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Behavioral Assessment and HPLC/MS/MS Identification of the Synthetic Cannabinoid, CP47,497, in Mice

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

by

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Clarification of Contribution

Without the technical and scientific contributions of those listed below, the data presented herein would not have been possible. All data included within this dissertation, aside from that described below, is exclusively my own.

Chapter 3

All samples prepared for HPLC/MS/MS analysis were run in the Pharmacology/Toxicology Mass Spectrometry Core at Virginia Commonwealth University (VCU) by Justin Poklis.

Chapter 4

Dr. Qing Tao (VCU) performed the [^{35}S]-GTPγS binding experiments presented in Figure 4.1. Drug-discrimination studies and data analysis presented in Figure 4.8 were completed by Dr. Bogna Ignatowska-Jankowska (VCU).

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List of Chemical Nomenclature

9-*nor*-9β-Hydroxyhexahydrocannabinol (HHC)

6,6-dimethyl-3-pentyl-6a,7,8,9,10,10a-hexahydrobenzo[c]chromene-1,9-diol

A796-260

1-(2-morpholin-4-ylethyl)-1H-indol-3-yl]-(2,2,3,3 tetramethylcyclopropyl)

methanone

ADB-PINACA

(S)-N-(1-amino-3-methyl-1-oxobutan-2-yl)-1-pentyl-1H-indazole-3-carboxamide

AKB48

1-pentyl-N-tricyclo[3.3.1.13,7]dec-1-yl-1H-indazole-3-carboxamide

AM-2201

[1-(5-fluoropentyl)-1H-indol-3-yl]-1-naphthalenyl-methanone

Cannabidiol (CBD)

2-[1R-3-methyl-6R-(1-methylethenyl)-2-cyclohexen-1-yl]-5-pentyl-1,3-benzenediol

Cannabinol

6,6,9-Trimethyl-3-pentyl-6*H*-benzo[*c*]chromen-1-ol

CP47,497

rel-2-[(1S,3R)-3-hydroxycyclohexyl]-5-(2-methyloctan-2-yl)-phenol

CP47,497-C8

(2-[(1R,3S)-3-hydroxycyclohexyl]- 5-(2-methylnonan-2-yl)phenol)

CP47,497-C8-d7

rel-2-[(1S,3R)-3-hydroxycyclohexyl]-5-(2-methylnonan-6,6,7,7,8,8,8-d_7-2-yl)- phenol

CP47,497-d11

rel-5-(1,1-dimethylheptyl-3,3,4,4,5,5,6,6,7,7,7-d_{11})-2-[(1R,3S)-3-hydroxycyclohexyl]-phenol

CP55,940

rel-5-(1,1-dimethylheptyl)-2-[(1R,2R,5R)-5-hydroxy-2-(3 hydroxypropyl)cyclohexyl]-phenol

Delta-9-tetrahydrocannabinol (**THC)**

6aR,7,8,10aR-tetrahydro-6,6,9-trimethyl-3-pentyl-6H-dibenzo[b,d]pyran-1-ol

JWH-018

(1-pentyl-1H-indol-3-yl)-1-naphthalenyl-methanone

JWH-073

(1-butyl-1H-indol-3-yl)-1-naphthalenyl-methanone

JWH-122

(4-methyl-1-naphthalenyl)(1-pentyl-1H-indol-3-yl)-methanone

JWH-210

(4-ethyl-1-naphthalenyl)(1-pentyl-1H-indol-3-yl)-methanone

JWH-250

1-(1-pentyl-1H-indol-3-yl)-2-(2-methoxyphenyl)-ethanone

JWH-250-d5

1-(1-pentyl-1H-indol-3-yl)-2-(2-methoxyphenyl)-ethanone-2,4,5,6,7-d_5

HU-210

3-(1,1'-dimethylheptyl)-6aR,7,10,10aR-tetrahydro-1-hydroxy-6,6-dimethyl-6H-dibenzo[b,d]pyran-9-methanol

HU-211

3-(1,1-dimethylheptyl)-6aS,7,10,10aS-tetrahydro-1-hydroxy-6,6-dimethyl-6H-dibenzo[b,d]pyran-9-methanol

Pravadoline

(4-methoxyphenyl)[2-methyl]-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-yl]-methanone

RCS-4

(4-methoxyphenyl)(1-pentyl-1H-indol-3-yl)methanone

Rimonabant (SR141617A)

5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-N-1-piperidinyl-1H-pyrazole-3-carboxamide

UR-144

(1-pentyl-1H-indol-3-yl)(2,2,3,3-tetramethylcyclopropyl)-methanone

WIN55,212-2

(+)-[2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-*de*]-1,4-benzoxazin-6-yl]-1napthalenylmethanone

XLR-11

(1-(5-fluoropentyl)-1H-indol-3-yl)(2,2,3,3-tetramethylcyclopropyl)methanone

List of Abbreviations

2-AG	2-arachidonylglycerol
[^{3}H]-CP55,940	tritium labeled rel-5-(1,1-dimethylheptyl)-2-[(1R,2R,5R)-5-hydroxy-2-(3 hydroxypropyl)cyclohexyl]-phenol
GTPγS	guanosine 5'-O-[thio-gamma]-triphosphate
%MPE	maximum percent effect
AC	adenylyl cyclase
AEA	anandamide
AKI	acute kidney injury
ANOVA	analysis of variance
cAMP	cyclic adenosine monophosphate
CB1	cannabinoid receptor type 1
CB2	cannabinoid receptor type 2
CHS	cannabinoid hyperemesis syndrome
CP47	CP47,497
CP55	CP55,940
CNS	central nervous system
CV	coefficient of variation
CYP450	superfamily of cytochrome monooxygenase

DSE	depolarization-induced suppression of excitation
DQC	dilution quality control
DSI	depolarization-induced suppression of inhibition
EC	endocannabinoid
ED_{50}	half maximal (50%) effective dose
EPSC	excitatory postsynaptic currents
FAAH	fatty-acid amide hydrolase
GC/MS	gas chromatography mass spectrometry
GIRK	G-protein inwardly rectifying potassium channel
G-protein	guanine nucleotide binding protein
GPCR	G-protein coupled receptor
GTP	guanosine triphosphate
HIP	herbal incense product
HPLC/MS/MS	high performance liquid chromatography tandem mass spectrometry
HQC	high quality control
i.p.	intraperitoneal
IPSC	inhibitory postsynaptic currents
ISTD	internal standard
KO	knockout

MAGL	monoacylglycerol lipase
MAPK	mitogen activated protein kinase(s)
MI	myocardial infarction
LLE	liquid-liquid extraction
LOQ	limit of quantitation
LQC	low quality control
LSD	lysergic acid diethylamide
MPE	maximum percent effect
MQC	medium quality control
PKA	protein kinase A
Rim	rimonabant (SR141617A)
SAR	structure activity relationship
SCB	synthetic cannabinoid
THC	delta-9-tetrahydrocannabinol
TLC	thin layer chromatography
VEH	vehicle
WT	wild type

Abstract

BEHAVIORAL ASSESSMENT AND HPLC/MS/MS IDENTIFICATION OF THE ABUSED SYNTHETIC CANNABINOID, CP47,497, IN MICE

By Kimberly Lynne Samano, M.S.

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

Virginia Commonwealth University, 2014.

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CP47,497 and other synthetic cannabinoid compounds were incipiently synthesized as research tools to investigate the mechanisms by which marijuana affects the brain and to aid in the development of therapeutic agents. Recently, these cannabinoid compounds have resurfaced in the designer drug market, marketed as “herbal incense products” (HIPs). Their popular use has resulted in an alarming rate of reported adverse effects and toxicities. Current legislation classified CP47,497 and several other synthetic

cannabinoids compounds as Schedule I agents, but abuse of these compounds persists with serious consequences to public health and safety. *In vivo* studies examining the behavioral consequences of abused synthetic cannabinoids are limited. As a result, the goals of this research were to elucidate the acute and chronic pharmacological effects of CP47,497 and to develop a bioanalytical method for CP47,497 drug detection in mice. Cannabimimetic effects were evaluated in well-established *in vivo* models, the tetrad paradigm and drug discrimination assay. The tetrad test is comprised of four outcome measures sensitive to the primary psychoactive cannabinoid present in marijuana, delta-9-tetrahydrocannabinol (THC): catalepsy (bar test), antinociception (tail withdrawal latency), hypothermia, and decreases in spontaneous locomotor activity. While many pharmacological agents can produce one or a subset of these tetrad effects, drugs that activate CB_1 receptors produce characteristic effects in all four parameters.

An HPLC/MS/MS method was developed and confirmed the presence of CP47,497 in brain. We investigated whether CB_1 receptors mediate the pharmacological effects of CP47,497. Cumulative dose-response experiments determined CP47,497 is more potent than THC *in vivo* in using multiple behavioral assays. Complementary pharmacological (CB_1 receptor antagonist, rimonabant) and genetic ($CB_1^{(-/-)}$ mice) approaches were used to investigate whether CB_1 receptors mediate the effects of CP47,497. Rimonabant (3 mg/kg or 10 mg/kg, depending on

independent measure) blocked all cannabinoid-like pharmacological effects of CP47,497. Supporting these findings, $CB_1^{(-/-)}$ mice were resistant to cannabimimetic effects of CP47,497. CP47,497 fully substituted for THC in the drug discrimination assay, with a potency of more than 5 times that of THC. Collectively, these results indicate that CP47,497 is markedly more potent (i.e. 5-8 fold) than THC, and its repeated administration produces tolerance to the cataleptic, antinociceptive, hypothermic and hypolocomotor effects in mice, with significant presentation of somatic withdrawal signs (paw flutter and head shakes) upon drug cessation. These findings are consistent with the high incidence of adverse events in humans abusing synthetic cannabinoids.

Chapter 1 – The Endogenous Cannabinoid System

1.1 Discovery and Isolation of THC

Cannabis sativa has been used for recreational and medicinal purposes for centuries (Mikuriya 1969; Schultes 1969) and marijuana is still today the most widely abused illicit drug worldwide (UNDOC 2013). Despite its prevalent use, most of our current knowledge about marijuana's effects in humans, and more specifically of delta-9-tetrahydrocannabinol (THC), has been largely elucidated over the last several decades. In the early 1930's, the structure of THC was first isolated in the laboratory of R.S. Cahn, and its subsequent synthesis occurred in 1940 by Adams *et al.* in the United States and Jacob *et al.* in Europe (Adams et al. 1940; Jacob & Todd 1940; Pertwee 2006). More than twenty years passed before Gaoni and Mechoulam identified THC as the major active constituent of hashish and published the first detailed description of its isolation, and confirmed the absolute structural configuration of THC including its stereospecific synthesis (Mechoulam et al. 1967; Mechoulam & Gaoni 1967). The isolation and synthesis of THC was a scientific breakthrough, and as a result, structure-activity relationship (SAR) studies, discussed further in Chapter 2, were performed to dissect which THC functional groups were important for binding and responsible for its biological activity.

These SAR studies were foundational to the exploration of the endocannabinoid (EC) system as they provided pharmacological tools that enabled the identification and cloning of cannabinoid receptors, discovery of endogenous cannabinoids, and a thorough examination of their physiological effect on neurotransmission in the central nervous system. Unfortunately, this research that allowed these important discoveries to be made has been commandeered in the illicit marketing of synthetic cannabinoid (SCB) compounds as described in Chapter 2. Synthetic cannabinoids were exclusively developed for research purposes and within the last several years have been falsely advertised as “natural” plant-derived components in herbal incense products (HIPs), most often called “K2” and “Spice”. While these “legal high” alternatives to marijuana were originally a misnomer, their chemical identification did not deter human users. More than 6,000 synthetic cannabinoid exposures were reported to poison control centers in 2011, versus less than 20 incidents in 2009 (Wood 2013). Consequently, abuse of SCB’s has dramatically escalated in the United States and Europe; reported toxicity and mortality make their consumption a significant public health concern.

1.2 Identification of Cannabinoid Receptors

Early experiments were aimed at understanding how THC elicits its actions on the central nervous system (CNS). Since cannabinoid compounds were unlike nitrogen-containing drugs of abuse (LSD, psilocybin), but similar to anesthetic compounds in

their lipophilicity profiles, it was hypothesized that the pharmacological effects of THC were due to ordered perturbations of lipid membranes (Lawrence & Gill 1975; Martin 1986). In one study, THC disordered cholesterol/lecithin lipid bilayers in a qualitative manner similar to a sub-effective dose of an anesthetic agent; therefore, it was believed THC and other cannabinoids could act as partial anesthetics (Lawrence & Gill 1975). Later studies interrogated the relationship between lipophilicity and *in vivo* pharmacological activity and concluded that while behaviorally active compounds exhibited high lipophilicity, as evidenced by water/octanol partition coefficients, there was no clear correlation between lipophilicity and behavioral potency (Thomas et al. 1990). Important physiochemical information was provided by this work, even though these experiments were unable to definitively characterize the pharmacological action of cannabinoids.

As a result, a concerted effort to identify the receptor responsible for cannabinoid actions included studies that investigated the stereoselectivity of cannabinoid effects using *in vitro* and *in vivo* approaches. Harris *et al.* provided initial evidence for the presence of specific cannabinoid binding sites that are displaceable and potentially stereospecific in cultured hepatoma cells and rat brain homogenates (Harris et al. 1978). Using neuroblastoma cells, Howlett *et al.* disproved the hypothesis that the stereoselective and specific inhibition of adenylate cyclase (AC) by psychoactive

cannabinoids was mediated by secretin, α-adrenergic, and muscarinic cholinergic receptors (Howlett & Fleming 1984). The effects of cannabinoids on AC inhibition were shown to require functional Gi-proteins as ADP-ribosylation of Gi-protein subunits by *pertussis toxin* coincided with cannabimimetic (of, or pertaining to THC-like pharmacological effects (Weissman et al. 1982)) inhibition of AC (Howlett et al. 1985). Subsequent studies postulated that the same receptor responsible for *in vitro* effects of AC inhibition by cannabimimetic compounds was also responsible for mediating analgesic effects across several pain assays *in vivo* (Howlett et al. 1987). Cannabinoid-induced behavioral effects by synthetic cannabinoid compounds levonantradol and CP55,940 were potent and stereoselective versus their respective enantiomers (dextronantradol and CP 56,677) (Little et al. 1988). Taken together, these findings provided strong pharmacological evidence that non-classical cannabinoid compounds' actions were receptor-mediated, thus, research around this time was published on the identification and characterization of a cannabinoid receptor (Devane et al. 1988). A series of elegant binding studies in rat membranes and synaptosomes were optimized and definitively illustrated displacement of [^{3}H]CP55,940 binding by THC, 11-OH-THC, cannabinol, and CP55,940 (Devane et al. 1988). Importantly, the data indicated this effect was saturable, rapid, reversible, and determined a single class of binding sites (Devane et al. 1988). Moreover, this work demonstrated using a non-hydrolyzable guanosine triphosphate (GTP) analog that [^{3}H]CP55,940 binding was affected in a

manner consistent with G-protein activation. Specificity of this binding interaction was observed using plant-derived (THC, 11-OH-THC) and synthetic cannabinoid compounds which each successfully displaced [^{3}H]CP55,940 from receptor binding in a concentration-dependent fashion, and inhibited adenylyl cyclase in a potent, reversible and stereoselective manner (Devane et al. 1988).

1.3 Cloning of Cannabinoid Receptors

In 1990, Matsuda and colleagues published the successful cloning of rat cerebral cDNA (complimentary DNA), 473 amino acids in length, and this sequence was found to belong to a G-protein-coupled receptor (GPCR) family identified as the cannabinoid receptor type 1 (CB_1). The human isoform (472 amino acids) which shares approximately 97% sequence homology to the cloned rat receptor was subsequently identified (Gerard et al. 1991). Furthermore, Chinese hamster ovary (CHO) K1 cells stably transfected with either the rat or human cloned receptor inhibited forskolin-stimulated accumulation of cAMP with THC and CP55,940 in a concentration-dependent and stereoselective fashion; inhibition of cAMP was observed with pertussis toxin, indicating Gi-protein signaling treatment (Matsuda et al. 1990; Gerard et al. 1991). *In situ* hybridization studies in rats revealed strong CB_1R mRNA (messenger RNA)

signal in cerebellum, hippocampus, amygdala, and hypothalamus and basal ganglia/striatum regions that correlate to known psychotropic cannabinoid actions on memory, cognition and complex motor behaviors (Matsuda et al. 1993).

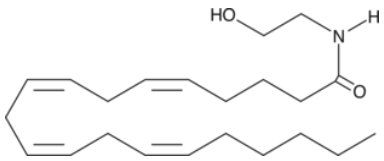
Since CB_1 receptors are expressed at high levels in the CNS, the non-psychoactive effects of cannabinoids were thought to be mediated by cannabinoid receptors in the periphery or indirectly by non-cannabinoid receptors. To test this hypothesis, Munro and colleagues used a human promyelocytic leukemia HL-60 cell cDNA library to screen for a peripheral cannabinoid receptor and identified what would later be termed cannabinoid receptor type 2 (CB_2). They reported a 360 amino acid sequence, demonstrating 44% sequence homology with the CB_1R, present in cells of leukocyte lineage including macrophage/monocytes, B-lymphocytes, NK cells and immune tissue (tonsils, spleen and lymph nodes). These findings suggested that in contrast to the CNS-mediated effects of CB_1, the CB_2 receptor was crucial in immune system regulation and inflammation (Munro et al. 1993). As we are interested in investigating the psychoactive effects of cannabinoids at CB_1 receptors, this document will focus primarily on actions at the CB_1 receptor.

1.4 Endogenous Cannabinoid Ligands: Anandamide and 2-Arachidonylglycerol

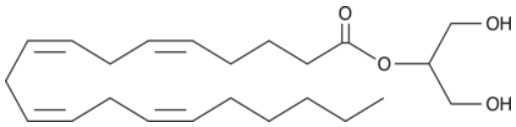
Since receptor-mediated actions of cannabinoids were stereospecific and selective depending on ligand, researchers predicted the existence of an endogenous cannabinoid compound. Shortly after cannabinoid receptors were cloned, the first endogenous cannabinoid ligand, anandamide (*N*-arachidonyl-ethanolamine, AEA), was isolated by Devane *et al*. in 1992. AEA (Figure 1) was separated from pig brain tissue fractions and purified by thin-layer chromatography (TLC), high performance liquid chromatography (HPLC), and gas-chromatography-mass spectrometry (GC/MS) methods (Devane et al. 1992). Binding of this endogenous cannabinoid was confirmed through the use of radioligand cannabinoid assays in which AEA to displaced [^{3}H]HU-243 from CB_1 receptors in the nanomolar range (Ki = 52 nM) (Devane et al. 1992). AEA behaved as a partial agonist at CB_1 receptors with varying efficacy dependent on the system, and acts as a weak partial agonist at CB_2 receptors (Felder et al. 1993). Biological cannabinoid activity of AEA was illustrated using vas deferens preparations, as AEA inhibited the electrically evoked mouse twitch response *ex vivo;* furthermore AEA produced decreases in spontaneous activity, induction of immobility, hypothermia and analgesia in mice (Devane et al. 1992; Smith et al. 1994).

Studies conducted by Di Marzo *et. al.* described the release of AEA and related *N*-acyl-ethanolamine species from cultured rat neurons after stimulation of intracellular Ca^{+2} with ionomycin using an [^{3}H]ethanolamine tracer that led to the elucidation of

AEA's structure by ^{1}H-NMR (nuclear magnetic resonance). These experiments supported AEA's *de novo* formation via a phosphodiesterase-mediated cleavage of an unidentified phospholipid precursor, *N*-arachidonyl-phosphatidylethanolamine (Di Marzo et al. 1994). Further work demonstrated AEA is synthesized via two pathways: directly by the *N*-acylation of ethanolamine or indirectly by phospholipase D-mediated synthesis (Di Marzo et al. 1994; Di Marzo 1998; Schmid et al. 1990). Degradation of AEA is achieved by the enzyme fatty acid-amide hydrolase (FAAH) to ethanolamine and arachidonic acid (Piomelli et al. 1998; Di Marzo 1998; Cravatt et al. 1996). Genetically modified mice which lack the catabolic FAAH enzyme show increased levels of AEA in brain accompanied by potent and hypersensitive CB_1 mediated behavioral effects (Cravatt et al. 2001).



Arachidonyl ethanolamide
(Anadamide)



2-Arachidonyl glycerol
(2-AG)

Figure 1.1 – Structure of endogenous cannabinoid compounds anandamide and 2-AG.

The discovery of a second endocannabinoid, 2-arachidonylglycerol (2-AG), was identified by GC/MS, with its structure (Figure 1) elucidated using NMR (Mechoulam et

al. 1995; Stella et al. 1997; Sugiura et al. 1995). Although 2-AG was first isolated from canine gut, it was later found in brain at higher levels than AEA (Mechoulam et al. 1995; Sugiura et al. 1995). In contrast to AEA, 2-AG acts as a full agonist at both CB_1 and CB_2 receptors. 2-AG competed with [^{3}H]HU-243 binding at the CB_1R with a Ki value of 472 ± 55 nM, whereas AEA exhibited a Ki value of 252 ± 47 nM. Effects of CB_1 activity were examined using *in vitro* and *in vivo* methods. 2-AG (IC_{50} = 4.8 μM) less potently inhibited electrically evoked twitch response in mouse vas deferens than AEA (IC_{50} = 52 nM) (Mechoulam et al. 1995). In mice, exogenous administration of 2-AG elicited classical CB_1-mediated behaviors (hypomotility, hypothermia, antinociception, catalepsy) equipotent to AEA but less potent than THC. The modest *in vivo* effects of 2-AG are likely due to its rapid degradation in animals (Willoughby et al. 1997; Vyvoda & Rowe 1973).

Cellular 2-AG rapidly increases upon stimulation from arachidonic acid rich membranes and is released on demand, which strongly argues it plays a pivotal role as a mediator of intracellular signaling. 2-AG is synthesized by digestion of phosphatidylinositol by phospholipase C (PLP C) into diacylglycerol (DAG) that is further metabolized by diacylglycerol lipase (DAGL). Another metabolic pathway includes degradation of phosphatidylinositol by phospholipase-A1 into lysophophatidlyinositol which is converted by PLC into 2-AG (Piomelli et al. 1998; Prescott & Majerus 1983;

Stella et al. 1997; Sugiura et al. 1995). Although FAAH is capable of hydrolyzing 2-AG to some extent *in vitro*, studies with FAAH (-/-) mice showed no significant difference in hydrolysis rates of 2-AG when compared to littermates which illustrates another enzyme is principally responsible for 2-AG degradation (Lichtman et al. 2002). A sophisticated series of experiments by Dinh *et al.* demonstrated 2-AG is metabolized into glycerol and free fatty acid by monoacylglycerol lipase (MAGL). They reported high expression of MAGL mRNA within the brain, specifically in regions containing high cannabinoid receptor density (cortex, hippocampus, cerebellum). Furthermore, overexpression of MAGL in cortical neurons by adenovirus delivery abolished receptor-stimulated production of 2-AG, but did not affect 2-AG synthesis nor AEA accumulation, indicating it selectively degraded 2-AG (Dinh et al. 2002).

1.5 Cannabinoid Receptor Activation and Signaling

An agonist is a drug that activates receptors in some measurable way, which is dependent on the assay; a full agonist elicits a maximal effect within set experimental parameters whereas a partial agonist (at full receptor occupancy) produces a detectable, but submaximal effect (Negus 2006). Furthermore, agonists possess variable intrinsic efficacies, which underlie differential effects of G-protein activation upon ligand binding

to GPCRs (Selley et al. 1997). Coupling of cannabinoid receptors to inhibitory Gi/o-proteins in rat membranes and whole brain using [^{35}S]-GTPγS autoradiography studies have demonstrated THC binds to CB_1 receptors as a low efficacy partial agonist, AEA behaves similar as a partial agonist, whereas 2-AG, WIN55,212-2, and CP55,940 act as full efficacy agonists (Breivogel et al. 1997; Breivogel & Childers 1998; Burkey, Quock, Consroe, Roeske, et al. 1997; Burkey, Quock, Consroe, Ehlert, et al. 1997; Selley et al. 1996; Sim et al. 1996).

Binding and subsequent stimulation of CB_1 receptors to Gi/o-proteins through endogenous or exogenous cannabinoid application inhibits adenylyl cyclase, and thus decreases cAMP (cyclic adenosine monophosphate) in a pertussis toxin sensitive manner (Felder et al. 1995; Howlett & Fleming 1984; Howlett 1985; Howlett et al. 1985). Cannabinoid receptor activation also promotes cyclic AMP-dependent phosphorylation events including activation of protein kinase A (PKA) activation resulting in modulation of potassium channels (Hampson et al. 1995; Mu et al. 2000). The resultant dissociation of Gi-proteins after CB_1 activation produces decreases in Ca^{+2} conductance of N-type voltage-gated ion channels (Caulfield & Brown 1992; Felder et al. 1993; Mackie et al. 1993; Pan et al. 1996), increases K^+ conductance of G-protein inwardly-rectifying (GIRK) and A-type channels (Deadwyler et al. 1995; Henry & Chavkin 1995; Mackie et al. 1995; Mu et al. 1999) and activates MAP kinase cascades (Bouaboula et al. 1995;

Wartmann et al. 1995). Cannabinoid-mediated activation of the MAP kinase pathway allows regulation of various cellular functions including cell growth and death through phosphorylation of nuclear proteins and activation of early immediate genes (Bouaboula et al. 1995), which shapes neuroplasticity within the endocannabinoid system.

The activation of CB_1 and subsequent fluctuations in intracellular Ca^{+2} concentrations results in events known as depolarization-induced suppression of inhibition (DSI) or excitation (DSE). These phenomena were discovered by Alger *et al.* and Llano *et al.*, who demonstrated through electrophysiological experiments that depolarization of cerebellar and hippocampal neurons produced a transient suppression of GABAergic synaptic input; and coined the term, “depolarization-induced suppression of inhibition”. Their studies demonstrated that an increase in intracellular Ca^{+2} is necessary for depolarization and that decreases of inhibitory post-synaptic current (IPSC) frequency are observed with no changes in peak magnitude. Additionally, recordings from neurons exposed to exogenous GABA did not induce DSI, which supports a presynaptic origin of signaling (Alger & Pitler 1995; Alger et al. 1996; Llano et al. 1991; Pitler & Alger 1992; Pitler & Alger 1994). Evidence was provided in 1994, by Pitler and Alger that this presynaptic phenomenon is Gi- or Go-coupled as DSI was nearly abolished in hippocampal slices from rats treated with pertussis toxin (Pitler & Alger 1994). These observations lead scientists to probe the involvement of the

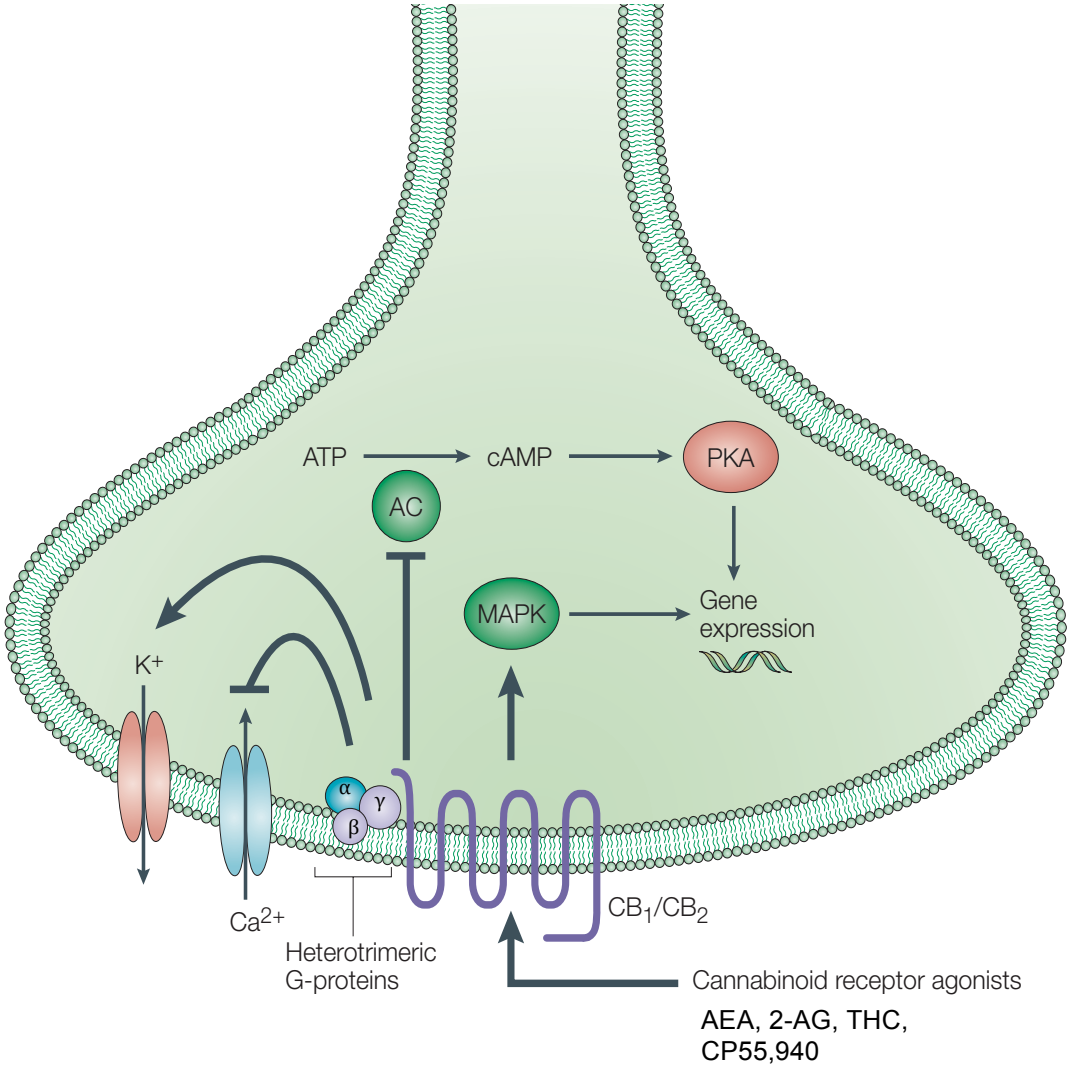


Figure 1.2 - **Schematic of Cannabinoid Receptor Activation and Signaling**. CB_1 and CB_2 receptor activation by endogenous, phytocannabinoid and synthetic cannabinoid agonists stimulate Gi/o heterotrimeric proteins; thus, inhibition of adenylyl cyclase (AC) prevents phosphorylation events of protein kinase A (PKA) and mitogen-activated protein kinase (MAPK) pathways. Dissociation of Gi/o-α subunit from Gβγ-complex elicits inhibition of voltage-gated Ca^{2+} channels and activation of inwardly-rectifying K^+ channels that results in the inhibition of excitatory and inhibitory neurotransmitter release in nerve cells. Image modified from (Di Marzo et al. 2004).

endocannabinoid system in this mechanism and, in 2001, three separate research groups made the exciting scientific discovery that the retrograde signaling molecule responsible for DSI was an endocannabinoid (Kreitzer & Regehr 2001; Ohno-Shosaku et al. 2001; Wilson & Nicoll 2001).

Wilson and Nicoll's work demonstrated that selective CB_1 antagonist drugs, SR141617A and AM251, both significantly attenuated the magnitude of DSI, whereas WIN55,212-2 recapitulated the effect of DSI on inhibitory transmission. Further studies illustrated that unlike classical neurotransmitter release, the release of this retrograde signal did not require vesicle fusion; the effect of Ca^{+2} influx via depolarization step or by liberation of Ca^{+2} from a photoliable chelator produced DSI; and finally, treatment with a metabotropic glutamate receptor (mGluR) antagonist did not affect DSI (Wilson & Nicoll 2001). Ohno-Shosaku *et al.* (2001) used synthetic cannabinoid agonists and antagonists that confirmed the presynaptic nature of DSI. Their work extended findings of Wilson *et al.* that a selective $GABA_B$ antagonist in addition to an mGluR antagonist were ineffective in eliciting DSI (Ohno-Shosaku et al. 2001). Concomitantly, Kreitzer *et al.* reported that depolarization-induced suppression of excitation (DSE) in glutamatergic excitatory synapses was endocannabinoid dependent. Experiments illustrated DSE occurs in purkinje cells after an inhibition of presynaptic calcium influx; and this is prevented by AM251 pretreatment, and occluded with WIN55,212-2 treatment (Kreitzer

& Regehr 2001). Taken together, this body of work provides scientifically sound evidence that DSI/DSE results from endocannabinoid production in postsynaptic neurons, which diffuse in a retrograde manner and bind to presynaptic cannabinoid receptors resulting in a suppression of GABA or glutamate release, respectively.

Studies performed at single-cell resolution utilizing double *in situ* hybridization of CB_1 mRNA and neurotransmitter mRNA demonstrate that CB_1 expressing cells within mouse forebrain regions exhibit clear subpopulation divisions; for example, cells expressing high mRNA levels of CB_1 are co-localized on GABAergic interneurons, whereas low, but physiologically relevant, expression of CB_1 mRNA is detected on non-GABAergic cells, considered to be principal projection neurons, in regions of the hippocampus, amygdala, entorhinal cortex (Marsicano & Lutz 1999). In contrast, activation of cannabinoid receptors in lateral amygdala can decrease glutamatergic as well as GABAergic neurotransmission, as WIN55,212-2 decreased the amplitude of AMPA-evoked EPSCs and IPSCs in $CB1^{(+/+)}$ mice, but not in $CB1^{(-/-)}$ mice (Azad et al. 2003). Studies have shown FAAH and MAGL are distinct in their subcellular loci, with FAAH located in presynaptic somato-dendritic compartments of the cerebellum and MAGL preferentially located postsynaptically in axons, which further supports a mechanism for 2-AG in retrograde signaling (Gulyas et al. 2004).

1.6 Cannabinoid Receptor Localization

CB_1 receptors are the most highly expressed GPCRs in the brain (Devane et al. 1988; Herkenham et al. 1991) and their localization was first described quantitatively in 1991 by Herkenham *et al.* using *in vitro* autoradiography with [H^3]-CP55,940. Experiments demonstrated coupling of CB_1 receptors with guanine nucleotide regulatory proteins in a concentration-dependent manner using nonhydrolyzable analogs of GTP. Anatomical distribution of receptor binding was observed in brain regions primarily responsible for motor function and cognition (Herkenham et al. 1991).

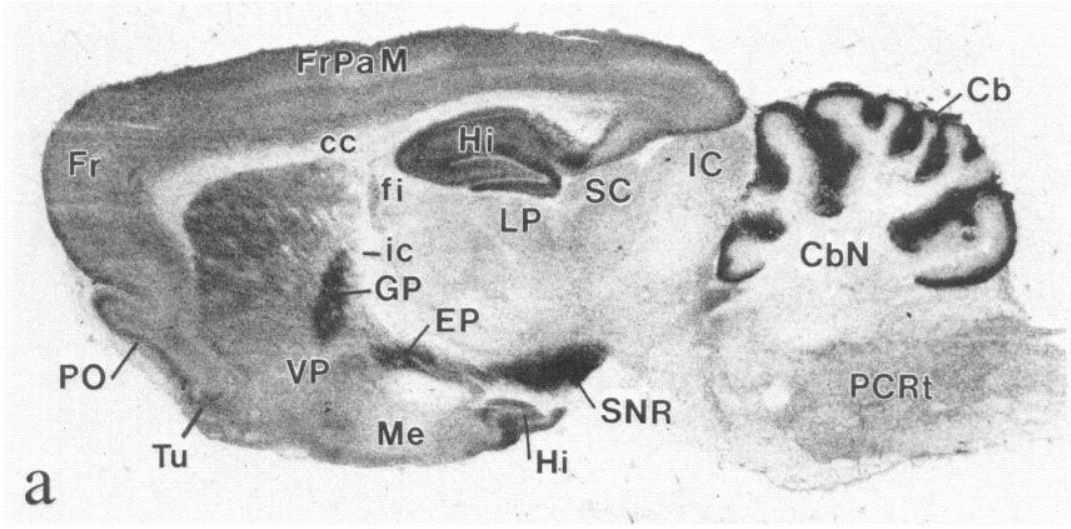


Figure 1.3 - Distribution of cannabinoid receptors in sagittal section of rat brain using [^{3}H]-CP55,940 autoradiography; receptor distribution is densest in regions of frontal cortex (Fr/FrPaM), hippocampus (Hi), cerebellum (Cb), and output nuclei of the basal ganglia (GP, EP, SNR); image from Herkenham et al. 1991.

Comprehensive evaluation of CB_1 receptor localization using mRNA expression and immunohistochemical approaches in rats extended earlier findings that CB_1 receptor populations are most dense in basal ganglia nuclei, substantia nigra (pars reticulate), and globus pallidus with high binding reported in the molecular layer of the cerebellum, hippocampus and dentate gyrus (Matsuda et al. 1993; Tsou et al. 1998). Investigation of fetal and adult human cannabinoid receptor distribution supports a similar CNS localization and distribution (Glass et al. 1997; Biegon & Kerman 2001).

Studies have identified cannabinoid receptor immunolabeling on most neuronal cell types which involves inhibitory and excitatory neuromodulation (Moldrich & Wenger 2000). For instance, the EC system has been implicated in learning/memory and emotion/motivation via presynaptic endocannabinoid release in cholecystokinin-positive GABAergic nerve terminals within hippocampal and amygdala brain areas (Katona et al. 1999; Katona et al. 2001). Additionally, bath application of WIN55,212-2 or CP55,940 produced a reduction in glutamatergic neurotransmission in nucleus accumbens of mice, a brain region implicated in both natural and drug associated rewards (Robbe et al. 2001). Modulation of excitatory and inhibitory neurotransmitter release by endocannabinoids through DSI and DSE mechanisms are well documented, as described above (Pitler & Alger 1992; Chevaleyre et al. 2006; Hermann et al. 2002;

Ohno-Shosaku et al. 2001; Wilson & Nicoll 2001). Taken together, these data describe several mechanisms by which the endocannabinoid system interacts with other neuronal receptor systems within the CNS; this neuronal modulation can be transient in nature or have long-term consequences on learning, memory, and habitual drug use.

1.7 Consequences of Acute and Chronic Cannabinoid Administration in Rodents

While continued exploration of the endocannabinoid system posits discovery of potential therapeutic applications, the clinical use of cannabinoids has largely been dismissed due to their undesirable side effects, including THC-like subjective high, abuse liability, and potential for tolerance development upon repeated administration. In humans THC affects mood and perception, delayed internal clock, memory impairment, anxiety, reward, and motor impairment and are consistent with associated function of brain regions expressing CB_1 receptors (Casswell & Marks 1973; Herkenham et al. 1991; Karniol & Carlini 1973).

Tetrad and Drug Discrimination Behavioral Assays

While it is difficult to measure affective components (perception, motivation, reward) resulting from THC use in laboratory animals, preclinical evaluation of putative cannabimimetic drugs can be easily accomplished. Administration of cannabinoids in rodents elicits a characteristic profile of behavioral effects called the “tetrad” including

catalepsy, antinociception, hypothermia, and hypomotility, which permits screening of putative CB_1 compounds (Martin et al. 1991). Tetrad outcome measures are sensitive to the primary psychoactive cannabinoid present in marijuana, THC, and have been extensively correlated to CB_1 receptor binding and activation (Compton et al. 1996; Little et al. 1988; Martin et al. 1991; Monory et al. 2007; Pertwee 2008; Smith et al. 1994; Wiley 2003). While many pharmacological agents can produce one or a subset of these tetrad effects, drugs that activate CB_1 receptors produce measurable effects in all four parameters (Wiley 2003). Moreover, these behaviors are prevented by the CB_1R antagonist, rimonabant (SR141617A), and in $CB_1^{(-/-)}$ mice, which supports a CB_1-mediated mechanism *in vivo* (Compton et al. 1996; Ledent et al. 1999; Monory et al. 2007; Rinaldi-Carmona et al. 1994; Rinaldi-Carmona et al. 1995; Zimmer et al. 1999). For decades, the tetrad has proven to be a rapid, reliable and reproducible screening tool to assess cannabimimetic effects, thus, it has been used extensively throughout this dissertation research.

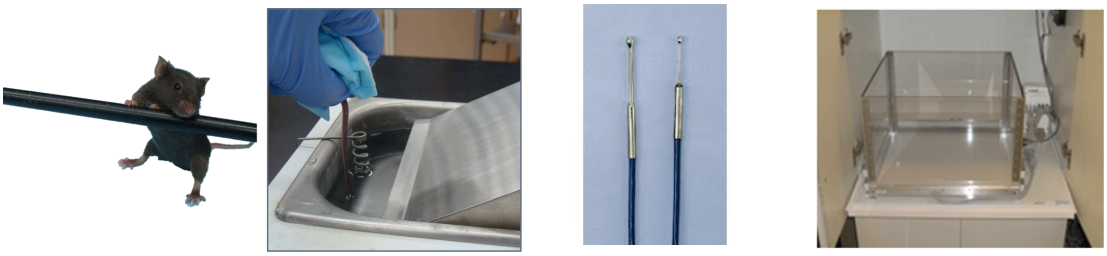


Figure 1.4 - **Behavioral Test Components of the Tetrad**. Catalepsy assessed with the bar test, antinociception measured by tail immersion in a 52.0 °C warm water bath, hypothermia determined used a rectal thermometer probe, and locomotor activity is monitored in activity chambers.

Examination of the endocannabinoid system, particularly in regards to the anatomical localization and activation of cannabinoid receptors, has corroborated those behavioral effects of THC with CB_1R activation in the brain. For example, functional studies in the rat indicate that the acute activation of brain regions after THC exposure, measured indirectly by glucose metabolism, correspond closely with temporally observed physiologic and behavioral effects (Whitlow et al. 2002). Additionally, THC administration produced dose-dependent effects in cerebral glucose metabolism, an effect that can be attenuated by rimonabant pretreatment (Freedland et al. 2002). Both dose- and time-dependent effects were regionally specific (Freedland et al. 2002; Whitlow et al. 2002), which supports the unique behavioral profile observed after cannabinoid administration.

Furthermore, a positron emission tomography (PET) study using the dopamine (D_2/D_3) receptor tracer [^{14}C]-raclopride, illustrated that THC inhalation exposure in seven healthy volunteers resulted in significant release of dopamine in the striatum, demonstrating activation of the mesolimbic reward pathway in humans (Bossong et al. 2009). This reward pathway projects from the ventral tegmental area to the nucleus accumbens and is a circuit involved in recognition of natural reward (i.e. food and sex); however, cannabinoids can activate this pathway and stimulate release of dopamine, which is thought to underlie the reinforcing and rewarding properties of known drugs of abuse (Oleson & Cheer 2012; Solinas et al. 2008). Experiments utilizing *in situ* hybridization in mouse forebrain demonstrate colocalization of CB_1 receptors with dopamine (D1 and D2) and serotonin (5-HT1B and 5-HT3) receptors (Hermann et al. 2002). A myriad of studies, including those utilizing $CB_1^{(-/-)}$ animals, have documented the functional interaction of the cannabinoid system with GABAergic, gluatamatergic, and dopaminergic neurotransmitter systems among others (Azad et al. 2003; Gardner 2005; Hungund et al. 2003; Katona et al. 1999; Katona et al. 2001; Kreitzer & Regehr 2001; Mascia et al. 1999; Monory et al. 2007; Ohno-Shosaku et al. 2001; Tanda & Goldberg 2003; Wilson & Nicoll 2001). Fundamentally, modulations of dopaminergic signaling in the mesolimbic pathway are believed to contribute to both the rewarding effects of THC as well as to the development of physical dependence seen with chronic exposure (Wise & Koob 2014; Solinas et al. 2008; Tanda & Goldberg 2003).

One approach to measuring a drug's "rewarding/reinforcing" effects is through self-administration studies. Whereas reinforcement can be positive (food) or negative (foot shock) in nature, a reward generally refers to positive reinforcement of a given behavior, as seen with repeated drug taking (Panlilio et al. 2010; Solinas et al. 2006; Solinas et al. 2008; Tanda & Goldberg 2003). In humans, continuous drug taking correlates with drug reward (Wise & Koob 2014), but THC self-administration studies in rodents have not been met with success (Gardner 2005; Justinova et al. 2003). However, drug naïve mice will self-administer synthetic cannabinoid agonists, HU-210, CP55,940, and WIN55,212-2 (Martellotta et al. 1998; Navarro et al. 2001). Interestingly, THC effects are species- dependent as squirrel monkeys have been reported to self-administer THC (Justinova et al. 2003; Tanda et al. 2000); which is blocked by CB_1R antagonist rimonabant (Tanda et al. 2000).

As laboratory animals do not readily self-administer THC, the drug discrimination paradigm serves as a useful tool for investigating drugs of abuse in preclinical research settings (Justinova et al. 2005). In humans, marijuana or THC exposure produces a unique profile of subjective (interoceptive) affects that including drug liking, pleasure and craving, and are often used as an indirect measure of a drugs reinforcing effect. Drug-discrimination procedures can be used to evaluate the presence or absence of interoceptive stimulus effects of drugs in animal models, and experiments have

established that rodents will readily learn to discriminate cannabinoids using a two-lever paradigm (Justinova et al. 2005; Wiley 1999). As an alternative to self-administration, drug discrimination serves as a pharmacologically selective behavioral model to infer subjective effects of drugs in rodents. Once trained, animals make an operant response (e.g. lever press or nose poke) to distinguish the discriminative cues between a training drug (e.g. THC) and a test compound (e.g. novel cannabinoid). The extent to which the test drug substitutes for the training drug provides insight into the interoceptive effects of novel drugs (Wiley 1999).

It has been well documented that pigeons, rats, and monkeys discriminate THC from vehicle; and in general, drugs that bind to CB_1 receptors substitute for THC. Conversely, drugs that do not bind to CB_1 receptors do not exhibit similar discriminative stimulus properties to THC, which has been demonstrated with cannabinol, CNS stimulants and depressants, and morphine among various other compounds examined (Järbe & Henriksson 1974; Wiley et al. 1995). An exception is seen with (+)-methamphetamine and diazepam which exhibit partial substitution in rats trained to discriminate 3.0 mg/kg THC from vehicle (Barrett et al. 1995). Crucially, experiments have demonstrated cannabinoid discriminative effects are prevented by pretreatment with rimonabant (Wiley et al. 1995). Other cannabinoids used to serve as the discriminative cue in mouse discrimination studies include CP55,940 and WIN55,212-2

(McMahon et al. 2008). Even though AEA is a partial agonist similar to THC, studies using AEA or its stable analog (methanandamide, MAEA) do not fully substitute for THC (Wiley, Matthew Walentiny, et al. 2011). This discrepancy can be explained by the rapid metabolism of AEA to arachidonic acid by FAAH in comparison to THC (Deutsch, DG & Chin 1993). Bolstering this hypothesis, a recent study reported FAAH $^{(-/-)}$ mice successfully discriminated between AEA and vehicle in a dose-dependent fashion (Walentiny et al. 2011). Rimonabant antagonized the discriminative cue of AEA, which implicates a CB_1 mediated receptor mechanism. Finally, THC fully substituted for AEA, which suggests these two share similar stimulus properties through a CB_1 receptor mechanism of action.

Tolerance

Due to the desired subjective "high" effects, people use marijuana on a continual basis; thus, repeated administration studies in animals are important to assess long-term drug effects that better resemble drug abuse in humans. Chronic cannabinoid exposure leads to tolerance of acute effects including locomotion (Abood et al. 1993; Oviedo et al. 1993), hypothermia (Pertwee et al. 1993; Fan et al. 1996), analgesia (Adams & Martin 1996) and memory disruption (Deadwyler et al. 1995; Wise et al. 2011).

In addition to behavioral tolerance, adaptations at the molecular level have been examined. In post-mortem human tissue, chronic use (defined in the study as daily use for multiple years) of marijuana elicited decreases in cannabinoid receptor binding in the hippocampus using [^{3}H]SR141617A when compared to normal brains (*cannabis* free) with decreased mRNA (CB_1-positive neuronal cells) expression observed caudate, putamen, nucleus accumbens and hippocampus regions of chronic *cannabis* smokers (Villares 2007). In a separate study, chronic *cannabis* users (daily consumption of multiple marijuana cigarettes or blunts for an average of 12 years) showed reversal of CB_1 brain receptor downregulation after 4 weeks of abstinence (Hirvonen et al. 2012). Desensitization of CB_1R-coupled G-proteins has been documented in several brain regions (Rubino et al. 1994; Rubino et al. 1997; Rubino, Vigano', et al. 2000; Rubino, Viganò, et al. 2000; Sim et al. 1996), with temporal development of downregulation and desensitization observed in a brain-region specific manner (Breivogel et al. 1999). Chronic CP55,940 treatment (0.4 mg/kg, 11 days) in rats produced decreases in $G\alpha_i$, $G\alpha_s$ and $G\alpha_o$ mRNA in a brain region specific manner, without concomitant changes in subunit protein levels (Rubino et al. 1997). Additionally, *in vivo* autoradiography of agonist-stimulated [^{35}S]-GTPγS binding was reduced in rats chronically administered THC (10 mg/kg, 21 days) (Sim et al. 1996). Furthermore, repeated THC induces adaptations at the receptor level and downstream at second messenger signaling of cAMP and PKA (Rubino, Vigano', et al. 2000). However, conflicting studies have

reported no alteration in cAMP/PKA levels (mRNA) after chronic CP55,940 in both mice (Fan et al. 1996) and rats (Rubino, Viganò, et al. 2000), which is thought to be a result of differences in efficacy with THC and CP55,940. In mice, long-term THC treatment (15 days) with escalating doses (10 mg/kg initial dose to 160 mg/kg final dose) produced CB_1 receptor downregulation and desensitization of cannabinoid-agonist stimulated G-protein activation coincident with inhibition of adenylyl cyclase (Selley et al. 2004). Overall, the magnitude of cannabinoid responses varies greatly (Burkey, Quock, Consroe, Ehlert, et al. 1997; Burkey, Quock, Consroe, Roeske, et al. 1997; Fan et al. 1996; Rubino et al. 1994; Rubino et al. 1997; Rubino, Vigano', et al. 2000; Selley et al. 1996; Sim et al. 1996) with complex and diverse alterations in intracellular signaling pathways after chronic cannabinoid treatment dependent on agonist efficacy, drug dosage, treatment interval, brain region and species. Interestingly, cross-tolerance to the tetrad behavioral effects have been observed between THC and cannabimimetic agents CP55,940, WIN55,212-2 and AEA (Fan et al. 1994; Pertwee et al. 1993).

Dependence

Neuroplasticity after repeated drug exposure is thought to underlie the behaviors that ultimately manifest into drug abuse and addiction. Upon cessation of marijuana, humans report restlessness and sleep disturbances, accompanied with symptoms of irritability, anxiety, nervousness, aggression, appetite suppression, and weight loss.

While underscored, this marijuana withdrawal syndrome has clinical importance and has been likened to tobacco (nicotine) discontinuation; as a result, detriments to daily life often lead to relapse of marijuana use (Budney et al. 2008).

Likewise, documenting THC withdrawal in laboratory animals in THC-dependent animals has been challenging to assess and quantify. Dependence is assessed in rodents by measuring somatic withdrawal signs (paw flutters/tremors, wet dog shakes, rearing, piloerection). Cannabinoid withdrawal can be determined after spontaneous (abrupt cessation of drug) or precipitated (by receptor antagonism) withdrawal. Spontaneous withdrawal occurs once the parent drug has been transformed to inactive metabolite(s), whereas precipitated withdrawal produced a rapid onset of somatic withdrawal signs. Spontaneous and precipitated somatic withdrawal signs in rats have been reported after treatment with WIN55,212-2 (Aceto et al. 2001). In contrast, THC withdrawal signs are more readily obtained with rimonabant challenge (Diana et al. 1998). As documented for other cannabinoid effects, there are species differences as cannabinoid antagonist administration produced withdrawal in morphine-dependent rats (Navarro et al. 1998) but not in mice (Lichtman et al. 2001).

The molecular mechanisms believed to underlie dependence include activation of second messenger signals such as cAMP, PKA and AC (Rubino, Vigano', et al. 2000; Tzavara et al. 2000). Interestingly, rats treated with chronic THC then with rimonabant

showed increases in cAMP and PKA activity in brain regions (cerebellum, striatum, cortex) that have also undergone CB_1 receptor downregulation (Rubino, Vigano', et al. 2000). Mice exhibit withdrawal signs after THC with concomitant increase in AC within cerebellum after rimonabant administration (Hutcheson et al. 1998).

Repeated exposure to THC and synthetic cannabinoid agonists WIN55,212-2 and CP55,940 causes desensitization of cannabinoid mediated G-protein activity, CB_1 receptor downregulation, and alterations in downstream signaling in rodents (Martin et al. 2004; Sim-Selley 2003; Sim-Selley & Martin 2002). Additionally, rats chronically treated with cannabinoid receptor agonists' display somatic withdrawal signs after rimonabant challenge (Rubino et al. 1998; Tsou et al. 1995). Not surprisingly, prolonged use of synthetic cannabinoids found in "K2" and "Spice" have led to the development of tolerance and withdrawal symptoms similar to, and potentially more disruptive, than those observed with prolonged *cannabis* use, due most likely to their increased potency and efficacy at CB_1 receptors (Berry-Cabán et al. 2013; Zimmermann et al. 2009). Therefore, it is relevant to preclinically evaluate the development of tolerance and withdrawal symptoms after repeated administration of abused synthetic cannabinoid compounds.

Chapter 2 - Synthetic Cannabinoid Compounds

2.1 Synthetic Cannabinoid Abuse is a Serious Public Health Concern

As a “legal” alternative to marijuana, synthetic cannabinoid (SCBs) compounds, intended for research uses only, are being added to herbal incense/botanical material and sold openly to the public, without age restrictions. This is dangerous to consumers as very few *in vivo* studies have been published regarding effects of these potent synthetic cannabinoids in laboratory animals, and no clinical studies have been conducted to test safety or hazardous effects after human consumption.

Despite serious toxicities reported and recent regulatory efforts to eliminate the use of synthetic cannabinoids in herbal incense products (HIPs), the abuse of synthetic cannabinoid compounds continue to threaten public health and safety (United States Congress 2012). In 2013, more than 2,500 calls were made to national poison control centers concerning adverse effects after SCB exposure which was a massive increase from the 14 calls received in 2009 (Wood 2013). In 2012, synthetic cannabinoids were the second most commonly used illicit drug among 10^{th} and 12^{th} grade high school students, and approximately 8% of high school and college students reported having ever used synthetic cannabinoids (Hu et al. 2011; Johnston et al. 2014). Additionally, the Drug Abuse Warning Network (DAWN) reported over 11,400 emergency department

visits as a result of synthetic cannabinoid ingestion, which mostly involved male patients 12 to 29 years old (SAMSHA 2012).

Furthermore, the consequences of inhaling pyrolysis products created from burning the herbal material are unknown. While case reports of toxicity and mortality associated with SCB exposure have been reported, lack of validated analytical screening and confirmation methods for the detection in biological samples have limited the identification of the specific synthetic cannabinoid compound(s) responsible for these untoward effects. SCB-containing products continue to pose challenges for clinicians, toxicologists, law enforcement and public health officials as they are readily available online, with rapidly evolving chemical constituents marketed in a variety of formulations. The research presented in this dissertation will provide much needed *in* vivo pharmacological and analytical toxicology data related to the synthetic cannabinoid, CP47,497, which is known to be contained in HIPs.

2.2 Development of Synthetic Cannabinoids as Research Tools

Synthetic cannabinoids were originally created as pharmacological tools to understand the effects of marijuana on the brain and to investigate the endogenous cannabinoid system. For decades, researchers have questioned how THC elicits its antinociceptive actions on the central nervous system (CNS). As a result, medicinal

chemists explored how alterations in the cannabinoid structure could be used to develop novel analgesic agents with the expectation that intelligently designed compounds could perform similar to morphine, but lack addictive properties (Lemberger 1980). Experiments first examined whether parent delta-9-THC or its primary active metabolite, 11-OH-THC, and structural derivatives such as 9-nor-9-hydroxyhexahydrocannabinol (HHC), were responsible for the analgesic effects of THC observed in animals (Wilson & May 1975; Wilson & May 1976). Results of these studies showed that 11-hydroxy and β-hydroxy metabolites of THC and HHC, respectively, acted as potent analgesics in mice; this subsequently fueled structure activity relationship studies for the development of novel cannabinoid compounds to be used as analgesics. In 1975, scientists at Pfizer initiated a program to design a prototypical drug that would include structural components necessary for analgesia, yet be structurally and pharmacologically unique from potent opioid and cannabinoid analgesics (Johnson et al. 1981; Lemberger 1980). While undesired cannabimimetic side effects prevented these SAR studies from creating new pharmacological drugs to treat pain, they provided novel cannabinoid compounds that served as useful tools to both understand THC effects in the brain and elucidate the endogenous cannabinoid system.

Cannabinoid receptor agonists have been classified according to their chemical structure and **Figure 2.1** illustrates pharmacophores of major cannabinoid agonists,

including the prototypical compounds from each classification. Dibenzopyran derivatives, or compounds that are structurally similar to THC compose the “classical” cannabinoid group. Compounds from this series were developed beginning in the 1960’s and examples include nabilone, with HU-210 and HU-211 synthesized by researchers at Hebrew University (HU) (Mechoulam et al. 1988). The “nonclassical” group is comprised of bicyclic and tricyclic analogs of THC that lack the pyran ring moiety. Commonly referred to as the cyclohexylphenols (CP) series, this class was developed by scientists at Pfizer chemical company and notably include the prototypical synthetic cannabinoid CP47,497, and the well-characterized compound CP55,940 (Weissman et al. 1982). Due to its increased potency at CB_1 receptors, CP55,940 has been an instrumental tool in elucidating the endogenous cannabinoid system and was used to demonstrate specific cannabinoid binding in the brain (Devane et al. 1988), which ultimately contributed to the discovery of cannabinoid receptors.

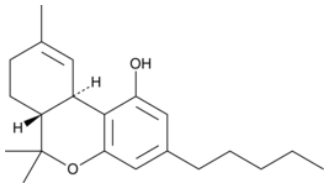
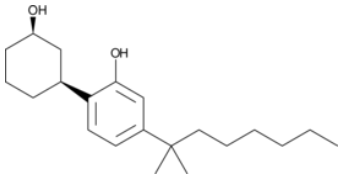
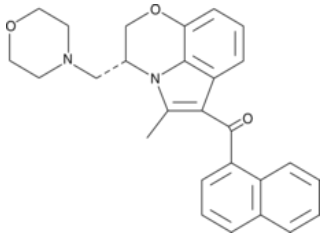
Classical	Non-Classical	Aminoalkylindole
		
Δ^9-Tetrahydrocannabinol	CP47,497	WIN55,212-2

Figure 2.1 – Prototypical cannabinoid compounds.

Preclinical research by scientists from the Winthrop-Sterling group on a new compound, pravadoline, showed analgesic promise with a mechanism of action separate from opioids and cyclooxygenases (Haubrich et al. 1990). Although pravadoline was found to cause nephrotoxicity, continued SAR studies led to the development of the novel aminoalkylindole cannabinoid, WIN55,212-2, which behaves as a high efficacy agonist at both CB_1 and CB_2 receptors (Compton, Gold, et al. 1992; Haubrich et al. 1990; Pacheco et al. 1991). Stemming from this research, John W. Huffman (JWH) continued development of an aminoalkylindole class of synthetic cannabinoids and derivatives which include compounds such as JWH-018, JWH-073, and JWH-250 (Aung et al. 2000; Huffman et al. 1994; Huffman et al. 2005; Wiley et al. 1998). These synthetic cannabinoid SAR studies yielded a spectrum of cyclohexylphenol and aminoalkylindole derived compounds that were intended for

research purposes, but recently emerged as novel psychoactive cannabinoid drugs of abuse.

2.3 Emergence of Synthetic Cannabinoids as Drugs of Abuse

Botanical Composition of Herbal Incense Products

HIPs were initially touted as herbal blends that produced a “natural” or “legal” high similar to marijuana, but lacked legal restrictions like the natural psychoactive botanicals khat (*Catha edulis*) and salvia (*Salvia divinorum*) (EMCDDA 2009). These products contain botanical material of diverse origins: damiana (*Turnera diffusa*), indian warrior (*Pedicularis densiflora*), lion’s ear (*Leonotis leonurus*), and dwarf skullcap (*Scuttelaria nana*) among others, and are reported as having mild anxiolytic, muscle relaxant, and aphrodisiac effects (Cornara et al. 2013; EMCDDA 2009; Ogata et al. 2013; Rosenbaum et al. 2012). Until laboratories identified the presence of synthetic cannabinoids, people largely assumed that the natural botanical ingredients were responsible for the marijuana-like high (EMCDDA 2009).

Cornaro *et al.* utilized DNA barcoding with microscopic examination of structural and morphological botanical characteristics coupled to GC/MS and LC/MS to identify

both the plant and chemical constituents of several HIPs (Cornara et al. 2013). The botanical material examined was a mixture of plants from several families with multiple synthetic cannabinoid compounds added exogenously. This multidisciplinary approach enabled differentiation of herbal/botanical material adulterated with synthetic cannabinoid drugs from other blends which contain naturally occurring plant-based alkaloids such as the mu-opioid receptor agonist, Kratom (*Mitragyna speciosa)* (Cornara et al. 2013). While Ogato *et al.* demonstrated that damiana and Lamiaceae herbs are commonly detected in HIPs, their analysis confirmed that listed ingredients are often inaccurate, as the herbs listed on the packaged material were often missing entirely (Ogata et al. 2013).

In addition, various SCB samples contained other bioactive ingredients not listed including THC, CBD, Kratom, salvia, caffeine, nicotine and *O*-desmethyltramadol, identified using GC/MS and LC/MS (EMCDDA 2009; Dresen et al. 2010; Ogata et al. 2013; Uchiyama et al. 2010). The sleep-inducing lipid signaling molecule, oleamide, and the lipophilic anti-oxidant α-tocopherol (vitamin E), have also been identified in numerous SCB-containing products. Oleamide is presumably added due to its publicly perceived cannabinoid-like effects, and the common and abundant presence of α-tocopherol is thought serve as a masking agent in these products (EMCDDA 2009; Ogata et al. 2013; Uchiyama et al. 2010). Further complicating the issue, botanical

material can also be adulterated with designer drugs of other classes including psychomotor stimulants (commonly referred to as “bath salts”) and hallucinogens (Seely et al. 2013). Product surveillance demonstrated no consistency in HIP composition between the same brands over time and disturbingly, drug distribution is non-homogenous within a single packet due to the haphazard method of adulterating bulk herbal material with SCB drugs (Griffiths et al. 2010; Hillebrand et al. 2010; Logan et al. 2012; Seely et al. 2013; Shanks et al. 2012). Accordingly, approximately 10% of survey respondents reported variable and unpredictable effects with multiple uses of the same brand of HIP (Vandrey et al. 2012).

Packaging, Availability, and Cost of Herbal Incense Products

Interestingly, the original name of “Spice” along with packages depicting a prominent open eye (Figure 5, Panel A) is thought to derive from Frank Herbert’s 1965 science-fiction series, “Dune”; where the highly addictive drug “mélange” was often referred to as “[the] Spice” (Lindigkeit et al. 2009). Initially sold as “Spice” and “K2”, the appearance of other brand name quickly ensued: “Spice Silver”, “Spice Gold”, “Spice Diamond”, “Yucatan Fire”, “Skunk”, “Genie”, etc. (EMCDDA 2009; Fattore & Fratta 2011; Rosenbaum et al. 2012). The products are marketed in attractive and colorful packages, which are sold in smoke shops (specializing in tobacco and smoke related products

including pipes, water bongs, hookahs, etc.), convenience stores, and online (Figure 4). Surreptitious packaging led users to believe these products were a natural, safe, and legal substitution for marijuana.

Figure 2.2 - Illicit synthetic cannabinoid herbal incense product (HIP) contents with "Not for consumption" disclaimer (Panel A, center photo) and varieties of decorative product packaging (Panel B). Images modified from (Spaderna et al. 2013) and National Medical Services, Inc. 2010 (www.nmslabs.com).

Synthetic cannabinoids are sprayed or laced onto bulk botanical material, which is sold in foil-packets containing 0.4-3.0 grams of product to buyers at retail or online with no minimum age requirements (EMCDDA 2009; Fattore & Fratta 2011). Due to

recent changes in drug legislation, HIPs are primarily purchased online, which may actually further facilitate the ease and anonymity of SCB drug use (UNDOC 2013; United States Congress 2012). As with *cannabis* preparations, herbal incense products (HIPs) are typically smoked (via pipe, cigarette, blunt, water pipe/bong) although vaporization, oral ingestion, and rectal routes of administration have been reported (Vandrey et al. 2012). However, HIPs are advertised as “potpourri” and “incense” that are “not for human consumption”.

In the U.S., Europe and Australia, SCBs are sold in 3 gram packages, with the average price of $10-12 (7-9 €) per gram of plant material, depending on the brand and source (Barratt et al. 2013; EMCDDA 2009; Wells & Ott 2011; Wiley, Marusich, et al. 2011). Recently, synthetic cannabinoid product composition has extended from herbal or plant-based materials to the availability of highly pure powder drug form which can be added to any plant material by users (Jankovics et al. 2012; Seely et al. 2013). This shift is partially attributed to cost-effectiveness, as preparing homemade HIP blends is less expensive than purchasing pre-packaged HIPs (Kikura-Hanajiri et al. 2011; Wells & Ott 2011). Additionally, creating homemade mixtures allows users to titrate their dose and combine multiple SCB drugs in specific ratios, which is presumably advantageous to experienced users, who believe they can control desired subjective effects and avoid

the development of tolerance from less-potent products available at retail (Wells & Ott 2011).

2.4 Synthetic Cannabinoid Compounds: Legislation and Rapid Evolution of HIP Constituents

The emergence of synthetic cannabinoids as drugs of abuse began in Europe around the mid-2000's, with the first identification of the synthetic cannabinoid compound, JWH-018, in several "Spice" products in December 2008 in Austria and Germany which was later corroborated by five other European countries (EMCDDA 2009). According to the United Nation's *World Drug Report,* the majority of reporting countries indicated Asia as the primary source for novel psychoactive compounds, including SCBs (UNDOC 2013).

In January 2009, Auwarter *et al.* published the identification of synthetic cannabinoid compounds CP47,497, its C8 homologue (CP47,497-C8 or cannabicyclohexanol), and the endogenous fatty acid amide, oleamide, in several products tested (Auwärter et al. 2009). Monitoring and analysis of HIPs by GC/MS and NMR identified the presence of CP47,497, CP47,497-C8, JWH-018, and JWH-073 as the most commonly encountered SCBs in 140 herbal products in Germany from 2008-2009 (Dresen et al. 2010).

Conclusive drug identification in these products prompted European countries to initiate legislative actions beginning in 2009. Despite regulatory efforts, in a short period of time HIPs gained popularity and their use spread to numerous European nations, Australia, and the United States. Consequently, by 2011, the U.S. Attorney General added five SCBs commonly encountered in HIPs (CP47,497 CP47,497-C8, JWH-018, JWH-073 and JWH-200) to Schedule I of the Controlled Substances Act (CSA) and by 2012, the United States Congress amended the Food and Drug Administration Innovation Act (S.3187) to permanently designate these drugs as Schedule I compounds (Department of Justice 2013; The Drug Enforcement Administration 2011; United States Congress 2012). This law prohibits all preparations containing cannabimimetic agents, their salts, isomers, and salts of isomers. Within the legislation a cannabimimetic is defined as, “any substance that is a cannabinoid receptor (CB_1) agonist, as demonstrated by binding studies and functional assays” (United States Congress 2012).

While legislative efforts were aimed at reducing SCB abuse, initial prohibition of specific SCBs led to the synthesis of a second- and third-generation SCBs, with each new SCB class masquerading as chemical iterations of the last generation in a concerted effort to evade legislative actions (Lindigkeit et al. 2009). For example, after CP47,497 and its isomers were banned, a new compound, JWH-073 was identified in

products within weeks, demonstrating that the drug manufacturers are cognizant of, and one step ahead of, government and law enforcement endeavors aimed at protecting public health and safety (Dresen et al. 2010; Lindigkeit et al. 2009).

The constant evolving nature of SCB drugs of abuse identified in HIPs precede the timeline of enacted legislation, with the first generation (2008-2011) including cyclohexylphenol (CP47,497; cannabicyclohexanol) and napthoylindole (JWH-018 and JWH-073) compounds; the second generation (2011-2012) phenylacetylindoles (JWH-250), napthoylindoles (AM-2201, JWH-210, JWH-211) and benzoylindoles (RCS-4); the third generation (2012-2013) compounds, tetramethylcyclopropylindoles (UR-144, XLR-11), with the most recently encountered fourth-generation (2013-present) adamantoylindoles (AKB48, ADB-PINACA) compounds (Dresen et al. 2010; Lindigkeit et al. 2009; Seely et al. 2013; Shanks et al. 2012; Vardakou et al. 2010). For complete description of each class with examples, please refer to the recent review by Presley et al. 2014 (see **Appendix II** for chemical structures).

Continuous analytical inspection and identification of chemical constituents in seized HIPs and powders over a three year period established the evolution of synthetic cannabinoid drugs, as JWH-018 was present in nearly all samples tested in 2010, then showed a downward trend while identification of structural analogs, AM2201, JWH-210, and JWH-211 steadily increased (Seely et al. 2013). The most recent seizure in

Germany identified the novel tetramethylcyclopropyl compound, commonly referred to as A796-260 (Westphal et al. 2014).

The European Monitoring Centre for Drugs and Drug's Addiction early warning system estimated that more than 50 new synthetic cannabinoid substances were identified from 2011-2012, up from approximately 20 substances documented within the previous five year period of 2005-2009 (UNDOC 2013). In addition, since 2008 more than 140 compounds have been identified in HIPs confiscated in Japan (Kikura-Hanajiri et al. 2011). Moreover, as of January 2014 in the United States, 15 cannabimimetic compounds have been placed in Schedule I nationally (and 7 temporarily scheduled), with various legislation enacted at the state level (United States Congress 2012).

2.5 Effects of Synthetic Cannabinoids in Humans

Synthetic Cannabinoid User Demographics

Data collected from internet-based surveys that include participants from an Australian cohort and worldwide indicate that the majority of HIP users are young (median age 25-27 years old), Caucasian (90-91%) males (77-83%) who have completed a university degree (30-48%) and were employed (47-78%) at the time of the questionnaire (Barratt et al. 2013; Vandrey et al. 2012; Winstock & Barratt 2013). Users report consumption of SCBs for reasons that include curiosity, positive recreational

effects, legal status and the inability to detect SCBs in traditional drug tests (Barratt et al. 2013; EMCDDA 2009; Vandrey et al. 2012).

While self-reports of drug abuse are not without limitations in sampling and interpretation, these data are in good agreement with available poison control data. From 2009-2012, approximately 11,500 synthetic cannabinoid exposures were reported to the American Association of Poison Control Centers (Wood 2013). National user demographics illustrate callers reported intentional inhalation of synthetic cannabinoids, and calls were predominately from males (74%) ages 13-29 years old (83%) (Forrester et al. 2012; Wood 2013). However, relying of information from poison control centers likely over-represents extreme adverse effects requiring medical intervention but underrepresents milder side effects in other users. For example, the Colorado Department of Public Health and Environment (CDPHE) identified 263 potential incidents of synthetic cannabinoid exposure statewide over an approximate one-month period, whereas the state poison control center received only 15 reports during this same time interval (Monte et al. 2014). Overall, self-reports along with poison center and emergency department data support the finding that young males comprise the majority of synthetic cannabinoid users worldwide.

Since many synthetic cannabinoids evade detection by traditional drug testing, their use has also been popular among members of the U.S. military and professional

athletes (Bebarta et al. 2012; Berry-Cabán et al. 2013; Heltsley et al. 2012; Johnson et al. 2011). Adverse effects after smoking synthetic cannabinoids have been documented in active military members with clinical presentations of paranoia, delusions, agitation, somnolence, and hyperreflexia accompanied with tachycardia and hypertension after both acute and chronic exposure (Bebarta et al. 2012; Berry-Cabán et al. 2013; Johnson et al. 2011). As a consequence of these reports, the United States military has established restrictions against the novel psychoactive substances in an attempt to limit their use in active military members (Johnson et al. 2011).

A retrospective examination of 7,500 urine specimens from German athletes showed a 0.03% positivity rate for JWH-018 and its major metabolites, whereas U.S. athlete use was more prevalent as 4.5% of nearly 6,000 urine samples were positive for synthetic cannabinoids (Heltsley et al. 2012; Möller et al. 2011). A reason for higher positivity rates in U.S. versus German athletes may be that more SCB compounds were included in the U.S. analysis. Additionally, the data may reflect regional differences in drug use or highlight different sensitivities in analytical methods.

Acute Synthetic Cannabinoid Use

When compared to the primary psychoactive ingredient in marijuana, THC, the enhanced potency and efficacy of synthetic cannabinoid activation of CB_1 receptors likely contributes significantly to the adverse effects and toxicities reported. Case reports from emergency departments, poison control centers and the military indicate that synthetic cannabinoid ingestion elicits anxiety, panic, disorientation, extreme agitation or somnolence, that is sometimes accompanied with visual and auditory disturbances (Auwärter et al. 2009; Forrester et al. 2012; Heltsley et al. 2012; Hoyte et al. 2010; Johnson et al. 2011; Simmons et al. 2011; Wood 2013). Physiological symptoms include conjunctival injection (bloodshot eyes), xerostomia (dry mouth), nausea/vomiting, hyperreflexia, cardiac arrhythmias, changes in blood pressure, and seizures (Castellanos & Thornton 2012; Harris & Brown 2013). Furthermore, these potent cannabimimetic compounds are thought to contribute to psychotic episodes in individuals with or without previous psychotic disposition (Every-Palmer 2011; Hurst et al. 2011; Müller et al. 2010).

Complex and life-threatening cardiac, renal, and seizure-related toxicities have been documented after synthetic cannabinoid use. Prolonged chest pain and abnormal heart rate was reported in a 17-year-old male shortly after “K9” consumption; subsequent analytical testing of the HIP positively identified JWH-018 and JWH-073 compounds (Young et al. 2012). Additionally, three incidents of myocardial infarction

(MI) were diagnosed in otherwise healthy 16 year-old male adolescents within a few days of ingestion of “K2” (Mir et al. 2011). While increased risk of MI after recent marijuana use have been reported and complaints of chest pain are common among adolescents in emergency settings, it is extremely rare to observe MI in adolescents (Mittleman et al. 2001). Cardiac arrest leading to death in a 58-year-old man who smoked synthetic cannabinoids was reported; however, the effect of drug on cardiac arrest was undocumented (Hoyte et al. 2010). Taken together, these data support reasonable concerns of synthetic cannabinoid use and related cardiotoxicites and emphasize the need for enhanced analytical methods to detect SCBs as well as understand their pharmacology and toxicology.

Adverse renal effects have been documented in conjunction with synthetic cannabinoid use (Bhanushali et al. 2013; CDC & Centers for Disease Control 2013; Thornton et al. 2013). Sixteen individuals (15 males, 15 to 33 years old) from six states in the US presented with abdominal pain accompanied with nausea and vomiting after synthetic cannabinoid ingestion, and were subsequently diagnosed with acute kidney injury (AKI). Clinical specimens were not tested in each case but those analyzed showed association between renal toxicity and the SCB, XLR-11 and its known metabolites (CDC & Centers for Disease Control 2013). A separate case report implicated XLR-11 and UR-144 and common N-pentanoic-acid metabolite with AKI in a

26-year-old male that admitted to use of an HIP, “Mr. Happy” (Thornton et al. 2013). In addition, renal biopsy results of acute tubular necrosis was observed in three young adult male patients known to have recently used “Spice”; of note, each individual had a history or prolonged synthetic cannabinoid use (several weeks or months), and had recently increased consumption or used a different brand prior to seeking medical attention (Bhanushali et al. 2013).

Two fatal occurrences have been published involving the SCB, AM-2201 as confirmed by toxicological analysis; one resulted in a drug-induced panic attack and suicide of a 23-year-old male and the other was an unintentional overdose in a 59-year-old male (Patton et al. 2013; Saito et al. 2013). The most recent outbreak affecting over 250 users in Colorado required serious medical intervention (intubation in several patients) after individuals presented with altered mental status, bradycardia and seizure activity; this toxicity has been linked to the fourth generation synthetic cannabinoid drug, ADB-PINACA identified in “Black Mamba” HIPs (Monte et al. 2014).

Chronic Synthetic Cannabinoid Use

Zimmerman *et al.* first reported the development of tolerance in a 20-year-old male who continually smoked “Spice Gold” over an 8-month period, reaching 3 grams consumption per day. Abstinence from drug produced withdrawal phenomena

characterized by drug craving, nervousness, irritability, nightmares, profuse sweating, headaches, nausea, tremors, and a sustained elevation in blood pressure (Zimmermann et al. 2009). Importantly, it should be noted that at least a subset of these symptoms have also been documented after marijuana cessation (Budney et al. 2008; Ramesh et al. 2011). Further, a user survey indicated that a subset of respondents met DSM-IV criteria of the American Psychiatric Association for abuse (37%) and dependence (17%), with users reporting tolerance (36%) and withdrawal symptoms including headaches, anxiety/nervousness, sleep disturbances, anger/irritability, difficulty concentrating, and restlessness (Vandrey et al. 2012).

While pulmonary dysfunction has been established in chronic marijuana users, pulmonary toxicity related to synthetic cannabinoid use has not been well documented. Severe pulmonary injury, characterized by diffuse lung infiltrates and inflammation was observed in a 21-year-old male who chronically smoked HIPs; analysis confirmed the presence of synthetic cannabinoid compounds, predominately AM-2201 and to a lesser extent JWH-122, JWH-210, and JWH-018 in blood, urine and saliva (Alhadi et al. 2013). Additionally, chronic and prolonged marijuana use results in an under-recognized cannabinoid hyperemesis syndrome (CHS), which causes gastrointestinal disturbances symptomatic of recurrent abdominal pain, nausea, and vomiting (Allen et al. 2004). A case study reported treatment of CHS in a 30-year-old as a consequence of smoking

synthetic cannabinoids almost hourly each day and several times at night over a 2 month period (Hopkins & Gilchrist 2013). Subsequent laboratory examination identified JWH-018, JWH-073, and AM-2201 in urine samples, and upon hospital discharge and a two-week abstinent period from synthetic cannabinoids, the patient's symptoms were completely resolved with good prognosis.

In contrast to THC, examples presented here illustrate the alarming rates of adverse effects and toxicities associated with synthetic cannabinoid use. As with most drugs of abuse, the severity and magnitude of toxicity is dependent on the specific synthetic cannabinoid compound(s) ingested, as well as user experience, and relative dosing. A more comprehensive discussion with specific details pertaining to clinical and toxicological case reports can be found in recently published review articles (Gurney et al. 2014; Hermanns-Clausen et al. 2012a).

2.6 Synthetic Cannabinoids Pharmacology and Toxicology

Previous research has characterized that synthetic cannabinoids bind to CB_1 and CB_2 receptors. While each ligand has a unique binding profile, compounds found within HIPs have been exploited for their ability to bind to and activate CB_1 receptors, thereby producing cannabimimetic effects similar to the major psychoactive compound in

marijuana, THC. What distinguishes abused synthetic cannabinoids from THC is their high CB_1 receptor binding affinity and increased efficacy in activating the receptor. These same properties are hypothesized to make them more dangerous than THC in humans.

Mechanism of Action

Numerous studies have characterized binding of classical (THC analogs), nonclassical (cyclohexyphenols) and aminoalkylindole compounds at CB_1 and CB_2 receptors (Aung et al. 2000; Compton et al. 1993; Huffman et al. 2005; Showalter et al. 1996; Wiley, Marusich, Lefever, et al. 2013). However, for synthetic cannabinoid constituents found in HIPs, fewer studies have characterized functional activation of CB_1 receptors via agonist-stimulated [^{35}S]-GTPγS binding assays. In contrast to the partial agonist efficacy of THC, several synthetic cannabinoids act as full efficacy ligands at CB_1 (Brents et al. 2011; Brents et al. 2012; Huffman et al. 2005; Nakajima et al. 2011; Wiley, Marusich & Huffman 2013). In a neuronal cell culture model, synthetic cannabinoids JWH-018, JWH-073 and CP47,497-C8 inhibited excitatory neurotransmission (EPSCs) with a magnitude similar to the well-characterized cannabinoid agonist, WIN55,212-2 (Atwood et al. 2010; Atwood et al. 2011). Moreover, these compounds caused CB_1 receptor internalization, albeit to different degrees, which supports the hypothesis that tolerance is likely to develop upon repeated use.

Metabolism and Pharmacokinetics

Metabolism of THC has been well elucidated (Agurell et al. 1986; Huestis 2007), and pharmacokinetic data are important to understanding the drug's course of action in the body. One published report has described *in vitro* CP47,497 metabolite formation using human liver microsomes; mainly Phase-I oxygenated and hydroxylated metabolites were identified by LC/MS (Jin et al. 2013). Several studies investigating the metabolic profile of aminoalkylindole (JWH series) synthetic cannabinoids qualitatively demonstrated that the prevalent urine metabolites of JWH-018, JWH-073 and JWH-250 in rodents were N-dealkylated and N-dealkyl monohydroxylated species (Grigoryev, Savchuk, et al. 2011; Grigoryev, Melnik, et al. 2011), whereas human metabolism involved production of monohydroxylated species that were further glucuronidated for excretion (Möller et al. 2011; Sobolevsky et al. 2010). These phase II glucuronidated metabolites have been identified *in vitro* and in authentic urine samples, but are not detected in whole blood specimens (Möller et al. 2011; Kacinko et al. 2011; Wintermeyer et al. 2010). Similar to THC metabolism, involvement of the superfamily of cytochrome monooxygenase (CYP450) enzymes in the formation of hydroxylated metabolites has been established in several synthetic cannabinoid compounds using human liver microsomal preparations (Chimalakonda et al. 2012; Chimalakonda, Bratton, et al. 2011; Sobolevsky et al. 2010; Wintermeyer et al. 2010). When SCB

metabolite internal standards became commercially available, several validated methods were published which examined the quantitative measurement of JWH-018 and JWH-073 glucuronic conjugates after self-administration studies (Chimalakonda, Moran, et al. 2011; Chimalakonda et al. 2012). Many subsequent studies have identified these metabolites in urine (Dowling & Regan 2011; Grigoryev, Savchuk, et al. 2011; Sobolevsky et al. 2012).

In contrast to THC and its major active metabolite 11-OH-THC, mono-hydroxylated but not carboxylate metabolites of SCBs JWH-018, JWH-073 and AM-2201, retain nanomolar binding affinity at CB_1 receptors (Brents et al. 2011; Brents et al. 2012; Chimalakonda et al. 2012). Furthermore, these monohydroxylated metabolites act as full efficacy agonists assessed *in vitro* by G-protein activation and *in vivo,* acting as cannabimimetics in mouse tetrad studies (Brents et al. 2011; Brents et al. 2012; Chimalakonda et al. 2012).

There is a lack of controlled pharmacokinetic studies evaluating peak drug effects and time-course, but users have anecdotally reported that synthetic cannabinoids have a shorter duration of action with a faster peak onset when compared to *cannabis* (Winstock & Barratt 2013). For example, two individuals who smoked a 0.3 g synthetic cannabinoid-containing cigarette reported alterations of mood and perception accompanied with conjunctival injection, xerostomia, and increased pulse rate, within

approximately 10 minutes after consumption (Auwärter et al. 2009). Not surprisingly, subjective effects were reported to vary depending on specific compound; when compared to THC, JWH-018 effects are short (1-2 h) while CP47,497-C8 effects are markedly longer lasting (5-6 h) (EMCDDA 2009).

Data from another anecdotal report attempted to quantify serum concentrations of JWH-018 with various pharmacological self-reports, such as feelings sickness, sedation, xerostomia and increased pulse rates (Teske et al. 2010). JWH-018 was successfully quantitated in serum from 5 minutes to 3 hours after smoking (< 10 ng/mL); while present at 6, 24, 48 hours, JWH-018 values were not reported because they were below analytical limit of quantitation.

2.7 Analytical Testing and Detection of Synthetic Cannabinoids

Because the chemical structures of SCBs greatly vary from THC and its metabolites, 11-OH-THC and THC-COOH, they are not detected via established drug testing methods. In order to assess the consequences of synthetic cannabinoid abuse on public health and safety, the development and validation of sensitive and selective analytical techniques are critical for successful evaluation of preclinical and clinical pharmacological studies. In order to identify SCB compound(s) present in HIPs, analytical techniques needed to quantify them as well their major metabolites following

consumption. Subsequent analysis will permit pharmacokinetic and pharmacodynamic investigations that are necessary for interpretation of suspected intoxication and overdose reports.

Identification and quantification of many SCB have been reported in herbal incense products using high performance liquid chromatography tandem mass spectrometry (HPLC/MS/MS) and gas-chromatography mass spectrometry (GC/MS) techniques (Dresen et al. 2010; Hudson & Ramsey 2011; Logan et al. 2012; Amy Leah Patton et al. 2013; Uchiyama et al. 2010). Ensuing studies examined the presence of SCB compounds confirmed in HIPs in biological matrices, but many of these early validated methods were applied to authentic urine specimens (Heltsley et al. 2012; Möller et al. 2011). While this approach is suitable for workplace drug-testing and to establish drug history, it is of limited utility for interpretation of SCB impairment or toxicity (Dowling & Regan 2011; Chimalakonda, Moran, et al. 2011; ElSohly et al. 2011; Musshoff et al. 2013; Sobolevsky et al. 2010). Even though several studies have described the analysis of synthetic cannabinoid compounds in serum and whole blood, more work is needed to understand the relationship between circulating drug levels and physiological symptoms (Ammann et al. 2012; Dresen et al. 2011; Kacinko et al. 2011; Musshoff et al. 2013).

Rationale and Hypothesis

Data sourced from the scientific research and recent case reports support the abuse of synthetic cannabinoids for their THC-like properties. Since these chemicals were intended specifically for laboratory research, there is limited information regarding their pharmacological and toxicological effects in animals and humans. Importantly, the White House's *National Drug Control Strategy* emphasized that synthetic cannabinoids are understudied substances due to limited scientific data; therefore, there is a need for further evaluation concerning the patterns and significance of abuse, as well as risk assessment for public health and safety (Sacco & Finklea 2013). To address these gaps in the literature, CP47,497 was chosen for evaluation in these studies as it was shown to be biologically active and has been prominently detected in HIPs. Additionally, information on its effects and identification in biological fluids can be used to address legal consequences of SCB use and abuse in humans (Compton, Johnson, et al. 1992; Hudson et al. 2010; Uchiyama et al. 2009; Uchiyama et al. 2010; Weissman et al. 1982).

It is hypothesized that CP47,497 elicits more potent and efficacious cannabimimetic effects than those of THC, through a CB_1 receptor mechanism; and

therefore has an inherent risk for abuse, tolerance, and dependence. As a result**,** the objectives of this dissertation are to investigate the bioavailability of the prototypical synthetic cannabinoid, CP47,497 in the CNS and to characterize *in vivo* pharmacological effects of the drug when compared to the predominant psychoactive compound in marijuana, THC.

Analytical Method Validation

In order to investigate drug actions in biological matrices, analytical methods for detection and quantification of SCB analytes need to be developed. Analyte detection in biological matrices requires validation of experimental, clinical, and forensic toxicological analyses to ensure the efficacy and reliability of an analytical method (Services & Administration 2001; SWGTOX 2013). To achieve this, an HPLC/MS/MS method for the detection of CP47,497 in brain was validated. To test selectivity of the method, the structural analogs CP47,497-C8, and the aminoalkylindole compound JWH-250, were also examined in the analysis. These synthetic cannabinoids were chosen as their presence has been confirmed in HIPs and case reports (Hermanns-Clausen et al. 2012b). To identify limitations of the HPLC/MS/MS method, experiments were designed to evaluate calibration model, bias, precision, matrix interference, ion suppression, carryover, stability, limit of detection, limit of quantification and dilution integrity which will be discussed further in Chapter 3 (SWGTOX 2013).

CP47,497 Mechanism of Action

Determination of synthetic cannabinoid concentration in brain will enable us to examine the relationship between the bioavailability of CP47,497 and resultant behavior. Pharmacokinetic data on THC, the main psychoactive component of marijuana are well established. As there are limited data regarding pharmacokinetics of CP47,497, the first series of experiments examined whether dose-dependent and temporal effects of CP47,497 are similar to those of THC. Because CP47,497 CB_1 receptor binding affinity is higher then the affinity of THC, we predicted that in mice, CP47,497 will produce more potent and efficacious cannabimimetic effects than THC. Next, we wanted to determine if CP47,497 achieved its neuropharmacological effects through a CB_1 mediated mechanism of action. Studies employed a complementary pharmacological approaches using the CB_1 antagonist, rimonabant, and combined with genetically modified mice lacking the CB_1 receptor to ascertain whether CB_1 receptors mediate the pharmacological effects of CP47,497. Given that CB_1 receptor agonists, such as THC, cause conformational changes upon receptor binding with functional downstream signaling consequences, studies employing non-hydrolyzable GTPγS permitted assessment of functional GPCR activation by CP47,497.

CP47,497-mediated Development of Tolerance and Dependence

Due to severe adverse effects reported in chronic users, the consequences of prolonged synthetic cannabinoid use need to be systematically investigated. Chronic marijuana use leads to tolerance and dependence; therefore studies were conducted to examine the untoward effects upon repeated CP47,497 administration. Since CP47,497 is known to bind to CB_1 receptors in the low nanomolar range and produce cannabimimetic effects *in vivo*, we predict that repeated administration of CP47,497 will cause tolerance to tetrad behavioral effects in mice. Additionally, we hypothesize that mice treated repeatedly with CP47,497 will exhibit somatic withdrawal signs when challenged with rimonabant. Lastly, anecdotal accounts describe a THC-like high after synthetic cannabinoid use; as a result, drug-discrimination experiments were performed to test whether CP47,497 substitutes for THC.

Chapter 3 – HPLC/MS/MS Identification and Method Validation of Synthetic Cannabinoids in Brain

3.1 Abstract

While Marijuana continues to be the most widely used illicit drug, abuse of synthetic cannabinoid compounds in “Spice” or “K2” herbal incense products has emerged as a significant public health concern in many European countries and in the United States. Several of these synthetic cannabinoids have been declared Schedule I controlled substances but detection and quantification in biological samples remains a challenge. Therefore, we present a liquid chromatography-tandem mass spectrometry method after liquid-liquid extraction for the quantitation of CP47,497, CP47,497-C8, and JWH-250 in mouse brain. We report data for linearity, LOQ, accuracy/bias, precision, recovery, selectivity, carryover, matrix effects and stability experiments which were developed and fully validated based on (SWGTOX) guidelines for forensic toxicology method validation. Acceptable coefficients of variation for accuracy/bias, within- and between-run precision and selectivity were determined, with all values within ± 15% of the target concentration. Validation experiments revealed degradation of CP47, 497 and CP47,497-C8 at different temperatures, and significant ion suppression was produced in

brain for all compounds tested. The method was successfully applied to detect and quantify CP47,497 in brains from mice demonstrating significant cannabimimetic behavioral effects as assessed by the classical tetrad paradigm.

3.2 Introduction

Abuse of synthetic cannabinoids (SCBs) in herbal incense products (HIPs) has recently emerged as a significant national public health and safety concern. In the United States, emergency bans were swiftly mandated by the Drug Enforcement Administration (DEA) to regulate the barrage of synthetic cannabinoid compounds available to the public (Department of Justice 2013). To date, dozens of SCBs have been detected in HIPs, as their structures are continually modified to circumvent legislative efforts (Choi et al. 2013). Anecdotal accounts from user forums (www.bluelight.ru; erowid.org) suggest that SCB effects are reminiscent of the effects of marijuana (Vardakou et al. 2010) and while published user reports of synthetic cannabinoid effects are limited, studies indicate SCBs produce alterations in perception, conjunctival injection, xerostomia, and increases in pulse rate similar to that of marijuana (Auwärter et al. 2009; Teske et al. 2010).

Synthetic cannabinoid compounds were incipiently developed as tools to gain insight on marijuana's effects on the brain and to investigate potential therapeutic

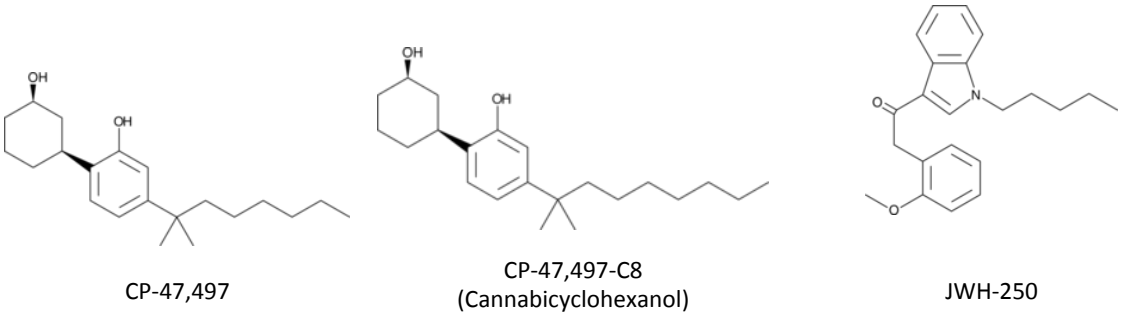
applications of the endogenous cannabinoid receptor system (CB1 and CB_2) modulation. The cyclohexylphenol (CP) series were synthesized by Pfizer beginning in the 1970's, with CP47,497 serving as a prototypical bicyclic analgesic compound (Weissman et al. 1982). Research from scientists at the Winthrop-Sterling company over two decades ago led to the development of the novel aminoalkylindole synthetic cannabinoid, WIN55,212-2, which behaves as a full receptor agonist at both CB1 and CB_2 receptors (Haubrich et al. 1990; Compton, Gold, et al. 1992). Stemming from this research, John W. Huffman continued development of an aminoalkylindole (or JWH) class of synthetic cannabinoids, which include compounds such as JWH-250 (Huffman et al. 2005). Over the last several years, these SCB compounds, intended solely for research purposes, have been sold as a "legal high" alternative to marijuana, with the first identification of CP 47,497 in 2008 from the Wisconsin State Crime Laboratory (The Drug Enforcement Administration 2009).

Marijuana remains the most widely used illicit substance worldwide (UNDOC 2013), but the fact that standard drug screens do not typically include SCBs, may contribute to the recent popularity of these substances. The sudden spike in SCB abuse will likely continue to threaten public health and safety. Repeated abuse of SCBs has led to tolerance and dependence symptoms after attempted drug cessation (Zimmermann et al. 2009; Nacca et al. 2013). Thus, there is a need for validated

methods to detect these SCB substances from biological material. In the present study, we present a high performance liquid chromatography tandem mass spectrometry (HPLC/MS/MS) method for the detection and quantification of JWH-250, CP47,497 and CP47,497-C8, in brain. We chose to include CP47,497, CP47,497-C8, and JWH-250 in this method as they are among the first synthetic cannabinoid compounds to be positively detected in herbal products (Vardakou et al. 2010; Lindigkeit et al. 2009; Auwärter et al. 2009; Hudson et al. 2010; Uchiyama et al. 2009; Uchiyama et al. 2010). Of relevance to their abuse, these three SCBs have also been identified in SCB exposure case reports (Hermanns-Clausen et al. 2012b; EMCDDA 2009).

It is hypothesized that the desired subjective effects of synthetic cannabinoids are mediated through activation of CB1 receptors in the central nervous system (CNS) similar to those of delta-9-tetrahydrocannabinol (THC), the primary psychoactive constituent of marijuana (EMCDDA 2009; Huffman et al. 2008; Atwood et al. 2011). In contrast to the partial-agonist activity of THC, many SCBs including CP47,497, CP47,497-C8, and JWH-250 possess enhanced binding affinity at CB1 receptors (Melvin et al. 1993). Despite this proposed mechanism of action, few published studies have identified or quantified the presence of synthetic cannabinoids in brain after drug exposure (Wiebelhaus et al. 2012; Poklis et al. 2012; Poklis et al. 2012). Of clinical significance, it has been previously demonstrated that THC can be detected in brain

after the window of detection in blood/plasma has lapsed (Mura et al. 2005). The method presented here demonstrates that SCBs from distinct cyclohexylphenol and aminoalkylindole structural classes (Figure 3.1) can be simultaneously detected in brain.



CP-47,497 | CP-47,497-C8 (Cannabicyclohexanol) | JWH-250

Figure 3.1 – Chemical structures of synthetic cannabinoid compounds CP47,497, CP47,497-C8 and JWH-250 included in HPLC/MS/MS method validation.

3.3 Materials and Methods

Drugs and reagents

CP47,497, CP47,497-C8, and JWH-250 drugs and CP47,497-d11, CP47,497-C8-d7 and JWH-250-d5 deuterated internal standards (ISTD) were purchased from Cayman Chemical (Ann Arbor, MI). HPLC grade or better acetonitrile, methanol, and ammonium formate were obtained from Fisher Scientific (Waltham, MA). Deionized (DI) water was obtained from an in-house water system (Millipore, Bedford MA). Nitrogen

gas (medical grade) was obtained from National Welders Supply Company (Richmond, Virginia).

Animals, behavioral measures and tissue collection

Drug naïve, male, ICR (CD-1) mice (Harlan Laboratories, Dublin, VA) weighing 25-35 grams were obtained and housed in Virginia Commonwealth University's (VCU) animal care facilities at 22 ± 2 °C on a 12-hour light/dark cycle with *ad libitum* water and standard rodent chow (7012 Teklad Mouse/Rat Diet, Harlan Laboratories). For proof of method experiments, mice received intraperitoneal (i.p.) injections of either vehicle (ethanol:emulphor:saline in a ratio of 1:1:18) or 30 mg/kg CP47,497 administered at a volume of 0.1mL per 10 g of body mass, as this SCB dose produced measurable cannabimimetic effects in previous dose-response studies *in vivo* (Samano et al. 2013).

For tetrad behavioral testing, mice were acclimated to the test environment for at least 1 h prior to testing for spontaneous activity, catalepsy, antinociception, and hypothermia (Wiley 2003; Martin et al. 1991). Locomotor activity was assessed by placing mice into individual photocell activity boxes (28 × 16.5 cm chambers containing two sets of eight photocells; Omnitech, Columbus, OH) five minutes after vehicle or drug treatment. Photocell beam interruptions were recorded for 10 minutes using Digiscan Animal Activity Monitor software (Med Associates, Inc., St. Albans, VT) with data

expressed as the average number of photocell beam breaks. Mice were assessed for catalepsy, antinociception and hypothermia thirty minutes after vehicle or drug treatment. Catalepsy was measured using the bar test in which both forelimbs of the mouse were placed on a horizontal bar, with the duration of a fixed and motionless posture recorded over a 60 second interval. Antinociception was determined using a warm water (52 °C) tail immersion test. The distal end (approximately 1 cm) of the tail was immersed in the water bath and the latency of the mouse to withdrawal its tail was recorded (to the nearest tenth of a second) using a 10 second cut-off to minimize tail damage. Antinociception data was transformed to represent a maximum percent effect (%MPE) by the following formula: %MPE = [(test latency – pretreatment latency)/(10–pretreatment latency)]×100. Body temperature measurements were collected by inserting a rectal probe attached to a telethermometer (Yellow Spring Industries Inc., Yellow Springs, OH) to a 2 cm depth with measurements recorded to the nearest 0.1 °C.

One hour post-injection, mice were deeply anesthetized via 3% isoflurane-containing oxygen induction and sacrificed by cervical dislocation. Whole brain was harvested and stored at -80 °C until analysis. All experiments were approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University in

accordance with the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals.*

Brain homogenate preparation

Brains from ICR and C57BL/J6 mice was harvested, pooled and used to obtain drug-free tissue for the method validation. Brain homogenates were prepared in a 4:1 ratio (w/w) using deionized water with a stand homogenizer (Janke and Kunkel Ultra Turrax T25). For selectivity experiments, 10 individual lots of tissue (each lot required two pooled brains) were used to prepare each of 10 separate homogenate tissue lots. For mouse studies, after homogenization of whole brain tissue, 0.5 mL aliquots of homogenate were used from drug-treated and vehicle-treated mice to isolate CP47,497 using the liquid-liquid extraction (LLE) method described herein.

Internal standard and stock solution preparation

Separate working stock solutions containing each CP47,497, CP47,497-C8 and JWH-250 at 250 ng/mL, 2,500 ng/mL, and 10,000 ng/mL were prepared by dilution with methanol of commercially purchased drug compounds. The deuterated ISTD mixture containing 2500 ng/mL of CP47,497-d11, CP47,497-C8-d7, and JWH-250-d5 was prepared by dilution of purchased ISTDs with methanol. Working stock and ISTD stock solutions were stored at -20 °C.

Calibrator and quality control preparation

Calibrators were prepared by fortifying 0.5 mL aliquots of drug-free mouse brain homogenates with appropriate drug and ISTD concentrations for each calibrator. Prior to every analytical run, fresh calibrators containing CP47,497, CP47,497-C8 and JWH-250 were prepared in duplicate at 20, 40, 80, 200, 400, 1000 and 2000 ng/g concentrations and used to construct a 7-point calibration curve. A single lot of quality control (QC) specimens containing CP47,497, CP47,497-C8 and JWH-250 were prepared at the start of the method validation and analyzed at the following levels: limit of quantification quality control (LOQ), target concentration of 20 ng/g; low quality control (LQC), target concentration 60 ng/g; medium quality control (MQC), target concentration 300 ng/g; and high quality control (HQC), target concentration 1600 ng/g. A dilution quality control (DQC), target concentration 400 ng/g upon dilution (1:10), was included to ensure accurate quantification of authentic samples in instances where quantified specimen concentrations surpass the established calibration range, or when the sample volume is insufficient for testing. Calibrators and QCs specimens each contained all three SCB analytes along with their respective deuterated ISTDs (final concentration 800 ng/g). A negative control (drug-free) containing ISTD, and a double negative control (drug-free without ISTD) were also included in each analytical run. All QC lots and standards were stored at 4 °C for the duration of the validation.

SCB extraction procedure

CP47,497, CP47,497-C8 and JWH-250 were isolated using a liquid-liquid extraction (LLE) method for isolation of cannabinoids from blood as described by Foltz. *et al.* (Foltz et al. 1983) and previously adapted specifically for cannabinoid isolation from brain tissue (Poklis et al. 2010). To each set of calibrators, QCs, or treated mouse tissue, 10 μL of ISTD mix consisting of 2500 ng/mL of each CP47,497-d11, CP47,497-C8-d7 and JWH-250-d5 were added and vortex-mixed thoroughly. Samples were then refrigerated overnight at 4 °C to ensure equilibration. The next day, 2 milliliters of "ice-cold" acetonitrile was added to homogenates in a drop-wise manner while samples were constantly vortex-mixed. Samples were then centrifuged for 10 minutes (2500 x g) to separate solid and liquid phases. Extracted samples were stored overnight at -20 °C to allow for complete phase resolution. The following day, the organic layer was isolated using a glass pipet, transferred to a clean glass tube, and evaporated to dryness in a Savant Speed Vac Concentrator (SPD1010, Thermo Scientific). Samples were reconstituted in 100 μL of 20/80 water/acetonitrile and transferred to auto-sampler vials. The vials were stored on the sample tray maintained at 4 °C until analyzed.

HPLC/MS/MS conditions

The HPLC/MS/MS system was an Applied Bio Systems 3200 Q Trap with Turbo V source for TurbolonSpray coupled to a Shimadzu SCL HPLC system with data acquisition controlled by Analyst 1.4.2 software. Chromatographic separation was achieved with a Zorbax Eclipse XBD-C18 (4.6 x 75 mm, 3.5 μm) column (Agilent Technologies). The mobile phase consisted of water/acetonitrile (20:80 v/v) with 0.1 mM ammonium formate and had an isocratic flow rate of 0.5 mL/min. The ionspray voltage was set at 5000V with ion source gases 1 and 2 maintained at a flow rate of 60 mL/min. The source temperature was programmed at 650 °C, with a curtain flow rate of 30 mL/min. Multiple reaction monitoring (MRM) acquisition mode was used. Transition ions monitored along with the corresponding deprotonation (DP) and collision energies (CE) utilized for each analyte are presented in Table I. The injection volume was 10 μL and the method had a total chromatographic run time of 12 minutes. Figure 3.2 shows the chromatographic method successfully resolved CP47,497, CP47,497-C8 and JWH-250.

Table I
Synthetic Cannabinoid Retention Time, Multiple Reaction Monitoring (MRM) Transition Ions, and Corresponding Collision Energies (CE)

Analyte	RT (min)	Quant Ion *(m/z)*	CE (eV)	Qual Ion (m/z)	CE (eV)
Positive Mode:					
JWH-250	4.3	336>121	27	336>91	61
JWH-250-d5	4.2	341>121	27	341>91	61
Negative Mode:					
CP-47,497	5.1	317>245	44	317>299	34
CP-47,497-d11	4.9	328>245	44	328>299	34
CP-47,497-C8	6.7	331>259	42	331>313	34
CP-47,497-C8-d7	6.3	338>266	42	338>320	34

Quantitative assay performance

The presented HPLC/MS/MS method validation for the detection and quantification of SCBs in brain was based on recommendations of the Scientific Working Group for Forensic Toxicology (SWGTOX) Standard Practices for Method Validation in Forensic Toxicology (SWGTOX 2013). The assay was evaluated over six days with each run including calibrators prepared in duplicate, a negative control, a double negative control, and batch prepared LOQ, LQC, MQC, HQC, and DQC samples in replicates of 3 or 6.

Linearity and LOQ

A 7-point calibration curve was prepared in duplicate at 20, 40, 80, 200, 400, 800, and 2000 ng/g in homogenized brain tissue for determination of SCB drug concentrations. The linear-regression calibration model was verified using the ratio of the peak area of the abundance quantification ion of SCBs to the peak area abundance quantification ion of each SCBs' deuterated ISTD versus the concentration. The lower limit of quantification (LOQ) and lower limit of detection (LOD) were administratively set at 20 ng/g. LOQ QCs were prepared for each run to verify that the LOQ was within ± 20% of the target value and had a response at least five times greater than the signal to noise ratio of drug-free brain.

Accuracy/bias and precision

Accuracy/bias and precision (within- and between-run) were determined for the method by analysis of prepared QC samples over six separate analytical runs. The method bias was determined by calculating the ratio of the mean SCB concentration of six aliquots of each QC (LOQ, LQC, MQC, HQC, and DQC) to the target concentration of the QC specimen, then multiplying by 100 to obtain a percentage. Data was considered acceptable when the accuracy/bias results were within ± 20% of the QC's target value. Precision was assessed at five concentrations (LOQ, LQC, MQC, HQC,

and DQC) in triplicate over six analytical runs. Within-run and between-run precision were calculated after performing a one-way Analysis of Variance (ANOVA) using GraphPad Prism® statistical software (Version 5.0; LA Jolla, CA) with calculated values from the analysis reported.

Absolute recovery and matrix effects

Absolute recovery was determined at 60, 300 and 1600 ng/g covering the linear range of the assay by comparing the mean SCB peak area of six aliquots where drug was added to homogenates prior to extraction (i.e. before, B) to six blank homogenate extracts to which drug was added to the extracted matrix (i.e. after, A). To calculate absolute recovery, the following equation was used: (Mean area of B/Mean area of A) X 100. For determination of matrix effects of LQC, MQC, and HQC specimens, the mean SCB peak area of unextracted (i.e. neat, N) samples were compared to the SCB peak area of blank homogenate samples fortified with drug after extraction (A). Percent ion suppression/enhancement was determined by use of the following equation: [(Mean area of N/Mean area of A) - 1] X 100.

Stability studies

Since little is known regarding the stability of SCB compounds in biological samples, SCB stability experiments were conducted to assess bench top stability,

freeze/thaw stability, storage parameters and post-preparative stability using LQC (60 ng/g), MQC (300 ng/g) and HQC (1600 ng/g) specimens. LOQ (20 ng/g) and DQC (400 ng/g) samples were also examined for post-preparative stability. For bench top stability studies, the effects of routine specimen transportation and laboratory handling were investigated. Six aliquots of each LQC, MQC, and HQC specimens were left exposed at room temperature for 72 hours, extracted as described herein, then analyzed. Brain tissue is commonly stored frozen prior to analysis so the consequences of freeze/thaw treatment were also evaluated. To account for reanalysis of frozen specimens, three freeze/thaw cycles were performed on six aliquots of each LQC, MQC and HQC. QC samples were frozen at -20 °C, allowed to thaw at room temperature unassisted, and refrozen for a minimum of 24 hours before the next thaw cycle. Upon the third thaw cycle, the QC specimens were extracted then analyzed using freshly prepared calibrators. Short-term storage was also assessed. Thirty days after QC batches were prepared for the validation study, six aliquots from each LQC, MQC, and HQC lots were extracted and analyzed using freshly prepared calibrators. Post-preparative stability of processed samples was examined after 72 hours, to simulate situations where prepared samples cannot be immediately analyzed. LQC, MQC, and HQC samples were extracted normally and quantified using freshly prepared calibrators. These same QC extracts were kept at room temperature, re-injected after 72 hours and quantified

against the original calibration curve. SCB analytes were considered stable if the concentrations were within ± 20% of the target concentrations.

Analyte and ISTD stock stability was assessed at LQC, MQC, and HQC concentrations after room temperature exposure over a 6 hour period as previously recommended (Services & Administration 2001), with stability determined by comparing instrument response of the stocks kept at room temperature to freshly prepared analyte and ISTD stocks. Stock standards were considered stable if analyte or ISTD peak area was within ± 20% of freshly prepared stock peak areas.

3.4 Results

For each analytical run, the calibrators analyzed in duplicate were back calculated and determined to be within ± 15% of their nominal value. Additionally, linear regression correlation coefficients (r^2) means (n = 21) across runs were 0.9964 ± 0.003 (CP47,497), 0.9963 ± 0.003 (CP47,497-C8) and 0.9960 ± 0.003 (JWH-250), with ranges of 0.9984-0.9992, 0.9904-0.9992, and 0.9932-0.9996, respectively. It was verified that the LOD of each SCB demonstrated at least five times the signal-to-noise ratio of the response seen for both qualifier and quantifier ions in drug-free brain.

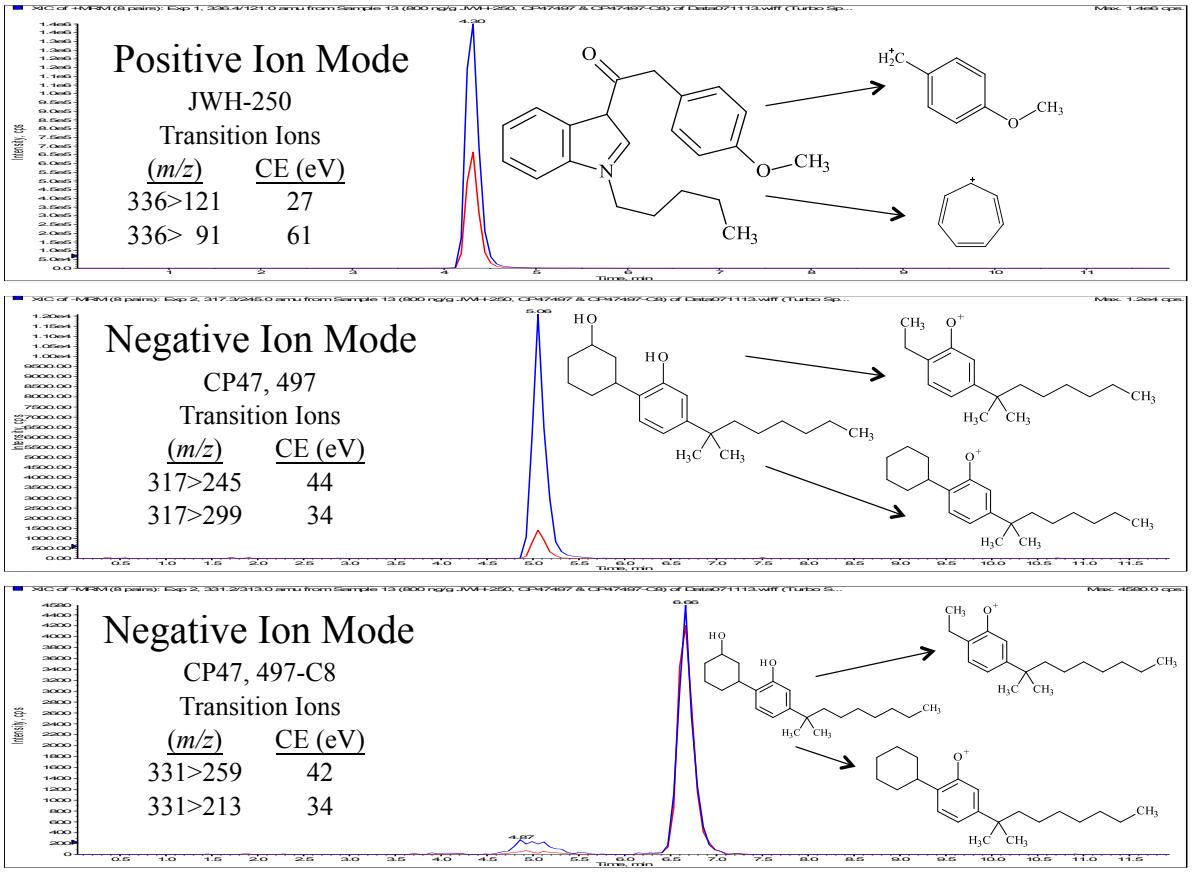


Figure 3.2 – Representative total ion chromatogram (TIC) and fragmentation pattern of CP47,497, CP47,497-C8 and JWH-250 analytes.

Accuracy/bias shown in Table II of the tested SCBs ranged at the LOQ (20 ng/g) from 90% to 98%, the LQC (60 ng/g) from 87% to 100%, the MQC (300 ng/g) from 93% to 101%, the HQC (1600 ng/g) from 96% to 100%, and the DQC (400 ng/g) from 86% to 97%. Values for the coefficient of variation (CV), expressed as a percentage, for LOQ, LQC, MQC, HQC, and DQC were all within ± 15% of the target concentration, with a

range of 3% to 12%. Accuracy/bias determined over the linear range varied from a low of 86% exhibited by CP47,497-C8 at the DQC target value of 400 ng/g to a high of 101% for CP47,497 at the MQC target value of 300 ng/g.

Table II
Accuracy/bias (n = 6) for JWH-250, CP-47,497 and CP-47,497-C8 in mouse brain; values represent mean ± SD (CV)

Murine Brain (ng/g):	LOQ (20 ng/g)	LQC (60 ng/g)	MQC (300 ng/g)	HQC (1600 ng/g)	DQC (400 ng/g)
JWH-250	22 ± 1.2 (6)	68 ± 2.3 (3)	319 ± 35 (11)	1601 ± 150 (9)	411 ± 22 (5)
CP-47,497	20 ± 0.7 (4)	60 ± 3.6 (6)	303 ± 26 (9)	1598 ± 161 (10)	369 ± 40 (11)
CP-47,497-C8	19 ± 1.5 (8)	56 ± 3.6 (6)	278 ± 17 (6)	1536 ± 182 (12)	344 ± 33 (10)

LOQ, Limit of quantitation; LQC, low quality control; MQC, medium quality control; HQC, high quality control
DQC, dilution quality control; CV, coefficient of variation

Within-run and between-run precision is shown in Table III for LOQ, LQC, MQC, HQC and DQC samples. Within-run precision was acceptable with all CV values within ± 15% the expected concentration and a range of 4.8% to 9.1% CV for JWH-250, 7.7% to 13.4% CV for CP47,497 and 6.3% to 11.2% CV for CP47,497-C8. The between-day precision across each analyte at five QC levels was within ± 15% of the expected CV, with a range of 5.2% to 15.3% across the SCB analytes tested.

Table III
Within- and between-run precision for JWH-250, CP-47,497 and CP-47,497-C8 in mouse brain (n = 21)

Analyte	Concentration	Within-run CV (%)	Between-run CV (%)
JWH-250	LOQ (20 ng/g)	7.1	8.0
	LQC (60 ng/g)	4.8	5.2
	MQC (300 ng/g)	5.2	11.9
	HQC (1600 ng/g)	6.0	11.6
	DQC (400 ng/g)	9.1	10.1
CP-47,497	LOQ (20 ng/g)	11.2	9.9
	LQC (60 ng/g)	7.9	7.9
	MQC (300 ng/g)	7.7	10.7
	HQC (1600 ng/g)	8.4	12.2
	DQC (400 ng/g)	13.4	15.3
CP-47,497-C8	LOQ (20 ng/g)	11.2	12.5
	LQC (60 ng/g)	7.5	8.9
	MQC (300 ng/g)	9.0	9.6
	HQC (1600 ng/g)	6.3	10.5
	DQC (400 ng/g)	9.7	12.4

LOQ, limit of quantitation; LQC, low quality control; MQC, medium quality control; HQC, high quality control DQC, dilution quality control; CV, coefficient of variation

Absolute recovery shown in Table IV for the SCBs at the LQC ranged from 94% to 117%, at the MQC from 98% to 116%, and at the HQC from 115% to 118%. Recovery determined over the linear range varied from a low of 94% exhibited by CP47,497-C8 at a target concentration of 60 ng/g (LQC) to a high of 118% for JWH-250 and CP47-497 both at target concentrations of 1600 ng/g (HQC). Matrix effects shown in Table IV demonstrated significant ion suppression across the SCBs tested. Matrix

effects for JWH-250 ranged from 35 to 45%, CP47,497 from 32 to 69% and CP47,497-C8 from 27 to 69%.

Table IV
Recovery and ion suppression/enhancement of synthetic cannabinoids (n = 6) and their respective internal standards (n = 18) in brain

		Mean ± CV	
Murine Brain:		% Recovery	Ion Suppression
Synthetic cannabinoid			
JWH-250	LQC (60 ng/g)	117 ± 20	35 ± 10
	MQC (300 ng/g)	116 ± 18	43 ± 12
	HQC (1600 ng/g)	118 ± 2	45 ± 8
CP-47,497	LQC (60 ng/g)	96 ± 16	48 ± 9
	MQC (300 ng/g)	105 ± 10	32 ± 17
	HQC (1600 ng/g)	118 ± 11	69 ± 28
CP-47,497-C8	LQC (60 ng/g)	94 ± 11	44 ± 20
	MQC (300 ng/g)	98 ± 11	27 ± 18
	HQC (1600 ng/g)	115 ± 5	69 ± 26
ISTD			
JWH-250-d5	800 ng/g	120 ± 19	42 ± 15
CP-47,497-d11	800 ng/g	102 ± 19	47 ± 18
CP-47,497-C8-d7	800 ng/g	93 ± 19	43 ± 26

LQC, low quality control; MQC, medium quality control; HQC, high quality control
ISTD, internal standard

Selectivity was assessed using 10 separate lots of drug-free brain. Samples from each lot included a drug-free aliquot with and without ISTD, and three aliquots fortified with 60 ng/g of each SCB analyte. Each fortified tissue lot was run in triplicate and calculated CVs were within ± 15% for CP47,497, CP47,497-C8 and JWH-250. The selectivity bias range was 100% to 116% for CP47,497, 80% to 116% for CP47,497-C8 and 104% to 119% for JWH-250. Bias for all samples were ± 20% of the target value with the exception of one tissue lot that demonstrated 130% bias for CP47,497.

Sample carryover was assessed using two different procedures. First, immediately following the injection of the 2000 ng/g calibrator, a negative control was injected. No carryover was detected in the negative control. Second, an injection of the HQC (1600 ng/g) was immediately followed by injection of the LQC (60 ng/g) sample. This procedure was routinely applied each time HQC and LQC samples were analyzed. Lack of carryover was confirmed, as the SCB LQC concentration bias was less than 20% of the LQC's target concentration.

Data in Table V demonstrate that CP47,497 is unstable after 72 hours at room temperature, with the MQC (300 ng/g) and LQC (60 ng/g) samples exhibiting only 56% and 74% of their target values, respectively. Stability of CP47,497, CP47,497-C8 and JWH-250 were unaffected by freeze/thaw treatment, and were considered acceptable as all QC samples were within ± 20% of the target concentration. JWH-250 and

CP47,497 QC samples were stable short-term (4° C), demonstrating values ± 20% of the target concentration. However, CP47,497-C8 fortified QCs exhibited significant analyte loss at all levels tested (Table V). All processed QC samples tested satisfied this criterion of ± 20% of the target concentration as seen in Table V. Finally, both ISTD and drug stock solutions were stable at room temperature for up to 6 hours, with all CV values within ± 15%.

Table V
Post-Preparative, Benchtop, Freeze/Thaw and Short-Term Stability of CP-47,497, CP-47,497-C8 and JWH-250 in mouse brain.
Results are expressed as the % target value ± SD (%CV); n = 6

		Stability Parameter			
Analyte	Sample	Post-preperative (72 h)	Benchtop (72 h)	Freeze/Thaw (3X)	Month (4° C)
JWH-250	LQC (60 ng/g)	98 ± 6.7 (11)	116 ± 1.9 (3)	118 ± 1. (2)	116 ± 6.5 (9)
	MQC (300 ng/g)	102 ± 41 (13)	111 ± 25 (7)	113 ± 18 (5)	105 ± 16 (5)
	HQC (1600 ng/g)	80 ± 141 (11)	107 ± 107 (6)	113 ± 73 (4)	91 ± 67 (5)
	DQC (400 ng/g)	87 ± 31 (9)			
	LOQ (20 ng/g)	95 ± 2.3 (12)			
CP47,497	LQC (60 ng/g)	111 ± 5 (7)	85 ± 14 (27)	107 ± 2.2 (3)	105 ± 3.5 (6)
	MQC (300 ng/g)	117 ± 18 (5)	56 ± 44 (26)	103 ± 26 (8)	92 ± 33 (12)
	HQC (1600 ng/g)	113 ± 134 (7)	74 ± 101 (9)	100 ± 140 (9)	92 ± 137 (9)
	DQC (400 ng/g)	102 ± 53 (13)			
	LOQ (20 ng/g)	100 ± 2.7 (13)			
CP47,497-C8	LQC (60 ng/g)	102 ± 6 (9)	84 ± 7.3 (15)	98 ± 8.3 (14)	50 ± 8.1 (27)
	MQC (300 ng/g)	105 ± 33 (11)	97 ± 43 (12)	98 ± 25 (8)	58 ± 18 (10)
	HQC (1600 ng/g)	98 ± 167 (11)	91 ± 98 (7)	102 ± 150 (9)	68 ± 115 (11)
	DQC (400 ng/g)	95 ± 43 (11)			
	LOQ (20 ng/g)	97 ± 2.2 (11)			

LOQ, limit of quantitation; LQC, low quality control; MQC, medium quality control; HQC, high quality control
DQC, dilution quality control; CV, coefficient of variation

Application of method to animal samples

Table VI illustrates locomotor activity (5-15 minutes post-injection), catalepsy, antinociception and temperature data (each taken at 30 min post-injection) with

corresponding brain concentrations (60 min post-injection) after a 30 mg/kg challenge of CP47,497 (Table VII). Drug-treated mice exhibited significant ($p < 0.05$) decreases in locomotor activity, expressed as number of beam breaks, compared to vehicle-treated mice. Catalepsy and antinociception (%MPE) were not detected after vehicle treatment, whereas CP47-497-treated mice displayed statistically significant catalepsy and antinociception ($p < 0.0001$ and $p < 0.05$, respectively). Pre-treatment (i.e. baseline) means ± SEM for body temperature (°C) were 37.0 ± 0.3 for vehicle-treated and 37.5 ± 0.3 for CP47,497-treated mice, demonstrating no significant differences in baseline measurements between vehicle and drug treated mice. CP47,497-treated mice showed significant ($p < 0.001$) hypothermia (i.e., a 6.9 ± 0.6 °C drop in temperature) compared to vehicle-treated (i.e., a 1.5 ± 0.4 °C increase in temperature) mice. Table VI illustrates the cannabimimetic behavioral effects observed from the same group of mice with corresponding brain concentrations of CP47,497 (Table VII) ranging from 1660 ng/g to 1890 ng/g (mean 1804 ± 93 ng/g), with no analyte peaks detected in brains from vehicle-treated mice.

Table VI

Significant cannabimimetic effects in mice after CP47,497 (30 mg/kg) in measures of spontaneous activity, catalepsy, antinociception and body temperature. Values represent the mean (± SEM); n= 4-5 mice per group.

Treatment	Spontaneous Activity (beam breaks), mean ± SEM	Catalepsy (s), mean ± SEM	Antinociception (MPE), mean ± SEM	Body temperature (°C), mean ± SEM
Vehicle (1:1:18)	601 ± 78	ND	ND	1.3 ± 0.3
CP47,497 (30 mg/kg)	194 ± 120	50.7 ± 2.2	51 ± 14	-6.9 ± 0.3
p-value	< 0.05	< 0.0001	< 0.05	< 0.0001

Pre-treatment (i.e. baseline) means ± SEM for temperature (°C) = 37.4 ± 0.2 vehicle-treated and 37.9 ± 0.2 CP47,497-treated mice.
Baseline tail withdrawal latencies (s) for vehicle-treated were 1.5 ± 0.4 and 1.6 ± 0.3 for CP47,497-treated mice.
MPE: maximum percent effect (10 second cut-off); ND: none detected

Table VII

Quantification of CP-47,497 (ng/g) in mouse brain 1-hour after vehicle or CP-47,497 (30 mg/kg) injection (i.p.)

	Treatment	
Animal	Vehicle	CP-47,497
1	ND	1890
2	ND	1660
3	ND	1840
4	ND	1780
5	ND	1850

Data represents the mean (± SD), n = 5 mice per group
ND = none detected; i.p. = intraperitoneal

3.5 Discussion

We have previously quantified JWH-018 in blood of mice exposed to synthetic cannabinoids in “Buzz” smoke (Haggerty et al. 2010; Wiebelhaus et al. 2012). Using the same LLE and HPLC/MS/MS parameters as the presented method, prior experiments demonstrated this analytical approach is capable of concurrently detecting THC, its carboxylic acid metabolite, JWH-073, JWH-250, JWH-398, CP47,497, and HU-210 in whole blood (Haggerty et al. 2010). Dresen *et al.* (Dresen et al. 2011) reported an HPLC/MS/MS method for detection of aminoalkylindole compounds in human serum using a LLE, with sensitivity ranging from 0.1 to 2.0 ng/mL. They concluded that cyclohexylphenyl-containing cannabimimetics should be analyzed by Gas Chromatography-Mass Spectrometry (GC-MS) methods, as both electrospray ionization and chemical ionization showed poor responses during method optimization. While our method used a less sensitive linear range, it was shown to be suitable for the objective of this study. Additionally, Kacinko *et al.* (Kacinko et al. 2011) published an HPLC/MS/MS method validation for quantification of JWH-018, JWH-073, JWH-019, but with only one transition ion found for JWH-250, reported this method was only appropriate for qualitative identification of this analyte. An HPLC/MS method was determined for the detection of twenty-five synthetic cannabinoid compounds, including CP47,497, CP47,497-C8 and JWH-250 in blood which required only a 100 μL sample

volume (Ammann et al. 2012). Similar to results presented in this study, significant matrix effects were seen with both CP47,497 (82-89%) and CP47,497-C8 (73-84%) in blood. Matrix effect experiments, summarized in Table IV, demonstrate that there were significant effects of ion suppression produced in brain specimens tested.

We have previously reported the phenomenon of ion suppression in brain (Poklis et al. 2010) without adverse effects on the method validation, but a literature search provided no other data for ion suppression/enhancement effects of brain in HPLC systems. Data obtained from accuracy/bias studies, shown in Table II, along with assessment of SCB selectivity using 10 separate tissue lots (fortified with 60 ng/g of each analyte), for a total sample size of 30, provide evidence that the observed ion suppression does not affect other validation parameters. No peaks were detected that co-eluted with the analyte or ISTD peaks of interest in any of the 10 lots of brain, which demonstrates a lack of interference from endogenous components and solidifies selectivity of the assay. Importantly, the same pattern and magnitude of ion suppression was observed for each of the deuterated ISTDs (Table IV). Taken together, the ion suppression produced by endogenous brain components does not appear to affect the overall method validation.

Adverse effects of synthetic cannabinoids in humans are communicated primarily as case reports. Thus, there is a gap in knowledge pertaining to synthetic cannabinoid

disposition and related toxicities. Mice were used for the present validation study in order to procure biological tissue and assess the pharmacological effects due to exposure of the synthetic cannabinoid, CP47-497. Following a 30 mg/kg injection of CP47,497, mice displayed cannabimimetic behavioral effects, consistent with CB1 receptor activation (Compton et al. 1993), including a decrease in spontaneous locomotor activity with the presentation of catalepsy, antinociception and hypothermia as previously reported in our laboratory (Wiebelhaus et al. 2012) and in other laboratories (Wiley et al. 2012). These cannabimimetic behavioral results are in agreement with previously published reports of quantified SCB concentrations in brain after inhalation exposure (Wiebelhaus et al. 2012; Poklis, Amira, Wise, Wiebelhaus, Haggerty & Poklis 2012; Poklis, Amira, Wise, Wiebelhaus, Haggerty, Lichtman, et al. 2012; Haggerty et al. 2010).

3.6 Conclusion

The HPLC/MS/MS method validation presented herein is a straightforward, selective, and accurate method for the determination of three synthetic cannabinoid drugs belonging to the cyclohexylphenol and aminoalkylindole structural classes. The assay required a simple liquid-liquid extraction procedure before HPLC/MS/MS analysis. Importantly, this is the first full method validation of CP47,497, CP47,497-C8 and JWH-

250 synthetic cannabinoid compounds in tissue. This study has important implications for preclinical research settings where it is pertinent to demonstrate that SCBs are present in the brain, bolstering support that cannabimimetic effects result from a CNS-mediated mechanism of action. Additionally, providing a method to detect and quantify SCBs in brain may assist in further understanding the complex interplay between behavioral responses and SCB drugs, which is of particular interest in post-mortem or impaired driving cases where SCB use is suspected (Yeakel & Logan 2013). The development and validation of analytical methods for the detection and quantification of SCBs in complex biological matrices is critical for future investigation of the pharmacokinetic, pharmacodynamic and toxicological profiles of SCBs. Information garnered from animal studies will provide insight and lay the foundation necessary for approval of controlled human SCB studies, both which will provide invaluable knowledge for forensic toxicologists and law enforcement.

Chapter 4 – Evaluation of *in vitro* and *in vivo* indices of CP47,497-mediated effects

4.1 Introduction

Cannabis has been utilized for medicinal and recreational purposes for centuries and is still today the most commonly used illicit drug in the world (Schultes 1969; UNDOC 2013). However, much of our understanding of marijuana and specifically the primary psychoactive phytocannabinoid in *cannabis*, THC, has been elucidated over the last several decades due to the development and study of synthetic cannabinoids (SCBs), as reviewed in Chapters 1 and 2. Synthetic cannabinoids were first identified in Europe as designer drug components in herbal incense products through continuous surveillance of the illicit drug market (EMCDDA 2009; Lindigkeit et al. 2009; Ogata et al. 2013; Uchiyama et al. 2009). In 2009 in the U.S., the prototypical non-classical cannabinoid, CP47,497 was one of the first synthetic cannabinoid drugs detected as a powdered material confiscated by the Wisconsin state police (The Drug Enforcement Administration 2009).

CP47,497 was synthesized by Pfizer in the 1970's and in contrast to THC, CP47,497 is a high affinity CB_1 receptor agonist (K_i = 40.2 ± 0.47 nM versus K_i = 2.2 ± 0.47 nM, respectively) (Compton, Johnson, et al. 1992; Compton et al. 1993; Melvin et

al. 1984; Melvin et al. 1993; Weissman et al. 1982). Initial studies demonstrated the analgesic activity of CP47,497 was equipotent to morphine across several pain models in mice and rats, and from this work it was established that the benzopyran ring of THC and related analogs are nonessential for analgesic activity (Melvin et al. 1984; Melvin et al. 1993; Weissman et al. 1982).

Although these SCB compounds were designated specifically for research purposes, SCBs have emerged as drugs of abuse, yet very few published studies have reported the pharmacological consequences of these compounds in controlled laboratory studies. This dissertation was specifically focused on studying the behavioral consequences of CP47,497 administration, as it is both known to bind at the CB_1 receptor and has been identified in biological specimens from clinical and toxicology reports, confirming its abuse in humans (Heltsley et al. 2012; Yeakel & Logan 2013). Importantly, in the time since the initial CP47,497 pharmacological studies were published, new research tools have been developed to investigate the mechanism of action including a selective CB_1 receptor antagonist (rimonabant, or SR141617A) (Compton et al. 1996; Rinaldi-Carmona et al. 1994; Rinaldi-carmona et al. 1995) and genetically modified mice devoid of the CB_1 receptor (Ledent et al. 1999; Zimmer et al. 1999).

Studies from our laboratory and other laboratories support the ability of cannabinoids to produce dose-dependent cataleptic, antinociceptive, hypothermic, and hypolocomotor effects in rodents (Agarwal et al. 2007; Cravatt et al. 2001; Lichtman et al. 1996; Martin et al. 1995; Meng et al. 1998; Monory et al. 2007; Varvel et al. 2006; Wiley et al. 2005). Additionally, CB_1 binding information for most synthetic cannabinoids has been published and illustrate that *in vitro* binding affinity of cannabinoid agonists is a reliable predictor of observing cannabimimetic effects *in vivo* (Compton, Gold, et al. 1992; Compton, Johnson, et al. 1992; Huffman et al. 1994; Huffman et al. 2005; Little et al. 1988; Wiley et al. 1998). Based on this correlate, and reports that CP47,497 is a higher affinity agonist compared to THC (Compton et al. 1993; Melvin et al. 1993), we predict that CP47,497 will demonstrate enhanced potency versus THC in behavioral assays, and this will be achieved through a CB_1 receptor mediated mechanism. Therefore, similar to THC, CP47,497 poses a significant and perhaps, greater, risk for abuse, tolerance, and dependence. Moreover, the abuse of potent synthetic cannabinoids such as CP47,497 as "legal" THC substitutes may be very hazardous to public health and further scientific evaluation is required to understand consequences of use in humans.

The purpose of the present study was to examine *in vitro* and *in vivo* pharmacological effects of CP47,497 relative to THC. To do so, CP47,497 was tested in

assays capable of predicting abuse liability including activation of CB_1 receptors (Compton et al. 1993; Wiley et al. 1998), evaluation of cannabimimetic activity in the tetrad (Compton, Johnson, et al. 1992; Little et al. 1988; Martin et al. 1991), and interrogation of CP47,497 subjective effects in mice trained to discriminate THC from vehicle (Järbe & Henriksson 1974; Justinova et al. 2003; Wiley, et al. 2011). Tolerance to cannabimimetic behavioral effects was evaluated after repeated administration of CP47,497, and additionally, cross-tolerance to CP55,940 and THC was explored. Lastly, mice repeatedly treated with CP47,497 were tested for the development of physical dependence, assessed by somatic withdrawal signs upon challenge with CB_1 antagonist treatment. The studies herein will improve basic scientific knowledge of the acute pharmacological effects of the synthetic cannabinoid compound CP47,497, provided insight into its mechanism of action, and set the framework for further evaluating the behavioral consequences following chronic abuse (i.e., tolerance development and dependence liability).

4.2 Materials and Methods

Subjects

Drug naïve, male, ICR (CD-1) mice (Harlan Laboratories, Dublin, VA) weighing 22-32 grams (g) were obtained for use in cumulative dose-response, time-course, antagonism, tolerance, and withdrawal experiments. Male and female, $CB_1^{(-/-)}$ and $CB_1^{(+/+)}$ mice (19-27 g) backcrossed at least 13 generations onto a C57BL/6J background were obtained from the Transgenic Colony at Virginia Commonwealth University (VCU). Male, C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) weighing 20–25 g were utilized in drug discrimination and cumulative dose-response experiments as described below.

Mice were housed in clear plastic cages in VCU's temperature-controlled (22 ± 2 °C) animal care facility on a 12-hour light/dark cycle with *ad libitum* water and standard rodent chow (7012 Teklad Mouse/Rat Diet, Harlan Laboratories), unless otherwise described. Mice were individually housed the day prior to behavioral testing for acute dosing and drug discrimination experiments, and housed 4-6 mice per cage for repeated administration (tolerance and dependence) experiments.

Drugs and Chemicals

CP47,497 was purchased from Cayman Chemical (Ann Arbor, MI), CP55,940 from Tocris Bioscience (Minneapolis, MN), and THC and rimonabant were obtained from the National Institute of Drug Abuse (NIDA) Drug Supply Program (Rockville, MD). All vehicle (ethanol:emulphor:saline in a ratio of 1:1:18) and drug (CP47,497, THC, CP55,940 and rimonabant; i.p. or s.c.) injections were administered at a volume of 0.1 mL per 10 g of body mass. For acute studies, mice (ICR, C57BL6/J, $CB_1^{(-/-)}$ and $CB_1^{(+/+)}$) received drug treatment via intraperitoneal injections. In antagonist and precipitated-withdrawal studies, rimonabant was delivered subcutaneously (3 and 10 mg/kg). For drug-discrimination and tolerance and dependence experiments, C57BL6/J and ICR mice, respectively, received vehicle, CP47,497, THC and CP55,940 drug treatments via subcutaneous route of administration.

For binding studies, [^{35}S]-GTPγS (1250 Ci/mmol) was purchased from PerkinElmer (Waltham, MA), scintillation fluid (ScintiSafe Econo 1) and Whatman GF/B glass fiber filters from Fisher Scientific (Pittsburgh, PA); and GDP, GTPγS, adenosine deaminase, and serum albumin (BSA) from Sigma-Aldrich (St. Louis, MO).

Agonist-Stimulated [^{35}S]- GTPγS Binding in Brain

Effects of CP47,497, CP47-497-C8 and JWH-250 compounds on

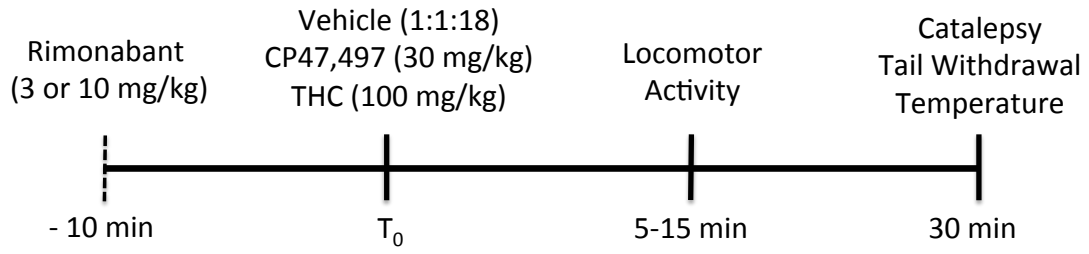
G-protein coupled signal transduction were investigated using agonist-stimulated guanosine-5'-O-(3-[35 S]thiotriphosphate) ([^{35}S]- GTPγS) activation. For each experiment, whole brain tissue was thawed from frozen storage (-80 °C) and homogenized in membrane buffer (50 mM Tris-HCl, 3 mM $MgCl_2$, 1 mM EGTA, pH 7.4), with resultant homogenates centrifuged at 50,000 x g for 10 min at 4°C. The supernatant was discarded and pellets were suspended with 5 mL of assay Buffer A (50 mM Tris-HCl, 3 mM $MgCl_2$, 0.2 mM EGTA, 100 mM NaCl, pH 7.4) with protein concentration determined by ultraviolet spectrophotometry. Samples were incubated for 15 minutes at 30 °C with adenosine deaminase (3 mU/ml), to limit basal activity by adenosine receptors. Concentration-effect curves were constructed by incubating 5 µg of membrane protein in assay while adding increasing concentrations of CP55,940 (0.001 µM to 30 µM), CP47,497 (0.0001 µM to 30 µM), CP47,497-C8 (0.0001 µM to 30 µM), and JWH-250 (0.0003 µM to 30 µM). Next, 30 µM GDP, 0.1 nM [^{35}S]-GTPγS and assay Buffer B (50 mM Tris-HCl, 3 mM $MgCl_2$, 0.2 mM EGTA, 100 mM NaCl, pH 7.4, and 1.25 g/L BSA) were added to a total volume of 0.5 mL. Nonspecific binding of membrane preparations was assessed in the presence of 20 µM unlabeled GTPγS, with basal activity determined in the absence of agonist. Samples were vortexed and the reaction (30°C) was terminated after 2 hours by rapid vacuum filtration through Whatman G/FB glass fiber filters, followed by three rinses in Tris buffer (50 mM Tris-HCl, pH 7.4, 4°C). Bound radioactivity was determined in triplicate after a 10-hour

extraction of filters in ScintiSafe Econo 1 scintillation fluid using liquid scintillation spectrophotometry as previously described (Falenski et al. 2010).

Tetrad Behavioral Assessment

Mice were acclimated to the test environment for at least 1 h prior to testing for tetrad components: spontaneous activity, catalepsy, antinociception, and hypothermia (Martin et al. 1991; Wiley 2003). For all locomotor studies, five minutes after vehicle or drug treatment, mice were placed into clear acrylic boxes (approximately 44.5 cm x 22.25 cm x 20.0 cm) contained within sound-attenuating cabinets equipped with an LED light source and fans for general air circulation and creation of white noise (customized and constructed at VCU) five minutes after vehicle or drug treatment. The distance traveled (meters) and time spent immobile (seconds) for each mouse was collected and recorded for 10 minutes using Fire-i™ digital cameras purchased from Unibrain (San Ramon, CA) and ANY-maze™ video tracking software purchased from Stoelting Company (Wood Dale, IL).

Schematic 4.1 – Timeline of acute drug treatment and behavioral testing in mice



Thirty minutes after vehicle or drug treatment, mice were assessed for catalepsy, followed by antinociception, and finally, hypothermia (see **Figure 1.4** and **Schematic 4.1**). Catalepsy was measured using the horizontal bar test in which both forelimbs of the mouse were placed on a horizontal bar (approximately 1.25 cm in diameter and 4.45 cm parallel to the bench top), with the duration of a fixed and motionless posture (except normal breathing) recorded by stopwatch over a 60 second interval. Antinociception was determined by warm water (52 °C) tail immersion whereby the distal end (approximately 1 cm) of the tail was immersed in the water bath and the latency of the mouse to withdrawal its tail recorded (to the nearest tenth of a second). A 10 second cut-off was used to minimize tail damage. Antinociception data was transformed to represent a maximum percent effect (%MPE) by the following formula: %MPE = [(test latency – pretreatment latency)/(10–pretreatment latency)] × 100. Body temperature measurements (recorded to the nearest 0.1 °C) were collected by inserting a rectal

probe, lubricated with mineral oil and attached to a telethermometer (Yellow Spring Industries Inc., Yellow Springs, OH), to a 2 cm depth.

Cumulative Dose-Response Studies

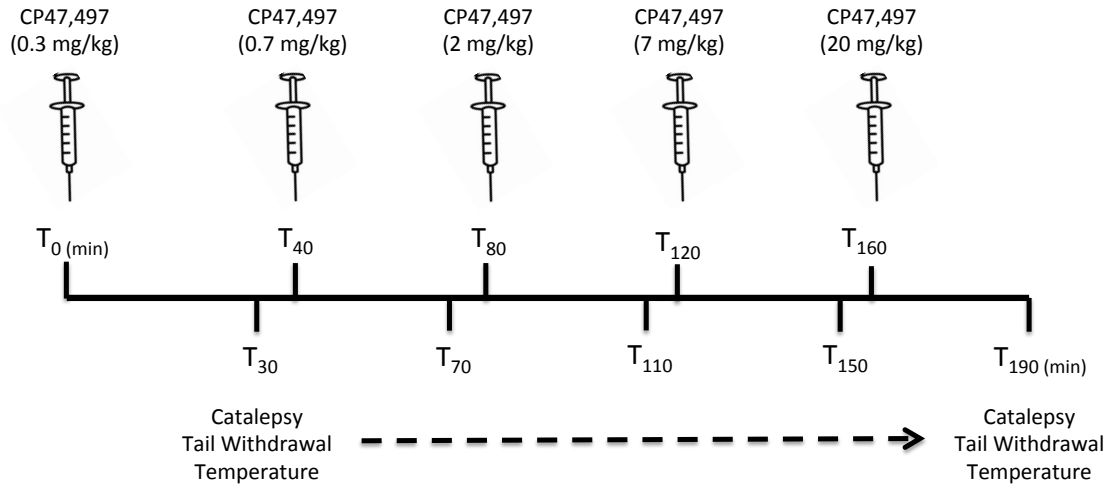
A cumulative dosing-paradigm was used to construct dose-response curves. ICR mice received increasing doses of drug (CP47,497 or THC) or repeated vehicle every 40 minutes, with behavioral testing performed 30 minutes after each injection. Therefore, results represent catalepsy, antinociception and hypothermia observations 30 minutes after each of the respective cumulative doses of CP47,497 (0.3, 1, 3, 10, and 30 mg/kg) and THC (3, 10, 30, 100 and 200 mg/kg). For example, 30 minutes after the cumulative dose of 30 mg/kg CP47,497 was injected at 160 minutes, behavioral assessment will be performed the 190 minute time point (see **Schematic 4.2** below).

Acute Drug Administration Studies

For acute drug administration studies, ICR mice received CP47,497 (30 mg/kg) or THC (100 mg/kg THC), which approximate the ED_{50} value calculated from antinociception dose-response experiments. The treatment doses (30 mg/kg CP47,497

and 100 mg/kg THC) were chosen based on calculated ED_{84} values derived from linear regression analysis of antinociceptive data (see Results, **Figure 4.2**). For antagonism studies, ICR mice received by subcutaneous (s.c.) injection either 3 or 10 mg/kg rimonabant 10-minutes before test drug, and were assessed in the tetrad as previously described. For tetrad dose-response evaluation of CP47,497 in C57BL6/J mice, single injections of 1, 3.2, and 10 mg/kg (i.p.) were administered, followed by tetrad testing as described. In complimentary receptor studies, $CB_1^{(-/-)}$ and $CB_1^{(+/+)}$ mice received vehicle or 30 mg/kg CP47,497 and were tested for tetrad behaviors as described.

Schematic 4.2 – Cumulative drug dosing and behavioral assessment timeline



Behavioral Evaluation of Somatic Withdrawal Signs

For dependence experiments, mice received CP47,497 (15 mg/kg), CP55,940 (2 mg/kg) or THC (50 mg/kg) twice daily (approximately 0900 and 1700 hours (h)) for 5.5 days, delivered subcutaneously. Half of the ED_{84} dose used for the acute experiments was administered during repeated administration experiments for CP47,497 and THC, and the dose of CP55,940 was chosen based on previously established literature examining tolerance of repeated CP55,940 administration in ICR mice (Fan et al. 1994; Fan et al. 1996). The same acrylic boxes and cabinets described for locomotor assessment were utilized. After final drug administration (day 6, 0900 h injection), mice were acclimated for 30 minutes to behavioral chambers, which consisted of an opaque acrylic box (20 x 20 x 20 cm), equipped with a clear front panel and mirrored back panel, enclosed inside of activity cabinets. After the 30-minute period, mice were removed from chambers, received rimonabant (10 mg/kg, i.p.), and were then immediately placed back into their boxes for a 1-hour observation period. The acrylic boxes were cleaned with water after the acclimation but before the observation period to remove urine and feces present. Somatic withdrawal signs (paw flutters/tremors/clapping, headshakes, and jumping) were captured through the clear front acrylic panel (and by mirrored reflection of the backside of box) by Fire-i™ digital cameras, and recorded by ANY-maze™ digital tracking software for one hour. Somatic

withdrawal signs were observed and scored by a researcher blinded to experimental treatment.

Tolerance Experiments

The ability of CP47,497 to produce tolerance and/or cross-tolerance to catalepsy, antinociception and hypothermia was assessed by treating mice with CP47,497 (15 mg/kg), CP55,940 (2 mg/kg) or THC (50 mg/kg THC) twice daily for five and a half days, with a cumulative CP47,497 (1, 3, 10, 30 and 56 mg/kg) dose-response regimen administered on day seven, 24-hours (to allow drug washout) after the last morning dose on day six.

Drug Discrimination Assay

C57BL/6J mice were maintained at 85–90% of free feeding body weight by restricting daily ration of standard rodent chow, with *ad libitum* water available except during training and testing sessions. Training and test sessions were conducted at similar times during the light phase of a 12 h light/dark cycle. Sound- and light-attenuated mouse operant conditioning chambers (MED Associates, St. Albans, VT)

were used for behavioral training and testing; each chamber housed an acrylic operant box (18 x 18 x 18 cm) equipped with a house light and two nose poke apertures (left and right) with a recessed well between the two apertures (pictured in **Appendix II**). Houselights were illuminated during all operant sessions and fan motors provided ventilation and masked outside noise. A computer with Logic ‘‘1’’ interface and MED-PC software (MED Associates) controlled contingency schedules and data recording. Mice were trained to respond in one aperture following administration of 5.6 mg/kg THC, and to respond in the opposite aperture following vehicle administration (**Figure 4.1**). A sweetened pellet served as reinforcement and was delivered on a fixed-ratio 10 (FR10) schedule. Each incorrect response reset the FR requirement. Daily 15-min training sessions were performed Monday–Friday with dosing following a double alternation sequence of drug or vehicle (e.g., drug, drug, vehicle, vehicle) until mice met two criteria during nine of 10 consecutive sessions: (criterion i) correct completion of the first FR10 (e.g., first 10 consecutive responses on condition appropriate aperture) and (criterion ii) >80% of the total condition (drug or vehicle) appropriate responding. During test sessions, responses in either aperture delivered reinforcement according to an FR10 schedule. When these two criteria were met, acquisition of the discrimination was established and substitution testing began. Stimulus substitution tests were conducted on Tuesdays and Fridays during 15-min test sessions with training continued on remaining weekdays. Control tests were conducted with the training dose of the drug

(5.6 mg/kg THC) or vehicle. For substitution tests, CP47,497 was administered subcutaneously (doses of 0.03, 0.1, 0.3, 1 and 3.2 mg/kg) 30 minutes before the test session.

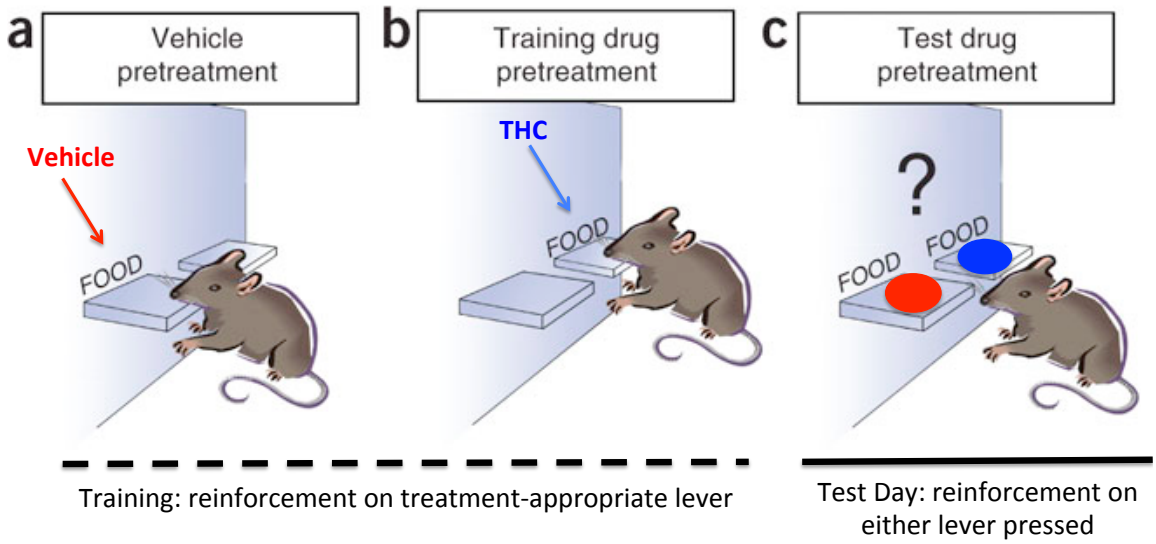


Figure 4.1 – Drug discrimination paradigm assays the subjective effects of drugs. C57BL/6J mice were trained to respond in one aperture following administration of 5.6 mg/kg THC and to respond in the opposite aperture following CP47,497 administration. During training sessions **(a,b)**, reinforcement is provided for responses on treatment-appropriate lever; in contrast, reinforcement is delivered on either lever pressed during test sessions **(c).** Image adapted from (Solinas et al. 2006).

Drug Extraction and Analysis from Biological Specimens

Blood, collected via cardiac puncture, and brain specimens were harvested one hour after drug administration unless otherwise stated, and placed in frozen storage (-80 °C) until analysis. Samples were thawed, homogenized if necessary, then extracted

and quantified by HPLC/MS/MS as described in Chapter 3, Materials and Methods section.

Data Analysis and Statistics

Unless specifically stated otherwise, presented data are reported as mean (± SEM). Experimental data were analyzed by one- and two- way analysis of variance (ANOVA) with time, treatment, dose or genotype as between-subjects factors, where appropriate. Significant ($p < 0.05$) ANOVAs were followed by Bonferroni's post-hoc test, unless stated otherwise, and used to make multiple comparisons to establish dose-dependency and evaluate effects between agonist drug treatments. In drug-discrimination studies, Dunnett's post-hoc analysis was used to make comparisons versus control. Determination of ED_{50} values and 95% confidence limits (CL) were calculated by standard linear regression analysis of generated drug dose-response curves. Following a method described by Colquhoun *et al.,* potency ratio with 95% CL were determined by comparing potency between drug treatments groups (Colquhoun, 1971). Significance was denoted for *p*-values less than 0.05 with all statistical analyses performed using GraphPad Prism® statistical software (Version 5.0; LA Jolla, CA).

4.3 Results

4.3.1 CP47,497, CP47,497-C8, and JWH-250 are high efficacy partial agonists at the CB_1 receptor

As with the HPLC/MS/MS validation studies in Chapter 3, we examined if representative cyclohexylphenol (CP47,497, CP47,497-C8) and aminoalkylindole (JWH-250) compounds would activate G-protein receptors in a similar manner to THC using an *in vitro* functional assay. The ability of the synthetic cannabinoids, CP47,497, CP47,497-C8, and JWH-250 to bind to CB_1 receptors has been previously reported (Compton et al. 1993; Huffman et al. 2005; Melvin et al. 1993). However, fewer data have been published regarding the ability of these abused synthetic cannabinoids to stimulate G-proteins upon CB_1 receptor activation (Gurney et al. 2014). Resultantly, we investigated receptor activation of G-proteins by CP47,497, CP47,497-C8, and JWH-250 using CP55,940-stimulated [^{35}S]-GTPγS (GTPγS) binding in mouse brain membrane preparations (Burkey, Quock, Consroe, Roeske, et al. 1997; Selley et al. 1996).

Figure 4.2 illustrates that all three SCBs - CP47,497, its C8 homologue, and JWH-250 behave similar to CP55,940 as partial agonists with a high level of activity in the GTPγS assay, which has previously been described as a high efficacy partial agonist (Burkey, Quock, Consroe, Ehlert, et al. 1997; Devane et al. 1988).

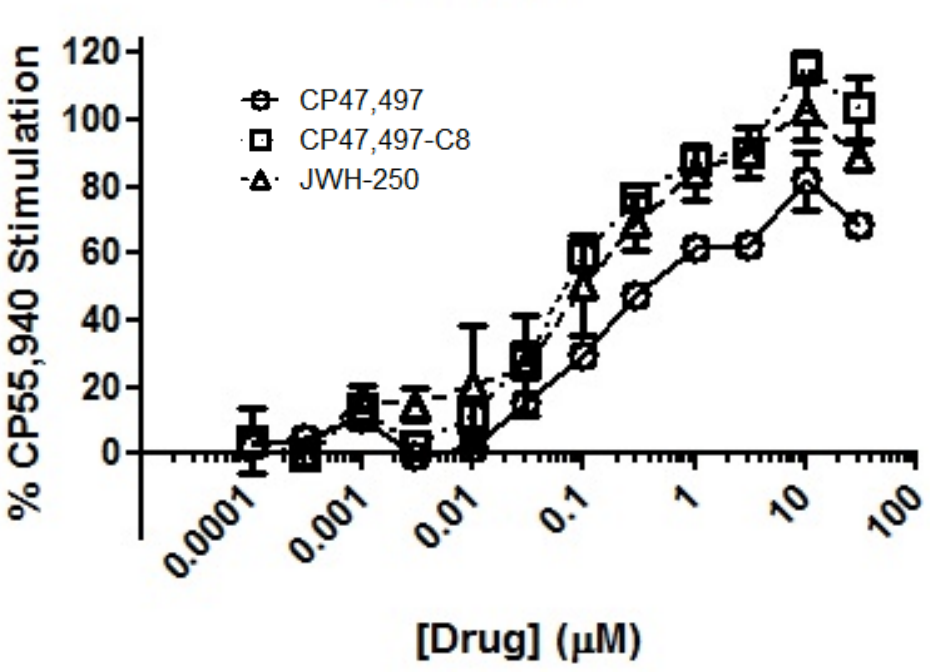


Figure 4.2 – Effects of synthetic cannabinoid agonist stimulation of [^{35}S]-GTPγS binding in whole brain membrane preparations. Concentration-effect curves of CP47,497 (circles), CP47,497-C8 (squares) and JWH-250 (triangles) represent mean ± SEM (Table 4.1) from triplicate analyses; n = 3 brains per treatment group.

A two-way ANOVA revealed main effects for drug treatment [$F(2,66) = 25.3$, $p < 0.0001$] and concentration [$F(10,66) = 89.4$, $p < 0.0001$], however, no significant drug treatment and concentration interaction effect was observed [$F(20,66) = 1.3$, $p < 0.21$]. One-way ANOVA with Bonferroni's *post-hoc* analyses revealed significant differences between drug EC_{50} (50% effective-concentration) values [$F(3,14) = 18.7$, $p < 0.0001$]. CP47,497, its C8 homologue, and JWH-250 had EC_{50} values that were higher than CP55,940 ($p < 0.05$). Significant differences in E_{Max} values were observed between drugs [$F(3,14) = 4.1$, $p < 0.03$]. And the *post-hoc* analysis revealed CP47,497-C8 had an E_{Max} significantly higher than CP55,940 ($p < 0.05$).

Table 4.1 – E_{max} and EC_{50} values of SCB agonist-stimulated [^{35}S]-GTPγS in brain membranes. Data represent mean ± SEM, from n = 3 experiments in triplicate.

Measure	CP55,940	CP47,497	CP47,497-C8	JWH-250
E_{max} (% stimulation)	73 ± 2.6	62 ± 4.7	84 ± 4.3[b]	68 ± 4.1
EC_{50} (nM)	12.6 ± 2	135 ± 2[a]	83 ± 7[a]	135 ± 4[a]

[a] $p < 0.05$ versus CP55,940 as determined by ANOVA with Bonferroni's *post-hoc* test

[b] $p < 0.05$ versus CP47,497 as determined by ANOVA with Bonferroni's *post-hoc* test

Table 4.2 – Mean ± SEM data from SCB agonist stimulated [^{35}S]-GTPγS binding experiments; values represent normalization to % stimulation of CP55,940 (Panel A) and calculated % stimulation for each analyte (Panel B).

A

	CP47,497		CP47,497-C8		JWH-250	
[Drug] (μM)	Mean	SEM	Mean	SEM	Mean	SEM
0.0001	2.27	2.76	3.70	9.72	--	--
0.0003	4.61	0.55	-0.65	1.38	0.05	3.74
0.001	10.47	1.85	13.84	6.38	16.44	3.41
0.003	-0.78	1.01	2.43	1.63	14.76	5.10
0.01	1.62	1.19	11.59	5.04	20.42	17.91
0.03	15.32	3.14	28.22	4.90	26.28	15.09
0.1	29.86	1.76	59.56	4.44	50.61	14.95
0.3	47.59	0.76	76.52	2.68	69.34	8.47
1	61.85	3.51	88.33	4.35	84.34	8.40
3	62.42	3.26	90.17	2.96	90.09	7.37
10	81.66	8.95	115.79	4.58	103.19	9.31
30	68.26	2.14	103.48	9.31	88.92	4.05
n	3	3	3	3	3	3

B

	CP55,940		CP47,497		CP47,497-C8		JWH-250	
[Drug] (μM)	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
0.0001	--	--	2.10	2.55	3.02	7.94	--	--
0.0003	--	--	2.47	1.60	-0.54	1.13	0.03	2.58
0.001	13.51	5.30	6.20	3.01	11.31	5.21	11.32	2.35
0.003	20.95	2.60	-0.21	0.81	1.99	1.33	10.16	3.51
0.01	30.44	3.99	2.81	1.22	9.47	4.12	14.05	12.33
0.03	49.43	4.66	17.80	3.34	23.05	4.00	18.09	10.39
0.1	60.35	4.50	26.50	1.44	48.65	3.63	34.83	10.29
0.3	69.60	5.43	39.71	3.97	62.52	2.19	47.72	5.83
1	71.01	4.96	53.69	3.36	72.16	3.55	58.05	5.78
3	77.88	7.85	53.35	3.86	73.67	2.42	62.01	5.07
10	74.71	4.95	69.18	7.48	94.59	3.74	71.02	6.41
30	71.69	2.83	61.69	1.76	84.54	7.60	61.20	2.78
n	9	9	3	3	3	3	3	3

4.3.2 CP47,497 elicits potent cannabimimetic effects in mice

After establishing functional *in vitro* responses, studies using CP47,497 were designed to investigate the physiological effects in mice using well-established behavioral models. In the present study, we investigated whether CP47,497 produces dose- and time-dependent cannabimimetic effects. **Figure 4.3** depicts that cumulative vehicle dosing (5 total injections) alone did not produce cannabimimetic behavioral effects in any of the behavioral outcome measures. Acute CP47,497 treatment produced significant catalepsy [F (5,35) = 91.6, $p < 0.0001$] **(Figure 4.3, Panel A),** as administration of 3, 10, and 30 mg/kg produced significant increases in catalepsy compared to vehicle (Veh) ($p < 0.05$). Additionally, 10 and 30 mg/kg induced significantly longer durations of catalepsy versus 3 mg/kg ($p < 0.05$). Congruent with previous literature (Compton, Johnson, et al. 1992; Compton et al. 1996; Martin et al. 1991; Monory et al. 2007; Weissman et al. 1982; Zimmer et al. 1999), THC treatments (30, 100, and 200 mg/kg) significantly induced catalepsy compared to vehicle treatment [F (5,35) = 2.7, $p < 0.0001$]. Furthermore, 30 mg/kg THC resulted in a significantly lower duration of catalepsy than 100 and 200 mg/kg ($p < 0.05$). These data demonstrate a dose-dependent relationship for CP47,497 and THC cataleptic effects. Comparison between CP47,497- and THC-induced catalepsy revealed a potency ratio of 7.2 (5.4-

9.6; 95% confidence limits (CL)), calculated from ED_{50} values; CP47,497 had an ED_{50} of 4.7 (3.8-5.9) mg/kg and THC had an ED_{50} of 33.8 (26.1-43.7) mg/kg. Drug effects for CP47,497-induced catalepsy were significantly higher than the THC-treated group at 3 mg/kg ($p < 0.0001$), 10 mg/kg ($p < 0.0001$) and 30 mg/kg ($p < 0.01$).

Antinociceptive data in **Figure 4.3, Panel B**, illustrate a significant effect [F (5,35) = 24.8, $p < 0.0001$], but only the highest CP47,497 dose tested, 30 mg/kg, significantly increased antinociception as compared to vehicle-treated mice. Dose-dependent antinociceptive effects were not observed for CP47,497, as no differences were observed versus vehicle for doses less than 30 mg/kg tested; however, 30 mg/kg CP47,497 produced significantly increased antinociception versus all other doses tested ($p<0.05$). Conversely, 30, 100, and 200 mg/kg THC doses significantly increased antinociception compared to vehicle [F (5,35) = 9.80, $p < 0.0001$]. Additionally, 100 and 200 mg/kg THC resulted in significantly higher levels of antinociception compared to 10 mg/kg ($p < 0.05$). Between drug comparisons showed that 30 mg/kg CP47,497 produced significantly more antinociception than THC ($p < 0.0001$) (**Figure 4.3, Panel B**).

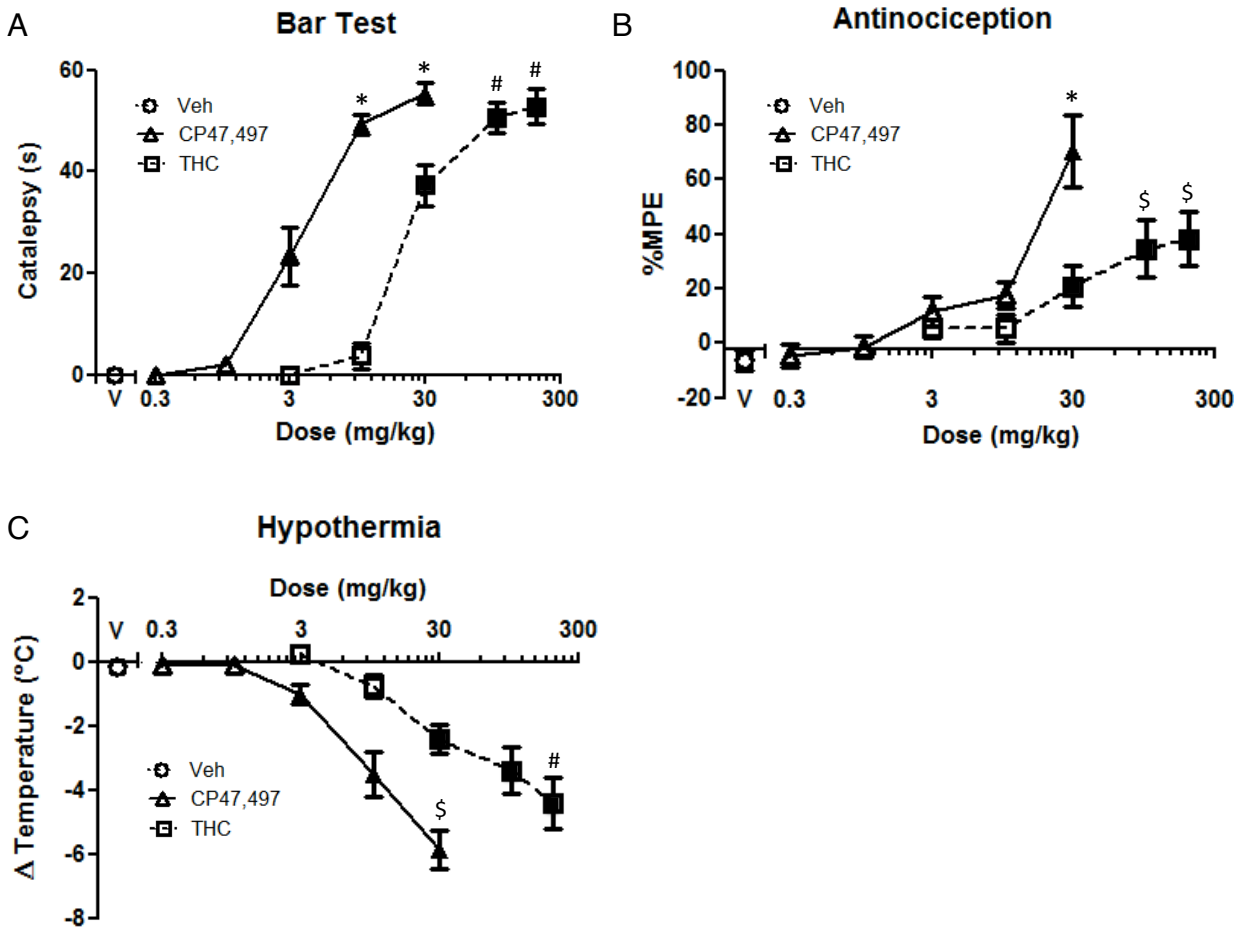


Figure 4.3 – CP47,497 and THC dose-responsiveness for catalepsy, antinociception, and hypothermia outcome measures. At the doses tested, both CP47,497 and THC produced dose-dependent effects of catalepsy in the horizontal bar test **(A)** and hypothermia **(C)**. Significant antinociceptive effects in the warm water tail withdrawal assay were observed at the highest doses of CP47,497 and THC tested **(B)**. Leftward shift in the catalepsy and hypothermia dose-response curves of CP47,497 (circles) from THC (squares) indicate increased potency of CP47,497 with calculated potency-ratios and 95% confidence limits of 7.2 (5.4- 9.6) and 7.7 (4.6-12.8), respectively. As THC antinociceptive data lacked a complete dose-response range at the doses tested, ED_{50} values and potency ratio could not be calculated. Filled symbols denote significance versus vehicle ($p < 0.05$); *$p < 0.05$ versus 3 mg/kg, \$$p < 0.05$ versus 10 mg/kg, #$p < 0.05$ versus 30 mg/kg. n = 8 per group. Pre-treatment (i.e. baseline) means ± SEM for tail withdrawal latencies (s) for vehicle-treated mice were 2.0 ± 0.2, 2.6 ± 0.3 for CP47,497-treated mice, and 2.0 ± 0.1 for THC-treated mice. Baseline temperatures (°C) were 37.7 ± 0.2 for vehicle-treated, 37.1 ± 0.2 for CP47,497-treated, and 36.9 ± 0.1 for THC-treated mice.

The antinociceptive ED_{50} value for CP47,497 was 26.2 (11.0 - 62.0) mg/kg. Since the doses of THC tested did not produce a 50% maximum effect, an ED_{50} value and potency ratio for THC could not be calculated.

CP47,497 produced significant hypothermic effects at 10 and 30 mg/kg doses compared to vehicle [F (5,35) = 55.42, $p < 0.0001$], with 10 mg/kg producing significantly less hypothermia than 30 mg/kg ($p<0.05$) **(Figure 4.3, Panel C)**. Similar to catalepsy and antinociception measures, THC (30,100, and 200 mg/kg) produced significant hypothermia versus vehicle [F (5,35) = 29.81, $p < 0.0001$], with 30 mg/kg producing significantly less hypothermia than 200 mg/kg THC ($p<0.05$). The potency ratio (95% confidence limits) for hypothermia was 7.7 (4.6-12.8) with a calculated ED_{50} of 12.1 (7.5-19.6) mg/kg for CP47,497 and an ED_{50} of 110.1 (69.7-172.7) mg/kg for THC. Determination of ED_{50} and potency ratio for hypothermia data were calculated using and Emax of -6 °C, which represents the maximal mean decrease in temperature from the CP47,497-treated group. Between drug comparisons revealed that CP47,497 produced significantly more hypothermia than THC at 10 mg/kg ($p < 0.0001$), and 30 mg/kg ($p < 0.0001$).

4.3.3 Cannabimimetic duration of action of CP47,497 is similar to THC

Next, we sought to examine if the potent effects of CP47,497 were maintained over time, with particular interest in comparison to the temporal effects of THC. In these studies, mice received a single bolus dose of CP47,497 (30 mg/kg), THC (100 mg/kg) or vehicle with measurements taken at 0.5, 1, 2, 4, 8, 12 and 24 hours. Drug doses were selected from dose-response experiments as described in Materials and Methods (Section 4.2).

CP47,497 showed significant effects of time [$F(6,126) = 201.4$], dose [$F(2,126) = 81.7$] and a significant interaction between time and dose [$F(12,126) = 78.2$], for catalepsy ($p < 0.0001$) **(Figure 4.4, Panel A)**. CP47,497 significantly increased catalepsy at 2 and 4 hours versus THC ($p < 0.05$). Both CP47,497 and THC induced significant catalepsy at 0.5, 1, 2, 4, and 8 hours versus vehicle ($p < 0.0001$). CP47,497 **(Figure 4.4, Panel B)** showed significant increases in antinociception across time [$F(6,120) = 23.9$, $p < 0.0001$], dose [$F(2,120) = 13.1$, $p = 0.0002$], and there was a significant interaction between time and dose [$F(12,120) = 5.4$, $p < 0.0001$]. Bonferroni's correction revealed that antinociception was produced by CP47,497 at 0.5, 1, 2 ($p< 0.0001$), and 4 hours ($p < 0.01$), and by THC at 1, 2, and 4 hours ($p< 0.01$). Finally, CP47,497 elicited a transient increase in antinociception versus THC at 0.5 hours ($p < 0.05$). Eight hours following drug administration, the nociceptive effects of the hot water bath were no different in CP47,497- and THC-treated mice compared to vehicle-treated

mice ($p > 0.05$). CP47,497-induced significant hypothermic effects across time [$F(6,126) = 201.4$], dose [$F(2,126) = 81.7$], and there was a significant interaction [$F(12,126) = 78.2$] between time and dose ($p < 0.0001$) **(Figure 4.4, Panel B)**. Both CP47,497 and THC caused significant hypothermia at 0.5, 1, 2 4 and 8 hours ($p < 0.0001$), compared to vehicle treatment.

One-way ANOVA for quantification of CP47,497 in blood revealed significant changes in concentration across the time points tested [$F(5,13) = 9.63$, $p = 0.0005$]. Specifically, blood concentrations of CP47,497 were significantly higher at 1 and 2 hours versus circulating drug concentration at 8 hours ($p < 0.05$*),* and CP47,497 concentrations were also significantly higher at 1 hour versus 4 hours ($p < 0.05$*)* **(Figure 4.4, Panel D)**.

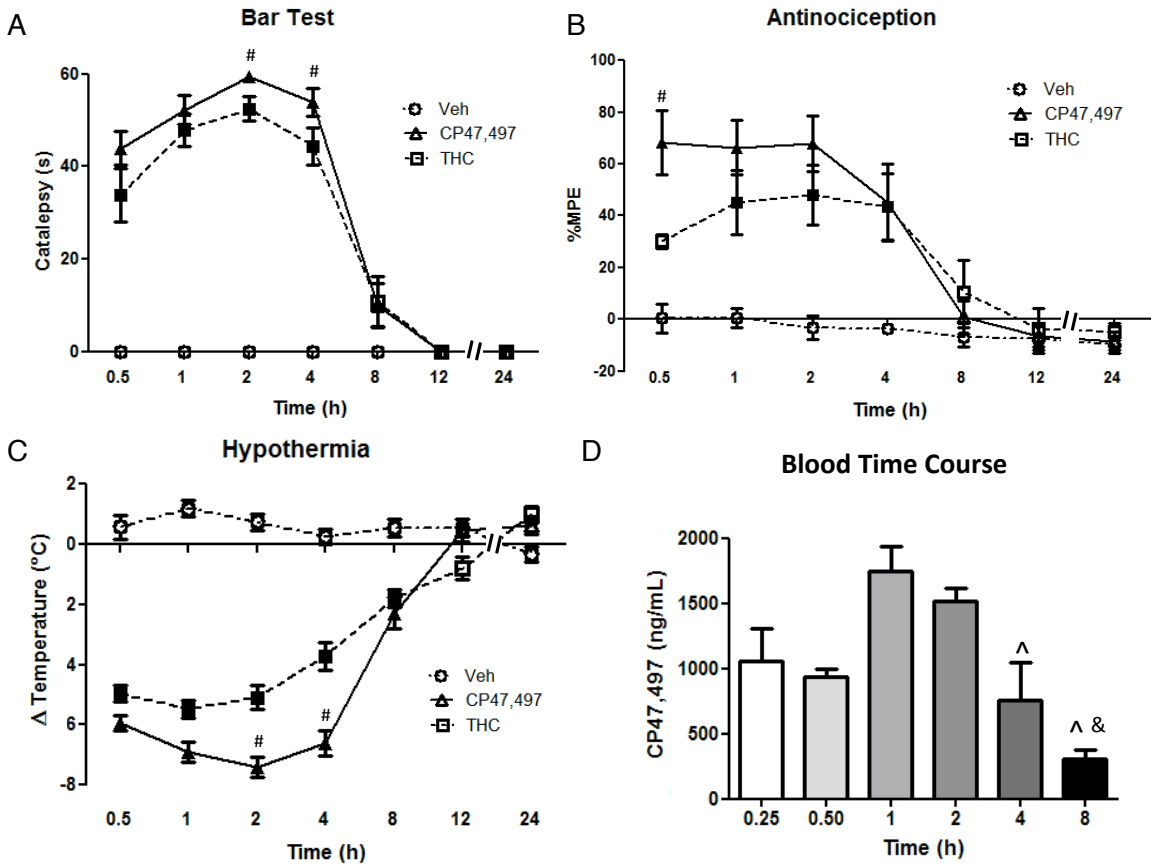


Figure 4.4 – Temporal cannabimimetic effects of CP47,497 and THC were significant from vehicle beginning at 0.5 hours and persisted up to 8 hours, depending on individual measure **(A, B, C)**. Two-way repeated measures ANOVA revealed significant interaction effects for **(A)** catalepsy [$F\ (12,108) = 29.49$, $p < 0.0001$], **(B)** antinociception [$F\ (12,120) = 5.39$, $p < 0.0001$], and **(C)** hypothermia [$F\ (12,126) = 78.16$, $p < 0.0001$] which follow a similar pattern for CP47,497 drug concentrations in whole blood **(D)** quantified by HPLC/MS/MS. Time and treatment effects were also significant ($p < 0.001$). Filled circles denote significance from vehicle. #$p < 0.05$ versus THC; ^$p < 0.05$ versus 1 hour time point; &$p < 0.05$ versus 2 hour time point. Data represent the mean ± SEM, n = 8 mice per group (A-C) or n = 2-4 mice per group (D).

4.3.4 CP47,497-induced catalepsy, antinociception, hypothermia and hypolocomotor effects are mediated by CB_1 receptors

To determine if CP47,497-elicited cannabimimetic effects were mediated through a CB_1 receptor mechanism, mice were pretreated with the selective CB_1 antagonist, rimonabant (3 or 10 mg/kg, s.c.), 10-minutes before CP47,497 (30 mg/kg, i.p.) or THC (100 mg/kg, i.p.) administration and assessed in the tetrad (**Schematic 4.1**). Significance was determined by a two-way ANOVA with Bonferroni post-hoc utilized for multiple comparisons. Experiments were designed to investigate the ability of low dose (3 mg/kg) or high dose rimonabant (10 mg/kg) to attenuate agonist-induced behavioral effects.

Statistical assessment using antagonist and drug treatment as between subject factors, revealed significant effects of antagonist [$F(2,69) = 122.90$, $p < 0.0001$] and drug treatment [$F(2,69) = 27.97$, $p < 0.0001$] for the presence of catalepsy, with a significant interaction effect observed [$F(4,69) = 33.35$, $p < 0.0001$]. **Figure 4.5, Panel A** illustrates that CP47,497 and THC produced significant catalepsy versus vehicle controls ($p < 0.0001$). Both 3 mg/kg and 10 mg/kg rimonabant (Rim) pretreatment significantly reduced the development of CP47,497- and THC-induced ($p < 0.0001$) catalepsy, with no differences

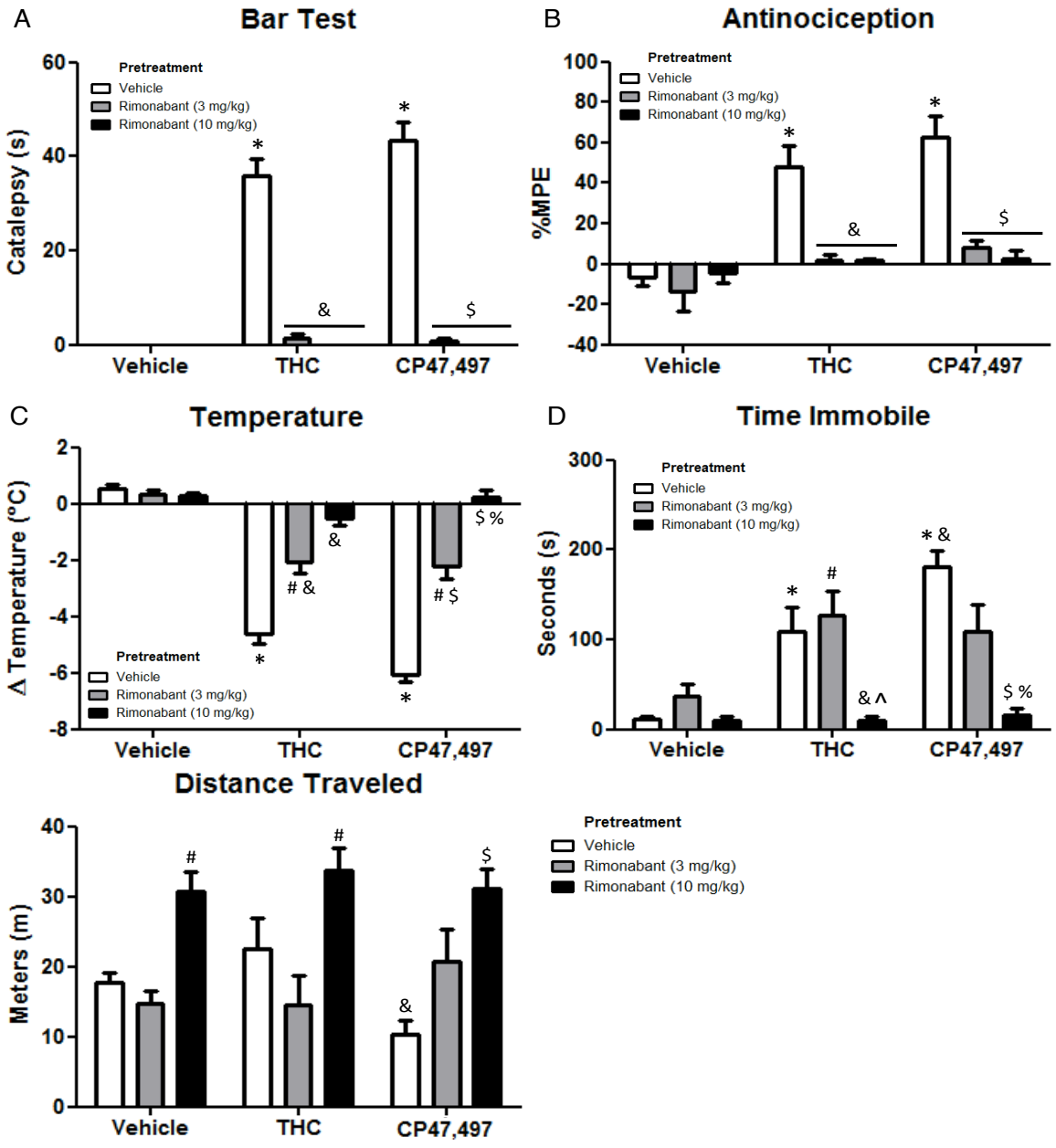


Figure 4.5 – Antagonism of cannabimimetic effects by low dose and high dose rimonabant. Pretreatment with low (3 mg/kg) and high (10 mg/kg) dose rimonabant reversed cataleptic **(A)** and antinociceptive **(B)** effects elicited by both CP47,497 (30 mg/kg) and THC (100 mg/kg). Significant CP47,497- and THC-induced hypothermic effects were observed versus control, and each were dose-dependently attenuated by rimonabant treatment **(C)**. High dose, but not low dose rimonabant produced significant reversal of time immobile elicited by CP47,497 and THC **(D)**. Compared to THC, administration

of CP47,497 caused a significant decrease in distance traveled, and was reversed by high dose rimonabant **(E)**. Significance denoted *$p < 0.05$ versus Veh/Veh, \$$p < 0.05$ vs. Veh/CP47,497, &$p < 0.05$ versus Veh/THC, #$p < 0.05$ vs. Rimonabant(3)/Veh, %$p<0.05$ vs. Rimonabant(3)/CP47,497, and ^$p < 0.05$ vs. Rimonabant(3)/THC. Data represent the mean ± SEM, n = 6-15 mice per group. Baseline means ± SEM for tail withdrawal latencies (s) and temperature (°C) are listed in Table 4.3.

observed between low and high dose rimonabant. During evaluation of catalepsy, 50% of CP47,497-treated mice (8 of 16) demonstrated hyperreflexia (or "popcorning") (Dewey 1986), three of which met criteria for data exclusion; that is, a hyperreflexic response each of four attempts to place the mouse on the horizontal bar. In contrast, only 7.7% of THC-treated mice (1 of 13) exhibited hyperreflexia and this mouse was excluded from the data set.

The ability of rimonabant to antagonize antinociceptive effects of CP47,497 and THC was next investigated. A two-way ANOVA revealed significant effects of drug [$F(2,73) = 12.29$, $p< 0.0001$] and antagonist treatment [$F(2,73) = 21.62$, $p < 0.0001$], as well as a significant interaction effect between drug and antagonist pretreatment [$F(4,73) = 4.86$, $p = 0.0016$]. Significant antinociception (**Figure 4.5, Panel B)** was observed after CP47,497 and THC treatment, compared to vehicle controls ($p < 0.0001$). Pretreatment with 3 mg/kg or 10 mg/kg rimonabant reversed agonist-induced antinociception by CP47,497 ($p < 0.001$) and THC ($p < 0.01$).

Rimonabant blocked the hypothermic effects of CP47,497 with significant effects observed for drug [$F(2,75)$ = 96.86, $p < 0.0001$], and antagonist treatment [$F(2,75)$ = 109.7, $p < 0.0001$], accompanied by a significant interaction of drug and antagonist effect [$F(4,75)$= 36.88, $p < 0.0001$] (**Figure 4.5, Panel C**). Compared to vehicle-treated mice, significant, dose-related hypothermic effects were observed with both CP47,497 and THC treatment ($p < 0.0001$) at 3 and 10 mg/kg rimonabant. Reversal of CP47,497- or THC-induced decreases in body temperature was observed with pretreatment of 3 mg/kg and 10 mg/kg rimonabant ($p < 0.0001$). Furthermore, pretreatment by rimonabant produced significant dose-related antagonism of hypothermia in both CP47,497 ($p < 0.0001$) and THC ($p < 0.05$) groups.

A two-way ANOVA of time immobile data, with antagonist and drug treatment as between subject factors, revealed main effects for antagonist [$F(2,75) = 14.1$, $p < 0.0001$] and drug treatment [$F(2,75) = 12.2$, $p < 0.0001$], with a significant interaction effect [$F(4,75)= 4.84$, $p < 0.01$] (**Figure 4.5, Panel D)**. CP47,497 ($p < 0.0001$) and THC ($p < 0.01$) groups significantly increased time immobile, compared to vehicle treatment. CP47,497 treatment significantly increased time immobile compared to THC ($p< 0.05$). CP47,497 ($p < 0.001$) or THC ($p < 0.05$) groups pretreated by high dose rimonabant (10 mg/kg) showed significant decreases in immobility from vehicle controls.

Assessment of distance traveled revealed a significant main effect for antagonist treatment [$F(2,75)= 16.9$, $p < 0.0001$], but no significant differences were observed with drug treatment [$F(2,75) = 0.6$, $p = 0.55$] nor was there an interaction between antagonist and drug treatment [$F(4,75) = 2.3$, $p = 0.06$]. CP47,497-treated mice displayed significant decreases in distance traveled versus THC-treated mice ($p < 0.05$). Significant increases in distance traveled were observed in vehicle ($p < 0.05$) and THC ($p < 0.01$) treated groups receiving high dose rimonabant versus the low dose rimonabant control group. Lastly, high dose rimonabant significantly increased distance traveled in CP47,497-treated mice as compared to CP47,497-treated mice without antagonist pretreatment ($p < 0.05$).

Table 4.3 – Baseline measurements for rimonabant antagonism experiments

Tail Withdrawal Latency (s)	Vehicle Pretreatment			Rimonabant (3 mg/kg) Pretreatment			Rimonabant (10 mg/kg) Pretreatment		
Drug	**Veh[a]**	**CP47,497[b]**	**THC[c]**	**Veh**	**CP47,497**	**THC**	**Veh**	**CP47,497**	**THC**
Mean	1.9	1.6	1.8	1.4	1.7	1.6	1.7	1.5	1.2
± SEM	0.3	0.2	0.2	0.2	0.2	0.2	0.4	0.3	0.2

Temperature (°C)	Vehicle Pretreatment			Rimonabant (3 mg/kg) Pretreatment			Rimonabant (10 mg/kg) Pretreatment		
Drug	**Veh**	**CP47,497**	**THC**	**Veh**	**CP47,497**	**THC**	**Veh**	**CP47,497**	**THC**
Mean	36.7	37.1	37.0	36.9	37.3	37.2	37.0	37.1	36.9
± SEM	0.1	0.2	0.1	0.1	0.3	0.2	0.1	0.2	0.2

[a]Vehicle (1:1:18), [b]CP47,497 (30 mg/kg), [c]THC (100 mg/kg)

To corroborate the pharmacological observations seen with the CB_1-selective antagonist, rimonabant, studies were designed using mice genetically devoid of the CB_1 receptor (Zimmer et al. 1999). Age-matched $CB_1^{(-/-)}$ mice and $CB_1^{(+/+)}$ littermate mice were assessed in the tetrad after receiving an acute dose of CP47,497 (30 mg/kg, i.p.). CP47,497-treated mice showed significant increases in catalepsy [$F(1,13) = 34.7$, $p < 0.0001$], antinociception [$F(1,16) = 36.9$, $p < 0.0001$], hypothermia [$F(1,16) = 142.3$, $p < 0.0001$], and immobility [$F(1,16) = 24.4$, $p = 0.0001$], demonstrating significant cannabimetic effects compared to vehicle-treated mice ($p < 0.0001$) (**Figure 4.6**). CP47,497 treatment significantly decreased distance traveled [$F(1,16) = 16.5$, $p = 0.0009$]. An interaction effect between CP47,497 treatment and genotype was observed [$F(1,16) = 31.04$, $p < 0.0001$], but significant effects were not seen across genotypes [$F(1,16) = 4.062$].

$CB_1^{(+/+)}$ mice demonstrated significant increases in catalepsy [$F(1,13) = 34.7$, $p < 0.0001$], antinociception [$F(1,16) = 33.5$, $p < 0.0001$], hypothermia [$F(1,16) = 105.5$, $p < 0.0001$], and immobility [$F(1,16) = 19.2$, $p = 0.0005$], compared to $CB_1^{(-/-)}$ mice, following CP47,497 administration. In fact, cannabimetic behavioral effects of CP47,497 were completely absent in $CB_1^{(-/-)}$ mice, and no significant differences were seen between CP47,497-treated and vehicle-treated $CB_1^{(-/-)}$ mice for any behavioral measure ($p < 0.001$). During catalepsy testing, 67% of CP47,497-treated $CB_1^{(+/+)}$ mice experienced

hyperreflexia, whereas this behavior was not displayed in CP47-497-treated $CB_1^{(-/-)}$ mice. Lastly, significant interaction effect of drug and genotype were also observed for catalepsy [$F(1,13) = 34.7$, $p < 0.0001$], antinociception [$F(1,16) = 33.1$, $p < 0.0001$], hypothermia [$F(1,16) = 89.2$, $p < 0.0001$], and time immobile [$F(1,16) = 29.3$, $p = 0.0005$].

Quantification of CP47,497 in brain tissue suggested there was a trend toward higher drug concentrations in wild type mice with values quantified (mean ± SEM) for $CB_1^{(+/+)}$ mice of 2575 ± 600 ng/g, and 1080 ± 120 ng/g in $CB_1^{(-/-)}$ mice. However, statistical analyses revealed no significant differences in CP47,497 drug concentration in brains of $CB_1^{(+/+)}$ mice versus $CB_1^{(-/-)}$ mice, which were harvested one hour after treatment with an acute dose of 30 mg/kg CP47,497 [$t = 2.4$, df= 6, p = 0.0511).

Table 4.4 – Baseline measurements in CB_1 knockout experiments

		Vehicle[a]		CP47,497[b]	
		CB1 (+/+)	CB1 (-/-)	CB1 (+/+)	CB1 (-/-)
Tail Withdrawal Latency (s)	Mean	1.8	2.4	2.5	1.9
	± SEM	0.3	0.5	0.4	0.4
		Vehicle[a]		CP47,497[b]	
		CB1 (+/+)	CB1 (-/-)	CB1 (+/+)	CB1 (-/-)
Temperature (°C)	Mean	37.1	36.8	36.8	37.0
	± SEM	0.2	0.1	0.1	0.4

[a]Vehicle (1:1:18), [b]CP47,497 (30 mg/kg)

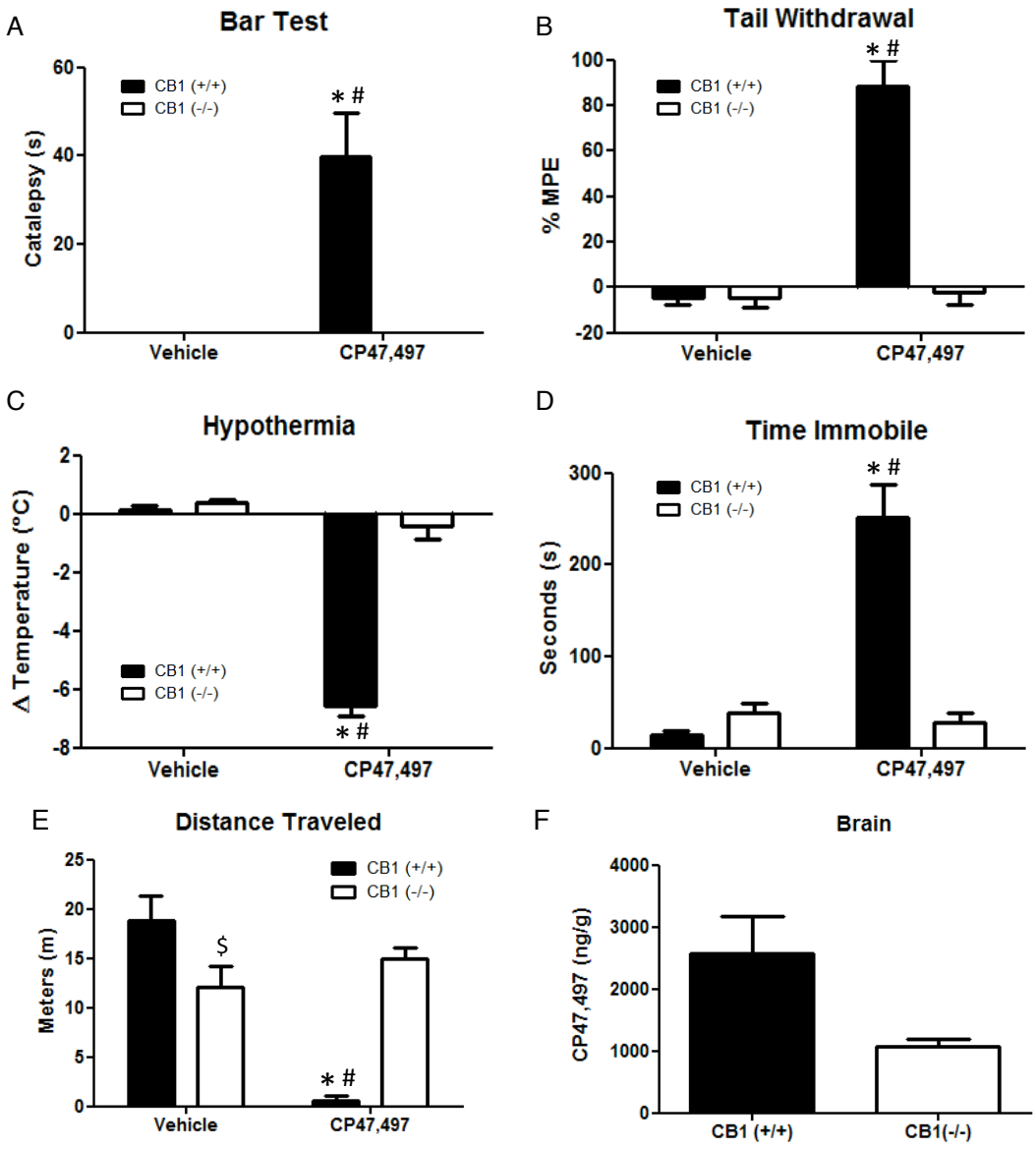


Figure 4.6 – CP47,497-induced effects of catalepsy (A), antinociception (B), hypothermia (C), and locomotor suppression (D, E) are abrogated in $CB_1^{(-/-)}$ mice. Significant effects of treatment and genotype were observed in all measures tested, but no significant differences in brain concentrations of CP47,497 **(F)** were observed as quantified by HPLC/MS/MS. Data represent the mean ± SEM, n = 4 mice per $CB_1^{(-/-)}$ group (male) and n = 6 mice per $CB_1^{(+/+)}$ group (sexes collapsed within group). Significance denoted as $*p < 0.0001$ vs. vehicle-treated $CB_1^{(+/+)}$ mice, $\#p < 0.0001$ vs. CP47,497-treated $CB_1^{(-/-)}$ mice and $\$p < 0.05$ vs. vehicle-treated $CB_1^{(+/+)}$ mice. Baseline means ± SEM for tail withdrawal latencies (s) and temperature (°C) are listed in Table 4.4.

4.3.5 CP47,497 potently substitutes for THC in the drug discrimination assay in C57BL6/J mice

Next, we evaluated whether CP47,497 would substitute for THC in the drug discrimination assay. These studies utilized C57BL6/J mice; therefore, a CP47,497 dose-effect study using cannabimimetic tetrad measures was performed to establish a behaviorally active dose range for CP47,497 **(Figure 4.7)**. In dose-response experiments, significant main effects were revealed for catalepsy in CP47,497-treated mice [$F(3,8) = 21.9$, $p < 0.001$], antinociception [$F(3,8)= 34.0$, $p < 0.0001$], hypothermia [$F(3,8)= 44.6$, $p < 0.0001$], time immobile [$F(3,8)= 31.27$, $p < 0.0001$] and distance traveled [$F(5,55)= 9.9$, $p< 0.01$] **(Figure 4.7)**. The doses of 3.2 and 10 mg/kg CP47,497 significantly increased catalepsy, hypothermia, time immobile, and significantly decreased distance traveled compared to vehicle-treated mice ($p<0.05$). Also, 1 and 3.2 mg/kg CP47,497 produced significantly less antinociception than 10 mg/kg CP47,497 ($p < 0.05$), but only 10 mg/kg CP47,497 produced significant antinociception versus vehicle ($p < 0.05$). Hypothermia was significantly lower following the 3.2 mg/kg dose, compared to 10 mg/kg CP47,497 ($p < 0.05$). From these results, we concluded that a 0.3 - 3.2 mg/kg dose range for CP47,497 would be sufficient to assess THC substitution in drug discrimination experiments while minimizing effects to normal motor activity.

Prior to the start of drug discrimination studies, C57BL6/J mice were trained to discriminate 5.6 mg/kg THC from vehicle. **Figure 4.8, Panel A** illustrates that THC dose-dependently substituted for the training dose with an ED_{50} of 1.6 mg/kg (95% CL: 1.2-2.1), with no observed decreases in response rates after vehicle treatment [$F(5,55)= 2.1$, $p=0.74$] (**Figure 4.8, Panel B**). During non-test days, nose poke responding on the appropriate treatment-paired side were established in vehicle and THC-treated mice (the left side of **Panel B, Figure 4.8)**. A one-way ANOVA with Dunnett's post-hoc comparison revealed significant drug-like responding in THC-treated mice [$F(5,55)=31.1$, $p<0.001$] for 3, 5.6 and 10 mg/kg doses, compared to vehicle ($p<0.001$). Significant increases in drug-like responding were observed in CP47,497-treated mice [$F(5,30)=19.5$, $p<0.001$], with 1 and 3.2 mg/kg doses significantly different than vehicle ($p<0.0001$).

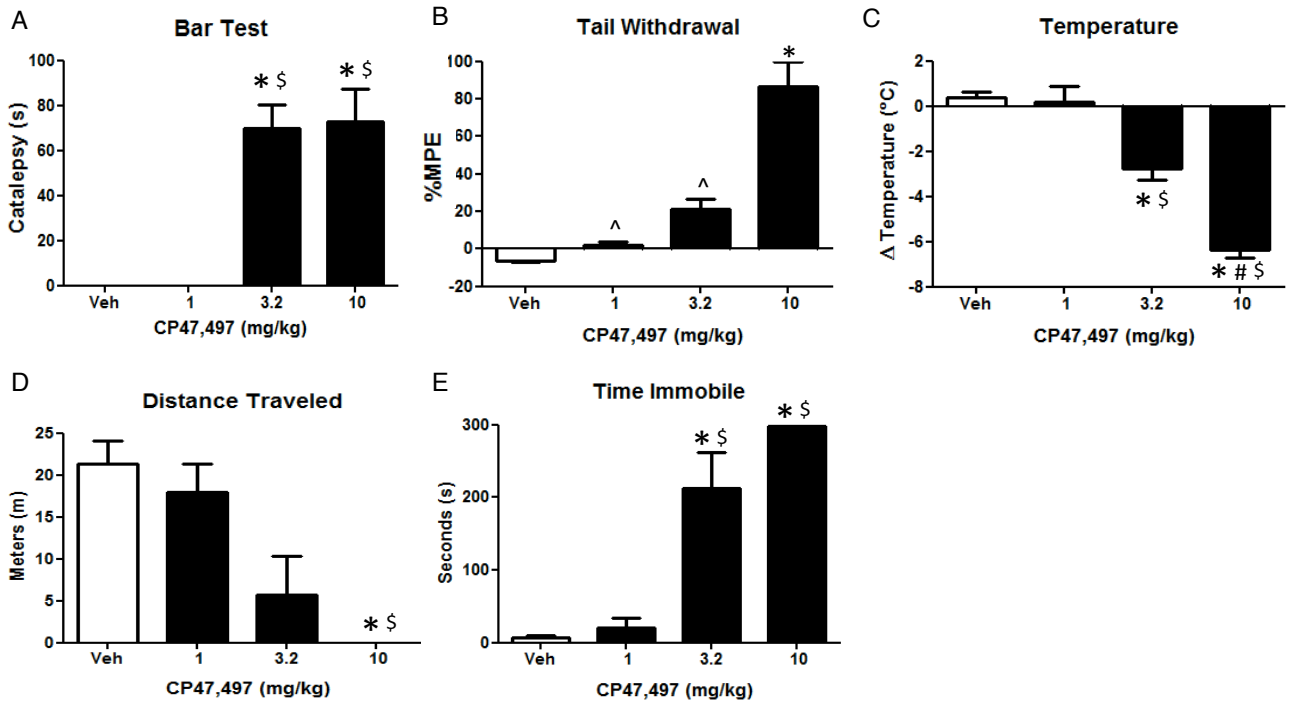


Figure 4.7 – CP47,497 dose-response in C57BL6/J mice. Significant effects of catalepsy **(A)**, antinociception **(B)**, hypothermia **(C)** and hypolocomotion **(D,E)** were observed after 3.2 or 10 mg/kg CP47,497 in C57BL6/J mice. Significance denoted $^{*}p < 0.05$ versus vehicle, $^{\$}p < 0.05$ versus 1 mg/kg CP47,497, $^{\#}p < 0.05$ versus 3.2 mg/kg CP47,497, $^{\wedge}p < 0.05$ versus 10 mg/kg CP47,497. Data represent the mean ± SEM, n = 3 mice per group. Baseline (means ± SEM) tail withdrawal latencies (s) for vehicle-treated mice were 1.6 ± 0.2, 1.5 ± 0.1 for 1 mg/kg CP47,497-treated mice, 1.8 ± 0.2 for 3.2 mg/kg CP47,497-treated mice, and 1.7 ± 0.2 for 10 mg/kg CP47,497-treated mice. Baseline means ± SEM for temperature (°C)= 37.1 ± 0.3 for vehicle-treated, 37.0 ± 0.5 for 1 mg/kg CP47,497-treated mice, 37.0 ± 0.1 for 3.2 mg/kg CP47,497-treated mice, and 37.4 ± 0.2 in 10 mg/kg CP47,497-treated mice.

CP47,497-treatment substituted for THC as seen in **Figure 4.8, Panel A** and exhibited more than 4-fold higher potency than THC, with a corresponding ED_{50} value of 0.26 mg/kg (95% CL: 0.15-0.45)]. Full substitution was observed at both 1 mg/kg and 3 mg/kg of CP47,497, with a significant effect of drug on response rates [$F(5,30)=4.23$, $p<0.01$]. *Post-hoc* analyses indicated that response rates only at the highest dose tested, 3 mg/kg CP47,497, were significantly ($p< 0.001$) reduced when compared to vehicle (**Figure 4.8, Panel B**).

4.3.6 Tolerance to CP47,497 and cross-tolerance between THC and CP55,940 effects are observed after repeated CP47,497 administration

Synthetic cannabinoids have varied binding characteristics and some possess greater affinity for CB_1 receptors versus THC. In rodents, repeated exposure to THC and synthetic cannabinoid agonists, WIN55,212-2 and CP55,940, produces desensitization of cannabinoid mediated G-protein activity, CB_1 receptor downregulation and diminished inhibition of adenylyl cyclase (Martin et al. 2004; Sim-Selley 2003). In humans, the development of tolerance and withdrawal symptoms after repeated oral administration of THC have been characterized (Jones et al. 1976), and has recently been documented for synthetic cannabinoid compounds (Nacca et al. 2013; Zimmermann et al. 2009).

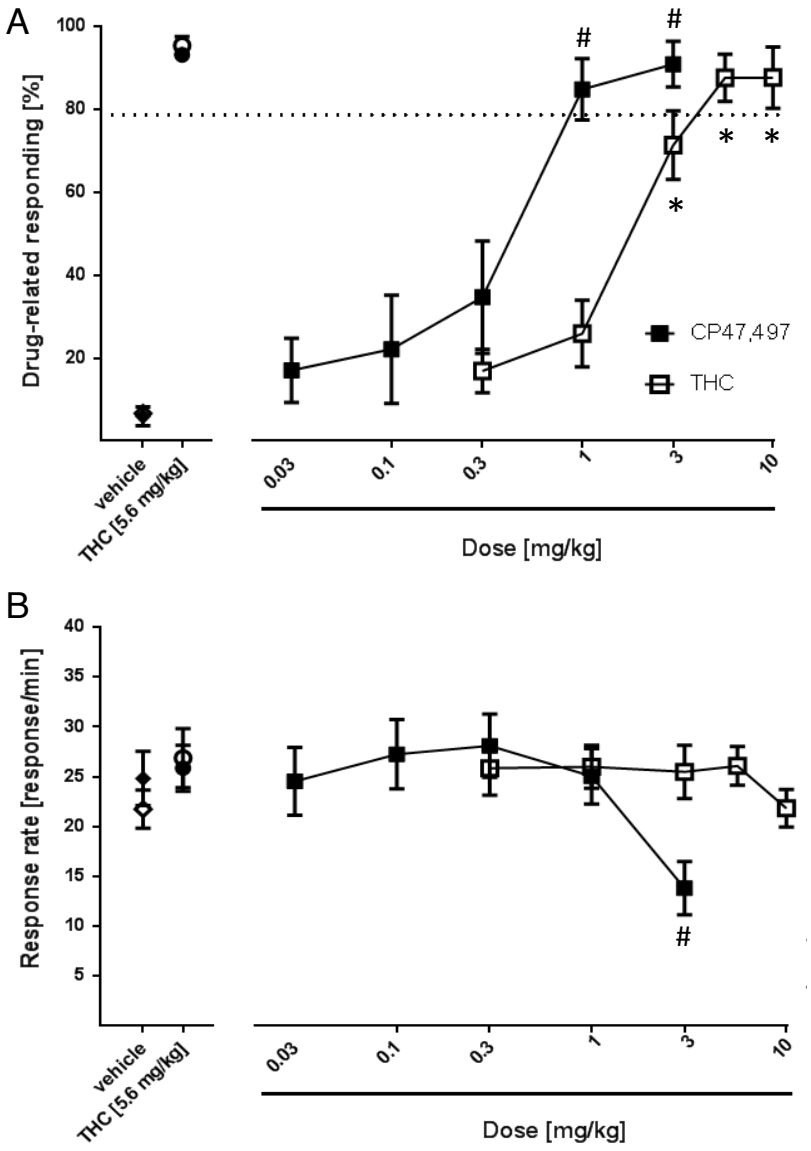


Figure 4.8 – CP47,497 drug discrimination in C57BL6/J mice. CP47,497 (1 and 3.2 mg/kg) fully substituted for THC in mice trained to discriminate 5.6 mg/kg THC from vehicle **(A)**. CP47,497 [ED_{50}= 0.26 mg/kg, 95% CL (0.15-0.45)] was 4.7 times more potent than THC [ED_{50}= 1.6 mg/kg, 95% CL (1.2-2.1)] in the drug discrimination assay. Response rates were affected only at the highest CP47,497 (3.2 mg/kg) dose tested **(B)**. Significance denoted CP47,497 versus vehicle (*#p*< 0.001), and THC versus vehicle (**p* < 0.0001). Data represent the mean ± SEM, n = 8-10 mice per group. Experiments and data analysis performed by Dr. B. Ignatowska-Jankowska.

Therefore, not only do these SCB drugs show potential for abuse, but also their repeated abuse may lead to the development of tolerance and dependence as these pharmacodynamic properties may promote increased drug consumption to sustain desired effects.

Based on the evidence that tolerance to the cannabimimetic effects of THC is seen upon chronic exposure (Abood et al. 1993; Fan et al. 1994; Hruba et al. 2012; Jones et al. 1976; Pertwee et al. 1993), we hypothesize that repeated administration of CP47,497 will produce tolerance to its cataleptic, antinociceptive and hypothermic effects. As is the case with THC, we predict that cross-tolerance to other synthetic cannabinoids, such as CP55,940 will develop (Fan et al. 1994; Pertwee et al. 1993). Our data indicate that acute administration of CP47,497 elicits more potent behavioral effects than THC; therefore, we predict that repeated CP47,497 administration will produce tolerance to tetrad behavioral effects.

Sim-Selley *et al.* has previously demonstrated tolerance and dependence using a twice daily sub chronic dosing regimen for 5.5 days with THC (Falenski et al. 2010, McKinney et al. 2008). Half of the dose of either CP47,497 or THC that produced approximately equal efficacy in acute tetrad behavioral experiments **(Figure 4.3),** were administered repeatedly. CP55,940 was used as a positive control with doses previously reported using ICR mice in the literature (Fan et al. 1994; Fan et al. 1996). In

these studies mice were treated (s.c.) twice daily for 5.5 days with either vehicle, CP47,497 (15 mg/kg), THC (50 mg/kg), or CP55,940 (2 mg/kg).

A two-way ANOVA of catalepsy tolerance data, with dose and drug treatment as between subject factors, revealed main effects for dose [$F(4,28)= 50.0$, $p<0.0001$], drug treatment [$F(3,28)= 37.1$, $p<0.0001$], and a significant interaction between drug and dose [$F(12,28)= 14.2$, $p<0.0001$]. As shown in **Figure 4.9, Panel A,** CP47,497-experienced animals displayed significant less catalepsy when challenged with CP47,497 (10, 30, and 56 mg/kg) versus vehicle-treated animals ($p <0.0001$). Likewise, animals repeatedly treated with THC and CP55,940 exhibited cross tolerance to CP47,497 at 10 mg/kg ($p <0.001$), 30 mg/kg ($p <0.0001$), and 56 mg/kg ($p <0.0001$) versus animals repeatedly given vehicle injections.

Assessment of tolerance to CP47,497-mediated antinociceptive effects revealed a significant main effect for dose [$F(4,28) = 36.9$, $p < 0.0001$], treatment [$F(3,28) = 11.9$, $p < 0.0001$], and interaction effect of dose and treatment [$F(12,28) = 10.5$, $p < 0.0001$]. Significant antinociceptive tolerance **(Figure 4.9, Panel B)** was detected for CP47,497, THC, and CP55,940 at cumulative doses of 30 and 56 mg/kg versus vehicle ($p < 0.0001$). No significant differences in tolerance to antinociceptive effects were observed across agonist treatments.

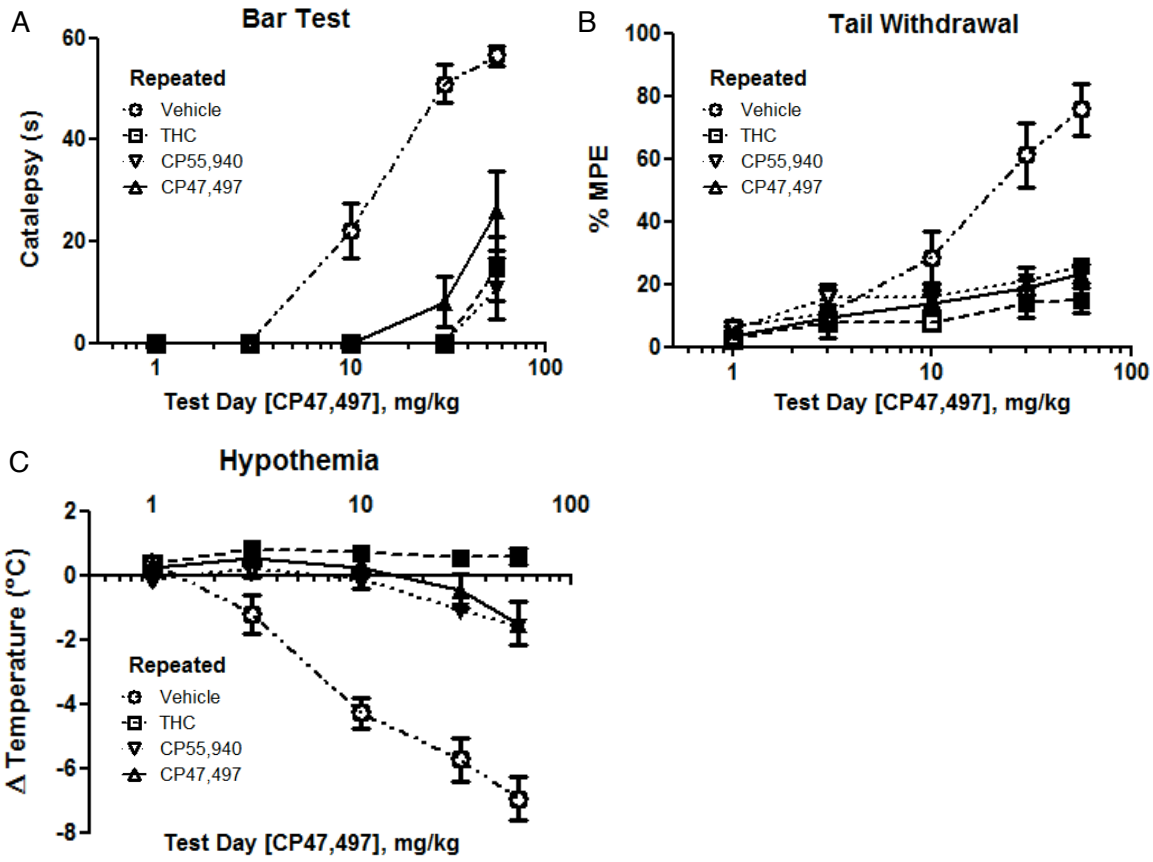


Figure 4.9 - Tolerance and cross-tolerance to CP47,497 was produced after repeated administration of CB_1 agonists THC, CP55,940 and CP47,497. Challenge with increasing doses of CP47,497 elicited significant tolerance to effects of catalepsy **(A)**, antinociception **(B)**, and hypothermia **(C)** in mice treated with twice-daily injections of vehicle, CP47,497 (15 mg/kg), THC (50 mg/kg) or CP55,940 (2 mg/kg) for 5.5 days. Filled symbols denote significance from vehicle. Data represent the mean ± SEM, n = 6-10 mice per group. Baseline (means ± SEM) tail withdrawal latencies (s) and temperatures (°C) are shown in Table 4.4.

Comparison of tolerance development to hypothermic effects in **Figure 4.9, Panel C** revealed significant differences for dose [$F(4,28)= 38.2$, $p < 0.0001$], drug treatment [$F(3,28)= 149.3$, $p < 0.0001$], and for an interaction effect of dose and drug [$F(12,28)= 17.3$, $p < 0.0001$]. Significant cross-tolerance was observed for THC at 3 mg/kg ($p < 0.05$), 10, 30, and 56 mg/kg ($p < 0.0001$) in comparison to repeated administration of vehicle. Significant tolerance developed to decreases in body temperature upon CP47,497 treatment in mice receiving repeated CP47,497 and CP55,940 (10, 30, 56 mg/kg) versus vehicle treated mice challenged with cumulative CP47,497 drug ($p < 0.0001$). At the highest dose tested, CP47,497 (56 mg/kg) elicited significantly less hypothermia than THC ($p < 0.05$). Overall, tolerance to CP47,497-induced catalepsy, antinociception, and hypothermia was produced, but statistical analyses revealed no significant differences in the magnitude of tolerance development between animals treated repeatedly with CP47,497, THC or CP55,940 as compared to control mice.

Table 4.5 – Baseline tail withdrawal latency and temperature measurements for tolerance experiments

Tail Withdrawal Latency (s)	Repeated Drug Treatment			
Drug	**Veh[a]**	**CP47,497[b]**	**CP55,940[c]**	**THC[d]**
Mean	1.5	1.0	0.9	1.6
± SEM	0.2	0.1	0.1	0.3

Temperature (°C)	Repeated Drug Treatment			
Drug	**Veh**	**CP47,497**	**CP55,940**	**THC**
Mean	37.2	37.7	38.1	37.5
± SEM	0.2	0.2	0.4	0.3

[a]Vehicle (1:1:18), [b]CP47,497 (15 mg/kg), [c]CP55,940 (2 mg/kg), [d]THC (50 mg/kg)

4.3.7 Rimonabant-precipitated somatic withdrawal signs are observed in mice treated repeatedly with CP47,497

Rodents repeatedly treated with cannabinoid receptor agonists display signs of precipitated withdrawal after challenge with rimonabant as previously reported (Rubino et al. 1998; Tsou et al. 1995). Therefore, we sought to determine if repeated CP47,497 exposure will give rise to dependence phenomena, characterized by presentation of somatic withdrawal signs. For this experiment, mice were treated twice daily for 5.5 days with subcutaneous delivery of vehicle, CP47,497 (15 mg/kg), THC (15 mg/kg), or CP55,940 (2 mg/kg), and on day 6, mice received a rimonabant injection (10 mg/kg)

and were monitored for somatic withdrawal signs (head shakes, paw flutters, and jumps).

Following a one-way ANOVA, significant main effects were observed for head shakes [$F(3,28)= 7.2$, $p < 0.001$] and paw flutters [$F(3,28)= 10.8$, $p < 0.0001$] as seen in **Figure 4.10**. For each treatment group, jumping was recorded but no significant differences were seen among any groups [$F(3,28)= 1.3$, $p = 0.29$]; these data are not shown as jumping was observed to a much lesser extent than head shakes and paw flutters. Significant increases in head shakes were observed in CP47,497-treated and THC-treated mice ($p < 0.05$) versus vehicle-treated mice, with CP47,497-treated mice displaying significantly more head shakes than CP55,940-treated mice ($p < 0.05$). Rimonabant-precipitated paw flutter withdrawal signs were significant in CP47,497- and THC-treated groups as compared to either CP55,940- or vehicle-treated groups ($p < 0.05$). No significant differences in head shakes or paw flutters were seen between CP55,940 and vehicle groups or CP47,497 and THC groups.

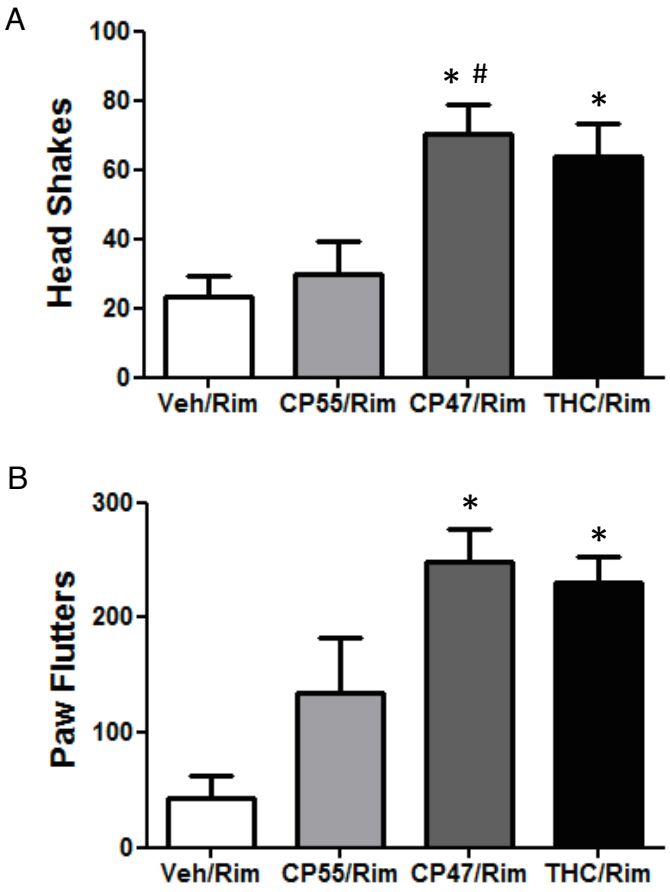


Figure 4.10 – Assessment of somatic withdrawal signs in mice following repeated CP47,497, THC and CP55,940 administration. Precipitated withdrawal by rimonabant (10 mg/kg) challenge elicited significant increases in both head shakes (A) and paw flutters (B) in CP47,497 and THC-treated, but not CP55,940-treated mice. No significant differences were observed between groups for jumping behavior. Significance denoted $*p < 0.05$ versus Veh/Rim and $\#p < 0.05$ versus CP55/Rim groups. Data represent the mean ± SEM, n = 6-10 mice per group.

4.4 Discussion

The body of work presented in this dissertation sought to determine if the synthetic cannabinoid, CP47,497, would produce potent and efficacious CB_1 receptor-mediated cannabimimetic effects as compared to the primary psychoactive component of marijuana, THC. Synthetic cannabinoids were insipiently created as biomedical research tools but they have been commandeered from their intended laboratory use and have surfaced as novel cannabinoid drugs of abuse. Because hundreds of SCBs have been synthesized, and since they are not detected in traditional drug screens, their popularity as "legal" alternatives to THC continues to increase. Furthermore, there is insufficient data regarding their abuse liability, and medical consequences of acute or repeated use including toxicity, pharmacodynamic, and pharmacokinetic effects remains largely unexplored. As a result**,** the objectives of this dissertation were to develop a bioanalytical assay for the extraction and quantification of CP47,497, to assess *in vitro* effects of CP47,497, and to examine *in vivo* pharmacological effects of acute and repeated CP47,497 drug administration, including determination of the discriminative stimulus effects of CP47,497 as compared to THC.

Previous studies have established that CP47,497 binds to CB_1 receptors with low nanomolar ($K_i < 10$ nM) affinity versus THC ($K_i \sim 40$ nM) (Compton et al. 1993; Melvin et al. 1993). Cannabinoid agonists bind to CB_1 receptors, the receptor undergoes

a conformational change that promotes activation of downstream signaling events (Howlett et al. 1999). To determine the first step in CB_1 receptor signaling, the effect of G-protein activation by synthetic cannabinoid CP47,497-, CP47,497-C8- and JWH-250-stimulated [^{35}S]-GTPγS binding was examined. Prior to cloning of cannabinoid receptors, Devane *et al.* used radiolabeled CP55,950 as a potent, efficacious, and stereoselective tool to characterize the binding site of the central cannabinoid receptor, CB_1 (Devane et al. 1988). Since then, CP55,940 remains a highly utilized pharmacological tool and is one of the best characterized SCBs (Compton, Johnson, et al. 1992; Fan et al. 1996; Little et al. 1988; Rubino, Viganò, et al. 2000). Therefore, CP55,950 was used as a comparison to the SCBs investigated in this thesis. At CB_1 and CB_2 receptors, CP55,940 exhibits both high efficacy, described as producing measurable responses at low receptor occupancies, and partial agonism, producing 80-90% of maximal percent stimulation above basal in the [^{35}S]-GTPγS binding assay. Agonist stimulated [^{35}S]-GTPγS binding of the SCBs of interest were normalized to maximum percent stimulation of CP55,940 to permit comparison across SCB compounds as each synthetic cannabinoid was assessed alongside CP55,940 in three separate experiments.

No significant differences were observed for maximum stimulation effect (E_{max}) between CP55,940, CP47,497, CP47,497-C8- and JWH-250 compounds tested, suggesting that the efficacy of each of the synthetic cannabinoids examined is similar to

the efficacy of CP55,940. Therefore, like CP55,940, these data strongly suggest that CP47,497, CP47,497-C8- and JWH-250 are high efficacy partial agonists. Additionally, EC_{50} values for CP47,497, CP47,497-C8- and JWH-250 were significantly higher than CP55,940. These results indicate that even though the SCBs tested are equally efficacious as CP55,940 in this assay, they demonstrate lower potencies. While the sample size used in these studies met the minimum threshold for statistical requirements (n = 3, run in triplicate), performing additional replicates would further strengthen the data.

Interestingly, the dimethyloctyl homologue of CP47,497, CP47,497-C8 elicited a significant increase in percent stimulation (E_{max}) when compared to CP47,497 in these studies, which illustrates a higher efficacy for CP47,497-C8 versus CP47,497. The efficacy of CP47,497-C8 has also translated to potent *in vivo* antinociceptive effects as addition of one methyl group confers higher CB_1 receptor binding affinity in rat brain membranes versus CP47,497 (Melvin et al. 1993). Moreover, our results in [^{35}S]-GTPγS studies authenticate a study reporting partial efficacy of CP47,497 in agonist stimulated [^{35}S]-GTPγS binding in both rat cerebellum and in CHO cell constructs expressing human cannabinoid receptors (hCB_1 and hCB_2) (Govaerts et al. 2004).

The SCB agonist [^{35}S]-GTPγS binding studies support our hypothesis that CP47,497 is an efficacious cannabimimetic agent that initiates signal transduction by

activating G-proteins associated with GPCRs. The data presented herein provide the first evidence of G-protein activation by CP47,497-C8 and JWH-250 synthetic cannabinoid compounds. The ability of bicyclic compounds, CP47,497 and CP47,497-C8, to activate G-proteins is not surprising based on their close structural resemblance to CP55,940. On the other hand, we might have expected the E_{max} of the aminoalkylindole, JWH-250, would be higher than those of the other compounds due to its structural resemblance to the CB_1 full-efficacy aminoalkylindole agonist, WIN55,212-2 (Breivogel & Childers 2000; Selley et al. 2004; Sim-Selley & Martin 2002). With no significant differences in E_{max} values, these data support that compounds with varying structures demonstrate similar efficacy in this assay. Based on available *in vitro* data presented here and in previously published work, we suspect that the lower nanomolar binding affinity of CP47,497 to CB_1 receptors, together with increased efficacy of G-protein activation may, at least, partially explain the potent THC-like behavioral effects observed by CP47,497.

CB_1 receptors are the most abundantly expressed GPCRs in the brain (Devane et al. 1988; Herkenham et al. 1991) and are, therefore, suspected to be responsible for G-protein activation of CP47,497 in this experiment. However, since many of the SCB compounds being abused are not well-studied, and because our studies do not definitively identify the GPCR eliciting G-protein activation, further research is warranted

to omit the possibility of off-targets of CP47,497 in brain. Exemplifying this possibility, the activation of G-proteins by WIN55,212-2 has been observed in brain membrane preparations from $CB_1^{(-/-)}$ mice (Breivogel et al. 2001; Nguyen et al. 2010), suggesting non-CB_1 binding activity by WIN55,212-2 in brain. To test whether CP47,497-stimulated G-protein activation is mediated by CB_1 receptors, SCB agonist stimulated [^{35}S]-GTPγS could be assessed in membranes treated with a CB_1 receptor selective antagonist, such as rimonabant, or with preparations from $CB_1^{(-/-)}$ mice. As an alternative to functional activation of G-proteins by CP47,497, species-specific differences could be determined by displacement of [^{3}H]-SR141617A binding (Breivogel & Childers 2000) by CP47,497 in Chinese hamster ovary (CHO) cells transfected with a construct expressing hCB_1 (Thomas et al. 2007). Furthermore, while CB_2 receptors play a role in immunity and are present primarily in the spleen and on circulating macrophage cells (Munro et al. 1993), but CB_2 expression is observed in the CNS as well (Atwood & Mackie 2010; Van Sickle et al. 2005). Additionally, stimulation of neuronal CB_2 receptors have been proposed to attenuate reward and the psychomotor stimulating effects of cocaine (Xi et al. 2011). Thus, CP47,497-stimulated [^{35}S]-GTPγS activity at CB_2 should be also be examined as CB_2 receptor affinity of CP47,497 has been previously published in CHO cells transfected with human CB_2 receptors (Govaerts et al. 2004).

Another important objective of this dissertation was to determine whether CP47,497 mimics the actions of THC *in vivo*, and whether CB_1 mediates these effects. This goal is important because SCB compounds are presently being abused by some individuals in lieu of THC (see Chapter 2 and Hillebrand et al. 2010; Vandrey et al. 2012), and research has established that the psychoactive effects of THC are mediated by CB_1 receptors (Martin et al. 1991; Matsuda et al. 1993). Moreover, the distribution of CB_1 receptors in the CNS via autoradiographic and immunohistochemical techniques in rodents have demonstrated that regions expressing high CB_1 receptor density (Herkenham et al. 1991; Matsuda et al. 1993; Tsou et al. 1998) overlap with brain areas well-known to mediate perception and cognition (cerebral cortex), memory (hippocampus), reward (nucleus accumbens), anxiety (amygdala), and motor functions (basal ganglia, cerebellum) (Casswell & Marks 1973; Herkenham et al. 1990; Karniol & Carlini 1973; Sim-Selley 2003; Varvel et al. 2001; Verrico et al. 2014). Human studies have shown that both developing and mature brains exhibit mostly similar receptor distribution, with cognitive and motor region-specific patterns of dense CB_1 expression (Biegon & Kerman 2001; Glass et al. 1997). These studies also demonstrate CB_1 receptor binding in areas associated with higher cognition (forebrain), movement (forebrain, midbrain, and hindbrain), and motor functioning (hindbrain), which demonstrate prominent CB_1 receptor expression in brain regions that correlate well with the known behavioral, psychomotor and psychological effects of THC.

Finally, to increase the understanding of CP47,497 efficacy, specific brain regions should be examined, since whole brain preparations were used for our agonist-stimulated [^{35}S]-GTPγS binding experiments and because agonist efficacy is shown to significantly vary across brain regions, depending on specific drug treatment and duration (Breivogel et al. 1997; Breivogel 1998; McKinney et al. 2008). Investigation of receptor desensitization, downregulation, or binding affinites in specific brain regions known to contain CB_1 receptors (i.e. cortex, hippocampus, cerebellum, striatum) could provide evidence for actions of CP47,497 distinct from other cannabinoid agonists, such as THC and WIN55,212-2 (Breivogel & Childers 1998; Selley et al. 1996). Reductions in agonist-stimulated [^{35}S]-GTPγS binding are seen in the hippocampus, cingulate cortex, periaqueductal gray, and cerebellum after repeated treatment with 10 mg/kg THC or with an escalating dosing paradigm, but higher doses are required to see decreases in caudate putamen, nucleus accumbens, and preoptic areas (McKinney et al. 2008). Based on behavioral observations of CP47,497 mice in the tetrad, we would predict that decreased [^{35}S]-GTPγS binding would occur in areas known to mediate cannabinoid behavioral effects and are high in CB_1 receptor expression, such as cortex, hippocampus, and cerebellum (Sim et al. 1996; Sim-Selley 2003). Additionally, hyperreflexia was prominent in CP47,497-treated mice in studies presented here, and has been observed in humans after SCB abuse (Harris & Brown 2013; Simmons et al. 2011). Therefore, differences in CB_1 activity may be observed in the cerebellum and

basal ganglia, as these areas are known to mediate movement and motor coordination behavior (Breivogel & Childers 1998; Patel & Hillard 2001). Examination of the hippocampus would reveal the potential role of CP47,497 effects on impairment of learning and memory, as has been observed with THC (Varvel et al. 2001; Wise et al. 2011) and the synthetic cannabinoid, JWH-081 (Basavarajappa 2014).

Extensive examination of CP47,497 in the tetrad behavioral assay was presented herein as it provides a sensitive index of cannabimimetic potency, and has been well-characterized for nonclassical (Compton et al. 1993; Little et al. 1988; Melvin et al. 1993), aminoalkylindole (Compton, Gold, et al. 1992; Wiley, Marusich & Huffman 2013) and structurally related synthetic cannabinoid compounds (Huffman et al. 2005; Wiley et al. 1998; Wiley et al. 2012). Perhaps the most convincing evidence that CB_1 receptors mediate actions of tetrad behaviors was the reversal of cannabimimetic effects via antagonism with rimonabant (Compton et al. 1996; Rinaldi-Carmona et al. 1994; Wiebelhaus et al. 2012) and the absence of cannabimimetic effects in genetically modified mice lacking CB_1 receptors (Ledent et al. 1999; Monory et al. 2007; Zimmer et al. 1999). Drugs from other classes (e.g. CNS depressants) can produce a subset of cannabimimetic effects, but in general, cannabimimetic compounds exhibit dose-dependent and reproducible behaviors in all four components (catalepsy, antinociception, hypothermia and locomotor suppression). For example, a

comprehensive examination of centrally acting drugs in the tetrad revealed that CNS depressants, such as ethanol, diazepam, and phenobarbital do not produce effects in all four tests; hypothermia was not observed with diazepam, catalepsy was absent in ethanol treated mice, and catalepsy nor antinociception was observed with phenobarbital (Wiley 2003). In this same series of experiments, antipsychotic agents, clozapine, haloperidol, and chlorpromazine exhibited effects in all four parameters; however, the magnitude of catalepsy produced exceeded the maximum response observed with THC. As verification of CB_1 receptor involvement, rimonabant completely antagonized the effects of THC, but not the effects of any of the other drugs examined (Wiley 2003). Thus, these data highlight that tetrad effects are not exclusive to cannabinoid compounds, as pharmacological drugs from different classes can exhibit tetrad behavioral effects. Therefore, the use of the tetrad assay in combination with pharmacological, genetic, and molecular tools to assess CB_1 receptor involvement represent a powerful approach to assess the effects of suspected novel cannabinoid compounds in the whole animal.

The experiments presented herein show that CP47,497, consistent with a cannabimimetic drug, produces all tetrad measures including catalepsy, antinociception, hypothermia, and locomotion, and displays increased potency (5- to 7- fold) over THC. Additionally, our results indicate CP47,497 is potentially more behaviorally efficacious

than THC, as 30 mg/kg of CP47,497 produced approximately 70% maximal antinociception versus approximately 20% maximal antinociception at 30 mg/kg THC. The ED_{50} value for antinociceptive effects of CP47,497 was 26.2 (11.0 -62.0), whereas, 200 mg/kg THC produced approximately 20% maximum antinociceptive effect in the warm water tail withdrawal assay. In order to discern whether the antinociceptive efficacy of THC is lower than that of CP47,497, it would be necessary to test higher doses of drug than those tested in the present study.

Determination of ED_{50} values for antinociception (radiant heat tail flick) for intravenous administration of THC (1.4 mg/kg) and CP47,497 (0.3 mg/kg) and subcutaneous administration of THC (72 mg/kg) and CP47,497 (4 mg/kg) have been reported previously (Compton et al. 1992; Little et al. 1988; Weissman et al. 1982). However, these previous results are discrepant from those presented here as the ED_{50} value for CP47,497 antinociceptive effects was 26.2 (11.0 - 62.0) mg/kg, and an ED_{50} value for THC could not be calculated because a 50% effect was not observed at doses as high as 200 mg/kg. The differences in data are likely due to several factors. First, the routes of administration varied. The present series of experiments used intraperitoneal administration, while previous publications used subcutaneous and tail vein (i.v.) routes of administration. Second, a variation of the (radiant heat) tail flick assay used in previous studies (Le Bars et al. 2001; King et al. 1997), the warm water (52 °C) tail

withdrawal assay, was used herein. The choice for these differences in experimental methods was intentional as they are more amenable to cumulative-dosing, which subsequently maximizes data collection, increases statistical sensitivity using a within-subjects experimental design, and reduces the number of animals necessary for each experiment. Differences in precise drug concentrations in cumulative versus bolus drug injections may also account for observed variability, as some drug metabolism occurs over the course of a cumulative-dose response experiment. Third, the specific strain of mice (Swiss CD or albino CF-1) used in each individual study was not clearly documented in this paper (Weissman et al. 1982). Resultantly, it is reasonable to conclude the differences in route of administration, nociceptive stimulus, and mouse strains explain the discrepant results.

It is important to note that in our studies, an additional behavior, known as hyperreflexia (Adams & Martin 1996; Dewey 1986), was prominent in CP47,497-treated (30 mg/kg), but not THC-treated mice, even at the high doses of THC (200 mg/kg) tested. Hyperreflexia was observed after CP47,497 treatment across acute drug administration experiments in ICR, C57BL6/J and $CB_1^{(+/+)}$ mice, but was absent in mice treated with either low or high dose rimonabant. The expression of hyperreflexia has been previously reported in dogs treated with THC (Wilson & May 1976), and in our laboratory in mice after inhalation exposure to synthetic cannabinoids, JWH-018 and

JWH-073 (Poklis, et al. 2012; Wiebelhaus et al. 2012). Cannabinoid-induced cerebellar dysfunction is characterized by motor incoordination, loss of muscle tone, and gait disturbances in mice as well as static ataxia in dogs (Dewey 1986; Patel & Hillard 2001; Weissman et al. 1982; Wilson & May 1976; Wilson & May 1979). Supporting a CB_1 component of these motor disturbances, hyperreflexia in mice (Patel & Hillard 2001) and ataxia in dogs (Lichtman et al. 1998) are blocked by rimonabant treatment. While the precise neuroanatomical mechanism(s) responsible for hyperreflexia and motor deficiency observed by endogenous, phytocannabinoid or synthetic cannabinoids remains to be elucidated (Patel & Hillard 2001; Weissman et al. 1982; Wilson & May 1976; Wilson & May 1979), human fMRI studies report decreased cerebellar function after THC exposure (Bloom et al. 1999), suggesting cerebellar changes are relevant to the cannabinoid-induced physiological motor impairments. Interestingly, commonly reported clinical features of SCB exposure include hyperreflexia, and to a lesser extent, jerking of limbs (Harris & Brown 2013; Simmons et al. 2011). As a result, these preclinical hyperreflexia data appear to demonstrate good face validity as a behavioral screen of abused synthetic cannabinoids in mice. However, further studies are needed to test this observation, specifically with SCB across aminoalkylindole and derivative structural classes, alongside evaluation of structurally similar non-cannabinoid compounds (control for false positives).

The quantification of CP47,497 in blood over time suggest that peak drug effects are achieved between 1 and 2 hours after intraperitoneal drug administration, although no significant differences are seen between drug present at 0.25-, 0.5-, 1- or 2- hour time points. Also, it is important to examine drug concentration at 12- and 24-hour time points against decreases in behavioral effects in future studies. We predict that CP47,497 concentrations would decrease at these later times as no significant behavioral effects are seen. These additional experiments will also assist in determining metabolic clearance of CP47,497 in blood. CP47,497 blood concentration appears to mirror effects within the behavioral time course, with decreases in catalepsy, antinociception, and hypothermia corresponding to a significant decrease in CP47,497 blood concentration at 8 hours.

While these data support the possibility that parent CP47,497 drug is likely responsible for the majority of behavioral effects seen, additional studies should examine drug brain concentrations to correlate CP47,497 behavioral effect with concentration of CP47,497 concomitantly present in brain. Metabolites of THC (Agurell et al. 1986; Foltz et al. 1983; Wilson & May 1975), as well as synthetic cannabinoid metabolites of JWH-018 and JWH-073 (Brents et al. 2011; Brents et al. 2012), are active at CB_1 receptors and responsible for cannabimimetic effects. Therefore, studies should also examine *in vivo* metabolic profiles of CP47,497 in blood and brain, as

Phase 1 hydroxylation and oxygenation reactions have been detected by human liver microsomal analysis (Jin et al. 2013). Furthermore, recent work implies this finding extends to cannabimimetic effects of SCB metabolites of JWH-018 (Brents et al. 2011) and JWH-073 (Brents et al. 2012). Data from these studies will allow us to determine if only the CP47,497 parent compound is mediating the behavioral effects or if, like THC, metabolites of CP47,497 possess bioactivity at CB_1 and play a role in the observed cannabimimetic actions.

Temporal effects of CP47,497 closely mirror those of THC, with sustained presentation of catalepsy, antinociception and hypothermia from 0.5 to 8 hours after drug administration. At 24-hours post-treatment, all behavioral effects returned to baseline levels. Maximum effects of catalepsy and hypothermia by CP47,497 were observed between 2 and 4 hours after administration, and levels were significantly higher than THC at these time points. Unexpectedly, a significant antinociceptive effect was not observed between THC and vehicle at 0.5 hours, as was shown in the dose-response experiments. A possible explanation is that slow absorption kinetics of THC via intraperitoneal administration could produce variable antinociceptive effects before peak drug distribution of THC are achieved. Nonetheless, CP47,497 produced significantly more antinociception than THC at this time point. From these data, we can

conclude that CP47,497-induced tetrad cannabimimetic effects display a similar duration as THC.

The identification of cannabinoid receptors, CB_1 (Matsuda et al. 1990) and CB_2 (Munro et al. 1993), permitted researchers to screen novel compounds for their ability to bind to cannabinoid receptors. Many structure activity relationship studies utilized a complementary approach to screen novel SCB compounds; specifically, compounds were tested *in vitro* for their CB_1 binding affinity and *in vivo* for their ability to elicit a characteristic cannabimimetic behavioral profile in the tetrad. Studies have illustrated that relative potencies of cannabinoid analogs, derived from *in vitro* CB_1 binding data, positively correlate with physiological effects observed from *in vivo* behavioral evaluation in animals (Compton et al. 1993; Wiley et al. 1998) and humans (Herkenham et al. 1990). Because SCB drugs are reportedly being abused for their THC-like psychoactive effects, studies were designed to investigate CB_1 receptor involvement *in vivo*. Importantly, pharmacological and genetic tools have been developed since initial CP47,497 studies were performed in the late 1980's. Therefore, to examine CB_1 receptor involvement in CP47,497-mediated effects, the CB_1 receptor antagonist, rimonabant, was utilized at both low (3mg/kg) and high (10 mg/kg) doses. Low dose rimonabant was sufficient to reverse cataleptic and antinociceptive effects of both CP47,497 and THC. For decreases in temperature, low dose rimonabant significantly

attenuated effects of CP47,497 and THC. High dose rimonabant was required for full reversal of hypothermia and time spent immobile. Treatment with high dose rimonabant blocked all measures of cannabimimetic tetrad activity produced by both THC and CP47,497. Lastly, high dose rimonabant was required to successfully inhibit time spent immobile produced by both CP47,497 and THC treatment. These data offer strong evidence confirming CP47,497 actions are CB_1 mediated.

Interestingly, rimonabant (10 mg/kg) elicited statistically significant increases in distance traveled across all treatment groups. This presents a potential confound of the experiment because it is not possible to decipher whether the increased distance traveled following the combination of rimonabant and CP47,497 are CB_1 mediated. Nevertheless, these effects of rimonabant are in agreement with published reports of locomotor stimulating effects of rimonabant produced at doses above 3 mg/kg (Compton et al. 1996). Additionally, these previously published studies reported an ED_{50} for rimonabant-induced locomotor stimulation of approximately 5 mg/kg; therefore, previous findings corroborate our results of significant locomotor activation at 10 mg/kg rimonabant, but not with 3 mg/kg. Comparison of CB_1 receptor binding affinities of CP47,497 (K_i = 2.2 ± 0.47 nM), THC (K_i = 40.7 ± 1.7 nM), and rimonabant (K_i = 1.98 ± 0.36 nM) support why increasing doses of the competitive antagonist are required to surmount the effects produced by the doses of CB_1 receptor agonists, 30 mg/kg

CP47,497 and 100 mg/kg, used in the experiment (Compton et al. 1993; Melvin et al. 1993; Rinaldi-Carmona et al. 1994). To overcome the confound in these experimental results and test the ability of rimonabant to block CP47,497-induced locomotor depressant effects, additional studies could utilize a lower dose of CP47,497 which still produces immobility, and examine if rimonabant, at doses less than 3 mg/kg, could effectively blunt the motor depressant effects.

A caveat to using rimonabant at the high doses required to antagonize our cannabinoid agonist effects is the potential of off-target effects. GTPγS studies in brains of $CB_1^{(-/-)}$ mice (Breivogel et al. 2001; Cinar & Szucs 2009) provide evidence of non-CB_1 targets for rimonabant. Therefore, to support these antagonism findings, we tested CP47,497 in mice genetically devoid of CB_1 receptors. To deconstruct whether CB_1 receptors were involved in the locomotor depressant effects of CP47,497, $CB_1^{(+/+)}$ and $CB_1^{(-/-)}$ mice were utilized in the next series of experiments. THC-like behavioral effects of catalepsy, antinociception, hypothermia, and immobility were completely abolished in $CB_1^{(-/-)}$ mice. Vehicle-treated $CB_1^{(-/-)}$ mice traveled less than their wild-type counterparts, which has been documented before in CB_1 mutant mice (Zimmer et al. 1999). Furthermore, HPLC/MS/MS quantitation of CP47,497 in brain confirmed the presence of drug concomitant with absence of cataleptic, antinociceptive, and hypothermic behavioral effects in $CB_1^{(-/-)}$ mice. Taken together, these complementary

pharmacological and genetic data support CB_1 mediation of THC-like effects of CP47,497 in the tetrad.

Based on dose-response studies in ICR and C57BL6/J mice, this dose of CP47,497 was a sufficient, if not a supramaximal dose, to elicit cannabimimetic effects in CB_1 genetically modified mice (backcrossed onto a C57BL6/J background). From these dose-response studies in ICR and C57BL6/J mice, we also observed that, depending on the measure, C57BL6/J mice exhibited greater cannabimimetic sensitivity to CP47,497 versus ICR mice; the differences in drug potency and efficacy suggest differential effects of CP47,497 across strains in the tetrad. This is not surprising, as behavioral responses to various pharmacological compounds have been found to vary among species and strains. For example, differential sensitivity to the endogenous cannabinoid, AEA, was observed in measures assessing motor function and emotionality, across ICR, DBA/2 and C57BL/6 mouse strains (Chakrabarti et al. 1998). Moreover, northern blot analysis identified increased CB_1 gene expression in ICR mice versus C57BL6/J after systemic and intracerebral administration of THC (Onaivi et al. 1995). This phenomena is not specific to cannabinoid drugs as an examination of morphine sensitivity across six mouse strains showed that morphine-pelleted mice demonstrated differential amounts of mortality, ranging from 5% in ICR, to 84% in A/J mice (Brase et al. 1977). Furthermore, differences in brain cannabinoid receptor binding

was observed between C57BL/6 and DBA/2 mice (Hungund & Basavarajappa 2000). Collectively, these data implicate that many drugs of abuse produce strain-specific phenotypes, and indicate potential neurobehavioral differences may be due, in part, to underlying genetic differences. Therefore, choice of mouse strain must be considered when designing experiments with synthetic cannabinoid compounds, such as CP47,497.

As previously discussed, drugs from many classes produce a subset, of behavioral effects in the tetrad assay and some (El-Alfy et al. 2009; Pava et al. 2012) even produce all four measures (Wiley 2003) . Historically, behavioral methods used to examine abused drugs include pharmacological comparison to other well-characterized drugs, self-administration, drug discrimination, and assessment of physical dependence (Wiley 1999). In contrast to other drugs of abuse, such as cocaine (Tanda et al. 2000), morphine (Navarro et al. 2001), and ethanol (Hungund et al. 2003), animals do not readily self-administer cannabinoids (Gardner 2005). However, THC self-administration has been reported in cocaine-experienced (Tanda et al. 2000) as well as drug-naïve (Justinova et al. 2003) squirrel monkeys. But this effect may be also be strain or species specific, as these findings are from the same research group and have not been reproduced across laboratories or in other self-administration animal models.

In contrast, numerous studies have shown that the discriminative stimulus effects of THC in animals are specific to marijuana-like drugs (Balster & Prescott 1992; Barrett

et al. 1995), a behavior that has also been demonstrated in humans (Lile et al. 2009). As a result, the drug discrimination assay is the most selective model available to study drug mechanism of action in terms of cannabinoid-like intoxicating effects (Balster & Prescott 1992; Wiley 1999). Discrimination of THC or other psychoactive drugs has been established using a two-lever paradigm in rodents, non-human primates, and humans (Barrett et al. 1995; Järbe & Henriksson 1974; Justinova et al. 2003; McMahon et al. 2008; Vann et al. 2009). Traditionally, these studies were conducted with rats and non-human primates, but recent examinations of drug discrimination in C57BL/6 mice have reported successful discriminative stimulus training (McMahon et al. 2008; Vann et al. 2009). The advantage of utilizing mice is the ability to test genetically modified mice, thus greatly enhancing the utility of the drug discrimination paradigm.

Drug discrimination experiments presented here revealed full substitution and dose-dependent effects of CP47,497 in C57BL/6J mice trained to discriminate THC from vehicle, illustrating subjective effects of CP47,497 are THC-like. Doses of 3.2 mg/kg CP47,497 in drug discrimination studies produced significant decreases in response rates, yet, this dose still fully displayed full substitution for 5.6 mg/kg THC. The drug discrimination data substantiate results from dose-response tetrad experiments in C57BL/6J mice, as 3.2 mg/kg CP47,497 caused significant immobility with a trend toward decreases in distance traveled. A remaining question of the drug discrimination

experiment is whether the discriminative stimulus effects of CP47,497 are CB_1 receptor mediated. For example, rimonabant has been systemically shown to block discriminative stimulus effects of endogenous (Wiley 1999; Wiley, et al. 2011), plant-derived (McMahon et al. 2008; Vann et al. 2009; Wiley 1999; Wiley, et al. 2011), and synthetic (Ginsburg et al. 2012; McMahon et al. 2008; Wiley et al. 1995; Wiley, et al. 2013) cannabinoids. Consequently, it would be of value to examine whether the THC-like effects of CP47,497 in the drug discrimination assay are blocked by rimonabant.

Our results are consistent with the structurally-related compound CP55,940, which fully substitutes for THC, and demonstrates enhanced potency in drug discrimination assays, which can be ameliorated by rimonabant treatment (McMahon et al. 2008; Wiley et al. 2011; Wiley et al. 2005). Further, these data are consistent with reports of other currently abused synthetic cannabinoids, primarily of the nonclassical (CP47,497 and HU-210) and aminoalkylindole (JWH-018 and JWH-073) classes, which have demonstrated complete substitution for THC in rats (Brents et al. 2013; Järbe & Gifford 2013; Weissman et al. 1982) and monkeys (Ginsburg et al. 2012). Importantly, rimonabant produced surmountable antagonism for the discriminative stimulus effects of JWH-018 and JWH-073 in monkeys (Ginsburg et al. 2012), as well as JWH-018 in rats (Järbe et al. 2012), but studies have not yet tested antagonism of CP47,497 by rimonabant. At the very least, our drug discrimination results substantiate that interoceptive effects of CP47,497 substitute for THC, which supports known abuse

potential of this synthetic cannabinoid in humans. However, additional studies are needed to assess whether rimonabant will block these discriminative stimulus effects. If rimonabant successfully blocks the discriminative stimulus effects of CP47,497, the animals would respond on the vehicle-appropriate lever, thus, strongly suggesting that CB_1 receptors are involved in the discriminative stimulus.

As discussed in detail in Chapter 2, when compared to different preparations of *cannabis,* SCB drugs have largely gained notoriety due to their magnitude and frequency of reported adverse effects including anxiety, hypertension, tachycardia, and seizures (Forrester et al. 2012; Hermanns-Clausen et al. 2012a; Seely et al. 2012; Wood 2013). Furthermore, reports of development of tolerance to the desired synthetic cannabinoid effects have been reported in humans (Gunderson et al. 2012; Zimmermann et al. 2009). Tolerance to behavioral effects of THC, and other cannabinoids has been well-characterized preclinically in rodents (Abood et al. 1993; Fan et al. 1994; Hruba et al. 2012; Jones et al. 1976; McKinney, et al. 2007; Pertwee et al. 1993; Sim-Selley, 2002). In particular, repeated cannabinoid administration leads to tolerance to acute effects of locomotion (Abood et al. 1993; Oviedo et al. 1993), hypothermia (Pertwee et al. 1993; Fan et al. 1996), and antinociception (Lee et al. 2003; Martin et al. 1996). Accordingly, studies were designed to test whether tolerance to the

CP47,497-induced cannabimimetic effects of catalepsy, antinociception and hypothermia develop with repeated administration in mice.

The data presented here show that repeated CP47,497, THC and CP55,940 treatment in mice resulted in the development of tolerance to CP47,497-induced catalepsy, antinociception, and hypothermia. Interestingly, CP47,497, THC and CP55,940 produced similar magnitudes of tolerance in antinociception, catalepsy and hypothermia, despite their differences in potency (binding affinity) and efficacy (as defined by agonist stimulation of [^{35}S]-GTPγS binding). Additionally, it was previously discussed that acute administration of CP47,497 produced pronounced hyperreflexia, but mice repeatedly given vehicle treatment and then administered increasing doses of CP47,497, did not exhibit hyperreflexic behavior. A possible explanation for this observed difference is that repeated handling, as well as exposure to repeated ethanol-containing vehicle injections, may have reduced sensitivity to CP47,497-induced hyperreflexic behavior. Studies have reported that mice treated chronically with ethanol demonstrate less sensitivity to hypothermic, locomotor depressant, and antinociceptive effects of WIN55,212-2 (Pava et al. 2012). The amount of ethanol was a minimal component of the vehicle used in our studies as compared to chronic ethanol exposure, but this may account for the modest decrease in hyperreflexia, with no other overt behavioral effects present.

Based on spare receptor theory, the magnitude of tolerance development to repeated CP47,497 administration is inversely related to spare receptor reserves (Kenakin 2004; Negus 2006). As a result, as a high efficacy partial agonist, CP47,497 should produce less tolerance than the low efficacy partial agonist, THC at equi-effective doses. However, in our experimental system, it appears that even though THC is a low efficacy agonist, no differences in tolerance may be due to the abundant cannabinoid receptor reserve in the CNS (Gifford et al. 1999). Thus, the ample number of spare receptors may account for the finding by which tolerance to the pharmacological effects of THC in the tetrad assay is indistinguishable from high efficacy partial agonists, CP55,4940 and CP47,497. It is also possible that testing additional points might reveal differences in tolerance among agonists, as tolerance was assessed only at as single time-point in these studies. For example, rats chronically treated with THC displayed a time-dependent loss of cannabinoid receptors and cannabinoid receptor-activated G proteins in both membranes and brain sections tested after 3, 7, 14, and 21 days of THC treatment (Breivogel et al. 1999). To rectify these two possibilities, time-course experiments are needed to examine time-dependent effects of tolerance to CP47,497 for each behavioral measure.

The localization of CB_1 receptors varies across brain regions (Herkenham et al. 1990), as does CB_1 receptor efficiency, defined as the number of G-proteins activated per CB_1 receptor (Breivogel et al. 1997; Burkey, Quock, Consroe, Ehlert, et al. 1997;

Howlett 2004). Studies have shown that repeated THC or WIN55,212-2 treatment produced tolerance to cannabinoid-induced behaviors concomitantly with receptor desensitization and downregulation, with brain-region specific desensitization observed (Sim-Selley & Martin 2002). Similarly, CB_1 receptor desensitization and downregulation effects were reproduced after repeated THC with diminished inhibition of adenylyl cyclase also observed (Selley et al. 2004). Studies with β-arrestin2 knockout mice reported enhancement of acute THC-mediated antinociception and hypothermia; upon repeated THC-administration, β-arrestin2 knockout mice showed reduced CB_1 receptor desensitization and downregulation in cerebellum, spinal cord and periaqueductal accompanied with reduced THC-mediated antinociceptive, but increased THC-mediated cataleptic tolerance (Nguyen et al. 2012). These data provide the best direct evidence of CB_1 receptor involvement of tolerance development to THC-mediated behaviors. Taken together, these data illustrate that molecular adaptations at the receptor and effector level underlie the presentation of behavioral tolerance. Moving forward, it is important to determine to what extent chronic CP47,497 administration causes CB_1 receptor desensitization and downregulation, with investigation of time-course, dose-response and brain-region specificity.

Our tolerance results are in agreement with previous studies that demonstrate cross-tolerance of CP55,940 and WIN55,212-2 develops to THC-induced

cannabimimetic effects, which suggests these drugs effects are mediated by the same receptor signaling mechanism(s) (Fan 1994, Pertwee 1993). Quantitative autoradiographic analysis of post-mortem brains obtained from chronic marijuana smokers showed decreases in CB_1 receptor binding in the dorsal striatum, nucleus accumbens and globus palladus, with pharmacodynamic adaptations including decreases in CB_1 cDNA expression and CB_1 receptor mRNA levels thought to contribute to the development of tolerance to THC effects in chronic *cannabis* users (Villares 2007). Furthermore, positron emission tomography (PET) imaging studies reported CB_1 downregulation in cortical, but not basal ganglia and midbrain structures, from chronic daily smokers (Hirvonen et al. 2012), which overlaps with observed CB_1 receptor downregulation in rodents. Although additional preclinical investigations of receptor adaptations following chronic synthetic cannabinoid exposure are needed, in the future, it may be possible to extrapolate data regarding tolerance to chronic THC to consequences of chronic abuse of CP47,497 or other full CB_1 receptor agonists in humans.

Consequently, prolonged use of cannabinoids and subsequent CB_1-mediated neuroadaptations promotes development of cannabis dependence, and is thought to be responsible for behaviors that lead to drug abuse and addiction. A withdrawal or abstinence syndrome, characterized by mood and sleep disturbances, with changes in

appetite have been previously described (Cooper & Haney 2008; Desai et al. 2013; Maldonado 2002; Ramesh et al. 2011). For rimonabant-precipitated withdrawal experiments, CP55,950 was used as a control with the dose of 2 mg/kg based on previously published studies that successfully achieved behavioral tolerance in ICR mice (Fan et al. 1994; Fan et al. 1996). To maximize the development of dependence, the dose of CP47,497 (15 mg/kg) and THC (50 mg/kg) was derived from catalepsy ED_{84} values from acute dose-response experiments. Using this approach, mice treated repeatedly with CP47,497 or THC, then challenged with rimonabant displayed significant increases in somatic withdrawal signs including paw flutters and head shakes versus vehicle-treated animals. Withdrawal from CP55,940 has been previously demonstrated in mice (Oliva et al. 2003) and rats (Rubino et al. 1998). Considering no differences in efficacy or potency were observed between CP47,497 or THC and CP55,940 in [^{35}S]-GTPγS binding, it is not likely that differences in G-protein activation are responsible for these effects. However, differences in downstream effector signaling may account for these observations, as basal cAMP and PKA levels were increased after repeated THC (15 mg/kg (i.p.) twice daily, 6.5 days) versus no alterations after repeated CP55,940 (0.4 mg/kg (i.p.) twice daily, 6.5 days) in rats (Rubino, Vigano', et al. 2000; Rubino, Viganò, et al. 2000). In one study, significant somatic withdrawal signs were observed after twice daily injections of CP55,940 (0.5 mg/kg) for 6.5 days (Oliva et al. 2003); however differences in mouse strain (Swiss albino vs. ICR), route of

administration (i.p. vs. s.c.), and agonist dose (0.5 vs. 2 mg/kg) may account for lack of significance observed in studies presented herein. Furthermore, as we are interested in THC-like effects of CP47,497, and significant somatic withdrawal were observed after repeated administration with CP47,497 and THC. Therefore, our studies suggest CP47,497 is more potent and efficacious (G-protein level) than THC, and when given repeatedly, produces significant withdrawal signs upon CB_1 antagonist treatment.

Conclusion

Considering that CP47,497 possesses low nanomolar CB_1 receptor binding affinity (Compton et al. 1993; Melvin et al. 1993), promotes functional activation of the CB_1 receptor upon ligand binding, produces cannabimimetic effects in the tetrad and drug discrimination assays, and these effects do not occur in $CB_1^{(-/-)}$ mice or wild type mice treated with a CB_1 receptor antagonist, it can be concluded that our results strongly support that CP47,497 achieves its potent and efficacious THC-like effects through CB_1 receptor involvement. Furthermore, our repeated administration studies demonstrated that similar to the consequences of repeated THC dosing (Abood et al. 1993; McKinney et al. 2008; Pertwee et al. 1993; Rubino, Vigano', et al. 2000), repeated CP47,497 led to behavioral tolerance of cannabimimetic effects in the tetrad assay and CP47,497-dependent mice exhibited somatic withdrawal signs upon rimonabant challenge.

Concluding Remarks and Future Directions

Data presented in this body of work developed a quantitative bioanalytical identification method and examined the pharmacological profile of acute and repeated administration of a new class of abused drugs, synthetic cannabinoids, and in particular, the prototypical bicyclic cannabinoid, CP47,497. Prior to the 1960's, much research exploring the effects of marijuana (medicinal and psychotropic) in humans were largely anecdotal. The decades of research following the isolation of THC and production of synthetic cannabinoids led to the discovery of an endogenous cannabinoid system, the elucidation of CB receptors (CB_1 and CB_2), their endogenous ligands (AEA and 2-AG), and elucidated molecular signaling events upon the subsequent activation of these receptors. However, with the expropriation of research tools as drugs of abuse, the scientific field finds itself challenged to examine pharmacological and toxicological consequences of their abuse in a timely and relevant manner. Synthetic cannabinoids, as a new class of designer drugs, present a persistent challenge to researchers, toxicologists, and law enforcement as the chemical constituents rapidly evolve to circumvent legislative bans and to evade biological detection.

Presented here are studies that investigated the mechanism(s) by which CP47,497 achieves THC-like effects, that is, through CB_1 receptor mediation, eliciting

potent behavioral effects in mice that develop tolerance and display dependence liability upon repeated administration. Equally important, we present an HPLC/MS/MS method previously used to quantify THC and its major metabolites, which has been adapted to detect several synthetic cannabinoids (CP47,497, CP47,497-C8, JWH-250) in biological tissues. Importantly, these experiments demonstrate bioavailability of CP47,497 in the brain, and with future method validation, traditional cannabinoid analytes as well as designer synthetic cannabinoids can be analyzed simultaneously. Taking into account the novelty of the analytical and pharmacological data presented in this dissertation, these studies provide the preclinical knowledge necessary for potential hypothesis-driven, scientific-based studies to be performed in humans in the future. Since tolerance to classical cannabinoid behavior was observed, with no difference in CP47,497-stimulated [^{35}S]-GTPγS binding observed, we propose that differences in brain region specific receptor downregulation, desensitization and second messenger adaptations are responsible for cannabimimetic effects observed**.** Furthermore, with the development and validation of an analytical method for detection of CP47,497 in biological tissues, studies can address the hypothesis that active metabolites of CP47,497 are involved in producing CP47,497-mediated behavioral effects in animals and humans. This is crucial as essentially all of the data collected regarding synthetic cannabinoid effects in humans has come from clinical case reports, poison control centers, and internet-based surveys. Data from the experiments presented herein may

assist clinicians, toxicologists and law enforcement by providing pharmacological and toxicological data, which can be used to extend what is known of THC to better understand THC-like effects of synthetic cannabinoids.

The clandestine synthesis and distribution of synthetic cannabinoids as designer drugs of abuse remains a continuously moving target. Therefore, in the future, studies need to be conducted that assess the: (1) activation of G-proteins in human cannabinoid receptor expression systems, (2) development of bioanalytical methods for detection of novel SCB compounds, and (3) screening of cannabimimetic behavioral effects in the tetrad and drug discrimination paradigms, with judicious use of the selective CB_1 antagonists, such as rimonabant, to determine the involvement of CB_1 receptors.

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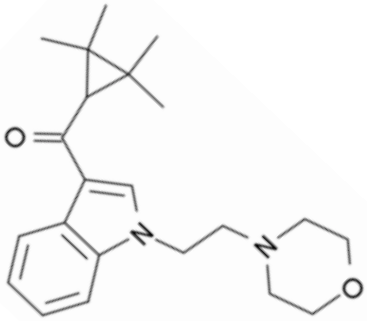
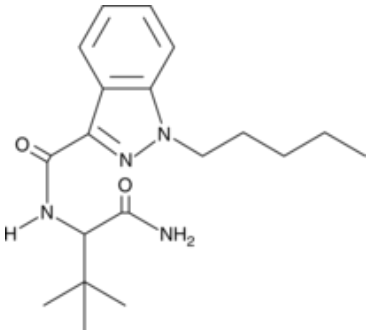
Appendix I – Chemical Structures

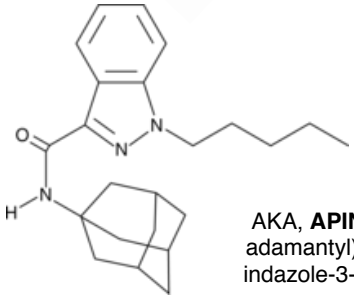
A796-260



ADB-PINACA

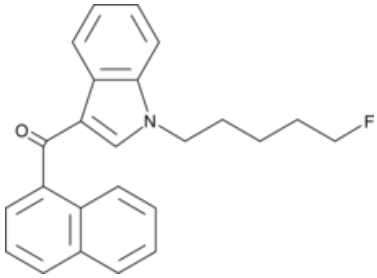


AKB48



AKA, **APINACA** (N-(1-adamantyl)-1-pentyl-1H-indazole-3-carboxamide)

AM-2201



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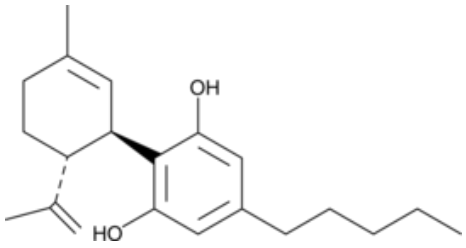
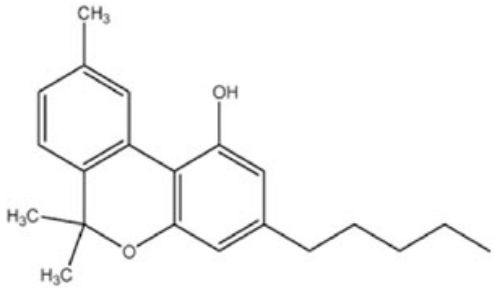
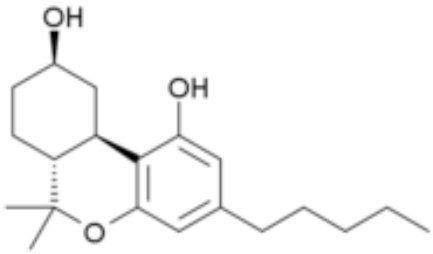


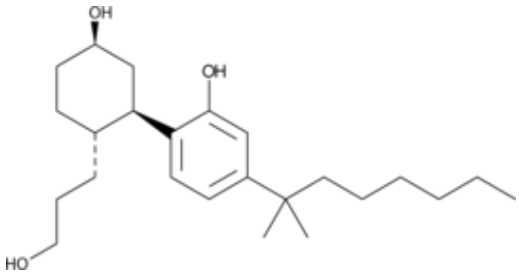
Cannabinol



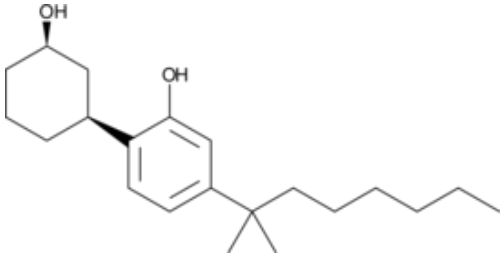
9-*nor*-9β-Hydroxyhexahydro-cannabinol (HHC)



(±)-CP55,940



CP47,497



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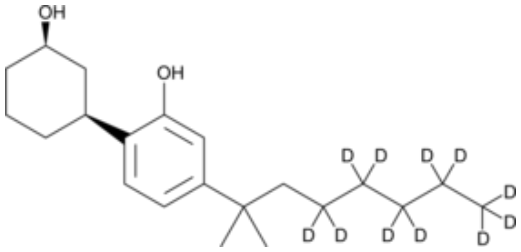

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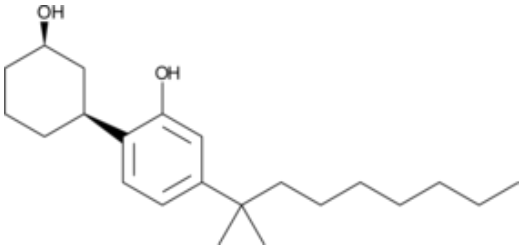

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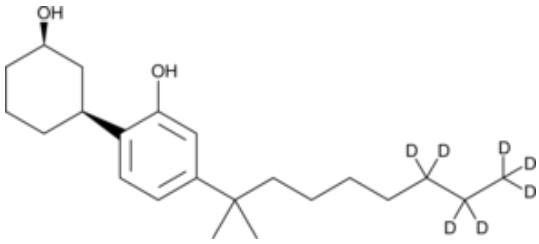
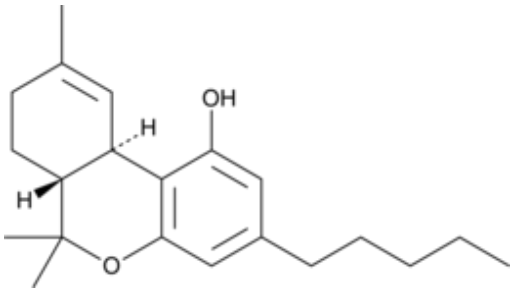
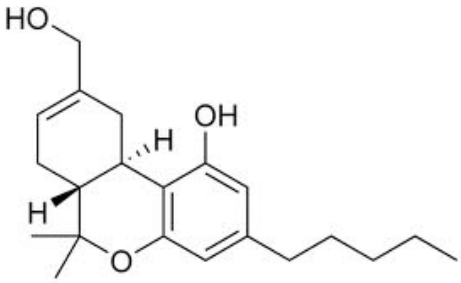


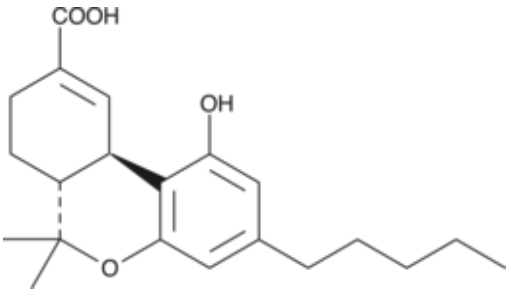
Δ^9-THC



11-OH-THC



(–)-11-nor-9-carboxy-Δ^9-THC



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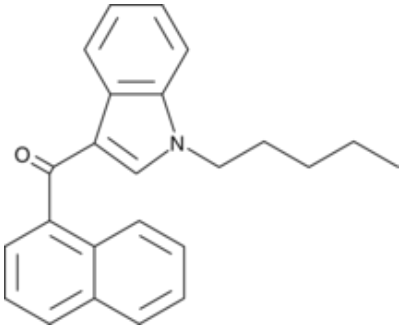

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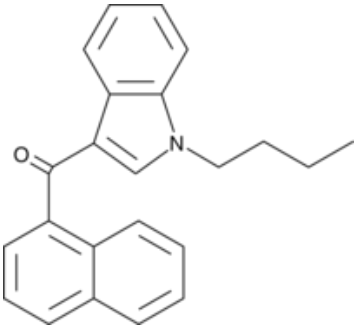

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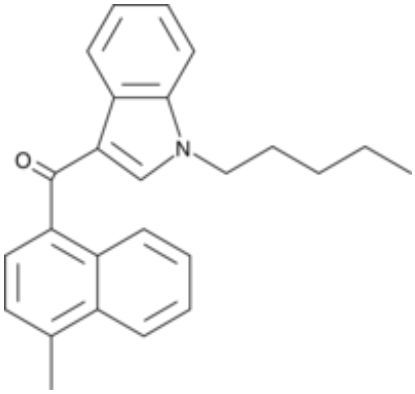

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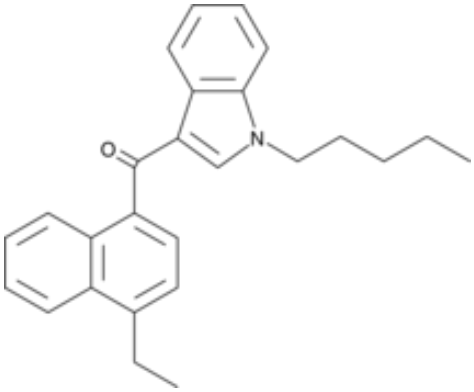

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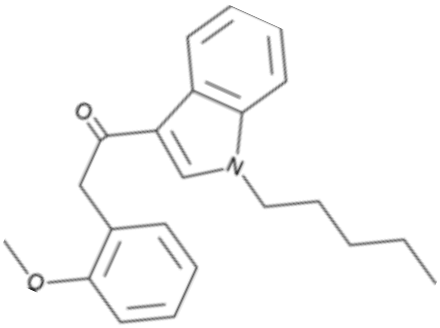

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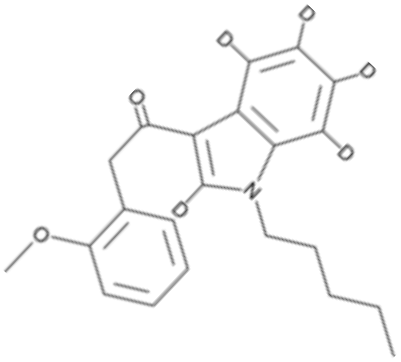

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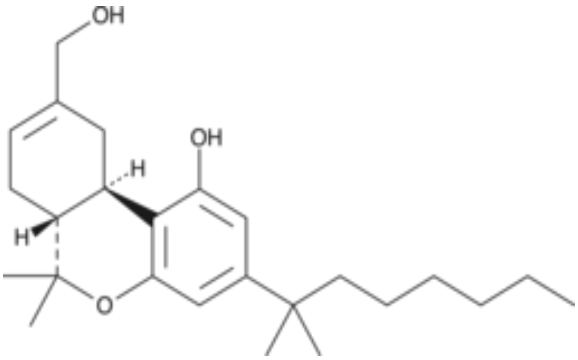

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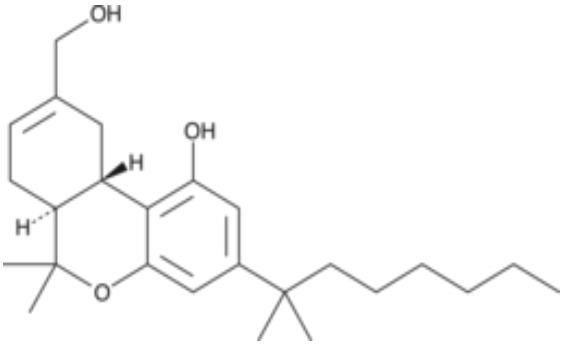
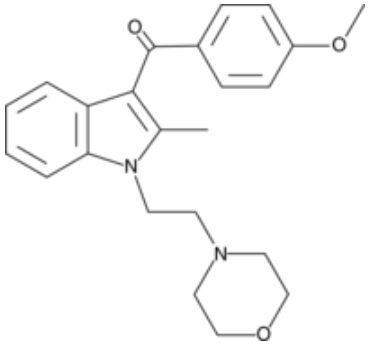
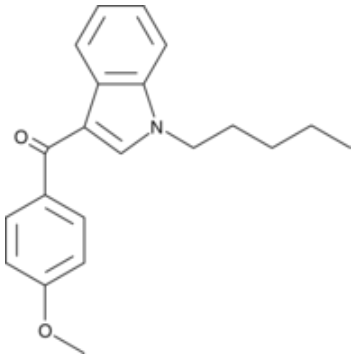


Pravadoline



RCS-4



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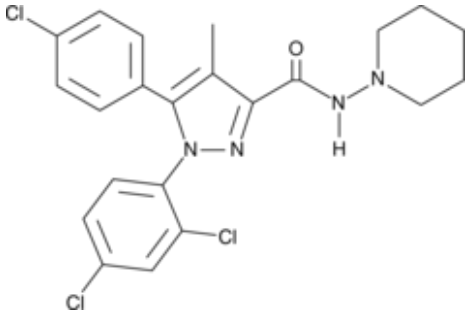
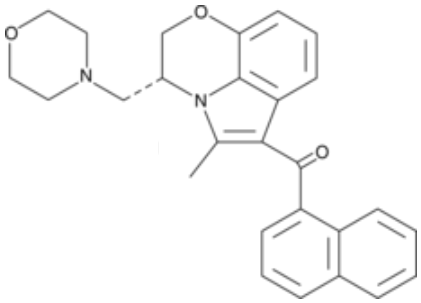


WIN55,212-2



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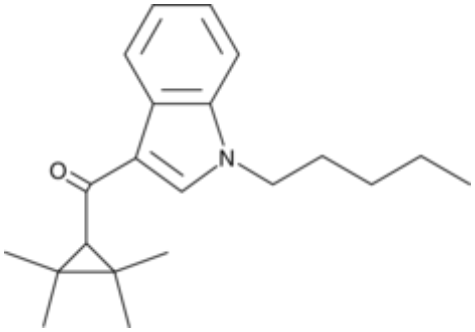

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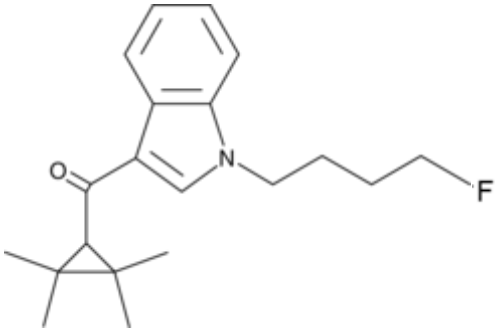
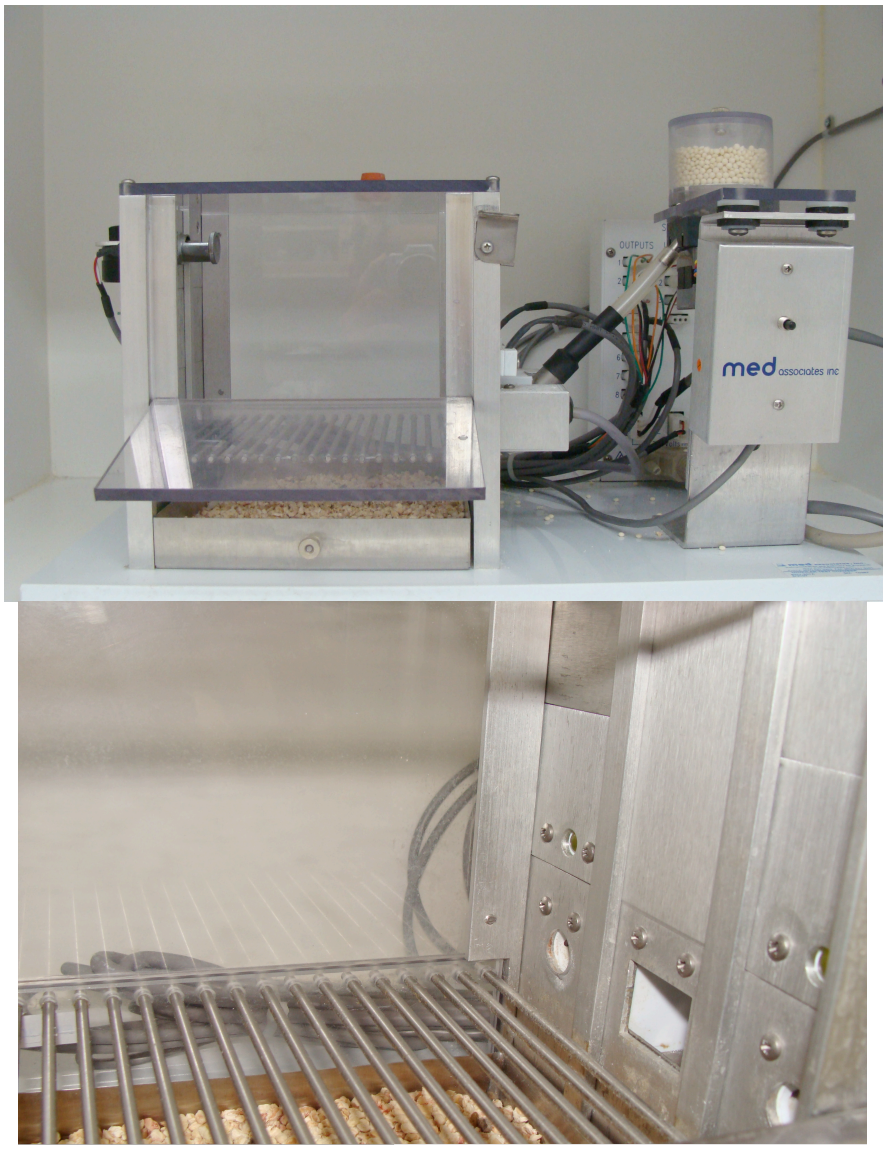


Appendix II – Drug Discrimination Apparatus



Picture credits, B. Ignatowska-Jankowska

Vita

Kimberly Lynne Samano was born on September 14, 1983 in Plantation, Florida and graduated from Piper High School (Sunrise, FL) in 2002. In 2006, she earned her Bachelors of Health Science with Chemistry and Criminology minors from the University of Miami (FL) and then earned her Masters of Science degree in Forensic Science from Arcadia University in Spring 2008. In the Fall, she matriculated into the Pharmacology and Toxicology doctoral program at Virginia Commonwealth University and in December of 2011, she began a collaborative research project under the guidance of Drs. Aron H. Lichtman and Alphonse Poklis. Upon acceptance of this dissertation, she will be awarded a Doctorate of Philosophy in Pharmacology and Toxicology.