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Evidence for a GPR18 role in chemotaxis, proliferation, and the course of wound closure in cornea

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Abstract

PURPOSE: We previously showed that cannabinoid-related GPR18 receptors are present in murine corneal epithelium but their function remains unknown. The related CB1 receptors regulate corneal healing, possibly via chemotaxis. We therefore examined a potential role for GPR18 in corneal epithelial chemotaxis and wound healing.

METHODS: We examined GPR18 mRNA and protein expression in cornea. We additionally examined the GPR18 action in cultured bovine corneal epithelial cells (bCECs) using Boyden and tracking assays, as well as proliferation and signaling. Finally, we examined wound closure in murine corneal explants.

RESULTS: GPR18 mRNA was upregulated with injury in mouse cornea. GPR18 protein was present in basal epithelial cells of the mouse and cow, and redistributed to the wound site upon injury. GPR18 ligand *N*-arachidonoylglycine (NAGly) induced bCEC chemotaxis. The endocannabinoid arachidonoylethanolamine (AEA) also induced chemotaxis *via* fatty acid amide hydrolase (FAAH) mediated metabolism to NAGly. GPR18 receptor activation additionally induced bCEC proliferation. In an explant model, the GPR18 antagonist O1918 slowed corneal epithelial cell migration and the rate of corneal wound closure.

CONCLUSIONS: Corneal GPR18 activation induced both chemotaxis and proliferation in CECs *in vitro*, and impacted wound healing. GPR18 may contribute to the maintenance of corneal integrity.

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Keywords

Cannabinoid; chemotaxis; cornea; wound healing; GPR18

INTRODUCTION

The process by which corneal wound healing occurs after superficial injury has been well described, occurring in a highly choreographed series of steps that involve complex multidirectional migration by multiple cell populations[1]. Migration is likely regulated by chemotaxis in response to a chemical gradient, and G protein coupled receptors are strongly implicated in cellular directional sensing,[2] but the guidance cues underlying corneal chemotaxis remain poorly understood.

We have shown that cannabinoid CB_1 receptors can mediate chemoattraction in corneal epithelial cells[3] while others have demonstrated that CB_1 knockout mice exhibit slower corneal wound healing.[4] We have previously shown that the related GPR18 receptors are present in murine eye where they play a role in diurnal regulation of ocular pressure[5] as well as retinal vascular function.[6] GPR18 is also expressed in the corneal epithelium.[7] The function of GPR18 in the cornea is unexplored but GPR18 has been implicated in both chemotaxis and proliferation *via* GPR18 in cell lines.[8, 9]

GPR18 is a $G_{\alpha i/o}$ coupled G-protein coupled receptor (GPCR)[10–12] that is distantly related to CB_1 and CB_2 and has been shown to be activated by Δ^9-tetrohydrocannabinol (Δ^9-THC).[13] The endocannabinoid-related lipid *N*-arachidonoylglycine (NAGly) has been proposed as an endogenous agonist at GPR18.[8] Activation of GPR18 inhibits cAMP production and stimulates p44/42 mitogen-activated protein kinase (MAPK) activation.[14, 15] NAGly synthesis likely occurs via hydrolysis of endocannabinoid arachidonoylethanolamine (AEA, anandamide) by fatty acid amide hydrolase (FAAH).[16, 17]

The current study details how GPR18 modulates corneal epithelial wound healing.

METHODS

Animals

The functional assays made use of bovine corneal epithelial cells harvested post-mortem from cows (see below). Explant, some immunohistochemistry and quantitative PCR experiments made use of mice. Experiments were conducted at the Indiana University Bloomington campus. All mice used for IOP experiments were handled according to the guidelines of the Indiana University animal care committee and in accordance with the ARVO animal statement. Mice (age 3–8 months) were kept on a 12 h (06:00–18:00) light dark cycle, and fed *ad libitum*. C57BL/6J (C57) mice were bred in our facility. Male mice were used for this study since we have seen sex-dependent ocular effects for both GPR119[18] and CB1[19] and because a sex-dependence of corneal wound healing has been described.[20] Tissue from global GPR18 knockout (KO) mice was kindly provided by Dr. Jason Cyster (UCSF, San Francisco, CA).

Bovine Corneal Epithelial Cell Harvesting

Our method for harvesting Bovine CECs have been described previously[3] though we emphasize that the SHEM medium did not contain dimethylsulfoxide.

Live-imaging migration assay

Our method for in vitro cell migration has been described previously.[3]

Immunohistochemistry and immunocytochemistry

Our methods for immunohistochemistry have been described previously.[18] Polyclonal antibodies against the GPR18 receptor were developed in the laboratory of our co-author Ken Mackie and used here; these antibodies have been characterized previously[7] but were also successfully validated here using knockout control tissue (see below). Phalloidin-Alexa488 (Thermo Scientific cat#: A12379) was employed as a counterstain to highlight outlines of corneal epithelial cells.

RT-PCR and QPCR Assays

Our methods for RT-PCR and QPCR have been described previously.[3, 19] Details specific to these experiments are included below. For RT-PCR, primers were designed against bovine GPR18 and β-Actin genes.

Nucleotide sequences of PCR primers (from 5’−3’) for RT-PCR were as follows:

GPR18 (forward): CCTCTGTTCATCATGATTGGGTGC

GPR18 (reverse): ACAGCATGACGCTAATGACTCGAG

B-actin (forward): CGTGAGAAGATGACCCAGATCATG

B-actin (reverse): CTTCTCCTTGATGTCACGGACGAT

Quantitative PCR was done using mouse tissue to compare the effects of corneal injury (3 hours post-injury). Primer sequences are listed below:

GPR18 S: 5’ AGC CAC AGA GCG AGG CTT GG 3’

GPR18 AS: 5’ CAG CCT TTG ACA GAC AGG AGG TTC 3’

A ~1mm diameter circular axial corneal epithelial defect was created in an isoflurane-anesthetized mouse using an Alger Brush under a stereomicroscope. The mouse was allowed to recover. After 3 hours the animal was euthanized after which eyes were extracted, the lens removed. Eyes were then promptly cooled to and stored at −80°C. RNA was extracted as previously described.[3]

Boyden Chamber Assay

Our methods for Boyden Chamber with bovine CEC have been described previously.[3]

Cell Proliferation Assay

bCECs were plated in a 96 well plate in serum-free SHEM medium. bCECs were plated at a concentration of 10,000 cells/well. After 30 hours incubation under various treatment conditions the cells were labeled with nuclear marker DRAQ5 and imaged on an Odyssey scanner (LiCOR Biosciences, Lincoln, NE).

Signaling Assays

Signaling assays used here have been described in our previous publications (e.g.[3]).

Corneal Explants

Our methods for preparation and use of murine corneal explant have been described recently elsewhere.[3]

Statistics

Values are expressed as mean ± SEM. Statistical analysis was done using GraphPad Prism version 6.0, GraphPad Software, San Diego CA, USA. Statistical tests used are indicated in the corresponding text and/or figure legend.

RESULTS

GPR18 mRNA is present in several bovine ocular structures and is upregulated by injury in murine cornea.

Though we have shown that GPR18 protein is widely expressed in the eye of the mouse,[6, 7] we used reverse transcriptase-PCR (RT-PCR) to test for the presence of GPR18 mRNA in several major ocular domains of the cow. We found that GPR18 is present in corneal epithelium, endothelium, and retina and trabecular meshwork (Fig. 1A) though the signal appears to be relatively weak in trabecular meshwork. In separate experiments we tested whether corneal debridement alters GPR18 mRNA expression in murine cornea using quantitative PCR (qPCR), finding that there was a statistically significant rise at 3 hours (Fig. 1B; ***, $p < 0.001$ by unpaired t-test).

GPR18 receptors are expressed in bovine and murine corneal epithelial cells and redistribute upon injury.

We have previously shown that GPR18 protein is expressed in cornea and elsewhere in the eye.[6, 7] We used the same GPR18 antibody to more closely examine protein expression in cornea of the mouse and cow and also to learn whether there are changes in protein distribution three hours after corneal debridement. We found that GPR18 protein is expressed in epithelial cells, with the highest levels in basal epithelial cells and have confirmed that the staining is absent in corneal tissue from GPR18 knockout mice (Fig. 2A–B). The staining appears to be intracellular and non-nuclear in uninjured cornea but redistributes upon injury. As shown in Figures 2C&D, GPR18 expression appears to be prominent in cells at the wound edge but also more superficial within the cell. Because we make use of bovine corneal epithelial cells for several experiments we also tested for GPR18

expression in bovine cornea (Fig. 2E). Here too the baseline expression in uninjured cornea is highest in basal epithelial cells and appears to be non-nuclear but intracellular.

Evidence for FAAH-mediated AEA conversion to NAGly to induce chemotaxis *via* GPR18

We tested whether the endocannabinoid AEA would promote migration of bovine CECs in the Boyden Chamber, finding that AEA induces a concentration-dependent migration, that was not blocked by the CB_1 antagonist SR141716 (SR1, 1μM) and therefore appears to be independent of CB_1 activation (Fig. 3, anandamide/AEA (100nM): 172 ± 3 cells; vehicle: 28 ± 1 cells, anandamide/AEA (100nM + 1μM SR1): 168 ± 3 cells; ns, $p > 0.05$, 1-way ANOVA with Bonferroni post hoc test). SR1 had no effect on migration on its own. That AEA acted independently of CB_1 was an unexpected result given that AEA is an agonist at CB_1[21] and that as noted previously CB_1 activation induces migration.

As noted in the introduction, AEA has been hypothesized to serve as a precursor for the GPR18 agonist NAGly and activation of GPR18 induces cell migration in other cell types.[22] If AEA acts *via* metabolism to NAGly that in turn activates GPR18, then AEA enhancement of bCEC migration should be blocked by a FAAH blocker as well as by a GPR18 antagonist. Consistent with this hypothesis, we found that treatment of CECs with 100 nM AEA together with a FAAH blocker URB597 (100 nM) prevented the AEA-induced migration (Fig. 4A, AEA (100 nM): 37 ± 5 cells, AEA + URB597: 6 ± 5 cells $p < 0.05$, 1-way ANOVA with Bonferroni post hoc test). Moreover, AEA induced migration is blocked by GPR18 antagonist O-1918 (1 μM, Fig. 4B, AEA (100 nM): 24 ± 7 cells, AEA + O-1918: 11 ± 6 cells $p < 0.05$, 1-way ANOVA with Bonferroni post hoc test). A related prediction is that NAGly itself should induce migration and that this would be blocked by O-1918. We therefore tested NAGly and found that it concentration-dependently induces CEC migration (Fig 4C, NAGly (100 nM): 209 ± 8; vehicle: 31 ± 1 cells $p < 0.05$, 1-way ANOVA with Bonferroni post hoc test). This effect was not blocked by 1 μM SR1 (Fig. 4C, NAGly (100 nM) + SR1: 214 ± 7 cells $p < 0.05$, 1-way ANOVA with Bonferroni post hoc test) but was blocked by 300 nM 0–1918 (Fig. 4D, NAGly (100 nm): 37 ± 8 cells; NAGly + O-1918: 2 ± 1 cells $p < 0.05$, 1-way ANOVA with Bonferroni post hoc test). Taken together these data implicate NAGly and/or AEA in GPR18-mediated migration of bovine CECs. We also tested the GPR55 agonist O-1602 at 100nM but did not observe migration as a result (O-1602 (100nM): 5 ± 1 cells, data not shown) though other compounds including Δ^9-THC induced migration in the same experiment. Our results also show that an endogenous cannabinoid, in this case AEA, can exert pronounced primary effects indirectly, *via* its metabolite. These results also lend support for the FAAH-mediated conversion of AEA to NAGly over the alternative alcohol-dehydrogenase-dependent model of endogenous NAGly synthesis in these cells.[16]

NAGly induces chemoattraction of bovine CECs in a dish migration assay.

Another approach to assess migration is to observe and track the migration of individual cells in real-time. We seeded bovine corneal epithelial cells in a petri dish and then placed a small agar block embedded with 300 nM NAGly on one side of the dish. We tracked the migration of cells before and after exposure to a gradient of NAGly. As summarized in

Figure 5, we found that cells moved toward the block containing NAGly over the course of 30 minutes.

GPR18 receptor activation increases proliferation in CECs

We separately tested whether NAGly altered CEC proliferation. We found that 100 nM NAGly increases proliferation in CECs (Fig. 6A; % increase in proliferation for NAGly (100 nM): 132 ± 3; $p < 0.05$, 1-way ANOVA with Bonferroni post hoc test vs. control). The GPR18 antagonist O-1918 (1 μM) does not affect CEC proliferation on its own but does block proliferative effects of 100 nM NAGly (Fig. 6A: proliferation for NAGly (100 nM): 93 ± 2; $p > 0.05$, 1-way ANOVA with Bonferroni post hoc test vs. control). These data suggest that GPR18 receptor activation enhances CEC proliferation.

We also tested whether AEA would induce CEC proliferation. We found that 500 nM AEA significantly increases proliferation in CECs (Fig. 6B; % change from control for AEA/anandamide (500 nM): 39 ± 9; $p < 0.05$, 1-way ANOVA with Bonferroni post hoc test). Interestingly when anandamide was combined with URB597 (100 nM) proliferation enhancement was abolished (Fig. 6B; % change from control for AEA/anandamide (500 nM) + URB597 (100 nM): 8 ± 5 vs. AEA (500 nM); *, $p < 0.05$ t-test vs. control) presumably by preventing conversion of anandamide into NAGly.

An unusual signaling profile for NAGly and AEA in bCECs

We previously reported that CB_1 activation in bCECs did not activate ERK1/2 signaling as expected but instead concentration-dependently suppressed ERK1/2.[3] We find a similar arrangement for AEA and NAGly, both of which dephosphorylate ERK1/2 in a concentration-dependent manner (Fig. 7A; EC_{50} [AEA]: 3.3 nM (CI 0.22 nM - 4.8 nM); EC_{50} [NAGly]: 1.1 nM (CI 0.12 nM - 2.9 nM)). We additionally examined the effect of AEA and NAGly on Akt signaling, finding that NAGly but not AEA activated Akt (Fig. 7B; EC_{50} [AEA]: 38 nM; EC_{50} [NAGly]: 12 nM). Finally, we tested the effect of GPR18 activation on cAMP levels after forskolin stimulation. Activation of the GPR18 receptor is expected to lower cAMP levels by inhibiting adenylyl cyclase due to $G_{i/o}$-coupling. Indeed, we found that NAGly lowered cAMP though AEA did not (Fig. 7C; EC_{50} [AEA]: 26.7 nM (CI 17.8 nM - 31.2 nM); EC_{50} [NAGly]: 21.7 nM (CI 15.1 nM - 29.5 nM).

GPR18 is expressed in a membrane-associated ring in bovine CECs.

Since many of our functional experiments involve bovine CECs we also tested for GPR18 expression using the knockout validated GPR18 antibody from Figure 2. As shown in Figure 8, using costaining with phalloidin to outline the cell, we found that GPR18 expression was in a ring-like membrane-associated pattern that is well-placed to receive a chemotaxic signal.

The GPR18 antagonist O-1918 slows migration and wound closure in murine corneal explants.

We have explored various aspects of GPR18-mediated cellular migration in cultured corneal epithelial cells, but there are several important reasons to examine healing in a more intact model system, not least because there is a reasonable concern whether the rolling migration

observed in the cultured CECs reflects a movement relevant for healing in an intact cornea. The classic model of migration of cells at the wound front involves essentially amoeboid movement and the chemotactic regulation of rolling migration observed may not be conserved for cells that are still part of a motile wound front. Mouse corneal explants replicate many of the hallmarks of wound closure with superficial wounds (those that do not expose the basement membrane) covered over in < 12 hours (and often less), while non-penetrating wounds that do remove the basal epithelial cells close in < 20hrs (e.g., Fig. 9E). We have calculated the velocity of the wound front in superficial wounds as well as rates of wound closure measured as % of wound area remaining (see Fig. 9A, C for schematic representations). Because superficial wounds are often healed by 8 hours, we observed velocity of the wound front only to 8 hours, at two-hour intervals. Using this approach, we find that O-1918 treatment (1μM) slows the superficial wound front (Fig. 9D). The velocity of cells is relatively constant in wild type animals at ~11 microns/hour whereas in O-1918-treated animals the value is half as much (n=9 per condition). Similarly, deeper debridement that removes the basal epithelial cells and requires more time for wound closure, is also slowed (Figure 9E, time course fitted with a nonlinear one-phase decay curve: (control ($t_{1/2}$ in hours(95% CI)): 4.4 (3.9–5.2), n=13; O-1918 (1 μM): 9.0 (7.4–11.6), n=8, 95% confidence intervals non-overlapping)).

DISCUSSION

Our central finding is that GPR18 receptors in corneal epithelium regulate both migration and proliferation in these cells *in vitro*, and that a GPR18 blocker slows corneal wound healing in murine corneal explants. GPR18 expression is upregulated upon injury and redistributes away from basal epithelial cells to cells at the wound margin. Our results also offer some insight into the synthetic pathway(s) for the endogenous GPR18 ligand NAGly since the endogenous cannabinoid AEA enhances migration and proliferation but appears to do so indirectly *via* FAAH-mediated conversion of AEA to NAGly. We find additionally that GPR18 signaling *suppresses* ERK1/2 signaling. As a $G\alpha_{i/o}$-coupled GPCR, GPR18 is predicted to activate ERK1/2[23–25] but the results are similar to the signaling seen for the CEC chemoattractive CB_1 receptors[3] which are also $G\alpha_{i/o}$-coupled.

The protein expression of GPR18 in both mouse and cow is most prominent in basal epithelial cells but redistributes to more superficial cells with injury and was seen in cells at the wound margin. The expression also appears to redistribute from intracellular to include membrane-associated staining (Fig. 2C–D) and is seen to be membrane-associated in solitary cultured epithelial cells (Fig. 8). This may be due to migration of epithelial cells toward the wound edge or due to upregulation in cells already at the wound margin. mRNA levels rise with injury, perhaps reflecting new production of GPR18 by cells associated with injury and migration.

We used a murine explant model to test the impact of GPR18 block on the behavior of cells in the course of wound healing. Explants recapitulate much of the course of wound healing and serve as an important bridge between our experiments involving cultured CECs and the course of healing in vivo. Explants also allow a more detailed visual time course than *in vivo* experiments that are as a rule before/after snapshots. Explants also separate out much of the

immune response that occurs with corneal injury, though there are resident immune cells that can undergo activation.[26] This has the advantage of providing a more isolated system but may miss an immune-related component of GPR18 action. We observed both a slowed migration of the superficial epithelial cell wound front (in the case of injuries that did not involve debridement of the basal epithelial cells) and a delayed wound closure (in injuries where basal epithelial cells were removed).

Our finding that AEA acts not directly *via* CB_1 but instead through a metabolite NAGly, supports a hypothesized FAAH-dependent pathway over an alcohol dehydrogenase-dependent synthetic pathway for NAGly. We have found AEA to be a full agonist at CB_1[21] and that CB_1 mediates chemotaxis.[3] However AEA has been found to be low efficacy in other systems (reviewed in[27]) and the ability of AEA to activate CB1 signaling may be signaling-pathway specific. Moreover, the intracellular expression of both CB_1 and GPR18 allow for a greater opportunity for enzymatic conversion of lipid messengers. AEA has been proposed to require a chaperone to transport it to intracellular compartments for metabolism.[28] Our data also support the proposed role of NAGly as an endogenous GPR18 ligand.

For a chemotaxic circuit to exist there must be a source of messengers. Given our experiments, in principle AEA or NAGly could serve in this role. It will be important to determine the ‘architecture’ of lipid signaling to determine the likely enzymatic source of these messengers. NAPE-PLD has been proposed to produce AEA and we have found that levels of these lipids are greatly diminished in the eyes of NAPE-PLD knockout mice.[5] If NAGly serves as the messenger, this raises a question of how and where NAGly is produced. Our results favor FAAH as an intermediate enzyme to convert AEA to NAGly. The corneal expression of FAAH is not known but tends to be intracellular, associated with the endoplasmic reticulum, requiring a chaperone protein such as a fatty acid binding protein to ferry the acylethanolamine to FAAH for conversion.[28] Though some GPR18 expression is seen to be membrane associated, particularly in cultured CECs (Figs. 2C, 8) the expression in uninjured cornea and in some cells post-injury is intracellular. Intracellular expression would position GPR18 closer to its ligand and intracellular GPR18 receptors in basal epithelial cells may serve some pro-proliferative role. It is possible that several lipid ligands activate GPR18 endogenously as is the case for the related cannabinoid CB1 receptors, activated by AEA[29] and 2-AG.[30]

Since GPR18 promotes proliferation of bCECs, GPR18 may have a second temporally and functionally distinct role in wound healing. Cell proliferation occurs after initial migration to allow the cornea to repopulate the corneal epithelium. The effect of GPR18 activation was modest relative to EGF and it remains to be seen whether GPR18 in fact plays a pro-proliferative role *in vivo* and thereby meaningfully contributes to corneal health.

We observed an unusual signaling profile for GPR18 with a suppression of ERK1/2 signaling in bCECs. Both AEA and NAGly dephosphorylated ERK1/2, hence deactivating the pathway. This is inconsistent with the expected signaling profile for GPR18 as a $G_{i/o}$ coupled receptor. It is also inconsistent with literature favoring ERK1/2 activation for cellular migration,[23–25] though it may be important to distinguish between chemokinesis and chemotaxis. The role of ERK signaling in chemotaxis has been less explicitly studied. We

have recently reported that CB2 receptors are upregulated in murine corneal epithelial cells upon injury and that these receptors mediate chemorepulsion, not chemoattraction.[31] Notably, the signaling profiles for ERK and cAMP are the reverse of those for GPR18 and CB1. That is, the two chemoattractant GPCRs dephosphorylate ERK1/2 and lower cAMP while the chemorepulsive GPCR phosphorylates ERK1/2 and raises cAMP levels. Either or both of these signaling pathways may therefore contribute to a push-pull regulation of CEC migration.

Though our findings implicate GPR18 in corneal function, until we have a clearer understanding of the circuitry of GPR18 signaling it is difficult to propose a model of GPR18 function in corneal function under normal or injured conditions. The corneal protein expression of the synthetic enzymes NAPE-PLD and FAAH remain to be determined. The simplest arrangement would involve production of AEA/OEA at the site of injury, creating a gradient that attracts a subpopulation of epithelial cells. However this raises a question of how such a sub-population of leading migrating cells interacts with neighboring cells since the epithelial cells are generally proposed move as a population or sheet.[32]

In summary, we find that GPR18 receptors are present chiefly in basal epithelial cells in intact cornea but that they are upregulated in cells near the wound area following corneal injury. GPR18 receptors induce both bCEC chemoattraction and proliferation, and are necessary for the normal course of healing in corneal explants. Our results raise the possibility that two related lipid-based GPCR signaling systems, GPR18 and CB_1, play functional roles in the corneal epithelium with implications for corneal wound healing.

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Abbreviations:

2-AG	2-arachidonoylglycerol
AEA	arachidonoyethanolamine
bCEC	bovine corneal epithelial cell
CEC	corneal epithelial cell
FAAH	fatty acid amide hydrolase
$G\alpha_{i/o}$	G-alpha G protein subunit, types *i* and *o*
GPCR	G protein-coupled receptor
IOP	intraocular pressure
MAGL	monoacylglycerol lipase
ERK	extracellular signal-regulated kinase
NAGly	N-arachidonoylglycine

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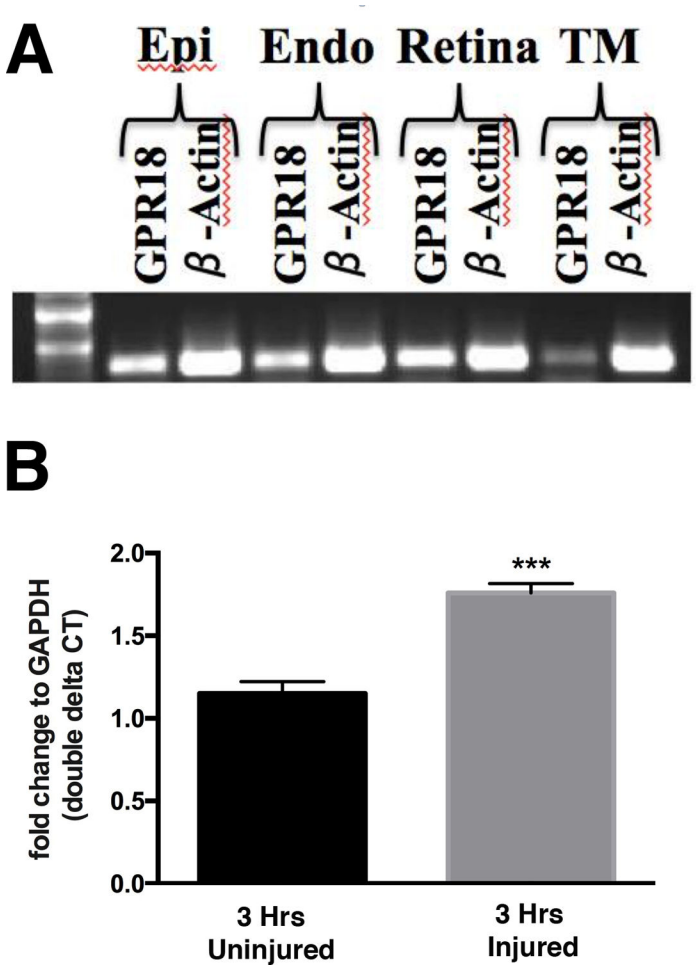

Figure 1. GPR18 mRNA is detected in several bovine ocular tissues and is upregulated with corneal debridement in murine cornea.

A) RT-PCR experiments reveal mRNA expression for GPR18 in corneal epithelium (Epi), corneal endothelium (Endo), retina, and trabecular meshwork (TM). B) Using qPCR in murine cornea we observed an upregulation of GPR18 mRNA at 3 hours post-injury. ***, $p < 0.001$ by unpaired t-test for injured vs. uninjured, n=12 for each condition.

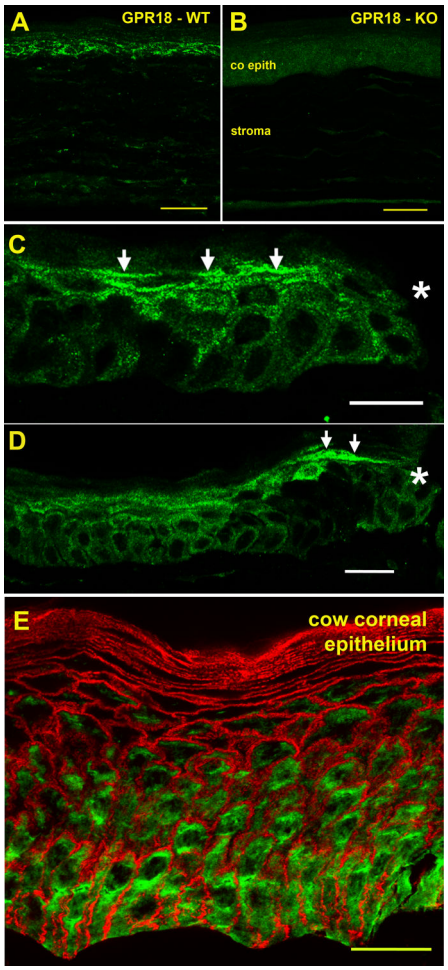

Figure 2. GPR18 protein is expressed in corneal epithelial cells of the mouse and redistributes near the wound site upon injury.

A) GPR18 staining is seen in epithelial layers of the murine cornea. B) The same antibody does not stain cornea from GPR18 knockout (KO) mice. C-D) 3 hours after injury GPR18 redistributes to more superficial cells in the cornea, and particularly in cells migrating toward the wound site (marked by asterisk). E) Sample GPR18 (green) staining in cow cornea, co-stained with phalloidin for actin staining (red). Scale bars: A-B) 30μm; C-E) 15μm

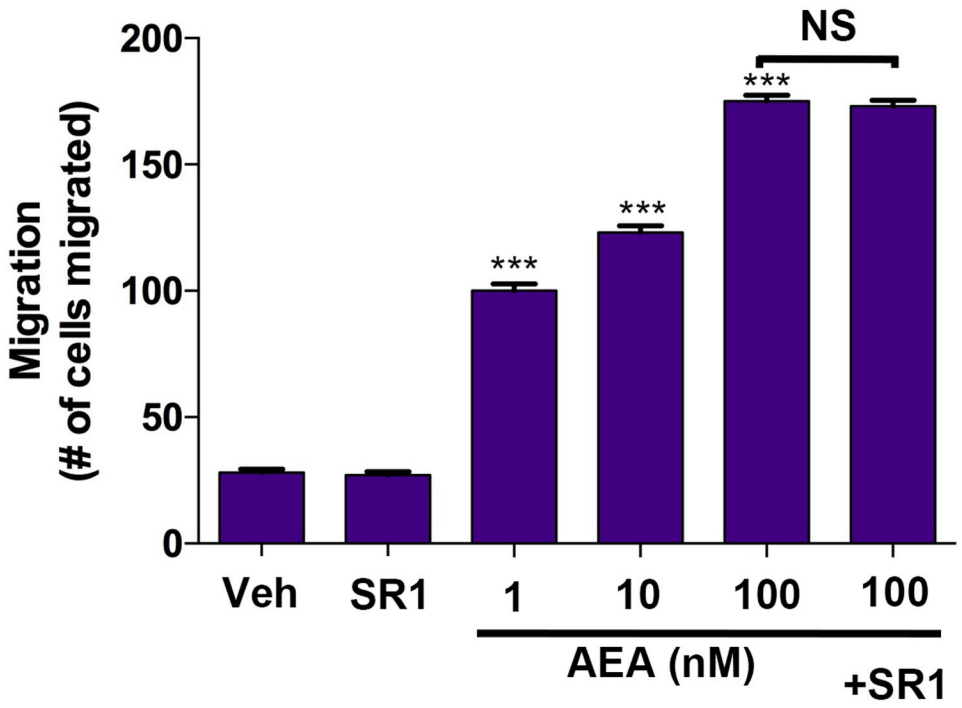

Figure 3. Anandamide induces migration of cultured bovine CECs independently of CB1.

In the Boyden chamber assay, the endocannabinoid AEA/anandamide concentration-dependently induces migration, but the effect is not blocked by CB_1 antagonist SR1 (1μM). SR1 alone does not induce migration in these cells. ***, p<0.005; ns, $p > 0.05$, 1-way ANOVA with Bonferroni post hoc test, n=3.

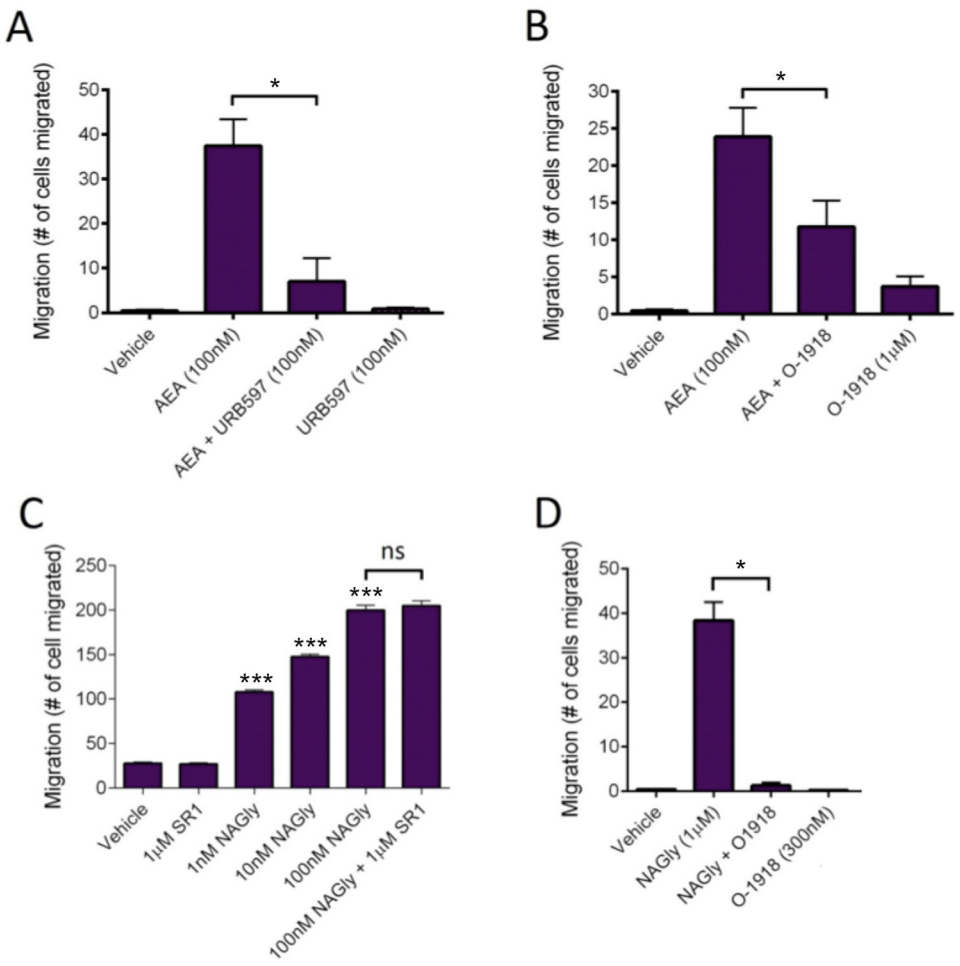

Figure 4. A potential role for GPR18 receptor in migration of bCECs.

A) AEA induced migration is blocked by pre-treatment with FAAH blocker URB597. URB597 does not induce bCEC migration on its own. n=8 B) AEA induced migration is blocked by GPR18 antagonist O-1918. O-1918 did no induce bCEC migration. n=8 C) Putative GPR18 endogenous ligand NAGly concentration-dependently induces migration that is not blocked by SR1. n=8 D) NAGly (1μM) concentration-dependently induces migration that is fully blocked by O-1918 (300nM), n=3. *, $p<0.05$; ***, $p<0.005$, 1 way ANOVA with Bonferroni post hoc test.

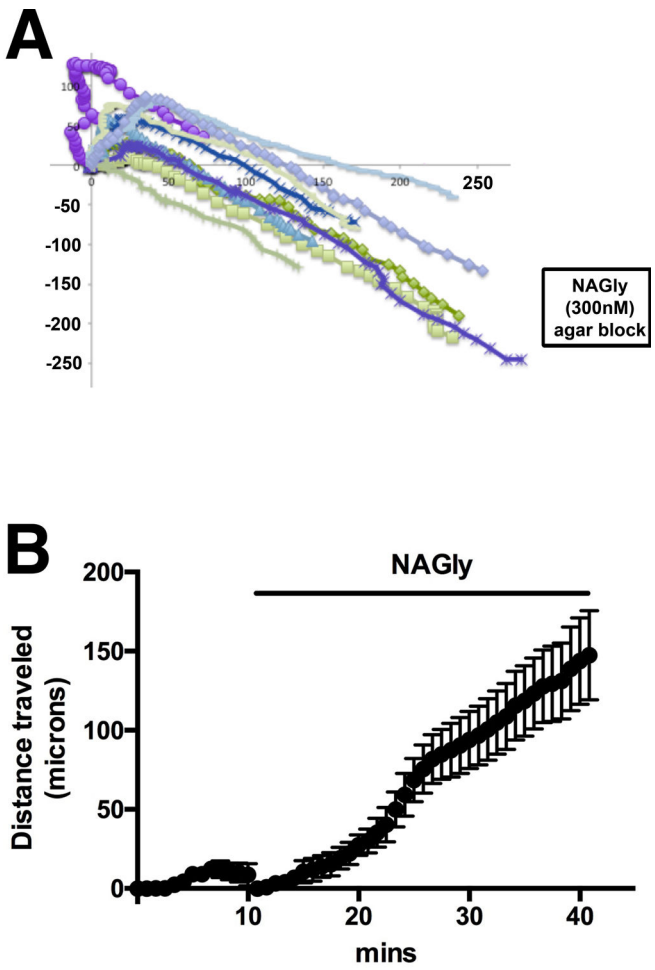

Figure 5. NAGly induces chemoattraction in a dish migration assay.

An agar block embedded with NAGly (300 nM) was placed at one side of the dish. The migration of cells was observed before and after introduction of the NAGly gradient. Summarized data from a representative experiment (n=10 cells, 4 experimental replicates) show average distance traveled. Values are normalized at beginning of NAGly treatment to show difference vs. untreated condition.

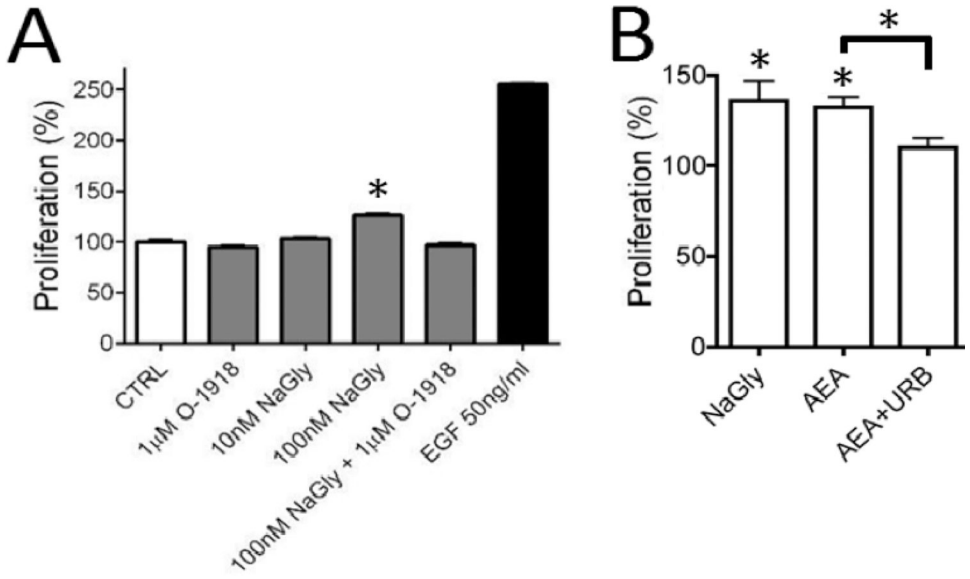

Figure 6. GPR18 activation enhances proliferation of bCECs.

A) In a proliferation assay, NAGly (100 nM) enhances bCEC proliferation. GPR18 antagonist O-1918 (1 μM) does not affect CEC proliferation. The combination of NAGly and O-1918 does not induce proliferation. EGF increases proliferation significantly. B) NAGly (100 nM) and AEA (500 nM) significantly enhance bCEC proliferation. Combination of AEA (500 nM) and URB597 (100 nM) does not induce proliferation. * $p <$ 0.05 1-way ANOVA with Bonferroni post hoc test vs. control, n=18–20.

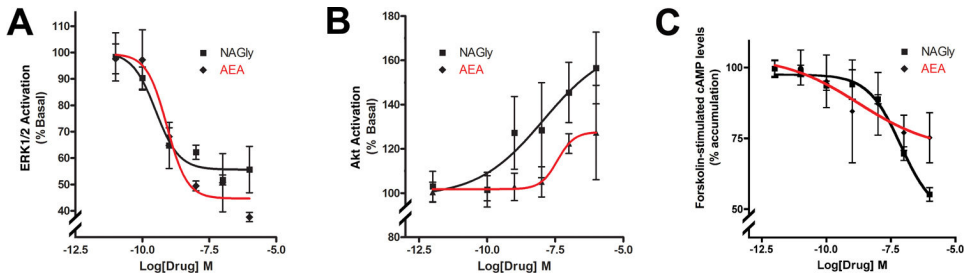

Figure 7: Cannabinoids exhibit an unexpected signaling activation profile in bovine corneal epithelial cells.

A) Instead of activating ERK1/2, AEA and NAGly dephosphorylate ERK1/2 in bCECs. B) NAGly activates Akt but AEA does not. C) NAGly reduces cAMP levels in forskolin-treated cells but AEA does not, n=3.

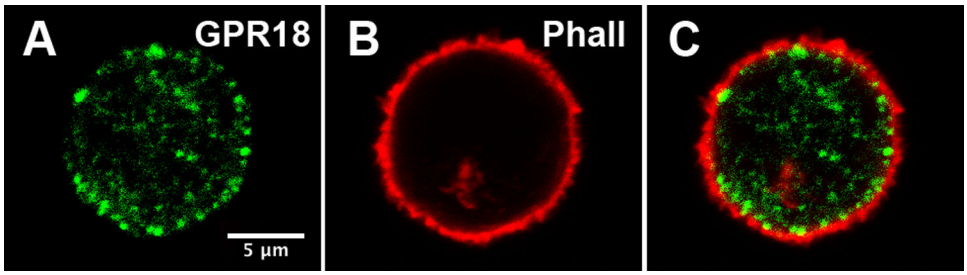

Figure 8. GPR18 staining in cultured bovine corneal epithelial cells.

A) GPR18 staining in a cultured bovine corneal epithelial cell. B) phalloidin in same cell. C) Overlay of GPR18 and phalloidin shows close association with membrane. Scale bar: 5µm.

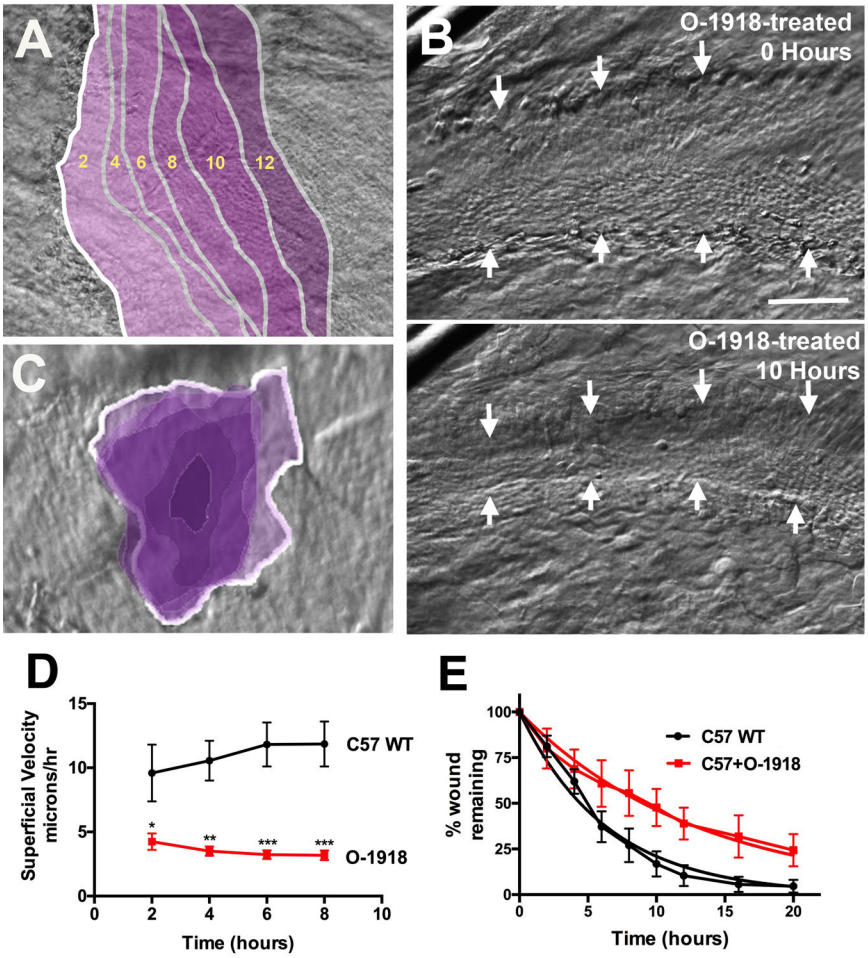

Figure 9. Wound-front migration is greatly slowed in WT explants treated with GPR18 antagonist O-1918.

A) Schematic shows wound front at two-hour intervals in sample wild-type cornea with superficial injury (i.e. basal epithelial cell layer is intact). B) Example of cornea with superficial injury treated with GPR18 antagonist O-1918. Lower panel shows position of the two wound fronts after 10 hours. C) Schematic showing area of progressive healing at two-hour intervals in wild-type cornea with “deep” injury where basal epithelial cells have been debrided. D) Time course of wound-front movement at 2-hour intervals shows velocity of wound-front toward the wound origin. GPR18 blocker O-1918 (1μM, n=9) slows velocity significantly relative to WT (n=9). E) Healing is also slowed in O-1918-treated explants (n=8) relative to wild type (n=13). *, $p < 0.05$, **, p<0.01, ***, p<0.005, two-way repeated-measure ANOVA. 95% CIs for decay curves in E non-overlapping. Scale bar: 33μm