Title: MUTEINS OF hNGF, THERAPEUTIC USES AND PHARMACEUTICAL COMPOSITIONS

Abstract: The present invention relates to muteins of hNGF, their therapeutic uses and pharmaceutical compositions. In particular, the muteins of the invention are selectively recognised by a specific reagent with regard to endogenous hNGF. The muteins are obtained by at least one amino acid substitution residing in loop III of hNGF.
**Muteins of hNGF, therapeutic uses and pharmaceutical compositions**

**TECHNICAL FIELD OF THE INVENTION**

The present invention relates to muteins of hNGF, their therapeutic uses and pharmaceutical compositions. In particular, the muteins of the invention are distinguishable from endogenous hNGF and can be recognised by specific antibodies. In particular, the invention relates to mutated coding sequences with point mutation(s) that do(es) not constitute an attack site for nucleases. The resulting protein does not provoke undesired immune reactions when administered for therapeutic or diagnostic purposes.

As an example, the mutation introduced in hNGF is the substitution of the proline amino acid in position 61 with a serine and/or a mutation in residue position 100, that provides further advantages to the mutein, with respect to hNGF.

**STATE OF THE ART**

The human “nerve growth factor” (NGF, SWISS PROT No P01138) protein has numerous potential therapeutic applications with regard to the central and peripheral nervous system. NGF could be used for treating Alzheimer’s Disease (Tuszynski et al., 2005), multiple sclerosis (Villoslada et al., 2004; Caggiula et al., 2006, ), schizophrenia (Shoval et al., 2005), Rett’s Syndrome (Riikonen et al., 1999), cerebral ischemia (Semkova et al., 1999), peripheral neuronal azotemia (Terenghi, 1999), diabetic neuropathies (Apfél, 1999) and paralyses of the facial nerve (Chao et al., 2006). Furthermore, NGF may be used in the treatment of ocular pathologies. In fact, NGF, in topical application, is used in the treatment of corneal ulcers (Lambiae et al., 1998), neuritis of the optic nerve and neuropathies of the optic nerve from traumatic or ischemic origins (Lambiae et al., 2005).

Finally, NGF is therapeutically beneficial in cutaneous and pressure ulcers (Landi et al, 2003) and other dermatology indications.

The implication of NGF in Alzheimer’s Disease derives from the fact that one of the neuron groups that degenerate during the course of the illness, the cholinergic neurons of the basal forebrain, depends on NGF to survive (Hefti, 1986). This concept has, moreover, been reinforced by the production and characterisation of a murine model, in which the deprivation of NGF, by using transgenic antibodies directly against NGF, induces the onset of a phenotype of progressive neurodegeneration, very similar to that encountered in
the brains of patients suffering from Alzheimer’s Disease (Capsoni et al., (2000; WO01/10203)).

The use of NGF as a therapeutic agent in the treatment of Alzheimer’s disease has already been analysed in patients suffering from this pathology. The first two clinical trials, realised by means of intra-cerebroventricular infusion of NGF, have demonstrated an improvement in the patients’ conditions indicated by an increase in the cerebral flow and nicotine receptors in the brain’s frontal and temporal cortices (Olson et al., 1992, 1993). However, the second trial, although reporting an improvement in the cerebral functions, also provided evidence of side effects, such as weight loss and the occurrence of pain in the lumbar region that resulted in the discontinuation of the treatment (Eriksdottir-Johangen et al., 1998).

A more recent approach sees the application of autologous fibroblasts, engineered to express NGF and implanted, by means of neurosurgery, in the brains of Alzheimer’s patients. During implantation, 2 patients demonstrated signs of subcortical haemorrhagia, with consequent hemiparesis in one patient and, in the other, death due to cardiac arrest. After 18 months from implantation, the 6 patients still undergoing the treatment reported an improvement in their cognitive functions, with their decline slowing down by 40-50% (Tuszynski et al., 2005).

Another potential use of NGF for other central nervous system (CNS) diseases is represented by Rett’s Syndrome. This is a progressive neurological disease, which mainly affects females from 6 months of age and for which there is still no cure. Its etiological cause is not well-defined. It is characterised by an arrest in the development of the CNS, characterised by microencephaly, accompanied by involuntary movements of the hands, the occurrence of stereotypical movements, problems in speaking, poor socialisation skills and learning difficulties (Hagberg, 1989; Witt-Engerstrom, 1992). Lack or reduced NGF concentration seems to be one of the causes of the disease. Indeed, Rett’s affected patients show a reduced NGF concentration either in the cerebrospinal liquid (Riikonen et al., 1999) or in the brain (Lipani et al., 2000) and the plasma (Calamandrei et al., 2001; Guideri et al, 2004). Rett’s syndrome patients, therefore, may benefit from having NGF administrations.

Finally, insensitivity to pain with anhidrosis (CIPA) is a rare autosomal recessive disorder, characterized by a lack of pain sensation and anhidrosis, which is caused by mutations in the genes encoding for the TRKA receptor for NGF (Indo, Y., Tsuruta, M., Hayashida, Y.,

However, notwithstanding the increased possibility of therapeutic effectiveness, the use of NGF to treat Alzheimer’s disease and other CNS diseases is made difficult, if not impossible in its current form, for the following reasons:

1. need for localised delivery, to avoid side effects due to noted (see below) actions of NGF on “peripheral” targets;
2. difficulty of accessing the CNS, due to the blood brain barrier;
3. difficulty of a selective dosing of exogenous, therapeutic NGF, compared to the dosing of endogenous NGF.

In fact, endogenous NGF levels are variable, both from individual to individual and in a single individual at various times, and according to stress conditions.

In addition, concerning its undesired side effects, NGF has a pro-nociceptive (or algogenic) action on the sensory neurons of dorsal root ganglia and spinal cord, on which it modulates the transmission of pain signals, and a pro-inflammatory action on inflammatory cells (including mastocytes), on which it activates the release of inflammation mediators (Bennett, 2001).

In rodents, NGF, administered topically or systemically, causes hyperalgesia in response to heat stimuli which lasts over time (Lewin et al., 1994; Della Seta et al., 1994)). In humans, the capacity of NGF to cause pain has been demonstrated in the clinical trials in AD patients, described above, as well as during clinical trials undertaken to explore the potential use of NGF in poly-neuropathies. The subcutaneous injection of NGF into the arms of healthy volunteers produces allodynia and hypersensitivity of the skin surrounding the point of injection. Furthermore, a general sensation of muscular pain is present in individuals treated with NGF and not with placebo (Dyck et al., 1997). This painful reaction lasts for up to 3 weeks after the injection (McArthur, et al., 2000). In a clinical study undertaken to study the effects of injecting NGF into the masseter muscle in healthy subjects, mechanical allodynia and hyperalgesia were observed, lasting at least a week (Svensson et al., Pain 104, pp. 241-247 (2003)). In addition, pain was observed during
movements of the mandible (Svensson et al., 2003). The direct administration of NGF into the sciatic nerve also causes hyperalgesia (Ruiz et al., 2004). The therapeutic use of NGF for Alzheimer’s disease and for other CNS pathologies, on the one hand is extremely advantageous, but is rendered very difficult by its limited access to the CNS. Therefore, its administration must be local in order to limit known undesirable effects due to too large NGF peripheral concentrations.

In humans, the maximum dose of NGF that does not cause hyperalgesia is 0.03 μg/kg (Petty et al., 1994). However, such dose is too low for NGF to access the CNS. In fact, only 2.4 ng out of 0.24 mg of NGF administered intravenously to a 250g rat (therefore, equal to 0.001% of the injected dose) will pass the blood brain barrier and reach the brain. This quantity is twenty times lower than the 55 ng that are found in the brain after intranasal administration of the same dose of 0.24 mg (Thorne and Frey, 2001).

To access the CNS by means of systemic delivery, very high, intolerable, doses must be given, in the order of mg/kg, far above the 0.03 μg/kg dose that does not cause hyperalgesia. Therefore a “treatment window” must be respected, NGF quantities must be sufficient to reach the CNS but not too high, to avoid painful side effects. The limits of this treatment window most likely vary from individual to individual. Therefore, there is a need to favour NGF access to the CNS whilst minimising the quantities that accumulate at the systemic level and that cause pain.

The problem with optimising NGF treatment window, or with minimising the systemic level distribution of NGF, and minimising its nociceptive algogenic effects also exists for the topical and local NGF treatment. In particular for applications for ocular pathologies: NGF is a treatment that is potentially effective for patients with corneal ulcers (Lambiase et al., 1998). Furthermore, in animal models of ocular diseases, the intraocular administration of NGF resulted in an improvement of the degeneration of the ganglia cells in the retina observed after transection of the optic nerve, ocular ischemia or induced ocular hypertension (Carmignoto et al, 1989; Siliprandi et al. 1993), putting NGF forward as a potential treatment for glaucoma.

A pharmacokinetic study of ophthalmic applications (Lambiase et al 2005) describes the application of NGF by the topical conjunctival route and demonstrates that NGF is vehiculated at the level of the optic nerve. However, the study shows that even in the case of conjunctival local administration of NGF drops, significant doses of NGF are found at the systemic level in the serum of the animals. This study demonstrates that, following the
conjunctival topical application of NGF into an eye, the levels of NGF accumulate in the
serum and in the other eye, demonstrating that NGF crosses the haematie ocular barrier.
It is, however, evident that there is a need to develop a method of NGF administration that
allows the treatment window to be optimised for each of NGF treatment applications, and
which allows NGF to access the CNS, or other targets (for example eyes and retinas) in
concentrations that do not induce side effects at the peripheral level. In particular, for all
NGF treatment applications, there is a need to optimise the treatment window by achieving
a compromise between the maximum dose that reaches the target and the minimum dose
that enters the systemic circulation and that causes pro-nociceptive effects.
To this goal, it is necessary to develop compositions and methods of administration that
facilitate access to the desired target and to design a therapeutic product that can easily be
measured, specifically and selectively, to optimise the therapeutic dose and to monitor it
under the various conditions, and against the variable background of endogenous NGF.
The state of the art describes methods of administration, in rodents preclinical studies, by
means of osmotic mini-pumps or intraventricular catheters. However, such methods
require repeated infusions into the brain, and mini-pumps must be refilled every time that
the reservoir needs to be substituted or the injection syringe reinserted into the catheters.
These methods may also introduce infection. Indeed, it has been reported that in intensive
care units, the intracerebroventricular catheters used to monitor intracranial pressure were
infected with bacteria as early as three days after implantation (Saffran, 1992). Infusions
into the ventricles may generate hydrocephaly (Saffran et al. 1989), whereas continued
infusions of solutions of parenchyma are associated with cellular necroses in the brain.
The state of the art also describes the use of foetal tissue to substitute the degenerate
neurons; such methods are not devoid of ethical limitations, and rejection by the host
organism or induction of tumour may occur, since the injected material is made up of non-
encapsulated cells.
The state of the art also describes direct injections of a host’s cells transformed with viral
vectors to induce the production of NGF by means of in vivo gene therapy. This technique
is limited since there is a risk of mutagenesis and tumorigenesis. In addition, this technique
does not allow NGF secretion regulation and can cause undesired side effects. The use of
cells engineered to secrete NGF was applied in clinical trials, during which coetaneous
fibroblasts secreting NGF were implanted into Alzheimer’s patients (Tuszynski et al.,
2005). Despite the encouraging therapeutic results obtained using this technique,
significant limits were demonstrated in connection with the extremely invasive method of administration that in 2 patients has lead to cerebral haemorrhages with consequent hemiparesis. In addition to these problems, that are specific to this case, there are more general intrinsic limits common to a cellular treatment with ex vivo engineered cells: the need for neurosurgical intervention to implant the cells, and the impossibility of interrupting the secretion of NGF by the cells or of interrupting the activity of the implanted cells.

The state of the art technique also describes cells encapsulated in order to produce NGF. Kordower et al. (1994) discloses the use of modified BHK cells expressing hNGF, but with the main limit of introducing animal proteins that represent potential immunogens for humans.

The international patent application WO2005/068498 describes a method for the implantation of encapsulated human cells in order to secrete a predetermined dose of NGF directly into the brain. Such a method requires general anaesthesia and an invasive neurosurgery and does not allow the NGF dose to be adjusted during the course of the treatment since the number of cells implanted is decided beforehand. Furthermore, all transplants of in vivo cells should allow treatment to be interrupted if needed, for instance in the case of side effects, the end of the treatment or a change of treatment strategy. However, in vivo cell treatment are irreversible which renders them unacceptable, unless there is a further surgical intervention or the insertion into the vector of expression of suicide genes, such as thymidine kinase derived from the herpes virus, that, upon administration of ganciclovir, kills the cell in which it is expressed. However, this remedy implies the presence of non-human genes.

Intranasal administration has been described in patents EP 0 504 263, EP 1 135 105 and EP 1 137 401. In particular, patent EP 1 137 401 relates to the use of a neurological agent or of one of its biologically active variants as a pharmaceutical product to be inoculated into the nasal cavity at a dose of 0.1 nmol up to 1000 nmol in order to protect and treat a CNS cell, in which the neurotrophic agent is selected from the group of neurotrophins (IGF, NGF, FGF and their biologically active variants).

The patent application CN 1616087 relates to NGF liposomes that have a greater intra-body stability than NGF, a quadruple half-life and a greater concentration in the cerebral tissue than NGF. The NGF liposomes are administered into the nasal cavity by means of gel spray.
The patent application EP 1 539 208 relates to compositions and methods that include a biologically active agent and a permeating agent that promotes the administration of the biologically active agent, i.e. NGF by the mucous route. The limit of NGF external administration lies in the fact that it is impossible to distinguish the administered NGF from endogenous NGF and this limit is particularly impeding, if one wants to overcome the problem with optimising NGF treatment window. An ideal treatment should allow a sensitive and safe quantification of the administered agent. This is particularly true in the case of NGF. In fact, endogenous NGF concentrations in the blood and tissue vary greatly between individuals, either because NGF is bound to plasmatic proteins, thus limiting its availability to the action site, or due to the variability in its production in relation to the individual conditions that influence the synthesis of this protein and the expression of its receptors, for example stress (Aloe et al., 2002) or the patient’s hormonal balance (Lanlua et al., 2001).

Furthermore, exogenous NGF protein administered needs to have a bioactivity that is similar in every respect to that of the endogenous NGF. The protein should not be immunogenic and, therefore as similar as possible in terms of its amino acid sequence to endogenous NGF. However such protein would not be distinguishable from endogenous NGF.

It is, therefore, necessary to identify an NGF for which an optimal therapeutic dose can be established and that can be detected.

An ideal NGF-based treatment for pathologies of the CNS, for example Alzheimer’s (AD), multiple sclerosis and Rett’s syndrome, and for ocular pathologies must overcome the problems mentioned above and allow the therapeutic dose of NGF to be optimised, on an individual basis and over time, both to maximise the therapeutic dose, for the various types of administration that may be on offer and to minimise the undesirable side effects. A therapeutic NGF, in addition to having the same biological activity of the wild type endogenous NGF should have at least one of the following properties and, ideally, all of them:

i) to be distinguishable from endogenous NGF by a sensitive and selective method, whilst being bioactive to the same degree as endogenous NGF;

ii) not to provoke undesired immune reactions if administered for therapeutic or diagnostic purposes;
iii) to be used with co-adjuvants for blocking any hyperalgesic effects; in the
course of optimising the dose or of the treatment;
iv) to be further mutated to avoid causing pain.

The idea of constructing a mutein hNGF by introducing a peptide tag that renders the
therapeutic NGF distinguishable from the endogenous NGF by the use of an antibody
against the peptide tag, presents numerous problems and disadvantages, both general and
specific to the case under consideration.

In general, peptide tags have the disadvantage of being immunogenic, because they alter
the protein’s immunological profile. Tags can also be easily proteolitically removed from
the protein, thus losing their function as indicators of such protein. Peptide tags are
relatively long sequences and, therefore, may alter the protein’s structural and functional
profile, particularly if inserted within the protein sequence. For this reason tags are usually
located at the N terminus or the C terminus of the protein, but in such positions they are
more easily proteolitically cleaved off. The smallest known antibody tag (epitope)
described is made up of three amino acids, such as, for example, the C-terminal GAE tag
recognised by th monoclonal antibody 423 (Khuebachova et al., 2002). In addition,
antibody 423 also recognises an epitope of the tau protein (Skrabana et al., 2004) therefore
cannot be used because of its non-specificity.

In addition to these general problems, there are specific problems. In the case of NGF, a
peptide tag would have to be inserted within the coding sequence for NGF since both the
N- and the C- terminal parts are proteolitically processed during the course of the
biosynthetic maturation of NGF (by N-terminal processing: Edwards et al., 1988; Seidah
et al., 1996 and by C-terminal processing: Kruttgen et al., 1997 ). Furthermore, the
insertion of a tag in the N-terminal portion would have the further disadvantage of
interfering with the binding between NGF and TrkA receptor (Woo et al., 1995;
Wiesmann et al., 1999; Robertson et al., 2001). Moreover, the insertion of a peptide tag
within the coding sequence of mature NGF would result in a drastic alteration to its
structure and, therefore, its function.

Patent-related and scientific literature cite numerous muteins of NGF with various
characteristics as listed below:

i) Pan neurotrophins: muteins that combine on NGF key residues of other
neurotrophins, to obtain a neurotrophin that acts on three receptors, TrkA,
TrkB and TrkC. In particular muteins in position 16, 18, 20, 23, 79, 81, 84,
86, 88, in various combinations are disclosed. These muteins are described in the international patent application WO2005068498;

ii) Mutant NGF with a reduced capacity to bind the p75 receptor. The involved residues are residues in positions 32, 34, 35, and 95. These muteins are described in US patent 5,349,055.

iii) Mutant NGF that does not bind TrkA (delta 1-9 and delta 117-118) (Woo et al., J Biol. Chem. 270, 6278, 1995);

iv) Mutant NGF with increased stability, common residuals between 25 and 36 are mutated (Ibanez Tibtech 13, 217-227, 1995);

v) Chimeras between NGF and BDNF. Muteins of more than 5 amino acids in sequence, taken from BDNF;

vi) Mutated NGF beta gene as described in the international patent application WO2005/040335 and identified from genomic DNA of patients with a pathology of slight insensitivity to pain. The corresponding peptide presents a mutation in position 100 of the deduced amino acid sequence of NGF (Einarsdottir et al., 2004).

The authors of the present invention have a panel of antibodies (directed against NGF, or its TrkA receptor). These antibodies are able to block the biological effects of NGF mediated by the binding to TrkA, including peripheral nociceptive effects. Two reagents alfaD11 (anti-NGF, Cattaneo et al 1988; WO 2005/061540) and MNAC13 (anti-TrkA, Cattaneo et al 1999, EP 1 181 318, WO 2005/061540) are of particular interest because they are available in a “humanised” form which can be used in humans. The comparison between the 2 antibodies, one directed against the binding and the other against the receptor, is of significant interest, since the inhibition of NGF binding cannot functionally be assimilated to the inhibition of the TrkA receptor. Such reagents may be used as “antidotes” to minimise, at the systemic level, any pro-nociceptive algogenic activity of the therapeutic NGF.

Finally, a NGF mutein further mutated to avoid causing pain, but having the same potency and neurotrophic activity as hNGF on its neuronal targets would be of great advantage, avoiding the unwanted side effects that limit its therapeutic uses.

SUMMARY OF THE INVENTION
It is an object of the instant invention a mutein of human NGF characterised by the fact that:

a) it is therapeutically active;

b) it does not induce undesired immune reactions;

c) it is selectively recognised by a specific reagent with regard to endogenous hNGF.

The term “mutein of human NGF” refers to muteins of the whole human pro-NGF or of the truncated mature human NGF, lacking first 121 amino acids.

The term “therapeutically active” refers to a biologically active molecule providing a therapeutic benefit in the following human diseases: Alzheimer’s disease, Parkinson’s disease, Chronic Insensitivity to pain with anhydrosis (CIPA), multiple sclerosis, peripheral nerve injury and neuropathies, Rett’s syndrome, Schizophrenia, pathologies of peripheral nervous system, ulcers from diabetis or decubitus, Vasculitis associated reumatoid arthritis, congestive heart failure, corneal ulcer and/or age-related immunodeficiency.

The term “undesired immune reactions” refers to a molecule lacking induction of antibodies against endogenous human NGF and against mutein of human NGF and lacking induction of allergic reactions.

Preferably the mutein is obtained by at least one amino acid substitution residing in loop III of hNGF, more the substituted amino acid in loop III is proline at position 61, most preferably the amino acid substitution of proline in position 61 is with a serine.

It is a further object of the invention the mutein of human NGF as above described further comprising at least one amino acid substitution able to substantially reduce its nociceptive activity. Preferably the mutein of human NGF further comprises at least one amino acid substitution leading to a reduced interaction of the mutein with the p75 receptor. More preferably the amino acid substitution is at any of positions 95-101. Most preferably the amino acid substitution is that of the arginine in position 100, even more preferably the amino acid substitution of the arginine in position 100 is with an acidic amino acid or tryptophane. The acidic amino acid may be glutamic or aspartic acid. In an alternative embodiment the amino acid substitution is that of the glutamine in position 96. In an alternative embodiment the amino acid substitution is that of the tryptophan in position 99. In an alternative embodiment the amino acid substitution is that of the phenylalanine in position 101.
It is a further object of the invention the mutein of human NGF as above described for therapeutic use.

It is a further object of the invention the use of the mutein of human NGF as above described for the preparation of a medicament for the treatment of pathologies of the central and peripheral nervous system. Preferably for Alzheimer’s disease, Parkinson’s disease, Chronic Insensitivity to pain with anhydrosis (CIPA), multiple sclerosis, peripheral nerve injury and neuropathies, Rett’s syndrome, schizophrenia, ulcers from diabetes or decubitus, vasculitis associated rheumatoid arthritis, congestive heart failure, corneal ulcer and/or age-related immunodeficiency.

It is a further object of the invention a pharmaceutical composition comprising a pharmaceutically acceptable and effective dose of the mutein of human NGF as above described and suitable diluent and/or carrier. Preferably the dose is determined according to the concentration of endogenous human NGF. More preferably the pharmaceutical composition as above described is for final vehiculation to the central nervous system, for intranasal administration, for intraocular or ophthalmic administration, for systemic administration or topical administration. Even more preferably the mutein of human NGF is associated with nanostructures or with liposomes. Preferably the pharmaceutical composition further comprises a pain-killer agent. More preferably the pain-killer agent is a neutralising anti-NGF or anti-TrkA antibody.

The neutralising anti-NGF or anti-TrkA antibody is an antibody that blocks the biological activity of NGF or TrkA, and in particular the nociceptive action. Preferably, the pain-killer agent is formulated separately.

It is a further object of the invention a specific reagent able to selectively recognise the mutein as above described with regard to endogenous hNGF. Preferably, the specific reagent is a monoclonal antibody or a synthetic or a biotechnological or a humanised antibody, or a fragment thereof. More preferably it is an antibody able to selectively recognise a mutein of hNGF having a serine at position 61.

It is a further object of the invention the use of the specific reagent as above described to monitor the amount of the mutein of human NGF of the invention in a biological sample.

It is another object of the invention a kit to monitor the amount of the mutein of human NGF as above described in a biological sample for therapy control comprising the specific reagent of the invention.
In the present invention reference is made to the following NGF proteins:

<table>
<thead>
<tr>
<th>denomination</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse pro-NGF</td>
<td>mPro-NGF</td>
</tr>
<tr>
<td>Mouse NGF</td>
<td>mNGF</td>
</tr>
<tr>
<td>Rat proNGF</td>
<td>rPro-NGF</td>
</tr>
<tr>
<td>Rat NGF</td>
<td>rNGF</td>
</tr>
<tr>
<td>Human pro-NGF</td>
<td>hPro-NGF</td>
</tr>
<tr>
<td>Human NGF</td>
<td>hNGF</td>
</tr>
<tr>
<td>Human NGF mutated in position 61 (corresponding to aa 182 of SEQ ID No 3)</td>
<td>NGF61, NGF-61, hNGF61</td>
</tr>
<tr>
<td>Human NGF mutated in position 100 (corresponding to aa 221 of SEQ ID No 3)</td>
<td>hNGFR100W</td>
</tr>
<tr>
<td>Human NGF mutated in position 100 (corresponding to aa 221 of SEQ ID No 3)</td>
<td>hNGFR100A</td>
</tr>
<tr>
<td>Human NGF mutated in position 100 (corresponding to aa 221 of SEQ ID No 3)</td>
<td>hNGFR100E</td>
</tr>
<tr>
<td>Human NGF mutated in position 100 (corresponding to aa 221 of SEQ ID No 3)</td>
<td>hNGFR100K</td>
</tr>
<tr>
<td>Human NGF mutated in position 100 (corresponding to aa 221 of SEQ ID No 3)</td>
<td>hNGFR100Q</td>
</tr>
<tr>
<td>——</td>
<td>——</td>
</tr>
<tr>
<td>Human NGF mutated in position 100 (corresponding to aa 221 of SEQ ID No 3)</td>
<td>hNGFR100V</td>
</tr>
<tr>
<td>Human NGF mutated in position 61 and 100 (corresponding to aa 182 and aa 221 of SEQ ID No 3, respectively)</td>
<td>hNGF61R100W</td>
</tr>
<tr>
<td>Human NGF mutated in position 61 and 100 (corresponding to aa 182 and aa 221 of SEQ ID No 3, respectively)</td>
<td>hNGF61R100A</td>
</tr>
<tr>
<td>Human NGF mutated in position 61 and 100 (corresponding to aa 182 and aa 221 of SEQ ID No 3, respectively)</td>
<td>hNGF61R100E</td>
</tr>
<tr>
<td>Human NGF mutated in position 61 and 100 (corresponding to aa 182 and aa 221 of SEQ ID No 3, respectively)</td>
<td>hNGF61R100K</td>
</tr>
</tbody>
</table>
Human NGF mutated in position 61 and 100 (corresponding to aa 182 and aa 221 of SEQ ID No 3, respectively)  

| hNGF61R100Q | P → S |
|             | R → Q |

The present invention will now be illustrated by means of non-limiting examples, with particular reference to the following figures:

FIGURE 1: (A) Multiple alignment of the primary structures of NGF from various analysed species: mouse NGF, mNGF, rat NGF, rNGF, human NGF, hNGF (B) and (C) Identification of NGF epitope bound by the antibody 4GA (evidenced by a dotted line) at the level of the tertiary structure of mNGF; figure produced with VMD (Visual Molecular Dynamics) (Humphrey et al., 1996): two different perspectives.

FIGURE 2: Structural alignment of the tertiary structure of mNGF (shown in grey) and the hNGF (in black): loop III is indicated by a dotted line; figures produced using Pymol (DeLano, 2002).

FIGURE 3: Absence of interaction between loop III (dotted area) and domain d5 of receptor TrkA (A) and P75 (B).

FIGURE 4: Comparison of binding activity of the monoclonal antibody 4GA to NGF in various species A) comparison between rat NGF (rNGF) and mouse (mNGF); B) comparison between mouse (mNGF) and human (hNGF) NGF and their precursors mouse (m proNGF) and human (h proNGF) proNGF. 4GA binding is revealed by a secondary anti rat antibody that is conjugated to the enzyme Horseradish Peroxidase (HPR), that converts the substrate TMB in a cromogen product whose OD (450nm) after blocking is correlated first to the product concentration by the Lambert-Beer law (OD=αlc, where α is the absorption coefficient, l is the path length and c the concentration of the product). The concentration of the product is correlated to the enzyme concentration that depends on 4GA binding.

FIGURE 5: Comparison of binding activity of the monoclonal antibody 4GA to point muteins of rNGF (rat wt) in non-conserved amino acid positions 40 (G40A), 61 (P61S) and 117 (A117T), demonstrated in comparative analyses between mNGF and rNGF. The P61S mutation of the rNGF confers reactivity towards the 4GA antibody that is fully
comparable with that of the natural antigen (mNGF). The measured OD(450nm) is related to 4GA binding activity as explained in Figure 4.

FIGURE 6: Comparison of binding activity of the monoclonal antibody 4GA to the point mutein of hNGF (human wt) in non-conserved amino acid position 61 (P61S) demonstrated in comparative analyses with wildtype human NGF. The point mutation Pro61Ser in hNGF is able to restore the reactivity of the monoclonal 4GA antibody, even with regards to the hNGF.

FIGURE 7: The affinity for the receptors TrkA and p75 of mutein form hNGF Pro61Ser results similar to that of wildtype hNGF.

Response curves of serial dilutions (500nM and 4nM) of A) hNGF and of B) hNGF Pro61Ser (in B) on a cell with 8000RU (Resonance Units) of immobilised TrkA immunoadhesin, Response curves of serial dilutions (500nM a 4nM) of C) hNGF and D) hNGF Pro61Ser in a cell with 3900RU of immobilised p75 immunoadhesin. All the graphs plot the resonance units (RU) on the y axis versus the time on the x axis.

FIGURE 8: The induction of TrkA phosphorylation of the mutein form hNGF Pro61Ser results similar to that of wild type hNGF. The phosphorylation levels of TrkA induced by serial dilutions (shown on x axis) of wild type hNGF and the mutein form hNGF Pro61Ser (indicated on y axis) are detected by a rabbit antibody that recognizes phospho-TrkA, which is in turn revealed by an anti-rabbit antibody that is conjugated to the enzyme Horseradish Peroxidase (HPR): The measured OD (450nm), plotted on z axis, is related to TrkA phosphorylation levels as explained in Figure 4.

FIGURE 9: Proliferative response of the TF1 cell line to NGF. The TF1 proliferation assay was performed in quadruplicate in microtiter plates by incubating 15,000 cells per well in the presence of the indicated concentrations of huNGF and hNGF-61. After a 40-hour culture period, 10 µl of MTT substrate solution was added for an additional 4 hours of incubation and the OD was determined at 570 nm in a microtiter plate reader, 16 h following the addition of detergent reagent (100 µl per well). huNGF and hNGF-61 dose/response curves were substantially super-imposable.

FIGURE 10: Scheme of TrkA signalling pathways. The picture depicts the interactions of TrkA receptor and major signalling pathways activated by this receptor. The arrows point to the proteins that were analyzed to assess the efficacy of hNGF-61 in activating the TrkA receptor.
FIGURE 11: Western blot analysis of TrkA and Akt phosphorylation on Y490 and S473, respectively, in extracts from BALB/C 3T3 cells incubated in presence of huNGF R&D (commercial source, produced in mammalian cells), hNGF (produced in E. coli following the same procedure as NGF-61, see examples) and hNGF-61. The three NGF variants induce the same degree of activation.

FIGURE 12: Position of the residue Arg100 (R100, dotted area) in the crystallographic structures of the complexes between wild type hNGF (shown in dark grey) and its receptors: A) p75 (left part of the complex) and B) domain d5 of receptor TrkA (right-hand part of the complex); figures produced using Pymol (DeLano, 2002).

FIGURE 13: Analysis of the binding interface between hNGF and p75 receptor, that involves the Arg100 residue (underlined and in bold). The table lists all the residues that mediates the binding interface, in particular first column: residue type; second column: residue number, third column: molecule (NGF monomer: NGF_A; p75 respectively); forth and fifth columns: interface mediated by each residue (respectively in absolute and % value); sixth column: hydrogen bonds mediated by each residue (if present, 1).

FIGURE 14: (A) Elution profile from Hitrap SP sepharose XL of huproNGF WT after pulsed refolding: the area below the curve is proportional to the total amount of purified protein. (B) Comparison of elution profiles from Hitrap SP sepharose XL of huproNGF muteins in position 100, after pulsed refolding: the areas below each curve is proportional to the total amount of each purified mutein.

FIGURE 15: Affinity to TrkA of hNGF muteins in position 100: response curves of serial dilutions (from 500nM to 4nM) of A) hNGF, B) hNGF Arg100Lys, C) hNGF Arg100Ala, D) hNGF Arg100Gln and E) hNGF Arg100Glu. All the graphs plot the resonance units (RU) on the y axis versus the time on the x axis.

FIGURE 16: Affinity to p75 of hNGF muteins in position 100: response curves of serial dilutions (from 500nM to 4nM) of A) hNGF, B) hNGF Arg100Lys, C) hNGF Arg100Ala, D) hNGF Arg100Gln and E) hNGF Arg100Glu. All the graphs plot the resonance units (RU) on the y axis versus the time on the x axis.

FIGURE 17: Analysis of the affinity of muteins NGF-61 R100 for TrkA (A, C, E, G, I) and p75 receptors (B, D, F, H, J). Analyses of serial dilutions (from 500nM to 4nM) of NGF-61 (A, B), NGF-61 Arg100Lys (C, D), NGF-61 Arg100Ala (E, F), NGF-61 Arg100Gln (G, H) and NGF-61 Arg100Glu (I, J). All the graphs plot the resonance units (RU) on the y axis versus the time on the x axis.
FIGURE 18: Induction of TrkA phosphorylation by hNGF muteins in position 100.

The phosphorylation levels of TrkA induced by serial dilutions (shown on x axis) of wild type hNGF and the mutein forms of hNGF in position 100 (indicated on y axis) are detected by measuring OD(450nm), plotted on z axis, as explained in Figure 8.

FIGURE 19: Western blot analysis of TrkA, Akt and PLC-γ1 phosphorylation on Y490, S473 and Y783, respectively, in extracts from BALB/C 3T3 cells incubated in presence of different R100 muteins. A = Arg100Ala; E = Arg100Glu; K = Arg100Lys, Q = Arg100Gln; V = Arg100Val; W = Arg100Trp.

FIGURE 20: Densitometric analysis of western blot analysis for (A) TrkA phosphorylated in position Y490, shc/Ras pathway, (B) Akt phosphorylated in position S473 and (C) PLC-γ1 phosphorylated in position Y783. Bars are representative of the ratio between values obtained for muteins and wild type huNGF.

FIGURE 21: The differentiation of PC12 cells is induced by 100 ng/ml (A) hNGF and (B) NGF-61. (C) No differentiation was shown in PC12 cultures grown in absence of NGF. The muteins (D) huNGF Arg100Lys and (E) NGF-61 Arg100Lys induce a similar differentiation with respect to huNGF and NGF-61, while the muteins (F) huNGF Arg100Glu and (G) NGF-61 Arg100Glu are less effective in inducing differentiation in PC12 cells.

FIGURE 22: Treatment with NGF-61 administered intranasally improves the number of cholinergic neurons in the basal forebrain in AD11 anti-NGF mice, a transgenic mouse model for Alzheimer’s disease. (*, P < 0.05 AD11 vs. non-transgenic mice; #, P < 0.05 AD11 treated with rhNGF or hNGF-61 vs. AD11 treated with placebo).

FIGURE 23: Treatment with NGF-61 administered intranasally reduces the number of cells that express phosphorylated tau in AD11 anti-NGF mice, a transgenic mouse model for Alzheimer’s disease. (*, P < 0.05 AD11 vs. non-transgenic mice; #, P < 0.05 AD11 treated with rhNGF or hNGF-61 vs. AD11 treated with placebo).

FIGURE 24: Treatment with NGF-61 administered intranasally reduces the number of dystrophic neurites that contain β amyloid. (*, P < 0.05 AD11 vs. non-transgenic mice; #, P < 0.05 AD11 treated with rhNGF or hNGF-61 vs. AD11 treated with placebo).

FIGURE 25: Discrimination index in the object recognition test in AD11 anti-NGF mice after treatment with hNGF-61. Non-transgenic mice mainly explore the new object and, consequently, have a positive discrimination index. AD11 mice treated with PBS do not discriminate between the new and old object and, therefore, have a negative discrimination.
index. The mice treated with rhNGF and NGF-61 display an significantly reduced deficit and have an intermediate discrimination index, whilst remaining significantly different from non-transgenic mice. (* P < 0.05 vs. AD11 + PBS; # P < 0.05 vs. non-transgenic mice).

FIGURE 26: The intranasal administration of huNGF and hNGF-61 to 15 months old AD11 mice determines: the increase in cholinergic neurons in (A) the medial septum (MS) and diagonal band of Broca (DBH); (B) the decrease of β-amyloid burden in the hippocampus and (C) the decrease of tangle-like cells over-expressing phosphorylated tau. (*, P < 0.05 AD11 vs WT mice; # P < 0.05 AD11 treated with huNGF or hNGF-61 vs. placebo-treated AD11 mice).

FIGURE 27: The monoclonal antibody 4Ga is able to distinguish different concentrations of exogenous hNGF-61 from endogenous wild type human NGF in human blood serum (A) The antibody 4GA allows to distinguish hNGF-61 from endogenous NGF in human serums, whereas (B) the antibody αD11 does not distinguish between hNGF-61 and endogenous NGF. Bars represent the mean value ± SEM for each exogenous hNGF-61 concentration.

FIGURE 28: Absence of production of anti-NGF IgG in mice treated with hNGF61. The level of anti-NGF IgG were measured by ELISA in different dilutions of the serum. The level of optical density (O.D.) represented on the Y axis directly correlates with the amount of IgG in the serum. Bars represent the mean value ± SEM for each serum dilution.

FIGURE 29: Absence of increase of IgE (expressed on the Y axis as μg/ml) in the serum of mice treated with hNGF61 intranasally with respect to mice treated with PBS. Bars represent the mean value ± SEM for each group of treatment.

FIGURE 30: Effects of intra-cerebroventricular injection (I.C.V.) of NGF-61 on the response to pain assessed using the hot plate test. I.C.V. NGF61 administration decreases the latency to paw licking in rats. The reduction in pain threshold is prevented by administration of the antibody αD11.

FIGURE 31: Intermittent dosing in AD11 mice. The intranasal administration of NGF-61 to 6 months old AD11 mice three times per week (3 W) determines the rescue of the object recognition memory deficit, indicated by a higher discrimination index (on the Y axis). A more delayed administration [once per week (1W) or twice per week (2W)] is not effective. (*, P < 0.05 AD11 vs WT mice; # P < 0.05 AD11 treated with huNGF or hNGF-
61 vs. placebo-treated AD11 mice). Bars represent the mean ± SEM discrimination index for each group of treatment.

FIGURE 32: The intranasal administration of NGF-61 to 6 months old AD11 mice three times per week determines the rescue all neurodegenerative markers, while a more delayed administration is effective only in rescuing the increase in Aβ deposits. Total number of number of cholinergic (ChAT-positive) neurons in (A) medial septum (MS) and diagonal band of Broca (DBH) and in (B) nucleus basalis of Meynert (NBM). (C) Percentage of hippocampal area occupied by β amyloid clusters and (D) Number of phosphotau-positive dystrophic neurites in the entorhinal cortex. (*, P < 0.05 AD11 vs WT mice; # P < 0.05 AD11 treated with huNGF or NGF-61 vs. placebo-treated AD11 mice). Bars represent the mean value ± SEM for each group of treatment.

FIGURE 33: (A) The intranasal administration of huNGF and NGF-61 to 15 months old AD11 mice three times per week determines the rescue object recognition deficit (a) and this amelioration persists after one month during which the mice were not treated (b). (B) After 1 month of was-out the number of cholinergic neurons in the basal forebrain was not improved both by huNGF and NGF-61. (C) After 1 month washout, a decrease in the percentage of hippocampal area occupied by β-amyloid was shown in AD11 mice treated with NGF-61, but not huNGF. (D) HuNGF and NGF-61 decreased the number of phosphotau-positive neurons in AD11 mice after 1 month of wash-out. (*, P < 0.05 AD11 treated with huNGF or NGF-61 vs. placebo-treated AD11 mice).

FIGURE 34: The intranasal (in) and conjunctival (oc) administration of NGF-61 to 6 months old AD11 mice three times per week determines the rescue of the object recognition memory deficit. (*, P < 0.05 AD11 vs WT mice; # P < 0.05 AD11 treated with huNGF or NGF-61 vs. placebo-treated AD11 mice).

FIGURE 35: The intranasal (in) administration of NGF-61 to 6 months old AD11 mice three times per week determines the rescue all neurodegenerative markers. The administration of the same dose of NGF-61 via the conjunctiva (oc) rescue only the β-amyloid degeneration, while a higher dose of the compound given through the same administration route was effective in decreasing also the tau hyperphosphorylation. Number of cholinergic neurons in (A) medial septum (MS) and diagonal band of Broca (DBH) and in (B) nucleus basalis of Meynert (NBM). (C) Percentage of hippocampal area occupied by β amyloid clusters and (D) Number of phosphotau-positive dystrophic neurites in the entorhinal cortex. (*, P < 0.05 AD11 vs WT mice; # P < 0.05 AD11 treated
with huNGF or NGF-61 vs. placebo-treated AD11 mice; \( P < \) conjunctiva route vs intranasal route).

FIGURE 36: Absence of loss of body weight in AD11 mice treated intranasally for 15 days with 480 ng/kg of hNGF-61.

FIGURE 37: Area in \( \mu \text{m}^2 \) (mean \( \pm \) S.E.M.) of calcitonin gene-related peptide-immunoreactive fibers in the spinal cord in AD11 mice treated intranasally with placebo, huNGF (480 ng/kg) or NGF-61 (480 ng/kg) for 1 month.

FIGURE 38: The intranasal administration of NGF-61 Arg100Glu and NGF-61 to 6 months old AD11 mice three times per week determines the rescue of the object recognition memory deficit (*, \( P < 0.05 \) AD11 vs WT mice; \# \( P < 0.05 \) AD11 treated with NGF-61 Arg100Glu or NGF-61 vs. placebo-treated AD11 mice).

FIGURE 39: The intranasal administration of NGF-61 Arg100Glu to 6 months old AD11 mice three times per week determines the rescue all neurodegenerative markers with the same efficacy as NGF-61. Number of cholinergic neurons in (A) MS and DBH and in (B) NBM. (C) Number of \( \beta \) amyloid clusters in hippocampus and (D) number of phosphotau-positive dystrophic neurites in the entorhinal cortex. (*, \( P < 0.05 \) AD11 vs WT mice; \# \( P < 0.05 \) AD11 mice treated with NGF-61 Arg100Glu or NGF-61 vs. placebo-treated AD11 mice; \( \$ \), \( P < \) AD11 mice treated with NGF-61 Arg100Glu vs WT mice).

FIGURE 40: Hot plate jumping response of male mice injected IV with either NGF-61 (2.5 \( \mu \text{g/g} \)), NGF-61 Arg100Glu (2.5 \( \mu \text{g/g} \)) or cytochrome c (CYT, 2.5 \( \mu \text{g/g} \)) in repeated tests (n = 10 animals/group). (A) Number of jumps. (B) Latency to the first jump. Data represent mean levels \( \pm \) SEM.

RESULTS

In order to solve the problems described above, a variant of NGF needs to be identified. It should have the following properties:

i) same bioactivity than wild human NGF;

ii) minimum possible number of differences in the composition and sequence of amino acids compared to wild human NGF, but selectively and quantitatively distinguishable from endogenous NGF.

To this end, in the present invention, a combination of phylogenetic and structural analyses were used.
It is known that NGF proteins of any known species are also bioactive on human receptors as measured in a large number of in vitro quantitative tests. In addition, it is also known that murine NGF is active on humans, under therapeutic conditions, for example in the treatment of corneal ulcers (Lambiasi et al. 1998). The reason for this similar bioactivity may be explained by the fact that NGF proteins of these species are highly conserved, both from the point of view of the primary sequence, especially in the protein regions involved in the interactions with TrkA e p75 receptors, and from the structural point of view. In particular, murine NGF is considered to be the golden standard in terms of bioactivity, also in respect to the bioactivity of recombinant human NGF.

Therefore, the authors proceeded to identify, by means of a primary sequence alignment, the difference in amino acid residues between NGF of three species, mice, rats and humans (mNGF, rNGF and hNGF). A multiple alignment of the primary structures of the mNGF, rNGF and hNGF was carried out, as reported in Figure 1A. The comparative analysis indicated a total of 5 mutations that differentiate mNGF from both hNGF and rNGF (indicated in bold and underlined in Figure 1A). Two of these mutations are considered conservative because maintaining the electrostatic charge (Arg or Glu substituted with Lys and Asp, respectively,) and have, therefore, not been considered. In the case of the remaining, not obviously conserved, differences (Gly40Ala, Pro61Ser, Ala 117Thr), position 61 was selected. The position of residue Ser61, within the three-dimensional structure of mNGF, as indicated in Figures 1B and 1C, is in a superficial region of the molecule, exposed to the solvent and not directly involved in the interaction surfaces of NGF with its receptors (Wiesmann et al., (1999); He and Garcia, (2004); Aurikko et al., (2005)). This region is classified as loop III of NGF.

**Example 1. Structural comparative analysis of mNGF and hNGF of the loop III region containing position 61**

The structural similarity of the polypeptide frameworks at the level of the identified epitope was analysed by means of alignment of the known tertiary structures of mNGF and hNGF. In particular, whereas the structure of mNGF was resolved (code PDB 1BET, McDonald et al., (1991); code PDB 1BTG, Holland et al., (1994)), the structure of hNGF is only known in a complex with its natural receptors. In particular, the structures of the complexes between hNGF and the domain d5 of TrkA (code PDB 1WWW, Wiesmann et al., (1999)) and between hNGF and the extra-cellular domain of p75 are known (code
PDB 1SG1, He and Garcia, (2004)). In the two complexes, the overall NGF structure is conserved and it lacks the entire loop III in both of them. The superimpose program (Diederichs, 1995) was used to align the tertiary structures of the frameworks of alpha carbon atoms of mNGF (light grey) and hNGF (black): the two superimposed structures are represented in Figure 2. As shown in this Figure, the region including position 61 is not defined in the structure of hNGF, in which the entire loop III is completely lacking (see black C-alpha trace underlined by a circle). The absence of loop III in the experimental structures of hNGF complexes indicates the scarce definition of the electronic density at this level of the crystallographic structures and is an index of disorder and flexibility, which is not found in the structure of mNGF, where such a region is perfectly defined. Considering that in loop III the only difference between mNGF and hNGF is represented by Pro 61Ser, it is reasonable to suppose that the presence of Pro in position 61 is responsible for the increased flexibility of this loop in the human form, compared to that of murine. It can also be considered that the introduction of the residue Ser into position 61 of hNGF, in place of residue Pro could stabilise the entire loop III, consequently facilitating its crystallisation.

The fact that loop III of NGF, which contains position 61, is not directly involved in the structural interaction between NGF and its two receptors TrkA and p75 is demonstrated in Figure 3. In this figure, mNGF was substituted in the experimental complexes between hNGF and the receptors, to indicate loop III given that, in the case of hNGF it cannot be seen. Figure 3 clearly shows that loop III does not significantly interact with either of the two receptors TrkA and p75.

From these phylogenetic and structural analyses, it may be concluded that position 61 of hNGF, and, in particular, the Pro61Ser substitution may lead to the engineering of an NGF variant capable of solving the problem described. The variant of NGF modified in position 61 has a bioactivity comparable to that of hNGF (and mNGF), since such a position is not involved in the interaction with its two receptors, and since the residue that is naturally present in position 61 (proline 61) in huNGF is substituted by a residue (serine 61) naturally present in mNGF, which represents the golden standard in terms of NGF bioactivity and potency.

Moreover, only position 61 distinguishes hNGF from mNGF. The most convenient method involve the use of a specific binder, i.e. an antibody, directed against an epitope comprising position 61.
To such end, antibodies that were capable of distinguishing a variant of hNGF comprising the Serine antibody in position 61 from the corresponding wild hNGF protein were selected.

The strategy used to obtain such antibodies was to implement a combined approach: immunising rats with the murine NGF protein (according to the already described protocol to isolate monoclonal antibodies directed against NGF (Cattaneo et al., 1988), benefiting from the fact that mNGF protein naturally contains the residue Ser 61, whilst the rat and human proteins (rNGF, hNGF) do not, and testing the antibodies obtained for their ability to differentiate between i) mNGF from rNGF and hNGF and ii) an hNGF-61 variant, containing Ser61, from wild hNGF.

The immunisation protocol and the immunisation methods used are implemented as described in Cattaneo et al 1988 and allowed the isolation of an antibody denominated 4GA.

Example 2. The monoclonal 4GA antibody is species-specific for mNGF

The immunoglobulins to be tested were expressed in the supernatant using culture of hybridoma cells deriving from the fusion of the lymphocytes obtained from the spleens of rats immunised with myeloma cells, and concentrated using precipitation with ammonium sulphate 29% followed by dialysis in PBS. Next, they were purified by means of affinity chromatography using a column of Protein G Sepharose (Pharmacy).

A two-site sandwich ELISA test was developed to compare the reactivity to murine NGF (mNGF) and the ortholog rat form (rNGF), immobilising the monoclonal 4GA antibody on Maxi sorb 96 well plates, by means of incubation at 37°C for 2 hours and in a concentration of 10 μg/ml in sodium carbonate buffer 0.1M at pH 9.6.

After an hour of blocking with PBS containing 3% milk (MPBS) at room temperature, plates were incubated overnight at 4°C with the same concentration of mNGF, rNGF (expressed in the supernatant of COS cells transiently transfected with an expression vector for rNGF in mammalian cells) and, in parallel, also with the supernatant of non-transfected COS cells (negative control).

After incubation with the primary anti-mNGF antibody and with the secondary antibody (anti-rabbit peroxidase conjugated), it is possible to detect the binding activity reading the optical density at 450 nm (OD450) by means of incubation with the substrate TMB
(TECNA). The colorimetric reaction is blocked with the addition of a volume of H₂SO₄, 0.5M and the spectrophotometric reading is taken using an ELISA Reader (Spectra). The resultant monoclonal 4GA antibody is able to interact only with mNGF and not with rNGF (Figure 4A).

Figure 4B shows the results of a similar ELISA test carried out to compare reactivities towards murine NGF and its precursor proNGF (mNGF and m-proNGF, respectively) with the ortolog human forms (hNGF and hproNGF, respectively). In this case, the aforementioned 4 antigens (mNGF, Alomone, Israel, or hNGF, hproNGF, mproNGF produced according to Rattenhall et al., (2001)) at a concentration of 10 µg/ml in sodium carbonate buffer 0.1M, pH 9.6 were directly immobilised on Maxi sorb 96 well plates by means of incubation overnight at 4°C.

After an hour of blocking with PBS containing 3% milk (MPBS) at room temperature, plates were incubated with two serial dilutions (1:1, 1:10) of the surpernatant of the hybridoma that expresses the monoclonal 4GA antibody. After incubation with the secondary antibody (anti-rat IgG peroxidase conjugated), the colorimetric test was carried out as described above. The monoclonal 4GA antibody is able to interact only with the murine NGF (mNGF) and its precursor (m-proNGF) and not with the human NGF (hNGF) or its precursor (h-proNGF, Figure 4B).

**Example 3: Mapping the epitope of the monoclonal 4GA antibody**

To identify the epitope recognised by the 4GA antibody, three point rat NGF muteins were generated by site-specific mutagenesis according to Stratagene’s protocol. Such mutations restore the murine amino acid in the backbone of r NGF: Gly40Ala, Pro61Ser, Ala117Thr. The resulting mutagenesis was controlled by DNA sequencing.

To express each mutein, including rNGF, 250,000 COS cells were transfected with 1 µg of coding plasmid DNA for each mutein by FuGENE, as advised in the protocol (Roche). 72 hours after the transfection, each supernatants containing the transfected mutein expressed by the host cells were collected, then concentrated using Centriprep 50 (Amicon): the quantity of each mutein was standardised by dot blot. Briefly, 5 µl of each concentrated supernatant was applied to nitrocellulose membrane, together with serial dilutions of known concentration of mNGF (positive control and standard) and in parallel to the concentrated supernatant of non-transfected COS cells (negative control). After one
hour of blocking with PBS containing 3% milk (MPBS) at room temperature, membranes were incubated overnight at 4°C with the primary anti-mNGF antibody (Sigma). After one hour incubation with the secondary antibody (anti-rabbit peroxidase conjugated), the detection was realised using an electrochemiluminescent (ECL) test (Amersham).

The two-site sandwich ELISA test, performed as described in example 2, Figure 4A, allowed to identify that the point mutation able to restore the reactivity of 4GA with regard to rNGF is the mutation Pro61Ser: the results are shown in Figure 5. Thus it can be concluded that 4GA recognizes selectively human NGF having a ser residue at position 61.

Example 4. Mutagenesis of hNGF

Recognition by 4GA antibody

In order to verify in a definitive and direct way that the antibody 4GA has indeed the property of recognising an epitope comprising loop III and position 61 and, in particular, whether it is capable of distinguishing if the position 61 is occupied or not by a residue Serine61 instead of a residue proline61, a variant of hNGF was engineered, in which residue Pro61 was substituted by Ser 61 (denominated hNGF P61S or hNGF61), leaving the rest of the backbone of the human protein unaltered. The residue Pro 61 was substituted in the hNGF protein with Ser by site-specific mutagenesis according to Stratagene’s protocol. The mutagenesis was controlled by DNA sequencing.

To obtain in parallel both the wild type human hNGF and the mutein (Pro61Ser), both cDNA were cloned in a vector for prokaryotic expression (pETM11) in the form of a precursor (hproNGF), and the corresponding proteins expressed in inclusion bodies and renatured by means of the pulsed refolding protocol of Rattenhoff et al., (2001). Each precursor underwent proteolytic cleavage with trypsin (enzyme substrate ratio of 1:250) to obtain mature NGF that, after addition of protease inhibitors, was purified by means of gel chromatography using a Superdex 75 column (Pharmacy) and quantified by a Lowry test (BIORAD).

The ELISA test, described in exemple 2, Figure 4B, allowed to confirm unequivocally that the Pro61Ser point mutation was able to confer to the corresponding hNGF-Ser61 protein complete and specific reactivity for the 4GA antibody. By contrast, 4GA is not able to recognise the wild type hNGF protein (Figure 6).

Affinity for TrkA and p75 receptors
The affinity of hNGF Pro61Ser mutein for the TrkA and p75 receptors was compared using BiaCore experiments, immobilizing through amine coupling 8000RU of TrkA immunoadhesin and 3900RU p75 immunoadhesin, respectively. The kinetic analyses (Figure 7) were realised at a flow rate of 30 µl/min injecting serial dilutions of hNGF (in A and C) and of hNGF Pro61Ser (in B and D) in the interval between 500nM to 4nM and regenerating the surfaces after each cycle using Glycine pH 1.5. The resultant dissociation constants KD for TrkA and p75 were substantially identical for hNGF Pro61Ser mutein and wild type hNGF, being in the 10⁻⁹M range (nM) for TrkA receptor and in the 10⁻⁸M range for p75 receptor.

**Biological activity of hNGF Pro61Ser**

*TrkA phosphorylation*

The biological activity of the mutein hNGF Pro61Ser was compared to that of the wild type hNGF by means of TrkA phosphorylation test in BALB/C 3T3 cells stably transfected with the human TrkA receptor. Briefly, 10⁶ cells from this stable line were plated for each experiment and incubated for an hour in DMEM with the serum removed and supplemented with 0.05% BSA. Therefore, serial equimolar dilutions of wild type hNGF and mutein hNGF Pro61Ser (from 1 to 20 nM) are incubated for 10 minutes at 37°C. After washing with PBS, the cells were collected in 0.25ml of cold RIPA buffer supplemented by protease inhibitors and phosphates (50mM Tris pH 7.4, 150mM NaCl, 1% Triton X100, 1% Na deoxycholate, 10mM EDTA, cocktails of protease inhibitors (Roche), 1 mM sodium orthovanadate, 50 mM NaF, 1 nM okadaic acid). The insoluble fraction was removed by centrifuging at 10,000 rpm for 5 minutes. The extracts were quantified using the Lowry test (BIORAD) and standardised in the subsequent Sandwich ELISA test. This test includes the immobilization of the monoclonal anti-TrkA MNAC13 antibody (Cattaneo et al., 1999) on Maxi sorb 96 well plates, by incubation at 4°C for 16 hours and a concentration of 10 µg/ml in sodium carbonate buffer 0.1M pH 9.6. After an hour of blocking with PBS containing 3% milk (MPBS) at room temperature, the standardised quantities of each extract were incubated for two hours at room temperature. After incubation with the primary anti-phosphotyrosine antibody TrkA (cell signalling) diluted 1:1000 in MPBS and with the secondary antibody (anti-rabbit peroxidase conjugated), it was possible to detect the phosphorylation activity after NGF mutant binding by reading the optical density at 450 nm (OD450) by incubation with the TMB
substratum (TECNA). The colorimetric reaction was blocked by addition of a volume of 0.5M H₂SO₄ and the spectrophotometric reading taken using an ELISA Reader (Spectra). The induction of TrkA phosphorylation by hNGF Pro61Ser is completely similar to that of wild type hNGF (Figure 8).

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PC12 cells neurite outgrowth

Neurite outgrowth in PC12 cells was also evaluated by treating for a week 25,000 cells plated on collagenated plates with 100 ng/ml of hNGF or of hNGF P61S, changing the culture medium every three days. The capacity of hNGFPro61Ser mutein to induce differentiation is indistinguishable from that of the wild type Hngf (Fig. 21).

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Survival and differentiation test of dorsal root ganglia (DRG) and superior cervical ganglia

The biological activity of hNGF61 protein has been assessed using a survival and differentiation test in dorsal root ganglia (DRG) from chicken embryos 7-9 days old and paravertebral sympathetic ganglia from chicken embryos aged 10-12 days, after incubation with wild hNGF and hNGF-61, at concentration intervals between 1 and 100 ng/ml. After dissection, the ganglia were left on a Hepes buffer (HBSS) for then to be cultivated on plates treated with poly-lysine (1%), in DMEM with 10% FBS, 2mM L-glutamine and antibiotics. The treatment with hNGF and hNGF-61 was performed at the beginning, then repeated every 48 hours for 2-3 weeks. The effect of hNGF and hNGF-61 on the neurite growth (time of appearance and length of neurites) after 24 hours was similar in the two treatments.

The biological activity of hNGF-61 on hypertrophy of the superior cervical ganglia (SCG) in neonate mice was evaluated. Mice were treated over 5 days with NGF-61 (5 micrograms/gram of body weight). After dissection, the ganglia were fixed in 4% paraformaldehyde on phosphate buffer and stained with toluidine blue. The dimension of the ganglia and the number of neurons in the ganglia was equivalent after treatment with hNGF or with hNGF-61.

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TF1 hematopoietic cell line expressing TrkA receptors

PC12 cells, DRG and SCG bioassays represent the classical assays to measure the potency of NGF activity. In order to compare the activity of hNGF and hNGF-61 on human
receptors, the biological activity of hNGF61 was analyzed in TF1 erythroleukemic cells. This human cell line express human TrkA receptors in the absence of detectable p75 (Chevalier et al., Blood 83: 1479-1485, 1994). NGF induces autophosphorylation of TrkA and could substitute for granulocyte-monocyte colony-stimulating factor (GM-CSF) to trigger the proliferation of the TF1 cell line. Thus, the assay provides a quantitative measure of NGF activity and potency on human TrkA receptors.

To perform the test, hNGF-61 and wild type hNGF (hNGF) were synthesized according to the procedure described above.

To test if hNGF-61 was equally potent as hNGF in inducing the proliferative response in TF1 cells, the latter were purchased from ATCC (ATCC #CRL-2003, United Kingdom) and cultured according to the protocol established by Kitamura T, et al. (J. Cell. Physiol. 140: 323-334, 1989). Cells were cultured for 1 week in the following medium (ATCC #30-2001): RPMI 1640 medium with 2 mM L-glutamine adjusted to contain 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 10 mM HEPES, and 1 mM sodium pyruvate (90 %), supplemented with 2 ng/ml recombinant human GM-CSF (R&D Systems #215-GM), and 10% fetal bovine serum. The cell culture were maintained at constant temperature of 37 °C under an atmosphere of 95% air and 5% CO2.

The TF1 proliferation assay was performed in 96 wells microtiter plates by incubating 15,000 cells per well in the presence of several doses of either huNGF or hNGF-61 ranging between 50,000 and 5 pg/ml. Cells were seeded 1h before adding treatments. A MTT Cell proliferation assay kit (ATCC #30-1010K) was employed to evaluate cell response: after a 40-hour culture period, 10 μl of MTT solution was added for an additional 4-hour incubation, according to the previously described colorimetric assay (Mosmann, J Immunol Methods 65:55, 1983). The optical density (OD) was determined at 570 nm in a microtiter plate reader, 16 h (O/N), following addition of detergent reagent (100μl per well).

The results showed that hNGF-61 was able to induce proliferation TF1 cells in a dose/dependent manner and that its potency was identical to the one showed by wild type hNGF (Figure 9) and to the one reported in literature (Chevalier et al., Blood 83: 1479-1485, 1994). Indeed, the concentration of NGF determining a half maximum effect on cell proliferation was equal to 0.80 ng/ml for hNGF-61, 0.78 for hNGF and to 1 ng/ml for mNGF (Chevalier et al., Blood 83: 1479-1485, 1994), which represent the golden standard for NGF potency and bioactivity. Thus the potency of hNGF-61 is identical to that of
huNGF and of mNGF also on a quantitative potency assay involving human TrkA receptors.

**Activation of TrkA pathway**

It is known that NGF induces cell survival through the activation of the Akt pathway and that the activation of cytoplasmic domain of TrkA Y490 promotes cell growth through the activation of the shear pathway (reviewed in Reichardt, Phil. Trans. R. Soc. B, 361: 1545-1564, 2006). The induction of cell growth and of cell differentiation and plasticity is also mediated by the activation of PKC pathway through PLCγ (reviewed in Reichardt, Phil. Trans. R. Soc. B, 361: 1545-1564, 2006). To show that hNGF-61 was able to activate different signalling pathways summarized in Figure 10, BALB/C 3T3 cells transfected with the human TrkA receptor were seeded and cultured in DMEM medium without serum and supplemented with 0.05% BSA. After 1 hour, commercial hNGF (hNGF R&D) purchased from R&D Systems Inc., hNGF and hNGF-61 produced from E. coli were added at different concentrations ranging from 5 to 25 ng/ml for 10 min at 37°C. After lysis in RIPA buffer as described above, the extracts were processed for Western blot analysis and incubated with antibodies recognizing the activated forms of TrkA (anti Pi TrkA Y490; Cell Signaling) and of Akt (anti-pi AkT Ser 473, Cell Signaling). The results were normalize for total TrkA receptor and total Akt using the antibody antiTrkA (R&D) and antiAkt (Cell Signaling), respectively.

Figure 11 shows that hNGF-61 induce a perfectly comparable activation of TrkAY490 domain and of Akt to that induced by other NGF species and sources tested.

**Example 5. Structural analyses of the complexes hNGF with TrkA and p75 receptors at the level of residue R100**

A mutation in hNGF at position 100 (Arg → Trp), R100W mutation, has been recently associated with syndromes of pain insensitivity by a genetic analysis (CIPA, Einarsdottir et al., 2004).

This constitute the basis and the starting point to develop a molecule of NGF with a reduced ability to activate unwanted pain pathways (see above).

To this aim, in the present invention, the position of residue Arg100 in hNGF has been analysed at the level of NGF known tertiary structures in complex with its natural receptors.
In particular, the structure of the complex between hNGF and the extra-cellular domain of p75 receptor was analysed (code PDB 1SG1, He and Garcia, (2004)); as demonstrated in Figure 12A, the residue Arg100 (dotted area shaded in grey) is directly involved in salt bridge with residue 75 of p75 (left-hand part of the complex) and is located within a large interface described in Figure 13. In particular, Figure 13 lists all the residues that mediate NGF-p75 interaction, pointing out the contribution of each residue to the binding interface. The mutation R100W is very likely to disrupt this salt bridge negatively affecting the interaction between NGF and p75 and concerning this position other mutations of hNGF could have a similar impact of the R100W substitution. In particular, it is revealed that among residues in sequence 95-101, in addition to position R100, residues Gln96, Trp99 and Phe101 interact more stringently with receptor p75 (see Figure 13).

Concerning the analyses of the structure of the complex between hNGF and the domain d5 of TrkA (code PDB 1WWW, Wiesmann et al., (1999)), Figure 12B demonstrates that the residue Arg100 of hNGF is located close to the C-terminus of domain d5. Considering that in the extracellular domain of the complete TrkA receptor, a linker of 58 amino acids which connects domain d5 to the transmembrane domain is also present, the present structure indicates the possibility that such linker may be in direct contact with the NGF residue Arg100 and with nearby residues (in particular residues contained in the interaction patch between NGF and p75, between positions 95 and 101). The alteration of such residues could have similar consequences than the Arg100Trp mutation.

**Example 6. Preparation of wild type huNGF or NGF-61 mutated in position 100**

To obtain a derivative of hNGF-61 carrying a mutation at position Arg100, with the aim of obtaining a comparable survival, differentiation and proliferative activity, but a reduced ability to activate pain-related signalling, variants of wild type hNGF or hNGF Pro61Ser were engineered in which the residue Arg100 was substituted with Trp, Ala, Glu, Lys and Gln respectively. The residue Arg100 was substituted in the wild type hNGF or hNGF-61 proteins by site-specific mutagenesis according to the Stratagene protocol. The occurrence of the mutagenesis was verified by DNA sequencing.

To obtain the protein, the cDNA was cloned in the prokaryotic vector pETM11 and expressed and purified according to the pulsed-refolding protocol described by Rattenholl et al. (2001).
The refolded protein yields of the different mutants were compared to those obtained for wild type huproNGF (Fig. 14A). Mutants Arg100Lys and Arg100Gln (Fig. 14B) gave the same yields as wild type huProNGF, while Arg100Trp gave a low expression yield (Fig. 14B). The expression of ARg100Ala and ARG100Glu gave an intermediate amount of protein with respect to huproNGF and Arg100Trp (Fig. 14B).

**Binding affinity of hNGF-100 muteins for TrkA and p75**

The affinity for TrkA and p75 of NGF muteins in position 100 was compared using BiaCore experiments, similar to those described above for wild type hNGF and mutein NGF Pro61Ser. Kinetic analyses for TrkA are reported in Figure 15 and in Figure 16 for p75. The affinities for TrkA are very similar for all the muteins (Figure 15). By contrast, the affinity for p75 is variable according to the mutein. Only the mutein Arg100Lys (Figure 16B) displays a comparable affinity to wild type hNGF (Figure 16A). The substitution of the positive charge with a negative one in mutein Arg100Glu (Figure 16E) causes a drastic reduction in the affinity for p75. A reduction in the affinity is also observed for the Arg100Ala mutein (C) and for Arg100Gln (D).

Similarly, the affinity of hNGF-61 mutants in position 100 for the TrkA and p75 receptors was compared to the one showed by hNGF-61 using the BiaCore system as described above, by immobilizing through amine coupling 8000RU of TrkA immunoadhesin and 3900RU of p75 immunoadhesin. The kinetic analyses are reported in Fig. 17 and confirm what shown for R100 mutants on the wild type huNGF backbone. From this figure, it is clear that the affinity for the TrkA receptor is unchanged for all the mutants analyzed (hNGF-61: Fig. 17A; hNGF-61 Arg100Lys: Fig. 17C; hNGF-61 Arg100Ala: Fig. 17E; hNGF-61 Arg100Gln: Fig. 17G; hNGF-61 Arg100Glu: Fig. 17I). On the contrary, the affinity for the p75 receptor was comparable only for the conservative mutation Arg100Lys (hNGF-61: Fig. 17B; hNGF-61 Arg100Lys Fig.17D), while a reduced affinity was shown by hNGF-61 mutants Arg100Ala (Fig. 17F) and hNGF-61 Arg100Gln (Fig. 17H). The substitution Arg100Glu, in analogy with what shown for R100 mutants on the wild type huNGF backbone, resulted in a drastic reduction of the affinity for the p75 receptor, probably due to the substitution of the positive charge with a negative one.

**TrkA phosphorylation**
The ability of the hNGF muteins in position 100 to induce overall TrkA phosphorylation in 3T3 BALB/C cells as described above was evaluated. hNGF muteins in position 100 show similar activity compared to that of the wild type hNGF (Figure 18). However, muteins Arg100Ala and Arg100Glu display a slight reduction in TrkA phosphorylation.

The PI3K/AKT and PCL-γ1 pathways are particularly interesting since the activation of TrkA receptor through these pathways results in hypersensitization of the TRP channel to thermal and mechanical stimuli and thus results in induction of pain (Chuang et al., Nature 411, 957–962, 2001; Prescott and Julius Science 300, 1284–1288, 2003; Bonnlington and McNaughton J Physiol (London) 551, 433-446, 2003). Thus, to induce cell survival and differentiation and to avoid the triggering of pain stimuli, an ideal NGF mutant should be able to activate Akt and Shc/Ras pathways, while having a reduced efficacy in activating the PCL-γ1 pathway.

To determine which pathways were activated by the various hNGF R100 mutants, BALB/C 3T3 cells expressing the human TrkA receptor were incubated with NGF R100 mutants under the conditions described before. All mutants were used in the range of 50-100 ng/ml. Data shown are for 100 ng/ml.

The results showed that while the mutants Arg100Ala (A), Arg100Lys (K), Arg100Gln (Q) and Arg100Val (V) activated the shc, Akt and PKC pathways (Fig. 19 and 20), the mutant Arg100Trp (W) and the mutant Arg100Glu (E) activated only the Akt pathway (Fig. 19 and 20).

Neurite outgrowth in PC12 cells
The interaction of the various hNGF-61 mutants was verified in the PC12 assay according to the protocol described above.

Figure 21 shows that both hNGF (Fig. 21A) and hNGF-61 (Fig 21B) were able to induce and differentiation in PC12 cells, while in the absence of hNGF or hNGF 61 PC12 cells did not differentiate (Fig. 21C). A pro-differentiation effect was obtained by hNGF Arg100Lys (Fig. 21D) and by hNGF-61 Arg100Lys (Fig. 21E). The mutation Arg100Glu, in line with the results obtained in the TrkA phosphorylation assay described above, showed a reduced differentiation activity with respect to hNGF and hNGF-61 (Fig. 21F and 21G, respectively).

TFI proliferation
The biological activity of hNGF mutants and hNGF-61 mutants in position R100 was also tested in the proliferation assay on TF1 cells, according to the protocol described above. As positive controls, hNGF R&D (commercial from R&D systems), hNGF synthetized according to the procedure described in example (hNGF) and hNGF-61 were used.

As in example 4, curves describing the proliferative response of TF1 cell line to the various mutants were obtained. The overall shape of the proliferative curve as a function of [mutein] concentration was similar in all cases.

Table 2 reports the concentrations of hNGF mutants at which the half-maximum (50%) proliferative response it was observed.

### Table 2. Concentration of single and double mutants necessary to achieve half-maximum (50%) TF1 cell proliferation

<table>
<thead>
<tr>
<th>Mutants</th>
<th>Concentration corresponding to 50% cell proliferation (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hNGF R&amp;D</td>
<td>0.89</td>
</tr>
<tr>
<td>hNGF</td>
<td>1.80</td>
</tr>
<tr>
<td>hNGF-61</td>
<td>1.68</td>
</tr>
<tr>
<td>hNGF Arg100Ala</td>
<td>4.27</td>
</tr>
<tr>
<td>hNGF-61 Arg100Ala</td>
<td>2.30</td>
</tr>
<tr>
<td>hNGF Arg100Glu</td>
<td>1.28</td>
</tr>
<tr>
<td>hNGF-61 Arg100Glu</td>
<td>1.89</td>
</tr>
<tr>
<td>hNGF Arg100Lys</td>
<td>0.92</td>
</tr>
<tr>
<td>hNGF-61 Arg100Lys</td>
<td>0.94</td>
</tr>
<tr>
<td>hNGF Arg100Gln</td>
<td>1.11</td>
</tr>
<tr>
<td>hNGF-61 ARg100Gln</td>
<td>3.23</td>
</tr>
</tbody>
</table>

The results showed that the value for hNGF R&D, hNGF and hNGF-61 was comprised between 1 and 2 ng/ml. The mutations hNGF Arg100Glu, hNGF Arg100Lys and hNGF Arg100Gln did not affect the rate of TF1 cell proliferation, since the concentration necessary to achieve 50% cell proliferation was comprised in the range of 1-2 ng/ml. When the mutation was performed on the NGF-61 background, the same result was obtained for hNGF-61 Arg100Glu and Arg100Lys, while the mutation hNGF-61 Arg100Gln provoked a shift of the concentration to 3.23 ng/ml. The mutation Arg100Ala decreased the ability of
hNGF and hNGF-61 molecules to achieve the 50% proliferation of TF1 cells, with a concentration of 4.27 and 2.30 ng/ml, respectively. Therefore, it can be concluded that all muteins tested at position 100, including those in the hNGF-61 backbone, show a comparable activity and potency on the proliferative TF1 assay, showing a 50% proliferative index between 0.9 and 4.27 ng/ml.

In conclusion, it is possible to obtain hNGF derivatives mutated in position 100 and possibly in other positions, showing an identical capacity than wild type hNGF in inducing of survival, differentiation and proliferation, but a differential ability in activating the signalling pathways of TrkA receptor linked to nociception. In particular, the mutants Arg100Trp and Arg100Glu do not activate the PCL-γ1 and Shc/Ras pathways. This conclusion has been verified both on wild type huNGF and NGF-61 backbone.

**Example 7. Efficacy of hNGF-61 in a murine model of Alzheimer’s disease**

The efficacy of hNGF-61 was evaluated after intranasal administration in 6 month old AD11 anti-NGF mice (Ruberti et al, 2000; Capsoni et al. 2000). hNGF-61 was administered every 2 days for 2 months. As a positive control, human recombinant NGF was administered (rhNGF; Alomone Laboratories, Jerusalem). For the administration, the mice were anæsthetised with an intraperitoneal injection of 2,2,2-tribromomethanol (400 mg/kg). NGF-61 and rhNGF were administered in a 10 μM dose, mixed into 40 mM PBS, pH 7.4 for a total volume of 48 μl. This volume was subdivided into 3 μl drops, which were then allowed to fall into nostrils, alternating between nostrils, for a period of 30 min. Control mice received an equal quantity of PBS.

ELISA was used to check that sufficient quantities of hNGF-61 had reached the brain. Briefly, homogenized samples of mice brains were added to the wells of a plate, in which had been adhered the monoclonal antibody alfaD11 (5 μg/ml). After an incubation period of 2 hours at ambient temperature and washing in PBS–0.05% Tween 20 and PBS, the quantity of hNGF and hNGF-61, not bound to the transgenic antibody (= free NGF), was detected using the monoclonal antibody 4GA.

The effects on the Alzheimer’s phenotype were assessed by incubating sections of alfaD11 mice brain with antibodies directed against the transfected acetycholine enzyme (Chemicon, Temecula, CA), against the phosphorylated tau protein (mAb anti-Ser202, Pierce, Rockford, IL) and against the N-terminal of the beta-amyloid peptide (Santa Cruz, Santa Cruz, CA).
The administration of hNGF-61 and rhNGF induced an increase in the levels of free NGF equal to double that observed in alfaD11 mice not treated with NGF-61 or rhNGF (mice treated with NGF-61: 23 ng/mg tissue; mice treated with rhNGF: 20 ng/mg tissue, mice treated with vehicle: 8 ng/mg tissue).

Number of cholinergic neurons, of neurons expressing phosphorylated tau and of dystrophic neurons
As with hNGF, the administration of hNGF 61 induced an increase in the number of cholinergic neurons in the basal proencephalon of alfaD11 mice, which have, therefore, recovered the cholinergic deficit (Figure 22). Furthermore, the administration of hNGF-61 induced a the reduction in the number of neurons that express the tau protein in its phosphorylated form (Figure 23) and the number of groups of dystrophic neurites in the hippocampus (Figure 24). Therefore, hNGF-61 effectively improves Alzheimer’s phenotype.

Cognitive deficits
The efficacy of hNGF-61 on cognitive deficits AD11 anti-NGF mice was assessed after intranasal administration in mice of 15 months of age. NGF-61 was administered every 2 days for 1 month. As a positive control, recombinant human NGF was administered (rhNGF; Alomone Laboratories, Jerusalem). For the administration, mice were anaesthetised with an intraperitoneal injection of 2,2,2-tribromoetanololu (400 mg/kg). hNGF-61 and rhNGF were administered at a dose of 10 µM, mixed in 40 mM PBS, pH 7.4 at a dose of 480 ng/kg. The control mice received an equal volume of PBS.

The effects of hNGF 61 on the cognitive deficit were assessed by means of the object recognition test. The test consists of three phases. During the first phase, the mice explored for 5 minutes a Plexiglas square labyrinth with black walls and washable surfaces. The session was repeated after 30-minutes. On the next day, the mice explored 2 identical objects (A1 and A2) for 10 minutes and were removed from the labyrinth. After twenty-four hours, the mice were placed again in the labyrinth in the presence of an object identical to those of the previous day (A1 or A2) and of a second object (B), never explored before and different from the previous one in terms of shape and colour. During this test phase, the mice that succeed in remembering the old object (A1 or A2), explored
more the new object (B), whilst the mice with cognitive deficits (such as AD11 anti-NGF) indifferently explored the old object (A1 or A2) and the new one (B).

The results of the test (Figure 25) demonstrate that AD11 mice treated with PBS have extensively explored the old object, resulting in a negative discrimination index, statistically different from that of non-transgenic mice. Mice treated with rhNGF and hNGF-61 have a discrimination index around zero. There is a significant difference between animals treated with hNGF-61 and those treated with PBS.

_Fully blown neurodegeneration_

At the end of the object recognition test, the efficacy of hNGF-61 and recombinant hNGF (hNGF, Alomone labs) treatment in 15 months old AD11 mice were studied by analyzing the neurodegeneration using immunohistochemical techniques described in above. The administration of hNGF-61 and hNGF determined a similar rescue of cholinergic neurons in the medial septum and diagonal band of Broca (Fig. 26A). The administration of hNGF-61 was also effective in reducing the percentage of hippocampal area occupied by beta amyloid plaques (Fig. 26B). The number of tau-positive neurons in the lateral entorhinal cortex was revealed using mAB AT8, recognizing phosphorylated form of Ser 202, and mAB AT270 (Pierce Endogen), a monoclonal antibody that recognizes the phosphorylated form of Thr181, known to be one of the phosphorylated aminoacids in paired helical filaments-tau proteins.

A decrease in the number of phosphotau-positive neurons was shown in AD11 mice treated with hNGF-61 (Fig 26C).

This example demonstrates that, in AD11 mice, hNGF-61 is effective in rescuing even a more severe neurodegeneration.

**Example 8. Efficacy of the 4GA antibody in discriminating between hNGF-61 and endogenous NGF.**

The efficacy of the 4GA antibody in detecting hNGF-61 in the human serum, therefore the presence of endogenous hNGF was evaluated as follows. Known quantities of hNGF-61 (concentration from 1 to 50 pg/ml) were added to 10 serums from healthy volunteers. A two-site ELISA was carried out, using 96-well plates. The solid phase was obtained by incubating the plates overnight at 4°C with a solution containing 1 µg/ml of monoclonal
alfaD11 antibody in an 0.1 M solution of sodium carbonate buffer (sol. A) at pH 9.5. The monoclonal alfaD11 antibody recognises both the endogenous NGF and NGF-61. After 3 washes in phosphate buffer (0.1 M, pH 7.4) and 3 washes in buffer and 0.1% Tween 20, the plate was incubated with a solution made up of a phosphate buffer and 2% milk (sol. B) for 2 hours at 37°C. After discarding solution B, for the standard curve, hNGF-61 was added at a range of concentrations from 1 to 1000 pg/ml. In wells containing the samples, human serum was added in quadruplicates. A duplicate contained only the human serum, the second duplicate contained the serum with known quantities of hNGF-61 equal to 5, 10, 25, 50 pg/ml. After incubating for 2 hours at ambient temperature and having washed in phosphate buffer and phosphate buffer and 0.1 % Tween 20, the serums were incubated (2 hours at ambient temperature) with the supernatant of the hybridoma secreting the detector antibody 4GA diluted 1:10 in phosphate buffer and 2% milk. After incubation with the secondary antibody (anti-rat peroxidase conjugated IgG, DAKO) the binding activity was measured by reading the optic density at 450 nm (OD450) by incubation with substrate TMB (Sigma). The colorimetric reaction is blocked by adding a volume of 0.5M H₂SO₄ and spectrophotometric reading is realised using an ELISA Reader. The experiment was carried out in parallel, using as a detecting antibody, the monoclonal alfaD11 antibody. The results demonstrate that 4GA is able to recognise only hNGF-61, and not endogenous NGF (Figure 27A). The alfaD11 antibody is unable to distinguish hNGF-61 from endogenous NGF. In this case, the values obtained in the serum correspond to the sum of the values for endogenous NGF plus the quantity of NGF-61 that was exogenously added to the sera (Figure 27B).

Example 9. Absence of immunogenic effects of hNGF-61 in mice.

The absence of immunogenic effects has been evaluated after intranasal administration over 2 months. At the end of the treatment, blood was collected from the mice, centrifuged to obtain the serum and the level of anti-NGF IgG was measured using ELISA. The solid phase was obtained by incubating the columns overnight at 4°C with a solution containing 5 µg/ml of murine NGF mixed in 0.1 M of pH 9.5 sodium carbonate buffer. After 3 washes in phosphate buffers (0.1 M, pH 7.4) and 3 washes in buffer and 0.1 % Tween 20, the column was incubated in a solution constituted by phosphate buffer and 2%
milk for 2 hours at 37°C. After removing the solution with the milk, the mouse blood was added in various dilutions. In some shafts, as a positive control, was added the monoclonal alfaD11 antibody diluted 1:1000, 1:5000, 1:10,000. After incubating for 2 hours at an ambient temperature and after washing in phosphate buffer and phosphate buffer and 0.1% Tween 20, the columns were incubated (2 hours at ambient temperature) with an anti-rat IgG antibody (in the case of shafts with mAb alfaD11) or with an anti-mouse IgG antibody. Both antibodies were connected with horseradish peroxides (HRP). After incubation with the secondary antibody, the reaction was revealed by incubation with substrate TMB (Sigma). The colorimetric reaction was blocked by adding a volume of 0.5M H₂SO₄ and the spectrophotometric reading realised with an ELISA Reader. The result show an absence of murine IgG recognising NGF (Figure 28). Therefore, the intranasal treatment does not promote the insurgence of undesired immunogenic reactions.

**Example 10. Absence of allergogenic effects.**

The absence of allergogenic effects has been verified by analysing the blood of mice after administration of hNGF-61 for 2 months. The blood was left to coagulate at an ambient temperature and then centrifuged at 1200 g for 10 min. The quantity of total IgE was measured using ELISA, as described by Braun et al., (Eur J Immunol 28, pp. 3240-3251 (1998)).

No increase in IgE production was observed when compared to the mice treated with PBS (Fig. 29).

**Example 11. Prevention of pain induced by hNGF-61**

The efficacy of the MNAC13 and alfaD11 antibodies in preventing/eliminating the pain induced by the administration of hNGF-61 was demonstrated using an experimental protocol and a concentration of NGF known to be able to induce allodynia (Hao et al., Neurosci. Lett. 286, pp. 208-212 (2000)).

hNGF-61 (7 μg/day; n = 10) was administered intracerebroventricularly (icv) for 14 days by osmotic pumps implanted under the skin and connected by a catheter (28 gauge, 0.64 mm) inserted into the right lateral cerebral ventricle.

The antibody MNAC13 was used to prevent any pain caused by the administration of hNGF-61. Therefore, a second group received MNAC13 subcutaneously in a dose of 0.4 mg/kg one hour before the administration of hNGF-61.
A third group of animals (n = 10) received, in addition to hNGF-61, and starting 1 day after the implantation of the osmotic pump, a dose of the monoclonal mAb alfaD11 antibody (2 mg/kg i.p.) for 8 consecutive days.

A fourth group of rats received PBS through the osmotic pump and is the control group.

The behavioural test performed to determine the effects on pain was the hot plate test. The rats underwent this test for 5 consecutive days prior to the osmotic pumps being implanted. The test was carried out with a hot plate temperature of 54°C and the latency time taken for the rats to start licking their rear paws was measured.

The results demonstrated that the I.C.V infusion of hNGF-61, at a concentration of 7 μg/day, induces a significant reduction in the latency time in response to heat (Fig. 30). The administration for 8 consecutive days of the antibody αD11 induces a significant decrease in the hyperalgesic response provoked by hNGF-61 (Fig.30).

**Example 12. Efficacy of hNGF-61 in a murine model of Rett’s syndrome**

The efficacy of hNGF-61 was evaluated after intranasal administration in 6-week old protein Mecp-2 knock-out mice (Chen et al., 2001). hNGF-61 was administered every day for 1 month at a dose of 1mg/kg in association with the antibody αD11 (2 mg/kg, i.v) or MNAC13 (0.4 mg/kg, s.c.).

The effects on the Rett phenotype were assessed by immunohistochemical and biochemical analysis of the expression of the following proteins: acetylcholine transferase, MAP2, synaptophysin, PS95, NR1, NR2A and 2B, GLUR1, NF200. From the behavioural point of view, the mice were assessed for alterations in socialisation and locomotor activities, for anxiety/fear-related behaviours and for any alterations in pain sensitivity.

The post-treatment analysis showed that NGF-61 was able to ameliorate the general behavioural alterations of the knockout mice, while the histological analysis of the brain showed no alterations of the basal levels cytoskeletal and synaptic proteins (such as MAP2, NF200, synaptophysin and PS95) in Mecp2 mice treated with placebo or NGF-61 with respect to wild type mice. In Mecp-2 mice treated with placebo, the expression of NR1 and GluR1 was unchanged with respect to wild type mice and mice treated with NGF-61. The number of neurons expressing glutamate receptors subunits NR2A was decreased in Mecp2 mice with respect to wild type mice, whereas the expression of the NR2B receptor was increased. The treatment with NGF-61 determined a recovery by the expression of this receptors, with levels of expression comparable to those of wild type
mice. Acetyl cholinesterase levels were also mildly decreased in Meep2 mice and the treatment with NGF-61 recovered the levels of this enzyme.

**Example 13. Intermittent dosing**

The frequency of administration is a critical parameter to obtain the desired therapeutic effect.

To determine the frequency of administration necessary to achieve the rescue of the neurodegenerative phenotype, hNGF-61 (480 ng/kg = 0.45 pmol) was administered to 6 months old anti-NGF AD11 mice according to the following schedule: one group of mice received hNGF-61 once a week, the second group of mice received hNGF-61 twice a week and the third group of mice received hNGF-61 three times per week. The treatment lasted for 15 days.

*Cognitive deficits*

At the end of the treatment, an object recognition test was performed to evaluate the efficacy of hNGF-61 in rescuing the memory deficit in AD11 mice. The results showed that only the group of AD11 mice which received hNGF-61 three times per week completely recovered the memory deficit (Fig. 31).

**Number of cholinergic neurons, of neurons expressing phosphorylated tau and of dystrophic neurons**

The analysis of the brains was performed as described above. The neurostereological counts of the number of cholinergic neurons in the basal forebrain nuclei confirmed that a statistically significant increase in the number of ChAT-positive neurons in MS/DBH and NBM was obtained only in AD11 mice treated three times per week (Fig. 32A and 32B), even if dosing twice a week gives the same trend even if not statistically significant because of higher variability. The calculation of the percentage of hippocampal area occupied by β-amyloid clusters was significantly decreased in all groups of AD11 treated with NGF-61 (Fig 32C). The analysis of phosphotau-positive dystrophic neurites revealed that only the treatment with a three times per week frequency was effective in decreasing their number (Fig. 32D). In conclusion, the frequency of administration should not be inferior than 3 times per week to obtain a full rescue of the Alzheimer-like neurodegeneration.
Example 14. The rescue of the neurodegenerative phenotype by hNGF-61 is maintained after 1 month of wash-out in a mouse model for Alzheimer’s disease

After the introduction of symptomatic treatment, the next target for therapeutic approaches in Alzheimer’s disease is the development of disease-modifying drugs (Scarpini et al., 2003). For a neurodegenerative disorder, such as Alzheimer’s disease, a disease-modifying intervention is typically considered to be the one that can reduce disease progression rate, due to an effect of the drug on the pathophysiological mechanism of the disease and resulting in long-lasting changes in disability (Vellas et al, 2007). This contrasts with interventions that are only able to relieve impairments as measured by the signs and symptoms of the disease (Vellas et al, 2007). Thus, a real disease modifying agent should not only improve cognitive deficit but also stop or slow down the progression of neurodegeneration, in a way that this improvement is maintained after the discontinuation of the administration of the therapeutic agent. Wash out studies in experimental models are essential to assess the disease modifying property of a molecule.

Cognitive deficits

To determine whether the effect of hNGF-61 was persistent after stopping its administration, 15 months old AD11 mice were treated with hNGF or hNGF-61 for 1 month and analyzed for memory in the object recognition test (ORT). The results show that there is a rescue of the memory deficit both when huNGF LLG and hNGF-61 were administered (Fig.33Aa). At the end of the ORT, mice were maintained for 1 month without treatment (wash-out) and tested again for memory deficits. Fig33Ab showed that the amelioration of the memory deficit was persistent.

Number of cholinergic neurons, of neurons expressing phosphorylated tau and of dystrophic neurons

At the end of the behavioural test, mice were sacrificed and the brain tissue processed for immunohistochemical analysis to quantify the number of cholinergic neurons in the basal forebrain, the percentage of hippocampal area occupied by β-amyloid plaques and the number of phosphor-tau positive neurons in the entorhinal cortex. The results showed that there was no difference among the three groups of AD11 mice in the number of cholinergic neurons in the basal forebrain (Fig. 33B) while a marked decrease in the
percentage of hippocampal area occupied by β-amyloid plaques was observed in mice treated with HNGF-61 but not with hNGF (Fig. 33C). The immunohistochemistry against phospho-tau revealed that the number of neurons expressing phosphorylated tau was decreased in both AD11 mice treated with hNGF and with NGF-61 (Fig. 33D).

Example 15. Comparison of efficacy of intranasal versus conjunctival delivery of hNGF-61 in mice

The possibility to deliver peptides to the brain by applying the peptide solution to the conjunctiva mucosa and ameliorate neurodegenerative syndromes such as Alzheimer's disease has been covered by scientific publications (Lambiase et al., Brain Res. 2007 Jan 5;1127(1):45-51) and patent or patent applications (EP1135105 and WO2007020672).

In this set of experiments, the efficacy of intranasal delivery was compared to that of conjunctival delivery. To this purpose, a group of 6 months old AD11 mice was treated with hNGF-61 (480 ng/kg = 0.48 µg/kg = 0.45 pmol for 15 days) via intranasal delivery, as previously described. Two other groups of AD11 mice were treated by applying hNGF-61 drops to the conjunctiva at the doses of 480 ng/kg (= 0.48 µg/kg = 0.45 pmol) and 4800 ng/kg (= 4.8 µg/kg = 4.5 pmol), respectively. To perform the application of NGF-61 solutions, mice were not anaesthetized and were placed on their belly with the head elevated with respect to the rest of the body. Drops were placed on the border of inferior eyelid. As controls, WT mice and a group of AD11 mice received eye drops of placebo.

Cognitive deficits

After 15 days of administration, with a frequency of 3 administrations per week, the mice were analysed in the object recognition test. The results showed that both intranasal and ocular treatment were able to rescue the behavioural deficit (Fig. 34). At the end of the behavioural test, mice were perfused with 4% paraformaldehyde and the brains processed for histological analysis as described before.

Histology

The histological analysis showed that the intranasal delivery of hNGF-61 induced the rescue of the cholinergic neurons loss in the MS + DBH (Fig. 35A) and in NBM (Fig. 35B), while the two doses of hNGF-61 given through the conjunctiva were completely ineffective in rescuing the cholinergic deficit in MS +DBH (Fig. 35A). In NBM, a partial
rescue, not statistically significant, was achieved only at the dose of 4.5 (4.8 µg/kg as in Fig. 35B) pmol. The dose of 0.45 (or 0.48 µg/kg as in Fig. 35B) pmol induced a number of ChAT-positive neurons that was statistically inferior to that achieved after intranasal delivery of the same amount of NGF-61 (Fig. 35B). Both intranasal and conjunctiva administration induced a significant decrease in the number of β-amyloid clusters in AD11 mice (Fig. 35C). The intranasal administration and the conjunctiva administration at the dose 4.5 pmol induce the rescue of phospho-tau positive dystrophic neurites in the entorhinal cortex (Fig. 35D). The dose of 0.45 pmol administered through the conjunctiva was not effective (Fig. 35D).

Thus, it can be concluded that the intranasal administration is more effective in rescuing the neurodegeneration in AD11 mice than the conjunctival route.

**Example 16. Absence of loss of body weight after intranasal administration of hNGF-61**

It is known that one of the side effects observed when NGF was administered to Alzheimer’s patients during a clinical trial is the loss of body weight (Eriksdotter et al., 1998). Such side effect lead to the end of the trial. To exclude that the intranasal administration of hNGF-61 provokes loss of body weight in 6 months old treated mice, the individual body weight of the animals was recorded before, during and at the end of the treatment.

The result is shown in Fig. 36. No statistically significant difference was observed among and within treatment groups.

**Example 17. Absence of induction of sensory hyper-innervation by hNGF-61 in mouse spinal cord**

The first clinical trial in AD patients was interrupted due to the onset, among other side effects, of back pain (Eriksdotter et al., 1998). Indeed, when infused into the brain ventricular system, NGF causes pain as a consequence of an hyper-innervation of the spinal cord leading to hyper stimulation of dorsal root ganglia nociceptive neurons (Tuszynski, *Lancet Neurol.* 1: 51–57, 2002).

The absence of sensory hyper-innervation of the spinal cord induced by the administration of huNGF (Alomone labs) or NGF-61 was verified in 15 months old AD11 mice after 1 month of treatment, according to the protocol described in example 7.
Spinal cords of the animals were dissected and the lumbar portion (which should receive the highest amount of NGF from the CSF, if present) was sectioned (40 µm) and the sections processed for immunohistochemistry against calcitonin gene-related peptide, a neurotransmitter marker of sensory fibers. The polyclonal antibody was purchased from Chemicon International Inc. (Temecula, CA).

The spinal cord sections were viewed using a Nikon microscope. The cumulative area occupied by calcitonin-gene relate peptide (CGRP)-positive fibers in regions of the gray matter of the spinal cord was obtained using the morphometry LUCIA program (Laboratory Imaging Ltd., Prague, Czechoslovakia). The numbers of stained pixels in each section were summed and converted into square micrometers via pre-programmed calibration standards. Each pixel represented an area of 0.24 µm². This degree of resolution allowed immunoreactivity to be detected in single fibers. The region of the spinal cord in which the areas of immunoreactive fibers were measured in a dorsal region including Rexed’s laminae III–V, that normally contains CGRP-IR afferent fibers.

The statistical analysis revealed that there were no differences between AD11 mice treated with placebo compared to either huNGF or NGF-61 (Fig. 37). Thus, it can be concluded that the intranasal administration of hNGF-61 does not induce a sensory hyper-innervation of the spinal cord. Therefore, no hypersensitivity to pain is expected to be observed in subject treated with intranasal hNGF-61.

**Example 18. Effects of treatment with hNGF-61 Arg100Glu on the neurodegenerative phenotype in AD11 mice**

The efficacy of the mutant hNGF-61 Arg100Glu in rescuing the Alzheimer-like phenotype in 6 months old AD11 mice was compared to the one showed by hNGF-61. To this aim, AD11 mice were treated for 15 days with intranasal hNGF-61 or hNGF-61 Arg100Glu at the dose of 480 ng/kg (0.45 pmol) and 540 ng/kg (0.51 pmol), respectively.

*Cognitive deficits*

At the end of the treatment the mice were analyzed for the cognitive deficit using the object recognition test, as described above.

The results showed that both hNGF-61 and hNGF-61 Arg100Glu rescued the cognitive deficit in AD11 mice (Fig. 38) and that there was no statistical difference between the degree of rescue obtained by hNGF-61 and hNGF-61 Arg100Glu (P = 0.65).
Thus, it can be concluded that hNGF-61 R100E is as effective as hNGF-61 in rescuing the cognitive deficit in AD11 mice.

**Histological analysis**

At the end of the behavioural test, the mice were perfused with 4% paraformaldehyde and the brains processed for histological analysis as described before. The histological analysis showed that the intranasal delivery of hNGF-61 Arg100Glu was as effective as hNGF-61 in determining the rescue of the cholinergic neurons in the MS + DBH (Fig. 39A) and in NBM (Fig. 39B). hNGF-61 Arg100Glu showed the same efficacy as hNGF-61 in diminishing the number of β-amyloid clusters of dystrophic neurites (Fig. 39C) and in decreasing the number of phosphotau-positive dystrophic neurites (Fig. 39D).

Thus, it can be concluded that the mutation in position Arg100Glu is comparable to hNGF61 and is effective in rescuing the neurodegeneration in AD11 mice.

**Example 19. Lack of hyperalgesia in mice treated with hNGF-61 Arg100Glu**

To assess the degree of hyperalgesia induced by hNGF-61 Arg100Glu, the compound was administered to CD-1 mice at the dose of 2.5 µg/g i.v. As positive control, the same dose of hNGF-61 was used. Cytochrome c was used as negative control since it is usually employed as a control treatment due to its similar physicochemical characteristics to NGF but without neurotrophic activity.

To assess the effects on thermal nociception, each mouse was placed in the center of a glass-covered cylinder (diameter 19 cm) hot plate apparatus (Ugo Basile Inc., Comerio, Italy) maintained at 52 ± 0.1 °C. Mice were tested on the hot plate 15, 30, 60, 180, and 360 min after the injection.

hNGF-61-treated animals showed increased nociceptive response when compared to cytochrome c-treated mice (Fig. 40). In particular, the number of jumps in hNGF-61-treated group was significantly higher than in the control group (Fig. 40A) and the latency to the first jump was shorter in hNGF-61-treated mice (Fig. 40B). Such an hyperalgesic profile emerged 30 min after injection, peaked at 60 min, and decreased slowly in the two subsequent assessments. The level of jumping response in hNGF-61 Arg100Glu was significantly lower than that shown by NGF-61 treated mice (Fig. 40 A,B) and comparable to what observed in the cytochrome c-treated mice.
The reduced nociceptive activity of mutein hNGF-61 Arg100Glu in comparison to hNGF-61 were further confirmed in the experimental protocol described in Example 11. Indeed results show that intracerebroventricular administration of mutein hNGF-61 Arg100Glu induces a reduced pain response, also with this alternative delivery route.

The reduced nociceptive activity of mutein hNGF-61 Arg100Glu allows to increase the amount of mutein deliverable by a systemic route without induction of pain. Thus, a higher systemic dosage can be used, allowing to increase the percentage of mutein crossing the blood brain barrier up to a therapeutical effective dose (Thorne and Frey, 2001).

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CLAIMS
1. A mutein of human NGF characterised by the fact that:
   a) it is therapeutically active;
   b) it does not induce undesired immune reactions;
   c) it is selectively recognised by a specific reagent with regard to endogenous hNGF.
2. The mutein of human NGF according to claim 1 in which the mutein is obtained by at least one amino acid substitution residing in loop III of hNGF.
3. The mutein of human NGF according to claim 2 in which the substituted amino acid in loop III is proline at position 61.
4. The mutein of human NGF according to claim 3 in which the amino acid substitution of proline in position 61 is with a serine.
5. The mutein of human NGF according to any one of previous claims further comprising at least one amino acid substitution able to substantially reduce its nociceptive activity.
6. The mutein of human NGF according to any one of previous claims further comprising at least one amino acid substitution leading to a reduced interaction of the mutein with the p75 receptor.
7. The mutein of human NGF according to claim 6 wherein the amino acid substitution is at any of positions 95-101.
8. The mutein of human NGF according to claim 7 wherein the amino acid substitution is that of the arginine in position 100.
9. The mutein of human NGF according to claim 8 in which the amino acid substitution of the arginine in position 100 is with an acidic amino acid or tryptophane.
10. The mutein of human NGF according to claim 9 in which the acidic amino acid is glutamic or aspartic acid.
11. The mutein of human NGF according to claim 7 in which the amino acid substitution is that of the glutamine in position 96.
12. The mutein of human NGF according to claim 7 in which the amino acid substitution is that of the tryptophan in position 99.
13. The mutein of human NGF according to claim 7 in which the amino acid substitution is that of the phenylalanine in position 101.
14. The mutein of human NGF according to any one of previous claims for therapeutic use.

15. Use of the mutein of human NGF according to any of claims 1 to 13 for the preparation of a medicament for the treatment of pathologies of the central and peripheral nervous system.

16. Use of the mutein of human NGF according to claim 15 in which the pathologies belong to the group of pathologies of the development of the nervous system, such as schizophrenia and Rett’s syndrome.

17. Use of the mutein of human NGF according to claim 15 in which the pathologies belong to the group of neurodegenerative pathologies, such as Alzheimer’s disease and multiple sclerosis.

18. Use of the mutein of human NGF according to claims 1 to 13 for the preparation of a medicament for the treatment of pathologies that belong to the group of ocular pathologies, such as corneal ulcers, neuritis of the optic nerve and neuropathies of the optic nerve of traumatic and ischemic origin.

19. Use of the mutein of human NGF according to claims 1 to 13 for the preparation of a medicament for the treatment or prevention of skin ulcers.

20. Use of the mutein of human NGF according to claims 19 wherein the skin ulcers occur in aging patients.

21. Use of the mutein of human NGF according to claims 1 to 13 for the preparation of a medicament for the treatment of age-related immunodeficiency.

22. Use of the mutein of human NGF according to claims 1 to 13 for the preparation of a medicament for the treatment of congenital insensitivity to pain and anhidrosis.

23. A pharmaceutical composition comprising a pharmaceutically acceptable and effective dose of the mutein of human NGF according to any of claims from 1 to 13 and suitable diluent and/or carrier.

24. The pharmaceutical composition according to claim 23 in which the dose is determined according to the concentration of endogenous human NGF.

25. The pharmaceutical composition according to claims 23 or 24 for final vehiculation to the central nervous system.

26. The pharmaceutical composition according to claims 23 to 25 for intranasal administration.
27. The pharmaceutical composition according to claims 23 to 25 for intraocular or ophthalmic administration.

28. The pharmaceutical composition according to claims 23 to 25 for systemic administration.

29. The pharmaceutical composition according to claim 23 for topical administration.

30. The pharmaceutical composition according to claims 23 to 29 in which the mutein of human NGF is associated with nanostructures.

31. The pharmaceutical composition according to claims 23 to 29 in which the mutein of human NGF is associated with liposomes.

32. The pharmaceutical composition according to any one of claims 23 to 31 further comprising a pain-killer agent.

33. The pharmaceutical composition according to claim 32 wherein the pain-killer agent is a neutralising anti-NGF or anti-TrkA antibody.

34. The pharmaceutical composition according to claims 32 or 33 wherein the pain-killer agent is formulated separately.

35. A specific reagent able to selectively recognise the mutein according to one of claims 1 to 13 with regard to endogenous hNGF.

36. The specific reagent according to claim 35 being a monoclonal antibody or a synthetic or a biotechnological or a humanised antibody, or a fragment thereof.

37. The specific reagent according to claim 36 being an antibody able to selectively recognise a mutein of hNGF having a serine at position 61.

38. Use of the specific reagent according to any one of claims from 35 to 37 to monitor the amount of the mutein of human NGF according to claims from 1 to 13 in a biological sample.

39. A kit to monitor the amount of the mutein of human NGF according to claims from 1 to 13 in a biological sample for therapy control comprising the specific reagent according to any one of claims from 35 to 37.
Fig. 1

Fig. 2
Fig. 3
Fig. 4

Fig. 5
Fig. 8

NGF dose response in TF1 cells

Fig. 9
Fig. 10

hNGF (ng/ml) - 5 10 25
hNGF R&D (ng/ml) - 5 10 25
hNGF P61S (ng/ml) - 5 10 25

anti PI TrkA (Y490)
anti TrkA total

anti PI Akt (S473)
anti Akt total

Fig. 11
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Fig. 13
Fig. 16
**Fig. 19**

**huNGF (100 ng/ml)**

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- anti PI TrkA (Y490)
- anti TrkA total
- anti PI Akt (S473)
- anti Akt total
- anti PI PLCγ (Y783)
- anti PLCγ total
Fig. 20
Fig. 26
Fig. 27
Fig. 30
Fig. 31
Fig. 32
Fig. 33
Fig. 34
Fig. 35
Fig. 36

Body weight (g.)

WT + placebo  AD11 + placebo  AD11 + NGF-61

Fig. 37

Area (μm²)

AD11 + placebo  AD11 + huNGF  AD11 + NGF61
Fig. 38
Fig. 39
Fig. 40
**INTERNATIONAL SEARCH REPORT**

**International application No**
PCT/EP2007/057215

**A. CLASSIFICATION OF SUBJECT MATTER**
INV. C07K14/48 A61K38/18

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)
C07K A61K

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 data base and, where practical, search terms used)
EPO-Internal, WPI Data, BIOSIS, EMBASE, Sequence Search, CHEM ABS Data

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

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<td>WO 2005/040335 A (HOLMBERG MONIKA [SE]; HOLMBERG DAN [SE]; MINDE JAN [SE]; HOLMGREN GOES) 6 May 2005 (2005-05-06) claims 1-16</td>
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Further documents are listed in the continuation of Box C.

See patent family annex.

* Special categories of cited documents:
  *A* document defining the general state of the art which is not considered to be of particular relevance
  *E* earlier document but published on or after the international filing date
  *L* document which may throw doubts on priority claims or which is cited to establish the publication date of another citation or other special reason (as specified)
  *O* document referring to an oral disclosure, use, exhibition or other means
  *P* document published prior to the international filing date but later than the priority date claimed

*"* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

*"* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

*"* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

*"* document member of the same patent family

Date of the actual completion of the international search
24 September 2007

Date of mailing of the international search report
08/10/2007

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Fax +31-70 340-3016

Authorized officer
Vollbach, Silke

Form PCT/ISA/210 (second sheet) (April 2005)

page 1 of 2
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<td>URFER R ET AL: &quot;SPECIFICITY DETERMINANTS IN NEUROTROPIN-3 AND DESIGN OF NERVE GROWTH FACTOR-BASED TRKC AGONISTS BY CHANGING CENTRAL BETA-STRAND BUNDLE RESIDUES TO THEIR NEUROTROPHIN-3 ANALOGS&quot; BIOCHEMISTRY, AMERICAN CHEMICAL SOCIETY. EASTON, PA, US, vol. 36, 1997, pages 4775-4781, XP002073889 ISSN: 0006-2960 table 1</td>
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**INTERNATIONAL SEARCH REPORT**

**Box II  Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [ ] Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:

2. [X] Claims Nos. 1-2, 5-39 partially

   because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

   see FURTHER INFORMATION sheet PCT/ISA/210

3. [ ] Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box III  Observations where unity of invention is lacking (Continuation of item 3 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims, it is covered by claims Nos.:

**Remark on Protest**

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.
Continuation of Box II.2

Claims Nos.: 1-2,5-39 partially

The search on the basis of the functional definition given in claim 1 does not allow a meaningful search to be carried out, since a comparison with the prior art is not possible. The search of the claims has been performed on the basis of the specific mutation at position 61 of NGF and those mutants which have additionally been mutated at other positions.

The applicant's attention is drawn to the fact that claims relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure. If the application proceeds into the regional phase before the EPO, the applicant is reminded that a search may be carried out during examination before the EPO (see EPO Guideline C-VI, 8.5), should the problems which led to the Article 17(2) declaration be overcome.
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