Validation and Quantification of Ligand-Biased Signalling and Allosteric Modulation at CB_1 Cannabinoid Receptors

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Abstract

The CB_1 cannabinoid receptor (CB_1R) is a potential target for the treatment of numerous central nervous system disorders. Although a large number of CB_1R ligands exist, their therapeutic applications are limited due to adverse on-target effects. Selective activation of the receptor signalling events that mediate desired therapeutic effects at the expense of those that mediate adverse effects may overcome adverse on-target effects. This could be achieved via a phenomenon referred to as ligand-biased signalling.

There is growing evidence that CB_1R ligands may activate selective signalling pathways and engender biased signalling (Bosier et al., 2008b). More strikingly, CB_1R allosteric modulators, such as Org27569 may also display pathway selective modulation or biased allosterism (Ahn et al., 2012). Several endogenous allosteric modulators at CB_1Rs have also been suggested, including pregnenolone (Vallee et al., 2014), lipoxin A4 (Pamplona et al., 2012) and CRIP1a (Niehaus et al., 2007).

The current investigation aimed to detect and quantify ligand-biased signalling and allosterism at CB_1Rs using sophisticated analytical methods, in order to establish potential CB_1R biased "fingerprints" that may guide structure-activity and drug discovery studies.

Our results showed that 2-AG and WIN55,212-2 had little preference for cAMP inhibition and pERK1/2 activation (bias factor not dissimilar from 1). However, anandamide, Δ9-THC, CP55940 and in particular HU-210 and methanandamide with bias factors of over 20 and 15, respectively were biased towards cAMP inhibition. We also demonstrated that Org27569 reduced the CB_1R inverse agonist [^{3}H]SR141716A binding, indicated by a binding cooperativity (α)

value close to 0. However, it had little effect on the binding of cannabinoid agonists (α close to 1). Org27569 completely abolished inhibition of cAMP by all the cannabinoids tested, indicated by functional cooperativity (β) values approaching 0. Interestingly, however, in pERK1/2 assays, Org27569 abolished the response to HU-210 and CP55940, had no significant effect on pERK1/2 activation by anandamide, methanandamide and Δ9-THC, and only partially inhibited 2-AG and WIN55,212-2-induced pERK1/2 activation, as indicated by β values ranging from 0 to 1. This clearly indicates strong probe-dependence and biased allosterism by Org27569. Furthermore, our results showed no inhibitory effects on Δ9-THC-induced pERK1/2 activation by pregnenolone, and no enhancing effects on anandamide-mediated inhibition of cAMP by lipoxin A4, in contrast to previous findings (Pamplona et al., 2012; Vallee et al., 2014). Our results also demonstrated that CRIP1a knockdown in NG108-15 cells abolished WIN55,212-2-induced cAMP inhibition and reduced KCl-induced Ca^{2+} influx. However, it had no effects on cannabinoid-mediated Ca^{2+} mobilisation. In recombinant HEK-CB_1-TREx CRIP1a cells, cannabinoid-mediated cAMP and pERK1/2 signalling was unchanged in the absence or presence of CRIP1a. Therefore, further research is required to verify the allosteric nature of these endogenous ligands.

In conclusion, this study quantifies, for the first time, ligand-biased signalling from CB_1Rs, provides quantitative insights into biased allosterism and probe-dependence by the small molecule Org27569 at CB_1Rs, provides evidence against the reported allosteric effects of the endogenous ligands lipoxin A4 and pregnenolone, and demonstrates the cell line-dependent effects of CRIP1a. These novel insights may contribute to the development of selective CB_1R-targeted therapies.

General Declaration

In accordance with Monash University Doctorate Regulation 17.2 Doctor of Philosophy and Research Master's regulations the following declarations are made:

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

The core theme of the thesis is "evaluation of ligand-biased signalling and allosteric modulation at CB_1 cannabinoid receptors". The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the candidate, working within the Drug Discovery Biology laboratory of the Monash Institute of Pharmaceutical Sciences under the supervision of Dr. Katie Leach and Dr. Daniel Malone.

Signed: 

Date: 07 May 2015

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Publications and Communications

Research Article

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Manuscripts in preparation

- Khajehali E., Malone D.T., Sexton P., Christopoulos A. and Leach K. Modulation of CB_1 receptor-mediated signalling pathways by CRIP1a. To be submitted to *Molecular Pharmacology*.

Communications

- Khajehali E., Malone D.T., Christopoulos A. and Leach K. Ligand-biased signalling and ligand-biased allosteric modulation at CB_1 cannabinoid receptors. *ASCEPT-MPGPCR*, Melbourne, Australia, 2014.

- Khajehali E., Malone D.T. and Leach K. Allosteric modulation and biased signalling at CB_1 cannabinoid receptors. *International Cannabinoid Research Society (ICRS)*, Baveno, Italy, 2014.

- Khajehali E., Malone D.T. and Leach K. Pathway-selective allosteric modulation of CB_1 receptor signalling. *Federation of European Neuroscience Society (FENS),* Milan, Italy, 2014.

- Khajehali E., Malone D.T. and Leach K. Pathway-selective modulation of CB_1 receptor signalling by the allosteric modulator Org27569. *ASCEPT*, Melbourne, Australia, 2013.

- Khajehali E., Malone D.T. and Leach K. Effects of the CB_1 cannabinoid receptor allosteric modulator, Org 27569, on agonist-induced ERK phosphorylation. *ComBio*, Perth, Australia, 2013.

- Khajehali E., Malone D.T. and Leach K. Pathway-specific modulation of CB_1 cannabinoid receptors. *Monash Institute of Pharmaceutical Sciences (MIPS) 8th Annual Postgraduate Research Symposium*, Melbourne, Australia, 2013.

- Khajehali E., Malone D.T. and Leach K. Org27569 displays pathway-selective allosteric modulation at CB_1 cannabinoid receptors. *Drug Discovery Biology 1st Annual Student Symposium*, Melbourne, Australia, 2013.

- Khajehali E., Malone D.T. and Leach K. Allosteric modulation of CB_1 cannabinoid receptor-mediated signalling pathways by Org 27569. *3rd Annual Student of Brain Research (SOBR) Symposium*, Melbourne, Australia, 2013.

- Khajehali E., Exintaris B., White P.J., Haynes JM and Malone D.T. Reduced expression of cannabinoid receptor interacting protein CRIP1a modulates CB_1 receptor-mediated downstream signalling events in neuroblastoma x glioma cells. *Australian Neuroscience Society* (*ANS)*, Melbourne, Australia, 2013.

- Khajehali E, Malone D, Haynes J, Exintaris B. Effects of cannabinoid receptor interacting protein (CRIP1a) on CB_1 receptor-mediated downstream signalling events. *Monash Institute of Pharmaceutical Sciences (MIPS) 7th Annual Postgraduate Research Symposium*, Melbourne, Australia, 2012.

- Khajehali E., Exintaris B., White P.J., Haynes JM and Malone D.T. Cannabinoid Receptor Interacting Protein (CRIP1a) modulates CB_1 receptor-mediated effects on intracellular levels of cAMP and Ca^{2+}. *Molecular Pharmacology of G Protein-Coupled Receptors 2012 meeting (MPGPCRs)*, Melbourne, Australia, 2012.

- Khajehali E, Malone D, Haynes J, Exintaris B. Modulation of CB_1 receptor-mediated downstream signalling events by Cannabinoid Receptor Interacting Protein (CRIP1a) in neural cells in culture. *2nd Annual Student of Brain Research (SOBR) symposium*, Melbourne Brain Centre, Australia, 2012.

Abbreviations

Δ9-THC	Δ9-tetrahydrocannabinol
[^{35}S]GTPγS	Guanosine 5'-O-[gamma-thio]triphosphate
2-AG	2-arachydonylglycerol
7TM	7 transmembrane domain
aa	Amino acid
AP	Activator protein
ATCM	Allosteric ternary complex model
BSA	Bovine serum albumin
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
CB_1	Cannabinoid type 1
CB_2	Cannabinoid type 2
CHO	Chinese hamster ovary
CRE	cAMP response element
CRIP	CB_1 receptor interacting protein
DAGL	Diacylglycerol lipase
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
E	Extracellular loops
E. coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic acid
ERK1/2	Extracellular signal-regulated kinase 1 and 2
FAAH	Fatty acid amide hydrolase
FAN	Factor associated with neutral sphingomyelinase

FBS	Foetal bovine serum
GASP1	GPCR-associated sorting protein
GDP	Guanosine diphosphate
GPCR	G protein-coupled receptor
GRK	G protein-coupled receptor kinase
GTP	Guanosine-5'-triphosphate
HEK	Human embryonic kidney
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
I	Intracellular loops
IBMX	3-isobutyl-1-methylxanthine
K^+	Potassium
KCl	Potassium chloride
LB	Luria Bertoni
LTP	Long term potentiation
MAGL	Monoacyl glycerol lipase
MAPK	Mitogen-activated protein kinase
mmsiRNA	Mismatch siRNA, Inactive negative control siRNA
NADA	N-arachidonyl-dopamine
NAPE-PLD	*N*-acylphosphatidyl-ethanolamine-specific phospholipase D
OLDA	*N*-oleoyl dopamine
PBS	Phosphate buffered saline
PI3K	Phosphatidylinositol-3-kinase
PKA	Protein kinase A
PLCβ	Phospholipase Cβ
PTX	Pertussis toxin
PWR	Plasmon-waveguide resonance

RFU	Relative fluorescence units
RIPA	Radio Immuno Precipitation Assay
SCG	Superior cervical ganglion
siRNA	Small interfering RNA
TREx	Tetracycline-regulated expression
TRPVR	Transient receptor potential cation channel vanilloid receptors
Virodhamine	*O*-arachidonoyl ethanolamine
Zn^{2+}	Zinc

Chapter 1

General Introduction

1.1. G protein-coupled receptors

G protein-coupled receptors (GPCRs) are one of the largest protein families in vertebrates, encoded by approximately 4% of the human genome (Foord, 2002). They are characterised by an extracellular N-terminus, seven transmembrane domains (TM1-TM7) that form a hydrophobic core, an intracellular amphipathic helix (H8), which contains palmitoylation sites in its C-terminus, three intracellular loops (I1-I3), three extracellular loops (E1-E3) and an intracellular C-terminus (Ji et al., 1998; Nathans and Hogness, 1983; Venkatakrishnan et al., 2013). Each of the TMs are generally composed of 20-27 amino acids, but the terminal and loop segments vary in size (Ji et al., 1995).

A large number of ligands, including hormones, neurotransmitters, peptides, ions, photons and chemotactic agents act at GPCRs to produce many physiological functions, including phenotypic differentiation, cell survival or death, organismal homeostasis, motility, learning and memory, and secretion (Callihan et al., 2011; Kristiansen, 2004; Neves et al., 2002; Radeff-Huang et al., 2004). Therefore, GPCRs are important therapeutic targets for the treatment of many diseases, and are currently the targets for 40% of drugs on the market (Drews, 2000; Hopkins and Groom, 2002; Overington et al., 2006). However, a large population of GPCRs have not been targeted as yet. Therefore, there is still huge potential for developing novel GPCR-based drugs (Lappano and Maggiolini, 2011).

Distinct regions of a GPCR are involved in its binding to endogenous and exogenous molecules depending on the family of receptors. Small molecule ligands often bind to the hydrophobic core of GPCRs, whereas peptide and protein ligands generally interact with the N terminus, extracellular loops and the region

formed by the top of TM3, 5, 6 and 7 (Schwartz and Rosenkilde, 1996; Wess, 1997; Yeagle et al., 2001).

Agonist binding to a GPCR stabilises a conformational state of the receptor that is open at the intracellular receptor surface, promoting its interaction with G proteins. The second and third intracellular loops and the C terminus are necessary for G protein interactions (Venkatakrishnan et al., 2013; Wess, 1997). This interaction leads to the exchange of GDP for GTP on the α-subunit of the G protein, which results in dissociation of the α-subunit from the βγ G protein complex. α-GTP and βγ mediate diverse physiological responses (Conklin and Bourne, 1993; Wess, 1997). GPCRs can additionally mediate signal transduction via alternative signalling molecules, such as β-arrestins and kinases (Ji et al., 1998).

The “GRAFS” classification system divides GPCRs into five groups based on sequence homology and receptor function: the glutamate, rhodopsin, adhesion, frizzled/taste 2 and secretin family (Davies et al., 2007; Jacoby et al., 2006; Kolakowski, 1994). The rhodopsin family comprises over 80% of all GPCRs, and are subdivided into α, β, γ and δ groups. Cannabinoid receptors that are the focus of the current project belong to the α group (Fredriksson et al., 2003).

1.2. The endocannabinoid system: A general overview

The endocannabinoid system comprises cannabinoid receptors, their endogenous ligands and the enzymatic systems involved in their synthesis, transport and degradation (Fig.1.1). It is involved in many important physiological functions, including neuronal development and neurogenesis (Jin et al., 2004; Parmentier-Batteur et al., 2002), inhibition of neurotransmitter release (Hashimotodani et al., 2007), long-term synaptic plasticity (Marsicano et al., 2003), energy metabolism and cardiovascular function (Cota, 2007; Matias and Di Marzo, 2007), bone formation (Ofek et al., 2006; Tam et al., 2006), and immune cell responses (Massi et al., 2000; Sacerdote et al., 2000).

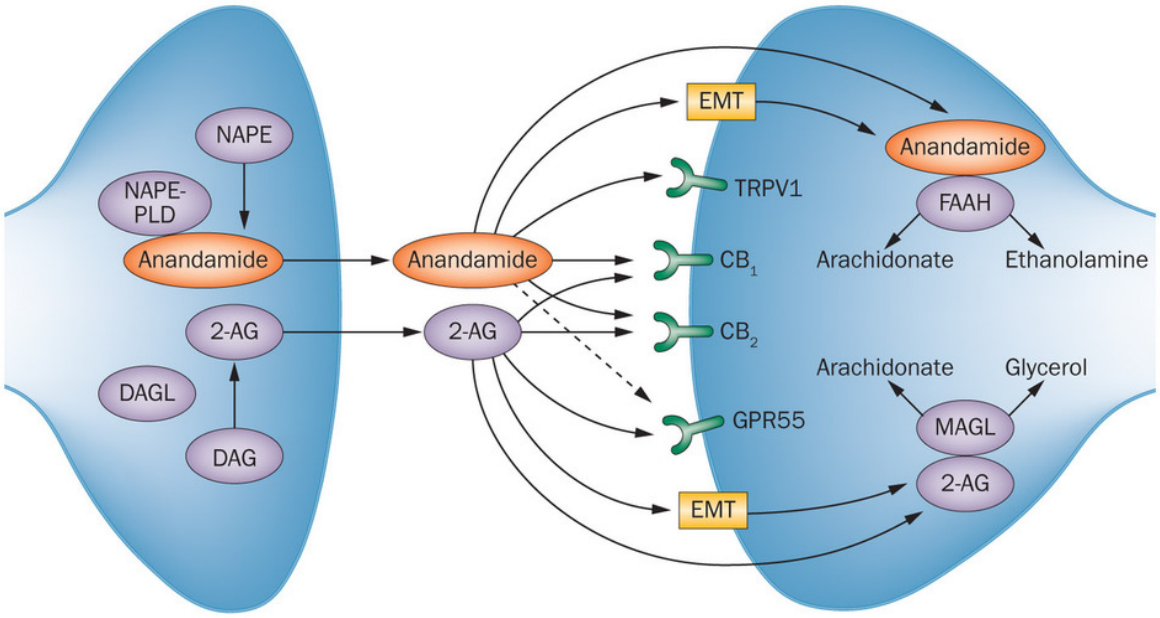


Fig. 1.1. The endocannabinoid system. Cannabinoid receptors, their endogenous ligands and the enzymes involved in the synthesis, transport and degradation of endogenous cannabinoids form the endocannabinoid system (from Schicho and Storr, 2014).

CB_1 and CB_2 cannabinoid receptors, which belong to the rhodopsin-like GPCRs (Fredriksson et al., 2003), mediate the majority of the effects of cannabis and other cannabinoid compounds, such as euphoria, appetite stimulation, sedation, altered perception, and impairments in memory and motor control (Adams and Martin, 1996). The CB_1 receptor is the most abundant GPCR in the brain; highly expressed in the cerebral cortex, hippocampus, substantia nigra, globus pallidus and cerebellum (Devane et al., 1988; Glass et al., 1997; Herkenham, 1991). However, it is also present at lower levels in the testes, spleen and in immune cells (Gerard et al., 1991; Kaminski et al., 1992). The other major cannabinoid receptor, the CB_2 receptor, is more abundant in immune cells; however, it is also expressed in low amounts in the brain (Atwood and Mackie, 2010; Munro et al., 1993; Onaivi et al., 2006). There is also evidence that cannabinoids may act at non-CB_1 and non-CB_2 receptors, including GPR55 (Ryberg et al., 2007), GPR18 (Kohno et al., 2006), and transient receptor potential cation channel vanilloid (TRPV) receptors (Zygmunt et al., 1999).

The CB_1 receptor shows 44% homology with the CB_2 receptor (Munro et al., 1993). There are several important structural differences between CB_1 and CB_2 receptors, mainly in the N-terminal domain, the second extracellular loop, the C-terminus of TM7 and the C-terminal tail of the receptor (Montero et al., 2005). These differences can be used for the development of receptor subtype-selective therapeutics. The CB_1 receptor has a long N-terminus, consisting of approximately 70 residues, which may be involved in the stabilisation and surface expression of the receptor (Montero et al., 2005).

The main endogenous ligands for cannabinoid receptors (endocannabinoids) are N-arachidonoylethanolamine or anandamide and 2-

arachydonylglycerol (2-AG). *N*-acylphosphatidyl-ethanolamine-specific phospholipase D (NAPE-PLD) and diacylglycerol lipases (DAGL-α and DAGL-β) are the main enzymes responsible for the biosynthesis of anandamide (Okamoto et al., 2004) and 2-AG (Bisogno et al., 2003), respectively. The two endocannabinoids are hydrolysed mainly by the enzymes fatty acid amide hydrolase (FAAH) and monoacyl glycerol lipase (MAGL), respectively (Di Marzo et al., 2004; Pertwee and Ross, 2002). In addition to these two endocannabinoids, a number of endogenous lipids have been shown to act as CB_1 and/or CB_2 receptor agonists or antagonists, including dihomo-γ-linolenylethanolamide, docosatetraenylethanol-amide, *O*-arachidonoyl ethanolamine (virodhamine), 2-arachidonylglyceryl ether (noladin ether), oleamide, *N*-oleoyl dopamine (OLDA) and N-arachidonyl-dopamine (NADA) (reviewed in Pertwee, 2005). However, their physiological roles have yet to be fully elucidated.

1.3. CB_1 cannabinoid receptors

1.3.1. Physiological roles of CB_1 receptors

Studies using CB_1 knockout mice or selective CB_1 receptor inverse agonists have demonstrated the physiological importance of CB_1 receptors. For instance, CB_1 knockout mice demonstrate defective adult neurogenesis (Jin et al., 2004), and increased neurological deficits are particularly apparent in these mice (Parmentier-Batteur et al., 2002). CB_1 knockout mice exhibit hypoactivity and hypoalgesia (Zimmer et al., 1999). These mice are also hypophagic and exhibit reduced body weight and fat mass (Cota et al., 2003). The role of CB_1 receptors in the rewarding effects of drugs of abuse is also well documented. In CB_1 knockout

mice, the antinociceptive and rewarding effects of nicotine are enhanced (Castane et al., 2002) and preference for ethanol and morphine is decreased (Cossu et al., 2001; Lallemand and de Witte, 2005).

1.3.2. CB_1 receptor-mediated signalling

Most of the central nervous system effects of cannabinoids are related to activation of distinct CB_1 receptor-mediated signalling pathways. The CB_1 receptor preferentially couples to Gi/o proteins to modulate multiple downstream signalling events, including inhibition of N- and P/Q-type calcium channels (Twitchell et al., 1997), activation of inwardly rectifying potassium channels (Vasquez et al., 2003), inhibition of adenylate cyclase (Howlett et al., 1986) and stimulation of mitogen-activated protein kinase (Bouaboula et al., 1995b). However, CB_1 receptors may also couple to Gs or Gq/11 proteins which may lead to stimulation of adenylate cyclase, and increases in intracellular levels of Ca^{2+} (Bash et al., 2003; Felder et al., 1995; Glass and Felder, 1997; Lauckner et al., 2005). Evidence for CB_1 receptor coupling specificity is provided below.

1.3.2.1. Regulation of adenylate cyclase

The CB_1 receptor-mediated cAMP signalling pathway is involved in regulation of neurite remodelling. This pathway, at least in part, mediates the psychoactive and neurotoxic effects of cannabinoids (Zhou and Song, 2001). Inhibition of adenylate cyclase activity through coupling to Gi/o proteins (Childers et al., 1993; Howlett et al., 1986) has been shown in both recombinant cell systems (Matsuda et al., 1990; Vogel et al., 1993) and in endogenously expressing

cells such as neuroblastoma cells (Howlett and Fleming, 1984). This effect was inhibited with the selective CB_1 inverse agonist SR141716 (Glass and Felder, 1997; Goodfellow et al., 2011), indicating the involvement of CB_1 receptors.

However, as mentioned above the CB_1 receptor can also couple to Gs. This is apparent when Gi/o proteins are inhibited with pertussis toxin (PTX) (Felder et al., 1995; Glass and Felder, 1997). Stimulation of cAMP production is also mediated by CB_1 receptors, as the response was blocked by SR141716 (Chen et al., 2010). However, in some instances, accumulation of cAMP may be attributable to the membrane-perturbing effects of high micromolar concentrations of cannabinoids (Glass and Felder, 1997; Hillard et al., 1995).

1.3.2.2. Modulation of intracellular Ca^{2+}

CB_1 receptor-mediated regulation of Ca^{2+} channel activity is involved in depolarization-induced suppression of synaptic activity (Chevaleyre et al., 2006; Kano et al., 2009). Cannabinoid-induced inhibition of N- and P/Q-type Ca^{2+} channels is mediated by Gi/o proteins acting directly on the channels, independently of cAMP, which conversely can enhance the activity of these channels (Caulfield and Brown, 1992; Mackie and Hille, 1992; Pan et al., 1996; Taguchi et al., 1997; Twitchell et al., 1997). Effects of cannabinoid agonists on other Ca^{2+} channels are controversial. CB_1 receptor agonists may inhibit L- or T-type voltage-gated Ca^{2+} currents in a CB_1 receptor-dependent (Hoddah et al., 2009) or –independent (Chemin et al., 2001) manner, and in some instances they display no inhibitory effects on these channels (Mackie and Hille, 1992). CB_1 receptor-mediated Ca^{2+} influx through L-type voltage gated Ca^{2+} channels could potentially contribute to cannabinoid-induced neurodegeneration (Ho et al., 2001).

Cannabinoids may also stimulate the release of Ca^{2+} from intracellular stores, and thereby increase intracellular Ca^{2+} levels (Netzeband et al., 1999). Different mechanisms are involved in this response, including coupling of CB_1 receptors to Gs (Bash et al., 2003) or Gq/11 proteins (Lauckner et al., 2005) or activation of phospholipase Cβ (PLCβ) enzymes by the βγ subunits of Gi/o proteins (Varga et al., 2008).

1.3.2.3. Activation of inwardly-rectifying K^+ channels

The CB_1 receptor-mediated activation of K^+ channels has been suggested to be involved in long-term depression of synaptic activity and the suppression of excitatory synaptic transmission (Daniel and Crepel, 2001; Kano et al., 2009). CB_1 receptor agonists activate inwardly rectifying K^+ currents via Gi/o in AtT-20 pituitary tumour cells and rat sympathetic neurons in a cAMP/PKA (protein kinase A) independent manner (Guo and Ikeda, 2004; Mackie et al., 1995) and in dissociated hippocampal neurons, through a cAMP/PKA-dependent pathway (Deadwyler et al., 1995).

1.3.2.4. Activation of mitogen-activated protein kinase

The involvement of the mitogen-activated protein kinase (MAPK) family, which includes extracellular signal-regulated kinase 1 and 2 (ERK1/2 or p42/p44 MAPK), p38 MAPK and c-JUN N-terminal kinases, in the development of tolerance and addiction to cannabinoids has been reported (Rubino et al., 2006). The ERK pathway is also involved in the regulation of neuronal migration and differentiation, and glucose metabolism by cannabinoids (Berghuis et al., 2005; Guzman and Sanchez, 1999; Rueda et al., 2002; Sanchez et al., 1998).

CB_1 receptor-mediated activation of MAPK via Gi/o has been demonstrated in several cell lines (Bouaboula et al., 1995a; Bouaboula et al., 1995b; Liu et al., 2000; Rueda et al., 2000; Wartmann et al., 1995). Different mechanisms are involved in the regulation of MAPK, such as recruitment of phosphatidylinositol-3-kinase (PI3K) and phosphorylation of protein kinase B (Sanchez et al., 1998). Phosphorylation of Raf by PKA is another mechanism involved in MAPK activation (Derkinderen et al., 2003). Cannabinoid agonists can also activate MAPK, independent of Gi/o, through CB_1 receptor-mediated ceramide synthesis from sphingomyelin hydrolysis (Sanchez et al., 2001).

1.3.2.5. G protein-independent signalling

Receptor internalisation, desensitisation and downregulation is an underlying mechanism for the development of tolerance to cannabinoids (Appleyard et al., 1997; Kouznetsova et al., 2002; Kovoor et al., 1997; Martini et al., 2007). β-arrestins (Jin et al., 1999) and G protein-coupled receptor kinases (GRKs) (Jin et al., 1999; Kouznetsova et al., 2002) are involved in CB_1 receptor desensitisation and internalisation, and GPCR-associated sorting protein (GASP1) (Martini et al., 2007) is involved in downregulation of CB_1 receptors. Factor associated with neutral sphingomyelinase (FAN) (Sanchez et al., 2001) is involved in CB_1 receptor-coupled sphingomyelin breakdown. Sphingomyelin hydrolysis is involved in the regulation of many physiological events related to cellular differentiation, proliferation, and apoptosis (Hannun and Luberto, 2000; Kolesnick and Krönke, 1998). Thus, activation of these proteins independent of G protein signalling is also important in the effects of CB_1 receptor agonists.

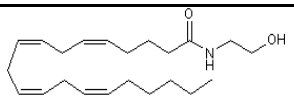
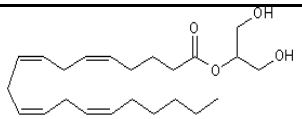
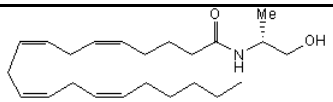
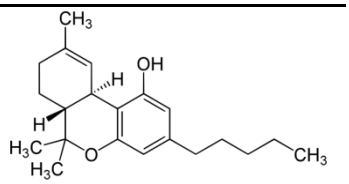
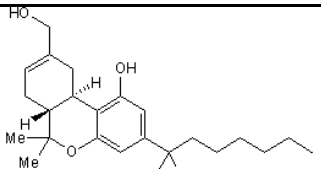
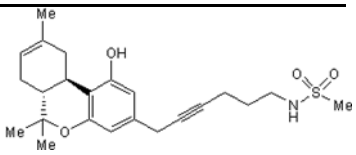
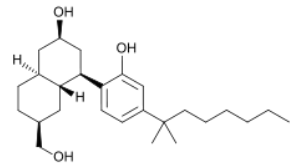
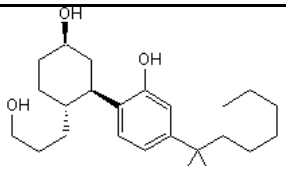
1.3.3. CB_1 receptors as therapeutic targets

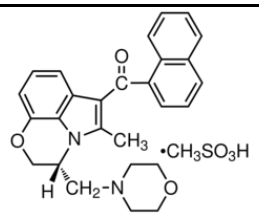
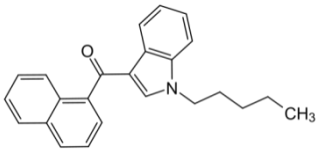
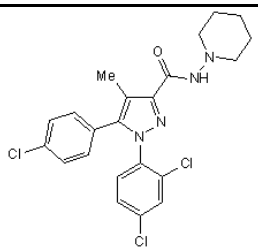
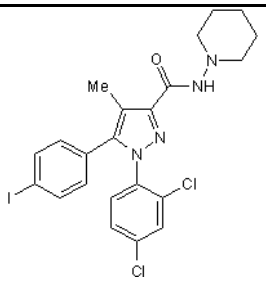
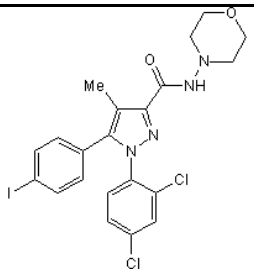
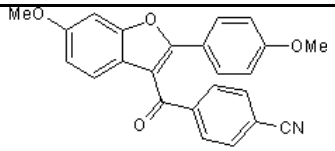
CB_1 receptors are involved in many physiological functions through coupling to various downstream signalling pathways; therefore, they are potential targets for the treatment of a number of diseases. CB_1 receptor antagonists have therapeutic potential for the treatment of a variety of conditions including obesity (Horvath, 2003), osteoporosis (Idris et al., 2005), nicotine and morphine addiction (Castane et al., 2002; Cossu et al., 2001; Le Foll and Goldberg, 2005) and Parkinson's disease (Segovia et al., 2003); and CB_1 receptor agonists for the treatment of pain (Iversen and Chapman, 2002), inflammation (Rice et al., 2002), cancer (Bifulco and Di Marzo, 2002), multiple sclerosis (Pertwee, 2002) and cardiovascular disease (Randall et al., 2002). However, therapeutic applications of cannabinoid compounds, in particular CB_1 receptor agonists, are limited mainly due to their psychotropic effects. For example, essentially all CB_1 receptor agonists that have been tested in humans have the potential to cause psychotic like effects and cognitive impairment (Castaneto et al., 2014). Also, the selective CB_1 antagonist/inverse agonist rimonabant (SR141716), which was approved by the European Medicines Agency (EMEA) in 2006 for the treatment of obesity, was withdrawn from the market due to an increased risk of serious psychiatric disorders such as depression and anxiety (Christensen et al., 2007; Mitchell and Morris, 2007). Therefore, there is a great need to identify CB_1 receptor ligands that display relative selectivity in terms of producing therapeutic effects without adverse effects.

1.3.4. Cannabinoid therapeutics

The search for cannabinoid agonists and antagonists has led to the identification of several exogenous cannabinoids. The structure of Δ9-tetrahydrocannabinol (Δ9-THC), the main psychoactive component of cannabis, was first elucidated in 1964 (Gaoni and Mechoulam, 1964). The chemical structures of different classes of cannabinoid ligands, as well as a brief description of the receptor activity, are shown in Table 1.1. Phytocannabinoids such as Δ9-THC and synthetic analogues such as HU-210 are classified as classical cannabinoids (tricyclic dibenzopyran derivatives). The non-classical cannabinoids include bicyclic (e.g. CP55940) and tricyclic (CP55244) analogues of Δ9-THC, which lack the dihydropyran ring found in classical cannabinoids. Synthetic cannabinoids also include aminoalkylindoles such as WIN55,212-2 and AM 678, which are structurally distinct from other classes of cannabinoids. The diarylpyrazoles are the most commonly used cannabinoid inverse agonists and include SR141716, AM 251 and AM 281. Neutral antagonists include compounds with similar structure to the classical cannabinoids, such as O-2050 (Howlett et al., 2002).

Table 1.1. Some commonly used cannabinoid ligands

Classification	Name	Activity	Chemical structure
Eicosanoids	Anandamide	Endogenous partial agonist at CB_1 and CB_2 receptors; exhibiting lower CB_2 than CB_1 efficacy	
	2-arachydonylglycerol	Endogenous agonist at CB_1 and CB_2 receptors. Also a potent agonist at GPR55	
	(R)-(+)-Methanandamide	Selective agonist at CB_1 receptors. Also an agonist at vanilloid receptors	
Classical	(-)-Δ9-THC	Partial agonist at CB_1 and CB_2 receptors	
	HU-210	Potent agonist at CB_1 and CB_2 receptors	
	O-2050	CB_1 receptor antagonist	
Non-classical	CP55244	Potent agonist at CB_1 and CB_2 receptors	
	CP55940	Potent agonist at CB_1 and CB_2 receptors	

Aminoalkylindole	WIN55,212-2	Potent agonist at CB_1 and CB_2 receptors	
	AM 678	Full agonist at CB_1 and CB_2 receptors	
Diarylpyrazoles	SR141716	Selective CB_1 inverse agonist/antagonist	
	AM 251	Selective CB_1 inverse agonist/antagonist. Also an agonist at GPR55	
	AM 281	Potent selective CB_1 inverse agonist/antagonist	
Benzofuran	LY320135	Selective CB_1 inverse agonist/antagonist	

1.4. Ligand-biased signalling

Although a large variety of CB_1 receptor ligands exist, adverse on-target effects have hampered their therapeutic application. However, selective activation of the receptor signalling events that mediate desired effects at the expense of those that cause adverse effects may overcome these problems. This may be achieved via a phenomenon referred to as ligand-biased signalling or biased agonism (Fig. 1.2).

Ligand-biased signalling is the ability of different ligands to stabilise a unique subset of receptor conformations, with each conformation being able to couple to distinct signalling pathways (Kenakin and Christopoulos, 2013). Therefore, the pharmacological properties of a ligand depend on the particular signal transduction pathway being studied. For instance, a ligand can behave as an agonist for one pathway, or antagonist or inverse agonist for another (Kenakin, 2007).

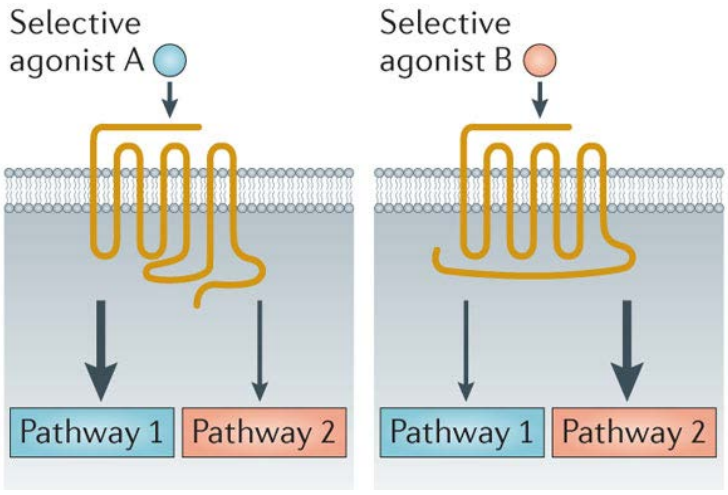


Fig. 1.2. Ligand-biased signalling. Agonist A preferentially activates pathways 1 whereas agonist B preferably activates pathway 2 (from Kenakin and Christopoulos, 2013).

1.4.1. Ligand-biased signalling at CB_1 receptors

There is growing evidence that ligand-biased signalling exists at CB_1 receptors. For example, HU-210 and CP55940 display differential regulation of gene transcription, because while they exhibit similar potencies and efficacies in terms of inhibition of cAMP response element (CRE)-mediated gene transcription, only HU-210 inhibits activator protein (AP)-1-mediated gene transcription in transfected N1E-115 cells (Bosier et al., 2008a). Reversals in cannabinoid efficacy or potency in activating different signal transduction pathways, which is a hallmark of ligand-biased signalling, has also been demonstrated. For example, in N1E-115 neuroblastoma cells, HU-210 was more efficacious than CP55940 in activating pERK1/2, while CP55940 displayed higher efficacy than HU-210 in activating JNK (Bosier et al., 2008b).

There is also evidence for activation of distinct Gi protein subtypes by different cannabinoids (Mukhopadhyay and Howlett, 2005). Further proof of ligand-biased signalling at CB_1 receptors was provided by plasmon-waveguide resonance (PWR) spectroscopy, a highly sensitive experimental method that can be used to detect and characterise protein-ligand or protein-protein interactions (for details refer to Varga et al., 2008). Varga and colleagues characterised the interaction of the CB_1 receptor with structurally different ligands and with individual G protein subtypes. The shift in the PWR spectra in opposite directions by WIN55,212-2 and CP55940 indicated that these ligands induce different conformational changes in the receptor. Furthermore, it was demonstrated that WIN55,212-2- and CP55940-occupied CB_1 receptors have different affinities and efficacies for the Gi_1 protein (Varga et al., 2008).

Most importantly, there is evidence that cannabinoid ligand-biased signalling may be operative *in vivo*. This was demonstrated by reversals in potency or efficacy of cannabinoid ligands in producing the “tetrad of effects”, consisting of hypoactivity, antinociception, hypothermia, and catalepsy (Elphick and Egertová, 2009; Wiley and Martin, 2009), through activation of the CB_1 receptor (Abood and Martin, 1992).

Therefore, the ability to selectively direct CB_1 receptor signalling towards therapeutically desirable signalling pathways at the exclusion of pathways linked to unwanted side-effects through ligand-biased signalling may aid the development of more successful CB_1 receptor-targeted therapies.

1.4.2. CB_1 receptor residues important for binding/signalling of cannabinoid ligands

The ability of different ligands to stabilise distinct receptor conformations and therefore engender bias is due to their ability to interact with different receptor regions and/or their ability to engage different amino acids when transmitting their response through the receptor. Therefore, in subsequent sections the CB_1 receptor amino acid residues that are involved in ligand binding or signalling are discussed.

It is now known that different, although overlapping, binding sites exist for different CB_1 receptor ligands. Therefore, ligand binding pocket for cannabinoid compounds has not been well established due to the variety of structurally different cannabinoids. Nonetheless, based on molecular dynamic simulation and mutagenesis studies, it has been proposed that the region formed by TM2, TM3, TM6 and TM7 is important for anandamide binding (McAllister et al., 2003), the

region formed by TM3, TM5, TM6 and TM7 for CP55940 (Reggio, 1999) and the region formed by TM3, TM4, TM5 and TM6 for WIN55,212-2 and SR141716 binding (McAllister et al., 2003).

There is however some overlap between the binding sites that have been defined for each class of ligand (Shim, 2010). In particular, the binding site residues of the aminoalkylindole WIN55,212-2 show some diversity but some overlap to other classes of cannabinoids (Shim and Howlett, 2002). For example, $Y275^{5.39}$ [the amino acids are numbered according to Ballesteros-Weinstein numbering system (Ballesteros and Weinstein, 1995)] is important for binding of WIN55,212-2, CP55940 and anandamide (McAllister et al., 2002), whereas $V282^{5.46}$, $F200^{3.36}$ and $W279^{5.43}$ are important for the binding of WIN55,212-2 but not other cannabinoids (McAllister et al., 2003), and $D176^{2.63}$ (Kapur et al., 2008) and $K192^{3.28}$ (Song and Bonner, 1996) are involved in binding of cannabinoid compounds other than WIN55,212-2. Figure 1.3 summarises the CB_1 receptor residues that are involved in ligand binding either directly, by forming the binding pocket, or indirectly, through inducing global conformational changes in the receptor, and thereby altering the binding pocket topology. Table 2.2 shows only the residues that directly interact with ligands.

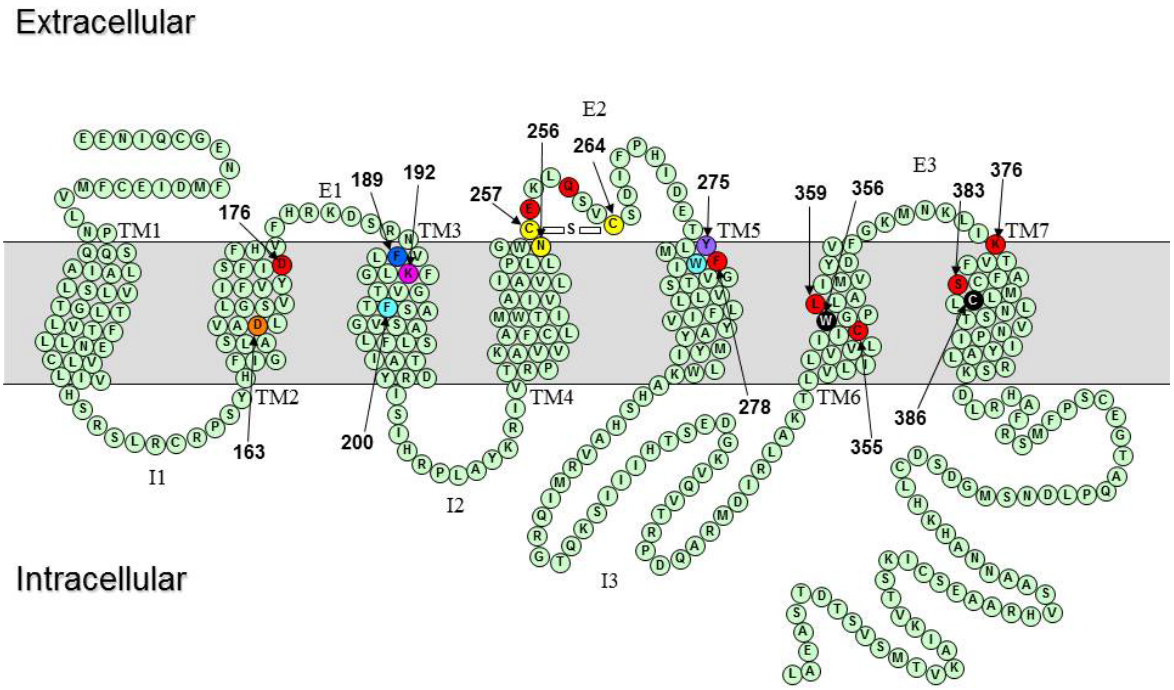


Fig 1.3. Two-dimensional structure of the human CB_1 receptor illustrating amino acid residues important for cannabinoid ligand binding. Seven TM helices (TM1-TM7), three extracellular loops (E1, E2, and E3), three intracellular loops (I1, I2, and I3), and N- and C-terminals are presented. Amino acid residues that have been shown through mutational and ligand docking studies to be critical for anandamide binding are in dark blue, for CP55940 in red, for WIN55,212-2 in orange, for anandamide, CP55940 and WIN55,212-2 in purple, for SR141716 in yellow, for SR141716 and WIN55,212-2 in light blue, for SR141716 and CP55940 in black and for SR141716, anandamide and CP55940 in pink.

Table 2.2. The key CB_1 receptor amino acid residues that directly contact with cannabinoid ligands

Ligand	Amino acid residue	Reference
Anandamide	$F189^{3.25}$	(McAllister et al., 2003)
	$Y275^{5.39}$	(McAllister et al., 2002)
CP55940	E2 residues F268/P269/H270/I271	(Ahn et al., 2009)
	$C355^{6.47}$	(Picone et al., 2005)
	$Y275^{5.39}$	(McAllister et al., 2002)
WIN55,212-2	$G195^{3.31}$	(Reggio et al., 1998)
	$W280^{5.43}$ $F201^{3.36}$ $W356^{6.48}$ $V282^{5.46}$	(McAllister et al., 2003)
	$W279^{5.43}$ $F200^{3.36}$	
	$Y275^{5.39}$	(McAllister et al., 2002)
SR141716	$F200^{3.36}$ $W279^{5.43}$ $W356^{6.48}$ $C386^{7.42}$	(Ahn et al., 2009)

Of the residues listed in Table 2.2, those in TM6 and TM7 of the inactive receptor are not believed to form initial contacts for agonist binding but become available when the receptor is activated by the inward movements of TM6 and TM7. However, these residues form initial contacts for the inverse agonist SR141716 (Shim, 2010). Therefore, it seems that the hydrophobic pocket that interacts with cannabinoids forms dynamically as the receptor equilibrium shifts toward the active state (Shim et al., 2011).

Therefore, differences in the interaction between cannabinoid ligands and the CB_1 receptor may reflect their distinct biased signalling profiles. In fact, it has been demonstrated that the binding of different cannabinoids can evoke coupling of the CB_1 receptor to different signalling proteins. For instance, the I3 loop of the CB_1 receptor is involved in the interaction of the receptor with $G\alpha_{i-1}$ and $G\alpha_{i-2}$ proteins, while the C-terminal domain regulates $G\alpha_o$ and $G\alpha_{i-3}$ (Mukhopadhyay et al., 2002). Varga *et al.* (2008) suggested that binding of cannabinoids to helices of

CB_1 receptors that extend to the I3 loop (TM5 and TM6) preferentially evokes $G\alpha_{i-1}$ and $G\alpha_{i-2}$ signalling, and interaction of ligands with TM7 of the receptor changes the conformation of the juxtamembrane C-terminal domain and promotes $G\alpha_o$ and $G\alpha_{i-3}$ coupling (Varga et al., 2008).

Mutagenesis studies have revealed that helix 8 of CB_1 receptors is involved in differential signalling of cannabinoids. The L7.60F mutation of the highly conserved $NPXXY(X)_{5,6}L$ motif, which links the binding pocket and the G protein binding interface (Shim and Padgett, 2013), attenuated [^{35}S]GTPγS stimulation by WIN55,212-2 and CP55940 but not HU-210, whereas the L7.60I mutation inhibited the response to all three agonists (Anavi-Goffer et al., 2007).

1.5. Allosteric modulation at CB_1 receptors

In addition to the orthosteric binding site, where endogenous agonists bind to the receptor, GPCRs including CB_1 receptors contain a topographically distinct binding site called the allosteric binding site. The binding of a ligand to an allosteric site may alter the binding and/or signalling properties of the orthosteric ligand, indicating that the sites are conformationally linked (May et al., 2007). Allosteric modulators may have divergence in their effects on ligand binding affinity and efficacy, for example being an enhancer of orthosteric ligand binding and a negative modulator of ligand efficacy (Price et al., 2005).

Allosteric modulators have several advantages over orthosteric ligands. Importantly, whereas orthosteric binding sites share high sequence conservation across receptor subtypes, allosteric sites are generally more diverse and therefore offer targets for more selective therapies (Christopoulos and Kenakin, 2002; Rees

et al., 2002). Furthermore, rather than directly mimicking or blocking the actions of the endogenous agonists that bind to the orthosteric site, allosteric modulators can fine-tune pharmacological agonist responses by altering the binding and/or signalling properties of orthosteric ligands (May et al., 2007). However, some allosteric modulators may also display agonist activity in their own right (May et al., 2007).

One of the characteristics of allosteric interactions is that their effects are saturable, with a limit in their effect which can be represented by a cooperativity factor, α. α describes the magnitude of the allosteric change in ligand affinity when the two sites are occupied. An α value >1 describes positive cooperativity (allosteric enhancement of binding), while an α value <1 (but >0) describes negative cooperativity (allosteric inhibition of binding) and an α = 1 describes neutral cooperativity, i.e., no net effect on binding affinity at equilibrium (Ehlert, 1988). Saturability leads to a ceiling effect, and therefore assuming no off-target effects, allosteric modulators may be used in larger doses without causing on-target toxicity, normally observed at high concentrations of orthosteric ligands (May et al., 2007).

Like orthosteric ligands, allosteric modulators may engender biased signalling (Fig. 1.4) by promoting unique conformational states of the receptor, which may result in modulation of specific orthosteric ligand-mediated signalling pathways (Kenakin and Christopoulos, 2013).

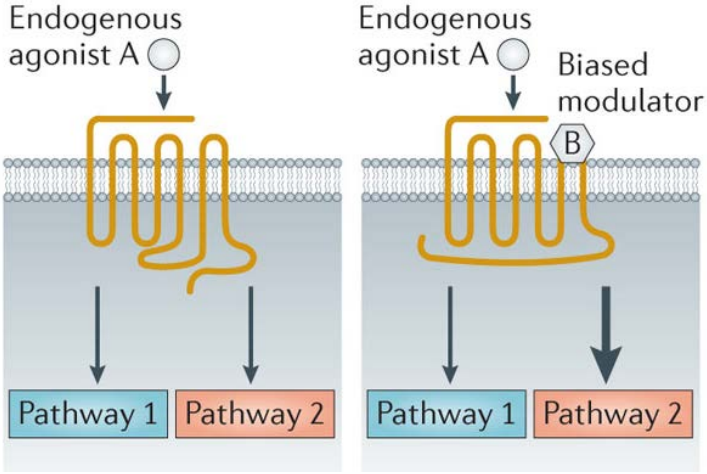


Fig. 1.4. Biased allosteric modulation. An allosteric modulator may selectively modulate specific signalling pathways activated by an orthosteric ligand (from Kenakin and Christopoulos, 2013).

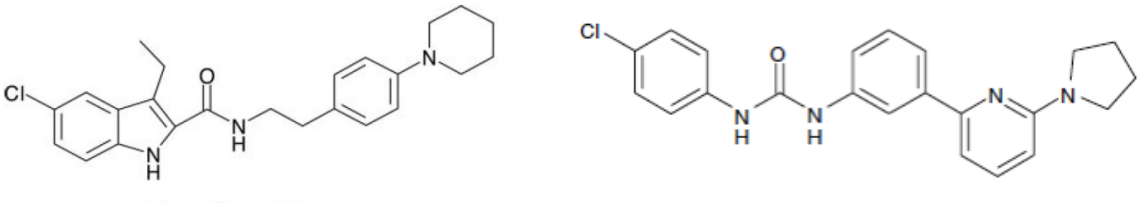
Another important feature of allosteric interactions is probe-dependence that is the effects of an allosteric modulator at a given receptor may change depending on the type of orthosteric ligand used (Leach et al., 2007; Valant et al., 2012). Therefore, the same allosteric modulator can act at the same receptor as an enhancer of one orthosteric ligand, an inhibitor of another, and exert no effect on a third (Leach et al., 2007; Valant et al., 2012). Thus, probe-dependence provides the opportunity to target selective signalling pathways using distinct combinations of allosteric and orthosteric ligands. A thorough understanding of allosterism at CB_1 receptors is therefore critical for the development of selective as well as effective therapeutics targeting these receptors.

1.5.1. Small molecule allosteric modulators of the CB_1 receptor

Price *et al.* (2005) reported that the cannabinoid CB_1 receptor contains an allosteric binding site for small molecule ligands. They identified three novel compounds, named Org27569, Org27759 and Org29647 with unique properties. These compounds act as allosteric inhibitors of agonist function at the CB_1 receptor while enhancing agonist binding and, on the other hand, reducing CB_1 receptor inverse agonist [^{3}H]SR141716A binding (Price et al., 2005).

Site-directed fluorescent labelling studies of agonist-occupied CB_1 receptors by Fay and Farrens (2012) showed that Org27569 blocked agonist-induced conformational changes at TM6. It also completely inhibited agonist-stimulated GTPγS binding in purified receptor reconstituted with $G\alpha_i$ while increasing agonist binding to the purified CB_1 receptor. Therefore, the authors suggested that in the presence of Org27569, the CB_1 receptor is stabilised in an intermediate agonist-bound but non-signalling conformation that lacks the movements in TM6 required for receptor activation (Fay and Farrens, 2012).

PSNCBAM-1, a novel compound with similar structure to the Org series of compounds (Fig. 1.5) was also reported to act as an allosteric modulator at the CB_1 receptor (Horswill et al., 2007). It displays a similar pharmacology to Org27569 by inhibiting cannabinoid agonist function while increasing binding (Horswill et al., 2007).



Org 27569 **PSNCBAM-1**

Fig. 1.5. Molecular structure of CB_1 allosteric modulators.

It has been demonstrated that Org27569 and PSNCBAM-1 display probe-dependence and engender biased allosteric effects (biased allosterism) (Ahn et al., 2012; Baillie et al., 2013; Horswill et al., 2007; Price et al., 2005; Wang et al., 2011). Org27569 also displays biased signalling on its own right (Baillie et al., 2013). These effects are discussed in more detail in Chapter 3.

1.5.1.1. Amino acid residues involved in binding/activity of CB_1 small molecule allosteric modulators

Limited studies have probed the CB_1 receptor allosteric binding site(s), and the amino acid residues involved in the transmission of cooperativity between the CB_1 allosteric and orthosteric ligands. Nonetheless, Org27569 and PSNCBAM-1 have been suggested to target the same binding site on the CB_1 receptor (Ross, 2007b). In a recent study using combined molecular modelling, mutagenesis and functional studies, Shore and colleagues identified a region involving TM3, 6, 7 of the CB_1 receptor to be important for binding of Org27569. This overlaps with the orthosteric binding site of SR141716 but extends extracellularly (Shore et al., 2014). Docking experiments by Barber et al. (2006) suggested that Org27569 may

interact at the TM5-6 interface of the receptor and an interaction that stabilises W356$^{6.48}$ may particularly be important for binding of Org27569 (Barber et al., 2006). However, mutagenesis studies by Shore and colleagues demonstrated the lack of effect of W356$^{6.48}$ on binding of Org27569 (Shore et al., 2014).

Mutations at K192$^{3.28}$ and W279$^{5.43}$ in the CB_1 receptor caused loss of the ability of Org27569 to inhibit CP55940 signalling, suggesting that these residues are essential for either binding of Org27569 or the transmission of cooperativity across the allosteric and orthosteric binding sites (Baillie et al., 2013; Shore et al., 2014). F189$^{3.25}$A eliminated inverse agonism of Org27569, whereas it retained its ability to inhibit CP55940 signalling (Shore et al., 2014).

To date, there is only one study quantifying the effects of mutations in CB_1 receptors on the binding of Org27569 or its transmission of cooperativity (Ahn et al., 2012). Ahn and colleagues demonstrated that the alanine mutation of T210$^{3.46}$ caused a 3-fold decrease in the binding affinity of Org27569. Furthermore, the binding affinity of [^{3}H]CP55940 was increased in the wild-type and T210$^{3.46}$A receptors, but not in T210$^{3.46}$I receptors, in the presence of Org27569 (Ahn et al., 2012). Figure 1.6 shows the CB_1 receptor amino acid residues that have been suggested to be involved in the binding of Org27569 or the transmission of cooperativity between Org27569 and CP55940.

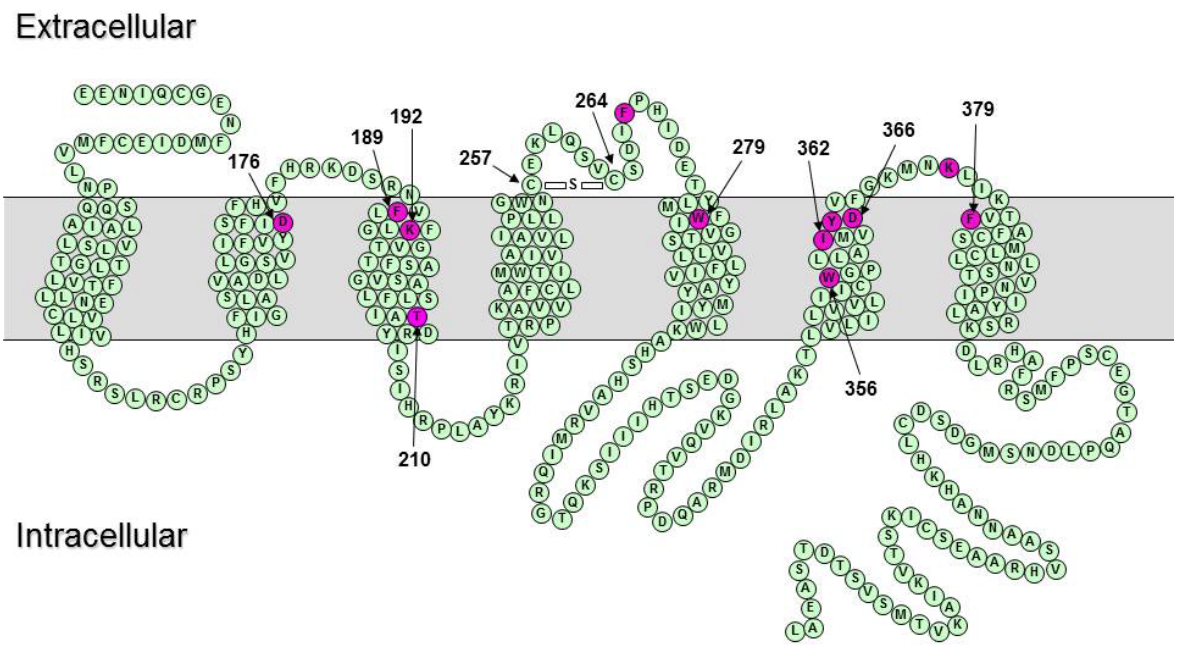


Fig. 1.6. CB_1 receptor amino acid residues involved in the binding of Org27569 or the transmission of cooperativity between Org27569 and CP55940. These residues are highlighted in pink.

Molecular modelling studies suggested that the interactions of Org27569 with F379$^{7.35}$, I362$^{6.54}$, Y365$^{6.57}$ and D366$^{6.58}$ prevent the conformational changes in the TM6 necessary for receptor activation. The movement of the E2 loop towards the transmembrane core is also blocked by interaction of Org27569 with F268; thereby the receptor remains in an inactive state. Org27569 also blocks the necessary conformational changes in the E3 loop by preventing the interaction between D176$^{2.63}$ and the E3 residue, K373. Taken together, Shore and colleagues suggested that blocking the necessary conformational changes in the TM6, E2 and E3 during receptor activation by Org27569 appears to be important for antagonising the efficacy of CP55940 (Shore et al., 2014).

Further molecular modelling and mutagenesis studies are required to determine the regions of the receptor involved in the binding, transmission of

cooperativity, and biased allosteric effects of the CB_1 receptor allosteric modulators.

1.5.2. Endogenous allosteric modulators of the CB_1 receptor

Several endogenous allosteric modulators of GPCRs have been implicated in physiological and pathophysiological processes. G proteins and other GPCR-interacting proteins are examples of endogenous allosteric modulators for GPCRs (May et al., 2007) because they can alter the conformation of a GPCR and therefore its ligand binding and signalling properties.

Some ions have been reported to have allosteric activity at different GPCRs. For example, Zn^{2+} allosterically modulates the activity of orthosteric ligands at D1, D2 (Schetz and Sibley, 1997), and D4 (Schetz and Sibley, 2001) dopamine receptors. Ca^{2+} can act both as an allosteric modulator and as an orthosteric ligand for some GPCRs (Conigrave et al., 2000; Galvez et al., 2000). Endogenous peptides (Bauer et al., 2012; Massot et al., 1996) and lipids (Gimpl et al., 1997; Grazzini et al., 1998; Thomas et al., 1997) may also act as allosteric modulators.

Several endogenous allosteric modulators have been suggested for CB_1 receptors. Lipoxin A4, an endogenous lipoxin that is largely involved in immune system regulation, was reported as an allosteric enhancer at CB_1 receptors as it enhanced agonist binding and functions whereas it partially inhibited inverse agonist binding (Pamplona et al., 2012). Bauer et al. (2102) identified pepcans (a new family of endogenous peptides) as allosteric inhibitors of agonist binding and function at CB_1 receptors (Bauer et al., 2012). More recently, the inactive precursor of neurosteroids, pregnenolone, was reported to act as an allosteric inhibitor of

agonist function without altering agonist equilibrium binding at CB_1 receptors (Vallee et al., 2014).

Another endogenous modulator at CB_1 receptors was identified by Niehaus and colleagues in 2007 (Niehaus et al., 2007). They reported the interaction of the last nine amino acids (aa 465-473) of the CB_1 receptor C-terminal tail with two structurally related CB_1 receptor interacting proteins (CRIP1a and CRIP1b). The 164-aa CRIP1a is encoded by a gene on human chromosome 2, which is alternatively spliced to encode the 128-aa CRIP1b. CRIP1a is conserved throughout vertebrates whereas CRIP1b has been identified only in primates (Niehaus et al., 2007). Modulatory effects of CRIP1a on CB_1 receptor-mediated signal transduction pathways were demonstrated by attenuation of inverse agonist but not agonist activity on Ca^{2+} currents in superior cervical ganglion (SCG) neurons over-expressing CRIP1a (Niehaus et al., 2007), suggesting that CRIP1a may act as an endogenous allosteric inhibitor at CB_1 receptors.

However, the allosteric effects of these endogenous modulators warrant further investigation.

1.6. Scope of thesis

As mentioned earlier, the CB_1 receptor is implicated in numerous CNS disorders. Selective activation of receptor signalling events that mediate desired therapeutic effects, at the expense of those that mediate adverse effects, via orthosteric or allosteric ligand-biased signalling is a promising approach to gaining selective therapies targeting these receptors. However, no study has directly quantified ligand-biased signalling at CB_1 receptors to date. The current study therefore aimed to detect and quantify ligand-biased signalling and allosterism at CB_1 receptors using sophisticated analytical methods.

To address this aim, in chapter 2 the binding affinities of several CB_1 receptor endogenous and exogenous ligands were determined in competition binding experiments by displacement of [^{3}H]SR141716A, and the potency and efficacy values were determined in two important CB_1 receptor-mediated signalling pathways, inhibition of cAMP formation and activation of pERK1/2, in FlpIn CHO-CB_1 cells. Ligand-biased signalling from the CB_1 receptor in cAMP and pERK1/2 pathways was quantified for each ligand. In chapters 3 and 4 the allosteric effects of the CB_1 receptor small molecule allosteric modulator Org27569 (Price et al., 2005), and endogenous allosteric modulators pregnenolone (Vallee et al., 2014), lipoxin A4 (Pamplona et al., 2012) and CRIP1a (Niehaus et al., 2007) were investigated. Binding interaction studies between Org27569 and several cannabinoid ligands were performed to quantify the binding cooperativity between the allosteric and orthosteric ligands. The functional cooperativity between the allosteric and orthosteric ligands was then quantified in cAMP and pERK1/2 interaction studies. To determine the activity of pregnenolone and lipoxin A4 at

CB_1 receptors, [^{3}H]SR141716A displacement by these ligands was studied. Furthermore, the ability of pregnenolone, lipoxin A4 and CRIP1a to modulate cannabinoid-mediated signalling was investigated in functional interaction experiments.

These studies provide novel insights into orthosteric ligand-biased signalling, allosteric modulator-biased signalling and probe-dependence at CB_1 receptors, which may help in the development of selective therapeutics targeting these receptors.

Chapter 2

Validation and Quantification of Ligand-Biased Signalling at CB_1 Receptors

2.1. Introduction

As discussed in the previous chapter, despite the immense potential for CB_1 receptor-based therapies in the treatment of numerous disorders (Zhang et al., 2009), many existing ligands that target these receptors have limited therapeutic value mainly due to their unwanted side effects. Thus, approaches to develop effective cannabinoid drugs with reduced adverse effects must be undertaken.

One drawback to many CB_1 receptor small molecule ligands is their tendency to interact with CB_2, as well as CB_1, receptors. Therefore, subtype selectivity is an important consideration for minimising adverse effects of cannabinoids. However, even when subtype selective drugs are available, some adverse effects may arise from on-target actions of drugs in undesirable tissues. Therefore, to avoid the central nervous system side effects of cannabinoid agonists, an approach to activate only the peripheral CB_1 receptor by developing ligands that do not cross the blood brain barrier may be useful (Hosking and Zajicek, 2008). However, this approach is unlikely to be successful as most of the therapeutic potential of cannabinoid agonists is associated with CB_1 receptors in the brain (Croxford, 2003).

A novel approach to dissociate on-target therapeutic effects from on-target adverse effects is through selective targeting of receptor signalling pathways (Mailman, 2007; Mukhopadhyay et al., 2002). This has now been attempted at a number of GPCRs through ligand-biased signalling (reviewed in Kenakin and Miller, 2010).

Early theories of GPCR signal transduction proposed that all agonists stabilise a single active receptor conformation, which activates a set of signalling

proteins. Therefore, the receptor exists in two conformations, inactive (R) and active (R*) (Leff, 1995). In this two-state model, the receptor isomerises between R and R* states represented as,

$$\begin{array}{ccc} R & \rightleftharpoons & R^* \\ +L \uparrow\downarrow & & \uparrow\downarrow +L \\ RL & \rightleftharpoons & R^*L \end{array}$$

According to this model, R and R* are in equilibrium in the absence of ligand (L). Ligand binding to the receptor drives the equilibrium towards one of these states, depending on the activity of the ligand. An agonist has a higher affinity for R* while an inverse agonist has a higher affinity for R, and an antagonist displays equal affinities for the two receptor states (Lefkowitz et al., 1993; Leff, 1995).

However, it is now known that distinct agonists can stabilise diverse receptor conformations rather than a single state, and each conformation may couple to a unique subset of signalling pathways (Kenakin, 1995a; Kenakin, 1995b). Therefore a single ligand may show diverse efficacy across different signalling pathways. For instance, a ligand may act as an agonist in one signalling pathway and as an antagonist in another. Therefore, the traditional classification of ligands as full agonists, partial agonists, antagonists and inverse agonists is no longer valid (Kenakin, 2011).

There is evidence that CB_1 receptor agonists engender ligand-biased signalling. For instance, WIN55,212-2 activates all Gi subtypes (Gi_1, Gi_2, and Gi_3), whereas (R)-methanandamide shows agonist activity only at Gi_3 and inverse agonism at Gi_1 and Gi_2 (Mukhopadhyay and Howlett, 2005). Furthermore, there is evidence for reversal in the rank order of cannabinoid efficacy in activating

different signalling pathways, also indicating ligand-biased signalling at these receptors (Bosier et al., 2008a; Bosier et al., 2008b). For instance, in a recent study, anandamide was reported to be more potent than Δ9-THC in activating Gi-mediated pERK1/2 activation, whereas Δ9-THC was more potent than anandamide in recruiting β-arrestin1 (Laprairie et al., 2014). Furthermore, different cannabinoid ligands may display different rank orders of potency or efficacy in producing the tetrad of cannabinoid effects in mice (consisting of hypoactivity, antinociception, hypothermia, and catalepsy) (Abood and Martin, 1992; Ryan et al., 1995; Smith et al., 1994; Wiley et al., 1998). For example, some novel indole derivatives of WIN55,212-2 are more potent in inducing antinociception than hypolocomotion, while Δ9-THC and WIN55,212-2 are more potent in producing hypolocomotion than antinociception (Wiley et al., 1998).

Although ligand-biased signalling has been observed at CB_1 receptors, no study has directly quantified it. Quantification of bias is important in order to determine the effects of structure-activity relationship studies on bias, and to link *in vitro* findings to potential therapeutic value. Furthermore, the classification of agonists already in the clinic based on their bias signalling profiles can be useful retrospectively to link their therapeutic profiles in humans to their *in vitro* bias profiles (Kenakin and Christopoulos, 2013). Useful methods for quantifying bias are described below.

The most convincing indication of ligand-biased signalling is a complete reversal in the rank order of agonist potency and/or efficacy in different signalling assays (Kenakin, 2003; Kenakin, 2012b). A comparison of the relative activity (RA), i.e. the ratio between the maximal agonist response and its potency, across different signalling pathways provides a useful method to calculate bias. However,

this scale has limited applications where the slope of concentration-response curves is significantly different from unity (Ehlert, 2008; Kenakin and Christopoulos, 2013).

The Black-Leff operational model (Black and Leff, 1983) provides an alternative method to quantify ligand-bias by estimating a “transduction coefficient” (tau/KA). In this model, the transduction coefficient incorporates agonist efficacy, receptor density, receptor stimulus-response coupling and an operational measure of affinity (KA) (which may differ from the KA value obtained from binding studies) (Kenakin et al., 2012).

To determine true ligand bias, the tau/KA ratio must be compared to a reference agonist in order to eliminate system and observational bias (Black and Leff, 1983; Kenakin et al., 2012; van der Westhuizen et al., 2014). System bias is the relative coupling efficiency of a receptor to different pathways. For example, a weak agonist may only activate the most efficiently coupled pathway. Observational bias is the result of the sensitivity of different assays, and may depend on the experimental conditions under which the assay is performed (Kenakin and Christopoulos, 2013).

As noted previously, ligand-biased signalling at CB_1 receptors has not been quantified to date. The present study therefore sought to quantify ligand-biased signalling by endogenous and exogenous cannabinoids in two important CB_1 receptor-mediated signalling pathways, inhibition of cAMP formation and activation of ERK1/2 phosphorylation. The cAMP pathway has been shown to be involved in neurite remodelling and facilitates psychoactive and neurotoxic effects of cannabinoids (Zhou and Song, 2001), and pERK1/2 is involved in neuronal migration and differentiation, glucose metabolism and the development of

tolerance and addiction to cannabinoids (Berghuis et al., 2005; Guzman and Sanchez, 1999; Rubino et al., 2006; Rueda et al., 2002; Sanchez et al., 1998). We used the operational model of agonism to detect the differences between tau/KA values (van der Westhuizen et al., 2014) for numerous cannabinoids to quantify bias.

2.2. Materials and Methods

2.2.1. Materials

(+)-WIN55,212-2, CP55940, HU-210, methanandamide, anandamide, LY320135 and JZL 184 were obtained from Tocris Bioscience, and Δ9-THC from THC pharm (Frankfurt, Germany). 2-AG, SR141716A, forskolin and fatty acid free bovine serum albumin (BSA) were obtained from Sigma Aldrich, and hygromycin B from Roche (Mannheim, Germany). Lipofectamine 2000, foetal bovine serum (FBS) and cell culture media were obtained from Invitrogen. The cAMP AlphaScreen® kit, *SureFire*® ERK1/2 phosphorylation kit and [^{3}H]SR141716A (35-60 Ci/mmol) were obtained from Perkin Elmer.

2.2.2. Generation of hCB_1 receptor expression vectors

DNA was purified from Luria Bertoni (LB) liquid cultures that were inoculated with *E. coli* containing hCB_1 in Gateway® P-DONR™201 cloning vector, using Promega Wizard® Plus SV Minipreps DNA Purification System, and the concentration of DNA was determined using a nanodrop™ (Thermo Scientific). A Gateway® LR clonase™ II reaction was performed according to manufacturer's

instructions (Invitrogen) to transfer hCB_1 in Gateway® P-DONR™201 into the Gateway® pEF5/FRT/V5™ destination vector. Briefly, 150 ng of hCB_1 in Gateway® P-DONR™201 was mixed with 150 ng of the destination vector in Tris-EDTA buffer, and incubated with Gateway® LR clonase™ II enzyme for at least 4 hours at room temperature. The reaction was then incubated with proteinase K solution for 10 minutes at 37 ºC. Chemically competent *E.coli* (DHα5) were transformed according to manufacturer's instructions (Invitrogen), and plated onto agar plates containing ampicillin (100 µg/ml). Colonies were picked and used to prepare bacterial cultures. DNA was extracted using the Wizard® Plus SV Miniprep DNA Purification kit and the concentration was determined using a nanodrop™ (Thermo Scientific). Sequencing results verified the presence of the hCB_1 receptor gene in the pEF5/FRT/V5-dest vector.

2.2.3. Generation of Flp-In CHO-CB_1 cell line

Flp-In™ Chinese Hamster Ovary (CHO) cells stably expressing human CB_1 cannabinoid receptors (CHO-hCB_1 cells) were generated according to the manufacturer's instructions for the generation of Flp-In cell lines (Invitrogen). Briefly, 0.8 µg of receptor DNA (or equivalent volume of media for a negative control) was co-transfected with 8 µg of the pOG44 Flp-In recombinase expression vector (Invitrogen, USA) using lipofectamine 2000. The transfected cells were selected with 700 µg/ml of hygromycin B. When confluent, cells were harvested using 2 mM EDTA in phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.5 mM KH_2PO_4) and passaged until the negative control cells were selected out. Following selection, cells were maintained in Dulbecco's

Modified Eagle Medium (DMEM) containing 10% FBS, 16 mM HEPES and 700 µg/ml of hygromycin B.

2.2.4. Ligand preparation

Cannabinoid ligands CP55940, HU-210, Δ9-THC, methanandamide, anandamide and 2-AG were dissolved in 100% ethanol, and WIN55,212-2 and forskolin in 100% DMSO to make stock solutions of 10 mM. Subsequently, the compounds were diluted in assay buffer/media to a concentration of up to 100 µM before addition into the assay to achieve a final concentration of up to 10 µM. The appropriate concentrations of ethanol or DMSO were used as negative controls. The final concentration of DMSO or ethanol in all assays did not exceed 0.1% (v/v).

2.2.5. Whole cell radioligand binding assays

[^{3}H]SR141716A was used as the radioligand in all binding experiments. CHO-hCB_1 cells were seeded at a density of 50,000 cells per well in 96-well tissue culture-treated isoplates, and incubated overnight at 37 ºC, 5% CO_2. The following day, media was removed and cells were washed twice with 100 µl ice-cold PBS. Cells were then incubated with [^{3}H]SR141716A (1 nM final concentration) and the appropriate concentrations of the test compounds in DMEM containing 5% w/v fatty acid free BSA in a final volume of 200 µl. Assays were terminated by 2 rapid washes with ice cold 0.9% NaCl to remove unbound ligand. After the final wash, 100 µl of Optiphase supermix® scintillation fluid (Perkin Elmer) was added to the

wells. Plates were shaken for 30 minutes and then radioactivity was measured for 1 min/well on a microbeta Counter (Perkin Elmer).

Non-specific binding was determined using 10 µM LY320135. Total binding was defined in the absence of the test ligand. All experiments were performed at 4 ºC in order to minimise cannabinoid agonist-induced internalisation or desensitisation.

2.2.5.1. [^{3}H]SR141716A association kinetic assays

[^{3}H]SR141716A association kinetic assays were performed to determine the time taken to reach [^{3}H]SR141716A equilibrium binding, by incubating cells with an approximate K_d concentration (1 nM) of [^{3}H]SR141716A in the absence or presence of 10 µM LY320135 for different time intervals (0-360 minutes and overnight).

2.2.5.2. Homologous competition binding assays

Homologous competition binding experiments were performed by incubating cells with [^{3}H]SR141716A at 1 nM and a wide concentration range of unlabelled SR141716A, for 6 hours, based on the time required to reach the equilibrium in association kinetic assays.

2.2.5.3. Heterologous competition binding assays

Competition binding assays were performed by incubating cells with 1 nM [^{3}H]SR141716A and increasing concentrations of cannabinoid ligands for 6 hours, based on the time determined in association kinetic assays.

2.2.6. Extracellular signal regulated kinase 1/2 phosphorylation (pERK1/2) assays

CHO-hCB_1 cells were seeded at 50,000 cells/well into 96-well clear bottom culture plates and grown overnight in complete medium. The following day, cells were washed twice with 100 µl of PBS and incubated in serum-free DMEM, supplemented by 16 mM HEPES, at 37 ºC for 5 hours to minimise FBS-stimulated pERK1/2 levels. Cells were incubated with varying concentrations of cannabinoid compounds at 37 ºC in a final volume of 200 µl. In all experiments, 10% FBS was used as a positive control, and vehicle controls were also included. The assays were terminated by the addition of 100 µl *SureFire*® lysis buffer. The lysates were agitated for at least 5 minutes, and then mixed with *SureFire*® activation buffer, *SureFire*® reaction buffer and AlphaScreen® beads in a 4:1:6:0.05 ratio in a 384-well white opaque proxiplate under low light conditions. Plates were read on an Envision® plate reader (Perkin Elmer) after 1.5 hours incubation at 37 ºC in the dark using standard AlphaScreen® settings.

2.2.6.1. pERK1/2 time courses

Initial time course experiments were performed to determine the time that the maximum signal was produced by each ligand. In this set of assays, a 10 µM final concentration of each ligand was used, and cells were incubated for different time intervals (0, 2.5, 5, 7, 10, 15, 20, 30 minutes) before termination of pERK1/2 stimulation.

2.2.6.2. Agonist concentration-response experiments

Agonist concentration-response curves were obtained by adding increasing concentrations of each ligand and terminating the assay at the time at which maximum pERK1/2 was stimulated in time course assays, which was 2.5 minutes for anandamide and methanandamide, and 5 minutes for 2-AG, CP55940, HU-210, WIN55,212-2, and Δ9-THC.

2.2.7. AlphaScreen® cAMP assays

CHO-hCB_1 cells were seeded at 50,000 cells per well into 96-well clear bottom culture plates, and incubated overnight at 37 ºC, 5% CO_2. The following day, cells were serum deprived for 1 hour to minimize basal cAMP signalling, by replacing the growth media with serum-free DMEM/F12, containing 1 mM IBMX (3-isobutyl-1-methylxanthine; a non-selective phosphodiesterase inhibitor, to prevent degradation of cAMP) and 0.5% w/v BSA. Cells were then incubated with each ligand in a final volume of 100 µl for 30 minutes at 37 ºC. The assays were terminated by adding 50 µl of 100% ethanol and cells were lysed using 0.1% BSA, 5 mM HEPES, 0.3% Tween20 in milliQ water. The lysates were agitated for at least 15 minutes, transferred to a 384-well optiplate, and then incubated with AlphaScreen® acceptor beads in stimulation buffer in a 2:1:100 ratio, for 30 minutes at 37 ºC. AlphaScreen donor beads and the biotinylated-cAMP were prepared in lysis buffer in a 1:0.075:100 ratio, 30 minutes before addition to the cell lysate. Plates were incubated for 2 hours in the dark at 37 ºC, and then read on an Envision® plate reader (Perkin Elmer) using standard AlphaScreen® settings.

2.2.7.1. Forskolin concentration-response experiments

Initial forskolin concentration-response experiments were performed to determine the optimal forskolin concentration for stimulation of adenylate cyclase. Cells were incubated with increasing concentrations of forskolin for 30 minutes at 37 ºC.

2.2.7.2. Agonist concentration-response experiments

Cells were incubated with increasing concentrations of each cannabinoid compound together with the optimal concentration of forskolin determined in forskolin concentration-response experiments (1 μM) for 30 minutes at 37 ºC. Control cells were treated with only forskolin or vehicle.

2.2.7.3. Antagonist interaction experiments

cAMP interaction studies with the antagonist were performed by pre-incubating cells with varying concentrations of SR141716A for 10 minutes, before the addition of increasing concentrations of the agonist.

2.2.8. Data analysis

Data were analysed using Prism 6 (GraphPad, San Diego, CA). In all radioligand binding assays, it was ascertained that ligand depletion (a condition in which more than 10% of the total radiloligand concentration binds to receptors, thereby leads to erroneous estimation of binding parameters (Hulme and Trevethick, 2010)) did not occur. [^{3}H]SR141716A association kinetic data were fitted to an exponential one-phase association equation to determine the time taken for the radioligand to reach equilibrium (Eq. 2.1),

$$Y = \mathrm{Y0} + (\mathrm{Plateau} - \mathrm{Y0})(1\text{-}e^{-\mathrm{Kt}}) \qquad \text{Eq. 2.1}$$

where Y0 is the specific radioligand binding when t (time) is zero, Plateau denotes the specific binding at infinite times, and K is the radioligand association rate constant.

Homologous competition binding data were fitted a one-site homologous binding equation to determine the radioligand K_d value (Eq. 2.2),

$$Y = \frac{\mathrm{B_{max}HotnM}}{\mathrm{HotnM+ColdnM+K_dnM}} + \mathrm{Bottom} \qquad \text{Eq. 2.2}$$

where B_{max} is maximum binding, Bottom denotes the minimal asymptotes of the curve, and K_d is the radioligand equilibrium dissociation constant.

T To calculate the B_{max} (binding sites/cell), the following equation was used (Eq. 2.3),

$$\mathrm{SA}_{\left(\frac{cpm}{fmol}\right)} = 2.22\left(\mathrm{SA}_{\left(\frac{Ci}{mmol}\right)}\mathrm{CE}\right)$$

$$\mathrm{B_{max}}_{\left(\frac{sites}{cell}\right)} = \left(\frac{\mathrm{B_{max}}_{(cpm)}}{\mathrm{SA}_{\left(\frac{cpm}{fmol}\right)}}\right)\left(\frac{6.02 \times 10^{8}}{\mathrm{n}}\right)$$

Where, SA is the specific activity for [^{3}H]SR141716A and CE is the counter efficiency. $B_{max\ (cpm)}$ is the B_{max} determined from homologous competition assays analysed using equation 2.2, and n is the cell number.

cAMP interaction studies between the orthosteric agonist and antagonist were fitted to a Gaddum/Schild equation (Eq. 2.4),

$$Y = \frac{(\mathrm{Top} - \mathrm{Bottom})}{1 + 10^{(\log \mathrm{EC}-\log[\mathrm{A}])\mathrm{n}}} + \mathrm{Bottom}$$

$$\mathrm{LogEC} = \mathrm{Log}\left(\mathrm{EC_{50}}\left(1 + \frac{[\mathrm{B}]}{10^{(\mathrm{pA_2})}}\right)^{m}\right) \qquad \text{Eq. 2.4}$$

Where Y is the response to agonist, Top and Bottom denote the maximal and minimal asymptotes of the curve, respectively; [A] and [B] are the concentration of agonis and antagonist, respectively, EC_{50} denotes the concentration of agonist that produces half the maximal response in the absence of antagonist, pA_2 is the negative logarithm of concentration of antagonist that shifts the EC_{50} by a factor of 2, n is the Hill slope, which describes the steepness of the curves, and m denotes the Schild slope, which quantifies how well the shifts correspond to the prediction of competitive interaction. If the antagonist is competitive, the Schild slope will equal 1.0.

For competition binding experiments with orthosteric ligands, a one-site binding equation (Motulsky and Christopoulos, 2004) was used to analyse specific binding of each ligand (Eq. 2.5),

$$Y = \frac{(\text{Top - Bottom})}{1 + (10^{[I]\text{-log}IC_{50}})} + \text{Bottom} \qquad \text{Eq. 2.5}$$

The equilibrium dissociation constant (K_i) of unlabelled ligand was calculated using the Cheng and Prusoff equation (Eq. 2.6) (Cheng and Prusoff, 1973),

$$K_i = \frac{IC_{50}}{1 + \frac{[\text{Radioligand}]}{K_d}} \qquad \text{Eq. 2.6}$$

where Y represents the percentage of specific binding; Top and Bottom denote the maximal and minimal asymptotes of the curve, respectively; [I] is the concentration of inhibitor; and IC_{50} is the concentration of competitor that produces half the maximal response. K_d is the equilibrium dissociation constant derived from homologous competition binding assays.

Concentration-response data generated from cAMP and pERK1/2 assays were fitted to a three parameter concentration response equation (Eq. 2.7)

$$Y = \frac{(\text{Top - Bottom})}{1 + (10^{\log EC_{50} - A})} + \text{Bottom} \qquad \text{Eq. 2.7}$$

where, Top and Bottom denote the maximal and minimal asymptotes of the curve, respectively; and log EC_{50} is the A value when the response is halfway between Bottom and Top; or to the following operational model of agonism (van der Westhuizen et al., 2014) to determine the transduction ratio (tau/KA, R) (Eq. 2.8),

$$E = \frac{(E_m - \text{Basal})}{1 + \left(\frac{\left(\frac{[A]}{10^{\log K_A}} + 1 \right)}{10^{\log R} \times [A]} \right)} + \text{Basal} \qquad \text{Eq. 2.8}$$

where Em is the maximal possible system response (the top plateau of the dose-response curve obtained for the full agonist, CP55940), Basal is the response in the absence of agonist, KA denotes the equilibrium dissociation constant of the agonist (A), LogR is the logarithm of the transduction ratio, which is an index of the coupling efficacy of the agonist and n is the unitless transducer slope.

2.2.9. Statistics

Values are expressed as means ± S.E.M. Mean values have been compared using one-way ANOVA with Bonferroni's multiple comparison test to determine the statistical differences in cannabinoid potency values between cAMP and pERK1/2 assays, or the differences in ΔΔτ/KA ratios. A P value < 0.05 was considered significant.

2.3. Results

2.3.1. [^{3}H]SR141716A displacement by cannabinoid ligands

We first set out to determine the affinity of cannabinoid ligands for the CB_1 receptor using whole cell radioligand binding assays, have the advantage of assessing the binding properties of the receptor under similar conditions as the functional assays conducted in the present study. Furthermore, membrane based assays do not reflect the native environment of the receptor in the cell. For example, the existing ion gradients across the plasma membrane will be disturbed and interactions of the receptor with cytoplasmic components such as effectors and nucleotides will not occur in membrane preparations (Bylund and Toews, 1993). To ensure that radioligand binding reached equilibrium, initial association kinetic assays were performed. By fitting the data to a one-phase association equation (Eq. 2.1), a half-time of the equilibration reaction ($t_{1/2}$) equal to 79 minutes was obtained. The time equivalent to 5 times the $t_{1/2}$ was considered sufficient for 1 nM [^{3}H]SR141716A to reach equilibrium (Fig. 2.1). Therefore, all subsequent experiments were terminated after a 6-hour incubation period.

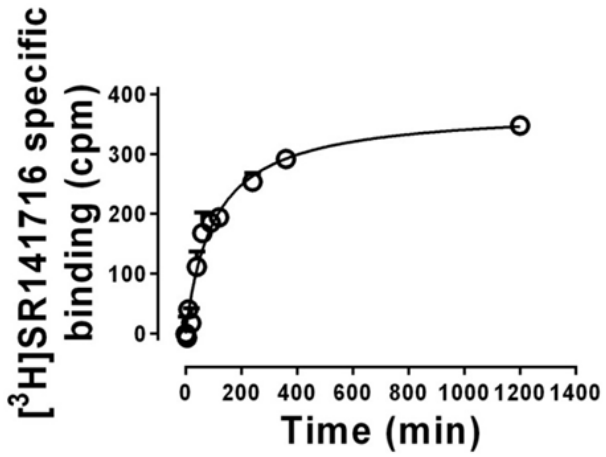


Fig. 2.1. [^{3}H]SR141716A association kinetic assays. CHO-hCB_1 cells were incubated with 1 nM [^{3}H]SR141716A in the absence or presence of 10 µM LY320135 (to define non-specific binding) for different time intervals at 4 ºC. Curves were generated by fitting the data to a one-phase association equation (Eq. 2.1). Data represent mean + S.E.M. of two experiments performed in triplicate.

Next, homologous competition binding assays between SR141716A and [^{3}H]SR141716A were performed to determine the equilibrium dissociation constant (pK_d) for [^{3}H]SR141716A. By fitting the data to a one-site homologous binding equation (Eq. 2.2), a pK_d value of 8.51±0.35 was estimated (Fig. 2.2). The total number of receptors expressed (B_{max}) in our system was estimated to be 800,098 ± 1868 sites/cell (Eq. 2.3).

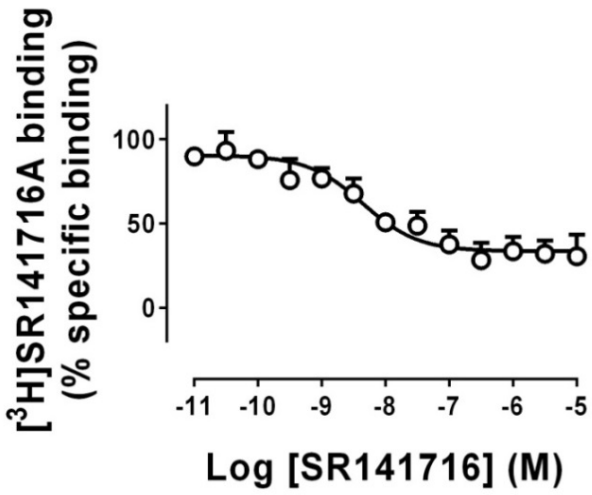


Fig. 2.2. Homologous competition binding assays. CHO-hCB_1 cells were incubated with 1 nM [^{3}H]SR141716A and a wide concentration range of unlabelled SR141716A, for 6 hours at 4 ºC. Curves were generated by fitting the data to a one-site homologous binding equation (Eq. 2.2). Data represent mean + S.E.M. of five experiments performed in triplicate.

Unlabelled SR141716A did not completely displace [^{3}H]SR141716A binding in whole cells. This was most likely due to non-receptor radioligand binding events, such as incorporation into the cell plasma membrane. This can occur even in the presence of an unlabelled competitive ligand (Wennerberg et al., 2010). Nonetheless, the pK_d calculated for [^{3}H]SR141716A in these assays (8.51 ± 0.35) was in agreement with its pK_d value determined previously in both membrane

preparations (Govaerts et al., 2004) and in whole HEK293-CB_1 cells (Wennerberg et al., 2011). To confirm the accuracy of the estimated pK_d value for [^{3}H]SR141716A in binding experiments, the interaction between SR141716A and CP55940 was determined in cAMP assays (Fig. 2.3). Data were analysed using the Gaddum/Schild equation (Eq. 2.4). The estimated pA_2 value of 8.89 ± 0.37 for SR141716A was close to the estimated value in binding studies. The pA_2 equals the pK_d when the Schild slope is equal to 1. Thus, [^{3}H]SR141716A was used as the radioligand in all subsequent heterologous binding experiments.

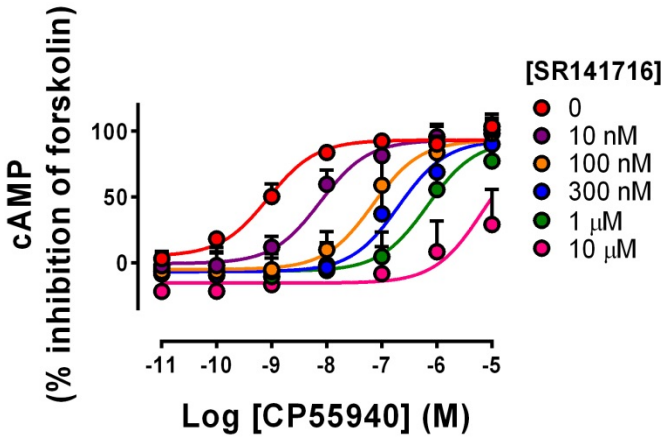


Fig. 2.3. Interaction between SR141716A and CP55940 in cAMP assays. CHO-hCB_1 cells were incubated with varying concentrations of SR141716A for 10 minutes before addition of CP55940. Curves were generated by fitting the data to Gaddum/Schild equation (Eq. 2.4). Data represent mean + S.E.M. of two experiments performed in triplicate.

Heterologous competition binding assays were next performed to determine the binding affinity (pK_i) of endogenous and exogenous cannabinoid agonists. Displacement of [^{3}H]SR141716A was analysed using a one-site binding equation (Eq. 2.5) (Motulsky and Christopoulos, 2004) (Fig. 2.4). The calculated pK_i values

(Eq. 2.6) were in agreement with previously published values, and are presented in Table 2.1. The rank order of agonist affinity was: HU-210 = CP55940 > WIN55,212-2 = Δ9-THC > methanandamide = anandamide = 2-AG. Displacement of [^{3}H]SR141716 by the CB_1 receptor inverse agonist, LY320135, yielded an estimated pK_i value of 7.4±0.1.

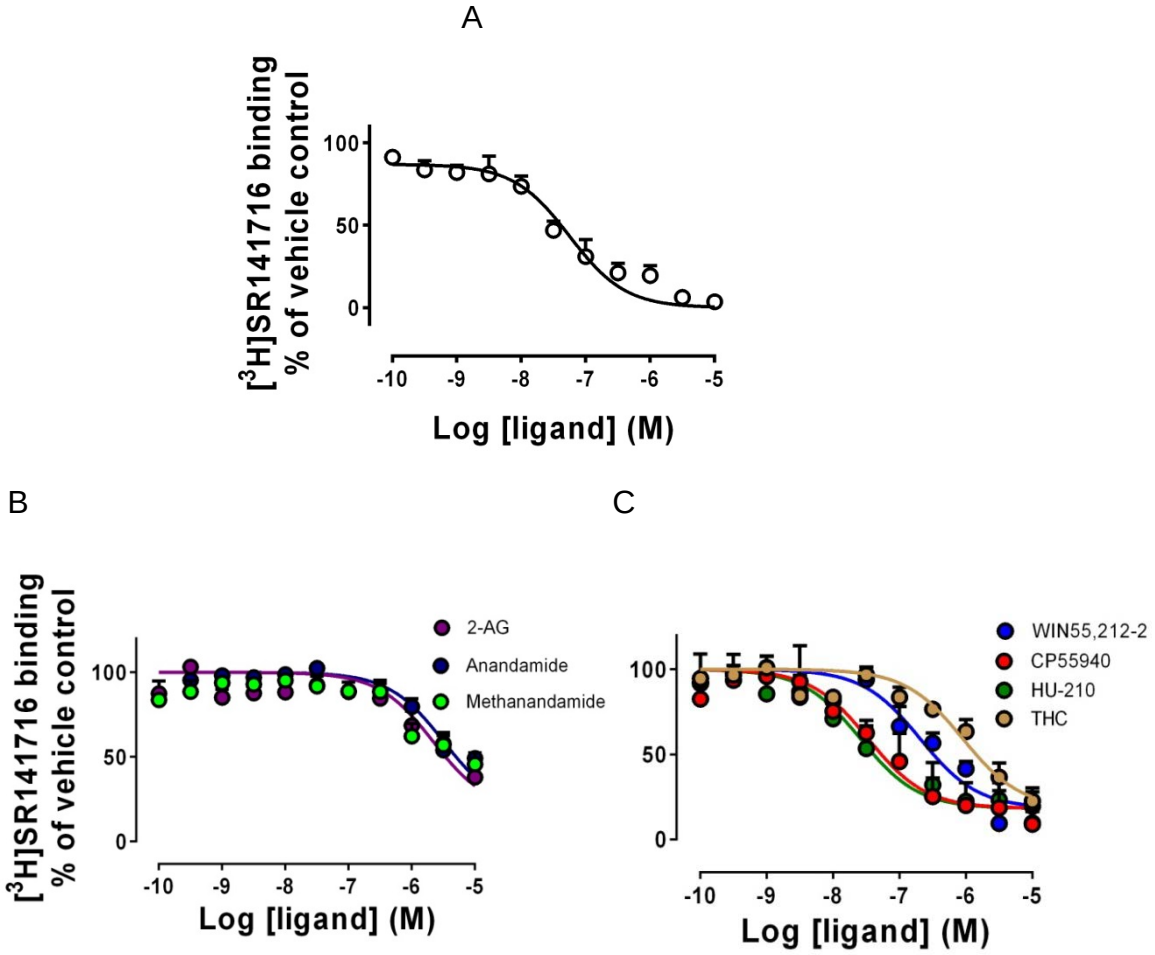


Fig. 2.4. Heterologous competition binding assays. CHO-hCB_1 cells were incubated with 1 nM [^{3}H]SR141716A and increasing concentrations of cannabinoid ligands for 6 hours at 4 ºC. [^{3}H]SR141716A displacement by (A) the CB1 inverse agonist, LY320135, (B) endogenous cannabinoid agonists and (C) exogenous cannabinoid agonists. Curves were generated by fitting the data to a one-site binding equation (Eq. 2.5). Data points represent mean values + S.E.M. from at least three experiments performed in triplicate.

Indeed, although in our experiments fatty acid free BSA was used to minimise the binding of ligands to non-specific sites, none of the cannabinoid agonists used in the current study fully displaced [^{3}H]SR141716A, with approximately 20-30% radioligand binding remaining (Figure 2.4). This suggests that the cannabinoid agonists (but not LY320135, which was used to define nonspecific binding) also displace [^{3}H]SR141716A from some non-specific binding sites such as the assay plates, BSA, or plasma membrane, resulting in an overestimation of specific radioligand binding.

Table 2.1. Binding affinity (pK_i) values for cannabinoid ligands determined by measuring [^{3}H]SR141716A displacement in competition binding assays in CHO-hCB_1 cells.
The previously reported pK_i values for cannabinoids in displacement of [^{3}H]SR141716A or [^{3}H]agonist, in either membrane preparations or whole cells, are also presented. Data were analysed using a one-site binding equation (Eq. 2.5). Values represent the mean ± S.E.M. from at least three experiments performed in triplicate.

Ligand	**pK_i**	**Published pK_i***	
		[^{3}H]SR141716A	[^{3}H]agonist
CP55,940	7.4±0.2	7.3-8.9	7.6-10.2
HU-210	7.7±0.2	7.7-9.6	8.5-10.6
WIN55,212-2	6.3±0.1	5.9-8.9	6.2-8.9
Δ9-THC	6.6±0.2	6.8-7.5	6.3-8.8
Methanandamide	5.9±0.1	5.2-6.5	6.2-7.7
Anandamide	5.5±0.2	6.1-7.4	5.6-7.6
2-AG	5.6±0.1	ND	5.6-7.0
SR141716A	8.5±0.3	7.9-9.9	

***** Reviewed in (McPartland et al., 2007)

2.3.2. Activation of pERK1/2

We next examined activation of pERK1/2 by cannabinoid ligands. The results of pERK1/2 time course experiments showed that the maximum signal for CP55940, WIN55,212-2, HU-210, Δ9-THC and 2-AG was stimulated at 5 minutes, and at 2.5 minutes for anandamide and methanandamide. The CB_1 receptor inverse agonists SR141716A and LY320135 did not produce any response on their own (Fig. 2.5).

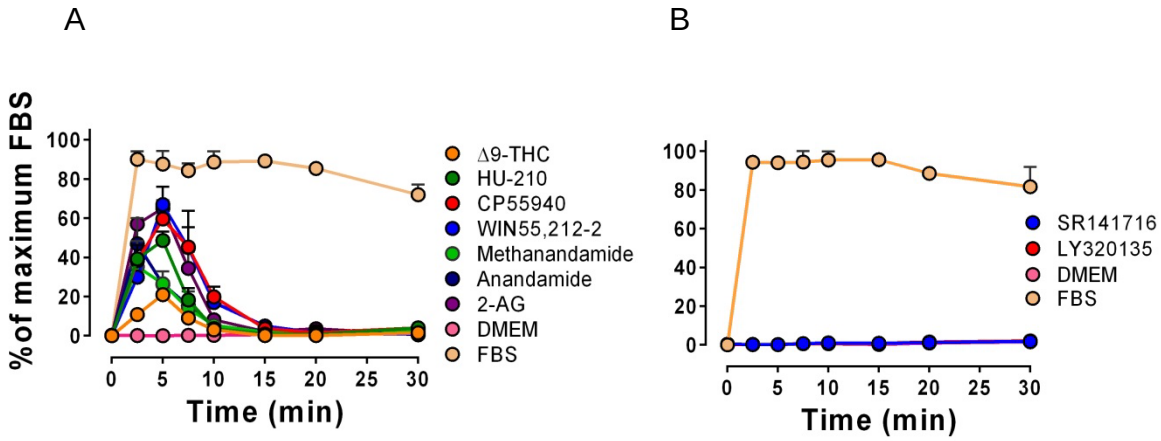


Fig. 2.5. pERK1/2 time course assays for (A) cannabinoid agonists and (B) antagonists/inverse agonists. CHO-hCB_1 cells were incubated with 10 µM of each ligand at 37 ºC for different time intervals before termination of pERK1/2 stimulation. Data points represent mean values + S.E.M. from three experiments performed in triplicate.

Endogenous and exogenous cannabinoids activated pERK1/2 in a dose-dependent manner (Fig. 2.6). Data were fitted to a three parameter concentration response equation (Eq. 2.7) to derive the functional potency (pEC_{50}) and efficacy (E_{max}) of the ligands (Table 2.2). In pERK1/2 assays, cannabinoid pEC_{50}s generally matched their estimated pK_i values. The rank order of potency was

therefore HU-210 = CP55940 > WIN55,212-2 = Δ9-THC > methanandamide = 2-AG = anandamide (Table 2.2).

The results also demonstrated that CP55940, HU-210, WIN55,212-2 and 2-AG were full agonists, while Δ9-THC and methanandamide acted as partial agonists in pERK1/2 assays (Fig. 2.6).

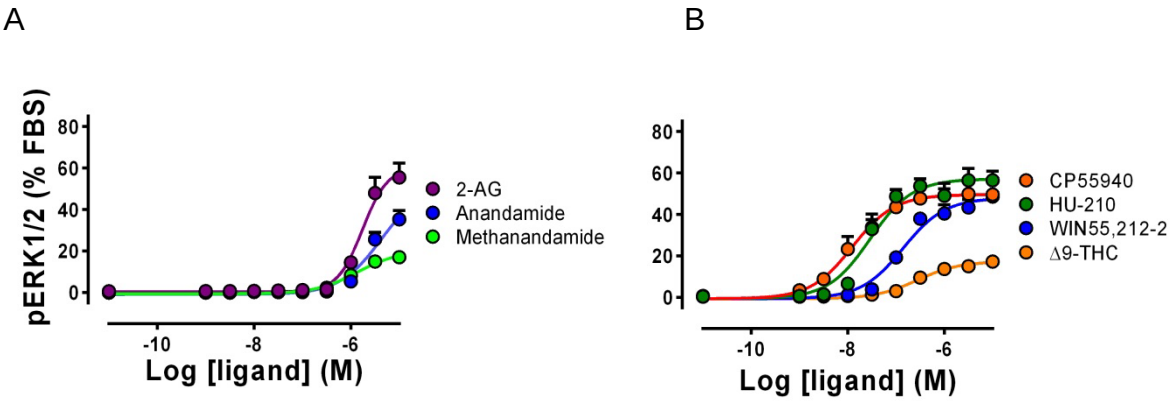


Fig. 2.6. Activation of pERK1/2 by (A) endogenous and (B) exogenous cannabinoids in CHO-hCB_1 cells. Cells were incubated with increasing concentrations of each ligand at 37 ºC for the time determined in time course assays. Curves were generated by fitting the data to a three parameter concentration response equation (Eq. 2.7). Data points represent mean values + S.E.M. from at least three experiments performed in triplicate.

Table 2.2. Potency (pEC_{50}) and relative efficacy (E_{max}) of cannabinoid ligands in pERK1/2 assays in CHO-hCB_1 cells.

Data were analysed using a three parameter concentration response equation (Eq. 2.7). Values represent the mean ± S.E.M. from at least three experiments performed in triplicate.

Ligand	**pERK1/2**	
	pEC_{50} (EC_{50} nM)	**E_{max}[a]**
CP55,940	7.7±0.2 (19)	51.4±1.9
HU-210	7.5±0.1 (31)	57.4±3.6
WIN55,212-2	7.0±0.2 (107)	52.2±4.6
Δ9-THC	6.4±0.1 (371)	19.3±3.0
Methanandamide	5.9±0.1 (1230)	26.3±5.7
Anandamide	5.5±0.2 (3162)	~ 40[b]
2-AG	5.8±0.1 (1585)	~ 60[b]

[a] % of maximum FBS response

[b] As the curves did not reach a plateau, the exact E_{max} values could not be defined and were estimated by constraining the value to be not more than the maximum effect produced by the full agonist, CP55940.

No functional response to cannabinoids was observed in untransfected Flp-In CHO cells, confirming the involvement of CB_1 receptors in activation of pERK1/2 by cannabinoid ligands (Fig. 2.7).

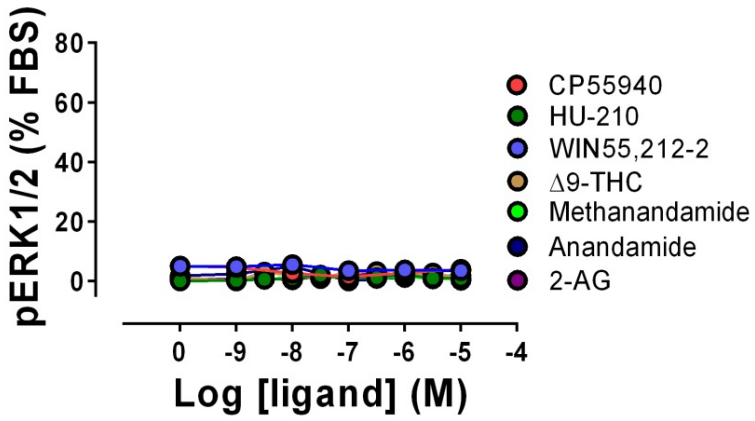


Fig. 2.7. Cannabinoid ligands have no effect on pERK1/2 levels in untransfected Flp-In CHO cells. Cells were incubated with increasing concentrations of each ligand at 37 ºC for the time determined in time course assays. Data points represent mean values + S.E.M. from two experiments performed in triplicate.

2.3.3. Inhibition of forskolin-stimulated cAMP formation

Next, CB_1 receptor-mediated inhibition of cAMP formation was investigated. Based on the results obtained in forskolin concentration-response experiments (Fig. 2.8), an EC_{50} concentration of 1 µM forskolin was used for subsequent agonist concentration response experiments.

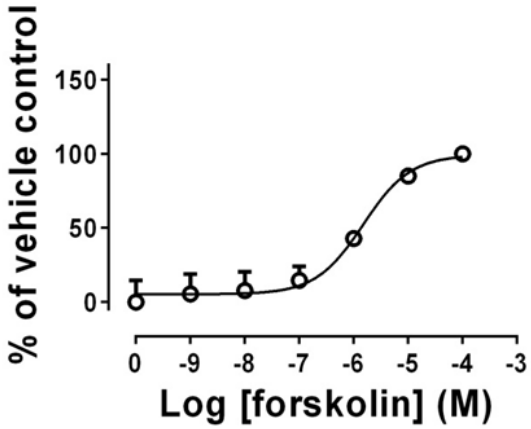


Fig. 2.8. Forskolin concentration-response curve. CHO-hCB_1 cells were incubated with increasing concentrations of forskolin for 30 minutes at 37 ºC. Data points represent mean values + S.E.M. from four experiments performed in triplicate.

As shown in figure 2.9, all endogenous and exogenous cannabinoids tested inhibited forskolin-stimulated cAMP formation in a dose-dependent manner in CHO-hCB_1 cells.

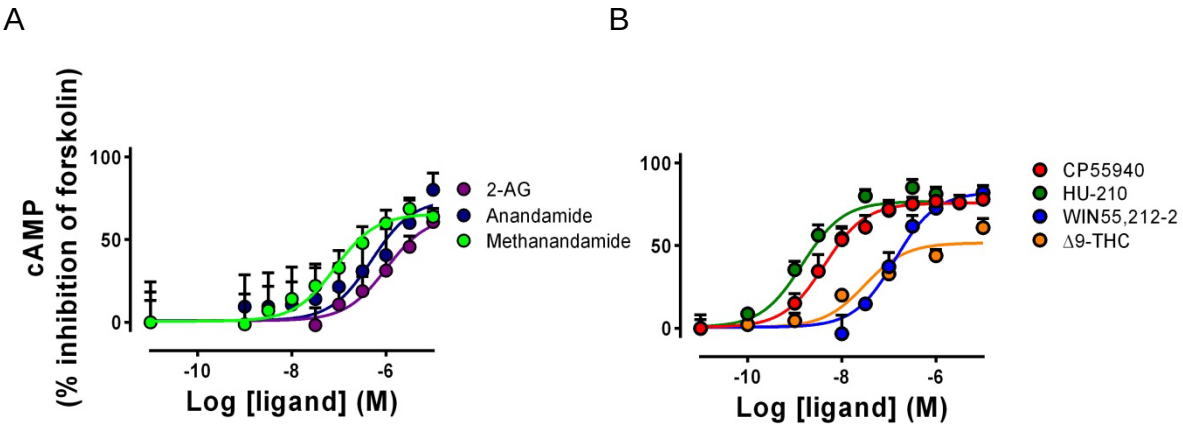


Fig. 2.9. Inhibition of 1 μM forskolin-stimulated cAMP formation by (A) endogenous and (B) exogenous cannabinoids in CHO-hCB_1 cells. Cells were incubated with increasing concentrations of each cannabinoid ligand in the presence of 1 μM forskolin for 30 minutes at 37 ºC. Curves were generated by fitting the data to a three parameter concentration response equation (Eq. 2.7). Data points represent mean values + S.E.M. from at least three experiments performed in triplicate.

Potency and efficacy of ligands in inhibition of 1 μM forskolin-stimulated cAMP formation were determined by fitting the data to a three parameter concentration response equation (Eq. 2.7). The potency and efficacy of WIN55,212-2 and 2-AG were comparable in both cAMP and pERK1/2 assays, suggesting that these cannabinoids showed no preference in activating either pathway. However, HU-210, Δ9-THC, methanandamide and anandamide showed greater potency and/or efficacy in cAMP versus pERK1/2 assays. The difference in potency between assays was particularly noticeable for HU-210 ($p< 0.0001$),

Δ9-THC ($p< 0.01$) and methanandamide ($p< 0.05$) (Table 2.2 and 2.3). CP55940 showed a tendency towards greater potency in cAMP than pERK1/2 assays, however, it did not reach statistical significance. Therefore, in contrast to pERK1/2 assays, HU-210 was significantly (10 fold; $p< 0.05$) more potent than CP55940 in assays measuring inhibition of cAMP (Table 2.3). Thus, the rank order of potency in cAMP was: HU-210 > CP55940 > WIN55,212-2 = Δ9-THC > methanandamide = anandamide = 2-AG. The results also showed that CP55940, HU-210, WIN55,212-2, 2-AG, anandamide and methanandamide behaved as full agonists while Δ9-THC was a partial agonist in cAMP assays (Table 2.3). Furthermore, whereas Δ9-THC was a partial agonist in both cAMP and pERK1/2 assays, methanandamide behaved as a partial agonist in pERK1/2 assays, but as a full agonist in cAMP assays, indicating its preference towards inhibition of cAMP formation. The enhanced potency and/or efficacy of some but not all agonists in cAMP versus pERK1/2 assays is indicative of ligand-biased signalling at CB_1 receptors.

Table 2.3. Potency (pEC_{50}) and relative efficacy (E_{max}) of cannabinoid ligands in cAMP assays in CHO-hCB_1 cells.
Data were analysed using a three parameter concentration response equation (Eq. 2.7). Values represent the mean ± S.E.M. from at least three experiments performed in triplicate.

Ligand	**cAMP**	
	pEC_{50} (EC_{50} nM)	**E_{max}[a]**
CP55940	8.1±0.2 (7.9)	81.8±5.5
HU-210	9.0±0.2 (1.0)	80.5±5.1
WIN55,212-2	7.1±0.1 (79)	86.2±3.9
Δ9-THC	7.6±0.1 (25)	51.9±4.5
Methanandamide	6.8±0.4 (158)	70.6±4.4
Anandamide	6.1±0.2 (794)	74.0±9.4
2-AG	5.9±0.2 (1259)	64.2±10.6

[a] % of maximum inhibition of forskolin response

To ensure that the low affinity and potency of 2-AG in our experiments is not due to the compound being old or due to its breakdown by monoacylglycerol lipase (MAGL), we performed competition binding assays and cAMP studies using a new batch of 2-AG in the presence or absence of 100 nM final concentration of JZL 184, a potent and selective MAGL inhibitor. The results showed that 30 minute pre-incubation of cells with JZL 184 did not alter the K_i value of 2-AG. Also, there was no significant difference between the potency of 2-AG in the presence or absence of JZL 184 in the inhibition of cAMP formation (Fig. 2.10, Table 2.4). The potency of the new 2-AG was also comparable to that of the old 2-AG.

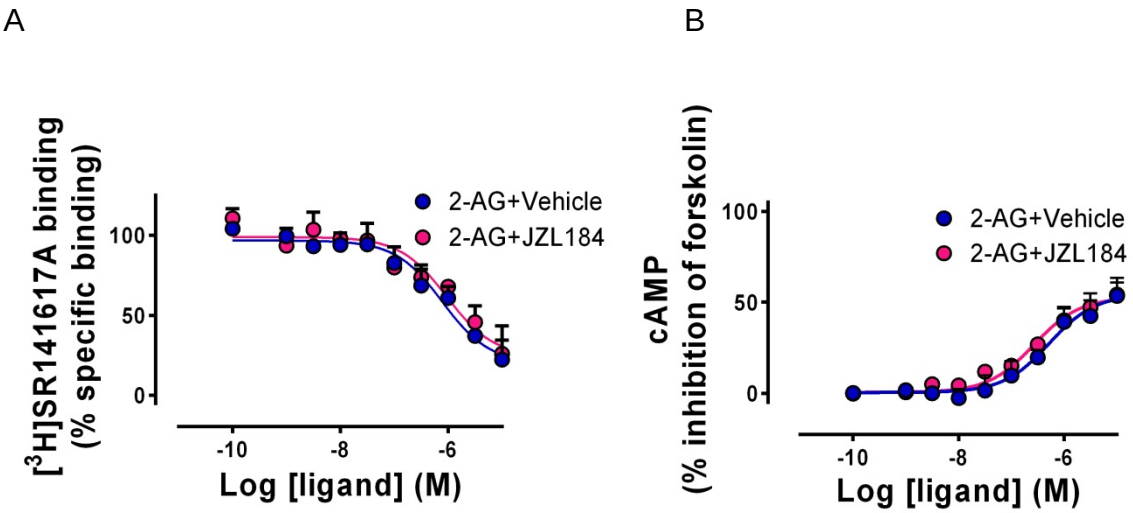


Fig. 2.10. Effects of JZL 184 on 2-AG binding and signalling in CHO-hCB$_1$ cells. [^{3}H]SR141716A displacement (A) and inhibition of 1 µM forskolin-stimulated cAMP formation (B) by 2-AG in the presence or absence of JZL 184. Cells were pre-incubated with 100 nM final concentration of JZL 184 for 30 minutes. Curves were generated by fitting the data to a one-site binding equation (Eq. 2.5) for binding experiments, and to a three parameter concentration response equation (Eq. 2.7) for cAMP studies. Data points represent mean values + S.E.M. from three experiments performed in triplicate.

Table. 2.4. Binding affinity (pK_i) and potency (pEC_{50}) of 2-AG in the presence or absence of 100 nM JZL 184 obtained from radioligand binding and cAMP assays in CHO-hCB$_1$ cells.

Data were analysed using a three parameter concentration response equation (Eq. 2.7). Values represent the mean ± S.E.M. from three experiments performed in triplicate.

Ligand	**pK_i**	**pEC_{50}**
2-AG+vehicle	6.4±0.2	6.2±0.2
2-AG+JZL 184	6.3±0.2	6.4±0.2

Furthermore, the lack of response in untransfected Flp-In CHO cells confirmed the involvement of CB_1 receptors in inhibition of cAMP formation by cannabinoid ligands (data not shown).

2.3.4. Quantification of ligand bias at CB_1 receptors

Due to differences in agonist potency between pERK1/2 and cAMP assays, we next set out to quantify ligand-biased signalling from CB_1 receptors. Bias plots (Fig. 2.11) were first constructed to more readily visualise the differences in signalling preference of each cannabinoid ligand. These plots represent the response of the receptor to equimolar concentrations of agonist in pERK1/2 (X-axis) versus inhibition of cAMP formation (Y-axis). Curves through the data sets were generated by plotting 150 points that defined each agonist concentration-response curve. Curves that lie either side of the line of identity (shown by the dotted line) highlight preferential coupling to one pathway over the other. It is again apparent from these plots that 2-AG and WIN55,212-2 show little preference for coupling to either pathway, whereas HU-210 and methanandamide, in particular, preferentially mediate inhibition of cAMP.

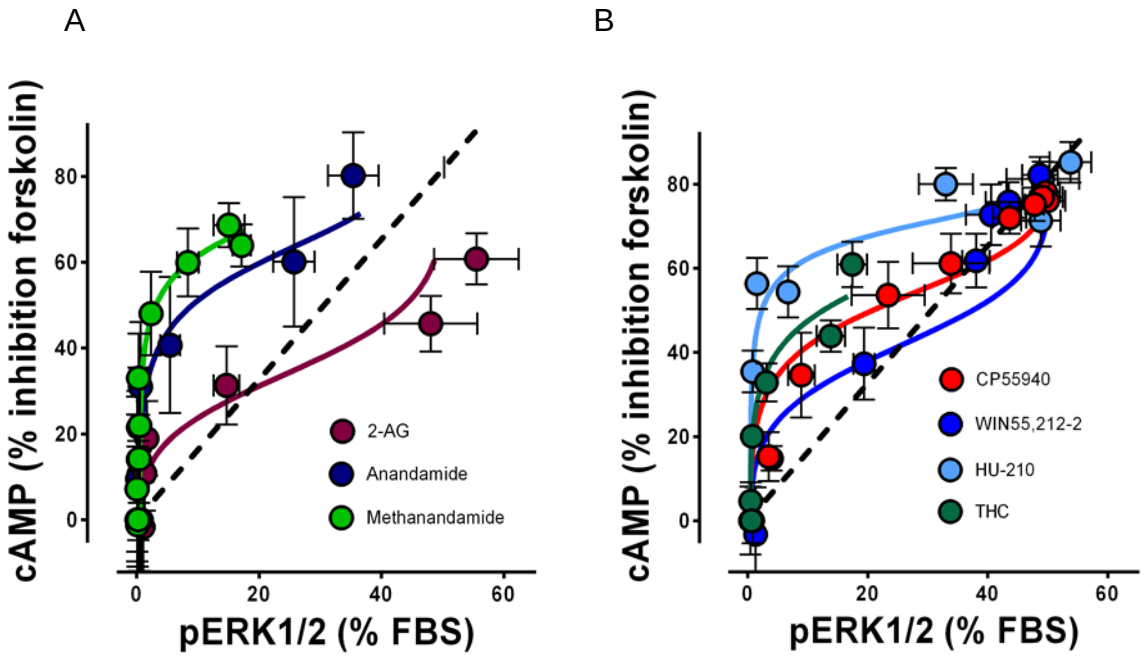


Fig. 2.11. Cannabinoids are biased agonists at CB_1 receptors. Bias plots for inhibition of cAMP formation and activation of pERK1/2 by (A) endogenous and (B) exogenous cannabinoids. The curves represent the response to equimolar concentrations of agonist in pERK1/2 (X-axis) versus inhibition of cAMP formation (Y-axis). Biased ligands fall either side of the line of identity (the dotted line), towards the preferred pathway.

To quantify ligand-biased signalling from the CB_1 receptor, data from cAMP and pERK1/2 agonist concentration-response experiments were next fitted to an operational model of agonism (van der Westhuizen et al., 2014) (Eq. 2.8), to determine the transduction ratio, tau/K_A, of each cannabinoid. To eliminate "system" or "observation bias", cannabinoid transduction ratios were compared to that of 2-AG, in part because this endogenous CB_1 receptor agonist exhibited little bias towards cAMP or pERK1/2 (Fig. 2.11. A). Thus, the relative effectiveness of cannabinoids in driving the CB_1 receptor towards cAMP or pERK1/2 were calculated as the difference in the transduction ratio between 2-AG and the

cannabinoids (ΔLogR, Table 2.5) (van der Westhuizen et al., 2014). Next, the strength of cannabinoid-mediated receptor coupling towards each pathway was calculated as the difference between the ΔLogR values for each pathway (ΔΔLogR) (van der Westhuizen et al., 2014). The anti-log of ΔΔLogR represents the bias factor of each agonist (Table 2.5). Bias factors in Table 2.5 equal to 1 demonstrate that the cannabinoid promotes the same coupling preference as 2-AG. Bias factors greater than 1 indicate the cannabinoid preferentially promotes receptor coupling towards cAMP inhibition over pERK1/2.

The bias factors highlight that whereas WIN55,212-2 demonstrates a similar profile to 2-AG with a bias factor not dissimilar from 1, HU-210 and methanandamide exhibit strong bias (over 20 and 15, respectively) towards cAMP inhibition ($p< 0.05$). CP55940, Δ9-THC and anandamide displayed a preference towards cAMP inhibition although they did not reach statistical significance (Table 2.5).

Table 2.5. LogR (τ/K_A), ΔLogR ($\Delta\tau/K_A$), ΔΔLogR ($\Delta\Delta\tau/K_A$) ratios and bias factors (BF) for cannabinoid ligands, relative to 2-AG, at the CB_1 receptors.

Values were obtained by fitting the data to an operational model of agonism (Eq. 2.8), and represent the mean ±S.E.M. from at least three experiments carried out in triplicate.

Ligand	cAMP		pERK1/2		cAMP/pERK1/2	
	LogR	ΔLogR	LogR	ΔLogR	ΔΔLogR	BF
CP55940	8.3±0.2	2.6±0.2	7.9±0.1	2.0±0.1	0.6±0.2	3.8
HU-210	8.8±0.2	3.1±0.2	7.6±0.1	1.8±0.1	1.3±0.2*	20.8
WIN55,212-2	6.9±0.2	1.2±0.2	6.8±0.1	1.0±0.1	0.2±0.2	1.6
Δ9-THC	7.1±0.3	1.4±0.3	6.5±0.2	0.6±0.2	0.8±0.4	5.6
Methanandamide	6.9±0.2	1.2±0.2	5.9±0.2	0.0±0.2	1.2±0.3*	15.1
Anandamide	6.3±0.2	0.6±0.2	5.6±0.1	-0.3±0.1	0.8±0.2	6.8
2-AG	5.7±0.2	0.0±0.2	5.9±0.1	0.0±0.1	0.0±0.2	1.0

*$p < 0.05$, statistically significant differences in the preference of 2-AG versus other cannabinoids for receptor signalling were determined using one-way ANOVA with Bonferroni's multiple comparison test on ΔΔLogR.

2.4. Discussion

This study quantifies for the first time ligand-biased signalling at the CB_1 receptor, and shows that certain cannabinoid agonists are biased towards inhibition of cAMP formation over pERK1/2 activation. Ligand-biased signalling is an important pharmacological phenomenon that may be used to achieve selective therapeutics through activation of receptor signalling pathways related to desired effects, at the expense of those that mediate adverse effects (Kenakin and Christopoulos, 2013; Valant et al., 2014). CB_1 receptor-mediated cAMP and pERK signalling may play distinct physiological roles and contribute to different *in vivo* effects produced by cannabinoids (Rubino et al., 2006; Zhou and Song, 2001). Therefore, agonists that activate only one of these two pathways may exhibit therapeutic advantages by avoiding the adverse effects related to the other pathway.

Binding studies were first performed to determine the affinity of cannabinoid ligands in displacing [^{3}H]SR141716A binding. The rank order of efficacy was HU-210 = CP55940 > WIN55,212-2 = Δ9-THC > methanandamide = 2-AG = anandamide. This is consistent with the reported rank order of binding affinity as: HU-210 > CP55940 > Δ9-THC = WIN55,212–2 > anandamide (Bonhaus et al., 1998; Song and Bonner, 1996). It should also be noted that the affinity values determined for cannabinoids are greatly dependent on the radioligand used, with tritiated inverse agonists resulting in lower affinities compared to tritiated agonists (Govaerts et al., 2004; Thomas et al., 1998). For instance, the K_i value reported for WIN55,212-2 using [^{3}H]CP55940 or [^{3}H]SR141716A was 2 and 22 nM, respectively (Thomas et al., 1998). Nonetheless, pK_i values calculated in the

present study for CP55940, HU-210, WIN55,212-2, Δ9-THC and methanandamide were in general agreement with those reported previously in membrane-based radioligand binding assays that employed [^{3}H]SR141716A (D'Antona et al., 2006; Muccioli et al., 2005; Thomas et al., 1998). There is no published pK_i values for 2-AG using [^{3}H]SR141716A. However, its estimated pK_i value in the present study is similar to a reported value using [^{3}H]CP55940 (Sugiura et al., 1995). The calculated pK_i value for anandamide was slightly lower than those reported previously using [^{3}H]CP55940 (Adams et al., 1998) or [^{3}H]SR141716A (Bisogno et al., 2000). Therefore, the tendency for affinity values of cannabinoid ligands to lie near the low borderline of reported values (Table 2.1) may be due to the presence of fewer receptors in the active state in whole cells compared to membrane preparations in previous studies, or the different experimental conditions, as well as the competition against [^{3}H]SR141716A rather than tritiated agonists.

Previous reports showed the rank order of potency of three commonly used cannabinoids for pERK1/2 activation as CP55940 > Δ9-THC > WIN55,212-2 (Bouaboula et al., 1995b). In the present study, the rank order of potency in activation of pERK1/2 was HU-210 = CP55940 > WIN55,212-2 = Δ9-THC > methanandamide = 2-AG = anandamide, which was the same as their rank order of affinity. The rank order of potency in inhibition of cAMP formation was HU-210 > CP55940 > WIN55,212-2 = Δ9-THC > methanandamide = 2-AG = anandamide. This is in general agreement with the rank order of potency reported in previous studies as HU-210 > CP55940 > Δ9-THC > WIN55,212-2 > anandamide (Bonhaus et al., 1998; Song and Bonner, 1996), and the EC_{50} values in the present study were similar to the reported values for these ligands in CHO-CB_1 cells (Bonhaus et al., 1998).

Interestingly, although CP55940 and HU-210 had similar potencies in activation of pERK1/2, HU-210 was approximately 10 times more potent than CP55940 in inhibition of cAMP formation, providing evidence for ligand-biased signalling at CB_1 receptors. Reversal in efficacy of CP55940 and HU-210 has also been demonstrated in previous studies measuring activation of pERK1/2 vs. JNK and in inhibition of gene transcription by CRE vs. AP-1 (Bosier et al., 2008a; Bosier et al., 2008b). If *in vitro* effects such as these are translated into *in vivo* effects, cannabinoid ligands that display different therapeutic or adverse effects *in vivo* may do so because they activate selective signalling pathways.

This study sought to quantify, for the first time, ligand-biased signalling at CB_1 receptors using an operational model of agonism (van der Westhuizen et al., 2014). It should be noted that some of the previously reported differential signalling by different ligands may not be true ligand bias. For instance, WIN55,212-2 and HU-210 produce similar efficacies for both Gi and Go, while CP55,940 and anandamide preferentially activate Gi proteins (Bonhaus et al., 1998). However, this could also be the result of strength of coupling as CB_1 receptors preferentially couple to Gi rather than Gs (Felder et al., 1998). HU-210 is a full agonist at both Gi and Go while WIN55,212-2 and anandamide are full agonists at Gi but partial agonists at Go. On the other hand, Δ9-THC partially activates both Gi and Go (Glass and Northup, 1999). However, again the effects of strength of coupling cannot be ruled out.

Quantifying bias will be highly important in the development of novel CB_1 receptor therapies to establish correlations between ligand structure, signalling bias profile and therapeutic activity. However, ligand-biased signalling has not been quantified at CB_1 receptors to date. Our quantitative analysis showed that

the exogenous cannabinoids in particular displayed biased agonism at CB_1 receptors. Whereas WIN55,212-2 displayed little bias towards cAMP inhibition or pERK1/2 activation, HU-210, CP55940 and Δ9-THC favoured inhibition of cAMP formation over activation of pERK1/2. In particular, HU-210 was over 20 times more biased towards inhibition of cAMP over activation of pERK1/2. This raises the possibility that cannabinoid agonists may produce different *in vivo* effects. In agreement with this, it has been demonstrated that whereas WIN55,212-2 is 10 times more potent than Δ9-THC in producing hypoactivity in mice, Δ9-THC is approximately 10 times more potent than WIN55,212-2 in producing hypothermia (Abood and Martin, 1992).

Ligand-biased signalling has particular significance where multiple endogenous ligands bind to the same receptor. CB_1 receptors bind multiple endogenous agonists, including anandamide and 2-AG. Although the functional significance of various endocannabinoids is not fully understood, they may mediate distinct or even opposing physiological roles via differential signalling through the same receptor (Basavarajappa et al., 2014; Pan et al., 2011). For instance, elevated anandamide levels reduce CB_1 receptor-mediated inhibition of long term potentiation (LTP) and impair learning and memory (Basavarajappa et al., 2014). In contrast, elevated levels of 2-AG enhance LTP, learning and memory (Pan et al., 2011). It has also been demonstrated that elevated 2-AG concentrations produce analgesia, hypomobility and hypothermia, whereas elevated anandamide concentrations only induce analgesia in mice (Long et al., 2009). Therefore, a striking finding of this study is that 2-AG and anandamide display distinct ligand-biased signalling profiles at CB_1 receptors. Whereas 2-AG shows little preference for inhibition of cAMP formation and activation of pERK1/2,

anandamide is approximately 7 times more biased towards cAMP inhibition than pERK1/2 activation. The bias profile of methanandamide, the more stable analogue of anandamide, was similar to the bias profile of anandamide, and was over 15 times more biased towards inhibition of cAMP. This rules out the possibility that metabolites produced from anandamide breakdown are responsible for the differential effects of anandamide.

Ligand-biased signalling may have real therapeutic application. For instance, the dopamine D2 receptor partial agonist, aripiprazole, does not stimulate receptor internalisation and is biased towards inhibition of cAMP formation over activation of MAPK phosphorylation. Aripiprazole therefore displays advantages in the treatment of schizophrenia by modulating dopamine activity without completely blocking D2 receptors unlike other anti-psychotic agents (Grady et al., 2003; Urban et al., 2007). Carvedilol, unlike most other β-adrenoceptor blockers, shows beneficial effects in congestive heart failure possibly because it activates β-arrestin-mediated ERK signalling while blocking Gs protein-mediated cAMP activation (Wisler et al., 2007). Opioid agonists such as herkinorin, which do not activate β-arrestin-mediated internalisation of the receptor, could offer better chronic analgesic effects with reduced adverse effects such as respiratory depression and constipation (Raehal et al., 2005; Varga et al., 2004). Likewise, potential application of ligand-biased signalling in the treatment of several other diseases such as Parkinson's disease, addiction, depression, hypertension, dyslipidemia, osteoporosis, small-cell lung cancer and HIV infection has also been proposed (reviewed in Kenakin, 2012a; Kenakin and Miller, 2010).

In the present study, ligand-biased signalling at CB_1 receptors was detected and quantified, providing a potential means to selectively promote CB_1 receptor

signalling pathways towards desired therapeutic effects. This may be relevant for treating a number of conditions, including pain (Iversen and Chapman, 2002), obesity (Horvath, 2003), nicotine addiction (Le Foll and Goldberg, 2005) and Parkinson's disease (Segovia et al., 2003).

However, in many instances, it is not yet known which signalling profiles may offer therapeutic advantages (Kenakin, 2012a). It is also possible that both the desired and unwanted effects may be mediated through the same CB_1 receptor signalling pathway in different cells or tissues (Mukhopadhyay et al., 2002). Furthermore, the translation of *in vitro* bias profiles into *in vivo* effects is difficult to predict for a number of reasons, including different expression levels of the receptor and signalling elements in different cells/tissues (Kenakin and Christopoulos, 2013). For instance, a recent study highlighted differences in bias factors for β2-adrenoceptor agonists in cells over-expressing or endogenously expressing the receptor (van der Westhuizen et al., 2014). Therefore, bias determined in CHO cells in the present study may not necessarily be the same as bias in physiologically relevant systems. Hence, assays that can detect and quantify selective signalling pathways need to be followed by *in vivo* studies to determine whether the phenotypic signalling *in vitro* translates to unique therapeutic phenotypes *in vivo* (Kenakin, 2012a; Kenakin and Christopoulos, 2013). Nonetheless, the approach used in the present study may help in the classification/selection of cannabinoids based on their signalling profiles, which may direct structure-activity studies and the drug discovery process towards the development of selective therapeutics with reduced adverse effects targeting the CB_1 receptor.

Chapter 3

Validation and Quantification of Allosteric Modulation at CB_1 Receptors

3.1. Introduction

In the previous chapter, ligand-biased signalling was detected and quantified at CB_1 receptors. In this chapter, allosteric modulation, which is an alternative approach to gaining greater selectively in targeting CB_1 receptors, is presented.

Although attempts have been made to develop selective ligands for CB_1 or CB_2 receptors, the issue of receptor subtype selectivity has not been adequately addressed (Ross, 2007a). Even when cannabinoid compounds selectively act on either of the cannabinoid receptor subtypes *in vitro*, they may activate the other subtype at doses used *in vivo*. For instance, studies using CB_1 knockout mice demonstrated that the CB_1 receptor was responsible for the antispastic effects of so called "selective" CB_2 receptor agonists (Pryce and Baker, 2007).

Targeting potential CB_1 receptor allosteric sites may provide a higher degree of selectivity as allosteric sites are often less conserved across receptor subtypes. Furthermore, even when allosteric modulators do not bind exclusively to one subtype, they may display specificity in action through selective modulation of agonist function at one receptor subtype but not the other (Leach et al., 2007; Ross, 2007a). For example, an allosteric ligand may act as an enhancer on some receptor subtypes, but may display no effect or inhibitory effects on other subtypes (Jensen and Spalding, 2004). Another advantage of allosteric ligands is that their effects are saturable and therefore, unlike orthosteric ligands, they have a reduced propensity to cause on-target toxicity even at high doses (May et al., 2007).

Allosteric ligands may also display biased signalling through stabilisation of specific receptor conformations, which may result in activation of distinct signalling pathways (Leach et al., 2007). Therefore, biased allosteric effects offer the

opportunity to reduce the on-target adverse effects of CB_1 receptor activation through differential modulation of signalling pathways that are associated with therapeutic or adverse effects of cannabinoids.

The nature and magnitude of allosteric effect may also vary depending on the orthosteric probe studied (Leach et al., 2007; Valant et al., 2012). Therefore, probe-dependence has important implications for the detection, validation and subsequent classification of allosteric ligands.

Furthermore, CB_1 receptor allosteric modulators that have no agonist activity on their own may have the potential to retain the spatial and temporal aspects of receptor function, thus producing a more physiological regulation of receptor activity. Therefore, as they act only when the receptor is bound to endogenous cannabinoids, these modulators may avoid the psychoactive effects that are characteristic of direct CB_1 receptor agonism (Cravatt and Lichtman, 2004; Piomelli et al., 2006; Price et al., 2005; Ross, 2007a).

In recent years, there has been remarkable progress in the discovery and development of allosteric modulators at multiple GPCRs for clinical application. The first GPCR allosteric modulators that found their way to the market were cinacalcet and maraviroc. Cinacalcet is an allosteric enhancer of the calcium sensing receptor, which is used in hyperparathyroidism (Harrington and Fotsch, 2007). Maraviroc is an allosteric inhibitor of the chemokine receptor CCR5, which is used in the treatment of HIV infection, by reducing the affinity of the HIV virus to bind to the chemokine CCR5 receptor and to enter the cells (Dorr et al., 2005). Allosteric modulators of mGlu receptors and muscarinic acetylcholine receptors have been proposed to be useful in the treatment of CNS disorders such as

anxiety, schizophrenia, Alzheimer's disease and Parkinson's disease (reviewed in Conn et al., 2009; Conn et al., 2014).

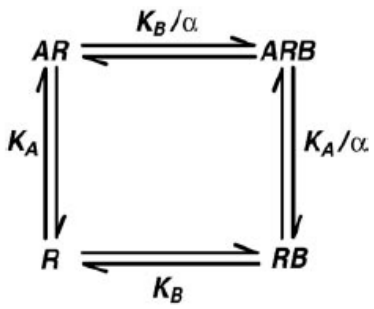
Similarly, allosteric modulation of CB_1 receptors may offer therapeutic advantages in the treatment of diseases where the orthosteric cannabinoid ligands fail to be useful. In 2005, the first CB_1 receptor allosteric modulators were identified, with Org27569 being the most potent among them. In addition to small molecule allosteric modulators, the endogenous ligands pregnenolone (Vallee et al., 2014) and lipoxin A4 (Pamplona et al., 2012) were suggested to act allosterically at CB_1 receptors.

Org27569 and a structurally related compound, PSNCBAM-1, (Fig. 1.4) displayed an intriguing profile by reducing inverse agonist binding and enhancing agonist binding while inhibiting agonist signalling in [^{35}S]GTPγS binding assays (Horswill et al., 2007; Price et al., 2005). The allosteric activity of Org27569 and PSNCBAM-1 is dependent on the orthosteric probe used; they fully inhibit CP55940 or anandamide efficacy in cAMP inhibition and [^{35}S]GTPγS binding assays (Baillie et al., 2013; Horswill et al., 2007; Price et al., 2005; Wang et al., 2011), but only partially inhibit the WIN55,212-2-induced responses (Baillie et al., 2013; Wang et al., 2011).

It has also been demonstrated that the allosteric activity of Org27569 is dependent on the signalling pathway studied, such that it displays biased allosteric modulation at CB_1 receptors, as previously shown by inhibition of CP55940-stimulated [^{35}S]GTPγS binding, JNK phosphorylation and cAMP inhibition but potentiation of CP55940-stimulated pERK1/2 (Ahn et al., 2012; Baillie et al., 2013; Cawston et al., 2013).

Pregnenolone inhibited Δ9-THC-induced activation of pERK1/2, whereas it had no effect on inhibition of cAMP formation by Δ9-THC, and did not modify equilibrium binding of [^{3}H]CP55940 and [^{3}H]WIN55,212-2 in CHO-hCB_1 cells (Vallee et al., 2014). Lipoxin A4 enhanced [^{3}H]CP55940 and to a lesser extent [^{3}H]WIN55,212-2 binding, and shifted the [^{3}H]SR141716A displacement curve by anandamide to the left, whereas it partially inhibited [^{3}H]SR141716A binding. It also enhanced anandamide-mediated inhibition of cAMP in HEK-CB_1 cells and activation of inwardly rectifying K^+ channels in *Xenopus laevis* oocytes injected with mouse CB_1 receptor. However, an interaction between lipoxin A4 and anandamide was not observed in [^{35}S]GTPγS binding assays (Pamplona et al., 2012).

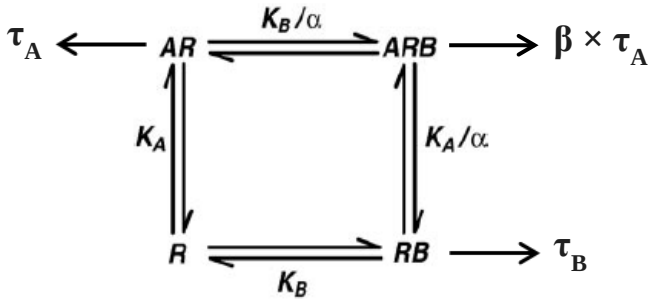
To predict the effects of drugs when tested in animal models, it is beneficial to differentiate allosteric from orthosteric modes of action. Quantification of allosteric effects can be used to select drugs for screening assays and *in vivo* studies. It also can direct structure-activity relationship studies that aim to develop compounds with improved receptor subtype selectivity and high binding affinity to the allosteric site, which can selectively modulate orthosteric agonist affinity and efficacy to only evoke the desired effects of CB_1 receptor activation (Ross, 2007a). The simplest model to quantify allosteric interactions is the allosteric ternary complex model (ATCM) (Ehlert, 1988).



Orthosteric and allosteric ligands bind to their respective binding sites on the receptor. R denotes the receptor; A and B denote the orthosteric and allosteric ligands, respectively; K_A and K_B denote their respective equilibrium dissociation constants. The cooperativity factor, α, describes the magnitude of the allosteric change in ligand affinity when both sites are occupied. Because of conformational linkage, the allosteric interaction between the orthosteric and allosteric sites is reciprocal.

The ATCM model can be applied to quantify allosteric effects on the binding affinity of each ligand, however, it does not account for modulator effects on the signalling properties of the receptor. Therefore the application of the ATCM is inappropriate when allosteric modulators alter orthosteric ligand efficacy (Ehlert, 1988; May et al., 2007).

An alternative operational model of allosterism can be used to quantify allosteric effects on both the affinity and efficacy of the orthosteric ligand (Leach et al., 2007).



Orthosteric ligand efficacy is defined by τ_A. Allosteric modulation of orthosteric ligand efficacy is defined by a cooperativity factor, β. τ_B represents the operational efficacy of the allosteric agonist. In this model, allosteric modulation is governed by both affinity modulation (α) and efficacy modulation (β), permitting allosteric modulators to have divergence in their effects on ligand binding affinity

and efficacy. For example Org27569 is a positive modulator of orthosteric agonist binding but a negative modulator of agonist efficacy (Price et al., 2005).

Research into the allosteric modulation of CB_1 receptors is relatively new and to date, has not been thoroughly quantified. Furthermore, previous observations of the modulatory effects of Org27569 were mainly obtained using CP55940 or WIN55,212-2 as the agonist probes, rather than endogenous cannabinoids. Therefore, the present study aimed to fully quantify the allosteric effects of Org27569 on binding and signalling properties of endogenous and exogenous cannabinoids, using the models mentioned above. Given the important implications of biased allosteric effects and probe-dependent effects of allosteric modulators, cAMP and pERK1/2 assays were performed to validate biased allosteric effects, and several cannabinoid ligands were tested to detect the probe-dependent effect of Org27569. It was hypothesised that Org27569 would display probe- and signalling pathway-selective modulation at CB_1 receptors.

Furthermore, an in-depth characterisation of the activity of pregnenolone and lipoxin A4 at CB_1 receptors has not been undertaken. Therefore, the binding of these ligands to CB_1 receptors and their functional interactions with cannabinoid agonists were investigated. Pregnenolone and lipoxin A4 were hypothesised to modulate the binding or activity of cannabinoid ligands through allosteric interactions.

Given the therapeutic advantages of allosteric modulators over orthosteric ligands, detection and quantification of allosteric interactions at CB_1 receptors is important in order to produce clinically useful compounds.

3.2. Materials and Methods

3.2.1. Materials

5(S), 6(R)-Lipoxin A4 was obtained from Tocris Bioscience. Org27569 and pregnenolone (3β-Hydroxy-5-pregnen-20-one) were purchased from Sigma Aldrich. Details for purchasing all other materials used in this chapter were the same as detailed in Chapter 2.

3.2.2. Ligand preparation

Ligands were prepared as previously described in Chapter 2, with the addition of Org27569 that was dissolved in 100% DMSO for a stock solution of 10 mM. Stock solutions of 10 mM and 250 μM were prepared in ethanol for pregnenolone and lipoxin A4 respectively.

3.2.3. Cell line

Flp-In CHO-hCB_1 cells were generated as described in Chapter 2, and maintained in DMEM containing 10% FBS, 16 mM HEPES and 700 μg/ml of hygromycin B.

3.2.4. Whole cell radioligand binding assays

The general protocol for the preparation of cells, termination of assays and detection of radioligand binding is described in Chapter 2.

3.2.4.1. Allosteric modulator titration assays

Allosteric modulator titration assays were performed by incubating cells with 1 nM [^{3}H]SR141716A and increasing concentrations of Org27569, pregnenolone or lipoxin A4, for 6 hours, based on the equilibrium time determined in association kinetic assays (Chapter 2).

3.2.4.2. Binding interaction studies

For interaction studies between the unlabelled competitor and allosteric modulators, cells were incubated with 1 nM [^{3}H]SR141716A and increasing concentrations of orthosteric ligands in the presence or absence of various concentrations of Org27569 for 6 hours.

3.2.5. AlphaScreen cAMP assays

The general protocol is described in Chapter 2.

3.2.5.1. cAMP interaction studies

For cAMP interaction studies with the allosteric modulators, cells were pre-incubated with varying concentrations of Org27569, pregnenolone or lipoxin A4 for 10-15 minutes, before the addition of different concentrations of orthosteric ligands.

3.2.6. pERK1/2 assays

The general protocol is described in Chapter 2.

3.2.6.1. pERK1/2 time courses

Initial time course experiments for allosteric modulators were performed, as described in chapter 2 for orthosteric ligands, by incubating the cells with 10 μM final concentration of each allosteric modulator for different time intervals to determine the time that the maximum signal was produced for subsequent concentration-response studies. The effects of allosteric modulators on the time course of cannabinoid agonists were investigated by incubating 10 μM final concentration of each agonist in the presence of 10 μM final concentration of each modulator.

3.2.6.2. pERK1/2 interaction studies

pERK1/2 interaction studies with the allosteric modulators were performed by pre-incubating cells with varying concentrations of Org27569, pregnenolone or lipoxin A4 for 10-15 minutes, before the addition of increasing concentrations of orthosteric ligands for the time determined in the time course experiments (Chapter 2).

3.2.7. Data analysis

Data were analysed using Prism 6 (GraphPad, San Diego, CA). Data from [^{3}H]SR141716A displacement by Org27569 were fitted to an allosteric ternary complex model (Price et al., 2005) (Eq. 3.1),

$$Y = \frac{[\mathrm{A}]}{[\mathrm{A}] + \left[\frac{K_a(1+[\mathrm{B}]/K_b)}{1+\alpha[\mathrm{B}]/K_b}\right]} \qquad \text{Eq. 3.1}$$

where Y is the fractional specific binding, K_a and K_b denote the equilibrium dissociation constants of the radioligand and the allosteric ligand, respectively, [A],

and [B] are their respective concentrations, α is the cooperativity factor between the allosteric ligand and radioligand. An α value >1 describes positive cooperativity (allosteric enhancement of radioligand binding), while an α value < 1 (but >0) describes negative cooperativity (allosteric inhibition of binding) and an α = 1 describes neutral cooperativity, i.e., no net effect on binding affinity at equilibrium (Ehlert, 1988).

Binding interaction experiments between cannabinoid agonists, Org27569 and [^{3}H]SR141716A were fitted to the following allosteric ternary complex model (Leach et al., 2010) (Eq. 3.2),

$$Y = \frac{B_{max}[A]}{[A] + [\frac{K_a K_b}{\alpha[B] + K_b}]\,[1 + \frac{[I]}{K_i} + \frac{[B]}{K_b} + \frac{\alpha'[I][B]}{K_i K_b}]} \qquad \text{Eq. 3.2}$$

where Y, K_a, K_b, [A] and [B] are as defined above, K_i denotes the equilibrium dissociation constant of unlabelled orthosteric ligand, and [I] is its concentration, B_{max} is the total number of receptors, and α and α' are the cooperativity factors between the allosteric ligand and radioligand or unlabelled ligand, respectively.

To define allosteric effects on intrinsic efficacy of orthosteric ligands (β), data from functional interaction experiments were fitted to the following operational model of allosterism (Leach et al., 2007) (Eq. 3.3),

$$\text{Effect} = \frac{E_m(\tau_A[A](K_b + \alpha\beta[B]) + \tau_B[B]K_a)^n}{(K_b[A] + K_a K_b + K_a[B] + \alpha[A][B])^n + (\tau_A[A](K_b + \alpha\beta[B]) + \tau_B[B]K_a)^n} \qquad \text{Eq. 3.3}$$

where τ_A and τ_B denote the efficacy of orthosteric and allosteric ligands respectively, α and β denote allosteric effects on binding affinity and efficacy of orthosteric ligands, respectively. K_a and K_b are the equilibrium dissociation constants of orthosteric and allosteric ligands, respectively; and [A] and [B] denote

their respective concentrations. Em is the maximal possible system response and n is the slope factor of the transducer function.

Competition binding experiments between pregnenolone or lipoxin A4 and the radiolabelled SR141716A were fitted to a one-site binding equation (Motulsky and Christopoulos, 2004) (Eq. 3.4),

$$Y = \frac{(\text{Top - Bottom})}{1 + (10^{[I]-\log IC_{50}})} + \text{Bottom} \quad \text{Eq. 3.4}$$

where Y represents the percentage of specific binding; Top and Bottom denote the maximal and minimal asymptotes of the curve, respectively; [I] is the concentration of inhibitor; and IC_{50} is the concentration of competitor that produces half the maximal response.

Functional interaction studies with pregnenolone or lipoxin A4 were fitted to a three parameter concentration response equation (Eq. 3.5),

$$Y = \frac{(\text{Top - Bottom})}{1 + (10^{\log EC_{50}-A})} + \text{Bottom} \quad \text{Eq. 3.5}$$

where Top and Bottom denote the maximal and minimal asymptotes of the curve, respectively, A is the concentration of agonist, and log EC_{50} is the concentration of agonist that produces half the maximal response.

3.3. Results

3.3.1. Org276529 displays probe-dependent allosteric modulation at CB_1 receptors

Previous studies have shown that Org27569 increases the binding of the CB_1 receptor agonist, [^{3}H]CP55940, has no effect on [^{3}H]WIN,55212 binding and displays negative binding cooperativity with the CB_1 receptor inverse agonist [^{3}H]SR141716 (Baillie et al., 2013; Price et al., 2005). Thus, we first validated the effects of Org27569 on [^{3}H]SR141716A binding and then on displacement of [^{3}H]SR141716A by cannabinoid agonists.

In agreement with previous findings (Price et al., 2005), our results showed that Org27569 completely displaced [^{3}H]SR141716A, indicating high negative allosteric cooperativity between these two ligands (Fig. 3.1). Thus, a pK_b value of 5.81±0.08 for Org27569 was determined by fitting the displacement data to an allosteric ternary complex model (Eq. 3.1). An α value less than 1 (α ~ 0), indicated negative allosteric modulation of [^{3}H]SR141716A binding. Due to the very high negative cooperativity estimated (Logα > -10), this model then effectively becomes a competitive binding model.

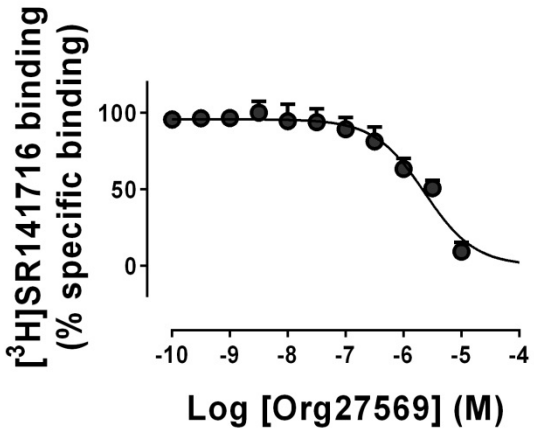


Fig. 3.1. [^{3}H]SR141716A displacement by the CB_1 receptor allosteric modulator Org27569. CHO-hCB_1 cells were incubated with 1 nM [^{3}H]SR141716A and increasing concentrations of Org27569 for 6 hours at 4 ºC. Curves were generated by fitting the data to an allosteric ternary complex model (Eq. 3.1). Data points represent mean values + S.E.M. from four experiments carried out in triplicate.

Binding interaction studies were next performed to determine the effects of Org27569 on the binding of cannabinoid agonists, by measuring agonist displacement of [^{3}H]SR141716A in the absence and presence of various Org27569 concentrations. The data were fitted to an allosteric ternary complex model (E.q. 3.2) to quantify the allosteric binding cooperativity between Org27569 and the cannabinoid agonists (Fig. 3.2. and Table 3.1).

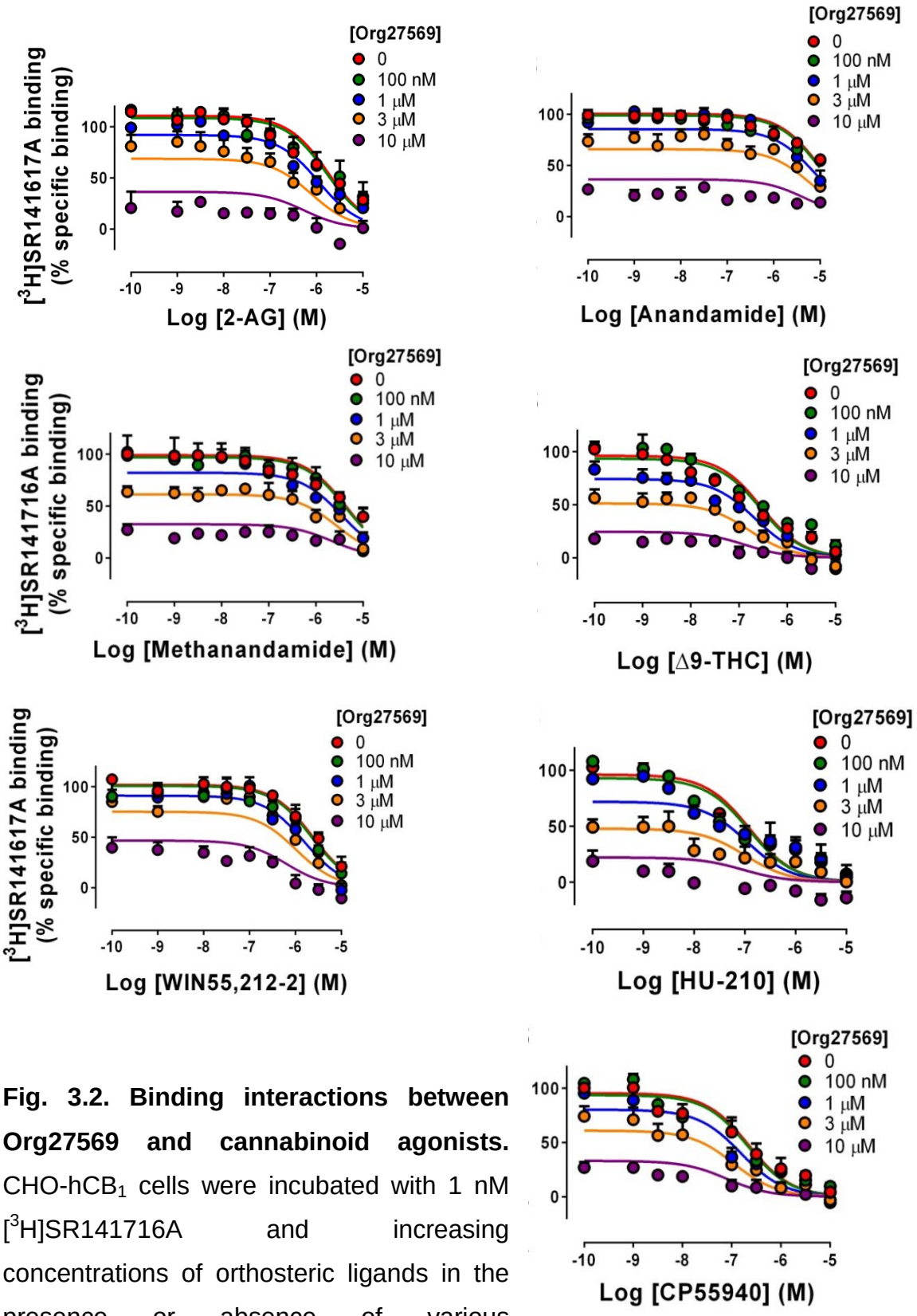


Fig. 3.2. Binding interactions between Org27569 and cannabinoid agonists. CHO-hCB_1 cells were incubated with 1 nM [^{3}H]SR141716A and increasing concentrations of orthosteric ligands in the presence or absence of various concentrations of Org27569 for 6 hours at 4 ºC. Curves were generated by fitting the data to an allosteric ternary complex model (Eq. 3.2). Data points represent mean values + S.E.M. from at least three experiments carried out in triplicate.

Table 3.1. Binding parameters for the allosteric interaction between Org27569 and cannabinoid agonists determined in binding interaction experiments.

Data were fitted to an allosteric ternary complex model (Eq. 3.2) to determine the cooperativity between Org27569 and cannabinoid agonists (Logα'). The binding cooperativity between [^{3}H]SR141716A and Org27569 (Logα) was highly negative (>-10). The negative logarithm of the radioligand dissociation constant was fixed to that determined from homologous competition binding experiments (pK_a 8.5). Values represent the mean ± S.E.M. from at least three experiments performed in triplicate.

Ligand	**Logα'[a] [α']**	**pK_b[b]**	**pK_i[c]**
CP55940	0.5±1.0 [3.2]	5.4±0.1	6.8±0.6
HU-210	0.0±0.7 [1.0]	5.6±0.1	7.0±0.3
WIN55,212-2	0.6±0.5 [4.0]	5.2±0.1	5.8±0.2
Δ9-THC	0.3±0.2 [2.0]	5.6±0.1	6.7±0.1
Methanandamide	0.3±0.6 [2.0]	5.4±0.1	5.5±0.2
Anandamide	0.3±0.4 [2.0]	5.4±0.0	5.2±0.1
2-AG	0.5±0.2 [3.2]	5.4±0.1	5.9±0.6

[a] Logarithm of the binding cooperativity factor between Org27569 and cannabinoid agonists
[b] Negative logarithm of the allosteric modulator dissociation constant
[c] Negative logarithm of the competing orthosteric ligand dissociation constant

Our quantitative analysis demonstrated that Org27569 had little effect on the binding of CP55940, indicated by only a small (3 fold) enhancement in CP55940-mediated displacement of [^{3}H]SR141716A. A similar observation was made for WIN55,212-2, HU-210, Δ9-THC, methanandamide, anandamide and 2-AG (Fig. 3.2). The analysis had difficulty determining binding cooperativity between Org27569 and cannabinoid ligands as seen by the large error, probably because it

was close to neutral. This can be shown by no significant change in the pIC_{50} values (Table 3. 2).

Table 3.2. Effects of various concentrations of Org27569 on pIC_{50} values of cannabinoid agonists in displacement of [^{3}H]SR141716A.
Data were analysed using a three parameter concentration response equation (Eq. 3.5). Values represent the mean ± S.E.M. from at least three experiments performed in triplicate.

Ligand	**Concentration of Org27569 (M)**				
	-5	**-5.5**	**-6**	**-7**	**0**
			pIC_{50}		
CP55940	-7.0±0.3	-7.1±0.3	-7.3±0.2	-7.0±0.1	-7.6±0.1
HU-210	-8.2±0.3	-7.8±0.4	-7.7±0.2	-7.7±0.2	-7.8±0.1
WIN55,212-2	-6.0±0.3	-5.8±0.2	-5.6±0.2	-5.6±0.3	-5.6±0.2
Δ9-THC	-6.5±0.3	-6.7±0.2	-6.9±0.2	-7.0±0.1	-7.0±0.1
Methanandamide	-5.7±0.4	-5.4±0.4	-6.0±0.3	-5.7±0.3	-6.0±0.2
Anandamide	-5.9±0.8	-5.3±0.4	-5.4±0.2	-5.8±0.3	-5.7±0.1
2-AG	-6.3±0.5	-6.9±0.3	-6.4±0.1	-6.4±0.3	-6.3±0.2

3.3.2. Determination of the optimum incubation time in functional studies

pERK1/2 time course experiments were performed to determine potential stimulation of pERK1/2 activation by Org27569 in its own right, and its effect on the time at which the maximum signal is produced by each cannabinoid agonist. The results showed that Org27569 by itself did not produce any response during the 30 minute incubation period, but it did abolish the peak pERK1/2 signal produced by CP55940, HU-210 and 2-AG. However, the time at which the maximum signal was produced by WIN55,212-2, Δ9-THC, methanandamide and anandamide did not alter in the presence of Org27569 (Fig. 3.3). Therefore, the time point determined in Chapter 2 for each agonist (in the absence of Org27569) was used in subsequent pERK1/2 interaction studies.

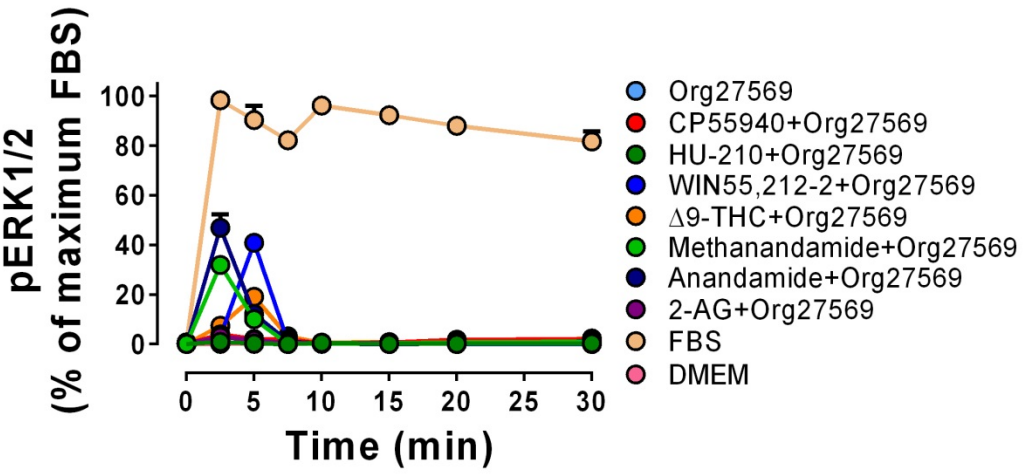


Fig. 3.3. pERK1/2 time course assays. CHO-hCB_1 cells were incubated with 10 µM final concentration of Org27569, or with 10 µM of each cannabinoid ligand in the presence of 10 µM final concentration of Org27569 at 37 ºC for different time intervals before termination of pERK1/2 stimulation. Data points represent mean values + S.E.M. from two experiments performed in triplicate.

Our initial pERK1/2 interaction studies demonstrated that when Org27569 and cannabinoid agonists, CP55940 or WIN55,212-2, were added together, weak or no modulatory effect by Org27569 was observed on pERK1/2 activation (Fig. 3.4.A). However, a 15 minute pre-incubation with Org27569 resulted in modulation of CB_1 receptor-mediated signalling (Fig. 3.4.B). Therefore, in all subsequent interaction experiments a 15 minute pre-incubation with Org27569 was performed prior to agonist addition.

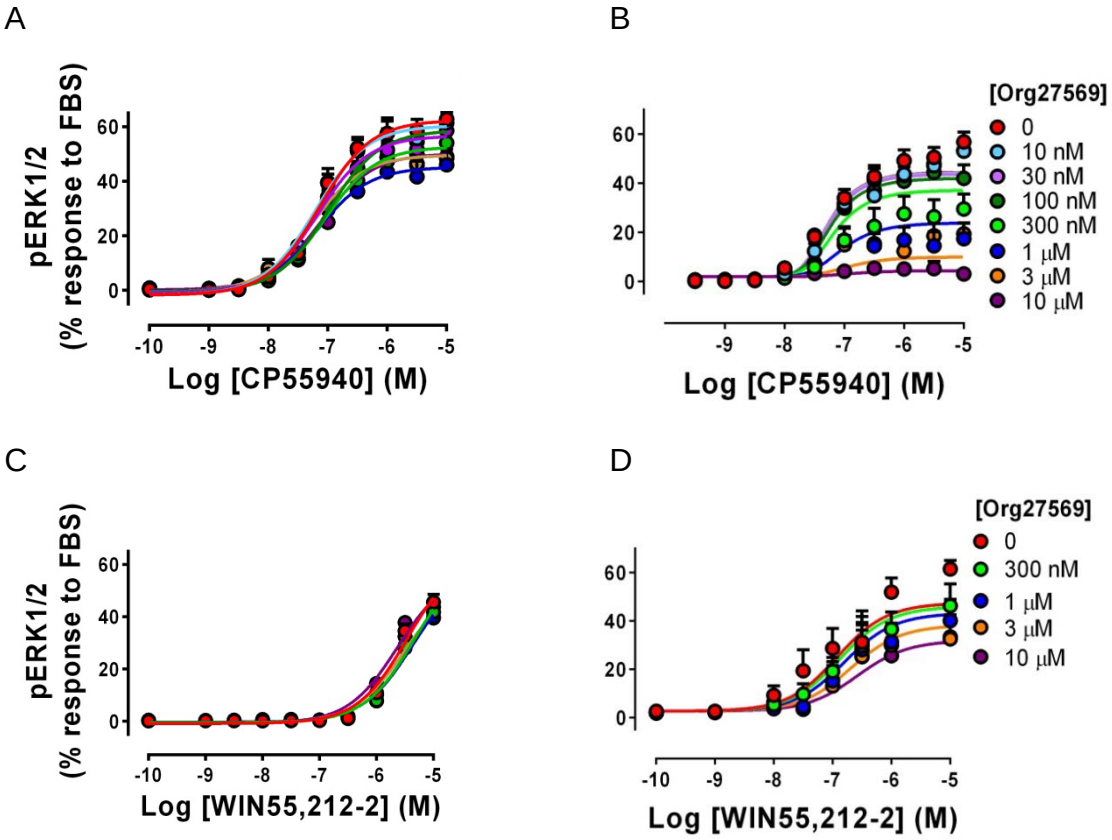


Fig. 3.4. Effects of incubation time with Org27569 on activation of pERK1/2 by cannabinoid agonists. Org27569 and CP55940 (A) or WIN55,212-2 (C) were co-added to CHO-hCB_1 cells. Org27569 was added to the cells 15 minutes prior to the addition of CP55940 (B) or WIN55,212-2 (D). Assays were terminated 5 minutes (the time determined in the time course experiments) after incubation of cells with CP55940 or WIN55,212-2. Data points represent mean values + S.E.M. from at least three experiments carried out in triplicate.

3.3.3. Org27569 displays probe- and signalling pathway-dependent allosteric modulation at CB_1 receptors

Functional interaction studies between Org27569 and various cannabinoid agonists were performed to detect and quantify the allosteric effects of Org27569 on CB_1 receptor-mediated signalling.

Org27569 completely abolished inhibition of cAMP formation by 2-AG, anandamide, methanandamide, Δ9-THC, WIN55,2212-2, HU-210 and almost completely abolished CP55940-mediated inhibition of cAMP (Fig. 3.5). Interestingly however, in pERK1/2 assays, Org27569 had no significant effects on activation of pERK1/2 by anandamide, methanandamide and Δ9-THC (Fig. 3.6). This indicates strong biased allosteric effects of Org27569. Our results also demonstrated striking probe-dependence by Org27569. Thus, while Org27569 did not modulate activation of pERK1/2 by anandamide, methanandamide and Δ9-THC, it partially inhibited 2-AG and WIN55,212-2-induced pERK1/2 activation and completely abolished pERK1/2 activation by HU-210 and CP55940 (Fig. 3.6).

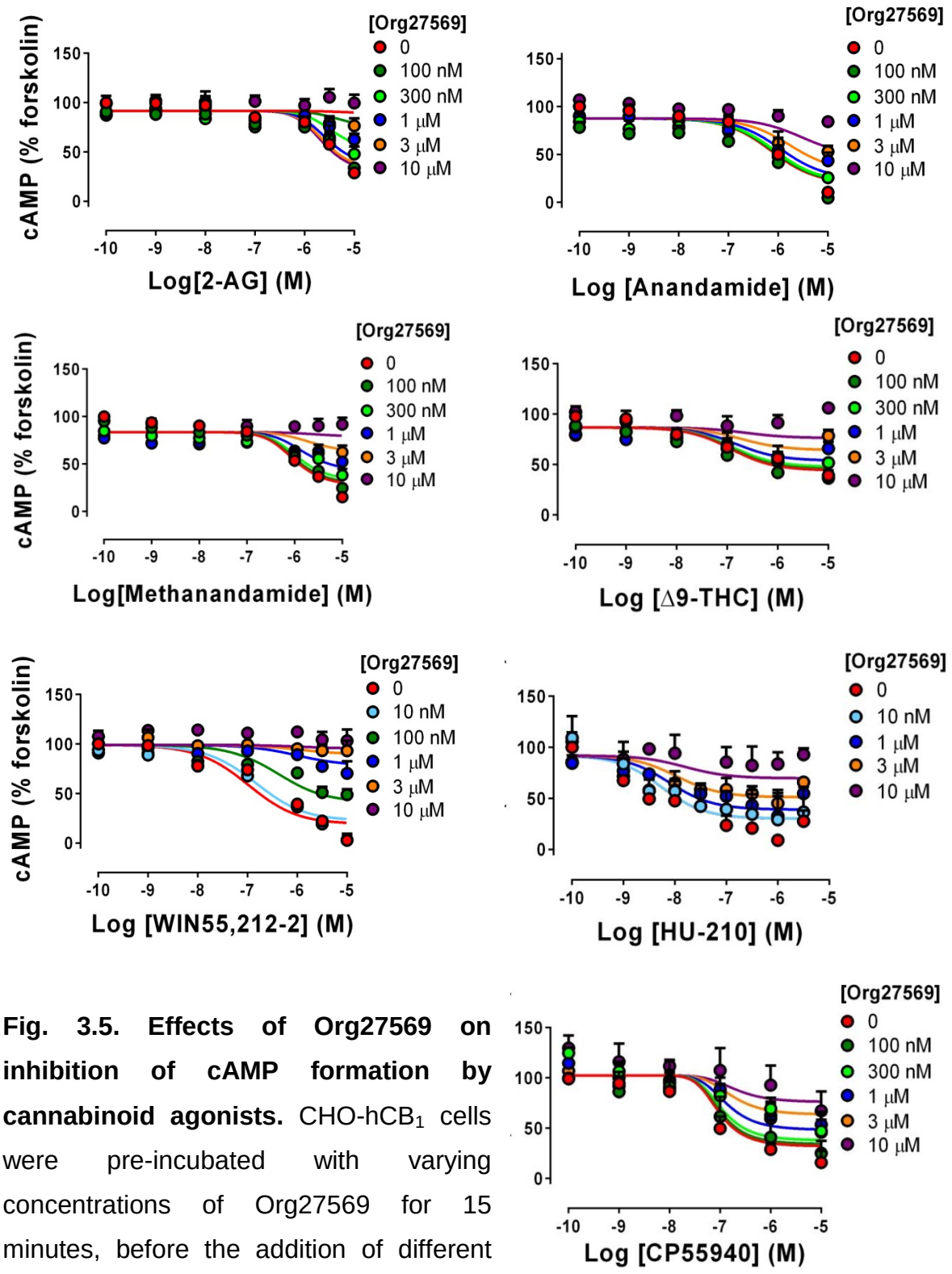


Fig. 3.5. Effects of Org27569 on inhibition of cAMP formation by cannabinoid agonists. CHO-hCB_1 cells were pre-incubated with varying concentrations of Org27569 for 15 minutes, before the addition of different concentrations of orthosteric ligands in the presence of 1 μM forskolin for 30 minutes at 37 ºC. Curves were generated by fitting the data to an operational model of allosterism (Eq. 3.3). Data points represent mean values + S.E.M. from at least three experiments carried out in triplicate.

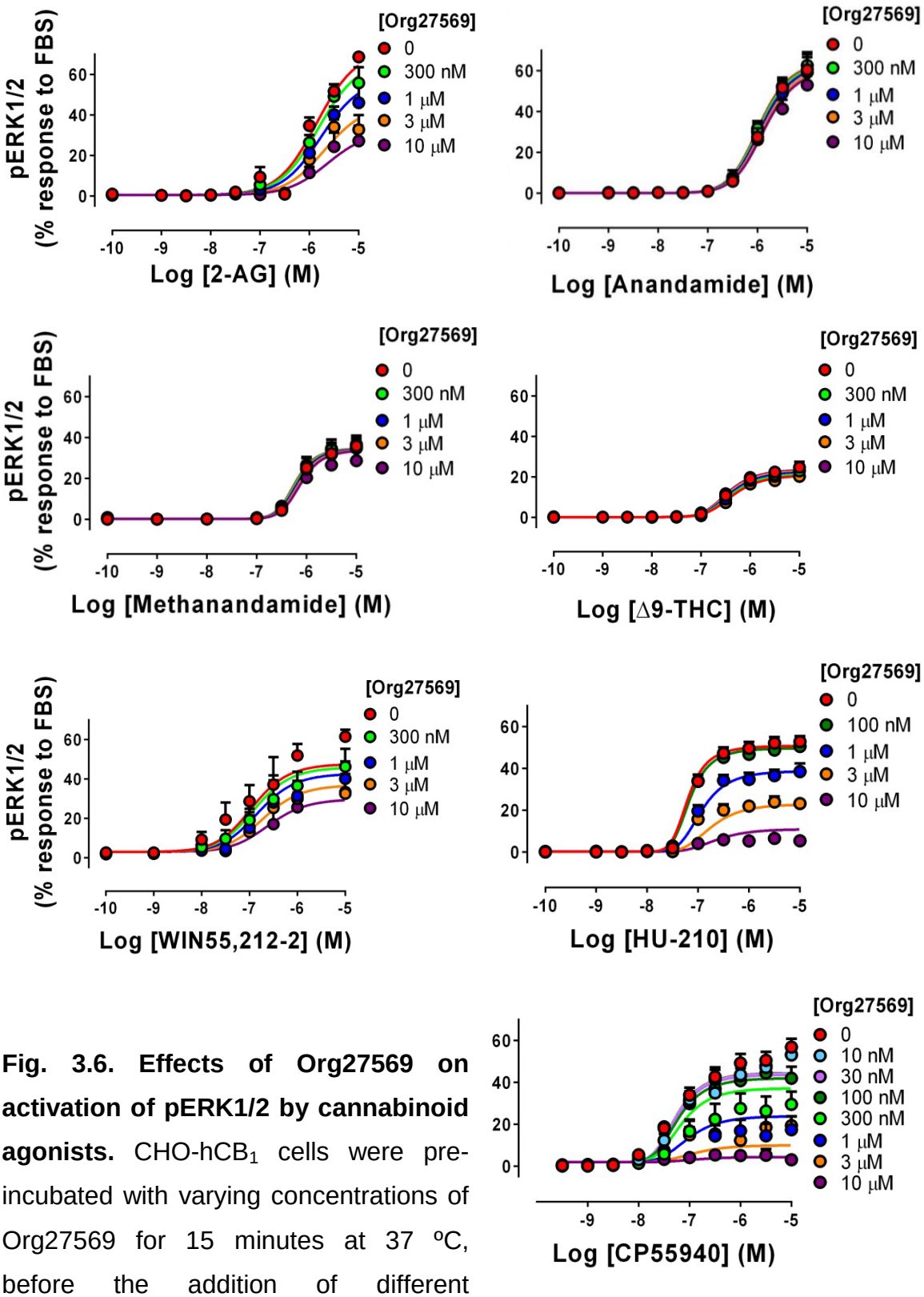


Fig. 3.6. Effects of Org27569 on activation of pERK1/2 by cannabinoid agonists. CHO-hCB_1 cells were pre-incubated with varying concentrations of Org27569 for 15 minutes at 37 ºC, before the addition of different concentrations of orthosteric ligands. Curves were generated by fitting the data to an operational model of allosterism (Eq. 3.3). Data points represent mean values + S.E.M. from at least three experiments carried out in triplicate.

The data from functional interaction studies were fitted to an operational model of allosterism (Eq. 3.3) to quantify the functional cooperativity between Org27569 and cannabinoid agonists (Table 3.3). The binding affinity of orthosteric ligands were fixed to the values determined in binding interaction assays (Table 3.1), the binding affinity of the modulator was fixed to the value obtained in competition binding assays (pK_b 5.8), which is close to its estimated pK_b values in binding interaction assays (Table 3.1), and α was fixed to 1 to reflect the neutral binding cooperativity. This enabled determination of the functional cooperativity (β) between Org27569 and the cannabinoids. In cAMP interaction studies, β between Org27569 and CP55940 was 0.11. For the interaction between Org27569 and all other tested cannabinoid agonists, β was close to 0. This indicates very strong negative functional cooperativity between Org27569 and cannabinoid agonists tested. In pERK1/2 interaction studies, β between Org27569 and cannabinoid agonists varied from close to 0 for CP55940 to close to 1 for Δ9-THC (Table 3.3). This indicates that depending on the cannabinoid agonist used, the effects of Org27569 on cannabinoid-mediated pERK1/2 activation vary from strong inhibition to no modulation. The strong negative functional cooperativity is demonstrated by the large effect of Org27569 on the maximum signalling capacity (E_{max}) of the cannabinoids, whereas the neutral cooperativity is shown by no change on the E_{max} (Fig. 3.5 and 3.6). The potency (EC_{50}) of all tested cannabinoid agonists was unchanged in cAMP and pERK1/2 assays, which is in agreement with the neutral binding cooperativity between Org27569 and cannabinoid agonists.

Taken together, our results clearly indicate strong probe-dependence and biased allosteric effects by Org27569, whereby it negatively modulated cAMP

inhibition by all cannabinoids tested, and some, but not all, cannabinoid-mediated pERK1/2 signalling.

Table 3.3. Operational model parameters (Eq. 3.3) for the functional interaction between Org27569 and cannabinoid agonists.

pK_a was fixed to values estimated in binding interaction assays and pK_b to 5.8 determined in allosteric titration assays. Logα was fixed to 0, and logτ_B to -1000. Values represent the mean ± S.E.M. from at least three experiments performed in triplicate.

Ligand	**Logβ^a [β]**		**Log$\tau_A{}^b$ [τ_A]**	
	pERK1/2	**cAMP**	**pERK1/2**	**cAMP**
CP55940	>-10 [~ 0]	-1.0±0.5 [0.1]	0.3±0.1 [2.0]	0.3±0.4 [2.0]
HU-210	-1.0±0.1 [0.1]	>-10 [~ 0]	0.3±0.0 [2.2]	0.7±0.8 [5.0]
WIN55,212-2	-0.6±0.2 [0.2]	>-10 [~ 0]	0.4± 0.3 [2.5]	0.3±0.1 [2.0]
Δ9-THC	-0.1±0.0 [0.9]	>-10 [~ 0]	0.1±0.1 [1.3]	0.1±0.8 [1.3]
Methanandamide	-0.1±0.1 [0.8]	>-10 [~ 0]	0.8±0.0 [6.3]	0.4±0.2 [2.5]
Anandamide	-0.1±0.1 [0.7]	>-10 [~ 0]	0.8±0.1 [6.6]	0.9±0.3 [7.9]
2-AG	-0.4±0.1 [0.4]	>-10 [~ 0]	0.0±0.2 [1.1]	0.2±0.2 [1.6]

[a] Logarithm of the activation cooperativity factor between Org27569 and cannabinoid agonists
[b] Logarithm of the functional efficacy of orthosteric ligands

3.3.4. Pregnenolone, but not lipoxin A, displays weak activity at CB_1 receptors

To verify the allosteric activity of pregnenolone at CB_1 receptors, we first investigated its effects on displacement of [^{3}H]SR141716A. Our results showed a concentration-dependent decrease in [^{3}H]SR141716A binding by pregnenolone (Fig. 3.7). However, as pregnenolone completely displaced [^{3}H]SR141716A, it cannot be distinguished whether pregnenolone acts as an allosteric inhibitor with very high negative cooperativity or acts as a competitive inhibitor. The data therefore were fitted to a one-site binding equation (Eq. 3.4).

pERK1/2 interaction studies were next performed between pregnenolone and Δ9-THC. Curves were generated by fitting the data to a three parameter concentration response equation (Eq. 3.5). In contrast to the previous study by Vallee and colleagues (Vallee et al., 2014), our results revealed a complete lack of modulation of Δ9-THC-induced signalling by pregnenolone (Fig. 3.7). As modulatory effects of allosteric ligands can be dependent on the agonist used to detect the effect, we also determined effects of pregnenolone on activation of pERK1/2 by WIN55,212-2. However, it also had no effect on the response to WIN55,212-2 (Fig. 3.7). The possibility that pregnenolone may change the time at which maximum pERK1/2 signal is produced by cannabinoid agonists was ruled out in our time course experiments (data not shown).

We next investigated the effects of lipoxin A4 on [^{3}H]SR141716A displacement and on the cAMP response to anandamide. In contrast to previous findings, lipoxin A4 did not alter the binding of [^{3}H]SR141716A (Fig. 3.7), and did

not modulate cAMP inhibition induced by anandamide. Similarly, there was no cAMP interaction between lipoxin A4 and CP55940 (Fig. 3.7).

Therefore, the previously reported allosteric effects of pregnenolone and lipoxin A4 were not verified in the present study.

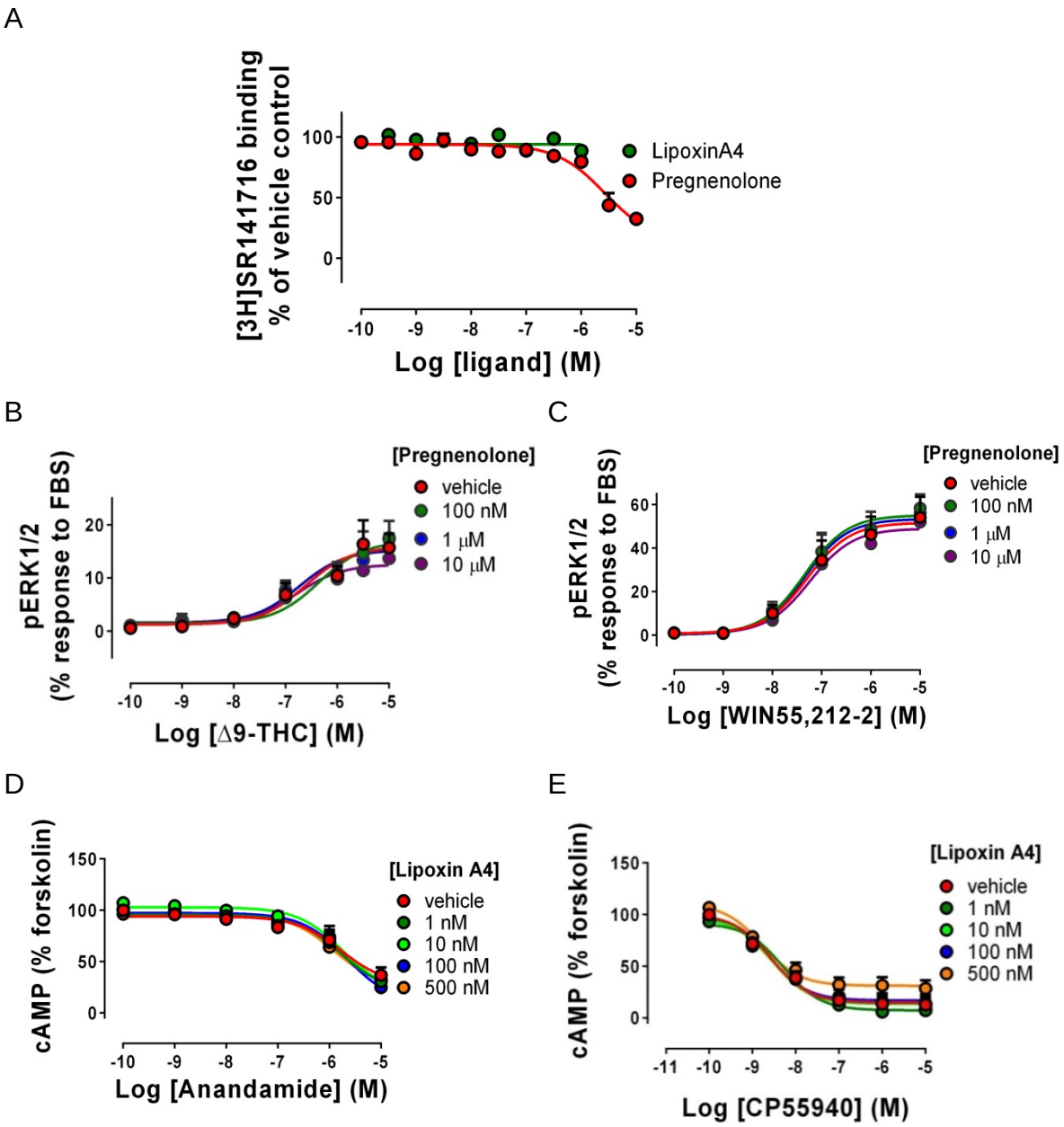


Fig. 3.7. Pregnenolone, but not lipoxin A4, binds to CB_1 receptors, but neither ligand modulates cannabinoid-mediated signalling. (A) [^{3}H]SR141716A displacement by CB_1 receptor allosteric modulators, pregnenolone and lipoxin A4. Interaction between pregnenolone and Δ9-THC (B) or WIN55,212-2 (C) in pERK1/2 assay, and interaction between lipoxin A4 and anandamide (D) or CP55940 (E) in cAMP assay in CHO-hCB_1 cells. Curves were generated by fitting the data to a three parameter concentration response equation (Eq. 3.4). Data points represent mean values + S.E.M. from four experiments carried out in triplicate.

3.4. Discussion

This study provides quantitative insight into biased allosterism and probe-dependence by the small molecule, Org27569, at CB_1 receptors, and demonstrates evidence against the previously reported allosteric effects of the endogenous ligands pregnenolone and lipoxin A4.

Quantification of allosteric effects may provide useful information for the development of better therapeutics. However, to date only a few studies have investigated allosteric interactions at CB_1 receptors, in which there are few quantitative findings reported. Therefore, the present study sought to validate and quantify allosteric modulation at these receptors.

We first performed radioligand binding experiments. Org27569 reduced the binding of [^{3}H]SR1417161A with a pK_b value of 5.81 and an α value close to 0, indicating strong negative binding cooperativity between Org27569 and [^{3}H]SR1417161A. This is in agreement with the previously reported pK_b and α values of 5.95 and 0.09, respectively (Price et al., 2005). However, the results of our binding interaction studies demonstrated little or no effect of Org27569 on [^{3}H]SR141716A displacement by CP55940, and other tested cannabinoid agonists. This may suggest that Org276529 differentially modulates binding of different cannabinoid ligands; thereby it affects inverse agonist binding but not agonist binding. The probe-dependent effect of Org27569 on cannabinoid ligand binding was previously demonstrated by its weak effect on the binding of [^{3}H]WIN55,212-2, while enhancing [^{3}H]CP55940 binding (Baillie et al., 2013).

The pK_b values estimated for Org27569 in our binding interaction studies were similar using different cannabinoid agonists, and were close to the estimated

pK_b value for Org27569 in [^{3}H]SR141716A displacement assays, confirming the validity of our model. Similarly, Price and colleagues reported a pK_b value for Org27569 in enhancing [^{3}H]CP55940 binding, close to that estimated from [^{3}H]SR141716A displacement assays (Price et al., 2005).

In contrast to the close to neutral cooperativity between Org27569 and CP55940 in the present study, Price et al. and Ahn et al. demonstrated positive cooperativity with α values of 14 and 7, respectively, for the displacement of [^{3}H]CP55940 by Org27569 (Ahn et al., 2012; Price et al., 2005). This may be explained by the different experimental approach used in our study. We studied [^{3}H]SR141716A displacement by cannabinoid agonists in the presence of Org27569, whereas in previous studies the direct effect of Org27569 on binding of tritiated agonists ([^{3}H]CP55940 or [^{3}H]WIN55,212-2) was investigated. Therefore, the presence of the inverse agonist in our study may change the equilibrium of high and low affinity binding sites, influencing the activity of Org27569. The difference may also be explained by the use of whole cells in the present study versus mouse brain membrane preparations used in previous studies (Baillie et al., 2013; Price et al., 2005). Different receptor populations may be present in whole cells versus membranes (e.g. different proportions of receptor-G protein complexes), reducing the positive cooperativity between Org27569 and cannabinoid agonists.

The probe-dependent effect of Org27569 extends to functional assays, demonstrated previously by its ability to modulate CP55940-induced activation of pERK1/2 and inhibition of cAMP formation, while having weak or no effect on responses to WIN55,212-2 (Baillie et al., 2013). We further investigated this by screening a wider range of cannabinoid ligands. Our results clearly show that

Org27569 displays probe-dependence by inhibiting pERK1/2 activation by HU-210 and CP55940, while only partially inhibiting 2-AG and WIN55,212-2 responses and having no significant effects on pERK1/2 activation by anandamide, methanandamide and Δ9-THC. The results of our quantitative analysis demonstrated β values ranging from close to 0 (indicating strong negative functional cooperativity) to close to 1 (indicating neutral functional cooperativity) between Org27569 and cannabinoid agonists in pERK1/2 interaction studies. This highlights the probe-dependent effect of Org27569.

Our findings that Org27569 either negatively modulates or has no effect on cannabinoid-induced pERK1/2 signalling, depending on the probe used, are in contrast to previous studies where it positively modulated this signalling pathway activated by CP55940 (Ahn et al., 2012; Baillie et al., 2013). This could be explained as the cell background and subsequent complement of intracellular signalling proteins may greatly influence the modulatory effects of Org27569 on CB_1 receptor-mediated signalling.

The present study confirmed that the allosteric activity of Org27569 is also dependent on the signalling pathway studied, such that it displays biased allosteric modulation at CB_1 receptors. Thus, whereas Org27569 had a partial inhibitory or no effect on pERK1/2 signalling induced by some of the cannabinoids tested, it completely abolished inhibition of cAMP formation by every agonist, highlighting the pathway-specific modulation of CB_1 receptor signalling. Therefore, our quantitative analysis provided β values close to 0 for the cAMP interaction between Org27569 and all cannabinoid agonists, except for CP55940, which was 0.11. This indicates strong negative modulation of cannabinoid-mediated inhibition of cAMP formation by Org27569. Previous studies reported biased allosteric

effects of Org27569 as it antagonised inhibition of cAMP formation, stimulation of [^{35}S]GTPγS binding and JNK phosphorylation by CP55490, while potentiating cannabinoid-induced activation of pERK1/2 (Ahn et al., 2012; Baillie et al., 2013).

This is the first study that provides a comprehensive quantitative analysis of the binding and functional parameters for the interaction between Org27569 and several cannabinoid agonists, including endocannabinoids. Taken together, our study demonstrates that Org27569 displays probe-dependence and biased allosterism.

We also evaluated the allosteric nature of the two previously reported endogenous allosteric ligands at CB_1 receptors, pregnenolone and lipoxin A4. Previous binding interaction studies demonstrated no effect of pregnenolone on equilibrium binding of [^{3}H]CP55940 and [^{3}H]WIN55,212-2 (Vallee et al., 2014). However, our results demonstrate a complete displacement of [^{3}H]SR141716A by pregnenolone, suggesting an interaction between these two ligands at CB_1 receptors, which may be through a competitive or an allosteric action. It also could be due to CB_1 receptor-independent mechanisms, such as cell membrane disruption by lipophilic pregnenolone. Similarly, highly lipophilic cannabinoid ligands display high levels of non-specific binding in radioligand binding experiments (Ashton et al., 2008). In contrast to previous findings (Vallee et al., 2014), we saw no inhibitory effect of pregnenolone on Δ9-THC-induced activation of pERK1/2. It also had no effect on the response to WIN55,212-2.

Also, in contrast to previous results, where lipoxin A4 partially inhibited [^{3}H]SR141716A binding and enhanced [^{3}H]SR141716A displacement by anandamide (Pamplona et al., 2012), it had no effect in our binding studies. Furthermore, our results demonstrated no effect on the cAMP response to

anandamide or CP55940 by lipoxin A4, in contrast to the previously reported enhancement of anandamide-mediated cAMP inhibition (Pamplona et al., 2012).

However, it may be necessary to test a wider range of cannabinoid ligands to ascertain that the potential allosteric effects of pregnenolone and lipoxin A4 are not masked by a probe-dependent nature. Nonetheless, the previously reported allosteric effects of pregnenolone and lipoxin A4 were not verified in the present study.

It should be noted that it might be difficult to predict the activity of allosteric modulators *in vivo,* in particular where the target receptor, such as the CB_1 receptor, interacts with multiple endogenous ligands, resulting in more than one set of pK_b, α and β values. Even for a single combination of orthosteric ligand and allosteric modulator, different pharmacological effects may be produced in different cells or tissues (Kenakin, 2012b; Leach et al., 2007). Nonetheless, the quantitative insight into allosteric modulation of CB_1 receptors provided in the present study may help in differentiation of allosteric and orthosteric actions of ligands, and guide structure-activity relationships and drug discovery studies towards developing successful therapeutics targeting CB_1 receptors.

Chapter 4

Modulation of CB_1 Receptor-Mediated Signalling Pathways by CRIP1a

4.1. Introduction

In addition to Org27569, pregnenolone and lipoxin A4, which were previously discussed in Chapter 3, an endogenous protein named cannabinoid receptor interacting protein 1a (CRIP1a) was reported to modulate CB_1 receptor function through an interaction with the receptor C-terminal tail (Niehaus et al., 2007). CRIP1a was shown to co-immunoprecipitate with the CB_1 receptor in membranes from rat brain, and co-localise at the plasma membrane in superior cervical ganglion (SCG) neurons (Niehaus et al., 2007). CRIP1a does not interact with CB_2 receptors (Niehaus et al., 2007).

Similar to the pattern of CB_1 receptor expression, CRIP1a is highly expressed in the brain; however it is also found in other tissues such as heart, lung, intestine, kidney, testis, spleen, liver and muscle. Endogenous expression of CRIP1a was also detected in multiple cell lines, such as rat cerebellar granule neurons, SCG neurons, N18TG2 neuroblastoma and pituitary adenoma cell line (AtT-20 cells) (Niehaus et al., 2007). Previous studies have demonstrated that modulation of CRIP1a expression in SCG neurons and N18TG2 cells does not alter CB_1 receptor expression (Blume et al., 2015; Niehaus et al., 2007).

CB_1 receptors are expressed on both excitatory glutamatergic and inhibitory GABAergic neurons (Katona and Freund, 2008), and activation of CB_1 receptors inhibits the release of both neurotransmitters in the brain. Interestingly, CRIP1a is selectively expressed on glutamatergic neurons but not GABAergic neurons (Ludanyi et al., 2008). Therefore, the most profound effect of CRIP1a is anticipated to be on CB_1 receptor-mediated glutamatergic neurotransmission. Thereby, selective modulation of CB_1 receptors by CRIP1a may retain the beneficial effects

of cannabinoids mediated by glutamatergic signalling, such as neuroprotection and reduced excitotoxicity, without causing the adverse effects associated with activation of GABAergic neurons such as memory impairment (Ludanyi et al., 2008; Puighermanal et al., 2009).

However, evidence for a role of CRIP1a in the brain is conflicting. For instance, a reduction in mRNA levels of CRIP1a and CB_1 receptors was detected in glutamatergic axon terminals in hippocampal tissues surgically removed from human epileptic patients, suggesting a neuroprotective role for the endocannabinoid system, which is diminished in epileptic hippocampi (Ludanyi et al., 2008). However, in hippocampal samples of epileptic rats, higher expression levels of the CB_1 receptor and CRIP1a compared to non-epileptic rats were reported. The authors suggested that increased expression of CB_1 receptors may be an adaptive neuroprotective mechanism (Bojnik et al., 2012). CRIP1a has been reported to prevent cannabinoid agonist-mediated neuroprotection, while inducing cannabinoid antagonist-mediated neuroprotection in primary neuronal cortical cultures over-expressing CRIP1a. In this instance, WIN55,212-2 did not protect neurons from glutamate-induced cell death, whereas SR141716 attenuated glutamate-induced cell death (Stauffer et al., 2011). Therefore, modulation of CB_1 receptors in specific tissues or neurons in a ligand-dependent manner by CRIP1a may provide an approach to develop more selective CB_1 receptor-targeted therapeutics in particular for diseases associated with excessive excitatory transmission, such as epilepsy (Ludanyi et al., 2008).

The mechanisms of CRIP1a function at CB_1 receptors have not been well investigated. The modulatory effect of CRIP1a on CB_1 receptor-mediated signal transduction pathways has been demonstrated by attenuation of SR141716

enhancement of Ca^{2+} currents (indicating attenuation of CB_1 receptor-mediated constitutive inhibition of Ca^{2+} channels), in SCG neurons over-expressing CRIP1a (Niehaus et al., 2007). This suggests that CRIP1a may act as an endogenous allosteric modulator at CB_1 receptors. The binding affinity and maximum binding of [^{3}H]SR141716A in membranes from HEK-hCB_1 cells were unaffected by the presence of CRIP1a (Niehaus et al., 2007), suggesting that it has specific effects on receptor signalling events without effects on ligand binding. Furthermore, neither the time course of inhibition nor recovery from inhibition of Ca^{2+} currents by WIN55212-2 was significantly altered in the presence of CRIP1a (Niehaus et al., 2007), suggesting CRIP1a does not alter agonist function. CRIP1a may selectively block CB_1 receptor coupling to $G\alpha_{i-3}$ that was reported to enhance the constitutive inhibition of Ca^{2+} channels, but not to $G\alpha_{oA}$ responsible for agonist-mediated inhibition of Ca^{2+} channels (Anavi-Goffer et al., 2007; Niehaus et al., 2007). CRIP1a over-expression in the striatum or in N18TG2 cells was reported to reduce pERK1/2 levels without altering phosphorylated cAMP response element-binding protein (pCREB) and forskolin-stimulated cAMP levels (Blume et al., 2013; Blume et al., 2015). Activation of pERK1/2 by WIN55212-2, CP55940 or methanandamide was unaltered in CRIP1a over-expressing cells. However, the response to CP55940 was enhanced in CRIP1a knockdown cells. The inhibition of forskolin-stimulated cAMP formation by CP55940, and to a lesser extent by WIN55212-2, was also enhanced in CRIP1a knockdown cells but was unchanged in over-expressing cells (Blume et al., 2015). However, CRIP1a over-expression abolished SR141716A-induced reduction of basal levels of pERK1/2 (Blume et al., 2015). The authors suggested that CRIP1a may act as a negative modulator of CB_1 receptor function in a ligand-specific manner (Blume et al., 2015). The effects

of CRIP1a on CB_1 receptor signalling are somewhat variable depending on the agonist and cell line used. For instance, a recent study demonstrated a reduction in the E_{max} of 2-AG, WIN55,212-2, HU-210 and CP55940 in [^{35}S]GTPγS binding assays when CRIP1a was over-expressed in HEK cells (Smith et al., 2015). However, CRIP1a over-expression did not alter methanandamide- or Δ9-THC-mediated [^{35}S]GTPγS binding (Smith et al., 2015). CRIP1a over-expression in mouse neuroblastoma N18TG2 cells did, however, reduce methanandamide-stimulated [^{35}S]GTPγS binding, whilst CRIP1a knockdown in these cells increased agonist-mediated G protein activity (Smith et al., 2015).

To date, there are only a few studies investigating the modulation of CB_1 receptor signalling pathways by CRIP1a, and the results are inconclusive. Therefore, the present study sought to further investigate CRIP1a's mechanism of action at CB_1 receptors. cAMP inhibition and pERK1/2 activation studies were performed in CB_1 receptor and CRIP1a over-expressing HEK293 cells. We hypothesised that CRIP1a may modulate CB_1 receptor inverse agonist, but not agonist-mediated signalling pathways. The effects of CRIP1a in cAMP and Ca^{2+} mobilisation assays were further investigated in a more physiologically relevant cell line (neuroblastoma × glioma, NG108-15 cells), which endogenously express CB_1 receptors and CRIP1a.

4.2. Materials and Methods

4.2.1. Materials

Poly-D-Lysine and tetracycline were purchased from Sigma Aldrich. Lipofectamine RNAiMAX, Opti-MEM reduced serum medium, blasticidin and HAT supplement were purchased from Invitrogen. EDTA-free protease inhibitor cocktail tablets were obtained from Roche Applied Science, and Laemmli buffer from Bio-Rad. Ambion CNRIP1 Silencer Select Pre-designed siRNA and mismatch siRNA were obtained from Applied Biosystems. Rabbit anti-CNRIP1 antibody was obtained from Abcam, and mouse anti-β-actin antibody from Santa Cruz Biotechnology. Donkey anti-rabbit IRDye 680 nm® and donkey anti-mouse IRDye 800 nm® were purchased from LI-COR Biosciences. Fura 2-AM was purchased from Merk Millipore, and puromycin from Integrated Sciences (Australia). All other materials used in this chapter were from the same suppliers as detailed in Chapter 2.

4.2.2. Ligand preparation

Ligands were prepared as previously described in Chapter 2.

4.2.3. Cell line

4.2.3.1. HEK-CB_1-TREx CRIP1a cells

HEK293 cells with stable expression of the CB_1 receptor and tetracycline-regulated expression of CRIP1a (HEK-CB_1-TREx CRIP1a), provided by Dr. Stewart Fabb and Ms. Nilushi Karunaratne (Monash Institute of Pharmaceutical Sciences), were maintained in DMEM containing 10% FBS, 16 mM HEPES,

3 µg/ml puromycin (for selective expression of CB_1 receptors), 200 µg/ml of hygromycin B (for selective expression of CRIP1a) and 15 µg/ml of blasticidin (tetracycline repressor).

4.2.3.2. Neuroblastoma × glioma hybrid cells

Neuroblastoma × glioma hybrid cells (NG108-15) were cultured in DMEM supplemented with 10% FBS and 1% HAT (10 mM hypoxanthine, 0.1 mM aminopterin, 1.6 mM thymidine), and incubated at 37 ºC in a humidified atmosphere of 5% CO_2, 95% O_2.

4.2.4. Induction of CRIP1a expression in HEK-CB_1 cells

HEK-CB_1-TREx CRIP1a cells were incubated with 1 µg/ml tetracycline for 18 hours to induce CRIP1a expression.

4.2.5. CRIP1a knockdown in NG108-15 cells

NG108-15 cells in poly-D-lysine coated 6-well plates or T-75 flasks at approximately 50% confluence were transfected with siRNA targeting CRIP1a (20 nM) or negative control mismatch siRNA (mmsiRNA) using Lipofectamine RNAiMAX (6:1 ratio of siRNA to RNAiMAX) in serum free medium. siRNAs and Lipofectamine RNAiMAX were separately diluted in Opti-MEM reduced serum medium, and after 20 minutes incubation at room temperature, were mixed together and further incubated for 30 minutes, after which they were gently added to the cells. Five hours after transfection, the medium was replaced with complete

growth medium and cells were maintained for 24 or 48 hours before being used for subsequent experiments.

4.2.6. Western blotting to detect CRIP1a over-expression or knockdown

Cells in poly-D-lysine coated 6-well plates were lysed by Radio Immuno Precipitation Assay (RIPA) lysis buffer, containing 150 mM NaCl, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS (sodium dodecyl sulphate), 50 mM Tris and freshly added protease inhibitors, and centrifuged at 12,000 rpm at 4 ºC for 20 minutes. The supernatant was collected and the pellet discarded. Protein concentration was quantified using the BCA or Bradford protein assay according to the manufacturer's instructions (Bio-Rad, Hercules, CA). Equal amounts of protein and Laemmli buffer, containing 4% SDS, 10% 2-mercaptoehtanol, 20% glycerol, 0.004% bromophenol blue and 0.125 M Tris HCl, were mixed and heated at 95 ºC for 10 minutes. Samples were loaded into 10% SDS-polyacrylamide gels and electrophoresis was performed at 150 V for 60 minutes. Electrophoretic transfer to nitrocellulose membranes was then carried out using a Transblot semi-dry electrophoretic transfer cell (Bio-Rad, Hercules, CA) at 10 V for 60 minutes. Membranes were blocked in 5% skim milk and incubated overnight with rabbit anti-CRIP1a antibody (1:1,000) against the full length of human CRIP1a, and mouse anti-β-actin (loading control) primary antibody (1:300) with constant shaking at 4 ºC. The following day, membranes were washed with TBST buffer (a mixture of Tris-Buffered Saline and Tween 20) three times for 5 minutes, and incubated with fluorescent-conjugated donkey anti-rabbit IRDye 680 nm®

(1:10,000) and donkey anti-mouse IRDye 800 nm® (1:30,000) secondary antibodies in TBST for 60 minutes at room temperature. After three 10 minute washes, the membrane blots were scanned by a densitometer (Odyssey model 9120, Li-COR Biosciences, Lincon, NE). To quantify band density, Photoshop software was used. Background was subtracted from CRIP1a and β-actin band density. Data were normalised to β-actin.

4.2.7. AlphaScreen® cAMP assays

HEK-CB_1-TREx CRIP1a cells were seeded at 100,000 cells/well into 96-well clear bottom culture plates and grown overnight in complete medium (as described earlier under 4.2.3. cell line) for un-induced cells, or complete medium containing 1 µg/ml tetracycline (for inducing CRIP1a expression). Untreated, mmsiRNA-treated or CRIP1a siRNA-treated NG108-15 cells were seeded at 20,000 cells/well into poly-D-lysine pre-coated 96-well clear bottom culture plates and grown overnight in complete medium. The general protocol for cAMP assays is described in Chapter 2.

4.2.7.1. Agonist concentration-response experiments

Agonist concentration-response studies were performed as described in Chapter 2, with or without induction of CRIP1a.

4.2.7.2. cAMP interaction studies

For cAMP interaction studies, cells in the presence or absence of CRIP1a were pre-incubated with varying concentrations of Org27569 for 15 minutes,

before the addition of different concentrations of orthosteric ligands. cAMP assays were subsequently performed as described in Chapter 2.

4.2.8. pERK1/2 assays

To investigate effects of CRIP1a on CB_1 receptor-mediated pERK1/2 activation, cells were seeded as mentioned above (under AlphaScreen cAMP assays). The general protocol for pERK1/2 assays is described in Chapter 2.

4.2.8.1. Agonist concentration-response experiments

Agonist concentration-response studies were performed as described in Chapter 2, with or without induction of CRIP1a.

4.2.8.2. pERK1/2 interaction studies

For pERK1/2 interaction studies, cells in the presence or absence of CRIP1a were pre-incubated with varying concentrations of Org27569 for 15 minutes, before the addition of different concentrations of orthosteric ligands. pERK1/2 assays were subsequently performed as described in Chapter 2.

4.2.9. Ca^{2+} mobilisation assays

4.2.9.1. Single cell Ca^{2+} imaging

NG108-15 cells were grown on coverslips pre-coated with poly-D-lysine. On the day of recording, media was removed and cells were washed with HEPES-based buffer (HEPES 10 mM; NaCl 145 mM; KCl 5 mM; $MgSO_4.6H_2O$ 1 mM; $CaCl_2$ 2.5 mM; glucose 10 mM; 0.5% BSA; pH 7.4), and incubated with 5 µM of

the Ca^{2+}-sensitive dye, fura-2 acetoxymethyl ester (fura-2 AM), for 30 minutes at 37 ºC in the dark. Cells were then washed three times with the dye free buffer, and incubated at 37 ºC for 30 minutes to allow for de-esterification of the dye. Live video images of individual neurons were acquired every 1-3 seconds using a Nikon inverted microscope. The dye was excited by 340 and 380 nm light and emitted light was collected at 510 nm. Changes in intracellular Ca^{2+} concentration were indicated by changes in the ratio of the fluorescence signals (340/380 nm) obtained using Metafluor software. Background fluorescence at 340 and 380 nm was acquired and subtracted from each series of recordings. Changes in Ca^{2+} levels were measured in 20-50 cells within a microscopic field of view. Each well of 24-well plates was used for only one experiment and each experiment included multiple stimuli.

4.2.9.2. High throughput Ca^{2+} mobilisation assays

NG108-15 cells were seeded at 20,000 cells/well into poly-D-lysine pre-coated 96-well clear bottom culture plates and grown overnight in DMEM/F12 containing 10% FBS and 1% HAT. The following day, media was removed and cells were washed with HEPES-based buffer containing 0.5% BSA, and incubated with 1 µM fura-2 AM for 1 hour at 37 ºC. Cells were then washed three times with dye free buffer, and incubated at 37 ºC for 30 minutes. Fluorescence signals (340/380 nm) were recorded for 6 minutes per well every 2 seconds using a Flexstation microplate reader (Molecular Devices, California).

4.2.10. Data analysis

Data were analysed using Prism 6 (GraphPad, San Diego, CA). Data from agonist concentration-response and functional interaction studies with Org27569, in the presence or absence of CRIP1a, were fitted to a three parameter concentration response equation (Eq. 4.1).

$$Y = \frac{(\text{Top - Bottom})}{1 + (10^{\log EC_{50} - A})} + \text{Bottom} \qquad \text{Eq. 4.1}$$

where Top and Bottom denote the maximal and minimal asymptotes of the curve, respectively, A is the concentration of agonist, and $\log EC_{50}$ is the agonist concentration that produces half the maximal response.

4.2.11. Statistics

Values are expressed as means ± S.E.M. Mean values were compared using one-way ANOVA with Bonferroni's multiple comparison test. A P value <0.05 was considered significant.

4.3. Results

HEK-CB_1-TREx CRIP1a cells were used to investigate the effects of CRIP1a over-expression on CB_1 receptor-mediated signalling pathways.

4.3.1. Validation of CRIP1a over-expression in HEK293 cells

Western blotting was performed on HEK-CB_1-TREx CRIP1a cells to confirm the induction of CRIP1a expression after treatment of cells with tetracycline for 18 hours. In Western blots of samples prepared from tetracycline-induced cells, a band of the expected molecular mass (18 KD) for CRIP1a was observed. However, there was no band labelled using non-induced cell membrane preparations (Fig. 4.1).

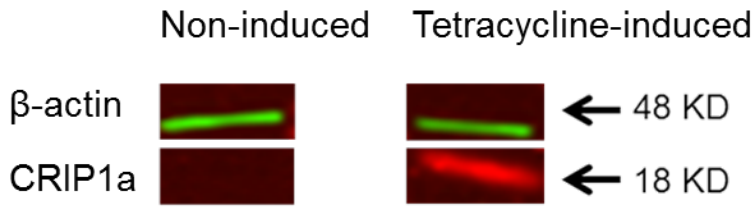


Fig. 4.1. Western blot of non-induced and tetracycline-induced HEK-CB_1-TREx CRIP1a cells. The incubation of cells with 1 µg/ml tetracycline for 18 hours induced CRIP1a expression, whereas no CRIP1a was detected in non-induced cells. β-actin was used as the loading control.

4.3.2. CRIP1a does not modulate cannabinoid-mediated signal transduction in HEK293 cells

4.3.2.1. Cannabinoid agonist-mediated cAMP inhibition and pERK1/2 activation

In a recent study, it was demonstrated that CRIP1a over-expression in N18TG2 cells (which endogenously express CB_1 receptors and CRIP1a) reduced basal pERK1/2 levels, whereas it did not alter forskolin-stimulated cAMP formation (Blume et al., 2015). Therefore, effects of CRIP1a over-expression in the absence of cannabinoid agonists on cAMP and pERK1/2 signalling were first investigated.

To induce expression of CRIP1a, HEK-CB_1-TREx CRIP1a cells were incubated with 1 µg/ml tetracycline for 18 hours. Our results showed that the basal levels of cAMP and pERK1/2 were not significantly different in tetracycline-induced HEK-CB_1-TREx CRIP1a cells compared to non-induced cells. CRIP1a also did not alter forskolin-stimulated intracellular levels of cAMP and FBS-stimulated pERK1/2 levels (Fig. 4.2).

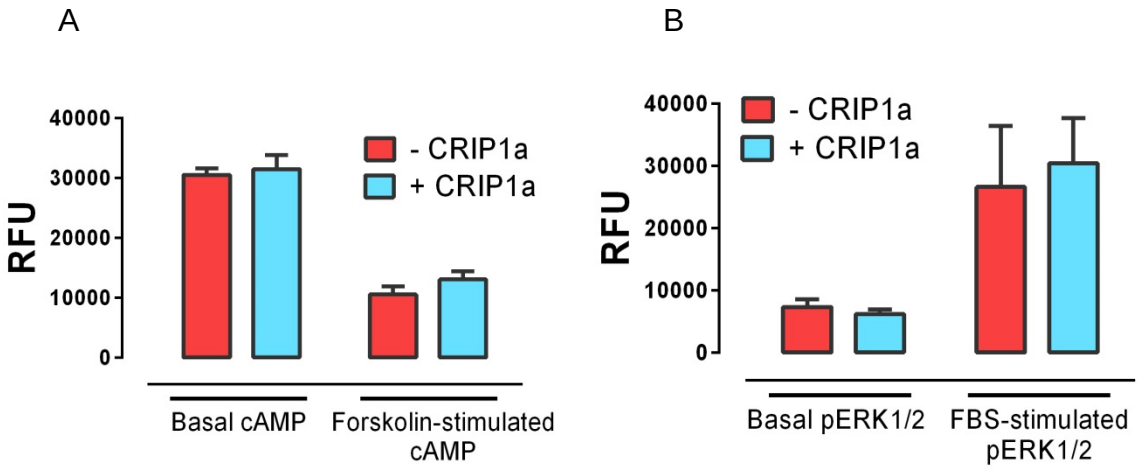


Fig. 4.2. CRIP1a over-expression does not alter cAMP and pERK1/2 levels in the absence of cannabinoid ligands. Non-induced or tetracycline-induced HEK-CB_1-TREx CRIP1a cells were incubated (A) with DMEM or 1 µM forskolin to determine the basal or forskolin-stimulated levels of cAMP, or (B) with DMEM or 10% FBS to determine the basal or FBS-stimulated pERK1/2 levels, in the absence and presence of CRIP1a. RFU: relative fluorescence units. In cAMP assays, the higher levels of cAMP will result in lower RFU. Data represents mean + S.E.M. of at least four experiments performed in triplicate.

Next, the effects of CRIP1a on agonist-mediated CB_1 receptor signalling pathways were studied. As shown in Fig. 4.3, cannabinoid agonists inhibited cAMP formation and activated pERK1/2 in a dose-dependent manner in non-induced HEK-CB_1-TREx CRIP1a cells. These effects were unaffected in the presence of CRIP1a in tetracycline-induced cells.

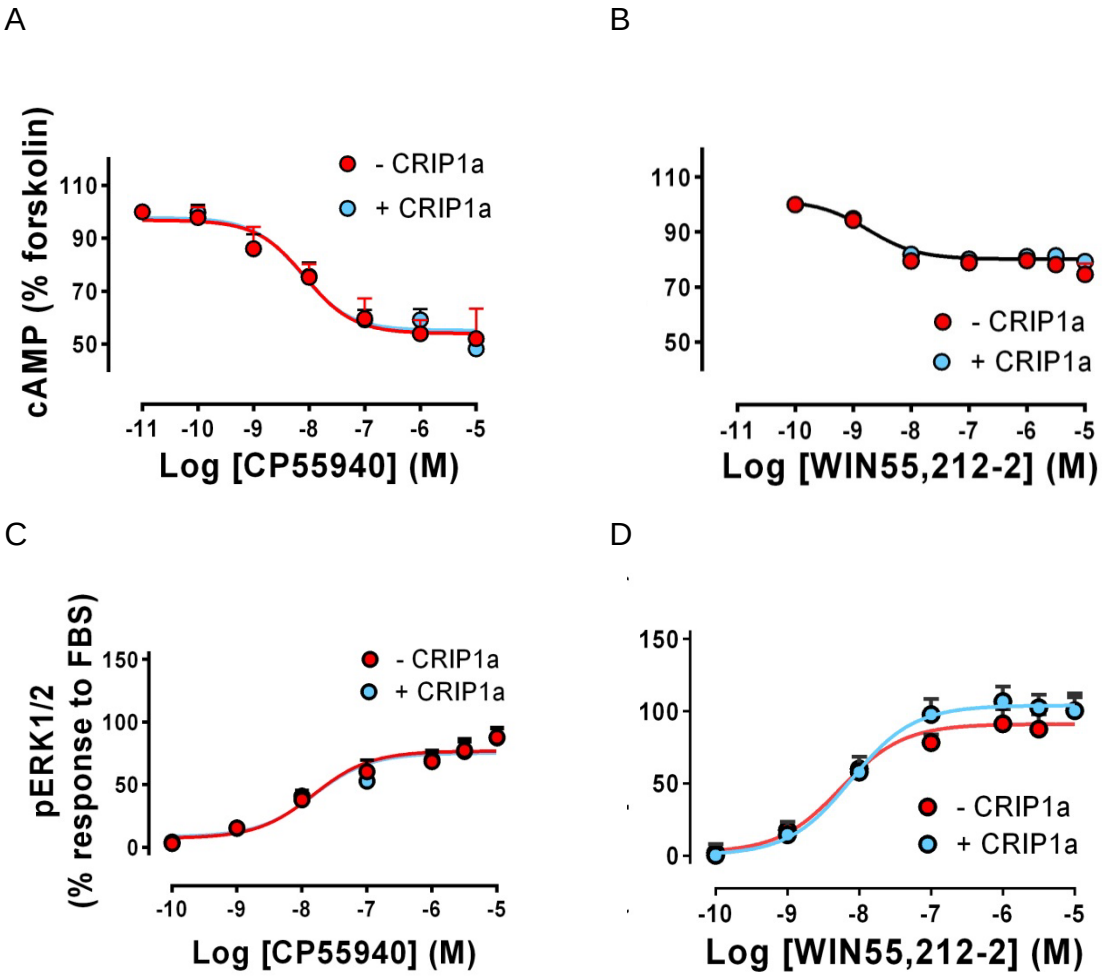


Fig. 4.3. CRIP1a expression does not modulate CB_1 receptor-mediated signalling in HEK-CB_1-TREx CRIP1a cells. (A) CP55940- and (B) WIN55,212-2-induced cAMP inhibition, (C) CP55940- and (D) WIN55,212-2-induced pERK1/2 activation, in the absence and presence of CRIP1a. Data represents mean + S.E.M. of at least three experiments performed in triplicate.

4.3.2.2. Modulation of CB_1 inverse agonist-mediated cAMP accumulation

It was previously shown that CRIP1a over-expression reduced the enhancement of Ca^{2+} currents by the CB_1 receptor inverse agonist, SR141716, in SCG neurons (Niehaus et al., 2007). Therefore, we hypothesised that it may also reduce SR141716-induced cAMP accumulation. Our results showed that in non-induced HEK-CB_1-TREx CRIP1a cells, SR141716 increased basal levels of cAMP. However, the response to SR141716 did not change in the presence of CRIP1a in tetracycline-induced cells (Fig. 4.4).

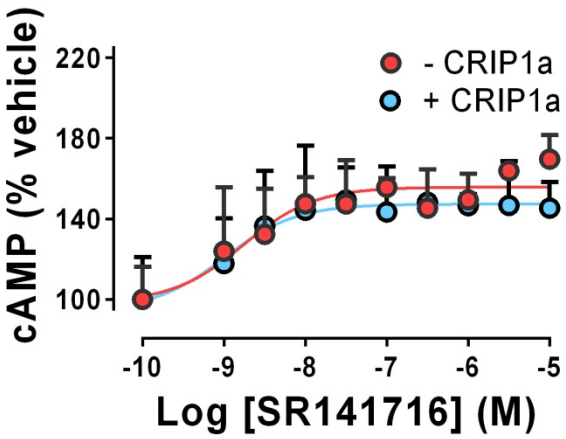


Fig. 4.4. CRIP1a expression does not modulate SR141716-induced cAMP accumulation in HEK-CB_1-TREx CRIP1a cells. Cells were incubated with increasing concentrations of SR141716 for 30 minutes at 37 ºC, in the presence or absence of CRIP1a. Data represents mean + S.E.M. of three experiments performed in triplicate.

4.3.3. CRIP1a does not alter Org27569 modulation of cannabinoid-mediated signal transduction

In Chapter 3, we demonstrated that Org27569 inhibited cAMP inhibition and pERK1/2 activation by some cannabinoid agonists in CHO-hCB_1 cells. To determine whether CRIP1a alters the modulatory effect of Org27569 at CB_1 receptors, cAMP and pERK1/2 interaction studies between cannabinoid agonists and Org27569 were investigated in the presence or absence of CRIP1a.

Org27569 completely abolished CP55940-induced inhibition of cAMP formation in non-induced HEK-CB_1-TREx CRIP1a cells. Inducing CRIP1a expression, however, had no effect on Org27569-induced inhibition of CP55940-mediated responses (Fig. 4.5). Similar results were obtained using WIN55,212-2 (data not shown).

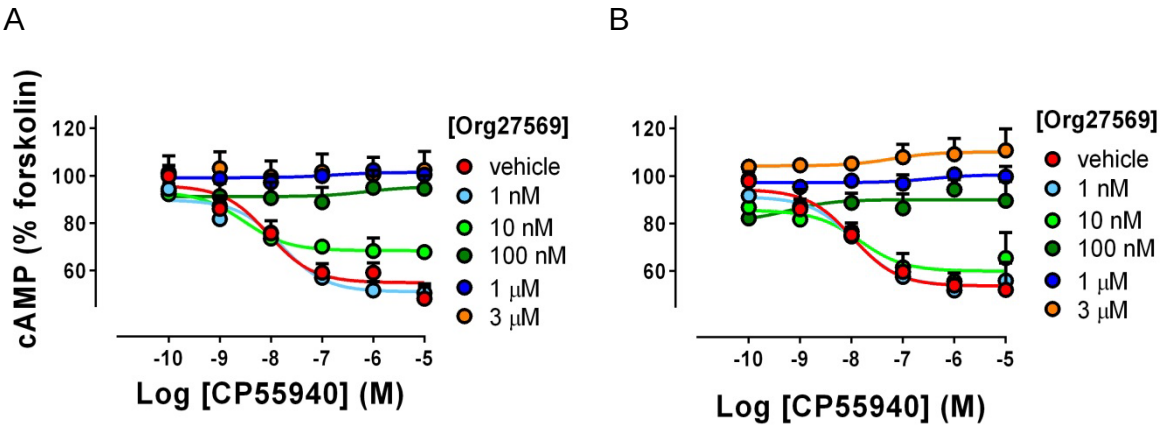


Fig. 4.5. CRIP1a expression does not alter modulatory effects of Org27569 on cannabinoid agonist-mediated cAMP inhibition. Inhibition of CP55940-induced cAMP inhibition by Org27569 in (A) non-induced and (B) tetracycline-induced HEK-CB_1-TREx CRIP1a cells. Data represents mean + S.E.M. of at least three experiments performed in triplicate.

Similarly, Org27569 inhibited CP55940-induced pERK1/2 activation in non-induced HEK-CB_1-TREx CRIP1a cells. The inhibitory effect of Org27569 was unchanged in the presence of CRIP1a in tetracycline-induced cells (Fig. 4.6). Similar results were obtained using WIN55,212-2 (data not shown).

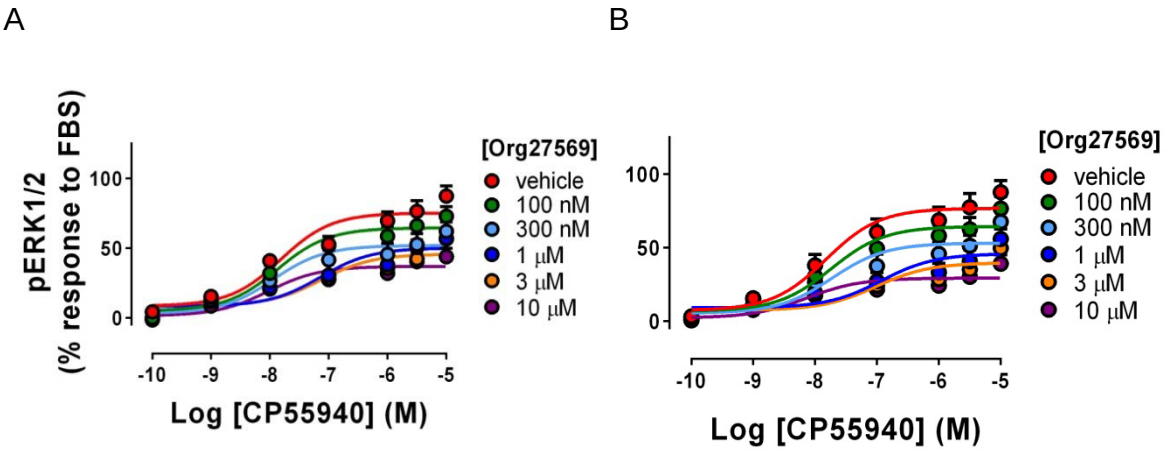


Fig. 4.6. CRIP1a expression does not alter the modulatory effects of Org27569 on cannabinoid agonist-mediated pER1/2 activation. Inhibition of CP55940- or WIN55,212-2-induced pERK1/2 activation by Org27569 in (A) non-induced and (B) in tetracycline-induced HEK-CB_1-TREx CRIP1a cells. Data represents mean + S.E.M. of at least three experiments performed in triplicate.

Taken together, the results demonstrate that CRIP1a does not modulate cannabinoid-mediated signalling or the modulatory effects of Org27569 on cannabinoid signalling in HEK-CB_1-TREx CRIP1a cells.

4.3.4. CRIP1a knockdown in NG108-15 cells

To ensure that the lack of effects of CRIP1a in HEK cells was not due to the use of a recombinant over-expressing cell line, we next sought to investigate effects of CRIP1a in an endogenously expressing cell line, neuroblastoma × glioma (NG108-15).

In order to determine the effects of CRIP1a on CB_1 receptor-mediated signalling in NG108-15 cells, siRNA technology was used to reduce the expression of CRIP1a. Western blotting was performed on lysates prepared from untreated, mmsiRNA-treated or CRIP1a siRNA-treated NG108-15 cells to confirm the reduced expression of CRIP1a after 24 or 48 hours treatment with siRNA.

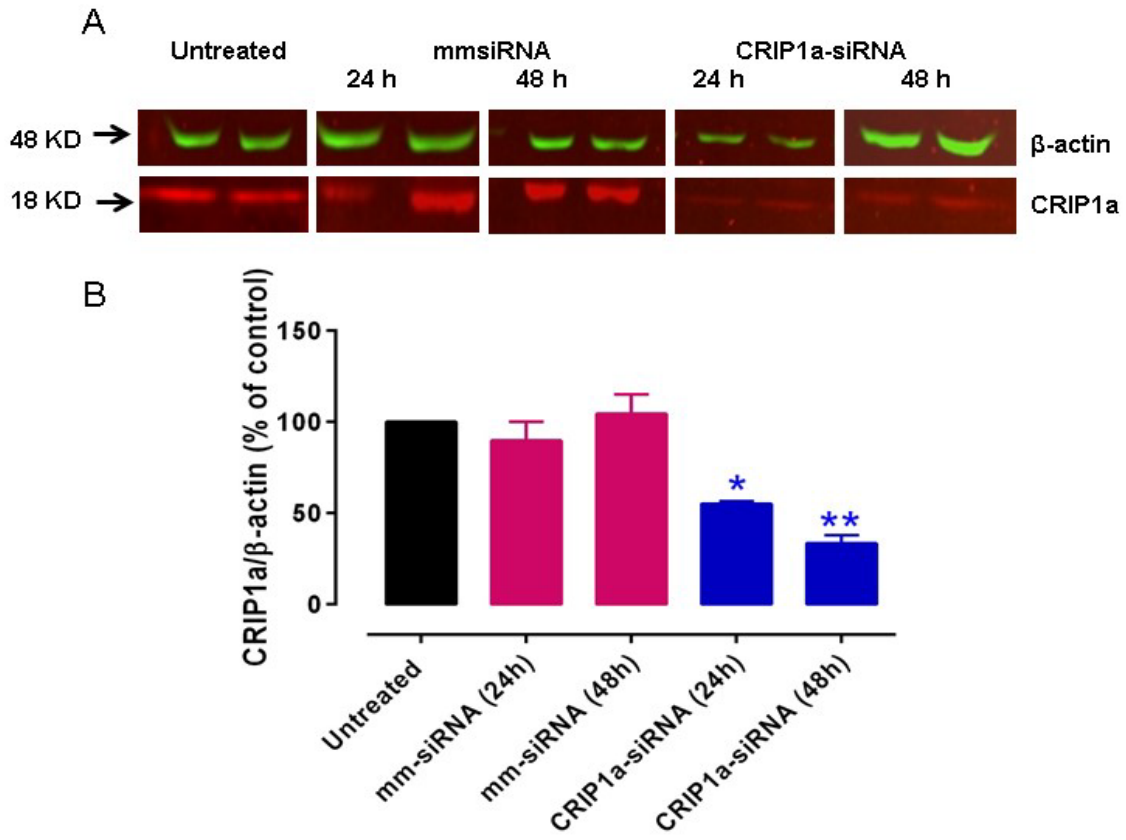


Fig. 4.7. CRIP1a knockdown in NG108-15 cells. (A) Western blot of cells expressing CRIP1a and cells with reduced expression of CRIP1a. (B) Densitometric analysis of CRIP1a expression levels, quantified from bands of western blot. Data were normalised to corresponding β-actin levels, and expressed as a percentage of CRIP1a levels in untreated samples. *p< 0.05, **p< 0.01, statistically significant differences using one-way ANOVA with Bonferroni's multiple comparison test.

A greater level of CRIP1a knockdown was observed in cells treated with 20 nM CRIP1a-siRNA, when measured 48 hours post transfection compared to 24 hours. Thus, 48 hours transfection was used for subsequent experiments. The inactive negative control siRNA (mmsiRNA) did not knockdown CRIP1a (Fig. 4.7).

4.3.5. CRIP1a knockdown reduces cannabinoid agonist-induced inhibition of cAMP

Cannabinoid agonist-induced inhibition of cAMP formation was studied in the presence and absence of CRIP1a. The results showed that WIN55,212-2 reduced 10 µM forskolin-stimulated cAMP inhibition in a concentration-dependent manner in untreated and mmsiRNA-treated NG108-15 cells. Interestingly, however, the response to WIN55,212-2 was abolished in CRIP1a knockdown cells (Fig. 4.8).

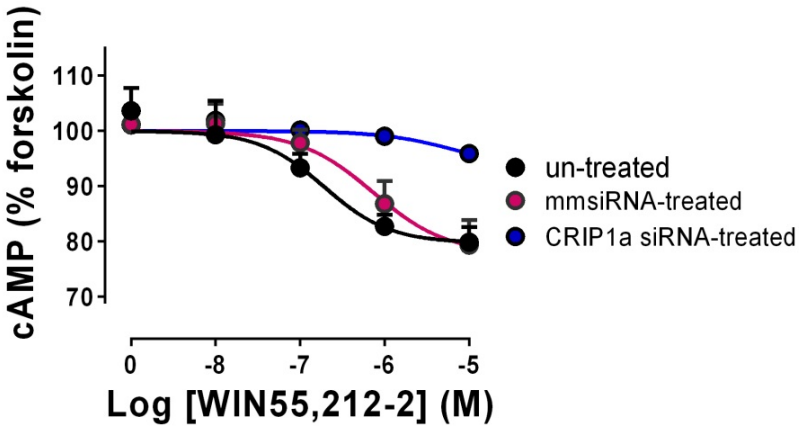


Fig. 4.8. CRIP1a knockdown in NG108-15 cells blocks WIN55,212-2-induced cAMP inhibition. Untreated, mmsiRNA-treated or CRIP1a siRNA-treated NG108-15 cells were incubated with increasing concentrations of WIN55,212-2 for 30 minutes at 37 ºC. Data represents mean + S.E.M. of at least three experiments performed in triplicate.

4.3.6. Single cell Ca^{2+} imaging

4.3.6.1. Effects of cannabinoids on Ca^{2+} levels in the presence and absence of CRIP1a

Effects of cannabinoid agonists on Ca^{2+} channels are controversial. In contrast to the study by Hoddah et al., where WIN55,212-2 inhibited L-type voltage-gated Ca^{2+} currents in hypothalamic neurons, L- and T-type Ca^{2+} channels were not affected by this ligand in NG108-15 cells (Mackie and Hille, 1992).

In this study, the effects of WIN55,212-2 on basal levels of Ca^{2+} in the absence or presence of CRIP1a were studied in NG108-15 cells. WIN55,212-2 (10 µM) alone appeared to increase basal levels of Ca^{2+} in untreated and mmsiRNA-treated cells, but the effect did not reach statistical significance. Similar results were obtained using CRIP1a-siRNA treated cells (Fig. 4.9), indicating that CRIP1a does not alter the basal or cannabinoid-elevated Ca^{2+}levels.

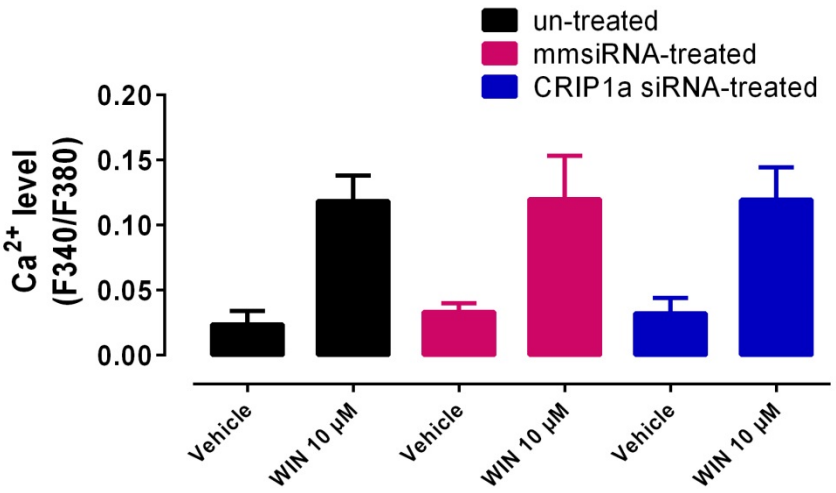


Fig. 4.9. CRIP1a knockdown does not alter basal and cannabinoid-elevated levels of Ca^{2+}. The basal levels of Ca^{2+} and the Ca^{2+} signal in response to WIN55,212-2 (WIN) were not significantly different in untreated, mmsiRNA-treated and CRIP1a siRNA-treated NG108-15 cells. Data represents mean + S.E.M. of at least 80 cells from four experiments.

Previous studies have shown that cannabinoid-induced inhibition of L-type voltage-gated Ca^{2+} currents is cAMP/PKA-dependent (Hoddah et al., 2009). NG108-15 cells mostly express L- and T-type channels (Lukyanetz, 1998). Therefore, we next investigated the effects of WIN55,212-2 on forskolin-stimulated levels of Ca^{2+}. Forskolin increases the intracellular levels of cAMP through activation of adenylate cyclase. Our results showed a concentration-dependent increase in intracellular levels of Ca^{2+} during the first 5 minutes after the addition of forskolin (Fig. 4.10).

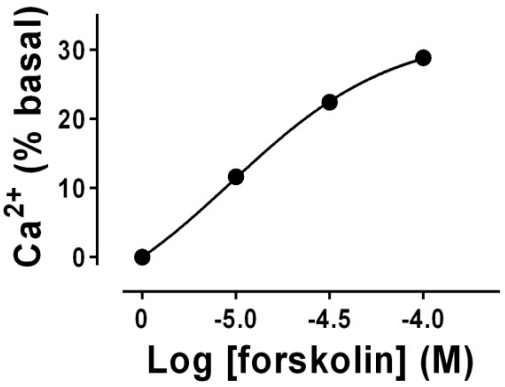


Fig. 4.10. Forskolin increases intracellular Ca^{2+} levels in a concentration-dependent manner. Data represents mean + S.E.M. of at least 60 cells from three experiments.

We next investigated effects of WIN55,212-2 or vehicle in the presence of 10 µM forskolin, in the absence or presence of CRIP1a. In untreated cells, WIN55,212-2 and forskolin together had an additive effect and significantly increased levels of Ca^{2+} compared to vehicle or WIN55,212-2 alone. Similar observations were made for cells treated with mmsiRNA and also for CRIP1a siRNA-treated cells (Fig. 4.11).

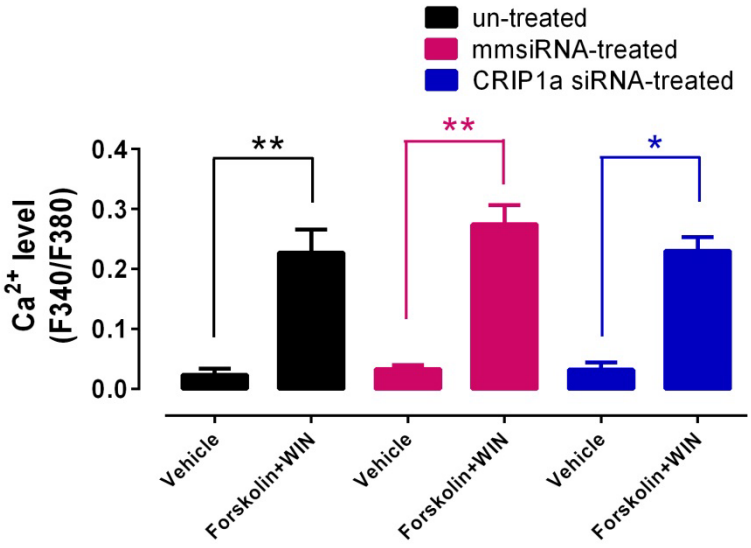


Fig. 4.11. CRIP1a knockdown does not alter Ca^{2+} signal in response to the combination of WIN55,212-2 and forskolin. The Ca^{2+} signals in response to 10 µM WIN55,212-2 (WIN) in the presence of 10 µM forskolin were not significantly different in untreated, mmsiRNA-treated and CRIP1a siRNA-treated NG108-15 cells. Data represents mean + S.E.M. of at least 60 cells from three experiments. *p< 0.05, **p< 0.01, statistically significant differences using one-way ANOVA with Bonferroni's multiple comparison test.

Our results demonstrate that cannabinoid agonists in combination with forskolin can cause a significant increase in Ca^{2+} levels in NG108-15 cells. This effect is unaffected by CRIP1a knockdown.

4.3.6.2. CRIP1a knockdown reduces depolarisation-induced Ca^{2+} influx

Previous studies have shown that cannabinoid agonists inhibit Ca^{2+} influx evoked by KCl-induced depolarisation in cerebellar granule neurons or in differentiated NG108-15 cells (Nogueron et al., 2001; Sugiura et al., 1997). We therefore investigated effects of CRIP1a knockdown on 30 mM KCl-induced Ca^{2+} influx.

KCl caused a significant increase in Ca^{2+} levels. WIN55,212-2 had a small effect on KCl-induced Ca^{2+} influx in undifferentiated NG108-15 cells, however, the effect did not reach statistical significance. Interestingly, CRIP1a knockdown reduced depolarisation-induced Ca^{2+} influx. Significance for this effect was only reached in WIN55,212-2-treated cells (Fig. 4.12).

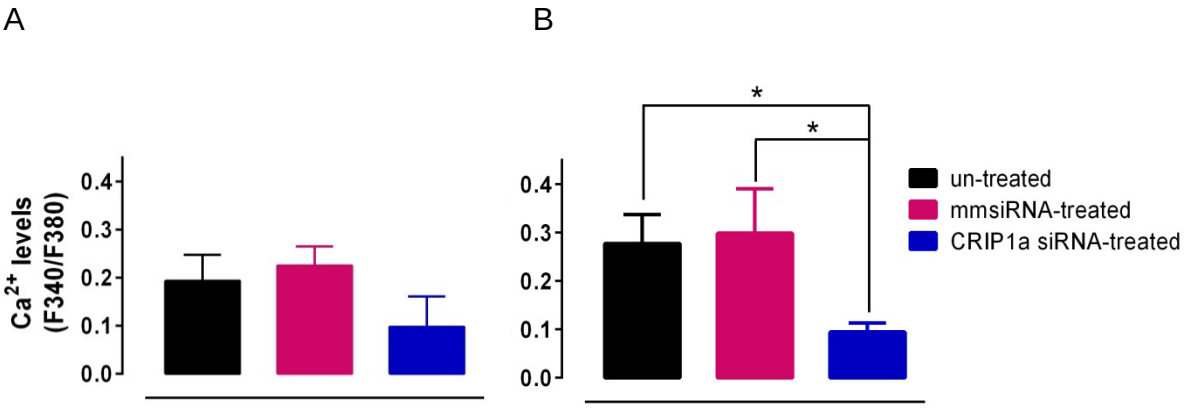


Fig. 4.12. CRIP1a knockdown reduces KCl-induced Ca^{2+} signal in the presence of a cannabinoid agonist. Ca^{2+} signal to 30 mM KCl in (A) vehicle-treated, and (B) WIN55,212-2-treated NG108-15 cells, in the presence or absence of CRIP1a. Data represents mean + S.E.M. of at least 60 cells from three experiments. *p< 0.05, statistically significant differences using one-way ANOVA with Bonferroni's multiple comparison test.

The results suggest that CRIP1a is involved in KCl-induced Ca^{2+} influx in undifferentiated NG108-15 cells in the presence of the cannabinoid agonist (Fig. 4.12 B). Although there was a reduction in KCl-induced Ca^{2+} influx in the absence of the agonist in CRIP1a knockdown cells, the effect did not reach statistical significance (Fig. 4.12 A). Therefore, the involvement of CB_1 receptors cannot be verified in this set of experiments.

4.3.7. High throughput Ca^{2+} mobilisation assays

To confirm the findings obtained from single cell Ca^{2+} imaging, high throughput Ca^{2+} imaging assays were performed.

4.3.7.1. Effects of cannabinoids on Ca^{2+} levels in the presence and absence of CRIP1a

The cannabinoid agonists WIN55,212-2 and methanandamide had no effect on basal levels of Ca^{2+} in NG108-15 cells, in agreement with previously published results (Mackie and Hille, 1992), neither in the absence nor presence of CRIP1a (Fig. 4.13).

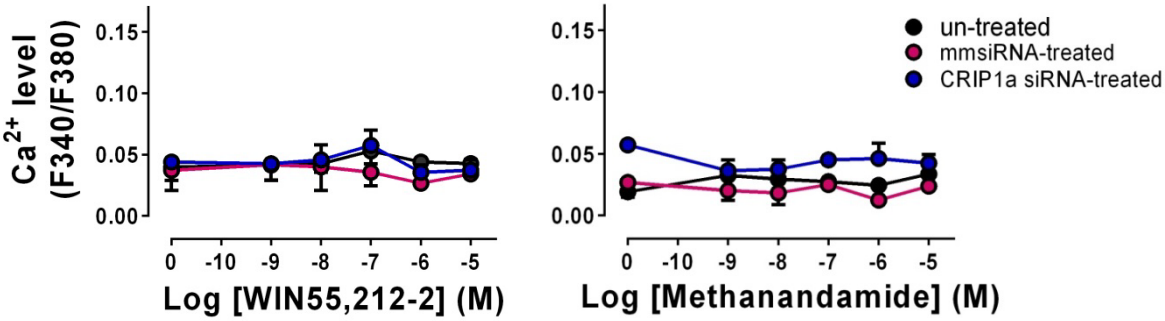


Fig. 4.13. Lack of effect of cannabinoid agonists on basal levels of Ca^{2+} in NG108-15 cells. Untreated, mmsiRNA-treated or CRIP1a siRNA-treated cells were incubated with WIN55,212-2 or methanandamide for 6 minutes. Data represents mean + S.E.M. of at least three experiments performed in triplicate.

4.3.7.2. CRIP1a knockdown reduces depolarisation-induced Ca^{2+} influx

Similar to the results of our single cell Ca^{2+} imaging experiments, KCl caused a significant increase in Ca^{2+} levels in untreated and mmsiRNA-treated NG108-15 cells. In CRIP1a knockdown cells, KCl-induced Ca^{2+} influx was significantly lower than in untreated and mmsiRNA-treated cells (Fig. 4.14). However, as opposed to results presented in Fig. 4.12, this effect was not dependent on the activation of CB_1 receptors by an agonist.

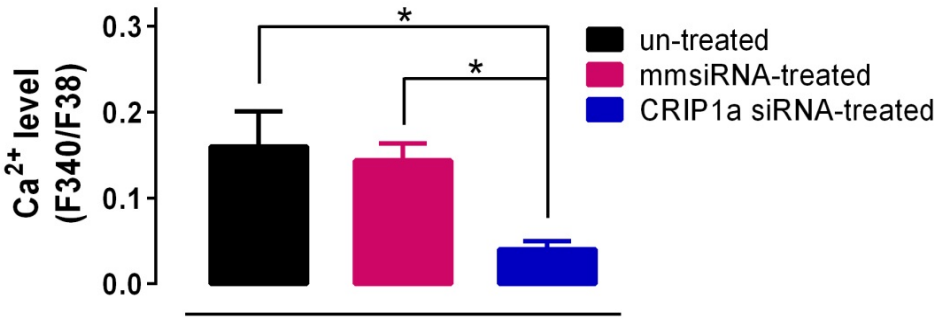


Fig. 4.14. CRIP1a knockdown reduces KCl-induced Ca^{2+} signal. Ca^{2+} signal to 30 mM KCl in NG108-15 cells, in the presence or absence of CRIP1a. Data represents mean + S.E.M. of five experiments performed in triplicate. *p< 0.05, statistically significant differences using one-way ANOVA with Bonferroni's multiple comparison test.

The results suggest that CRIP1a may have a non-CB_1 receptor-mediated effect on KCl-induced Ca^{2+} influx, or that the effect may be mediated by agonist-independent, constitutive activity on CB_1 receptors.

4.4. Discussion

This study provides further insight into the modulation of CB_1 receptor-mediated signalling pathways by an endogenous protein interacting with CB_1 receptors, named CRIP1a, in cell lines that over-express or endogenously express this protein. The results demonstrated that the effects of CRIP1a vary depending on the signalling pathway studied and the cell line used. However, a non-CB_1 receptor-mediated mechanism might be involved in the observed effects.

As previously described in Chapter 3, CB_1 receptor allosteric modulators offer an intriguing approach to obtaining selective therapeutics, by modulating only the desired therapeutic pathways, through biased allosterism and probe-dependence. The attenuation of SR141716 enhancement of, but not WIN55,212-2-induced inhibition of, Ca^{2+} currents in SCG neurons (Niehaus et al., 2007) suggests that CRIP1a may allosterically modulate CB_1 receptors. Therefore, CRIP1a may be a potential target for the development of selective CB_1 receptor-based therapies.

However, there are limited studies that have explored the modulation of CB_1 receptor downstream signalling pathways by CRIP1a. We demonstrated that the basal levels of cAMP and pERK1/2 were unchanged in the presence of CRIP1a in HEK-CB_1-TREx CRIP1a cells. CRIP1a had no effect on enhancement of cAMP accumulation by SR141716, or on cAMP inhibition or pERK1/2 activation induced by WIN55212-2 or CP55940 in HEK-CB_1-TREx CRIP1a cells. Surprisingly, in NG108-15 cells, which endogenously express CB_1 receptors and CRIP1a, WIN55,212-2-induced cAMP inhibition was abolished by knocking down CRIP1a, indicating that CRIP1a may be required for the cAMP response to WIN55,212-2 in

these cells. The results suggest the importance of the cell line used in the modulation of cannabinoid-activated signal transduction pathways by CRIP1a. However, a direct effect of CRIP1a on the CB_1 receptor could not be verified in this study. Similarly, Blume et al. (2015) demonstrated differences in modulatory effects of CRIP1a using CRIP1a over-expressing or knockdown N18TG2 cells. Whereas pERK1/2 and cAMP responses to CP55940 were unaltered in CRIP1a over-expressing N18TG2 cells, they were enhanced in CRIP1a knockdown cells (Blume et al., 2015). In a recent study, CRIP1a knockdown enhanced CB_1 receptor-mediated G protein activation in N18TG2 cells endogenously expressing CB_1 receptors and CRIP1a. Furthermore, CRIP1a inhibited CB_1 receptor-mediated [^{35}S]GTPγS binding by cannabinoid agonists in HEK cells stably expressing CB_1 receptors and CRIP1a (Smith et al., 2015). Therefore our observed lack of effect of CRIP1a in HEK cells is unlikely related to over-expression of these proteins.

The differences between the results of the present study and the previous findings may be explained by the presence of different isoforms of adenylate cyclase and G protein populations in different cells (Mukhopadhyay et al., 2002). CRIP1a has been shown to bias the G protein-CB_1 receptor pool. For instance, CP55940-induced Gi_3 and Go activation was attenuated, whereas Gi_1 and Gi_2 activation was enhanced in cells over-expressing CRIP1a. CRIP1a knockdown enhanced CP55940-mediated Gi_3 and Go activation, whereas it had no effect on Gi_1 and Gi_2 activation (Blume et al., 2015). Therefore, the effects of CRIP1a on the G protein-CB_1 receptor pool may vary in different cell lines (Blume et al., 2015).

In Chapter 3, we showed that the CB_1 receptor small allosteric modulator Org27569 inhibits cAMP or pERK1/2 signalling by some cannabinoids in CHO-hCB_1 cells. An allosteric interaction of CRIP1a with CB_1 receptors could alter the

receptor conformation such that it might change the response to allosteric ligands as well as orthosteric ligands at CB_1 receptors. However, our results demonstrated that CRIP1a expression had no effect on small molecule allosteric modulation of CB_1 receptors in HEK-CB_1-TREx CRIP1a cells. This suggests a lack of effect of CRIP1a on binding of Org27569 or on the transmission of cooperativity between Org27569 and cannabinoid ligands.

Pathway-specific modulation of cannabinoid-mediated signalling by CRIP1a was demonstrated by its lack of effect on Ca^{2+} signalling as opposed to cAMP signalling by WIN55,212-2, in NG108-15 cells. NG108-15 cells express only low-voltage sensitive calcium channels (activated at -30 mV and completely inactivated at holding potentials of -60 to -50 mV), mostly of the L- and T-types, whereas after differentiation they also express high voltage N and P/Q type calcium channels (activated by larger depolarisations and slower inactivation) (Lukyanetz, 1998). Unlike inhibition of N and P/Q type calcium channels, inhibition of L-type Ca^{2+} currents is cAMP/PKA-dependent (Caulfield and Brown, 1992; Hoddah et al., 2009; Mackie and Hille, 1992; Pan et al., 1996; Taguchi et al., 1997; Twitchell et al., 1997), as demonstrated in our study by the forskolin-induced increase in Ca^{2+} currents. However, WIN55,212-2 was reported to have no effect on L-type voltage-gated Ca^{2+} currents in NG108-15 cells (Mackie and Hille, 1992). We observed a non-significant increase in Ca^{2+} levels by WIN55,212-2 and a significant increase by WIN55,212-2 and forskolin together. This is in agreement with the observation that the inhibitory effect of cannabinoids on Ca^{2+} channels may be overcome by the release of Ca^{2+} from intracellular stores when channels are not highly active (Netzeband et al., 1999), such as in undifferentiated NG108-15 cells in the present study. Furthermore, the response to WIN55,212-2 and/or

forskolin was unaltered by knockdown of CRIP1a in these cells. This is in agreement with the lack of modulation of WIN55,212-2-induced inhibition of Ca^{2+} currents by CRIP1a over-expression in SCG neurons (Niehaus et al., 2007).

Additionally, WIN55,212-2 had no significant effect on KCl-induced Ca^{2+} influx in our undifferentiated NG108-15 cells, whereas previous studies demonstrated inhibition of the Ca^{2+} signal to KCl by cannabinoid agonists in cerebellar granule neurons or in differentiated NG108-15 cells (Nogueron et al., 2001; Sugiura et al., 1997). The difference might be explained by the presence of different types of Ca^{2+} channels in differentiated or undifferentiated cells. Interestingly, CRIP1a knockdown significantly reduced depolarisation-induced Ca^{2+} influx. Further investigation is required to explain the mechanisms by which CRIP1a knockdown reduces KCl-induced Ca^{2+} signals. However, this may be due to a non-CB_1 receptor-mediated effect. Alternatively, this may be due to a global effect of CRIP1a on CB_1 receptor signalling. CB_1 receptors exhibit high levels of constitutive activity (reviewed in Meye et al., 2014), and CRIP1a over-expression has been shown to reduce CB_1 receptor constitutive activity (Niehaus et al., 2007). Therefore, the higher constitutive activity of CB_1 receptors in CRIP1a knockdown cells may result in lower levels of K^+ or Ca^{2+} inside the cells.

The present study demonstrates that CRIP1a modulates cannabinoid-mediated signalling in a pathway-dependent manner in neuron-like cells while having no effects on cannabinoid-mediated signalling in HEK cells over-expressing CB_1 and CRIP1a. However, our results did not verify whether the modulatory effects of CRIP1a are mediated through its action on CB_1 receptors or through non-specific effects. Therefore, further research is required to elucidate CRIP1a

mechanisms of action, and its therapeutic advantages in a number of CB_1 receptor-associated central nervous system disorders.

Chapter 5

General Discussion

The widespread expression and biological role of CB_1 receptors in the brain makes them intriguing targets for the treatment of numerous central nervous system disorders (Zhang et al., 2009). Several exogenous cannabinoid ligands have therefore been developed; however, attempts to develop selective CB_1 receptor-targeted therapeutics with minimal adverse effects have failed. This is in part due to loss in selectivity at high concentrations of CB_1 receptor ligands and subsequent activity at CB_2 receptors. Also the same receptor subtype can mediate both therapeutic and adverse effects (Mukhopadhyay et al., 2002). Therefore, due to extensive off-target and on-target adverse effects, currently there are only a few cannabinoid-based therapeutics on the market, including dronabinol, nabilone, levonantradol and Sativex®.

In the present study, two novel approaches to obtaining more selective therapeutics at CB_1 receptors were investigated; ligand-biased signalling and allosteric modulation. Ligand-biased signalling has proved useful in the treatment of several pathophysiological conditions, such as schizophrenia, Parkinson's disease, addiction, depression, cardiovascular disorders, dyslipidemia, osteoporosis and cancer, through selective activation of receptor-mediated signalling pathways related to the therapeutic effects, at the exclusion of those related to the adverse effects (reviewed in Kenakin and Miller, 2010).

Allosteric modulators may offer several advantages over orthosteric ligands. If they have no intrinsic efficacy, they may only act in the presence of the endogenous ligand, thus producing a more physiological response. They may also offer receptor subtype specificity as allosteric sites are often less conserved across receptor subtypes (May et al., 2007). Allosteric modulators may display probe-dependence (May et al., 2007; Valant et al., 2012), which is depending on

the orthosteric probe used, different effects can be produced by the same allosteric modulator (Leach et al., 2007; Valant et al., 2012). This has particular importance where the receptor binds to various endogenous ligands involved in diverse physiological effects. Therefore, allosteric ligands that modulate binding or signalling of one endogenous ligand but not the other may be used to enhance or reduce specific physiological responses where necessary. Allosteric modulators may also display biased signalling by promoting unique conformational states of the receptor, which may lead to modulation of distinct signalling pathways activated by orthosteric ligands (Kenakin and Christopoulos, 2013). In fact, there are several allosteric modulators with therapeutic advantages in clinical trials or on the market for the treatment of several central nervous system disorders (including anxiety, cognitive disorders, schizophrenia, Alzheimer's and Parkinson's disease), gastroesophageal reflux, HIV infection and hyperparathyroidism (reviewed in Conn et al., 2009; Conn et al., 2014).

Ligand-biased signalling and allosteric modulation at CB_1 receptors has not been thoroughly investigated to date. However, there is evidence for differential activation of distinct G proteins and signalling pathways by different cannabinoid ligands (reviewed in Bosier et al., 2010; Varga et al., 2008). CB_1 receptor allosteric modulators such as Org27569 have also been identified, and demonstrated to engender ligand-biased signalling and probe-dependence (Baillie et al., 2013; Price et al., 2005).

However, in order to link *in vitro* findings to potential therapeutic outcomes, robust methods that can quantify ligand-biased signalling and/or allosteric modulation must be applied. This has not been done at CB_1 receptors to date, and therefore, the present study aimed to use sophisticated analytical methods to

quantify these phenomena using two important CB_1 receptor-mediated signalling pathways, inhibition of cAMP formation and activation of pERK1/2, which are involved in different CB_1 receptor-mediated physiological and pathophysiological functions (Berghuis et al., 2005; Guzman and Sanchez, 1999; Rubino et al., 2006; Rueda et al., 2002; Sanchez et al., 1998; Zhou and Song, 2001). CB_1 receptor modulation of cAMP signalling has been linked to neurite remodelling, which has been suggested to facilitate some of the psychoactive and neurotoxic effects of cannabinoids (Zhou and Song, 2001). The ERK1/2 pathway is involved in regulation of cannabinoid-mediated neuronal migration and differentiation (Berghuis et al., 2005; Rueda et al., 2002), and may play an important role in the development of tolerance and addiction to cannabinoids (Rubino et al., 2006). Furthermore, pCREB and pERK1/2 are key regulators of synaptic plasticity, learning and memory (Basavarajappa et al., 2014).

Herein, results have been presented that provide quantitative insight into orthosteric and allosteric ligand-biased signalling using an operational model of agonism (van der Westhuizen et al., 2014) or allosterism (Leach et al., 2007), respectively. The first important finding of the present study is that the endocannabinoids 2-AG and anandamide display different biased signalling profiles in pERK and cAMP assays. Thus, whereas 2-AG shows little bias between pathways, anandamide is more biased towards cAMP inhibition over pERK1/2 activation. Our quantitative analysis estimated a bias factor of 7 for anandamide towards cAMP inhibition. This is the first study that detects and quantifies biased agonism by endocannabinoids. It is well established that 2-AG and anandamide are involved in different physiological and pathophysiological functions (Bernabo et al., 2013; Luchicchi and Pistis, 2012). For instance, most studies have determined

anandamide as a principal modulator of learning and memory, whereas it appears that 2-AG is the predominant agonist involved in synaptic plasticity and neuroprotection (reviewed in Luchicchi and Pistis, 2012). There is evidence that some of the different effects of these two endocannabinoids are mediated through CB_1 receptors (Basavarajappa et al., 2014; Long et al., 2009; Pan et al., 2011). Therefore, our findings suggest that the distinct physiological roles of 2-AG and anandamide may be associated with activation of distinct CB_1 receptor signalling pathways by these endogenous agonists. Thus, selective modulation of 2-AG- or anandamide-mediated signalling in different tissues may provide an approach to achieve a desirable therapeutic outcome.

Another important finding of this study is that the CB_1 receptor allosteric modulator Org27569 does not modulate endocannabinoid binding, indicated by almost neutral (α close to 1) binding cooperativity factors, quantified using an allosteric ternary complex model (Leach et al., 2010). To our knowledge this is the first study that has investigated the effects of Org27569 on binding of these two main endocannabinoids. Our results also demonstrated that Org27569 partially inhibited 2-AG-induced pERK1/2 activation, while having no effect on the response to anandamide, indicating its probe-dependent effect. Using an operational model of allosterism (Leach et al., 2007), functional cooperativity factors, β, of 0.36 and 0.72 were estimated between Org27569 and 2-AG or anandamide respectively, in pERK1/2 assays. In contrast, Org27569 blocked cAMP inhibition by both anandamide and 2-AG, indicated by β values approaching 0. This provides a striking example of biased allosteric effects of Org27569 between pathways. Previous findings demonstrated that Org27569 inhibited cAMP inhibition (Cawston et al., 2013), [^{35}S]GTPγs binding and β-arrestin recruitment by anandamide (Baillie

et al., 2013). However, these effects were not quantified. Also, there is no other published study on modulation of 2-AG signalling by Org27569. This is the first study that demonstrates differential modulation of 2-AG and anandamide signalling by Org27569. Allosteric modulators that display differential binding or functional cooperativity with endocannabinoids may therefore be used to obtain desired pharmacological effects.

Similar to the endocannabinoids, exogenous cannabinoids also displayed distinct biased profiles in pERK1/2 and cAMP assays. The present study demonstrated that HU-210, Δ9-THC and to a lesser extent CP55940 favoured inhibition of cAMP formation over activation of pERK1/2. Interestingly, WIN55,212-2 displayed an unbiased profile similar to that of 2-AG. Using an operational model of agonism (van der Westhuizen et al., 2014) bias factors of 21, 6, 4 and 2 were estimated for HU-210, Δ9-THC, CP55940 and WIN55,212-2, respectively. These findings may aid the development of biased ligands that could mediate only desirable therapeutic effects. In particular, ligands with biased profiles similar to the endocannabinoids may have potential to produce effects close to the natural physiological response. Previous studies have provided evidence that cannabinoid agonists may engender bias. For instance, differential activation of different G proteins by cannabinoid ligands (Laprairie et al., 2014; Mukhopadhyay and Howlett, 2005), or reversal in efficacy of CP55940 and HU-210 in activation of pERK1/2 vs. JNK and inhibition of gene transcription by CRE vs. AP-1 was demonstrated (Bosier et al., 2008a; Bosier et al., 2008b). However, this is the first study that used a systematic approach to detect and quantify biased agonism by various cannabinoid ligands.

Similar to the close to neutral binding cooperativity between Org27569 and the endocannabinoids, the binding of exogenous cannabinoid agonists was weakly modulated by Org27569, whereas there was a high negative cooperativity between the CB_1 inverse agonist, [^{3}H]SR141716A, and Org27569. This indicates that Org276529 modulates cannabinoid binding in a ligand-specific manner. Previous findings also demonstrated negative binding cooperativity between [^{3}H]SR141716A and Org27569. However, strong positive cooperativity ($\alpha = 14$) was reported for the displacement of [^{3}H]CP55940 by Org27569 (Price et al., 2005). The differences between our results and the previous findings could be due to the different experimental conditions, or the use of whole cells compared to membrane preparations in the previous studies.

Similar to biased allosteric effects between Org27569 and endocannabinoids, the present study demonstrates differential modulation of exogenous cannabinoid-mediated signalling between pathways. Thus, Org27569 inhibited cAMP signalling by CP55940, HU-210, WIN55,212-2 and Δ9-THC, whereas it only inhibited pERK1/2 activation by CP55940 and HU-210, partially inhibited the response to WIN55,212-2, and had no effect on pERK1/2 signalling by Δ9-THC, as indicated by β values ranging from 0 to 1 depending on the agonist used. The biased allosteric effects and probe-dependence by Org27569 was previously demonstrated as it had weak or no effect on the pERK1/2 or cAMP response to WIN55,212-2, but potentiated CP55940-induced pERK1/2 activation while inhibiting CP55940-induced cAMP inhibition (Baillie et al., 2013). The negative cooperativity between Org27569 and some of the tested cannabinoids in pERK1/2 assays is in contrast to the previously reported potentiation of CP55940-activated pERK1/2 by Org27569 (Ahn et al., 2012; Baillie et al., 2013). This could be due to

the presence of different types or proportions of intracellular signalling proteins in different cells. Nonetheless, our findings provide further evidence for the probe-dependent and biased allosteric effects of Org27569 and for the first time thoroughly quantifies binding and functional parameters for the interaction between Org27569 and several cannabinoid agonists. Therefore, the information provided herein suggest that a unique combination of allosteric and orthosteric ligands may be used to selectively activate desired CB_1 receptor signalling pathways.

Although Org27569 acts as a potent allosteric modulator of CB_1 receptor functions *in vitro*, it has been reported that the effects do not necessarily translate into *in vivo* effects. In mice, Org27569 did not modulate analgesia, catalepsy, or hypothermia induced by anandamide, CP55940 or Δ9-THC, and although Org27569 reduced food intake, it did so independently of CB_1 receptors (Gamage et al., 2014). In rats, however, Org27569 inhibited hypothermia produced by CP55940 and anandamide. In contrast, it had no effect on CP55940-induced catalepsy and antinociception, and did not modulate SR141716A-induced grooming and scratching behaviours. Org27569 also decreased food intake in rats. However, the involvement of CB_1 receptors in these effects is unclear (Ding et al., 2014). Nonetheless, differential modulation of behavioural effects of cannabinoids *in vivo* by Org27569 may be explained by differential modulation of cannabinoid-mediated signalling pathways. Overall, these findings indicate that *in vitro* drug activity at CB_1 receptors must be further validated in relevant cell lines, tissues and whole animals to assess the correlation between observed cell-based pharmacology and subsequent *in vivo* effects.

An interesting observation is that WIN55212-2 and 2-AG, despite being structurally very different, display a similar pattern of modulation by Org27569, as well as having a similar pattern of ligand biased signalling. This may help in the prediction of *in vivo* effects of small molecule cannabinoids or their effects in the presence of an allosteric modulator. It may also facilitate the development of cannabinoid-based drugs that may produce effects close to the physiological responses produced by endocannabinoids.

In addition to the small molecule allosteric modulator, Org27569, the present study further investigated the effects of putative endogenous allosteric modulators at CB_1 receptors, pregnenolone, lipoxin A4 and CRIP1a. In contrast to previous findings (Vallee et al., 2014), in the present study, pregnenolone did not modulate CB_1 receptor-mediated pERK1/2 signalling although it reduced the binding of radiolabelled SR141716A. It was unclear whether inhibition of [^{3}H]SR141716A binding was via a competitive or an allosteric interaction at CB_1 receptors or even a non-specific effect. Therefore, further research is necessary to validate whether these effects are mediated through an allosteric site on CB_1 receptors. Similarly, the present study did not verify the previously reported (Pamplona et al., 2012) allosteric action of lipoxin A4 at CB_1 receptors. In contrast to previous findings in HEK-CB_1 cells (Pamplona et al., 2012), lipoxin A4 did not inhibit [^{3}H]SR141716A binding, and did not enhance anandamide-mediated inhibition of cAMP in our CHO-hCB_1 cells.

Previous studies in mice demonstrated that pregnenolone inhibited the “cannabinoid tetrad” of effects (analgesia, catalepsy, hypoactivity and hypothermia), increased food intake and memory impairment produced by Δ9-THC, and reduced self-administration of WIN55,212-2 (Vallee et al., 2014).

Lipoxin A4 has also been shown to potentiate the cataleptic effect of anandamide in mice, and to a lesser extent CP55940, without altering the response to 2-AG (Pamplona et al., 2012). Thus, it appears that pregnenolone and lipoxin A4 modulate behavioural effects of cannabinoids. However, this is not conclusive evidence of an allosteric interaction of these ligands at CB_1 receptors. Therefore, further research is required to validate their mechanism of action.

The present study further investigated the effects of CRIP1a over-expression or knockdown on cannabinoid-mediated signalling pathways. It was previously reported that CRIP1a over-expression in superior cervical ganglion (SCG) neurons attenuated SR141716 enhancement of Ca^{2+} currents while leaving the inhibition of Ca^{2+} currents by WIN55212-2 unaltered (Niehaus et al., 2007). In the present study, a reduction in CRIP1a expression did not alter cannabinoid-mediated Ca^{2+} mobilisation, but it reduced KCl-induced Ca^{2+} influx in NG108-15 cells. We also demonstrated that unlike its lack of effect in recombinant HEK cells, CRIP1a was required for WIN55,212-2-induced cAMP inhibition in NG108-15 cells, which endogenously express CB_1 receptors and CRIP1a. Thus, another important finding of the present study is that the modulatory effect of CRIP1a depends on the expression levels of CRIP1a and the cell line used. In a previous study, different effects of CRIP1a were observed in CRIP1a over-expressing or knockdown N18TG2 cells (Blume et al., 2015). In N18TG2 cells endogenously expressing CB_1 receptors and CRIP1a, CRIP1a knockdown enhanced CB_1 receptor-mediated G protein activation and specifically increased CB_1 receptor-coupling to Gi_3 and Go proteins. In contrast, CRIP1a over-expression switched CB_1 receptor signalling towards interactions with Gi_1 and Gi_2 (Blume et al., 2015).

Thus, this may reflect the importance of the types and proportions of G proteins and other signalling elements in cells.

The expression levels of CB_1 receptors in HEK and NG108-15 cells have not been determined in the present study. Given that CB_1 receptor signalling properties (potency, E_{max}) were similar in CHO and HEK cells, and that both cell lines represent an over-expressing recombinant cell system, CB_1 receptor expression levels would likely be comparable in CHO and HEK cells. Indeed, previous studies have demonstrated comparable levels of CB_1 receptor expression in different cell preparations. For instance, the B_{max} was estimated to be 0.95 pmol/mg in HEK293-hCB_1 and 0.83 pmol/mg in CHO-hCB1 (Tao and Abood, 1998). Encouragingly, these values are not dissimilar to those determined in mouse brain (1.81 pmol/mg) (Abood et al., 1997). CB_1 receptor expression levels are likely lower in endogenously expressing cell lines. For instance, mouse neuroblastoma N18TG2 cells were reported to express 0.2 pmol/mg CB_1 receptors (Abood et al., 1997), and a similar expression level would be expected in our related NG180-15 cells. Furthermore, according to a recently published study, the B_{max} value for CB_1 receptors is unaltered by CRIP1a over-expression or knockdown (Smith et al., 2015).

CRIP1a also did not alter the modulatory effects of Org27569 on cannabinoid-mediated cAMP or pERK1/2 signalling in recombinant HEK cells. Thus, although CRIP1a may modulate some cannabinoid-mediated signalling pathways, this study cannot conclude that its effects are mediated through a direct effect on the CB_1 receptor. Nonetheless, the results suggest that CRIP1a is involved in activation of some of the intracellular signalling events in NG108-15 cells.

Taken together, the present study provides quantitative insight into CB_1 receptor function, which may aid the development of orthosteric and allosteric CB_1 receptor ligands that are biased towards activation of therapeutically relevant signalling pathways.

However, many challenges remain in the area of CB_1 receptor ligand-biased signalling and allosteric modulation. For instance, in most cases, it is not yet known which signalling pathways are associated with therapeutic or adverse effects. It is also possible that overlapping signalling pathways may mediate both types of effect (Kenakin, 2012a; Mukhopadhyay et al., 2002). Furthermore, it is difficult to predict the ligand biased effects or allosteric activities in different cells or tissues (Kenakin and Christopoulos, 2013; Kenakin, 2012b), and the effects in recombinant cell lines may not translate into the same effects in primary cell lines, tissues or whole animals. Therefore, one might argue that the results obtained using over-expressing cell lines might be physiologically irrelevant. However, recombinant systems provide a robust pharmacological tool to detect the ability of ligands to display bias or allosterism, and can inform future mechanistic studies. Ligands can then be selected for further research in physiologically relevant and model disease systems (Kenakin, 2011; Valant et al., 2014). Ultimately, as more biased ligands and allosteric modulators enter the clinic, further opportunities will be provided to establish the correlation between their *in vitro* and therapeutic effects (Kenakin, 2011).

To conclude, the present study demonstrates an approach that can quantitatively evaluate ligand-biased signalling and allosteric modulation at CB_1 receptors. This serves as an initial step in determining ligand-biased “fingerprints” that can guide structure-activity studies and the selection of drug candidates for *in*

vivo studies, which may ultimately aid the development of more successful CB_1 receptor-targeted therapies.

References

Abood ME, Ditto KE, Noel MA, Showalter VM and Tao Q (1997) Isolation and expression of a mouse CB1 cannabinoid receptor gene. Comparison of binding properties with those of native CB1 receptors in mouse brain and N18TG2 neuroblastoma cells. *Biochem Pharmacol* **53**(2): 207-214.

Abood ME and Martin BR (1992) Neurobiology of marijuana abuse. *Trends Pharmacol Sci* **13**(5): 201-206.

Adams IB, Compton DR and Martin BR (1998) Assessment of anandamide interaction with the cannabinoid brain receptor: SR 141716A antagonism studies in mice and autoradiographic analysis of receptor binding in rat brain. *J Pharmacol Exp Ther* **284**(3): 1209-1217.

Adams IB and Martin BR (1996) Cannabis: pharmacology and toxicology in animals and humans. *Addiction* **91**(11): 1585-1614.

Ahn KH, Bertalovitz AC, Mierke DF and Kendall DA (2009) Dual role of the second extracellular loop of the cannabinoid receptor 1: ligand binding and receptor localization. *Mol Pharmacol* **76**(4): 833-842.

Ahn KH, Mahmoud MM and 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. *J Biol Chem* **287**(15): 12070-12082.

Anavi-Goffer S, Fleischer D, Hurst DP, Lynch DL, Barnett-Norris J, Shi S, Lewis DL, Mukhopadhyay S, Howlett AC, Reggio PH and Abood ME (2007) Helix 8 Leu in the CB1 cannabinoid receptor contributes to selective signal transduction mechanisms. *J Biol Chem* **282**(34): 25100-25113.

Appleyard SM, Patterson TA, Jin W and Chavkin C (1997) Agonist-Induced Phosphorylation of the κ-Opioid Receptor. *J Neurochem* **69**(6): 2405-2412.

Ashton JC, Wright JL, McPartland JM and Tyndall JD (2008) Cannabinoid CB1 and CB2 receptor ligand specificity and the development of CB2-selective agonists. *Curr Med Chem* **15**(14): 1428-1443.

Atwood BK and Mackie K (2010) CB2: a cannabinoid receptor with an identity crisis. *Br J Pharmacol* **160**(3): 467-479.

Baillie GL, Horswill JG, Anavi-Goffer S, Reggio PH, Bolognini D, Abood ME, McAllister S, Strange PG, Stephens GJ, Pertwee RG and Ross RA (2013)

CB1 Receptor Allosteric Modulators Display Both Agonist and Signaling Pathway Specificity. *Mol Pharmacol* **83**(2): 322-338.

Ballesteros JA and Weinstein H (1995) Integrated methods for modeling G-protein coupled receptors, in *Methods in neuroscience* (Conn PM and Sealfon SC eds) pp 366-428, Academic Press, San Francisco.

Barber TS, Hurst DP and Reggio PH (2006) Identification of an allosteric binding site at the cannabinoid CB1 receptor. *American Chemical Society Abstracts* P130.

Basavarajappa BS, Nagre NN, Xie S and Subbanna S (2014) Elevation of endogenous anandamide impairs LTP, learning, and memory through CB1 receptor signaling in mice. *Hippocampus* **24**(7): 808-818.

Bash R, Rubovitch V, Gafni M and Sarne Y (2003) The stimulatory effect of cannabinoids on calcium uptake is mediated by Gs GTP-binding proteins and cAMP formation. *Neurosignals* **12**(1): 39-44.

Bauer M, Chicca A, Tamborrini M, Eisen D, Lerner R, Lutz B, Poetz O, Pluschke G and Gertsch J (2012) Identification and quantification of a new family of peptide endocannabinoids (Pepcans) showing negative allosteric modulation at CB1 receptors. *J Biol Chem* **287**(44): 36944-36967.

Berghuis P, Dobszay MB, Wang X, Spano S, Ledda F, Sousa KM, Schulte G, Ernfors P, Mackie K, Paratcha G, Hurd YL and Harkany T (2005) Endocannabinoids regulate interneuron migration and morphogenesis by transactivating the TrkB receptor. *Proc Natl Acad Sci U S A* **102**(52): 19115-19120.

Bernabo N, Barboni B and Maccarrone M (2013) Systems biology analysis of the endocannabinoid system reveals a scale-free network with distinct roles for anandamide and 2-arachidonoylglycerol. *OMICS* **17**(12): 646-654.

Bifulco M and Di Marzo V (2002) Targeting the endocannabinoid system in cancer therapy: a call for further research. *Nat Med* **8**(6): 547-550.

Bisogno T, Howell F, Williams G, Minassi A, Cascio MG, Ligresti A, Matias I, Schiano-Moriello A, Paul P, Williams EJ, Gangadharan U, Hobbs C, Di Marzo V and Doherty P (2003) Cloning of the first sn1-DAG lipases points to the spatial and temporal regulation of endocannabinoid signaling in the brain. *J Cell Biol* **163**(3): 463-468.

Bisogno T, Melck D, Bobrov M, Gretskaya NM, Bezuglov VV, De Petrocellis L and Di Marzo V (2000) N-acyl-dopamines: novel synthetic CB(1) cannabinoid-receptor ligands and inhibitors of anandamide inactivation with cannabimimetic activity in vitro and in vivo. *Biochem J* **351 Pt 3**: 817-824.

Black JW and Leff P (1983) Operational models of pharmacological agonism. *Proc R Soc Lond B Biol Sci* **220**(1219): 141-162.

Blume LC, Bass CE, Childers SR, Dalton GD, Roberts DC, Richardson JM, Xiao R, Selley DE and Howlett AC (2013) Striatal CB1 and D2 receptors regulate expression of each other, CRIP1A and delta opioid systems. *J Neurochem* **124**(6): 808-820.

Blume LC, Eldeeb K, Bass CE, Selley DE and Howlett AC (2015) Cannabinoid receptor interacting protein (CRIP1a) attenuates CB1R signaling in neuronal cells. *Cell Signal* **27**(3): 716-726.

Bojnik E, Turunc E, Armagan G, Kanit L, Benyhe S, Yalcin A and Borsodi A (2012) Changes in the cannabinoid (CB1) receptor expression level and G-protein activation in kainic acid induced seizures. *Epilepsy Res* **99**(1-2): 64-68.

Bonhaus DW, Chang LK, Kwan J and Martin GR (1998) Dual activation and inhibition of adenylyl cyclase by cannabinoid receptor agonists: evidence for agonist-specific trafficking of intracellular responses. *J Pharmacol Exp Ther* **287**(3): 884-888.

Bosier B, Hermans E and Lambert D (2008a) Differential modulation of AP-1- and CRE-driven transcription by cannabinoid agonists emphasizes functional selectivity at the CB1 receptor. *Br J Pharmacol* **155**(1): 24-33.

Bosier B, Lambert DM and Hermans E (2008b) Reciprocal influences of CB1 cannabinoid receptor agonists on ERK and JNK signalling in N1E-115 cells. *FEBS Lett* **582**(28): 3861-3867.

Bosier B, Muccioli GG, Hermans E and Lambert DM (2010) Functionally selective cannabinoid receptor signalling: therapeutic implications and opportunities. *Biochem Pharmacol* **80**(1): 1-12.

Bouaboula M, Bourrie B, Rinaldi-Carmona M, Shire D, Le Fur G and Casellas P (1995a) Stimulation of cannabinoid receptor CB1 induces krox-24 expression in human astrocytoma cells. *J Biol Chem* **270**(23): 13973-13980.

Bouaboula M, Poinot-Chazel C, Bourrie B, Canat X, Calandra B, Rinaldi-Carmona M, Le Fur G and Casellas P (1995b) Activation of mitogen-activated protein kinases by stimulation of the central cannabinoid receptor CB1. *Biochem J* **312**(Pt 2): 637-641.

Bylund DB and Toews ML (1993) Radioligand binding methods: practical guide and tips. *Am J Physiol* **265**(5 Pt 1): L421-429.

Callihan P, Mumaw J, Machacek DW, Stice SL and Hooks SB (2011) Regulation of stem cell pluripotency and differentiation by G protein coupled receptors. *Pharmacol Ther* **129**(3): 290-306.

Castane A, Valjent E, Ledent C, Parmentier M, Maldonado R and Valverde O (2002) Lack of CB1 cannabinoid receptors modifies nicotine behavioural responses, but not nicotine abstinence. *Neuropharmacology* **43**(5): 857-867.

Castaneto MS, Gorelick DA, Desrosiers NA, Hartman RL, Pirard S and Huestis MA (2014) Synthetic cannabinoids: Epidemiology, pharmacodynamics, and clinical implications. *Drug Alcohol Depend* **144**(1): 12-41.

Caulfield MP and Brown DA (1992) Cannabinoid receptor agonists inhibit Ca current in NG108-15 neuroblastoma cells via a pertussis toxin-sensitive mechanism. *Br J Pharmacol* **106**(2): 231-232.

Cawston EE, Redmond WJ, Breen CM, Grimsey NL, Connor M and Glass M (2013) Real-time characterization of cannabinoid receptor 1 (CB1) allosteric modulators reveals novel mechanism of action. *Br J Pharmacol* **170**(4): 893-907.

Chemin J, Monteil A, Perez-Reyes E, Nargeot J and Lory P (2001) Direct inhibition of T-type calcium channels by the endogenous cannabinoid anandamide. *EMBO J* **20**(24): 7033-7040.

Chen XP, Yang W, Fan Y, Luo JS, Hong K, Wang Z, Yan JF, Chen X, Lu JX, Benovic JL and Zhou NM (2010) Structural determinants in the second intracellular loop of the human cannabinoid CB1 receptor mediate selective coupling to G(s) and G(i). *Br J Pharmacol* **161**(8): 1817-1834.

Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. *Biochem Pharmacol* **22**(23): 3099-3108.

Chevaleyre V, Takahashi KA and Castillo PE (2006) Endocannabinoid-mediated synaptic plasticity in the CNS. *Annu Rev Neurosci* **29**: 37-76.

Childers SR, Pacheco MA, Bennett BA, Edwards TA, Hampson RE, Mu J and Deadwyler SA (1993) Cannabinoid receptors: G-protein-mediated signal transduction mechanisms. *Biochem Soc Symp* **59**: 27-50.

Christensen R, Kristensen PK, Bartels EM, Bliddal H and Astrup A (2007) Efficacy and safety of the weight-loss drug rimonabant: a meta-analysis of randomised trials. *Lancet* **370**(9600): 1706-1713.

Christopoulos A and Kenakin T (2002) G protein-coupled receptor allosterism and complexing. *Pharmacol Rev* **54**(2): 323-374.

Conigrave AD, Quinn SJ and Brown EM (2000) L-amino acid sensing by the extracellular Ca2+-sensing receptor. *Proc Natl Acad Sci U S A* **97**(9): 4814-4819.

Conklin BR and Bourne HR (1993) Structural elements of G alpha subunits that interact with G beta gamma, receptors, and effectors. *Cell* **73**(4): 631-641.

Conn PJ, Christopoulos A and Lindsley CW (2009) Allosteric modulators of GPCRs: a novel approach for the treatment of CNS disorders. *Nat Rev Drug Discov* **8**(1): 41-54.

Conn PJ, Lindsley CW, Meiler J and Niswender CM (2014) Opportunities and challenges in the discovery of allosteric modulators of GPCRs for treating CNS disorders. *Nat Rev Drug Discov* **13**(9): 692-708.

Cossu G, Ledent C, Fattore L, Imperato A, Bohme GA, Parmentier M and Fratta W (2001) Cannabinoid CB1 receptor knockout mice fail to self-administer morphine but not other drugs of abuse. *Behav Brain Res* **118**(1): 61-65.

Cota D (2007) CB1 receptors: emerging evidence for central and peripheral mechanisms that regulate energy balance, metabolism, and cardiovascular health. *Diabetes Metab Res Rev* **23**(7): 507-517.

Cota D, Marsicano G, Tschop M, Grubler Y, Flachskamm C, Schubert M, Auer D, Yassouridis A, Thone-Reineke C, Ortmann S, Tomassoni F, Cervino C, Nisoli E, Linthorst AC, Pasquali R, Lutz B, Stalla GK and Pagotto U (2003) The endogenous cannabinoid system affects energy balance via central orexigenic drive and peripheral lipogenesis. *J Clin Invest* **112**(3): 423-431.

Cravatt BF and Lichtman AH (2004) The endogenous cannabinoid system and its role in nociceptive behavior. *J Neurobiol* **61**(1): 149-160.

Croxford JL (2003) Therapeutic potential of cannabinoids in CNS disease. *CNS Drugs* **17**(3): 179-202.

D'Antona AM, Ahn KH and Kendall DA (2006) Mutations of CB1 T210 produce active and inactive receptor forms: correlations with ligand affinity, receptor stability, and cellular localization. *Biochemistry* **45**(17): 5606-5617.

Daniel H and Crepel F (2001) Control of Ca(2+) influx by cannabinoid and metabotropic glutamate receptors in rat cerebellar cortex requires K(+) channels. *J Physiol* **537**(Pt 3): 793-800.

Davies MN, Gloriam DE, Secker A, Freitas AA, Mendao M, Timmis J and Flower DR (2007) Proteomic applications of automated GPCR classification. *Proteomics* **7**(16): 2800-2814.

Deadwyler SA, Hampson RE, Mu J, Whyte A and Childers S (1995) Cannabinoids modulate voltage sensitive potassium A-current in hippocampal neurons via a cAMP-dependent process. *J Pharmacol Exp Ther* **273**(2): 734-743.

Derkinderen P, Valjent E, Toutant M, Corvol JC, Enslen H, Ledent C, Trzaskos J, Caboche J and Girault JA (2003) Regulation of extracellular signal-regulated kinase by cannabinoids in hippocampus. *J Neurosci* **23**(6): 2371-2382.

Devane WA, Dysarz FA, 3rd, Johnson MR, Melvin LS and Howlett AC (1988) Determination and characterization of a cannabinoid receptor in rat brain. *Mol Pharmacol* **34**(5): 605-613.

Di Marzo V, Bifulco M and De Petrocellis L (2004) The endocannabinoid system and its therapeutic exploitation. *Nat Rev Drug Discov* **3**(9): 771-784.

Ding Y, Qiu Y, Jing L, Thorn DA, Zhang Y and Li JX (2014) Behavioral effects of the cannabinoid CB1 receptor allosteric modulator ORG27569 in rats. *Pharmacol Res Perspect* **2**(6): e00069.

Dorr P, Westby M, Dobbs S, Griffin P, Irvine B, Macartney M, Mori J, Rickett G, Smith-Burchnell C, Napier C, Webster R, Armour D, Price D, Stammen B, Wood A and Perros M (2005) Maraviroc (UK-427,857), a potent, orally bioavailable, and selective small-molecule inhibitor of chemokine receptor

CCR5 with broad-spectrum anti-human immunodeficiency virus type 1 activity. *Antimicrob Agents Chemother* **49**(11): 4721-4732.

Drews J (2000) Drug discovery: a historical perspective. *Science* **287**(5460): 1960-1964.

Ehlert FJ (1988) Estimation of the affinities of allosteric ligands using radioligand binding and pharmacological null methods. *Mol Pharmacol* **33**(2): 187-194.

Ehlert FJ (2008) On the analysis of ligand-directed signaling at G protein-coupled receptors. *Naunyn Schmiedebergs Arch Pharmacol* **377**(4-6): 549-577.

Elphick MR and Egertová M (2009) Cannabinoid receptor genetics and evolution, in *The cannabinoid receptors* (Reggio PH ed) pp 123-149, Springer, New York.

Fay JF and Farrens DL (2012) A key agonist-induced conformational change in the cannabinoid receptor CB1 is blocked by the allosteric ligand Org 27569. *J Biol Chem* **287**(40): 33873-33882.

Felder CC, Joyce KE, Briley EM, Glass M, Mackie KP, Fahey KJ, Cullinan GJ, Hunden DC, Johnson DW, Chaney MO, Koppel GA and Brownstein M (1998) LY320135, a novel cannabinoid CB1 receptor antagonist, unmasks coupling of the CB1 receptor to stimulation of cAMP accumulation. *J Pharmacol Exp Ther* **284**(1): 291-297.

Felder CC, Joyce KE, Briley EM, Mansouri J, Mackie K, Blond O, Lai Y, Ma AL and Mitchell RL (1995) Comparison of the pharmacology and signal transduction of the human cannabinoid CB1 and CB2 receptors. *Mol Pharmacol* **48**(3): 443-450.

Foord SM (2002) Receptor classification: post genome. *Curr Opin Pharmacol* **2**(5): 561-566.

Fredriksson R, Lagerstrom MC, Lundin LG and Schioth HB (2003) The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints. *Mol Pharmacol* **63**(6): 1256-1272.

Galvez T, Urwyler S, Prezeau L, Mosbacher J, Joly C, Malitschek B, Heid J, Brabet I, Froestl W, Bettler B, Kaupmann K and Pin JP (2000) Ca(2+) requirement for high-affinity gamma-aminobutyric acid (GABA) binding at GABA(B)

receptors: involvement of serine 269 of the GABA(B)R1 subunit. *Mol Pharmacol* **57**(3): 419-426.

Gamage TF, Ignatowska-Jankowska BM, Wiley JL, Abdelrahman M, Trembleau L, Greig IR, Thakur GA, Tichkule R, Poklis J, Ross RA, Pertwee RG and Lichtman AH (2014) In-vivo pharmacological evaluation of the CB1-receptor allosteric modulator Org-27569. *Behav Pharmacol* **25**(2): 182-185.

Gaoni Y and Mechoulam R (1964) Isolation, structure, and partial synthesis of an active constituent of hashish. *J Am Chem Soc* **86**(8): 1646-1647.

Gerard CM, Mollereau C, Vassart G and Parmentier M (1991) Molecular cloning of a human cannabinoid receptor which is also expressed in testis. *Biochem J* **279**: 129-134.

Gimpl G, Burger K and Fahrenholz F (1997) Cholesterol as modulator of receptor function. *Biochemistry* **36**(36): 10959-10974.

Glass M, Dragunow M and Faull RL (1997) Cannabinoid receptors in the human brain: a detailed anatomical and quantitative autoradiographic study in the fetal, neonatal and adult human brain. *Neuroscience* **77**(2): 299-318.

Glass M and 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. *J Neurosci* **17**(14): 5327-5333.

Glass M and Northup JK (1999) Agonist selective regulation of G proteins by cannabinoid CB(1) and CB(2) receptors. *Mol Pharmacol* **56**(6): 1362-1369.

Goodfellow CE, Graham SE, Dragunow M and Glass M (2011) Characterization of NTera2/D1 cells as a model system for the investigation of cannabinoid function in human neurons and astrocytes. *J Neurosci Res* **89**(10): 1685-1697.

Govaerts SJ, Hermans E and Lambert DM (2004) Comparison of cannabinoid ligands affinities and efficacies in murine tissues and in transfected cells expressing human recombinant cannabinoid receptors. *Eur J Pharm Sci* **23**(3): 233-243.

Grady MA, Gasperoni TL and Kirkpatrick P (2003) Aripiprazole. *Nat Rev Drug Discov* **2**(6): 427-428.

Grazzini E, Guillon G, Mouillac B and Zingg HH (1998) Inhibition of oxytocin receptor function by direct binding of progesterone. *Nature* **392**(6675): 509-512.

Guo J and Ikeda SR (2004) Endocannabinoids modulate N-type calcium channels and G-protein-coupled inwardly rectifying potassium channels via CB1 cannabinoid receptors heterologously expressed in mammalian neurons. *Mol Pharmacol* **65**(3): 665-674.

Guzman M and Sanchez C (1999) Effects of cannabinoids on energy metabolism. *Life Sci* **65**(6-7): 657-664.

Hannun YA and Luberto C (2000) Ceramide in the eukaryotic stress response. *Trends Cell Biol* **10**(2): 73-80.

Harrington PE and Fotsch C (2007) Calcium sensing receptor activators: calcimimetics. *Curr Med Chem* **14**(28): 3027-3034.

Hashimotodani Y, Ohno-Shosaku T and Kano M (2007) Endocannabinoids and synaptic function in the CNS. *Neuroscientist* **13**(2): 127-137.

Herkenham M (1991) Characterization and localization of cannabinoid receptors in brain: an in vitro technique using slide-mounted tissue sections. *NIDA Res Monogr* **112**: 129-145.

Hillard CJ, Edgemond WS and Campbell WB (1995) Characterization of ligand binding to the cannabinoid receptor of rat brain membranes using a novel method: application to anandamide. *J Neurochem* **64**(2): 677-683.

Ho R, Ortiz D and Shea TB (2001) Amyloid-beta promotes calcium influx and neurodegeneration via stimulation of L voltage-sensitive calcium channels rather than NMDA channels in cultured neurons. *J Alzheimers Dis* **3**(5): 479-483.

Hoddah H, Marcantoni A, Comunanza V, Carabelli V and Carbone E (2009) L-type channel inhibition by CB1 cannabinoid receptors is mediated by PTX-sensitive G proteins and cAMP/PKA in GT1-7 hypothalamic neurons. *Cell Calcium* **46**(5-6): 303-312.

Hopkins AL and Groom CR (2002) The druggable genome. *Nat Rev Drug Discov* **1**(9): 727-730.

Horswill JG, Bali U, Shaaban S, Keily JF, Jeevaratnam P, Babbs AJ, Reynet C and Wong Kai In P (2007) PSNCBAM-1, a novel allosteric antagonist at

cannabinoid CB1 receptors with hypophagic effects in rats. *Br J Pharmacol* **152**(5): 805-814.

Horvath TL (2003) Endocannabinoids and the regulation of body fat: the smoke is clearing. *J Clin Invest* **112**(3): 323-326.

Hosking RD and Zajicek JP (2008) Therapeutic potential of cannabis in pain medicine. *Br J Anaesth* **101**(1): 59-68.

Howlett AC, Barth F, Bonner TI, Cabral G, Casellas P, Devane WA, Felder CC, Herkenham M, Mackie K, Martin BR, Mechoulam R and Pertwee RG (2002) International Union of Pharmacology. XXVII. Classification of cannabinoid receptors. *Pharmacol Rev* **54**(2): 161-202.

Howlett AC and Fleming RM (1984) Cannabinoid inhibition of adenylate cyclase. Pharmacology of the response in neuroblastoma cell membranes. *Mol Pharmacol* **26**(3): 532-538.

Howlett AC, Qualy JM and Khachatrian LL (1986) Involvement of Gi in the inhibition of adenylate cyclase by cannabimimetic drugs. *Mol Pharmacol* **29**(3): 307-313.

Hulme EC and Trevethick MA (2010) Ligand binding assays at equilibrium: validation and interpretation. *Br J Pharmacol* **161**(6): 1219-1237.

Idris AI, van 't Hof RJ, Greig IR, Ridge SA, Baker D, Ross RA and Ralston SH (2005) Regulation of bone mass, bone loss and osteoclast activity by cannabinoid receptors. *Nat Med* **11**(7): 774-779.

Iversen L and Chapman V (2002) Cannabinoids: a real prospect for pain relief? *Curr Opin Pharmacol* **2**(1): 50-55.

Jacoby E, Bouhelal R, Gerspacher M and Seuwen K (2006) The 7 TM G-protein-coupled receptor target family. *Chem Med Chem* **1**(8): 761-782.

Jensen AA and Spalding TA (2004) Allosteric modulation of G-protein coupled receptors. *Eur J Pharm Sci* **21**(4): 407-420.

Ji TH, Grossmann M and Ji I (1998) G protein-coupled receptors. I. Diversity of receptor-ligand interactions. *J Biol Chem* **273**(28): 17299-17302.

Ji TH, Murdoch WJ and Ji I (1995) Activation of membrane receptors. *Endocrine* **3**(3): 187-194.

Jin K, Xie L, Kim SH, Parmentier-Batteur S, Sun Y, Mao XO, Childs J and Greenberg DA (2004) Defective adult neurogenesis in CB1 cannabinoid receptor knockout mice. *Mol Pharmacol* **66**(2): 204-208.

Jin W, Brown S, Roche JP, Hsieh C, Celver JP, Kovoor A, Chavkin C and Mackie K (1999) Distinct domains of the CB1 cannabinoid receptor mediate desensitization and internalization. *J Neurosci* **19**(10): 3773-3780.

Kaminski NE, Abood ME, Kessler FK, Martin BR and Schatz AR (1992) Identification of a functionally relevant cannabinoid receptor on mouse spleen cells that is involved in cannabinoid-mediated immune modulation. *Mol Pharmacol* **42**(5): 736-742.

Kano M, Ohno-Shosaku T, Hashimotodani Y, Uchigashima M and Watanabe M (2009) Endocannabinoid-mediated control of synaptic transmission. *Physiol Rev* **89**(1): 309-380.

Kapur A, Samaniego P, Thakur GA, Makriyannis A and Abood ME (2008) Mapping the structural requirements in the CB1 cannabinoid receptor transmembrane helix II for signal transduction. *J Pharmacol Exp Ther* **325**(1): 341-348.

Katona I and Freund TF (2008) Endocannabinoid signaling as a synaptic circuit breaker in neurological disease. *Nat Med* **14**(9): 923-930.

Kenakin T (1995a) Agonist-receptor efficacy I: mechanisms of efficacy and receptor promiscuity. *Trends Pharmacol Sci* **16**(6): 188-192.

Kenakin T (1995b) Agonist-receptor efficacy. II. Agonist trafficking of receptor signals. *Trends Pharmacol Sci* **16**(7): 232-238.

Kenakin T (2003) Ligand-selective receptor conformations revisited: the promise and the problem. *Trends Pharmacol Sci* **24**(7): 346-354.

Kenakin T (2007) Functional selectivity through protean and biased agonism: who steers the ship? *Mol Pharmacol* **72**(6): 1393-1401.

Kenakin T (2011) Functional selectivity and biased receptor signaling. *J Pharmacol Exp Ther* **336**(2): 296-302.

Kenakin T (2012a) The potential for selective pharmacological therapies through biased receptor signaling. *BMC Pharmacol Toxicol* **13**: 3.

Kenakin T and Christopoulos A (2013) Signalling bias in new drug discovery: detection, quantification and therapeutic impact. *Nat Rev Drug Discov* **12**(3): 205-216.

Kenakin T and Miller LJ (2010) Seven transmembrane receptors as shapeshifting proteins: the impact of allosteric modulation and functional selectivity on new drug discovery. *Pharmacol Rev* **62**(2): 265-304.

Kenakin T, Watson C, Muniz-Medina V, Christopoulos A and Novick S (2012) A simple method for quantifying functional selectivity and agonist bias. *ACS Chem Neurosci* **3**(3): 193-203.

Kenakin TP (2012b) Biased signalling and allosteric machines: new vistas and challenges for drug discovery. *Br J Pharmacol* **165**(6): 1659-1669.

Kohno M, Hasegawa H, Inoue A, Muraoka M, Miyazaki T, Oka K and Yasukawa M (2006) Identification of N-arachidonylglycine as the endogenous ligand for orphan G-protein-coupled receptor GPR18. *Biochem Biophys Res Commun* **347**(3): 827-832.

Kolakowski LF, Jr. (1994) GCRDb: a G-protein-coupled receptor database. *Receptors Channels* **2**(1): 1-7.

Kolesnick RN and Krönke M (1998) Regulation of ceramide production and apoptosis. *Annu Rev Physiol* **60**(1): 643-665.

Kouznetsova M, Kelley B, Shen M and Thayer SA (2002) Desensitization of cannabinoid-mediated presynaptic inhibition of neurotransmission between rat hippocampal neurons in culture. *Mol Pharmacol* **61**(3): 477-485.

Kovoor A, Nappey V, Kieffer BL and Chavkin C (1997) Mu and delta opioid receptors are differentially desensitized by the coexpression of beta-adrenergic receptor kinase 2 and beta-arrestin 2 in xenopus oocytes. *J Biol Chem* **272**(44): 27605-27611.

Kristiansen K (2004) Molecular mechanisms of ligand binding, signaling, and regulation within the superfamily of G-protein-coupled receptors: molecular modeling and mutagenesis approaches to receptor structure and function. *Pharmacol Ther* **103**(1): 21-80.

Lallemand F and de Witte P (2005) Ethanol induces higher BEC in CB1 cannabinoid receptor knockout mice while decreasing ethanol preference. *Alcohol Alcohol* **40**(1): 54-62.

Lappano R and Maggiolini M (2011) G protein-coupled receptors: novel targets for drug discovery in cancer. *Nat Rev Drug Discov* **10**(1): 47-60.

Laprairie RB, Bagher AM, Kelly ME, Dupre DJ and Denovan-Wright EM (2014) Type 1 cannabinoid receptor ligands display functional selectivity in a cell culture model of striatal medium spiny projection neurons. *J Biol Chem* **289**(36): 24845-24862.

Lauckner JE, Hille B and Mackie K (2005) 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* **102**(52): 19144-19149.

Le Foll B and Goldberg SR (2005) Cannabinoid CB1 receptor antagonists as promising new medications for drug dependence. *J Pharmacol Exp Ther* **312**(3): 875-883.

Leach K, Loiacono RE, Felder CC, McKinzie DL, Mogg A, Shaw DB, Sexton PM and Christopoulos A (2010) Molecular mechanisms of action and in vivo validation of an M4 muscarinic acetylcholine receptor allosteric modulator with potential antipsychotic properties. *Neuropsychopharmacology* **35**(4): 855-869.

Leach K, Sexton PM and Christopoulos A (2007) Allosteric GPCR modulators: taking advantage of permissive receptor pharmacology. *Trends Pharmacol Sci* **28**(8): 382-389.

Leff P (1995) The two-state model of receptor activation. *Trends Pharmacol Sci* **16**(3): 89-97.

Liu J, Gao B, Mirshahi F, Sanyal AJ, Khanolkar AD, Makriyannis A and Kunos G (2000) Functional CB1 cannabinoid receptors in human vascular endothelial cells. *Biochem J* **346** (Pt 3): 835-840.

Long JZ, Li W, Booker L, Burston JJ, Kinsey SG, Schlosburg JE, Pavon FJ, Serrano AM, Selley DE, Parsons LH, Lichtman AH and Cravatt BF (2009) Selective blockade of 2-arachidonoylglycerol hydrolysis produces cannabinoid behavioral effects. *Nat Chem Biol* **5**(1): 37-44.

Luchicchi A and Pistis M (2012) Anandamide and 2-arachidonoylglycerol: pharmacological properties, functional features, and emerging specificities of the two major endocannabinoids. *Mol Neurobiol* **46**(2): 374-392.

Ludanyi A, Eross L, Czirjak S, Vajda J, Halasz P, Watanabe M, Palkovits M, Magloczky Z, Freund TF and Katona I (2008) Downregulation of the CB1 cannabinoid receptor and related molecular elements of the endocannabinoid system in epileptic human hippocampus. *J Neurosci* **28**(12): 2976-2990.

Lukyanetz EA (1998) Diversity and properties of calcium channel types in NG108-15 hybrid cells. *Neuroscience* **87**(1): 265-274.

Mackie K and Hille B (1992) Cannabinoids inhibit N-type calcium channels in neuroblastoma-glioma cells. *Proc Natl Acad Sci U S A* **89**(9): 3825-3829.

Mackie K, Lai Y, Westenbroek R and 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. *J Neurosci* **15**(10): 6552-6561.

Mailman RB (2007) GPCR functional selectivity has therapeutic impact. *Trends Pharmacol Sci* **28**(8): 390-396.

Marsicano G, Goodenough S, Monory K, Hermann H, Eder M, Cannich A, Azad SC, Cascio MG, Gutierrez SO, van der Stelt M, Lopez-Rodriguez ML, Casanova E, Schutz G, Zieglgansberger W, Di Marzo V, Behl C and Lutz B (2003) CB1 cannabinoid receptors and on-demand defense against excitotoxicity. *Science* **302**(5642): 84-88.

Martini L, Waldhoer M, Pusch M, Kharazia V, Fong J, Lee JH, Freissmuth C and Whistler JL (2007) Ligand-induced down-regulation of the cannabinoid 1 receptor is mediated by the G-protein-coupled receptor-associated sorting protein GASP1. *FASEB J* **21**(3): 802-811.

Massi P, Fuzio D, Vigano D, Sacerdote P and Parolaro D (2000) Relative involvement of cannabinoid CB(1) and CB(2) receptors in the Delta(9)-tetrahydrocannabinol-induced inhibition of natural killer activity. *Eur J Pharmacol* **387**(3): 343-347.

Massot O, Rousselle JC, Fillion MP, Grimaldi B, Cloez-Tayarani I, Fugelli A, Prudhomme N, Seguin L, Rousseau B, Plantefol M, Hen R and Fillion G (1996) 5-hydroxytryptamine-moduline, a new endogenous cerebral peptide, controls the serotonergic activity via its specific interaction with 5-hydroxytryptamine1B/1D receptors. *Mol Pharmacol* **50**(4): 752-762.

Matias I and Di Marzo V (2007) Endocannabinoids and the control of energy balance. *Trends Endocrinol Metab* **18**(1): 27-37.

Matsuda LA, Lolait SJ, Brownstein MJ, Young AC and Bonner TI (1990) Structure of a cannabinoid receptor and functional expression of the cloned cDNA. *Nature* **346**(6284): 561-564.

May LT, Leach K, Sexton PM and Christopoulos A (2007) Allosteric modulation of G protein-coupled receptors. *Annu Rev Pharmacol Toxicol* **47**: 1-51.

McAllister SD, Rizvi G, Anavi-Goffer S, Hurst DP, Barnett-Norris J, Lynch DL, Reggio PH and Abood ME (2003) An aromatic microdomain at the cannabinoid CB(1) receptor constitutes an agonist/inverse agonist binding region. *J Med Chem* **46**(24): 5139-5152.

McAllister SD, Tao Q, Barnett-Norris J, Buehner K, Hurst DP, Guarnieri F, Reggio PH, Nowell Harmon KW, Cabral GA and Abood ME (2002) A critical role for a tyrosine residue in the cannabinoid receptors for ligand recognition. *Biochem Pharmacol* **63**(12): 2121-2136.

McPartland JM, Glass M and Pertwee RG (2007) Meta-analysis of cannabinoid ligand binding affinity and receptor distribution: interspecies differences. *Br J Pharmacol* **152**(5): 583-593.

Meye FJ, Ramakers GM and Adan RA (2014) The vital role of constitutive GPCR activity in the mesolimbic dopamine system. *Transl Psychiatry* **4**: e361.

Mitchell PB and Morris MJ (2007) Depression and anxiety with rimonabant. *Lancet* **370**(9600): 1671-1672.

Montero C, Campillo NE, Goya P and Paez JA (2005) Homology models of the cannabinoid CB1 and CB2 receptors. A docking analysis study. *Eur J Med Chem* **40**(1): 75-83.

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

Muccioli GG, Martin D, Scriba GK, Poppitz W, Poupaert JH, Wouters J and Lambert DM (2005) Substituted 5,5'-diphenyl-2-thioxoimidazolidin-4-one as CB1 cannabinoid receptor ligands: synthesis and pharmacological evaluation. *J Med Chem* **48**(7): 2509-2517.

Mukhopadhyay S and Howlett AC (2005) Chemically distinct ligands promote differential CB1 cannabinoid receptor-Gi protein interactions. *Mol Pharmacol* **67**(6): 2016-2024.

Mukhopadhyay S, Shim JY, Assi AA, Norford D and Howlett AC (2002) CB(1) cannabinoid receptor-G protein association: a possible mechanism for differential signaling. *Chem Phys Lipids* **121**(1-2): 91-109.

Munro S, Thomas KL and Abu-Shaar M (1993) Molecular characterization of a peripheral receptor for cannabinoids. *Nature* **365**(6441): 61-65.

Nathans J and Hogness DS (1983) Isolation, sequence analysis, and intron-exon arrangement of the gene encoding bovine rhodopsin. *Cell* **34**(3): 807-814.

Netzeband JG, Conroy SM, Parsons KL and Gruol DL (1999) Cannabinoids enhance NMDA-elicited Ca2+ signals in cerebellar granule neurons in culture. *J Neurosci* **19**(20): 8765-8777.

Neves SR, Ram PT and Iyengar R (2002) G protein pathways. *Science* **296**(5573): 1636-1639.

Niehaus JL, Liu Y, Wallis KT, Egertova M, Bhartur SG, Mukhopadhyay S, Shi S, He H, Selley DE, Howlett AC, Elphick MR and Lewis DL (2007) CB1 cannabinoid receptor activity is modulated by the cannabinoid receptor interacting protein CRIP 1a. *Mol Pharmacol* **72**(6): 1557-1566.

Nogueron MI, Porgilsson B, Schneider WE, Stucky CL and Hillard CJ (2001) Cannabinoid receptor agonists inhibit depolarization-induced calcium influx in cerebellar granule neurons. *J Neurochem* **79**(2): 371-381.

Ofek O, Karsak M, Leclerc N, Fogel M, Frenkel B, Wright K, Tam J, Attar-Namdar M, Kram V, Shohami E, Mechoulam R, Zimmer A and Bab I (2006) Peripheral cannabinoid receptor, CB2, regulates bone mass. *Proc Natl Acad Sci U S A* **103**(3): 696-701.

Okamoto Y, Morishita J, Tsuboi K, Tonai T and Ueda N (2004) Molecular characterization of a phospholipase D generating anandamide and its congeners. *J Biol Chem* **279**(7): 5298-5305.

Onaivi ES, Ishiguro H, Gong JP, Patel S, Perchuk A, Meozzi PA, Myers L, Mora Z, Tagliaferro P, Gardner E, Brusco A, Akinshola BE, Liu QR, Hope B, Iwasaki S, Arinami T, Teasenfitz L and Uhl GR (2006) Discovery of the presence and

functional expression of cannabinoid CB2 receptors in brain. *Ann N Y Acad Sci* **1074**: 514-536.

Overington JP, Al-Lazikani B and Hopkins AL (2006) How many drug targets are there? *Nat Rev Drug Discov* **5**(12): 993-996.

Pamplona FA, Ferreira J, Menezes de Lima O, Jr., Duarte FS, Bento AF, Forner S, Villarinho JG, Bellocchio L, Wotjak CT, Lerner R, Monory K, Lutz B, Canetti C, Matias I, Calixto JB, Marsicano G, Guimaraes MZ and Takahashi RN (2012) Anti-inflammatory lipoxin A4 is an endogenous allosteric enhancer of CB1 cannabinoid receptor. *Proc Natl Acad Sci U S A* **109**(51): 21134-21139.

Pan B, Wang W, Zhong P, Blankman JL, Cravatt BF and Liu QS (2011) Alterations of endocannabinoid signaling, synaptic plasticity, learning, and memory in monoacylglycerol lipase knock-out mice. *J Neurosci* **31**(38): 13420-13430.

Pan X, Ikeda SR and Lewis DL (1996) Rat brain cannabinoid receptor modulates N-type Ca2+ channels in a neuronal expression system. *Mol Pharmacol* **49**(4): 707-714.

Parmentier-Batteur S, Jin K, Mao XO, Xie L and Greenberg DA (2002) Increased severity of stroke in CB1 cannabinoid receptor knock-out mice. *J Neurosci* **22**(22): 9771-9775.

Pertwee RG (2002) Cannabinoids and multiple sclerosis. *Pharmacol Ther* **95**(2): 165-174.

Pertwee RG (2005) The therapeutic potential of drugs that target cannabinoid receptors or modulate the tissue levels or actions of endocannabinoids. *AAPS J* **7**(3): E625-654.

Pertwee RG and Ross RA (2002) Cannabinoid receptors and their ligands. *Prostaglandins Leukot Essent Fatty Acids* **66**(2-3): 101-121.

Picone RP, Khanolkar AD, Xu W, Ayotte LA, Thakur GA, Hurst DP, Abood ME, Reggio PH, Fournier DJ and Makriyannis A (2005) (-)-7'-Isothiocyanato-11-hydroxy-1',1'-dimethylheptylhexahydrocannabinol (AM841), a high-affinity electrophilic ligand, interacts covalently with a cysteine in helix six and activates the CB1 cannabinoid receptor. *Mol Pharmacol* **68**(6): 1623-1635.

Piomelli D, Tarzia G, Duranti A, Tontini A, Mor M, Compton TR, Dasse O, Monaghan EP, Parrott JA and Putman D (2006) Pharmacological profile of

the selective FAAH inhibitor KDS-4103 (URB597). *CNS Drug Rev* **12**(1): 21-38.

Price MR, Baillie GL, Thomas A, Stevenson LA, Easson M, Goodwin R, McLean A, McIntosh L, Goodwin G, Walker G, Westwood P, Marrs J, Thomson F, Cowley P, Christopoulos A, Pertwee RG and Ross RA (2005) Allosteric modulation of the cannabinoid CB1 receptor. *Mol Pharmacol* **68**(5): 1484-1495.

Pryce G and Baker D (2007) Control of spasticity in a multiple sclerosis model is mediated by CB1, not CB2, cannabinoid receptors. *Br J Pharmacol* **150**(4): 519-525.

Puighermanal E, Marsicano G, Busquets-Garcia A, Lutz B, Maldonado R and Ozaita A (2009) Cannabinoid modulation of hippocampal long-term memory is mediated by mTOR signaling. *Nat Neurosci* **12**(9): 1152-1158.

Radeff-Huang J, Seasholtz TM, Matteo RG and Brown JH (2004) G protein mediated signaling pathways in lysophospholipid induced cell proliferation and survival. *J Cell Biochem* **92**(5): 949-966.

Raehal KM, Walker JK and Bohn LM (2005) Morphine side effects in beta-arrestin 2 knockout mice. *J Pharmacol Exp Ther* **314**(3): 1195-1201.

Randall MD, Harris D, Kendall DA and Ralevic V (2002) Cardiovascular effects of cannabinoids. *Pharmacol Ther* **95**(2): 191-202.

Rees S, Morrow D and Kenakin T (2002) GPCR drug discovery through the exploitation of allosteric drug binding sites. *Receptors Channels* **8**(5-6): 261-268.

Reggio PH (1999) Ligand-ligand and ligand-receptor approaches to modeling the cannabinoid CB1 and CB2 receptors: achievements and challenges. *Curr Med Chem* **6**(8): 665-683.

Reggio PH, Basu-Dutt S, Barnett-Norris J, Castro MT, Hurst DP, Seltzman HH, Roche MJ, Gilliam AF, Thomas BF, Stevenson LA, Pertwee RG and Abood ME (1998) The bioactive conformation of aminoalkylindoles at the cannabinoid CB1 and CB2 receptors: insights gained from (E)- and (Z)-naphthylidene indenes. *J Med Chem* **41**(26): 5177-5187.

Rice AS, Farquhar-Smith WP and Nagy I (2002) Endocannabinoids and pain: spinal and peripheral analgesia in inflammation and neuropathy. *Prostaglandins Leukot Essent Fatty Acids* **66**(2-3): 243-256.

Ross RA (2007a) Allosterism and cannabinoid CB(1) receptors: the shape of things to come. *Trends Pharmacol Sci* **28**(11): 567-572.

Ross RA (2007b) Tuning the endocannabinoid system: allosteric modulators of the CB1 receptor. *Br J Pharmacol* **152**(5): 565-566.

Rubino T, Vigano D, Premoli F, Castiglioni C, Bianchessi S, Zippel R and Parolaro D (2006) Changes in the expression of G protein-coupled receptor kinases and beta-arrestins in mouse brain during cannabinoid tolerance: a role for RAS-ERK cascade. *Mol Neurobiol* **33**(3): 199-213.

Rueda D, Galve-Roperh I, Haro A and Guzman M (2000) The CB(1) cannabinoid receptor is coupled to the activation of c-Jun N-terminal kinase. *Mol Pharmacol* **58**(4): 814-820.

Rueda D, Navarro B, Martinez-Serrano A, Guzman M and Galve-Roperh I (2002) The endocannabinoid anandamide inhibits neuronal progenitor cell differentiation through attenuation of the Rap1/B-Raf/ERK pathway. *J Biol Chem* **277**(48): 46645-46650.

Ryan W, Singer M, Razdan RK, Compton DR and Martin BR (1995) A novel class of potent tetrahydrocannabinols (THCS): 2'-yne-delta 8- and delta 9-THCS. *Life Sci* **56**(23-24): 2013-2020.

Ryberg E, Larsson N, Sjogren S, Hjorth S, Hermansson NO, Leonova J, Elebring T, Nilsson K, Drmota T and Greasley PJ (2007) The orphan receptor GPR55 is a novel cannabinoid receptor. *Br J Pharmacol* **152**(7): 1092-1101.

Sacerdote P, Massi P, Panerai AE and Parolaro D (2000) In vivo and in vitro treatment with the synthetic cannabinoid CP55, 940 decreases the in vitro migration of macrophages in the rat: involvement of both CB1 and CB2 receptors. *J Neuroimmunol* **109**(2): 155-163.

Sanchez C, Galve-Roperh I, Rueda D and Guzman M (1998) Involvement of sphingomyelin hydrolysis and the mitogen-activated protein kinase cascade in the Delta9-tetrahydrocannabinol-induced stimulation of glucose metabolism in primary astrocytes. *Mol Pharmacol* **54**(5): 834-843.

Sanchez C, Rueda D, Segui B, Galve-Roperh I, Levade T and Guzman M (2001) The CB(1) cannabinoid receptor of astrocytes is coupled to sphingomyelin hydrolysis through the adaptor protein fan. *Mol Pharmacol* **59**(5): 955-959.

Schetz JA and Sibley DR (1997) Zinc allosterically modulates antagonist binding to cloned D1 and D2 dopamine receptors. *J Neurochem* **68**(5): 1990-1997.

Schetz JA and Sibley DR (2001) The binding-site crevice of the D4 dopamine receptor is coupled to three distinct sites of allosteric modulation. *J Pharmacol Exp Ther* **296**(2): 359-363.

Schicho R and Storr M (2014) IBD: Patients with IBD find symptom relief in the Cannabis field. *Nat Rev Gastroenterol Hepatol* **11**(3): 142-143.

Schwartz TW and Rosenkilde MM (1996) Is there a 'lock' for all agonist 'keys' in 7TM receptors? *Trends Pharmacol Sci* **17**(6): 213-216.

Segovia G, Mora F, Crossman AR and Brotchie JM (2003) Effects of CB1 cannabinoid receptor modulating compounds on the hyperkinesia induced by high-dose levodopa in the reserpine-treated rat model of Parkinson's disease. *Mov Disord* **18**(2): 138-149.

Shim JY (2010) Understanding functional residues of the cannabinoid CB1. *Curr Top Med Chem* **10**(8): 779-798.

Shim JY, Bertalovitz AC and Kendall DA (2011) Identification of essential cannabinoid-binding domains: structural insights into early dynamic events in receptor activation. *J Biol Chem* **286**(38): 33422-33435.

Shim JY and Howlett AC (2002) Prediction of the binding conformation of the potent aminialkylindole agonist WIN55212-2 in the brain cannabinoid receptor. *American Chemical Society Abstracts*.

Shim JY and Padgett L (2013) Functional Residues Essential for the Activation of the CB1 Cannabinoid Receptor. *Methods Enzymol* **520**(1): 337-355.

Shore DM, Baillie GL, Hurst DH, Navas F, 3rd, Seltzman HH, Marcu JP, Abood ME, Ross RA and Reggio PH (2014) Allosteric modulation of a cannabinoid G protein-coupled receptor: Binding site elucidation and relationship to G protein signaling. *J Biol Chem* **289**(9): 5828-5845.

Smith PB, Compton DR, Welch SP, Razdan RK, Mechoulam R and Martin BR (1994) The pharmacological activity of anandamide, a putative endogenous cannabinoid, in mice. *J Pharmacol Exp Ther* **270**(1): 219-227.

Smith TH, Blume LC, Straiker A, Cox JO, David BG, McVoy JR, Sayers KW, Poklis JL, Abdullah RA, Egertova M, Chen CK, Mackie K, Elphick MR, Howlett AC and Selley DE (2015) Cannabinoid receptor-interacting protein 1a modulates CB1 receptor signaling and regulation. *Mol Pharmacol* **87**(4): 747-765.

Song ZH and Bonner TI (1996) A lysine residue of the cannabinoid receptor is critical for receptor recognition by several agonists but not WIN55212-2. *Mol Pharmacol* **49**(5): 891-896.

Stauffer B, Wallis KT, Wilson SP, Egertova M, Elphick MR, Lewis DL and Hardy LR (2011) CRIP1a switches cannabinoid receptor agonist/antagonist-mediated protection from glutamate excitotoxicity. *Neurosci Lett* **503**(3): 224-228.

Sugiura T, Kodaka T, Kondo S, Tonegawa T, Nakane S, Kishimoto S, Yamashita A and Waku K (1997) Inhibition by 2-arachidonoylglycerol, a novel type of possible neuromodulator, of the depolarization-induced increase in intracellular free calcium in neuroblastoma x glioma hybrid NG108-15 cells. *Biochem Biophys Res Commun* **233**(1): 207-210.

Sugiura T, Kondo S, Sukagawa A, Nakane S, Shinoda A, Itoh K, Yamashita A and Waku K (1995) 2-Arachidonoylglycerol: a possible endogenous cannabinoid receptor ligand in brain. *Biochem Biophys Res Commun* **215**(1): 89-97.

Taguchi K, Ueda M and Kubo T (1997) Effects of cAMP and cGMP on L-type calcium channel currents in rat mesenteric artery cells. *Jpn J Pharmacol* **74**(2): 179-186.

Tam J, Ofek O, Fride E, Ledent C, Gabet Y, Muller R, Zimmer A, Mackie K, Mechoulam R, Shohami E and Bab I (2006) Involvement of neuronal cannabinoid receptor CB1 in regulation of bone mass and bone remodeling. *Mol Pharmacol* **70**(3): 786-792.

Tao Q and Abood ME (1998) Mutation of a highly conserved aspartate residue in the second transmembrane domain of the cannabinoid receptors, CB1 and CB2, disrupts G-protein coupling. *J Pharmacol Exp Ther* **285**(2): 651-658.

Thomas BF, Gilliam AF, Burch DF, Roche MJ and Seltzman HH (1998) Comparative receptor binding analyses of cannabinoid agonists and antagonists. *J Pharmacol Exp Ther* **285**(1): 285-292.

Thomas EA, Carson MJ, Neal MJ and Sutcliffe JG (1997) Unique allosteric regulation of 5-hydroxytryptamine receptor-mediated signal transduction by oleamide. *Proc Natl Acad Sci U S A* **94**(25): 14115-14119.

Twitchell W, Brown S and Mackie K (1997) Cannabinoids inhibit N- and P/Q-type calcium channels in cultured rat hippocampal neurons. *J Neurophysiol* **78**(1): 43-50.

Urban JD, Vargas GA, von Zastrow M and Mailman RB (2007) Aripiprazole has functionally selective actions at dopamine D2 receptor-mediated signaling pathways. *Neuropsychopharmacology* **32**(1): 67-77.

Valant C, Felder CC, Sexton PM and Christopoulos A (2012) Probe dependence in the allosteric modulation of a G protein-coupled receptor: implications for detection and validation of allosteric ligand effects. *Mol Pharmacol* **81**(1): 41-52.

Valant C, May LT, Aurelio L, Chuo CH, White PJ, Baltos JA, Sexton PM, Scammells PJ and Christopoulos A (2014) Separation of on-target efficacy from adverse effects through rational design of a bitopic adenosine receptor agonist. *Proc Natl Acad Sci U S A* **111**(12): 4614-4619.

Vallee M, Vitiello S, Bellocchio L, Hebert-Chatelain E, Monlezun S, Martin-Garcia E, Kasanetz F, Baillie GL, Panin F, Cathala A, Roullot-Lacarriere V, Fabre S, Hurst DP, Lynch DL, Shore DM, Deroche-Gamonet V, Spampinato U, Revest JM, Maldonado R, Reggio PH, Ross RA, Marsicano G and Piazza PV (2014) Pregnenolone can protect the brain from cannabis intoxication. *Science* **343**(6166): 94-98.

van der Westhuizen ET, Breton B, Christopoulos A and Bouvier M (2014) Quantification of ligand bias for clinically relevant beta2-adrenergic receptor ligands: implications for drug taxonomy. *Mol Pharmacol* **85**(3): 492-509.

Varga EV, Georgieva T, Tumati S, Alves I, Salamon Z, Tollin G, Yamamura HI and Roeske WR (2008) Functional selectivity in cannabinoid signaling. *Curr Mol Pharmacol* **1**(3): 273-284.

Varga EV, Navratilova E, Stropova D, Jambrosic J, Roeske WR and Yamamura HI (2004) Agonist-specific regulation of the delta-opioid receptor. *Life Sci* **76**(6): 599-612.

Vasquez C, Navarro-Polanco RA, Huerta M, Trujillo X, Andrade F, Trujillo-Hernandez B and Hernandez L (2003) Effects of cannabinoids on endogenous K+ and Ca2+ currents in HEK293 cells. *Can J Physiol Pharmacol* **81**(5): 436-442.

Venkatakrishnan AJ, Deupi X, Lebon G, Tate CG, Schertler GF and Babu MM (2013) Molecular signatures of G-protein-coupled receptors. *Nature* **494**(7436): 185-194.

Vogel Z, Barg J, Levy R, Saya D, Heldman E and Mechoulam R (1993) Anandamide, a brain endogenous compound, interacts specifically with cannabinoid receptors and inhibits adenylate cyclase. *J Neurochem* **61**(1): 352-355.

Wang X, Horswill JG, Whalley BJ and Stephens GJ (2011) Effects of the allosteric antagonist 1-(4-chlorophenyl)-3-[3-(6-pyrrolidin-1-ylpyridin-2-yl)phenyl]urea (PSNCBAM-1) on CB1 receptor modulation in the cerebellum. *Mol Pharmacol* **79**(4): 758-767.

Wartmann M, Campbell D, Subramanian A, Burstein SH and Davis RJ (1995) The MAP kinase signal transduction pathway is activated by the endogenous cannabinoid anandamide. *FEBS Lett* **359**(2-3): 133-136.

Wennerberg M, Balendran A, Clapham JC and Vauquelin G (2010) Unravelling the complex dissociation of [(3)H]-rimonabant from plated CB(1) cannabinoid receptor-expressing cells. *Fundam Clin Pharmacol* **24**(2): 181-187.

Wennerberg M, Cheng L, Hjorth S, Clapham JC, Balendran A and Vauquelin G (2011) Binding properties of antagonists to cannabinoid receptors in intact cells. *Fundam Clin Pharmacol* **25**(2): 200-210.

Wess J (1997) G-protein-coupled receptors: molecular mechanisms involved in receptor activation and selectivity of G-protein recognition. *FASEB J* **11**(5): 346-354.

Wiley JL, Compton DR, Dai D, Lainton JA, Phillips M, Huffman JW and Martin BR (1998) Structure-activity relationships of indole- and pyrrole-derived cannabinoids. *J Pharmacol Exp Ther* **285**(3): 995-1004.

Wiley JL and Martin BR (2009) Preclinical pharmacological and brain bioassay systems for CB1 cannabinoid receptors, in *The Cannabinoid Receptors* pp 329-360, Springer, New York.

Wisler JW, DeWire SM, Whalen EJ, Violin JD, Drake MT, Ahn S, Shenoy SK and Lefkowitz RJ (2007) A unique mechanism of β-blocker action: carvedilol stimulates β-arrestin signaling. *Proc Natl Acad Sci U S A* **104**(42): 16657-16662.

Yeagle PL, Choi G and Albert AD (2001) Studies on the structure of the G-protein-coupled receptor rhodopsin including the putative G-protein binding site in unactivated and activated forms. *Biochemistry* **40**(39): 11932-11937.

Zhang Y, Seltzman HH, Brackeen M and Thomas BF (2009) Structure–activity relationships and conformational freedom of CB1 receptor antagonists and inverse agonists, in *The cannabinoid receptors* pp 95-119, Springer, New York.

Zhou D and Song ZH (2001) CB1 cannabinoid receptor-mediated neurite remodeling in mouse neuroblastoma N1E-115 cells. *J Neurosci Res* **65**(4): 346-353.

Zimmer A, Zimmer AM, Hohmann AG, Herkenham M and Bonner TI (1999) Increased mortality, hypoactivity, and hypoalgesia in cannabinoid CB1 receptor knockout mice. *Proc Natl Acad Sci U S A* **96**(10): 5780-5785.

Zygmunt PM, Petersson J, Andersson DA, Chuang H, Sorgard M, Di Marzo V, Julius D and Hogestatt ED (1999) Vanilloid receptors on sensory nerves mediate the vasodilator action of anandamide. *Nature* **400**(6743): 452-457.