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Glaucoma alters the expression of NGF and NGF receptors in visual cortex and geniculate nucleus of rats: Effect of eye NGF application

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ABSTRACT

We investigated the effect of glaucoma (GL) on nerve growth factor (NGF) presence in two brain visual areas. Rats with elevated intraocular pressure (EIOP), induced by hypertonic saline injection in the episcleral vein, were treated with eye topical application of saline or NGF. Rats were subsequently sacrificed, and brain tissues were used for immunohistochemical, biochemical, and molecular analyses. We found that GL alters the basal level of NGF and NGF receptors in brain visual centers and that NGF eye application normalized these deficits. These findings demonstrate that the reduced presence of NGF can arise due to degenerative events in retinal and brain visual areas.

1. Introduction

Glaucoma (GL) is an ocular disorder characterized by optic nerve (ON) degeneration and loss of retinal ganglion cells (RGCs) (Domenici, Berardi, Carmignoto, Vantini, & Maffei, 1991; Gupta & Yucel, 2001; Morrison, Moore, Deppmeier, Gold, Meshul, & Johnson, 1997). Although elevated intraocular pressure (IOP) is the main risk factor for GL, other mechanisms appear to be implicated in this ocular disorder since GL can develop in the absence of elevated IOP (Sommer, 1989). GL is characterized not only by RGC loss but also by degeneration in the post-retinal pathways. It has been observed that neural cells localized in the lateral geniculate nucleus (LGN) and visual cortex (VC) display degenerative changes in animal models of GL (Glovinsky, Quigley, & Dunkelberger, 1991; Gupta & Yucel, 2001; Gupta & Yucel, 2003; Yucel, Zhang, Gupta, Kaufman, & Weinreb, 2000; Yucel, Zhang, Weinreb, Kaufman, & Gupta, 2001). Moreover, electrophysiological studies in humans indicate that both retinal cells and post-retinal structures are functionally impaired in GL (Parisi, 2001). Other studies have shown that the reduced presence of biological mediators produced and released by these brain regions and/or by retinal cells might be implicated in RGC death and loss of ON axons (Nucci, Morrone, Rombola, Nistico, Piccirilli, & Cerulli, 2003; Yucel et al., 2000; Yucel et al., 2001). This hypothesis is supported by findings showing that the administration of neurotrophic factors, such as nerve growth factor (NGF), can differentially modulate the development of the geniculate-cortical pathway (Kaushik, Pandav, & Ram, 2003; Riddle, Lo, & Katz, 1995) (Schoups, Elliott, Friedman, & Black, 1995) and promote regeneration of axons and damaged retinal ganglion cells (RGCs) (Pearson & Stoffler, 1992; Schoups et al., 1995; Watanabe, Tokita, Kato, & Fukuda, 2003; Yip & So, 2000).

NGF is the first discovered and best characterized member of the growth factor family that includes brain derived neurotrophic factor (BDNF) and neurotrophin-3/4 (Connor & Dragunow, 1998; Sofroniew, Howe, & Mobley, 2001). The biological effects of NGF are mediated by two NGF receptors: the high-affinity receptor, tyrosine kinase (TrkA), and the low-affinity receptor, p75, located on the surface of NGF-responsive cells (Aloe, Tirassa, & Bracci-Laudiero, 2001; Casaccia-Bonnefil, Gu, & Chao, 1999; Sofroniew et al., 2001). Impairment in the expression of these receptors and/or their ligands can alter the functional activity of NGF-responsive cells (e.g. retinal cells, Müller cells, photoreceptors, and RGCs of developing and adult rodents) in the nervous system (Connor & Dragunow, 1998; Sofroniew et al., 2001) and visual system (Caminos, Becker, Martin-Zanca, & Vecino, 1999; Karlsson, Clary, Lefcort, Reichardt, Karten, & Hallbook, 1998; Lambiase & Aloe, 1996; Lenzi, Coassin, Lambiase, Bonini, Amendola, & Aloe, 2005). A possible functional role for NGF in cells of the visual system is also suggested by findings that intraocular administration of NGF reduces RGC degeneration following rat ON section (Carmignoto, Maffei, Candeo, Canella, & Comelli, 1989), reduces

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RGC damage induced in rabbit ocular hypertension (Lambiase, Centofanti, Micera, Manni, Mattei, De Gregorio, de Feo, Bucci, & Aloe, 1997), and prevents the shift in ocular dominance distribution of visual cortical neurons in monocular deprived rats (Domenici, Parisi, & Maffei, 1992; Domenici et al., 1991; Maffei, Berardi, Domenici, Parisi, & Pizzorusso, 1992; Pizzorusso, Porciatti, Tseng, Aebischer, & Maffei, 1997). However, no investigation into the role of NGF in this animal model of GL has been performed to date.

We have recently reported that conjunctival application of NGF can reach brain cholinergic neurons and suggested that high molecular weight protein can be safely delivered into the brain via the ocular surface to promote the recovery of damaged brain cells (Chen, Fawcett, Rahman, Ala, & Frey, 1998; Di Fausto, Fiore, Tirassa, Lambiase, & Aloe, 2007; Koevary, 2003; Koevary, Lam, Patsiopoulos, & Lake, 2003; Lambiase, Pagani, Di Fausto, Sposato, Coassin, Bonini, 2007). This evidence raised questions about whether eye-applied NGF can reach the brain's visual regions and whether NGF affects the deficits induced by GL on cells of the VC and LGN.

In the present study, we used this rat model of GL as an approach to investigate the effects of GL on cells of the LGN and VC and for understanding the role of eye-applied NGF on possible neurodegenerative events linked to NGF-responsive cells in these brain visual regions.

2. Materials and methods

2.1. Animals, surgical procedures, and treatment

GL was induced in right eye of adult male Sprague Dawley rats (n = 135) following the procedure described previously (Morrison, Johnson, Cepurna, & Jia, 2005; Morrison et al., 1997). Briefly, deeply anesthetized rats received 50 μl of hypertonic saline injected into the right episcleral vein, causing elevated ocular hypertension by sclerosis of the aqueous outflow pathways. Rats were housed in a constant low-light environment (40–90 lux) to minimize IOP circadian oscillation. A calibrated TonoPen XL tonometer (Mentor, Norwell, MA) was used for daily monitoring of IOP under topical anesthesia. Each daily IOP value was determined as the mean of ten valid readings. Twelve rats (about 9%) of hypertonic saline injected with an IOP less that 30 mmHg were classified as non-glaucomatous rats and were not used for this study. The left eye was used as a non-glaucomatous control.

Five rats died within 24 h after the induction of IOP elevation. The remaining rats were divided two groups (n = 65 each), housed in single cages in a constant low-light environment, and treated daily with NGF or vehicle applied as collyrium (see also Ref. (Di Fausto et al., 2007)). The first group of rats (GL + V) received 20 μl of vehicle solution (V) consisting of 0.9% NaCl twice a day in both eyes. The left non-glaucomatous eye is indicated as (CNTL + V), and the right glaucomatous eye is indicated as (IPSL + V). The second group of rats (GL + N) received 20 μl of purified NGF (N) dissolved in V at a concentration of 200 μg/ml in both eyes. In this group of rats, the left non-glaucomatous eye is indicated as CNTL + N and the right glaucomatous eye as IPSL + N. Thus, control (left) eyes (CNTL + V and CNTL + N) project to the right LGN and VC, whereas glaucomatous (right) eyes (IPSL + V and IPSL + N) project to the left LGN and VC. Two other groups of 20 healthy (not glaucomatous) adult rats received either: a) no treatment at all (group C) or b) NGF in the right eye (NGF-C) and vehicle in the left eye (V-C). These groups were used as additional controls.

Ocular treatments began one day after the induction of GL and continued for 35 consecutive days. To measure IOP, we used TonoPen XL tonometer (Mentor, Norwell, MA) under topical anesthesia. IOP values were evaluated weekly and determined as the mean of ten valid readings as previously described (Morrison et al., 2005). Animals were handled routinely prior to injection to allow reproducible baseline IOP measurements. Intraocular pressures following episcleral vein injections of hypertonic saline were determined in awake animals using one drop of 0.5% proparacaine hydrochloride instilled in each eye. Animals were gently restrained with light hand pressure, and TonoPen measurements obtained using the above criteria.

Mean intraocular pressure changes over the time of pressure elevation were calculated for each eye, together with the standard error of the mean. Rats were sacrificed 1, 2, 3, 4, and 5 weeks after the induction of GL and/or NGF administration. We used five rats per group for morphological analysis and eight rats per group for biochemical analysis. Since no statistically significant structural or biochemical differences in NGF or NGF receptor expression was found in the LGN and VC during the first 4 weeks (data not shown) and our previous studies indicated that the expression of anti-apoptotic signals are markedly down-regulated and pro-apoptotic signal up-regulated 5 weeks after the induction of GL (Coassin, Lambiase, Sposato, Micera, Bonini, & Aloe, 2008), we will present and discuss the results obtained with rats 5 weeks after the induction of GL.

All experiments were performed in compliance with the intramural Ethical Committee for the use of Animal care, National and International laws (EEC council directive 86/609, OJ L 358, 1, December 12, 1987), ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

2.2. Chemicals

The ELISA "NGF Emax™ Immunoassay System number G7631" was purchased by Promega (Madison, WI, USA); anti-p75 receptor and anti-TrkA receptor were purchased from UPSTATE (Temecula, CA, USA); and β-actin from Sigma (SIGMA, Saint Louis, MO, USA). A monoclonal antibody against choline acetyl-transferase (anti-ChAT) was generously provided by Dr. Costantino Cozzari, Institute of Cellular Biology, CNR, Italy (Lambiase & Aloe, 1996; Lenzi et al., 2005); Horseradish peroxidase-conjugated anti-rabbit IgG and anti-mouse IgG were purchased from Cell Signaling Technology (Danvers, MA, USA); and ECL chemiluminescent HRP substrate from Millipore Corporation (Billerica, MA, USA).

The 2.5 S purified NGF obtained from Microlab Company, Roma, Italy or was purified from adult male mouse submaxillary salivary glands following the described method (Bocchini & Angeletti, 1969).

2.3. Tissue collection and extraction

Rats were deeply anesthetized with an overdose of Nembutal, and cerebrospinal fluid (CSF) and blood samples were collected separately from each rat. Brain tissues (right and left LGN and VC) were dissected out as previously reported (Amendola, Fiore, & Aloe, 2003). The CSF was removed from brain 3rd ventricle with glass micropipettes, blood collected after decapitation, and serum separated from blood cells by centrifugation 5 min at 5.000 rpm. For LGN and VC dissection, brains were placed on a cold glass dish under a stereomicroscope. The right and left LGN and VC areas were identified as previously reported (Amendola et al., 2003; Palkovits & Brownstein, 1988), quickly removed, and then stored at −70 °C until used for biochemical analysis of NGF and NGFR presence.

For NGF determination, brain tissues were homogenized with ultrasonication in extraction buffer (20 mM Tris-acetate, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium-pyrophosphate, 1 mM orthovanadate, 1 mM β-glycerolphosphate,

100 mM NaF, 1 mM PMSF, 1 μg/ml leupeptin) and centrifuged at 4° C for 10 min at 13,000 rpm. The resultant supernatants were recovered and used for NGF and NGF receptor determination.

2.4. Histological analysis of the retinal damage

Rats were sacrificed with an overdose of Nembutal, and eye cups (n = 5 rats from each experimental group) were fixed in Bouin fluid for 1 week. After several washes, sections of the eye were cut at 20 μm and stained with hematoxylin and eosin.

To estimate the number of RGCs, we used sections (ten for each eye) located approximately 350 mm distant from the ON head. The mean numbers of retinal ganglion cells present in 0.25 mm^2 were counted under 200× magnifications with the aid of a square grid. The results were then averaged to generate data for one eye.

2.5. Ultra structural analysis

To assess the existence of ON fiber loss, the ONs of control (n = 4) and glaucomatous (n = 4) eyes were fixed in 4% glutaraldehyde in 0.1 phosphate buffer, pH 7.2 for 24 h, washed with fresh buffer, and then post-fixed in 1% OsO4 in the same buffer. ONs were then dehydrated with ascending ethanol and acetone and embedded in 812/Spurr's low viscosity resin. Thin sections were then cut with an ultramicrotome and collected on 200 mesh grids. Sections were stained with uranyl acetate and lead citrate and viewed under an electron microscope.

2.6. NGF assay

The tissue concentration of NGF was measured in the ON (n = 8) of each experimental group by a highly sensitive and specific two-site enzyme immunoassay ELISA kit "NGF Emax™ Immunoassay System number G7631" from Promega, (Madison, WI, USA) following the instructions provided by the manufacturer. The amount of rat NGF was determined from the regression line for the NGF standard (ranging from 7.8 to 500 pg/ml of purified mouse NGF), Incubation conditions were similar for each assay, and all assays were performed in duplicate.

2.7. Immunohistochemistry

Rats (n = 5 per experimental group) were anesthetized and transcardially perfused with 4% paraformaldeyde in 0.1 M phosphate-buffered saline (PBS), pH 7.4; brain and eye cups removed and post-fixed overnight at room temperature with the same fixative. Tissues were then left overnight in 0.1 M PBS containing 20% sucrose, and coded 20 μm thick sections were then cut with a cryostat (Leica CM 1850 UV, Germany) at −20 °C and used for immunohistochemical analysis. Free-floating sections were exposed to 3% hydrogen peroxide (H_2O_2) and 10% methanol (W/V) for 20 min, blocked in 0.1 M PBS containing 10% horse serum for 1 h, and then incubated overnight at 4 °C with antibodies against TrkA, p75, or ChAT. Sections were exposed to biotinylated anti-mouse IgG (1:300, Vector Laboratories) with 2% horse serum for 2 h at room temperature and then to an avidin-biotinylated horseradish peroxidase complex (Vector Laboratories) in 0.1 M PBS with 0.1% Triton X-100 for another 2 h at room temperature. This was followed by treatment for 15′ with a 0.1 M solution of 3,3′-diaminobenzidine (DAB) (Sigma Chemical Company) in 0.1 M PBS 0.1% Triton X-100, to which 0.01% of H_2O_2, 0.025% cobalt chloride, and 0.02% nickel ammonium sulfate were added as chromogens. All sections studied passed through all procedures simultaneously to minimize any differences due to immunohistochemical staining procedures. Staining specificity was assessed by omission of primary antibodies.

To estimate the number of immunostained ChAT nerve cells, we examined ipsilateral (IPSL) and controlateral (CNTL) coronal brain sections (n = 7) containing the LGN, spanning from −3.80 mm to −5.20 mm relative to bregma (see brain atlas by (Paxinos, 1982)). ChAT-positive cells were counted in non-overlapping fields using the Nikon-Lucia image processing and analysis program, which automatically selects only cell bodies but not small fragments or cells that do not have a complete soma.

2.8. Western blotting analysis

ONs (n = 6) from each experimental group were homogenized in lysis buffer (0.01 M Tris–HCl buffer (pH 7.4) containing 0.1 M NaCl, 1 mM EDTA, 1 mM EGTA, 2 mM PMSF, 50 μM leupeptin, 100 μg/ml pepstatin, and 100 μg/ml aprotinin) at 4 °C. After 8000 *g* centrifugation for 20 min, the supernatants were used for Western blotting. Samples (30 μg of total protein) were dissolved with loading buffer (0.1 M Tris–HCl buffer (pH 6.8) containing 0.2 M DTT, 4% SDS, 20% glycerol, and 0.1% bromophenol blue), separated by 8% SDS-PAGE, and electrophoretically transferred to PVDF membrane overnight. The membranes were incubated for 1 h at room temperature with blocking buffer (5% non-fat dry milk, 10 mM Tris, pH 7.5, 100 mM NaCl, and 0.1% Tween-20). Membranes were washed three times for 10 min each at room temperature in TTBS (10 mM Tris, pH 7.5, 100 mM NaCl, and 0.1% Tween-20), followed by incubation at 4 °C with primary antibodies overnight. Membranes were washed three times for 10 min each at room temperature in TTBS and incubated for 1 h with either horseradish peroxidase-conjugated anti-rabbit IgG or horseradish peroxidase-conjugated anti-mouse IgG as the secondary antibody. The blots were developed with ECL chemiluminescent HRP substrate (Millipore Corporation, Billerica, USA) as the chromophore. A personal computer and the public domain (http://www.rsb.info.nih.gov/ij/) Image J software were used to evaluate band density, which was expressed as arbitrary units of gray level. The ImageJ software determines the optical density of the bands using a gray scale thresholding operation. The optical density of β-actin bands was used as a normalizing factor. For each gel/blot, the normalized values were then expressed as a percentage of the relative normalized controls and used for statistical evaluation.

2.9. Statistical analyses

Statistical evaluations were performed using the StatView package for Windows, and data were expressed as mean and S.E.M. TrkA and p75 optical density data from Western blot analyses were evaluated using an analysis of variance (one-way ANOVA) followed by a Tukey-Kramer post-hoc test. A p-value less than 0.05 was considered to be statistically significant.

3. Results

We first assessed whether the experimental approach we used induced elevated IOP and loss of cells in the retinal ganglion layer. Time-course analysis revealed progressive elevation of IOP (Fig. 1A). One week after hypertonic saline injection, the IOP of the glaucomatous eye (IPSL + V) reached the highest value (42.7 ± 3.7 mmHg). It then decreased during the second week post-treatment to 35.7 ± 2.5 mmHg, compared to the IOP of the untreated eyes (CNTL + V) of 26.9 ± 2.6 mmHg (mean ± SEM). NGF administration has no statistically significant effect on the EIOP of glaucomatous eye.

Histological analysis of retinas revealed that elevated IOP caused loss of cells in the RGL (Fig. 1B and C). In glaucomatous rats, a reduction in the number of RGCs was first observed the second week after

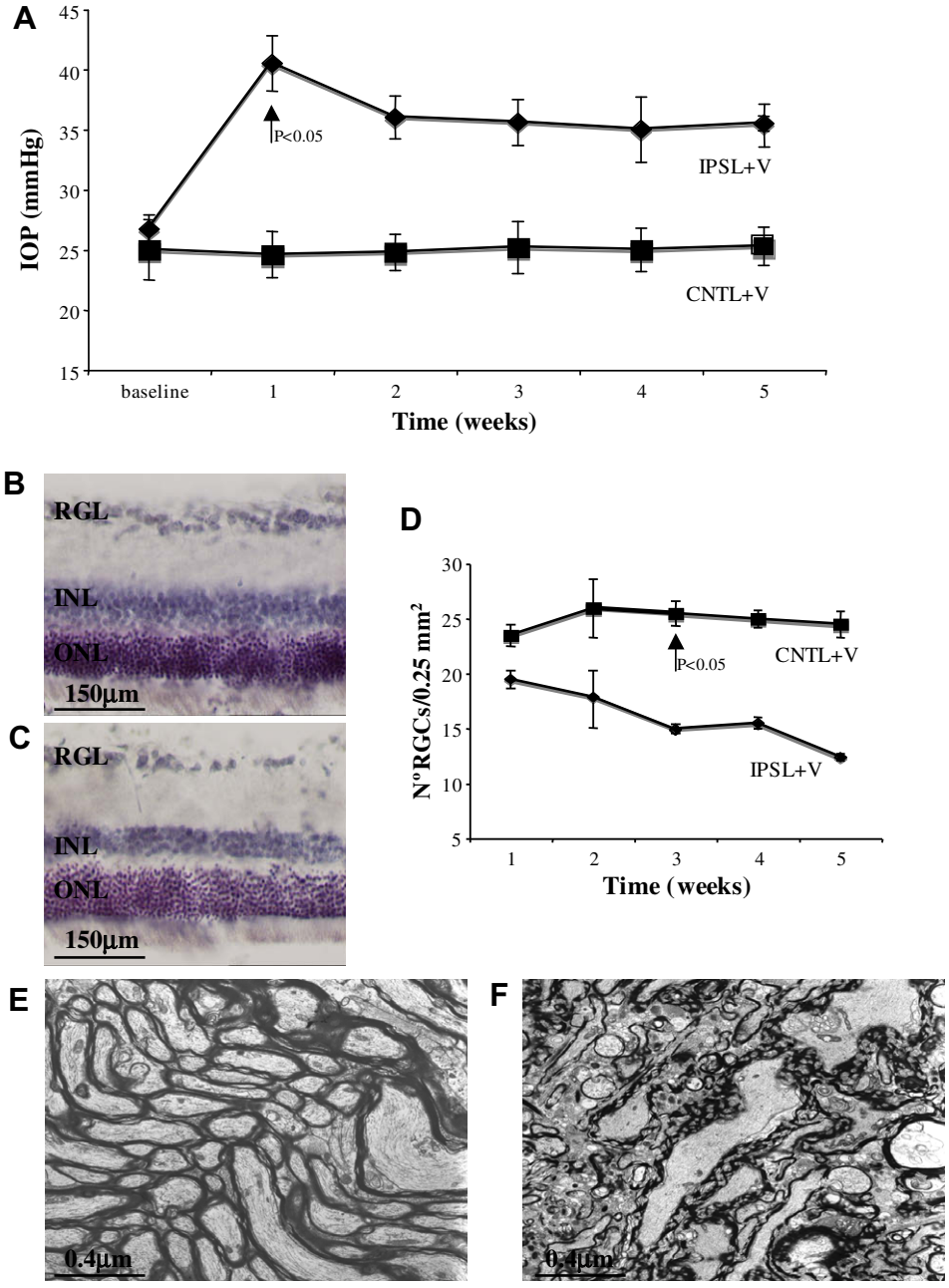

Fig. 1. The intraocular pressure of adult rats under normal conditions and during the progression of the experimentally-induced glaucoma (A). IOP increased constantly during the first week post-GL, decreased moderately during the second week, and then remained significantly ($p < 0.05$) higher than that of not glaucomatous eyes treated with vehicle or NGF. Group IPSL + V = glaucomatous right eye treated with vehicle; CTNL + V = left controlateral eye treated with vehicle. Histological preparation of a retina stained with hematoxylin-eosin showing the reduced presence of retinal ganglion cells of control (B) and glaucomatous (C) retinas. Retinal ganglion layer (RGL), Inner retinal layer (INL); Outer retina layer (ONL). Bars: 150 µm. Time-course analysis and the progressive loss (arrow) of retinal ganglion cells in glaucomatous eye, in 0.5 µm/length, compared to control (D). The difference reached statistical significance ($p < 0.05$) during the second week after GL induction. Representative section at the ultrastructural level of the ON of glaucomatous (F) and control (E) retinas showing axon swelling and myelin debris. Bars: 0.4 µm.

induction of GL. Thereafter the number of these cells decreases steadily ($p < 0.05$) and 5 weeks later the RGC reduction in glaucomatous eye was about 50% compared to control eye (Fig. 1D). Ultrastructural analysis revealed that elevated IOP caused axon swelling and myelin debris in the ON after 5 weeks (Fig. 1E and F).

3.1. Effect of GL and topical NGF on serum and CSF levels of NGF

As reported in Fig. 2A, GL (GL + V) induces no changes in serum NGF protein levels but lowers NGF content in the CSF (Fig. 2B) in comparison to control rats. This finding suggests that the NGF present in the CSF is taken up by damaged retinal or brain neurons. Moreover, topical eye NGF application in glaucomatous rats (GL + N) had no effect on IOP or concentrations of NGF in the serum but enhanced NGF presence in the CSF (GL + N versus GL + V: $p < 0.05$). This latter increase is most likely due to the accumulation of administered NGF.

3.2. Effect of NGF and NGFR in the LGN of glaucomatous rats

The topographic region of the LGN of rats removed for measuring the concentration of NGF is illustrated in Fig. 3A (dark area). As shown in Fig. 3B, GL caused a statistically significant reduction in the concentration of NGF in the LGN (IPSL + V versus CNTL + V;

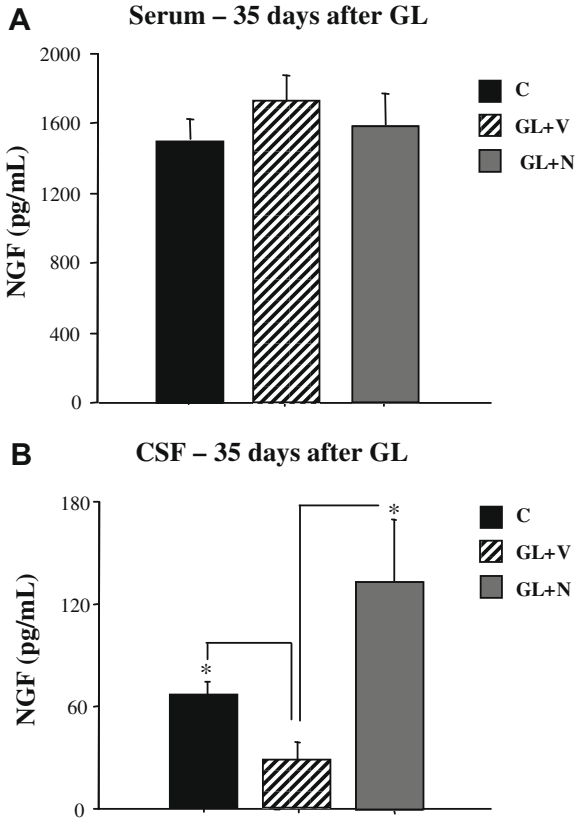

Fig. 2. Concentration of NGF in the serum (A) and CSF (B) of glaucomatous rats treated and untreated with topical NGF eye drops. Note that glaucoma caused a decrease of NGF contents in the CSF but not serum (C versus GL + V: $p < 0.05$). This decrease is most likely due to a major requirement for NGF in degenerating retinal or brain NGF-responsive cells. Alternatively, it may be due to reduced synthesis and release by local NGF-producing cells. Topical application of NGF increased the NGF levels in the CSF (GL + V versus GL + N: $p < 0.05$) but had no effect on these levels in the serum.

$p \leqslant 0.01$), whereas eye NGF applications for 35 consecutive days restored the presence of NGF to nearly normal levels (IPSL + N versus IPSL + V; $p \leqslant 0.01$). This figure also indicates that NGF administration has no statistically relevant effect ($p > 0.05$) on normal eyes (CNTL + N versus CNTL + V).

We next investigated whether NGF receptive cells are present in the LGN. Immunohistochemical studies carried out on serial brain sections showed that both high-affinity (TrkA, Fig. 3C) and low-affinity (p75, Fig. 3D) NGF receptors are expressed by cells of the LGN. TrkA is markedly expressed by nerve fibers, whereas p75 is found mainly in nerve cell bodies. As reported in Fig. 3E–H, Western blot analyses revealed that GL enhanced the presence of TrkA protein in the LGN (Fig. 3E, G IPSL + V versus CNTL + V; $^{*}p < 0.05$) and that eye NGF application enhanced this expression (Fig. 3E, G IPSL + N versus IPSL + V; $^{**}p \leqslant 0.01$). On the contrary, GL had no effect on the expression of p75 in the LGN (Fig. 3F, H IPSL + V versus CNTL + V), and eye NGF application enhanced p75 protein expression (Fig. 3F, H IPSL + N versus IPSL + V; $p < 0.05$). NGF administration had no effect on the LGN of normal rats (CNTL + N versus CNTL + V, $p > 0.05$).

3.3. NGF and NGFR in the VC of glaucomatous rats

The topographic region of the VC of rats removed for measuring the concentration of NGF is illustrated in Fig. 4A (dark area). Up to the forth week after GL induction, no statistically significant differences were observed (data not shown). Fig. 4B shows that GL significantly reduced the concentration of NGF in the VC, (IPSL + V versus CNTL + V, $p \leqslant 0.01$), and topical NGF application for 35 consecutive days normalized the NGF concentration (Fig. 4B, IPSL + N versus IPSL + V; $p \leqslant 0.01$). Immunohistochemical analysis of VC sections indicated that both TrkA (Fig. 4C) and p75 (Fig. 4D) receptors were expressed by cells of the VC, suggesting that NGF was produced by and acted upon cells of the VC. As demonstrated in Fig. 4E–H, Western blot analyses indicated that GL down-regulated the expression of TrkA in the VC (Fig. 4E, G IPSL + V versus CNTL + V) and that eye NGF application was unable to normalize this decrease (Fig. 4E, G IPSL + N versus IPSL + V). Moreover, GL significantly reduced the expression of p75 in the VC (Fig. 4F and H, IPSL + V versus CNTL + V; ($^{*}p < 0.05$)), and eye NGF application further reduced the expression of p75 protein (Fig. 4F and H, IPSL + N versus CNTL + V; $^{**}p \leqslant 0.01$).

3.4. Effect of eye NGF application in naive rats

The above observations raised questions about the effect of eye NGF application in the LGN and VC of healthy (non-operated) rats. As shown in Fig. 5, the concentration of NGF in the LGN and VC of control (C), vehicle (C-V), and NGF-treated (NGF-C) rats after 35 days was nearly the same. This observation suggests that NGF exerts its effect in injured or degenerating, but not intact tissues. No statistically significant differences were found upon comparison to the levels in controls (Figs. 3 and 4).

3.5. GL reduces the number of ChAT-immunopositive fibers in the LGN

As NGF plays a crucial role in preventing degeneration of brain cholinergic neurons (Connor & Dragunow, 1998; Sofroniew et al., 2001) and brain cholinergic projections exert an important modulator of visual activity in the LGN and VC (Carden, Datskovskaia, Guido, Godwin, & Bickford, 2000), we examined the distribution of ChAT immuno-labeled cells in the IPSL and CNTL LGN and VC of glaucomatous rats that had been treated or untreated with NGF for 35 days. Fig. 6A–C show that GL markedly reduced the number of ChAT-positive cells in the LGN receiving input from the glaucomatous eye (B) in comparison to the LGN corresponding to the healthy eye (A); NGF application prevented the loss of ChAT-positive neurons (C). Quantitative analysis in Fig. 6D indicated that experimental GL markedly reduced the number of ChAT-positive cells in the LGN (IPSL + V versus CNTL + V; $p \leqslant 0.01$). This study revealed that eye NGF administrations normalized these deficits (IPSL + N versus IPSL + V; $p \leqslant 0.01$) (Fig. 6D). No difference in the number of ChAT-immuno-positive cells was found in the VC of glaucomatous rats compared to controls (data not shown).

4. Discussion

In this study, we investigated whether (i) experimentally-induced GL in adult rats alters the presence of NGF and its receptors in the LGN and VC and (ii) eye topical NGF application can prevent these deficits. We found that GL reduces the concentration of NGF in the CSF, LGN, and VC and causes no significant changes in serum NGF levels. Moreover, immunohistochemical and biochemical analyses revealed that GL enhances the presence of the TrkA receptor in the LGN and decreases the expression of this receptor in the VC, whereas the presence of the p75 receptor is unaffected in the LGN and down-regulated in the VC. Topical eye NGF application for 35 consecutive days enhances the concentration of NGF in the CSF of glaucomatous rats and normalizes its presence in the VC and LGN. NGF application induced up-regulation of TrkA in the LGN but not VC, and it enhanced the expression of p75 in the LGN and reduced its presence in the VC. A summary of all results is given in Table 1.

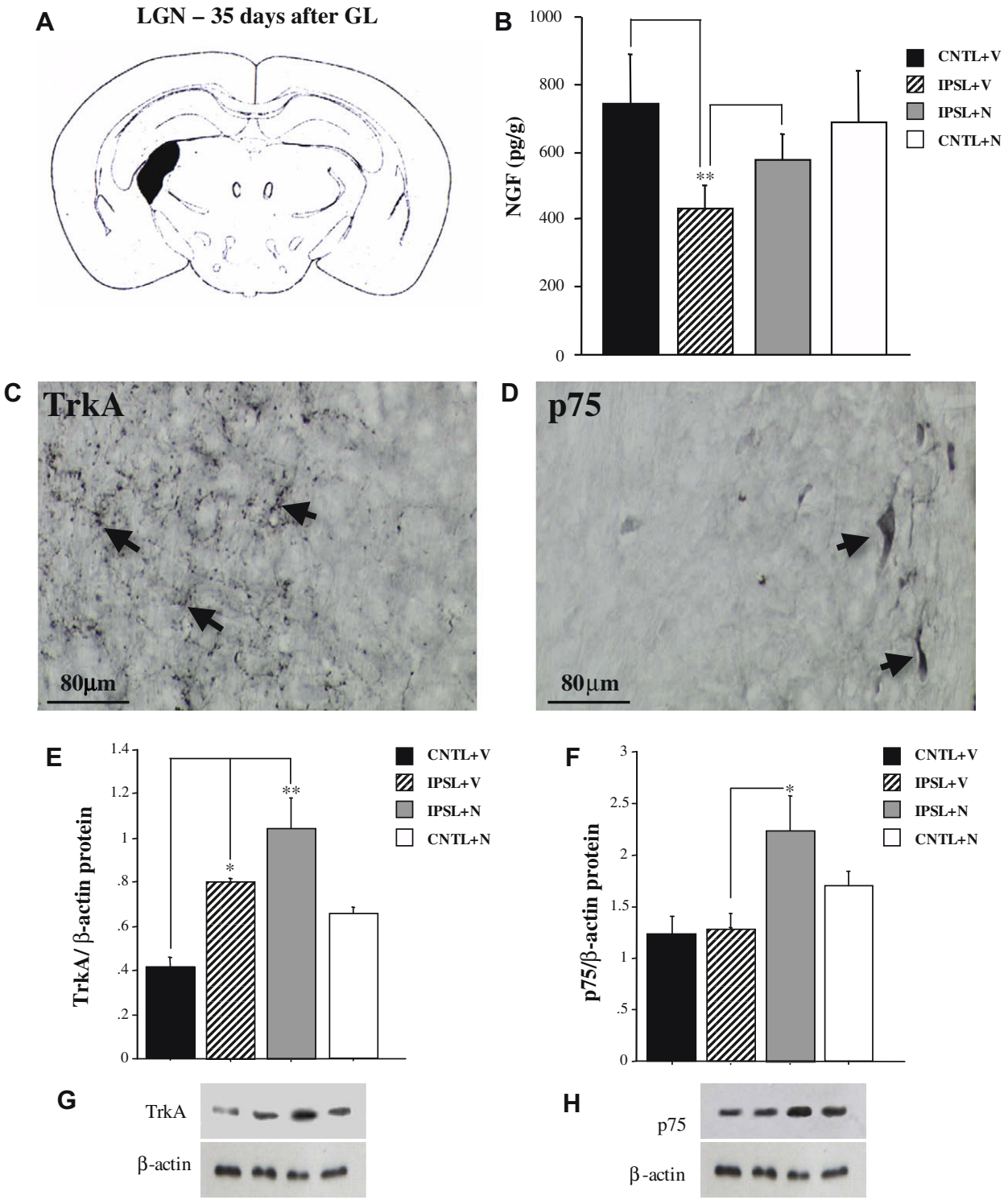

Fig. 3. (A) Schematic representation of rat brain sections indicating the LGN area used for biochemical analysis. (B) The concentration of NGF in the LGN of control and glaucomatous rats treated and untreated with NGF. Note the significant ($p < 0.01$) decrease of NGF presence in the LGN of glaucomatous rats (group IPSL + V) compared to controls (group CNTL + V) and nearly complete normalization after daily NGF treatment (group IPSL + N). No significant changes in NGF levels were found in non-glaucomatous rats after NGF administration (CNTL + N versus CNTL + V: $p > 0.05$). TrkA (C) or p75 (D) immunopositivity in the LGN of normal rats. Note that TrkA is mainly localized to nerve fibers (arrows), whereas p75 is in cell bodies (arrows). Bars: 80 µm. Western blot analysis of TrkA (E and G) and p75 (F and H) proteins in the LGN. GL caused up-regulation of TrkA in the LGN (IPSL + V versus CNTL + V), and eye NGF application further enhanced this increase (IPSL + N versus IPSL + V). In control rats, eye NGF administration augmented TrkA expression (CNTL + N versus CNTL + V). GL induced no changes (F, H) in p75 protein expression (IPSL + V versus CNTL + V), whereas eye NGF application up-regulated p75 expression in comparison to glaucomatous (IPSL + N versus IPSL + V) and control (IPSL + N versus CNTL + V) eyes. No significant NGF effect was found in control eyes (CNTL + N versus CNTL + V: $p > 0.05$).

The functional significance and mechanism(s) causing the different expression of low and high-affinity NGF receptors in glaucomatous VC and LGN are not known. Most probably, they are related to the effects induced by GL on apoptotic cell death and the different role played by NGF receptors in cell death and survival (Casaccia-Bonnefil et al., 1999; Middleton & Davies, 2001;

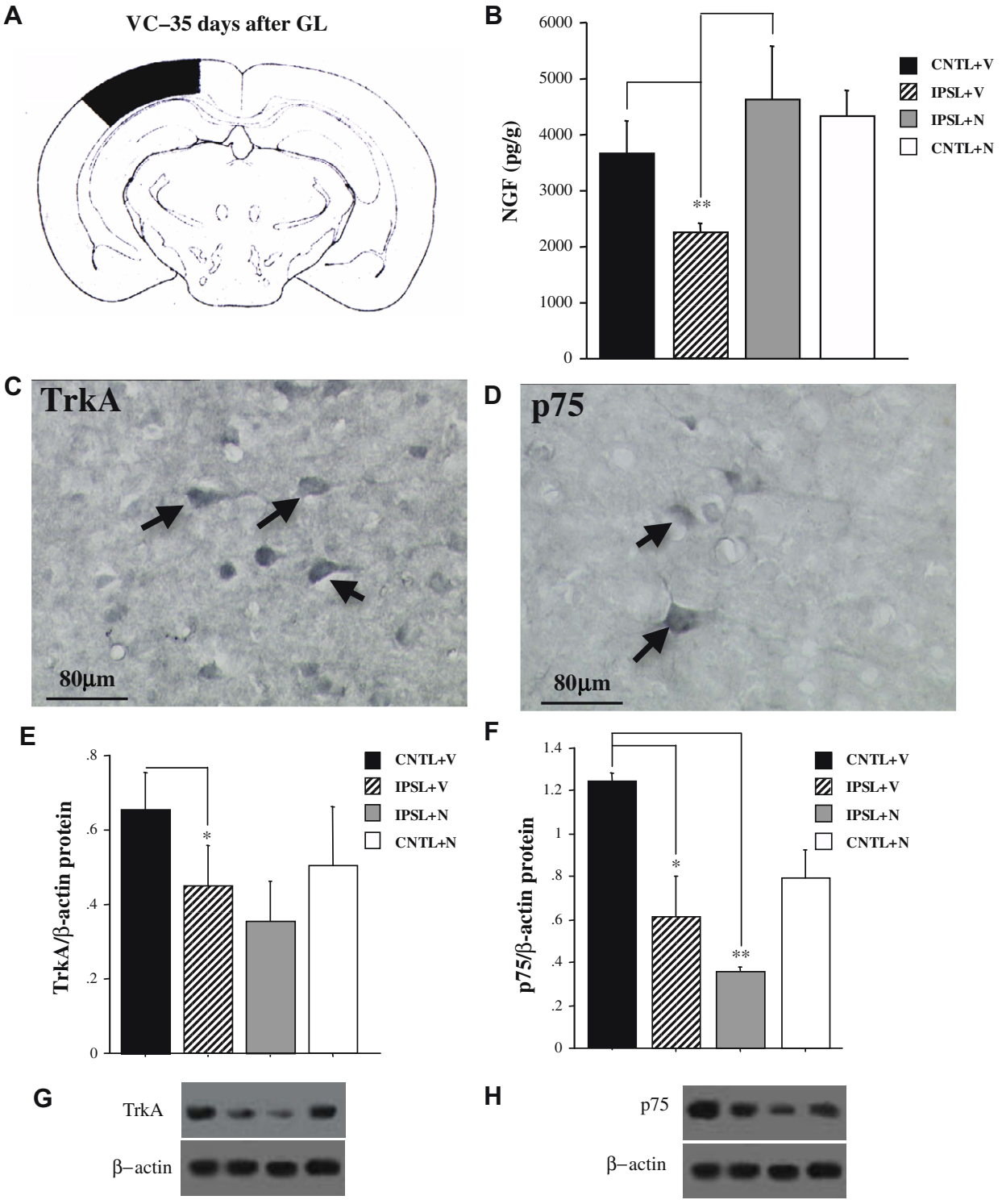

Fig. 4. Schematic representation of rat brain sections (A) indicating the VC area removed for biochemical analysis. Fig. B shows a significant ($p < 0.01$) decrease of NGF protein in the VC of glaucomatous rats (IPSL + V versus CNTL + V) and its normalization after eye administration of NGF (IPSL + N versus IPSL + V). Immunopositivity for TrkA (C) and p75 (D) in cells of the VC of normal rats. Both TrkA and p75 were predominantly expressed in cell bodies (arrows). Bars: 80 µm. GL down-regulates, though not markedly (IPSL + V versus CNTL + V), the TrkA band in the VC samples upon Western blotting (E, G). Eye NGF administration was unable to normalize this deficit (IPSL + N versus IPSL + V). GL significantly down-regulated the expression of p75 (F, H) (IPSL + V versus CNTL + V), and NGF administration further reduced the expression of p75 (IPSL + N versus CNTL + V).

Sofroniew et al., 2001). This hypothesis is in line with the observation that a major mechanism of neuron loss during GL is related to an increase in RGC susceptibility to apoptotic mechanisms (Nucci et al., 2003), because NGF/NGF receptors play a marked role in this process (Ambati, Canakis, Miller, Gragoudas, Edwards, Weissgold, Kim, Delori, & Adamis, 2000).

One question raised by our observations is how eye NGF application reaches NGF receptive cells of the VC and LGN. As demon-

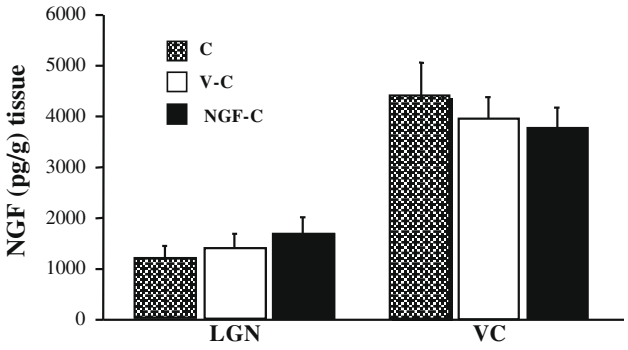

Fig. 5. NGF levels in extracts from the LGN and VC of naive control rats (C) and healthy rats treated with eye drops of vehicle (V-C) or NGF (NGF-C) for 35 consecutive days. No statistically significant differences ($p > 0.05$) in NGF concentration were found in brain visual areas in Vehicle (V-C) or NGF-treated (NGF-C) rats, suggesting that NGF does not accumulate in the brain after topical eye application.

strated for insulin and other molecules, the mechanism through which NGF ocular application reaches the brain neurons the existence of the anatomical connections between the eye and the brain or via nasal mucosa, naso-lacrimal duct, brain ventricles, or cerebrospinal fluid diffusion (Ambati et al., 2000; Koevary, 2003; Koevary et al., 2003; Lambiase et al., 2007; Schoenwald, Deshpande, Rethwisch, & Barfknecht, 1997). This observation is in line with our recent study (Lambiase et al., 2007) showing that NGF applied to the ocular surface may reach some brain regions, such as the nucleus basalis and septum, and promote the recovery of damaged brain cells (Di Fausto et al., 2007). NGF and other members of the neurotrophin family are known to play a marked role not only in peripheral nerve cells, but also in brain cells via retrograde and anterograde mechanisms. Moreover, axonal anterograde transport of neurotrophins, including NGF, appears to be a widespread mechanism (Altar & DiStefano, 1998; Conner, Lauterborn, & Gall, 1998; Cui, Tang, Hu, So, & Yip, 2002; Sofroniew et al., 2001; Wahle, Di Cristo, Schwerdtfeger, Engelhardt, Berardi, & Maffei, 2003). These suggest that deficits in this mechanism could compromise the structural and neurochemical plasticity of brain visual neurons. Our observation that the presence of NGF and NGFRs in cells of the LGN and VC is significantly impaired in experimentally-induced GL suggests that these NGF signaling are functionally involved in neurodegenerative events occurring in retinal and visual brain regions (Morrison et al., 2005; Parisi, 2001; Vickers, Hof, Schumer, Wang, Podos, & Morrison, 1997).

An other question raised by our findings is the functional significance of NGF in the LGN and VC. Neurotrophins, and in particular NGF, have been shown to modulate cell development, survival, and

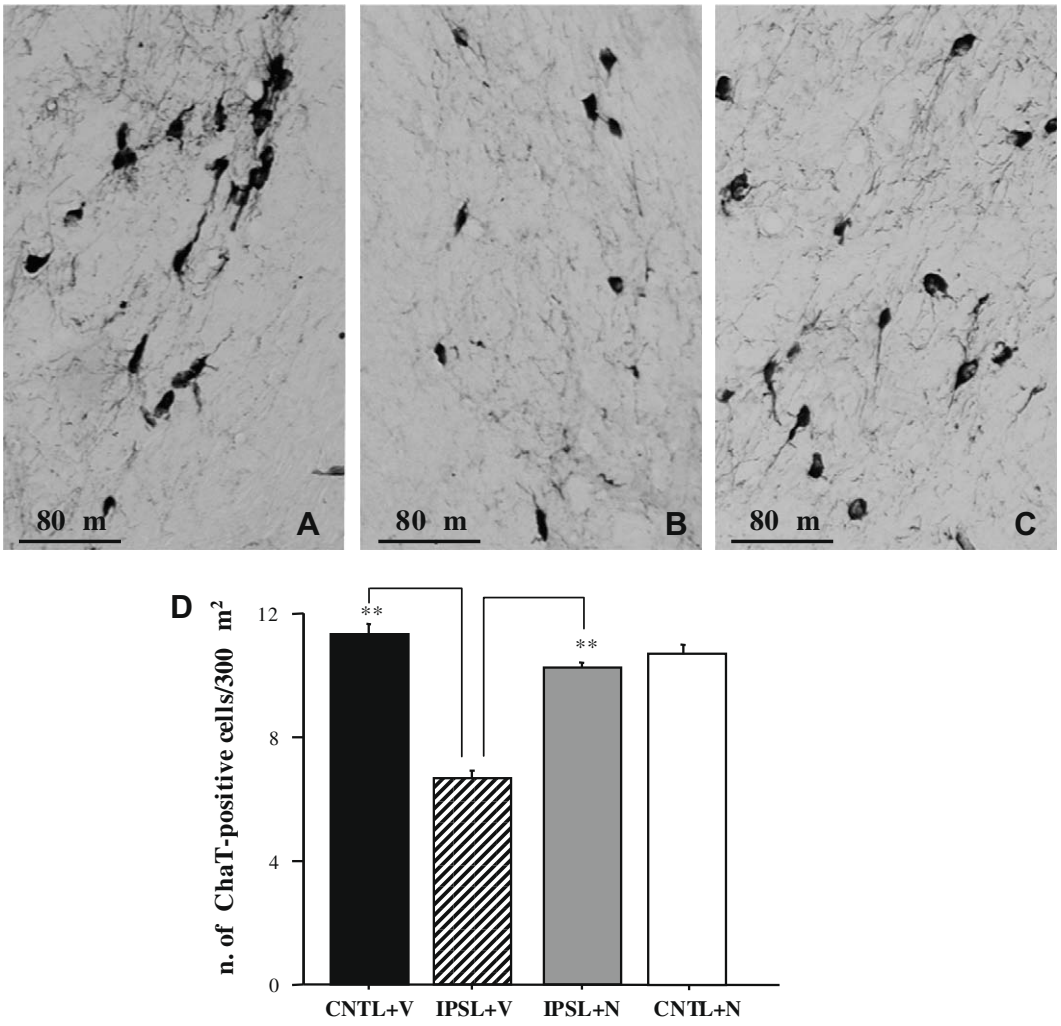

Fig. 6. The effect of GL and eye vehicle or NGF treatment on ChAT immuno-labeled cells in the lateral LGN of glaucomatous rats. Representative sections of the lateral LGN immunohistochemically stained for ChAT in the CNTL + V (A), IPSL + V (B), and IPSL + N (C) groups. No differences of ChAT immunostaining were found when the CNTL + V group was compared to the CNTL + N group ($p > 0.05$). Bars: 80 μm Quantitative determination showed that experimental glaucoma markedly reduced the number of ChAT-positive cells in the IPSL + V LGN compared to the CNTL + V LGN ($p < 0.01$). Eye NGF administration normalized this decline (IPSL + N, versus IPSL + V; $p < 0.01$), D.

Table 1
Summary of results.

	CSF			Serum			LGN			VC		
	NGF	TrkA	p75	NGF	TrkA	p75	NGF	TrkA	p75	NGF	TrkA	p75
GL/normal IOP	↓	–	–	0	–	–	↓↓	↑	0	↓↓	↓	↓
GL + NGF/GL	↑↑	–	–	0	–	–	↑↑	↑↑	↑	↑↑	0	↓↓

0: no change; –: not done.

plasticity of both LGN and VC neurons (Pizzorusso et al., 1997; Schoenwald et al., 1997). Present data showing that NGF administration in glaucomatous rats enhances the presence of ChAT-positive neurons in the LGN suggest that NGF may also modulate the synthesis and/or release of biological mediators in cholinergic neurons localized not only in the forebrain (Connor & Dragunow, 1998) but also in the LGN (Carden et al., 2000).

The possibility of a role for NGF in cells of the visual system is suggested by a number of other recent findings. For example, intraocular administration of NGF reduces RGC degeneration following rat ON section (Carmignoto et al., 1989) and rabbit ocular hypertension (Lambiase et al., 1997), and it prevents the shift in ocular dominance distribution of visual cortical neurons after monocular deprivation in rats (Domenici et al., 1991; Domenici et al., 1992; Maffei et al., 1992; Pizzorusso et al., 1997) and in rats with inherited retinopathy (Lambiase & Aloe, 1996; Lenzi et al., 2005). The effects of NGF eye drops, a simple non-invasive treatment, in normalizing NGF and modulating p75 and TrkA expression during GL encourage further studies to investigate the potential therapeutic use of NGF in degenerative diseases of the visual pathway. Altogether, our findings are in line with the hypothesis that neurotrophin function extends from retrograde, target-derived trophic factors to anterograde, afferent and local, paracrine actions in the VC, LGN, and retina (Rossi, Sala, & Maffei, 2002). It remains to be established, however, whether prolonged NGF application could provide a functional benefit for the RGC, LGN, and VC damage induced by GL.

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References

Aloe, L., Tirassa, P., & Bracci-Laudiero, L. (2001). Nerve growth factor in neurological and non-neurological diseases: Basic findings and emerging pharmacological prospectives. *Current Pharmaceutical Design, 7*(2), 113–123.

Altar, C. A., & DiStefano, P. S. (1998). Neurotrophin trafficking by anterograde transport. *Trends Neuroscience, 21*(10), 433–437.

Ambati, J., Canakis, C. S., Miller, J. W., Gragoudas, E. S., Edwards, A., Weissgold, D. J., et al. (2000). Diffusion of high molecular weight compounds through sclera. *Investigative Ophthalmology & Visual Science, 41*(5), 1181–1185.

Amendola, T., Fiore, M., & Aloe, L. (2003). Postnatal changes in nerve growth factor and brain derived neurotrophic factor levels in the retina, visual cortex, and geniculate nucleus in rats with retinitis pigmentosa. *Neuroscience Letters, 345*(1), 37–40.

Bocchini, V., & Angeletti, P. U. (1969). The nerve growth factor: purification as a 30,000-molecular-weight protein. *Proceedings of the National Academic Sciences of the United States of America, 64*(2), 787–794.

Caminos, E., Becker, E., Martin-Zanca, D., & Vecino, E. (1999). Neurotrophins and their receptors in the tench retina during optic nerve regeneration. *The Journal of Comparative Neurology, 404*(3), 321–331.

Carden, W. B., Datskovskaia, A., Guido, W., Godwin, D. W., & Bickford, M. E. (2000). Development of the cholinergic, nitrergic, and GABAergic innervation of the cat dorsal lateral geniculate nucleus. *The Journal of Comparative Neurology, 418*(1), 65–80.

Carmignoto, G., Maffei, L., Candeo, P., Canella, R., & Comelli, C. (1989). Effect of NGF on the survival of rat retinal ganglion cells following optic nerve section. *Journal of Neuroscience, 9*(4), 1263–1272.

Casaccia-Bonnefil, P., Gu, C., & Chao, M. V. (1999). Neurotrophins in cell survival/death decisions. *Advances in Experimental Medicine and Biology, 468*, 275–282.

Chen, X. Q., Fawcett, J. R., Rahman, Y. E., Ala, T. A., & Frey, I. W. (1998). Delivery of nerve growth factor to the brain via the olfactory pathway. *Journal of Alzheimer's Disease, 1*(1), 35–44.

Coassin, M., Lambiase, A., Sposato, V., Micera, A., Bonini, S., Aloe, L., et al. (2008). Retinal p75 and bax overexpression is associated with retinal ganglion cells apoptosis in a rat model of glaucoma. *Graefe's Archive for Clinical and Experimental Ophthalmology*.

Conner, J. M., Lauterborn, J. C., & Gall, C. M. (1998). Anterograde transport of neurotrophin proteins in the CNS—a reassessment of the neurotrophic hypothesis. *Reviews Neuroscience, 9*(2), 91–103.

Connor, B., & Dragunow, M. (1998). The role of neuronal growth factors in neurodegenerative disorders of the human brain. *Brain Research Brain Research Reviews, 27*(1), 1–39.

Cui, Q., Tang, L. S., Hu, B., So, K. F., & Yip, H. K. (2002). Expression of trkA, trkB, and trkC in injured and regenerating retinal ganglion cells of adult rats. *Investigative Ophthalmology & Visual Science, 43*(6), 1954–1964.

Di Fausto, V., Fiore, M., Tirassa, P., Lambiase, A., & Aloe, L. (2007). Eye drop NGF administration promotes the recovery of chemically injured cholinergic neurons of adult mouse forebrain. *European Journal of Neuroscience, 26*(9), 2473–2480.

Domenici, L., Berardi, N., Carmignoto, G., Vantini, G., & Maffei, L. (1991). Nerve growth factor prevents the amblyopic effects of monocular deprivation. *Proceedings of the National Academic Sciences of the United States of America, 88*(19), 8811–8815.

Domenici, L., Parisi, V., & Maffei, L. (1992). Exogenous supply of nerve growth factor prevents the effects of strabismus in the rat. *Neuroscience, 51*(1), 19–24.

Glovinsky, Y., Quigley, H. A., & Dunkelberger, G. R. (1991). Retinal ganglion cell loss is size dependent in experimental glaucoma. *Investigative Ophthalmology & Visual Science, 32*(3), 484–491.

Gupta, N., & Yucel, Y. H. (2001). Glaucoma and the brain. *Journal of Glaucoma, 10*(5 Suppl. 1), S28–S29.

Gupta, N., & Yucel, Y. H. (2003). Brain changes in glaucoma. *European Journal of Ophthalmology, 13 Suppl. 3*, S32–S35.

Karlsson, M., Clary, D. O., Lefcort, F. B., Reichardt, L. F., Karten, H. J., Hallbook, F., et al. (1998). Nerve growth factor receptor TrkA is expressed by horizontal and amacrine cells during chicken retinal development. *The Journal of Comparative Neurology, 400*(3), 408–416.

Kaushik, S., Pandav, S. S., & Ram, J. (2003). Neuroprotection in glaucoma. *Journal of Postgraduate Medicine, 49*(1), 90–95.

Koevary, S. B. (2003). Pharmacokinetics of topical ocular drug delivery: Potential uses for the treatment of diseases of the posterior segment and beyond. *Current Drug Metabolism, 4*(3), 213–222.

Koevary, S. B., Lam, V., Patsiopoulos, G., & Lake, S. (2003). Accumulation of porcine insulin in the rat brain and cerebrospinal fluid following ocular application. *Journal of Ocular Pharmacology and Therapeutics, 19*(4), 377–384.

Lambiase, A., & Aloe, L. (1996). Nerve growth factor delays retinal degeneration in C3H mice. *Graefe's Archive for Clinical and Experimental Ophthalmology, 234*(Suppl. 1), S96–S100.

Lambiase, A., Centofanti, M., Micera, A., Manni, G. L., Mattei, E., De Gregorio, A., et al. (1997). Nerve growth factor (NGF) reduces and NGF antibody exacerbates retinal damage induced in rabbit by experimental ocular hypertension. *Graefe's Archive for Clinical and Experimental Ophthalmology, 235*(12), 780–785.

Lambiase, A., Pagani, L., Di Fausto, V., Sposato, V., Coassin, M., Bonini, S., et al. (2007). Nerve growth factor eye drop administrated on the ocular surface of rodents affects the nucleus basalis and septum: biochemical and structural evidence. *Brain Research, 1127*(1), 45–51.

Lenzi, L., Coassin, M., Lambiase, A., Bonini, S., Amendola, T., Aloe, L., et al. (2005). Effect of exogenous administration of nerve growth factor in the retina of rats with inherited retinitis pigmentosa. *Vision Research, 45*(12), 1491–1500.

Maffei, L., Berardi, N., Domenici, L., Parisi, V., & Pizzorusso, T. (1992). Nerve growth factor (NGF) prevents the shift in ocular dominance distribution of visual cortical neurons in monocularly deprived rats. *Journal of Neuroscience, 12*(12), 4651–4662.

Middleton, G., & Davies, A. M. (2001). Populations of NGF-dependent neurones differ in their requirement for BAX to undergo apoptosis in the absence of NGF/TrkA signalling in vivo. *Development, 128*(23), 4715–4728.

Morrison, J. C., Johnson, E. C., Cepurna, W., & Jia, L. (2005). Understanding mechanisms of pressure-induced optic nerve damage. *Progress in Retinal and Eye Research, 24*(2), 217–240.

Morrison, J. C., Moore, C. G., Deppmeier, L. M., Gold, B. G., Meshul, C. K., Johnson, E. C., et al. (1997). A rat model of chronic pressure-induced optic nerve damage. *Experimental Eye Research, 64*(1), 85–96.

Nucci, C., Morrone, L., Rombola, L., Nistico, R., Piccirilli, S., Cerulli, L., et al. (2003). Multifaceted roles of nitric oxide in the lateral geniculate nucleus: from visual signal transduction to neuronal apoptosis. *Toxicology Letters, 139*(2-3), 163–173.

Palkovits, M., & Brownstein, M. J. (1988). *Maps and guide to microdissection of the rat brain*. New York: Elsevier.

Parisi, V. (2001). Impaired visual function in glaucoma. *Clinical Neurophysiology, 112*(2), 351–358.

Paxinos, G. (1982). *The rat brain in stereotaxic coordinates*. Sydney: Academic Press.

Pearson, H. E., & Stoffler, D. J. (1992). Retinal ganglion cell degeneration following loss of postsynaptic target neurons in the dorsal lateral geniculate nucleus of the adult cat. *Experimental Neurology, 116*(2), 163–171.

Pizzorusso, T., Porciatti, V., Tseng, J. L., Aebischer, P., & Maffei, L. (1997). Transplant of polymer-encapsulated cells genetically engineered to release nerve growth factor allows a normal functional development of the visual cortex in dark-reared rats. *Neuroscience, 80*(2), 307–311.

Riddle, D. R., Lo, D. C., & Katz, L. C. (1995). NT-4-mediated rescue of lateral geniculate neurons from effects of monocular deprivation. *Nature, 378*(6553), 189–191.

Rossi, F. M., Sala, R., & Maffei, L. (2002). Expression of the nerve growth factor receptors TrkA and p75NTR in the visual cortex of the rat: development and regulation by the cholinergic input. *Journal of Neuroscience, 22*(3), 912–919.

Schoenwald, R. D., Deshpande, G. S., Rethwisch, D. G., & Barfknecht, C. F. (1997). Penetration into the anterior chamber via the conjunctival/scleral pathway. *Journal of Ocular Pharmacology and Therapeutics, 13*(1), 41–59.

Schoups, A. A., Elliott, R. C., Friedman, W. J., & Black, I. B. (1995). NGF and BDNF are differentially modulated by visual experience in the developing geniculocortical pathway. *Brain Research Development Brain Research, 86*(1-2), 326–334.

Sofroniew, M. V., Howe, C. L., & Mobley, W. C. (2001). Nerve growth factor signaling, neuroprotection, and neural repair. *Annual Review of Neuroscience, 24*, 1217–1281.

Sommer, A. (1989). Intraocular pressure and glaucoma. *American Journal of Ophthalmology, 107*(2), 186–188.

Vickers, J. C., Hof, P. R., Schumer, R. A., Wang, R. F., Podos, S. M., Morrison, J. H., et al. (1997). Magnocellular and parvocellular visual pathways are both affected in a macaque monkey model of glaucoma. *The Australian & New Zealand Journal of Ophthalmology, 25*(3), 239–243.

Wahle, P., Di Cristo, G., Schwerdtfeger, G., Engelhardt, M., Berardi, N., Maffei, L., et al. (2003). Differential effects of cortical neurotrophic factors on development of lateral geniculate nucleus and superior colliculus neurons: anterograde and retrograde actions. *Development, 130*(3), 611–622.

Watanabe, M., Tokita, Y., Kato, M., & Fukuda, Y. (2003). Intravitreal injections of neurotrophic factors and forskolin enhance survival and axonal regeneration of axotomized beta ganglion cells in cat retina. *Neuroscience, 116*(3), 733–742.

Yip, H. K., & So, K. F. (2000). Axonal regeneration of retinal ganglion cells: Effect of trophic factors. *Progress in Retinal and Eye Research, 19*(5), 559–575.

Yucel, Y. H., Zhang, Q., Gupta, N., Kaufman, P. L., & Weinreb, R. N. (2000). Loss of neurons in magnocellular and parvocellular layers of the lateral geniculate nucleus in glaucoma. *Archives of Ophthalmology, 118*(3), 378–384.

Yucel, Y. H., Zhang, Q., Weinreb, R. N., Kaufman, P. L., & Gupta, N. (2001). Atrophy of relay neurons in magno- and parvocellular layers in the lateral geniculate nucleus in experimental glaucoma. *Investigative Ophthalmology & Visual Science, 42*(13), 3216–3222.