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Effects of Cannabinoid Receptor Interacting Protein (CRIP1a) on Cannabinoid Receptor (CB1) Function

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Effects of Cannabinoid Receptor Interacting Protein ($CRIP_{1a}$) on Cannabinoid (CB_1) Receptor Function.

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

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List of Abbreviations

2-AG	2-arachidonoyl glycerol
[^{35}S]GTPγS	guanosine 5'-O-[gamma-thio]triphosphate
AC	adenylyl cyclase
ADP	adenosine diphosphate
AEA	anandamide
ANOVA	analysis of variance
AP-2	clathrin adaptor protein complex
AtT20	pituitary adenoma cell line
AUC	area under the curve
B_{max}	binding sites (max)
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CB	cannabinoid
CBD	cannabidiol
CBV	cannabivarin
cDNA	complementary deoxyribonucleic acid
CHAPS	3-[3(-cholamindopropyl) dimethylammonio] propanesulfonate
CHO	Chinese hamster ovary
CNS	central nervous system
CP	CP 55,940
CRIP	cannabinoid receptor interacting protein
DA	dopamine

DAGL	diacylglycerol lipase
DALN	desacetyllevonantradol
DMEM	Dulbecco's modified eagle medium
DNA	deoxyribonucleic acid
DTT	dithiothreitol
E_{max}	maximal effect
EC_{50}	half maximal (50%) effective concentration
EDTA	ethylenediaminetetraacetic acid
ERK	extracellular signal regulated kinase
FAN	factor associated with neutral sphingomyelinase activation
FBS	fetal bovine serum
G-protein	guanine nucleotide binding protein
G418	Geneticin
GASP	G-protein coupled receptor associated sorting protein
GDP	guanosine diphosphate
GPCR	G-protein coupled receptor
GRK	G-protein coupled receptor kinase
GST	glutathione S transferase
GTP	guanosine triphosphate
GTPase	guanosine triphosphate hydrolase
HEK	human embryonic kidney
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IBMX	isobutylmethylxanthine

IPTG	isopropyl thiogalactoside
IR	infrared
JNK	c-Jun N-terminal kinase
K_D	equilibrium dissociation constant
LAMP	lysosomal associated membrane protein
LC-ESI-MS-MS	liquid chromatography electrospray ionization tandem mass spectrometry
Levo	levonantradol
MAPK	mitogen activated protein kinase
MGL	monoacylcyclyceride lipase
MethA	methanandamide
mRNA	messenger ribonucleic acid
Nol Eth	noladin ether
NTR	neurotensin receptor
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDZ	PSD-95, Disc large protein and ZO-1
PLA2	phospholipase A2
PLC	phospholipase C
P/S	penicillian/streptomycin
PTX	pertussis toxin
PVDF	polyvinylidene difluoride
RT	room temperature

SCG	superior cervical ganglion
SDS	sodium dodecyl sulfate
Sf9	*Spodoptera frugiperda*
shRNA	short hairpin ribonucleic acid
SR1	SR141716A
SR2	SR144528
TBST	Tris buffered saline with Tween-20
THC	Δ^9-tetrahydrocannabinol
Tris	tris(hydroxymethyl)aminomethane
VTA	ventral tegmental area
WIN	WIN 55,212-2

Abstract

EFFECTS OF CANNABINOID RECEPTOR INTERACTING PROTEIN ($CRIP_{1a}$) ON CANNABINOID (CB_1) RECEPTOR FUNCTION.

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Major Director: Dana E. Selley, Ph.D., Department of Pharmacology and Toxicology

This dissertation examines modulation of cannabinoid CB_1 receptor function by Cannabinoid Receptor Interacting Protein ($CRIP_{1a}$), a novel protein that binds the C-terminus of CB_1 receptors. In Human embryonic kidney cells expressing human CB_1 receptors (hCB_1-HEK) and hCB_1-HEK cells stably co-expressing $CRIP_{1a}$ (hCB_1-HEK-$CRIP_{1a}$), quantitative immunoblotting revealed a $CRIP_{1a}/CB_1$ molar ratio of 5.4 and 0.37, respectively, with no difference in CB_1 receptor expression. To test the hypothesis that $CRIP_{1a}$ modulates CB_1 receptor signaling, G-protein and effector activity were examined with and without full, partial and inverse agonists. [^{35}S]GTPγS binding, which measures G-protein-coupled receptor (GPCR)-mediated G-protein activation, showed that $CRIP_{1a}$ inhibited constitutive CB_1 receptor activity, as indicated by the decreased effect of the inverse agonist SR141716A. $CRIP_{1a}$ also decreased CB_1 receptor-mediated G-protein activation by high efficacy agonists, whereas moderate and low efficacy agonists were unaffected. In experiments varying Na^+ concentration, $CRIP_{1a}$ decreased spontaneous G-protein activation at low Na^+ concentrations, where spontaneous GPCR activity is highest. This effect was eliminated by pertussis toxin pre-treatment, indicating that $CRIP_{1a}$ only inhibits GPCR-mediated activity. To determine whether $CRIP_{1a}$ modulates

receptor adaptation, hCB_1-HEK (±$CRIP_{1a}$) cells were pretreated with WIN or THC. Both ligands desensitized CB_1 receptor-mediated G-protein activation, but desensitization was unaffected by $CRIP_{1a}$. In contrast, $CRIP_{1a}$ attenuated downregulation of CB_1 receptor binding sites by WIN, but not THC. Downstream, $CRIP_{1a}$ attenuated constitutive CB_1 receptor-mediated inhibition of cAMP, as indicated by elimination of SR141716A-stimulated cAMP, without affecting agonist-induced cAMP inhibition. Constitutive inhibition was not due to endocannabinoids because LC-ESI-MS-MS did not detect endocannabinoids in hCB_1-HEK (±$CRIP_{1a}$) cells. To determine whether effects of $CRIP_{1a}$ were conserved among cell types, Chinese Hamster Ovary cells expressing CB_1 receptors were stably co-transfected with $CRIP_{1a}$, and had a $CRIP_{1a}/CB_1$ receptor molar ratio of 15 and 1900 with and without $CRIP_{1a}$ over-expression, respectively. In this model, $CRIP_{1a}$ inhibited constitutive CB_1 receptor-mediated G-protein activity, but activation by agonists was enhanced, suggesting $CRIP_{1a}$ effects were dependent on stoichiometry of $CRIP_{1a}/CB_1$ receptor or cell type. Overall, these results indicate that $CRIP_{1a}$ decreases constitutive CB_1 receptor activity, modulates agonist efficacy, and inhibits CB_1 receptor downregulation, in a ligand- and cellular environment-dependent manner.

Chapter 1. Introduction

1.1 Introduction to Cannabinoids and the Cannabinoid Receptors

Cannabis, or marijuana, use has been documented in human history as early as 2600 B.C. when in ancient Chinese texts, such as the Nei Ching, its use was recommended in cramping, pain, and to stop hemorrhage during childbirth. Cannabis was also used in ancient Egypt for a variety of medical reasons, including childbirth, enemas, eye medications and ointments in bandages. Ancient Indian texts, as early as 1200 B.C., describe cannabis as a sacred grass, from which bhang, the drink prepared from cannabis, is used for its anxiolytic properties, 'antiphlegmatic' properties and in religious rites. In the 19th century its use spread to Europe and the Americas as a psychoactive substance. The medical utilization of cannabis also flourished during these times, until cannabis was banned in 1937 due to concerns over its abuse potential (Mechoulam 1986). Recently, an 11 g sample of ancient cannabis was found in a 2700-year-old tomb excavated in China. High performance liquid chromatography confirmed the presence of psychoactive constituents, verifying cannabis use for medicinal or divinatory purposes early in human history (Russo et al. 2008).

In 1988, the main psychoactive component of marijuana, Δ^9-tetrahydrocannabinol (THC) was found to exert its affects by binding to cannabinoid (CB) receptors in the brain (Devane et al. 1988). Currently, the potential medical benefits of cannabinoids and

the endocannabinoid system are on the forefront of drug discovery (Mackie 2006). Dronabinol, a synthetic form of THC, has been proven affective in cancer patients for chemotherapy-induced nausea and vomiting. Dronabinol is also used to treat AIDS-related anorexia. Other therapeutic effects of the drug include analgesia, antitumor effects, mood elevation, muscle relaxation, and relief of insomnia (Walsh et al. 2003). Human placebo-controlled trials of the drug Sativex, a buccal spray containing THC and another cannabinoid cannabidiol, have proven effective for the spasticity and neuropathic pain associated with multiple sclerosis (Barnes 2006). The upregulation of the endocannabinoid pathway in response to neuronal injury has sparked interest in the use of cannabinoids in neurodegenerative disorders (Bahr et al. 2006). CB_1 receptor antagonists have shown promising results in anti-obesity studies (Palamara et al. 2006). Overall, the CB_1 receptor is an important therapeutic target that warrants further study. Understanding how the CB_1 receptor is regulated may allow the selective targeting of beneficial effects while decreasing unwanted side effects, such as anxiety, short-term memory impairment and decreased motor coordination.

Cannabinoid receptors are of particular importance in the arena of drug abuse. Cannabis is the most commonly used illicit drug in the United States, with more than 40% of Americans age 12 and older having tried cannabis at least once (Administration 2003). In addition to mediating the effects of THC, cannabinoid receptors are also involved in mediating the rewarding properties of other drugs. The cannabinoid and opioid system modulate each other's ability to activate dopamine (DA) release in the ascending mesocorticolimbic projections of the ventral tegmental area (VTA), which is activated by most drugs of abuse (Maldonado and Rodriguez de Fonseca 2002;

Maldonado et al. 2006). In mice with a genetic knockout of CB_1, the major cannabinoid receptor in the CNS, the reinforcing effects of morphine were reduced in intravenous self-administration tests (Ledent et al. 1999). The CB_1 receptor is also of importance in nicotine addiction, as CB_1 knockout in mice blocked the rewarding properties of nicotine in conditioned place preference studies (Castane et al. 2002). In addition, cannabinoid agonists stimulate voluntary alcohol consumption in alcohol-preferring Sardinian and Wistar rats (Gallate et al. 1999; Colombo et al. 2002). Although CB_1 knockout mice did not exhibit decreased self-administration of cocaine and amphetamine acutely (Cossu et al. 2001), they did show evidence of lower cocaine reinforcing efficacy on a progressive ratio schedule of self-administration (Soria et al. 2005). This type of schedule evaluates the reinforcing strength of a drug, and lower break points indicate a decreased motivation for maintaining cocaine-seeking behavior.

Rimonabant, a CB_1 antagonist also known as SR141716A, has shown considerable promise as a drug addiction treatment. Rimonabant decreased opioid self-administration (Navarro et al. 2001) and conditioned place preference in rodents (De Vries et al. 2003). In human studies, rimonabant was significantly effective in tobacco cessation studies in North America [Studies with Rimonabant and Tobacco use in North America (STRATUS-North America)] and Europe (Fernandez and Allison 2004). Rimonabant reduced conditioned reinstatement of ethanol-seeking behavior in rats (Cippitelli et al. 2005), and decreased cocaine relapse after cocaine re-exposure (De Vries and Schoffelmeer 2005).

1.2 Cannabinoid Receptor Location, Function and Ligands

Two cannabinoid receptors have been cloned to date; CB_1 (Matsuda et al. 1990) and CB_2 (Munro et al. 1993). CB_1 is one of the most abundant receptors in the brain (Ester Fride 2003), and it is also found in the periphery (Devane et al. 1988). CB_2 is found mainly within the immune system (Munro et al. 1993) and has limited expression in the central nervous system (CNS) (Van Sickle et al. 2005).

CB_1 receptors are found throughout the central nervous system, with particularly high expression densities in the hippocampus, cortex, caudate-putamen, globus pallidus, substantia nigra and cerebellum, and with moderate expression in periaquidctal grey, spinal cord and hypothalamus (Herkenham et al. 1991; Sim et al. 1996; Tsou et al. 1998). The immediate effects of marijuana intoxication reflect the functions of these brain regions, including impairment of short term memory, emotional disruption, hypomotility, reward, catalepsy, decreased motor coordination, antinociception and hypothermia (Dewey 1986; Hollister 1986).

The various cannabinoid ligands show a strong correlation between their affinity for CB_1 receptor binding and their potency for *in vivo* effects (Compton et al. 1993). CB_1 receptors have also been linked to a myriad of processes including brain development (Rueda et al. 2002), short-term synaptic plasticity (depolarized-induced suppression of inhibition (DSI) (Wilson and Nicoll 2001) and depolarized-induced suppression of excitation (DSE) (Kreitzer and Regehr 2001), and facilitation of long term potentiation (Carlson et al. 2002).

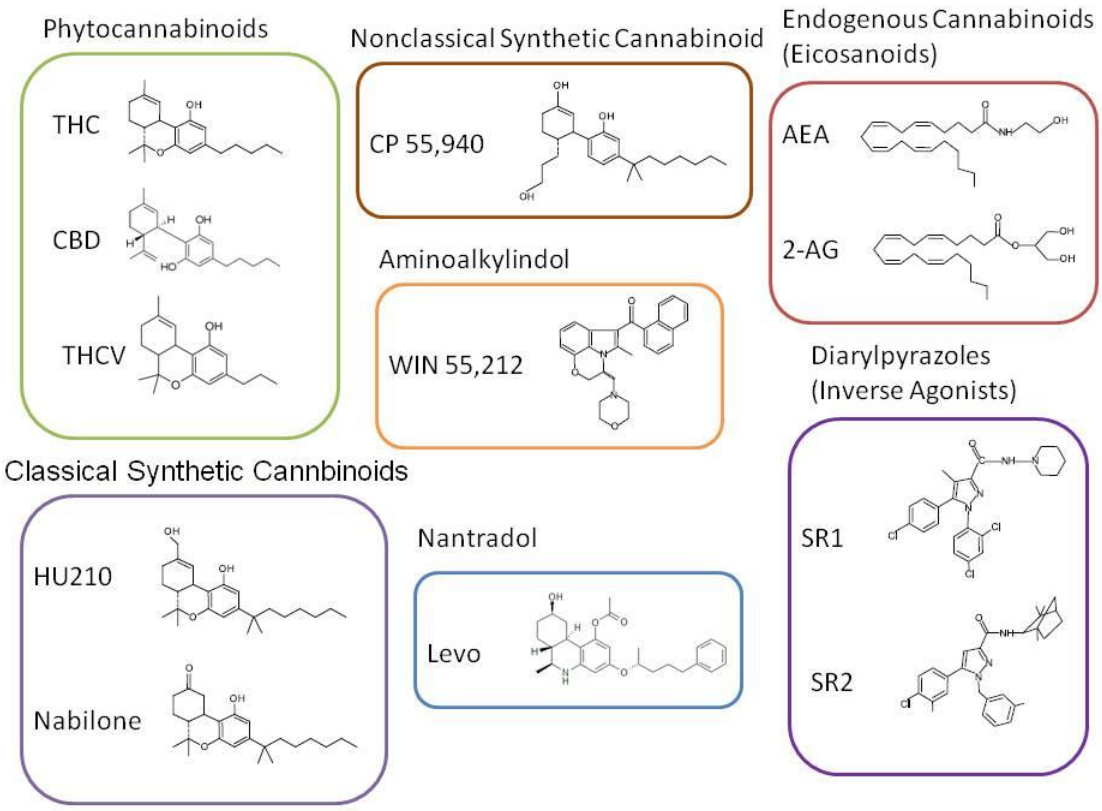

Figure 1. The chemical structures of cannabinoid ligands. Phytocannabinoids include Δ^9-tetrahydrocannabinol (THC), cannabidiol (CBD), and Δ^9-tetrahydrocannabivarin (THCV). Classic synthetic cannabinoids include HU210 and nabilone. A non-classical synthetic cannabinoid is CP 55,940. An aminoalkykindol is WIN 55,212-2. A nantradol is levonantradol. Endogenous cannabinoids (eicosanoids) include anandamide (AEA) and 2-arachidonoyl glycerol (2-AG). The diarylpyrazoles, which are inverse agonists, include SR141716A (SR1) and SR144528 (SR2).

The first ligands discovered for the CB_1 receptor were isolated from the Cannabis plant, and are termed phytocannabinoids; the main psychoactive component isolated from Cannabis being Δ^9-tetrahydrocannabinol (THC) (Gaoni and Mechoulam 1971) (Figure 1). The discovery that the l-stereoisomer of THC, and not the d-stereoisomer, was responsible for the biological activity of THC suggested the involvement of a receptor, as drug-receptor interactions are stereoselective (Hollister 1974). The involvement of a receptor was later confirmed by saturable, high affinity, stereospecific binding sites for the cannabinoid radioligand [^{3}H]CP 55,940 in mouse brain membranes (Devane et al. 1988). Cannabidiol (CBD), a phytocannabinoid found in high abundance in Cannabis, has a very low affinity for cannabinoid receptors but does act as an antagonist of cannabinoid receptor agonists (Thomas et al. 2007). Therapeutically, cannabidiol is not psychoactive, but is effective in the treatment of convulsions, inflammation, nausea and anxiety (Mechoulam et al. 2007). Δ^9-Tetrahydrocannabivarin (THCV), also highly abundant in Cannabis, is a behaviorally active phytocannabinoid that acts as an weak partial agonist at CB_1 receptors, and is able to antagonize the effects of THC (Pertwee et al. 2007).

A myriad of synthetic cannabinoids have been synthesized (Figure 1), including synthetic classical cannabinoids that are structurally similar to THC, including HU210, which is greater than 100 times more potent than THC and has a longer duration of action (Felder et al. 1995). Another synthetic cannabinoid similar to THC, nabilone, marketed as Cesmet, is approved for use nausea and vomiting associated with cancer chemotherapy (Slatkin 2007). Non-classical synthetic cannabinoids that are somewhat structurally dissimilar to classical cannabinoids have also been synthesized, including the bicyclic CP

55,940 (CP) which is greater than 100 times more potent than THC in behavioral tests (Wiley et al. 1995) and CB_1 receptor binding (Gatley et al. 1997). Another non-classical, synthetic class of cannabinoids is the aminoalkylindoles, such as WIN 55,212,2 (WIN), which are structurally dissimilar to classical or bicyclic cannabinoids, yet bind potently to CB receptors and elicit strong behavioral effects (Kuster et al. 1993; Pacher et al. 2006). In the 1980s, Pfizer Pharmaceuticals introduced another structural class somewhat similar to the THC class termed the nantradols, which includes levonantradol and dextronantradol. Levonantradol (Levo) is equipotent to 30 times more potent than THC in behavioral tests, while dextronantradol is weak to inactive (Little et al. 1988).

In 1992, the first endogenous ligand for the CB_1 receptor was discovered, N-arachidonoyl ethanolamine. This compound was also named anandamide, after the Sanskrit word *ananda*, meaning "bliss, delight". (Devane et al. 1992). Three years later another endogenous cannabinoid, 2-arachidonoylglycerol (2-AG) was independently co-discovered (Mechoulam et al. 1995; Sugiura et al. 1995). Anandamide is modestly selective for the CB_1 receptor. Anandamide may be partially synthesized *in vivo* via N-acyl phosphatidylethanolamine-selective phospholipase D (NAPE-PLD) in response to elevations of intracellular Ca^{+2}. However, NAPE-PLD knock-out mice were still able to produce reduced levels of N-acyl ethanolamines, suggesting that alternative pathways for the synthesis of anandamide exist (Leung et al. 2006). Anandamide is broken down by fatty acid amide hyrdrolase (Cravatt et al. 2001). 2-AG binds to CB_1 and CB_2 with similar affinities, and is synthesized from diacylglycerol by diacyglycerol lipase (DAGL) (Bisogno et al. 2003), and is broken down primarily by monoacylcyclyceride lipase (MGL) (Dinh et al. 2002). Many other endogenous lipids with endocannabinoid activity

have also been reported (Bisogno et al. 2005; Pacher et al. 2006). One example is 2-arachidonyl glyceryl ether, commonly known as noladin ether (Nol Eth), which was isolated from porcine brain in 2001. It is psychoactive and binds strongly to the CB_1 receptor and weakly to the CB_2 receptor (Hanus et al. 2001).

The first CB_1 receptor antagonist, discovered by Sanofi Aventis, was the diarylpyrazole SR141716A (SR1) (Rinaldi-Carmona et al. 1994). This development was followed by the discovery of another diarylpyrazole selective for the CB_2 receptor, SR144528 (SR2) (Rinaldi-Carmona et al. 1998). Both antagonists were later shown to have inverse agonist properties, meaning that they inhibit the basal, agonist independent activity of the receptor to which they bind (Bouaboula et al. 1997; Bouaboula et al. 1999).

1.3 Cannabinoid Receptor Signaling

Cannabinoid receptors are members of the G-protein coupled receptor (GPCR) superfamily, which are proteins with seven transmembrane alpha-helical domains, as well as an extracellular N terminus and an intracellular C-terminus (Matsuda et al. 1990). These membrane bound receptors are activated by extracellular ligands, transducing the signal to the cell interior by activation of heterotrimeric guanine nucleotide bindin G-proteins (G-proteins) (Gilman 1987). The cannabinoid receptors specifically activate the pertussis (PTX) toxin sensitive G_i/G_o subfamily of proteins, (Howlett et al. 1986), of which there are four types: $G\alpha_i1$, $G\alpha_i2$, $G\alpha_i3$, and $G\alpha_o$ (Albert and Robillard 2002).

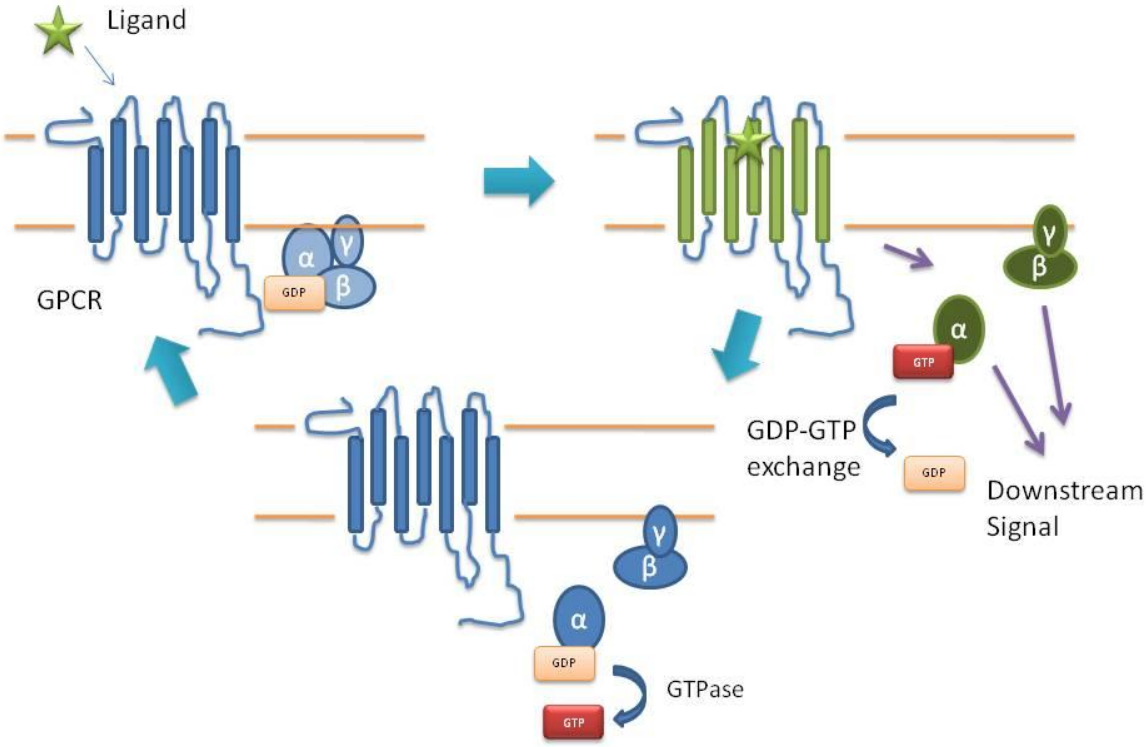

Figure 2. G-protein coupled receptor signaling. Prior to ligand binding, the receptor is inactive and G-proteins exist in the form of an αβγ heterotrimer. The inactive Gα subunit is bound to GDP. Upon ligand activation, the receptor changes to an active conformation (green), thereby activating G-proteins. GDP is exchanged for GTP and the Gα and Gβγ dissociate from one another and the receptor and are free to activate downstream signaling events. The cycle concludes when the GTPase activity of the Gα subunit dephosphorylates GTP to GDP, allowing the Gα subunit to return to its resting confirmation and reassociate with Gβγ.

A basic introduction to GPCR signaling is as follows (Figure 2). Prior to activation, G-proteins exist in the form of an αβγ heterotrimer, and are associated with the membrane via post-translational lipid moieties (Chen and Manning 2001). The Gα subunit is bound to GDP. Upon ligand binding, the receptor changes conformation, and becomes activated, thereby activating the G-protein. GDP is exchanged for GTP, and the Gα and the Gβγ subunits dissociate from one another and the receptor and are free to activate downstream signaling events. The Gβ and Gγ remain bound to each other as a dimer. The cycle concludes when the GTPase activity of the Gα subunit dephosphorylates GTP to GDP, allowing the Gα subunit to return to its resting conformation. Gα and Gβγ reassociate, returning the G-protein to its original inactivated state (Rockhold 2002). GPCRs act catalytically; each GPCR can activate multiple G-proteins over time. For instance the catalytic amplification factor of the cannabinoid CB_1 receptor has been calculated as 3 in striatum, as determined by comparing the B_{max} value of agonist binding to the B_{max} value of agonist-simulated [^{35}S]GTPγS binding. It is important to note that although this calculated amplification factor might not be true under all conditions, it is useful to compare among different receptors in the same tissue when assayed under identical conditions. For example, cannabinoid receptors exhibited low catalytic amplification factors compared to the μ-opioid and δ-opioid receptors in the striatum, which exhibited an amplification factor of 17 and 22, respectively (Sim et al. 1996). Moreover, the amplification factor of CB_1 receptors, calculated by this same approach, varied among different brain regions (Breivogel et al. 1997).

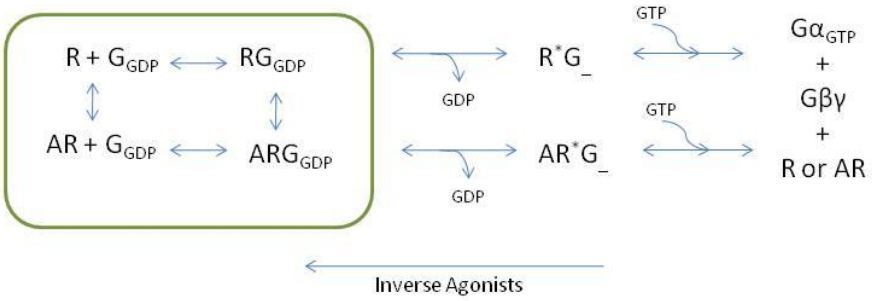

Figure 3. Equilibrium ternary complex model of ligand-receptor-G-protein interaction. GPCRs exist in equilibrium between an inactive (R) and active (R*) state. The isomerization from R to R* enables GPCRs to dissociate from GDP from G-proteins (G), which is the rate limiting step in GPCR signaling. The conversion from R to R* may happen in the presence of an agonist (A) or spontaneously, in the case of constitutively active receptors. After GDP dissociation, GTP binds to the Gα subunit of the G-protein heterotrimer, causing its dissociation into the active components of $G\alpha_{GTP}$, Gβγ, and its dissociation from the receptor.

Despite the seeming simplicity of the G-protein activation cycle, the nuances generated by the multitude of binding equilibria for all the possible protein-protein and ligand-protein interactions are more accurately represented in the equilibrium ternary complex model of ligand-receptor-G-protein interaction (Figure 3) (Leff 1995; Leff et al. 1997; Howlett 2004; Mukhopadhyay and Howlett 2005). GPCRs exist in equilibrium between an inactivate (R) and active state (R*) (Leff 1995). The isomerization from R to R* enables GPCRs to promote GDP dissociation from G-proteins, which is the rate-limiting step in GPCR signaling (Gilman 1987). Agonist (A) binds to the receptor, also an equilibrium event, which may or may not be precoupled to the G-protein heterotrimer bound to GDP (G_{GDP}). Agonist bound receptor enters into equilibrium with the "ternary complex", which is agonist bound to receptor coupled with a GDP-bound G-protein heterotrimer. This ternary complex stabilizes the receptor in its active conformation (R*) that promotes the dissociation of GDP from the Gα subunit in the G-protein heterotrimer. After GDP dissociation, GTP binds to the Gα in the G-protein heterotrimer causing its dissociation into its active components of $G\alpha_{GTP}$, Gβγ and its dissociation from the receptor. Full agonists are maximally able to shift the equilibrium from R to R*, partial agonists less so, and neutral antagonists do not affect this equilibrium. Inverse agonists, however, stabilize the inactivated R state of the receptor, thereby reducing GDP/GTP exchange. Additionally, constitutively active receptor spontaneously isomerizes from R to R* in the absence of ligand (Seifert and Wenzel-Seifert 2002).

The ability of a cannabinoid ligand to act as a full, partial, or inverse agonist is frequently measured as efficacy in [^{35}S]GTPγS binding assays. In these assays, a non-hydrolysable form of GTP, in which an oxygen on the γ phosphate is replaced by a

radioactive sulfur ([^{35}S]GTPγS), is incubated with cannabinoid ligands, excess GDP and membrane homogenates containing the CB_1 receptor and G-proteins. Receptor-activated Gα releases GDP and binds [^{35}S]GTPγS, and the accumulation of this radioactive complex is a measure of receptor-mediated G-protein activation in response to ligand. Concentration effect curves for CB_1-mediated G-protein activation are generated with each ligand, and the E_{max} values of the curves are taken as measures of efficacy. Examples of CB_1 receptor high-efficacy, full agonists include WIN 55,212-2 (WIN) and CP 55,940 (CP), CB_1 partial agonists include Δ^9-tetrahydrocannabinol (THC) and methanandamide (MethA) and a CB_1 receptor inverse agonist is SR141716A (Breivogel et al. 1998; Breivogel and Childers 2000; Childers 2006). Efficacy of partial agonists can also be shown indirectly by competitive antagonism; THC was first shown to be a partial agonist when high concentrations of THC were shown to antagonize the full effects of WIN when the two drugs were added together in a [^{35}S]GTPγS binding study in cerebellar membranes (Sim et al. 1996).

G-proteins interact with the C-terminal tail of the CB_1 receptor (Nie and Lewis 2001) and the third intracellular loop (Mukhopadhyay et al. 2000). Demonstrated through the use of cannabinoid receptor intracellular domain mimicking peptides to disrupt specific CB_1 receptor-G-protein associations, inhibitory G-protein subtypes interact specifically with certain regions of the CB_1 receptor; $G\alpha_i1$ and $G\alpha_i2$ interact with third cytosolic loop of the CB_1 receptor (Mukhopadhyay et al. 2000; Mukhopadhyay and Howlett 2001) and $G\alpha_i3$ and $G\alpha_o$ interact with the C-terminal tail (Mukhopadhyay et al. 2000). Furthermore, specific agonists have the ability to differentially activate specific $G\alpha_i$ proteins. CB_1 receptors solubilized from membranes using detergent (3-[3(-

cholamindopropyl) dimethylammonio] propanesulfonate, i.e. CHAPS) were co-immunoprecipitated with various types of G-proteins ($G\alpha_i1$, $G\alpha_i2$, $G\alpha_i3$) in the absence of exogenously added cannabinoid ligands (Mukhopadhyay and Howlett 2005). Addition of specific ligands caused the differential dissociation of the various types of G-proteins, demonstrating ligand-specific G-protein activation. WIN stimulates all three $G\alpha_i$ subtypes, whereas desacetyllevonantradol (DALN) stimulates $G\alpha_i1$ and $G\alpha_i2$ while acting as inverse agonist for $G\alpha_i3$, and MethA stimulates $G\alpha_i3$ while acting as an inverse agonist for $G\alpha_i1$ and $G\alpha_i2$. SR1 acts as an inverse agonist for all three subtypes (Mukhopadhyay and Howlett 2005). The synthetic analog of THC, HU210, produces maximal stimulation of both $G\alpha_i$ and $G\alpha_o$ proteins, as measured by [^{35}S]GTPγS binding assays of purified $G\alpha$ proteins reconstituted with CB_1 receptors recombinantly expressed in *Spodoptera frugiperda* (Sf9) cells. WIN maximally activated $G\alpha_i$ but not $G\alpha_o$. THC caused only partial $G\alpha_i$ and $G\alpha_o$ activation (Glass and Northup 1999). Overall, this evidence implies that different ligands can invoke various conformations of the CB_1 receptor. Utilizing different ligands exogenously or the presence of CB_1 receptor-interacting G-proteins endogenously could change the conformation of the CB_1 receptor to allow the promotion of differential signal transduction pathways. In theory, selective pharmacological targeting of CB_1 receptors could be used to promote therapeutic pharmacological effects while minimizing unwanted side effects induced by the CB_1 receptor, such as disruption of short-term memory and sedation (Mukhopadhyay et al. 2002). Moreover, if CB_1 receptor G-protein coupling specificity is modulated by endogenous proteins, then these proteins can also be pharmacologically targeted for the same purpose.

CB_1 receptors are constitutively active (Bouaboula et al. 1997; Pan et al. 1998; Nie and Lewis 2001), meaning that they can spontaneously shift conformation from R to R* in the absence of ligand. This constitutive activity can increase basal G-protein activity and modulation of effector system activity, and is reversible by inverse agonists (Seifert and Wenzel-Seifert 2002). Lewis and Nie (2001) found that truncation of the distal C-terminal tail of the CB_1 receptor at amino acid 417 enhanced the constitutive activity of the receptor. This raises the possibility that a protein binds to the distal C-terminal tail that attenuates the constitutive activity of the CB_1 receptor.

A highly conserved aspartate in the second transmembrane domain, denoted II:14D (transmembrane domain II, amino acid position 14, aspartate (D)) (Baldwin et al. 1997) or D164 (aspartate at CB_1 amino acid position 164) is critical to CB_1 receptor constitutive activity. A mutation in this aspartate abolished constitutive activity without disrupting agonist-mediated inhibition of Ca^{+2} channels (Nie and Lewis 2001) or inhibition of cAMP production. However, disruption of this aspartate can disrupt the coupling of the CB_1 receptor to the potentiation of inward rectifying potassium channels, and prevent the internalization of the receptor after exposure to agonist (Roche et al. 1999). When analyzing a GPCR for constitutive activity, determination of endogenous ligands within the study system is important to rule out endogenous ligand-mediated stimulation (Morisset et al. 2000). Furthermore, CB_1 receptors are constitutively internalized and recycled back to the cell surface, in a manner dependent on the constitutive activity of the receptor (Leterrier et al. 2004; Leterrier et al. 2006), although one group has suggested that constitutive activity may not be necessary for constitutive internalization of CB_1 receptors (McDonald et al. 2007).

In addition, GPCRs coupled to inhibitory G_i/G_o proteins are affected by sodium concentrations (Jakobs 1979). Sodium stabilizes the inactivated R state of the receptor and diminished basal G-protein activity, thus acting as an allosteric inverse agonist and decreasing the apparent efficacy of inverse agonists (Koski et al. 1982) (Seifert and Wenzel-Seifert 2002). Specifically, sodium has been shown to decrease CB_1 receptor agonist binding and G-protein interactions with the CB_1 receptor (Devane et al. 1988; Kuster et al. 1993; Houston and Howlett 1998). Additionally, an optimal sodium concentration is required for the coupling of CB_1 receptor to adenylyl cyclase inhibition in specific brain regions, such as striatum. Interestingly, inhibition of adenylyl cyclase in rat cerebellar second transmembrane spanning domain of GPCRs, II:14D, is responsible for the allosteric regulation by sodium, which was first discovered via site-directed mutagenesis of the α2-adrenergic receptor (Horstman et al. 1990) and has since been demonstrated for a plethora of other GPCRs (Horstman et al. 1990; Kong et al. 1993; Ceresa and Limbird 1994). The physiological sodium concentration at this aspartate is unknown (Seifert and Wenzel-Seifert 2002).

Another hallmark of G_i/G_o coupled receptor signaling is sensitivity to pertussis toxin (PTX), derived from *Bordetella pertussis* (whopping cough). PTX ribosylates a cysteine in the heterotrimeric forms of inhibitory $G\alpha_{i/o}$ proteins (Locht and Antoine 1995), thus blocking all interaction with activated R* GPCRs (Seifert and Wenzel-Seifert 2002). PTX is used to confirm the involvement of inhibitory $G\alpha_{i/o}$ proteins in CB_1 receptor mediated downstream signals, including adenylyl cyclase inhibition (Howlett et al. 1986; Pacheco et al. 1994) and N-type Ca^{+2}current inhibition (Guo and Ikeda 2004). CB_1 receptor-mediated downstream signals not blocked by PTX do not involve inhibitory

$G\alpha_{i/o}$ proteins, rather they signal through alternative CB_1-mediated pathways, possibly G-protein-coupled receptor kinases (GRKs), β-arrestin, PDZ domain-containing G-proteins (Hall et al. 1999), or other G-proteins types, such as the signal switching to $G\alpha_s$ family members seen when CB_1 receptors are stimulated concurrently with dopamine (D_2) receptors (Glass and Felder 1997; Kearn et al. 2005).

Once activated, CB_1 receptors modulate multiple downstream signaling events, including inhibition of adenylyl cyclase (AC) (Howlett et al. 1986), phosphorylation of p42/p44 mitogen activated protein kinases (MAPK), which are also known as extracellular signal regulated kinases (ERK1/2) (Bouaboula et al. 1995; Derkinderen et al. 2001; Galve-Roperh et al. 2002), inhibition of N-type and P/Q type voltage dependent Ca^{+2} channels (Pan et al. 1996) (Twitchell et al. 1997), stimulation of inward rectifying K^+ channel (Mackie et al. 1995; Vasquez et al. 2003), inhibition of Na^+ channels (Nicholson et al. 2003), stimulation of phospholipases C and A2 (PLC, PLA2) (Hunter et al. 1986), activation of c-Jun N-terminal kinase (JNK), and activation of p38 mitogen-activated protein kinase (Rueda et al. 2000). CB_1 receptors generally inhibit AC (types 1, 3, 5, 6 and 8), but they also have the ability to stimulate certain AC isoforms (types 2, 4, and 7) via Gβγ (Rhee et al. 1998).

The modulation of downstream signaling events by activated G-proteins is usually accomplished through the Gβγ subunit (Clapham and Neer 1997; Offermanns 2003). Such is the case for MAPK (Inglese et al. 1995), N and P/Q type voltage dependent Ca^{+2} channels (Herlitze et al. 1996), inwardly rectifying K^+ channel (Logothetis et al. 1987), and phospholipases C (Camps et al. 1992) and A2 (Jelsema and Axelrod 1987). Adenylyl cyclase is regulated by both $G\alpha_i$ and Gβγ, dependent on the subtype of AC; for

the subtypes of AC inhibited by CB_1 receptors (isoforms 1, 3, 5, 6 and 8) (Howlett et al. 2002), type 1 is inhibited by both $G\alpha_i$ and $G\beta\gamma$, whereas types 3, 5, 6 and 8 are inhibited by $G\alpha_i$ (Rhee et al. 2000; Offermanns 2003).

1.4 Cannabinoid Receptor Regulation & Cellular Localization

CB_1 receptors signal are regulated in their native environments not only through G-protein interactions, but also by modulation and trafficking of the receptors themselves. Following agonist stimulation, CB_1 receptors may be desensitized, internalized and then recycled or downregulated, similarly to other GPCRs (Figure 4) (Lefkowitz 1998). Ultimately these processes lead to a reduced CB_1 receptor signal, which could contribute to the development of tolerance (Maldonado 2002; Sim-Selley and Martin 2002; Sim-Selley 2003).

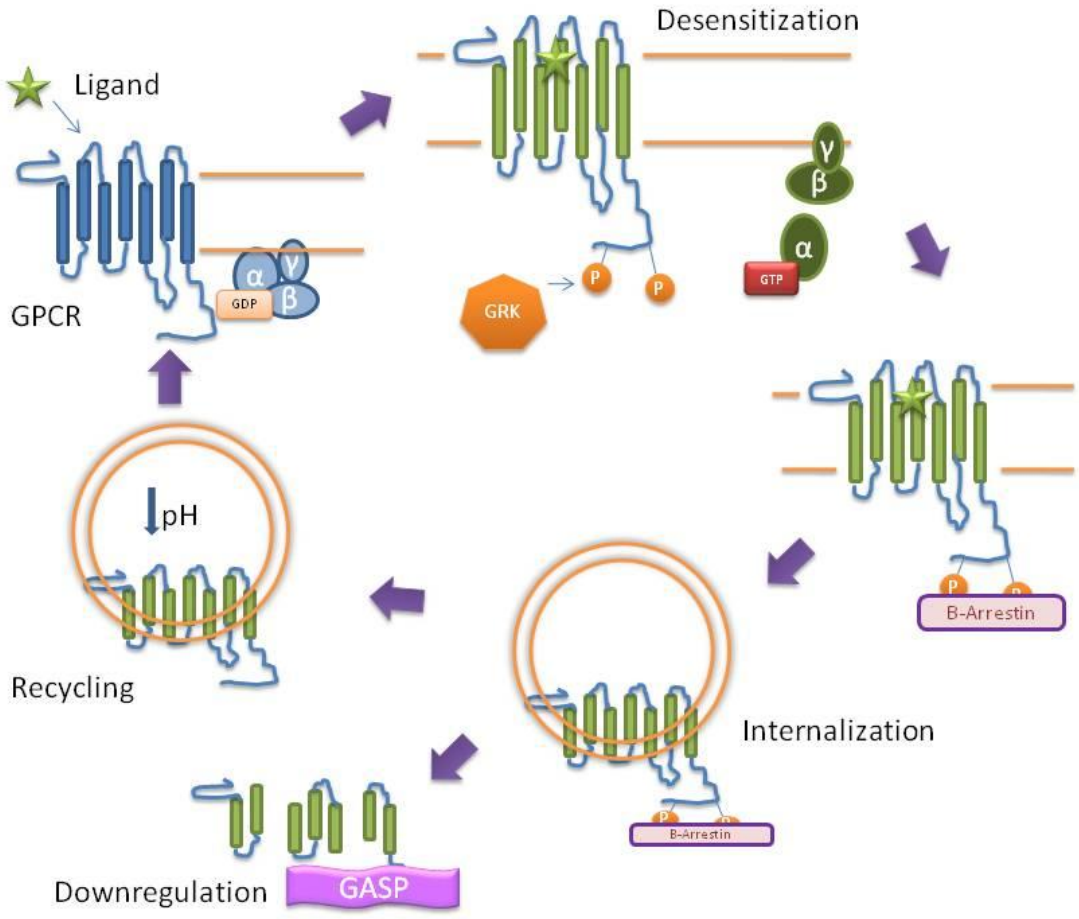

Figure 4. G-protein coupled receptor regulation and cellular location. Upon agonist stimulation, G-protein receptor kinase (GRK) phosphorylates the GPCR, impairing the ability of the receptor to interact with G-proteins. This process is known as desensitization. Once phosphorylated, β-arrestin can bind to the GPCR, stabilizing the desensitized state and causing the receptor to internalize via clathrin-coated pits. Once internalized, the GPCR is either degraded, otherwise known as downregulation, or recycled back to the cell surface following dephosphorylation in acidified endosome.

Upon agonist stimulation, CB_1 receptors desensitize, making them less sensitive to subsequent stimulation. Agonist binding causes a conformational change in the receptor, activates Gβγ, which recruits G-protein coupled receptor kinases (GRKs) (Pitcher et al. 1992), allowing G-protein coupled receptor kinase 3 (GRK3) (Hsieh et al. 1999; Jin et al. 1999) or G-protein coupled receptor kinase (GRK2) (Kouznetsova et al. 2002; Rubino et al. 2006) to phosphorylate serine 426 and/or 430 in the CB_1 receptor C-terminal tail (Hsieh et al. 1999; Jin et al. 1999). GRK binding substantially impairs the ability of the receptor to interact with G-proteins (Pitcher et al. 1998; Reiter and Lefkowitz 2006). GRK phosphorylation recruits the binding of β-arrestin, which further increases desensitization (Reiter and Lefkowitz 2006). CB_1 receptor desensitization can be seen as a decrease in the E_{max} of agonist stimulated [^{35}S]GTPγS binding in brain membrane homogenates, (Sim et al. 1996; Sim-Selley and Martin 2002; Selley et al. 2004), and cannabinoid-stimulated [^{35}S]GTPγS autoradiography in brain slices (Sim et al. 1995; Sim et al. 1996), both of which measure GPCR-mediated G-protein activation. THC treated rats (10 mg/kg/day, 21 days) subjected to [^{35}S]GTPγS binding and autoradiography experiments show a decrease in WIN-stimulated binding in all brain regions. However, the magnitude of desensitization varied by region, with the largest decreases seen in hippocampus and cortex, followed by cerebellum, caudate-putamen, globus pallidus, periaqueductal gray, and the smallest decrease in substantia nigra (Sim et al. 1996). In addition, the time course required for desensitization varied by brain region; hippocampus reached maximal desensitization follwing 7 days of THC treatment, whereas cerebellum took 14 days to fully desensitize, and caudate-putamen required 21 days (Breivogel et al. 1999).

CB_1 receptor signal is further lost upon agonist binding when the desensitized receptor is internalized into the cell interior (Hsieh et al. 1999; Coutts et al. 2001; Wu et al. 2008). The CB_1 receptor may then be degraded, a process termed downregulation, and can be measured as decreased binding of radioactive cannabinoid ligands in autoradiographic studies of brain slices (Oviedo et al. 1993; Breivogel et al. 1999; Sim-Selley and Martin 2002) or as decreased binding (B_{max} values) in [^{3}H]SR141716A saturation binding analyses of brain tissue homogenates (Breivogel et al. 1999; Sim-Selley et al. 2006). The magnitude of CB_1 receptor downregulation varies from brain region to brain region; downregulation is greatest in cortex, cerebellum, hippocampus and caudate-putamen, and less downregulation is seen for substantia nigra and globus pallidus. (Rodriguez de Fonseca et al. 1994; Breivogel et al. 1999; Sim-Selley and Martin 2002). Downregulation in different brain regions is also time course dependent. Maximal downregulation of CB_1 receptors in the rat hippocampus and cerebellum was achieved after 7 days of THC administration, but downregulation in the caudate/putamen and globus pallidus was not detectable until 21 days (Breivogel et al. 1999).

The cause(s) of differential regional desensitization and downregulation of CB_1 receptors in the CNS remains unknown, but may be caused by differential expression of proteins that interact with the CB_1 receptor, such as the region-specific expression of Gγ7 (Watson et al. 1994), differences in the formation of CB_1 receptor dimers from region to region (Wager-Miller et al. 2002), differential changes in CB_1 receptor mRNA levels in response to chronic cannabinoid treatment (Zhuang et al. 1998; Romero et al. 1999; Sim-Selley et al. 2006) or variations in other unknown and uncharacterized proteins. Additionally, proteins that are known to regulate CB_1 receptor adaptation respond to drug

treatment; GRK2, GRK4, β-arrestin 1 and β-arrestin 2 are all upregulated by chronic THC treatment (Rubino et al. 2006). These regulatory proteins may exhibit differential regional localization and/or respond differently to chronic THC treatment in various brain regions, and thus may account for differential CB_1 receptor adaptation.

Downregulation occurs when, following GRK phosphorylation, β-arrestin can bind to the CB_1 receptor, stabilizing desensitization and causing the receptor to internalize via clathrin-coated pits (Reiter and Lefkowitz 2006). Once bound, β-arrestin associates with the AP-2 complex, the heterotetrameric clathrin adaptor protein that targets the receptor to clathrin-coated pits (Laporte et al. 1999), and then binds directly to clathrin to initiate internalization (Goodman et al. 1996). Specifically, CB_1 receptors have been shown to rapidly internalize in AtT20 cells via clathrin coated pits, because sucrose pretreatment, which disrupts clathrin-coated pit formation (Heuser and Anderson 1989), prevented CB_1 receptor internalization (Hsieh et al. 1999). Residues 460-463 for the CB_1 receptor are required for β-arrestin-mediated internalization (Hsieh et al. 1999; Daigle et al. 2008).

Alternatively, CB_1 receptors can internalize into caveolae (Keren and Sarne 2003; Bari et al. 2008; Wu et al. 2008), which are a specialized subclass of lipid rafts involved in cholesterol trafficking, endocytosis of external molecules, and regulation of several signal transduction pathways (Razani et al. 2002). CB_1 receptors can co-localize with caveolin-1, a major component of the caveolae protein coat (Bari et al. 2008). Interestingly, when both clathrin-dependent and caveolae-dependent internalization were blocked, CB_1 receptors were still able to internalize, to a lesser extent, suggesting another

endocyctic pathway remains to be discovered (Keren and Sarne 2003). An alternate endocytic pathway may be flotillins, which are a clathrin and caveolin-independent enodocytic pathway found in mammalian cells (Glebov et al. 2006)

Exposure time is an important factor in determining the fate of internalized CB_1 receptors. Once internalized, CB_1 receptors are either dephosphorylated, resensitized and recycled back to the cell surface or targeted to the lysosomes for degradation (downregulated) (Reiter and Lefkowitz 2006). Rapid recycling of the CB_1 receptor occurs after short agonist exposures, and requires dephosphorylation by an okadaic acid-sensitive phosphatase and endosomal acidification. Resensitization of the receptors occurs more rapidly for high (WIN) vs. low (THC) endocytotic agonists, suggesting the CB_1 receptor endocytosis is an important facilitator in the resensitization of CB_1 receptors (Bohn 2007; Wu et al. 2008). Long duration agonist exposure leads to downregulation (Hsieh et al. 1999). In addition, shorter duration exposure to very high doses of CB_1 agonists can also lead to downregulation (Keren and Sarne 2003; Martini et al. 2007).

G-protein-coupled receptor-associated sorting protein (GASP1) is responsible for sorting the CB_1 receptor into the downregulation pathway, increasing the associating between CB_1 receptors and lysosomal markers LAMP1 and LAMP2 (Martini et al. 2007). WIN-induced CB_1 receptor downregulation is attenuated by a dominant-negative mutant of GASP1 (cGASP) in primary cultured rat spinal neurons. Additionally, the dominant negative GASP1 mutant attenuated analgesic tolerance and CB_1 receptor downregulation *in vivo* following its injection into the spinal dorsal horn of mice expressed in a recombinant chimeric adeno-associated virus 1/2 viron (Tappe-Theodor et al. 2007).

Once downregulated, new protein synthesis is required for the recovery of the CB_1 receptors to the cell surface (Hsieh et al. 1999).

The specific agonist used to desenstitize/downregulate the CB_1 receptor is another important factor in determining the fate of the CB_1 receptor. For instance, THC, a low endocytotic agonist exhibited greater desensitization than the high endocytic agonist WIN, as measured by inhibition of intracellular cAMP accumulation (Wu et al. 2008). Therefore, THC may robustly stimulate desensitization of CB_1 receptors, while less robustly inducing their internalization. Indeed, β-arrestin 2 knockout mice show a selective enhancement of THC-induced behavioral effects. β-arrestin 2 knockout did not affect sensitivity to other CB_1 agonists tested, including CP, MethA, JWH-073 and O-1812 (Breivogel et al. 2008). This finding suggests that THC-occupied CB_1 receptors are strongly influenced by β-arrestin 2, possibly recruiting β-arrestin 2 only or more strongly than β-arrestin 1, which may affect the processes of CB_1 receptor desensitization and/or downregulation.

Localization of the CB_1 receptor within the plasma membrane itself may also be important. Plasma membranes contain discrete regions of proteins and lipids, composed largely of cholesterol and sphingolipds in the outer leaflet of the lipid bilayer, termed lipid rafts (Barnett-Norris et al. 2005). Many GPCRs localize to the lipid rafts, and agonist stimulation of these GPCRs can promote entry into, or exit from, the lipid raft microenvironments (Patel et al. 2008). Importantly, many proteins associated with the signaling of GPCRs, such as heterotrimeric G-proteins, may be targeted to lipid rafts due to their lipid modifications, namely by the myrsotylation and/or palmitoylation of Gα

subunits and prenylation of Gα subunits (Wedegaertner et al. 1995). Thus, lipid rafts may aggregate GPCRs and their signaling proteins to increase the specificity and efficiency of signal transduction (Moffett et al. 2000). Lipid rafts may also serve to limit the signal transduction of CB_1 receptors, because treatment of rat C6 glioma cells with the lipid raft disruptor methyl-β-cyclodextrin (MCD) increases the stimulation of [^{35}S]GTPγS binding by the endocannabinoid AEA and downstream signaling to AC and p42/p44 MAPK (Bari et al. 2005). Thus exit or entry into lipid rafts via interactions with agonists, or CB_1 interacting proteins, could be important modifiers of CB_1 receptor activity.

1.5 Proteins that Interact with the CB_1 Receptor

GPCRs do not interact exclusively with G-proteins. Rather, GPCRs exist in tandem with numerous protein interacting partners, collectively termed a receptosome, that control processes such as ligand specificity, signal amplification, constitutive activity, GPCR localization, desensitization, internalization, recycling and downregulation (for review see (Tilakaratne and Sexton 2005)).

In addition to G_i/G_o proteins, the CB_1 receptosome can contain numerous proteins, including Factor Associated with Neutral sphingomyelinase activation (FAN), involved in CB_1-evoked sphingomyelin breakdown (Sanchez et al. 2001), Rab5 and Rab4 GTPases that regulate constitutive endocytosis and recycling, respectively, (Leterrier et al. 2004), GRK2 (Kouznetsova et al. 2002) and GRK3 (Jin et al. 1999), which are involved in CB_1 receptor desensitization, β-arrestin 2, which is involved in CB_1 receptor desensitization and internalization (Jin et al. 1999), and G-protein-coupled-receptor-Associated Sorting Protein (GASP1), which plays a role in CB_1 receptor downregulation

(Martini et al. 2007). This study will examine the function of the novel and as yet relatively uncharacterized CB_1 receptor interaction protein; Cannabinoid Receptor Interacting Protein 1a ($CRIP_{1a}$)

Like G-proteins, GRKs, β-arrestin, and GASP1 discussed earlier, FAN interacts directly with the CB_1 receptor. The interaction of the CB_1 receptor and FAN was discovered after the observation that THC induces sphingomyelin breakdown in primary astrocytes (Sanchez et al. 1998). FAN interacts directly with CB_1 in primary astrocytes, but not U373 MG astrocytoma cells, because FAN is immunoprecipitated from these cells with anti-CB_1 antibody. Interestingly, THC increased the binding of FAN to CB_1 receptors, an effect that was blocked by SR1. Furthermore the role of FAN in CB_1 receptor coupled sphingomyelin breakdown was confirmed using cells expressing a dominant negative form of FAN, in which sphingomyelin breakdown was attenuated (Sanchez et al. 2001).

Contrariwise, Rab4 and Rab5 have not been shown to directly interact with the CB_1 receptor, but functionally colocalize with the receptor during receptor trafficking. Rab GTPases organize membrane trafficking in eukaryotic cells and are associated with the cell membrane by hydrophobic geranylgeranyl groups that are attached to one or two carboxy-terminal cysteine(s). Specifically, Rab4 and Rab5 are involved in endosomal fusion and endocytic recycling (Stenmark 2009). Dominant negative (GDP-bound) and dominant active (GTP bound) mutants of Rab4 and Rab5, but not Rab11, disrupt constitutive endocytosis and recycling of the CB_1 receptor, confirming their involvement in these processes (Leterrier et al. 2004). Although they do not directly interact with the receptor, these proteins may be considered part of the larger CB_1 receptosome. This

broad definition of receptosome could include other proteins that might be involved in the signaling and regulation of the CB_1 receptor, including proteins involved in scaffolding, signal modulation or other GPCRs (e.g. CB_1-GPCR heterodimers), and are too numerous for discussion here.

A common protein-protein interaction motif between GPCR interacting proteins is the PDZ domain. The acronym 'PDZ' is derived from the first three proteins in which these motifs were identified (PSD-95, Disc large protein and ZO-1) (Tilakaratne and Sexton 2005). The vast majority of PDZ-containing proteins are associated with the plasma membrane. In addition, multiple copies of the PDZ domains commonly occur within a single polypeptide chain (Jelen et al. 2003). These ubiquitous protein interaction modules are commonly found in proteins involved in receptor trafficking, receptor anchoring/stabilization, scaffolding for assembly of signaling molecules, and modulation of receptor ligand specificity (Tilakaratne and Sexton 2005). An example of a PDZ containing protein is spinophilin, which contains one PDZ domain and is known to interact with the D_2 dopamine receptor (Smith et al. 1999). In addition, spinophilin is known to interact with other protein binding partners, including other GPCRs, cytoskeletal and cell adhesion molecules, ion channels and enzymes. The function of spinophilin has not been fully elucidated, but is proposed to provide a link between GPCRs and mitogenic signaling events, as well as regulate synaptic plasticity, spine morphology, and neuronal migration (Sarrouilhe et al. 2006).

1.6 Cannabinoid Receptor Interacting protein ($CRIP_{1a}$)

The discovery that the truncation of the distal C-terminal tail of the CB_1 receptor enhanced the constitutive activity of the CB_1 receptor (Nie and Lewis 2001) lead to a search for a protein that binds to the CB_1 receptor C-terminus and inhibits the constitutive activity of the CB_1 receptor. Two novel proteins, coined cannabinoid receptor interacting proteins, ($CRIP_{1a}$ & $CRIP_{1b}$) were recently discovered via yeast two-hybrid screening of a human brain cDNA library using the last 55 amino acids of the C-terminal tail of the CB_1 receptor (amino acids 418-472) as bait (Niehaus et al. 2007).

A CB_1 receptor interacting gene was found on human chromosome 22 that alternatively splices to form $CRIP_{1a}$ (exons 1, 2 and 3a) and $CRIP_{1b}$ (exons 1, 2, and 3b), which are 164 and 128 amino acids, respectively. The GenBank accession numbers for the nucleotide sequences of $CRIP_{1a}$ and $CRIP_{1b}$ are AY883936 and AY144596, respectively. The role of $CRIP_{1b}$ is unclear; it is found only in primates and its effects on CB_1 receptor function remain undiscovered. However, $CRIP_{1a}$ appears to decrease the constitutive activity of the CB_1 receptor.

The region of the CB_1 receptor required for CRIP interaction was determined using a yeast two-hybrid screening of various CB_1 receptor C-terminal tail mutants as bait and $CRIP_{1b}$ as prey (Figure 6). The last nine amino acids of the CB_1 receptor were required for $CRIP_{1b}$ interaction. CRIP did not interact with either the desensitization (aa 419-438) or internalization (aa 460-463) regions of the CB_1 receptor. Furthermore, Western blot analysis of in vitro binding assays confirmed $CRIP_{1a}$/CB_1 interaction, as bacterially expressed $CRIP_{1a}$ bound specifically to immobilized GST-CB_1 C-terminal tail and not to the negative control, GST.

In vivo interaction of $CRIP_{1a}$ and the CB_1 receptor was confirmed using co-immunoprecipitation of $CRIP_{1a}$ with the CB_1 receptor from rat brain homogenates. $CRIP_{1a}$ was highly expressed in mouse brain homogenates, and also detectable in heart, lung and intestine via Western blot analysis, as well as in mouse brain and cerebellar homogenates. Confocal microscopy of cDNA microinjected rat superior cervical ganglion (SCG) neurons found that $CRIP_{1a}$, $CRIP_{1b}$ and the CB_1 receptor are enriched near the plasma membrane and overlap in orthogonal perspectives.

Comparative genomic analyses indicate that $CRIP_{1a}$ is conserved throughout the vertebrates. $CRIP_{1a}$ contains no transmembrane domains, as determined by hydropathy analysis, but does contain a predicted palmyitoylation site, which may aid its association with the plasma membrane. The C-terminal tail of $CRIP_{1a}$ contains a predicted PDZ Class I ligand, which could allow it to interact with PDZ-containing G-proteins. This finding suggests that $CRIP_{1a}$, like many other proteins that interact with PDZ modules, may be important for regulating GPCR signaling, scaffolding or trafficking.

Electrophysiological voltage-step protocol of calcium current recordings in rat SCG neurons microinjected with cDNA encoding the CB_1 receptor, with and without co-microinjection of cDNA encoding $CRIP_{1a}$, showed that $CRIP_{1a}$ attenuated the constitutive CB_1-mediated inhibition of calcium channels, as revealed by an elimination of the inverse agonist activity of SR1 (Figure 7) . However, the CB_1-receptor mediated decrease in calcium current elicited by the CB_1 agonist WIN was unaffected by co-expression of $CRIP_{1a}$ (Niehaus et al. 2007).

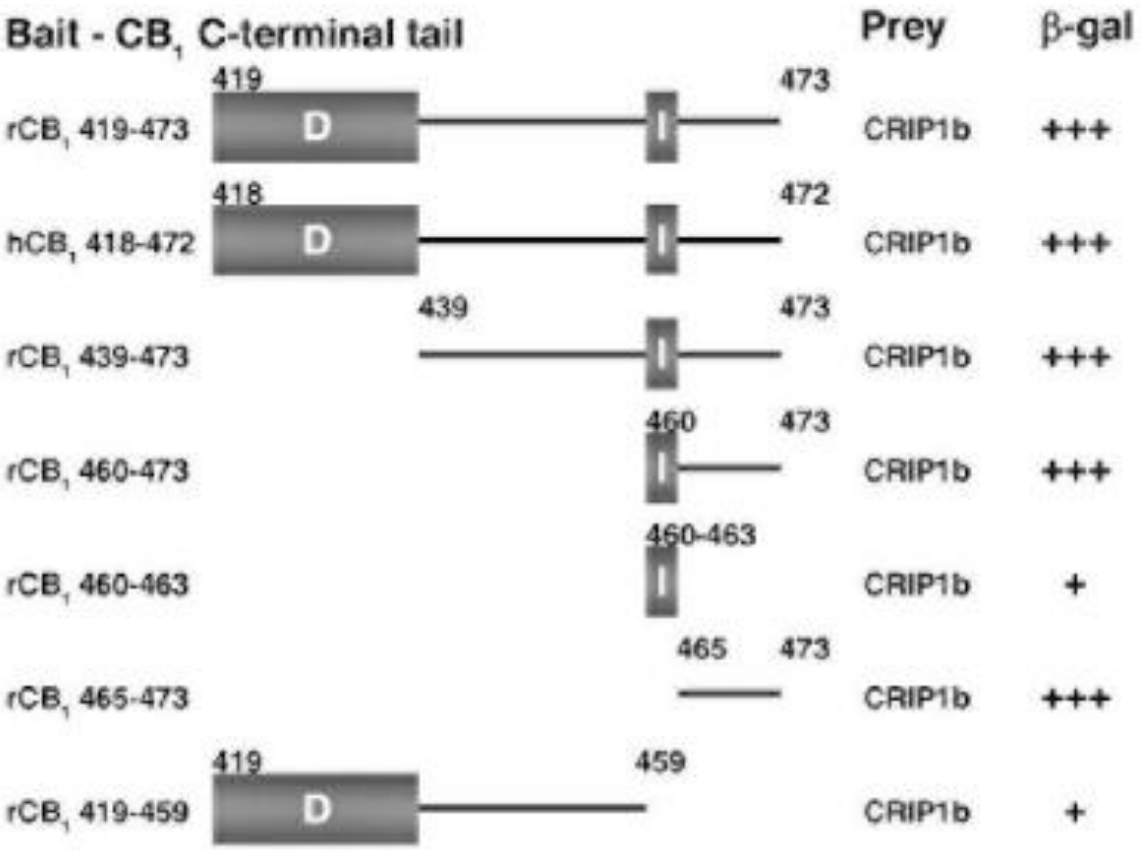

Figure 6. CB_1 receptor interaction with CRIP requires the last nine amino acids of the CB_1 C-terminal tail. Desensitization (D) and internalization (I) regions of the CB_1 receptor are depicted as boxes. Numbers indicate the amino acids residues of the rat (r) CB_1 receptor used as bait in yeast two-hybrid screening. Reprinted from Niehaus et al. (2007).

Only one other study has examined $CRIP_{1a}$; Ludanyi et. al. (2008) postulated that endocannabinoid signaling has an important protective role against pathologic neuronal excitability. To address this hypothesis, they utilized quantitative PCR measurements to evaluate mRNA levels of CB_1 receptor related molecular elements in epileptic versus healthy postmortem human hippocampal tissue. Human sclerotic hippocampi show a reduction in $CRIP_{1a}$ gene expression in tandem with reduction in CB_1 receptor expression, suggesting that downregulation of the CB_1 receptor, in tandem with $CRIP_{1a}$ may facilitate the harmful effects of increased neuronal excitability (Ludanyi et al. 2008).

The known effects of $CRIP_{1a}$ on activity modulated by the CB_1 receptor are limited (Table 1). According to Neihaus et al. (2007), $CRIP_{1a}$ does not affect CB_1 receptor expression, but does inhibit the constitutive inhibition of Ca^{+2} channels by the CB_1 receptor without affecting agonist-induced inhibition of this effector. This dissertation further examines the effect of $CRIP_{1a}$ on constitutive and agonist-mediated CB_1 receptor function using human embryonic kidney (HEK) cells stably transfected with the human CB_1 (hCB_1 HEK) compared to cells containing a stable co-transfection of $CRIP_{1a}$ (hCB_1-HEK $CRIP_{1a}$). Several $CRIP_{1a}$ effects will be examined (Figure 8), including effects of $CRIP_{1a}$ on ligand specific CB_1-receptor mediated G-protein activation, constitutive activity of the CB_1 receptor, CB_1 receptor desensitization and downregulation, downstream signaling events, and cell type specificity of effects. In addition, the stoichiometric relationship of $CRIP_{1a}$ to the CB_1 receptor in cell and animal models will be examined.

I hypothesize that $CRIP_{1a}$ will attenuate constitutive CB_1 receptor-mediated G-protein activation, thus explaining the effects on downstream signaling by Neihaus et al.

(2007). In addition, I expect to see a similar inhibition of CB_1 receptor constitutive activity on other downstream signaling events, including the constitutive inhibition of adenylyl cylase and constitutive phosphorylation of ERK 1/2. However, I do not expect $CRIP_{1a}$ to modulate ligand stimulated effects of the CB_1 receptor at the G-protein or at the downstream effector level. Additionally, because $CRIP_{1a}$ binds to the C-terminal tail of the CB_1 receptor, where proteins that affect CB_1 receptor trafficking also bind, it is possible that $CRIP_{1a}$ will modulate ligand-induced desensitization and downregulation of the CB_1 receptor. However, as the $CRIP_{1a}$ binding site does not directly overlap with the CB_1 C-terminal tail region specifically required for desensitization or downregulation, there may be no effect. In addition, I expect $CRIP_{1a}$ to be upregulated in response to chronic cannabinoid administration in the whole animal, as seen with other CB_1 receptor interacting proteins such as GRKs and β-arrestins. My overall hypothesis is that $CRIP_{1a}$ is the member of the CB_1 receptosome that determines the constitutive activation of the CB_1 receptor.

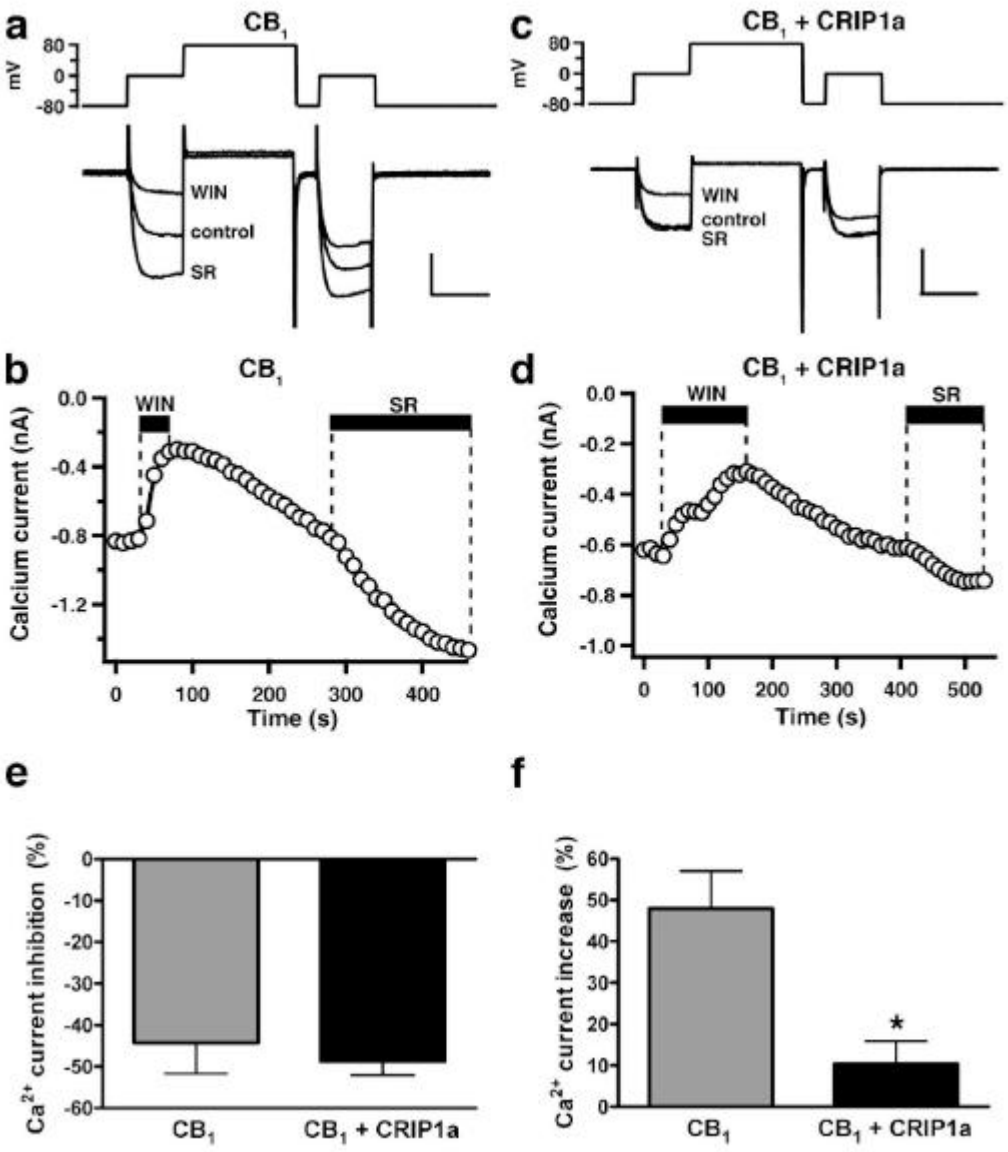

Figure 7. $CRIP_{1a}$ decreases CB_1 receptor-mediated tonic inhibition of voltage-gated Ca^{+2} channels. Panel a, top, voltage-step protocol used to elicit Ca^{+2} current. Bottom, superimposed Ca^{+2} current traces perfusion of control solution (middle trace), 1 μM WIN (top trace) or 1 μM SR1 (bottom trace) for a representative SCG neuron expressing CB_1 receptor. Panel b. Ca^{+2} current amplitude from a SCG neuron expressing CB_1 receptor plotted over time course of a representative experiment. Application of the CB_1 agonist WIN decreased Ca^{+2} current, whereas the CB_1 inverse agonist increased Ca^{+2} current. Panel c. top, voltage step protocol used to elicit Ca^{+2} current traces during perfusion of control solution (middle trace), WIN (top trace) or SR1 (bottom trace) for a representative neuron co-expressing CB_1 receptor and $CRIP_{1a}$. Panel d. Ca^{+2} current amplitude from a SCG neuron co-expressing CB_1 and $CRIP_{1a}$ plotted over the time course for a representative experiment. Application of the CB_1 agonist WIN decreased Ca^{+2} current; however, the ability of the CB_1 agonist to increase Ca^{+2} current was

impaired. Panel e, the ability of the CB_1 agonist WIN to inhibit Ca^{+2} current is unaffected by $CRIP_{1a}$. Panel f, CB_1-mediated enhancement of Ca^{+2} current by inverse agonist SR1 is significantly attenuated by $CRIP_{1a}$ (* $p < 0.05$). Scale bars in panels a and c, 500 pA, 25 ms.

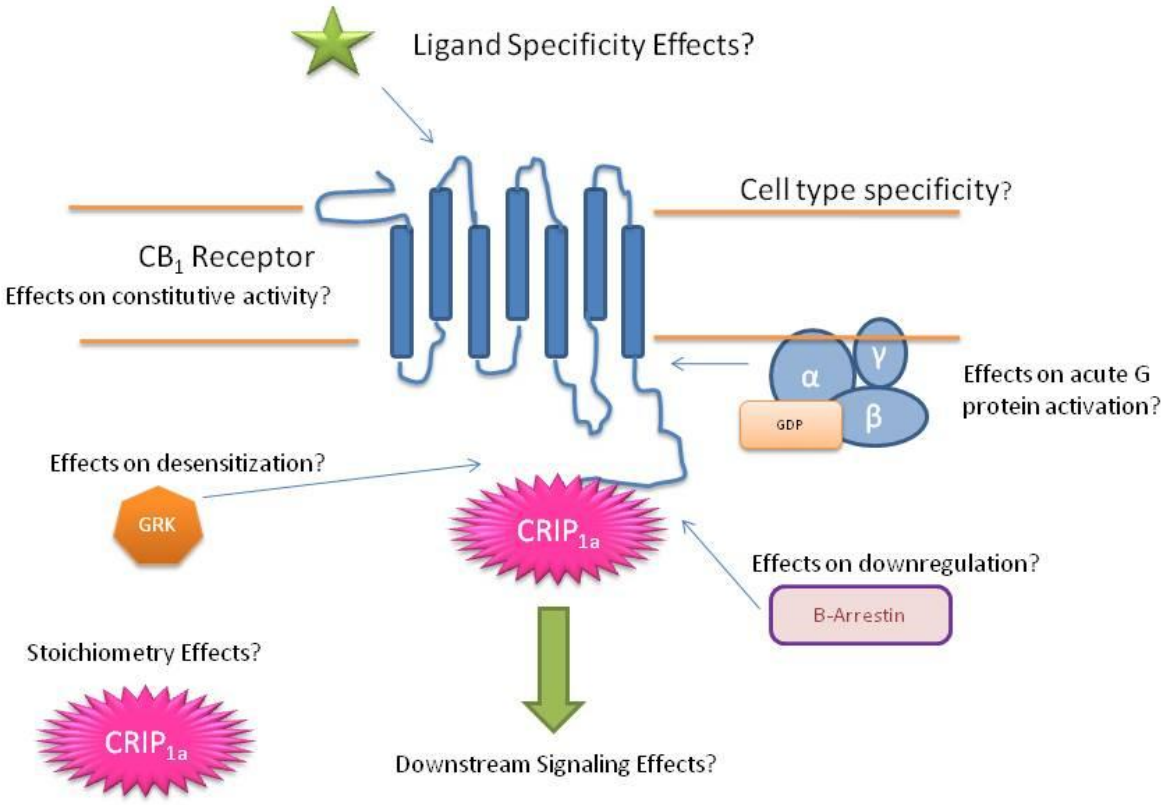

Figure 8. $CRIP_{1a}$ effects to be examined in this dissertation. This study will address the effects of $CRIP_{1a}$ on the constitutive activity of the CB_1 receptor, on acute ligand-specific effects on CB_1 receptor mediated G-protein activation, on CB_1 receptor desensitization and downregulation in response to prolonged agonist treatment, and on CB_1 receptor downstream signaling to effectors. Additionally, this study will examine the stoichiometric relationship between $CRIP_{1a}$ and the CB_1 receptor in CB_1 receptor expressing cells and rat cerebellum.

$CRIP_{1a}$ Effects?	Constitutive	Agonist-Mediated
CB_1 Receptor Expression	No (1)	None apparent.
G-protein Activation	?	?
Receptor Desensitization	None apparent.	?
Receptor Downregulation	None apparent.	?
Ca^{+2} Channel Inhibition	Yes (1)	No (1)
Adenylyl Cyclase Inhibition	?	?
ERK 1/2	?	?

Table 1. Summary of the effects of $CRIP_{1a}$ on CB_1 receptor modulated activity. Prior to this dissertation, known effects of $CRIP_{1a}$ are listed. References: (1) Neihaus et al. (2007).

Chapter 2. Methodology

2.1 Chemicals.

[^{35}S]GTPγS (1150-1300 Ci/mmol) was obtained from PerkineElmer Life and Analytical Sciences (Whaltman, MA). [^{3}H]SR141716A (44.0 Ci/mmol) was purchased from GE Healthcare (Buckinghamshire, UK). WIN 55,212-2 (dissolved in ethanol), GDP (H_2O), pertussis toxin (H_2O) and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO). THC (ethanol), CP 55,940 (ethanol), and SR141716A (ethanol) were provided by the Drug Supply Program of the National Institute of Drug Abuse (NIDA, Rockville, MD). Methananamide (ethanol) was purchased from Cayman Chemical (Ann Arbor, MI). Levonanatradol (ethanol), HU210 (H_2O) and Noladin Ether (ethanol) were supplied by the Department of Pharmacology & Toxicology (Virginia Commonwealth University, Richmond, VA) via the NIDA Drug Supply Program. GST-tagged $CRIP_{1a}$ construct in pGEX vector was provided by Dr. Kathleen Wallis (Medical College of Georgia, GA). $CRIP_{1a}$ antisera 077.4 was provided by Dr. Maurice Elphick (Queen Mary, University of London). Licor Odyssey infrared dye secondary antibodies were purchased from Li-Cor Biosciences (Lincoln, NE). Alpha-tubulin antibody and phosphorylated ERK 1/2 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). ERK 1 antibody was purchased from Chemicon (Billerica, MA). All other reagent grade chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

2.2 Cell culture.

Human embryonic kidney (HEK 293) cells stably expressing the human CB_1 receptor (hCB_1-HEK) were cultured in Dulbecco's Modified Eagle Medium, 1x high glucose (DMEM) containing 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (P/S), 0.25 mg/ml Geneticin (G418) and 15mM HEPES. hCB_1-HEK cells stably co-transfected with $CRIP_{1a}$ (hCB_1-HEK-$CRIP_{1a}$) were cultured in the same media with the addition of 0.1 mg/ml zeocin. Both cell lines were provided by Dr. Deborah Lewis (formerly of the Medical College of Georgia). Dr. Mary Abood (Temple University) created the hCB_1-HEK cell line.

Chinese hamster ovary (CHO) cells stably expressing the mouse CB_1 receptor (mCB_1-CHO) were cultured in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) containing 10% FBS, 1% P/S, and 0.25 mg/ml Hygromycin B. mCB_1-CHO stably co-transfected with $CRIP_{1a}$ (CB_1-CHO-$CRIP_{1a}$) were cultured in the same media with the addition of 1 mg/ml G418. mCB_1-CHO cells were provided by Dr. Billy R. Martin (Virginia Commonwealth University).

2.3 Cell Culture Drug Treatment

Cells were grown to greater than 95% confluency, except where otherwise indicated. Appropriate concentrations of drugs were added to drug treatment media (DMEM, 1% FBS, 1% P/S) and sterile filtered. Drug treatment media was added to cells and incubated for the appropriate time period. To terminate drug treatments, cells were rinsed twice for 2 min with warm rinse media (DMEM, 1% FBS), and harvested, as in section 2.5, for use in assay.

2.4 Cell Transfection

Cells were transfected at 50-80% confluency. Plasmid DNA was added to DMEM media and Plus Reagent (Invitrogen) in the following ratio: per 1 μg DNA, use 60 μl DMEM and 10 μl Plus reagent, and incubated at room temperature (RT) for 15 min. DNA/Plus Reagent mixture was then combined with an equal volume mixture of Lipofectamine (Invitrogen) in the ratio of 2.5 μl Lipofectamine per 1 μg DNA and incubated for 15 min at RT. Cells were rinsed 2x with DMEM, and covered with DMEM at the transfection medium volume recommended by the manufacturer (Invitrogen). The DNA complex was added and the cells incubated for 4-6 hours. Media were removed and replaced with complete culture media specific to the untransfected cell type. Transiently transfected cells were harvested and used after 48 hours.

For stable $CRIP_{1a}$ cell transfection, cells were trypsinized and incubated with increasing doses of selection antibiotic known as a 'kill curve'. After one week, the concentration of selection antibiotic in which the cells were 50% confluent was the concentration used for all complete cell media preparations. The 50% confluent cells were grown to 100% confluency. Cells were typsinized and plated in 96-well plates at a density of 1 cell for every 3 wells, insuring that each colony was grown from a single cell. Each week the colonies with surviving cells were moved into larger plates (24 well plate, 6 well plate, 10 cm dish). The colonies were then harvested and stored for use in assays. Cells were screened via immunoblot (see section 2.8) for $CRIP_{1a}$ expression and [^{3}H]SR141716A saturation analysis (see section 2.9) for CB_1 receptor expression. The colony in which $CRIP_{1a}$ expression was the highest and had a CB_1 receptor level most

similar to the CB_1-CHO cells without $CRIP_{1a}$ transfection was selected and cultured for future experiments.

2.5 Membrane Homogenate Preparation.

Cells were harvested in PBS + 0.4% (w, v) EDTA or scraped and centrifuged at 1000 x g for 10 min to remove media. Cells were homogenized in ice-cold assay buffer (50 mM Tris-HCl, 3 mM $MgCl_2$, 0.2 mM EGTA, 100 mM, pH 7.4), centrifuged at 50,000 g for 10 min, and protein content was determined by the Bradford method (Bradford 1976).

2.6 Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry (LC-ESI-MS-MS) Analysis of Endocannabinoids.

The endocannabinoids, arachidonoyl ethanolamide (AEA) and 2-arachidonoyl glycerol (2-AG) were measured using a method modified from Di Marzo et al. (Di Marzo et al. 2000). Briefly, 1 nmol of AEA-d8 and 2 nmol 2AG-d8 as deuterated internal standards were added to each sample. The endocannabinoids were extracted from the samples with 3 volumes chloroform/methanol (2/1, v,v) and a 0.73% (w,v) sodium chloride mixture. The chloroform was collected and evaporated to dryness with nitrogen. The extracts were reconstituted with 100 µL methanol and placed in autosample vials for LC-ESI-MS-MS analysis. The AEA and 2-AG were separated and detected using a Shumadzu SCL HPLC system (Kyoto, Japan) with a Discovery® HS C18 Column 15cm x 2.1mm, 3µm (Supelco: Bellefonte, PA) kept at 40°C and an Applied Bio systems 3200 Q trap with a turbo V source for TurbolonSpray (Ontario, Canada) run in multiple reaction monitoring (MRM) mode. The mobile phase was 10 % water with 1g/L ammonium acetate and 0.1% (v,v) formic acid, and 90% (v,v) methanol with 1 g/L

ammonium acetate and 0.1% (v,v) formic acid The flow rate was 0.3 mL/min and total run time was 10.00 min. The injection volume was 20 µL and the auto sampler temperature was set at 5°C. The following transition ions for AEA, AEA-d8, 2-AG and 2-AG-d8 were monitored: 348 > 62, 356 > 62, 387 > 96 and 379 > 287 m/z, respectively. The standard curves for the samples were 0.039 – 1.25 pmol AEA and 0.063- 2.0 nmol 2-AG. The limit of detection and limit of quantification were set at 0.039 pmol for AEA and 0.063 nmol for 2-AG.

2.7 $CRIP_{1a}$ Generation, Purification, and Determination of Stoichiometry.

Glutathione-S-transferase (GST)-tagged $CRIP_{1a}$ vector (GST tag-thrombin cleavage site-$CRIP_{1a}$) was provided in pGEX-4T-1 vector (GE Healthcare, Piscataway, NJ), cloned into the BAM HI and XHO sites of the vector. Plasmid DNA containing GST-tagged $CRIP_{1a}$ was transformed into E. coli BL21-DE3 competent cells. E. coli were grown to OD(600) = 0.6 from a single colony, and then GST-tagged $CRIP_{1a}$ expression was induced via addition of isopropyl thiogalactoside (IPTG, 1 mM) for 6 hours. E. coli were collected via centrifugation (1,000 g, 10 min, 4° C) and a bacterial lysate produced via sonication with lysozyme (25 µg/ml). $CRIP_{1a}$ induction and solubility tests were performed by polyacrylamide gel electrophoresis (PAGE) on harvest lysates (crude lysate, which was then separated into soluable and insoluable lysates) on 10% polyacrlamide gels and stained with Coomassie blue to verify protein expression. GST-tagged $CRIP_{1a}$ was isolated from bacterial lysate using a GSTrap FF column (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's instruction. Briefly, the column was equilibrated with binding buffer (0.1 M phosphate buffered saline, PBS), bacterial lysate was added to allow GST-CRIP fusion, the column was

washed (PBS), and the GST tag was cleaved via thrombin (500 units in 0.5 ml PBS). Following elution with PBS, the eluate contained protein and thrombin. The thrombin was subsequently removed by HiTrap Benzamidine column purification according the manufacturer's instruction. Briefly, the column was equilibrated with binding buffer (0.05 M Tris-HCl, 0.5 M NaCl, pH 7.4). Sample was added to the column and eluted with binding buffer. Eluates were collected and pooled. $CRIP_{1a}$ pools and a BSA protein concentration curve were subject to PAGE using 15% polyacrylamide gels, and visualized by Coomassie blue stain. Stained gel images were captured via ImageJ, and $CRIP_{1a}$ concentration was determined by subsequent linear regression analysis (Windows Excel). $CRIP_{1a}$ concentration curves were then generated in tandem with hCB_1-HEK (± $CRIP_{1a}$) or mCB_1-CHO (± $CRIP_{1a}$) cell membrane preparations or mouse cerebellum to determine $CRIP_{1a}$ concentration via immunoblot analysis on 15% polyacrylamide gels, visualized by the Licor Odyssey system. From this data, the relationship between $CRIP_{1a}$ concentration in the cells and CB_1 receptor levels, determined by [^{3}H]SR141716A saturation binding, was calculated.

2.8 Mouse THC Treatment

Male ICR mice were housed in an animal care facility maintained at 22 ± 2° C on a 12 hour light/dark cycle. Food and water were available ad libitum. All experiments were conducted according to the guidelines established by the Institutional Animal Care and Use Committee of Virginia Commonwealth University Medical Center. THC was dissolved in a 1:1:18 solution of ethanol, castor oil 40 mole ethoxylate (Emulphor) and saline. Mice received subcutaneous injections of THC or vehicle twice daily (7:00 am and 3:00 pm) for 6.5 days. Mice were injected with 10 mg/kg THC that was increased

every other day to 30 and 60 mg/kg THC, respectively. Twenty-four hours after final injection, mice were sacrificed and decapitated. Hippocampi were harvested from brains and were immediately frozen in isopentane at -30° C and stored at -80° C.

2.9 Immunoblotting.

Samples (70 μg) of cell membrane homogenates were added to sample buffer (1 M TRIS, 20% SDS, 1 M DTT, 60% sucrose, bromophenol blue) and boiled for 10 min. Samples were loaded into 15% SDS polyacrylamide gels, and electrophoresis was conducted at 120 V for 1.5 hours. Proteins were electrophorectically transferred to polyvinylidene difluoride (PVDF) membranes at 70 V for 70 min. Blots were blocked for 1 hour at room temperature (RT) with 5% (w/v) nonfat dry milk and then rinsed with TRIS buffered saline with 0.1% (v/v) Tween-20 (TBST). Primary antibody (anti-$CRIP_{1a}$ antisera 077.4 (rabbit), 1:500) was incubated overnight at 4° C, followed by TBST rinse. Secondary antibody (Licor goat anti-rabbit 800 CW IR dye, 1:5,000) was then incubated at RT for 1 hr, followed by TBST rinse. Blots were visualized with the Licor Odyssey system.

2.10 [^{3}H]SR141716A Binding.

Saturation analysis of [^{3}H]SR141716A ([^{3}H]SR1) binding was performed by incubating 30 μg of membrane protein with 0.5-10 nM [^{3}H]SR1 in TME (50 mM TRIS, 3 mM $MgCl_2$, 0.2 mM EGTA) with 0.5% (w/v) BSA, in a total volume of 0.5 ml ± 5 μM unlabeled SR1 to determine non-specific binding. The assay was incubated for 90 min at 30° C and terminated by vacuum filtration through GF/B glass fiber filters that were presoaked in Tris buffer containing 0.5% (w/v) BSA. Bound radioactivity was determined using liquid scintillation spectrophotometry at 45% efficiency for [^{3}H].

2.11 [^{35}S]GTPγS Binding.

Cell membrane preparations (10 μg protein) were incubated with various drugs, 100 mM NaCl, 0.1 % BSA, 10 μM GDP and 0.1 nM [^{35}S]GTPγS in TME, 0.5 ml total volume, for 2 hr at 30° C. In some experiments, 100 mM NaCl was replaced by varying concentrations of NaCl. Basal binding was assessed in the absence of agonist, and nonspecific binding was measured with 10 μM unlabeled GTPγS. The reaction was terminated by vacuum filtration through GF/B glass fiber filters. Bound radioactivity was determined by liquid scintillation spectrophotometry at 95% efficiency for [^{35}S].

2.12 [^{3}H]cAMP Whole Cell Assay

Cells were seeded on 24-well plates the previous evening to reach 100% confluency on day of assay. Cells were treated with phosphodiesterase inhibitor mix (1 M HEPES, 10 mM RO 20-1724, 100 mM IBMX, 100 mg/ml BSA in DMEM) for 30 min at 37° C. Cells were then incubated in the presence of 10 μM forskolin, with and without drugs, for 8 min, in a total reaction volume of 200 μl. Following drug treatment, cells were placed on ice and drug-containing media removed to terminate the reaction. Cells were lysed with 3% perchloric acid for 30 min, and neutralized with 15% potassium bicarbonate.

Cell lysate supernatant was assayed for cAMP formation using the Liquid Phase Cyclic AMP radioassay (Diagnostic Products Corporation), which determines cAMP concentration through competitive displacement of labeled versus unlabeled cAMP binding to a cAMP binding protein, according to the manufacturer's instruction. Briefly, cell supernatant or cAMP standards are combined with [^{3}H]cAMP and cAMP binding protein in a TRIS-EDTA buffer and incubated on ice for 90 min. A blank (no cells), cell

blank (with cells), and total binding were assayed in the absence of cAMP binding protein. Unbound cAMP was removed through the addition and subsequent centrifugation (15,000 g, 10 min, 4° C) of 100 ml of a charcoal/dextran suspension. Radioactivity of the supernatant was determined using liquid scintillation spectrophotometry at 45% efficiency for [^{3}H]. A log transformation calibration curve of radioactivity versus standards was generated on Microsoft Excel from which unknown cAMP concentrations were determined.

2.13 MAP Kinase Assay

Cells were seeded on 6 well plates the previous evening to reach 100% confluency on the day of assay. Cells were serum starved (no FBS) for 6 hours prior to drug treatment. Cells were then incubated for 8 min with either WIN (2 μM), THC (3 μM), SR1 (0.2 μM) or vehicle in a reaction volume of 1 ml. Following drug treatment, cells were placed on ice to terminate the reaction and treated with 200 μl cell lysis buffer (0.5% NP40, 1% Triton X-100, 20 mM Tris-HCl, 135 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 1 mM PMSF, 1 mM Na Vandate, and 1:1000 aprotonin) for 15 min. Cell lysates were centrifuged for 15 min at 13,000 rpm at 4° C. The protein concentration of the resultant membrane preparations was determined via Bradford assay. Membrane preparations were subject to immunblot analysis of pERK 1/2 and ERK 1/2 levels as described in Methods 2.9. Membrane preparations of hCB_1-HEK cells (±$CRIP_{1a}$ transfection) (50 μg) were probed using phosphorylated ERK 1/2 antibody (1:200 Santa Cruz) and ERK 1 antibody for loading control (1:250, Chemicon).

2.14 Data Analysis.

Unless otherwise noted, all binding data are reported as mean values ± standard error of the mean (SEM) of at least three independent experiments that were each performed in duplicate ([^{3}H]SR141716A) or triplicate ([^{35}S]GTPγS). Data were analyzed using Graph Pad Prism v4.0c software. B_{max}, K_D, E_{max} and EC_{50}, values were determined by non-linear regression analysis. Non-linear regression was used to fit the data to the following equation: $y = (B_{max})(L)/(K_D + L)$ where y is equal to the amount of [^{3}H]SR141716A or [^{35}S]GTPγS bound at receptor ligand concentration L. E_{max} and EC_{50} was substituted for B_{max} and K_D respectively where appropriate. B_{max} and E_{max} is the amount of [^{3}H]SR141716A and [^{35}S]GTPγS bound at maximally effective concentrations of receptor ligand, respectively. K_D and EC_{50} values are the concentration of receptor ligand producing half maximal binding of [^{3}H]SR141716A and modulation of [^{35}S]GTPγS binding, respectively. Basal binding is determined in the absence of ligand. Net stimulated binding is defined as agonist-stimulated minus basal binding. Percentage of stimulation is defined as (net stimulated binding/basal binding) x 100%.

Measurements of cAMP levels and MAPK activity were the results of at least 3 independent experiments. cAMP levels were measured in duplicate in each experiment. Data are reported as percentage of control, which is defined as (ligand modulated value/vehicle value) x 100%.

Significance was determined using ANOVA and the post-hoc Newman-Keuls Multiple Comparison Test for comparison of three or more conditions or by students t-test for comparison of two conditions. Two way ANOVA and the post-hoc Bonferroni Test was used in experiments examining the effects of $CRIP_{1a}$ on CB_1 receptor function

in conjunction with NaCl effects, and on CB_1 receptor desensitization. Results were considered statistically significant when the p value ≤ 0.05.

Chapter 3. Results

3.1 CB_1 Receptor Expression in hCB_1 HEK, hCB_1 HEK-$CRIP_{1a}$ Cell Lines and Rat Cerebellum.

Previous studies have shown that $CRIP_{1a}$ localizes to the cell membrane and interacts with the C-terminal tail of CB_1 receptors, without affecting CB_1 receptor expression levels (see Introduction 1.6). Initial experiments were performed to confirm that lack of effect of $CRIP_{1a}$ on CB_1 receptor levels. Human embryonic kidney cells (HEK) stably transfected with the human CB_1 receptor (hCB_1-HEK) cells were created by Dr. Mary Abood (Temple University). hCB_1-HEK with a stable co-transfection of $CRIP_{1a}$ (hCB_1-HEK-$CRIP_{1a}$) were co-transfected and provided by Dr. Deborah Lewis (formerly of the Medical College of Georgia).

To determine whether the stable co-expression of $CRIP_{1a}$ affected CB_1 receptor expression levels, [^{3}H]SR141716A saturation binding analyses were performed in hCB_1-HEK and hCB_1-HEK-$CRIP_{1a}$ cells (Table 2). There were no statistically significant differences between hCB_1-HEK, with and without $CRIP_{1a}$ co-expression with regard to CB_1 receptor number (B_{max} = 1.64 ± 0.29 pmol/mg in cells without $CRIP_{1a}$ versus 1.51 ± 0.30 pmol/mg in cells with $CRIP_{1a}$) or the equilibrium dissociation constant, (K_D = 3.02 ± 1.59 nM in cells without $CRIP_{1a}$ versus 3.82 ± 1.34 nM in cells with $CRIP_{1a}$), according to students t-test (signficance reached at $p < 0.05$).

	B_{max} (pmol/mg)	K_D (nM)
hCB_1-HEK	1.64 ± 0.298	3.02 ± 1.59
hCB_1-HEK-$CRIP_{1a}$	1.51 ± 0.308	3.82 ± 1.34
Rat Cerebellum	3.63 ± 0.372	0.452 ± .077

Table 2. [^{3}H]SR141716A saturation analysis of hCB_1-HEK cells, with and without $CRIP_{1a}$ co-expression, and rat cerebellum. Data are mean values ± SEM (n=6). B_{max} values represent total cell membrane receptor levels. No significant differences between the two hCB_1-HEK cell types were observed. K_D values represent the reciprocal of the affinity of [^{3}H]SR141716A for the receptor. No significant effect of $CRIP_{1a}$ on the affinity of [^{3}H]SR141716A binding was observed.

In addition, CB_1 expression levels were determined in rat cerebellum, for later stoichiometric comparison to the hCB_1-HEK (± $CRIP_{1a}$) cell lines. Rat cerebellum expressed CB_1 receptors at a B_{max} value of 3.63 ± 0.372 pmol/mg.

3.2 CB_1 Receptor Expression Relative to Cell Confluency in hCB_1-HEK (± $CRIP_{1a}$) Cell Lines.

The level of cell confluency can affect the expression level of cell surface receptors, which might be modulated by $CRIP_{1a}$. To determine whether cell confluency affected the expression of stably transfected CB_1 receptor in the hCB_1-HEK (± $CRIP_{1a}$) cell lines, cells were grown to low (50%), high (95%), and ultra-confluence (100+ %) and assayed for CB_1 receptor expression using saturation analysis of [^{3}H]SR141716A binding (Figure 9). High and ultra-confluence did not affect CB_1 receptor expression in hCB_1-HEK with and without $CRIP_{1a}$ transfection. However, low confluency significantly decreased CB_1 receptor expression in hCB_1-HEK cells compared to hCB_1-HEK-$CRIP_{1a}$ cells (ANOVA, Newman Keuls Multiple Comparison post-hoc test). hCB_1-HEK cells at 50% confluency expressed a B_{max} value of 0.90 ± 0.29 pmol/mg compared to a B_{max} value of 1.43 ± 0.29 and 1.64 ± 0.29 pmol/mg for high and ultra-confluency, respectively. Low confluency did not affect CB_1 receptor expression in hCB_1-HEK-$CRIP_{1a}$ cells, with a B_{max} value of 1.68 ± 0.17 pmol/mg compared to 1.63 ± 0.26 and 1.51 ± 0.30 pmol/mg for high and ultra-confluency, respectively. In all subsequent radioligand binding experiments, cells were harvested and utilized at a greater than 95% confluence.

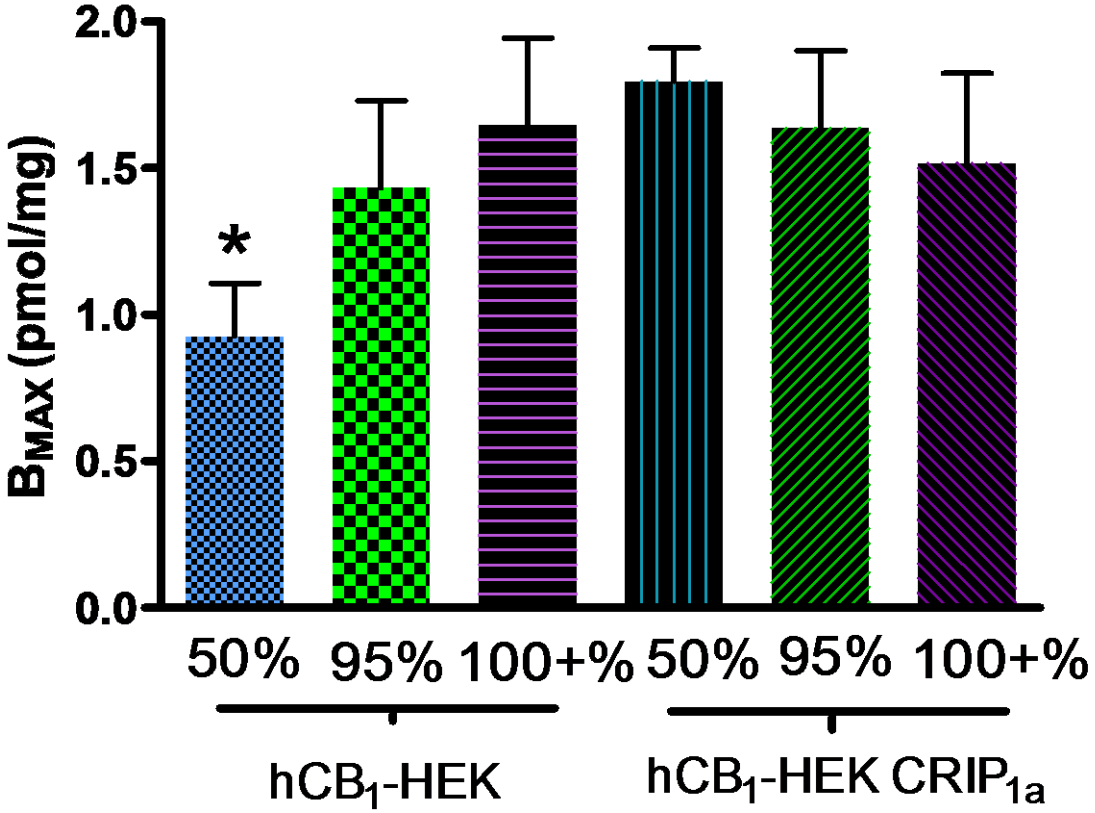

Figure 9. CB_1 receptor expression relative to cell confluency in hCB_1-HEK cells ($\pm CRIP_{1a}$ co-expression). Data are mean B_{max} values from saturation analysis of [^{3}H]SR141716A binding ± SEM (n=4). hCB_1-HEK cells ($\pm CRIP_{1a}$ transfection) were grown to low (50%), high (95%) and ultra-confluence (100+%) and subject to [^{3}H]SR141716A. High and ultra confluence did not affect CB_1 receptor expression in hCB_1-HEK cells ($\pm CRIP_{1a}$ transfection). Low confluency significantly decreased CB_1 receptor expression in hCB_1-HEK cells compared to hCB_1-HEK-$CRIP_{1a}$ cells. (ANOVA, Newman Keuls Multiple Comparison post-hoc test, $p < 0.05$).

3.3 $CRIP_{1a}$ Expression and Stoichiometric Relationship of $CRIP_{1a}/CB_1$ in hCB_1 HEK Cell Lines (± $CRIP_{1a}$ co-transfection) and Mouse Cerebellum.

The effect of $CRIP_{1a}$ on CB_1 receptor function is likely to be determined in part by the molar ratio of $CRIP_{1a}$ to CB_1 receptor. To determine the stoichiometric relationship of the $CRIP_{1a}$ to CB_1 receptor, $CRIP_{1a}$ was generated and purified using GST-pulldown methodology. Purified $CRIP_{1a}$ was used to generate $CRIP_{1a}$ concentration curves. hCB_1-HEK (± $CRIP_{1a}$) membrane preparations (70 μg) were compared to $CRIP_{1a}$ concentrations curves via immunoblot of 15% polyacrylamide gels and visualized by the Licor Odyssey system to elucidate unknown $CRIP_{1a}$ concentrations (Figure 10). $CRIP_{1a}$ concentrations were determined for male mouse cerebellum in the same manner. Experimentally determined $CRIP_{1a}$ concentrations were compared to CB_1 receptor expression (Table 2) to determine the molar stoichiometric relationship of $CRIP_{1a}/CB_1$ receptors (Table 3). In hCB_1-HEK cells lacking $CRIP_{1a}$ transfection, the molar ratio of $CRIP_{1a}/CB_1$ is less than 1 (0.376 ± 0.875), indicating that the CB_1 receptor is in molar excess relative to amount of $CRIP_{1a}$ natively expressed in hCB_1-HEK cells. In hCB_1-HEK-$CRIP_{1a}$ cells, $CRIP_{1a}$ is in molar excess to the CB_1 receptor, with a molar ratio of $CRIP_{1a}/CB_1$ receptor of 5.47 ± 0.429. These $CRIP_{1a}/CB_1$ receptor molar ratios are statically different (t-test, $p < 0.001$). Interestingly, mouse cerebellum has a $CRIP_{1a}/CB_1$ receptor molar ratio of 33.6 ± 5.19, which indicates a molar excess of $CRIP_{1a}$ compared to the CB_1 receptor, similar to the hCB_1-HEK cell line, although somewhat greater in magnitude.

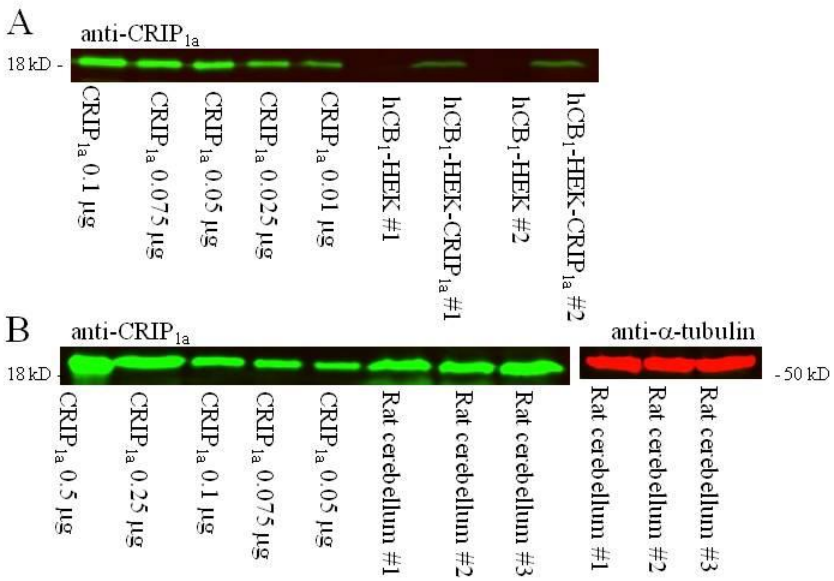

Figure 10. Quantitative western blot analysis of $CRIP_{1a}$ concentration in hCB_1-HEK cells (±$CRIP_{1a}$ transfection) and rat cerebellum. $CRIP_{1a}$ purified via GST-pulldown methodology generated a $CRIP_{1a}$ concentration curve. Probed membrane preparations (A) hCB_1-HEK cells (±$CRIP_{1a}$ transfection) (70 µg/sample) and (B) mouse cerebellum (100 µg/sample) using anti-$CRIP_{1a}$ antisera 077.4 (Elphick, rabbit, 1:500) followed by Licor Odyssey goat anti-rabbit 800 CW IR dye (1:5,000). Mouse cerebellum loading control probed using anti-α-tubulin (Santa Cruz Biotechnology, 1:500) followed by Licor Odyssey goat anti-mouse 680 IR dye (1:5,000). Images were analyzed via Licor Odyssey Infrared Imaging System. Unknown $CRIP_{1a}$ concentrations were calculated by linear regression analysis using Microsoft Excel. Images are from a representative immunoblot of 3 replicates.

	CB_1 (pmol/mg)	**$CRIP_{1a}$ (pmol/mg)**	**Molar Ratio ($CRIP_{1a}/CB_1$)**
hCB_1-HEK	**1.64 ± 0.298**	**0.564 ± 0.131**	**0.376 ± 0.875**
hCB_1-HEK-$CRIP_{1a}$	**1.51 ± 0.308**	**8.20 ± 0.643**	**5.47 ± 0.429***
Rat Cerebellum	**3.63 ± 0.371**	**115 ± 12.2**	**33.6 ± 5.19**

Table 3. Stoichiometric molar ratio of $CRIP_{1a}/CB_1$ in hCB_1-HEK cells (±$CRIP_{1a}$ transfection) and rat cerebellum. Data are mean B_{max} values from saturation analysis of [^{3}H]SR141716A binding ± SEM for CB_1 receptor expression, $CRIP_{1a}$ concentration (pmol/mg) determined via immunoblot ± SEM, and resulting molar ratio ± SEM (n=4). Stable $CRIP_{1a}$ transfection caused a significant increase in $CRIP_{1a}$ expression, creating a significant molar excess relative to CB_1 receptor expression (* = $p < 0.001$ different from hCB_1-HEK cells lacking $CRIP_{1a}$ transfection).

3.4 $CRIP_{1a}$ Modulation of Ligand Specific CB_1 Receptor-Generated G-protein Activation; [^{35}S]GTPγS Binding in hCB_1-HEK (± $CRIP_{1a}$) Lines.

The interaction of $CRIP_{1a}$ with the intracellular surface of CB_1 receptors could affect the ability of the receptor to activate G-proteins. Indeed, Neihaus et al. (2007) have reported that co-expression of $CRIP_{1a}$ with CB_1 receptors in SCG neurons decreased the basal activity of CB_1 receptors without altering WIN-stimulated activity (see Introduction 1.6). I hypothesize that the effects of $CRIP_{1a}$ on CB_1 receptor basal activity downstream are generated at the level of acute G-protein activation, and that $CRIP_{1a}$ co-expression will decrease constitutive CB_1 receptor-mediated G-protein activation, but not in G-protein activation induced by agonist ligands. Therefore, to determine the effects of $CRIP_{1a}$ on basal and agonist stimulated CB_1 receptor mediated G-protein activity, [^{35}S]GTPγS binding was performed in hCB_1-HEK (± $CRIP_{1a}$) cells (Figure 11). A wide variety of CB_1 ligands were tested, including the classical phytocannabinoid THC, and its synthetic nantradol analog Levo, the aminoalkylindole WIN, the nonclassical bicyclic CP, the eicosanoid MethA (a stable analog of the endocannabinoid anandamide), the diarylpyrazole inverse agonist SR1, the classical synthetic cannabinoid HU210, and the putative endogenous cannabinoid Nol Eth.

In hCB_1-HEK cells, Nol Eth appeared to act as a full agonist, while WIN, CP and HU210 also acted as high efficacy agonists. MethA and Levo acted as high to moderate efficacy partial agonists, THC acted as a low efficacy partial agonist and SR1 acted as an inverse agonist. Interestingly, $CRIP_{1a}$ co-expression reduced the apparent inverse agonism of SR1 (Figure 16), in agreement with Neihuas et al. (2007). SR1 produced –13.34 ± 1.65% stimulation which was reduced to –7.33 ± 1.21 in the presence of $CRIP_{1a}$.

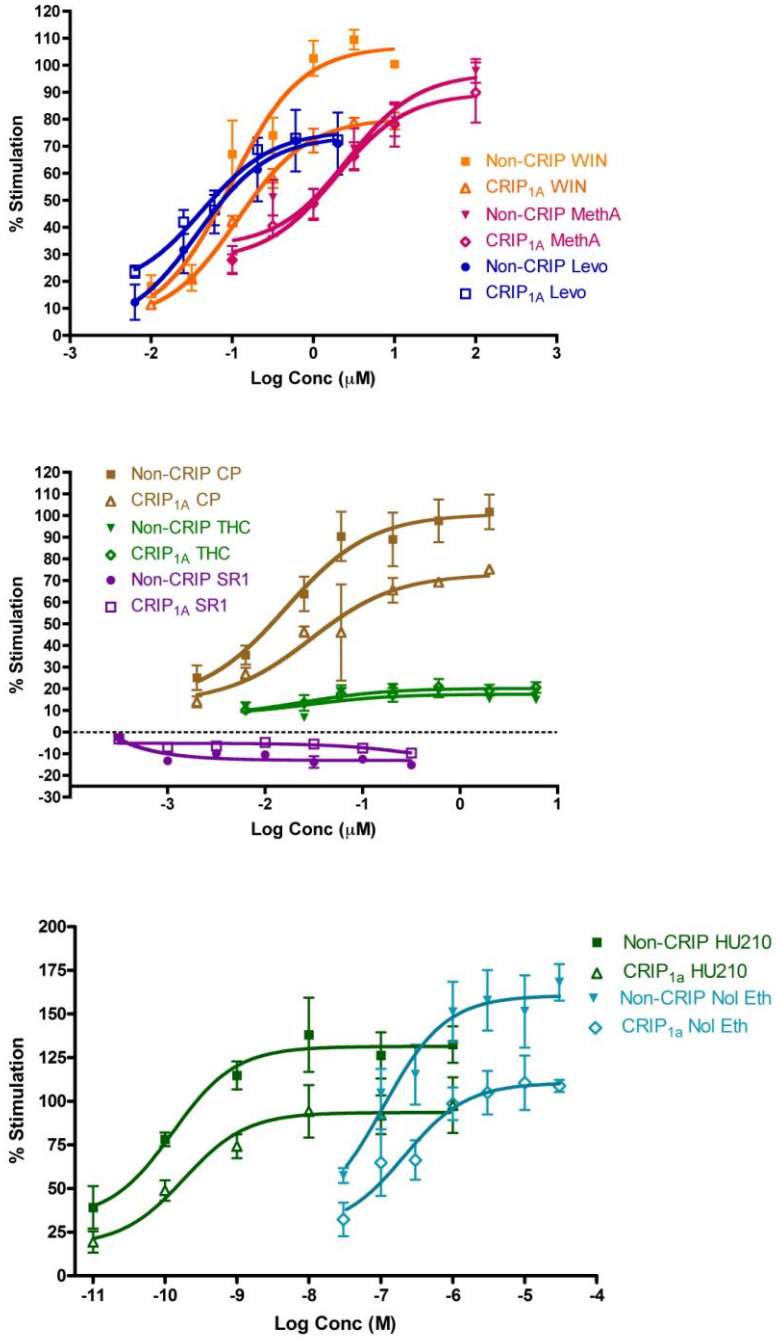

Figure 11. Ligand-induced [^{35}S]GTPγS binding in hCB$_1$-HEK and hCB$_1$-HEK-CRIP$_{1a}$ cells. The top panel shows concentration-effect curves for WIN, MethA, and Levo, the middle panel shows curves for CP, THC and SR1, and the bottom panel shows curves for HU210 and Nol Eth. Data points are mean % stimulation ± SEM (n=3). All experiments were performed in the presence of 100 mM NaCl.

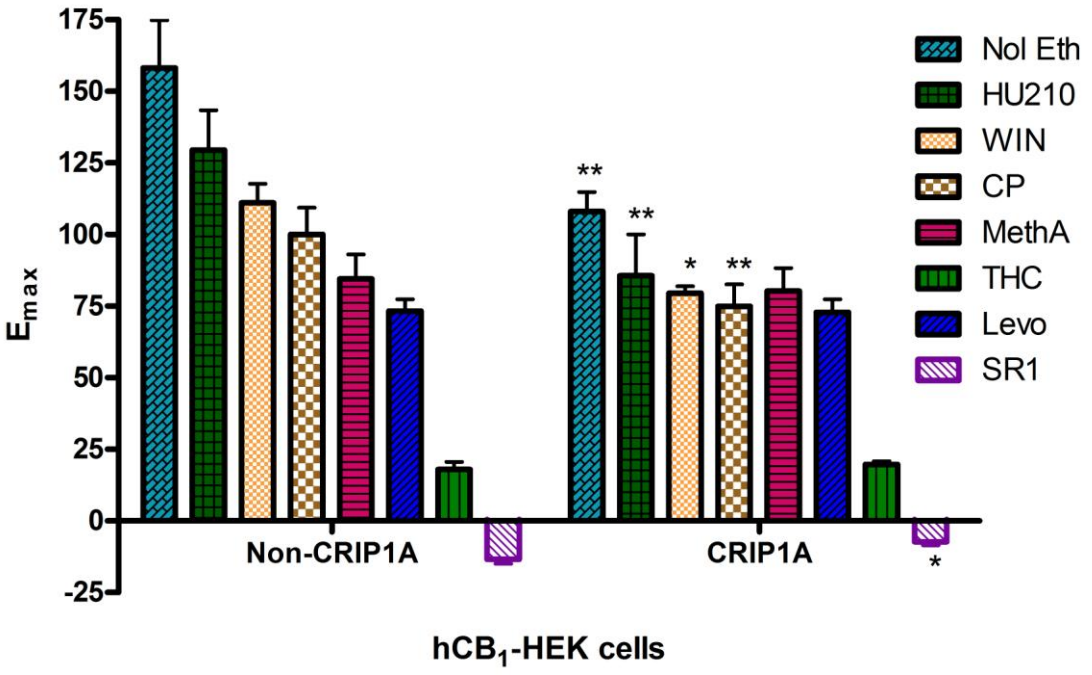

Figure 12. E_{max} values for Nol Eth, HU210, WIN, CP, MethA, THC, Levo and SR1 in membranes from hCB_1-HEK cells with and without co-expression of $CRIP_{1a}$. Data are mean E_{max} values derived from the concentration-effect curves shown in Figure 11 ± SEM (n=3). Significant differences were found for the E_{max} values of Nol Eth, HU210, WIN, CP and SR1 in cells with $CRIP_{1a}$ co-expression compared to those without. *,**: $p < 0.05$, 0.01 different from the corresponding drug in non-$CRIP_{1a}$ expressing cells by ANOVA with post-hoc Newman-Keuls Test.

$CRIP_{1a}$ also reduced CB_1 receptor-mediated G-protein activation of the high efficacy agonists Nol Eth, HU210, WIN and CP. Nol Eth produced an E_{max} value of 158.2 ± 16.6% in hCB_1-HEK cells, which was significantly reduced to an E_{max} value of 108.1 ± 6.7% in hCB_1-HEK-$CRIP_{1a}$ cells. Nol Eth produced an E_{max} value of 129.5 ± 13.9% in hCB_1-HEK cells, which was significantly reduced to an E_{max} value of 85.6 ± 14.5% in hCB_1-HEK-$CRIP_{1a}$ cells. WIN produced an E_{max} value of 111.10 ± 6.66% stimulation in hCB_1-HEK cells, which was significantly reduced to an E_{max} value of 79.47 ± 2.46% in hCB_1-HEK-$CRIP_{1a}$ cells. CP stimulated hCB_1-HEK cells to an E_{max} value of 100.10 ± 9.32% which $CRIP_{1a}$ co-expression significantly reduced to an E_{max} value of 74.95 ±7.69%.

Stimulation by MethA, Levo, and THC were unaffected by $CRIP_{1a}$ (E_{max} value of 84.55 ± 8.48% stimulation by MethA in hCB_1-HEK cells compared to E_{max} value of 80.23 ± 8.02% in hCB_1-HEK-$CRIP_{1a}$). Levo produced an E_{max} value of 73.28 ± 4.07% in hCB_1-HEK compared to an E_{max} value of 72.84 ± 4.52% in hCB_1-HEK $CRIP_{1a}$. THC produced an E_{max} value of 17.90 ± 2.68% in hCB_1-HEK compared to an E_{max} value of 19.74 ± 1.07 in hCB_1-HEK $CRIP_{1a}$. All data were analyzed via ANOVA ($p < 0.05$, $n = 16$, $F = 84.87$, $R^2 = 0.9725$, and $df = 17$), with post-hoc Neuman Keuls Multiple Comparison Test post-hoc. Significance was reached at $p < 0.05$.

3.5 The Effects of $CRIP_{1a}$ on Spontaneous CB_1 G-protein Activation; [^{35}S]GTPγS Binding in hCB_1-HEK (± $CRIP_{1a}$) Cell Lines with Varying Na^+ Levels.

Spontaneous CB_1 receptor mediated G-protein activity in hCB_1-HEK cells was relatively small in magnitude, as determined by SR1 inhibition of [^{35}S]GTPγS binding (see Results 3.4). Therefore, it was of interest to examine the effects of $CRIP_{1a}$ under varying conditions of spontaneous CB_1 receptor activity. I hypothesize that as spontaneous CB_1 receptor activity increases, the ability of $CRIP_{1a}$ to decrease constitutive activity will become more pronounced. In addition, the magnitude of attenuation of the net stimulatory effects of WIN on the CB_1 receptor-mediated G-protein activity by $CRIP_{1a}$ is hypothesized to decrease as a function of spontaneous receptor activity, whereas attenuation of net SR1-inhibited G-protein activity is expected to increase as a function of spontaneous receptor activity. Because Na^+ is a negative allosteric modulator of spontaneous GPCR activity, the effects of $CRIP_{1a}$ on spontaneous CB_1 receptor-mediated G-protein activity were examined in [^{35}S]GTPγS binding studies with varying NaCl concentrations. hCB_1-HEK (± $CRIP_{1a}$) cells were incubated with maximally effective concentrations of WIN (10 μM), THC (6 μM), SR1 (0.05 μM) or under basal conditions and increasing doses of NaCl (0-175 mM).

For hCB_1-HEK cells with and without $CRIP_{1a}$ co-expression, increasing sodium concentrations lead to a decrease in overall G-protein activation for all conditions tested (Figure 13).

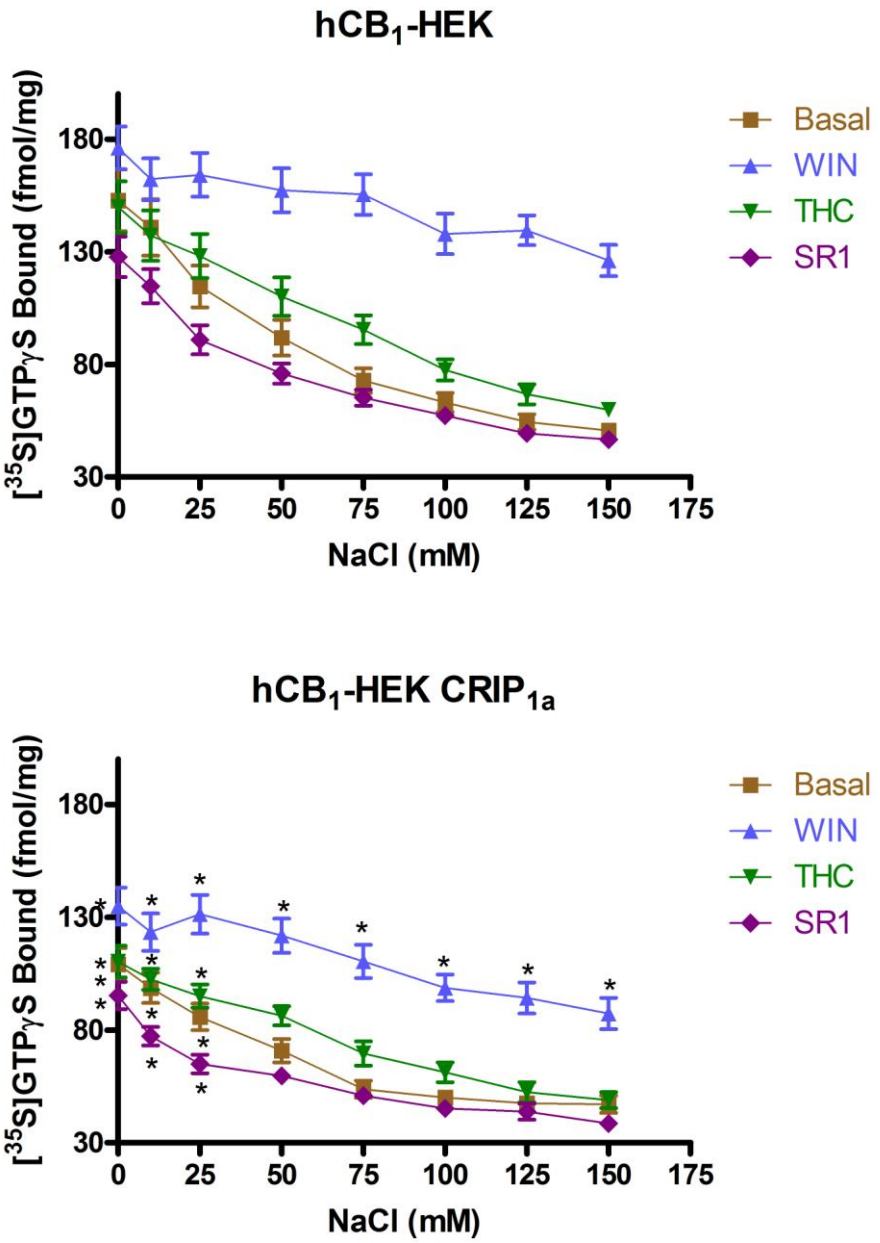

Figure 13. [^{35}S]GTPγS binding in hCB$_1$-HEK cells (±CRIP$_{1a}$ co-expression) with varying NaCl concentrations. Cells were incubated with maximally effective concentrations agonist concentrations (10 μM WIN, 6 μM THC) and a maximally inhibiting concentration of the inverse agonist SR1 (0.5 μM SR1) in the presence of varying concentrations of NaCl (0-150 mM). Data points are mean [^{35}S]GTPγS bound (fmol/mg) ± SEM (n=5). * indicates statistically significant difference in hCB$_1$-HEK-CRIP$_{1a}$ cells compared to identical conditions in hCB$_1$-HEK cells via two-way ANOVA with post-hoc Bonferroni Test ($p < 0.05$).

Furthermore, $CRIP_{1a}$ co-expression decreased WIN-stimulated G-protein activation at all NaCl concentrations tested, similar to the earlier experiments under 100 mM NaCl (Figure 16). At high NaCl concentrations (> 50 mM NaCl), THC was unaffected by $CRIP_{1a}$ co-expression, also as in earlier experiments (Figure 12). $CRIP_{1a}$ significantly decreased GPCR- mediated G-protein activation when spontaneous CB_1 activity was high (0, 10 & 25 mM NaCl) for all conditions tested. Data were analyzed using two-way ANOVA with Bonferroni post-hoc test, which found both a significant interaction of $CRIP_{1a}$ and NaCl ($p < 0.0001$, DF = 7), and both $CRIP_{1a}$ and NaCl were a significant source of variation for all treatment groups ($p < 0.05$, DF = 7).

Additionally, basal activity was subtracted from all the conditions and the data were expressed as net fmol/mg (Figure 14). When examining net [^{35}S]GTPγS binding, the apparent $CRIP_{1a}$ effects on spontaneous CB_1 receptor mediated G-protein activation at low NaCl concentrations are lost. However, the decrease in WIN-stimulated G-protein activation in the presence of $CRIP_{1a}$ remains significant. Data were analyzed using two-way ANOVA with Bonferroni post-hoc test, which found a significant interaction of $CRIP_{1a}$ and NaCl in WIN treated cells ($p < 0.0001$, DF = 56). NaCl was a significant source of variation for all treatments ($p < 0.0001$). However, $CRIP_{1a}$ was only a significant source of variation for WIN treatment ($p = 0.0098$). For each analysis, DF = 7.

Lastly, net fmol/mg data were analyzed for area under the curve (AUC) (Figure 19). $CRIP_{1a}$ co-expression significantly decreased AUC for WIN stimulated [^{35}S]GTPγS binding (10000 ± 579 fmol/mg in hCB_1-HEK versus 6920 ± 590 fmol/mg in hCB_1-HEK-$CRIP_{1a}$ cells, two-way ANOVA, $p < 0.001$). The presence of $CRIP_{1a}$ did not statistically

affect AUC in the presence of THC (2010 ± 143 fmol/mg in hCB_1-HEK versus 1440 ± 189 fmol/mg in hCB_1-HEK-$CRIP_{1a}$ cells) or SR1 (-1840 ± 362 fmol/mg in hCB_1-HEK versus -1420 ± 343 fmol/mg in hCB_1-HEK-$CRIP_{1a}$ cells). However, there was a trend for $CRIP_{1a}$ to decrease the apparent inverse agonism of SR1. Data were analyzed via two-way ANOVA, with post-hoc Bonferroni Test ($df = 2$, $F = 9.910$). Significance was reached at $p < 0.05$.

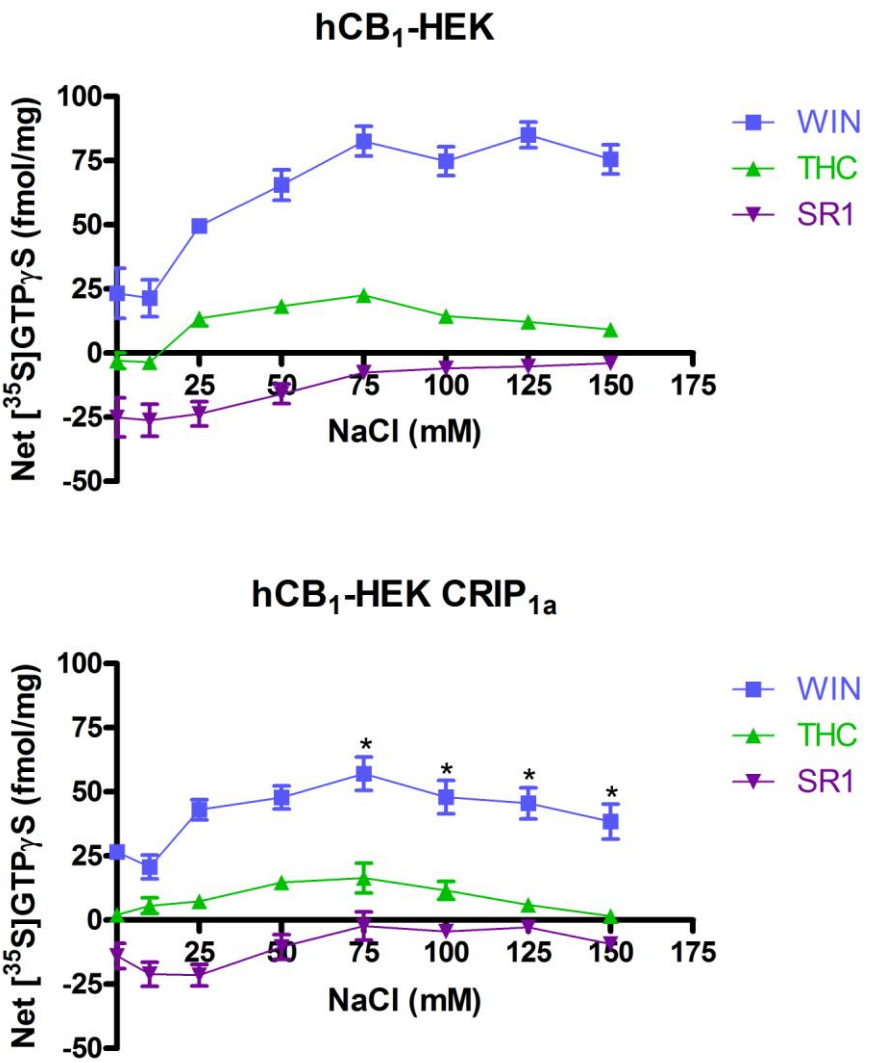

Figure 14. Net-stimulated [^{35}S]GTPγS binding in hCB_1-HEK cells (±$CRIP_{1a}$ co-expression) with varying NaCl concentrations. Cells were incubated with maximally effective concentrations of cannabinoid ligands (10 μM WIN, 6 μM THC) and a maximally inhibiting concentration of the inverse agonist SR1 (0.5 μM SR1) in the presence of varying concentrations of NaCl (0-150 mM). Data are mean net-stimulated fmol/mg ± SEM (n=5). * indicates statistically significant difference in hCB_1-HEK-$CRIP_{1a}$ cells compared to identical conditions in hCB_1-HEK cells via two-way ANOVA with post-hoc Bonferroni Test ($p < 0.05$).

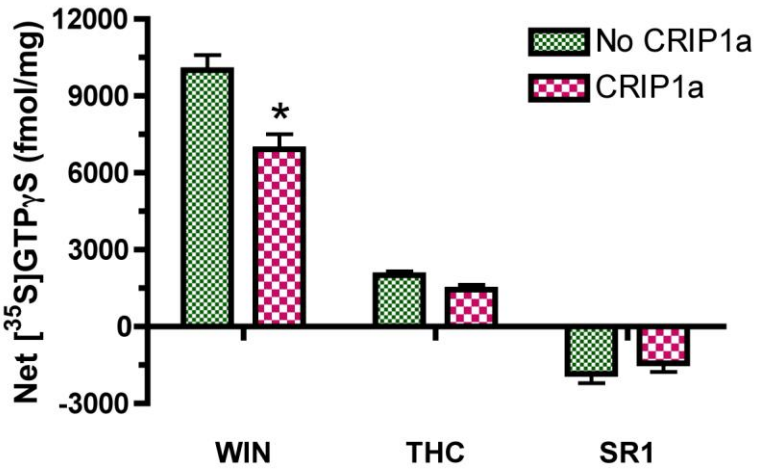

Figure 15. Area under the curve (AUC) analysis of net-stimulated [^{35}S]GTPγS binding studies in hCB_1-HEK cells (±$CRIP_{1a}$ co-expression) with varying NaCl concentrations. Cells were incubated with maximally effective concentrations of cannabinoid ligands (10 μM WIN, 6 μM THC) and a maximally inhibiting concentration of the inverse agonist SR1 (0.5 μM SR1) in the presence of varying concentrations of NaCl (0-150 mM) (see figure 14). Data are mean AUC of net fmol/mg caculated from the curves shown in Figure 14 ± SEM (n=5). * indicates statistically significant difference in hCB_1-HEK-$CRIP_{1a}$ cells compared to identical conditions in hCB_1-HEK cells (ANOVA with post-hoc Newman-Keuls Test, $p < 0.05$).

3.6 [^{35}S]GTPγS in hCB$_1$-HEK (± CRIP$_{1a}$) Cell Lines with PTX Pre-treatment, with and without 100 mM NaCl.

Given that basal and agonist G-protein activity was negatively modulated by Na^+ concentrations, the previous results question whether CRIP$_{1a}$ modulates GPCR-mediated G-protein activity or directly affects G-proteins. I hypothesize that CRIP$_{1a}$ acts exclusively on the CB$_1$ receptor, to which it binds, without directly interacting with G-proteins. To determine the effect of CRIP$_{1a}$ on GPCR-specific G-protein activation versus non-GPCR mediated G-protein activity, [^{35}S]GTPγS binding was performed on hCB$_1$-HEK (± CRIP$_{1a}$) cells with and without pertussis toxin (PTX) pretreatment, and in the presence and absence of sodium (Figure 16). Under normal 100 mM Na^+ conditions, CRIP$_{1a}$ significantly reduced WIN stimulated [^{35}S]GTPγS binding, as seen in the previous experiments (149.7 ± 19.2 fmol/mg in hCB$_1$-HEK vs. 121.5 ± 17.1 fmol/mg in hCB$_1$-HEK-CRIP$_{1a}$ cells). In the hCB$_1$-HEK cells lacking CRIP$_{1a}$, PTX pre-treatment significantly reduced GPCR-dependent G-protein activation. Interestingly, in CRIP$_{1a}$-containing cells, there was no significant difference between cells with or without PTX pre-treatment under basal or SR1 inhibited conditions. Data were analyzed via ANOVA (n = 12, $F = 20.28$, $R^2 = 0.8711$), with post-hoc Neuman Keuls Multiple Comparison Test, significance reached at $p < 0.05$.

Spontaneous G-protein activity was enhanced by the lack of Na^+; under these conditions CRIP$_{1a}$ decreased G-protein activation under all conditions tested. Furthermore, in CRIP$_{1a}$-containing cells, there was a significant difference between G-protein activation with and without PTX pre-treatment for all conditions tested, suggesting that while CRIP$_{1a}$ does decrease G-protein activity, it is not as effective as

PTX when spontaneous GPCR activity is high. All data were analyzed via ANOVA ($n = 12$, $F = 70.24$, $R^2 = 0.9590$), with post-hoc Neuman Keuls Multiple Comparison Test, significance reached at $p < 0.05$.

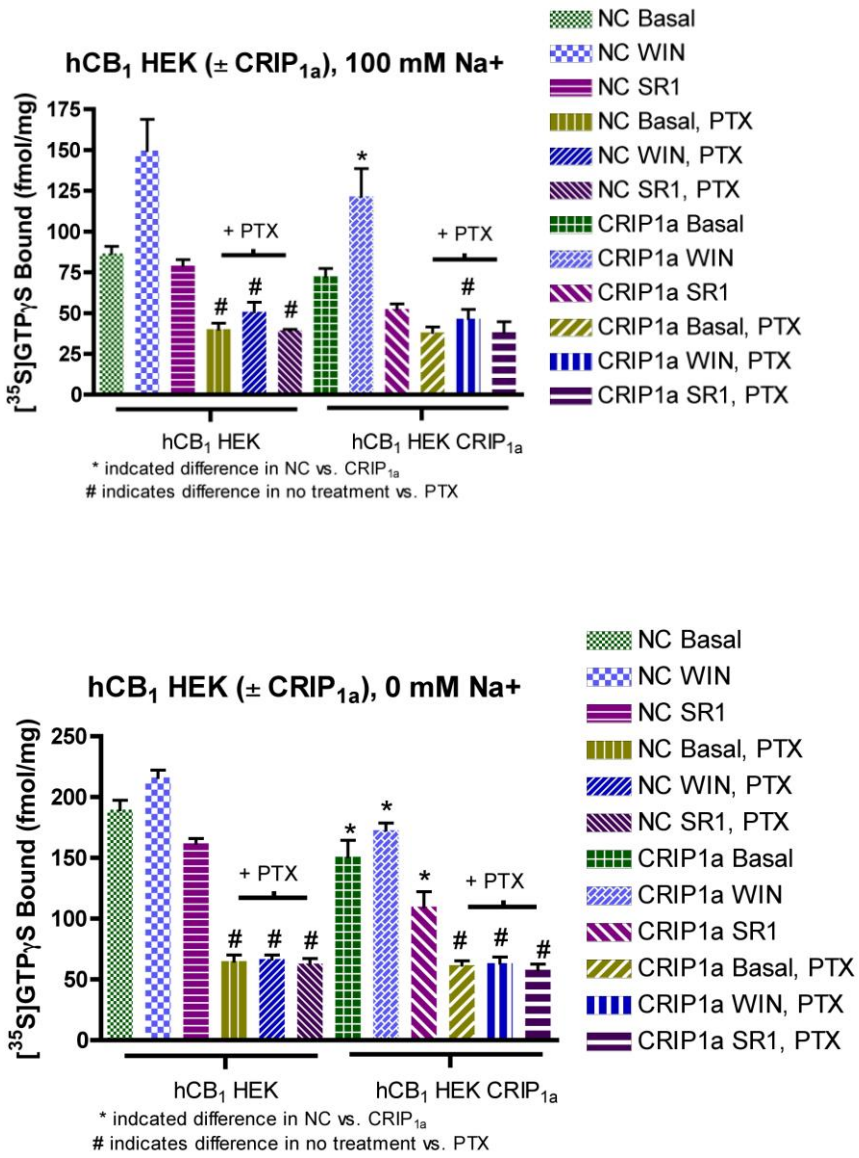

Figure 16. [^{35}S]GTPγS binding in hCB_1-HEK cells (±$CRIP_{1a}$ co-expression) with varying NaCl concentrations, with and without PTX pre-treatment. NC indicates no $CRIP_{1a}$. Cells were incubated with maximally effective concentrations agonist concentrations (10 μM WIN, 6 μM THC) and a maximally inhibiting concentration of the inverse agonist SR1 (0.5 μM SR1) in the presence (top) or absence (bottom) of NaCl and with or without PTX pre-treatment. Data points are averaged from at least independent three experiments containing measurements made in triplicate ± SEM. * indicates statistically significant difference in hCB_1-HEK-$CRIP_{1a}$ cells compared to identical conditions in hCB_1-HEK cells, # indicates statistically significant difference between no treatment versus PTX pre-treatment within the cell type (ANOVA with post-hoc Newman-Keuls Test, $p < 0.05$).

3.7 Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry (LC-ESI-MS-MS) Analysis of Endocannabinoids in hCB_1-HEK (± $CRIP_{1a}$) Cell Lines.

Basal G-protein activity in CB_1 receptor expressing cells can be due in part to the presence of endocannabinoids or to actual spontaneous GPCR-mediated G-protein activity. To determine whether endocannabinoids were present in sufficient levels to activate CB_1 receptors, the levels of the two established endocannabinoids, AEA and 2-AG, were measured via liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS-MS) in hCB_1-HEK (± $CRIP_{1a}$) cell lines (Table 4). For both cells types, 3 samples of whole cells (10×10^6 cells) or membrane preparations (1,000 μg) were analyzed. There were no detectable levels of AEA or 2-AG in whole cell or membrane preparations for either hCB_1-HEK (± $CRIP_{1a}$) cell lines.

	AEA (pmol)	2-AG (nmol)
hCB_1-HEK Whole cells (10 x 10^6)	None Detected	None Detected
hCB_1-HEK Membrane prep (1,000 μg)	None Detected	None Detected
hCB_1-HEK-$CRIP_{1a}$ Whole cells (10 x 10^6)	None Detected	None Detected
hCB_1-HEK-$CRIP_{1a}$ Membrane prep (1,000 μg)	None Detected	None Detected

Table 4. Endocannabinoid levels in CB_1 in hCB_1-HEK cells (±$CRIP_{1a}$ co-expression). Liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS-MS) was used to determine the levels of two endocannabinoids, AEA and 2-AG in whole cell and membrane preparations of hCB_1-HEK cells (±$CRIP_{1a}$ co-expression). Data are mean values (pmol/nmol) ± SEM of three independent experiments. No AEA or 2-AG were detected in either cell line or either preparation type.

3.8 $CRIP_{1a}$ Effects on CB_1 Receptor Desensitization; [^{35}S]GTPγS Binding in Drug-treated hCB_1-HEK (± $CRIP_{1a}$) Cell Lines.

The CB_1 receptor C-terminus is important in the regulation of CB_1 receptor signaling by the GRK/β-arrestin pathway, as described in Introduction 1.4. $CRIP_{1a}$ is known to interact with the CB_1 receptor C-terminus and therefore could modulate the response of this receptor to prolonged agonist occupancy, such as desensitization or downregulation. I hypothesize that $CRIP_{1a}$ will affect CB_1 receptor desensitization; $CRIP_{1a}$ may only affect high efficacy ligand desensitization induced by WIN treatment as a result of reduced CB_1 receptor-mediated G-protein activation, or could affect the desensitization induced by all full and partial agonist ligands as a result of steric hindrance with receptor regulatory proteins such as GRK or β-arrestin. To determine the effects of $CRIP_{1a}$ on CB_1 receptor desensitization, hCB_1-HEK (± $CRIP_{1a}$) cells were pre-incubated with WIN (10 μM), THC (6 μM) or vehicle for 4 hours, followed by MethA-stimulated [^{35}S]GTPγS binding to assess CB_1 receptor function (Figure 17). MethA was used to assess CB_1 activation because acute stimulation of [^{35}S]GTPγS binding by this ligand was unaffected by $CRIP_{1a}$ (Figure 12).

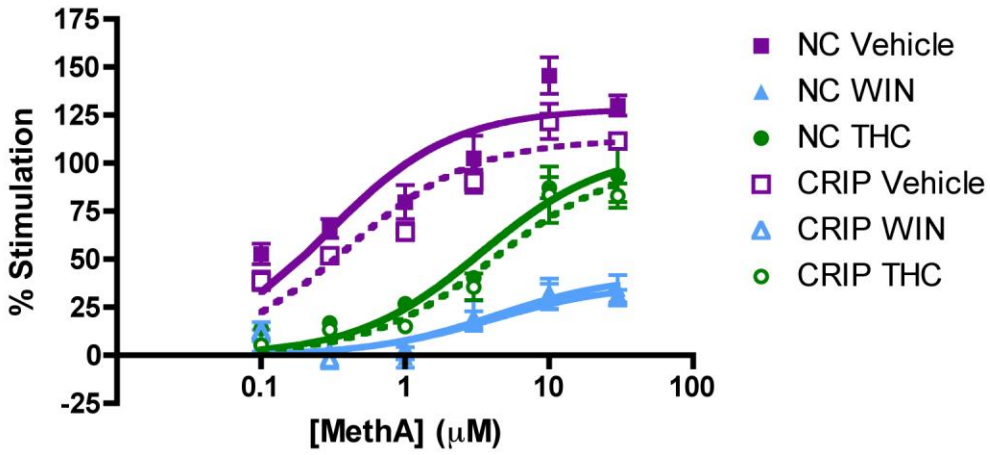

Figure 17. [^{35}S]GTPγS binding in hCB$_1$-HEK cells (±CRIP$_{1a}$ co-expression) following four hour drug pretreatment. Cells were pre-treated with maximally effective concentrations of agonist (10 μM WIN, 5 μM THC) or vehicle and subjected to MethA-stimulated [^{35}S]GTPγS binding. Concentration effect curves were generated for vehicle, WIN and THC-treated cells. Data points are mean % stimulation ± SEM (n=4).

Two-way ANOVA of E_{max} values indicate that drug pretreatment was a significant source of variation (df = 2, F = 73.69, significance reached at $p < 0.05$), suggesting that pretreatment with either WIN or THC caused significant desensitization of the CB_1 receptor. However, there was no significant effect of $CRIP_{1a}$ or an interaction between $CRIP_{1a}$ and drug treatment. Similarly, as reveled by two-way ANOVA, EC_{50} values were affected by drug treatment (df = 2, F = 7.418 significance reached at $p < 0.05$), but there was no effect of $CRIP_{1a}$ nor an interaction between the two. Therefore, $CRIP_{1a}$ did not affect cannabinoid-induced CB_1 receptor desensitization.

Subsequent 1-way ANOVA with Newman-Keuls Multiple Comparison Test (significance reached at $p < 0.05$, n = 6) revealed that both E_{max} and EC_{50} values for WIN and THC pre-treatment were significantly different from vehicle (Table 5), except for the E_{max} value of THC pre-treated hCB_1-HEK-$CRIP_{1a}$ cells. ANOVA of E_{max} yielded F = 30.11 and $R^2 = 0.8931$, and ANOVA of EC_{50} yielded F = 6.442 and $R^2 = 06823$. Pretreatment with WIN decreased the E_{max} value of MethA by 66.5 % in hCB1-HEK cells and by 67.0 % in hCB1-HEK-$CRIP_{1a}$ cells compared to vehicle. Pretreatment with THC decreased the Emax value of MethA by 21.9 % in hCB1-HEK cells and by 7.2 % in hCB1-HEK-CRIP1a cells compared to vehicle. Pretreatment with WIN or THC resulted in an approximately 10-fold increase in EC_{50} values.

The basal values of G-protein activation were unchanged by $CRIP_{1a}$ for all treatment groups (48.9 ± 1.92 fmol/mg, 47.3 ± 2.71 fmol/mg and 55.6 ± 1.83 fmol/mg in hCB_1-HEK cells for vehicle, WIN and THC treatment, respectively, and 43.9 ± 4.21 fmol/mg, 55.0 ± 10.5 fmol/mg and 45.5 ± 1.98 fmol/mg in hCB_1-HEK-$CRIP_{1a}$ cells for

vehicle, WIN and THC treatment, respectively). Data were analyzed via ANOVA, with post-hoc Neuman Keuls Multiple Comparison Test. Significance was reached at $p < 0.05$.

	E_{max} (% Stimulation)	EC_{50} (μM)
hCB_1-HEK Vehicle Treated	**128 ± 8.04**	**0.308 ± 0.071**
hCB_1-HEK WIN Treated	**43.1 ± 7.07 ***	**5.70 ± 2.11 ***
hCB_1-HEK THC Treated	**100 ± 8.55 ***	**3.00 ± 0.049 ***
hCB_1-HEK-$CRIP_{1a}$ Vehicle Treated	**112 ± 5.10**	**0.423 ± 0.082**
hCB_1-HEK-$CRIP_{1a}$ WIN Treated	**37.0 ± 4.73 ***	**4.16 ± 0.595***
hCB_1-HEK-$CRIP_{1a}$ THC Treated	**104 ± 7.37**	**5.17 ± 1.31***

Table 5. E_{max} and EC_{50} values from concentration-effect curves of MethA-stimulated [^{35}S]GTPγS binding in hCB_1-HEK cells (±$CRIP_{1a}$) following four hour pre-treatment with vehicle, WIN or THC. Data are means values derived from the concentration-effect curves shown in Figure 21 ± SEM (n=4). * indicates statistically significant difference from vehicle treatment within cell type via one-way ANOVA with post-hoc Newman-Keuls Multiple Comparison Test ($p < 0.05$).

3.9 $CRIP_{1a}$ Effects on CB_1 Receptor Downregulation; [^{3}H]SR141716A Saturation Analysis in Drug-treated hCB_1-HEK (± $CRIP_{1a}$) Cell Lines.

Although $CRIP_{1a}$ did not have a significant effect on CB_1 receptor desensitization, the $CRIP_{1a}$ binding site on the CB_1 receptor is known to be near the binding site for GASP1, a protein involved in CB_1 receptor downregulation (see Introduction 1.4). Therefore, I hypothesize that $CRIP_{1a}$ may be able to affect CB_1 receptor downregulation without affecting desensitization. To determine the effects of $CRIP_{1a}$ on CB_1 receptor downregulation, hCB_1-HEK (± $CRIP_{1a}$) cells were pre-incubated with WIN (10 μM), THC (6 μM) or vehicle for 4 hours, followed by [^{3}H]SR141716A saturation analysis to assess CB_1 receptor expression (Figure 18).

In hCB_1-HEK cells, both WIN and THC caused significant downregulation of the CB_1 receptor. WIN pretreatment decreased the [^{3}H]SR141716A B_{max} value to 48.2 ± 7.9% of vehicle control and pretreatment with THC decreased the B_{max} value to 23.6 ± 7.8% of vehicle control for hCB_1-HEK cells. Absolute B_{max} values for vehicle, WIN and THC treated hCB_1-HEK cells were 1.09 ± 0.06 pmol/mg, 0.53 ± 0.08 pmol/mg, and 0.26 ± 0.08 pmol/mg, respectively.

$CRIP_{1a}$ attenuated WIN-induced CB_1 receptor downregulation. WIN pretreatment decreased the B_{max} value to 85.9 ± 22.1% of vehicle control in hCB_1-HEK-$CRIP_{1a}$ cells, which was not significantly different from control. However, $CRIP_{1a}$ did not significantly decrease THC-induced downregulation. The B_{max} value (50.4 ± 25.6% of vehicle control) in THC-treated hCB_1-HEK-$CRIP_{1a}$ cells was not significantly different from the B_{max} value of THC-treated hCB_1-HEK cells (23.6 ± 7.8%). Absolute B_{max}

values for vehicle, WIN and THC treated hCB_1-HEK-$CRIP_{1a}$ cells were 1.28 ± 0.22 pmol/mg, 0.99 ± 0.22 pmol/mg, and 0.49 ± 0.21 pmol/mg, respectively. Data were analyzed via one-way ANOVA (n= 6, F = 7.493, R^2 = 0.6408) with post-hoc Neuman Keuls Multiple Comparison Test. Significance was reached at $p < 0.05$.

Further analysis examined absolute B_{max} and K_d values (Table 6). In agreement with % vehicle data, WIN and THC caused significant downregulation of the CB_1 receptor in hCB_1-HEK cells, which $CRIP_{1a}$ co-expression prevented in WIN pre-treated cells. Furthermore, the K_D values were not significantly different between vehicle and drug pretreated groups in either cell line, indicating effective removal of the drug following pretreatment.

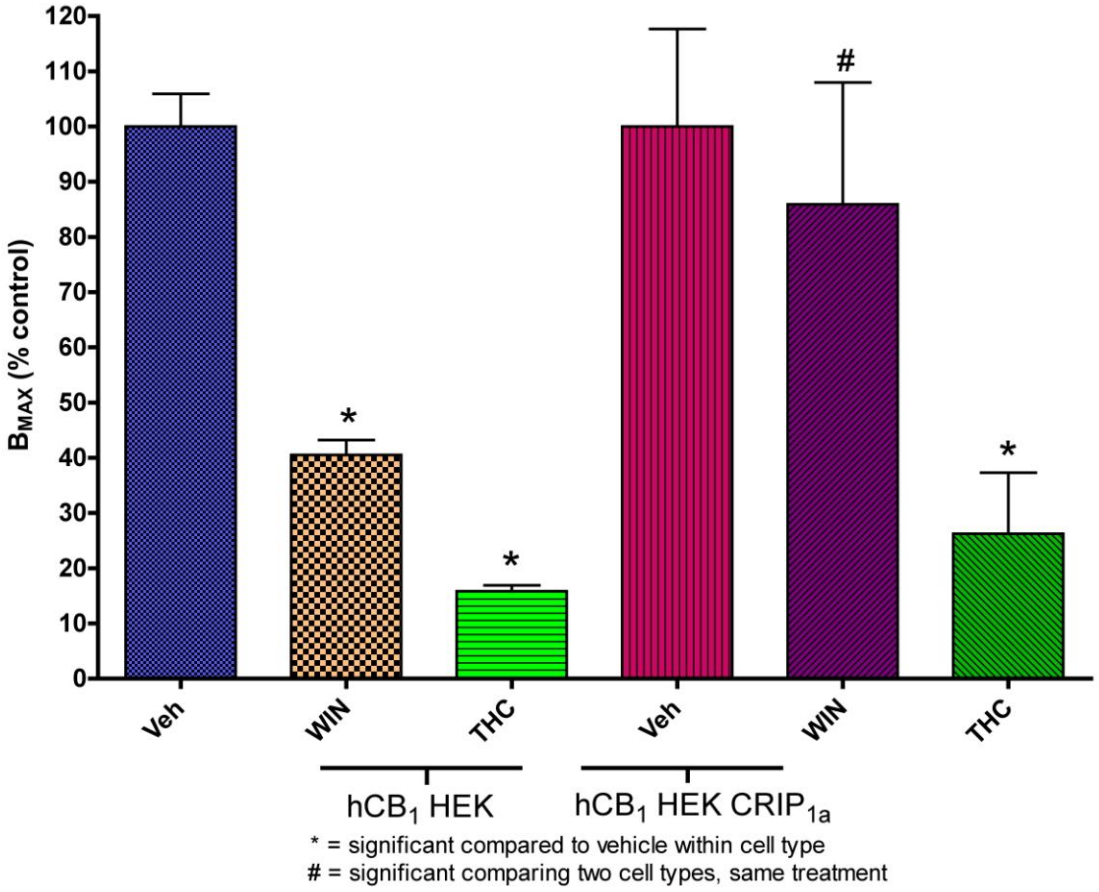

Figure 18. CB_1 receptor levels in hCB_1-HEK cells ($\pm CRIP_{1a}$ co-expression) following four hour drug pretreatment. Cells were pre-treated with maximally effective concentrations agonist concentrations (10 μM WIN, 5 μM THC) or vehicle. B_{max} (pmol/mg) values were obtained by [^{3}H]SR141716A saturation analysis and are expressed as mean % of vehicle control ± SEM (n=4). * indicates statistically significant difference from vehicle within each cell type, # indicates statistically significant difference comparing two cell types with the same treatment (ANOVA with post-hoc Newman-Keuls Test, $p < 0.05$).

	B_{max} (pmol/mg)	K_D (nM)
hCB_1-HEK Vehicle Treated	1.09 ± 0.065	1.48 ± 0.346
hCB_1-HEK WIN Treated	0.531 ± 0.089*	0.984 ± 0.272
hCB_1-HEK THC Treated	0.260 ± 0.087*	1.12 ± 0.997
hCB_1-HEK-$CRIP_{1a}$ Vehicle Treated	1.28 ± 0.227	2.93 ± 0.675
hCB_1-HEK-$CRIP_{1a}$ WIN Treated	0.994 ± 0.220	3.11 ± 1.16
hCB_1-HEK-$CRIP_{1a}$ THC Treated	0.499 ± 0.214*	2.42 ± 1.24

Table 6. B_{max} and K_D values from [^{3}H]SR141716A binding studies using hCB_1-HEK cells (±$CRIP_{1a}$) following four hour pre-treatment with vehicle, 5 µM WIN or 10 µM THC. Data are mean values ± SEM (n=4). * indicates statistically significant difference from vehicle treatment within cell type via one-way ANOVA ($p < 0.05$) with post-hoc Neuman Keuls Multiple Comparison Test.

3.10 Effect of $CRIP_{1a}$ on CB_1 receptor mediated modulation of cAMP generation in hCB_1-HEK (± $CRIP_{1a}$) cell lines.

$CRIP_{1a}$ acutely decreased spontaneous G-protein activation and the G-protein activation induced by certain cannabinoid ligands. Therefore, it is of interest to determine if the acute modulation of CB_1 receptor mediated G-protein activity translates into effects on CB_1 receptor mediated downstream signaling events. I hypothesize that $CRIP_{1a}$ will attenuate the constitutive inhibition of cAMP, but not agonist-mediated effects on cAMP, by the CB_1 receptor, in agreement with results found by Neihaus et al. on the voltage-gated Ca^{+2} channels.

Modulation of 10μM forskolin-stimulated cAMP generation in hCB_1-HEK (± $CRIP_{1a}$) cell lines was examined in intact cells incubated with maximally effective concentrations of 2 μM WIN, 3 μM THC, 0.2 μM SR1 or vehicle (Figure 19). In hCB_1-HEK cells, CB_1 activation by the full agonist WIN caused a significant decrease in cAMP levels (32.7 ± 2.96 pM cAMP in vehicle vs. 12.7 ± 2.88 pM cAMP for WIN treatment). THC also appeared to cause a decrease in cAMP, but it was not significantly different from vehicle (20.1 ± 1.06 pM). The inverse agonist SR1 nearly doubled cAMP levels compared to control (62.0 ± 1.9 pM). The presence of $CRIP_{1a}$ did not significantly alter cAMP levels in the presence of vehicle alone (21.9 ± 3.34 pM), nor the significant decrease in cAMP levels due to the full agonist WIN (12.7 ± 2.77 pM, $p < 0.05$ different from vehicle condition) or cAMP levels observed with THC treatment (23.4 ± 1.7 pM). Notably, $CRIP_{1a}$ co-expression abolished the inverse agonism of SR1, such that the levels of cAMP in the presence of SR1 were not significantly different from vehicle-treatment

in hCB_1-HEK-$CRIP_{1a}$ cells (27.5 ± 5.7 pM cAMP following SR1 treatment versus 21.9 ± 3.34 pM in vehicle treated cells). Moreover, the levels of cAMP in the presence of SR1 in hCB_1-HEK-$CRIP_{1a}$ cells were significantly lower than in hCB_1-HEK cells without $CRIP_{1a}$ co-expression. Results were analyzed via one-way ANOVA, with significance reached at $p < 0.05$ ($n = 8$, $F = 17.51$, $R^2 = 0.8845$).

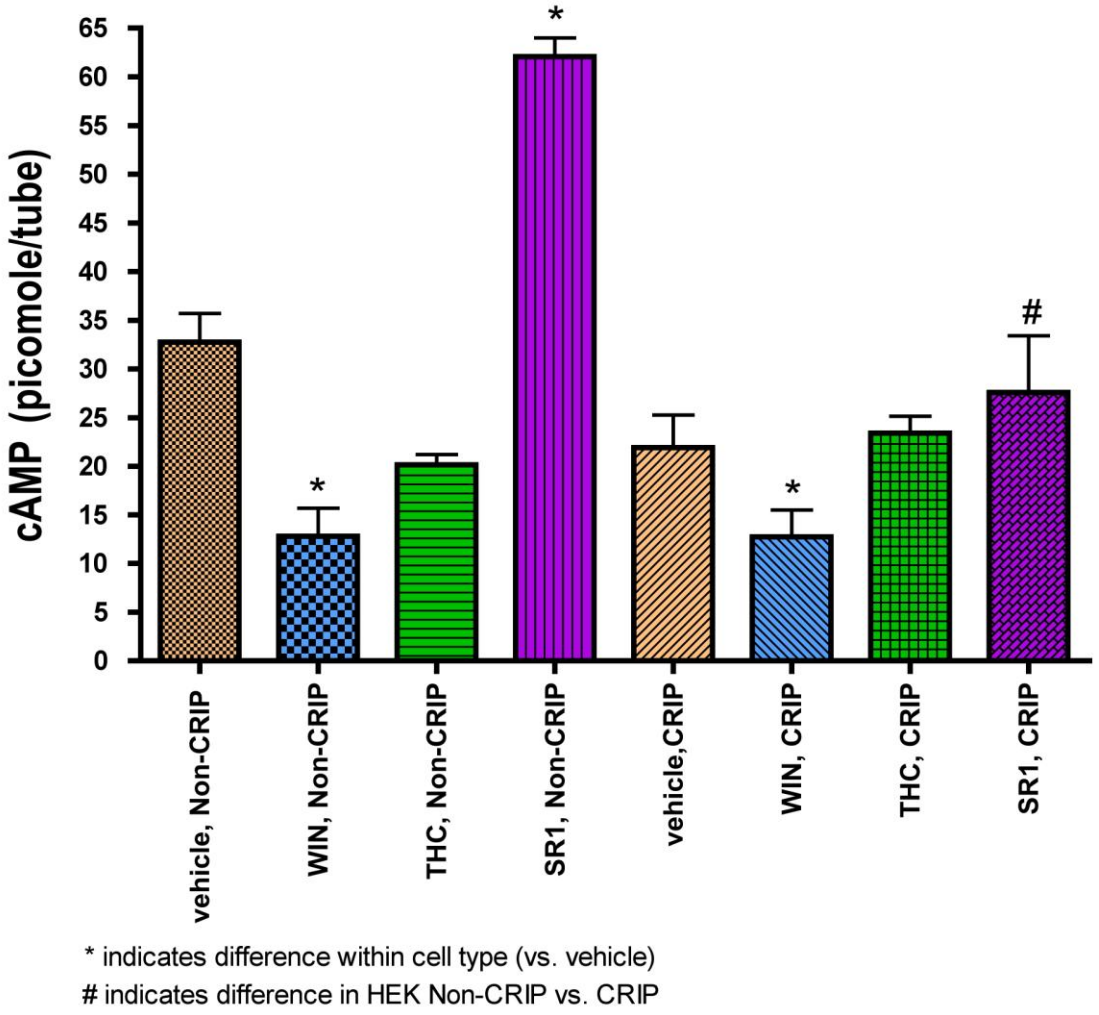

Figure 19. Forskolin-stimulated cAMP generation in hCB_1-HEK cells ($\pm CRIP_{1a}$ co-expression). Cells were incubated with either 2 µM WIN, 3 µM THC, 0.2 µM SR1 or vehicle in the presence of 10 µM forskolin and phosphodiesterase inhibitors for 8 min. cAMP concentrations were determined using a [^{3}H]cAMP kit [Liquid phase Cyclic AMP (PIKAPH-2). Data are mean cAMP levels (pmol/tube) ± SEM (n=4). * indicates statistically significant difference from vehicle within each cell type, # indicates statistically significant difference comparing two cell types with the same treatment (ANOVA with post-hoc Newman-Keuls Test, $p < 0.05$).

3.11 Immunoblot Analysis of CB_1 Receptor-mediated ERK Phosphorylation in hCB_1-HEK (± $CRIP_{1a}$) Cell Lines.

The modulation of intracellular kinases downstream of CB_1 receptor-mediated generation of free Gβγ is another effector cascade that plays a role in CB_1 receptor signaling. I hypothesize that CRIP1a will attenuate the constitutive CB_1 receptor-mediated phosphorylation of ERK 1/2, similarly to cAMP inhibition (Figure 19). The effect of $CRIP_{1a}$ on the ability of the CB_1 receptor to modulate p42/p44 MAP kinase (ERK 1/2) phosphorylation was examined using immunoblot techniques. hCB_1-HEK (± $CRIP_{1a}$) cells were serum starved for 6 hours, followed by an 8 min drug treatment with either 2 μM WIN, 3 μM THC, 0.2 μM SR1 or vehicle. Cells were lysed with cell lysis buffer containing protease and phosphatase inhibitors and samples were immunoblotted and probed for ERK and p-ERK (Figure 20). WIN, and to a lesser extent, THC, stimulated phosphorylation of ERK1 and ERK2 (Figure 21). In hCB_1-HEK cells, WIN increased ERK 1 phosphorylation to 148.64 ± 15.61% vehicle control, and THC increased ERK 1 phosphorylation by 117.61 ± 10.61% vehicle control. No differences were seen in results between hCB_1-HEK for hCB_1-HEK-$CRIP_{1a}$ cells, as WIN and THC stimulated ERK1 phosphorylation by 140.15 ± 1.85% vehicle control and 133.71 ± 10.25% vehicle control in hCB_1-HEK-$CRIP_{1a}$ cells, respectively.

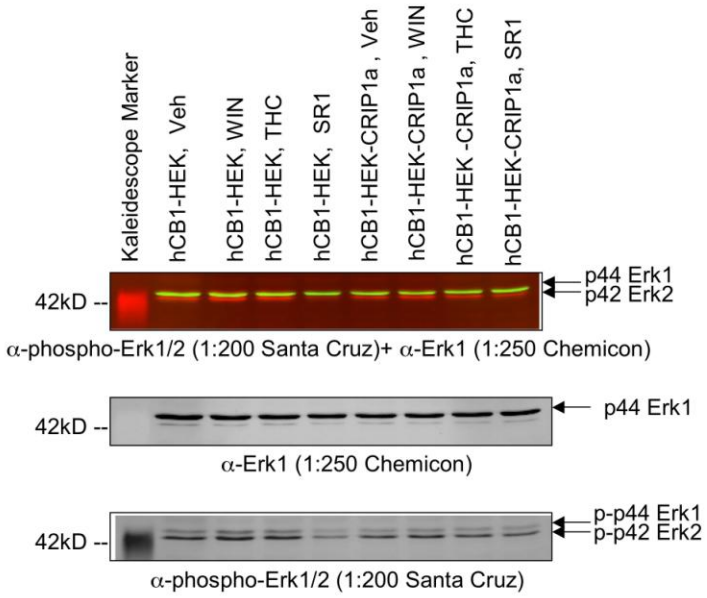

Figure 20. Western blot of p44/p42 ERK phosphorylation in hCB_1-HEK cells (±$CRIP_{1a}$ transfection). Membrane preparations of hCB_1-HEK cells (±$CRIP_{1a}$ transfection) (50 μg) were probed using phosphorylated ERK 1/2 antibody (1:200 Santa Cruz) and ERK 1 antibody for loading control (1:250, Chemicon). Images are from one representative experiment of 3 replicates, and were generated using Licor Odyssey software.

SR1 inhibited ERK1 phosphorylation in hCB_1-HEK cells, decreasing phosphorylation to 69.48 ± 25.92% vehicle control. In hCB_1-HEK-$CRIP_{1a}$ cells, SR1 inhibited ERK1 phosphorylation did not appear to decrease from vehicle (90.05 ± 14.01% vehicle control), however, there was no statistically significant differences between p-ERK levels obtained in hCB_1-HEK with and without $CRIP_{1a}$ co-expression.

CB_1 receptor mediated phosphorylation of ERK2 followed a similar pattern to ERK1. WIN and to a lesser extent, THC, stimulated ERK 2 phosphorylation (147.44 ± 10.66% vehicle control and 124.34 ± 7.78% vehicle control for WIN and THC, respectively) in hCB_1-HEK cells, with no significant differences in results seen between hCB_1-HEK and hCB_1-HEK-$CRIP_{1a}$ cells (145.18 ± 10.36% vehicle control and 140.38 ± 5.11% vehicle control for WIN and THC, respectively, in HEK-$CRIP_{1a}$ cells). Once again, SR1 appeared to decrease ERK2 phosphorylation to a greater exent in hCB_1-HEK compared to hCB_1-HEK-$CRIP_{1a}$ cells, (84.37 ± 14.77% vehicle control in hCB_1-HEK vs. 95.56 ±10.89% vehicle control), however, there were no significant differences in hCB_1-HEK cells with and without $CRIP_{1a}$ co-expression.

Lysates were also immunoblotted for non-phosphorylated ERK1 and ERK2 levels, to serve as loading controls. No significant differences were for observed for ERK1 or ERK2 levels in the presence or absence of any CB_1 receptor ligand.

All data were analyzed via ANOVA, significance achieved at $p < 0.05$, followed by a post-hoc Newman-Keuls Multiple Comparison Test ($n = 8$, $F = 4.468$, $R^2 = 0.6615$).

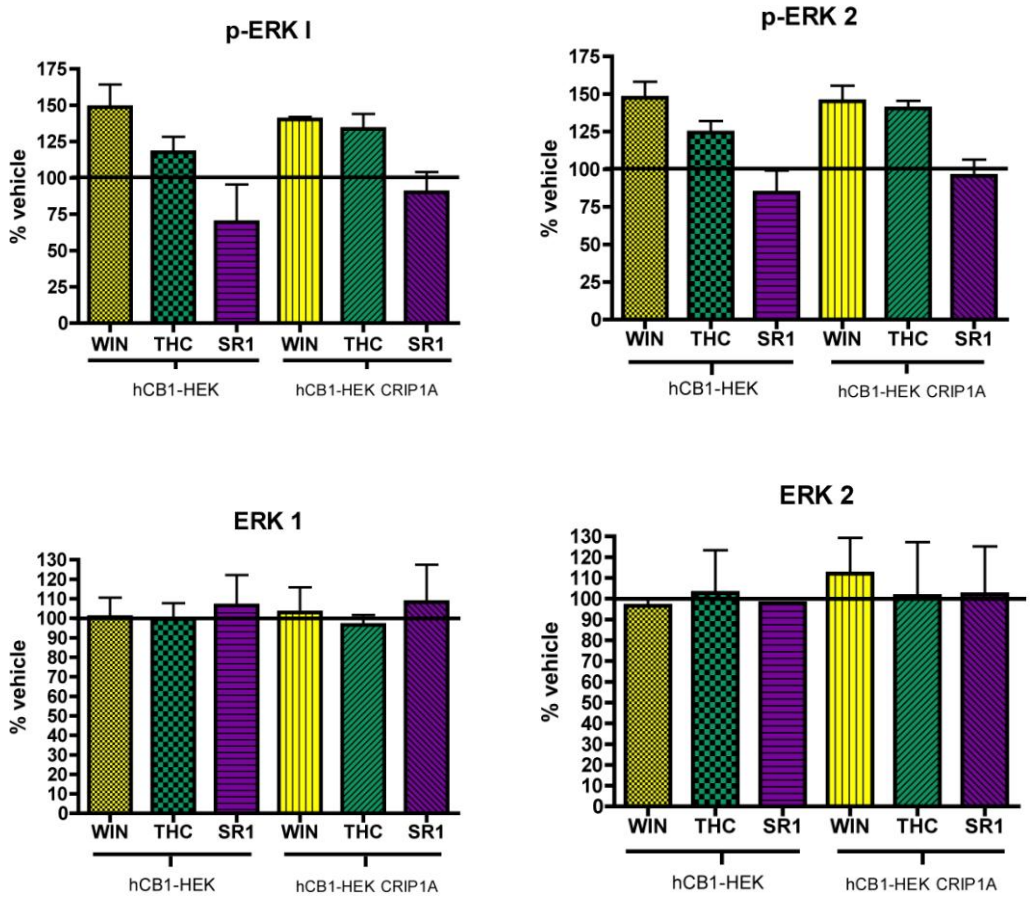

Figure 21. Densitometric analysis of p44/p42 ERK 1/2 immunoblot. The upper two panels show the effect of 2 µM WIN, 3 µM THC, or 0.2 µM SR1 on phosphorylation levels of p-44 ERK 1 (p-ERK 1, left) and p-42 ERK 2 (p-ERK 2, right). The lower two panels display the loading control values of p44 ERK 1 (ERK1) and p42 ERK 2 (ERK2). All data are mean values ± SEM expressed as normalized to vehicle alone (100%) (n=3). All values were derived from integrated intensity values obtained using Licor Odyssey software. No statistically significant differences between the two cell types were observed (ANOVA with post-hoc Newman-Keuls Test, $p < 0.05$).

3.12 $CRIP_{1a}$ Immunoblot Analysis of Hippocampi from THC Treated Mice.

Results in the HEK cell model indicate that $CRIP_{1a}$ co-expression could alter the adaptation of CB_1 receptors to prolonged agonist treatment in that $CRIP_{1a}$ inhibited WIN-mediated CB_1 receptor downregulation. It was of interest to determine whether prolonged administration of cannabinoid agonists could modulate $CRIP_{1a}$ expression. I hypothesize that $CRIP_{1a}$ levels will be altered in response to THC exposure, similarly to other CB_1 receptor interacting proteins involved in receptor regulation, such as the GRKs and β-arrestins (Rubino et al. 2006). $CRIP_{1a}$ expression in the HEK cells was driven by a viral promoter, and thus changes in $CRIP_{1a}$ expression may not be physiological relevant to mammalian models. To determine if cannabinoid exposure alters $CRIP_{1a}$ expression *in vivo*, an animal model was used. Hippocampus was chosen for examination, as $CRIP_{1a}$ expression in this region is very dense according to immunohistochemical staining of rodent brain by Dr. Maurice Elphick (unpublished data).

To determine whether chronic THC administration in the mice alters $CRIP_{1a}$ expression in the hippocampus, mice were treated with chronic, ramping doses (10, 20, 30 mg/kg, 2 injections per day for 6.5 days) of THC or vehicle. Hippocampi were harvested and subjected to immunoblot analysis for $CRIP_{1a}$ expression (Figure 22). Prolonged THC administration did not alter $CRIP_{1a}$ expression in mouse hippocampi. Integrated intensity of $CRIP_{1a}$ bands generated by the Licor Odyssey system were 24.73 ± 1.31 for THC treated mice and 26.64 ± 0.80 for vehicle treated mice, which were not significantly different according to a two-tailed t-test ($p > 0.05$, $t = 1.240$, $df = 6$). In addition, mouse hippocampi were immunoblotted for the presences of α-tubulin as a loading control. There were no significant differences in α-tubulin levels (integrated

intensity of 48.50 ± 2.29 for THC treated mice and 55.01 ± 2.43 for vehicle treated mice), according to a two-tailed t-test ($p > 0.05$, $t = 1.944$, $df = 6$).

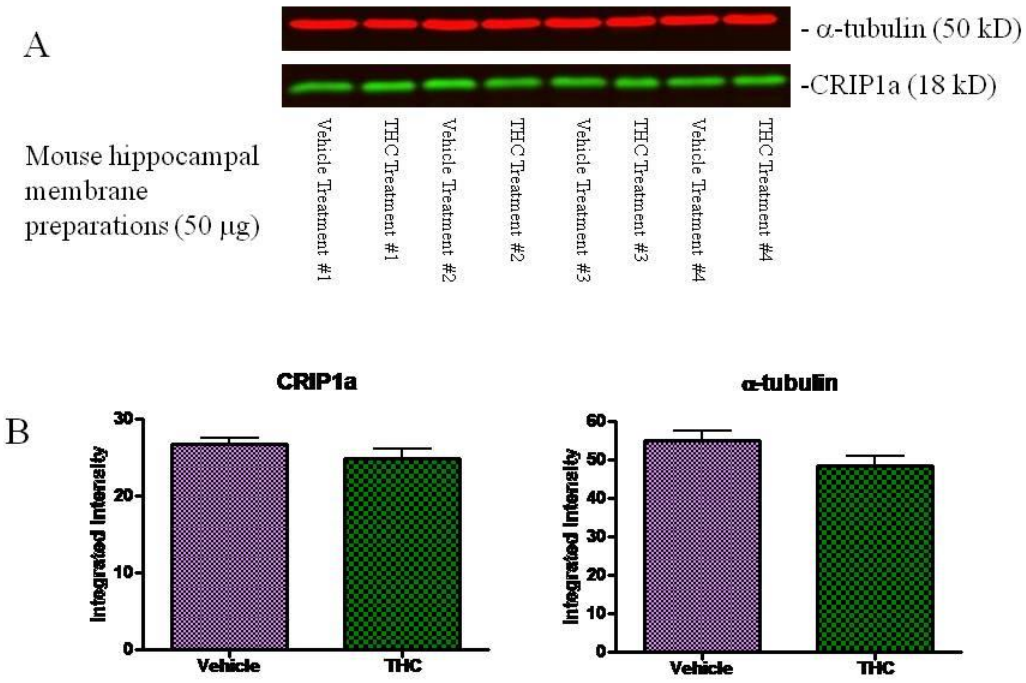

Figure 22. $CRIP_{1a}$ immunoblot analysis of hippocampi from THC treated male ICR mice. Mice were treated with chronic, ramping doses of THC or vehicle. A) Hippocampi were harvested and membrane preparations (100 µg protein) were probed using anti-$CRIP_{1a}$ antisera 077.4 (Elphick, rabbit, 1:500) followed by Licor Odyssey goat anti-rabbit 800 CW IR dye (1:5,000). Loading control was probed using anti-α-tubulin (Santa Cruz Biotechnology, 1:500) followed by Licor Odyssey goat anti-mouse 680 IR dye (1:5,000). Images were analyzed via Licor Odyssey Infrared Imaging System. B) Data are mean densitometric values ± SEM (n = 4 animals for each treatment group, and the immunoblot was replicated twice with similar results). There were no significant differences in levels of $CRIP_{1a}$ or α-tubulin loading control between mice treated with THC or vehicle (students t test, $p < 0.05$).

3.13 CB_1 Receptor Expression in mCB_1-CHO and mCB_1-CHO-$CRIP_{1a}$ Cell Lines.

Due to the modulatory effects of $CRIP_{1a}$ co-expression on CB_1 receptor signaling and adaptation in the HEK cell model, it was of interest to determine whether $CRIP_{1a}$ co-expression would have similar effects on CB_1 receptor function in a different cell line. To address this question, CHO cells stably transfected with the CB_1 receptor were stably co-transfected with $CRIP_{1a}$. Cells expressing high levels of $CRIP_{1a}$ were chosen for further study. I hypothesize that $CRIP_{1a}$ will not affect CB_1 receptor expression, as seen in the HEK cell model.

CB_1 receptor expression in mCB_1-CHO and mCB_1-CHO-$CRIP_{1a}$ cell lines were quantified using [^{3}H]SR141716A saturation analysis. Stable transfection of $CRIP_{1a}$ did not affect CB_1 receptor number (Table 7) (B_{max} value of 7.71 ± 1.63 pmol/mg in mCB_1-CHO cells versus B_{max} value of 5.48 ± 0.72 pmol/mg in mCB_1-CHO-$CRIP_{1a}$ cells). Furthermore, no significant differences were seen in [^{3}H]SR141716A K_D values (2.08 ± 0.16 nM in mCB_1-CHO cells versus 1.23 ± 0.39 nM in mCB_1-CHO-$CRIP_{1a}$ cells). Data were analyzed using two-tailed t-test with significance reached at $p < 0.05$ ($n = 4$, $df = 3$, $t = 1.537$).

	B_{max} (pmol/mg)	K_D (nM)
mCB_1-CHO	**7.72 ± 1.63**	**2.08 ± 0.16**
mCB_1-CHO-$CRIP_{1a}$	**5.48 ± 1.23**	**1.23 ± 0.39**

Table 7. B_{max} and K_D values from saturation analysis of [^{3}H]SR141716A binding in mCB_1-CHO cells with and without $CRIP_{1a}$ co-expression. B_{max} values represent total cell membrane receptor levels. Data are mean values ± SEM (n=4). No significant differences between the two CHO cell types were observed (two-tailed t-test, $p > 0.05$).

3.14 Stoichiometric Relationship of $CRIP_{1a}$ to CB_1 Receptor in mCB_1-CHO and mCB_1-CHO-$CRIP_{1a}$ Cell Lines.

To determine the stoichiometric relationship of $CRIP_{1a}$ to CB_1 receptor in mCB_1-CHO and mCB_1-CHO-$CRIP_{1a}$ cell lines, immunoblot analysis of $CRIP_{1a}$ was performed using a GST-pulldown purified $CRIP_{1a}$ concentration curve for comparison to cell line samples and analyzed on the Licor Odyssey system (Figure 23). I hypothesize, that like HEK cells, $CRIP_{1a}$ transfection will increase the stoichiometric relationship of $CRIP_{1a}$ to CB_1 receptor. $CRIP_{1a}$ concentrations were inferred from a linear regression of the concentration curve in Micrsoft Excel, and the unknown $CRIP_{1a}$ concentrations of mCB_1-CHO and mCB_1-CHO-$CRIP_{1a}$ membrane preparations were determined (Table 8). mCB_1-CHO-$CRIP_{1a}$ cells express significantly more $CRIP_{1a}$ than mCB_1-CHO cells (7720 ± 1370 pmol/mg in mCB_1-CHO-$CRIP_{1a}$ cells versus 63.1 ± 18.0 pmol/mg in mCB_1-CHO cells) according to two-tailed t-test ($p = 0.0021$, $df = 8$, $t = 4.461$).

CB_1 receptor levels and $CRIP_{1a}$ concentrations were used to generate stoichiometric ratios for mCB_1-CHO and mCB_1-CHO-$CRIP_{1a}$ cell lines (Table 8). The molar ratio of $CRIP_{1a}/CB_1$ receptor was significantly higher in mCB_1-CHO-$CRIP_{1a}$ cells (1930 ± 343 pmol/mg) than in mCB_1-CHO-$CRIP_{1a}$ cells (15.7 ± 4.52) as analyzed by two-tailed t-test ($p = 0.0021$, $df = 8$, $t = 4.461$).

Comparison of the molar ratio of $CRIP_{1a}/CB_1$ receptors in rat cerebellum (33.6 ± 5.19) and mCB_1-CHO cells (± $CRIP_{1a}$ transfection), revealed that the molar ratio of $CRIP_{1a}/CB_1$ receptors found in mCB_1-CHO cells without $CRIP_{1a}$ transfection (15.7 ± 4.52) was closer to that found in rat cerebellum than the ratio found in the mCB_1-CHO-$CRIP_{1a}$ cell line (1930 ± 343 pmol/mg). Additionally the molar ratio of $CRIP_{1a}/CB_1$

receptors in mCB_1-CHO cells was greater than the molar ratio of $CRIP_{1a}/CB_1$ receptors found in either hCB_1-HEK or hCB_1-HEK-$CRIP_{1a}$ cell lines (ANOVA with Newman-Keuls Multiple Comparisons post-hoc test, $p < 0.05$).

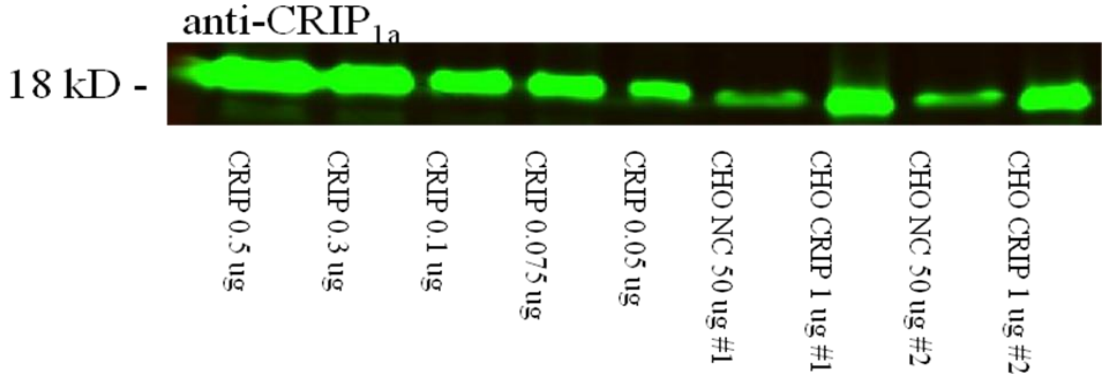

Figure 23. Quantitative western blot analysis of $CRIP_{1a}$ concentration in mCB_1-CHO cells (±$CRIP_{1a}$ transfection). $CRIP_{1a}$ purified via GST-pulldown methodology generated a $CRIP_{1a}$ concentration curve. Membrane preparations of mCB_1-CHO cells (±$CRIP_{1a}$ transfection) (70 μg/sample) were probed using anti-$CRIP_{1a}$ antisera 077.4 (Elphick, rabbit, 1:500) followed by Licor Odyssey goat anti-rabbit 800 CW IR dye (1:5,000). Images were analyzed via Licor Odyssey Infrared Imaging System. Unknown $CRIP_{1a}$ concentrations were calculated using linear regression on Microsoft Excel. Image is representative of immunoblot of 3 replicates.

	CB_1 (pmol/mg)	$CRIP_{1a}$ (pmol/mg)	Molar Ratio ($CRIP_{1a}/CB_1$)
mCB_1-CHO	7.72 ± 1.63	63.1 ± 18.0	15.7 ± 4.52
mCB_1-CHO-$CRIP_{1a}$	5.48 ± 1.23	7720 ± 1370*	1930 ± 343*

Table 8. Stoichiometric molar ratio of $CRIP_{1a}/CB_1$ receptor in mCB_1-CHO cells (±$CRIP_{1a}$ transfection). Data are mean values ± SEM (n=4). B_{max} values represent total cell membrane CB_1 receptor levels. Stable $CRIP_{1a}$ transfection caused a significant increase in $CRIP_{1a}$ expression, creating a significant molar excess relative to CB_1 receptor expression (* = $p < 0.05$ different from mCB_1-CHO cells lacking $CRIP_{1a}$ transfection in two-tailed t-test).

3.15 $CRIP_{1a}$ Modulation of Ligand-Specific CB_1 Receptor-Mediated G-protein Activation; [^{35}S]GTPγS Binding Studies in mCB_1-CHO (± $CRIP_{1a}$) Cell Lines.

To determine the effects of $CRIP_{1a}$ over-expression on CB_1 receptor mediated G-protein activation by specific cannabinoid ligands, [^{35}S]GTPγS binding assays were performed in mCB_1-CHO (± $CRIP_{1a}$) cell lines with WIN, THC and SR1 (Figure 24). I hypothesize that greater $CRIP_{1a}$ expression will decrease the constitutive and high efficacy ligand activation of CB_1 receptor-mediated G-protein activity, similarly to the HEK cell model.

Unlike results in hCB_1-HEK-$CRIP_{1a}$ cell line, the $CRIP_{1a}$ significantly increased WIN stimulated E_{max} values (228.0 ± 26.58% in mCB_1-CHO-$CRIP_{1a}$ cells versus 122.61 ± 36.50% in mCB_1-CHO cells) according to two-tailed t-test ($p < 0.05$, $n = 5$, $df = 4$, $t = 3.731$) (Figure 29). Over-expression of $CRIP_{1a}$ also significantly increased the percent stimulation by a single, maximally effective concentration (6 μM) of THC (125.7 ± 11.4% in mCB_1-CHO-$CRIP_{1a}$ cells vs. 83.6 ± 19.9% in mCB_1-CHO cells) according to two-tailed t-test analysis ($p < 0.05$, $n = 4$, $df = 3$, $t = 4.033$).

Notably, $CRIP_{1a}$ significantly decreased the inverse agonism of SR1 (E_{max} value of 27.33 ± 7.18% in mCB_1-CHO-$CRIP_{1a}$ cells versus 39.10 ± 4.31% in mCB_1-CHO cells, as indicated by two-tailed t-test ($p < 0.05$, $n = 4$, $df = 3$, $t = 5.644$). This finding is in agreement with results seen in hCB_1-HEK cells with and without $CRIP_{1a}$ co-expression.

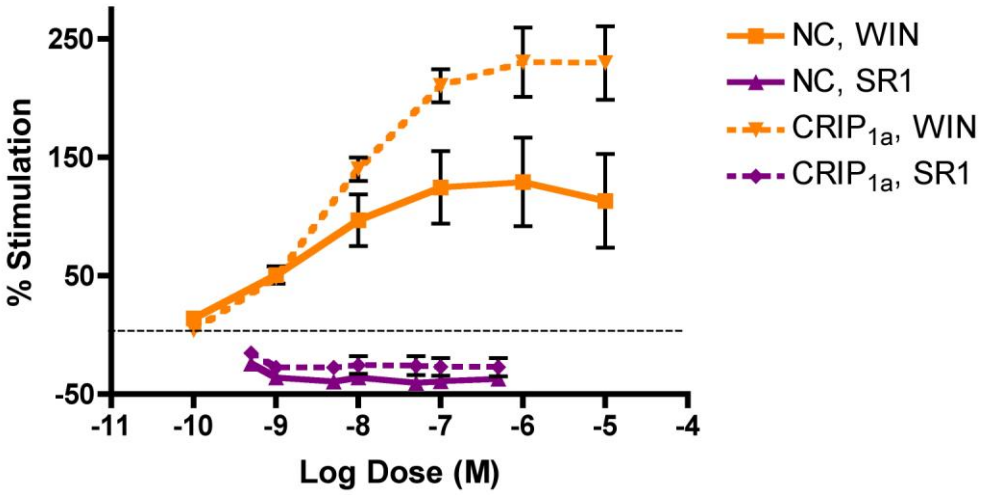

Figure 24. [^{35}S]GTPγS binding in mCB_1-CHO and mCB_1-CHO-$CRIP_{1a}$ cells. Concentration effect curves were generated for WIN and SR1. Data points are mean % stimulation ± SEM (n=4). All experiments performed in the presence of 100 mM NaCl.

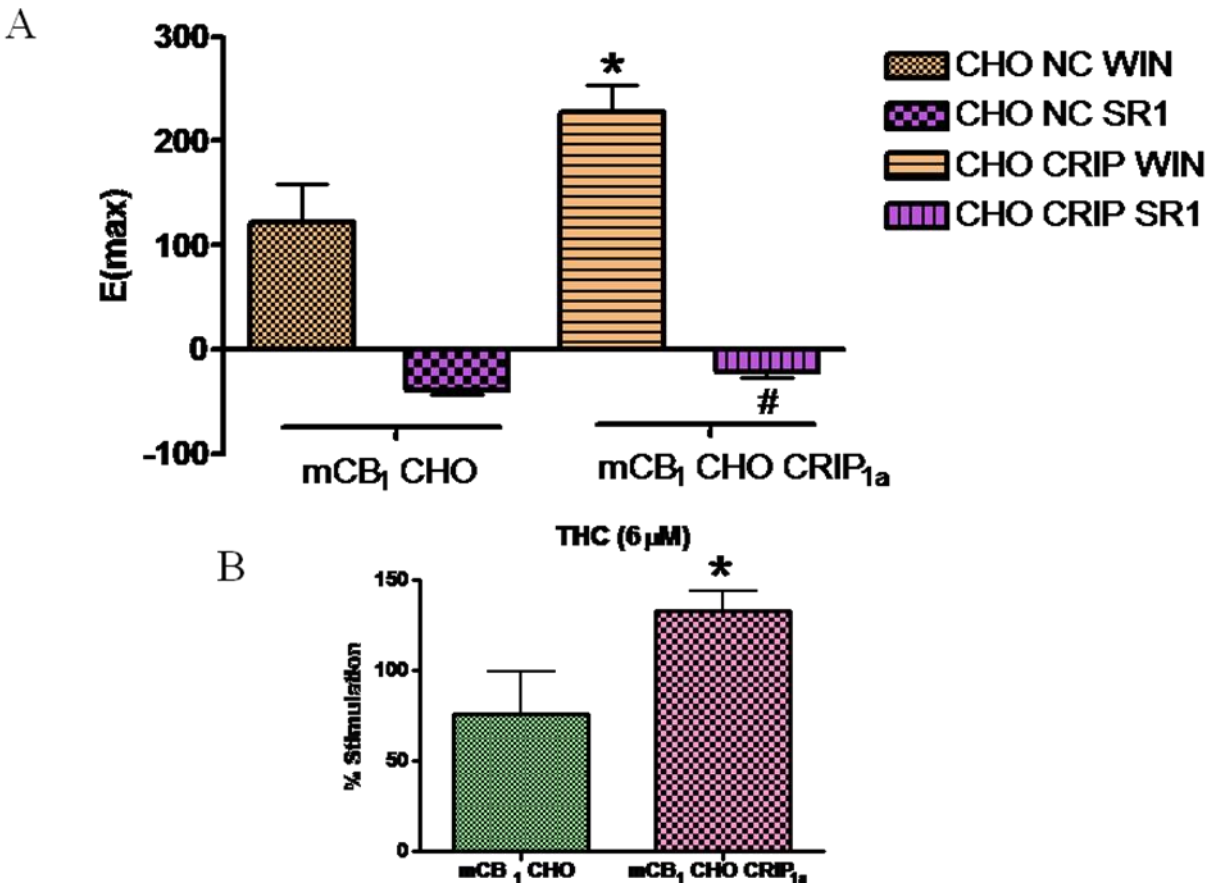

Figure 25. E_{max} values for WIN and SR1 and percent stimulation for THC in membranes from mCB_1-CHO cells with and without over-expression of $CRIP_{1a}$. Data are mean values ± SEM (n=4). A) $CRIP_{1a}$ expression significantly increased the E_{max} value of WIN and decreased the negative E_{max} value of the inverse agonist SR1. B) $CRIP_{1a}$ significantly increased the % stimulation by 6 µM THC. * $p < 0.05$ different from the corresponding drug in non-$CRIP_{1a}$ expressing cells by two-tailed t-test. # $p < 0.01$ different from the corresponding drug in non-$CRIP_{1a}$ expressing cells by two tailed t-test.

3.16 The Effects of $CRIP_{1a}$ on Spontaneous CB_1 G-protein Activation; [^{35}S]GTPγS in mCB_1-CHO (± $CRIP_{1a}$) Cell Lines with Varying Na^+ Levels.

To examine the effects of $CRIP_{1a}$ on spontaneous CB_1 G-protein activation, mCB_1-CHO cells were incubated with maximally effective concentrations of WIN (10 μM), THC (6 μM), SR1 (0.05 μM) or under basal conditions as well as incubated with increasing concentrations of NaCl (0-175 mM) (Figure 26). Decreasing NaCl concentrations lead to greater overall G-protein activity for all treatment conditions. However, $CRIP_{1a}$ had no effect on G-protein activation at the various NaCl concentrations in the presence or absence of WIN, THC or SR1 as analyzed by two-way ANOVA, significance reached at $p < 0.05$.

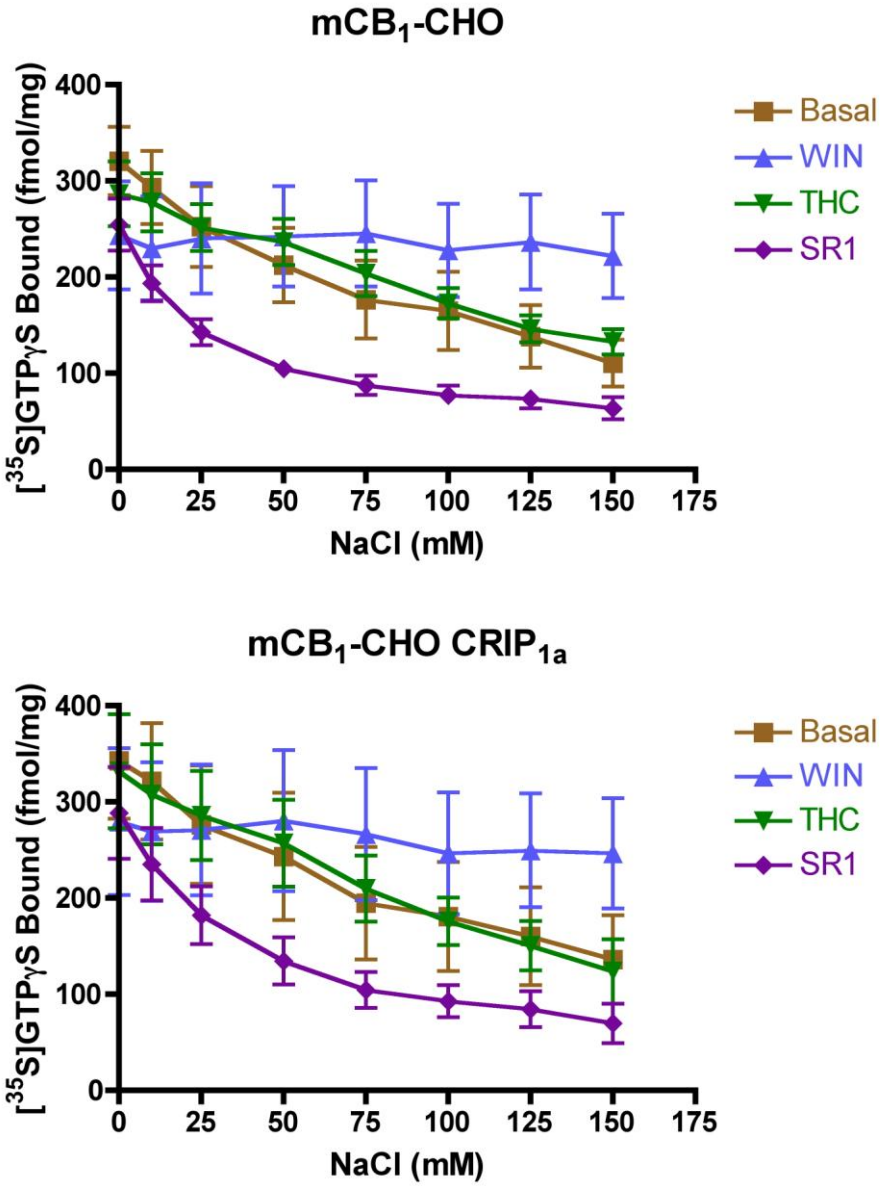

Figure 26. [^{35}S]GTPγS binding in mCB$_1$-CHO cells (±CRIP$_{1a}$ over-expression) with varying NaCl concentrations. Cells were incubated with maximally effective concentrations agonist concentrations (10 μM WIN, 6 μM THC) and a maximally inhibiting concentration of the inverse agonist SR1 (0.5 μM SR1) in the presence of varying concentrations of NaCl (0-150 mM). Data points are mean [^{35}S]GTPγS bound (fmol/mg) ± SEM (n=6). No statistical differences were found between the two cell types (ANOVA with post-hoc Newman-Keuls Test ($p < 0.05$).

3.17 Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry (LC-ESI-MS-MS) Analysis of Endocannabinoids in mCB_1-CHO (± $CRIP_{1a}$) Cell Lines.

LC-ESI-MS-MS was utilized to measure the endocannabinoid levels of of AEA or 2-AG. I hypothesize that $CRIP_{1a}$ will not affect endocannabinoid levels, as in the HEK cell model. For mCB_1-CHO cells with and without $CRIP_{1a}$ over-expression, 3 samples of whole cells (10×10^6 cells) or membrane preparations (1,000 μg) were analyzed (Table 9). No detectable levels of AEA were found in whole cell or membrane preparations of mCB_1-CHO (± $CRIP_{1a}$) cell lines. No detectable levels of 2-AG were found in the membrane preparations of mCB_1-CHO (± $CRIP_{1a}$) cell lines. However, 2-AG was detected in whole cell extracts of mCB_1-CHO cells (0.074 ± 0.0020 nmol), which was significantly increased by the over-expression of $CRIP_{1a}$ (0.22 ± 0.020 nmol) (two-tailed t-test, $p = 0.0023$, $df = 4$, $t = 6.949$).

	AEA (pmol)	2-AG (nmol)
mCB_1-CHO **Whole cells (10 x 10^6)**	**None Detected**	**0.074 ± 0.0020**
mCB_1-CHO **Membrane prep (1,000 μg)**	**None Detected**	**None Detected**
mCB_1-CHO -$CRIP_{1a}$ **Whole cells (10 x 10^6)**	**None Detected**	**0.22 ± 0.020***
mCB_1-CHO -$CRIP_{1a}$ **Membrane prep (1,000 μg)**	**None Detected**	**None Detected**

Table 9. Endocannabinoid levels in mCB_1-CHO cells (±$CRIP_{1a}$ over-expression). Liquid chromatography electrospray ionization tandem mass spectrometry determined the levels of two endocannabinoids, AEA and 2-AG, in whole cell and membrane preparations of mCB_1-CHO cells (±$CRIP_{1a}$ over-expression). Data are mean values (nmols) ± SEM (n=3). No AEA was detected in either cell line or either preparation type. $CRIP_{1a}$ over-expression significantly up-regulated 2-AG levels in whole cell preparations of mCB_1-CHO cells (students t-test, $p = 0.0023$), however no 2-AG was detected in membrane preparations of either cell type.

Chapter 4. Conclusions.

4.1 CB_1 Receptor Expression; Effect of $CRIP_{1a}$ Transfection and Cell Confluency

CB_1 receptor expression was not affected by $CRIP_{1a}$ co-transfection when comparing hCB_1-HEK and hCB_1-HEK-$CRIP_{1a}$ cell lines at 100% confluency (Table 2), in agreement with Neihaus et al., and the proposed hypothesis that $CRIP_{1a}$ does not affect total expression levels of the CB_1 receptor. This lack of effect of $CRIP_{1a}$ on CB_1 receptor expression at high confluency is particularly convenient for studies comparing effects on CB_1 receptor function in HEK cells, as alterations in CB_1 receptor signaling can be directly attributed to $CRIP_{1a}$ and not to alterations in CB_1 receptor levels.

Furthermore, K_D values were unaffected by the presence of $CRIP_{1a}$, indicating that $CRIP_{1a}$ did not alter the binding of [^{3}H]SR141716A to the CB_1 receptor. Therefore, $CRIP_{1a}$ effects on inverse agonist properties of SR1 are unlikely to be due to changes in affinity of the CB_1 receptor for SR1.

Low cell confluency decreased CB_1 receptor expression in hCB_1-HEK cells, but not hCB_1-HEK-$CRIP_{1a}$ cells (Figure 9). Cell confluency affects protein expression in cultured cells (Wolthuis et al. 1993). Low CB_1 receptor expression in low confluency hCB_1-HEK cells may be due to the greater growth rate of low confluency cells. During rapid cell proliferation, expression of transfected proteins may be suppressed, as

proposed in the hypothesis that CB_1 receptor expression would be negatively influenced by cell confluency. However, this effect was not seen in cells co-transfected with $CRIP_{1a}$, suggesting that $CRIP_{1a}$ co-expression may stabilize the expression of the CB_1 receptor in rapidly dividing cells. CB_1 receptor levels were not affected by cell confluences above 95% in either cell line. Therefore, cells were harvested for use at 95% confluency or above for all further experiments.

4.2 Stoichiometric Relationship of $CRIP_{1a}/CB_1$ Receptor in hCB_1-HEK (± $CRIP_{1a}$) Cell Lines and Mouse Cerebellum.

The stoichiometric molar ratio of $CRIP_{1a}/CB_1$ receptor in hCB_1-HEK cells was less than one (0.376), compared to hCB_1-HEK-$CRIP_{1a}$ cells in which the ratio was greater than one (5.47), and was increased by $CRIP_{1a}$ transfection as hypothesized (Table 3). This allowed for the comparison of a cell line in which the CB_1 receptor is in excess over $CRIP_{1a}$ in hCB_1-HEK cells compared to a cell line in which $CRIP_{1a}$ is in excess to the CB_1 receptor in hCB_1-HEK-$CRIP_{1a}$ cells. The physiological relevance of the $CRIP_{1a}/CB_1$ receptor ratio in hCB-HEK-$CRIP_{1a}$ cells was verified in mouse cerebellum, where the native stoichiometric molar ratio of $CRIP_{1a}/CB_1$ receptor was significantly greater than one (33.6), indicating that under physiological conditions, $CRIP_{1a}$ exerts its biological effect when there are more than sufficient $CRIP_{1a}$ molecules per CB_1 receptor. Cerebellum was chosen for this comparison because dense, uniform CB_1 receptor expression in the molecular layer of the cerebellar cortex (Herkenham et al. 1991; Tsou et al. 1998), closely resembles a similar dense uniform immunoreactivity of $CRIP_{1a}$ in the cerebellar cortex (Elphick et. al, unpublished data), compared to other brain regions. Furthermore, the molecular layer of the cerebellum contains one of the densest CB_1

receptor expressions in the brain, suggesting a uniform distribution of these receptors, making the cerebellum an appropriate location for stoichiometric comparison (Herkenham et al. 1991). However, whole cerebellum was used for experiments, due to the difficulty of anatomical dissection of the molecular layer. Even if only one-third of the $CRIP_{1a}$ expressed in the cerebellum was co-localized in the same cells as the CB_1 receptor, the $CRIP_{1a}/CB_1$ ratio would be no less than in the stably co-transfected HEK cell model used the present studies. Therefore, it seems likely that hCB_1-HEK-$CRIP_{1a}$ cells express $CRIP_{1a}$ in a molar excess relative to the CB_1 receptor that is no greater than that found in native rat cerebellum. When compared to hCB_1-HEK cells which lack sufficient $CRIP_{1a}$ to interact with each CB_1 receptor molecule, the hCB_1-HEK-$CRIP_{1a}$ cell line can serve as a reasonable model system to assess the effects of $CRIP_{1a}$ on CB_1 receptor function.

4.3 $CRIP_{1a}$ Modulation of Acute CB_1 Receptor Mediated G-protein Activation in HEK Cells.

$CRIP_{1a}$ decreased acute CB_1 receptor-mediated G-protein activation by the high efficacy agonists Nol Eth, HU210, WIN and CP, while leaving acute receptor-mediated G-protein activation by MethA, Levo, and THC unaffected in [^{35}S]GTPγS binding studies (Figure 11 and Figure 12). All experiments were performed in the presence of 100 mM NaCl. Under these conditions, $CRIP_{1a}$ affects certain cannabinoid ligands, but not others. The first obvious difference between Nol Eth, HU210, WIN, CP and the unaffected cannabinoid ligands is efficacy. Nol Eth, HU210, WIN and CP all behave as high efficacy agonists in these studies, with E_{max} values of 100% or greater in hCB_1-HEK cells without $CRIP_{1a}$ transfection (Figure 16). In the presence of $CRIP_{1a}$ the E_{max} value of Nol

Eth to approximately 100%, whereas those of HU210, WIN and CP were reduced to ≤ 80%. Incidentally, this value is approximately equivalent to the E_{max} values of the next most efficacious ligands, MethA and Levo. One possibility is that $CRIP_{1a}$ reduces G-protein activation by the CB_1 receptor only when receptor activity is near maximal, thus preferentially affecting high efficacy cannabinoid ligands.

Another, less likely, possibility is that $CRIP_{1a}$ affects cannabinoid ligands with certain structural characteristics. WIN is a structurally distinct aminoalkylindol that binds differentially to the CB_1 receptor. A mutation at position 5.46 from Val to Phe in the CB_1 receptor alters the affinity of WIN for the CB_1 receptor, while leaving the CB_1 receptor affinity of HU210, CP, and AEA unaffected (Song et al. 1999). However, CP is a nonclassical synthetic cannabinoid that is different from classical synthetic cannabinoids but still resembles THC to a degree, making this hypothesis slightly less probable than the efficacy hypothesis stated above. Furhermore, HU210 is a high efficacy analog of the low efficacy partial agonist THC (Breivogel et al. 2001) and Nol Eth is a high efficacy eicosanoid that is structurally related to the moderate efficacy cannabinoid MethA (Sugiura et al. 1999). These structure-activity relationships argue against structural features as the main determining factor in the effects of $CRIP_{1a}$. Thus, the effects of $CRIP_{1a}$ on acute CB_1 receptor-mediated G-protein activation are more likely to be dependent on the efficacy of the ligand.

$CRIP_{1a}$ may also affect ligands that stimulate certain $G\alpha_i$ protein subtypes. WIN activates all three $G\alpha_i$ subtypes (see Introduction 1.3), whereas MethA only stimulates $G\alpha_i3$ and acts as an inverse agonist for $G\alpha_i1$ and $G\alpha_i2$ (Mukhopadhyay and Howlett 2005). It is not known what $G\alpha_i$ subtypes are specifically activated by the other

cannabinoid ligands used in the [^{35}S]GTPγS binding experiments. $CRIP_{1a}$ may only affect ligands that interact with $G\alpha_i1$, $G\alpha_i2$, or $G\alpha_o$ such as WIN, while leaving ligands that primarily activate $G\alpha_i3$, such as MethA, unaffected.

$CRIP_{1a}$ attenuated the apparent ability of SR1 to act as an inverse agonist in the [^{35}S]GTPγS binding studies. This finding suggests that $CRIP_{1a}$ inhibits the constitutive activity of the CB_1 receptor, in agreement with studies by Neihaus et al. (2007), which found that $CRIP_{1a}$ inhibited constitutive inhibition of voltage gated Ca^{+2} channels (See Introduction 1.6). Another possibility is that $CRIP_{1a}$ affects SR1 due to the $G\alpha_i$ subtypes it affects. SR1 acts as an inverse agonists for all three $G\alpha_i$ subtypes (Mukhopadhyay and Howlett 2005). One of these three subtypes may be preferentially affected by $CRIP_{1a}$. The juxtamembrane CB_1 receptor C-terminus recognizes $G\alpha_i3$ and $G\alpha_o$, making them more likely candidates for interference by $CRIP_{1a}$ than $G\alpha_i1$ and $G\alpha_i2$, which interact with the C3 loop (Mukhopadhyay and Howlett 2001; Mukhopadhyay et al. 2002). Furthermore, $G\alpha_i3$ is associated with constitutive activity of the CB_1 receptor, further suggesting it may be a candidate for $CRIP_{1a}$ interference (Anavi-Goffer et al. 2007). However, this hypotheses is mutually exclusive to the hypothesis that MethA is unaffected by $CRIP_{1a}$ because it only activates $G\alpha_i3$. Future experiments examining the effects of $CRIP_{1a}$ on CB_1 receptor-Gα intereactions will need to be done to test these hypotheses.

4.4 $CRIP_{1a}$ Attenuates Constitutive CB_1 Receptor Activity & Spontaneous CB_1 Receptor Mediated G-protein Activation in HEK Cells.

$CRIP_{1a}$ decreased the ability of SR1 to act as an inverse agonist in [^{35}S]GTPγS binding studies, which suggests that $CRIP_{1a}$ decreases the constitutive activity of the

receptor. Importantly, this is in agreement with the earlier Neihaus et. al. study, which demonstrated a decrease in the inverse agonism of SR1 in electrophysiology studies using a voltage-step Ca^{+2} current protocol. To further examine the effects of $CRIP_{1a}$ on spontaneous CB_1 receptor G-protein activation, [^{35}S]GTPγS binding experiments were conducted over a range of Na^+ concentrations (0-150 mM NaCl) (Figure 13). In addition, several levels of ligand-induced CB_1 receptor activation were examined [basal activity, occupancy by the high effcicay agonist WIN (10 μM), occupancy by the partial agonist THC (6 μM) and occupancy by the full inverse agonist SR1 (0.5 μM SR1)]. Na^+ acts as a negative allosteric modulator of GPCR activity (Koski et al. 1982). As NaCl concentrations decreased, CB_1 receptor-mediated G-protein activation spontaneously increased for both cell types for all conditions tested, as indicated by increased net inhibition by SR1. $CRIP_{1a}$ decreased WIN simulated CB_1 receptor activation at all NaCl concentrations, in agreement with previous results obtained in the present studies using 100 mM NaCl (Fig 15 & 16)

The effects of $CRIP_{1a}$ became more evident as spontaneous CB_1 receptor activity increased, in agreement with the proposed hypothesis that $CRIP_{1a}$ inhibits spontaneous CB_1 receptor-mediated activity. $CRIP_{1a}$ significantly decreased G-protein activation for all conditions tested at low NaCl concentrations (0, 10 & 25 mM NaCl). This finding suggests that $CRIP_{1a}$ is able to affect the CB_1 receptor, not only for when it is stimulated by certain ligands, but under any conditions when the CB_1 receptor is highly active. This includes THC and basal conditions, which were not affected by CRIP at 100 mM NaCl (Figure 11 & Figure 12).

When basal [^{35}S]GTPγS binding was subtracted from the data at all NaCl concentrations, the significant effects of $CRIP_{1a}$ at lower NaCl concentrations were lost for all conditions (Figure 14). This finding suggests that $CRIP_{1a}$ decreased an equal amount of spontaneous G-protein activity under all conditions tested, including basal. This spontaneous G-protein activity seen at low Na^+ concentrations was in addition to the CB_1 receptor-mediated G-protein activation that was modulated by the cannabinoid ligands, as represented by the difference between the levels of [^{35}S]GTPγS binding in the presence of a full agonist and an inverse agonist. However, the decrease in net WIN-stimulated CB_1 receptor-mediated G-protein activation in the presence of $CRIP_{1a}$ was statistically significant at higher NaCl concentrations (75, 100, 125 & 150 mM NaCl). This finding suggests that $CRIP_{1a}$ can decrease G-protein activity in more than one fashion. Not only does $CRIP_{1a}$ decrease spontaneous G-protein activation as seen at low NaCl concentrations, but $CRIP_{1a}$ also net decreases WIN-stimulated CB_1 receptor-mediated G-protein activation at moderate to high NaCl concentrations as seen in Figure 14.

Furthermore, these net stimulated [^{35}S]GTPγS binding data were subject to AUC analysis (Figure 15). $CRIP_{1a}$ significantly decreased the AUC for WIN-stimulated CB_1 receptor-mediated G-protein activation in hCB-HEK cells compared to hCB-HEK-$CRIP_{1a}$ cells. The AUC was not significantly different for THC or SR1 treatment between the two cell types. However, there was a trend for $CRIP_{1a}$ to decrease the apparent inverse agonism by SR1. The lack of SR1 effect may be due to the small apparent constitutive CB_1 receptor-mediated G-protein activity seen in hCB-HEK cells (SR1 E_{max} value of $-13.34 \pm 1.65\%$ in hCB-HEK cells in the presence of 100 mM NaCl,

Figure 16). Also, since Na^+ and inverse agonists both stabilize the R state of the receptor, the efficacy of inverse agonists is reduced in the presence of Na^+ (Seifert and Wenzel Seifert, 2002), which may also account for the lack of statistically significant effects of $CRIP_{1a}$ on SR1.

To further examine the effect of $CRIP_{1a}$ on spontaneous CB_1 receptor activity, the same conditions were tested (basal, WIN, THC and SR1), at normal (100 mM) and low (0 mM) NaCl concentrations, with the additional test condition of PTX pretreatment (Fig 16). PTX treatment ADP-ribosylates inhibitory $G_{i/o}$ protein α subunits (Locht and Antoine 1995), inhibiting their interaction with GPCRs, thus allowing a distinction between GPCR-dependent G-protein activation and spontaneously active G-proteins. Under normal, 100 mM NaCl, conditions, $CRIP_{1a}$ significantly decreased WIN-stimulated G-protein activity, in as seen in previous experiments. There were no statistical differences for basal G-protein activity and SR1 inhibited G-protein activity under 100 mM NaCl conditions between hCB_1-HEK cells with and without $CRIP_{1a}$ co-expression. The lack of difference under basal conditions is in agreement with earlier experiments. However, earlier experiments showed a decrease in inverse agonism by SR1 in the presence of $CRIP_{1a}$. This may be due to the very small amount of constitutive activity seen in hCB_1-HEK cells, which is generally 13% at 100 mM NaCl. Previous experiments utilized an SR1 concentration-effect curve (Figure 11 & 12), which added statistical power to the SR1 analysis that the current experiment lacked. PTX pretreatment significantly decreased G-protein activation for all conditions in hCB_1-HEK cells. Interestingly, PTX pretreatment only significantly decreased WIN-stimulated G-protein activity in hCB_1-HEK-$CRIP_{1a}$ cells, while PTX pretreatment did not make a

significant difference for basal or SR1-inhibited conditions. This finding suggests that the presence of $CRIP_{1a}$ in hCB_1-HEK-$CRIP_{1a}$ cells reduces G-protein activity to levels indistinguishable from PTX pretreatment, further supporting the theory that $CRIP_{1a}$ reduces spontaneous GPCR-dependent G-protein activation.

Once again, the absence of NaCl magnified the effect of $CRIP_{1a}$. Under 0 mM NaCl concentration, $CRIP_{1a}$ significantly decreased G-protein activation for the basal, WIN and SR1 conditions, in agreement with the previous experiment (Figure 13). The increase of spontaneous GPCR activity due to the absence of NaCl created a significant difference for all conditions with and without PTX in hCB_1-HEK-$CRIP_{1a}$ cells, indicating that $CRIP_{1a}$ is not as effective as PTX in ameliorating spontaneous G-protein activation under these conditions. Importantly, the presence of $CRIP_{1a}$ had no effect in cells that were pretreated with PTX, such that G-protein activity in hCB_1-HEK was not different from that in hCB_1-HEK-$CRIP_{1a}$ cells after PTX pretreatment PTX regardless of the presence or absence of agonist or inverse agonist, indicating that all inhibitory effects of $CRIP_{1a}$ are on GPCR-dependent G-protein activity, in agreement with the proposed hypothesis.

In these experiments, the ability of $CRIP_{1a}$ to reverse the apparent inverse agonism of SR1 was not detected as in earlier experiments (Figure 12). As stated earlier, this may be due to the relatively low amount of constitutive CB_1 receptor activity in hCB-HEK cells. Furthermore, the concentration of SR1 used, which was maximally effective, may have been high enough to affect GPCRs other than the CB_1 receptor under certain conditions. At concentrations over 1 μM, SR1 is able to affect both basal and WIN or AEA-induced stimulation of [^{35}S]GTPγS binding in CB_1 receptor knockout mouse brain

membranes (Breivogel et al. 2001). This finding raises the possibility that SR1 affects GPCRs other than the CB_1 receptor near this concentration. Additionally, in a study characterizing SR1-inhibited G-protein activity by measuring [^{35}S]GTPγS binding in rat cerebellar membranes found that SR1 acts as a competitive antagonist at nM concentrations, whereas it inhibits basal receptor-mediated G-protein activity at micromolar concentrations (Sim-Selley et al. 2001). This finding also suggests that SR1 may affect GPCRs other than the CB_1 receptor, and/or that SR1 binds to different sites on the CB_1 receptor to produce inverse agonist versus competitive agonist effects. It is conceivable that at the concentration used in these studies, SR1 may be partially affecting receptor activity other than that of the CB_1 receptor, confounding interpretation of the effect of $CRIP_{1a}$ on CB_1 receptor-mediated G-protein activation. Some evidence for this possibility is seen in the present study. [^{35}S]GTPγS binding studies utilizing a concentration effect curve for SR1 show a reduced ability for $CRIP_{1a}$ to inhibit the apparent inverse agonist effects of SR1 at the highest concentrations examined (Figure 11). This effect may be due to interference by other non-CB_1 receptor-mediated effects of SR1. Therefore, the ability of $CRIP_{1a}$ to inhibit the constitutive activity of the CB_1 receptor may not be as apparent at high SR1 concentrations.

Another potentially confounding variable in experiments examining effects of $CRIP_{1a}$ on constitutive CB_1 receptor activity is the possibility of the presence of endogenous cannabinoids. The effects of $CRIP_{1a}$ on apparently spontaneous CB_1 receptor-mediated G-protein activity could be misinterpreted if in fact endogenous cannabinoids are contributing to this basal activity. To ensure that differences in basal G-protein activation seen between hCB_1-HEK and hCB_1-HEK-$CRIP_{1a}$ cells were due to the

presence of $CRIP_{1a}$ and not differences in endogenous cannabinoid expression, endocannabinoid levels were measured in whole cells and membrane preparations from each cell line (Table 4). No detectable levels of AEA or 2-AG were found in either preparation of either cell line.

Collectively, the data indicate that $CRIP_{1a}$ decreases not only constitutive activity, and high efficacy agonist-stimulated stimulated CB_1 receptor-mediated G-protein activity, but possibly non CB_1 receptor-mediated G-protein activity under any conditions when spontaneous GPCR activation is high.

4.5 $CRIP_{1a}$ Decreases CB_1 Receptor Downregulation, but not CB_1 Receptor Desensitization.

To assess the effect of $CRIP_{1a}$ on CB_1 receptor regulation by prolonged agonist treatment, hCB_1-HEK cells (± $CRIP_{1a}$ co-expression) were pretreated for four hours with agonist (10 μM WIN, 6 μM THC or vehicle), followed by MethA-stimulated [^{35}S]GTPγS binding (Figure 17). MethA was chosen as the stimulating ligand in these studies because $CRIP_{1a}$ did not affect its ability to acutely stimulate CB_1 receptor-mediated G-protein activation in earlier studies (Figure 11 and Figure 12). Both the high efficacy agonist WIN, and to a lesser extent, the partial agonist THC, caused significant desensitization of the CB_1 receptor, as indicated by decreased E_{max} values for MethA stimulated G-protein activation in agonist pretreated cells. However, the presence of $CRIP_{1a}$ did not significantly affect this desensitization, in opposition to the proposed hypothesis that $CRIP_{1a}$ could sterically interfere with GPCR C-terminal interacting proteins that mediate homologous desensitization, such as GRKs or β-arrestins. Nonetheless, the inability of $CRIP_{1a}$ to affect CB_1 receptor desensitization is not

surprising. $CRIP_{1a}$ binds to the last nine amino acids of the CB_1 receptor C-terminal tail, which is some distance from the CB_1 C-terminal region required for GRK or β-arrestin binding and CB_1 receptor desensitizaiton (aa 419-438) (Jin et al. 1999), therefore making steric hinderance of GRK or β-arrestin binding less likely.

Interestingly, the MethA EC_{50} values for WIN and THC pre-treated cells were significantly increased in both hCB_1-HEK (± $CRIP_{1a}$) cell lines (Table 5), as a result of rightward shifts of the MethA concentration-effect curves (Figure 17). For many GPCRs, only a fraction of available receptors need to be occupied for an agonist to produce a full functional response. Further receptor occupancy beyond the maximal response produces no further stimulation, and this situation is referred to as receptor reserve. Receptor reserve can be measured in a biological system by inactivating an increasing percentage of receptors, such as with an irreversible antagonist, which will produce a progressive rightward shift in the dose-response curve that is subsequently followed by a depression in the maximal response. CB_1 receptors in particular are known for their large receptor reserve for downstream cannabinoid actions in the central nervous system (Gifford et al. 1999). However, the receptor reserve for CB_1 receptor mediated G-protein activation may not be as large, as demonstrated in heterozygous CB_1 knockout mice (Selley et al. 2001), but there could be receptor reserve for G-protein activation in transfected cell lines, as shown with the μ opioid system comparing CHO cells transfected with μ opioid receptor to rat brain (Selley et al. 1998). In this study, the decrease in MethA EC_{50} values is indicative of loss of receptor reserve due to downregulation of the CB_1 receptor, as seen in the next set of experiments measuring loss of receptors in response to WIN and THC pretreatment.

The effects of $CRIP_{1a}$ on CB_1 receptor downregulation was determined using the same four hour drug pretreatment followed by [^{3}H]SR141716A saturation analysis to determine changes in CB_1 receptor number (Figure 18). In hCB_1-HEK cells, the partial agonist THC caused a robust CB_1 receptor downregulation, while the full agonist WIN caused less downregulation. $CRIP_{1a}$ attenuated the less substantial CB_1 downregulation induced by WIN, but did not significantly attenuate the robust downregulation induced by THC, although there appeared to be a trend toward a decrease. [^{3}H]SR141716A K_D values were unaffected by drug treatment (Table 6), indicating the effective removal of WIN and THC that were used for the pretreatment prior to assay. This finding suggests that rightward shifts in the MethA concentration-effect curves induced by drug pretreatment were due to a loss in receptor reserve and not residual pretreatment drug in the membranes. One possible explanation for the differential ability of $CRIP_{1a}$ to inhibit WIN- versus THC-induced CB_1 receptor downregulation may be that $CRIP_{1a}$ weakly sterically hinders the interaction of β-arrestin with the phosphorylated receptor. β-arrestins are sizable (~48 kDa) proteins that interact near the C-terminal tail of the CB_1 receptor and interact with GPCRs strongly enough to relocate them from signaling complexes to clathrin-coated pits (Reiter and Lefkowitz 2006). It is possible that in this cell model $CRIP_{1a}$ is able to sterically interference with WIN-stimulated interactions of β-arrestin and the CB_1 receptor in a way that THC is able to overcome.

Another possibility is differential induction of the β-arrestin types by THC and WIN. HEK 293 cells contain both β-arrestin 1 and 2. In HEK 293 cells transfected with the μ opiod receptor (μOR), the μOR interacts with β-arrestin 2 only when the partial agonist morphine is used. However, when the full agonist etorphine is utilized, the μOR

receptor interacts with both β-arrestin 2 and 1 (Bohn et al. 2004). Furthermore, the genetic knockout of β-arrestin 2 in mice selectively enhanced the behavioral effects of THC, while leaving the effects of CP, MethA, JWH-073 and O-1812 unaffected (Breivogel et al. 2008). Therefore, THC may be preferentially affect β-arrestin 2 binding to the CB_1 receptor. It is possible that with the CB_1 receptor, THC and WIN cause differential recruitment of β-arrestin 2 and 1, and that $CRIP_{1a}$ preferentially interferes with the binding of one of these isoforms, namely β-arrestin 1.

Alternatively, $CRIP_{1a}$ may attenuate WIN-induced CB_1 receptor downregulation by interfering with GASP1 binding to the CB_1 receptor. GASP1 is required for ligand-induced downregulation of the CB_1 receptor (Martini et al. 2007), but is not directly involved in CB_1 receptor desensitization, thus potentially explaining the ability of $CRIP_{1a}$ to interfere with downregulation but not desensitization, as hypothesized. The robust downregulation induced by THC compared to WIN may indicate that THC induces greater CB_1 receptor/GASP1 interactions than WIN. $CRIP_{1a}$ may not sterically hinder this stronger interaction induced by THC as effectively as the putatively weaker interaction of CB_1 receptor to GASP1 induced by WIN. This difference may account for $CRIP_{1a}$ preferentially affecting WIN over THC-mediated downregulation.

Downregulation by WIN could also be more greatly affected by $CRIP_{1a}$ because $CRIP_{1a}$ inhibits acute G-protein activation by WIN, which could result in impairment of Gβγ recruitment of GRK 2/3. Another related possibility is that $CRIP_{1a}$ affects WIN-stimulated CB_1 receptor regulation due to the specific Gα subtypes that WIN activates, as hypothesized for acute CB_1 receptor-mediated G-protein activation (see Conclusions 4.3).

However, these explanations seem less likely as $CRIP_{1a}$ affected downregulation of the CB_1 receptor without affecting CB_1 receptor desensitization.

The time course used in the CB_1 receptor desensitization and downregulation experiments may have played a part in the observed results. $CRIP_{1a}$ had no effect on desensitization of the CB_1 receptor after four hours of drug exposure (Figure 17). Maximal endocytosis of the CB_1 receptor is observed in desacetyllevonantradol (DALN) exposed CB_1-HEK cells after 1 hour (Keren and Sarne 2003). $CRIP_{1a}$ may affect desensitization on a shorter time scale, before maximal desensitization is reached. Thus, if $CRIP_{1a}$ inhibits the rate of desensitization but not the maximal level of desensitization, then it is possible that maximal desensitization was achieved well before 4 hours, and the effect of $CRIP_{1a}$ was not evident at this time point. Future experiments should examine the effects of $CRIP_{1a}$ on desensitization in time increments less than 1 hour. Additionally, effects of $CRIP_{1a}$ on THC-induced downregulation may have been apparent on a shorter time scale. CB_1 receptors are downregulated by more than 75% in WIN-exposed CB_1-HEK cells after 3 hours (Martini et al. 2007). Subsequent experiments examining the effects of $CRIP_{1a}$ on downregulation may yield significant results at one and two hour time points, regardless of the agonist occupying the receptor.

Lastly, the cannabinoid ligand concentrations used may have played a role in the observed effects of $CRIP_{1a}$ on CB_1 receptor desensitization and downregulation. Maximally effective concentrations of WIN (10 μM) and THC (6 μM) were used in both experiments. Desensitization is increased by increasing WIN concentrations as measured by cannabinoid inhibition of Ca^{+2} spiking activity in hippocampal neurons (Kouznetsova et al. 2002). Also, increasing concentrations of DALN increased CB_1 receptor

endocytosis in CB_1-HEK cells (Keren and Sarne 2003). Therefore, lower cannabinoid concentrations may be used to reveal subtle effects of $CRIP_{1a}$ on the potency of agonists to induce CB_1 receptor desensitization and downregulation. Future experiments examining the effects of $CRIP_{1a}$ on CB_1 receptor regulation should include concentration-effect curves that begin at sub-EC_{50} ligand concentrations.

4.6 $CRIP_{1a}$ and Downstream Signaling of CB_1 Receptors

The effects of $CRIP_{1a}$ on downstream CB_1 receptor mediated inhibition of forskolin stimulated cAMP generation were measured via liquid phase cAMP radioassay (Figure 19). $CRIP_{1a}$ did not alter cAMP levels in cells treated with WIN, THC or vehicle. However, interestingly, $CRIP_{1a}$ reversed the apparent inverse agonism of SR1. The ability of $CRIP_{1a}$ to decrease inverse agonism, a measure of the constitutive activity of the CB_1 receptor, in cAMP experiments agrees with the results obtained when measuring the effects of $CRIP_{1a}$ on constitutive CB_1 receptor activity in [^{35}S]GTPγS binding experiments. Moreover, these results are in agreement with the ability of $CRIP_{1a}$ to decrease CB_1 receptor-mediated tonic inhibition of voltage-gated Ca^{+2} channels by Neihaus et al. (2007). Therefore, $CRIP_{1a}$ appears to decrease the spontaneous modulation of certain downstream effectors by CB_1 receptors, as hypothesized.

The effects of $CRIP_{1a}$ on downstream CB_1 receptor-stimulated ERK 1/2 phosphorylation were examined via immunoblot analysis using an antibody that is specific for phosphorylated ERK 1/2 (Figure 20). WIN, and to a lesser extent THC, increased ERK 1 and 2 phorsphorylation, and SR1 inhibited ERK 1 and 2 phosphorylation (Figure 21). There was no statistical differences in agonist-stimulated ERK 1 or 2 phosphorylation between hCB_1-HEK cells with and without $CRIP_{1a}$ co-

expression, suggesting that $CRIP_{1a}$ had no significant effect on downstream ERK 1/2 signaling by CB_1 receptors.

However, it should be noted that there was a trend for $CRIP_{1a}$ to reverse the ability of SR1 to produce inverse agonism in the phosphorylation of ERK 1 and 2. However, this effect was also not statistically significant. It is possible that this immunblot assay protocol was not sensitive enough to detect differences between hCB_1-HEK cells with and without $CRIP_{1a}$ co-expression. Future protocols more sensitive to changes in CB_1 receptor-dependent phorsphorylation of ERK 1 and 2 may yet detect an effect by $CRIP_{1a}$. Furthermore, future experiments could utilize a concentration-effect curve, as $CRIP_{1a}$ may lead to potency changes in the ability of cannabinoids to phosphorylate ERK 1/2. A similar argument could be made for agonist-inhibited cAMP generation above.

Furthermore, the concentration of SR1 used, which was maximally effective, may have been too high to see significant effects of $CRIP_{1a}$ on the CB_1 receptor-mediated phosphorylation of ERI 1/2. At this dose it might have GPCR effects not specific to the CB_1 receptor that may be affecting the results, as discussed for PTX pre-treatment experiments in Conclusions 4.4.

Considering the effect of $CRIP_{1a}$ on downstream signaling to both cAMP and ERK 1/2, it seems probable that $CRIP_{1a}$ affects some downstream signals but not others. In addition, $CRIP_{1a}$ could differentially modulate $G\alpha$ versus $G\beta\gamma$-mediated signaling. However, $CRIP_{1a}$ decreased the constitutive CB_1-mediated inhibition of cAMP, which is modulated mainly by $G\alpha_1$ (Howlett et al. 2002), and decreased the constitutive inhibition of voltage-gated Ca^{+2} channels (Niehaus et al. 2007), which is modulated by $G\beta\gamma$

(Herlitze et al. 1996). Thus $CRIP_{1a}$ is apparently able to modulate G-protein mediated downstream signals despite whether Gα or Gβγ is required for downstream signaling. Therefore, it remains uncertain why SR1-stimulated cAMP generation was significantly inhibited by $CRIP_{1a}$ co-expression, whereas SR1-inhibited ERK 1/2 phosphorylation was not. Aside from methodological issues, it is possible that there is greater amplification of the cAMP signal provided by the catalytic action of the adenylyl cyclase enzyme, relative to less amplification in the signaling cascade leading to ERK 1/2 phosphorylation. If so, then this differential amplification might explain the greater signal for SR1-modulated activity in the cAMP than in the ERK 1/2 experiments.

4.7 Mouse hippocampal $CRIP_{1a}$ expression in response to THC administration.

Expressions of various regulators of CB_1 receptor signaling are altered in response to chronic cannabinoid treatment. Chronic, but not acute, THC treatment upregulates GRK2, GRK4, β-arrestin 1, and β-arrestin 2 (Rubino et al. 2006). To determine if chronic drug administration could alter $CRIP_{1a}$ levels in the hippocampus, mice were administered ramping doses of THC and their hippocampi were dissected and immunoblotted for $CRIP_{1a}$ expression (Figure 22). $CRIP_{1a}$ levels were not different between mice administered THC or vehicle. Chronic THC administration did not alter $CRIP_{1a}$ expression in the hippocampus of mice in response to repeated ramping doses of THC administration. This was not in agreement with the predicted hypothesis that THC treatment would affect $CRIP_{1a}$ levels. GRKs and β-arrestins are proteins involved in CB_1 receptor adaptation to prolonged agonist treatment, and therefore it is not surprising that prolonged agonist treatment affects their expression. $CRIP_{1a}$ regulates the acute and constitutive responsiveness of the CB_1 receptor; therefore regulation of $CRIP_{1a}$

expression in response to prolonged agonist occupancy might be unnecessary. However, it would be interesting to examine the effects of WIN on $CRIP_{1a}$ expression, as the downregulation induced by the CB_1 receptor occupancy by WIN was affected by the co-expression of $CRIP_{1a}$.

4.8 Stoichiometric Relationship of $CRIP_{1a}/CB_1$ Receptor in mCB_1-CHO Cells with and without $CRIP_{1a}$ Co-expression.

CB_1 receptor (Table 7) and $CRIP_{1a}$ levels (Figure 22) were quantified in mCB_1-CHO cells with and without stable co-transfection of $CRIP_{1a}$, and the $CRIP_{1a}/CB_1$ receptor molar stoichiometric relationship was calculated from these values (Table 8). mCB_1-CHO cells without $CRIP_{1a}$ transfection contained a substantial amount of $CRIP_{1a}$. The stoichiometric molar ratio in mCB_1-CHO cells without $CRIP_{1a}$ transfection was greater than hCB_1-HEK cells with stable $CRIP_{1a}$ transfection (Table 3). Stable $CRIP_{1a}$ transfection further increased $CRIP_{1a}$ expression dramatically in mCB_1-CHO cells, as hypothesized, creating a nearly 2,000-fold molar excess of $CRIP_{1a}/CB_1$ receptor, which can be viewed as $CRIP_{1a}$ over-expression.

4.9 $CRIP_{1a}$ Modulation of Acute CB_1 Receptor Mediated G-protein Activation in CHO Cells.

The effects of $CRIP_{1a}$ on acute CB_1 receptor-mediated G-protein activation were examined in [^{35}S]GTPγS binding studies using WIN, THC and SR1 in mCB_1-CHO cell membranes with and without $CRIP_{1a}$ over-expression (Figure 24 and Figure 25). Notably, further over-expression of $CRIP_{1a}$ in mCB_1-CHO-$CRIP_{1a}$ cells further decreased constitutive activity of CB_1 receptors compared to mCB_1-CHO cells which already contained ample levels of $CRIP_{1a}$, as hypothesized. This finding raises the further

possibility that $CRIP_{1a}$ did not reliably reduce constitutive CB_1 receptor activity in all experiments in hCB_1-HEK-$CRIP_{1a}$ cells (especially under low sodium concentrations) because it was not expressed at a sufficiently high molar ratio compared to CB_1 receptor levels.

Surprisingly, $CRIP_{1a}$ over-expression in mCB_1-CHO cells increased maximal CB_1 receptor-mediated G-protein activation by both WIN and THC, in opposition to the proposed hypothesis. Therefore, stoichiometric relationship could be a critical determinant in how CB_1 receptor-mediated G-protein activation responds acutely to the presence of $CRIP_{1a}$. Over-expression of $CRIP_{1a}$ in co-transfected CB_1-CHO cells caused augmentation of acute agonist-stimulated CB_1 receptor-mediated G-protein activation, rather than the attenuation seen at near physiological $CRIP_{1a}$ levels present in $CRIP_{1a}$ co-transfected CB_1-HEK cells.

Alternatively, the effects of $CRIP_{1a}$ may be cell type dependent. CHO and HEK cell lines are two of the most commonly used cell lines in biomedical research. CHO cells, derived from Chinese Hamster Ovary cells (Puck et al. 1958) are the most utilized cell line in the pharmaceutical large scale production of therapeutic protein products, accounting for billions of dollars in products annually (Andersen and Krummen 2002). However, the genome of CHO cells is not well characterized (Wlaschin et al. 2005). In contrast, HEK cells, derived from Human Embryonic Kidney cells (Graham et al. 1977) are postulated to be derived from a neuronal cell lineage (Shaw et al. 2002). Notably, a HEK genomic database at the University of Florida (http://www.mbi.ufl.edu/~shaw/293.html) reveals that HEK cells contain a surprising amount of neuron-specific proteins including several neurofilament subunits and alpha

internexin, as well as GPCRs, such as dopamine (D_2) receptors and neurotensin (NTR_2) receptors. Additionally, CHO and HEK cells are different in the subtypes of $G\alpha_i$ that they express. CHO cells express $G\alpha_i2$ and $G\alpha_i3$, but not $G\alpha_i1$ (Gettys et al. 1994). On the other hand, HEK cells express $G\alpha_i1$ and $G\alpha_i3$ but not $G\alpha_i2$ (Law et al. 1993). Therefore, $CRIP_{1a}$'s differential effects on acute agonist stimulated G-protein activity may be due to the differences in the proteome of HEK and CHO cell types, rather than to differences in the stoichiometric relationships between $CRIP_{1a}$ and CB_1 receptor in the two cell lines.

Future experiments examining the effects of genetic silencing of $CRIP_{1a}$ expression using shRNA in CHO cells may be able to answer the question of cell type specificity. In this case $CRIP_{1a}$ silencing could be expected to reverse both the attenuation of constitutive activity and augmentation of agonist stimulated CB_1 receptor-mediated G-protein activation in mCB_1-CHO cells. It is also possible that partial silencing of $CRIP_{1a}$ expression in mCB_1-CHO cells, to a molar ratio similar to that obtained in hCB_1-HEK-$CRIP_{1a}$ cells, could produce attenuation of full agonist-stimulated G-protein activation.

Another explanation may be that the $CRIP_{1a}$ differentially affects the human and mouse CB_1 receptor. However, this explanation is less likely due to expression of $CRIP_{1a}$ throughout the vertebrates and the identical amino acid composition of the last 9 amino acids of both murine and human CB_1 receptors, which compromise the $CRIP_{1a}$ binding site.

4.10 $CRIP_{1a}$ Does Not Affect Spontaneous CB_1 Receptor Mediated G-protein Activation in CHO Cells.

Varying NaCl concentrations were used to determine the effect of $CRIP_{1a}$ on spontaneous G-protein activation in mCB_1-CHO cells with and without $CRIP_{1a}$ over-expression in the presence of WIN, THC, SR1 and basal conditions (Figure 26). No effect of $CRIP_{1a}$ on spontaneous or agonist-stimulated G-protein activation was found, thus rejecting the proposed hypothesis. This was in contrast to results seen in earlier ligand concentration-effect curves in the presence of 100 mM NaCl, in which $CRIP_{1a}$ over-expression suppressed the inverse agonist effects of SR1 and augmented CB_1 receptor-mediated G-protein activation by agonists.

The most likely explanation for these different results involves the manner in which the membrane preparations for these experiments were prepared. Unlike all other experiments in this dissertation, mCB_1-CHO cells ($\pm CRIP_{1a}$) were grown in flasks, drug-treated, homogenized and frozen back into aliquots which were later used in [^{35}S]GTPγS binding studies. Freeze-thawing membrane preparations may impair the acute signaling of the CB_1 receptor or its modulation by $CRIP_{1a}$ and explain the variability and lack of effect of $CRIP_{1a}$ seen in these results. Also notable, fresh membrane preparations were used in initial [^{3}H]SR141716A saturation analysis of hCB_1-HEK and mCB_1-CHO cell lines with and without $CRIP_{1a}$ transfections (Table 3 and Table 8) . However, excess remaining protein from [^{35}S]GTPγS binding studies was often frozen back and used in [^{3}H]SR141716A saturation analysis to confirm unchanging CB_1 receptor numbers. No differences between fresh and frozen membrane preparations were seen in the results of [^{3}H]SR141716A saturation analysis. This finding is unsurprising as freezing back

membrane preparations is more likely to upset the more delicate signaling process assayed in receptor-stimulated [^{35}S]GTPγS binding studies rather than the number of CB_1 receptors determined by high affinity [^{3}H]SR141716A saturation analysis.

Another potential explanation is the comparison of stoichiometric relationship between the HEK and CHO cell line models. mCB_1-CHO cells, unlike hCB_1-HEK cells contain a substantial amount of $CRIP_{1a}$, resulting in a molar excess of $CRIP_{1a}$ over CB_1 receptor that is merely enhanced by $CRIP_{1a}$ over-expression in this cell line. Therefore, comparing an excess of $CRIP_{1a}$ to an even greater excess of $CRIP_{1a}$ relative to the CB_1 receptor might result in no discernable effects of $CRIP_{1a}$ over a range of Na^+ concentration. However, this explanation is less likely, as an effect of $CRIP_{1a}$ on constitutive and agonist-induced CB_1 receptor-mediated G-protein activity was seen at 100 mM NaCl.

4.11 $CRIP_{1a}$ Transfection Affects 2-AG Expression in mCB_1-CHO (±$CRIP_{1a}$) Cell Lines.

Levels of two endocannabiniods, AEA and 2-AG were determined via LC-ESI-MS-MS for mCB_1-CHO cells with and without $CRIP_{1a}$ over-expression (Table 9). No detectable levels of AEA were found in either cell line in either whole cell or membrane preparations. Contrariwise, whole cell preparations of mCB_1-CHO cells contained small amounts of 2-AG which were significantly increased in $CRIP_{1a}$ over-expressing mCB_1-CHO-$CRIP_{1a}$ cells, which was not predicted by the hypothesis. 2-AG can act as an autocrine mediator, as it is responsible for the postsynaptic slow self-inhibition of neocortical low-threshold spiking interneurons (Marinelli et al. 2008). $CRIP_{1a}$ co-expression may feedback onto 2-AG synthesis, thus increasing 2-AG levels in mCB_1-

CHO- $CRIP_{1a}$ cells. However, this difference cannot explain the results seen in earlier [^{35}S]GTPγS binding studies comparing the two CHO cell lines, as no detectable levels of 2-AG were found in the membrane preparations of either cell line. Nonetheless, downstream signaling experiments utilizing mCB_1-CHO cells with and without $CRIP_{1a}$ over-expression may not accurately reflect the effects of $CRIP_{1a}$ on basal CB_1 receptor activity due to the increase in 2-AG in $CRIP_{1a}$ over-expressing whole cells. Downstream experiments utilized whole cells which were drug treated and harvested for analysis of cAMP and pERK 1/2 levels, which could be affected by the endogenous presence of 2-AG. Conversely, endogenous cannabinoids could not have affected downstream cAMP and phosphorylated ERK 1/2 signals in hCB_1-HEK cells with and without $CRIP_{1a}$ transfection, as no endogenous cannabinoids were detected in these cell lines.

4.12 Summary of $CRIP_{1a}$ Conclusions

In summary, $CRIP_{1a}$ inhibits the constitutive activity of the CB_1 receptor (Figure 27, Table 10). Additionally, $CRIP_{1a}$ modulates acute CB_1 receptor mediated G-protein activation of high efficacy ligands in a manner that is cell type dependent and/or dependent on the stoichiometric relationship of $CRIP_{1a}$ to the CB_1 receptor. $CRIP_{1a}$ does not affect the maximal level of CB_1 receptor desensitization, but does modify CB_1 receptor downregulation induced by WIN. Lastly, $CRIP_{1a}$ affects downstream constitutive CB_1 receptor-mediated signaling for cAMP inhibition, but not the phosphorylation of ERK 1/2. $CRIP_{1a}$ also appears to modulate 2-AG production only in certain cell types (i.e. CHO) when expressed as sufficiently high levels.

Overall, $CRIP_{1a}$ mediates numerous CB_1 receptor effects while being a small 17 kD protein. This observation, in tandem with the fact that $CRIP_{1a}$ contains a PDZ Class I

ligand, suggests that $CRIP_{1a}$ mediates it effects in concert with other proteins to which it binds. For example, $CRIP_{1a}$ may bind to members of the cell cytoskeleton, thus stabilizing the CB_1 receptor and preventing its constitutive activation and activation by high efficacy ligands. A possible parallel example to this may be the recently discovered Homer proteins, which bind to metabotropic glutamate (mGlu) receptors and cytoskeletal Shank proteins. Homer proteins decrease the constitutive activity of the mGlu receptors, much like $CRIP_{1a}$ decreases the constitutive activity of the CB_1 receptor (Ango et al. 2001; Bockaert et al. 2004). Future studies should seek to discover other $CRIP_{1a}$ protein binding partners.

Physiologically, the ability of $CRIP_{1a}$ to affect high efficacy ligands while leaving moderate and low efficacy ligands unaffected may be important in the differential regulation of endocannabinoid signals generated by the CB_1 receptor. For instance, AEA is a partial agonist (Breivogel et al. 1998), as is its stable analog, MethA, used in the present study. $CRIP_{1a}$ did not affect MethA stimulation of the CB_1 receptor, and presumably will not affect stimulation by its partial efficacy analog AEA. However, 2-AG is a full agonist (Stella et al. 1997), and noladin ether, another high efficacy agonist that is structurally similar to 2-AG, was affected by $CRIP_{1a}$ in this study. Therefore, it is reasonable to speculate that $CRIP_{1a}$ will modify the CB_1 receptor-mediated G-protein signal generated by 2-AG but not by AEA. Therefore, under physiological conditions, $CRIP_{1a}$ may affect the signaling of certain endogenous cannabinoids but not others.

Overall, $CRIP_{1a}$ may be key to modulating CB_1 receptor functions; allowing the generation of certain therapeutic responses while minimizing deleterious side effects. For example, pharmacological modulation of $CRIP_{1a}$, rather than direct modulation of the

CB_1 receptor, may allow the therapeutic modulation of high efficacy endocannbinoid effects while avoiding any side effects generated by the modulation of partial efficacy endocannabinoids.

In conclusion, $CRIP_{1a}$ is an important member of the CB_1 receptor receptosome that regulates both constitutive activity of the CB_1 receptor and ligand-mediated CB_1 receptor activity in a manner that differentially affects high efficacy and partial agonists, both of exogenous and endogenous origin. Additional evidence suggests that $CRIP_{1a}$ can also attenuate agonist-induced CB_1 receptor downregulation and constitutive CB_1 receptor modulation of a subset of effector pathways. These properties suggest that $CRIP_{1a}$ may someday be targeted pharmacologically to allow more effective manipulation of CB_1 receptor activities to therapeutic advantage.

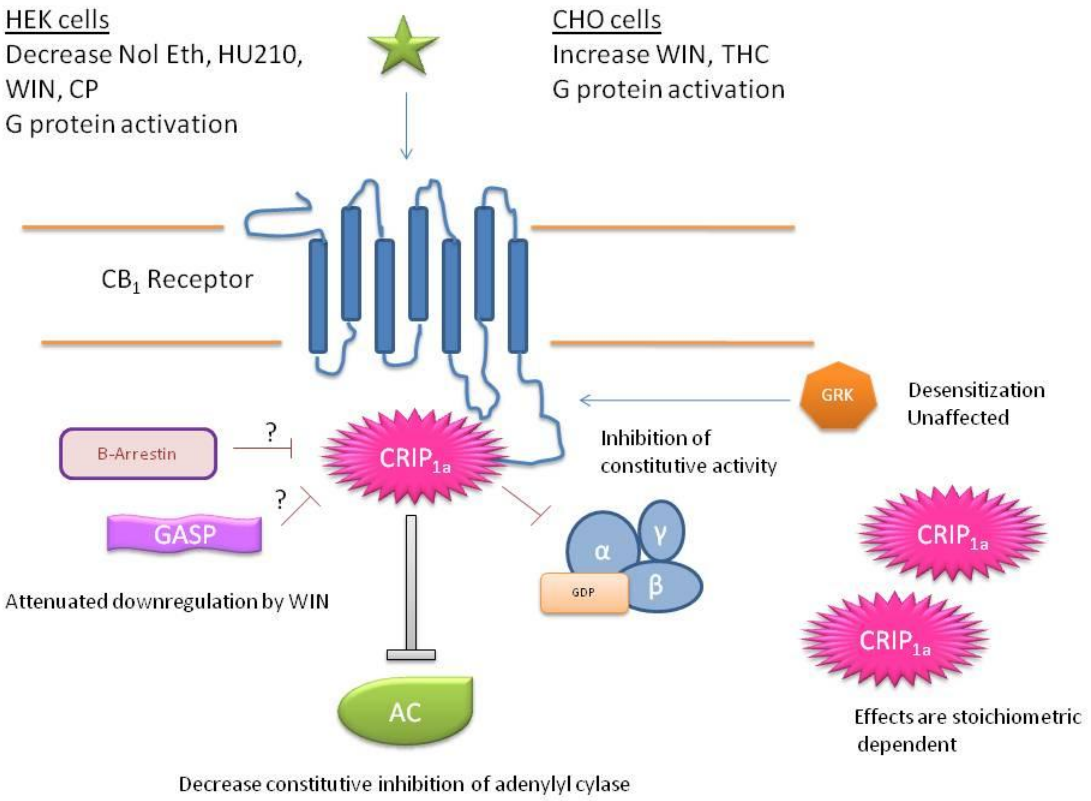

Figure 27. The effects of Cannabinoid Receptor Interacting protein ($CRIP_{1a}$) on CB_1 receptor function. $CRIP_{1a}$ inhibited constitutive G-protein activation by the CB_1 receptor. $CRIP_{1a}$ decreased WIN and CP-stimulated acute G-protein activation by the CB_1 receptor in HEK cells and increased WIN and THC-stimulated acute G-protein activation by the CB_1 receptor in CHO cells. $CRIP_{1a}$ did not affect desensitization, but did attenuate downregulation induced by WIN occupancy of the CB_1 receptor. Downstream, $CRIP_{1a}$ decreased the constitutive inhibition of adenylyl cylase by the CB_1 receptor. The effects of $CRIP_{1a}$ were dependent on the stoichiometric ratio of $CRIP_{1a}/CB_1$ receptors.

$CRIP_{1a}$ Effects?	Constitutive	Agonist-Mediated
CB_1 Receptor Expression	No (1, 2)	None apparent.
G-protein Activation	Yes (2)	Yes (2)
Receptor Desensitization	None apparent.	No (2)
Receptor Downregulation	None apparent.	Yes (2)
Ca^{+2} Channel Inhibition	Yes (1)	No (1)
Adenylyl Cyclase Inhibition	Yes (2)	No (2)
ERK 1/2	No (2)	No (2)

Table 10. Concluding summary of the effects of $CRIP_{1a}$ on CB_1 receptor mediated activity. References: (1) Neihaus et al. (2007), (2) Results from the current dissertation.

List of References

Administration, S. A. a. M. H. S. (2003). Results from the 2003 National Survey on Drug Use and Health: National Findings. NHSDA Series H-25. Rockville, MD, Substance Abuse and Mental Health Services Administration.

Albert, P. R. and L. Robillard (2002). "G protein specificity: traffic direction required." Cell Signal **14**(5): 407-18.

Anavi-Goffer, S., D. Fleischer, D. P. Hurst, D. L. Lynch, J. Barnett-Norris, S. Shi, D. L. Lewis, S. Mukhopadhyay, A. C. Howlett, P. H. Reggio and M. E. Abood (2007). "Helix 8 Leu in the CB1 cannabinoid receptor contributes to selective signal transduction mechanisms." J Biol Chem **282**(34): 25100-13.

Andersen, D. C. and L. Krummen (2002). "Recombinant protein expression for therapeutic applications." Curr Opin Biotechnol **13**(2): 117-23.

Ango, F., L. Prezeau, T. Muller, J. C. Tu, B. Xiao, P. F. Worley, J. P. Pin, J. Bockaert and L. Fagni (2001). "Agonist-independent activation of metabotropic glutamate receptors by the intracellular protein Homer." Nature **411**(6840): 962-5.

Bahr, B. A., D. A. Karanian, S. S. Makanji and A. Makriyannis (2006). "Targeting the endocannabinoid system in treating brain disorders." Expert Opin Investig Drugs **15**(4): 351-65.

Baldwin, J. M., G. F. Schertler and V. M. Unger (1997). "An alpha-carbon template for the transmembrane helices in the rhodopsin family of G-protein-coupled receptors." J Mol Biol **272**(1): 144-64.

Bari, M., N. Battista, F. Fezza, A. Finazzi-Agro and M. Maccarrone (2005). "Lipid rafts control signaling of type-1 cannabinoid receptors in neuronal cells. Implications for anandamide-induced apoptosis." J Biol Chem **280**(13): 12212-20.

Bari, M., S. Oddi, C. De Simone, P. Spagnolo, V. Gasperi, N. Battista, D. Centonze and M. Maccarrone (2008). "Type-1 cannabinoid receptors colocalize with caveolin-1 in neuronal cells." Neuropharmacology **54**(1): 45-50.

Barnes, M. P. (2006). "Sativex: clinical efficacy and tolerability in the treatment of symptoms of multiple sclerosis and neuropathic pain." Expert Opin Pharmacother **7**(5): 607-15.

Barnett-Norris, J., D. Lynch and P. H. Reggio (2005). "Lipids, lipid rafts and caveolae: their importance for GPCR signaling and their centrality to the endocannabinoid system." Life Sci **77**(14): 1625-39.

Bisogno, T., F. Howell, G. Williams, A. Minassi, M. G. Cascio, A. Ligresti, I. Matias, A. Schiano-Moriello, P. Paul, E. J. Williams, U. Gangadharan, C. Hobbs, V. Di Marzo and P. Doherty (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): 463-8.

Bisogno, T., A. Ligresti and V. Di Marzo (2005). "The endocannabinoid signalling system: biochemical aspects." Pharmacol Biochem Behav **81**(2): 224-38.

Bockaert, J., L. Fagni, A. Dumuis and P. Marin (2004). "GPCR interactinG proteins (GIP)." Pharmacol Ther **103**(3): 203-21.

Bohn, L. M. (2007). "Constitutive trafficking--more than just running in circles?" Mol Pharmacol **71**(4): 957-8.

Bohn, L. M., L. A. Dykstra, R. J. Lefkowitz, M. G. Caron and L. S. Barak (2004). "Relative opioid efficacy is determined by the complements of the G-protein-coupled receptor desensitization machinery." Mol Pharmacol **66**(1): 106-12.

Bouaboula, M., D. Dussossoy and P. Casellas (1999). "Regulation of peripheral cannabinoid receptor CB2 phosphorylation by the inverse agonist SR 144528. Implications for receptor biological responses." J Biol Chem **274**(29): 20397-405.

Bouaboula, M., S. Perrachon, L. Milligan, X. Canat, M. Rinaldi-Carmona, M. Portier, F. Barth, B. Calandra, F. Pecceu, J. Lupker, J. P. Maffrand, G. Le Fur and P. Casellas (1997). "A selective inverse agonist for central cannabinoid receptor inhibits mitogen-activated protein kinase activation stimulated by insulin or insulin-like growth factor 1. Evidence for a new model of receptor/ligand interactions." J Biol Chem **272**(35): 22330-9.

Bouaboula, M., C. Poinot-Chazel, B. Bourrie, X. Canat, B. Calandra, M. Rinaldi-Carmona, G. Le Fur and P. Casellas (1995). "Activation of mitogen-activated protein kinases by stimulation of the central cannabinoid receptor CB1." Biochem J **312 (Pt 2)**: 637-41.

Bradford, M. M. (1976). "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding." Anal Biochem **72**: 248-54.

Breivogel, C. S. and S. R. Childers (2000). "Cannabinoid agonist signal transduction in rat brain: comparison of cannabinoid agonists in receptor binding, G-protein activation, and adenylyl cyclase inhibition." J Pharmacol Exp Ther **295**(1): 328-36.

Breivogel, C. S., S. R. Childers, S. A. Deadwyler, R. E. Hampson, L. J. Vogt and L. J. Sim-Selley (1999). "Chronic delta9-tetrahydrocannabinol treatment produces a time-dependent loss of cannabinoid receptors and cannabinoid receptor-activated G-proteins in rat brain." J Neurochem **73**(6): 2447-59.

Breivogel, C. S., G. Griffin, V. Di Marzo and B. R. Martin (2001). "Evidence for a new G-protein-coupled cannabinoid receptor in mouse brain." Mol Pharmacol **60**(1): 155-63.

Breivogel, C. S., J. M. Lambert, S. Gerfin, J. W. Huffman and R. K. Razdan (2008). "Sensitivity to delta9-tetrahydrocannabinol is selectively enhanced in beta-arrestin2 -/- mice." Behav Pharmacol **19**(4): 298-307.

Breivogel, C. S., D. E. Selley and S. R. Childers (1998). "Cannabinoid receptor agonist efficacy for stimulating [35S]GTPgammaS binding to rat cerebellar membranes correlates with agonist-induced decreases in GDP affinity." J Biol Chem **273**(27): 16865-73.

Breivogel, C. S., L. J. Sim and S. R. Childers (1997). "Regional differences in cannabinoid receptor/G protein coupling in rat brain." J Pharmacol Exp Ther **282**(3): 1632-42.

Camps, M., C. Hou, D. Sidiropoulos, J. B. Stock, K. H. Jakobs and P. Gierschik (1992). "Stimulation of phospholipase C by guanine-nucleotide-bindinG protein beta gamma subunits." Eur J Biochem **206**(3): 821-31.

Carlson, G., Y. Wang and B. E. Alger (2002). "Endocannabinoids facilitate the induction of LTP in the hippocampus." Nat Neurosci **5**(8): 723-4.

Castane, A., E. Valjent, C. Ledent, M. Parmentier, R. Maldonado and O. Valverde (2002). "Lack of CB1 cannabinoid receptors modifies nicotine behavioural responses, but not nicotine abstinence." Neuropharmacology **43**(5): 857-67.

Ceresa, B. P. and L. E. Limbird (1994). "Mutation of an aspartate residue highly conserved among G-protein-coupled receptors results in nonreciprocal disruption of alpha 2-adrenergic receptor-G protein interactions. A negative charge at amino acid residue 79 forecasts alpha 2A-adrenergic receptor sensitivity to allosteric modulation by monovalent cations and fully effective receptor/G protein coupling." J Biol Chem **269**(47): 29557-64.

Chen, C. A. and D. R. Manning (2001). "Regulation of G-proteins by covalent modification." Oncogene **20**(13): 1643-52.

Childers, S. R. (2006). "Activation of G-proteins in brain by endogenous and exogenous cannabinoids." Aaps J **8**(1): E112-7.

Cippitelli, A., A. Bilbao, A. C. Hansson, I. del Arco, W. Sommer, M. Heilig, M. Massi, F. J. Bermudez-Silva, M. Navarro, R. Ciccocioppo and F. R. de Fonseca (2005). "Cannabinoid CB1 receptor antagonism reduces conditioned reinstatement of ethanol-seeking behavior in rats." Eur J Neurosci **21**(8): 2243-51.

Clapham, D. E. and E. J. Neer (1997). "G protein beta gamma subunits." Annu Rev Pharmacol Toxicol **37**: 167-203.

Colombo, G., S. Serra, G. Brunetti, R. Gomez, S. Melis, G. Vacca, M. M. Carai and L. Gessa (2002). "Stimulation of voluntary ethanol intake by cannabinoid receptor agonists in ethanol-preferring sP rats." Psychopharmacology (Berl) **159**(2): 181-7.

Compton, D. R., K. C. Rice, B. R. De Costa, R. K. Razdan, L. S. Melvin, M. R. Johnson and B. R. Martin (1993). "Cannabinoid structure-activity relationships: correlation of receptor binding and in vivo activities." J Pharmacol Exp Ther **265**(1): 218-26.

Cossu, G., C. Ledent, L. Fattore, A. Imperato, G. A. Bohme, M. Parmentier and W. Fratta (2001). "Cannabinoid CB1 receptor knockout mice fail to self-administer morphine but not other drugs of abuse." Behav Brain Res **118**(1): 61-5.

Coutts, A. A., S. Anavi-Goffer, R. A. Ross, D. J. MacEwan, K. Mackie, R. G. Pertwee and A. J. Irving (2001). "Agonist-induced internalization and trafficking of cannabinoid CB1 receptors in hippocampal neurons." J Neurosci **21**(7): 2425-33.

Cravatt, B. F., K. Demarest, M. P. Patricelli, M. H. Bracey, D. K. Giang, B. R. Martin and A. H. Lichtman (2001). "Supersensitivity to anandamide and enhanced endogenous cannabinoid signaling in mice lacking fatty acid amide hydrolase." Proc Natl Acad Sci U S A **98**(16): 9371-6.

Daigle, T. L., M. L. Kwok and K. Mackie (2008). "Regulation of CB1 cannabinoid receptor internalization by a promiscuous phosphorylation-dependent mechanism." J Neurochem **106**(1): 70-82.

De Vries, T. J., J. R. Homberg, R. Binnekade, H. Raaso and A. N. Schoffelmeer (2003). "Cannabinoid modulation of the reinforcing and motivational properties of heroin and heroin-associated cues in rats." Psychopharmacology (Berl) **168**(1-2): 164-9.

De Vries, T. J. and A. N. Schoffelmeer (2005). "Cannabinoid CB1 receptors control conditioned drug seeking." Trends Pharmacol Sci **26**(8): 420-6.

Derkinderen, P., C. Ledent, M. Parmentier and J. A. Girault (2001). "Cannabinoids activate p38 mitogen-activated protein kinases through CB1 receptors in hippocampus." J Neurochem **77**(3): 957-60.

Devane, W. A., F. A. Dysarz, 3rd, M. R. Johnson, L. S. Melvin and A. C. Howlett (1988). "Determination and characterization of a cannabinoid receptor in rat brain." Mol Pharmacol **34**(5): 605-13.

Devane, W. A., L. Hanus, A. Breuer, R. G. Pertwee, L. A. Stevenson, G. Griffin, D. Gibson, A. Mandelbaum, A. Etinger and R. Mechoulam (1992). "Isolation and structure of a brain constituent that binds to the cannabinoid receptor." Science **258**(5090): 1946-9.

Dewey, W. L. (1986). "Cannabinoid pharmacology." Pharmacol Rev **38**(2): 151-78.

Di Marzo, V., C. S. Breivogel, Q. Tao, D. T. Bridgen, R. K. Razdan, A. M. Zimmer, A. Zimmer and B. R. Martin (2000). "Levels, metabolism, and pharmacological activity of anandamide in CB(1) cannabinoid receptor knockout mice: evidence for non-CB(1), non-CB(2) receptor-mediated actions of anandamide in mouse brain." J Neurochem **75**(6): 2434-44.

Dinh, T. P., D. Carpenter, F. M. Leslie, T. F. Freund, I. Katona, S. L. Sensi, S. Kathuria and D. Piomelli (2002). "Brain monoglyceride lipase participating in endocannabinoid inactivation." Proc Natl Acad Sci U S A **99**(16): 10819-24.

Ester Fride, R. M. (2003). New advances in the identification and physiological role of the different componenets of the endogenous cannabinoid system. Molecular Biology of Drug Addiction. R. Maldonado, Humana Press**:** 173-198.

Felder, C. C., K. E. Joyce, E. M. Briley, J. Mansouri, K. Mackie, O. Blond, Y. Lai, A. L. Ma and R. L. Mitchell (1995). "Comparison of the pharmacology and signal transduction of the human cannabinoid CB1 and CB2 receptors." Mol Pharmacol **48**(3): 443-50.

Fernandez, J. R. and D. B. Allison (2004). "Rimonabant Sanofi-Synthelabo." Curr Opin Investig Drugs **5**(4): 430-5.

Gallate, J. E., T. Saharov, P. E. Mallet and I. S. McGregor (1999). "Increased motivation for beer in rats following administration of a cannabinoid CB1 receptor agonist." Eur J Pharmacol **370**(3): 233-40.

Galve-Roperh, I., D. Rueda, T. Gomez del Pulgar, G. Velasco and M. Guzman (2002). "Mechanism of extracellular signal-regulated kinase activation by the CB(1) cannabinoid receptor." Mol Pharmacol **62**(6): 1385-92.

Gaoni, Y. and R. Mechoulam (1971). "The isolation and structure of delta-1-tetrahydrocannabinol and other neutral cannabinoids from hashish." J Am Chem Soc **93**(1): 217-24.

Gatley, S. J., R. Lan, B. Pyatt, A. N. Gifford, N. D. Volkow and A. Makriyannis (1997). "Binding of the non-classical cannabinoid CP 55,940, and the diarylpyrazole AM251 to rodent brain cannabinoid receptors." Life Sci **61**(14): PL 191-7.

Gettys, T. W., K. Sheriff-Carter, J. Moomaw, I. L. Taylor and J. R. Raymond (1994). "Characterization and use of crude alpha-subunit preparations for quantitative immunoblotting of G-proteins." Anal Biochem **220**(1): 82-91.

Gifford, A. N., M. Bruneus, S. J. Gatley, R. Lan, A. Makriyannis and N. D. Volkow (1999). "Large receptor reserve for cannabinoid actions in the central nervous system." J Pharmacol Exp Ther **288**(2): 478-83.

Gilman, A. G. (1987). "G proteins: transducers of receptor-generated signals." Annu Rev Biochem **56**: 615-49.

Glass, M. and C. C. Felder (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): 5327-33.

Glass, M. and J. K. Northup (1999). "Agonist selective regulation of G-proteins by cannabinoid CB(1) and CB(2) receptors." Mol Pharmacol **56**(6): 1362-9.

Glebov, O. O., N. A. Bright and B. J. Nichols (2006). "Flotillin-1 defines a clathrin-independent endocytic pathway in mammalian cells." Nat Cell Biol **8**(1): 46-54.

Goodman, O. B., Jr., J. G. Krupnick, F. Santini, V. V. Gurevich, R. B. Penn, A. W. Gagnon, J. H. Keen and J. L. Benovic (1996). "Beta-arrestin acts as a clathrin adaptor in endocytosis of the beta2-adrenergic receptor." Nature **383**(6599): 447-50.

Graham, F. L., J. Smiley, W. C. Russell and R. Nairn (1977). "Characteristics of a human cell line transformed by DNA from human adenovirus type 5." J Gen Virol **36**(1): 59-74.

Guo, J. and S. R. Ikeda (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): 665-74.

Hall, R. A., R. T. Premont and R. J. Lefkowitz (1999). "Heptahelical receptor signaling: beyond the G-protein paradigm." J Cell Biol **145**(5): 927-32.

Hanus, L., S. Abu-Lafi, E. Fride, A. Breuer, Z. Vogel, D. E. Shalev, I. Kustanovich and R. Mechoulam (2001). "2-arachidonyl glyceryl ether, an endogenous agonist of the cannabinoid CB1 receptor." Proc Natl Acad Sci U S A **98**(7): 3662-5.

Herkenham, M., A. B. Lynn, M. R. Johnson, L. S. Melvin, B. R. de Costa and K. C. Rice (1991). "Characterization and localization of cannabinoid receptors in rat brain: a quantitative in vitro autoradiographic study." J Neurosci **11**(2): 563-83.

Herlitze, S., D. E. Garcia, K. Mackie, B. Hille, T. Scheuer and W. A. Catterall (1996). "Modulation of Ca2+ channels by G-protein beta gamma subunits." Nature **380**(6571): 258-62.

Heuser, J. E. and R. G. Anderson (1989). "Hypertonic media inhibit receptor-mediated endocytosis by blocking clathrin-coated pit formation." J Cell Biol **108**(2): 389-400.

Hollister, L. E. (1974). "Structure-activity relationships in man of cannabis constituents, and homologs and metabolites of delta9-tetrahydrocannabinol." Pharmacology **11**(1): 3-11.

Hollister, L. E. (1986). "Health aspects of cannabis." Pharmacol Rev **38**(1): 1-20.

Horstman, D. A., S. Brandon, A. L. Wilson, C. A. Guyer, E. J. Cragoe, Jr. and L. E. Limbird (1990). "An aspartate conserved among G-protein receptors confers allosteric regulation of alpha 2-adrenergic receptors by sodium." J Biol Chem **265**(35): 21590-5.

Houston, D. B. and A. C. Howlett (1998). "Differential receptor-G protein coupling evoked by dissimilar cannabinoid receptor agonists." Cell Signal **10**(9): 667-74.

Howlett, A. C. (2004). "Efficacy in CB1 receptor-mediated signal transduction." Br J Pharmacol **142**(8): 1209-18.

Howlett, A. C., F. Barth, T. I. Bonner, G. Cabral, P. Casellas, W. A. Devane, C. C. Felder, M. Herkenham, K. Mackie, B. R. Martin, R. Mechoulam and R. G.

Pertwee (2002). "International Union of Pharmacology. XXVII. Classification of cannabinoid receptors." Pharmacol Rev **54**(2): 161-202.
Howlett, A. C., J. M. Qualy and L. L. Khachatrian (1986). "Involvement of Gi in the inhibition of adenylate cyclase by cannabimimetic drugs." Mol Pharmacol **29**(3): 307-13.
Hsieh, C., S. Brown, C. Derleth and K. Mackie (1999). "Internalization and recycling of the CB1 cannabinoid receptor." J Neurochem **73**(2): 493-501.
Hunter, S. A., S. Burstein and L. Renzulli (1986). "Effects of cannabinoids on the activities of mouse brain lipases." Neurochem Res **11**(9): 1273-88.
Inglese, J., W. J. Koch, K. Touhara and R. J. Lefkowitz (1995). "G beta gamma interactions with PH domains and Ras-MAPK signaling pathways." Trends Biochem Sci **20**(4): 151-6.
Jakobs, K. H. (1979). "Inhibition of adenylate cyclase by hormones and neurotransmitters." Mol Cell Endocrinol **16**(3): 147-56.
Jelen, F., A. Oleksy, K. Smietana and J. Otlewski (2003). "PDZ domains - common players in the cell signaling." Acta Biochim Pol **50**(4): 985-1017.
Jelsema, C. L. and J. Axelrod (1987). "Stimulation of phospholipase A2 activity in bovine rod outer segments by the beta gamma subunits of transducin and its inhibition by the alpha subunit." Proc Natl Acad Sci U S A **84**(11): 3623-7.
Jin, W., S. Brown, J. P. Roche, C. Hsieh, J. P. Celver, A. Kovoor, C. Chavkin and K. Mackie (1999). "Distinct domains of the CB1 cannabinoid receptor mediate desensitization and internalization." J Neurosci **19**(10): 3773-80.
Kearn, C. S., K. Blake-Palmer, E. Daniel, K. Mackie and M. Glass (2005). "Concurrent stimulation of cannabinoid CB1 and dopamine D2 receptors enhances heterodimer formation: a mechanism for receptor cross-talk?" Mol Pharmacol **67**(5): 1697-704.
Keren, O. and Y. Sarne (2003). "Multiple mechanisms of CB1 cannabinoid receptors regulation." Brain Res **980**(2): 197-205.
Kong, H., K. Raynor, K. Yasuda, G. I. Bell and T. Reisine (1993). "Mutation of an aspartate at residue 89 in somatostatin receptor subtype 2 prevents Na+ regulation of agonist binding but does not alter receptor-G protein association." Mol Pharmacol **44**(2): 380-4.
Koski, G., R. A. Streaty and W. A. Klee (1982). "Modulation of sodium-sensitive GTPase by partial opiate agonists. An explanation for the dual requirement for Na+ and GTP in inhibitory regulation of adenylate cyclase." J Biol Chem **257**(23): 14035-40.
Kouznetsova, M., B. Kelley, M. Shen and S. A. Thayer (2002). "Desensitization of cannabinoid-mediated presynaptic inhibition of neurotransmission between rat hippocampal neurons in culture." Mol Pharmacol **61**(3): 477-85.
Kreitzer, A. C. and W. G. Regehr (2001). "Retrograde inhibition of presynaptic calcium influx by endogenous cannabinoids at excitatory synapses onto Purkinje cells." Neuron **29**(3): 717-27.
Kuster, J. E., J. I. Stevenson, S. J. Ward, T. E. D'Ambra and D. A. Haycock (1993). "Aminoalkylindole binding in rat cerebellum: selective displacement by natural and synthetic cannabinoids." J Pharmacol Exp Ther **264**(3): 1352-63.

Laporte, S. A., R. H. Oakley, J. Zhang, J. A. Holt, S. S. Ferguson, M. G. Caron and L. S. Barak (1999). "The beta2-adrenergic receptor/betaarrestin complex recruits the clathrin adaptor AP-2 during endocytosis." Proc Natl Acad Sci U S A **96**(7): 3712-7.

Law, S. F., K. Yasuda, G. I. Bell and T. Reisine (1993). "Gi alpha 3 and G(o) alpha selectively associate with the cloned somatostatin receptor subtype SSTR2." J Biol Chem **268**(15): 10721-7.

Ledent, C., O. Valverde, G. Cossu, F. Petitet, J. F. Aubert, F. Beslot, G. A. Bohme, A. Imperato, T. Pedrazzini, B. P. Roques, G. Vassart, W. Fratta and M. Parmentier (1999). "Unresponsiveness to cannabinoids and reduced addictive effects of opiates in CB1 receptor knockout mice." Science **283**(5400): 401-4.

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

Leff, P., C. Scaramellini, C. Law and K. McKechnie (1997). "A three-state receptor model of agonist action." Trends Pharmacol Sci **18**(10): 355-62.

Lefkowitz, R. J. (1998). "G protein-coupled receptors. III. New roles for receptor kinases and beta-arrestins in receptor signaling and desensitization." J Biol Chem **273**(30): 18677-80.

Leterrier, C., D. Bonnard, D. Carrel, J. Rossier and Z. Lenkei (2004). "Constitutive endocytic cycle of the CB1 cannabinoid receptor." J Biol Chem **279**(34): 36013-21.

Leterrier, C., J. Laine, M. Darmon, H. Boudin, J. Rossier and Z. Lenkei (2006). "Constitutive activation drives compartment-selective endocytosis and axonal targeting of type 1 cannabinoid receptors." J Neurosci **26**(12): 3141-53.

Leung, D., A. Saghatelian, G. M. Simon and B. F. Cravatt (2006). "Inactivation of N-acyl phosphatidylethanolamine phospholipase D reveals multiple mechanisms for the biosynthesis of endocannabinoids." Biochemistry **45**(15): 4720-6.

Little, P. J., D. R. Compton, M. R. Johnson, L. S. Melvin and B. R. Martin (1988). "Pharmacology and stereoselectivity of structurally novel cannabinoids in mice." J Pharmacol Exp Ther **247**(3): 1046-51.

Locht, C. and R. Antoine (1995). "A proposed mechanism of ADP-ribosylation catalyzed by the pertussis toxin S1 subunit." Biochimie **77**(5): 333-40.

Logothetis, D. E., Y. Kurachi, J. Galper, E. J. Neer and D. E. Clapham (1987). "The beta gamma subunits of GTP-bindinG proteins activate the muscarinic K+ channel in heart." Nature **325**(6102): 321-6.

Ludanyi, A., L. Eross, S. Czirjak, J. Vajda, P. Halasz, M. Watanabe, M. Palkovits, Z. Magloczky, T. F. Freund and I. Katona (2008). "Downregulation of the CB1 cannabinoid receptor and related molecular elements of the endocannabinoid system in epileptic human hippocampus." J Neurosci **28**(12): 2976-90.

Mackie, K. (2006). "Cannabinoid receptors as therapeutic targets." Annu Rev Pharmacol Toxicol **46**: 101-22.

Mackie, K., Y. Lai, R. Westenbroek and R. Mitchell (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): 6552-61.

Maldonado, R. (2002). "Study of cannabinoid dependence in animals." Pharmacol Ther **95**(2): 153-64.

Maldonado, R. and F. Rodriguez de Fonseca (2002). "Cannabinoid addiction: behavioral models and neural correlates." J Neurosci **22**(9): 3326-31.

Maldonado, R., O. Valverde and F. Berrendero (2006). "Involvement of the endocannabinoid system in drug addiction." Trends Neurosci **29**(4): 225-32.

Marinelli, S., S. Pacioni, T. Bisogno, V. Di Marzo, D. A. Prince, J. R. Huguenard and A. Bacci (2008). "The endocannabinoid 2-arachidonoylglycerol is responsible for the slow self-inhibition in neocortical interneurons." J Neurosci **28**(50): 13532-41.

Martini, L., M. Waldhoer, M. Pusch, V. Kharazia, J. Fong, J. H. Lee, C. Freissmuth and J. L. Whistler (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): 802-11.

Matsuda, L. A., S. J. Lolait, M. J. Brownstein, A. C. Young and T. I. Bonner (1990). "Structure of a cannabinoid receptor and functional expression of the cloned cDNA." Nature **346**(6284): 561-4.

McDonald, N. A., C. M. Henstridge, C. N. Connolly and A. J. Irving (2007). "An essential role for constitutive endocytosis, but not activity, in the axonal targeting of the CB1 cannabinoid receptor." Mol Pharmacol **71**(4): 976-84.

Mechoulam, R. (1986). The Pharmacohistory of *Cannabis Sativa*. Jerusalem, Israel, CRC Press, Inc.

Mechoulam, R., S. Ben-Shabat, L. Hanus, M. Ligumsky, N. E. Kaminski, A. R. Schatz, A. Gopher, S. Almog, B. R. Martin, D. R. Compton and et al. (1995). "Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors." Biochem Pharmacol **50**(1): 83-90.

Mechoulam, R., M. Peters, E. Murillo-Rodriguez and L. O. Hanus (2007). "Cannabidiol--recent advances." Chem Biodivers **4**(8): 1678-92.

Moffett, S., D. A. Brown and M. E. Linder (2000). "Lipid-dependent targeting of G-proteins into rafts." J Biol Chem **275**(3): 2191-8.

Morisset, S., A. Rouleau, X. Ligneau, F. Gbahou, J. Tardivel-Lacombe, H. Stark, W. Schunack, C. R. Ganellin, J. C. Schwartz and J. M. Arrang (2000). "High constitutive activity of native H3 receptors regulates histamine neurons in brain." Nature **408**(6814): 860-4.

Mukhopadhyay, S. and A. C. Howlett (2001). "CB1 receptor-G protein association. Subtype selectivity is determined by distinct intracellular domains." Eur J Biochem **268**(3): 499-505.

Mukhopadhyay, S. and A. C. Howlett (2005). "Chemically distinct ligands promote differential CB1 cannabinoid receptor-Gi protein interactions." Mol Pharmacol **67**(6): 2016-24.

Mukhopadhyay, S., H. H. McIntosh, D. B. Houston and A. C. Howlett (2000). "The CB(1) cannabinoid receptor juxtamembrane C-terminal peptide confers activation to specific G-proteins in brain." Mol Pharmacol **57**(1): 162-70.

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

Munro, S., K. L. Thomas and M. Abu-Shaar (1993). "Molecular characterization of a peripheral receptor for cannabinoids." Nature **365**(6441): 61-5.

Navarro, M., M. R. Carrera, W. Fratta, O. Valverde, G. Cossu, L. Fattore, J. A. Chowen, R. Gomez, I. del Arco, M. A. Villanua, R. Maldonado, G. F. Koob and F. Rodriguez de Fonseca (2001). "Functional interaction between opioid and cannabinoid receptors in drug self-administration." J Neurosci **21**(14): 5344-50.

Nicholson, R. A., C. Liao, J. Zheng, L. S. David, L. Coyne, A. C. Errington, G. Singh and G. Lees (2003). "Sodium channel inhibition by anandamide and synthetic cannabimimetics in brain." Brain Res **978**(1-2): 194-204.

Nie, J. and D. L. Lewis (2001). "The proximal and distal C-terminal tail domains of the CB1 cannabinoid receptor mediate G-protein coupling." Neuroscience **107**(1): 161-7.

Nie, J. and D. L. Lewis (2001). "Structural domains of the CB1 cannabinoid receptor that contribute to constitutive activity and G-protein sequestration." J Neurosci **21**(22): 8758-64.

Niehaus, J. L., Y. Liu, K. T. Wallis, M. Egertova, S. G. Bhartur, S. Mukhopadhyay, S. Shi, H. He, D. E. Selley, A. C. Howlett, M. R. Elphick and D. L. Lewis (2007). "CB1 cannabinoid receptor activity is modulated by the cannabinoid receptor interactinG protein CRIP 1a." Mol Pharmacol **72**(6): 1557-66.

Offermanns, S. (2003). "G proteins as transducers in transmembrane signalling." Prog Biophys Mol Biol **83**(2): 101-30.

Oviedo, A., J. Glowa and M. Herkenham (1993). "Chronic cannabinoid administration alters cannabinoid receptor binding in rat brain: a quantitative autoradiographic study." Brain Res **616**(1-2): 293-302.

Pacheco, M. A., S. J. Ward and S. R. Childers (1994). "Differential requirements of sodium for coupling of cannabinoid receptors to adenylyl cyclase in rat brain membranes." J Neurochem **62**(5): 1773-82.

Pacher, P., S. Batkai and G. Kunos (2006). "The endocannabinoid system as an emerging target of pharmacotherapy." Pharmacol Rev **58**(3): 389-462.

Palamara, K. L., H. R. Mogul, S. J. Peterson and W. H. Frishman (2006). "Obesity: new perspectives and pharmacotherapies." Cardiol Rev **14**(5): 238-58.

Pan, X., S. R. Ikeda and D. L. Lewis (1996). "Rat brain cannabinoid receptor modulates N-type Ca2+ channels in a neuronal expression system." Mol Pharmacol **49**(4): 707-14.

Pan, X., S. R. Ikeda and D. L. Lewis (1998). "SR 141716A acts as an inverse agonist to increase neuronal voltage-dependent Ca2+ currents by reversal of tonic CB1 cannabinoid receptor activity." Mol Pharmacol **54**(6): 1064-72.

Patel, H. H., F. Murray and P. A. Insel (2008). "G protein-coupled receptor-signaling components in membrane raft and caveolae microdomains." Handb Exp Pharmacol(186): 167-84.

Pertwee, R. G., A. Thomas, L. A. Stevenson, R. A. Ross, S. A. Varvel, A. H. Lichtman, B. R. Martin and R. K. Razdan (2007). "The psychoactive plant cannabinoid, Delta9-tetrahydrocannabinol, is antagonized by Delta8- and Delta9-tetrahydrocannabivarin in mice in vivo." Br J Pharmacol **150**(5): 586-94.

Pitcher, J. A., N. J. Freedman and R. J. Lefkowitz (1998). "G protein-coupled receptor kinases." Annu Rev Biochem **67**: 653-92.

Pitcher, J. A., J. Inglese, J. B. Higgins, J. L. Arriza, P. J. Casey, C. Kim, J. L. Benovic, M. M. Kwatra, M. G. Caron and R. J. Lefkowitz (1992). "Role of beta gamma subunits of G-proteins in targeting the beta-adrenergic receptor kinase to membrane-bound receptors." Science **257**(5074): 1264-7.

Puck, T. T., S. J. Cieciura and A. Robinson (1958). "Genetics of somatic mammalian cells. III. Long-term cultivation of euploid cells from human and animal subjects." J Exp Med **108**(6): 945-56.

Razani, B., S. E. Woodman and M. P. Lisanti (2002). "Caveolae: from cell biology to animal physiology." Pharmacol Rev **54**(3): 431-67.

Reiter, E. and R. J. Lefkowitz (2006). "GRKs and beta-arrestins: roles in receptor silencing, trafficking and signaling." Trends Endocrinol Metab **17**(4): 159-65.

Rhee, M. H., M. Bayewitch, T. Avidor-Reiss, R. Levy and Z. Vogel (1998). "Cannabinoid receptor activation differentially regulates the various adenylyl cyclase isozymes." J Neurochem **71**(4): 1525-34.

Rhee, M. H., I. Nevo, T. Avidor-Reiss, R. Levy and Z. Vogel (2000). "Differential superactivation of adenylyl cyclase isozymes after chronic activation of the CB(1) cannabinoid receptor." Mol Pharmacol **57**(4): 746-52.

Rinaldi-Carmona, M., F. Barth, M. Heaulme, D. Shire, B. Calandra, C. Congy, S. Martinez, J. Maruani, G. Neliat, D. Caput and et al. (1994). "SR141716A, a potent and selective antagonist of the brain cannabinoid receptor." FEBS Lett **350**(2-3): 240-4.

Rinaldi-Carmona, M., F. Barth, J. Millan, J. M. Derocq, P. Casellas, C. Congy, D. Oustric, M. Sarran, M. Bouaboula, B. Calandra, M. Portier, D. Shire, J. C. Breliere and G. L. Le Fur (1998). "SR 144528, the first potent and selective antagonist of the CB2 cannabinoid receptor." J Pharmacol Exp Ther **284**(2): 644-50.

Roche, J. P., S. Bounds, S. Brown and K. Mackie (1999). "A mutation in the second transmembrane region of the CB1 receptor selectively disrupts G-protein signaling and prevents receptor internalization." Mol Pharmacol **56**(3): 611-8.

Rockhold, R. W. (2002). The Chemical Basis for Neuronal Communication. Fundamental Neuroscience. D. E. Haines. New York, Churchill Livingstone**:** 57-70.

Rodriguez de Fonseca, F., M. A. Gorriti, J. J. Fernandez-Ruiz, T. Palomo and J. A. Ramos (1994). "Downregulation of rat brain cannabinoid binding sites after chronic delta 9-tetrahydrocannabinol treatment." Pharmacol Biochem Behav **47**(1): 33-40.

Romero, J., F. Berrendero, L. Garcia-Gil, S. Y. Lin, A. Makriyannis, J. A. Ramos and J. J. Fernandez-Ruiz (1999). "Cannabinoid receptor and WIN-55,212-2-stimulated [35S]GTPgammaS binding and cannabinoid receptor mRNA levels in several brain structures of adult male rats chronically exposed to R-methanandamide." Neurochem Int **34**(6): 473-82.

Rubino, T., D. Vigano, F. Premoli, C. Castiglioni, S. Bianchessi, R. Zippel and D. Parolaro (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., I. Galve-Roperh, A. Haro and M. Guzman (2000). "The CB(1) cannabinoid receptor is coupled to the activation of c-Jun N-terminal kinase." Mol Pharmacol **58**(4): 814-20.

Rueda, D., B. Navarro, A. Martinez-Serrano, M. Guzman and I. Galve-Roperh (2002). "The endocannabinoid anandamide inhibits neuronal progenitor cell differentiation through attenuation of the Rap1/B-Raf/ERK pathway." J Biol Chem **277**(48): 46645-50.

Russo, E. B., H. E. Jiang, X. Li, A. Sutton, A. Carboni, F. del Bianco, G. Mandolino, D. J. Potter, Y. X. Zhao, S. Bera, Y. B. Zhang, E. G. Lu, D. K. Ferguson, F. Hueber, L. C. Zhao, C. J. Liu, Y. F. Wang and C. S. Li (2008). "Phytochemical and genetic analyses of ancient cannabis from Central Asia." J Exp Bot **59**(15): 4171-82.

Sanchez, C., I. Galve-Roperh, D. Rueda and M. Guzman (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): 834-43.

Sanchez, C., D. Rueda, B. Segui, I. Galve-Roperh, T. Levade and M. Guzman (2001). "The CB(1) cannabinoid receptor of astrocytes is coupled to sphingomyelin hydrolysis through the adaptor protein fan." Mol Pharmacol **59**(5): 955-9.

Sarrouilhe, D., A. di Tommaso, T. Metaye and V. Ladeveze (2006). "Spinophilin: from partners to functions." Biochimie **88**(9): 1099-113.

Seifert, R. and K. Wenzel-Seifert (2002). "Constitutive activity of G-protein-coupled receptors: cause of disease and common property of wild-type receptors." Naunyn Schmiedebergs Arch Pharmacol **366**(5): 381-416.

Selley, D. E., M. P. Cassidy, B. R. Martin and L. J. Sim-Selley (2004). "Long-term administration of Delta9-tetrahydrocannabinol desensitizes CB1-, adenosine A1-, and GABAB-mediated inhibition of adenylyl cyclase in mouse cerebellum." Mol Pharmacol **66**(5): 1275-84.

Selley, D. E., Q. Liu and S. R. Childers (1998). "Signal transduction correlates of mu opioid agonist intrinsic efficacy: receptor-stimulated [35S]GTP gamma S binding in mMOR-CHO cells and rat thalamus." J Pharmacol Exp Ther **285**(2): 496-505.

Selley, D. E., W. K. Rorrer, C. S. Breivogel, A. M. Zimmer, A. Zimmer, B. R. Martin and L. J. Sim-Selley (2001). "Agonist efficacy and receptor efficiency in heterozygous CB1 knockout mice: relationship of reduced CB1 receptor density to G-protein activation." J Neurochem **77**(4): 1048-57.

Shaw, G., S. Morse, M. Ararat and F. L. Graham (2002). "Preferential transformation of human neuronal cells by human adenoviruses and the origin of HEK 293 cells." Faseb J **16**(8): 869-71.

Sim-Selley, L. J. (2003). "Regulation of cannabinoid CB1 receptors in the central nervous system by chronic cannabinoids." Crit Rev Neurobiol **15**(2): 91-119.

Sim-Selley, L. J., L. K. Brunk and D. E. Selley (2001). "Inhibitory effects of SR141716A on G-protein activation in rat brain." Eur J Pharmacol **414**(2-3): 135-43.

Sim-Selley, L. J. and B. R. Martin (2002). "Effect of chronic administration of R-(+)-[2,3-Dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolo[1,2,3-de]-1,4-b enzoxazinyl]-(1-naphthalenyl)methanone mesylate (WIN55,212-2) or delta(9)-

tetrahydrocannabinol on cannabinoid receptor adaptation in mice." J Pharmacol Exp Ther **303**(1): 36-44.

Sim-Selley, L. J., N. S. Schechter, W. K. Rorrer, G. D. Dalton, J. Hernandez, B. R. Martin and D. E. Selley (2006). "Prolonged recovery rate of CB1 receptor adaptation after cessation of long-term cannabinoid administration." Mol Pharmacol **70**(3): 986-96.

Sim, L. J., R. E. Hampson, S. A. Deadwyler and S. R. Childers (1996). "Effects of chronic treatment with delta9-tetrahydrocannabinol on cannabinoid-stimulated [35S]GTPgammaS autoradiography in rat brain." J Neurosci **16**(24): 8057-66.

Sim, L. J., D. E. Selley and S. R. Childers (1995). "In vitro autoradiography of receptor-activated G-proteins in rat brain by agonist-stimulated guanylyl 5'-[gamma-[35S]thio]-triphosphate binding." Proc Natl Acad Sci U S A **92**(16): 7242-6.

Sim, L. J., D. E. Selley, R. Xiao and S. R. Childers (1996). "Differences in G-protein activation by mu- and delta-opioid, and cannabinoid, receptors in rat striatum." Eur J Pharmacol **307**(1): 97-105.

Slatkin, N. E. (2007). "Cannabinoids in the treatment of chemotherapy-induced nausea and vomiting: beyond prevention of acute emesis." J Support Oncol **5**(5 Suppl 3): 1-9.

Smith, F. D., G. S. Oxford and S. L. Milgram (1999). "Association of the D2 dopamine receptor third cytoplasmic loop with spinophilin, a protein phosphatase-1-interactinG protein." J Biol Chem **274**(28): 19894-900.

Song, Z. H., C. A. Slowey, D. P. Hurst and P. H. Reggio (1999). "The difference between the CB(1) and CB(2) cannabinoid receptors at position 5.46 is crucial for the selectivity of WIN55212-2 for CB(2)." Mol Pharmacol **56**(4): 834-40.

Soria, G., V. Mendizabal, C. Tourino, P. Robledo, C. Ledent, M. Parmentier, R. Maldonado and O. Valverde (2005). "Lack of CB1 cannabinoid receptor impairs cocaine self-administration." Neuropsychopharmacology **30**(9): 1670-80.

Stella, N., P. Schweitzer and D. Piomelli (1997). "A second endogenous cannabinoid that modulates long-term potentiation." Nature **388**(6644): 773-8.

Stenmark, H. (2009). "Rab GTPases as coordinators of vesicle traffic." Nat Rev Mol Cell Biol **10**(8): 513-25.

Sugiura, T., T. Kodaka, S. Nakane, T. Miyashita, S. Kondo, Y. Suhara, H. Takayama, K. Waku, C. Seki, N. Baba and Y. Ishima (1999). "Evidence that the cannabinoid CB1 receptor is a 2-arachidonoylglycerol receptor. Structure-activity relationship of 2-arachidonoylglycerol, ether-linked analogues, and related compounds." J Biol Chem **274**(5): 2794-801.

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

Tappe-Theodor, A., N. Agarwal, I. Katona, T. Rubino, L. Martini, J. Swiercz, K. Mackie, H. Monyer, D. Parolaro, J. Whistler, T. Kuner and R. Kuner (2007). "A molecular basis of analgesic tolerance to cannabinoids." J Neurosci **27**(15): 4165-77.

Thomas, A., G. L. Baillie, A. M. Phillips, R. K. Razdan, R. A. Ross and R. G. Pertwee (2007). "Cannabidiol displays unexpectedly high potency as an antagonist of CB1 and CB2 receptor agonists in vitro." Br J Pharmacol **150**(5): 613-23.

Tilakaratne, N. and P. M. Sexton (2005). "G protein-coupled receptor-protein interactions: basis for new concepts on receptor structure and function." Clin Exp Pharmacol Physiol **32**(11): 979-87.

Tsou, K., S. Brown, M. C. Sanudo-Pena, K. Mackie and J. M. Walker (1998). "Immunohistochemical distribution of cannabinoid CB1 receptors in the rat central nervous system." Neuroscience **83**(2): 393-411.

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

Van Sickle, M. D., M. Duncan, P. J. Kingsley, A. Mouihate, P. Urbani, K. Mackie, N. Stella, A. Makriyannis, D. Piomelli, J. S. Davison, L. J. Marnett, V. Di Marzo, Q. J. Pittman, K. D. Patel and K. A. Sharkey (2005). "Identification and functional characterization of brainstem cannabinoid CB2 receptors." Science **310**(5746): 329-32.

Vasquez, C., R. A. Navarro-Polanco, M. Huerta, X. Trujillo, F. Andrade, B. Trujillo-Hernandez and L. Hernandez (2003). "Effects of cannabinoids on endogenous K+ and Ca2+ currents in HEK293 cells." Can J Physiol Pharmacol **81**(5): 436-42.

Wager-Miller, J., R. Westenbroek and K. Mackie (2002). "Dimerization of G-protein-coupled receptors: CB1 cannabinoid receptors as an example." Chem Phys Lipids **121**(1-2): 83-9.

Walsh, D., K. A. Nelson and F. A. Mahmoud (2003). "Established and potential therapeutic applications of cannabinoids in oncology." Support Care Cancer **11**(3): 137-43.

Watson, J. B., P. M. Coulter, 2nd, J. E. Margulies, L. de Lecea, P. E. Danielson, M. G. Erlander and J. G. Sutcliffe (1994). "G protein gamma 7 subunit is selectively expressed in medium-sized neurons and dendrites of the rat neostriatum." J Neurosci Res **39**(1): 108-16.

Wedegaertner, P. B., P. T. Wilson and H. R. Bourne (1995). "Lipid modifications of trimeric G-proteins." J Biol Chem **270**(2): 503-6.

Wiley, J. L., R. L. Barrett, J. Lowe, R. L. Balster and B. R. Martin (1995). "Discriminative stimulus effects of CP 55,940 and structurally dissimilar cannabinoids in rats." Neuropharmacology **34**(6): 669-76.

Wilson, R. I. and R. A. Nicoll (2001). "Endogenous cannabinoids mediate retrograde signalling at hippocampal synapses." Nature **410**(6828): 588-92.

Wlaschin, K. F., P. M. Nissom, L. Gatti Mde, P. F. Ong, S. Arleen, K. S. Tan, A. Rink, B. Cham, K. Wong, M. Yap and W. S. Hu (2005). "EST sequencing for gene discovery in Chinese hamster ovary cells." Biotechnol Bioeng **91**(5): 592-606.

Wolthuis, A., A. Boes and J. Grond (1993). "Cell density modulates growth, extracellular matrix, and protein synthesis of cultured rat mesangial cells." Am J Pathol **143**(4): 1209-19.

Wu, D. F., L. Q. Yang, A. Goschke, R. Stumm, L. O. Brandenburg, Y. J. Liang, V. Hollt and T. Koch (2008). "Role of receptor internalization in the agonist-induced desensitization of cannabinoid type 1 receptors." J Neurochem **104**(4): 1132-43.

Zhuang, S., J. Kittler, E. V. Grigorenko, M. T. Kirby, L. J. Sim, R. E. Hampson, S. R. Childers and S. A. Deadwyler (1998). "Effects of long-term exposure to delta9-

THC on expression of cannabinoid receptor (CB1) mRNA in different rat brain regions." Brain Res Mol Brain Res **62**(2): 141-9.

Vita

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