BJP British Journal of Pharmacology

DOI:10.1111/bph.12136
www.brjpharmacol.org

Themed Section: Cannabinoids 2012, Part Two

RESEARCH PAPER

A GPR18-based signalling system regulates IOP in murine eye

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Keywords
GPR18; cannabinoid; THC; intraocular pressure; mouse; eye

Received
22 April 2012
Revised
17 January 2013
Accepted
7 February 2013

BACKGROUND AND PURPOSE

GPR18 is a recently deorphaned lipid receptor that is activated by the endogenous lipid *N*-arachidonoyl glycine (NAGly) as well the behaviourally inactive atypical cannabinoid, abnormal cannabidiol (Abn-CBD). The presence and/or function of any GPR18-based ocular signalling system remain essentially unstudied. The objectives of this research are: (i) to determine the disposition of GPR18 receptors and ligands in anterior murine eye, (ii) examine the effect of GPR18 activation on intraocular pressure (IOP) in a murine model, including knockout mice for CB_1, CB_2 and GPR55.

EXPERIMENTAL APPROACH

IOP was measured in mice following topical application of Abn-CBD, NAGly or the GPR55/GPR18 agonist O-1602, alone or with injection of the GPR18 antagonist, O-1918. GPR18 protein localization was assessed with immunohistochemistry. Endocannabinoids were measured using LC/MS-MS.

KEY RESULTS

GPR18 protein was expressed most prominently in the ciliary epithelium and the corneal epithelium and, interestingly, in the trabecular meshwork. The GPR18 ligand, NAGly, was also detected in mouse eye at a level comparable to that seen in the brain. Abn-CBD and NAGly, but not O-1602, significantly reduced IOP in all mice tested. The antagonist, O-1918, blocked the effects of Abn-CBD and NAGly.

CONCLUSIONS AND IMPLICATIONS

We present evidence for a functional GPR18-based signalling system in the murine anterior eye, including receptors and ligands. GPR18 agonists, Abn-CBD and NAGly, reduce IOP independently of CB_1, CB_2 or GPR55. These findings suggest that GPR18 may serve as a desirable target for the development of novel ocular hypotensive medications.

LINKED ARTICLES

This article is part of a themed section on Cannabinoids. To view the other articles in this section visit http://dx.doi.org/10.1111/bph.2013.169.issue-4 & http://dx.doi.org/10.1111/bph.2012.167.issue-8

Abbreviations

Abn-CBD, abnormal cannabidiol; AEA, arachidonoyl ethanolamide, anandamide; eCB, endocannabinoid; FAAH, fatty acid amide hydrolase; IOP, intraocular pressure; NAGly, *N*-arachidonoyl glycine; THC, tetrahydrocannabinol

Introduction

The human genome encodes ~1000 G protein-coupled receptors (GPCRs), a large family of transmembrane receptors that transduce extracellular signals into intracellular responses via coupling to G proteins. GPCRs are the target of a large percentage of modern medicinal drugs and are activated by a diversity of endogenous ligands including photons, odours, pheromones, hormones, neurotransmitters, as well as lipids.

One such endogenous lipid, anandamide (*N*-arachidonoyl ethanolamine; AEA), is produced throughout the body and was identified as the first endogenous cannabinoid (eCB) to activate cannabinoid CB_1 and CB_2 GPCRs (Devane *et al.*, 1992). It is established that eCBs and CB_1 receptors are abundant in the eye (Porcella *et al.*, 1998; 2000; Straiker *et al.*, 1999a,b; Chen *et al.*, 2005; Hu *et al.*, 2010), as are assorted cannabinoid-related proteins (Hu *et al.*, 2010). Importantly, cannabinoids have been shown to regulate intraocular pressure and are implicated in retinal signalling and health (Calignano *et al.*, 1998; Crandall *et al.*, 2007; Straiker *et al.*, 1999a; Straiker and Sullivan, 2003). Enzymatic production and inactivation of anandamide has been detected in several ocular tissues as well as the lacrimal gland (Matsuda *et al.*, 1997; Bisogno *et al.*, 1999). However, it has now become clear that 'cannabinoid' signalling consists of more than anandamide and CB_1/CB_2 [reviewed in (Pertwee, 2010)]. Several families of eCB-like lipids are present in the body at physiologically relevant levels and have been shown to induce functional effects (Calignano *et al.*, 1998; Lauffer *et al.*, 2009). Evidence also points to physiological roles for several cannabinoid-like orphan GPCRs, most notably GPR55, GPR119 and GPR18 (Mackie and Stella, 2006; Pertwee, 2010). The physiological role(s) these GPCRs fulfil in the eye remains essentially unstudied.

Recently, we have shown that *N*-arachidonoyl glycine (NAGly), an endogenous anandamide metabolite, is itself an extremely powerful signalling lipid that activates GPR18; NAGly is produced throughout the body (Huang *et al.*, 2001; Bradshaw *et al.*, 2009) by either of two distinct biosynthetic pathways, one of which occurs via fatty acid amide hydrolase (FAAH; Bradshaw *et al.*, 2009). Sub-nanomolar concentrations of NAGly potently drive directed migration, proliferation, and MAP kinase activation in both BV-2 microglia and HEC-1B endometrial cells via GPR18 receptors (McHugh *et al.*, 2010; 2012a). Given that both anandamide and FAAH have been detected in the eye, we explored whether a GPR18-based signalling system may be present in the anterior eye. Using immunohistochemical tools, we found that GPR18 is expressed in murine eye, in a pattern consistent with a potential role in regulation of intraocular pressure (IOP). Elevated IOP is indicated in many forms of glaucoma, a leading cause of blindness worldwide (Quigley and Broman, 2006). We now report the presence of a GPR18-based signalling system in the anterior eye of the mouse and demonstrate that this system regulates IOP.

Methods

Animals

Experiments were conducted at both the Dalhousie University and Indiana University campuses. All mice used for IOP experiments were handled according to the guidelines of the respective institutes' animal care committees. Mice were kept on a 12 h (07:00–19:00) light dark cycle, and fed *ad libitum*. C57BL/6J (C57) mice were obtained from Charles River Laboratories International Inc. (Wilmington, MA). Mice were 6–8 weeks of age and allowed to acclimatize to the animal care facility for at least a week prior to their use in experiments. A total of 136 animals were used in these experiments. $CB_1^{-/-}$, $CB_2^{-/-}$ and $GPR55^{-/-}$ mice were kindly provided by Dr. Ken Mackie (Indiana University, Bloomington, IN, USA). The authors declare that they have consulted the ARRIVE guidelines for *in vivo* research. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals. (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

Intraocular pressure measurements

IOP was measured in mice by rebound tonometry, using a Tonolab (Icare Finland Oy, Helsinki, Finland). This instrument uses a light plastic tipped probe to briefly make contact with the cornea; after the probe hits the eye, the instrument measures the speed at which it rebounds in order to calculate IOP (Cervino, 2006).

To obtain reproducible IOP measurements, mice were anaesthetized with isoflurane (4% induction). The anaesthetized mouse was then placed on a platform in a prone position, where anaesthesia was maintained with 2% isoflurane. IOP measurements were then made with 10 individual pressure readings taken from each eye. The pressure from each eye was then recorded as the average of these 10 measurements.

For diurnal IOP experiments, IOP was measured on the same day and from the same animal both early in the light cycle, between 09:00 and 09:30 (reported as 09:00), or early in the dark cycle, between 21:00–21:30 (reported as 21:00). Statistical analysis of these data was carried for each eye independently by paired Student's *t*-test, comparing the 09:00 and 21:00 measurements.

All IOP measurements following drug administration were recorded between 16:00 and 18:00 in order to reduce any variability in IOP resulting from diurnal changes. Drugs were applied topically to one randomly assigned eye, while the other eye received the appropriate vehicle. IOP measurements following drug administration were analysed by paired Student's *t*-test comparing the drug-treated eye to the vehicle-treated eye of the same animal.

Immunohistochemistry

After the animals were killed, their eyes were removed, and the anterior or posterior eye section cut away to form a posterior or anterior eyecup; the eyecup was fixed in 4% paraformaldehyde followed by a 30% sucrose immersion for 24–72 h at 4°C. Tissue was then frozen in OCT compound and sectioned (15–25 μm) using a Leica CM1850 cryostat. Tissue sections were mounted onto Superfrost-plus slides, washed, treated with a detergent (Triton-X100, 0.3% or saponin, 0.1%) and milk (5%), followed by primary antibodies overnight at 4°C. Secondary antibodies (Alexa 594 or Alexa 488, 1:500, Invitrogen, Inc., Carlsbad, CA, USA) were subsequently applied at room temperature for 1.5 h. The tyrosine hydroxylase (TH) antibody (Sigma-Aldrich, St. Louis, MO, USA; 1:500) is well-characterized (Gastinger *et al.*, 2006). TH retinal staining was limited to a characteristic population of amacrine cells and their processes in the distal inner plexiform layer (data not shown). The GPR18 antibody was the generous gift of Dr. Ken Mackie (Indiana University). The specificity of the GPR18 antibody (1:300) was characterized

by pre-incubation with the immunizing protein (3 μg mL^{-1}). Images were acquired with a Leica TCS SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany) using Leica LAS AF software and a 63X oil objective. Images were processed using ImageJ (available at http://rsbweb.nih.gov/ij/) and/or Photoshop (Adobe Inc., San Jose, CA, USA). Images were modified only in terms of brightness and contrast.

LC/MS-MS

Lipids were extracted as previously described (Bradshaw *et al.*, 2006). In brief, tissue was weighed, and placed on ice for 1 h in methanol (50 volumes to wet weight) and spiked with deuterium-labelled NAGly (Cayman Chemical, Ann Arbor, MI, USA). Samples were sonicated and then centrifuged for 20 min at 19 000× *g* at 24°C. HPLC-grade water (75% volume) was then added to the supernatant. Partial purification was achieved through the use of 100 mg Empore C18 solid phase extraction columns preconditioned with 5 mL HPLC methanol and 2.5 mL HPLC water. The cartridges were washed with 2.5 mL HPLC water and 1.5 mL 40% methanol and eluted with 1.5 mL 70%, 85% and 100% methanol. NAGly was detected in the 85% elution fraction. Each elution was vortexed before mass spectrometric analysis. Levels of NAGly were analysed by multiple reactions monitoring mode using an applied Biosystems/MDS Sciex triple quadruple mass spectrometer API 3000 (Foster City, CA, USA) with electrospray ionization. Samples were loaded using Shimadzu SCL10Avp auto-sampler and chromatographed on a 210 mm Zorbax Eclipse XDB-C18 reversed-phase HPLC column (3.5 μm internal diameter) maintained at 40°C. The flow rate was 200 μL min^{-1} achieved by a system comprised of a Shimadzu controller and two Shimadzu LC10ADvp pumps. The Shimadzu LC10ADvp HPLC pumps operated with a starting gradient of 0% mobile phase B that was increased to 100% before returning back to 0%. The mobile phase A consisted of 20/80 MeOH/water containing 1 mM ammonium acetate and mobile phase B consisted of 100% MeOH containing 1 mM ammonium acetate and 0.5% acetic acid. Levels of NAGly in tissue were determined by comparing the area of the curve for each chromatographic peak to a standard curve generated with synthesized standards. This value was then converted to moles per elution and then moles per gram of tissue.

Drugs

Abnormal cannabidiol (Abn-CBD). NAGly, O-1602, O-1918, Capsazepine and Tocrisolve were obtained from Tocris (Ellisville, MO, USA). Topically applied drugs were prepared 1% or 2% w/v by dilution in Tocrisolve. O-1918 and capsazepine were prepared as a 10 mM and 100 mM stock in DMSO, respectively, then diluted in a saline solution. O-1918 and capsazepine were administered intraperitoneally at 2 mg kg^{-1} 30 min prior to topical treatment with agonists. Injection volumes were 100 μL mouse^{-1} with maximal final DMSO volumes of 20 μL mouse^{-1}.

Results

GPR18 protein is expressed in cornea and ciliary epithelium

We explored whether GPR18 is expressed in anterior eye using an antibody developed against the GPR18 receptor. This antibody has been previously characterized (McHugh *et al.*, 2010). We found that GPR18 is abundantly expressed in several tissues of the anterior chamber of the mouse eye, with staining most prominent in ciliary and corneal epithelium. Staining was absent in sections pre-incubated with immunizing protein (3 μg mL^{-1}) (Figure 1). Closer examination of staining in the ciliary epithelium showed that the staining for GPR18 is present in both epithelial layers (Figure 2A-B).

We also examined the relative expression patterns of GPR18 and CB_1 receptors since both receptors are present in the same tissues of the anterior eye. On a gross scale, the expression pattern of GPR18 is similar to that of CB_1 but differs from that of CB_1 on closer examination. For instance, GPR18 staining is more restricted to the epithelial layers than CB_1, which, as we have previously reported, is also closely associated with blood vessels of the ciliary body in both the

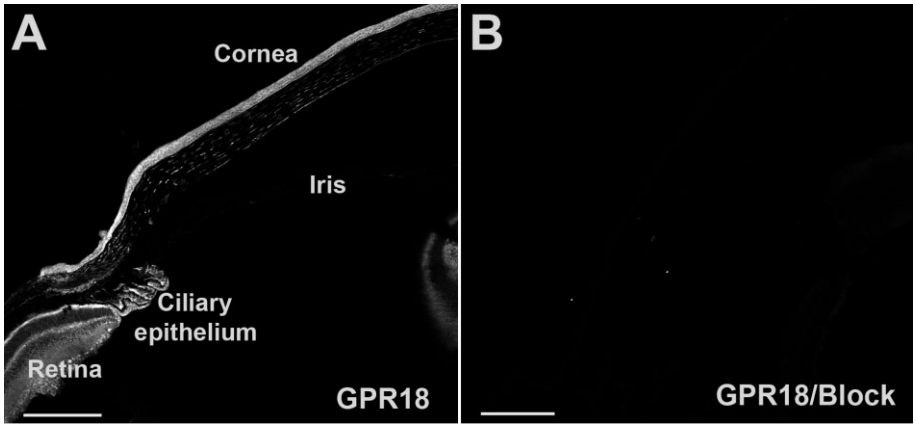

Figure 1

GPR18 staining is prominent in ciliary epithelium and corneal epithelium. (A) Low-magnification micrograph shows GPR18 staining in murine anterior eye with prominent signal in ciliary epithelium, cornea and retina. (B) GPR18 staining is absent in sections pre- and co-incubated with the immunizing protein (IP). Scale bars: 150 μm.

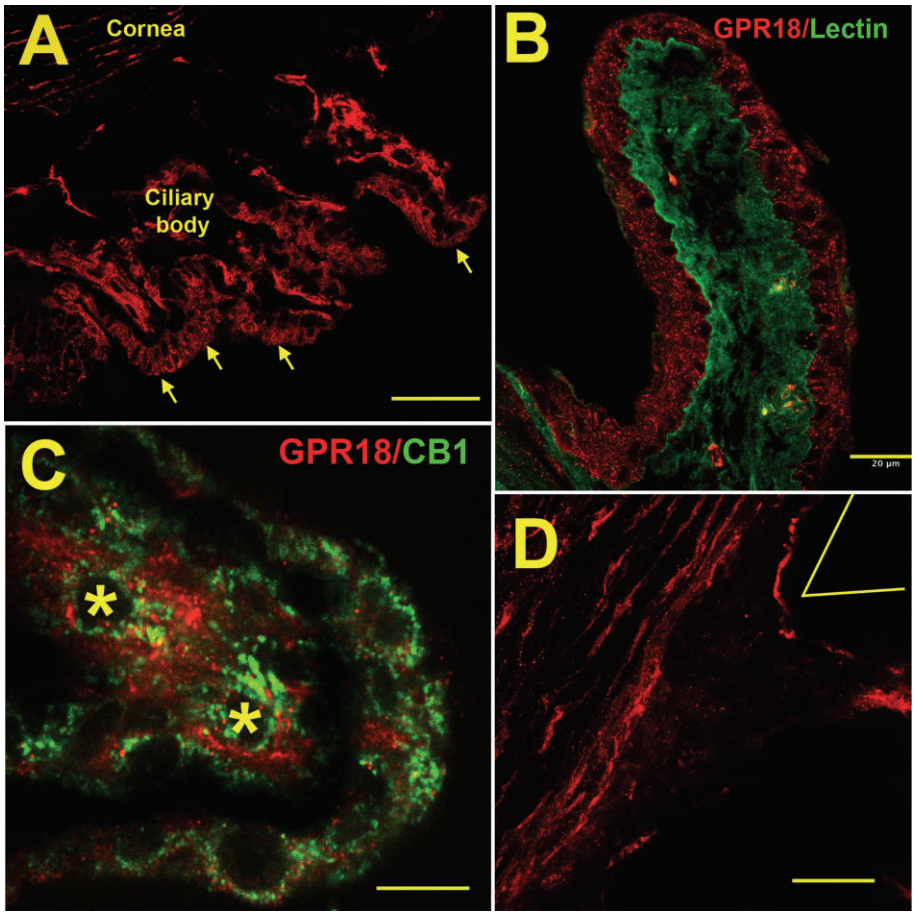

Figure 2

GPR18 staining is present in both layers of ciliary epithelium and in the angle. (A) GPR18 staining is detected in both the inner and outer layers of the ciliary epithelium (arrows). (B) GPR18 with ciliary body counterstain (green) highlights predominantly epithelial staining in ciliary body. (C) Co-staining for GPR18 and CB_1 (green) indicates that CB_1 is more closely associated with blood vessels (asterisk). (D) GPR18 staining is also seen in the angle, with a localization corresponding to trabecular meshwork (arrow). Scale bars: A: 40 µm B: 20 µm, C: 10 µm, D: 25 µm.

mouse and human (Figure 2C; Straiker *et al.*, 1999a; Hudson *et al.*, 2011). In the angle, GPR18 is associated with structures that correspond to trabecular meshwork (Figure 2D, arrow). This means that GPR18 localization is consistent with a potential role modulating either inflow or outflow of aqueous humour.

GPR18 is also prominently expressed in cornea, particularly the corneal epithelium, but also in the stroma and the endothelium (Figure 3). Stromal labelling overlaps somewhat with that for TH (Figure 3A) but not with CGRP (data not shown). The expression pattern is similar to that for CB_1, although complete overlap is seen only in the corneal endothelium (Figure 3B). Staining in iris was much less pronounced than in other regions of the anterior eye (Figure 3C). Both ciliary epithelial and corneal staining for GPR18 were similar in $CB1^{-/-}$ tissue (Supporting Information Figure S1).

GPR18 ligand NAGly is detected in anterior eye

The second component of a *bona fide* signalling system is the presence of endogenous ligands. As mentioned earlier, there is substantial evidence that NAGly acts via GPR18 and that it can be produced from anandamide precursors. We have previously measured eCB levels in rat retina (Straiker *et al.*, 1999a) and have developed lipid extraction and LC/MS-MS methods to reliably quantify lipids from the murine eye, which is technically more difficult than other tissues. Figure 4A shows the results for NAGly in murine anterior eye. The values (9.7×10 picomoles g^{-1}) are comparable to the 6×10^{-12} picomoles g^{-1} seen in whole brain (Bradshaw *et al.*, 2009). We also tested for the presence of anandamide (AEA) in the same samples. As shown in Figure 4B, anandamide is present in anterior eye tissues. Indeed, the levels (1.03 ± 0.06 nanomoles g^{-1} of tissue) are surprisingly high, since anandamide is generally found in picomoles g^{-1} of tissue in brain (Cravatt *et al.*, 2001).

GPR18 agonists reduce intraocular pressure in the mouse

We have recently shown that CB_1 receptor agonists reduce intraocular pressure in a normotensive murine model (Hudson *et al.*, 2011). Using cannabinoid receptor and related

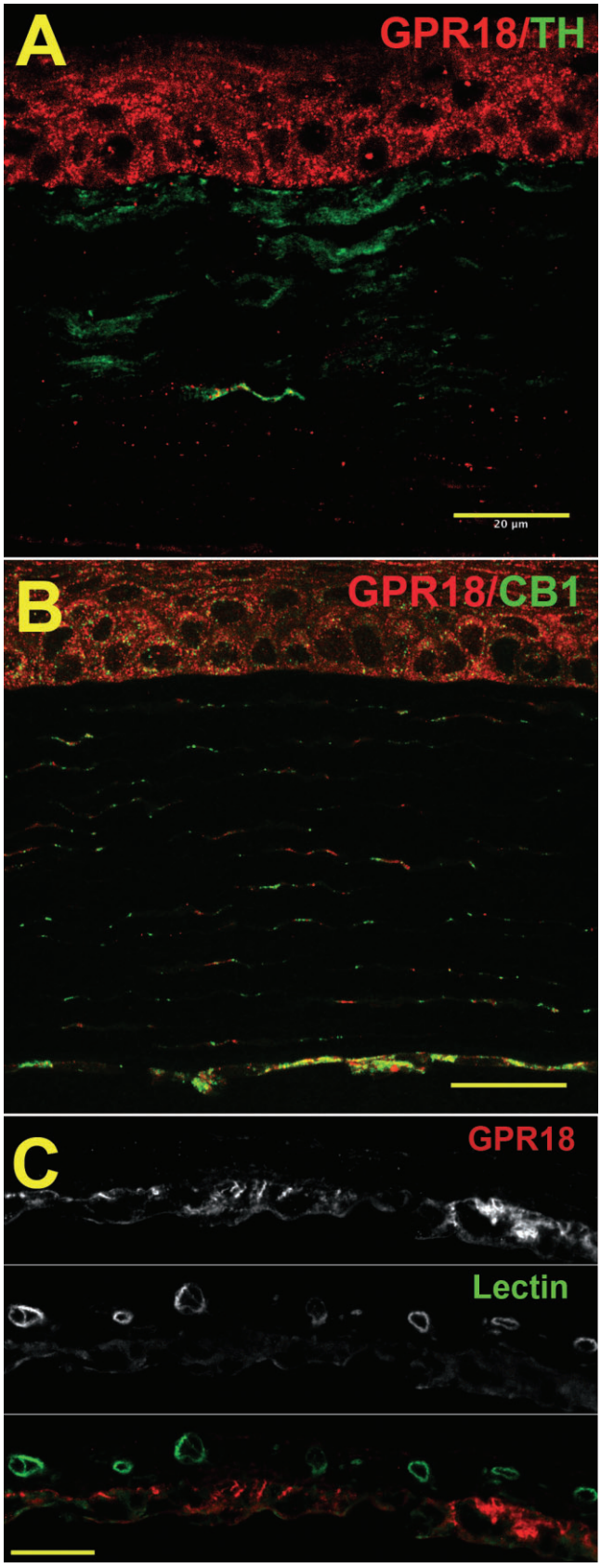

Figure 3

Strong GPR18 staining is present in corneal epithelium with modest staining in corneal stroma and iris. (A) GPR18 staining is prominent in all ciliary epithelial cells but is also present in the corneal stroma. This image shows association with TH positive staining in the stroma suggesting location at or near TH positive innervation of the cornea. (B) GPR18 is expressed in many of the same corneal cells as CB_1 including the corneal endothelium. (C) Modest staining in iris for GPR18 is not associated with blood vessels as labelled by lectin. Scale bars: A-B: 20 µm, C: 25 µm.

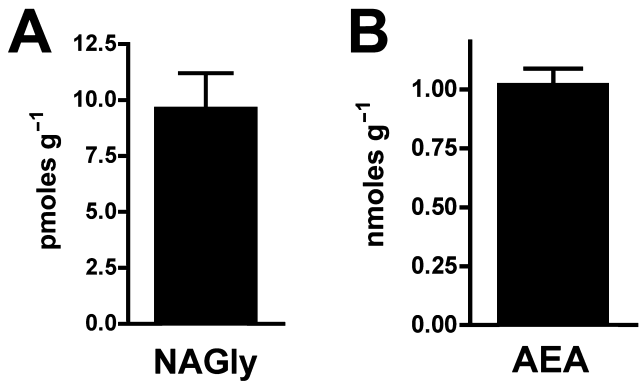

Figure 4

NAGly and anandamide are present in the anterior eye of the mouse. (A) NAGly levels measured in mouse anterior eye homogenate. (B) Anandamide (AEA) levels measured in mouse anterior eye homogenate.

knockouts for $CB_1^{-/-}$, $CB_2^{-/-}$ and $GPR55^{-/-}$, we confirmed the CB_1 receptor-dependence of this action. However, the identification of GPR18 receptors in locations associated with regulation of intraocular pressure led us to hypothesize that GPR18 activation may also alter IOP. Accordingly, we tested the GPR18 agonists, NAGly and Abn-CBD, as well as an assortment of related lipid receptor agonists and antagonists, for IOP effects.

We found that GPR18 agonists, Abn-CBD (2% w/v) and NAGly (1% w/v), significantly reduced IOP in C57 wild-type (WT) mice (Figure 5A–D; Abn-CBD: 1.17 ± 0.15 mmHg; NAGly: 0.72 ± 0.18 mmHg). A 2% w/v concentration was used for Abn-CBD but not O-1602 (see below) because at 1% the Abn-CBD trended towards an effect while O-1602 showed no sign of deviation from baseline IOP. NAGly, at 1% w/v, was sufficient to induce a clear effect. We also tested O-1602, a GPR55 agonist (Johns *et al.*, 2007) that has recently been shown to have activity at GPR18 (McHugh *et al.*, 2012c), but found that O-1602 (1%) did not reduce IOP (Figure 5E–F; 0.10 ± 0.10 mmHg). These reductions in IOP are very similar to those recently reported by us for commonly used glaucoma treatments such as timolol and latanoprost (Hudson *et al.*, 2011). The results further suggest that GPR18 activation may serve to reduce IOP in this normotensive murine model. It is possible that the actions of Abn-CBD and/or NAGly may be occurring through cannabinoid family lipid receptors: CB_1 or CB_2. To test this possibility, we examined the effects of

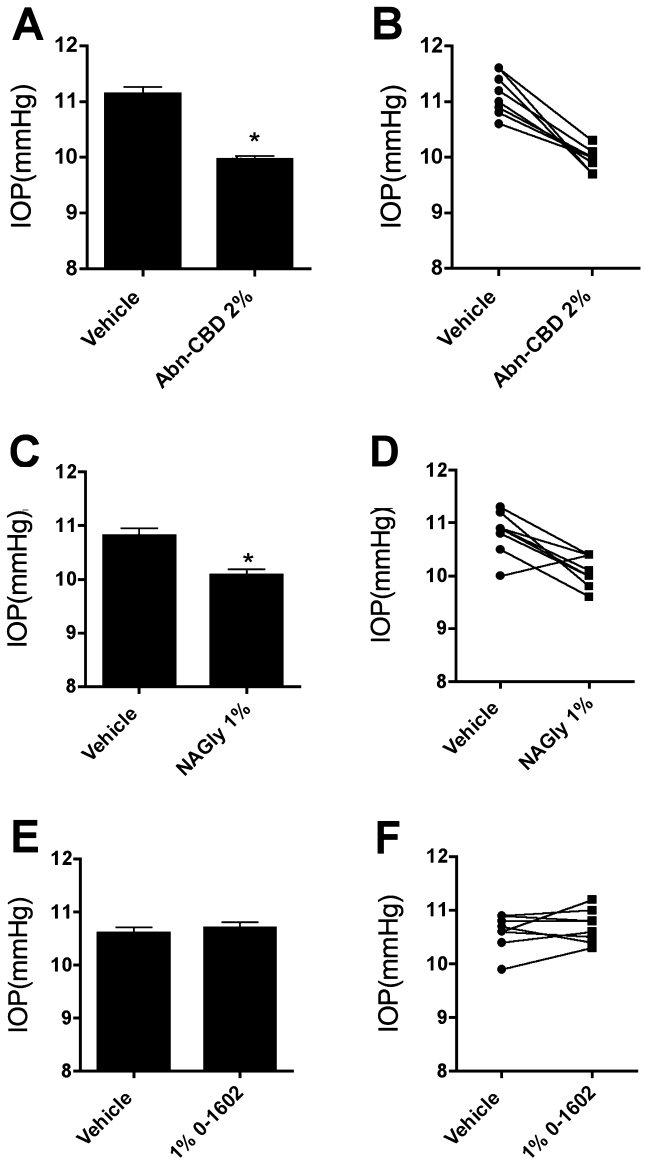

Figure 5

Topical application of GPR18 agonists *N*-arachidonylglycine (NAGly) or Abn-CBD reduces IOP whereas GPR55/GPR18 agonist O-1602 does not. (A) Mean IOP taken from *C57* mice 30 min after topical administration of 2.0% Abn-CBD or vehicle. $P < 0.001$, $n = 8$. (B) Individual variation in IOP between vehicle and Abn-CBD treated eyes. (C) Average IOP responses for GPR18 agonist NAGly (1%) as above $P < 0.005$, $n = 8$. (D) Raw values for NAGly, (E) Average IOP responses for GPR55 agonist O-1602 (1%). $P = 0.366$, $n = 8$. (F) Raw values for O-1602.

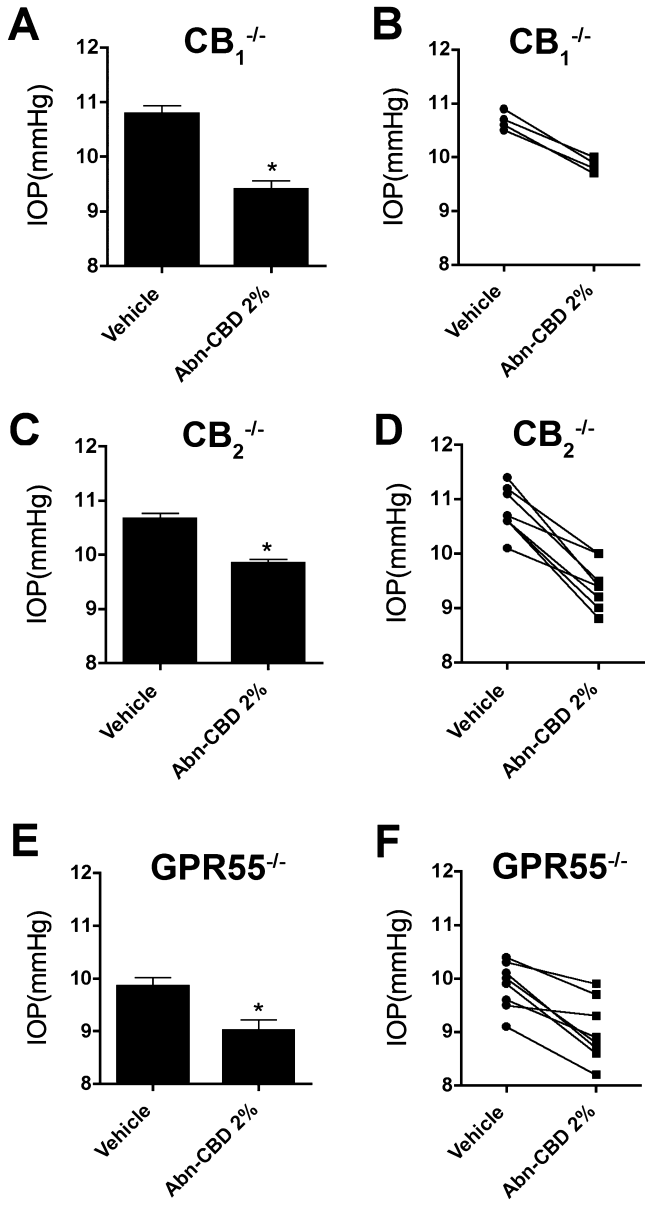

Figure 6

Abnormal cannabidiol (Abn-CBD) does not lower IOP via cannabinoid receptors or GPR55. (A) Average values for Abn-CBD in $CB_1^{-/-}$ mice. $P < 0.001$, $n = 4$. (B) Raw values from A. (C) Average values for Abn-CBD in $CB_2^{-/-}$ mice. $P < 0.0001$. $n = 8$. (D) Raw values for C. (E) Average values for Abn-CBD in $GPR55^{-/-}$ mice. $P < 0.05$. $n = 8$. (F) Raw values for E.

Abn-CBD on IOP in $CB_1^{-/-}$ and $CB_2^{-/-}$ mice, which produced decreases of 1.37 ± 0.16 mmHg and 0.675 ± 0.08 mmHg, respectively (Figure 6A–D). NAGly (1%) produced similar decreases in IOP of 1.41 ± 0.08 mmHg and 1.22 ± 0.23 mmHg in respective knockout control groups (Figure 7A–D). The use of knockout controls argues strongly against the participation of these receptors in the action of NAGly or Abn-CBD. Because the CB_1 knockout mice we used are in a CD1 background strain, we tested the effect of Abn-CBD in CD1 WT animals. Our results demonstrate that Abn-CBD still reduces IOP independent of the background strain (1.10 ± 0.16 mmHg; $P < 0.0001$, $n = 8$). Tests of either Abn-CBD or NAGly in GPR55 knockout mice showed that both of these drugs are still able to lower IOP (Figures 6E–F, 7E–F; Abn-CBD: 0.85 ± 0.15 mmHg; NAGly: 0.86 ± 0.18 mmHg). Baseline IOP values of $GPR55^{-/-}$ do not differ from those of WT (data not shown). These data argue against a role for GPR55 in mediating the effects of NAGly/Abn-CBD on IOP in this normotensive murine model.

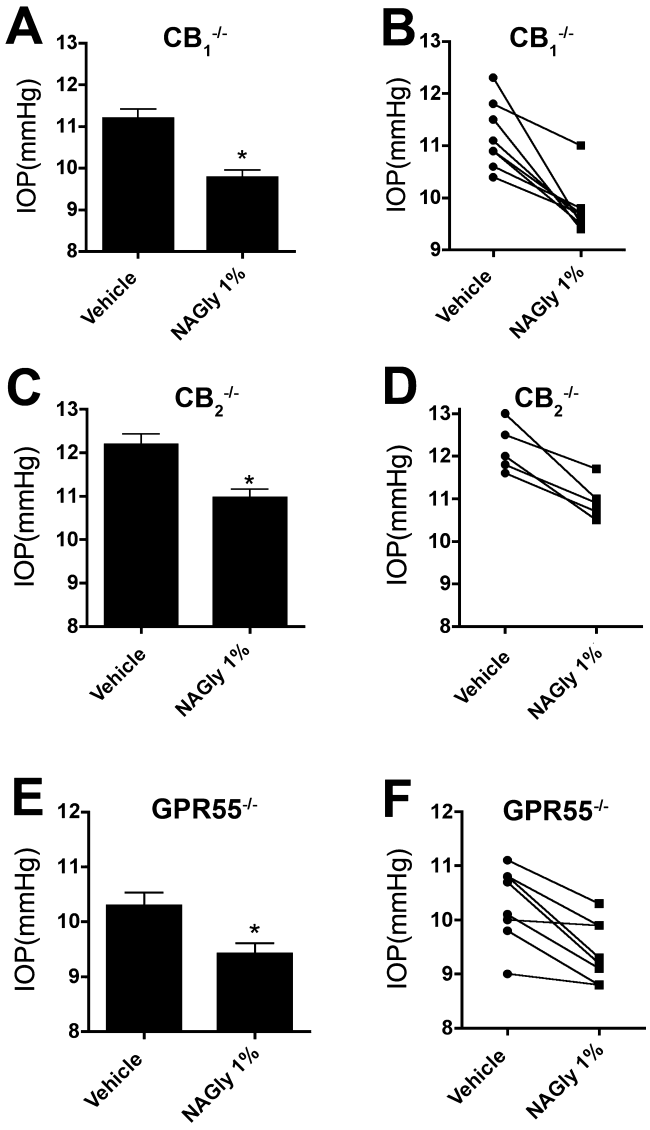

Figure 7

NAGly does not lower IOP via cannabinoid receptors or GPR55. (A) Average IOP values for NAGly in $CB_1^{-/-}$ mice. $P < 0.001$. $n = 8$. (B) Raw values from A. (C) Average IOP values for NAGly in $CB_2^{-/-}$ mice. $P = 0.0062$. $n = 5$. (D) Raw values for C. (E) Average values for NAGly in $GPR55^{-/-}$ mice. $P < 0.05$. $n = 8$. (F) Raw values for E.

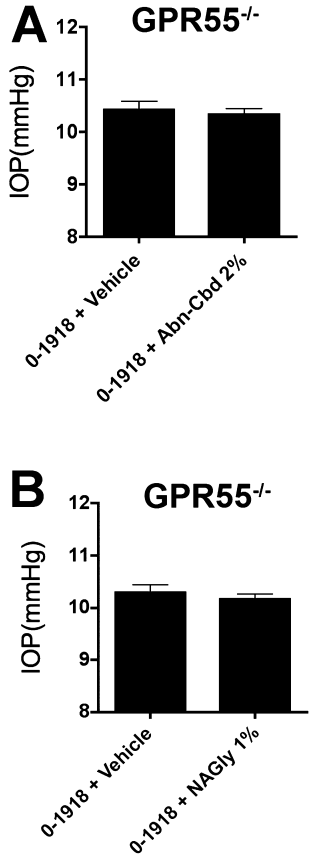

Figure 8

The GPR18 antagonist O-1918 blocks Abn-CBD and NAGly action in GPR55-/- mice. (A) Mean IOP of Abn-CBD 2% in $GPR55^{-/-}$ pre-treated with 0–1918 taken after 30 min after topical administration of 2% Abn-CBD or vehicle, $P > 0.05$, $n = 8$. (B) Mean IOP of 1% NAGly in $GPR55^{-/-}$ pre-treated with 0–1918 taken after 30 min after topical administration of 1% NAGly or vehicle, $P > 0.05$, $n = 8$.

To rule out O-1918 action via GPR55, we tested the ability of O-1918 to block the effects of Abn-CBD and/or NAGly in $GPR55^{-/-}$ mice. Consistent with GPR18 as a site of action, we found that Abn-CBD and NAGly did not significantly reduce IOP when co-administered with O-1918 in $GPR55^{-/-}$ mice (Figure 8; $P > 0.05$ per respective group). Taken together, this evidence provides a compelling argument that GPR18 mediates a reduction in IOP in this mouse model. We separately noted whether IOP varies diurnally in the $GPR55^{-/-}$ mice. We previously tested WT, $CB_1^{-/-}$ and $CB_2^{-/-}$ mice, with the conclusion that the diurnal variation was intact in those animals. Diurnal variation was also intact in $GPR55^{-/-}$ mice (data not shown; 21:00 h: 11.85 ± 0.22 mmHg; 09:00 h: 10.03 ± 0.12 mmHg, $n = 8$, $P < 0.0001$).

We have recently shown that CB_1 receptor-mediated reduction of IOP occurs via modulation of beta-adrenoceptor (βAR) signalling (Hudson *et al.*, 2011). The reduction of IOP seen with CB_1 agonists such as WIN55212-2 is absent in a βAR1/βAR2 double knockout mouse. To examine whether the GPR18 hypotensive action occurs via a similar mechanism, we tested the effects of Abn-CBD and NAGly in βAR1/βAR2 double knockout mice. We found that both drugs still retained their IOP-reducing properties in the absence of βAR1/βAR2 (Figure 9; Abn-CBD 1.22 ± 0.079 mmHg, $n = 8$, $P < 0.0001$; NAGly 0.71 ± 0.12 mmHg, $n = 8$, $P = 0.0006$).

Lastly, transient receptor potential receptors, most notably TRPV1, have been shown to be activated by some endocannabinoids, particularly AEA (Smart *et al.*, 2000). Since AEA may be a precursor for NAGly, we tested whether NAGly acts via TRPV1 by treating animals with a TRPV1 antagonist, capsazepine. We found that hypotensive responses to both agents were intact in animals that were injected with capsazepine (2 mg kg^{-1}, IP, 1.04 ± 0.08 mmHg; $P < 0.0001$, $n = 8$).

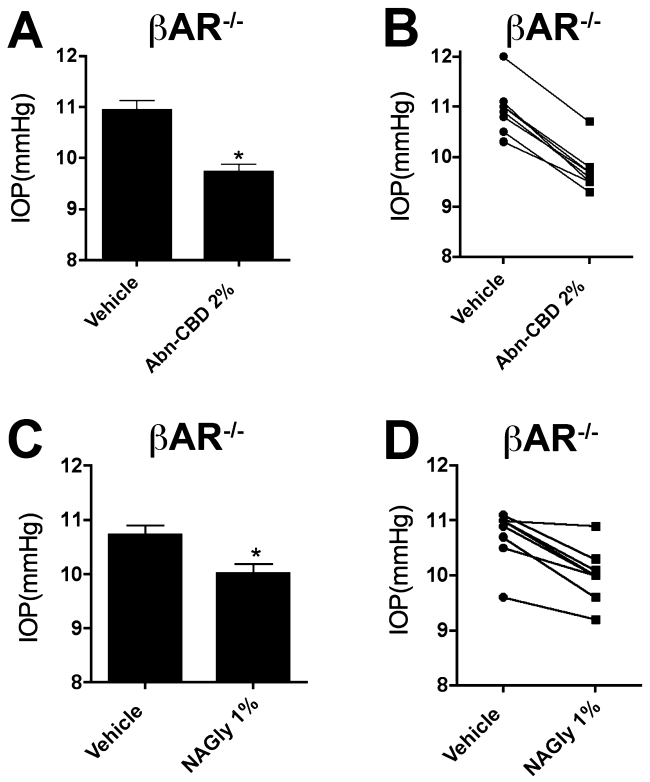

Figure 9

Abn-CBD and NAGly lower IOP independent of beta adrenoceptors. (A) Mean IOP taken from β-AR$^{-/-}$ male mice 30 min after topical administration of 2% Abn-CBD or vehicle. $P < 0.0001$. $n = 8$. (B) Individual variation in IOP between vehicle and Abn-CBD treated eyes in β-AR$^{-/-}$ mice. (C) Mean IOP taken from β-AR$^{-/-}$ male mice 30 min after topical administration of 1% NAGly or vehicle. $P < 0.0005$. $n = 8$. (D) Individual variation in IOP between vehicle and NAGly treated eyes in β-AR$^{-/-}$ mice.

Discussion

We have offered evidence for a functional GPR18-based signalling system in the anterior eye. This includes expression of GPR18 protein, detection and measurement of the endogenous ligand NAGly and, most notably, the observation that GPR18 agonists reduce IOP in mouse. GPR18 is a recently deorphanized lipid receptor that belongs to a larger class of cannabinoid-related receptors. GPR18 is also activated by the atypical cannabinoid, Abn-CBD and the chief psychoactive ingredient of marijuana, Δ^9-tetrahydrocannabinol (THC; McHugh *et al.*, 2012b). However, GPR18 does not mediate the psychoactive effects of marijuana and is therefore a more attractive therapeutic target.

Our most notable finding is that GPR18 agonists are equieffective with CB1 agonists and other currently prescribed ocular hypotensives in reducing IOP in normotensive mice (Hudson *et al.*, 2011). This action of GPR18 agonists was mediated independent of cannabinoid CB_1 and CB_2 receptors and also of the candidate cannabinoid receptor, GPR55. Our results nonetheless leave open the possibility that this action occurs via another receptor distinct from GPR18. NAGly has been shown to have activity at GPR92 (Kohno *et al.*, 2006; Oh *et al.*, 2008; Williams *et al.*, 2009). However, NAGly was found to have both poor potency and efficacy at GPR92 relative to farnesyl pyrophosphate, often requiring 50 μM or more to reach maximal effect. This is in contrast to the subnanomolar NAGly concentrations found sufficient to activate GPR18 (McHugh *et al.*, 2010). Indeed, the evidence that NAGly is the endogenous ligand for GPR18, combined with the activation profile of assorted agonists and antagonists, strongly supports our contention that the observed effects on IOP occur via GPR18. As such, our results offer a novel therapeutic target for the lowering of IOP. GPR18 should next be evaluated as a potential target in other model systems and the GPR18 expression profile should be examined in human tissue.

The complete lack of effect for the mixed GPR55/GPR18 agonist, O-1602, was unexpected given its reported activity at GPR18 (McHugh *et al.*, 2012c). O-1602 is structurally similar to Abn-CBD and O-1918 and should not therefore have greater difficulty crossing the cornea. It is possible that the O-1602 has a secondary effect at another target, such as the IOP-potentiating effect we recently observed for the CB_1 receptor agonist, WIN55212 (Hudson *et al.*, 2011). This potentiation was only unmasked when WIN55212 was tested in CB1$^{-/-}$ animals.

The GPR18 expression profile, with prominent labelling in ciliary epithelium and the trabecular meshwork, is consistent with a site of action at either (or both) the site of aqueous humour inflow or outflow. However, GPR18 is also expressed in several additional anterior eye tissues, most notably in the corneal epithelium, but also at a lower level in other anterior eye tissues such as the iris, suggesting that it may have other distinct roles at these sites.

Our data suggest that a NAGly/GPR18 signalling system plays at least one role within the anterior eye, but also raises questions about the relationship between this system and the known CB_1-based signalling system, activation of which also reduces IOP (Hepler and Frank, 1971). Although AEA was the first identified endocannabinoid (Devane *et al.*, 1992), the relationship between AEA and CB_1 signalling is still being explored. Certainly, a substantial portion of CB_1-based cannabinoid signalling occurs via 2-AG (Stella *et al.*, 1997; Kano *et al.*, 2009). AEA is unusual insofar as it is a full agonist at the unrelated TRPV1 receptors (Smart *et al.*, 2000). The distribution of GPR18 is, like CB_1, widespread in the anterior eye, with broad overlap including the ciliary epithelium, and like CB_1, GPR18 is likely a $G_{i/o}$-coupled GPCR (Walter *et al.*, 2003). We recently determined that CB_1 reduces IOP via the beta adrenergic system, through inhibition of the release of norepinephrine (Hudson *et al.*, 2011). However, we have shown here that GPR18 does not act via this system. And although present, GPR18 is not as strongly associated with the large ciliary epithelial blood vessels as CB_1 (Hudson *et al.*, 2011). Because $G_{i/o}$-coupled GPCRs are linked to numerous potential signalling pathways including the opening and closing of ion channels, it is difficult to predict the form that such a mechanism may take. However, the finding that GPR18 mediates changes in cell morphology and migration rates (McHugh *et al.*, 2010) raises the possibility that the alterations in IOP occur via morphological changes in key cell populations.

The relationship between GPR18 and CB_1 signalling is rendered more complicated because NAGly is a natural

metabolite of the endocannabinoid, AEA. In principle, an anandamide-based treatment might offer the promise of acting via two targets. However, Pate *et al.*, (1998) found that AEA action on IOP was substantially CB_1-independent in a normotensive rabbit model. It is possible that AEA is metabolized too quickly to reach CB1 receptors, although this is somewhat unexpected given our finding that AEA levels are unusually high in the anterior eye; this result suggests that there is already a substantial reservoir of this NAGly precursor. The manner and extent to which AEA is converted to NAGly will depend in part on the distribution of FAAH and NAAA (Tsuboi *et al.*, 2005) in the anterior eye, neither of which is as yet known. Similarly, because both THC and 2-AG (and to a lesser extent AEA itself) have been reported to activate GPR18 (McHugh *et al.*, 2010), our results raise the interesting possibility that some IOP-lowering effects of either of these agonists may occur via GPR18. These will be important subjects of future research. However, it is safe to conclude that given the assorted links between the CB1- and GPR18-based signalling systems that any studies of potential ocular therapy involving either system will likely have to take into account the other.

In summary, we have collected several lines of evidence for the presence of a functional GPR18-based signalling system in the murine anterior eye. Although the presence of a novel lipid receptor signalling system is inherently interesting, the finding that activation of this system reduces murine intraocular pressure has considerable therapeutic implications, since elevated intraocular pressure is indicated in glaucoma, a leading cause of blindness. The mechanism by which GPR18 reduces IOP will be an important subject of further study as will investigation of GPR18 function in other anterior eye tissues.

Acknowledgements

This work was supported by the following grants: EY021831(AS), DA24122(AS), CIHR operating grant FRN 97768 (MK). We would also like to acknowledge the support of the Indiana University Light Microscopy Imaging Center.

Conflict of interest

The authors declare that they have no competing interests with respect to the manuscript.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1 GPR18 distribution in $CB_1^{-/-}$ mouse ciliary epithelium and cornea. (A) GPR18 distribution in $CB_1^{-/-}$ ciliary epithelium shows a broadly similar distribution to WT, with strong diffuse labelling throughout the outer epithelial layer. (B) GPR18 distribution is unaltered in $CB_1^{-/-}$ cornea despite strong co-localization with CB_1 in WT cornea. Scale bars. A: 15 μm, B: 30 μm.