT mice, both p75NTR and total TrkA were undetectable in vivo, while they were readily expressed in cultured WT primary microglia. Both receptors were instead well detectable in the cortex of PBS-treated 5xFAD mice (Capsoni et al., 2017). Interestingly, a similar increase in microglial p75NTR and TrkA immunoreactivity could be shown in brain sections from Alzheimer's disease patients (Capsoni et al., 2017).

After hNGFp treatment, p75NTR expression in 5xFAD cortical microglia was decreased, while TrkA immunoreactivity remained high. 5xFAD mice showed a higher number of amoeboid microglial cells, characterized by a lower number of ramifications, with respect to WT mice. hNGFp reduced microglia number and soma size and partially restored ramification complexity.

5xFAD microglia cells are engulfed by A β and A β oligomers and hNGFp induced a robust decrease of both A β species

Similarly to microglia, p75NTR nor TrkA are not expressed in cortical astrocytes from WT mice, but they are in culture. A significant increase astrocytic p75NTR expression was found in PBS-treated 5xFAD mice and was reduced after hNGFp administration. TrkA expression in astrocytes was undetectable both in WT and PBS-treated 5xFAD mice, but a robust increase in the expression of this NGF receptor was observed after hNGFp administration (Capsoni et al., 2017). Most importantly, an increased expression of both p75NTR and TrkA in astrocytes could also be detected in Alzheimer's disease brain sections (Capsoni et al., 2017).

As for astrocyte morphology, 5xFAD mice showed massive astrogliosis, characterized by an increase in the volume of single astrocytes, which was significantly reduced after hNGFp administration. In addition, similarly to microglia, the treatment with hNGFp decreased A β and A β oligomer immunoreactivity in 5xFAD astrocytes.

In a previous article we showed that hNGFp preferentially binds to TrkA, with respect to p75NTR (Covaceuszach et al, 2010). Since, in PBS-treated 5xFAD mice, TrkA is first expressed on microglia and not on astrocytes, we adduce that microglia cells might represent the primary target of hNGFp action and astrocytes may represent a secondary target (Fig 2).

Thus, while TrkA is not expressed in cortical microglia or astrocytes from WT brains, in 5xFAD

brain, both glial cells express TrkA and p75NTR receptors with a p75NTR/TrkA ratio in favor of p75NTR. The balance is restored by hNGFp, which shifts the expression of NGF receptors towards TrkA on both cell types.

In any event, an increased degradation of cellular A β forms uptaken by microglia and astrocytes might significantly contribute to the overall decrease of A β deposition after hNGFp delivery.

The chemokine CXCL12 mediates hNGFp neuroprotective and anti-amyloidogenic actions in 5xFAD mice

What is the mechanism whereby hNGFp-targeted microglia protects neurons in this system? By acting on microglia, hNGFp might change the expression of microglia-derived cytokines and chemokines. A cytokine and chemokine profiling of total brain extracts revealed that hNGFp treatment specifically increases the amount of the soluble TNF α receptor II (sTNFRII) (an extracellular decoy of TNF α), of macrophage inflammatory protein 1 α and 1 γ (MIP-1 α and MIP-1 γ) and of CXCL12 (also known as SDF-1 α) (Capsoni et al., 2017).

The focus was put on the CXCL12 chemokine, which is known to modulate A β expression (Parachikova and Cotman, 2007; Laske et al, 2008a; Laske et al, 2008b). Surprisingly, it was found that CXCL12 expression was not up-regulated by hNGFp treatment in 5XFAD microglia, but in neurons. Which microglia-derived factor could modulate CXCL12 expression in neurons? A possible microglia-derived candidate was identified in TNF α , since its levels are known to be regulated by NGF (Marshall et al, 1999; Prencipe et al, 2014). It is known that CXCL12 can be upregulated by reduced levels of TNF α (Zhang et al, 2008; Bockstal et al, 2011) in non-neuronal cells and, consistently, Capsoni et al (2017) showed that the incubation of WT and 5xFAD

cultured cortical neurons with TNF α reduces CXCL12 immunoreactivity, providing a link between TNF signaling and neuronal CXCL12. Also, hNGFp treatment decreased the expression of TNF α in 5xFAD microglia. Similarly, in WT microglia cultures treated with A β O_s TNF α expression was reduced by hNGFp treatment. Altogether, data point to the reduction of TNF α signaling as the mechanism whereby hNGFp treatment increases neuronal CXCL12 expression.

Does CXCL12 mediate the ameliorative effects of hNGFp on memory deficits and on pro-amyloidogenic APP processing in 5xFAD mice? 5xFAD mice were co-treated with hNGFp and AMD3100, an inhibitor of CXCL12 receptor CXCR4, which is detectable on neurons, astrocytes and microglia (Guyon, 2014). The co-treatment of 5xFAD mice with AMD3100 completely occluded and counteracted the rescuing effects of hNGFp on memory deficits, on A β plaque load and on the levels of A β O. AMD3100 was also found to block the hNGFp-induced modulation of presenilin 1 and BACE1 levels and the formation of APP processing products, as well as the reduction of intracellular A β induced by hNGFp in microglia cells (Capsoni et al., 2017).

These data support the fact that CXCL12 mediates most of the actions of intranasally delivered hNGFp in 5xFAD mice. In keeping with this conclusion, CXCL12 treatment of 5xFAD cultured cortical neurons also decreased A β O immunoreactivity in neurons, while it protected WT neurons from cell death induced by incubation with A β O_s.

In conclusion, Capsoni et al (2017) showed that hNGFp exerts its neuroprotective actions in 5xFAD mice via microglia and astrocytes, which under neurodegenerative conditions express TrkA (Fig 2). More in detail, hNGFp would act on microglia as its primary cellular target in the brain, lowering the overall TNF α signaling by microglia, thereby inducing neuronal CXCL12.

This chemokine would then be an mandatory mediator of the neuroprotective and anti-

amyloidogenic actions of hNGFp, inhibiting APP pathological processing in neurons from 5xFAD mice and acting on astrocytes, making them responsive to hNGFp by increasing astrocyte TrkA receptor (Figure 2). In turn, microglia and astrocytes exposed to hNGFp increase uptake and degradation of A β forms. Thus, the dramatic overall reduction of A β deposition after hNGFp nasal administration is due, at least partially, to a reduced APP amyloidogenic processing and, also, to an increased uptake and degradation of cellular A β forms by microglia and astrocytes.

NGF steers microglia towards a neuroprotective phenotype

Overall, these data set the basis of the emerging concept that the neuroprotective actions of NGF in the brain go well beyond BFCNs (Capsoni and Cattaneo, 2006; Capsoni et al, 2011; Cattaneo and Calissano, 2012) and demonstrate that the potent anti-amyloidogenic actions of hNGFp are exerted also in the absence of an overt cholinergic deficit of BFCNs. Most importantly, the study establishes glial cells (both astrocytes and microglia) as a broad target for hNGFp actions in the brain, and identifies the chemokine CXCL12 as a key factor of the anti-neurodegenerative and anti-amyloidogenic actions of hNGFp, with significant clinical and therapeutic implications.

Can we obtain independent evidence that microglia are target cells of NGF in the brain?

Microglia cells are the sentinels of the brain, but a clear understanding of the physiological and pathological consequences of their activation in response to different signals is still lacking. It would be very important to learn how to selectively modulate a neuroprotective subset of the functional activities of microglial cells, particularly in microglia cells activated by pathological stimuli. The results described above prospect hNGFp (and by extension possibly also NGF) as a potential modulator of microglia functions. A recent paper (Rizzi et al 2018) explored this

possibility and clearly demonstrates that NGF acts on microglia cells by steering them towards a neuroprotective phenotype. A transcriptomic analysis of NGF treated primary microglia showed that NGF treatment induces a modulation of motility, phagocytosis and degradation pathways. On that note, NGF induced an increase in membrane dynamics and macropinocytosis, but not phagocytosis, in primary microglial cells. Since microglia are supposed to be a major player in A β amyloid peptide clearance in the brain, the effects of NGF on the uptake of different forms of A β peptide by microglia cells were investigated. NGF not only promotes the TrkA-mediated engulfment of soluble A β peptide and soluble A β oligomers by microglia, but also enhances the degradation of the peptide. Importantly, the proinflammatory transition induced in microglia by A β treatment is fully counteracted by the concomitant administration of NGF, that completely reverts the proinflammatory cytokine profile induced by A β (Fig 3A). Thus, NGF is very effective in reverting the pro-inflammatory state of microglia induced by A β , while it has only a moderate effect on the basal cytokine profile of naïve microglia cells (Fig 3 A).

One prominent action of A β oligomers is to decrease spine density both in vitro and in vivo, and to reduce synaptic long term potentiation (LTP). Rizzi et al (2018) show that NGF, by acting specifically on microglia cells, prevents the A β induced loss of dendritic spines and the A β induced inhibition of LTP (Fig 3 B). Indeed, these neuroprotective effects by NGF are not observed when NGF is delivered directly to neurons, in the absence of microglia cells and are only seen when NGF and microglia are both added to the A β treated neuronal cultures (Fig 3 B). In conclusion, this work supports a role for NGF in the regulation of microglial physiology and characterizes this neurotrophin as a neuroprotective agent against neurodegeneration of different forms, including, but not limited to, A β accumulation pathologies. It remains to be seen to what extent hNGFp recapitulates these properties shown by hNGF.

Conclusions

Despite a strong rationale, the clinical uses of NGF are hampered by NGF potent pro-nociceptive activity, forcing current clinical trials in Alzheimer's disease patients to an invasive intraparenchymal route of administration (Tuszynski et al, 2005; Eriksson-Jonhagen et al, 2012). The design of hNGFp, with similar neurotrophic properties to wild type hNGF, but a significantly reduced pain-sensitizing activity, offers a new avenue for a new generation of neuroprotective therapies for AD and other dementias. The remarkable efficacy of intranasally delivered hNGFp in counteracting neurodegeneration and behavioral deficits in different neurodegeneration mouse models contrasts with the ineffectiveness of local hNGFp delivery to the basal forebrain. The neuroprotection activated by hNGFp is not mediated by the cholinergic target system, and involves a newly discovered mechanism mediated by microglia cells. In any case, the pro-cholinergic activity of hNGFp (in common with that of NGF) is an additional bonus of this neurotrophic and neuroprotective treatment.

The classical concept of neurotrophic factors posited that each neurotrophin acts on a specific set of target neurons (e.g NGF and its target basal forebrain cholinergic neurons and GDNF and catecholaminergic nigrostriatal neurons (Pascual et al 2008) and this paradigm has guided the clinical trials with neurotrophic factors. The initial disappointing results obtained with neurotrophic factor therapies for different neurodegenerative diseases led to suggestions for "improved methods for regulated local supply of NTs to specific populations of neurons" (Thoenen and Sendtner 2002). However, this more precisely targeted neurotrophic factor therapeutic approach led to continuing disappointing failures, for different diseases. We show now that the potent neuroprotective and anti-amyloidogenic actions of hNGFp necessarily

require a widespread and global biodistribution of hNGFp in relevant brain regions, such as that obtained by intranasal delivery neurotrophic factors. We further show that hNGFp has a broader target cell basis, calling into action astroglial cells, that exert broad neuroprotective actions.

This has important implications for the current NGF-based clinical trials, that are forcedly being performed by the local stereotaxic delivery of NGF secreting cells or of viruses encoding NGF.

Thus, the outcome of those trials could have been much more positive, if a more global exposure of the brain to NGF could have been achieved. Of course, the “dark side of the moon” of NGF, namely its pain sensitizing activity, does not allow for this more global exposure.

An important point in the discussion between targeted versus diffuse biodistribution in neurotrophic factor therapies is the fact that often the corresponding neurotrophic factor receptors are down regulated in the target neurons from the diseased brain, and this can limit the neuroprotective efficacy by the locally delivered neurotrophic factor. Previous studies have demonstrated that microglia can take up both soluble and insoluble forms of A β in vitro and in vivo (Bolmont et al, 2008; Lee and Landreth, 2010) but also that phagocytosis may not be so efficient in compensating the continuous deposition of A β (Bolmont et al, 2008; Sierra et al, 2013). Similarly, astrocytes have been shown to uptake A β in vitro and in vivo (Wyss-Coray et al, 2003) with a feed-forward mechanism driven by cytokines and A β 42, which might lead to A β production also in these glial cells (Zhao et al, 2011). The dramatic overall reduction of A β deposition after hNGFp is linked, at least partially, to a reduced APP amyloidogenic processing and, also, to an increased uptake and degradation of cellular A β by microglia and astrocytes.

The observed increase in the microglia-derived TNF α decoy sTNF α RII, leads to a reduced TNF α signaling by microglia, which upregulates the neuronal expression of the chemokine CXCL12, which has well established neuroprotective (Guyon and Nahon, 2007; Li et al, 2012) and anti-

amyloidogenic actions (Parachikova and Cotman, 2007; Laske et al, 2008a; Laske et al, 2008b). Most importantly, CXCL12 expression is reduced in Alzheimer's disease brains (Parachikova and Cotman, 2007; Laske et al, 2008a; Laske et al, 2008b). Capsoni et al (2017) provided compelling evidence that CXCL12 is necessary for the pharmacological effects of hNGFp in 5xFAD mice.

These results provide a very important motivation for the use and further development of painless NGF, thanks to its increased therapeutic window and to the cellular basis for its actions in the brain. Thus, based on data discussed above, we can draw a picture whereby hNGFp would act primarily on microglia and astrocytes in the neurodegenerating brain, leading to a reduced availability of TNF α that, possibly with other unknown factors, provokes an increase of neuronal CXCL12 levels and the downstream decrease of APP processing, increased clearance of A β and widespread neuroprotection and restoration of neuron-to-glia homeostasis (Fig. 2). How exactly does CXCL12 exert these downstream neuroprotective actions remains to be seen. One could envisage that this chemokine, produced by neurons, acts on microglia and astrocytes, to re-establish a correct neuron-glia homeostasis. In any event, one could predict that this chemokine should mimic many of the actions of hNGFp in the 5XFAD, as well as in other neurodegeneration models.

In more general terms, one can envisage that in the normal brain, the neuronal well being is ensured by neuroprotective astrocyte-to-neuron and microglia-to-neuron interactions (Fig 4 top). In the course of neurodegeneration, the homeostatic well being loop between astroglia and neurons is broken, with insulted neurons inducing activation of microglia and/or induction of astrocyte A1 neurotoxic phenotype (Liddlelow and Barres 2017), and the latter, in turn reducing their neuroprotective astroglia-to-neuron interactions, or possibly, releasing neurotoxic

substances (Liddlelow and Barres 2017) (Fig 4, middle). Remarkably, by acting directly on microglia and astrocytes, hNGFp re-establishes the neuronal homeostasis (Fig 4, bottom). Thus, besides being a neurotrophic factor, NGF can be considered a neurokine, a concept put forward two decades ago by Levi-Montalcini (Levi-Montalcini et al 1996). The direct actions of hNGFp on its target BFCNs represents an additional bonus in its comprehensive neuroprotective mechanism. NGF appears therefore to be strategically positioned at the center of a homeostatic loop between neurons (target and non target), microglia and astrocytes.

Altogether, we have argued a strong mechanistic rationale for the clinical uses of hNGFp in neurodegenerative diseases, and demonstrated a new anti-neurodegenerative pathway, under clinically very relevant conditions. This pathway might become a target for new therapeutic opportunities for Alzheimer's and other neurodegenerative diseases. The broad neuroprotective actions of hNGFp, via glial cells, open the therapeutic potential of painless hNGFp to a large number of neurodegenerative conditions, beyond, but including, Alzheimer's disease, where an action through astrocytes and microglia cells could exert beneficial actions to restore a correct neuronal homeostasis.

Conflict of Interest

Antonino Cattaneo and Simona Capsoni declare no conflict of interest regarding this manuscript

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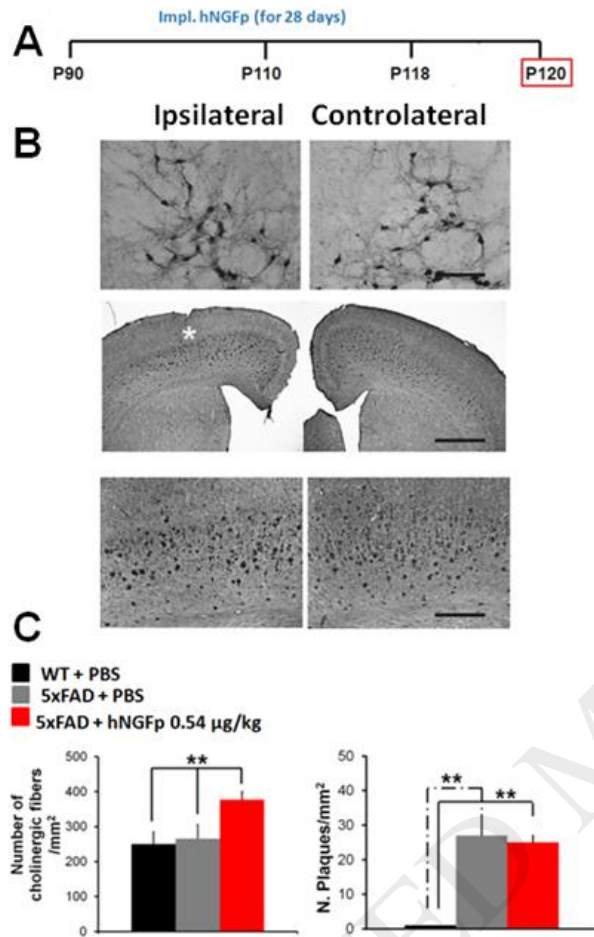
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Intraparenchymal Injection in NBM



Intranasal delivery

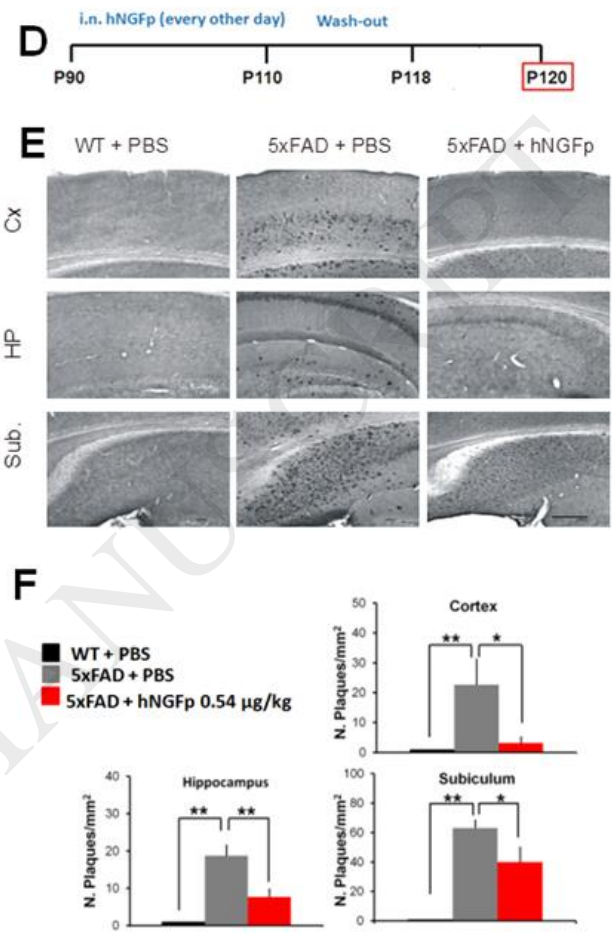


Figure 1 Comparison of local delivery of hNGFp to the NBM versus the nasal delivery of hNGFp in 5xFAD mice.

Local delivery of hNGFp by an osmotic minipump close to the NBM of 3-month-old 5xFAD mice does not decrease amyloid plaque load in 5xFAD mice. A) Scheme of local delivery of hNGFp. B) hNGFp caused cholinergic sprouting in the ipsilateral NBM, but not in the contralateral NBM. No decrease of the amyloid- β plaque load is observed in the cerebral cortex (B).

Quantification of cholinergic sprouting and of amyloid plaque load with anti Ab immunocytochemistry in C.

B-D-E) Intranasal delivery of hNGFp decreases Ab plaque load in 5xFAD mice and determines a strong neuroprotection (B) Scheme of intranasal delivery to 5xFAD mice. (D)

Immunohistochemistry for amyloid- β in cerebral cortex, hippocampus and subiculum and (E) quantification of amyloid- β plaque load in cerebral cortex (Cx), hippocampus (HP) and subiculum (Sub.) after intranasal delivery of hNGFp. A surprisingly robust decrease of plaque load in different brain areas is observed.

Adapted from Capsoni et al (21017) BRAIN, 140: 201-217.

hNGFp neuroprotection is mediated by astroglial cells

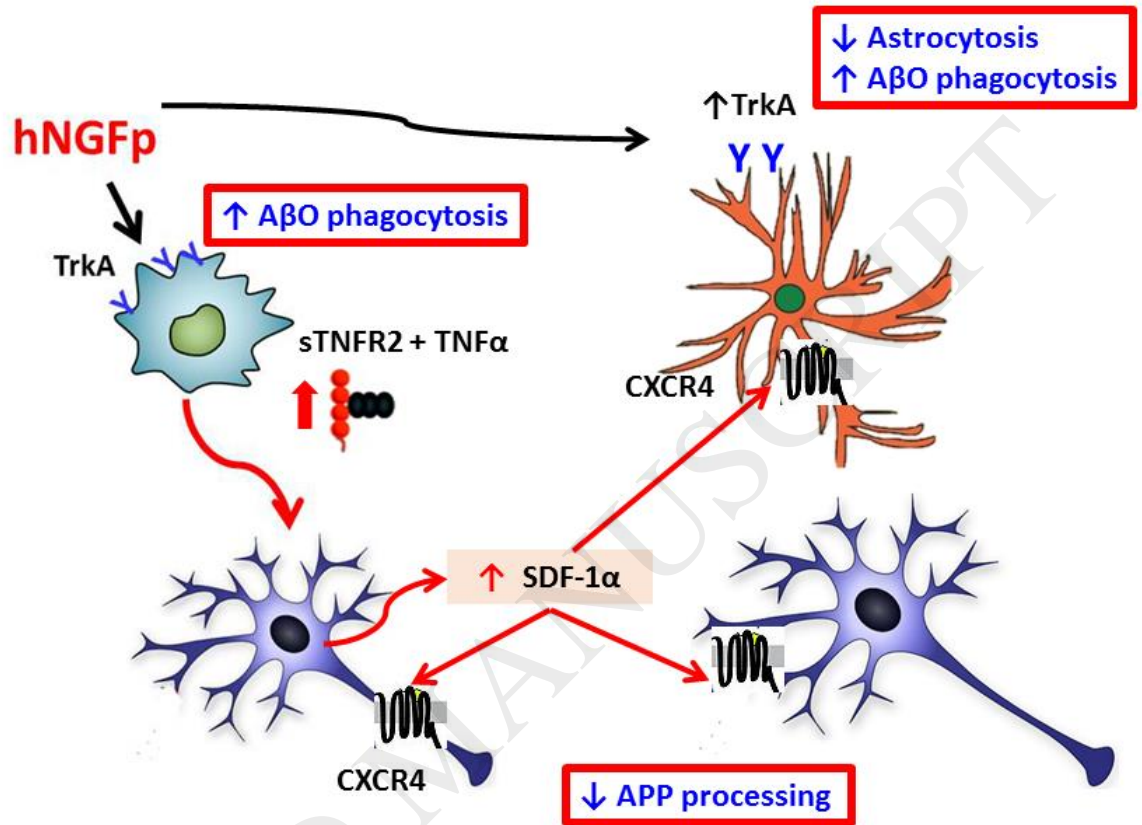


Figure 2 hNGFp exerts neuroprotective actions in 5xFAD mice via microglia and astrocytes.

Under neurodegenerative conditions microglia express TrkA. hNGFp acts on microglia as its primary cellular target in the brain, lowering the overall TNF α signaling by microglia, thereby inducing neuronal CXCL12. This chemokine would then be an obligatory mediator of the neuroprotective and anti-amyloidogenic actions of hNGFp, inhibiting APP pathological processing in neurons from 5xFAD mice and acting on astrocytes, making them responsive to hNGFp by increasing astrocyte TrkA receptor. In turn, microglia and astrocytes exposed to hNGFp increase uptake and degradation of A β forms.

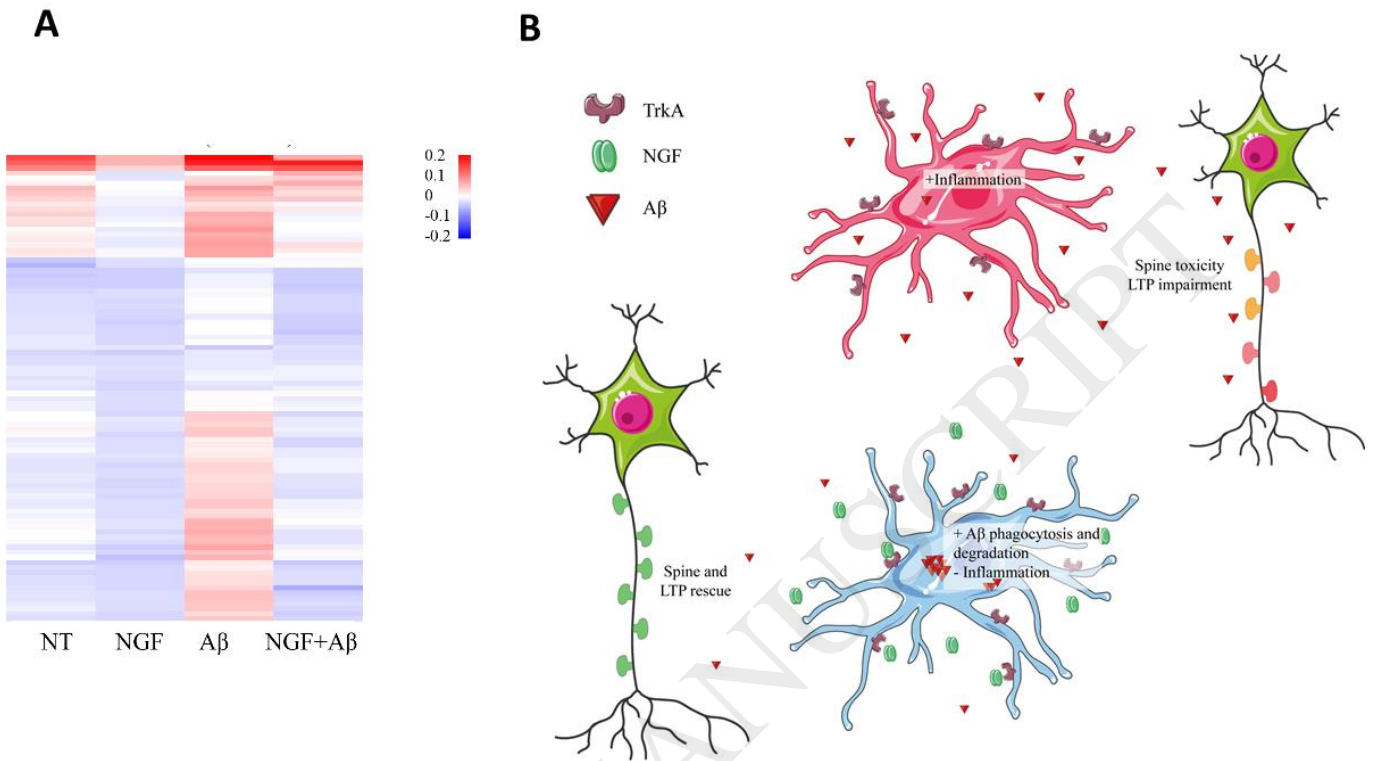


Figure 3 NGF steers microglia towards a neuroprotective phenotype

- A) Heat map of the cytokine profile from primary mouse microglia cultures treated with A β , with or without NGF. The proinflammatory transition induced in microglia by A β treatment is fully counteracted by the concomitant administration of NGF. Thus, NGF is very effective in reverting the pro-inflammatory state of microglia induced by A β , while it has only a moderate effect on the basal cytokine profile of naïve microglia cells.
- B) One prominent action of A β oligomers is to decrease spine density both in vitro and in vivo, and to impair synaptic LTP. NGF, by acting specifically on microglia cells, protects neurons from the A β induced loss of dendritic spines and from the A β induced inhibition of LTP (B). Indeed, these neuroprotective effects by NGF are not observed

when NGF is delivered directly to neurons, in the absence of microglia cells and are only seen when NGF and microglia are both added to the A β treated neuronal cultures (B).

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Restoration of neuronal homeostasis by hNGFp

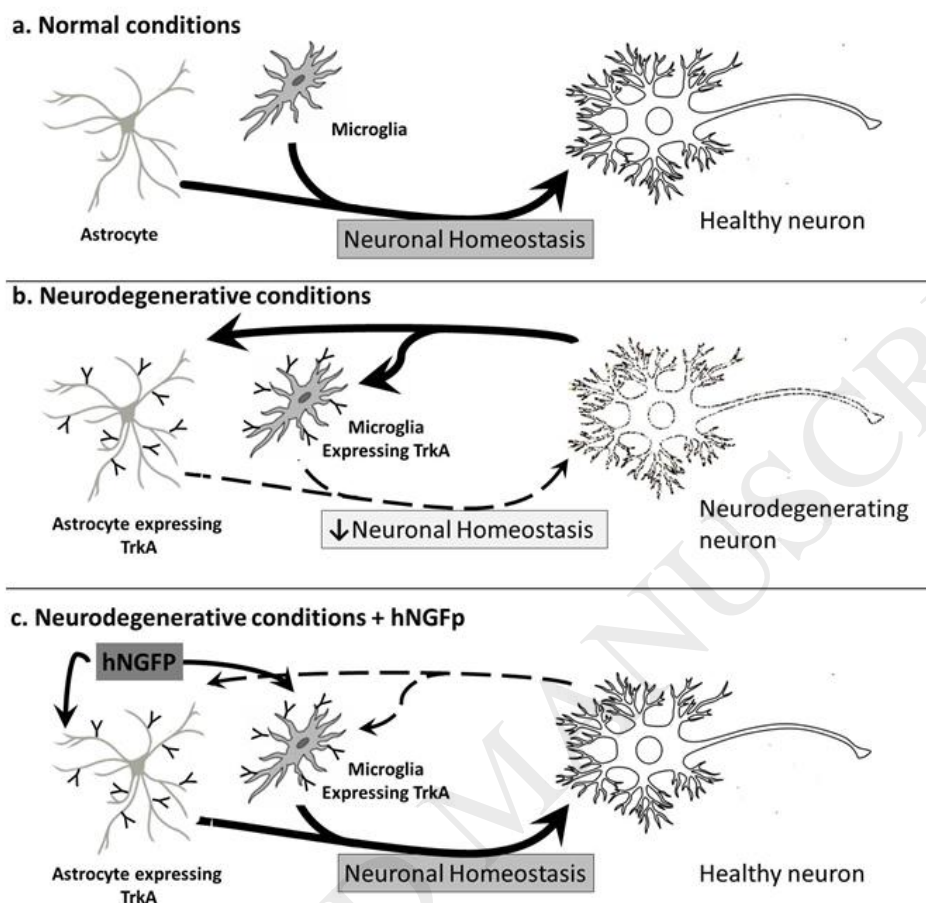


Figure 4

Scheme of proposed neuroprotective mechanism by hNGFp

Under normal conditions (a), astrocytes and microglia do not express TrkA receptors and contribute to maintain neuronal homeostasis and welfare. In the neurodegenerating brain (b), astrocytes and microglia start expressing TrkA and changing the p75NTR/TrkA ratio in favor of p75NTR, possibly as a protective reaction against neurodegeneration. After intranasal administration, hNGFp acts primarily on microglia and astrocytes (c), leading to a decreased availability of TNF α to neurons, that, possibly together with other undefined factors, in turn

mediate an increase of neuronal CXCL12 levels. This results, by as yet undefined mechanisms, in the downstream reduction of APP processing and broad neuroprotection and restoration of neuron-to-glia homeostasis.

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