TARGETING THE ENDOCANNABINOID SYSTEM WITH ORTHOSTERIC AND ALLOSTERIC CANNABINOID LIGANDS TO REDUCE CORNEAL PAIN AND INFLAMMATION

by

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I am very much thankful to my mother, Ambika Thapa, for always motivating and inspiring me to become a nice human. Her unconditional love and support have always helped me to move forward in life, no matter how hard the situation is. It was a very tough time for me two years before to leave my mother in Nepal who was suffering from chronic corneal pain resulted from bilateral cataract surgery.

I hope my research presented in this thesis would contribute to the development of a new pharmacotherapy for people suffering from chronic corneal pain, including my mother.

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ABSTRACT

The endocannabinoid system, including cannabinoid 1 (CB_1R) and cannabinoid 2 (CB_2R) receptor, is an emerging target for treating pain and inflammation. This research investigated the effects of orthosteric and allosteric cannabinoid receptor ligands using a mouse model of corneal hyperalgesia. Behavioral pain responses were quantified 6 h after corneal chemical injury. Neutrophil infiltration was assessed by immunohistochemistry in post-mortem corneas 6-12 h post-injury. Tetrahydrocannabinol (Δ^8THC) mediated its anti-nociceptive and anti-inflammatory effects via CB_1R whereas, cannabidiol (CBD) mediated anti-nociceptive and anti-inflammatory actions via 5-HT_{1A} receptor. Anti-nociceptive and anti-inflammatory effects of the CBD-derivative, HU-308, were mediated via CB_2R. Allosteric ligands can minimize side-effects associated with orthosteric receptor activation. The CB_1R positive allosteric modulator (PAM), GAT229, and the ago-PAM, GAT211 (plus Δ^8THC), or the CB_1R allosteric agonist, GAT228, were anti-nociceptive and anti-inflammatory in cornea. Cannabinoids, together with allosteric ligands, could be a novel therapy for corneal pain and inflammation.

LIST OF ABBREVIATIONS USED

Δ^8 THC	delta 8 tetrahydrocannabinol
Δ^9 THC	delta 9 tetrahydrocannabinol
2-AG	2-arachidonoyl glycerol
5-HT	5-hydroxytryptamine
AC	adenylyl cyclase
ACEA	arachidonoyl-2'- chloroethylamide
AEA	arachidonoyl ethanolamide or anandamide
Akt	protein kinase B
AM251	*N*-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*- pyrazole-3-carboxamide
ANOVA	analysis of variance
AP-1	activator protein-1
ARRIVE	animal research: reporting of in vivo experiments
BAC	benzalkonium chloride
bp	base pairs
Ca^{2+}	calcium
cAMP	cyclic adenosine monophosphate
CB_1R	cannabinoid 1 receptor
$CB_1R^{-/-}$	cannabinoid 2 receptor knock-out
CB_2R	cannabinoid 2 receptor
$CB_2R^{-/-}$	cannabinoid 2 receptor knock-out
CBRs	cannabinoid receptors
CBD	cannabidiol
CCI	chronic constrictive injury
CCL	CC chemokine ligand
CCR	CC chemokine receptor
CFA	colonization factor antigens
CINC-1	cytokine-induced neutrophil chemoattractant-1
CNP	corneal neuropathic pain

CNS	central nervous system
CN V	cranial nerve V
COX	cyclooxygenase
CXCL	CXC chemokine ligand
DAGL	diacylglycerol lipase
DEWS	dry eye workshop
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EAU	experimental autoimmune uveoretinitis
eCBs	endocannabinoids
ECS	endocannabinoid system
ERK	extracellular signal regulated kinase
FAAH	fatty acid amide hydrolase
GIRK	G-protein inwardly rectifying K^+-channel
GPCR	G-protein coupled receptor
GPI	glycosylphosphatidylinositol
GTPγS	guanosine 5'-O-[gamma-thio] triphosphate
i.p.	intraperitoneal
i.v.	intravenous
ICAM	intracellular adhesion molecule;
IFN	interferon
IL	interleukin
IOP	intraocular pressure
IVM	intravital microscopy
JNK	c-Jun NH_2-terminal kinase
JWH133	3-(1′1′-dimethylbutyl)-1-deoxy-Δ^8-THC
KC	keratinocyte derived chemokine
LPS	lipopolysaccharide
MAGL	monoacylglycerol lipase
MAPK	mitogen activated protein kinase
MCP	monocyte chemoattractant protein

MIP	macrophage inflammatory protein
MS	multiple sclerosis
NAM	negative allosteric modulator
NAPE-PLD	N-acyl phosphatidylethanolamine phospholipase-D
NF-κB	nuclear factor kappa-light-chain enhancer of activated B cells
NGF	nerve growth factor
NK	natural killer
NSAIDS	non-steroidal anti-inflammatory drugs
p38	p38 mitogen activated protein kinase
PAM	positive allosteric modulator
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PD	pharmacodynamic
PE	phosphatidylethanolamine
PFA	paraformaldehyde
PK	pharmacokinetic
PFATs	preservative free artificial tears
PIP_2	phosphatidylinositol
PI3K	phosphatidyl inositol 3 kinase
PKA	protein kinase A
PLC	phospholipase C
PVR	proliferative vitreoretinopathy
SAR	structure-activity based relationship
SLN	solid lipid nanoparticles
SD	standard deviation
SNRIs	serotonin norepinephrine reuptake inhibitors
TCAs	tricyclic antidepressants
TCR	T-cell receptor
TGF- β	transforming growth factor beta
TMS	transcranial magnetic stimulation
TNF-α	tumor necrosis factor alpha

TRP	transient receptor potential
TRPA1	transient receptor potential cation channel subfamily A member 1
TRPV1	transient receptor potential cation channel subfamily V member 1
TRPM8	transient receptor potential cation channel subfamily M member 8
VCAM	vascular cell adhesion molecule
VEGF	vascular endothelial growth factor
Vi/Vc	trigeminal subnucleus interpolaris/caudalis
Vc/C1	trigeminal subnucleus caudalis-upper cervical spinal cord
WT	wild type

ACKNOWLEDGEMENTS

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CHAPTER I: INTRODUCTION

1.1 The Cornea

1.1.1 Anatomy and physiology of the cornea

The cornea is a transparent, dome-shaped avascular tissue that has an average diameter of 11.3-12.1 mm in humans and covers the anterior chamber of the eye with a thickness ranging from 500-600 μm in the center, to 600–800 μm at the corneal periphery (Meek and Knupp, 2015; Pinero et al., 2008; Rufer et al., 2005). In mice, the corneal diameter varies from 2.3 -2.6 mm and the thickness ranges from 155-170 μm (Henriksson et al., 2009). The cornea acts as a barrier to foreign particles, absorbs oxygen and nutrients from the tear fluid and aqueous humor, and provides around two-thirds of the eye's refractive power (De Miguel et al., 2010). The cornea is considered a unique tissue in the body because of its immunologic privilege (i.e. the cornea prevents tissue damage and vision loss by supressing inflammation) (Treacy et al., 2016). Several factors are involved in maintaining this privilege. These include physical boundaries such as the blood-aqueous and blood retinal barriers, and immunomodulatory factors such as transforming growth factor-β (TGF-β) (Zhou and Caspi, 2010; Toguri et al., 2016).

There are 5 layers that form the cornea, listed here in order from most superficial (contacting air) to the deepest (contacting aqueous humor): the epithelium, Bowman's layer, the stroma, Descemet's membrane, and the endothelium (Figure 1). The outermost layer, the epithelium (50-90 μm thick), is comprised of epithelial cells with tight junctions that prevents foreign matter entering the inner layers. Following minor injury or abrasion, the epithelium can repair quickly. Depending on the severity of the insult, corneal scarring may occur and result in permanent vision loss via loss of transparency (Ramponi, 2017). Bowman's layer is a thin (8-14 μm) modified region of the anterior stroma which divides

the epithelium and stroma. Unlike the epithelium, Bowman's layer is relatively resistant to mechanical or infective trauma. The stroma is the thickest layer (500 μm) and consists of collagen fibrils and keratocytes, constituting around 90% of corneal thickness. The transparency of the cornea results from lattice arrangements of collagen fibrils in the stroma, as well from the cornea being avascular (Maurice, 1957). Descemet's membrane is the thinnest layer (2.2-4.5 μm) and is located below the stroma and above the endothelium. Descemet's membrane functions to support the endothelial cells that line the posterior cornea (endothelium). The endothelium (~5 μm thick) is formed of a single layer of mitochondria-rich hexagonal or cuboidal cells which regulate fluid and solute transport between the aqueous humor in the anterior chamber and the corneal stroma. Unlike the epithelium, cells of the endothelium do not regenerate upon injury (Bourne, 2003). The transparent and avascular cornea is separated from vascular and opaque sclera (the white of the eye) by the corneoscleral junction, called the limbus (Figure 1). The limbus forms the pathway for the outflow of aqueous humor and is the site of incision for cataract and glaucoma surgery (Van Buskirk, 1989).

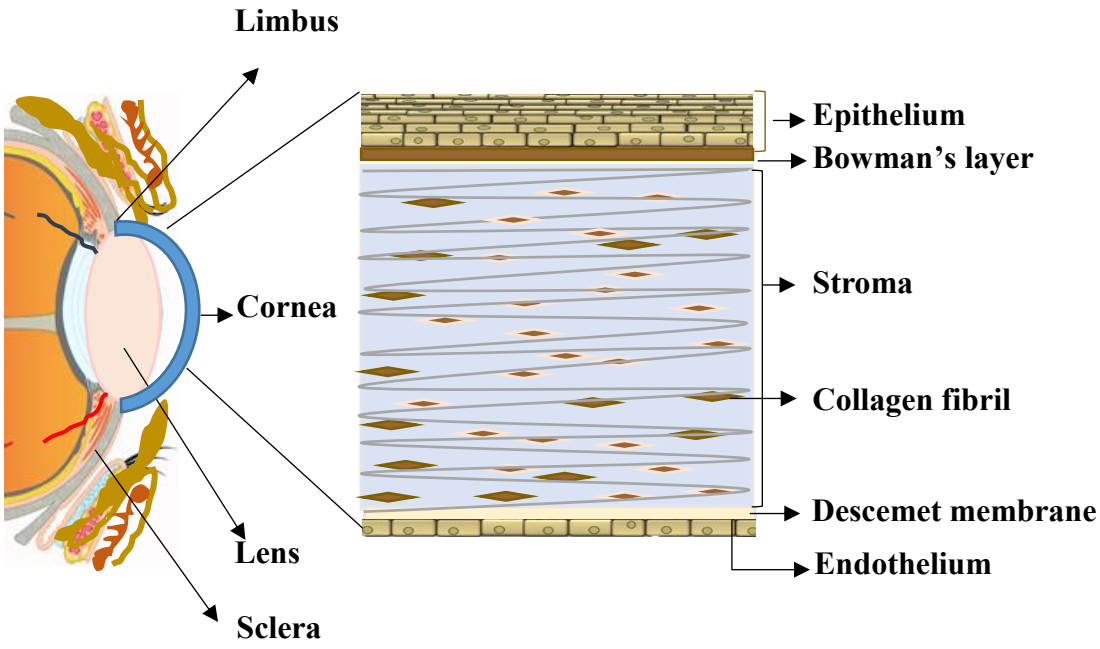

Figure 1. Cross section of the cornea. Corneal layers: the epithelium, Bowman's layer, stroma, Descemet's membrane and endothelium.

1.1.2 Sensory innervation of the cornea

The cornea contains the densest concentrations of sensory nerve endings in the body (Müller et al., 2003). The corneal sensory nerves are highly sensitive to touch, temperature and various chemical mediators. Damage or irritation to corneal sensory nerves results in a robust pain response (Ahmed et al., 2015; Azher et al., 2017; Beuerman and Tanelian, 1979)

The sensory nerve endings of the cornea are the peripheral axons of neurons that originate from the ophthalmic division of the trigeminal nerve, cranial nerve V (CN V), that is located in the trigeminal ganglion (Belmonte et al., 2004; Müller et al., 2003). Nociceptors are sensory receptors located at the ends of axons that act as detectors for injurious stimuli. Stimulation of these receptors through physical, mechanical or chemical insult produces pain sensation (Dubin and Patapoutian, 2010). Nociceptors can be categorized into mechanical, thermal or chemical depending on the type of stimuli to which they respond. Nociceptors that respond to multiple types of stimuli are termed as polymodal. These polymodal nociceptors comprise 70-80% of corneal nerve fibres (Belmonte et al., 2015; Goyal and Hamrah, 2016).

Corneal sensory neurons can be also classified as either thin myelinated (A-δ type) or unmyelinated (C-type) fibers based on the presence of a myelin sheath surrounding the axon and conduction velocity (Belmonte et al., 2004; Gallar et al., 1993). Majority of polymodal nociceptors in human cornea belong to the group of unmyelinated C fibers while some of them are thin myelinated (A-δ type) nerve fibers (Belmonte et al., 2004).

1.1.3 Higher-order secondary ocular neurons

Sensory information from corneal nociceptors is carried to higher areas in the brain by trigeminal ganglion neurons. The central axons of primary corneal neurons terminate in two region of trigeminal brainstem complex: the trigeminal subnucleus interpolaris/caudalis (Vi/Vc) transition region, and the subnucleus caudalis-upper cervical spinal cord (Vc/C1) region, through which direct or interneuron-mediated contacts are established with second order neurons (Gallar et al., 2004; Linna et al., 2000; Stapleton et al., 2006). Axons of second order neurons that are primarily located in the Vi/Vc region have a role in reflexive actions of eye (such as blink and tear reflexes), whereas Vc/C1 neurons mediate aspects of pain sensation (Hirata, Hu, & Bereiter, 1999). In addition, a specific set of moisture-sensitive neurons have been identified at the Vi/Vc region that is suggested to play a role in tear reflexes and in maintenance of fluid homeostasis on the ocular surface (Hirata et al., 2004)

1.2 Epidemiology of corneal pain and inflammation

1.2.1 Corneal pain

Ocular surface pain is an unpleasant sensory and emotional experience associated with actual or potential damage of ocular surface (Belmonte et al., 2004; Belmonte et al., 2004). Events leading to ocular pain range from the improper use of contact lenses, environmental or chemical exposure (air pollutants, hazardous chemicals, air pressure etc.) or surgical procedures such as cataract surgery (Belmonte et al., 2015; Cho et al., 2009).

Diseases of the cornea, or corneal damage, that affect corneal sensory neurons may lead to long-term dysregulation of peripheral nociceptive input and result in corneal

neuropathic pain (CNP) (Rosenthal and Borsook, 2012; Rosenthal and Borsook, 2016; Rosenthal et al., 2016). A recently published clinical review on CNP reported an increased number of patients presenting to clinics with unexplained ocular surface pain and symptoms such as burning, stinging, eye-ache, photophobia or severe eye-pain (Goyal and Hamrah, 2016). CNP can be severe and is frequently characterized by ocular pruritus, irritation, dryness, burning, aching, and photophobia, which are patient-specific and are integrated at higher brain centers (Galor et al., 2015; Shaheen et al., 2014). Etiologies of CNP include ocular pathologies such as dry eye disease or infectious keratitis (e.g. herpetic keratitis), surgical procedures such as corneal transplantations or refractive surgery, or recurrent erosions or tissue damage resulting from traumatic injury, chemical burns, or systemic treatment with chemotherapy. Other ocular pathologies associated with ocular pain include uveitis, retinitis, acute congestive glaucoma or endophthalmitis (Belmonte et al., 2015; Borsook and Rosenthal, 2011; Launay et al., 2016; Toguri et al., 2016).

1.2.2 Corneal inflammation

Corneal pain and inflammation often persist mutually (Namavari et al., 2012). Following corneal injury or infection, inflammatory substances such as prostaglandins and bradykinins are released locally by damaged tissues and can lead to corneal inflammation (Akpek and Gottsch, 2003). These substances activate polymodal nociceptors, and include actions at ligand-gated ion channels, G Protein-coupled Receptors (GPCRs), and cytokine receptors (Belmonte et al., 2004; Tumpey et al., 1998). Activation of ligand-gated ion channels including transient receptor potential cation channel subfamily V member 1 (TRPV1), transient receptor potential cation channel subfamily A member 1 (TRPA1) and

transient receptor potential cation channel subfamily M member 8 (TRPM8) channels, results in membrane depolarization and subsequent production of neuropeptides and cytokines (Akpek and Gottsch, 2003; Azher et al., 2017; Song et al., 2016). These mediators can sensitize nociceptors resulting in local neurogenic pain and inflammation (Belmonte et al., 2004). Severe corneal injury can produce intense ocular pain and lead to CNP, because of permanent changes (distorted neuronal excitability) in nociceptive excitability (Belmonte et al., 2015; Galor et al., 2015).

In normal homeostatic conditions, the cornea maintains its avascularity due to a balance between angiogenic and anti-angiogenic factors (Voiculescu et al., 2015). However, in conditions such as inflammation, hypoxia, trauma or limbal stem cell deficiency, this balance gets disrupted and lead to corneal neovascularization (Chang et al., 2012). The upregulation of vascular endothelial growth factor (VEGF), one of the most important mediators of angiogenesis, is responsible for inducing corneal neovascularization (Chang et al., 2012). Corneal neovascularization is one of the most prominent causes of temporary or permanent vision loss.

Cytokines and chemokines are small protein molecules, and are the most commonly released inflammatory mediators following corneal injury. They are released from the vasculature surrounding the cornea. Cytokines such as interleukin-7 (IL-7), IL-6, IL-1α, and interferon gamma (IFN-γ) as well as chemokines such as macrophage inflammatory protein-2 (MIP-2), MIP-1α, MIP-1β, and monocyte chemoattractant protein-1 (MCP-1), have a pro-inflammatory role in neutrophil infiltration and corneal inflammation (Azher et al., 2017). There are, however, some cytokines and chemokines that have a protective role in corneal inflammation, namely IL-10 and CCl3. Other cytokines such as tumor necrosis

factor-alpha (TNF-α), keratinocyte derived chemokine (KC) and IL-1β have been reported to produce hyper-nociception in a mouse model of carrageenan-induced inflammation (Cunha et al., 2005). Furthermore, it has been reported that TNF-α, acutely released following injury, triggers the subsequent release of the other major pro-inflammatory cytokines such as IL-6/IL-1β, leading to an increase in synthesis of prostaglandins (Cunha et al., 2005; Lorenzetti et al., 2002). TNF-α also leads to the release of cytokine-induced neutrophil chemoattractant-1 (CINC-1), which results in an increase in the release of sympathetic amines (Lorenzetti et al., 2002). Together, pro-inflammatory cytokines and chemokines that released following ocular inflammation as demonstrated in figure 2, are responsible for ocular pain (Launay et al., 2016).

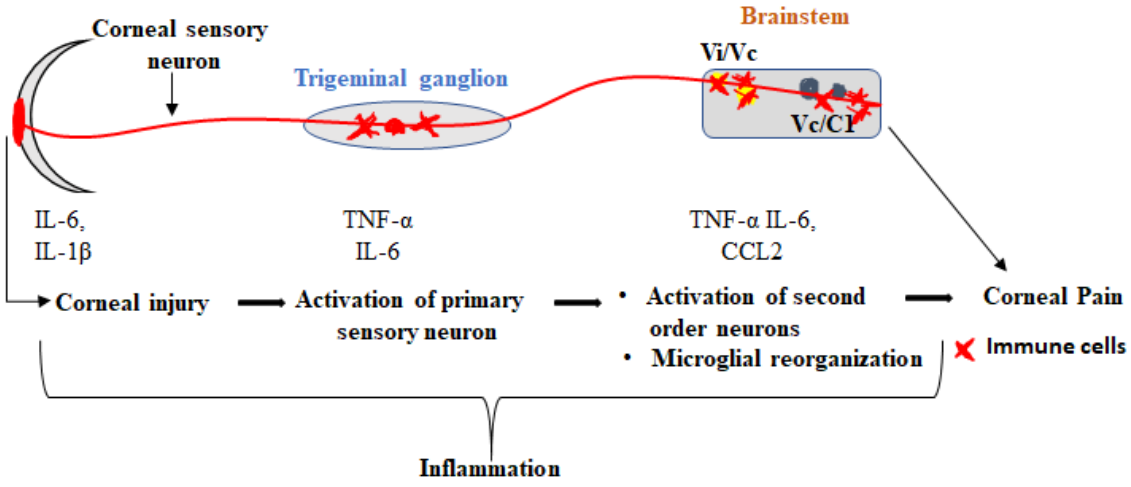

Figure 2. Schematic diagram showing injury-induced corneal inflammatory pain. Acute/chronic corneal injury leads to over-expression of pro-inflammatory cytokines (e.g. TNF-α, IL-6, IL-1β) which results in the recruitment and infiltration of immune cells and inflammation in the ophthalmic region of the trigeminal nerve (increased TNF-α and IL-6 mRNA expression). This neuroinflammation transfers to the sensory trigeminal complex (Vi/Vc, Vc/C1) of the brainstem, and is characterized by activated second order neurons as well as microglial cells reorganization and an increase in the release of pro-inflammatory mediators (TNF-α, IL-6, FOS, CCL2) which ultimately leads to corneal pain. Figure modified from (Launay et al., 2016).

1.2.3 Neutrophil infiltration into the corneal tissue

Neutrophils are among the various immune cells that infiltrate into the cornea from the surrounding blood vasculature (e.g. limbus) following injury, and are the first line of innate immune defense to fight against the infection or pathogens. Promoters of neutrophil chemotaxis, such as the neuropeptide secretoneurin, are released from damaged nerve terminals (Figure 3) and drive this process, representing the first phase of neutrophil infiltration (Oh et al., 2012). In the second phase, chemokines such as, IL-8, MIP-2 etc. and cytokines such as IL-1α, IL-1β etc., are released from the keratocytes and macrophages, respectively, and further drive the infiltration of neutrophils to the site of corneal injury (Oh et al., 2012) . Neutrophil infiltration is considered the hall mark of acute inflammation (Harada et al., 1994).

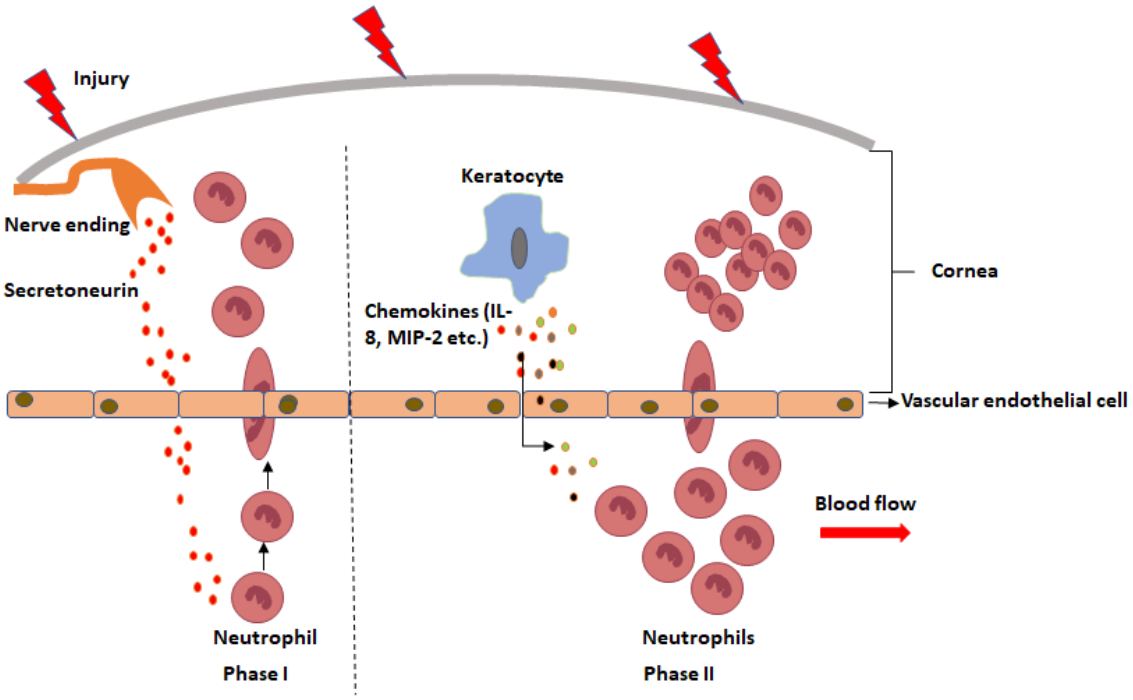

Figure 3. Schematic diagram showing corneal neutrophil infiltration following injury. Corneal injury induces nerve irritation, resulting in the release of neutrophil chemotaxis-promoting agents, such as secretoneurin, which drive the migration of neutrophils from the vasculature surrounding the cornea into corneal tissues (phase I). IL-8; Interleukin-8, MIP-2; macrophage inflammatory protein-2. Damaged keratocytes release pro-inflammatory cytokines and chemokines (e.g. IL-8, MIP-2) which further drive neutrophil migration towards injured areas of the cornea (phase II). Figure modified from (Oh et al., 2012).

1.3 Current therapies for corneal pain and inflammation

Current pharmacotherapies target diseases of the ocular surface, co-morbidities, nerve damage that leads to neuropathic pain, or act as analgesics and/or anti-inflammatories. Treatment of inflammatory ocular pain involves topical and/or oral anti-inflammatory agents. Common topical agents include corticosteroids *e.g*. loteprednol, prednisolone etc. (Goyal and Hamrah, 2016), non-steroidal anti-inflammatory drugs (NSAIDs) *e.g*. bromfenac, nepafenac, ketorolac etc. (Donnenfeld et al., 2011; Henderson et al., 2011)), or antibiotics, *e.g*. azithromycin, doxycycline etc. These treatments, however, may not be effective in treating corneal pain resulting from peripheral and/or central sensitization (Goyal and Hamrah, 2016). Systemic pharmacotherapies that are used for neuropathic pain, such as tricyclic antidepressants (TCAs) *e.g*. nortriptyline, amitriptyline, GABAergic drugs *e.g.* gabapentin, pregabalin, or antidepressants such as serotonin reuptake inhibitors (SNRIs) *e.g*. duloxetine, venlafaxine, are reported to be beneficial in the treatment of CNP (Dieckmann et al., 2017). Opioid analgesics *e.g.* fentanyl, codeine etc. are used for short-term treatment of ocular pain (*e.g.* post-operative) but are associated with adverse effects, including nausea and vomiting (Pereira et al., 2017). Preservative-free artificial tears (PFATs) and scleral contact lenses are used for the protection of the ocular surface as needed (Goyal and Hamrah, 2016).

Corticosteroids act through the inhibition of transcription of various genes including nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB) and activator protein-1 (AP-1). This results in an up-regulation of anti-inflammatory cytokines and decreased production of pro-inflammatory cytokines (van der Velden, 1998). However, corticosteroids carry a substantial side effect profile, potentially leading to

corneal and scleral thinning, increased intraocular pressure (glaucoma), opacification of lens (precipitating the development of cataracts), hypertension, osteoporosis and hyperglycemia (Faktorovich and Melwani, 2014; Smith et al., 1998). Long term use of corticosteroids supresses the host immune response and as a result produces increased susceptibility to microbial infections and retardation in corneal wound healing (McGhee et al., 2002).

Cyclosporine is a potent immunosuppressive drug that binds to a cytosolic protein cyclophilin in activated T-cells. The cyclosporine-cyclophilin complex inhibits the phosphatase activity of calcineurin (serine/threonine protein phosphatase) and consequently leads to a decrease in production of inflammatory cytokines such as IL-2 and IL-4 (Matsuda and Koyasu, 2000). In addition to reducing ocular surface inflammation, cyclosporine treatment results in an increased tear production, with applications in dry eye disease (Donnenfeld and Pflugfelder, 2009). Cyclosporine has, however, been shown to slow nerve fibre regeneration, induce hypertension and is nephrotoxic (Adams et al., 1977; Merida et al., 2015).

NSAIDs derive their analgesic and anti-inflammatory actions by blocking the formation of prostaglandins through inhibition of cyclooxygenase 1 and 2 (COX-1 and COX-2) enzymes (Cashman, 1996). NSAIDs offer several benefits over corticosteroids, including analgesia, an intra-ocular pressure (IOP)-stabilizing effect, as well as reducing the risk of secondary infection (Cho et al., 2009). However, long-term use of NSAIDs are associated with serious complications such as corneal melting and corneal ulceration (Gaynes and Fiscella, 2002).

It has been reported that people with dry eye syndrome often experience blurred vision, irritation and ocular pain (Galor et al., 2017). Definition and Classification Subcommittee of the International Dry Eye Workshop (DEWS) has defined dry eye as *"a multifactorial disease of the ocular surface characterized by a loss of homeostasis of the tear film, and accompanied by ocular symptoms, in which tear film instability and hyperosmolarity, ocular surface inflammation and damage, and neurosensory abnormalities play etiological roles"* (Belmonte et al., 2017). Low levels of carotenoids in meibum (an oily substance supplied by the meibomian gland in the eye that prevents evaporation of the eye's tear film) have been suggested to result in an imbalance in tear film contributing to the symptoms of dry eye (Foulks et al., 2013). Topical application of antibiotics such as azithromycin and doxycycline, have been shown to restore carotenoids in meibum, thereby stabilizing the tear film and contributing to the resolution of some symptoms of dry eye (Foulks et al., 2013). Azithromycin is a macrolide antibiotic that has strong gram-negative bacterial coverage, as well as some gram-positive bacterial coverage (Williams, 1991). Doxycycline is a tetracycline antibiotic that has both gram-positive and gram-negative (broad spectrum) bacterial coverage (Cunha et al., 1982). Oral administration of doxycycline at a dose of 100 mg once/twice a day for 2–3 months followed by daily dosing for another 3 months have been shown to be effective in the treatment of ocular rosacea (inflammation that causes redness, burning and itching of the eyes) and increasing tear film stability (Golub et al., 1998).

Systemic pharmacotherapies are associated with various CNS and peripheral side effects (Finnerup et al., 2005; Goyal and Hamrah, 2016). For tricyclic antidepressants, such as nortriptyline, these side-effects are related to their anticholinergic profile and include

symptoms such as, sedation, dizziness, dry mouth and eyes, and blurred vision (Dieckmann et al., 2017). GABAergic drugs, such as pregabalin, are associated with side-effects including somnolence, dizziness, ataxia, dry mouth, weight gain and blurred vision (Kremer et al., 2016). The side-effects of opioid therapies include rapidly developing tachyphylaxis, sedation and other anticholinergic effects such as dry mouth, constipation, potentially severe respiratory depression, as well as potential for addiction (Dieckmann et al., 2017).

Because of the multifactorial nature of ocular surface diseases that involve corneal inflammation and pain leading to dysfunctional corneal sensory neurotransmission, a single drug therapy may not be effective in producing adequate pain relief. Therefore, some non-pharmacological treatment modalities are also being used as adjuncts in addition to drug therapy. These include diet modification, cardio-exercise, neuromodulation, and transcranial magnetic stimulation (TMS) etc. (Dieckmann et al., 2017). Daily intake of omega-3 fatty acid has been shown to decrease corneal dryness (Liu and Ji, 2014). Cardio-exercises have been shown to inhibit pain pathways, thereby improving allodynia and hyperalgesia (Kami et al., 2017). Taken together, it is of great importance to develop novel drugs and pharmacological strategies that target both ocular pain and inflammation, with favourable side effect profiles and efficacy in order to treat acute and chronic ocular surface disease.

1.4 The endocannabinoid system (ECS)

The ECS has been reported to be an important endogenous regulatory system in the modulation of pain, inflammation, and neurodegenerative disorders and may be an important therapeutic target for human disease (Bisogno et al., 2016; Huang et al., 2016). The ECS is an endogenous lipid signaling system that includes two G-protein coupled receptors (GPCRs), cannabinoid 1 receptor (CB_1R) and cannabinoid 2 receptor (CB_2R), endocannabinoids (eCBs), and the cognate enzymes responsible for the synthesis and degradation of eCBs (Kaur et al., 2016; Lu and Mackie, 2016).

N-arachidonoylethanolamine (AEA) and 2-arachidonogylcerol (2-AG) are the two most studied eCBs, and both activate CB_1R and CB_2R, producing analgesic as well as anti-inflammatory effects (Woodhams et al., 2017; Zogopoulos et al., 2013). As demonstrated in figure 4, the enzymatic action of N-acyl phosphatidylethanolamine phospholipase-D (NAPE-PLD) on membrane lipids result in ‘on-demand’ synthesis of AEA, which gets degraded by fatty acid amide hydrolase (FAAH) into arachidonic acid and ethanolamine (Di Marzo, 2008). Diacylglycerol-lipase (DAGL) is responsible for synthesis of 2-AG whereas, monoacylglycerol lipase (MAGL) degrades 2-AG into arachidonic acid and glycerol (Di Marzo, 2008).

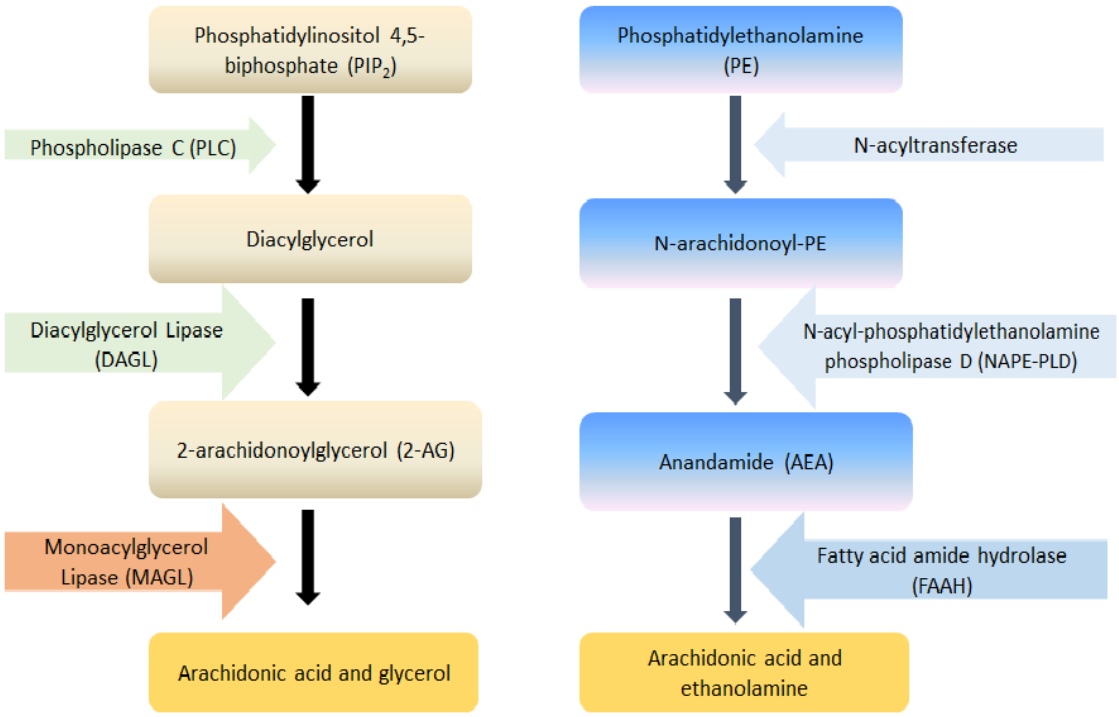

Figure 4: Synthesis and degradation pathway of endocannabinoids.

Endocannabinoids in the body are released on-demand in response to various physiological and pathological states including: pain, inflammation, stress, emotion, anxiety, depression etc. (Crowe et al., 2014; Hillard, 2018; Huang et al., 2016; Jenniches et al., 2016). On-demand Ca^{2+}-dependent synthesis of eCBs in the postsynaptic neuron results in retrograde activation of CB_1R in the presynaptic neuron, and inhibition of the release of neurotransmitters (Figure 5) (Diana and Marty, 2004; Freund et al., 2003; Howlett et al., 2002; Howlett et al., 2004). Although, the eCBs mediate their pharmacological actions through CB_1R or CB_2R (Kaur et al., 2016; Starowicz and Finn, 2017), activity at non-cannabinoid receptors such as TRPV1 and 5-HT_{1A} have been also reported (Morales and Reggio, 2017; Starowicz and Finn, 2017; Ward et al., 2014).

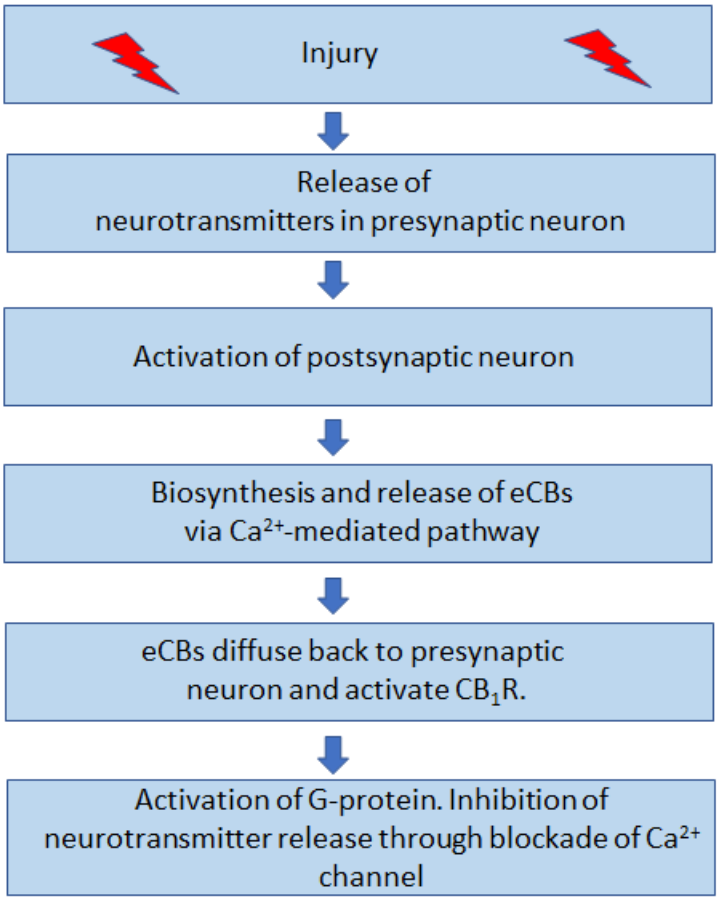

Figure 5. Inhibition of neurotransmitter release following CB_1R activation. eCBs; endocannabinoids, Ca^{2+}; calcium, CB_1R; cannabinoid 1 receptor.

1.4.1 Distribution and pharmacological role of CB_1R

CB_1R is a member of the family A GPCRs that couples to $G_{i/o}$ proteins (Howlett et al., 2002). CB_1R is expressed in and highly localized to neurons, in both the central and peripheral nervous systems (Kaur et al., 2016; Porcella et al., 2000; Stamer et al., 2001). CB_1R activation leads to inhibition of adenylyl cyclase (AC) and protein kinase A (PKA) signaling, inhibition of voltage-gated Ca^{2+}-channels and stimulation of G-protein inwardly rectifying K^+-channel (GIRK) with resultant reductions in neurotransmitter release (Lu and Mackie, 2016; Mackie, 2006). With respect to pain transmission, CB_1R-mediated inhibition of cAMP-dependent PKA-dependent TRPV1 receptor phosphorylation in nociceptors leads to blunting of the TRPV1 receptor-mediated inflammatory response and decreased sensitization (Khasabova et al., 2012; Moriello and De Petrocellis, 2016; Rosenbaum and Simon, 2007; Wang et al., 2014; Yang et al., 2013).

CB_1R activation has been reported to have a beneficial effect in the treatment of several neurological disorders including pain and inflammation (Iversen and Chapman, 2002; Cravatt and Lichtman, 2004; Pertwee, 2002; Rice et al., 2002). However, the actions of clinical drugs that target CB_1R are limited by the cognitive deficits, memory impairment, motor disturbances and tolerance. Peripheral activation of CB_1R reduces pain and inflammation in animal models of hyperalgesia and inflammation (Richardson et al., 1998). Therefore, targeting CB_1R with local/regional application of CB_1R agonist(s) may be a useful therapeutic strategy to mitigate CNS side-effects in order to reduce inflammatory-mediated peripheral pain and sensitization. In support of this, a study carried out in mice with CB_1R knockout only in their peripheral nociceptive neurons, and with preservation of expression of CB_1R in the CNS, demonstrated a reduction in analgesia produced by local

and systemic, but not intrathecal, delivery of cannabinoids (Agarwal et al., 2007). This strengthens the evidence supporting peripheral activation of CB_1R to achieve analgesia without central side-effects.

Recently, the agonist-bound crystal structures of human CB_1R has been described (Hua et al., 2016; Hua et al., 2017; Shao et al., 2016). These crystal structures will aid in the development of more selective CB_1R ligands and provide novel insights into the structure-activity based relationship (SAR) of these ligands (Laprairie et al., 2015). Based on these studies, ligands have been shown to bind to distinct orthosteric and allosteric sites on CB_1R. Such allosteric sites are topographically distinct from the orthosteric binding site (Christopoulos and Kenakin, 2002). Allosteric ligand-binding results in conformational changes to the receptor that alter affinity and/or the efficacy of orthosteric ligands (Laprairie et al., 2017; Ross, 2007). CB_1R-related side effects associated with orthosteric activation such as supraphysiological receptor activation and desensitization may be reduced via allosteric activation of CB_1R (Ross, 2007).

1.4.2 Distribution and pharmacological role of CB_2R

Like CB_1R, CB_2R is a family A GPCR that couples to $G_{i/o}$ proteins, and inhibits AC (Felder et al., 1995). Additionally, activation of CB_2R modulates phosphorylation of extracellular signal-regulated kinase (ERK), c-Jun NH_2-terminal kinase (JNK), and p38 (p38 mitogen activated protein kinase) (Dhopeshwarkar and Mackie, 2014; Kishimoto et al., 2003). CB_2R is often referred to as the 'peripheral' cannabinoid receptor, and is expressed primarily on immune cells (Croxford and Yamamura, 2005). Among the various immune cells present in the body, B cells, natural killer (NK) cells, monocytes, neutrophils,

CD8+ T cells, CD4+ T cells express CB_2R-mRNA in highest order, respectively (Galiegue et al., 1995). CB_2Rs are also expressed on monocyte-derived cells such as microglia, circulating macrophages, osteocytes, osteoclasts and hepatic Kupffer cells (Atwood & Mackie, 2010). The expression of CB_2R on immune cells coupled to lack of psychotropic effects associated with receptor activation suggests that the CB_2R is a promising drug target for immunomodulation and to achieve analgesia (Dhopeshwarkar and Mackie, 2014; Starowicz and Finn, 2017).

Activation of CB_2R modulates the release of various inflammatory mediators and has been shown to lead to a reduction in inflammation (Berdyshev, 2000; Munro et al., 1993; Onaivi et al., 2006). Among various immune cells that are activated following inflammation, neutrophils are considered the hallmarks of acute inflammation (Harada et al., 1994). Given evidence that neutrophils make a crucial contribution to a number of autoimmune, autoinflammatory, and neoplastic disorders (Nathan, 2006), drugs that modify neutrophil activation, such as CB_2R agonists, have the potential to reduce the inflammatory response (Kapellos et al., 2017; Wang et al., 2016). In line with this, CB_2R activation has been shown to protect against cerebral ischemia through inhibition of neutrophil recruitment in a mouse model of ischemic stroke (Murikinati et al., 2010), and cannabinoids that act at CB_2R inhibit the release and migration of neutrophils in various *in vitro* and *in vivo* studies (McHugh et al., 2008; Nilsson et al., 2006). In addition to anti-inflammatory effects, CB_2R activation has also been reported to be neuroprotective and analgesic, with a role in wound-healing (Dhopeshwarkar and Mackie, 2014; Wang et al., 2016).

1.4.3 Distribution and pharmacological role of 5-HT$_{1A}$ receptor, a non-cannabinoid receptor

5-HT$_{1A}$ receptors are GPCRs that bind serotonin (5-HT) and when activated couple to G$_{i/o}$ proteins. Like CB$_1$R and CB$_2$R, activation of 5-HT$_{1A}$-G$_{i/o}$ signaling leads to inhibition of AC (De Vivo and Maayani, 1986). The presence of 5-HT$_{1A}$ receptors have not been reported in the cornea, however, 5-HT$_{1A}$ receptors have been shown to be highly expressed in the CNS, with the highest densities in the hippocampus, lateral septum frontal cortex, and lowest densities in the thalamic or hypothalamic nuclei (Banerjee et al., 2007). Dysregulation of 5-HT$_{1A}$ receptors in these locations of brain have been correlated with mood disorders such as depression and anxiety, and schizophrenia (Garcia-Garcia et al., 2014; Lanfumey and Hamon, 2000). 5-HT$_{1A}$ receptors have been also implicated in modulation of pain (Bardin et al., 2003). The activation of 5-HT$_{1A}$ receptors by CBD, a non-psychoactive phytocannabinoid, has been shown to reduce neuropathic pain, depression and acute stress in animal models (Linge et al., 2016; Resstel et al., 2009; Ward et al., 2014). Given that activation of 5-HT$_{1A}$ receptor is beneficial in several pathophysiological states, it could serve as an important therapeutic target for the anti-inflammatory and analgesic actions of some cannabinoids.

1.4.4 Endocannabinoids and their pharmacological role

There are two major endocannabinoids that have been frequently studied, AEA and 2-AG. AEA and 2-AG can activate both CB$_1$R and CB$_2$R and produce analgesic as well as anti-inflammatory effects (Rice et al., 2002; Woodhams et al., 2017). Both AEA and 2-AG have been shown to produce analgesic effects in animal models of acute pain (Khasabova

et al., 2011; Zubrzycki et al., 2017), inflammation and painful neuropathy (reviewed in Fine and Rosenfeld, 2013; Ulugol, 2014; Zogopoulos et al., 2013). 2-AG has been reported to reduce levels of pro-inflammatory cytokines i.e. TNF- α, IL- 1β and IL-6 in a mouse model of closed head injury (Panikashvili et al., 2006). It has been also shown that 2-AG has a neuroprotective role via inhibition of NF-kappa B in a mouse model of closed head injury (Panikashvili et al., 2005). This study also showed that a neuroprotective effect of 2-AG was blocked in $CB_1R^{-/-}$ mice. A study performed in a chronic constrictive injury (CCI)-induced neuropathic pain model in rats found elevated levels of AEA and 2-AG; the author has suggested that the elevation of AEA and 2-AG could play a role in inhibiting pain (Petrosino et al., 2007).

AEA and 2-AG have poor pharmacokinetic profiles in the context of their therapeutic use. Namely, they are rapidly degraded through the endogenous enzymatic action of fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL) for AEA and 2-AG, respectively (Anderson et al., 2014b; Di Marzo, 2008). Recently, research has focused on the use of FAAH and MAGL inhibitors to increase levels of AEA and 2-AG, to reduce pain and inflammation. For example, in a mouse model of sciatic nerve injury Kinsey at al., (2009) showed that the inhibition of FAAH or MAGL, leads to increased levels of endocannabinoids and reduces neuropathic pain (Kinsey et al., 2009). This suggests that therapeutic approaches that result in enhanced eCBs actions may also be beneficial for alleviating pain in various pathologies.

1.4.5 Phytocannabinoids and their pharmacological role

Phytocannabinoids are cannabis plant ("phyto-")-derived cannabinoids. There are more than 400 active chemicals present in the cannabis plant (Atakan, 2012) and over 120 of these are active phytocannabinoids (Morales et al., 2017). The pharmacological interest in phytocannabinoids began with the isolation and identification of the two most abundant and therapeutically active constituents of the cannabis plant: (–)-*trans*-Δ^9–tetrahydrocannabinol (Δ^9THC) and (-)-cannabidiol (CBD). Δ^9THC is well known primarily for the psychoactive effects of cannabis, and was first isolated and identified in 1964 by Raphael Mechoulam's group (Mechoulam and Gaoni, 1967; Russo, 2011; Schafroth and Carreira, 2017). CBD is non-psychoactive and has diverse pharmacology, but also has reported therapeutic actions including: analgesic, anti-inflammatory, neuroprotective, anti-tumor, antiepileptic, cardioprotectant, and anxiolytic effects. (Morales et al., 2017).

Δ^9THC produces anti-nociceptive effects in various animal models of pain, including tail-flick, hotplate, inflammatory, cancer, neuropathic, and visceral nociceptive model (Burstein et al., 1988; Compton et al., 1991; Formukong et al., 1988; Martin et al., 1984; Varvel et al., 2005). Δ^8THC, a less psychotropic analogue of Δ^9THC, has been shown to reduce intraocular pressure when delivered topically to rabbit eyes (Jarvinen et al., 2002; Muchtar et al., 1992). The pharmacological responses produced by THC are through activation of both CB_1R and CB_2R, whereas the actions of CBD are independent of the direct activation of CB_1R or CB_2R. Several non-cannabinoid receptors such as TRPV1 and 5-HT_{1A} have been reported to be involved in the analgesic and anti-inflammatory effects of CBD (Starowicz and Finn, 2017).

1.4.6 Synthetic cannabinoids and their pharmacological role

Identification of eCBs and phytocannabinoids has led to synthesis of other classical and non-classical cannabinoids. Most of the synthetic cannabinoids are developed based on modifications to the C3 side chain of classical phytocannabinoids. For example, the synthetic classical cannabinoid, CP55940, was developed from Δ^9THC through C3 side chain modification. Cannabinoids such as WIN 55,212-2, JWH-018, and SR141716 are examples of non-classical cannabinoids that are not based on phytocannabinoid structure (Bow and Rimoldi, 2016). Synthetic cannabinoids such as CP55940 and WIN55,212-2, activate both CB_1R and CB_2R (Felder et al., 1995; Florek-Luszczki et al., 2015; Kapur et al., 2009; Kuster et al., 1993); the activation of CB_1R may produce behavioral side-effects. Therefore, synthesis of CB_2R selective cannabinoids may enable certain therapeutic effects, without producing psychotropic effects associated with CB_1R activation. For example, a selective CB_2R agonist, HU-308 (a CBD-derivative), has been shown to elicit anti-inflammatory and peripheral anti-nociceptive activity in a rat model of formalin-induced peripheral pain, an effect which was blocked by CB_2R antagonist (SR-144528) (Hanus et al., 1999). Similarly, a synthetic Δ^9THC-derivative and a selective CB_2R agonist, O-3223, reduced nociceptive behavior in the formalin test, thermal hyperalgesia in the chronic constriction injury of the sciatic nerve (CCI) model, and reduced edema and thermal hyperalgesia elicited by intraplantar injection of LPS, without psychoactive side-effects, in preclinical pain models (Kinsey et al., 2011).

1.4.7 CB_1R allosteric modulators and their pharmacological role

Allosteric modulators are small molecules that bind to allosteric sites and potentiate or inhibit the action of an orthosteric agonist (Christopoulos and Kenakin, 2002; Kenakin, 2012). As demonstrated in figure 6, if the allosteric modulator enhances receptor signaling, it is termed a positive allosteric modulator (PAM) and conversely if it inhibits receptor signaling, it is termed a negative allosteric modulator (NAM) (Gregory et al., 2013). Allosteric modulators do not produce pharmacological response in the absence of orthosteric ligands (Figure 6). In addition to allosteric modulators, some allosteric ligands can directly activate or inhibit the receptor are referred to as allosteric agonists or antagonists, respectively (Christopoulos and Kenakin, 2002; Kenakin, 2012; Wootten et al., 2013). Identification of the allosteric site on CB_1R was facilitated by identification of three indole compounds that modulated the action of an orthosteric agonist CP55,940 (Price et al., 2005). Following the identification of an allosteric site at the CB_1R, several allosteric modulators have now been synthesized and studied in animal models of pain and inflammation (Khurana et al., 2017; Nguyen et al., 2017). Orthosteric activation of CB_1R has been reported to produce side-effects such as tolerance, psychosis and physical dependence (Biala, 2008; Compton et al., 1990; Gonzalez et al., 2005). CB_1R allosteric modulators binds at the distinct, allosteric site and may stabilize the GPCR conformations in such a way that they can modulate the affinity and/or efficacy of orthosteric binding (Ross, 2007). These properties of an allosteric modulators may be useful in minimizing the side-effects associated with direct orthosteric activation of CB_1R (Khurana et al., 2017; Changeux and Christopoulos, 2017). Further, as suggested by Khurana et al., allosteric

modulators also have potential in the development of pathway-specific or biased therapeutics. The CB_1R PAM, ZCZ011 has been shown to increase the potency of orthosteric agonists, CP55,940 and AEA, and reduce neuropathic and inflammatory pain using chronic constriction nerve injury-induced neuropathic pain and carrageenan-induced inflammatory pain models in mice, with minimal or no cannabimimetic side-effects (Ignatowska-Jankowska et al., 2015). In a recent *in vitro* study, the phytocannabinoid CBD was shown to act as a NAM at the CB_1R, where it reduced the efficacy and potency of 2-AG, and Δ^9THC-mediated ERK1/2 signaling (Laprairie et al., 2015). The NAM property of CBD could explain its use as an anti-psychotic phytocannabinoid. Use of CBD in combination with THC would be beneficial to minimize the unwanted side-effects associated with THC such as desensitization.

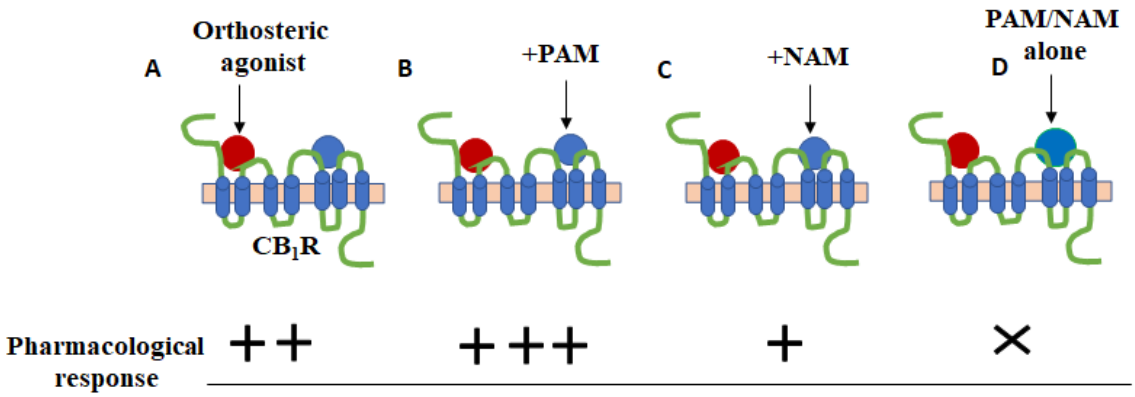

Figure 6. Signaling of CB_1R with orthosteric and/or allosteric site activation. (A) Activation of the orthosteric site (red circle) of CB_1R leads to downstream signaling. (B) Activation of the allosteric site (blue circle) by PAM potentiate the potency and/or efficacy of orthosteric ligands. (C) Negative allosteric modulators (NAM) decrease the potency and/or efficacy of orthosteric ligands. (D) Allosteric modulators, alone, do not produce pharmacological response.

Recently, work on synthesis and *in vitro* and *ex vivo* pharmacology of the novel CB_1R modulators: GAT211, GAT228 and GAT229 was published (Figure 7) (Laprairie et al., 2017). GAT228 (R) and GAT229 (S) are the resolved enantiomers of the racemic compound GAT211 (Laprairie et al., 2017). GAT211, acted as a CB_1R-selective ago-PAM (displayed both PAM and agonist activity) in HEK293A and Neuro2a cells expressing human recombinant CB_1R (hCB_1R) (Laprairie et al., 2017). Further, GAT211 also exhibited ago-PAM activity in mouse-brain membranes rich in native CB_1R. GAT228 is responsible for the allosteric agonist activity of GAT211, whereas PAM activity of GAT211 is GAT229-dependant (Laprairie et al., 2017).

A recent study by Cairns et al. in normotensive mice and a murine model of ocular hypertension has shown that GAT229 acted as a CB_1R-PAM (Cairns et al., 2017). Further, GAT229 enhanced a CB_1R-mediated reduction in IOP, in combination with a sub-threshold dose of an orthosteric agonist WIN55,212-2 (Cairns et al., 2017). The *in vivo* profile of the racemate, GAT211, has been recently reported (Slivicki et al., 2017). This study found that GAT211 suppressed both neuropathic and inflammatory pain without producing typical CB_1R-mediated cannabimimetic side-effects (Slivicki et al., 2017). Unlike orthosteric CB_1R agonists, tachyphylaxis was not observed over a 20-day interval of once daily dosing of GAT211 (Slivicki et al., 2017). GAT211, GAT228 and GAT229 have been studied *in vitro*, however, to-date there has been limited *in vivo* studies.

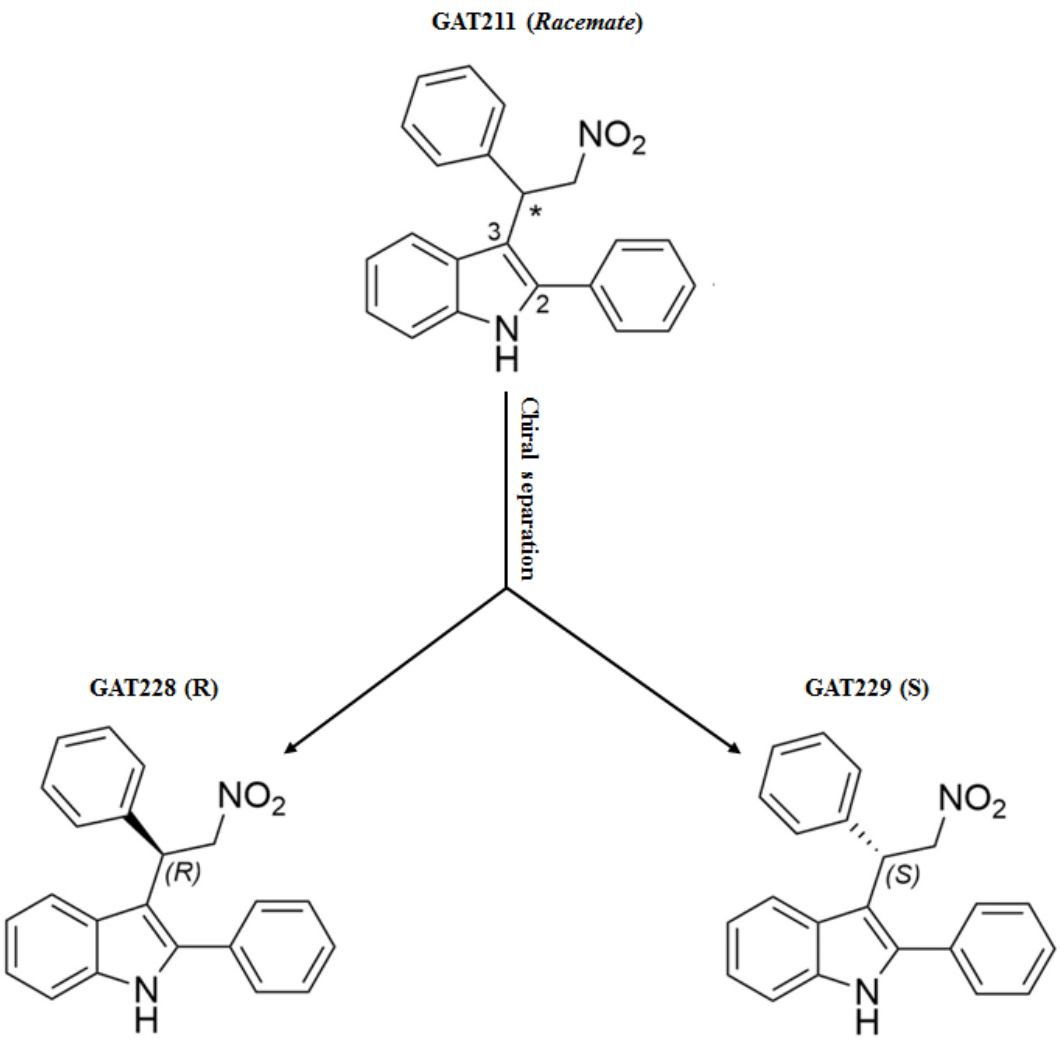

Figure 7. Chiral separation of GAT228 (R) and GAT229 (S) from its racemate GAT211. GAT211 is a CB_1R agonist & positive allosteric modulator (ago-PAM), GAT228 is a CB_1R allosteric agonist and GAT229 is a pure CB_1R positive allosteric modulator (PAM). Reprinted (adapted) with permission from Laprairie et al., 2017. Copyright (2017) American Chemical Society.

1.5 Cannabinoids in corneal pain and inflammation

1.5.1 Cannabinoids in corneal pain

The presence of CB_1R has been reported in the cornea, retina and trabecular meshwork of human and non-human primates, while CB_2R expression is limited under non-pathological conditions (Straiker et al., 1999; Toguri et al., 2016). A recent study has reported the presence of CB_1R in bovine corneal epithelial cells (Murataeva et al., 2015). However, this study did not detect the expression of CB_2Rs. While several studies have demonstrated reductions in both acute/chronic pain and inflammation with cannabinoid treatments via activation of both CB_1Rs and/or CB_2Rs (Clapper et al., 2010; Guindon and Hohmann, 2008; Guindon and Hohmann, 2009; Mackie, 2006), there are limited studies reporting the effects of cannabinoids on ocular pain. One such study investigated the effects of the non-selective cannabinoid agonist, WIN55212-2, on rat trigeminal brainstem complex (Bereiter et al., 2002). The topical application of WIN55212-2 reduced the number of Fos-like immunoreactive neuronal nuclei (Fos-LI) at the Vi/Vc transition but not at the Vc/C1 junction region; the effect prevented by selective CB_1R antagonist, SR141716A (1mg/kg, i.v). This study concluded that activation of CB_1R affects the activity of corneal-responsive neurons that preferentially contribute to homeostasis of the anterior eye and/or reflexive aspects (Vi/Vc) rather than the sensory-discriminative aspects of corneal nociception (Vc/C1). As this study used only an acute stimulus (topical mustard oil or a CO_2 air puff) to activate corneal nociceptors, further research is needed to understand the role of the ECS in chronic models of corneal pain. In a study with chemical irritation and mechanical scratching of corneal epithelium, elevated expression of c-Fos in the trigeminal nucleus neuron was reported (Martinez and Belmonte, 1996). These c-Fos

can induce chemo-sensitivity of the nociceptive terminals which may result in ocular pain. Cannabinoids can reduce the expression of c-Fos leading to attenuation of c-Fos-induced pain.

Targeting ECS for wound healing and the treatment of corneal neuropathies has shown promising results. For example, following treatment with WIN55212-2 (a non-selective cannabinoid agonist) full recovery of the corneal epithelial layer was seen at 5 days post-corneal alkali burn in WT mice compared to $CB_1R^{-/-}$ mice; WIN55212-2 improved wound healing and reduced stromal scarring (Yang et al., 2013). This study also reported co-expression of CB_1R and TRPV1 receptors in the corneal epithelium and activation of CB_1R by WIN55212-2 has been shown to reduce TRPV1 sensitization and reduction of corneal inflammation and scarring (Yang et al., 2013). It has been also reported that the activation of CB_1R inhibits NGF-induced TRPV1 sensitization in mouse dorsal root ganglion (DRG) afferent neurons (Wang et al., 2014). This reduction in TRPV1 sensitization and inflammation by activation of CB_1R could be beneficial in reducing corneal pain.

1.5.2 Cannabinoids in corneal inflammation

Normally, CB_2R expression is low in anterior ocular structures including the cornea, however these levels increased during ocular pathologies, such as uveitis (Toguri et al., 2014; Xu et al., 2007). It has been reported that the selective activation of CB_2R reduces inflammation in the anterior ocular chamber (Toguri et al., 2014; Toguri et al., 2015; Xu et al., 2007). The selective CB_2R agonist, JWH-133, reduced ocular inflammation by decreasing leukocyte infiltration into the inflamed retina, which was associated with the

decrease in pro-inflammatory cytokines, including TNF-α, interferon-γ (IFN-γ) and IL-6 (Xu et al., 2007). Recent work using rat models of inflammation reported reductions in leukocyte-endothelial interactions following CB_2R activation, in addition to reduction in the levels of several pro-inflammatory cytokines, such as IL-1β, IL-6, TNF-α, and INF-γ, and chemokines such as CCL5 and CXCL2 (Toguri et al., 2014; Toguri et al., 2015). This reduction in cytokines and chemokines was shown to be mediated by a reduction in transcription factors (NF-κB and AP-1). This study also demonstrated that CB_2R agonists were more efficacious than clinically used topical corticosteroids and NSAIDs in a single dose regimen (Toguri et al., 2014).

A recent study from our lab has also shown that selective activation of CB_2R with the CB_2R-selective agonist, HU-308, decreases retinal histopathological scores, the number of activated microglia, and leukocyte adhesion compared to vehicle-treated eyes in a model of chronic ocular inflammation (Szczesniak et al., 2017). Beneficial effects of CB_2R activation were absent in $CB_2R^{-/-}$ mice. It has been also reported that the activation of CB_1R by the non-selective cannabinoids, WIN55,212-2, reduced TRPV1 receptor-mediated inflammatory response in a mouse model of corneal injury (Yang et al., 2013). Given the evidence that activation of both CB_1R and CB_2R are beneficial in ocular surface (corneal) injury and intraocular inflammation, targeting the cannabinoid receptors could be beneficial in mitigating corneal pain and inflammation.

1.6 Experimental models of corneal pain and inflammation

Animal models are frequently used to study corneal hyperalgesia and inflammation following corneal injuries that result from some of the most common causes i.e. chemical,

mechanical (surgery) or radiation insults (Acosta et al., 2014; Wenk and Honda, 2003; Wenk et al., 2003). Use of animal models provide opportunities to explore mechanisms contributing to disease as well as examine putative novel therapeutic approaches for treating corneal disease. Chronic topical instillations of 0.2% benzalkonium chloride (BAC) in male C57BL/6J mice has been shown to increase pro-inflammatory and neuronal markers in trigeminal ganglion resulting in ocular pain and inflammation (Launay et al., 2016).

In my thesis, I used a mouse model of acute corneal hyperalgesia and inflammation, resulting from superficial chemical injury to the cornea. This is an adaption of an established rat model of corneal hyperalgesia and inflammation, in which central corneal cauterization results in corneal hyperalgesia and acute inflammation (Wenk and Honda, 2003). In the rat model described by Wenk and Honda (2003), the central cornea was cauterized using a silver nitrate applicator stick (75% silver nitrate, 25% potassium nitrate), producing a discrete lesion of 1 mm in diameter. Edema of the corneal stroma and neutrophil counts were assessed as parameters of corneal inflammation, and an increase in blinks resulting from the corneal application of 1μM capsaicin was indicative of corneal hyperalgesia. A significant increase in capsaicin-induced blinking was evident 2 h after cauterization, peaked at 6 h, and was no longer significant at 12 h. Similarly, corneal neutrophil infiltration was significant until 48 h post-injury.

Transient receptor potential (TRP) channels are a group of ion channels expressed in animal cell membranes that can respond to various types of stimuli such as heat, cold, chemicals, light and touch (Clapham et al., 2001; Ramsey et al., 2006; Venkatachalam and Montell, 2007; Zheng, 2013). The activation of TRP-channels expressed in corneal nerve

fibres (C and Aδ-fibres), such as TRPV1, TRPA1 and TRPM8 can induce a pain response (Belmonte et al., 2004; Eguchi et al., 2017). TRPV1 is a non-selective ligand-gated cation channel that can be activated by chemicals such as capsaicin whereas, TRPA1 and TRPM8 are reported to be activated by cold (Frias and Merighi, 2016; Obata et al., 2005; Selescu et al., 2013). TRPV1 receptor is highly expressed in primary sensory neurons, including corneal sensory neurons that mediates pain sensation (Moriello and De Petrocellis, 2016; Murata & Masuko, 2006). TRPV1 receptors are also present in the plasma membrane of immune cells, keratinocytes, smooth muscle cells, and in the urothelium (Moriello and De Petrocellis, 2016). The TRPM8 receptor has been shown to be activated by several factors including menthol, icilin, acetone, and cold-temperature (Andersson et al., 2004; Caspani et al., 2009; Colburn et al., 2007). Cold-stimulation applied to the cornea is another model used to study corneal sensitivity. It has been shown that cold nociceptors located at the periphery of the cat's cornea were excited by decrease in temperature ranging from 30°C to 8°C (Gallar et al., 1993). Cold-stimulation activates cold-sensitive channel TRPM8 and elicits a pain response (Selescu et al., 2013; Su et al., 2017).

Application of physical stimuli to the cornea, elicited by corneal esthesiometers, is frequently used to study corneal sensitivity in a clinical ophthalmology setting. This instrument measures the qualitative sensations of eye, based on the response to pressure applied at the corneal surface and is interpreted as normal, reduced or absent. In an observational tear dysfunction studies (an ocular surface diseases), reduced corneal sensitivity has been associated with greater eye irritation, tear instability, and increased blink rate. The more severe the ocular surface diseases were, the more rapid blinking was reported (Rahman et al., 2015).

Consistent with these studies, I hypothesize that superficial corneal chemical injury produces acute corneal inflammation and hyperalgesia in mice, and that this may be a useful model in examining novel pharmacological agents and strategies for the modulation of corneal hyperalgesia and inflammation. In my model, I assessed eye-wipe behavior and eye squints, in addition to the blink response, as an indication of the corneal pain response. Two types of stimulation were used to elicit a pain response, chemical (capsaicin) and thermal (cold saline). Additionally, corneal neutrophil infiltration was measured at 6 and 12 h after cauterization as an indication of the magnitude and duration of the corneal inflammatory response.

1.7 Hypothesis and objectives

The overall purpose of this thesis is to investigate the antinociceptive and anti-inflammatory effects of phytocannabinoids and synthetic cannabinoids in a new mouse model of corneal hyperalgesia and inflammation.

The hypothesis of my research is that topical application of cannabinoids can reduce corneal pain and inflammation via actions at both CB_1R and CB_2R.

The main objectives of my research were as follows:

i. To develop an acute model of corneal hyperalgesia in mice using two types of pain modalities (chemical and cold stimulation)
ii. To test the antinociceptive and anti-inflammatory effects of the phytocannabinoids, Δ^8THC and CBD, and synthetic CB_2R-selective cannabinoid, HU-308, in a mouse model of corneal hyperalgesia and inflammation.

iii. To investigate the corneal receptor-mediated mechanisms involved in the antinociceptive and anti-inflammatory actions of Δ^8THC, CBD and HU-308.

iv. To determine the effects of the CB_1R allosteric ligands (GAT211, GAT229 and GAT228) alone or in combination with CB_1R orthosteric agonist in a mouse model of corneal hyperalgesia and inflammation.

CHAPTER II: MATERIALS AND METHODS

2.1 Experimental animals

All animal care and experimental procedures followed the Canadian Council for Animal Care guidelines (http://www.ccac.ca/), and were approved by the Dalhousie University Committee on Laboratory Animals. All studies involving animals are reported in accordance with the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines [http://www.nc3rs.org.uk/; (Kilkenny et al., 2010; McGrath et al., 2010)].

Male BALB/c wild-type (WT; 25-30 g; Charles River, QC, Canada) and age-matched (8-12 weeks) CB_2R knockout mice ($CB_2R^{-/-}$) were used in this study. $CB_2R^{-/-}$ mice were bred in Dalhousie animal care facilities by crossing male C57BL/6J $CB_2R^{-/-}$ mice (strain B6.129P2-Cnr2tm1Dgen/J; Jackson Laboratory, Bar Harbour, ME, USA) with inbred BALB/c female mice (Charles River) for ten generations. Mice were housed in groups of 3-5, kept on a light/dark cycle (07:00 – 19:00/19:00 – 07:00) with unrestricted access to water and standard rodent food. A total of 217 mice were used in this study that includes 174 BALB/c mice and 43 $CB_2R^{-/-}$ mice.

2.2 Genotyping of $CB_2R^{-/-}$ mice

Genetic knockout of CB_2R was confirmed by Dr. Toguri using polymerase chain reaction (PCR), as per standard protocol (Toguri, 2015). Tissue was collected from ear punches using an Accustart II Mouse Genotyping Kit (Quanta Biosciences, MD, USA) and following the manufacturer's instructions. The following PCR primers were used: moIMR0086 (5'-GGGGATCGATCCGTCCTGTAAGTCT-3'; mutant forward), oIMR7552 (5'-GACTAGAGCTTTGTAGGTAGGCGGG-3'; common reverse), and oIMR7565 (5'-GGAGTTCAACCCCATGAAGGAGTAC-3'; WT forward). The

following PCR products were expected: single product at ~550 bp for $CB_2R^{-/-}$ mice, single product at ~385 bp for WT mice, and two products at ~550 and 385 bp for heterozygous animals.

2.3 Induction of corneal injury

Corneal injury was induced using a protocol adapted from a model of corneal hyperalgesia previously described in rats by Wenk and Honda (Wenk and Honda, 2003). Briefly, mice were anaesthetized using 2-3% isoflurane gas (1L/min) carried in oxygen. The central cornea of both eyes were cauterized using MedPro® (75% silver nitrate, 25% potassium nitrate; AMG Medical Inc., Montreal, Canada), applied with a micro-applicator brush (Centrix Inc., Shelton, USA). The micro-applicator brush was held in contact with the cornea for 2 seconds (s), resulting in the formation of a discrete superficial white lesion of 1 mm diameter in the epithelial cell layer. Cauterized eyes were then rinsed three times with room temperature saline. An ocular lubricant (Systane®, Alcon Canada Inc., ON, Canada) was applied to reduce corneal drying. Mice recovered fully from anesthesia within 3-5 min post-cauterization. Mice that kept their eyes closed completely for more than 15 min following cauterization were excluded from the study. Mice were euthanized using euthanyl over-dose (0.2 ml i.p.) at 6 or 12 h post-cauterization and the eyes were enucleated and stored at -20°C for immunohistochemical analysis.

2.4 Pharmacological treatments

For topical administration, the following cannabinoids were dissolved in soybean oil (Sigma-Aldrich, Ontario, Canada) at different concentrations {(0.2-5.0%

weight/volume (w/v)}, using gentle heat (37˚C): Δ^8THC ([6aR,10aR]-6,6,9-trimethyl-3-pentyl-6a,7,8,10a-tetrahydrobenzo[c]chromen-1-ol; Cayman Chemical, Ann Arbor, MI, USA), CBD (2-[(1*R*,6*R*)-3-Methyl-6-(1-methylethenyl)-2-cyclohexen-1-yl]-5-pentyl-1,3-benzenediol); Cayman Chemical), and 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, USA). The CB_1R antagonist, AM251 (*N*-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide; Tocris Bioscience) was suspended in 10% dimethyl sulfoxide (DMSO, Sigma-Aldrich, Ontario, Canada) in 0.9% normal saline with gentle heat (37°C) before use. AM251 was delivered intraperitoneally (i.p.) at a dose of 2.0 mg/kg 15 min pre-cauterization. 1 µM capsaicin (Tocris Bioscience) was prepared in 0.002% DMSO in sterile saline. The 5-hydroxytryptamine 1A ($5\text{-}HT_{1A}$) receptor antagonist, WAY100635 (*N*-[2-[4-(2-Methoxyphenyl)-1-piperazinyl]ethyl]-*N*-2-pyridinylcyclohexanecarboxamide maleate; Tocris Bioscience) was dissolved in 0.9% sterile saline and delivered i.p. at a dose of 1.0 mg/kg 15 min pre-cauterization. The CB_1R allosteric ligands GAT211, GAT228, and GAT229 were synthesized and provided by the laboratory of Dr. Ganesh A. Thakur (Northeastern University, USA) (Laprairie et al., 2017). GAT compounds were dissolved in 2% DMSO and 4% Tween-20 (Sigma-Aldrich) in soybean oil and were delivered topically. All topical drugs were administered (5 µl) to cauterized corneas at 30, 60 and 120 min post-cauterization.

2.5 Assessment of behavioral pain sensitization

The corneal sensory neurons in the mouse are classified as thin myelinated (A-δ type, 30%) or unmyelinated (C-type, 70%) fibres based on the size and presence of myelin

sheath in the axon (Belmonte et al., 2004). About 70% of these corneal sensory fibres are polymodal nociceptors and can be activated by variety of mechanical energy, exogenous and endogenous chemical irritants. About 10-15% of corneal nerve fibres are cold-sensitive nociceptors and can be activated by cold temperature (below corneal surface temperature i.e. 33°C) (Belmonte et al., 2004). Corneal sensory nerves express transient receptor potential (TRP)-channels such as TRPV1, TRPA1 and TRPM8 and their activation has been shown to induce pain responses (Belmonte et al., 2015; Reinach et al., 2015). TRPV1 receptors can be activated by chemicals such as capsaicin, whereas TRPA1 and TRPM8 are reported to be activated by cold (Frias and Merighi, 2016; Obata et al., 2005; Selescu et al., 2013). Both chemical and cold stimuli were used in this study to assess pain responses resulting from activation of different polymodal pain receptor populations. At 6 or 12 h after corneal cauterization, chemical (1 µM capsaicin) or cold stimuli (4, 10, and 15°C saline) were topically applied to both eyes (right eye first followed by left eye) to elicit a pain response. Pain behaviors were recorded in the tested eye using an iPhone 5S (8 megapixel*)*. Videos were analyzed offline in slow motion (play speed 0.5, Windows Media Player version 10) and the pain response was scored by adding the total number of blinks, squints and eye wipes recorded in 30 s (capsaicin stimulation) or 60 s (cold-stimulation). A sham control group was set up by touching the micro-applicator brush to the cornea for 2 s in the absence of sliver nitrate and with all other parameters held constant.

2.5.1 Capsaicin stimulation

At 6 or 12 h post-cauterization, 5 µl of 1 µM capsaicin was applied topically to the cauterized or control eyes to elicit a pain response. One µM concentration of capsaicin was chosen based on the previous study where it elicited an increased pain response in injured

cornea compared to non-injured cornea (Wenk and Honda, 2003). Immediately following the application of capsaicin, the mice were video recorded, and the videos were analyzed for 30 s; the pain response was minimal after 30 s. Each animal was given a single dose of capsaicin to avoid the desensitization which may occur from repeated application (Wenk and Honda, 2003).

2.5.2 Cold stimulation

Cold hyperalgesia represents well documented signs and symptoms of inflammatory and neuropathic pain (Obata et al., 2005). Therefore, while developing novel analgesics for corneal pain, in addition to test the activity against chemical stimulation, it is important to see the activity against cold hypersensitivity (Allchorne et al., 2005). At 6 h post-cauterization, injured or control eyes were stimulated with 5 µl of topically applied cold saline (4, 10 or 15°C) to see the pain response at different temperature in control and cauterized corneas. There was an increased pain response in all these temperature in cauterized eyes compared to control eyes. Corneal pain response was assessed using a 60-s video clip, recorded following stimulation with topical cold saline. The pain response was minimal after 60 s.

2.6 Immunohistochemistry

At 6 or 12 h following corneal cauterization, eyes were enucleated and fixed in 4% paraformaldehyde (PFA) for 24 h followed by 30% sucrose overnight. Corneal sagittal sections (12 µm) were prepared using a Leica CM1850 cryostat (Wetzlar, Germany) and washed 4 times in Phosphate Buffer Saline (PBS), before being incubated for 2 h with

blocking medium (10% normal goat serum 0.5% Triton X-100/PBS) in order to block non-specific binding. Following this, the sections were incubated for 2 nights at 4°C in purified rat anti-Ly-6G antibody (1:200, Abcam, Cambridge, MA, USA). Ly-6G is a GPI-anchor protein expressed predominantly on neutrophils; the anti- Ly-6G antibody allows for detection of these cells. Sections were then washed 4 times for 10 min with PBS, followed by an overnight incubation with a secondary antibody (1:500, goat anti-rat Alexa Fluro® 488, Jackson ImmunoResearch Laboratories, Inc., USA). Stained sections were washed 4 times in PBS and mounted in superfrost slides (Fisher Scientific, Ontario, Canada) using Fluoromount (Sigma, Ontario, Canada).

2.7 Quantification of neutrophil migration

Neutrophil migration was quantified in corneal sections at 20X magnification using a Zeiss Axiovert 200M microscope with a Hamamatsu Orca R2 Camera (Zeiss, Canada). Three representative images were taken from each section; the right and left peripheral cornea and one at the center of the cornea, respectively. Neutrophils counted from these three images were summed to provide the total neutrophil count per section. Five-twelve sections from each eye were analyzed and averaged to represent neutrophil per section in an eye. Sections were chosen at an interval of 120µm. For each experimental group, 5-7 eyes were analyzed.

2.8 Data analysis

Individual animals in each treatment group were coded and the experimental data was analyzed blinded. Statistical analysis was performed in GraphPad Prism version 6

using one-way analysis of variance (ANOVA) with either Dunnett's or Tukey's multiple comparison *post hoc* test for groups of three or more than three. A T- test was carried out to test the statistical significance between two groups. All data reported are represented as group mean ± standard deviation. Data were considered significant at $p < 0.05$.

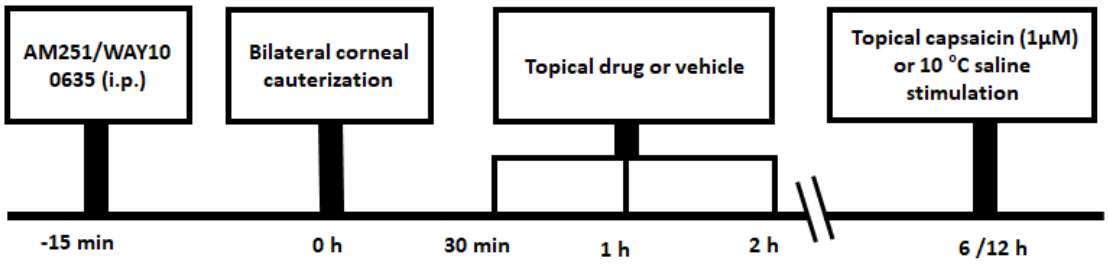

Figure 8: Experimental timeline used for assessment of murine corneal hyperalgesia and inflammation. Mice were treated topically either with cannabinoids or vehicle at 30 min, 1 h and 2 h post-cauterization. CB_1R (AM251) or 5-HT_{1A} receptor (WAY100635) antagonists were given intraperitoneally (i.p.) for some groups 15 min prior to cauterization.

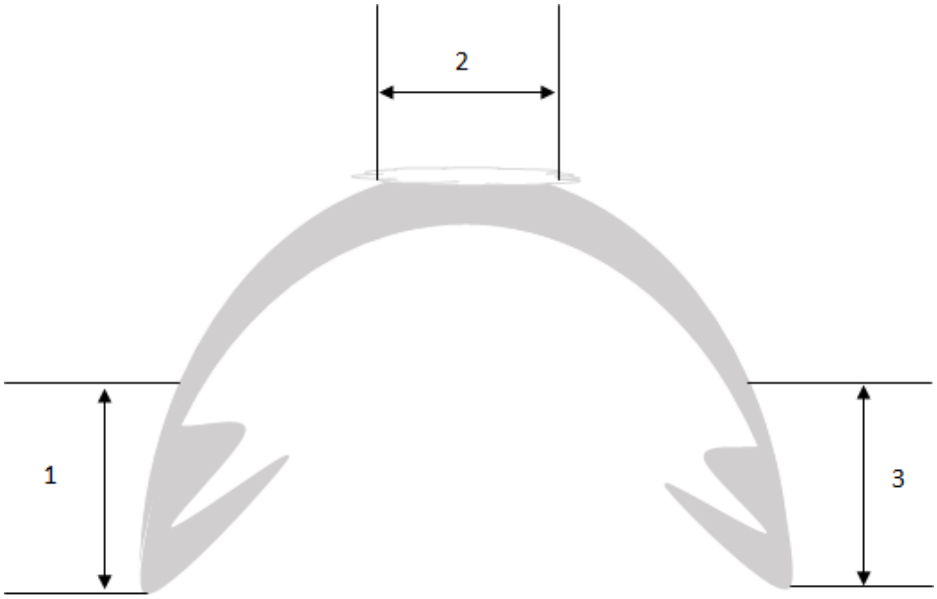

Figure 9: Quantification of neutrophils on corneal sections. Three representative images (1, 2 and 3) from a corneal section were captured and the total number of neutrophils was summed.

CHAPTER III: THE CANNABINOIDS, Δ^8THC, CBD and HU-308, ACT VIA DISTINCT RECEPTORS TO REDUCE CORNEAL PAIN AND INFLAMMATION

3.1 Manuscript status and student contribution

This chapter is accepted to Cannabis and Cannabinoid Research for publication. The manuscript has been modified to meet the Dalhousie FGS formatting requirements. Dinesh Thapa, Elizabeth A. Cairns, Anna-Maria Szczesniak, J. Thomas Toguri, Meggie Caldwell, Melanie EM. Kelly (2017). The cannabinoids, Δ^8THC, CBD and HU-308, act via distinct receptors to reduce corneal pain and inflammation. *Cannabis and Cannabinoid Research*.

As first author on this article, I performed all experiments, collected, analyzed and interpreted data. I wrote the manuscript myself in close guidance from Drs. Melanie Kelly, Elizabeth A. Cairns and Anna-Maria Szczesniak and Tom Toguri. Meggie Caldwell initially helped to set up the experimental model.

3.2 Abstract

Background and purpose: Corneal injury can result in dysfunction of corneal nociceptive signaling and corneal sensitization. Activation of the ECS has been reported to be analgesic and anti-inflammatory. The purpose of this research was to investigate the anti-nociceptive and anti-inflammatory effects of cannabinoids with reported actions at CB_1R, CB_2R receptor and/or non-cannabinoid 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^8THC or 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 post-injury were quantified from video recordings. Mice were euthanized at 6 h and 12 h post corneal injury for immunohistochemical analysis to quantify corneal neutrophil infiltration.

Results: Corneal cauterization resulted in hyperalgesia to capsaicin at 6 h post-injury compared to sham control eyes. Neutrophil infiltration, indicative of inflammation, was apparent at 6 and 12 h post-injury in WT mice. Application of Δ^8THC, CBD, and HU-308 reduced the pain score and neutrophil infiltration in WT mice. The anti-nociceptive 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 anti-nociceptive and anti-inflammatory actions of HU-308 were absent in the $CB_2R^{-/-}$ mice. The

anti-nociceptive and anti-inflammatory effects of CBD were blocked by the 5-HT_{1A} antagonist WAY100635.

Conclusion: Topical cannabinoids reduce corneal hyperalgesia and inflammation. The anti-nociceptive 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.

3.3 Introduction

The cornea is a thin, transparent dome-shaped avascular tissue that is densely innervated by sensory nerve endings ((Belmonte et al., 2004a; Belmonte et al., 2004b Belmonte et al., 2015). Damage to these nerve endings, resulting from surgery, trauma, neurological disease, or infection may develop into CNP (Belmonte et al., 2015). CNP is a clinically significant problem, characterized by persistent hyperalgesia, debilitating pain, photo-allodynia, burning, stinging, dryness, and inflammation (Goyal and Hamrah, 2016). Corneal damage can also result in an inflammatory response that involves the production of pro-inflammatory cytokines, neovascularization, recruitment of leukocytes, and release of neuropeptides that results in ocular pain and inflammation (Rosenthal & Borsook, 2012; Lai et al., 2015; Voiculescu et al., 2015; Launay et al., 2016; Okada et al., 2015; Toguri, Caldwell, & Kelly, 2016) (for review see Toguri et al.,2015).

Existing pharmacotherapies for ocular pain, inflammation and CNP include topical corticosteroids, tricyclic antidepressants, GABAergic drugs, and opioids (Donnenfeld et al., 2011; Henderson et al., 2011; Faktorovich & Melwani, 2014; Goyal & Hamrah, 2016; Dieckmann et al., 2017; Pereira et al., 2017). These treatments, however, frequently fail to provide adequate pain relief, and are associated with side-effects (Faktorovich and Melwani, 2014; Dieckmann et al., 2017; Pereira et al., 2017). 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) (Crowe et al.,2014; Kaur et al., 2016; Huang et al., 2016; Woodhams et al., 2017).

The ECS is an endogenous lipid signaling system that includes two GPCRs, CB_1R and CB_2R, eCBs, and cognate enzymes for biosynthesis and degradation of eCBs (Mechoulam and Parker, 2013; Kaur et al., 2016; Maldonado et al., 2016). CB_1Rs are widely expressed in many tissues, including the central and peripheral nervous systems, where activation of CB_1R modulates neurotransmitter release (Matsuda et al., 1990; Porcella et al., 2000; Kaur et al., 2016; Lu and Mackie, 2016). CB_2R is highly expressed on immune cells and its activation is anti-inflammatory, resulting in decreased production of pro-inflammatory mediators, and a reduction in leukocyte recruitment (Munro et al., 1993; Galiegue et al., 1995; Berdyshev, 2000; Onaivi et al., 2006). 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 (Fine and Rosenfeld, 2014; Kaur et al., 2016; Wang et al., 2016).

Cannabinoids have not been extensively studied in ocular surface pain and inflammation. CB_1R is expressed in the corneal epithelium and endothelium in rodents and primates (Straiker et al., 1999), and activation of CB_1R has been reported to inhibit neuropeptide-induced sensitization of TRPV1 in afferent neurons (Wang et al., 2014). Under non-pathological 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 (Toguri et al., 2014; Toguri et al., 2015) (for review see Toguri et al., 2016). 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 anti-nociceptive and anti-inflammatory effects of several cannabinoids that act at either CB_1R and/or CB_2R, or non-cannabinoid receptors. These included Δ^8THC, a more stable isomer Δ^9THC, CBD, and the CBD derivative HU-308. Both Δ^8THC and Δ^9THC produce anti-nociceptive effects in preclinical models with similar potency via activation of CB_1R (Burstein et al., 1988; Compton et al., 1991; Formukong et al., 1988; Martin et al., 1984; Varvel et al., 2005). CBD lacks the behavioral effects of THC at CB_1R, and may produce pharmacological actions through the activation of non-cannabinoid receptors (Linge et al., 2016; Pertwee, 2004; Russo et al., 2005). HU-308 is a selective and highly potent agonist at CB_2R (Hanus et al., 1999), and has previously been shown to reduce LPS-induced intraocular inflammation (Toguri et al., 2014).

3.4 Materials and methods

3.4.1 Experimental animals and corneal injury model

All animal care and experimental procedures complied with the Canadian Council for Animal Care guidelines (http://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, USA) 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 Harbour, ME, USA) with inbred BALB/c female mice (Charles River) for ten generations. Genetic loss of CB_2R (*Cnr2*) was confirmed by Dr. Toguri via PCR genotyping using DNA extracted from ear punches with an Accustart II Mouse Genotyping Kit (Quanta Biosciences, MD, USA), according to manufacturer's instructions (Toguri, 2015). Primer sequences were: mouse CB_2 mutant forward (moIMR0086) 5'- GGG GAT CGA TCC GTC CTG TAA GTC T-3'; mouse CB_2 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 wild-type 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 (Wenk and Honda, 2003). Briefly, mice were anaesthetized using 2-3% isoflurane gas. The center of the cornea on both eyes was cauterized with silver nitrate (75% silver nitrate, 25% potassium nitrate; MedPro®,

England) using a micro-applicator brush (Centrix Inc., Shelton, USA). The micro-applicator brush was held in contact with the cornea for 2 s, 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., Canada) was applied to reduce corneal drying. Mice recovered fully from anesthesia within 3-5 min post-cauterization. Mice were euthanized at 6 or 12 h post-cauterization, and the eyes were enucleated for immunohistochemical analysis.

3.4.2 Assessment of behavioral pain sensitization

At 6 or 12 h post-cauterization, 5 µl of 1 µM capsaicin was applied topically to the cauterized eyes to elicit a pain response (Wenk et al., 2003). A sham control group was induced by touching the micro-applicator brush to the cornea for 2 s 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 s. Videos were analyzed offline in slow motion, where the number of blinks, squints and eye wipes to capsaicin challenge were summed to give a pain score.

3.4.3 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 PBS and blocked for non-specific binding (10% normal goat serum in 0.5% Triton-X/PBS; Sigma-Aldrich, Oakville, ON, Canada) for 2 h, followed by 48 h incubation in purified rat anti-Ly-6G antibody

(1:200; Abcam, Cambridge, MA, USA). Ly-6G is a GPI-anchor protein expressed predominantly on neutrophils; the anti-Ly-6G antibody allows for detection of these cells (Marrazzo et al., 2011). Sections were then washed with PBS and incubated with the secondary antibody (1:500, goat anti-rat Alexa Fluro® 488**,** Jackson ImmunoResearch Laboratories, Inc., USA**)**. 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 20X magnification using a Zeiss Axiovert 200M microscope (Zeiss, Thornwood, NY, USA). 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.

3.4.4 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, USA), 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, USA) 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 post-

cauterization. The CB_1R antagonist AM251 (*N*-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-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 post-injury. The $5\text{-}HT_{1A}$ 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.

3.4.5 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 were used, as appropriate, to compare data between experimental groups of three or more. A T-test was 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 $p < 0.05$.

3.5 Results

3.5.1 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). Additionally, the pain score in cauterized eyes at 12 h was significantly lower than the pain score at 6 h ($p < 0.05$; Figure 10A).

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 (Figure 10C). Figure 10B demonstrates, however, the presence of neutrophils at 6 and 12 h following cauterization (126 ± 33, n = 5, and 156 ± 28, n = 6, respectively; Figure 10B, D, E).

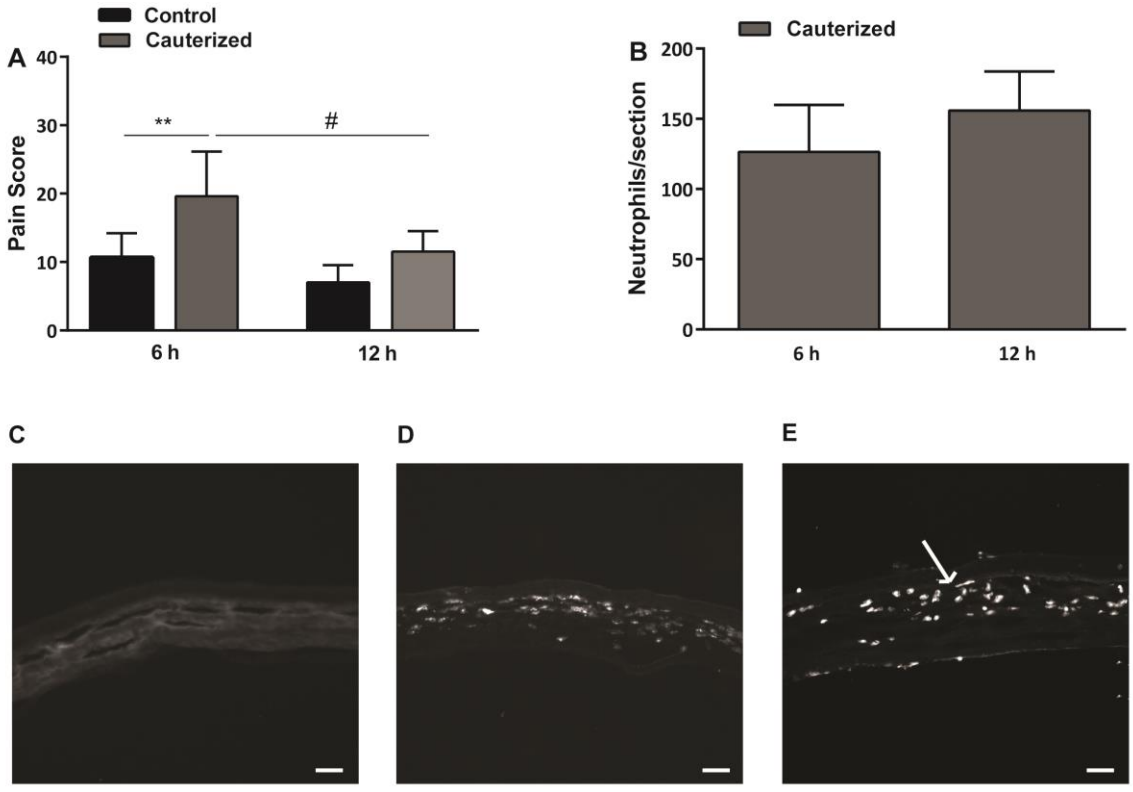

Figure 10. Corneal chemical injury results in hyperalgesia and inflammation. **(A)** Pain responses to topical capsaicin challenge (1 μM) in non-cauterized sham control eyes (n = 5-6 per group) and cauterized eyes (n = 6-10 per group) at 6 h and 12 h post-injury. **(B)** Neutrophil expression in cauterized corneas at 6 h and 12 h post-injury (n = 5-6 per group). **(C-E)** Representative images of transverse sections of the central cornea from **(C)** sham control (non-cauterized) corneas and cauterized corneas at **(D)** 6 h and **(E)** 12 h post-injury. 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. **$p < 0.01$, # $p < 0.05$.

3.5.2 Topical application of Δ^{8}THC, 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^{8}THC, CBD, and HU-308 were examined in WT mice in order to establish the effective drug concentrations required to reduce corneal pain compared to the vehicle-treated group (Figure 11A). Administration of 0.5% and 1% Δ^{8}THC produced a significant reduction in pain scores (18 ± 6, n = 6, $p < 0.05$; 12 ± 5, n = 12, $p < 0.0001$, respectively). At the lower concentrations (0.2% and 0.4%), Δ^{8}THC 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 was not effective at reducing the pain score (n = 6, $p > 0.05$). Additionally, 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 anti-nociceptive (17 ± 4, n = 6, $p < 0.01$). Therefore, given their efficacy at reducing the pain response, 1% Δ^{8}THC, 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 either 1% Δ^{8}THC, 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).

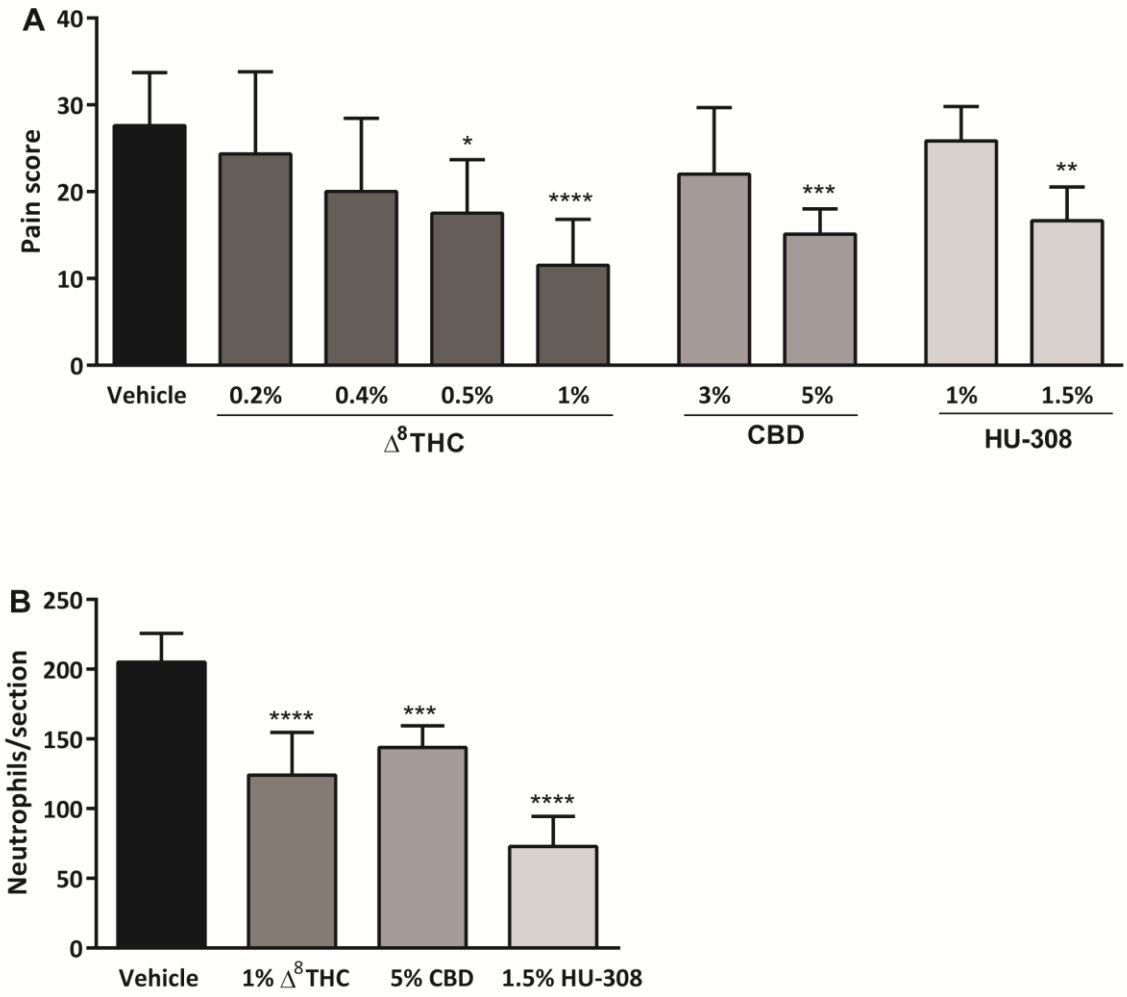

Figure 11. Topical administration of Δ^8THC, CBD, or HU-308 reduces corneal hyperalgesia and neutrophil infiltration in WT mice after corneal cauterization. **(A)** Dose-response for anti-nociceptive effects of Δ^8THC (0.2-1.0%, n = 6-12 per group), CBD (3 and 5%, n = 6 and 10, respectively) and HU-308 (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 post-injury 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. ****$p < 0.0001$, *** $p < 0.001$, **$p < 0.01$, *$p < 0.05$.

3.5.3 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.), prior to corneal cauterization and capsaicin stimulation, blocked the anti-nociceptive actions of Δ^8THC (Figure 12A; $n = 8$, $p > 0.05$), suggesting that Δ^8THC acts via CB_1R to reduce corneal pain. However, the anti-nociceptive actions of 5% CBD were maintained in eyes pre-treated with CB_1R antagonist AM251 (23 ± 6, $n = 8$), compared to vehicle-treated eyes plus AM251 (35 ± 4, $n = 8$, $p < 0.001$; Figure 12A).

Likewise, the number of neutrophils in corneas from mice treated with AM251 and either 1% Δ^8THC or vehicle were not significantly different ($n = 6$, $p > 0.05$). In contrast, 5% CBD treatment was still able to reduce neutrophils in corneas from mice treated with AM251 (95 ± 44, $n = 6$) versus vehicle-treated cauterized eyes from mice receiving AM251 (202 ± 31, $n = 6$, $p < 0.01$; Figure 12B)

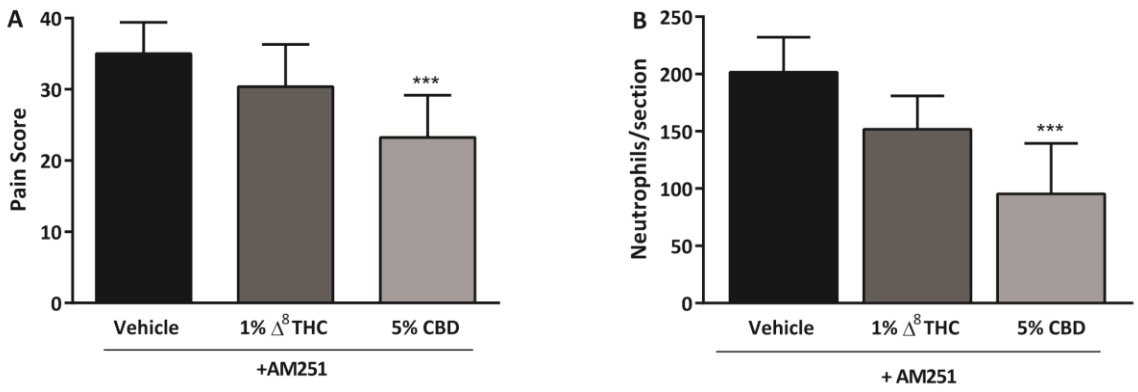

Figure 12. The CB_1R antagonist AM251 reduces the antinociceptive and anti-inflammatory actions of Δ^8THC but not CBD. **(A)** Pain score measured in WT mice at 6 h post-cauterization and following administration of 5 µl of topical vehicle, 1% Δ^8THC, or 5% CBD (n = 8 per group) in mice pre-administered with AM251 (2.0 mg/kg i.p.). **(B)** The number of neutrophils per section at 12 h post-cauterization in corneas from WT mice pre-administered 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. ***$p < 0.001$, ** $p < 0.01$.

3.5.4 The anti-nociceptive 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 anti-nociceptive and anti-inflammatory effects of Δ^8THC, CBD, and HU-308 were examined using $CB_2R^{-/-}$ mice. Compared to WT mice receiving vehicle, neutrophils in vehicle-treated $CB_2R^{-/-}$ mice were significantly increased ($p < 0.01$); 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 post-cauterization, 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 anti-nociceptive effect of 1.5% HU-308 ($n = 7$) was not significantly different compared to vehicle-treated animals ($p > 0.05$; Figure 13A), confirming the involvement of CB_2R in the anti-nociceptive effects of HU-308, but not THC or CBD.

Consistently, at 12 h post-cauterization 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 ($p > 0.05$; Figure 13B).

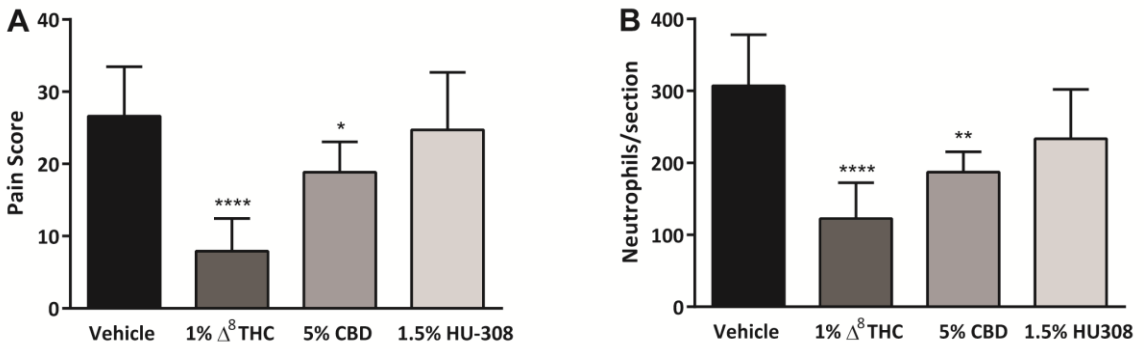

Figure 13. The corneal anti-nociceptive and anti-inflammatory effects of HU-308, but not Δ^8THC or CBD, are mediated via CB_2R. **(A)** Pain scores measured at 6 h post-corneal cauterization in $CB_2R^{-/-}$ mice treated with 5 µl topical vehicle (n = 8), or either 1% Δ^8THC (n = 12), 5% CBD (n = 7) or 1.5% HU-308 (n = 7). **(B)** The number of neutrophils per section measured at 12 h post-cauterization in corneas from $CB_2R^{-/-}$ mice treated with 5 µl of vehicle (n = 7), or either 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. ****$p < 0.0001$, ** $p < 0.01$, *$p < 0.05$.

3.5.5 CBD acts at 5-HT$_{1A}$ receptors to reduce corneal pain and inflammation

The corneal anti-nociceptive 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 (Russo et al., 2005). Treatment of mice with the 5-HT_{1A} receptor antagonist WAY100635 (1.0 mg/kg i.p.) was able to completely eliminate the anti-nociceptive actions of CBD in cauterized cornea (Figure 14A; $n = 8$ 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 anti-nociceptive and anti-inflammatory actions in cornea (Figure 14B).

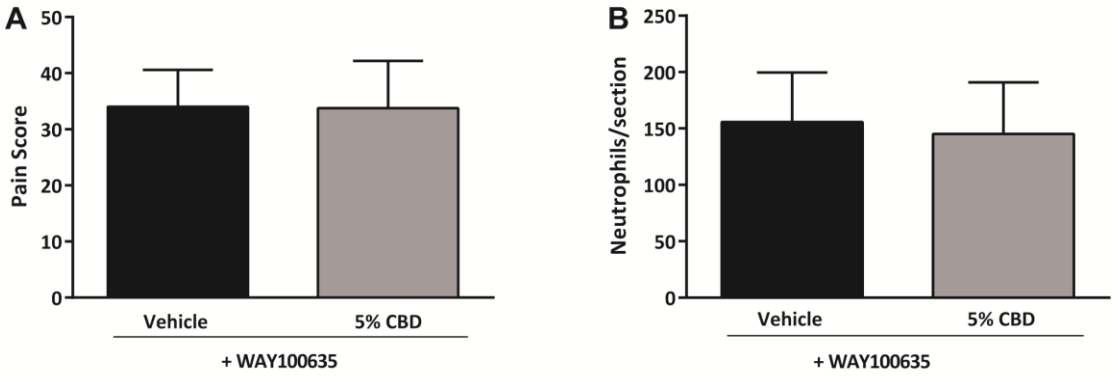

Figure 14. The corneal anti-nociceptive and anti-inflammatory effects of CBD are mediated through 5-HT_{1A} receptor. **(A)** Pain score measured at 6 h post-cauterization in WT mice pre-administered with the 5-HT_{1A} receptor antagonist, WAY100635 (1.0 mg/kg i.p.) and treated topically with 5 µl of either vehicle (n = 8) or 5% CBD (n = 8). **(B)** The number of neutrophils per section measured at 12 h post-cauterization 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-test was used.

3.6 Discussion

Our results provide novel evidence that the phytocannabinoids Δ^8THC and CBD, and synthetic cannabinoid derivative HU-308, are anti-nociceptive and 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 the CB_1R and CB_2R, as well as the 5-HT_{1A} receptor.

We have shown that the topical application of Δ^8THC reduces hyperalgesia following corneal injury. These effects were blocked in mice treated with a CB_1R antagonist but remained unaffected in $CB_2R^{-/-}$ mice, suggesting that the anti-nociceptive effects of Δ^8THC are mediated via CB_1R. Additionally, 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 treatment with the 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. Given that inflammation following corneal injury contributes to corneal nerve sensitization, activation of CB_1R would therefore be expected to reduce 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 anti-nociceptive effects, which were blocked by the CB_1R antagonist AM281, and to a lesser extent by the CB_2R antagonist AM630 (0.5 mg/kg i.p.) (Bagüés et al., 2014). 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 (Agarwal et al., 2007).

The anti-nociceptive effect of CB_1R activation, reported in this study, may involve the modulation of TRPV1 receptor activity. In the mammalian cornea, expression of CB_1R has been reported to be co-localized with TRPV1 (Yang et al., 2013), the latter of which is expressed in corneal epithelium (Zhang et al., 2007) and endothelium (Mergler et al., 2010), and sensory nerve endings of the ophthalmic branch of the trigeminal nerve innervating the cornea (Murata & Masuko, 2006). TRPV1 is activated following damage to cornea, culminating in activation of corneal nerves and local inflammation. The release of pro-inflammatory cytokines and neuropeptides, including nerve growth factor (NGF) and substance P, contribute to neurogenic inflammation, and can lead to corneal nerve sensitization (Belmonte et al., 2004; Yang et al., 2013). In sensory neurons isolated from rat dorsal root ganglia, activation of CB_1R by the cannabinoid agonist ACEA (arachidonoyl-2'- chloroethylamide) prevented NGF-induced sensitization of the TRPV1 receptor. This action was blocked by the CB_1R antagonist AM251, suggesting that the activation of CB_1R may produce analgesia by desensitization of TRPV1 receptors (McDowell et al., 2013).

Our data implicate $5\text{-}HT_{1A}$ receptors, and not cannabinoid receptors, in the both anti-nociceptive and anti-inflammatory actions of CBD in experimental models of corneal injury. We showed that the actions of CBD were completely blocked by the $5\text{-}HT_{1A}$

receptor 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 (Linge et al., 2016; Resstel et al., 2009; Russo et al., 2005). Using a heterologous cell expression system, Russo et al. reported that CBD bound to both human and rat 5-HT_{1A} receptor with micromolar affinity, and displaced the agonist [^{3}H]8-OH-DPAT in a concentration-dependent manner (Russo et al., 2005). Additionally, CBD increased [^{35}S]GTPγS binding, and decreased forskolin-stimulated cAMP production, which was blocked by the specific 5-HT_{1A} receptor antagonist NAN-190 (Russo et al., 2005).

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 (Ward et al., 2014). 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 sub-chronic 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 (Ward et al., 2014).

HU-308 has been reported as a selective CB_2R agonist (Hanus et al., 1999). In our model of corneal hyperalgesia, the anti-nociceptive 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 (Szczesniak et al., 2017; Toguri et al., 2015). In experimental uveitis, it has been reported that CB_2R activation reduced leukocyte-endothelial adhesion in the iridial microvasculature as well as inhibited release of pro-inflammatory mediators including TNFα, IL1β, Il6, CCL5 and CXCL2 (Toguri et al., 2014). Conversely, a CB_2R antagonist, AM630, increased leukocyte-endothelial adhesion in experimental uveitis (Toguri et al., 2014), 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 (Szczesniak et al., 2017). Another study, in a mouse model of endotoxemia, has shown increased neutrophil recruitment to the spleen in $CB_2R^{-/-}$ mice compared to WT control (Kapellos et al., 2017). 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 pro-inflammatory in ocular tissues (Szczesniak et al., 2017; Toguri et al., 2015).

Reports of the anti-nociceptive and anti-allodynic efficacy of CB_2R agonists have also been reported in other experimental models of hyperalgesia and chronic inflammatory neuropathic pain (Kinsey et al., 2011) (For review, see ref. Anand et al., 2009; Piomelli & Sasso, 2014; Piomelli et al., 2014; Starowicz & Finn, 2017; Woodhams et al., 2017). 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 (Deng et al., 2015). The authors reported that chronic CB_2R activation with the CB_2R 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 AM1710. Furthermore, using intrathecal cannabinoid administration, this study identified a possible role for spinal CB_2R in the anti-allodynic actions of AM1710, as well as a reduction in pro-inflammatory cytokines, in paclitaxel-treated mice (Deng et al., 2015). 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 (Anand et al., 2008). Our data demonstrating the anti-nociceptive and anti-inflammatory actions of CB_2R activation in cornea, together with these studies, further supports the utility of CB_2R agonists for treating inflammatory pain.

3.7 Conclusions

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.

3.8 Acknowledgement

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3.9 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. M.D.C. have no existing competing financial interests.

CHAPTER IV: ALLOSTERIC MODULATION OF CANNABINOID 1 RECEPTOR REDUCES OCULAR PAIN AND INFLAMMATION

4.1 Manuscript status and student contribution

The figures and text presented in this chapter are from a manuscript to be submitted with the following authors. Dinesh Thapa, Elizabeth A. Cairns, Anna-Maria Szczesniak, Ganesh A. Thakur, Melanie EM. Kelly (2017).

As a first author on this article, I performed all experiments, collected, analyzed and interpreted data. I wrote the manuscript myself in close guidance with Drs. Melanie Kelly, Elizabeth Cairns and Anna-Maria Szczesniak. Ganesh A. Thakur provided the drugs for the experiments.

4.2 Abstract

Aim: Current treatments for corneal pain are not always effective in producing adequate pain relief, and have side effects. Cannabinoid receptor 1 (CB_1R) orthosteric activation has anti-nociceptive effects, however, therapeutic applications may be limited due to behavioral side-effects and tolerance. CB_1R allosteric ligands bind to site distinct from the orthosteric site at CB_1R and either activate or inhibit the receptor directly or modulate the affinity and/or efficacy of the orthosteric ligand (allosteric modulator). Allosteric ligands may offer an alternative approach to reduce pain with improved specificity and reduced side-effects. This research investigated the anti-nociceptive properties of a novel racemic CB_1R allosteric ligand, GAT211, and its resolved enantiomers, GAT228 and GAT229, in a mouse model of corneal hyperalgesia**.**

Methods: Corneal hyperalgesia was generated using chemical cauterization of the cornea in wildtype (WT) and CB_2R knockout ($CB_2R^{-/-}$) mice. Cauterized eyes were treated topically with GAT211, GAT229 and GAT228 (0.5-2% w/v) alone or in combination with the orthosteric CB_1R agonist, Δ^8- tetrahydrocannabinol (Δ^8THC, 0.4% w/v), in the presence or absence of the CB_1R antagonist AM251 (2.0 mg/kg i.p). The ocular pain score was analyzed from 30-60 s video recordings of the behavioral pain response following chemical or cold stimulation of cornea at 6 h post-cauterization. Corneal neutrophil infiltration, an indicative of corneal inflammation, were analyzed at 6 h post-cauterization.

Results: GAT228 (1 & 2%), but not GAT229 and GAT211 (0.5 - 2%), reduced corneal hyperalgesia. Topical application of 0.4% Δ^8THC alone was subthreshold to reduce corneal pain, but increasing the concentration of Δ^8THC from 0.5% to 1% significantly reduced the pain response. Combination treatments of 0.5% GAT229 or 1% GAT211 with 0.4%

Δ^8THC was able to significantly reduce corneal hyperalgesia. The anti-nociceptive effects of both GAT229 and GAT228 were blocked by the CB_1R antagonist, AM251, but remained unaffected in $CB_2R^{-/-}$ mice. 2% GAT 228, 1% Δ^8THC or the combination of 0.4% Δ^8THC with 1% GAT211 or 0.5% GAT229, in the cold stimulation model, also significantly reduced corneal inflammation.

Conclusion: The CB_1R allosteric ligands GAT211, GAT229 and GAT 228 reduce corneal pain and inflammation either through potentiating the orthosteric activity of Δ^8THC at CB_1R (GAT211 and GAT229) or independently through allosteric activation of CB_1R (GAT228). Allosteric modulation of CB_1R could offer a novel approach for treating corneal pain and inflammation, and may reduce the side-effects associated with orthosteric activation of CB_1R.

4.3 Introduction

The cornea is considered a unique tissue in the body because of its transparency, avascularity, and immunologic privilege (Treacy et al., 2016). The cornea has the densest concentrations of unmyelinated sensory nerve endings in the body (Müller et al., 2003), which are highly sensitive to touch, temperature, and various chemicals mediators. Damage or irritation to these nerve endings results in strong ocular pain, often characterized by allodynia and hyperalgesia (Ahmed et al., 2015; Azher et al., 2017; Beuerman and Tanelian, 1979).

Ocular pain is an unpleasant emotional, sensory and behavioral experience that can result from ocular surface manipulations such as cataract surgery (Assam et al., 2018), long-term, and improper use of contact lenses (Millodot, 1978; Patel et al., 2002; Tabatabaei et al., 2017), and frequent exposure to irritating environmental and chemical stimuli (air pollutants, hazardous chemicals, air pressure etc.) (Belmonte et al., 2004a; Belmonte et al., 2004b; Belmonte et al., 2015). Following corneal injury or infection, inflammatory mediators are released locally in the eye, leading to corneal inflammation (Akpek and Gottsch, 2003). Ocular surface injury, inflammation and dryness often persists together resulting in intense ocular pain (Belmonte et al., 2015; Belmonte et al., 2017). The current pharmacotherapies for corneal pain and inflammation include topical steroids, NSAIDS and antibiotics, while tricyclic antidepressants (TCA), GABAergic drugs (gabapentin), opioids etc. are may be prescribed for corneal neuropathic pain (CNP) (Dieckmann et al., 2017; Galor et al., 2017; Namavari et al., 2012). However, these treatments are not always effective enough to produce adequate pain relief, and in the case

of TCAs, GABAergic drugs and opioids, they do not treat the inflammation that contributes to corneal sensitization (Goyal and Hamrah, 2016).

Targeting the endocannabinoid system (ECS) has emerged as a novel approach to modulate pain, inflammation, and neurodegenerative disorders (Bisogno et al., 2016; Huang et al., 2016). The ECS is comprised of G-protein GPCRs, CB_1R and CB_2R, endocannabinoids, and the enzymes that catalyze the synthesis and degradation of endocannabinoids (Alger and Kim, 2011; Di Marzo, 2011; Kaur et al., 2016; McPartland et al., 2015; Mechoulam and Parker, 2013). Orthosteric activation of CB_1R can provide analgesia (Seltzman et al., 2016; Starowicz and Finn, 2017; Woodhams et al., 2017), including in corneal pain model (Thapa et al., 2017, accepted); however, the clinical usefulness of this approach may be limited due to behavioral side effects, supraphysiological CB_1R activation, and tolerance with repeated administration (Biala, 2008; Burston et al., 2010; Gonzalez et al., 2005; Lichtman and Martin, 2005). Drugs that target an allosteric binding site at CB_1R (Price et al., 2005), a topographically distinct site from the orthosteric binding site (Christopoulos and Kenakin, 2002), could offer a novel approach to modulate CB_1R and reduce corneal pain, while minimizing limitations of CB_1 orthosteric ligands alone (Khurana et al., 2017; Ross, 2007).

GAT211, GAT228, and GAT229 are a group of novel compounds that modulate CB_1R through the allosteric site, as demonstrated by *in vitro* data (Laprairie et al., 2017). GAT228 (R) and GAT229 (S) are the resolved enantiomers of GAT211 (racemate) (Khurana et al., 2017; Laprairie et al., 2017). GAT211 has been reported to have both agonist and positive allosteric modulator (PAM) activity at CB_1R; the allosteric agonist activity of GAT211 is dependent on GAT228, while its PAM activity is GAT229-

dependent (Laprairie et al., 2017). A recently published study has shown that topical or systemic GAT229 reduces intraocular pressure when combined with sub-therapeutic dose of an orthosteric agonist in normotensive mice, or alone in ocular hypertensive mice (Cairns et al., 2017). Additionally, in a paclitaxel model of neuropathic pain, GAT211 when delivered alone reduced neuropathic and inflammatory pain without producing typical CB_1R-mediated cannabimimetic side-effects (Slivicki et al., 2017), as did a related CB_1R PAM, ZCZ011 (Ignatowska-Jankowska et al., 2015). In addition, unlike an orthosteric CB_1R agonist WIN55,212-2, GAT211 did not produce tolerance over a 19-day interval of once-daily dosing (Slivicki et al., 2017).

This paper has explored the effects of GAT211, GAT228 or GAT229 alone or in combination with the CB_1R orthosteric agonist, Δ^8THC, in a mouse model of corneal pain and inflammation. When combined with a subthreshold orthosteric ligand, administration of GAT211 and GAT229, or GAT228 alone, reduced the pain response in response to chemical or cold challenge following corneal injury, as well as reduced the number of infiltrating neutrophils into the cornea. Our findings suggest that CB_1R allosteric modulation may provide a novel strategy to reduce both corneal pain and inflammation.

4.4 Materials and methods

4.4.1 Experimental animals

All animal care and experimental procedures complied with the Canadian Council for Animal Care guidelines (http://www.ccac.ca/) and were approved by the Dalhousie University Committee on Laboratory Animals. Animals studies are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (http://www.nc3rs.org.uk/; (Kilkenny et al., 2010; McGrath et al., 2010).

Male BALB/c (25-30 g; 8-12 weeks; Charles River, QC, Canada) and age-matched $CB_2R^{-/-}$ were used in this study. $CB_2R^{-/-}$ mice were bred in the Dalhousie animal care facilities by crossing male C57BL/6J $CB_2R^{-/-}$ mice (strain B6.129P2-Cnr2tm1Dgen/J; Jackson Laboratory, Bar Harbour, ME, USA) with inbred BALB/c female mice (Charles River) for ten generations (Toguri, 2015). $CB_2R^{-/-}$ was confirmed by Dr. Toguri via genotyping, as reported in Toguri (2015). Animals were kept on a 12 h light/dark cycle with unrestricted access to food and water.

4.4.2 Induction of corneal injury

Corneal injury in mice was induced as previously described in Thapa et al. (2017, accepted), using a protocol adapted from a rat model of corneal hyperalgesia (Wenk and Honda, 2003). Briefly, mice were anesthetized using 2-3% isoflurane gas, and the corneal injury was induced in both eyes using a micro-applicator brush (Centrix Inc., Shelton, USA) coated with 75% silver nitrate and 25% potassium nitrate (MedPro®, AMG Medical Inc., Montreal, Canada). The micro-brush was held in contact with the cornea for 2 s to produce a distinct superficial white lesion of 1 mm diameter in the epithelial cell layer.

Cauterized eyes were then rinsed several times with room temperature saline. An ocular lubricant (Systane®, Alcon Canada Inc., ON, Canada) was applied to the corneal epithelial surface to reduce corneal drying. Mice recovered fully from anesthesia within 3-5 min post-cauterization, and 6 h later were challenged with either chemical (capsaicin) or cold stimulation to elicit a behavioral response.

4.4.3 Assessment of behavioral pain response

Following corneal injury, chemical or cold exposure can elicit a heightened pain response. Six h following cauterization, injured corneas were challenged with either chemical (capsaicin; 1 µM) or cold (4/10/15°C saline) stimulation topically (5 µl), and behavior was recorded for 30 or 60 s, respectively. Offline analysis of these responses was carried out by an experimenter blinded to the treatments given to quantify the number of blinks, squints and eye wipes, which were summed to give a corneal pain score.

4.4.4 Neutrophil migration

Mice were sacrificed, and eyes were enucleated and fixed in 4% PFA followed by 30% sucrose overnight, following behavioral pain assessments at 6 h. Corneal sections (12 µm) were prepared using a Leica CM1850 cryostat (Wetzlar, Germany). Sections were washed for 4 times in Phosphate Buffer Saline (PBS, Sigma-Aldrich, Oakville, ON, Canada) and blocked for non-specific binding (10% normal goat serum 0.5% Triton-X/PBS, Sigma-Aldrich) for 2 h, followed by incubation for 2 nights at 4°C in purified rat-anti Ly-6G antibody (1:200 diluted in 0.5% Triton-X/PBS; Abcam, Cambridge, MA, USA). Sections were then washed with PBS 4 times for 10 min, followed by an overnight

incubation with the secondary antibody (1:500, goat anti-rat Alexa Fluro® 488, Jackson ImmunoResearch Laboratories, Inc., USA). Stained sections were washed 4 times with PBS and mounted on superfrost slides (Fisher Scientific, Ottawa, ON, Canada) using Fluoromount (Sigma-Aldrich).

Neutrophil migration was quantified in corneal sections at 20X magnification using a Zeiss Axiovert 200M microscope with a Hamamatsu Orca R2 Camera (Zeiss, Thornwood, NY, USA). Three representative images were taken from each section of the right and left corneal peripheries and from the center of the cornea, respectively. Neutrophils from these three images were counted and summed to represent the total neutrophil per section. A total of 6-8 sections with 120 µm intervals were analyzed from each sample and were averaged. For each experimental group, 6-7 eyes were analyzed.

4.4.5 Pharmacological treatments

Δ^8THC (6aR,10aR]-6,6,9-trimethyl-3-pentyl-6a,7,8,10a-tetrahydrobenzo [c]chromen-1-ol; Cayman Chemical, Ann Arbor, MI, USA) was dissolved in soybean oil (Sigma-Aldrich) at different concentrations (0.2-5.0% w/v). The CB_1R allosteric ligands, GAT211, GAT228, and GAT229, were synthesized and provided by Dr. Ganesh A. Thakur (Northeastern University, USA)(Laprairie et al., 2017). GAT211, GAT228, and GAT229 were dissolved in soybean oil with 2% dimethyl sulfoxide DMSO (Sigma-Aldrich) and 4% Tween-20 (Sigma-Aldrich). DMSO is an organosulfur compound that dissolves both polar and non-polar compounds. Drugs were topically administered (5 µl) to cauterized corneas 30, 60 and 120 min post-cauterization. The CB_1R antagonist, AM251 (*N*-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide; Tocris

Bioscience, Minneapolis, MN, USA), was suspended in 10% DMSO in saline. AM251 was injected at a dose of 2.0 mg/kg intraperitoneally (i.p.) 15 min before cauterization. Capsaicin (1 μM; Tocris Bioscience) was prepared in 0.002% DMSO.

4.4.6 Data analysis

Statistical analysis was performed in GraphPad Prism version 6. One-way analysis of variance (ANOVA) with Dunnett's *post hoc* was used to compare data between groups of three or more. Analysis between two groups was performed using t-test. All data are represented as group mean ± standard deviation, and were considered significant at $p < 0.05$.

4.5 Results

4.5.1 GAT211 and GAT229 potentiated the corneal anti-nociceptive effects of Δ^8THC, whereas GAT228 was directly efficacious in reducing capsaicin-induced corneal pain

Different concentrations of topical GAT211, GAT229, and GAT228 were examined in WT mice to establish the effective concentrations required to reduce the corneal pain score compared to the vehicle-treated group (27 ± 8, n = 6; Figure 15). For GAT211, topical concentrations of 0.5%, 1%, or 2% were examined: none of these concentrations were effective in reducing corneal pain compared to vehicle-treated eyes ($p > 0.05$, n = 6 per group). However, topical treatment of animals with 0.4% Δ^8THC plus 0.5% GAT211 significantly reduced the corneal pain score (17 ± 6, n = 6) compared to vehicle treated eyes ($p < 0.05$). Likewise, topical application of GAT229 (0.5%, 1%, or 2%, n = 6-7 per group) alone did not reduce corneal pain ($p > 0.05$), but the combination of 0.4% Δ^8THC with 0.5% GAT229 (n = 6) significantly reduced the corneal pain response (17 ± 2, n = 6) compared to vehicle-treated eyes ($p < 0.05$). For GAT228, mice that received concentrations of GAT228 of <1% (e.g. 0.5%) did not have a significant reduction in pain score compared to vehicle-treated mice ($p > 0.05$). Increasing the concentration of GAT228 to 1% and 2%, however, significantly reduced the pain score (12 ± 5, n = 6, and 12 ± 4, n = 5, respectively, $p < 0.001$, Figure 15).

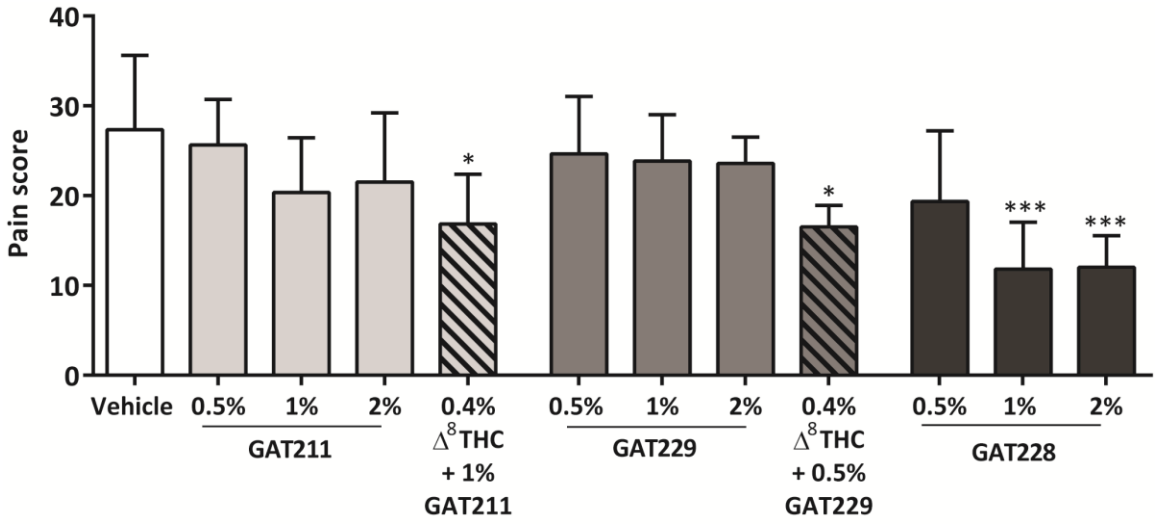

Figure 15. Topical administration of GAT211 or GAT229 in combination with 0.4% Δ^8THC, or GAT228 alone or reduces corneal hyperalgesia in WT mice following chemical cauterization. Dose-response for GAT211 (0.5-2.0%, n = 6 per group), GAT229 (0.5-2%, n = 6 per group) and GAT228 (0.5-2%, n = 5-6 per group) following capsaicin challenge. Antinociceptive effects of 1% GAT211 or 0.5% GAT229 in combination with 0.4% Δ^8THC (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. *$p < 0.05$, ***$p < 0.001$.

4.5.2 GAT229 and GAT228 reduce corneal pain via activation of CB_1R

Administration of the CB_1R antagonist, AM251 (2.0 mg/kg, i.p.), prior to corneal cauterization and capsaicin stimulation, blocked the anti-nociceptive actions of 0.4% Δ^8THC plus 0.5% GAT229 (28 ± 10, n = 6) compared to vehicle-treated eyes plus AM251 (33 ± 6, n = 7, $p > 0.05$, Figure 16A), suggesting that the actions of Δ^8THC plus 0.5% GAT229 are mediated via CB_1R. Likewise, the anti-nocieptive effects of 2% GAT228 are absent in mice pre-treated with CB_1R antagonist AM251 (30 ± 7, n = 6) compared to vehicle-treated eyes plus AM251 ($p > 0.05$, Figure 16A). Figure 16B shows the pain score measured in cauterized eyes in $CB_2R^{-/-}$ mice following treatment with vehicle, or 0.4% Δ^8THC plus 0.5% GAT229, or 2% GAT228. Either 0.4% Δ8THC plus 0.5% GAT229 or 2% GAT228 reduced the corneal pain score (18 ± 4 and 14 ± 6, respectively, n = 6 in each group) compared to vehicle-treated eyes (30 ± 5, n = 8; $p < 0.001$ and $p < 0.0001$, respectively), suggesting that the GAT-mediated reduction of corneal pain seen with GAT229 + Δ^8THC and GAT228 is independent of CB_2R.

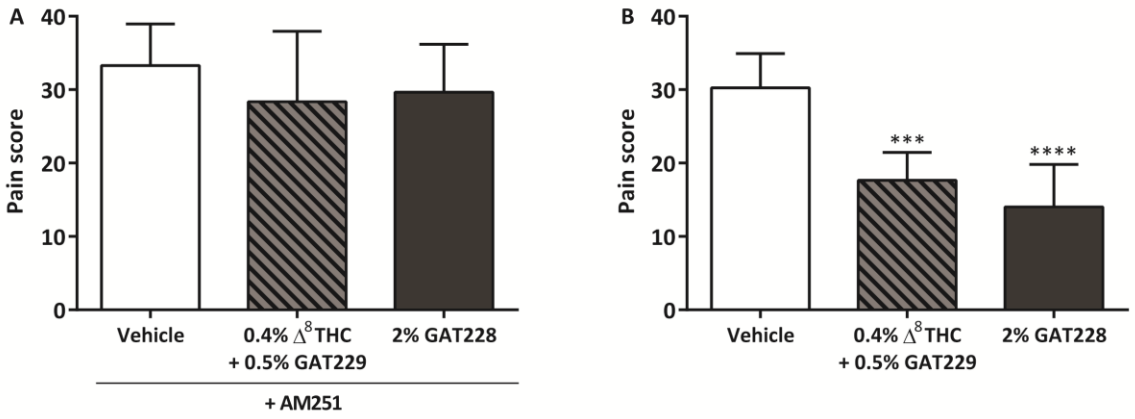

Figure 16. The antinociceptive effects of GAT229 and GAT228 are blocked by antagonism of CB_1R by AM251 (2.0mg/kg i.p.). **(A)** Pain score measured in WT mice at 6 h post-cauterization and following administration of 5 µl of topical vehicle, 0.4% Δ^8THC + 0.5% GAT229 or 2% GAT228 (n = 8 per group) in mice pre-administered with AM251 **(B)** Pain score measured in $CB_2R^{-/-}$ mice following administration of 5 µl of topical vehicle, 0.4% Δ^8THC + 0.5% GAT229 or 2% GAT228 (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. *** $p < 0.001$, ** $p < 0.01$.

4.5.3 Δ^8THC potentiated the anti-nociceptive effects of both GAT211 and GAT229, GAT228 reduces corneal pain on its own, following cold challenge

To see the effects of GAT compounds in modulating cold-induced pain, a model of corneal cold hyperalgesia was established using cold (4-15°C) saline challenge. The corneal pain response was recorded following topical cold saline-stimulation (5 µl) at 6 h post-cauterization. There was an increased pain response at 4°C (47 ± 16), 10°C (42 ± 16), and 15°C (42 ± 14) saline stimulation in cauterized cornea compared to their respective sham control eyes (18 ± 2, 10 ± 1, 10 ± 3, $p < 0.01$, $p < 0.001$ and $p < 0.001$, n = 4-6 per group, Figure 17A). A 10°C saline stimulation was then used to measure corneal pain for experiments with drug or vehicle.

Corneal pain response was recorded in WT mice following topical treatment of vehicle, 0.4% Δ^8THC, or 1% Δ^8THC (5 µl) to cauterized corneas at 30, 60 and 120 min post-injury, and cold stimulation. There was no significant reduction in pain score in mice treated with 0.4% Δ^8THC (29 ± 7) compared to vehicle-treated eyes (37 ± 8, $p > 0.05$, n = 6 per group). Increasing the concentration of Δ^8THC to 1% (n = 7) significantly reduced the corneal pain score (16 ± 5) compared to the pain score in vehicle-treated eyes ($p < 0.0001$, Figure 17B). Effective concentrations of GAT compounds that reduced corneal pain with the capsaicin challenge were chosen to see their effects in the cold-stimulation model. Topical application of 0.4% Δ^8THC plus 1% GAT211 reduced the corneal pain score (22 ± 5, n = 6) compared to vehicle-treated eyes (39 ± 12, n = 7, $p < 0.01$). Similarly, combination 0.4% Δ^8THC plus 0.5% GAT229 produced a significant reduction in the pain score (20 ± 7, n = 7) compared to vehicle-treated eyes ($p < 0.001$, Figure 17C). The

allosteric agonist, 2% GAT228, significantly reduced pain score (14 ± 5, n = 6) compared to vehicle-treated eyes ($p < 0.0001$, Figure 17C).

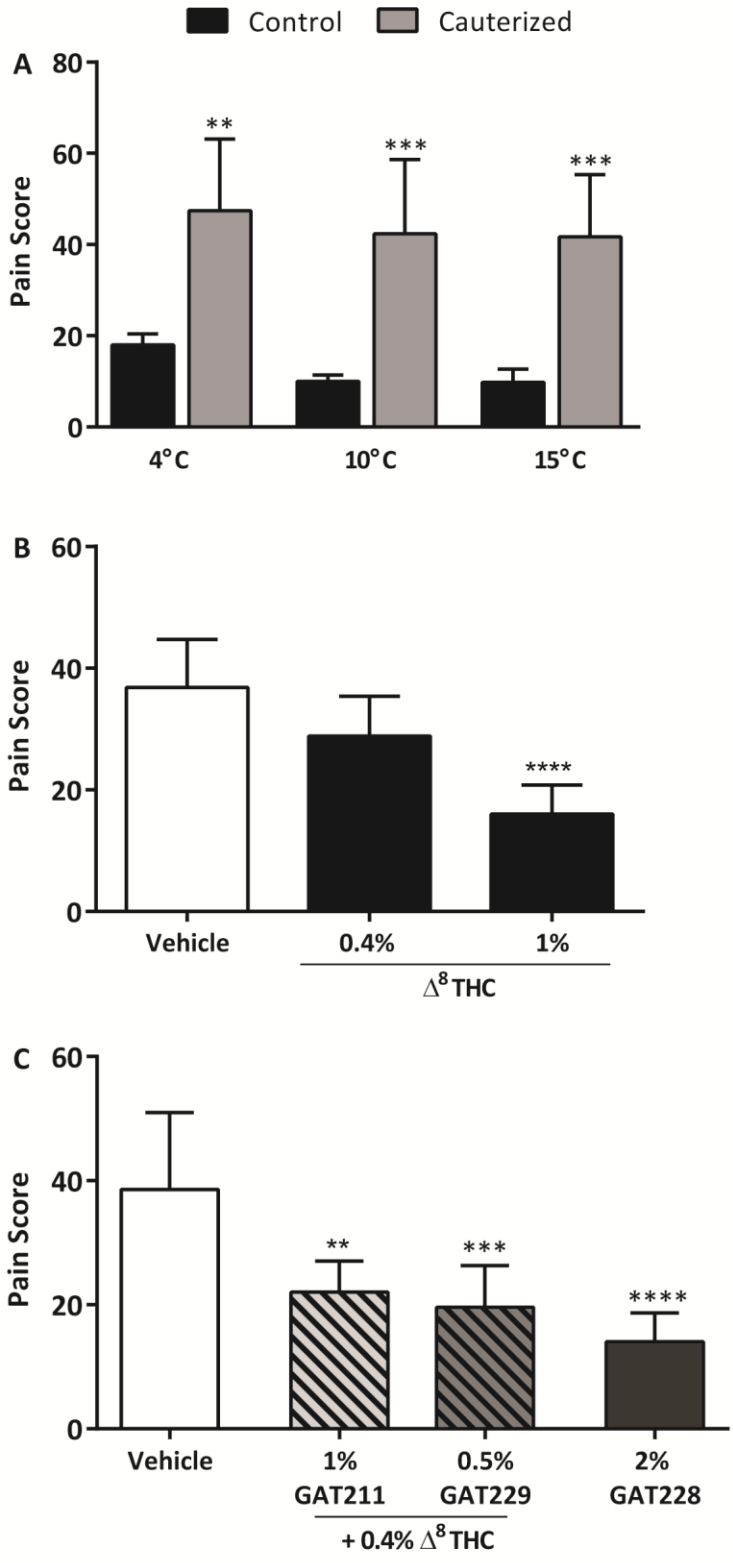
Control
Cauterized
A
Pain Score
80
60
40
20
0
**
***
***
4°C
10°C
15°C
B
Pain Score
60
40
20
0
****
Vehicle
0.4%
1%
Δ8 THC
C
Pain Score
60
40
20
0
**
***
****
Vehicle
1%
GAT211
0.5%
GAT229
2%
GAT228
+ 0.4% Δ8 THC

Figure 17. Corneal pain response to cold-stimulation following injury. **(A)** Pain responses to topical cold saline-stimulation (4/10/15°C) in non-cauterized control eyes and cauterized eyes at 6 h post-injury (n = 5-6 per group). **(B)** Effects of the topical administration of vehicle or Δ^8THC (0.4% or 1%) in corneal hyperalgesia in WT mice following chemical injury and 10°C saline-stimulation (n = 7). **(C)** Effects of combination of 0.4% Δ^8THC plus (0.5% GAT229 or 1% GAT211) or GAT228 (2%) alone (n = 6 per group). Values represent mean ± SD. For statistical analysis T-test (A) and (B) one-way ANOVA with Dunnett's *post hoc* test (compared to vehicle) was used. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

4.5.4 GAT229 in combination with Δ^8THC, and GAT228 alone, reduces neutrophil infiltration to the cornea

Neutrophil infiltration into the cornea of WT mice was examined at 6 h following treatment with topical vehicle, Δ^8THC (0.4%), 2% GAT228, or 0.4% Δ^8THC plus 0.5% GAT229. In WT mice, topical treatment of 0.4% Δ^8THC did not reduce neutrophil infiltration (231 ± 26) compared to vehicle treated eyes (216 ± 35, $p > 0.05$, n = 6 per group, Figure 18A). However, the combination of 0.4% Δ^8THC with 0.5% GAT229 significantly reduced neutrophil infiltration (137 ± 23, n = 6) compared to vehicle treated group (248 ± 47, n = 7, $p < 0.0001$). 2% GAT228 also significantly reduced neutrophil infiltration (149 ± 24, n = 6) compared to vehicle-treated eyes ($p < 0.001$, Figure 18B).

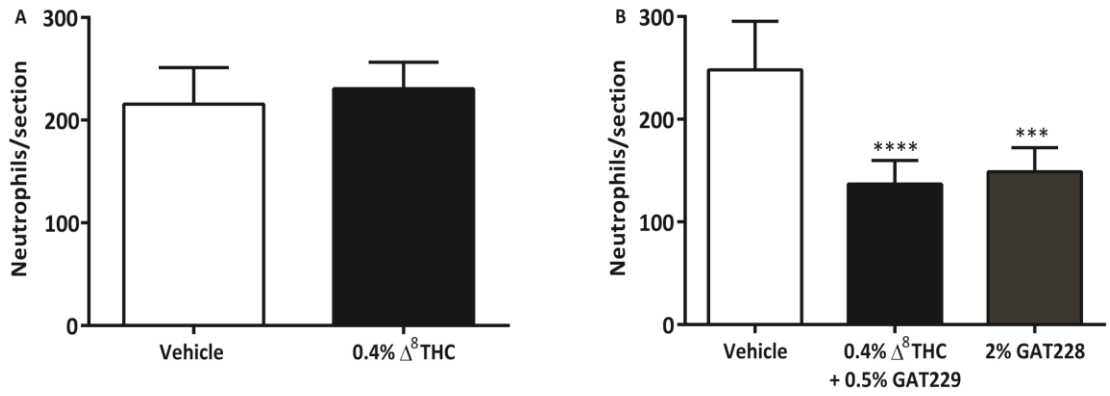

Figure 18. Neutrophil expression in cauterized corneas at 6 h post injury following the topical treatments of drug or vehicle, and cold-stimulation. **(A)** Effects of topical treatment of WT cauterized eyes with 0.4% Δ^8THC (n = 6) in neutrophil infiltration compared to vehicle (soybean only, n = 6) treated eyes. **(B)** Neutrophils expression in WT mice treated with 0.4% Δ^8THC + 0.5% GAT229 or 2% GAT228 compared to vehicle (Soybean + 2% DMSO + 4% Tween80) treated eyes (n = 6 per group). Values represent mean ± SD. For statistical analysis t-test (A) and (B) one-way ANOVA with Dunnett's *post hoc* test (compared to vehicle) was used. ****$p < 0.0001$, ***$p < 0.001$.

4.6 Discussion

Our results provide novel evidence demonstrating that topical application of CB_1R allosteric modulators can reduce corneal pain and inflammation. The pain response, following corneal injury, can be induced by different environmental stimuli including chemical and cold stimuli. We showed that the CB_1R allosteric modulators, GAT211 and GAT229, when combined with an orthosteric agonist Δ^8THC at a subthreshold dose, or the allosteric agonist, GAT228, on its own, reduce both chemical, and cold-induced corneal pain in a mouse model of corneal hyperalgesia and inflammation. Furthermore, GAT229 plus Δ^8THC, or GAT228 alone, decreased corneal neutrophil infiltration, a sign of corneal inflammation.

We have previously shown that topical application of the CB_1R orthosteric agonist Δ^8THC, reduced corneal pain and inflammation, measured at 12 h following corneal injury and capsaicin stimulation (Thapa et al., 2017, accepted). Similar findings were also reported in the rat model of neuropathic pain, where the cannabinoid agonist WIN55,212-2 reduced cold-induced allodynia and hyperalgesia (Bridges et al., 2001). The analgesic effect of WIN55,212-2 was blocked by treatment of animals with CB_1R antagonist but not the CB_2R antagonist, further implicating a role of CB_1R in analgesic effect of WIN55,212-2 (Bridges et al., 2001).

However, the therapeutic use of CB_1R orthosteric agonists may be limited due to side-effects such as tolerance, receptor desensitization and psychosis (Biala, 2008; Compton et al., 1990; Gonzalez et al., 2005; Lichtman & Martin, 2005). In an *in vitro* model of acquired epilepsy in the hippocampal neuronal culture, prolonged exposure of WIN55,212,-2 has been reported to produce tolerance to its anti-convulsant effects (Blair

et al., 2009). Consistently, repeated administration of THC, an orthosteric agonist, has been shown to produce desensitization and downregulation of CB_1R in rat brain (Burston et al., 2010), which may lead to loss of therapeutic efficacy over time. Given these limitations, we hypothesized that allosteric modulators may provide an alternate means to modulate CB_1R, but with fewer limitations.

Allosteric modulators may stabilize GPCR conformations in such a way that they can fine-tune the effects of orthosteric ligands (Kenakin, 2012; Laprairie et al., 2016), increasing affinity and/or efficacy of binding, and ultimately resulting in changes in downstream signaling. We have shown that topical application of CB_1R PAMs, GAT211 or GAT229, potentiated the corneal anti-nociceptive effects of a subthreshold dose of an orthosteric agonist, Δ^8THC, irrespective of the pain modalities tested. Additionally, GAT211, and GAT229 also potentiated the anti-inflammatory effects of a subthreshold dose Δ^8THC measured at 6 h following cold-stimulation. Previous studies have reported that administration of GAT211 and ZCZ011, a related CB_1R PAM, reduced mechanical and cold allodynia in mouse models of neuropathic and inflammatory pain (Ignatowska-Jankowska et al., 2015; Slivicki et al., 2017). GAT211 treatment did not show evidence of anti-nociceptive tolerance throughout the entire 19-day dosing period, or physical dependence (measured by paw tremors) at 20 days following the treatment (Slivicki et al., 2017). Similarly, there were no difference in the anti-nociceptive effect of ZCZ011 at 6 days of chronic dosing (40mg/kg, i.p. b.i.d) compared to acute dosing at day 1 (40mg/kg, i.p), further confirming the lack of tolerance to the CB_1R PAM (Ignatowska-Jankowska et al., 2015).

Allosteric modulation may also provide an opportunity to modulate CB_1R, but with reduced potential to produce psychotropic side-effects (Ross, 2007). It has been reported that the administration of GAT211 alone did not produce side-effects that are frequently associated with activation of CB_1R with orthosteric agonists such as Δ^9THC, or WIN55,212-2 (Slivicki et al., 2017). However, Ignatoawaska-Jankowska et al. (2015) reported potentiation of cannabimimetic side-effects (anti-nociception, hypothermia and catalepsy) of CB_1R orthosteric ligands when administered in combination with the CB_1R PAM ZCZ011. Similarly, Slivicki et al. (2017) showed that administration of CB_1R PAM GAT211 enhanced cataleptic effects in mice where FAAH and MAGL enzymes are genetically deleted.

Increased local release of endocannabinoids has been reported in several pathologies including pain and inflammation (Rice et al., 2002; Fine and Rosenfeld, 2013). Local increases of eCBs at the site of pathology that is sufficient to potentate the actions of CB_1R PAM may be more desirable as a treatment paradigm than using a CB_1R PAM with an exogenous orthosteric agonist. Such was the case in models of neuropathic and inflammatory pain, where the CB_1R PAMs, GAT211 and ZCZ011, reduced pain on their own (Slivicki et al., 2017; Ignatowska-Jankowska et al., 2015). Similarly, the CB_1R PAM GAT229, on its own, reduced intraocular pressure in a mouse model of ocular hypertension, but not in normotensive mice (Cairns et al., 2017).

In our corneal hyperalgesia model, administration of GAT211 or GAT229 reduced corneal pain when combined with subthreshold Δ^8THC, but not on their own. This may be due to lack of, or insufficient, local eCB production at the time point we measured the pain response. Unlike previous studies investigating the *in vivo* effects of CB_1R PAMs, our

model is relatively acute, with drug administration occurring shortly after cauterization, and chemical or cold challenge occurring only 6 h later. In contrast, in a rat model of chronic constrictive nerve injury, a chronic model of pain, both the eCBs, AEA and 2-AG, were significantly increased in the brain and spinal cord at 3 days following injury, with a 1.3-3-fold increase at 7 days following injury (Petrosino et al., 2007). Further studies using a more chronic model of corneal pain would therefore be useful to investigate if increases in eCB levels following injury are sufficient to permit PAM potentiation of CB_1R activation by eCBs.

Unlike GAT211 or GAT229, GAT228 significantly reduced corneal pain and inflammation on its own which is consistent with actions of an allosteric agonist as previously demonstrated in an *in vitro* study (Laprairie et al., 2017). As allosteric sites tend to be more unique than the receptor orthosteric site, drugs targeting at the allosteric site may produce less off-target effects compared to the drugs that target orthosteric site (Changeux & Christopoulos, 2017; Nguyen et al., 2017).

4.7 Conclusions

This study provides further evidence supporting the role of CB_1R in modulating either chemical or cold-evoked corneal pain responses and inflammation following corneal injury. Allosteric activation of CB_1R using the PAMs, GAT211 or GAT229, in combination with subthreshold dose of CB_1R orthosteric agonist Δ^8THC, or a CB_1R allosteric agonist GAT228 alone, reduced both corneal pain and inflammation. CB_1R PAMs in combination with subtherapeutic dose of orthosteric agonists, or CB_1 allosteric

agonists alone, could be a novel approach for the treatment of corneal pain and inflammation.

CHAPTER V: GENERAL DISCUSSION

5.1 Objectives of the research

The overall aim of this thesis was to investigate the pharmacology of phytocannabinoids and synthetic cannabinoids in modulating corneal pain and inflammation. The long-term goals of this work are to identify effective pharmacotherapies for the treatment of corneal hyperalgesia and neuropathic pain.

Specific objectives that I addressed in my work included: 1) the development of a mouse model of corneal hyperalgesia, 2) the identification of anti-nociceptive and anti-inflammatory cannabinoid receptor ligands and the receptor targets for these ligands for ameliorating pain and inflammation in the cornea, 3) further determination of the pharmacology of the novel racemic allosteric molecule, GAT211, and its resolved enantiomers, the positive allosteric modulator GAT229, and the allosteric agonist, GAT228.

5.2 Summary of the key findings

The work presented in this thesis demonstrated that the topical application of phytocannabinoids and synthetic cannabinoids reduces corneal pain and inflammation in a novel mouse model of corneal hyperalgesia and inflammation. Each of the tested cannabinoids, Δ^8THC, CBD, HU-308, exerted their effects through distinct receptors. The phytocannabinoid, Δ^8THC, reduced corneal pain and inflammation primarily through the activation of CB_1R; the actions of THC were blocked by a selective CB_1R antagonist (AM251) and absent in $CB_2R^{-/-}$ mice. The non-psychoactive phytocannabinoid, CBD, exerted its anti-nociceptive and anti-inflammatory effects independent of cannabinoid receptors via the serotonin 5-HT_{1A} receptor, and the actions of CBD were eliminated by

the 5-HT_{1A} receptor antagonist, WAY100635. The cannabinoid, HU-308, a synthetic derivative of CBD, reduced corneal pain and inflammation via selective activation of CB_2R which was absent in $CB_2R^{-/-}$ mice.

Additionally, in my thesis, I examined allosteric ligands that act at CB_1R. Allosteric ligands, by binding to a topographical distinct site to the orthosteric ligand, can activate/inhibit the receptor directly (allosteric agonist/antagonist) or modulate the receptor activity when the orthosteric ligand is bound (positive allosteric modulator/negative allosteric modulator). Allosteric ligands can also be biased with PAM activity in one pathway (e.g. G-protein coupled signaling) and NAM activity in another (e.g. beta-arrestin signaling) (Ahn et al., 2013; Bertini et al., 2017; Changeux and Christopoulos, 2017; Kenakin, 2017; Laprairie et al., 2015; Nguyen et al., 2017). Allosteric ligands afford several advantages with respect to CB_1R, including a potential reduction in behavioral side-effects and tolerance and the possibility of developing selective, novel receptor and site-specific drugs; allosteric sites tend to be more distinct with respect to individual receptors compared to orthosteric sites (Nguyen et al., 2017; Mohr et al., 2013; Price et al., 2005; Ross, 2007).

I examined the novel racemic ago-PAM, GAT211 and both of it's resolved enantiomers, GAT228 (R) and GAT229 (S) in the mouse model of corneal hyperalgesia and inflammation. I found that GAT211 and GAT229 potentiated the corneal antinociceptive effects of an orthosteric agonist Δ^8THC, indicating PAM actions whereas, GAT228 acted as a direct allosteric agonist and reduced corneal pain independent of addition of orthosteric agonist. The CB_1R antagonist, rimonabant, has been reported to block allosteric activation of CB_1R (Slivicki et al., 2017). Using AM251 (structurally like

rimonabant), I demonstrated that the effects of GAT211, GAT229 and GAT228 were mediated through activation of CB_1R. In keeping with a CB_1R mediated mechanism of action, the PAM, GAT229, also potentiated the corneal inflammatory action of Δ^8THC whereas, the allosteric agonist GAT228 reduced corneal inflammation on its own.

Taken together, these results provide novel data on cannabinoid modulation of corneal pain and inflammation, and indicate that cannabinoids that act at both CB_1R and CB_2R, as well as 5-HT_{1A} receptor, may be useful as a novel therapeutics for ocular surface disease.

5.2.1 Antinociceptive and anti-inflammatory effects of phytocannabinoids

Cannabinoids such as THC and CBD have been shown to be beneficial in treating a range of pathological conditions, including pain and neurological disorders (Scharf, 2017; Booker et al., 2009; Campos et al., 2012; Russo and Guy, 2006; Russo, 2008). However, to-date there is no published data specifically examining the antinociceptive and anti-inflammatory actions of individual phytocannabinoids in corneal pain and inflammation. The work presented in this thesis demonstrated that the topical application of Δ^8THC reduced corneal pain in a mouse model of superficial corneal injury. This reduction in corneal pain was mediated through CB_1R. The co-localization of CB_1R and TRPV1 receptors in the human cornea has been reported in an earlier study that showed that the stimulation of CB_1R using a non-selective cannabinoid agonist, WIN55212-2, suppressed a TRPV1-dependent inflammatory response following corneal injury (Yang et al., 2013). Increased corneal stromal thickness, infiltration of immune cells (neutrophils and macrophages) and corneal scaring were used as parameters of corneal inflammation.

Furthermore, WIN55212-2 treatment also resulted in enhanced corneal wound healing in WT versus $CB_1R^{-/-}$ mice. In line with their findings, I showed a reduction in corneal neutrophil infiltration (a parameter of corneal inflammation) with Δ^8THC treatment. This effect was present in $CB_2R^{-/-}$ mice but was inhibited by the pretreatment of WT mice with the CB_1R antagonist, AM251. The pharmacological benefits of CB_1R activation have been studied in animal models of both acute inflammatory and neuropathic pain. For example, Δ^9THC (1 mg/kg i.p.) has been shown to produce an antinociceptive effect in a rat model of acute muscle pain; this effect was blocked by the CB_1R antagonist, AM251 (0.5mg/kg i.p.), and to a lesser extent by a CB_2R antagonist, AM630 (0.5mg/kg i.p.). In another study using a rat model of muscular pain, administration of 10 µl of Δ^9THC (1 mg/ml i.m.) decreased masticatory muscle sensitization in female rats through peripheral CB_1R activation, an effect which was blocked by AM251 (CB_1R antagonist), but not by the CB_2R antagonist AM630 (Wong et al., 2017). Furthermore, in a mouse model of inflammatory and neuropathic pain, the analgesic effects of WIN55212-2 were dependent upon peripheral activation of CB_1R (Agarwal et al., 2007). Based on the fact that CB_1Rs are highly expressed in the corneal epithelium (Straiker et al., 1999), topical Δ^8THC could be beneficial in minimizing acute corneal pain and inflammation. Ocular topical delivery of cannabinoids with central CB_1R-activating potential would minimize their systemic bioavailability and therefore minimize the possibility of psychotropic effects.

My work has also shown that topical CBD (5% w/v) reduces corneal pain and neutrophil infiltration through activation of $5\text{-}HT_{1A}$ receptor, without activity at the CBRs. The $5\text{-}HT_{1A}$ receptor-mediated mechanism by which CBD modulates pain is also in line with the study by Ward and colleagues (2014), who showed that the chronic dosing of CBD

(2.5-10 mg/kg i.p) for 14 days in mice prevented chemotherapy-induced neuropathic pain, from treatment with paclitaxel (Ward et al., 2014). This effect was blocked by WAY100635 but not by CB_1R (SR141716) or CB_2R (SR144528) antagonists.

Drugs based on THC and CBD have been used successfully in humans for treatment of several disorders including pain, nausea and vomiting (Russo et al., 2016). For example, Sativex® (oromucosal spray), an equimolar mixture of THC and CBD, is approved to manage spasticity and neuropathic pain in patient suffering from multiple sclerosis (Nurmikko et al., 2007; Patti et al., 2016; Russo et al., 2016). Oral forms (tablets and capsules) of THC, such as dronabinol and nabilone, are also available for the treatment of chemotherapy-induced nausea and vomiting in cancer patients who have failed to response to conventional therapies (Badowski, 2017). Oral administration of dronabinol (5 mg, b.i.d) was shown to reduce the frequency and intensity of functional chest pain in a small double-blind placebo-controlled trial without any adverse effects (Malik et al., 2017). A larger multi-centered clinical trial is still necessary to further assess the efficacy and safety of dronabinol for these specific indications.

Taken together, the evidence supports the use of THC and CBD to reduce pain and inflammation in both preclinical (Booker et al., 2009; Morales et al., 2017), as well as clinical models (Patti et al., 2016). My findings provide additional novel findings that indicate the anti-inflammatory and anti-nociceptive effects of topical THC and CBD in corneal injury, pointing to the potential for further investigation of these phytocannabinoids for clinical use in painful inflammatory eye diseases.

5.2.2 CB_2R as an important therapeutic target for corneal pain and inflammation

One of the findings from my work was that cannabinoids that are selective for CB_2R, like CB_1R agonists, show efficacy for reducing corneal pain and inflammation. CB_2R modulation does not produce behavioral side-effects that are associated with CB_1R activation, and therefore, holds the potential for clinical use (Ligresti et al., 2009). Localization of CB_2R on immune cells makes CB_2R a promising therapeutic target in modulating the inflammation of different tissues, including the eye (Kaur et al., 2016; Toguri et al., 2016). An earlier study examining CB_2R expression in various immune cells reported that highest levels of CB_2R mRNA is present in order of: B cells > NK cells > monocytes > neutrophils > CD8+ T cells > CD4+ T cells (Galiègue et al., 1995). Our lab has also demonstrated that CB_2R is upregulated in ocular inflammation and activation of CB_2R with selective agonists is an efficacious treatment for ocular inflammation in experimental models of anterior and posterior (retina) inflammation (Szczesniak et al., 2017; Toguri et al., 2014; Toguri et al., 2015).

Several studies have now reported the immunosuppressive effects of CB_2R agonists in experimental intraocular inflammatory diseases. For example, the CB_2R agonist, JWH 133, was demonstrated by Xu et al. (2007) to reduce leukocyte-endothelial adhesion in the retina in an experimental autoimmune uveoretinitis (EAU) model in mice, an animal model that is characterized by retinal infiltration of inflammatory cells, as well as increased choroidal and ciliary body thickness, contributing to ocular inflammation (Chan et al., 1990). The synthetic cannabinoid, HU-308, used in my studies is a selective CB_2R agonist that produces peripheral analgesic and anti-inflammatory effects with no behavioral effects in mice (Hanus et al., 1999). HU-308 was shown to reduce ocular inflammation and retinal

disease severity in a mouse model of proliferative vitreoretinopathy (PVR) (Szczesniak et al., 2017; Toguri et al., 2014). Furthermore, topical application of HU-308 (1.5% w/v) is more efficacious in reducing anterior ocular inflammation in rats compared to corticosteroids (dexamethasone or prednisolone) or treatment with an NSAID (nepafenac); both steroids and NSAIDs are routinely employed pharmacological modalities in the clinical management of ocular inflammation (Toguri et al., 2014). In keeping with my thesis findings of the efficacy of CB_2R agonists to reduce pain and inflammation in corneal hyperalgesia, CB_2R agonists have been also reported to be analgesic and anti-inflammatory effects in other experimental models of hyperalgesia and chronic inflammatory neuropathic pain (Dhopeshwarkar and Mackie, 2014; Kinsey et al., 2011; Woodhams et al., 2017). For example, chronic activation of CB_2R with AM1710 (CB_2R agonist) was able to reverse paclitaxel-induced allodynia – an effect that was blocked in WT mice treated with the CB_2R antagonist AM630, as well as in $CB_2R^{-/-}$ mice (Deng et al., 2015). In addition, this treatment did not produce the tolerance or withdrawal effects associated with CB_1R activation (Deng et al., 2015). Similarly, my results have demonstrated a reduction in corneal hyperalgesia and inflammation following topical treatment with HU-308 in a model of superficial corneal chemical injury. The anti-nociceptive and anti-inflammatory actions of HU-308 were absent in $CB_2R^{-/-}$ mice, further validating the CB_2R-mediated actions of HU-308. CB_2R-selective agonists, such as HU-308, could offer a novel drug in the treatment of ocular pain and inflammation given that these drugs are devoid of behavioral side-effects of CB_1R agonists but still maintain analgesic and anti-inflammatory efficacy.

5.2.3 CB_1R allosteric modulation as an emerging therapeutic strategy for reducing corneal pain and inflammation

Several side effects associated with orthosteric activation of the CB_1R limit the use of drugs that activate this receptor (Bertini et al., 2017). These side effects include receptor desensitization or internalization, addiction, and cognitive impairment (Pertwee, 2009; Skosnik et al., 2012). The first cannabinoid-based clinical drug, Rimonabant (an orthosteric CB_1R antagonist), was marketed in the European Union as an orally administered treatment for obesity, and was withdrawn from the market after two years following post-marketing reports of serious adverse effects including psychiatric disorders and suicidal thoughts (McNaughton et al., 2014). This being said, the bioavailability and potential CNS effects of topical application of CB_1R agonists have yet to be assessed.

My research has examined the topical application of CB_1R allosteric modulators as a means to circumvent some of the disadvantages associated with CB_1R orthosteric agonism (Ignatowska-Jankowska et al., 2015; Khurana et al., 2017). I assessed several novel CB_1R allosteric ligands including: the ago-PAM, GAT211, the PAM, GAT229 and the allosteric agonist, GAT228, in a preclinical model of corneal hyperalgesia. Administration of these compounds has been shown to enhance the potency and efficacy of the endocannabinoids, 2-AG and AEA, at CB_1R in HEK293A cells (heterologously expressing CB_1R) and Neuro2a cells (endogenously expressing CB_1R) (Laprairie et al., 2017). Of these compounds, GAT211 acted as an ago-PAM at CB_1R i.e. displayed the activity of a mixed PAM and partial allosteric agonist at CB_1R. GAT229 acted as a "true" PAM at CB_1R, while GAT228 acted as an allosteric agonist at CB_1R (Laprairie et al., 2017).

My research has shown that topical application of GAT211 alone, up to the concentration of 2% (w/v), did not reduce corneal pain at 6 h post-cauterization. However, the combination of 1% GAT211 with 0.4% Δ^8 THC, concentrations that are individually ineffective, significantly reduce corneal pain when given concurrently following either chemical or cold stimulation of an injured cornea. A recently published study using GAT211 has shown that GAT211 in combination with orthosteric CB_1R activation produced CB_1R-dependent antinociceptive effects in a mouse model of inflammatory and neuropathic pain (Slivicki et al., 2017). GAT211 potentiated the antinociceptive effects of an orthosteric agonist, WIN55,212-2, a FAAH-inhibitor (URB597), or a MAGL-inhibitor (JZL184). Additionally, GAT211, on its own, also reduced complete Freund's adjuvant-induced mechanical hypersensitivity at a dose of 20 and 30mg/kg i.p., measured at 30 and 90 min post-injection, but failed to produce anti-nociception after 150 min of injection of GAT211. CFA is an emulsified solution of antigen that are often used in animal research to induce painful reaction. Similarly, chronic dosing of GAT211 alone, at a dose of 20mg/kg i.p given daily up to 19 days, reduced paclitaxel-induced mechanical and cold allodynia. Moreover, GAT211 did not produce physical dependence, tolerance or cardinal signs of CB_1R activation (alteration in motor coordination, body temperature, etc.) (Slivicki et al., 2017). The absence of anti-nociceptive effect of GAT211 alone in our model may be due to lack of, or insufficient, production of eCBs at the time point of our pain measurement. To further understand the pharmacology of GAT211, individual enantiomers of GAT211 (GAT229 and GAT228) were investigated. In our study, GAT229, like GAT211, did not reduce corneal pain on its own but did in combination with a subthreshold dose (0.4%) of Δ^8THC. These effects of GAT229 were extinguished in mice

pretreated with the CB_1R antagonist AM251, however remained unaffected in $CB_2R^{-/-}$ mice. GAT229 acted as a PAM; it potentiated the antinociceptive effects of an orthosteric CB_1R agonist, Δ^8THC. Another enantiomer, GAT228, reduced corneal pain on its own which is consistent with its action as an allosteric agonist (Laprairie et al., 2017). The absence of anti-nociceptive effects of GAT228 in mice pretreated with CB_1R antagonist, but not in $CB_2R^{-/-}$ mice, validates its action a CB_1R allosteric agonist (Laprairie et al., 2017).

There are only a few studies available to-date that evaluate the *in vivo* efficacy of CB_1R PAMs. The synthetic CB_1R PAM, ZCZ011, produced analgesic effects in a mouse model of chronic constriction nerve injury-induced neuropathic pain, and of carrageenan-induced inflammatory pain, without tetrad effects (hypomotility, catalepsy, hypothermia, and analgesia) when administered acutely (40 mg/kg i.p.) (Ignatowska-Jankowska et al., 2015). Furthermore, daily dosing of ZCZ011 up to 6 days did not produce tolerance. Similarly, lipoxin A4, an endogenous anti-inflammatory lipid molecule that is present in brain tissues and acts as an endogenous allosteric enhancer of CB_1R, did not compete for the orthosteric binding site of CB_1R but enhanced the affinity of the endocannabinoid AEA at CB_1R and potentiated the effects of AEA (Pamplona et al., 2012).

The pharmacological role of CB_1R PAMs have been reported in the eye in only one other study. Cairns et al. reported that the PAM, GAT229, potentiated the intraocular pressure (IOP)-lowering effects of non-selective CB_1R orthosteric agonists WIN55,212-2 and Δ^9THC in a mouse model of ocular hypertension (Cairns et al., 2017). This study also reported an increased duration of action of subthreshold, topical WIN55,212-2 (0.25%) when combined with GAT229 compared to previous studies where the orthosteric

activation of CB_1R by WIN55,212-2 exhibited shorter duration of action in lowering IOP (Porcella et al., 2001; Song and Slowey, 2000). This increase in duration of action, resulting from PAM activation of CB_1R, has an important application for human use; lowering the frequency of drug application may increase patient compliance.

In my work examining the effects of CB_1R allosteric modulators in corneal inflammation, I found that the PAM, GAT229, potentiated the anti-inflammatory effects of CB_1R orthosteric agonist, Δ^8THC, and that the allosteric CB_1R agonist, GAT228, was anti-nociceptive and anti-inflammatory in the absence of orthosteric CB_1R activation, irrespective of stimulation modalities. The effects of both these allosteric ligands were mediated through activation of CB_1R. Development of improved drug-like CB_1R PAMs for corneal pain and inflammation, either as stand-alone therapies or in combination with low dose orthosteric CB_1R agonists, would likely increase the success of new clinical trials for cannabinoids for treatment of chronic pain conditions given that allosteric modulation provides a means to "revisit" the potential of CB_1R agonists while minimizing unwanted side-effects.

5.3 Conclusions

Modulation of CBRs offers a promising alternative therapeutic target for corneal pain and inflammation and may circumvent the lack of efficacy, undesirable side-effects and costs associated with currently available pharmacological treatments for ocular surface disease. My research has demonstrated that topical application of the phytocannabinoids, Δ^8THC and CBD, and the CBD derivative, HU-308, can reduce corneal hyperalgesia and neutrophil infiltration in models of superficial corneal chemical injury. The effects of Δ^8THC, CBD, and HU-308 were mediated by distinct receptors, CB_1R, 5-HT_{1A}, and CB_2R, respectively. The involvement of these distinct receptors in the analgesic and anti-inflammatory actions of cannabinoids may be of value in the development of combination drugs that involve CB_1R agonists. Combination drug treatments would allow the use of lower doses of the individual drugs, for example, THC and CBD. The options for use of non-psychotropic cannabinoids, that target non-CB_1R, such as CBD and HU-308, would also circumvent the behavioral side-effects of CB_1R activation and, with respect to HU-308, would allow the use of drugs which are target-specific with reduced off-target effects. Additionally, the topical or systemic use of CB_1R allosteric ligands could also offer novel opportunities to maximize the analgesic and anti-inflammatory actions of CB_1R activation but minimize side-effects that may be associated with chronic use of CB_1R agonists, including tolerance and CNS behavioral actions on systemic exposure. As the allosteric sites of G-protein coupled receptors tend to be more unique than the orthosteric sites, drugs targeting these sites would be expected to have less off-target effects.

Taken together, my thesis work provides new insights into the use of cannabinoids as a therapy to treat ocular surface disease and suggests that cannabinoid drugs should be

explored further for their efficacy in alleviating the pain and inflammation that results from injuries to the corneal surface, as well as chronic pain conditions such as corneal neuropathic pain.

5.4 Considerations and future work

My thesis has explored the preclinical pharmacology of cannabinoid molecules, both phytocannabinoids and their synthetic derivatives, in the treatment of acute corneal pain and inflammation. The study has demonstrated that the topical application of cannabinoids reduces behavioral pain response, as well as corneal neutrophil infiltration, indicative of inflammation, in the mouse model of corneal injury. These findings are novel, and have potential for the future development of cannabinoid-based therapeutics for corneal pain and inflammation. The following proposed work would be valuable in expanding my research and addressing the limitations of the present study.

5.4.1 Sex differences and limitations stemming from size

The present study was carried out in adult male mice (8-12 weeks). It has been reported that corneal sensitivity/pain decreases with age due to decreased corneal nerve density (Gipson, 2013). Therefore, it would be interesting to see if cannabinoid treatment can alter corneal sensitivity in older animals. Further, it has been reported that adult female humans experience more pain, greater pain-related distress and heightened sensitivity to external stimuli compared to males (Paller et al., 2009). Several factors are involved in this difference including genetics, hormones, psychology etc. Therefore, it would also be

important to carry out these studies in female mice to assess gender-based differences in corneal pain sensation that may impact drug development and treatment.

Limitations exist for the use of a mouse model in studies of corneal pain and inflammation. Inhalation of marijuana has been reported to produce transient hyperemia in the eye and decreased tear production in human (Brown et al., 1977) reviewed in (Tomida et al., 2004; Yazulla, 2008). It would be important to see if topical delivery of cannabinoids produces such changes in our model, but these changes are more difficult to access in mice due to the smaller eye size. Rat or rabbit models of corneal injury may be more suitable to study the effects of these compounds on these parameters, as they have bigger ocular size. In addition, these models could be beneficial in studying corneal sensation that results from physical or mechanical stimulation, using devices such as corneal esthesiometer. Corneal esthesiometers are used routinely in clinics to measure corneal pain sensation by contact with cotton-tipped applicator on the ocular surface, however their use would be better applied to larger animals for applicability to findings in humans.

5.4.2 Markers of inflammation

In this thesis, inflammation has been quantified through assessing corneal neutrophil infiltration. It would be valuable to see how topical treatment of selective or non-selective cannabinoids modulates the mRNA and protein levels of pro-inflammatory cytokines and cellular adhesion molecules such as IL-1β, TNF-α, IL-6, TGF-β, CXCL2, CCL5 AP-1, ICAM-1, ICAM-2, VCAM-1, L-selectin, E-selectin, and P-selectin. These can be measured by using techniques like qPCR and multiplex assay (Toguri et al., 2014; Toguri et al., 2015). Corneal injury also leads to corneal edema and neovascularization

which affects the corneal transparency, thereby resulting in temporary or permanent vision loss (Anderson et al., 2014a; Shi et al., 2011; Wenk and Honda, 2003). Corneal edema can be measured either *in-vivo* by using confocal microscopy, or by Hematoxylin and Eosin (H and E) histochemical staining in post-mortem corneal tissues (Alomar et al., 2011). Similarly, corneal neovascularization can be studied by using *in-vivo* intravital microscopy (IVM) or by immunohistochemistry using endothelial blood markers (Anderson et al., 2014a; Giacomini et al., 2014; Voiculescu et al., 2015).

5.4.3 Positive control experiments

In this thesis, the effects of cannabinoids were compared to the vehicle the drug was dissolved in. In future studies, it would be important to compare the efficacy of topical cannabinoids with drugs that are the clinical standards of care in the treatment of corneal pain, namely corticosteroids (1% topical prednisolone) and NSAIDs (0.5% ketorolac, or 0.09% bromfenac, or 0.1% nepafenac) (Donnenfeld et al., 2011; Malik et al., 2016).

5.4.4 Pharmacokinetic (PK) and systemic effects

The systemic bio-availability of cannabinoids such as THC can produce CNS-mediated side effects that result from central CB_1R activation. Therefore, the study of PK parameters and safety profiles of topically delivered cannabinoids is crucial. Cannabinoids present in the aqueous humor, cornea or in systemic circulation can be assessed by using gas chromatography and/or mass spectrometry. The efficacy of oral THC and CBD in treating symptoms associated with multiple sclerosis, such as spasticity and neuropathic pain, has been frequently studied in humans (Nurmikko et al., 2007; Russo et al., 2016). The oral mucosal spray application of Sativex® (combination of THC and CBD) has been shown to reduce indices of neuropathic pain in a randomised, double blind, placebo-controlled trial in multiple sclerosis patient (Nurmikko et al., 2007). Sedation and gastrointestinal discomfort were the major side-effects reported. To date, no studies have been carried out to see if the topically delivered cannabinoids produce CNS-mediated side effects. This can be studied using standard tetrad tests which measure hypomotility, catalepsy, hypothermia, and analgesia produced from the administration of cannabinoids in rodents (Hanus et al., 1999). This thesis has investigated the efficacy of CB_1R allosteric modulators applied topically in the treatment of corneal pain and inflammation, the PK and pharmacodynamic (PD) parameters have yet to be assessed in addition to a thorough evaluation of their ocular safety profiles. The allosteric modulators of CB_1R would not be expected to produce tetrad effects. However, it is important to see if the chronic dosing of CB_1R allosteric modulators produce tetrad effects.

5.4.5 Topical drug delivery

One of the major concerns associated with topical drug delivery is the precorneal loss of drug that results in poor bioavailability (Agrahari et al., 2016). Several factors such as solution drainage, tear dilution, lacrimation, and conjunctival absorption limit the absorption of drugs across the cornea. Topically delivered drugs bind with the protein present in the tear fluid and may decrease the concentration of free drug required to produce a pharmacological response at the target site (Gaudana et al., 2010). In addition, the anatomy of the corneal tissue, specifically, the lipid bilayer of cornea, plays an important role in drug permeability. The outermost epithelial layer of the cornea is lipophilic in nature, while the stroma (covers about 90% thickness of cornea) is hydrophilic. Therefore, while formulating topical drugs, the vehicle must be chosen cautiously so that there is proper balance of lipophilicity and hydrophilicity (Kaur et al., 2004). This can be achieved by using some emerging drug delivery techniques. One such technique is the use of solid lipid nanoparticles (SLN) which are made up of a solid lipid core that is stabilized by surfactants in aqueous dispersion (Sanchez-Lopez et al., 2017). Given that cannabinoids are lipophilic molecules, use of SLN can enhance their permeability across the epithelial and stromal layer of the cornea.

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