

The Pharmacology of Cannabinoids and Cannabimimetic Ligands in the Eye and
their Effects on Intraocular Pressure

By

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Abstract

Cannabinoids produce ocular hypotension via activation of cannabinoid receptor type 1 (CB1). Adrenergic receptors (ARs) may contribute to this effect. Recently de-orphaned receptors, GPR55 and GPR18, are activated by some cannabinoids, but their role in IOP modulation is unknown. This research examined CB1-mediated IOP reduction by cannabinoids and tested whether ligands that activate GPR55 and GPR18 reduce IOP. Measurement of IOP was made using rebound tonometry in wild-type (WT) mice, and mice lacking β ARs, GPR55, or cannabinoid receptors. CB1 or β AR knock-out, or β AR desensitization and catecholamine depletion in WT mice eliminated IOP reduction by cannabinoids. CB2 and GPR55 activation in WT mice failed to reduce IOP. Activation of GPR18 by selective and non-selective agonists reduced IOP and the GPR55/GPR18 antagonist, 0-1918, blocked this effect. These findings suggest that cannabinoids reduce IOP via a CB1-mediated reduction in catecholamine release and that GPR18 activation reduces IOP independent of CB1/CB.

List of Abbreviations Used

Δ^9 -THC	Δ 9-tetrahydrocannabinol
2-AG	2-arachidonyl glycerol
Abn-CBD	Abnormal cannabidiol
AEA	N-arachidonylethanolamine /anandamide
AH	Aqueous Humour
β -AR	β -Adrenergic receptor
β 1AR	β -Adrenergic receptor type 1
β 2AR	β -Adrenergic receptor type 2
β -AR ^{-/-}	β -Adrenergic receptor type 1 and 2 null mice
BID	Twice Daily
C. sativa	Cannabis sativa
C57	C57BL/6N strain of mice
CAI	Carbonic Anhydrase Inhibitor
CB1 ^{-/-}	Mice lacking Cannabinoid receptor type 1
CB1	Cannabinoid receptor type 1
CB2 ^{-/-}	Mice lacking Cannabinoid receptor type 2
CB2	Cannabinoid receptor type 2
COX-2	Cooxygenase-2
CP	CP 55,940
DAG	Diacylglycerol
DGL α	Diacylglycerol lipase α
DGL β	diacylglycerol lipases β
ECS	Endocannabinoid system
eCB	endogenous ligands AEA and 2-AG
GPCR	G-protein coupled receptor

GPR55 ^{-/-}	Mice lacking GPR55
IOP	Intraocular pressure
ISO	Isoproterenol
LPI	Lysophosphatidylinositol
MAP	Mitogen activated kinase
MGL	Monoglycerol lipase
NAGly	<i>N</i> -Arachidony glycine
NAPE-PLD	<i>N</i> -acyl phosphatidylethanolamine phospholipase D
NAPE	<i>N</i> -acyl phosphatidylethanolamine
NAT	<i>N</i> -acyltransferase
NE	Norepinephrine
NT	Neurotransmitter
PE	phosphatidylethanolamine
PLD	phospholipase D
qPCR	quantitative polymerase chain reaction
RGC	Retinal ganglion cell
TID	Three times a day
TRPV1	Transient receptor potential vanilloid 1 cation channel
WIN	WIN 55212-2
WT	Wild-Type Mice

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Chapter 1 : Introduction

1.1: Historical Overview of Marijuana and Cannabinoids

Marijuana (*Cannabis sativa*) is known for its therapeutic applications and illicit abuse of its well-established psychoactive properties. Ingestion and inhaling of *C. sativa* causes euphoria, sedation, reduced cognitive function, increase in appetite and motor incoordination (Howlett et al., 2004). The first therapeutic descriptions of *cannabis* use date back 5000 years ago in Ancient China, and by the latter half of the 19th century *cannabis* preparations were widely used and prescribed for range of conditions including: muscle spasms associated with tetanus, insomnia, dysmenorrhea, gonorrhoea, migraine, and rheumatic disease (Mikuriya, 1969; Hanuš, 2009). The rise of opiates as a therapeutic analgesic and an increase in recreational misuse of *cannabis* led to it being removed from the national formulary and pharmacopedia in the United States in 1941 (Mikuriya, 1969).

The chemical structures of the components of *C. sativa* that mediate the pharmacological effects of *cannabis* did not start emerging until the late half of the 19th century with the identification and isolation of cannabidiol, a psychoactive component of *C. sativa* (Di Marzo, 2006; Elsohly & Slade, 2005; Wood et al., 1896). Δ^9 -tetrahydrocannabinol (Δ^9 -THC), the most widely known psychoactive cannabinoid, was not isolated until 1964 (Gaoni & Mechoulam, 1964).

The identification of Δ^9 -THC led to a number of discoveries that identified how these bioactive phytocannabinoids of *C. sativa* mediate their therapeutic and

psychoactive effects (Pertwee et al., 2010). These included the identification of receptors that bind phytocannabinoids and development of synthetic cannabinoids, as well as the existence of an endogenous endocannabinoid system (see Section 1.2). Despite this knowledge, the use of cannabis-based medicines, natural or synthetic, still remains limited with only about a handful of cannabinoids approved for use worldwide (Gaoni & Mechoulam, 1964; Pertwee et al., 2010; Yazulla, 2008).

1.2: Cannabinoid System

1.2.A. Cannabinoid Receptors

The phytocannabinoid Δ^9 -THC was initially thought to mediate psychotropic and medicinal effects of *C. sativa* by directly interacting with plasma membranes (Mikuriya, 1969). However, there is now evidence that the effects of Δ^9 -THC are due to the activation of specific G-protein coupled receptors (GPCR) known as cannabinoid receptor type 1 (CB1) and cannabinoid receptor type 2 (CB2) (Howlett, 1985; Matsuda, et al. 1990; Mikuriya, 1969; Munro, et al., 1993). The expression pattern and physiologic function differ between these two cannabinoid receptors and these differences may allow for unique pharmacological targets. To-date, accumulated evidence indicates that cannabinoid receptors can be activated by plant-derived cannabinoids as well as a variety of diverse synthetic analogs and endogenous ligands (Mackie, 2008; Pertwee, 2012).

CB1 receptors are localized on presynaptic neurons throughout the central nervous system (CNS)(Glass et al., 1997; Herkenham et al., 1991; Moldrich & Wenger, 2000; Pertwee, 2006). CB1 has an abundant expression throughout the

brain in cortical areas, the limbic system, and sub-cortical areas (Howlett et al., 2007). Consistent with this expression pattern, it is accepted that most of the psychotropic actions of *C. sativa*, and more specifically Δ^9 -THC, are mediated through the activation of the CB1 receptor in the CNS (Pertwee et al., 2010). The psychotropic action of CB1 was further validated when Huestis and colleagues (2001) found that ingestion of a CB1 antagonist blocked subjective reports of psychoactivity reported by participants after smoking marijuana (Huestis et al., 2001). Cannabinoid effects including, hypotension, analgesia, and psychomotor activity, were also determined to be CB1 mediated; cannabinoid agonists administered intravenously to mice lacking the CB1 receptor failed to show an effect (Ledent et al., 1999).

CB1 is also present in several organ systems outside the CNS including: cardiac vasculature, lung, bladder adrenal glands, immunological tissue, adipose tissue, liver and in the eye (Cota et al., 2003; Herkenham et al., 1991; Stella, 2010; Straiker et al., 1999a; Straiker et al., 1999b).

Three years after the identification of CB1, CB2 was identified and cloned (Munro et al., 1993). CB2 was located principally throughout the immune system but was also found in selective areas of the CNS (Galiègue et al., 1995; Munro et al., 1993; Pertwee, 2006). CB2 regulates cell migration, cytokine production and antigen presentation (Mackie et al., 1993; Mackie et al., 1995; Miller & Stella, 2008). There is an abundant expression of CB2 in tissues such as the spleen, thymus gland, macrophages, B-cells and T-cells. Given this receptor's distribution, CB2 is a potential research target for novel anti-inflammatory therapeutics, both

systemically and in the eye (Lehmann et al., 2012; Pertwee, 2005; Stella, 2010; Toguri et al., 2014).

CB1 and CB2 receptors belong to the Rhodopsin Class A group of GPCR's. Their activation results in modulation of many G protein coupled signaling pathways and proteins including, ion channels, protein kinases, and various enzymes (Mackie & Stella, 2006; Reviewed by Stella, 2010). Both CB1 and CB2 couple predominately to inhibitory $G_{i/o}$ proteins, resulting in inhibition of adenylyl cyclase, decreases in cyclic AMP and activation of MAP kinase pathways (Demuth & Molleman, 2007).

CB1 modulates cell signal transmission by inhibiting presynaptic N, and P/Q-type calcium channels and increasing inwardly rectifying potassium current, with resultant inhibition of neurotransmitter (NT) release (Twitchell et al., 1997). CB1-mediated alteration of neurotransmission has been well-documented with respect to NT such as: GABA, glutamate, noradrenaline, dopamine, serotonin and acetylcholine (Cheer et al., 1999; Fitzgerald, Shobin, & Pickel, 2012; Jennings et al., 2001; Oz, et al., 2014; Pakdeechote, et al., 2007; Schultheiß, et al., 2005; Vaughan et al., 1999). Through modulating NT release, CB1 receptors are capable of affecting multiple homeostatic systems including bone formation and intraocular pressure (IOP) (Hosseini et al., 2006; Oltmanns et al., 2008; Tam et al., 2008). Selective activation of peripheral CB1 or activation of CB2, is devoid of psychoactivity but has many potential therapeutic actions, including immunosuppression and analgesia. This suggests that peripherally restricted cannabinoid drugs or localized cannabinoid receptor activation may have

therapeutic utility in the absence of behavioral effects (Green, 1998; Yang et al., 2012).

1.2.B. Endocannabinoid System

The endocannabinoid system (ECS) encompasses known cannabinoid receptors, endogenous ligands known as endocannabinoids (eCBs) and the enzymes required for their synthesis and degradation. In addition, several cannabimimetic ligands have been described that may be part of the ECS, and display similar effects to cannabinoids, including a decrease in IOP, yet do not bind to CB1 or CB2 but instead activate distinct receptors (see Section 1.4) (Ho & Hiley, 2003; Qiao et al., 2012; Szczesniak, et al, 2011).

To date several eCBs have been isolated, with most research focus being on N-arachidonylethanolamine or anandamide (AEA) and 2-arachidonyl glycerol, (2-AG) (Mackie et al., 1993; Mechoulam et al., 1995; Stella, 1997). AEA and 2-AG were discovered in the early 1990's after identification of the cannabinoid receptors and both activate CB1 and CB2 receptors (Devane et al., 1992; Mechoulam et al., 1995; Pertwee et al., 2010). In addition, AEA is capable of activating non-cannabinoid receptors, including the transient receptor potential vanilloid 1 (cation) channel (TRPV1) and other targets (see Section 1.4) (Al-Hayani et al., 2001; Begg et al., 2007; Gertsch et al.,2010; Kreitzer & Stella, 2009).

AEA and 2-AG are highly lipophilic and are not stored in vesicles(Pertwee et al., 2010). eCBs are synthesized from arachidonic acid and released on demand from the post-synaptic cell in response to increases in intracellular calcium. They act in a

retrograde manner on the presynaptic cell to inhibit NT release through negative feedback (Figure 1.1)(Kano et al., 2009).

Synthesis of these eCB's occurs via 2 complex and distinct pathways. Briefly, arachidonic acid containing diacylglycerol (DAG) is hydrolyzed by diacylglycerol lipases, DGL α and DGL β , to produce 2-AG (Yazulla, 2008). AEA can be synthesized via a 2-step pathway whereby calcium-dependent N-acyltransferases (NAT), namely N-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD), move the *sn*-1 acyl chain of a phospholipid onto the amine of phosphatidylethanolamine (PE) creating N-acyl PE (NAPE). Secondly NAPE is hydrolyzed by phospholipase D (PLD) into AEA and phosphatidic acid (Hu et al., 2010; Leung, et al.,2006).

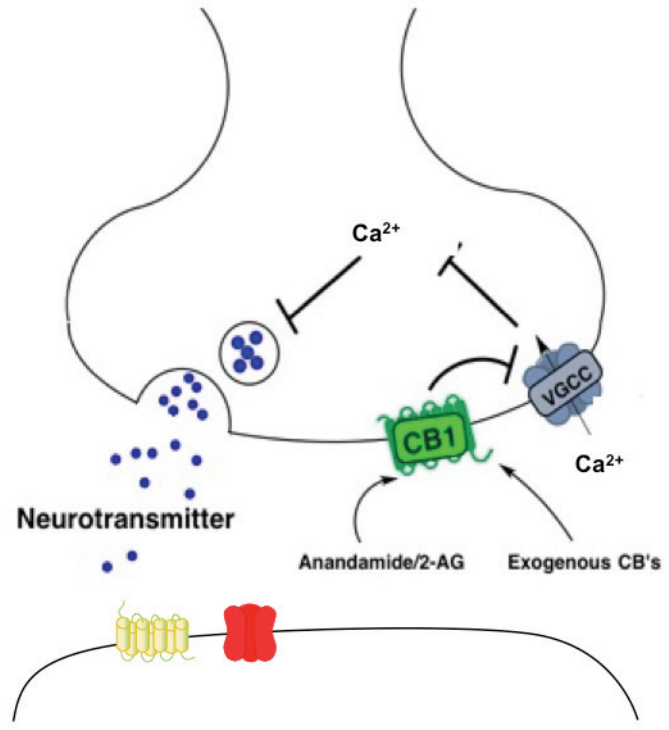
Degradation of AEA is carried out by uptake into the post-synaptic cell, where the catabolic enzymes fatty acid amide hydrolase (FAAH) or, to a lesser extent, cyclooxygenase-2 (COX-2), breaks AEA down into arachidonic acid and ethanolamine (Cravatt et al., 1996; Howlett et al., 2002; Nucci et al., 2008). 2-AG is metabolized primarily by monoacylglycerol lipase (MGL) but can be degraded by COX-2 and FAAH into arachidonic acid and glycerol (Pertwee et al., 2010; Yazulla, 2008).

The ECS has been identified in neural and non-neural tissues and is capable of mediating a wide range of physiological and pathological processes in major organ systems including: the heart (Bátkai & Pacher, 2009), liver (Kunos & Osei-Hyiaman, 2008), reproductive (Meccariello et al., 2014) and digestive systems, (Sharkey et al., 2014), immune system (Chiurchiù et al., 2015; Lehmann et al., 2012) and the eye (Chen et al., 2005; Toguri et al., 2014; Yazulla, 2008).

Figure 1.1: *CB1 mediated mechanism of inhibition of neurotransmitter release.*

Endocannabinoids are released post-synaptically by passive and/or facilitated diffusion. AEA and 2-AG are synthesized by N-acyltransferase (NAT) or diacylglycerol lipase, respectively, in response to depolarization via calcium influx by various mechanisms. Acting in a retrograde manner, AEA and 2-AG, or exogenous cannabinoids, activate CB1, which inhibits subsequent calcium-dependent neurotransmitter release by inhibition of voltage-dependent calcium channels.

Modified from Hudson, B. D. (2010). Interactions between the cannabinoid and the β -Adrenergic Systems: Receptor Heterodimerization, Signalling Interactions and the Regulation of Intraocular Pressure. *ProQuest Dissertations & Theses Full Text*, 310.



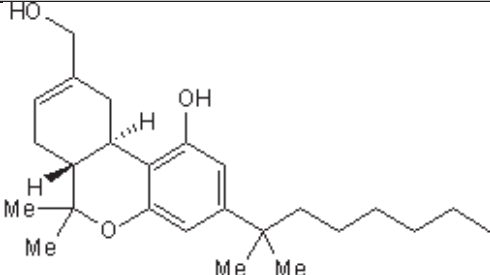
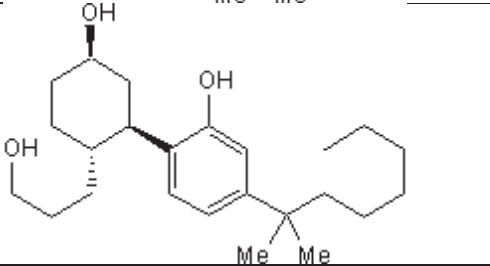
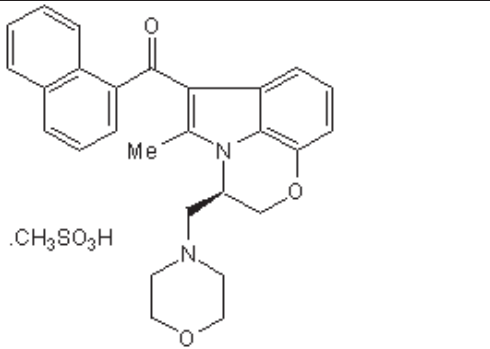
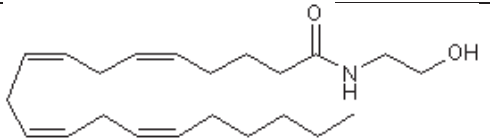
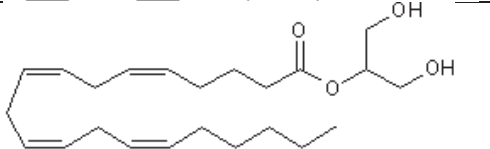
1.2.C. Ligands

Δ^9 -THC is a major component of *C. sativa*. However, there are >480 bioactive components that exist in the cannabis plant and about 60 compounds that have been classified as cannabinoids (Hanuš, 2009; Hosseini et al., 2006; Tomida et al., 2004). Besides natural plant cannabinoids or phytocannabinoids, various synthetic cannabinoids have been synthesized and can activate or block CB1 and CB2, in addition to non-cannabinoid receptors (Howlett et al., 2004).

Cannabinoid ligands are structurally classified into four groups: classical, non-classical, aminoalkylindole and eicosanoid (Pertwee, 2006) (Table 1.1). Δ^9 -THC and CBD as with other plant derived or synthetic analogues belongs to the classical group. Non-classical cannabinoids are structurally similar to plant derived cannabinoids, but lack the dihydropyran ring and include the potent CB1/CB2 agonist CP 55,940 (CP) (Pertwee, 2006).

Synthetic cannabinoids belonging to non-classical and aminoalkylindole groups were developed by the pharmaceutical industry as non-opioid and non-NSAID analgesics but were never pursued to market. These synthetic cannabinoids have been valuable research tools in understanding the ECS (Howlett et al., 2002; Howlett et al., 2004). Lastly, the eicosanoid group is a group of endogenous ligands or eCBs of which, AEA and 2-AG were the first to be identified and, to-date, have been the most heavily researched (Mackie et al., 1993; Mechoulam et al., 1995; Pertwee, 2006; Stella et al., 1997).

Table 1.1: Different classes of cannabinoid ligands and prototypical examples

Class	Chemical Structure	Prototypical Example
Classical		HU-210
Non-Classical		CP55,940 (CP)
Aminoalkylindole		WIN55212-2 (WIN)
Eicosanoid		Anandamide
		2-Arachidonyl Glycerol

Cannabinoids have different activity profiles for CB1 and CB2 and can either selectively or non-selectively activate/block cannabinoid receptors. For example, the aminoalkylindole, WIN, and the non-classical cannabinoid, CP, can activate both CB1 and CB2, but are slightly more effective at CB1. This also holds true for the eCBs, AEA and 2-AG; AEA, like Δ^9 -THC, shows partial agonist activity at both CB1 and CB2 receptors, while 2-AG has full agonist activity at both cannabinoid receptors (Pertwee et al., 2010). A list of commonly used cannabinoid ligands in cannabinoid research is outlined in Table 1.2. (Reviewed by Pertwee et al. (2010).

1.2.D. Ocular Endocannabinoid System

The eye is one organ that could allow for local targeting of the cannabinoid system; topical application or intraocular injection of cannabinoids would result in reduced systemic absorption and minimal CNS exposure (Green, 1998). Ocular distribution of CB1 receptors are located in all major eye structures (reviewed by Yazulla, 2008). Throughout the eye, CB1 has been identified in the ciliary body, trabecular meshwork, iris and cornea, and in retinal tissue (Porcella et al., 2000; Straiker et al., 1999a; Straiker et al., 1999b). The abundant expression of CB1 in tissues that are involved with aqueous humour (AH) secretion and outflow indicates a potential role in modulating IOP (Porcella et al., 2000; Straiker et al., 1999; Yazulla, 2008). Additionally, enzymes required for 2-AG synthesis, DGL α and DGL β , and degradation, namely MAGL and FAAH, have been located in the retina (Hu et al., 2010). Further, in the case of AEA, several AEA precursors, including PE, have

Table 1.2: Common Cannabinoid ligands used in research that interact with CB1 or CB2 Receptors (Reviewed by Pertwee et al., 2010).

Ligand	Action
CB1 Selective ligands	
(R)-(+)-Methanandamide	Agonist
ACEA	Agonist
SR141716A	Antagonist
AM251	Antagonist
AM281	Antagonist
CB2 Selective ligands	
HU308	Agonist
AM630	Antagonist
Nonselective CB1 and CB2 Ligands	
WIN55212-2	Agonist
CP55940	Agonist
Δ^9 -THC	Agonist
Endogenous Ligands	
2AG	Agonist
AEA	Agonist

been identified in retinal tissue in addition to FAAH, the enzyme dominantly responsible for the breakdown of AEA (Bisogno et al., 1999; Hu et al., 2010). Identification of CB1 and CB2, in addition to eCBs and respective enzymes for synthesis and degradation, provide support for a local ECS system in the eye (Bisogno et al., 1999; Chen et al., 2005; Nucci et al., 2008; Stamer et al., 2001; Yazulla et al., 1999).

1.3: Non- Cannabinoid Receptors & Cannabinoid Ligands

It is now accepted that cannabinoids, both exogenous and endogenous, can activate either CB1 or CB2 receptors or both. However, cannabinoid ligands are often described as promiscuous due to their ability to activate not only CB1 and CB2 but also non-cannabinoid receptors as well as orphan GPCRS that have yet to be classified (Pertwee et al., 2010). For example, the TRPV1 cation channel can be activated by cannabinoids (Pertwee et al., 2010; Smart et al., 2000; Yazulla, 2008) such as AEA, WIN, THC, and cannabidiol (CBD), while CP fails to activate TRPV1 (Kreitzer & Stella, 2009; Pertwee & Ross, 2002; Stella, 2010).

Beyond TRPV1, there is a growing body of evidence that supports the existence of additional novel non-CB1 and CB2 receptors that can also bind some cannabinoids and endocannabinoids and these are frequently referred to as cannabinoid-associated or cannabinoid-related receptors (Begg et al., 2007; McHugh et al., 2012)). Further, the use of transgenic mice lacking cannabinoid receptors and selective CB1 and CB2 receptor antagonists has highlighted additional not yet identified targets of cannabinoids (Begg et al., 2007; Kreitzer & Stella, 2009;

Pate et al., 1995). Specifically, AEA causes CB1-like vasodilation in the presence of the CB1 and CB2 selective antagonists and in transgenic mice lacking both cannabinoid receptors and only suprathreshold doses of the CB1 receptor antagonist, SR141716A were able to block this effect (Begg et al., 2007; Járai et al., 1999, 2007; White et al., 2001). The putative cannabinoid receptor site is suggested to be vascular. In addition to AEA, the cannabidiol derivative, abnormal cannabidiol (Abn-CBD) mimics the effects of AEA at this site. The lack of binding of Abn-CBD to CB1 or CB2 has led to the classification of Abn-CBD as an atypical cannabinoid. In the vasculature, the vasodilation produced by AEA, and the atypical cannabinoid, Abn-CBD, occurs independent of CB1 receptors, and is endothelium-dependent and pertussis-toxin sensitive, suggesting a distinct cannabinoid-like vascular GPCR target (Offertáler et al., 2003).

Several candidates for this putative cannabinoid receptor have been presented in the literature, of particular interest are the recently de-orphanized GPCRs, GPR55 and GPR18 (Reviewed by Pertwee et al., 2010). Demonstration of novel, cannabinoid-related receptor(s) has renewed interest in the therapeutic properties of ECS modulation, and in particular the promise of development of cannabimimetic ligands that might engage receptor targets distinct to CB1, thus circumventing CNS side-effects.

1.4: GPR55 & GPR18

1.4.A. GPR55

A significant amount of research has been pursued on GPR55 as a candidate cannabinoid receptor, which was first isolated in 1998 by Swardargo and colleagues (1999), GPR55 ligands include endogenous lipids, lysophosphatidylinositol (LPI), synthetic compounds, O-1602 and abnormal cannabidiol (Abn-CBD), that are structurally related to cannabinoids, as well as cannabinoids that bind to both CB1 and CB2, including THC (Table 1.3) (Hiley & Kaup, 2007; Schuelert & McDougall, 2011),

In addition to eCBs, AEA and the AEA derivative, methanadamide, and the synthetic agonist, CP, also activate GPR55 (Kreitzer & Stella, 2009; Oka et al., 2007; Pertwee, 2007; Pertwee et al., 2010; Ryberg et al., 2007; Sawzdargo et al., 1999; Sharir & Abood, 2010).

Furthermore, there are emerging classes of novel ligands that display cannabimimetic activity at GPR55 and either have no or low affinity for CB1 and CB2 (Begg et al., 2007; Kreitzer & Stella, 2009). One class, of what is now termed atypical cannabinoids, includes several ligands outlined in Table 1.3. Of particular interest is Abn-CBD, a synthetic analog of cannabidiol, which does not activate CB1 but, like AEA, does activate non-cannabinoid and non-TRPV1 receptors in endothelial cells of blood vessels (Járai et al., 2007; Ryberg et al., 2007; Wagner et al., 1999). Abn-CBD produces systemic hypotension and analgesia devoid of psychoactive side effects, unlike CB1 agonist activation (Adams et al., 1977; Begg et al., 2007; Huestis et al., 2001).

Table 1.3: Cannabimimetic ligands that have activity at GPR55 and GPR18

Ligand	Action	Receptor Target	Reference
GPR55 Ligands			
O-1602	Agonist	GPR55	Ashton et al., (2012); Console-Bram (2014);McHugh et al. (2012)
Lysophosphatidylinositol (LPI)	Agonist	GPR55	Oka et al., (2007)
GPR18 Ligands			
N-Arachidonyl glycine (NAGly)	Agonist	GPR18	Kohono et al., (2006); McHugh et al., (2011);McHugh et al. (2012)
Non-selective GPR55 & GPR18 Ligands			
Abnormal Cannabidiol (Abn-CBD)	Agonist	GPR55/GPR18	McHigh et al., (2010); McHugh et al. (2012)
$\Delta(9)$ -THC	Agonist	GPR18/CB1/CB2	McHugh et al. (2012)
AEA	Agonist	GPR18/CB1/CB2	McHugh et al. (2012)
CP55,940	Agonist	GPR55/GPR18	Console-Bram et al. (2014)
O-1918	Antagonist	GPR55/GPR18/CB1/CB2	Rhyburg et al., (2007)

The hypotensive effect of Abn-CBD is not mediated through CB1 or CB2, but is partly antagonized by the CB1 selective cannabinoid antagonist, SR141716A, at suprathreshold concentrations for CB1 block (Ho & Hiley, 2003; Járai et al., 1999, 2007). The vasodilation effects of AEA and Abn-CBD were initially thought to be mediated through GPR55 (Brown et al., 2009; Ryberg et al., 2007). However, other studies utilizing GPR55 null mice have shown that although Abn-CBD and 0-1602 are capable of activating GPR55, this receptor does not appear to be involved in mediating the vasodilation effects (Johns et al., 2007; Kreitzer & Stella, 2009; Staton et al., 2008). The effect of Abn-CBD is mediated via a pertussis-toxin-sensitive $G_{i/o}$ signaling pathway and cannot be blocked by known CB1 antagonists, AM251, or CB2 antagonists, SR144528 and AM630, but is abolished by 0-1918, an analogue of Abn-CBD (Ho & Hiley, 2003; Kreitzer & Stella, 2009). 0-1918 is a non-selective antagonist for both GPR55 and GPR18, suggesting that Abn-CBD-mediated hypotensive actions may be mediated by GPR18 (Bondarenko, 2014; Ho & Hiley, 2003; McHugh et al., 2012; Szczesniak et al., 2011).

1.4. B: GPR18 and Cannabimimetic Lipids

In comparison to GPR55, very little research has explored GPR18 as a potential cannabinoid receptor. GPR18 was identified using polymerase chain reaction and genomic library screening by Gantz and colleagues (1997), and cloned in 2006 (Gantz et al., 1997; Kohno et al., 2006; Pertwee et al., 2010). GPR18 has been localized in neuronal and immunological tissues such as: spinal cord, testis, small intestine, cerebellum, spleen, bone marrow, thymus, lung, and the eye (Caldwell et al., 2013; Gantz et al., 1997; McHugh et al., 2012; McHugh et al., 2010; Regard et

al.,2008). Activation of GPR18 drives directed cell migration, proliferation, and MAP kinase activation (Kohno et al., 2006; McHugh et al., 2012b; McHugh et al., 2012; McHugh et al., 2010).

Several studies have indicated that the endocannabinoid AEA, and the phytocannabinoid, Δ 9-THC, are capable of activating GPR18, suggesting that GPR18 may be a novel cannabinoid receptor. Furthermore, N-arachidonoyl glycine (NAGly), a cannabimimetic lipid, is considered a ligand for GPR18 and is produced throughout the body and found in the eye (Huang et al., 2001; Bradshaw et al., 2009). NAGly is a metabolite of AEA and can be produced by the conjunction of arachidonic acid and glycerol via FAAH, which hydrolyzes AEA into arachidonic acid and glycine (Bradshaw et al., 2009). NAGly is capable of producing anti-inflammatory and anti-nociceptive effects through GPR18 and may also additionally act at other non-GPR18 targets (Bradshaw et al.,2009; Console-Bram et al.,2014; Hanuš et al., 2014; McHugh, et al., 2012; Pertwee et al., 2010).

There is some debate in the literature as to whether NAGly is the endogenous ligand for GPR18. For example, a study by Lu and colleagues (2013) was not able to demonstrate GPR18 activation by NAGly, Abn-CBD, or AEA using *in vitro* assays (Hanuš et al., 2014; Lu et al.,2013). This discrepancy could be the result of inter-species and tissue differences, as several other studies support GPR18 activation by NAGly (Bradshaw et al., 2009; Huang et al., 2001; McHugh et al., 2012a; McHugh et al., 2012b; McHugh et al., 2010).

Evidence also implicates Abn-CBD, a ligand for GPR55, as an agonist at GPR18 (McHugh et al., 2012). This suggests that the vasodilation and hypotensive

effects reported for Abn-CBD, both systemically and at the ocular level, could be mediated by GPR18 (Ho & Hiley, 2003; Szczesniak et al., 2011). Therefore, it follows that selective GPR18 activation by NAGly could produce systemic and ocular hypotension.

1.5 Cannabinoid-Adrenergic Interactions

The endocannabinoid system is capable of modulating other neurochemical systems. In addition to the well-documented ability of certain cannabinoid ligands to activate TRPV1, CB1 receptors can interact with other GPCR's and modify their activity (Reviewed by Mackie, 2005). CB1 can physically interact with dopamine D2 receptors and μ -, κ - and δ -opioid receptors (Bushlin et al., 2012; Katia, 2015; Mackie, 2005). CB1 also has physical and functional interactions with β 1 and β 2-adrenergic receptors (Hudson et al., 2010; Hudson, 2010).

There are two major types of adrenoreceptors (AR), which are further subclassified into: α (α 1, α 2), and β (β 1, β 2, β 3) (Reviewed by Bylund et al., 1994). As with CB1 and CB2, adrenergic receptors belong to the Class A rhodopsin family of GPCR's, and are distributed throughout the body, including the eye (Bylund et al., 1994; McAuliffe-Curtin & Buckley, 1989; Wax & Molinoff, 1987). β -AR couple primarily through G_s , but also $G_{i/o}$, transduction pathways. β 1 and β 2 receptor activation stimulates adenylate cyclase, leading to increases in cAMP, activation of protein kinase A, phosphorylation of target cell proteins and activation of L-type calcium channels (Chen-Izu et al., 2000; Li, De Godoy, & Rattan, 2004; Stiles et al., 1984).

β -receptors are commonly targeted by therapeutics for cardiopulmonary disorders such as asthma, chronic obstructive pulmonary disease, chronic heart failure, hypertension, cardiac arrhythmias (Azuma & Nonen, 2009; Malerba et al., 2015; Poirier & Tobe, 2014; Spina, 2014; Tobe, 2014). Adrenergic receptors are also ocular therapeutic targets in the treatment of glaucoma, a blinding eye disease for which elevated IOP is a risk factor (Coleman, 1999; Nemesure et al., 2007; Willis, 2004).

In the ciliary body of the eye, there is abundant adrenergic innervation mediated via β and α receptors (Gabelt & Kaufman, 2011); β_2 receptor activation enhances aqueous humor formation, a fluid that maintains shape and refractive properties of the eye (Goel et al., 2010). In contrast, α_2 -receptor activation inhibits aqueous humor formation. Drugs targeting these two adrenergic receptors are first line therapeutics for glaucoma, where modification IOP is warranted (see Section 1.6-1.7). (Marquis & Whitson, 2005). CB1 and specifically β_1/β_2 receptors are co-expressed in the ciliary epithelium and evidence suggests functional interactions between these receptors (Hudson et al., 2010; Jampel et al., 1987; Stamer et al., 2001; Straiker et al., 1999; Wax & Molinoff, 1987). Cannabinoid-adrenergic interactions in the eye are of interest given the role of adrenergic receptors in maintaining the equilibrium of AH secretion and outflow (Jampel et al., 1987; McAuliffe-Curtin & Buckley, 1989; Yoshitomi & Gregory, 1991).

Marijuana smoking and CB1 agonists have been well established to lower IOP when administered in isolation or in combination with β -AR antagonists (Green, 1998; Hosseini et al., 2006; Oltmanns et al., 2008). However, when the

administration of cannabinoid and adrenergic ligands occurs concurrently, no additive effect is observed suggesting possible overlapping mechanisms of lowering IOP (Green et al., 1977; Oltmanns et al., 2008). Further evidence for cannabinoid-adrenergic interactions has come from studies that have abrogated adrenergic input through sympathectomy e.g. surgical ciliary ganglionectomy, or chemical ganglionic blocking agents. In these studies cannabinoid agonists, specifically Δ^9 -THC, have a reduced effect on lowering IOP (Green & Kim, 1976; Green et al., 1977). This effect may be species dependent because it was not observed in cats (Colasanti & Powell, 1985). Furthermore, Hudson (2010) found that the cannabinoid agonist, WIN, failed to reduce IOP in mice genetically lacking $\beta 1/\beta 2$ or CB1 receptors, and, in CB1 null mice, WIN actually caused an increase in IOP. This suggests additional targets for WIN besides CB1 and CB2.

Overall the interactions between CB1 and adrenergic systems appear to be complex and could be species dependent or ligand specific. Whether or not these findings are strain specific or isolated to WIN55212-2 remain unknown.

1.6. Aqueous Humor Dynamics

Intraocular pressure is maintained by AH, which has a low viscosity. AH serves several vital functions: (1) to nourish the avascular structures of the eye, which include the cornea and lens. (2) To remove metabolic waste from the anterior portion of the eye. (3) To maintains the shape of the globe, and (4) To retains a refractive index, and helps refract light in the eye (Goel et al., 2010).

AH is secreted by non-pigmented cells of the bi-layered ciliary epithelium lining the ciliary processes of the ciliary body in the posterior chamber of the eye.

The AH secreted by these cells surrounds the lens and enters the anterior chamber through the pupil. There are two outflow pathways that regulate IOP: (1) the conventional trabecular meshwork pathway (Tamm, 2009), and (2) the unconventional uveoscleral pathway (Alm & Nilsson, 2009) (Figure 1.2).

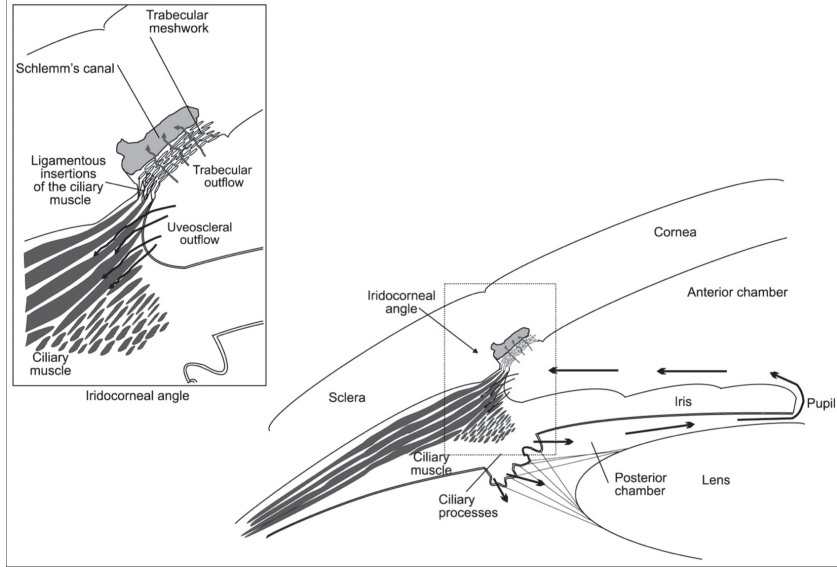
Through the conventional pathway, AH exits through the trabecular meshwork and into Schlemm's canal (SC). SC is surrounded by connective tissue and is composed of several collector channels that are connected to episcleral and conjunctival veins. AH exits by passive flow from these collector channels into aqueous veins and finally into the episcleral veins (Goel et al., 2010; Tamm, 2009).

In the uveoscleral pathway or the unconventional route, AH exits the eye through the extracellular spaces between muscle bundles in the ciliary muscle, through suprachoroidal space and out through the sclera (Alm & Nilsson, 2009). This pathway is a secondary pathway in humans, accounting for approximately 20% of outflow, but there are variations between 5-40% depending on species (Goel et al., 2010). Additionally, there is a strong effect of age on outflow due to an increase in extracellular fibrous connective tissue, which decreases outflow (Alm & Nilsson, 2009; Beltran-Agullo et al., 2011; Goel et al., 2010).

1.6: Glaucoma

Glaucoma is an optic neuropathy disorder with a multifactorial etiology characterized by loss of retinal ganglion cells, loss of axons in the optic nerve, and thinning of the retinal nerve fiber layer in the eye (Goel et al., 2010; Quigley, 2011). Once damaged, these cells cause loss of the peripheral visual field. Visual defects

Figure 1.2: *Schematic Representation of Aqueous Humour formation and Outflow.* AH is secreted by non-pigmented cells in the ciliary epithelium located in the posterior chamber of the eye. The fluid then travels around the lens and enters the anterior chamber of the eye through the pupil. AH serves many vital functions in the eye including: providing nutrients to the avascular structures of the eye, removes waste, maintains shape of the globe and aids in the refraction of light. Reprinted from: Llobet, Gasull, & Gual (2003). Understanding trabecular meshwork physiology: a key to the control of intraocular pressure? *News in Physiological Sciences : An International Journal of Physiology Produced Jointly by the International Union of Physiological Sciences and the American Physiological Society*, 18, 205–209.
doi:10.1152/nips.01443.2003



include restrictive fields, arcuate scotomas, nasal steps, depending on where the lesion occurs (Drance, 1972).

As a disease that has an average age of onset after 60 years, longer life expectancies and aging populations will increase the number of patients worldwide with glaucoma from 60.5 million in 2010 to 79.6 million in 2020 (Bagnis, Papadia, Scotto, & Traverso, 2011; Quigley & Broman, 2006; Harry. Quigley, 2011). The number of cases that will involve bilateral blindness will reach approximately 5 million people by 2020 (Quigley & Broman, 2006).

The two categories of primary glaucoma are closed angle glaucoma and open angle glaucoma (POAG). Angle closure glaucoma is a relatively asymptomatic disease characterized by: peripheral anterior synichiae, high IOP and decreased vision, while remaining asymptomatic (Emanuel et al., 2014; Quigley, 2011; Sihota, 2011). Risk factors for developing angle-closure glaucoma include having a small eye, small anterior chamber and a large lens (Quigley, 2011). Diagnosis of a narrow angle can be made via gonioscopy where the iris blocks 50% of the trabecular meshwork in 360° angle viewed (Friedman et al., 2012). Additionally, acute angle-closure crises can occur with a sudden rise in IOP, pain, corneal edema, decreased vision, vascular congestion, and a mid- dilated pupil(Emanuel et al., 2014; Sihota, 2011).

POAG is characterized by a slow painless loss of vision, which can occur unilateral or bilateral over months to years. Typically it occurs under asymmetric bilateral conditions. Risk factors for developing open-angle glaucoma include age,

axial myopia, thin central corneal thickness, family history of the disease, and elevated IOP (Chauhan et al., 2010; Distelhorst & Hughes, 2003; Nemesure et al., 2007; Quigley, 2011). IOP is the only factor for glaucoma that can be modified; however, its exact relation to the progression of the disease has been debated in recent literature (Chauhan et al., 2010; Nemesure et al., 2007; Quigley, 2011). The relationship between IOP and glaucoma is not a linear relationship as previously thought. It is now widely accepted that higher IOP is not necessarily a defining characteristic of glaucoma, which can be best illustrated by patients who continue to show progression of visual field loss despite well-controlled IOP (Chauhan et al., 2010; Marquis & Whitson, 2005; Nakazawa et al., 2012). Likewise, ocular hypertension (IOP >22 mmHg) is not synonymous with glaucoma unless signs such as damages to retinal nerve fiber layer and visual field pathologies co-exist. However, despite a multi-factorial etiology, medical treatment is still aimed at reducing IOP, even with pressure in the established normal range (IOP >22 mmHg), because IOP reduction is linked with slowed progression of the disease (Kass et al., 2002).

1.7: Medical therapy of glaucoma

The goal of medical therapy for glaucoma is to prevent any further loss of visual function by means of reducing IOP. Numerous pharmacotherapies exist for the treatment of glaucoma, that either reduce AH secretion, increase AH outflow or a combination of the two (Willis, 2004)(Table 1.4).

Table 1.4: Common ocular hypotensive medications used in glaucoma treatment

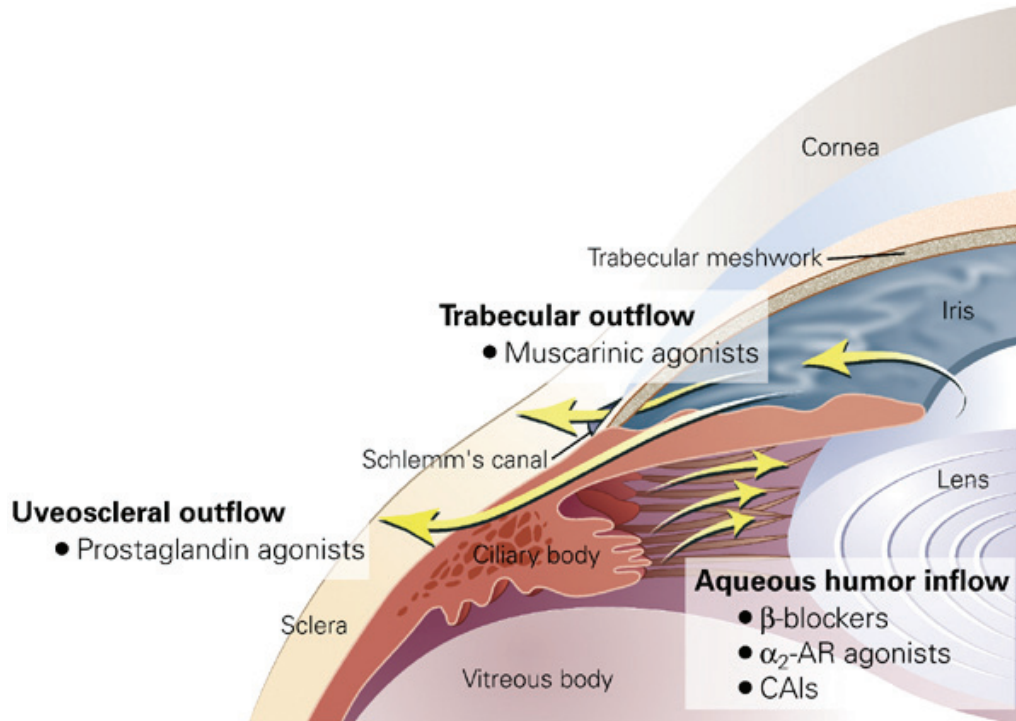
Drug Class	Effect of AH	Examples
β -Antagonists	Decrease Secretion	Timolol Levobrunolol Betaxolol (β 1 selective)
α 2-agonists	Decrease Secretion	Brimo idine Apraclonidine
Carbonic Anhydrase Inhibitors	Decrease Secretion	Dorzolamide Brinzolamide Acetazolamide
Cholinergic agents	Increase Trabecular Meshwork Outflow	Pilocarpine Carbachol Phospholine Iodide Physostigmine
Prostaglandin Analogues	Increase Uveoscleral Outflow	Latanoprost Travoprost Bimatoprost

Different classes of ocular hypotensive drugs target IOP by reducing AH secretion in the ciliary body or increasing outflow through conventional and uveoscleral pathways. With regards to AH outflow modulation, prostaglandin analogues increase outflow through the uveoscleral pathway, while cholinergic agents known as miotics increase outflow through the trabecular meshwork pathway (Figure 1.3) (Woodward & Gil, 2004).

Of agents that modulate inflow by influencing AH secretion, there are α and β -adrenergic drugs, and carbonic anhydrase inhibitors (CAI's) (Marquis & Whitson, 2005; Watanabe & Chiou, 1983). α and β -adrenergic receptor activation results in a decreased and increased AH secretion respectively (Gabelt & Kaufman, 2011). The β -AR antagonists, reduce AH secretion by blocking the effect of NE in the ciliary body and include timolol, which is considered to be the gold standard of treatment. β -AR antagonists are used either alone or in combination with other drug classes, unless medical history is positive for cardiorespiratory disease (Diggory et al., 1993; Marquis & Whitson, 2005; Tarkkanen et al., 2008). Additionally, due to the distinct mechanisms of actions between these drug classes, therapies can be co-administered to produce additive reductions in IOP (Holló et al., 2014). Despite the existence of different classes of drugs, glaucoma continues to be a challenging disease to treat from both clinical and pharmacotherapy approaches. POAG is characterized by a slow, painless loss of visual function, patients are not aware of the loss of visual field under binocular conditions (Quigley, 2005). This results in poor adherence to treatment and is linked to higher morbidity (Budenz, 2009; Okeke et al., 2009).

Figure 1.3: *Classes of Glaucoma Medications that target Aqueous Humour Dynamics.*

AH is maintained by the balance of inflow from the ciliary epithelium and outflow through trabecular meshwork or uveoscleral pathways. Different classes of ocular hypotensive actions target each of anatomical targets. Drugs that reduce AH secretion include β -Blockers, α 2-agonists, and carbonic anhydrase inhibitors (CAI's). Prostaglandin analogues are the only agents clinically used to increase outflow through the uveoscleral pathway, while cholinergic muscarinic agents, known as miotics, increase outflow through the trabecular meshwork. Reprinted by permission from Macmillan Publishers Ltd: McLaren & Moroi. (2003). Clinical implications of pharmacogenetics for glaucoma therapeutics. *The Pharmacogenomics Journal*, 3(4), 197–201. doi:10.1038/sj.tpj.6500181. ©2003. See appendix 1 for license.



1.8: Cannabinoids, Intraocular Pressure and Glaucoma

The therapeutic use of cannabinoids in the eye, particularly for the treatment of glaucoma, is an area that has attracted considerable attention since Hepler and Frank (1971) reported that smoking marijuana lowered IOP. The reported response to marijuana, however, was of short duration and was accompanied by a reduction in blood pressure and psychoactive side effects (Hepler & Frank, 1971).

Since the Hepler and Frank (1971) study, the ability of marijuana and various cannabinoid ligand preparations to reduce IOP has been extensively investigated in animal and clinical trials. Several different routes of administration of marijuana and cannabinoid ligands have been tested for their ocular hypotensive effects, these include: inhaled (Merritt et al., 1980; Merritt et al., 1981), oral (Merritt et al., 1981), sublingual (Tomida et al., 2006), intravenous (Purnell & Gregg, 1975), and topical routes (Jay & Green, 1983; Porcella et al., 2001). The observation of a cannabinoid-mediated reduction in IOP was initially variable, with some producing a significant decrease in IOP (Porcella et al., 2001; Tomida et al., 2006), while others not (Jay & Green, 1983). Those that did decrease IOP did so for a short duration or were accompanied by adverse systemic and ocular effects including cognitive and psychoactive side effects (Hepler & Frank, 1971; Huestis et al., 2001; Merritt et al., 1980; Tomida et al., 2006). Despite the early conflicting reports on the IOP reduction effect of cannabinoids, the consensus in the field accepts that cannabinoids through topical and systemic formulations are capable of reducing IOP (Hepler & Frank, 1971; Jay & Green, 1983; Merritt et al., 1980; Szczesniak et al., 2006; Tomida et al., 2004; Yazulla, 2008).

The reduction in IOP seen on exposure to cannabinoids was initially thought to be mediated through the CNS, as a direct result of systemic hypotension (Green et al., 1977; Hepler & Frank, 1971; Merritt et al., 1980). However, since the identification of an ocular ECS (see Section 1.2), it is now accepted that the IOP reducing effects of cannabinoids are mediated locally in the eye. The distribution of CB1 throughout the ciliary body and trabecular meshwork suggests that cannabinoids decrease IOP by decreasing AH secretion in the ciliary body and increasing outflow through the trabecular meshwork (Merritt et al., 1981; Oltmanns et al., 2008; Porcella et al., 2000; Stamer et al., 2001; Straiker et al., 1999a; Tomida et al., 2004; Yazulla, 2008). Furthermore, in addition to IOP maintenance, targeting the ECS could offer more complete treatment with additional neuroprotective effects (Yazulla, 2008). Increasing the level of eCB's in the eye, either directly with application of cannabinoid agonist such as WIN or indirectly by using FAAH inhibitors, has been shown to be neuroprotective and prevent RGC loss in rat models of optic nerve axotomy and acute rise in IOP induced ischemia (Pinar-Sueiro et al., 2013; Slusar et al., 2013).

Marijuana smoking, although effective at reducing IOP, is not advised due to toxic effects, short duration and availability of better therapeutics (Buys & Rafuse, 2010; Mechoulam & Feigenbaum, 1987). One study found that oral and inhaled doses were poorly tolerated in patients with open-angle glaucoma, with all patients electing to discontinue THC treatment for glaucoma due to cognitive and psychological side effects. Furthermore from a patient compliance perspective, one patient particularly, discontinued treatment because of effort required given the

short duration of action (Flach, 2002). Furthermore, cannabinoids are highly lipophilic and have the poor aqueous solubility, which creates a disadvantage for corneal penetration and tissue absorption via the topical route (Pertwee et al., 2010; Prausnitz & Noonan, 1998; Yazulla, 2008). Ocular side effects included: lid inflammation, reduced tear secretion, changes in pupil diameter, conjunctival hyperemia, corneal opacification and were also linked to vehicle treatments, highlighting the difficulty with a topical delivery system (Hepler & Frank, 1971; Jay & Green, 1983).

Currently, there are no prescription ocular cannabinoid medications, including for the treatment of glaucoma. In part, this has been attributed to potential adverse side effects including psychoactivity associated with CB1 activation (Colasanti et al., 1984; Green, 1998; Huestis et al., 2001; Oltmanns et al., 2008; Yazulla, 2008). However, the identification of CB1 receptors in the eye allows for ocular activation of CB1 receptors located on tissues involved with AH secretion and outflow (Straiker et al., 1999). This localization could alleviate the issue of psychoactivity associated with systemic administration (Green, 1998; Straiker et al., 1999). Additionally, the relatively new discovery of behaviorally inactive cannabinoids and associated lipids that activate novel non CB1 receptors to reduce IOP highlights the existence of additional novel targets that may be exploited for IOP reduction (Szczeniak et al., 2011). Therefore, the use of alternative approaches and novel drugs to target the ECS will not only will increase our understanding of the ocular endocannabinoid system but may renew the feasibility of targeting the ECS as a future treatment for glaucoma and ocular disease.

1.9: Rationale & Objectives

Identifying the mechanisms of IOP reduction by ECS modulation is required to determine if targeting the ECS, using cannabinoids or novel cannabimimetic ligands, provides a feasible approach for the development of ocular hypotensive drugs for glaucoma. As cannabinoids have demonstrated neuroprotective properties in experimental models of RGC loss, a(Pinar-Sueiro et al., 2013; Slusar et al., 2013; Yazulla, 2008), in addition to their IOP-lowering actions, therapeutic use of cannabinoids or ECS modulating drugs in glaucoma remains of considerable interest.

It is well established that cannabinoids are capable of lowering IOP (Green, 1998). Specifically, the aminoalkindole CB1/CB2 agonist, WIN, reduces IOP through CB1 activation and this mechanism may also involve alterations in β AR signaling (Hudson, 2010; Oltmanns et al., 2008). However, to-date no other cannabinoid ligands have been evaluated to determine if this mechanism of action is specific to the aminoalkylindole class of cannabinoids i.e. WIN, or can be applied more broadly to other classes of cannabinoids such as the classical and non-classical cannabinoids, including THC and CP, respectively. Furthermore, the mechanisms by which CB1 and β ARs interact to reduce IOP remains to be clarified but may involve CB1-mediated inhibition of NE release. Additionally, the contribution of CB2 to the ocular hypotensive actions of cannabinoids requires further verification.

Secondly, the development of cannabimimetic ligands that do not bind directly to CB1 or CB2 but produce cannabinoid-like effects has been reported and identification of receptor targets for these ligands could offer potential new avenues

for the development of ocular hypotensive drugs. For example, Szczesniak and colleagues (2011) reported a CB1-independent IOP reduction by the cannabidiol analog, Abn-CBD, in rats. Abn-CBD, as well as other cannabinoids and endocannabinoids have been reported to activate GPR55, originally thought to be a novel cannabinoid receptor, but subsequently shown to be the target for the endogenous lipid, LPI, (Oka et al., 2007). In addition to GPR55, cannabinoids and cannabimimetic lipids may produce ocular hypotension via another cannabinoid-candidate receptor- GPR18. This receptor is also activated by Abn-CBD and the phytocannabinoid, THC. To-date, the involvement of both GPR55 and GPR18 in IOP regulation in the eye has not extensively examined.

Therefore the research objectives of my thesis are to:

- 1) Compare the IOP reducing properties of the non-classical cannabinoid, CP, to the aminoalkylindole cannabinoid, WIN, in order to determine if these structurally diverse cannabinoids reduce IOP via similar mechanisms i.e. CB1-mediated reduction in IOP.
- 2) Further investigate the interactions between CB1 and the adrenergic nervous system in the IOP-reducing effects of cannabinoids.
- 3) Examine the role of CB2 in the IOP-reducing effects of cannabinoids.
- 4) Investigate the pharmacology of the atypical cannabinoid, Abn-CBD, and cannabimimetic lipid, NAGly, in reducing IOP.

Chapter 2 : Methodology

2.1. Animals

Six-week C57BL/6J mice were obtained from Charles River Laboratories International Inc. (Wilmington, MA). Mice were given a minimum of one week to acclimatize to the animal care facility before the first experimental manipulation.

CB₁^{+/-} mice were obtained from Dr. Carl Lupica (NIH). Heterozygotes were bred in-house at the Carlton Animal Care Facility. Offspring were genotyped using PCR. CB₁^{-/-} mice were identified by using the CB₁ forward primer: 5'-GTACCATCACCACAGACCTCCT-3', and the CB₁ reverse primer: 5'-AAGAACGAGATCAGCAGCCTCT-3'.

β₁AR^{-/-}/β₂AR^{-/-} (βAR^{-/-}), in addition to the CB₂^{-/-} mice, were obtained from Jackson Laboratories (Bar Harbor, Maine); both strains were bred in house in homozygous pairs. Strain background for βAR^{-/-} is a mixed stock background (C57BL/6J, DBA/2, 129, FVB/N, and CD1). CB₂^{-/-} mice were on a C57BL/6J background.

All mice were kept in the Carlton Animal Care Facility and handled according to the Canadian Council for Animal Care guidelines (<http://www.ccac.ca/>). All experimental protocols were approved by the Dalhousie University Committee on Laboratory Animals and were designed to ensure that the minimum number of animals used. Mice were kept on a 12-hour light dark cycle and given unrestricted access to food and water except during experiments.

Additional CB₁^{-/-} (CD1 backstrain) and GPR55^{-/-} (C57BL6 backstrain) mice were kindly provided by Dr. Ken Mackie and Dr. Alex Straiker (Indiana University,

Bloomington IN). Experiments with these mice were carried out at Indiana University (Bloomington, IN, USA) with ethical approval provided by the Bloomington Institutional Animal Care and Use Committee and according to the same guidelines set out by the Canadian Council for Animal Care.

2.2 Intraocular Pressure Measurements:

Intraocular pressure (IOP) measurements were made noninvasively using rebound tonometry (Tono-lab tonometer; Colonial Medical Supply Company, Franconia, NH). The technique of rebound tonometry involves placing a magnetized probe on the cornea of the eye; this in turn determines IOP by changes in the motion parameters of the probe (Nissirios et al., 2007). The Tono-lab has been shown to be reliable instrument for measuring IOP in different mouse strains (Nissirios et al., 2007).

In order to obtain reproducible IOP measurements, mice were anesthetised with 3-4% isoflurane in oxygen. The mouse was then placed on a secure stage in the prone position and anaesthesia was maintained with 2% isoflurane. Ten IOP measurements were taken from each eye and the average taken as the absolute pressure. All IOP measurements following drug administration were taken between 16:00 and 18:00 in order to reduce any variability in IOP resulting from diurnal changes.

For diurnal IOP experiments, IOP was measured by using the method described above on the same day and from the same animal at 9:00 and 21:00. Diurnal variation experiments were carried out to insure that IOP regulation was intact, recent evidence suggests that circadian rhythms are altered in eye which have glaucoma or ocular hypertension (Aihara, 2003; Aihara et al., 2007). Diurnal variation of IOP is well established in humans, primates and rodents (Aihara et al., 2007; Moore et al.,1996;

Nickla et al., 2002; Nickla, 2013). In mice IOP is higher in the dark cycle and for this reason IOP measurements were carried out at the same time of day between 16:00 and 18:00 (Aihara, 2003; Aihara et al., 2007).

2.2: Materials:

Capsazepine, 0-1602, WIN, 55212-2, Abn-CBD, HU308, AM281, AM630, and NAGly were all obtained from Tocris Bioscience (Ellisville, MO, USA). Isoproterenol (ISO), reserpine, timolol, metoprolol were obtained from Sigma-Aldrich (Oakville, ON, Canada). Ophthalmic solutions latanoprost and dorzolamide were obtained from Pfizer (Kirkland, QC, Canada), and from Merck Frost (Kirkland, QC, Canada), respectively.

For all receptor agonists, drugs were applied topically to one randomly assigned eye, while the other eye received the appropriate vehicle. All receptor antagonists were injected intraperitoneally (i.p.) at a dose of 2mg/kg, 30 minutes prior to topical application of the agonist, in order to control for topical washout and allow for time to get to the ocular site of action. The vehicle used for the cannabinoids: WIN, Abn-CBD, NAGly, 0-1602 and CP 55,940 was Tocrisolve™. Saline was used as a vehicle for timolol, latanoprost, dorzolamide and isoproterenol.

2.3. β -Receptor desensitization:

Desensitization of β -adrenergic receptors (β -AR) was achieved by using the non-selective β -AR agonist ISO. ISO was dissolved in saline at 2.5% and applied to both eyes in the experimental animals; control animals received saline. Drops were installed twice a day between 9:00-10:00 and 21:00-22:00 for a time period of 7 days.

In order to demonstrate β -AR desensitization, the actions of the non-selective β -

AR antagonist timolol 0.5% was tested after 7 days of treatment, 7 hours after last ISO topical administration. In treated animals, one eye received saline and the experimental eye received timolol.

In addition, a two-week trial of daily ISO treatments at an increased dose and concentration was also performed. Experimental animals received 5% ISO topically three times a day at 9:00, 15:00 and 21:00 for a time period of 14 days. Control animals received saline. At the end of the treatment period, animals were again tested for the ocular hypotensive actions of timolol. After this, ISO treatments were resumed for an additional three days and the ocular hypotensive actions of WIN were then tested in the same manner as timolol.

2.4: Chemical Sympathectomy:

Depletion of catecholamine levels was based on the methods of Olfe and colleagues (2010). Briefly, reserpine was dissolved in 5% glacial acetic acid (at 2 µg/µl). All mice used in these experiments were approximately 25g. Animals received reserpine at 1 µg/g body weight: 12.5 µl of the reserpine stock solution was combined with 100 µl saline for a total of 112.5ul, which was injected subcutaneously 24 hours prior to experimental manipulation. Control animals received the same injection without reserpine (12.5 µl 5% glacial acetic acid + 100 µl saline).

At 24 hours post-reserpine/saline injection, animals were anesthetized using pentobarbital at 60 mg/kg. Baseline IOP under phenobarbital anesthesia was first measured using the Tono-lab. Ten measures were taken from each eye and the average taken as the absolute pressure. Animals then received the drug treatment ,

WIN 1% or NAGly 1%, which was applied to one randomly assigned eye, while the contralateral eye served as the control and received Tocrisolve™. IOP was then measured 20-30 minutes following the drug or vehicle administration.

2.5: Evaluating Sympathetic Tone:

Innervation to the eyelid tarsal muscle is supplied by sympathetic innervation. In order to determine if reserpine affected sympathetic activity to the ocular structures animals were evaluated for ptosis (lower upper eyelid).

Animals receiving either reserpine or vehicle control treatment, were evaluated 24 hours later by both a blinded researcher and a researcher who administered treatments. An scale was used from one to three: Zero =no observable ptosis, 1 = mild ptosis, not interfering with the visual axis, 2 = moderate ptosis, covering visual axis, and 3 = complete ptosis. Half values were also evaluated. For these evaluations, animals were non-anesthetized and both researchers handled the mice in order to effectively evaluate the presence of a ptosis.

2.6. Statistical Analysis:

All data are represented as mean and standard error of mean. All analysis were carried out using Graphpad Prism V.6 (Graphpad Software Inc. San Diago). For statistical tests used included: paired t-tests when comparing 2 groups or by one-way ANOVA when comparing multiple groups, with Tukey's post-hoc analysis used for multiple comparisons when appropriate. Statistical significance was deemed when $p < 0.05$ for both tests. All graphs represent mean \pm standard error of the mean, unless otherwise noted.

Chapter 3 : Results

Some of the figures in this chapter have been modified and reproduced from publications (Hudson, B. D., Beazley, M., Szczesniak, A., Straiker, A., & Kelly, M. E. M. (2011). Indirect sympatholytic actions at β -adrenoceptors account for the ocular hypotensive actions of cannabinoid receptor agonists. *The Journal of Pharmacology and Experimental Therapeutics*, 339(3), 757–67. doi:10.1124/jpet.111.185769 & Caldwell, M. D., Hu, S. S.-J., Viswanathan, S., Bradshaw, H., Kelly, M. E. M., & Straiker, A. (2013). A GPR18-based signalling system regulates IOP in murine eye. *British Journal of Pharmacology*, 169(4), 834–43. doi:10.1111/bph.12136. Licenses for reuse from each publication are located in appendix II and III respectively.

As second author on Hudson et al., (2011), I performed the majority of IOP experiments. I also participated to the data analysis and data interpretation of this manuscript.

As first author on Caldwell et al., (2013) I performed all IOP experiments, participated in research design, data analysis, data interpretation and contributed to writing of the manuscript.

As first author on Caldwell et al., (2013) I performed all IOP experiments, participated in research design, data analysis, data interpretation and contributed to writing of the manuscript.

3.1. Baseline Parameters

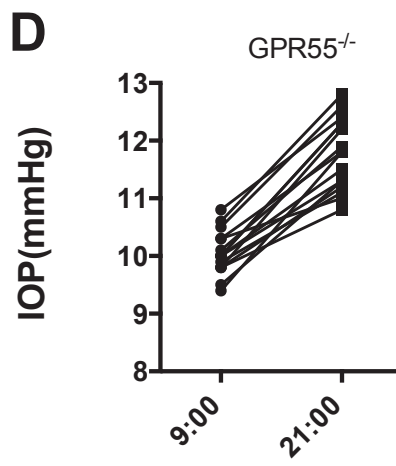
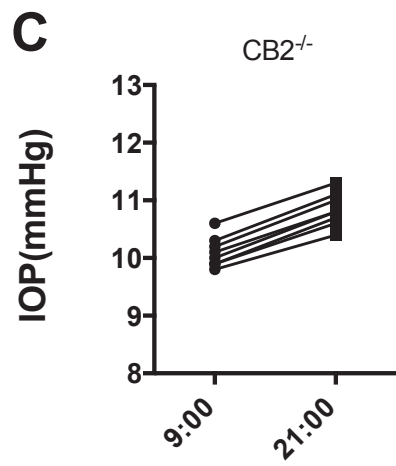
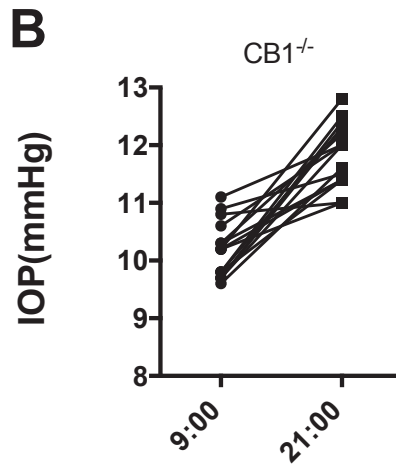
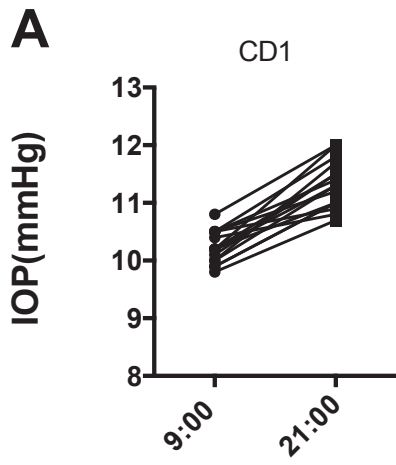
Intraocular pressure has a diurnal variation that oscillates between higher and lower pressure. Diurnal variation of IOP is well established in humans, primates and rodents (reviewed by Nickla, 2013). In mice, IOP is higher

at the beginning of the dark cycle and lower at the beginning of the light cycle (Aihara et al., 2007; Maeda et al., 2007).

Previous studies have demonstrated intact diurnal rhythms in C57 WT and mice genetically lacking either β -AR or CB1 (C57 background) receptors. They reported that IOP was significantly higher near the beginning of the dark cycle at 21:00 compared to 9:00 (Hudson et al.,2010). Initial studies determined the presence of diurnal regulation of IOP in several mouse strains, which included male CD1 WT, CB1^{-/-} (CD1 background), CB2^{-/-} (C57 background), and GPR55^{-/-} (C57 background). The presence of diurnal regulation served as an indicator that aqueous humor production and outflow was not altered between WT and transgenic mice used in this study. IOP was measured in each animal at 9:00 and 21:00 within the same 24 hour time period. Consistent with previous reports IOP was significantly higher at 21:00 when compared to 9:00. Figure 3.1.A shows the presence of diurnal variation of CD1 WT with higher IOP near the start of the dark cycle when compared to the start of the light cycle (1.09 ± 0.19 , $p < 0.0001$, $n = 16$). The CB1^{-/-} mice showed higher IOP at night when compared to IOP taken in the morning (Figure 3.1.B; 1.70 ± 0.02 , $p < 0.0001$, $n = 16$). Figure 3.1.C showed the same diurnal oscillation as previous research in CB2^{-/-} mice with significantly higher IOP in the late evening when compared to morning (0.74 ± 0.03 , $p < 0.0001$, $n = 8$). Lastly, the GPR55^{-/-} mice paralleled these results, with significantly higher IOP at 21:00 when compared to 9:00 (Figure 3.1.D; 1.7 ± 0.13 , $p < 0.0001$, $n = 16$).

Figure 3.1: *Diurnal variation of IOP in CD1 wildtype and transgenic mouse strains.*

A. Individual variation in IOP of male CD1 mice measured at 9:00 and 21:00 within a 24 hour time period ($p < 0.0001$, $n = 16$). B. Individual diurnal changes in IOP of male CB1^{-/-} (CD1 background) ($p < 0.0001$, $n = 16$). C. Diurnal variation in IOP of CB2^{-/-} (C57 background) mice taken at 9:00 and 21:00 ($p < 0.0001$, $n = 8$). D. Individual variation in IOP of male GPR55^{-/-} taken at 9:00 and 21:00 within a 24 hour time period ($p < 0.0001$, $n = 16$).



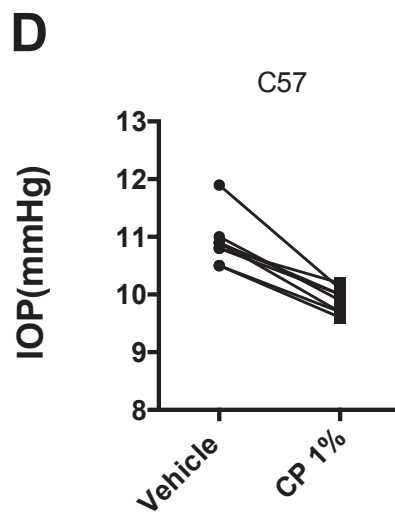
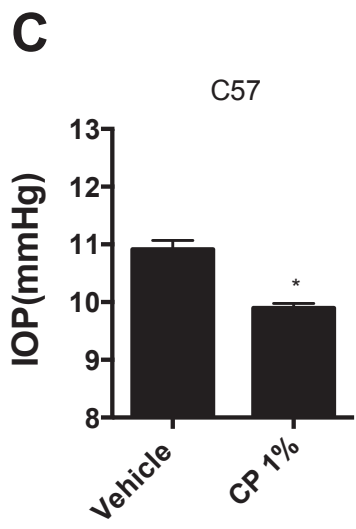
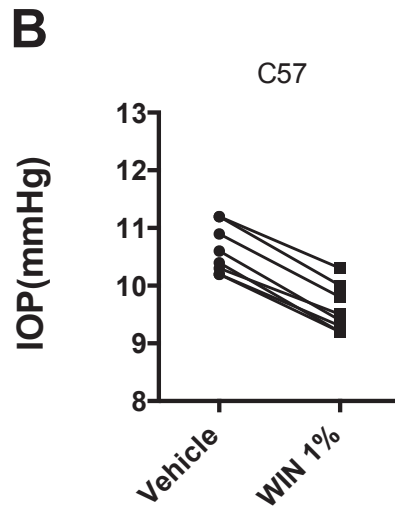
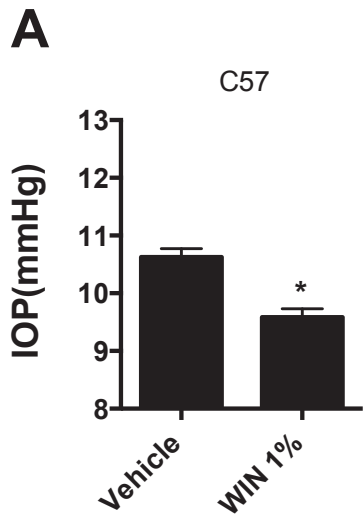
3.2. Psychotropic Cannabinoids

Ingestion of marijuana and dosing with individual phytocannabinoids and synthetic cannabinoid drugs reduces IOP (Hepler & Frank, 1971; Oltmanns et al., 2008). While the mechanisms of action may vary with individual cannabinoids, it has been suggested that activation of CB1 receptors accounts for the ocular hypotensive action of psychotropic cannabinoids such as the phytocannabinoid, Δ^9 -THC, in addition to synthetic cannabinoids such as WIN (Oltmanns et al., 2008; Pate et al., 1998; Szczesniak et al., 2006). In addition, recent evidence has suggested that some cannabimimetic ligands, such as atypical cannabinoids and cannabimimetic lipids, can lower IOP by mechanism(s) of action that are independent of CB1 receptors. This would theoretically avoid many of the behaviorally active side-effects associated with *cannabis* smoking and CB1-activating drugs (Qiao et al., 2012; Szczesniak et al., 2011). In order to delineate the mechanisms of action by different cannabinoids reducing IOP, my experiments used both pharmacology and transgenic approaches.

Behaviorally-active synthetic cannabinoids reduce Intraocular Pressure

The ocular hypotensive actions of behaviorally-active synthetic cannabinoid agonists, WIN and CP, were first re-examined in C57 WT mice to confirm that these agents were able to lower IOP under the experimental conditions used. Firstly, the effects of WIN 1% on IOP were assessed 30 minutes after topical application (Figure 3.2A). WIN treated eyes had a lower IOP (-1.04 ± 0.06) when compared to tocrisolve-vehicle treated eyes ($p < 0.0001$, $n = 8$). The reduction in IOP was

Figure 3.2: *Behaviorally-active cannabinoids reduce IOP in C57 wildtype mice* A. Mean IOP taken from C57 male mice 30 minutes after topical administration of WIN 1% or vehicle ($p < 0.0001$, $n = 8$). B. Individual variation in IOP between vehicle and WIN treated eyes C. Mean IOP taken from C57 male mice 1 Hour after topical administration of CP 1% or vehicle ($p < 0.001$, $n = 8$). D. Individual variation in IOP between vehicle and CP treated eyes.



independent of the absolute IOP value observed in the control eye (Figure 3.2.B). Similar to WIN, Figure 3.2.C shows CP 1% reduced IOP of -1.01 ± 0.13 when measured at 1 hour ($p < 0.0001$, $n = 8$). In these experiments IOP was lower in each CP 1% treated eye when compared to the vehicle treated eye (Figure 3.2.D).

From a clinical perspective, it was important to establish that cannabinoids lower IOP, in addition, doing so in a comparable manner to clinically available glaucoma therapeutics. Figure 3.3 shows no significant differences were detected in the change in IOP of WIN 1% and CP 1% and the ocular hypotensive actions of clinically available ocular hypotensive agents. These included: the β -adrenergic antagonist timolol malate, the prostaglandin analogue latanoprost, and the carbonic anhydrase inhibitor dorzolamide ([F (4,39)=0.4809, $p = 0.27$].

As psychotropic cannabinoids, WIN and CP activate both CB1 and CB2 receptors, selective receptor ligands and receptor knock-out mice were used to determine if the IOP decrease seen with these cannabinoids might involve CB1 and CB2 collectively or individually or other non-cannabinoid receptors.

The involvement of CB2 was assessed using topical application of the CB2 agonist, HU308 1%, in male C57 WT mice (Figure 3.4. A). In Figure 3.4.B, HU308 1% had no significant effect on IOP (Δ IOP of 0.02 ± 0.07 , $n = 5$) when compared to the tocrisolve-vehicle topically treated eye ($p > 0.05$). Consistent with the distinct involvement of CB1, when WIN 1% was applied topically in CB2^{-/-} mice there continued to be a statistically significant reduction in IOP ($p < 0.005$, $n = 4$) of -0.73 ± 0.11 mmHg (Figure 3.4. C & D). Moreover, figure 3.4.E and F shows the

Figure 3.3: *Behaviorally-active cannabinoid agonists reduce IOP comparable to known ocular hypotensive agents.* A. Mean change in IOP following treatment of WIN, CP, timolol, latanoprost and dorzolamide, $n=8$ for each respective group. No significant difference between each treatment on IOP (1-way ANOVA; $p>0.05$).

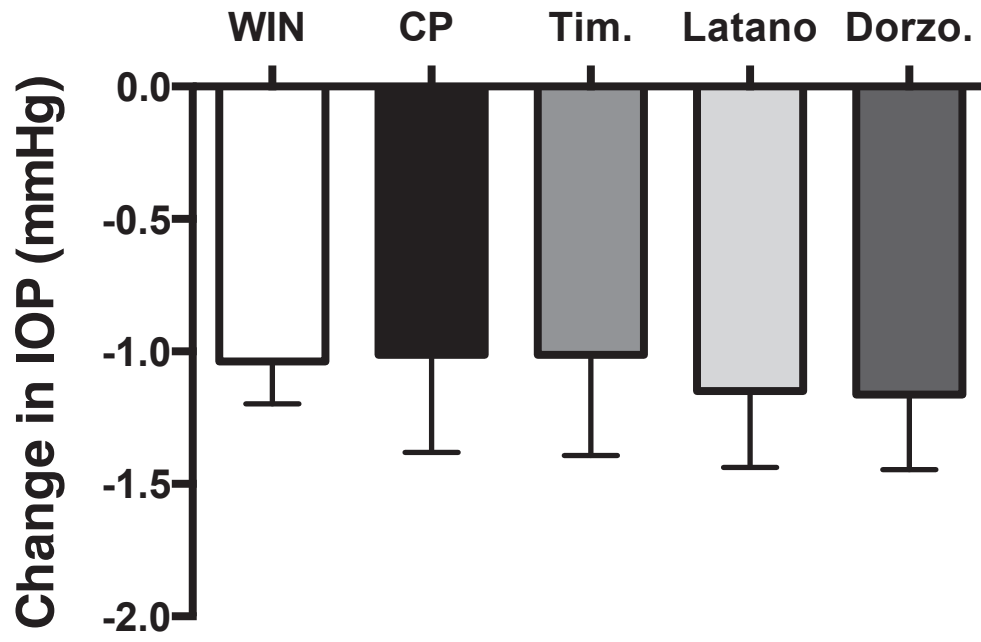
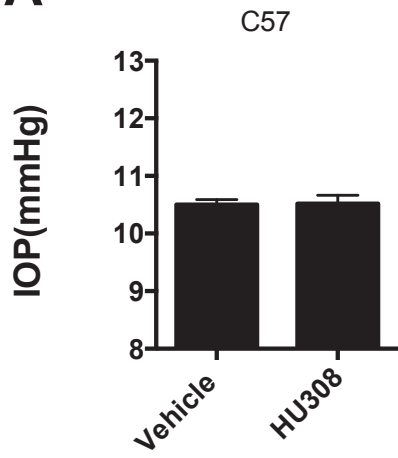
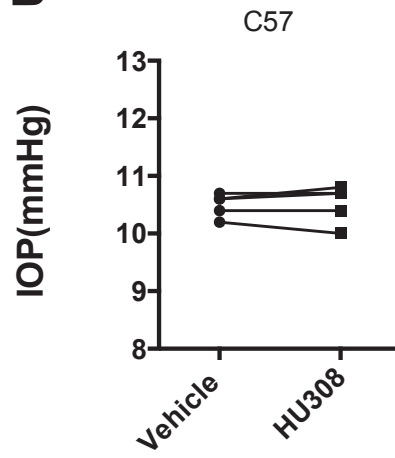
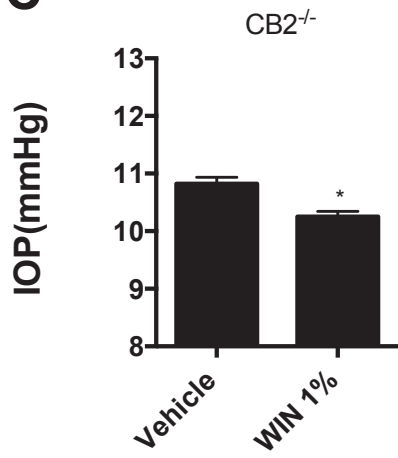
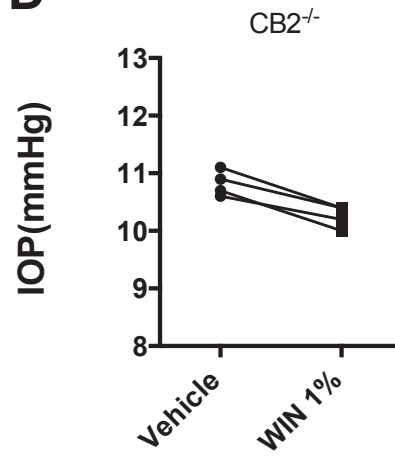
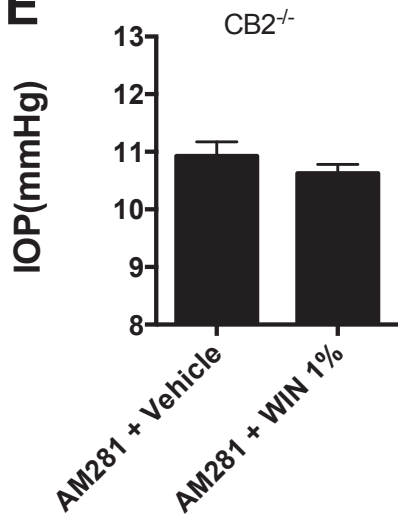
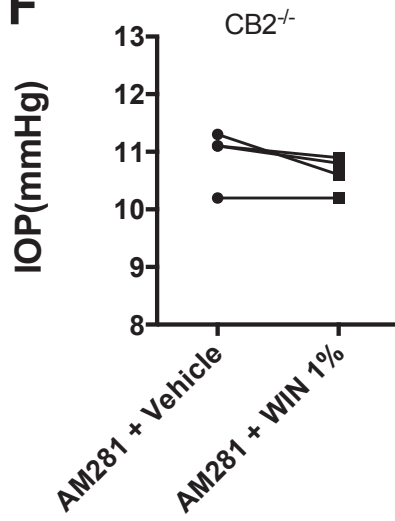


Figure 3.4: *WIN-mediated ocular hypotension occurs through selectively through cannabinoid receptor type 1* A. Mean IOP taken from C57 mice 1 Hour after topical administration of HU308 1% or vehicle ($p>0.05$, $n=5$). B. Individual variation in IOP between vehicle and HU 308 treated eyes C. Mean IOP taken from CB2^{-/-} (C57 background) mice 30 minutes after topical administration of WIN 1% or vehicle, ($p<0.005$, $n=4$). D. Individual variation in IOP of WIN1% or Vehicle. E. Mean IOP taken from CB2^{-/-} mice pre-treated with AM281 taken after 30 minutes after topical administration of 1.0% WIN or vehicle ($p>0.05$, $n=4$). F. Individual variation of IOP with WIN 1% in CB2^{-/-} mice pre-treated with AM281.

A**B****C****D****E****F**

reduction in IOP of WIN 1% was blocked by the CB1 antagonist, AM281 (Δ IOP of -0.30 ± 0.15 mmHg; $p > 0.05$ $n=4$). To further confirm the involvement of CB1 in the ocular hypotensive effect of psychotropic cannabinoid agonists, WIN and CP were examined in CB1^{-/-} (CD1-background) mice. In contrast to CD1 WT mice, figure 3.5.A shows CB1^{-/-} mice had a significant increase in IOP of 0.80 ± 0.48 in the WIN 1% topically treated eye compared to the Tocrisolve-vehicle treated eye when measured at 30 minutes ($p < 0.001$, $n=8$). When observing the individual differences all CB1^{-/-} mice showed an increase in the WIN treated eye (Figure 3.5.B). Unlike WIN, CP did not show a potentiation in IOP and did not produce any significant alterations in IOP (0.01 ± 0.22 , $n=8$) in CB1^{-/-} mice (Figure 3.5.C). With CP 1%, Figure 3.5.D shows there was no visible trend in IOP of individual mice ($p > 0.05$).

To determine if the WIN-mediated increase in IOP observed in the CB1^{-/-} mice was mediated through CB2, a group of CB1^{-/-} animals ($n=8$) were pre-treated with the CB2 antagonist, AM630 (i.p.2mg/kg), 30 minutes prior to topical treatment with WIN 1% or tocrisolve-vehicle (Figure 3.6.A.). The WIN-mediated increase in IOP in CB1^{-/-} was still apparent in the mice pretreated with the CB2 antagonist AM630 and resulted in an increase in IOP of 1.14 ± 0.17 mmHg ($p < 0.0001$) (Figure 3.6.B). This result suggests that the increase in IOP unmasked by WIN treatment in CB1^{-/-} mice is unlikely to be mediated through CB2. Furthermore, the increase in IOP was not observed in CB1^{-/-} mice with treatment of the non-selective CB1/CB2 cannabinoid agonist, CP. This finding suggests the potentiation IOP seen with WIN is likely specific to the aminoalkylindole class of cannabinoids and may occur through a yet-to-be-identified receptor target.

Figure 3.5: *Potentiation of IOP with the synthetic cannabinoid agonist WIN.* A. Mean IOP taken from CB1^{-/-} (CD1 background) mice 30 minutes after topical administration of WIN 1% or vehicle ($p < 0.001$, $n = 8$). B. Individual variation in IOP between vehicle and WIN treated eyes. C. Mean IOP taken from CB1^{-/-} mice (CD1 background) 1 hour after topical administration of CP 1.0% or vehicle ($p > 0.05$, $n = 8$). D. Individual variation in IOP between vehicle and CP treated eyes.

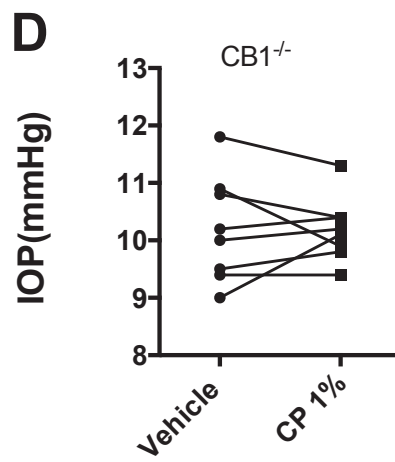
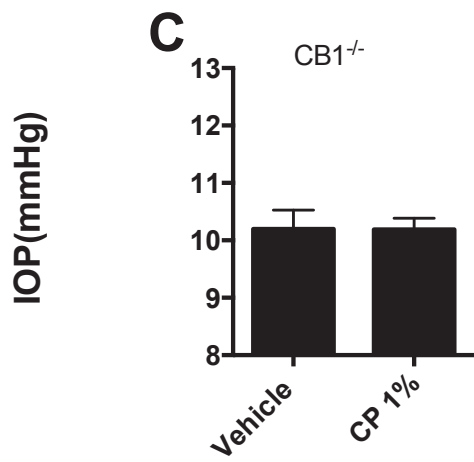
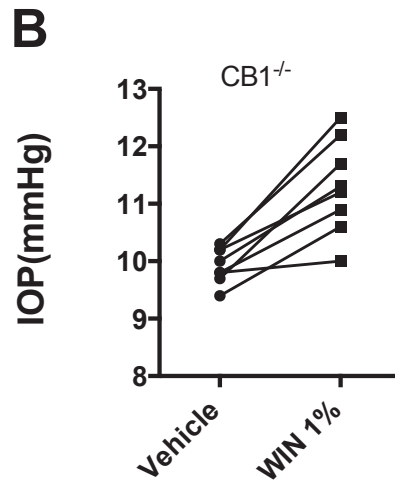
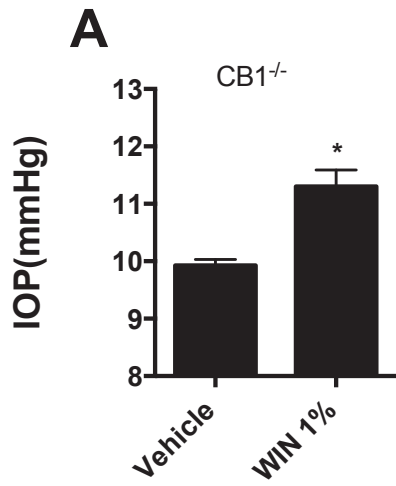
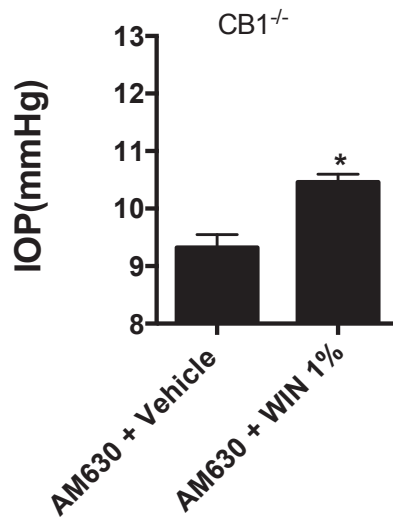
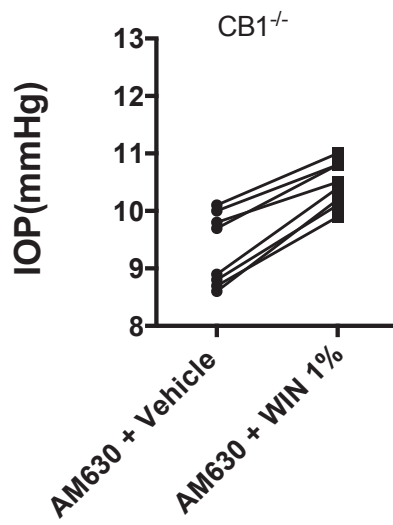


Figure 3.6: *WIN 55212-potentiates IOP independent of cannabinoid receptor type 2*. A. Mean IOP taken from CB1^{-/-} (CD1 background) mice pre-treated with AM630 30 minutes after topical administration of 1.0% WIN or vehicle ($p > 0.0001$, $n = 8$). B. Individual variation of IOP with WIN 1% in CB1^{-/-} mice pre-treated with AM630.

A



B



Taken together, these findings suggest that the reduction in IOP produced by the psychotropic cannabinoids, WIN and CP, is mediated distinctly through the CB1 receptor, independent of CB2.

Effects of behaviourally-active cannabinoid ligands are dependent on an intact β -adrenergic system

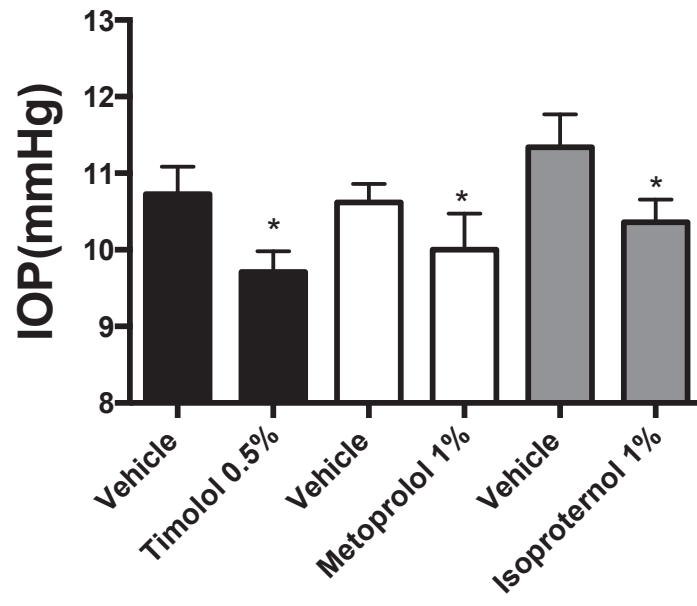
β -AR ligands have long been established as ocular hypotensive agents, and are regularly used clinically to treat ocular hypertension and primary open angle glaucoma (Bagnis et al., 2011). IOP is determined by the balance of AH secretion versus outflow. With β -AR antagonists, they reduce IOP by decreasing AH secretion in the ciliary epithelium and are first line therapeutics in the treatment glaucoma. In contrast, β -AR agonists can increase AH outflow but are not readily used in the clinical setting.

The ocular hypotensive actions of cannabinoids were suggested to be mediated, at least in part, through the adrenergic nervous system (Hudson et al., 2011; Hudson, 2010; Oltmanns et al., 2008) . Therefore, in order to determine whether the hypotensive actions of the behaviourally-active cannabinoid agonists, WIN and CP involve β -AR receptors, experiments were carried out using both β -AR and cannabinoid ligands in C57 WT, β -AR^{-/-} and CB1^{-/-} mice.

Initial experiments first determined the ocular hypotensive effects of the β 1AR selective antagonist, metoprolol, the non-selective β 1AR and β 2-AR antagonist timolol, and the non-selective β 1AR and β 2-AR agonist, isoproterenol (ISO) in C57 WT mice. Figure 3.7.A. demonstrates that all three ligands significantly lowered IOP with reductions of -0.62 ± 0.19 ($p < 0.05$, $n=5$), -1.01 ± 0.13 ($p < 0.001$, $n=8$) and

Figure 3.7: β -Adrenergic antagonists agonist lower IOP in C57BL6 mice A. IOP data from C57 wildtype mice treated topically with either vehicle or timolol 0.5% (Solid bars) $p < 0.001$, metoprolol 1% (open bars) $p < 0.05$, isoproterenol 1%, $p < 0.005$ (grey bars).

A



-0.98±0.12 ($p<0.005$, $n=5$), respectively. The change in IOP was slightly greater with the β 1AR and β 2-AR antagonist, timolol; suggesting that the IOP-lowering effect of β AR antagonists is mediated in part by both the β 1AR and the β 2AR. However, there were no statistically significant differences found between adrenergic agents tested [$F(2,17)=1.87, p=0.67$].

To determine putative cannabinoid-adrenergic interactions on IOP, the effect of WIN 1% was examined in β -AR $^{-/-}$ mice. Figure 3.8.A and B shows WIN 1% does not significantly reduce IOP (0.13±0.16mmHg) when measured 30 minutes after drop instillation ($p>0.05$, $n=8$). To determine that the lack of decrease in IOP was a property of all CB1 agonists, not just the aminoalkylindole class of cannabinoids to which WIN belongs, the non-classical cannabinoid, CP, was also assessed. As seen with WIN 1%, Figure 3.8.C and D shows there was no significant alterations in IOP (-0.41±0.27mmHg) measured in β -AR $^{-/-}$ mice treated with CP 1% when measured after 1 hour ($p>0.05$, $n=8$). IOP was then measured 1 hour after timolol 0.5% administration in CD1 WT and CB1 $^{-/-}$ (CD1 background) mice.

Contrary to expectation, figure 3.9.A demonstrates that timolol 0.5% failed to significantly reduce IOP when measured 1 hour after drop instillation in CB1 $^{-/-}$ mice (0.16±0.17, $n=8$). There was no clear trend in IOP observed in individual animals (Figure 3.9.B, $p>0.05$). To ensure that strain was not a factor with this unexpected finding, timolol 0.5% was also assessed in CD1 wildtype and as observed with C57 WT mice, Figure 3.9.C and D shows timolol 0.5% was able to reduce IOP with reductions of -0.83±0.09 mmHg ($p<0.0001$, $n=8$).

Figure 3.8: *Cannabinoid agonists do not lower IOP in male β -AR^{-/-} mice* A. Mean IOP taken from β -AR^{-/-} mice 30 minutes after topical administration of WIN 1% or vehicle ($p > 0.05$, $n=8$). B. Individual variation in IOP between vehicle and WIN treated eyes. C. Mean IOP taken from β -AR^{-/-} mice 1 Hour after topical administration 1% CP 55,940 or vehicle ($p > 0.05$, $n=8$). D. Individual variation in IOP between vehicle and CP 55,940 treated eyes.

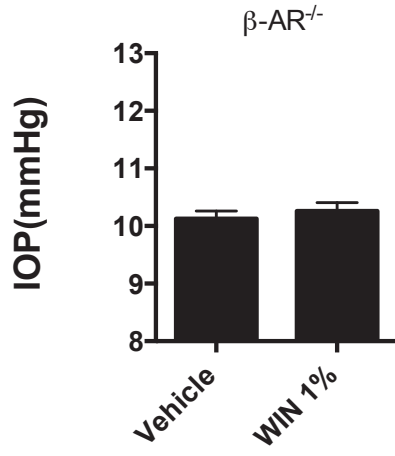
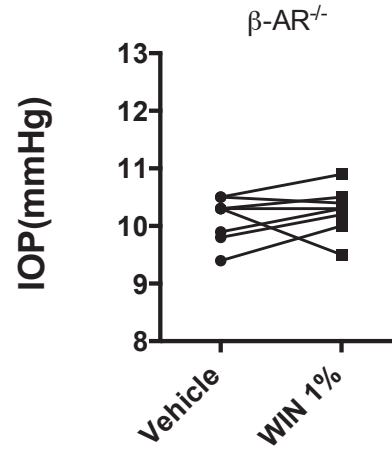
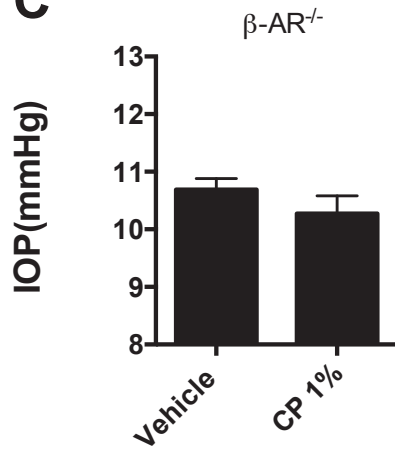
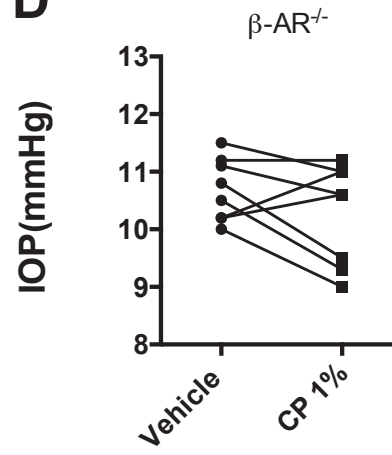
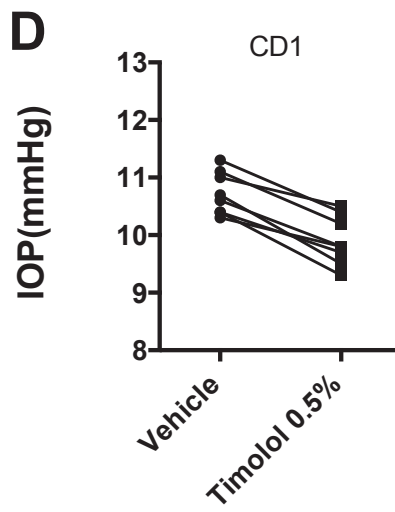
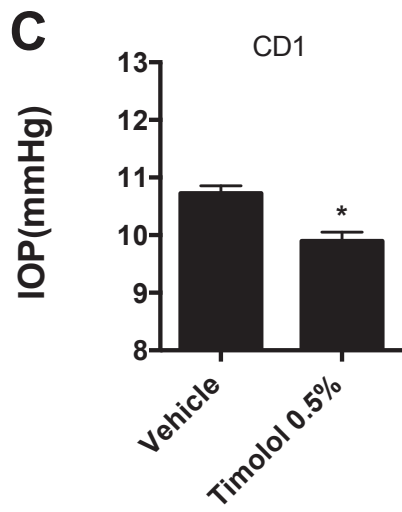
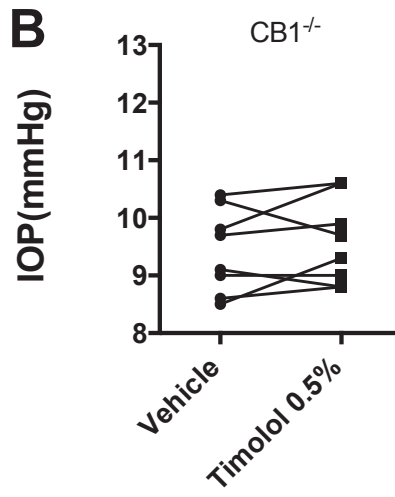
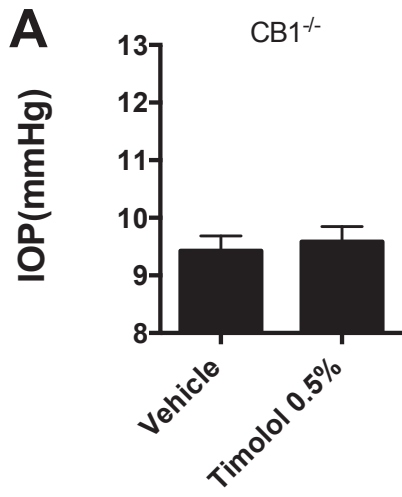
A**B****C****D**

Figure 3.9: *Timolol malate 0.5% fails to reduce IOP in CB1^{-/-} mice.* A. Mean IOP taken from CD1 mice 1 Hour after topical administration of timolol 0.5% or vehicle ($p < 0.0001$, $n = 8$). B. Individual variation in IOP between vehicle and timolol treated eyes in CD1 mice. C. Mean IOP taken from CB1^{-/-} mice 1 Hour after topical administration of timolol 0.5% or vehicle ($p > 0.3738$, $n = 8$). D. Individual variation in IOP between vehicle and timolol treated eyes in CB1^{-/-} mice.



Chronic treatment with the β_1 AR and the β_2 AR agonist Isoproterenol blocks the IOP reducing effect of Timolol and WIN

The finding that adrenergic agents failed to reduce IOP in CB1^{-/-} was unexpected and suggested changes in noradrenergic signaling with genetic elimination of CB1. Therefore additional adrenergic agents were assessed to determine whether lack of IOP reduction by timolol 0.5% in CB1^{-/-} mice was isolated to this compound. Figures 3.10 A & B shows that the selective β_1 - antagonist metoprolol (1%) failed to significantly reduce IOP when measured 1 hour after drop instillation in CB1^{-/-} mice (-0.10±0.32, n=3). The inability of adrenergic agents was not isolated to β_1 - antagonists with similar findings observed with the non-selective agonist ISO 1% (Figure 3.10.C&D). Figure 3.10. C& D shows that topical application of 1% isoproterenol did not have ocular hypotensive abilities in CB1 CB1^{-/-} (C57 background) mice (-0.03±0.32, n=3).

Previous studies have demonstrated that genetic disruption of CB1 receptors results in an increase in endogenous noradrenergic tone (Szabo & Schlicker, 2005). This increase in noradrenergic tone could result in a compensatory desensitization of β -AR's. To test this hypothesis, in a proof-of principle experiment, C57 WT mice were chronically pre-treated with topical application of the β_1 AR and the β_2 AR non-selective agonist, ISO 2.5%, or saline BID for a period of 7 days, after which the effect of timolol was assessed topically (Figure 3.11.A). In this experiment, timolol 0.5% significantly reduced IOP in both the saline-treated animals ($p<0.0005$) and ISO-treated animals ($p<0.0005$).

Figure 3.10: Metoprolol 1% and Isoproterenol 1% both fail to reduce IOP in CB1^{-/-} mice. A. Mean IOP taken from CB1^{-/-} mice 1 Hour after topical administration of metoprolol or vehicle ($p>0.05$, $n=3$). B. Individual variation in IOP between vehicle and metoprolol treated eyes in CB1^{-/-} mice. C. Mean IOP taken from CB1^{-/-} mice 1 Hour after topical administration of isoproterenol 1% or vehicle ($p>0.05$, $n=3$). D. Individual variation in IOP between vehicle and isoproterenol treated eyes in CB1^{-/-} mice.

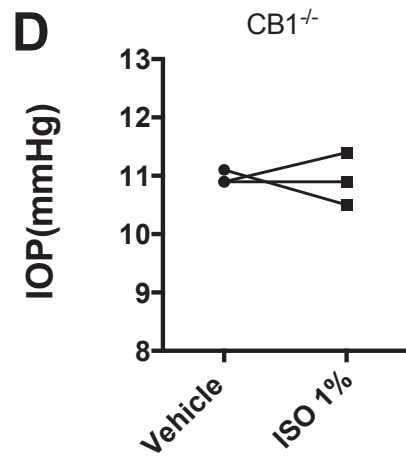
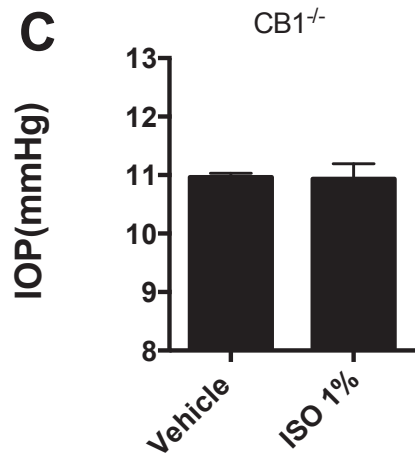
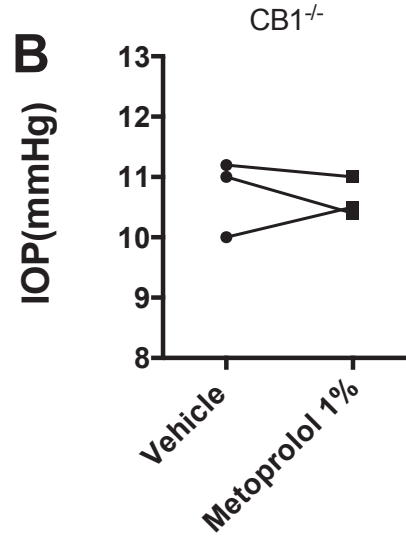
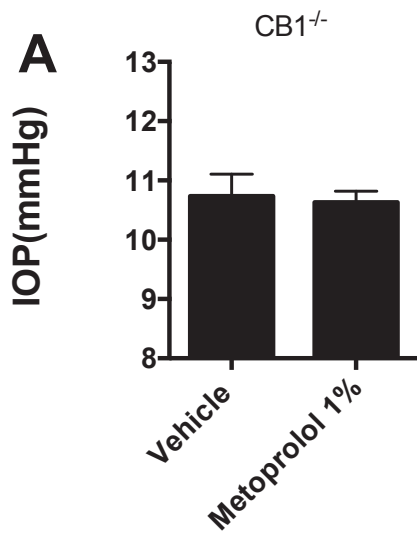
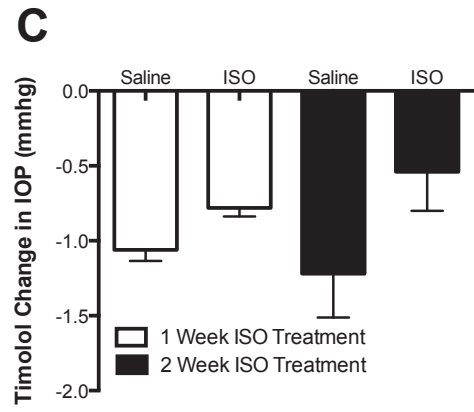
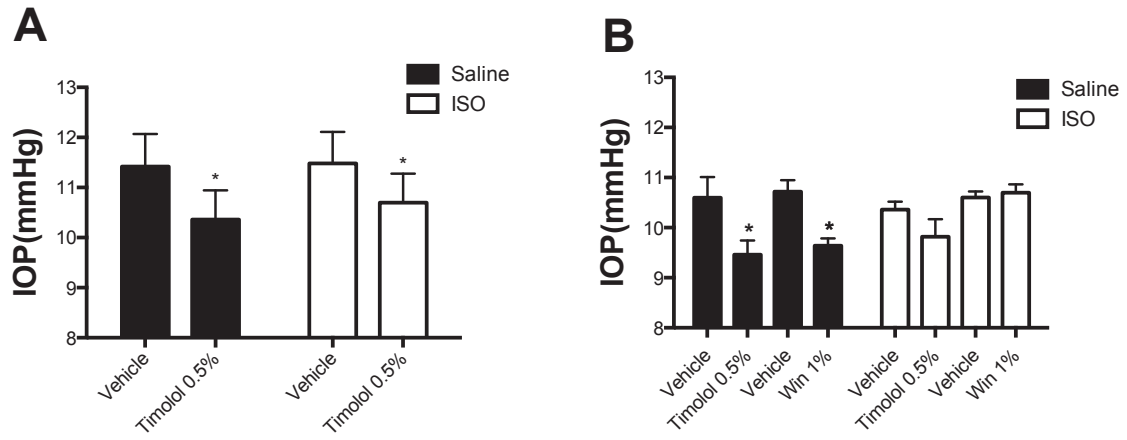


Figure 3.11: Chronic topical application of the β -adrenergic agonist isoproterenol, desensitizes the ocular hypotensive response to timolol in C57BL6 mice. A. Mean IOP data following timolol 0.5% treatment in C57 wildtype mice treated for one week with saline vehicle (solid bars; n=5;p<0.001) or isoproterenol 2.5%(open bars; n=5; p<0.001). B. Mean IOP data following timolol 0.5% or WIN treatment in C57 wildtype mice pretreated for two week with saline vehicle (solid bars; n=5;p<0.01 and p<0.005 respectively) or isoproterenol 5% (open bars; n=5; p>0.05 and p>0.05 respectively). C. Change in IOP following treatment with timolol 0.5% in C57 wildtype mice treated for 1 (solid bars) or 2 (open bars) week with either saline vehicle or isoproterenol 2.5% and 5% for 1 and 2 weeks respectively..



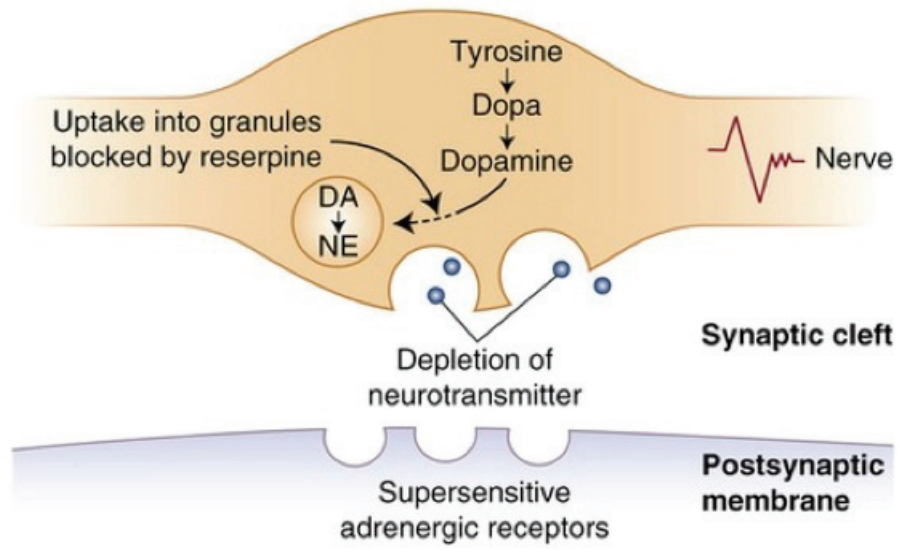
Although significance was achieved in both groups the degree of ocular hypotension was slightly less in the ISO-treated animals when compared to the saline-treated animals with reductions of -1.06 ± 0.07 mmHg and -0.78 ± 0.06 mmHg respectively. Therefore to further investigate this effect, a two-week trial was initiated with some changes to the existing parameters. First, the concentration of ISO was increased from 2.5% to 5% and secondly, the dosage was increased to TID for 14 days. Figure 3.11.B shows timolol continued to reduce IOP after 14 days in the saline-treated animals, producing a decrease of -1.14 ± 0.28 , ($p < 0.05$ $n=5$). However, in contrast, the treated animals no longer showed a significant reduction in IOP in response to timolol 0.5% (-0.54 ± 0.26 , $n=5$) consistent with receptor desensitization (Figure 3.11.B). Figure 3.11.C summarizes the effects of timolol in the 2 groups that were treated for 1-week and 2-weeks respectively. Additionally, the effectiveness of cannabinoid agonists the presence of adrenergic receptor desensitization, ISO 5% or saline was continued for an additional 3 days after the timolol experiment, following which, the effect of WIN 1% was evaluated (Figure 3.11.B). In this experiment, topical WIN 1% produced a reduction in IOP of -1.26 ± 0.27 in the saline-treated animals ($p < 0.01$, $n=5$). However, in the ISO-treated group, WIN failed to significantly lower IOP (0.100 ± 0.14 , $n=5$) (Figure 3.11.B). Figure 3.11.C demonstrates change in IOP following treatment with timolol 0.5% in C57 wild type mice treated for 1 and 2 weeks respectively. This finding implicates adrenergic receptors in the ocular hypotensive response of cannabinoid agonists.

Depletion of catecholamines diminishes ocular hypotensive effects of WIN

Previous studies have demonstrated that presynaptic CB1 receptors can inhibit norepinephrine (NE) release in other tissues (Ishac et al., 1996; Quarta et al., 2010). If this also occurs within the eye, it suggests that cannabinoid agonists acting at CB1 could reduce IOP by indirectly inhibiting NE release in the ciliary body. To directly assess this, catecholamines were depleted by a chemical sympathectomy, using the drug reserpine (Brodie et al., 1957; Olfe et al., 2010; Orlans et al., 1960; Sheppard & Zimmerman, 1960). Reserpine produces its effects by depleting stores of catecholamines and 5-hydroxytryptamine thus depressing the sympathetic nervous system (Figure 3.12.) (Martínez-Olivares et al., 2006).

In order to determine if reserpine was capable of modulating sympathetic tone, animals received either reserpine or saline and were subsequently evaluated after 24 hours for the presence of a ptosis. Chemical sympathectomy by reserpine has previously been shown to create a ptosis as a result of alterations in sympathetic innervation to Müllers muscle (Fischer & Heller, 1967; Tedeschi, et al., 1967). Figure 3.13.A shows the eye of an animal that received saline-control. In contrast figure 3.13. B shows the eye of an animal, which received reserpine-treatment. The level of ptosis was evaluated by 2 orthoptists, with one orthoptist unaware of which animals received reserpine. A grading scale was used where: 0 represented no ptosis, 1 represented partial ptosis where the eyelid did not cover 50% of the globe, 2 represented partial ptosis where the eyelid did cover the axis of vision, and 3 represented complete ptosis. Half values were also assigned.

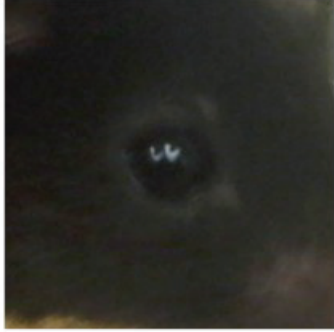
Figure 3.12: *Chemical sympathectomy by reserpine treatment results in decreased global adrenergic tone.* A. Reserpine acts by interacting with aminergic transportation by inhibiting uptake and storage of amines into vesicles. This results in leakage of currently stored amines such as norepinephrine, serotonin, and dopamine, into the cytoplasm effectively depleting catecholamines in both central and peripheral neurons. This starts to occur at one hour and reaches a maximum depletion 24 hours from a single dose at 2ug/g body weight as in Olf et al. (2010). Figure was reproduced with permission from SpringerImages (Sica, 2009; Atlas of Hypertension: Antihypertensive Agents: Mechanisms of Drug Action). See Appendix II for license



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Figure 3.13: *Chemical sympathectomy by reserpine treatment results in decreased global adrenergic and creates a ptosis. A. Eyelid position in a saline treated C57 mouse showing a clear visual axis B. Eyelid position in a reserpine treated mouse results in a ptosis that obstructs the visual axis.*

A



B



Figure 3.14.A shows a mean ptosis grade of 0 and 0.5 in saline-treated animals ($n=5$) by orthoptist A and orthoptist B respectively (median of 0 by both orthoptists). The reserpine-treated animals received a mean grade of 2 and 2.1 by orthoptist A and orthoptist B respectively (median of 2 by both orthoptists). There was a significant difference in ptosis between saline and reserpine-treated animals ($p<0.0001$). Reliability of scoring is evident in Figure 3.14.B with scoring grades from both independent researchers were positively correlated ($r=0.86$). The presence of the ptosis demonstrates that reserpine treatment in mice is capable of chemically depleting catecholamines and thus reducing adrenergic tone.

Before any experimental IOP data could be collected it was important to rule out the possibility that the systemic hypotensive actions of reserpine alone can significantly alter IOP between saline and reserpine treated animals. To determine this, baseline mean IOP measurements were recorded after 24 hours in both saline and reserpine pretreated animals (Figure 3.15.A). There were no detectable differences in IOP. Mean IOP recorded from saline-control and reserpine animals were 9.70 ± 0.26 mmHg ($n=18$) and 9.70 ± 0.25 mmHg ($n=18$), respectively ($p>0.05$). To further demonstrate that reserpine was capable of producing a chemical sympathectomy the effect of timolol 0.5% was assessed in reserpine and saline pretreated animals (figure 3.15B). Saline-pretreated animals ($n=6$,) had a significant reduction of -0.85 ± 0.05 in IOP in response to topically applied timolol 0.5% when compared to saline-vehicle ($p<0.0005$). In contrast, the reserpine-treated animals did not show any significant alterations in IOP in response to timolol 0.5% (-0.06 ± 0.09 , $n=5$)

Figure 3.14: *Chemical sympathectomy by reserpine produces ptosis.* A. Saline treated animals ($n=5$) produce a mean ptosis grade of 0, while reserpine treated animals ($n=4$) received a mean ptosis grade of 2 by researcher A (closed bars). Saline treated animals produce a mean ptosis grade of 0.5, while reserpine treated animals received a mean ptosis grade of 2.1 by researcher B (open bars). Reserpine treated animals were likely to have a ptosis compared to saline ($p<0.01$) B. raw grading values of ptosis of each animal across both researchers, correlation coefficient, $r=0.86$.

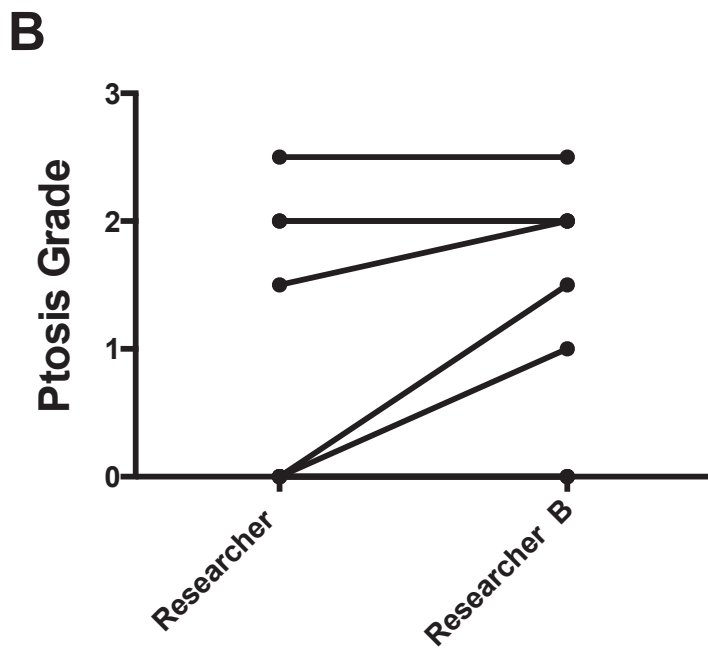
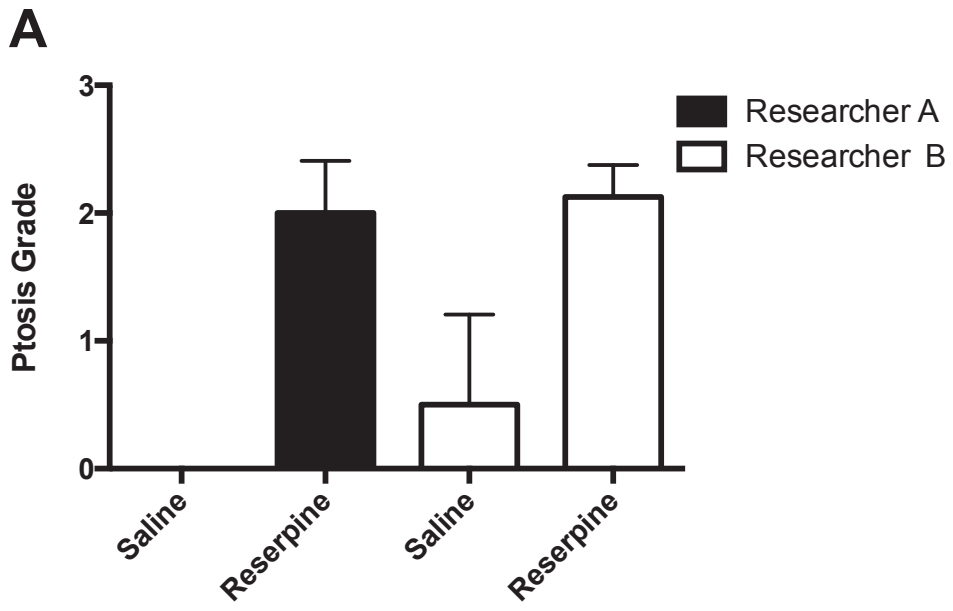
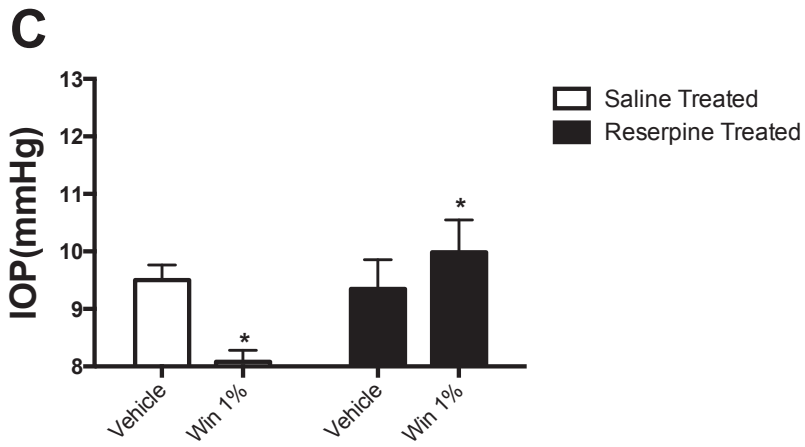
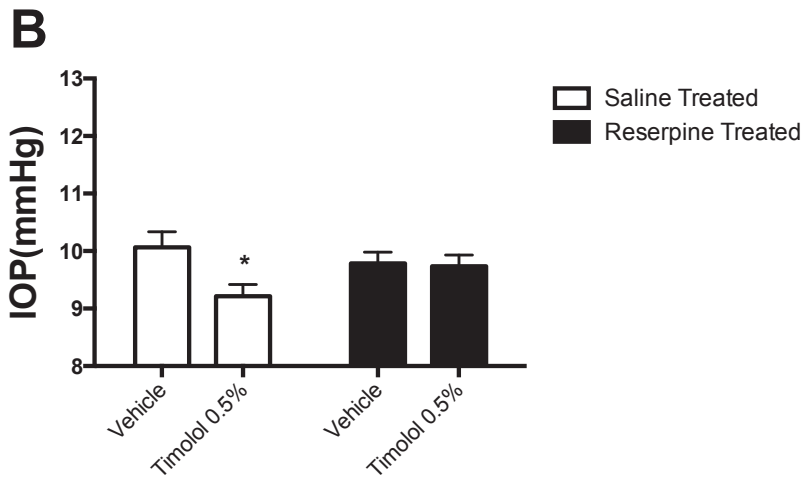
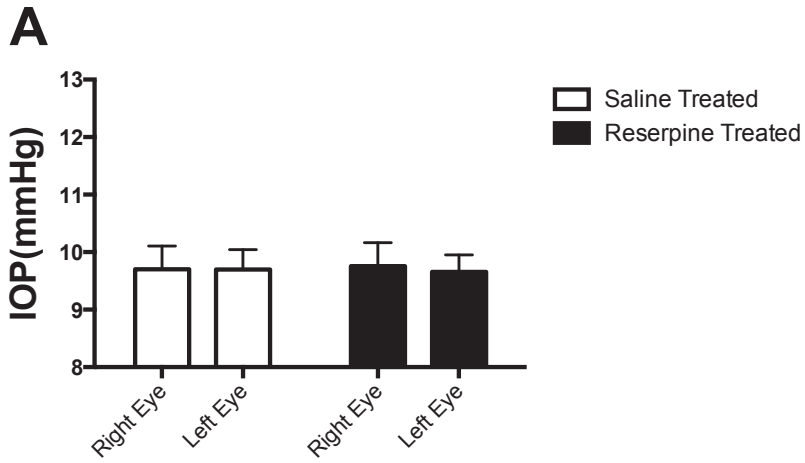


Figure 3.15: Chemical sympathectomy by reserpine abolishes ocular *hypotensive responses to timolol and WIN*. A. Mean IOP taken from C57 mice 24 hours after pretreatment with saline or reserpine. B. Mean IOP taken 1 hour after topical timolol treatment of timolol from animals that were pretreated with either saline ($p < 0.001$, $n = 6$) or reserpine ($p > 0.05$, $n = 5$). C. Mean IOP taken 30 minutes after topical treatment of WIN from animals that were pretreated with either saline ($p < 0.001$, $n = 6$), or reserpine ($p < 0.05$, $n = 6$).



With an effective chemical sympathectomy via reserpine established, the involvement of the sympathetic system in cannabinoid-mediated ocular hypotension was assessed. Figure 3.15.C demonstrates treatment with WIN 1% failed to reduce IOP in reserpine-pretreated animals when measured 24 hours after reserpine ($n=6$). Unexpectedly, when applied topically WIN,1% produced a significant increase in IOP of 0.633 ± 0.20 ($p<0.05$). In the saline pre-treated animals, WIN 1% continued to reduce IOP with reductions of -1.417 ± 0.168 ($n=6$) with the saline-pretreated animals ($p<0.005$).

Taken together, these results demonstrate that reserpine was effective at creating a chemical sympathectomy by depleting catecholamines. Furthermore, the ocular hypotensive actions of cannabinoid agonists were absent in animals that received a sympathectomy, therefore requiring the presence of catecholamines. This evidence suggests that CB1 cannabinoid agonists lower IOP via indirect sympatholytic actions.

3.3. Non-Psychotropic Cannabinoids

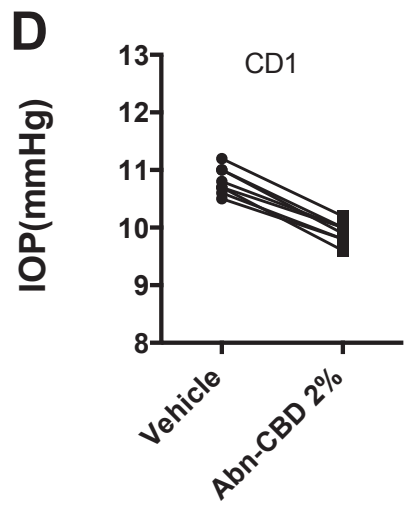
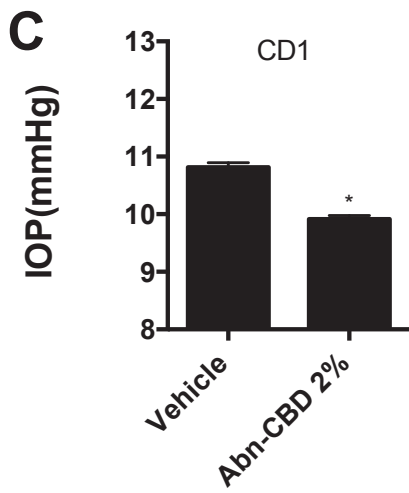
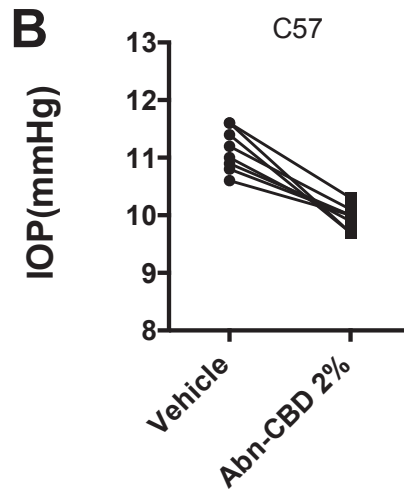
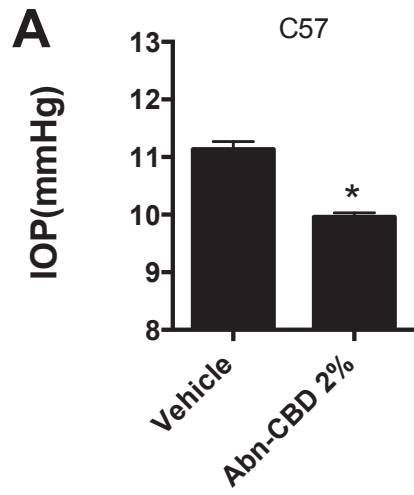
The reduction in IOP by psychotropic cannabinoids is mediated through CB1 and is dependent on an intact sympathetic nervous system. This finding provides evidence that CB1 ligands do not provide a novel mechanism of action of reducing IOP compared to clinically available therapeutics such as the non-selective β -blocker timolol, which also don't have the associating potential of psychoactivity with CB1 activation. Recently however, behaviourally-inactive cannabinoids and cannabimimetic lipids have been identified that exert cannabinoid-like actions but

have no affinity for CB1 and CB2 thus lacking psychoactive side-effects(Járai et al., 1999; Szczesniak et al., 2011)

However, the pharmacology of these atypical cannabinoids and cannabimimetic lipids has not been extensively explored and both receptor and tissue targets are still to be determined. In addition, the therapeutic potential of these compounds in the treatment of ocular hypertension and glaucoma remains unknown. Using pharmacological and transgenic mouse strains, my experiments will provide evidence that both atypical cannabinoids and cannabimimetic lipids are capable of reducing IOP when applied topically to eye.

The atypical cannabinoid, Abn-CBD, an analogue of the phytocannabinoid, cannabidiol was the first compound tested. Previous research has shown that Abn-CBD was capable of producing systemic hypotension and ocular hypotension in the rat, but it is unknown whether this is the case in a mouse model (Adams et al., 1977; Ho & Hiley, 2003; Szczesniak et al., 2011) When applied topically Abn-CBD 2% significantly lowered IOP when compared to Tocrisolve™-vehicle in C57 WT animals (Figure 3.16.A). Figure 3.16.B shows the reduction in IOP by Abn-CBD 2% (-1.18 ± 0.16 mmHg) was measured in all C57 animals tested ($p < 0.001$, $n=8$). As observed with the C57 WT mice, Figure 3.16C shows IOP was reduced (-0.90 ± 0.06 mmHg) with the topical application of Abn-CBD 2% in CD1 animals ($p < 0.0001$, $n=8$). There was clear ocular hypotensive effect of Abn-CBD 2% across all CD1 WT animals (Figure 3.16.D). These findings are in agreement with previous studies that carried out similar experiments in the rat (Szczesniak et al., 2011).

Figure 3.16: *Abnormal cannabidiol lowers IOP in C57 and CD1 wildtype mice.* A. Mean IOP taken from C57 mice 30 minutes after topical administration of Abn-CBD 2.0% or vehicle ($p < 0.001$, $n = 8$). B. Individual variation in IOP between vehicle and Abn-CBD treated eyes C. Mean IOP taken from CD1 mice 30 minutes after topical administration of Abn-CBD 2% or vehicle ($p < 0.0001$, $n = 8$). D. Individual variation in IOP between vehicle and Abn-CBD treated eyes.



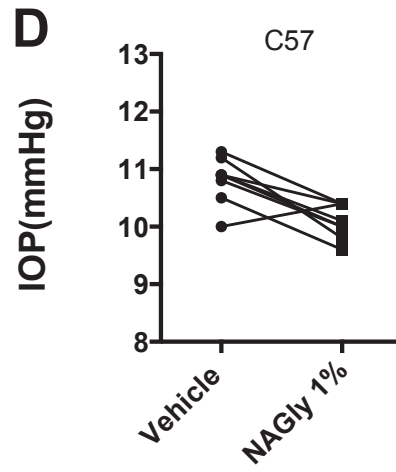
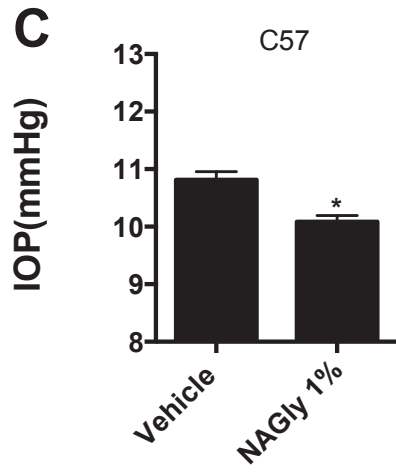
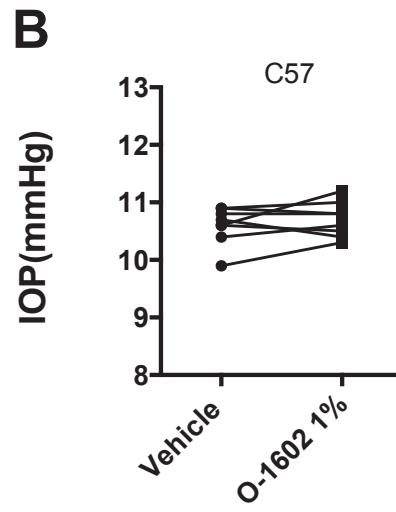
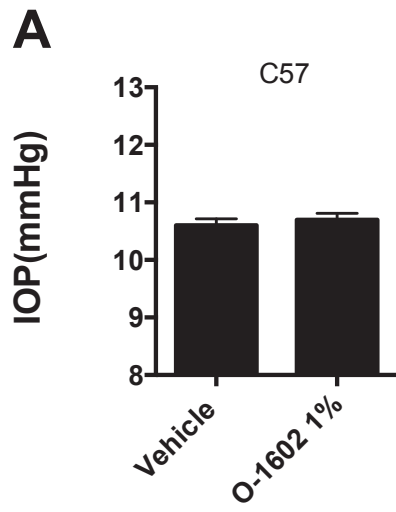
With the ocular hypotensive effects of Abn-CBD established in a wild-type mouse model, next it was important to discern which potential receptors were potentially involved. Although currently, CB1 and CB2 are the only classified cannabinoid receptors, some cannabinoids and cannabimimetic lipids have shown activity at other receptor targets, particularly transient receptor potential ion channels (TRPV1) and GPCR's, GPR18 and GPR55 (De Petrocellis et al., 2012; Lam et al., 2005; McHugh, et al., 2012; McHugh et al., 2010). Previous research has suggested that Abn-CBD lowers IOP independent of CB1 and CB2 receptors and is capable of activating both GPR18 and GPR55, two orphan GPCR's that have been implicated as putative cannabinoid receptors themselves (Johns et al., 2007; McHugh, et al.2012; Szczesniak et al., 2011).

Non-CB1 and CB2 receptors GPR55, TRPV1 do not account for IOP reducing effects of Abn-CBD and NAGly

To determine if GPR55 or GPR18 activation collectively or independently was capable of lowering IOP, selective GPR55 and GPR18 ligands 0-1605 and NAGly, respectively, were assessed (Figure 3.17). The first ligand tested was 0-1605 1% did not show any significant alteration in IOP (0.10 ± 0.10 , $n=8$) compared to tocrisolve-vehicle when measured after topical application in C57 WT mice (Figure 3.17A). Figure 3.17.B shows no clear observable trend in IOP in response to topical 0-1602 1% when compared to tocrisolve-vehicle when IOP was measured 30 minutes afterdrop installation. In contrast, Figure 3.17C and D shows that when the GPR18 selective ligand NAGly 1% was applied topically there showed a significant reduction in IOP of -0.73 ± 0.18 compared to the vehicle treated eye ($p < 0.01$, $n=8$).

Figure 3.17: Selective GPR55 agonist 0-1602 fails to reduce IOP in C57BL6 mice while the selective GPR18 agonist N-arachidonyl glycine causes ocular hypotension.

A. Mean IOP taken from C57BL6 wildtype mice 1 Hour after topical administration of 0-1602 or vehicle ($p>0.05$ $n=8$). B. Individual variation in IOP between vehicle and 0-1602 treated eyes in C57 mice. C. Mean IOP taken from C57 mice 30 minutes after topical administration of NAGly 1% or vehicle ($p<0.001$, $n=8$). D. Individual variation in IOP between vehicle and NAGly treated eyes in C57 mice.



To more directly assess the role of GPR55 in the ocular hypotensive effects of Abn-CBD 2% and NAGly 1%, these compounds were subsequently assessed in GPR55^{-/-} mice. Similarly to WT mice, figure 3.18. A and B show Abn-CBD 2% continued to lower IOP (-0.85±0.15) with topical application after 30 minutes ($p < 0.001$, $n = 8$). NAGly 1% again reliably reduced IOP when applied topically (Figure 3.18.C and D) After 30 minutes NAGly 1% lowered IOP with a reductions of -0.88±0.18 in GPR55^{-/-} mice ($p < 0.01$, $n = 8$). The continued reduction in IOP by NAGly in GPR55^{-/-} mice suggests the involvement of GPR18. 0-1918 is an analogue of the cannabinoid cannabidiol and has been shown to antagonize the effects of Abn-CBD and NAGly (McHugh et al., 2010; Szczesniak et al., 2011). To determine if this antagonist was capable of blocking the ocular hypotensive effects produced by Abn-CBD and NAGly, C57 WT mice were pretreated with 0-1918 (i.p. 2mg/kg) 30 minutes before topical application of Abn-CBD 2% or NAGly 1%. In the presence of the 0-1918, neither Abn-CBD 2% (Figure 3.19.A. and B) or NAGly 1% (Figure 3.19. C and D) had a significant alteration in IOP in C57 WT mice (-0.07±0.11, $n = 8$, and 0.00±0.09 $n = 8$, respectively). To provide further evidence that GPR18 activation is responsible for the IOP-reducing effects of Abn-CBD and NAGly, these compounds were also evaluated in GPR55^{-/-} animals ($n = 8$). As observed in C57 mice, pretreatment with 0-1918 blocked the reduction in IOP produced by both Abn-CBD (Figure 3.20.A and B) and NAGly (Figure 3.20.C and D), with IOP alterations of -0.09±0.14, and -0.13±0.09 respectively.

Another potential target for behaviourally inactive cannabinoids is TRPV1. Previous research has shown that TRPV receptors are capable of being activated by

Figure 3.18: *Abnormal cannabidiol and N-arachidonyl glycine in GPR55^{-/-} mice.* A. Mean IOP measured from GPR55^{-/-} male mice 30 minutes after topical administration of Abn-CBD 2%, ($p>0.001$. $n=8$). B. Individual variation in IOP between vehicle and Abn-CBD treated eyes in GPR55^{-/-} mice. C. Mean IOP measured from GPR55^{-/-} male mice 30 minutes after topical administration of NAGly 1% or vehicle ($p<0.01$. $n=8$). D. Individual variation in IOP between vehicle and NAGly treated eyes in GPR55^{-/-} mice.

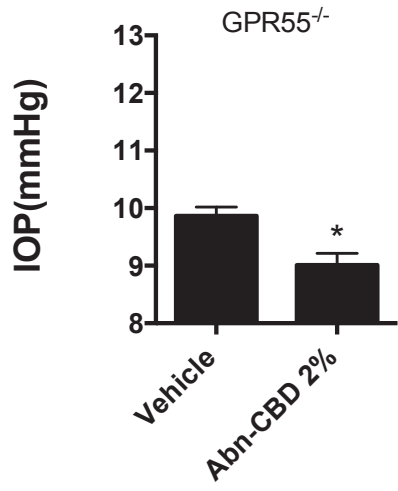
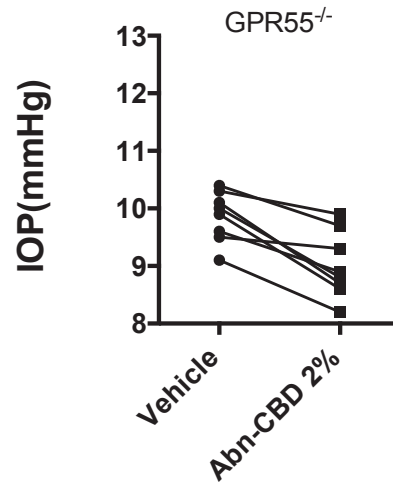
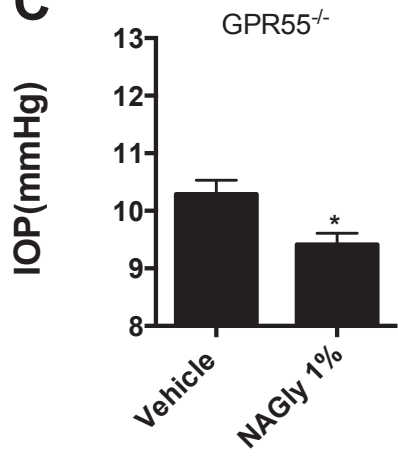
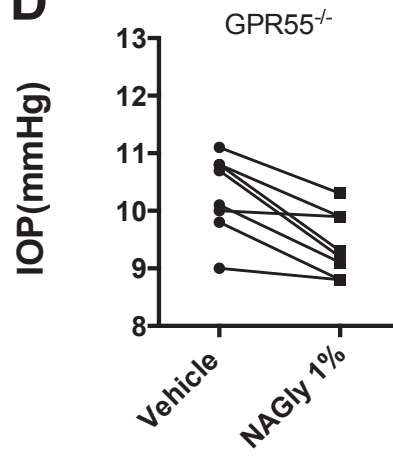
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Figure 3.19: *Abnormal cannabidiol receptor antagonist 0-1918 blocks Abn-CBD and NAGly ocular hypotensive actions in C57 wildtype mice.* A. Mean IOP of C57 wildtype mice pre-treated with 0-1918 taken after 30 minutes after topical administration of Abn-CBD 2% or vehicle ($p>0.05$, $n=8$). B. Individual variation of IOP with Abn-CBD 2% in C57 mice pre-treated with 0-1918. C. Mean IOP of C57 wildtype mice pre-treated with 0-1918 taken after 30 minutes after topical administration of NAGly 1% or vehicle ($p>0.05$, $n=8$). D. Individual variation of IOP with NAGly 1% in C57 mice pre-treated with 0-1918.

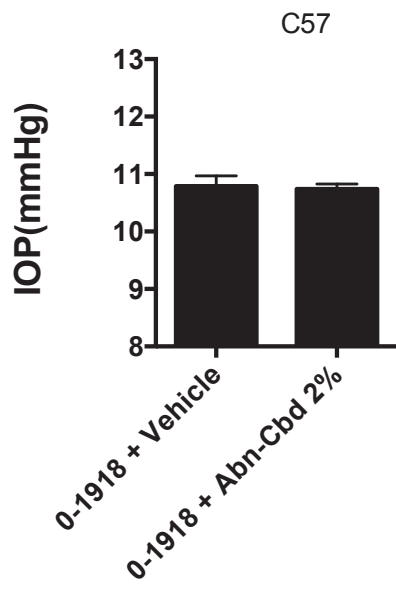
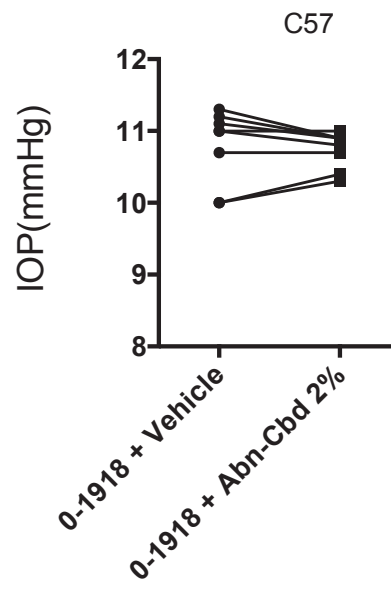
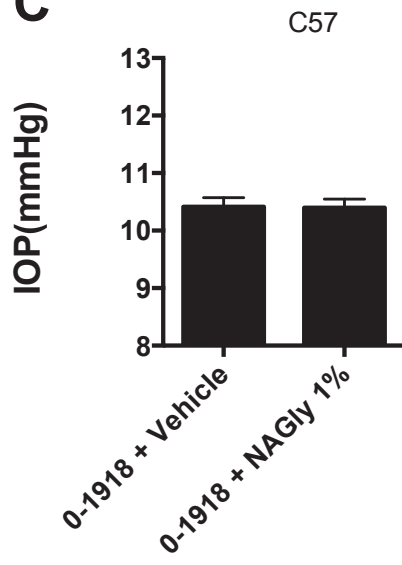
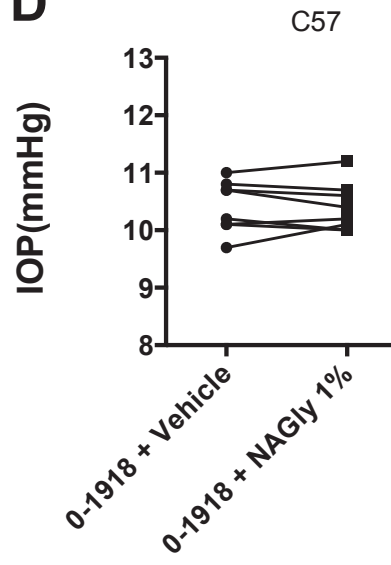
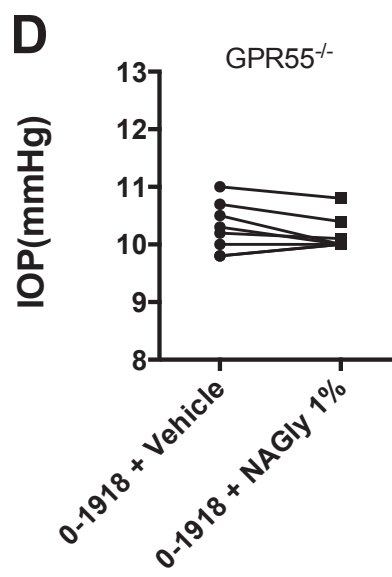
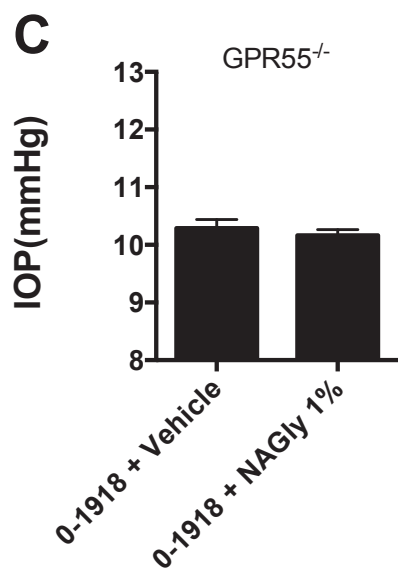
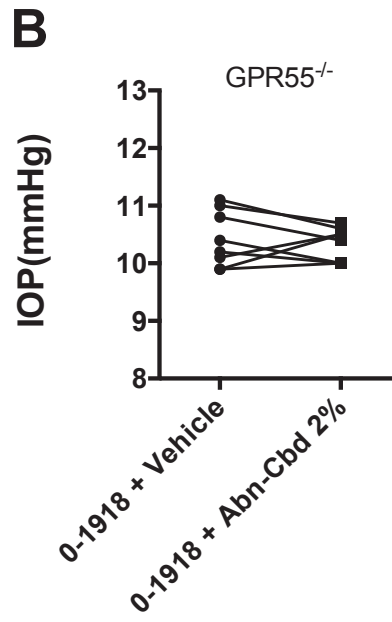
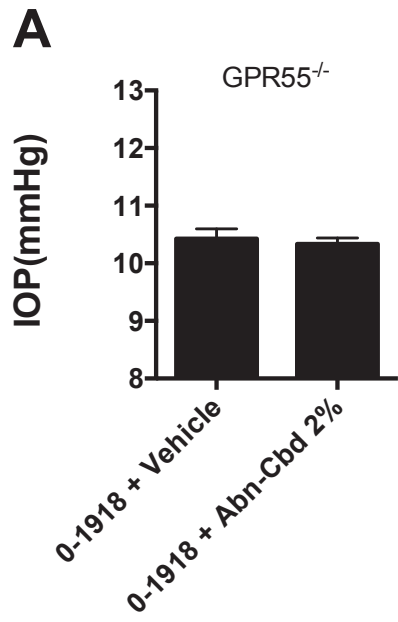
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Figure 3.20: *Abnormal cannabidiol receptor antagonist 0-1918 blocks Abn-CBD and NAGly ocular hypotensive actions in GPR55^{-/-} mice.* A. Mean IOP of Abn-CBD 2% in GPR55^{-/-} pre-treated with 0-1918 taken after 30 minutes after topical administration of Abn-CBD 2% or vehicle ($p>0.05$, $n=8$). B. Individual variation of IOP with Abn-CBD 2% in GPR55^{-/-} mice pre-treated with 0-1918. C. Mean IOP of NAGly 1% in GPR55^{-/-} pre-treated with 0-1918 taken after 30 minutes after topical administration of 1% NAGly or vehicle ($p>0.05$, $n=8$). D. Individual variation of IOP with NAGly 1% in GPR55^{-/-} mice pre-treated with 0-1918.



cannabinoids, particularly endocannabinoids such as AEA and the phytocannabinoid Δ^9 -THC (Lam et al., 2005; Smart et al., 2000). Therefore the involvement of TRPV1 in the IOP-lowering action of the GPR18 selective ligand NAGly was assessed in C57 WT mice using a pretreatment of the selective TRPV1 antagonist capsazepine (Figure 3.21.A and B). When capsazepine pre-treatment was given 30 minutes prior to NAGly 1% topical treatment (i.p.2mg/kg), there continued to be a reduction in IOP of -1.08 ± 0.12 ($p < 0.0001$, $n=8$).

Collectively, these findings provide evidence in support of GPR18 as a candidate receptor for the IOP reducing effect of Abn-CBD and NAGly. Moreover, these results also provide additional verification that 0-1918 is capable of blocking Abn-CBD and NAGly ocular hypotensive actions and is an antagonist for GPR18.

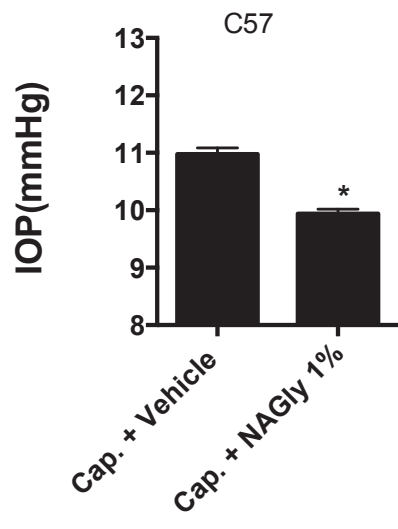
Behaviourally-inactive cannabinoids Abn-CBD and NAGly reduce IOP distinct for CB1 and CB2 receptors

To rule out the involvement of CB1 and CB2 receptors, the ocular hypotensive actions of Abn-CBD 2% and NAGly 1% were assessed in both CB1^{-/-} and CB2^{-/-} mice.

In CB1^{-/-} mice, Figure 3.22.A and B demonstrate that there was a significant reduction in IOP of -1.38 ± 0.17 mmHg in response to topically applied Abn-CBD 2% when measured 30 minutes after application ($p < 0.0001$, $n=8$). Topical application of NAGly 1% was also examined in CB1^{-/-} mice 30 minutes after topical application (Figure 3.22.C and D). Like Abn-CBD 2% there was a significant reduction in IOP of -1.41 ± 0.26 mmHg ($p < 0.001$, $n=8$).

Figure 3.21: *N-arachidonylglycine* lower IOP independent of TRPV1. A. Mean IOP of 1% NAGly in C57BL6 pre-treated with capsazepine taken after 30 minutes after topical administration of 1% NAGly or vehicle, $p < 0.0001$, $n = 8$. D. Individual variation of IOP with 1% NAGly in C57BL6 mice pre-treated with 0-1918.

A



B

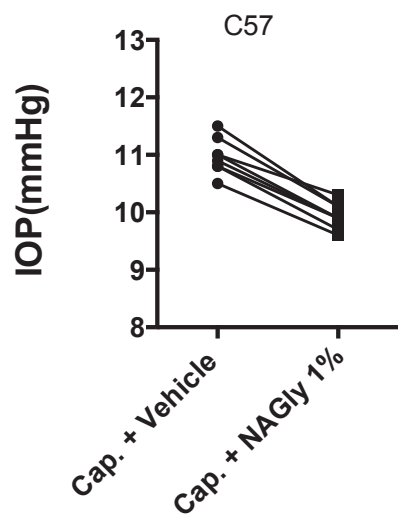
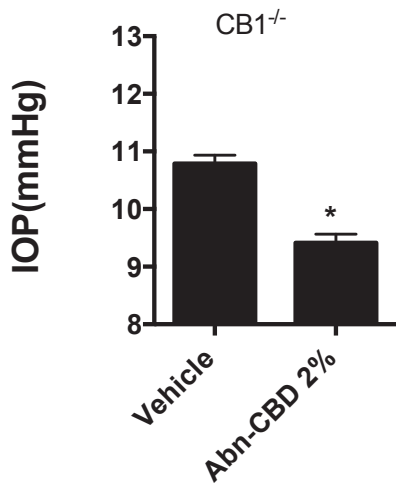
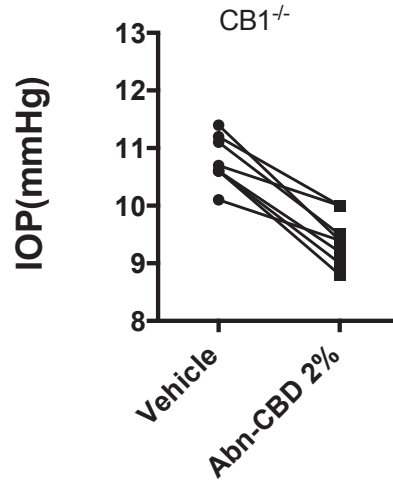
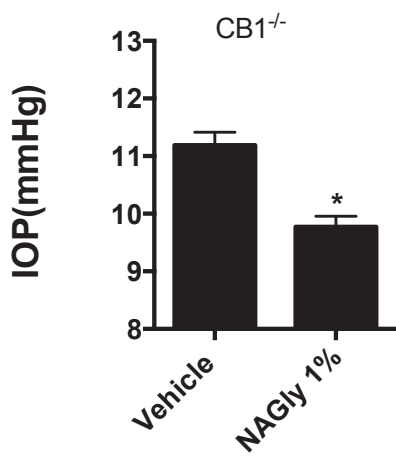
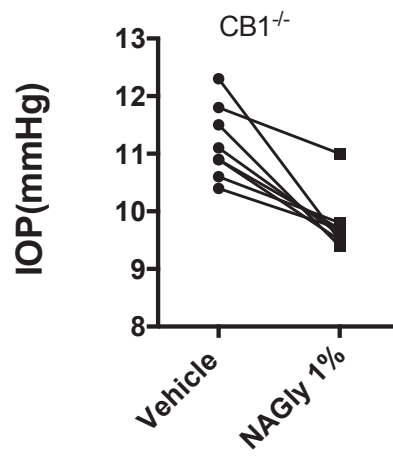


Figure 3.22: *Abnormal cannabidiol and N-arachidonyl glycine in CB1^{-/-} mice.* A. Mean IOP taken from CB1^{-/-} male mice 30 minutes after topical administration of Abn-CBD 2%, ($p < 0.0001$, $n = 8$). B. Individual variation in IOP between vehicle and Abn-CBD treated eyes in CB1^{-/-} mice. C. Mean IOP taken from CB1^{-/-} male mice 30 minutes after topical administration of 1% NAGly or vehicle ($p < 0.001$, $n = 8$). D. Individual variation in IOP between vehicle and NAGly treated eyes in CB1^{-/-} mice.

A**B****C****D**

This finding provides evidence that CB1 is not accountable for any of the ocular hypotensive effects of Abn-CBD and NAGly. The involvement of CB2 was also assessed in transgenic mice lacking the CB2 receptor. As seen in the CB1^{-/-} mice, Abn-CBD 2% continued to reduce IOP (-0.68±0.09 mmHg) significantly ($p<0.01$, $n=4$) with topical application when measured at the same time point of 30 minutes (Figure 3.23.A and B). NAGly 1% consistently showed ocular hypotensive actions of -1.22±0.23mmHg ($p<0.01$, $n=5$) in CB2^{-/-} mice (Figure 3.23.C and D). These data provide evidence that the behaviorally-inactive cannabinoids, Abn-CBD and NAGly are capable of reducing IOP independent of CB1 or CB2 receptors.

Non-psychotropic cannabinoids Abn-CBD and NAGly lower IOP independent of the sympathetic nervous system

I have previously shown that behaviourally-active cannabinoids activate CB1 receptors to lower IOP. This CB1-mediated ocular hypotensive effect of behaviourally-active cannabinoids also depends on an intact sympathetic nervous system requiring the presence of β -AR receptors and catecholamines. To determine if behaviourally inactive cannabinoids reduce IOP in a similar mechanism, Abn-CBD 2% and NAGly 1% were examined in β AR^{-/-} mice mechanism, Abn-CBD 2% and NAGly 1% were examined in β AR^{-/-} mice. Figure 3.24.A and B show Abn-CBD 2% was still able to reduce IOP (-1.22±0.08 mmHg) in β AR^{-/-} mice using the same experimental parameters as CB1^{-/-} mice ($p<0.0001$, $n=8$). Similarly, although to a lesser magnitude NAGly 1% continued to reduce IOP (-0.71±0.12 mmHg, $p<0.001$, $n=8$) in β AR^{-/-} mice (Figure 3.24. C and D).

Figure 3.23: *Abnormal cannabidiol and N-arachidonyl glycine in CB2^{-/-} mice.* A. Mean IOP taken from CB2^{-/-} male mice 30 minutes after topical administration of Abn-CBD 2%, ($p < 0.01$, $n = 4$). B. Individual variation in IOP between vehicle and Abn-CBD treated eyes in CB2^{-/-} mice. C. Mean IOP taken from CB2^{-/-} male mice 30 minutes after topical administration of NAGly 1% or vehicle ($p < 0.01$, $n = 5$). D. Individual variation in IOP between vehicle and NAGly treated eyes in CB2^{-/-} mice.

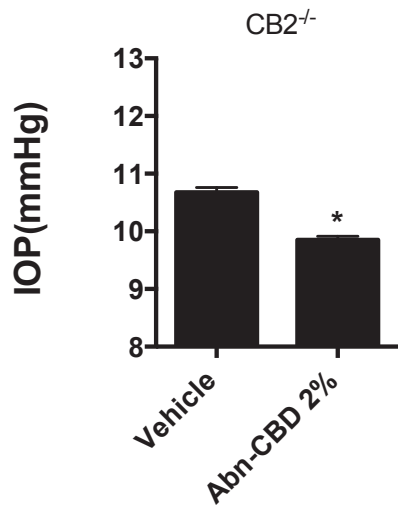
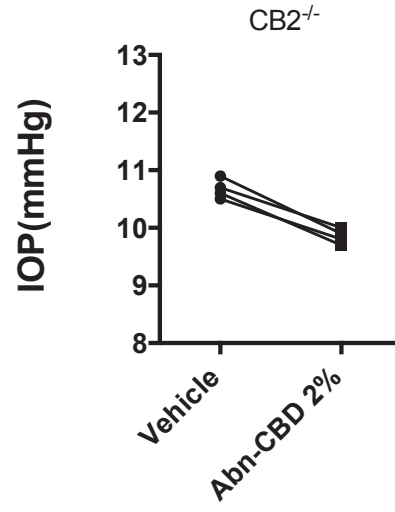
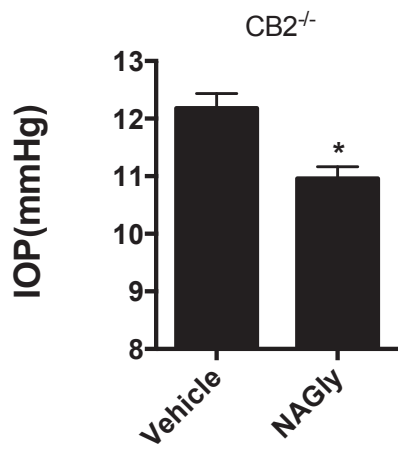
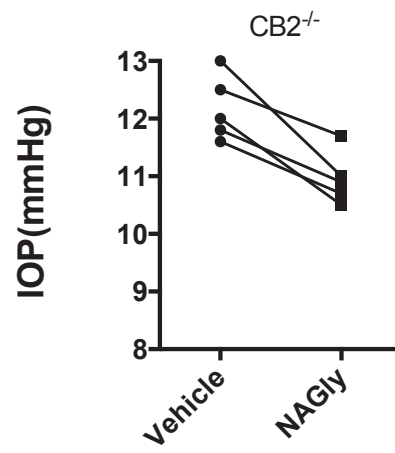
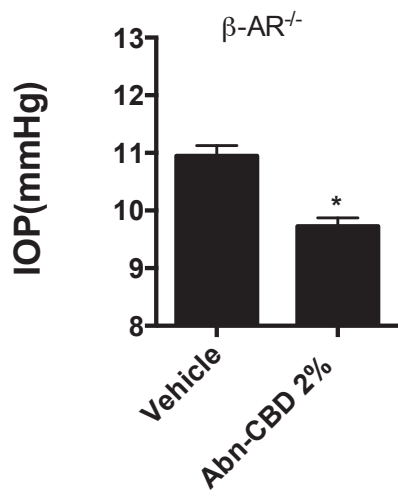
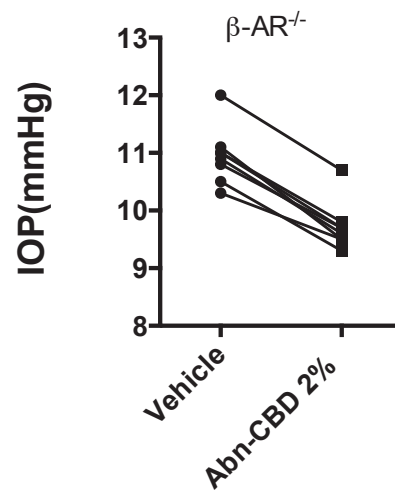
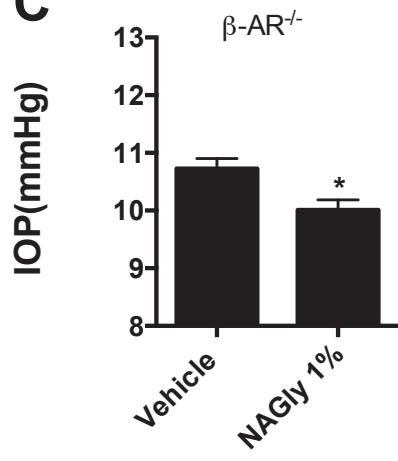
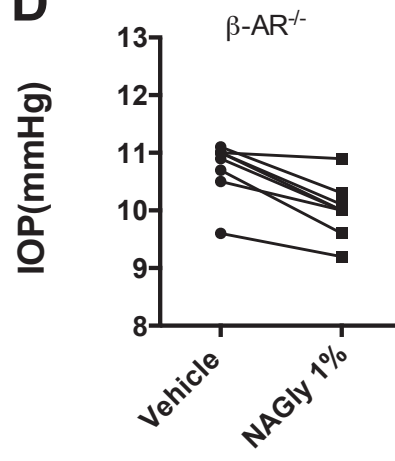
A**B****C****D**

Figure 3.24: *Abnormal cannabidiol and N-arachidonyl glycine lower IOP independent of the adrenergic nervous system.* A. Mean IOP taken from β -AR^{-/-} male mice 30 minutes after topical administration of Abn-CBD 2% or vehicle ($p < 0.0001$, $n = 8$). B. Individual variation in IOP between vehicle and Abn-CBD treated eyes in β -AR^{-/-} mice. C. Mean IOP taken from β -AR^{-/-} male mice 30 minutes after topical administration of 1% NAGly or vehicle ($p < 0.001$, $n = 8$). D. Individual variation in IOP between vehicle and NAGly treated eyes in β -AR^{-/-} mice.

A**B****C****D**

To further assess if alternations in sympathetic nervous system activity play a role in the GPR18 mediated ocular hypotensive actions of NAGly, the effect of a chemical sympathectomy via reserpine pre-treatment was assessed (Figure 3.25). In both saline ($n=5$) and reserpine ($n=5$) pre-treated animals, the topical application of NAGly1% significantly ($p<0.05$) lowered IOP compared to the vehicle-treated eye. There was a reduction of -0.58 ± 0.18 for the saline group and -0.62 ± 0.22 for the reserpine group. These findings are in contrast to cannabinoids that lower IOP via a CB1 mechanism and suggest that ocular hypotensive effects of the behaviourally-inactive cannabinoids, Abn-CBD and NAGly, exert their IOP lowering effects independent of the BAR and sympathetic nervous system.

Comparison of behaviourally-inactive cannabinoids to clinically available ocular hypotensive therapeutics and behaviourally-active cannabinoids

I have demonstrated that the behaviourally-inactive cannabinoid, Abn-CBD and the cannabimimetic lipid NAGly are able to produce statistically significant levels of ocular hypotension. However to determine whether there is a potential clinical significance, the reduction in IOP produced by Abn-CBD and NAGly were compared to clinically available drugs. Figure 3.26.A shows the IOP-lowering effect of Abn-CBD compared with the beta-blocker timolol, the prostaglandin analogue, latanoprost, and the carbonic anhydrase inhibitor, dorzolamide, in addition to the non-selective cannabinoid CB1 and CB2 receptor agonists, WIN and CP. No statistically significant differences in the reduction of IOP were observed between any of the groups in C57 WT mice [F (5,47)=0.4456, $p=0.81$].

Figure 3.25: *Chemical sympathectomy by reserpine does not affect ocular hypotensive actions of NAGly.* Mean IOP taken 30 minutes after topical NAGly 1% treatment from animals that were pretreated with either saline ($p < 0.05$, $n = 5$) or reserpine ($p < 0.05$, $n = 5$).

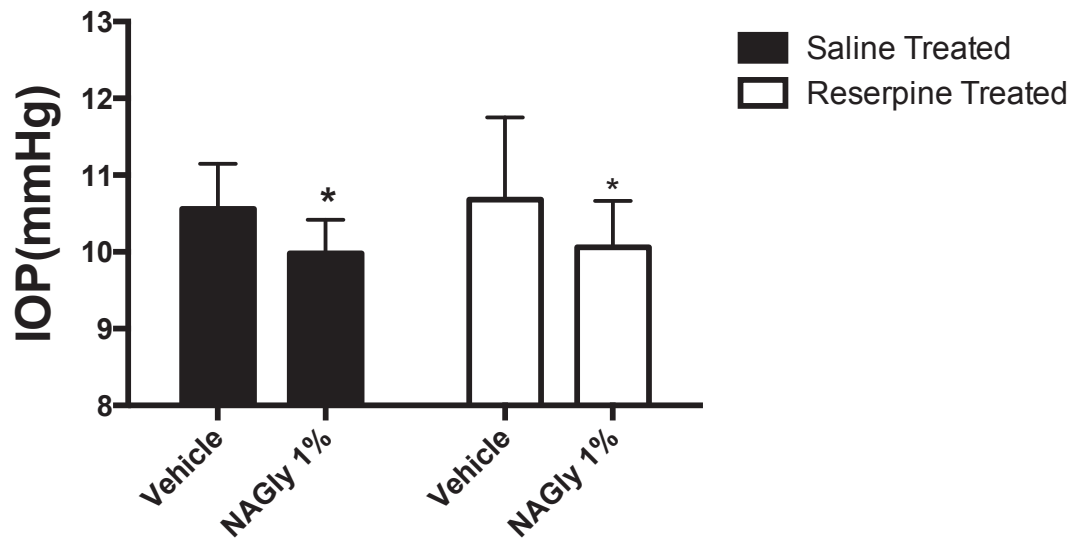
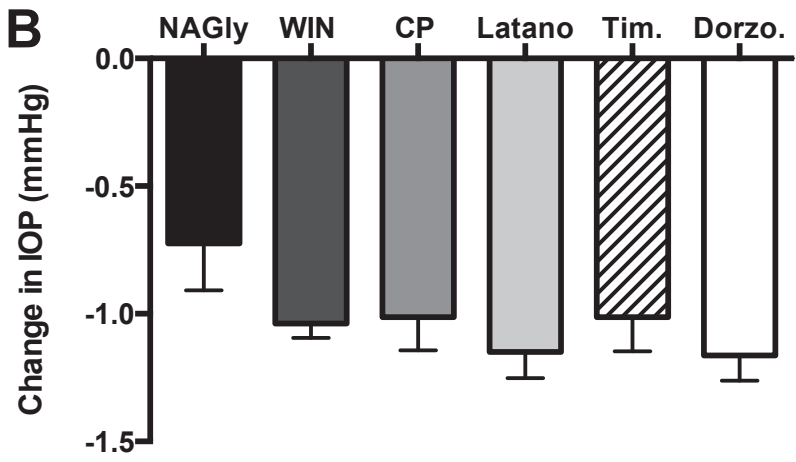
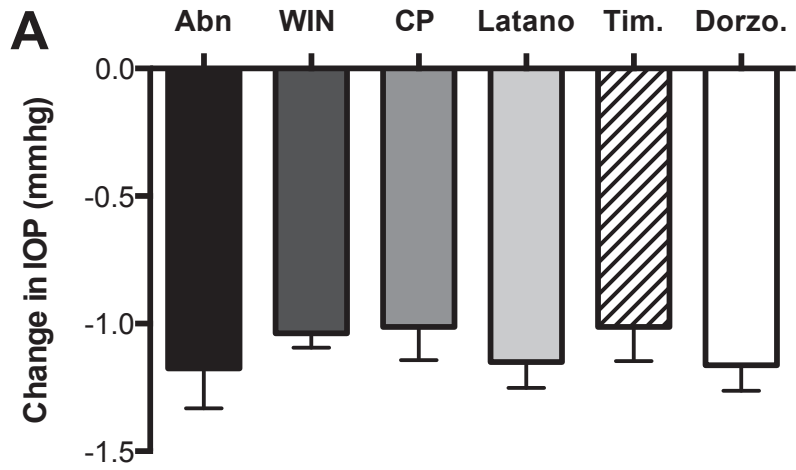


Figure 3.26: *Topical application of abnormal cannabidiol and N-arachidonyl glycine reduces IOP comparable to known ocular hypotensive drugs.* A. Mean change in IOP following treatment of Abn-CBD, WIN, CP, , latanoprost, timolol and dorzolamide, $n=8$ for each respective group. No significant difference between each treatment on IOP (1-way ANOVA; $p>0.05$). B. Mean change in IOP following treatment of NAGly, WIN, CP, , latanoprost, timolol and dorzolamide, , $n=8$ for each group respectively. No significant difference between each treatment on IOP (1-way ANOVA; $p>0.05$).



Similarly as observed with Abn-CBD 2%, Figure 3.26.B demonstrates that the reduction in IOP by NAGly 1% was not significantly different to other glaucoma medications tested in this study (timolol, latanoprost, & dorzolamide) or behaviourally-active cannabinoids (WIN & CP) when assessed in C57 WT mice [F(5,47)=1.62, p=0.11]. Together these findings suggest that behaviourally-inactive cannabinoids and cannabimimetic lipids are capable of producing a therapeutic reduction in IOP, which potentially could be beneficial in the clinical setting of glaucoma medical management.

Chapter 4 : Discussion

4.1 Overview

IOP is the principal modifiable risk factor for the progression of glaucoma (Chauhan et al., 2010; Nemesure et al., 2007; Quigley, 2011). Although IOP is not necessarily increased in all cases of glaucoma, reducing IOP, irrespective of baseline values, has been shown to slow progression of the disease (Kass et al., 2002). Therefore, current medical and surgical treatment is aimed at reducing IOP, and drugs that are capable of reducing IOP (ocular hypotensives) are potential anti-glaucoma agents (Marquis & Whitson, 2005).

The identification of a local ECS system in the eye, including endocannabinoid ligands, AEA and 2-AG, cannabinoid receptors, CB1 and CB2, and enzymes that synthesize and degrade endocannabinoids, has renewed interest in further examining the potential of targeting the ECS for glaucoma (Bisogno et al., 1999; Chen et al., 2005; Hu et al., 2010; Straiker et al., 1999). My thesis examined the mechanisms underlying the IOP reduction by cannabinoid agonists that act as CB1 and CB2, including WIN and CP, and the endogenous lipid NAGly which activates recently deorphanized non-cannabinoid receptor, GPR18; this receptor is also activated by the atypical cannabinoid, Abn-CBD.

Through the use of transgenic mice and pharmacological approaches, I demonstrated that β -adrenergic receptors and catecholamines are required for CB1-mediated IOP reduction. Cannabinoids that act at CB1 reduce IOP through an adrenergic-dependent and CB2-independent mechanism. (Hudson et al., 2011).

Additionally, I demonstrated that the endogenous lipid, NAGly, and the atypical cannabinoid, Abn-CBD, are able to produce ocular hypotension through GPR18 activation (Caldwell et al., 2013). The following sections discuss these findings as well as the limitations and potential for the use of cannabinoids and cannabimimetic lipids as ocular hypotensive agents in the treatment of glaucoma.

4.2 Cannabinoids lower IOP

I used rebound tonometry in various strains of mice to examine the effects of cannabinoids and lipids on IOP (Hudson et al., 2011; Hudson, 2010). Importantly, given the strain variability presented in the literature, I first validate diurnal variation in all strains of mice used in this study (Savinova et al., 2001). As with previous reports of diurnal variation in mice, all strains, including wild-type and transgenic mice, had higher IOP at 21:00 compared to 9:00 (Hudson, 2010). These results are consistent with previous reports in rodents and contrast diurnal IOP variation observed in humans, where IOP is higher in the morning compared to evening (Aihara, 2003; Goel et al., 2010).

My results demonstrated that non-selective cannabinoid agonists that act at both CB1 and CB2 are capable of producing ocular hypotension (see Section 3.2). In C57 WT mice, the synthetic cannabinoids WIN 1% and CP 1% reduced IOP when applied topically. These findings are in-line with previous reports of ocular hypotension produced by cannabinoid agonists (Hosseini et al., 2006; Szczesniak et al., 2006). As the cannabinoids used in my study have activity at both CB1 and CB2, it was then important to determine whether both receptors are involved in the IOP

reduction or whether the effects were primarily mediated by CB1, as has been suggested by other studies (Hudson, 2010; Pate et al., 1998). Using transgenic mice lacking CB2, WIN and CP still produced a decrease in IOP, and the CB1 selective antagonist, AM281, blocked this effect. This finding, therefore, confirmed that CB2 was not involved in the IOP lowering actions of non-selective cannabinoids in mice. This result was further substantiated by the lack of efficacy seen with the CB2 selective agonist, HU308, applied topically in wildtype mice, in which there was no observable effect on IOP. Consistent with my results, Laine et al. (2003) examined the topical effect of CB2 selective agonist JWH133 in rabbit eyes and did not find any reduction in IOP. In contrast, Zhong et al. (2005) found that JWH015 was able to reduce IOP and these authors hypothesized that this was mediated by CB2 receptors expressed in trabecular meshwork cells. The inconsistency of the role of CB2 in the literature could be due to different species studied and *in vivo* versus *in vitro* models (Laine et al., 2003; Zhong et al., 2005). Additionally, dose-dependent off-target CB1 effects of CB2 agonists have been reported *in vivo* and may have accounted for the JWH015 mediated ocular hypotensive actions reported by Zhong et al. (Murataeva et al., 2012). To date, no further follow up work has examined CB2 expression in tissues involved with AH secretion or outflow, and lack of useful antibodies has hampered detailed anatomical studies of CB2 protein localization in the anterior eye.

The effect of CB1 was directly examined in my studies using genetically modified CD1 mice lacking the CB1 receptor. My results showed that the ocular hypotensive effect of WIN was lost in CB1^{-/-} mice and further unmasked a

potentiation of IOP by WIN compared to CD1 wildtype control. These findings are consistent with the work of Hudson (2010), who also saw the potentiation of IOP with WIN in the C57 strain of mice lacking the CB1 receptor. These findings, therefore, suggest that the WIN-mediated IOP increase in mice lacking CB1 is not strain dependent.

WIN belongs to the aminoalkylindole class of cannabinoids (Table 1.1; reviewed by Pertwee et al., 2010). I also examined the non-classical cannabinoid, CP, in order to determine if the results obtained with WIN were representative of other classes of cannabinoid ligands. Like WIN, CP failed to reduce IOP in CB1^{-/-} mice. However, unlike the aminoalkylindole, WIN, there was no substantial increase in IOP seen with CP. This suggests that the increase in IOP in CB1^{-/-} mice is isolated to the aminoalkylindole class of cannabinoids. The cannabinoid-mediated increase in IOP in CB1^{-/-} mice also appears to be independent of CB2, persisting in the presence of the CB2 antagonist, AM630. Further studies are required to explore additional mechanisms for this effect.

4.3 CB1 mediated ocular hypotension occurs through adrenergic mechanisms

I assessed several adrenergic agents in WT mice, all reduced IOP, consistent with their use in clinical practice as systemic and ocular hypotensive agents (Alvarado et al., 1998; Kolko, 2014; Willis, 2004; Woodward & Gil, 2004). The β -agonist, ISO, was also able to lower IOP, an effect comparable to timolol treatment (figure 3.7B). However, although both β -antagonists and agonists reduce IOP, they

do so by contrasting mechanisms by reducing AH secretion and increasing outflow, respectively (Alvarado et al., 1998; Kolko, 2014; Woodward & Gil, 2004). These findings are consistent with clinical practice where β -antagonists are prescribed as a first line treatment for glaucoma, and also as an adjunct therapy with CAI's, and prostaglandin analogues (Marquis & Whitson, 2005; Radcliffe, 2014).

Recently, Hudson et al. (2010) reported that the cannabinoid agonist, WIN, failed to reduce IOP in transgenic mice lacking both β 1 and β 2 adrenergic receptors, suggesting that the IOP-lowering actions of cannabinoids involved β -adrenergic receptors. My research work corroborated these findings (Section 3,2; figure 3.5). I was able to show that the effects of the cannabinoids, WIN and CP, given topically, failed to reduce IOP in β 1/ β 2^{-/-} mice. I hypothesized that CB1 agonists may act at pre-synaptic CB1 receptors to reduce IOP via inhibition of NE release. Presynaptic CB1 activation has shown to decrease NE release and modulate neurotransmission in other tissues. To test this hypothesis, I used reserpine to deplete catecholamines and create a chemical sympathectomy (Martínez-Olivares et al., 2006; Olfe et al., 2010). Under these conditions, the CB1 agonist WIN was unable to reduce IOP. Decreased sympathetic tone following reserpine treatment was confirmed by the lack of IOP reduction via timolol (i.e no decrease in IOP) and by the presence of a ptosis. Alternation of sympathetic innervation is seen in Horner's syndrome, characterized by miosis, anhidrosis, and ptosis (Elkington & Khaw, 1988). However, as miosis and anhidrosis are difficult to assess in a mouse model, I used the presence of a ptosis to confirm decreased sympathetic innervation.

There are several lines of evidence supporting cannabinoid-adrenergic

interactions. One of the earlier studies implicating cannabinoid-adrenergic interactions showed that Δ^9 -THC had a reduced effect in animals that received adrenergic blocking drugs or sympathetic ganglionectomy (Green and Kim (1976). Furthermore, from an anatomical point of view, CB1 and β -AR's have been shown to be co-localized in the ciliary epithelium of the ciliary body and angle (Hudson et al., 2011). Therefore, CB1 activation could decrease AH secretion in the ciliary epithelium through presynaptic inhibition of NE release, thus acting like a β -antagonist. However, it is also possible that similar mechanisms could operate to cause an increase in IOP. Given that activation of β ARs in the trabecular meshwork increases outflow, CB1 mediated sympatholytic activity could decrease outflow through the TM pathway. As IOP represents a balance of AH inflow and outflow, the net reduction in IOP with CB1 activation suggests a greater effect on AH secretion (Goel et al., 2010). This is supported by primate studies showing that cannabinoids acting at CB1 decrease AH secretion (Chien et al.,2003). Additionally, adrenergic innervation in the ciliary epithelium is greater than in TM outflow pathways; therefore effect of CB1 modulation on adrenergic activity would be expected to have greater effect in the ciliary epithelium, by decreasing secretion of AH and consequently reducing IOP (Akagi et al.,, 1976).

Further support for overlapping mechanisms between cannabinoid and adrenergic systems on IOP has also come from studies showing an absence of additive effects with the β -antagonist, timolol and CB1 agonist WIN (Green et al., 1977; Hudson, 2010; Oltmanns et al., 2008). Additive effects on IOP reduction would

suggests that the mechanism(s) by which IOP is reduced occurs through independent pathways/mechanisms in both rodents and humans (Akaishi et al., 2009).

One unexpected finding of my study was that timolol failed to reduce IOP in CB1^{-/-} mice (Section 3.2; Figure 3.9). Hudson (2010) proposed that timolol should produce a greater reduction in IOP in CB1^{-/-} than WT control mice. The elimination of CB1 could theoretically contribute to increased NE tone because there would be no endogenous endocannabinoid activation of CB1 contributing to inhibition of NE release. This would result in enhanced efficacy of β -antagonists. Furthermore, increased NE tone has been already reported in CB1^{-/-} mice (Quarta et al., 2010; Schlicker et al., 2003). However, despite this evidence, my studies found no significant effect of timolol in CB1^{-/-} mice. I then examined other β -adrenergic agents, including the β_1 antagonist, metoprolol, and non-selective β agonist, ISO, all of which failed to produce ocular hypotensive actions in CB1^{-/-} mice (Section 3.2; Figure 3.9, 3.10). This is an interesting finding because the β -agonist, ISO, and β -antagonists metoprolol and timolol lower IOP by different mechanisms. The former lowers IOP by increasing outflow through the trabecular meshwork pathway whereas β -antagonists decrease secretion in the ciliary epithelium. The only commonality between the 2 groups of agents is that they involve β -adrenergic receptors.

One explanation for lack of effect of β adrenergic drugs in CB1^{-/-} mice is that lack of endogenous CB1 activation leading to increased adrenergic tone results in

compensatory desensitization of ARs (Chao & Walkenbach, 1985). This hypothesis was tested by experiments examining β -receptor desensitization of WT mice following chronic administration with ISO (Section 3.2; Figure 3.11.) Following 2 weeks of chronic treatment with the β -agonist, ISO, both timolol and WIN, failed to reduce IOP, thus supporting my hypothesis that timolol failed to reduce IOP in CB1^{-/-} mice due to β -receptor desensitization.

Taken together, my results, and previous findings reported by Green and Kim (1978), Oltmans et al., 2008, Hudson (2010) and Hudson et al.(2011), provide evidence for the involvement of β -adrenergic receptors and modulation of catecholamine release in the ocular hypotensive actions of cannabinoids.

4.4 Atypical cannabinoids and cannabimimetic lipids lower IOP

In addition to cannabinoids that act at cannabinoid receptors, I also examined the mechanisms of action by which the atypical cannabinoid, Abn-CBD, and the endogenous lipid NAGly reduce IOP. Both Abn-CBD and NAGly are non-psychotropic and have been reported to act at non-CB1/CB2 receptors (Begg et al., 2007; McHugh et al., 2010). My research demonstrated for the first time in mice that NAGly has ocular hypotensive properties and that both Abn-CBD and NAGly, applied topically in mice, can reduce IOP (Section 3.3, figures 3.16,-3.17).

The IOP-lowering actions of Abn-CBD and NAGly were independent of CB1 and CB2 activation, as indicated by the continued reduction in IOP in transgenic mice lacking CB1 and CB2 receptors. This finding is consistent with one previous report in rats that demonstrated that the reduction in IOP seen with the non-

psychotropic cannabinoids, Abn-CBD and CBG-DMH, were not blocked by a CB1 antagonist but were antagonized by O-1918, an antagonist for GPR55 and GPR18 (Szczesniak et al., 2011). Unlike CB1-agonists, NAGly and Abn-CBD continued to reduce IOP in $\beta 1/\beta 2^{-/-}$ mice (Hudson et al., 2011). Furthermore, chemical sympathectomy by reserpine also failed to affect the ability of NAGly to reduce IOP. These findings suggest a distinct mechanism of ocular hypotension for some non-psychotropic cannabinoids and lipids. This mechanism is independent of CB1.

The receptor target mediating the ocular hypotensive properties of the non-psychotropic cannabinoid, Abn-CBD, and the cannabimimetic lipid, NAGly, has remained somewhat elusive. The affinity of Abn-CBD for the LPI receptor, GPR55, made this receptor a logical candidate to initially assess potential hypotensive properties of Abn-CBD and NAGly (Oka et al., 2007). However, when the selective GPR55 ligand, O-1602, was applied topically, I saw no significant effect on IOP and Abn-CBD and NAGly continued to reduce IOP in GPR55^{-/-} mice. These findings excluded GPR55 as the receptor mediating the ocular hypotension actions of Abn-CBD and NAGly.

The finding that Abn-CBD and NAGly can decrease IOP independent of GPR55 is also supported by other *in vitro* studies. Abn-CBD and NAGly can cause endothelium-dependent vasodilation in mesenteric artery beds and this vasodilation is sensitive to O-1918, a synthetic analog of the cannabidiol which acts as an antagonist at both GPR55 and GPR18 (Ho & Hiley, 2003; Járai et al., 1999; Offertáler et al., 2003; Parmar & Ho, 2010; Wagner, Varga, Jarai, & Kunos, 1999). These vasodilator effects are not mediated via GPR55 as the vasodilation produced

by Abn-CBD persisted in GPR55^{-/-} (Johns et al., 2007; Ryberg, Larsson, Sjögren, et al., 2007). More specifically in the eye, MacIntyre and colleagues (2014) determined that Abn- CBD and NAGly cause O-1918-sensitive vasorelaxation of endothelin- vasoconstricted retinal arterioles and this action is independent of GPR55 but involves GPR18. They assessed several GPR55 ligands including O-1602, LPI and AM251, all of which did not have any vasorelaxant properties (MacIntyre et al., 2014).

In the eye GPR18 has been identified in several ocular tissues with expression throughout the cornea, retina and more importantly the ciliary epithelium and trabecular meshwork (Caldwell et al., 2013; Macintyre et al., 2014). The expression pattern in the eye indicates IOP could be modified by decreasing AH secretion in the ciliary epithelium, increasing AH outflow or could be a combination of the two. Additionally, this expression pattern could account for the findings from Qiao et al, (2012) who used isolated perfused porcine eyes to demonstrate that Abn- CBD increased aqueous humor outflow via a novel non-CB1/CB2 target that was sensitive to O-1918.

The fact that NAGly is a metabolite of AEA, an eCB that also shows activity at TRPV1, could indicate that the effects of NAGly are mediated through TRPV1 (Lam et al., 2005). However, my results showed that the IOP reduction produced by NAGly persisted in the presence of the TRPV1 antagonist, capsazepine (Section 3, figure 3.21). These results are consistent with *in vitro* work, where NAGly continued to produce vasodilation via nitric oxide release in rat small mesenteric arteries

following pretreatment with the TRPV1 antagonist, SB366791 (Parmar & Ho, 2010). These findings indicate that TRPV1 does not account for NAGly-mediated actions in both *in vitro* and *in vivo* models.

4.5. Clinical applications of atypical cannabinoids and lipids

Abn-CBD and NAGly produce similar magnitudes of IOP reduction in experimental models to CB1 agonists and various classes of glaucoma therapeutics including: β -blockers (timolol), prostaglandin analogues (latanoprost), and CAI's (Dorzolamide). This is an important finding given that many patients have contraindications for current therapies or require multi-drug therapy in order to maintain an appropriate level of IOP (Diggory et al., 1993; Holló et al., 2014). Ligands such as NAGly and Abn-CBD that at a novel non-CB1/CB2 receptor targets are behaviorally-inactive and offer a potential treatment, either as monotherapy, or in fixed-combination with other classes of ocular hypotensives, for the treatment of glaucoma. Abn-CBD has also been reported to provide neuroprotection for RGC's in models of experimental optic nerve injury (Sczcesniak et al., 2011). The additional neuroprotective benefits of GPR18 ligands, such as Abn-CBD, in addition to their ocular hypotensive properties, suggests that GPR18 may be a promising therapeutic target for further development of glaucoma therapeutics.

One barrier for the use of cannabinoids as topical ocular hypotensive agents has been poor corneal penetration due to lipophilic structure and poor aqueous solubility. Lack of adequate corneal penetration could cause limited efficacy, with drugs failing to reach therapeutic concentrations at the proper site of action.

Oltmanns et al (2008) reported that the cannabinoid, WIN dissolved in Tocrisolve™, was still detectable in the posterior chamber of the eye 80 minutes post-topical application in rats. Corneal penetrance was not directly assessed in my study. However, the use of Tocrisolve™, as the vehicle for delivery of cannabinoids and lipid ligands in my research, produced statistically significant drops in IOP that were comparable to clinically prescribed medications in a mouse model, suggesting adequate corneal ocular penetrance (Chapter 3: figure 3.2, 3.26). Additionally, drop placement in my studies was carried out perpendicular to the corneal apex; Green and Kearse (2000) determined that there was greater penetration of lipophilic Δ^9 -THC when the drop was placed over the corneal surface as opposed to the lower cul-de-sac using rabbits.

4.5 Limitations and Future Studies

My results indicated that behaviorally active cannabinoids reduce IOP via a CB1-mediated reduction in catecholamine release and that ligands that activate GPR18 activation produce ocular hypotensive actions that are independent of CB1 or CB2. However, there are limitations to my findings as outlined below.

I relied on the use of rebound tonometry for measurement of IOP in mice. This methodology has been found to be relatively non-invasive and allows for *in vivo* measurement (Nissirios et al., 2007). However, it does not directly measure IOP but rather provides an estimate of IOP (see Methods). Corneal thickness was not accounted for across the different strains of mice used in this study, and could have contributed to the IOP readings, with thick corneas over-estimating IOP (Doughty & Zaman, 2000). This is unlikely to be a significant factor in this study, with previous

studies demonstrating that mouse corneal thickness had little effect on IOP with the use of rebound tonometry (Nissirios et al., 2007;Shepard et al., 2010).

I presented a novel mechanism of IOP reduction occurring through GPR18 using selective and non-selective GPR18 ligands. However, due to the lack of specific GPR18 antagonists, it will be important to further validate the role of GPR18 with the use of transgenic mice lacking the GPR18 receptor, pending availability, or using intraocular RNAi injection to knock-down GPR18.

Flach (2002) highlighted the limitations set forth for cannabinoid treatment of glaucoma, including issues of patients' compliance and pharmacologic issues with the short duration of action of most cannabinoids. The duration of IOP reduction by Abn-CBD, like CB1 agonists, has been reported to be brief with the duration of IOP reduction lasting less than 2 hours (Szczesniak et al., 2011). I assessed IOP at 30 minutes after Abn-CBD or NAGly drop instillation. Therefore, it is unknown whether or not NAGly creates a sustained reduction in IOP. Time course experiments using various topical dosing regimens of NAGly have not been explored and further studies are required to determine the how long the ocular hypotensive actions of NAGly persist.

Furthermore, the IOP reducing properties of NAGly and Abn-CBD have not been assessed in intraocular hypertensive models; the magnitude of IOP reduction in a glaucomatous state remains unknown. Additionally, Abn-CBD has been reported to be neuroprotective in the retina, but the receptor target and signaling pathways responsible for this action remain to be confirmed. The potential neuroprotective effects of NAGly at this time remain unknown.

As NAGly continued to reduce IOP in reserpine-pretreated mice, unlike the CB1 cannabinoid agonist, WIN, and both Abn-CBD and NAGly reduced IOP in $\beta 1/\beta 2$ /, it is not unreasonable to hypothesize that NAGly and Abn-CBD effects should be additive with timolol. In addition, NAGly or Abn-CBD with timolol would be a potential favorable combination drop due to the excellent ocular tolerability of timolol when used with other classes of drugs, such as prostaglandin analogues (Radcliffe, 2014).

Future studies to further identify the effects of Abn-CBD and NAGly on AH dynamics will be instrumental in understanding the mechanisms of action of GPR18 in regulating IOP. Fluorophotometry and anterior segment perfusion models could help determine if NAGly causes increased AH outflow as previously described for Abn-CBD, as well as effects of Abn-CBD or NAGly on AH inflow (Avila, Mitchell, Stone, & Civan, 2003; Gabelt & ; Kaufman, 2011; Qiao et al., 2012).

4.6 Conclusions

The results of this study provide further support that cannabinoids and cannabimimetic lipids are capable of producing reductions in IOP; albeit through distinct mechanisms of action. The first stage of this study used transgenic mice and unique pharmacologic agents to demonstrate that cannabinoids, WIN and CP, reduce IOP via a CB1 mechanism dependent on an intact adrenergic system. My findings suggest cannabinoids from non-classical and aminoalkylindole groups inhibit NE release and act as indirect sympatholytic agents. In the light of this finding, these cannabinoids would not necessarily afford advantages for decreasing

IOP over β -antagonists, the current standard of clinical care. Furthermore, β -antagonists have fewer adverse side effects and are devoid of psychoactivity (Colasanti, Brown, & Craig, 1984; Uusitalo & Palkama, 1989; Yazulla, 2008). Additionally, the aminoalkylindole class of cannabinoid agonists, compared to the non-classical cannabinoids, is least likely to be effective as glaucoma agents given the off-target non-CB1 /CB2 effect increase in IOP seen in CB1^{-/-} mice. However, cannabinoid treatment of glaucoma should not be discounted. The neuroprotective properties of cannabinoids and the development of various new cannabinoid analogues and derivatives that modulate IOP via CB1 and non-CB1 receptor targets remains promising and may provide useful adjunct therapies to mitigate ganglion cell loss in glaucoma.

The second part of my thesis evaluated atypical cannabinoids and cannabimimetic lipids that act at non-cannabinoid receptors for their ability to reduce IOP. I identified that NAGly and Abn-CBD are capable of reducing IOP independent of CB1 and CB2 and that this action is mediated via the novel cannabinoid candidate receptor, GPR18. As GPR18 is localized in the anterior eye and retina, this receptor may be a promising therapeutic target for glaucoma and drugs acting at GPR18 should be considered either as monotherapy or in combination with other ocular hypotensives.

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