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Investigating Cannabinoid Type-1 Receptor (CB1R) Positive Allosteric Modulators (PAMs) in Mouse Models of Overt Cannabimimetic Activity, Subjective Drug Effects, and Neuropathic Pain

Jayden Elmer
Virginia Commonwealth University

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2021

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Jayden A. Elmer
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A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University

By

Jayden Aric Elmer

Bachelor of Science, University of Virginia, 2018

Director: Dr. Aron Lichtman, Professor, Department of Pharmacology & Toxicology; Associate Dean of Research and Graduate Studies, School of Pharmacy

Virginia Commonwealth University
Richmond, Virginia
July 2021
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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>2-AG</td>
<td>2-arachidonoylglycerol</td>
</tr>
<tr>
<td>AA</td>
<td>arachidonic acid</td>
</tr>
<tr>
<td>AAI</td>
<td>aminoalkylindole</td>
</tr>
<tr>
<td>AEA</td>
<td>anandamide</td>
</tr>
<tr>
<td>CB₁</td>
<td>cannabinoid receptor, subtype 1</td>
</tr>
<tr>
<td>CB₂</td>
<td>cannabinoid receptor, subtype 2</td>
</tr>
<tr>
<td>CBD</td>
<td>cannabidiol</td>
</tr>
<tr>
<td>CBN</td>
<td>cannabinol</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CTC</td>
<td>cubic ternary complex model</td>
</tr>
<tr>
<td>DAGL</td>
<td>diacylglycerol lipase</td>
</tr>
<tr>
<td>DEA</td>
<td>Drug Enforcement Administration</td>
</tr>
<tr>
<td>DLR</td>
<td>drug-like responding</td>
</tr>
<tr>
<td>DRL</td>
<td>differential reinforcement of low rate</td>
</tr>
<tr>
<td>eCB</td>
<td>endocannabinoid</td>
</tr>
<tr>
<td>ETC</td>
<td>extended ternary complex model</td>
</tr>
<tr>
<td>FAAH</td>
<td>fatty acid amide hydrolase</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FR</td>
<td>fixed ratio</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
</tr>
<tr>
<td>G&lt;sub&gt;i&lt;/sub&gt;</td>
<td>cAMP inhibitory G-protein</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>i.c.v</td>
<td>intracerebroventricular</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>i.t.</td>
<td>intrathecal</td>
</tr>
<tr>
<td>LSD</td>
<td>lysergic acid diethylamide</td>
</tr>
<tr>
<td>MAGL</td>
<td>monoacylglycerol lipase</td>
</tr>
<tr>
<td>MDA</td>
<td>3, 4-methylenedioxyamphetamine</td>
</tr>
<tr>
<td>MDMA</td>
<td>3, 4-methylenedioxyamphetamine</td>
</tr>
<tr>
<td>MOR</td>
<td>µ-opioid receptor</td>
</tr>
<tr>
<td>MULT FR</td>
<td>multiple fixed ratio</td>
</tr>
<tr>
<td>nAChR</td>
<td>nicotinic acetylcholine receptor</td>
</tr>
<tr>
<td>NAM</td>
<td>negative allosteric modulator</td>
</tr>
<tr>
<td>NAPE-PLD</td>
<td>N-acyl phosphatidylethanolamine-specific phospholipase D</td>
</tr>
<tr>
<td>NMDAR</td>
<td>n-methyl-d-aspartate receptor</td>
</tr>
<tr>
<td>PAG</td>
<td>periaqueductal grey</td>
</tr>
<tr>
<td>PAM</td>
<td>positive allosteric modulator</td>
</tr>
<tr>
<td>pSNL</td>
<td>partial sciatic nerve ligation</td>
</tr>
<tr>
<td>PTX</td>
<td>pertussis toxin</td>
</tr>
<tr>
<td>s.c.</td>
<td>subcutaneous</td>
</tr>
<tr>
<td>SD rats</td>
<td>Sprague-Dawley rats</td>
</tr>
<tr>
<td>SDL</td>
<td>state dependent learning</td>
</tr>
<tr>
<td>THC</td>
<td>tetrahydrocannabinol</td>
</tr>
<tr>
<td>TL</td>
<td>two-lever</td>
</tr>
</tbody>
</table>
Abstract

Investigating Cannabinoid Type-1 Receptor (CB1R) Positive Allosteric Modulators (PAMs) in Mouse Models of Overt Cannabimimetic Activity, Subjective Drug Effects and Neuropathic Pain

By

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Chronic pain affects between 20 and 30 percent of the adult population in western countries and represents a wide array of specific etiologies (Berge, 2011). Neuropathic pain secondary to traumatic nerve injury, chemotherapeutic toxicity, or diseases (e.g., diabetes mellitus) is often refractory to conventional analgesics, with patients receiving less than 50% pain relief compared to placebo (Finnerup et al. 2010). The endocannabinoid system has shown potential as a therapeutic target for neuropathic pain wherein CB1 agonism via administration of exogenous agonists or pharmacological blockade of endocannabinoid catabolic enzymes exhibits efficacy in reversing allodynia in the chronic constriction injury (CCI) model of neuropathic pain (Rahn & Hohmann, 2009). More recently CB1 positive allosteric modulators (PAMs) have shown antinociceptive efficacy in CCI with ZCZ011 and its analogs, GAT211 and ABD1236, producing dose and time-dependent reversal of alldynia (Ignatowska-Jankowska et al. 2015; Slivicki et al. 2018; Tseng et al. 2019). This study reports the activity of the 2-phenyl indole
class of CB₁ PAMs represented by ZCZ011 in behavioral paradigms for overt and subjective cannabimimetic side effects, and neuropathic pain. The goal of the study was to examine the relationship between the antiallodynic effects, overt and subjective cannabimimetic effects of CB₁ PAMs. Overt cannabimimetic activity was assessed in the tetrad assay which consists of the measures: locomotor activity, catalepsy, antinociception, and hypothermia). Subjective cannabimimetic effects were measured in the drug discrimination paradigm. ZCZ011 analogs were either tested alone in these assays to screen for agonist activity or in combination with CB₁ orthosteric agonist CP55,940 to screen for PAM effects. ZCZ011 analogs did not exhibit CB₁ agonist activity as measured in the tetrad assay and drug discrimination paradigm when administered alone. ZCZ011 was the only PAM to potentiate all three measures of the triad assay (catalepsy, antinociception, hypothermia) whereas the remaining analogs potentiated only a subset of those effects. ZCZ011, GAT211, LDK1747, and LDK1752 were evaluated in the drug discrimination paradigm. Of these compounds only ZCZ011 and LDK1752 had a potentiating effect on subjective responding to CP55,940. Lastly ZCZ011 analogs were tested for antiallodynic activity in a chronic constriction injury (CCI) model of neuropathic pain. ZCZ011, ABD1236, and GAT211 produced full reversal of allodynia in CCI-mice whereas the remaining analogs had no effect. Comparing results from this study, ZCZ011 is the only compound which exhibits PAM activity in each of the three behavioral paradigms. The remaining analogs show disparate effects with respect to overt and/or subjective cannabimimetic effects and antiallodynic activity. The results of this study indicate that there is no correlation for CB₁ PAM activity between the three behavioral paradigms and that it is possible for CB₁ PAMs to affect only a subset of cannabinoid-related behaviors.
Endogenous Cannabinoid System

Endogenous Cannabinoid Receptors

Cannabinoid receptors are highly conserved in vertebrates and have also been identified in some invertebrate species (Elphick, 2012; McPartland, 2004). Human and rodent CB₁ receptors have a high degree of sequence similarity sharing greater than 90% nucleic acid identity and 97% amino acid identity (Abood et al. 1997; Chakrabarti et al. 1995; Ho & Zhao, 1996). A strong line of evidence for the existence of cannabinoid receptors came from studies showing that cannabinoids inhibit adenylyl cyclase activity in neuronal cell models (Howlett & Fleming, 1984). The cannabinoid type-1 (CB₁) receptor was then identified through radioligand binding studies using [³H]-CP55,940 and subsequently cloned from rat and human brain (Devane et al. 1988; Gérard et al. 1991; Matsuda et al. 1990). A second subtype, cannabinoid type-2 (CB₂) receptor was identified and cloned using a PCR-based approach in differentiated myeloid cell lines (Munro et al. 1993). Both CB₁ and CB₂ receptors are the primary targets of Δ⁹-THC, the primary psychoactive constituent of Cannabis sativa (Rinaldi-Carmona et al. 1994). In conjunction with receptor binding data, in vivo behavioral measures such as the tetrad assay and drug discrimination paradigm have helped confirm that CB₁ receptors mediate the effects of cannabinoids in the CNS (Compton et al. 1993; Wiley et al. 1995a-c).
Anatomic Distribution of Cannabinoid Receptors

The CB$_1$ receptor is the most highly expressed G-protein coupled receptor (GPCR) in the CNS, with highest expression in the cerebral cortex, cerebellum, hippocampus, and basal ganglia nuclei (Glass et al. 1997; Herkenham et al. 1990; Tsou et al. 1998). The effects of cannabinoids on memory and cognition, motor control, and analgesia are correlated with the distribution of CB$_1$ receptors in the cerebral cortex and hippocampus, cerebellum, and basal ganglia, respectively. CB$_1$ receptors are expressed presynaptically on GABAergic neurons and to a lesser extent on glutamatergic neurons where the mediate inhibition of neurotransmitter release (Katona et al. 1999; Puighermanal et al. 2009; Straiker & Mackie, 2005). CB$_1$ receptors are also expressed in astrocytes where they play a role in modulating synaptic transmission and plasticity (Han et al. 2012). In the periphery, CB$_1$ receptors are expressed in circulating immune cells and several tissues including adrenal gland, bone marrow, heart, liver, lung, prostate, ovary, testis, thymus, tonsil, uterus, and vas deferens (Bouaboula et al. 1999; Galiegue et al. 1995). The CB$_2$ receptor is mainly expressed in cells associated with immune system function including lung, spleen, testis, thymus, tonsil, leukocytes and macrophages (Brown et al. 2002; Galiegue et al. 1995; Munro et al. 1993). In the CNS, CB$_2$ receptors are primarily expressed in microglia and are upregulated during immune responses to stimulate chemotactic responding (Cabral et al. 2008; Palazuelos et al. 2009). CB$_2$ expression has also been reported in neuronal cells where they may modulate dopamine-related behaviors in mice as well as synaptic plasticity in hippocampal neurons (Stempel et al. 2016; Zhang et al. 2014; Xi et al. 2011).
Endocannabinoids

The discovery of cannabinoid receptors prompted research efforts to identify the putative endogenous ligands of CB\textsubscript{1} and CB\textsubscript{2}. The first candidate molecule was N-arachidonoyl ethanolamine (anandamide; AEA), isolated from porcine brain, which was shown to displace \textsuperscript{3}H-U243 from rat membranes and inhibit electrically contractions of isolated mouse vas deferens (Devane \textit{et al.} 1992). The second endocannabinoid identified was 2-arachidonoyl glycerol (2-AG). Similar to AEA, 2-AG was found to displace synthetic cannabinoids in competitive inhibition experiments and also produced tetrad effects \textit{in vivo} when administered exogenously (Mechoulam \textit{et al.} 1995; Sugiura \textit{et al.} 1995). Arachidonic acid and N-palmitoylethanolamine lack cannabimimetic activity indicating that free arachidonic acid, of which AEA and 2-AG are precursors, does not activate cannabinoid receptors and that the arachidonic acid moiety is required of N-acylethanolamines and N-acylglycerols for receptor activation (Sugiura \textit{et al.} 1995). Hence, AEA and 2-AG are degraded enzymatically and represent the putative endogenous ligands of cannabinoid receptors. Both the synthesis and degradation of endocannabinoids are subject to enzymatic regulation. AEA is synthesized by N-acyl phosphatidylethanolamine-specific phospholipase D (NAPE-PLD) (Cadas \textit{et al.} 1996; Schmid \textit{et al.} 1983). 2-AG is synthesized by diacylglycerol lipase (DAGL), of which there exists two isoforms: DAGL\textalpha, expressed in neuronal cells and DAGL\textbeta, expressed in microglia and macrophages (Bell \textit{et al.} 1979; Prescott & Majerus 1983; Viader \textit{et al.} 2016). In neuronal cells, the endocannabinoids are synthesized on demand from membrane phospholipids and signal retrograde to CB\textsubscript{1} receptors on the presynaptic membrane to inhibit neurotransmitter release (Bisogno \textit{et al.} 2003; Katona \textit{et al.} 2006). AEA and 2-AG are subsequently degraded by fatty
Cannabinoid Pharmacology

Cannabinoid Receptor Structure and Binding Sites

The CB₁ and CB₂ receptors belong to the G-protein coupled receptor (GPCR) family and are primarily coupled to Gαᵢ/o heterotrimeric G proteins but also signal through β-arrestins (Howlett et al. 1985; Jin et al. 1999). The crystal structures of CB₁ and CB₂ have been obtained in complex with stabilizing antagonists AM6538 and AM10257, respectively (Hua et al. 2016, Li et al. 2019). Characteristic of GPCRs, cannabinoid receptors possess seven transmembrane domains connected by three extracellular and intracellular loops each, an extracellular N-terminal tail, and an intracellular C-terminal tail. Extracellular loop 2 (ECL2) of CB₁ is notably involved in agonist/inverse agonist binding (Ahn et al. 2009). Docking studies have shown that CB₁ agonists including AEA, 2-AG, Δ₉-THC, CP55,940, JWH-018, and WIN55,212-2 interact mainly with ECL2, the N-terminal loop, and transmembrane helices III, VI, and VII (Hua et al. 2016). Together these domains make up the orthosteric binding site which recognizes agonist and inverse agonist ligands. In addition to the orthosteric binding site, other allosteric binding sites have been identified on CB₁ receptors (Hurst et al. 2019; Shao et al. 2019). Studies on CB₂ receptors have identified various similarities and differences between CB₁ and CB₂ receptors which help to explain differences in selectivity of cannabinoid receptor agonists and antagonists (Li et al. 2019). The CB₂ receptor also possesses multiple binding sites as evidenced by reports on selective CB₂ allosteric modulators (Gado et al. 2019).
Cannabinoid Receptor Signaling Pathways

GPCR signaling occurs in three separate spatiotemporal waves (Lohse & Hofmann, 2015; Nogueras-Ortiz & Yudowski, 2016). The first wave of CB₁ signaling is mediated through Gαᵢ/o which leads to decreased cAMP through inhibition of adenylyl cyclase, decreased Ca²⁺ conductance, and increased K⁺ conductance (Mackie et al. 1995; Twitchell et al. 1997; Guo & Ikeda, 2004). The second wave occurs after ligand-induced receptor phosphorylation which leads to receptor desensitization and β-arrestin recruitment. β-arrestins in this wave of signaling function in receptor internalization as well as activation of several downstream effectors (Ahn et al. 2013; Breivogel et al. 2008; Delgado-Peraza et al. 2016; Laprairie et al. 2014). The third wave of cannabinoid signaling is mediated through CB₁ receptors localized to intracellular compartments (Brailoiu et al. 2011; Rozenfeld & Devi, 2008).

Allosteric Modulation of Cannabinoid Type I Receptors

Allosteric Modulation and Biased Signaling

The operational model of receptor theory (Eq. 1.1) holds that a pharmacological response is given by the concentration of agonist, its dissociation constant for the receptor, the maximal response, and a tissue-specific component τ which represents the concentration of agonist bound receptor that produces a half-maximal tissue response (Black & Leff, 1983; Kenakin, 2004). The operational model allows for comparison of agonist responses between different receptor systems. The site to which an agonist binds a receptor to elicit a response is termed the
orthosteric site. Allosteric modulators bind to topologically distinct or allosteric sites where they induce conformational changes that alter receptor activity (Kenakin, 2004).

\[
\text{Response} = \left( [A] \times \tau \times E_{\text{Max}} \right) / \left( [A](\tau+1) + K_A \right) \quad \text{Equation 1.1}
\]

To understand allosteric modulation of GPCRs one must take into account ligand binding at both orthosteric and allosteric sites, receptor activation states, and G protein association/activation. Models for the biochemical mechanisms of GPCRs include the extended ternary complex (ETC) model and cubic ternary complex (CTC) model (De Leen et al. 1980; Weiss et al. 1996). The ETC and CTC models are shown in Figure 1.1. In the ETC model the receptor can exist in an active (R\(_a\)) or inactive state (R\(_i\)). These receptor states may coexist according to the allosteric constant L given by L = [R\(_a\)] / [R\(_i\)]. Constitutive receptor activity is given by the concentration of active state R\(_a\)G species. Allosteric modulators can then, according to this model, affect receptor activity through changes in receptor affinity towards orthosteric ligands or G proteins; that is allosteric modulators may affect both affinity and efficacy (Kenakin, 2013). Not shown in the ETC or CTC model is the association of the receptor with β-arrestins which mediate arrest of G protein signaling, receptor internalization, and intracellular signaling (Lefkowitz et al. 1998; Lohse et al. 1990; Luttrell et al. 1999). Allosteric modulators may impart bias towards either G protein or β-arrestin signaling in the presence of an orthosteric agonist, which even alone may exhibit signaling bias (Kenakin, 2019).
A. Extended ternary complex (ETC) model  

B. Cubic ternary complex (CTC) model

**Figure 1.1** Pictorial representations of the extended ternary complex (ETC) model (A) and the cubic ternary complex (CTC) model (B). Figures drawn by author using ChemDraw Professional 16.0. 1.4

**CB1 Negative Allosteric Modulators**

Org27569, Org27759, and Org29647 were the first CB1 allosteric modulators characterized. These compounds were shown to increase the binding of $[^{3}H]$-CP55,940 but caused significant reductions in the $E_{\text{Max}}$ values for CP55,940- and AEA-induced $[^{35}S]$ GTP$\gamma$S binding (Baillie et al. 2013; Price et al. 2005). Despite enhancement of orthosteric agonist binding, the decrease in CB1 signaling functionally classifies these compounds as negative allosteric modulators (NAMs) of CB1 receptors. Org27569 reduces inhibition of cAMP accumulation for several natural and synthetic CB1 agonists (Khajehali et al. 2015). Org27569 also reduces CP55,940-induced β-arrestin recruitment and downstream signaling (Ahn et al.
2012). *In vivo* Org27569 decreases feeding behavior in rats and mice, as well as decreases CP55,940-induced hypothermia in rats in the triad assay (Gamage et al. 2014; Ding et al. 2014). CB₁ NAMs have also been studied in the drug discrimination paradigm (see Chapter 2).

**CB1 Positive Allosteric Modulators**

Using *in vivo* measures such as the tetrad assay and drug discrimination paradigm, CB₁ PAMs have been shown to augment the overt and subjective cannabinoid effects of orthosteric CB₁ agonists but lack intrinsic activity in these assays when administered alone (Table 1.2). Three classes of CB₁ positive allosteric modulators (PAMs) include the tropane derivatives (analogs of RTI-371), lipoxin A4, and 2-phenyl indoles (analogs of ZCZ011) (Ignatowska-Jankowska et al. 2015; Navarro et al. 2009; Pamplona et al. 2012). In contrast to CB₁ NAMs, these compounds enhance both the binding and signaling of orthosteric agonists. ZCZ011 is fairly well characterized both *in vitro* and *in vivo* and several of its analogs have been tested in the CCI model of neuropathic pain, tetrad/triad assay, and drug discrimination paradigm (Table 1.1. and 1.2). Compounds including ZCZ011, GAT211, and ABD1236 have demonstrated antiallodynic effects in models of neuropathic pain (Ignatowska-Jankowska et al. 2015; Slivicki et al. 2018; Tseng et al. 2019). The LDK series of ZCZ011 analogs lack antiallodynic activity but demonstrate some PAM activity with CP55,940 in the triad assay. In drug discrimination, only ZCZ011 has been studied where it has been shown to potentiate the subjective effects of AEA and CP55,940. The subjective drug effects of the remaining analogs have yet to be evaluated.
The Endogenous Cannabinoid System in Acute and Chronic Pain

**Ascending and Descending Pain Pathways**

The spinothalamic and spinoparabrachial tracts are two major ascending pathways in mammals, which relay noxious stimulation (Sun et al. 2020; Yam et al. 2018). Nociception is relayed through a series of neurons starting with primary sensory afferents which originate in the periphery and run through the dorsal root ganglion (DRG) into the dorsal horn of the spinal cord where they synapse with second-order neurons. These second-order neurons decussate in the spinal cord, ascend the contralateral ventral column then synapse with third-order neurons in the thalamus. Finally, the third-order neurons project to the primary somatosensory cortex of the postcentral gyrus. The spinoparabrachial tract originates in the spinal cord and relays pain information to the parabrachial nuclei of the pons where the synapse with third-order neurons which then project to the hypothalamus and amygdala which mediate the emotional response to pain. Modulation of pain is mediated by the descending pain pathway, which originates in the cortex, hypothalamus, and amygdala and projects down through the brainstem and spinal cord via the periaqueductal grey (PAG). The endocannabinoid system is expressed throughout both ascending and descending pain pathways and modulates pain through peripheral, spinal, and supraspinal mechanisms of action. CB$_1$ receptors are expressed by primary afferent neurons on their peripheral endings, central terminals, and in the DRG (Hohmann et al. 1999; Hohmann & Herkham 1998, 1999a). In the spinal cord, CB$_1$ receptors are located within the superficial laminae of the dorsal horn (Glass et al. 1997; Tsou et al. 1998). In the brain, CB$_1$ receptors are found in all brain regions associated with pain processing including the cerebral cortex, thalamus, hypothalamus, amygdala, basal ganglia, and PAG (Glass et al. 1997). CB$_2$ receptors
have also been found in the same brain regions involved in pain as the CB\textsubscript{1}, although to a lesser extent (Brusco \textit{et al.} 2008; Gong \textit{et al.} 2006; Onaivi \textit{et al.} 2006). Lastly, the biosynthetic and degradative enzymes of the endocannabinoids are expressed throughout both ascending and descending pain pathways in tissues innervated by primary afferents as well as the spinal cord and brain (Di Marzo \textit{et al.} 2000; Egertová \textit{et al.} 1998; Felder \textit{et al.} 1996; Stella \textit{et al.} 1997).

Supraspinal mechanisms of action for cannabinoids have been demonstrated by studies which show antinociception in rodent models of acute and chronic pain following administration of CB\textsubscript{1} receptor ligands via intracerebroventricular (i.c.v.) injection, intrathecal (i.t.) injection or microinjection into specific brain regions. $\Delta^9$-THC and synthetic CB\textsubscript{1} receptor agonists WIN55,212-2 and CP55,940 (i.c.v.) produce thermal antinociception in the tail-flick test at doses that do not significantly alter motor activity, indicating that increased tail-withdrawal latencies were not the result of motor impairment (Martin \textit{et al.} 1993; Raffa \textit{et al.} 1999). Antinociception in the tail-flick test is also produced following microinjection of CB\textsubscript{1} agonists into the amygdala, thalamus, rostral ventromedial medulla (RVM), and PAG (Lichtman \textit{et al.} 1996; Martin \textit{et al.} 1998, 1999). The CB\textsubscript{1} receptor antagonist/inverse agonist rimonabant (i.c.v. or i.p.) blocks the antinociceptive effects of $\Delta^9$-THC and CP55,940 (i.c.v. or i.t.), thus demonstrating a CB\textsubscript{1}-dependent mechanism of action (Welch \textit{et al.} 1998). Additionally, cannabinoids produce antinociception through interactions with the endogenous opioid system (Fang \textit{et al.} 2012; Welch \textit{et al.} 1994, 1995). Alterations in the endocannabinoid system occur in certain brain regions following nerve injury in models of neuropathic pain. For example, AEA and 2-AG levels are increased following sciatic nerve injury in the chronic constriction injury (CCI) model of neuropathic pain in the PAG and RVM (Petrosino \textit{et al.} 2007). Formalin-evoked pain behaviors are depressed in rats after partial sciatic nerve ligation (pSNL) and this effect is
blocked by administration of rimonabant into the nucleus reticularis gigantocellularis, a region involved in descending pain modulation (Monhemius et al. 2001). Lastly, upregulation of thalamic CB₁ receptor mRNA in pSNL rats suggests that increased CB₁ receptor density in certain brain regions may serve to increase the analgesic effects of endocannabinoids during neuropathic pain states (Siegling et al. 2001).

Both in vivo and in vitro measures have been used to demonstrate spinal mechanisms of action for the antinociceptive effects of cannabinoids. Levonantradol (i.t.) produces increases in response latencies of rats in both hot-plate and tail-flick tests (Yaksh, 1981). Δ⁹-THC (i.t.) produces thermal antinociception in the tail-flick test in mice following spinal cord transection at T12, which indicates the antinociceptive effects are not due solely to supraspinal mechanisms (Smith et al. 1992). Topical application of WIN55,212-2 to the dorsal aspect of the spinal cord reduces heat-evoked activity in isolated neurons of the hind paw of rats (Hohmann et al. 1998). CP55,940 (i.t.) attenuates capsaicin-induced sensitization of spinal nociceptive neurons (Johanek et al. 2005). In the chronic constriction injury (CCI) model of neuropathic pain, spinal AEA and 2-AG levels are elevated and Inhibition of FAAH or MAGL has been shown to reduce mechanical hypersensitivity (Ignatowska-Jankowska et al. 2015; Kinsey et al. 2009; Petrosino et al. 2007; Starowicz et al. 2012).

The peripheral antinociceptive effects of cannabinoids involve both CB₁- and CB₂-dependent mechanisms. CB₁ receptors undergo peripheral axon flow in DRG neurons and are expressed on peripheral terminals of nociceptors (Agarwal et al. 2007; Hohmann & Herkenham 1999b). Direct administration of AEA into the hind paw of rats reduces formalin-evoked nociceptive behavior (Calignano et al. 1998). Local administration of WIN55,212-2 into the ipsilateral hind paw reduces mechanical hypersensitivity in pSNL rats (Fox et al. 2001). The
CB2-selective agonist AM1241 (local or i.p.) produces thermal analgesia in rats and this effect is blocked by the CB2-selective antagonist AM630 but not AM251, a CB1-selective antagonist (Malan et al. 2001). Moreover, AM1241 injected locally to the contralateral hind paw does not produce thermal antinociception in the ipsilateral paw and AM630 injected locally to the ipsilateral paw blocks the antinociceptive effects of AM1241 administered systemically (i.p.). These findings strongly implicate CB2 receptors as the peripheral site of action of cannabinoids.

**Chronic Constriction Injury Model of Neuropathic Pain**

Neuropathic pain can be modeled through spinal nerve ligation (SNL), partial sciatic nerve ligation (pSNL), and the chronic constriction injury (CCI) (Bennett & Xie, 1988; Kim & Chung, 1992; Seltzer et al. 1990). This thesis employs the CCI model of neuropathic pain, which involves the tying of loose ligatures around the sciatic nerve which results in mechanical and thermal hypersensitivity in the hind paw either unilaterally or bilaterally, depending on the technique (De Vry et al. 2004). This model has been used to study the antinociceptive effects of CB1 and CB2 receptor agonists, allosteric modulators, and inhibitors of biosynthetic or catabolic enzymes of the endocannabinoids (Donvito et al. 2018).

**Investigating the Properties of Cannabinoids in vitro & in vivo**

**Binding and Functional Assays In Vitro**

The binding properties of novel cannabinoid receptor ligands are determined through binding assays measuring the displacement of a tritium labeled CB1/CB1 agonist such as [3H]-CP55,940 (Howlett et al. 2002). The [35S] GTPγS binding assay can be used to measure agonist-
stimulated G protein activation as well as visualize receptor binding in autoradiography experiments (Howlett et al. 2002; Sim et al. 1995). Inhibition of adenylyl cyclase activity can be measured through changes in cAMP concentration inside model cells transfected with CB1 or CB2 receptors (Howlett et al. 1985; Pertwee, 1997). There also exist other functional assays to measure β-arrestin recruitment and ERK phosphorylation (Osmond et al. 2005; Zhao et al. 2008).

Behavioral Paradigms for Cannabinimetic Activity

Early studies on cannabinoids involved measuring overt behaviors such as static ataxia in dogs or ptosis and sedation in monkeys (Walton et al. 1937; Edery et al. 1971). More commonly used models at present include the tetrad assay and drug discrimination paradigm. The tetrad assay is a measure of cannabinimetic activity wherein direct CB1 agonists produce a full subset of effects including hypomotility, catalepsy, thermal antinociception, and hypothermia which strongly correlates with psychoactivity in humans (Martin et al. 1991). The drug discrimination paradigm is used to measure the subjective effects of CB1 receptor agonists and has been used extensively to characterize cannabinoid receptor ligands in rodents and non-human primates (reviewed in Chapter 2). Both the tetrad assay and drug discrimination paradigm have been useful in drawing structure-activity relationships between cannabinoid receptor ligands and assessing abuse liability.

Overview of 2-phenyl indoles Represented by ZCZ011

The class of CB1 PAMs represented by ZCZ011 are all characterized by their 2-phenyl indole backbone (Fig. 1.2). ZCZ011 represents a racemic mixture of its (R)-isomer (ZCZ011A)
and (S)-isomer (ZCZ011B). Other racemates include GAT211, ABD1236 and ABD1236. Compounds such as LDK1747 and LDK1752 possess a trisubstituted amine which by inversion renders them achiral. Additions or substitutions at the chiral center or on the indole ring distinguish each analog and produce varying degrees of change in their activity *in vitro* or *in vivo* summarized in Table 1.1 and 1.2, respectively.
**Figure 1.2** Structures of ZCZ011, its structural isomers and analogs.

**Pharmacological Effects In Vitro**

**Table 1.1** Summary of *in vitro* activity for ZCZ011 analogs and CP55,940

<table>
<thead>
<tr>
<th>Compound</th>
<th>Equilibrium Binding</th>
<th>[^{35}S] GTP(\gamma)S Binding</th>
<th>cAMP Inhibition</th>
<th>(\beta)-arrestin Recruitment</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZCZ011</td>
<td>enhances binding: [^{3}H]-CP55,940 &amp; [^{3}H]-WIN55,212-2 decreases binding: [^{3}H]-SR141617A</td>
<td>enhances binding (AEA)</td>
<td>32</td>
<td>94</td>
<td>777</td>
</tr>
<tr>
<td>ABD1236</td>
<td>enhances [^{3}H]-CP55,940 binding</td>
<td>-</td>
<td>29</td>
<td>84</td>
<td>828</td>
</tr>
<tr>
<td>GAT211</td>
<td>enhances [^{3}H]-CP55,940 binding</td>
<td>-</td>
<td>-</td>
<td>129.54</td>
<td></td>
</tr>
</tbody>
</table>
Pharmacological Effects In Vivo

Table 1.2 Summary of *in vivo* activity for ZCZ011

<table>
<thead>
<tr>
<th>Compound</th>
<th>CCI Model of Neuropathic Pain(^a)</th>
<th>Tetrad(^b)</th>
<th>Triad(^c)</th>
<th>Drug Discrimination(^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZCZ011</td>
<td>full reversal</td>
<td>no effect</td>
<td>potentiation(3/3)</td>
<td>no substitution or rate suppression</td>
</tr>
</tbody>
</table>

\(^a\) indicates not reported

\(^1\) personal communication
Chapter 2: Cannabinoid Drug Discrimination - From Agonism to Allosteric Modulation

Historical Origins

A more detailed account on the origins of drug discrimination has been written by Donald Overton; this section provides a briefer summary of his work (Overton, 1991). Drug discrimination developed from studies on state dependent memory, or state dependent learning (SDL). SDL is the phenomenon where memory recall performance is dependent on the state of consciousness or overall physiological state at the time of acquisition and time of recall. When a behavioral response in an animal is learned under either condition of drug (D) or no drug (N), the animal performs the behavior most efficiently when the drug condition at the time of training is reestablished (Overton, 1984). SDL is related to context dependent learning although the key difference is that context in SDL is the cognitive state of the individual in terms of organic mood states or synthetic mood states as induced by drugs (Bower, 1981; Grant et al., 1998; Overton 1964). Whereas context dependent learning involves exteroceptive cues (i.e., the environment), SDL involves interoceptive cues (i.e., cognitive state of the organism). The first case report of the effects of drugs on memory retrieval came from George Combe who remarked on a story of a man who when sober could not recall where he had placed a package but upon becoming intoxicated again was able to relocate the lost item. “The only conclusion which seems to arise … is that before memory can exist, the organs [have] to be affected in the same manner, or to be in a state analogous to that in which they were, when the impression was first received” (Combe, 1835). Combe’s report appeared throughout medical literature in the decades following and the idea that a drug state (e.g., alcohol intoxication) could produce SDL was generally accepted (Elliotson, 1840; Macnish 1834, 1835; Overton, 1991; Winslow, 1860). In the later part of the
century French physiologist Théodule-Armand Ribot refined this idea by proposing that bodily “organic sensations”, or interoceptive stimuli, play an important role in memory retrieval (Ribot, 1882, 1891). Investigations involving SDL in the 19th and early 20th centuries helped lay the conceptual groundwork for the study of drug discrimination. After 1950, advances in the field and procedural changes marked a transition to the use of what are now considered modern drug discrimination procedures (Overton 1971, 1982, 1991; Schuster and Balster 1977).

One of the earliest drug discrimination studies involved an approach/avoidance task in which rats learned to approach an object while intoxicated or avoid while sober, and vice versa (Conger, 1951). Conger noted that the change in avoidance behavior may have been due solely to the change in the animals’ internal state (intoxicated vs. sober) rather than to any intrinsic effects of alcohol intoxication. To demonstrate that drugs can serve as discriminative stimuli, subsequent studies employed a 2X2 experimental design in which subjects are trained and tested for recall in groups representing all possible transitions: D (drug) → D, D → N (no drug), N → N, and N → D (Auld, 1951; Grossman and Miller, 1961; Miller, 1957; Miller and Barry, 1960; Murphy and Miller, 1955). The 2X2 experimental design proved useful for detecting drug stimulus effects as it takes into account each possible change of state of the subject though it was not without limitations. The 2X2 design assumes that SDL is symmetrical in that D → N and N → D state changes should produce equally large deficits and it cannot distinguish memory impairment from depressant effects of a drug on performance. An improvement to drug discrimination methodology came with the incorporation of symmetrical tasks such as the two-choice T-maze. In using a symmetrical drug discrimination task, drug stimulus properties are measured according to response selection rather than response occurrence (Overton 1961, 1964).
In 1968, Harris and Balster applied operant conditioning to the drug discrimination paradigm whereby they trained rats to discriminate DL-amphetamine from saline on two-lever (TL) multiple fixed ratio 50/differential-reinforcement-of-low-rate 20s (MULT FR50, DRL 20s). They showed that the rats acquired the amphetamine discriminative cue and demonstrated condition-appropriate responding under extinction conditions (Harris & Balster, 1968). Studies employing the operant drug discrimination paradigm generated ED$_{50}$ values for dose-response curves much lower than reported in T-maze paradigms (Kubena & Barry, 1969a, b; Morrison & Stephenson 1969). A major step that helped standardize the two-lever drug discrimination procedure was the introduction of fixed ratio (FR) schedules of reinforcement where a pellet reward is given, for example, every tenth condition-appropriate response (FR10). A study in 1975, in which rats trained to discriminate fentanyl using a TL FR10 schedule of reinforcement, showed that the discriminative stimulus effects of fentanyl were dose-dependent and pharmacologically specific (i.e., generalized only to drugs of the same class) and measurement of response rates could detect inhibitory or stimulatory effects of a drug (Colpaert & Niemegeers, 1975).

The standard approaches and utility of two-lever operant drug discrimination procedures were apparent by the mid-1970s. In 1976, Shannon and Holtzman trained rats to discriminate morphine (3.0 mg/kg) from saline in a shock avoidance procedure. They demonstrated that the discriminative stimulus effects of morphine were time and dose-dependent, pharmacologically specific, stereoselective, and cross-tolerant with the discriminative cue of methadone (Shannon & Holtzman, 1976). These characteristic features of receptor-mediated pharmacology have also been demonstrated with other classes of drugs aside from opioids, highlighting the utility of discrimination paradigms for the classification of drugs according to their discriminative
stimulus effects. Major drug classes which produce a discriminative stimulus include stimulants, depressants, opioids, hallucinogens, dissociative anesthetics, and cannabinoids. Drug discrimination became increasingly popular among behavioral pharmacologists as a tool to investigate drugs of abuse. Table 1 gives a partial list of drugs that have been used as a discriminative stimulus in drug discrimination paradigms. From the body of literature on drug discrimination arise a few core principles. The first is that the discriminative stimulus properties of a drug are generally considered to be reflective of its subjective effects. For example, a drug that substitutes for the discriminative stimulus of ∆⁹-THC in rodents is likely to produce the same subjective ‘high’ feeling in humans as ∆⁹-THC. Sensitivity to discriminative stimuli may also vary between individuals in human and nonhuman subjects. Second, that drugs may be classified according to their discriminative stimulus properties and this feature may be used to study tolerance and cross-tolerance among drugs of the same class. Lastly, that the discriminative stimulus properties of a drug are stereospecific, and their action reflects CNS activity at specific neurotransmitter receptors.

<table>
<thead>
<tr>
<th><strong>Table 2.1</strong></th>
<th>Partial list of drugs exhibiting discriminative stimulus properties†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drug</td>
<td>Class or Receptor Mechanism</td>
</tr>
<tr>
<td>amphetamine</td>
<td>stimulant</td>
</tr>
<tr>
<td>apomorphine</td>
<td>dopamine receptor agonist</td>
</tr>
<tr>
<td>atropine</td>
<td>antimuscarinic</td>
</tr>
<tr>
<td>buprenorphine</td>
<td>opioid analgesic, MOR partial agonist</td>
</tr>
<tr>
<td>Substance</td>
<td>Category</td>
</tr>
<tr>
<td>-----------------</td>
<td>--------------------</td>
</tr>
<tr>
<td>buspirone</td>
<td>anxiolytic</td>
</tr>
<tr>
<td>caffeine</td>
<td>stimulant</td>
</tr>
<tr>
<td>cholecystokinin</td>
<td>neuropeptide hormone</td>
</tr>
<tr>
<td>chlorpromazine</td>
<td>antipsychotic</td>
</tr>
<tr>
<td>clozapine</td>
<td>antipsychotic</td>
</tr>
<tr>
<td>cocaine</td>
<td>stimulant</td>
</tr>
<tr>
<td>desipramine</td>
<td>antidepressant</td>
</tr>
<tr>
<td>dextromethorphan</td>
<td>antitussive</td>
</tr>
<tr>
<td>diazepam</td>
<td>anxiolytic</td>
</tr>
<tr>
<td>diphenhydramine</td>
<td>antihistamine</td>
</tr>
<tr>
<td>DOM</td>
<td>hallucinogen</td>
</tr>
<tr>
<td>ephedrine</td>
<td>sympathomimetic</td>
</tr>
<tr>
<td>ethanol</td>
<td>sedative</td>
</tr>
<tr>
<td>fenfluramine</td>
<td>anorectic</td>
</tr>
<tr>
<td>fentanyl</td>
<td>opioid analgesic, MOR partial agonist</td>
</tr>
<tr>
<td>imipramine</td>
<td>tricyclic antidepressant</td>
</tr>
<tr>
<td>LSD-25</td>
<td>hallucinogen</td>
</tr>
<tr>
<td>MDA</td>
<td>empathogen-entactogen</td>
</tr>
<tr>
<td>MDMA</td>
<td>empathogen-entactogen</td>
</tr>
<tr>
<td>Substance</td>
<td>Classification</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>morphine</td>
<td>opioid analgesic, MOR agonist</td>
</tr>
<tr>
<td>naloxone</td>
<td>MOR antagonist</td>
</tr>
<tr>
<td>nicotine</td>
<td>nAChR agonist</td>
</tr>
<tr>
<td>NMDA</td>
<td>NMDAR agonist</td>
</tr>
<tr>
<td>pentazocine</td>
<td>opioid analgesic, MOR agonist</td>
</tr>
<tr>
<td>pentobarbital</td>
<td>sedative</td>
</tr>
<tr>
<td>phencyclidine</td>
<td>dissociative anesthetic</td>
</tr>
<tr>
<td>pregnenolone</td>
<td>neurosteroid hormone</td>
</tr>
<tr>
<td>Δ⁹-THC</td>
<td>CB₁ receptor agonist</td>
</tr>
<tr>
<td>Toluene</td>
<td>abused inhalant</td>
</tr>
</tbody>
</table>

*MOR μ-opioid receptor, LSD lysergic acid diethylamide, MDA 3, 4-methylenedioxyamphetamine, MDMA 3, 4-methylenedioxymethamphetamine, nAChR nicotinic acetylcholine receptor, CB₁ cannabinoid receptor subtype-1, Δ⁹-THC delta-9-tetrahydrocannabinol

† Source: Richard Young in chapter 3 of Methods of Behavior Analysis in Neuroscience, 2nd edition

The first behavioral pharmacologists investigating in vivo effects of cannabinoids employed drug discrimination procedures developed in the 1960s and 1970s. Cannabinol (CBN) was the first structurally confirmed phytocannabinoid isolated from cannabis. Early pharmacological studies showed that tetrahydrocannabinols, compared to other compounds such as cannabidiol (CBD) are the primary psychoactive constituents of cannabis (Cahn, 1933; Loewe 1946). It was not until the 1960s however that advances in separation techniques and structural determination led to the isolated structures of Δ⁹-THC and CBD (Gaoni & Mechoulam, 1964; Mechoulam & Shvo, 1963). By the 1970s the techniques for isolation, structural confirmation,
and synthesis of major and other phytocannabinoids were well established (Mechoulam & Gaoni, 1965, 1967, 1971). Research around the time focused primarily on developing bioassays to quantify the behavioral effects of cannabis and its individual cannabinoid constituents, establishing pharmacokinetic pathways for those compounds, and constructing structure-activity relationships (Agurell, 1986; Edery et al., 1971; Pertwee, 1972). The first animal model used to study the effects of cannabinoids was measuring static ataxia in dogs which consists of sedation, catalepsy, motor impairment, and hyperexcitability (Walton, 1937). Another useful model was studying overt behaviors in monkeys (e.g., sedation, ptosis, slouched posture, hyperexcitability). Static ataxia and overt behavior models were reliable for assessing structure activity relationships for cannabinoids and novel structural analogs, demonstrating both stereospecificity, shifts in potency, and high correlation with psychoactivity (Edery et al., 1971; Martin et al., 1975).

Concurrent with the advances in cannabinoid pharmacology following the discoveries of Mechoulam, was the development of drug discrimination procedures. By the time $\Delta^9$-THC had been isolated and synthesized, operant drug discrimination tasks had already proven useful for characterizing the discriminative stimulus effects of drugs of abuse such as amphetamine or alcohol (Harris & Balster, 1968; Kubena & Barry, 1969a, b; Morrison & Stephenson, 1969).

Cannabinoid researchers now had a reliable tool for measuring the *subjective effects* of cannabinoids in addition to predicting psychoactivity whereas previous behavioral measures were strictly models of intoxication.

In 1971, $\Delta^9$-THC was shown to produce SDL in rats under a conditioned avoidance paradigm, suggesting that it might also serve as a discriminative stimulus as had been demonstrated with other drugs known to produce SDL (Henriksson & Järbe, 1971). $\Delta^9$-THC was used as a discriminative stimulus the following year in a study, which trained male Wistar rats in
an operant-lever shock avoidance procedure to discriminate the effects of Δ⁹-THC from vehicle and a variety of other pharmacological agents (Kubena & Barry, 1972). It was successfully demonstrated that Δ⁹-THC produces a discriminative cue that does not generalize to drugs of other classes or to non-psychoactive cannabinoids such as CBD. That same year Henriksson & Järbe successfully trained Sprague-Dawley rats to use Δ⁹-THC as a discriminative stimulus in a water T-maze position learning task (Henriksson & Järbe, 1972). Järbe and collaborators published much of the early literature on cannabinoid drug discrimination in the following years (Järbe & Henriksson, 1974; Järbe et al. 1975, 1977; Järbe & McMillan 1979, 1980). These studies corroborated evidence from other behavioral models showing Δ⁹-THC is the primary psychoactive component of cannabis in addition to novel generalization tests. Important structure activity relationships were also demonstrated, with analogs of Δ⁹-THC (e.g., Δ⁸-THC) or its 11-hydroxy metabolites generalizing to the discriminative cue of Δ⁹-THC. Lastly, these studies showed that other species including gerbils and pigeons learn to discriminate Δ⁹-THC from vehicle and other compounds (Table 2.2). The work on cannabinoid drug discrimination strongly suggested a common mechanism of action for these drugs. However, the mechanism of action mediating the pharmacological effects of cannabinoids remained under debate until the development of a cannabinoid receptor radioligand binding assay and the cloning of cannabinoid receptors from rat brain and later from human brain (Devane et al. 1998; Matsuda et al. 1990; Munro et al. 1993).

Drug discrimination remained a useful and reliable tool in the cannabinoid field following the discovery of cannabinoid receptors. The model was extended to Rhesus monkeys which was used to characterize novel cannabinoids such as CP55,940 and WIN55,212-2 with the intention of providing a more accurate correlate of psychoactivity in humans than the rodent and
avian drug discrimination studies (Gold et al. 1992; Compton et al. 1992). The discriminative stimulus effects of the endogenous cannabinoid AEA and/or its metabolically stable analogs were characterized following their discovery, effectively showing that CB₁ receptors mediate their discriminative stimulus effects (Wiley et al. 1995a; Burkey and Nation, 1997). The discriminative stimulus effects of the other endocannabinoid 2-AG were characterized indirectly by using inhibitors of its degradative enzyme, MAGL (Wiley et al. 1995b, c; Solinas et al. 2007; Walentiny et al. 2011). Hence the role of the endocannabinoid system in cannabinoid drug discrimination can be studied through administration of CB₁ receptor agonists or indirectly by pharmacological blockade of endocannabinoid degradation. Whether by administration of exogenous agonist or pharmacologic blockade of MAGL and/or FAAH, the common feature among all previously mentioned studies is that they quantify discriminative stimulus effects in terms of CB₁ agonism and antagonism, through generalization and antagonism tests. More recently however, CB₁ receptor allosteric modulators have been investigated in the drug discrimination paradigm, CB₁ receptor allosteric modulators which do not substitute for CB₁ receptor orthosteric agonist training drugs but can produce shifts in the dose-response curves of the generalization curves of the training drugs (Gamage et al. 2014; Ignatowska-Jankowska et al. 2015). Published reports investigating CB₁ receptor allosteric modulators suggest potential therapeutic applications and advantages over direct agonism or antagonism making CB₁ receptor allosteric modulation an exciting area for drug development to which the drug discrimination paradigm lends itself well. ZCZ011 for example is a CB₁ positive allosteric modulator (PAM), which reverses alldynia in a mouse chronic constriction injury (CCI) model of neuropathic pain but lacks intrinsic cannabimimetic activity similar to Δ⁹-THC (Ignatowska-Jankwoska et al. 2015). Moving forward, cannabinoid drug discrimination offers a reliable in vivo model to screen
novel CB₁ receptor allosteric modulators and their structural analogs. The remainder of this review will cover important concepts in drug discrimination, both theoretical and methodological as well as discuss separately the discriminative stimulus effects of phytocannabinoids, endogenous cannabinoids, cannabinoid antagonists, and cannabinoid allosteric modulators. Table 2 provides a summary of substitution profiles for cannabinoid and non-cannabinoid drugs discussed in this review.

### Table 2.2 Substitution profiles of various drugs under cannabinoid discrimination procedures

<table>
<thead>
<tr>
<th>Training drug, dose (mg/kg), route&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Test drug, dose (mg/kg), route&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Species</th>
<th>Procedure&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Result, %DLR&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Response rates&lt;sup&gt;d&lt;/sup&gt;</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Δ⁹-THC, 3.0</td>
<td>amphetamine, 0.3-1.8</td>
<td>Male, SD rats</td>
<td>TL, FR10</td>
<td>N.S.</td>
<td>decrease</td>
<td>Solinas et al. (2010)</td>
</tr>
<tr>
<td>Δ⁹-THC, 3.2</td>
<td>amphetamine, 0.32</td>
<td>Male, SD rats</td>
<td>TL, FR10, CS-reinforced</td>
<td>N.S.</td>
<td>no effect</td>
<td>Browne and Weissman (1981)</td>
</tr>
<tr>
<td>Δ⁹-THC, 5.0</td>
<td>amphetamine, 2.5-5.0</td>
<td>Male, SD rats</td>
<td>water T-maze, escape reinforced</td>
<td>N.S.</td>
<td>n/a</td>
<td>Järbe and Henriksson (1974)</td>
</tr>
<tr>
<td>Δ⁹-THC, 3.0</td>
<td>cocaine, 1-10</td>
<td>Male, SD rats</td>
<td>TL, FR10</td>
<td>N.S.</td>
<td>decrease</td>
<td>Solinas et al. (2010)</td>
</tr>
<tr>
<td>Δ⁹-THC, 5.0</td>
<td>cocaine, 5-20</td>
<td>Male, SD rats</td>
<td>water T-maze, escape reinforced</td>
<td>N.S.</td>
<td>n/a</td>
<td>Järbe and Henriksson (1974)</td>
</tr>
<tr>
<td>Δ²-THC, 10</td>
<td>10</td>
<td>Male, C57BL6/ J mice</td>
<td>cocaine, 10-56</td>
<td>TH, FR30, CM-reinforced</td>
<td>N.S.</td>
<td>decrease</td>
</tr>
<tr>
<td>Δ²-THC, 0.04-0.17 IM</td>
<td>0.04-0.17</td>
<td>Male, Rhesus monkeys</td>
<td>diazepam, 0.025-1.2</td>
<td>TL, FR50</td>
<td>P.S.</td>
<td>no effect</td>
</tr>
<tr>
<td>Δ²-THC, 3.0</td>
<td>3.0</td>
<td>Male, SD rats</td>
<td>diazepam, 0.1-10</td>
<td>TL, FR10, SM-reinforced</td>
<td>P.S.</td>
<td>decrease</td>
</tr>
<tr>
<td>Δ²-THC, 5.0</td>
<td>5.0</td>
<td>Male, SD rats</td>
<td>ethanol, 1000-2000</td>
<td>water T-maze, escape reinforced</td>
<td>N.S.</td>
<td>n/a</td>
</tr>
<tr>
<td>Δ²-THC, 1.8</td>
<td>1.8</td>
<td>Male, SD rats</td>
<td>ethanol, 300-1000</td>
<td>TL, FR10</td>
<td>P.S.</td>
<td>decrease</td>
</tr>
<tr>
<td>Δ²-THC, 10</td>
<td>10</td>
<td>Male, C57BL6/ J mice</td>
<td>ethanol, 320-1000</td>
<td>TH, FR30, CM-reinforced</td>
<td>P.S.</td>
<td>decrease</td>
</tr>
<tr>
<td>Δ²-THC, 10</td>
<td>10</td>
<td>Male, C57BL6/ J mice</td>
<td>ketamine, 3.2-32</td>
<td>TH, FR30, CM-reinforced</td>
<td>P.S.</td>
<td>decrease</td>
</tr>
<tr>
<td>Δ²-THC, 3.2</td>
<td>3.2</td>
<td>Male, SD rats</td>
<td>LSD, 0.1</td>
<td>TL, FR10, CS-reinforced</td>
<td>N.S.</td>
<td>decrease</td>
</tr>
<tr>
<td>Δ²-THC 5.0 or hashish smoke</td>
<td>5.0 or</td>
<td>Male, SD rats</td>
<td>morphine, 1.25-10</td>
<td>water T-maze, escape reinforced</td>
<td>N.S.</td>
<td>n/a</td>
</tr>
<tr>
<td>Δ²-THC, 0.04-0.17 IM</td>
<td>0.04-0.17</td>
<td>Male, Rhesus monkeys</td>
<td>morphine, 0.1-1.0</td>
<td>TL, FR50</td>
<td>N.S.</td>
<td>no effect</td>
</tr>
<tr>
<td>Compound</td>
<td>Dose</td>
<td>Species</td>
<td>Condition</td>
<td>Effect</td>
<td>Reference</td>
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<tr>
<td>Δ⁹-THC, 3.2</td>
<td>morphine, 3.2</td>
<td>Male, SD rats</td>
<td>TL, FR10, CS-reinforced</td>
<td>P.S. 20</td>
<td>no effect</td>
<td>Browne and Weissman (1981)</td>
</tr>
<tr>
<td>Δ⁹-THC, 0.04-0.17 IM</td>
<td>phencyclidine, 0.03-0.3</td>
<td>Rhesus monkeys</td>
<td>TL, FR50, CS-reinforced</td>
<td>N.S.</td>
<td>no effect</td>
<td>Wiley et al. (1995b)</td>
</tr>
<tr>
<td>Phytocannabinoids and Endogenous Cannabinoids</td>
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<tr>
<td>Δ⁹-THC, 3.0</td>
<td>anandamide, 45</td>
<td>Male, SD rats</td>
<td>TL, FR10</td>
<td>F.S. decrease</td>
<td>Wiley et al. (1995a)</td>
<td></td>
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<tr>
<td>Δ⁹-THC, 0.08-0.16 IM</td>
<td>anandamide, 0.1-0.16 IM</td>
<td>Rhesus monkeys</td>
<td>TL, FR50-100</td>
<td>inconsistent</td>
<td>no effect</td>
<td>Wiley et al. (1997)</td>
</tr>
<tr>
<td>Δ⁹-THC, 2.0</td>
<td>anandamide, 0.5-16</td>
<td>Male, SD rats</td>
<td>TL, FR10, SW</td>
<td>P.S. 37.7</td>
<td>decrease</td>
<td>Burkey and Nation (1997)</td>
</tr>
<tr>
<td>Δ⁹-THC, 1.8-5.6</td>
<td>anandamide, 10-18</td>
<td>Male, SD rats</td>
<td>TL, FR10</td>
<td>N.S. decrease</td>
<td>Järbe et al. (2001)</td>
<td></td>
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<tr>
<td>Δ⁹-THC, 30</td>
<td>anandamide, 3.0-100</td>
<td>Male, C57BL/6/J mice</td>
<td>TH, FR10, CS-reinforced</td>
<td>N.S.</td>
<td>no effect</td>
<td>Wiley et al. (2011)</td>
</tr>
<tr>
<td>methanandamide, 10-18</td>
<td>anandamide, 10-18</td>
<td>Male, SD rats</td>
<td>TL, FR10</td>
<td>F.S. decrease</td>
<td>Järbe et al. (2001)</td>
<td></td>
</tr>
<tr>
<td>Δ⁹-THC, 3.2</td>
<td>cannabinol, 6.77</td>
<td>Male, SD rats</td>
<td>TL, FR10, CS-reinforced</td>
<td>F.S.</td>
<td>no effect</td>
<td>Browne and Weissman (1981)</td>
</tr>
<tr>
<td>Compound</td>
<td>Dose</td>
<td>Species</td>
<td>Treatment</td>
<td>Outcome</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
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<td></td>
</tr>
<tr>
<td>Δ⁹-THC 5.0 or hashish smoke</td>
<td></td>
<td>Male, SD rats</td>
<td>water T-maze, escape reinforced</td>
<td>F.S.</td>
<td>n/a</td>
<td>Henriksson (1974)</td>
</tr>
<tr>
<td>Δ⁹-THC, 12-16 hashish smoke</td>
<td></td>
<td>Male, Mongolia gerbils</td>
<td>T-maze, escape reinforced</td>
<td>F.S.</td>
<td>n/a</td>
<td>Järbe et al. (1975)</td>
</tr>
<tr>
<td>Amphetamine, 1.6 IM</td>
<td>Δ⁹-THC, 0.125-0.5 IM</td>
<td>mixed strain pigeons</td>
<td>TK, FR15</td>
<td>N.S.</td>
<td>decrease</td>
<td>Järbe (1982)</td>
</tr>
<tr>
<td>Cocaine, 3.0 IM</td>
<td>Δ⁹-THC, 0.3 IM</td>
<td>Carneau pigeons</td>
<td>TK, FR15</td>
<td>N.S.</td>
<td>decrease</td>
<td>Järbe (1984)</td>
</tr>
<tr>
<td>Methanandamide, 10</td>
<td>Δ⁹-THC, 0.1-3.0</td>
<td>Male, SD rats</td>
<td>TL, FR10</td>
<td>F.S.</td>
<td>decrease</td>
<td>Järbe et al. (2001)</td>
</tr>
<tr>
<td>Δ⁹-THC, 0.15-0.20 IM</td>
<td>Δ⁹-THC, 0.2 IM</td>
<td>mixed strain pigeons</td>
<td>TK, FR15</td>
<td>F.S.</td>
<td>decrease</td>
<td>Henriksson et al. (1975)</td>
</tr>
<tr>
<td>Δ⁹-THC, 5.6 s.c.</td>
<td>Δ⁹-THC, 5.6</td>
<td>C57BL6/ J mice</td>
<td>TH, FR10</td>
<td>F.S.</td>
<td>not reported</td>
<td>Long et al. (2009)</td>
</tr>
<tr>
<td>Δ⁹-THC, 5.6 s.c.</td>
<td>Δ⁹-THC, 5.6</td>
<td>FAAH (−/−) mice</td>
<td>TH, FR10</td>
<td>F.S.</td>
<td>not reported</td>
<td>Long et al. (2009)</td>
</tr>
<tr>
<td>Δ⁹-THC, 5.6 s.c.</td>
<td>anandamide, 1.0-30 s.c.</td>
<td>Male, FAAH (−/−) mice</td>
<td>TH, FR10</td>
<td>F.S.</td>
<td>decrease</td>
<td>Walentiny et al. (2015)</td>
</tr>
<tr>
<td>Δ⁹-THC, 3.0</td>
<td>11-OH-Δ⁹-THC, 11-OH-Δ⁸-THC, 0.3-3.0</td>
<td>Male, SD rats</td>
<td>TL, FR10</td>
<td>F.S.</td>
<td>decrease</td>
<td>Järbe and McMillan (1980)</td>
</tr>
<tr>
<td>Δ⁹-THC, 1.0</td>
<td>11-OH-Δ⁹-THC 0.03-1.0, 11-OH-Δ⁸-THC 0.1-1.0</td>
<td>Male, white Carneau pigeons</td>
<td>TK, FR15</td>
<td>F.S.</td>
<td>decrease</td>
<td>Järbe and McMillan (1980)</td>
</tr>
<tr>
<td>pentobarbital, 20</td>
<td>Δ⁹-THC, 2.0-8.0</td>
<td>Male, T-maze, Mongolian gerbils</td>
<td>N.S.</td>
<td>n/a</td>
<td></td>
<td>Järbe et al. (1975)</td>
</tr>
</tbody>
</table>

### Selective and Dual FAAH/MAGL Inhibitors

| Δ⁹-THC, 3.0 | anandamide, 0.3-3.0 i.v. + URB597, 0.3 | Male, SD rats | TL, FR10 | F.S. | decrease | Solinas et al. (2007) |
| Δ⁹-THC, 5.6 | JZL184, 40 | Male, C57BL6/ J mice | P.S. | not | | Long et al. (2009) |
| Δ⁹-THC, 5.6 | JZL195, 40 | Male, C57BL6/ J mice | F.S. | not | | Long et al. (2009) |
| Δ⁹-THC, 5.6 | JZL184, 40 | Male, FAAH<sup>−/−</sup> mice | F.S. | not | | Long et al. (2009) |
| Δ⁹-THC, 5.6 | JZL195, 40 | Male, FAAH<sup>−/−</sup> mice | F.S. | not | | Long et al. (2009) |

[Note: The table represents different studies involving Δ⁹-THC and its effects in various species and conditions.]
<table>
<thead>
<tr>
<th>Compound</th>
<th>Dose</th>
<th>Species</th>
<th>Condition</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Δ⁹-THC, 5.6 s.c.</td>
<td>KML29, 40 s.c.</td>
<td>Male, C57BL6/ J mice</td>
<td>TH, FR10, N.S.</td>
<td>no effect</td>
<td>Ignatowska-Jankowska et al. (2014)</td>
</tr>
<tr>
<td>Δ⁹-THC, 5.6 s.c.</td>
<td>KML29, 40 s.c.</td>
<td>Male, FAAH&lt;sup&gt;−/−&lt;/sup&gt; mice</td>
<td>TH, FR10, F.S.</td>
<td>no effect</td>
<td>Ignatowska-Jankowska et al. (2014)</td>
</tr>
<tr>
<td>Δ⁹-THC, 5.6 s.c.</td>
<td>JZL184, 120 + PF3845, 10</td>
<td>Male, C57BL6/ J mice</td>
<td>TH, FR10, SM-reinforced</td>
<td>not reported</td>
<td>Hruba et al. (2015)</td>
</tr>
<tr>
<td>Δ⁹-THC, 5.6</td>
<td>SA-57, 10</td>
<td>Male, C57BL6/ J mice</td>
<td>TH, FR10, SM-reinforced</td>
<td>not reported</td>
<td>Hruba et al. (2015)</td>
</tr>
<tr>
<td>Δ⁹-THC, 5.6</td>
<td>JZL195, 120</td>
<td>Male, C57BL6/ J mice</td>
<td>TH, FR10, SM-reinforced</td>
<td>not reported</td>
<td>Hruba et al. (2015)</td>
</tr>
<tr>
<td>CP55,940, 0.1, s.c.</td>
<td>MJN110, 0.25-2.5 s.c.</td>
<td>Male, C57BL6/ J mice</td>
<td>TH, FR10, SM-reinforced</td>
<td>increase</td>
<td>Ignatowska-Jankowska et al. (2015)</td>
</tr>
<tr>
<td>CP55,940, 0.1, s.c.</td>
<td>JZL184, 4.0-100</td>
<td>Male, C57BL6/ J mice</td>
<td>TH, FR10, SM-reinforced</td>
<td>no effect</td>
<td>Ignatowska-Jankowska et al. (2015)</td>
</tr>
<tr>
<td>Δ⁹-THC, 5.6 s.c.</td>
<td>JZL184, 1.0-40</td>
<td>Male, C57BL6/ J mice</td>
<td>TH, FR10, P.S.</td>
<td>no effect</td>
<td>Walentiny et al. (2015)</td>
</tr>
<tr>
<td>Δ⁹-THC, 5.6 s.c.</td>
<td>JZL195, 40 s.c.</td>
<td>Male, C57BL6/ J mice</td>
<td>TH, FR10, F.S.</td>
<td>no effect</td>
<td>Walentiny et al. (2015)</td>
</tr>
<tr>
<td>Compound</td>
<td>Dose (μg/kg)</td>
<td>Species, Strain</td>
<td>Condition</td>
<td>F.S.</td>
<td>Result</td>
</tr>
<tr>
<td>-------------------</td>
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<td>--------------</td>
</tr>
<tr>
<td>∆⁹-THC, 5.6</td>
<td>JZL184, 1.0-40</td>
<td>Male, FAAH&lt;sup&gt;(−/−)&lt;/sup&gt; mice</td>
<td>TH, FR10</td>
<td>F.S.</td>
<td>no effect</td>
</tr>
<tr>
<td></td>
<td>JZL195, 40 s.c.</td>
<td>Male, FAAH&lt;sup&gt;(−/−)&lt;/sup&gt; mice</td>
<td>TH, FR10</td>
<td>P.S.</td>
<td>74</td>
</tr>
</tbody>
</table>

**Synthetic Cannabinoids**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dose (μg/kg)</th>
<th>Species, Strain</th>
<th>Condition</th>
<th>F.S.</th>
<th>Result</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>∆⁹-THC, 2.0</td>
<td>CP55,940, 0.05-0.8</td>
<td>Male, SD rats</td>
<td>TL, FR10, SW</td>
<td>F.S.</td>
<td>decrease</td>
<td>Burkey and Nation (1997)</td>
</tr>
<tr>
<td></td>
<td>CP55,940, 0.01-3.0</td>
<td>Male, C57BL/6 J mice</td>
<td>TH, FR10, SM-reinforced</td>
<td>F.S.</td>
<td>decrease</td>
<td>Wiley &lt;i&gt;et al.&lt;/i&gt; (2011)</td>
</tr>
<tr>
<td></td>
<td>methanandamide, 0.5-8.0</td>
<td>Male, SD rats</td>
<td>TL, FR10, SW</td>
<td>F.S.</td>
<td>decrease</td>
<td>Burkey and Nation (1997)</td>
</tr>
<tr>
<td></td>
<td>methanandamide, 10-18</td>
<td>Male, SD rats</td>
<td>TL, FR10</td>
<td>F.S.</td>
<td>decrease</td>
<td>Järbe &lt;i&gt;et al.&lt;/i&gt; (2001)</td>
</tr>
<tr>
<td></td>
<td>methanandamide, 30-100</td>
<td>Male, C57BL/6 J mice</td>
<td>TH, FR10, SM-reinforced</td>
<td>N.S.</td>
<td>decrease</td>
<td>Wiley &lt;i&gt;et al.&lt;/i&gt; (2011)</td>
</tr>
<tr>
<td></td>
<td>rimonabant, 0.3-5.6</td>
<td>Male, SD rats</td>
<td>TL, FR10</td>
<td>N.S.</td>
<td>no effect</td>
<td>Wiley &lt;i&gt;et al.&lt;/i&gt; (1995c)</td>
</tr>
<tr>
<td></td>
<td>WIN55,212-2, 0.1-3.0</td>
<td>Male, SD rats</td>
<td>TL, FR10</td>
<td>F.S.</td>
<td>decrease</td>
<td>Wiley &lt;i&gt;et al.&lt;/i&gt; (1995c)</td>
</tr>
<tr>
<td>∆⁹-THC, 3.0</td>
<td>AM2201, 0.03-1.0</td>
<td>Male, SD rats</td>
<td>TL, FR10</td>
<td>F.S.</td>
<td>no effect</td>
<td>Gatch and Forster (2014)</td>
</tr>
<tr>
<td>∆⁹-THC, 1.8</td>
<td>JWH018 (AM678), 0.03-1.0</td>
<td>Male, SD rats</td>
<td>TL, FR10</td>
<td>F.S.</td>
<td>no effect</td>
<td>Järbe et al. (2010)</td>
</tr>
<tr>
<td>∆⁹-THC, 3.0</td>
<td>JWH073, 0.1-10</td>
<td>Male, SD rats</td>
<td>TL, FR10</td>
<td>F.S.</td>
<td>decrease</td>
<td>Gatch and Forster (2014)</td>
</tr>
<tr>
<td>∆⁹-THC, 3.0</td>
<td>JWH200, 0.1-10</td>
<td>Male, SD rats</td>
<td>TL, FR10</td>
<td>F.S.</td>
<td>no effect</td>
<td>Gatch and Forster (2014)</td>
</tr>
<tr>
<td>∆⁹-THC, 10 s.c.</td>
<td>JWH-202, 1.0-30 s.c.</td>
<td>Male, C57BL6/ J mice</td>
<td>TL, FR10, SM- reinforced</td>
<td>N.S.</td>
<td>no effect</td>
<td>Vann et al. (2009)</td>
</tr>
<tr>
<td>∆⁹-THC, 10 s.c.</td>
<td>JWH-204, 0.3-10 s.c.</td>
<td>Male, C57BL6/ J mice</td>
<td>TL, FR10, SM- reinforced</td>
<td>F.S.</td>
<td>no effect</td>
<td>Vann et al. (2009)</td>
</tr>
<tr>
<td>∆⁹-THC, 10 s.c.</td>
<td>JWH-205, 1.0-56 s.c.</td>
<td>Male, C57BL6/ J mice</td>
<td>TL, FR10, SM- reinforced</td>
<td>F.S.</td>
<td>decrease</td>
<td>Vann et al. (2009)</td>
</tr>
<tr>
<td>∆⁹-THC, 3.0</td>
<td>SP111 1.0-10</td>
<td>Male, SD rats</td>
<td>TL, FR10</td>
<td>F.S.</td>
<td>decrease</td>
<td>Järbe and McMillan (1980)</td>
</tr>
<tr>
<td>Dose</td>
<td>Route</td>
<td>Species</td>
<td>Responding</td>
<td>Effect</td>
<td>Reference</td>
<td></td>
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<tr>
<td>methanandamide, 70</td>
<td>s.c.</td>
<td>Male, C57BL6/ J mice</td>
<td>C57BL6/ J mice</td>
<td>SM- reinforced</td>
<td>TH, FR10</td>
<td>N.S. decrease</td>
</tr>
</tbody>
</table>

F.S. full substitution, P.S. partial substitution, N.S. no substitution, IM intramuscular route of administration, s.c. subcutaneous route of administration, DLR drug lever or drug-like responding, TL two-lever choice, TH two-hole choice nose-poke, TK two-key choice, SD Sprague-Dawley rats, FR fixed ratio, CM 1:1 condensed milk and water, CS 1:1 carnation slender and water, SW dilute (0.4%) saccharin solution, SM sweetened milk

(a) Intraperitoneal route of administration unless otherwise stated
(b) Food-pellet reinforced unless otherwise stated
(c) Full substitution defined as ≥80% DLR; No substitution defined as <20% DLR
(d) Effect on rate of responding with respect to the highest dose tested
Core Concepts and Principles of Drug Discrimination

The studies listed in Table 2 demonstrate important concepts that make drug discrimination a reliable preclinical tool for studying cannabinoids *in vivo*. It is evident that drug discrimination can be used to classify drugs according to their discriminative stimulus properties and that these effects are mediated through receptor specific neural mechanisms. Morphine for example which acts via \( \mu \)-opioid receptors does not substitute for the discriminative stimulus of \( \Delta^9 \)-THC in rats or monkeys trained to discriminate \( \Delta^9 \)-THC from vehicle (Järbe & Henriksson, 1974; Browne & Weissman, 1981; Wiley *et al.* 1995d). Overall, drug discrimination is useful for investigating novel compounds of structurally similar and dissimilar cannabinoids including CB\(_1\) receptor agonists, inverse agonists, and allosteric modulators. Drug discrimination also clearly demonstrates aspects of receptor mediated pharmacology in that the discriminative stimulus effects of cannabinoids are dose-dependent, stereospecific, antagonizable, and subject to tolerance with cross-tolerance seen between cannabinoids with similar activity (i.e. CB\(_1\) receptor agonists are cross tolerant with one another). Although these features can be demonstrated with other bioassays, various aspects of drug discrimination make it an excellent model for drug development in the cannabinoid field.

Subjective Effects of Drugs

There is a general consensus in the field of drug discrimination that the model provides a means by which the *subjective effects* of drugs can be quantified (Colpaert *et al.* 1976; Hirschhorn & Rosecrans, 1976; Shannon & Holtzman, 1976). Subjective effects of drugs refer to the effects which are mediated through a drug’s action on the CNS rather than any peripheral effects. The subjective effects of morphine for example can be attributed to its agonist action at
μ-opioid receptors in the CNS rather versus peripheral analgesic mechanisms in dorsal root ganglia (DRG) (Colpaert & Niemegeers, 1975; Colpaert et al. 1975). One should use caution when interpreting subjective drug effects between animal and human models of drug discrimination. For humans, oral reports can provide a detailed description of how a drug makes them feel and how its subjective effects correlate with its discriminative stimulus properties (Chait et al. 1984, 1985, 1986a, 1986b, 1988). Discrimination studies in humans have also revealed sex differences in the subjective effects of Δ⁹-THC in cannabis users (Fogel et al. 2017). The same degree of qualitative information on the subjective effects of drugs cannot be ascertained from animals; however, the key point is that drug discrimination tasks do not ask the subjects how they feel but rather quantitatively ascertains the interoceptive effects of a drug.

As a reliable model for measuring subjective drug effects, drug discrimination is also used as preclinical model for assessing abuse liability of novel cannabinoids. Should a compound substitute for the discriminate stimulus of a known drug of abuse, that compound is inferred to have potential liability. Drug self-administration has largely overtaken drug discrimination as a model for drug abuse in the last twenty years as the neural circuitry involved in drug-reinforced behavior is more well defined than the mechanisms responsible for discriminative stimulus effects and that it has high face-validity for drug abuse in humans (reviewed in McMahon, 2015). However, few studies have demonstrated cannabinoid self-administration in nonhuman animals (Carney et al., 1977; Young et al., 1981; Tanda et al., 2000). Additionally, drug discrimination compared to other behavioral paradigms is highly sensitive to cannabinoids. Δ⁹-THC is more potent in regard to its subjective effects as measured by drug discrimination than it is in producing the full tetrad set of cannabimimetic effects consisting of hypomotility, catalepsy, antinociception, and hypothermia (Long et al. 2009; Marshell et al. 2014). CB₁ receptor agonists
may also produce full substitution at doses that do not negatively affect response rates (Vann et al., 2009) whereby the subjective effects of cannabinoids therefore are detected prior to alteration of overt behaviors such as locomotion. The subjective effects of drugs (contrary to their namesake) are an objectively useful quality which investigators analogously quantify as a discriminative stimulus.

Receptor Mechanisms of Discriminative Stimulus Effects

The CB₁ receptor is the most abundant GPCRs in the CNS and is highly expressed throughout the neocortex, basal ganglia, hippocampus, cerebellum, and brainstem (Herkenham et al. 1990, 1991; Glass et al. 1997; Tsou et al. 1998). The neuroanatomic distribution of CB₁ receptors correlates with its role in the endocannabinoid system and is responsible for mediating the effects of exogenous agonists such as ∆⁹-THC or synthetic cannabinoids. Pharmacological and genetic techniques have been used in conjunction with various behavioral paradigms to parse out the role of CB₁ receptors in different brain regions (Zimmer et al. 1999; Wilson-Poe et al. 2012). CB₁ receptors are primarily coupled to pertussis toxin (PTX)-sensitive Gaᵢ/₀ proteins and activation by agonists leads to inhibition of adenyl cyclase as well as modulation of voltage-gated calcium channels and potassium channels (Mackie et al. 1995; Twitchell et al. 1997; Guo & Ikeda, 2004). The net cellular effect of CB₁ receptor activation is dependent on the cell-type on which they are expressed and the cell's dominant neurotransmitter product.

CB₁ receptors are primarily localized to presynaptic axon terminals of GABAergic and glutamatergic neurons where they mediate inhibition of neurotransmitter release (Straiker & Mackie, 2005). Astrocytes express CB₁ receptors to a lesser extent, though understanding how they play an important role in modulating synaptic transmission and plasticity is increasing (Han
et al. 2012). The endocannabinoids 2-AG and AEA are synthesized on demand from membrane phospholipids in the postsynaptic membrane and signal in a retrograde fashion (Bisogno et al. 2003; Katona et al. 2006). The discriminative stimulus effects of cannabinoids are produced as a result of their neuromodulatory capacity. Other sedative drugs which modulate GABAergic neurotransmission including barbiturates and benzodiazepines may partially substitute for Δ⁹-THC in drug discrimination paradigms but full substitution is only achieved from CB₁ receptor activation (see Table 2).

CB₁ receptor agonists both endogenous and synthetic produce a discriminative stimulus in a dose dependent manner that can be generalized. Differences in structure may impart differences in potency. 11-OH-Δ⁹-THC and 11-OH-Δ⁸-THC (Δ⁹-THC metabolites) both generalize to Δ⁹-THC with the Δ⁹ isomer being more potent, thereby demonstrating stereoselectivity (Järbe & McMillan, 1979). Discriminative stimulus effects of cannabinoids are also stereospecific. The (+) configuration of WIN55,212-2 produces a discriminative stimulus similar to Δ⁹-THC whereas (-)-WIN55,212-2 has no effect (Compton et al. 1992). The CB₁ receptor inverse agonist rimonabant (SR141617A) dose-dependently antagonizes the discriminative stimulus effects of CB₁ receptor agonists (Wiley et al. 1995c; Pério et al. 1996; Järbe et al. 2001; Walentiny et al. 2015). Finally, CB₁ receptor allosteric modulators may enhance or diminish the potency of the discriminative stimulus of CB₁ receptor agonists. The positive allosteric modulator (PAM) ZCZ011 for example does not elicit a discriminative stimulus alone but produces a leftward shift in the dose-response curves of CP55,940 and AEA (Ignatowska-Jankowska et al. 2015).
Tolerance and Cross-Tolerance

The subjective effects of Δ⁹-THC and other CB₁ receptor agonists are only modestly affected by tolerance in that full substitution can be seen at doses that do not impair response rates (Hruba et al. 2012; Vann et al. 2009). This was demonstrated almost 40 years ago in a study that used trained rats to discriminate Δ⁹-THC from vehicle and used rope-climbing performance as a measure of tolerance (Bueno et al. 1972). They found that after daily training sessions with Δ⁹-THC, the rats became tolerant to the drugs impairing effects on rope-climbing but still discriminated Δ⁹-THC from vehicle, suggesting that its discriminative stimulus properties were not subject to tolerance. Furthermore, lever response rates remain stable throughout chronic administration and it has been shown that chronic administration of Δ⁹-THC at higher doses than the training dose produces only minor decreases in the degree of differential responding (Hirschhorn & Rosecrans, 1974). Under standard drug discrimination acquisition training, subjects do not develop appreciable tolerance to the discriminative stimulus of the training drug apart from under certain modifications. For example, rats trained to discriminate 3.0 mg/kg Δ⁹-THC were then administered either vehicle or a high dose of Δ⁹-THC during a period of suspended training (Wiley et al. 1993). Rats subjected to the high dose treatment of Δ⁹-THC showed a 40-fold rightward shift in their dose-response compared to the dose-response curve generated under the prior training dose regimen. This shift in the potency suggested the rats developed tolerance to the discriminative stimulus effects of Δ⁹-THC. This result is consistent with the effect that higher training dose produces higher ED₅₀ values (Schechter, 1983; Solinas et al. 2006). As such, the discriminative stimulus effects of CB₁ receptor agonists can undergo tolerance but solely through repeated administration of high doses, atypical for drug discrimination training or testing. The lack of cannabinoid compound tolerance under standard
training doses makes drug discrimination a very reliable model for studying CB₁ receptor pharmacology in vivo.

**Drug Discrimination in Drug Development**

Drug discrimination has demonstrated pharmacological specificity for a variety of drug classes including cannabinoids and has been employed by pharmaceutical companies, government agencies, and academic institutions for the classification of novel compounds. Olanzapine, an atypical antipsychotic was approved by the FDA four years following a study, which demonstrated that it fully substituted for the cue in animals trained to discriminate the atypical antipsychotic clozapine (Moore *et al.* 1992). The DEA has made use of drug discrimination for scheduling purposes in assessing abuse liability of illicit drugs such as substituted cathinones. Drug discrimination studies have also provided the DEA with evidence for the scheduling of synthetic cannabinoids commonly found on the gray market (Gatch & Forster 2014, 2015). With respect to cannabinoids, drug discrimination has proven useful in establishing structure activity relationships of novel cannabinoids both structurally similar and dissimilar to Δ⁹-THC such as CP47,497 and those frequently used in research, CP55,940 and WIN55,212-2 (Grim *et al.* 2016; Wiley *et al.* 1995c; Compton *et al.* 1992). In addition to exogenous cannabinoid agonists, drug discrimination has also been used in the development of metabolically stable analogs of the endocannabinoids 2-AG and AEA as well as inhibitors of their respective catabolic enzymes, MAGL and FAAH (Wiley *et al.* 1997; Solinas *et al.* 2007; Long *et al.* 2009; Hruba *et al.* 2015; Walentiny *et al.* 2015). Most recently drug discrimination has been used to assess the activity of CB₁ receptor allosteric modulators such as ZCZ011,
Org27569, and novel analogs of those compounds (Gamage et al. 2014; Ignatowska-Jankowska et al. 2015).

**Discriminative Stimulus Properties Cannabinoid Agonists**

Cannabinoids are derived from three primary sources: species of the genus *Cannabis* (phytocannabinoids), organic synthesis in laboratories (synthetic cannabinoids), and endogenous biosynthesis (endocannabinoids). Cannabinoids within either category exhibit some structural similarity though comparison between categories shows marked differences in structure. The common factor among these cannabinoids is that they are agonists at CB$_1$ receptors and that is the primary mechanism by which they produce their discriminative stimulus effects (Wiley et al. 1995a-d).

*Phytocannabinoids*

![Chemical structures of major cannabinoids found in Cannabis sativa and related compounds](image)

**Figure 2.1** Chemical structures of major cannabinoids found in Cannabis sativa and related compounds (all structures drawn by author using ChemDraw Professional 16.0. 1.4)
In addition to Δ⁹-THC, Cannabis sativa contains myriad cannabinoid compounds including CBD, CBN, Δ⁸-THC as well as non-cannabinoid constituents such as terpenoids (Mechoulam et al. 1972; Fischedick et al. 2010; Radwan et al. 2015). The interactions of these compounds, psychoactive and non-psychoactive at the receptor and behavioral levels has not been fully determined and represents a burgeoning area of research (reviewed in Russo, 2011). Δ⁹-THC being the primary psychoactive component of Cannabis sativa, is the most well characterized phytocannabinoid, and was almost the exclusive focus of cannabinoid drug discrimination research until the development of synthetic CB₁ receptor agonists. Early discrimination studies demonstrated specificity of the Δ⁹-THC cue in that pharmacologically distinct compounds failed to either substitute for, or antagonize the discriminative stimulus produced by Δ⁹-THC (Järbe & Henriksson, 1974; Järbe et al. 1976; Browne & Weissman, 1981). Phytocannabinoids have been shown to display both stereoselectivity and stereospecificity in their ability to serve as discriminative stimuli. The (+)-Δ⁹-THC isomer does not substitute for the (-)-isomer (i.e., stereoselectivity) whereas (-)-Δ⁸-THC, (-)-Δ⁹,₁₁-THC, CBN, and Δ⁹-THC’s 11-hydroxy metabolites all substitute in animals trained to discriminate Δ⁹-THC, albeit with varying potencies (i.e., stereospecificity) (Järbe et al. 1981, 1987; Järbe & McMillan, 1980; Semjonow & Binder, 1985). CBD, the major non-psychoactive phytocannabinoid does not substitute for Δ⁹-THC nor does it alter Δ⁹-THC’s substitution patterns or rate of responding (Hiltunen & Järbe, 1986; Järbe et al. 1977, 1986; Vann et al. 2008). Much of the early literature on cannabinoid drug discrimination was published before the existence of endogenous cannabinoid receptors was definitively proven but the findings were consistent with a specific receptor mediated mechanism. And indeed, it was later shown that in vivo potencies of phytocannabinoids correlated with their binding affinities for CB₁ receptors (Compton et al. 1993). In lieu of
synthetic CB₁ receptor agonists, Δ⁹-THC discrimination has been a reliable animal model of cannabis intoxication and the standard for studying the receptor mechanisms of cannabinoids and predicting cannabis-like abuse potential of novel compounds.

_Synthetic Cannabinoids_

![Chemical structures of synthetic cannabinoid CB₁ receptor agonists](image)

_Fig. 2.2 Chemical structures of synthetic cannabinoid CB₁ receptor agonists (all structures drawn by author using ChemDraw Professional 16.0. 1.4)_
Five major categories of synthetic cannabinoids that are classified according to their structure include: *classical, non-classical, hybrids, aminoalkylindoles, and eicosanoids* (Howlett *et al.* 2002; Thakur *et al.* 2005). Within each category, there are also multiple families of synthetic cannabinoids (e.g., HU-#, CP-#, AM-#, WIN-#, JWH-#). Efforts to synthesize novel cannabinoids followed after the synthetic routes for $\Delta^9$-THC established by Mechoulam and Gaoni in 1965. The first compounds initially synthesized were classical cannabinoids (i.e. analogs of $\Delta^9$-THC based on the dibenzopyran ring) including nabilone, levonantradol, and HU-210 (Archer *et al.* 1977; Koe, 1981; Mechoulam *et al.* 1990). These compounds elicit similar discriminative stimulus properties compared to that of $\Delta^9$-THC in drug discrimination in both human and non-human animal models with relatively higher potencies (Young *et al.* 1981; Lile *et al.* 2010; Hruba *et al.* 2014). The first non-classical cannabinoids were those of the cyclohexylphenol (CP) series developed by Pfizer in the 1970s and 1980s as prototypical analgesics (Melvin *et al.* 1984; Compton *et al.* 1992). Notable examples include CP47,497, its C8 homologue (CP47,497-C8), and CP55,940. Synthetic hybrids exhibit structural features common to both classical and non-classical cannabinoids. AM-4030, an analog of HU-210 has both a dibenzopyran ring common among classical cannabinoids and an aliphatic hydroxyl group common among cyclohexylphenols, Aminoalkylindoles (AAIs) are structurally dissimilar from $\Delta^9$-THC and classical synthetic cannabinoids and include compounds such as WIN55,212-2 and JWH-018, the first synthetic cannabinoid identified in smokable “spice” blends (Gatch & Forster, 2014). Eicosanoid synthetics were developed subsequent to the discovery of the endocannabinoids 2-AG and AEA. Compounds such as methanandamide and methylated fluoroanandamide (2-arachidonoyl-2'-fluoroanandamide) were developed for their increased metabolic stability compared to the putative endocannabinoids (Burkey & Nation,
Eicosanoid derivatives have not been diverted towards abuse as other classes of synthetic cannabinoids and have largely been used to study the discriminative stimulus properties of the putative endocannabinoids 2-AG and AEA. As such they will be discussed with the endocannabinoids in the following subsection. Discrimination studies have demonstrated that synthetic cannabinoids dose-dependently substitute for Δ⁹-THC in both rodents and Rhesus monkeys and have ED50 values which correlate well with their CB₁ receptor binding affinities (Compton et al. 1992; Gold et al. 1992; Lainton et al. 1995; McMahon et al. 2008; Järbe et al. 2011; Ginsburg et al. 2012). Synthetic cannabinoids have been particularly useful in establishing structure activity relationships with respect to both in vitro and in vivo potencies (Wiley et al. 1998; 2014). But given the limited therapeutic applications of cannabinoid agonists, drug discrimination has largely been used to assess abuse liability of novel cannabinoids.

The first wave of abused synthetic cannabinoids included structural analogs of JWH-018 (naphthoylindoles) such as JWH-073, JWH-203, JWH-204, and JWH-250 (Huffman et al. 2005; Wiley et al. 2012). These compounds have high affinity for CB₁ receptors and produce full substitution in rodents trained to discriminate Δ⁹-THC via intraperitoneal administration as well as inhalation (Vann et al. 2009; Gatch & Forster, 2014; Marshall et al. 2014). JWH-018 discrimination has been demonstrated in both rodents and Rhesus monkeys and has been the basis for studying the discriminative stimulus properties of XLR-11 and UR-144 which were derived from a series of tetramethylcyclopropyl ketone indoles developed by Abbot Laboratories (Uchiyama et al. 2013; Frost et al. 2008, 2010; Rodriguez et al. 2014; Wiley et al. 2014). Both XLR-11 and UR-144 showed dose-dependent substitution for Δ⁹-THC in mice and rats (Wiley et al. 2013; Gatch & Forster, 2015). Indazole cannabinoids have begun to replace naphthoylindole derivatives in products marketed for abuse. Indazoles which have been identified recently
include AB-CHMINACA, AB-FUBINACA, and AB-PINACA all of which fully substitute in rodents trained to discriminate $\Delta^9$-THC from vehicle (Karinen et al. 2015; Shevyrin et al. 2014; Uchiyama et al. 2015; Wiley et al. 2015). As new synthetic cannabinoids infiltrate the gray market, drug discrimination remains a reliable predictor of abuse liability for cannabimimetics.

_Endocannabinoids_

![Chemical structures of the endogenous cannabinoids anandamide (AEA) and 2-arachidonoylglycerol (2-AG)](all structures drawn by author using ChemDraw Professional 16.0. 1.4)

*Fig. 2.3* Chemical structures of the endogenous cannabinoids anandamide (AEA) and 2-arachidonoylglycerol (2-AG) (all structures drawn by author using ChemDraw Professional 16.0. 1.4)

Initial attempts to train rodents to discriminate AEA failed and evaluation of substitution for $\Delta^9$-THC or CP55,940 showed mixed results. One study reported substitution in rats, but not in mice and only at dose which significantly reduced rate of responding (Wiley et al. 1995a). Other studies showed no substitution for $\Delta^9$-THC (Burkey & Nation, 1997; Wiley et al. 1998). Similarly, exogenous administration of AEA does not substitute for $\Delta^9$-THC in mice (Wiley et al. 2014). The rapid hydrolysis of AEA (Deutsch & Chin, 1993) accounted for the challenge to achieve consistent substitution of this endogenous ligand in drug discrimination studies. The rapid degradation of AEA was corroborated in subsequent studies showing enzymatic inactivation of the endocannabinoids (Boger et al. 2000; Dinh et al. 2002; Cravatt et al. 2002). Highly selective catabolic enzyme inhibitors would not become available until several years later
and so investigators relied on metabolically stable analogs of AEA. These compounds were useful in establishing structure activity relationships to determine how the endocannabinoids, which are eicosanoid derivatives, interact with CB1 receptors in comparison to classical, non-classical cannabinoids and their derivatives (Adams et al. 1995, 1998). Evaluation of their discriminative stimulus properties was also useful in exploring the physiological and behavioral roles of the endocannabinoids.

The synthetic analog (R)-methanandamide substitutes for ∆9-THC but only in rats trained to discriminate a relatively lower training dose (3.0mg/kg or less) whereas no generalization occurs in rats trained to discriminate 5.6mg/kg or 30mg/kg (Burkey & Nation; 1997; Järbe et al. 1998, 2000; Wiley et al. 2011). Explanations for these dissimilar substitution patterns include the possibility that higher dose (R)-methanandamide produces a discriminative stimulus via TRPV1 receptors based on the observation (R)-methanandamide induces TRPV1-dependent locomotor depression (Millns et al. 2006). Another possibility is that ∆9-THC is more potent than (R)-methanandamide. ∆9-THC is more potent than both O-1812 and methylated fluoroanandamide in mice trained to discriminate O-1812 and that it produces a higher degree of substitution than exogenous AEA in rats and monkeys trained to discriminate ∆9-THC (Wiley et al. 1997, 2004). Despite these differences in potency and receptor mechanisms, it is apparent that AEA shares CB1-dependent discriminative stimulus properties with ∆9-THC considering that rimonabant completely attenuates substitution of (R)-methanandamide for ∆9-THC (Järbe et al. 2001).

Analogs of AEA have also been used as the training drug in discrimination studies (Järbe et al. 2001, 2009, 2010; Wiley et al. 2004, 2011). In rats trained to discriminate (R)-methanandamide from vehicle, AEA produced a higher degree of substitution than ∆9-THC. Of note, these results occurred when the interval between treatment and testing was reduced, reflecting
pharmacokinetic differences, or when the training dose of methanandamide was sufficiently high that Δ⁹-THC yielded no substitution, indicating a CB₁-independent mechanism (Järbe et al. 2001; Wiley et al. 2011).

Considering the differences between Δ⁹-THC and AEA or its analogs, genetic approaches have come into favor for studying the discriminative stimulus properties of endocannabinoids. Genetic deletion of FAAH or MAGL (i.e., FAAH(−/−) and MAGL(−/−) mice) results insignificantly elevated levels of AEA and 2-AG, respectively. This increased endocannabinoid tone imparts distinct phenotypes allowing researchers to study the implications of the endocannabinoid system in various physiological and behavioral processes such as metabolism, pain, and cognition (Lichtman et al. 2004; Petrenko et al. 2014; Kishimoto et al. 2015; Pan et al. 2011; Varvel et al. 2007; Tourino et al. 2010; Taschler et al. 2011). In particular, FAAH(−/−) mice have been trained to discriminate exogenously administered AEA from vehicle in two-lever and T-maze procedures (Walentiny et al. 2011; Wiley et al. 2016). O-1812 was shown to substitute in both FAAH(−/−) and wildtype mice but was more potent in FAAH(−/−) mice suggesting that increased brain levels of AEA contributed to the discriminative stimulus effects of O-1812 (Walentiny et al. 2015). So, in addition to pharmacological approaches using analogs of AEA, genetic deletion of FAAH has provided further evidence that the endocannabinoids similar to Δ⁹-THC elicit their discriminative stimulus via CB₁ receptor activation.
Discriminative Stimulus Properties of Endocannabinoid Degradative Enzyme Inhibitors

Fig. 2.4 Chemical structures of selective and dual inhibitors of FAAH/MAGL (all structures drawn by author using ChemDraw Professional 16.0. 1.4)

Endocannabinoid discrimination studies focusing on AEA have used both pharmacological and genetic approaches to prevent its hydrolysis. It should be reasonable to predict that 2-AG has the similar discriminative stimulus properties as AEA given it too binds CB₁ receptors with high affinity and is present in the CNS at concentrations up to 170 times that
of AEA (Devane et al. 1992; Stella et al. 1997). SAR studies on the endocannabinoids have also revealed that the arachidonoyl acid moiety common to both AEA and 2-AG has the least amount of viable substitutions so the activity of either endocannabinoid at CB1 receptors would not be expected to differ greatly (Rabinovich and Ripatti, 1991; Rich, 1993). An approach to study the discriminative stimulus properties of 2-AG specifically is nevertheless required to make conclusions as to the role of either endocannabinoid in the subjective effects associated with CB1 activation. Compared to FAAH (-/-) mice, MAGL (-/-) mice present certain confounders in that they exhibit reduced CB1 receptor expression and function as well as anxiety-like behaviors (Deng et al. 2020; Imperatore et al. 2015; Schlosburg et al. 2010). As such, MAGL(-/-) mice have not been evaluated as FAAH(-/-) mice in discrimination studies but the development of selective and dual inhibitors of MAGL or FAAH has allowed investigators to study the discriminative stimulus properties of the primary endocannabinoid hydrolytic enzymes. Both MAGL and FAAH inhibitors induce large increases in levels of 2-AG and AEA in mouse brain, respectively which produces behavioral effects (Fegley et al. 2005; Anh et al. 2009; Long et al. 2009; Chang et al. 2012; Niphakis et al. 2012, 2013).

**Monoacylglycerol Lipase (MAGL) Inhibitors**

Similar to the case of AEA, exogenous 2-AG is rapidly degraded and fails to substitute for ∆9-THC (Matuszak et al. 2009; Wiley et al. 2014). Selective MAGL inhibitor JZL184 has been evaluated for discriminative stimulus effects and has been shown to partially substitute for ∆9-THC in rodents (Wiley et al. 2014, 2016; Walentiny et al. 2015; Long et al. 2009). One study showed that mice receiving JZL184 produced no greater than 25% ∆9-THC appropriate responding although the rate of responding was reduced to less than that of vehicle (Hruba et al.
2015). Other studies similarly have shown mixed results for selective MAGL inhibitors. KML29 fails to substitute in mice trained to discriminate Δ⁹-THC from vehicle, suggesting that pharmacological blockade of MAGL alone is insufficient to produce subjective effects similar to Δ⁹-THC (Ignatowska-Jankowska et al. 2014). MJN110 produces full substitution in mice trained on CP55,940 and interestingly JZL184 fully substitutes for CP55,940 whereas it had only partially substituted Δ⁹-THC in prior studies (Ignatowska-Jankowska et al. 2015).

**Fatty Acid Amide Hydrolase (FAAH) Inhibitors**

The selective FAAH inhibitors URB-597 and PF-3845 do not substitute for Δ⁹-THC in rodent models of drug discrimination (Wiley et al. 2014; Hruba et al. 2015). URB-597 also fails to substitute for Δ⁹-THC in Rhesus monkeys (Stewart & McMahon, 2011). Putative AEA reuptake inhibitors AM-404 and UCM-707 similarly do not substitute for Δ⁹-THC in rodents (Solinas et al. 2007). Whereas these drugs are not sufficient to substitute for cannabinoids, combination of FAAH inhibitors and exogenous AEA produces behavioral effects. URB-597 and AEA produce full substitution for Δ⁹-THC in rats and Rhesus monkeys (Solinas et al. 2007; Stewart et al. 2011). In mice, PF-3845 in combination with AEA produces partial substitution up to 64% Δ⁹-THC lever responding (Wiley et al. 2014). These results suggest that elevating endogenous levels of AEA through FAAH inhibition are not high enough to produce subjective cannabinoid effects but FAAH blockade reveals the cannabinoid subjective effects of AEA.

**Dual MAGL & FAAH Inhibitors**

Administration of the selective MAGL inhibitor JZL184 in FAAH(-/-) mice fully substitutes for Δ⁹-THC in food-reinforced discrimination procedures and partially substitutes in a
water T-maze discrimination procedure (Walentiny et al. 2015; Long et al. 2009; Wiley et al. 2016). JZL184 also partially substitutes for AEA in FAAH\(^{(-/-)}\) mice in a water T-maze procedure. Similarly, the MAGL inhibitor KML29 produces full substitution for AEA in a food-reinforced discrimination procedure (Ignatowska-Jankowska et al. 2014). Another method for endocannabinoid discrimination involves administration of both MAGL and FAAH inhibitors or a single dual FAAH/MAGL inhibitor in wildtype mice. The combination of PF-3845 and JZL184 produces full substitution for \(\Delta^9\)-THC in wildtype mice (Hruba et al. 2015). In the same study it was shown that another FAAH inhibitor URB-597 in combination with JZL184 resulted in mainly vehicle-lever responding. The disparate activities of PF-3845 and URB-597 in concert with JZL184 could be due to species differences as URB-597 and JZL184 co-administration in rats fully substitutes for \(\Delta^9\)-THC (Wiley et al. 2014). Dual FAAH/MAGL inhibitors JZL195 and SA-57 both fully substitute for \(\Delta^9\)-THC in wildtype mice trained to discriminate \(\Delta^9\)-THC from vehicle (Walentiny et al. 2015; Hruba et al. 2015; Long et al. 2009). SA-57 has also been used as a training drug in wild-type mice, exhibiting both dose- and time-dependent discriminative stimulus effects (Owens et al. 2016). Cross substitution was also demonstrated between SA-57 and CP55,940 with either drug producing full substitution for the other in mice trained to discriminate SA-57 or CP55,940. SA-57 also produced full substitution for AEA in FAAH\(^{(-/-)}\) mice, indicating involvement of 2-AG in producing a CB\(_1\)-mediated discriminative stimulus. Taken together, the results from selective and dual inhibitors of FAAH and MAGL in both wildtype and FAAH\(^{(-/-)}\) mice provide sufficient evidence to implicate both AEA and 2-AG as mediators of the cannabinoid discriminative stimulus in whole animals.
Discriminative Stimulus Properties of Cannabinoid Antagonists

![Chemical structures of CB1 receptor inverse agonists rimonabant and AM-251](image)

Fig. 2.5 Chemical structures of CB1 receptor inverse agonists rimonabant and AM-251 (all structures drawn by author using ChemDraw Professional 16.0. 1.4)

The pharmacological properties of the CB1 receptor antagonist/inverse agonist rimonabant were first described in 1994 (Rinaldi-Carmona et al. 1994). In vitro, rimonabant antagonizes CB1 receptor agonist mediated inhibition of mouse vas deferens contraction and adenylyl cyclase activity. In vivo, rimonabant antagonizes the behavioral effects of CB1 receptor agonists in the mouse triad assay (three of the four measures in the tetrad assay excluding locomotor activity). Rimonabant was initially approved in the European Union as weight loss medication for the treatment of obesity but was recalled due to its anxiogenic and depression side effects, as well as reports of suicide ideation, but was not approved in the U.S. (reviewed in Sam et al. 2011). Despite its failure as a therapeutic, rimonabant has proven invaluable to the study of cannabinoid pharmacology by providing insight into receptor mechanisms of action.

With respect to cannabinoid drug discrimination, the discriminative stimulus effects of CB1 receptor agonists are considered CB1-dependent if rimonabant completely antagonizes their substitution under test conditions. Antagonism of the Δ⁹-THC discriminative stimulus has been
demonstrated in numerous studies in several species including rodents, pigeons and monkeys (Wiley et al. 1995c, d; Mansbach et al. 1996; Pério et al. 1996; Järbe et al. 2001, 2006; Solinas et al. 2004; McMahon et al. 2006; Walentiny et al. 2015). CB1 receptor antagonism with rimonabant was used to demonstrate that the discriminative stimulus effects of synthetic cannabinoids share the same CB1-dependent mechanism as Δ9-THC (Wiley et al. 1995c, d, 2013; Pério et al. 1996; Järbe et al. 2001, 2006; DeVry & Jentzsch, 2004). The discriminative stimulus effects of the endocannabinoids were also proven to be CB1-dependent using rimonabant.

Rimonabant reverses substitution of AEA in rats trained to discriminate methanandamide from vehicle (Järbe et al. 2001). The discriminative stimulus effects of both selective and dual FAAH/MAGL inhibitors in wildtype and FAAH(-/-) mice are also antagonized by rimonabant (Solinas et al. 2007; Walentiny et al. 2011, 2015; Wiley et al. 2016; Hruba et al. 2015; Stewart & McMahon, 2011; Long et al. 2009; Owens et al. 2016).

Given anxiogenic effects of rimonabant in humans it would seem plausible that rimonabant alone may elicit a unique discriminative stimulus. However, attempts to establish rimonabant discrimination in pigeons and rats through food-reinforced discrimination procedures were unsuccessful (Mansbach et al. 1996; Pério et al. 1996). Rimonabant discrimination has been demonstrated in rats through a taste aversion paradigm in which lithium chloride was paired with rimonabant administration and not vehicle such that absence of the rimonabant discriminative stimulus served as cue to the rats that the solution was safe to drink (Järbe et al. 2004, 2008). Rats on the same treatment schedule of rimonabant and vehicle without coadministration of lithium chloride did not demonstrate acquisition of the rimonabant cue. Substitution tests showed that AM-251, an analog of rimonabant fully substituted for rimonabant whereas the CB2 inverse agonists SR144528 and AM-630 failed to substitute indicating that the
Discriminative stimulus effects of rimonabant are CB₁-mediated (Järbe et al. 2008). ∆⁹-THC failed to substitute for rimonabant when given alone but when administered in combination with rimonabant, ∆⁹-THC attenuated rimonabant-induced taste aversion thereby demonstrating the opposing actions of CB₁ receptor agonists and inverse agonists (Järbe et al. 2008).

Shock avoidance in Rhesus monkeys receiving daily injections of ∆⁹-THC is another method for establishing rimonabant discrimination (McMahon & France 2003; McMahon, 2006). Using this model, it has been shown that discontinuation of daily ∆⁹-THC injections induces rimonabant-lever responding with monkeys exhibiting overt behaviors indicative of cannabinoid withdrawal (Stewart & McMahon, 2010). Monkeys which did not receive daily injections of ∆⁹-THC did not acquire the rimonabant cue essentially making this version of rimonabant discrimination a precipitated withdrawal model. Consistent with this observation CB₁ receptor inverse agonist AM-251 substitutes for rimonabant in Rhesus monkeys and pretreatment with CB₁ receptor agonists including AEA, ∆⁹-THC, CP55,940 and WIN55,212-2 prior to the rimonabant training dose attenuates rimonabant-lever responding (McMahon, 2006; Stewart & McMahon, 2011).

Whereas rimonabant discrimination is difficult to establish under traditional procedures applied to CB₁ agonists, discrimination of its analog O-6629 has been demonstrated in wildtype mice under FR10 food-reinforced conditions (Walentiny et al. 2013). Although O-6629 showed dose-dependent substitution for the training dose under test conditions it did not antagonize the discriminative stimulus of ∆⁹-THC in mice trained to discriminate ∆⁹-THC from vehicle and there was no cross-substitution between the two drugs in mice trained to discriminate either O-6629 or ∆⁹-THC. Rimonabant also did not substitute for nor antagonize O-6629. The only drug which dose-dependently substituted for O-6629 was another pyrazole 3-substituent analog of
rimonabant O-6658. These findings are consistent with another study which previously showed the series of analogs including O-6629 and O-6658 produced agonist-like effects in both wildtype and CB$_1^{(-/-)}$ mice which were not antagonized by rimonabant, suggesting this series represents a novel class of compounds with non-CB$_1$ mediated mechanisms of action (Wiley et al. 2013).

**Discriminative Stimulus Properties of CB$_1$ Receptor Allosteric Modulators**

![Chemical structures of CB$_1$ receptor allosteric modulators](image)

*Fig. 2.6* Chemical structures of CB$_1$ receptor allosteric modulators (all structures drawn by author using ChemDraw Professional 16.0. 1.4)
Efforts to develop therapeutics which target the endocannabinoid system have largely focused on ligands which bind CB₁ receptors at the orthosteric site (i.e. the principal site of action of CB₁ receptor agonists) or which augment endocannabinoid tone through pharmacological blockade of FAAH and/or MAGL. Advances in functional screening of GPCR ligands in vitro has led to an increase in the availability of novel compounds which interact non-competitively with orthosteric ligands through a distinct allosteric binding site (Rees et al. 2002). Allosteric modulators may impart functional selectivity and/or biased signaling with respect to orthosteric ligands by inducing conformational changes of the receptor which alter certain parameters according to the extended ternary complex model for GPCRs (reviewed in Kenakin 2013, 2019). The first CB₁ receptor allosteric modulators described were a series of compounds developed by Organon: Org27569, Org27759, and Org29647 (Price et al. 2005). Org27569 was shown to be a CB₁ receptor negative allosteric modulator (NAM) in that it antagonized agonist-induced [³⁵S]GTPγS binding with significant decreases in the $E_{\text{max}}$ values of CP55,940 and AEA (Price et al. 2005; Baillie et al. 2013). In vivo Org27569 decreased feeding behavior in rats and mice and attenuation of CP55,940-induced hypothermia in rats (Gamage et al. 2014; Ding et al. 2014). In drug discrimination however Org27569 did not alter the discriminative stimulus effects of ∆⁹-THC in C57BL6/J mice or AEA in FAAH (−/−) mice (Gamage et al. 2014). LDK1258, an analog of Org27569 similarly had no effect in drug discrimination when administered alone and neither altered the dose response curves for CP55,940 in C57BL6/J mice nor AEA in FAAH (−/−) mice (Mustafa, 2020). ZCZ011 has been well characterized as a CB₁ receptor positive allosteric modulator (PAM) both in vitro and in vivo (Ignatowska-Jankowska et al. 2015). ZCZ011 displayed robust PAM activity in a battery of in vitro functional assays. In vivo ZCZ011 potentiated the pharmacological effects of CP55,940
and AEA, showing significant increases in CP55,940-induced antinociception, catalepsy, and hypothermia in C57BL6/J mice as well as an increase in AEA-induced hypothermia in FAAH\(^{(-/-)}\) mice. In drug discrimination ZCZ011 significantly increased the potency of both CP55,940 and AEA in C57BL6/J and FAAH\(^{(-/-)}\) respectively without eliciting any discriminative stimulus effects or rate suppression when administered alone. ZCZ011 has garnered much interest in recent years primarily due to its antiallodynic effects in the chronic constriction injury (CCI) model of neuropathic pain which has spawned the development of analogs with similar antiallodynic activity including GAT211 and ABD1236 (Slivicki et al. 2018; Tseng et al. 2019). The discriminative stimulus effects of GAT211 and ABD1236 are not currently known.

**Future Directions in Cannabinoid Drug Discrimination**

Cannabinoid discrimination has demonstrated several aspects of translation efficacy following its inception in the 1970s. Studies on \(\Delta^9\)-THC discrimination in several species provided convincing evidence for the existence of an endogenous cannabinoid receptor well before the development of *in vitro* assays to measure adenylyl cyclase activity the subsequent isolation and cloning of CB\(_1\) and CB\(_2\) receptors (Järbe et al. 1974; Devane et al. 1988, 1992; Matsuda et al. 1990; Munro et al. 1993). Following the development of synthetic CB\(_1\) receptor agonists drug discrimination found utility in assessment of abuse liability and in SAR studies which demonstrated correlation between CB\(_1\) receptor binding affinity and potency of discriminative stimulus effects *in vivo* (Compton et al. 1992; Gold et al. 1992; Lainton et al. 1995; McMahon et al. 2008; Järbe et al. 2011; Ginsburg et al. 2012; Wiley et al. 1998; 2014; Gatch & Forster 2014, 2015). Reliable preclinical models for cannabimimetic activity such as drug discrimination were necessary in generating sufficient data to move CB\(_1\) receptor ligands to
clinical trials in humans. Pharmaceutical formulations of Δ<sup>9</sup>-THC such as dronabinol have been approved by the FDA as an orexigenic for the treatment of cachexia in HIV/AIDS patients and as an antiemetic for the treatment chemotherapy-induced nausea and vomiting (Badowski, 2017; Wang et al. 2019). Discrimination studies involving FAAH and MAGL inhibitors were pivotal in characterizing the behavioral effects of the endocannabinoids and conversely showing that pharmacological blockade of these enzymes produces discernable behavioral effects. Correlation between discriminative stimulus effects of such compounds may be useful in identifying compounds with analgesic properties such as PF-3845 which reduces inflammatory pain and MJN110 which reduces neuropathic pain (Ahn et al. 2009; Ignatowska-Jankowska et al. 2015; Wilkerson et al. 2016). Drug discrimination remains a reliable measure of cannabimimetic side-effects. Lastly, cannabinoid drug discrimination is useful assay to quantify CB<sub>1</sub> receptor allosteric modulation. Considering the complex neural circuitry which mediates the discriminative stimulus effects of orthosteric CB<sub>1</sub> receptor ligands, drug discrimination will be useful in examining whether allosteric modulator activity in vitro translates to behavioral effects in whole organisms. As such, drug discrimination may help identify novel compounds with useful properties such as ZCZ011, GAT211, and ABD1236 that are both potent and resistant to tolerance and dependence (Ignatowska-Jankowska et al. 2015; Slivicki et al 2018; Tseng et al. 2019). Aside from potential use as analgesics CB<sub>1</sub> receptor PAMs may also have utility in the treatment of cannabinoid use disorder (CUD) and substance use disorders involving non-cannabinoid drugs of abuse (Trexler et al. 2019; Slivicki et al. 2020; Jing et al. 2014).
Chapter 3: Divergent effects of ZCZ011 analogs in mouse models of overt cannabimimetic activity, subjective drug effects, and neuropathic pain

Rationale

The rationale behind this study is to evaluate the 2-phenyl indole class of CB₁ PAMs represented by ZCZ011 for antiallodynic effects in the CCI model of neuropathic pain, overt cannabimimetic effects in the tetrad assay, and subjective drug effects in the drug discrimination paradigm. Using the tetrad and drug discrimination assay we will test analogs of ZCZ011 for agonist activity alone and for PAM activity in combination with the orthosteric CB₁ agonist CP55,940. Considering the activity of ZCZ011 analogs in CCI, tetrad, and drug discrimination we can examine the relationship between the antiallodynic effects, overt and subjective cannabimimetic effects of CB₁ PAMs. Should any correlation exist between CCI and drug discrimination, then the drug discrimination paradigm may serve as a predictive model for antiallodynic activity of CB₁ PAMs.

Hypothesis

The tetrad and drug discrimination paradigms serve as predictive tools for the antiallodynic activity of CB₁ PAMs in the CCI model of neuropathic pain. CB₁ PAMs will produce no overt or subjective cannabimimetic effects when administered alone but will potentiate the *in vivo* pharmacological effects of the CB₁ orthosteric agonist CP55,940. ZCZ011 and analogs, which behave as CB₁ PAMs will show positive correlations between their potentiation of the pharmacological effects of CP55,940 in the triad and drug discrimination assays and their antiallodynic effects in the CCI model of neuropathic pain.
Materials and Methods

Drug Discrimination

1. Subjects

Twenty-four male and female C57BL/6J mice (Jackson Laboratory, Bar Harbor, Maine, USA) were housed individually in clear plastic cages (18x29x30cm) with steel wire fitted tops and wood-chip bedding in a temperature controlled (20-22° C) vivarium. Training and test sessions were conducted at similar times during the light phase of a 12-hour light/dark cycle. Water was available ad libitum except during training and test sessions. Mice were maintained at 85-90% of free-feeding body weights by restricting daily rations of standard rodent chow (supplied by Harlan labs, Frederick, MD. Rodent diet 7912). Mice were given food ad libitum for a period of at least two weeks once every six months. Animals used in this study were cared for in accordance with the guidelines of the Institutional Animal Care and Use Committee of the Virginia Commonwealth University and the ‘Guide for the Care and Use of Laboratory Animals’ (National Research Council, 1996).

2. Apparatus

Experimental sessions were conducted in sound- and light-attenuated operant conditioning chambers (Med Associates, St. Albans, VT). Each chamber (18x18x18 cm) was equipped with a house light, two nose-poke apertures, and a recessed food receptacle centered between the apertures that was connected to a pellet hopper. Fans in the chambers provided ventilation and white noise. The house light remained off during training and test sessions. The chambers were connected to a computer running Med-PC software (Med Associates) used for scheduling contingencies and recording data.

3. Procedures
3.1. *Overnight interactive FR training*

Mice were placed in designated operant chambers and trained to respond at one aperture according to a fixed ratio (FR) 1 schedule of reinforcement. A food pellet reinforcement was delivered after every response. After one hour or 50-100 reinforcements the FR value was increased to FR2 for the remainder of the overnight session. Overnight training was concluded after 10-12 hours and the aperture with the most responses for each mouse was designated as the preferred-side aperture.

3.2. *Interactive FR10 training*

Mice were placed in designated operant chambers and trained to respond at their preferred-side aperture at a FR2 schedule of reinforcement. The FR value was gradually increased to the final FR10 schedule of reinforcement in which 10 consecutive responses were required for delivery of food reinforcement. After mice were trained at one aperture, the contingency requirements were switched to the other aperture. Training at the second aperture proceeded identically to that at the first aperture. When responding at the second aperture under a FR10 schedule of reinforcement was acquired, discrimination training was initiated.

3.3. *Discrimination training*

Mice were trained to respond at one aperture following administration of 0.1 mg/kg CP55,940 s.c. (30-min pretreatment time) and to respond at the other lever following vehicle s.c. injection according to a FR10 schedule of reinforcement. Each response at the incorrect aperture reset the response requirement at the correct aperture. Daily injections were administered on a double alternation sequence of CP55,940 and vehicle (e.g. drug, drug, vehicle, vehicle). Daily 15-min training sessions were held until the mice had met three
criteria during 9 of 10 consecutive training sessions: (1) the first completed FR10 (FFR) was at the correct aperture, (2) ≥80% of the total responding was at the correct aperture and (3) the rate of responding was ≥10min⁻¹. When these criteria were met, acquisition of the discrimination was established, after which substitution and combination testing began.

3.4. Substitution and Combination tests

Discrimination training was continued 5-7 days per week with stimulus substitution or combination tests occurring up to two days per week with no less than 72 hours between tests. To be eligible for testing, mice must have passed discrimination criteria during their last drug and vehicle training sessions. Prior to substitution or combination tests, generalization curves for CP55,940 were generated for all mice. During test sessions, responses at either aperture delivered reinforcement according to an FR10 schedule. Substitution and combination tests were conducted with several 2-phenyl indole analogs of ZCZ011 including GAT211, LDK1747, and LDK1752. For substitution tests, the test compound (40 mg/kg) or vehicle i.p. was administered 30-min prior to the test session. For combination tests, the test compound (40 mg/kg) or vehicle i.p. was administered 15-min prior to treatment with CP55,940 (0.01, 0.03, 0.056, 0.1, 0.3, or 1.0 mg/kg) or vehicle s.c. and 45-min prior to the test session. To ensure maintenance of CP55,940’s discriminative stimulus effects, control tests with the training dose of CP55,940 and vehicle were repeated before conducting substitution or combination tests with novel compounds.

4. Drugs

ZCZ011 and its analogs have been previously characterized in vitro with CP55,940 (Table 1.1). Therefore CP55,940 will serve as the orthosteric probe for the present studies. CP55,940 was supplied by the National Institute on Drug Abuse (NIDA). GAT211,
LDK1747, and LDK1752 were synthesized at the Rangel College of Pharmacy Health Science Center at Texas A&M University (Kingsville, TX, USA). Drugs were dissolved via sonication in a vehicle consisting of ethanol, Alkamuls-620 (Sanofi-Aventis) and saline in a ratio of 1:1:18. All drugs were administered at an injection volume of 10μl per gram of body mass. Subcutaneous route of administration was used for CP55,940 and vehicle injections in the drug discrimination studies. Intraperitoneal route of administration was used for all other drugs.

5. Dose selection

ZCZ011 and all analogs were tested at a dose of 40 mg/kg in the drug discrimination, tetrad, and CCI experiments. ZCZ011 is analgesic in the CCI model at 40 mg/kg and so that dose will serve as the basis for comparison for ZCZ011 analogs.

6. Data analysis

Acquisition indices were the percentage of animals meeting the discrimination training criteria (1.3.3). For each test session, the percentage of responses at the drug-side aperture and response rate (responses/min) were calculated. Mice that responded less than 10 times during a test session did not receive a reinforcement and so were excluded from analysis of aperture selection. All mice were included in analysis of response rate. Full substitution for CP55,940 was defined as ≥80% CP55,940-appropriate responding. Partial substitution for CP55,940 was defined as ≥20% and <80% CP55,940-appropriate responding.

Tetrad

1. Subjects

12 Male and female C57BL/6J mice (Jackson Laboratory, Bar Harbor, Maine, USA) were housed in clear plastic cages (18x29x30cm) with steel wire fitted tops and
wood-chip bedding in a temperature controlled (20-22º C) vivarium. Food and water were available ad libitum. Mice were tested during the day on a normal 12-hour light-dark cycle. Animals used in this study were cared for in accordance with the guidelines of the Institutional Animal Care and Use Committee of the Virginia Commonwealth University and the ‘Guide for the Care and Use of Laboratory Animals’ (National Research Council, 1996).

2. Procedures

2.1. Baseline measurements

Prior to injection, mice were weighed and baseline measurements were taken of tail withdrawal latency and rectal temperature. Mice were injected following baseline measurements and then tested 30-min later in the order which follows.

2.2. Locomotor activity assessment

Locomotor effects were assessed by placing mice in clear Plexiglas enclosures (43x21x20 cm) housed in sound- and light-attenuated chambers equipped with a house light and Fire-i™ digital cameras (Unibrain, San Ramon, CA, USA). Fans in the chambers provided ventilation and white noise. The house light remained on during the 300 second test sessions. The chambers were connected to a computer running Anymaze (Stoelting, Wood Dale, IL) software for session parameter control and data collection. Distance traveled (m), time immobile (s), and mean speed were recorded over 300 seconds.

2.3. Catalepsy bar test

Catalepsy was assessed on a metal bar attached to a ring-stand placed 4.5 cm above the platform. The mice were placed with their front paws resting on the bar and
time spent immobile was measured over 60 seconds. If the mouse climbed onto the bar or moved from its fixed position it was replaced for a maximum of three tries.

2.4. Warm-water tail withdrawal assay

Thermal nociceptive behavior was assessed by immersing the distal portion (approximately 1 cm) of the mice tails in a water bath held at 52º C. Tail withdrawal latency was measured up to a maximum of 10 seconds. Data were expressed as percent change from baseline or maximum percent effect (%MPE) according to the formula: %MPE = [(test latency – preinjection latency) / (10 – preinjection latency)] ×100.

2.5. Rectal temperature

Hypothermic effects were assessed by measuring rectal temperature with a thermometer probe (Physitemp Instruments, Clifton, NJ) inserted 2 cm into the rectum. Data were expressed as a change in temperature (ΔT) from baseline measured in ºC.

3. Drugs

See Drug Discrimination section 4.0

4. Dose selection

See Drug Discrimination section 5.0

Chronic Constriction Injury (CCI) Model of Neuropathic Pain

1. Subjects

See Tetrad section 2.1

2. Apparatus

Mice were placed individually in Plexiglas cylinders (8 cm diameter, 15 cm height) situated over a wire screen mesh surface. A blanket was draped over the setup to
blind mice to visual distractions in the laboratory. Mice were observed from below the surface of the screen mesh.

3. Procedures

3.1. Sciatic nerve ligation

Surgery was performed according to techniques described previously (Bennett & Xie et al. 1988; Ignatowska-Jankowska et al. 2015). Mice were anesthetized with isoflurane and the surgical site was prepared using aseptic technique. The sciatic nerve was isolated and loosely ligated. The sham surgery was performed identically but without nerve ligation.

3.2. Von Frey test for mechanical hypersensitivity

Mechanical hypersensitivity following surgery was measured using von Frey calibrated filaments as previously described (Murphy et al. 1999). Von Frey filaments were applied to the hind paws ipsilateral and contralateral to the surgery at 30-min postinjection. The stimulus threshold which evoked a response as defined by either lifting, licking, or shaking of the paw was recorded.

4. Drugs

See Drug Discrimination section 4.0

5. Dose selection

See Drug Discrimination section 5.0
Results

Evaluation of ZCZ011 analogs in the tetrad assay

The tetrad assay was performed in a series of four experiments (detailed in section 2.2). ZCZ011 analogs did not elicit full cannabimimetic effects in the tetrad assay. LDK1729 (40 mg/kg) suppressed locomotor activity with respect to distance traveled and time spent immobile (p < 0.01; unpaired t-test) (Table 3.1) and showed small but significant hypothermic effects at 30 min post-administration (F (2, 20) = 9.292; P = 0.001). However, LDK1729 (40 mg/kg) did not produce significant cataleptic or antinociceptive effects. The remaining analogs (ZCZ011, ABD1236, GAT211, LDK1730, LDK1747, LDK1750, LDK1752) did not produce significant effects in any of the tetrad measures (Table 3.1).

<table>
<thead>
<tr>
<th>Table 3.1 Summary of ZCZ011 analog activity in the tetrad assay</th>
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<tr>
<td>Locomotor immobility (s)</td>
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<td>--------------------------</td>
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<tr>
<td></td>
</tr>
<tr>
<td>VEH</td>
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<tr>
<td>ZCZ011</td>
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Acquisition and discrimination of the CP55,940 discriminative stimulus shows no sex differences

Cannabinoid drug discrimination studies in rodents have traditionally used male subjects. Therefore, the first set of experiments examined whether there were sex differences in acquisition of CP55,940 discrimination. Discrimination acquisition curves were constructed for male and female C57BL/6J mice training to discriminate CP55,940 (0.1 mg/kg) from vehicle (Fig. 3.1A). All mice acquired the discriminative stimulus of CP55,940 (0.1 mg/kg) within 90 training days. Unpaired t-test of male and female learning curves (Fig. 3.1B) showed no significant difference in acquisition according to sex (t=1.613). Dose-response curves for

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<th>0</th>
<th>0</th>
<th>0.91 ±</th>
<th>1.01 ±</th>
<th>38.35 ±</th>
<th>38.23 ±</th>
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<tbody>
<tr>
<td>LDK1747</td>
<td>10.93 ±</td>
<td>4.16</td>
<td>0.10</td>
<td>0.173</td>
<td>0.17</td>
<td>0.07</td>
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<tr>
<td>VEH</td>
<td>7.67 ±</td>
<td>2.03</td>
<td>0.10</td>
<td>0.10</td>
<td>0.16</td>
<td>0.10</td>
<td></td>
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<tr>
<td>LDK1750</td>
<td>16.33 ±</td>
<td>10.26</td>
<td>0.26</td>
<td>0.24</td>
<td>0.13</td>
<td>0.16</td>
<td></td>
</tr>
<tr>
<td>VEH</td>
<td>6.22 ±</td>
<td>1.730</td>
<td>0.24</td>
<td>0.09</td>
<td>0.21</td>
<td>0.38</td>
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<tr>
<td>LDK1752</td>
<td>5.38 ±</td>
<td>0.95</td>
<td>0.12</td>
<td>0.19</td>
<td>5.86</td>
<td>0.15</td>
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(*) indicates significant results
responding on the CP55,940 associated aperture and response rates were constructed using CP55,940 (0.01-1.0 mg/kg). Responding on the drug aperture was statistically significant at 0.1 mg/kg (CP55,940 main effect: F (DFn, DFd) 3.668 (5, 95); P < 0.0001; Fig. 3.1C). Rate of responding was significantly decreased at and above 0.3 mg/kg (CP55,940 main effect: F (DFn, DFd) 8.685 (5, 122); P < 0.0001; Fig. 3.1E). The data were also plotted according to sex (Fig. 3.1D, F) and analyzed using two-way ANOVA. No significant difference was observed between males and females for CP55,940 drug-like responding or response rates (interaction between sex and dose: F (5, 90) = 0.5777, P = 0.7169; CP55,940 main effect: F (5, 90) = 32.19, P < 0.0001; sex main effect: F (1, 90) = 2.585, P = 0.1114).

(A)  (B)

![Discrimination Learning Curve](image)

(C)  (D)

![Drug-side aperture (%)](image)
Figure 3.1 Male and female C57BL6/J mice show identical acquisition rates of CP55,940 in the drug discrimination paradigm similar generalization curves of CP55,940. (A) Discrimination learning curve shows 100% of total C57BL6/J mice acquired the discriminative stimulus of CP55,940 within 90 training days. (B) Discrimination learning curve for males and females reveal no sex differences for acquisition of the CP55,940 discriminative stimulus. (C) CP55,940 (0.01-1.0 mg/kg) produces a dose-dependent discriminative stimulus in C57/BL6J mice. (D) No sex differences exist for CP55,940 (0.01-1.0 mg/kg) drug-like responding. (E) CP55,940 (0.01-1.0 mg/kg) produces dose-dependent suppression of response rates in C57BL6/J mice. (F) No sex differences exist for CP55,940 (0.01-1.0 mg/kg) effect on response rates. N = 23 C57BL6/J mice; n = 11-12 mice per sex. All data were collected 30 min after treatment administration and reported as mean ± SEM. Data were analyzed via unpaired t-test (B), one-way ANOVA (C) & (D), or two-way ANOVA (E) & (F).

Evaluation of GAT211, LDK1747, and LDK1752 substitution for CP55,940 in the drug discrimination paradigm

ZCZ011 analogs were screened for subjective cannabimimetic effects by measuring substitution for the discriminative stimulus of CP55,940 (0.1 mg/kg) in the drug discrimination paradigm. GAT211 (20-40 mg/kg) did not substitute for CP55,940 (0.1 mg/kg) and had no effect on response rates at 30 min post-injection (Fig 3.2A). LDK1747 (40 mg/kg) did not substitute for the discriminative stimulus of CP55,940 (0.1 mg/kg) and had no effect on response rates at 45
min post-injection (Fig. 3.2B). LDK1752 (40 mg/kg) did not substitute for CP55,940 (0.1 mg/kg) and had no effect on response rates at 45 min post-injection (Fig. 3.2C).

**Figure 3.2** Evaluation of GAT211, LDK1747, and LDK1752 substitution for CP55,940 in the drug discrimination paradigm. (A) GAT211 (20 or 40 mg/kg) did not substitute for the discriminative stimulus of CP55,940 (0.1 mg/kg) and did not suppress the rate of responding. (B) LDK1747 (40 mg/kg) did not substitute for the discriminative stimulus of CP55,940 (0.1 mg/kg) and did not alter the rate of responding. (C) LDK1752 did not substitute for the discriminative stimulus of CP55,940 (0.1 mg/kg) and did not alter the rate of responding. All data were collected 30 min after treatment and reported as mean ± SEM. All data were analyzed using one-way ANOVA.
Evaluation of ZCZ011 analogs in augmenting the pharmacological effects of CP55,940: triad assay

ZCZ011 analogs were tested in combination with CP55,940 in the triad assay to screen for allosteric modulator effects. ZCZ011 (40 mg/kg) potentiated the cataleptic, antinociceptive, and hypothermic effects of CP55,940 (0.3 mg/kg) (Fig. 3.3A). ABD1236 (40 mg/kg) potentiated CP55,940-induced antinociception and hypothermia (Fig. 3.3B). GAT211 (20-40 mg/kg) potentiated CP55,940-induced catalepsy, antinociception, and hypothermia (Fig. 3.3C).

LDK1729 (40 mg/kg) potentiates CP55,940-induced hypothermia (Fig. 3.3D). LDK1730 slightly potentiated CP55,940-induced hypothermia with significance at 1.0 mg/kg CP55,940 (Fig. 3.3E). LDK1747 (40 mg/kg) potentiated CP55,940-induced antinociception and hypothermia (Fig. 3.3F). LDK1750 (40 mg/kg) does not affect the dose-effect relationships of CP55,940 (Fig. 3.3G). LDK1752 (40 mg/kg) produced a small but significant potentiation of CP55,940-induced catalepsy (F (3, 30) = 4.013; P <0.0001; Fig. 3.3H), but only at 0.3 mg/kg CP55,940. LDK1752 (40 mg/kg) failed to alter the dose-response relationships of CP55,940 (0.1-3.0 mg/kg) for antinociception and hypothermia (Fig. 3.3H). The ED$_{50}$ values of CP55,940 as well as potency ratios were calculated for each measure of the triad assay. Table 3.2 shows the ED$_{50}$ and potency ratio comparisons between vehicle and ZCZ011 analog pretreatment groups.
(B) Catalepsy, Antinociception, Hypothermia

(C) Catalepsy, Antinociception, Hypothermia

(D) Catalepsy, Antinociception, Hypothermia

(E) Catalepsy, Antinociception, Hypothermia
Figure 3.3 Evaluation of ZCZ011 analogs in combination with CP55,940 in the triad assay. (A) ZCZ011 (40 mg/kg) potentiates CP55,940-induced catalepsy, antinociception, and hypothermia. (B) ABD1236 (40 mg/kg) potentiates CP55,940-induced antinociception and hypothermia. (C) GAT211 (20-40 mg/kg) potentiates CP55,940-induced catalepsy, antinociception, and hypothermia. (D) LDK1729 (40 mg/kg) slightly potentiated CP55,940-induced antinociception. (E) LDK1730 (40 mg/kg) slightly potentiated CP55,940-induced hypothermia. (F) LDK1747 (40 mg/kg) potentiates CP55,940-induced antinociception and hypothermia. (G) LDK1750 (40 mg/kg) does not affect the dose-effect relationships of CP55,940. (H) LDK1752 (40 mg/kg) slightly potentiated CP55,940-induced catalepsy with significance at 0.3 mg/kg dose of CP55,940 but did not alter the antinociceptive or hypothermic effects of CP55,940 (0.1-3.0 mg/kg). All data are reported as mean ± SEM and were analyzed via two-way ANOVA. Experiments (A-G) conducted by Julien Dodu and Mohammed Mustafa.
Table 3.2 Effects of ZCZ011 analogs on CP55,940 ED\textsubscript{50} and potency ratio values in the triad assay

<table>
<thead>
<tr>
<th>Compound</th>
<th>Measure</th>
<th>Vehicle ED\textsubscript{50} (CL 95%)</th>
<th>Vehicle CP55,940 (mg/kg)</th>
<th>PAM ED\textsubscript{50} (CL 95%)</th>
<th>PAM CP55,940 (mg/kg)</th>
<th>Potency Ratio (CL 95%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZCZ011</td>
<td>catalepsy</td>
<td>0.55 (0.36-0.85)</td>
<td>0.31 (0.21-0.46)</td>
<td>1.66 (0.95-3.17)</td>
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<td></td>
<td>antinociception</td>
<td>0.42 (0.29-0.63)</td>
<td>0.30 (0.21-0.42)</td>
<td>1.40 (0.84-2.48)</td>
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<tr>
<td></td>
<td>hypothermia</td>
<td>1.19 (0.93-1.53)</td>
<td>0.44 (0.35-0.55)</td>
<td>2.08 (1.38-3.36)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ABD1236</td>
<td>catalepsy</td>
<td>0.36 (0.28-0.46)</td>
<td>0.41 (0.31-0.55)</td>
<td>0.89 (0.65-1.22)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>antinociception</td>
<td>0.58 (0.40-0.84)</td>
<td>0.18 (0.12-0.27)</td>
<td>3.26 (1.77-8.49)</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>hypothermia</td>
<td>0.35 (0.31-0.41)</td>
<td>0.22 (0.18-0.27)</td>
<td>1.57 (1.23-2.04)</td>
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<tr>
<td>GAT211</td>
<td>catalepsy</td>
<td>0.53 (0.34-0.84)</td>
<td>0.47 (0.26-0.83)</td>
<td>1.12 (0.77-1.64)</td>
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<tr>
<td></td>
<td>antinociception</td>
<td>0.57 (0.42-0.79)</td>
<td>0.21 (0.17-0.27)</td>
<td>2.48 (1.63-4.03)</td>
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<tr>
<td></td>
<td>hypothermia</td>
<td>0.33 (0.27-0.39)</td>
<td>0.14 (0.12-0.18)</td>
<td>1.87 (1.45-2.45)</td>
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<tr>
<td>LDK1729</td>
<td>catalepsy</td>
<td>0.53 (0.37-0.78)</td>
<td>0.48 (0.33-0.71)</td>
<td>1.15 (0.72-1.86)</td>
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<tr>
<td></td>
<td>antinociception</td>
<td>0.44 (0.28-0.51)</td>
<td>0.38 (0.28-0.51)</td>
<td>1.16 (1.01-1.33)</td>
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<td></td>
<td>hypothermia</td>
<td>0.29 (0.23-0.37)</td>
<td>0.18 (0.14-0.24)</td>
<td>1.54 (1.17-2.04)</td>
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<tr>
<td>LDK1730</td>
<td>catalepsy</td>
<td>0.33 (0.19-0.58)</td>
<td>0.43 (0.21-0.87)</td>
<td>0.88 (0.33-2.32)</td>
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<tr>
<td></td>
<td>antinociception</td>
<td>0.13 (0.05-0.36)</td>
<td>0.18 (0.06-0.51)</td>
<td>0.71 (0.15-3.32)</td>
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<tr>
<td></td>
<td>hypothermia</td>
<td>catalepsy</td>
<td>antinociception</td>
<td>hypothermia</td>
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<tr>
<td>LDK1747</td>
<td>0.33 (0.21-0.52)</td>
<td>0.14 (0.10-0.19)</td>
<td>2.31 (1.41-3.86)</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>0.28 (0.21-0.37)</td>
<td>0.21 (0.14-0.31)</td>
<td>1.32 (0.84-2.11)</td>
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<tr>
<td></td>
<td>0.40 (0.30-0.55)</td>
<td>0.33 (0.24-0.44)</td>
<td>1.25 (0.89-1.77)</td>
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<tr>
<td></td>
<td>0.26 (0.22-0.31)</td>
<td>0.16 (0.13-0.19)</td>
<td>1.58 (1.35-1.84)</td>
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<tr>
<td>LDK1750</td>
<td>0.32 (0.17-0.60)</td>
<td>0.63 (0.38-1.09)</td>
<td>1.80 (0.71-4.94)</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>0.07 (0.04-0.14)</td>
<td>0.04 (0.02-0.09)</td>
<td>0.78 (0.24-2.49)</td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>0.13 (0.09-0.18)</td>
<td>0.13 (0.10-0.16)</td>
<td>0.98 (0.67)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>LDK1752</td>
<td>0.53 (0.29-0.96)</td>
<td>0.35 (0.13-0.91)</td>
<td>0.56 (0.31-1.00)</td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>0.48 (0.38-0.61)</td>
<td>0.40 (0.31-0.51)</td>
<td>0.82 (0.60-1.13)</td>
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<tr>
<td></td>
<td>0.36 (0.28-0.46)</td>
<td>0.32 (0.25-0.40)</td>
<td>0.85 (0.60-1.19)</td>
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</tbody>
</table>

*Evaluation of GAT211, LDK1747, and LDK1752 in combination with CP55,940 in the drug discrimination paradigm*

In the combination studies, GAT211 (40 mg/kg) did not alter the dose-response relationship of CP55,940 (0.01-1.0 mg/kg) in drug-like responding or response rates at 45 min post-injection (Fig. 3.4A, B). Separate analysis of male and female data using 2-way ANOVA showed no significant difference in drug-like responding (Table 3.3) or response rates (Table 3.4) following GAT211 (40 mg/kg) administration. LDK1747 (40 mg/kg) did not alter the dose-response relationship of CP55,940 (0.01-1.0 mg/kg) in drug-like responding or response rates at 45 min post-injection (Fig. 3.4C, D). Separate analysis of male and female data using 2-way
ANOVA showed no significant difference in drug-like responding (Table 3.3) or response rates (Table 3.4) following LDK1747 (40 mg/kg) administration. LDK1752 (40 mg/kg) slightly potentiated the discriminative stimulus of CP55,940 with significance at 0.03 mg/kg (interaction between LDK1752 and CP55,940: F (2, 10) = 6.486, P = 0.0156; CP55,940 main effect: F (2, 10) = 26.64, P < 0.0001; LDK1752 main effect: F (1, 5) = 5.673, P = 0.0630; Fig. 3.4E). LDK1752 (40 mg/kg) did not alter the dose-response relationship of CP55,940 with respect to response rates (Fig. 3.4F). Separate analysis of male and female data using 2-way ANOVA showed no significant difference in drug-like responding (Table 3.4) or response rates (Table 3.4) following LDK1752 (40 mg/kg) administration.
Figure 3.4 Evaluation of GAT211, LDK1747, and LDK1752 in combination with CP55,940 in the drug discrimination paradigm. (A) GAT211 (40 mg/kg) did not alter the dose-response curve of CP55,940 drug-like responding (B) GAT211 (40 mg/kg) did not alter the dose-response curve of CP55,940 rate of responding. (C) LDK1747 (40 mg/kg) did not alter the dose-response curve of CP55,940 drug-like responding (D) LDK1747 (40 mg/kg) did not alter the dose-response curve of CP55,940 rate of responding. (E) LDK1752 potentiated CP55,940 drug-like responding with significance at 0.03 mg/kg (F) LDK1752 (40 mg/kg) did not alter the dose-response curve of CP55,940 (0.01-1.0 mg/kg) in rate of responding.

<table>
<thead>
<tr>
<th>Interaction</th>
<th>CP55,940 main effect</th>
<th>Sex main effect</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F (DFn, DFD) P value</td>
<td>F (DFn, DFD) P value</td>
</tr>
<tr>
<td>GAT211</td>
<td>5, 17 = 1.123</td>
<td>ns</td>
</tr>
<tr>
<td>LDK1747</td>
<td>4, 8 = 0.1930</td>
<td>ns</td>
</tr>
<tr>
<td>LDK1752</td>
<td>2, 4 = 0.0869</td>
<td>ns</td>
</tr>
</tbody>
</table>
### Table 3.4 Summary of two-way ANOVA between sex and allosteric pretreatment in rate of responding

<table>
<thead>
<tr>
<th>Interaction</th>
<th>CP55,940 main effect</th>
<th>Sex main effect</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F (DFn, DFd)</td>
<td>P value</td>
</tr>
<tr>
<td>GAT211</td>
<td>6, 21 = 1.397</td>
<td>0.2619</td>
</tr>
<tr>
<td>LDK1747</td>
<td>6, 12 = 0.8609</td>
<td>0.5495</td>
</tr>
<tr>
<td>LDK1752</td>
<td>6, 12 = 1.111</td>
<td>0.4107</td>
</tr>
</tbody>
</table>

**Evaluation of ZCZ011 analogs in the chronic constriction injury (CCI) model of neuropathic pain**

The ZCZ011 analogs, ABD1236, GAT211, LDK1729, LDK1730, LDK1747, LDK1750, and LDK1752 (Fig. 3.5) were evaluated in the CCI model of neuropathic pain. ZCZ011, ABD1236, and GAT211 produced full reversal of allodynia, whereas the LDK series of compounds lacked activity. ZCZ011 (40 mg/kg) produces time-dependent reversal of allodynia for up to twelve hours following administration and this effect is not subject to tolerance under repeated administration (Fig. 3.6A). ABD1236 (40 mg/kg) produces significant increases in paw withdrawal thresholds for up to four hours post-administration (Fig. 3.5). GAT211 (40 mg/kg) was shown to reverse allodynia for up to eight hours following administration (Fig. 3.5). The
remaining analogs LDK1729, LDK1730, LDK1747, LDK1750, and LDK1752 at 40 mg/kg had no effect on paw withdrawal thresholds at any of the time points tested (Fig. 3.5).
Figure 3.5 Evaluation of ZCZ011 analogs in the CCI model of neuropathic pain. (A) ZCZ011 (40 mg/kg) reverses allodynia up to 12 hours post administration and maintains its effect following repeated administration. (B) ABD1236 (40 mg/kg) reverses allodynia up to 4 hours post administration. (C) GAT211 (40 mg/kg) reverses allodynia up to 8 hours post administration. (D-H) LDK series of ZCZ011 analogs fail to reverse allodynia at a dose of 40 mg/kg. n = 6-8 C57BL6/J mice per treatment group; mixed sex. All data are reported as mean ± SEM. Surgeries and experiments conducted by Lauren Moncayo and Rebecca Moncayo.
Chapter 4. Discussion and Conclusions

Summary of Results

The results obtained from studies on GAT211, LDK1747 and LDK1752 are summarized along with those of previous *in vivo* experiments on ZCZ011 and its analogs in Table 4.1. Almost no activity was seen for any compound in the tetrad assay except for minor locomotor suppression and hypothermic effects following LDK1729 administration. In the triad assay, only ZCZ011 and GAT211 produced leftward shifts in the dose-response relationship of CP55,940 for all three measures. ABD1236 and LDK1747 did not alter the cataleptic effects of CP55,940. LDK1729 and LDK1730 potentiated only hypothermic effects of CP55,940, whereas LDK1752 potentiated catalepsy, only. ZCZ011 was the only 2-phenyl indole characterized in drug discrimination prior to this study. This study examined GAT211, LDK1747, and LDK1752 in the drug discrimination paradigm. ZCZ011 analogs all failed to substitute for the discriminative stimulus of CP55,940 and had no effect on response rates. In the combination studies, only ZCZ011 and LDK1752 potentiated the discriminative stimulus effects of CP55,940 and none of the compounds had any effect on response rates. In the CCI model of neuropathic pain, only ZCZ011, ABD1236, and GAT211 had antiallodynic activity whereas the LDK series of compounds were ineffective. In summary, ZCZ011 is the only 2-phenyl indole, which elicits leftwards shifts in the CP55,940 dose response relationships in the triad assay and drug discrimination paradigm, as well as reduces CCI-induced allodynia.
<table>
<thead>
<tr>
<th>Compound</th>
<th>CCI Model of Neuropathic Pain&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Tetrad&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Triad&lt;sup&gt;c&lt;/sup&gt; [CP55,940]</th>
<th>Drug Discrimination&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZCZ011</td>
<td>full reversal</td>
<td>no effect</td>
<td>potentiation (3/3)</td>
<td>no substitution or rate suppression</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>potentiates DLR%</td>
</tr>
<tr>
<td>ABD1236</td>
<td></td>
<td></td>
<td>potentiation (2/3); no effect on catalepsy</td>
<td>not tested</td>
</tr>
<tr>
<td>GAT211</td>
<td></td>
<td></td>
<td>potentiation (3/3)</td>
<td>no substitution or rate suppression</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>no effect on CP55,940 dose response</td>
</tr>
<tr>
<td>LDK1729</td>
<td>no effect</td>
<td>locomotor</td>
<td>potentiates hypothermia</td>
<td>not tested</td>
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<td></td>
<td></td>
<td>suppression,</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>hypothermia</td>
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<td>LDK1730</td>
<td>no effect</td>
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<td>potentiates hypothermia</td>
<td>not tested</td>
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<tr>
<td>LDK1747</td>
<td>no effect</td>
<td></td>
<td>potentiation (2/3); no effect on catalepsy</td>
<td>no substitution or rate suppression</td>
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<td>no effect on CP55,940 dose response</td>
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<td>no effect</td>
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<td>no substitution or rate suppression</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>potentiates DLR% without effect on response rates</td>
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</table>

<sup>a.</sup> Surgeries and experiments conducted by Lauren Moncayo and Rebecca Moncayo.
<sup>b.</sup> (c) Experiments conducted by Julien Dodu and Mohammad Mustafa. GAT211, LDK1747, and LDK1752 studies were conducted by the author.

**In Vivo activity of ZC011 analogs**

Before interpreting the results, the rationale behind dose selection and use of male and female subjects in the drug discrimination paradigm should be discussed. The dose chosen for each CB<sub>1</sub> PAM evaluated was based on the effective dose of ZCZ011 in previous studies that produced full reversal of allodynia in the CCI model (i.e., 40 mg/kg; i.p.) (Ignatowska-
Jankowska et al. 2015). At this dose, ZCZ011 also produced leftward shifts in the dose-response curves of CP55,940 in the triad assay and drug discrimination paradigm and so this parent compound was considered the gold standard by which to compare the other analogs. Therefore, should an analog not exhibit the same degree of PAM activity in vivo as ZCZ011, one could test a higher dose but having to do so would mean the same compound has no therapeutic advantage over ZCZ011. Furthermore, solubility presents more of a challenge when preparing doses greater than 40 mg/kg. Drug discrimination studies have largely used only male subjects however sex differences in subjective responses to cannabinoids have been demonstrated in humans and rodents. In humans, females have reported greater subjective responses to Δ⁹-THC at 3 mg (p.o.) than males, whereas males reported greater sensitivity at higher doses of Δ⁹-THC (15 mg/kg p.o.) (Fogel et al. 2017). Female Sprague-Dawley rats learn to discriminate Δ⁹-THC faster and at lower doses (1 mg/kg vs 3 mg/kg) than their male counterparts (Wiley et al. 2017). One study using C57BL6/J trained to discriminate Δ⁹-THC (0.56 mg/kg) for food reinforcement showed that CP55,940 was more potent in male subjects than in females (Wiley et al. 2019). Therefore, it was important to evaluate sex differences in the cannabinoid discrimination paradigm used in this study. Here, we report that the potency of the CP55,940 discriminative stimulus did not differ between male and female C57BL6/J mice and response rates similarly showed no differences (Table 4.1).

**ZCZ011 analogs do not elicit overt or subjective cannabimimetic effects**

The purpose of the tetrad assay was to screen ZCZ011 analogs for CB₁ agonist-mediated cannabimimetic effects. Consistent with the assertion that PAMs do not activate the orthosteric site of their respective receptors, ZCZ011 analogs do not elicit the full tetrad set of behavioral
effects seen with CB\textsubscript{1} agonists. LDK1729 elicited only minor locomotor and hypothermic effects and lacked activity in the other behavioral measures. The drug discrimination paradigm was used to screen ZCZ011 analogs for discriminative stimulus effects similar to CP55,940. When tested for substitution, none of the ZCZ011 analogs substituted for the discriminative stimulus of CP55,940 (0.1 mg/kg). Accordingly, ZCZ011 analogs do elicit in vivo pharmacological effects associated with CB\textsubscript{1} orthosteric agonists. These results seem consistent with the activity of ZCZ011 analogs in vitro. The \( E_{\text{max}} \) values for ZCZ011 analogs with respect to cAMP-inhibition and \( \beta \)-arrestin recruitment would indicate these compounds have some agonist activity, however their \( EC_{50} \) values are orders of magnitude higher in comparison to CP55,940 (Table 1.1). ZCZ011 analogs are then considerably lacking in potency at the orthosteric site of CB\textsubscript{1} receptors compared to direct agonists both in vitro and in vivo, as would be expected for purported CB\textsubscript{1} PAMs. Whether any ZCZ011 analog acts at the orthosteric site or functions as an allosteric agonist at CB\textsubscript{1} receptors in vitro, it is not behaviorally relevant as cannabimimetic effects were not observed in the tetrad assay or substitution tests.

*Activity comparisons between ZCZ011 analogs across behavioral paradigms*

The overall purpose of this study was to examine the relationship between the antiallodynic, overt and subjective cannabimimetic effects of CB\textsubscript{1} PAMs represented by ZCZ011. According to the hypothesis, antiallodynic analogs of ZCZ011 should have also elicited leftward shifts of the CP55,940 dose-response curves in the tetrad assay and drug discrimination paradigm. In other words, positive allosteric modulation of the overt and subjective effects of cannabinoids was expected to be positively associated with reversal of alldynia in the CCI.
model of neuropathic pain. The data from this study did not suggest a relationship among the CCI, tetrad, and drug discrimination paradigms for CB₁ PAMs.

On the other hand, three compounds that produced antinociception in the CCI assay also augmented the pharmacological effects of CP55,940 in the triad assay. In the triad assay, ZCZ011, ABD1236, and GAT211 had in general greater effects on CP55,940 potency than the LDK series. LDK1747, similar to ABD1236, potentiated CP55,940-induced antinociception and hypothermia however LDK1747 was less potent by comparison. These results suggest that CB₁ PAMs, which augment the overt cannabimimetic effects of CP55,940 in the triad assay, may be predictive of antinociceptive activity in the CCI model of neuropathic pain. However, this relationship does not extend to the drug discrimination paradigm. GAT211 which has been shown to produce antiallodynic effects in a CIPN model of neuropathic pain at a dose of 20 mg/kg (Slivicki et al 2018) did not potentiate the discriminative stimulus effects of CP55,940 and also had no effect on response rates even at 40 mg/kg. As expected for CB₁ PAMs, neither GAT211, LDK1747, nor LDK1752 substituted for the discriminative stimulus of CP55,940. Of these compounds only LDK1752 potentiated the CP55,940 discriminative stimulus. This result was surprising considering LDK1752 had only a minor effect in the triad assay and lacked any effects in CCI or the tetrad assay. Additionally, GAT211 which behaved as a PAM in the triad assay failed to do so in drug discrimination. Taken together, these results suggest that antiallodynic activity in CCI is not correlated with PAM activity in the triad assay and drug discrimination paradigm.

The disparate effects of ZCZ011 analogs in vivo merits discussion on the separate neurological mechanisms which govern the behaviors each measured in CCI, tetrad, and drug discrimination. The CCI model and tail-flick test of the tetrad assay are both measures of pain
and so both involve pathways mediating pain transmission and modulation. The drug discrimination paradigm, however, is a measure of subjective drug effects and is performed in the absence of painful stimuli. The discriminative stimulus effects of a drug involve a combination of sensory and affective components, either of which can be modulated to produce antinociception. Whether the antiallodynic or antinociceptive properties of a drug depends more on its modulation of the sensory or affective components of pain may also affect its discriminative stimulus properties. That is to say selective modulation of pathways mediating the sensory or affective components of pain may explain why antiallodynic analogs of ZCZ011 such as GAT211 have no effect in the drug discrimination paradigm. Additionally, it is known that models of neuropathic pain can lead to phenotypic alterations in CB₁ receptor and endocannabinoid levels (Ignatowska-Jankowska et al. 2015; Petrosino et al. 2007; Siegling et al. 2001). Therefore, it is possible that the CCI surgery could lead to changes in the subjective responding of mice to CP55,940 which could produce different results for ZCZ011 analogs in drug discrimination.

At the receptor level, there may be pharmacodynamic differences between ZCZ011 analogs. The activity of ZCZ011 analogs in vivo may differ depending on the orthosteric probe being coadministered. The analogs examined in this study were tested using CP55,940 whereas ZCZ011 has previously been tested in combination with AEA in FAAH (-/-) mice as well as in combination with CP55,940 in C57BL6/J mice (Ignatowska-Jankowska et al. 2015). Every compound was shown to have some effect in combination with CP55,940 in the triad assay suggesting that each compound reaches the CNS within the time points tested to produce a measurable interaction with CP55,940. No conclusion can be made however without quantifying drug levels in CNS as there remains the possibility of peripheral mechanisms of action.
In the CCI model, the effects of ZCZ011 are presumed to be mediated through enhancement of endocannabinoid signaling at CB$_1$ receptors (Ignatowska-Jankowska et al. 2015). Upregulation of the endocannabinoid system with respect to AEA or 2-AG levels as well as CB$_1$ receptor expression is known to occur in response to neuropathic pain (Petrosino et al. 2007; Siegling et al. 2001). Thus, for ZCZ011, ABD1236, and GAT211, their antiallodynic effects may depend on increased endocannabinoid tone that results from the neuropathic pain state induced in the CCI model. Accordingly, the CB$_1$ PAM may enhance the signaling of AEA and/or 2-AG at CB$_1$ receptors. Alternatively, these modulators may act as allosteric agonists. Based on in vitro data, analogs of ZCZ011 possess some intrinsic agonist activity at CB$_1$ receptors. Studies on GAT211 have shown that its (R)- and (S)-isomers GAT228 and GAT229, respectively, bind to distinct allosteric sites on CB$_1$ receptors where GAT228 behaves as an allosteric agonist and GAT229 is a PAM (Hurst et al. 2019; Laprairie et al. 2017). ZCZ011 and ABD1236 also feature a chiral center, which is lacking in the LDK series of compounds. Most LDK compounds are comparable to ZCZ011 in inhibition of cAMP accumulation and so the structural differences do not significantly affect this activity. None of the compounds tested behaved in a similar fashion as CB$_1$ orthosteric agonist in the tetrad assay or drug discrimination paradigm. This result could be a function of dose in that some compounds may have CB$_1$ agonist activity, but not at the doses tested. The potency of ZCZ011 analogs in vitro were significantly lower in comparison to CP55,940 so it is likely that a dose of 40 mg/kg did not result in drug concentration sufficient for agonist effects in vivo. LDK1729 produced small but significant effects on locomotor activity and body temperature; however, it did not produce significant effects in all four tetrad measures. A compound would need to induce all four tetrad effects to be considered a cannabinoid agonist. None of the compounds tested in this study substituted for the
discriminative stimulus effects of CP55,940 in the drug discrimination paradigm. The allosteric agonist activity of ZCZ011 analogs does not predict agonist activity in vivo. The extent however to which ZCZ011 analogs function as PAMs or allosteric agonists may result in differences in PAM activity in vivo. Differential activity at CB1 receptor sites which mediate PAM or allosteric agonist effects may contribute to differences between ZCZ011 analogs in the triad and drug discrimination assay. For example, only ZCZ011 potentiated the effects of CP55,940 in all three measures of the triad assay whereas the other analogs only potentiated a subset of those effects. To attribute differences in activity in vivo to activity at specific binding sites however would require site-directed mutagenesis and transgenic expression of CB1 receptors.

In the triad and drug discrimination paradigms, only CP55,940 was used as an exogenous CB1 orthosteric agonist. It is difficult to draw comparisons among these two assays and the CCI model for several reasons. First, in the CCI model, although ZCZ011 may augment endocannabinoid signaling through its actions as a CB1 PAM, the concentration of endocannabinoids at CB1 receptors is unknown. AEA or 2-AG could have been used in the tetrad and drug discrimination studies however it would be draw comparisons between assays in which the orthosteric agonists at work are administered exogenously or produced endogenously. The second reason is that neuropathic pain and antiallodynic effects in CCI are dependent on specific pathways which modulate pain. Drug discrimination is a measure of subjective drug effects and so has more to do with the affective component of cannabinoid-related behaviors. With respect to pain however, there is a sensory component in addition to the affective component. Therefore, CB1 PAMs may have a differential effect on the overt and subjective effects of cannabinoids. That is to say CB1 PAMs may affect only a subset of cannabinoid-related behaviors. GAT211 potentiates the effects of CP55,940 in the triad assay but does not alter the potency of its
discriminative stimulus. LDK1752 potentiated the discriminative stimulus of CP55,940 but had very little effect on its potency in the triad assay. Overall, the 2-phenyl indole class of CB₁ PAMs show differential modulation of the antiallodynic, overt-behavioral, and subjective drug effects of cannabinoids as measured in the CCI model of neuropathic pain, tetrad assay, and drug discrimination paradigm, respectively.

**Limitations**

The first limitation of this study is that only CP55,940 was used as the orthosteric probe to study the effects of CB₁ PAMs. ZCZ011 has been characterized in the triad assay and drug discrimination paradigm with both AEA and CP55,940 and there are ligand specific effects. In the triad assay, ZCZ011 potentiates the effect of CP55,940 in all three measures whereas it only potentiates the hypothermic effects of AEA (Ignatowska-Jankowska et al. 2015). Therefore, differences in activity compared to ZCZ011 or a total lack of activity for the other analogs may be attributed to ligand-dependent effects. Using either endocannabinoid AEA or 2-AG would have better allowed for comparison to CCI results wherein the mechanism of action of CB₁ PAMs are endocannabinoid-dependent. If ZCZ011 reduces CCI-induced mechanical allodynia by potentiating endocannabinoid signaling at CB₁ receptors, then an endocannabinoid ligand should have been used in the triad assay and drug discrimination paradigm. The same applies for the *in vitro* characterization as it would be beneficial to know whether any ZCZ011 analogs exhibit ligand bias towards endogenous or exogenous cannabinoids. Doing so could help control for ligand-dependent effects however the difference still remains that in CCI the endocannabinoids would be produced endogenously rather than administered exogenously. The challenge however with studying the endocannabinoids is that AEA and 2-AG are rapidly
hydrolyzed \textit{in vivo}. AEA has been studied successfully with the use of FAAH (-/-) mice. Although MAGL (-/-) mice can be generated for study, they present some confounds in that they exhibit reduced CB$_1$ receptor expression and function as well as anxiety-like behaviors (Deng \textit{et al.} 2020; Imperatore \textit{et al.} 2015; Schlosburg \textit{et al.} 2010).

In relation to the allosteric ligands, it is not known precisely how each interacts with CB$_1$ receptors. Structural and \textit{in vitro} data suggest the isomers of GAT211 bind at distinct sites to mediate either PAM or allosteric agonist effects. Applying site-directed mutagenesis to CB$_1$ receptors and expressing those receptors in transgenic mice could offer a model to compare ZCZ011 analogs in terms of their CB$_1$ binding interactions. The differences seen \textit{in vivo} between the ZCZ011 analogs may depend on which binding sites are occupied and which residues are involved in binding.

Another limitation in this study was in relation to dose selection. No effect was seen for GAT211 or LDK1747 at 40 mg/kg in drug discrimination and so it’s possible a higher dose could have had an effect however solubility then becomes an issue. A dose of 40 mg/kg for a ZCZ011 analog is approximately 10mM in terms of concentration. Studies on ZCZ011 \textit{in vitro} used concentrations no higher than 1\(\mu\)M which potentiated the pharmacological effects of AEA and CP55,940 (Ignatowska-Janowksa \textit{et al.} 2015). Thus, it seems 40 mg/kg of a ZCZ011 analog should be sufficient to produce PAM effects however the exact concentration achieved at CB$_1$ receptors \textit{in vivo} is unknown. Regardless, using another analog and having to exceed the dose of ZCZ011 which reverses allodynia would make that compound less relevant from a therapeutic standpoint and so it is perhaps unnecessary to test doses higher than 40 mg/kg.

Lastly this study was limited in the compounds available for study such as ABD1236 which could not be obtained. ABD1236 was among the three compounds which exhibited
antiallodynic activity in CCI and PAM activity in the triad assay (along with ZCZ011 and GAT211). Therefore, it would have been beneficial in testing the hypothesis to evaluate ABD1236 in the drug discrimination paradigm. LDK1729 and LDK1730 similarly remain to be evaluated in drug discrimination with CP55,940. Despite this limitation there appears to be sufficient evidence that the pharmacological effects of ZCZ011 analogs in vitro and in vivo do not predict their antinociceptive effects in the CCI model of neuropathic pain.

**Future Directions**

Future directions for this study involve characterization of the remaining ZCZ011 analogs (ABD1236, LDK1729, LDK1730) in the drug discrimination paradigm. It would be of interest to test whether ABD1236 potentiates CP55,940 dose-response generalization curve in drug discrimination. Further experimentation in drug discrimination could be done using either AEA in FAAH (−/−) mice or MAGL inhibitors in C57BL6/J mice to examine whether ZCZ011 analogs differentially potentiate the subjective effects of either endocannabinoid. This experiment could help evaluate probe dependence for ZCZ011 analogs in drug discrimination. For the compounds evaluated in this study (GAT211, LDK1747, LDK1752), 40 mg/kg was the highest dose used and so future studies should examine whether these compounds alter the discriminative stimulus properties of CP55,940 at higher doses. An interesting question which arises from this study is whether any changes in the endocannabinoid system induced by CCI surgery alter the overt or subjective effects of cannabinoids as measured in the tetrad assay and drug discrimination paradigm. In other words, it is not known whether the CCI model produces any changes in the endocannabinoid system which modulate non-pain-related behaviors. If sciatic nerve injury in the CCI model results in an upregulation of the endocannabinoid system
that enhances the sensory component of pain modulation, it may also produce changes in the affective component of cannabinoid-related behaviors. To test this hypothesis, it would require combining the CCI model with the drug discrimination paradigm in such a way that one can assess whether the CCI surgery alters the discriminative stimulus properties of a CB₁ orthosteric agonist. To do this would require obtaining a dose-response for a CB₁ agonist in drug-like responding and response rates then subjecting that cohort of animals to the CCI surgery or a sham surgery and see whether the dose-response relationships are altered. Subsequent to the surgery would be re-constructing the same dose-response curves to see if the CCI surgery produced any leftward or rightward shifts in CB₁ agonist potency and whether that effect was any different from the sham surgery group. A challenge to this model would be that in the weeks it takes to build a dose-response curve in drug discrimination, the pain response thresholds of the mice would increase over time as they healed, making the model inconsistent. This problem could be overcome by using a between-subjects design, however that would require more subjects. Another question to examine in the future is whether the hypothesis holds true for a separate class of CB₁ PAMs that are structurally distinct from the 2-phenyl indoles represented by ZCZ011.

**Conclusion**

The purpose of this study was to examine the relationship between CB₁ PAMs in the CCI model of neuropathic pain, tetrad assay, and drug discrimination paradigm. The results of this study indicate that there is no correlation for CB₁ PAM activity between the three behavioral paradigms. The pathways which mediate the overt and subjective effects of cannabinoids are separate and distinct to the extent that compounds in the same class of CB₁ PAMs exhibit
activity which does not always translate between behavioral measures \textit{in vivo}. The tetrad and drug discrimination paradigms are useful for inferring cannabimimetic effects and have been used to infer potential abuse liability of CB\(_1\) receptor ligands administered alone. However, the results from studies investigating CB\(_1\) PAMs in combination with CP55,940 in these assays did not predict efficacy in predicting antiallodynic effects in the CCI model. Similarly, a CB\(_1\) PAM which is effective in the CCI model may exhibit no PAM activity in the tetrad or drug discrimination assays in that they do not potentiate the dose-response relationships of orthosteric agonists; GAT211 is one such example. For CB\(_1\) receptor ligands tested in the CCI model of neuropathic pain, it remains important to assess those compounds in tetrad and drug discrimination to rule out any possible psychoactive effects. Thus, all three measures should be employed when studying the \textit{in vivo} pharmacological effects of CB\(_1\) receptor ligands. Although the triad and drug discrimination assays do not appear to not offer a means to predict antinociceptive effects in the CCI neuropathic pain model, these assays possess utility to investigate the efficacy of CB\(_1\) ago-PAMs in neuropathic pain models, which lack cannabimimetic side effects associated with CB\(_1\) orthosteric agonists. Nevertheless, for ligands acting at CB\(_1\) receptors, the tetrad assay and drug discrimination paradigm should still be employed to screen for abuse liability. These assays are also required from a mechanistic standpoint. The CCI model does not yield any information about the mechanism of action of ZCZ011 analogs and whether they are acting as an agonist or PAM at CB\(_1\) receptors. The tetrad and drug discrimination paradigms therefore represent an indispensable tool to pharmacologists studying the effects of CB\(_1\) PAMs.
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