

Retrobulbar administration of purified anti-nerve growth factor in developing rats induces structural and biochemical changes in the retina and cornea

Luigi Aloe^{1,2}, Maria Luisa Rocco^{2,3}, Bijorn Omar Balzamino⁴, Graziana Esposito⁴, Alessandra Micera⁴

¹Institute of Cell Biology and Neurobiology, CNR, Lazio 00143, Rome, Italy

²Fondazione IRET, Ozzano Emilia, Bologna 40064, Italy

³Institute of Translational Pharmacology, CNR, Lazio 00133, Rome, Italy

⁴Research and Development Laboratory for Biochemical, Molecular and Cellular Applications in Ophthalmological Science, IRCCS, Fondazione Bietti, Rome 00182, Italy

Correspondence to: Luigi Aloe. Fondazione IRET, Ozzano Emilia, Bologna 40064, Italy. luigi.aloe@cnr.it

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Abstract

• **AIM:** To develop an experimental model of endogenous nerve growth factor (NGF) deprivation by retrobulbar administration of purified neutralizing anti-NGF antibodies in young Sprague-Dawley rats and provide further information on NGF expression in the retina and cornea.

• **METHODS:** Sixty old pathogen-free Sprague Dawley rats (p14, post-natal days) were treated with repeated retrobulbar injections of neutralizing anti-NGF (2 µL, 100 µg/mL, every 3d). After 2wk (p28), retinal and corneal tissues were investigated for morphological, biochemical, and molecular expression of trkA^{NGFR} by using Western blotting or immunofluorescence. Rhodopsin as well as protein profile expression were also investigated.

• **RESULTS:** Chronic retrobulbar neutralizing anti-NGF antibodies changed the distribution of trkA^{NGFR} immunoreactivity at retinal level, while no changes were detected for global trkA^{NGFR} protein expression. By contrary, the treatment resulted in the increase of corneal trkA^{NGFR} expression. Retinal tissues showed a decreased rhodopsin expression as well as reduced number of both rhodopsin expressing and total retinal cells, as observed after single cell extraction. A decreased expression of ICAM-1, IL-17 and IL-13 as well as an increased expression of IL-21 typified retinal extracts. No significant changes were observed for corneal tissues.

• **CONCLUSION:** The reduced availability of endogenous NGF, as produced by chronic retrobulbar anti-NGF administration, produce a quick response from retinal tissues, with

respect to corneal ones, suggesting the presence of early compensatory mechanisms to protect retinal networking.

• **KEYWORDS:** anti-nerve growth factor; trkA^{NGFR}; retina; cornea; photoreceptors; tissue response

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INTRODUCTION

Nerve growth factor (NGF), the prototype of neurotrophins' family, plays pleiotropic effects in differentiation, growth and maintenance of neurons during development and adulthood^[1]. Since the first observation of NGF activity on mast cells, a sequela of works corroborated to define NGF as a crucial player in the neuro-endocrine-immune system interactions^[2-3]. Several studies focused on the developing of neuroprotective and immunomodulatory strategies for the cure of neurodegenerative and chronic immune related disorders^[4]. First, the intracerebral administration of purified NGF showed a protective action of NGF on damaged forebrain cholinergic neurons, preventing cell death from apoptotic mechanisms^[5-7]. At the same time, the administration of neutralizing anti-NGF antibodies in developing and adult rodents triggered the degeneration of peripheral sensory and sympathetic neurons due to a reduced availability of endogenous NGF^[8-9].

Several old and recent findings highlighted the potential role of NGF as therapeutic agent for neurodegenerative eye disorders, by means of a direct effect on corneal and retinal cells^[10-11]. The topical administration of NGF protected corneal cells, retinal ganglion cells (RGCs) and photoreceptors from the deleterious effects of pathological insults, as observed in neurotrophic ulcers, glaucoma, retinitis pigmentosa and maculopathy^[4,12-18]. On the other side, the neutralizing anti-NGF antibodies exacerbated the suffering of RGCs and promoted vascularization and angiogenesis inside retina, lacrimal gland and brain due to a direct depletion of endogenous NGF and an increased

expression of vascular endothelial growth factor (VEGF)^[13,19-20]. As stated in previous studies, the biological action of NGF is mediated by $\text{trkA}^{\text{NGFR}}$ (a tyrosine kinase receptor) and p75^{NTR} (the pan-neurotrophin receptor, a member of the tumor necrosis factor receptor superfamily), and the selective binding to the high affinity receptor ($\text{trkA}^{\text{NGFR}}$ and p75^{NTR}) is strictly dependent on $\text{trkA}^{\text{NGFR}}$ - p75^{NTR} expression ratio at cellular membrane of target cells^[21-23].

Some aspects regarding the influence of limited or complete absence of NGF in the balanced protein profile regulating homeostasis at retinal and corneal tissues, from parainflammation to inflammation, are still unknown. Therefore, we sought to examine potential changes at retinal and corneal structure due to chronic exposure to neutralizing anti-NGF antibodies. Overall, $\text{trkA}^{\text{NGFR}}$, rhodopsin (specific photoreceptor marker) and some inflammatory mediators were investigated. This experimental approach to reduce endogenous NGF levels might allow to understand initial biochemical events occurring at both retina and cornea from young rats, in case of partial/initial defect in NGF local availability.

MATERIALS AND METHODS

Animals and Reagents Sixty (30F/30M) pathogen-free Sprague Dawley rats (Charles River, Wilmington, MA, USA) were used at postnatal day 14 (p14). Housing and handling procedures were approved by the Ethical Commission on animal experimentation (CNR, Rome, Italy). All the experimental procedure complied with the directive 2010/63/EU guidelines. All efforts were carried out to reduce number and sorrow of treated animals.

Purified neutralizing anti-NGF antibodies were raised in goats by multiple 2.5S β NGF injections to develop circulating antibodies that were purified by affinity chromatography, according to a standard procedure^[24]. Non-immune goat IgG antibodies were obtained according to a standard procedure^[25].

Target antibodies were as follows: polyclonal anti- $\text{trkA}^{\text{NGFR}}$ (sc-118) and monoclonal anti-GAPDH from Santa Cruz Biotech (Santa Cruz, CA, USA), and monoclonal anti-rhodopsin antibodies (Neomarkers, Fremont, CA, USA).

Plastic-wares and analytical grade chemicals were purchased from EuroClone (Milan, Italy), Sigma Aldrich (St. Louis, MO, USA), ICN (Milan, Italy) and Carlo Erba (Milan, Italy) or elsewhere, as specified in the text. RNase-free demineralized MilliQ water was daily produced (Direct Q5 apparatus; Millipore Corp., Billerica, MA, USA).

Subgroups and Experimental Procedure Rats (p14) were grouped as anti-NGF ($n=30$; 15F/15M) and control ($n=30$; 15F/15M) and housed in the same conditions with food/tap water available *ad libitum*. Anti-NGF treated rats received a retrobulbar injection of 2 μL purified neutralizing anti-NGF antibodies diluted in balanced-sodium saline (BSS). Control

rats received non-immune goat IgG serum administration. A total of 5 retrobulbar injections were performed, one every 3d, for a 14-day range time, by using a 28G needle Hamilton syringe^[18]. Two weeks later, rats (p28) were sacrificed with an overdose of intraperitoneally delivered anesthetic mixture of 2 mg/mL ketamine (0.2 mL/10 g body-weight; Ketavet, Gellini Pharmaceuticals, Italy) and 0.23 mg/mL medetomidine (0.24 mL/10 g body-weight; Domitor, Orion Corp., Espoo, Finland). No significant changes were produced during delivery. Eyes were quickly removed and ocular tissues (cornea and retina) were dissected out under a stereo microscope (SMZ645; Nikon, Tokyo, Japan) equipped with cold-light optic fibers (PL2000 photonic; Axon, Vienna, Austria), according to a standard procedure^[26-27]. Microscopical [$n=12$ /assay type; immunohistochemistry (IHC) and Immunofluorescence (IF)] or biomolecular [$n=18$ /assay type; enzyme-linked immunosorbent assay (ELISA), Western blotting, protein array or real time polymerase chain reaction (PCR)] analysis were performed.

Previous works report the procedure of NGF neutralization by using a neutralizing anti-NGF antibody produced according to the 2.5S β NGF boosting, extraction, purification and testing of activity, specificity and purity^[8-9,24].

Immunohistochemistry for $\text{trkA}^{\text{NGFR}}$ and Rhodopsin

Whole eyes (3M-3F/assay type) were post-fixed in 4% buffered paraformaldehyde for 48h. Coded serial sections (10- μm thickness) were produced (CM3050; Leica Microsystems, Rijswijk, The Netherlands), placed on glass-slides (Merck-BDH, Poole, UK), left to adhere for 1h and stored at -20°C . Both $\text{trkA}^{\text{NGFR}}$ (1:100) and rhodopsin (1:100) immunostainings were performed after a brief blocking/permeabilizing step. The specific binding was detected by using biotinylated anti-rabbit (for $\text{trkA}^{\text{NGFR}}$; 1:400, BA-2000) or anti-mouse (for rhodopsin; 1:40, BA-1000; Vector) antibodies and labeled according to the Avidin-Biotin-Peroxidase method, using DAB (brown) as substrate (ABC-Vectastain Elite; Vector Laboratories, Burlingame, CA, USA). A slight haematoxylin counterstaining (Bio-Optica, Milan, Italy) was performed before mounting in anti-fading medium (Vector). Sections were observed under a direct microscope equipped with a digital camera for image acquisitions (Carl-Zeiss, Oberkochen, Germany). Parallel sections were incubated with purified non-specific IgG antibodies (isotypes; Vector). No color intensity adjustment was performed during panel assembling (Adobe Photoshop ver.7; Adobe System, San Jose, CA, USA). Integrated densitometric analysis (IntDen values) was performed according to a standard procedure and differences between groups were statistically analyzed.

Single Retinal Cell Collection and Rhodopsin Immunofluorescence Retinas (3M-3F/assay type) were quickly dissociated in a 0.025% Trypsin-0.02% EDTA solution

(Lonza, Basel, Switzerland) at 37°C for 10min. Single cells were collected, centrifuged (3500 rpm, 7min) and equilibrated in cold Hank's BSS (without Ca²⁺/Mg²⁺) supplemented with EDTA (HBSS; Lonza, Milan, Italy). Cell aliquots (5000 cells/well) were seeded on pre-coated (0.5% poly-lysine; P2636; Sigma) round-glass coverslips sited in 24-well plates (Nunc, Roskilde, Denmark). After 48h in CO₂ incubator, adhered cells were probed with anti-rhodopsin specific antibodies (1:100; 4°C, 2h), washed in PBS-TW (10 mmol/L phosphate in 0.9% saline containing 0.03% Tween-20) and labeled with cy3-conjugated specie-specific secondary antibodies (1:200; 4°C, 1h). Coverslips were mounted in the presence of nuclear stainer (DAPI, blue; D9542; Sigma-Aldrich). Specific and isotype signals were analyzed under a confocal inverted microscope (TSP SP5; Leica Microsystems, Mannheim, Germany). No intensity-image adjustments were performed while for exhibition purposes color-image changes were achieved after assembling the panels (red to magenta color; Adobe Photoshop). Rhodopsin immunoreactive cells (cy3/red/magenta), over a total nuclear monolayer (blue), were counted from RGB-split images in grayscale conversion, using the Image J Software (Image J v1.43; NIH <http://rsb.info.nih.gov/ij/>).

Western Blotting for trkA^{NGFR} and Rhodopsin Tissues (3F-3M/assay-type) were homogenized by ultra-sonication in modified RIPA buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1% Triton-X100, 5 mmol/L EDTA, 100 mmol/L NaF and 1 mmol/L PMSF; pH 7.5). After centrifugation (13 000 rpm, 20min, 4°C), the clarified supernatants were collected and total protein amounts were spectrophotometrically quantified (A280; Nanodrop, Celbio, Milan, Italy). No-pooled samples were stored at -20°C. Normalized samples (30 µg total protein/lane) were boiled in loading buffer (100 mmol/L Tris-HCl buffer containing 0.2 mol/L DTT, 0.5% SDS, 20% glycerol and 0.1% bromophenol blue; pH 6.8), separated in 4%-20% SDS-PAGE minigels (vertical apparatus; Biorad, Hercules, California, USA) and electrophoretically transferred (overnight) to PVDF membrane (vertical apparatus; Biorad). Membranes were incubated for 60min at room temperature with blocking buffer containing 5% BSA (targets) or non-fat dry milk (housekeeping) in TBS-TW (10 mmol/L Tris-100 mmol/L NaCl containing 0.1% Tween-20; pH 7.5). Membranes were then probed under gentle shaking with the primary antibodies specific for trkA^{NGFR} (1:1000), rhodopsin (1:1000) or anti-GAPDH (1:4000) and then incubated with horseradish peroxidase-conjugated secondary antibodies (1:4000, 60min, under gentle shaking). Specific binding was developed with ECL chemiluminescent horseradish peroxidase substrate (Millipore, MA, USA). Washing and incubation steps were according to standard procedures. Band density (IntDen)

was calculated by Image J Software and data were expressed as arbitrary units of gray level. IntDen values from GAPDH immunoblots were used as an additional normalizing factor. Data are expressed as a percentage of expression relative normalized controls.

Chip-based Protein Array Analysis Protein extracts (3F-3M/assay type) were obtained from not pooled tissues, as previously reported^[28]. Clarified supernatants (13 000 rpm, 20min) were collected and total proteins were spectrophotometrically quantified (A280, nanodrop). Normalized protein extracts (60 µg; 70 µL/well) were hybridized in chip-based arrays (glass-slides comprising 14 identical sub-arrays). A total of 60 factors per sample were analyzed simultaneously (G-series arrays, Ray Biotech, Norcross, CA). Incubation, washing, detection and labeling steps were according to the manufacturer's recommendation. Glass-slides were scanned in a GenePix 4400 Microarray platform (Molecular Devices LLC, Sunnyvale, Silicon-Valley, CA, USA). Acquisitions were carried out with the Axon GenePix Pro 6.0 software and images were provided as 8-bit tiff converted format. The minimum sensitivity range of the array was 3.8-56 pg/mL, as provided by the manufacturer.

ELISA for NGF Tissues (3M-3F/assay type) were homogenized by ultra-sonication in extraction buffer (50 mmol/L tris-HCl, pH 7.5; 150 mmol/L NaCl; 5 mmol/L EDTA; 1% Triton X-100; 0.1% SDS and 0.5% DOC; sodium deoxycholate; 1 mmol/L PMSF; 1 µg/mL leupeptin; AppliChem, Darmstadt, Germany) and protein extracts were clarified by centrifugation (13 000 rpm, 20min, 4°C). NGF assay was carried out following the instructions of the manufacturer's (Emax Immunoassay System ELISA kit; Promega Corp., Madison, WI, USA), using a precoated plate and all solutions/buffers required. Assay sensitivity (10 pg/mL NGF) and specificity (no cross-reactivity for BDNF/NT3/NT4) were as reported in instruction guidelines. The optical density (OD) was measured at 575 nm using an ELISA reader (multiskan EX, Thermo electron corporation, Madison, USA). Total protein amounts were determined by DC protein assay kit (Biorad Laboratories, Hercules, CA, USA) and quantified by using a 96-well plate ELISA reader (Sunrise, Tecan, Milan, Italy). Standard and sample OD values were corrected by subtracting the related non-specific binding values. Polynomial curve was produced and used for gaining quantitative values. The assays were performed in duplicate and data are shown as pg/mg of total protein.

Molecular Assessment of Transcripts Tissue extracts were subjected to total RNA extraction (TRIzol, Sigma Aldrich). The 2-step PCR amplification technique was performed by using a programmable thermal cycler (LifePRO/BIOER; Euroclone, Milan, Italy) and the EcoTM Illumina real time PCR platform

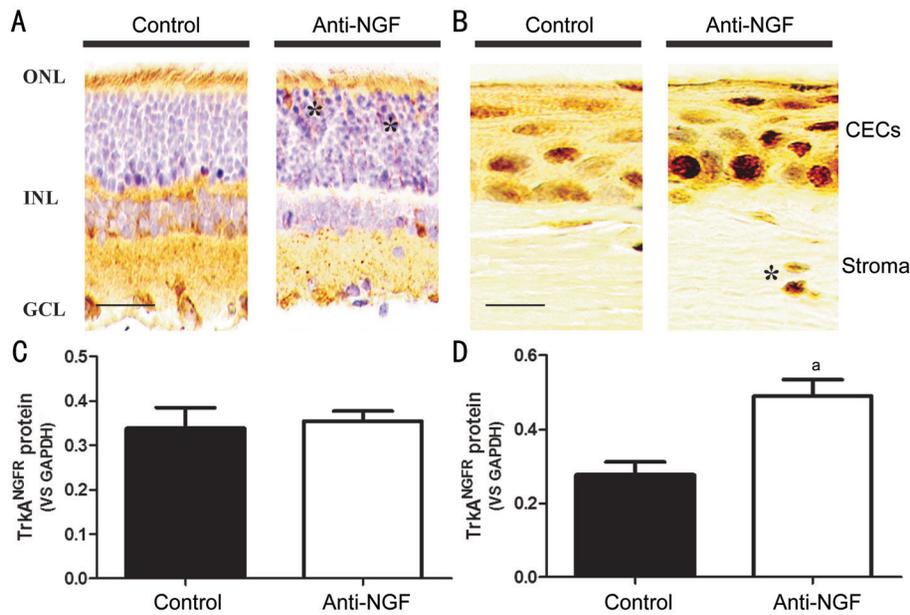


Figure 1 $trkA^{NGFR}$ expression in retinal and corneal tissues Rats (p14) were subjected to multiple retrobulbar injections (2 μ L, every 3d, 100 μ g/mL anti-NGF) and after 2wk (p28), tissues were processed for immunohistochemistry and Western blotting analysis. Representative immunohistochemical images from control and anti-NGF retrobulbar treated retinal and corneal tissues are shown (A-B). A different $trkA^{NGFR}$ immunoreactivity was observed in retinal sections (asterisk at ONL layer; A). As well, a different $trkA^{NGFR}$ immunoreactivity was observed in corneal sections (asterisk pointed stroma cells; B). Western blotting quantifications showed unchanged retinal $trkA^{NGFR}$ protein levels in extracts from anti-NGF treated rats (C; $P>0.05$, ANOVA). Corneal $trkA^{NGFR}$ protein levels were significantly increased upon anti-NGF administration (D; $^aP<0.05$, ANOVA). Magnification: A, $\times 20$ and B, $\times 40$. Scale bar: 50 μ m (A) and 20 μ m (B). Histograms (C-D) show ratios as mean \pm SEM values ($trkA^{NGFR}/GAPDH$). ONL: Outer nuclear layer; INL: Inner nuclear layer; GCL: Ganglion cell layer; CECs: Corneal epithelial cells.

(San Diego, CA). The cDNA synthesis was carried out with the GoScript™ reverse transcriptase (Promega, Madison, CA, USA). Specific target/referring gene amplifications were performed according to the SYBRgreen AmpliTaq protocol (Applied Biosystem, Foster City, CA). All steps in the procedure, including primers and amplification profile, as well as target gene expression analysis (fold-changes; $2^{-\Delta\Delta Ct}$, Rest analysis) were as previously reported^[27].

Data Analysis All statistical evaluations were performed using the StatView II package for Windows (Abacus Concepts, Inc., Barkley, CA, USA). Data are expressed as mean \pm standard deviation (SD; results) or mean \pm standard error of mean (SEM; graphics). Graphics were assembled by the GraphPad Prism package for Windows (San Diego, CA, USA). For OD (ELISA) and IntDen (IHC and WB) comparisons, ANOVA followed by Tukey-Kramer post-hoc analysis was carried out and a P value ≤ 0.05 was considered statistically significant. For protein (fluorescent intensity, FI) and molecular (FC, fold changes) analysis, all values were analyzed by ANOVA Benjamini-Hochberg procedure for multiple testing and Tukey-Kramer post-hoc test for multiple comparisons. A P value ≤ 0.001 (0.05/50) was considered.

RESULTS

Chronic retrobulbar anti-NGF administration did not produce any structural changes, as detected at light microscopy.

Effect of Anti-NGF Administration on $trkA^{NGFR}$ Expression in Retina and Cornea The effect of chronic retrobulbar anti-NGF administration on retinal and corneal $trkA^{NGFR}$ protein expression is shown in Figure 1.

Changes in retinal $trkA^{NGFR}$ immunoreactivity were observed upon anti-NGF treatment, and particularly some immunoreactive cells occurred at the outer nuclear layer (ONL; Figure 1A). This different receptor distribution was not associated with changes in total $trkA^{NGFR}$ protein expression, as quantified by Western blotting analysis (0.354 \pm 0.023 vs 0.339 \pm 0.046 IntDen; anti-NGF vs control retinal extracts; $P>0.05$; Figure 1C). By contrary, chronic retrobulbar anti-NGF administration induced an increased $trkA^{NGFR}$ immunoreactivity at the corneal epithelial cell layers, as compared to control counterparts (Figure 1B). Few $trkA^{NGFR}$ immunoreactive cells were also detected at the stroma level. This increased $trkA^{NGFR}$ immunoreactivity was confirmed by specific Western blotting analysis (0.4900 \pm 0.0437 vs 0.2767 \pm 0.0345 IntDen; anti-NGF vs control; $P<0.05$; Figure 1D).

Effect of Anti-NGF Treatment on Rhodopsin Expression in Retinal Tissues and Dissociated Cells A reduced rhodopsin immunoreactivity was observed at the photoreceptor layer of anti-NGF treated retinas, as compared to controls (Figure 2A). A slight reduction of rhodopsin protein was detected in anti-NGF treated retinal extracts (Western blotting analysis:

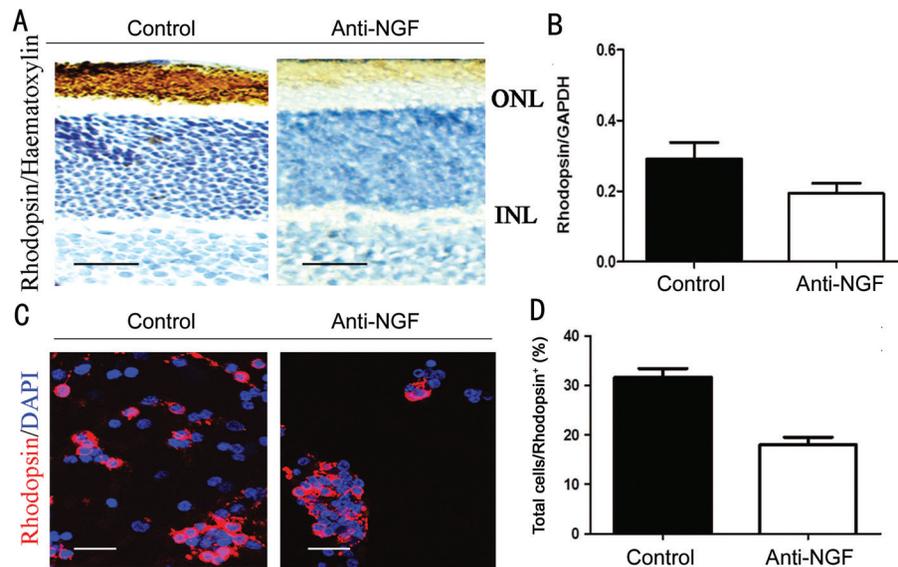


Figure 2 Retinal rhodopsin expression Whole eyes were post fixed (A) or dissected retinas (C) were enzymatically digested to release single retinal cells. Rhodopsin expression was quantified as described in S&M. A decreased immunoreactivity (brown) was observed in retinal sections (photoreceptor layer) after anti-NGF treatment, as compared to control counterparts (A). Densitometric analysis from Western blotting analysis confirmed the rhodopsin decrease in anti-NGF treated retinas, with respect to control ones ($P=0.0186$ ANOVA; B). Specific rhodopsin immunoreactivity (red) was observed in single retinal cells (blue counterstained nuclei, DAPI) from both anti-NGF treated and control rats by immunofluorescent analysis (C). The percentage (%) of rhodopsin positive cells was less in anti-NGF treated retinas, as compared to control ones ($P<0.05$; D). Magnification: A-C, $\times 20$. Scale bar: 50 μm . ONL: Outer nuclear layer; INL: Inner nuclear layer.

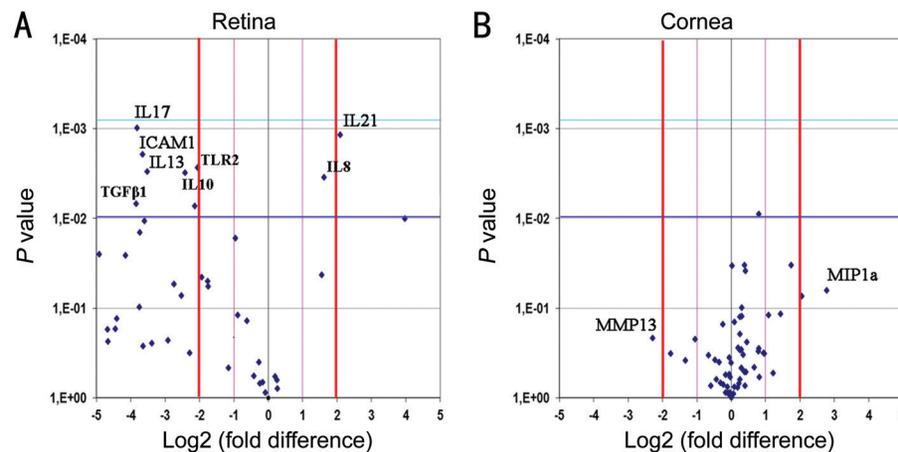


Figure 3 Protein array analysis Volcano plots showing the protein expression in retina (A) and cornea (B) from anti-NGF treated rats, with respect to control counterparts. A: Merely, ICAM-1, IL-17, IL-13 and TLR-2 were significantly decreased in anti-NGF retinal extracts, while IL-21 was increased; B: No significant difference was observed for corneal extracts. Fold differences (\log_2) and P values were as produced by the t test analysis (as previous studies^[28]). A $P<0.01$ ($P=0.05/50$) was used as statistically significant value.

0.292 \pm 0.046 vs 0.193 \pm 0.028 IntDen; anti-NGF vs control; $P=0.0186$; Figure 2B). To better clarify, retinas were enzymatically digested and 24h-adhered single cells were postfixed for IF. A reduced rhodopsin immunoreactivity was also observed in the total retinal cell suspension from anti-NGF treated retinas (Figure 2C). Interesting, the number of rhodopsin immunoreactive cells over a total of blue nuclear stained retinal cells (DAPI) decreased in anti-NGF treated retinas, as compared to control ones. Moreover, the total number of living retinal cells was decreased (51%) in retinal cells from anti-NGF treated retinas, as compared to control (Figure 2D).

Inflammatory Profile in Anti-NGF Exposed Retinas and Corneas To a better understand the inflammatory profile resulting from this NGF deprivation, a chip-based protein array study was performed on retinal and corneal extracts. As shown by Volcano plot in Figure 3A, lower expression of ICAM-1 (-2.86 FC; $P=0.001$), IL-17 (-2.54 FC; $P=0.001$), IL-13 (-2.10 FC; $P=0.003$) and TLR-2 (-2.13 FC; $P=0.006$), as well as a trend to a decrease for IL-10 (-1.80 FC; $P=0.007$) and TGF- β 1 (-1.92 FC; $P=0.003$) were detected in retinal extracts from anti-NGF treated rats. A significant upregulation was observed for IL-21 (+4.21 FC; $P=0.001$; Figure 3A). No significant changes

were detected in corneal extracts (Figure 3B).

Effect of Chronic Retrobulbar Anti-NGF Administration on Local NGF Expression in Retina and Cornea The real time PCR analysis showed an upregulation of NGF mRNA in retinal extracts from chronic retrobulbar anti-NGF treated rats (4.57 ± 2.55 ; $P < 0.05$), while no changes were detected for corneal NGF mRNA expression (0.24 ± 0.15 ; $P > 0.05$). Although with some limitations, NGF protein levels were also quantified in these tissues (ELISA). Retinal tissues from both anti-NGF treated and control rats showed no significant changes of NGF protein levels (284.60 ± 61.83 vs 224.40 ± 13.35 pg/mg total protein; anti-NGF vs control; $P > 0.05$). By the way, decreased NGF protein levels were observed in corneal extracts from anti-NGF treated rats (302.00 ± 13.12 vs 448.20 ± 36.58 pg/mg total protein; anti-NGF vs control; $P = 0.0062$).

DISCUSSION

Herein, chronic (multiple) retrobulbar administration of purified neutralizing anti-NGF antibodies was carried out to examine the biochemical changes in retinal and corneal sections and tissue extracts as well as on isolated retinal cells. This slow and continuous neutralization leading to a decrease of local NGF availability might be of interest to investigate the early effects of NGF depletion on local homeostasis.

In the visual system, NGF is synthesized, released and utilized in an autocrine and/or paracrine mode by several structural and accessory cells taking part in eye development (retinogenesis) and homeostasis^[11,27]. NGF administration can induce modification of pre-synaptic elements in adult visual system, prevent the shift in ocular dominance distribution of visual cortical neurons, promote functional recovery of RGCs after ischemia and delay retinal cell degeneration in rodents with inherited retinopathy^[5,15-16,29,30]. Topically applied NGF can reach the retina, preventing cell degeneration, improving the inner retinal layer functionality and post-retinal conduction parameters (visual acuity), as observed in several experimental models and human clinical trials^[4,13-15]. Moreover, topical, intravitreal and retrobulbar delivered NGF can reach optic nerve and brain neurons, opening to other therapeutically purposes^[4,13-15]. By contrary, NGF sequestration, the outcome of local delivery of neutralizing anti-NGF antibodies, can influence the tidy organized cell-to-cell and cell-to-mediator interplay as the result of unbalanced growth factor availability^[8,31]. Balanced NGF levels might contribute to tissue homeostasis and counterbalance inflammation, angiogenesis and tissue remodeling activities. In previous studies, the reduced expression of endogenous NGF at both peripheral neurons and retina of old mice was associated with VEGF upregulation in the retina^[20,32]. VEGF is a well-known angiogenic factor, working in concert with other growth factors and even NGF, to allow a good quality retinal activity.

Therefore, it might be possible to highlight that reduced NGF levels coupled to changes in the expression of other mediators (VEGF, transforming growth factor β 1, platelet derived growth factor) can influence the microenvironment, and even contribute to parainflammation. Overall, neutralizing procedures were also used to better characterize the “developing stages” in which a neurotrophic-based support might be required for retina and brain plasticity^[19,33].

From these studies, the continuous NGF delay did not show structural changes at both retina and corneas as detected by light microscopy. By the way, retinal and corneal tissues showed different distribution and expression of $\text{trkA}^{\text{NGFR}}$ upon anti-NGF delivery. As stated, NGF promotes growth, differentiation and survival of different structural and functional cells by binding to $\text{trkA}^{\text{NGFR}}$ or p75^{NTR} , either alone or in combination^[21]. Expressed by RGCs (expressing $\text{trkA}^{\text{NGFR}}$) and glia cells (expressing p75^{NTR}), both receptors can regulate opposing cell functions in healthy and insulted retina, from survival to apoptosis^[34-35]. Upon NGF neutralization, rhodopsin bearing cells were significantly reduced as well as the total retinal cell number. The specific increased $\text{trkA}^{\text{NGFR}}$ immunoreactivity observed at the ONL of NGF deprived retinas might be consistent with a prompt retinal cell response to retrobulbar anti-NGF conveyance. Both localized $\text{trkA}^{\text{NGFR}}$ expression, reduced amount of retinal living cells and a decreased rhodopsin expression might be consistent with this hypothesis. These findings further support the hypothesis of a different response between retinal and corneal cells to retrobulbar anti-NGF treatment. Actually, corneal $\text{trkA}^{\text{NGFR}}$ changes are missing of explanation. The increased expression of both $\text{trkA}^{\text{NGFR}}$ and NGF proteins might be consistent with a local distress due to retrobulbar surgery and potential recirculation of neutralizing NGF antibodies.

The possibility that anti-NGF administration can induce changes in other biological mediators cannot be excluded. Therefore, some proteins known to play key roles in tissue inflammation were explored in both tissue extracts. Few inflammatory markers were found decreased (ICAM-1, IL-17 and IL-13), while only IL-21 (pleiotropic type 1 cytokine) was found increased in retinal extracts. This finding would suggest the absence of local inflammation upon NGF deprivation but the potential activation of an adaptive response (parainflammation)^[36]. Merely to IL-21 expression in anti-NGF treated retinas, we can suggest a possible activation of Müller cells or microglial cells^[37]. In this context, TLR-2 and IL-21 changes need further investigation.

Overall NGF levels were unchanged in retinal extracts and significantly high in corneal extracts. Different results between retina and cornea might find an explanation in the possibility that retinal cells are more vulnerable to anti-NGF than

corneal ones or alternatively that retinal cells express wide concentration of NGF or even the different cell population or even the retrobulbar space. This different response would imply that visual cells as well as their microenvironment (accessory cells) might counteract the exogenous-linked perturbation (reduced NGF availability) by increasing NGF amount. Molecular analysis corroborated the increased NGF levels in retinal extracts.

Open questions include: 1) the selective deregulation of $\text{trkA}^{\text{NGFR}}$ and p75^{NTR} receptors by means of specific old and recent inhibitors (GNF kinases); 2) the confirmation of Müller cells activation and release of protective mediators (*in vitro*) and finally; 3) the possible contribution of other growth factors that can be influenced upon this specific deregulation (BDNF, NT3, VEGF and GDNF). Current *in vivo* and *in vitro* studies are under investigation.

In conclusion, while several information's are available regarding exogenous NGF administration, few data exist with respect to a reduced intraocular NGF availability that might harm physiological cell-to-cell and cell-to-mediator networks. Taken together, the herein reported findings highlight the pivotal role of NGF in retaining physiological function of retinal and corneal structures (homeostasis). The different response of retinal and corneal tissue to neutralizing anti-NGF antibodies seems from the specific $\text{trkA}^{\text{NGFR}}$ expression in areas close to retrobulbar delivery (photoreceptors and other structural and accessory cells at the ONL layer). Balanced NGF levels are required to provide a tidy protein profile inside the tissue. Tissue protection occurs by deregulation of some inflammatory mediators. The overexpression of IL-21 cytokine would suggest the activation of accessory glial cells, most probably Müller cells^[37-38]. The initial NGF deprivation appears quickly regulated in the retina, a neuronal network, reinforcing the concept that NGF is fundamental for the steady and tidy activity of entire visual network and particularly for photoreceptors. Further investigation is required to understand the mechanisms underlying Müller cell-neuron unit protection under NGF deprivation.

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Conflicts of Interest: Aloe L, None; Rocco ML, None; Balzamino BO, None; Esposito G, None; Micera A, None.

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