Abstract: Human mutated form of NGF comprising two mutations, a first mutation being represented by the substitution of the proline amino acid in position 61 with a serine, a second mutation being represented by the substitution of an amino acid in any one of the positions 95-101, for simultaneous use as agent for the activation of the chemokine SDF-1alpha and as agent for the inhibition of the activity of the cytokine TNF alpha.

Figure I

**INHIBITOR OF ASTROCYTE TNF ALPHA FOR USE IN THE TREATMENT OF NEUROLOGICAL DISSEASES**

**Agents:** FIAMMENGHI, Eva et al; Via delle Quattro Fontane, 31, 1-00184 Roma (IT).


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"Inhibitor of astrocyte tnf alpha for use in the treatment of neurological diseases"

Description

Field of the art
The present invention refers to the medical and biological fields. More in detail the present invention refers to a known mutated form of NGF for a new use in the therapeutic and prophylaxis fields.

State of the art
Neuroinflammation is an aspect of neurodegeneration which, in various neurological diseases, including Alzheimer's disease, has become a target for the development of new therapies (Frank-Cannon et al 2009; Latta et al., 2015). Neuroinflammation is mediated by glial lineage cells (astrocytes and microglia). Among the different mediators of the immune response, and of neuroinflammation, SDF-lalpha (CXCL12) has received great interest; this is a member of the chemokine family CXC and the only physiological ligand of the receptor CXCR4 (Guyon, 2014). In the central nervous system, SDF-lalpha transcripts are mainly expressed in oligodendrocytes, astrocytes and neurons of the cortex, hippocampus and cerebellum (Stumm et al., 2007). Various studies have demonstrated that SDF-1 promotes neurogenesis and plays an important role in processes of synaptic plasticity, since it promotes and regulates the release of glutamate and GABA (Guyon, 2014).

Regarding neurodegeneration, it was demonstrated that SDF-lalpha promotes the remyelination in multiple sclerosis models (Patel et al., 2010) and the recruitment of neuronal stem cells in cerebral ischemia models (Imitola et al., 2004, Wang et al., 2008). In the case of Alzheimer's disease, the levels of SDF-lalpha are diminished in patients (Parachikova and Cotman, 2007, Zhang et al., 2012) and in transgenic mice Tg2576 which express the human form of APP with the Swedish mutation (Parachikova and Cotman, 2007). In non-transgenic mice, the chronic treatment with the antagonist AMD3100 of the
receptor CXCR4 causes learning and memory deficits (Parachikova and Cotman, 2007).
The pre-treatment with SDF-1 alpha in cell cultures protects the neurons from the apoptosis
induced by amyloid beta (Raman et al., 2011). In vivo, the administration of SDF-lalpha
inhibits the decrease of the length of the dendrites and spines induced by amyloid beta in the
hippocampus (Raman et al., 2011). All these studies suggest that an increase of the
concentration of SDF-lalpha could be a potential therapeutic agent for treating Alzheimer's
disease and other neurodegenerative and neurological pathologies. (SDF-lalpha Patent). The
document WO2007/051785 in fact describes the use of SDF 1 or of an agonist thereof, and
in particular the use of SDF-lalpha for producing a medication for the treatment and/or
prevention of neurological diseases, even if some diseases like Alzheimer's disease are not
expressly claimed.
However, SDF-lalpha is a pleiotropic molecule, with numerous contrasting actions over
various systems, including the immune system. SDF-lalpha has numerous pleiotropic
effects, also at the level of the central nervous system. In particular, SDF-lalpha is a
chemoattractant for lymphocytes T and B, monocytes and neutrophils during the immune
response to pathogenic agents (Bleul et al., 1996, Petty et al., 2007). For the purpose of a
possible therapeutic use of SDF-lalpha and of its receptor CXCR4 in neurological and
neurodegenerative pathologies, this action can have damaging effects, attracting cells of
inflammatory and immune nature into a neuronal context already subjected to inflammatory
stress (neuroinflammation associated with neurodegeneration), as well as other contrasting
pleiotropic effects (Nayak et al 2014).
In addition, specifically in the context of the nervous system, SDF-lalpha increases neuronal
death, after bonding with the receptor CXCR4, due to an excessive release of glutamate by
astrocytes, mediated by the increased release of the cytokine TNF alpha (Bezzi et al., 2001).
In the central nervous system, SDF-lalpha, and its receptor CXCR4, are part of a glia-glia
and glia-neurons signaling pathway whose excessive activation is the cause of neurotoxicity
and neuronal death (Call and Bezzi 2010). The activation of the astrocyte receptor CXCR4
by its natural ligand SDF-lalpha (or CXCL12) activates a long and branched chain of
intracellular and extracellular events (including the release of the pro-inflammatory cytokine TNFalpha and prostaglandins) which lead to the increase of the release of glutamate by astrocytes (Cali and Bezzi 2010). Generally, it can be stated that an increase of the release of glutamate by the astrocytes can be beneficial, or damaging, depending on the level of synaptic glutamatergic transmission. Nevertheless, an increase of the pro-inflammatory cytokine TNF alpha is certainly damaging, even more so in a neuronal context already affected by neuroinflammation (Habbas et al 2015). Indeed, increased levels of the cytokine TNFalpha cause persistent synaptic alterations in the hippocampus and consequent grave cognitive deficits (Santello and Volterra 2012; Habbas et al 2015).

Hence, it can be included that there are two effects induced by the activation of the SDF1-alpha/CXCR4 pathway, with a certainly negative action represented by the induction of the astrocyte TNFalpha (Fig. 1).

For such purpose, the object of the present invention is to provide a local topical activator of the SDF-1 alpha pathway, and in particular activators of its expression or of its action, which allow exerting the positive effects of the chemokine SDF-1 alpha, but that do not have the side effects thereof such as the induction of chemotaxis of cells of the immune system, nor the induction of an excessive release of glutamate and consequent neuronal death, nor even the induction of the cytokine TNFalpha.

Before commencing the following detailed description, and for the purpose of a clearer comprehension thereof, it should be noted that a mutated form of the human NGF was characterized in a series of preceding studies: hNGFP61S/R100E (NGF painless hNGFp) (Covaceuszach et al 2010; Capsoni et al PlosOne 2011; Capsoni and Cattaneo Patent). The molecule hNGFp is a variant of the molecule of wild type NGF. The protein NGF, even if having neurotrophic properties of therapeutic relevance for various neurodegenerative and neurological pathologies, is negatively affected by the undesired property of having a powerful nociceptive, algogenic and pain sensitization action (Reference Apfel; Pezet and Macmahon 2006;). In order to overcome this problem, which limits the therapeutic actions of the wild type NGF, the molecule hNGFp was developed that is the object of the document
WO2008006893: mutations were introduced at the level of two amino acids in the sequence of the mature NGF. The first substitution, from proline 61 to serine, renders the mutated NGF detectable by means of ELISA assays, with regard to the endogenous NGF (Covaceuszach et al., 2009, Malerba et al., 2015). The second mutation is based on the mutation R100W of the NGF gene present in patients affected by sensory and autonomic neuropathy type V (Einarsdottir et al., 2004). These patients are affected by pain insensitivity (Einarsdottir et al., 2004). By introducing the mutation R100E, which has effects similar to the pathogenic mutation in terms of signal transduction, a double mutant hNGFP61S/R100E (hNGF painless, hNGFp) was obtained, characterized by identical neurotrophic properties but also by a capacity to induce pain that is 10 times less than that of wild type NGF (Malerba et al., 2015). With regard to its interactions with the membrane receptors, hNGFp is a selective agonist of the receptor TrkA, while it binds the receptor p75NTR with an affinity 100 times less than the wild type NGF (Covaceuszach et al. 2010). It is necessary to underline that according to the prior art, all the neurotrophic properties of wild type NGF are identically preserved by hNGFp, and only the capacity of nociceptive sensitization is 10 times less in NGFp than in wild type NGF. The object of the present invention is therefore to promote the activation of the SDF-1-alpha pathway, simultaneously avoiding the side effects associated with this chemokine. This occurs by means of particular administrations of hNGFp.

Description of the invention

The present description refers to a new use of a known mutated human form of NGF. Said mutated human form will be indicated, in the course of the present description, as hNGFp. More in detail, by hNGFp it is intended a human mutated form of NGF comprising two mutations with respect to the form of the wild type NGF. The main mutation is the aforesaid mutation R100E, which confers to the mutated form the property of not activating the pain sensitization pathways (the indication "p" refers to the "painless" condition), but being equally neurotrophic. The other mutation, P61S, does not at all alter any of the known
properties of the wild type NGF; it is therefore "neutral" from the biological standpoint, but renders the molecule hNGFp recognizable by the endogenous molecules of NGF, by means of a specific immunoassay which was suitably developed. More clearly, this allows distinguishing the forms of therapeutic NGF from the endogenous forms. Since the doses of endogenous NGF vary from person to person, and in the same individual in accordance with many factors, stress and approach, the possibility of specifically dosing therapeutic NGF with respect to endogenous NGF is a very advantageous condition.

Still more in detail, the two mutations regard: the first: the substitution of the proline amino acid in position 61, in particular the substitution of the proline 61 with a serine; the second: the substitution of an amino acid in any one of the positions 95-101. By way of a non-limiting example, the substitution of the arginine in position 100. Preferably, said arginine is substituted with tryptophan or with an amino acid selected between glutamic acid and aspartic acid.

More in detail, the present description will refer to the sequences hNGFP61SR100E and hNGFP61SR100W, regarding which the following accession numbers are reported:

BankItl936238  hNGFP61SR100E   KX548900
BankItl936247  hproNGFP61SR100W   KX548901

More specifically, the object of the present industrial invention patent application is that of describing and claiming hNGFp (object of said prior art document (WO2008/006893)) for simultaneous use as agent for the activation of the SDF-1 alpha pathway and as agent for the inhibition of the activity of astrocyte TNF alpha. The object of the present invention is also to claim hNGFp for use in preventing Alzheimer's disease as well as for use in treating said Alzheimer's disease. Such uses, and specifically that relative to the treatment of Alzheimer's disease - even if already claimed in the aforesaid prior art document - in fact relates herein to a new technical effect encountered following the particular administration of hNGFp.

Another object of the present industrial invention patent application is to describe and claim a particular administration path for hNGFp in order to obtain said new technical effect. More specifically, the latter consists of attaining all the benefits deriving from the activation of the
pathway of the chemokine SDF-1 alpha - a chemokine known for its anti-amyloidogenic and anti-neurodegenerative activity - and from the inhibition of astrocyte TNF alpha whose presence mediates an excessive release of glutamate by the astrocytes, as well as other negative effects. More in detail said technical effect consists of promoting the neurogenesis and anti-amyloidogenic activity and of inhibiting the excessive and normally-encountered side effect of glutamate release. The technical effect is thus seen in the dual action of hNGFp which, as will be described hereinbelow, is unexpected and decidedly effective for the abovementioned uses in the prophylaxis and in the therapy of Alzheimer's disease.

More in detail, in the course of the studies pertaining to the present invention, it was found that the painless NGF (hNGFp) is a local activator of the SDF-1alpha pathway, and that through the activation of this pathway it exerts a powerful anti-amyloidogenic and anti-neurodegenerative action. Indeed, by blocking this pathway, with an inhibitor that blocks the interaction between SDF-1alpha and its receptor CXCR4, these positive effects of hNGFp are blocked. Nevertheless, surprisingly, and unexpectedly, it was found that an increased expression of SDF-1alpha by hNGFp does not induce an corresponding increase of the levels, nor of the activity of the proinflammatory cytokine TNFalpha, an event that would be undesired and counter-productive. This is doubly unexpected, since i) SDF-1alpha activates the production of astrocyte TNF-alpha and ii) NGF wild type is a powerful inducer of the expression of TNFalpha (Barouch et al 2001; Takei and Laskey 2008). Hence, the property of not inducing TNF alpha is a new unexpected property of hNGFp, that could not be predicted beforehand, and useful in light of that stated above.

**Detailed description of the invention**

The invention will be described in detail hereinbelow with reference to the enclosed figures, in which:

FIGURE 1 shows: a scheme of the negative effects of SDF-1alpha on astrocytes due to the excessive production of TNFalpha and glutamate. These effects lead to neuronal death.

FIGURE 2 shows: (A) the immunohistochemistry for amyloid beta Aβ in the cerebral cortex,
hippocampus and subiculum of 5xFAD mice after intranasal administration of hNGFp, (B) the quantification of the quantity of amyloid beta plaques in the cerebral cortex, hippocampus and subiculum after intranasal treatment with hNGFP (the bars of the histograms are representative of mean ± standard error, n = 8 per group, one-way analysis of the variance (ANOVA), Bonferroni post-hoc test, P < 0.001).

FIGURE 3 shows: hNGFp promotes the reduction of the pro-amyloidogenic processing of APR Western blots for (a) PS1, anterior pharynx 1a (APh1) and Pen-2, (b) Nicastrin (NCT), (c) BACE1 (d) Neprilysin (NEP) (e) full length APP (fAPP), (f) c-terminal fragments and (h) insoluble Aβ. The images are representative of 3 independent experiments, (g) Dot blot for verifying the expression of the amyloid beta oligomers (the bars of the histograms are representative of mean ± standard error, one-way analysis of the variance (ANOVA), Bonferroni post-hoc test, P < 0.001).

FIGURE 4 shows: (A) Increase of the cholinergic innervation after intranasal administration of hBDNF. (B) Lack of decrease of the number of amyloid beta plaques after intranasal administration of hBDNF (one-way analysis of the variance (ANOVA), n = 8 per group, Bonferroni post-hoc test, P < 0.001).

FIGURE 5 shows: in the 5xFAD mice, the cortical neurons which produce amyloid beta (in a) do not express the receptor for NGF TrkA. Markers for the neurofilament M and for TrkA.

FIGURE 6 shows: scheme of the intraparenchymal injection site of hNGFp.

FIGURE 7 shows: (A) immunohistochemistry for the choline acetyltransferase (ChAT) in the cholinergic nucleus basalis of Meynert and for amyloid beta Aβ in the cerebral cortex of the 5xFAD mice after intraparenchymal administration of hNGFp. (B) Quantification of the cholinergic fibers and of the quantity of amyloid beta plaques in the cerebral cortex after intraparenchymal treatment with hNGFP (the bars of the histograms are representative of mean ± standard error, n = 4 per group, one-way analysis of the variance (ANOVA), Bonferroni, P < 0.001).

FIGURE 8 shows: (a) Confocal images and quantification of the co-localization of p75NTR and TrkA in the microglia cells (n = 8). (b) Confocal images and quantification of the co-
localization of p75NTR and TrkA in the astrocytes (n = 5). (c) Confocal images for showing
the microglial marker IBA-1 and quantification of the number of microglial cells, of the area
occupied by these cells and of the number of ramifications (non-transgenic mice n = 71 cells;
5xFAD mice treated with PBS n = 99 and 5xFAD mice treated with hNGFp n = 61). (d)
Confocal images for showing the astrocyte marker GFAP and quantification of the volume of
astrocytes (n = 20 cells per treatment group). (e) Confocal images and co-localization of
IBA-1, oligomers of amyloid beta and total amyloid beta in the microglia (n = 7 cells per
group). (f) Confocal images and co-localization of GFAP, oligomers of amyloid beta and of
total amyloid beta in the astrocytes (n = 7 cells per group) (* P < 0.05, ** P < 0.001, *** P<
0.0001).

FIGURE 9: shows the expression of the receptors for NGF in the cortex of Alzheimer (AD)
patients and non-demented patients (CTRL). (A) TrkA and (B) p75 in microglia marked with
an antibody against IBA-1. (C) TrkA and (D) P75 in astrocytes marked with an antibody
directed against GFAP.

FIGURE 10 shows: increased phagocytosis of the oligomers of amyloid beta by the microglia
in the presence of hNGFp and not of hwild type NGF.

FIGURE 11 shows: increased phagocytosis of the amyloid beta oligomers by the astrocytes in
the presence of hNGFp.

FIGURE 12 shows: Quantification of the immunoblot used for the analysis of the
inflammatory markers, (one-way analysis of the variance (ANOVA), n = 4 per group,
Bonferroni post-hoc test, P < 0.05).

FIGURE 13 shows: Confocal images and quantification of the co-localization of amyloid
beta, NF-M and SDF-1α in cortical neurons (one-way analysis of the variance (ANOVA), n =
9 per group, Bonferroni post-hoc test, P < 0.0001).

FIGURE 14 shows: (A) Quantification of the percentage of neurons that express SDF-lalpha
after treatment with hwild type NGF; (B) Orofacial test for measuring the mechanical
alldynia in the region of the nose after administration of increasing doses hNGFp or hwild
type NGF (n = 10 per group, one-way analysis of the variance (ANOVA), Bonferroni post-
hoc test, *P < 0.001).

FIGURE 15 shows: decrease of the expression of SDF-1alpha in cortical neurons after incubation with 50ng/ml of TNFalpha.

FIGURE 16 shows: the administration of the inhibitor of the receptor CXCR4 of SDF-1alpha prevents the improvement of the neurodegenerative phenotype determined by the intranasal administration of hNGFp (a) Scheme of the treatment (b) Test of the Y maze for evaluating the spatial memory (n = 10; one-way ANOVA, Bonferroni post-hoc test, * P < 0.05, ** P < 0.001). (c) Immunohistochemistry for amyloid beta in the cerebral cortex and quantification of the number of plaques (d) Western blots for presenilin 1, BACE 1, full length APP and C-terminal fragments, (e) Dot blot for the quantification of the oligomers of amyloid beta.

FIGURE 17 shows: the co-incubation of hNGFp with the inhibitor AMD3100 does not affect the decrease of oligomers in the microglia (A) and the decrease of non-oligomer Abeta in the astrocytes (B) observed after treatment with hNGFp on its own.

FIGURE 18 shows: (A-C) recovers the neurogenesis in Down syndrome model mice after administration of hNGFp. (D) quantification of the recovery of the neurogenesis deficit.

FIGURE 19 shows: (A) quantification of the volume of astrocytes in Down syndrome model mice after treatment with hNGFp. (B) Western blot for GFAP and (C) quantification of the GFAP level reduction after treatment with hNGFp.

FIGURE 20 shows: (A) after intranasal administration, hNGFp determines the recovery of the memory deficit in recognizing objects; (B) after subcutaneous administration, hNGFp determines the recovery of the sensitivity to painful stimulus of thermal type.

**Experimental data:**

hNGFp was administered intranasally with a very aggressive murine model of Alzheimer's disease, the 5xFAD mouse (Oakley et al., 2006), which has 5 gene mutations corresponding to the same number of mutations that lead to familiar forms of Alzheimer's disease. After
intranasal delivery of hNGFp, a powerful anti-amyloidogenic action is observed at the dose of 0.54 μg/kg (Fig. 2), an action characterized by a dramatic reduction of the number of amyloid beta plaques (Fig. 2). The mechanism of this anti-amyloidogenic action of hNGFp is determined by the fact that this neurotrophin diminishes the expression of presenilin 1 (Fig. 3a), nicastrin (Fig. 3b) and BACE1 (Fig. 3), i.e. of proteins of the enzymatic complexes that cut the precursor protein of the amyloid APP, forming the amyloid beta peptide (Fig. 3d, e). The result of this action is a lower production both of oligomers of the amyloid beta peptide (Fig. 3g) and of amyloid beta aggregates (Fig. 3f). The expression of degradation of amyloid beta, neprilysin, is instead not significantly varied (Fig. 3d).

Surprisingly, this action of the hNGFp is specific for this neurotrophin. Indeed, the administration of doses up to 100 times greater of the neurotrophin hBDNF, very similar to NGF (and hence also to hNGFp), determine an increase of the cholinergic innervation (Fig. 4A) but does not decrease the number of amyloid beta plaques (Fig. 4B). Hence, the action of hNGFp is not generically neuroprotective, but involves a specific mechanism that leads to the reduction of the formation of amyloid beta peptide.

This very effective anti-amyloidogenic and anti-neurodegenerative action of hNGFp poses a question: which are the cells of the brain that mediate the actions of hNGFp? In fact, the neurons that produce amyloid beta do not express TrkA (Fig. 5), the main receptor for hNGFp (Covaceuszach et al., 2010). The only cell population in the CNS that is known and validated as target of the NGF action (Rosenberg et al., 1988, Smith et al., 1999, Tuszynski, 2000, Salehi et al., 2006, Nagahara et al., 2009) is constituted by the cholinergic neurons of the basal forebrain, which innervate all the cortical areas in a distributed and diffused manner. Other neuronal populations, or other cell types (microglia and astrocytes) do not express TrkA receptors in the brain in physiological conditions (Table 1).

**Table 1:**

Modulation of the expression of the receptors p75NTR and TrkA in the microglia and in the astrocytes after treatment with hNGFp.
In order to verify if the activation of the cholinergic neurons by the hNGFp is responsible for the observed anti-amyloidogenic effects, we have administered hNGFp via intraparenchymal administration by means of osmotic minipumps, which release the neurotrophin directly in proximity to the cholinergic neurons of the basal forebrain (Fig. 6). The hNGFp was administered at the same dose used intranasally (0.54 µg/kg). We have observed that the hNGFp determines, as expected, the increase of the cholinergic innervation (Fig. 7A,B), but surprisingly does not determine a reduction of the number of amyloid beta plaques (Fig. 7A,B) and results entirely ineffective after this localized administration. It follows that i) the action of the hNGFp requires a diffused biodistribution and that ii) surprisingly and unexpectedly the anti-amyloidogenic action of the hNGFp is not mediated by the cholinergic neurons but rather by the cells of the microglia and by the astrocytes. Indeed, contrary to what occurs in physiological conditions (Table 1), the microglia cells, in neurodegeneration conditions, express both the receptors TrkA and p75NTR (Fig. 8a and Table 1). In the same conditions, the astrocytes express only p75NTR (Fig. 8b and Table 1). Therefore, it can be concluded that in neurodegeneration and neuroinflammation conditions, microglial and astrocyte cells acquire the capacity to respond to hNGFp.

The expression of these receptors is modulated by the hNGFp itself (Table 1): following the treatment with hNGFp, the expression of p75NTR is decreased in both cell types, while the expression of TrkA remains constant in the microglia and increases in the astrocytes.

In these cells, the intranasal administration of hNGFp to 5xFAD mice determines:
(1) in the microglia cells: a decrease of the number, of their area and an increase of the number of ramifications, with respect to that detected in the brains of the control 5xFAD mice, treated with PBS (Fig. 8c). In addition, a reduction is observed of the quantity of immunoreactive cells for the different species of amyloid beta and in particular of oligomers (Fig. 8e).

(2) in the astrocytes: a decrease of the volume of the single cells, signifying a decrease of astrocytosis (Fig. 8d). In addition, also for the astrocytes, a reduction is observed of the quantity of immunoreactive cells for the different species of amyloid beta, and in particular of oligomers (Fig. 8f).

The expression of the receptors for NGF was also verified post-mortem in parietal cortex tissues of Alzheimer patients. In this cerebral area, the expression of the receptors TrkA and p75 results increased in the microglia (Fig. 9A, B) and in the astrocytes (Fig. 9C, D). Therefore, also in the human cerebral tissue affected by Alzheimer's disease, the astrocyte and microglial cells become responsive to NGF, by means of expression of the corresponding receptors (which do not express in physiological conditions in vivo). Such cells therefore become mediators of the specific actions of hNGFp.

In cultured cells, the treatment with hNGFp determines an increase of the phagocytosis of the oligomers of amyloid beta by cultured microglial cells (Fig. 10) and by cultured astrocytes (Fig. 11). In an unexpected manner, and not expected based on the known or predictable properties of hNGFp, hNGFp is more effective than the wild type NGF in inducing phagocytosis of amyloid beta oligomers by the microglial (Fig. 10).

These results demonstrate that the treatment with hNGFp determines an increase of the capacity to remove pathological forms of the amyloid beta peptide. The verification of this event poses the question: how can the action of hNGFp on the glial cells also determine the observed reduction of the production of amyloid beta in the neurons (Fig. 2)?

The microglia can be considered the primary cellular target of hNGFp in the brain of the 5xFAD mice, since: (1) hNGFp is bonded nearly exclusively to the receptor TrkA and does not effectively bond the other receptor p75NTR (Covaceuszach et al., 2010); (2) the
microglia is the only non-neuronal cell type that expresses TrkA, in the brain of the 5xFAD mice that are not treated or treated with PBS. Since one of the functions of the microglia is that of secreting soluble factors that act in an autocrine and paracrine manner (Nayak et al 2014), it is assumed that a soluble factor can be secreted by the microglia and decrease the production of Abeta.

In order to identify this factor, an array of antibodies was used that allows identifying the main cytokines and chemokines modulated during the inflammation states. Among those analyzed, 5 factors are modulated by hNGFp:

1. The expression of the soluble receptor 2 of TNFalpha (sTNFRII), and of the chemokines MIP-lalpha (CCL3), MIP-lgamma (CCL9) and SDF-lalpha (CxCL12) is increased (Fig. 12 and Table 2).

2. The expression of the cytokine IL-lalpha is decreased (Fig. 12 and Table 2).

**Table 2:**

<p>| Modulation of inflammatory cytokines and chemokines in extracts of 5xFAD mice after treatment with hNGFp |</p>
<table>
<thead>
<tr>
<th>Marker</th>
<th>WT + PBS mean + SEM</th>
<th>5xFAD + PBS mean + SEM</th>
<th>5xFAD + hNGFp mean + SEM</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eotaxin</td>
<td>0.01 ± 0.013</td>
<td>0.03 ± 0.014</td>
<td>0.02 ± 0.010</td>
<td>&gt; 0.05</td>
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<tr>
<td>Eotaxin-2</td>
<td>0.12 ± 0.015</td>
<td>0.07 ± 0.024</td>
<td>0.09 ± 0.030</td>
<td>&gt; 0.05</td>
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<td>Fas Ligand</td>
<td>0.02 ± 0.008</td>
<td>0.06 ± 0.011</td>
<td>0.04 ± 0.007</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>Fractalkine</td>
<td>0.004 ± 0.007</td>
<td>0.04 ± 0.008</td>
<td>0.05 ± 0.016</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>GCSF*</td>
<td>0.00 ± 0.002</td>
<td>0.06 ± 0.016</td>
<td>0.05 ± 0.011</td>
<td>0.009</td>
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<tr>
<td>GM-CSF</td>
<td>0.01 ± 0.002</td>
<td>0.07 ± 0.005</td>
<td>0.08 ± 0.010</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>IFN-γ*</td>
<td>0.02 ± 0.008</td>
<td>0.06 ± 0.005</td>
<td>0.06 ± 0.007</td>
<td>0.002</td>
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<tr>
<td>IL-1β**</td>
<td>0.11 ± 0.012</td>
<td>0.25 ± 0.013</td>
<td>0.15 ± 0.026</td>
<td>0.0012</td>
</tr>
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<td>IL-1β</td>
<td>0.04 ± 0.017</td>
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<td>0.04 ± 0.013</td>
<td>&gt; 0.05</td>
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<tr>
<td>IL-2</td>
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<td>&gt; 0.05</td>
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<tr>
<td>IL-4</td>
<td>0.05 ± 0.001</td>
<td>0.08 ± 0.006</td>
<td>0.08 ± 0.011</td>
<td>0.006</td>
</tr>
<tr>
<td>IL-6</td>
<td>0.01 ± 0.002</td>
<td>0.03 ± 0.010</td>
<td>0.03 ± 0.008</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>IL-9</td>
<td>0.05 ± 0.018</td>
<td>0.04 ± 0.013</td>
<td>0.07 ± 0.015</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>IL-10</td>
<td>0.02 ± 0.010</td>
<td>0.03 ± 0.012</td>
<td>0.04 ± 0.017</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>IL-12p40p70</td>
<td>0.01 ± 0.012</td>
<td>0.03 ± 0.015</td>
<td>0.05 ± 0.002</td>
<td>&gt; 0.05</td>
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<td>IL-12p70*</td>
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<td>IL-13</td>
<td>0.03 ± 0.008</td>
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<td>&gt; 0.05</td>
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<td>IL-17</td>
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<td>0.02 ± 0.014</td>
<td>0.04 ± 0.006</td>
<td>&gt; 0.05</td>
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<td>I-TAC</td>
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<td>0.03 ± 0.019</td>
<td>0.04 ± 0.013</td>
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<td>KC</td>
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<td>0.06 ± 0.017</td>
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<td>Leptin</td>
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<td>LIX*</td>
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<td>0.29 ± 0.027</td>
<td>0.31 ± 0.051</td>
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<td>Lymphotactin</td>
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<td>0.25 ± 0.041</td>
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<td>0.06 ± 0.009</td>
<td>0.09 ± 0.009</td>
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<td>M-CSF</td>
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<td>0.16 ± 0.056</td>
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<td>MIG</td>
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<td>0.02 ± 0.017</td>
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<td>MIP-1α**</td>
<td>0.03 ± 0.002</td>
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<td>MIP-1γ**</td>
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<td>0.03 ± 0.016</td>
<td>0.11 ± 0.023</td>
<td>0.019</td>
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<td>Rantes</td>
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<td>SDF-1**</td>
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<td>0.04 ± 0.016</td>
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<td>0.27 ± 0.064</td>
<td>0.28 ± 0.021</td>
<td>&gt; 0.05</td>
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<td>TECK</td>
<td>0.06 ± 0.006</td>
<td>0.04 ± 0.008</td>
<td>0.05 ± 0.008</td>
<td>&gt; 0.05</td>
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<tr>
<td>TIMP-1</td>
<td>0.13 ± 0.004</td>
<td>0.16 ± 0.005</td>
<td>0.16 ± 0.021</td>
<td>&gt; 0.05</td>
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<tr>
<td>TIMP-2</td>
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<td>0.05 ± 0.029</td>
<td>0.03 ± 0.008</td>
<td>&gt; 0.05</td>
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<td>TNFa***</td>
<td>0.02 ± 0.008</td>
<td>0.03 ± 0.006</td>
<td>0.02 ± 0.008</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>sTNFR1</td>
<td>0.03 ± 0.010</td>
<td>0.03 ± 0.002</td>
<td>0.03 ± 0.006</td>
<td>&gt; 0.05</td>
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<tr>
<td>sTNFR2**</td>
<td>0.12 ± 0.007</td>
<td>0.05 ± 0.009</td>
<td>0.09 ± 0.01</td>
<td>0.005</td>
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</table>

* = markers modulated by hNGFp with respect to the non-transgenic mice

** = markers which are modulated by hNGFp with respect to 5xFAD mice treated with PBS

*** = TNFalpha

In addition, by means of immunohistochemistry, it is observed that the expression of SPF-lalpha is specifically increased in the neurons that produce amyloid Abeta (Fig. 13).
Surprisingly, an analogous treatment with hNGF wildtype at the same dose of hNGFp (0.54 µg/kg) does not determine an increase of SDF-lalpha (Fig. 14A). This induction can only be obtained at very high doses (da 1 µg/Kg), which have as negative effect the activation of the nociceptors of the trigeminal nerve and the consequent induction of pain in the orofacial region (Fig. 14B).

It is particularly significant that among the cytokines that are instead not modulated by hNGFp, there is TNFalpha. This is surprising and unexpected, i) since it is known that the wild type NGF is, on the contrary, a powerful inducer of TNFalpha (Barouch et al 2001; Takei and Laskey 2008), ii) and in light of the fact that hNGFp instead induces SDF-lalpha (Fig. 12 and Table 2), which in turn is a known inducer and activator of TNFalpha (see above). Hence, the property of not inducing TNF alpha is a new unexpected property of hNGFp, not predictable beforehand, and useful in light of that stated above. In addition, the fact that hNGFp instead stimulates the production of the soluble receptor 2 of TNFalpha (sTNFRII) (a known decoy inhibitor of the TNFalpha action) contributes to ensuring that the final result of the treatment with hNGFp is that of reducing the general activity of TNFalpha.

Since it is known that in tissues of non-neuronal origin an increase of TNFalpha causes a decrease of the expression of SDF-lalpha (Zhang et al., 2008, Bockstal et al., 2011), the reduced tone of TNFalpha correlates with the observed increase of SDF-lalpha (Fig. 12 and Table 2). It is verified that this relation also existed in cultured neurons, where it is confirmed that TNFalpha reduces the expression of SDF-lalpha (Fig. 15).

Overall, all these results propose the possibility that, surprisingly and unexpectedly, the neuroprotective, anti-amyloidogenic and anti-neurodegenerative actions of hNGFp are mediated by the chemkine SDF-lalpha, without however there being the onset of undesired involvement of the cytokine TNFalpha, which normally is in the cascade activated by SDF-lalpha.

In order to demonstrate this assumption, thus passing from a correlation to a demonstration, in the course of the experiments pertaining to the present invention a known inhibitor of the receptor CXCR4 of the chemokine SDF-lalpha was used, AMD3100. The co-treatment of
the 5xFAD mice with hNGFp (0.54 µg/kg) and AMD3100 (1 mg/kg twice per day) completely blocks the anti-amyloidogenic action of hNGFp, since the decrease of the number of amyloid beta plaques is no longer observed (Fig. 16a,c), nor is the reduction observed of the expression of presenilin 1, nicastrin and BACE1 and of the oligomers of Abeta (Fig. 16d,e) which would have been obtained following treatment with hNGFp on its own.

Unexpectedly, not all the parameters measured in the microglia and in the astrocytes and improved after intranasal administration of hNGFp are blocked by the co-administration of AMD3100.

Table 3:

Modulation of the expression of the receptors p75NTR and TrkA in the microglia and in the astrocytes after treatment with hNGFp.

<table>
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<th>Cell type</th>
<th>hNGFp</th>
<th>block after co-treatment with AMD3100</th>
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<tr>
<td>Abeta deposition diminution</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>APP processing diminution</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Diminution of Abeta oligomers in neurons</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Diminution of spatial memory deficit</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Increased expression of TrkA in astrocytes</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Diminution of Abeta oligomers in microglia</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Diminution of Abeta oligomers in astrocytes</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Diminution of non-oligomeric Abeta in microglia</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Diminution of non-oligomeric Abeta in astrocytes</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

Indeed, the reduction of the immunoreactivity for the Abeta oligomers in the microglia and
of the immunoreactivity for non-oligomer forms of Abeta in the astrocytes, by the hNGFP, is not affected by the simultaneous administration of the inhibitor AMD3 100 (Fig. 17). This demonstrates that hNGFP is an activator of SDF-1 alpha and that it selectively activates most, but not all, the pathways activated by this chemokine, showing - in a manner that absolutely could not have been predicted beforehand - a more favorable action spectrum. The anti-amyloidogenic effect of hNGFP must necessarily be mediated by the cells of the glia and not by the cholinergic neurons. Microglia and astrocytes are thus unexpectedly a new target of hNGFP, selectively mediated by the chemokine SDF-1 alpha. This extends the possibility of therapeutic use thereof to other clinical indications. As evidence thereof, the effectiveness of hNGFP was verified in improving some deficits observed in the model of the Down syndrome, Ts65Dn (Wisniewski et al, 1984; Colombo et al., 2005). Among these, deficits of neurogenesis and alteration of the morphology of the astrocytes. Following intranasal treatment of hNGFP in the Ts65Dn mice, a rescue of the neurogenesis deficits was verified (Fig. 18) along with a return of the volume of the astrocytes by hNGFP to the control levels (Fig. 19), similar to that observed in the model AD, and an accompanying reduction of the expression of the fibrillary acidic protein (GFAP) (Fig. 19).

The Ts65Dn mice also have other aspects of the Down syndrome pathology, including learning and memory deficits and many other typical signs of Alzheimer's neuropathology, including an increase of the peptide Ab, starting from the age of 6 months. It is therefore of interest to verify if the treatment with hNGFP is also capable of improving these aspects of neurodegeneration in the Ts65Dn mice. Ts65Dn mice of 6 months age were treated for three weeks with the same dose of hNGFP, and the expression of the peptide Ab was observed via immunohistochemistry with the antibody 4G8 anti Ab, demonstrating a considerable reduction of the expression of this peptide in the brain. The levels of the peptide Ab, after the intranasal treatment with hNGFP, were measured in the brain of the Ts65Dn mice, by means of ELISA and Western blot, also demonstrating in this case a considerable reduction of the peptide Ab. At the behavioral level, the reduction of the peptide Ab correlates with the observation of a recovery of the behavioral memory deficit, present in these mice, measured
with the Y maze protocol. In conclusion, the intranasal administration of hNGFp to Ts65Dn mice significantly improves numerous aspects of the deficits observed in these mice, and of the neuropathology of Alzheimer type, characteristic of Down syndrome.

The most generalized neuroprotective effect of hNGFp is also shown in animal models of a rare human disease, the sensory and autonomic neuropathy type IV (also termed HSAN IV). This rare disease is due to mutations of the gene that encodes the receptor TrkA (Indo et al., 2014). The mutations HSAN IV render the receptor TrkA hypofunctional and determine a decreased sensitivity to painful stimuli and learning and memory deficits. The functionality of the receptor p75NTR is however unaltered in such pathology. The supplementation with hNGFp, used in place of the h wild type NGF as preferred agonist of the receptor TrkA, with respect to p75NTR, without however activating the signaling through the receptor p75NTR in an undesired manner, determined: 1) after intranasal administration for a month, the recovery of the cognitive deficits, as demonstrated by the object discrimination test in Fig. 20 A, and 2) the recovery of pain sensitivity, as demonstrated by the hot plate test in Fig. 20 B.

In light of the aforesaid experimental data, pharmaceutical compositions were prepared comprising hNGFp and excipients and/or carriers pharmaceutically acceptable for use in the aforesaid treatments were prepared and in particular: for use in a prophylaxis method for Alzheimer's disease and in a therapeutic treatment method for said disease which are such to allow a diffused biodistribution of said composition at the nervous system level such that the glia and microglia cells can mediate the anti-amyloidogenic action of hNGFp. Such pharmaceutical compositions can be administered, by way of a non-limiting example, intranasally.
Bibliography


Claims

1. Human mutated form of NGF comprising two mutations, a first mutation being represented by the substitution of the proline amino acid in position 61 with a serine, a second mutation being represented by the substitution of an amino acid in any one of the positions 95-101, for simultaneous use as agent for the activation of the chemokine SDF-1alpha and as agent for the inhibition of the activity of the cytokine TNF alpha.

2. Human mutated form of NGF comprising two mutations, a first mutation being represented by the substitution of the proline amino acid in position 61 with a serine, a second mutation being represented by the substitution of an amino acid in any one of the positions 95-101, for simultaneous use as agent for the activation of the chemokines SDF-1alpha, MIP-1alpha and as agent for the inhibition of the activity of the cytokine TNF alpha.

3. Human mutated form of NGF comprising two mutations, a first mutation being represented by the substitution of the proline amino acid in position 61 with a serine, a second mutation being represented by the substitution of an amino acid in any one of the positions 95-101, for simultaneous use as agent for the activation of the chemokines SDF-1alpha, MIP-1alpha, MIP-lgamma and as agent for the inhibition of the activity of the cytokine TNF alpha.

4. Human mutated form of NGF according to the preceding claims wherein the second mutation regards the substitution of the arginine amino acid in position 100 with an amino acid selected in the group constituted by tryptophan and glutamic acid.

5. Human mutated form of NGF according to any one of the preceding claims for use in a treatment method for Alzheimer's disease for biodistribution diffused at the level of the
Nervous System.

6. Human mutated form of NGF as defined in claim 1, 4 for use in a prophylaxis method for Alzheimer's disease for biodistribution diffused at the level of the Nervous System.

7. Human mutated form of NGF as defined according to any one of the claims 1, 2 for use in a prophylaxis method and/or treatment method for Alzheimer's disease for biodistribution diffused at the level of the glia and microglia cells.

8. Pharmaceutical composition comprising a human mutated form of NGF as defined in any one of the claims 1, 2 comprising excipients and/or carriers pharmaceutically acceptable for use in a treatment method for Alzheimer's disease for biodistribution diffused at the level of the glia and microglia cells.

9. Pharmaceutical composition comprising a human mutated form of NGF as defined in any one of the claims 1, 2 comprising excipients and/or carriers pharmaceutically acceptable for use in a treatment method for Alzheimer's disease for biodistribution diffused at the level of the glia and microglia cells intranasally.

10. Pharmaceutical composition comprising a human mutated form of NGF as defined in any one of the claims 1, 2 comprising excipients and/or carriers pharmaceutically acceptable for use in a prophylaxis method for Alzheimer's disease for biodistribution diffused at the level of the glia and microglia cells.

11. Pharmaceutical composition comprising a human mutated form of NGF as defined in any one of the claims 1, 2 comprising excipients and/or carriers pharmaceutically acceptable for use in a prophylaxis method for Alzheimer's disease for biodistribution diffused at the level of the glia and microglia cells intranasally.
12. Human mutated form of NGF as defined in claim 1 or 2 for use in a treatment method for Down syndrome.

13. Human mutated form of NGF as defined in claim 1 or 2 for use in a treatment method for Sensory and Autonomic Neuropathy type IV.
Figure 1

SDF-1α

Astrocyte

Release of TNFα

Release of glutamate

Neuronal death
Figure 2

A

WT + PBS  5xFAD + PBS  5xFAD + hNGFp

Cx

HP

Sub.

B

WT + PBS

5xFAD + PBS

5xFAD + hNGFp 0.54 µg/kg/in.

Cortex

Hippocampus

Subiculum

N. Plaques/mm²

**  *
Figure 4

A

WT + PBS  5xFAD + PBS  5xFAD + hBDNF 54 μg/kg i.n.

5xFAD + PBS  5xFAD + BDNF

Number of cholinergic fibers/mm²

B

WT + PBS  5xFAD + PBS  5xFAD + hBDNF

N. Plaques/mm²

Figure 5

a

b

NF-M  TrkA  NF-M/TrkA
Figure 12

- WT + PBS
- 5xFAD + PBS
- 5xFAD + hNGFp 0.54 g/kg

Relative levels

- sTNFR2
- IL-α1
- MIP-1α
- MIP-1γ
- SDF-1α

** **
Figure 13

[Images of microscopic slides with annotations]

% SDF-1a IR neurons

---

**Significant difference**
Figure 15

Medium

Medium + TNFα
### A. CLASSIFICATION OF SUBJECT MATTER

INV. A61K38/00 A61K38/16 A61K38/17 A61K38/18 A61P25/00
ADD.

According to International Patent Classification (IPC) and national classification.

### B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols): A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched:

Electronic database consulted during the international search (name of database and, where practicable, search terms used):

**EPO-Internal, WPI Data, BIOSIS, EMBASE**

### C. DOCUMENTS CONSIDERED TO BE RELEVANT

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Date of the actual completion of the international search: 9 May 2017

Date of mailing of the international search report: 22/05/2017

Name and mailing address of the ISA:
European Patent Office, P.B. 5818 Patentlaan 2
NL-2280 HV Rijswijk
Tel. (+31-70) 340-2040,
Fax: (+31-70) 340-3016

Authorized officer: Zel Lner, Eveline

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