Published in final edited form as:
Neurobiol Dis. 2014 November ; 71: 44–52. doi:10.1016/j.nbd.2014.07.016.

Combinatorial targeting of early pathways profoundly inhibits neurodegeneration in a mouse model of glaucoma

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Abstract

The endothelin system is implicated in various human and animal glaucomas. Targeting the endothelin system has great promise as a treatment for human glaucoma, but the cell types involved and the exact mechanisms of action are not clearly elucidated. Here, we report a detailed characterization of the endothelin system in specific cell types of the optic nerve head (ONH) during glaucoma in DBA/2J mice. First, we show that key components of the endothelin system are expressed in multiple cell types. We discover that endothelin 2 (EDN2) is expressed in astrocytes as well as microglia/monocytes in the ONH. The endothelin receptor type A (*Ednra*) is expressed in vascular endothelial cells, while the endothelin receptor type B (*Ednrb*) receptor is expressed in ONH astrocytes. Second, we show that Macitentan treatment protects from glaucoma. Macitentan is a novel, orally administered, dual endothelin receptor antagonist with greater affinity, efficacy and safety than previous antagonists. Finally, we test the combinatorial effect of targeting both the endothelin and complement systems as a treatment for glaucoma. Similar to endothelin, the complement system is implicated in a variety of human and animal glaucomas, and has great promise as a treatment target. We discovered that combined targeting of the endothelin (Bosentan) and complement (*C1qa* mutation) systems is profoundly protective. Remarkably, 80% of DBA/2J eyes subjected to this combined inhibition developed no detectable glaucoma. This opens an exciting new avenue for neuroprotection in glaucoma.

Introduction

Glaucoma is one of the most common neurodegenerative diseases (Quigley, 1996) characterized by the death of retinal ganglion cells (RGCs) and degeneration of the optic

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nerve (reviewed in (Burgoyne, 2011; Nickells *et al.*, 2012)). The optic nerve head (ONH) is a key site in glaucoma (e.g. (Anderson & Hendrickson, 1974; Quigley & Anderson, 1976; Anderson & Hendrickson, 1977; Quigley & Anderson, 1977; Quigley & Addicks, 1980; Howell *et al.*, 2007a)). More effective therapies, particularly those that target damaging processes in the optic nerve head are required. A critical and early insult damages RGC axons in the ONH (Schlamp *et al.*, 2006; Howell *et al.*, 2007a; Burgoyne, 2011). Nevertheless, the earliest processes that damage RGC axons are not well defined. To better understand these early stages, we performed gene expression profiling from DBA/2J mice, a widely used mouse model of glaucoma (Howell *et al.*, 2011a; Howell *et al.*, 2012b). Our gene expression analyses identified a temporally ordered series of early glaucoma stages. Previous studies had suggested the importance of the endothelin system in glaucoma (reviewed in (Chauhan, 2008; Good & Kahook, 2010; Prasanna *et al.*, 2011)) and our data showed that endothelin-2 (*Edn2*) was significantly upregulated compared to non-glaucoma eyes at early stages of the disease, prior to significant axon damage (Howell *et al.*, 2011a). Cumulatively, this data suggest that components of the endothelin system maybe critical in the early progression of glaucoma in the optic nerve head.

The endothelin system is comprised of three ligands (endothelin 1, EDN1; EDN2 and endothelin 3, EDN3) that interact with two receptors, endothelin receptor type A (EDNRA) and endothelin receptor type B (EDNRB) (Kedzierski & Yanagisawa, 2001). Endothelin ligands binding either to one or both of the endothelin receptors activates a variety of different responses within tissues (reviewed in (Kedzierski & Yanagisawa, 2001). Upregulation of components of the endothelin system is described in human glaucoma and in animal models relevant to glaucoma. Endothelin 1 (EDN1) was elevated in the aqueous humor of primary open angle glaucoma (POAG) patients compared to normotensive individuals (Noske *et al.*, 1997; Tezel *et al.*, 1997). Injections of EDN1 or endothelin 2 (EDN2) can induce RGC loss in the retina and optic nerve head (Chauhan *et al.*, 2004; Cioffi, 2005; Stokely *et al.*, 2005; Sasaoka *et al.*, 2006; Howell *et al.*, 2011a). Intravitreous injections of EDN1 cause a dose-related decrease in the number of retrogradely labeled RGCs (Taniguchi *et al.*, 2006). Furthermore, in response to optic nerve crush in rabbits, EDNRB is upregulated in activated astrocytes (Rogers *et al.*, 2003). Infusion of Bosentan (an inhibitor of endothelin receptor type A, EDNRA and endothelin receptor type B, EDNRB) reduced astrocyte activation in crushed optic nerves (Rogers *et al.*, 2003). Also, EDNRB deficiency lessened neurodegeneration in a rat model with experimentally induced elevation of IOP (Minton *et al.*, 2012).

Targeting early events in glaucoma is likely to have better therapeutic efficacy than targeting later events. However, the exact roles of the endothelin system in early stages of glaucoma have not been elucidated. Therefore, to begin to understand these roles, we have performed a detailed characterization of the endothelin system in DBA/2J glaucoma. As combinatorial treatment regimens targeted against multiple early events are likely to be more effective than monotherapy, we have also tested the effects of targeting the endothelin pathway alone and in combination with the complement pathway. The complement pathway is another promising target as it has been widely implicated in human and animal models relevant to glaucoma (Stasi *et al.*, 2006; Steele *et al.*, 2006; Johnson *et al.*, 2007; Stevens *et al.*, 2007).

Furthermore, like the endothelin system, we have shown that the complement cascade is upregulated very early in DBA/2J glaucoma, prior to significant RGC axon damage (Howell *et al.*, 2011a). Here, we show that combinatorial targeting of the endothelin system and the classical pathway of the complement cascade is more effective at reducing glaucomatous damage than separately inhibiting either process.

Materials and Methods

Mouse Strains, Breeding and Husbandry

All experiments were conducted in accordance with the Association for Research in Vision and Ophthalmology statement on the use of animals in ophthalmic research and approved by The Jackson Laboratory Animal Care and Use Committee. Mice were housed in a 14-hour light/10-hour dark cycle under previously described conditions (Smith *et al.*, 2000). All DBA/2J mice used were obtained from either The Jackson Laboratory production facility (Bar Harbor, ME) or from the John Lab research colony. This colony is routinely crossed with DBA/2J mice from The Jackson Laboratory production facility to prevent genetic drift. Details for D2.*C1qa*$^{+/-}$ mice have been described previously (Botto *et al.*, 1998; Howell *et al.*, 2011a). Cohorts of *C1qa*-deficient DBA/2J mice (D2.*C1qa*$^{-/-}$) were generated by intercrossing D2.*C1qa*$^{+/-}$ mice. Endothelin deficient mice for assessment of the *Edn2* riboprobes were obtained from our colony (Reinholdt *et al.*, 2012).

Gene expression analysis

We previously collected a large dataset of gene expression changes in the ONH across different stages of glaucoma (Howell *et al.*, 2011a; Howell *et al.*, 2011b; Howell *et al.*, 2012a). Briefly, we previously performed gene expression analysis on eyes from female DBA/2J mice at 4, 8 and 10.5 months of age. Eyes from mice at 4 and 8 months of age showed no glaucoma where as eyes at 10.5 months of age had a range of glaucoma from no glaucoma to severe glaucoma (see *Analysis of glaucomatous damage* below). We used hierarchical clustering to determine temporally ordered, molecular stages of disease that were termed stage 1a, 1b, 1c, 2, 3, 4 and 5. Stage 1a was most similar to no glaucoma controls. Stages 1a-2 contained eyes with no morphological signs of axon damage (as judged by PPD staining). This dataset can be interrogated for specific genes to support many studies and is publicly available at GEO DataSets (GSE26299). We interrogated this dataset to determine the expression levels of *Edn1*, *Edn2*, endothelin 3 (*Edn3)*, *Ednra* and *Ednrb*.

RNA in situ hybridization and immunofluorescence

RNA in situ hybridization was performed as previously described (Soto *et al.*, 2008; Howell *et al.*, 2011a), using 4% PFA perfusion-fixed, 12-μm-thick tissue sections. Digoxigenin-labeled (DIG-labeled) riboprobes for *Ednra*, *Edrnb* and *Edn2* were transcribed from cDNA clones (Open Biosystems clone ID: 2812426, 4971909, and 4512195 respectively). For anti-sense probes, *Ednra* and *Ednrb* plasmids were digested with *Sal*I, and the *Edn2* plasmid was digested with *Eco*R1. In vitro transcription was performed with T7 polymerase. For sense control probes, *Ednra* and *Ednrb* plasmids were digested with *NotI* and the *Edn2* plasmid was digested with *Hind*III. In vitro transcription was performed with SP6 polymerase. No signal was observed for the *Edn2* antisense probe in mice lacking *Edn2 (Supplemental*

Figure S1) (Saida *et al.*, 2002). In all cases, the sense control probe showed no signal (see Supplemental Figures S1 and S2). Details for the *C1qa* riboprobe have been described previously (Howell *et al.*, 2011a).

The detection of hybridized mRNA in sections was performed using the Cy-3 Tyramide Signal Amplification System (PerkinElmer). After in situ hybridization, the sections were incubated in the primary antibodies: rabbit anti-IBA1 (1:500, Wako), chicken anti-GFAP (1:500, Abcam), rat anti-EMCN (1:50, Santa Cruz). Primary antibodies were diluted in a solution of 10% normal goat serum, 0.5% Triton X-100, and 0.5% BSA in 0.1 M PBS. For the secondary antibodies, goat anti-mouse Alexa Fluor 647, goat anti-rabbit Alexa Fluor 488, goat anti-chicken Alexa Flour 633 or goat anti-rat Alexa Flour 488 were used at a 1:1000 dilution (Invitrogen). The sections were then incubated with DAPI (Invitrogen) and mounted in Fluoromount (Sigma-Aldrich). Fluorescence was visualized using a SP5 confocal microscope (Leica). Images were processed in Fiji (formerly ImageJ). For each probe/antibody at least three sections taken from at least six eyes were assessed. With the exception of the control eyes from the D2.*Edn2*$^{-/-}$ mice (which were collected from pre-wean pups), all eyes assessed had no glaucoma and were from DBA/2J mice between 9 and 10.5 months of age (as judged by PPD staining, see *Analysis of glaucomatous damage* below).

Bosentan and Macitenan administration

Bosentan or Macitentan (Actelion Pharmaceuticals) was incorporated into standard mouse chow (Bosentan: 100 mg/kg, Test Diet; Macitentan: 30 mg/kg, Test Diet). DBA/2J mice were administered Macitentan from 6 months of age and assessed for glaucoma at 10.5 and 12 months of age. Results were compared to our previous study using Bosentan (Howell *et al.*, 2011a). Number of eyes were: 10.5 months; Control=42, Bos=54, Mac=50. 12 months; Control=58, Bos=58, Mac=54. D2.*C1q*$^{-/-}$ mice were administered Bosentan from 6 months of age and assessed for glaucoma at 12 months of age and compared to our previous study of D2.*C1qa*$^{-/-}$ mice on regular chow and DBA/2J mice fed Bosentan-contain chow (Howell *et al.*, 2011a). Number of eyes were: Control=58, Bos=54, *C1qa* deficient = 56, Bosentan treated *C1qa* deficient = 40. As endothelin receptor antagonists can decrease blood pressure in hypertensive individuals, a separate cohort of 11 mice were administered Macitentan for 2 weeks, and blood pressures were measured using previously described procedures (Sugiyama *et al.*, 2002). Blood pressure was slightly lower in the Macitentan treated group (BP, mmHg ± SEM: 108.18 ± 1.2 treated; 111.97 ± 1.6 control, $P = 0.034$), but the slight decrease is not expected to affect neurodegeneration. Bosentan has no effect on blood pressure (Howell*et al.*, 2011a).

Clinical examination and IOP measurements

DBA/2J mice develop an iris disease that results in elevation of intraocular pressure and glaucoma. They develop high IOP as a result of an iris pigment disease in the front of the eye (Anderson *et al.*, 2002). IOP elevation leads to glaucoma in approximately 65-80% of eyes by 12-14 months of age (reviewed in (Libby *et al.*, 2005a)). At least 40 IOP measurements at each time point are collected and the IOP profile assessed at each age/genotype/treatment group as a population. This is because no single IOP measurement is

indicative of total IOP insult or glaucoma status. It is not possible to assess the IOP profile of a single eye across multiple ages in DBA/2J mice as the IOP measurement involves cannulation (John *et al.*, 1997; Savinova *et al.*, 2001). Measurements with the Tonolab are not reliable in aged DBA/2J mice due to corneal changes that develop with age. Previous studies have confirmed that glaucoma in DBA/2J mice is as a result of ocular hypertension because lowering IOP protects from glaucomatous damage (Schuettauf *et al.*, 2002; Matsubara *et al.*, 2006; Wong & Brown, 2012; 2013).

Clinical examinations assessing the iris disease were via slit lamp (Anderson *et al.*, 2002) and cannulated IOP measurements were performed as previously described (John *et al.*, 1997; Savinova *et al.*, 2001). For clinical examinations, at least 40 eyes from each genotype or treatment group were examined at 8.5, 10.5, and 12 months of age. IOP measurements were taken at 4.0, 10.5 and 12.0 months of age.

Analysis of glaucomatous damage

Intracranial portions of optic nerves were processed and analyzed as previously described (Anderson *et al.*, 2005; Libby *et al.*, 2005a; Libby *et al.*, 2005b; Anderson *et al.*, 2006). Briefly, optic nerves were fixed in 4% paraformaldehyde for 48 hours, dissected free, processed, and embedded in plastic. One micrometer-thick cross sections of optic nerve from behind the orbit were cut and stained with paraphenylenediamine (PPD). PPD darkly stains the myelin sheaths and axoplasm of sick or dying axons, but only lightly stains healthy axons. Two masked investigators determined the degree of nerve damage as previously described (Anderson *et al.*, 2005; Libby *et al.*, 2005b; Howell *et al.*, 2007a; Howell *et al.*, 2007b). If necessary, a third masked investigator settled any instances of disparity between the first two investigators with the consensus damage level being assigned (Anderson *et al.*, 2005; Libby *et al.*, 2005b; Howell *et al.*, 2007a; Howell *et al.*, 2007b). Damage level was assigned based on axon damage and scarring as the result of gliosis. In nerves with no glaucoma, there is no detectable axon loss and the number of damaged axons is indistinguishable from age-matched control mice that do not develop glaucoma (Anderson *et al.*, 2005; Libby *et al.*, 2005b; Howell *et al.*, 2007a; Howell *et al.*, 2007b). Nerves with moderate glaucoma have conspicuous damage, but the majority of axons are healthy, with an average of 30% axon loss. Nerves with severe glaucoma have substantial damage, with more than 50% of the axons lost. Each age group contained samples from males and females, as well as left and right nerves.

Results

Multiple cell types in the ONH express Edn2

We have previously used gene expression profiling combined with computational approaches to identify molecular stages of disease in DBA/2J mice that precede conventionally detectable damage (Howell *et al.*, 2011a; Howell *et al.*, 2012b). This identified seven, temporally ordered stages of glaucoma that occur in the ONH (see Methods for more detail). The first five stages (termed stage 1a, 1b, 1c, 2 and 3) are early stages that result from expression changes that precede axon loss and other conventional signs of glaucoma including gliosis. Stage 3 is noteworthy as eyes at this stage are about to

experience damage and axon loss. Stage 4 and 5 are largely comprised of eyes with moderate and severe glaucoma, respectively. We interrogated this dataset for expression levels of the three endothelin ligands (Figure 1). *Edn2* was first significantly upregulated in stage 2. By stage 3, when eyes are about to experience damage, *Edn2* had undergone a greater than 10 fold increase in expression compared to the no glaucoma control group (D2-*Gpnmb*$^{+}$, (Howell *et al.*, 2007b). This high expression is maintained through stages 4 and 5 (Figure 1). *Edn1* and *Edn3* only increased very modestly in expression (<1.5 fold) compared to controls and in only one or two stages (1c and 5 for *Edn1*, and 1c for *Edn3*). Therefore, *Edn2* is the major endothelin ligand upregulated in the ONH during DBA2J glaucoma.

To determine the cell types in the ONH expressing *Edn2*, we preformed RNA in situ hybridization using a riboprobe for *Edn2* (Supplemental Figure S1). Many *Edn2*+ cells were observed in the optic nerve head of at least 6 eyes prior to glaucomatous damage from 9-10.5 months old DBA/2J mice. Using RNA in situ hybridization in combination with immunofluorescence we determined that *Edn2* was expressed in IBA1+ cells (Figure 2) and GFAP+ cells (Figure 3). This is the first observation of EDN2+ astrocytes in DBA/2J glaucoma. As expected given the regional nature of glaucoma (May & Mittag, 2006; Schlamp *et al.*, 2006; Howell *et al.*, 2007b) and the plasticity of glial cells (Sofroniew, 2009; Perry *et al.*, 2010), Edn2 was expressed in only a subset of glial cells.

Endothelin receptors localize to astrocytes and endothelial cells in the ONH

To begin to understand the impact of an upregulation of *Edn2* in the ONH in early stages of DBA/2J glaucoma, we assessed the expression of its receptors *Ednra* and *Ednrb*. Both receptors are significantly upregulated compared to controls during early stages of glaucoma in the ONH (*Ednra*, >1.5 fold stages 1c and 2; *Ednrb*, ≥ 1.5 fold stages 1b, 1c and 2; Figure 4). To determine the cell types expressing the endothelin receptors, we performed RNA in situ hybridization using riborobes for *Ednra* and *Ednrb* in combination with immunofluorescence. We assessed expression in at least three sections from 6 eyes with no glaucoma obtained from 9-10.5 months old DBA/2J mice. *Ednra* is expressed by endothelial cells in the ONH (Figure 5) consistent with a role in mediating early vascular changes that we have previously reported in DBA/2J mice (Howell *et al.*, 2011a). No significant expression of *Ednra* was observed in other cells types including astrocytes or microglia. *Ednrb* is expressed in astrocytes in the ONH (Figure 6).

Targeting the endothelin system as a possible treatment for glaucoma

Targeting the endothelin system can lessen RGC loss in animal models relevant to glaucoma (Howell *et al.*, 2011a; Minton *et al.*, 2012). To further explore the potential for targeting the endothelin system for glaucoma, we first tested Macitentan (Iglarz *et al.*, 2008; Bolli *et al.*, 2012). Macitentan is a derivative of the well-established dual endothelin receptor antagonist Bosentan. Macitentan was developed to be more efficacious at lower concentrations than Bosentan. When considering therapeutic strategies, lower efficacious doses often improve safety, as there is a greater window between the effective dose and the first non-tolerated dose. Therefore, we tested the potential for Macitentan to protect DBA/2J mice from glaucoma at a dose three times lower than that used for Bosentan (see Methods). Macitentan

was administered to DBA/2J mice from 6 months of age and clinical examinations, IOP levels and RGC loss were assessed between 10.5 and 12 months of age.

Macitentan had no effect on either the development or progression of the DBA/2J iris disease that leads to IOP elevation and glaucoma (data not shown). Furthermore, IOP profiles of mice fed Macitentan were similar to DBA/2J mice fed control chow (Figure 7A). In contrast, DBA/2J mice fed Macitenan had significantly less RGC loss as evident by less optic nerve damage compared to DBA/2J mice on control chow (Figure 7B-C). At 10.5 months of age, approximately 80% of eyes from mice fed Macitentan showed no glaucoma compared to approximately 50% of eyes for mice fed control chow ($P=1.4\times10^{-7}$). By 12 months of age, approximately 45% of eyes from mice fed Macitentan showed no glaucoma compared to approximately 35% of eyes from mice fed control chow (P=0.05). These results further support the potential for targeting the endothelin system as a therapy for human glaucoma.

Targeting the endothelin system alone is not sufficient to protect all eyes from glaucoma. Inhibiting multiple processes that occur during early stages of glaucoma is likely to be a more powerful strategy to protect from glaucoma. The complement cascade is one of the earliest processes upregulated in glaucoma (Stasi *et al.*, 2006; Steele *et al.*, 2006; Johnson*et al.*, 2007; Stevens *et al.*, 2007) but again its blockade using mutants for complement component C1QA is not sufficient to prevent all glaucoma (Howell *et al.*, 2011a). IBA1+ cells in the ONH express both *Edn2* and *C1qa* (Figure 2 and (Howell *et al.*, 2011a) and using two color RNA in situ hybridization we show that a subset set of cells express both *Edn2* and *C1qa* (Figures 8A-F). However, many cells express either *Edn2* or *C1qa*, providing further support for different subpopulations of IBA1+ cells (including resident microglia and infiltrating monocytes) being present in the ONH in glaucoma (Howell *et al.*, 2012b). To determine if combinatorial blockade of the endothelin and complement pathways is more effective than either single intervention, we administered Bosentan to *C1qa* mutant DBA/2J mice. Blocking both the endothelin system and the classical complement cascade is profoundly protective at 12 months of age (80% of eyes had no detectable glaucoma), significantly more protective than blocking either pathway alone (Figure 8G-H).

Discussion

The endothelin system has been implicated in human and animal models of glaucoma but its exact roles are not clear. Here, we assess expression and cell type localization of key components of the endothelin system during early stages of glaucoma in DBA/2J mice. The major ligand that is upregulated in DBA/2J mice prior to significant RGC loss is EDN2. In the retina, EDN2 is uniquely expressed in IBA1+ cells in DBA/2J mice (Howell *et al.*, 2011a). Interestingly EDN2 is expressed in two different cell types in the ONH including IBA1+ microglia/monocytes (Figure 2) and GFAP+ astrocytes (Figure 3). IBA1+ cells include resident microglia and blood-derived monocytes. Blocking the entry of infiltrating monocytes appears protective against glaucoma and this may be in part due to the reduction in cells expressing damaging molecules such as EDN2 (Howell*et al.*, 2012b). However, the current study is the first report of EDN2 in ONH astrocytes in glaucoma. Previous studies have shown the presence of endothelin ligands and their receptors in astrocytes in the brain

(Ehrenreich, 1999; Blomstrand *et al.*, 2004). It is thought that the 'astrocytic endothelin system' may modulate astroglial activation, proliferation and differentiation (Supattapone *et al.*, 1989; Hama *et al.*, 1992; Koyama *et al.*, 1993; Stanimirovic *et al.*, 1995; Cazaubon *et al.*, 1997), processes shown to occur in the ONH early in glaucoma (Hernandez *et al.*, 2002; Johnson *et al.*, 2010).

The endothelin ligands act through binding to two receptors, EDNRA and/or EDNRB. Our gene expression data suggest both *Ednra* and *Ednrb* are upregulated prior to significant RGC axon damage in the ONH and so both could play key roles in glaucoma. Here, we show that *Ednra* is expressed in endothelial cells in DBA/2J mice. The endothelin system can impact vascular-related diseases including hypertension, atherosclerosis and vasospasm after subarachnoid hemorrhage (reviewed in (Kedzierski & Yanagisawa, 2001). We have also shown previously that vascular dysfunction occurs early in DBA/2J glaucoma (Howell *et al.*, 2011a) and our data here suggest this could be mediated through EDNRA expressed on vascular endothelial cells. *Ednrb* is expressed in astrocytes in the ONH in early stages of DBA/2J glaucoma. The endothelin system can play a role in the activation of astrocytes (Rogers *et al.*, 2003; Prasanna *et al.*, 2011) and astrocyte activation is thought to be critical in early changes in the ONH in response to IOP elevation (reviewed in (Nickells *et al.*, 2012). Others have shown the presence of EDNRB on RGCs suggesting an upregulation of the endothelin system in response to IOP elevation could directly impact RGCs (Minton *et al.*, 2012). In support of this, injections of EDN1 peptide into the vitreous can directly affect axon transport in RGCs (Stokely *et al.*, 2002; Taniguchi *et al.*, 2006). Axon transport is reported to be a very early sign of dysfunction to RGCs in glaucoma (e.g. (Anderson & Hendrickson, 1974; Almasieh *et al.*, 2012), although not necessarily prior to glial cell activation. *Ednrb*-deficient rats show reduced RGC loss compared to their wild-type counterparts in an inducible model of glaucoma (Minton *et al.*, 2012). Critically, it is not known whether the protection is mediated directly by signaling through EDNRB on RGCs or indirectly through, endothelin receptors on other cell types such as astrocytes or endothelial cells. To fully determine this, it would be necessary to ablate endothelin ligands and receptors in specific cell types and assess axon transport, RGC loss and other glaucoma relevant phenotypes.

Both Bosentan (Tracleer®) and Macitentan (Opsumit®) are approved by the food and drug administration (FDA) for the treatment of pulmonary arterial hypertension (PAH) (Patel & McKeage, 2014). In our studies, these endothelin receptor antagonists have been provided *ad libitum* in the mouse chow (100 mg/kg for Bosentan and 30 mg/kg for Macitentan). At these doses, significant protection against glaucoma is observed but they appear to slow glaucoma more effectively than prevent it completely. A delay in the progression of glaucoma for even a few years would greatly improve the lives of many glaucoma patients. However, it may also be possible to improve on the current treatment regimen. For instance, higher concentrations of Macitentan or Bosentan may protect more eyes from glaucoma for a longer period of time. Also different routes of administration could be explored. For patients with PAH, Tracleer and Opsumit are provided in tablet form. This could be an effective treatment route for glaucoma, but an alternative route for glaucoma would be in the form of eye drops. It remains to be seen whether administering Bosentan or Macitentan to

the eye directly is more protective than administration in food. Although more research is needed, it is clear that targeting the endothelin system with receptor antagonists such as Tracleer or Opsumit is a promising avenue for the treatment of human glaucoma.

Given the complexity of glaucoma, with involvement of diverse cell types and pathways, it is reasonable that combinatorial treatments will prove more efficacious than monotherapy. Combinatorial treatments that target multiple early pathways are likely to be the most effective, having great potential to transform the treatment of glaucoma. In this study, we show that targeting both the classical pathway of the complement cascade and the endothelin system protects more eyes from glaucoma than targeting either pathway individually. In fact, this combined targeting is profoundly protective with over 80% of eyes having no detectable glaucoma. However, the increased protection from the dual therapy maybe as a result of either a boosted response targeting a single process, or each therapy working independently. Inhibiting the endothelin system, either separately, or in combination with other pathways, offers exciting possibilities for developing novel neuroprotective therapies for glaucoma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank Marc Iglarz and Actelion for providing Bosentan and Macitentan and for help with experimental design. We thank Richard Libby for advice and reading this manuscript and Mimi de Vries for help with this study. This work was supported by NIH EY021525 (G.R.H.), donors of National Glaucoma Research – a program of the BrightFocus Foundation (G.R.H), NIH EY011721 (S.W.M.J.), and the Barbara and Joseph Cohen Foundation (S.W.M.J.). S.W.M.J. is an Investigator of the Howard Hughes Medical Institute.

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Research highlights

- Endothelin system components expressed in multiple cell types in glaucoma
- The endothelin system inhibitor Macitentan protects RGCs from glaucoma
- Combined inhibition of endothelin and complement systems protects from glaucoma

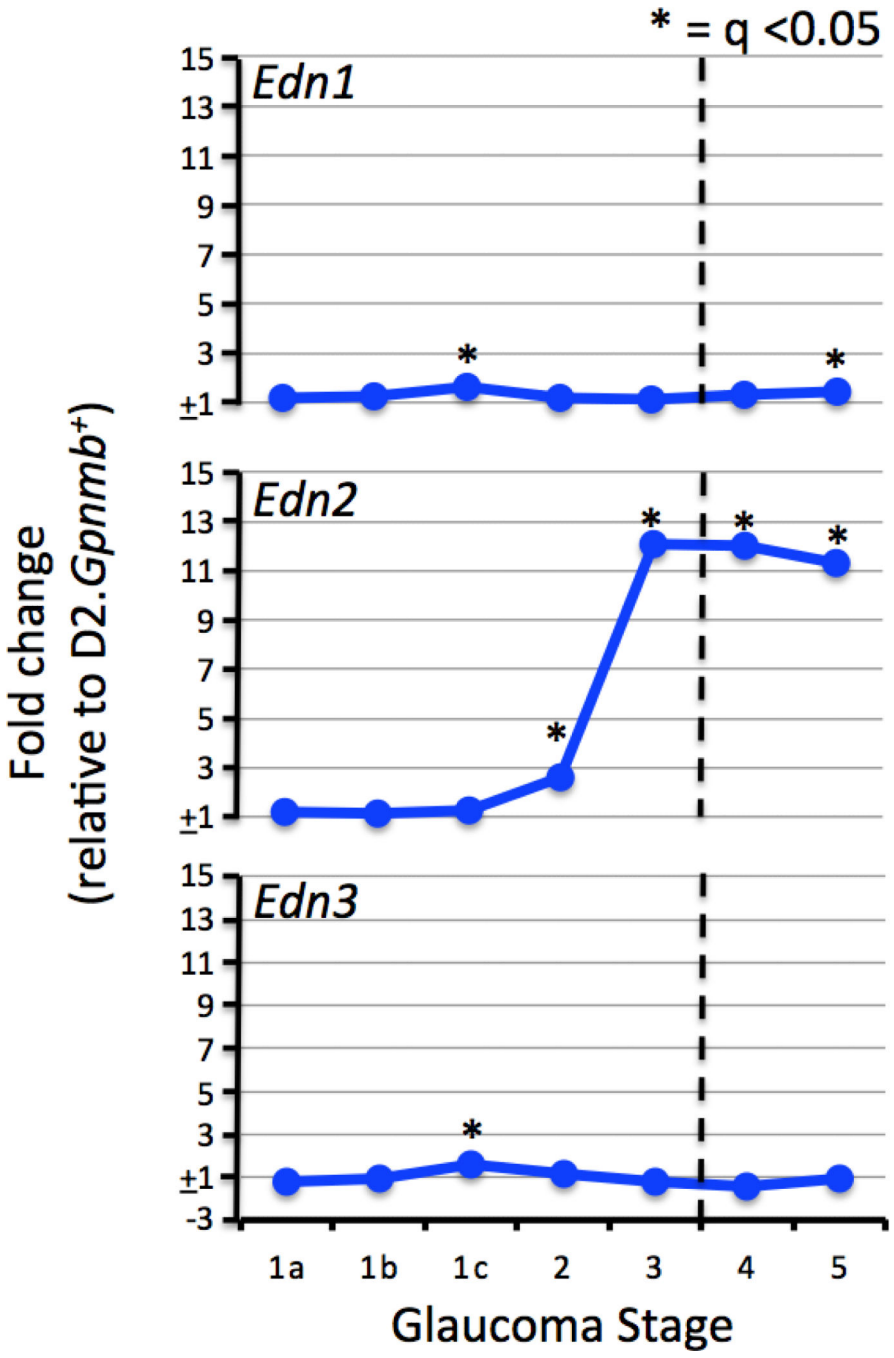

Figure 1. *Edn2* is the major ligand upregulated in the ONH in DBA/2J glaucoma

We interrogated our gene expression data (see Methods) to determine the expression levels of the three endothelin ligands (*Edn1*, *Edn2* and *Edn3*) at each stage (relative to the D2-*Gpnmb*$^+$ control group). *Edn2* was significantly upregulated (q<0.05, *) from stage 2 (2.5X) and continued to be significantly upregulated through stage 5, reaching a maximum of 12X in stage 2. *Edn1* and *Edn3* are only significantly upregulated in stage 1c. Stages 1a-2 represent stages of glaucoma that precede axon loss and axon transport deficiency (Howell *et al.*, 2012b; Williams *et al.*, 2013).

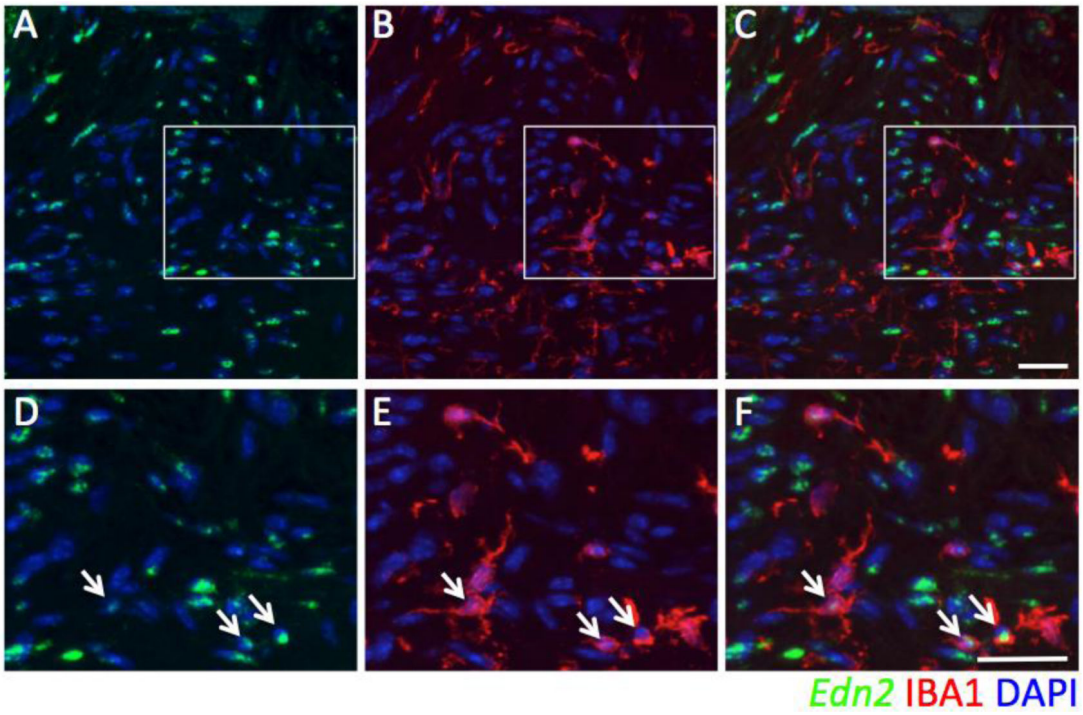

Figure 2. *Edn2* is localized to some IBA1+ cells in the ONH in DBA/2J mice

RNA *in situ* hybridization combined with immunofluorescence shows that *Edn2* is located in multiple cell types in the ONH in glaucoma (see also Figure 3). *Edn2* expression (green, riboprobe) occurs in some IBA1+ cells (marker for microglia/monocytes, red, antibody) in the ONH during DBA/2J glaucoma. All panels show the same eye from a DBA/2J mouse at 10.5 months of age. The boxed area in panels A-C is enlarged in panels D-F respectively. Arrows indicate IBA1+ cells that express EDN2. Bars = 25 μm.

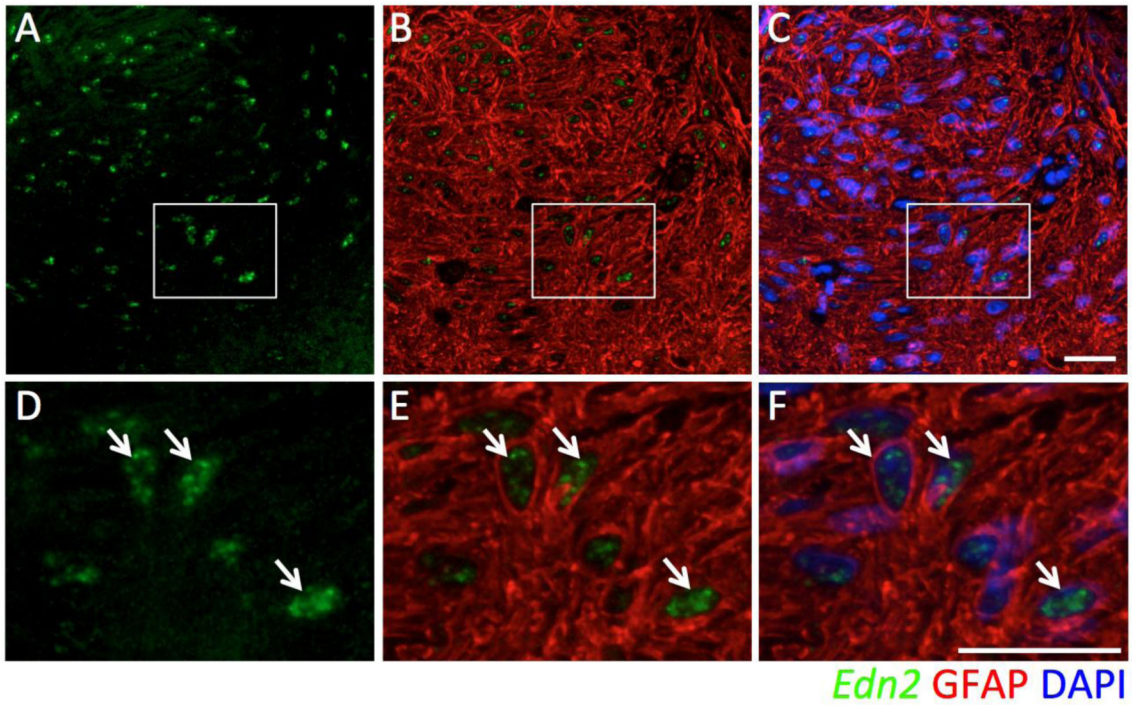

Figure 3. *Edn2* is localized to some GFAP+ cells in the ONH in DBA/2J mice

RNA in situ hybridization combined with immunofluorescence shows that *Edn2* expression (green, riboprobe) occurs in some GFAP+ cells (astrocyte marker, red, antibody) in the ONH during DBA/2J glaucoma. All panels show the same eye from a DBA/2J mouse at 10.5 months of age (same eye as shown in Figure 2). The boxed area in panels A-C is enlarged in panels D-F respectively. Arrows indicate GFAP+ cells that express EDN2. Bars = 25 μm.

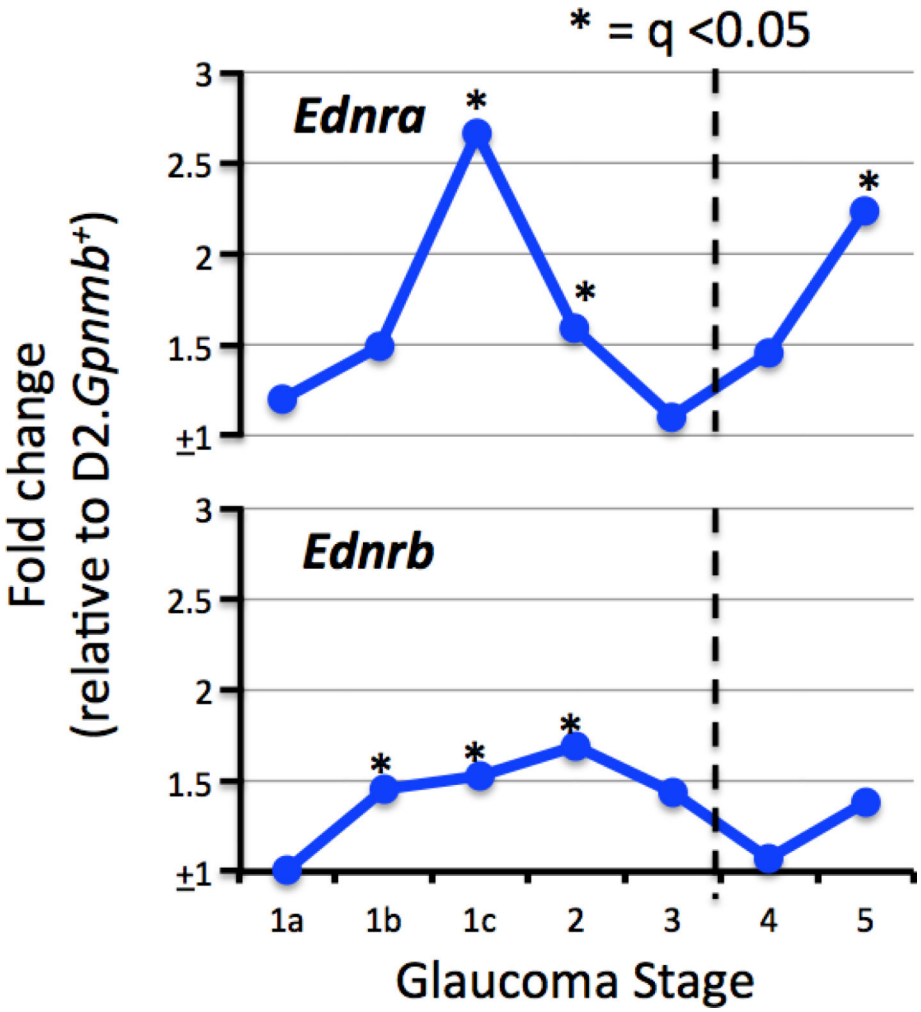

Figure 4. *Ednra* and *Endrb* are upregulated in multiple stages in the ONH in DBA/2J glaucoma

We interrogated our gene expression data (see Methods) to determine the expression levels of both endothelin receptors (*Ednra* and *Ednrb*) at each stage (relative to the D2-*Gpnmb*$^{+}$ control group). *Ednra* was significantly upregulated ($q<0.05$, *) in stage 1c (>2.5X), stage 2 (>1.5) and stage 5 (>2.0). *Ednrb* was greater than 1.5 fold upregulated in stages 1b, 1c and 2. This data suggests that both receptors may play key roles in mediating early changes in the ONH in DBA/2J glaucoma.

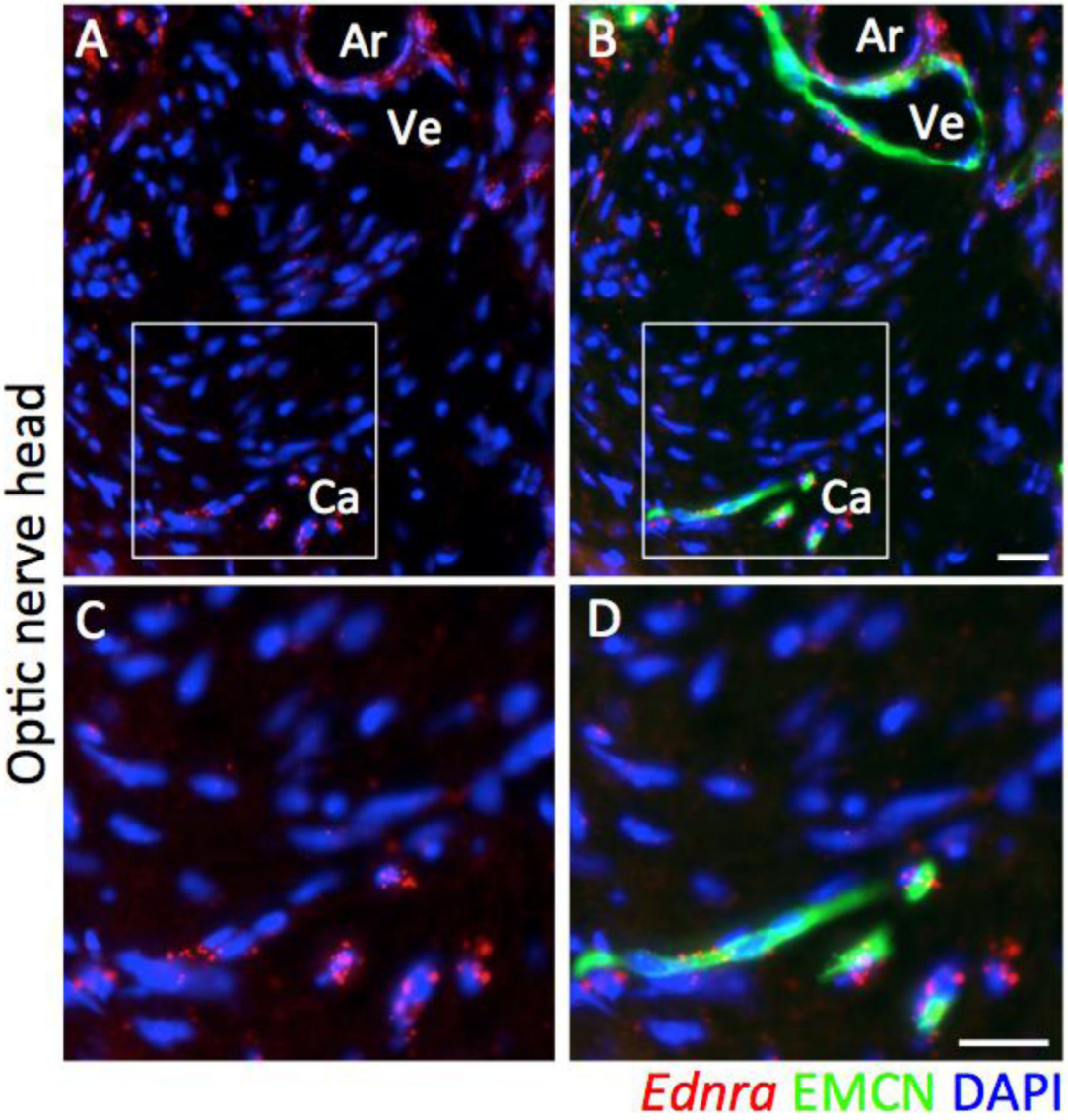

Figure 5. *Ednra* is expressed in vascular endothelial cells in the ONH

(**A-D**) *Ednra* (red, riboprobe) localizes to cells around the central retina artery (Ar, Endomucin negative, EMCN-), the central retinal vein (Ve, EMCN+) and capillaries (Ca) in the ONH. All panels show the same eye from a DBA/2J mouse at 10.5 months of age. The boxed area in panels A and B is enlarged in panels C and D respectively. Bars: A-B = 30 μm; C-D = 20 μm.

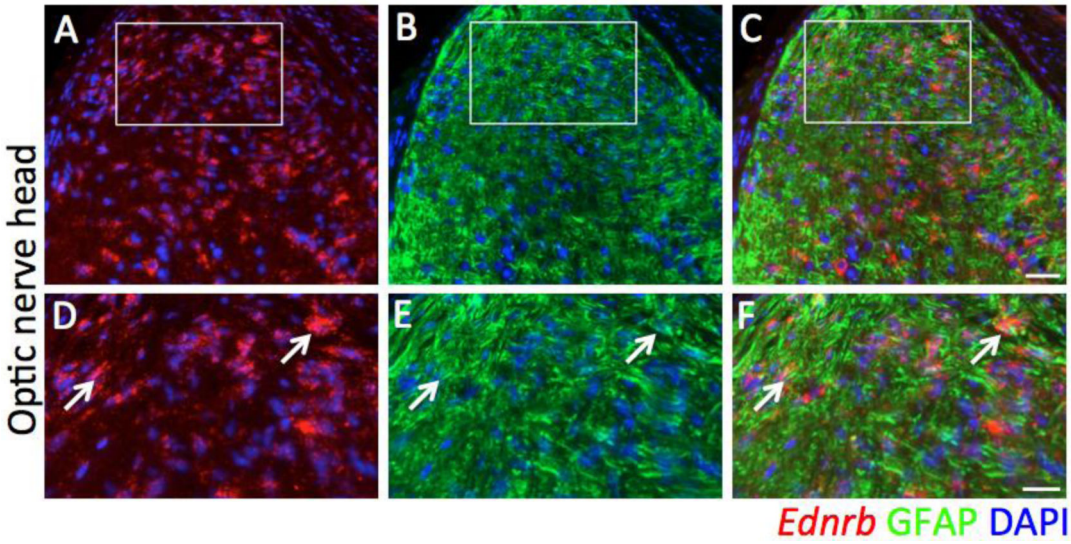

Figure 6. *Ednrb* is expressed in astrocytes in the ONH

Ednrb expression (red, riborobe) colocalizes with GFAP+ cells (astrocyte marker, green, antibody) in the optic nerve head (A-F). All panels show the same eye from a DBA/2J mouse at 10.5 months of age. The boxed area in panels A-C is enlarged in panels D-F respectively. Arrows indicate examples of cells expressing *Ednrb*. Bars: A-C = 30 μm; D-F = 10 μm.

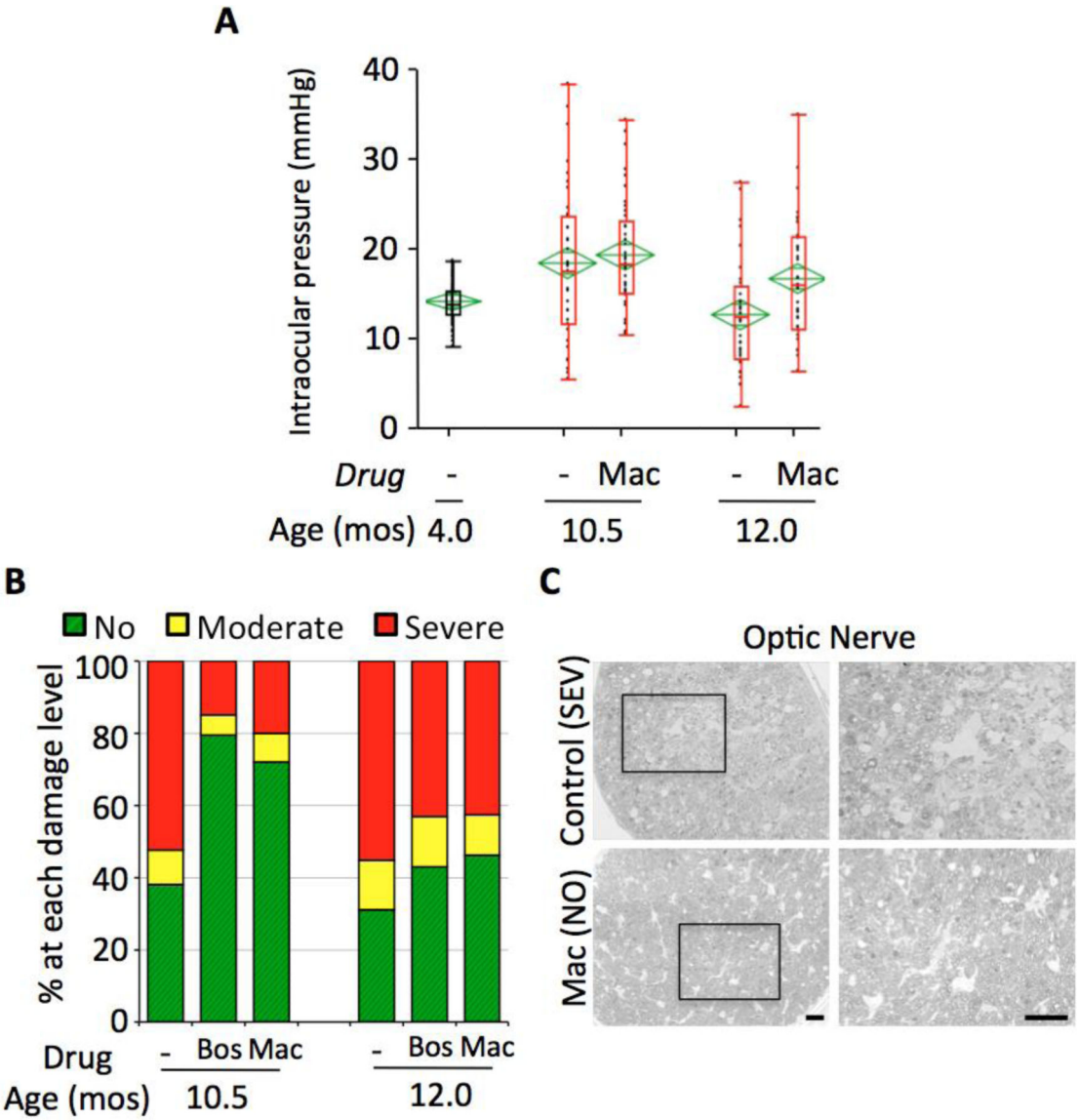

Figure 7. Macitentan protects DBA/2J eyes from glaucoma

(A) Age dependent elevation of IOP was found in both DBA/2J mice that received control chow (-) and chow enriched with 30 mg/kg of Macitentan (Mac). Boxplots were generated using JMP v7.0. The ends of each box represent the 75^{th} and 25^{th} percentile. The lines across the middle of each box indicate the median value. The whiskers extending from either end indicate the extent of the data points. Values falling outside the whiskers are considered outliers. The green diamonds indicate the mean and the 95% confidence interval. (**B**) Distributions of optic nerve damage show a significant increase in the number of eyes with no glaucoma (No) in eyes from Macitentan (Mac) mice compared to eyes from DBA/2J mice provided regular chow at 10.5 months ($P=1.4x10^{-7}$) and tending towards significance at 12.0 months (P=0.05). The protection was similar to that seen from our previous study using Bosentan (Bos, (Howell *et al.*, 2011a)) (**C**) Examples of the most common category of nerve damage for mice fed control and Macitentan chow. SEV = Severe. Scale bar = 50 μm.

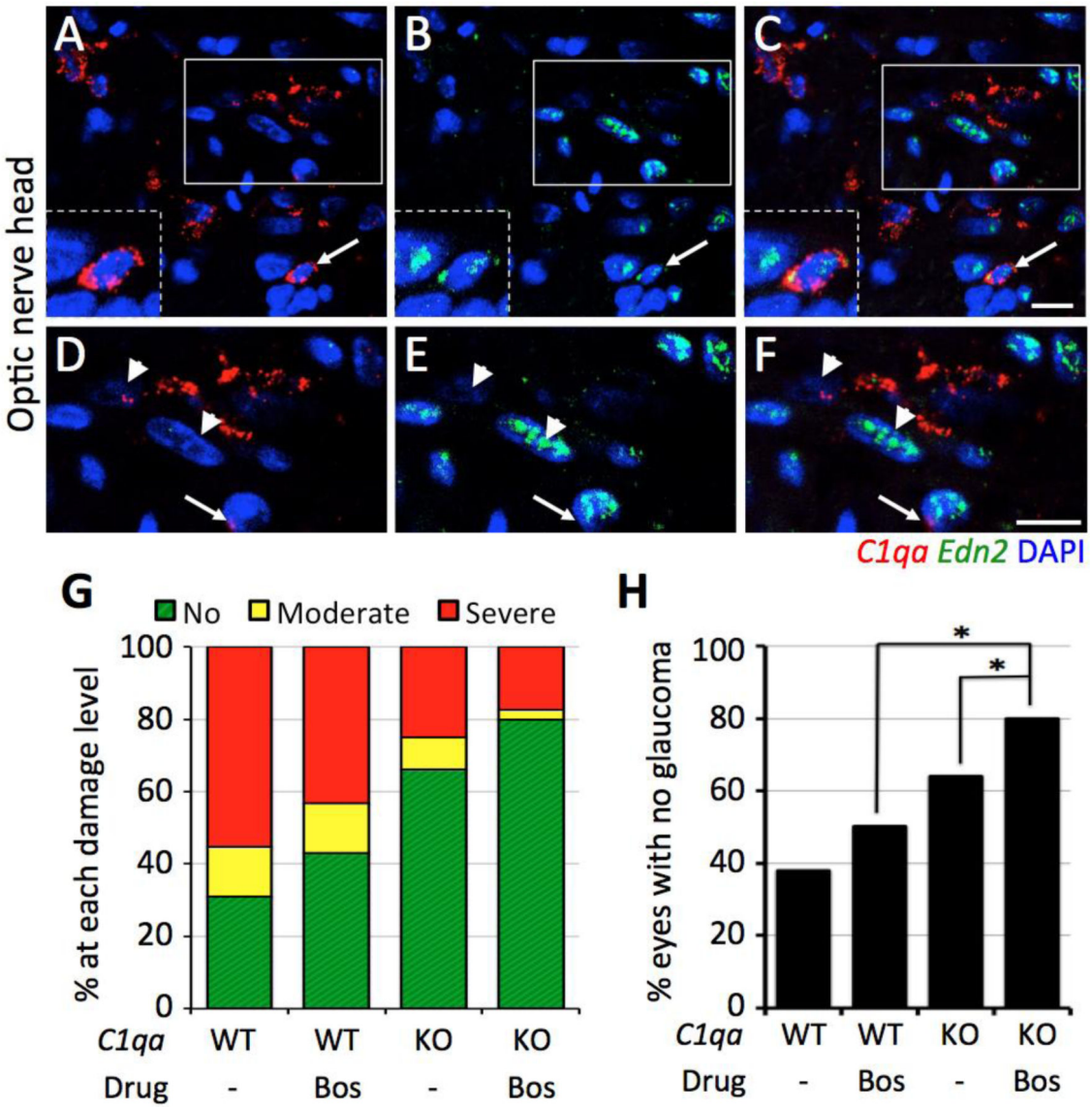

Figure 8. Targeting *C1qa* and the endothelin system together shows a greater protection than targeting them separately

(**A-F**) We have previously shown that *C1qa* is expressed in IBA1+ in the optic nerve head (Howell *et al.*, 2011a). Here, we show that *Edn2* is expressed in both IBA1+ and GFAP+ cells in the optic nerve head (Figure 2-3). Interestingly, *Edn2* (green, riboprobe) and *C1qa* (red, riboprobe) appear to be expressed in different cells (arrowheads) as well as the same cells (arrows). The dotted boxed area in panels A-C is an enlargement of the cell expressing both *C1qa* and *Edn2* (indicated by the arrow). The solid line boxed area in panels A-C is enlarged in panels D-F respectively. (**G**) Distribution of optic nerve damage at 12.0 months of age shows a significant increase in the number of eyes with no glaucoma (No) in eyes from Bosentan (Bos)-treated, C1qa-deficient (KO) mice compared to either eyes from DBA/2J mice ($P=1.2\times10^{-8}$), Bosentan-treated DBA/2J mice ($P=2.7\times10^{-5}$) or *C1qa*-

deficient mice (P=0.02). (H) The percentage of eyes with no glaucoma showing the significant protection in Bosentan-treated *C1qa* mutant eyes. * P<0.05. Bars = 10 μm.