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Research paper

Retinoid x receptor modulation protects against ER stress response and rescues glaucoma phenotypes in adult mice

Yogita Dheer[a,*], Nitin Chitranshi[a], Veer Gupta[b], Samridhi Sharma[a], Kanishka Pushpitha[a], Mojdeh Abbasi[a], Mehdi Mirzaei[c], Yuyi You[d], Stuart L. Graham[a,d], Vivek Gupta[a,*]

[a] Faculty of Medicine and Health Sciences, Macquarie University, F10A, 2 Technology Place, North Ryde, NSW 2109, Australia
[b] School of Medicine, Deakin University, Melbourne, Australia
[c] Department of Molecular Science, Macquarie University, North Ryde, NSW 2109, Australia
[d] Save Sight Institute, Sydney University, Sydney, NSW 2000, Australia

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ABSTRACT

Retinoid X receptors (RXRs) play an important role in transcription, are involved in numerous cellular networks from cell proliferation to lipid metabolism and are essential for normal eye development. RXRs form homo or heterodimers with other nuclear receptors, bind to DNA response elements and regulate several biological processes including neurogenesis. Mounting evidence suggests that RXR activation by selective RXR modulators (sRXRms) may be neuroprotective in the central nervous system. However, their potential neuroprotective role in the retina and specifically in glaucoma remains unexplored. This study investigated changes in RXR expression in the human and mouse retina under glaucomatous stress conditions and investigated the effect of RXR modulation on the RGCs using pharmacological approaches. RXR protein levels in retina were downregulated in both human glaucoma and experimental RGC injury models while RXR agonist, bexarotene treatment resulted in upregulation of RXR expression particularly in the inner retinal layers. Retinal electrophysiological recordings and histological analysis indicated that inner retinal function and retinal laminar structure were preserved upon treatment with bexarotene. These protective effects were associated with downregulation of ER stress marker response upon bexarotene treatment under glaucoma conditions. Overall, retinal RXR modulation by bexarotene significantly protected RGCs in vivo in both acute and chronic glaucoma models.

1. Introduction

Glaucoma is the second most common cause of vision loss after cataract (Quigley and Broman, 2006; Sappington et al., 2010) and the disease is characterised by slow, progressive degeneration of retinal ganglion cells (RGCs) and optic nerve atrophy. Retinoid receptors play an important role in the course of eye development and are localised to specific cell types in the adult retina (Mori et al., 2001). RXR activation has been shown to be beneficial in rodent models of neurodegenerative disorders affecting the brain (Friling et al., 2009; Nam et al., 2016). However, the neuroprotective role of RXR activation on RGC in glaucoma has not been explored. RXR plays an important role in mediating the Docosahexaenoic acid (DHA) induced protection of photoreceptors and retinal pigment epithelium (Ayala-Pena et al., 2016; Shimazawa et al., 2009). RXR activation was found to protect retina pigment epithelial cells (RPE) from oxidative stress-induced apoptosis suggesting RXR agonists might be protective in the retina (Ayala-Pena et al., 2016). There are also reports indicating that RXR activation may be protective against amyloid beta (Aβ) toxicity (Bachmeier et al., 2013), mitochondrial dysfunction (Lee et al., 2015) and glutamate excitotoxicity (Mariani et al., 2017). All these factors are reported to be involved in RGC death in glaucoma.

RXRs belong to the superfamily of steroid/thyroid receptors, are involved in a number of cellular and metabolic processes, and are classified into α, β, and γ subtypes (Dawson and Xia, 2012). These can form both homodimer as well as heterodimer complexes with other nuclear receptors (NRs) making it a unique member of nuclear hormone receptor family that includes peroxisome proliferator-activated receptor (PPAR), liver-X-receptor (LXR), farnesoid X receptor (FXR), pregnane X receptor (PXR), retinoic acid receptor (RAR), vitamin D receptors (VDR), thyroid hormone receptor (TR) etc. (Forman et al., 1995; Kurokawa et al., 1993). This versatile role of RXR to serve as a common heterodimer partner for multiple NRs enables the pleiotropic effects of the receptor to be involved in several key cellular signalling

* Corresponding authors at: Faculty of Medicine and Health Sciences, 75, Talavera Road, Macquarie University, Sydney, NSW 2109, Australia.
E-mail addresses: yogita.dheer@hdr.mq.edu.au (Y. Dheer), vivek.gupta@mq.edu.au (V. Gupta).

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pathways (Ayala-Pena et al., 2016; Germain et al., 2006). RXRs also regulate gene expression by forming homodimers upon being activated by selective agonists.

The RXR agonist bexarotene is approved for the treatment of cutaneous T-cell lymphoma and more recently, therapeutically beneficial effects of the drug are being explored for metabolic disorders like diabetes, obesity, inflammatory disorders, atherosclerosis, and other cardiovascular diseases (Altucci et al., 2007; Claudel et al., 2001; Desreumaux et al., 2001). Various studies have also shown structure activity relationship for bexarotene and its analogs in cutaneous T-cell lymphoma (CTCL) (Atigadda et al., 2015; Jurutka et al., 2013; Wagner et al., 2009). Evidence from recent reports strongly suggests a neuroprotective role of the drug in other neurodegenerative diseases such as Parkinson's disease (McFarland et al., 2013), multiple sclerosis (Natrajan et al., 2015) and Alzheimer's disease (Crunkhorn, 2012). RXR activation by bexarotene is also a promising neuroprotective therapy for amyotrophic lateral sclerosis (ALS) as it delays motor neuron degeneration and maintains neuronal survival in transgenic SOD1G93A mice. Recently, RXR activation by bexarotene was reported to decrease neuronal hyperexcitability in mouse models of epilepsy (Bomben et al., 2014).

Recent in vitro studies demonstrated that bexarotene treatment protected neurons from glutamate-induced excitotoxicity (Huuskonen et al., 2016), Aβ induced endoplasmic reticulum (ER) stress and BAD (Bcl-2-associated death promoter) protein activation (Dheer et al., 2018). It is a highly selective synthetic retinoid which has high blood brain permeability and activates all the three isoforms of RXRs i.e. RXRα, RXRβ and RXRγ (Cramer et al., 2012; Dheer et al., 2018). Understanding of the role of RXRs in the retina is rather limited and the potential effects of RXR activation in glaucoma have not been previously investigated.

In this study, we sought to investigate the involvement of RXRs in the retina with particular focus on their role under glaucoma conditions. The effects of bexarotene treatment on RXR expression and other biochemical effects in the retina were studied. We probed the potential protective effects of RXR modulation within the retina utilising two different experimental models of RGC injury. A glutamate mediated increased excitotoxicity model was investigated to understand relatively acute effects of the RGC injury and a microbead injection model with chronically increased intraocular pressure (IOP) was utilised to study the IOP related effects. Our findings highlight that pharmacological modulators of RXR provide protection of the inner retinal function in glaucoma by suppressing the detrimental effect caused by ER stress. This study reports a novel molecular target suggesting RXR agonist might be beneficial therapeutic agent in treatment of glaucoma and possibly other neurodegenerative disorder.

2. Materials and methods

2.1. Chemicals

Primary antibodies anti-RXR α (5388S), anti-RXR β (8715S), anti-RXR γ (5629S) and anti-BAD (9268S) were purchased from cell signalling technology. Anti-GADD-153 (sc-7351), anti-p-PERK (sc-3257) antibodies were obtained from Santa Cruz Biotechnology, CA, USA. Anti-beta III tubulin (ab78078), anti-NeuN (ab104225) were obtained from Abcam, VIC, Australia. β-actin antibody (AC-40) and bexarotene were obtained from Sigma. All other chemicals and reagents were of analytical grade (Sigma or Invitrogen).

2.2. Ethics and Bexarotene treatment

The animal experimental procedures were performed in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and conformed to the ARVO guidelines for the Use of Animals in Ophthalmic and Vision Research. Male C57BL/6 mice from animal research centre, Perth Australia were used in this study. All the study was approved by the Animal Ethics Committee of Macquarie University, NSW, Australia. The animals were reared on a 12 h light /dark cycle with free access to water and diet. Prior to their use in experiments including injections and electrophysiological recordings, animals were anesthetized using cocktail of ketamine (75 mg/kg) and medetomidine (0.5 mg/kg) and anaesthesia reversal was achieved using atipamazole (1 mg/kg) at the end of the experiment. Bexarotene was initially dissolved in Dimethyl sulfoxide (DMSO) followed by saline (10% DMSO final concentration). Bexarotene (100 mg/kg) (Casali et al., 2018; Huuskonen et al., 2016; Mariani et al., 2017; Riancho et al., 2015; Tachibana et al., 2016; Washington and Burns, 2016) was administered by oral feeding using syringe to C57BL/6 mice once daily in drug treatment groups and continued for one week in glutamate model and for two weeks in microbead model of glaucoma. The dose selected for bexarotene was selected based on previous pharmacokinetic studies done by Landreth et al. using two different doses of bexarotene (25 mg/kg and 100 mg/kg) (Landreth et al., 2013). Bexarotene treatment was started at the same time as experimental stress was initiated. For human tissue analysis, the freshly frozen human donor eye samples were obtained from the Sydney Eye Bank, Australia. Samples of glaucoma eyes were compared with age matched control tissues. The ethics approval was obtained from the Macquarie University Human Research Ethics committee. The donor's age ranged 67–82 years with a mean of 74 years (SD ± 7.4 years). A history of primary open angle glaucoma was obtained from the donor's medical records. Subjects with neurodegenerative conditions, other ocular disorders or ocular surgery were excluded. Frozen eyes were allowed to thaw and washed with PBS and retinal tissues excised carefully under the surgical microscope (Carl Zeiss, Germany).

2.3. Animal models of glaucoma

A single intravitreal injection with volume of 1 μl solution containing glutamate (200 nmol) dissolved in saline (Schori et al., 2001) was performed into the vitreous chamber behind the lens. Intracameral injections of microbeads (Fluospheres, Molecular Probes, 10 μm) were made into the anterior chamber of eye avoiding needle contact with the iris or lens. The microbead injections were repeated every week for up to 2 months to bring about a sustainably increased IOP. Both glutamate and microbead injection were performed using Hamilton syringe with a 33 gauge needle under surgical microscope. IOP was measured by using a handheld electronic tonometer (Icare Tonovet, Helsinki, Finland) during anaesthesia prior to each injection and was the mean of six consecutive measurements. Three consecutive IOP readings were obtained from each eye and the average number was taken as the IOP.

2.4. Electroretinogram recordings

To assess the functional changes of RGCs, ERG recordings were done at the initial point to get the baseline data and at the final time point 7 days afterwards in glutamate model and after 2 months in microbead model. Animals were dark-adapted overnight and anesthetized as described earlier (Gupta et al., 2014). The body temperature of animals was maintained at 37 °C from the start of experiment till the final recovery. A total of three electrodes were used for ERG recordings: 1) Reference electrode-placed in the forehead of the animal 2) Ground electrode-placed in the tail of the animal. Both these electrodes were placed subcutaneously 3) Recording electrode (solid gold ring) - placed on the surface of each eye contacting cornea. All ERG recordings were done using a flash intensity of 3 log cd·s/m^2 (Ocusciences, Xenotec, Inc., USA) and for analysing both a-wave and b-wave amplitudes. The measurements were done from baseline to the a-wave trough and from the a-wave trough to the peak of b-wave for a-wave and b-wave amplitudes respectively. For the positive scotopic threshold responses (pSTRs) dim stimulation (−3.4 log cd·s/m^2) was delivered 30 times

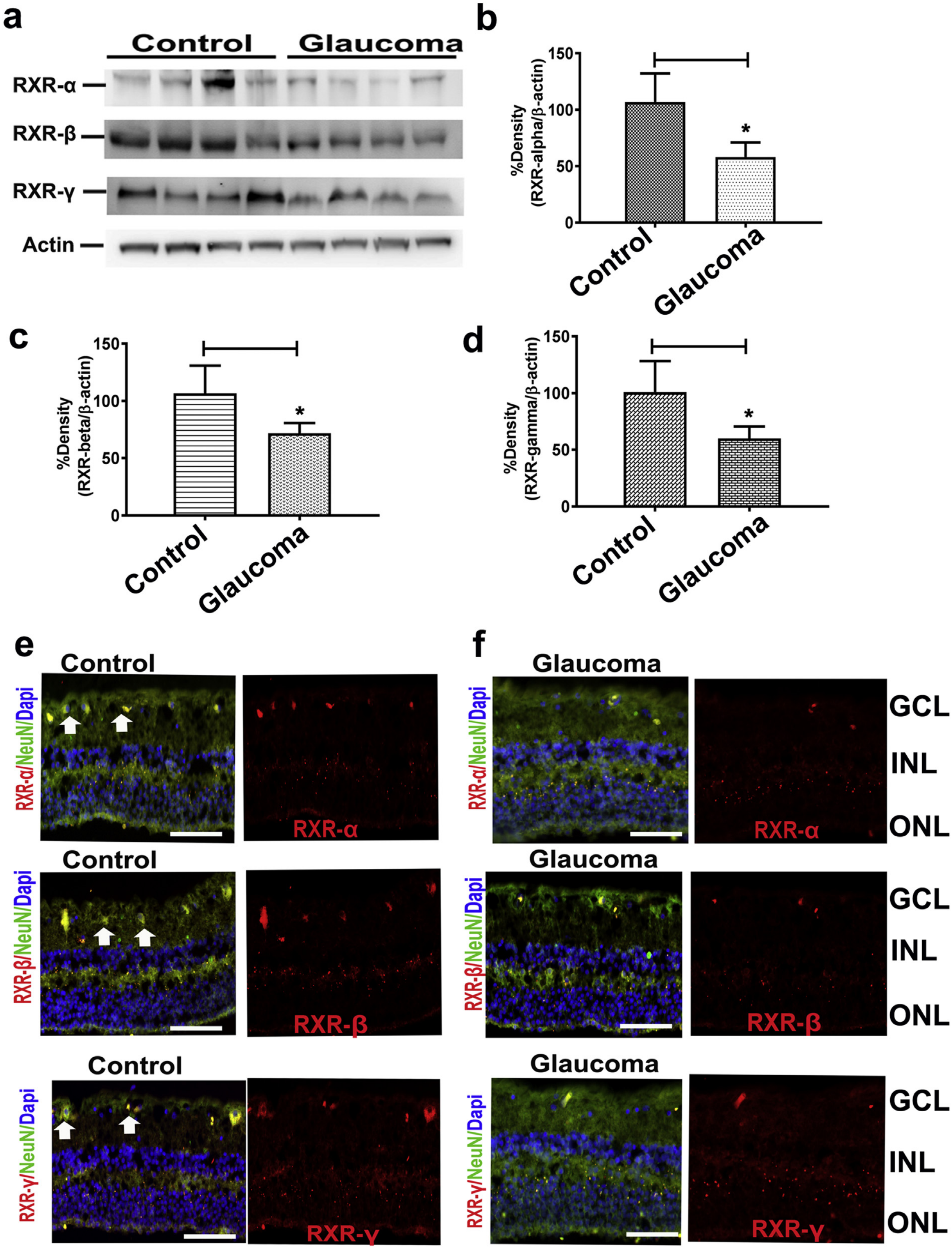

Fig. 1. Differential expression of RXR (α, β and γ) in control and glaucoma human retinas:
(a) The expression of RXRs (α, β and γ) in control and glaucoma human ONH lysates were detected by western blotting. (b), (c) and (d) Densitometric quantification of immunoblots relative to β-actin shows significant suppression of RXR expression in tissue lysates of glaucoma subjects as compared to the age-matched controls.*$p < .05$.
(e) and (f) Immunohistochemical images of control and glaucoma retinal sections stained with anti-RXR (α, β and γ) antibodies and anti-NeuN followed by secondary antibodies to detect RXR α, β and γ (red) and NeuN (green). Nuclei were stained with DAPI (blue). Scale bar = 50 μm. Human retinal sections from control subjects showed enhanced expression of RXR receptors as compared to glaucoma patients as indicated by white arrows. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

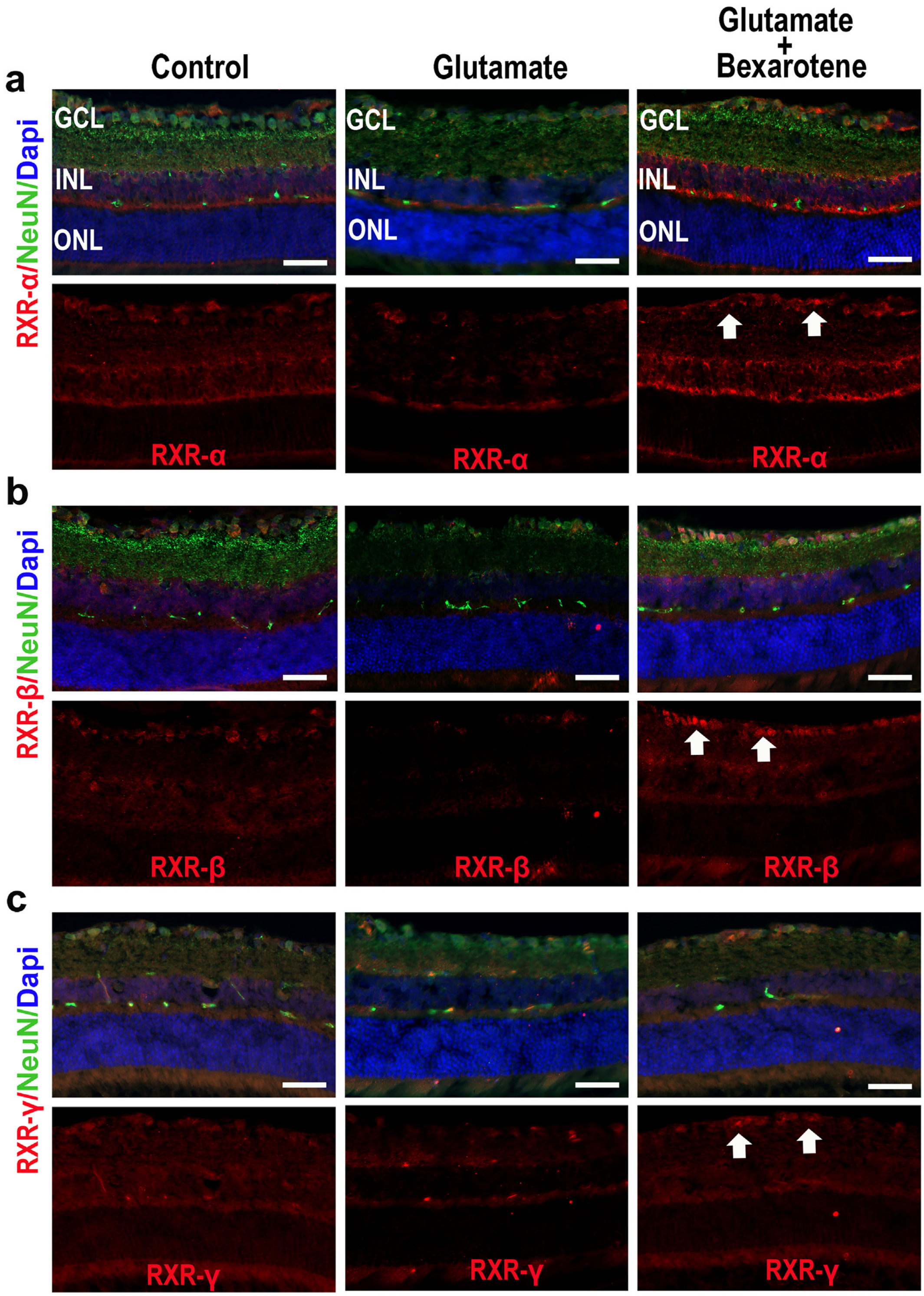

Fig. 2. Changes in expression of RXR (α, β, and γ) in control, glutamate and glutamate + bexarotene group:
(a), (b) and (c) Immunohistochemical images of mice retinal cryosections with anti-RXRs (α, β, and γ) and anti NeuN (neuronal marker) from different groups. Red colour and green colour indicates staining for RXR and NeuN respectively while blue nuclei is for DAPI; GCL, retinal ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. Microscopic images after immunostaining revealed that bexarotene treatment in mice exposed to intravitreal glutamate upregulates expression of all the three isoforms of RXR as compared to glutamate group. Scale bar = 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(0.5 Hz). For all positive STR responses, measurements were done from baseline to the positive peak observed around 120 ms as reported previously (Gupta et al., 2014; You et al., 2014).

2.5. Histology

Animals were euthanized with an overdose of intraperitoneal injection of sodium pentobarbitone (100 mg/kg Lethabarb, Virbac Pty, Australia) and then perfused transcardially with 4% paraformaldehyde

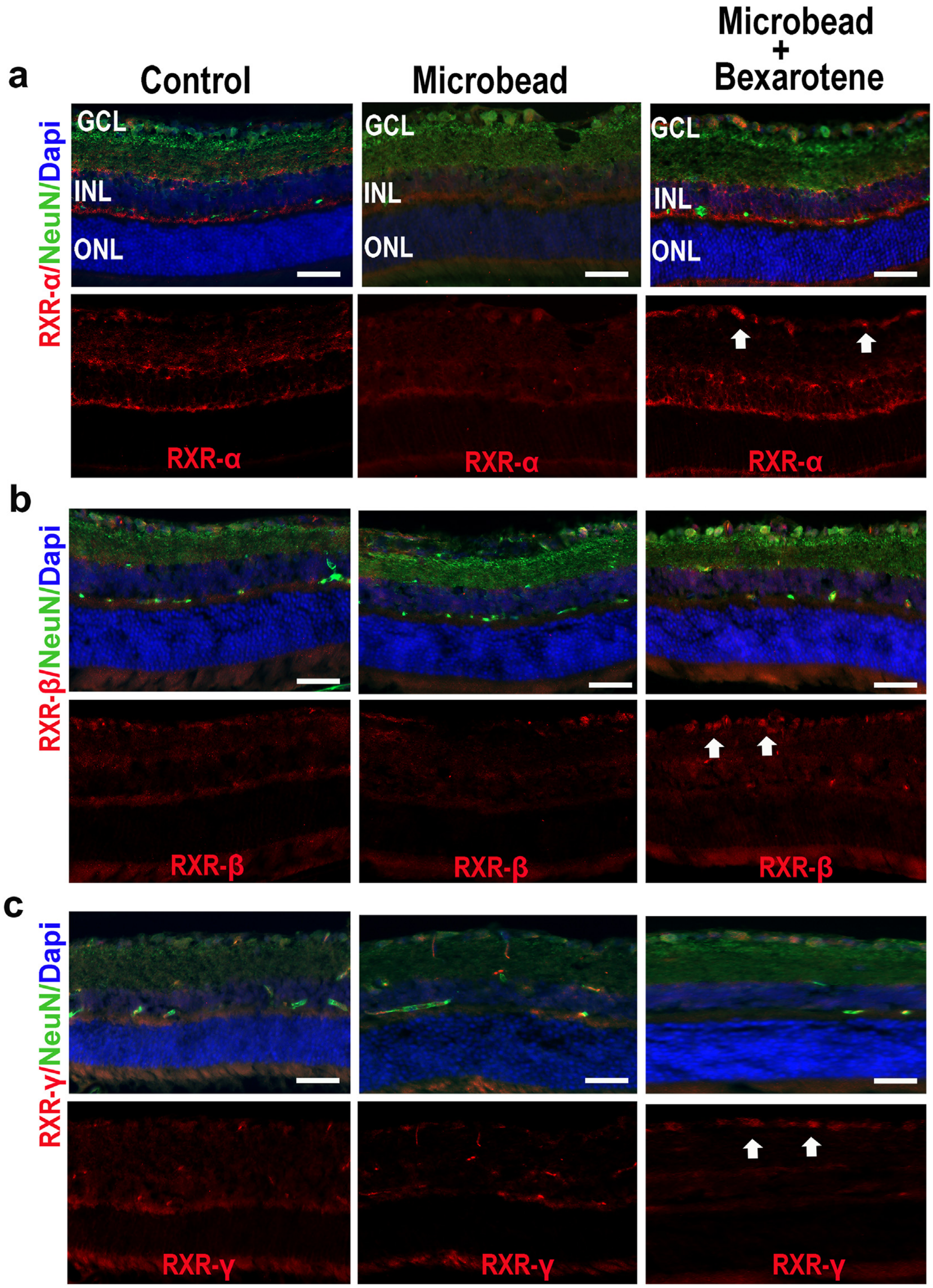

Fig. 3. Changes in expression of RXR (α, β, and γ) in control, microbead and microbead + bexarotene group:
(a), (b) and (c) Immunohistochemical images of mice retinal cryosections with anti-RXRs (α, β, and γ) and anti NeuN (neuronal marker) from different groups. Red colour and green colour indicates staining for RXR and NeuN respectively while blue nuclei is for DAPI; GCL, retinal ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. Microscopic images after immunostaining revealed that bexarotene treatment in mice exposed to intracameral microbeads upregulates expression of all the three isoforms of RXR as compared to microbead group. Scale bar = 50 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Sigma). Tissues (eyes, optic nerve and brain) were harvested for morphometric and immunofluorescence studies. For morphometric analysis, the eyes were fixed for 2 h in 4% PFA and then incubated in 70% ethanol overnight at 4 °C. Eyeballs were placed in embedding cassette and processed via automatic tissue processor (ASP200S, Leica). Eyes were subsequently embedded in molten paraffin wax ensuring their similar orientation using tissue marking dye. Using a rotator microtome, 7 µm thick paraffin embedded sections involving the whole retina as well as the optic disc, were cut from the vertical meridian of each eye and mounted onto Superfrost Plus slides (Menzel-Glaser Lomb). Retinal tissue sections were subjected to haematoxylin and eosin staining. The number of cells in the ganglion cell layer was

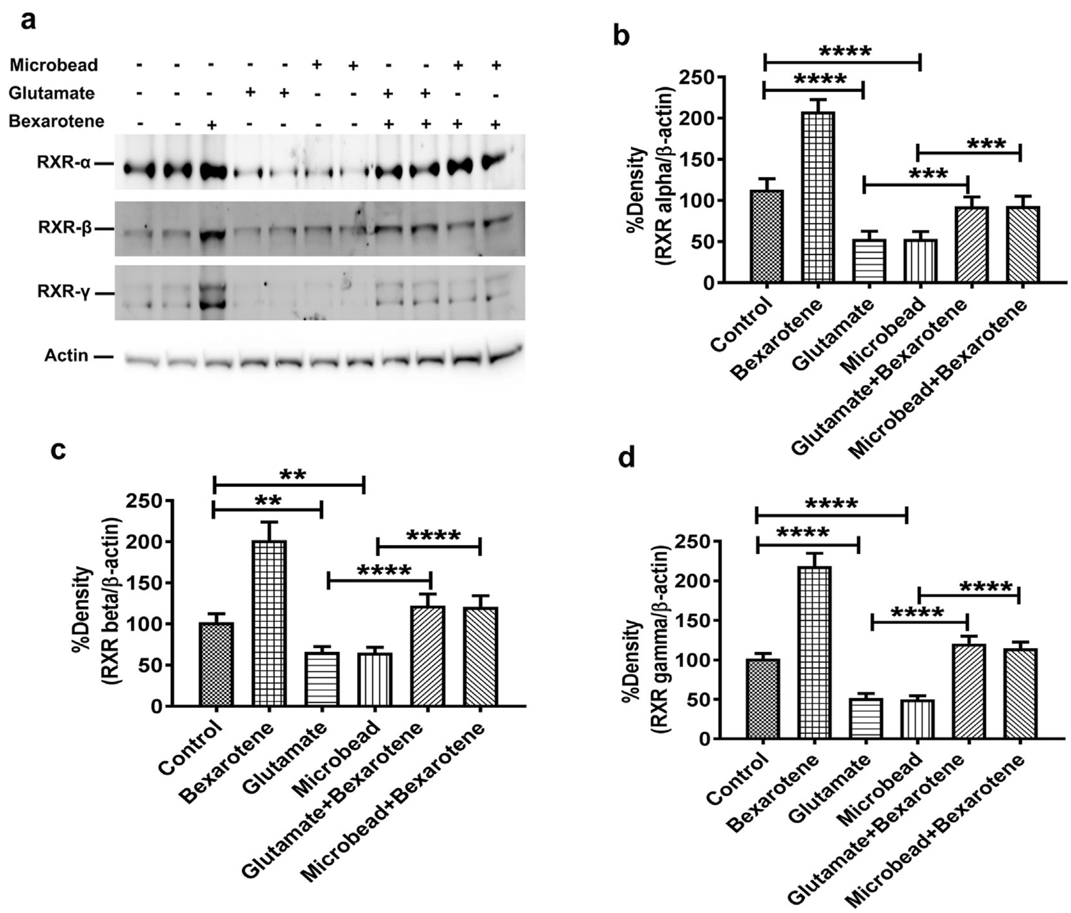

Fig. 4. Bexarotene mediated upregulation of RXRs (α, β, and γ) in control, glaucoma and glaucoma + bexarotene group:
(a) Immunoblotting of RXR (α, β, and γ) expression in optic nerve lysates of control, bexarotene, glaucoma and glaucoma + bexarotene group.
(b), (c) and (d) Densitometric analysis showed a significant upregulation of RXRs in glaucoma + bexarotene as compared to glaucoma. **$p < .05$, ***$p < .001$, ****$p < .0001$.

counted over a length of 300 μm (100 to 400 μm from the edge of the optic disc). For each eye, cell counts were averaged over three consecutive sections to analyse the number of cells in the retinal ganglion cell layer (GCL). Bielschowsky's silver staining was carried out to evaluate axonal density in the optic nerve. The axonal density was determined for each optic nerve by counting the number of axons per mm^2 under the microscope. The evaluators who counted the cells were blinded to eliminate potential bias. For immunofluorescence analysis, eyes and optic nerve were kept in 4% PFA for 1–2 h at room temperature, followed by three subsequent washings with 1 ×-PBS. Tissues were cryopreserved in 30% sucrose solution (in 1× PBS with 0.01% sodium azide) in 4 °C until the tissue sank to the bottom. Following this, the samples were embedded in OCT cryostat embedding medium as described previously (Gupta et al., 2014). Tissue sections were prepared using a cryostat and sections were permeabilized in 0.1% Triton X-100 in PBS (Gupta et al., 2014; You et al., 2014). This was followed by incubating the sections with the indicated primary antibodies overnight at 4 °C. Further, the slides were incubated with either of the secondary antibodies for 1 h at room temperature followed by extensive washing and coverslips were mounted on to the slides with mounting medium containing DAPI. Images were acquired using a Zeiss fluorescence microscope (Rajala et al., 2013).

2.6. Western blotting

Briefly, ONH lysates of control, glaucoma and drug treated groups were lysed in lysis buffer (HEPES buffer) including 20 mM HEPES, pH 7.4, 1% Triton X-100, 1 mM EDTA) containing PhosSTOP and protease inhibitor cocktails, followed by sonication. The lysates were centrifuged at 1200 rpm for 10 min to remove any insoluble material. The supernatants obtained and protein concentrations were determined by a BCA kit (Pierce).

Proteins were subjected to SDS-PAGE and transferred to PVDF. Following protein transfer, the PVDF membranes were washed with TTBS [20 mM Tris–HCl (pH 7.4), 100 mM NaCl, and 0.1% Tween 20] and blocked in 5% skimmed milk in TTBS for 1 h at 25 °C (Gupta et al., 2010). The blots were incubated with primary antibody against anti-RXRα (1:1000), anti-RXRβ (1:1000), anti-RXRγ (1:1000), anti-GADD 153 (1:1200), anti-p-PERK (1:200), anti-BAD (1:1000) and anti-β-actin (1:5000) at 4 °C overnight and next day after three washes (10 min each) in TBST buffer, the immunoblots were incubated with horseradish peroxidase (HRP)-labelled secondary antibodies for 1 h at room temperature. Proteins were detected using Super signal West Pico Chemiluminescent substrate (Pierce). Signals were detected using an automated luminescent image analyser (ImageQuant LAS 4000; GE Healthcare, UK). The densitometric analysis of the band intensities was performed using the Image J software (NIH, USA) and the results normalized by reprobing the membranes with β-actin, which was used as internal loading control and relative density of the peaks was plotted (Gupta et al., 2017).

2.7. HDAC activity

HDAC activity was measured using Pherastar microplate reader with the fluorometric HDAC Activity Assay kit (Abcam, #ab156064) involving black coloured 96-well plates as per the manufacturer's

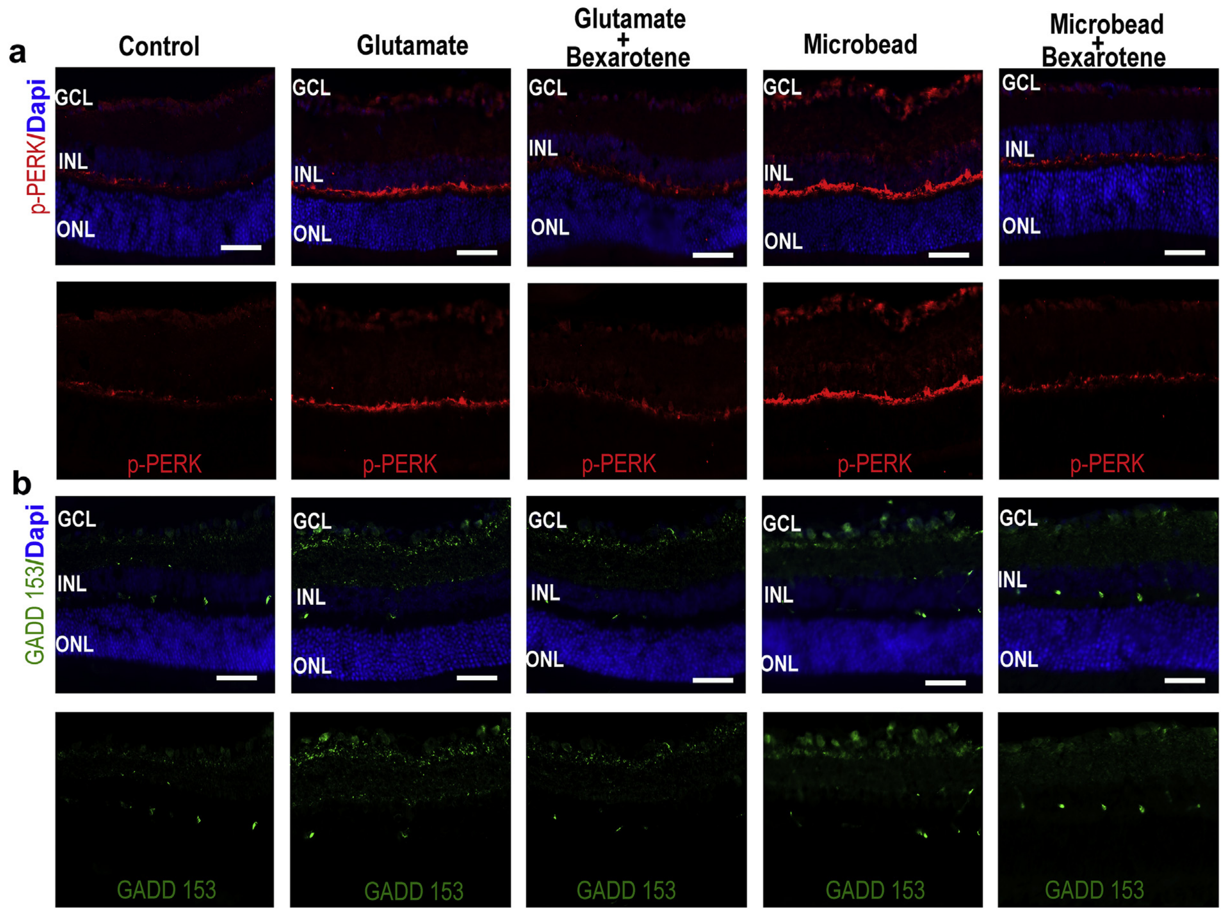

Fig. 5. Effect of bexarotene on glutamate and microbead injections induced ER stress by Immunofluorescence:
(a) and (b) Immunohistochemical images of mice retinal sections stained with p-PERK (red) and GADD 153 (green), revealed that there is upregulation of p-PERK and GADD 153 (ER stress markers) in glaucoma (glutamate and microbead). Bexarotene treatment suppressed the expression of ER stress markers. DAPI was used as nucleus marker. Scale bar = 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

instructions. ONH lysates of control, glaucoma and drug treated groups were prepared as discussed earlier in HEPES buffer. After protein estimation, tissue homogenates were used to perform the HDAC assay. The fluorescence intensity changes during the HDAC assay were identified using with the excitation wavelength at 360 nm and emission wavelength at 460 nm and data plotted with respect to time.

2.8. Statistical analysis

GraphPad Prism software was used to analyse HDAC activity, ERG/pSTR amplitudes, GCL density, axonal density as well as protein expression. All values with error bars are presented as mean ± SD for given n sizes and compared using one way ANOVA followed by Bonferroni post-hoc test for multiple-comparison and student's t-test for unpaired data. HDAC activity assay was statistically analysed by multiple t-tests (multiple comparisons using the Holm-Šídák method). The significance was set at $p < .05$.

3. Results

3.1. Glaucoma downregulates RXR expression in the retina

The expression of various RXR isoforms (α, β, γ) in optic nerve head (ONH) region lysates of human donor tissues was evaluated using immunoblotting. Densitometric quantification revealed significant downregulation of all the three RXR isoforms (*p < .05) in tissues from glaucoma subjects compared to the age matched controls (Figs. 1 a-d). These findings were further evaluated in the human donor eye sections subjected to isoform specific immunohistochemistry. All the three isoforms were well expressed in the GCL although a decrease in expression was evident in glaucoma tissues with no remarkable differences in localisation of RXR immunoreactivity within the retinal layers (Fig. 1e and f).

In order to correlate human glaucoma findings with the mice models of RGC injury, we assessed the expression of RXR receptor isoforms in the mice retinas and evaluated the effects of glaucomatous injury on RXR expression levels using two different experimental glaucoma models. Immunostaining of the retinal sections from both glutamate administration and microbead induced increased IOP models revealed that RXR α was well expressed in all the retinal layers while RXR β staining was predominantly localised in the GCL. RXR γ immunoreactivity was also identified in all the retinal layers (Figs. 2 and 3). The expression of various RXR receptors was also studied in the ONH lysates using western blotting in the control and experimental glaucoma groups in glutamate excitotoxicity and microbead induced increased IOP models (Fig. 4a). Microbead administration resulted in chronic increase in IOP i.e. 20.11 ± 2.63 mmHg (mean ± SD) compared to control group with an average IOP of 9.16 ± 0.43 ($p < .001$) (Supplementary Fig. 1a). No IOP changes were observed in the glutamate treated group (Supplementary Fig. 1b). Results from immunofluorescence and densitometric quantification of the immunoblotting determined that RXR expression was significantly downregulated in both the experimental glaucoma models compared to the control groups (RXR α, control 113 ± 6.699 vs glutamate 53.23 ± 4.657, ****$p < .0001$; microbead 53.21 ± 4.446, ****p < .0001; RXR β, control 101.8 ± 5.27 vs glutamate 65.96 ± 3.303, **$p < .05$;

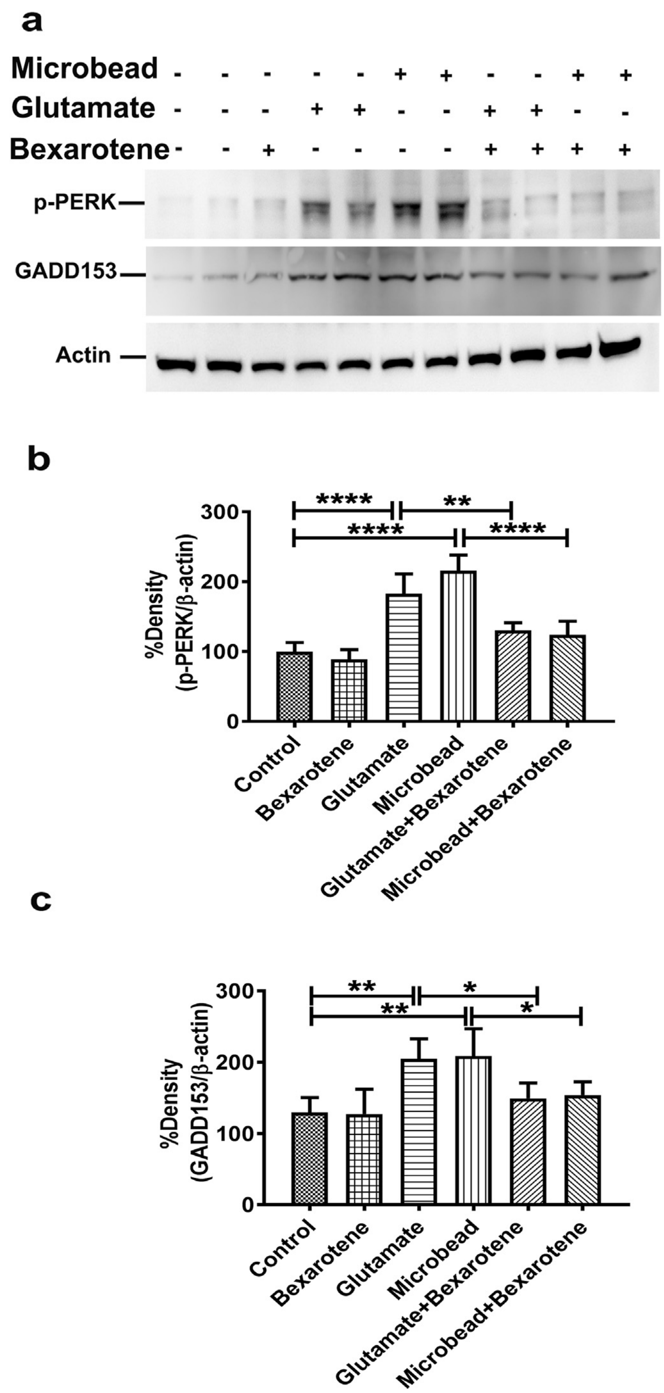

Fig. 6. Effect of bexarotene on glutamate and microbead injections mediated ER stress marker response by Immunoblotting:
(a)Western blot of p-PERK and GADD 153 expression in mice optic nerve lysates. (b) and (c) Statistical analysis of western blot results indicates that the protein level of p-PERK and GADD 153 was significantly decreased with bexarotene treatment as compared to glaucoma group. (*,**p < .05, ****p < .0001)

microbead 65.33 ± 3.186, **p < .05; RXR γ, control 101.7 ± 3.258 vs glutamate 51.58 ± 2.982, ****p < .0001; microbead 49.95 ± 2.373, ****p < .0001 mean ± SD %) (*n* = 3 mice per group) (Fig. 4b, c and d).

3.2. Treatment with RXR agonist bexarotene enhanced retinal RXR expression

To investigate the effects of bexarotene drug treatment on the RXR expression in the retinas under control and experimental glaucoma conditions, ONH lysates were subjected to immunoblotting analysis. Results demonstrated increased expression of RXR α, β and γ isoforms in the bexarotene treated groups as compared to glaucoma conditions (RXR α, bexarotene 92.51 ± 5.869, ***p < .001 (glutamate), 93.23 ± 5.931, ***p < .001 (microbead); RXR β, bexarotene 122.4 ± 6.912, ****p < .0001, (glutamate), 120.6 ± 6.83, ****p < .0001 (microbead); RXR γ, bexarotene 120.7 ± 4.722, ****p < .0001 (glutamate), 114.6 ± 3.96,****p < .0001 (microbead) mean ± SD %) (n = 3 mice per group) (Fig. 4b, c and d). Drug treatment as such did not have any effect on the IOP in control or experimental glaucoma groups. The retinal sections from the bexarotene treated animals were also subjected to immunofluorescence analysis. IF results substantiated the WB findings and highlighted that bexarotene treatment promoted RXR expression in the retinas exposed to glaucomatous injury (Figs. 2 and 3). The increased expression was predominantly associated with the inner retina partcularly GCL and colocalised with the NeuN expressing ganglion cells. Staining with non-immune IgGs was used as control. RXR isoforms were observed to colocalise with NeuN neuronal marker in the GCL at higher magnification (63×) in both the control and bexarotene alone treated animals (Supplementary Fig. 2).

3.3. RXR activation reduces ER stress response in experimental glaucoma

Taking into account that ER stress is associated with RGC death, we investigated ER stress associated changes in our acute (glutamate) and chronic (microbead) mouse model of experimental glaucoma (Doh et al., 2010; Shimazawa et al., 2007). Expression of ER stress protein markers p-PERK and GADD 153 was evaluated in retinal sections using immunofluorescence and data indicated increased staining under the glaucoma conditions. The reactivity of p-PERK was mainly localised to the GCL, outer plexiform layer and INL while for GADD153 it was mainly localised to the GCL (Fig. 5). DAPI was used for orientation and to identify various cellular layers in the retina. Further, we evaluated the effects of the RXR agonist bexarotene on the ER stress protein localisation and protein expression. Drug treatment of the glaucoma animals suppressed both p-PERK and GADD 153 protein expression. No remarkable changes were noticed in the localisation of the proteins with respect to retinal layers under glaucoma conditions or in tissues from drug treated mice (Fig. 5a and b).

The changes in ER stress protein markers p-PERK and GADD 153 was also evaluated in the ONH lysates using western blotting and data quantified using band intensities. A significant increase in the ER stress proteins was identified in both glutamate and microbead models compared to the control tissues (p-PERK, control 99.6 ± 6.6 vs glutamate 182.9 ± 14.08, ****p < .0001; microbead 216 ± 11.13, ****p < .0001; GADD153, control 129 ± 10.38 vs glutamate 204 ± 14.09, **p < .05; microbead 208 ± 19.02, **p < .05; mean ± SD %) (*n* = 3 mice per group) (Fig. 6 a-c). Tissue from glaucoma mice treated with the RXR agonist demonstrated significant loss of p-PERK and GADD153 protein activation compared to the glaucomatous tissues alone (p-PERK, (mean ± SD %) 130.5 ± 5.37, **p < .05 (glutamate), 123.6 ± 9.94, ****p < .0001 (microbead); GADD153, (mean ± SD %) 149.4 ± 12.3, *p < .05 (glutamate),153.9 ± 10.08, *p < .05 (microbead) (n = 3 mice per group) (Fig. 6a-c). Together, these experiments established that bexarotene treatment prevented ER stress activation in the glaucoma models in vivo.

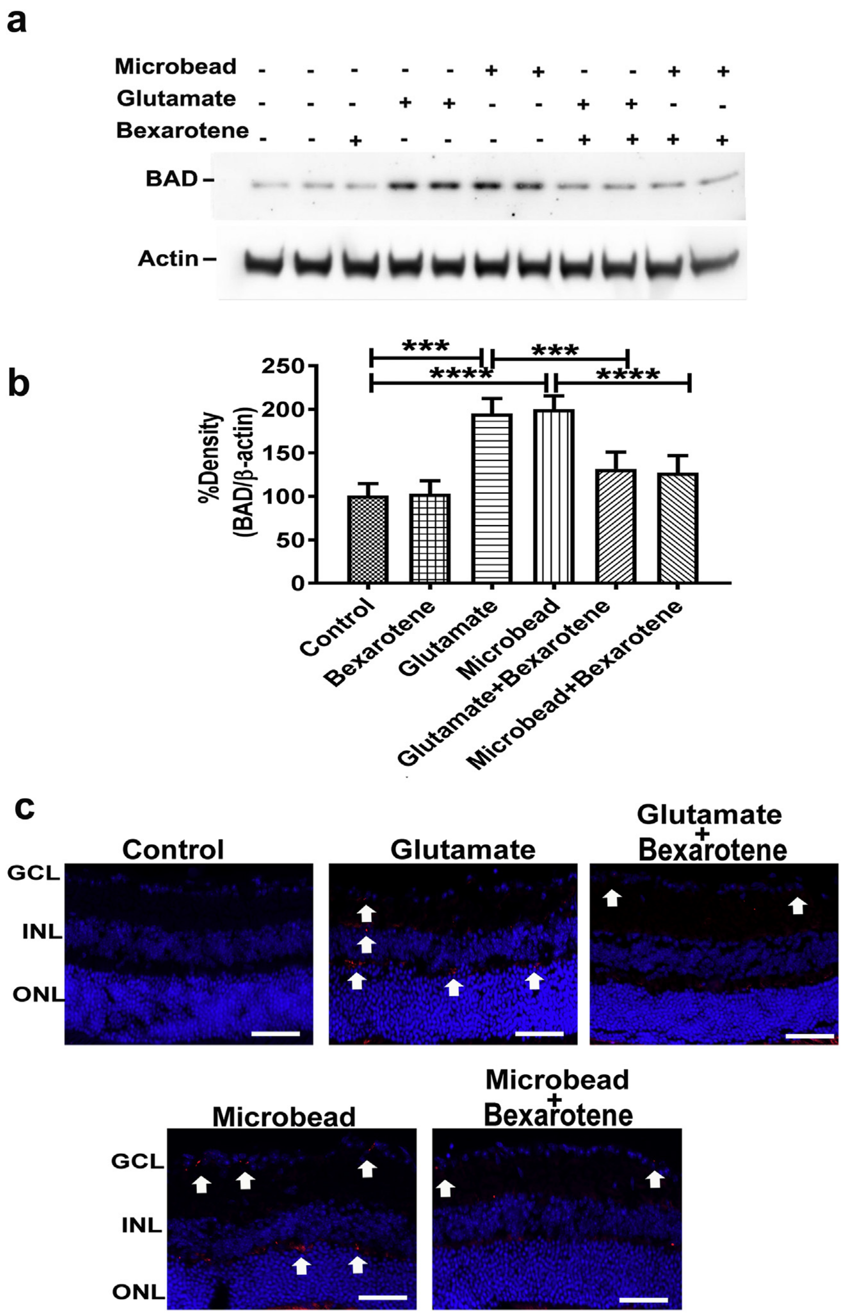

Fig. 7. Bexarotene mediated suppression of apoptosis:
(a)Western blot of BAD expression in mice optic nerve lysates.
(b) Statistical analysis of western blot results indicates that the protein level of BAD was significantly decreased with bexarotene treatment as compared to glaucoma group. (***p < .001, ****p < .0001).
(c) Immunohistochemical images of mice retinal sections stained with TUNEL assay kit for apoptotic changes. Images showing increased TUNEL reactivity (red) in glaucoma. DAPI was used as nucleus marker. Scale bar = 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. Reduced apoptotic pathway activation in glaucoma upon RXR activation

We evaluated the expression of BAD protein which is an apoptotic marker in both glutamate and microbead induced glaucoma model using immunoblotting. Quantification determined significant BAD protein upregulation in both glaucoma models compared to that observed in the control tissues (Control 101 ± 6.83 (mean ± SD %), glutamate 195.1 ± 8.6, ****p* < .001; microbead, 200.4 ± 7.5, ****p < .0001) (n = 3 mice per group). Bexarotene treatment of the glaucoma mice resulted in BAD suppression in vivo as demonstrated by significantly reduced band intensities (glutamate, 131.5 ± 9.6, ***p < .001; microbead, 125.3 ± 9.8, ****p < .0001) (n = 3 mice per group). Actin was used as loading control in each case. Bexarotene treatment of the control mice did not result in any significant changes in BAD protein expression suggesting that RXR activation was effective in suppressing BAD once it is activated such as observed under the experimental glaucoma conditions (Fig. 7a and b). Mice retinal sections were also investigated for apoptotic changes using TUNEL staining (Fig. 7c). Glutamate and microbead injected mice models revealed increased number of TUNEL positive cells in the retina. Bexarotene treatment however resulted in reduced TUNEL staining, corroborating the BAD protein findings. These results suggested that apoptotic pathway activation associated with ER stress protein upregulation in glaucoma can be suppressed by pharmacological activation of RXR receptors in the retina.

3.5. Bexarotene treatment suppressed the elevated HDAC activity in glaucoma models

An inverse relationship between the RXR activation and HDAC (Histone deacetylase) regulation has been suggested in various studies previously (Dummer et al., 2012; Papi et al., 2012; Wang et al., 2015). Increased HDAC activity has been shown to play important role in

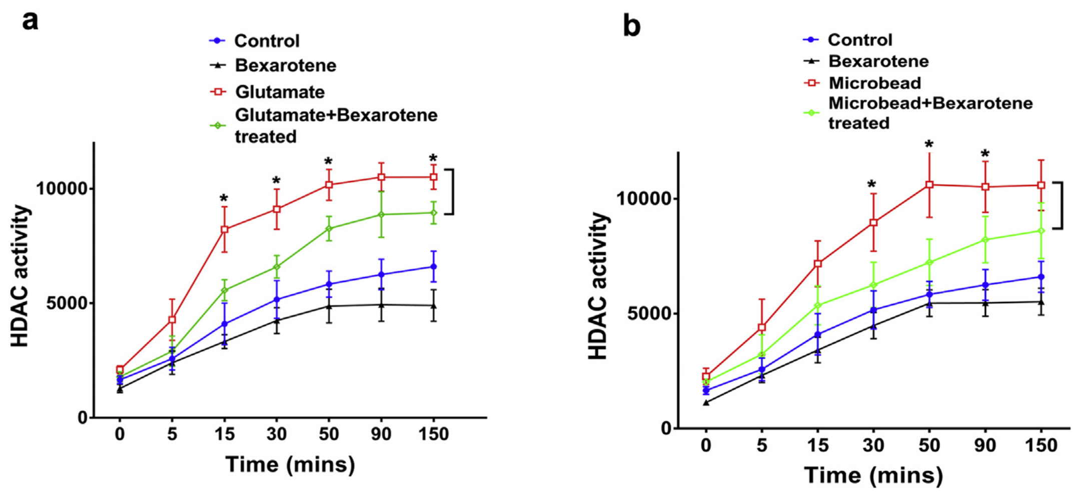

Fig. 8. Effects of bexarotene on HDAC inhibition:
Time dependent HDAC enzyme activity was measured in optic nerve lysates from control, bexarotene, glaucoma (glutamate and microbead) and glaucoma + bexarotene groups and data plotted. a) and (b) Graph of both glaucoma models (glutamate and microbead) showed an increased HDAC activity whereas bexarotene treatment in glaucoma conditions resulted in reduction of HDAC activity. (*p < .05).

inducing RGC death after acute insult to the optic nerve (Schmitt et al., 2014) and its inhibition shown to protect the retina from ischemic injury (Crosson et al., 2010). To explore the effect of glutamate induced excitotoxicity and microbead induced increased IOP, we analysed the ONH region of these two mice models of RGC injury for changes in HDAC activity. Corroborating the previous reports of HDAC activation in RGC injury models, we observed increased HDAC activity in both glutamate ($p < .05$) and chronically increased IOP ($p < .05$) models. Interestingly, bexarotene treatment was effective in bringing about a significant reduction in the HDAC activity in both the RGC injury models (Glutamate injury-p < .05, chronically increased IOP injury-p < .05). Bexarotene alone treated animals were used as controls and while these animals demonstrated a trend of slightly decreased HDAC activity compared to the vehicle treated animals; the differences were not statistically significant (Fig. 8). Therefore, RXR activation by bexarotene seems to be negatively associated with HDAC activity in the retina under experimental glaucoma conditions.

3.6. RXR activation ameliorated inner retinal functional deficits associated with glaucomatous stress

Both glutamate excitotoxicity and experimental glaucoma models are reported to be associated with inner retinal functional deficits characterised by a reduced positive scotopic threshold response (pSTR) (Bui et al., 2009; Della Santina et al., 2013). In this study both intravitreal glutamate injection and microbead administration resulted in reduction of pSTR amplitudes (29.35 ± 2.71 μV; glutamate model and 22.82 ± 1.80 μV; microbead model) as compared to control mice (55.97 ± 6.92 μV) (mean ± SD) ($n = 24$ mice) (Fig. 9a, b, e and f). We investigated whether treatment with the RXR agonist and resultant increased expression of RXR isoforms correlated with potential effects on the retinal functional recordings. The loss of pSTR amplitude was significantly prevented upon bexarotene treatment in both the glutamate (40.82 ± 3.14 μV; *$p < .05$; $n = 9$ mice) and microbead (41.28 ± 3.078 μV; ****p < .0001; n = 9 mice) groups as compared to the corresponding glaucoma groups. Scotopic ERG recordings from whole retina demonstrated no significant changes in a- or b- wave amplitudes in either glaucoma or RXR agonist treatment groups suggesting that effects of glaucoma injury and protective effects of RXR agonist treatment were mainly localised to the inner retina (Fig. 9c,d,g and h). These results demonstrated that RXR upregulation by its pharmacological agonist partially rescued the retinal functional impairment caused by glutamate excitotoxicity and microbead induced increased IOP injury.

3.7. Effects of RXR agonist on the ganglion cell layer and optic nerve

We investigated whether the protective effects of the drug on electrophysiological recordings were supported by protection of cellular morphology in the retinal laminar structure. Retinal sagittal sections were subjected to H and E staining (Fu and Sretavan, 2010) and analysed using light microscopy (Fig. 10a). A significant decrease in the number of cells in GCL was observed in both glutamate and microbead induced glaucoma injury models (79.49 ± 3.42% cells in control versus 43.83 ± 2.17% cells in glutamate, ****$p < .0001$, and 33.15 ± 1.87 in microbead model, ****p < .0001) ($n = 4$ mice per group) (Fig. 10). Interestingly, RXR agonist significantly prevented the loss of GCL density and increased number of cells were identified in both the experimental glaucoma injury models (58.17 ± 2.66% cells in glutamate *$p < .05$, and 57.41 ± 2.8 in microbead model, ***$p < .001$) (n = 4 mice per group) (Fig. 10b and c) compared to controls. The retinal sections were also subjected to immunofluorescence staining using beta-III tubulin which is a ganglion cell specific marker in the retina (Supplementary Fig. 3a) (Jiang et al., 2015). These results corroborated H and E staining observations and demonstrated significantly reduced β-III tubulin positive ganglion cells in the retinas of glutamate and microbead models (p < .001). An increased number of β-III tubulin positive cells were observed in the bexarotene treated mice groups indicating a protective effect of the drug on the RGCs in experimental glaucoma conditions (p < .05) (Supplementary Fig. 3b and c).

Axons of the RGCs converge to the optic nerve through which signals from retina are transmitted to higher visual centres in the brain. The protective effect of the RXR agonist on the RGCs was evaluated by monitoring axonal density of the optic nerve sections in experimental glaucoma and drug treated groups. Optic nerve sections were stained using Bielschowsky's silver staining and axonal density analysed using light microscopy (Fig. 11a) (You et al., 2011). Whereas both the experimental glaucomatous injury models showed a loss of axonal density in the optic nerve (control 85.12 ± 6.27, glutamate 58.95 ± 5.22%, ****p < .0001, microbead 42.2 ± 4.78% (mean ± SD), ****p < .0001), the extent of loss was lower in the mice that were pharmacologically treated with RXR agonist (glutamate 71.82 ± 5.88%;**p < .05, microbead 60.4 ± 4.29%;**** p < .0001 (n = 4 mice per group) (Fig. 11b and c). Results delineated that treatment with RXR agonist helped in preventing RGC and optic

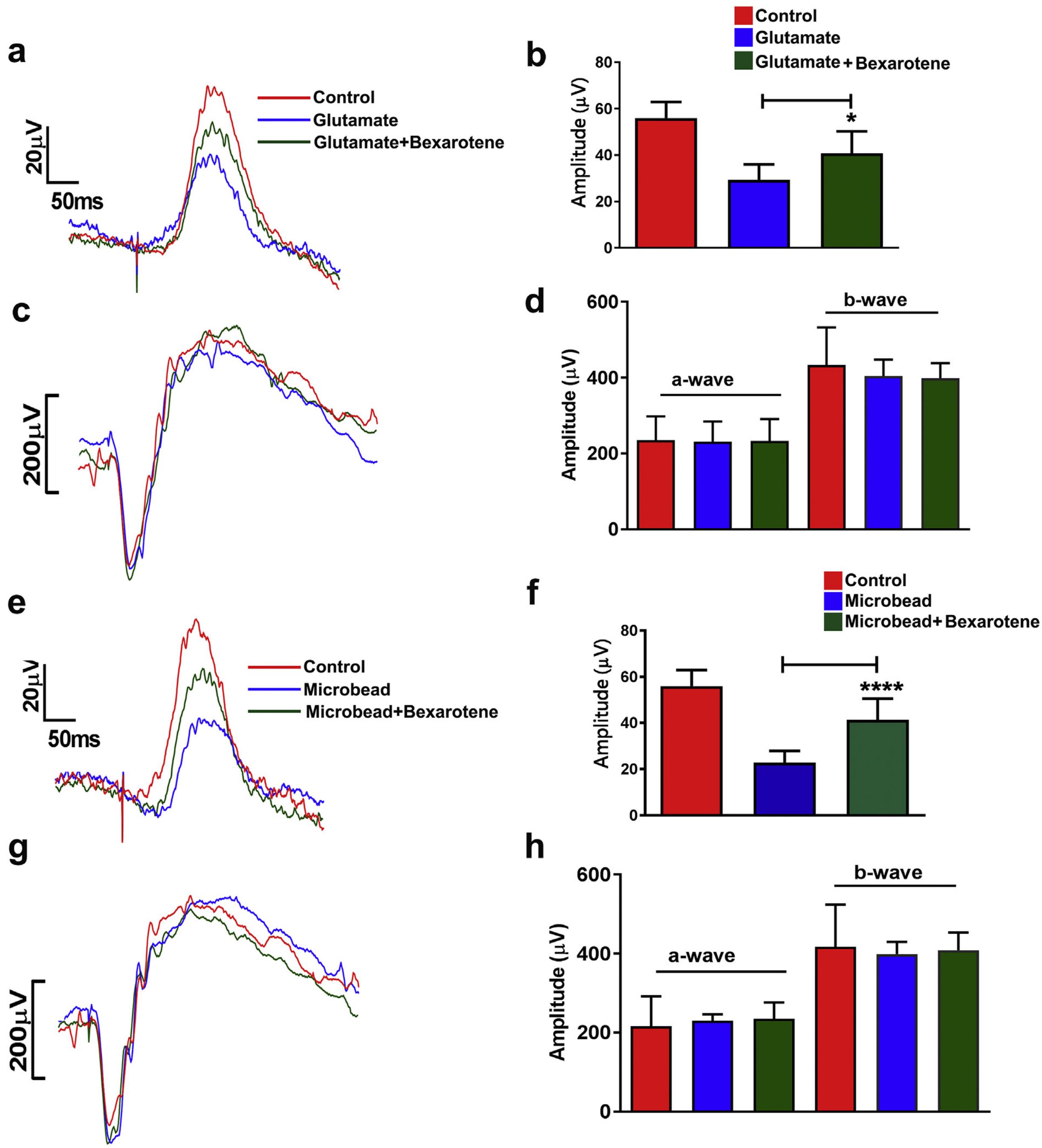

Fig. 9. Effects of bexarotene on electroretinography (ERG) and positive scotopic threshold response (pSTR) responses:
(a) and (e) Average traces of positive scotopic threshold responses of all groups (both glutamate and microbead model) (b) and (f) Quantification of pSTR amplitudes indicates a significant elevated amplitude of pSTR in the bexarotene treated mice as compared to the glaucoma mice. $^{*}p < .05$, $^{****}p < .0001$. (c) and (g) Average ERG traces for the control, glaucoma and glaucoma + bexarotene group (d) and (h) Data analyses of ERG indicates no significant change in a-wave and b-wave.

nerve axonal loss in glaucoma conditions that also correlated with retinal electrophysiological protection conferred by the drug (Fig. 9, 10 and 11).

4. Discussion

This study examined the role of RXR activation in the retina in glaucoma using its pharmacological agonist bexarotene. Emerging evidence indicates that bexarotene plays a neuroprotective role in the CNS and has particularly been shown to reverse cognitive and behavioural deficits in AD models, although the extent of protection conferred by the drug requires further investigations (Bachmeier et al., 2013; Certo et al., 2015; Tousi, 2015). Glaucoma has been shown to exhibit biochemical similarities in cellular neurodegenerative pathways with AD (Ghiso et al., 2013) and we and others have shown increased accumulation of Aβ species in the retina in glaucoma conditions (Gupta et al., 2016). The objective of this study was to examine various biochemical pathways affected by the drug in the retina to enhance our understanding of the role of RXR in the retina in healthy and glaucoma conditions.

RXRs are well expressed in the retina (Mori et al., 2001) and recent studies indicate that activation of these receptors promotes photoreceptor survival and suppresses apoptosis (German et al., 2013). This study demonstrated that glaucoma leads to a decrease in the expression

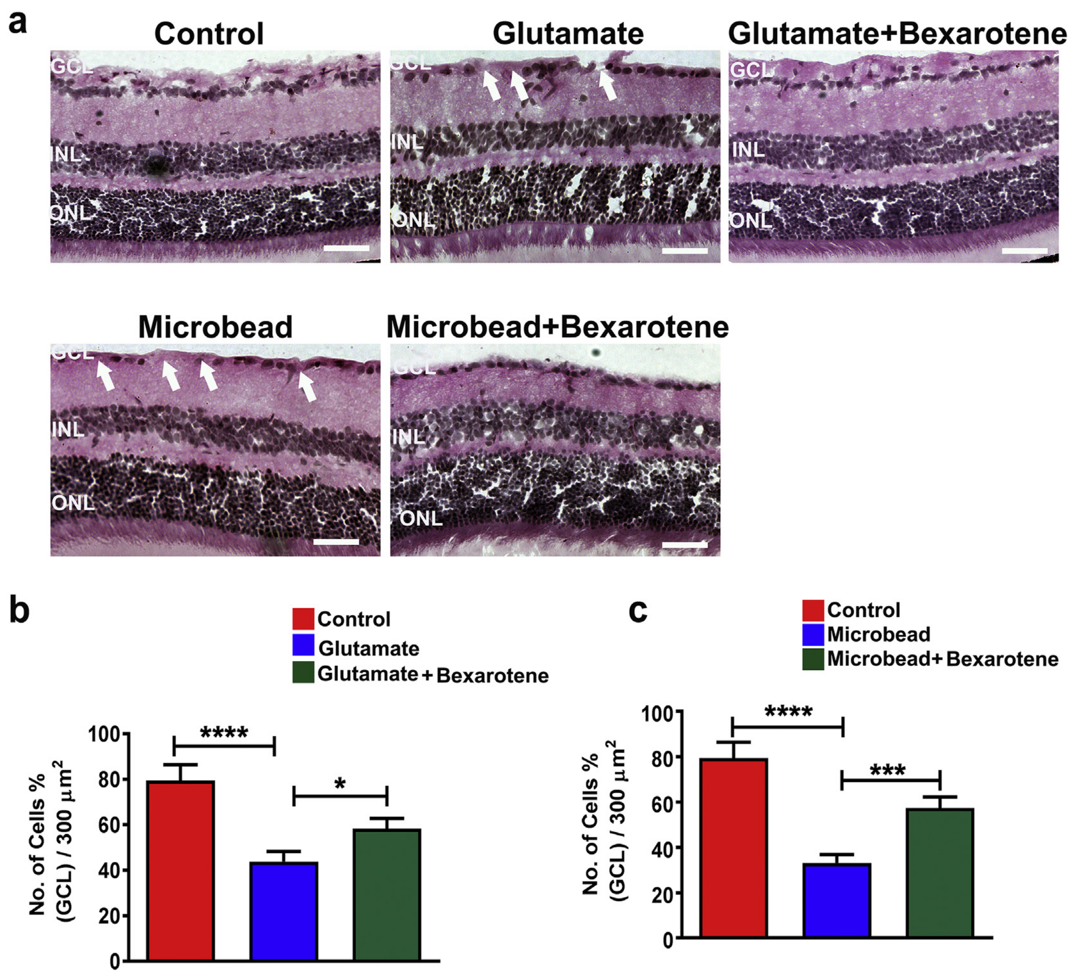

Fig. 10. Histological changes in control, glaucoma and glaucoma + bexarotene group using H and E staining to reveal retinal morphology:
(a) Representative images of H and E stained mice retinal sections showing a decline in number of retinal ganglion cells in glaucoma (both glutamate and microbead) (b) and (c) Quantification indicating significant preservation of GCL with bexarotene treatment as compared to glaucoma in mice retinal sections (*$p < .05$, ***$p < .001$). Scale bar = 50 μm.

of various RXRs in the retina, particularly in the inner retinal layers. Treatment with a dose of 100 mg/kg of bexarotene during initial stages of the glaucomatous injury is effective in inducing an increased expression of all the three RXR isoforms in the retina. The 100 mg/kg dose levels of bexarotene has been extensively reported in the literature (Casali et al., 2018; Cramer et al., 2012; Riancho et al., 2015). Wang et al. (2016) observed bexarotene concentrations in brain homogenates of rats dosed at 100 mg/kg to be 6.26 μM with a plasma concentration of 5.74 μM whereas another study by Ghosal et al. (2016) reported 1–2 μM plasma concentrations and nM levels of the drug in CSF after oral administration of bexarotene (225 mg) in a phase 1b clinical trial (Ghosal et al., 2016; Wang et al., 2016). Differential distribution of the drug observed in these two studies might be attributed to tissue specific differences in CSF versus brain tissue. Species specific blood-brain barrier permeability differences might also alter the pharmacokinetics of the drug in CNS (Syvänen et al., 2009). Our study however, is focussed on determining the protective effects of the drug on the retina in two different glaucoma models. This is the first study of its kind in the retina to establish whether RXR modulation could be effective in eliciting a protective response in glaucoma. Further, RXR activation reduced the ER stress marker proteins in the inner retina in both of these models and suppressed the apoptotic pathway activation. Pharmacological upregulation of RXRs was demonstrated to be effective in protecting against the inner retinal functional and anatomical deficits induced by glaucoma in both of these experimental models.

Our results indicating loss of RXR expression in the inner retina in glaucoma are novel and RXR activation caused by bexarotene treatment is in agreement with previous reports (Janakiram et al., 2012; Mounier et al., 2015; Riancho et al., 2015). RXR expression was decreased in both the mouse models of RGC injury demonstrating that effects on RXR expression were independent of IOP changes. In addition, we also identified that RXR expression was downregulated in human glaucoma retinal tissues. We observed increased expression of RXR α, β and γ isoforms in the inner retina in bexarotene treated mice tissues using both western blotting and immunofluorescence approaches. ONH tissue lysates were used for WB as they are mainly comprised of RGC axons with astrocytes comprising other resident cell types in the tissue.

As in other neurodegenerative diseases, many studies have shown that ER stress pathway gets activated in the retina of glaucoma mice model and plays a key role in RGC death (Yang et al., 2016; Yasuda et al., 2014). Drugs showing anti-ER stress properties have been shown to prevent RGC death (Nakamura et al., 2017; Tsuruma et al., 2012). Bexarotene has been demonstrated to reduce protein aggregation and inhibit cytoplasmic inclusion body formation and maintaining neuronal proteostasis (Riancho et al., 2015). Any disturbances in homoeostasis result in improper protein processing leading to ER stress with subsequent accumulation of structurally abnormal or unfolded proteins in the ER lumen (Zhang and Kaufman, 2006). Unfolded protein response eliminates misfolded proteins and decreases translocation of protein into the ER lumen (Schroder and Kaufman, 2005). PERK (protein kinase

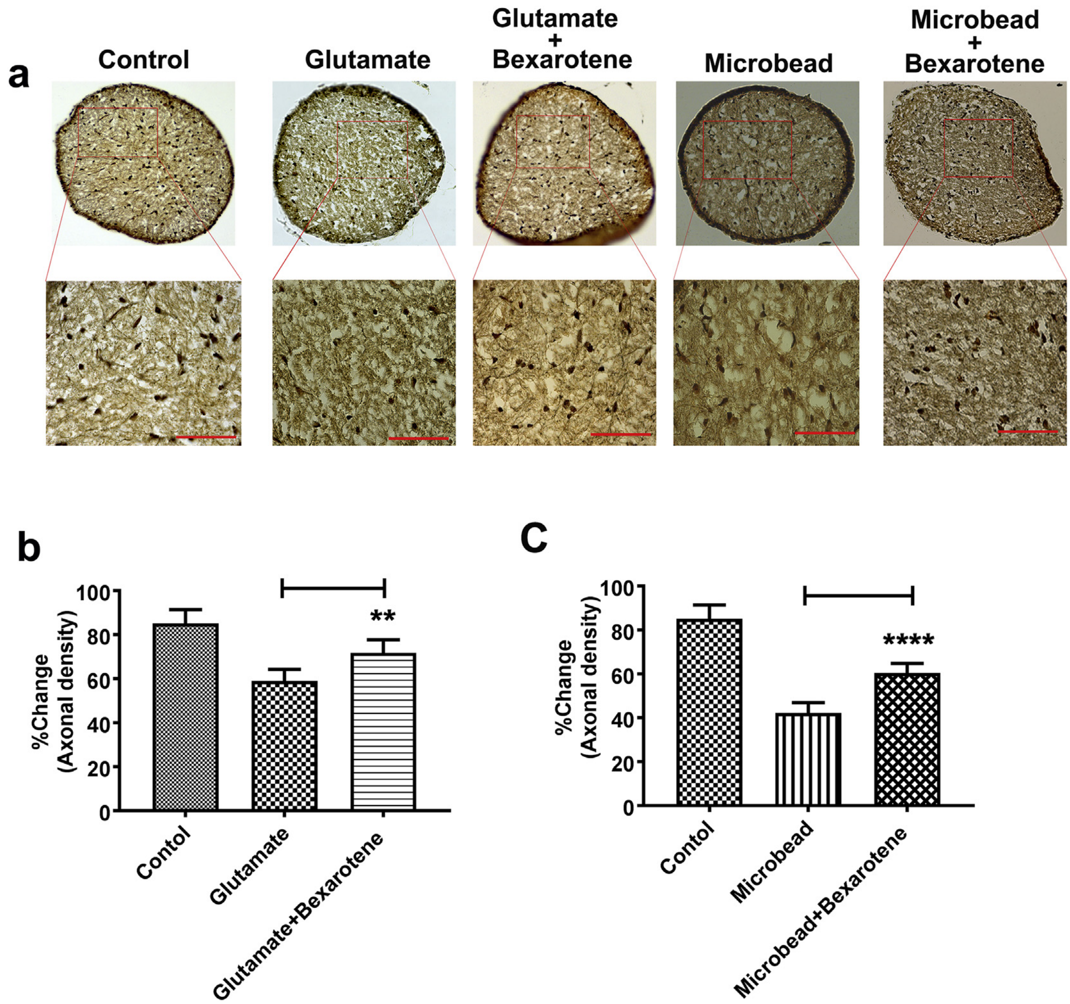

Fig. 11. Prevention of axonal loss in optic nerve by bexarotene treatment:
(a) Representative images of Bielschowsky's silver staining of optic nerve sections in control, glaucoma and glaucoma + bexarotene group. (b) and (c) Quantification revealed that bexarotene significantly rescued axonal loss caused by glaucoma. $^{**}p < .05$, $^{****}p < .0001$. Scale bar = 10 μm.

RNA (PKR)-like ER kinase), is a major ER stress sensor (Harding et al., 1999; Mori et al., 1993; Yoshida et al., 1998) and previous studies found that the CHOP (transcriptional factor C/EBP homologous protein, also known as GADD 153) signal pathway plays a role in RGC death associated with ER stress. Increased endoplasmic reticulum (ER) stress was demonstrated to contribute to the glaucoma pathology (Zode et al., 2011; Zode et al., 2014) and prolonged ER stress has been shown to play a key role in retinal apoptosis and cell death (Anholt and Carbone, 2013; Jing et al., 2012; Shimazawa et al., 2007; Yang et al., 2011). ER stress and unfolded protein response (UPR) has been implicated in both in the experimental acute (Awai et al., 2006) and chronic glaucoma models (Doh et al., 2010). Our studies found that bexarotene treatment diminished the expression of p-PERK and GADD 153 in both the models of experimental glaucoma. Activation of the p-PERK and GADD153 during chronic ER stress promotes pro-apoptotic pathways (Dheer et al., 2018; Wang et al., 1996; Zinszner et al., 1998). We also observed reduced BAD activation in the retina in drug treated animals.

Further, class 1 HDAC enzyme is well expressed in the retina including in the GCL and activation of cellular apoptotic processes has been shown to associate with epigenetic processes including HDAC activation in the RGCs (Fan et al., 2013; Lagali and Picketts, 2011). Pharmacological inhibition of HDAC in the retina as well as conditional ablation of *Hdac3* were demonstrated to protect the RGC against optic nerve crush injury (Pelzel et al., 2010). Here we observed that HDAC activity was increased in both the acute and chronic models of RGC damage suggesting its possible role in glaucomatous injury. HDAC and RXR have been suggested to reciprocally regulate their actions and bexarotene was shown to induce transcriptional repression through its effects on the HDAC (Li et al., 2011). An RXR agonist, DW22 was also demonstrated to promote RXR activation while leading to inhibitory effects on the HDAC in cancer cells (Wang et al., 2015). In this study, we observed that systemic treatment of RXR agonist bexarotene resulted in loss of HDAC activity in both of these RGC injury models suggesting its potentially protective role in glaucomatous injury.

In order to understand, whether these biochemical changes were associated with any effects on retinal function, we tested the control and drug treated animals for electrophysiological recordings and then examined retinal and optic nerve structure histologically. We used male mice in this study to decrease variables potentially associated with hormonal differences. Selection of one gender further helped to minimise variables with respect to the two glaucoma models used and the drug effects. Our in vivo retinal functional and retinal laminar structural analyses demonstrated that RXR targeting was effective in ameliorating inner retinal dysfunction caused by both acute and chronic models of experimental glaucoma. The functional protection identified in the drug treated animals corresponded with protective effects on retinal laminar structure. Histological analysis indicated significant loss of the GCL in both of these glaucoma models and RXR activation led to significant protection against this loss in both of these models. Consequently, these results suggested that RXR activation protects the

RGCs against both excitotoxic and IOP induced injury and is able to protect the retina under both acute and chronic glaucoma conditions. Immunological staining of the retinal sections by β-III tubulin also indicated reduced numbers of RGCs in the glaucoma which were partially protected in RXR activated conditions. Furthermore, optic nerve axonal density evaluation using Bielschowsky's silver staining substantiated the fact that the drug was effective in significantly protecting the RGC axons in glaucoma conditions. Summarising, protective effects of RXR modulation suggest these receptors as novel drug targets that can be modulated to impart protection to the RGCs in glaucoma. The results obtained from functional and structural data and supported by its anti-ER stress role demonstrate that bexarotene exerts neuroprotective effects on retina and optic nerve in excitotoxicity as well as increased IOP glaucoma models in mice. To this extent, RXR activation could be a novel strategy to manage glaucoma as a complementary therapy, possibly in combination with surgical intervention and IOP lowering therapies.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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