Molecular Pharmacology of Cannabinoids: Beyond THC

Shivani Sachdev

Master of Research (Biomedical)

Bachelor of Technology (Biotechnology)

Department of Biomedical Sciences

Faculty of Medicine and Health Sciences

Macquarie University, Australia

Submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

2020

Table of Contents

Statement of Originality

I certify that the work in this thesis entitled "Molecular Pharmacology of Cannabinoids: Beyond THC" has not previously been submitted for a degree nor has it been submitted as part of requirements for a degree to any other university or institution other than Macquarie University. I also certify that the thesis is an original piece of research and it has been written by me, except where due references is stated otherwise. Any help and assistance that I have received in my research work and the preparation of the thesis itself have been appropriately acknowledged.

In addition, I certify that all information sources and literature used are indicated in the thesis.

The research presented in this thesis was approved by the Macquarie University Biosafety Committee, reference number: 5201700579

Shivani Sachdev
Department of Biomedical Sciences
Faculty of Medicine and Health Sciences
Macquarie University

31 January 2020

Abstract

Cannabinoids from plants are some of the oldest human medicine, while synthetic cannabinoid receptor agonists (SCRAs) new psychoactive substances have been responsible for hundreds of deaths all over the world. The studies presented in this thesis were undertaken to further the understanding of the molecular pharmacology of cannabinoids, and how structurally diverse cannabinoids have greatly varied outcomes when acting via the same target. The characterisation of cannabinoids at activating cannabinoid receptors (CB1 and CB2) combined with operational model, functional selectivity and allosteric modulation together form the basis of the current thesis to better understand the molecular contributions to the toxic effects of SCRAs and therapeutic effects of phytocannabinoids.

The first major finding of this thesis was the quantitative determination of the efficacy of SCRAs downstream of CB1 using the operational model of pharmacological agonism. A panel of 17 cannabinoids were compared for their ability to induce response after the pharmacological knockdown of CB1 receptor using AM6544 (irreversible CB1 antagonist). The operational efficacy of cannabinoids ranged from 233 (5F-MDMB-PICA) to 0.9 (THC), with CP55940 in the middle of the efficacy rank order. SCRAs generally demonstrated substantially higher efficacy at activating CB1 receptors than THC. This work is the first of its kind to provide a framework to quantify the efficacy of chemically diverse cannabinoids and help identify some of the potential underlying molecular mechanisms regarding the SCRAs-related adverse effect on CB1 activation.

The functional activity of the same panel of cannabinoids was also characterised in two signalling endpoints - $G_{\alpha i/o}$ (inhibition) and $G_{\alpha s}$ (stimulation) of cAMP signalling. The rank order of potency of the cannabinoids to stimulate $G_{\alpha s}$-like signalling compared to $G_{\alpha i/o}$ signalling was significantly different. This suggests the differing ability of cannabinoids to preferentially induce CB1-dependent cAMP inhibition over stimulation of cAMP (functional selectivity). Cannabinoids showed diverse signalling profile (wide range of E_{MAX}) at $G_{\alpha s}$-like pathway than their activity at canonical G_i-mediated signalling pathway. Evaluating the differences in G protein preference among SCRAs may contribute to unravelling their complex effects in humans.

Another important concept in molecular pharmacology is the allosteric modulation of receptor signalling. The fourth chapter of this thesis was a natural continuation of the original investigation of the SCRA-associated toxicity, where we utilised the concept of allosteric modulation to understand mechanisms underlying the adverse effects related to the mixing of brodifacoum (superwarfarin) with SCRAs. Results revealed that cannabinoid signalling was not affected by brodifacoum, indicating that combining SCRA with brodifacoum is not likely to enhance user experience through interactions with cannabinoid receptors.

Allosteric modulation of cannabinoid receptors was also studied in the context of the 'entourage effect' - an emerging idea amongst users of cannabis is that the whole plant must be used in order to achieve the maximal therapeutic effect. However, Chapter 5 determined that aromatic compounds called terpenoids that are commonly found in cannabis had no additive effect on cannabinoid receptor function in the presence of a high efficacy agonist (CPP5940) or a low efficacy agonist (THC), suggesting the absence of 'entourage effect'.

While the earlier chapters are focused on the pathways involved in the on-targets effects associated with SCRA toxicity, Chapter 6 featured an exploratory study primarily concerned with the pharmacological effects underlying the potential therapeutic effects of cannabidiol (CBD) signalling on multiple receptors. We demonstrate the specific inhibitory effects of CBD on CB1 responses at physiologically relevant temperature, however, the effect of CBD on CB2, or mu-opioid receptor appears relatively non-specific in nature. The outcome of this chapter indicates that more work is required to understand whether the promiscuity of CBD interaction with multiple receptors is due to the membrane interaction.

The finding of this thesis provides new mechanistic insight into cannabinoid receptor function, and would hopefully direct future investigations into design of cannabinoids with high specificity and improved pharmaceutical properties.

List of Original Publications

This thesis comprises a body of three years of doctoral investigation presented in publication format. The publications arising from this work are listed here.

Papers presented as results chapters

I **Sachdev S**, Vemuri K, Banister SD, Longworth M, Kassiou M, Santiago M, Makriyannis A, Connor M. In vitro determination of the efficacy of illicit synthetic cannabinoids at CB1 receptors. 2019. British Journal of Pharmacology. 2019;1–13. https://doi.org/10.1111/bph.14829

II **Sachdev S**, Boyd R, Grimsey NL, Santiago M, Connor M. Brodifacoum does not modulate human cannabinoid receptor-mediated hyperpolarization of AtT20 cells or inhibition of adenylyl cyclase in HEK 293 cells. 2019. PeerJ. 7:e7733 https://doi.org/10.7717/peerj.7733

III Santiago M, **Sachdev S**, Arnold JC, McGregor IS, and Connor M. Absence of entourage: terpenoids commonly found in *Cannabis sativa* do not modulate the functional activity of Δ^9-THC at human CB1 and CB2 receptors. 2019. Cannabis and Cannabinoid Research. 4:3, 165-176, http://doi.org/10.1089/can.2019.0016

IV **Sachdev S**, Banister SD, Santiago M, Bladen C, Kassiou M, Connor M. Differential activation of G-protein-mediated signalling by synthetic cannabinoid receptor agonists. 2019. Accepted January 2020. Pharmacology Research & Perspectives.

V **Sachdev S**, Santiago M, Patel M, Manandhar P, Udoh M, Glass M, Connor M. Investigating the specificity of cannabidiol signalling. *In preparation.*

Book chapter

I Manandhar P, **Sachdev S**, Santiago M. Evaluating opioid mediated adenylyl cyclase inhibition in live cells using a BRET based assay. 2019. *Submitted for publication in 2019.*

Conference Presentations

Selected oral presentations

I **Sachdev S**, Santiago M, Patel M, Manandhar P, Udoh M, Glass M, Connor M. Investigating the specificity of cannabidiol signalling. 2019. ASCEPT-PAGANZ. Queenstown, New Zealand.

II **Sachdev S.** Pharmacology of synthetic cannabinoid new psychoactive substances. 2019. Invited talk at Pint of Science Sydney, Australia.

III **Sachdev S**. Pharmacology of synthetic cannabinoids receptor agonists new psychoactive substances. 2019. Invited seminar at Gill Center for Biomolecular Science, Indiana University, U.S.A.

IV **Sachdev S**, Vemuri K, Banister SD, Kassiou M, Makriyannis A, Connor M. Synthetic cannabinoid efficacy may predict its toxicity at CB1. 2018. GPCR Symposium Sydney, Australia.

Selected poster presentations

I **Sachdev S**, Vemuri K, Banister SD, Kassiou M, Makriyannis A, Connor M. In vitro determination of the CB1 efficacy of illicit synthetic cannabinoids. 2019. Experimental Biology, published in FASEB journal, Volume 33, U.S.A.

II **Sachdev S**, Vemuri K, Banister SD, Kassiou M, Makriyannis A, Connor M. Differential desensitization of CB1 receptor by high efficacy synthetic cannabinoid receptor agonists. 2018. British Pharmacological Society- Molecular Pharmacology of G-protein coupled receptors conference, Monash University, Melbourne, Australia.

III Santiago M, **Sachdev S**, Longworth M, Kassiou M, Connor M. How ZCZ011 compare to its analogues on modulating and activating cannabinoid CB1 receptor. 2018. Australasian Society of Clinical and Experimental Pharmacologist and Toxicologist Annual Scientific Meeting, Adelaide, Australia.

IV **Sachdev S**, Bladen C, Vemuri K, Banister SD, Kassiou M, Makriyannis A, Connor M. Use of novel CB1 irreversible antagonist to determine the efficacy of illicit synthetic cannabinoids. 2018. Neuroscience, U.S.A.

V **Sachdev S**, Santiago M, Banister SD, Longworth M, Kassiou M, Connor M. Desensitization of CB1 receptor signalling by valinate and tert-leucinate synthetic cannabinoids, ASCEPT- MPGPCR, Melbourne, 2016.

VI. Connor M, Santiago M, **Sachdev S**, Arnold JC, McGregor IS. Monoterpenes from Cannabis sativa do not affect human CB1 or CB2 receptor activation of K channels in AtT-20 cells. 2016. ASCEPT- MPGPCR, Melbourne, Australia.

Awards during candidature

2019 - Skipper Foundation - Skipper Postgraduate Travel Award

2019 - Australian Society of Clinical and Experimental Pharmacology and Toxicology society conference travel award

2019 - Cayman Chemical Conference Travel Grant, U.S.A.

2018 - Macquarie University Postgraduate Research Fund, Australia

2018 - Minomic International Ltd. Sponsor Travel Grant, Australia

Acknowledgement

The following PhD thesis would not have been possible without the financial and institutional support of Macquarie University and the Australian Government Research Training Program Scholarship.

First of all, I would like to thank my supervisor, Professor Mark Connor for all the tremendous help and support throughout my time as a research student. It is a rare thing to find in a supervisor who not only has excellent scientific grounding, but also his great sense of humour has made this whole process 60x less stressful. I could not have imagined a better advisor and mentor for my PhD study, and he has certainly been an integral part of my scientific career.

To Dr. Marina Santiago, Dr. Samuel Banister, and Dr. Chris Bladen, thank you for all your contributions to my project and for providing me valuable advice at key moments throughout my candidature.

I thank all the present and past members of the Connor group - including Preeti Manandhar, Somayeh Mirlohi, Michael Udoh, and Rochelle Boyd for providing a stimulating environment to learn and grow, and they undoubtedly have been a major factor in my enjoyment of the last four years.

My sincere thanks to Professor Alex Makriyannis, and his group for providing us with the AM6544 compound. Professor Michelle Glass and her team over at the University of Otago, for providing scientific and technical assistance, and for contributing data to Chapter 6 of the thesis. Dr. Natasha Grimsey for providing helpful advice in establishing CAMYEL assay. Professor Michael Kassiou for providing the synthetic compounds that allowed me to complete this work. Dr. Varun Srinivasan for many interesting discussions and insights into chapter 6 of this thesis. The Lambert Initiative for Cannabinoid Therapeutics at the University of Sydney for their scientific expertise, and synthesis of phytocannabinoids used in this thesis. I hope to continue our collaborations in the years ahead.

Still others, especially Dr. Annemarie Nadort and Dr. Anna Guller helped with enthusiasm, moral support and for always believing in me. For always being in the stands cheering for me.

I also extend thanks to Professor Brad Walsh, who has been kind enough to advise and help me throughout this process.

I would like to individually thank Dr. Jen Rowland and Dr. Maria Mempin who helped me out with thesis formatting, Joel Berliner for proofreading in the crucial moments, and Dmitry Polikarpov for keeping me accompanied along the way, and Sally Purcell for always being there for me.

Beyond the academic world, I would like to thank Tim, Gemma, and Kawsar for many fruitful years of friendship and I am so glad you are in my life.

A very special thanks goes to my family, particularly mum and dad, for their unflagging love and for all the sacrifices you have made in order to help me reach this stage in my life. I could not have made it through this process without you. I love you.

List of Figures

Chapter I.

Chapter II.

Chapter III.

Chapter IV.

Chapter V.

Chapter VI.

Appendix A.

Appendix B.

List of Tables

Chapter I.

Chapter II.

Chapter III.

List of Contributors

Several researchers contributed to the publication of the work presented in this thesis. Relative contribution to different components of the work is outlined in the table below.

DIVISION OF LABOUR IN CO-AUTHORED ARTICLES

JA - Jonathan C. Arnold; SB - Samuel D. Banister; RB - Rochelle Boyd; MC - Mark Connor, MK - Michael Kassiou; MG - Michelle Glass; NG - Natasha L. Grimsey; ML - Mitchell Longworth; AM - Alexandros Makriyannis; PM - Preeti Manandhar; IM - Iain I. McGregor; MP - Monica Patel; SS - Shivani Sachdev; MS - Marina Santiago; MU - Michael Udoh; KV - Kiran Vemuri.

	I	II	III	IV	V
Conception & design	MC, SS	MC, SS	MC, SS	MC, MS	MC, SS
Planning & implementation	MC, SS	MC, SS	RB, MC, SS	MC, IM, MS	MC, SS
Data collection	SS, MS	SS	RB, SS	SS, MS	PM, MP, SS, MS, MU
Chemical synthesis of drugs	SB, ML, KV	SB	-	-	-
Analysis & interpretation	MC, SS, MS	CB, MC, SS, MS	MC, NG, SS, MS	MC, SS, MS	MC, MG, PM, MP, SS, MS, MU
Writing the article	SB, MC, MK, ML, AM, SS, MS, KV	SB, CB, MC, MK, SS, MS	RB, MC, NG, SS, MS	JA, MC, IM, SS, MS	MC, SS
Overall responsibility	MC, SS	MC, SS	MC, SS	MC, MS	MC, SS

All authors of these manuscripts have provided their permission to include the work into this thesis.

Abbreviations

2-AG	2-arachidonoylglycerol
AC	Adenylyl cyclase
AEA	N-arachidonoylethanolamine
AIDS	Acquired Immune Deficiency Syndrome
AtT20	Pituitary adenoma cell line
ATCC	American Type Culture Collection
ANOVA	Analysis of Variance
AUC	Area under the curve
BRET	Bioluminescence Resonance Energy Transfer
BFC	Brodifacoum
BSA	Bovine serum albumin
cAMP	3'-5'-Cyclic Adenosine Monophosphate
CAMYEL	cAMP with YFP, Epac, RLuc
CB1/CB2	Cannabinoid type 1/2 receptor
CBD	Cannabidiol
CHO	Chinese Hamster Ovary cells
$Ca_v3.1$-3.3	T-type calcium channel
CTX	Cholera toxin
CRC	Concentration response curve
CYPs	cytochrome450s
D2	Dopamine receptor
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
EMCDDA	European Monitoring Centre for Drugs and Drug Addiction
EC_{50}	Half maximal effective concentration
E_{MAX}	Maximal effect
ERK	Extracellular signal-Regulated Kinase
FAAH	Fatty acid amide hydrolase

FBS	Foetal Bovine Serum
FLIPR	Fluorometric imaging plate reader
FSK	Forskolin
$GABA_A$	γ-Aminobutyric acid type A receptors
GIRK/ K_{ir}	G protein coupled inwardly rectifying potassium channel
G protein	GTP binding protein
GPCR	G Protein-Coupled Receptor
GRK	GPCR kinase
GTPγS	Guanosine 5'-O-[gamma-thio] triphosphate
GPR 55	G protein-coupled receptor 55
HA	Haemagglutinin (human influenza)
HBSS	Hank's Balanced Salt Solution
HEK	Human Embryonic Kidney
HIV	Human Immunodeficiency Virus
HMIP	Her Majesties Inspectorate of Prisons
IC_{50}	Half maximal inhibitory concentration
K_i / K_B	equilibrium dissociation constant
MPA	Membrane Potential Assay
MOR	mu-opioid receptor
NAMs	Negative Allosteric Modulators
NPS	New Psychoactive Substances
NanoBit®	NanoLuc® Binary Technology
PAMs	Positive Allosteric Modulators
PBS	Phosphate Buffer Saline
PDL	Poly-D-lysine
PEI	Polyethyleneimine (linear or branched)
PPAR	Peroxisome proliferator-activated receptor
PLC	Phospholipase C
PKB	Protein Kinase B
PI3K	Phosphatidylinositol-3-kinase
PTX	Pertussis toxin
RLuc	*Renilla* Luciferase

RAi	Relative agonist activity
RFU	Relative fluorescence units
RT	Room temperature
SCRAs	Synthetic Cannabinoid Receptor Agonists
SEM	Standard error of the mean
SRIF	Somatotropin release-inhibiting factor
SST	Somatostatin
TM6	Transmembrane helix 6
Tau (τ)	Trasnsducer constant (efficacy)
WT	Wildtype
YFP	Yellow Fluorescent Protein
THC	Δ^9-tetrahydrocannabinol
TRP	Transient receptor potential channel
UNODC	United Nations Office on Drugs and Crime

Chapter I. General Introduction

This chapter will address, in general, the pharmacology of cannabinoids, and particularly will summarise the history of medical cannabis from ancient Chinese pharmacopoeias to the incremental development of the current understanding of the human endocannabinoid system involved in many brain functions. The endocannabinoid system comprises of endogenous cannabinoids, the enzymes which produce or metabolise them, and the cannabinoid receptors (CB1 and CB2). Each of these components will be introduced in this chapter, with emphasis on the spectrum of the CB1 signalling responses. The concept of phytocannabinoid-terpenoid entourage effect will then be introduced, with focus on their *in vitro* activity profiles in cannabinoid receptor function. Although cannabinoids have therapeutic potential, recent decades have seen emergence of vast numbers of new psychoactive substances - synthetic cannabinoids. This chapter will also focus on the emergence of synthetic cannabinoids as drugs of abuse, their nefarious adverse effects and their potential mechanism of action. These themes will then be combined in a brief discussion about unlocking the activity of chemically diverse cannabinoids to associate the signalling dynamics of CB1 with potentially useful therapeutic properties (i.e. biased agonism) and the use of operational model to quantify agonist efficacy. This thesis presents a comprehensive and up-to-date summary of many aspects of cannabinoid research, and it will be of interest to biomedical researchers, as well as chemists and toxicologists.

1.1. Cannabis - Over 5000 years ago

The first evidence of the use of *Cannabis Sativa* (cannabis) can be traced back to China by at least 5000 years ago, where archaeological evidence indicated that the plant was first used for fibre and textile (Li, 1974, Touw, 1981, Grotenhermen and Russo, 2002, Ren et al., 2019). It is difficult to exactly identify where and when the medical use of cannabis first came into existence. While the oldest journal articles available suggest that the medical use of cannabis was first documented in Chinese Pharmacopoeia (credited to Emperor Shen Nung), the Rh-Ya (Touw, 1981, Li, 1974, Zuardi, 2006). Since then the therapeutic indications of cannabis are mentioned in the medical texts of civilisations throughout Asia and North Africa (reviewed in Zuardi (2006)) (Figure 1-1). The widely documented medicinal uses of cannabis include anti-nociception, anti-inflammatory, anticonvulsant, and anti-emetic properties (Mechoulam, 1986b, Iversen, 2001). Specific mention of cannabis for

the treatment of seizures and epilepsy was first reported in Arabic and Islamic writings (Lozano, 2001). While the first detailed description of the anti-convulsant effect of cannabis-based products tested for safety and toxicological profile in animals was reported by William O′Shaughnessy (O'shaughnessy, 1843, Friedman and Sirven, 2017, Perucca, 2017). In addition to the safety profile of cannabis, the effectiveness of cannabis extracts was also evaluated for the treatment of infantile convulsions (O'shaughnessy, 1843, Friedman and Sirven, 2017, Perucca, 2017). He wrote a brief qualitative description of a 40-day-old child who developed nocturnal convulsive episodes and was treated repeatedly for weeks with cannabis extracts until the convulsions stopped; he concluded, "the child is now in the enjoyment of robust health, and has regained her natural plump and happy appearance" (O'shaughnessy, 1843).

Though the ancient texts mainly discuss medicinal applications of cannabis, there was a peculiar silence on the psychoactive effects of cannabis (Li, 1974, Touw, 1981). It was not until 10th century A.D/A.C.E. that the first definitive record of the psychological effect of cannabis was observed, and they stated that:

"Ma-fen (the fruit of cannabis)... if taken in excess will produce visions of devils … over a long term, it makes one communicate with spirits and lightens one's body…"

There is a considerable difference of opinion concerning the psychological consequences of the recreational use of cannabis – those that underestimate and others who exaggerate the psychotropic effects related to cannabis use (Rubin, 2011). Epidemiologic and preclinical data suggest that the recreational use of cannabis is associated with euphoria, sedation, stimulation of appetite, hallucinations, impaired short-term memory, altered perception of time and space, and aphrodisiac effects (Lee, 2012). The use of cannabis, whether for medicinal or recreational purposes, is a polarising concept shrouded in myths and misconceptions (Friedman and Sirven, 2017). The effect of recreational cannabis use during adolescence is of particular concern, since adolescents are more vulnerable than adults to deleterious cognitive effects produced by cannabis (Tortoriello et al., 2014). One study showed that the adults who smoked cannabis during adolescence have impaired neural connectivity in specific brain regions (Zalesky et al., 2012). However, an ever-increasing number of studies examine the psychological effects of cannabis, the long term behavioural and biochemical data for cannabis remain an emerging area of research (Volkow et al., 2014). Some studies reported little to no change in cognitive functions between the long-

term cannabis users and the people who have never used cannabis in their lifetime (reviewed in Earleywine (2002)). These results, however, may have been confounded by many factors including sample size (in this case 10 people), biased sampling that may include chronic users who were not experiencing negative consequences, and the tests employed often are too simple to detect the detrimental effects of long-term cannabis use (Earleywine, 2002). On the other hand, largest set of studies accessed over 1600 Egyptian prisoners revealed cannabis-related cognitive in users compared to nonusers (reviewed in Earleywine (2002)). It was also perhaps difficult to interpret the distinctive effects (medical and recreational) of cannabis use. For example, a study presented the long-term use of cannabis in patients with human immunodeficiency syndrome (HIV) infection or AIDS, they found that cannabis use could alleviate neurocognitive impairment in people with HIV (Cristiani et al., 2004). While another study found that smoking cannabis helped patients with HIV gain weight by stimulating their appetite with no effect on the viral load (Watson et al., 2000). Investigation into the extreme varying effects of cannabis spurred the discovery of its' active component, (-)-trans-Δ^9- Tetrahydrocannabinol (THC).

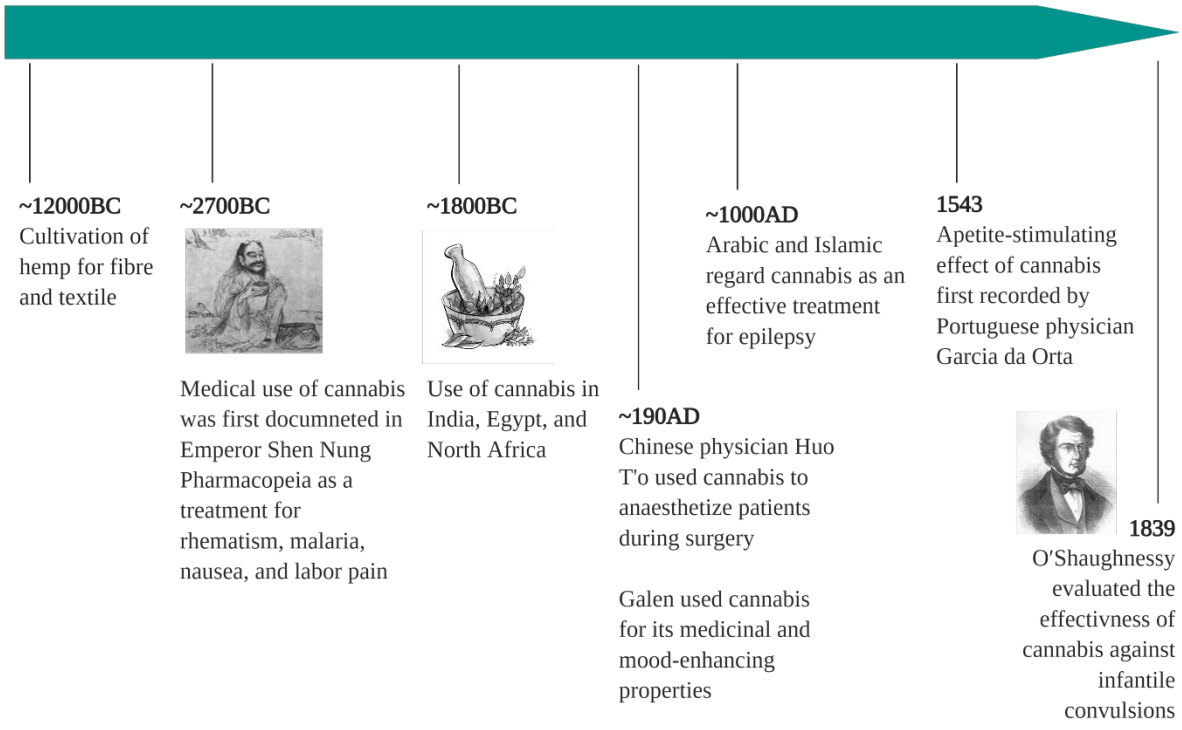


Figure 1-1. History of Medical Cannabis

The use of medical cannabis has a long history. A brief timeline illustrating the periods in which the medical uses of cannabis began. The picture incorporated in the figure is under the terms of the Creative Commons Attribution License. Figure created with BioRender.

1.2. Phytocannabinoids

Cannabinoid was originally the collective term given to a group of oxygen-containing C_{21} aromatic hydrocarbon compounds from Cannabis sativa, and their derivatives and synthetic analogues (Huestis, 2005). Since the synthesis of synthetic cannabinoids, a separate term "phytocannabinoids" has been coined to those that originate from plant itself (Table 1-1). Beyond the phytocannabinoids, cannabis produces an extensive range of terpenoids that are synthesised alongside phytocannabinoids and concentrated mainly in the trichomes of the plant (discussed in Chapter 5).

Table 1-1. Types of Phytocannabinoids

More than 100 phytocannabinoids across 10 different classes listed in this table have been isolated and identified to date. Adapted from (Pertwee, 2006)

Table 1 Plant-derived cannabinoids (phytocannabinoids)

- Δ^9-tetrahydrocannabinol-type (9)
- Δ^8-tetrahydrocannabinol-type (2)
- Cannabidiol-type (7)
- Cannabigerol-type (6)
- Cannabichromene-type (5)
- Cannabicyclol-type (3)
- Cannabielsoin-type (5)
- Cannabitriol-type (9)
- Miscellaneous-type (11)
- Cannabinol and cannabinodiol-types (air-oxidation artefacts)

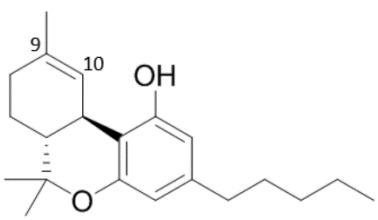
The number of each of the listed types of cannabinoid that has been found in cannabis is shown in parenthesis (reviewed in ElSohly, 2002). Tincture of cannabis (right hand panel) was a commercial product that was prepared from *Cannabis sativa* grown in Pakistan and imported into Britain under licence (Gill *et al.*, 1970).

1.2.1. Δ^9- Tetrahydrocannabinol (THC)

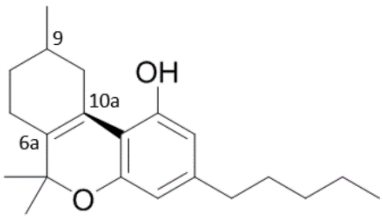
1.2.1.1. Discovery of Δ^9-THC

The 19th century saw dramatic advances in the chemical research for active natural products. Numerous alkaloids were identified from various plants - including morphine, cocaine, strychnine and their derivatives of potential therapeutic interest (Mechoulam and Hanuš,

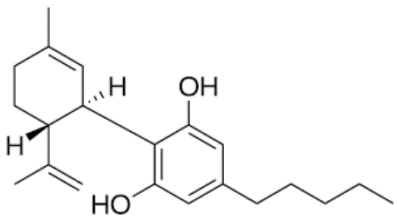
2000). However, the active constituent of cannabis was not identified until the 1960s; unlike alkaloids crystallised as salts, cannabinoids are usually present in mixtures and many are similar in structure making separation of individual components more difficult (reviewed in Mechoulam and Hanuš, (2000)). A group in Cambridge, Wood et al. (1896), developed a method for the purification of compounds in cannabis. Using fractional distillation from the ethereal extract of the plant, they obtained four distinct chemical fractions – a terpene, a sesquiterpene, a paraffin and a red oil extract (Wood et al., 1896). In a further elaboration, authors proposed that the actions of terpenes have very little to no physiological effect ('0.5 gram of terpene produced none of the characteristic symptoms of cannabis action'), while the toxic red oil extract (regarded as active constituent of the cannabis) when taken in a dose of 0.05 grams 'induces decided intoxication followed by sleep' (Marshall, 1898, Wood et al., 1896). Later it was shown that the red oil fraction was not homogeneous, and the first phytocannabinoid cannabinol was isolated, which was believed to be the psychoactive component of cannabis (Dunstan and Henry, 1898, Wood et al., 1899). In continuing Wood's characterisation of cannabinol, Cahn (1933) discovered that pure cannabinol was only weakly psychoactive in dog ataxia test, and hence the quest for the active component of cannabis continued (reviewed in Pertwee (2006)). Several groups (Roger Adams in USA, and Alexander Todd in England) reported initial attempts to isolate and identify active compound in cannabis (Adams et al., 1940, Todd, 1946) - they published the preparation and evaluation of synthetic compounds, such as $\Delta^{6a,10a}$-THC (Figure1-2); while the pharmacological activity of this synthetic racemate mixture was investigated in collaboration with (Loewe, 1950), who noted that $\Delta^{6a,10a}$-THC showed cannabis-like effects (i.e. increase in pulse rate and reddening of the eyes) in humans with potency 15-30% of THC (reviewed in (Banister et al., 2019b)). Subsequently, Adams and colleagues synthesised a series of analogues of THC - one of the compounds synthesised had a potency significantly greater than that of natural THC (Adams et al., 1949).



Δ^{9}-THC



$\Delta^{6a,10a}$-THC



CBD

Figure 1-2. Chemical structure of selected phytocannabinoids (Δ^9-THC and CBD), and synthetic cannabinoid ($\Delta^{6a,10a}$-THC). Chemical structures were drawn using ChemDraw Professional 18.2.

Ultimately, the credit for identifying Δ^9-THC from cannabis plant, and that can be isolated (in pure form) by using column chromatography, was carried out in Raphael Mechoulam' laboratory in a study published in 1964 (Gaoni and Mechoulam, 1964). In it, Gaoni and Mechoulam demonstrated that repeated chromatography of hexane extract of cannabis on alumina can induce separation of the following compounds - cannabidiol (CBD), Δ^9-THC, cannabinol (CBN), cannabichromene (CBC), cannabigerol (CBG), and polar constituents and polymers (Gaoni and Mechoulam, 1964, Mechoulam and Shvo, 1963, Gaoni and Mechoulam, 1971). The absolute configuration of Δ^9-THC was then established by correlation with known terpenoids (Mechoulam and Shvo, 1963). Later, the pharmacological profile of the purified compounds was evaluated in rhesus monkey – they noted that the psychotropic-like effects in monkeys (including akinesia, apathy, reddening of eyes, drowsiness, and tameness) was largely attributed to Δ^9 -THC (Mechoulam et al., 1970). Studies in humans have demonstrated dose-dependent impairment of motor coordination and performance, loss of muscle strength, and rapid heart rate following inhalation of THC (reviewed in Mechoulam (1986a), Reyes et al. (1973)). While the early research provided a more detailed description of the chemistry and psychopharmacological effects of Δ^9-THC, the mechanisms by which these effects were produced were not known during this time.

1.2.1.2 Molecular pharmacology of THC

THC binds to both cannabinoid-type-1 (CB1) and type-2 (CB2) receptors in the nanomolar range, although it exhibits lower intrinsic activities at these receptors when compared to CP55940, a prototype high-efficacy cannabinoid agonist (see below, cannabinoid receptors). While it behaves as a partial agonist at both these receptors types, it activates CB2 with apparently lower efficacy as compared to CB1 (the mediator of cannabinoid-related psychoactivity) (reviewed in Huestis (2005), Pertwee (2008)). Previous studies using molecular characterisation have reported that THC also exerts its activity at non-canonical endocannabinoid system, summarised in Table 1-2 (reviewed in Huestis (2005), Turner et al. (2017)). However, only a few studies have been published examining the physiological role of this non-cannabinoid target effect of THC responses (Turner et al., 2017).

Table 1-2. Pharmacological profile of THC at multiple targets based on results of studies in different experimental systems.

Receptor/target	Pharmacology
CB1	Low efficacy agonist (Pertwee, 1999)
CB2	Low efficacy agonist (Pertwee, 1999)
GPR55	*Conflicting (agonist or NAM) (Ryberg et al., 2007, Anavi-Goffer et al., 2012)
GPR18	Agonist (< 1 μM) (Mchugh et al., 2012)
Serotonin 3A receptors	NAM (< 1 μM) (Barann et al., 2002)
PPARγ nuclear receptor	Agonist (O'Sullivan et al., 2005)
TRPA1	Low efficacy agonist (De Petrocellis et al., 2011)
TRPV2	Low efficacy agonist (De Petrocellis et al., 2011)
TRPV3-4	Low efficacy agonist (De Petrocellis et al., 2012)
α1, α1β1 glycine	PAM (< 1 μM) (Hejazi et al., 2006)
$Ca_v3.1$, $Ca_v3.2$, or $Ca_v3.3$	Inhibitor (IC_{50} 1-3 μM) (Ross et al., 2008)

1.2.2. Cannabidiol (CBD)

Here, I must disrupt the chronological sequence. While CBD was discovered more than 20 years before THC, cannabinoid research was overwhelmingly dominated by THC (Castle and Murray, 2004) (Figure 1-3). There was no detailed pharmacological report on CBD until the 1970s, except that CBD lacks the THC-like psychoactive effects altogether *in vivo* (Reyes et al., 1973). Although the interest in medical cannabis and its individual constituents persisted throughout the years, it is only in the last decade that clinical and preclinical research has focused on the anti-convulsant profile of CBD (Perez-Reyes and Wingfield, 1974, Perucca, 2017). Cunha et al. (1980) became the first researchers to report that pure CBD was effective in the treatment of seizures in humans at an initial dose of 200 mg/day. To date, the largest exploratory study on the safety and effectiveness of CBD against drug-resistant epilepsy in Dravet syndrome was tested in a double-blinded, placebo-controlled trial, involving 120 children and young adults (sponsored by GW Pharmaceuticals) (Devinsky et al., 2017). This trial for the first time provided robust evidence that the use of CBD results in a greater reduction in convulsive seizure frequency than the placebo group. Note that the patients were under stable-antiepileptic regime during this trial, suggesting that some effects of CBD may be due to drug-drug interaction (additive effect) between CBD and other anti-epileptic medication (Devinsky et al., 2017). A recent study systematically characterised the pharmacodynamic and pharmacokinetic interaction between CBD and clobazam (conventional anti-epileptic drug) (Anderson et al., 2019). The authors found that CBD and clobazam coadministration produced a greater anticonvulsant effect than that of

the drug alone, while pharmacodynamic interactions revealed novel mechanism of action where CBD and clobazam together enhanced inhibitory $GABA_A$ receptor activation (Anderson et al., 2019). Beyond epilepsy, CBD has been suggested to possess therapeutic effects for a wide range of health conditions, attributed to its activity at multiple targets – G protein coupled receptors (GPCRs), ion channels and membrane bound-enzymes, but nearly all at micromolar potency (Perucca, 2017). Recent research has included a randomised double-blind controlled trial to examine the effect of CBD on the permeability of the gastrointestinal tract (Couch et al., 2019). The aspirin-induced gut hyperpermeability was reduced when treated with CBD, and that these effects were mediated by CB1 receptors (Couch et al., 2019). While CBD has emerged as an important cannabinoid for its wide range of possible therapeutic activities, our current understanding of CBD interactions with multiple targets are limited by assay conditions. Frequently, CBD assays are performed at room temperature without the appropriate control for effector modulation by CBD, and thus the results of these studies are difficult to extrapolate to normal human physiology (e.g Laprairie et al. (2015), Navarro et al. (2018)). This picks up on the idea discussed in Chapter 6; where the specificity of CBD signalling across multiple GPCRs was investigated using uniform *in vitro* assays, and would hopefully direct future investigations into the biological relevance of assays with physiological face validity.

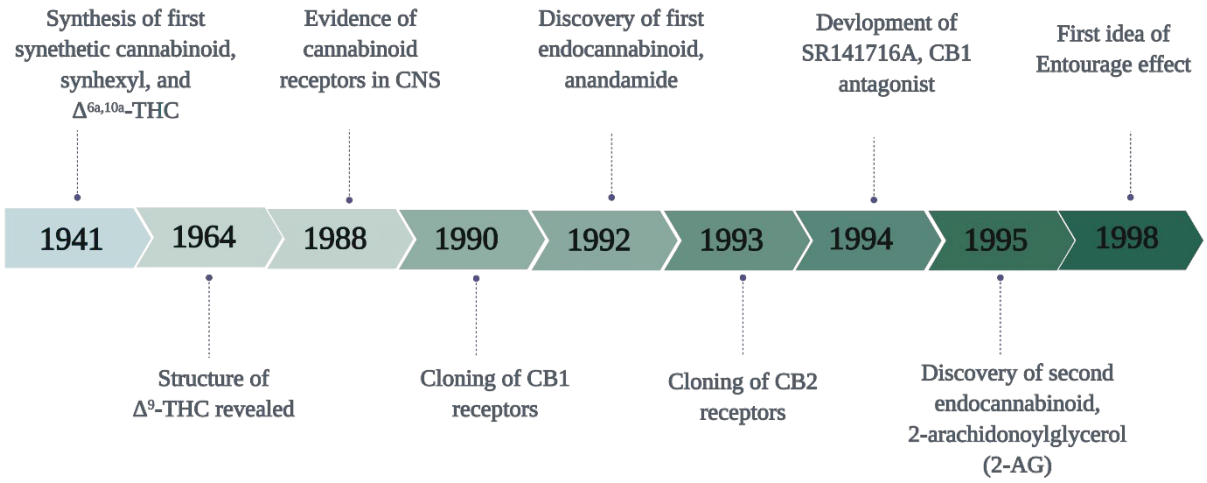


Figure 1-3. Cannabinoid research timeline

A brief timeline illustrating the periods in which the cannabinoids and the endocannabinoid system were first discovered. Figure created with BioRender.

1.3. Cannabinoid receptors

Two class A (Rhodopsin-like) GPCRs have been identified through which cannabinoids primarily exert their pharmacological effects – CB1 and CB2. CB1 is one of the most abundant GPCRs in the mammalian central nervous system. The distribution of CB1 in peripheral and central nervous system accounts for therapeutic effects of cannabinoids for conditions as diverse as pain, nausea, neurological diseases, and cancer (reviewed in Zou and Kumar (2018)). CB2 receptors are expressed primarily in the immune system, and as such may be a therapeutic target for immune modulation in a wide range of disorders (reviewed in Pacher and Kunos (2013)). Thus, because activation of these receptors regulates fundamental physiological processes, effort has been made towards understanding the signalling phenotypes of cannabinoid receptor activation.

1.3.1. Discovery of cannabinoid receptors

The fundamental idea that a cannabinoid (THC, in this case) exerts their pharmacological activity by binding to discrete molecular entities (a receptor) was weakened by the non-experimental opinion of Paton (1975). In it, he proposed that THC belongs to a group of biologically active lipophiles (anaesthetics and solvents), and that these compounds exert their effects by non-specific interaction with the phospholipid constituents of the membrane (Paton, 1975, Pertwee, 1988). It is worth noting that the idea of discrete receptors in general was not well accepted at the time. Gill and Lawrence (1976), went further to provide experimental evidence to support the above suggestion, concluding that:

> "it is unnecessary to postulate the existence of a more complex macromolecular receptor substance to account for the observed structure-activity relationships"

A second conceptual problem was the lack of stereoselectivity of THC – synthetic (+) isomer demonstrated cannabimimetic activity, although to a much lesser extent as compared to natural (-) isomer of THC (see Castle and Murray (2004)). However, later it was established that cannabinoids with chiral centres exhibit high stereoselectivity (this picks up on the indication of the existence of cannabinoid receptor), and that the previous observation resulted from an impure sample (Mechoulam et al., 1988, Howlett et al., 1990). In 1974, the pharmaceutical company Pfizer launched a medicinal chemistry campaign to synthesise a new class of cannabinoids - CP55940, CP47497, and CP55240 in order to determine the molecular target for cannabinoids (structure-activity relationship analysis) (reviewed in Pertwee (2014)).

In 1988, Allyn Howlett's laboratory at St Louis University provided the first direct evidence of the existence of cannabinoid receptors in brain (Devane et al., 1988). Howlett observed that cannabinoids inhibit adenylyl cyclase enzyme in neuroblastoma N18TH2 cell line, indicated by reductions in cAMP levels (Howlett and Fleming, 1984). Subsequently, the tritium labelled [^{3}H] CP55940 was used to identify and characterize CB1 in rat brain membranes (Devane et al., 1988). Autoradiographic studies showed the heterogenous distribution pattern of CB1 receptors within the central nervous system – presence of large population of CB1 receptors in cortex, basal ganglia, hippocampus, and cerebellum ((Herkenham et al., 1990), reviewed in Howlett (2005), Mackie (2005)) (Figure 1-4). The authors note that the previous attempts to characterise the CB1 receptors was unsuccessful due to low binding affinity and specificity of THC, thus CP55940 was used to circumvent some of these technical issues (Herkenham et al., 1990). Additional studies to further investigate the activity of cannabinoids on CB1 were made in collaboration with Devane et al. (1988). The authors found that the ability of unlabelled cannabinoids to displace [^{3}H] CP55940 from discrete binding sites of receptor was shown to correlate with their cannabimimetic effects *in vivo* in mice (reviewed in (Howlett, 2005, Pertwee, 2006)). The discovery of the CB1 receptor was further confirmed upon cloning of rat CB1 (473 amino acids) in Tom Bonner's laboratory at NIH and of the human CB_1 (472 amino acids) by Gérard and colleagues in Brussels (Matsuda et al., 1990, Gerard et al., 1991). Shortly thereafter, the molecular identity of CB2 was revealed in Sean Munro's laboratory in Cambridge (360 amino acids, 44% homology with CB1 (Munro et al., 1993)). However, CB2 receptors are largely found in the peripheral tissues of immune cells (Galiègue et al., 1995), and have also been identified in brain (expressed mainly in glial cells (Pazos et al., 2004)). More recent studies have focused on whether CB2 is expressed in the neurons, and if they present distinct physiological characteristics depending on where they are expressed. Molecular biological studies have demonstrated CB2 mRNA expression in neurons in brain regions (including cerebellum, hippocampus, cerebral cortex) (Zhang et al., 2015), although studies using CB2 antibodies are somewhat controversial as the commercially available anti-CB2 antibodies have poor specificity for their intended target (reviewed in Li and Kim (2017)).

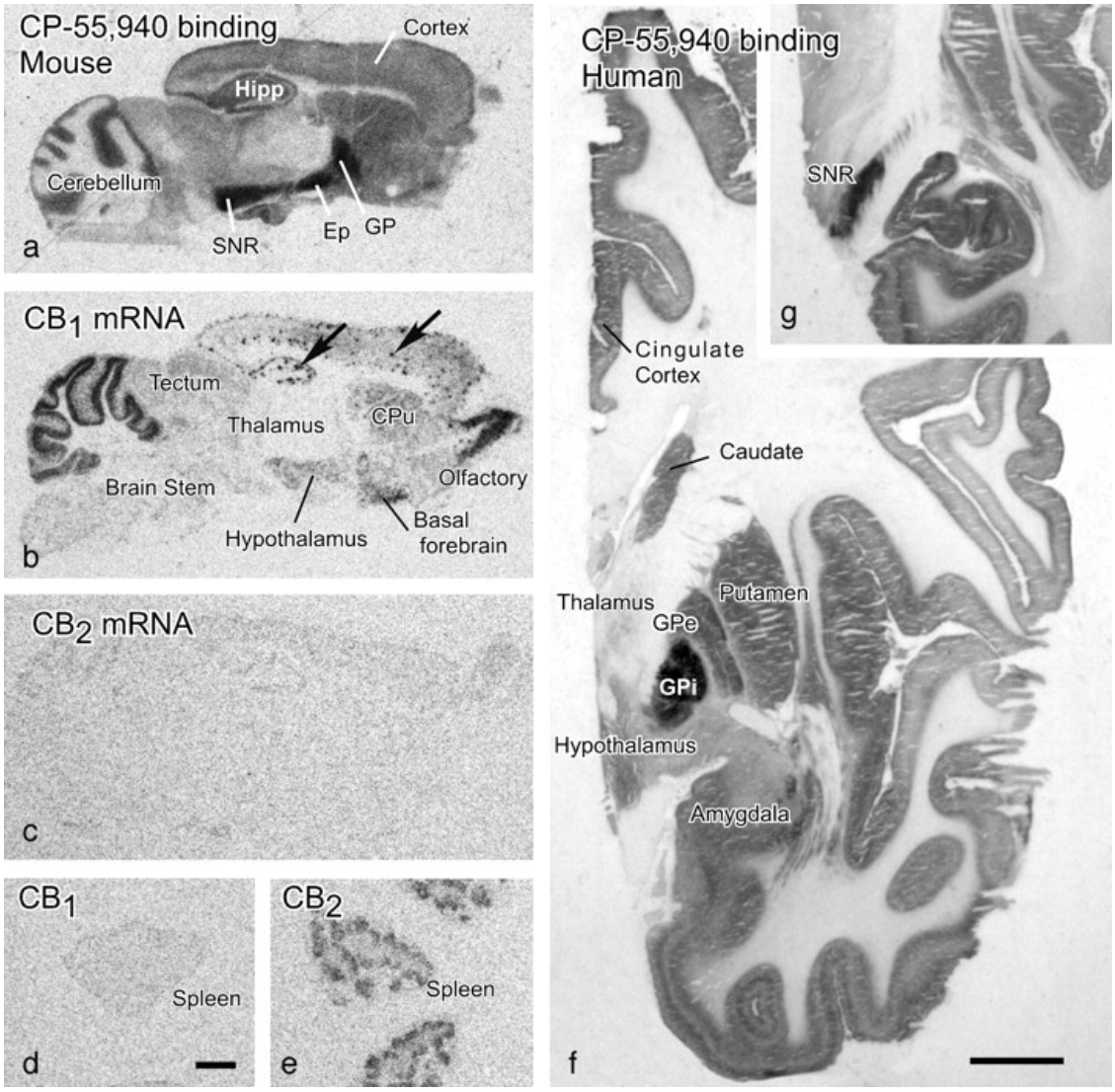


Figure 1-4. Autoradiographs showing cannabinoid receptor binding and expression in sections from mouse and human brain, and mouse spleen

(a,b) showing binding of [^{3}H] CP55940 to the cannabinoid receptor in the basal ganglia, cerebellum, hippocampal, and cerebral cortex; (b,d) expression of CB1 mRNA in the sections from brain (b) and spleen (d); (c,e) expression of CB2 mRNA in the sections from brain (c) and spleen (e) respectively. Adapted from (Howlett et al., 2002).

1.3.2. Cannabinoid CB1 and CB2 receptor signalling

The molecular identity of CB1 and CB2 receptor was soon followed by the development of a number of *in vitro* bioassays (except that the cAMP assay guided the discovery of CB1) to understand the mechanism of action of these receptors (reviewed in Huestis (2005)). Both CB1 and CB2 receptors were initially described to inhibit adenylyl cyclase in a pertussis toxin-sensitive manner, implicating a cannabinoid receptor coupling to a $G_{i/o}$ protein (Felder et al., 1995). Additional characterisation of cannabinoid-mediated inhibition of adenylyl

cyclase (and thus inhibition of cAMP production) was demonstrated in slices of rat brain (hippocampus, striatum, cerebral cortex and cerebellum) (Bidaut-Russell et al., 1990), and in CHO cells expressing exogeneous CB1 (Matsuda et al., 1990, Felder et al., 1993, Hillard et al., 1999). In recent years, the cAMP assay has become an important pharmacological technique for studying cannabinoid-mediated modulation of adenylyl cyclase in a high-throughput environment (Cawston et al., 2013). The functional characterisation of cannabinoids in this thesis have employed a kinetic Bioluminescence Resonance Energy Transfer (BRET) assay to assess cAMP levels in intact cells (method described in Appendix. B).

An year later, a more direct measure of CB1-G-protein coupling, an early event after GPCR activation, was determined using membrane-bound assay, involving GTPγS (Selley et al., 1996). Both CP55940 and WIN5512-2 stimulated [^{35}S]GTPγS binding to a similar extent in an SR141716A (CB1 antagonist) sensitive-manner, giving a relatively simple mechanistic understanding of cannabinoid specific effect (Selley et al., 1996). Another study showed the WIN55212-2-mediated stimulation of [^{35}S]GTPγS in CB1 knockout mice, and that this effect was not blocked by SR141716A, possibly hinting on the involvement of other molecular targets (of which CB2 is an example) (Breivogel et al., 2001). Moreover, the first indication of the constitutive activity of CB1 was observed in the previous study, where SR141716A alone produced a small inhibition of basal [^{35}S]GTPγS binding (Selley et al., 1996), although this activity was examined in more detail by others (Seifert and Wenzel-Seifert, 2002, Leterrier et al., 2004, Grimsey et al., 2010).

The modulation of ion channels is another significant pathway downstream of the cannabinoid receptor activation through a $G_{i/o}$-dependent mechanism (Demuth and Molleman, 2006). An early study showed the cannabinoid-mediated inhibition of N-type voltage-gated Ca^{2+} channels in differentiated N18 neuroblastoma and NG108-15 neuroblastoma-glioma hybrid cells (Mackie and Hille, 1992, Mackie et al., 1993). Further investigation on the mechanism of inhibition of N-type currents revealed that this effect was mediated by $G_{i/o}$ proteins (PTX-sensitive) and was independent of the cAMP pathway (unaffected by 8-Bromo-cAMP treatment) (Mackie et al., 1993). In rat cervical ganglion neurons microinjected with CB1 mRNA, WIN55212-mediated inhibition of Ca^{2+} currents were both PTX- and ω-conotoxin-sensitive, demonstrating its mediation by N-type Ca^{2+} channels via $G_{i/o}$ protein subunits (Pan et al., 1996). When CB1 was expressed in AtT-20 cells, cannabinoids mediated inhibition of P/Q-type calcium channels and an activation of

G protein-gated inwardly rectifying potassium channels (GIRKs) was observed (Mackie et al., 1995). Again, this process was mediated via $G_{i/o}$-dependent mechanism (Mackie et al., 1995). Further elaboration into cannabinoid-mediated regulation of these channels was demonstrated in Xenopus oocytes and rat sympathetic neurons co-expressing the CB1 receptor and GIRK1 and GIRK4 channels (Mcallister et al., 1999). By contrast, early reports in AtT-20-CB2 cells were unable to find evidence of CB2 coupling to either Q-type Ca or K_{ir} current, but inhibition of adenylyl cyclase was reported (Felder et al., 1995). This may be because of the poor coupling of WIN55212-2 to CB2 (i.e. ligand-specific effects on the conformation states of the receptor), as later CP55940 was shown to inhibit voltage-gated Ca^{2+} channels downstream of CB2 activation, but a later study found that WIN55212-2 also failed to modulate Ca^{2+} channel in the same cells expressing mouse CB2 (Atwood et al., 2012, Ibsen et al., 2017), suggesting ligand bias. However, a recent detailed study on human CB2 receptor signalling profile showed the ability of WIN55212-2 and CP55940 to stimulate canonical CB2 receptor $G_{i/o}$-mediated activation of GIRK channel in AtT20 cells (Soethoudt et al., 2017). Assaying the activity of GIRK channel has become more common in recent years with the development of a high-throughput assay by the Connor group in Macquarie University to readily measure the real-time activation of GIRK channel in AtT-20 cells expressing cannabinoid receptors (Knapman et al., 2014, Banister et al., 2016, Sachdev et al., 2019c).

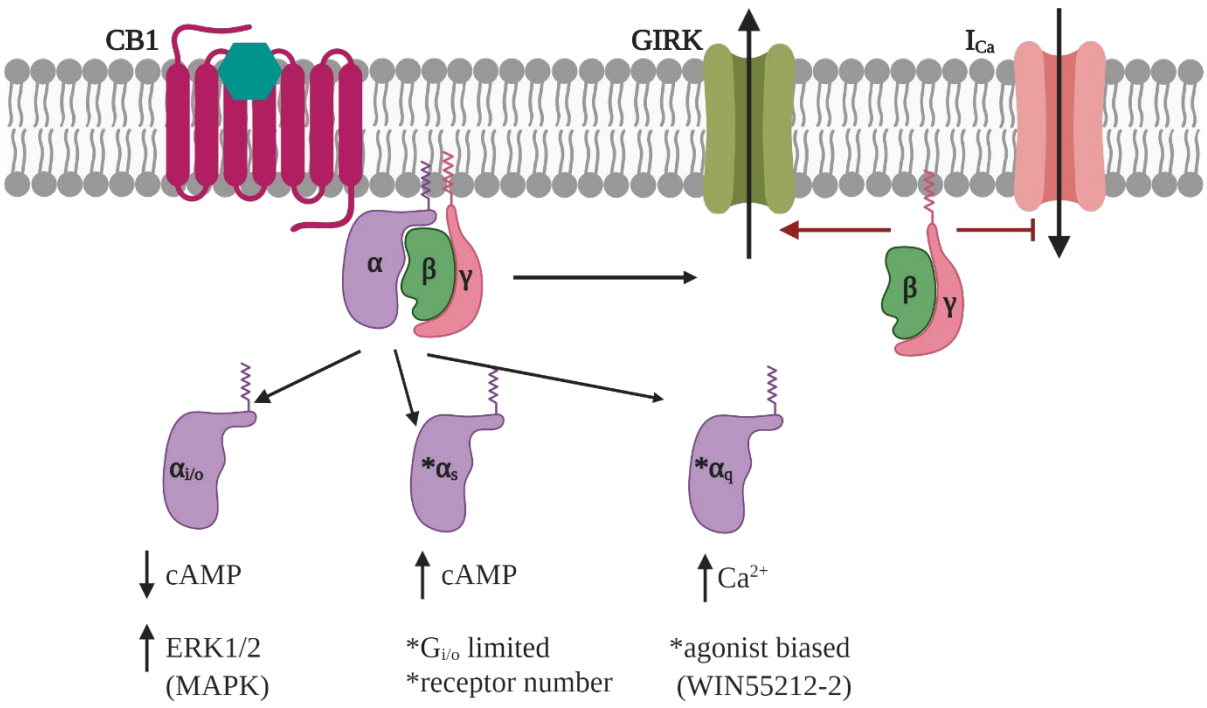


Figure 1-5. CB1 receptor signalling with a focus on the Gα proteins

Signalling through CB1 can be mediated via more than one G protein subtypes. $G_{i/o}$ is canonical CB1 signalling pathway; G_s is the second most characterised G protein alternative signalling pathway; while CB1 coupling to G_q has also been observed under various circumstances (represented as *). Figure created with BioRender.

CB1 coupling to G_s (stimulation of cAMP) has also been observed under certain circumstances. Glass and Northup (1999) demonstrated the ability of cannabinoids to stimulate cAMP on co-expression with D2 receptor. The mechanism likely involves sequestration of $G_{i/o}$ by D2 receptor – such that $G_{i/o}$ is no longer available for coupling to CB1, and thus drives the coupling to G_s (Glass and Felder, 1997, Jarrahian et al., 2004). G_s-like signalling of CB1 has also been suggested under circumstances where G_i is limited; pre-treatment of cells with PTX to prevent G_i coupling to the CB1 is used readily to measure CB1-G_s coupling (Jarrahian et al., 2004). A recent study has observed G_s-like signalling of CB1 in assays with very high receptor expression levels (Finlay et al., 2017). The ability of cannabinoids to activate G_s-like signalling of CB1 is discussed in detail in Chapter 3 summarised in Figure 1-5 (Differential activation of G-protein-mediated signalling by synthetic cannabinoid receptor agonists). In contrast to CB1 receptors, CB2 does not couple to G_s, HU-210 and CP55940 failed to increase cAMP levels in CHO-CB2 cells pre-treated with PTX (Glass and Felder, 1997, Calandra et al., 1999). In studies with native CB2, however HU-308 mediated stimulation of cAMP was observed in human primary leukocytes (Saroz et al., 2019a). G_s-protein was specifically implicated in this effect, as a selective inhibitor of this G protein, NF-449 abolished the net cAMP response such that CB2 agonist (HU-308) can no longer drive effects (Saroz et al., 2019a).

A few reports also suggest that cannabinoid-mediated increase in intracellular Ca^{2+} influx may be due to CB1 coupling to G_q protein (Lauckner et al., 2005). The first report of CB1-G_q involved a fura-2 fluorescence assay in HEK cells transfected with CB1 as well as in cultured hippocampal neurons (which endogenously expresses CB1). WIN55212-2 (but not THC, HU-210, and 2-AG) increased intracellular Ca^{2+} in a PTX-insensitive manner. On treatment with U73122 (PLC inhibitor), the effect was blocked suggesting a potential involvement of G_q pathway in this response (Lauckner et al., 2005). This was further confirmed when WIN55212-2 failed to change the intracellular Ca^{2+} levels in cells expressing dominant negative G_q proteins (Q209L/D277N) (Lauckner et al., 2005). An opportunity therefore existed in the current study to better understand the agonist-dependent

coupling to different G proteins (may be explained by different receptor conformation) - an example of biased signalling (Ibsen et al., 2017). The earliest reports with CB1 have otherwise suggested PTX-sensitive ($G_{i/o}$ mediated) increase of Ca^{2+} release from internal stores (Sugiura et al., 1997, Netzeband et al., 1999). Recently, the Connor group has shown another example of significant bias in CB1 coupling to G_q - functional characterisation of *N*-arachidonoyl dopamine (NADA, an endocannabinoid) revealed G_q-mediated increase in intracellular Ca^{2+} levels, while NADA failed to affect cAMP, ERK, or GIRK signalling (Redmond et al., 2016). To date, the literature does not present strong evidence for CB2 coupling to G_q (Figure 1-6). One study suggests anandamide-mediated increase in cytosolic Ca^{2+} was blocked by SR144528 (CB2 antagonist) and PLC inhibitor, indicative of a CB2 receptor-mediated PLC mechanism for Ca^{2+} mobilisation from endoplasmic reticulum stores (Zoratti et al., 2003).

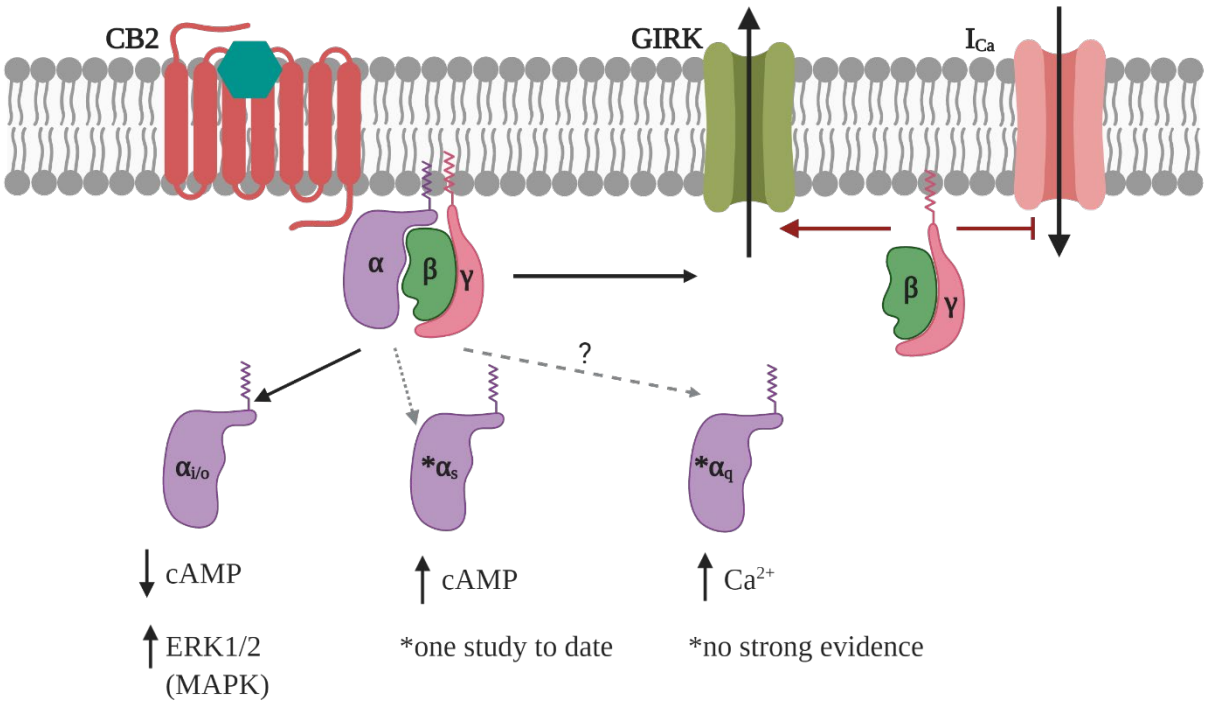


Figure 1-6. CB2 receptor signalling with a focus on the Gα proteins

Signalling through CB2 can be mediated via more than one G-protein subtypes. $G_{i/o}$ is canonical CB2 signalling pathway; and there is limited evidence for CB2 coupling to G_s and G_q (represented as *). Figure created with BioRender.

Activation of mitogen-activated protein kinase (MAPK, also known as the Ras-Raf-MEK-ERK) pathway downstream of CB1 is an important pharmacological endpoint as it is involved in many cellular processes (including proliferation, differentiation, and apoptosis), although the precise mechanism for the induction of MAPK remains to be elucidated (Ibsen et al., 2017). CB1 is believed to regulate MAPK pathway via many different mechanisms - activation of phosphatidylinositol-3-kinase (PI3K) (Del Pulgar et al., 2000), stimulation of protein kinase B (PKB) (Sánchez et al., 2003), production of lipid second messenger ceramide (Sánchez et al., 1998), inhibition of cAMP levels (Derkinderen et al., 2003), and modulation by transcription factors (Krox-24) (Bouaboula et al., 1995) (reviewed elsewhere Sachdev (2016)). The diverse responses of CB1-MAPK activation are unique to the whole-cell systems being studied, indicating a degree of system bias effect. While CB2-mediated activation of MAPK pathway has not been extensively studied, two publications exist on the mechanism underlying MAPK pathway activation (PTX-sensitive phosphorylation of ERK1/2 (Bouaboula et al., 1996), and a study primarily focused on CB2-mediated signalling in human primary leukocytes suggests Gβγ involvement in ERK signalling of CB2 (Saroz et al., 2019a)).

Although the studies presented in this thesis only investigated the signalling dynamics of CB1 and CB2 receptors, it should be noted that several studies have also reported the ability of certain cannabinoids to interact with the putative non-CB1/2 target, particularly G protein coupled receptor (GPR55), and transient receptor potential (TRP) channels (Ryberg et al., 2007). Initially, cannabinoids have been shown to interact with GPR55 in a yeast expression system in a GlaxoSmithKline patent, where the CB1 antagonists SR141716, and AM251 showed agonistic profile for GPR55 activating the system at a micromolar concentration range (Brown and Hiley, 2009). While, AstraZeneca group demonstrated the stimulatory effect of a diverse group of cannabinoids (including endocannabinoids, CP55940, and THC) on GPR55 in an assay of GTPγS binding in HEK cells (Ryberg et al., 2007). Although, based on data from various cell types and functional readouts, some studies have reported that endogenous and synthetic cannabinoids, including many mentioned above, failed to affect the signalling transduction pathway downstream to GPR55 (Oka et al., 2007). Given the large variation in signalling readouts - suggesting a form of observational bias based on relative sensitivity of assays, GPR55 has been considered to have a unique response profile compared to CB1 and CB2 receptor, highlighting the enigmatic pharmacology of GPR55 (Sharir and Abood, 2010). Although it's physiological role has yet to be fully elucidated, it

has been implicated in neuropathic and inflammatory pain, and bone remodelling (Staton et al., 2008). Pharmacological evidence also suggests that cannabinoids can modulate the activity of TRP channels implicated in sensation of temperature, pH, smell, taste, vision, and pain perception (Ramsey et al., 2006, Pertwee et al., 2010). Channels of Transient Receptor Superfamily (TRP), such as TRPV1-4, TRPM8, and TRPA1, are non-selective channels that conducts calcium and sodium into range of cell types in mammals (Ramsey et al., 2006). A suite of cannabinoids have been investigated for their differential responses upon activation of TRP channels (Morales et al., 2017). For example, the endocannabinoid, anandamide and NADA, were identified as endogeneous antagonist of TRPM8 (De Petrocellis et al., 2007), while anandamide was identified as an agonist of TRPV1 in an assay of vasodilatior action of AEA and electrophysiology (Zygmunt et al., 1999). CBD and THC were both found to modulate different subtypes of TRP channels – TRPA1 and TRPV2 (discussed below). These observations highlight the complexity of the pharmacology of cannabinoids interaction with TRP channels, and their involvement in chronic pain and inflammation merits further study (reviewed in Muller et al., 2019).

1.3.3. Regulation of cannabinoid receptors - Desensitisation, arrestins and internalisation

The continuous stimulation of receptor with ligand generally results in the reduction of receptor activity referred to as desensitisation. In brief, desensitisation involves phosphorylation of the receptor by GPCR kinases (GRKs), which increases the affinity of β-arrestins for receptor. Binding of β-arrestins to receptor facilitates the recruitment of another protein called clathrin, which is followed by receptor internalisation into cytoplasmic vesicles. Lastly, the receptor is either recycled to the membrane or degraded, depending on the receptor studied (Von Zastrow, 2003). These steps highlighting regulation of GPCRs are rather generalisations as not all receptors rely on these mechanisms.

An early study conducted during the 1990s indicated no change in receptor number or mRNA levels on repetitive administration of THC in whole-brain homogenates prepared from male mice, although a 27-fold tolerance to THC was observed in the behavioural assay suggesting that tolerance can develop to many of the effects of THC (Abood et al., 1993). However, the authors noted that the possibility remains that the cannabinoid receptor signal transduction system may cause desensitisation without having an effect on the receptor number (Abood et al., 1993). Other studies to date, following daily THC treatment in developing rats at different time-points, showed extensive reduction in cannabinoid receptor

binding in cerebellum, hippocampus, caudate-putamen, and globus pallidus in a time-dependent manner (Oviedo et al., 1993, Breivogel et al., 1999). These effects have been reviewed in detail (Huestis, 2005). Soon after that, Mackie and colleagues, studied the role of regulatory proteins involved in the desensitisation of rat CB1 transfected in Xenopus oocyte and AtT-20 cells (Jin et al., 1999, Roche et al., 1999). They demonstrated that both GRK3 and β-arrestins were required for desensitisation of CB1-mediated activation of GIRK channel injected in oocytes, but not affected by either protein alone (Jin et al., 1999). Further investigation into the region of CB1 involved in receptor regulation showed that mutation of two sites (S426A or S430A) in the C-terminal tail of CB1 significantly attenuated desensitisation in oocytes; however, these sites had no influence on CB1 internalisation when tested in AtT20-CB1 cells (Jin et al., 1999). When the C-terminal of rat CB1 was truncated at residue 418, WIN55212-2-mediated desensitisation was abolished, suggesting GRK-dependent desensitisation of CB1 (Jin et al., 1999). Another study was conceived by the same group to understand the differences in receptor signalling between rat- and human-CB1 expressed in autaptic hippocampal neurons, while human CB1 differs from rat only by 13 residues, significant differences in their signalling profile was observed when depolarisation-induced suppression of excitation (DSE) was induced (Straiker et al., 2012). Although clearly more research is required to assess distinct amino acid residues involved in GRK- or arrestin-mediated cannabinoid receptor regulation - as the cellular trafficking observed in oocyte is different to those observed in native tissues or neurons (expressing multiple native channels) (Goldin, 2006). Our group has studied the mechanism underlying the CB1 desensitisation by more recent catalogues of synthetic cannabinoids in AtT20 cells that had been stably transfected with human CB1 in assays examining the sensitivity of the process to Compound 101 (a potent selective inhibitor of GRK2/3) (Sachdev, 2016). Our data suggest GRK-dependent and independent mechanisms for CB1 receptor desensitisation by the highest efficacy agonists, but only GRK-independent mechanisms for lower efficacy agonists (Appendix A.) (Sachdev, 2016). To date there is little evidence on CB2 receptor desensitisation, Bouaboula et al. (1999) showed that CB2 receptor is also desensitised and internalised following agonist treatment *in vitro* - phosphorylation by S352 may be a key factor for CB2 receptor regulation.

Like other GPCRs, CB1 also undergoes internalisation after brief exposure to agonist as observed in AtT-20-CB1 cells, where WIN55212-2 caused rapid internalisation of CB1 receptor while THC only caused modest CB1 internalisation (Hsieh et al., 1999). In CHO

cells transfected with CB1, chronic treatment with an inverse agonist resulted in alteration in cellular distribution profile by upregulating the cell surface receptors (Rinaldi-Carmona et al., 1998). Direct evidence of the involvement of β-arrestins1/2 in CB1 internalisation has been assessed using confocal imaging of β-arrestin-2-RFP recruitment to CB1 receptor (Daigle et al., 2008, Gyombolai et al., 2013). Studies using β-arrestin 2 knockout mice revealed greater antinociception and hypothermia following treatment with THC compared to their wild-type counterparts, while CP55940 failed to affect *in vivo* activity in either β-arrestin 2 -/- or +/+ (indicating the role of other arrestins, and protein kinases in the regulation of CB1) (Breivogel et al., 2008). It is suggested that β-arrestin 2 may regulate CB1 receptor signalling in an agonist-specific manner (Breivogel et al., 2008). Further studies revealed that CB1 may recycle or degrade following internalisation depending on the duration of agonist stimulation (Hsieh et al., 1999, Martini et al., 2007). However, another study performed a detailed quantification of CB1 trafficking in four different cell lines (Grimsey et al., 2010). They found that CB1 does not recycle following agonist-induced internalisation, but rather exhibits a primarily degradative phenotype (Grimsey et al., 2010) (summarised in Figure 1-7). A more detailed study on CB1 receptor trafficking can be found in (PhD Thesis, Grimsey (2010)). Recently, Ibsen et al. (2019) investigated the ability of CB1 and CB2 to induce β-arrestins 1/2 translocation to the membrane on stimulation with range of cannabinoid ligands - using a new method that does not require any modification of receptors. The authors found diverse range of efficacy profile between ligands for translocation of arrestins, with endocannabinoid 2-arachidonoyl glycerol showing higher efficacy for translocation of β-arrestin 2 via CB1, while THC was unable to induce translocation of either arrestin via CB1 or CB2 (Ibsen et al., 2019). These studies also suggest that the important area for further investigation include differential regulation of cannabinoid receptor activation by agonists with diverse efficacy profile, a systematic investigation of the protein kinases involved in cannabinoid receptor desensitisation, identification of the key residues required for receptor desensitisation and internalisation, and modulation of CB1 targeting to the degradation pathway.

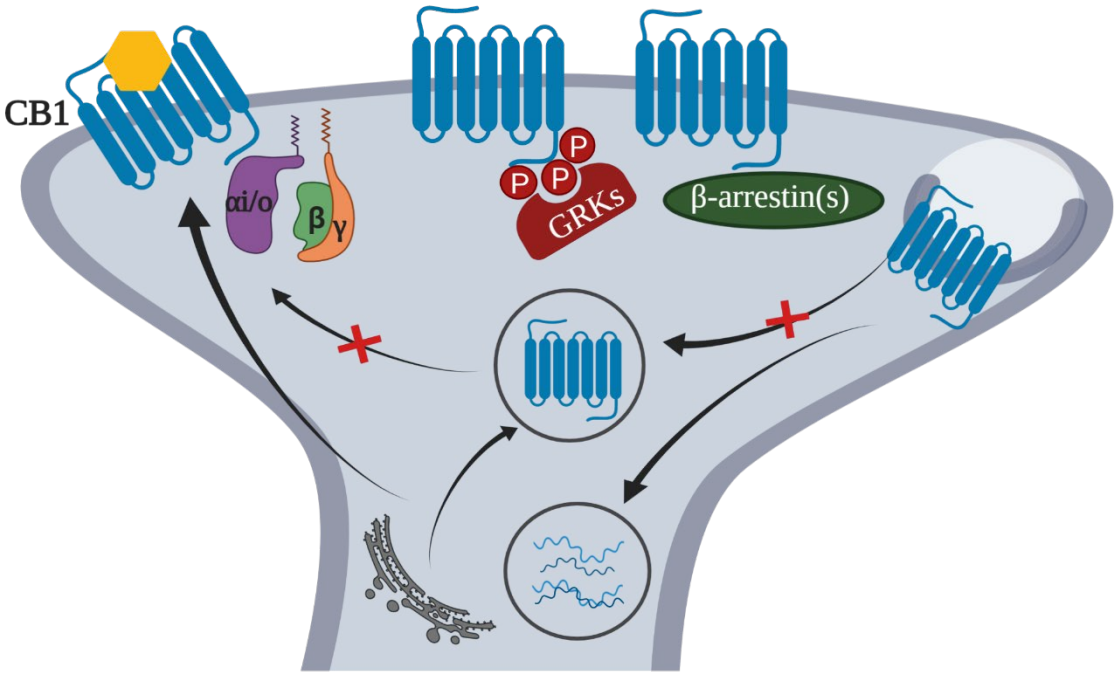


Figure 1-7. Regulation of CB1 receptors

The whole process is initiated by binding of an agonist to the CB1 for an extended period of time, which triggers a signalling (coupling of G_i to CB1). Prolonged receptor activation also results in phosphorylation by GRKs. This post-translational modification leads to CB1 desensitisation and the recruitment of β-arrestins, resulting in CB1 internalisation. Unlike other GPCRs, CB1 intracellular pool is not delivered back to the cell surface (presented as **X**) and is instead degraded. Thus, the newly synthesised receptors are transported from the endoplasmic reticulum to the cell surface. Figure created with BioRender.

1.4. Endocannabinoids

Endogenous cannabinoids are generally referred to as 'endocannabinoids', and together with cannabinoid receptors, and the enzymes responsible for their synthesis and degradation, form the endocannabinoid system. The endocannabinoid system modulates a wide range of physiological processes in mammals, including learning, memory, motor control, and neurodegenerative diseases (reviewed in (Hillard, 2018, Maccarrone, 2019)).

1.4.1. Discovery of endocannabinoids

The identification of CB1 receptors in the brain suggested the presence of an endogenous cannabinoid that activate these receptors. Professor Mechoulam presented the early ideas of the existence of endogenous ligands, proposing that:

"It was quite unacceptable to most neuroscientists that the brain will waste its resources to synthesize a receptor in order to bind a constituent of a plant."

Devane et al. (1992) and his group developed tritium-labelled probe [^{3}H]HU-243 (CB1 agonist) to test the ability of porcine brain extract to displace the probe-receptor complex in a ligand-binding assay. The constituents of the fraction that displaced the radioactive probe were purified using column chromatography, which was then isolated as one main peak on gas chromatography-mass spectrometry (GC-MS). This lipophilic compound represented the first brain constituent that exhibited cannabis-like (or, cannabimimetic) activity – binding of [^{3}H]HU-243 was displaced in a manner consistent with that of competitive ligand (K_i similar to THC) (Devane et al., 1992). They named this endogenous cannabinoid 'anandamide' from '*ananda*' the Sanskrit word for bliss. Subsequently, Pertwee et al. (1992) demonstrated the inhibitory effect of anandamide (AEA) on twitch response in electrically-stimulated mouse vas deferens in a result which further confirmed the cannabimimetic activity of AEA at CB1. In the late 1990s, several groups initiated work aimed at investigating the pharmacological profile of AEA in bioassays for cannabinoid receptor agonists (reviewed in Huestis (2005)). The discovery of a second endogenous cannabinoid, again an arachidonic acid derivative, 2-arachidonoyl glycerol (2-AG), was followed soon after (Sugiura et al., 1995). Although both AEA and 2-AG contain arachidonic acid, they are synthesised and degraded by almost completely distinct enzymatic pathways. The best characterised endocannabinoid enzymes include diacylglycerol lipase (synthesis of 2-AG), monoacylglycerol lipase (degradation of 2-AG), N-acyl phophatidylethanolamine phospholipase D (synthesis of AEA), and fatty acid amide hydrolase (degradation of AEA) (reviewed in Zou and Kumar (2018), Maccarrone (2019)).

1.4.2. Endocannabinoid Signalling

Although produced by the same cell-types in central nervous system, the endocannabinoids 2-AG and AEA have unique pharmacological profiles at CB1 and CB2, exhibiting different efficacies and affinities (Hillard, 2000, Savinainen et al., 2001, Luk et al., 2004). It is well established that 2-AG has high intrinsic efficacy at CB1 compared to AEA, which is a partial agonist of CB1 (Horne and Stella, 2008). A systematic characterisation of the endocannabinoids in oocytes transfected with CB1 showed that 2-AG activated the GIRK currents to a similar extent as the reference compound, WIN55212-2 (100% maximal WIN response); AEA was less efficacious at activating GIRK currents, achieving only 65% of the

WIN response (Luk et al., 2004). Consistent with this, our laboratory recently performed a quantitative pharmacological study to measure the efficacy of a range of cannabinoids, where 2-AG was found to be 12-fold more efficacious in activating native GIRK channels in AtT-20-CB1 cells than AEA ((Sachdev et al., 2019c), Chapter 2). Some evidence suggests that AEA (or THC) may antagonise 2-AG-mediated CB1 receptor signalling under certain circumstances, such as low receptor density or limiting post-receptor effectors (Kelley and Thayer, 2004, Kellogg et al., 2009). Studies in brain homogenates of CB1 knock-out mice demonstrate that AEA was still able to stimulate GTPγS binding (Hájos et al., 2001), this finding was added to and largely confirmed in a later study which examined the activity of AEA and 2-AG in HEK293 cells expressing GPR55 (putative cannabinoid receptor) (Lauckner et al., 2008, Alexander et al., 2011). In addition, AEA has also been shown to activate TRPV1, and inhibit L-type Ca^{2+} channel (Van Der Stelt et al., 2005, Puente et al., 2011, Zou and Kumar, 2018). A list of molecular target and actions reported for endocannabinoids to date is summarised in the Table 1-3.

Table 1-3. Pharmacological profile of endocannabinoids at multiple targets based on results of studies in different experimental systems.

Endocannabinoid	Receptor/target	Pharmacology
AEA	CB1	low efficacy agonist (Hillard, 2000)
	CB2	low efficacy agonist (Hillard, 2000)
	GPR55	low efficacy agonist (Ross, 2009)
	TRPV1	low efficacy agonist (Roberts et al., 2002)
2-AG	CB1	high efficacy agonist (Sachdev et al., 2019c)
	CB2	high efficacy agonist (Luk et al., 2004)
	GPR55	agonist* (only in [^{35}S]GTPγS assay) (Ross, 2009)
	PPARγ	agonist (Kozak et al., 2002)

1.5. Entourage effect: the first ideas

The 'entourage effect' was first proposed by Raphael Mechoulam in relation to endocannabinoid system in which the activity of the primary endogenous cannabinoid, 2-AG, is largely regulated (inhibited or augmented) by the formation and presence of related, inactive, fatty acid esters of glycerol (Ben-Shabat et al., 1998). This theory of 'entourage effect' was ultimately advanced to explain the idea that cannabinoids and other components of cannabis have a greater therapeutic or psychotropic effect combined than when consumed individually (Mechoulam and Ben-Shabat, 1999, Russo, 2018). This theory can be broken

down into two main classes: cannabinoid-cannabinoid interaction and cannabinoid-terpenoid interaction. The idea of cannabinoid-cannabinoid interaction was first implied by Russo and Guy (2006), and Thomas et al. (2007) in an observation that CBD was able to antagonise THC-mediated effect of CB1 receptor, despite the low affinity of CBD for the CB1 orthosteric site. In line with this observation, CBD has been reported to negatively modulate the CB1 signalling, which is likely to contribute to its anti-psychotic effect (Laprairie et al., 2015). This finding, however, remains controversial with some studies producing contradictory results (discussed in Chapter 6). In a clinical trial in multiple sclerosis patients with spasticity, CBD mitigated some of the adverse effects caused by THC when CBD and THC (Sativex) was administered together, suggesting the possibility of synergistic benefit of a THC-CBD preparation (Russo, 2011, Koppel et al., 2014, Benbadis et al., 2014).

Over 200 terpenoids have been isolated from the cannabis plant (Hendriks et al., 1975, Ross and Elsohly, 1996, Brenneisen, 2007). Terpenoids, the collective name given to large number of monoterpenes (limonene, myrcene, pinene, etc.) and sesquiterpenoids (especially caryophyllene), are lyophilic compounds that can permeate the cell membrane, and are responsible for the unique aroma of cannabis (Russo, 2011). Unlike for cannabinoid-cannabinoid interaction, there is very little scientific evidence on the cannabinoid-terpenoid interaction (Santiago et al., 2019). Terpenoids are widely professed to exhibit anti-inflammatory, antioxidant, analgesic, anticonvulsive, antidepressant, anxiolytic, anticancer, and neuroprotective properties (mostly derived from non-scientific literature). Moreover, terpenoids are believed to exert their therapeutic effects through interactions with multiple targets, including CB1, CB2, GPR55, acetylcholine receptors, serotonin receptors, and GABA receptors (summarized in (Nahler et al., 2019)). A single publication also exists on the selective agonist activity of β-caryophyllene on CB2 (Gertsch et al., 2008). This interesting facet of the hypothesised cannabinoid-terpenoid ‘entourage effect’ has been investigated by our group in a uniform in vitro study. We found that the cannabinoid receptors were not altered by any of the six major terpenoids tested (including β-caryophyllene), neither when used individually nor when mixed, suggesting the absence of an ‘entourage effect’ (discussed in more detail in Chapter 5, (Santiago et al., 2019)). Still, more research is needed to conclusively determine if terpenoids produce physiologically-relevant interactive effects with other targets.

1.6. Synthetic cannabinoid receptor agonists (SCRAs)

1.6.1. Discovery of synthetic cannabinoids

Since the precise determination of the structure of THC in the early 1960s, synthetic cannabinoids were synthesised in order to investigate the endocannabinoid system (Harbert et al., 1979, Compton et al., 1992b). THC served as a template for generation of the first synthetic cannabinoids, HU-210 and CP47497; however, over time the structure of synthetic cannabinoids have deviated further from THC (Wiley et al., 2011) (Figure 1-8). Subsequent studies synthesised CP55940 (a bicyclic compound) to assess potential cannabinoid receptor binding (Harbert et al., 1979). For example, the tritium labelled [^{3}H] CP55940 was used to identify and characterise the CB1 in rat brain membranes (Devane et al., 1988). Martin et al. (1991) categorised CP55940, a bicyclic compound, as a non-classical cannabinoid (as opposed to classical cannabinoids such as THC). Importantly, the authors fully characterised the pharmacological similarities and differences between CP55940 and THC. CP55940 can produce the full spectrum of tetrad effects (hypoactivity, hypothermia, antinociception and catalepsy) in mice that is typical of cannabinoids (Martin et al., 1991), though this phenomenon has been explicated in more detail in other studies (Little et al., 1988, Fox et al., 2001). Further, the behavioural potency of CP55940 was found to be 30 times that of THC (Gold et al., 1992, Wiley et al., 1995). The finding of the *in vivo* reinforcing effects of CP55940 was determined by the conditioned place preference and conditioned taste avoidance paradigms (McGregor et al., 1996). Indeed, the more that is known about these compounds, the more question that arise.

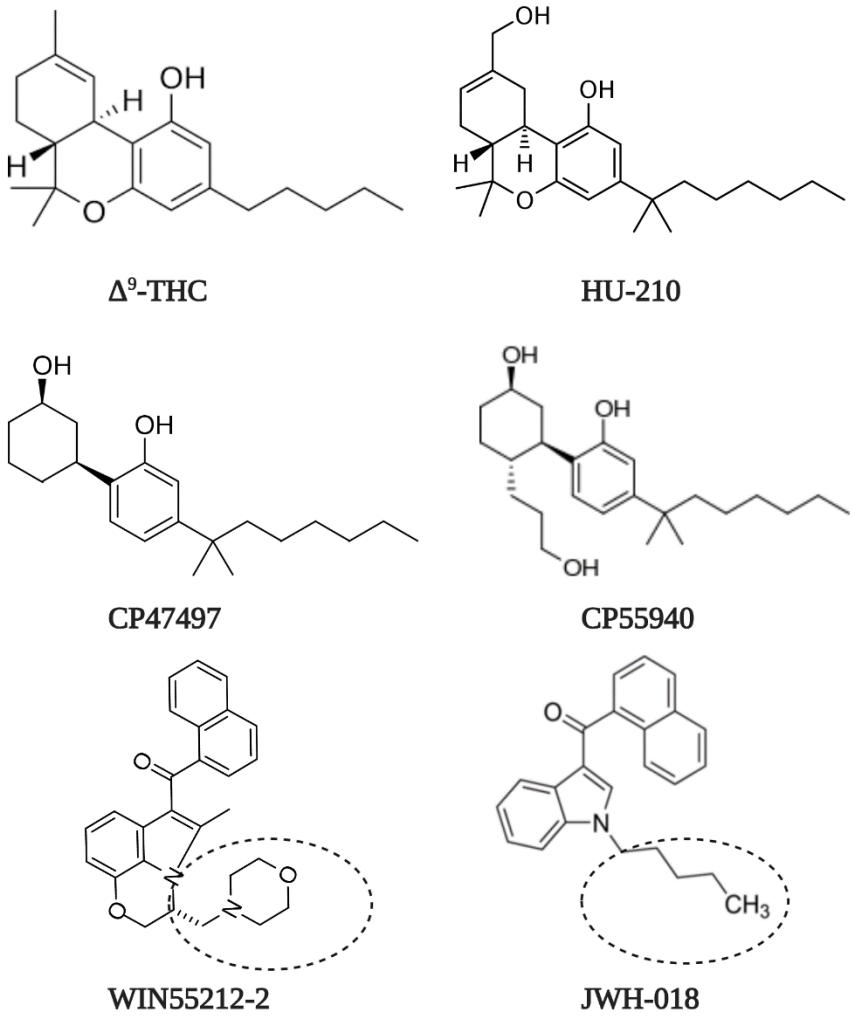


Figure 1-8. Molecular structures of THC, and early synthetic cannabinoids identified in herbal incense products

Molecular structures of THC (a phytocannabinoid), HU-210 (classical cannabinoid based on the template of THC), CP47497 and CP55940 (non-classical cannabinoids characterised as bi- and tri-cyclic THC analogues lacking pyran ring), WIN55212-2 (aminoalkylindole), JWH-018 (first generation synthetic cannabinoids). Structures were drawn using ChemDraw Professional 18.2.

Considerable effort was made into modifying the structure of cannabinoids to further explore the structural requirements of CB1 receptor binding - the work at Sterling-Winthrop led to some aminoalkylindole analogues (originally designed as non-steroidal anti-inflammatory drugs) (Haubrich et al., 1990, D'ambra et al., 1992). Researchers later discovered that this group of synthetic aminoalkylindole compounds, including WIN55212-2, produce strong analgesic effects at relatively lower doses (D'ambra et al., 1992). Further research into the pharmacological profile of aminoalkylindole analogues suggested that these compounds have nanomolar potency in a cannabinoid receptor binding assay and corresponding

cannabimimetic activity in *in vivo* assay (Compton et al., 1992a, Eissenstat et al., 1995). In an effort to dissect the analgesic properties of cannabinoids from unwanted psychotropic effects, a whole new library of synthetic cannabinoids were developed (based on WIN55212-2 scaffold) in the lab of John Huffman at Clemson University (Huffman et al., 1994). These compounds (including JWH-018) were relatively easy to synthesise with carbon chains attached to the nitrogen in the indole substituent (Wiley et al., 2011) (Figure 1-8). Later, studies reported the binding affinity and efficacy of JWH-018 for CB1 and CB2 receptors. In contrast to THC, which is a low efficacy agonist of CB1 (meaning it cannot activate CB1 receptor to the same extent irrespective of concentration), JWH-018 showed maximal agonist activity at the CB1 receptor compared to THC (Atwood et al., 2010, Paronis et al., 2012, Sachdev et al., 2019c). Huffman and his colleagues eventually created more than 300 new compounds with the incremental development of understanding of cannabinoid receptor pharmacology (Wiley et al., 1998). Some years later, Professor Huffman received a call from law enforcement agencies as JWH-018 was identified in a herbal mixture being marketed as a recreational drug in the new psychoactive substances (NPS) market (Wiley et al., 2011). Basically, in Huffman's own words (Money, 2019):

"It was the 18th compound that we synthesized, and it was pretty potent - never thought anything of it".

Recreational use of synthetic cannabinoids was first reported in Europe in the early 2000s, and shortly thereafter it was introduced in the NPS market in USA in 2008 (Dresen et al., 2010). These herbal products were made by dissolving synthetic cannabinoids in a solvent (acetone) and sprayed onto a dried-plant derived base which is then packaged for retail. SPICE was the brand name for the herbal product, and contained several synthetic cannabinoids, yet primarily JWH-018 (Auwärter et al., 2009, Griffiths et al., 2010, Logan et al., 2012). Often products were labelled "not for human consumption" in an attempt to circumvent legislation (Bretteville-Jensen et al., 2013). These products spread in popularity by word of mouth and drug-user blogs, were thought to produce similar highs to that of cannabis (words such as, "safe", "natural", and "legal" were used). In consequence, synthetic cannabinoid products quickly spread to other markets, and were readily accessible on the internet, head-shops, gas station, convenience stores etc (Bretteville-Jensen et al., 2013). For example, an increase in the headshops up from 314 in 2011 to 693 in 2012 in Ireland was reported to the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA, 2012). Subsequently, more structurally diverse (and some unknown) synthetic

cannabinoids have been identified in herbal mixtures with brand names such as K2, Cloud 9, Kronic, Black Mamba, Rasta King, etc., accounting for 30% of the 888 NPS reported to the United Nations Office on Drugs and Crime (UNODC, 2018) Early Warning Advisory (EWA) up to December 2018. Whereas most of the early compounds were derived from prototypic napthoylindole JWH-018, several chemical innovation of the napthoyl group soon began to appear (Wiley at al., 2016). Unlike the substitution at other positions, additions to the napthoyl group is less likely to interfere with the optimal aromatic stacking, important for cannabinoid receptor recognition (binding affinity of the compounds may be greatly enhanced by this modification) (Huffman et al., 2003). Later, the SCRAs resulting from the substitution of napthoyl core with tetramethylcyclopropyl ketone (UR-144, XLR-11) have been identified in the NPS market (Kavanagh et al., 2013). It was also recognised that the addition of the fluorine group at the terminal end of the n-alkyl substituent of UR144 (i.e. XLR-11) displayed more potent activation of CB1 receptor (Bansiter at al., 2015b). The substitution of indole group with indazole core (AB-PINACA, AB-CHMINACA, etc.) was another major pharmacophore for CB1 activation (Wiley at al., 2016, Bansiter at al., 2015a); indazole-derived cannabinoids were reported to demonstrate drastic changes in their pharmacological abilities (greater efficacies of these compounds compared to the full agonist activity of CP55940 or WIN55212-2 at CB1) (Wiley et al., 2015). Figure 1-9 provides a summary of the structurally diverse and rapidly changing compounds that have been found since 2009, highlighting the continued evaluation and sophistication of the chemical modifications in response to regulation (reviewed in Banister and Connor (2018a), Worob and Wenthur (2019)). Given the structural heterogenicity of synthetic cannabinoids with very little known about their pharmacology and toxicology at time of identification, substantial conflicts remain in the literature regarding the name and classification of synthetic cannabinoids (Potts et al., 2019). As synthetic cannabinoids are high efficacy agonist of CB1 and CB2 receptors and produce psychoactive effects attributed mainly to the activation of CB1, these compounds are referred to as synthetic cannabinoid receptor agonists (SCRAs) in the rest of this thesis (Whiting, 2015, Helander, 2017).

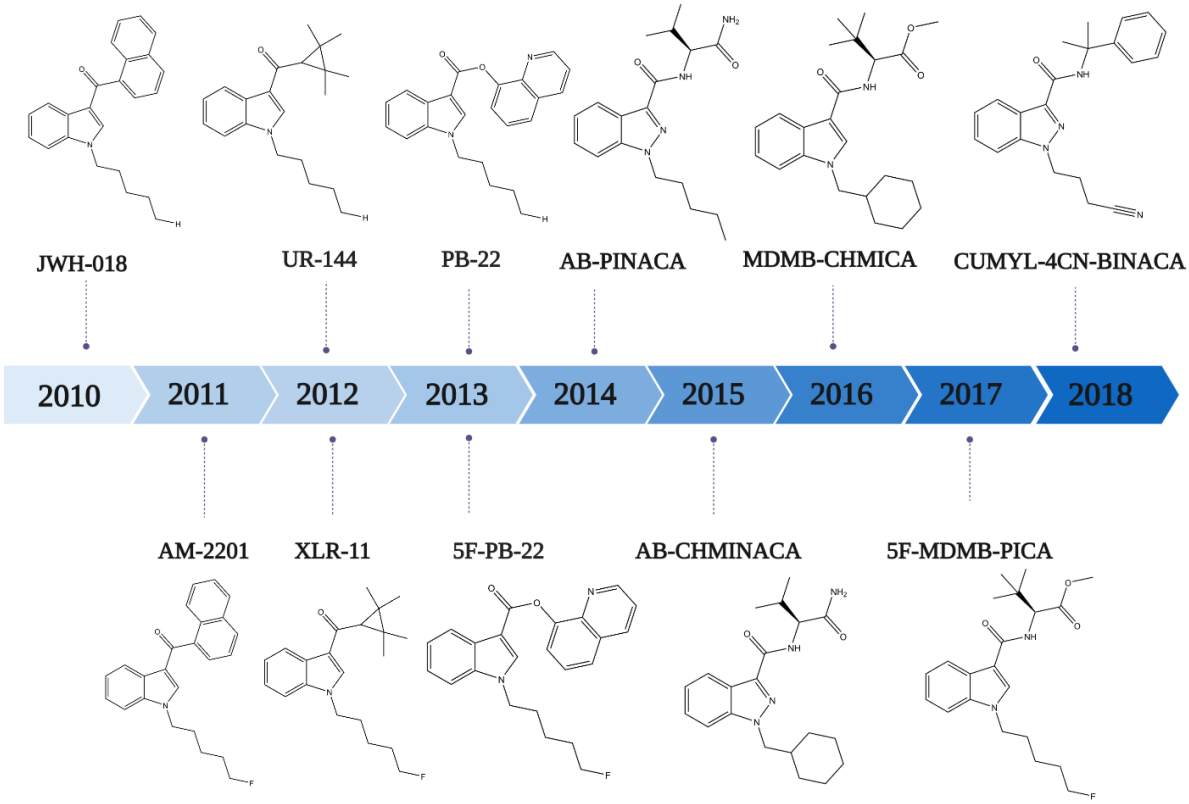


Figure 1-9. Evolution of SCRAs over time

Structurally diverse and rapidly changing SCRAs beyond aminoalkylindole compounds since 2009. Structures were drawn using ChemDraw Professional 18.2. Figure created with BioRender.

1.6.2. Synthetic cannabinoid receptor agonists toxicity

SCRAs, unlike their natural counterpart (THC), produce a wide range of acute effects – physiological and psychological (Schneir et al., 2011, Castaneto et al., 2014). The acute effects of SCRAs include changes in mood, anxiety, perception, thinking, and memory, often result in more amplified neuropsychiatric effect as compared to THC (reviewed in Spaderna et al., 2013). A clinical case report of acutely intoxicated patients described a variety of other effects including nausea, tachycardia, respiratory difficulties and generalized convulsions (Hermanns-Clausen et al., 2013). A very common psychological reaction was fear and paranoia (Soussan and Kjellgren, 2014). For example, one user reported anecdotal account of his first experience trying K2, saying, “It felt like my body was just failing on me, my organs were not working any further, it was just shutting down” (similar reports are abundant across various internet drug forums) (Speiser, 2015). The acute cognitive effect of SCRAs have also been studied in relation to driving. Consumption of

SCRAs showed clear symptoms of impairment (sedating effects, retarded movements and impairment of fine motor skills) typically described as somewhat similar to that of cannabis (Musshoff et al., 2014, Cohen and Weinstein, 2018). There have been a number of case studies with SCRA-associated impaired driving, where JWH-018, AM-2201, JWH-210, and JWH-122 were all found in drivers' systems ranging from 0.1-9.9 ng/ml (Musshoff et al., 2014). The author suggested that analytical procedures should include lower limits of quantification (below 1 ng/ml) as SCRAs were detected at lower serum levels than THC, indicating high potency of these compounds (Musshoff et al., 2014). In addition to the psychological effects, users also reported tachycardia and hypertension (Spaderna et al., 2013, Alipour et al., 2019). In one case, supraventricular tachycardia with a heart rate as high as 172 beats per minute was noted in a 24-year-old after ingestion of e-cigarette fluid containing AB-FUBINACA and ADB-FUBINACA, confirmed by quantitative analysis of the serum drug concentrations and urine toxicology screening, although the presence of AB-FUBINACA was undetected in the patient's urine (Lam et al., 2017). This highlights the challenges faced by clinicians to accurately identify and confirm human urinary SCRAs and their metabolites, as may be the case for closely structurally-related SCRAs that produce identical metabolites (Andersson et al., 2016). Another study determined the clinical characteristics of SCRA toxicity in a cohort of patients (from the ToxIC registry) (Monte et al., 2017). The authors identified 321 cases of SCRA use, in this analysis, they reported that 44 patients (12.5%) had heart rates above 140 beats per minute. While, bradycardia was the second most commonly found abnormality with 20 patients (5.7%) having heart rates less than 50 beats per minute (Monte et al., 2017). The clinical history provided by medical professional suggest that there is no consistent SCRA toxidrome, and every single case has the potential to be completely different from previously reported cases.

Several reports have documented various and substantial adverse effects associated with SCRAs, including psychosis, cardiac arrest, nephrotoxicity, gastrointestinal problems including hyperemesis, hyperthermia, cerebral ischemia, and seizures (reviewed in Cooper (2016)). Mass intoxication with use of AMB-FUBINACA in 2016 was described at the time as a "zombie outbreak', due to altered mental status of users, with reports by medical emergency services as users "being slow to respond to questioning and as having blank stare" (Adams et al., 2017). Multiple cases also detail cerebral seizures following use of SCRAs. For example, a high frequency of seizures was observed in 12 of 44 patients after using MDMB-CHMICA and AB-CHMINACA, which was detected in both blood and urine

samples (Hermanns-Clausen et al., 2018). In another case, unfortunately, an 8-year-old male was brought to an emergency department for tonic-clonic episodes, following consumption of non-pharmaceutical graded CBD oil contaminated with the SCRA (AB-FUBINACA) (Rianprakaisang et al., 2019). Acute kidney injury (AKI) has also been documented in over ten cases after smoking SCRA-product, where nine patients were hospitalised, and one required haemodialysis. The authors concluded that a new SCRA, XLR-11, may have been the cause of AKI, which was detected in both suspected product and clinical samples, reinforcing the importance of vigilance to detect unique toxidrome caused by SCRAs (Buser et al., 2014, Bhanushali et al., 2013). Cardiovascular fatalities have also been reported in the literature. For example, a 41-year-old female died due to fatal coronary thrombosis after smoking an SCRA-product, Mojo, containing ADB-FUBINACA (Shanks et al., 2016). Unexpectedly, in 2018 an outbreak of severe bleeding and abnormal coagulation profile (including four deaths) was reported following use of SCRA (Riley et al., 2019). Clinical investigation reported 81 cases testing positive for brodifacoum (superwarfarin) (Moritz et al., 2018), and AB-FUBINACA was recently detected in toxicological analyses of blood and urine samples in a single case study in the USA, suggesting apparent mixing of brodifacoum with SCRAs (Riley et al., 2019). There exists a number of very important and unanswered questions. Why has brodifacoum been mixed with SCRA? Was this an accident or deliberate? And how is it metabolised? (discussed in detail in Chapter 4 (Sachdev et al., 2019b)).

The increasing use of SCRA in prison over recent years is a crucial area of research (EMCDDA, 2018). Earlier reports have suggested high level of drug (like heroin, cannabis, opioid analgesics, etc.) consumption in prison (70%), although, the pattern of drug use in prison is changing from traditional drugs to NPS (Ralphs et al., 2017). While, a total of 670 NPS has been reported to EMCDDA, with SCRAs being the most common group of NPS used in prison (EMCDDA, 2018). The earliest report of the use of SCRA among prisoners in UK comes from Her Majesty's Inspectorate of Prisons (HMIP) in 2015, where it was observed that "spice and black mamba…had had a severe impact and...led to debt and serious violence" (HMIP, 2015). A 2016 survey led by user Voice found that one in three prisoners in England and Wales have tried SCRA in the past month, where, at the time, over 85,000 people were living in nine prisons across England and Wales (Voice, 2016). Specific motivation of SCRA use in prison has also been investigated by (EMCDDA) - as would be expected SCRA is undetectable in urine toxicology screening (at the time of identification),

effect of SCRAs themselves (boredom and escapism from prison life), the legal perception, value for money, and availability compared to natural cannabis. The actual number of deaths resulting from SCRA consumption has not been documented (for a number of technical and practical reasons reviewed in EMCDDA (2018)). Of the 79 deaths reported by EMCDDA for the years 2013-2016, 56 were self-inflicted, highlighting the likely possibility of abuse potential of SCRAs (considering the earlier reports of suicidal thoughts and self-harm after consumption of SCRA-based product). Studies have also reported some of the methods for supplying SCRAs in prison - the use of drones to delivery "packages", drugs smuggled by body packers, or paper sheets ("letters") impregnated with SCRAs (Angerer et al., 2018, EMCDDA, 2018, Norman et al., 2019). This latter issue was further investigated to provide analytical support to test 354 individual paper seized from three Scottish prisons (Norman et al., 2020). The authors confirmed that 41% (146 sa/mples) contained at least one SCRA, while 23% (33 samples) contained multiple SCRAs, of which the most prevalent SCRAs detected include 5F-MDMB-PICA and 4F-MDMDB-BINACA. It is hoped that the close monitoring of SCRAs supplied in prison may help to minimise their adverse effects to this end.

1.6.3. The pharmacology of synthetic cannabinoid receptor agonists

The pharmacological evaluation of SCRAs is essential to understand the mechanism by which SCRAs exert adverse effects, and to determine if their activity through CB1 receptor is sufficient to contribute to their ongoing impact across clinical aspects. Similar to THC, SCRAs have high binding affinity for CB1 and CB2 receptors - homogenate binding competition-displacement assays revealed nanomolar-range affinities (reviewed in Castaneto et al. (2014)). A battery of standard *in vitro* assay have been used to study the pharmacological profile (potency and efficacy) of SCRAs at CB1 and CB2 receptors. Qualitative assessment of the activity profiles of SCRAs suggests that they are far more potent and efficacious at CB1 than THC (Banister and Connor, 2018b, Wouters et al., 2019c), a finding which this thesis aims to explore further. Earlier studies revealed that JWH-018 potently inhibited excitatory postsynaptic neurons when tested in autaptic hippocampal neurons, and acted with essentially equivalent potency in multiple CB1 signalling pathways (Atwood et al., 2010). Wiley et al. (2012) used a GTPγS assay to determine the ability of the first generation of SCRAs to activate CB1. The authors concluded that all the SCRAs tested exhibited higher potency, except JWH-415 that had lower potency and efficacy at CB1 (Wiley et al., 2012). With the identification of

tetramethylcyclopropyl ketone indoles in the NPS market, researchers investigated the functional activity of UR144 and XLR-11 in an assay of GTPγS binding in HEK cells (Wiley et al., 2013), and a membrane potential assay in AtT-20 cells (Banister et al., 2015b). They found that both UR-144 and XLR-11 activated CB1 with a higher potency (within 90-160 nM range) compared to THC (>10μM) in the GTPγS binding assay (Wiley et al., 2013), while Banister et al. (2015b) found that UR-144 had 2- and 4-times lower potency compared to THC and XLR-11, respectively. This discrepancy could be due to a system bias effect (bias relating to the pathway or experimental model). A popular design trend in the NPS market is the addition of a fluorine group in the end of the side chain, with evidence suggesting a higher functional activity of fluorinated analogues at CB1 receptor (Banister et al., 2015b, Sobolevsky et al., 2015). For example, the terminally-fluorinated analogue of PB-22, 5F-PB-22, roughly showed 2-times greater potency compared to its corresponding non-fluorinated parent. This effect was more pronounced for fluoro/non-fluoro UR144/XLR11 pair, focusing our attention to structure-activity relationship - linking the chemical structure with the observed cellular signalling profile (reviewed in Banister et al. (2015b), Worob and Wenthur (2019)). More recently, indole and indazole SCRAs have been identified featuring a valinate or a tert-leucinate group, and/or a carboxamide group, that activate the CB1 receptor with sub-nanomolar potencies in a classical signalling cascade (assessed in GTPγS assay (Gamage et al., 2018), cAMP signalling (Gamage et al., 2018, Banister et al., 2019a), membrane potential assay (Banister et al., 2015a, Banister et al., 2016, Banister et al., 2019a), and recruitment of β-arrestins using the NanoBiT® complementation assay (Antonides et al., 2019, Wouters et al., 2019b, Fabregat-Safont et al., 2019)). Despite the chemical diversity of the ligands characterised, these SCRAs exhibited similar maximal effect to that of the reference compound CP55940 (though NanoBit® assay was able to differentiate between the E_{MAX} of SCRAs, indicating a low level of receptor reserve for SCRAs coupling to β-arrestin (Wouters et al., 2019c)). These studies did not appear to consider the confounding influence on efficacy measurement that arise from the "spare receptors" in the system, where only submaximal receptor occupancy is required for the agonist to achieve system maximum. This also means that qualitative measurement of SCRAs efficacy may not be transferable between different assay systems, and would explain why several studies disagree in their efficacy findings (Kenakin and Christopoulos, 2013). The most appealing starting point for this would be to quantitatively measure the efficacy of SCRAs using an operational model of agonism to understand the diverse physiological consequences resulting from CB1 receptor activation by high and low

efficacy agonists (discussed in more detail in Chapter 2, (Sachdev et al., 2019c)). Some evidence exists on SCRAs activity at targets other than CB1 and CB2 - partial agonist activity at TRPA1(Stuart, 2015), potent activity of CP55940 and HU-210 at GPR55 (De Petrocellis and Di Marzo, 2010), binding affinities of some SCRAs for serotonin 2B receptors (Wiley et al., 2012), indole-derived SCRAs-mediated inhibition of alpha subunit of K channels (Wiley et al., 2012), and SCRA-mediated inhibition of $Ca_v3.2$ T-type Ca channel (Bladen et al., 2018), although these remain poorly characterised. These interactions alone, or in combination with CB1/2, may explain the range of physiological and toxicological effects produced by these compounds. Indeed, the seizurogenic effect of SCRAs may arise from strong activation of CB1 receptors (Malyshevskaya et al., 2017, Funada and Takebayashi-Ohsawa, 2018).

The cannabinoid tetrad effect has been extremely useful approach for characterising the cannabimimetic activity of SCRAs in rodent studies (Fantegrossi et al., 2014). Qualitative assessment of cluster of four classical tetrad endpoints for cannabinoids are characterised by dose-dependent decrease in body temperature and motor activity, and dose-dependent increase in measures of analgesia and catalepsy (reviewed in Tai and Fantegrossi (2014)). However, tetrad effect does not necessarily reflect the "cannabis-like high" effect. The first generation of SCRAs, JWH-018 and JWH-073, produced tetrad effects of similar magnitude compared to THC (Wiley et al., 2012). Similarly, UR-144 and XLR-11 exhibited a complete profile of tetrad effects in mice, with potencies up to 15- and 17- fold greater than THC respectively (Wiley et al., 2013). A recent study compared THC- and XLR-11-induced tetrad effects between wildtype and CB1-, CB2- and GPR55-knock-out mice (Wang et al., 2019). Genetic deletion of CB1 blocked the THC- and XLR-11-induced tetrad effects; while genetic deletion of CB2 had no effect on the XLR-11-induced tetrad effects but selectively blocked THC-induced analgesia and catalepsy. However, genetic deletion of GPR55 receptors appears to produce opposing effect on THC-induced tetrad effects, but not XLR-11, suggesting distinct receptor mechanism underlie the classical tetrad effects produced by phytocannabinoid or SCRAs (Wang et al., 2019). Studies with recent groups of SCRAs have utilised drug discrimination paradigms in which animals are usually trained to distinguish administration of a dose of a particular drug from administration of the vehicle (Glennon and Young, 2011, Tai and Fantegrossi, 2014). In a study by (Gamage et al., 2018) rodents were trained to lever press on a particular lever or poke nose on a particular aperture when administered with THC. The authors found that UR-144, XLR-11, AB-CHMINACA,

MDMB-FUBINACA, 5F-CUYL-PICA and many other SCRAs fully and dose-dependently substituted for THC with significantly greater potency. Application of radiotelemetric probes have enabled the real-time measurement of body temperature and heart rate, and hold the capacity to show both the magnitude and time-course of cannabinoid effects in rat physiology (Banister et al., 2016). Bradycardia and hypothermia were induced by UR-144, XLR-11, PB-22, AB-PINACA, AB-FUBINACA, MDMB-FUBINACA, and many other structurally diverse SCRAs with much greater potency than THC *in vivo* (Banister et al., 2015a, Banister et al., 2015b, Banister et al., 2016, Banister et al., 2019a). However, SCRAs failed to produce a tachycardiac effect in the biotelemetry assay *in vivo*, this is surprising, as tachycardia is one of the common adverse effects seen with these drugs. This means that the effects may be confounded by other factors in the system.

To gain further mechanistic insight into the wide-ranging adverse effects of SCRAs, it is important to understand the extent to which these effects are caused by either their parent compounds or metabolites and thermolytic degradants. It has been shown that SCRAs are generally metabolised in two-step process - oxidation by cytochrome450s (CYPs) enzymes, followed by conjugation with UDP-glucuronosyltransferase (UGT) (Tai and Fantegrossi, 2016). While THC is metabolised via oxidation to form a major active metabolite, 11-hydroxy-THC, which may undergo further oxidation to produce a carboxylic group at several positions (11-nor-9-carboxy-THC, an inactive metabolite) (reviewed in Elsohly et al. (2014), Tai and Fantegrossi (2016)). This was first demonstrated in a study from Brents et al. (2012), where five potential Phase I metabolites of JWH-018 showed high levels of activation with high affinity and efficacy at CB1, relative to THC. Interestingly, only a few studies have examined the pharmacology of SCRAs metabolites (the aforementioned study, plus Brents et al. (2012), Rajasekaran et al. (2013), Longworth et al. (2017), Cannaert et al. (2017), Gamage et al. (2019)). One example is the *in vitro* pharmacological profiling of hydroxypentyl metabolites of selected SCRAs in a GTPγS binding assay (Gamage et al., 2019). The authors concluded that majority of hydroxypentyl metabolites retained the same level of efficacy as their parent compound that may suggest that these metabolites are likely to contribute to the cannabimimetic effects in vivo, although the metabolites had relatively lower potency and affinity for CB1 (Gamage et al., 2019). Another group investigated the functional activity of seven common hydrolysis metabolites of fifteen SCRAs using the NanoBiT® complementation assay (Wouters et al., 2019a). They found that the metabolites of some selected SCRAs had little to no activity on CB1 at concentrations up to 1 μM, yet

the metabolites of other closely related SCRAs retained activity at CB1, although potency was significantly less than the parent compound (Wouters et al., 2019a). There appears to be slight discrepancy between two studies, further studies are needed to build a full pharmacological profile of metabolites of SCRAs in uniform in vitro assay to circumvent system-dependent agonist pharmacology. Characterising the functional activity of thermolytic degradants of SCRAs is also important for understanding how thermolysis products, produced during smoking, may have different pharmacological profiles compared to their parent compound. For example, UR-144 and XLR-11 contain a tetramethylcyclopropyl ring system which opens when heated to 800°C (the temperature in burning cigarette) (Thomas et al., 2017). This thermolysis product had higher functional activity (affinity and efficacy) and was more potent in producing cannabimimetic activity *in vivo* as compared to their parent compound (Thomas et al., 2017). Researchers are increasingly aware of the need to explore the pharmacological profile of SCRAs as we get closer to understanding the mechanisms through which they exert life-threatening effects.

1.7. Unlocking the pot-ential activity of cannabinoids

As our understanding of pharmacological processes evolves, so is the tool to facilitate mechanistic insight into selective receptor-agonist responses. The key component of drug discovery process is the pharmacological characterisation of an agonist to induce a cellular response. As first defined by Stephenson (1956), efficacy is the property of an agonist to elicit different intensities of responses as consequence of different proportions of receptors occupied by agonists. Importantly, he also went further to expand the efficacy parameter by adding a stimulus function (system properties) to indicate the amount of receptor activated by a given agonist in the system (Stephenson, 1956). Mathematical models were developed to link together two fundamental phenomenon of receptor function – receptor occupancy and efficacy (reviewed in Finlay et al., 2020). Mathematical model of receptor-agonist function is employed in this thesis to understand the extent of CB1 activation by distinct classes of ligands, and consequently such studies can aid in the design of drugs with better safety profile. For example, the high efficacy agonist of CB1, MDMB-FUBINACA has a rigid C shape geometry (indazole scaffold), which has been shown to interact with $F200^{3.36}$-$W356^{6.48}$ (toggle twin switch) in the binding pocket of CB1, and forms a cavity for G protein binding (Kumar et al., 2019). While, the lack of the toggle twin interaction has been suggested for the low efficacy agonist profile of THC, a characteristic that presumably makes it safer compared to high efficacy synthetic cannabinoids (discussed in Chapter 2 and

3). Studies have shown that distinct ligands stabilise different active receptor conformations, developing the early ideas of biased agonists (i.e. some ligands can selectively stabilise a particular receptor conformation to drive differential response) (Perez and Karnik, 2005). The key to discovering biased agonist is to further our understanding of the signalling dynamics of distinct receptor activation state and even provide therapeutic benefits without adverse consequences (Kenakin and Christopoulos, 2013).

Historically, the drug discovery programmes for GPCR ligands have been dominated by efforts to develop agonist, and antagonist for the orthosteric site of the receptor, but their utility for therapeutic potential has been challenging due to their detrimental side effects (Conn et al., 2009). This has facilitated investigation into development of selective allosteric modulators - these molecules do not bind on the orthostertic ligand binding site but instead bind to a site which is topographically distinct to orthostreic site (Conn et al., 2009). Allosteric modulators can impact receptor activity in several ways – positive allosteric modulators (PAMs) increases the response of the receptor to agonist by influencing the binding affinity and efficacy of the orthosteric agonist, negative allosteric modulators (NAMs), which reduce the receptor responsiveness, and/or these modulators can also cause its own signalling effects separate from the effects of orthosteric ligands (reviewed in Dopart et al., 2018). The emerging field of positive and negative allosteric modulation of the cannabinoid receptors offers considerable promise for the development of drugs which may have less side effects (Dopart et al., 2018). For example, ZCZ011, a PAM of CB1, has been shown to reduce nociceptive behaviour in the chronic constriction sciatic nerve injury model of neuropathic pain and carrageenan model of inflammatory pain with minimal or no cannabimimetic psychoactive effects (Ignatowska-Jankowska et al., 2015).

In the domain of cannabinoids, CB1 and CB2 receptors are involved in regulation of broad panel of central and physiological processes (Kogan and Mechoulam, 2007), while the adverse activity profile of SCRAs (to some extent) is also associated with CB1 activation (reviewed in Worob and Wenthur (2019)). The structural and pharmacological features contributing to cannabinoid therapeutic or toxicity profile downstream of CB1 have not yet been dissected. Deciphering the molecular features of CB1 receptor signalling pathways that confer therapeutic cannabinoid effect from their unwanted side effect is important within the context of cannabinoid research. Thus, examining the functional activity of molecules on a range of different receptor responses using high-throughput assays can provide new insights into receptor function (Kenakin, 2005, Wouters et al., 2019c). However, the

qualitative analysis of cannabinoids might be less interpretable – as agonist outcomes might be affected by system properties (such as receptor density). Further extension of the receptor function seeks to use operational model of pharmacological agonism (proposed by Black and Leff (1983)) to quantify agonist activity in a system-independent manner. Therefore, the ultimate hope of this work is to quantitatively determine the differential ability of structurally diverse cannabinoids to induce functional response in canonical endpoints downstream of CB1 activation (discussed in Chapter 2 and 3).

1.8. Aims of this Thesis

Given the increase in the number and prevalence of structurally diverse SCRAs, often associated with significant morbidity and mortality, and the therapeutic application of natural cannabis for the treatment of wide range of health conditions; the purpose of this thesis was to further the understanding of the molecular pharmacology of cannabinoids from two divergent viewpoints: the toxic effects of synthetic cannabinoids and the therapeutic effects of medical cannabis. This work is presented in the following chapters outlined below.

Chapter 2	Study I	Quantitatively determine the efficacy of a library of most prevalent class of SCRAs using operational model of pharmacological agonism
Chapter 3	Study II	Determine the distinct effects of chemically diverse SCRAs downstream of CB1 in two signalling endpoints - $G_{\alpha i/o}$ (inhibition) and $G_{\alpha s}$ (stimulation) of cAMP signalling to understand the apparent differences in effect between these drugs in humans
Chapter 4	Study III	Examine whether mixing of brodifacoum (superwarfarin) with SCRAs exhibits an additive effect on cannabinoid receptor activity potentially illuminating the adverse effects associated with their consumption
Chapter 5	Study IV	Elucidation of the possible entourage effect applied to medical cannabis by examining cannabinoid-terpenoid interaction on cannabinoid receptor signalling
Chapter 6	Study V	Investigation the molecular effects of CBD on the signalling of multiple GPCRs to provide a greater understanding of CBD function and its corresponding therapeutic advantages

Chapter II.

Study I. In vitro determination of the efficacy of illicit synthetic cannabinoids at CB1 receptors

Synthetic cannabinoid receptor agonists (SCRAs) are gaining notoriety as an illicit class of recreational drugs associated with significant toxicity and even death, consequences very different from herbal cannabis and other forms of Δ^9-THC. To investigate whether efficacy can explain the toxicity related to SCRAs, we have systematically quantified the efficacy of panel of 17 cannabinoids using operational model of pharmacological agonism. This was done using well-studied phytocannabinoid (THC), endocannabinoid (2-AG and AEA), and non-classical cannabinoid (CP55940) as reference drug for comparison with the newer, and chemically diverse class of SCRAs.

Contributions to the work

This paper represents a collaborative work hosted in Mark Connor's laboratory at Macquarie University Australia. I took the lead role in experimental design, conducting the experiments, data analysis and writing the paper with some support from my co-investigators: Mark Connor oversaw the work in this research group; Samuel Banister and Mitchell Longworth created all the SCRAs with guidance from Michael Kassiou; Kiran Vemuri and Alexandros Makriyannis designed and shared an irreversible antagonist of CB1; and Marina Santiago provided assistance in the experimental work. All authors reviewed the final manuscript.

Received: 25 February 2019 | Revised: 10 June 2019 | Accepted: 5 August 2019

DOI: 10.1111/bph.14829

RESEARCH PAPER

In vitro determination of the efficacy of illicit synthetic cannabinoids at CB_1 receptors

Shivani Sachdev[1] | Kiran Vemuri[2] | Samuel D. Banister[3,4] | Mitchell Longworth[4] | Michael Kassiou[4] | Marina Santiago[1] | Alexandros Makriyannis[2] | Mark Connor[1]

[1] Department of Biomedical Sciences, Macquarie University, Sydney, NSW, Australia

[2] Center for Drug Discovery, Department of Pharmaceutical Sciences and Chemical Biology, Northeastern University, Boston, Massachusetts

[3] The Lambert Initiative for Cannabinoid Therapeutics, Brain and Mind Centre, The University of Sydney, Sydney, NSW, Australia

[4] School of Chemistry, The University of Sydney, NSW, Australia

Correspondence
Mark Connor, Department of Biomedical Sciences, Macquarie University, Sydney, NSW, Australia.
Email: mark.connor@mq.edu.au

Funding information
European Union's Seventh Framework Programme, Grant/Award Number: Grant Agreement No. HEALTH-F2-2011-278850; National Health and Medical Research Council, Grant/Award Number: APP1107088; National Institutes of Health, Grant/Award Numbers: P01DA009158 and R21DA045882; NHMRC, Grant/Award Number: 1107088

Background and Purpose: The morbidity and mortality associated with recreational use of synthetic cannabinoid receptor agonists (SCRAs) may reflect strong activation of CB_1 receptors and is a major health concern. The properties of SCRA at CB_1 receptors are not well defined. Here we have developed an assay to determine acute CB_1 receptor efficacy using receptor depletion with the irreversible CB_1 receptor antagonist AM6544, with application of the Black and Leff operational model to calculate efficacy.

Experimental Approach: Receptor depletion in mouse AtT-20 pituitary adenoma cells stably expressing human CB_1 receptors was achieved by pretreatment of cells with AM6544 (10 μM, 60 min). The CB_1 receptor-mediated hyperpolarisation of AtT-20 cells was measured using fluorescence-based membrane potential dye. From data fit to the operational model, the efficacy (τ) and affinity (K_A) parameters were obtained for each drug.

Key Results: AM6544 did not affect the potency or maximal effect of native somatostatin receptor-induced hyperpolarization. The τ value of Δ^9-THC was 80-fold less than the reference CB receptor agonist CP55940 and 260-fold less than the highest efficacy SCRA, 5F-MDMB-PICA. The operational efficacy of SCRAs ranged from 233 (5F-MDMB-PICA) to 28 (AB-PINACA), with CP55940 in the middle of the efficacy rank order. There was no correlation between the τ and K_A values.

Conclusions and Implications: All SCRAs tested showed substantially higher efficacy at CB_1 receptors than Δ^9-THC, which may contribute to the adverse effects seen with these drugs but not Δ^9-THC.

1 | INTRODUCTION

Synthetic cannabinoid receptor agonists (SCRAs) are a large class of new psychoactive substances (NPS), notionally designed to mimic the effects of Δ^9-tetrahydrocannabinol (Δ^9-THC), the main psychoactive ingredient in cannabis (Wiley, Marusich, & Huffman, 2014). SCRAs have been marketed as herbal incense blends (often known as Spice or K2) and legal cannabis substitutes which are undetectable using conventional drug tests (Auwärter et al., 2009). Since the first generation of generally available SCRAs (including JWH-018, JWH-073, JWH-200, and CP47497) were detected in herbal blends in 2008, more than 250 SCRAs have been reported in over 100 countries (Banister & Connor, 2018; United Nations Office on Drugs and Crime, 2018). SCRA use has been associated with adverse health effects including hundreds of hospitalisations and dozens of

Abbreviations: AtT-20-CB1, mouse pituitary tumour cells stably transfected with HA-tagged human CB_1 receptors; GIRK, G protein-coupled inwardly rectifying potassium channel; NPS, new psychoactive substances; PTX, Pertussis toxin; RA_i, relative agonist activity; SCRAs, synthetic cannabinoid receptor agonists; SRIF, somatotropin release-inhibiting factor

fatalities (Adams et al., 2017; Trecki, Gerona, & Schwartz, 2015). The most commonly reported adverse effects are psychosis, anxiety, agitation, seizures, tachycardia, hypothermia, and kidney injury (Tait, Caldicott, Mountain, Hill, & Lenton, 2016). In addition to these life-threatening effects, daily SCRA use has been linked to dependence and withdrawal (Cooper, 2016).

SCRAs activate **cannabinoid CB_1 and CB_2 receptors**, with their psychoactive effects caused by activation of CB_1 receptors (Pacher, Bátkai, & Kunos, 2006). While cannabinoids, including SCRA, have been reported to have activity at a variety of ion channels and GPCRs other than CB_1 and CB_2 receptors (De Petrocellis & Di Marzo, 2010), the relevance of these interactions to the effects of cannabinoids in humans remains to be established, and their potential role in SCRA toxicity is unknown. In rodents, both JWH-018- and **AM-2201**-induced seizures are mediated by CB_1 receptors (Funada & Takebayashi-Ohsawa, 2018; Malyshevskaya et al., 2017; Vigolo et al., 2015). SCRA-induced hypothermia and bradycardia are also CB_1 receptor-dependent (Banister et al., 2013; Banister et al., 2015; Banister et al., 2016). Intriguingly, a recent report suggests that the hypertensive effects of some SCRAs in rats may be independent of CB_1 receptors (Schindler, Gramling, Justinova, Thorndike, & Baumann, 2017).

Most SCRAs studied to date activate CB_1 receptors with greater potency and efficacy than Δ^9-THC in [^{35}S]GTPγS binding assay (Gamage et al., 2018; Wiley et al., 2015), **AC** assay (Costain et al., 2018; Hess, Schoeder, Pillaiyar, Madea, & Müller, 2016), β-arrestin 2 recrutiment assay using NanoLuc binary technology (Cannaert, Storme, Franz, Auwärter, & Stove, 2016), and fluorescence-based membrane potential assay (Banister et al., 2013; Banister et al., 2015; Banister et al., 2016; Banister, Stuart, et al., 2015). However, there is little quantitative information about the efficacy of SCRA at CB_1 receptors. A substantial component of SCRA toxicity may be mediated through activation of CB_1 receptors (Krishna Kumar et al., 2019), and defining the efficacy of SCRAs is an important step towards understanding possible mechanisms of CB_1 receptor-mediated toxicity. The similar maximal effects of several SCRAs reported in these assays may reflect receptor reserve, with only submaximal receptor occupancy by agonists needed to achieve their maximal response. Depleting receptor reserve can allow for quantitative determination of efficacy, by fitting concentration–response data before and after receptor depletion to the operational model of Black and Leff (Black & Leff, 1983). We have used the irreversible CB_1 receptor antagonist AM6544 (Finlay et al., 2017) to facilitate quantitative measure of SCRAs efficacy to produce CB_1 receptor-dependent hyperpolarisation of intact AtT-20 cells expressing human CB_1 receptors. We determined the efficacy of a library of the most prevalent SCRAs identified in the NPS market since 2008 and found that the SCRAs we tested had up to 300 times the efficacy of Δ^9-THC, which may contribute to the apparently greater toxicity of these drugs. In this study, we have established an assay that can be used to quantitate CB_1 receptor efficacy efficiently, and which is readily adaptable to the study of other CB_1 receptor signalling pathways.

2 | METHODS

2.1 | Cell culture

Experiments used mouse AtT-20 pituitary tumour cells (RRID: CVCL_4109) engineered to express FLP recombination site were transfected with human CB_1 receptors as previously described (Banister et al., 2016). Cells were cultured in DMEM (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% FBS (Sigma-Aldrich, St. Louis, MO, USA), 100 units·ml^{-1} of penicillin, 100 μg·ml^{-1} of streptomycin (Thermo Fischer Scientific, Waltham, MA, USA), and 80 μg·ml^{-1} of hygromycin (InvivoGen, San Diego, CA, USA). The cells were grown and maintained in 75 cm^2 flask and passaged at 80% confluency or grown to 90% confluency for assay. Cells were incubated at 37°C in a humidified 5% CO_2 atmosphere.

2.2 | Achieving receptor depletion in a membrane potential assay

Cannabinoid receptors couple to **G protein-coupled inwardly rectifying potassium** (GIRK) channels in several types of neurons (Bacci, Huguenard, & Prince, 2004; Marinelli, Pacioni, Cannich, Marsicano, & Bacci, 2009). This coupling reflects a close association between CB receptors, G proteins, and channels (Guo & Ikeda, 2004), and all the components, other than the receptor, are naturally expressed in the AtT-20 cells. Endogenous expression of GIRK channels (**K_{ir}3.1** and **Kir3.2**) in AtT-20 cells is crucial for the main signalling assay performed during this work, and the direct activation of these channels in AtT-20 cells by CB_1 receptors has been studied repeatedly (Garcia, Brown, Hille, & Mackie, 1998; Mackie, Lai, Westenbroek, & Mitchell, 1995). Changes in the membrane potential of cells in response to GIRK activation were measured using the fluorometric imaging plate reader (FLIPR) membrane potential (blue) assay kit (Molecular Devices, Sunnyvale, CA) as previously described (Knapman et al., 2013). Cells were detached from the flask using trypsin/EDTA (Sigma-Aldrich), and the pellet was resuspended in 10 ml Leibovitz's (L-15) media supplemented with 1% FBS, 100 units·ml^{-1} of penicillin, 100 μg·ml^{-1} of streptomycin, and 15 mM glucose. The cells were seeded in a volume of 90 μl in poly-D-lysine (Sigma-Aldrich) coated, black wall, clear bottom 96 well microplates. Cells were incubated overnight at 37°C in ambient CO_2.

We used a receptor depletion assay to quantitatively determine the efficacy of a range of SCRAs. This approach involves irreversible binding of an antagonist to the orthosteric binding site of the receptors, thus permanently occluding a fraction of functional receptors available to an orthosteric ligand (Besse & Furchgott, 1976). We used the new CB_1 receptor irreversible antagonist AM6544, synthesised at the Center for Drug Discovery, Northeastern University (Patent US8084451, 2011) to systematically reduce active receptor number in the AtT-20-CB1 cell expression system. The day after plating, AM6544 (10 μM) was prepared in HBSS composed of (mM) NaCl 145, HEPES 22, Na_2HPO_4 0.338, $NaHCO_3$ 4.17, KH_2PO_4 0.441,

$MgSO_4$ 0.407, $MgCl_2$ 0.493, $CaCl_2$ 1.26, glucose 5.56 (pH 7.4, osmolarity 315 ± 15), and supplemented with 0.1% BSA. Receptor depletion was achieved following pretreatment of cells with AM6544 (10 μM) in parallel to the vehicle (control) for 60 min after removal of the L-15, at 37°C in ambient CO_2. The concentration of DMSO (0.1%) was kept constant for AM6544-treated and control cells. Cells were then washed twice with warm HBSS and loaded with 90 μl per well of L-15 media and 90 μl per well of reconstituted FLIPR dye. The cell plate was incubated at 37°C in ambient CO_2 for 1 hr prior to measuring the fluorescence using a FlexStation 3 microplate reader (Molecular Devices). The AM6544-treated and control cells were compared side by side. The cells were excited at a wavelength of 530 nm and emission measured at 565 nm, with cut-off at 550 nm, and the readings were made every 2 s. Baseline readings were taken for 2 min after which 20 μl of drug (10×) was added to each well to give the desired concentration. The drugs of various concentrations were prepared in HBSS containing 0.1% BSA and 1% DMSO. The final concentration of DMSO in each well was always 0.1%. A concentration–response curve (CRC) for **CP55940** was performed each day for quality control. On rare occasions where AM6544 pretreatment failed to produce a substantial shift in responses to CP55940, results were discarded, as they probably indicated experimenter error.

SCRAs were synthesised as previously described by Banister, Moir, et al. (2015), Banister, Stuart, et al. (2015), and Banister et al. (2016). Chemical structure of SCRAs can be found in Figure S1. The functional activity (EC_{50}) of SCRAs at CB_1 receptors were compared to Δ^9-THC and CP55940 (see Table S1). We have previously shown that the effects of SCRAs in AtT-20-CB1 cells were blocked by **SR141716A**, a CB_1 receptor antagonist, and that none of the SCRAs produced a significant change in the membrane potential of AtT-20 wild-type cells (Banister et al., 2016; Banister, Moir, et al., 2015; Banister, Stuart, et al., 2015). SCRA-mediated hyperpolarisation of AtT-20-CB1 cells is also Pertussis toxin (PTX) sensitive, confirming that the response is $G_{i/o}$-dependent (Banister et al., 2016, Banister, Moir, et al., 2015, Banister, Stuart, et al., 2015).

2.3 | Data analysis

2.3.1 | Operational model analysis

Drug responses are reported as percentage change of baseline fluorescence, following correction for the vehicle responses (0.1% DMSO). The hyperpolarisation of the cells produces a decrease in fluorescence. For convenience, values are expressed such that a change of 30% means a reduction in fluorescence of 30%. Data for individual experiments were analysed and the CRC before and after receptor depletion was fitted with the Black and Leff operational model in PRISM (Graph Pad Software Inc., San Diego, CA; RRID:SCR_002798), using five-parameter non-linear regression (Basal, K_A, $Effect_{max}$, τ, and transducer slope) to fit the operational model-receptor depletion equation (Motulsky & Christopoulos, 2004).

The equation for operational model-depletion presented in the same style as Prism:

$$operate = \left[\frac{10^{\log K_A} + 10^X}{10^{\log\tau + X}}\right]^n,$$

$$Y = \frac{Basal + (Effect_{max} - Basal)}{1 + operate},$$

where the maximal response of the system is given by $Effect_{max.}$ The parameter, τ, equals the total concentration of receptor in the system divided by the concentration of agonists occupied receptors that are required to produce half-maximal tissue response. The parameter, K_A, is define as the equilibrium K_D for agonist binding to the receptors, while n is the slope factor of the transducer function.

From the operational model, efficacy (τ) and affinity (K_A) parameters were obtained for each drug. The basal parameter was constrained to zero as the basal activity (without drug) was routinely subtracted from the measurements. The transducer slope n of all the agonist CRCs was constrained to 1 (after initial fits showed this to be a good approximation). The parameter $Effect_{max}$ is tissue specific and thus shared by all agonists acting on CB_1 receptors through a given pathway for that day. The parameter, K_A, is ligand–receptor specific, whereas τ has ligand-specific elements (efficacy of ligand) and system-specific elements (coupling efficiency of receptors to signalling pathway). Thus, for individual drugs, K_A was shared between the AM6544-treated and control state, but the separate best fit values of τ were determined for each data set. The τ value in the control state was used to measure the CB_1 receptor agonist efficacy. This procedure serves to measure the efficacy of a group of agonists on a per-day basis obtained from fitting data simultaneously to the operational model, with mean and SEM calculated using individual values for each experiment.

2.4 | Estimate of relative agonist activity (RA_i)

After determining the efficacy and affinity of SCRAs from the operational model under control and AM6544-treated conditions, the data were used for the calculation of the initial estimate of RA_i value. The relative affinities of agonists for the active state of a receptor (RA_i) expressed relative to that of a reference agonist as described previously by Ehlert (2008) was calculated.

$$RA_i = \frac{\tau_B K_A}{\tau_A K_B}$$

In this equation, τ_A and τ_B denote the intrinsic efficacies, and K_A and K_B denote the K_D of reference and test agonist, obtained earlier from the operational model. CP55940 was used as the reference agonist to define the RA_i of the SCRAs.

We also estimated RA_i values of eight SCRAs from four published studies to compare these with the initial estimate of RA_i values determined from the data generated in our laboratory. RA_i values were estimated from studies on the [^{35}S]GTPγS binding assay in HEK cells (RRID:CVCL_0045) by Ford et al. (2017), Thomas et al. (2017), Gamage et al. (2018), and Wiley et al. (2015). We used a simple

calculation for the estimation of RA_i as only the EC_{50} and the E_{max} values of SCRAs were available from the literature (Ehlert, Griffin, Sawyer, & Bailon, 1999; Griffin, Figueroa, Liller, & Ehlert, 2007).

$$RA_i = \frac{E_{maxB}\ EC_{50A}}{E_{maxA}\ EC_{50B}}$$

in which the subscript refers to the parameters of reference (A) and tests (B) agonists. In all instances, it was impossible to extract SEM for these data sets, as we do not have access to their raw data. The rank order of agonist activity based upon RA_i values calculated from the literature for [^{35}S]GTPγS binding assay was compared to our results for membrane potential assay. Finally, for each agonist, the RA_i value for membrane potential assay was divided by the RA_i value of GTPγS binding assay to estimate the bias factor.

Unless otherwise stated, the data represent mean ± SEM of at least six independent experiments, each conducted in duplicate. The data and statistical analysis comply with the recommendations of the *British Journal of Pharmacology* on experimental design and analysis in pharmacology (Curtis et al., 2018). Statistical significance is defined as $P < .05$.

2.5 | Materials

CP55940, **2-arachidonolylglycerol**, **anandamide**, and CUMYL-4CN-BINACA were purchased from Cayman Chemical Company (Ann Arbour, MI, USA); Δ^9-THC was obtained from The Lambert Initiative (Sydney, NSW, Australia). AM6544 was a gift from laboratory of Professor Alexandros Makriyannis (Northeastern University, Massachusetts, USA). All the SCRAs, unless otherwise stated, were synthesised by Samuel D. Banister and Mitchell Longworth in the lab of Professor Michael Kassiou at Sydney University (Sydney, NSW, Australia). All the drugs were stored in aliquots of 30 mM at −80°C until needed.

2.6 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org/, the common portal of data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding et al., 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/2018 (Alexander, Christopoulos, et al., 2017; Alexander, Fabbro et al., 2017).

3 | RESULTS

3.1 | Specificity of AM6544: a new irreversible antagonist of CB_1 receptors

In vitro, AM6544 behaves as an irreversible antagonist of CB_1 receptors , as established by Finlay et al. (2017) using radioligand binding assays in whole cells (pEC_{50} 5.45 ± 0.11). To confirm that AM6544 does not non-specifically interfere with receptor signalling mechanisms in AtT-20-CB1 cells, we examined the effect of AM6544 on the activation of native **somatotropin release-inhibiting factor (SRIF) receptors**. Pretreatment with AM6544 (10 μM, 60 min) had no effect on the potency or maximal effect of SRIF-induced hyperpolarisation when compared to vehicle-treated cells (Control, pEC_{50} 9.13 ± 0.05, E_{max} 38 ± 1%; AM6544-treated pEC_{50} 9.18 ± 0.04, E_{max} 39 ± 0.7%, Figure 1), indicating that AM6544 did not interfere with either SRIF receptors or their signalling pathways

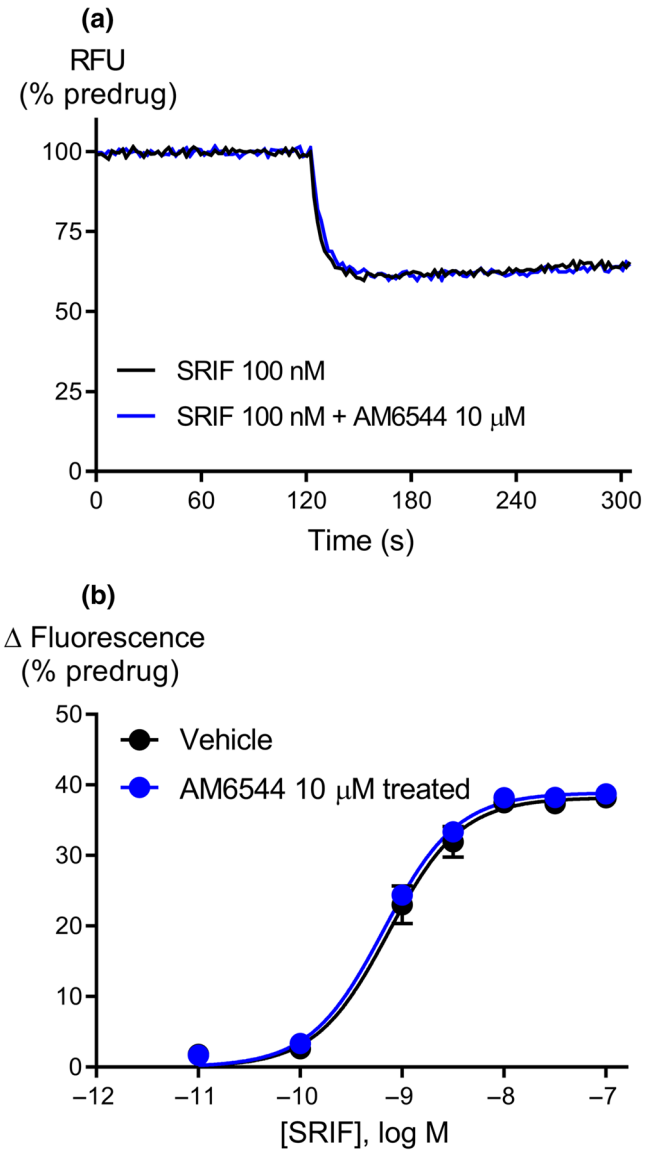


FIGURE 1 AM6544 is a specific irreversible antagonist of CB_1 receptors. (a) Raw trace showing the change in fluorescence normalised to the predrug baseline for SRIF on AtT-20-CB1 cells pretreated for 60 min with vehicle or AM6544 (10 μM) and then washed twice before incubation with MPA dye. The traces are representative of at least six independent experiments. (b) Concentration–response curve for SRIF mediated hyperpolarisation of AtT-20-CB1 cells following pretreatment with AM6544 (10 μM) or vehicle. Data represent the mean ± SEM of six independent determinations performed in duplicate. There was no difference in the potency or maximal effect of SRIF between vehicle or following pretreatment with AM6544.

likely to be shared with CB_1 receptors in AtT-20 cells. We also examined the possibility that AM6544 could affect the membrane potential of the cells prior to agonist addition. Application of AM6544 for 60 min at concentration up to 10 μM did not significantly affect the membrane potential of the AtT20-CB1 cells by itself, nor did it modify the GIRK-mediated hyperpolarisation produced by SRIF (Figure S2, $P > .05$).

3.2 | Functional activity of cannabinoids after receptor depletion with AM6544

The efficacy of the classical CB_1 receptor agonist, CP55940, was measured after the pharmacological knockdown of CB_1 receptors with AM6544 (10 μM, 60 min). The maximal response of CP55940 (10 μM) was reduced after AM6544 pretreatment compared to vehicle-treated cells (Control, E_{max} 33 ± 2; AM6544-treated E_{max} 26 ± 2, $P < .05$, Figure 2a). The τ value for CP55940 was reduced 10-fold in AM6544 pretreated cells compared to vehicle cells (Table 1), suggesting that AM6544 can effectively deplete the receptors available to high efficacy SCRAs. From the operational model, the pK_A of CP55940 was estimated to be 5.8 ± 0.1 (Table 1, $n = 20$).

We also determined the efficacy of some frequently used research cannabinoids - **WIN55212-2**, the main psychoactive phytocannabinoid Δ^9-THC, and the endogenous cannabinoids 2-arachidonolylglycerol and anandamide - on CB_1 receptors after receptor depletion with AM6544. The hyperpolarisation produced by WIN55212-2 was strongly inhibited by AM6544 pretreatment (10 μM, 60 min) compared to vehicle-treated cells (Figure 2). The τ for WIN55212-2 was reduced 1.5-fold compared to CP55940 but was 63-fold greater than Δ^9-THC (Table 1). The efficacy of endogenous cannabinoids, 2-arachidonolylglycerol and anandamide, was respectively 1.2- and 14-fold less than that of CP55940 (Figure 3; Table 1).

We assessed the relative efficacy of SCRAs to provide insight into potential mechanisms of toxicity and the functional consequences of the evolution of SCRA structures over time. The efficacy of SCRAs was determined following receptor depletion with AM6544. Example traces and CRC are shown for JWH-018, **MDMB-FUBINACA**, and XLR-11 (Figure 4). The efficacy for all the drugs we examined are found in Table 1. The τ of SCRAs tested ranged from 28 to 233, with two of the 13 CRAs having τ values greater than 150 (5F-MDMB-PICA and XLR-11). The first SCRA to be identified in Spice, JWH-018, exhibited 2-fold less τ than CP55940 but 43-fold higher τ than Δ^9-THC. Only two of the SCRAs (MDMB-CHMICA and CUMYL-4CN-BINACA) exhibited similar efficacy to CP55940 (Table 1), whereas four of the SCRAs (PB-22, UR-144, AM-2201, and AB-PINACA) had approximately 50% of the efficacy of CP55940. The least efficacious SCRA, AB-PINACA, showed 3-fold less τ than CP55940 while the most efficacious SCRA, 5F-MDMB-PICA, showed 3-fold higher τ than CP55940 (Table 1).

We calculated the functional affinity of SCRAs at CB_1 receptors using the operational model (Table 1). In the present study, the K_A of SCRAs ranged from 33 nM to 31 μM, where three of the 13 SCRAs had K_A values less than 100 nM (CUMYL-4CN-BINACA, MDMB-FUBINACA, and 5F-PB-22) and three demonstrated micromolar affinities (JWH-018, XLR-11, and UR-144). Most of the SCRAs had a higher affinity for CB_1 receptors compared to CP55940, with the exception of UR-144 and XLR-11, which had 19- and 13-fold lower affinity respectively (Table 1). No correlation was found between the operational efficacy and affinity obtained for SCRAs (Figure 5, $r^2 = .0004$, $P > .05$).

To determine the percentage of CB_1 receptors available after AM6544 pretreatment, the ratio of τ post- and pre-receptor depletion were measured for each SCRA. The ratio of $\tau_{(depleted)}$ to $\tau_{(control)}$ for each drug reflects the reduction in the total functional receptor concentration [R_0] due to AM6544-treatment. The average value of τ post- and pre-receptor depletion curves for SCRAs tested was found to be 0.07 ± 0.005, indicating that AM6544 caused an overall 93% reduction in receptors available to CB_1 receptor agonists.

3.3 | Quantification of relative agonist activity (RA_i)

RA_i values of the SCRAs for stimulating [^{35}S]GTPγS binding assay and membrane potential assay were calculated in an attempt to compare the relative efficacy of these compounds in two very different assays of receptor activation. Activity was calculated with reference to that of the CP55940 and summarised in Table 2. A rank order of agonist activity, based on selectivity for the [^{35}S]GTPγS binding assay is AB-CHMINACA > PB-22 > MDMB-FUBINACA > AB-PINACA > JWH-018 > XLR-11 > UR-144 > Δ^9-THC. By contrast, relative activity of SCRAs in the membrane potential assay is MDMB-FUBINACA > AB-CHMINACA > PB-22 > AB-PINACA > JWH-018 > XLR-11 > Δ^9-THC > UR-144. The pattern of selectivity that we observed in our studies is consistent with the data from the literature for [^{35}S]GTPγS binding assay, with the striking exception of MDMB-FUBINACA, which exhibited 37-fold greater RA_i value at membrane potential assay than those calculated for [^{35}S]GTPγS binding assay. Most of the SCRAs had a higher RA_i value at membrane potential assay compared to [^{35}S]GTPγS binding assay, with the exception of UR144, which had a higher activity at [^{35}S]GTPγS binding assay with a bias factor of 0.21 (Table 2). UR144 had a RA_i value lower than Δ^9-THC in the membrane potential assay, despite the much higher efficacy of UR144 calculated using the operational model; this presumably relates to the very low functional affinity of UR144 in membrane potential assay.

4 | DISCUSSION

We have measured the efficacy of a wide range of SCRA-induced activation of native GIRK channels in an intact AtT-20-CB1 cells. To achieve this, we have employed high throughput assay technology to construct full concentration–response data following receptor depletion with the irreversible CB_1 receptor antagonist AM6544 fitted to the operational model of pharmacological agonism to calculate the efficacy (τ) and affinity (K_A) of these SCRAs. The principal finding of this study is that all the SCRAs tested showed substantially higher

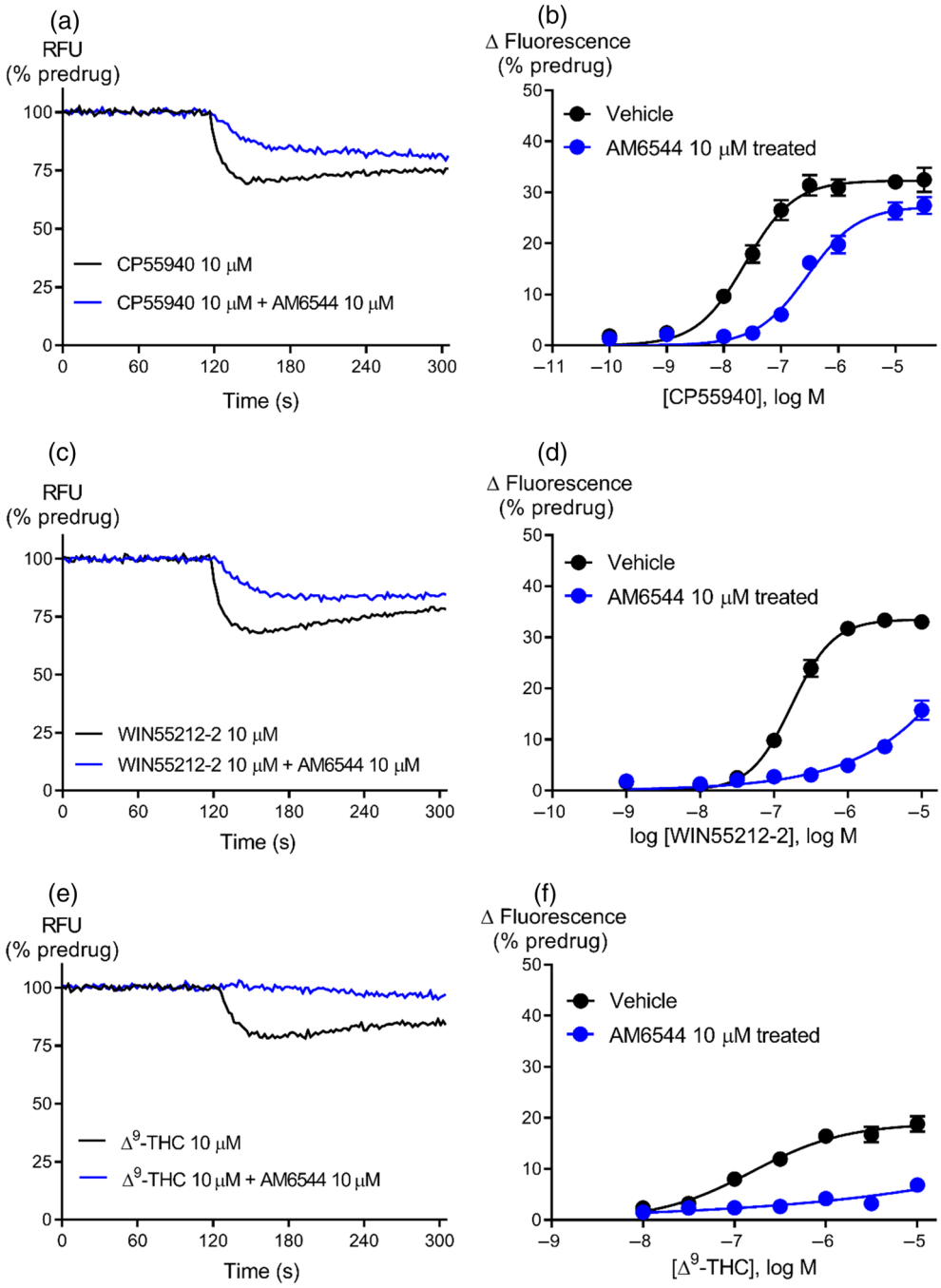


FIGURE 2 Representative traces for research cannabinoids CP55940 (a), WIN55212-2 (c), and ?^{9}-THC (e) following pretreatment with vehicle or AM6544 (10 μM) on AtT-20-CB1 cells. Raw trace showing reduction in hyperpolarisation induced by maximally effective concentration (10 μM) of CP55940, WIN55212-2, and ?^{9}-THC after AM6544 pretreatment compared to vehicle. Concentration–response curves for (b) CP55940 (n = 20), (d) WIN55212-2 (n = 7), and (f) ?^{9}-THC (n = 6) were plotted using five-parameter non-linear regression to fit the operational model-receptor depletion equation with basal constrained to 0. Data represent the mean ± SEM of technical replicates. For some points, the error bars are smaller than the height of the symbol.

agonist activity at CB_1 receptors than Δ^9-THC (τ, 0.9 ± 0.1), with τ that ranged between 28 and 233. 5F-MDMB-PICA and XLR-11 exhibited the highest efficacies from the SCRAs tested. However, there was no correlation between the τ and K_A of SCRAs, and no obvious trend for decreasing/increasing τ over time.

We have used the new CB_1 receptor irreversible antagonist, AM6544, to specifically deplete the CB_1 receptor reserve from the pool available for orthosteric agonist binding. The specificity of AM6544 was confirmed by showing the lack of effect of AM6544 pretreatment on the activation of native SRIF receptors in the same cells. AM6544 treatment effectively blocked CB_1 receptors in AtT20 cells, although as it does not have a high affinity at these receptors on intact cells (pEC_{50} 5.45, Finlay et al., 2017), a relatively high concentration had to be used. Under these conditions, the receptors are

TABLE 1 Efficacy and functional affinity of CP55940, □^{9}-THC, and other SCRAs

Compound	Operational efficacy τ		Functional affinity pK_A (±SEM)
	Control (±SEM)	AM6544-treated (±SEM)	
CP55940	72 (30)	7 (3)	5.78 (0.09)
WIN55212-2	57 (41)	3 (1)	4.84 (0.24)
□^{9}-THC	0.9 (0.1)	0.3 (0.1)	6.54 (0.07)
2-AG	60 (27)	3 (1)	5.16 (0.08)
AEA	5 (2)	2 (2)	5.17 (0.10)
JWH-018	43 (21)	2.5 (1)	5.72 (0.21)
AM-2201	32 (6)	2 (0.4)	6.53 (0.08)
UR-144	36 (7)	3 (0.5)	4.51 (0.15)
XLR-11	152 (73)	13 (9)	4.68 (0.26)
PB-22	44 (10)	3.2 (1)	6.73 (0.10)
5F-PB-22	102 (48)	7 (5)	7.05 (0.09)
AB-CHMINACA	92 (42)	4 (1)	6.98 (0.12)
AB-PINACA	28 (7)	2 (0.4)	6.90 (0.21)
MDMB-CHMICA	79 (32)	8 (4)	6.35 (0.17)
MDMB-FUBINACA	103 (62)	5 (1.4)	7.12 (0.10)
5F-MDMB-PICA	233 (65)	14 (3)	6.71 (0.13)
CUMYL-4CN-BINACA	70 (28)	4 (1)	7.48 (0.07)

Note. Values were calculated using the operational model of pharmacological agonism following CB_1 receptor depletion with AM6544, as outlined in Section 2.

sufficiently depleted to ensure that the high efficacy agonist can no longer yield a system maximum at saturating concentrations. Other irreversible CB_1 receptor antagonists have been identified, but they are not well suited for use in the kind of studies described here. Methyl arachidonyl flurophosphate can act at several components of the cannabinoid system including **fatty acid amide hydrolase**, an enzyme involved in the degradation of endogenous cannabinoids (Fernando & Pertwee, 1997); falcarinol is very unstable (Leonti et al., 2010), and previously known irreversible analogues of SR141716A displayed lower affinities for CB_1 receptors or acted as partial agonists (Howlett et al., 2000). AM6544 was used by Finlay et al. (2017) as an irreversible antagonist to deplete CB_1 receptors in order to study the effect of receptor number on G protein preference in coupling to AC and, here, we have shown that it can also be used to study coupling to K channels through PTX-sensitive G proteins. These kinds of quantitative pharmacological studies were not possible for CB_1 receptors before AM6544 became available, and it is likely to be a useful compound in future experiments examining CB_1 receptors.

The efficacy of SCRAs has principally been measured using [^{35}S]GTPγS binding assays, which measures the accumulated activation of G proteins in membranes over a period of 30–60 min (De Luca et al., 2016; Gamage et al., 2018; Thomas et al., 2017; Wiley et al., 2015). In these assays, the maximum response is used as the measure of efficacy, with the assumption that this maximum response is not constrained—that there is an excess of G-proteins relative to CB_1 receptors. Given the high levels of receptor expression that can be achieved in recombinant systems, this assumption may not be valid (Gamage et al., 2018). In our study, we have circumvented this limitation by reducing receptor number, and we have been able to measure a very wide range of apparent efficacies to produce acute hyperpolarisation of AtT-20-CB1 cells (>250-fold), compared with a twofold to threefold difference in the maximum response to agonists in CB_1 receptor GTPγS assays (Table 2). Estimation of RA_i values of SCRAs in GTPγS and membrane potential assay was undertaken in order to further observe an effect of different assays on the relative agonist activity of these compounds and also to quantify the functional selectivity of SCRAs for different receptor active states (Table 2). The rank order of agonist activity that we observed in our studies is generally similar with the data from the GTPγS assay. Only three of the SCRAs (AB-CHMINACA, PB-22, and MDMB-FUBINACA) exhibited higher E_{max} relative to CP55940, whereas all the other SCRAs presented very similar maximal response as CP55940 in GTPγS assays. Our assay seems sensitive to differences in efficacy, probably because GIRK activation is relatively poorly amplified, probably requiring 4 Gβγ subunits to simultaneously bind to each channel complex to open it (Whorton & MacKinnon, 2013), in contrast to the single ligand–receptor G protein complex required for stimulation of effectively irreversible GTPγS binding. In contrast to the 4 Gβγ subunits required to fully activate GIRK, inhibition of AC or voltage-gated calcium channels by CB receptor activation requires only 1 Gα or Gβγ subunit respectively. Given that each ligand-bound receptor is likely to activate multiple G protein heterotrimers, there will be significantly greater signal amplification when AC or I_{Ca} are used as readouts. Activation of kinases such as **ERK** are usually multistep processes, and ERK activation can also be stimulated by several upstream signalling pathways (Jain, Watson, Vasudevan, & Saini, 2018), meaning that amplification can occur at several points. CB_1 receptor-dependent activation of GIRK has been reported in several types of neuron, and it represents a naturalistic, if understudied, signalling pathway for CB receptors (Azad et al., 2003; Daniel, Rancillac, & Crepel, 2004; Marinelli et al., 2009). Our work represents acute activation of one pathway; GTPγS assays provide a more general measure of $G\alpha_{i/o}$-subunit activation, but uncoupled from signalling pathways (Ibsen, Connor, & Glass, 2017). Neither assay effectively captures CB_1 receptor coupling to $G\alpha_s$ or $G\alpha_q$, or non-G protein mediated pathways, such as those dependent on arrestin, but together they re-enforce the quantitative differences in receptor activity between Δ^9-THC and more recently encountered cannabinoid agonists. The activity of SCRA-induced arrestin recruitment by CB_1 receptors has been studied using NanoLuc binary technology, and many SCRAs showed strong activation of CB_1 receptors in this assay compared to Δ^9-THC (Cannaert et al., 2016; Noble, Cannaert, Linnet, & Stove, 2018), consistent with its low relative efficacy reported in the present work and GTPγS binding assays.

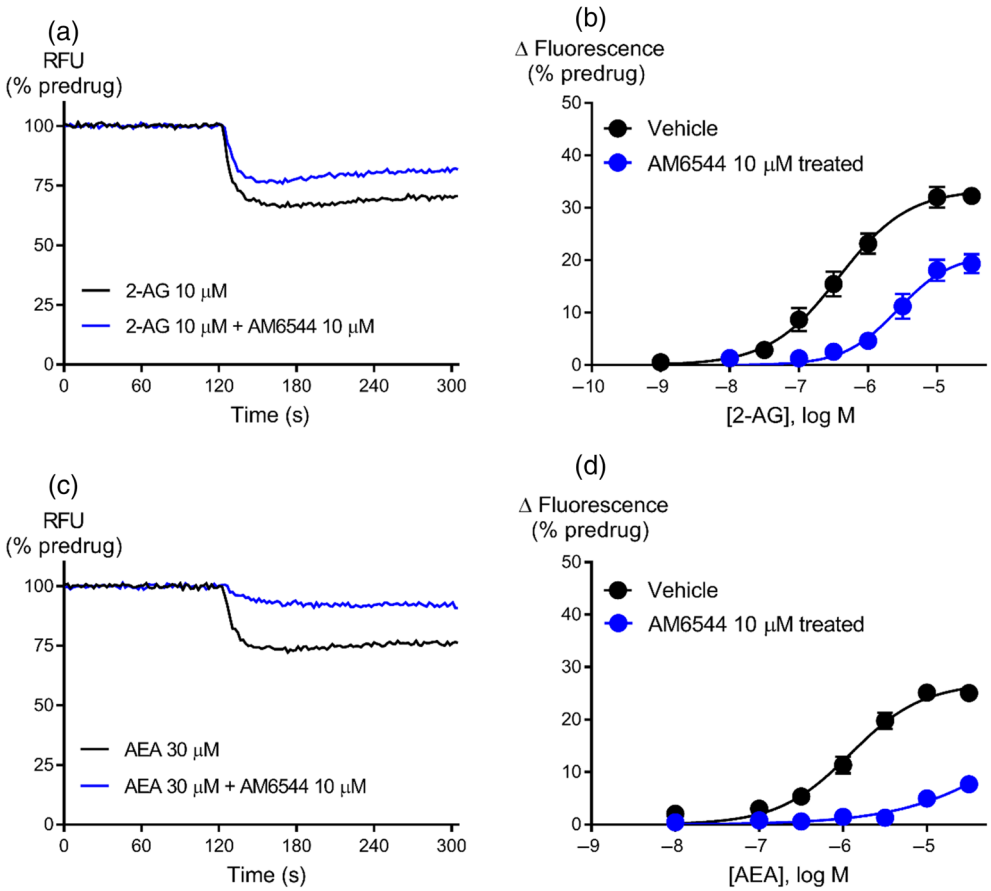


FIGURE 3 Representative traces for endogenous cannabinoids (a) 2-arachidonolylglycerol (2-AG) and (c) anandamide (AEA) after pretreatment with vehicle or AM6544 (10 μM) on AtT-20-CB1 cells. Raw trace showing reduction in hyperpolarisation induced by maximally effective concentration of 2-AG (10 μM) and AEA (30 μM) after AM6544 pretreatment compared to vehicle. Concentration–response curves for (b) 2-AG (n = 7) and (d) AEA (n = 7) were plotted using four parameter non-linear regression to fit the operational model-receptor depletion equation with basal constrained to 0. Illustrates the increase in efficacy of 2-AG as compared to AEA. Data represent the mean ± SEM of technical replicates. For some points, the error bars are smaller than the height of the symbol.

The first generation of SCRAs, JWH-018, JWH-073, JWH-200, and CP47497 were detected in herbal blends in 2008 (Auwärter et al., 2009; Banister & Connor, 2018), and since then, there has been a rapid increase in structurally diverse sets of compounds with relatively unknown pharmacology and toxicology that continues to this day (European Monitoring Centre for Drugs and Drug Addiction, 2018; United Nations Office on Drugs and Crime, 2018). The toxicity associated with emerging SCRA has been reviewed elsewhere (Hermanns-Clausen, Kneisel, Szabo, & Auwärter, 2013). There is no information as to whether the toxic effects of SCRAs may be mediated via direct CB_1 receptor activation in humans, but studies in animals and cell lines indicate that seizures and effects on the kidney associated with SCRA may depend on CB_1 receptor activation (Silva, Carmo, & Carvalho, 2018; Wiley, Barrett, Lowe, Balster, & Martin, 1995). All 13 SCRAs tested in this study had a much higher efficacy than Δ^9-THC, suggesting that adverse effects produced by Δ^9-THC intake may provide a limited guide to the potential consequences of CB_1 receptor activation with high efficacy agonists. SCRAs produce CB_1 receptor-mediated seizures in animals, in addition to the well-characterised CB_1 receptor-mediated "tetrad" of hypolocomotion, catalepsy, anti-nociception, and hypothermia, and these could conceivably account for some of the adverse effects of SCRAs in humans (Vigolo et al., 2015). We also measured the efficacy of two principal endocannabinoids: 2-AG and anandamide. Our results are consistent with previous reports, showing that 2-AG is a higher efficacy agonist of CB_1 receptors compared to anandamide (Di Marzo & De Petrocellis, 2012) and that both have a higher efficacy than Δ^9-THC (Pertwee, 1997). Thus, Δ^9-THC, but not most of the SCRAs investigated here, is likely to act as an antagonist of 2-AG modulation of neuronal activity in vivo (Pertwee, 2008; Straiker & Mackie, 2005).

Cannabinoid interactions with renal and cardiovascular systems have also been described (Pacher, Steffens, Haskó, Schindler, & Kunos, 2018), but the degree to which these interactions are influenced by agonist efficacy is unknown. A specific toxicity attributed to a particular SCRA was the acute kidney injury linked to the use of XLR-11 (Thornton, Wood, Friesen, & Gerona, 2013). The present study shows that XLR-11 had a high operational efficacy, which together with its relative non-selectivity for CB_1 over CB_2 receptors (Banister, Stuart, et al., 2015) may contribute to its unique toxicological profile. XLR-11 affects kidney cells via CB receptors on mitochondria, rather than

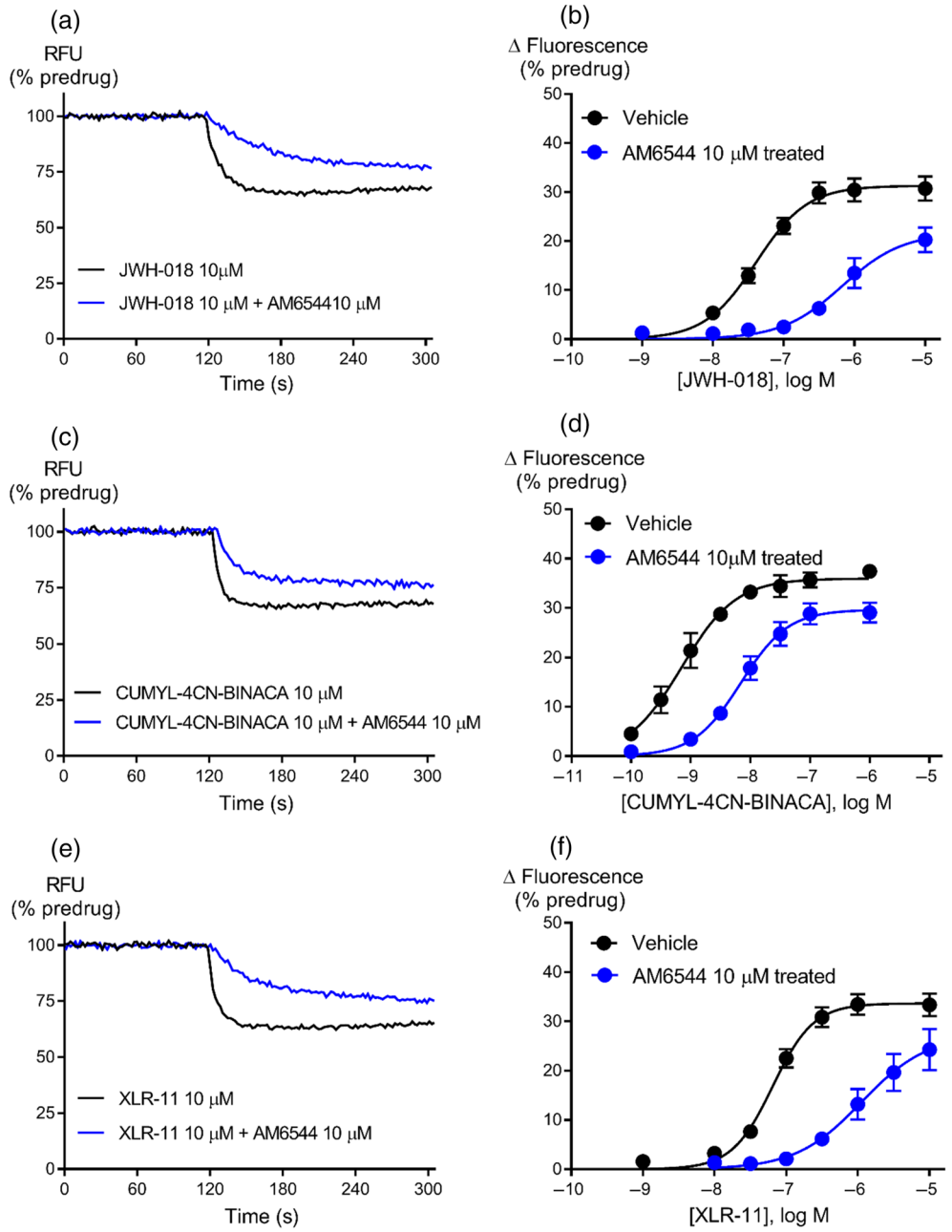


FIGURE 4 Representative traces for JWH-018 (a), CUMYL-4CN-BINACA (c), and XLR-11 (e) following pretreatment with vehicle or AM6544 (10 μM) on AtT-20-CB1 cells. Raw trace showing reduction in hyperpolarisation induced by maximally effective concentration of JWH-018 (10 μM), CUMYL-4CN-BINACA (10 μM), and XLR-11 (10 μM) after AM6544 pretreatment compared to vehicle. Concentration–response curves for (b) JWH-018 (*n* = 8), (d) CUMYL-4CN-BINACA (n = 7), and (f) XLR-11 (n = 7) were plotted using four parameter non-linear regression to fit the operational model-receptor depletion equation with basal constrained to 0. Data represent the mean ± SEM of technical replicates. For some points, the error bars are smaller than the height of the symbol.

through the plasma-membrane delimited pathway we have examined, and both CB_1 and CB_2 receptors were reported to be involved in the toxic effects of XLR-11 in vitro (Silva et al., 2018). Thus, toxicity for individual SCRAs potentially involves a complex interplay between activity at both CB_1 and CB_2 receptors as well as efficacy at CB_1 receptors, cellular and subcellular distribution, access to receptors to different body and cellular compartments, and the formation of bioactive drug metabolites (Fantegrossi, Moran, Radominska-pandya, & Prather, 2014).

The emergence of new psychoactive substances provides a continual challenge to the development of targeted interventions and novel therapeutics to help minimise the adverse effects associated with their use (European Monitoring Centre for Drugs and Drug Addiction, 2018). Although some SCRAs were mined from older patents (AM2201, AB-CHMINACA, AB-FUBINACA, UR144, etc.), newer drugs have unprecedented structures (Banister & Connor, 2018). We assessed a diversity of SCRAs identified in the NPS market, from the earliest to most recent examples. There was no obvious trend for

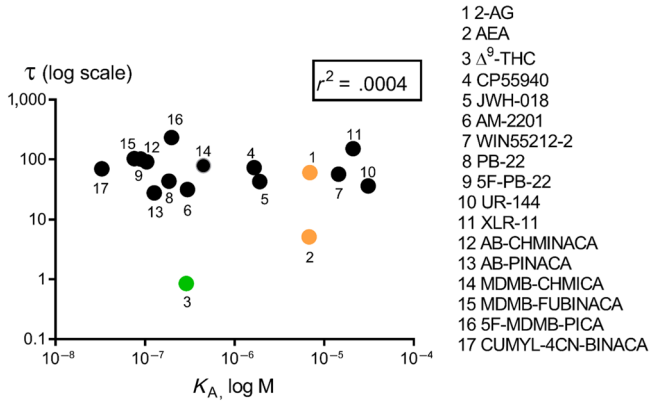


FIGURE 5 Correlation of operational efficacy (τ) and functional affinity (K_A) for CB_1 receptor agonists on G_i-dependent activation of GIRK channel in AtT-20 cells. Representative data are presented, demonstrating a non-significant value of r^2 of .0004, where τ and K_A values shown are the fitted values from the operational analysis.

decreasing/increasing τ over time and τ or functional affinity, suggesting that SCRAs are not designed to be more efficacious over time. Our data also show no obvious relationship between the efficacy of SCRA to activate native GIRK channels and their reported adverse effects. It is not immediately apparent what causes the toxic effects of SCRAs and whether signalling of SCRAs at $G\alpha_s$, $G\alpha_q$ or arrestins, rather than $G\alpha_{i/o}$-dependent CB_1 receptor signalling is important. However, it is clear that these drugs are likely to have very different pharmacological profiles to the commonly consumed cannabinoid, Δ^9-THC. This was highlighted in a recent study where the crystal structure of CB_1 receptors bound to MDMB-FUBINACA demonstrated a "toggle twin switch" interaction that Δ^9-THC did not. This might explain the low efficacy activity of Δ^9-THC compared to the high efficacy of MDMB-FUBINACA when activating CB_1 receptors (Krishna Kumar et al., 2019). Furthermore, CB_1 receptors are known to exert pleotropic effects by virtue of its ability to interact with multiple G-proteins. A recent study reported that AB-CHMINACA showed

TABLE 2 Comparison of human CB_1 receptor functional efficacy for selected SCRAs at CB_1 receptors, measured using [^{35}S]GTPγS binding assay and membrane potential assay

Compound	GTPγS binding assay, RA_i	Membrane potential assay, RA_i	Bias factor
Δ9-THC	32 (27–36)[a] **0.02** *3*	1 (0.1) **0.1** *6*	**5.0**
JWH-018	1.02 (±0.10)[b] **0.45** *4*	43 (21) **0.5** *8*	**1.1**
UR-144	193 (164–221)[c] **0.14** *5*	36 (7) **0.03** *6*	**0.21**
XLR-11	205 (177–233)[c] **0.16** *5*	152 (73) **0.2** *7*	**1.3**
PB-22	415 (373–458)[c] **0.91** *2*	44 (10) **5.4** *6*	**5.9**
AB-CHMINACA	205 (±14)[d] **5.21** *6*	92 (42) **20** *7*	**3.8**
AB-PINACA	192 (±25)[d] **0.51** *6*	28 (7) **5.0** *7*	**9.8**
MDMB-FUBINACA	75 (68–82)[a] **0.83** *3*	103 (62) **31** *9*	**37**

Note. Agonist activity (RA_i) of SCRAs for eliciting different responses in assays for CB_1 receptors are expressed relative to CP55940 is shown in bold below the E_{max} (±SEM) for GTPγS binding assay or τ (±SEM) for membrane potential assay. The bias factor is expressed as the ratio of RA_i-membrane potential assay to RA_i-GTPγS binding assay. For each measure, the number of replicates *n* is shown below the relative efficacy.

[a]Values from Gamage et al (2018) represent E_{max} (95% confidence interval) for percentage increase over basal stimulation.

[b]Values from Ford et al. (2017) represent E_{max} (±SEM) are presented as the fraction of the effect produced by reference agonist CP55940.

[c]Values from Thomas et al (2017) represent E_{max} (95% confidence interval) for percentage [^{35}S]GTPγS with basal globally shared at 100%.

[d]Values from Wiley et al (2015) represent E_{max} (±SEM) for percentage increase over basal stimulation.

specific CB_1 receptor-dependent activation of $G\alpha_s$ signalling (Costain et al., 2018). These observations highlight the complexity of the pharmacology of SCRAs-mediated activation of different signalling pathways downstream of CB_1 receptors . Structural examination of CB_1 receptors for ligand efficacy and G-protein recruitment provides molecular insights into the active state of the receptor (Krishna Kumar et al., 2019) and is a first step in informing us about the diverse physiological consequences resulting from CB_1 receptor activation by high efficacy agonists.

ACKNOWLEDGEMENTS

This work was supported by NHMRC Project Grant 1107088 awarded to M.K. and M.C. and National Institutes of Health Grant P01DA009158 to A.M. Work was also supported in part by the European Union's Seventh Framework Programme (FP7/2007-103) InMind (Grant Agreement HEALTH-F2-2011-278850) and R21DA045882 to K.V. S.S. was supported by a Macquarie University Doctoral Scholarship. We would like to thank Dr Chris Bladen and Alexander Gillis for their insightful discussions regarding work described in the draft manuscript.

AUTHOR CONTRIBUTIONS

S.S. designed and performed experiments, analysed the data, and wrote the manuscript. Data analysis was performed by M.C. and S.S. K.V. and A.M. generated the AM6544 compound. The synthesis of drugs was carried out by S.D.B. and M.L. with direction from M.K. M.S. made and characterised CB1 cells. The manuscript was drafted by S.S. and M.C. with contributions from S.D.B., K.V., and M.K. M.C. supervised the study and revised the manuscript. All the authors have given approval to the final version of manuscript.

CONFLICT OF INTEREST

Two authors (A.M. and K.V.) are co-inventors for a patent which encompasses AM6544-heteropyrrole analogues acting on cannabinoid receptors, US Patent US8084451B2, 2011. There are no other conflicts of interest to declare.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design & Analysis, and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

ORCID

Shivani Sachdev https://orcid.org/0000-0003-2514-9189
Kiran Vemuri https://orcid.org/0000-0003-3371-8185
Samuel D. Banister https://orcid.org/0000-0002-4690-4318
Michael Kassiou https://orcid.org/0000-0002-6655-0529
Marina Santiago https://orcid.org/0000-0001-9388-8309
Alexandros Makriyannis https://orcid.org/0000-0003-3272-3687
Mark Connor https://orcid.org/0000-0003-2538-2001

REFERENCES

Adams, A. J., Banister, S. D., Irizarry, L., Trecki, J., Schwartz, M., & Gerona, R. (2017). "Zombie" outbreak caused by the synthetic cannabinoid AMB-FUBINACA in New York. *New England Journal of Medicine*, *376*, 235–242. https://doi.org/10.1056/NEJMoa1610300

Alexander, S. P. H., Christopoulos, A., Davenport, A. P., Kelly, E., Marrion, N. V., Peters, J. A., … CGTP Collaborators (2017). The Concise Guide to PHARMACOLOGY 2017/18: G protein-coupled receptors. *British Journal of Pharmacology*, *174*, S17–S129. https://doi.org/10.1111/bph.13878

Alexander, S. P. H., Fabbro, D., Kelly, E., Marrion, N. V., Peters, J. A., Faccenda, E., … CGTP Collaborators (2017). The Concise Guide to PHARMACOLOGY 2017/18: Enzymes. *British Journal of Pharmacology*, *174*, S272–S359. https://doi.org/10.1111/bph.13877

Auwärter, V., Dresen, S., Weinmann, W., Müller, M., Pütz, M., & Ferreirós, N. (2009). Spice'and other herbal blends: Harmless incense or cannabinoid designer drugs? *Journal of Mass Spectrometry*, *44*, 832–837. https://doi.org/10.1002/jms.1558

Azad, S. C., Eder, M., Marsicano, G., Lutz, B., Zieglgänsberger, W., & Rammes, G. (2003). Activation of the cannabinoid receptor type 1 decreases glutamatergic and GABAergic synaptic transmission in the lateral amygdala of the mouse. *Learning & Memory*, *10*, 116–128. https://doi.org/10.1101/lm.53303

Bacci, A., Huguenard, J. R., & Prince, D. A. (2004). Long-lasting self-inhibition of neocortical interneurons mediated by endocannabinoids. *Nature*, *431*, 312–316. https://doi.org/10.1038/nature02913

Banister, S. D., & Connor, M. (2018). The chemistry and pharmacology of synthetic cannabinoid receptor agonists as new psychoactive substances: Origins. *Handbook of experimental pharmacology*, *252*, 165–190. https://doi.org/10.1007/164_2018_144

Banister, S. D., Longworth, M., Kevin, R., Sachdev, S., Santiago, M., Stuart, J., … Kassiou, M. (2016). Pharmacology of valinate and tert-leucinate synthetic cannabinoids 5F-AMBICA, 5F-AMB, 5F-ADB, AMB-FUBINACA, MDMB-FUBINACA, MDMB-CHMICA, and their analogues. *ACS Chemical Neuroscience*, *7*, 1241–1254. https://doi.org/10.1021/acschemneuro.6b00137

Banister, S. D., Moir, M., Stuart, J., Kevin, R. C., Wood, K. E., Longworth, M., … Kassiou, M. (2015). Pharmacology of Indole and Indazole Synthetic Cannabinoid Designer Drugs AB-FUBINACA, ADB-FUBINACA, AB-PINACA, ADB-PINACA, 5F-AB-PINACA, 5F-ADB-PINACA, ADBICA, and 5F-ADBICA. *ACS Chemical Neuroscience*, *6*, 1546–1559. https://doi.org/10.1021/acschemneuro.5b00112

Banister, S. D., Stuart, J., Kevin, R. C., Edington, A., Longworth, M., Wilkinson, S. M., … Kassiou, M. (2015). Effects of bioisosteric fluorine in synthetic cannabinoid designer drugs JWH-018, AM-2201, UR-144, XLR-11, PB-22, 5F-PB-22, APICA, and STS-135. *ACS Chemical Neuroscience*, *6*, 1445–1458. https://doi.org/10.1021/acschemneuro.5b00107

Banister, S. D., Wilkinson, S. M., Longworth, M., Stuart, J., Apetz, N., English, K., … Kassiou, M. (2013). The synthesis and pharmacological evaluation of adamantane-derived indoles: Cannabimimetic drugs of abuse. *ACS Chemical Neuroscience*, *4*, 1081–1092. https://doi.org/10.1021/cn400035r

Besse, J. C., & Furchgott, R. F. (1976). Dissociation constants and relative efficacies of agonists acting on alpha adrenergic receptors in rabbit aorta. *Journal of Pharmacology and Experimental Therapeutics*, *197*, 66–78.

Black, J. W., & Leff, P. (1983). Operational models of pharmacological agonism. *Proceedings of the Royal Society of London B*, *220*, 141–162. https://doi.org/10.1098/rspb.1983.0093

Cannaert, A., Storme, J., Franz, F., Auwärter, V., & Stove, C. P. (2016). Detection and activity profiling of synthetic cannabinoids and their metabolites with a newly developed bioassay. *Analytical Chemistry*, *88*, 11476–11485. https://doi.org/10.1021/acs.analchem.6b02600

Cooper, Z. D. (2016). Adverse effects of synthetic cannabinoids: Management of acute toxicity and withdrawal. *Current Psychiatry Reports*, *18*, 52. https://doi.org/10.1007/s11920-016-0694-1

Costain, W. J., Rasquinha, I., Comas, T., Hewitt, M., Aylsworth, A., Rouleau, Y., … Tauskela, J. S. (2018). Analysis of the pharmacological properties of JWH-122 isomers and THJ-2201, RCS-4 and AB-CHMINACA in HEK293T cells and hippocampal neurons. *European Journal of Pharmacology*, *823*, 96–104. https://doi.org/10.1016/j.ejphar.2018.01.043

Curtis, M. J., Alexander, S., Cirino, G., Docherty, J. R., George, C. H., Giembycz, M. A., … Ahluwalia, A. (2018). Experimental design and analysis and their reporting II: Updated and simplified guidance for authors and peer reviewers. *British Journal of Pharmacology*, *175*, 987–993. https://doi.org/10.1111/bph.14153

Daniel, H., Rancillac, A., & Crepel, F. (2004). Mechanisms underlying cannabinoid inhibition of presynaptic Ca2+ influx at parallel fibre synapses of the rat cerebellum. *The Journal of Physiology*, *557*, 159–174. https://doi.org/10.1113/jphysiol.2004.063263

De Luca, M. A., Castelli, M. P., Loi, B., Porcu, A., Martorelli, M., Miliano, C., … Di Chiara, G. (2016). Native CB1 receptor affinity, intrinsic activity and accumbens shell dopamine stimulant properties of third generation SPICE/K2 cannabinoids: BB-22, 5F-PB-22, 5F-AKB-48 and STS-135. *Neuropharmacology*, *105*, 630–638. https://doi.org/10.1016/j.neuropharm.2015.11.017

De Petrocellis, L., & Di Marzo, V. (2010). Non-CB 1, non-CB 2 receptors for endocannabinoids, plant cannabinoids, and synthetic cannabimimetics: Focus on G-protein-coupled receptors and transient receptor potential channels. *Journal of Neuroimmune Pharmacology*, *5*, 103–121. https://doi.org/10.1007/s11481-009-9177-z

Di Marzo, V., & De Petrocellis, L. (2012). Why do cannabinoid receptors have more than one endogenous ligand? *Phil. Trans. R. Soc. B*, *367*, 3216–3228. https://doi.org/10.1098/rstb.2011.0382

Ehlert, F. J. (2008). On the analysis of ligand-directed signaling at G protein-coupled receptors. *Naunyn-Schmiedeberg's Archives of Pharmacology*, *377*, 549–577. https://doi.org/10.1007/s00210-008-0260-4

Ehlert, F. J., Griffin, M. T., Sawyer, G. W., & Bailon, R. (1999). A simple method for estimation of agonist activity at receptor subtypes: Comparison of native and cloned M3 muscarinic receptors in guinea pig ileum and transfected cells. *Journal of Pharmacology and Experimental Therapeutics*, *289*, 981–992.

European Monitoring Centre for Drugs and Drug Addiction (2018). Fentanils and synthetic cannabinoids: Driving greater complexity into the drug situation. An update from the EU early warning system June 2018, Publications Office of the European Union, Luxembourg.

Fantegrossi, W. E., Moran, J. H., Radominska-pandya, A., & Prather, P. L. (2014). Distinct pharmacology and metabolism of K2 synthetic cannabinoids compared to Δ^9-THC: Mechanism underlying greater toxicity? *Life Sciences*, *97*, 45–54. https://doi.org/10.1016/j.lfs.2013.09.017

Fernando, S. R., & Pertwee, R. G. (1997). Evidence that methyl arachidonyl fluorophosphonate is an irreversible cannabinoid receptor antagonist. *British Journal of Pharmacology*, *121*, 1716–1720. https://doi.org/10.1038/sj.bjp.0701303

Finlay, D. B., Cawston, E. E., Grimsey, N. L., Hunter, M. R., Korde, A., Vemuri, V. K., … Glass, M. (2017). $G\alpha_s$ signalling of the CB1 receptor and the influence of receptor number. *British Journal of Pharmacology*, *174*, 2545–2562. https://doi.org/10.1111/bph.13866

Ford, B. M., Franks, L. N., Tai, S., Fantegrossi, W. E., Stahl, E. L., Berquist, M. D., … Prather, P. L. (2017). Characterization of structurally novel G protein biased CB1 agonists: Implications for drug development. *Pharmacological Research*, *125*, 161–177. https://doi.org/10.1016/j.phrs.2017.08.008

Funada, M., & Takebayashi-Ohsawa, M. (2018). Synthetic cannabinoid AM2201 induces seizures: Involvement of cannabinoid CB1 receptors and glutamatergic transmission. *Toxicology and Applied Pharmacology*, *338*, 1–8. https://doi.org/10.1016/j.taap.2017.10.007

Gamage, T. F., Farquhar, C. E., Lefever, T. W., Marusich, J. A., Kevin, R. C., McGregor, I. S., … Thomas, B. F. (2018). Molecular and behavioral pharmacological characterization of abused synthetic cannabinoids MMB- and MDMB-FUBINACA, MN-18, NNEI, CUMYL-PICA, and 5-Fluoro-CUMYL-PICA. *Journal of Pharmacology and Experimental Therapeutics*, *365*, 437–446. https://doi.org/10.1124/jpet.117.246983

Garcia, D., Brown, S., Hille, B., & Mackie, K. (1998). Protein kinase C disrupts cannabinoid actions by phosphorylation of the CB1 cannabinoid receptor. *Journal of Neuroscience*, *18*, 2834–2841. https://doi.org/10.1523/JNEUROSCI.18-08-02834

Griffin, M. T., Figueroa, K. W., Liller, S., & Ehlert, F. J. (2007). Estimation of agonist activity at G protein-coupled receptors: Analysis of M2 muscarinic receptor signaling through $G_{i/o}$, G_s, and G_{15}. *Journal of Pharmacology and Experimental Therapeutics*, *321*, 1193–1207. https://doi.org/10.1124/jpet.107.120857

Guo, J., & Ikeda, S. R. (2004). Endocannabinoids modulate N-type calcium channels and G-protein-coupled inwardly rectifying potassium channels via CB1 cannabinoid receptors heterologously expressed in mammalian neurons. *Molecular Pharmacology*, *65*, 665–674. https://doi.org/10.1124/mol.65.3.665

Harding, S. D., Sharman, J. L., Faccenda, E., Southan, C., Pawson, A. J., Ireland, S., … NC-IUPHAR (2018). The IUPHAR/BPS Guide to PHARMACOLOGY in 2018: Updates and expansion to encompass the new guide to IMMUNOPHARMACOLOGY. *Nucleic Acids Research*, *46*, D1091–D1106. https://doi.org/10.1093/nar/gkx1121

Hermanns-Clausen, M., Kneisel, S., Szabo, B., & Auwärter, V. (2013). Acute toxicity due to the confirmed consumption of synthetic cannabinoids: Clinical and laboratory findings. *Addiction*, *108*, 534–544. https://doi.org/10.1111/j.1360-0443.2012.04078.x

Hess, C., Schoeder, C. T., Pillaiyar, T., Madea, B., & Müller, C. E. (2016). Pharmacological evaluation of synthetic cannabinoids identified as constituents of spice. *Forensic Toxicology*, *34*, 329–343. https://doi.org/10.1007/s11419-016-0320-2

Howlett, A. C., Wilken, G. H., Pigg, J. J., Houston, D. B., Lan, R., Liu, Q., & Makriyannis, A. (2000). Azido- and isothiocyanato-substituted aryl pyrazoles bind covalently to the CB1 cannabinoid receptor and impair signal transduction. *Journal of Neurochemistry*, *74*, 2174–2181.

Ibsen, M. S., Connor, M., & Glass, M. (2017). Cannabinoid CB1 and CB2 receptor signaling and bias. *Cannabis and Cannabinoid Research*, *2*, 48–60. https://doi.org/10.1089/can.2016.0037

Jain, R., Watson, U., Vasudevan, L., & Saini, D. K. (2018). ERK activation pathways downstream of GPCRs. *International Review of Cell and Molecular Biology*, *338*, 79–109. https://doi.org/10.1016/bs.ircmb.2018.02.003

Knapman, A., Santiago, M., Du, Y. P., Bennallack, P. R., Christie, M. J., & Connor, M. (2013). A continuous, fluorescence-based assay of μ-opioid receptor activation in AtT-20 cells. *Journal of Biomolecular Screening*, *18*, 269–276. https://doi.org/10.1177/1087057112461376

Krishna Kumar, K., Shalev-Benami, M., Robertson, M. J., Hu, H., Banister, S. D., Hollingsworth, S. A., … Skiniotis, G. (2019). Structure of a signaling

cannabinoid receptor 1-G protein complex. *Cell*, *176*, 448–458.e12. https://doi.org/10.1016/j.cell.2018.11.040

Leonti, M., Casu, L., Raduner, S., Cottiglia, F., Floris, C., Altmann, K.-H., & Gertsch, J. (2010). Falcarinol is a covalent cannabinoid CB1 receptor antagonist and induces pro-allergic effects in skin. *Biochemical Pharmacology*, *79*, 1815–1826. https://doi.org/10.1016/j.bcp.2010.02.015

Mackie, K., Lai, Y., Westenbroek, R., & Mitchell, R. (1995). Cannabinoids activate an inwardly rectifying potassium conductance and inhibit Q-type calcium currents in AtT20 cells transfected with rat brain cannabinoid receptor. *Journal of Neuroscience*, *15*, 6552–6561. https://doi.org/10.1523/JNEUROSCI.15-10-06552.1995

Malyshevskaya, O., Aritake, K., Kaushik, M. K., Uchiyama, N., Cherasse, Y., Kikura-Hanajiri, R., & Urade, Y. (2017). Natural (Δ^9-THC) and synthetic (JWH-018) cannabinoids induce seizures by acting through the cannabinoid CB 1 receptor. *Scientific Reports*, *7*, 10516. https://doi.org/10.1038/s41598-017-10447-2

Marinelli, S., Pacioni, S., Cannich, A., Marsicano, G., & Bacci, A. (2009). Self-modulation of neocortical pyramidal neurons by endocannabinoids. *Nature Neuroscience*, *12*, 1488–1490. https://doi.org/10.1038/nn.2430

Motulsky, H., & Christopoulos, A. (2004). *Fitting models to biological data using linear and nonlinear regression: A practical guide to curve fitting*. San Diego, CA, USA: Oxford University Press.

Noble, C., Cannaert, A., Linnet, K., & Stove, C. P. (2018). Application of an activity-based receptor bioassay to investigate the in vitro activity of selected indole-and indazole-3-carboxamide-based synthetic cannabinoids at CB1 and CB2 receptors. *Drug Testing and Analysis*, *11*, 501–511. https://doi.org/10.1002/dta.2517

Pacher, P., Bátkai, S., & Kunos, G. (2006). The endocannabinoid system as an emerging target of pharmacotherapy. *Pharmacological Reviews*, *58*, 389–462. https://doi.org/10.1124/pr.58.3.2

Pacher, P., Steffens, S., Haskó, G., Schindler, T. H., & Kunos, G. (2018). Cardiovascular effects of marijuana and synthetic cannabinoids: the good, the bad, and the ugly. *Nature Reviews Cardiology*, *15*, 151–166. https://doi.org/10.1038/nrcardio.2017.130

Pertwee, R. G. (1997). Pharmacology of cannabinoid CB1 and CB2 receptors. *Pharmacology & Therapeutics*, *74*, 129–180. https://doi.org/10.1016/S0163-7258(97)82001-3

Pertwee, R. G. (2008). The diverse CB1 and CB2 receptor pharmacology of three plant cannabinoids: Δ^9-tetrahydrocannabinol, cannabidiol and Δ^9-tetrahydrocannabivarin. *British Journal of Pharmacology*, *153*, 199–215. https://doi.org/10.1038/sj.bjp.0707442

Schindler, C. W., Gramling, B. R., Justinova, Z., Thorndike, E. B., & Baumann, M. H. (2017). Synthetic cannabinoids found in "spice" products alter body temperature and cardiovascular parameters in conscious male rats. *Drug & Alcohol Dependence*, *179*, 387–394. https://doi.org/10.1016/j.drugalcdep.2017.07.029

Silva, J. P., Carmo, H., & Carvalho, F. (2018). The synthetic cannabinoid XLR-11 induces in vitro nephrotoxicity by impairment of endocannabinoid-mediated regulation of mitochondrial function homeostasis and triggering of apoptosis. *Toxicology Letters*, *287*, 59–69. https://doi.org/10.1016/j.toxlet.2018.01.023

Straiker, A., & Mackie, K. (2005). Depolarization-induced suppression of excitation in murine autaptic hippocampal neurones. *The Journal of Physiology*, *569*, 501–517. https://doi.org/10.1113/jphysiol.2005.091918

Tait, R. J., Caldicott, D., Mountain, D., Hill, S. L., & Lenton, S. (2016). A systematic review of adverse events arising from the use of synthetic cannabinoids and their associated treatment. *Clinical Toxicology*, *54*, 1–13. https://doi.org/10.3109/15563650.2015.1110590

Thomas, B. F., Lefever, T. W., Cortes, R. A., Grabenauer, M., Kovach, A. L., Cox, A. O., … Wiley, J. L. (2017). Thermolytic degradation of synthetic cannabinoids: Chemical exposures and pharmacological consequences. *Journal of Pharmacology and Experimental Therapeutics*, *361*, 162–171. https://doi.org/10.1124/jpet.116.238717

Thornton, S. L., Wood, C., Friesen, M. W., & Gerona, R. R. (2013). Synthetic cannabinoid use associated with acute kidney injury. *Clinical Toxicology*, *51*, 189–190. https://doi.org/10.3109/15563650.2013.770870

Trecki, J., Gerona, R. R., & Schwartz, M. D. (2015). Synthetic cannabinoid-related illnesses and deaths. *The New England Journal of Medicine*, *373*, 103–107. https://doi.org/10.1056/NEJMp1505328

United Nations Office on Drugs and Crime (2018). World Drug Report. United Nations publication.978–92–1-148291-1.

Vigolo, A., Ossato, A., Trapella, C., Vincenzi, F., Rimondo, C., Seri, C., … Marti, M. (2015). Novel halogenated derivates of JWH-018: Behavioral and binding studies in mice. *Neuropharmacology*, *95*, 68–82. https://doi.org/10.1016/j.neuropharm.2015.02.008

Whorton, M. R., & MacKinnon, R. (2013). X-ray structure of the mammalian GIRK2–βγ G-protein complex. *Nature*, *498*, 190–197. https://doi.org/10.1038/nature12241

Wiley, J., Barrett, R., Lowe, J., Balster, R., & Martin, B. (1995). Discriminative stimulus effects of CP 55,940 and structurally dissimilar cannabinoids in rats. *Neuropharmacology*, *34*, 669–676. https://doi.org/10.1016/0028-3908(95)00027-4

Wiley, J. L., Marusich, J. A., & Huffman, J. W. (2014). Moving around the molecule: Relationship between chemical structure and in vivo activity of synthetic cannabinoids. *Life Sciences*, *97*, 55–63. https://doi.org/10.1016/j.lfs.2013.09.011

Wiley, J. L., Marusich, J. A., Lefever, T. W., Antonazzo, K. R., Wallgren, M. T., Cortes, R. A., … Thomas, B. F. (2015). AB-CHMINACA, AB-PINACA, and FUBIMINA: Affinity and potency of novel synthetic cannabinoids in producing Δ^9-tetrahydrocannabinol–like effects in mice. *Journal of Pharmacology and Experimental Therapeutics*, *354*, 328–339. https://doi.org/10.1124/jpet.115.225326

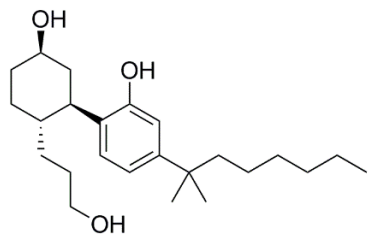
SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

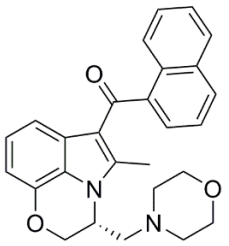
How to cite this article: Sachdev S, Vemuri K, Banister SD, et al. In vitro determination of the efficacy of illicit synthetic cannabinoids at CB_1 receptors. *Br J Pharmacol*. 2019;1–13. https://doi.org/10.1111/bph.14829

Supplementary Figure 1

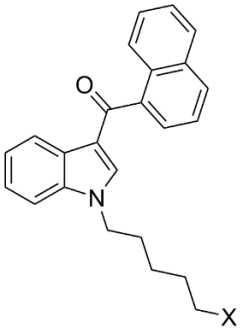
Structure of selected SCRAs



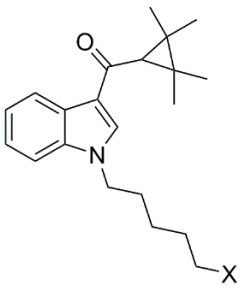
CP55940



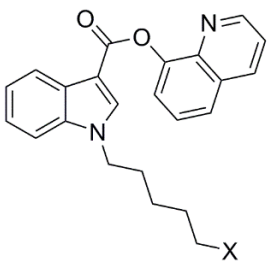
WIN55212-2



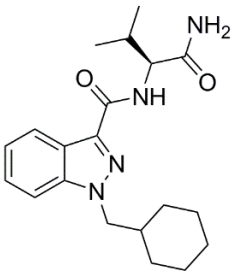
JWH-018 (X = H)
AM-2201 (X = F)



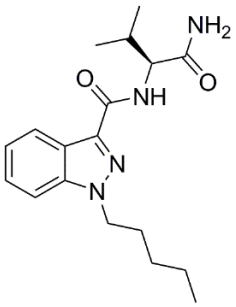
UR-144 (X = H)
XLR-11 (X = F)



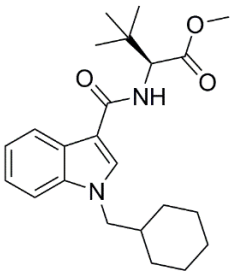
PB-22 (X = H)
5F-PB-22 (X = F)



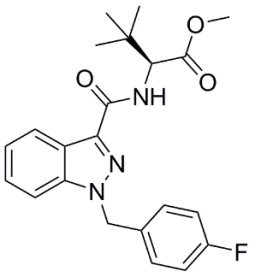
AB-CHMINACA



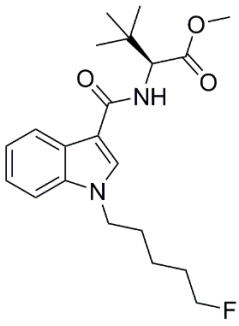
AB-PINACA



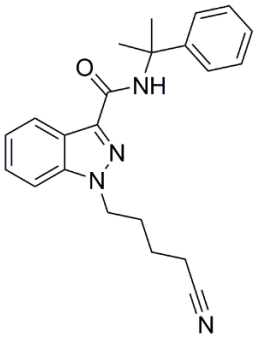
MDMB-CHMICA



MDMB-FUBINACA

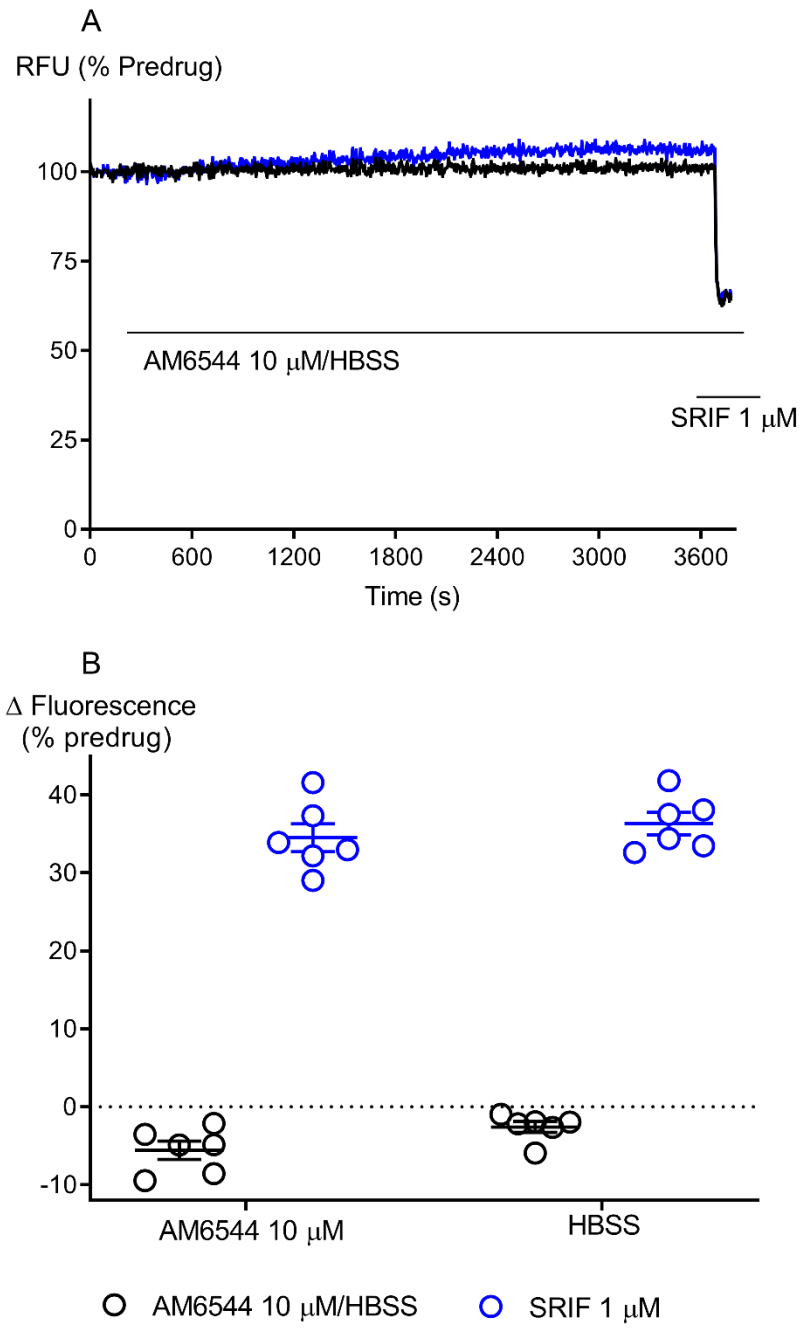


5F-MDMB-PICA



CUMYL-4CN-BINACA

Supplementary Figure 2



AM6544 does not activate GIRK channels in AtT20 cells. (A) Raw trace showing minimal changes in hyperpolarisation induced by maximally effective concentration (10 μM) of AM6544 or vehicle on At-T20-CB1 cells (B) Scatter dot plot showing the percentage change in fluorescence of SRIF (1 μM) on AtT20-CB1 cells in the presence of HBSS or AM6544 (10

μM) for 60 mins. Data represents the mean ± SEM of 6 independent determinants performed in duplicate.

Supplementary Table 1

Functional activity of CP55940, Δ^9-THC, and other SCRAs. Values were calculated using the four-parameter nonlinear regression to fit the concentration response curves.

Compound	pEC_{50} ± SEM (EC_{50}, nM)
CP55940	7.62 ± 0.07 (23)
WIN55212-2	6.7 ± 0.05 (200)
Δ^9-THC	6.81 ± 0.12 (156)
2-AG	6.47 ± 0.07 (338)
AEA	5.91 ± 0.07 (1229)
JWH-018	7.37 ± 0.08 (43)
AM-2201	8.02 ± 0.1 (9.6)
UR-144	6.3 ± 0.08 (503)
XLR-11	7.14 ± 0.08 (72)
PB-22	8.36 ± 0.05 (4.4)
5F-PB-22	9.02 ± 0.08 (0.95)
AB-CHMINACA	7.67 ± 0.14 (21)
AB-PINACA	8.68 ± 0.08 (2.1)
MDMB-CHMICA	8.33 ± 0.09 (4.7)
MDMB-FUBINACA	9.1 ± 0.13 (0.8)
5F-MDMB-PICA	9.15 ± 0.08 (0.7)
CUMYL-4CN-BINACA	9.16 ± 0.08 (0.68)

Chapter III.

Study II. Differential activation of G-protein-mediated signalling by synthetic cannabinoid receptor agonists

The third chapter of this thesis is a natural continuation of the original investigation of SCRA-associated toxicity, where the functional activity of same panel of cannabinoids was also characterised in two signalling endpoints - $G_{\alpha i/o}$ (inhibition) and $G_{\alpha s}$ (stimulation) of cAMP signalling. Pharmacological assessment was done to study whether different agonists produce different profiles of effects depending on the functional endpoint, and for better understanding of CB1 functional selectivity in general.

Contributions to the work

This paper represents a collaborative work hosted in Mark Connor's laboratory at Macquarie University Australia. I took the lead role in experimental design, conducting the experiments, data analysis and writing the paper with some support from my co-investigators: Mark Connor oversaw the work in this research group; Samuel Banister generated all the synthetic cannabinoids with guidance from Michael Kassiou; Marina Santiago and Chris Bladen provided assistance with the statistical analysis. All authors reviewed the final manuscript.

Received: 21 November 2019 | Revised: 23 January 2020 | Accepted: 27 January 2020

DOI: 10.1002/prp2.566

ORIGINAL ARTICLE

Differential activation of G protein-mediated signaling by synthetic cannabinoid receptor agonists

Shivani Sachdev[1] | Samuel D. Banister[2,3] | Marina Santiago[1] | Chris Bladen[1] | Michael Kassiou[3] | Mark Connor[1]

[1]Department of Biomedical Sciences, Macquarie University, Sydney, NSW, Australia

[2]The Lambert Initiative for Cannabinoid Therapeutics, Brain and Mind Centre, The University of Sydney, Sydney, NSW, Australia

[3]School of Chemistry, The University of Sydney, Sydney, NSW, Australia

Correspondence
Mark Connor, Department of Biomedical Sciences, Macquarie University, Sydney, NSW, Australia.
Email: mark.connor@mq.edu.au

Funding information
National Health and Medical Research Council, Grant/Award Number: APP1107088; Macquarie University Research Excellence Scholarship

Abstract

Synthetic cannabinoid receptor agonists (SCRAs) are new psychoactive substances associated with acute intoxication and even death. However, the molecular mechanisms through which SCRAs may exert their toxic effects remain unclear—including the potential differential activation of G protein subtypes by cannabinoid receptor type 1 (CB1), a major target of SCRA. We measured CB1-mediated activation of $G\alpha_s$ and $G\alpha_{i/o}$ proteins by SCRAs by examining stimulation (pertussis toxin, PTX treated) as well as inhibition (non-PTX treated) of forskolin (FSK)-induced cyclic adenosine monophosphate (cAMP) accumulation in human embryonic kidney (HEK) cells stably expressing CB1. Real-time measurements of stimulation and inhibition of cAMP levels were made using a BRET biosensor. We found that the maximum concentration of SCRAs tested (10 µmol L^{-1}), increased cAMP levels 12%-45% above that produced by FSK alone, while the phytocannabinoid THC did not significantly alter cAMP levels in PTX-treated HEK-CB1 cells. All SCRAs had greater potency to inhibit FSK-induced cAMP levels than to stimulate cAMP levels. The rank order of potencies for SCRA stimulation of cAMP ($G\alpha_s$) was PB-22 > 5F-MDMB-PICA > JWH-018 ≈ AB-FUBINACA > XLR-11. By contrast, the potency of SCRAs for inhibition of cAMP ($G\alpha_{i/o}$) was 5F-MDMB-PICA > AB-FUBINACA > PB-22 > JWH-018 > XLR-11. The different rank order of potency and E_{Max} of the SCRAs to stimulate $G\alpha_s$-like signaling compared to $G\alpha_{i/o}$ signaling suggests differences in G protein preference between SCRAs. Understanding the apparent differences among these drugs may contribute to unravelling their complex effects in humans.

KEYWORDS

cannabinoid receptor, G protein, signaling, synthetic cannabinoid receptor agonist, toxicity

Abbreviations: 2-AG, 2-arachidonoyl glycerol; AC, adenylyl cyclase; BRET, bioluminescence resonance energy transfer; CAMYEL, cAMP sensor YFP-Epac-Rluc; CB1, cannabinoid receptor type 1; GIRK, G protein-gated inwardly rectifying K^+ channel; HA, hemagglutinin; HEK-CB1, human embryonic kidney cells stably transfected with HA tagged human CB1 receptors; NPS, novel psychoactive substances; PEI, polyethylenimine; PTX, pertussis toxin; SCRA, synthetic cannabinoid receptor agonists; THC, Δ^9-tetrahydrocannabinol.

1 | INTRODUCTION

The use of synthetic cannabinoid receptor agonist (SCRA) new psychoactive substances (NPS) is associated with significant morbidity and mortality compared to use of Δ^9-tetrahydrocannabinol (THC), the main psychoactive ingredient of cannabis.[1,2] SCRAs are linked to a wide range of toxic effects including seizures, agitation, hypertension, cardiotoxicity, kidney damage, and sometimes death.[3,4] There has been a rapid increase in the number of structurally diverse SCRAs since 2010, with little known about their pharmacology and toxicology at time of identification.[5] The constant evolution of SCRA structures occurs in response to legislative restriction and development of urine drug screens for existing compounds.[6-8] A time-series of seizures (by tonnage) of NPS reported to the United Nations Office on Drug and Crime[9] showed that the SCRAs dominated the synthetic NPS market over the period 2011-2017.

SCRAs are usually agonists at both cannabinoid type-1 and type-2 receptors (CB1 and CB2, respectively[10]); with the psychoactive effects attributed to the activation of CB1.[11] We have previously described the in vitro quantitative measurement of SCRA efficacy at CB1, where all SCRAs tested showed between 20- and 300-fold greater agonist activity at CB1 compared to THC.[12] Cannabinoid receptor-mediated G protein signaling is predominantly through the $G\alpha_{i/o}$ protein family[13]; however, under some circumstances, CB1 can also stimulate adenylyl cyclase (AC) through $G\alpha_s$-proteins.[14-16] For example, blockade of the canonical CB1-$G\alpha_i$ pathway with pertussis toxin (PTX) or sequestration of CB1-$G\alpha_i$ protein in the primary striatal rat neurons on coexpression with D2 results in an augmentation of cyclic adenosine monophosphate (cAMP) levels by cannabinoids, suggesting that CB1 couples to $G\alpha_s$.[14,15] A recent study characterized the relationship between CB1 receptor expression and signaling, and showed that at very high receptor expression levels, the effect of CB1 activation on cAMP signaling was stimulatory, a phenotype that was reversed by systematic pharmacological knockdown at the receptor level.[17] The idea that certain SCRAs may preferentially activate different CB1 Gα subtypes is not unprecedented[18-20]; in a study by Costain et al[21] AB-CHMINACA elicited an elevation in cAMP levels in both the absence and presence of forskolin (FSK) in human embryonic kidney (HEK) cells transiently expressing CB1, suggesting an AB-CHMINACA-specific CB1-mediated activation of $G\alpha_s$ signaling.

The mechanism(s) through which SCRAs exert different behavioral and physiological effects remains unclear, and which pathways modulated by CB1 activation mediate the specific pharmacological effects of SCRAs is also unknown. Similarly, the question of whether these pathways are activated in a quantitatively or qualitatively similar way by SCRAs and THC is only beginning to be addressed.[22] Finally, the question of whether SCRA activity at noncannabinoid receptors is also important for their pharmacological effects is very much open.[23-25] With more than 250 SCRAs identified in the NPS market,[9] elucidation of the differential molecular mechanisms by which these compounds can exert distinct pharmacology, including their signaling via CB1, is essential for understanding their adverse effects. This study examined whether SCRAs that are representative of structural classes confirmed in patients admitted to emergency departments with presumed SCRA toxicity stimulate $G\alpha_s$-like cAMP signaling via CB1. We measured the SCRA-mediated stimulation as well as inhibition of FSK-induced cAMP accumulation in HEK cells stably expressing CB1. We have observed SCRA-specific CB1-dependent activation of the two signaling pathways, but THC only coupled to inhibition, not stimulation of cAMP. While AB-CHIMINACA, previously identified as having a unique profile among SCRAs for elevating cAMP, appeared to signal, in part, through non-CB1 mechanisms.

2 | MATERIALS AND METHODS

2.1 | CB1 receptor transfection and cell culture

HEK 293 FlpIn cells with homogeneous G protein-gated inwardly rectifying K^+ (GIRK4) channel expression (the construction of these cells by Grimsey et al will be described elsewhere) were cotransfected with pcDNA5/FRT construct encoding hemagglutinin (HA)-tagged human CB1 receptor cDNA and pOG44 (Flp recombinase plasmid) using the same random incorporation method of stable transfection as described previously for AtT-20 pituitary tumor cells.[26] Cells stably expressing the CB1 receptor were cultured in Dulbecco's Modified Eagle Media (Thermo Fischer Scientific) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich), 100 units mL^{-1} penicillin, 100 μg mL^{-1} streptomycin (Thermo Fischer Scientific), 400 μg mL^{-1} G418 (GIRK4 selection antibiotic) and 100 μg mL^{-1} hygromycin (CB1 selection antibiotic) up to passage 5 (selection phase). Hygromycin concentration was reduced to 80 μg mL^{-1} beyond passage 5 (maintenance phase). Cells were grown in 75 cm^2 flask at 37°C/5% CO_2 and passaged at 80% confluency as required. Assays were carried out on cells up to 25 passages.

2.2 | Assay for cAMP measurement

Intracellular cAMP levels were measured using pcDNA3L-His-CAMYEL plasmid, which encodes the cAMP sensor YFP-Epac-RLuc (CAMYEL) as outlined in Ref. [27,28] Cells were detached from the flask using trypsin/EDTA (Sigma-Aldrich), and resuspended in DMEM supplemented with 10% FBS, 100 units mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin. Cells were seeded in 10 cm dishes at a density of 7 000 000 such that they would be 60%-70% confluent the next day. On the following day, the cells were transiently transfected with 5 μg of pcDNA3L-His-CAMYEL plasmid using the linear polyethylenimine (PEI, m.w. 25 kDa) (Polysciences). The PEI/DNA complex mixture was sequentially added to the cells at the ratio of 1:6, and cells were incubated in 5% CO_2 at 37°C. Approximately 24 hours after transfection, the cells were then detached from the dish and the pellet was resuspended in Leibovitz's (L-15—Thermo Fischer Scientific) media supplemented with 1% FBS, 100 units mL^{-1} penicillin, 100 μg mL^{-1} streptomycin and 15 mmol L^{-1} glucose. In the experiments with PTX to irreversibly uncouple $G\alpha_i$ proteins, the cells were resuspended in the media containing 200 ng mL^{-1} PTX. The PTX-treated and control (non-PTX treated) cells were plated

at a density of 100 000 cells per well in poly D-lysine (Sigma-Aldrich) coated, white wall, clear bottomed 96-well microplates. Cells were incubated overnight at 37°C in ambient CO_2.

The day after plating, FSK (an activator of AC) was prepared in Hanks' balanced salt solution (HBSS) composed of (mmol L^{-1}) NaCl 145, HEPES 22, Na_2HPO_4 0.338, $NaHCO_3$ 4.17, KH_2PO_4 0.441, $MgSO_4$ 0.407, $MgCl_2$ 0.493, $CaCl_2$ 1.26, glucose 5.56 (pH 7.4, osmolarity 315 ± 15), and supplemented with 0.1% bovine serum albumin. All the drugs used for the series of real-time measurements of stimulation and inhibition of cAMP levels were made in 3 µmol L^{-1} of FSK immediately before the assay. The concentration of DMSO (0.10%-0.13%) was kept constant for all experiments, however this limited the maximum drug concentration that could be tested. Coelenterazine H substrate (NanoLight Technologies) was made in HBSS, and added to a final concentration of 5 µmol L^{-1} (10 µL per well) to the cells, and incubated for 5 minutes after which 10 µL of (10×) drug was added to each well to obtain the desired concentration. A vehicle (HBSS plus DMSO alone) was included in each column of a 96-well microplate and routinely subtracted from the measurements. The PTX-treated and control cells were compared side by side. Luminescence was measured using a PHERAstar plate reader (BMG Labtech) at 37°C. The cell signaling was measured at an emission wavelength of 475 and 535 nm simultaneously, and the readings were made every 40 seconds for approximately 20 minutes. A concentration response curve (CRC) for CP55940 and WIN55212-2 inhibition of cAMP accumulation was performed for each experimental replicate as a reference standard (Figure 1). Day to day variation in the degree of Gs-stimulation was observed, presumably arising in part from the transient transfections and subsequent PTX treatment required for each assay.

2.3 | Data analysis

Raw data are presented as inverse bioluminescence resonance energy transfer (BRET) ratio of emission at 475/535 nm, such that an increase in ratio corresponds with increase in cAMP production. Real-time (raw) cAMP time course data were then analyzed using area under curve analysis in GraphPad PRISM (Graph Pad Software Inc). Data were normalized to the change produced by FSK over 20 minutes (set as 100%) for each experiment. The percent change values were fit to three or four-parameter non-linear regression curves in PRISM to derive EC_{50} and E_{max}. In the three parameter fit the Hill slope was constrained to 1, in the four parameter fit it was free to vary. All final datasets passed the Shapiro-Wilk test for normality. Unless otherwise stated, the data represent mean ± SEM of at least five independent experiments, each conducted in duplicate.

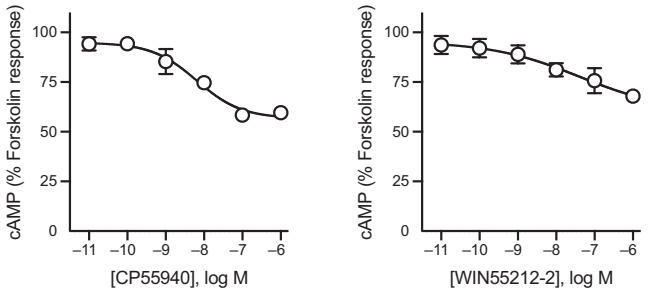


FIGURE 1 Concentration response curve for CP55940 and WIN55212-2. Treatment with CP55940 or WIN55212-2 produced a concentration-dependent inhibition of forskolin-mediated cAMP production in human embryonic kidney 293-cannabinoid receptor type 1. Curves were generated by area under the curve analysis for CP55940 or WIN55212-2 in the presence of 3 µmol L^{-1} forskolin. Data were normalized to forskolin (100%) and vehicle (0%), and plotted as mean ± SEM for at least five independent experiments performed in duplicate. cAMP, cyclic adenosine monophosphate

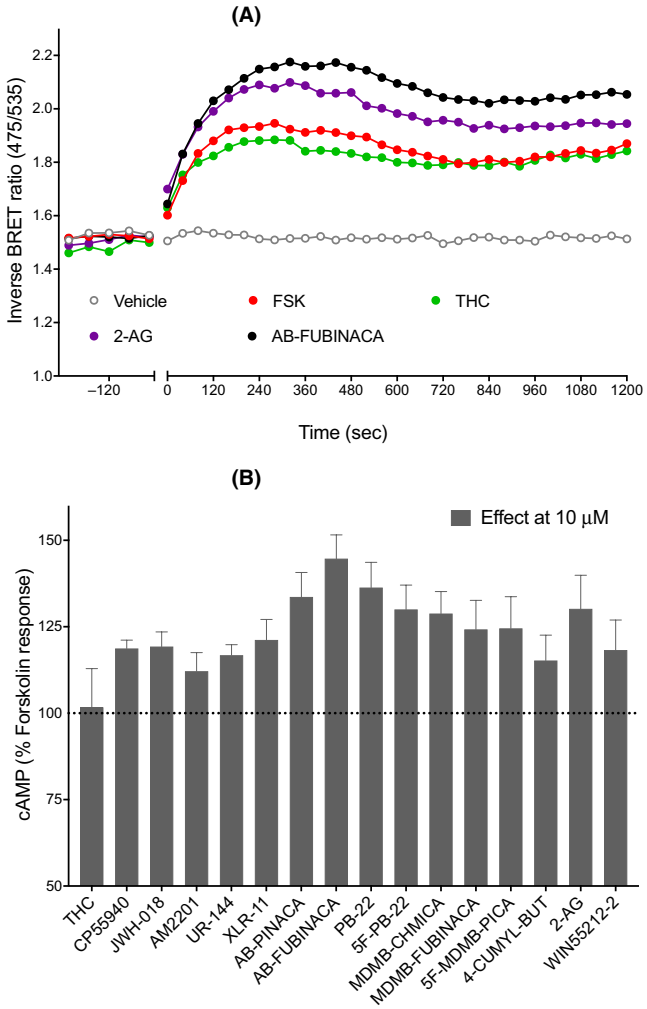


FIGURE 2 $G\alpha_s$-mediated signaling of synthetic cannabinoid receptor agonists. A, Representative data for real-time measurement of stimulation of cAMP levels by 10 µmol L^{-1} of cannabinoids (THC, 2-arachidinoylglycerol, and AB-FUBINACA) in human embryonic kidney cells expressing cannabinoid receptor type 1 receptors, an increase in inverse BRET ratio (emission at 475/535 nm) corresponds to an increase in cAMP. B, A bar chart summarizing the cAMP signaling peaks for 16 cannabinoids (excluding AB-CHMINACA) showing an increase in cAMP levels above that of FSK (3 µmol L^{-1}) alone (FSK, 100%). Graphs show mean + SEM for at least five independent experiments performed in duplicate. BRET, bioluminescence resonance energy transfer; cAMP, cyclic adenosine monophosphate; FSK, forskolin; THC, Δ9-tetrahydrocannabinol

The differences between groups were tested using unpaired Student's *t* test, and one-way ANOVA as appropriate when comparing multiple groups (PRISM). Statistical significance is defined as $P < .05$.

2.4 | Materials

CP55940, WIN55212-2, 2-arachidonoylglycerol (2-AG), CUMYL-4CN-BINACA, and SR141716A were purchased from Cayman Chemical, THC was from THC Pharm GmbH and was a kind gift from the Lambert Initiative for Cannabis Therapeutics (University of Sydney). PTX was from HelloBio, and FSK was from Ascent Scientific Ltd. All the SCRAs, unless otherwise stated, were synthesized by Dr Samuel D. Banister in the lab of Professor Michael Kassiou at Sydney University. Chemical structure of SCRAs can be found elsewhere.[12] All the SCRAs were prepared in DMSO and stored in aliquots of 30 mmol L^{-1} in −30°C until needed.

3 | RESULTS

3.1 | Real-time cAMP BRET measurement of the $G\alpha_s$-mediated signaling of SCRAs

Using the CAMYEL assay, we measured the effect of seventeen cannabinoids (10 µmol L^{-1} each) on the FSK-stimulated cellular cAMP levels in HEK-CB1 cells following pretreatment with PTX. All the SCRAs produced an increase in cAMP levels above that produced by FSK alone (100%). Examples of raw traces are shown for some SCRAs (Figure 2A), note that the stimulation of cAMP by SCRAs in the presence of FSK and PTX plateaued approximately after 12 minutes, and maintained at that level for the entire course of the assay (20 minutes). The effects of SCRAs tested ranged from 12% to 45% increase in signal relative to FSK alone. Most of the SCRAs had approximately 1.5 times higher effect than CP55940 (19%) or WIN55212-2 (18%), except for JWH-018, UR-144, AM-2201, and CUMYL-4CN-BINACA, which showed similar or lower effect (Figure 2B). AB-FUBINACA had up to 2.5 times higher effect than CP55940. In PTX-treated cells, the endocannabinoid 2-AG (10 µmol L^{-1}) produced an increase in FSK-stimulated cAMP levels approximately twice that of CP55940, while the phytocannabinoid THC did not significantly alter cAMP levels in the presence of FSK (compared to FSK alone Figure 2B, $P > .05$).

3.2 | Differential SCRAs-induced stimulation and inhibition of cAMP signaling in HEK-CB1

To assess whether there was any evidence of preferential coupling to $G\alpha_{i/o}$ over $G\alpha_s$ among SCRAs, we assessed the pharmacological activity (EC_{50} and E_{max}) of a selection of SCRAs belonging to different structural classes (JWH-018, PB-22, AB-FUBINACA, XLR-11, and 5F-MDMB-PICA), to stimulate and inhibit cAMP in HEK-CB1 cells. All the SCRAs tested activated CB1 through $G\alpha_{i/o}$ (inhibitory, non-PTX treated), and $G\alpha_s$ (stimulatory, PTX treated) in a concentration-dependent manner (Figure 3). As previously reported,[29] treatment with CP55940 and WIN55212-2 produced an immediate concentration-dependent inhibition of FSK-mediated cAMP production (pEC_{50} CP55940 8.1 ± 0.4, pEC_{50} WIN55212-2 7.9 ± 0.4). All SCRAs had greater potency (0.62-63 nmol L^{-1}) for inhibition of FSK-induced cAMP levels in non-PTX-treated HEK cells compared to their potency to stimulate cAMP levels (69-4720 nmol L^{-1}) (Table 1). The activation of CB1-$G\alpha_s$ by SCRAs showed a wide variation in E_{max} values, and there was a significant difference in efficacy between AB-FUBINACA, XLR-11 and JWH-018 (one-way ANOVA, $P < .05$). The rank order of efficacy for stimulation of Gαs was AB-FUBINACA ≈ PB-22 > 5F-MDMB-PICA > XLR-11 > JWH-018, whereas all the SCRAs were similarly effective at inhibiting cAMP production (Table 1). It should be noted that the CRC for the most efficacious compound tested at $G\alpha_s$ pathway, AB-FUBINACA, may not have reached a plateau at highest concentration we could test, 30 µmol L^{-1}, and that of XLR-11 almost certainly had not. The first SCRA to be identified in spice, JWH-018, caused partial (14% increase over FSK alone) activation of $G\alpha_s$ pathway, but produced greater inhibition of the FSK-induced cAMP response (64% of FSK response). Whereas other SCRAs tested in this study induce moderate activation of $G\alpha_s$ pathway (26%-36% relative to FSK) compared to their activity at $G\alpha_{i/o}$ inhibitory pathway (Figure 3). The rank order of potencies for SCRAs for inhibition of cAMP ($G\alpha_{i/o}$) is 5F-MDMB-PICA > AB-FUBINACA > PB-22 > JWH-018 > XLR-11. By contrast, the potency of SCRAs for stimulation of cAMP ($G\alpha_s$) is PB-22 > 5F-MDMB-PICA > JWH-018 ≈ ≥ AB-FUBINACA > XLR-11. The most efficacious SCRA at $G\alpha_s$ pathway (AB-FUBINACA) was roughly 300 times less potent at $G\alpha_s$ than the $G\alpha_{i/o}$-pathway, while JWH-018 was only 18 times less potent. XLR-11 had much lower potency compared to all the other SCRAs for both $G\alpha_s$ pathway and $G\alpha_{i/o}$ pathway (Table 1).

We then tested if the SCRA-induced observed stimulatory effects were mediated through CB1 receptors. Pretreatment of HEK-CB1 with SR141716A (3 µmol L^{-1}, 5 minutes), a potent and selective CB1 antagonist,[30] prevented the subsequent SCRA (10 µmol L^{-1})-mediated stimulation of FSK-induced cAMP response compared to the vehicle-treated cells (Figure 4; $P < .05$). Consistent with $G\alpha_s$ CB1-specific responses of SCRAs, pretreatment with SR141716A also blocked the inhibitory cAMP signaling induced by SCRAs (Figure S1; $P < .05$).

AB-CHMINACA has previously been reported to stimulate $G\alpha_s$-like cAMP signaling pathway in a concentration-dependent manner in HEK-CB1 cells.[21] Following PTX treatment, AB-CHMINACA increased cAMP levels above that of FSK alone (Figure 5A) in a concentration-dependent manner, with an increase of 86 ± 21% at 30 µmol L^{-1}. However, in cells pretreated with SR141716A (3 µmol L^{-1}, 5 minutes), the stimulatory effects of AB-CHMINACA (10 µmol L^{-1}) were only partially inhibited, in contrast to other SCRAs tested in this study (Figure 5B). To confirm that this response was at least in part non-CB1-mediated, AB-CHMINACA was tested in HEK 293 wild-type cells; in these cells, AB-CHMINACA (10 µmol L^{-1}) also

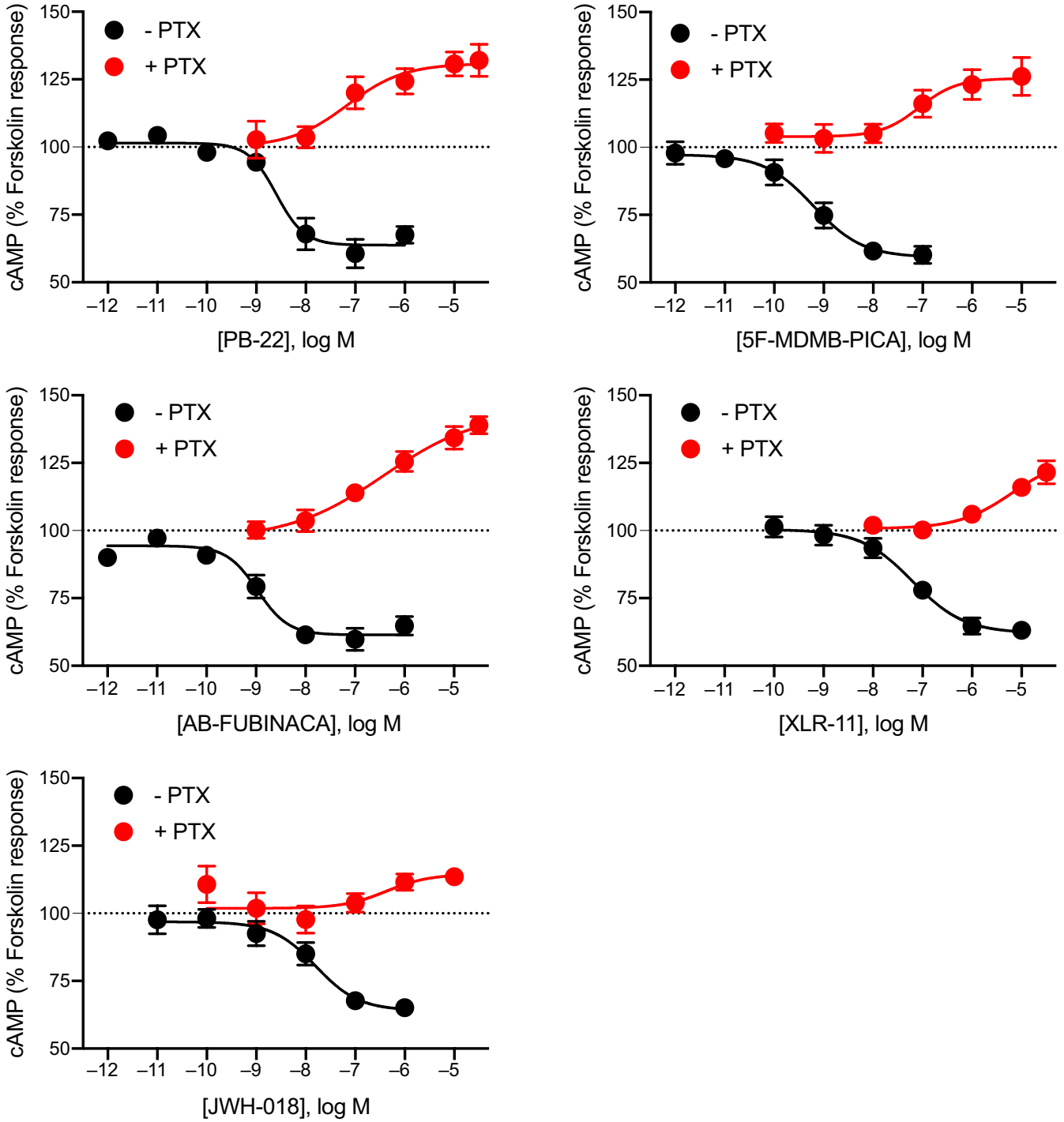


FIGURE 3 Concentration response curves for SCRAs-induced stimulation and inhibition of cAMP signaling. Pooled concentration response relationship for five SCRAs (PB-22, 5F-MDMB-PICA, AB-FUBINACA, XLR-11, and JWH-018) for two signaling outputs of cannabinoid receptor type 1—stimulation and inhibition of cAMP levels following overnight treatment in the absence (−PTX, black), or presence (+PTX, red) of PTX. Data were normalized to forskolin (FSK, 100%) and vehicle (0%), and plotted as mean ± SEM for at least five independent experiments performed in duplicate. For some points, the error bars are shorter than the height of the symbol. BRET, bioluminescence resonance energy transfer; cAMP, cyclic adenosine monophosphate; PTX, pertussis toxin; SCRA, synthetic cannabinoid receptor agonist

produced a small increase in FSK-stimulated cAMP accumulation (Figure 5C, 29 ± 10%), suggesting that some of these stimulatory effects were occurring via mechanism(s) unrelated to CB1 receptor activity.

4 | DISCUSSION

In this study, we set out to systematically characterize the ability of several SCRAs to activate $G\alpha_s$ and $G\alpha_{i/o}$ proteins by examining

TABLE 1 Comparison of pharmacological activity (EC_{50} and E_{max}) of SCRAs-induced stimulation (G_s (+PTX)) and inhibition (G_i (−PTX)) of cAMP signaling in HEK-CB1 cells

Compound	G_i (-PTX)		G_s (+PTX)		
	pEC_{50} (EC_{50}, nmol L^{-1})	E_{max} (% FSK)	pEC_{50} (EC_{50}, nmol L^{-1}	E_{max} (% FSK)	G_i (−PTX) selectivity
CP55940	8.1 ± 0.4 (7)	58 ± 3	—	—	—
WIN55212-2	7.9 ± 0.4 (11)	70 ± 4	—	—	—
JWH-018	7.8 ± 0.2 (16)	64 ± 3	6.5 ± 0.7 (288)	114 ± 4	18
XLR-11	7.2 ± 0.2 (63)	63 ± 2	5.3 ± 0.8 (4720)	124 ± 5	75
PB-22	8.6 ± 0.2 (2.5)	64 ± 3	7.2 ± 0.5 (69)	130 ± 3	28
AB-FUBINACA	9.0 ± 0.2 (0.96)	61 ± 2	6.4 ± 0.5 (278)	144 ± 12	290
5F-MDMB-PICA	9.2 ± 0.2 (0.62)	60 ± 4	7.1 ± 0.4 (85)	126 ± 5	137

Note: The selectivity is expressed as the ratio of G_s (+PTX) EC_{50} to G_i (−PTX) EC_{50}. Pooled data from at least five independent experiments was fit to a three parameter logistic equation in PRISM. Data are presented ± SEM.

Abbreviations: cAMP, cyclic adenosine monophosphate; CB1, cannabinoid receptor type 1; FSK, forskolin; HEK, human embryonic kidney; PTX, pertussis toxin; SCRA, synthetic cannabinoid receptor agonist.

stimulation as well as inhibition of FSK-induced cAMP accumulation in HEK cells stably expressing CB1. Assays of cAMP signaling revealed that the maximum concentration of SCRAs tested (10 µmol L^{-1}), increased cAMP levels 12%-45% above that produced by FSK alone, while THC failed to increase cAMP levels, an observation consistent with the findings of Finlay et al[17] To further investigate the differential response of SCRA-induced activation and inhibition of cAMP production, we constructed the CRCs for SCRAs belonging to different structural classes (JWH-018, PB-22, AB-FUBINACA, XLR-11, and 5F-MDMB-PICA); the rank order of potency of these SCRAs to stimulate $G\alpha_s$-like cAMP signaling pathway was different from their activity in $G\alpha_{i/o}$-pathway (inhibition of cAMP), suggesting that some of these drugs differentially regulate G protein coupling to CB1.

SCRA-mediated inhibition of cAMP has been extensively studied in cell models expressing cannabinoid receptors[21,24] but some studies have also demonstrated the ability of cannabinoids to stimulate $G\alpha_s$-like cAMP signaling downstream of CB1.[14-17] We found that, at a concentration of 10 µmol L^{-1}, three of the fifteen SCRAs tested, AB-FUBINACA, PB-22, and AB-PINACA, activated $G\alpha_s$-like CB1 signaling to more than 30% above the FSK response. In a previous study using AB-CHMINACA, Costain et al[21] showed similar increases in cAMP levels to that seen in this study without the need for FSK or PTX pretreatment. In our cells, none of the cannabinoids tested altered cAMP levels in the absence of FSK (data not shown). Costain et al[21] performed their assays on HEK293T cells transiently transfected with CB1. Transient transfection of CB1 may have led to a higher level of receptor expression than in our cells, and high levels of CB1 receptor expression are sufficient to result in a switch in cAMP signaling from $G\alpha_i$-mediated (inhibitory) to $G\alpha_s$-mediated (stimulatory) nett effect.[17] Costain *et al*[21] also used a GloSensor cAMP assay, wherein cannabinoid was added for 12 minutes prior to the addition of FSK (10 µmol L^{-1}), and luminescence was monitored for 30 minutes.[21] This may have contributed to the differences in the results of the two studies, but it is not immediately obvious why this would be. Finally, the HEK-293 "T" subclone used in the previous study harbors considerable genomic differences to the parental HEK 293 cell line used in this study,[31,32] which may also contribute to altered cAMP responses (via different AC isoforms). However, our data, together with that of Costain et al[21] suggest potentially different receptor/effector coupling pathways in the presence of some SCRAs (AB-FUBINACA, PB-22, and AB-PINACA, AB-CHMINACA) compared to other CB1 ligands.

We further sought to investigate SCRA differential activation of distinctive G protein subsets—inhibition and stimulation of FSK-mediated cAMP signaling. The relative ability of SCRAs to induce inhibition of cAMP production via $G\alpha_{i/o}$ is very similar to that observed in previous studies in assays of membrane potential and [^{35}S]GTPγS binding.[12,25,33,34] The similar E_{max} observed for the SCRA-mediated activation of $G\alpha_{i/o}$-CB1 signaling in this study probably reflects receptor reserve for inhibition of cAMP accumulation in these cells, wherein maximal responses are elicited at less than maximal receptor occupancy because the system maximum is already achieved.[12] SCRA-induced stimulation of cAMP showed significant differences in E_{max} (Table 1), suggesting an absence of receptor reserve for most of the Gs-dependent signaling we observed for the SCRA in these conditions. This may (at least for the drugs with a lower E_{max}) reflect an accurate representation of intrinsic efficacy of the ligands at this pathway.[35] The observed dynamic range of E_{max} for cannabinoids is consistent with CB1 having low coupling efficiency to both $G\alpha_s$ pathway and β-arrestin-2 (as observed previously[32]), compared to

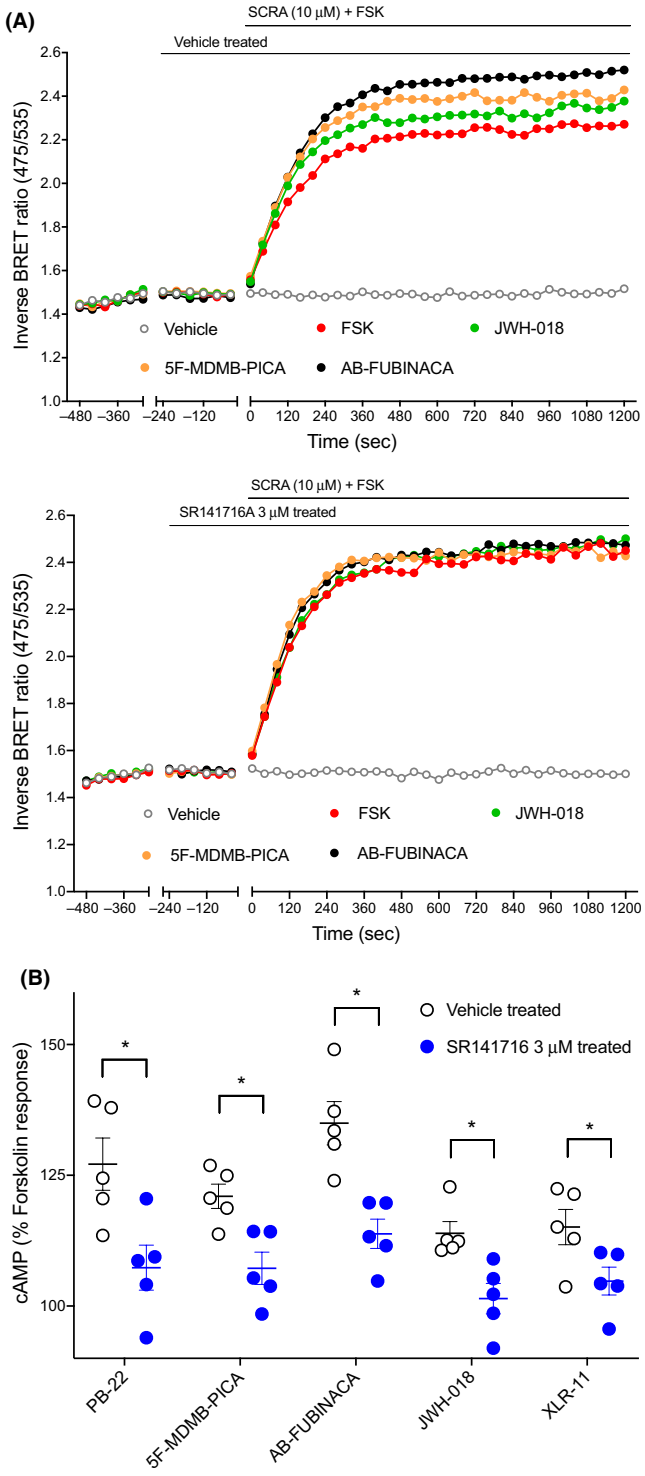


FIGURE 4 Effect of CB1 antagonist on the SCRA-mediated cAMP signaling peaks in HEK-CB1 cells. A, Traces from a representative experiment showing that SCRA (JWH-018, 5F-MDMB-PICA, and AB-FUBINACA) induced observed stimulatory effects were inhibited by SR141716A (CB1 antagonist, 3 μmol L^{-1}) pretreatment. B, Scatter dot plot representing SCRAs-mediated stimulation of forskolin (3 μmol L^{-1})-induced cAMP response in presence and absence of SR141716A 3 μmol L^{-1} on HEK 293 cells expressing CB1. Within each set SCRAs (10 μmol L^{-1}) were compared to SCRAs + SR141716 (unpaired Student's t test, $P < .05$ marked with *). Data were normalized to forskolin (FSK, 100%) and vehicle (0%), and plotted as mean ± SEM for at least five independent experiments performed in duplicate. cAMP, cyclic adenosine monophosphate; CB1, cannabinoid receptor type 1; HEK, human embryonic kidney; SCRA, synthetic cannabinoid receptor agonist

that of $G\alpha_i$ pathway.[17,36,37] Future studies could examine the structure of SCRA-bound CB1-$G\alpha_s$ complexes, which might assist in explaining the observed cAMP signaling profiles. This is particularly interesting given that the interaction of SCRA MDMB-FUBINACA with the "toggle twin switch" in the CB1 binding pocket coupled to $G\alpha_i$ was recently studied.[38] The rigid C-shape geometry of MDMB-FUBINACA along with the strong pi-pi interaction of its indazole ring with "toggle twin switch" residues, might help distinguish the high efficacy agonist activity of SCRA from partial agonists like THC lacking "toggle twin switch" interaction.[38] Promiscuous coupling to both $G\alpha_i$ and $G\alpha_s$ has been reported for multiple GPCRs (eg β_2-adrenergic receptor),[39] while some receptors couple predominantly to one G protein subtype (eg μ-opioid receptor coupling to the $G\alpha_{i/o}$ family[40]). The potential of cannabinoids to differentially activate one signaling cascade over another (functional selectivity[41]) may aid the development of new therapeutic compounds with reduced psychoactive effects; a research domain that has attracted much recent interest.[42]

Considering the adverse effects associated with SCRA use, it is important to continue characterizing the pharmacological profile of these compounds in order to understand the mechanisms driving their toxicity.[43,44] Although this study does not identify which pathway contributes to the toxic effects observed following SCRA consumption, our data do provide valuable insights into SCRA-mediated stimulation and inhibition of cAMP signaling in vitro. Previous studies have shown that JWH-018- AM-2201-, 5F-AB-PINACA-, and CUMYL-4CN-BINACA-induced seizures are CB1-mediated in mice, which might explain some of the toxicity experienced by recreational users of these drugs.[43-49] Our data shows that SCRA-induced cAMP increase was abolished after SR141716A treatment, supporting the hypothesis that SCRAs $G\alpha_s$-like effects were mediated through CB1 receptor. All the SCRAs tested in this study exhibited greater potency at $G\alpha_i$- than $G\alpha_s$-like pathways, and the efficacies of these SCRAs have previously been measured in response to $G\alpha_i$-mediated activation of GIRK channel in AtT20-CB1 cells.[12] The rank order of SCRA efficacy based on selectivity for $G\alpha_i$-GIRK signaling was found to be 5F-MDMB-PICA > XLR-11 > AB-FUBINACA > PB-22 ≈ JWH-018.[12] 5F-MDMB-PICA showed the highest efficacy for modulation of K channel activity via $G\alpha_i$ pathway in the former study, in contrast to the intermediate efficacy of 5F-MDMB-PICA to stimulate the $G\alpha_s$-like cAMP signaling pathway in this study. AB-FUBINACA exhibited greater efficacy for the $G\alpha_s$ pathway compared to its $G\alpha_i$-mediated activity profile in the membrane potential assay.[12] Evaluating the differences in G protein preference between SCRAs may be an important part of understanding the apparent differences in effect between these drugs in humans. However, the biological significance of SCRA-mediated differential coupling of CB1 to $G_{i/o}$ and G_s is not well

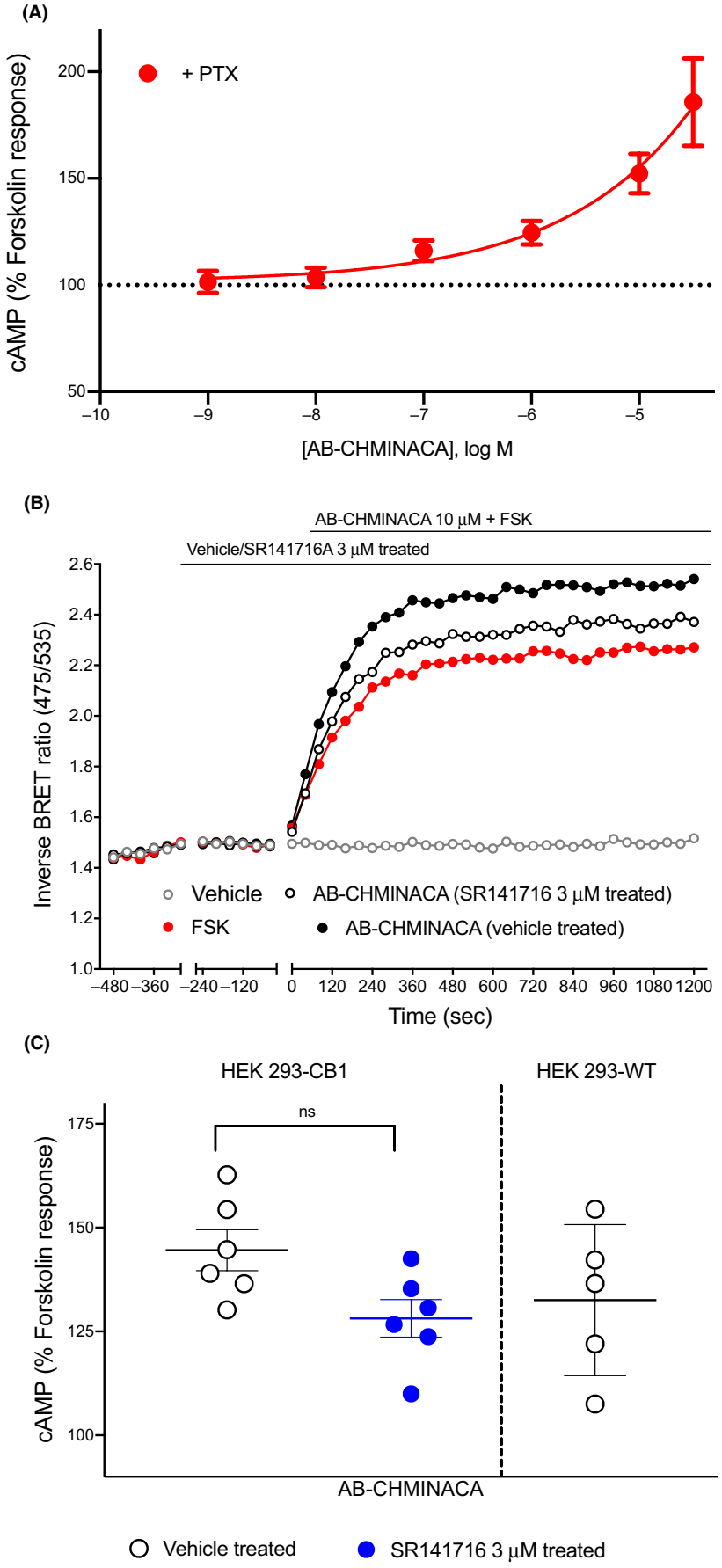


FIGURE 5 AB-CHMINACA does not modulate cAMP levels via CB1 receptors in HEK 293 cells. A, Treatment with AB-CHMINACA produced a concentration-dependent increase in forskolin-mediated cAMP production in HEK 293-CB1 in presence of PTX. B, Traces from a representative experiment showing that AB-CHMINACA (10 μmol L^{-1}) induced observed stimulatory effects were only partially inhibited by SR141716A 3 μmol L^{-1}. C, Scatter dot plot comparing AB-CHMINACA-mediated stimulation of forskolin (3 μmol L^{-1})-induced cAMP response in presence and absence of SR141716 3 μmol L^{-1} in HEK 293-CB1 cells, and the data were not significantly different. AB-CHMINACA (10 μmol L^{-1}) also modestly augmented forskolin-stimulated cAMP levels in HEK-wild-type cells (not containing CB1 receptors). Graphs show mean ± SEM for at least five independent experiments performed in duplicate. cAMP, cyclic adenosine monophosphate; CB1, cannabinoid receptor type 1; HEK, human embryonic kidney; PTX, pertussis toxin

understood. The G_s signaling of CB1 arises in circumstances where $G_{i/o}$ is exhausted or sequestered, and has been measured after PTX treatment or when other G_i-coupled receptors are concomitantly activated. The phenomenon was first observed in primary rat striatal neurons natively expressing CB1 and D2 receptors,[14,15] while a switch in G_i-G_s signaling due to high CB1 expression has subsequently been defined in recombinant systems.[17] The phenomenon of CB1-Gs coupling may be relevant in specific cancer conditions where upregulation in CB1 receptor was reported (eg colorectal cancer, human epithelia ovarian tumors, and prostate cancer).[17]

Our study showed that SCRAs have significantly different pharmacological profiles (maximal activities and potencies) for the activation of CB1-mediated G protein-stimulation and -inhibition of FSK-mediated cAMP signaling. Although it is speculated that the adverse effects of SCRAs are mediated by CB1,[49,50] based on the results presented here we wonder how the differential responses of SCRAs are related to the physiological effects resulting from the activation of each intracellular pathway, and if these may be correlated with the in vivo toxicity of SCRAs. The unique toxicological profile of SCRAs may result from a combination of factors; pharmacokinetic differences, activity at both cannabinoid and noncannabinoid targets, pharmacological activity of metabolites and thermolytic degradants.[25,37,51-53] These findings may provide a starting point to help predict the pharmacological characteristics of SCRAs that demonstrate differential activation of $G\alpha_i$ vs $G\alpha_s$ coupling to CB1.

ACKNOWLEDGEMENTS

This work was supported by NHMRC Project Grant 1107088 awarded to MK, and MC. SS was supported by a Macquarie University Research Excellence Scholarship. The authors thank Dr Natasha Grimsey (Auckland University) for helpful advice on establishing the CAMYEL assay.

DISCLOSURES

The authors declare that they have no conflict of interest related to this work.

AUTHORS' CONTRIBUTIONS

SS designed and performed experiments, analyzed the data and wrote the manuscript. SDB synthesized SCRAs supervised by MK. MS and CB advised on the CAMYEL assay and data analysis. MC provided critical feedback and helped shape the research, analysis, and manuscript. All authors reviewed and edited the manuscript.

ORCID

Shivani Sachdev https://orcid.org/0000-0003-2514-9189
Mark Connor https://orcid.org/0000-0003-2538-2001

REFERENCES

1. Brandt SD, King LA, Evans-Brown M. The new drug phenomenon. *Drug Test Anal*. 2014;6:587-597.
2. Wiley JL, Marusich JA, Lefever TW, et al. AB-CHMINACA, AB-PINACA, and FUBIMINA: affinity and potency of novel synthetic cannabinoids in producing Δ9-tetrahydrocannabinol–like effects in mice. *J Pharmacol Exp Ther*. 2015;354:328-339.
3. Adams AJ, Banister SD, Irizarry L, Trecki J, Schwartz M, Gerona R. "Zombie" outbreak caused by the synthetic cannabinoid AMB-FUBINACA in New York. *N Engl J Med*. 2017;376:235-242.
4. Trecki J, Gerona RR, Schwartz MD. Synthetic cannabinoid-related illnesses and deaths. *N Engl J Med*. 2015;373:103-107.
5. Banister SD, Connor M. The chemistry and pharmacology of synthetic cannabinoid receptor agonists as new psychoactive substances: origins. *Handb Exp Pharmacol*. 2018;252:165-190.
6. Auwärter V, Dresen S, Weinmann W, Müller M, Pütz M, Ferreirós N. "Spice" and other herbal blends: harmless incense or cannabinoid designer drugs? *J Mass Spectrom*. 2009;44:832-837.
7. Thakur GA, Tichkule R, Bajaj S, Makriyannis A. Latest advances in cannabinoid receptor agonists. *Expert Opin Ther Pat*. 2009;19:1647-1673.
8. Aghazadeh Tabrizi M, Baraldi PG, Borea PA, Varani K. Medicinal chemistry, pharmacology, and potential therapeutic benefits of cannabinoid CB2 receptor agonists. *Chem Rev*. 2016;116:519-560.
9. United Nations Office on Drug and Crime. *Global Overview of Drug Demand and Supply*. New York, NY: United Nations; 2019.
10. Alexander SPH, Kelly E, Marrion NV, et al. The concise guide to pharmacology 2017/18: overview. *Br J Pharmacol*. 2017;174:S1-S16.
11. Huestis MA, Gorelick DA, Heishman SJ, et al. Blockade of effects of smoked marijuana by the CB1-selective cannabinoid receptor antagonist SR141716. *Arch Gen Psychiatry*. 2001;58:322-328.
12. Sachdev S, Vemuri K, Banister S, et al. In vitro determination of the CB1 efficacy of illicit synthetic cannabinoids. *Br J Pharmacol*. 2019. https://doi.org/10.1111/bph.14829.
13. Mallipeddi S, Janero DR, Zvonok N, Makriyannis A. Functional selectivity at G-protein coupled receptors: advancing cannabinoid receptors as drug targets. *Biochem Pharmacol*. 2017;128:1-11.
14. Glass M, Felder CC. Concurrent stimulation of cannabinoid CB1 and dopamine D2 receptors augments cAMP accumulation in striatal neurons: evidence for a Gs linkage to the CB1 receptor. *J Neurosci*. 1997;17:5327-5333.
15. Bonhaus D, Chang L, Kwan J, Martin G. Dual activation and inhibition of adenylyl cyclase by cannabinoid receptor agonists: evidence for agonist-specific trafficking of intracellular responses. *J Pharmacol Exp Ther*. 1998;287:884-888.
16. Scotter E, Goodfellow C, Graham E, Dragunow M, Glass M. Neuroprotective potential of CB1 receptor agonists in an in vitro model of Huntington's disease. *Br J Pharmacol*. 2010;160:747-761.
17. Finlay DB, Cawston EE, Grimsey NL, et al. Gαs signalling of the CB1 receptor and the influence of receptor number. *Br J Pharmacol*. 2017;174:2545-2562.
18. Laprairie RB, Bagher AM, Kelly ME, Dupré DJ, Denovan-Wright EM. Type 1 cannabinoid receptor ligands display functional selectivity in a cell culture model of striatal medium spiny projection neurons. *J Biol Chem*. 2014;289:24845-24862.
19. Lauckner JE, Hille B, Mackie K. The cannabinoid agonist WIN55, 212–2 increases intracellular calcium via CB1 receptor coupling to Gq/11 G proteins. *Proc Natl Acad Sci U S A*. 2005;102:19144-19149.
20. Mukhopadhyay S, Howlett AC. Chemically distinct ligands promote differential CB1 cannabinoid receptor-Gi protein interactions. *Mol Pharmacol*. 2005;67:2016-2024.
21. Costain WJ, Rasquinha I, Comas T, et al. Analysis of the pharmacological properties of JWH-122 isomers and THJ-2201, RCS-4 and AB-CHMINACA in HEK293T cells and hippocampal neurons. *Eur J Pharmacol*. 2018;823:96-104.
22. Finlay DB, Manning JJ, Ibsen MS, et al. Do toxic synthetic cannabinoid receptor agonists have signature in vitro activity profiles? A case study of AMB-FUBINACA. *ACS Chem Neurosci*. 2019;10:4350-4360.

23. Schoeder CT, Hess C, Madea B, Meiler J, Müller CE. Pharmacological evaluation of new constituents of "Spice": synthetic cannabinoids based on indole, indazole, benzimidazole and carbazole scaffolds. *Forensic Toxicol*. 2018;36:385-403.
24. Hess C, Schoeder CT, Pillaiyar T, Madea B, Müller CE. Pharmacological evaluation of synthetic cannabinoids identified as constituents of spice. *Forensic Toxicol*. 2016;34:329-343.
25. Wiley JL, Lefever TW, Marusich JA, et al. Evaluation of first generation synthetic cannabinoids on binding at non-cannabinoid receptors and in a battery of in vivo assays in mice. *Neuropharmacology*. 2016;110:143-153.
26. Knapman A, Abogadie F, McIntrye P, Connor M. A real-time, fluorescence-based assay for measuring μ-opioid receptor modulation of adenylyl cyclase activity in Chinese hamster ovary cells. *J Biomol Screen*. 2014;19:223-231.
27. Jiang LI, Collins J, Davis R, et al. Use of a cAMP BRET sensor to characterize a novel regulation of cAMP by the sphingosine 1-phosphate/G13 pathway. *J Biol Chem*. 2007;282:10576-10584.
28. Sachdev S, Boyd R, Grimsey NL, Santiago M, Connor M. Brodifacoum does not modulate human cannabinoid receptor-mediated hyperpolarization of AtT20 cells or inhibition of adenylyl cyclase in HEK 293 cells. *PeerJ*. 2019;7:e7733.
29. Cawston EE, Redmond WJ, Breen CM, Grimsey NL, Connor M, Glass M. Real-time characterization of cannabinoid receptor 1 (CB 1) allosteric modulators reveals novel mechanism of action. *Br J Pharmacol*. 2013;170:893-907.
30. Rinaldi-Carmona M, Barth F, Héaulme M, et al. SR141716A, a potent and selective antagonist of the brain cannabinoid receptor. *FEBS Lett*. 1994;350:240-244.
31. Lin Y-C, Boone M, Meuris L, et al. Genome dynamics of the human embryonic kidney 293 lineage in response to cell biology manipulations. *Nat Comm*. 2014;5:4767.
32. Ibsen MS, Finlay DB, Patel M, Javitch JA, Glass M, Grimsey NL. Cannabinoid CB1 and CB2 receptor-mediated arrestin translocation: species, subtype, and agonist-dependence. *Front Pharmacol*. 2019;10:350.
33. Banister SD, Longworth M, Kevin R, et al. Pharmacology of valinate and tert-leucinate synthetic cannabinoids 5F-AMBICA, 5F-AMB, 5F-ADB, AMB-FUBINACA, MDMB-FUBINACA, MDMB-CHMICA, and their analogues. *ACS Chem Neurosci*. 2016;7:1241-1254.
34. Gamage TF, Farquhar CE, Lefever TW, et al. Molecular and behavioral pharmacological characterization of abused synthetic cannabinoids MMB-and MDMB-FUBINACA, MN-18, NNEI, CUMYL-PICA, and 5-Fluoro-CUMYL-PICA. *J Pharmacol Exp Ther*. 2018;365:437-446.
35. Kenakin T. New concepts in pharmacological efficacy at 7 TM receptors: IUPHAR R eview 2. *Br J Pharmacol*. 2013;168:554-575.
36. Wouters E, Walraed J, Banister SD, Stove CP. Insights into biased signaling at cannabinoid receptors: synthetic cannabinoid receptor agonists. *Biochem Pharmacol*. 2019;169:113623.
37. Cannaert A, Storme J, Franz F, Auwärter V, Stove CP. Detection and activity profiling of synthetic cannabinoids and their metabolites with a newly developed bioassay. *Anal Chem*. 2016;88:11476-11485.
38. Krishna Kumar K, Shalev-Benami M, Robertson MJ, et al. Structure of a signaling cannabinoid receptor 1-G protein complex. *Cell*. 2019;176:448-458.
39. Westfield GH, Rasmussen SG, Su M, et al. Structural flexibility of the Gαs α-helical domain in the β2-adrenoceptor Gs complex. *Proc Natl Acad Sci U S A*. 2011;108:16086-16091.
40. Connor M, Christie MJ. Opioid receptor signalling mechanisms. *Clin Exp Pharmacol Physiol*. 1999;26:493-499.
41. Kenakin T. Functional selectivity and biased receptor signaling. *J Pharmacol Exp Ther*. 2011;336:296-302.
42. Wouters E, Walraed J, Robertson MJ, et al. Assessment of biased agonism amongst distinct synthetic cannabinoid receptor agonist scaffolds. *ACS Pharmacol Transl Sci*. 2019. https://doi.org/10.1021/acsptsci.9b00069.
43. Drummer OH, Gerostamoulos D, Woodford NW. Cannabis as a cause of death: a review. *Forensic Sci Int*. 2019;298:298-306.
44. Labay LM, Caruso JL, Gilson TP, et al. Synthetic cannabinoid drug use as a cause or contributory cause of death. *Forensic Sci Int*. 2016;260:31-39.
45. Funada M, Takebayashi-Ohsawa M. Synthetic cannabinoid AM2201 induces seizures: involvement of cannabinoid CB1 receptors and glutamatergic transmission. *Toxicol Appl Pharmacol*. 2018;338:1-8.
46. Malyshevskaya O, Aritake K, Kaushik MK, et al. Natural (Δ 9-THC) and synthetic (JWH-018) cannabinoids induce seizures by acting through the cannabinoid CB 1 receptor. *Sci Rep*. 2017;7:10516.
47. Vigolo A, Ossato A, Trapella C, et al. Novel halogenated derivates of JWH-018: behavioral and binding studies in mice. *Neuropharmacology*. 2015;95:68-82.
48. Wilson CD, Tai S, Ewing L, et al. Convulsant effects of abused synthetic cannabinoids jwh-018 and 5f-ab-pinaca are mediated by agonist actions at cb1 receptors in mice. *J Pharmacol Exp Ther*. 2019;368:146-156.
49. Kevin RC, Anderson L, McGregor IS, et al. CUMYL-4CN-BINACA is an efficacious and potent pro-convulsant synthetic cannabinoid receptor agonist. *Front Pharmacol*. 2019;10. https://doi.org/10.3389/fphar.2019.00595.
50. Wallace MJ, Wiley JL, Martin BR, DeLorenzo RJ. Assessment of the role of CB1 receptors in cannabinoid anticonvulsant effects. *Eur J Pharmacol*. 2001;428:51-57.
51. Schindler CW, Gramling BR, Justinova Z, Thorndike EB, Baumann MH. Synthetic cannabinoids found in "spice" products alter body temperature and cardiovascular parameters in conscious male rats. *Drug Alch Dep*. 2017;179:387-394.
52. Presley BC, Logan BK, Jansen-Varnum SA. In-vitro phase i metabolism of indazole carboxamide synthetic cannabinoid MDMB-CHMINACA via human liver microsome incubation and high-resolution mass spectrometry. *Drug Test Anal*. 2019;2019:1942-7603.
53. Thomas BF, Lefever TW, Cortes RA, et al. Thermolytic degradation of synthetic cannabinoids: chemical exposures and pharmacological consequences. *J Pharmacol Exp Ther*. 2017;361:162-171.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

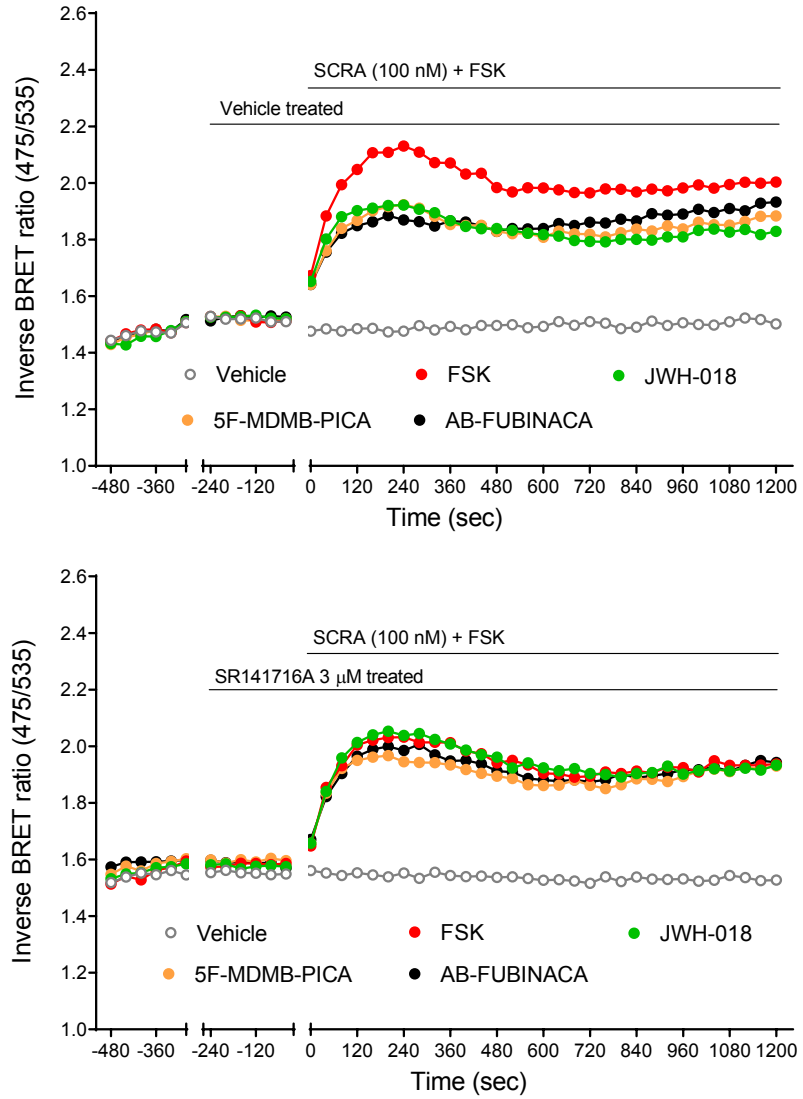
How to cite this article: Sachdev S, Banister SD, Santiago M, Bladen C, Kassiou M, Connor M. Differential activation of G protein-mediated signaling by synthetic cannabinoid receptor agonists. *Pharmacol Res Perspect*. 2020;00:e00566. https://doi.org/10.1002/prp2.566

Supplementary Data

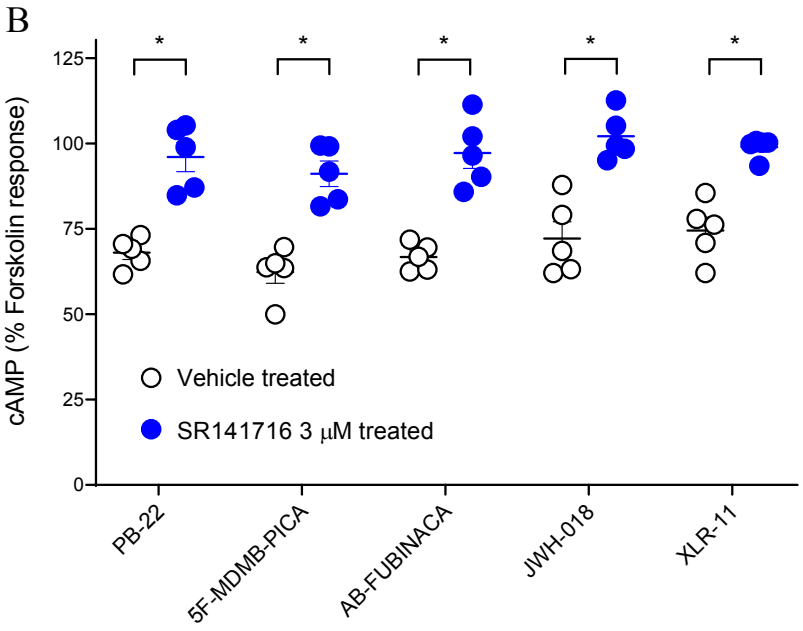
Supplementary Figure 3-1

Effect of CB1 antagonist on the SCRA induced inhibition of cAMP signalling. A. Traces from a representative experiment showing that SCRA (JWH-018, 5F-MDMB-PICA, and AB-FUBINACA) induced inhibitory effects were completely blocked by SR141716A (CB1 antagonist, 3 μM) pre-treatment. **B**. Scatter dot plot representing SCRAs-mediated inhibition of forskolin-induced cAMP response in presence and absence of SR141716A 3 μM on HEK 293 cells expressing CB1. Within each set SCRAs (100 nM) were compared to SCRAs + SR141716. Data were normalized to forskolin (100%) and vehicle (0%), and plotted as mean ± SEM for at least 5 independent experiments performed in duplicate.

A


SCRA (100 nM) + FSK
Vehicle treated
Inverse BRET ratio (475/535)
Vehicle
FSK
JWH-018
5F-MDMB-PICA
AB-FUBINACA
Time (sec)
SCRA (100 nM) + FSK
SR141716A 3 μM treated
Inverse BRET ratio (475/535)
Vehicle
FSK
JWH-018
5F-MDMB-PICA
AB-FUBINACA
Time (sec)

B


cAMP (% Forskolin response)
Vehicle treated
SR141716 3 μM treated
PB-22
5F-MDMB-PICA
AB-FUBINACA
JWH-018
XLR-11

Supplementary Table 3-1.

Comparison of pharmacological activity (EC_{50} and E_{MAX}) of SCRAs-induced stimulation (G_s (+PTX)) and inhibition (G_i (-PTX)) of cAMP signalling in HEK-CB1 cells. Data was fit to a 4-parameter logistic equation in PRISM. The selectivity is expressed as the ratio of G_s (+PTX) EC_{50} to G_i (-PTX) EC_{50}. Data is presented mean ± S.E.M.

Compound	G_i (-PTX)		G_s (+PTX)		*Gi* (-PTX) selectivity
	pEC_{50} (EC_{50}, nM)	E_{max} (% FSK) (nH)	pEC_{50} (EC_{50}, nM)	E_{max} (% FSK) (nH)	
CP55940	8.2 ± 0.3 (6.4)	57 ± 5 *-0.7 ± 0.3*	-	-	-
WIN55212-2	7.4 ± 2 (40)	60 ± 9 *-0.4 ± 0.5*	-	-	-
JWH-018	7.8 ± 0.3 (16)	64 ± 4 *-0.9 ± 0.5*	6.7 ± 0.7 (221)	113 ± 4 *1.4 ± 2*	14
XLR-11	7.2 ± 0.2 (63)	62 ± 3 *-0.8± 0.3*	5.2 ± 0.8 (6490)	127 ± 16 *0.8 ± 0.6*	103
PB-22	8.6 ± 0.2 (2.5)	64 ± 3 *-1.5 ± 0.6*	7.2 ± 0.5 (69)	131 ± 5 *0.8± 0.7*	28
AB-FUBINACA	9.0 ± 0.2 (1.1)	61 ± 2 *-1.4 ± 1*	6.4 ±0.5 (383)	144 ± 12 *0.5 ± 0.3*	348
5F-MDMB-PICA	9.2 ± 0.2 (0.62)	59 ± 4 *-0.9 ± 0.4*	7.1 ± 0.4 (85)	126 ± 5 *1.1 ± 1.5*	137

Chapter IV.

Study III. Brodifacoum does not modulate human cannabinoid receptor-mediated hyperpolarization of AtT20 cells or inhibition of adenylyl cyclase in HEK 293 cells

In the U.S., a disturbing trend towards using SCRAs mixed with Brodifacoum (superwarfarin) began to appear in consumers of these illicit drugs. In molecular pharmacology, the concept of allosteric modulation may help explain how some drugs can modify the receptor activity toward the agonist in a myriad of different ways from increasing affinity and/or efficacy to decreasing it. This chapter investigated whether brodifacoum, an anticoagulant associated with a recent outbreak of SCRA poisoning, is likely to affect the agonist responses through allosteric modulation of cannabinoid receptors.

Contributions to the work

This paper represents a collaborative work hosted in Mark Connor's laboratory at Macquarie University Australia. I took the lead role in experimental design, conducting the experiments, data analysis and writing the paper with support from my co-investigators: Mark Connor oversaw the work in this research group; Rochelle Boyd performed some of the preliminary experiments exploring the activity of brodifacoum on cannabinoid receptors using hyperpolarisation (GIRK) assay; Marina Santiago and Natasha Grimsey provided invaluable input into the design and analyses of the cAMP experiments. All authors reviewed the final manuscript.

Brodifacoum does not modulate human cannabinoid receptor-mediated hyperpolarization of AtT20 cells or inhibition of adenylyl cyclase in HEK 293 cells

Shivani Sachdev[1,*], Rochelle Boyd[1,2,*], Natasha L. Grimsey[3], Marina Santiago[1] and Mark Connor[1]

[1] Department of Biomedical Sciences, Faculty of Medicine and Health Sciences, Macquarie University, Sydney, New South Wales, Australia

[2] Cancer Research Unit, Children's Medical Research Institute, Sydney, NSW, Australia

[3] Department of Pharmacology and Clinical Pharmacology, and Centre for Brain Research School of Medical Sciences, Faculty of Medical and Health Sciences, University of Auckland, Auckland, New Zealand

[*] These authors contributed equally to this work.

ABSTRACT

Background. Synthetic cannabinoids are a commonly used class of recreational drugs that can have significant adverse effects. There have been sporadic reports of co-consumption of illicit drugs with rodenticides such as warfarin and brodifacoum (BFC) over the past 20 years but recently, hundreds of people have been reported to have been poisoned with a mixture of synthetic cannabinoids and BFC. We have sought to establish whether BFC directly affects cannabinoid receptors, or their activation by the synthetic cannabinoid CP55940 or the phytocannabinoid Δ^9-tetrahydrocannabinol (Δ^9-THC).

Methods. The effects of BFC on the hyperpolarization of wild type AtT20 cells, or AtT20 cells stably expressing human CB_1- or CB_2- receptors, were studied using a fluorescent assay of membrane potential. The effect of BFC on CB_1- and CB_2-mediated inhibition of forskolin-stimulated adenylyl cyclase (AC) activation was measured using a BRET assay of cAMP levels in HEK 293 cells stably expressing human CB_1 or CB_2.

Results. BFC did not activate CB_1 or CB_2 receptors, or affect the hyperpolarization of wild type AtT20 cells produced by somatostatin. BFC (1 μM) did not affect the hyperpolarization of AtT20-CB_1 or AtT20-CB_2 cells produced by CP55940 or Δ^9-THC. BFC (1 μM) did not affect the inhibition of forskolin-stimulated AC activity by CP55940 in HEK 293 cells expressing CB_1 or CB_2. BFC (1 μM) also failed to affect the desensitization of CB_1 and CB_2 signaling produced by prolonged (30 min) application of CP55940 or Δ^9-THC to AtT20 cells.

Discussion. BFC is not a cannabinoid receptor agonist, and appeared not to affect cannabinoid receptor activation. Our data suggests there is no pharmacodynamic rationale for mixing BFC with synthetic cannabinoids; however, it does not speak to whether BFC may affect synthetic cannabinoid metabolism or biodistribution. The reasons underlying the mixing of BFC with synthetic cannabinoids are unknown, and it remains to be established whether the "contamination" was deliberate or accidental. However, the consequences for people who ingested the mixture were often serious,

Submitted 27 March 2019
Accepted 23 August 2019
Published 25 September 2019

Corresponding author
Mark Connor,
mark.connor@mq.edu.au

Academic editor
Jörg Oehlmann

Additional Information and Declarations can be found on page 11

DOI **10.7717/peerj.7733**

How to cite this article Sachdev S, Boyd R, Grimsey NL, Santiago M, Connor M. 2019. Brodifacoum does not modulate human cannabinoid receptor-mediated hyperpolarization of AtT20 cells or inhibition of adenylyl cyclase in HEK 293 cells. *PeerJ* **7**:e7733
http://doi.org/10.7717/peerj.7733

and sometimes fatal, but this seems unlikely to be due to BFC action at cannabinoid receptors.

Subjects Biochemistry, Cell Biology, Toxicology, Pharmacology
Keywords Synthetic cannabinoid, Superwarfarin, Overdose, Cannabinoid receptor signaling

INTRODUCTION

Brodifacoum (BFC) is an inhibitor of vitamin K epoxide reductase and active ingredient of rodenticides (*King & Tran, 2015*). There have been sporadic reports of brodifacoum consumption with drugs such as cocaine and cannabis (*La Rosa, Clarke & Lefkowitz, 1997*; *Waien, Hayes Jr & Leonardo, 2001*; *Spahr, Maul & Rodgers, 2007*), however, a large number of people were recently hospitalized with poisoning by brodifacoum and related compounds following ingestion of what are believed to be synthetic cannabinoid receptor agonists (SCRAs) (*Kelkar et al., 2018*; *Riley et al., 2019*; *Moritz et al., 2018*; *Panigrahi, Jones & Rowe, 2018*). There is limited evidence to suggest that people have on occasions deliberately combined brodifacoum with cannabis (*La Rosa, Clarke & Lefkowitz, 1997*; *Spahr, Maul & Rodgers, 2007*), and the apparent mixing of brodifacoum with a variety of different SCRA could be a deliberate attempt to enhance the effects of the drugs through either a pharmacokinetic or pharmacodynamic mechanism. In this study, we have examined the effects of brodifacoum on the acute signalling of human CB_1 and CB_2 receptors in AtT20 and HEK 293 cells. In AtT20 cells, activation of heterologously expressed CB_1 or CB_2 produces a hyperpolarization, mediated by activation of G protein-gated inwardly rectifying K channels (*Mackie et al., 1995*; *Banister et al., 2016*). In CB1- or CB2-expressing HEK 293 cells, we measured the real time modulation of forskolin-stimulated cAMP accumulation (*Cawston et al., 2013*). We found that cannabinoid-induced signaling was not affected by brodifacoum, indicating that combining SCRA with brodifacoum is not likely to enhance user experience through interactions with cannabinoid receptors.

METHODS

Drugs

(-) CP 55940 was from Cayman Chemical (#90084; Ann Arbor MI, USA), Δ^9-tetrahydrocannabinol (THC) was from THCPharm (Frankfurt, Germany) and was a kind gift from the Lambert Initiative for Cannabis Therapeutics (University of Sydney). Brodifacoum was from Sigma-Aldrich (#46036), and forskolin was from Ascent Scientific Ltd.

Hyperpolarization assay

Experiments on AtT20FlpIn cells stably transfected with human CB_1 (AtT20-CB_1) or CB_2 (AtT20-CB_2) were carried out essentially as described in *Banister et al. (2016)*. The AtT20FlpIn cells were created in our laboratory from wild type AtT20 cells we purchased from the American Type Culture Collection (ATCC CRL-1795). The assay

method is based on that outlined in detail in *Knapman et al. (2013)*. Cells were grown in DMEM (#D6429; Sigma-Aldrich, Castle Hill, NSW) supplemented with 10% fetal bovine serum (FBS, #12003C; SAFC Biosciences, Brooklyn, Victoria, Australia), 100 units penicillin/100 μg ml^{-1} streptomycin (1%, #15140122; Life Technologies, Scoresby, Victoria, Australia), hygromycin gold (80 μg ml^{-1}, #ant-hg; Invivogen, San Diego, CA). Cells were grown in 75 cm^2 flasks and passaged when 80–90% confluent. On the evening before experiments, cells were detached using trypsin/EDTA solution (#T3924; Sigma-Aldrich), resuspended in L-15 media (#11415064; Life Technologies) supplemented with 1% FBS, penicillin/streptomycin, and glucose (15 mM, SIGMA #G7021) and plated onto 96 well black walled, clear bottomed, culture plates which had been previously coated with poly-D-lysine (SIGMA #P6407). Cells were incubated overnight at 37 °C in a humidified incubator in room air.

Proprietary FLIPR membrane potential dye (blue, #R8034, Molecular Devices, Sunnyvale CA) was dissolved in Hank's Balanced Salt Solution (HBSS) of composition (mM) NaCl 145, HEPES 22, Na_2HPO_4 0.338, $NaHCO_3$ 4.17, KH_2PO_4 0.441, $MgSO_4$ 0.407, $MgCl_2$ 0.493, $CaCl_2$ 1.26, glucose 5.56 (pH 7.4, osmolarity 315 $\pm$ 15) and added to the cells an hour before fluorescence reading began. Dye was used at 50% of the manufacturers recommended concentration, and cells were incubated at 37 °C in humidified room air for loading. Plates were read using a Flexstation 3 (Molecular Devices) plate reader at 37 °C. Plates were excited at a wavelength of 530 nm, emission was measured at 565 nm, with cut-off filter at 550 nm. Drugs were added using the pipetting function of the Flexstation in a volume of 20 μl after recording 60–120 s of baseline fluorescence. Readings were made every 2 s. Drug stocks were made up in DMSO (#D8418, Sigma-Aldrich) and diluted on the day of experiment, the final concentration of DMSO in the assay was 0.1%.

Data were expressed as the percentage change in baseline fluorescence produced by drug addition. The change in fluorescence produced by vehicle (0.1% DMSO) addition was subtracted from the traces before this calculation. Data is expressed as the mean $\pm$ SEM of at least 5 independent determinations performed in duplicate, unless otherwise noted. Pooled data was fit to a four-parameter logistic equation in Graphpad PRISM 7 (GraphPad Software, San Diego CA, USA).

Assay of cAMP levels

Human embryonic kidney (HEK) 293 FlpIn cells stably transfected with human CB_1 or CB_2 receptors tagged with three haemagglutinin epitopes at the amino terminus and human G protein gated inwardly rectifying potassium channel 4 (GIRK4) were used (the construction of these cells will be described in another place, and we did not assay CB receptor coupling to GIRK4 in this study). Cells were grown in DMEM containing 10% FBS and 100 units/ml/penicillin, 100 μg/ml streptomycin and were maintained under selection with hygromycin (80 μg ml^{-1}) and G418 (400 μg ml^{-1}). HEK 293 FlpIn cells were originally obtained from Life Technologies (now Thermofisher, #75007).

Cellular cAMP levels were measured using the pcDNA3L-His-CAMYEL plasmid, which encodes the cAMP sensor YFP-Epac-RLuc (CAMYEL), (*Cawston et al., 2013*). The pcDNA3L-His-CAMYEL was a kind gift from Dr. Angela Finch (The University of

New South Wales, NSW, Australia), and originally obtained from American Type Culture Collection (Manassas, VI, USA). Cells were seeded in 10 cm dishes at a density of 6,000,0000 such that they would be 60–70% confluent the next day. The day after seeding, pcDNA3L-His-CAMYEL plasmid was transiently transfected into cells using linear polyethyleneimine (PEI, m.w. 25 kDa) (#23966, Polysciences, Warrington, PA, USA). The DNA-PEI complex mixture was added to the cells at the ratio of 1:6, and incubated for 24 h in 5% CO_2 at 37 °C. After the incubation, cells were detached from the dish using trypsin/EDTA and the pellet was resuspended in 10 ml Leibovitz's L-15, no phenol red (#21083027; Gibco) media supplemented with 1% FBS, 100 units/ml/penicillin, 100 μg/ml streptomycin and 15 mM glucose. The cells were seeded at a density of 100,000 cells per well in poly D-lysine (Sigma-Aldrich) coated, white wall, clear bottom 96 well microplates. Cells were incubated overnight at 37 °C in ambient CO_2.

On the following day, drugs were prepared in HBSS containing 0.1 mg ml^{-1} BSA. For measurement of cAMP inhibition, all the drugs were made in 3 μM of forskolin. Coelenterazine-h substrate (2.5 μM) (#S2011; Promega, Madison, WI, USA) was added to the cells, and incubated for 5 mins prior to the addition of drugs or vehicle. Luminescence was measured using a Flexstation 3 (Molecular Devices) microplate reader at 37 °C at an emission wavelength of 461 nm and 542 nm simultaneously, with an integration time of 1 s. Drugs were added in a volume of 10 μl (10×) to each well to give the desired concentration. The final concentration of DMSO in each well was always 0.1%. Raw data are presented as inverse BRET ratio of emission at 461/542. Background reading (no substrate) was subtracted from raw values before calculating ratios. For convenience, values are expressed such that an increase in ratio correlates with increase in cAMP production. Area under the curve (AUC) analysis was performed in GraphPad prism (Graph Pad Software Inc., San Diego, CA, USA), and data were expressed as percentage of the difference between basal (vehicle, 0%) and forskolin (100%) values over a 5-minute period after forskolin addition.

For experiments examining the potential interaction between brodifacoum and cannabinoids, the cells were pre-treated with 1 μM of brodifacoum (or vehicle) and the response to a subsequent addition of SCRAs was measured. The concentration of DMSO (0.1%) was kept constant for the brodifacoum-treated and control cells. Data was normally distributed (D'Agostino and Pearson normality test, PRISM), differences between groups were tested using unpaired Student's *t*-Test (PRISM). Statistical significance was defined as $P < 0.05$.

RESULTS

Acute application of brodifacoum for 5 min at concentrations up to 30 μM did not significantly affect the fluorescence of AtT20 cells expressing CB_1 or CB_2 receptors (Fig. 1). Prolonged exposure to brodifacoum at concentrations greater than 10 μM produced decreases in fluorescence in AtT20 cells expressing CB receptors as well as wild type cells, and so for experiments examining the potential interaction between brodifacoum and cannabinoids we used a concentration of 1 μM.

We generated concentration–response curves for the high efficacy cannabinoid agonist CP55940 and the lower efficacy agonist THC after 5 min of exposure to brodifacoum

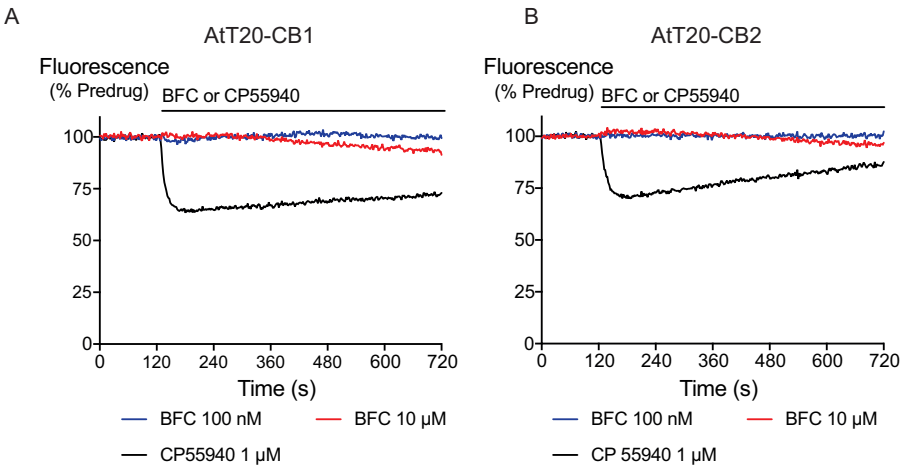


Figure 1 The effects of brodifacoum (BFC) and CP55940 in AtT20 cell expressing CB_1 or CB_2. Representative traces showing the change in fluorescence induced by application of CP55940 (1 μM) but not BFC (10 μM) in (A) AtT20-CB_1 and (B) AtT20-CB_2 cells. Values are expressed as a percentage of predrug baseline. A reduction in fluorescence indicates a hyperpolarization. The prolonged application of BFC (10 μM) produces small changes in the fluorescence in AtT20 cells expressing cannabinoid receptors. Drug was added for the duration of the bar; the traces are representative of at least five independent experiments.

Full-size DOI: 10.7717/peerj.7733/fig-1

(Fig. 2). In AtT20-CB_1 cells, application of CP55940 produced a maximum change in fluorescence of 33 ± 1%, with a pEC_{50} of 7.7 ± 0.04; with the addition of brodifacoum the maximum change in fluorescence was 33 ± 1%, with a pEC50 of 7.7 ± 0.06 ($P = 0.97$). In AtT20-CB_2 cells, application of CP55940 produced a maximum change in fluorescence of 29 ± 1.1%, with a pEC_{50} of 7.3 ± 0.1; with the addition of brodifacoum the maximum change in fluorescence was 31 ± 1.2%, with a pEC_{50} of 7.4 ± 0.1 (Fig. 2, $P = 0.85$). Brodifacoum failed to affect the hyperpolarization produced by THC in AtT20-CB_1 cells (control, pEC50 6.4 ± 0.6, maximum change in fluorescence 18 ± 5%; in brodifacoum, pEC_{50} 6.5 ± 0.5, max 18 ± 5%, $P = 0.95$). In AtT20-CB_2 cells THC only produced a small hyperpolarization, the response to 10 μM THC was unchanged in the presence of brodifacoum (6.4 ± 1.2% in control, 7.4 ± 1.8% in brodifacoum, $P = 0.65$) (Fig. 2). Application of brodifacoum (10 μM) or CP55940 (1 μM) for 5 min produced very small changes in the fluorescence of wild type AtT20 cells, and neither drug affected the response to subsequently applied somatostatin (100 nM), which activates native SST receptors in AtT20 cells (*Günther, Culler & Schulz, 2016*) (Fig. S1).

Inhibition of adenylyl cyclase activity is another significant biological effect of cannabinoid receptor activation. Brodifacoum (300 nM–30 μM) co-applied with forskolin (3 μM) for 10 min did not affect increases in cAMP levels in HEK 293 cells expressing CB_1 or CB_2 (Fig. 3). Brodifacoum (1 μM) incubation for 5 min also failed to affect the CP55940 inhibition of forskolin-stimulated cAMP elevation. In cells expressing CB_1, CP55940 inhibited cAMP with a pEC_{50} of 7.5 ± 0.3, to a minimum of 52 ± 12% of forskolin alone; in the presence of brodifacoum these were pEC_{50} 7.4 ± 0.2 and minimum of 52 ± 7% of the forskolin response. Brodifacoum also did not affect forskolin-stimulated cAMP levels

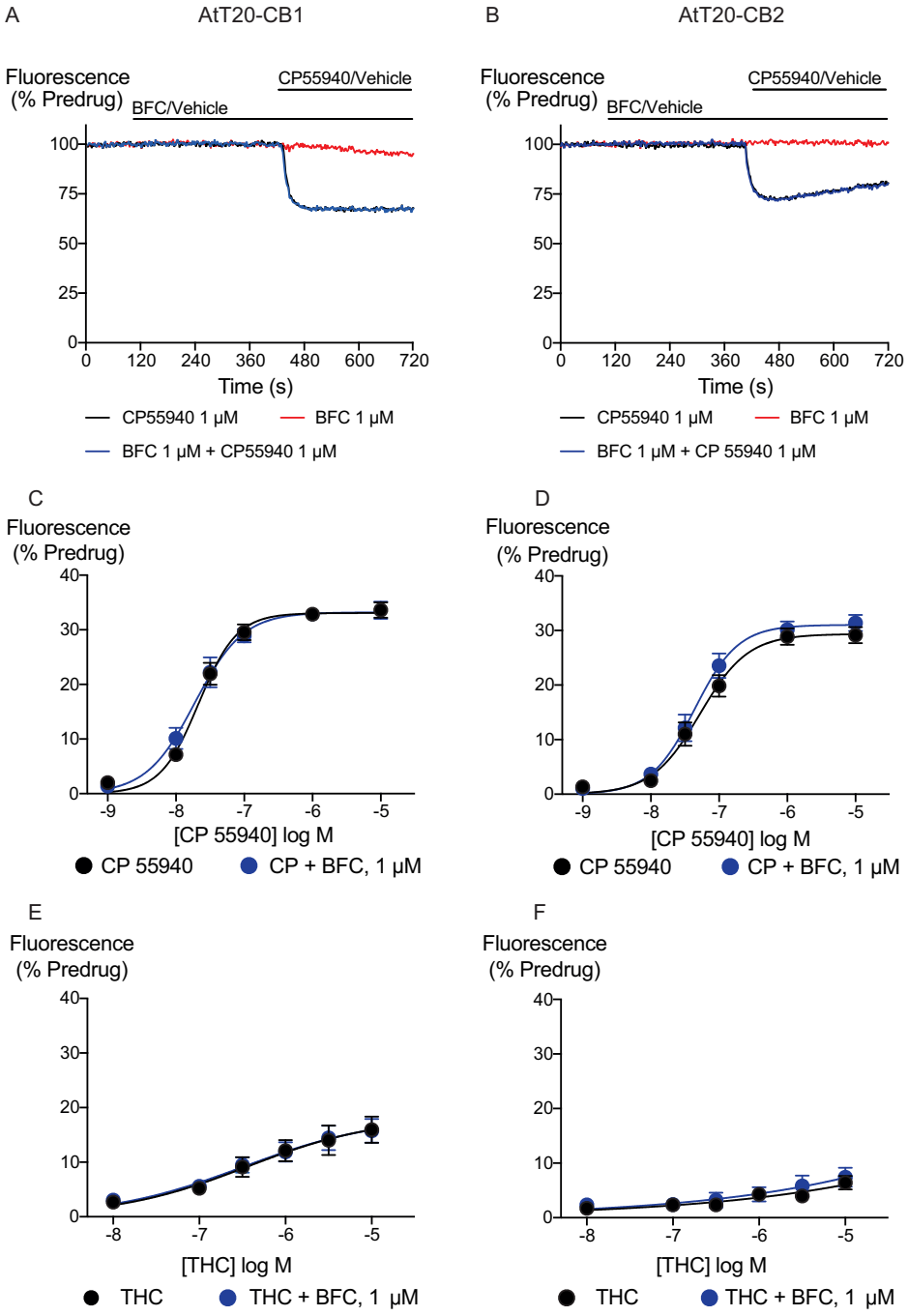


Figure 2 Brodifacoum (BFC) effect on CP55940 and Δ^9-THC induced hyperpolarization of AtT20 cell expressing CB_1 or CB_2. Representative traces showing the change in fluorescence for CP55940 on (A) AtT20-CB_1, and (B) AtT20-CB_2 in the presence of BFC 1 μM or vehicle. Values are expressed as a percentage of predrug baseline. A reduction in fluorescence indicates a hyperpolarization. Drugs were added for the duration of the bar; the traces are representative of at least five independent experiments. Concentration response curve of hyperpolarization of AtT20-CB_1 or AtT20-CB_2 cells stimulated with (C), (D) CP55940 or (E), (F) Δ^9-THC in the continued presence of either HBSS or BFC. Data represents the mean ± SEM of five independent experiments performed in duplicate. There was no difference in the potency or maximal effect of CP55940 and Δ^9-THC between HBSS or in presence of BFC.

Full-size DOI: 10.7717/peerj.7733/fig-2

in HEK293 cells expressing CB_2 (Fig. 3), or CP55940 inhibition of cAMP levels (pEC_{50} in control cells expressing CB_2 7.4 ± 0.2, to a minimum of 39 ± 7%; in brodifacoum pEC_{50} of 7.5 ± 0.1; to a minimum of 45 ± 4%).

We also examined the possibility that brodifacoum could affect the sustained responses to CP55940 or THC. As previously described (*Cawston et al., 2013*), prolonged application of cannabinoids in AtT20-CB_1 cells produces a response that wanes over time, reflecting desensitization of receptor signaling. The degree to which this desensitization reflects changes in signaling specific to cannabinoid receptors is tested by application of somatostatin, which activates receptors native to AtT20 cells (*Günther, Culler & Schulz, 2016*; *Heblinski, Bladen & Connor, 2019*). In these experiments, CP55940 (100 nM) or THC (10 μM) were applied 2 min after addition of brodifacoum (1 μM), and the fluorescence monitored for 30 min before the addition of somatostatin (100 nM) (Fig. 4). Desensitization was quantified after 30 min of agonist application, and was expressed as the % decline from the peak response. We did not observe any significant difference in the desensitization of CB_1 signaling mediated by CP55940 (100 nM) when co-applied with brodifacoum (Control, 71 ± 4%; brodifacoum treated, 66 ± 7%, $P = 0.55$). The presence of brodifacoum had no effect on the somatostatin (100 nM) induced hyperpolarization alone, or after 30 mins of CP55940 treatment ($P = 0.75$) (Fig. S2). The desensitization produced by THC (10 μM, 30 mins) in AtT20-CB_1 cells was not different when co-applied with brodifacoum, (Control, 65 ± 6%; brodifacoum treated, 53 ± 8%, $P = 0.3$) (Fig. 4). A similar reversal of the hyperpolarization produced by CP55940 (100 nM) in AtT20-CB_2 cells was also observed. Treatment with brodifacoum did not significantly affect the desensitization produced by CP55940 compared to control cells (Control, 77 ± 6%; brodifacoum treated, 63 ± 8%, $P = 0.2$). THC (10 μM, 30 mins) signaling at CB_2, although modest, also declined during continuous drug exposure, and this was also not affected by co-application of brodifacoum (37 ± 14% in control, 20 ± 7% in brodifacoum treated, $P = 0.3$) (Fig. 4). The hyperpolarization induced by somatostatin after prolonged application of CP55940 ($P = 0.56$) or THC ($P = 0.87$) to AtT20-CB_2 cells was also not significantly different in the presence of brodifacoum (Fig. S2).

DISCUSSION

The principal finding of this study is that brodifacoum does not affect CB_1 or CB_2 signaling, either to K channels in AtT20 cells or adenylyl cyclase in HEK 293 cells. In the assay of K channel activation, there was no effect on the concentration response relationship for CP55940 or THC, and brodifacoum did not affect the desensitization of signaling produced by prolonged application of either drug. Brodifacoum had no effect on the potency, maximum effect or time-dependence of the actions of the high efficacy synthetic cannabinoid CP55940 or the lower efficacy phytocannabinoid THC, indicating that it is unlikely to act as modulator of the pharmacodynamic effects of cannabinoids.

Activation of GIRK is mediated by the $G\beta\gamma$ subunits of G protein heterotrimers, and many Gi/Go coupled receptors effectively signal through this pathway in AtT20 cells (e.g., *Mackie et al., 1995*; *Günther, Culler & Schulz, 2016*; *Knapman et al., 2013*; *Heblinski, Bladen*

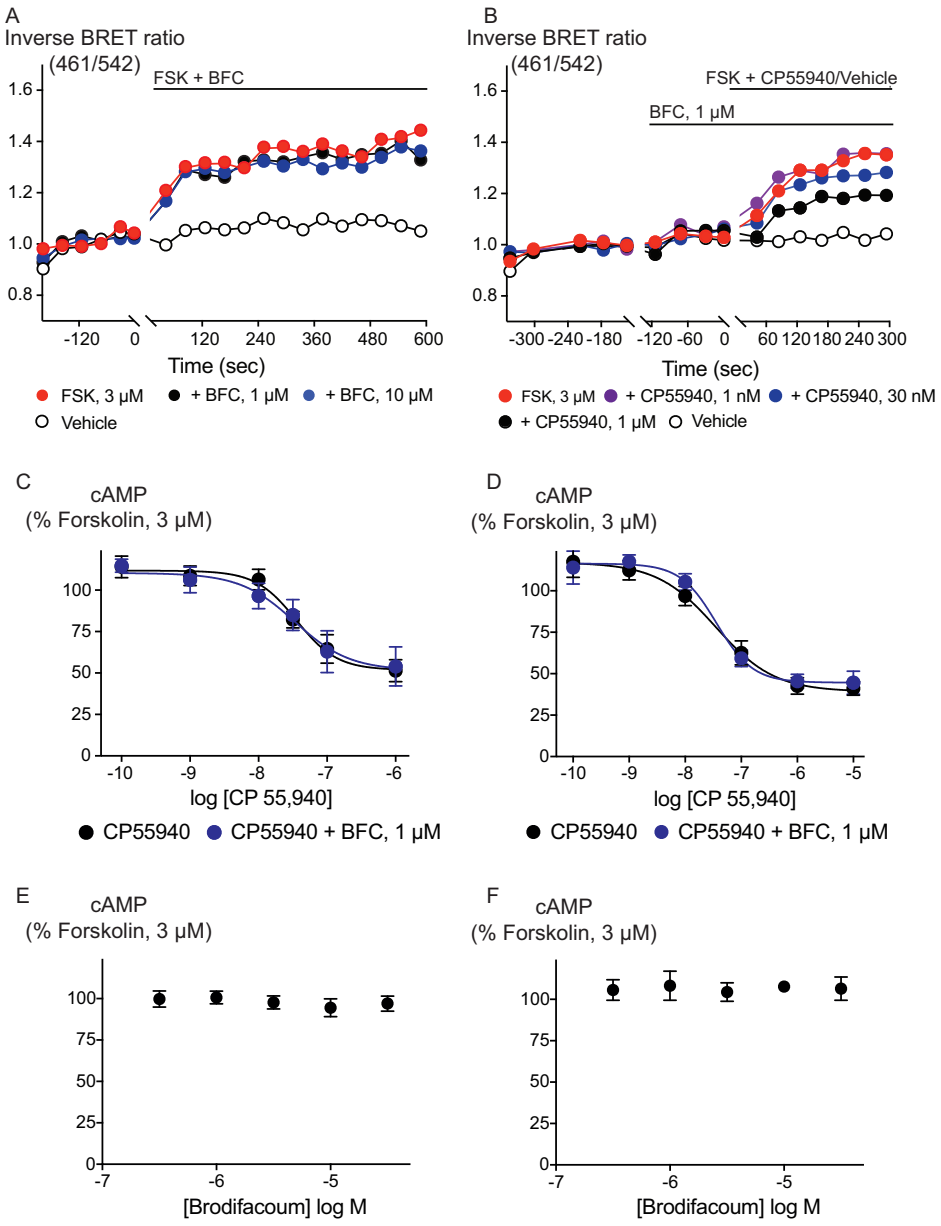


Figure 3 Brodifacoum (BFC) does not modulate cAMP accumulation via CB_1 or CB_2 receptors expressed in HEK 293 cells. Representative data from the CAMYEL assay for HEK 293 cells expressing CB1 receptors, an increase in inverse BRET ratio (emission at 461/542 nm) corresponds to an increase in cAMP. (A) BFC does not affect the rapid increase in cAMP production produced by forskolin (3 μM); (B) BFC (1 μM) does not affect responses to forskolin (3 μM) applied in the presence of CP55940. Data are representative of at least five independent experiments. Concentration response curve showing CP55940 induced inhibition of forskolin-stimulated cAMP elevation in presence and absence of BFC 1 μM on HEK 293 cells expressing (C) CB_1 or (D) CB_2. Data are expressed as a percentage of response produced by forskolin (3 μM), and plotted as mean ± SEM of five independent determinants performed in duplicate. Concentration response curve showing the effect of BFC on forskolin (3 μM)-stimulated cAMP elevations in HEK 293 cells expressing (E) CB_1 or (F) CB_2. Data are expressed as a percentage of response produced by forskolin (3 μM), and plotted as mean ± SEM of five independent experiments performed in duplicate.

Full-size DOI: 10.7717/peerj.7733/fig-3

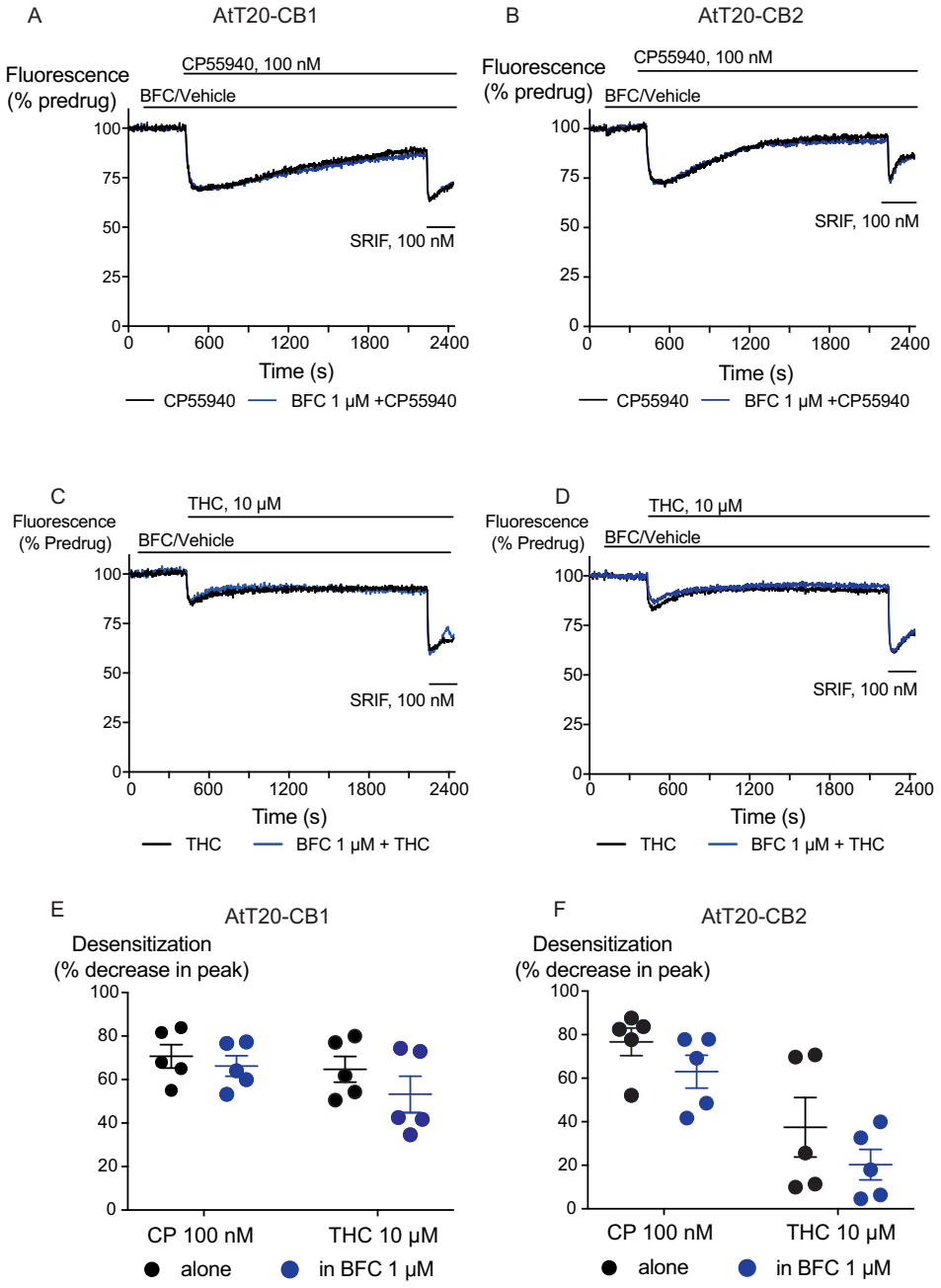


Figure 4 The effect of brodifacoum (BFC) on CP55940 and Δ^9-THC mediated desensitization of signalling in AtT20-CB_1 and -CB_2. Representative traces showing desensitization of signalling in AtT20-CB_1 and AtT20-CB_2 on prolonged stimulation with (A), (C) CP55940 (100 nM) or (B), (D) Δ^9-THC (10 μM) in the presence of BFC 1 μM or vehicle. Cells were challenged with somatostatin (100 nM) after 30 minutes of CP55940 or Δ^9-THC. Drugs were added for the duration of the bar; the traces are representative of at least five independent experiments. Scatter dot plot representing desensitization of (E) CB_1 and (F) CB_2 on exposure to CP55940 or Δ^9-THC for 30 mins in the presence of BFC 1 μM or vehicle. This plot shows percentage desensitization comparing peak fluorescence after the addition of drugs and 30 mins post addition. Data represents the mean ± SEM of five independent experiments performed in duplicate.

Full-size DOI: 10.7717/peerj.7733/fig-4

& Connor, 2019). We have previously used the fluorescent measurement of membrane potential to study CB_1 and CB_2 agonists, antagonists, and allosteric modulators of CB_1 (*Cawston et al., 2013*). Inhibition of adenylyl cyclase activity by CB receptors is mediated via the Gα subunits of G protein heterotrimers, and brodifacoum also failed to affect this signal transduction pathway. The precise cellular signaling mechanisms responsible for the subjective effects of *Cannabis* and synthetic cannabinoid agonists are not established, although the signal transduction of cannabinoid receptors has been extensively studied (*Howlett & Abood, 2017*; *Ibsen, Connor & Glass, 2017*) and it is unlikely that any one pathway is responsible. It remains formally possible that brodifacoum could selectively modulate pathways other than G$\beta\gamma$-mediated activation of GIRK or Gα-mediated inhibition of cAMP accumulation, but the lack of any effect whatsoever on the effects of CP55940 or THC suggests that ligand interactions with cannabinoid receptors are unaffected by brodifacoum.

The concentration of brodifacoum in blood or brain after co-ingestion with synthetic cannabinoids is unknown. However, concentrations of up to 3 μM have been reported in the serum of people who have deliberately ingested large quantities of rat poison (*Weitzel et al., 1990*; *Hollinger & Pastoor, 1993*), and inhalation of BFC via smoked synthetic cannabinoids may produce higher serum concentrations of BFC than ingestion. Brodifacoum at 1 μM failed to affect CB_1 or CB_2 receptor signaling when measured continuously over a period of 30 min, and 10 μM brodifacoum failed to mimic or affect the acute response to a maximally effective concentration of CP 55940, although at this concentration prolonged application of brodifacoum produced a decrease in the fluorescence of wild type AtT20 cells, as well as those expressing CB_1 and CB_2 receptors. This effect at higher concentrations may reflect direct interactions of brodifacoum with cell membranes (*Marangoni et al., 2016*). Concentrations of brodifacoum in the upper range of what we tested are achieved only after ingestion of large amounts of rat bait, it is possible that they could be achieved while ingesting contaminated synthetic cannabinoids, but this remains unreported.

Several case reports suggest an interaction between therapeutic warfarin and cannabis or cannabidiol (*Grayson et al., 2018*; *Yamreudeewong et al., 2009*; *Damkier et al., 2019*). It has been suggested that cannabinoid inhibition of enzymes responsible for the metabolism of warfarin can increase blood levels of the drug, and while these studies have focussed on potentially dangerous changes in warfarin concentration, levels of cannabinoids could also be reciprocally elevated. Such interactions may inform a decision to deliberately combine "superwarfarin" with SCRA, as has been previously suggested for cannabis (*La Rosa, Clarke & Lefkowitz, 1997*; *Spahr, Maul & Rodgers, 2007*), although whether brodifacoum is metabolized by pathways shared with SCRA in humans is unknown. Information about how or even whether BFC is metabolized in humans is very sparse, although available evidence suggests metabolism is very limited or absent (*Hauck, Feinstein & Van Breeman, 2016*). Apart from the obvious danger of ingesting brodifacoum, altering the metabolism of SCRA is likely to have unpredictable consequences, as some metabolites of SCRA retain cannabinoid receptor activity (e.g., *Brents et al., 2011*; *Chimalakonda et al., 2012*; *Longworth et al., 2017*; *Cannaert et al., 2016*), and may contribute to the overall SCRA experience.

Ingestion of brodifacoum is relatively common, while death from exposure is rare, owing to ready treatment with vitamin K (*King & Tran, 2015*; *Gummin et al., 2018*). The high number of deaths associated with the combination of SCRA and anticoagulants in 2018 (at least eight; *Connors, 2018*) may point to an interaction between the drugs. It may also reflect the identity and dose of the synthetic cannabinoid(s) consumed, as well as the general health status of the drug users. Deaths from synthetic cannabinoid exposure are uncommon, but well documented (e.g., *Kasper et al., 2015*; *Trecki, Gerona & Schwartz, 2015*). While there is a general acceptance that brodifacoum or a similar agent is responsible for the coagulopathies associated with synthetic cannabinoid ingestion, identification of the synthetic cannabinoid has not been reported in most cases, but a recent report identified a metabolite of AB-FUBINACA in one patient following ingestion of "King Kong", a brodifacoum laced SCRA (*Riley et al., 2019*). It seems unlikely, though, that brodifacoum would interact with higher efficacy or potency SCRAs at cannabinoid receptors when it clearly does not interact with CP55940 or THC signaling (*Noble et al., 2019*; *Sachdev et al., in press*). Intriguingly, several groups have reported cannabinoid receptor ligands based on a coumarin scaffold (*Behrenswerth et al., 2009*; *Han et al., 2015*). While these drugs have been reported to be either antagonists/inverse agonists (*Behrenswerth et al., 2009*) or CB_2-selective agonists (*Han et al., 2015*), they remain largely uncharacterized. Given the propensity of chemists producing and, in some cases, designing cannabinoids for the recreational market, it cannot be ruled out that coagulopathy may be an unanticipated adverse effect of a synthetic cannabinoid, which may have arisen from a novel, coumarin-based cannabinoid that retains some of the vitamin K epoxide reductase inhibitory of warfarin and brodifacoum.

In conclusion, we report that brodifacoum does not appear to be an agonist or antagonist of human cannabinoid receptors, and it also does not appear to be an allosteric modulator of CB_1 or CB_2 activation of K channels or inhibition of adenylyl cyclase. Why brodifacoum has been mixed with synthetic cannabinoid receptor agonists remains a matter for speculation, although an intended effect on synthetic cannabinoid drug pharmacokinetics cannot entirely be ruled out.

ACKNOWLEDGEMENTS

We thank Dr Sam Banister for his insight, especially around the possibility of poisoning with coumarin-derived synthetic cannabinoids. The authors declare they have no conflicts of interest with regard to this work.

ADDITIONAL INFORMATION AND DECLARATIONS

Funding

This work was supported by the National Health and Medical Research Council of Australia (APP1107088). Shivani Sachdev is supported by an International Research Excellence Scholarship from Macquarie University. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Grant Disclosures

The following grant information was disclosed by the authors:
National Health and Medical Research Council of Australia: APP1107088.
International Research Excellence Scholarship from Macquarie University.

Competing Interests

Mark Connor is an Academic Editor for PeerJ. None of the authors have any other competing interests.

Author Contributions

- Shivani Sachdev conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.
- Rochelle Boyd conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, approved the final draft.
- Natasha L. Grimsey conceived and designed the experiments, contributed reagents/materials/analysis tools, authored or reviewed drafts of the paper, approved the final draft.
- Marina Santiago conceived and designed the experiments, analyzed the data, contributed reagents/materials/analysis tools, approved the final draft.
- Mark Connor conceived and designed the experiments, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.

Data Availability

The following information was supplied regarding data availability:

The data used to generate the concentration-response curves and dot plots are available as Supplemental File.

Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.7733#supplemental-information.

REFERENCES

Banister SD, Longworth M, Kevin R, Sachdev S, Santiago M, Stuart J, Mack JB, Glass M, McGregor IS, Connor M, Kassiou M. 2016. Pharmacology of valinate and tert-leucinate synthetic cannabinoids 5F-AMBICA, 5F-AMB, 5F-ADB, AMB-FUBINACA, MDMB-FUBINACA, MDMB-CHMICA, and their analogues. *ACS Chemical Neuroscience* **7**:1241–1254 DOI 10.1021/acschemneuro.6b00137.

Behrenswerth A, Volz N, Toräng J, Hinz S, Bräse S, Müller CE. 2009. Synthesis and pharmacological evaluation of coumarin derivatives as cannabinoid receptor antagonists and inverse agonists. *Bioorganic & Medicinal Chemistry* **17**:2842–2851 DOI 10.1016/j.bmc.2009.02.027.

Brents LK, Reichard EE, Zimmerman SM, Moran JH, Fantegrossi WE, Prather PL. 2011. Phase I hydroxylated metabolites of the K2 synthetic cannabinoid JWH-018 retain in vitro and in vivo cannabinoid 1 receptor affinity and activity. *PLOS ONE* **6**:e21917 DOI 10.1371/journal.pone.0021917.

Cannaert A, Storme J, Franz F, Auwärter V, Stove CP. 2016. Detection and activity profiling of synthetic cannabinoids and their metabolites with a newly developed bioassay. *Analytical Chemistry* **88**:11476–11485 DOI 10.1021/acs.analchem.6b02600.

Cawston EE, Redmond WJ, Breen CM, Grimsey NL, Connor M, Glass M. 2013. Real-time characterization of cannabinoid receptor 1 (CB 1) allosteric modulators reveals novel mechanism of action. *British Journal of Pharmacology* **170**:893–907 DOI 10.1111/bph.12329.

Chimalakonda KC, Seely KA, Bratton SM, Brents LK, Moran CL, Endres GW, James LP, Hollenberg PF, Prather PL, Radominska-Pandya A, Moran JH. 2012. Cytochrome P450-mediated oxidative metabolism of abused synthetic cannabinoids found in K2/Spice: identification of novel cannabinoid receptor ligands. *Drug Metabolism and Disposition* **40**:2174–2184 DOI 10.1124/dmd.112.047530.

Connors JM. 2018. Hemorrhagic highs from synthetic cannabinoids—a new epidemic. *New England Journal of Medicine* **379**:1275–1277 DOI 10.1056/NEJMe1810418.

Damkier P, Lassen D, Christensen MMH, Madsen KG, Hellfritzsch M, Pottegård A. 2019. Interaction between warfarin and cannabis. *Basic & Clinical Pharmacology & Toxicology* **124**:28–31 DOI 10.1111/bcpt.13152.

Grayson L, Vines B, Nichol K, Szaflarski JP. 2018. An interaction between warfarin and cannabidiol, a case report. *Epilepsy & Behavior Case Reports* **9**:10–11 DOI 10.1016/j.ebcr.2017.10.001.

Gummin DD, Mowry JB, Spyker DA, Brooks DE, Osterthaler KM, Banner W. 2018. 2017 annual report of the American association of poison control centers' National Poison Data System (NPDS): 35th annual report. *Clinical Toxicology* **56**:1213–1415 DOI 10.1080/15563650.2018.1533727.

Günther T, Culler M, Schulz S. 2016. Research resource: real-time analysis of somatostatin and dopamine receptor signaling in pituitary cells using a fluorescence-based membrane potential assay. *Molecular Endocrinology* **30**:479–490 DOI 10.1210/me.2015-1241.

Han S, Zhang F-F, Qian H-Y, Chen L-L, Pu J-B, Xie X, Chen JZ. 2015. Design, syntheses, structure–activity relationships and docking studies of coumarin derivatives as novel selective ligands for the CB2 receptor. *European Journal of Medicinal Chemistry* **93**:16–32 DOI 10.1016/j.ejmech.2015.01.054.

Hauck ZZ, Feinstein DL, Van Breeman RB. 2016. LC-MS-MS analysis of brodifacoum isomers in rat tissue. *Journal of Analytical Toxicology* **40**:304–309 DOI 10.1093/jat/bkw008.

Heblinski M, Bladen C, Connor M. 2019. Regulation of heterologously expressed 5-HT1B receptors coupling to potassium channels in AtT-20 cells. *British Journal of Pharmacology* **176**:451–465 DOI 10.1111/bph.14547.

Hollinger BR, Pastoor TP. 1993. Case management and plasma half-life in a case of brodifacoum poisoning. *Archives of Internal Medicine* **153**:1925–1928 DOI 10.1001/archinte.1993.00410160099010.

Howlett AC, Abood ME. 2017. CB1 and CB2 receptor pharmacology. *Advances in Pharmacology* **80**:169–206 DOI 10.1016/bs.apha.2017.03.007.

Ibsen MS, Connor M, Glass M. 2017. Cannabinoid CB1 and CB2 receptor signaling and bias. *Cannabis and Cannabinoid Research* **2**:48–60 DOI 10.1089/can.2016.0037.

Kasper AM, Ridpath AD, Arnold JK, Chatham-Stephens K, Morrison M, Olayinka O, Parker C, Galli R, Cox R, Preacely N, Anderson J, Kyle PB, Gerona R, Martin C, Schier J, Wolkin A, Dobbs T. 2015. Notes from the field: Severe illness associated with reported use of synthetic cannabinoids—Mississippi, 2015. *Morbidity and Mortality Weekly Report* **64**:1121–1122 DOI 10.15585/mmwr.mm6439a7.

Kelkar AH, Smith NA, Martial A, Moole H, Tarantino MD, Roberts JC. 2018. An outbreak of synthetic cannabinoid–associated coagulopathy in Illinois. *New England Journal of Medicine* **379**:1216–1223 DOI 10.1056/NEJMoa1807652.

King N, Tran M-H. 2015. Long-acting anticoagulant rodenticide (superwarfarin) poisoning: a review of its historical development, epidemiology, and clinical management. *Transfusion Medicine Reviews* **29**:250–258 DOI 10.1016/j.tmrv.2015.06.002.

Knapman A, Santiago M, Du YP, Bennallack PR, Christie MJ, Connor M. 2013. A continuous, fluorescence-based assay of μ—opioid receptor activation in AtT-20 cells. *Journal of Biomolecular Screening* **18**:269–276 DOI 10.1177/1087057112461376.

La Rosa FG, Clarke SH, Lefkowitz JB. 1997. Brodifacoum intoxication with marijuana smoking. *Archives of Pathology and Laboratory Medicine* **121**:67–69.

Longworth M, Connor M, Banister SD, Kassiou M. 2017. Synthesis and pharmacological profiling of the metabolites of synthetic cannabinoid drugs APICA, STS-135, ADB-PINACA, and 5F-ADB-PINACA. *ACS Chemical Neuroscience* **8**:1673–1680 DOI 10.1021/acschemneuro.7b00116.

Mackie K, Lai Y, Westenbroek R, Mitchell R. 1995. Cannabinoids activate an inwardly rectifying potassium conductance and inhibit Q-type calcium currents in AtT20 cells transfected with rat brain cannabinoid receptor. *Journal of Neuroscience* **15**:6552–6561 DOI 10.1523/JNEUROSCI.15-10-06552.1995.

Marangoni MN, Martynowycz MW, Kuzmenko I, Braun D, Polak PE, Weinberg G, Rubinstein I, Gidalevitz D, Feinstein DL. 2016. Membrane cholesterol modulates superwarfarin toxicity. *Biophysical Journal* **110**:1777–1788 DOI 10.1016/j.bpj.2016.03.004.

Moritz E, Austin C, Wahl M, DesLauriers C, Navon L, Walblay K, Hendrickson M, Phillips A, Kerins J, Pennington AF, Lavery AM, El Zahran T, Kauerauf J, Yip L, Thomas J, Layden J. 2018. Notes from the field: outbreak of severe illness linked to the vitamin K antagonist brodifacoum and use of synthetic cannabinoids—Illinois, March–2018. *Morbidity and Mortality Weekly Report* **67**:607–608 DOI 10.15585/mmwr.mm6721a4.

Noble C, Cannaert A, Linnet K, Stove CP. 2019. Application of an activity-based receptor bioassay to investigate the in vitro activity of selected indole- and indazole-3-carboxamide-based synthetic cannabinoids at CB1 and CB2 receptors. *Drug Testing and Analysis* **11**:501–511 DOI 10.1002/dta.2517.

Panigrahi B, Jones BC, Rowe SP. 2018. Brodifacoum-contaminated synthetic marijuana: clinical and radiologic manifestations of a public health outbreak causing life-threatening coagulopathy. *Emergency Radiology* **25**:715–718 DOI 10.1007/s10140-018-1628-5.

Riley SB, Sochat M, Moser K, Lynch KL, Tochtrop R, Isbell TS, Scalzo A. 2019. Case of brodifacoum-contaminated synthetic cannabinoid. *Clinical Toxicology* **57**:143–144 DOI 10.1080/15563650.2018.1502444.

Sachdev S, Vemuri K, Banister S, Longworth M, Kassiou M, Santiago MJ, Makriyannis A, Connor M. 2019. In vitro determination of the CB1 efficacy of illicit synthetic cannabinoids. *British Journal of Pharmaoclogy* In Press DOI 10.1111/bph.14829.

Spahr JE, Maul JS, Rodgers GM. 2007. Superwarfarin poisoning: a report of two cases and review of the literature. *American Journal of Hematology* **82**:656–660 DOI 10.1002/ajh.20784.

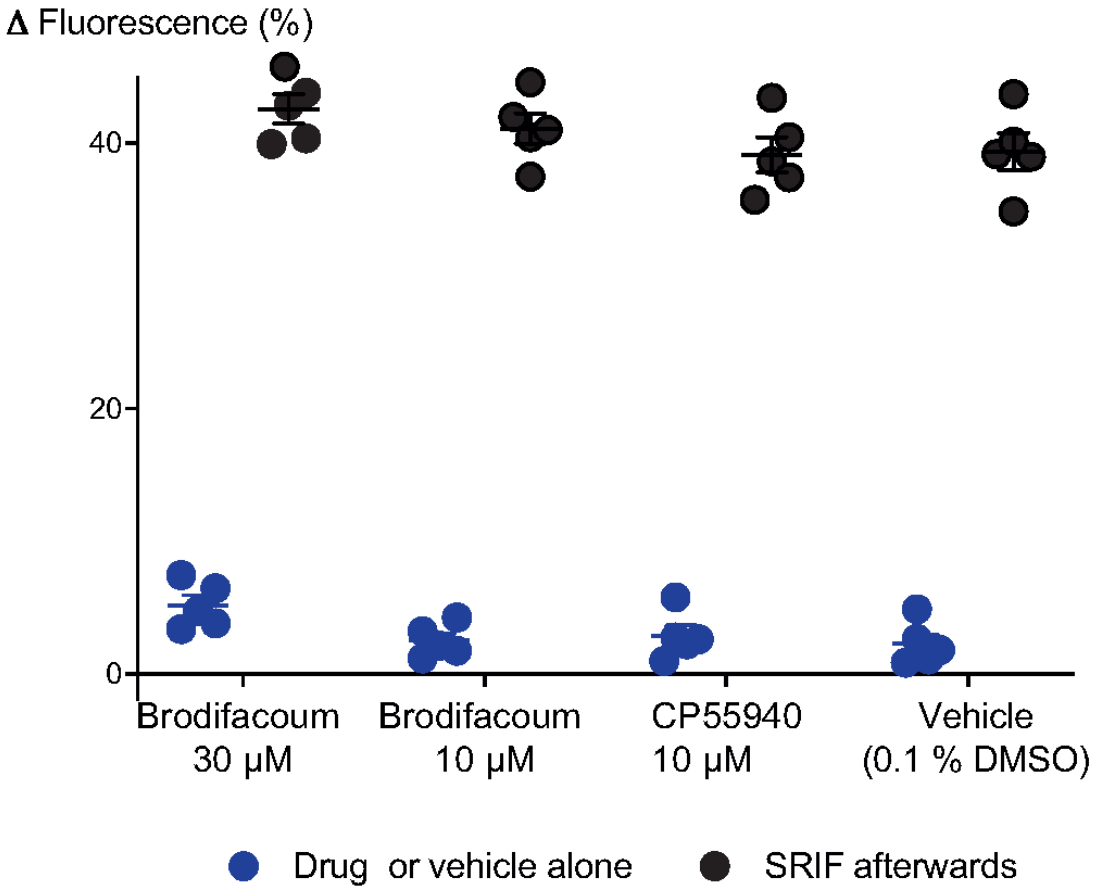
Trecki J, Gerona RR, Schwartz MD. 2015. Synthetic cannabinoid-related illnesses and deaths. *New England Journal of Medicine* **373**:103–107 DOI 10.1056/NEJMp1505328.

Waien SA, Hayes Jr D, Leonardo JM. 2001. Severe coagulopathy as a consequence of smoking crack cocaine laced with rodenticide. *New England Journal of Medicine* **345**:700–701.

Weitzel JN, Sadowski JA, Furie BC, Moroose R, Kim H, Mount ME, Murphy MJ, Furie B. 1990. Surreptitious ingestion of a long-acting vitamin K antagonist/rodenticide, brodifacoum: clinical and metabolic studies of three cases. *Blood* **76**:2555–2559.

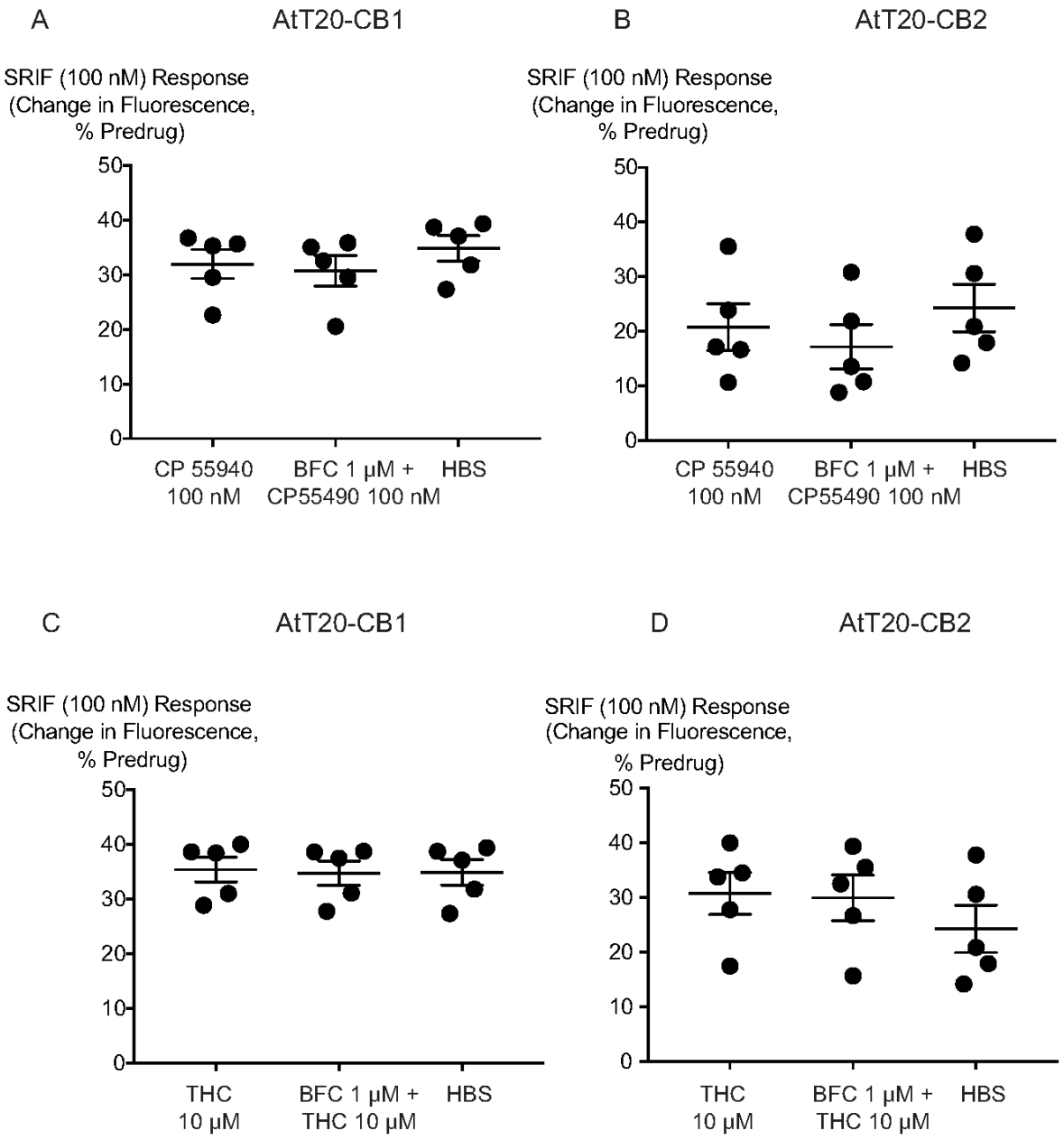
Yamreudeewong W, Wong HK, Brausch LM, Pulley KR. 2009. Probable interaction between warfarin and marijuana smoking. *Annals of Pharmacotherapy* **43**:1347–1353 DOI 10.1345/aph.1M064.

4.1. Supplementary Data



Supplementary Figure 4-1 Effects of brodifacoum (BFC) and CP55940 in wild type AtT20 cells

Scatter dot plot representing the percentage change in fluorescence for BFC (30 µM), BFC (10 µM), CP55940 (10 µM), and Vehicle (0.1% DMSO) alone (blue dots), and the response to the subsequent addition of Somatostatin (100 nM) to AtT20-WT cells (black dots). Data represents the mean ± SEM of five independent experiments performed in duplicate ($p > 0.05$).



Supplementary Figure 4-2 Effect of brodifacoum (BFC) on somatostatin (SRIF) challenge after 30 minutes of drugs on AtT20-CB_1 and -CB_2 cells

Comparison of percentage change in fluorescence after SRIF (100 nM) challenge on AtT20-CB_1, and AtT20-CB_2 in the continuous presence of (A), (C) CP55940 or (B), (D) Δ^9-THC added with either HBS or BFC (1 µM). BFC did not affect the hyperpolarization induced by SRIF after prolonged application of CP55940 or Δ^9-THC. Data represents the mean ± SEM of five independent experiments performed in duplicate ($p > 0.05$).

Chapter V.

Study IV. Absence of Entourage: Terpenoids Commonly Found in Cannabis sativa Do Not Modulate the Functional Activity of Δ^9-THC at Human CB1 and CB2 Receptors

Allosteric modulation of cannabinoid receptors was also studied in the context of the "entourage effect" – a belief that the components within the cannabis plant will produce much greater pharmacological effects when combined as opposed to the individual components. As a preliminary approach to investigating the terpenoid-cannabinoid interaction, this chapter examined whether the effects of low efficacy phytocannabinoid (THC), or high efficacy synthetic cannabinoid (CP55940) on the acute signalling of human cannabinoid receptors would be modified in the presence of terpenoids, either alone or in combination. At the least, this study might dispel notions of "entourage effect" at the pharmacodynamic level of cannabinoid receptor function.

Contributions to the work

This paper represents a collaborative work hosted in Mark Connor's laboratory at Macquarie University Australia. Marina Santiago designed and performed the experiments related to the effect of terpenoid on the acute signalling of cannabinoid receptor, while I assisted with the desensitisation experiments to examine the potential effects of terpenoids on prolong cannabinoid receptor activation, and analysed the data. All the authors discussed the results and contributed to the manuscript.

Cannabis and Cannabinoid Research
Volume 4, Number 3, 2019
Mary Ann Liebert, Inc.
DOI: 10.1089/can.2019.0016

ORIGINAL RESEARCH

Absence of Entourage: Terpenoids Commonly Found in *Cannabis sativa* Do Not Modulate the Functional Activity of Δ^9-THC at Human CB_1 and CB_2 Receptors

Marina Santiago,[1,*] Shivani Sachdev,[1] Jonathon C. Arnold,[2,3] Iain S. McGregor,[2,4] and Mark Connor[1]

Abstract

Introduction: Compounds present in *Cannabis sativa* such as phytocannabinoids and terpenoids may act in concert to elicit therapeutic effects. Cannabinoids such as Δ^9-tetrahydrocannabinol (Δ^9-THC) directly activate cannabinoid receptor 1 (CB_1) and cannabinoid receptor 2 (CB_2); however, it is not known if terpenoids present in *Cannabis* also affect cannabinoid receptor signaling. Therefore, we examined six common terpenoids alone, and in combination with cannabinoid receptor agonists, on CB_1 and CB_2 signaling *in vitro*.

Materials and Methods: Potassium channel activity in AtT20 FlpIn cells transfected with human CB_1 or CB_2 receptors was measured in real time using FLIPR® membrane potential dye in a FlexStation 3 plate reader. Terpenoids were tested individually and in combination for periods up to 30 min. Endogenous somatostatin receptors served as a control for direct effects of drugs on potassium channels.

Results: α-Pinene, β-pinene, β-caryophyllene, linalool, limonene, and β-myrcene (up to 30–100 μM) did not change membrane potential in AtT20 cells expressing CB_1 or CB_2, or affect the response to a maximally effective concentration of the synthetic cannabinoid CP55,940. The presence of individual or a combination of terpenoids did not affect the hyperpolarization produced by Δ^9-THC (10 μM): (CB_1: control, 59% ± 7%; with terpenoids (10 μM each) 55% ± 4%; CB_2: Δ^9-THC 16% ± 5%, with terpenoids (10 μM each) 17% ± 4%). To investigate possible effect on desensitization of CB_1 responses, all six terpenoids were added together with Δ^9-THC and signaling measured continuously over 30 min. Terpenoids did not affect desensitization, after 30 min the control hyperpolarization recovered by 63% ± 6% in the presence of the terpenoids recovery was 61% ± 5%.

Discussion: None of the six of the most common terpenoids in *Cannabis* directly activated CB_1 or CB_2, or modulated the signaling of the phytocannabinoid agonist Δ^9-THC. These results suggest that if a phytocannabinoid–terpenoid entourage effect exists, it is not at the CB_1 or CB_2 receptor level. It remains possible that terpenoids activate CB_1 and CB_2 signaling pathways that do not involve potassium channels; however, it seems more likely that they may act at different molecular target(s) in the neuronal circuits important for the behavioral effect of *Cannabis*.

Keywords: phytocannabinoid; cannabinoid receptor; terpenoid; entourage effect; THC; signaling

[1]Department of Biomedical Sciences, Macquarie University, Sydney, New South Wales, Australia.
[2]The Lambert Initiative for Cannabinoid Therapeutics, [3]Discipline of Pharmacology, and [4]School of Psychology, The University of Sydney, Sydney, New South Wales, Australia.
Some of these data were presented at The Australasian Society of Clinical and Experimental Pharmacologists and Toxicologists Conference in Melbourne, Australia (2016).

*Address correspondence to: Marina Santiago, PhD, Department of Biomedical Sciences, Macquarie University, Sydney 2109, New South Wales, Australia, E-mail: marina.junqueirasantiago@mq.edu.au

Introduction

An enduring notion in the medicinal *Cannabis* and cannabinoid field is that of entourage: the idea that use of the whole plant may exert substantially greater effects than the sum of its individual parts.[1] Entourage is usually construed as a positive attribute, with the assumption that superior therapeutic actions, or a more favorable "high," will be obtained from consuming the whole *Cannabis* plant rather than individual components such as Δ^9-tetrahydrocannabinol (Δ^9-THC). Somewhat surprisingly, the evidence for this widely cited notion is relatively sparse.

Cannabis contains ~150 phytocannabinoids, the most common of which are Δ^9-THC and cannabidiol (CBD), together with their acid precursors THCA and CBDA.[2] *Cannabis* also contains a large number of monoterpene and sesquiterpene compounds (together called terpenoids), the most common of which include α-pinene, β-pinene, linalool, limonene and β-myrcene (monoterpenes) and β-caryophyllene and caryophyllene oxide (sesquiterpenes).[3] Terpenoids are volatile compounds that are synthesized alongside phytocannabinoids mainly in the trichomes of the cannabis plant, and provide cannabis with its distinctive aroma and flavor.[4] Terpenoids are often lost if the extraction process involves heating.[5]

The entourage concept applied to cannabis can encompass the potential for both cannabinoid–cannabinoid and cannabinoid–terpenoid interactions. With regard to the former, Δ^9-THC-CBD synergy in producing analgesia was reported in an animal model of neuropathic pain[6] while in humans, CBD has been proposed to ameliorate some of the adverse psychotomimetic and anxiogenic effects of Δ^9-THC.[7,8] This claim is controversial, however, with a number of contrary findings.[9,10] CBD may modulate Δ^9-THC effects at the receptor level acting as a CB_1 negative allosteric modulator,[11] providing some biological plausibility to a modulatory interaction.

Scientific evidence for cannabinoid–terpenoid interactions is essentially absent, and mostly comes from websites and dispensaries extolling the virtues of proprietary *Cannabis* chemical varieties, or chemovars.[12,13] However, some terpenoids do have intrinsic psychoactive and physiological effects, and modulatory effects on Δ^9-THC actions are not farfetched.[1,14] For example, in studies with laboratory animals, limonene displayed anxiolytic effects, pinene increased gastrointestinal motility, linalool was sedative, anticonvulsant, and anxiolytic, while myrcene produced sedation, analgesia, and muscle relaxant effects (summarized in Russo and Marcu[14]). Lewis et al.[13] reported that in a low terpenoids variety (1.1% terpenoids) myrcene concentration is 0.45%, while in a high variety (4.8% total) myrcene concentration is as high as 3.44%. Compelling evidence for cannabinoid–terpenoid interactions or synergy does not yet exist. A report on perceived efficacy of Cannabis for childhood epilepsy identified the presence of three predominant terpenoids (β-caryophyllene, β-myrcene, and α-pinene); however, when extracts perceived as "effective" were compared with "ineffective" extracts, differences in terpenoid profile/content were not significant.[15]

With so many bioactive components present in cannabis, the systematic, granular elucidation of possible entourage effects poses a substantial combinatorial puzzle and scientific challenge. As a preliminary approach to addressing this challenge, this study examined whether the effects of Δ^9-THC on its cognate cannabinoid receptors (CB_1 and CB_2) would be modified in the presence of terpenoids that are commonly found in cannabis, either alone or in combination. The demonstration of such a receptor-level entourage effect might lead to predictions regarding functional cannabinoid–terpenoid interaction effects that could be tested *in vivo*.

Materials and Methods

Cell culture

Experiments used mouse wild-type AtT20 FlpIn cells (AtT20-WT), or these cells stably transfected with human CB_1 or CB_2 receptors with 3×N-terminus hemagglutinin tags (AtT20-CB_1 and AtT20-CB_2, respectively).[16] Cells were cultivated in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS; Sigma/SAFC) and 100 U penicillin/100 μg streptomycin mL^{-1} (Gibco). Selection antibiotics were 80 μg mL^{-1} Zeocin (Invivogen) for AtT20-WT or 80 μg mL^{-1} hygromycin B Gold (Invivogen) for transfected cells.

Cells were grown in 75 mm^2 flasks at 37°C/5% CO_2 and passaged when 80–90% confluent. Assays were carried out on cells up to 20 passages in culture.

Potassium channel activity measurements

Changes in membrane potential were measured using the FLIPR® blue membrane potential dye (Molecular Devices) in a FlexStation 3, as outlined in Knapman 2013.[17] Cells from a 90–100% confluent 75 mm^2 flask were resuspended in Leibovitz's L-15 Medium (Gibco) supplemented with 1% FBS, 100 U penicillin/100 μg streptomycin mL^{-1}, and glucose (15 mM) and plated in 96-well black-walled clear bottom microplates (Costar)

in a volume of 90 μL per well. Cells were incubated overnight in humidified ambient air at 37°C incubator. Membrane potential dye, used at 50% of the manufacturer-recommended concentration, was resuspended in Hank's Balanced Salt Solution (HBSS) of composition (in mM): NaCl 145, HEPES 22, Na_2HPO_4 0.338, $NaHCO_3$ 4.17, KH_2PO_4 0.441, $MgSO_4$ 0.407, $MgCl_2$ 0.493, $CaCl_2$ 1.26, glucose 5.55 (pH 7.4, osmolarity 315 ± 15). Dye was loaded onto each well (90 μL per well) and equilibrated at 37°C for ~1 h before assay. Fluorescence was measured every 2 sec (λ excitation = 530 nm, λ emission = 565 nm, λ emission cutoff = 550 nm). Assays were carried out at 37°C, and drugs were automatically added in volumes of 20 μL.

Determining the effects of terpenoids on acute hyperpolarization. Terpenoids were added after ~60 sec of baseline recording and incubated for 5 min before cannabinoid (CP55,940 or Δ^9-THC) addition. In AtT20-WT cells, somatostatin (SST) was added instead of cannabinoid.

Determining the effects of terpenoids on signaling desensitization. Homologous desensitization was measured by simultaneously adding Δ^9-THC with

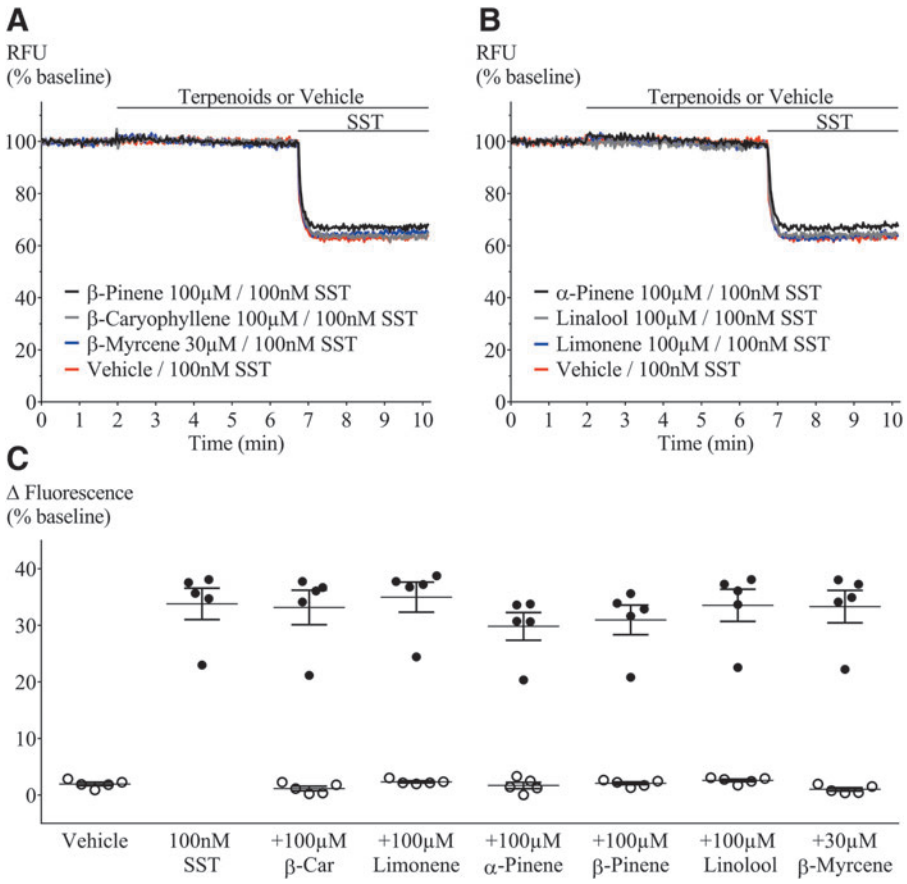


FIG. 1. Terpenoid- and SST-mediated fluorescence change in AtT20-WT. Representative traces showing change in fluorescence signal after terpenoid and SST (100 nM) application. A decrease in signal corresponds to membrane hyperpolarization. Addition of terpenoids **(A)** β-pinene, β-caryophyllene, and β-myrcene; **(B)** α-pinene, linalool, and limonene did not change baseline fluorescence, while SST mediated a clear hyperpolarization. **(C)** Percentage change of fluorescence from baseline after each terpenoid (open circles) and SST (closed circles) application. Terpenoids were added at 2 min; 5 min before SST. When compared with positive (SST) or negative (vehicle) controls, none of the terpenoids tested affected baseline membrane potential or peak SST response. β-Car = β-caryophyllene. $n = 5$, SEM, one-way ANOVA $p > 0.05$. Drugs were added for the duration of the bar. ANOVA, analysis of variance; SEM, standard error of the mean; SST, somatostatin.

terpenoids after 120 sec of baseline recording. Signaling desensitization was calculated as percentage decrease from peak Δ^9-THC response after 30 min in drugs. SST (100 nM) was added 30 min after Δ^9-THC addition to examine the potential effects of prolonged cannabinoid receptor activation on native SST receptors (heterologous desensitization). The SST response was compared between groups (with or without terpenoids).

Drug dilution. All drugs (except SST) were prepared in dimethyl sulfoxide (DMSO) and stored as frozen stocks at a concentration of 10–100 mM. Terpenoid stock solution concentrations were 100 mM, with the exception of β-myrcene (30 mM), which was insoluble at 100 mM. SST was dissolved in water. Fresh aliquots were used each day, with the drugs diluted in HBSS containing 0.1% bovine serum albumin (Sigma-Aldrich) immediately before the assay. The final concentration of DMSO in each well was 0.1–0.11%; this limited the maximum concentration of terpenoids able to be tested. A vehicle (HBSS plus solvent alone) well was included in each column of the 96-well plate, and the changes in fluorescence produced by vehicle alone were subtracted before determining the maximum hyperpolarization after each drug exposure.

Drugs and reagents

Δ^9-THC was obtained from THCPharm (Frankfurt, Germany). Terpenoids were obtained from Sigma-Aldrich; (+)-α-pinene, (+)-β-pinene, (–)-β-caryophyllene, (+/–)-linalool, (R)-(+)-limonene, and β-myrcene. SST was obtained from Auspep and CP55,940 from Cayman. Unless otherwise indicated, the other chemicals and reagents were obtained from Sigma-Aldrich.

Data analysis

Each experiment was independently repeated at least five times, with two technical replicates in each determination. Data are expressed as a percentage change in the fluorescence compared with the predrug baseline (30 sec before drug addition) or as a percentage of 1 μM CP55,940 response. Graphs were plotted using Graphpad Prism 7.02, and scatter dot plots show means with standard error of the mean. Means were compared using unpaired Student's *t*-test or no matching one-way analysis of variance, followed by correction for multiple comparisons (Dunnett); and null hypothesis was rejected if *p*-value was <0.05 ($p > 0.05$ = not significant).

Results

Terpenoids in AtT20-WT cells

We first examined terpenoid action in nontransfected AtT20 cells. We used SST (100 nM) as a positive control because it hyperpolarizes AtT20-WT cells through activation of endogenous SST receptors (Fig. 1A, B).[17,18] Addition of α-pinene, β-pinene, β-caryophyllene, linalool, limonene (100 μM), or β-myrcene (30 μM) did

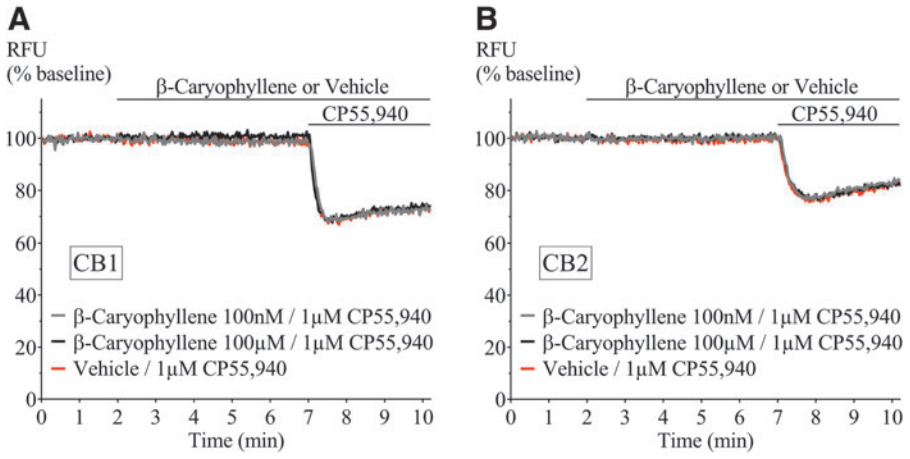


FIG. 2. Representative traces of β-caryophyllene and CP55,940 in AtT20-CB_1 and -CB_2. Fluorescence was recorded for 10 min where β-caryophyllene (100 nM and 100 μM) was added at 2 min followed by incubation for 5 min, before 1 μM CP55,940 application. β-caryophyllene did not hyperpolarize **(A)** AtT20-CB_1 and **(B)** AtT20-CB_2 cells, or affect the response to CP55,940 (1 μM). Drugs were added for the duration of the bar. CB_1, cannabinoid receptor 1, CB_2, cannabinoid receptor 2.

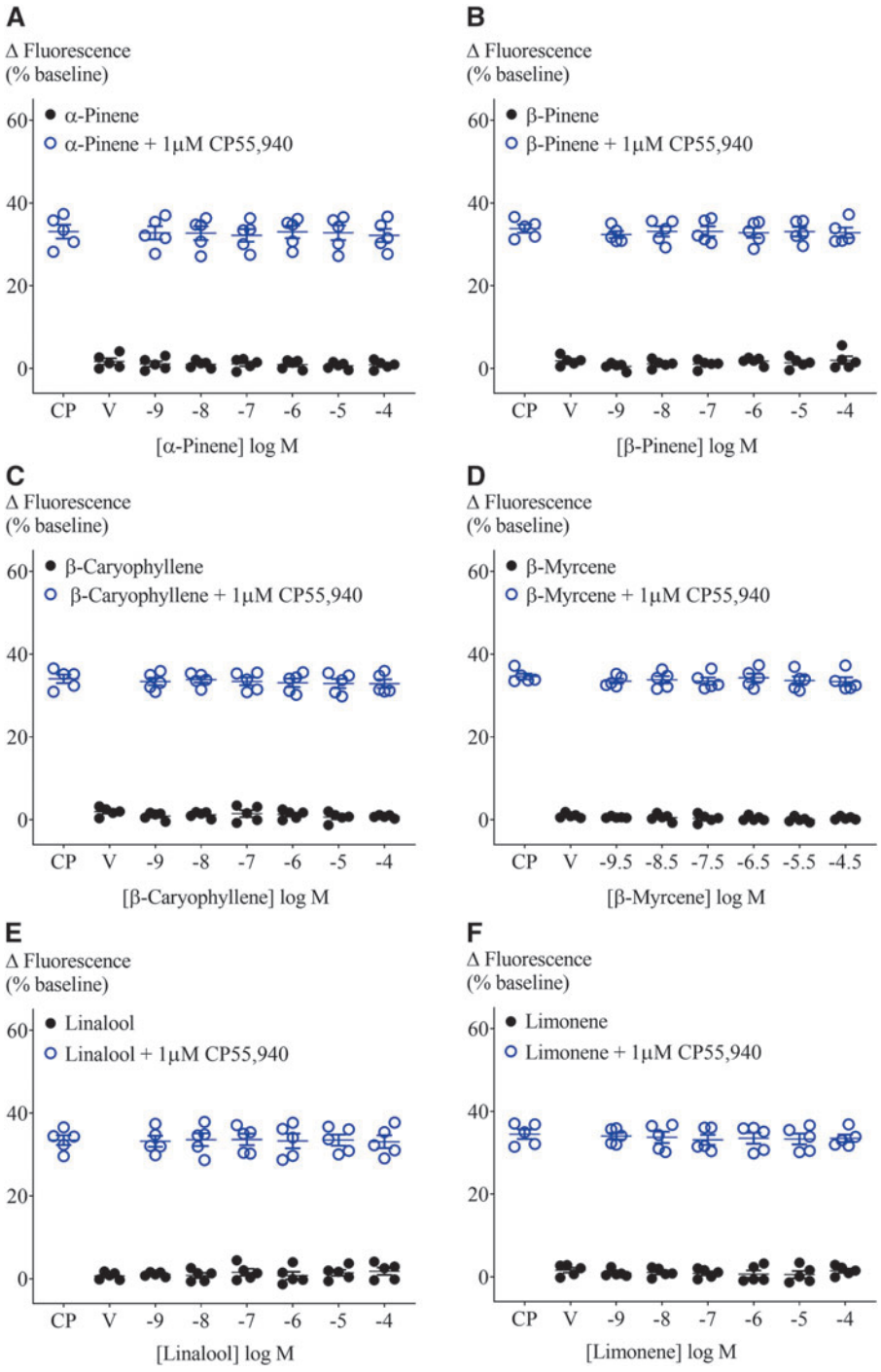


FIG. 3. Effect of terpenoids at varying concentrations on AtT20-CB_1 membrane potential and on 1 μM CP55,940-induced hyperpolarization. Terpenoids **(A)** α-pinene, **(B)** β-pinene, **(C)** β-caryophyllene, **(D)** β-myrcene, **(E)** linalool, and **(F)** limonene were added to AtT20-CB_1 cells and incubated for 5 min. Maximum fluorescence changes were not different from negative control (closed circles, $n = 5$, SEM, one-way ANOVA $p > 0.05$). CP55,940 (1 μM) addition to AtT20-CB_1 cells induced fluorescence changes from 33.1% ± 1.7% to 34.6% ± 0.7%. Peak CP55,940 responses were not affected by the presence of terpenoids (open circles, $n = 5$, SEM, one-way ANOVA $p > 0.05$). V, vehicle.

not affect the membrane potential of AtT20-WT cells (Fig. 1C, open circles). The presence of terpenoids (100 μM/30 μM) had no effect on the subsequent SST response (Fig. 1C).

Terpenoids in AtT20-CB_1 and -CB_2 cells

The absence of a terpenoid response in AtT20-WT cells enabled the study of their effect on membrane potential in AtT20 cells expressing human CB_1 or CB_2. We examined whether terpenoids (1 nM–100 μM, β-myrcene 300 pM–30 μM) hyperpolarized cells through these receptors and, in parallel, whether they affected a subsequent response to a maximally effective concentration of CP55,940 (1 μM; Fig. 2).[16] A summary of the fluorescence change after terpenoid addition to AtT20-CB_1 cells is shown in Figure 3 (closed circles). No difference between vehicle and terpenoids was observed. Further, none of the terpenoids changed the membrane potential of cells expressing CB_2 (Supplementary Fig. S1). The change in fluorescence produced by the subsequent addition of the nonselective cannabinoid agonist CP55,940 (1 μM) was also unaffected in both AtT20-CB_1 and -CB_2 (Fig. 3 and Supplementary Fig. S1, open circles).

CP55,940 is a high-efficacy agonist of both CB_1 and CB_2 receptors.[19] However, in *Cannabis*, Δ^9-THC is the principle cannabinoid agonist, and it has a lower efficacy than CP55,940, which is apparent in the hyperpolarization assay as a lower maximal response.[19] We next tested the effect of a low and high concentration of terpenoids (100 nM and 10 μM) on the hyperpolarization produced by three concentrations of Δ^9-THC (100 nM, 1 and 10 μM). Application of Δ^9-THC, after 5 min of individual terpenoid application, produced a fluorescence change (Fig. 4) that was not significantly different from that produced by Δ^9-THC alone in both AtT20-CB_1 and -CB_2 cells (10 μM Δ^9-THC, Figs. 5 and 6; 100 nM Δ^9-THC, Supplementary Figs. S2 and S3). To explore the possibility of an emergent entourage effect, we combined all six terpenoids (10 μM each) and tested the effect of the mixture on the Δ^9-THC-induced hyperpolarization. Similar to individually tested terpenoids, the effects of Δ^9-THC were not changed by the mixture (Fig. 7).

Terpenoids and desensitization in AtT20-CB_1

We have previously reported desensitization cannabinoid-mediated cellular hyperpolarization in AtT20 cells expressing rat or human CB_1 receptors,[20,21] and we found that this reversal of CP55,940-induced hyperpolarization was accelerated by negative allosteric modulators such as ORG27569 and PSNCBAM-1. Therefore, we tested whether terpenoids may act in a similar way to ORG27569 and other negative allosteric modulators, altering desensitization time course. We used Δ^9-THC instead of CP55,940, as Δ^9-THC is the main phytocannabinoid agonist. Prolonged application of Δ^9-THC (10 μM) produced a hyperpolarization that reversed substantially over 30 min. Representative traces

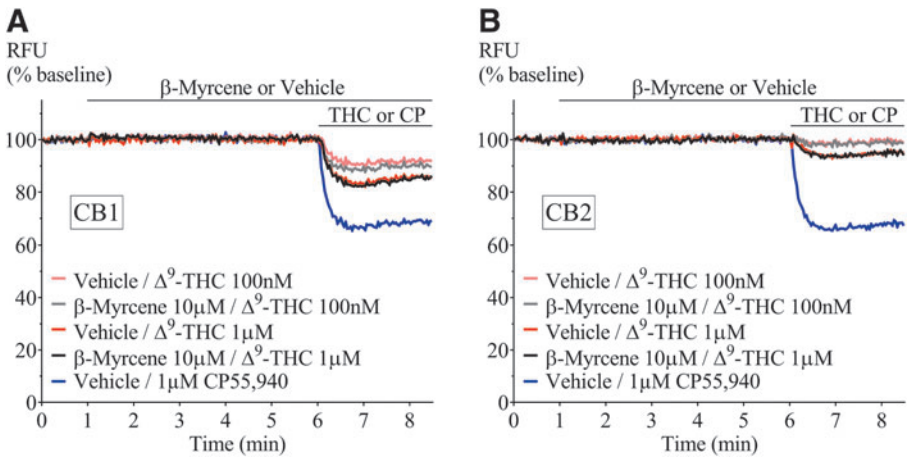


FIG. 4. Representative traces of β-myrcene and Δ^9-THC in **(A)** AtT20-CB_1 and **(B)** AtT20-CB_2. Fluorescence change mediated by two submaximal concentrations of Δ^9-THC (100 nM and 1 μM) in the presence of β-myrcene (10 μM). Terpenoid was added at 1 min and incubated for 5 min before Δ^9-THC application. CP55,940 added as positive control. Drugs were added for the duration of the bar. Δ^9-THC, Δ9-tetrahydrocannabinol.

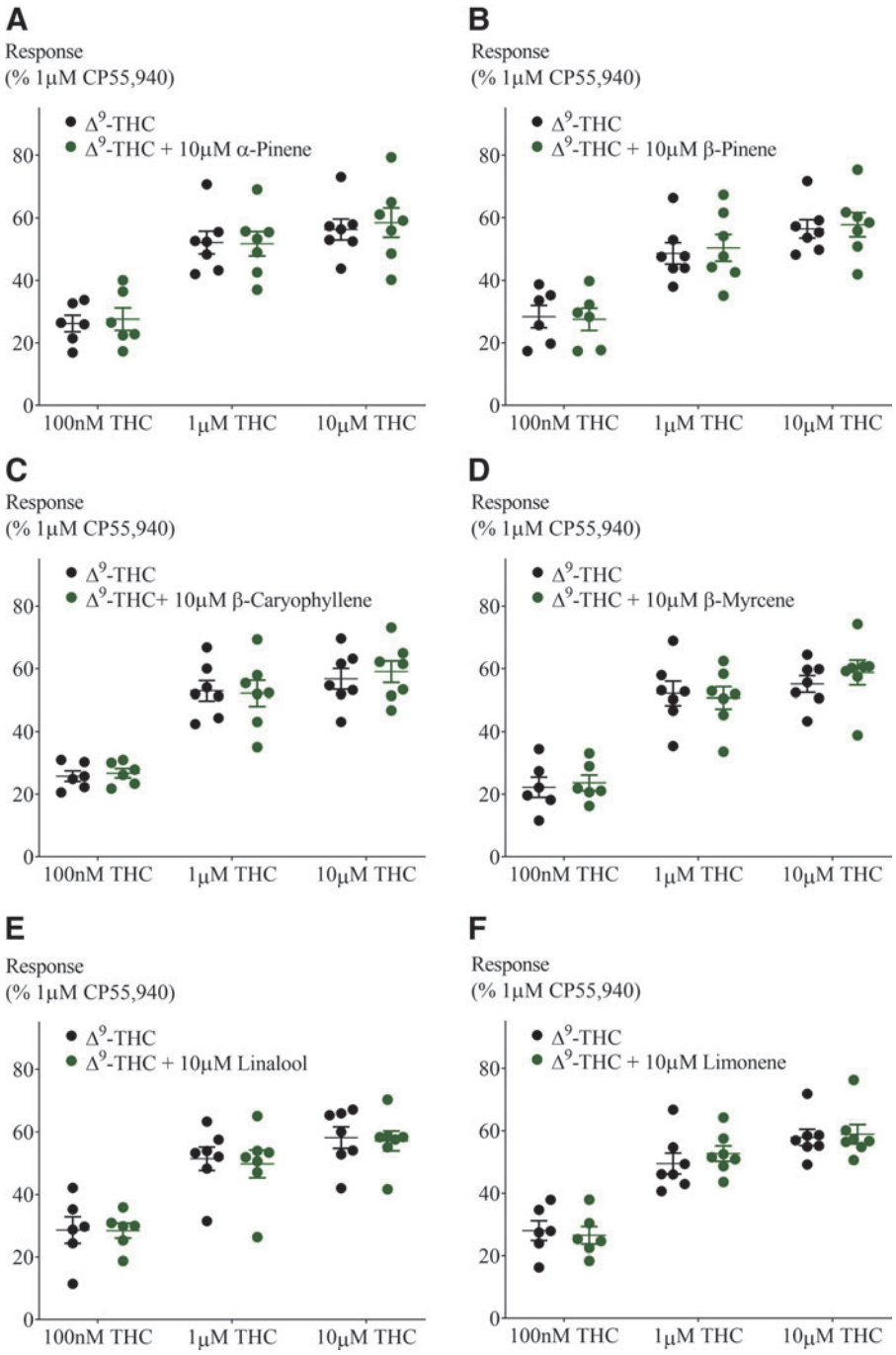


FIG. 5. Effect of 10 μM terpenoids on Δ^9-THC-induced hyperpolarization in AtT20-CB_1. Terpenoids tested were **(A)** α-pinene, **(B)** β-pinene, **(C)** β-caryophyllene, **(D)** β-myrcene, **(E)** linalool, and **(F)** limonene. Response to Δ^9-THC at two submaximal and one maximal concentration ($n = 6$–7, SEM, unpaired t-test $p > 0.13$). Data presented as % of maximum CP55,940 (1 μM) response.

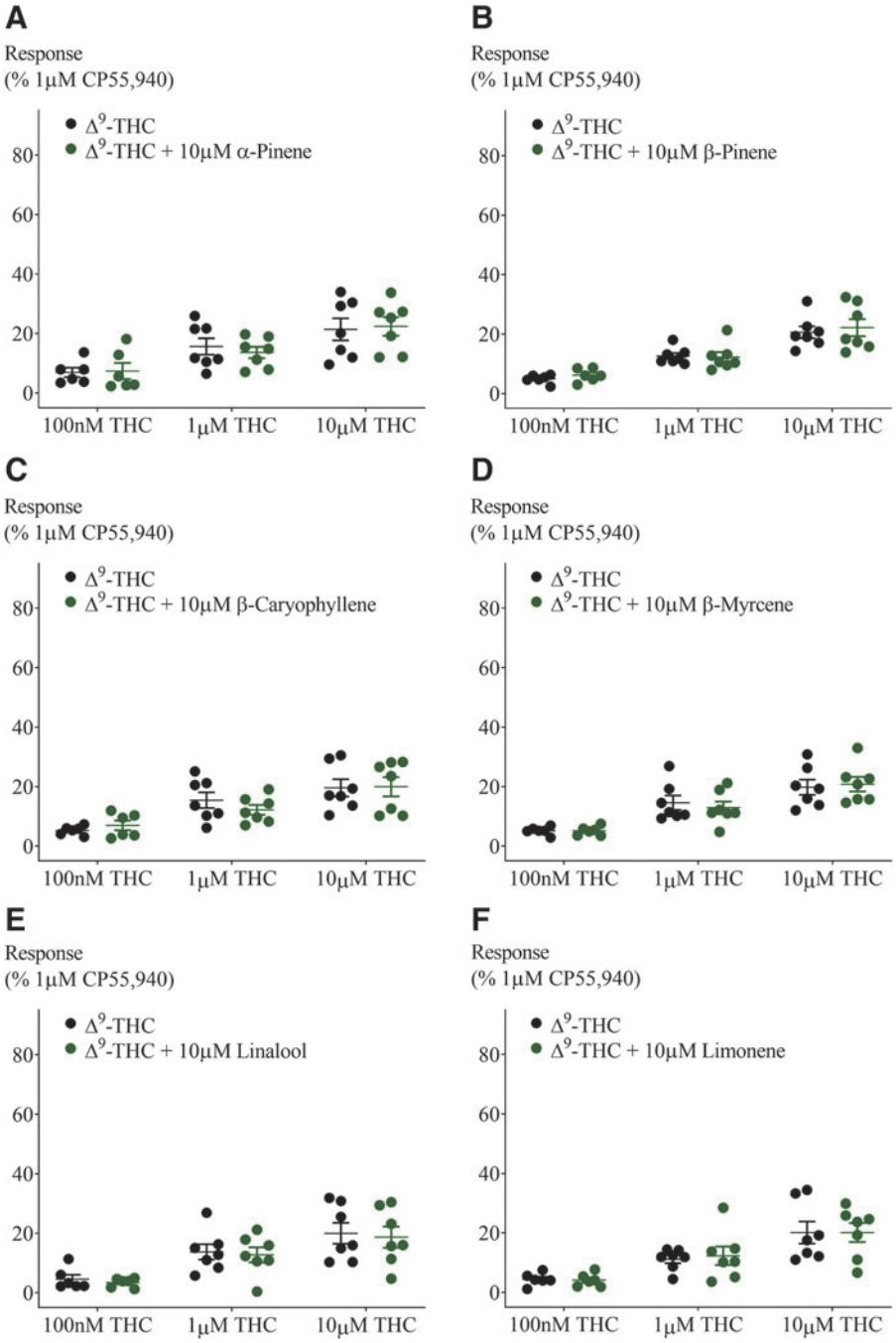


FIG. 6. Effect of 10 μM terpenoids on Δ^9-THC-induced hyperpolarization in AtT20-CB_2. Terpenoids tested were **(A)** α-pinene, **(B)** β-pinene, **(C)** β-caryophyllene, **(D)** β-myrcene, **(E)** linalool, and **(F)** limonene. Response to Δ^9-THC at two submaximal and one maximal concentration ($n = 6$–7, SEM, unpaired t-test $p > 0.26$). Data presented as % of maximum CP55,940 (1 μM) response.

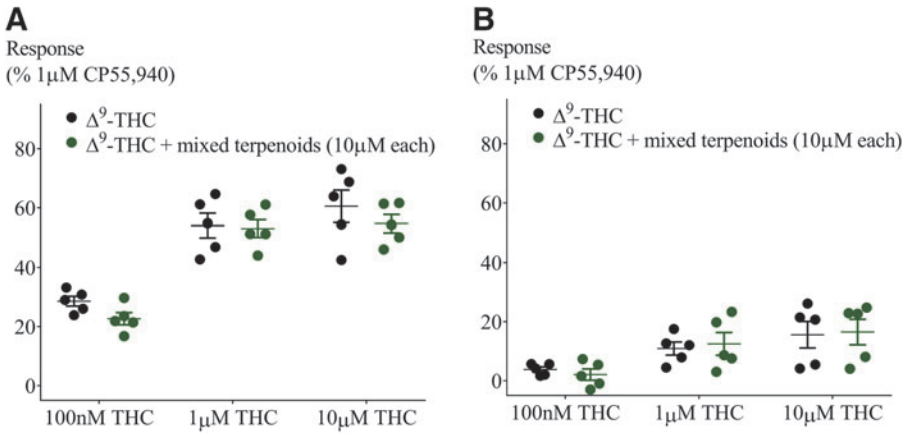


FIG. 7. Testing the "Entourage effect." Effect of combination of six terpenoids at 10 μM each on Δ^9-THC-induced hyperpolarization in **(A)** AtT20-CB_1 and **(B)** AtT20-CB_2. Response to Δ^9-THC at two submaximal and one maximal concentration ($n=5$, SEM, unpaired t-test $p>0.13$). Data presented as % of maximum CP55,940 (1 μM) response.

for this experiment are illustrated in Figure 8A. We measured the peak response to Δ^9-THC and the signal remaining 30 min after agonist exposure, and quantified desensitization as a percentage decline in the peak response. The Δ^9-THC (10 μM) signal desensitized by 63% ± 6%, in the presence of the terpenoid mix desensitization, was 61% ± 5% (Fig. 8B). Thus, terpenoids did not interfere with desensitization of CB_1 signaling produced by Δ^9-THC. We also assessed the capacity of Δ^9-THC alone, terpenoids alone (10 μM each), or terpenoids combined with Δ^9-THC to affect SST receptor signaling in AtT20-CB_1 cells (heterologous

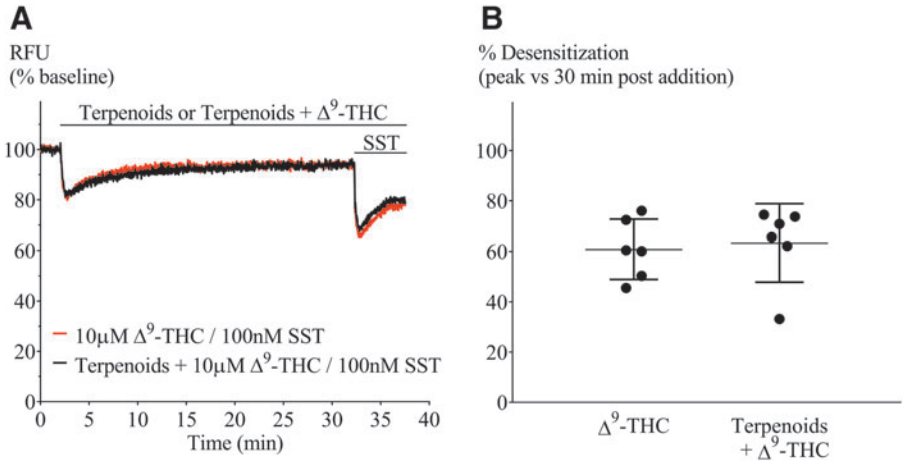


FIG. 8. Terpenoids on Δ^9-THC-mediated desensitization in AtT20-CB_1. **(A)** Representative traces of hyperpolarization and signal desensitization mediated by Δ^9-THC alone (10 μM, black) or with terpenoids (10 μM each, red). Cells were then challenged with SST (100 nM) after 30 min to examine heterologous desensitization. **(B)** Percentage desensitization after 30 min exposure to Δ^9-THC alone (10 μM) or in the presence of terpenoids (10 μM each), compared with peak fluorescence response. Terpenoids did not affect Δ^9-THC-mediated desensitization ($n=5$, SEM, unpaired t-test $p=0.76$). Drugs were added for the duration of the bar.

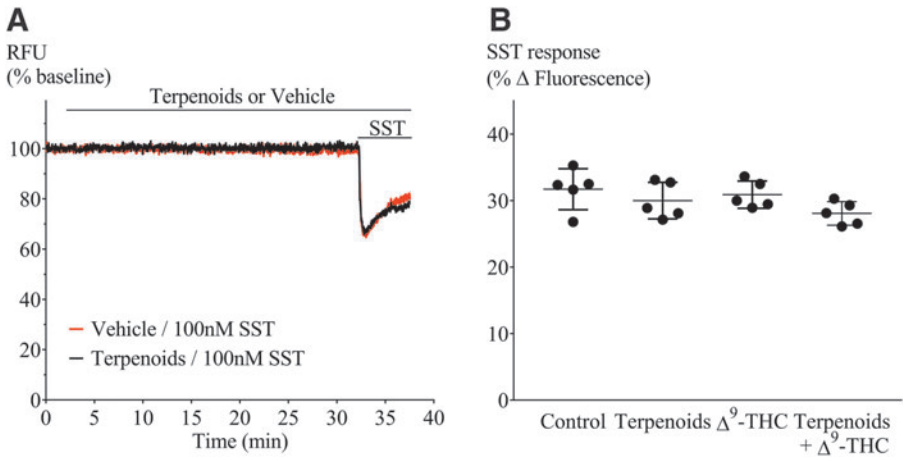


FIG. 9. SST challenge of AtT20-CB_1 cells to investigate heterologous desensitization. **(A)** Representative traces of cells preincubated with (black) or without (red) terpenoids for 30 min before SST (100 nM) challenge. **(B)** Comparison of peak hyperpolarization (% fluorescence change) obtained after SST (100 nM) challenge ($n = 5$, one-way ANOVA $p > 0.05$). Drugs were added for the duration of the bar.

desensitization). SST (100 nM) was applied 30 min after first drug application (Figs. 8A and 9A), and the hyperpolarization produced by SST after Δ^9-THC, terpenoids alone, or Δ^9-THC with terpenoids was not significantly different from that produced by SST alone ($p > 0.05$, Fig. 9B).

Discussion

The principal finding of this study is that agonist activation of CB_1 and CB_2 receptors is not obviously altered by any or all of the six major terpenoids from *Cannabis sativa*. The terpenoids tested did not activate CB_1 or CB_2 by themselves, nor did they modify the signaling of the high-efficacy agonist CP55,940 or the lower efficacy agonist Δ^9-THC. In particular, Δ^9-THC effects would be expected to be very sensitive to the presence of drugs that inhibited (or enhanced) signaling at the receptor. There are no spare receptors for Δ^9-THC in this assay, and changes in ligand binding would be directly reflected as a change in the maximum response. The lack of effect of terpenoids on the response to the synthetic cannabinoid CP55,940 indicates that terpenoids do not interfere with maximal cannabinoid receptor-mediated hyperpolarization, suggesting no direct modulation of the potassium channel response. This was confirmed by the lack of effect of terpenoids on the response to SST.

A previous study showed that β-caryophyllene is a CB_2 agonist.[22] However, we were unable to detect any effect of β-caryophyllene on CB_2 signaling in this study. The reasons for this are unclear, but the efficacy of β-caryophyllene has not been defined in cellular assays and may be lower than that of Δ^9-THC. The CB_2 response to even high concentrations of Δ^9-THC in our assay is small, suggesting that productive coupling of CB_2 to endogenous potassium channels in AtT20 cells requires high-efficacy agonists. The affinity of β-caryophyllene for CB_2 (155 nM) has been determined in membranes from HEK293 cells heterologously expressing CB_2,[22] but is not known in intact cells. Its EC_{50} for inhibition of forskolin-induced adenylyl cyclase in CHO-K1 expressing CB_2 was $\sim 2\,\mu$M,[22] suggesting a low functional affinity, which may not be sufficient to significantly affect the rapid response to the higher affinity agonist Δ^9-THC.

The role of terpenoids in cannabis-induced analgesia in rats was recently evaluated by Harris et al.[23] They tested THC, isolated terpenoids, extract without terpenoids, and full extract, and suggested that the analgesic effect of cannabis is mainly due to THC presence and proposed that terpenoids do not contribute to cannabis-mediated analgesia. These findings support our results, and interestingly their extract had a very high percentage of β-caryophyllene.

Positive and negative allosteric modulators have been reported for CB_1,[24,25] and the effects of several negative allosteric modulators have been defined in the hyperpolarization assay used here.[20] Both PSNCBAM-1 and ORG27569 enhanced CP55,940 signal desensitization,

while PSNCBAM-1 also inhibited the initial CP55,940 hyperpolarization. Coapplication of the terpenoids with Δ^9-THC failed to affect the peak response, or the degree of tachyphylaxis observed over a 30-min exposure to drug, suggesting that they are not acting as allosteric modulators of this CB_1 signaling pathway.

Limitations

A limitation of this study is that we only examined CB_1 and CB_2 signaling through one pathway, involving Gi/o. The hyperpolarization of the AtT20 cells likely represents G-protein-mediated activation of inwardly rectifying potassium channels (GIRK), as previously described for CB_1 and other GPCR in these cells as well as in several different neurons.[26–28] Cannabinoid receptors couple to multiple G proteins as well as signaling through other pathways such as those dependent on arrestins, and it is possible that entourage effects of terpenoids are mediated through modulation of a subset of the cannabinoid receptor signaling repertoire.[26] CB_1 and CB_2 receptors can be activated in a ligand-biased manner—the phenomenon where a drug preferentially activates a subset of the signaling pathways that the receptor can access.[29] In general, this bias has been best defined for G protein coupling versus activation of arrestin-mediated signaling, but to our knowledge there are no examples of cannabinoid ligands only affecting arrestin-mediated signaling.[19,30] It remains possible that terpenoids have such an absolute bias, but this would be unprecedented, and in any case recruitment of arrestin would be expected to produce enhanced desensitization of the CB_1 responses to prolonged agonist exposure.[20,29] Any subtle change to receptor signaling should be clear with use of the low-efficacy agonist Δ^9-THC.

Overall, our data suggest that it is unlikely that the terpenoids studied here affect Δ^9-THC interactions with cannabinoid receptors. However, this is not a definitive rebuttal of the entourage effect. Our study cannot address the possibility of entourage effects emerging through effects of terpenoids on cannabinoid metabolism and distribution as well as interaction with other G-protein-coupled receptors, ligand-gated ion channels, signaling cascades present on the same cells that express cannabinoid receptors, or on other cells up or downstream of the cannabinoid receptor expressing cells. There are many other ways that these molecules could interact with cannabinoids to influence the overall therapeutic and subjective outcomes of cannabis administration, and it should be acknowledged that Δ^9-THC influences signaling at a wide variety of other noncannabinoid receptor targets (see Banister et al.[31] for a review). Terpenoids may even have primary effects on distinct functional modules that together with cannabinoid receptor-modulated pathways are ultimately integrated into a behavioral or physiological output. So the quest for entourage does not end here; in many ways, it has only just begun.

Acknowledgments

S.S. is a recipient of the Macquarie University Research Excellence Scholarship for International Students. We thank Lambert Initiative for Cannabinoid Therapeutics for funding this project.

Author Disclosure Statement

M.S., S.S., and M.C. have no competing financial interests to disclose. I.S.M. currently acts as a consultant to Kinoxis Therapeutics. J.C.A. has acted as a consultant to the World Health Organization in the last 12 months in its review of cannabis and the cannabinoids. I.S.M. and J.C.A. are inventors on several patents involving cannabinoid therapeutics.

Supplementary Material

Supplementary Figure S1
Supplementary Figure S2
Supplementary Figure S3

References

1. Russo EB. Taming THC: potential cannabis synergy and phytocannabinoid-terpenoid entourage effects. Br J Pharmacol. 2011; 163:1344–1364.
2. Hanuš LO, Meyer SM, Muñoz E, et al. Phytocannabinoids: a unified critical inventory. Nat Prod Rep. 2016;33:1357–1392.
3. Ibrahim EA, Wang M, Radwan MM, et al. Analysis of Terpenes in *Cannabis sativa* L. Using GC/MS: Method Development, Validation, and Application. Planta Med. 2019;85:431–438.
4. Pertwee RG. Handbook of cannabis. 1st ed. Oxford University Press: Oxford, United Kingdom, 2014.
5. Sexton M, Shelton K, Haley P, et al. Evaluation of Cannabinoid and terpenoid content: Cannabis flower compared to supercritical CO_2 concentrate. Planta Med. 2018;84:234–241.
6. Casey SL, Atwal N, Vaughan CW. Cannabis constituent synergy in a mouse neuropathic pain model. Pain. 2017;158:2452–2460.
7. Englund A, Freeman TP, Murray RM, et al. Can we make cannabis safer? Lancet Psychiatry. 2017;4:643–648.
8. Russo E, Guy GW. A tale of two cannabinoids: the therapeutic rationale for combining tetrahydrocannabinol and cannabidiol. Med Hypotheses. 2006;66:234–246.
9. Haney M, Malcolm RJ, Babalonis S, et al. Oral Cannabidiol does not alter the subjective, reinforcing or cardiovascular effects of smoked Cannabis. Neuropsychopharmacology. 2016;41:1974–1982.
10. Ilan AB, Gevins A, Coleman M, et al. Neurophysiological and subjective profile of marijuana with varying concentrations of cannabinoids. Behav Pharmacol. 2005;16:487–496.
11. Laprairie RB, Bagher AM, Kelly ME, et al. Cannabidiol is a negative allosteric modulator of the cannabinoid CB1 receptor. Br J Pharmacol. 2015; 172:4790–4805.
12. Russo EB. The case for the entourage effect and conventional breeding of clinical Cannabis: no "Strain," no gain. Front Plant Sci. 2018;9: 1969.

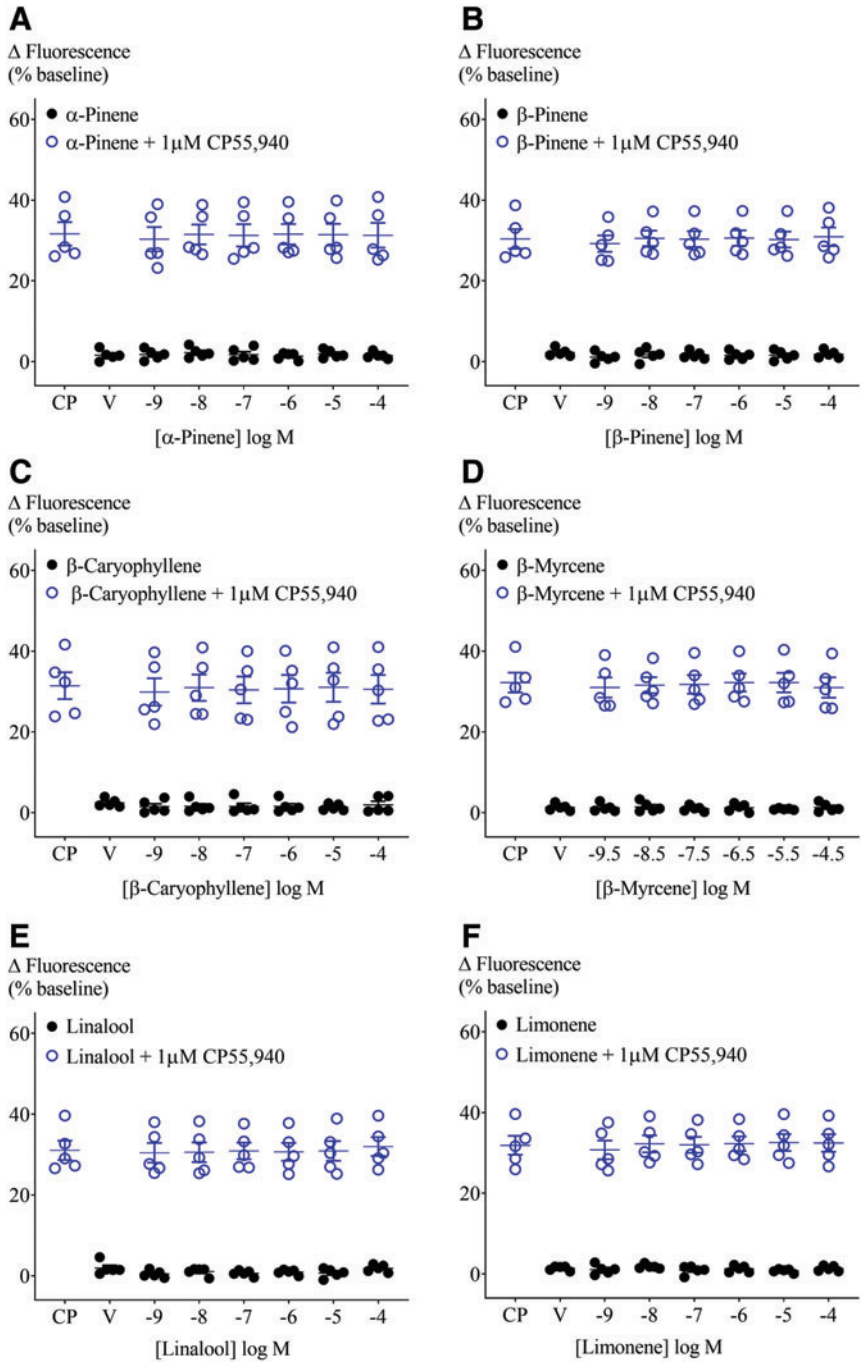
13. Lewis MA, Russo EB, Smith KM. Pharmacological foundations of Cannabis chemovars. Planta Med. 2018;84:225–233.
14. Russo EB, Marcu J. Cannabis pharmacology: the usual suspects and a few promising leads. Adv Pharmacol. 2017;80:67–134.
15. Suraev A, Lintzeris N, Stuart J, et al. Composition and use of Cannabis extracts for childhood epilepsy in the Australian community. Sci Rep. 2018;8:10154.
16. Banister SD, Longworth M, Kevin R, et al. Pharmacology of valinate and tert-leucinate synthetic Cannabinoids 5F-AMBICA, 5F-AMB, 5F-ADB, AMB-FUBINACA, MDMB-FUBINACA, MDMB-CHMICA, and their analogues. ACS Chem Neurosci. 2016;7:1241–1254.
17. Knapman A, Santiago M, Du YP, et al. A continuous, fluorescence-based assay of mu-opioid receptor activation in AtT-20 cells. J Biomol Screen. 2013;18:269–276.
18. Gunther T, Culler M, Schulz S. Research resource: real-time analysis of somatostatin and dopamine receptor signaling in pituitary cells using a fluorescence-based membrane potential assay. Mol Endocrinol. 2016;30: 479–490.
19. Soethoudt M, Grether U, Fingerle J, et al. Cannabinoid CB2 receptor ligand profiling reveals biased signalling and off-target activity. Nat Commun. 2017;8:13958.
20. Cawston EE, Redmond WJ, Breen CM, et al. Real-time characterization of cannabinoid receptor 1 (CB1) allosteric modulators reveals novel mechanism of action. Br J Pharmacol. 2013;170:893–907.
21. Cawston EE, Connor M, Di Marzo V, et al. Distinct temporal fingerprint for cyclic adenosine monophosphate (cAMP) signaling of indole-2-carboxamides as allosteric modulators of the Cannabinoid receptors. J Med Chem. 2015;58:5979–5988.
22. Gertsch J, Leonti M, Raduner S, et al. Beta-caryophyllene is a dietary cannabinoid. Proc Natl Acad Sci U S A. 2008;105:9099–9104.
23. Harris HM, Rousseau MA, Wanas AS, et al. Role of cannabinoids and terpenes in cannabis-mediated analgesia in rats. Cannabis and Cannabinoid Research. 2019;4:177–182.
24. Price MR, Baillie GL, Thomas A, et al. Allosteric modulation of the cannabinoid CB1 receptor. Mol Pharmacol. 2005;68:1484–1495.
25. Ignatowska-Jankowska BM, Baillie GL, Kinsey S, et al. A Cannabinoid CB1 receptor-positive allosteric modulator reduces neuropathic pain in the mouse with no psychoactive effects. Neuropsychopharmacology. 2015; 40:2948–2959.
26. Bacci A, Huguenard JR, Prince DA. Long-lasting self-inhibition of neocortical interneurons mediated by endocannabinoids. Nature. 2004;431:312–316.
27. Marinelli S, Pacioni S, Cannich A, et al. Self-modulation of neocortical pyramidal neurons by endocannabinoids. Nat Neurosci. 2009;12: 1488–1490.
28. Felder CC, Joyce KE, Briley EM, et al. Comparison of the pharmacology and signal transduction of the human cannabinoid CB1 and CB2 receptors. Mol Pharmacol. 1995;48:443–450.
29. Ibsen MS, Connor M, Glass M. Cannabinoid CB1 and CB2 receptor signaling and bias. Cannabis Cannabinoid Res. 2017;2:48–60.
30. Atwood BK, Wager-Miller J, Haskins C, et al. Functional selectivity in CB(2) cannabinoid receptor signaling and regulation: implications for the therapeutic potential of CB(2) ligands. Mol Pharmacol. 2012;81: 250–263.
31. Banister SD, Arnold JC, Connor M, et al. Dark classics in chemical neuroscience: delta(9)-tetrahydrocannabinol. ACS Chem Neurosci. 2019; 10:2160–2175.

Cite this article as: Santiago M, Sachdev S, Arnold JC, McGregor IS, Connor M (2019) Absence of entourage: terpenoids commonly found in *Cannabis sativa* do not modulate the functional activity of Δ^9-THC at human CB_1 and CB_2 receptors, *Cannabis and Cannabinoid Research* 4:3, 165–176, DOI: 10.1089/can.2019.0016.

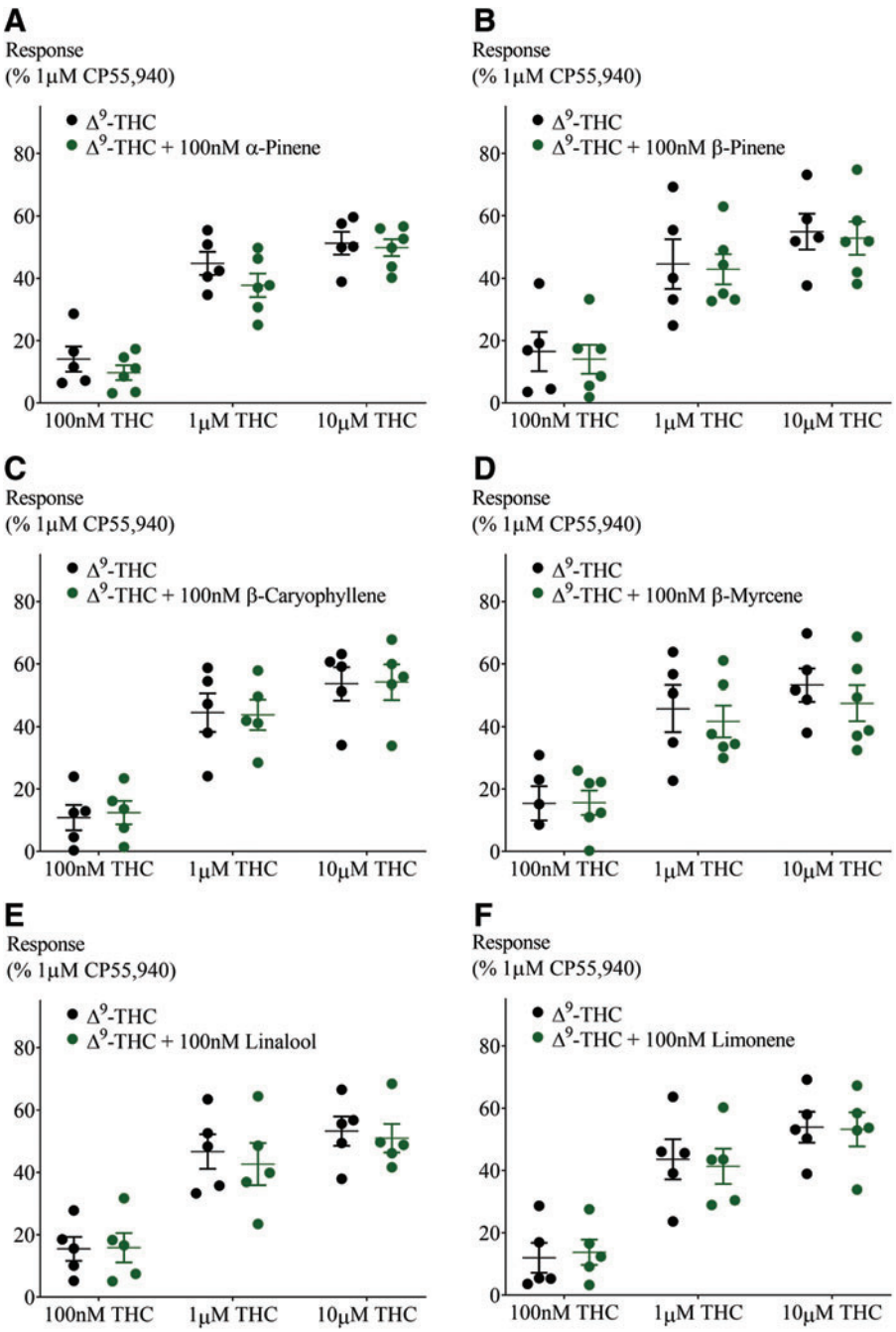
Abbreviations Used

Δ^9-THC = Δ^9-tetrahydrocannabinol
β-Car = β-caryophyllene
ANOVA = analysis of variance
CBD = cannabidiol
DMEM = Dulbecco's modified Eagle's medium
DMSO = dimethyl sulfoxide
FBS = fetal bovine serum
HBSS = Hank's Balanced Salt Solution
RFU = relative fluorescence units
SEM = standard error of the mean
SST = somatostatin
V = vehicle

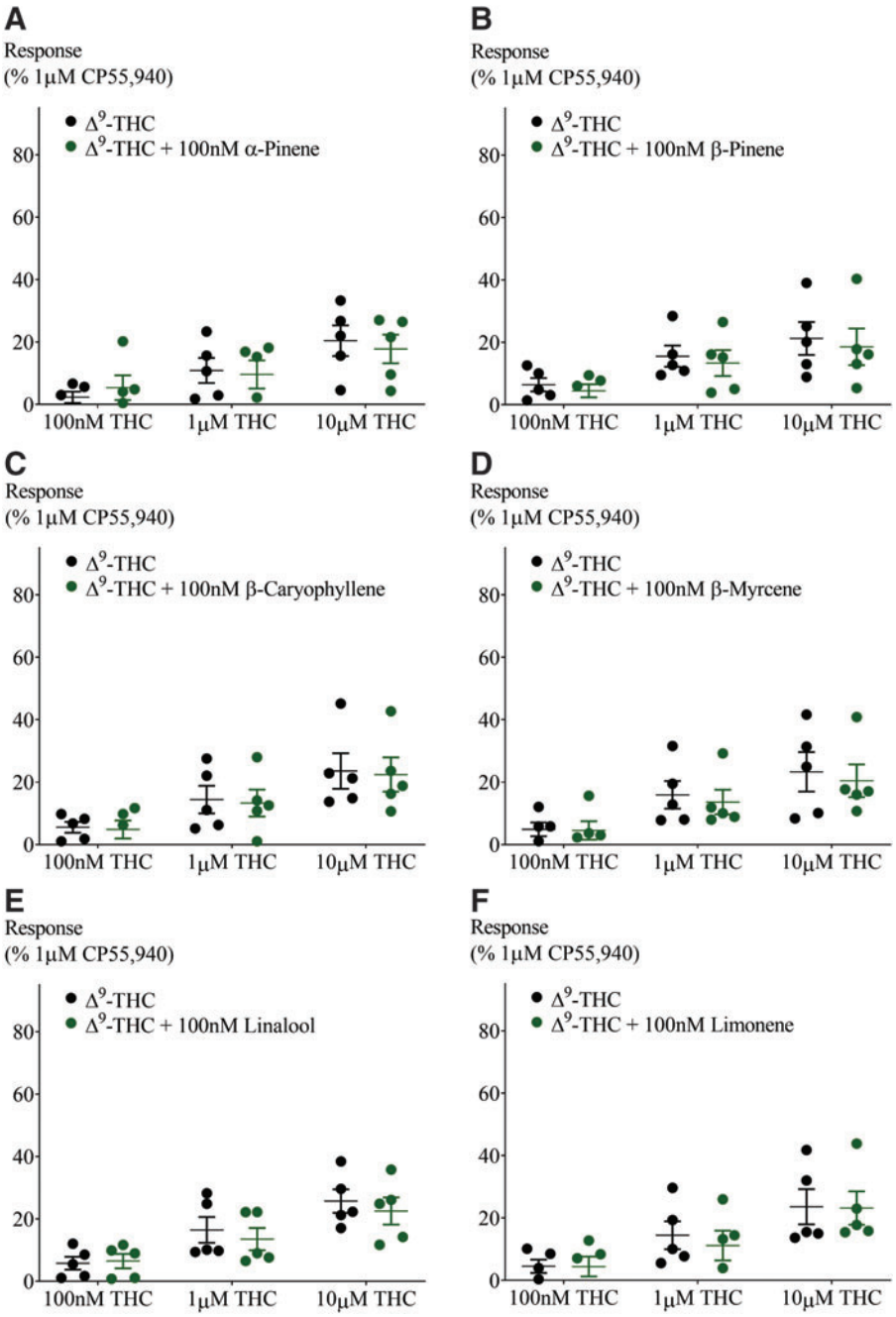
Supplementary Data



SUPPLEMENTARY FIG. S1. Effect of terpenoids at varying concentrations on AtT20-CB_2 membrane potential and on 1 μM CP55,940-induced hyperpolarization. Terpenoids **(A)** α-pinene, **(B)** β-pinene, **(C)** β-caryophyllene, **(D)** β-myrcene, **(E)** linalool, and **(F)** limonene were added to AtT20-CB_2 cells and incubated for 5 min. Maximum fluorescence changes were determined and compared with negative control (HBSS, closed circles). No significant fluorescence difference was observed when comparing means of terpenoids and HBSS ($n = 5$, SEM, unpaired t-test $p = 0.72$). CP55,940 (1 μM) addition to AtT20-CB_2 cells induced fluorescence changes from 30.4% ± 2.4% to 32.2% ± 2.5%. Peak CP55,940 responses were not affected by the presence of terpenoids (open circles, $n = 5$, SEM, unpaired t-test $p > 0.09$). CB_2, cannabinoid receptor 2; HBSS, Hank's Balanced Salt solution; SEM, standard error of the mean; V, vehicle



SUPPLEMENTARY FIG. S2. Effect of 100 nM terpenoids on peak hyperpolarization induced by Δ^9-THC in AtT20-CB_1 cells. Terpenoids tested were **(A)** α-pinene, **(B)** β-pinene, **(C)** β-caryophyllene, **(D)** β-myrcene, **(E)** linalool, and **(F)** limonene. Response to Δ^9-THC at two submaximal and one maximal concentration ($n = 5$, SEM, unpaired t-test $p > 0.24$). Data presented as % of maximum CP55,940 (1 μM) response. Δ^9-THC, Δ9-tetrahydrocannabinol; CB_1, cannabinoid receptor 1.



SUPPLEMENTARY FIG. S3. Effect of 100 nM terpenoids on peak hyperpolarization induced by Δ^9-THC in AtT20-CB_2 cells. Terpenoids tested were **(A)** α-pinene, **(B)** β-pinene, **(C)** β-caryophyllene, **(D)** β-myrcene, **(E)** linalool, and **(F)** limonene. Response to Δ^9-THC at two submaximal and one maximal concentration ($n = 5$, SEM, unpaired t-test $p > 0.50$). Data presented as % of maximum CP55,940 (1 μM) response.

Chapter VI.

Study V. Investigating the specificity of cannabidiol signalling

While the earlier chapters are focused on the pathways involved in the on-target effects associated with SCRA toxicity, Chapter 6 features an exploratory study of the pharmacological effects of cannabidiol (CBD) signalling on multiple receptors, and their corresponding therapeutic advantages. This Chapter presents a detailed molecular pharmacological study of CBD across a range of GPCRs (including CB1, CB2, D2, and MOR) at 37 °C or at room temperature (to mimic the significant number of studies reported to date). The signalling pathways investigated included the $G_{i/o}$-mediated activation of GIRK channels, and inhibition of forskolin-induced cAMP production. Additionally, attempts were made to characterise the negative allosteric-like activity of CBD at CB1. Therefore, the ultimate aim of this work was to determine whether CBD specifically modulates receptor(s) signalling at a physiologically relevant temperature or the effects observed in vitro are confounded by variables such as temperature.

Contributions to the work

This paper represents a collaborative work hosted in Mark Connor's laboratory at Macquarie University Australia. I took the lead role in experimental design, conducting the experiments, data analysis and writing the paper with support from my co-investigators: Mark Connor oversaw the work in this research group; Marina Santiago assisted with the experiment related to acute effect of CBD on CB1 and CB2; Preeti Manandhar performed the experiments concerned with the CBD effect on MOR; Michael Udoh assisted with the experiment related to CBD effect on CB2. Cyclic AMP signalling assay were carried out independently in Michelle Glass laboratory to investigate the effect of CBD on CB1 and D2 signalling. All the authors discussed the results and contributed to the manuscript.

Investigation of the specificity of cannabidiol signalling

Shivani Sachdev[1], Marina Santiago[1], Monica Patel[2], Preeti Manandhar[1], Michael Udoh[1], Michelle Glass[2], Mark Connor[1]

[1]Department of Biomedical Sciences, Macquarie University, NSW, Australia; [2]Department of Pharmacology and Toxicology, University of Otago, New Zealand.

6.1. Introduction

The *Cannabis sativa* plant contains approximately 150 phytocannabinoids, including the non-psychoactive compound cannabidiol (CBD) (Hanuš et al., 2016). Unlike Δ^9-tetrahydrocannabinol (THC), which is responsible for the unique psychoactive effects of cannabis, CBD shows acceptable safety and tolerability profile in humans (Machado Bergamaschi et al., 2011), and therefore has gained popularity as a "natural treatment" for a wide range of health conditions (Russo, 2017). There is growing interest in the potential therapeutic effects of CBD as a result of its purported anxiolytic, antipsychotic, antiemetic and anti-inflammatory properties (Zhornitsky and Potvin, 2012, Fernández-Ruiz et al., 2013, Oláh et al., 2014). Currently, the US Food and Drug Administration and the European Medicines Agency have approved CBD (Epidiolex®, GW Pharmaceuticals) for the treatment of Dravet and Lennox-Gastaut Syndromes (rare types of epilepsy in children) (GW Pharmaceuticals, 2018). Sativex, a medication that combines both CBD and THC has also been approved for the alleviation of neuropathic pain in adults suffering from multiple sclerosis (Pertwee, 2012). Given the potential for wide-ranging therapeutic effects of CBD, it is important to understand the pharmacological profile of CBD across potential molecular targets, including G-protein coupled receptors (GPCRs).

The therapeutic interest in CBD has been accompanied by several studies investigating the fundamental signalling mechanism of CBD at multiple targets. These include the activity of CBD at cannabinoid receptors; despite the low binding affinity of CBD for the orthosteric sites of both CB1 and CB2 (as concluded from the binding studies mean Ki was found to be 3245 ± 803 nM – 40-fold less than the binding affinity of THC at CB1 (McPartland et al., 2015)). While some in vitro studies have suggested that CBD may antagonise the cannabinoid-induced effect with K_B values 37-times more potent than their binding affinities

at CB1 (reviewed in (Pertwee, 2008, McPartland et al., 2015)). Laprairie et al. (2014) reported that CBD reduced the potency and efficacy of THC and 2-arachidonoylglycerol on G_q (PLCβ3)- and G_i (ERK1/2)-dependent signalling of CB1, suggesting the negative allosteric activity of CB1 by CBD. In autaptic hippocampal neurons, CBD treatment has also been found to suppress endogenous cannabinoid/CB1-mediated signalling in a fashion consistent with negative allosteric modulation of CB1 (Straiker et al., 2018). However, early studies indicated that the ability of CBD to antagonise CB1-mediated effects may be due to its inhibitory effect on enzymatic hydrolysis of the endocannabinoid anandamide (McPartland et al., 2015). For example, one study showed that CBD inhibits anandamide hydrolysis by FAAH in a rat brain membrane with an IC_{50} of ~10 μM (Leweke et al., 2012). The initial report of opioid receptor binding data led Kathmann et al. (2006) to propose that CBD is an allosteric modulator of mu-opioid receptor, they found that CBD (30 μM) was able to fully inhibit DAMGO binding to rat brain cortical membranes when incubated for 45 min at 25 °C. CBD may also regulate the serotonin $5HT_{1A;}$ GTPγS assay data revealed that 16 μM of CBD increased [^{35}S]GTPγS incorporation by 67 ± 6% in membranes containing $5HT_{1A}$ receptor (though with an efficacy which may not be physiologically relevant) (Russo et al., 2005). At GPR55, several studies have suggested that CBD antagonises HU210- or CP55940-induced [^{35}S]GTPγS binding in HEK293 cell membranes (Drmota et al., 2006, Ryberg et al., 2007). The negative allosteric activity of CBD has also been reported for dopamine receptors in mouse striatal membranes (Bloom and Hillard, 1985). Around a dozen studies exist on the inhibitory effect of CBD on ion channels such as $GABA_A$, α3 glycine receptors, TRPV1, and T-type calcium channels (e.g. Long et al., 2012, Ahrens et al., 2009, Di Marzo et al., 2002, Ross et al., 2008).

Given the promiscuity of CBD interactions with multiple targets, our current understanding of CBD signalling may depend on the system in which it is studied - several of the studies were performed at room temperature without appropriate controls for CBD modulation of effectors, thus the results of these studies are difficult to extrapolate to normal human physiology (reviewed in (Bisogno et al., 2001)). Therefore, we have undertaken a detailed molecular pharmacological study of CBD effects on the signalling of multiple GPCR using uniform *in vitro* assays. In this study, we examined the effects of CBD on acute signalling of multiple receptors (CB1, CB2, D2, and mu-opioid receptor (MOR)) stably transfected into AtT20 or HEK 293 cells. Intriguingly, we found a temperature-dependent effect of CBD modulation of $G_{i/o}$-mediated activation of GIRK channel or inhibition of adenylyl cyclase in

cells expressing CB1, CB2, D2 or MOR (i.e. greater inhibitory effect of CBD at RT compared to physiologically relevant temperature). In fact, we have observed a disparate pharmacological profile of CBD at CB1. We found that CBD selectively inhibited the CB1-mediated cellular hyperpolarisation at physiologically relevant temperature, and that the effect of the submaximally effective concentration of the CB1 allosteric ligand (ORG27569) on desensitisation of receptor signalling was significantly reversed when co-applied with CBD. Obtaining an accurate understanding of CBD activity profile is critical to the meaningful interpretation of CBD function across multiple targets and in assessing its therapeutic potential.

6.2. Methods

6.2.1. Stable cell lines and cell maintenance

Experiments utilized human CB1, CB2, D2, and MOR stably transfected into AtT20 or HEK 293 cells. AtT20 Flp-In cells transfected with human CB1, CB2, or mu-opioid receptor (MOR) (previously described in (Knapman et al., 2014, Banister et al., 2016)) were used for assays of G-protein inwardly rectifying potassium channel (GIRK)-mediated hyperpolarisation. Clonally-isolated HEK 293 cells expressing human CB_1 N terminally-tagged with three haemagglutinin motifs (first described in (Cawston et al., 2013)) or human D2 receptors N terminally-tagged with a FLAG motif (not previously described) were used to measure changes in cAMP. AtT20 and HEK 293 cells were cultured in Dulbecco's Modified Eagle Media (DMEM, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich, St. Louis, MO, USA), 100 units/ml penicillin, 100 μg/ml streptomycin (Thermo Fischer Scientific, Waltham, MA, USA). ATt20 cell media contained 80 μg/ml hygromycin (InvivoGen, San Diego, CA, USA), while media for HEK 293 cells was supplemented with 250 μg/ml zeocin. Cells were grown in 75 cm^2 flasks at 37 °C in a humidified 5% CO_2 atmosphere and passaged when 80-90% confluent. Assays were carried out on cells up to 25 passages.

6.2.2. Potassium channel activity measurements

Changes in the membrane potential of cells in response to GIRK activation was measured with the automated fluorometric imaging plate reader (FLIPR) membrane potential (blue) assay kit (Molecular Devices, Sunnyvale, CA) in FlexStation 3 as previously described (Knapman et al., 2013). AtT20 cells from a 90-100% confluent flask was resuspended in 10

ml Leibovitz's (L-15) media supplemented with 1% FBS, 100 units/ml penicillin, 100 µg/ml streptomycin and 15 mM glucose. The cells were seeded in a volume of 90 µl in poly-D-lysine (Sigma-Aldrich) coated clear bottom 96 well microplates. Cells were incubated overnight at 37 °C in humidified ambient CO_2.

On the following day, 90 µl of membrane potential dye diluted in HBSS composed of (mM) NaCl 145, HEPES 22, Na_2HPO_4 0.338, $NaHCO_3$ 4.17, KH_2PO_4 0.441, $MgSO_4$ 0.407, $MgCl_2$ 0.493, $CaCl_2$ 1.26, glucose 5.56 (pH 7.4, osmolarity 315 ± 15), was loaded into each well of the plate 1 hour prior to the measuring fluorescence using the microplate reader. Meanwhile, the drugs (serial dilution of CP55940, or morphine for CB1, CB2 or MOR, respectively) of various concentration were prepared in HBSS supplemented with 0.1% bovine serum albumin (BSA) and 1% DMSO. The cells were excited at a wavelength of 530 nm and emission measured at 565 nm, with cut-off at 550 nm, and the fluorescence was measured every 2s. CBD (10 µM or 100 nM) was added after 2 min of baseline recording and incubated for 5 min after which the drug (10X) was added to give the desired concentrations. The final concentration of DMSO in each well was 0.1-0.11%; which limited the maximum concentration of drug used in the assay. A vehicle (HBSS plus DMSO alone) was included in each column of 96-well microplate to enable correction for any vehicle-dependent change in fluorescence measurements. For experiments examining the prolonged effects of CBD on these receptors, cells were pre-treated with CBD (10 µM or 100 nM) in parallel to the vehicle (control, non-CBD treated) for 60 min (incubated with the membrane potential dye). The percentage change in fluorescence was measured for the cells treated with CBD and vehicle. The measurements were obtained at both physiological (37 °C) and room temperature (24±1°C).

CB1 signalling desensitisation was measured during prolonged activation of GIRK in AtT20 cells as previously described (Cawston et al., 2013, Sachdev et al., 2019). AtT20-CB1 cells were pre-treated with CBD, ORG27569 (ORG, CB1 NAM (Price et al., 2005)), CBD + ORG, or vehicle for 5 min after which CP55940 was added. Desensitisation was calculated as % decline from peak response after 30 min of CP55940 application. The raw data were also fitted to 'plateau followed by one phase association curves' in PRISM (Graph Pad Software Inc., San Diego, CA). The parameter, X0, is the length (in seconds) of the initial plateau phase that represented the minimum hyperpolarisation induced by CP55940 (Y0), and 'plateau' which indicated the recovery of signalling after 30 min in CP55940. The parameter, *tau*, is defined as time constant, expressed in units of time, seconds. The

estimated *tau* value for each data set was compared between the different experimental conditions. On rare occasions where CP55940 failed to produce a substantial CB1 desensitisation, the *tau* was constrained to the time at which the association curve ended.

6.2.3. cAMP measurement

BRET-CAMYEL assays to measure cellular cAMP levels were performed as previously described by Jiang et al. (2007) and Cawston et al. (2013). HEK 293 cells expressing human CB_1 or D2 receptors were seeded in 10 cm culture dishes (Corning®, NY, USA) at densities such that confluency would be approximately 50% after either 24 or 48 hours. At this point, the culture medium was replaced, and cells were transfected with 5 µg pcDNA-His-CAMYEL plasmid with 30 µg linear polyethyleneimine (PEI, Polysciences, Warrington, PA, U.S.A.). Cells were incubated overnight before being lifted and plated at a density of 70,00-80,000 cells/well in poly-D-lysine (0.05 mg/mL, PDL; Sigma-Aldrich) coated, white 96-well CulturPlate plates (PerkinElmer, Waltham MA, U.S.A.), and grown overnight. For assaying, culture medium was aspirated, cells were washed once with PBS and then serum-starved for at least 30 minutes in phenol-red free DMEM (Thermo Fisher Scientific, Waltham MA, U.S.A.), supplemented with 1 mg/mL fatty acid-free BSA (ICPBio, Auckland, NZ) and 10 mM HEPES (Thermo Fisher Scientific) ("assay buffer"). Cells were then pre-incubated with various concentrations of CBD for 15 minutes at either 25 °C or 37 °C in a LUMIstar® Omega luminometer plate reader (BMG Labtech GmbH, Ortenberg, Germany). Coelenterazine-h (final concentration 5 µM; NanoLight Technologies, Pinetop, AZ) was then dispensed and incubated for five minutes in darkness. Drugs (final concentrations 5 µM forskolin with serial dilutions of CP55,940 or quinpirole for CB_1 or D_2, respectively) prepared in assay buffer were then dispensed into assay wells. Luminescence was simultaneously detected at 475 nm and 535 nm for approximately one hour at 25 °C or 20 minutes at 37 °C. Raw data are presented as inverse BRET ratio of emission at 475/535.

6.2.4. Data Analysis

Analysis of all the experiments was performed in GraphPad Prism (Graph Pad Software Inc., San Diego, CA). Membrane potential assay were represented as a percentage of baseline fluorescence, following correction of the vehicle responses. Concentration response curves were fit to the four-parameter logistic equation in PRISM v8 to obtain the EC_{50} and E_{max} values. For cAMP measurement, inverse BRET ratios (475/535 nm) were exported

from Omega MARS software (V3.1 R5, BMG Labtech GmbH) and data were further analysed in Prism v8, with responses normalised to forskolin (100%) and vehicle (0%). Unpaired *t*-tests were used when comparing two data points, and one-way ANOVA for more than two data points with one independent variable. Data are expressed as the mean ± SEM of at least 5 independent determinations performed in duplicate, unless otherwise noted. Effects with *p* values less than 0.05 were considered to be statistically significant.

6.2.5. Materials

Drugs stocks were made up in DMSO and diluted on the day of the assay. CP55940, SR141716A, AM630, and quinpirole was purchased from Cayman Chemical Company (Ann Arbour, MI, USA); CBD was purchased from National Measurement Institute (Sydney, NSW, Australia), ORG27569 and somatostatin were from Hello Bio (Bristol, UK) or Auspep (VIC, Australia). Morphine was a kind gift from Department of Pharmacology, University of Sydney. All the drugs were stored in aliquots of 30 mM at –30 °C until needed.

6.3. Results

6.3.1. Studies of K channel activation

6.3.1.1. Effect of CBD on CB1 receptor signalling

Previous reports suggested that CBD could act as a negative allosteric modulator of CB1 receptor signalling at concentrations well below the reported affinity (K_i) of CBD at the CB1 orthosteric site (Thomas et al., 2007, Hayakawa et al., 2008, Laprairie et al., 2015, Straiker et al., 2018). Therefore, we initially examined the ability of CBD (10 μM or 100 nM) to modify canonical CB1 receptor $G_{i/o}$-mediated activation of GIRK channel in AtT20 cells. Application of CBD (10 μM or 100 nM) for 5 min did not significantly affect the hyperpolarisation induced by CP59940 compared to vehicle treated cells at physiologically relevant temperature (Control, pEC$_{50}$ 7.5 ± 0.1, max 34 ± 1.7%; in CBD 100 nM pEC$_{50}$ 7.5 ± 0.1, max 34 ± 1.9%; in CBD 10 μM pEC$_{50}$ 7.3 ± 0.3, max 32 ± 1.8%) (Figure 6-1, $P > 0.05$). However, before agonist addition, CBD (10 μM) modestly but significantly produced a consistent decrease in the resting membrane potential of the cells by itself (Supplementary Figure 6-1A, $P < 0.05$). We performed the same experiments at 24 °C to closely mimic a significant number of key studies to date. In comparison with the assay performed at 37 °C, pre-treatment with CBD (10 μM, 5 min) at 24 °C inhibited the hyperpolarisation induced by CP55940 in the same cells (Control, pEC$_{50}$ 7.2 ± 0.1, max 29 ± 1.3%; in CBD 10 μM pEC$_{50}$

6.6 ± 0.2, max 21 ± 1.8%) (Figure 6-1). At 25 °C 100 nM CBD had no effect on the CP55940 signalling (Control, pEC_{50} 7.2 ± 0.1, max 29 ± 1.3%; in CBD 100 nM pEC_{50} 7.1 ± 0.1, max 27 ± 1.2%). Unlike 37 °C, brief CBD treatment at 24 °C did not produce a change in the membrane potential by itself. To further understand the effects of CBD alone at 37 °C on the membrane potential of the AtT20-CB1 cells prior to agonist addition, we applied CBD for 60 min at a concentration up to 10 µM, and found that CBD produced a maximum change in fluorescence of 9 ± 1.2%; however when co-applied with SR141716A 1 µM (CB1 antagonist), we did not observe any significant difference in the CBD response (Supplementary Figure 6-1B), implicating that these effects were occurring via mechanism independent of CB1 orthosteric site.

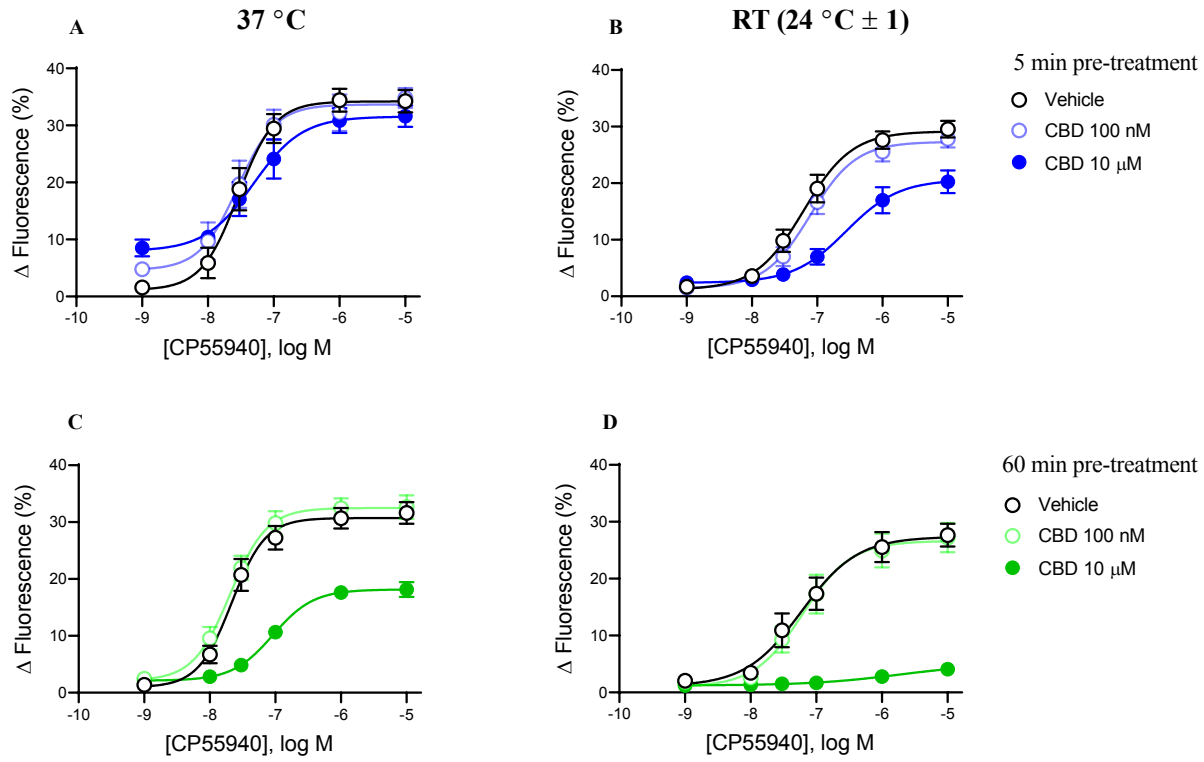


Figure 6-1. Effect of CBD on CP55940-mediated CB1 signalling

Concentration response curve showing CP55940 induced hyperpolarisation of CB1 pre-treated with vehicle (○), or CBD 100 nM (○), or CBD 10 µM (●) for 5 min at 37 °C (A) and at RT (B). Second panel of concentration response curve of CP55940 pre-treated with vehicle (○), or CBD 100 nM (○), or CBD 10 µM (●) for 60 min at 37 °C (C) and at RT (D). Data represents mean ± SEM of six independent determinants performed in duplicate.

To determine whether more prolonged application of CBD can affect the CB1 receptor signalling, we pre-treated AtT20-CB1 cells with CBD (10 μM or 100 nM) for 60 min prior to the subsequent addition of CP55940 at 37 °C and 24 °C. Pre-treatment of cells at 37 °C with CBD (10 μM, 60 min) inhibited the hyperpolarisation induced by CP55940 (Control, pEC$_{50}$ 7.7 ± 0.1, max 31 ± 1.3%; in CBD 10 μM pEC$_{50}$ 7 ± 0.1, max 18 ± 0.7%) (Figure 6-1), consistent with a negative allosteric-like activity of CBD at CB1. By contrast, CBD (100 nM) did not affect the CP55940 induced hyperpolarisation in AtT20-CB1 cells (Control, pEC$_{50}$ 7.7 ± 0.1, max 31 ± 1.3%; in CBD 100 nM pEC$_{50}$ 7.7 ± 0.1, max 32 ± 1.2%). When the experiments were performed at 24 °C, CBD (10 μM, 60 min) abolished the effects of CP55940 compared with vehicle treated cells (Control, pEC$_{50}$ 7.2 ± 0.2, max 27 ± 2%; in CBD 10 μM pEC$_{50}$ 5.7 ± 2, max 5 ± 4%) (Figure 6-1), while 100 nM of CBD was without effect (Control, pEC$_{50}$ 7.2 ± 0.2, max 27 ± 2%; in CBD 100 nM pEC$_{50}$ 7.2 ± 0.2, max 27 ± 2%).

Based on the functional profile of CBD to inhibit CP55940-induced signalling of CB1; we hypothesised that CBD is an allosteric modulator of CB1 and that may behave in a similar way as ORG, altering CB1 desensitisation time course. We investigated this theory by pre-treating the AtT20-CB1 cells with CBD or ORG individually, in combination (CBD + ORG), or vehicle for 5 min prior to the subsequent application of CP55940 for 30 min. The presence of CBD (10 μM – 100 nM, 5 min) alone did not significantly affect the desensitisation produced by CP55940 (100 nM) compared to control cells (Control 64 ± 4%, CBD 10 μM treated 64 ±7%, CBD 1 μM treated 74 ± 6%, CBD 100 nM treated 61 ± 5%) (Supplementary Figure 6-2). Consistent with our previous results (Cawston et al., 2013), the desensitisation of CP55940 induced hyperpolarisation was potentiated in the continuous presence of ORG (10 μM and 1 μM) compared to corresponding CP55940 response without ORG (Control 64 ± 4%, ORG 10 μM treated 120 ± 4%, ORG 1 μM treated 100 ± 3%) (Supplementary Figure 6-2). However, when CBD and ORG was added together, the hyperpolarisation produced by CP55940 reversed faster in the presence of 'CBD (10 μM) + ORG (1 μM)' compared to ORG alone (CBD + ORG treated *tau* 405s, 92 ± 6%; ORG treated *tau* 791s, 100 ± 3%) (Figure 6-2). It was also observed that the CB1 receptor signal also desensitised to CP55940 at a much slower rate without CBD and ORG mix (Control *tau* 1257s, 64 ± 4%). Meaning that CBD exerts its effect at a faster rate in presence of ORG, while desensitisation (peak response at 30 min) of receptor signalling was reduced when

CBD and ORG were added together, might therefore, suggest a complex signalling profile of CBD different to negative allosteric behaviour of ORG at CB1.

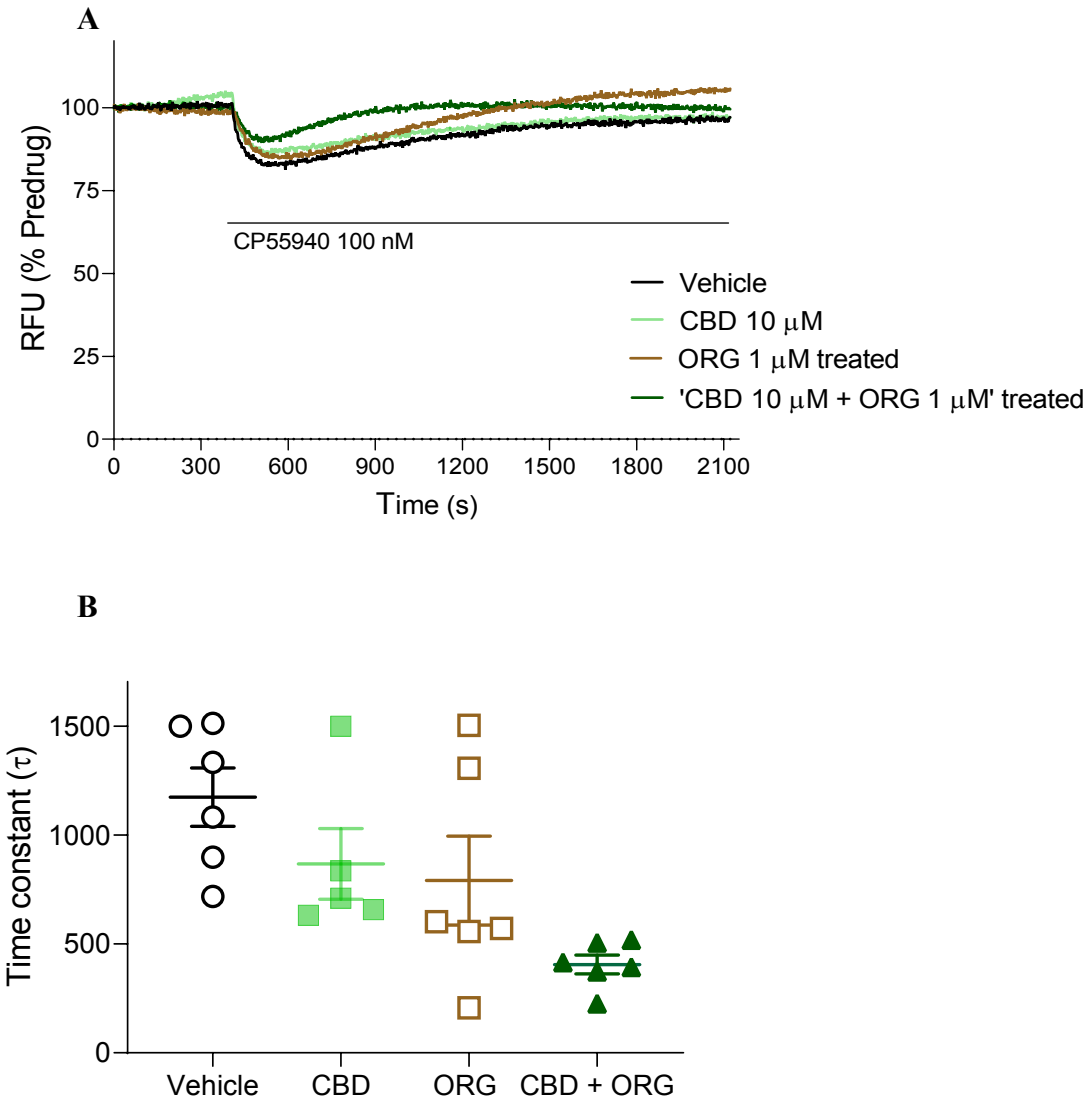


Figure 6-2. Effect of CBD on CB1 signalling during continuous CP55940 exposure

(A) Representative trace showing AtT20-CB1 cells pre-treated with vehicle, or ORG (1 μM), or in combination (CBD 10 μM + ORG 1 μM) for 5 min prior to the subsequent application of CP55940 for 30 min. (B) Within each set, condition 'Vehicle' was compared to 'CBD', or 'ORG', or 'CBD + ORG'. Data represent mean ± SEM of at least five independent determinants performed in duplicate.

6.3.1.2. Effect of CBD on CB2 receptor signalling

CBD has previously, though inconsistently, being reported as an antagonist of CB2 receptor (Thomas et al., 2007). In AtT20-CB2 cells, CP55940 produced concentration-dependent hyperpolarisation (pEC_{50} 7.2 ± 0.1, max 30 ± 2%); pre-treatment with CBD (10 μM or 100 nM) for 5 min at 37 °C had no effect on the observed CP55940 signalling (in CBD 100 nM pEC_{50} 7.2 ± 0.2, max 30 ± 2%; in CBD 10 μM pEC_{50} 7 ± 0.1, max 31 ± 1.4%) (Figure 6-3). Furthermore, we observed no significant difference in CP55940 signalling at 24 °C when co-applied with CBD (Control, pEC_{50} 7.1 ± 0.1, max 28 ± 1.9%; in CBD 100 nM pEC_{50} 6.9 ± 0.1, max 29 ± 2%; in CBD 10 μM pEC_{50} 6.7 ± 0.1, max 26 ± 1.6%). When applied as a 60 min pre-treatment, CBD 10 μM inhibited the CP55940 induced hyperpolarisation at both temperatures (at 37 °C Control, pEC_{50} 7.4 ± 0.1, max 35 ± 1.3%; in CBD 10 μM pEC_{50} 7.1 ± 0.1, max 29 ± 0.8%; at 24 °C Control, pEC_{50} 6.9 ± 0.1, max 31 ± 1.4%; in CBD 10 μM pEC_{50} 6.3 ± 0.1, max 21 ± 1%) (Figure 6-3); however, the extent of inhibition was small in comparison with the effect on CB1 receptors at the same concentration (CB2 receptor 14 ± 4%, CB1 receptor 37 ± 6%, summarised for all receptors in Figure 6-6). CBD 100 nM (60 min) had no measurable effects on the CP55940 signalling at either temperature. Although CBD alone (10 μM, 60 min) produced a small decrease in fluorescence in AtT20-CB2 cells (5 ± 1.4%), this was not blocked by CB2 antagonist (AM630, 3 μM, $P > 0.05$), indicating that this was unlikely to represent the CB2 orthosteric-agonist effect (Supplementary Figure 6-3).

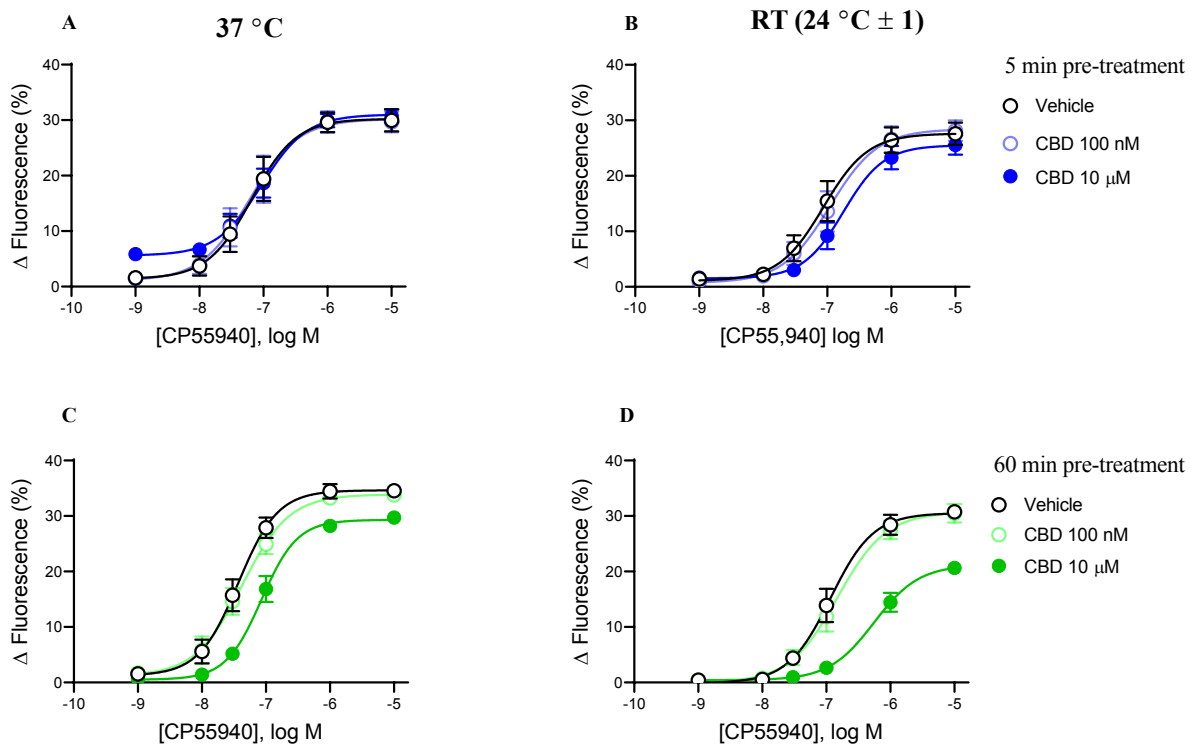


Figure 6-3. Effect of CBD on CP55940-mediated CB2 signalling

Concentration response curve showing CP55940 induced hyperpolarisation of CB2 pre-treated with vehicle (○), or CBD 100 nM (○), or CBD 10 µM (●) for 5 min at 37 °C (A) and at RT (B). Second panel of concentration response curve of CP55940 pre-treated with vehicle (○), or CBD 100 nM (○), or CBD 10 µM (●) for 60 min at 37 °C (C) and at RT (D). Data represents mean ± SEM of six independent determinants performed in duplicate.

6.3.1.3. Effect of CBD on MOR signalling

As previously reported (Kathmann et al., 2006), CBD possesses an allosteric effect on ligand binding at the mu-opioid receptor (MOR). In order to study the functional consequences of CBD on MOR, we pre-treated the AtT20-MOR with CBD for 5 min and 60 min respectively before the addition of morphine (Figure 6-4). Treatment with CBD (10 µM or 100 nM) for 5 min at 37 °C or 24 °C failed to affect the morphine-induced hyperpolarisation of MOR compared to vehicle treated cells (at 37 °C Control, pEC_{50} 7.4 ± 0.1, max 30 ± 1%; in CBD 10 µM pEC_{50} 7.5 ± 0.1, max 32 ± 2%; at 24 °C Control, pEC_{50} 6.7 ± 0.4, max 24 ± 5%; in CBD 10 µM pEC_{50} 6.7 ± 0.6, max 26 ± 7%) (Figure 6-4).

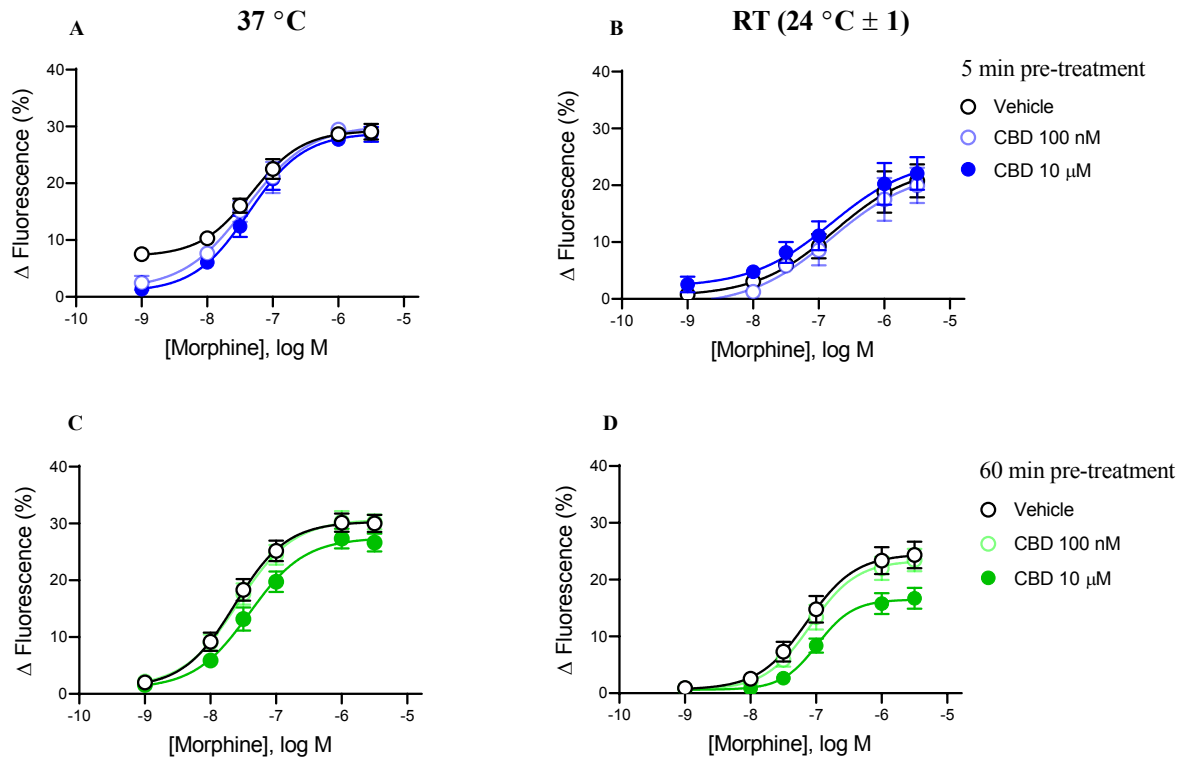


Figure 6-4. Effect of CBD on Morphine-mediated mu-opioid receptor signalling

Concentration response curve showing Morphine induced hyperpolarisation of MOR pre-treated with vehicle (⚬), or CBD 100 nM (◦), or CBD 10 μM (•) for 5 min at 37 °C (A) and at RT (B). Second panel of concentration response curve of Morphine pre-treated with vehicle (⚬), or CBD 100 nM (◦), or CBD 10 μM (•) for 60 min at 37 °C (C) and at RT (D). Data represent mean ± SEM of six independent determinants performed in duplicate.

Application of CBD (10 μM or 100 nM) for 60 min at 37 °C also failed to affect the morphine-mediated MOR activation, showing the lack of effect of CBD on MOR signalling at physiologically relevant temperature (Control, pEC_{50} 7.7 ± 0.1, max 31 ± 1.2%; in CBD 100 nM pEC_{50} 7.6 ± 0.2, max 31 ± 1.3%; in CBD 10 μM pEC_{50} 7.4 ± 0.1, max 28 ± 1.4%). By contrast, pre-treatment with CBD 10 μM for 60 min at 24 °C inhibited the morphine induced hyperpolarisation (Control, pEC_{50} 7.2 ± 0.1, max 25 ± 2%; in CBD 10 μM pEC_{50} 7 ± 0.1, max 17 ± 1%) (Figure 6-4). Thus, CBD (10 μM, 60 min) inhibits CB2 and MOR signalling to a similar extent at 24 °C (MOR 30± 3%, CB2 31 ± 4%), however under these conditions the effect of CBD on these receptors was much less than the effect on CB1 receptor signalling (84 ± 2% inhibition) (Figure 6-6).

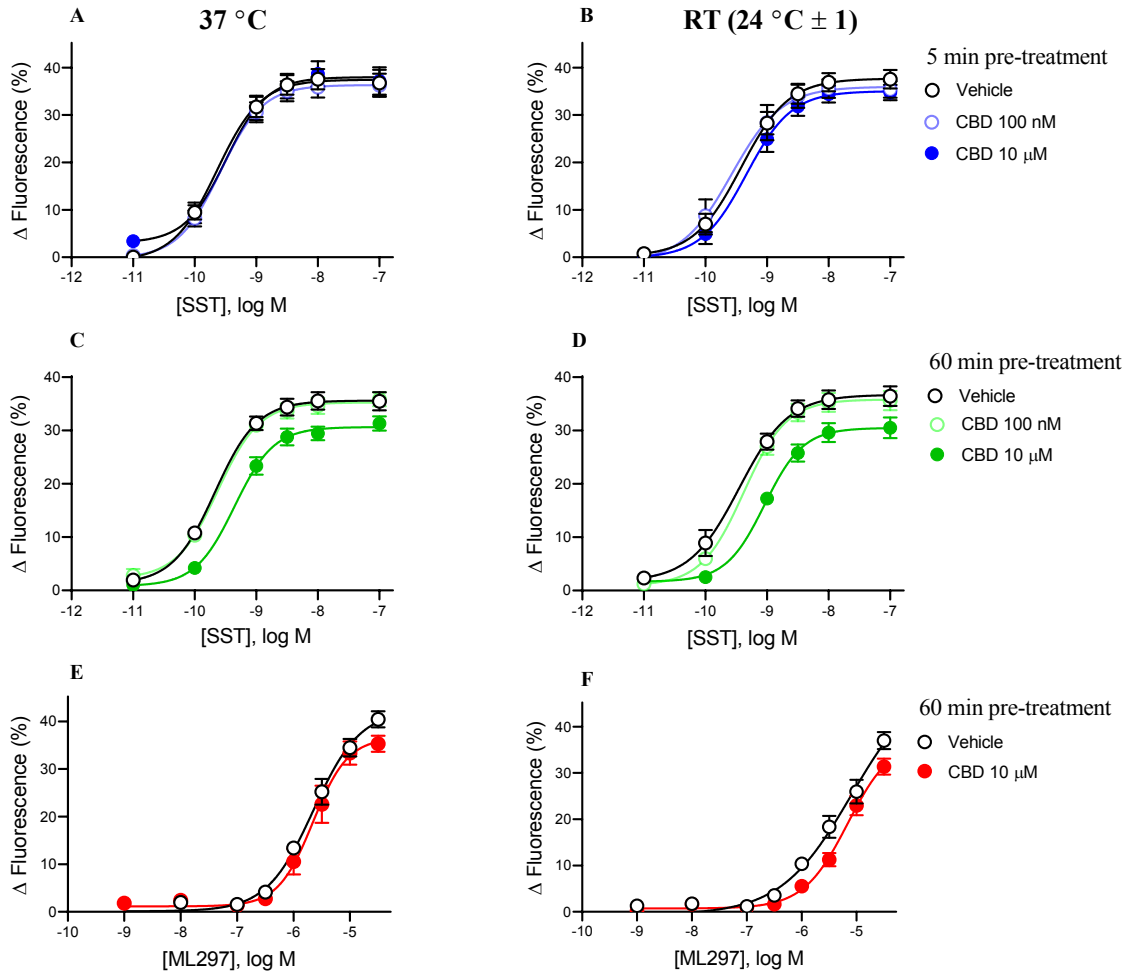


Figure 6-5. Effect of CBD on AtT20-WT signalling

Concentration response curve showing native Somatostatin (SST) induced hyperpolarisation of AtT20 WT pre-treated with vehicle (○), or CBD 100 nM (○), or CBD 10 μM (●) for 5 min at 37 °C (A) and at RT (B). Second panel of concentration response curve of SST pre-treated with vehicle (○), or CBD 100 nM (○), or CBD 10 μM (●) for 60 min at 37 °C (C) and at RT (D). the third panel showing the concentration response curve of ML297 pre-treated with vehicle (○), or CBD 10 μM (●) for 60 min at 37 °C (E) and at RT (F). Data represent mean ± SEM of six independent determinants performed in duplicate.

6.3.1.4. Effect of CBD interaction with wild-type AtT20 cells

To determine whether CBD non-specifically interferes with GPCR signalling in AtT20 cells, we tested the effect of CBD on native somatostatin receptors (SST), which hyperpolarise AtT20-WT cells via GIRKs (Cervia et al., 2003) (Figure 6-5). Application of CBD (10 μM or 100 nM) for 5 min did not affect the hyperpolarisation to the subsequently added SST at both 37 °C (Control, pEC_{50} 9.6 ± 0.1, max 38 ± 1.4%; in CBD 100 nM pEC_{50} 9.6 ± 0.1, max 36 ± 1.4%; in CBD 10 μM pEC_{50} 9.5 ± 0.2, max 38 ± 2%), and 24 °C (Control, pEC_{50} 9.4 ± 0.1, max 38 ± 1.5%; in CBD 100 nM pEC_{50} 9.6 ± 0.2, max 36 ± 2%; in CBD 10 μM pEC_{50} 9.3 ± 0.1, max 35 ± 1.5%) respectively (Figure 6-5). Similarly to the effects of CBD at CB2, pre-treatment with CBD 10 μM for 60 min, modestly, but significantly inhibited the hyperpolarisation produced by SST alone (at 37 °C Control, pEC_{50} 9.7 ± 0.1, max 36 ± 1%; in CBD 10 μM pEC_{50} 9.4 ± 0.1, max 31 ± 1%; at 24 °C Control, pEC_{50} 9.5 ± 0.1, max 37 ± 1.3%; in CBD 10 μM pEC_{50} 9 ± 0.1, max 31 ± 1%); however CBD 100 nM produced no change in the SST signalling (Figure 6-5). We also note that CBD 10 μM inhibits CB2 and SST signalling to a similar degree (CB2 14 ± 4%, SST 14 ± 4%). Because CBD 10 μM, 60 min non-specifically interferes with the receptor signalling in AtT20 cells, we sought to establish whether the decline in signalling was due to its effect on GIRK channel. To address this, we assessed the effect of CBD on the hyperpolarisation produced by ML297, a direct activator of GIRK channel. The results indicate that 60 min treatment with CBD 10 μM at 37 °C or 24 °C produced a minimal change in ML297-mediated activation of GIRK channel (Figure 6-5, $P > 0.05$), indicating that GIRK channel does not have direct consequences on the signalling profile of CBD.

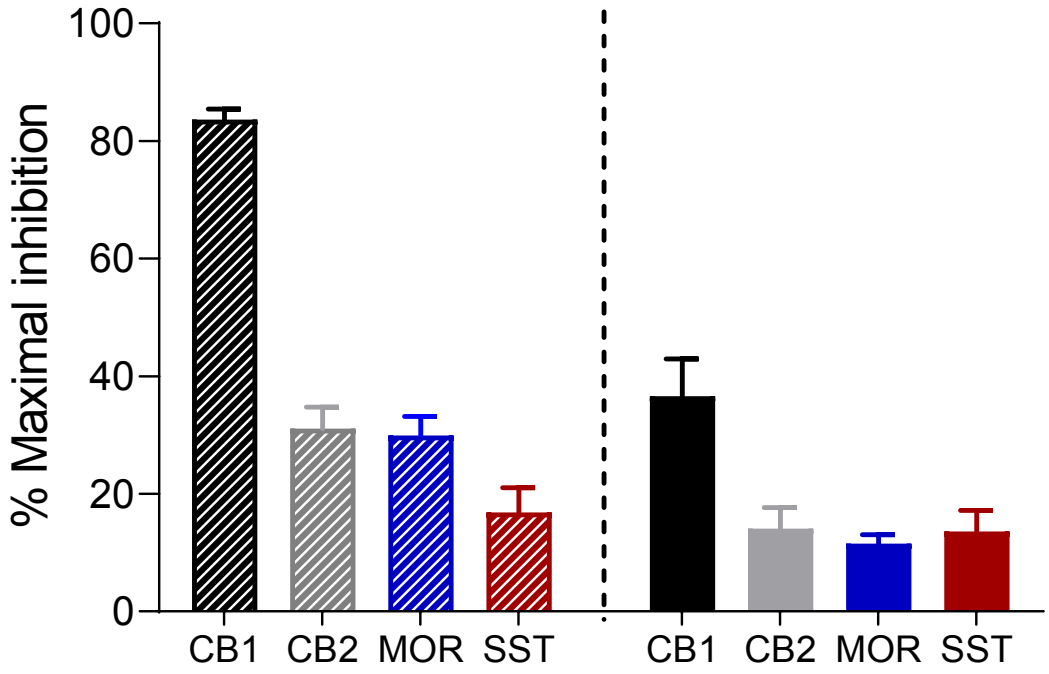


Figure 6-6. Specificity of 10 µM CBD signalling

Summarised data comparing CBD-induced % maximal inhibition of multiple receptors (including CB1, CB2, MOR (µ-receptor), SST) signalling when treated for 60 mins at RT (left panel), and 37 °C (right panel). Data represent mean ± SEM of six independent determinants performed in duplicate.

6.3.2. Studies of cyclic AMP measurements

6.3.2.1.Effect of CBD on CB1 signalling

Inhibition of cAMP is another significant signalling pathway associated with cannabinoid receptor activation (Howlett and Fleming, 1984). Therefore, we examined the ability of CBD to modify cAMP levels in HEK 293-CB1 cells. CBD (10 µM or 100 nM) pre-treatment for 15 min at 37 °C failed to affect the CP55940-mediated inhibition of forskolin-induced cAMP response (Control, pEC_{50} 7.7 ± 0.1, to a minimum of 39 ± 1%; in CBD 100 nM pEC_{50} 7.7 ± 0.1, to a minimum of 38 ± 1.3%; in CBD 10 µM pEC_{50} 7.3 ± 0.1, to a minimum of 45 ± 3%) (Figure 6-7). However, when the experiments were performed at 25 °C, CBD 10 µM appeared to block the inhibition of FSK-stimulated cAMP activity by CP55940, though a definite change in baseline was also observed (Control, pEC_{50} 7.9 ± 0.1, to a minimum of

40 ± 1.3%; in CBD 10 µM pEC_{50} 7.4 ± 0.2, to a minimum of 64 ± 4%); while CBD 100 nM had no impact on the CP55940 inhibition of cAMP levels (pEC_{50} 7.9 ± 0.1, to a minimum of 40 ± 1.5%) (Figure 6-7).

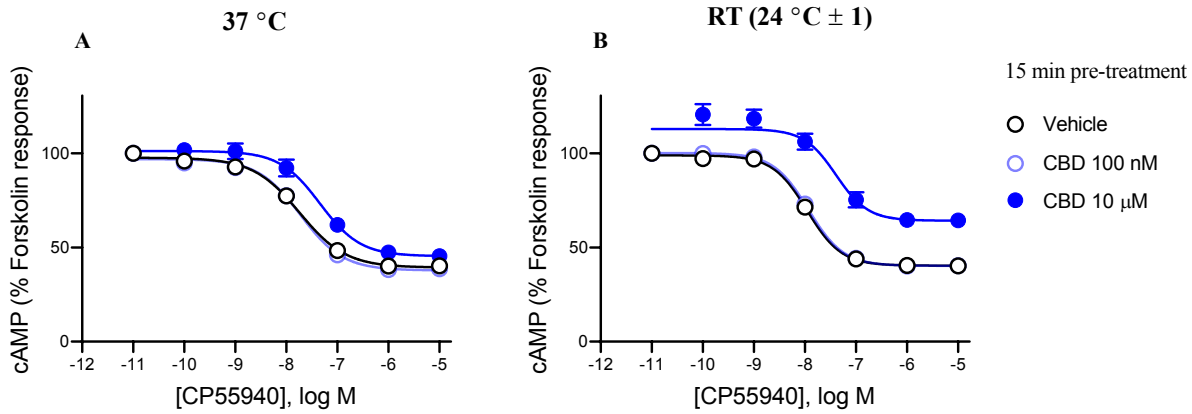


Figure 6-7. Concentration response curve of CP55940-induced inhibition of cAMP levels in HEK-CB1 pre-treated with vehicle (⚬), or CBD 100 nM (○), or CBD 10 µM (●) for 15 min at 37 °C (A) and at RT (B). Data represent mean ± SEM of six independent determinants performed in duplicate.

Data from Glass laboratory

6.3.2.2. Effect of CBD on D2 signalling

CBD has also been reported to act as an allosteric modulator of dopamine D2 (Bloom and Hillard, 1985). Therefore, CBD was tested for its ability to antagonize quinpirole-mediated inhibition of cAMP in HEK cells expressing D2. At 10 µM of CBD (15 min, 25 °C), apparent blockade of quinpirole-mediated inhibition of forskolin-induced cAMP response was observed (Control, pEC_{50} 9.1 ± 0.1, to a minimum of 45 ± 1%; in CBD 10 µM pEC_{50} 9.1 ± 0.1, to a minimum of 57 ± 2%) with the extent of the effect being smaller than that of CB1 receptors at the same concentration of CBD (D2 13 ± 2%, CB1 24 ± 3%). It was also noted that CBD produced an apparent shift in the baseline similar to that in HEK-CB1. While CBD (100 nM) did not affect the inhibition of cAMP accumulation by quinpirole (in CBD 100 nM pEC_{50} 9.1 ± 0.1, to a minimum of 45 ± 2%) nor shifted the baseline (Figure 6-8). Application of CBD (10 µM or 100 nM) for 15 min at 37 °C failed to affect the quinpirole inhibition of cAMP levels (Control, pEC_{50} 9.1 ± 0.1, to a minimum of 41 ± 1%; in CBD 100 nM pEC_{50} 9.1 ± 0.1, to a minimum of 44 ± 1.3%; in CBD 10 µM pEC_{50} 9 ± 0.1, to a minimum of 48 ± 1.4%) (Figure 6-8), suggesting a weak interaction of CBD with D2 receptors.

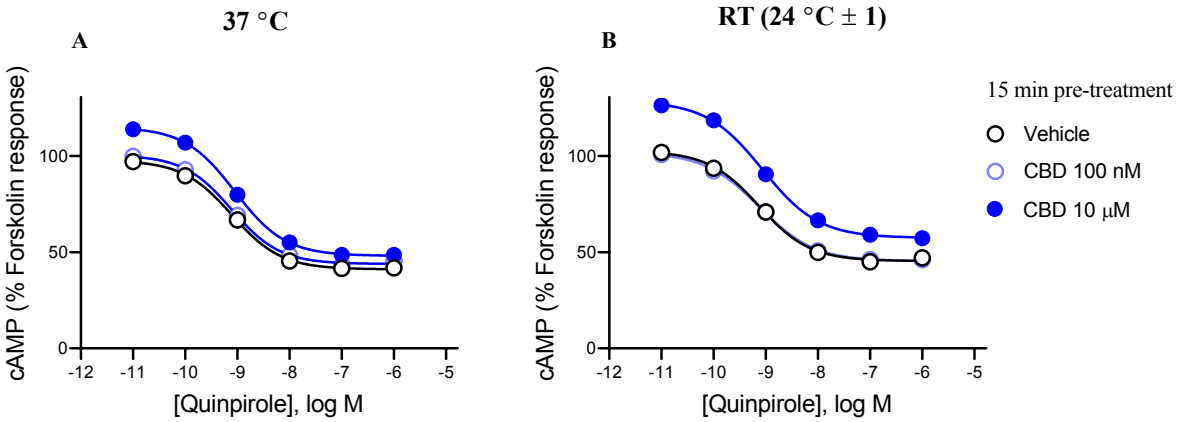


Figure 6-8. Concentration response curve of Quinpirole-induced inhibition of cAMP levels in HEK-D2 pre-treated with vehicle (ʘ), or CBD 100 nM (○), or CBD 10 μM (●) for 15 min at 37 °C (A) and at RT (B). Data represent mean ± SEM of six independent determinants performed in duplicate.

Data from Glass laboratory

6.4. Discussion

CBD is widely touted to possess therapeutic effects for a wide range of health conditions attributed to its' interaction with multiple receptor targets (reviewed in (Devinsky et al., 2014, Peres et al., 2018, Morales et al., 2017)). The primary aim of the current study was to systematically compare the signalling profile of CBD across multiple receptor targets to provide a greater understanding of CBD function and its corresponding therapeutic advantages. The effect of CBD was examined using real-time assays of receptor signalling in AtT20 or HEK 293 cells expressing CB1, CB2, D2, and mu-opioid receptor (MOR) at both room temperature (RT 24 °C ± 1; to closely mimic a significant number of CBD studies to date), and 37 °C. We have provided novel insights into the promiscuous interaction of CBD with multiple GPCRs, including the temperature-dependent effect (given the greater effect of CBD to inhibit CB2, MOR, or D2 signalling at RT compared to 37 °C). However, our data confirms the specific inhibitory effects of CBD on CB1 responses. The non-specific effect of CBD at RT compared to physiological temperature highlights the value of assays with physiological face validity, and also re-enforce the need to include a range of receptors.

Previous studies have demonstrated a broad pharmacological profile of CBD, including interaction with several GPCRs. These include the antagonistic effect of CBD on the CB1

and CB2 receptor (Thomas et al., 2007), allosteric modulation of MOR (Kathmann et al., 2006), and partial agonism of D2R (Bloom and Hillard, 1985). CBD action was first attributed to the cannabinoid receptor - the nanomolar-range antagonistic activity of CBD on CB1 despite its low affinity binding for CB1 orthosteric site (Pertwee, 2008, Thomas et al., 2007, McPartland et al., 2015). This finding gave credence to the idea that CBD may be exerting its antagonistic effect on CB1 by binding to a site different from an orthosteric site (i.e. allosteric site), and thus led to an investigation into the allosteric activity of CBD on CB1. CBD negative allosteric modulation of CB1 has been studied in the past in a battery of in vitro assays for example; arrestin, $G\alpha_q$ (PLCβ3) and $G\alpha_{i/o}$ (ERK1/2) pathways (Laprairie et al., 2015), electrophysiology data from cultural autaptic hippocampal neurons (Straiker et al., 2018), and comparative study of the biased agonism of selective cannabinoids in the presence of CBD (Navarro et al., 2018). A significant limitation to these studies is that the assays were performed at RT - this means that the observed allosteric effect of CBD is confounded by homeostasis, presumably due to change in membrane fluidity as a function of temperature (Shinitzky, 1984, Otto et al., 1984). These considerations have led us to investigate the effect of CBD at a physiologically relevant temperature as well as RT.

Decreasing the temperature from 37 °C to RT (24 °C ± 1) enhanced the inhibitory effects of CBD on multiple receptor (including CB1, CB2, D2, and MOR) signalling, indicating that the observed inhibitory effects of CBD is temperature-dependent and nonspecific. A possible explanation for this is that at a lower temperature the overall fluidity of the membrane decreases, and thereby affects the bilayer composition (Shinitzky, 1984). The decrease in membrane fluidity (i.e. more close and regular packing of the lipid bilayer) is likely to have an indirect effect on the conformational transitions of the active state (ligand-receptor-G protein ternary complex) of the receptors, and indeed would affect the overall signalling profile of receptors (Altiere et al., 1981, Wei and Sulakhe, 1982); this is observed in our data when comparing maximum hyperpolarisation of CP55940 response at 24 °C and 37 °C. CBD is a lipid-soluble drug which may further modify lipid membrane characteristics by affecting cholesterol's role in maintaining membrane fluidity at low temperatures. One possible explanation of our experimental observation is that in the presence of CBD, phospholipids may be clustered together to a larger extent which further effects the ability of the receptor to stabilise an active conformation. A second possible explanation is that the ligand-receptor environment is destabilised (reflecting the differences in thermodynamics of

the binding site of receptor) in the presence of CBD and as a result, it might be difficult for CBD to find interaction sites that may exist in thermally volatile regions of the receptor (leading to reduced activity of CBD at 37 °C) (Hitzemann, 1988, IngóLfsson et al., 2014, Ghovanloo et al., 2018). However, our results indicate that the specific inhibition of CB1 signalling which only occurred after pre-treatment with 10 μM of CBD over a period of 60 min (effect of CBD 1 μM was not determined in the current study – may be worth validating in future studies), while CBD 100 nM had no measurable effect on CB1 signalling.

Moving forward, the specific inhibition of CB1 signalling by CBD is consistent with previous reports that specifically report the negative allosteric-like activity of CBD at CB1 (e.g. (Laprairie et al., 2015, Straiker et al., 2018)). It is tempting to speculate that this notion - 'specific allosteric effect' of CBD on CB1 may be unduly restrictive; we hypothesise that it may be that CB1 function is simply more intimately sculpted by the membrane composition/properties than other receptors. Nevertheless, an intended effect of CBD on the CB1 allosteric site cannot be ruled out. We then investigated whether CBD behaves in a similar way to the well-characterized CB1 NAM, ORG27569 (ORG) (Cawston et al., 2013, Price et al., 2005, Ahn et al., 2012). Our data demonstrate that CBD had no effect on the CP55940-mediated desensitisation of CB1 receptor signalling (it is possible that differences between these groups may be masked by error), but their application together with ORG results in much faster reversal of CP55940-mediated hyperpolarisation in AtT20-CB1 above that produced by ORG alone (Figure 6-2). A more *prima facie* interpretation, however, would be that the mode of regulation of ORG and CBD on CB1 is different in our system, and presumably acts on a different allosteric site at CB1. While some studies have suggested that CBD might bind to a similar site as ORG (Laprairie et al., 2015, Chung et al., 2019). Recently, Shao et al., 2019 determined the crystal structure of CB1 bound to ORG. The structure reveals that the ORG binds to an extrahelical site in the inner leaflet of the membrane, which overlaps with a conserved site of cholesterol interaction in many GPCRs (Shao et al., 2019)). It would be technically straightforward to show whether structurally distinct NAMs (CBD or ORG) share similar binding sites at CB1. Creating point mutation at the ORG-binding pocket of CB1 may provide mechanistic insight into the allosteric nature of CBD. In future studies, it would be of interest to quantify the affinity, cooperativity, and intrinsic efficacy of CBD on CB1, and whether the NAM activity of CBD in *in vitro* system can be correlated to its efficacy *in vivo* (Gregory et al., 2019).

Besides the reported allosteric-like activity of CBD at CB1, studies have shown that CBD also affects the signalling of CB2 (Thomas et al., 2007). This is supported by the finding that CBD (1 μM) produced a downward displacement of CP55940 in the [^{35}S]GTPγS-binding assay, suggesting the antagonistic effect of CBD on CB2 (Thomas et al., 2007). Another study has suggested the orthosteric partial agonism of CBD at CB2 – using cAMP inhibition and β-arrestin 1 recruitment assay (note temperature of experimental conditions was not indicated) (Tham et al., 2019). While the present study observed a minimal inhibition of CB2 signalling following CBD pre-treatment at 37 °C, suggesting that the discrepancy between results may be due to differences in experimental temperature. Similar inhibitory effects to CB2 were detected in AtT20 cells expressing SST (natively) or MOR when pre-treated with CBD (10 μM, 60 min). The ability of CBD to induce inhibition of multiple receptor signalling suggests a high degree of promiscuity consistent with the activity profile of phenolic phytochemicals (IngóLfsson et al., 2014, Hu and Bajorath, 2013). These phytochemicals tend to localise in the lipid bilayer interfaces, and thereby alter bilayer properties by reducing membrane stiffness, increase membrane elasticity, or increase lateral pressure in the headgroup region – thus altering the conformation equilibrium of receptors or the cholesterol that are embedded in the membrane (reviewed in (Andersen and Koeppe, 2007, Lundbæk et al., 2009)). The idea that CBD may mediate its affect by altering the membrane biophysical properties rather than acting through a discrete binding site is not unprecedented (first evaluated using fluorescent probes in brain synaptic plasma membranes and phospholipid vesicles, where CBD treatment decreased the polarisation of the fluorescent probe in various phosphatidylcholines suggesting a general ability of CBD to alter lipid fluidity to indirectly affect protein properties (Hillard et al., 1985)).

An opportunity, therefore, exists in the future to examine the effects of CBD on viscosity/stiffness of membrane bilayer. One way to approach this question is to study whether the lateral diffusion constant of the receptors or lipids embedded in the membrane changes following CBD treatment – this can be measured by using DOPE-biotin labelled with GFP via streptavidin (personal correspondence with Dr. Varun Sreenivasan, University of New South Wales). A fluorescent probe (laurdan) approach can also be employed to detect changes in the membrane phase properties by taking into consideration the results of fluorescence emission and anisotropy measurements ((Harris et al., 2002), originally reported in (Sheffield et al., 1995)).

Overall, our data suggest that the specific inhibitory effects of CBD on CB1 responses occurs at a physiologically relevant temperature, however, the effect of CBD on CB2, MOR or native SST signalling at physiological temperatures appears relatively non-specific in nature. Our study cannot pin down the exact mechanism of action of CBD's non-specific effects on multiple receptor signalling, as there are many ways that CBD could modulate the effects of GPCRs – direct interaction with the conserved site among class A GPCRs, alteration of membrane biophysical properties, or a combination of both. This is an important area for continued study to provide a greater understanding of CBD function across multiple receptor targets given the increasing medical and commercial interest in CBD.

6.5. References

Ahn KH, Mahmoud MM, & Kendall DA (2012). Allosteric modulator ORG27569 induces CB1 cannabinoid receptor high affinity agonist binding state, receptor internalization, and Gi protein-independent ERK1/2 kinase activation. Journal of Biological Chemistry, 287**,** 12070-12082.

Ahrens J, Demir R, Leuwer M, De La Roche J, Krampfl K, Foadi N, et al. (2009). The nonpsychotropic cannabinoid cannabidiol modulates and directly activates alpha-1 and alpha-1-Beta glycine receptor function. Pharmacology, 83**,** 217-222.

Altiere RJ, Douglas JS, & Gillis C (1981). Temperature related effects on the binding characteristics of beta-adrenergic receptor agonists and antagonists by rabbit lung. Naunyn-Schmiedeberg's Archives of Pharmacology, 316**,** 278-287.

Andersen OS, & Koeppe RE (2007). Bilayer thickness and membrane protein function: an energetic perspective. Annual Review of Biophysics and Biomolecular Structure, 36.

Banister SD, Longworth M, Kevin R, Sachdev S, Santiago M, Stuart J, et al. (2016). Pharmacology of valinate and tert-leucinate synthetic cannabinoids 5F-AMBICA, 5F-AMB, 5F-ADB, AMB-FUBINACA, MDMB-FUBINACA, MDMB-CHMICA, and their analogues. ACS Chemical Neuroscience, 7**,** 1241-1254.

Bisogno T, Hanuš L, De Petrocellis L, Tchilibon S, Ponde DE, Brandi I, et al. (2001). Molecular targets for cannabidiol and its synthetic analogues: effect on vanilloid VR1 receptors and on the cellular uptake and enzymatic hydrolysis of anandamide. British Journal of Pharmacology, 134**,** 845-852.

Bloom A, & Hillard C. Cannabinoids, neurotransmitter receptors and brain membranes. Marihuana'84: proceedings of the Oxford Symposium on Cannabis: 9th International Congress of Pharmacology, 3rd Satellite Symposium on Cannabis/edited by DJ Harvey; assistant editors Sir William Paton, GG Nahas, 1985. Oxford: IRL Press, c1985.

Cawston EE, Redmond WJ, Breen CM, Grimsey NL, Connor M, & Glass M (2013). Real-time characterization of cannabinoid receptor 1 (CB 1) allosteric modulators reveals novel mechanism of action. British Journal of Pharmacology, 170**,** 893-907.

Cervia D, Fehlmann D, & Hoyer D (2003). Native somatostatin sst 2 and sst 5 receptors functionally coupled to G i/o-protein, but not to the serum response element in AtT-20 mouse tumour corticotrophs. Naunyn-Schmiedeberg's Archives of Pharmacology, 367**,** 578-587.

Chung H, Fierro A, & Pessoa-Mahana CD (2019). Cannabidiol binding and negative allosteric modulation at the cannabinoid type 1 receptor in the presence of delta-9-tetrahydrocannabinol: An In Silico study. PloS one, 14.

Devinsky O, Cilio MR, Cross H, Fernandez-Ruiz J, French J, Hill C, et al. (2014). Cannabidiol: pharmacology and potential therapeutic role in epilepsy and other neuropsychiatric disorders. Epilepsia, 55**,** 791-802.

Di Marzo V, De Petrocellis L, Fezza F, Ligresti A, & Bisogno T (2002). Anandamide receptors. Prostaglandins, Leukotrienes and Essential Fatty Acids (PLEFA), 66**,** 377-391.

Drmota T, Greasley P, & Groblewski T (2006). Screening assays for cannabinoid-ligand-type modulators of GPR55. Google Patents

Fernández-Ruiz J, Sagredo O, Pazos MR, García C, Pertwee R, Mechoulam R, et al. (2013). Cannabidiol for neurodegenerative disorders: important new clinical applications for this phytocannabinoid? British Journal of Clinical Pharmacology, 75**,** 323-333.

Ghovanloo M-R, Shuart NG, Mezeyova J, Dean RA, Ruben PC, & Goodchild SJ (2018). Inhibitory effects of cannabidiol on voltage-dependent sodium currents. Journal of Biological Chemistry, 293**,** 16546-16558.

Gregory KJ, Bridges TM, Gogliotti RG, Stauffer SR, Noetzel MJ, Jones CK, et al. (2019). In Vitro to in Vivo Translation of Allosteric Modulator Concentration-Effect Relationships: Implications for Drug Discovery. ACS Pharmacology & Translational Science, 2**,** 442-452.

GW Pharmaceuticals. (2018). Subsidiary Greenwich Biosciences Announces FDA Approval of EPIDIOLEX® (Cannabidiol) Oral Solution–The First Plant-Derived Cannabinoid Prescription Medicine.

Hanuš LO, Meyer SM, Muñoz E, Taglialatela-Scafati O, & Appendino G (2016). Phytocannabinoids: a unified critical inventory. Natural Product Reports, 33**,** 1357-1392.

Harris FM, Best KB, & Bell JD (2002). Use of laurdan fluorescence intensity and polarization to distinguish between changes in membrane fluidity and phospholipid order. Biochimica et Biophysica Acta (BBA)-Biomembranes, 1565**,** 123-128.

Hayakawa K, Mishima K, Hazekawa M, Sano K, Irie K, Orito K, et al. (2008). Cannabidiol potentiates pharmacological effects of Δ9-tetrahydrocannabinol via CB1 receptor-dependent mechanism. Brain Research, 1188**,** 157-164.

Hillard CJ, Harris RA, & Blooms AS (1985). Effects of the cannbinoids on the physical properties of brain membranes and phospholipid vesicles: fluorescent studies. Journal of Pharmacology and Experimental Therapeutics, 232 (3) 579-588.

Hitzemann R (1988). Thermodynamic aspects of drug-receptor interactions. Trends in Pharmacological Sciences, 9**,** 408-411.

Howlett A, & Fleming R (1984). Cannabinoid inhibition of adenylate cyclase. Pharmacology of the response
in neuroblastoma cell membranes. Molecular Pharmacology, 26**,** 532-538.

Hu Y, & Bajorath J (2013). Activity profile relationships between structurally similar promiscuous compounds. European Journal of Medicinal Chemistry, 69**,** 393-398.

Ingólfsson HI, Thakur P, Herold KF, Hobart EA, Ramsey NB, Periole X, et al. (2014). Phytochemicals perturb membranes and promiscuously alter protein function. ACS Chemical Biology, 9**,** 1788-1798.

Jiang LI, Collins J, Davis R, Lin K-M, DeCamp D, Roach T, et al. (2007). Use of a cAMP BRET sensor to characterize a novel regulation of cAMP by the sphingosine 1-phosphate/G13 pathway. Journal of Biological Chemistry, 282**,** 10576-10584.

Kathmann M, Flau K, Redmer A, Tränkle C, & Schlicker E (2006). Cannabidiol is an allosteric modulator at mu-and delta-opioid receptors. Naunyn-Schmiedeberg's Archives of Pharmacology, 372**,** 354-361.

Knapman A, Santiago M, & Connor M (2014). Buprenorphine signalling is compromised at the N40D polymorphism of the human μ opioid receptor in vitro. British Journal of Pharmacology, 171**,** 4273-4288.

Knapman A, Santiago M, Du YP, Bennallack PR, Christie MJ, & Connor M (2013). A continuous, fluorescence-based assay of μ-opioid receptor activation in AtT-20 cells. Journal of Biomolecular Screening, 18**,** 269-276.

Laprairie R, Bagher A, Kelly M, & Denovan-Wright E (2014). Cannabidiol is a negative allosteric modulator of the type 1 cannabinoid receptor (Brit. J. Pharmacol. Submitted).

Laprairie R, Bagher A, Kelly M, & Denovan-Wright E (2015). Cannabidiol is a negative allosteric modulator of the cannabinoid CB1 receptor. British Journal of Pharmacology, 172**,** 4790-4805.

Leweke F, Piomelli D, Pahlisch F, Muhl D, Gerth C, Hoyer C, et al. (2012). Cannabidiol enhances anandamide signaling and alleviates psychotic symptoms of schizophrenia. Translational Psychiatry, 2**,** e94.

Long LE, Chesworth R, Huang X-F, Wong A, Spiro A, McGregor IS, et al. (2012). Distinct neurobehavioural effects of cannabidiol in transmembrane domain neuregulin 1 mutant mice. PloS one, 7**,** e34129.

Lundbæk JA, Collingwood SA, Ingólfsson HI, Kapoor R, & Andersen OS (2009). Lipid bilayer regulation of membrane protein function: gramicidin channels as molecular force probes. Journal of the Royal Society Interface, 7**,** 373-395.

Machado Bergamaschi M, Helena Costa Queiroz R, Waldo Zuardi A, & Crippa AS (2011). Safety and side effects of cannabidiol, a Cannabis sativa constituent. Current Drug Safety, 6**,** 237-249.

McPartland JM, Duncan M, Di Marzo V, & Pertwee RG (2015). Are cannabidiol and Δ9-tetrahydrocannabivarin negative modulators of the endocannabinoid system? A systematic review. British Journal of Pharmacology, 172**,** 737-753.

Morales P, Hurst DP, & Reggio PH (2017). Molecular targets of the phytocannabinoids: a complex picture. Phytocannabinoids. Springer.

Navarro G, Reyes-Resina I, Rivas-Santisteban R, de Medina VS, Morales P, Casano S, et al. (2018). Cannabidiol skews biased agonism at cannabinoid CB1 and CB2 receptors with smaller effect in CB1-CB2 heteroreceptor complexes. Biochemical Pharmacology, 157**,** 148-158.

Oláh A, Tóth BI, Borbíró I, Sugawara K, Szöllõsi AG, Czifra G, et al. (2014). Cannabidiol exerts sebostatic and antiinflammatory effects on human sebocytes. The Journal of Clinical Investigation, 124**,** 3713-3724.

Otto MK, Krabichler G, Thiele J, Ölkrug D, & Schultz JE (1984). Effect of temperature on membrane fluidity and calcium conductance of the excitable ciliary membrane from Paramecium. Biochimica et Biophysica Acta (BBA)-Biomembranes, 769**,** 253-260.

Peres FF, Lima AC, Hallak JE, Crippa JA, Silva RH, & Abílio VC (2018). Cannabidiol as a promising strategy to treat and prevent movement disorders? Frontiers in Pharmacology, 9.

Pertwee R (2008). The diverse CB1 and CB2 receptor pharmacology of three plant cannabinoids: Δ9-tetrahydrocannabinol, cannabidiol and Δ9-tetrahydrocannabivarin. British Journal of Pharmacology, 153**,** 199-215.

Pertwee RG (2012). Targeting the endocannabinoid system with cannabinoid receptor agonists: pharmacological strategies and therapeutic possibilities. Philosophical Transactions of the Royal Society B: Biological Sciences, 367**,** 3353-3363.

Price MR, Baillie GL, Thomas A, Stevenson LA, Easson M, Goodwin R, et al. (2005). Allosteric modulation of the cannabinoid CB1 receptor. Molecular Pharmacology, 68**,** 1484-1495.

Ross HR, Napier I, & Connor M (2008). Inhibition of recombinant human T-type calcium channels by Δ9-tetrahydrocannabinol and cannabidiol. Journal of Biological Chemistry, 283**,** 16124-16134.

Russo EB (2017). Cannabidiol claims and misconceptions. Trends in Pharmacological Sciences, 38**,** 198-201.

Russo EB, Burnett A, Hall B, & Parker KK (2005). Agonistic properties of cannabidiol at 5-HT1a receptors. Neurochemical Research, 30**,** 1037-1043.

Ryberg E, Larsson N, Sjögren S, Hjorth S, Hermansson NO, Leonova J, et al. (2007). The orphan receptor GPR55 is a novel cannabinoid receptor. British Journal of Pharmacology, 152**,** 1092-1101.

Sachdev S, Boyd R, Grimsey NL, Santiago M, & Connor M (2019). Brodifacoum does not modulate human cannabinoid receptor-mediated hyperpolarization of AtT20 cells or inhibition of adenylyl cyclase in HEK 293 cells. PeerJ, 7**,** e7733.

Shao Z, Yan W, Chapman K, Ramesh K, Ferrell AJ, Yin J, et al. (2019). Structure of an allosteric modulator bound to the CB1 cannabinoid receptor. Nature Chemical Biology, 15**,** 1199-1205.

Sheffield MJ, Baker BL, Li D, Owen NL, Baker ML, & Bell JD (1995). Enhancement of Agkistrodon piscivorus piscivorus venom phospholipase A2 activity toward phosphatidylcholine vesicles by lysolecithin and palmitic acid: studies with fluorescent probes of membrane structure. Biochemistry, 34**,** 7796-7806.

Shinitzky M (1984). Membrane fluidity and receptor function. Membrane Fluidity. Springer.

Straiker A, Dvorakova M, Zimmowitch A, & Mackie K (2018). Cannabidiol inhibits endocannabinoid signaling in autaptic hippocampal neurons. Molecular Pharmacology, 94**,** 743-748.

Tham M, Yilmaz O, Alaverdashvili M, Kelly ME, Denovan-Wright EM, & Laprairie RB (2019). Allosteric and orthosteric pharmacology of cannabidiol and cannabidiol-dimethylheptyl at the type 1 and type 2 cannabinoid receptors. British Journal of Pharmacology, 176**,** 1455-1469.

Thomas A, Baillie G, Phillips A, Razdan R, Ross RA, & Pertwee RG (2007). Cannabidiol displays unexpectedly high potency as an antagonist of CB1 and CB2 receptor agonists in vitro. British Journal of Pharmacology, 150**,** 613-623.

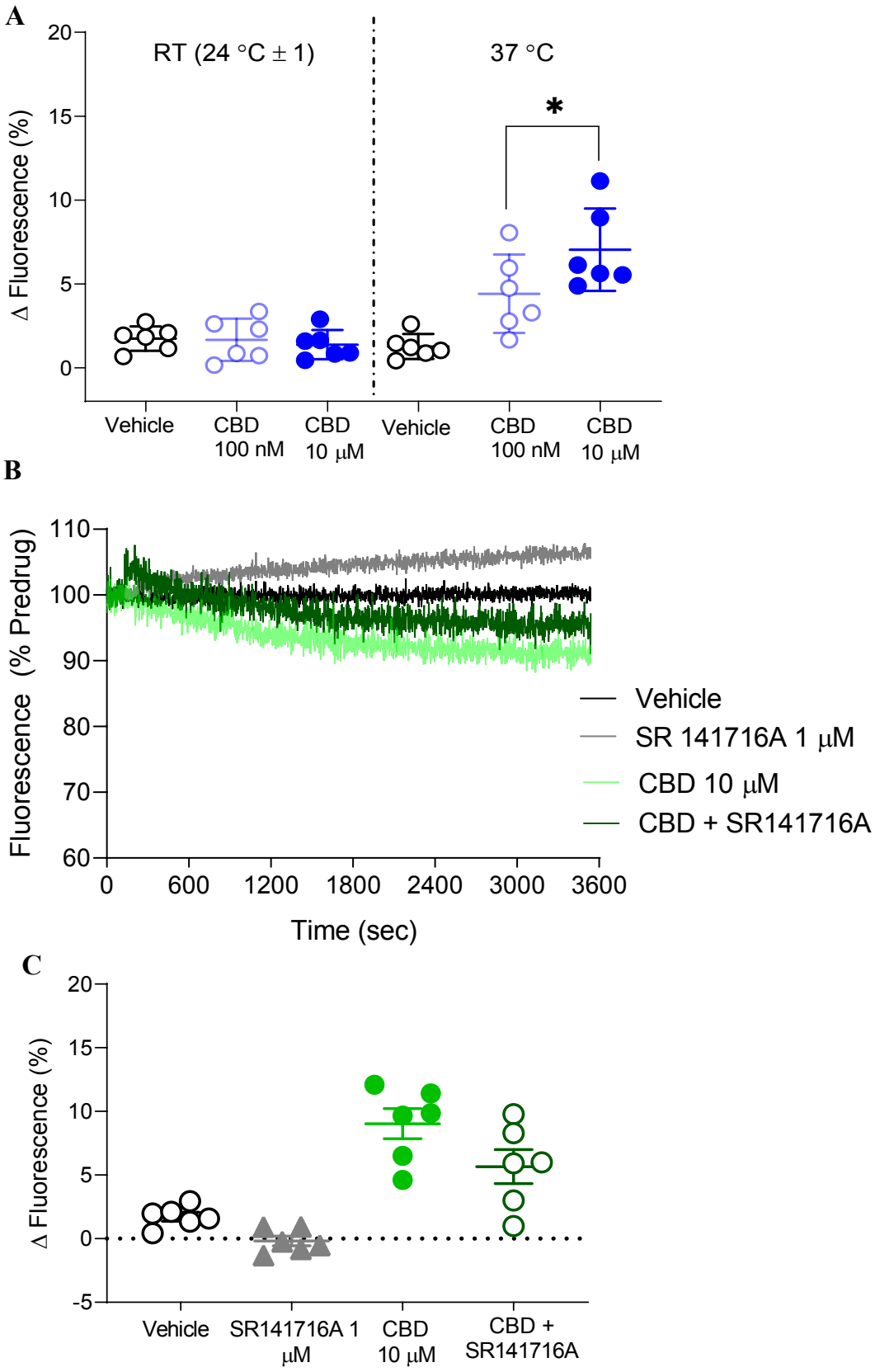
Wei J-W, & Sulakhe PV (1982). Lowering temperature increases cardiac muscarinic receptor site affinity towards agonists and antagonists. General Pharmacology, 13**,** 413-419.

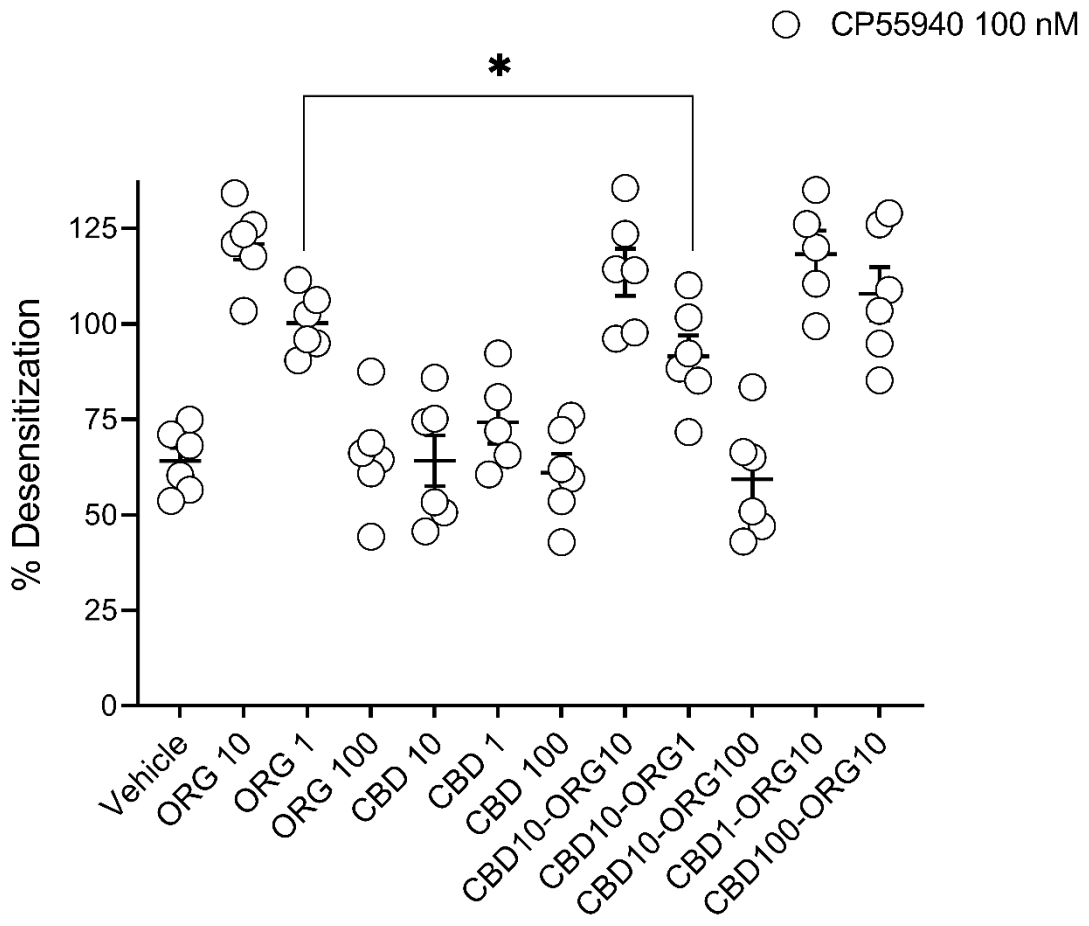
Zhornitsky S, & Potvin S (2012). Cannabidiol in humans—the quest for therapeutic targets. Pharmaceuticals, 5**,** 529-552.

6.6. Supplementary Data

Supplementary Figure 6-1. Effect of CBD on AtT20-CB1 signalling

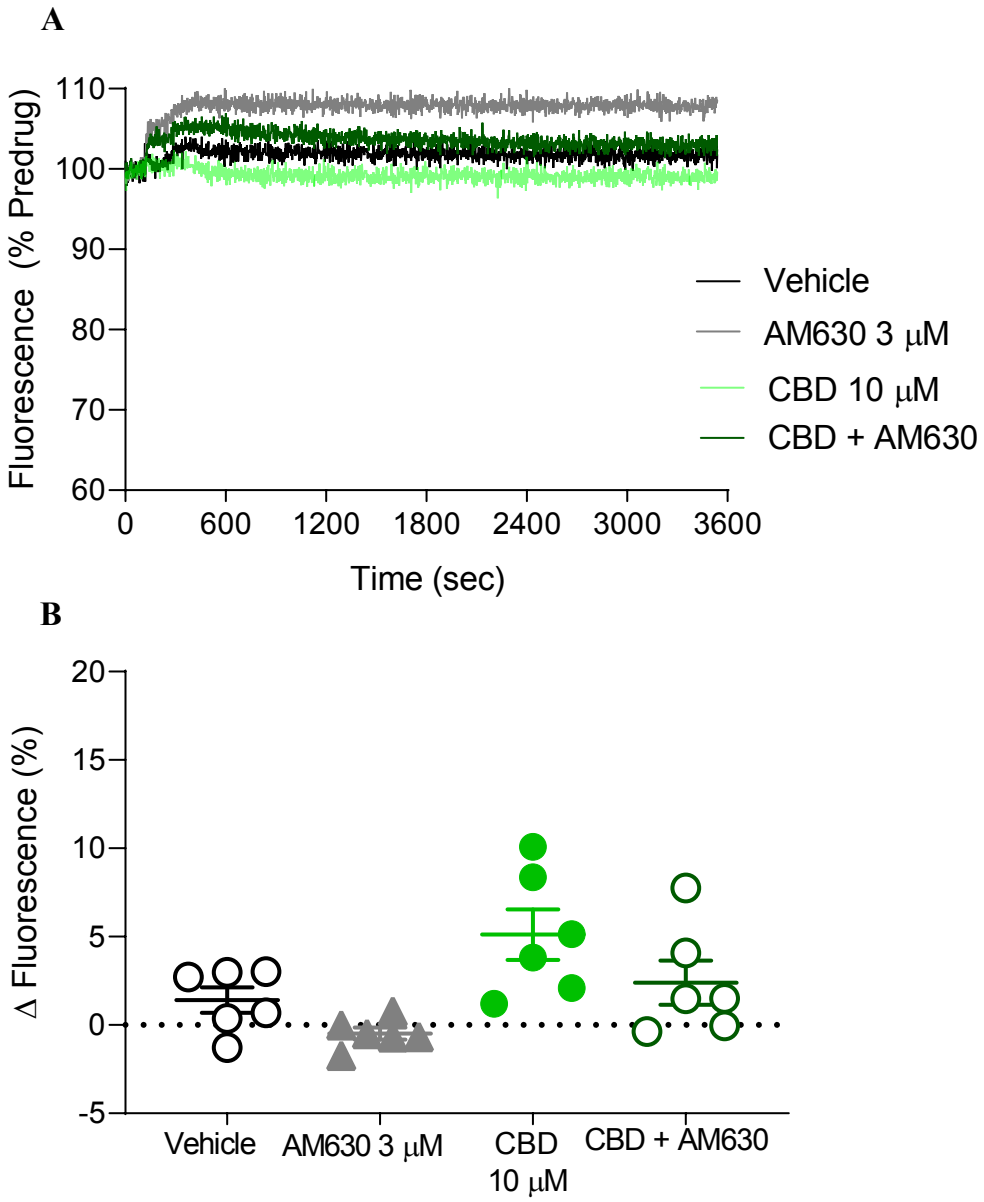
(A) Summarised data comparing pre-treatment of AtT20-CB1 cells with vehicle, or CBD (10 μM or 100 nM) for 5 min before addition of CP55940 at RT and 37 °C (B) An individual representative trace showing change in fluorescence of AtT20-CB1 produced during continuous exposure of vehicle, CBD (10 μM), SR141716A (1 μM), or in combination (CBD + SR141716) (C) Within each set, condition 'CBD' was compared to 'SR141716', or 'CBD + SR141716', and had no significant difference ($P < 0.05$). Data represents mean ± SEM of six independent determinants performed in duplicate.


A
RT (24 °C ± 1)
37 °C
*
Δ Fluorescence (%)
20
15
10
5
0
Vehicle
CBD 100 nM
CBD 10 μM
Vehicle
CBD 100 nM
CBD 10 μM
B
Fluorescence (% Predrug)
110
100
90
80
70
60
0
600
1200
1800
2400
3000
3600
Time (sec)
Vehicle
SR 141716A 1 μM
CBD 10 μM
CBD + SR141716A
C
Δ Fluorescence (%)
20
15
10
5
0
-5
Vehicle
SR141716A 1 μM
CBD 10 μM
CBD + SR141716A



Supplementary Figure 6-2. CBD on CP55940-mediated CB1 desensitisation

(A) Summarised data for percentage desensitization after 30 min exposure to CP55940 (100 nM) alone or in presence of CBD or ORG individually, or in combination (CBD + ORG), compared with peak fluorescence response. Within each set, condition 'ORG 10 (10 μM)' was compared to 'CBD (10 μM-100 nM)', 'ORG (1 μM-100 nM)' or 'CBD + ORG'. Data represents mean ± SEM of at least independent determinants performed in duplicate.



Supplementary Figure 6-3. Effect of CBD on AtT20-CB2 signalling

(A) An individual representative trace showing change in fluorescence of AtT20-CB1 produced during continuous exposure of vehicle, CBD (10 μM), AM630 (3 μM), or in combination (CBD + AM630) at 37 °C (B) Within each set, condition 'CBD' was compared to 'AM630', or 'CBD + AM630', and had no significant difference ($P < 0.05$). Data represents mean ± SEM of six independent determinants performed in duplicate.

Chapter VII. General Discussion

The studies presented in this thesis were undertaken to further the understanding of the molecular pharmacology of cannabinoids and how traditional, and structurally diverse synthetic cannabinoids have greatly varied outcomes when acting via the same target. This discussion will seek to consider the wider implications of the work reported in this thesis and provide potential directions for future research.

The characterisation of the functional activation of CB1 by synthetic cannabinoid receptor agonist (SCRAs) was a natural starting point to examine the SCRA toxicity at molecular level (in comparison to THC, CP55940, WIN55212-2 etc). Intrinsic efficacy combined with an operational model of pharmacological agonism was found to be a new determinant for the quantitative measurement of the activity profile of different cannabinoids downstream of CB1 receptors. Results revealed that some of the SCRAs had up to 300 times the efficacy of THC, and this molecular effect was systematically characterised in the first results chapter of this thesis (Chapter 2, (Sachdev et al., 2019c)). In recent years, concerns over the current operational analysis, particularly, for the quantification of bias have been raised. Here, the assumption that all the assay endpoints are in a state of equilibrium (i.e. the receptor density will remain constant with time upon internalisation), does not actually reflect real biological states (Herenbrink et al., 2016, Zhu et al., 2019, Finlay, 2018). Changes in the number of receptors at the cell surface over time may therefore reflect changes in the responsiveness of the system (efficacy). This is particularly relevant in the case of cannabinoids, and thus kinetic aspects of cannabinoid signalling should be considered in future studies (Zhu et al., 2019). However, this may not be problematic in our study as we are confident that maximum effect of SCRAs measured captures the efficacy appropriately – we measured the real-time activation of K channel as soon as the drug was added to the system (within 3 min). This portion of time course would certainly have no dramatic change in the receptor density. Our study compared the relative agonist activity of SCRAs in GTPγS binding and membrane potential assays, and highlighted the differences in response of some SCRAs between the two assay systems, suggesting a form of observational bias based on the relative sensitivity of assays (Kenakin and Christopoulos, 2013).

An emerging idea in molecular pharmacology is the differing ability of agonists to activate multiple signalling pathways of the receptor (functional selectivity). This means that

agonists stabilise different (or unique) active conformational states of the receptor, which in turn modulates the extent (efficacy) to which different downstream pathways are activated (Kenakin et al., 2012). The functional selectivity of SCRAs was explored recently in a case study of AMB-FUBINACA (Finlay et al., 2019). The authors found that AMB-FUBINACA was highly efficacious and potent in the pathways assayed including cAMP inhibition, pERK activation, CB1 internalisation, and translocation of β-arrestin1/2 (a finding consistent with the high efficacy of MDMB-FUBINACA, an analogue of AMB-FUBINACA, characterised in the Chapter 2). However, AMB-FUBINACA did not appear to be biased for any pathways in the operational analysis (Finlay et al., 2019). While THC activated all these pathways with lower potency and efficacy than AMB-FUBINACA, operational analysis suggested that THC may be a biased ligand, and displays lower activity for arrestin pathways (Finlay et al., 2019). Another study assessed the biased agonism among 21 structurally different SCRAs in an assay of G_i or β-arrestin recruitment to CB1 (Wouters et al., 2019d). The authors used relative agonist activity (i.e. E_{MAX} and potency) to compare the two signalling pathways. A limitation to this method arises from the fact that the CB1-G_i pathway has a substantial degree of receptor reserve (e.g. see Finlay et al. (2017), Sachdev et al. (2019c)) compared to lower levels of receptor reserve for CB1-β-arrestin pathway (Ibsen et al., 2019). This implies that even a low efficacy agonist can have a higher activity profile at G_i pathway, as there are sufficient receptors available to yield a system maximum at saturating concentration and this might have a confounding influence on bias conclusion that arise from "full agonist" activity of ligands. Nevertheless, the variety of SCRAs presented in this study exert highly variable profile of potency and E_{MAX} across different pathways (Wouters et al., 2019d). The physiological implication of different signalling pathways downstream to CB1 have yet to be determined. This phenomenon has been more closely examined for opioid receptor; for example, mu-opioid receptor is thought to mediate the analgesic effect via activation of G protein-coupled signalling, but adverse effects (including respiratory depression) exist through arrestin-coupled signalling (Bohn et al., 1999, Xu et al., 2007). Even though the characterisation of this system continues to be assessed, it appears that researchers have begun to accept that it is the low intrinsic G-protein efficacy of opioids (not biased agonism) that is linked to the improved safety profile of new opioid-based treatments (such as TRV-130) (Gondin et al., 2019). It is also important to look at the physiological implications of the pathways involved in cannabinoid receptor signalling - this can be achieved by examining the CB1/2 signalling and behaviour

(antinociception, hyperthymia, catalepsy) in β-arrestin(s) knock-out mice following administration of structurally diverse selected cannabinoids.

The concept of 'biased agonism' was further explored in the third chapter of this thesis, where SCRA-specific modulation of two signalling endpoints was investigated - $G_{\alpha i/o}$ (inhibition) and $G_{\alpha s}$ (stimulation) of cAMP signalling. The rank order of potency of the SCRAs to stimulate $G_{\alpha s}$-like signalling compared to $G_{\alpha i/o}$ signalling as demonstrated was significantly different. This suggests that differences in G-protein preferences between SCRAs are likely due to receptor conformation differences in the presence of different ligands, a key determinant of receptor bias (Sachdev et al., 2019a). Recently, a cryogenic electron microscopy study was published on the structure of the SCRA, MDMB-FUBINACA, bound to CB1-G_i complex, and the promiscuous nature of CB1 coupling to both G_i and G_s was also investigated (Kumar et al., 2019). Previous work has shown that L222 residue in the ICL2 is critical for mediating G_s protein coupling to GPCRs (Chen et al., 2010), and the ability of TM6 domain to move to a greater extent outward to accommodate the C terminus of G_s is also one of the determinants of G_s coupling specificity (Kumar et al., 2019). The authors suggested that glycine residue at the position 357 of CB1 may add extra flexibility to TM6, driving CB1 coupling to G_s - a prototypical example is β2-adrenergic receptor, which couples to G_s by larger displacement of TM6 attributed to $G^{6.38}$ and $G^{6.42}$ (Kumar et al., 2019). However, the biological significance of SCRA-mediated differential coupling of CB1 to $G_{i/o}$ and G_s are not well understood. One of the greatest obstacles in biomedical science is the difficulty in relating the *in vitro* findings of signalling phenotype of cannabinoids to their physiological role in normal and in disease states. Some studies have investigated the ability of cannabinoids to induce apoptosis in tumours, and the link between high CB1 expression and its functional consequence in cancer cells (such as prostate cancer) (Galve-Roperh et al., 2000, Cudaback et al., 2010, Cipriano et al., 2013). For example, one study to date has revealed the ability of cannabinoid (CP55940) to regulate signalling endpoints (kinases) in astrocytoma expressing low, medium and high levels of CB1 and CB2 (Cudaback et al., 2010). They found that increased expression of CB1 and CB2 receptor allowed for additional coupling to Akt signalling pathway (survival pathway regulating cell proliferation and apoptosis), which abolished the ability of cannabinoids to induce apoptosis, unless Akt was concomitantly inhibited (Cudaback et al., 2010). It is not yet known, however, whether the increase in CB1 expression would modify the G_i-G_s signalling switch in cancer cells.

Another important domain in molecular pharmacology is the allosteric modulation of receptor signalling. By its simplest definition, allosteric modulators bind to the receptor at a site topographically separate from the orthosteric site, and globally alter the conformation of the receptor, thereby modulating the receptor function in a positive or negative way. These molecules act at receptors as either positive allosteric modulators (PAMs) or negative allosteric modulators (NAMs) (Gregory et al., 2010). Kenakin and Christopoulos (2013) note that allosteric modulators can also induce receptor activation on their own by altering the basal signalling state of the receptor (called Ago-PAM). Allosteric modulators hold great therapeutic potential by allowing receptor activation to be tuned in a direction of maximal benefit. For example, ZCZ011 (a CB1 PAM) enhanced antinociceptive effects in neuropathy and inflammatory pain models, but held limited cannabimimetic side effects (Ignatowska-Jankowska et al., 2015). While ORG27569 remains the most characterised allosteric modulator of CB1, increasing the CB1 agonist binding with 'insurmountable antagonism of receptor function' - meaning that ORG27569 increases the binding of CB1 agonist CPP5940 but producing a concentration-dependent decrease in CB1 agonist function, a rare phenotype of NAM (Price et al., 2005). The third chapter of this thesis was a natural continuation of the original investigation into SCRA-associated toxicity, where the concept of allosteric modulation was utilised to understand whether or not there is a pharmacological reason for mixing brodifacoum (superwarfarin) with synthetic cannabinoids. Although cannabinoid-induced signalling was not different in the presence of brodifacoum, this study suggests that mixing SCRAs with brodifacoum are not likely to enhance user experience through acute interactions with cannabinoid receptors (Sachdev et al., 2019b). Allosteric modulation of CB1 using ORG27569 was previously reported by Professor Michelle Glass in collaboration with our lab at Macquarie University (Cawston et al., 2013), where evidence of ORG27569-mediated inhibition of the agonist response was observed after 5 min following drug addition (in contrast to immediate antagonism observed with SR141716), as well as a delay in ability to antagonise the agonist-mediated hyperpolarisation of K channel was seen (Cawston et al., 2013). Similar experimental conditions were used to investigate the effect of brodifacoum on the sustained responses to cannabinoid-mediated hyperpolarisation of K channel; however, the presence of brodifacoum failed to affect the signalling produced by prolonged application of cannabinoids (Sachdev et al., 2019b). Future studies could determine the pharmacokinetic profile of brodifacoum-laced SCRAs (especially their metabolic pathway), and determine if the findings in the present study can be correlated to an *in vivo* model.

Allosteric modulation of cannabinoid receptors was also studied in the context of the "entourage effect" – an emerging idea that the components within the cannabis plant will produce much stronger effect when combined as opposed to the individual components (an assumption that many researchers would reject). Substantial conflicts remain in the literature and emphasise the complex interaction between terpenoids and cannabinoids at molecular or physiological level (Russo, 2011). The terpenoids tested in this study neither activated cannabinoid receptors alone nor modulate the response to a high efficacy agonist (CPP5940) and a low efficacy agonist (THC) (Santiago et al., 2019). It is noted that in a previous publication from Gertsch et al. (2008) β-caryophyllene appears to be a CB2 agonist, however this was not replicated in the current study (Chapter 5, (Santiago et al., 2019)). Very recently, a second study to reveal the agonist activity of β-caryophyllene at CB2 was achieved in a mixed human primary leukocyte (Saroz et al., 2019b). This emphasises the importance of using primary culture which may allow the characterisation of cannabinoids in an environment closer to in vivo settings, although it would be difficult to entirely isolate a pure CB2 response from a heterogenous cell population.

While Chapter 2, Chapter 3 and Chapter 4 are focused on the pathways involved in the on-target effects associated with SCRA toxicity, Chapter 6 described an exploratory study primarily concerned with the molecular mechanism underlying the therapeutic effects of the phytocannabinoid, cannabidiol (CBD). The function of CBD is believed to be attributed to allosteric modulation of a variety potential molecular targets. The signalling profile of CBD across a range of targets has been extensively studied over the recent years (reviewed in Perucca (2017)), however, reporting of the diverse responses of CBD is generally compromised by the use of physiologically irrelevant temperature and dose conditions in the experimental design (e.g. Laprairie et al. (2015), Navarro et al. (2018), Straiker et al. (2018)). Temperature-dependent effects are a frequently encountered phenomenon, and we cannot assume that the effect of CBD at room temperature is similar to that at body temperature. The current study demonstrated that the interaction of CBD with the multiple receptors is temperature dependent (greater effect at 24 °C ± 1 as compared to 37 °C), while specific inhibition of CB1 signalling by CBD was confirmed (consistent with the negative allosteric-like activity of CBD at CB1). As discussed in Chapter 6, the non-specific interaction of CBD with multiple receptors is presumably due to change in membrane fluidity as a function of temperature. Given the ability of CBD to alter membrane bilayer fluidity attributed to its physiochemical properties, development of synthetic CBD

derivatives is a key aim of drug design researchers in order to improve the pharmacodynamic or pharmacokinetic properties of CBD (Morales et al., 2017).

Other than the experiments outlined in each chapter, future plans for the continuation of this project include the full pharmacological assessment of diverse class of cannabinoids at signalling pathway(s) downstream to cannabinoid receptor activation to help identify the biased signalling profiles of these compounds, and particular structural conformations responsible for their effects (Patel et al., 2020). Indeed, many SCRAs have demonstrated the apparent ability to stimulate $G_{\alpha s}$-like cAMP signalling via CB1 in the present study. Looking at the CB1-G_s signalling profile of cannabinoids in specific cancer conditions may be relevant under conditions where upregulation in CB1 was reported (Finlay et al., 2017), querying the relative effect of these cannabinoids on the molecular markers involved in the prognosis of cancer, and warranting further investigation. A future direction may also involve examining the functional activity (particularly, efficacy) of this panel of cannabinoids on CB2 receptor as this receptor does not mediate the psychotropic effect of cannabis. It is hoped that targeting CB2 might be therapeutically useful in wide range of health conditions, including Parkinson's disease, multiple sclerosis, stroke, neurodegeneration, and various cancer states (Navarro et al., 2016). Given that the use of cannabis and cannabis extracts is becoming more prevalent, it remains important to access the pharmacological profile of terpenoids in conjunction with cannabinoids. Future studies will investigate the possibility of entourage effects emerging through terpenoid-cannabinoid interaction acting via non-cannabinoid receptor mechanism. This hypothesis also has precedent; for example, a recent study determined whether terpenoids influence the effect of cannabinoids on human TRPA1 and TRPV1 channels (Heblinski et al., 2020). However, this study could not find any evidence of terpenoid-cannabinoid interaction at TRPA1 or TRPV1, but given the promiscuous nature of cannabinoids, the search should continue by exploring this complex interaction on additional molecular targets (e.g. GABA, GPR55, GPR18, glycine, etc.) (Heblinski et al., 2020). Finally, with over 65 molecular target identified for CBD, an accurate understanding of CBD activity profile across range of receptor targets is critical to the meaningful interpretation of its therapeutic potential. We and others are pursuing research in this area currently as evident from Chapter 6. Although, our data provides insights into the promiscuous interaction of CBD with multiple GPCRs, including the temperature-dependent effect (given the greater effect of CBD to inhibit CB1, CB2, MOR, or D2 signalling at RT compared to 37 °C), it does not provide a universal

molecular or structural basis of CBD's non-specific interaction with multiple receptor signalling. Thus, given the ability of CBD to alter the membrane biophysical properties, an important initial study to follow-on from this significant finding will be use of a fluorescent probe (Harris et al., 2002), biotinylation of biological membranes (Henry et al., 2018), or a gramicidin-based assay (IngóLfsson et al., 2014) to understand how CBD or other membrane-embedded ligands can modulate the activity of range of medically-important GPCR.

In conclusion, the research presented in this thesis has investigated aspects of chemically diverse cannabinoids to produce different profiles of activity downstream of cannabinoid receptors. The differing abilities of cannabinoids to activate signalling responses not only provides mechanistic insight into cannabinoid receptor activation that may be able to be linked to clinically confirmed effects of particular SCRAs, but will also aid in design of drugs with high specificity and improved pharmaceutical properties. The insight gained by this thesis will certainly serve as an important foundation for further investigations into the molecular contributions to the toxic effects of synthetic cannabinoids and therapeutic effects of phytocannabinoids. Exciting opportunities exist in future research to understand the consequences of cannabinoid receptor activation by a vast array of available drugs in normal state physiology and in disease.

Appendices

Appendix A. Chapter 2 related results

Appendix B. Method Chapter: Evaluating opioid mediated adenylyl cyclase inhibition in live cells using a BRET based assay

Appendix C. Permissions for reproducing published material in this thesis

Appendix D. Biosafety application

Appendix A.

In the Chapter 2 of this thesis, we initially examined the ability of the irreversible CB1 antagonist methyl arachidonyl fluorophosphonate (MAFP) (reported by Fernando and Pertwee (1997)) to pharmacologically knockdown CB1 receptor function. On the day of the assay, cells were incubated with MAFP (10 μM) for 20 mins at 37 ºC, after which MAFP was removed and the cells were washed twice with HBSS. Pre-treatment with MAFP resulted in a reduction in the hyperpolarisation induced by subsequent application of CP55940 compared to untreated cells (Figure A-1, Control E_{max} 36 ± 1.3% and MAFP treated E_{max} 29 ± 2.2%), consistent with reduction in receptor number. MAFP pre-treatment did not significantly affect the hyperpolarisation induced by SST (100 nM) in the same cells (Figure A-2, $P > 0.05$), suggesting that it did not interfere with G-protein coupled receptor signalling to G-protein gated inward rectifying potassium (GIRK) channels *per se*. However, MAFP inhibits a range of enzymes, including those that degrade endocannabinoids such as, fatty acid amide hydrolase (FAAH) and monacylglycerol lipase (MAGL) (Goparaju et al., 1999). To assess whether the decrease in response of CP55940 may have been related to changes in endocannabinoid levels, we also investigated the effects of the structurally unrelated, non-selective inhibitor of FAAH and MAGL, JZL195 (Long et al., 2009), on CP55940 signalling. Pre-treatment of cells with JZL195 (1 μM, 60 min) also inhibited the hyperpolarisation induced by CP55940 (Figure A-1, Control E_{max} 32 ± 0.8% and JZL treated E_{max} 27 ± 1.8%), suggesting that altering endocannabinoid degradation can alter CB1 signalling; therefore, due to uncertainty surrounding the mechanism through which MAFP alters CP55940 responses, we turned to AM6544 to deplete the CB1 receptors.

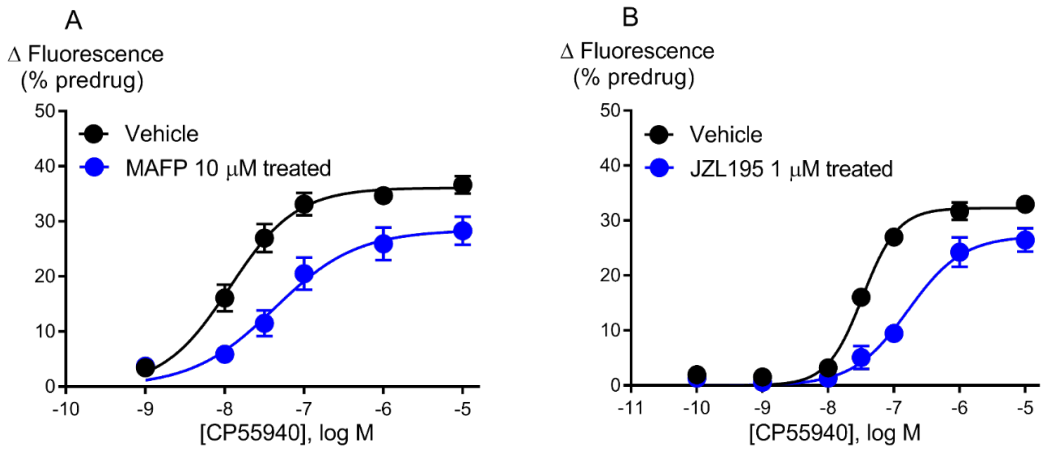


Figure A-1. Effect of MAFP and JZL-195 pre-treatment on CP55940 signalling on CB1

Concentration response curves for CP55940 mediated hyperpolarisation of AtT20-CB1 following pre-treatment with 10 μM of MAFP (A) and 1 μM of JZL195 (B). Representative data are presented as percentage in fluorescence corresponding to the hyperpolarisation of the cells. Each point represents the mean ± SEM of 6 independent determinants performed in duplicate and pooled data was fitted with four parametric logistic equation.

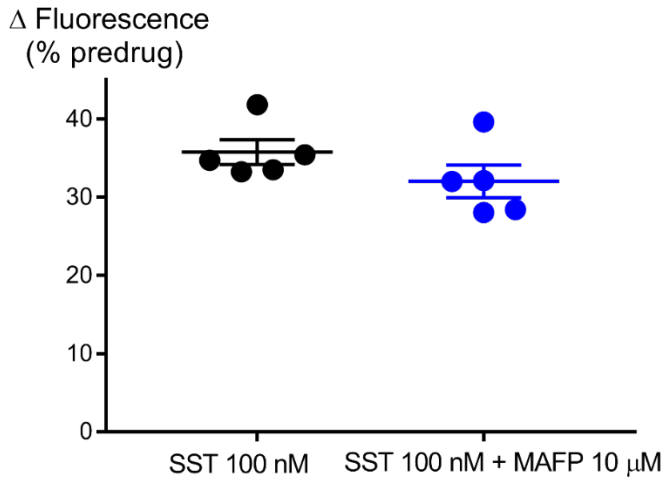


Figure A-2. Effect of MAFP pre-treatment on native Somatostatin (SST) signalling

Scatter dot plot showing the percentage change in fluorescence of SST (100 nM) on AtT20-CB1 cells pre-treated for 20 min with vehicle or MAFP (10 μM) and then washed twice before incubation with membrane assay dye. Data represents the mean ± SEM of 5 independent determinants performed in duplicate. There was no difference in the maximal response of SST between vehicle or following pre-treatment with MAFP.

Differential regulation of CB1 receptor by high efficacy CB1 agonists

Long term effect of cannabinoids is still largely unknown. An especially significant unknown is the molecular mechanism underlying the prolong effect of cannabinoids (desensitisation) on CB1 signalling. The desensitisation of CB1 signalling was accessed in the continued presence of 5F-MDMB-PICA (most efficacious SCRA) or CP55940 (10 μM, 30 min), after depletion of CB1 receptor reserve with AM6544. Compound 101, a potent and selective GRK 2/3 inhibitor was used to study the potential involvement of these kinases in CB1 desensitisation. The desensitisation produced by CP55,940 or 5F-MDMB-PICA, measured as percentage decline in CB1 receptor activity, was found to be 39±4% and 86±2.4% respectively. Compound 101 (10 μM) did not affect the desensitisation evoked by CP55,940, but reduced the CB1 desensitisation by 5F-MDMB-PICA from 86±2.4% to 63±3.2% (Figure A-3, $P<0.05$). Receptor depletion had no effect on the magnitude or Compound 101-sensitivity of CB1 desensitisation induced by CP55,940 or 5F-MDMB-PICA. Our data suggest GRK-dependent and independent mechanisms for CB1 receptor desensitisation by the highest efficacy agonist (5F-MDMDB-PICA), but only GRK-independent mechanisms for lower efficacy agonist (CP55940).

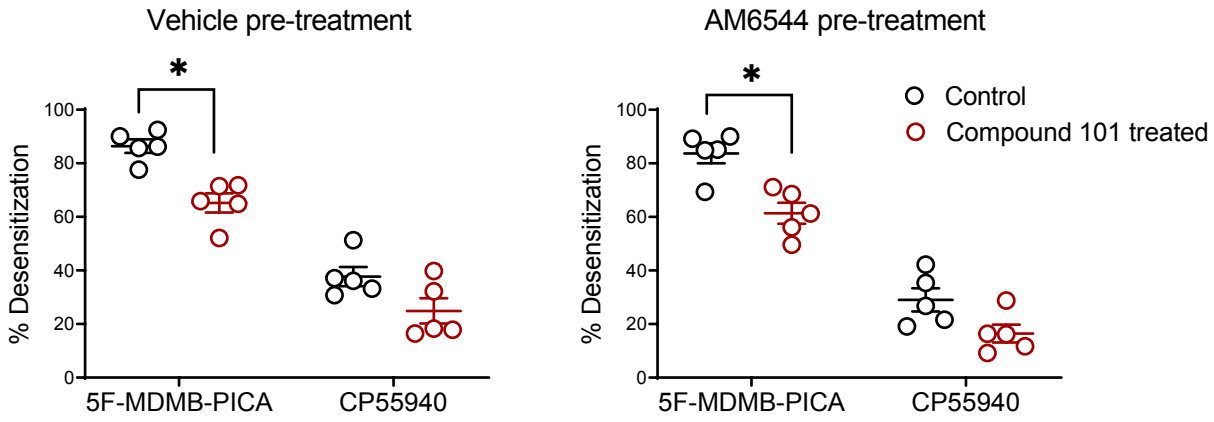


Figure A-3 Effect of Compound 101 on CB1 signalling during continuous exposure of 5F-MDMB-PICA or CP55940

Summarised data comparing the CP55940- or 5F-MDMB-PICA-mediated desensitisation of CB1 signalling when pre-treated with vehicle (left panel) or AM6544 (right panel) for 60 mins. Within each set, condition 'Control' was compared to 'Compound 101'. Data represents mean ± SEM of at least five independent determinants performed in duplicate.

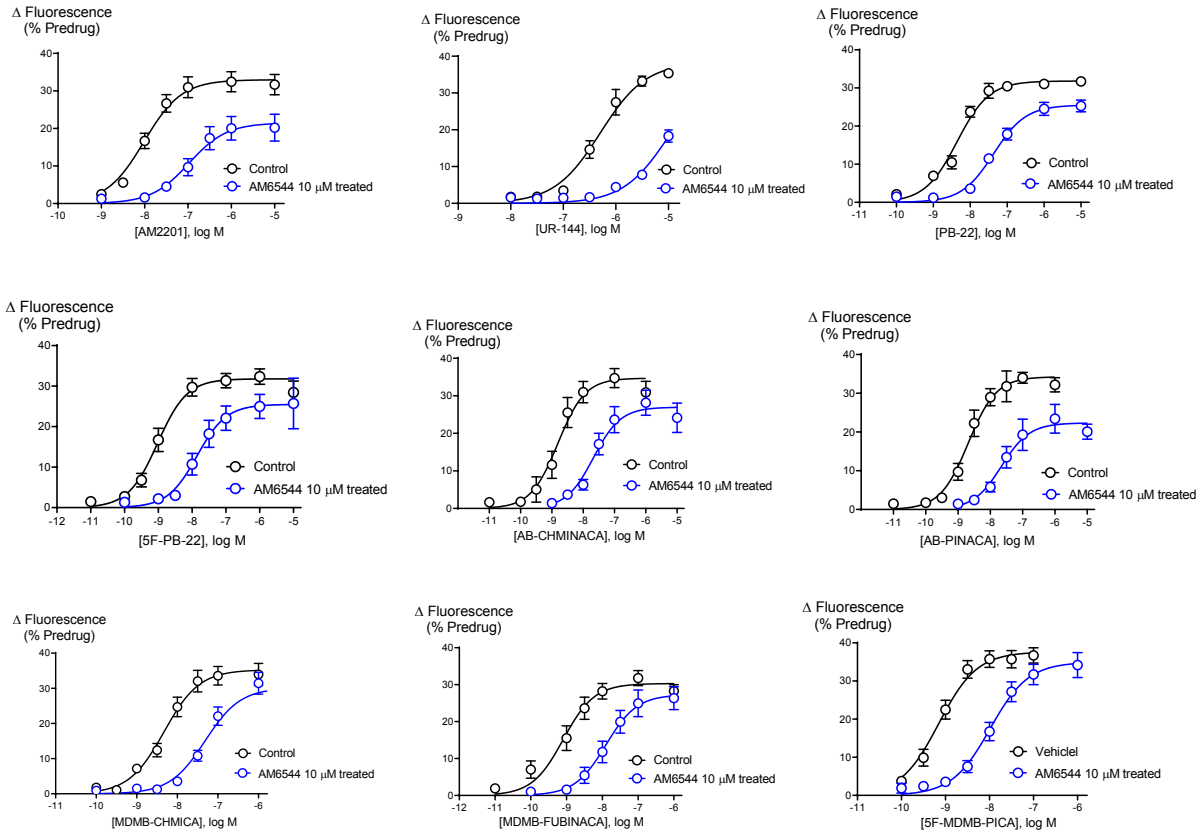


Figure A-4. ***Functional activity of SCRAs following receptor depletion with AM6544***

Concentration response curves for all the nine SCRAs (AM2201, UR-144, XLR-11, PB-22, 5F-PB-22, AB-CHMINACA, AB-PINACA, MDMB-CHMICA, MDMB-FUBINACA, and 5F-MDMB-PICA) missing from the Chapter 2 were plotted using five-parameter non-linear regression to fit the operational model receptor depletion equation with basal constrained to 0. Data represents mean ± SEM of technical replicates. For some points, the error bars are smaller than the height of the symbol.

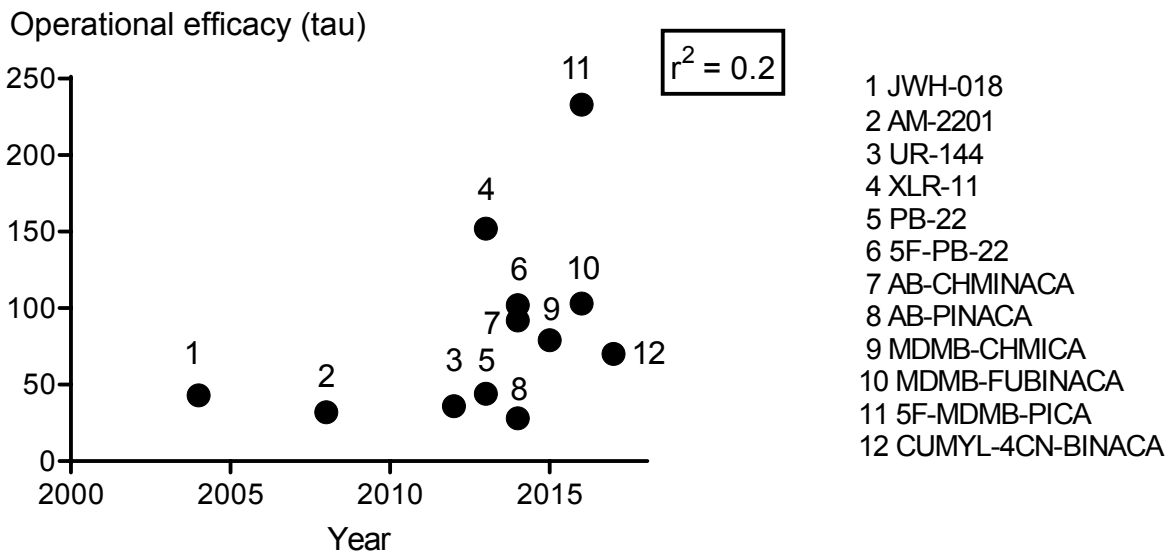


Figure A-5. Correlation of operational efficacy (τ) and evolution of SCRAs over time.

Representative data are presented demonstrating a non-significant value of r^2 of 0.2, where there was no obvious trend for increasing/decreasing tau over time. Please see Figure 1-9 for more information regarding the order of each SCRAs from the first time they appeared in the NPS market to the most recent ones (Evolution of SCRAs over time).

Appendix B.

Title: Evaluating opioid mediated adenylyl cyclase inhibition in live cells using a BRET based assay

Authors:

Preeti Manandhar[1], Shivani Sachdev[1], and Marina Santiago[1]

1 – Department of Biomedical Sciences. Faculty of Medicine and Health Sciences. Macquarie University. NSW, Australia

Corresponding Author:

Marina Santiago

Email address of corresponding author:

marina.junqueirasantiago@mq.edu.au

Contributions to the work

This method chapter represents a collaborative work hosted in Mark Connor's laboratory at Macquarie University Australia. Preeti Manandahar and Marina Santiago developed and designed the structure of this chapter to assess opioid-mediated inhibition of cAMP signalling response using BRET-based assay, while I assisted with the preliminary experiments to optimise the assay to measure the canonical CB1 cAMP signalling phenotype. All the authors discussed the results and contributed to the manuscript.

Evaluating opioid mediated adenylyl cyclase inhibition in live cells using a BRET based assay

Preeti Manandhar, Shivani Sachdev, and Marina Santiago

Running head: Real-time BRET based cAMP measurement

Abstract

Quantitative measurement of receptor signalling by different ligands is important for understanding the mechanism of drug action and screening of drugs. Here, we describe a simple and cost-effective method of measuring adenylyl cyclase inhibition, one of the hallmarks of opioid receptor activation. The assay is based on Bioluminescence Resonance Energy Transfer (BRET) that involves transfection of a biosensor in human embryonic kidney (HEK) 293 cells stably transfected with μ-opioid receptor (μ receptor), enabling real-time measurement of cAMP levels.

Key Words: cAMP, Forskolin, BRET Assay, Adenylyl cyclase, μ-opioid receptor, HEK, CAMYEL

1 Introduction

Monitoring changes in cAMP levels is a reliable approach of assessing receptor signalling as it is a proximal signalling step after receptor activation. cAMP serves as a secondary messenger that activates other effectors downstream of receptor signalling. Activation of μ receptor catalyses exchange of guanidine diphosphate (GDP) for guanidine triphosphate (GTP), this causes dissociation of heterotrimeric G protein. μ receptor mediates downstream signalling predominantly through the $G\alpha_i$ protein family, which leads to inhibition of adenylyl cyclase [1], an enzyme that converts adenosine triphosphate to cyclic adenosine monophosphate (cAMP) [2].

A novel method of cAMP measurement incorporates the use of Bioluminescence Resonance Energy Transfer (BRET) based biosensor. It is a simple, robust and real time-based assay that is easily adapted to an automated microplate reader. This sensitive and reproducible assay uses a specially designed biosensor, cAMP sensor using YFP-Epac-RLuc (CAMYEL) capable of detecting intracellular cAMP in live cells. The biosensor consists of a cAMP

binding protein, Epac flanked by the BRET pair - *Renilla* luciferase (RLuc- the donor luminescence enzyme) and yellow fluorescent protein (YFP- acceptor fluorescent protein) [3, 4]. Similar to other adenylyl cyclase inhibition assays [5, 6], forskolin (an activator of adenylyl cyclase) is added which causes increase in cAMP that binds to Epac and results in less energy transfer between Rluc and YFP (Figure 1). Upon addition of a μ-opioid agonist, forskolin stimulated cAMP production decreases, thus, less cAMP binds to Epac resulting in more energy transfer between the BRET pair. In contrast to most of the cAMP assays that rely on end point measurement after single agonist incubation, this assay can be used to study the temporal dimensions of prolonged exposure to agonists. In this chapter, we describe in detail how to perform a CAMYEL assay to determine real time cAMP level changes due to μ receptor activation in HEK cells.

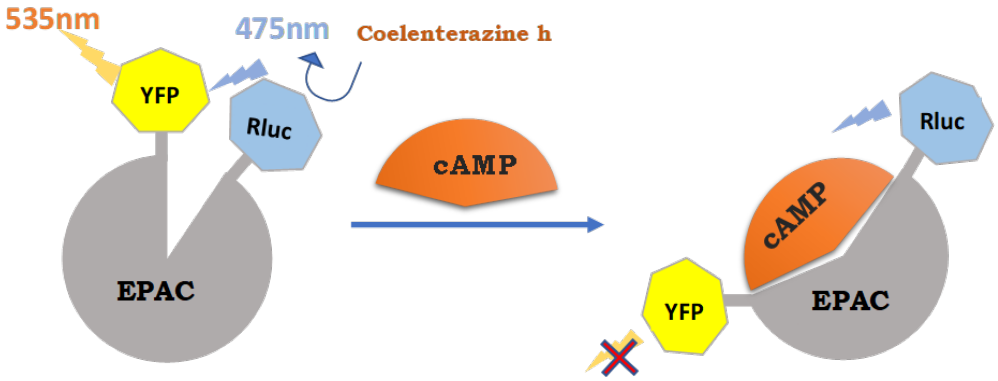


Figure B-1. Representative diagram of CAMYEL BRET biosensor. In the absence of cAMP, resonance energy is transferred between the BRET pair (Rluc and YFP). In the presence of cAMP, it binds to EPAC hindering energy transfer between the pair, resulting in loss of BRET signal.

2 Materials

2.1 DAY 1:

1. HEK293-GIRK4 cells stably expressing human μ receptor at 80-90% confluency in 75 cm^2 tissue culture flask
2. A 10 cm cell culture dish
3. Maintenance media: DMEM supplemented with 10% fetal bovine serum (FBS) and 100 units/ml penicillin, streptomycin 100 μg/ml
4. Automated cell counter/ haemocytometer

2.2 DAY 2:

1. CAMYEL plasmid (originally obtained from American Type Culture Collection (Manassas, VI, USA) [4]
2. 1mg/ml PEI: Polyethylenimine
3. 150 mM sterile NaCl
4. Maintenance media
5. Vortex Mixer

2.3 DAY 3:

1. Plating media: Leibovitz's L-15 media without phenol red supplemented with 1% FBS, 100 units/ml penicillin, streptomycin 100 µg/ml and glucose to 15 mM final concentration
2. PDL coated white wall clear bottomed 96 well microplates
3. Automated cell counter/ haemocytometer
4. 8 channel multi-pipette

2.4 DAY 4:

1. PheraSTAR FS Plate Reader (BMG Labtech)
2. Coelenterazine h (see NOTE 1)
3. Forskolin
4. Opioid ligands
5. 96-Well clear, v-bottomed microplates
6. Hank's Balanced Salt Solution (HBSS): 145 mM NaCl, 22 mM HEPES, 0.338 mM Na_2HPO_4, 4.17 mM $NaHCO_3$, 0.441 mM KH_2PO_4 , 0.407 mM $MgSO_4$, 0.493 mM $MgCl_2$, 1.26mM $CaCl_2$, 5.56 mM Glucose. Adjusted to pH 7.4 and osmolarity 310-320

3 Methods

3.1 DAY 1: Plating the cells in a 10 cm dish

1. Detach the cells from tissue culture flask using trypsin and centrifuge at 1000 rpm for 5 minutes.
2. Resuspend the cell pellet in 4 ml media and count cells using automated cell counter or haemocytometer.

3. Seed 6 million cells in 10 cm culture dish containing at least 7 ml of maintenance media (*see* NOTE 2).
4. Incubate the dish overnight at 37 °C in a humidified atmosphere containing 5% CO_2.

3.2 DAY 2: **Transfection of Biosensor (*see* NOTE 3)**

1. The DNA:PEI ratio for transfection is 1:6. The concentration of DNA is 5 µg, hence concentration of PEI required is 30 µg.
2. First, calculate the amount of DNA and PEI to be added from the stock solution to achieve the desired concentration. Then, determine the required volume of 150 mM sterile NaCl solution to make the final volume of 500 µl.
   For example: if concentration of stock DNA is 2 µg/µl, 5 µg DNA will be in 2.5 µl of the stock. Likewise, if the concentration of PEI in stock solution is 1 mg/ml, then use 30 µl to get 30 µg PEI. Combine 2.5 µl DNA and 30 µl PEI to 467.5 µl of NaCl solution. (*see* NOTE 4)
3. Immediately vortex the PEI/DNA mixture for approximately 10 seconds and incubate for 10 minutes at room temperature. Meanwhile, replace the media from the dish containing cells to be transfected with 10 ml of fresh maintenance media. Once incubation is over, add 500 µl PEI/DNA dropwise while gently swirling the plate. Incubate the plate at 37°C in a humidified atmosphere containing 5% CO_2.
4. Record the time of transfection. (*see* NOTE 5)

3.3 DAY 3: Replating cells in assay plate

1. Visually inspect viability of the cells and after 24 hours of cell transfection, detach cells from the plate using trypsin/EDTA and centrifuge. Resuspend the cell pellet in 5 ml of L-15 and count the cells using automated cell counter or haemocytometer. Dilute the cell suspension to get 100,000 cells per 80 µl (volume per well) (*see* NOTE 6 for calculation). Mix and dispense 80 µl cell suspension in each well using a multichannel pipette.
2. Incubate the plate overnight at 37°C in ambient CO_2 (*see* NOTE 7).

3.4 DAY 4: cAMP Assay

Step 1. Prepare drug solutions

Forskolin and opioids are added to the cells at the same time point. Prepare forskolin in HBSS and use this solution to prepare different opioid concentrations. Ensure the final concentration of forskolin remains constant (30 µM). (*see* NOTE 8)

All drug solutions are made at 10x the desired concentration. This is because the cell plate already contains 80 µl of cell suspension and 10µl substrate, thus adding 10 µl of drug in forskolin results in dilution of both drug and forskolin by 10 times.

For example, if the final desired concentration of forskolin is 3 µM and drug A is 1 µM, then the concentration after preparation of drug solutions should be 10 µM drug A and 30 µM forskolin.

Step 2. Prepare substrate

Prepare 50 µM coelenterazine h in cold HBSS in an amber coloured tube in small batches due to the low stability at room temperature.

Step 3. Load drugs in the drug plate

Load 100 µl of all the concentrations of drugs and vehicle in a V-bottom clear plate. Reserve one well for forskolin by itself and another for vehicle (*see* NOTE 9).

Step 4. Set up the parameters in PHERAstarFS (*see* NOTE 10).

Turn on the plate reader and set it at 37 °C. Upload the cell plate in the machine and set up the assay parameters in the reader as follows:

BASIC SETTTING PARAMETERS	
Measurement type	Luminescence (dual emission)
Microplate name	COSTAR 96
Type of optic used	Top optic
Settling time [s]	0.1
Reading direction	↓↑↓
Target temperature [°C]	37
PLATE MODE SETTINGS	
No. of cycles	7
Cycle time	40
Measurement interval time [s]	0.50
OPTIC SETTINGS	
Optic module	BRET 1 plus
Emission A	535-30
Emission B	475-30
Gain A	3300
Gain B	3600
Focal height [mm]	10.0
Ratio multiplier	1
SHAKING SETTINGS	
Shaking frequency [rpm]	500
Shaking mode	Linear
Additional shaking time	10s before first cycle

Step 5. Run the assay

First add 10 µl of 50 µM coelenterazine h in the first two columns (performing duplicates) of cell plate using a multichannel pipette and read luminescence signal for 5 minutes (baseline). After the first run is over, immediately add 10µl of different drug concentrations from V-bottomed plate to the same two columns of the cell plate and measure the luminescence for approximately 5 minutes.

Step 6. Collect the data and analyse

Calculate the inverse BRET ratio (461/542) in the MARS data analysis software. *See* NOTES 11-14 for further analysis details.

4 Notes:

1. Keep the resuspended stock in -30°C freezer and amber containers because coelenterazine h is temperature and light sensitive.

2. Having cells at higher confluency may affect the efficiency of transfection so make sure to inspect the cells before transfection. This protocol is for HEK 293 human μ receptor cells, the number of cells to be added to plate on day 1 would be different for different cell lines. Thus, optimisation is highly recommended.

3. Cells can be transfected a day or two after plating depending on the cell type and number of cells seeded on day 1. Different cells grow at different rate so add less cells if you plan to transfect 2 days after plating.

4. Both CAMYEL DNA and PEI must be stored at -30°C. Thaw PEI at room temperature, to avoid precipitation, before adding to the mixture. Refreezing excess PEI is not recommended.

5. Recording time of transfection is important, as the plating of cells into 96 well plate should be done at least 24 hours after transfection.

6. In the assay plate, seed 100,000 cells per well (total volume per well is 80 μl). Cell numbers must be optimised for different cell lines to obtain a well at approximately 80% confluency.

   For a full plate, 10 ml of total suspension is required therefore 10 ml of suspension should have 12.5 million cells.

   80 μl suspension would have 100,000 cells.

   1000 μl (1 ml) would have (100,000/80) x 1000 cells = 1,250,000 cells

   Thus, 10ml would have 1,250,000 x 10 = 12,500,000 cells = 12.5 million cells. If the cell counter reads 2.5 million cells/ml then, add 5 ml of cell suspension to 5 ml media to make total volume of 10 ml containing 12.5 million cells.

   If 2.5 million cells are in 1 ml suspension, then

   12.5 million cells would be in (1/2.5) x 12.5 = 5 ml suspension.

   So, add 5 ml cell suspension in (10-5) = 5 ml of fresh L-15 media.

7. It is important to incubate the cells at right temperature and environment. The assay plate containing cells in L-15 should be kept in incubator maintained at ambient CO2

as higher CO2 content changes the pH of the media, making the media toxic to the cells. Where incubators maintained at less CO2 is inaccessible, resuspend the cells in DMEM and incubate at 5% CO2. However, on the day of the assay, remove DMEM and serum starve cells in HBSS for 30 minutes prior to the assay.

8. The concentration of forskolin used in this assay is 3 μM (approximate EC_{50} value). Some studies have used 5 μM [7], hence, it is highly recommended to obtain a forskolin concentration-response curve and determine EC_{50} value before starting the experiment.

9. Forskolin and some of the ligands are dissolved in DMSO so make sure to keep the final concentration of DMSO constant when preparing drugs and vehicle.

10. We have previously used FlexStation 3 plate reader for this assay [4]. We followed kinetics settings in the machine and measured luminescence on a top read mode. The parameters for FlexStation 3 is as follows:

	Setting 1 (baseline)	Setting 2 (drug)
Read mode	Luminescence, Top read	
Wavelength 1	461	
Wavelength 2	542	
Integration	1000	
Run time	5 minutes	5 minutes
Interval	1 minute (4 reads)	0.46 (7 reads)
Automix	Yes (5 s after drug addition)	

The major difference in using Flex Station 3 to PHERAstar FS is the amplitude of signal. The window between background reading (no substrate) to substrate signal is very low in FlexStation 3. Flex Station 3 is unable to read more than one column at a time, while, in PHERAstar FS, it is possible to run more than one column.

11. For data analysis:

Calculate the inverse BRET ratio (475/535) in MARS Pherastar data analysis software. Plot the inverse BRET ratio in GraphPad Prism and calculate area under curve (AUC) for each concentration of drug and vehicle (Figure 2). When setting data analysis, take mean of first reading (before drug addition) as baseline, hence the response calculated is AUC after drug or forskolin addition. Subtract background

value (vehicle) from readings and present data as percentage difference between forskolin response (set as 100%) and forskolin with opioid response (Figure 3).

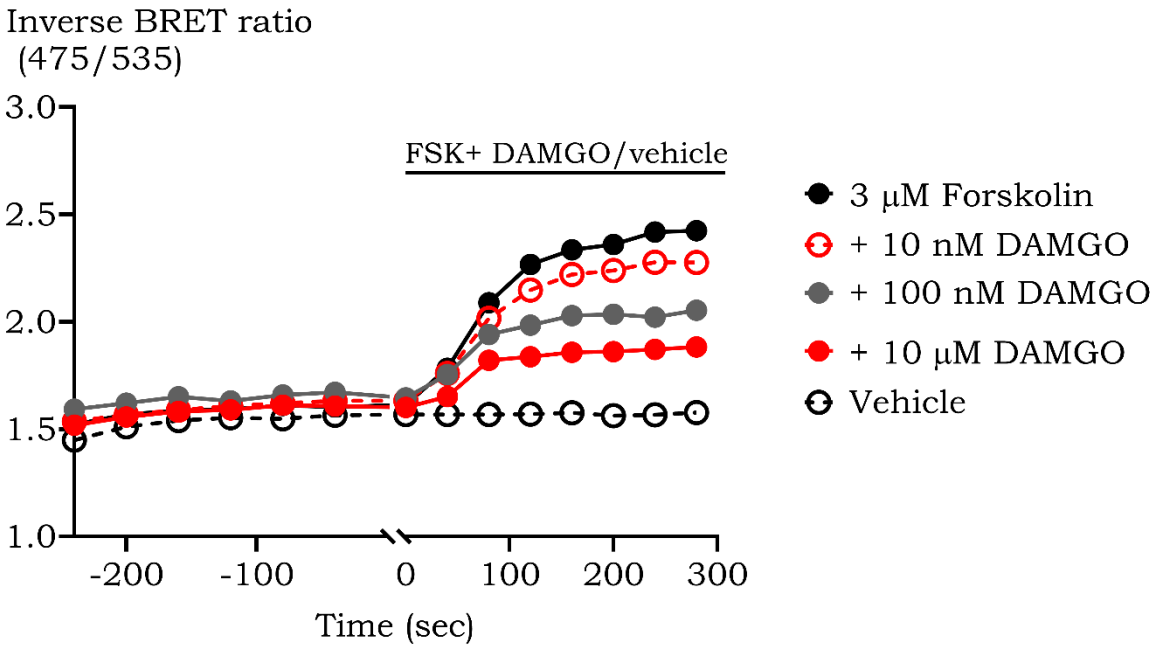


Figure B-2. Representative traces for CAMYEL assay for HEK 293 cells expressing μ receptors. The baseline is measured for 5 minutes before addition of various concentration of opioids with forskolin. An increase in BRET ratio (emission at 461/542 nm) corresponds to an increase in cAMP.

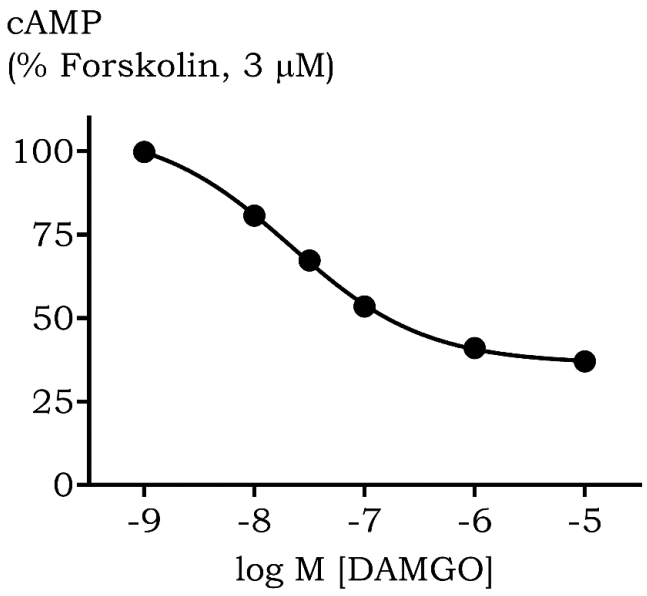


Figure B-3. Concentration response curve showing DAMGO mediated inhibition of forskolin stimulated cAMP production. Data is expressed as percentage of forskolin response (3 μM).

12. Standard BRET ratio (535/475) can also be used instead of inverse BRET ratio [7]. The only difference is the interpretation of data. When using inverse BRET ratio, increase in ratio corresponds to increase in cAMP and vice versa, however, in standard BRET ratio, increase in ratio corresponds to decrease in cAMP. Using either of the ratio does not change the final response of the drug.

13. The data can be analysed in a different way as percentage inhibition of forskolin response. The concentration response curve is as shown in figure 4.

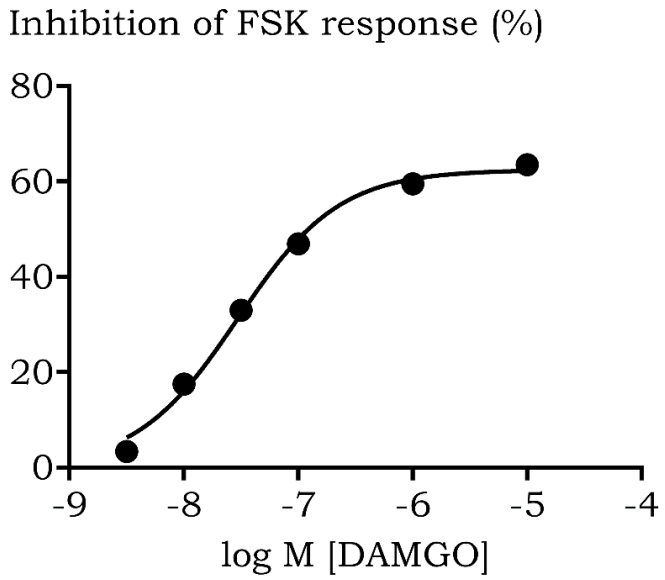


Figure B-4. Concentration response curve for DAMGO inhibition of elevated cAMP levels produced by forskolin (3 μM). Data is expressed as percentage inhibition of forskolin response.

14. This assay can be used to study the kinetics of adenylyl cyclase inhibition by pre-treatment of cells with forskolin instead of adding opioids and forskolin together [3]. For instance, measure baseline for 5 minutes after adding substrate, then add forskolin and read for another 5 minutes before adding opioids.

References:

1. Al-Hasani R, Bruchas MR (2011) Molecular mechanisms of opioid receptor-dependent signaling and behavior. Anesthesiology: The Journal of the American Society of Anesthesiologists, 115(6), 1363-1381.
2. Hill SJ, Williams C, May LT (2010) Insights into GPCR pharmacology from the measurement of changes in intracellular cyclic AMP; advantages and pitfalls of differing methodologies. British journal of pharmacology, 161(6), 1266-1275.
3. Thompson, G. (2018). Measurement of Intracellular cAMP using a BRET Biosensor.
4. Sachdev S, Boyd R, Grimsey NL, Santiago M, Connor M (2019) Brodifacoum does not modulate human cannabinoid receptor-mediated hyperpolarization of AtT20 cells or inhibition of adenylyl cyclase in HEK 293 cells. PeerJ. 7:e7733.
5. Knapman A, Connor M (2015) Fluorescence-based, high-throughput assays for μ-opioid receptor activation using a membrane potential-sensitive dye. In Opioid Receptors (pp. 177-185). Humana Press, New York, NY.
6. Buccioni M, Marucci G, Dal Ben D, Giacobbe D, Lambertucci C, Soverchia L, Cristalli G (2011) Innovative functional cAMP assay for studying G protein-coupled receptors: application to the pharmacological characterization of GPR17. Purinergic signalling, 7(4), 463-468
7. Oyagawa CR, Sara M, Saroz Y, Glass M, Vernall AJ, & Grimsey NL (2018) Cannabinoid Receptor 2 Signalling Bias Elicited by 2, 4, 6-Trisubstituted 1, 3, 5-Triazines. Frontiers in pharmacology, 9.

Appendix C & D of this thesis have been removed as they may contain sensitive/confidential content

References

Abood ME, Sauss C, Fan F, Tilton CL, & Martin BR (1993). Development of behavioral tolerance to Δ9-THC without alteration of cannabinoid receptor binding or mRNA levels in whole brain. Pharmacology Biochemistry and Behavior, 46**,** 575-579. https://psycnet.apa.org/doi/10.1016/0091-3057(93)90546-6

Adams AJ, Banister SD, Irizarry L, Trecki J, Schwartz M, & Gerona R (2017). "Zombie" outbreak caused by the synthetic cannabinoid AMB-FUBINACA in New York. New England journal of medicine, 376**,** 235-242.

Adams R, Baker B, & Wearn R (1940). Structure of cannabinol. III. Synthesis of cannabinol, 1-Hydroxy-3-n-amyl-6, 6, 9-trimethyl-6-dibenzopyran1. Journal of the American Chemical Society, 62**,** 2204-2207. https://doi.org/10.1021/ja01865a083

Adams R, Harfenist M, & Loewe S (1949). New analogs of tetrahydrocannabinol. XIX. Journal of the American Chemical Society, 71**,** 1624-1628. https://doi.org/10.1021/ja01173a023

Alexander SP, Mathie A, & Peters JA (2011). Guide to receptors and channels (GRAC). British Journal of Pharmacology, 164**,** S1-S2. https://doi.org/10.1111/j.1476-5381.2011.01649_1.x

Alipour A, Patel PB, Shabbir Z, & Gabrielson S (2019). Review of the many faces of synthetic cannabinoid toxicities. Mental Health Clinician, 9**,** 93-99. https://doi.org/10.9740/mhc.2019.03.093

Anavi-Goffer S, Baillie G, Irving AJ, Gertsch J, Greig IR, Pertwee RG, et al. (2012). Modulation of L-α-lysophosphatidylinositol/GPR55 mitogen-activated protein kinase (MAPK) signaling by cannabinoids. Journal of biological chemistry, 287**,** 91-104.

Anderson LL, Absalom NL, Abelev SV, Low IK, Doohan PT, Martin LJ, et al. (2019). Coadministered cannabidiol and clobazam: Preclinical evidence for both pharmacodynamic and pharmacokinetic interactions. Epilepsia, 60**,** 2224-2234.

Andersson M, Diao X, Wohlfarth A, Scheidweiler KB, & Huestis MA (2016). Metabolic profiling of new synthetic cannabinoids AMB and 5F-AMB by human hepatocyte and liver microsome incubations and high-resolution mass spectrometry. Rapid Communications in Mass Spectrometry, 30**,** 1067-1078. https://doi.org/10.1002/rcm.7538

Angerer V, Möller C, & Auwärter V. Synthetic cannabinoids in prisons – invisibly impregnated paper sheets as a Trojan horse. 2018. Forensic Toxicology, Institute of Forensic Medicine, Medical Center – University of Freiburg, Freiburg, Germany.

Antonides LH, Cannaert A, Norman C, Vives L, Harrison A, Costello A, et al. (2019). Enantiospecific synthesis, chiral separation and biological activity of four indazole-3-carboxamide-type synthetic cannabinoid receptor agonists and their detection in seized drug samples. Frontiers in Chemistry, 7**,** 321. https://doi.org/10.3389/fchem.2019.00321

Atwood BK, Huffman J, Straiker A, & Mackie K (2010). JWH018, a common constituent of 'Spice'herbal blends, is a potent and efficacious cannabinoid CB1 receptor agonist. British Journal of Pharmacology, 160**,** 585-593. https://doi.org/10.1111/j.1476-5381.2009.00582.x

Atwood BK, Wager-Miller J, Haskins C, Straiker A, & Mackie K (2012). Functional selectivity in CB2 cannabinoid receptor signaling and regulation: implications for the therapeutic potential of CB2 ligands. Molecular Pharmacology, 81**,** 250-263. https://doi.org/10.1124/mol.111.074013

Auwärter V, Dresen S, Weinmann W, Müller M, Pütz M, & Ferreirós N (2009). 'Spice'and other herbal blends: harmless incense or cannabinoid designer drugs? Journal of Mass Spectrometry, 44**,** 832-837.

Banister SD, Adams A, Kevin RC, Macdonald C, Glass M, Boyd R, et al. (2019a). Synthesis and pharmacology of new psychoactive substance 5F-CUMYL-P7AICA, a scaffold-hopping analog of synthetic cannabinoid receptor agonists 5F-CUMYL-PICA and 5F-CUMYL-PINACA. Drug Testing and Analysis, 11**,** 279-291. https://doi.org/10.1002/dta.2491

Banister SD, Arnold JC, Connor M, Glass M, & McGregor IS (2019b). Dark Classics in Chemical Neuroscience: Δ9-Tetrahydrocannabinol. ACS Chemical Neuroscience, 10**,** 2160-2175. https://doi.org/10.1021/acschemneuro.8b00651

Banister SD, & Connor M (2018a). The chemistry and pharmacology of synthetic cannabinoid receptor agonist new psychoactive substances: evolution. New Psychoactive Substances. Springer.

Banister SD, & Connor M (2018b). The chemistry and pharmacology of synthetic cannabinoid receptor agonists as new psychoactive substances: origins. New Psychoactive Substances. Springer.

Banister SD, Longworth M, Kevin R, Sachdev S, Santiago M, Stuart J, et al. (2016). Pharmacology of valinate and tert-leucinate synthetic cannabinoids 5F-AMBICA, 5F-AMB, 5F-ADB, AMB-FUBINACA, MDMB-FUBINACA, MDMB-CHMICA, and their analogues. ACS Chemical Neuroscience, 7**,** 1241-1254.

Banister SD, Moir M, Stuart J, Kevin RC, Wood KE, Longworth M, et al. (2015a). Pharmacology of Indole and Indazole Synthetic Cannabinoid Designer Drugs AB-FUBINACA, ADB-FUBINACA, AB-PINACA, ADB-PINACA, 5F-AB-PINACA, 5F-ADB-PINACA, ADBICA, and 5F-ADBICA. ACS Chemical Neuroscience, 6**,** 1546-1559. https://doi.org/10.1021/acschemneuro.5b00112

Banister SD, Stuart J, Kevin RC, Edington A, Longworth M, Wilkinson SM, et al. (2015b). Effects of bioisosteric fluorine in synthetic cannabinoid designer drugs JWH-018, AM-2201, UR-144, XLR-11, PB-22, 5F-PB-22, APICA, and STS-135. ACS Chemical Neuroscience, 6**,** 1445-1458. https://doi.org/10.1021/acschemneuro.5b00107

Barann M, Molderings G, Brüss M, Bönisch H, Urban B, & Göthert M (2002). Direct inhibition by cannabinoids of human 5-HT3A receptors: probable involvement of an allosteric modulatory site. British journal of pharmacology, 137**,** 589-596.

Ben-Shabat S, Fride E, Sheskin T, Tamiri T, Rhee M-H, Vogel Z, et al. (1998). An entourage effect: inactive endogenous fatty acid glycerol esters enhance 2-arachidonoyl-glycerol cannabinoid activity. European Journal of Pharmacology, 353**,** 23-31. https://doi.org/10.1016/s0014-2999(98)00392-6

Benbadis SR, Sanchez-Ramos J, Bozorg A, Giarratano M, Kalidas K, Katzin L, et al. (2014). Medical marijuana in neurology. Expert Review of Neurotherapeutics, 14**,** 1453-1465. https://doi.org/10.1586/14737175.2014.985209

Bhanushali GK, Jain G, Fatima H, Leisch LJ, & Thornley-Brown D (2013). AKI associated with synthetic cannabinoids: a case series. Clinical Journal of the American Society of Nephrology, 8**,** 523-526. https://doi.org/10.2215/CJN.05690612

Bidaut-Russell M, Devane WA, & Howlett AC (1990). Cannabinoid receptors and modulation of cyclic AMP accumulation in the rat brain. Journal of Neurochemistry, 55**,** 21-26. https://doi.org/10.1111/j.1471-4159.1990.tb08815.x

Black JW, & Leff P (1983). Operational models of pharmacological agonism. Proc. R. Soc. Lond. B, 220**,** 141-162. https://doi.org/10.1098/rspb.1983.0093

Bladen C, Santiago M, Longworth M, Kassiou M, Banister S, & Connor M. Characterization and modulation of human Cav3.2 channels by synthetic cannabinoids in vitro. International Cannabinoid Research Sciety, 2018 Leiden, Netherlands.

Bohn LM, Lefkowitz RJ, Gainetdinov RR, Peppel K, Caron MG, & Lin F-T (1999). Enhanced morphine analgesia in mice lacking β-arrestin 2. Science, 286**,** 2495-2498. https://doi.org/10.1126/science.286.5449.2495

Bouaboula M, Bourrié B, Rinaldi-Carmona M, Shire D, Le Fur G, & Casellas P (1995). Stimulation of cannabinoid receptor CB1 induces krox-24 expression in human astrocytoma cells. Journal of Biological Chemistry, 270**,** 13973-13980. https://doi.org/10.1074/jbc.270.23.13973

Bouaboula M, Desnoyer N, Carayon P, Combes T, & Casellas P (1999). Gi protein modulation induced by a selective inverse agonist for the peripheral cannabinoid receptor CB2: implication for intracellular signalization cross-regulation. Molecular Pharmacology, 55**,** 473-480.

Bouaboula M, Poinot-Chazel C, Marchand J, Canat X, Bourrié B, Rinaldi-Carmona M, et al. (1996). Signaling pathway associated with stimulation of CB2 peripheral cannabinoid receptor: Involvement of both mitogen-activated protein kinase and induction of Krox-24 expression. European Journal of Biochemistry, 237**,** 704-711. https://doi.org/10.1111/j.1432-1033.1996.0704p.x

Breivogel CS, Childers SR, Deadwyler SA, Hampson RE, Vogt LJ, & Sim-Selley LJ (1999). Chronic Δ^9-Tetrahydrocannabinol Treatment Produces a Time-Dependent Loss of Cannabinoid Receptors and Cannabinoid Receptor-Activated G Proteins in Rat Brain. Journal of Neurochemistry, 73**,** 2447-2459. https://doi.org/10.1046/j.1471-4159.1999.0732447.x

Breivogel CS, Griffin G, Di Marzo V, & Martin BR (2001). Evidence for a new G protein-coupled cannabinoid receptor in mouse brain. Molecular Pharmacology, 60**,** 155-163. https://doi.org/10.1124/mol.60.1.155

Breivogel CS, Lambert JM, Gerfin S, Huffman JW, & Razdan RK (2008). Sensitivity to Δ9-tetrahydrocannabinol is selectively enhanced in beta-arrestin2-/-mice. Behavioural Pharmacology, 19**,** 298. https://dx.doi.org/10.1097%2FFBP.0b013e328308f1e6

Brenneisen R (2007). Chemistry and analysis of phytocannabinoids and other Cannabis constituents. Marijuana and the Cannabinoids. Springer.

Brents LK, Gallus-Zawada A, Radominska-Pandya A, Vasiljevik T, Prisinzano TE, Fantegrossi WE, et al. (2012). Monohydroxylated metabolites of the K2 synthetic cannabinoid JWH-073 retain intermediate to high cannabinoid 1 receptor (CB1R) affinity and exhibit neutral antagonist to partial agonist activity. Biochemical Pharmacology, 83**,** 952-961. https://doi.org/10.1016/j.bcp.2012.01.004

Bretteville-Jensen AL, Tuv SS, Bilgrei OR, Fjeld B, & Bachs L (2013). Synthetic cannabinoids and cathinones: prevalence and markets. Forensic Science Review, 25(1-2):7-26.

Brwon AJ, and Hiley CR (2009). Is GPR55 an andamaide receptor? Vitamins and Hormones, 81, 111-137. https://doi.org/10.1016/S0083-6729(09)81005-4

Buser G, Gerona R, Horowitz BZ, Vian K, Troxell M, Hendrickson R, et al. (2014). Acute kidney injury associated with smoking synthetic cannabinoid. Clinical Toxicology, 52**,** 664-673. https://doi.org/10.3109/15563650.2014.932365

Cahn R (1933). 326. Cannabis indica resin. Part IV. The synthesis of some 2: 2-dimethyldibenzopyrans, and confirmation of the structure of cannabinol. Journal of the Chemical Society (Resumed)**,** 1400-1405. https://doi.org/10.1039/JR9330001400

Calandra B, Portier M, Kernéis A, Delpech M, Carillon C, Le Fur G, et al. (1999). Dual intracellular signaling pathways mediated by the human cannabinoid CB1 receptor. European Journal of Pharmacology, 374**,** 445-455. https://doi.org/10.1016/s0014-2999(99)00349-0

Cannaert A, Franz F, Auwärter V, & Stove CP (2017). Activity-based detection of consumption of synthetic cannabinoids in authentic urine samples using a stable cannabinoid reporter system. Analytical Chemistry, 89**,** 9527-9536. https://doi.org/10.1021/acs.analchem.7b02552

Castaneto MS, Gorelick DA, Desrosiers NA, Hartman RL, Pirard S, & Huestis MA (2014). Synthetic cannabinoids: epidemiology, pharmacodynamics, and clinical implications. Drug and alcohol dependence, 144**,** 12-41.

Castle D, & Murray R 2004. *Marijuana and Madness: Psychiatry and Neurobiology*, Cambridge University Press.

Cawston EE, Redmond WJ, Breen CM, Grimsey NL, Connor M, & Glass M (2013). Real-time characterization of cannabinoid receptor 1 (CB 1) allosteric modulators reveals novel mechanism of action. British Journal of Pharmacology, 170**,** 893-907.

Chen X, Yang W, Fan Y, Luo J, Hong K, Wang Z, et al. (2010). Structural determinants in the second intracellular loop of the human cannabinoid CB1 receptor mediate selective coupling to Gs and Gi. British Journal of Pharmacology, 161**,** 1817-1834. https://doi.org/10.1111/j.1476-5381.2010.01006.x

Cipriano M, Häggström J, Hammarsten P, & Fowler CJ (2013). Association between cannabinoid CB1 receptor expression and Akt signalling in prostate cancer. PLOS ONE, 8**,** e65798. https://dx.doi.org/10.1371%2Fjournal.pone.0065798

Cohen K, & Weinstein AM (2018). Synthetic and non-synthetic cannabinoid drugs and their adverse effects-a review from public health prospective. Frontiers in Public Health, 6**,** 162. https://dx.doi.org/10.3389%2Ffpubh.2018.00162

Compton DR, Gold LH, Ward SJ, Balster RL, & Martin BR (1992a). Aminoalkylindole analogs: cannabimimetic activity of a class of compounds structurally distinct from delta 9-tetrahydrocannabinol. Journal of Pharmacology and Experimental Therapeutics, 263**,** 1118-1126.

Compton DR, Johnson MR, Melvin LS, & Martin BR (1992b). Pharmacological profile of a series of bicyclic cannabinoid analogs: classification as cannabimimetic agents. Journal of Pharmacology and Experimental Therapeutics, 260**,** 201-209.

Cooper ZD (2016). Adverse effects of synthetic cannabinoids: management of acute toxicity and withdrawal. Current psychiatry reports, 18**,** 52. https://doi.org/10.1007/s11920-016-0694-1

Conn PJ, Christopoulous A, & Lindsley LW (2009). Allosteric modulators of GPCRs: a novel approach for the treatment of CNS disorders. Nature reviews Drug discovery, 8(1), 41-54. https://dx.doi.org/10.1038%2Fnrd2760.

Couch DG, Cook H, Ortori C, Barrett D, Lund JN, & O'Sullivan SE (2019). Palmitoylethanolamide and Cannabidiol Prevent Inflammation-induced Hyperpermeability of the Human Gut In Vitro and In Vivo—A Randomized, Placebo-controlled, Double-blind Controlled Trial. Inflammatory Bowel Diseases, 25**,** 1006-1018. https://doi.org/10.1093/ibd/izz017

Cristiani SA, Pukay-Martin ND, & Bornstein RA (2004). Marijuana use and cognitive function in HIV-infected people. The Journal of neuropsychiatry and clinical neurosciences, 16**,** 330-335. https://doi.org/10.1176/jnp.16.3.330

Cudaback E, Marrs W, Moeller T, & Stella N (2010). The expression level of CB1 and CB2 receptors determines their efficacy at inducing apoptosis in astrocytomas. PLoS One, 5**,** e8702. https://doi.org/10.1371/journal.pone.0008702

Cunha JM, Carlini E, Pereira AE, Ramos OL, Pimentel C, Gagliardi R, et al. (1980). Chronic Administration of Cannabidiol to Healthy Volunteers and Epileptic Patients Pharmacology, 21**,** 175-185. https://doi.org/10.1159/000137430

D'Ambra TE, Estep KG, Bell MR, Eissenstat MA, Josef KA, Ward SJ, et al. (1992). Conformationally restrained analogs of pravadoline: nanomolar potent, enantioselective,(aminoalkyl) indole agonists of the cannabinoid receptor. Journal of Medicinal Chemistry, 35**,** 124-135. https://doi.org/10.1021/jm00079a016

Daigle TL, Kearn CS, & Mackie K (2008). Rapid CB1 cannabinoid receptor desensitization defines the time course of ERK1/2 MAP kinase signaling. Neuropharmacology, 54**,** 36-44. https://doi.org/10.1016/j.neuropharm.2007.06.005

De Petrocellis L, & Di Marzo V (2010). Non-CB 1, non-CB 2 receptors for endocannabinoids, plant cannabinoids, and synthetic cannabimimetics: Focus on G-protein-coupled receptors and transient receptor potential channels. Journal of Neuroimmune Pharmacology, 5**,** 103-121. https://doi.org/10.1007/s11481-009-9177-z

De Petrocellis L, Starowicz K, Moriello AS, Vivese M, Orlando P, and Di Marzo V (2007). Regulation of transient receptor potential channels of melastatin type 8 (TRPM8): effect of cAMP, cannabinoid CB_1 receptors and endovanilloids. Experimental Cell Research, 313, 1911–1920. doi: 10.1016/j.yexcr.2007.01.008

De Petrocellis L, Ligresti A, Moriello AS, Allarà M, Bisogno T, Petrosino S, et al. (2011). Effects of cannabinoids and cannabinoid-enriched Cannabis extracts on TRP channels and endocannabinoid metabolic enzymes. British Journal of Pharmacology, 163**,** 1479-1494. https://doi.org/10.1111/j.1476-5381.2010.01166.x

De Petrocellis L, Orlando P, Moriello AS, Aviello G, Stott C, Izzo A, et al. (2012). Cannabinoid actions at TRPV channels: effects on TRPV3 and TRPV4 and their potential relevance to gastrointestinal inflammation. Acta Physiologica, 204**,** 255-266. https://doi.org/10.1111/j.1748-1716.2011.02338.x

Del Pulgar TG, Velasco G, & Guzman M (2000). The CB1 cannabinoid receptor is coupled to the activation of protein kinase B/Akt. Biochemical Journal, 347**,** 369-373. https://doi.org/10.1042/0264-6021:3470369

Demuth DG, & Molleman A (2006). Cannabinoid signalling. Life Sciences, 78**,** 549-563. https://doi.org/10.1016/j.lfs.2005.05.055

Derkinderen P, Valjent E, Toutant M, Corvol J-C, Enslen H, Ledent C, et al. (2003). Regulation of extracellular signal-regulated kinase by cannabinoids in hippocampus. Journal of Neuroscience, 23**,** 2371-2382. https://dx.doi.org/10.1523%2FJNEUROSCI.23-06-02371.2003

Devane WA, Breuer A, Sheskin T, Jaerbe TU, Eisen MS, & Mechoulam R (1992). A novel probe for the cannabinoid receptor. Journal of Medicinal Chemistry, 35**,** 2065-2069. https://doi.org/10.1021/jm00089a018

Devane WA, Dysarz Fr, Johnson MR, Melvin LS, & Howlett AC (1988). Determination and characterization of a cannabinoid receptor in rat brain. Molecular Pharmacology, 34**,** 605-613.

Devinsky O, Cross JH, Laux L, Marsh E, Miller I, Nabbout R, et al. (2017). Trial of cannabidiol for drug-resistant seizures in the Dravet syndrome. New England Journal of Medicine, 376**,** 2011-2020. 10.1056/NEJMoa1611618.

Dopart R, Lu D, Litchman AH, & Kendall DA (2018). Allosteric modulators of cannabinoid receptor 1: developing compounds for improved specificity. Drug metabolism reviews, 50 (1), 3-13. https://doi.org/10.1080/03602532.2018.1428342.

Dresen S, Ferreirós N, Pütz M, Westphal F, Zimmermann R, & Auwärter V (2010). Monitoring of herbal mixtures potentially containing synthetic cannabinoids as psychoactive compounds. Journal of Mass Spectrometry, 45**,** 1186-1194.

Dunstan W, & Henry T. On oxycannabin from Indian hemp. Proc. Chem. Soc, 1898. 44-45.

Earleywine M 2002. *Understanding marijuana: A new look at the scientific evidence*, Oxford University Press.

Eissenstat MA, Bell MR, D'Ambra TE, Alexander EJ, Daum SJ, Ackerman JH, et al. (1995). Aminoalkylindoles: structure-activity relationships of novel cannabinoid mimetics. Journal of Medicinal Chemistry, 38**,** 3094-3105. https://doi.org/10.1021/jm00016a013

ElSohly MA, Gul W, Wanas AS, & Radwan MM (2014). Synthetic cannabinoids: analysis and metabolites. Life Sciences, 97**,** 78-90. https://doi.org/10.1016/j.lfs.2013.12.212

EMCDDA (2012). New drugs in Europe, 2012. EMCDDA–Europol 2012 Annual Report on the implementation of Council Decision 2005/387/JHA**,** Luxembourg: Publications Office of the European Union. 10.2810/99367

EMCDDA (2018). New psychoactive substances in prison. Publications Office of the European Union, Luxembourg. http://www.emcdda.europa.eu/system/files/publications/8869/nps-in-prison.pdf

Fabregat-Safont D, Mardal M, Noble C, Cannaert A, Stove CP, Sancho JV, et al. (2019). Comprehensive investigation on synthetic cannabinoids: metabolic behaviour and potency testing, using 5F-APP-PICA and AMB-FUBINACA as model compounds. Drug Testing and Analysis. https://doi.org/10.1002/dta.2659

Fantegrossi WE, Moran JH, Radominska-Pandya A, & Prather PL (2014). Distinct pharmacology and metabolism of K2 synthetic cannabinoids compared to Δ9-THC: mechanism underlying greater toxicity? Life Sciences, 97**,** 45-54. https://doi.org/10.1016/j.lfs.2013.09.017

Felder CC, Briley EM, Axelrod J, Simpson JT, Mackie K, & Devane WA (1993). Anandamide, an endogenous cannabimimetic eicosanoid, binds to the cloned human cannabinoid receptor and stimulates receptor-mediated signal transduction. Proceedings of the National Academy of Sciences, 90**,** 7656-7660. https://doi.org/10.1073/pnas.90.16.7656

Felder CC, Joyce KE, Briley EM, Mansouri J, Mackie K, Blond O, et al. (1995). Comparison of the pharmacology and signal transduction of the human cannabinoid CB1 and CB2 receptors. Molecular Pharmacology, 48**,** 443-450.

Fernando SR, & Pertwee RG (1997). Evidence that methyl arachidonyl fluorophosphonate is an irreversible cannabinoid receptor antagonist. British Journal of Pharmacology, 121**,** 1716-1720. https://doi.org/10.1038/sj.bjp.0701303

Finlay, D., 2018. Factors influencing the specificity and bias of CB1 cannabinoid receptor signalling. PhD Thesis, The University of Auckland.

Finlay DB, Cawston EE, Grimsey NL, Hunter MR, Korde A, Kiran Vemuri V, et al. (2017). Gαs signalling of the CB1 receptor and the influence of receptor number. British Journal of Pharmacology. https://doi.org/10.1111/bph.13866

Finlay DB, Manning JJ, Ibsen MS, Macdonald CE, Patel M, Javitch JA, et al. (2019). Do toxic synthetic cannabinoid receptor agonists have signature in vitro activity profiles? A case study of AMB-FUBINACA. ACS Chemical Neuroscience, 10**,** 4350-4360. https://doi.org/10.1021/acschemneuro.9b00429

Finlay DB, Duffull SB, & Glass M (2020). 100 years of modelling ligand–receptor binding and response: A focus on GPCRs. British Journal of Pharmacology, 177(7), 1472-1484. https://doi.org/10.1111/bph.14988.

Fox A, Kesingland A, Gentry C, McNair K, Patel S, Urban L, et al. (2001). The role of central and peripheral Cannabinoid1 receptors in the antihyperalgesic activity of cannabinoids in a model of neuropathic pain. Pain, 92**,** 91-100. https://doi.org/10.1016/s0304-3959(00)00474-7

Friedman D, & Sirven JI (2017). Historical perspective on the medical use of cannabis for epilepsy: ancient times to the 1980s. Epilepsy & Behavior, 70**,** 298-301. https://doi.org/10.1016/j.yebeh.2016.11.033

Funada M, & Takebayashi-Ohsawa M (2018). Synthetic cannabinoid AM2201 induces seizures: Involvement of cannabinoid CB1 receptors and glutamatergic transmission. Toxicology and Applied Pharmacology, 338**,** 1-8. https://doi.org/10.1016/j.taap.2017.10.007

Galiègue S, Mary S, Marchand J, Dussossoy D, Carrière D, Carayon P, et al. (1995). Expression of central and peripheral cannabinoid receptors in human immune tissues and leukocyte subpopulations. European Journal of Biochemistry, 232**,** 54-61. https://doi.org/10.1111/j.1432-1033.1995.tb20780.x

Galve-Roperh I, Sánchez C, Cortés ML, del Pulgar TG, Izquierdo M, & Guzmán M (2000). Anti-tumoral action of cannabinoids: involvement of sustained ceramide accumulation and extracellular signal-regulated kinase activation. Nature Medicine, 6**,** 313. https://doi.org/10.1038/73171

Gamage TF, Farquhar CE, Lefever TW, Marusich JA, Kevin RC, McGregor IS, et al. (2018). Molecular and Behavioral Pharmacological Characterization of Abused Synthetic Cannabinoids MMB-and MDMB-FUBINACA, MN-18, NNEI, CUMYL-PICA, and 5-Fluoro-CUMYL-PICA. Journal of Pharmacology and Experimental Therapeutics, 365**,** 437-446. https://doi.org/10.1124/jpet.117.246983

Gamage TF, Farquhar CE, McKinnie RJ, Kevin RC, McGregor IS, Trudell ML, et al. (2019). Synthetic cannabinoid hydroxypentyl metabolites retain efficacy at human cannabinoid receptors. Journal of Pharmacology and Experimental Therapeutics, 368**,** 414-422. https://doi.org/10.1124/jpet.118.254425

Gaoni Y, & Mechoulam R (1964). Isolation, structure, and partial synthesis of an active constituent of hashish. Journal of the American Chemical Society, 86**,** 1646-1647. https://doi.org/10.1021/ja01062a046

Gaoni Y, & Mechoulam R (1971). Isolation and structure of. DELTA.+-tetrahydrocannabinol and other neutral cannabinoids from hashish. Journal of the American Chemical Society, 93**,** 217-224. https://doi.org/10.1021/ja00730a036

Gerard CM, Mollereau C, Vassart G, & Parmentier M (1991). Molecular cloning of a human cannabinoid receptor which is also expressed in testis. Biochemical Journal, 279**,** 129-134. https://dx.doi.org/10.1042%2Fbj2790129

Gertsch J, Leonti M, Raduner S, Racz I, Chen J-Z, Xie X-Q, et al. (2008). Beta-caryophyllene is a dietary cannabinoid. Proceedings of the National Academy of Sciences, 105**,** 9099-9104. https://doi.org/10.1073/pnas.0803601105

Gill E, & Lawrence D (1976). The physiological mode of action of tetrahydrocannabinol on cell membranes. Pharmacol Marihuana, 1**,** 147-55. https://doi.org/10.1007/978-1-59259-710-9_78

Glass M, & Felder CC (1997). Concurrent stimulation of cannabinoid CB1 and dopamine D2 receptors augments cAMP accumulation in striatal neurons: evidence for a Gs linkage to the CB1 receptor. Journal of Neuroscience, 17**,** 5327-5333.

Glass M, & Northup JK (1999). Agonist selective regulation of G proteins by cannabinoid CB1 and CB2 receptors. Molecular Pharmacology, 56**,** 1362-1369. https://doi.org/10.1124/mol.56.6.1362

Glennon RA, & Young R 2011. *Drug discrimination: applications to medicinal chemistry and drug studies*, John Wiley & Sons.

Gold LH, Balster RL, Barrett RL, Britt DT, & Martin BR (1992). A comparison of the discriminative stimulus properties of delta 9-tetrahydrocannabinol and CP 55,940 in rats and rhesus monkeys. Journal of Pharmacology and Experimental Therapeutics, 262**,** 479-486.

Goldin AL (2006). Expression of ion channels in Xenopus oocytes. Expression and Analysis of Recombinant Ion Channels: From Structural Studies to Pharmacological Screening.

Gondin AB, Gillis A, Sanchez J, Lim HD, Halls ML, Christie MJ, et al. Low intrinsic G protein efficacy can explain the improved side effect profile of novel opioid ligands. ASCEPT-PAGANZ 2019, 2019 Queenstown, New Zealand.

Goparaju S, Ueda N, Taniguchi K, & Yamamoto S (1999). Enzymes of porcine brain hydrolyzing 2-arachidonoylglycerol, an endogenous ligand of cannabinoid receptors. Biochemical Pharmacology, 57**,** 417-423. https://doi.org/10.1016/s0006-2952(98)00314-1

Gregory KJ, Valant C, Simms J, Sexton PM, & Christopoulos A (2010). The emergence of allosteric modulators for G protein-coupled receptors. GPCR Molecular Pharmacology and Drug Targeting**,** 61-87.

Griffiths P, Sedefov R, Gallegos A, & Lopez D (2010). How globalization and market innovation challenge how we think about and respond to drug use:'Spice'a case study. Addiction, 105**,** 951-953. https://doi.org/10.1111/j.1360-0443.2009.02874.x

Grimsey NL. 2010. Intracellular trafficking of cannabinoid receptor 1: a thorough characterisation and investigation into the role of the intracellular pool. PhD Thesis, The University of Auckland.

Grimsey NL, Graham ES, Dragunow M, & Glass M (2010). Cannabinoid receptor 1 trafficking and the role of the intracellular pool: implications for therapeutics. Biochemical Pharmacology, 80**,** 1050-1062. https://doi.org/10.1016/j.bcp.2010.06.007

Grotenhermen F, & Russo E 2002. Cannabis and cannabinoids: pharmacology, toxicology, and therapeutic potential, Psychology Press.

Gyombolai P, Boros E, Hunyady L, & Turu G (2013). Differential β-arrestin2 requirements for constitutive and agonist-induced internalization of the CB1 cannabinoid receptor. Molecular and Cellular Endocrinology, 372**,** 116-127. https://doi.org/10.1016/j.mce.2013.03.013

Hájos N, Ledent C, & Freund TF (2001). Novel cannabinoid-sensitive receptor mediates inhibition of glutamatergic synaptic transmission in the hippocampus. Neuroscience, 106**,** 1-4. https://doi.org/10.1016/s0306-4522(01)00287-1

Harbert C, Johnson M, & Melvin Jr L (1979). 3-[Hydroxy-4-(substituted)-phenyl]-cycloalkanone and cycloalkanol derivatives. DE Patent, 2839836.

Harris FM, Best KB, & Bell JD (2002). Use of laurdan fluorescence intensity and polarization to distinguish between changes in membrane fluidity and phospholipid order. Biochimica et Biophysica Acta (BBA)-Biomembranes, 1565**,** 123-128. https://doi.org/10.1016/s0005-2736(02)00514-x

Haubrich D, Ward S, Baizman E, Bell M, Bradford J, Ferrari R, et al. (1990). Pharmacology of pravadoline: a new analgesic agent. Journal of Pharmacology and Experimental Therapeutics, 255**,** 511-522.

Heblinski M, Santiago M, Fletcher C, Stuart J, Connor M, McGregor IS, et al. (2020). Terpenoids Commonly Found in Cannabis sativa Do Not Modulate the Actions of Phytocannabinoids or Endocannabinoids on TRPA1 and TRPV1 Channels. Cannabis and Cannabinoid Research. https://doi.org/10.1089/can.2019.0099.

Hejazi N, Zhou C, Oz M, Sun H, Ye JH, & Zhang L (2006). Δ9-Tetrahydrocannabinol and endogenous cannabinoid anandamide directly potentiate the function of glycine receptors. Molecular Pharmacology, 69**,** 991-997. https://doi.org/10.1124/mol.105.019174

Helander A (2017). Synthetic Cannabinoid Receptor Agonists (Spice) as New Recreational Psychoactive Substances. Handbook of Cannabis and Related Pathologies. Elsevier.

Hendriks H, Malingré TM, Batterman S, & Bos R (1975). Mono-and sesqui-terpene hydrocarbons of the essential oil of Cannabis sativa. Phytochemistry.

Henry S, Williams E, Barr K, Korchagina E, Tuzikov A, Ilyushina N, et al. (2018). Rapid one-step biotinylation of biological and non-biological surfaces. Scientific Reports, 8**,** 2845. https://doi.org/10.1038/s41598-018-21186-3

Herenbrink CK, Sykes DA, Donthamsetti P, Canals M, Coudrat T, Shonberg J, et al. (2016). The role of kinetic context in apparent biased agonism at GPCRs. Nature Communications, 7**,** 10842. https://doi.org/10.1038/ncomms10842

Herkenham M, Lynn AB, Little MD, Johnson MR, Melvin LS, De Costa BR, et al. (1990). Cannabinoid receptor localization in brain. Proceedings of the National Academy of Sciences, 87**,** 1932-1936. https://doi.org/10.1073/pnas.87.5.1932

Hermanns-Clausen M, Müller D, Kithinji J, Angerer V, Franz F, Eyer F, et al. (2018). Acute side effects after consumption of the new synthetic cannabinoids AB-CHMINACA and MDMB-CHMICA. Clinical Toxicology, 56**,** 404-411. https://doi.org/10.1080/15563650.2017.1393082

Hermanns-Clausen M, Kneisel S, Hutter M, Szabo B, & Auwärter V (2013). Acute intoxication by synthetic cannabinoids–four case reports. Drug Testing and Analysis, 5**,** 790-794. https://doi.org/10.1002/dta.1483

Hillard CJ (2000). Biochemistry and pharmacology of the endocannabinoids arachidonylethanolamide and 2-arachidonylglycerol. Prostaglandins & Other Lipid Mediators, 61**,** 3-18. https://doi.org/10.1016/s0090-6980(00)00051-4

Hillard CJ (2018). Circulating endocannabinoids: from whence do they come and where are they going? Neuropsychopharmacology, 43**,** 155-172.

Hillard CJ, Manna S, Greenberg MJ, DiCamelli R, Ross RA, Stevenson LA, et al. (1999). Synthesis and characterization of potent and selective agonists of the neuronal cannabinoid receptor (CB1). Journal of Pharmacology and Experimental Therapeutics, 289**,** 1427-1433.

HMIP (2015). Changing patterns of substance misuse in adult prisons and service responses - a thematic review. https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/444785/hmip-2014-15.pdf

Horne E, & Stella N (2008). The ins and outs of endocannabinoid signaling in healthy and diseased brain. Future Lipidology, 3**,** 435-452. https://doi.org/10.2217/17460875.3.4.435

Howlett A (2005). Cannabinoid receptor signaling. Cannabinoids. Springer.

Howlett A, Barth F, Bonner T, Cabral G, Casellas P, Devane W, et al. (2002). International Union of Pharmacology. XXVII. Classification of cannabinoid receptors. Pharmacological Reviews, 54**,** 161-202.

Howlett A, Champion T, Wilken G, & Mechoulam R (1990). Stereochemical effects of 11-OH-Δ8-tetrahydrocannabinol-dimethylheptyl to inhibit adenylate cyclase and bind to the cannabinoid receptor. Neuropharmacology, 29**,** 161-165. https://doi.org/10.1016/0028-3908(90)90056-W

Howlett A, & Fleming R (1984). Cannabinoid inhibition of adenylate cyclase. Pharmacology of the response in neuroblastoma cell membranes. Molecular Pharmacology, 26**,** 532-538.

Hsieh C, Brown S, Derleth C, & Mackie K (1999). Internalization and recycling of the CB1 cannabinoid receptor. Journal of Neurochemistry, 73**,** 493-501. https://doi.org/10.1046/j.1471-4159.1999.0730493.x

Huestis M (2005). Handbook of Experimental Pharmacology. Pertwee, RG., editor. Vol. vol. 168. New York: Springer

Huffman JW, Dai D, Martin BR, & Compton DR (1994). Design, synthesis and pharmacology of cannabimimetic indoles. Bioorganic & Medicinal Chemistry Letters, 4**,** 563-566. https://doi.org/10.1016/S0960-894X(01)80155-4

Huffman JW, Mabon R, Wu MJ, Lu J, Hart R, Hurst DP, et. al. (2011). 3-Indolyl-1-naphthylmethanes: new cannabimimetic indoles provide evidence for aromatic stacking interactions with the CB1 cannabinoid receptor. Bioorganic & medicinal chemistry. 20;11(4):539-49.

Ibsen MS, Connor M, & Glass M (2017). Cannabinoid CB1 and CB2 receptor signaling and bias. Cannabis and Cannabinoid Research, 2**,** 48-60. https://doi.org/10.1089/can.2016.0037

Ibsen MS, Finlay DB, Patel M, Javitch JA, Glass M, & Grimsey NL (2019). Cannabinoid CB1 and CB2 receptor-mediated arrestin translocation: species, subtype, and agonist-dependence. Frontiers in Pharmacology, 10**,** 350. https://doi.org/10.3389/fphar.2019.00350

Ignatowska-Jankowska BM, Baillie GL, Kinsey S, Crowe M, Ghosh S, Owens RA, et al. (2015). A cannabinoid CB 1 receptor-positive allosteric modulator reduces neuropathic pain in the mouse with no psychoactive effects. Neuropsychopharmacology, 40**,** 2948. https://doi.org/10.1038/npp.2015.148

Ingólfsson HI, Thakur P, Herold KF, Hobart EA, Ramsey NB, Periole X, et al. (2014). Phytochemicals perturb membranes and promiscuously alter protein function. ACS Chemical Biology, 9**,** 1788-1798. https://doi.org/10.1021/cb500086e

Iversen LL 2001. *The science of marijuana*, Oxford University Press.

Jarrahian A, Watts VJ, & Barker EL (2004). D2 dopamine receptors modulate Gα-subunit coupling of the CB1 cannabinoid receptor. Journal of Pharmacology and Experimental Therapeutics, 308**,** 880-886. https://doi.org/10.1124/jpet.103.057620

Jin W, Brown S, Roche JP, Hsieh C, Celver JP, Kovoor A, et al. (1999). Distinct domains of the CB1 cannabinoid receptor mediate desensitization and internalization. Journal of Neuroscience, 19**,** 3773-3780. https://dx.doi.org/10.1523%2FJNEUROSCI.19-10-03773.1999

Kavanagh P, Grigoryev A, Savchuk S, Mikhura I, Formanovsky A (2013). UR-144 in products sold via the Internet: identification of related compounds and characterization of pyrolysis products. Drug Testing and Analysis, 5:683–692.

Kelley BG, & Thayer SA (2004). Δ9-Tetrahydrocannabinol antagonizes endocannabinoid modulation of synaptic transmission between hippocampal neurons in culture. Neuropharmacology, 46**,** 709-715. https://doi.org/10.1016/j.neuropharm.2003.11.005

Kellogg R, Mackie K, & Straiker A (2009). Cannabinoid CB1 receptor-dependent long-term depression in autaptic excitatory neurons. Journal of Neurophysiology, 102**,** 1160-1171. https://doi.org/10.1152/jn.00266.2009

Kenakin T (2005). New concepts in drug discovery: collateral efficacy and permissive antagonism. Nature reviews Drug discovery, 4**,** 919. https://doi.org/10.1038/nrd1875

Kenakin T, & Christopoulos A (2013). Signalling bias in new drug discovery: detection, quantification and therapeutic impact. Nature Reviews Drug discovery, 12**,** 205. https://doi.org/10.1038/nrd3954

Kenakin T, Watson C, Muniz-Medina V, Christopoulos A, & Novick S (2012). A simple method for quantifying functional selectivity and agonist bias. ACS Chemical Neuroscience, 3**,** 193-203. https://doi.org/10.1021/cn200111m

Knapman A, Abogadie F, McIntrye P, & Connor M (2014). A Real-Time, Fluorescence-Based Assay for Measuring μ-Opioid Receptor Modulation of Adenylyl Cyclase Activity in Chinese Hamster Ovary Cells. Journal of biomolecular screening, 19**,** 223-231.

Kogan NM, & Mechoulam R (2007). Cannabinoids in health and disease. Dialogues in Clinical Neuroscience, 9**,** 413.

Koppel BS, Brust JC, Fife T, Bronstein J, Youssof S, Gronseth G, et al. (2014). Systematic review: efficacy and safety of medical marijuana in selected neurologic disorders: report of the Guideline Development Subcommittee of the American Academy of Neurology. Neurology, 82**,** 1556-1563. 10.1212/WNL.0000000000000363

Kozak KR, Gupta RA, Moody JS, Ji C, Boeglin WE, DuBois RN, et al. (2002). 15-Lipoxygenase metabolism of 2-arachidonylglycerol generation of a peroxisome proliferator-activated receptor α agonist. Journal of Biological Chemistry, 277**,** 23278-23286. https://doi.org/10.1074/jbc.M201084200

Kumar KK, Shalev-Benami M, Robertson MJ, Hu H, Banister SD, Hollingsworth SA, et al. (2019). Structure of a signaling cannabinoid receptor 1-G protein complex. Cell, 176**,** 448-458. e12. https://doi.org/10.1016/j.cell.2018.11.040

Lam RPK, Tang MHY, Leung SC, Chong YK, Tsui MSH, & Mak TWL (2017). Supraventricular tachycardia and acute confusion following ingestion of e-cigarette fluid containing AB-FUBINACA and ADB-FUBINACA: a case report with quantitative analysis of serum drug concentrations. Clinical Toxicology, 55**,** 662-667. https://doi.org/10.1080/15563650.2017.1307385

Laprairie R, Bagher A, Kelly M, & Denovan-Wright E (2015). Cannabidiol is a negative allosteric modulator of the cannabinoid CB1 receptor. British Journal of Pharmacology, 172**,** 4790-4805. https://doi.org/10.1111/bph.13250

Lauckner JE, Hille B, & Mackie K (2005). The cannabinoid agonist WIN55, 212-2 increases intracellular calcium via CB1 receptor coupling to Gq/11 G proteins. Proceedings of the National Academy of Sciences, 102**,** 19144-19149. https://doi.org/10.1073/pnas.0509588102

Lauckner JE, Jensen JB, Chen H-Y, Lu H-C, Hille B, & Mackie K (2008). GPR55 is a cannabinoid receptor that increases intracellular calcium and inhibits M current.

Proceedings of the National Academy of Sciences, 105, 2699-2704. https://doi.org/10.1073/pnas.0711278105

Lee MA 2012. *Smoke signals: A social history of Marijuana-Medical, Recreational and Scientific*, Simon and Schuster.

Leterrier C, Bonnard D, Carrel D, Rossier J, & Lenkei Z (2004). Constitutive endocytic cycle of the CB1 cannabinoid receptor. Journal of Biological Chemistry, 279, 36013-36021. https://doi.org/10.1074/jbc.M403990200

Li H-L (1974). An archaeological and historical account of cannabis in China. Economic Botany, 28, 437-448. https://www.jstor.org/stable/4253540

Li Y, & Kim J (2017). Distinct roles of neuronal and microglial CB2 cannabinoid receptors in the mouse hippocampus. Neuroscience, 363, 11-25. https://doi.org/10.1016/j.neuroscience.2017.08.053

Little P, Compton D, Johnson M, Melvin L, & Martin B (1988). Pharmacology and stereoselectivity of structurally novel cannabinoids in mice. Journal of Pharmacology and Experimental Therapeutics, 247, 1046-1051.

Loewe S (1950). Cannabiswirkstoffe und Pharmakologie der Cannabinole. Naunyn-Schmiedeberg's Archives of Pharmacology, 211, 175-193.

Logan BK, Reinhold LE, Xu A, & Diamond FX (2012). Identification of synthetic cannabinoids in herbal incense blends in the United States. Journal of Forensic Sciences, 57, 1168-1180. https://doi.org/10.1111/j.1556-4029.2012.02207.x

Long JZ, Nomura DK, Vann RE, Walentiny DM, Booker L, Jin X, et al. (2009). Dual blockade of FAAH and MAGL identifies behavioral processes regulated by endocannabinoid crosstalk in vivo. Proceedings of the National Academy of Sciences, 106, 20270-20275. https://doi.org/10.1073/pnas.0909411106

Longworth M, Connor M, Banister SD, & Kassiou M (2017). Synthesis and pharmacological profiling of the metabolites of synthetic cannabinoid drugs APICA, STS-135, ADB-PINACA, and 5F-ADB-PINACA. ACS chemical neuroscience, 8, 1673-1680.

Lozano I (2001). The therapeutic use of Cannabis sativa (L.) in Arabic medicine. Journal of Cannabis Therapeutics, 1, 63-70. https://doi.org/10.3989/asclepio.1997.v49.i2.373

Luk T, Jin W, Zvonok A, Lu D, Lin XZ, Chavkin C, et al. (2004). Identification of a potent and highly efficacious, yet slowly desensitizing CB1 cannabinoid receptor agonist. British Journal of Pharmacology, 142, 495-500. https://doi.org/10.1038/sj.bjp.0705792

Maccarrone M (2019). Missing Pieces to the Endocannabinoid Puzzle. Trends in Molecular Medicine. https://doi.org/10.1016/j.molmed.2019.11.002

Mackie K (2005). Distribution of cannabinoid receptors in the central and peripheral nervous system. Cannabinoids. Springer.

Mackie K, Devane WA, & Hille B (1993). Anandamide, an endogenous cannabinoid, inhibits calcium currents as a partial agonist in N18 neuroblastoma cells. Molecular Pharmacology, 44**,** 498-503.

Mackie K, & Hille B (1992). Cannabinoids inhibit N-type calcium channels in neuroblastoma-glioma cells. Proceedings of the National Academy of Sciences, 89**,** 3825-3829. https://dx.doi.org/10.1073%2Fpnas.89.9.3825

Mackie K, Lai Y, Westenbroek R, & Mitchell R (1995). Cannabinoids activate an inwardly rectifying potassium conductance and inhibit Q-type calcium currents in AtT20 cells transfected with rat brain cannabinoid receptor. Journal of Neuroscience, 15**,** 6552-6561. https://dx.doi.org/10.1523%2FJNEUROSCI.15-10-06552.1995

Malyshevskaya O, Aritake K, Kaushik MK, Uchiyama N, Cherasse Y, Kikura-Hanajiri R, et al. (2017). Natural (Δ 9-THC) and synthetic (JWH-018) cannabinoids induce seizures by acting through the cannabinoid CB 1 receptor. Scientific Reports, 7**,** 10516. https://doi.org/10.1038/s41598-017-10447-2

Marshall CR. Proceedings of the Cambridge Philosophical Society: Mathematical and physical sciences. 1898. Cambridge Philosophical Society.

Martin BR, Compton DR, Thomas BF, Prescott WR, Little PJ, Razdan RK, et al. (1991). Behavioral, biochemical, and molecular modeling evaluations of cannabinoid analogs. Pharmacology Biochemistry and Behavior, 40**,** 471-478. https://doi.org/10.1016/0091-3057(91)90349-7

Martini L, Waldhoer M, Pusch M, Kharazia V, Fong J, Lee JH, et al. (2007). Ligand-induced down-regulation of the cannabinoid 1 receptor is mediated by the G-protein-coupled receptor-associated sorting protein GASP1. The FASEB Journal, 21**,** 802-811. https://doi.org/10.1096/fj.06-7132com

Matsuda LA, Lolait SJ, Brownstein MJ, Young AC, & Bonner TI (1990). Structure of a cannabinoid receptor and functional expression of the cloned cDNA. Nature, 346**,** 561. https://doi.org/10.1038/346561a0

McAllister SD, Griffin G, Satin LS, & Abood ME (1999). Cannabinoid receptors can activate and inhibit G protein-coupled inwardly rectifying potassium channels in a

xenopus oocyte expression system. Journal of Pharmacology and Experimental Therapeutics, 291**,** 618-626.

McGregor IS, Issakidis CN, & Prior G (1996). Aversive effects of the synthetic cannabinoid CP 55,940 in rats. Pharmacology Biochemistry and Behavior, 53**,** 657-664. https://doi.org/10.1016/0091-3057(95)02066-7

McHugh D, Page J, Dunn E, & Bradshaw HB (2012). Δ9-Tetrahydrocannabinol and N-arachidonyl glycine are full agonists at GPR18 receptors and induce migration in human endometrial HEC-1B cells. British journal of pharmacology, 165**,** 2414-2424. https://doi.org/10.1111/j.1476-5381.2011.01497.x

Mechoulam R 1986a. Cannabinoids as therapeutic agents, Boca Raton, FL: CRC Press.

Mechoulam R 1986b. The pharmacohistory of Cannabis sativa. In Cannabinoids as therapeutic agents, Boca Raton, FL: CRC Press, pp. 1-19.

Mechoulam R, & Ben-Shabat S (1999). From gan-zi-gun-nu to anandamide and 2-arachidonoylglycerol: the ongoing story of cannabis. Natural Product Reports, 16**,** 131-143. https://doi.org/10.1039/a703973e

Mechoulam R, Feigenbaum J, Lander N, Segal M, Järbe T, Hiltunen A, et al. (1988). Enantiomeric cannabinoids: stereospecificity of psychotropic activity. Experientia, 44**,** 762-764.

Mechoulam R, & Hanuš Lr (2000). A historical overview of chemical research on cannabinoids. Chemistry and Physics of Lipids, 108**,** 1-13. https://doi.org/10.1016/s0009-3084(00)00184-5

Mechoulam R, Shani A, Edery H, & Grunfeld Y (1970). Chemical basis of hashish activity. Science, 169**,** 611-612. https://doi.org/10.1126/science.169.3945.611

Mechoulam R, & Shvo Y (1963). Hashish—I: the structure of cannabidiol. Tetrahedron, 19**,** 2073-2078. https://doi.org/10.1016/0040-4020(63)85022-X

Money NP (2019). How The Wave Of Synthetic Cannabinoids Got Started.

Monte AA, Calello DP, Gerona RR, Hamad E, Campleman SL, Brent J, et al. (2017). Characteristics and treatment of patients with clinical illness due to synthetic cannabinoid inhalation reported by medical toxicologists: a ToxIC database study. Journal of Medical Toxicology, 13**,** 146-152. https://doi.org/10.1007/s13181-017-0605-9

Morales P, Hurst DP, & Reggio PH (2017). Molecular targets of the phytocannabinoids: a complex picture. Phytocannabinoids. Springer.

Morales P, Reggio PH, & Jagerovic N (2017). An overview on medicinal chemistry of synthetic and natural derivatives of cannabidiol. Frontiers in Pharmacology, 8**,** 422. https://doi.org/10.3389/fphar.2017.00422

Moritz E, Austin C, Wahl M, DesLauriers C, Navon L, Walblay K, et al. (2018). Notes from the field: outbreak of severe illness linked to the vitamin K antagonist brodifacoum and use of synthetic cannabinoids—Illinois, March–April 2018. Morbidity and Mortality Weekly Report, 67**,** 607.

Muller C, Morales P, & Reggio PH (2019).Cannabinoid lignands targeting TRP channels. Frontiers in Molecular Neuroscience, 11, 487. https://doi.org/10.3389/fnmol.2018.00487

Munro S, Thomas KL, & Abu-Shaar M (1993). Molecular characterization of a peripheral receptor for cannabinoids. Nature, 365**,** 61. https://doi.org/10.1038/365061a0

Musshoff F, Madea B, Kernbach-Wighton G, Bicker W, Kneisel S, Hutter M, et al. (2014). Driving under the influence of synthetic cannabinoids ("Spice"): a case series. International Journal of Legal Medicine, 128**,** 59-64. https://doi.org/10.1007/s00414-013-0864-1

Nahler G, Jones T, & Russo E (2019). Cannabidiol and Contributions of Major Hemp Phytocompounds to the "Entourage Effect"; Possible Mecha-nisms. Journal of Alternative, Complementary & Integrative Medicine, 5**,** 070.

Navarro G, Morales P, Rodríguez-Cueto C, Fernández-Ruiz J, Jagerovic N, Franco R (2016). Targeting cannabinoid CB2 receptors in the central nervous system. Medicinal chemistry approaches with focus on neurodegenerative disorders. Frontiers in neuroscience, 10, 406.

Navarro G, Reyes-Resina I, Rivas-Santisteban R, de Medina VS, Morales P, Casano S, et al. (2018). Cannabidiol skews biased agonism at cannabinoid CB1 and CB2 receptors with smaller effect in CB1-CB2 heteroreceptor complexes. Biochemical Pharmacology, 157**,** 148-158. https://doi.org/10.1016/j.bcp.2018.08.046

Netzeband JG, Conroy SM, Parsons KL, & Gruol DL (1999). Cannabinoids enhance NMDA-elicited Ca2+ signals in cerebellar granule neurons in culture. Journal of Neuroscience, 19**,** 8765-8777. https://dx.doi.org/10.1523%2FJNEUROSCI.19-20-08765.1999

Norman C, MacDonald C, Walker G, McKirdy B, Nic Daeid N, & McKenzie C. The Qualitative and Quantitative Analysis of Synthetic Cannabinoid Receptor Agonists

in Infused Papers within Prison Settings - A Temporal Study 2018-2019. The 57th Annual Meeting of the International Association of Forensic Toxicologists, 2019.

Norman C, Walker G, McKirdy B, McDonald C, Fletcher D, Antonides LH, et al. (2020). Detection and quantitation of synthetic cannabinoid receptor agonists in infused papers from prisons in a constantly evolving illicit market. Drug Testing and Analysis.

Oka S, Nakajima K, Yamashita A, Kishimoto S, & Sugiura T (2007).Identification of GPR55 as a lysophosphatidylinositol receptor. Biochemical and biophysical research communications, 362 (4), 928-934. https://doi.org/10.1016/j.bbrc.2007.08.078

O'Shaughnessy WB (1843). On the preparations of the Indian hemp, or Gunjah: Cannabis indica their effects on the animal system in health, and their utility in the treatment of tetanus and other convulsive diseases. Provincial Medical Journal and Retrospect of the Medical Sciences, 5, 363.

O'Sullivan SE, Tarling EJ, Bennett AJ, Kendall DA, & Randall MD (2005). Novel time-dependent vascular actions of Δ9-tetrahydrocannabinol mediated by peroxisome proliferator-activated receptor gamma. Biochemical and biophysical research communications, 337, 824-831.

Oviedo A, Glowa J, & Herkenham M (1993). Chronic cannabinoid administration alters cannabinoid receptor binding in rat brain: a quantitative autoradiographic study. Brain Research, 616, 293-302. https://doi.org/10.1016/0006-8993(93)90220-h

Pacher P, & Kunos G (2013). Modulating the endocannabinoid system in human health and disease–successes and failures. The FEBS journal, 280, 1918-1943. https://doi.org/10.1111/febs.12260

Pan X, Ikeda SR, & Lewis DL (1996). Rat brain cannabinoid receptor modulates N-type Ca2+ channels in a neuronal expression system. Molecular Pharmacology, 49, 707-714.

Paronis CA, Nikas SP, Shukla VG, & Makriyannis A (2012). Δ9-Tetrahydrocannabinol acts as a partial agonist/antagonist in mice. Behavioural Pharmacology, 23, 802. https://doi.org/10.1097/FBP.0b013e32835a7c4d

Patel M, Manning JJ, Finlay DB, Javitch JA, Banister SD, Grimsey NL, et al. (2020). Signalling profiles of a structurally diverse panel of synthetic cannabinoid receptor agonists. Biochemical Pharmacology, 175, 113871.

Paton WD (1975). Pharmacology of marijuana. Annual Review of Pharmacology, 15, 191-220.

Pazos M, Nunez E, Benito C, Tolon R, & Romero J (2004). Role of the endocannabinoid system in Alzheimer's disease: new perspectives. Life Sciences, 75**,** 1907-1915. https://doi.org/10.1016/j.lfs.2004.03.026

Perez-Reyes M, & Wingfield M (1974). Cannabidiol and electroencephalographic epileptic activity. JAMA, 230**,** 1635-1635. 10.1001/jama.1974.03240120017007

Perez DM, & Karnik SS (2005). Multiple signaling states of G-protein-coupled receptors. Pharmacological Reviews, 57**,** 147-161. https://doi.org/10.1124/pr.57.2.2

Pertwee RG (1988). The central neuropharmcology of psychotropic cannabinoids. Pharmacology & Therapeutics, 36**,** 189-261. https://doi.org/10.1016/0163-7258(88)90106-4

Pertwee RG (1999). Pharmacology of cannabinoid receptor ligands. Current Medicinal Chemistry, 6**,** 635-664.

Pertwee RG (2006). Cannabinoid pharmacology: the first 66 years. British Journal of Pharmacology, 147**,** S163-S171. https://doi.org/10.1038/sj.bjp.0706406

Pertwee RG (2008). The diverse CB1 nad CB2 receptor pharmacology of three plant cannabinoids: Δ^9-tetrahydrocannabinol, cannabidiol and Δ^9-tetrahydrocannabivarin. British journal of pharmacology, *153*(2), pp.199-215. 10.1038/sj.bjp.0707442

Pertwee RG, Howlett AC, Abood ME, Alexander SP, Di Marzo V, Elphick MR., et al. (2010). International union of basic and clinical pharmacology. LXXIX. Cannabinoid receptors and their ligands: beyond CB1 and CB2. Pharmacological Reviews, 62, 588–631. doi: 10.1124/pr.110.003004

Pertwee RG 2014. *Handbook of Cannabis*, Oxford University Press, USA.

Pertwee RG, Stevenson LA, Elrick D, Mechoulam R, & Corbett A (1992). Inhibitory effects of certain enantiomeric cannabinoids in the mouse vas deferens and the myenteric plexus preparation of guinea-pig small intestine. British Journal of Pharmacology, 105**,** 980-984. https://doi.org/10.1111/j.1476-5381.1992.tb09088.x

Perucca E (2017). Cannabinoids in the treatment of epilepsy: hard evidence at last? Journal of Epilepsy Research, 7**,** 61. https://doi.org/10.14581/jer.17012

Potts A, Cano C, Thomas S, & Hill S (2019). Synthetic cannabinoid receptor agonists: classification and nomenclature. Clinical Toxicology**,** 1-17. https://doi.org/10.1080/15563650.2019.1661425

Price MR, Baillie GL, Thomas A, Stevenson LA, Easson M, Goodwin R, et al. (2005). Allosteric modulation of the cannabinoid CB1 receptor. Molecular Pharmacology, 68**,** 1484-1495. https://doi.org/10.1124/mol.105.016162

Puente N, Cui Y, Lassalle O, Lafourcade M, Georges F, Venance L, et al. (2011). Polymodal activation of the endocannabinoid system in the extended amygdala. Nature Neuroscience, 14**,** 1542. https://doi.org/10.1038/nn.2974

Rajasekaran M, Brents LK, Franks LN, Moran JH, & Prather PL (2013). Human metabolites of synthetic cannabinoids JWH-018 and JWH-073 bind with high affinity and act as potent agonists at cannabinoid type-2 receptors. Toxicology and Applied Pharmacology, 269**,** 100-108. https://doi.org/10.1016/j.taap.2013.03.012

Ralphs R, Williams L, Askew R, & Norton A (2017). Adding spice to the porridge: The development of a synthetic cannabinoid market in an English prison. International Journal of Drug Policy, 40**,** 57-69. https://doi.org/10.1016/j.drugpo.2016.10.003

Ramsey IS, Delling M., & Clapham DE (2006). An introduction to TRP channels. Annual Review of Physiology, 68, 619-647. 10.1146/annurev.physiol.68.040204.100431

Redmond WJ, Cawston EE, Grimsey NL, Stuart J, Edington AR, Glass M, et al. (2016). Identification of N-arachidonoyl dopamine as a highly biased ligand at cannabinoid CB1 receptors. British Journal of Pharmacology, 173**,** 115-127. https://doi.org/10.1111/bph.13341

Ren M, Tang Z, Wu X, Spengler R, Jiang H, Yang Y, et al. (2019). The origins of cannabis smoking: Chemical residue evidence from the first millennium BCE in the Pamirs. Science Advances, 5**,** eaaw1391.

Reyes MP, Lipton MA, Timmons MC, Wall ME, Brine DR, & Davis KH (1973). Pharmacology of orally administered Δ9-tetrahydrocannabinol. Clinical Pharmacology & Therapeutics, 14**,** 48-55.

Rianprakaisang T, Gerona R, & Hendrickson RG (2019). Commercial cannabidiol oil contaminated with the synthetic cannabinoid AB-FUBINACA given to a pediatric patient. Clinical Toxicology. https://doi.org/10.1080/15563650.2019.1619758

Riley SB, Sochat M, Moser K, Lynch KL, Tochtrop R, Isbell TS, et al. (2019). Case of brodifacoum-contaminated synthetic cannabinoid. Clinical Toxicology, 57**,** 143-144. https://doi.org/10.1080/15563650.2018.1502444

Rinaldi-Carmona M, Le Duigou A, Oustric D, Barth F, Bouaboula M, Carayon P, et al. (1998). Modulation of CB1 cannabinoid receptor functions after a long-term

exposure to agonist or inverse agonist in the Chinese hamster ovary cell expression system. Journal of Pharmacology and Experimental Therapeutics, 287**,** 1038-1047.

Roberts LA, Christie MJ, & Connor M (2002). Anandamide is a partial agonist at native vanilloid receptors in acutely isolated mouse trigeminal sensory neurons. British Journal of Pharmacology, 137**,** 421-428. https://dx.doi.org/10.1038%2Fsj.bjp.0704904

Roche JP, Bounds S, Brown S, & Mackie K (1999). A mutation in the second transmembrane region of the CB1 receptor selectively disrupts G protein signaling and prevents receptor internalization. Molecular Pharmacology, 56**,** 611-618. https://doi.org/10.1124/mol.56.3.611

Ross HR, Napier I, & Connor M (2008). Inhibition of recombinant human T-type calcium channels by Δ9-tetrahydrocannabinol and cannabidiol. Journal of Biological Chemistry, 283**,** 16124-16134.

Ross RA (2009). The enigmatic pharmacology of GPR55. Trends in Pharmacological Sciences, 30**,** 156-163. https://doi.org/10.1016/j.tips.2008.12.004

Ross SA, & ElSohly MA (1996). The volatile oil composition of fresh and air-dried buds of Cannabis sativa. Journal of Natural Products, 59**,** 49-51.

Rubin V 2011. *Cannabis and Culture*, Walter de Gruyter.

Russo E, & Guy GW (2006). A tale of two cannabinoids: the therapeutic rationale for combining tetrahydrocannabinol and cannabidiol. Medical Hypotheses, 66**,** 234-246. https://doi.org/10.1016/j.mehy.2005.08.026

Russo EB (2011). Taming THC: potential cannabis synergy and phytocannabinoid-terpenoid entourage effects. British Journal of Pharmacology, 163**,** 1344-1364. https://doi.org/10.1111/j.1476-5381.2011.01238.x

Russo EB (2018). The Case for the Entourage Effect and Conventional Breeding of Clinical Cannabis: No "Strain," No Gain. Frontiers in Plant Science, 9**,** 1969. https://doi.org/10.3389/fpls.2018.01969

Ryberg E, Larsson N, Sjögren S, Hjorth S, Hermansson NO, Leonova J, et al. (2007). The orphan receptor GPR55 is a novel cannabinoid receptor. British journal of pharmacology, 152**,** 1092-1101.

Sachdev S. 2016. *The pharmacology of valinate and tert-leucinate synthetic cannabinoids.* Master of Research, Macquarie University.

Sachdev S, Banister S, Santiago M, Bladen C, & Connor M (2019a). Differential activation of G protein-mediated signalling by synthetic cannabinoid receptor agonists. BioRxiv**,** 850651.

Sachdev S, Boyd R, Grimsey NL, Santiago M, & Connor M (2019b). Brodifacoum does not modulate human cannabinoid receptor-mediated hyperpolarization of AtT20 cells or inhibition of adenylyl cyclase in HEK 293 cells. PeerJ, 7**,** e7733. https://doi.org/10.7717/peerj.7733

Sachdev S, Vemuri K, Banister S, Longworth M, Kassiou M, Santiago MJ, et al. (2019c). In vitro determination of the CB1 efficacy of illicit synthetic cannabinoids. British Journal of Pharmacology. 10.1111/bph.14829

Sánchez C, Galve-Roperh I, Rueda D, & Guzmán M (1998). Involvement of sphingomyelin hydrolysis and the mitogen-activated protein kinase cascade in the Δ9-tetrahydrocannabinol-induced stimulation of glucose metabolism in primary astrocytes. Molecular Pharmacology, 54**,** 834-843. https://doi.org/10.1124/mol.54.5.834

Sánchez MaG, Ruiz-Llorente L, Sánchez AM, & Díaz-Laviada I (2003). Activation of phosphoinositide 3-kinase/PKB pathway by CB1 and CB2 cannabinoid receptors expressed in prostate PC-3 cells. Involvement in Raf-1 stimulation and NGF induction. Cellular Signalling, 15**,** 851-859. https://doi.org/10.1016/s0898-6568(03)00036-6

Santiago MJ, Sachdev S, Arnold JC, McGregor IS, & Connor M (2019). Absence of entourage: Terpenoids commonly found in Cannabis sativa do not modulate the functional activity of Δ9-THC at human CB1 and CB2 receptors. Cannabis and Cannabinoid Research 4:3, 165–176. 10.1089/can.2019.0016

Saroz Y, Kho DT, Glass M, Graham ES, & Grimsey NL (2019a). Cannabinoid Receptor 2 (CB2) Signals via G-alpha-s and Induces IL-6 and IL-10 Cytokine Secretion in Human Primary Leukocytes. ACS Pharmacology & Translational Science, 2**,** 414-428. 10.1021/acsptsci.9b00049

Saroz Y, Kho DT, Glass M, Graham SE, & Grimsey NL. Molecular Pharmacology of Cannabinoid Receptor 2, a Promising Immunomodulatory Target. ASCEPT-PAGANZ 2019, 2019b Queenstown, New Zealand.

Savinainen JR, Järvinen T, Laine K, & Laitinen JT (2001). Despite substantial degradation, 2-arachidonoylglycerol is a potent full efficacy agonist mediating CB1 receptor-

dependent G-protein activation in rat cerebellar membranes. British Journal of Pharmacology, 134**,** 664-672. https://dx.doi.org/10.1038%2Fsj.bjp.0704297

Schneir AB, Cullen J, & Ly BT (2011). “Spice” girls: synthetic cannabinoid intoxication. The Journal of Emergency Medicine, 40**,** 296-299. https://doi.org/10.1016/j.jemermed.2010.10.014

Seifert R, & Wenzel-Seifert K (2002). Constitutive activity of G-protein-coupled receptors: cause of disease and common property of wild-type receptors. Naunyn-Schmiedeberg's Archives of Pharmacology, 366**,** 381-416. https://doi.org/10.1007/s00210-002-0588-0

Selley DE, Stark S, Sim LJ, & Childers SR (1996). Cannabinoid receptor stimulation of guanosine-5′-O-(3-[35S] thio) triphosphate binding in rat brain membranes. Life Sciences, 59**,** 659-668. https://doi.org/10.1016/0024-3205(96)00347-5

Shanks KG, Clark W, & Behonick G (2016). Death associated with the use of the synthetic cannabinoid ADB-FUBINACA. Journal of Analytical Toxicology, 40**,** 236-239. https://doi.org/10.1093/jat/bkv142

Sharir H, Abood ME (2010). Pharmacological characterization of GPR55, a putative cannabinoid receptor. Pharmacology & therapeutics, 126 (3), 301-313. https://dx.doi.org/10.1016%2Fj.pharmthera.2010.02.004

Sobolevsky T, Prasolov I, & Rodchenkov G (2015). Study on the phase I metabolism of novel synthetic cannabinoids, APICA and its fluorinated analogue. Drug Testing and Analysis, 7**,** 131-142. https://doi.org/10.1002/dta.1756

Soethoudt M, Grether U, Fingerle J, Grim TW, Fezza F, De Petrocellis L, et al. (2017). Cannabinoid CB 2 receptor ligand profiling reveals biased signalling and off-target activity. Nature Communications, 8**,** 13958. https://doi.org/10.1038/ncomms13958

Soussan C, & Kjellgren A (2014). The flip side of “spice”: the adverse effects of synthetic cannabinoids as discussed on a Swedish internet forum. Nordic Studies on Alcohol and Drugs, 31**,** 207-220.

Spaderna M, Addy PH, & D’Souza DC (2013). Spicing things up: synthetic cannabinoids. Psychopharmacology, 228**,** 525-540. https://doi.org/10.1007/s00213-013-3188-4

Speiser M. 2015. Users describe the terrifying effects of the drug some are calling 'weaponised marijuana'. *Business Insider Australia*.

Staton PC, Hatcher JP, Walker DJ, Morrison AD, Shapland EM, Hughes JP, et al (2008). The putative cannabinoid receptor GPR55 plays a role in mechanical

hyperalgesia associated with inflammatory and neuropathic pain. Pain, 139 (1), 225-236. https://doi.org/10.1016/j.pain.2008.04.006

Stephenson R (1956). A modification of receptor theory. British Journal of Pharmacology, 11**,** 379-393. https://dx.doi.org/10.1111%2Fj.1476-5381.1956.tb00006.x

Straiker A, Dvorakova M, Zimmowitch A, & Mackie K (2018). Cannabidiol inhibits endocannabinoid signaling in autaptic hippocampal neurons. Molecular Pharmacology, 94**,** 743-748. https://dx.doi.org/10.1124%2Fmol.118.111864

Straiker A, Wager-Miller J, Hutchens J, & Mackie K (2012). Differential signalling in human cannabinoid CB1 receptors and their splice variants in autaptic hippocampal neurones. British Journal of Pharmacology, 165**,** 2660-2671. https://doi.org/10.1111/j.1476-5381.2011.01744.x

Stuart JM. 2015. The pharmacology of novel illicit synthetic cannabinoids. PhD Thesis, Macquarie University.

Sugiura T, Kodaka T, Kondo S, Tonegawa T, Nakane S, Kishimoto S, et al. (1997). Inhibition by 2-arachidonoylglycerol, a novel type of possible neuromodulator, of the depolarization-induced increase in intracellular free calcium in neuroblastoma× glioma hybrid NG108-15 cells. Biochemical and Biophysical Research Communications, 233**,** 207-210. https://doi.org/10.1006/bbrc.1997.6425

Sugiura T, Kondo S, Sukagawa A, Nakane S, Shinoda A, Itoh K, et al. (1995). 2-Arachidonoylgylcerol: a possible endogenous cannabinoid receptor ligand in brain. Biochemical and Biophysical Research Communications, 215**,** 89-97. https://doi.org/10.1006/bbrc.1995.2437

Tai S, & Fantegrossi WE (2014). Synthetic cannabinoids: pharmacology, behavioral effects, and abuse potential. Current Addiction Reports, 1**,** 129-136. https://dx.doi.org/10.1007%2Fs40429-014-0014-y

Tai S, & Fantegrossi WE (2016). Pharmacological and toxicological effects of synthetic cannabinoids and their metabolites. Neuropharmacology of New Psychoactive Substances (NPS). Springer.

Thomas A, Baillie G, Phillips A, Razdan R, Ross RA, & Pertwee RG (2007). Cannabidiol displays unexpectedly high potency as an antagonist of CB1 and CB2 receptor agonists in vitro. British Journal of Pharmacology, 150**,** 613-623. https://doi.org/10.1038/sj.bjp.0707133

Thomas BF, Lefever TW, Cortes RA, Grabenauer M, Kovach AL, Cox AO, et al. (2017). Thermolytic degradation of synthetic cannabinoids: chemical exposures and

pharmacological consequences. Journal of Pharmacology and Experimental Therapeutics, 361**,** 162-171. https://doi.org/10.1124/jpet.116.238717err

Todd A (1946). Hashish. Experientia, 2**,** 55-60.

Tortoriello G, Morris CV, Alpar A, Fuzik J, Shirran SL, Calvigioni D, et al. (2014). Miswiring the brain: Δ9-tetrahydrocannabinol disrupts cortical development by inducing an SCG10/stathmin-2 degradation pathway. The EMBO journal, 33**,** 668-685. https://doi.org/10.1002/embj.201386035

Touw M (1981). The religious and medicinal uses of Cannabis in China, India and Tibet. Journal of Psychoactive Drugs, 13**,** 23-34. https://doi.org/10.1080/02791072.1981.10471447

Turner SE, Williams CM, Iversen L, & Whalley BJ (2017). Molecular Pharmacology of Phytocannabinoids. Phytocannabinoids. Springer.

UNODC (2018). Understanding the synthetic drug market: the NPS factor. Global SMART Update Volume 19. https://www.unodc.org/documents/scientific/Global_Smart_Update_2018_Vol.19.pdf

Van der Stelt M, Trevisani M, Vellani V, De Petrocellis L, Moriello AS, Campi B, et al. (2005). Anandamide acts as an intracellular messenger amplifying Ca2+ influx via TRPV1 channels. The EMBO journal, 24**,** 3026-3037. https://doi.org/10.1038/sj.emboj.7600784

Voice U (2016). Spice: the bird killer. What prisoners think about the use of spice and other legal highs in prison. http://www.uservoice.org/wp-content/uploads/2016/05/User-Voice-Spice-The-Bird-Killer-Report-Low-Res.pdf

Volkow ND, Baler RD, Compton WM, & Weiss SR (2014). Adverse health effects of marijuana use. New England Journal of Medicine, 370**,** 2219-2227. https://dx.doi.org/10.1056%2FNEJMra1402309

von Zastrow M (2003). Mechanisms regulating membrane trafficking of G protein-coupled receptors in the endocytic pathway. Life Sciences, 74**,** 217-224. https://doi.org/10.1016/j.lfs.2003.09.008

Wang XF, Galaj E, Bi GH, Zhang C, He Y, Zhan J, et al. (2019). Different receptor mechanisms underlying phytocannabinoid-versus synthetic cannabinoid-induced tetrad effects: Opposite roles of CB1/CB2 versus GPR55 receptors. British Journal of Pharmacology. https://doi.org/10.1111/bph.14958

Watson SJ, Benson JA, & Joy JE (2000). Marijuana and medicine: assessing the science base: a summary of the 1999 Institute of Medicine report. Archives of general psychiatry, 57**,** 547-552. https://doi.org/10.1001/archpsyc.57.6.547

Whiting D (2015). Synthetic Cannabinoid Receptor Agonists: a heterogeneous class of novel psychoactive substance with emerging risk of psychosis. Evidence-Based Mental Health, 18**,** 110-110. http://dx.doi.org/10.1136/eb-2015-102188

Wiley J, Barrett R, Lowe J, Balster R, & Martin B (1995). Discriminative stimulus effects of CP 55,940 and structurally dissimilar cannabinoids in rats. Neuropharmacology, 34**,** 669-676.

Wiley JL, Compton DR, Dai D, Lainton JA, Phillips M, Huffman JW, et al. (1998). Structure-activity relationships of indole-and pyrrole-derived cannabinoids. Journal of Pharmacology and Experimental Therapeutics, 285**,** 995-1004.

Wiley JL, Marusich JA, Huffman JW, Balster RL, & Thomas BF (2011). Hijacking of basic research: the case of synthetic cannabinoids. Methods Report (RTI Press), 2011. https://dx.doi.org/10.3768%2Frtipress.2011.op.0007.1111

Wiley JL, Marusich JA, Lefever TW, Grabenauer M, Moore KN, & Thomas BF (2013). Cannabinoids in disguise: Δ9-tetrahydrocannabinol-like effects of tetramethylcyclopropyl ketone indoles. Neuropharmacology, 75**,** 145-154. https://doi.org/10.1016/j.neuropharm.2013.07.022

Wiley JL, Smith VJ, Chen J, Martin BR, & Huffman JW (2012). Synthesis and pharmacology of 1-alkyl-3-(1-naphthoyl) indoles: Steric and electronic effects of 4- and 8-halogenated naphthoyl substituents. Bioorganic & Medicinal Chemistry, 20**,** 2067-2081. https://doi.org/10.1016/j.bmc.2012.01.038.

Wiley JL, Marusich JA, Lefever TW, Antonazzo KR, Wallgren MT, Cortes RA, et al. (2015) AB-CHMINACA, AB-PINACA, and FUBIMINA: affinity and potency of novel synthetic cannabinoids in producing delta9-tetrahydrocannabinol-like effects in mice. Journal of Pharmacology and Experimental Therapeutics, 354:328–339.

Wiley Jl, Marusich JA, & Thomas BF (2016). Combination chemistry: structure–activity relationships of novel psychoactive cannabinoids. In Neuropharmacology of New Psychoactive Substances (NPS), Springer, Cham, 231-248.

Wood TB, Spivey WN, & Easterfield TH (1896). XL.—Charas. The resin of Indian hemp. Journal of the Chemical Society, Transactions, 69**,** 539-546. 10.1039/CT8966900539

Wood TB, Spivey WN, & Easterfield TH (1899). III.—Cannabinol. Part I. Journal of the Chemical Society, Transactions, 75**,** 20-36. 10.1039/CT8997500020

Worob A, & Wenthur C (2019). Dark Classics in Chemical Neuroscience: Synthetic Cannabinoids (Spice/K2). ACS Chemical Neuroscience.

Wouters E, Mogler L, Cannaert A, Auwärter V, & Stove C (2019a). Functional evaluation of carboxy metabolites of synthetic cannabinoid receptor agonists featuring scaffolds based on L-valine or L-tert-leucine. Drug Testing and Analysis. https://doi.org/10.1002/dta.2607

Wouters E, Mogler L, Cannaert A, Auwärter V, & Stove C (2019b). Functional evaluation of metabolites of synthetic cannabinoid receptor agonists. Toxicologie Analytique et Clinique, 31**,** S64-S65. https://doi.org/10.1016/j.toxac.2019.03.097

Wouters E, Walraed J, Banister SD, & Stove CP (2019c). Insights into biased signaling at cannabinoid receptors: synthetic cannabinoid receptor agonists. Biochemical Pharmacology. https://doi.org/10.1016/j.bcp.2019.08.025

Wouters E, Walraed J, Robertson MJ, Meyrath M, Szpakowska M, Chevigné A, et al. (2019d). Assessment of Biased Agonism among Distinct Synthetic Cannabinoid Receptor Agonist Scaffolds. ACS Pharmacology & Translational Science.

Xu H, Partilla JS, Wang X, Rutherford JM, Tidgewell K, Prisinzano TE, et al. (2007). A comparison of noninternalizing (herkinorin) and internalizing (DAMGO) μ-opioid agonists on cellular markers related to opioid tolerance and dependence. Synapse, 61**,** 166-175. https://doi.org/10.1002/syn.20356

Zalesky A, Solowij N, Yücel M, Lubman DI, Takagi M, Harding IH, et al. (2012). Effect of long-term cannabis use on axonal fibre connectivity. Brain, 135**,** 2245-2255. https://doi.org/10.1093/brain/aws136

Zhang H-Y, Bi G-H, Li X, Li J, Qu H, Zhang S-J, et al. (2015). Species differences in cannabinoid receptor 2 and receptor responses to cocaine self-administration in mice and rats. Neuropsychopharmacology, 40**,** 1037. https://doi.org/10.1038/npp.2014.297

Zhu X, Finlay DB, Glass M, & Duffull SB (2019). Model-free and kinetic modelling approaches for characterising non-equilibrium pharmacological pathway activity: Internalisation of cannabinoid CB1 receptors. British Journal of Pharmacology, 176**,** 2593-2607. https://doi.org/10.1111/bph.14684

Zoratti C, Kipmen-Korgun D, Osibow K, Malli R, & Graier WF (2003). Anandamide initiates Ca2+ signaling via CB2 receptor linked to phospholipase C in calf pulmonary endothelial cells. British Journal of Pharmacology, 140**,** 1351-1362. https://doi.org/10.1038/sj.bjp.0705529

Zou S, & Kumar U (2018). Cannabinoid receptors and the endocannabinoid system: signaling and function in the central nervous system. International Journal of Molecular Sciences, 19**,** 833. https://doi.org/10.3390/ijms19030833

Zuardi AW (2006). History of cannabis as a medicine: a review. Brazilian Journal of Psychiatry, 28**,** 153-157. https://doi.org/10.1590/s1516-44462006000200015

Zygmunt PM, Petersson J, Andersson DA, Chuang H, Sørgård M, Di Marzo V, et al. (1999). Vanilloid receptors on sensory nerves mediate the vasodilator action of anandamide. Nature, 400, 452–457. doi: 10.1038/22761