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# A Comparative Study of Cannabinoids & CB1 Receptor GI Signaling

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# A COMPARATIVE STUDY OF CANNABINOIDS & CB1 RECEPTOR $G_i$ SIGNALING

by

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## DEDICATION

A diary entry reporting on the day I graduated Lockhart Elementary (Knightdale, NC). At the time, I was eleven years old. *name changed

June 8, 1997

Sunday 6:05 P.M.

Dear Me:

Today is the second day out from school. Thank you, God! Gradutration was great but a youuuuuu Dummy of a day. Cold and Cloudy. YUCK! After, me and 15 girls rode for two hours in the limo. Also, Lindsey Adams* liked the driver, Mike! After, that we had a party. And after that, Lindsey, Emily, Brittany, ~~and~~ Tiffany, and me had a sleepover in the camper. COOL. They tryed to fool me by saying It is Morning, that [the] sun is out. Nope, it was at list [least] 3:00 A.m In the morning. I had a Blast.

Glad To Be Me

Haley

This dissertation is dedicated to all who supported me. Thank you for all the love, advice, and proof-reading. *Fortīs Fortūna Adiuvat*

## ACKNOWLEDGEMENTS

First, I would like to acknowledge my mentor, Dr. Kenneth Walsh. It's hard to express my appreciation without the use of understatements and clichés, but I'll do my best. My confidence is the result of your guidance. My interests formed from your knowledge. My future developed from your laboratory. Thank you.

To my illustrious committee members, I am beyond fortunate to have you all. Dr. Stephen Cutler, thank you for sharing the same excitement about cannabinoid research. I enjoyed hearing you speak about interesting cannabinoid research. Dr. Mitzi Nagarkatti, your perspective of cannabinoids and cannabinoid receptors in the body has been invaluable. I appreciate you taking the time to talk with me, be it about research, travel, or Teslas. Dr. David Mott, thank you for taking the time to talk with me about everything needed to become a successful graduate student. Your jovial nature is a source of positivity, much welcomed. Dr. Janet Fisher, your knowledge of the electrochemical properties of neurons served as the "Gold Standard" by which I aspire to achieve. A special thank you for all the times you've lent me your key because I had locked myself out of a laboratory or the graduate lounge. Again, I am fortunate to have such a phenomenal committee and I am grateful for all you have done.

On a personal level, I would like to acknowledge my husband, Steven Beck; you deserve that motorcycle. I'd like to thank my best friend, Frank Scalise and my brother, Matt Andersen. Most importantly, thank you, Mom and Dad. I love you.

## ABSTRACT

The legalization of medical marijuana has highlighted cannabinoids as a potential, opioid-free therapeutic option for pain management; however, the rise in illicit synthetic cannabinoid-induced toxicity has demonstrated the need to outline cannabinoid molecular signaling. The cannabinoid-type 1 (CB1) receptor is an endogenous G protein-gated receptor (GPCR), well-expressed in the central nervous system (CNS) associated with modulating neuronal activity. Cannabinoid agonists bind to the CB1 receptor resulting in the inhibitory G protein ($G_i$) complex to dissociate into two subunits, $G\beta\gamma_i$ and $G\alpha_i$. The $G\alpha_i$ subunit inhibits adenylyl cyclase, leading to a decrease in cyclic adenosine monophosphate (cAMP). The $G\beta\gamma_i$ subunit activates G protein-gated inwardly-rectifying (GIRK) channels, resulting in the efflux of potassium ($K^+$) ions and the subsequent hyperpolarizing of the neuron.

Cannabinoids are a group of compounds with a diverse range of chemical structures. The primary cannabinoid classes are eicosanoid, classical, non-classical, and aminoalkylindole. The aminoalkylindole cannabinoids represent a large portion of illicit cannabinoids, or synthetic cannabinoid receptor agonists (SCRAs), marketed as marijuana alternatives. Contrary to marijuana, intake of SCRAs has toxic and sometimes, lethal consequences.

The following studies report: 1) a fluorescent GIRK channel assay sensitive to CB1 receptor-mediated decrease in membrane potential. 2) analysis of the

GIRK channel response to cannabinoids representative of the four cannabinoid classes 3) investigation of GIRK channel response to a selection of illicit SCRAs. The cAMP levels were compared for AEA, THC, CP 55, 940, and WIN 55, 212-2, in which all effectively suppressed cAMP. Cannabinoid potency across the primary cannabinoid classes ranked: CP 55, 940 > WIN 55, 212-2 > THC > AEA > THCA-A ≈ CBD. WIN 55, 212-2 (aminoalkylindole) was significantly more effective at activating the GIRK channel response compared to AEA (eicosanoid) and THC (classical). SCRAs had a rank order potency of 5-fluoro MDMB-PICA > 4-fluoro MDMB-BUTINACA > AB-FUBINACA > MDMB-4en-PINACA > JWH-018 > AM1220 > XLR-11 > JWH-122 N-(5-chloropentyl) > WIN 55, 212-2 > UR-144 > AM1248. CBD did not induce a GIRK channel response. Synthetic cannabinoids were more potent and effective at stimulating a GIRK channel response. Indole/Indazole carboxamide substitutions displayed higher potencies. Only 4-fluoro-MDMB-BUTINACA was significantly more efficacious at stimulating a GIRK channel response compared to WIN 55, 212-2. Overall, synthetic cannabinoids have greater GIRK channel potency and efficacy.

## TABLE OF CONTENTS

## LIST OF TABLES

## LIST OF FIGURES

## LIST OF ABBREVIATIONS

$5\text{-}HT_{1A}$ ..... Serotonin 1A receptor

AEA..... Anandamide

ANOVA ..... Analysis of variance

$BaCl_2$..... Barium chloride

βarr1 ..... Beta-arrestin 1

βarr2 ..... Beta-arrestin 2

$Ca^{2+}$ ..... Calcium

CAMP ..... Cyclic-AMP

CB1 receptor..... Cannabinoid-type 1 receptor

CB2 receptor..... Cannabinoid-type 2 receptor

CBD ..... Cannabidiol

D1 .....Dopamine-type 1 receptor

ECL..... Extracellular loop

GDP .....Guanosine Diphosphate

GIRK channels.....G protein-gated, inwardly-rectifying potassium channels

$G_i$ ..... Inhibitory G protein signaling pathway

$G\alpha_i$ ..... Inhibitory G protein alpha subunit

$G\beta\gamma_i$ ..... Inhibitory G protein beta gamma subunit

GPCR ..... G protein-gated receptor

GPR55 ..... G protein-gated receptor 55

GRK ..... G protein-coupled kinases

GTP ..... Guanosine triphosphate

IBMX ..... 3-isobutyl-1-methylxanthine

ICL ..... Intracellular loop

$K^+$ ..... Potassium

Kir ..... Inward rectifier $K^+$ channel

PIP2 ..... Phosphatidylinositol 4,5-bisphosphate

PLCβ ..... Phospholipase Cβ

PPARs ..... Peroxisome proliferator-activated receptors

PWR ..... Plasmon-waveguide resonance

SCRA ..... Synthetic cannabinoid receptor agonist

MOR ..... μ-opioid receptor

M2R ..... Muscarinic acetylcholine M2 receptor

MP-sensitive ..... Membrane potential sensitive

S.E.M ..... Standard error of means

SST ..... Somatostatin receptor

THC ..... $\Delta^9$-Tetrahydrocannabidol

THCA ..... $\Delta^9$-Tetrahydrocannabinolic acid

THCA-A ..... $\Delta^9$-Tetrahydrocannabinolic acid A

TMH ..... Transmembrane helices

TRPV1 ..... Transient receptor potential cation channel subfamily V member 1

# CHAPTER 1: *INTRODUCTION*

## 1.1 The cannabinoid dichotomy

Chronic pain reduces the quality of life for many Americans, and the standard treatment, opioids, are highly addictive.[3, 4] Prescription misuse accounts for 40% of the opioid deaths in the United States, highlighting the need for an alternative treatment.[3, 5, 6] Medical marijuana and cannabinoids have become a popular candidate for opioid-free pain management.[7, 8] Since 1996, 33 states have legalized medical marijuana based on the reported therapeutic effects in a variety of maladies, such as chemotherapy-induced emesis, glaucoma, anxiety, and multiple sclerosis-related pain.[9, 10] In the early 2000s, synthetic cannabinoid receptor agonists (SCRAs) began to appear on the drug market as legal alternatives to marijuana.[11, 12] Academic laboratories developed many of these compounds for the study of CB1 receptor binding and not for human use.[13] Marijuana use has a high margin of safety; however, synthetic cannabinoid use can cause stroke, acute kidney damage, psychosis, and seizures.[14, 15] Cannabinoids have emerged as a forerunner in the race to develop better pain management; however, the toxicity of synthetic cannabinoids has demonstrated the need to understand how cannabinoids work.

*Cannabinoids as therapeutic targets*

Humans have made products from the plant genus *Cannabis* for millennia, with evidence of smoking *Cannabis* dating back to the first millennium BCE.[16-18] The genus *Cannabis* is divided into three species: 1. *Cannabis ruderalis* 2. *Cannabis indica* 3. *Cannabis sativa*.[19] Cannabidiol (CBD) and $\Delta^9$-tetrahydrocannabinol (THC) are the two most prominent compounds found in Cannabis, with THC being the primary psychoactive and CBD the primary non-psychoactive compound.[20, 21] *Cannabis ruderalis* contains low amounts of THC, whereas *Cannabis sativa* and *Cannabis indica* have higher levels of THC.[19] Marijuana is dried *Cannabis sativa*, *Cannabis indica*, or a combination of both, with varying ratios of THC to CBD.[22] Tetrahydrocannabinolic acid (THCA) is another cannabinoid found in *Cannabis sativa*. Fresh, unprocessed *Cannabis sativa* has high THCA concentrations that are partially converted to THC when exposed to heat.[23] Studies of THCA show potential therapeutic effects in pain, metabolic, and neurological disorders.[22, 24, 25]

When surveyed, people reported a decrease in pain after smoking or inhaling vaporized marijuana with low (≈4mg) to moderate (≈16mg) levels of THC.[26-28] Currently, there are three cannabinoid pharmaceuticals reported to decrease chronic pain.[29-32] Dronabinol and nabiximol are FDA-approved pharmaceuticals derived from the *Cannabis* plant. Nabiximol contains THC and CBD, whereas dronabinol only contains THC. [33] Nabilone is a synthetic analog of THC approved as an adjunct treatment for pain.[10, 29] While some studies report effective chronic pain relief with cannabinoid therapies, others report little to

no anti-nociceptive effects when tested on other forms of pain, such as acute post-operative and abdominal pain.[10] A recent meta-analysis determined that the effectiveness of cannabinoid-based pain therapeutics in humans inconclusive, mainly due to studies lacking a positive control; however, research utilizing animal models has found cannabinoids reduce pain reflexes and opioids self-administration.[8, 10, 33, 34]

*Adverse effects of illicit synthetic cannabinoid intake*

In the early 2000s, SCRAs began appearing in shops as herbal incenses under the names "K2", "Spice," and "Black Mamba."[35] Today, SCRAs products are sold and distributed through the dark web, social media platforms, and smartphone apps.[36] These products are created by spraying a mixture of SCRAs on dried plant material, typically thyme or lemon balm, and then smoked like marijuana.[12] Additionally, SCRAs are available in liquid formulations for use in electronic cigarettes and other vaping devices.[37]

SCRAs bind to the same receptor as THC, the cannabinoid-type 1 (CB1) receptor.[38] Unlike THC, SCRA intake can cause serious bodily harm such as impairment of fine motor skills, increased blood pressure, tachycardia, tremors, respiratory depression, seizures, ataxia, nausea, vomiting, acute kidney injury, and death. [39-41] SCRAs typically produce more adverse psychological effects than those experienced with THC, including impairments of attention and concentration, anxiety, panic, agitation, paranoia, hallucinations, violent or aggressive behavior, short-term memory loss and lack of responsiveness.[11, 42, 43] Researchers are interested in the mechanisms underlying the differences between THC and SCRAs

pharmacological effects, particularly regarding the CB1 receptor. Structural activity relationship (SAR) studies show variation in SCRAs binding to the CB1 receptor.[2, 44, 45] These studies suggest that differences in CB1 receptor binding could mediate SCRA potency and efficacy.

## 1.2 Cannabinoid-type 1 (CB1) receptor

The CB1 and cannabinoid-type 2 (CB2) receptors are the two predominant receptors in the endocannabinoid system. The CB2 receptor is associated with the peripheral nervous system (PNS) and immune response mechanism (i.e., glia) in the central nervous system (CNS).[21, 46] The CB1 receptor is well-expressed in CNS, particularly in the neocortex, hippocampus, basal ganglia, cerebellum, and brainstem.[47] Primarily located on neuron axons, the CB1 receptor modulates neuronal activity, such as inhibiting excitatory neurotransmitter release, through the downstream effects of G protein signaling.[48-50]

### *CB1 receptor signaling*

The CB1 receptor is a type-A, G protein-coupled receptor (GPCR) consisting of seven transmembrane helices (TMH 1-7), three extracellular loops (ECL1-3), three intracellular loops (ICL1-3), an N-terminus, and an intracellular C-terminus.[51] When the CB1 receptor is inactive, a heterotrimeric G protein complex, Gαβγ, interacts with TMH5, TMH6, ICL2, and the c-terminus.[2] (Figure 1.1) A small opening between TMH1 and TMH7 allows for ligand entry into the CB1 receptor. The suggested location of CB1 receptor orthosteric ligand-binding pocket is in an area interacting with TMH2-3 and TMH6-7.[2, 45, 52] When an agonist binds to the CB1 receptor, a conformation change occurs, and GDP is

exchanged for guanosine triphosphate (GTP) at the Gα subunit.[53] The complex dissociates into Gα and Gβγ subunits. These subunits initiate a series of intracellular processes that mediate neuronal response.[54] There are three principle G protein signaling pathways, $G_s$, $G_i$, and $G_q$. The actions of the Gα subunit define the G protein signaling pathways. $G_s$ stimulates the production of cyclic-AMP (cAMP), whereas $G_i$ inhibits the production of CAMP. $G_q$ activates phospholipase Cβ (PLCβ), leading to an increase in intracellular calcium ($Ca^{2+}$) levels.[55] Also, signaling from the $G_q$ pathway can initiate the synthesis of endocannabinoids, anandamide (AEA) and 2-AG. [56]

Ligands binding to the CB1 receptor cause the recruitment of G protein-gated receptor kinases (GRKs), which phosphorylate the receptor's intracellular c-terminus.[57] The phosphorylation of the CB1 receptor initiates the recruitment of β-arrestin.[58, 59] (Figure 1.1C) The CB1 receptor recruits are two major β-arrestin isoforms, β-arrestin 1 (βarr) and β-arrestin 2(βarr2). βarr1 activates downstream signaling pathways that mediate gene expression and protein synthesis. βarr2 is associated with CB1 receptor internalization and desensitization.[60-62] These processes function to disrupt ongoing $G_i$ signaling and stimulate cell apoptosis. β-arrestins are often associated with adverse behavioral outcomes seen in prolonged opioid and *Cannabis* use.[62-64] -

*The CB1 receptor is defined as a $G_i$-coupled receptor*

The CB1 receptor primarily couples the $G_i$ signaling complex.[53, 65-67] CB1 receptor agonists facilitate the release of $G\alpha_i$, thus inhibiting adenylyl cyclase, a key enzyme for the production of cAMP.[54] (Figure 1.2A) Both $G\alpha_i$ and $G\beta\gamma_i$ can

regulate cellular processes like apoptosis, cell differentiation, and proliferation by stimulating the mitogen-activated protein kinase (MAPK) signaling cascade.[68] The $G\beta\gamma_i$ subunit inhibits N-type $Ca^{2+}$ channels and activates G protein-gated, inwardly-rectifying potassium (GIRK) channels. Activation of GIRK channels allows for the efflux of potassium ($K^+$) ions, driving the membrane potential towards $K^+$ equilibrium close to -90mV in neurons. (Figure 1.2B).[69-71]

1.3 Targeting GIRK channels

GIRK channels are ion channels composed of four inward rectifier $K^+$ (Kir) channel subunits, GIRK1 – GIRK4.[72] The GIRK1/2 subunit arrangement is well-expressed throughout the CNS, specifically the hippocampus, cerebellum, and spinal cord.[73] GPCRs activate GIRK channels through the $G\beta\gamma_i$ subunit, which include muscarinic acetylcholine M2 (M2), dopamine-type 1 (D1), somatostatin (SST), serotonin ($5\text{-}HT_{1A}$), μ-opioid (MOR), and CB1 receptors.[74-78]

Ligands binding to GPCRs, such as MOR and CB1, initiate the release of $G\beta\gamma_i$ from the $G\alpha_i$ subunit. The $G\beta\gamma_i$ subunit binds directly on the cytosolic side of the GIRK channel to the c-terminus, which activates the channel.[79, 80] The GIRK channel c-terminus can bind up to four subunits.[81] One $G\beta\gamma_i$ subunit binding to the c-terminus will activate the GIRK channel, and the addition of each subsequent $G\beta\gamma_i$ subunits will potentiate the GIRK channel response. The GIRK channel response is deactivated when the $G\alpha_i$, recouples to the $G\beta\gamma_i$ subunit.[76, 82]

Active GIRK channels will decrease spontaneous action potential formation and inhibited the release of excitatory neurotransmitters.[72, 83] Analgesia is the inability to perceive pain. Opioids, such as morphine, activate GIRK channels,

which mediate analgesic effects.[64, 84] The MOR-induced, GIRK channel activation mediating analgesia serves as a potential mechanism by which CB1 receptor agonism could relieve pain.[84, 85]

## 1.4 Cannabinoids

### *Cannabinoid classification*

Cannabinoids are compounds that act on receptors of the endocannabinoid system. The CB1 and CB2 receptors are considered the canonical endocannabinoid receptors; however, cannabinoids can bind to other receptors such as orphan G protein-gated receptor 55 (GPR55), Transient receptor potential cation channel subfamily V member 1 (TRPV1), and Peroxisome proliferator-activated receptors (PPARs).[21, 46, 86] Cannabinoids are categorized into groups by their originating source and their chemical structure. Endocannabinoids are cannabinoids synthesized within the body, in which the two primary ones are AEA and 2-AG. The phytocannabinoid class consists of plant-derived cannabinoids, such as THC and CBD. Synthetic cannabinoids are human-made cannabinoids, be it in legitimate laboratories or illicit manufacturing outlets. The first cannabinoids to have their chemical structure defined were from *Cannabis* and, therefore, classified as classical cannabinoids. [87] Non-classical cannabinoids share a similar structure to classical compounds; however, they are synthetic cannabinoids. Eicosanoids are the structural class that includes endocannabinoids. Aminoalkylindoles are synthetic cannabinoids that have a unique chemical structure, unlike the previously listed.[88] (Table 1.1)

*Cannabinoid signaling*

Cannabinoids signal through G protein-dependent mechanisms and β-arrestin recruitment. The initial wave of cannabinoid signaling is primarily G protein regulated.[62] In addition to the $G_i$ pathway, cannabinoids also initiate other G protein signaling pathways. While WIN 55, 212-2 signals mostly through the $G_i$ pathway, it can also induce the $G_q$ signaling pathway.[89] In studies where pertussis toxin (PTX) inhibited $G_i$, the aminoalkylindole, WIN 55, 212-2, could recruit $G_s$ and $G_q$ signaling.[53, 66] Another study reported the illicit aminoalkylindole derivatives, 5 fluoro-MDMB-PICA, JWH-018, and AB-FUBINACA could also recruit the $G_s$ signaling pathway.[90]

Cannabinoids show bias in recruiting either βarr1 or βarr2, which can mediate different CB1 receptor signaling outcomes. For example, WIN 55, 212-2 bound CB1 receptors and β-arrestin briefly interact in the clathrin-coated pits before receptor internalization, resulting in little to no βarr1 signaling. In comparison, the endocannabinoid, 2-AG, has prolonged contact with the clathrin coated-pits resulting in enhanced βarr1 signaling.[60, 61] Other studies demonstrate that chronic exposure to THC induces CB1 receptor internalization via βarr2 recruitment.[91, 92]

Recently, there is a growing interest in cannabinoid biased agonism. Biased agonism is a concept stating that agonists can stabilize a receptor in different active confirmations that allows them to preferential couple to the signaling molecule.[93] Studies show THC is biased towards βarr1 recruitment over $G\beta\gamma_i$ signaling; whereas AEA is biased toward $G\beta\gamma_i$ over βarr1.[94] For example, THC

bound to the CB1 receptor alternates between two binding confirmations and resulting in a less stable active confirmation.[2, 44] SCRAs do not fluctuate like THC when bound; therefore, they are much more effective at stabilizing the CB1 receptor in the active conformation. Additionally, SCRAs bind deeper than THC in the receptor which enables interacts with the twin toggle switch, a pair of residues located on TMH3 and TMH6. Interaction with the twin toggle switch results in a change in TMH6 that potentiates the exchange of GDP to GTP; and thus, initiating CB1 receptor intracellular signaling.[2, 45, 52] It is postulated that cannabinoids can stabilize active confirmations preferential to a specific G protein signaling pathways or to a specific β-arrestin recruitment. Since pharmacological outcomes, such as anti-nociception and tolerance, are associated with specific intracellular signaling pathways, researchers are interested in identifying the active confirmations that result in biased signaling.[95]

1.5 Synthetic cannabinoids

SCRAs are a diverse group of compounds with notably high affinity for the CB1 receptor. In the 1990s, the three-point attachment hypothesis stated cannabinoid receptor binding was contingent upon three THC moieties: 1) C9 methyl group 2) phenolic alcohol 3) pentyl side chain extending from C3. To test the three-point attachment hypothesis, WIN 55, 212-2 was created and found to have a higher affinity for the CB1 receptor in comparison to THC.[96] Because the chemical structure of WIN 55, 212-2 was unlike the other cannabinoid classes, it became the prototype cannabinoid for the aminoalkylindole class.[81] The chemical structure of WIN 55, 212-2 served as a model for John W. Huffman and

colleagues when they began synthesizing a group of naphthoylindole SCRAs. The naphthoylindole, JWH-018, made headlines in the early 2000s when it was identified in illicit substances marketed as legal alternatives to marijuana. Since then, there has been an ongoing, legal battle between scheduling SCRA compounds as illegal and the appearance of newly modified SCRAs that subvert the law.

Today, the SCRA class is the most chemically diverse group of cannabinoids. The vast number of SCRAs is attributed to two factors: 1) CB1 receptor binding is tolerant of structural modifications. 2) Synthesizing new aminoalkylindole-derived SCRAs is, relatively, easy compared to other compounds.[12, 97, 98] This has allowed for multiple reiterations of SCRAs, tweaked to increase CB1 receptor potency and efficacy with unknown consequences to human health. While categorizing such a large group of compounds would seem a daunting task, SCRAs consists of four basic pharmacophores: 1) core 2) linker 3) head group 4) tail group.[95] (Figure 1.3) SCRAs cores are commonly either indole or indazole substitutions because this increases the CB1 receptor potency.[99, 100]. Linkers are usually an amide, ketone, or ester and do not appear to alter CB1 receptor potency. There are many different head groups, but most contain a naphthyl, quinolinyl, adamantly, or tetramethylcyclopropyl moiety.[101] Tail groups, typically, consist of a hydrophobic alkyl group attached to the nitrogen atom of the head group.[11] The tail group can have different substitutions, with the most common being the terminal fluorination because it increases potency.[97, 102] (Table 1.2)

*Synthetic cannabinoid signaling*

SCRAs are pharmacologically similar to THC; for example, JWH-018 decreases the probability of neurotransmitter release and stimulates MAPK kinase, characteristics of CB1 receptor agonism.[103] Unlike THC, the use of SCRAs increases the likelihood of seizures.[15] Like the cannabinoids discussed in the previous section, SCRAs mediate their effects through CB1 receptor $G_i$ signaling.[11] Almost all SCRAs are considered full agonists, as demonstrated by enhanced $G_i$ signaling effects such as inhibition of cAMP production and GIRK channel activation in comparison to THC, a partial agonist.[99, 102, 104, 105] The highly potent and toxic SCRA, MDMB-FUBINACA, is shown to hold the twin toggle switch in a manner that stabilizes the CB1 receptor in the active conformation.[2, 45] The binding dynamics of cannabinoids are thought to mediate biased agonism, which states a receptor can have multiple active state confirmations.[51] Increased ligand interaction with` the twin toggle switch, located within the CB1 receptor, increases the surface binding area critical for G protein binding.[45]

## 1.6 The impetus for this work

Cannabinoid research aims to bridge the gap between the receptor signaling and the pharmacological response. Agonists of the CB1 receptor stimulates $G_i$ signaling, in particular, GIRK channels. Both cannabinoids and GIRK channels have therapeutic promise when regarding pain management. However the emergence of structurally diverse, illicit SCRAs highlights that there is much still unknown about cannabinoid signaling.

*Hypothesis:* Cannabinoid class and structure will differentially affect the GIRK channel signaling via the CB1 receptor.

Aim 1: Establish a CB1 receptor GIRK channel assay

Aim 2: Determine the GIRK channel response to cannabinoids representative of the eicosanoid, classical, non-classical, and aminoalkylindole classes.

Aim 3: Investigate the effect of illicit SCRAs on GIRK channel activation

Each of these aims will be addressed in the following chapters.

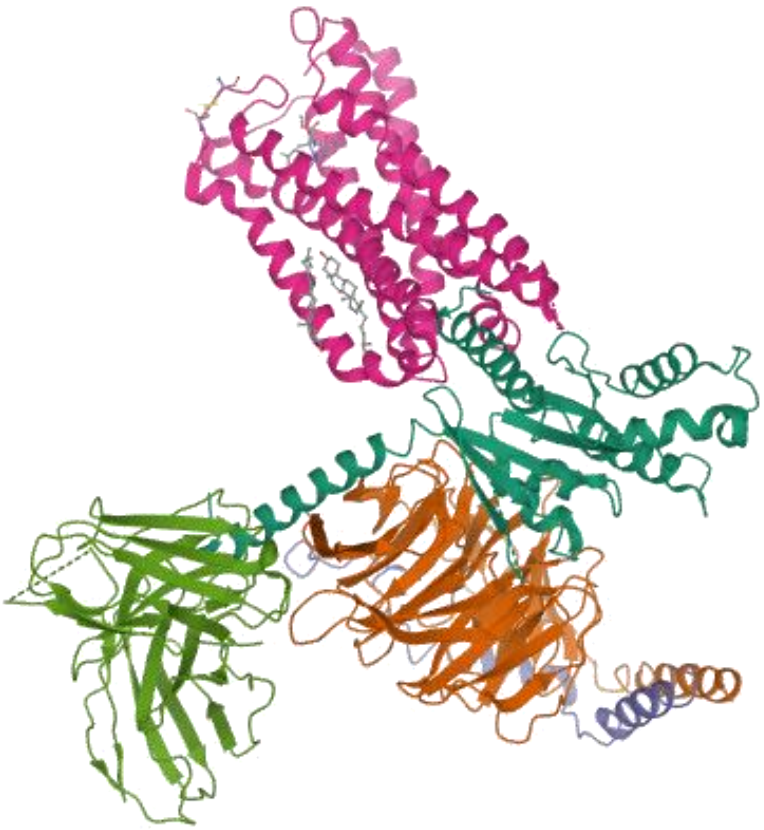


**Figure 1.1:** *Structure of CB1 receptor with G protein complex* Magenta: CB1 receptor Cyan: $G\alpha_i$ subunit Orange: $G\beta_i$ subunit Purple: $G\gamma_i$ subunit Green: scFv16 fragment used to stabilize CB1 receptor[2]

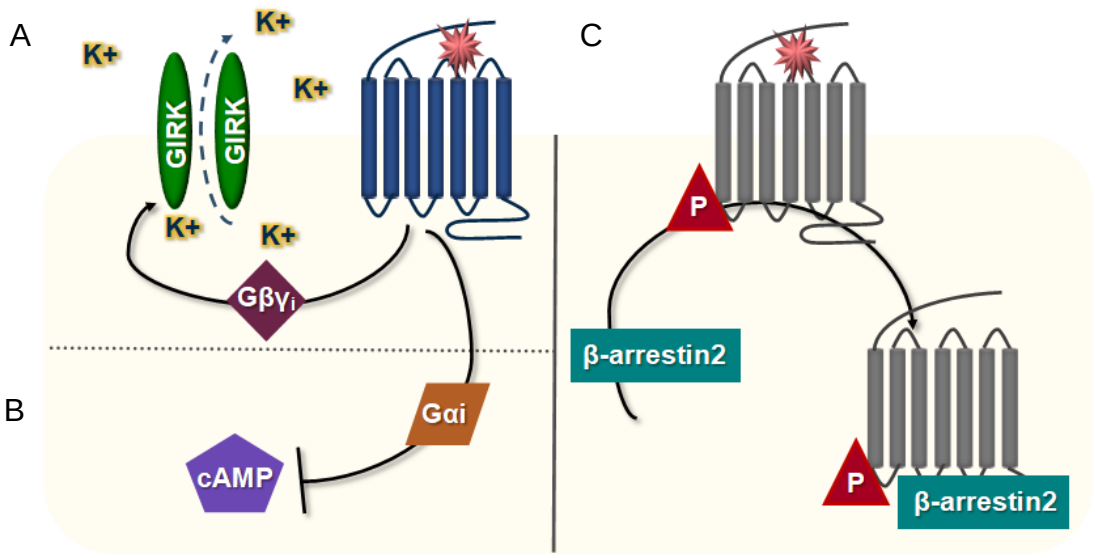
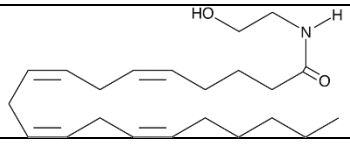
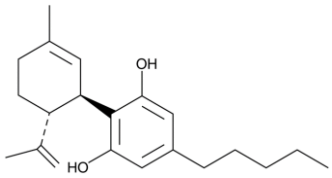
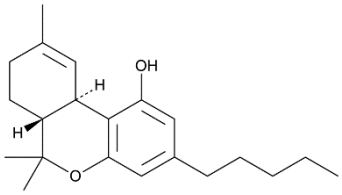
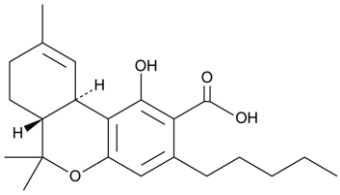
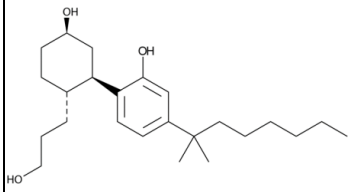
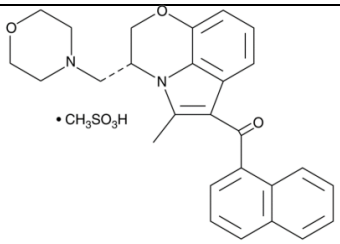



**Figure 1.2:** *CB1 receptor $G_i$ & β- arrestin signaling*. Cannabinoid (✸) bound to the CB1 receptor releases intracellular $G\beta\gamma_i$ from $G\alpha_i$ and recruits β-arrestin2 through receptor phosphorylation (▲). (A) $G\beta\gamma_i$ binds to and activates GIRK channels, causing an efflux of potassium, (B) Gαi inhibits adenylyl cyclase, leading to a decrease in cAMP. (C) β – arrestin2 is recruited to phosphorylated CB1 receptor, causing a decrease in receptor signaling (grey), and followed by receptor removal from the surface

**Table 1.1: *Cannabinoid class and structure***

| Cannabinoid Group | Cannabinoid Class | Cannabinoid | Structure |
|---|---|---|---|
| Endogenous | Eicosanoid | AEA |  |
| Phytocannabinoids | Classical | CBD |  |
| | | THC |  |
| | | THCA-A |  |
| Synthetics | Non-classical | CP 55, 940 |  |
| | Aminoalkylindole | WIN 55,<br>212-2 |  |

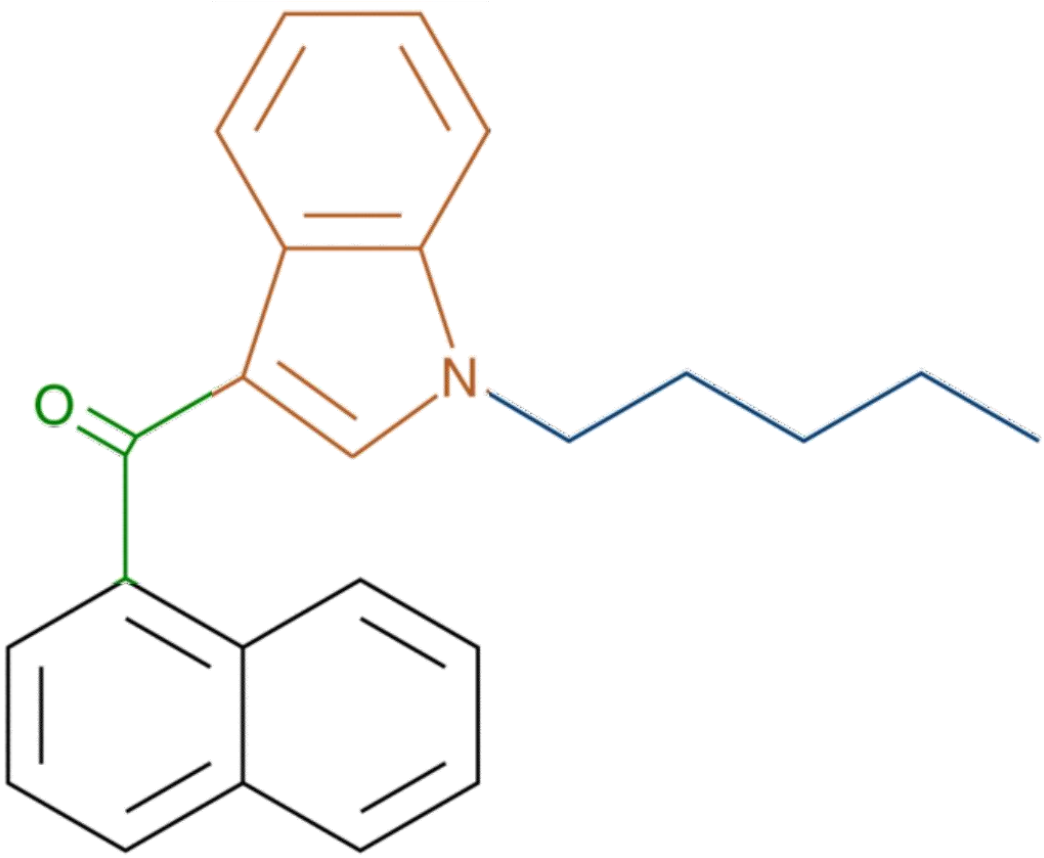
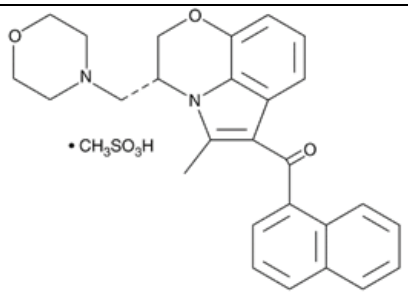
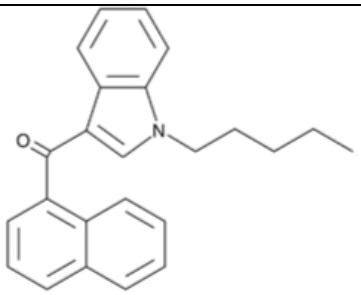
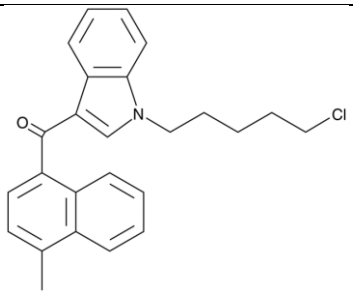
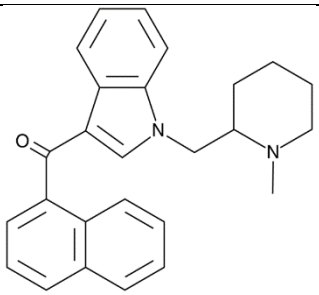
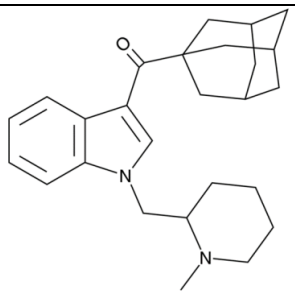
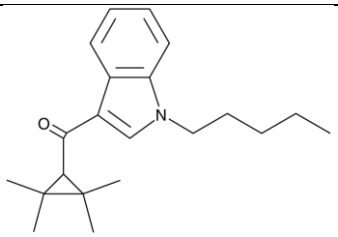
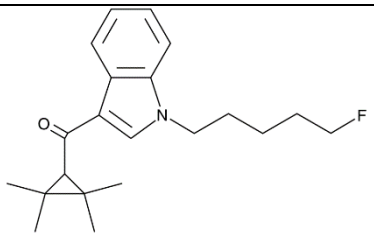
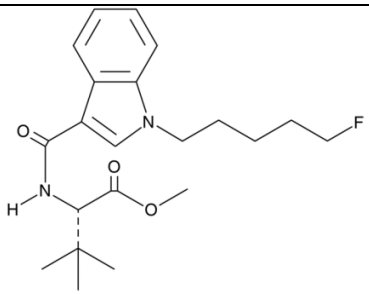
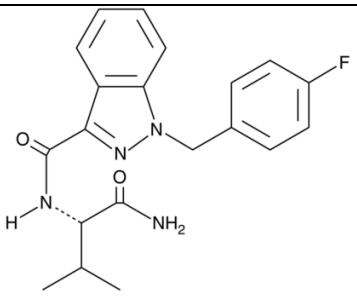
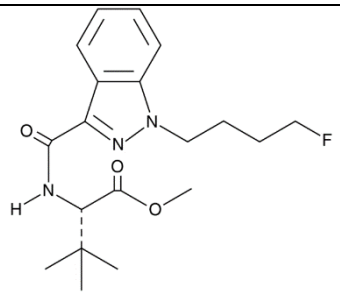
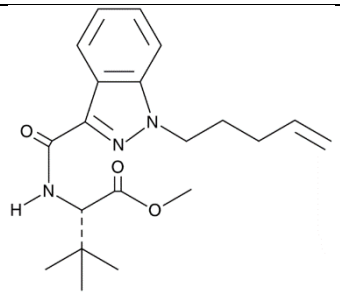



**Figure 1.3:** *Pharmacophore components of JWH-018.* Commonly altered areas on chemical structure that help categorize SCRA. Blue: Tail group Orange: Core Green: Linker Black: Head group Modified from EMCDDA 2017[1]

**Table 1.2: *SCRAs class and structure***

| Aminoalkylindole-derived SCRAs | Cannabinoid | Structure |
|---|---|---|
| Aminoalkylindole Prototype | WIN 55, 212-2 |  |
| Naphthoylindoles | JWH-018 |  |
| | JHW-122 N(-5-Chloropentyl) |  |
| | AM1220 |  |

| | | |
|---|---|---|
| Adamantoyloindole | AM1248 |  |
| Tetramethylcyclopropylindoles | UR-144 |  |
| | XLR-11 |  |
| Indole carboxamide | 5 fluoro-MDMB-PICA |  |
| Indazole carboxamide | AB-FUBINACA |  |

| | | |
|---|---|---|
| | 4 fluoro-MDMB-BUTINACA |  |
| | MDMB-4en-PINACA |  |

## CHAPTER 2: *ESTABLISHING THE CB1 RECEPTOR GIRK CHANNEL ASSAY*


### 2.1 Abstract

The CB1 receptor can regulate neuronal activity through activating GIRK channels. The Walsh laboratory developed a real-time membrane potential fluorescent assay for cannabinoids using pituitary AtT20 cells that endogenously expressed GIRK channels and were stably transfected either with the CB1 receptor using a recombinant lentivirus (AtT20/CB1) or the CB1 receptor tagged with super-ecliptic pHluorin (AtT20/SEPCB1). In whole-cell patch-clamp experiments application of the cannabinoid agonist WIN 55,212-2 to AtT20 cells expressing the CB1 receptor (AtT20/CB1) activated GIRK currents that were blocked by barium. WIN 55,212-2 activation of the GIRK channels was associated with a time- and concentration-dependent (AtT20/CB1: $EC_{50}$ 309 nM, AtT20/SEPCB1: $EC_{50}$ 523nM) hyperpolarization of the membrane potential in the cells when monitored using a fluorescent membrane potential-sensitive dye. WIN 55,212-2 induced fluorescent signal was inhibited by pretreatment of AtT20/CB1 cells with the GIRK channel blocker tertiapin and in both AtT20/CB1 and AtT20/SEPCB1 cells with the CB1 receptor antagonist SR141716. DMSO, the solvent used in cannabinoid stock concentrations, did not elicit a GIRK channel response. Together, this data supports the CB1 receptor, GIRK channel assay, as an effective method for measuring cannabinoid-mediated, GIRK channel signaling.

## 2.2 Introduction

The GIRK channel response is measured in a variety of cell types, with the most recognized being the human embryonic kidney, or HEK-293, cells. There is research suggesting that HEK-293 cells have different G protein expression levels when compared to the CNS; hence, this study's use of the AtT20 cell line.[106] AtT20 cells are an immortalized, mouse pituitary cell-line shown to have neuronal properties and endogenously express the GIRK1/2 subunits.[78, 107]

Cannabinoids can suppress neuronal activity by stimulating GIRK channels.[78] Changes in membrane potential can be measured by using a microplate reader capable of detecting excitation and emission wavelengths and fluorescent, membrane-potential sensitive (MP-sensitive) dye applied to cell cultures.[108-110] The MP-sensitive dye molecules emit a fluorescent signal when expressed in the intracellular space. (Figure 2.1A) Applying a CB1 receptor agonist activates GIRK channels via the $G\beta\gamma_i$ subunit, resulting in the efflux $K^+$ ions. (Figures 2.1B & 2.1C) The efflux of the positive $K^+$ ions cause the MP-sensitive dye molecules to move to the extracellular space where the fluorescent signal is quenched. (Figure 2.1D) The microplate reader records the change in membrane-potential-dependent fluorescence in real-time which is then quantified for analysis.[108, 111, 112]

This study reports the development of a real-time assay for studying cannabinoid-mediated $G\beta\gamma_i$ stimulation using MP-sensitive fluorescent dye. Clonal AtT20 pituitary cells, which endogenously express the GIRK1/2 subunits, were stably transfected with the unlabeled human CB1 receptor (AtT20/CB1) or the

human CB1 receptor tagged with super-ecliptic pHluorin construct (AtT20/SEPCB1) using lentivirus and subsequently studied using the whole-cell arrangement of the patch-clamp technique and a fluorescent plate reader. Application of WIN 55,212-2 to the AtT20/CB1 cells caused a time- and concentration-dependent hyperpolarization of the AtT20 cell membrane potential consistent with GIRK channel activation. The fluorescent signal produced by WIN 55,212-2 was inhibited by pretreating the cells with either the GIRK channel blocker tertiapin or the CB1 receptor antagonist SR141716. Therefore, the AtT20/CB1 cell fluorescent assay will provide a valuable methodology for determining the ability of various cannabinoid ligands to stimulate $G_i$ and activate GIRK channels.

## 2.3 Material and methods

### *AtT20 cell culture and plating*

The AtT20 pituitary cell line was obtained from ATCC (AtT-20/D16y-F2, CRL-1795) and grown in DMEM media with 10 % fetal bovine serum + Pen-Strep. Cells were plated on uncoated glass coverslips (5,000 cells per coverslip) (patch-clamp recording) and in poly-l-lysine-coated wells of black 96-well plates (Corning or Greiner) (30,000 cells per well) (fluorescent measurements). AtT20 cells were stably transfected with lentivirus vectors containing either green fluorescent protein (GFP) or the human cannabinoid type-1 (CB1) receptor (cDNA Resource Center) that were supplied by Dr. Seungjin Shin (Viral Core Facility, University of South Carolina). Cells were stored in an incubator at 37$^o$ C (5 % $O_2$ / 95 % $CO_2$) and used on days 1-3 after plating. Cells expressing GFP were imaged using a Leica

DM IL inverted microscope (Vashaw Scientific) and CoolSNAP EZ camera (Photometrics) as described previously.[109] An additional set of AtT20 cells were stably transduced with a lentivirus containing the SEPCB1 construct (courtesy of Dr. Andrew Irving, University College Dublin) by Dr. Seungjin Shin (Viral Core Facility, University of South Carolina). Cells were stored in an incubator at 37º C (5 % $O_2$ / 95 % $CO_2$) and used on days 1-3 after plating as described previously. [110]

*Drugs and chemicals*

Tertiapin was purchased from Alomone Laboratories (Jerusalem, Israel). Cannabinoids were purchased for Cayman Chemical (Ann Arbor, Michagan). Cannabinoid ligands were dissolved in DMSO at stock concentrations of 10-50mM and diluted to various concentrations in 1 mM KCl buffer solution containing the dye.

*CB1 receptor immunoblot analysis.*

AtT20 cells were harvested in RIPA lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, pH 7.4), and a protease inhibitor cocktail (Pierce Scientific). The cell lysate was pulse sonicated at 2 watts using a model 100 sonic dismembrator (Fisher Scientific) for two periods of 10 s separated by 1 min. The protein content was determined by a Lowry assay. Western blotting was performed by Dr. Gerardo G. Piroli, as described previously, with minor modifications.[113] Briefly, cell lysates were added to the loading buffer and incubated for 5 min at 60°C. Samples were then resolved by SDS/PAGE, transferred to PVDF

membranes and blocked for 1 hr at room temperature (RT) with 5% non-fat dry milk in wash buffer (20 mM Tris pH 7.4 containing 0.05% Tween 20). Membranes were then incubated at 4°C with a CB1 receptor Ab (guinea pig [gp] L15, a generous gift of Dr. Ken Mackie, University of Indiana) in a 1:2000 dilution in 2% non-fat dry milk in wash buffer. After 3 x 5 min washes with wash buffer, membranes were incubated with a secondary Ab (rabbit anti-gp HRP, Thermofisher) for 1 hr at RT, washed 3 x 5 min with wash buffer, developed with Pierce ECL2 and exposed on X-ray films. Membranes were then incubated with 62.5 mM Tris stripping solution (pH 6.8) containing 2% SDS and 0.7% 2-mercapto ethanol for 10 min at 65°C prior to re-probing with an Ab to actin (loading control) (Santa Cruz Biotechnology).

*Fluorescent, membrane-potential sensitive assay*

GIRK channel activation was monitored in the 96-well plates by fluorescently recording the cell membrane potential. [114, 115] and has been described in detail [109]. For the membrane potential measurements, cells were incubated for 30 min in normal buffer solution consisting of; 132 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM dextrose, 5 mM HEPES, pH 7.4 (with NaOH), with a MP-sensitive fluorescent dye (FLIPR Membrane Potential kit RED or BLUE; Molecular Devices). Prior to the fluorescent measurements, the cells were loaded with dye in buffer solution containing 1 mM KCl and incubated for an additional 5 min. Fluorescent signals were recorded using a Synergy2 microplate reader (Biotek) at 28$^{o}$ C [115]. The cannabinoids or control solution (10 or 20 µl) were added to each well (total volume = 110 or 220 µl) at time zero using an injector.

Data points were collected at 5 s intervals over a 250 s sampling period at excitation and emission wavelengths of 520 and 560 nm, respectively.

*Patch-clamp recording.*

The patch-clamp method was used to record the whole-cell, GIRK currents using L/M EPC-7 (Adams & List Associates) and Axopatch 200 (Molecular Devices) amplifiers.[116] Pipettes were made from borosilicate glass capillaries (World Precision Instruments) and had resistances of 2-3 Mohms when filled with an internal solution. All experiments were conducted on isolated, non-coupled cells at room temperature (22-24 $^{o}$C). GIRK currents were measured in external solution consisting of; 95 mM NaCl, 50 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM dextrose, 5 mM HEPES, pH 7.4 (with NaOH). High external $K^+$ was used in order to increase the driving force for $K^+$ movement through the GIRK channel and allow the measurements on inward GIRK currents. The internal solution consisted of; 50 mM KCl, 60 mM $K^+$-Glutamate, 2 mM $MgCl_2$, 1 mM EGTA, 2 mM ATP, 0.1 mM GTP, 10 mM HEPES, pH 7.3 (with KOH). Following the measurement of the cell background current, GIRK channels were activated by the addition of 2-5 μM WIN 55,212-2 using a perfusion system. In each experiment, the GIRK current was defined as the $BaCl_2$-sensitive current [115].

*Data analysis*

The WIN 55, 212-2 concentration versus response curve for the AtT20/CB1 cells was determined by fitting the data to a curve using a three-parameter, non-linear regression (listed below) where the $EC_{50}$ is the concentration producing a 50 % increase of the maximal response ($E_{max}$) and k is the slope factor.

Three-parameter non-linear equation

$$y = \frac{E_{max}}{(1 + \frac{Drug}{EC_{50}})^{k}}$$

The WIN 55, 212-2 concentration versus response curve for the AtT20/SEPCB1 cells was determined by fitting the data to a curve using a four-parameter, non-linear regression (listed below) where the $EC_{50}$ is the concentration producing a 50 % increase of the $y_{max}$ ($E_{max}$). $Y_{min}$ is defined as a minimum fluorescent GIRK channel response. Drug is the concentration, and hillslope is the slope factor.

Four-parameter non-linear equation

$$y = y_{min} + \frac{y_{max} - y_{min}}{1 + (\frac{Drug}{EC_{50}})^{-Hillslope}}$$

## 2.4 Results

*Development of a CB1 receptor MP-sensitive fluorescent assay*

In previous studies, transfection of the AtT20 cells with the CB1 receptor using traditional, liposome-based transfection procedures resulted in only weak cannabinoid-stimulated GIRK currents and fluorescent signals [78, 117]. Therefore, in this study, the ATt20 cells were stably transfected with the CB1 receptor (AtT20/CB1 cells) using a recombinant lentivirus vector. As shown in Figure 2.2A, the transfection of the AtT20 cells with a lentivirus vector containing GFP resulted in fluorescence labeling in over 50 % of the imaged cells (n = 3 cell cultures). The viral infection did not affect the morphology or viability of the cells. Also, the viral-transfected cells stably expressed GFP for several months at a time

with no additional treatment (virus, antibiotic, etc.) required. Immunoblot analysis was carried out to confirm the expression of the CB1 receptor in the AtT20/CB1 cells (Figure 2.2B). An immunoreactive band of approximately 50 kDa, corresponding to the size of the full CB1 receptor, was identified in cell lysates obtained from the AtT20/CB1 cells but not from untransfected, wild-type AtT20 cells (Figure 2.2B). A lower weight molecular weight band (≈ 30 kDa) was also measured in the AtT20/CB1 cells (Figure 2.2B).

The AtT20/CB1 cells were next cultured in 96-well plates and loaded with an MP-sensitive dye in 1 mM KCl buffer solution. The presence of 1 mM extracellular $K^+$ established a gradient for $K^+$ efflux out of the cells during GIRK channel activation. Figure 2.1C plots the MP-sensitive fluorescent dye signal measured over time in the AtT20/CB1 cells. The addition of WIN 55,212-2 to the AtT20/CB1 cells caused a rapid, time-dependent hyperpolarization of the resting membrane potential by allowing $K^+$ efflux through the opened GIRK channels. As anticipated, the application of WIN 55,212 to the wild-type AtT20 cells had no hyperpolarizing effect on the membrane potential (Figure 2.2C).

Whole-cell patch-clamp experiments were performed to confirm that the cannabinoid fluorescent signal resulted specifically from the opening of the GIRK channels. Application of WIN 55,212-2 to the AtT20/CB1 cells resulted in the activation of an inward rectifying $K^+$ current, which was inhibited by 1 mM $BaCl_2$, a GIRK channel blocker (n = 7 cells) (Figure 2.3 A & B). The properties of the cannabinoid-activated Kir current are consistent with GIRK channels previously recorded during somatostatin stimulation.[109, 114, 117]

*Establishing the CB1 receptor-induced GIRK channel assay using AtT20/CB1 cells*

Using the fluorescent assay for measuring cannabinoid signaling, the pharmacological properties of the expressed CB1 receptor were examined. As shown in Figure 2.4B, WIN 55,212 activated the AtT20/CB1 cell fluorescent signal with a half-maximal effective concentration ($EC_{50}$) of 309nM. Maximal stimulation occurred in the presence of a 2-5μM WIN 55, 212-2 (Figure 2.4A). The fluorescent signal was also measured following pretreatment of the cells with either tertiapin, a selective blocker of Kir channels, or the CB1 receptor antagonist SR141716 [118, 119]. As shown in Figures 2.4C and 2.4D, five minutes of exposure of the AtT20/CB1 cells to either tertiapin or SR141716 strongly inhibited the subsequent WIN 55,212-2 fluorescent signal.

It is worth noting the increase in fluorescent signal in Figure 2.2C and 2.3A has been documented in previous research.[108, 117] As state in the MP-Red dye manual (Molecular Devices), this is most likely a result of cells detaching from the bottom of the wells. This issue was later resolved using AtT20/SEPCB1 and MD-Blue dye discussed in the next subsection.

*Confirming CB1 receptor-induced, GIRK channel response using the AtT20/SEPCB1 cell line*

The previous experiment was repeated to confirm that the GIRK channel signal could be reliably measured in AtT20/SEPCB1 through CB1 receptor agonism. A concentration of 10μM WIN 55, 212-2 produced the largest GIRK channel signal in AtT20/SEPCB1 cells using the MP-BLUE dye. (Figure 2.5A) The WIN 55, 212-2 mediated, GIRK channel response had an $EC_{50}$ of 523 nM.

(Figure2.5B) The pretreatment of 1μM SR141716 inhibited the WIN 55, 212-2 induced, GIRK channel. (Figure2.5C) A concentration representing the largest volume of DMSO exposed to AtT20/SEPCB1 cells in the following chapters was tested to screen for off-target effects. DMSO (0.1%) did not elicit a GIRK channel response. (Figure 2.5D)

## 2.5 Discussion

This study describes a novel methodology for examining the action of CB1 ligands on $G_i$ signaling. This assay is based on the use of a fluorescent MP-sensitive dye to measure cell membrane hyperpolarization during $G_i$-mediated activation of GIRK1/2 channels in pituitary AtT20 cells. [114, 115] Previous studies have demonstrated that the CB1 receptor can be expressed in the AtT20 cells using liposome-based transfection procedures and used to measure GIRK channels in the presence of WIN 55,212-2. [78] However, both the reported WIN 55,212-2 activated GIRK currents and MP-sensitive fluorescent signals were small compared with those measured during stimulation of the endogenous SSTR with somatostatin [114, 115, 117]. To overcome this problem, the CB1 receptor was expressed using a recombinant lentiviral vector. With this approach, sizable inward rectifier $K^+$ currents (mean = 9 pA/pF at -100 mV) and fluorescent signals were measured in the AtT20/CB1 cells during activation with WIN 55,212-2 (Figures 2.2 & 2.3). The inhibition of the cannabinoid fluorescent signal by the GIRK channel blocker, tertiapin, strongly suggests that the hyperpolarization caused by WIN 55,212-2 results from the opening of GIRK channels.

WIN 55,212-2 is a synthetic aminoalkylindole cannabinoid that has been widely utilized in the study of the CB1 receptor. [53, 66, 88, 94] Van der Lee et al. (2009) carried out several cellular assays, including β-arrestin recruitment and cAMP accumulation assays with the CB1 receptor expressed in heterologous cell lines.[120] The $EC_{50}$ for WIN 55,212-2 in these assays ranged from 6 to 213 nM with maximum cannabinoid effects occurring at a concentration of 1 μM and above. More recently, cannabinoid signaling was studied in a mouse cell culture model of striatal medium spiny projection neurons that endogenously express CB1 receptors.[121] Bioluminescence resonance energy transfer (BRET) was utilized to quantify direct interactions of the CB1 receptor and βarr2 following ligand binding. Using this system, the BRET $EC_{50}$ for WIN 55,212-2 was 650 nM with maximal effects requiring concentrations of WIN 55,212-2 between 1 and 10 μM. [121] Thus, the $EC_{50}$ and maximal effects of WIN 55,212-2 observed in both the establishing and modified GIRK channel assays are consistent with those reported using other methodologies. Also, cannabinoid antagonist SR141716 sufficiently inhibited the WIN 55,212-2-induced GIRK channel response in both assays. (Figure 2.3).

In summary, a MP-sensitive, fluorescent GIRK channel assay was developed to measure $G_i$- activation of GIRK channels in AtT20 cells expressing the CB1 receptor. Biased receptor agonists, drugs that preferentially activate G protein signaling or β-arrestin recruitment, have become a primary focus of therapeutic research efforts.[122] Recently, the compound TRV130 was identified as a μ-opioid agonist that displays an efficacy for $G_i$ stimulation equal to that of

morphine, but without promoting morphine-mediated β-arrestin recruitment and μ-opioid receptor internalization.[64] In the mouse brain, different CB1 receptor agonists act in a biased manner to selectively activate different inhibitory and non-inhibitory G protein subunits.[89] Therefore, the CB1 receptor, GIRK channel fluorescent assay may be useful for identifying CB1 receptor agonists that are biased for $G_i$ signaling.

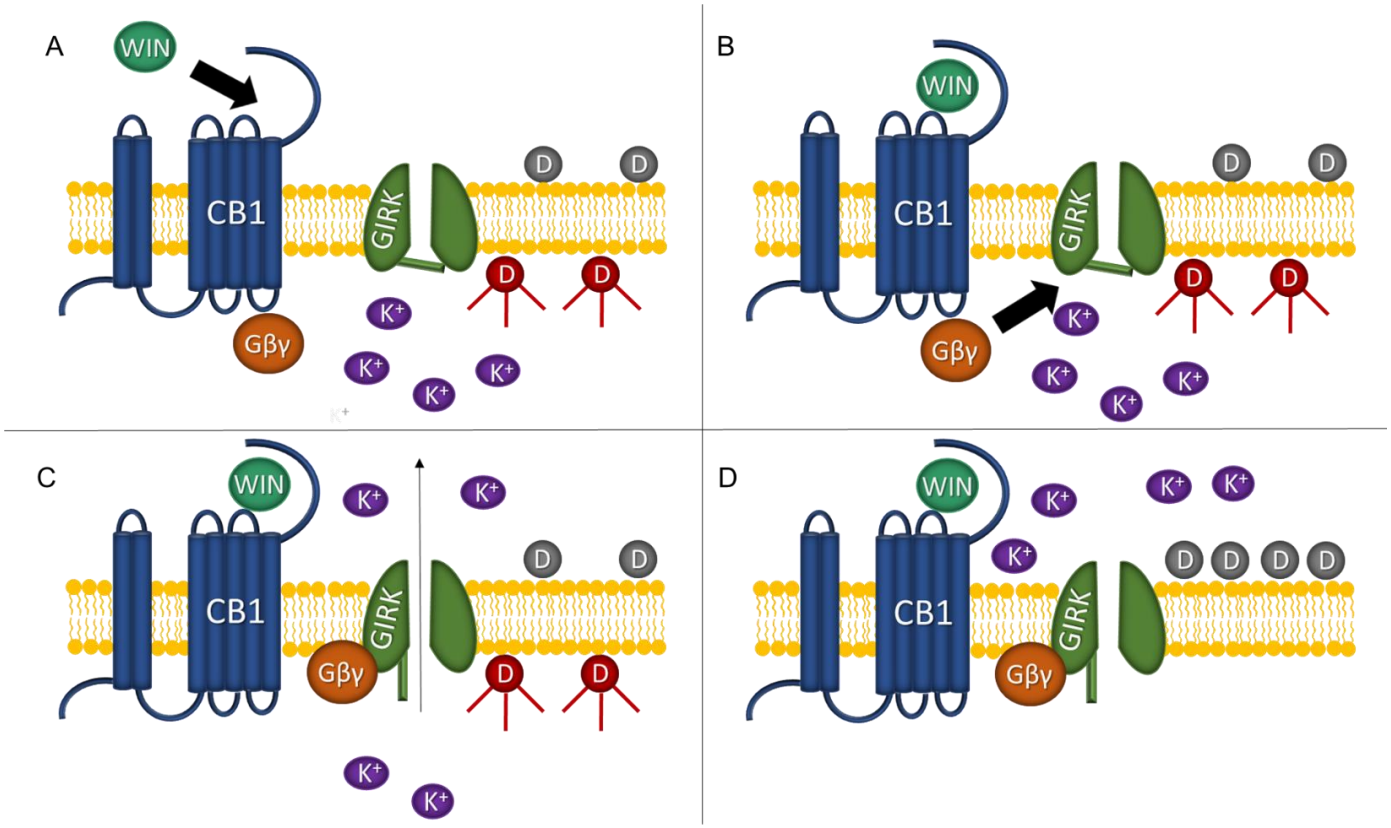



**Figure 2.1.** *Schematic of fluorescent, membrane potential GIRK channel assay.*(A) During AtT20/CB1 or AtT20/SEPCB1 cell resting state, Molecular Devices dye D is distributed intracellularly and produces a fluorescent signal. (B)*:* Binding of CB1 agonist, WIN 55, 212 (WIN) results in the intracellular, Gβγ protein subunit to disassociate and bind to GIRK, thus activating the channel. (C) Activation of GIRK channels results in an efflux of potassium ions ($K^+$), thus causing resting membrane potential to become more negative, hyperpolarizing the cell. (D) The shift towards a more negative membrane potential causes the dye to move to the extracellular space, thus quenching the fluorescent signal.

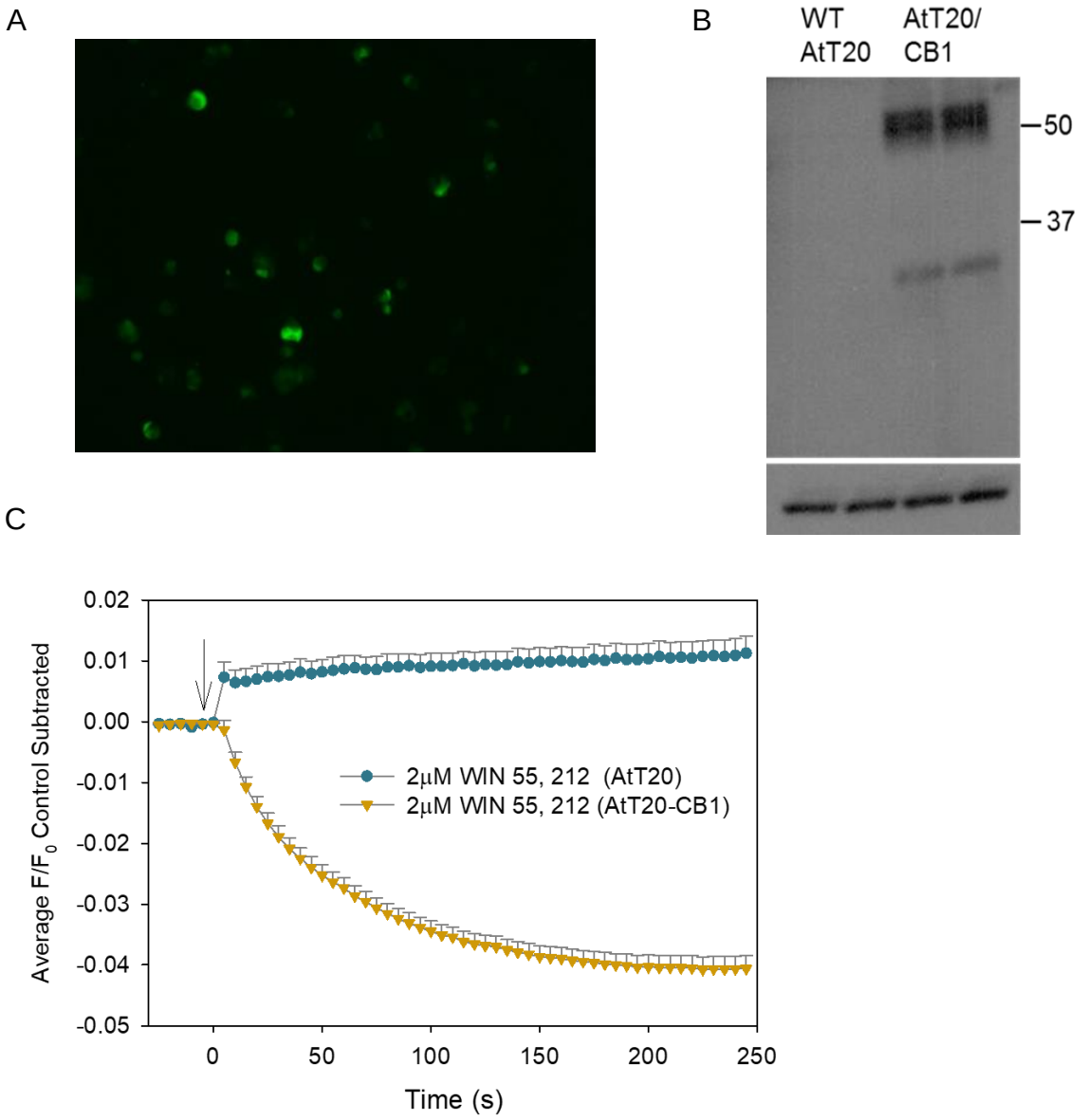



**Figure 2.2:** *Stable expression of GFP and the CB1 receptor in AtT20 cells using lentivirus vectors.* A: Fluorescent image of AtT20 cells infected with lentivirus expressing did not impact cell viability or cause abnormal cellular changes. B: Immunoblots for the CB1 receptor in AtT20 cells infected with lentivirus expressing the CB1 receptor (AtT20/CB1 cells) or non-transfected wild-type cells (wt AtT20 cells). Immunoblots obtained with an Ab to actin (bottom panel) demonstrated equal protein loading in the lanes. C: MP-sensitive dye fluorescent intensities obtained n AtT20 cells in the presence of absence of WIN 55, 212-2 (2μM) (WIN). The ratio of the fluorescent intensity ($F/F_0$) was calculated by dividing the signal measured in the presence (F) of WIN by the baseline signal measured before ($F_0$) addition of WIN. Each point represents the mean ± S.E.M obtained in 87 wells containing AtT20/CB1 cells and 12 wells containing wt AtT20 cells. WIN was added at time zero (↓)

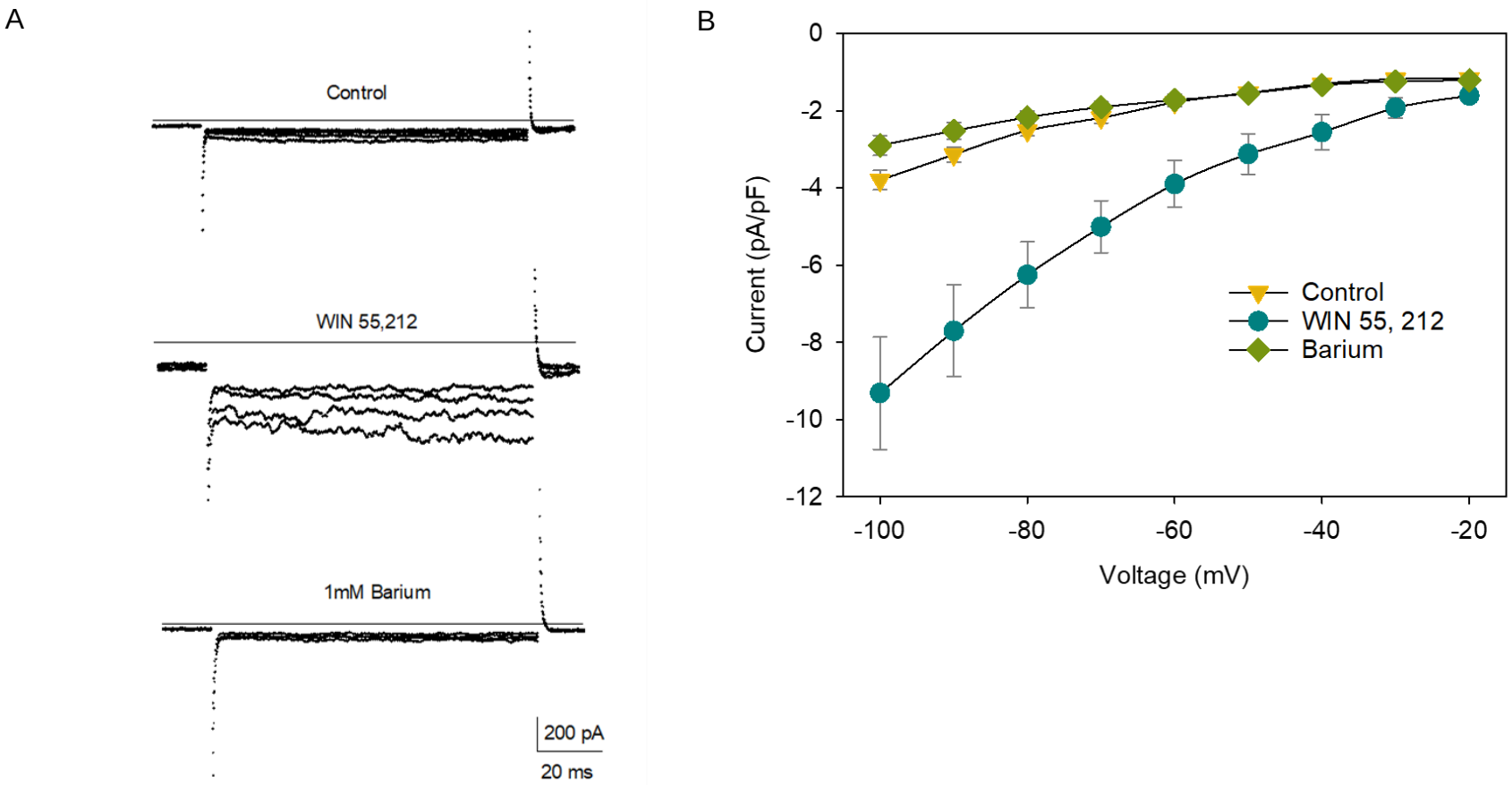



**Figure 2.3:** *CB1 receptor stimulation activates GIRK channels in AtT20/CB1 cells.* A: Representative AtT20/CB1 whole-cell current traces obtained during voltage steps applied from a holding potential of −40 mV to −100, −90, −80, & −70 mV before (control) and after the addition of 5 μM WIN 55,212-1. B: Current versus voltage relationship illustrating WIN 55,212-1 activation of an inward rectifying K+ current that was blocked by Barium chloride. Each point represents the mean ± S.E.M obtained in seven AtT20/CB1 cells.

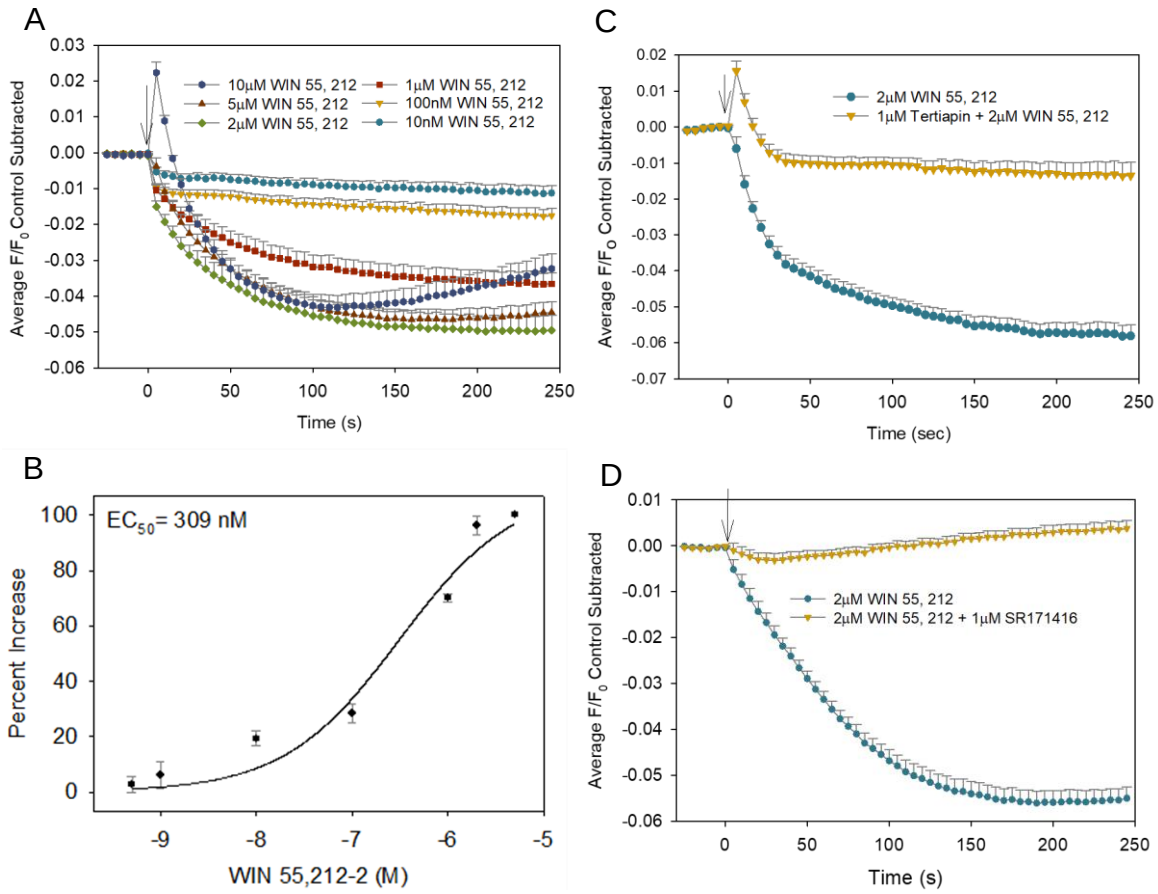



**Figure 2.4:** *WIN 55,212-2 stimulates the GIRK channel fluorescent signal in a concentration-dependent manner.* (A) Changes in the MP-sensitive dye (MP-RED) fluorescent signal following injection of WIN 55,212-2 in wells containing the AtT20/CB1 cells. Each point represents the mean ± S.E.M obtained in 9–12 wells. (B) Concentration versus response curve for the WIN-sensitive fluorescent signal. The concentration-response curve was obtained by fitting the maximum responses from A to a three-parameter non-linear regression, where the $EC_{50}$ is the concentration of WIN producing a 50% increase in the maximal response.(C) Cells pretreated with GIRK channel blocker, tertiapin, inhibited GIRK signal. (D) Cells pretreated with CB1 receptor antagonist, SR141716, inhibited GIRK channel signal. WIN was added at time zero (↓).

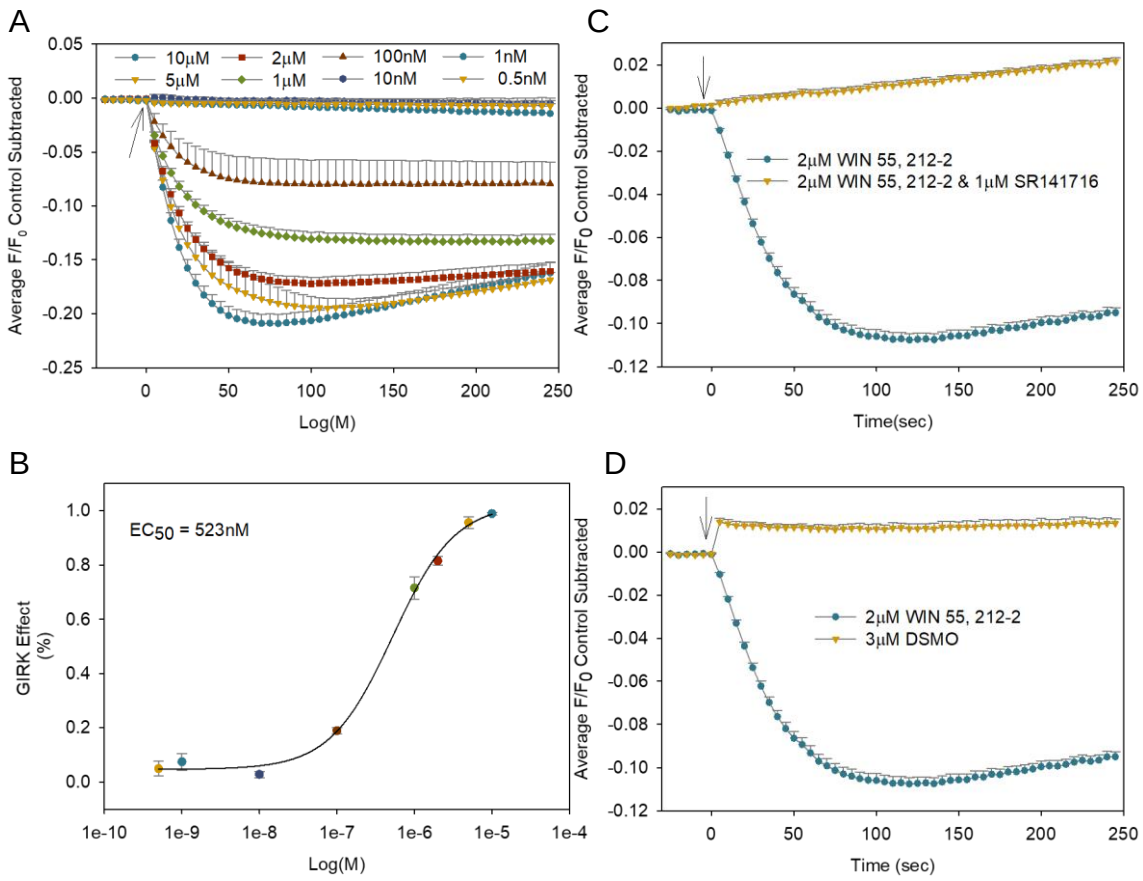



**Figure 2.5** *GIRK channel assay using MP-Blue dye and AtT20/SEPCB1 cells***.** (A) Changes in the MP-sensitive dye (MP-BLUE) fluorescent signal following injection of WIN 55,212-2 in wells containing the AtT20/SEPCB1 cells. Each point represents the mean ± S.E.M obtained in 4–8 wells. (B) Concentration versus response curve for the WIN-sensitive fluorescent signal. The concentration-response curve was obtained by fitting the maximum responses from A to a four-parameter non-linear regression, where the $EC_{50}$ is the concentration of WIN producing a 50% increase in the maximal response.(C) Cells pretreated with CB1 receptor antagonist, SR141716, inhibited GIRK channel signal. (D) DMSO does not induce a GIRK channel signal. Error bars are presented as ± S.E.M. WIN 55-212-2 or DMSO was added at time zero (↓)

## CHAPTER 3: *ANALYSIS OF GIRK CHANNEL RESPONSE TO CANNABINOIDS REPRESENTATIVE OF THE FOUR CLASSES*

### 3.1 Abstract

Cannabinoids uniquely bind to the CB1 receptor, potentially mediating the int. CB1 receptor agonists precipitate the release of the $G\beta\gamma_i$ subunit, which then binds to and activates GIRK channels. This study investigates GIRK channel responses to representatives from the following cannabinoid classes: eicosanoid, classical, non-classical, and aminoalkylindole. Using cultured AtT20/SEPCB1 cells, GIRK channel assays were performed with the following cannabinoids: anandamide (AEA), CP 55, 940, $\Delta^9$-tetrahydrocannabinol (THC), $\Delta^9$-tetrahydrocannabinolic A (THCA-A), cannabidiol (CBD), and WIN 55, 212-2. Also, cAMP levels were recorded for AEA, CP 55, 940, THC, and WIN 55, 212-2 using AtT20/CB1 cells. All of the cannabinoids were effective at suppressing cAMP production. When measuring the GIRK channel response, the cannabinoids displayed a rank order potency of CP 55, 940 > WIN 55, 212-2 > AEA > THC. Phytocannabinoids, THCA-A and CBD, were excluded from further analysis because both failed to stimulate a GIRK channel response. $E_{max}$ values were determined by normalizing the cannabinoids peak GIRK channel signal to WIN 55, 212-2. The only comparison to not reach significance was CP 55, 940 vs. WIN 55,

212-2. This study demonstrates that synthetic cannabinoids elicit a stronger GIRK channel response, which could potentially be due to CB1 receptor binding.

## 3.2 Introduction

While cannabinoids are commonly defined by their source (i.e., plants, body, or laboratory), the chemical structure is an equally important characteristic used to categorize the diverse range of compounds.[88] The eicosanoid, AEA, is a unique neurotransmitter as it is produced as needed and targets presynaptic CB1 receptors where it functions to decrease neurotransmitter release.[21] Recent changes to legal policies regarding marijuana have highlighted many classical cannabinoids, including THC, CBD, and THCA.[123] CP 55, 940 is a non-classical cannabinoid that lacks the pyran ring seen in classical cannabinoids.[104] Initially, Pfizer developed CP 55, 940 in 1974 as a non-opiate analgesic; however, it has since become a commonly used tool in cannabinoid studies.[124] WIN 55, 212-2 was developed by Sterling Research Institute as a tool to study CB1 receptor binding and is the prototype cannabinoid in the aminoalkylindole class. Like CP 55, 940, WIN 55, 212-2 is a well-known cannabinoid in cannabinoid research. [124-126]

CB1 receptor agonists can stimulate different G protein signaling pathways and β-arrestin recruitment and also vary in the magnitude of stimulation for these signaling pathways.[53, 121, 127] WIN 55, 212-2 and AEA are considered full CB1 receptor agonists. THC is partial agonist due to being less effective initiating $G\alpha_i$ signaling when compared to WIN 55, 212-2 and AEA.[53] Despite both being full agonists at the CB1 receptor, WIN 55, 212-2 and CP 55, 940 had plasmon-

waveguide resonance (PWR) shifts in opposite directions, which suggested that they had unique CB1 receptor active confirmations.[128] This was later supported by Hua et al. (2016) that demonstrated that WIN 55, 212-2 and CP 55, 940 had unique CB1 receptor binding characteristics.[44] In the presence of PTX, many cannabinoids can stimulate $G_s$.[90] Specifically, CP 55, 950 can recruit $G_s$ signaling and is more effective at recruiting $G_s$ over WIN 55, 212-2.[121, 129] Since the $G_s$ signaling pathway opposes the $G_i$ signaling pathway; therefore, downstream effects of active Gs signaling would attenuate the $G_i$-dependent intracellular responses. In the context of these experiments, more $G_s$ signaling could proportionately suppress the GIRK channel response by counteracting the decrease in membrane potential.

These sets of experiments aimed to establish an outline of CB1 receptor-induced, GIRK channel responses to cannabinoid compounds selected from the four major structural classes: eicosanoid, classical, non-classical, and aminoalkylindole. These results highlighted potential differences in $G\beta\gamma_i$ subunit signaling, which served as a resource in delineating the results in the following chapter.

## 3.3 Materials and methods

*Cell culture and assay*

AtT20/SEPCB1 cells were cultured in 96-well plates (Greiner), as previously discussed in chapter 2. AtT20/CB1 cells were cultured in clear, 6-well plates and stored in an incubator at 37º C (5 % $O_2$ / 95 % $CO_2$) and then used for cAMP measurements 3 to 4 days later.

*cAMP assay*

Forskolin is a compound used in experiments to increase cellular cAMP levels. 3-isobutyl-1-methylxanthine (IBMX) is added to inhibit phosphodiesterase degradation of cAMP to potentiate forskolin-induced cAMP production.[130, 131] Using a cAMP enzyme-linked immunosorbent assay (Direct cAMP ELISA kit, Enzo), AtT20/CB1 cells were first exposed to a cannabinoid, then forskolin and IBMX. cAMP levels were measured in the following conditions: 1) Control 2) cAMP generation (forskolin & IBMX) 3) CB1R stimulated (Cannabinoid + Forskolin & IBMX). Absorbance was read on a Synergy2 microplate reader (Biotek). N values in this assay represent the number of wells tested.

*Drugs and chemicals*

Arachidonoyl ethanolamide (Anandamide/AEA), CP 55, 212, cannabidiol (CBD), $\Delta^9$-tetrahydrocannabinol (THC), $\Delta^9$-Tetrahydrocannabinolic acid A (THCA-A), and WIN 55, 212-2 were all purchased from Cayman Chemicals (Ann Arbor, Michigan). CBD and THC were purchased with the Walsh Laboratory DEA license.

Cannabinoids were dissolved in DMSO at stock concentrations of 40mM - 50mM. AEA was purchased pre-diluted in alcohol at 145mM. All cannabinoids were diluted to working concentrations in 1 mM KCl buffer solution containing MD-BLUE dye (Molecular Devices).

*Data analysis*

Concentration curves were determined using four-parameter, non-linear regression analysis where $EC_{50}$ is the concentration producing a 50 % increase in the maximal response $y_{max}$ ($E_{max}$). (listed below) $Y_{min}$ is defined as a minimum

fluorescent GIRK channel response. Drug is the concentration, and hillslope is the slope factor.

Relative $E_{max}$ values were determined by normalizing each cannabinoid maximal GIRK channel response to the maximal GIRK channel response of WIN 55, 212-2 (10μM). "n" represents the number of wells in which that cannabinoid concentration was tested.

Four-parameter Equation

$$y = y_{min} + \frac{y_{max} - y_{min}}{1 + (\frac{Drug}{EC_{50}})^{-Hillslope}}$$

*Statistical analysis*

Statistical analysis of maximal GIRK channel response was completed using one-way, multiple measures ANOVA. Significance was set at $p < .05$. Post-hoc analysis was completed using Holms-Ŝídák. All data analyses were performed using Sigmaplot 14.0.

3.4 Results

All cannabinoids tested suppressed cAMP in the presence of forskolin and IMBX, which is consistent with previous studies.[78] (Figure 3.1) It is worth noting the cAMP levels were measured using AtT20/CB1 cells and were recorded prior to the GIRK channel assays. Later cAMP experiments were inconsistent and were further complicated by problems with the AtT20/CB1 cells; however, the reported results align with previous studies and are presented here to demonstrate $G\alpha_i$ signaling.[78, 129]

Maximum GIRK channel responses for THC, THCA-A, and CBD were compared to 2μM WIN 55, 212-2. (Figure 3.2 A-D) The concentration of WIN 55,

212-2 was set at 2μM, a reference concentration determined by previous experiments performed in the Walsh laboratory.[111] CBD did not activate GIRK channels at any concentration, whereas the THCA-A GIRK channel response was marginal. (THCA-A: 1nM - 1μM, CBD: 1nM - 10μM) (Figure 3.2E).

A dose-dependent, GIRK channel response was recorded for the following cannabinoids: AEA (n = 4), CP 55 940 (n = 7), THC (n = 9), and WIN 55, 212-2 (n = 5). (Figure 3.3 A-E) An $EC_{50}$ figure displaying WIN 55, 212-2, THC, AEA, and CP 55, 940 was created to demonstrate the differences in cannabinoid potencies better. (Figure 3.3 F). $E_{max}$ curves generated by normalizing the peak GIRK channel response for the cannabinoids to the maximum WIN 55, 212-2 concentration (10μM). The cannabinoid curves in the $E_{max}$ comparison graph was generated by running a four-parameter global regression analysis with minimum values shared.[132] (Figure 3.3F).

The rank order potency was CP 55, 940 > WIN 55, 212-2 > AEA > THC > THCA-A ≈ CBD. WIN 55, 212-2 produced the maximum GIRK channel response at a concentration of 10μM. Maximal AEA and THC GIRK channel responses were significantly lower when compared to WIN 55, 212-2. CP 55, 940 produced a maximal GIRK channel response slightly less, but not significantly different, than WIN 55, 212-2. (Table 3.1)

## 3.5 Discussion

The cannabinoids in this study exhibited differential GIRK channel responses. The synthetic cannabinoids, WIN 55, 212-2, and CP 55, 940, were the most efficacious at eliciting a GIRK channel response. The absence of a CBD-

induced, GIRK channel signal suggests CBD is not a CB1 receptor agonist. Research suggests that CBD does not directly activate the CB1 receptor; instead, evidence supports CBD as a negative allosteric modulator of CB1 effective for regulating CB1 receptor agonist effects.[51, 133] THCA-A exhibited a slight GIRK channel signal; however, this is likely THC. THCA-A undergoes decarboxylation into THC, and previous studies report THC contamination as a result of THCA-A instability.[123]

The results of this study are reflective of CB1 receptor binding and signaling across the four primary cannabinoid classes. In this study, the THC-induced, GIRK channel response is consistent with CB1 receptor partial agonism.[88] AEA was less potent than THC, suggestive of less $G\beta\gamma_i$ subunit signaling; however, it was significantly more effective than THC at stimulating the GIRK channel response. Previous research has reported AEA as having a greater $G\beta\gamma_i$ signaling-dependent response compared to THC.[53, 94, 128] This could account for the more effective GIRK channel response for AEA, despite the lower potency.

AEA was significantly less effective at stimulating a GIRK channel response in comparison to CP 55, 940, and WIN 55, 212-2. In a study by Laprairie et al. (2014), AEA was preferential towards the $G_q$ signaling pathway over THC, WIN 55, 212-2, and CP 55, 940.[121] The reduced GIRK channel response could be attributed to $G_q$ -related increases intracellular $Ca^{2+}$ levels causing an increase in the membrane potential in contrast to GIRK channel activity.[134] While CP 55, 940 was approximately 4x more potent that WIN 55, 212-2, it was not significantly more effective at stimulating a GIRK response. βarr2 recruitment modulates

agonist-induced, CB1 receptor internalization and desensitization.[135] In a study by Ford et al. (2017), βarr2 was actively recruited by CP 55, 940 [136] The rapid desensitization would extinguish the GIRK channel signal; therefore, reducing the effectiveness of CP 55, 940.

In conclusion, the analysis of the cannabinoids revealed that synthetic cannabinoids elicited a more significant GIRK channel response compared to phytocannabinoids and AEA. Of interest, CP 55, 940 was more potent than WIN 55, 212-2, but equally as effective in activating the GIRK channel response. Observing these results together suggests that the cannabinoid chemical structure could modulate the GIRK channel response. Investigation of synthetic cannabinoid mediated, GIRK channel response could provide further insight into the relationship between cannabinoid structure and cellular response.

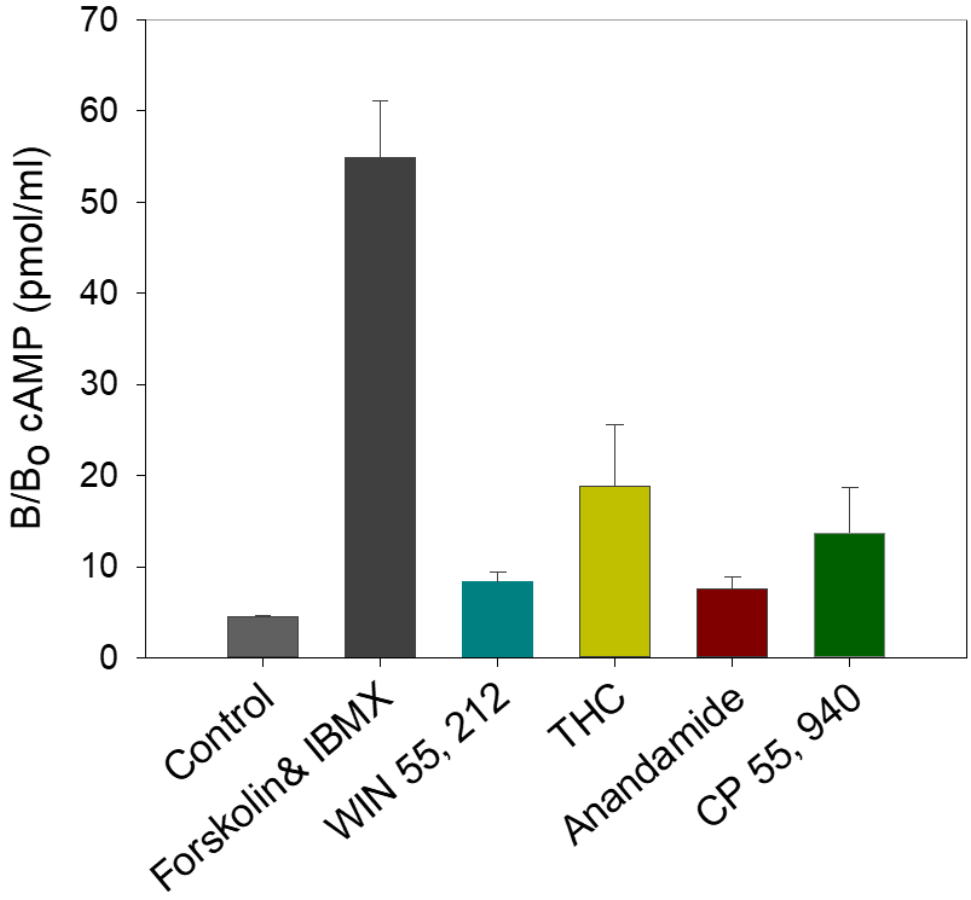



**Figure 3.1***: cAMP levels across cannabinoids***.** WIN 55, 212 and AEA suppress cAMP more effectively than CP 55, 940 suggestive of potential Gi signaling differences. Error bars represent standard error of mean. Control (*n = 24*) Forskolin & IBMX (*n = 24*), 2μM WIN 55, 212 (*n = 24*) 10μM THC (*n = 6*) 10μM AEA (*n = 6*) 1μM CP 55, 940 (*n = 6*)

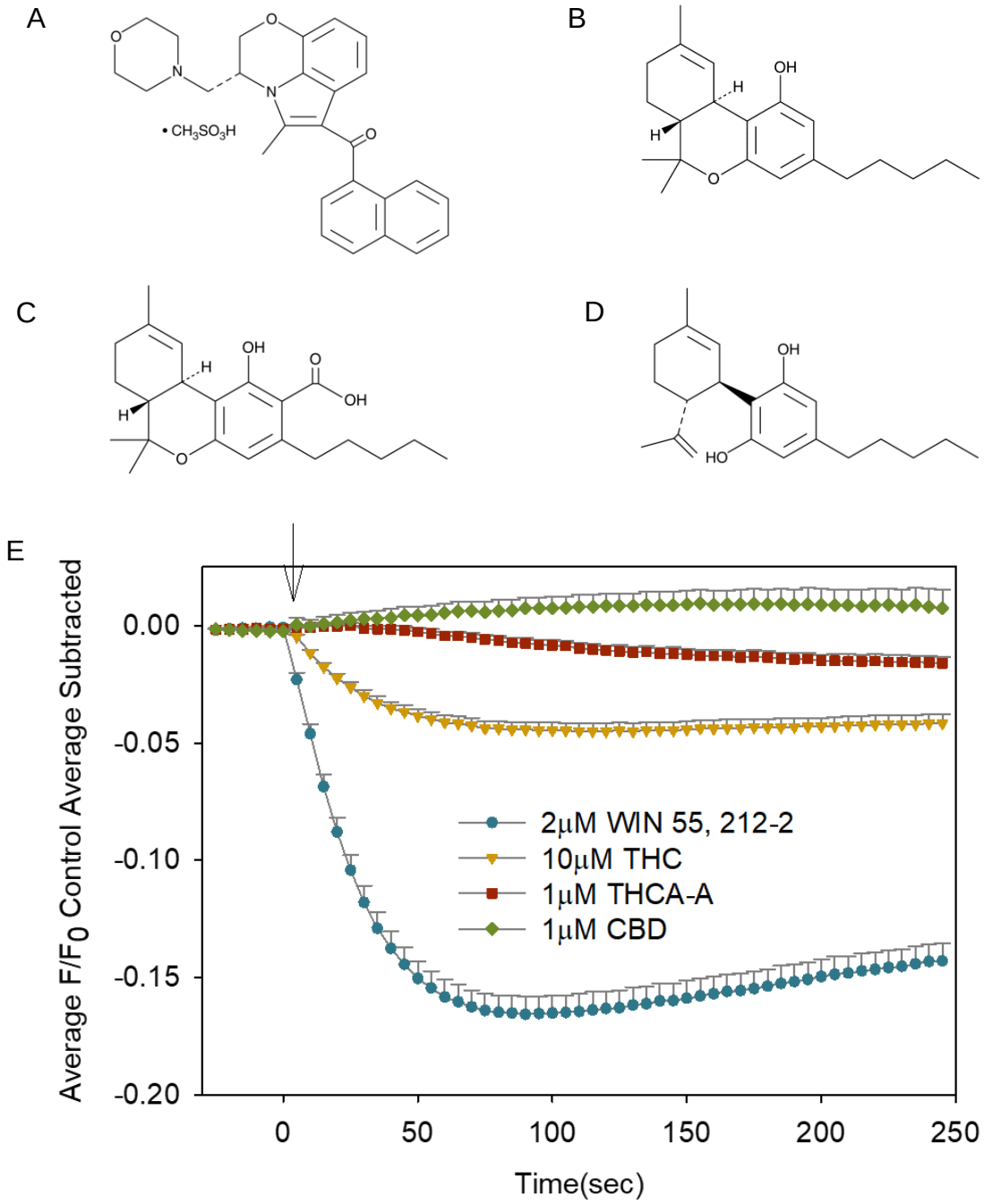



**Figure 3.2:** *Non-psychoactive, classical cannabinoids do not induce GIRK response.* Stronger GIRK channel response with structurally unique aminoalkylindole, WIN 55, 212-2, than psychoactive classical cannabinoid, THC. A) WIN 55, 212-2 B) THC C) THCA-A D) CBD E) GIRK channel responses comparing classical non-psychoactive and psychoactive cannabinoids to an aminoalkylindole Error bars represent standard error of mean.↓ Indicates drug application THC (*n* = *9*), CBD (*n* = *6*), THCA-A (*n* = *5*), WIN 55, 212-2 (*n* = *6*)

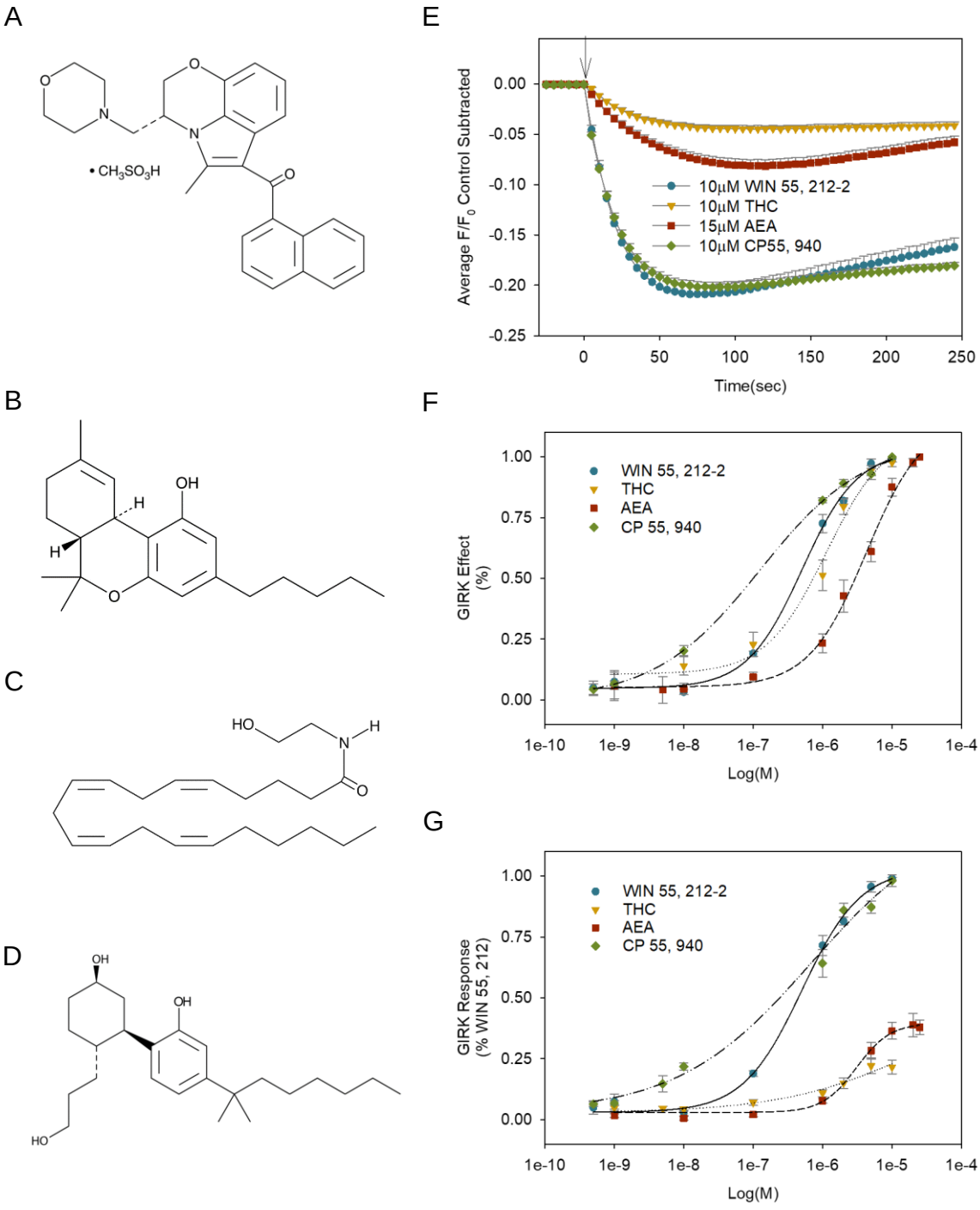



**Figure 3.3:** *GIRK channel responses for general cannabinoids and cannabinoid structures.* (A) WIN 55, 212-2 (B) THC (C) AEA (D) CP 55, 940 (E) Comparison of max GIRK channel responses. (F) Comparison of $EC_{50}$ demonstrating cannabinoid potency G) Comparison of cannabinoid $E_{max}$ relative to WIN 55, 212-2 Error bars represent ±S.E.M.↓ Indicates application of WIN 55, 212-2 (*n = 5*), THC (*n = 9*), AEA (*n = 4*), CP 55, 940 (*n = 6*)

**Table 3.1:** ***Potency and efficacy of four general cannabinoids***

| ***Class*** | ***Cannabinoid*** | ***$EC_{50}$ (nM) ± S.E.M*** | ***$E_{max}$ (%) ± S.E.M.*** |
|---|---|---|---|
| **Aminoalkylindole** | WIN 55, 212-2 | 522.9 ± 68.0 | Reference |
| **Eicosanoid** | AEA | 4252.0 ± 801 | 31.2 ± 4.27* |
| **Classical** | THC | 1140.7 ± 139 | 21.6 ± 2.78* |
| **Non-classical** | CP 55, 940 | 124.9 ± 41.2 | 98.3 ± 2.39 |

* $p < 0.001$ compared to WIN 55, 212-2

# CHAPTER 4: *GIRK CHANNEL RESPONSES TO SYNTHETIC CANNABINOIDS*

## 4.1 Abstract

The discovery of new synthetic cannabinoid receptor agonists (SCRAs) in illicit products marketed as alternatives to marijuana continues today. The intake of these substances adversely affects human health. Currently, there is no treatment for SCRA overdose. Illegal manufacturers will produce SCRAs with increased cannabinoid-type 1 (CB1) receptor potency and efficacy due to alterations made to the chemical structure. The $G\beta\gamma_i$ subunit released from the CB1 receptor G protein complex triggers a G protein-gated, inward-rectifier potassium (GIRK) channel response. In this study, the GIRK channel responses of 11 SCRAs were measured using AtT20/SEPCB1 cells and the GIRK channel fluorescent assay described in chapter 2. Potency was determined to be 5-fluoro MDMB-PICA > 4-fluoro MDMB-BUTINACA, AB-FUBINACA > MDMB-4en-PINACA > JWH-018 > AM1220 > XLR-11 > JWH-122 N-(5-chloropentyl) > WIN 55, 212-2 > UR-144 > AM1248. These experiments demonstrate that SCRA indole or indazole carboxamides increase GIRK channel potency. Additionally, the terminal fluorination of XLR-11 increases the GIRK channel response compared to parent compound, UR-144. This study demonstrates the effects of SCRA

structure on GIRK channel potency and efficacy, which can be applicable to future studies investigating how CB1 efficacy can be regulated by intracellular signaling.

## 4.2 Introduction

SCRAs emerged on the market in the early 2000s as a legal alternative to marijuana that would not appear on standard drug tests. Like THC, SCRAs bind to the cannabinoid-type 1 (CB1) receptor; however, SCRAs are known to induce psychosis, kidney failure, stroke, and even death.[11, 39, 137] SCRA toxicity is hypothesized to be a result of higher CB1 receptor potency and efficacy. In animal models, SCRAs administration enhance hypothermia onset and duration when compared to THC, reflective of increased SCRA efficacy.[138-140]

JWH-018 was one of the first SCRAs to be identified on the illicit drug market.[13] Its increased affinity and efficacy at the CB1 receptor are attributed to the replacement of the morpholino group on WIN 55, 212-2 with the C3 pentyl side chain of THC.[103, 141, 142] Clandestine manufacturers mostly produce SCRAs with an indole or indazole core substitution because this increases the cannabinoid potency at the CB1 receptor. These SCRAs are derivatives of WIN 55, 212-2, the prototype aminoalkylindole with an indole core and an high affinity for the CB1 receptor.[97, 143]

UR-144 is a tetramethylcyclopropylindole developed by Abbott laboratories and, in 2012, identified in SCRA products purchased online.[144-146] The terminal fluorination of UR-144 resulted in the compound, XLR-11, which displayed increased potency at the CB1 receptor.[102] XLR-11 is one of the SCRAs identified

in reports of fatal overdose, is established to mediate cell apoptosis, and precipitate acute kidney toxicity.[41, 147-149]

In 2018, Scotland reported higher incidences of prisoner aggression partially attributed to paper mail sprayed with illicit SCRAs. A study by Norman et al. (2020) tested some of these papers and found they contained SCRAs 5-fluoro PICA and MDMB-4en-PINACA with a variety of other high potency SCRAs.[150] These SCRAs are amongst the newer compounds that contain L-valinamide or L-*tert*-leucinamide substitutions and an indole or indazole core. When analyzed, these SCRAs display a very high affinity for the CB1 receptor.[99, 100, 105] AB-FUBINACA is another such SCRA identified in liquid formulations intended for e-cigarettes.[40] Interestingly, in an adolescent animal model, AB-FUBINACA and THC shared similar behavioral effects during drug administration; however, AB-FUBINACA exposure enhanced long-term deficient in cognitive processes and suggested to be the result of altered cannabinoid receptor signaling.[151]

The effect SCRAs have on CB1 receptor molecular mechanisms is understudied, a consequence of an ever-expanding catalog of SCRAs. The laboratory GIRK channel fluorescent assay described in chapter 2 can effectively measure CB1 receptor agonism.[108, 111] This study compared 10 illicit SCRAs to reference compound, WIN 55, 212-2, to elucidate SCRA structure on CB1 receptor-induced, GIRK channel potency, and efficacy. WIN 55, 212-2 was selected as the reference compound because it served as the prototype for JWH-018 and is well-represented in cannabinoid research.[124, 152]

## 4.3 Materials and methods

*Drugs and chemicals*

The following compounds were purchased from Cayman Chemical (Ann Arbor, Michigan): 4-fluoro MDMB-BUTINACA, 5-fluoro MDMB-PICA, AB-FUBINACA, AM1220, AM1248, , JWH-018, JWH-122 N-(5-chloropentyl),MDMB-4en-PINACA, WIN 55, 212-2, UR-144, and XLR-11. Compounds were dissolved in DMSO at stock concentrations of 20mM to 50mM. All cannabinoids were diluted to working concentrations in 1 mM KCl buffer solution containing MD-BLUE dye (Molecular Devices). Controlled substances were purchased using the Walsh Laboratory DEA license.

*Data analysis*

Concentration curves were determined using four-parameter, non-linear regression analysis where $EC_{50}$ is the concentration producing a 50 % increase in the maximal response $y_{max}$ ($E_{max}$). (listed below) $Y_{min}$ is defined as a minimum fluorescent GIRK channel response. Drug is the concentration, and hillslope is the slope factor.

Relative $E_{max}$ values were determined by normalizing each cannabinoid's peak GIRK channel response to the maximal GIRK channel response of WIN 55, 212-2 (10μM). $E_{max}$ graphs represents the SCRA concentration-response normalized to peak WIN 55, 212-2 GIRK channel response. Because the minimum GIRK channel response can vary between SCRAs and obscure the $E_{max}$ effect, minimum values were standardized. "n" represents the number of wells in which that cannabinoid concentration was tested.

Four-parameter Equation

$$y = y_{min} + \frac{y_{max} - y_{min}}{1 + (\frac{Drug}{EC_{50}})^{-Hillslope}}$$

*Statistical analysis*

Statistical significance of the comparison of cannabinoid GIRK channel $E_{maxs}$ was determined using a one-way, multiple measures ANOVA with significance set at $p < .05$. Significant differences were identified using a Holms-Ŝídák posthoc analysis. Data and statistical analyses were performed using Sigmaplot 14.0.

4.4 Results

GIRK channel activity for 11 cannabinoids was measured using our fluorescent, membrane-potential sensitive assay, and AtT20 cells transfected with the human CB1 receptor.[108, 111] The SCRAs were divided into two primary groups: Indole-based and Indazole-based. The Indole-based group was subdivided into the following subgroups: 1) Naphthoylindoles 2) Adamantoylindoles 3) Tetracyclopropylindoles 4) Indole and Indazole carboxamides. GIRK channel potency was defined as the SCRA concentration producing half the maximal GIRK channel response or the $EC_{50}$. All SCRAs activated GIRK channels in a concentration-dependent manner. Figures 4.1- 4.5 compare the results of structurally similar SCRAs to WIN 55, 212-2 regarding GIRK channel response, $EC_{50}$, and $E_{max}$. Only UR-144 and AM1248 were less potent than WIN 55, 212-2, with respective $EC_{50}$s of 532nM and 2530nM. Overall, the rank order of potencies were 5-fluoro MDMB-PICA > 4-fluoro MDMB-BUTINACA

> AB-FUBINACA > MDMB-4en-PINACA > JWH-018 > AM1220 > XLR-11 > JWH-122 N-(5-chloropentyl) > WIN 55, 212-2 > UR-144 > AM1248.

The $E_{max}$ values for each SCRA were normalized to the $E_{max}$ of WIN 55, 212-2, with the relative percentages available in Table 4.1. To identify $E_{max}$ differences compared to WIN 55, 212-2, a one-way ANOVA comparing the SCRA maximum fluorescent values to WIN 55, 212-2 maximum fluorescent values revealed most SCRAs were significantly less effective at stimulating a GIRK channel response. The indazole carboxamide, 4-fluoro MDMB-BUTINACA, was the only SCRA to significantly stimulate a GIRK channel response greater than WIN 55, 212-2. $E_{max}$ comparisons within-group revealed the effect of structural changes between two similar SCRAs discussed further below.

*Naphthoylindoles*

Both JWH-018 and JWH-122 N-(5-chloropentyl) had smaller GIRK channel responses compared to WIN 55, 212-2. JWH-122 N-(5-chloropentyl) was visibly slower to reach peak GIRK channel response but maintained a stable GIRK channel response until the end of the measurement. (Figure4.1 D) JWH-018 and JWH-122 N-(5-chloropentyl), had higher GIRK channel potency compared to WIN 55, 212-2. (Figure 4.1E). JWH-122 N-(5-chloropentyl) was significantly more effective at stimulating a GIRK channel signal compared to JWH-018, which indicates the addition of a methyl group and terminal chlorination increases GIRK channel efficacy. ($p < .05$) (Figure 4.1F)

*Adamantoylindoles*

The naphthoylindole, AM1220, shares a similar structure to the adamantoylindole, AM1248, and, therefore, were compared to each other. AM1220 and AM1248 had similar GIRK channel responses. (Figure 4.2D); however, AM1248 ($EC_{50}$: 2530nM) was much less potent than AM1220 ($EC_{50}$: 172nM) and WIN 55, 212-2 ($EC_{50}$: 523nM). (Figure 4.2 E) AM1220 was significantly more effective at stimulating a GIRK channel response compared to AM1248; however, both were significantly less effective when compared to WIN 55, 212-2. ($p < .001$) (Figure 4.2F).

*Tetracyclopropylindoles*

The GIRK channel response to XLR-11 decreased towards the end of the measurements, whereas the GIRK channel response to UR-144 appeared more stable. (Figure 5.3D) As mentioned above in the naphthoylindole section, different CB1 receptor kinetics could potentially explain the difference between UR-144 and XLR-11 GIRK channel response. XLR-11 ($EC_{50}$: 214nM) was more potent at eliciting a GIRK channel response when compared to UR-144 ($EC_{50}$: 532nM) and WIN 55, 212-2 ($EC_{50}$: 523nM) (Figure 5.3E). Interestingly, the $E_{max}$ results for UR-144 and XLR-11 were not significantly different from each other, despite the latter being more potent. (Figure 5.3F)

*Indole Carboxamides*

5-fluoro MDMB-PICA was the only indole carboxamide tested and was compared with fluorinated indazole carboxamide, AB-FUBINACA. 5-fluoro MDMB-PICA ($EC_{50}$: 3.33nM) was more potent compared to WIN 55, 212-2 ($EC_{50}$: 523nM)

and AB-FUBINACA ($EC_{50}$: 18.1nM). (Figure 5.4D & Figure 5.4 E) The $E_{max}$ of 5-fluoro MDMB-PICA was significantly larger than AB-FUBINACA; however, it was not significantly different from WIN 55, 212-2. Additionally, the $E_{max}$ of AB-FUBINACA was not significantly different from WIN 55, 212-2, despite being less than 5-fluoro MDMB-PICA. (Figure 5.4 F)

*Indazole Carboxamides*

In a recent analysis of SCRA products, the indazole carboxamides, 4 fluoro-MDMB-BUTINACA and MDMB-4en-PINACA, were identified. Both stimulated a more robust GIRK channel response that peaked at lower concentrations than other SCRAs. (Figure 4.5D). 4-fluoro MDMB-BUTINACA ($EC_{50}$: 4.97nM) was more potent than MDMB-4en-PINACA ($EC_{50}$: 19.7nM) (Figure 4.5E), with an $EC_{50}$ value similar to 5-fluoro MDMB-PICA ($EC_{50}$: 3.33nM). (Table 4.1) 4-fluoro MDMB-BUTINACA was significantly more efficacious at stimulating a GIRK channel response in comparison to MDMB-4en-PICA ($p < .05$) and WIN 55, 212-2. ($p < .001$)(Figure 4.5F) It is worth noting the $E_{max}$ values of 4-fluoro MDMB-BUTINACA and 5-fluoro MDMB-PICA were not significantly different from each other, and they shared similar potency; however, only 4-fluoro MDMB-BUTINACA produced a significantly larger GIRK channel response in comparison to WIN 55, 212-2. ($p < .001$)

*Comparison to THC and AEA*

A one-way ANOVA was performed to determine significant differences in $E_{max}$ values comparing SCRAs to the endocannabinoid, AEA, and the phytocannabinoid, THC. The analysis revealed that AEA and THC were

significantly less effective at stimulating the GIRK channel response in comparison to all SCRAs. ($p < .001$)

4.5 Discussion

The growing list of novel SCRAs and the ease of purchasing these products online has accumulated into baffling overdose cases in which medical personnel struggle to develop a treatment plan. To better treat the impact of SCRA toxicity, researchers study the molecular mechanisms driven by CB1 receptor agonism.[12] These experiments investigate the CB1 receptor-induced GIRK channel response to a series of SCRAs with different structural characteristics. Overall, most SCRAs tested had a higher affinity for the CB1 receptor when compared to WIN 55, 212-2; however, only 4-fluoro MDMB-BUTINACA had an $E_{max}$ that was significantly greater than WIN 55, 212-2.

There is increasing evidence that cannabinoids, SCRAs included, can induce biased signaling.[94, 121, 153, 154] In this study, XLR-11 was more potent than its parent compound, UR-144; therefore, supporting the fluorination of a SCRA increases potency. Interestingly, the GIRK channel response for XLR-11 has a greater decrease in fluorescence when compared to UR-144. This effect could be CB1 receptor rapid desensitization modulated by the recruitment of βarr2, as the binding of βarr2 to the CB1 receptor prevents the binding of G proteins.[58, 155].

CB1 receptor desensitization occurs when βarr2 is recruited to the phosphorylated, proximal c-terminus located on the intracellular side of the cell.[156, 157] An electrophysiology study by Jin et al. (1999) reported

approximately a 40% decrease in GIRK channel current at 80 seconds post WIN 55, 212-2 exposure. The decrease in GIRK channel current was attributed to βarr2 binding to the phosphorylated CB1 receptor results in the attenuation of the WIN 55, 212-2 induced GIRK channel signal via receptor desensitization. [51] The attenuation of the GIRK channel response would decrease $E_{max}$ values, such as the case of the SCRAs tested in this current study. Explicitly, XLR-11 biased towards βarr2 recruitment over $G\alpha_{i,}$ and the recruitment of βarr2 does not mediate CB1 receptor internalization. [153, 154] Although AB-FUBINACA and 5-fluoro MDMB-PICA had lower $EC_{50}$ values, the GIRK channel response was not significantly different from WIN 55, 212-2. In a study by Patel et al. (2020), both 5-fluoro MDMB-PICA and AB-FUBINACA were more potent recruiters of βarr2 when compared to WIN 55, 212-2, and thus, more likely to induce CB1 receptor desensitization.[153]

In conclusion, the structure of a SCRA can modulate the of outcome CB1 receptor signaling through potential βarr2 recruitment leading to receptor desensitization. Along with being expressed in the CNS, GIRK channels are expressed in the heart.[69, 72] This is particularly relevant to the physiological effects seen in SCRA (i.e.seizures and cardiovascular events), as the decline in GIRK channel activity can translate to the increase in excitatory neurotransmitter release.[15, 158, 159]

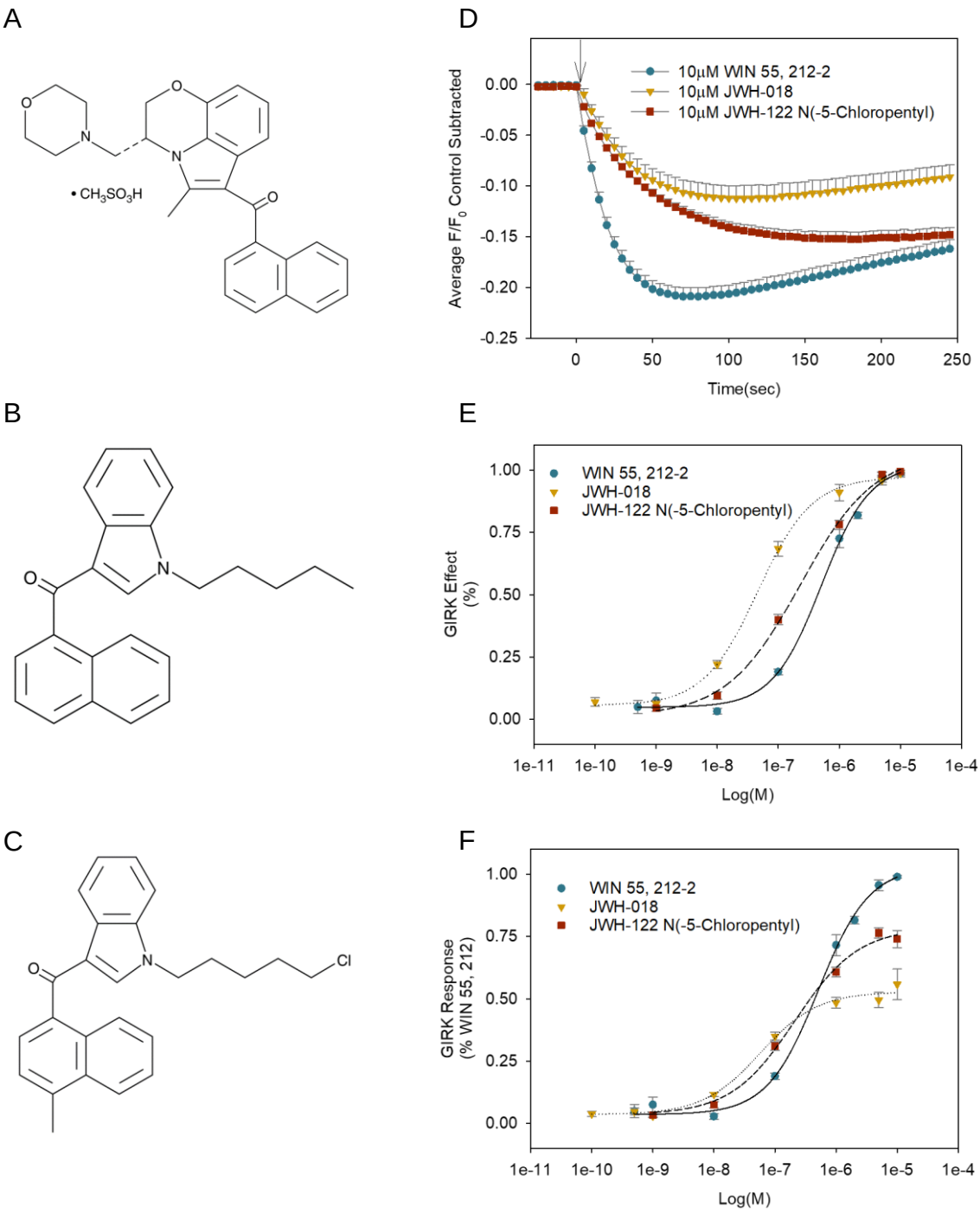



**Figure 4.1:** *GIRK channel responses and cannabinoid structures (JWH compounds)*. (A) WIN 55, 212-2 (B) JWH-018 (C) JWH-122 N-(5-chloropentyl) (D) Comparison of max GIRK channel responses. (E) Comparison of EC50s demonstrating cannabinoid potency (F)Comparison of cannabinoid $E_{max}$ relative to WIN 55, 212-2 Error bars represent standard error of mean.↓ Indicates application of WIN 55, 212-2 (*n = 5*), JWH-018 (*n = 4*), JWH-122 N-(5-chloropentyl) (*n = 4*).

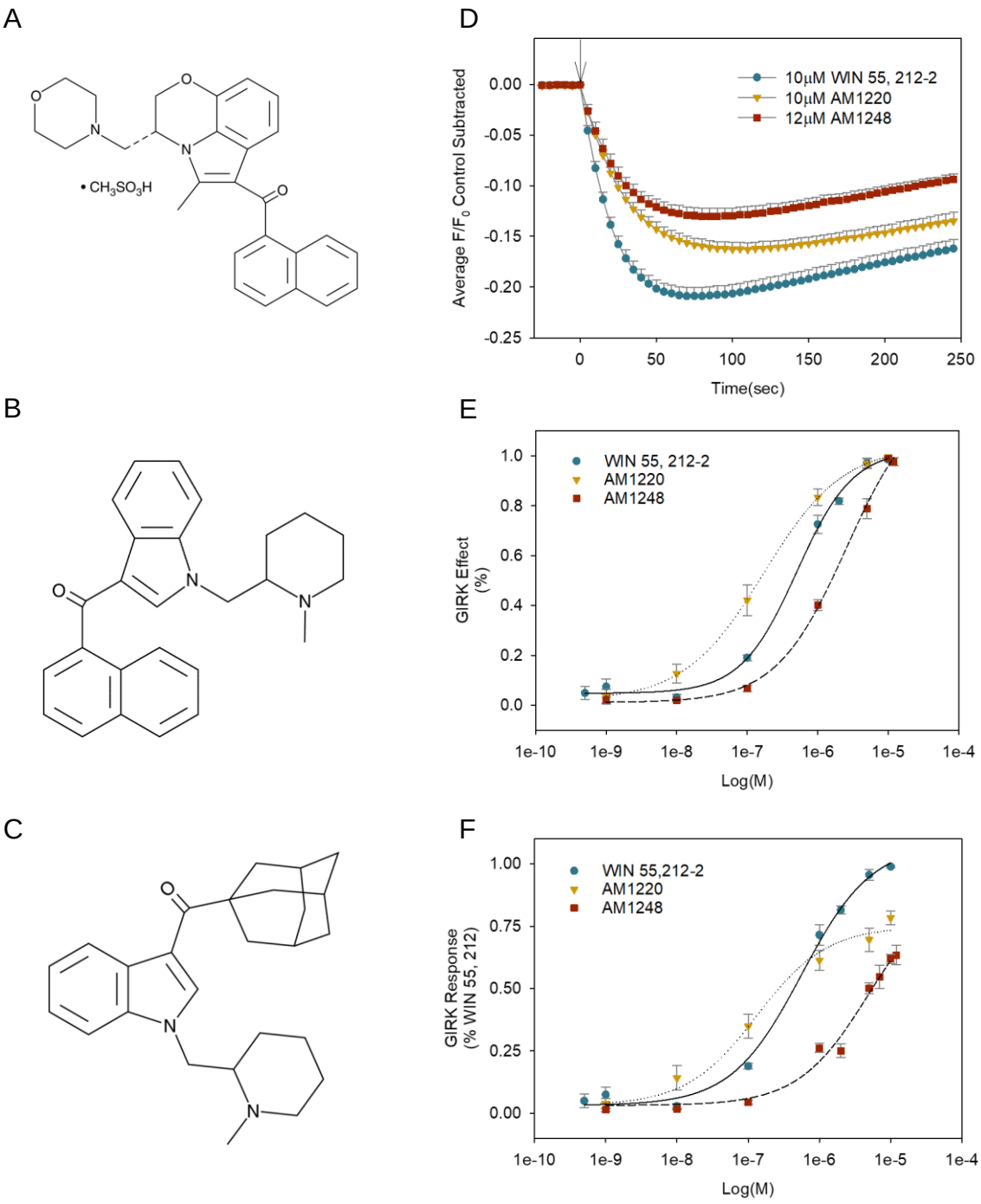



**Figure 4.2:** *GIRK channel responses and cannabinoid structures (AM compounds).* A) WIN 55, 212-2 B) AM1220 C) AM1248 D) Comparison of max GIRK channel responses. (E) Comparison of $EC_{50}$s demonstrating cannabinoid potency (F)Comparison of cannabinoid $E_{max}$ relative to WIN 55, 212-2 Error bars represent standard error of mean.↓ Indicates application of WIN 55, 212-2 (*n* = *5*), AM1220 (*n* = *5*), AM1248 (*n* = *5*)

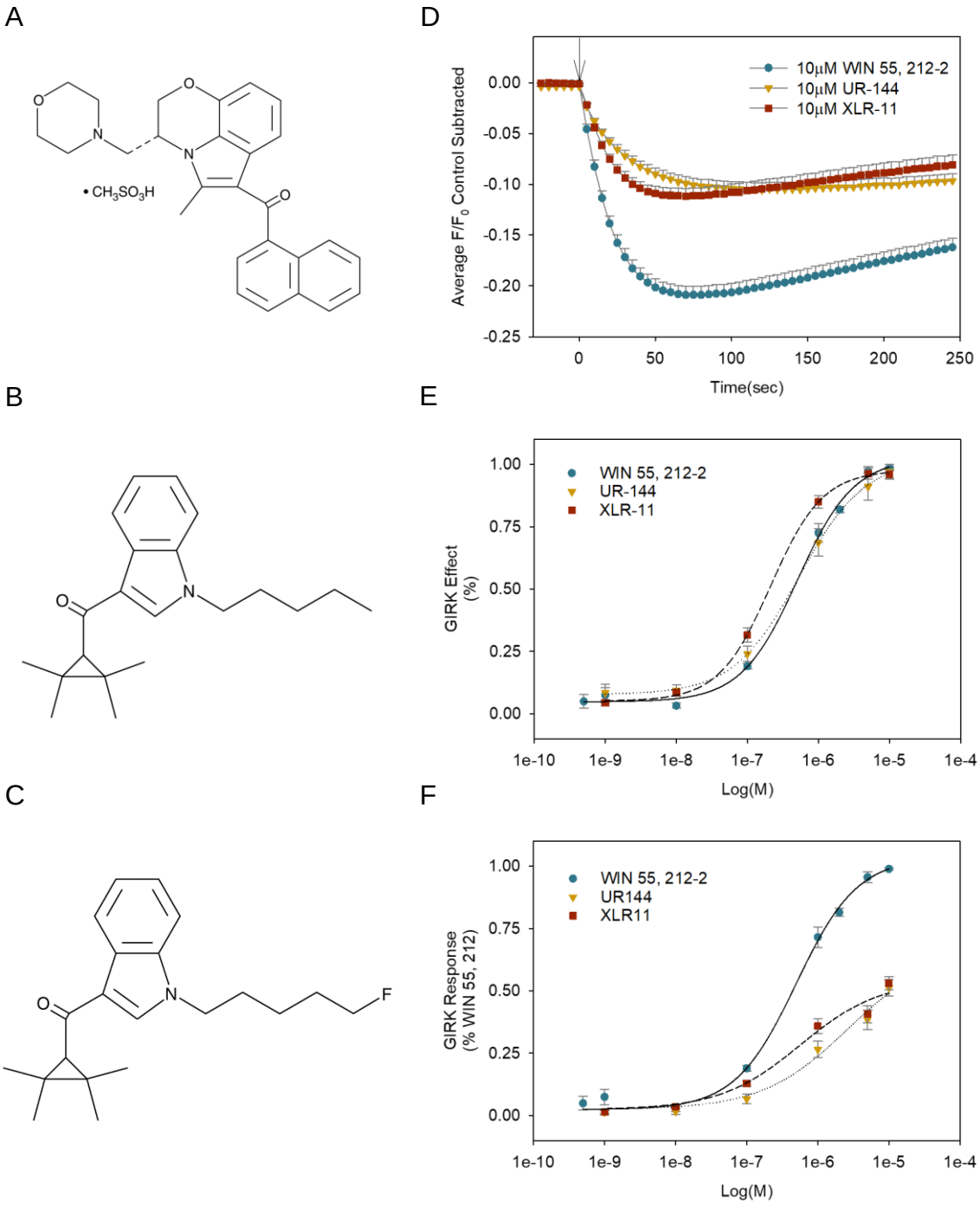



**Figure 4.3:** *GIRK channel responses and cannabinoid structures (Tetracyclopropylindoles)*. A) WIN 55, 212-2 B) UR-144 C) XLR-11 (D) Comparison of max GIRK channel responses. (E) Comparison of $EC_{50}$s demonstrating cannabinoid potency (F) Comparison of cannabinoid $E_{max}$ relative to WIN 55, 212-2 Error bars represent standard error of mean.↓ Indicates application of WIN 55, 212-2 (*n = 5*), UR-144 (*n = 4*), XLR-11 (*n = 5*)

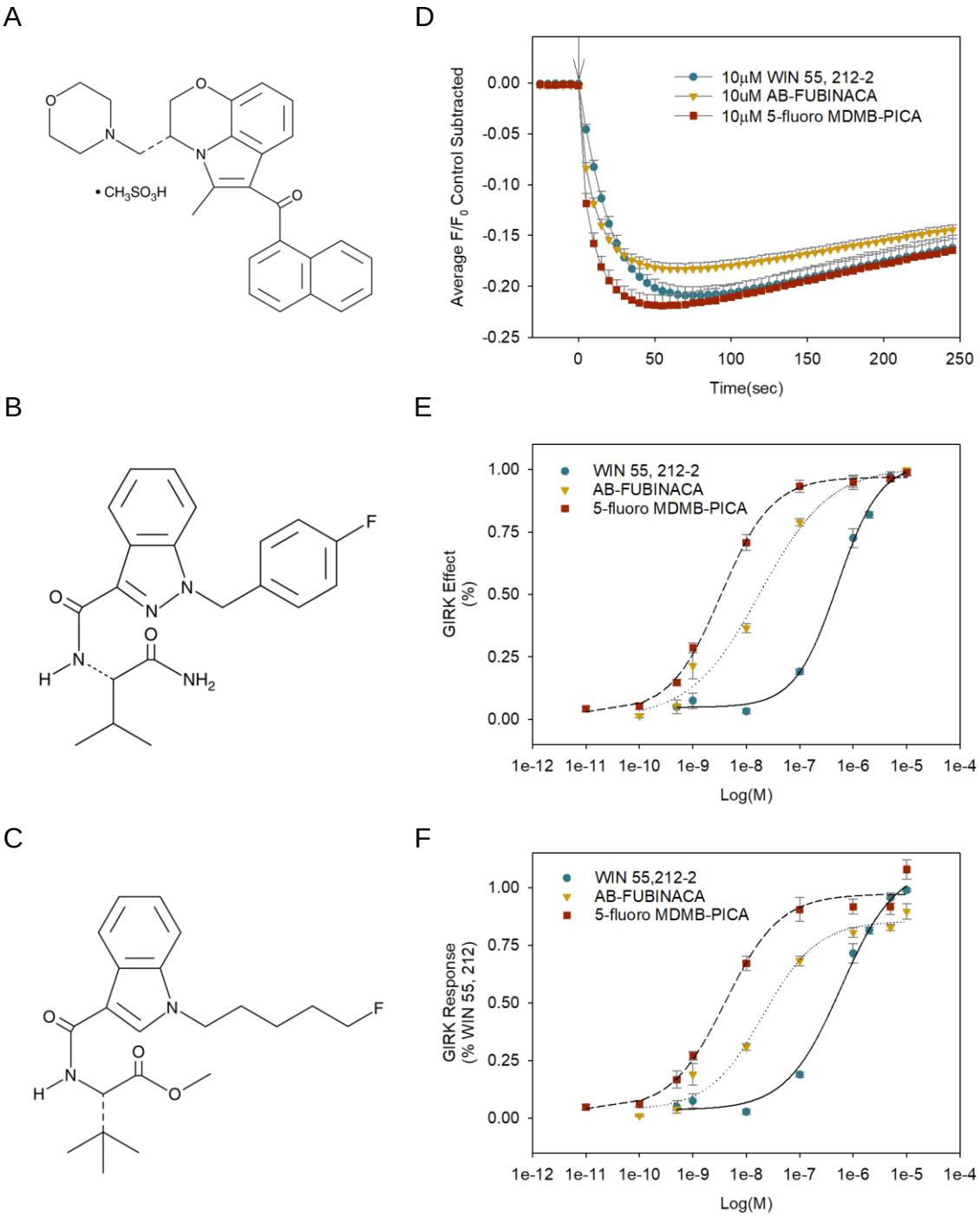



**Figure 4.4** *GIRK channel responses and cannabinoid structures (Carboxamides 1)*. (A) WIN 55, 212-2 (B) AB-FUBINACA (C) 5-fluoro MDMB-PICA (D) Comparison of max GIRK channel responses. (E) Comparison of $EC_{50}$s demonstrating cannabinoid potency (F)Comparison of cannabinoid $E_{max}$ relative to WIN 55, 212-2 Error bars represent standard error of mean.↓ Indicates application of WIN 55, 212-2 (n = 5), AB-FUBINACA (*n = 6*), 5-fluoro MDMB-PICA (*n = 4*)

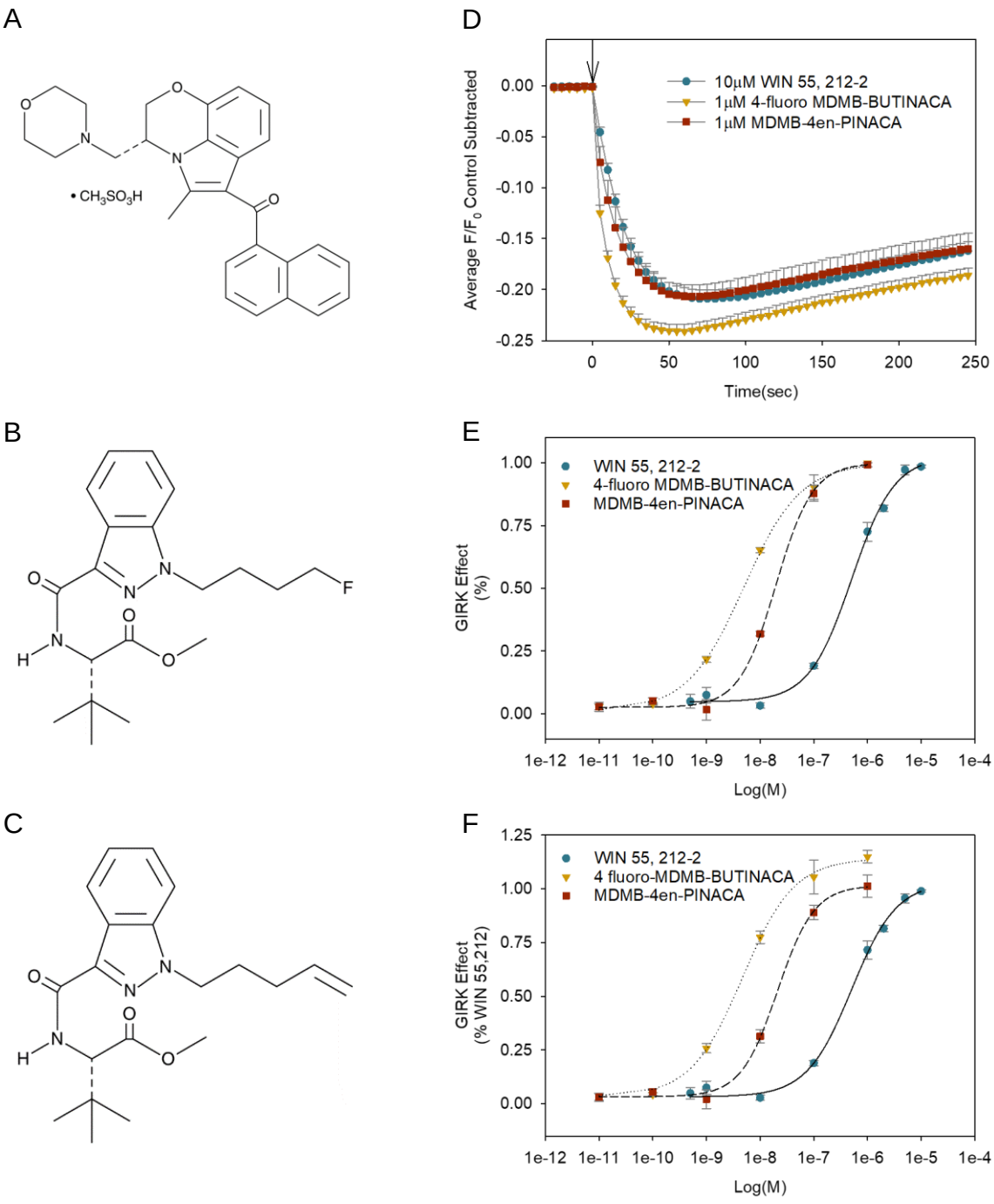



**Figure 4.5:** *GIRK channel responses and cannabinoid structures (Carboxamides 2)*. (A) WIN 55, 212-2 (B) 4-fluoro MDMB-BUTINACA (C) MDMB-4en-PINACA D) Comparison of max GIRK channel responses. (E) Comparison of $EC_{50}$s demonstrating cannabinoid potency (F) Comparison of cannabinoid $E_{max}$ relative to WIN 55, 212-2 Error bars represent standard error of mean.↓ Indicates application of WIN 55, 212-2 (*n* = *5*), 4-fluoro MDMB-BUTINACA (*n* = *6*), MDMB-4en-PINACA (*n* = *6*)

**Table 4.1: *SCRAs potency and efficacy***

| ***Class*** | ***Cannabinoid*** | ***$EC_{50}$ (nM) ± S.E.M*** | ***$E_{max}$ (%) ± S.E.M*** |
|---|---|---|---|
| **Indole-Based** | | | |
| *Aminoalkylindole Prototype* | WIN 55, 212-2 | 523 ± 68.0 | Reference |
| *Napthoylindoles* | JWH-018 | 46.3 ± 4.8 | 55.8 ± 6.13* |
| | JWH-122 N-(5-chloropentyl) | 231 ± 35.4 | 74.0 ± 3.41* |
| | AM1220 | 172 ± 6.2 | 78.3 ± 2.72* |
| *Adamantoylindole* | AM1248 | 2530 ± 805 | 63.5 ± 3.90* |
| *Tetracyclopropylindoles* | UR-144 | 532 ± 33.9 | 51.3 ± 3.26* |
| | XLR-11 | 214 ± 14.9 | 53.0 ± 2.59* |
| *Indole Carboxamide* | 5-fluoro-MDMB-PICA | 3.33 ± 0 .3 | 108 ± 4.27 |
| **Indazole-Based** | | | |
| *Indazole Carboxamide* | AB-FUBINACA | 18.1 ± 7.0 | 89 ± 2.93 |
| | 4-fluoro-MDMB-BUTINACA | 4.97 ± 2.6 | 115 ± 2.92† |
| | MDMB-4en-PINACA | 19.7 ± 2.9 | 101 ± 5.21 |

* $p < .001$ compared to WIN 55, 212-2
† $p < .05$ compared to WIN 55, 212-2

# CHAPTER 5: *CONCLUSIONS AND FUTURE DIRECTIONS*

## 5.1 Conclusion

The changing legal and social perception of *Cannabis* has precipitated interest in cannabinoid therapeutics, with a particular focus on pain management. The ease of online shopping and the legal loopholes facilitating the manufacturing of novel SCRAs have resulted in numerous overdose cases with potentially lethal outcomes. Together, these realizations draw attention to the need to understand the molecular mechanisms underlying CB1 receptor signaling.

This thesis elucidates CB1 receptor-induced, GIRK channel activity by a series of chemically distinct and similar cannabinoids by addressing the following: 1) establishing a GIRK channel assay 2) outlining the GIRK channel response to cannabinoids representative of the eicosanoid, classical, non-classical, and aminoalkylindole classes. 3) investigating the effect of illicit SCRAs on GIRK channel activation

GIRK channels are activated by agonists binding to the CB1 receptor. The use of fluorescent, MP-sensitive dye allowed for the GIRK channel response to be recorded in real-time. CB1 receptor-induced, GIRK channel activity was verified by inhibiting the WIN 55, 212-2 response by either pretreating the AtT20/CB1 cells with GIRK channel blocker, tertiapin, or the CB1 receptor antagonist, SR141716. Overall, chapter 2 reports a capable GIRK channel assay sensitive to CB1 receptor activity.

A similar structure defines the four primary groups of cannabinoids. The classical cannabinoids include compounds derived from the *Cannabis* plant (i.e., THC, CBD, and THCA-A). The eicosanoid, AEA, is synthesized endogenously in humans. CP 55, 940 is a notable synthetic, non-classical cannabinoid, and WIN 55, 212-2, is the aminoalkylindole prototype. Chapter 3 reports that neither THCA-A nor CBD produces a GIRK channel response reflective of activation. CP 55, 940 and WIN 55, 212-2 displayed a lower $EC_{50}$ values for the CB1 receptor compared to AEA and THC. Additionally, both synthetic cannabinoids were more effective at generating a GIRK channel response, potentially due to differential G protein signaling.

Through applying the GIRK channel assay to the study of SCRA-modulated CB1 receptor signaling, differences were uncovered regarding cannabinoid structure. Chapter 4 revealed that an indole or indazole core in a SCRA greatly increased CB1 receptor potency in addition to terminal fluorinations. Also, SCRA potency did not determine the magnitude of the GIRK channel response, potentially due to CB1 receptor desensitization.

In conclusion, the cannabinoids tested in these studies underscore the importance of understanding CB1 receptor signaling. Factors such as CB1 receptor internalization and desensitization can modulate the effectiveness of a cannabinoid at signaling the GIRK channel response. These factors play a crucial role in determining the physiological response to cannabinoids, and therefore should be further investigated.

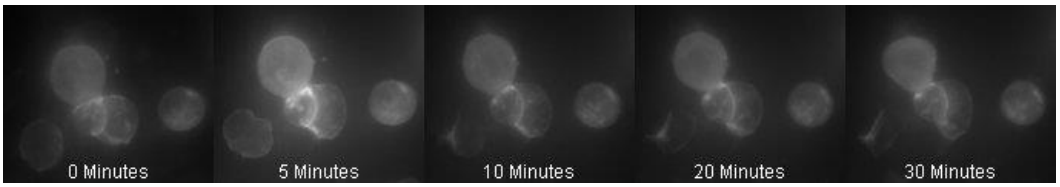
## 5.2 Future directions

The SEPCB1 receptor construct was developed by Dr. Andrew Irving (University College Dublin) to measure cannabinoid-induced, CB1 receptor internalization.[160] The SEPCB1 construct is a pH-sensitive variant of GFP located on the N-terminus of the receptor. This construct will emit a fluorescent signal when expressed on the surface of the cells; however, as the receptor internalizes, the signal will decrease due to the acidic environment located within the cells.[160, 161] The Walsh laboratory received the SEPCB1 construct, which was used to create the AtT20/SEPCB1 cells used in chapters 3 and 4. Additionally, the SEPCB1 construct was transfected into HEK293 cells (HEK293/CB1). A pilot experiment was conducted measuring CB1 receptor internalization over time in response to cannabinoids. Using the EVOS FL2 Auto live cell imaging system, fluorescent images were taken of HEK293/SEPCB1 cells in control, 2μM WIN 55, 212-2, or 1μM CP 55, 940 conditions at 0, 5, 10, 20, and 30 minute time points. (Figure 5.1)

For future directions, I propose expanding these experiments to include THC, JWH-018, XLR-11, AB-FUBINACA, and 5-fluoro MDMB PICA based on the previous experiments and their relevance to the field of research. The change in fluorescence in these images can be quantified using imaging programs like Image J. Data from these experiments could help define cannabinoid-dependent, CB1 receptor internalization which will determine signaling bias within β-arrestin recruitment and between $G_i$ protein signaling. Additional GIRK channel assays

using HEK293/SEPCB1 cells will be necessary to maintain consistency between the different methods.

A

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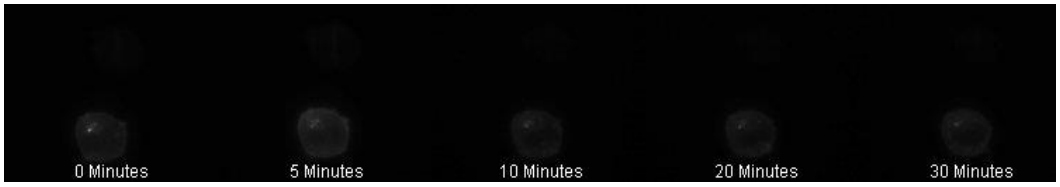


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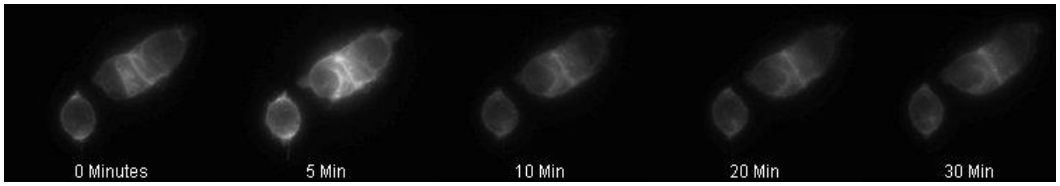


**Figure 5.1:** *Prelimary data from HEK293/SEPCB1 cannabinoid experiments.* Change in fluorescence over 30 minutes representative of CB1 receptor internalization. (A) Control (B) 2μM WIN 55, 212 (C) 1μM CP 55, 940

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