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17 β -Estradiol eye drops protect the retinal ganglion cell layer and preserve visual function in an *in vivo* model of glaucoma

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Abstract

Neuroprotection in glaucoma as a curative strategy complementary to current therapies to lower intraocular pressure (IOP) is highly desirable. This study was designed to investigate neuroprotection by 17 β -estradiol (E2) to prevent retinal ganglion cell (RGC) death in a glaucoma model of surgically elevated IOP in rats. We found that daily treatment with E2 containing eye drops resulted in significant E2 concentration in the retina with concomitant profound neuroprotective therapeutic benefits, even in the presence of continually elevated IOP. The number of apoptotic cells in the RGC layer was significantly decreased in the E2-treated group, when compared to the vehicle-treated controls. Deterioration in visual acuity in these animals was also markedly prevented. Using mass spectrometry-based proteomics, beneficial changes in the expression of several proteins implicated in the maintenance of retinal health were also found in the retina of E2-treated animals. On the other hand, systemic side-effects could not be avoided with the eye drops, as confirmed by the measured high circulating estrogen levels and through the assessment of the uterus representing a typical hormone-sensitive peripheral organ. Collectively, the demonstrated significant neuroprotective effect of topical E2 in the selected animal model of glaucoma provides a clear rationale for further studies aiming at targeting E2 into the eye while avoiding systemic E2 exposure to diminish undesirable off target side-effects.

Keywords

17 β -estradiol; eye drop; glaucomatous retinopathy; rat model; estrogen quantitation; retina proteomics; neuroprotection; visual acuity; contrast sensitivity; LC-MS/MS

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INTRODUCTION

Glaucoma, the second leading cause of blindness worldwide,¹ is a group of complex and slow progressing neurodegenerative diseases characterized by the gradual loss of retinal ganglion cells (RGC) and their axons.² While elevated intraocular pressure (IOP) is a major risk factor for developing the disease, visual field loss and RGC death also occur in cases where IOP is controlled or normal.^{3,4} Therefore, and since lowering elevated IOP is currently the only available treatment target for glaucoma, complementary strategies independent of IOP lowering are also urgently needed to manage the associated ocular pathology.

Due to many mechanistic properties qualifying glaucoma as a neurodegenerative disease and similarities to other chronic degenerative diseases affecting the brain,⁵⁻⁸ pursuing neuroprotection as a therapeutic target in glaucoma is a convincing and rational strategy.^{2-4,9,10} With this approach the goal is not only to prevent or slow down neuronal cell death, but also to preserve neuronal functions. In the context of glaucoma, neuroprotective approaches that are capable of successfully promoting RGC survival are needed. While our understanding of retinal pathophysiology involving glaucomatous neurodegeneration is incomplete due to the multi factorial nature of the disease, excitotoxicity, inflammation, oxidative stress and mitochondrial dysfunction have been implicated, among others, as potential factors contributing to the initiation and progression of the disease.¹¹⁻¹⁴ Development of multifunctional neuroprotective agents to impact multiple mechanisms in a single drug delivery and therapy approach would, therefore, be of great clinical value.¹⁵ This type of agents would also decrease undesirable drug-drug interactions and lessen disease burden, thereby improving the well-known compliance issues associated with currently available glaucoma medications.¹⁶

Several lines of evidence have demonstrated that the most potent human estrogen, 17 β -estradiol (E2, Figure 1), elicits broad spectrum neuroprotection in various *in vitro* and *in vivo* paradigms due to its pleiotropic genomic and non genomic actions.^{17,18} As such, E2 is capable of protecting neurons *via* a variety of mechanisms; e.g., by interfering with cellular death signaling cascades, altering neurotransmitters levels, scavenging free radicals, promoting synaptic plasticity as well as preventing axonal and dendritic pruning. Beside basic science studies, epidemiological observations also argue for the neuroprotective role of endogenous estrogens.^{19,20}

E2 has been shown to be beneficial for the health of various ocular structures.²¹⁻²⁴ These findings are not surprising considering that estrogen receptors (ER α and β) are abundantly expressed throughout the eye, and in particular in the retina.^{25,26} In animal models of various retinal diseases, E2 treatments have elicited protection of RGCs, photoreceptor cells, optic nerve and retinal pigment epithelium.²⁷⁻²⁹ The attenuation of osmotic swelling of RGCs³⁰ and of the breakdown of the blood-retina barrier³¹ by this steroid has also been reported. Additionally, a very recent clinical review specifically discusses detrimental consequences of estrogen deficiency after menopause as a causative factor for the increased susceptibility to glaucomatous damage with age.³²

Treatment regimens using topical administration are the current standard of care in clinical glaucoma therapy.¹⁶ E2's significant lipophilicity and, thus, extremely low water-solubility do not allow for its direct formulation in aqueous solution. Cyclodextrins have gained attention when difficulties in drug formulation of lipophilic agents arise.^{33,34} Complexation with a cyclodextrin not only allows for formulations of poorly water-soluble agents in aqueous vehicles, but also offers specific advantages in terms of improving transcorneal penetration and reducing corneal irritation seen with other excipients.³⁴ In particular, a

chemically modified cyclodextrin, hydroxypropyl- β -cyclodextrin (HPBCD), has frequently been used due to its favorable toxicity profile and enhanced water-solubility compared to the unmodified cyclic oligosaccharide.³⁵

The goal of the present study was to determine for the first time the effectiveness of topical E2 for alleviating RGC death by measuring functional effects resulting from neuroprotection in a surgically induced *in vivo* model of glaucomatous retinopathy³⁶. We specifically focused on monitoring the attenuation of losses in visual acuity and contrast sensitivity upon exposing the animals to a daily E2 eye drop dosing regimen. By using ovariectomized (OVX) rats lacking significant endogenous E2 sources, additional mechanistic and pharmacokinetic information were also derived; we measured E2 levels by a validated liquid chromatography–tandem mass spectrometry (LC-MS/MS) assay³⁷ in the retina, blood and uterus. The latter represents a typical E2-sensitive peripheral organ that respond with rapid structural changes after exposure to E2. Changes in retinal protein expression were also recognized as a potential mechanistic outcome for E2's neuroprotection in the retina using MS-based proteomics.³⁸

EXPERIMENTAL SECTION

Materials

17 β -estradiol (E2) was purchased from Steroids, Inc. (New Port, RI). ¹³C-labeled E2-(¹³C₆ E2) with an isotopic purity of 99% was purchased from Cambridge Isotope Laboratories (Andover, MA, USA). All other chemicals were obtained from Sigma Chemical Company (St. Louis, MO, USA), unless otherwise noted. The solvents were of analytical grade. Formulation of E2 for eye drops was done in 20% w/v HPBCD in saline. E2 was applied in 0.05% (w/v) solution.

Animals

OVX adult Brown Norway rats weighing 200-250 g were obtained from Charles Rivers Laboratories (Wilmington, DE, USA). Animals were kept under standard 12-h light/12-h dark cycle and room temperature was maintained at 21 °C. Animals had full access to standard diet and water. Rats were treated according to institutional animal care and use guidelines.

Topical E2 Treatment Protocol

For E2 quantitation and retina proteomics studies, nine animals per group received 10 μ L of E2 eye drops once daily (*q.d.*) in both eyes for 19 days. Dosing started after IOP was stably elevated at 10 days; 24 h after the last topical treatment concluding a 30 day observational period, *in vivo* studies specified below were conducted. The control group received the vehicle (20% w/v HPBCD in saline) only. After 24 h of the last treatment animals were euthanized by CO₂ overexposure. Blood was drawn through intracardiac puncture into heparinized tubes; clotted on ice and spun at 3000 rpm for 20 min to obtain serum samples. The eyes were immediately enucleated and processed; cornea, lens and retina were isolated, rinsed with saline, blotted dry and weighed. The brain and uterus were also collected. For E2 quantitation by LC-MS/MS, tissues from six animals were used. One retina per animal was used for the proteomic survey and three retina samples were processed from each treatment group. For neuroprotection studies using a rat model of surgically elevated intraocular pressure (IOP),³⁶ after stabilization of the elevated IOP in twelve rats, animals were randomly divided into 3 groups (surgery control, vehicle control and E2-treatment, n=4). The control group was treated with the vehicle only and the treatment group received 10 μ L of E2 eye drops into the ipsilateral eye, *q.d.*, for 19 days, analogously to those of drug distribution/proteomics studies.

Sample Preparation for E2 Quantitation

Tissues were frozen by a BioSqueezer (Biospec Products, Bartlesville, OK) and, then, ground up in a Cryo-Cup Grinder (Biospec Products, Bartlesville, OK, USA). From the collected frozen powders, 20% (w/v) homogenates were made in pH 7.4 phosphate buffer. The homogenates were spiked with 100 pg internal standard (IS, $^{13}\text{C}_6\text{-E2}$), diluted two fold with saline and extracted twice with 2 volumes of methyl *tert* butyl ether. The organic layers obtained from the liquid liquid extraction were removed, transferred to reacti-vials (Supelco, Bellefonte, PA, USA) and evaporated under a nitrogen stream to yield samples for derivatization for subsequent LC-MS/MS analysis. Derivatization was done by dansyl chloride (Dns-Cl, 30 μL of 1 mg/mL in acetonitrile) and 20 μL of sodium bicarbonate buffer (100 mM, pH 10.5) according to our recently reported method.³⁷ The samples were vortexed and then incubated in a heating block at 60 °C for 10 min. Thereafter, the samples were centrifuged at 14,500 rpm for 3 min, transferred to autosampler vials, sealed, and assayed by LC-MS/MS. Serum (100 μL) obtained from blood was extracted analogously.

LC-MS/MS Analysis

E2 levels in sera and tissues were measured by a validated LC-MS/MS assay applying isotope-dilution quantification.³⁷ The assay was performed on a TSQ Quantum Ultra triple-quadrupole instrument operated in positive ion mode and interfaced via a heated electrospray ionization (H-ESI) probe to a Surveyor LC System (Thermo Electron Corporation, Trace Chemical Analysis, Austin, TX, USA). Separations were carried out on a Phenomenex (Torrance, CA, USA) Kinetex Phenyl Hexyl column (50 \times 2.1 mm i.d., 2.6 μm “core shell” particles) using gradient elution with a flow rate of 0.4 mL/min. The eluent system consisted of (A) 0.1% (v/v) formic acid in water and (B) 0.1% (v/v) formic acid in acetonitrile. The eluent composition was initially set at 50% B and was linearly increased after sample injection to 87% B in 6 min. Then the column was flushed with 100% B for 1.8 min; and finally, was equilibrated with 50% B for 3 min. The autosampler injection volume was 5 μL . H-ESI spray voltage, H-ESI temperature, and capillary temperature were maintained at 3.5 kV, 350 °C, and 325 °C, respectively. Selected reaction monitoring (SRM) with unit mass resolution for the precursor and product ions was used for quantification. SRM transitions of m/z 506 \rightarrow 171, and 512 \rightarrow 171 were set up for Dns-E2, and Dns- $^{13}\text{C}_6\text{-E2}$, respectively. Data acquisition and processing were controlled by the XCalibur software (version 2.1) of the instrument.

Mass Spectrometry-Based Retina Proteomics

The extracted proteins were digested with trypsin and analyzed by data-dependent nanoflow liquid chromatography coupled with electrospray ionization mass spectrometry and tandem mass spectrometry (LC-ESI-MS/MS) according to a previously published protocol.³⁸ Separation was performed on an Eksigent nano LC-2D system (Dublin, CA, USA) using a 2.5 cm \times 75 μm i.d. IntegraFrit™ sample trap (New Objective, Woburn, MA, USA) and a 15 cm \times 75 μm i.d. PepMap C18 column (LC Packings, Dionex, San Francisco, CA, USA). The mobile phase for gradient elution was mixed from solvent A [0.1% acetic acid and 99.9% water (v/v)] and solvent B [0.1% acetic acid and 99.9% acetonitrile (v/v)] raising solvent B from 5% to 40% in 90 min after keeping the initial composition for 5 min during sample injection. Injection volume and flow rates were 5 μL and 250 nL/min, respectively. The effluent from the PepMap column was analyzed by data-dependent ESI MS/MS on a hybrid linear ion trap-Fourier transform ion cyclotron resonance mass spectrometer (LTQ-FT, Thermo Fisher, San Jose, CA, USA) equipped with the manufacturer’s nanoelectrospray ionization source and operated with the Xcalibur (version 2.0) and Tune Plus (version 2.2) data acquisition programs. After searching a protein database (SwissProt IPI, *Rattus norvegicus*) using the Mascot software (Matrix Science, Boston, MA), differentially

expressed proteins were identified *via* spectral counting option of Scaffold (Proteome Software, Portland, OR, USA) followed by summation-based G-tests, as described earlier.³⁸

Rat Model of Glaucoma and IOP Measurements

The procedure that was used to elevate the rat IOP was described previously.^{36,39} Briefly, surgery for IOP elevation was performed on anesthetized animals (1 mL/kg body weight *i.p.* injection of a standard rat anesthesia cocktail: mixture of ketamine at 5 mL of 100 mg/mL, xylazine at 0.5 mL of 100 mg/mL, acepromazine at 1 mL of 10 mg/mL, and 0.5 mL of water). One eye (left side) of each animal was used for the IOP elevation. 50 μ L of a sterile 1.8 M hypertonic saline solution was injected into the episcleral vein with a micro glass needle connected to an injection pump. After stabilization of IOP elevation to 25% above that of the contralateral control eye, IOP measurements were taken with a Tono-Pen XL tonometer (Mentor, Norwell, MA, USA) on conscious animals in the presence of topical anesthesia with eye drops containing proparacaine (0.1%, w/v) twice a week for up to 30 days post-surgery. Following the experimentation period, rats were euthanized by an *i.p.* injection of pentobarbital (120 mg/kg body weight).

Behavioral Testing of Rat Vision and Visual Acuity

Visual performance of rats was measured non-invasively by trained observers blinded to the identity of the individual experimental groups and using a behavioral assay determining the animal's optomotor reflex response to a moving visual stimulus.⁴⁰ Animals exhibit tracking behavior by following the rotating visual stimulus with small head movements in the direction of the rotation. This behavior was quantified with a computerized system that collects visual acuity and contrast sensitivity data (OptoMotry; CerebralMechanics, Lethbride, Alberta, Canada) as described previously.⁴¹⁻⁴⁴ Ipsi- and contralateral eyes were assessed independently by means of changing the direction of the rotating stimulus pattern while the contralateral eye served as an intrinsic control.^{41,42}

TUNEL Assay for Detection of Apoptosis in *Ex Vivo* Retina Material

After the behavioral testing of visual performance, animals were euthanized. Eyes were dissected and immersion-fixed in 4% paraformaldehyde overnight at 4 °C. Subsequently, after three washes in PBS, eyes were embedded in OCT compound (Sakura Finetek USA Inc., Torrance, CA, USA) and were sectioned vertically at 12 μ m thickness on a cryostat microtome.⁴⁵ Apoptotic cells in eye sections were visualized with the DeadEnd Fluorometric TUNEL System (Promega, Madison, WI, USA) following the manufacturer's instructions. Sections were mounted on cover slips with Prolong Gold antifade reagent mounting medium containing 1.5 μ g/mL DAPI (Molecular Probes, Eugene, OR, USA). Negative controls included omission of the TdT enzyme incubation, while positive controls were performed using DNase I incubation on the sections. Fluorescently labeled, TUNEL-positive cells were identified using standard epi-fluorescence microscopy, digital microphotography and quantitative stereotaxis to determine the number of TUNEL-positive cells in the ganglion cell layer (GCL) as stained profile counts (SimplePCI, Compix Inc., Image Systems, Sewickley, PA, USA). The TUNEL assay was chosen to identify late-stage apoptotic cells in the GCL as the most conservative assessment of cell death with apoptosis being the predominant form of cell death in the RGC.⁴⁶ Specifically, this enzyme-based assessment of cellular commitment to the final stage of programmed cell death generates an extremely reliable assay for complex tissues when combined with markers for nucleic acids as shown by Kelly *et al.*⁴⁷

Statistical Analysis

Descriptive statistics were calculated for each group and for all outcomes. Unless otherwise noted, determination of the difference between mean values for each experimental group was assessed by Student's *t*-test or with standard one-way ANOVA followed by *post hoc* Dunnett's test for significant differences among groups, with $p < 0.05$ considered statistically significant.

RESULTS AND DISCUSSION

Topical Ocular Delivery of E2 into the Retina of OVX Rats

E2 quantifications with isotope dilutions and SRM were performed using a validated LC-MS/MS assay after derivatization with dansyl chloride. We used $^{13}\text{C}_6$ -labeled E2 as the internal standard (IS)³⁷ to avoid a potential matrix effect arising from the differential suppression or enhancement of ionization upon the use of a traditionally applied deuterium-labeled E2-IS.⁴⁸ The analytical method employed also permitted measuring the very low E2 contents in the corresponding OVX control eye tissues. E2 formation in the eye, and prominently in the retina, has been proposed previously,⁴⁹ but to our knowledge, our study is the first to report the actual endogenous E2 contents in the OVX rat eye that were obtained *via* a reliable and sensitive LC-MS/MS assay.³⁷ In Figure 2, we show representative SRM chromatograms of retina extracts from the OVX control (Figure 2A) and the E2-treated (Figure 2B) groups. In animals exposed to daily E2 eye drop treatments, we measured high E2 concentrations (ng/g range), as shown in Table 1. In particular, we detected about 20 ng/g E2 in the retina and, expectedly, a significant quantity of E2 was also present in other selected eye segments, such as the lens and the cornea.

Mass Spectrometry-Based Retina Proteomics

As shown in Figure 3, proteomic analyses not only supported data shown in Table 1 for retinal delivery of E2, but they also demonstrated that E2 affected the expression of various retinal proteins that could be linked to important retinal functions with potential implications to neuroprotective ocular drug therapy. In particular, αA - and βB -crystallins⁵⁰ have been linked to the survival of RGCs after optic nerve axotomy⁵¹, a model of glaucoma-mediated injury. In addition, increased oxidative stress-induced retinal degeneration has been associated with decreased levels of α -crystallin expression.⁵² Upregulation of a specific isoform of this protein (αA crystallin (Figure 3), has also been obtained upon intravitreal injection of E2,⁵³ which further supports distribution of the hormone into the retina after topical application as eye drops in our study. CRYAA is a small heat shock protein with chaperone-like activity that prevents the aggregation of denatured or unfolded proteins.⁵⁰ CRYAA is involved in cytoskeleton remodeling, which inhibits apoptosis and enhances cellular resistance to stress. Altogether, upregulation of α -crystallins by E2 treatment should have RGC-protective effects. We also obtained an upregulation of β -crystallin b2(CRYBB2), which has been proposed as a novel class of neurite-promoting factors likely to operate through an autocrine mechanism and involved in the elongation of axons during retinal regeneration.⁵⁴

This identification of potential RGC-protective effects mediated by an upregulation of crystallines was mirrored by the upregulation of another group of proteins: E2 dosing also resulted in the upregulation of histones (Figure 3). An increased availability of histones for histone-dependent processes such as eu- and heterochromatin remodeling has been implicated in the proper function of diurnally regulated retinal activity and anti-apoptotic processes.^{55,57} Therefore, E2 may also exert positive effects by this mechanism, which is different from but potentially synergistic with the crystalline-mediated protection. The same analyses also identified critical molecules in the outer retina being modulated by the delivery

of E2 to the retina. Specifically, we observed a hitherto not reported E2-induced downregulation of rhodopsin (RHO) and of a rod outer segment membrane protein (ROM1) (Figure 3). RHO expression levels affect rod outer segment morphology and the kinetics of the light response,⁵⁸ and lower concentrations of RHO may lead to expanded disk membrane incisures that promote the longitudinal diffusion of soluble substances such as cGMP and Ca²⁺ and thereby disturb the gain and reproducibility of the single photon response.⁵⁹ ROM1 may function as an adhesion molecule involved in stabilization and compaction of outer segment disks and, therefore, it is essential for rod photoreceptor viability and disk morphogenesis.⁶⁰ On the other hand, a reduction in creatine kinase levels (Figure 3) may be an indicator of improved normal metabolic function and a decreased need for supplementary metabolic pathways typically activated by cellular and oxidative stress.⁶¹ Altogether, tissue proteomics provided additional evidence that a physiologically and potentially also therapeutically relevant amount of E2 reached the back of the eye after administration in eye drops thereby inducing significant changes in retinal protein expression with relevance to viability of retinal neurons.

Topical E2 Treatment in the Morrison Rat Model of Glaucoma

After establishing that the above treatment regimen allowed the generation of a significant E2 concentration in the retina (Table 1) with a concomitant profound change in retinal protein expression linked to signaling pathways relevant for neuroprotection (Figure 3), the functional consequences of these findings on RGC viability were investigated in a widely accepted rat model of glaucoma in which aqueous humor outflow is partially inhibited by surgical means to produce a gradually elevated IOP.³⁶

As shown in Figure 4, a consistent IOP elevation was seen in the ipsilateral eyes when compared to contralateral eyes that served as untreated controls and it remained elevated ipsilaterally for up to 30 days. Average baseline IOP (2 days before induction of IOP elevation) was 19 ± 3 mmHg, however, post surgery, average IOP in the injected ipsilateral eyes was 29 ± 4 mmHg for the duration of up to 19 days (Figure 4). Neither E2 nor vehicle treatment significantly altered IOP in comparison to the untreated contralateral eye when the animals received 10 μ L of E2 or vehicle eye drops, *q.d.* for 19 days, analogous to the experiments on E2 quantitation in ocular tissues and retina proteomics (Table 1 and Figure 3, respectively). Altogether, the expected consistent IOP elevation in the ipsilateral eye in the rat model,³⁶ was not significantly influenced by topical E2-treatment. Since our study aimed at investigating the neuroprotective potential of topical E2 in this glaucoma model, the finding that E2 does not ameliorate IOP is not a contraindication in the present context. It has been strongly indicated, that preservation of neuronal viability in the glaucomatous retina is needed independently from the management of the IOP, as neurodegeneration may also be significant when IOP is normal or controlled.^{3,4,7,9}

E2 Treatment Significantly Reduces Apoptotic Cell Death in the Ganglion Cell Layer

Apoptotic cells in sections from eyes with elevated IOP and treated with either the vehicle or E2 eye drops were detected by TUNEL staining. TUNEL histochemistry shows that in normotensive, contralateral controls the number of apoptotic cells in the ganglion cell layer (GCL) is extremely low (Figure 5A), was elevated in the vehicle-treated ipsilateral group that experienced elevated IOP (Figure 5B) and was significantly reduced in the E2-treated group when compared to vehicle (Figure 5C). Quantification of TUNEL-positive cells in the GCL (Figure 5D) confirmed significantly reduced apoptosis in the GCL of the E2-treated groups compared to the vehicle treated group. While RGC loss in the vehicle treated group was $34 \pm 5\%$, in the E2-treated animals only $19 \pm 6\%$ of RGCs underwent cell death. Accordingly, approximately 50% less RGC death was detected in the E2 treatment group, than without the neuroprotective intervention (i.e., with vehicle only). Interestingly, the

present study also indicates that the number of apoptotic cells in the GCL was small even in the vehicle treated group. This is potentially caused by the fact that the TUNEL assay used to detect apoptosis assayed only one time point at the end of the experimental regimen and the likely possibility exists that RGC undergoing cell death might have already passed or not reached detectability by TUNEL labeling system.

Behavioral Testing of Rat Visual Performance

In the animal model of glaucoma generated by surgically induced elevated IOP,³⁶ we also measured the functional consequences of the neuroprotective effect on RGC degeneration elicited by topical E2 treatments (Figure 5). Using the OptoMotry system developed by Prusky *et al.*,⁴² we determined the animals' visual performance by testing their optomotor response to moving visual stimuli. Figure 6 shows, that topical delivery of E2 via eye drops significantly preserved visual function and prevented loss of vision, measured as contrast sensitivity at a given spatial frequency when compared to vehicle control. This E2-mediated profound preservation of contrast sensitivity, leading to better visual function when compared to vehicle controls mirrors our histological quantitative measurements of RGC degeneration (Figure 5).

Off-Target Peripheral Effects of Topical E2 Treatments

Even subchronic systemic administration of E2 produces undesirable and harmful peripheral hormonal exposure.⁶² Therefore, it is imperative to address this issue when E2 is used as eye drops, considering that a significant portion of drug will end up in the circulatory and gastrointestinal system and therefore the periphery.⁶³ In Table 2 we show that the circulating E2 level in the hormone treated group was significantly higher than in the OVX control group (463 ± 29 versus 9 ± 3 pg/mL). Due to this substantial peripheral hormonal burden, we also observed a significant uterotrophic effect in the treatment group. The uterus is one of the most estrogen-sensitive organs in the periphery; therefore, its weight gain due to fluid imbibition triggered by exogenously administered estrogenic compounds to OVX animals has been a frequently used marker for the presence of estrogens in the circulation.⁶⁴ Indeed, we found that the wet uterus weight in the E2 group was approximately five-times higher than in the control group, indicating that a significant uterine stimulation was brought about by E2 eye drop treatments. Previously we have shown⁶⁵ that the uterotrophic effect triggered by high levels of circulating E2 also results in significantly increased E2 content in the uterus that may be directly responsible for the increased risk for developing endometrial cancer.

Altogether, our data shown in Table 2 raise the concern that, even though significant RGC protection was provided against IOP elevation-induced neurodegeneration (Figure 5) with concomitantly high functional protection (Figure 6), undesirable hormonal peripheral side-effects could outweigh the benefits of topical E2 therapy. Therefore, appropriate strategies need to be developed for selective ocular delivery of the hormone to take advantage of its potent neuroprotective actions to protect the retina, while minimizing the observed and other potential side-effects. These efforts are especially relevant since a recent report⁶⁶ further hypothesized that E2 has the potential to contribute to the prevention of glaucoma in menopausal women.

CONCLUSIONS

The main finding of this study showed that E2 formulated as eye drops generated significant E2 concentration in the retina and elicited profound, structurally and functionally measurable neuroprotective effects in an established *in vivo* rat model of glaucoma. Using mass spectrometry-based proteomics, several proteins involved in retinal health and

potentially mediating E2 neuroprotection were identified. At the same time, our study also provides a clear rationale for the need to target E2 only to the retina to avoid systemic hormonal exposure and off-target side-effects.

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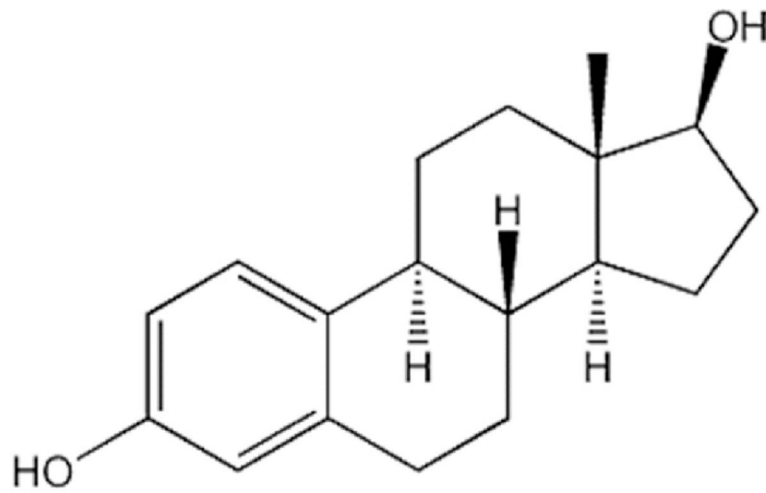


Figure 1.
Chemical structure of 17β-estradiol (E2).

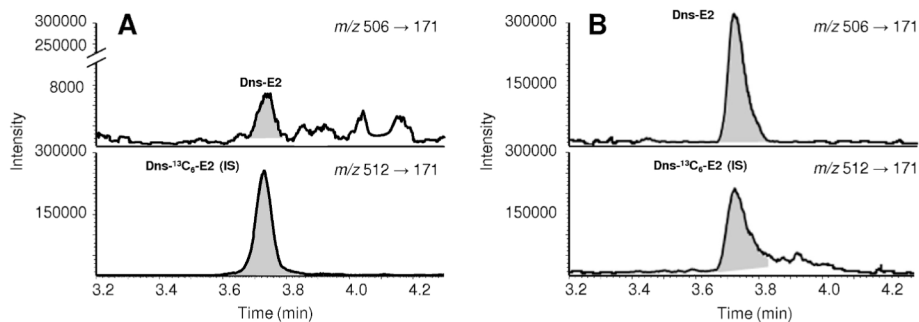


Figure 2. Representative LC-MS/MS selected reaction monitoring (SRM) traces of retinal extracts obtained from **A**) OVX rats after vehicle (20% w/v HPBCD in saline); and **B**) E2-eye drops (0.05% w/v) treatments, respectively (10 μ L, *q.d.*, for 19 days, n=6). Quantitation was done by isotope dilution using $^{13}\text{C}_6$ -E2 as internal standard (IS) and SRM (with transitions indicated) of the dansyl- (Dns-) derivatized analyte and IS.

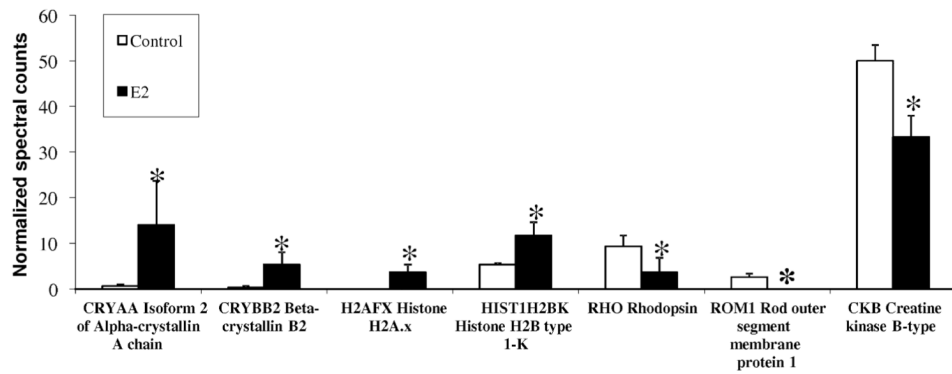


Figure 3.

Mass spectrometry-based retina proteomics. Normalized spectral counts obtained from data-dependent LC-MS/MS analyses are measures of protein abundance. Asterisks indicate statistically significant difference from control (summation-based G-test, $p < 0.05$; three biological replicates per group and two analytical replicates per sample). Several differentially expressed proteins (up- and downregulated compared to vehicle control) were found in the retina of OVX rats after 19-day *q.d.* treatments with E2 (0.05%, w/v in 20% w/v HPCD-containing saline vehicle).

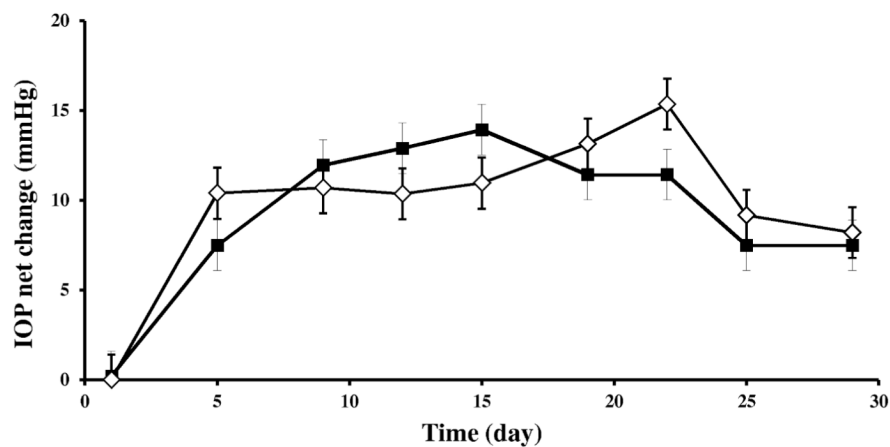


Figure 4. IOP changes in a rat model of glaucoma after vehicle (20% w/v, HPBCD in saline, \diamond) and E2 (0.05%, w/v in vehicle, \blacksquare) eye drop treatments (10 μ L, *q.d.*, for 19 days) in the ipsilateral eye of OVX rats. The contralateral eye served as untreated control. Each graph represents mean IOP values in mmHg \pm S.D. for net changes between the experimental (ipsilateral) eye and the control (contralateral) eye for four individual animals per group maintained for 30 days. Day zero was the day of initiation of IOP elevation.

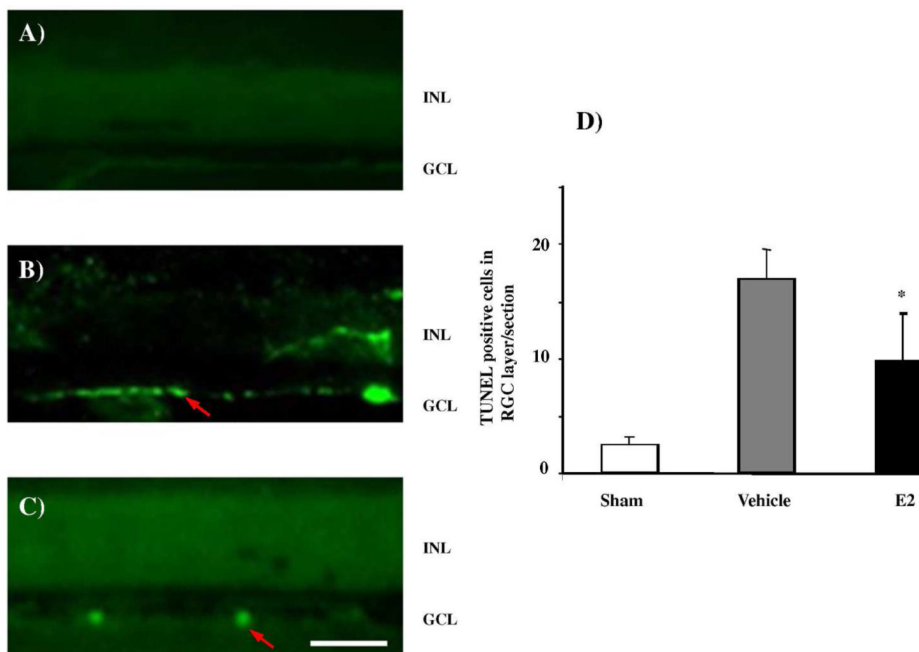


Figure 5. Fluorescence micrographs of 12 μm thick vertical cryo sections of rat eyes stained for TUNEL-positive cells. Arrows identify some representative TUNEL-positive cells in ganglion cell layer. (GCL). INL denotes inner nuclear layer. (A) No specific cellular staining and only fluorescence background was detected in the contralateral control (B) The number of TUNEL-positive cells in GCL (bright green fluorescence) increased in the vehicle treated rat eye, while fewer TUNEL-positive cells were found in the GCL of the E2-treated group (C); scale bar in (C) for (A) through (C), 50 μm . (D) Summary diagram of analyses of TUNEL-positive, apoptotic cells in the GCL per section of glaucomatous retina with and without E2 eye drop treatments (regimen specified in legends of Figures 2 – 4). Asterisk (*) indicates statistically significant difference from the vehicle treated group, $p < 0.05$, $n = 4$.

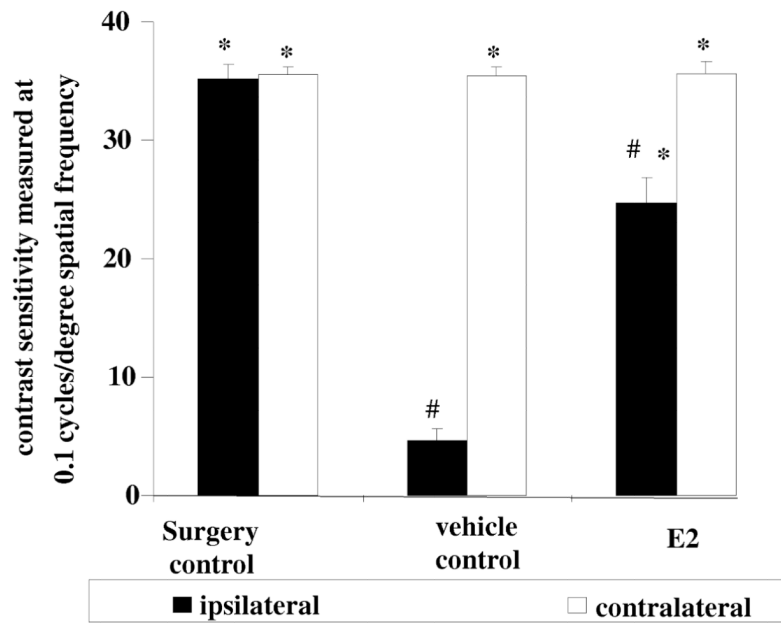


Figure 6.

Protective effect of E2 on visual function measured as contrast sensitivity at a given spatial frequency in the rat model of glaucoma. Either vehicle (20% w/v HPCD in saline) or E2 (0.5% w/v in vehicle) were administered topically as eye-drops for intervention treatments (same regimen as for Figures 2-5). Animals were tested prior to euthanasia and histological assessment. Contrast sensitivity was measured at a given spatial frequency for each eye individually and compared to the ipsilateral vehicle treated eye (*; $p < 0.05$, $n = 3$ per group) or to the sham surgery control (#; $p < 0.05$; $n = 3$ per group).

Table 1
E2 concentrations in selected eye compartments in OVX rats after E2 (0.05% w/v) or vehicle eye drop treatment (10 μ L, *q.d.* for 19 days)

Tissue	E2	
	Vehicle (control) (ng/g)	E2-treated (ng/g)
Retina	0.13 \pm 0.02	22 \pm 2 *
Cornea	0.40 \pm 0.01	78 \pm 12 *
Lens	0.03 \pm 0.003	3 \pm 1 *

^aData are expressed as mean \pm SEM for n=6.

* Significant difference compared to control, $p < 0.05$, n=6).

Table 2
Serum E2 concentration and uterotrophic effect in OVX rats after E2 (0.05% w/v) or vehicle eye drop treatments (10 μ L, *q.d.* for 19 days)

Treatment	Serum E2 (pg/mL) ^a	Uterus wet weight (mg) ^a
Vehicle (control)	9 \pm 3	83 \pm 2
E2	463 \pm 29 *	449 \pm 13 *

^aData are expressed as mean \pm SEM for n=6.

* Significant difference compared to control, p<0.05, n=6).