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The Cannabinoids Δ^8THC, CBD, and HU-308 Act via Distinct Receptors to Reduce Corneal Pain and Inflammation

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Abstract

Background and Purpose: Corneal injury can result in dysfunction of corneal nociceptive signaling and corneal sensitization. Activation of the endocannabinoid system has been reported to be analgesic and anti-inflammatory. The purpose of this research was to investigate the antinociceptive and anti-inflammatory effects of cannabinoids with reported actions at cannabinoid 1 (CB_1R) and cannabinoid 2 (CB_2R) receptors and/or noncannabinoid receptors in an experimental model of corneal hyperalgesia.

Methods: Corneal hyperalgesia (increased pain response) was generated using chemical cauterization of the corneal epithelium in wild-type (WT) and CB_2R knockout ($CB_2R^{-/-}$) mice. Cauterized eyes were treated topically with the phytocannabinoids Δ^8-tetrahydrocannabinol (Δ^8THC) or cannabidiol (CBD), or the CBD derivative HU-308, in the presence or absence of the CB_1R antagonist AM251 (2.0 mg/kg i.p.), or the 5-HT_{1A} receptor antagonist WAY100635 (1 mg/kg i.p.). Behavioral pain responses to a topical capsaicin challenge at 6 h postinjury were quantified from video recordings. Mice were euthanized at 6 and 12 h postcorneal injury for immunohistochemical analysis to quantify corneal neutrophil infiltration.

Results: Corneal cauterization resulted in hyperalgesia to capsaicin at 6 h postinjury compared to sham control eyes. Neutrophil infiltration, indicative of inflammation, was apparent at 6 and 12 h postinjury in WT mice. Application of Δ^8THC, CBD, and HU-308 reduced the pain score and neutrophil infiltration in WT mice. The antinociceptive and anti-inflammatory actions of Δ^8THC, but not CBD, were blocked by the CB_1R antagonist AM251, but were still apparent, for both cannabinoids, in $CB_2R^{-/-}$ mice. However, the antinociceptive and anti-inflammatory actions of HU-308 were absent in the $CB_2R^{-/-}$ mice. The antinociceptive and anti-inflammatory effects of CBD were blocked by the 5-HT_{1A} antagonist WAY100635.

Conclusion: Topical cannabinoids reduce corneal hyperalgesia and inflammation. The antinociceptive and anti-inflammatory effects of Δ^8THC are mediated primarily via CB_1R, whereas that of the cannabinoids CBD and HU-308, involve activation of 5-HT_{1A} receptors and CB_2Rs, respectively. Cannabinoids could be a novel clinical therapy for corneal pain and inflammation resulting from ocular surface injury.

Keywords: cannabinoids; cornea; pain; inflammation; hyperalgesia

Introduction

The cornea is a thin, transparent dome-shaped avascular tissue that is densely innervated by sensory nerve endings.[1,2] Damage to these nerve endings, resulting from surgery, trauma, neurological disease, or infection, may develop into corneal neuropathic pain (CNP).[2] CNP is a clinically significant problem characterized by persistent hyperalgesia, debilitating pain, photoallodynia, burning, stinging, dryness, and inflammation.[3] Corneal damage can also result in an inflammatory response

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that involves the production of proinflammatory cytokines, neovascularization, recruitment of leukocytes, and release of neuropeptides producing inflammatory pain.[4,5]

Existing pharmacotherapies for ocular pain, inflammation, and CNP include topical corticosteroids, tricyclic antidepressants, GABAergic drugs, and opioids.[3,6] These treatments, however, frequently fail to provide adequate pain relief and are associated with side effects.[3,6] Therefore, new therapies that can alleviate pain and symptoms associated with CNP have fewer side effects and can resolve corneal inflammation are urgently required. One drug target that may have a role in the modulation of pain and inflammation is the endocannabinoid system (ECS).[7,8]

The ECS is an endogenous lipid signaling system that includes two G-protein-coupled receptors, cannabinoid 1 receptor (CB_1R) and cannabinoid 2 receptor (CB_2R), endocannabinoids, and cognate enzymes for biosynthesis and degradation of endocannabinoids.[9,10] CB_1Rs are widely expressed in many tissues, including in the central and peripheral nervous systems, where activation of CB_1R modulates neurotransmitter release, and nerve activity.[11,12] CB_2R is highly expressed on immune cells and its activation is anti-inflammatory, resulting in decreased production of proinflammatory mediators and a reduction in leukocyte recruitment.[13–15] Drugs that enhance activation of the ECS, including activation of both CB_1R and CB_2R, have shown efficacy in experimental models of pain and inflammation, including neuropathic pain.[7,9,16,17]

Cannabinoids have not been extensively studied in ocular surface pain and inflammation; however, CB_1R is expressed in the corneal epithelium and endothelium in rodents and primates,[18] and activation of CB_1R has been reported to inhibit neuropeptide-induced sensitization of transient receptor potential cation channel subfamily V member 1 (TRPV1) in afferent neurons.[11] Under nonpathological conditions, CB_2R expression is low in the cornea and anterior ocular structures; however, increased CB_2R expression in anterior ocular tissues has been suggested in experimental uveitis, where CB_2R activation produces anti-inflammatory effects.[19–21] Taken together, these studies suggest that cannabinoids that activate CB_1R and/or CB_2R may be useful for mitigating corneal pain and inflammation.

In this study, we used a mouse model of corneal hyperalgesia to investigate the antinociceptive and anti-inflammatory effects of several cannabinoids that act at CB_1R and/or CB_2R, or noncannabinoid receptors. These included Δ^8-tetrahydrocannabinol (Δ^8THC), a more stable isomer of Δ^9-tetrahydrocannabinol (Δ^9THC), cannabidiol (CBD), and the CBD derivative HU-308. Both Δ^8THC and Δ^9THC produce antinociceptive effects in pre-clinical models with similar potency via activation of CB_1R.[22–26] CBD lacks the behavioral effects of THC at CB_1R, and may produce pharmacological actions through the activation of noncannabinoid receptors.[27–29] HU-308 is a selective and highly potent agonist at CB_2R,[30] and has previously been shown to reduce lipopolysaccharide-induced intraocular inflammation.[19]

Materials and Methods

Experimental animals and corneal injury model

All animal care and experimental procedures complied with the Canadian Council for Animal Care guidelines (www.ccac.ca/) and were approved by the Dalhousie University Committee on Laboratory Animals. Male BALB/c (20–30 g; Charles River Laboratories International, Inc., Wilmington, MA) and CB_2R knockout mice ($CB_2R^{-/-}$) were used for experiments. $CB_2R^{-/-}$ mice were obtained by crossing male C57BL/6J $CB_2R^{-/-}$ mice (strain B6.129P2-Cnr2tm1Dgen/J; Jackson Laboratory, Bar Harbor, ME) with inbred BALB/c female mice (Charles River) for 10 generations. Genetic loss of CB_2R (*Cnr2*) was confirmed via polymerase chain reaction genotyping using DNA extracted from ear punches with an Accustart II Mouse Genotyping Kit (Quanta Biosciences, Gaithersburg, MD) according to the manufacturer's instructions. Primer sequences were as follows: mouse CB_2 mutant forward (moIMR0086) 5′- GGG GAT CGA TCC GTC CTG TAA GTC T-3′; mouse CB_2 wild-type (WT) forward (oIMR7552) 5′- GGA GTT CAA CCC CAT GAA GGA GTA C-3′; mouse CB_2 common reverse (oIMR7552) 5′- GAC TAG AGC TTT GTA GGT AGG CGG G−3′ with a single product at ~550 bp for $CB_2R^{-/-}$, a single product at ~385 bp for WT, and two products at ~550 and 385 bp for heterozygous mice. Mice were housed in groups of 3–5, kept on a light/dark cycle (07:00–19:00/19:00–07:00), and fed *ad libitum*.

Corneal injury was induced using a protocol adapted from a model of corneal hyperalgesia previously described in rats by Wenk and Honda.[31] Briefly, mice were anesthetized using 2–3% isoflurane gas. The center of the cornea on both eyes was cauterized with silver nitrate (MedPro®, 75% silver nitrate, 25% potassium nitrate; AMG Medical Inc., Montreal, QC, Canada) using a micro-applicator brush (Centrix, Inc., Shelton, CT). The micro-applicator brush was

held in contact with the cornea for 2 sec, producing a distinct superficial white lesion of 1 mm in diameter, injuring the epithelial cell layer only. The cauterized eyes were then rinsed with saline and an ocular lubricant (Systane®; Alcon Canada, Inc., Dorval, QC, Canada) was applied to reduce corneal drying. Mice recovered fully from anesthesia within 3–5 min postcauterization. Mice were euthanized at 6 or 12 h postcauterization, and the eyes were enucleated for immunohistochemical analysis.

Assessment of behavioral pain sensitization

At 6 or 12 h postcauterization, 5 μL of 1 μM capsaicin was applied topically to the cauterized eyes to elicit a pain response.[32] A sham control group was induced by touching the micro-applicator brush to the cornea for 2 sec in the absence of sliver nitrate, keeping all other parameters the same. Immediately following the application of a single dose of capsaicin, the behavioral response was video recorded for 30 sec. Videos were analyzed offline in slow motion, where the number of blinks, squints, and eye wipes to capsaicin challenge was summed to give a pain score.

Immunohistochemistry

At 12 h following corneal cauterization, eyes were enucleated and fixed in 4% PFA, followed by 30% sucrose overnight. Corneal sections (12 μm) were cut using a Leica CM1850 cryostat (Wetzlar, Germany). Sections were washed in phosphate-buffered saline (PBS) and blocked for nonspecific binding (10% normal goat serum in 0.5% Triton-X/PBS; Sigma-Aldrich, Oakville, ON, Canada) for 2 h, followed by a 48-h incubation in purified rat anti-Ly-6G antibody (1:200; Abcam, Cambridge, MA). Ly-6G is a glycosylphosphatidylinositol-anchor protein expressed predominantly on neutrophils; the anti-Ly-6G antibody allows for detection of these cells.[33] Sections were then washed with PBS and incubated with a secondary antibody (1:500, goat anti-rat Alexa Fluor® 488, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Stained sections were washed in PBS and mounted on Superfrost slides (Fisher Scientific, ON, Canada) using Fluoromount (Sigma-Aldrich).

Neutrophil migration, indicative of an innate immune response, was quantified in corneal sections at 20× magnification using a Zeiss Axiovert 200 M microscope (Zeiss, Thornwood, NY). Three representative images were taken from each section of the right and left corneal peripheries and from the center of the cornea, respectively. The total number of neutrophils from these three images was counted for each section, and summed to represent the total neutrophil number for a single corneal section. A total of 5–12 sections with 120 μM intervals from each eye were analyzed and the neutrophil number was averaged.

Drugs and solutions

Δ^8THC ([6aR,10aR]-6,6,9-trimethyl-3-pentyl-6a,7,8,10a-tetrahydrobenzo[c]chromen-1-ol; Cayman Chemical, Ann Arbor, MI), CBD (2-[(1*R*,6*R*)-3-Methyl-6-(1-methylethenyl)-2-cyclohexen-1-yl]-5-pentyl-1,3-benzenediol; Cayman Chemical), HU-308 (4-[4-(1,1-Dimethylheptyl)-2,6-dimethoxyphenyl]-6,6-dimethylbicyclo[3.1.1]hept-2-ene-2-methanol; Tocris Bioscience, Minneapolis, MN) were dissolved in soybean oil (Sigma-Aldrich) at different concentrations (0.2–5.0% w/v). Drugs were topically administered (5 μL) to cauterized corneas at 30, 60, and 120 min postcauterization. The CB_1R antagonist AM251 (N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide; Tocris Bioscience) was suspended in 10% dimethyl sulfoxide (DMSO, Sigma-Aldrich) and diluted in sterile saline. AM251 was injected at 2.0 mg/kg intraperitoneally (i.p.) fifteen min before cauterization. Capsaicin (1 μM, in 0.002% DMSO in sterile saline) was applied topically to eyes (5 μL) 6 h postinjury. The 5-HT_{1A} receptor antagonist WAY100635 (*N*-[2-[4-(2-Methoxyphenyl)-1-piperazinyl]ethyl]-*N*-2-pyridinylcyclohexanecarboxamide maleate; Tocris Bioscience) was dissolved in sterile saline and injected at 1.0 mg/kg i.p., 15 min before cauterization.

Data analysis

Individual animals in each treatment group were coded and experimental data were analyzed blinded. One-way analysis of variance (ANOVA) with Dunnett's or Tukey's multiple comparison *post hoc* tests was used, as appropriate, to compare data between experimental groups of three or more. *t*-Tests were used to compare two experimental groups. The number of animals in each group was 5–12. All data reported are represented as group mean ± standard deviation. Data were considered significant at $p < 0.05$.

Results

Corneal chemical injury results in hyperalgesia and inflammation

At 6 h, the pain score to 1 μM capsaicin was significantly increased in cauterized eyes (20 ± 7, $n = 10$) compared to

sham control (11 ± 4, $n = 6$; $p < 0.01$). At 12 h, no significant difference ($p > 0.05$) was observed in pain score between sham control animals (7 ± 3, $n = 5$) compared to cauterized eyes (12 ± 3, $n = 6$). In addition, the pain score in cauterized eyes at 12 h was significantly lower than the pain score at 6 h ($p < 0.05$; Fig. 1A).

Immunohistochemical analysis was carried out to examine neutrophil migration, indicative of an inflammatory response, in the cornea 6 and 12 h after capsaicin challenge in either cauterized or sham eyes. Neutrophils were not observed in sham control corneas at 12 h (Fig. 1C). Figure 1B demonstrates, however, the presence of neutrophils at 6 and 12 h following cauterization (126 ± 33, $n = 5$, and 156 ± 28, $n = 6$, respectively; Fig. 1B, D, E).

Topical application of Δ^8THC, CBD, and HU-308 reduces corneal pain and inflammation

Vehicle treatment produced an average pain score of 28 ± 6 ($n = 8$). Different doses of topical Δ^8THC, CBD, and HU-308 were examined in WT mice to establish the effective drug concentrations required to reduce corneal pain compared to the vehicle-treated group (Fig. 2A). Administration of 0.5% and 1% Δ^8THC produced a significant reduction in pain scores (18 ± 6, $n = 6$, $p < 0.05$; 12 ± 5, $n = 12$, $p < 0.0001$, respectively). Although at lower concentrations (0.2% and 0.4%) Δ^8THC did not significantly affect the pain score ($n = 6$ in each group; $p > 0.05$). Topical application of 5% CBD also significantly reduced the pain score (15 ± 3, $n = 10$, $p < 0.001$); however, 3% CBD

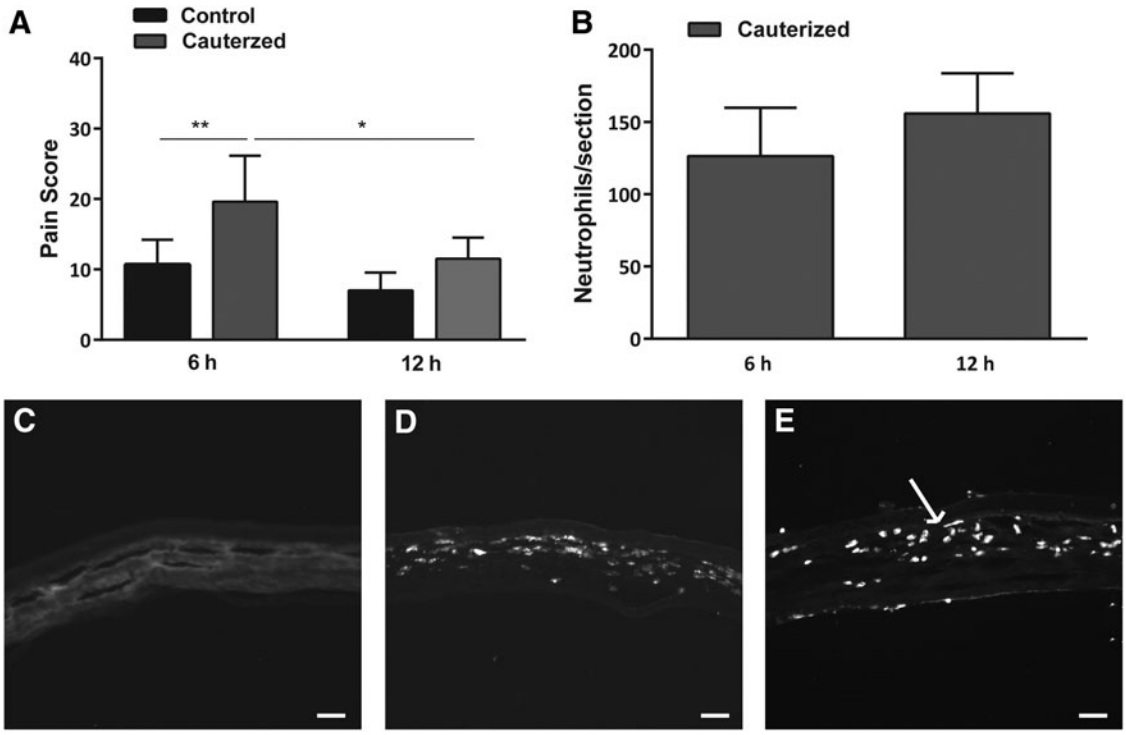

FIG. 1. Corneal chemical injury results in hyperalgesia and inflammation. **(A)** Pain responses to topical capsaicin challenge (1 μM) in noncauterized sham control eyes ($n = 5$–6 per group) and cauterized eyes ($n = 6$–10 per group) at 6 and 12 h postinjury. **(B)** Neutrophil expression in cauterized corneas at 6 and 12 h postinjury ($n = 5$–6 per group). **(C–E)** Representative images of transverse sections of the central cornea from **(C)** sham control (noncauterized) corneas and cauterized corneas at **(D)** 6 h and **(E)** 12 h postinjury. Arrow in **(E)** points to one of many infiltrating neutrophils. Scale bar = 50 μm. Values represent mean ± SD. For statistical analysis, one-way ANOVA with Tukey's multiple comparison *post hoc* test was used. **$p < 0.01$, *$p < 0.05$. ANOVA, analysis of variance.

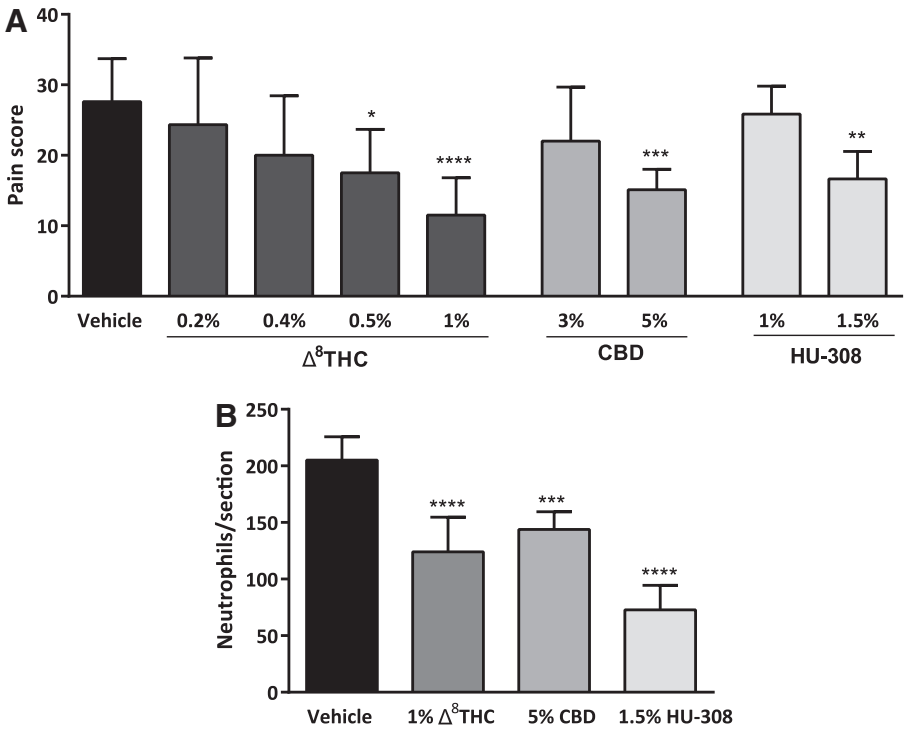

FIG. 2. Topical administration of Δ^8THC, CBD, or HU-308 reduces corneal hyperalgesia and neutrophil infiltration in WT mice after corneal cauterization. **(A)** Dose-response for antinociceptive effects of Δ^8THC (0.2–1.0%, $n=6$–12 per group), CBD (3% and 5%, $n=6$ and10, respectively), and HU-308 (1% and 1.5%, $n=6$ per group) following capsaicin challenge. **(B)** The number of neutrophils per section in corneas from WT mice treated with 1% Δ^8THC, 5% CBD, or 1.5% HU-308 at 12 h postinjury compared to vehicle-treated eyes ($n=6$ per group). Values represent mean ± SD. For statistical analysis, one-way ANOVA with Dunnett's *post hoc* test (compared to vehicle) was used. ****$p<0.0001$, ***$p<0.001$, **$p<0.01$, *$p<0.05$. Δ^8THC, Δ^8-tetrahydrocannabinol; CBD, cannabidiol; WT, wild-type.

was not effective at reducing the pain score ($n=6$, $p>0.05$). In addition, while 1% HU-308 did not produce a significant reduction in pain score ($n=6$, $p>0.05$), administration of 1.5% HU-308 was antinociceptive (17 ± 4, $n=6$, $p<0.01$). Therefore, given their efficacy at reducing the pain response, 1% Δ^8THC, 5% CBD, and 1.5% HU-308 were used for all further experiments.

Neutrophil infiltration into the cornea following treatment with cannabinoids was examined. Topical administration of 1% Δ^8THC, 5% CBD, or 1.5% HU-308 significantly reduced neutrophil number (124 ± 31, 144 ± 16, and 73 ± 22, respectively) compared to vehicle-treated eyes (205 ± 21; $p<0.0001$, $p<0.001$, and $p<0.0001$, respectively; $n=6$ per group).

The antinociceptive and anti-inflammatory effects of Δ^8THC, but not CBD, were mediated through CB_1R

Administration of the CB_1R antagonist AM251 (2.0 mg/kg, i.p.), before corneal cauterization and capsaicin stimulation, blocked the antinociceptive actions of Δ^8THC (Fig. 3A; $n=8$, $p>0.05$), suggesting that Δ^8THC acts via CB_1R to reduce corneal pain. However, the antinociceptive actions of 5% CBD were maintained in eyes pretreated with CB_1R antagonist AM251 (23 ± 6, $n=8$), compared to vehicle-treated eyes plus AM251 (35 ± 4, $n=8$, $p<0.001$; Fig. 3A).

Likewise, the number of neutrophils in corneas from mice treated with AM251 and either 1% Δ^8THC or vehicle was not significantly different ($n=6$, $p>0.05$). In contrast, 5% CBD treatment was still able to reduce

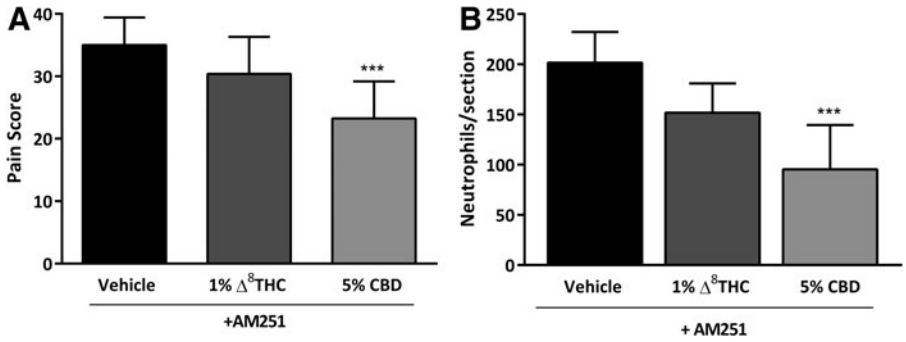

FIG. 3. The CB_1R antagonist AM251 reduces the antinociceptive and anti-inflammatory actions of Δ^8THC but not CBD. **(A)** Pain score measured in WT mice at 6 h postcauterization and following administration of 5 μL of topical vehicle, 1% Δ^8THC, or 5% CBD ($n=8$ per group) in mice preadministered with AM251 (2.0 mg/kg i.p.). **(B)** The number of neutrophils per section at 12 h postcauterization in corneas from WT mice preadministered with AM251 (2.0 mg/kg i.p.) and treated with 5 μL of vehicle, or either 1% Δ^8THC or 5% CBD ($n=6$ per group). Values represent mean ± SD. For statistical analysis, one-way ANOVA with Dunnett's *post hoc* test (compared to vehicle) was used. ***$p<0.001$. CB_1R, cannabinoid 1 receptor.

neutrophils in corneas from mice treated with AM251 (95 ± 44, $n=6$) versus vehicle-treated cauterized eyes from mice receiving AM251 (202 ± 31, $n=6$, $p<0.01$; Fig. 3B).

The antinociceptive and anti-inflammatory effects of HU-308, but not Δ^8THC or CBD, were mediated through CB_2R

The involvement of CB_2R in the antinocicpetive and anti-inflammatory effects of Δ^8THC, CBD, and HU-308 was examined using $CB_2R^{-/-}$ mice. Compared to WT mice receiving vehicle, the mean number of neutrophils in vehicle-treated $CB_2R^{-/-}$ mice was significantly increased (mean difference 102 ± 30, $n=6$ and 7, respectively, $p<0.01$); however, there was no significant difference in the pain score ($n=8$ in each group, $p>0.05$).

In $CB_2R^{-/-}$ mice at 6 h postcauterization, compared to vehicle-treated eyes (27 ± 7, $n=8$), application of 1% Δ^8THC (8 ± 5, $n=12$) or 5% CBD (19 ± 4, $n=7$) significantly decreased the pain score ($p<0.0001$ and $p<0.05$, respectively). However, the antinociceptive effect of 1.5% HU-308 ($n=7$) was not significantly different compared to vehicle-treated animals ($p>0.05$; Fig. 4A), confirming the involvement of CB_2R in the antinociceptive effects of HU-308, but not THC or CBD.

Consistently, at 12 h postcauterization in $CB_2R^{-/-}$ mice, the number of neutrophils in corneas receiving either 1% Δ^8THC (123 ± 50, $n=6$) or 5% CBD (187 ± 28, $n=6$) was significantly less than vehicle-treated corneas (307 ± 71, $n=7$; $p<0.0001$ and $p<0.01$, respectively). In HU-308-treated corneas (1.5%) from $CB_2R^{-/-}$ mice ($n=6$), there was no significant difference in neutrophil numbers compared to vehicle-treated eyes ($p>0.05$; Fig. 4B).

CBD acts at 5-HT_{1A} receptors to reduce corneal pain and inflammation

The corneal antinociceptive and anti-inflammatory effects of CBD were independent of CB_1R or CB_2R. Therefore, we examined an alternative non-cannabinoid receptor, 5-HT_{1A}, which has been reported as a target for CBD in other tissues.[29] Treatment of mice with the 5-HT_{1A} receptor antagonist WAY100635 (1.0 mg/kg i.p.) was able to completely eliminate the antinociceptive actions of CBD in cauterized cornea (Fig. 5A; $n=8$ in each group, $p>0.05$). In mice treated with WAY100635, the reduction in corneal neutrophils in cauterized eyes seen with CBD treatment was also blocked ($n=6$ in each group, $p>0.05$), suggesting that 5-HT_{1A} is the target receptor for CBD-mediated antinociceptive and anti-inflammatory actions in the cornea (Fig. 5B).

Discussion

Our results provide novel evidence that the phytocannabinoids Δ^8THC and CBD and synthetic cannabinoid derivative HU-308 are antinociceptive and

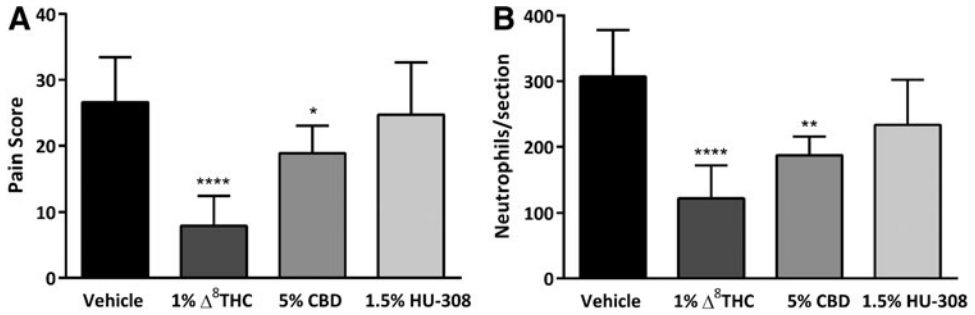

FIG. 4. The corneal antinociceptive and anti-inflammatory effects of HU-308, but not Δ^8THC or CBD, are mediated via CB_2R. **(A)** Pain scores measured at 6 h postcorneal cauterization in $CB_2R^{-/-}$ mice treated with 5 μL topical vehicle ($n=8$), or 1% Δ^8THC ($n=12$), 5% CBD ($n=7$), or 1.5% HU-308 ($n=7$). **(B)** The number of neutrophils per section measured at 12 h postcauterization in corneas from $CB_2R^{-/-}$ mice treated with 5 μL of vehicle ($n=7$), or 1% Δ^8THC ($n=6$), 5% CBD ($n=6$), or 1.5% HU-308 ($n=6$). Values represent mean ± SD. For statistical analysis, one-way ANOVA with Dunnett's *post hoc* test (compared to vehicle) was used. ****$p<0.0001$, **$p<0.01$, *$p<0.05$. CB_2R, cannabinoid 2 receptor.

anti-inflammatory in an experimental model of corneal hyperalgesia. Furthermore, we demonstrate that the actions of these cannabinoids are mediated via distinct receptor targets that include CB_1R and CB_2R, as well as 5-HT_{1A} receptor.

In the mammalian cornea, expression of CB_1R has been reported to colocalize with TRPV1,[34] the latter of which is expressed in corneal epithelium[35] and endothelium,[36] and sensory nerve endings of the ophthalmic branch of the trigeminal nerve innervating the cornea.[37] TRPV1 is activated following damage to cornea, culminating in activation of corneal nerves and local inflammation. The release of proinflammatory cytokines and neuropeptides, including nerve growth factor and substance P, contributes to neurogenic inflammation and can lead to corneal nerve sensitization.[34,38] In sensory neurons isolated from rat dorsal root ganglia, activation of CB_1R by the cannabinoid agonist ACEA (arachidonoyl-2′- chloroethylamide) prevented nerve growth factor-induced sensitization of the TRPV1 receptor. This action was blocked by the CB_1R antagonist AM251, suggesting that the

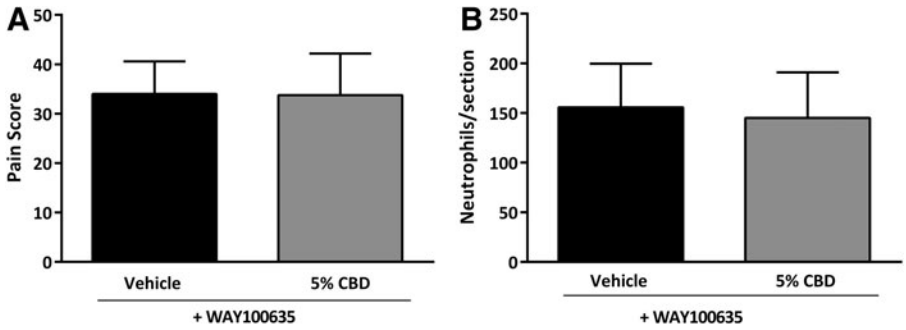

FIG. 5. The corneal antinociceptive and anti-inflammatory effects of CBD are mediated through 5-HT_{1A} receptor. **(A)** Pain score measured at 6 h postcauterization in WT mice preadministered with the 5-HT_{1A} receptor antagonist, WAY100635 (1.0 mg/kg i.p.), and treated topically with 5 μL of either vehicle ($n=8$) or 5% CBD ($n=8$). **(B)** The number of neutrophils per section measured at 12 h postcauterization in corneas from WT mice treated with 5 μL of either vehicle ($n=6$) or 5% CBD ($n=6$). Values represent mean ± SD. For statistical analysis, unpaired *t*-tests were used.

activation of CB_1R may produce analgesia by desensitization of TRPV1 receptors.[39]

Our results demonstrating that topical Δ^8THC, acting at CB_1R, reduces hyperalgesia following corneal injury are in line with these findings. In addition, we also demonstrated that Δ^8THC was able to reduce the neutrophil recruitment to the cornea observed at later time points following corneal epithelial damage. The inhibition of neutrophil recruitment was blocked with the treatment of CB_1R antagonist AM251, but was still present in $CB_2R^{-/-}$ mice. This suggests that the activation of CB_1R by Δ^8THC is important in mitigating the innate immune response following corneal injury, which may contribute to corneal nerve sensitization.

The importance of peripheral CB_1R in our study is also consistent with the actions of Δ^9THC reported in other models of both acute inflammatory and neuropathic pain. For example, administration of Δ^9THC (1 mg/kg i.p.) in a rat model of acute muscle pain produced antinociceptive effects, which was blocked by the CB_1R antagonist AM281 and to a lesser extent by the CB_2R antagonist AM630 (0.5 mg/kg i.p.).[40] Furthermore, in a model of inflammatory and neuropathic pain, mice lacking CB_1R in peripheral nociceptive neurons showed a reduced analgesic effect to local and systemic administration of the cannabinoid WIN55,212-2. With intrathecal application, the analgesic effect of WIN55,212-2 was absent, suggesting that peripheral CB_1R in nociceptive neurons plays an important role in producing the analgesic effects of cannabinoids.[41]

Our data implicate 5-HT_{1A} receptors, and not cannabinoid receptors, in both antinociceptive and anti-inflammatory actions of CBD in an experimental model of corneal injury. We showed that the actions of CBD were completely blocked by the 5-HT_{1A} antagonist WAY100635, but were still present after CB_1R block or in $CB_2R^{-/-}$ mice. CBD has been reported in other *in vitro* and *in vivo* models to bind to 5-HT_{1A} receptors.[27,29,42] Using a heterologous cell expression system, Russo et al. reported that CBD bound to both human and rat 5-HT_{1A} receptors with micromolar affinity, and displaced the agonist [^{3}H]8-OH-DPAT in a concentration-dependent manner.[29] In addition, CBD increased [^{35}S]GTPγS binding, and decreased forskolin-stimulated cAMP production, which was blocked by the specific 5-HT_{1A} antagonist NAN-190.[29]

In line with our findings of CBD activity at the 5-HT_{1A} receptor, a study by Ward et al. reported that CBD administration could prevent chemotherapy-induced neuropathic pain associated with paclitaxel treatment.[43] In this study, CBD was administered chronically for 14 days and prevented the onset of paclitaxel-induced mechanical and thermal sensitivity in female mice. A subsequent report showed that a subchronic dosing regimen of 2.5–10 mg/kg CBD (i.p.) was also effective in preventing paclitaxel-induced mechanical sensitivity. This effect was blocked by a 5-HT_{1A} antagonist (WAY100635), but not a CB_1R (SR141716) or CB_2R antagonist (SR144528), further supporting the role of 5-HT_{1A} in mediating the actions of CBD in preventing neuropathic pain.[43]

HU-308 has been reported as a selective CB_2R agonist.[30] In our model of corneal hyperalgesia, the antinociceptive and anti-inflammatory actions of HU-308, unlike Δ^8THC and CBD, were absent in $CB_2R^{-/-}$ mice, validating target specificity for this cannabinoid at CB_2R. This is the first time a CB_2R agonist has been demonstrated to reduce corneal pain, although CB_2R activation has been reported to reduce ocular inflammation.[20,44] In experimental uveitis, Toguri et al.[19] reported that CB_2R activation reduced leukocyte/endothelial adhesion in the iridial microvasculature as well as inhibited release of proinflammatory mediators, including TNFα, IL1β, Il6, CCL5, and CXCL2. Conversely, a CB_2R antagonist, AM630, increased leukocyte/endothelial adhesion in experimental uveitis,[19] suggesting that CB_2R activity in the eye is immunosuppressive during inflammation. In a mouse model of proliferative vitreoretinopathy, $CB_2R^{-/-}$ or pharmacological block of CB_2R, also produced increased inflammation and a more severe pathology.[44] Another study, in a mouse model of endotoxemia, has shown increased neutrophil recruitment to the spleen in $CB_2R^{-/-}$ mice compared to WT control.[45] In line with these results, in our experiments we observed an increase in the mean number of neutrophils in cauterized corneas in $CB_2R^{-/-}$ mice, suggesting that loss of constitutive CB_2R activity is proinflammatory in ocular tissues.[20,44]

Reports of the antinociceptive and antiallodynic efficacy of CB_2R agonists have also been reported in other experimental models of hyperalgesia and chronic inflammatory neuropathic pain.[46] While our study in cornea used a relatively acute dosing regimen, the utility of CB_2R agonists used chronically was previously reported in a mouse model of paclitaxel-induced neuropathic pain.[47] The authors reported that chronic CB_2R activation with the CB_2R-preferring agonist AM1710 was able to reverse paclitaxel-induced allodynia, an effect that was blocked in WT mice treated with the CB_2R antagonist AM630, or in $CB_2R^{-/-}$ mice. In comparison to

repeated dosing with agonists such as THC that produced behavioral effects and tolerance via CB_1R activation, no similar effects were observed with the CB_2R-preferring agonist AM1710. Furthermore, using intrathecal cannabinoid administration, this study identified a possible role for spinal CB_2R in the antiallodynic actions of AM1710, as well as a reduction in proinflammatory cytokines, in paclitaxel-treated mice.[47] Increased CB_2R expression has also been reported in human peripheral nerves after injury, and CB_2R agonist-mediated inhibition of capsaicin responses was observed in cultured human dorsal root ganglion sensory neurons.[48] Our data demonstrating the antinociceptive and anti-inflammatory actions of CB_2R activation in cornea, together with these studies, further support the utility of CB_2R agonists for treating inflammatory pain.

Conclusion

Our study showed that topical application of the phytocannabinoids Δ^8THC and CBD, and the cannabinoid derivative HU-308, reduced corneal hyperalgesia and neutrophil infiltration resulting from superficial chemical injury of corneal epithelium. The effects of these cannabinoids were mediated by distinct receptors, including CB_1R and CB_2R, as well as 5-HT_{1A} receptors. This suggests that when used either as sole agents or in combination, these cannabinoids could be effective agents in the treatment of ocular pain and inflammation resulting from corneal surface injuries.

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Author Disclosure Statement

M.E.M.K. is the founder and director of Panag Pharma, Inc. Panag develops phytotherapeutics for local and regional treatment of pain and inflammation. D.T., E.A.C., J.T.T., A.-M.S., and M.D.C. have no existing competing financial interests.

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Abbreviations Used

Δ^8THC = Δ^8-tetrahydrocannabinol
Δ^9THC = Δ^9-tetrahydrocannabinol
CBD = cannabidiol
CB_1R = cannabinoid 1 receptor
CB_2R = cannabinoid 2 receptor
CNP = corneal neuropathic pain
DMSO = dimethyl sulfoxide
ECS = endocannabinoid system
PBS = phosphate-buffered saline
TRPV1 = transient receptor potential cation channel subfamily V member 1
WT = wild-type