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The nerve growth factor administrated as eye drops activates mature and precursor cells in subventricular zone of adult rats

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ABSTRACT

The possibility to take advantage from the nerve growth factor (NGF) ability to induce recovery of damaged tissue has been largely explored in animal models and humans. Recently, the successful use of the ocular administration of NGF in ophthalmology, and the evidences that from the eyes NGF can access to the brain have stimulated new fields of research and open further perspectives to the clinical application of this neurotrophin. In our previous studies we have demonstrated the efficacy of NGF eye drop treatment to improved behavioural deficits and recover structural and biochemical alterations occurring follow brain lesion in animals. Since NGF exerts neuroreparative effects in brain by acting on mature neurons and neuronal precursors localised in germinal subventricular zone (SVZ), the present study has been aimed to evaluate the effects of NGF eye drop administration on the expression of the mitotic marker Ki67 in brain of adult rats. We found that a single ocular administration (10 µl) of 200 µg/mL NGF solution is sufficient to enhance the distribution of Ki67-positive cells also expressing p75 neurotrophin receptors in the proliferating layer of the SVZ. In addition, NGF treatment induces an increase of levels of brain-derived neurotrophic factor (BDNF) in forebrain. This data further supports the efficacy of ocular applied NGF to affect brain activities and suggests that NGF also by inducing local factors, including BDNF, can activate the machinery regulating the proliferation and maturation of neuronal precursor in brain.

Key words

Neurotrophins • Eye drops • p75NTR • Subventricular zone • Neuronal precursors

Introduction

The neurotrophins nerve growth factor (NGF) is well known as a survival, differentiative and protective factors for peripheral and central neural cells, which acts all life long, form development to adulthood and aging (Sofroniew et al., 2001). Since its discovery in the 1950s, the NGF ability to promote the recovery of neuronal damage in several animal models have attracted the interest of clinicians for its potential application as treatment in progressive neurodegenerative diseases (Connor and Dragunow, 1998; Aloe et al., 2001). In humans, NGF has showed to exert neuroprotective actions when administrated in: nasal cavity (Zhao et al., 2004; De Rosa et al., 2005; Vaka et al., 2009), intracerebroventricularly in subjects affected by Alzheimer's Disease (Eriksdotter Jonhagen et al., 1998; Tuszynski et al., 2005), subcutaneously and intravenously in patients with peripheral neuropathies (Apfel et al., 2000), or topically as treatment of skin and ocular ulcers (Aloe et al., 2008).

More recently, the application of NGF as eye drops has been successfully used in ophthalmology and proved to exert reparative actions on ocular damaged tissues also restoring visual capacity (Lambiase et al., 1998; Lambiase et al., 2009a; Lambiase et al., 2009b). Histological and autoradiographic evidences indicates

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that NGF eye drops act both at the retinal and post-retinal visual pathway levels, and exert its reparative effects also in primary brain visual areas (Lambiase et al., 1997; Lambiase et al., 2005; Sposato et al., 2009; Colafrancesco et al., 2010). In addition, ocular administrated NGF up-regulates the expression of the cholinergic markers in the NGF-target brain areas and promote the recovery of injured cholinergic neurons in adult mouse forebrain (Di Fausto et al., 2007; Lambiase et al., 2007), supporting the idea that from and throughout the eye tissues NGF can also enter the brain and/or exert central effects.

As reported, NGF promotes neuroprotection by acting on pre-existing mature neurons and guiding the differentiation and migration of neural precursors localised in germinative brain zones such as the dentate gyrus (DG) of the hippocampus and the subventricular zone (SVZ) (Fiore et al., 2002; Tirassa et al., 2003). Increased neurogenesis is also associated with the NGF-induced enhancement of cognitive performance in healthy and aged or brain lesion animals (Fiore et al., 2003; Triaca and Tirassa, 2003) further supporting the role played by NGF in the brain plasticity.

Whether NGF eye drops affect the proliferation or maturation neural precursors in brain is at present not known. These questions have been addressed in the present study by evaluating the effects of ocular applied NGF on the distribution of new cells in DG and SVZ of healthy adult rats. In addition, since other factors, including the NGF-family related neurotrophin the brain-derived neurotrophic factor (BDNF), promote proliferation and neurogenesis in germinal brain zones (Zigova et al., 1998; Pencea et al., 2001; Bath and Lee, 2010), the effects of NGF eye drop administration on the BDNF levels in different brain areas have been also investigated.

Materials and methods

Animals

Pathogen-free, adult male Sprague-Dawley rats (200-250 g) were maintained on a 12-hour light-dark cycle and provided with food and water *ad libitum*. For the housing, care and experimental procedures, we followed the guidelines indicated and approved by Intramural Committee and Institutional Guidelines of the Italian National Research Council in conformity with international laws (EEC council directive 86/609, 12 December 1987). All efforts were taken to limit the number of experimental rats.

NGF purification and administration

NGF was isolated from male mouse submaxillary salivary glands from animals and purified following a modified method of Bocchini and Angeletti (Bocchini and Angeletti, 1969). Purified NGF was dissolved in a physiological solution (0.9% sodium chloride) at a concentration of 200 μg/mL and stored at 4°C for less than a week.

The rats were divided into two groups: the first group of animals (NGF group n = 11) was treated with two drops (10 μl) of NGF solution – applied as collyrium to both eyes – containing 200 μg/mL of NGF; the second group of animals (Saline group n = 11) was treated on both eyes with two drops of physiological solution.

For immunohistochemical studies, the rats were sacrificed at 8 (n = 3 per group) and 24 hours (n = 3 per group) post treatment, with a dose of cloralium hydrate (400 mg/ml) and the cerebral tissues fixed through intracardiac perfusion with phosphate-buffered saline (PBS), followed by buffered 4% paraformaldehyde. After scalps were removed, the fixed brains were post-fixed with a solution of 4% paraformaldehyde for 24 hrs and then kept in 20% sucrose solution at 4°C until use.

For BDNF detections, the rats (n = 5 per group) were sacrificed at 24 hours post-treatment by decapitation and the fresh brains were removed from the scalp and dissected. The regions of the hippocampus (HI), septum (SE), entorhinal (EC) and frontal cortex (FC), and hypothalamus (HYP) were placed in sterile Eppendorf tubes and stored at -70°C until use.

Immunohistochemistry

Free floating immunohistochemistry was performed on 20-μm cryostat (ASI Instruments, USA) sections pretreated for 1 h with a solution containing 0.1% phenylidrazine, BSA (1 mg/ml) and 5% goat serum to block the endogenous peroxidase. Primary rabbit monoclonal anti c-fos (Cell Signaling Technology Inc., USA), rabbit polyclonal antibody against Ki67 (Signaling Technology Inc., USA) and homemade monoclonal antibody against the Neurotrophin receptor p75 ($p75^{NTR}$) were used to study the proliferating cells in germinal brain areas by single or double

staining technique. The sections were incubated overnight with the primary mentioned antibody and, following incubation in secondary antibody (biotynilated goat anti-rabbit, 1:200, two hours), processed for immunoperoxidase technique with the ABC Vectastain Kit (Vector Lab. Inc. Burlingame, USA) following the manufacturer's instructions. Staining specificity was assessed by omission of the primary antibody and by isotypic IgG. For double staining, after primary reactions, the sections were well swashed with PBS and then saturated with a solution of Avidin and Biotin (Avidin/Biotin Blocking Kit, Vector Lab. Inc. Burlingame, USA), to prevent the interaction of the second set of labeling reagents with the first ones. After blocking step the sections were processed as described for the fist reaction. DAB (orange/broen), DAB/Ni (brown or black) or Vector ®SG (blue/gray) were used as peroxidase substrates in single or double immunohistochemistry.
The immunohistochemical preparations were visualized on the Nikon Eclypse E600 equipped with the Nikon DMX 1200 digital camera connected to a PC computer.

Measurement of BDNF levels in brain

Brain tissue samples were homogenate by ultrasonication in buffer pH 7.00 (Tris-acetate 20 mM pH 7.5, NaCl 150 mM), EDTA 1 mM, EGTA 1 mM, sodium-pyrophosphate 2.5 mM, ortovanadate 1 mM, (R)-glycerolphosphate 1 mM, NaF 100 mM, PMSF (phenylmethylsulfonil fluoride) 1 mM, leupeptin 1 g/ml) and then centrifuged at 10,000°-g for 30 min at 4°C. The supernatants used for total protein and BDNF determination. The brain concentrations of BDNF were measured using an ELISA kit (BDNF Emax™ ImmunoAssay System G6891; Promega, Madison, WI) following the manufacture's instructions the colorimetric reaction product was measured at 450 nm using a microplate reader (Dynatech MR 5000, PBI International).
The BDNF data is expressed as UI/mg protein and pg/mg protein, respectively, and presented as mean SD. Total protein concentration was measured by the Bradford method.

Statistical analysis

Data were subjected to analysis of variance (ANOVA) using the SuperANOVA package for Macintosh (Abacus Concepts, Berkeley, CA) considering as variables the treatments with saline and NGF. Difference between groups was determined by Tukey-Kramer comparison; P 0.05 was considered statistically significant.

Results

NGF treatment affects the distribution of proliferating and neurotrophin-responding cells in germinal brain region. These effects were appreciable already at 8 hours post-NGF administration and persist at 24 hours, as reported in Fig. 1A-H.
The main effects were noted in the SVZ, where the cells expressing the proliferating marker Ki67 are normally abundantly localised (Fig. 1A-D). Compared to saline treatment (Fig. 1A), a large number of immunoreactive cells are localised along the border of the lateral ventricular and in the dorsolateral corner in rats receiving NGF eight and twenty-four hours before the sacrifice (Fig. 1B and C respectively). Magnification in Fig. 1D shows that Ki67-positive cells were most exclusively found in proliferating layer of the SVZ of NGF-treated rats, but larger cells with neural shape were also seen (indicated with an arrow in figure). Ki67 immunoreactive cells were also detected in the DG of both saline and NGF treated rats (data not showed)
Using double immohistochemistry, $p75^{NTR}$ and Ki67 markers were co-localised in cells of the SVZ (Fig. 1E-H). Round cells expressing the two markers were found in the layer II of the SVZ closely to other cells expressing only $p75^{NTR}$ (see Fig. 1E). Many double stained cells were localised in the dorsolateral corner (Fig. 1F). Magnifications in Fig. 1G and 1H show Ki67/p75NGFR cells with prominent processes in the dorsolateral corner of the ventricle and group of cells along the *corpus callosum*, respectively.
At 24 hours post NGF administration, numerous c-fos immunopositive cells were localised in the area surrounding the lateral ventricles at the suptum and fibria levels, see Fig. 2A and B, respectively. This data is in line with our very recent observation that NGF administrated as collyrium activates neurons in different brain areas, including the hippocampus and the hypothalamus (Calzà et al. data submitted for publication) further supporting the idea that from the eyes NGF can activates retinal pathways which innervate the limbic structures, like

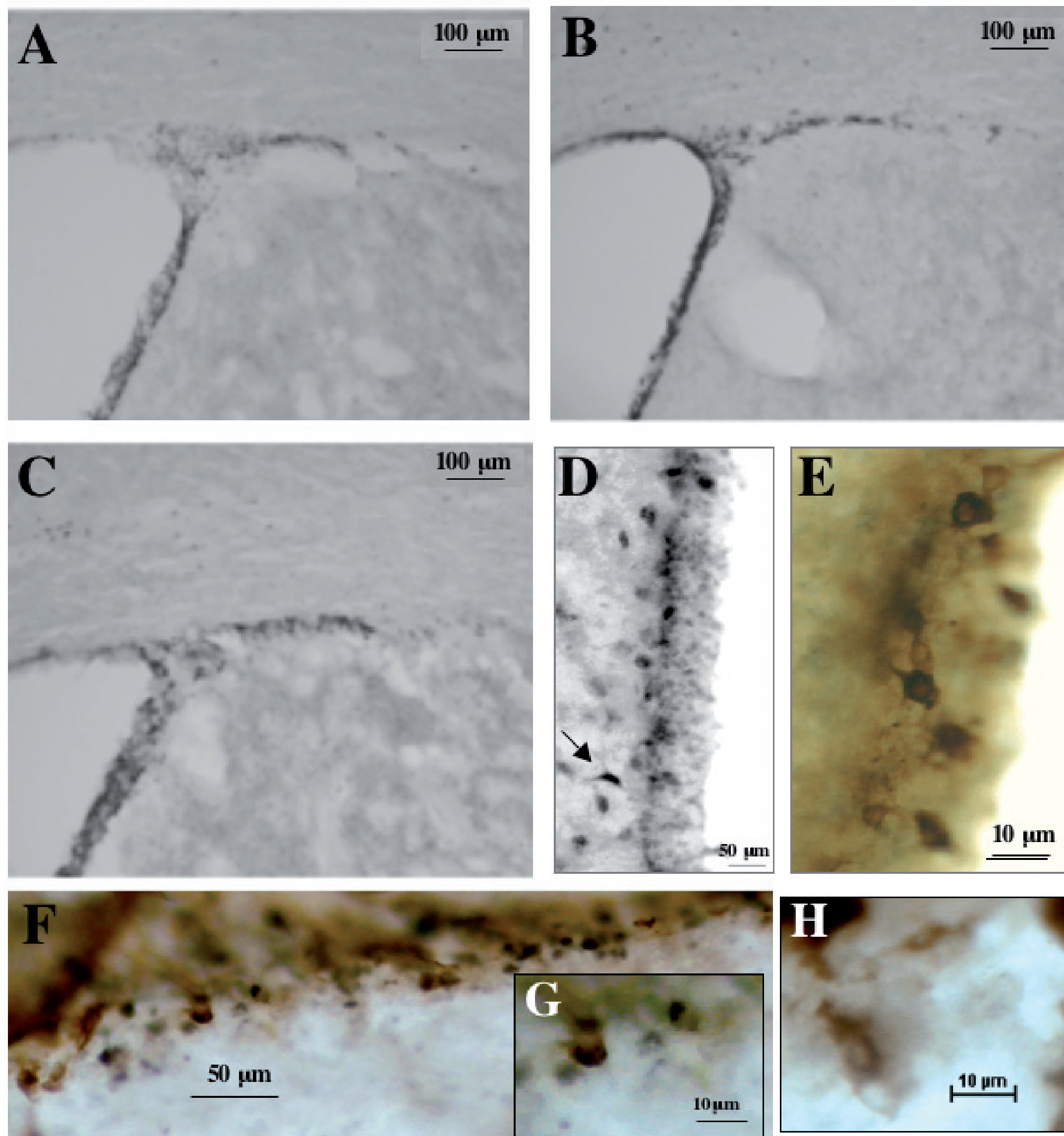

Fig. 1. - A-H. Distribution of Ki67 positive cells in the SVZ of adult rats receiving Saline (A) or NGF (B and C) as eye drops. Increase number Ki67 cells are observable at 8 and 24 hours post NGF administration, see pictures B and C respectively. The magnification in picture 1D shows that the Ki67 is mainly localised in round cells in the II layer of the SVZ, but morphologically different cells (see arrow) can be also found following NGF administration. Coexpression of Ki67 and $p75^{NTR}$ is showed in pictures E-H. Mostly of the round cells in the II layer express the neurotrophin receptors (E). Double stained cells presenting elongated morphology and leading processes are visible at levels of the dorsolateral corner (F and G) and in the area surrounding the *corpus callosum* (H).

described by Vereczki et al. (Vereczki et al., 2006), and represented in the Fig. 2C.

Parallelly to the stimulation of proliferating cells, NGF eye drop treatment also affects the brain BDNF content (Fig. 3). Compared to rats treated with saline, significant increase of BDNF protein was revealed in the septum ($p = 0.02$), and hippocampus ($p = 0.03$) of rats receiving NGF as eye drops 24 hours prior the sacrifice. A slight significant ($p = 0.0495$) BDNF decrease was found in the hypothalamus, while no changes were detected in entorhinal and frontal cortex.

Discussion

Beside its crucial role during development, NGF has also proved to influence brain plasticity and

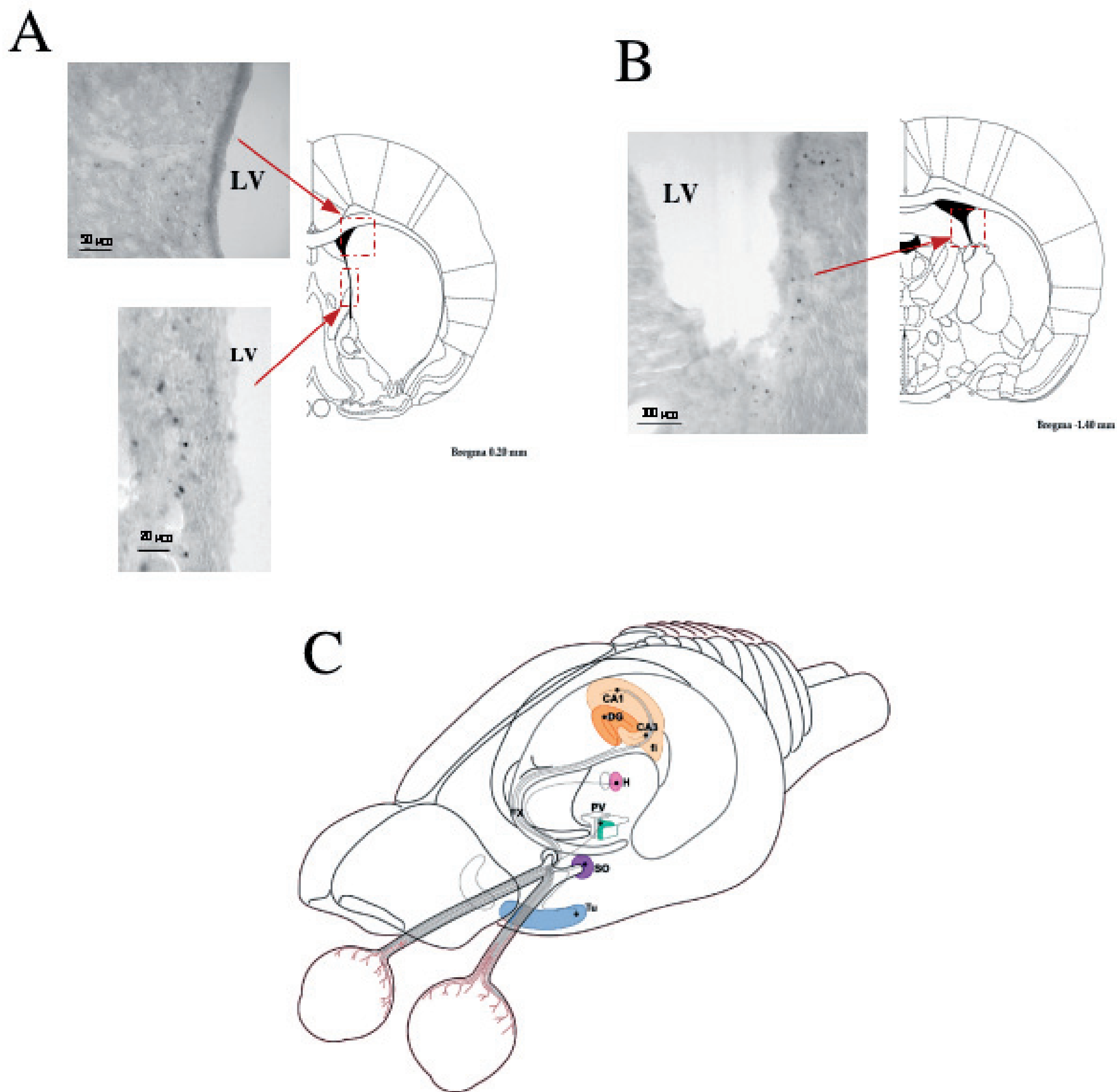

Fig. 2. - In panels A and B is showed the distribution of c-fos positive cells in subventricular zone at levels of lateral ventricles surrounding the septum (A) and the fimbria (B) of NGF-treated rats. The diagram below (C) shows the retinal projections to the limbic regions, which can be activated by NGF administrated as collyrium. Modified from Vereczki et al., 2006 (Vereczki et al., 2006).

neurogenesis in adulthood and during aging. These actions contribute to the mechanism by which NGF protects brain from insults and recovers damaged brain tissue, so that mature neurons and newly generated cells are targets for the exogenous NGF when administrated intracerebrally in animals (Fiore et al., 2002; Tirassa et al., 2003).

Recently, the topical application of NGF on the ocular surface has also showed to exert neuroreparative actions in humans and animals (Lambiase et al., 2009b) with ocular and brain diseases by restoring the structural and functional capacity of lesioned tissues. The data presented herein show that ocular administration of NGF affects the distribution of subventricular proliferating and neurotrophin-responding cells, and induces BDNF increase in forebrain, further supporting the efficacy of NGF eye drops to influence brain activity and function-sThe daily generation of new cell in adult SVZ, which is one of the most active germinal brain

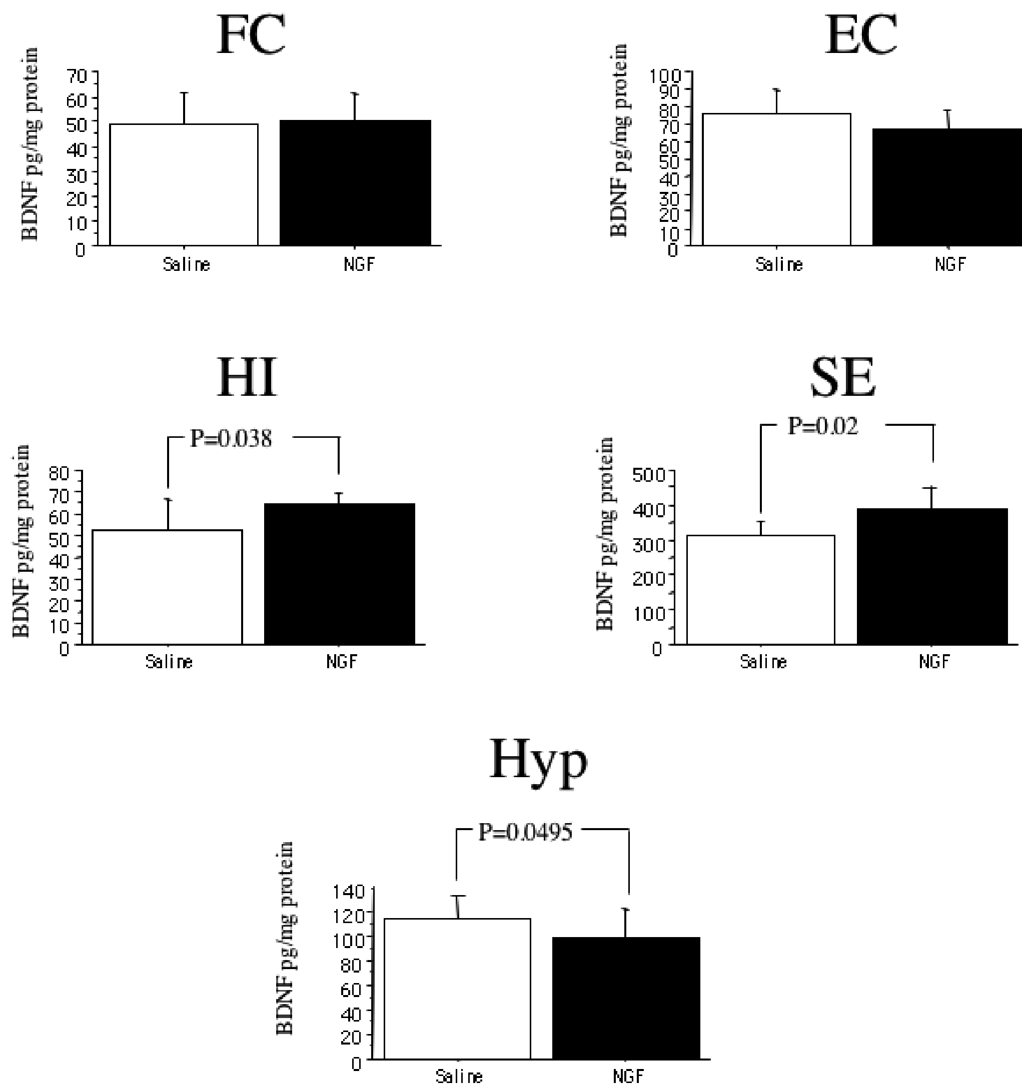

Fig. 3. - The graphs represent the BDNF levels (expressed as pg/mg total protein) measured in the enthorinal (EC) and frontal cortex (FC), hippocampus (HI), septum (SE) and hypothalamus (HYP) of adult rats receiving saline (□) or NGF (■) as eye drops 24 hours before the sacrifice. Statistical significance is showed in graphs.

area, and the length of cell cycle of about 15 hours (Cameron and McKay, 2001; Winner et al., 2002) corroborate the fact that NGF effects were observable at both 8 and 24 hours after administration and support our previous observations that NGF eye drop is a fast inducer of brain activities. Indeed, we have showed that: i) ocular applied radiolabelled NGF takes less than 6 hours to rich the retina and the optic nerve (Lambiase et al., 2005); ii) the activation of c-fos in diverse limbic regions, including the hippocampus and the hypothalamus, is already detectable a eight hours post-NGF ocular administration and persists at 24 hours (Calzà et al. data submitted for publication); iii) the brain levels of endogenous NGF are also increased at 6 hours following ocular NGF application and return to basal levels after on day (Di Fausto et al., 2007).

A similar time course is observable in SVZ, where the distribution of cells expressing the mitotic marker Ki67 increases respect to saline treated group and from 8 to 24 hours post NGF administration. Immunopositive cells were mainly found in the Layer II of the SVZ where the neuroblast-forming type A cells are found (Alvarez-Buylla and Garcia-Verdugo, 2002) indicating that the NGF treatment is affecting the production of new neural cells. The

colocalisation of $p75^{NTR}$ and the proliferative markers confirm the existence of neurotrophin-responding cells in the SVZ (Giuliani et al., 2004; Young et al., 2007) and further support the suggestion that NGF eye drop administration influences the formation and migration of neuronal precursor from the SVZ toward the parenchyma.

The ability of exogenous NGF to influences the proliferation of SVZ has been demonstrated in vivo following intracerebroventricular administration (Fiore et al., 2002; Tirassa et al., 2003), while *in vitro*, the application of NGF augments the survival and dendritic outgrowth of cells derived from the SVZ of rats (Gascon et al., 2005). More than stimulate proliferation *per se* NGF exerts a survival action and favourites the migration and differentiation of the newborn neurons (Cattaneo and McKay, 1990; Benoit et al., 2001), also acting cooperatively with other brain growth factors, including BDNF.

In vivo and *in vitro* evidences demonstrated that BDNF is capable to induce proliferation and neurogenesis through the $p75^{NTR}$ (Hosomi et al., 2003). In both postnatal and adult animals, the basal and BDNF-stimulated SVZ neuron production is dependent on the expression levels of $p75^{NTR}$, as showed in $p75^{NTR}$ null mice (Young et al., 2007). More, the population of $p75^{NTR}$-cells in SVZ is the same responding at the variety of environmental and diseases states characterised by altered levels of BDNF and brain neurogenesis (Duman and Monteggia, 2006; Curtis et al., 2007).

In our study, an NGF-induced enhancement of BDNF levels in the hippocampus and septum has been found. This data is in line with previous finding that a single NGF eye drop administration increases levels of BDNF protein and mRNA in the retina of adult rats (Lambiase et al., 2005), and indicate that from and throughout the eyes NGF modulates the BDNF synthesis in brain. Although BDNF levels were not analysed in SVZ in this study, the anatomical contiguity of the lateral ventricle with the septum and hippocampus led us to speculate that NGF treatment can result in a local increase of BDNF sufficient to drive neuronal precursors in the subventricular area. The evidences that following a single NGF intracerebroventricular administration the increased proliferation, migration and differentiation of subventricular neural cells are associated with the enhancement of BDNF protein and mRNA in the SVZ of mice (Tirassa et al., 2003) support this hypothesis.

However, since NGF administrated as collyrium also stimulates other growth factors, including beta-fibroblast growth factor, transforming growth factor-beta, vascular endothelial factor (Lenzi et al., 2005), which are as well involved in the proliferation and migration of the newly SVZ formed cells (Bath and Lee, 2010), the possibility that the NGF effects on SVZ are also mediated by other mediators need to be explored. In addition, as only the 40% of the new cells produce in the SVZ survive and can acquire neuronal phenotype to be functionally integrate into an existing circuitry (Lois and Alvarez-Buylla, 1994), the possibility that a single NGF application is not sufficient for completing the neurodifferentiative process cannot be excluded in our experimental condition. The effects of repeated and prolonged treatment with NGF eye drops in healthy animals and models of brain neuropathy are at present under investigation in our laboratory.

In conclusion, the findings that NGF ocular administration affects the levels of BDNF in forebrain areas and the number of proliferating cells expressing $p75^{NTR}$ in SVZ indicate the efficacy of ocular NGF to influence the machinery regulating neurogenesis in the SVZ and contribute to strengthen the idea of the NGF eye drop use for the treatment of brain neurological diseases.

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