European Review for Medical and Pharmacological Sciences 2015; 19: 3822-3832

High-pressure physiological saline isotonic solution administration enhances brain NGF and NGF-receptors expression

F. SALVINELLI, V. FRARI, M.L. ROCCO[1], A. D'ERAMO[2], E. SALVINELLI[2], B. SALVINELLI[3], L. ALOE[1]

Department of Otolaryngology, Campus Bio-Medico University, School of Medicine, Rome, Italy
[1]Institute of Neurobiology and Molecular Medicine, National Research Council, Rome, Italy
[2]Private Practice, Rome, Italy
[3]Department of Odontology, Universitad Europea de Madrid, Madrid, Spain

Abstract. – OBJECTIVE: Nerve growth factor (NGF) is a neurotrophin which promotes and regulates the survival of neurons in the peripheral nervous system. The aim of this study was to investigate the effect of high-pressure administration of sterile physiological saline isotonic solution (HpPSIS) into nasal cavity of laboratory animals on NGF levels and NGF-receptor expression in the olfactory bulbs and brain.

MATERIALS AND METHODS: For this study we used three weeks old female Sprague Dawley SD rats (n=48). Rats were divided into two groups, the first one treated delivering physiological saline solution with a normal syringe modified at the extremity to fit the rats' nostril (5 ml) (n=24) and the second one treated spray with HpPSIS (n=24 rats). Rats were treated three times a day either for 5 consecutive days (shorth term treatment) or 10 consecutive days (longer treatment) in both nostrils of HpPSIS delivered at high pressure (pression emission level: PEL: 7 g/sec for emission time ET: 0.5 sec) with a specific forced spray erogator. Untreated rats received a similar manipulation three times a day through a syringe in the nostrils, but no HpPSIS administration.

RESULTS: The results of these studies highlight the possibility that endogenous enhancement of NGF by stimulation of NGF-producing cells within the nasal cavities and also in the CNS represent a novel experimental approach to enhance the brain NGF levels with a new therapy.

HpPSIS treatment further enhances the presence of NGF in the four brains examined. Indeed, a significant increase of NGF was first observed after 5 days of HpPSIS treatment, compared to HpPSIS untreated rats. The increase was over 25% in the OB, ST, HI and in CX, while 10 days after HpPSIS treatments the levels of NGF were even higher. These differences were statistically significant, $p < 0.05$.

CONCLUSIONS: It was found that forced administration of HpPSIS enhances the presence of these neurotrophic signals, not only in the olfactory bulbs, but also in forebrain cholinergic neurons, which are known to degenerate as result of memory loss and brain aging, including Alzheimer Disease. These findings for the first time in the literature demonstrate the possibility of enhancing the endogenous NGF to protect NGF-damaged neurons. Since the enhanced expression of NGF was first observed after 5 days of treatment and higher after 10 days of treatment, a reasonable hypothesis is that longer HpPSIS treatment might further enhance the level of NGF in brain and olfactory bulbs.

Key Words:
High pressure physiological saline isotonic solution (HpPSIS), Nasal cavity (NC), Nerve growth factor (NGF), Olfactory bulb, Brain.

Abbreviations

AD = Alzhaimer disease; ABC = Avidin-Biotin complex; BSA = Bovine Serum Albumin; DAB = Diaminobenzidine; DOC = Sodium doexycholate; DTT = Dithiothreitol; EDTA = Ethylenediaminetetraacetic acid; ELISA = Enzyme-Linked ImmunoSorbent Assay; GAPDH = Glyceraldehyde 3-phosphate dehydrogenase; HRP = Horseradish peroxidase; HpPSIS = sterile physiological saline isotonic solution; IgG = Immunoglobulin G; NC = Nasal cavity; NGF = Nerve growth factor; NGFRs = NGF-receptor; p75NTR = p75 neurotrophin receptor; SDS = Sodium Dodecyl Sulphate; SDS-PAGE = Sodium Dodecyl Sulphate-PolyAcrylamide Gel Electrophoresis; SNHL = sensorineural hearing loss; TBS = Tris-buffered saline; TBS-T = Tris-buffered saline-tween.

Corresponding Author: Aloe Luigi, MD; e-mail: luigialoe@cnr.it

Introduction

Nerve growth factor (NGF) is known to play a critical protective action on survival of peripheral sensory neurons[1], including those innervating cells of the nasal cavity and auditory cells[2]. Its biological action is mediated by two distinct receptors: TrkA, a tyrosine kinase receptor, and p75 receptor (a member of the tumor necrosis factor receptor superfamily and their effect depend of the ratio of TrkA and p75 present on the surface of NGF-target cells[3,4]. It has been demonstrated that deficit of NGF synthesis and release and/or binding activity may lead to functional deficits of NGF-target cells, while exogenous NGF administration is able to rescue damaged nerve cells[5] and stimulate growth guidance of nerve fibers, including auditory nerve fibers toward their targets tissues. NGF is produced and released by brain cells and by a number of other cells, including olfactory epithelial cells, mucosal cells and mast cells[6,7] and by neurons of the auditory part of the inner hear[8]. Moreover, NGF is a leading neurotrophic factor required for the development, survival and neuroprotection of degenerating brain neurons, particularly of neurons that are known to degenerate in brain aging, including AD[9,10]. We have recently shown that autologous stimulation induced by nasal forced stress with isotonic solution causes an increase of NGF into nasal cavity in humans. The clinical and audiometric improvement in patients with SNHL and tinnitus treated with our new therapy represent a clinical confirm to our hypothesis and a new therapy of SNHL, tinnitus and hearing disorders[11]. However, whether the endogenous produced and released NGF within the nasal cavity reaches brain NGF-target cells is not known. The aim of the present study was, therefore, to investigate this effect. To address this specific question young-adult rats were treated with sterilized isotonic solution delivered at high pressure (pression emission level: PEL: 7 g/sec for emission time ET: 0.5 sec) through a specific spray erogator in both nostrils**,** three times a days for 5 and 10 consecutive days.

Materials and Methos

Drugs

Specific erogator delivering sterile physiological isotonic saline at high pressure PEL: 7 g/sec for emission time ET: 0.5 sec.

Chemicals

Primary antibodies: anti-trkA^{NGFR} (sc-118; Santa Cruz Biotech, Santa Cruz, CA, USA), anti-phospho trkA^{NGFR} (p-trkA^{NGFR}; sc-130222; Santa Cruz, Santa Cruz, CA, USA), anti-p75^{NTR} (sc-6188; Santa Cruz, Santa Cruz, CA, USA), and anti-GAPDH (sc-365062; Santa Cruz, Santa Cruz, CA, USA). Secondary antibodies: Vector anti-rabbit and Vector anti-mouse as well as horseradish peroxidase-conjugated anti-rabbit/mouse specie-specific secondary antibodies (Cell Signaling Technology, Danvers, MA, USA).

Primary antibodies: anti-trkA^{NGFR} (sc-118; Santa Cruz Biotech, Santa Cruz, CA, USA), anti-phospho trkA^{NGFR} (p-trkA^{NGFR}; sc-130222; Santa Cruz Biotech, Santa Cruz, CA, USA), anti-p75^{NTR} (sc-6188; Santa Cruz, CA, USA), and anti-GAPDH (sc-365062; Santa Cruz Biotech, Santa Cruz, CA, USA). Secondary antibodies: vector anti-rabbit and vector anti-mouse as well as horseradish peroxidase-conjugated anti-rabbit/mouse specie-specific secondary antibodies (Cell Signaling Technology, Danvers, MA, USA).

Animals

For this study we used three weeks old female Sprague Dawley SD rats (n=48). They were housed in polypropylene cages and animals kept under standard conditions (12 h light: 12 h dark cycle) with free access to water and food (Purina chow food). For the housing, care and experimental procedures, we followed the guidelines indicated and approved by CNR, Italian National Research Council and in conformity with the Intramural Committee and Institutional Guidelines in accordance with national and international laws (EEC council directive 86/609, OJ L 358, 1, December 12, 1987). All efforts were made to reduce the number of animals and to minimize animal suffering.

Treatment

Rats were divided into two groups, the first one treated delivering physiological saline solution with a normal syringe modified at the extremity to fit the rat's nostril (5 ml) (n=24) and the second one treated with a specific spray with HpPSIS (n=24 rats). Rats were treated three times a day either for 5 consecutive days (shorth term treatment) or 10 consecutive days (longer treatment) in both nostrils of HpPSIS delivered at high pressure (pression emission level: PEL: 7 g/sec for time ET: 0.5 sec emission) with a spe-

cific forced spray erogator. Untreated rats received a similar manipulation three times a day through a syringe in the nostrils, but no HpPSIS administration.

Tissue Dissection

At the end of the experimental protocol, animals were deeply anesthetized with an overdose of carbon oxide vapor and then sacrificed with an overdose of 3% pentobarbital sodium (30 mg/kg), brain removed and olfactory bulbs, cortex, hippocampus, and septum dissected out and stored at –70°C for biochemical and molecular analyses. Four rats of each group were perfused through the ascending aorta with 4% paraformaldehyde in 0.1 M-phosphate buffer, pH 7.4, brain removed, post-fixed overnight, and then left for 24 hrs in 0.1 M phosphate buffer containing 20% of sucrose, Serial brain sections were then cutted with cryostat and stained for localization of NGF receptors in olfactory bulb and forebrain NGF-target neurons.

NGF Determination

The tissue (olfactory bulb, cortex, septum and hippocampus) were homogenized with ultra-sonication in RIPA buffer (50 mM tris-HCl, pH 7.5; 150 mM NaCl; 5 mM EDTA; 1% Triton X-100; 0.1% SDS and 0.5% DOC; Sodium deoxycholate; 1 mM PMSF; 1 µg/mL leupeptin; Applichem, Darmstadt, Germany) and centrifuged at 13000 rpm (4°C) for 20 min. The supernatant was collected for NGF analysis (Emax ImmunoAssay System ELISA kit; Promega Corp., Madison, WI, USA). The assay sensitivity was 3 pg/mL and the NGF recovery ranged from 80% to 90%. No BDNF/NT3/NT4 cross-reactivity has been reported by manufacturers. The Optical Density (OD) was measured at 575 nm using an ELISA reader (Multiskan EX, Thermo Electron Corporation, Madison, WI, USA) and both standard and sample values were corrected by taking into consideration the non-specific binding. The assays were performed in duplicate and data (pg/µg of total proteins) are shown as fold increase in NGF-treated with respect to untreated one. For normalization, total proteins were determined by using a spectrophotometer and a DC protein assay reagent (Biorad Laboratories, Hercules, CA, USA).

Western Blot Analysis

Brain tissues were homogenized with ultrasonication in RIPA buffer (50 mM tris-HCl, pH7.4; 150 mM NaCl; 5 mM EDTA; 1% Triton X-100; 0.1% SDS; 0.5% DOC (Sodium deoxycholate; 1 mM PMSF; 1 µg/ml leupeptin), centrifuged at 4° C for 20 min at 13000 rpm, then supernatant was storage at –20°C. Samples (30 µg of total protein) were dissolved in loading buffer (0.1 M Tris-HCl buffer, pH 6.8, containing 0.2 M dithiothreitol, DTT, 4% sodium-dodecil-phosphate, SDS, 20% glycerol, and 0.1% bromophenol blue), separated by 8% or 12% SDS-PAGE, and electrophoretically transferred to PVDF membrane overnight. The membranes were incubated for 1 hr. at room temperature with blocking buffer constituted by 5% BSA (for TrkA and pTrkA) or non-fat dry milk (for p75, GAPDH) in TBS-T (10 mM Tris, pH 7.5, 100 mM NaCl, and 0.1% Tween-20). Membranes were washed three times for 10 minutes each at room temperature in TBS-T followed by incubation at 4°C with primary antibodies overnight polyclonal rabbit anti-TrkA 1:1000 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), monoclonal mouse anti-p75 1:1000 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), monoclonal mouse anti-pTrkA 1:1000 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Membranes were washed three times for 10 minutes each at room temperature in TBS-T and incubated for 1 hour with horseradish peroxidase-conjugated anti-rabbit IgG 1:4000 or horseradish peroxidase-conjugated anti-mouse IgG as the secondary antibody (Cell Signaling Technology, Danvers, MA, USA) at room temperature. The blots were developed with an ECL chemiluminescent horseradish peroxidase (HRP) substrate as the chromophore (Millipore, Billerica, MA, USA). The public Image J Software was used to evaluate band density, which was expressed as arbitrary units of grey level. The Image J program determines the optical density of the bands using a grey scale shareholding operation. The optical density of polyclonal rabbit anti-GAPDH 1:4000 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) bands was used as a normalizing factor. For each gel blot, the normalized values were then expressed as percentage of relative normalized controls and used for statistical evaluation. Corneas of rats in both experimental groups were used and experiments were performed in triplicate. Statistical evaluations were performed using the GraphPad Prism package for Windows and data expressed as means ± SEM of eight different corneas.

Total RNA Extraction, cDNA Synthesis a Relative Real-Time PCR

Total RNA was extracted from the tissue (olfactory bulb, cortex, septum and hippocampus) with TRIfast (1:1 v/v; EuroClone, Milan, Italy) and suspended in 10 µL fresh (daily-provided) RNase free water (Direct Q5, Millipore Corporation, Billerica, MA, USA). To eliminate any DNA contamination, all total RNA samples were treated with RNase-free DNaseI, according to the supplier's protocol (2 U/µL; AM-1907; Turbo DNA free kit; Ambion Ltd., Huntingdon, Cambridgeshire, UK). Total RNA samples were checked for RNA quantity/purity (> 1.8; A280, Nanodrop, Thermo Fisher Scientific Inc., Wilmington, DE, USA) and for absence of RNA degradation (1% agarose gel analysis). Equivalent amounts of RNA (1µg) were used as template to generate cDNAs in a one-cycler programmable thermocycler (PeqLab Biotech, Erlangen, Germany), according to the IMPROM manufacturer's procedure (Promega). The resulting cDNAs were amplified using the SYBR Green PCR core reagent kit (Applied Biosystems, Foster City, CA, USA) in a programmable real-time thermocycler (Opticon2; MJ Research, Watertown, MA, USA), according to a standard procedure. Samples were amplified in duplicate and in parallel with negative controls (either without template or with mRNA as template). Real cycle thresholds (Cts) were recorded during linear amplification and normalized to those of referring genes run in parallel ($nCts=Ct_{target}-Ct_{referring}$). Averages were calculated from replicates and expressed as normalized Ct or as expression ratio of a normalized target gene (absolute fold changes), according to the REST© analysis. The specific primers and amplification profiles were according to previous studies. Primer specificities were confirmed by single melting curves monitored during amplification and provided at the end of running by Opticon2 software. Some PCR products were randomly tested for single-band separation on 2.5% agarose gels (SaeKem LE-agarose; Lonza, Milan, Italy).

Total RNA Extraction, cDNA Synthesis and Relative Real-time PCR

Total RNA was extracted from pooled retinas with TRIfast (1:1 v/v; EuroClone, Milan, Italy) and resuspended in 10 µL fresh (daily-provided) RNase free water (Direct Q5, Millipore Corporation, Billerica, MA, USA). To eliminate any DNA contamination, all total RNA samples were treated with RNase-free DNaseI, according to the supplier's protocol (2 U/µL; AM-1907; Turbo DNA free kit; Ambion Ltd., Huntingdon, Cambridgeshire, UK). Total RNA samples were checked for RNA quantity/purity (> 1.8; A280, Nanodrop, Thermo Fisher Scientific Inc., Wilmington, DE, USA) and for absence of RNA degradation (1% agarose gel analysis). Equivalent amounts of RNA (1 µg) were used as template to generate cDNAs in a one-cycler programmable thermocycler (PeqLab Biotech, Erlangen, Germany), according to the IMPROM manufacturer's procedure (Promega, Madison, WI, USA). The resulting cDNAs were amplified using the SYBR Green PCR core reagent kit (Applied Biosystems, Foster City, CA, USA) in a programmable real-time thermocycler (Opticon2; MJ Research, Watertown, MA, USA), according to a standard procedure. Samples were amplified in duplicate and in parallel with negative controls (either without template or with mRNA as template). Real cycle thresholds (Cts) were recorded during linear amplification and normalized to those of referring genes run in parallel ($nCts=Ct_{target}-Ct_{referring}$). Averages were calculated from replicates and expressed as normalized Ct or as expression ratio of a normalized target gene (absolute fold changes), according to the REST© analysis[12]. The specific primers and amplification profiles were according to previous studies[13]. Primer specificities were confirmed by single melting curves monitored during amplification and provided at the end of running by Opticon2 software. Some PCR products were randomly tested for single-band separation on 2.5% agarose gels (SaeKem LE-agarose; Lonza, Milan, Italy). All experiments were performed in duplicate and provided as mean±SEM in the graphics. Unpaired Student t test analysis was performed using the StatView software (Abacus Concepts Inc., Barkley, CA, USA). A p-value ≤ 0.05 was considered significant. The REST/ANOVA-coupled analysis was carried out for molecular comparisons[12].

Immunohistochemical Analysis

To further evaluate the effect of HpPSIS administration on brain NGF-receptor expression, coded fixed brain sections were cut with a cryostat (Leica CM1850, Leica Microsystems Srl, Milan, Italy). For histological studies, sections were stained with toluidine blue and for im-

munohistochemistry, sections were first incubated in PBS containing 10% horse or goat serum for 1 hour, and then left overnight at 4°C with mouse monoclonal anti-p75NTR (sc-56331, Santa Cruz Biotechnology, Santa Cruz, CA, USA; working concentration: 1:100). Sections were then exposed to biotinylated rabbit anti-goat IgG Antibody (Vector Laboratories, Burlingame, CA, USA; working con- centration: 1:500) for 2 hours at room temperature, and the immunoperoxidase staining was performed using an ABC reagent (Avidin-Biotin Complex solution, Vectastain Elite Kit, Vector Laboratories, Burlingame, CA, USA). The sections incubated with normal IgG were used as non-specific staining controls. Immunostained signals were then visualized with 3,3′-diaminobenzidine (DAB) Peroxidase (HRP) Substrate Kit (Vector Laboratories, Burlingame, CA, USA). Differences between treated and control rats were determined with a computerized image analysis program (IAS 200, Delta System, Rome, Italy) attached to with a computerized controlled motorized stage and eyepiece with square grids.

Epifluorescent Studies

Brain tissues were post fixed in paraformaldehyde 4% (PFA) fixative at +4° for 15 days and cryopreserved in 10% buffered sucrose, according to a standard procedure, and sectioned at 20 µm thickness (cryostat, Leica CM1850 UV, Leica Microsystems, Wetzlar, Germany). Sections were placed on glass slides and incubated with the primary antibody specific $p75^{NTR}$ (single staining) (4°C/18 hrs; 1:300). To assess for staining specificity, parallel sections were incubated with purified non-specific rabbit IgG antibodies (isotype; Vector, Burlingame, CA, USA). After appropriate washing, the slides were incubated with Alexa Fluor®-488/Alexa Fluor®-594 specie-specific IgG secondary antibodies (1:400; 1hr on bench-top). Nuclear counterstaining was performed with Dapi (Molecular Probes, Eugene, OR, USA). Sections were mounted in anti-fading, observed under a confocal microscope (CLSM; SP5 Leica Microsystems, Wetzlar, Germany) and acquired at X60/objective (1024x1024 pixels; 10 Hz acquisition speed; 1 µm pine-hole exposure). No signal adjustments were carried out while color-image changes were performed for presentation purposes (Adobe Photoshop ver.7; Adobe System, San Jose, CA, USA).

Statistical Analysis

All experiments were performed in duplicate and provided as mean±SEM in the graphics. Unpaired Student t test analysis was performed using the StatView software (Abacus Concepts Inc., Barkley, CA, USA). A p-value ≤ 0.05 was considered significant. The REST/ANOVA-coupled analysis was carried out for molecular comparisons.

Results

Basal Brain NGF Levels Increase with Age

Figure 1 A-D and Figure 2 A-D report the concentration of NGF in four different brain regions of rats at 25 and 30 days of age respectively. This result indicates that constitutive levels of NGF in the olfactory bulbs, Cortex, HI and septum increase significantly from 25 to 30 days of age in non-treated rats. These differences are, most probably, suggesting that maturation and differention of brain nerve cells from pre- to post-puberty and increase learning abilities, NGF related, as NGF play a critical role in this process[1,5].

Treatment with HpPSIS and Brain NGF Levels

As shown in Figure 1 A-D and 2 A-D, HpPSIS treatment further enhances the presence of NGF in the four brains examined**.** Indeed, a significant increase of NGF was first observed after 5 days of HpPSIS treatment, compared to HpPSIS untreated rats. The increase was over 25% in the OB, ST, HI and in CX, while 10 days after HpPSIS treatments the levels of NGF was even higher. These differences were statistically significant, $p < 0.05$. This result suggests that further treatment with HpPSIS would be able to stimulate further the local presence of NGF.

Effect of NGF Receptor Expression.

Since the biological action of NGF is mediated by NGF receptors expressed by brain NGF-target cells**,** the expression of the low p75 and high-affinity NGF receptor, TrkA were determined with western blot and immunohistochemistry. As shown in Figure 3 A-D, it was found that both the presence of p75 and TrkA receptor (Figure 4 A-D), are significantly overexpressed in the OB, ST, HI and CX of HpPSIS treated rats compared to untreated rats.

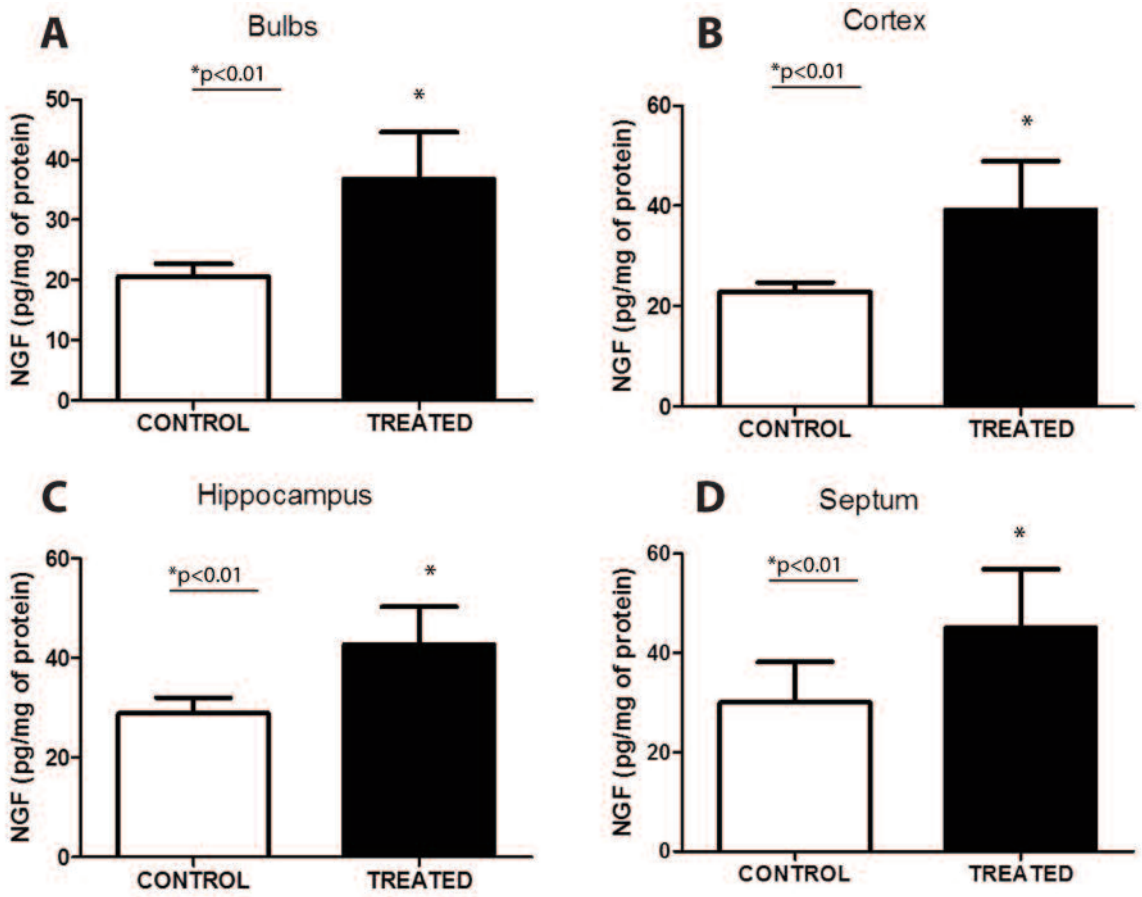

Figure 1. ***A-D,*** Levels of NGF in the HI (A), ST (B) and OB (C) of three weeks old rats treated for ten consecutive days with nasal spray or untreated. See the significant (*$p < 0.05$), increase of NGF levels of NGF after HpPSIS nasal spray compared to untreated rats. Abbreviations: NGF, Nerve Growth Factor; OB, olfactory bulb; HI, hippocampus; ST, septum.

NGF-Receptor Localization.

To further explore the effect of HpPSIS treatment on brain neurons, brain sections were immunostained against the low-affinity NGF-receptor that is widely expressed by brain neurons, both under normal and pathological conditions. As illustrated in Figure 5 A-D and Figure 6 A-D, neurons present in the OB, ST, HI and CX are more markedly positive for NGF receptors, as compared to control neurons untreated with HpPSIS.

Effect of HpPSIS in TrakA mRNA

Quantitative mRNA levels of NGF and TrkA receptor in cortex and OB using Real-time PCR revealed that expression of NGF and TrkA mRNA is not altered after HpPSIS administration. Table I indicated differences of response between different brain areas.

Discussion

We have recently reported that forced nasal isotonic solution administration enhances the presence of NGF-producing cells and the expression of NGF and NGF-producing cells[11] in the nasal cavity, suggesting that NGF plays a critical role in nerve endings of SNHL and tinnus in patients affected by hear sensorineural deficits, and that this therapy is useful in the treatment of these hearing disorders. In fact with our therapy mast cells in the nasal fluid were positive to NGF. So, we demonstrated for the first time that NGF produced by mast cells is enhanced thanks to this novel therapy based on a saline physiological saline solution delivered at high pressure[11].

We observed that stress induced by forced intranasal administration of physiological solution

can be used to enhance the constitutive amount of NGF to reinforce and protect the damaged sensory nerve endings in SNHL and tinnitus[2,11]. These observations raised the question as to whether isotonic solution administered at high pressure via nasal cavity would be able to reach brain neurons and alter the brain NGF levels and NGF-receptor expression.

In the present study, we address the question as whether forced nasal administration at high pressure of sterile physiological saline solution affects the basal concentration of NGF in the OB and BFCN that undergo degeneration in Alzheimer' disease (AD). The results of these studies indicate that five consecutive isotonic saline administrations at high pressure in the nasal cavity and more markedly 10 daily administrations enhance the brain NGF in the OB, cortex, ST and HI and NGF receptors in forebrain cholinergic neurons. Since these receptors are known to mediate the biological and functional activities of NGF, it is reasonable to hypothesize a critical important role of the enhanced nasal NGF levels for the brain neurons. We found that the effect is not restricted to NGF and NGF-receptors increase, but also to their RNA messengers expression, further suggesting that the described experimental approach might be useful non only for SNHL and tinnitus deficits, but also for enhancing brain NGF to brain neurons. These experimental observations suggest a novel strategy for protecting damaged brain NGF-target neurons and improving memory deficits and behavior performances. Indeed, there are a number of experimental data demonstrating that enhanced presence of NGF in fore-

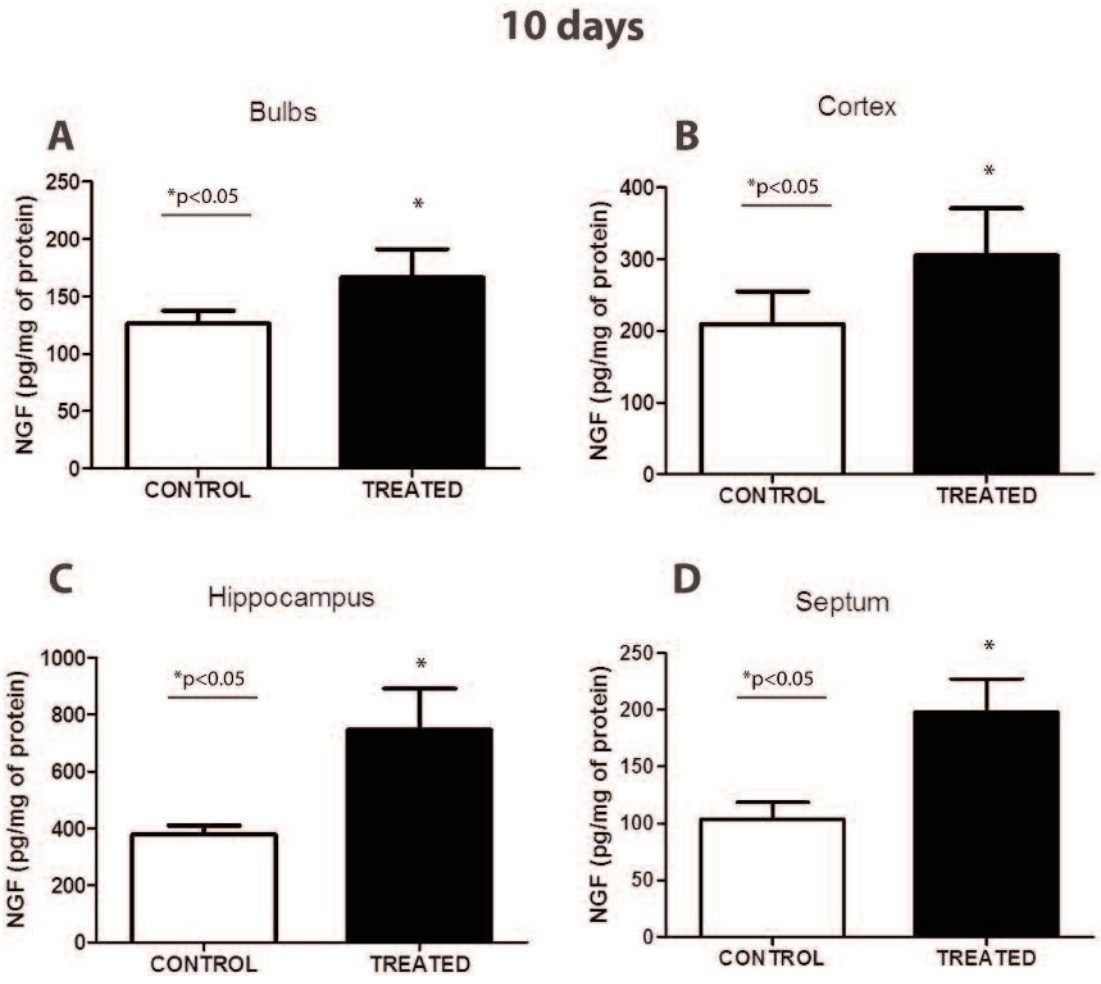

Figure 2. ***A-D,*** Western blot analysis of the low-affinity NGF-receptr, p75 protein after ten consecutive days with nasal spray, compared to untreated rats showing the expression of p75 receptor. See the significant (*$p < 0.01$), increase in the OB, olfactory bulb; HI, hippocampus; ST, septum.

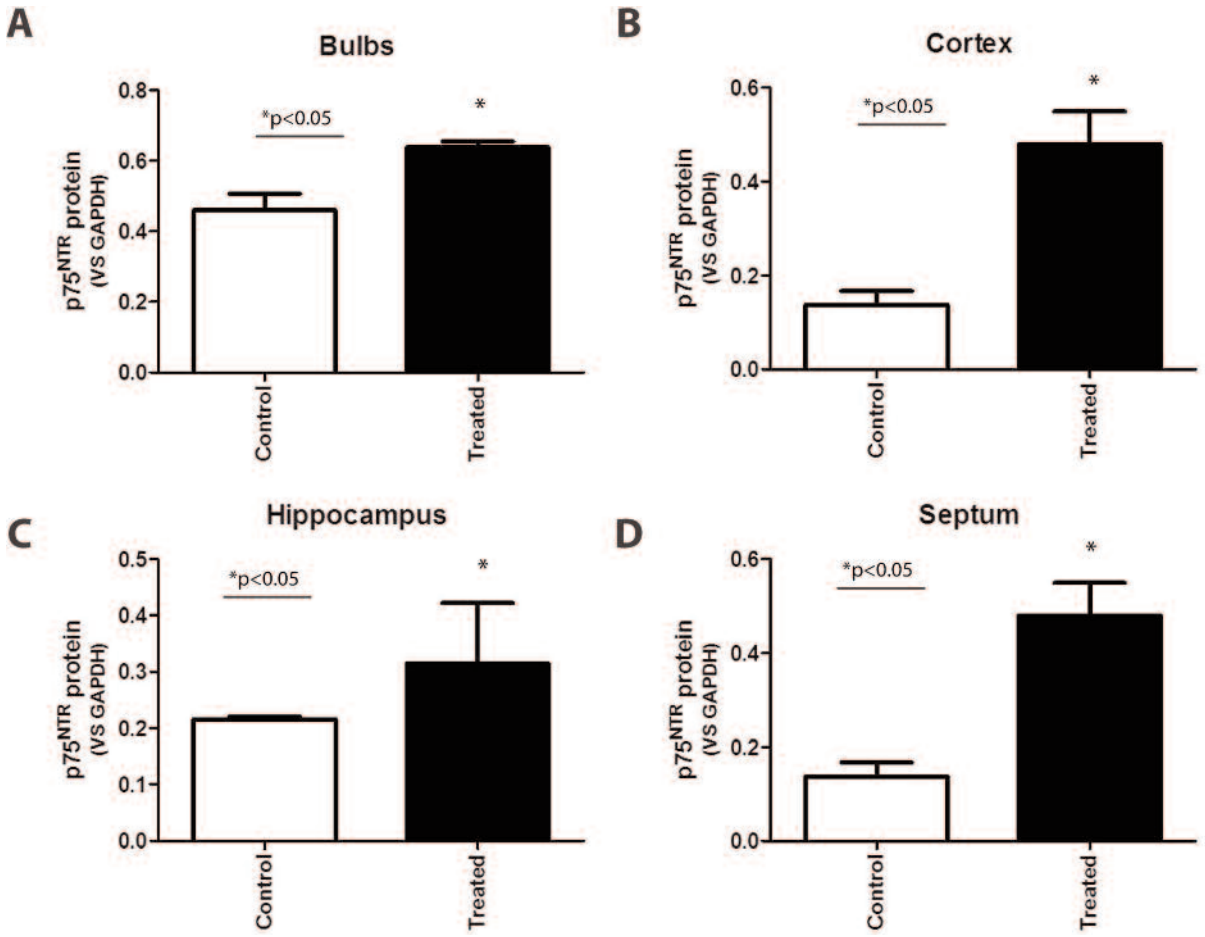

Figure 3. ***A-D,*** Western blot analysis of the high-affinity NGF receptor, TrkA protein after ten consecutive days with nasal spray, compared to untreated rats. See the significant (*$p < 0.01$), increase in the OB, olfactory bulb; HI, hippocampus; ST, septum.

brain neurons plays a protective critical role in these deficits, both in laboratory animals[14,10] and in humans[15,16]. The present observations indicate that nasal forced stress enhances the NGF not only in the nasal cavity, as previously demonstrated[11], but also the level of NGF in the olfactory bulbs, and also the NGF receptor expression in basal forebrain neurons. We have found evidence that the increased level of NGF in the nasal cavity is transported directly to brain NGF-target neurons and activate NGF-receptors localized on the surface of these neurons in the OB, septum and and other brain NGF-responsive neurons. This experimental approach represents a non-invasive strategy that may be of particular benefit for long-term treatments for damaged brain NGF-responsive neurons. Since the first observation with laboratory animals showing the critical properties of NGF to protect degenerating brain cholinergic neurons[14,17] including neurons that degenerate in AD[18], diverse experimental strategies were devised to deliver NGF into the brain, in the absence of side-effects. Thus, exogenous NGF was administered through nasal cavity[19,20] or gene delivery[22], but the possibility that exogenous NGF would induce local undesidered side-effects was not excluded. Recent demonstration that NGF administration can protect damaged brain neurons in humans[16] raised the need to search new strategies to enhance the endogenous synthesis and release of NGF. Indeed, NGF administration has been proved effective in protecting and/or reducing the number of degenerating neurons in patients with AD. However, limited aspect of exogenous administration of NGF administration is the high cost of treatment and undesired side effects.

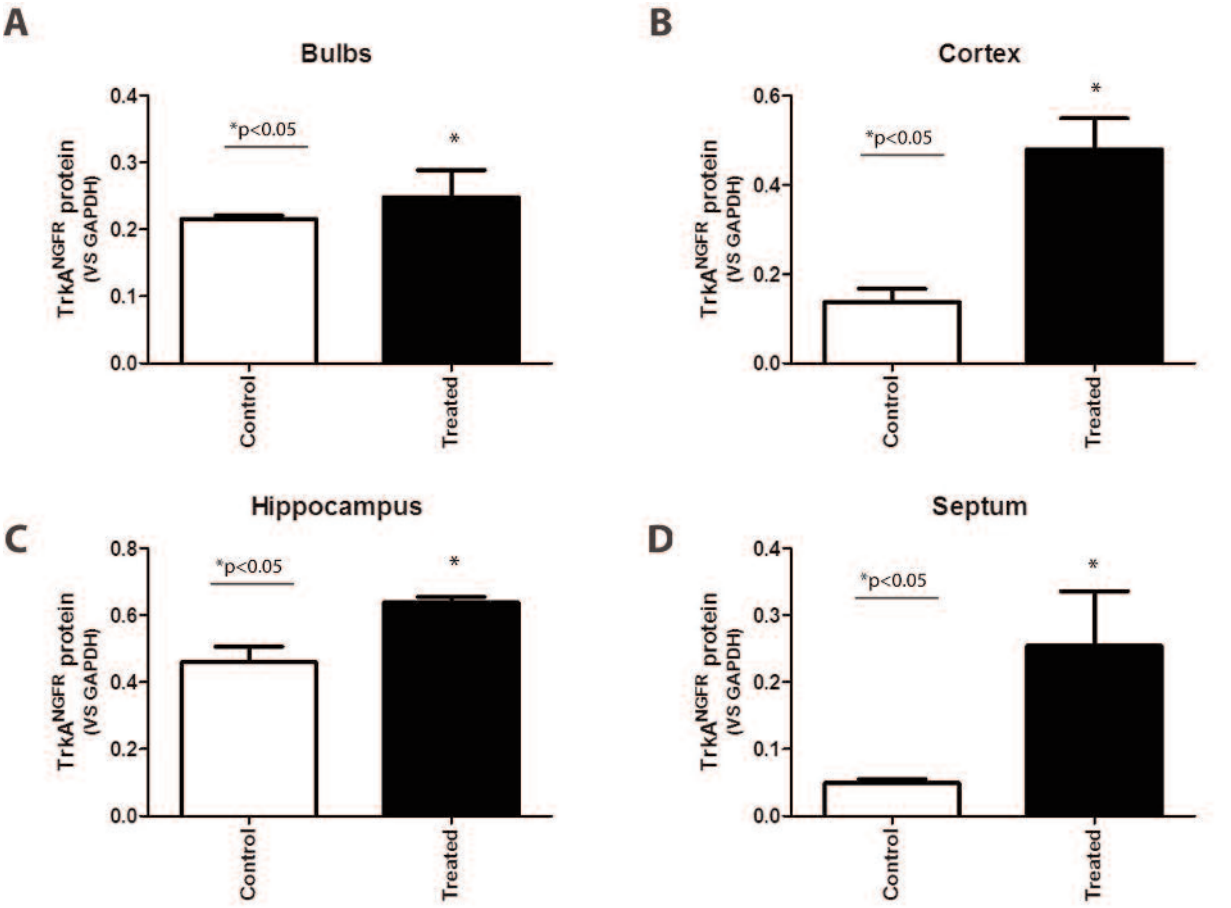

Figure 4. ***A-D,*** NGF receptors (TrkA and pTrkA) expression. Western blot analysis of the high-affinity NGF receptor protein showing the expression of inactivated (TrkA) and activate form (pTrkA) of TrkA receptor in the cortex ***(A,B)***, HI ***(C,D)*** and OB ***(E,F)*** of IgG and ANA-treated rats, suggesting that the NGF reaches brain NGF target cells and alters the biologic activity of these neurons. The effect is statistically significant (*$p < 0.05$). Abbreviations: OB, olfactory bulb; HI, hippocampus; ANA, anti-NGF antibody; TrkA, A Receptor Tyrosine Kinase

Conclusions

It was found that forced administration of HpPSIS enhances the presence of these neurotrophic signals, not only in the olfactory bulbs, but also in forebrain cholinergic neurons, which are known to degenerate as result of memory loss and brain aging, including Alzheimer Disease. These findings for the first time demonstrate the possibility of enhancing the endogenous NGF to protect NGF-damaged neurons. Since the enhanced expression of NGF was first observed after 5 days of treatment and higher after 10 days of treatment, a reasonable hypothesis is that longer HpPSIS treatment might further enhance the level of NGF in brain and olfactory bulbs. This novel therapy has already been proved effective in pathologies of the inner ear, as sensorineural hearing loss and tinnitus[11]. The observation reported in the present study with rats treated with this new therapy opens the way to a new valid strategy to enhance the level of brain endogenous NGF to protect NGF-damaged neurons in neurological diseases like Parkinson and Alzheimer.

Acknowledgements

This study was supported by the Italian National Research Council (CNR) and Association NGF-ONLUS to L. Aloe.

Conflict of Interest

The Authors declare that they have no conflict of interests.

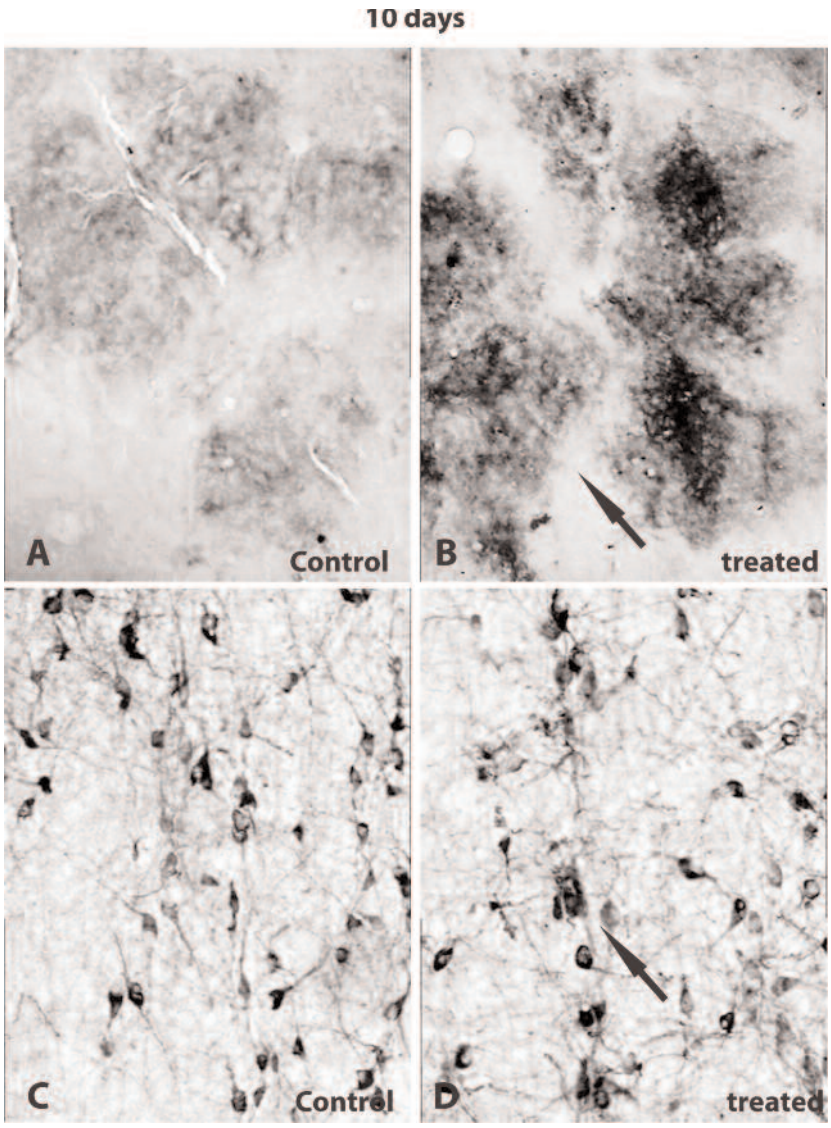

Figure 5. ***A-D,*** Representative photomicrographs showing p75-immunostained cells in ST ***(A),*** FBCN (C) and OB (E) of rats treated with ten consecutive days with nasal spray, compared to untreated rats ***(B-F)***. Note the increae of p75 immunostained cells in brain areas of rats treated for ten consecutive days with ANA (*see arrows*). Magnification: X-110. Abbreviations: ST, septum; OB, olfactory bulb.

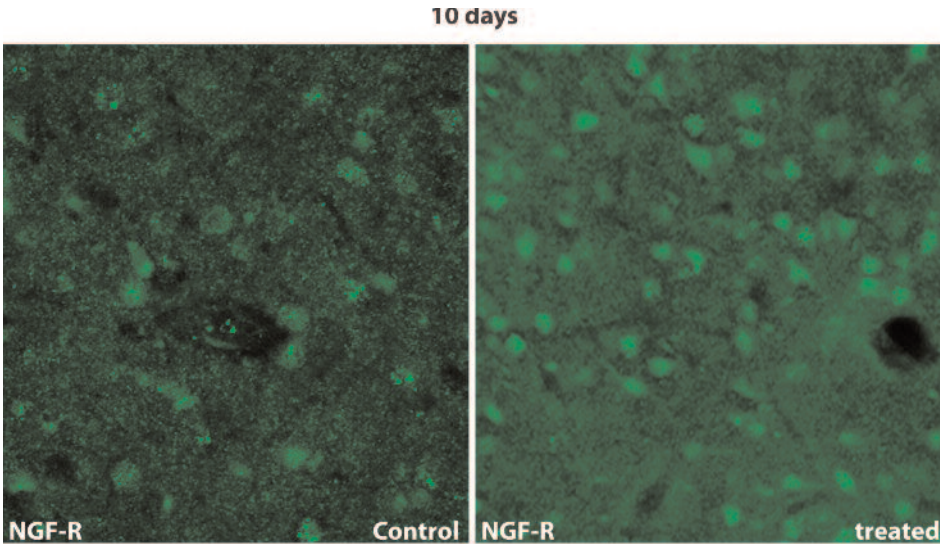

Figure 6. ***A-B,*** Brain sections of the frontal CT of untreated and HpPSIS treated rats showing p75 that immunofluorescent positive neurons are more numerous in HpPSIS treated compared to the CX of untreated rats.

Table I. mRNA levels of TrkA receptor expression in four brain NGF target regions of control and 10 days- treated rats.

Brain tissue	Control	Treated
OB	0.97±0.02	0.98±0.02
HI	0.91±0.02	0.99±0.02
CX	0.96±0.02	0.97±0.02
SE	0.96±0.02	1.02±0.02

Data presented as mean ± SEM where each bar represents four determination of different experiment animals. The differences between controls and treated rats is not statistical significant, but only a tendency to an increase mRNA-TrkA NGFR in treated compared to controls.

References

1) Levi-Montalcini R. The nerve growth factor 35 years later. Science 1987; 237: 1154-1162.
2) Dai CF, Steyger PS, Wang ZM, Vass Z, Nuttall AL. Expression of Trk A receptors in the mammalian inner ear. Hear Res 2004; 187: 1-11.
3) Chao MV. Hempstead, p75 and Trk: a two-receptor system. Trends Neurosci 1995; 18: 321-326.
4) Meakin SO, Shooter EM. The nerve growth factor family of receptors. Trends Neurosci 1992; 15: 323-331.
5) Aloe L, Calzà L, editors. NGF and Related Molecules in Health and Disease. Progr Brain Res Elsevier Editor, 2004; p. 146.
6) Aiba T, Mori J, and Nakai Y. Nerve growth factor (NGF) and its receptor in rat olfactory epithelium. Acta Otolaryngol Suppl 1993; 506: 37-40.
7) Groger M. Eosinophils and mast cells: a comparison of nasal mucosa histology and cytology to markers in nasal discharge in patients with chronic sino-nasal diseases. Eur Arch Otorhinolaryngol 2013; 270: 2667-2676.
8) Malgrade B, Rigo JM, Van De Walter TR, Staecker H, Moonen G, Lefebvre PP. Growth factor therapy to the damaged inner ear: clinical prospects. Int J Pediatr Otorhinolaryngol 1999; 49: 19-25.
9) Salvinelli F. Nerve growth factor serum level is reduced in patients with sensorineural hearing impairment: possible clinical implications. J Biol Regul Homeost Agents 2002; 16: 176-180.
10) Connor B, Dragunow M. The role of neuronal growth factors in neurodegenerative disorders of the human brain. Brain Res Brain Res Rev 1998; 27: 1-39.
11) Salvinelli F, Frari V, Rocco ML, Rosso P, Aloe L. Enhanced presence of NGF and mast cells number in nasal cavity after autologous stimulation: relation with sensorineural hearing deficit. Eur Rev Med Pharmacol Sci 2015; 19: 381-391.
12) Tuszynski MH, Thal L, Pay M, Salmon DP, U HS, Bakay R, Patel P, Blesch A, Vahlsing HL, Ho G, Tong G, Potkin SG, Fallon J, Hansen L, Mufson EJ, Kordower JH, Gall C, Conner J. A phase 1 clinical trial of nerve growth factor gene therapy for Alzheimer disease. Nat Med 2005; 11: 551-555.
13) Eriksdotter Jonhagen M, Nordberg A, Amberla K, Backman L, Ebendal T, Meyerson B, Olson L, Seiger, Shigeta M, Theodorsson E, Viitanen M, Winblad B, Wahlund LO. Intracerebroventricular infusion of nerve growth factor in three patients with Alzheimer's disease. Dement Geriatr Cogn Disord 1998; 9: 246-257.
14) Fischer W, Wictorin K, Bjorklund A, Williams LR, Varon S, Gage FH. Amelioration of cholinergic neuron atrophy and spatial memory impairment in aged rats by nerve growth factor. Nature 1987; 329: 65-68.
15) Tuszynski MH, Sang H, Yoshida K, Gage FH. Recombinant human nerve growth factor infusions prevent cholinergic neuronal degeneration in the adult primate brain. Ann Neurol 1991; 30: 625-636.
16) Ferreira D, Westman E, Eyjolfsdottir H, Almqvist P, Lind G, Linderoth B, Seiger A, Blennow K, Karami A, Darreh-Shori T, Wiberg M, Simmons A, Wahlund LO, Wahlberg L, Eriksdotter M. Brain changes in Alzheimer's disease patients with implanted encapsulated cells releasing nerve growth factor. J Alzheimers Dis 2015; 43: 1059-1072.
17) Hefti F. Nerve growth factor promotes survival of septal cholinergic neurons after fimbrial transections. J Neurosci 1986; 6: 2155-2162.
18) Yuen EC, Howe CL, Li Y, Holtzman DM, Mobley WC. Nerve growth factor and the neurotrophic factor hypothesis. Brain Dev 1996; 18: 362-368.
19) Chen XQ. Delivery of Nerve Growth Factor to the Brain via the Olfactory Pathway. J Alzheimers Dis 1998; 1: 35-44.
20) Vaka SR, Sammeta SM, Day LB, Murthy SN. Delivery of nerve growth factor to the brain via intranasal administration and enhancement of brain uptake. J Pharm Sci 2009; 21: 3640-3646.