Vascular and Neuronal Protection Induced by the Ocular Administration of Nerve Growth Factor in Diabetic-Induced Rat Encephalopathy

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SUMMARY

Background: Based on our previous findings on the efficacy of ocular applied nerve growth factor as eye drops (oNGF) to act in brain and counteract neuronal damage, we hypothesized that oNGF treatment might revert neuronal atrophy occurring in diabetic brain also by controlling neurotrophin system changes. The major NGF brain target areas, such as the septum and the hippocampus, were used as an experimental paradigm to test this hypothesis. **Methods:** Bilateral oNGF treatment was performed twice a day for 2 weeks in full-blown streptozotocin-treated adult male rats. The forebrain distribution of cholinergic and endothelial cell markers and NGF receptors were studied by confocal microscopy. The septo-hippocampal content of NGF mature and precursor form and NGF receptors expression were also analyzed by Elisa and Western blot. **Results:** oNGF treatment recovers the morphological alterations and the neuronal atrophy in septum and normalized the expression of mature and pro-NGF, as well as NGF receptors in the septum and hippocampus of diabetic rats. In addition, oNGF stimulated brain vascularization and up-regulated the TRKA receptor in vessel endothelium. **Conclusions:** Our findings confirm that reduced availability of mature NGF and NGF signaling impairment favors vascular and neuronal alterations in diabetic septo-hippocampal areas and corroborate the ability of oNGF to act as a neuroprotective agent in brain.

Introduction

In diabetic patients, the insulin deficiency and/or impaired insulin signaling is thought of as being responsible for inducing or precipitating neurodegeneration in brain vulnerable areas, including the septum and hippocampus [1] and, in turn, producing cognitive deficits [1,2].

A similar scenario is observable in streptozotocin-induced (STZ) diabetic rodents, in which the insulin depletion produced by pancreatic β -cell destruction causes encephalopathy [3]. In adult rats, the white and gray matter degeneration occurs relatively early (1–2 months) after the STZ-induced disease onset [4,5] and progressively results in spatial and long-term memory deficits accompanied by severe neuronal loss in forebrain. This evidence indicates that the detrimental effects of diabetes on brain are related to disease duration and suggests that STZ-induced diabetes is a useful model to identify temporal windows and efficacy of new therapeutic interventions [5]. Because of the emerging importance of diabetes as risk factor for dementia and Alzheimer's disease, the STZ model is also appealing to investigate causative common mechanisms for forebrain vulnerability like those related to the

perturbation in the brain expression of the neurotrophin nerve growth factor (NGF) [3,6].

NGF is an essential survival and differentiative factor for both mature and precursor nervous cells, from development to aging, and in pathological conditions [7]. Altered production and signaling of NGF are recognized as pathological markers for both peripheral and central nervous system and as responsible for the progression and worsening of neurological diseases [8], including diabetic neuropathy [9]. The identification of NGF precursor molecules (proNGF) and the impact of NGF dysmetabolism on neurodegeneration [10,11] have further supported the key role of mature NGF (mNGF) in the control and maintenance of brain cells. Indeed, the increased proNGF accumulation in hippocampus is today considered as part of a pathological cycle, which induces cholinergic neuron degeneration by affecting the mNGF/proNGF ratio and the NGF receptor balance [12–14].

Whether NGF production and signaling are altered and/or correlated with neurodegeneration in the forebrain of STZ diabetic rats is at the present not fully investigated. These two questions have been addressed in this study by analyzing the expression of pro and mature NGF and their receptors in the septum and hippo-

campus of STZ-induced diabetic rats. In addition, the effects of NGF treatment during the early stage of diabetes were also evaluated using NGF eye drops (oNGF) administration as a means to deliver NGF and/or activates its signals in brain.

oNGF has been successfully used in ophthalmology and in animal models of ocular pathologies, including diabetic retinopathy and glaucoma [15]. Beside this, when applied as eye drops, NGF activates c-fos in several areas of the limbic system in a time-dependent manner [16], stimulates brain choline acetyltransferase (ChAT) and induces neuronal recovery following chemical lesion in the forebrain [17], indicating that oNGF biological actions are extended to brain structures other than the primary visual ones and are similar to those observed following NGF intracerebral injection [18]. Thus, this study was aimed to verify the hypothesis that oNGF treatment performed during the early disease stage might influence the insurgence or progression of diabetic encephalopathy. Further, as the integrity of ocular structures and pathways is compromised in diabetes, our experimental approach might offer the double opportunity to enlarge the knowledge on oNGF-induced neuroprotection and evaluate the brain efficacy of oNGF in pathological conditions which potentially alter NGF transportation from the eyes to the brain [19,20].

Materials and Methods

Animals, Diabetes Induction, and NGF Treatment

Diabetes was induced in adult male Sprague-Dawley rats (b.w. 200–250 g) rats ($n = 30$) by a single injection of streptozotocin (STZ; Sigma-Aldrich, St. Louis, MO, USA; 60 mg/kg b.w.). Rats with blood glucose levels above 17 mmol/L – checked with an Accutrend GC glucose analyser (Roche Diagnostic, Mannheim, Germany) – were allocated to the streptozotocin experimental groups. Control rats received an injection of physiological solution ($n = 10$).

Purified NGF isolated from male mouse submaxillary salivary glands [21] dissolved in a physiological solution (0.9% sodium chloride) at a concentration of 200 $\mu\text{g}/\text{mL}$ was used during treatment.

A preliminary experiment was performed to further validate the safety of oNGF treatment and its efficacy to act in adult brain as trophic factor for cholinergic neurons. Increased expression of cholinergic markers in forebrain and no side effects, that is, decreased body weight or increased anxiety levels were observable following 2 weeks of oNGF treatment – two drops (10 μL) of NGF solution (200 $\mu\text{g}/\text{mL}$) in each eyes – in healthy adult male rats (Figure S1).

Based on this, the efficacy of oNGF to act in pathological conditions was investigated in the diabetic rats. One group of STZ rats (STZ+NGF) received two drops (10 μL) of NGF solution (200 $\mu\text{g}/\text{mL}$) in each eyes; a second group of diabetic rats (STZ) received two drops of physiological solution per eye. Healthy controls also received ocular treatment with saline. The saline/NGF treatment started 6 weeks from the diabetes induction and was repeated twice a day for 2 weeks. All rats were killed at 8 weeks post-diabetes induction.

For the housing, care, and experimental procedures, we followed the guidelines indicated and approved by the Intramural

Committee and Institutional Guidelines in conformity with international laws (EEC council Directive 2010/63/EU revising Directive 86/609/EEC, 12 December 1987).

Histological Preparations

Four rats per group were anaesthetized (chloralium hydrate at 400 mg/Kg b.w.) and intracardially perfused with phosphate-buffered saline (PBS), followed by buffered 4% paraformaldehyde. After removing, the brains were postfixed for one day and then kept in 20% sucrose solution at 4°C until use. Free floating immunohistochemistry and immunofluorescence were performed on 30- μm cryostat (ASI Instruments, Houston, TX, USA) coronal brain sections, including the septum and the hippocampus. Toluidine blue staining was performed to analyze brain histology.

Septal Cholinergic Cell Detection and Count

ChAT immunohistochemistry was performed on free floating sections using a primary monoclonal antibody against ChAT kindly provided by dr. C.Cozzari (IBCN, Italy) and ABC Vectastain Kit (Vector Lab. Inc. Burlingame, CA, USA) for reaction development.

The immunohistochemical preparations were visualized on the Nikon Eclipse E600 equipped with the Nikon DMX 1200 digital camera connected to a PC computer. Images from sections including the medial septum – plates 13–16 according to Paxinos and Watson [22] – were coded, and positive cells were counted in three regularly spaced sections per rat ($n = 4$ per group). Cell count was carried out using the image processing and analysis program Nikon NIS-Elements AR 2.30, and measurements were standardized between the experimental groups using the same calibration system and thresholding. The number of immunoreactive neurons (expressed as mean $\pm$ S.D. per mm^2) was determined in $10 \times$ magnification images. A morphological program selecting only cell bodies – but not small fragments, dendritic trees and cells without complete soma – was used to quantify immunopositive cells. Each object having higher or lower area and/or under threshold stain intensity was not considered.

Analysis of Brain Vasculature

Isolectin GS-IB4 from Griffonia simplicifolia, biotin-conjugate (Invitrogen Corporation, Carlsbad, CA, USA), and Streptavidin-AV-Alexa 594 (1:100 in PBS; Invitrogen Corporation) were used to identify blood vessels on brain sections. The brain immunofluorescence preparations were used for imaging analysis by Nikon NIS-Elements AR 2.30 program. For brain, images from sections including the medial septum and hippocampus – plates 13–15 and 28–32 according to Paxinos and Watson (1986), respectively – were coded, and vessels were counted in three regularly spaced sections per rat ($n = 4$ per group).

Vasculature assessment was based on the method of area fraction previously described [23] by counting the area occupied by blood vessels with respect to the total measured area. The total and vessel areas were determined in $10 \times$ magnification images. The stain intensity and the area range were set to exclude any fibroblasts, macrophages, and background noise in the image. The values were expressed as mean vessel area fraction $\pm$ S.D.

Confocal Study

NGF receptor distribution and cell characterization in the septum and hippocampus were studied by immunofluorescence using rabbit anti-TRKA (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA); monoclonal anti-p75NTR (clone 192 kindly provided by dr. E.M. Johnson, USA); monoclonal or polyclonal anti-ChAT (Chemicon, Temecula-CA, USA); and rabbit anti-CD34 (Santa Cruz Biotechnology Inc.). The AlexaFluor 488 or 555 anti-rabbit and AlexaFluor anti-mouse 594 (Invitrogen Corporation) were used as fluorochrome-conjugated secondary antibodies.

Images were acquired through a confocal laser scanning microscope (CLSM; SP5 Leica Microsystems, Wetzlar, Germany) equipped with four laser lines. Violet diode laser emitting at 405 nm, argon laser emitting at 488 nm, a helium/neon laser

emitting at 543 nm, and a helium/neon laser emitting at 633 nm. Images were acquired in sequential mode using objectives 20 $\times$ and 63 $\times$, pinhole value of 1 Airy units. Format resolution was 1024 $\times$ 1024 pixels and the acquisition speed 10 Hz. Figures were generated by adjusting only image brightness and contrast and composed using Adobe Illustrator 10 or Photoshop.

Protein Extraction

Six/eight rats per group were sacrificed by decapitation, the brains removed, and the hippocampus and septum were quickly dissected on ice and then maintained at -80°C until used. The tissue samples were homogenated 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, orthovanadate 1 mM,

Figure 1 (A–H) Cell atrophy/death and vascularization in the septum. (A–C) shows the distribution of ChAT immunopositive cells in the medial septum – indicated as gray in A – of CTR (B), STZ (C), and STZ+NGF (D). Bar = 100 μm . The visible reduction in stained cells in STZ and increased immunoreactivity in STZ+NGF are confirmed by the cell count reported in graph (E). The levels of DNA fragmentation reported in graph F (expressed as arbitrary OD units) indicate the effects of diabetes and oNGF treatment on cell mortality in the septum. The pictures at the bottom show the distribution of IB4-positive vessels in the septum of STZ (G) and STZ+NGF (H) rats (Bar = 50 μm). The graph on the right side (I) shows the difference in area fraction occupied by vessels in the three experimental rat groups. * $P < 0.05$; ** $P < 0.01$ statistically significant changes.

(R)-glycerol phosphate 1 mM, NaF 100 mM, PMSF (phenylmethylsulfonyl fluoride) 1 mM, leupeptin (1 g/mL) and then centrifuged at 10,000 rpm for 30 min at 4°C. The supernatants were used for total protein concentration – measured by the Bradford method – Elisa assays and Western blot studies as described.

Cell death in Septum and Hippocampus

Tissue extracts were used to determine the levels of DNA fragments by Cell Death Detection ELISA kit (Roche Inc.) following the manufacturer's instruction. The assay is based on a quantitative sandwich-enzyme-immunoassay principle, using mouse monoclonal antibodies directed against DNA and histones, respectively. This allows the specific detection and quantitation of mono- and oligonucleosomes that are released into the cytoplasm of cells that die from apoptosis. The colorimetric reaction product was measured at 690 nm using a microplate reader (Dynatech

MR 5000, PBI International). Results are expressed as OD 690 arbitrary units and reported as mean $\pm$ S.D.

Western Blot Studies

Samples (20–70 μ g total protein) were separated by SDS-PAGE, and electrophoretically transferred on PVDF membranes. After blocking, the membranes were incubated overnight at 4°C with primary antibody, such as anti p75NTR, TRKA and proNGF (anti-rabbit ANT005 purchased by Alomone Labs, Israel). The horseradish peroxidase conjugated anti-rabbit IgG (Cell Signalling Technology, Danvers, MA, USA) or anti-mouse IgG (Cell Signalling Technology) served as secondary antibodies. The blots were developed with ECL Chemiluminescent HRP Substrate (Millipore) as the chromophore.

The public domain Image J software (<http://rsb.info.nih.gov/ij/>) and the method described at <http://lukemiller.org/index.php/2010/11/>

Figure 2 (A–D) NGF receptor localization in the septum. The TRKA and p75NTR single stain and co-stain in the CTR, STZ and STZ+NGF septum are showed in **A**: note the reduction in cells expressing the NGF receptors in STZ and the recovering effects of oNGF on TRKA/p75NTR cell number and morphology. Magnification shows the different localization of NGF receptors on blood vessels and surrounding area. Specifically, single p75NTR and p75NTR/TRKA co-stain were found in cells inside and around the blood vessel (**B**), while only TRKA stains the basal endothelium and co-localizes with CD34 (**C** and **D**). Bar = 20 μ m.

analyzing-gels-and-western-blot-with-image-j/ were used for gel densitometry and protein quantification, respectively. The integrated density of β -actin bands was used as normalizing factor. Statistical evaluation was performed on separate gel runs/blots carried out using three different sets of samples ($n = 6$ or 8 for each experimental group).

Neurotrophin Concentrations

The brain concentrations of NGF were measured using the rat beta NGF Duoset Elisa (R&D) and following the manufacturer's instructions. The colorimetric reaction product was measured at 450 nm using a microplate reader (Dynatech MR 5000, PBI International). The NGF data are expressed as pg/mg protein and presented as mean $\pm$ S.D.

Statistical Analysis

Data were subjected to analysis of variance (ANOVA) using the Super ANOVA package for Macintosh (Abacus Concepts, Berkeley, CA, USA) considering diabetes and NGF treatment as variables. Difference between groups was determined by Tukey-Kramer multi-comparison; $P < 0.05$ was considered statistically significant.

Results

Cholinergic and Vascular Markers in the Septum

A reduction in the cellular density accompanied by a decreased stain of the cell body and terminals is observable in the medial

Figure 3 Effects of diabetes and oNGF on the hippocampal histology and vasculature. Toluidine blue stain reveals changes in the pyramidal layer of CA2/CA3 (A) of STZ rats when compared with STZ+NGF or healthy control rats (not showed). Many picnotic and ghost cells were localized in the DG area of STZ, while no apparent differences between CTR and STZ+NGF were observed (B). Representative pictures of the GS-IB4 (red stain) labeled large and small blood vessels in the CA2/3 and DG of CTR, STZ and STZ+NGF rats. A reduced vessel density and a relative augment of small vessels are observable in both the hippocampal areas of STZ rats compared with control rats and diabetic rats receiving NGF, see C. The graphs in D, reporting the computer analysis of the vessel density (expressed as fraction area occupied by vessels, see Materials and Methods and Results section for details) confirm the microscopy observations and show differences between the experimental groups. $**P < 0.01$ statistically significant changes.

septum of STZ rats (Figure 1C) compared with control (Figure 1B) and STZ+NGF group (Figure 1D). Quantitative analysis reported in the graph of Figure 1E validates these observations showing a significant decrease in ChAT cell number in STZ septum (203 ± 35 cells/mm²; $P < 0.05$) when compared with CTR (360 ± 45 cells/mm²) and STZ+NGF (297 ± 25 cells/mm²).

The measurement of the cytosolic levels of DNA fragments also confirms diabetes and oNGF effects on septal cell viability. As showed in the graph (Figure 1F), an increase in DNA fragments – expressed as optical density level – was detectable in the septum supernatants of STZ rats (415 ± 78 OD₆₉₀ $\times$ 1000), respect to that found in CTR (240 ± 46 OD₆₉₀ $\times$ 1000; $P = 0.003$ versus STZ) and STZ+NGF (261 ± 22 OD₆₉₀ $\times$ 1000; $P = 0.002$ versus STZ).

The analysis of the septal vessel density shows the difference between the STZ and the STZ+NGF group. The immunoreactive vessels in the medial septum of STZ (Figure 1G) are reduced in

number, size, and stain intensity when compared with those detectable in the same area of STZ+NGF rats (Figure 1H). Quantitative analysis substantiates this observation showing a significant decrease in the vessel area fraction in STZ septum (0.049 ± 0.031 vessel area/total area; $P < 0.01$ versus CTR and NGF groups) compared with both CTR (0.151 ± 0.028 vessel area/total area) and STZ+NGF (0.119 ± 0.015 vessel area/total area), see Figure 2I. The septal vessel distribution in CTR and oNGF treated rats was not statistically different.

NGF Receptor Distribution in the Septum of Diabetic and oNGF Treated Rats

In the medial septum of diabetic rats, immunoreactive cells presenting neuronal shape are reduced in number and size with respect to the other two rat groups and are mainly stained for p75NTR only (Figure 2A). In the STZ+NGF group, the TRKA/

Figure 4 (A–D) Distribution of TRKA and p75NTR in the CA2/3 area of the hippocampus. The effects of diabetes and oNGF on the NGF receptor distribution in CA layers are showed in **A**. Compared with CTR, TRKA expression is reduced in stratum pyramidalis (SP), and p75NTR immunoreactivity is shifted from the pyramidalis to the stratum oriens (SO) in diabetic rats, while in STZ+NGF rats, an increase in TRKA immunofluorescence is observable in stratum oriens (SO), pyramidalis (SP), and lucidum (SL). Cells co-localizing TRKA and p75NTR are identifiable. TRKA-positive blood vessels were scarcely found in STZ CA2/3 area but largely increased in STZ+NGF with respect to CTR. The magnification in **B** and **C** shows the TRKA-positive vessels and the p75NTR expression on vessels surrounding cells or intralumen cells. The co-expression of TRKA and CD34 is showed in Figure 5D. Bar = 20 μ m.

p75NTR- and TRKA-positive cells show increased cell body size and neuronal soma and process stain intensity when compared with CTR and STZ (Figure 2A).

A reduced vessel stain is also noted in the septum of diabetic rats compared with CTR or STZ+NGF rats where TRKA and p75NTR are also localized perivascularly or marking endothelial cells. Specifically, p75NTR stains cells are present closely to the vessels, and in the vessel lumen, while TRKA profiles the basal endothelium (Figure 2B). The co-stain with CD34 marker indicates the TRKA expression on endothelial cells (Figure 2C and D).

Effects of Diabetes and oNGF Treatment on Hippocampal Cell Viability and Vasculature

No significant differences in DNA fragmentation level were found between rat groups (data not shown), but the histological examination reveals cell loss in the CA3 pyramidal layer (Figure 3A), and ghost cells – characterized by loss of nuclear staining (black arrows) – and small cells with condensed highly stained cytoplasm (red arrows) – in the DG area of STZ rats (Figure 3B).

Figure 5 (A–D) NGF receptors in *Dentate gyrus* of hippocampus. In **A**, the effects of diabetes and oNGF on TRKA and p75 expression in DG are shown. Note the increased expression of TRKA on vessels and the TRKA/p75-positive cells in the hilus of STZ+NGF rats. The white frames indicate the area of magnifications at the bottom panel in which it is possible to observe the different distribution of NGF receptors in the granular layer of the three rat groups. Particulars of p75NTR immunoreactive cells localized closely to TRKA-stained vessels in STZ+NGF DG are shown in Figure 6B and C. Co-expression of TRKA and CD34 endothelial marker in vessels is demonstrated in Figure 6D. Bar = 20 μ m.

Figure 6 Expression of TRKA and p75NTR in the septum. The figure on the left shows the representative Western blots for the two NGF receptors. Two bands at 140 and 110 kDa identifying the mature and immature form of TRKA, respectively, were recognized, while a single band was found for p75NTR. Band intensities were normalized to β -actin, and the receptor expression was quantified from three or four independent experiments, and average levels in septum of CTR, STZ, and STZ+NGF rats are showed in the graphs. * $P < 0.05$; ** $P < 0.01$ statistically significant changes.

Small or fragmented blood vessels are also localizable in the CA3 and DG areas of diabetic rats, while vessels with increased size and stain intensity are found in the same hippocampal regions of diabetic rats receiving oNGF treatments (Figure 3C). Congruently, quantitative analysis (graph in Figure 3D) shows a significant reduction in vessel area fraction in CA3 (0.045 ± 0.009 vessel area/total area) and DG (0.012 ± 0.018 vessel area/total area) of STZ rats when comparing to CTR (CA3 = 0.082 ± 0.015 ; DG = 0.062 ± 0.018 vessel area/total area; $P < 0.01$ versus STZ); and STZ+NGF groups (CA3 = 0.092 ± 0.008 ; DG = 0.033 ± 0.012 vessel area/total area; $P < 0.01$ versus STZ).

TRKA and p75NTR Localization in Hippocampus

Similar to the septum, in the hippocampus, the NGF receptor antibodies mark both neuronal shaped cells and blood vessels, and differences in the subregional receptor distribution are observable in the CA2/3 and the DG area.

In the CA2/3 area of controls, the expression levels of the two NGF receptors appear similar and mostly concentrated in the pyramidal stratum. Many TRKA-positive vessels distributed along

the entire CA2/3 area are also observable (Figure 4A). In comparison, TRKA immunoreactivity is reduced in the CA2/3 pyramidal stratum of diabetic rats, and a more marked expression of p75NTR is localizable in the stratum oriens, where p75NTR/TRKA-stained cells are also observable (white arrows). What's more, although present, the TRKA-positive blood vessels are less notable.

In STZ+NGF rats, TRKA is distributed in the stratum oriens, pyramidal, and lucidum, where the p75NTR/TRKA-double-stained cells (white arrows in panel A) also is in larger number than the other rat group. Numerous TRKA-positive blood vessels are also present in the CA2/3 area of STZ+NGF rats, particularly in the stratum oriens (blue arrows in panel A). The p75NTR immunoreactivity is scarcely detectable in vessels localized in CA2/3, while TRKA stains almost the entire vessel wall (Figure 4B and C). Figure 4D shows the co-expression of CD34 and TRKA in vessels indicating the receptor localization on endothelial cells.

In dentate gyrus (Figure 5A), diabetic rats show a reduction in TRKA immunoreactivity and enhanced p75NTR single stain in cells in the granular layer and hilar region. In the STZ+NGF rat, the expression of p75NTR is also slightly increased compared with

Figure 7 Expression of TRKA and p75NTR in the hippocampus. The effects of diabetes and oNGF on the expression levels of TRKA (110 and 140 kDa), and p75 receptors are visible in the representative Western blot (figure on upper left side), and graphs. * $P < 0.05$; ** $P < 0.01$ statistically significant changes. Quantifications of three or four independent experiments were performed, and β -actin expression was used to normalize the sample variability.

CTR, but at variance with STZ, augmented TRKA immunoreactivity and cells co-expressing the two NGF receptors are observable in DG granular hilus and the molecular layers.

TRKA immunoreactivity is also found to delimit vessel circumference or mark the vessel long axis (Figure 5B–D), while no endothelial cells p75NTR immunopositive were localizable close to TRKA-positive vessels (Figure 5B and C). Co-expression with CD34 confirms the TRKA localization on vascular epithelial cells (Figure 5D).

Western Blot Analysis of NGF Receptors

Microscopy observations are confirmed by Western blot analysis. Indeed, significant increase in p75NTR and p75NTR/TRKA ratio and reduced levels of mature 140 kDa TRKA form are detectable in the septum (Figure 6) and hippocampus (Figure 7) of STZ rats, compared with CTR and STZ+NGF groups. No differences are observed in the expression levels of 110 kDa TRKA. In the septum of STZ+NGF rats, p75NTR is significantly increased compared with CTR and STZ, however, because 140 kDa TRKA is higher than in CTR, the p75NTR/TRKA ratio resulted comparable to CTR (Figure 6).

Similarly, in the hippocampus, the increased levels of TRKA detectable following oNGF treatment help to normalize the p75NTR/TRKA ratio also in the presence of the high but not significant levels of p75NTR (Figure 7).

Mature and pro-NGF Content in the Septum and Hippocampus

The concentrations of mature NGF in the rat septum and hippocampus were analyzed using an Elisa assay specific to recognize rat β -NGF in tissue extracts (Figure 8). Compared with CTR (56 ± 8 pg NGF/mg total protein), a significant NGF decrease is detected in the septum of STZ rats (38 ± 14 pg NGF/mg total protein; $P = 0.03$), while no significant variations are found in STZ+NGF (42 ± 7 pg NGF/mg total protein). In hippocampus, NGF levels tend to increase in diabetic rats (164 ± 37 pg NGF/mg total protein), but the change was significant only versus the STZ+NGF group (CTR = 123 ± 31 ; STZ+NGF = 106 ± 20 pg NGF/mg total protein, $P = 0.005$).

The comparative analysis of the proNGF expression levels in STZ reveals no significant changes in the septum and a significant increase in hippocampus when compared with CTR ($P < 0.0001$).

Figure 8 NGF content in the septum and hippocampus. The effects of diabetes and oNGF on the NGF and proNGF content in the septum and hippocampus were analyzed by ELISA and Western blot, respectively.

* $P < 0.05$; ** $P < 0.01$ statistically significant changes.

and STZ+NGF ($P = 0.0004$). No variations of septal and hippocampal proNGF were found comparing CTR and STZ+NGF.

Discussion

The involvement of NGF and NGF receptors in diabetic encephalopathy and the efficacy of ocular administrated NGF to counteract the diabetes-induced cerebral neurodegeneration have been analyzed in this study using STZ-induced diabetes in adult rats as experimental model. The findings reported herein confirm and extend the information on oNGF efficacy to affect brain in pathological conditions and suggest potential mechanisms of action showing that oNGF rebalances the mature and proNGF ratio, and up-regulates the TRKA expression in brain blood vessels.

Focusing our attention on NGF primary brain target areas, such as the septum and the hippocampus, we demonstrated that diabetes-induced cholinergic deficit is associated with the up-regulation of the P75NTR with respect to the TRKA receptor and, a reduced concentration of mature NGF in favor of its precursor form. A reduction in NGF and TRKA in diabetic hippocampus has been recently reported by Sima et al. [5], supporting that altered NGF synthesis/processing or signaling might contribute to encephalopathy. The increased proNGF/P75NTR binding, which it has proved to down-regulate TRKA maturation [24], might also explain our observation that 140 kDa TRKA form is reduced with respect to the immature non-glycosylated 110 kDa form in diabetic septum and the hippocampus and thus favors neuronal degeneration.

The altered relationship between the NGF forms and the two NGF receptor types is considered early events in the neurodegenerative process [25]. For example, the reduction in forebrain NGF [8] and TRKA [26] and the increase in levels of proNGF [27] characterize patients with mild cognitive impairments or in the early AD stage. Increased neurodegeneration and further impairment of mature NGF retro-transport or utilization has been shown to occur during AD progression and might continue on severe stages with significant cognitive dysfunction [8]. These facts indicate the existence of temporal windows during the course of the disease suitable for treatments addressed at alienating or obstructing neurodegeneration, reducing disease progression or facilitating repair of damaged tissue.

In this study, we hypothesized that daily treatment with oNGF during the early stage of diabetes might interfere with the events concurring to generate encephalopathy. Actually, we were able to validate this hypothesis showing reduced signs of brain cell atrophy and death and reduced cholinergic damage in diabetic rats receiving oNGF. We also demonstrate that, following treatment with oNGF, the unbalance between NGF forms and NGF receptors observable in the septum and hippocampus of diabetic rats is recovered. The mechanism by which these events occur is only speculative in our experimental conditions.

One possibility is that oNGF contributes to increase the availability of mature NGF in diabetic brains as observed in an other experimental model [17] or induces the brain-derived neurotrophic factor (BDNF) as demonstrated in the retina [19] and brain of healthy rats [28].

Whether brain BDNF synthesis or release is affected by diabetes, and/or repeated daily NGF administration was not determined in this study. However, as BDNF acts as survival and trophic factor on forebrain neurons [29], and reduced levels of BDNF have been previously found in the hippocampus of diabetic rats [30], the possibility that this neurotrophin might be also involved in the NGF-induced recovery of cholinergic deficit in STZ is highly plausible.

In addition, we observed that oNGF treatment up-regulates TRKA expression in blood vessels and stimulates revascularization in septo-hippocampal area. The co-expression with CD34 reveals that TRKA labels capillary endothelial cells and suggests that this cell type is able to respond to ocular administrated NGF.

Moser et collaborators [31] proved the autocrine/paracrine action of NGF on the brain capillary endothelial cells showing that these cells: preferentially express TRKA, respond to NGF by increasing proliferation, and produce NGF following inflammation. The same authors found that NGF also acts as a protective factor on cholinergic neuronal degeneration induced by dysfunctional endothelial cells by a NGF-activated TRKA mechanism [32].

Based on this evidence, endothelial cells might participate in the degenerative and NGF-mediated reparative events occurring in brain of STZ rats. The evidences that altered BBB permeability [33] and endothelial cell dysfunctions [34] have been described in diabetic patients and animal models support the suggestion that the impaired cerebrovascular system contributes to brain alterations, including the cholinergic neuronal damage observed by us in the STZ rats.

In this condition, NGF treatment might stimulate TRKA expression in endothelial cells and favor reparative brain angiogenesis

and/or obstacle neurodegeneration, like that observed following cutaneous wounds in type 1 diabetic mice [35]. In addition, TRKA-activated endothelial cells producing NGF might contribute to enhance the brain NGF content to counteract the diabetic-induced imbalance between the mature and proNGF. Future investigations are necessary to validate this intriguing hypothesis.

In conclusion, our study confirms that decreased availability of mature NGF and altered NGF receptors contribute to forebrain neuronal atrophy and demonstrate as oNGF's is able to counteracts diabetes-induced vascular and neuronal injury. As diabetes affects the retina and optic nerve, and these structures partially mediate the oNGF activity in brain [19], our findings indirectly indicate that the retina damage does not compromise the cerebral neuroprotective effects of oNGF. This fact, in addition to the findings that brain epithelial cells respond to oNGF treatment and might contribute to oNGF-mediated reparative effects, offer a new prospective to the neurotrophic factor-based therapy and support the efficacy and feasibility of ophthalmic drug delivery.

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Conflict of Interest

The authors declare no conflict of interest.

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Supporting Information

The following supplementary material is available for this article:

Figure S1. To validate the efficacy of ocular applied NGF to exert biological action in brain, healthy Sprague Dawley adult male rats received daily oNGF treatment for 2 weeks.