Subconjunctival Delivery of p75^{NTR} Antagonists Reduces the Inflammatory, Vascular, and Neurodegenerative Pathologies of Diabetic Retinopathy

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Purpose. The p75^{NTR} is a novel therapeutic target validated in a streptozotocin mouse model of diabetic retinopathy. Intravitreal (IVT) injection of small molecule p75^{NTR} antagonist THX-B was therapeutic and resolved the inflammatory, vascular, and neurodegenerative phases of the retinal pathology. To simplify clinical translation, we sought a superior drug delivery method that circumvents risks associated with IVT injections.

Methods. We compared the pharmacokinetics of a single 40 μg subconjunctival (SCJ) depot to the reported effective 5 μg IVT injections of THX-B. We quantified therapeutic efficacy, with endpoints of inflammation, edema, and neuronal death.

Results. The subconjunctival depot affords retinal exposure equal to IVT injection, without resulting in detectable drug in circulation. At week 2 of diabetic retinopathy, the SCJ depot provided therapeutic efficacy similar to IVT injections, with reduced inflammation, reduced edema, reduced neuronal death, and a long-lasting protection of the retinal structure.

Conclusions. Subconjunctival injections are a safe and effective route for retinal delivery of p75^{NTR} antagonists. The subconjunctival route offers an advantageous, less-invasive, more compliant, and nonsystemic method to deliver p75^{NTR} antagonists for the treatment of retinal diseases.

Keywords: p75 receptor, target, subconjunctival, diabetic retinopathy, antagonist

Ocular diseases affecting the posterior segment of the retina, such as diabetic retinopathy (DR), AMD, and glaucoma are the major causes of blindness.[1,2] Treatment of these chronic pathologies has been challenging and very few new drugs have been developed.

One challenge relates to the unique anatomic barriers and physiology of the eye. To ensure that a drug reaches the retina, specialists have used intravitreal (IVT) drug injections. However, complications can occur in 1:300 injections (retinal tears or detachment, vitreous hemorrhage and lens damage, infections, elevated pressure, glaucoma, or cataracts[3]). In patients with DR and AMD that often receive monthly IVT injections,[4] these can be serious and lead to poor compliance.

As an alternative, subconjunctival (SCJ) delivery may achieve many drug delivery goals.[5,6] However, few comprehensive studies have been published on drug pharmacokinetics and eye tissue distribution following SCJ injections (stability, retention time, rate of elimination, drug concentration at the target tissue).[6–9] These parameters are likely to be drug specific or target specific.

A second key challenge has been the lack of validated druggable targets and lack of new mechanisms of action to target.[8,10–12] For diabetic retinopathy, current accepted therapies include laser photocoagulation, intravitreal injections of corticosteroids, anti-VEGF antibodies, and vitreoretinal surgery.[11–16] All these approaches are effective but are invasive, costly, and are not novel mechanisms. No new drugs targeting novel targets or disease mechanisms have been approved in over 10 years.

The discovery and validation of novel therapeutic targets in DR, with novel mechanisms of action combined with improved drug delivery methods, would be an important contribution solving these two problems.

The receptor p75^{NTR} is a novel therapeutic target implicated in many retinal degenerative diseases such as DR,[17] experimental glaucoma,[18] and optic nerve damage.[19] Specifically, studies using a streptozotocin (STZ) mouse model of type I diabetes demonstrated p75^{NTR}-dependent induction of pro-inflammatory agents TNFα and α2M, disruption of the neuroglia vascular unit, blood–retina barrier (BRB) breakdown, edema, and ganglion cell neuronal death. Moreover, in a model of oxygen-induced retinopathy (OIR), the vaso-obliteration and pathologic neovascularization also occur in a p75^{NTR}-dependent manner. Antagonizing p75^{NTR} using IVT delivery of small molecule THX-B diminished all these pathologic features in DR and OIR.[17]

Here we report pharmacokinetic studies of the small molecule p75^{NTR} antagonist THX-B, and demonstrate that SCJ delivery of THX-B attains retinal exposure at therapeutic levels in the mouse model of diabetic retinopathy. Subconjunctival

THX-B curbed expression of TNFα and α2M proinflammatory agents decreased edema, and protected the ganglion cell layer and nerve fiber layer with the same efficacy as THX-B IVT injections.

Subconjunctival delivery of $p75^{NTR}$ inhibiting agents, which afford long-lasting therapeutic benefits and no systemic exposure, represent a promising novel approach for the treatment of DR, and perhaps other chronic retinal pathologies including glaucoma and AMD.

Methods

Animals

All studies adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and were approved by the McGill University Animal Care Committee. We purchased C57Bl/6 wild-type mice (~25 g) and Sprague-Dawley rats (~300 g) from Charles River Laboratories, Inc. (St. Zotique, Quebec, Canada).

Ocular Drug Delivery

Mice were anesthetized with 3% isoflurane during IVT or SCJ injections of a clear solution of THX-B $p75^{NTR}$ antagonist, or vehicle. THX-B is a pharmacologic small molecule antagonist of $p75^{NTR}$[19,20] and a competitive blocker of proNGF binding to $p75^{NTR}$.

For pharmacokinetic studies, we performed IVT injections in the left eye, and SCJ injections in the right eye of the same animal. In therapeutic studies of the diabetes mouse model, an SCJ depot of THX-B $p75^{NTR}$ antagonist (right eye) or PBS vehicle (left eye) were done in adult diabetic mice at 2.5 weeks of ongoing disease.

Intravitreal Injections. Intravitreal injections were performed as previously described.[17] Briefly, a volume of 2.5 μL containing a total of 5 μg THX-B was slowly delivered into the mouse's vitreous chamber using a Hamilton syringe, and confirmed microscopically. After the injection, the syringe was left in place for 30 seconds and slowly withdrawn to prevent efflux.

Subconjuctival Injections. A volume of 20 μL containing a total of 40 μg THX-B or 30 μL containing a total of 15 μg THX-B, or corresponding volumes of vehicle (PBS) were injected. The conjunctiva was gently pulled from the sclera using tweezers. Half of the total volume of THX-B was delivered into the superior subconjunctival space and the other half into the nasal subconjunctival region using a microsyringe with a 33 gauge needle.

Sample Tissue Preparation and Organic Extraction

For retinal tissue preparation mice were humanely sacrificed at different time points (5, 30, 60, 120, 180, and 360 minutes) after the ocular injections. Then, the eyecups were enucleated, rinsed in PBS, and transferred to a clean Petri dish. An incision was made in the sclera at the posterior part of the eye, and vitreous humor was aspirated with a micropipette. Afterward, the cornea and lens were removed and the whole retina was dissected. All samples were frozen in dry ice and stored at –80°C or immediately processed. Retina tissue was homogenized in 80 μL of methanol using a mini-homogenizer for 5 minutes at 4°C. The homogenate was vortexed for 30 seconds and left for 2 to 3 minutes on ice. Then, the samples were centrifuged for 10 minutes at 8,000*g*. The supernatants were collected and transferred into HPLC vials with insert to be analyzed by HPLC.

Determination of THX-B Stability in Plasma

For plasma preparation, whole blood was collected from rats by cardiac puncture under isoflurane anesthesia, and blood was transferred into commercial heparinized tubes (Covidien, Mansfield, MA, USA). Cells and platelets were removed and the plasma supernatant was processed for organic extraction. We incubated 2 μL of THX-B (2 mM) in 38 μL of plasma for 1, 30, and 60 minutes at 4°C or at 37°C. For extraction of the compound from plasma samples, 160 μL of methanol was added to each tube and vortexed. Then, the samples were centrifuged for 10 minutes at 8,000*g*. The supernatants were collected and analyzed by HPLC.

Determination of THX-B Stability in Vitreous

Rats were euthanized and the eyes were enucleated. An incision through the sclera was done at the posterior part of the eyeball and the vitreous was quickly aspirated using a pipette. To determine vitreous humor stability, 1 μl of THX-B (4 mM) was added to 19 μl of vitreous humor and incubated for 30, 60, 120, 180 and 240 min at 4°C or 37°C. The samples were extracted in solvent as above, and analyzed by HPLC.

HPLC and Analysis

The concentration of THX-B was determined by HPLC. For each sample, 20 μL of the supernatant collected after the organic extraction was analyzed by HPLC (Waters Corp., Milford, MA, USA) with a Harmony C18 analytical column (4.6 mm × 7.5 cm, 5.5 μm, 100A), injected with 20 μL/sample. Precolumn and mobile phase A consisted of 10% acetonitrile (ACN), 90% water (0.1% trifluoroacetic acid [TFA]); and B: 70% ACN, 30% water (0.1% TFA) at a flow rate of 1 mL/minute. The gradient was linear from 0 to 100% B for 10 minutes and UV detection at 275 nm. The retention time was 5.5 minutes. Quantification was done by a linear regression analysis of peak area from a standard curve. The calibration curve of THX-B showed excellent linearity between peak areas and concentrations over the range of 1 μM (0.74 μg/mL) to 100 μM (59.2 μg/mL) in water, with R^2 value over 0.99. The limit of quantification of the present method was 0.7 μM (0.52 μg/mL) corresponding to 10 ng in samples. The recovery was close to 100%. Within-day and interday coefficient of variation was lower than 3%.

Induction of Diabetes

Male 10-week-old mice received an intraperitoneal injection (IP) of streptozotocin (STZ) (60 mg/kg; Sigma-Aldrich Corp., St. Louis, MO, USA) dissolved in sodium citrate buffer (0.01 M, pH 4.5) on 5 consecutive days. After 1 and 6 weeks of injection with STZ, blood glucose was measured using a glucometer (Abbott Laboratories, Chicago, IL, USA), with fasting blood glucose >17 mmol/l (300 mg/dL) considered as diabetic. Age-matched, nondiabetic C57BL/6 mice injected with sodium citrate buffer were used as controls.

Optic Coherence Tomography (OCT) Imaging

A noninvasive spectrometer-based Fourier-domain (FD)-OCT system was used to acquire retinal images. Fourier domain OCT is a noninvasive method that allows time-kinetic studies in the same animal, with axial resolution in tissue nominally better than 3 μm.[21,22] In each B-scan, the thickness of the nerve fiber layer–ganglion cell layer (GCL)–inner plexiform layer (IPL), hereafter referred to as NGI, and the outer nuclear layer (ONL) was measured at three adjacent points, with ImageJ software (http://imagej.nih.gov/ij/; provided in the public domain by the

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National Institutes of Health, Bethesda, MD, USA) as described.[17] Data are shown as average thickness in μm ± SEM (absolute values) in control versus diabetic mice ± treatments.

Fluorescence "In Situ" Hybridization (FISH)

Retinal sections 20-μm thick were mounted onto gelatin-coated glass slides, refixed in 4% paraformaldehyde, permeabilized with proteinase K, and acetylated. Hybridization was carried by incubating the slides with either 200 ng/mL of digoxigenin-labeled TNFα or α_2M antisense RNAs probes overnight at 72°C in a hybridization oven (Robbins Scientific, San Diego, CA, USA), as previously described.[17] As controls, hybridization with either 200 ng/mL of digoxigenin-labeled TNFα or α_2M sense RNAs probes was performed on parallel slides using the same experimental conditions. Images were obtained using a confocal microscope (IX81; Olympus America, Inc., Center Valley, PA, USA) equipped with commercial software (Fluoview 281 3.1; Olympus America, Inc.).

Immunohistochemistry

Whole eyes were enucleated and processed for immunohistochemistry as previously described.[17] Retinal cross-sections (20 μm) were sectioned using a cryostat (Leica Microsystems, Wetzlar, Germany), and stained with antibodies specific for anti-$p75^{NTR}$ (1:1000; a gift from P. Barker); anti-CRALBP (15051, 1:1000; Abcam, Cambridge, UK); anti-glial fibrillary acidic protein (AB5804, 1:1000; Merck Millipore, Billerica, MA, USA); anti-proNGF/NGF mAb (1:2000[19,23]; prepared in-house); anti-TNFα (AB2148P, 1:1000; Merck Millipore); or anti-alpha 2 macroglobulin (1:1000, Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Labeled secondary antibodies (goat anti-mouse IgG AlexaFluor 488, and/or goat anti-rabbit IgG AlexaFluor 594; Invitrogen Corp., Carlsbad, CA, USA) were used for colocalization studies. Images were obtained using a confocal microscope (Olympus America, Inc.) equipped with commercial software (Olympus America, Inc.). As immunostaining controls, adjacent sections were processed by substituting primary with irrelevant primary or by omitting the primary, followed by the proper secondary. In all cases, background levels were undetectable.

Western Blotting

Dissected whole retinas were solubilized and samples (30 μg) were resolved on SDS-PAGE gels (8%–12%), and transferred by Western blotting. Membranes were incubated overnight at 4°C with mouse anti-β-actin (sc-47778, 1:1000; Santa Cruz Biotechnology); rabbit antibody to $p75^{NTR}$ (1:1000; a gift from P. Barker); rabbit anti-TNFα (AB2148P, 1:2000; Merck Millipore), rabbit anti-alpha 2 macroglobulin (1:2000; Santa Cruz Biotechnology); or rat anti-pro-NGF/NGF (1:200). After washing, membranes were incubated with 1:5000 horseradish peroxidase-conjugated anti-mouse or anti-goat or anti-rabbit secondary antibodies (Merck Millipore) for 2 hours at room temperature. Membranes were imaged with a commercial imager (LAS-3000; Fujifilm, Tokyo, Japan) and bands were assessed using densitometry plugins in ImageJ 1.47 software. The molecular weight (M_r) of bands were calculated using standard markers.

Reverse-Transcription PCR and Quantitative Real-Time PCR

Retina samples were processed as previously described.[17] Total RNA (1 μg) was reverse-transcribed to cDNA and analyzed by quantitative real-time PCR on a real-time PCR system (ABI 7500; Applied Biosystems, Foster City, CA, USA) and using a master mix (iQTM SYBR® Green Supermix; Bio-Rad Laboratories, Hercules, CA, USA) with primers targeting, TNFα and GAPDH. Mouse TNFα forward 5′ GAGTCCCAGGTCTACTTT 3′ reverse 5′ CAGGTCACTGTCCCAGCATCT 3′ primers were used to generate specific fragments. All primers were used at 100 nM. The reaction was performed in triplicate with a quantitative (q)PCR system (Mx3000P; Agilent Technologies, Santa Clara, CA, USA). Amplification reaction data were analyzed using the complementary analysis software (Agilent Technologies). Target gene expression was normalized to the average expression of the housekeeping gene GAPDH forward 5′ CACCACCTTGATG TCATC 3′ reverse 5′AGCCCAGAACATCCCTG 3′. Quantitative analysis of gene expression was assessed using the ΔΔCT quantification.

A.

Temperature	4° C						
Sample	Plasma			Vitreous			
Time	1'	30'	60'	30'	60'	120'	180'
THX-B (μM)	58	56	63	48	47	54	54
Temperature	37° C						
Sample	Plasma			Vitreous			
Time	1'	30'	60'	30'	60'	120'	180'
THX-B (μM)	53	48	52	49	47	48	46

B.

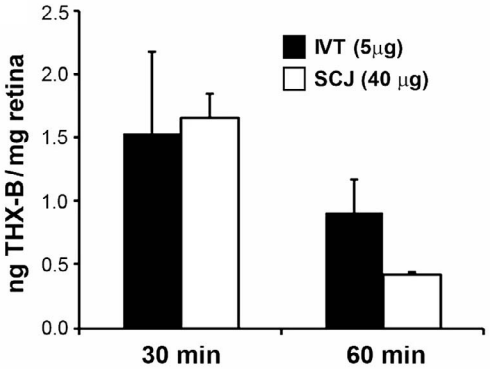

Figure 1. Stability and pharmacokinetics THX-B. (**A**) Plasma and vitreous stability of THX-B was quantified by HPCL. The concentration of THX-B in plasma were measured after spiking the plasma with THX-B (2 mM) and incubating at 4°C or 37°C for the indicated times, followed by precipitation with 4x MeOH volume. (**B**) Concentration of THX-B detected in healthy mice retinas, at the indicated times after delivery of 5 μg THX-B (IVT) or 40 μg THX-B (SCJ). The maximal concentration of THX-B was observed at 30 minutes after injection and decreased at 60 minutes. At both time points, the concentration of THX-B in the retina observed was very similar regardless of the method of delivery used.

Vascular Permeability Assay

Evans blue permeation was analyzed by measuring albumin-Evans blue complex leakage from retinal vessels as previously described.[17] Briefly, animals were injected intravenously with a solution of Evans blue (2% wt/vol dissolved in PBS). After 48 hours, animals were killed, the whole eyes enucleated, immediately embedded in OCT compound, and cross-sections were prepared. Images by immunofluorescence microscopy were obtained using identical exposure time, brightness, and contrast settings. For each experimental condition, at least six images were acquired from three sections cut from different areas of the retina ($n = 3$ retinas per group). The area of the Evans blue permeation was measured using ImageJ software; an arbitrary rectangle that included all layers of the retina—

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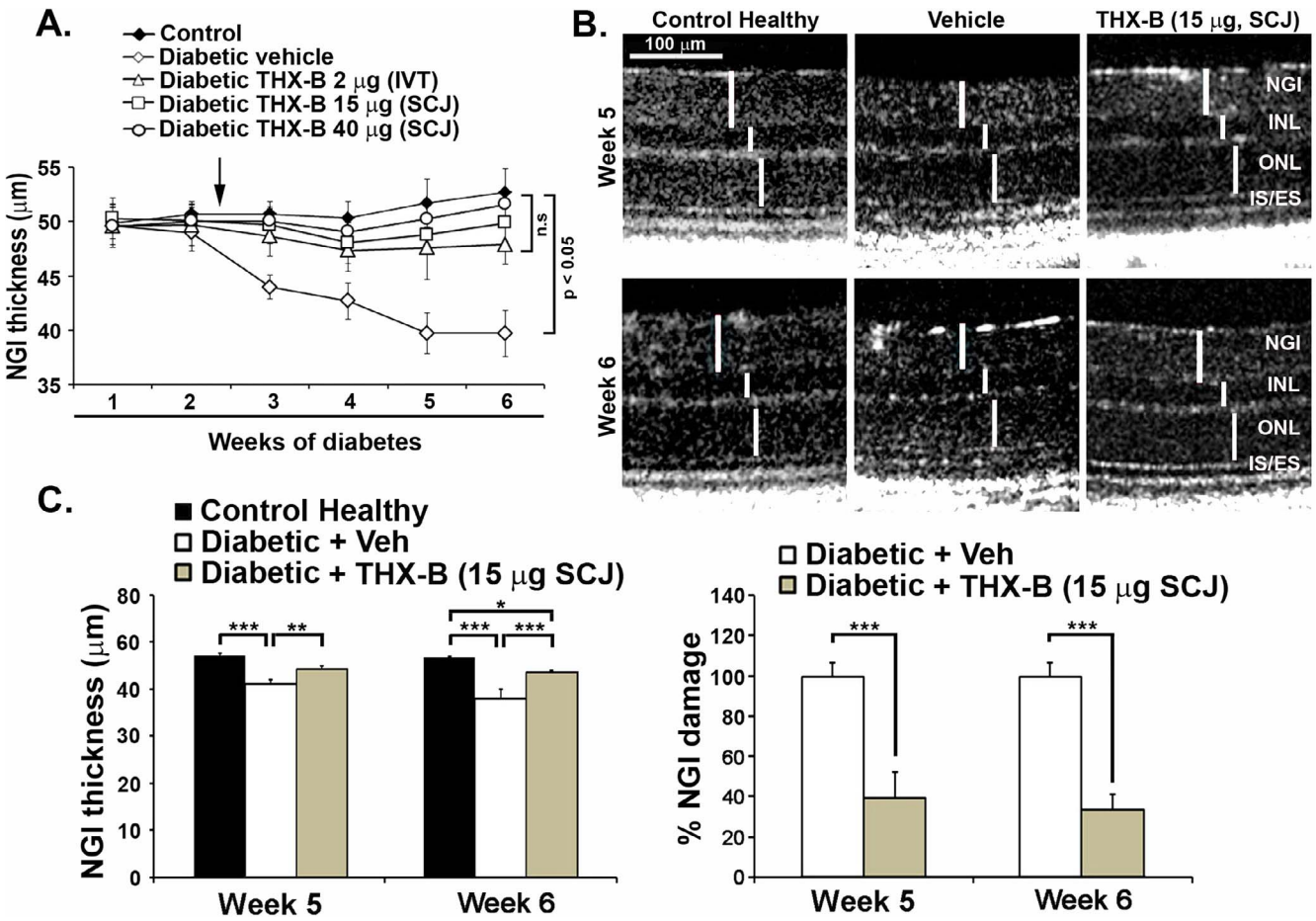

Figure 2. Subconjuctival delivery of THX-B in diabetes exerts the same protective effect as intravitreal administration. (**A**) Time-dependent changes in NGI thickness ± SD ($n = 4$ eyes). Thickness of NGI is lost progressively in the diabetic retina treated with vehicle. Subconjunctival THX-B treatment significantly preserved the NGI structure at weeks 5 and 6 ($P < 0.05$). (**B**) Representative sections of B-scans OCT images in 6-week-diabetic mice ± subconjunctival THX-B or vehicle (PBS; injections at week 2.5 of diabetes); compared to age-matched control healthy animals. *Vertical white lines* delimit the thickness of the NGI, INL, and ONL layers. *Scale bar*: 100 μm. (**C**) *Upper panel*: quantification of NGI layer thickness from B-scans OCT images at weeks 5 and 6 after onset of diabetes. Subconjunctival THX-B treatment was significantly protective (**$P < 0.01$ at week 5 and ***$P < 0.001$ at week 6; $n = 3$ independent experiments, total of 9 mice per group) in the NGI. *Lower panel*: percentage of NGI loss in diabetes comparing THX-B relative to vehicle-treated retinas (set at maximal NGI loss). Treatment with THX-B significantly reduced NGI damage at weeks 5 and 6 (***$P < 0.001$, $n =$ three independent experiments, total of nine mice per group). All statistical analyses are 2-way ANOVA with significance $\alpha < 0.05$, followed by Bonferroni's correction for multi-comparisons.

GCL; IPL; inner nuclear layer [INL]; ONL; photoreceptor layer [PhR]; and RPE—was drawn and the area in pixels quantified. Data are shown as the average area (in pixels) ± SEM in control versus diabetic injected once with vehicle or THX-B.

Statistical Analysis

Results are presented as mean ± SEM for all studies. We used 2-way ANOVA (for OCT) and 1-way ANOVA (for all other experiments), with significance of $\alpha = 0.05$ or higher for processing data. Bonferroni post hoc analysis was used for calculating significance between groups. Changes in NGI thickness for treated and untreated retinas were analyzed by 2-tailed Student's *t*-tests for significance between two means.

Results

Stability of THX-B in Plasma

We measured the THX-B plasma levels at 4°C and 36°C by spiking plasma with THX-B (2 mM), followed by precipitation with 4x MeOH after 1, 30, and 60 minutes of incubation. The results showed very small fluctuations of the THX-B concentration in plasma at the time points tested, regardless of the temperature (Fig. 1A). The recovery of THX-B was close to 100%. These data indicate that the THX-B compound is stable in plasma, and that it does not bind to proteins. Similar data was obtained when testing for THX-B stability in vitreous, for up to 4 hours (Fig. 1A).

Detection of THX-B in Retina After IVT and SCJ Injections

Next, we studied the pharmacokinetics of THX-B in the retina at 30 and 60 minutes after IVT or SCJ injections. The right eyes were injected with 5 μg of THX-B intravitreally, and the left eyes of the same animals were injected with 40 μg of THX-B by the subconjunctival route.

For both, IVT or SCJ injections, the maximum concentration of THX-B in retina tissue was detected 30 minutes after delivery of the compound. At 30 minutes, THX-B concentrations of 1.5 ± 0.645 ng/mg of retinal protein following IVT injections, and 1.7 ± 0.179 ng/mg of retinal protein following

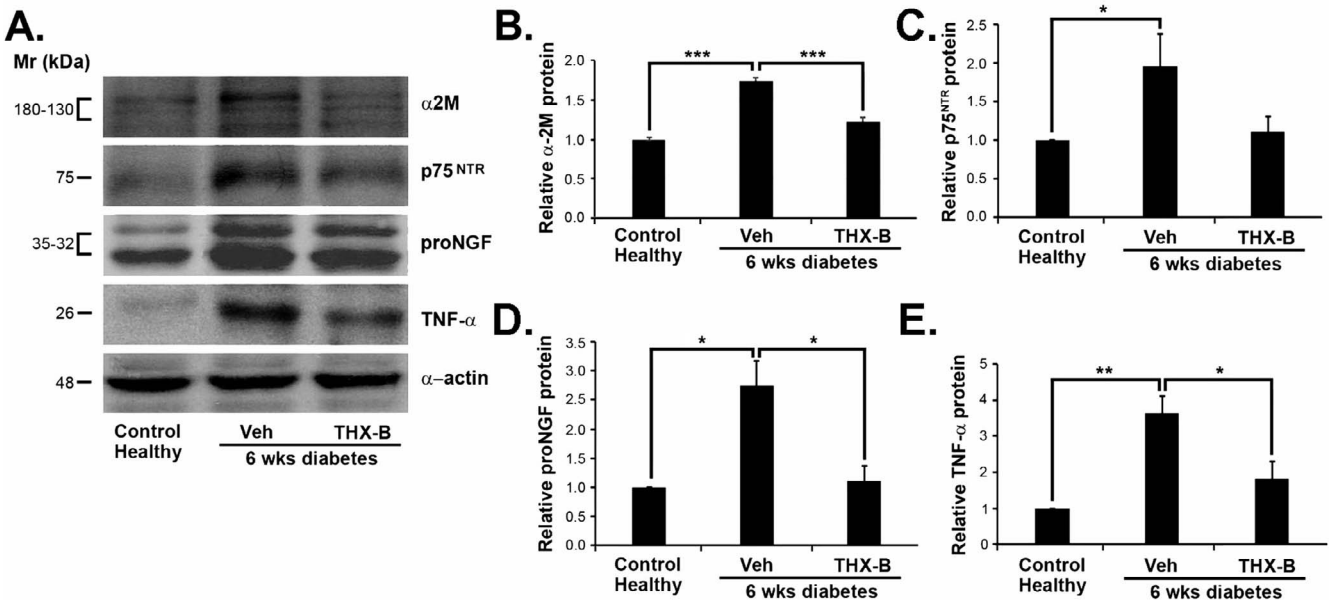

Figure 3. Subconjunctival THX-B reduces neurotoxic factors α2M and TNFα proteins. (**A**) Expression of α2M, $p75^{NTR}$, proNGF, and TNFα in whole retinas analyzed by Western blots of retinal samples from week-6 diabetic mice ± treated at week 2.5 of diabetes, compared to age-matched control healthy mice. (**B–E**) Histograms show quantification of Western blots. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ relative to control healthy animals ($n = 3$ independent experiments). Treatments were at week 2.5 of diabetes and sample collection and analyses were done at week 6. THX-B prevented the increase of α2M, proNGF, and TNFα without affecting $p75^{NTR}$ levels.

SCJ injections were detected. At 60 minutes, the amount of THX-B decayed to 0.9 ± 0.267 ng/mg after IVT injections and to 0.4 ± 0.027 ng/mg after SCJ delivery of THX-B (Fig. 1B). At 120 minutes, the concentration of THX-B in the retina was under the limit of detection for both routes of administration (data not shown), suggesting clearance due to vitreous exchange and/or draining into systemic circulation. These data were replicated in two independent experiments ($n = 2$ or 3 mice per group per experiment).

These data indicate that administration of THX-B by the SCJ route delivers to the retina the similar amount of THX-B as the IVT route. In intravitreal delivery, there is a clearance mechanism likely through exchange or renewal of vitreous fluid that reduces the THX-B concentration available to permeate from the vitreous to the retina. In subconjunctival delivery, clearance may be associated with choroid vasculature or the continuous migration of the compound through the sclera.

Subconjuctival Administration of THX-B in Diabetes Preserves Retinal Structure

We previously demonstrated early $p75^{NTR}$ upregulation in the retina, ~1 week after the onset in diabetes; and that antagonizing $p75^{NTR}$ with intravitreal THX-B preserved retinal structure and prevented diabetes-induced neuronal loss.[17] We investigated whether SCJ injection of THX-B achieved the same protection. At 2.5 weeks after onset of diabetes THX-B was injected into the conjunctiva of one eye and the contralateral eye was injected with control PBS vehicle. The structural changes of the retina were quantified in longitudinal studies by OCT, a noninvasive technique validated to correlate with retinal ganglion cell (RGC) numbers and nerve fiber layer health. In each group, comprised of 4 animals, we compared the right and left eyes of each animal. We performed OCT longitudinally for up to 6 weeks of diabetes, measuring RGC cell bodies and fibers in the NGI (Fig. 2A).

In diabetic retinas (injected SCJ with PBS vehicle control) the NGI thickness decreased progressively and was significantly lower after 5 weeks of diabetes compared to age-matched nondiabetic eyes ($P < 0.05$). Treatment of diabetic eyes with either 2 μg THX-B IVT or with two different doses of THX-B SCJ (15 or 40 μg) protected the NGI from thinning even at week 6 of diabetes ($P < 0.05$ to control diabetic retina). There were no differences in preservation of retinal structures achieved by SCJ and IVT deliveries (Fig. 2A). Protection by subconjunctival THX-B was achieved by the lower dose 15 μg THX-B SCJ, and this dose was used for further experiments.

A more detailed longitudinal study by OCT confirmed the protective effect of NGI by THX-B (15 μg, SCJ) with significant protection even at 6 weeks after the onset of diabetes (44.49 ± 0.69 μm versus 38.16 ± 2.7 μm; $P < 0.001$; Figs. 2B, 2C). Setting the NGI damage in vehicle-treated diabetic eyes as maximal (100%), the protection conveyed by THX-B to the NGI of diabetic eyes was ~50% at week 5 ($P < 0.001$) and 70% at week 6 ($P < 0.001$; Fig. 2C).

Diabetic retinopathy causes damage specifically to the NGI layers, and within the time frame of the experiments there were no structural alterations to the INL (where the Müller and bipolar cell bodies reside) or to the ONL (containing cone and rods photoreceptor cell bodies). These unaffected layers are useful as internal control of specific damage and specific rescue by delivery of $p75^{NTR}$ antagonists.

Together these data indicate that in ongoing diabetic retinopathy a single subconjunctival THX-B has a significant long-lasting protective effect of the NGI, comparable to a single injection of intravitreal THX-B.

Subconjuctival Injection of THX-B Reduces the Production of Neurotoxic Factors

A previous study in our laboratory showed that in diabetic retinopathy there is an increase of cytotoxic factors α2M and TNFα, associated with the upregulation of $p75^{NTR}$ and pro-NGF. Production of these neurotoxic factors are inhibited by

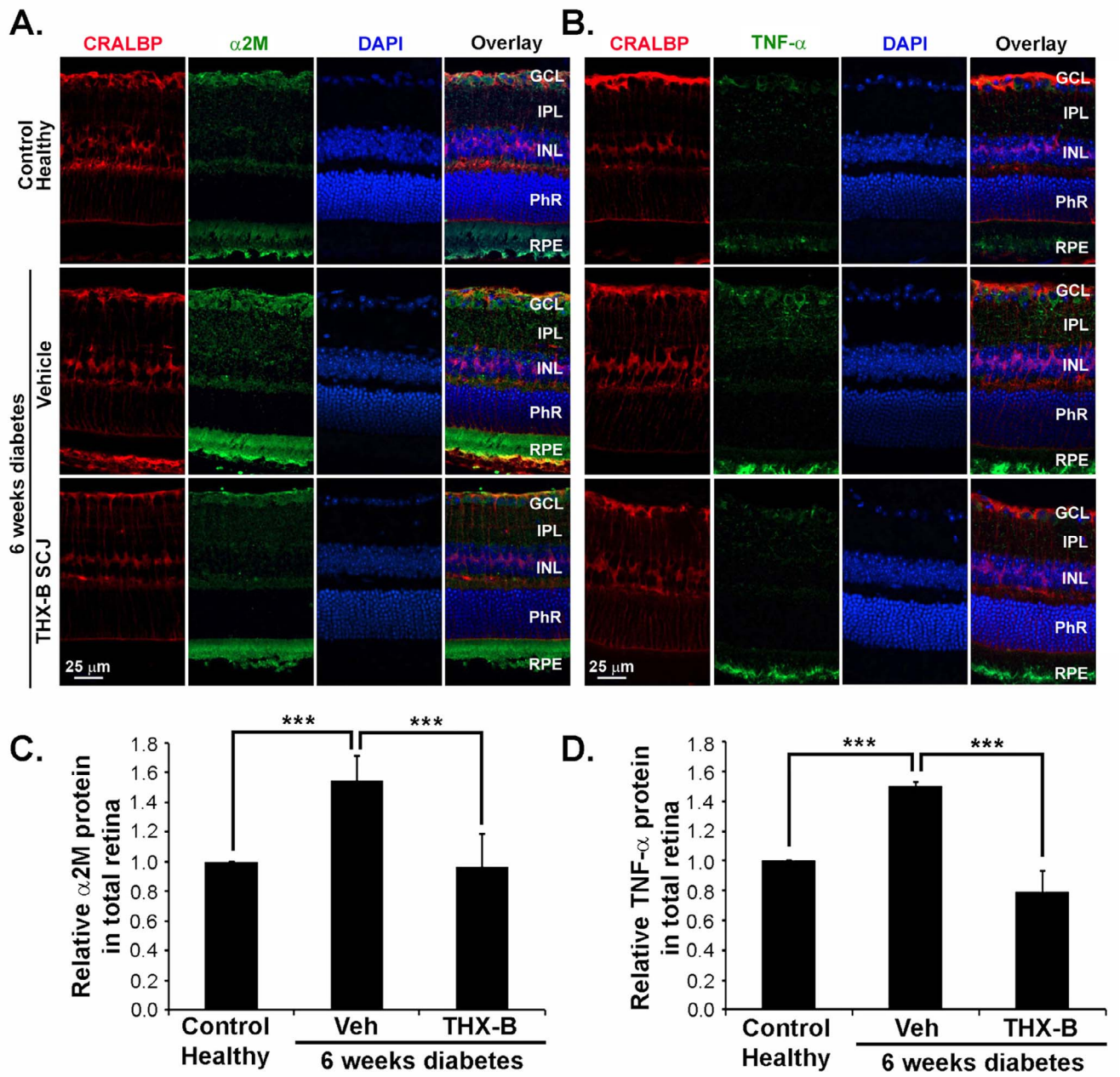

Figure 4. Proteins α2M and TNFα are specifically increased in retinal layers affected in diabetes, and subconjunctival THX-B reduces these neurotoxic factors. Confocal microscopy images showing the induction and distribution of (**A**) α2M or (**B**) TNFα proteins in retina at week 6 of diabetes ± treatment; compared to age-matched control healthy animals. Treatments were at week 2.5 of diabetes. Protein α2M (*green*) was preferentially expressed in the INL and the end-feet of Müller cells (*red*), as revealed by coexpression with CRALBP. Tumor necrosis factor alpha (*green*) was almost exclusively expressed in cells located in the GCL, surrounded by the end-feet of Müller cells (*red*). Nuclei are counterstained with DAPI. Images are representative of three independent experiments. *Scale bar*: 25 μm. (**C**, **D**) Quantification of the expression of α2M and TNFα protein in total retina relative to control healthy. GCL, INL, OPL, and PhR layers were quantified. THX-B significantly decreased α2M and TNFα compared to contralateral diabetic–vehicle (Veh)-treated retinas. $^{***}P < 0.001$; $n = 3$ independent experiments each with two animals/group.

intravitreal THX-B.[17] Here, we aimed to study the effect of subconjunctival THX-B using the same experimental paradigm, at week 6 after the induction of diabetes.

Compared to healthy controls, diabetic retinas increased α2M protein ~2-fold (α2M, 1.74 ± 0.0147; $P < 0.001$, $n = 3$), increased pro-NGF protein ~3-fold (pro-NGF, 2.75 ± 0.424; $P < 0.05$, $n = 3$) and increased TNFα protein ~3.5-fold (TNFα, 3.65 ± 0.454; $P < 0.01$, $n = 3$; Fig. 3A, quantified in Figs. 3B, 3D, 3E).

The increases in α2M, TNFα, and pro-NGF were significantly normalized by treatment with subconjunctival THX-B, compared to vehicle-contralateral diabetic eyes ($n = 3$; α2M, 1.23 ± 0.057, $P < 0.001$; pro-NGF, 1.10 ± 0.259, $P < 0.05$; and TNFα, 1.82 ± 0.461, $P < 0.05$; Fig. 3A, quantified in Figs. 3B, 3D, 3E). It is noteworthy that THX-B subconjunctival injection did not significantly decrease $p75^{NTR}$ protein levels (Fig. 3A, quantified in Fig. 3C).

Immunohistochemical studies in retinal sections demonstrated that in diabetic retinas, the α2M protein was mainly

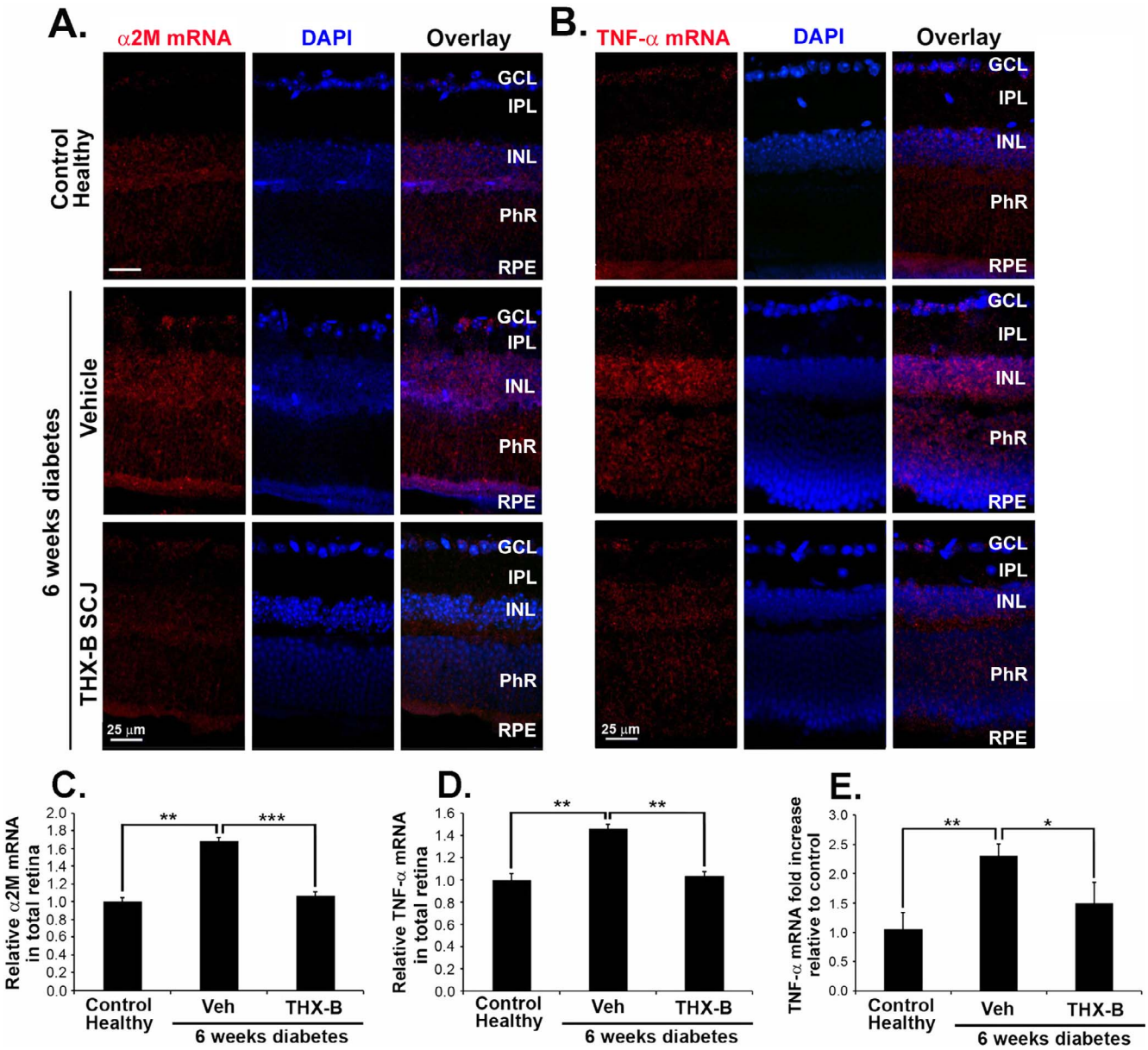

Figure 5. Subconjunctival THX-B reduces the mRNA levels of neurotoxic factors α2M and TNFα Fluorescence in situ hybridization shows induction and distribution of (**A**) DIG-labeled α2M or (**B**) TNFα antisense RNAs probes (*red*) in mice retina after 6 weeks of diabetes ± treatment; compared to age-matched control healthy animals. Treatments were at week 2.5 of diabetes. Nuclei are counterstained with DAPI. Images are representative of three independent experiments. *Scale bar*: 25 μm. (**C**, **D**) Quantification of the expression of α2M and TNFα mRNAs in total retina, relative to control healthy retinas. Layers of GCL, INL, OPL, and PhR were quantified. THX-B significantly decreased α2M and TNFα mRNAs compared to contralateral diabetic–vehicle-treated retinas. $^{*}P < 0.05$, $^{**}P < 0.01$; $n = 3$ independent experiments each with two animals/group. (**E**) Quantification of the levels of TNFα transcript by qPCR on whole retinas after 6 weeks of diabetes ± treatment; compared to age-matched control healthy animals. Treatments were at week 2.5 of diabetes. $^{*}P < 0.05$, $^{**}P < 0.01$; $n = 3$.

located in the end-feet of Müller cells identified by cellular retinaldehyde-binding protein (CRALBP) and in the INL (Fig. 4A), whereas TNFα protein was almost exclusively in the GCL (Fig. 4B). Parallel immunohistochemical studies using sections of contralateral diabetic retinas treated with subconjunctival THX-B show almost completely decreased α2M and TNFα expression to the basal levels detected in control healthy retinas (Figs. 4A–D).

Since α2M and TNFα are soluble factors, we further verified their mRNA cellular expression pattern. Retinal sections of diabetic mice 6 weeks after STZ injection were studied by in situ mRNA hybridization. Our data show significant increases in α2M mRNA ($P < 0.01$) and TNFα mRNA ($P < 0.01$) in the GCL and INL of diabetic retinas compared to control healthy retinas. As technical control for the specificity of in situ hybridization, using sense probes for TNFα or α2M showed undetectable levels (data not shown). Hence, most of the de novo upregulation of both cytokines was observed in the GCL and INL, the main retinal layers affected in the disease. In contralateral diabetic eyes α2M and TNFα mRNA levels were significantly reduced by subconjunctival THX-B treatment ($P < 0.001$ and $P < 0.01$, respectively; Figs. 5A, 5B, quantified in Figs. 5C, 5D).

In situ hybridization results were corroborated by qPCR quantification of TNFα-mRNA at 6 weeks after the induction of diabetes. We found TNFα-mRNA was significantly increased in retinas of diabetic mice compared to control healthy retinas

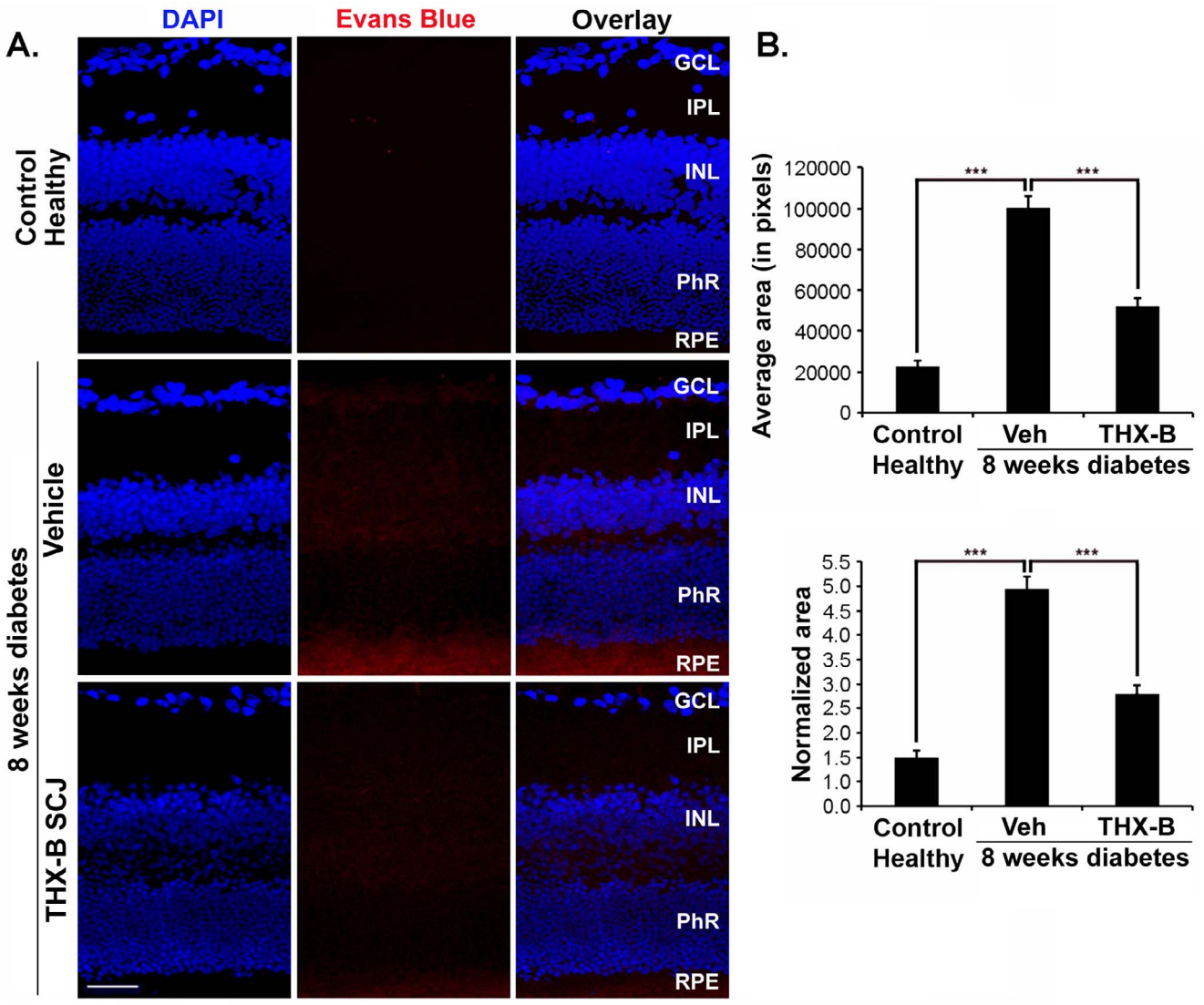

Figure 6. Subconjuctival administration of THX-B reduces pathologic vascular permeability in diabetes. (**A**) Confocal images of retinal sections from 8-week-diabetic mice ± treatment; compared to age-matched control healthy animals. Treatments were at week 2.5 of diabetes. *Red signal* depicts leakage of Evan blue into the retina. Nuclei are counterstained with DAPI. *Scale bar*: 25 μm. (**B**) Quantification of Evan blue images. Data are shown as the average area (in pixels) ± SEM and as normalized area relative to control healthy mice. Treatment with subconjunctival THX-B significantly reduces Evans blue extravasation compared to age-matched diabetic untreated mice. $^{*}P < 0.05$, $^{***}P < 0.001$, $n = 3$.

(2.3 ± 0.196; $P < 0.01$, $n = 3$; Fig. 5E); and the increase was significantly diminished by treatment with subconjunctival THX-B (1.4 ± 0.358; $P < 0.05$, $n = 3$; Fig. 5E).

Together, these data suggest that pharmacologic inhibition of $p75^{NTR}$, including a relatively benign SCJ delivery, attenuates the production of pro-inflammatory cytokines in the retina and affords structural preservation of retina with the same efficacy as previously reported IVT treatments.

Subconjuctival Administration of THX-B Reduces Vascular Permeability in Diabetes

The pathophysiology of diabetes is characterized by a cascade of events that include loss of blood–retinal barrier function causing extravasation and retinal edema. Here, we analyzed the effect of subconjunctival THX-B on extravasation in vivo, using the Evans blue vascular permeability assay, in mice at 8 weeks post-STZ. Our results demonstrate that subconjunctival THX-B treatment caused a significant decrease in vascular leakage compared to age-matched diabetic nontreated mice (2.31 ± 0.17 vs. 4.45 ± 0.25 normalized area values relative to control healthy, $P < 0.01$; Fig. 6A, quantified in Fig. 6B).

These data suggest that subconjunctival THX-B reduces extravasation, which is beneficial to prevent key aspects of the diabetic pathology in the retina. In addition, it is likely that ameliorating vascular permeability prevents extravasation of α2M and other inflammatory factors present in serum, hence further improving the environment of the diseased retina.

Subconjuctival Administration of THX-B in Diabetes Affords a Long-Lasting Preservation of Retinal Structure

Given that a single injection of THX-B (SCJ at week 2.5 of diabetes) had detectable physiologic benefits at week 8 of diabetes, we investigated benefits at longer time-points.

A quantitative longitudinal study by OCT examined the effect of THX-B (SCJ at week 2.5 of diabetes) on the NGI up to

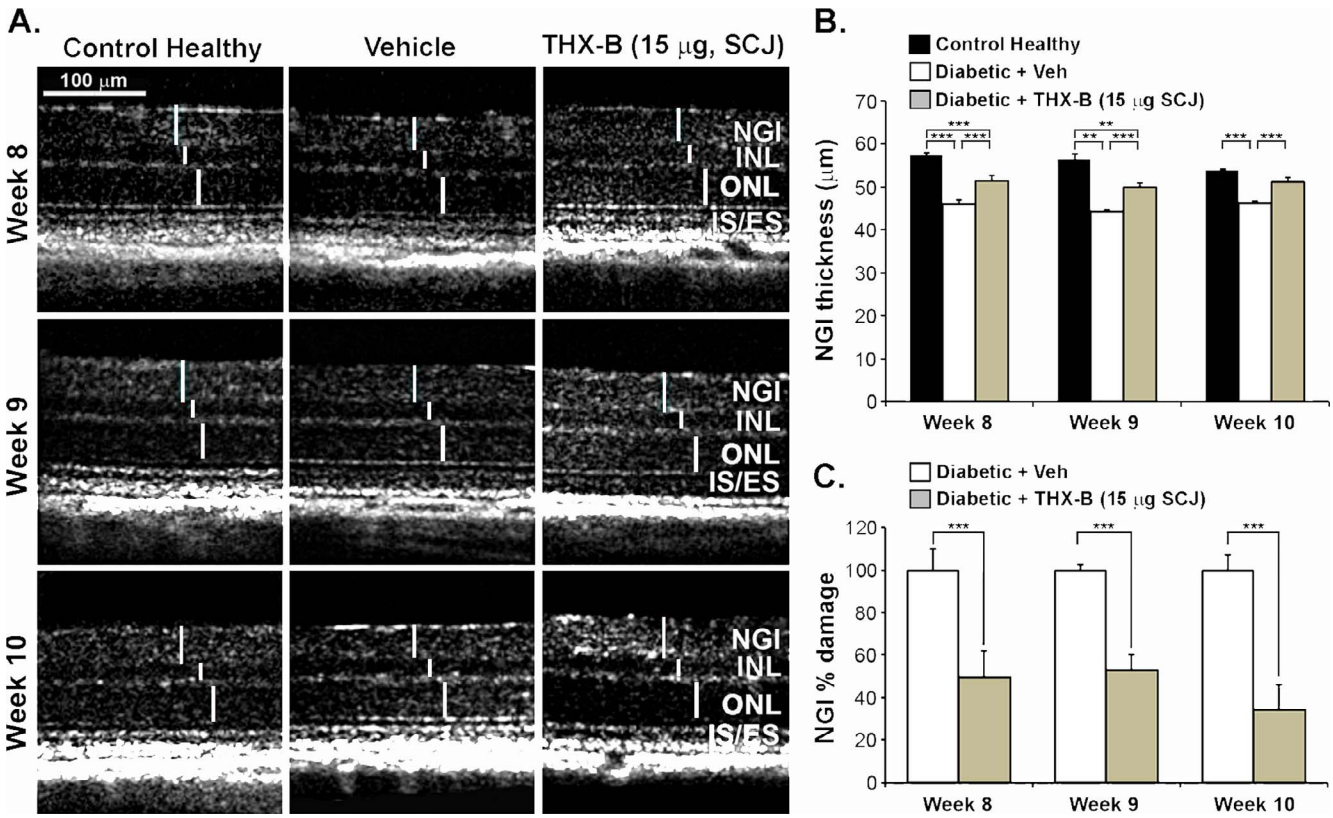

FIGURE 7. Subconjuctival administration of THX-B affords a long-lasting protective effect on the retinal structure during diabetes. (**A**) Representative sections of B-scan OCT images from diabetic mice treated with either vehicle (PBS) or subconjunctival THX-B (treatments at week 2.5 of diabetes). Quantifications were done after 8, 9, and 10 weeks following the onset of diabetes, and compared to age-matched control healthy animals. *Scale bar*: 100 μm. *Vertical white lines* delimit the thickness of the NGI, INL, and ONL layers. (**B**) Time-dependent changes in NGI thickness ± SEM ($n = 4$ eyes). Thickness of NGI is lost progressively in the diabetic retina treated with vehicle. Subconjuctival THX-B treatment significantly preserved the NGI structure from weeks 8 to 10 (***$P < 0.001$, **$P < 0.01$). (**C**) Percent NGI loss comparing THX-B relative to vehicle. Treatment with THX-B significantly reduced NGI damage at all time points studied (***$P < 0.001$, $n = 4$ mice per group). Two-way ANOVA with significance at $\alpha < 0.05$, followed by Bonferroni's correction for multi-comparisons.

week 10 of diabetes. In control diabetic retinas, thinning of the NGI progressed from weeks 8 to 10, compared to age-matched nondiabetic retinas ($P < 0.05$). Treatment of diabetic mice with THX-B SCJ (15 μg) prevented thinning of the NGI until week 10 of diabetes ($P < 0.05$ to control diabetic retina; Figs. 7A, 7B). It was not possible to test the mice past week 10 due to systemic consequences of diabetes.

Setting the NGI damage in vehicle-treated diabetic eyes as maximal (100%), the reduction in NGI damage achieved by THX-B at each week was ~50% for weeks 8 and 9 ($P < 0.001$) and ~35 % for week 10 ($P < 0.001$; Fig. 7C).

As internal control, indicating that diabetes specifically affects RGCs and their fibers, there were no structural alterations to the INL or to the ONL even at 10 weeks of diabetes (data not shown).

These data indicate that the NGI structure (containing RGCs and their fibers) degenerates chronically during diabetes. The $p75^{NTR}$ inhibitor THX-B specifically protects the NGI, and in ongoing diabetic retinopathy a single dose of subconjunctival THX-B has a significant long-lasting protective effect of that retinal structure.

DISCUSSION

We report on a novel disease-modifying target $p75^{NTR}$, and studies leading to the therapeutic use of small molecule antagonist THX-B delivered as a relatively benign SCJ depot, for treatment of serious, chronic, and still incurable diseases such as DR but also potentially AMD and glaucoma.[8,10–12]

The unique anatomy of the eye and the blood–retinal barrier protects the retina from the internal and the external environments, while maintaining the needed optical transparency and light detection by retinal neurons. This poses challenges to drug delivery to the retina. Systemic drug delivery often does not afford permeation to retina achieving therapeutic concentrations, or this route may require high drug doses, or cause undesirable effects elsewhere in the body.

Topical administration is useful, but very few agents achieve therapeutic concentrations in the vitreous humor or the retina. Hence, intravitreal injection has become a commonly used route for administration of compounds targeting the retina. Although this route is effective, it is invasive and repeated interventions carry a high burden.[3,9,24]

In addition to the retinal drug delivery challenge, there are very few new targets and mechanisms validated. This is especially significant given that there are very few targets that play an etiologic role in the neuroglia-vascular DR pathology comprising inflammatory, edema, and neuronal death.[17] Here, we demonstrate that acute subconjunctival administration of THX-B achieved concentrations in the retina sufficient to reduce edema, to decrease the production of retinal inflammatory cytokines TNFα and α2M, and to preserve the neuronal retinal structure for prolonged periods of time. The benefit is comparable to or better than IVT injection of THX-B.

We could not find relevant documentation regarding the concentrations of small molecule drugs to treat posterior-segment pathologies, using the SCJ route. However, detection of agents in the vitreous humor after SCJ delivery has been reported as early as 15 to 30 minutes.[3] Data documenting fast kinetics and accumulation of agents in the aqueous and vitreous following SCJ injection could explain the rapid entry of THX-B into the retina. For example, an SCJ injection of mannitol achieves maximum concentration in the aqueous and in the vitreous between 30 and 60 minutes, and disappears from the vitreous after 3 hours.[9] In humans, the maximum dexamethasone concentration in aqueous humor was detected at 2.5 hours after SCJ injection and in the vitreous at 3 hours.[7] A study using AlexaFluor 647–labeled pRNA nanoparticles reached the retina 6 hours after SCJ delivery.[25] The large and accessible surface area of the sclera and high degree of hydration and relatively absence of cells[5,26] may facilitate the diffusion of small water-soluble molecules such as THX-B.

The chemical properties of THX-B would account for its fast diffusion from the vitreous to the retina. Based on these data, we hypothesize that this may be the route for subconjunctival THX-B. Even if a much faster delivery of the drug to the retinal tissue in case of IVT administration is expected to occur, both routes have comparable efficiency to target the retina.

Regarding drug elimination, compounds delivered by IVT and SCJ injections can be cleared quickly. Upon IVT administration, agents can be cleared by diffusion or filtration to the aqueous humor, or by permeation across the BRB due to passive or active transport.[5] Upon SCJ administration, agents can be cleared from the depot by diffusion into surrounding tissues and also by rapid elimination via lymphatic and blood vessels of the conjunctiva.[5,6,26,27]

We would postulate that THX-B rapidly reaches the retina most likely by direct transcleral diffusion.[7] The sclera has a large and accessible area and it is highly hydrated, which renders it conducive to water-soluble or amphipathic agent such as THX-B. Moreover, the sclera has few proteolytic enzymes or protein-binding sites that can degrade or sequester compounds.[3] The absorption of the SCJ drug into the systemic circulation and then recirculation back to the retina is highly unlikely given the relatively low doses administered, the large volume of distribution that would result in this case, and the fact that after SCJ delivery we did not detect THX-B in circulation to a quantitative limit of detection of 5 ng.

Remarkably, acute delivery of THX-B at 2.5 weeks after the onset of diabetes affords a long-term therapeutic effect for multiple pathologic endpoints of DR, lasting up to 10 weeks, even though the THX-B is cleared from the SCJ depot and the retina. Most likely, inhibition of the multifactorial activities of $p75^{NTR}$ resets the retinal environment to homeostasis.

A therapeutic effect of $p75^{NTR}$ inhibition has been reported after IVT delivery in DR[17] and in experimental glaucoma,[18] optic nerve axotomy.[19] Hence, it will be important to test the newly reported SCJ delivery route for $p75^{NTR}$ antagonists in glaucoma, retinitis pigmentosa, AMD, and other chronic retinal diseases that require long-lasting therapeutic efficacy with reduced frequency of intervention.

In sum, our experimental observations demonstrate the efficacy of subconjunctival THX-B delivery, and support the view that $p75^{NTR}$ antagonists may be novel therapeutics with a wide spectrum of action. Subconjunctival delivery of $p75^{NTR}$ inhibiting agents that yield long-lasting therapeutic benefits represent a less-invasive approach to modify disease through a promising novel target.

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