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Effect Of CBD On D2 Dopamine Receptor And CB1 Cannabinoid Receptor Function And Localization

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BY

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ABSTRACT

Cannabidiol (CBD) is the second most abundant cannabinoid found in *Cannabis sativa* and its array of therapeutic effects make it a popular target of pharmacological research. Though its properties are psychoactive in nature, abuse and dependence of CBD has never been reported. Its beneficial side effects are mediated through the endocannabinoid system (ECS), as it functions as an antagonist at cannabinoid receptor 1 (CB1). However, recent evidence suggests that CBD may also be a CB1 negative allosteric modulator of (−)-*trans*-Δ^9-tetrahydrocannabinol (Δ^9-THC). Interestingly, the ECS is linked to the main circuit thought to modulate reward signaling when taking substances of abuse, the dopamine (DA) pathway. The DA pathway contains dopaminergic cell bodies originating in the ventral tegmental area (VTA), where reward-related information is carried to the nucleus accumbens (Acb). CB1 receptors are shown densely located across these regions, causing speculation as to the role of the ECS in reward functioning. In addition to these two systems interacting, one study found that CBD acts as a partial agonist at dopamine D2 receptors. Although experiments have shown CBD and its behavior at CB1 and D2 receptors, no studies exhibit what CBD does at the receptor-level when given in combination with CB1 and D2 receptor agonists during acute or chronic treatment. Therefore, the objective of this thesis was to examine the effect CBD has on signaling alone and in combination with CB1 and D2 receptor agonists at 5-minutes (acute) and 21-hours (chronic). SH-SY5Y cells were used as the model due to the native presence of both CB1 and D2 receptors. Western blot analyses were performed by probing protein samples for CB1 and D2 receptors and quantifying protein concentrations for each treatment. Confocal

microscopy was utilized to show CB1 and D2 receptor localization. Lastly, cAMP assays were run to measure levels of cAMP production during each drug treatment since levels have been shown to increase at the D2 receptor but decrease at the CB1 receptor. From representative Western blot analyses, acute treatments did not appear to alter CB1 or D2 receptor concentrations. However, for chronic treatments, CB1 receptor concentration seemed to be changed at treatment groups: WIN + QUIN, WIN + CBD, WIN + CBD + QUIN, and QUIN + CBD. No apparent alterations were seen for D2 receptor concentration levels for chronic treatments. Preliminary data obtained from confocal microscopy and cAMP assays will need to be further examined and compared before results can be deemed significant. Overall, by understanding CBD and its functioning at the CB1 and D2 receptors, future clinical treatment paradigms can be better informed.

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Chapter 1
Background

1.1 The Endocannabinoid System

The endocannabinoid system (ECS) has been well-established in its role of maintaining homeostasis in the mammalian body (1). Furthermore, its extensive network connects the body's organs and systems (1). The ECS contains cannabinoid receptors, endogenous ligands, and enzymes (2). The cannabinoid receptors include cannabinoid receptor 1 (CB1) and cannabinoid receptor 2 (CB2) (2). The naturally occurring endogenous cannabinoids found in the ECS include anandamide and 2-arachidonulglycerol (1). However, phytocannabinoids were also found to modulate the ECS, with (−)-*trans*-Δ^9-tetrahydrocannabinol (Δ^9-THC) and cannabidiol (CBD) being the most understood (1). Enzymes within the ECS needed for the biosynthesis and degradation of the two endogenous cannabinoids are fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL) (2). A particular point of interest in studying the ECS is its contribution in reward signaling (2). Research suggests the rewarding effect after taking abused substances is largely mediated through the mesolimbic dopamine (DA) pathway (2). Dopaminergic cell bodies originate in the ventral tegmental area (VTA) and carry reward-related information to the ventral striatum, specifically the nucleus accumbens (Acb) (2). It is thought that the acute reinforcing effect seen with addictive substances is by the direct or indirect activation of DA neurons throughout this pathway (2). Furthermore, DA activity is inherently connected to the activity of cannabinoids (2). Interestingly, CB1 receptors are found in

clusters among striatal regions, like the VTA and Acb, connecting the two systems together (2).

1.2 The History of Cannabis

The use of cannabis for medicinal, religious, and social practices can be dated as far back as 5000 years ago (3). The multifaceted plant served several purposes during Ancient Middle Eastern and European times (3). Medically, cannabis was used to bandage wounds and served as a key ingredient in ointments involving contusions or swelling (3). The fumes of the plant acted as a drug for those suffering from what was considered possible arthritis at the time (3). It was not till much more recent that cannabis was found to be the wellspring for over 60 compounds called cannabinoids (4). Cannabinol (CBN) was the first isolated plant cannabinoid (phytocannabinoid) discovered in the early 1930s, with the phytocannabinoid CBD discovered approximately 10 years later (4) (5). In addition to the recent discovery of cannabinoids, Δ^9-THC (THC) was found to be the main psychotropic compound in cannabis in 1964 (4). CBD and THC were found in the cannabis plant as acids, undergoing decarboxylation when heated (4). However, their effects were found to be in stark contrast with each other in several animal studies (4). THC induced symptoms of catalepsy in mouse studies, while CBD did not (4). CBN also induced catalepsy in mice, however, only at lethally high doses (4). Furthermore, THC proved to have a central excitant action and corneal areflexia induced behavior in rabbits, as well as mice (4). In contrast, CBD did not elicit any of the responses seen with THC involving animals (4). These observations led researchers to uncover a relationship between the catalepsy seen in rodents and level of psychotropic activity; that is, the more catalepsy seen in

mice at lower doses, the higher the psychotropic effect (4). Another finding related to the animal study observations was the evidence that CBD displays no psychotropic activity (4). It is this single observation which has made CBD the focus of many studies to come regarding this unique characteristic (4).

1.3 Overview of Cannabidiol

CBD is the main non-psychotomimetic component and second most abundant cannabinoid in *Cannabis sativa* (6). This compound's effects seen in animals and humans range from anti-inflammatory, analgesic, anti-anxiety, to anti-tumor (7). Although it was first discovered in the 1940s at the University of Illinois, its structure was not determined till 1963 (8). As more research is conducted surrounding the ECS, pharmacological research involving CBD has increased (8). Its numerous therapeutic properties are regarded as highly beneficial, making it the center of research for psychotic disorders, epilepsy, and chronic pain studies for treatment (8). CBD inhibits cyclooxygenase and lipoxygenase, which contributes to its analgesic and anti-inflammatory qualities (9). Additionally, studies have shown CBD to exhibit anxiolytic, antiemetic, antipsychotic, and neuroprotective antioxidant characteristics (9). The way CBD exerts its effects are still not fully understood (9). One hypothesis for how CBD exerts its pharmacological effects is the possible agonist effect at PPARγ, ultimately affecting intracellular calcium release (9). CBD can be inhaled or taken orally by capsule and oil (9). Clinically, patients 2 years and older receive an initial dose of 2.5 mg/kg per day (9). The dose is then increased to 5 mg/kg twice per day if the initial dose is tolerated (9). To avoid liver damage, CBD dosing is typically begun at a lower dose and titrated upwards for those with hepatic impairment (9). Patients taking CBD to control

seizures have shown positive results using a dose of 20 mg/kg per day, but with adverse reactions (9). Although incidence of adverse reactions is low, CBD may cause dose-related liver damage, somnolence, and increased suicidal thoughts (9). However, side effects that are mostly mild and infrequent including fatigue, diarrhea, or increased temperature (10). To date, no evidence supports dependence or abuse with the use of CBD (10). The diversity of CBD and its wide array of therapeutic targets have contributed to its increased commercial usage in recent years (10).

1.4 Regulation of Cannabidiol

Currently, the way CBD is regulated around the world is highly variable and ever-changing (11). However, its regulatory status has not affected its popularity on the consumer market (11). In the United States alone, hemp-derived CBD products accumulated 170 million in sales in 2016 (11). This number was expected to increase by a 55% compound annual growth rate for the next 5 years, amassing to over one billion dollars (11). In one study, surveys indicated that approximately 62% of all CBD users reported using CBD to treat a medical condition (11). Of the medical conditions reported by participants, pain, anxiety, and depression were the most frequent (11). Around 36% of individuals reported CBD treating their medical condition(s) on its own, and one out of every three CBD users reported experiencing nonserious adverse effects (11). In that same survey, 30% of participants answered, “Well in combination with conventional medicine.” in response to how they felt CBD treated their medical condition when combined with other medication (11). CBD use significantly increased between 2013 and 2015 when several states in the US approved medical cannabis usage (12). During this time, individuals with treatment-resistant epilepsy began to use CBD-

enriched cannabis to treat their seizures (12). Remarkably, patients saw a reduction in the frequency of their seizures and treatment was deemed successful (12). So far, only one CBD product has been FDA approved, treating two serious forms of epilepsy (13). Epidiolex® is a 99% pure oral CBD extract that can help decrease seizure frequency in those with refractory epilepsy (12). Children ages two and older who have been diagnosed with Lennox-Gaustaut syndrome or Dravet syndrome may be prescribed Epidiolex® by a licensed healthcare provider (14). Following FDA approval, the Drug Enforcement Administration placed Epidiolex® into schedule 5 of the Controlled Substances Act (CSA) (12). Schedule 5 is the least restrictive schedule in the CSA (12). Due to the insufficient data concerning CBD safety and risks, it is illegal to add CBD to food, or labels as a dietary supplement (13). Despite CBD and its category as a Schedule I controlled substance, ruled by the U.S. Drug Enforcement Administration, CBD products can be purchased online, over the counter, or at cannabis-specific dispensaries around the United States (15). Presently, CBD products sold in stores are not regulated (15). Determining its legal status relies heavily on the source of CBD extraction (15). *Cannabis sativa* (*C.sativa*) contains cannabis and hemp (15). Hemp is referred as a chemovar of *C. sativa*, with low concentration*s* of THC (15). CBD can be extracted from hemp, and the advent of the 2018 Hemp Bill legalized hemp-derived cannabinoids (15). This new ruling and distinction caused a novel legal market for unregulated CBD products (15).

1.5 FDA-Approved Cannabis Products

The limited amount of evidence surrounding CBD and its effects on the body have caused researchers to be skeptical of its use and practicality (16). However, some

cannabis-based products are FDA approved for certain uses (14). Sativex is a 1:1 ratio of THC and CBD in the form of an oral spray (16). It is a licensed treatment for spasticity in multiple sclerosis in 29 countries (16). Additionally, dronabinol and nabilone are synthetic forms of THC used for treating weight loss (wasting) in patients who have AIDS, as well as nausea or vomiting in cancer patients undergoing chemotherapy (16). The opposing effects of THC and CBD on the ECS may be the reason for the therapeutic effects seen in the mammalian central and peripheral nervous system (16). In one study, participants in a clinical trial involving the effect of CBD on schizophrenia showed increased plasma endocannabinoid levels and symptom improvement with CBD alone (16). Furthermore, when participants received CBD and THC together, adverse effects, like memory impairment and paranoia, of THC were reduced (16). Systematic reviews of cannabis-based products or cannabinoids strongly indicate their promise for treatment of chronic pain, multiple sclerosis, and treatment-resistant epilepsy (16). However, evidence of their effectiveness in randomized trials are limited partly due to the complex and inconsistent regulatory statuses of unregulated cannabis-based products (16). Additionally, limited, and vague descriptions of allocation, mechanisms of action, and receptor binding contribute to the unknown effectiveness of CBD and its potential use for treatment in specific disease states (16).

1.6 Cannabidiol at Cannabinoid Receptor CB1 and Dopamine Receptor D2

Despite demonstration of CBD effectiveness in several different disorders and disease states, its mechanism of action has not been fully elucidated. Research has demonstrated that CBD has a low affinity for CB1 and CB2 receptors (9). CBD is a partial agonist at CB2 and functions as a CB1 antagonist (17). However, recent

evidence suggests that CBD, at low concentrations, may also be a CB1 negative allosteric modulator of THC (17). To support this assumption, Sabatucci and colleagues discovered three putative allosteric sites for CBD on the CB1 receptor (17). It is possible that THC and CBD may bind to the CB1 receptor at the same time (17). Research also shows CBD as a partial agonist at serotonin 5-HT1A receptor and allosteric modulator of opioid receptors (9). Allosteric modulation of opioid receptors occurs at mu and delta receptor subtypes (9). CB1 and CB2 receptors belong to the G-protein coupled receptor superfamily (18). CB1 receptors are mostly located in the central nervous system but can be found throughout the body (18). Because of its location, psychoactivity seen with cannabinoids is thought to be modulated by CB1 receptors, since CB2 receptors are found mostly in the immune system (18). CB1 receptor activation occurs through cell signaling by G_i and G_o G-protein activation, decreasing cyclic adenosine monophosphate (cAMP) within cells (**Figure 1**) (18). Interestingly, one study showed that CB1 receptors stimulate cAMP by coupling to G_s when the dopamine receptor 2 (D2) is activated concurrently in cultured striatal neurons (**Figure 1**) (19). Furthermore, CBD was found to be a partial agonist at D2 receptors (20). Psychotic symptoms of schizophrenic patients decreased when given 800mg to 1000mg of CBD per day (20). Prior to this study, CBD was not known to interact with dopamine receptors (20). CBD inhibited the binding of radio-domperidone, a label for rat brain striatal D2 receptors, at D2 receptors (20). The results were compared with aripiprazole, which is a D2 partial agonist and antipsychotic drug, which demonstrated similar behavior at D2 (20). Additionally, D2 receptors have been shown to form heterodimeric complexes with CB1 receptors in the Acb, modulating the release of dopamine and endocannabinoids (21).

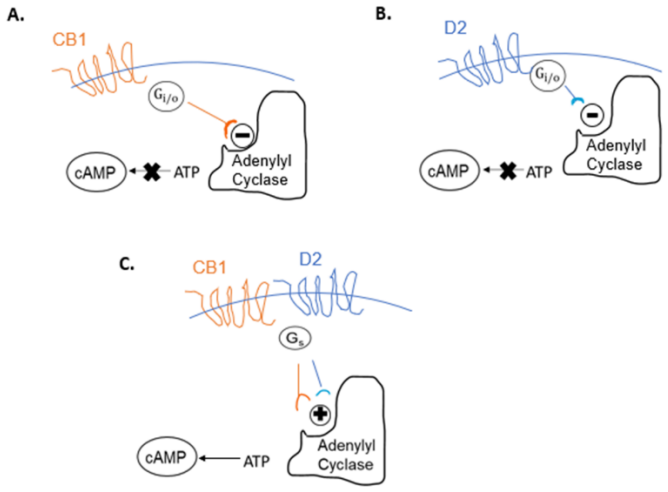

Figure 1. Signaling Pathway for CB1 and D2 Receptor Activation. A) demonstrates signaling of CB1 receptors. Activation of CB1 receptors activates the Gi/o pathway which inhibits adenylyl cyclase and therefore inhibits cAMP production B) demonstrates signaling of D2 receptors. Activation of D2 receptors activates the Gi/o pathway which inhibits adenylyl cyclase and therefore inhibits cAMP production. C) Activation of both CB1 and D2 receptors at the same time switches the signaling pathway to a Gs pathway, activating adenylyl cyclase, and increasing cAMP production.

1.7 Objectives of Study

Researchers have conducted studies observing the chronic dose of THC alone or THC and CBD together (22). Currently, literature exploring the binding of CBD at CB1 and D2 exist, but no studies have identified what is observed when CBD is given concurrently (acutely or chronic) with CB1 and D2 agonists. Additionally, no studies suggest what effect on signaling CBD has alone, or in combination with CB1 and D2 agonists when given at a chronic dosage. The objective of this project, therefore, is to examine the effect of CBD on D2 dopamine receptor and CB1 cannabinoid receptor function and localization using SH-SY5Y cells as the model. Understanding the

mechanisms of drug interactions will not only aid in understanding the molecular level of signaling pathways but could inform future clinical treatment paradigms.

Chapter 2
Experimental Methods

2.1 Cell Culture

2.1.1 Cell Type and Storage Conditions

A single line of SH-SY5Y; Neuroblastoma; Human (Homo sapiens) cells were purchased from ATCC, Manassas, VA. Upon arrival, cells were immediately stored in Eastern Kentucky University's cold room (Science Building 5210) in liquid nitrogen vapor phase until usage.

2.1.2 Handling Procedure for Frozen SH-SY5Y Cells and Incubation Conditions

The frozen vial of SH-SY5Y cells was taken out of liquid nitrogen vapor phase and placed in a bucket of ice for approximately 10 minutes. Next, gentle agitation by thawing the vial in a 37°C water bath was performed by keeping the O-ring of the vial out of the water. Rapid thawing did not exceed 2 minutes. Then, the vial was taken to the cell culture room where aseptic cell culture technique was executed. All lab equipment and reagents were sterilized using 70% ethanol. The contents of the vial were pipetted into a 15mL conical centrifuge tube containing 9mL of room temperature basic culture medium (Eagle's Minimum Essential Medium/ F12 Medium containing 10% fetal bovine serum). With the cap secured tightly, the tube was then inverted 2-3 times. Then, the tube was centrifuged at 1,000 x g for 2 minutes at 20°C. After centrifugation, the 15mL conical centrifuge tube was inspected for a pellet of cells. Once a pellet was visualized, the supernatant was discarded, leaving only the pellet of cells. The pellet was then resuspended in 10mL of room temperature basic culture medium.

The cells were then pipetted out of the 15mL conical centrifuge tube and into a sterile 75cm^2 cell culture flask with a vented cap. An additional 10mL of basic culture medium was added to the flask of cells. The cap was secured tightly onto the flask and placed in a cell culture incubator (New Brunswick™ Galaxy® 170 R High-Capacity CO_2 Incubators, Eppendorf) at 37°C and 5% CO_2 air atmosphere.

2.1.3 Basic Culture Medium Preparation and SH-SY5Y Cell Medium Changes

Complete growth medium conditions recommended by ATCC were followed. Cells were cultured in Eagle's Minimum Essential Medium (EMEM)/ F12 Medium containing 10% fetal bovine serum (FBS). Additionally, 1% Penicillin Streptomycin was added to basic culture medium to account for possible bacterial and fungal contaminations. Using aseptic cell culture technique inside a cell culture hood, sterile 50mL conical centrifuge tubes of basic culture medium aliquots were prepared for SH-SY5Y cells. Within each tube, the following concentrations were pipetted: 20mL of EMEM, 20mL of F12 Medium, 4mL of FBS, and 400µL of Penicillin Streptomycin. Each 50mL conical centrifuge tube of basic culture medium was labeled accordingly, "EMEM/ F12, 10% FBS, 1% Pen/ Strep" with the appropriate date and initials of the individual preparing the medium aliquots.

To conduct medium changes on SH-SY5Y cells, aseptic cell culture technique was followed inside a cell culture hood, sterilized with 70% ethanol. An aliquot of SH-SY5Y cell medium was warmed in a cell culture incubator for a minimum of 20 minutes. Once the cell medium was adequately warmed, the tube was inverted 2-3 times. Before the medium tube was placed inside the cell culture hood for use, 70% ethanol was sprayed on the tube and wiped dry with paper towels. Then, a 75cm^2 vented flask of

SH-SY5Y cells was taken out of the cell incubator and placed inside the cell culture hood. The cap on the flask was loosened and placed on its side inside the cell culture hood. The basic culture medium was pipetted out of the flask and discarded. Next, 20mL of warm, unused basic culture medium was pipetted into the same 75cm^2 flask of SH-SY5Y cells. The vented cap was then secured tightly on the flask and placed back inside the cell culture incubator at 37°C at 5% CO_2 air atmosphere to incubate. Medium changes on SH-SY5Y cells occurred approximately every 2 days.

2.1.4 Passaging/ Splitting SH-SY5Y Cells

As the SH-SY5Y cells began to grow exponentially, passaging or splitting flasks was necessary for maintenance. Aliquots of SH-SY5Y basic culture medium, phosphate-buffer saline 1X (PBS), and Trypsin-EDTA were warmed in the cell culture incubator (37°C) for a minimum of 20 minutes. Using aseptic cell culture technique and sterilizing with 70% ethanol, “old” media was removed from the cell flask and discarded. Cells were then washed in 3mL of PBS by capping the flask and using rocking motions 3-4 times. The PBS was then pipetted out of the flask and discarded. Next, 2mL of Trypsin-EDTA was pipetted into the flask of cells and incubated in the cell culture incubator (37°C) for 2 minutes. Once the incubation time was over, the flask was gently agitated by tapping the sides, and then visualized under a microscope (Nikon Inverted Routine Microscope ECLIPSE Ts2). When loose cells were confirmed by visualizing under the microscope, the flask was placed back inside the cell culture hood where 8mL of “new” basic culture medium was added to the flask. The flask was rocked back and forth 3-4 times. Again, using aseptic cell culture technique, 5mL of cells were added to another sterile 75cm^2 flask. The remaining 5mL of cells was added to a second sterile

75cm^2 flask. Then, 15mL of new basic culture medium was added to each flask. Each vented cap was secured tightly onto the two flasks and placed back in the cell incubator. Cells were passaged/ split once confluency reached ~90%.

2.1.5 Freezing SH-SY5Y Cells

First, aliquots of PBS and Trypsin-EDTA were warmed in the cell culture incubator (37°C) for a minimum of 20 minutes. Simultaneously, an aliquot of SH-SY5Y basic culture medium and 1mL cryogenic storage vial were placed in a bucket of ice for a minimum of 20 minutes. Using aseptic cell culture technique, old media was removed from an SH-SY5Y 75cm^2 flask and discarded. Cells were rinsed with 3mL of warm PBS, using rocking motions 3-4 times. The PBS was pipetted out of the flask and discarded. Next, 2mL of Trypsin-EDTA was added to the flask and incubated in the cell culture incubator (37°C) for 2 minutes. After the incubation time was completed, the flask was agitated by gently tapping the sides. Loose cells were visualized and confirmed under a microscope (Nikon Inverted Routine Microscope ECLIPSE Ts2). The flask was placed back inside the cell culture hood, where 7mL of cold SH-SY5Y basic culture medium was added. The cap was secured, and the flask was gently rocked 3-4 times. Then, cells were pipetted into a 15mL conical centrifuge tube and inverted 3-4 times. The tube of cells was centrifuged at 1,000 x g for 2 minutes at 20°C. After 2 minutes of centrifugation, a pellet of cells was seen at the bottom of the tube. The tube was placed back inside the cell culture hood, where the supernatant was discarded. The pellet was resuspended in 900µL of cold basic culture medium. Then, 45µL of dimethyl sulfoxide (DMSO) was added to the 15mL conical centrifuge tube. Cells were pipetted into a chilled cryogenic storage vial, securing the lid tightly, and placed in an ice bucket for at

least 10 minutes. Once the cryogenic vial of cells had been in ice for 10 minutes, the vial was placed in -20°C for at least 20 minutes. Following 20 minutes, the vial of cells was then placed in the -80°C for a minimum of 24 hours before being stored in liquid nitrogen vapor phase in Eastern Kentucky University's cold room (Science Building 5210). Freezing vials of SH-SY5Y cells were done when flask confluency was ~90%.

2.2 Treatment Conditions

2.2.1 Treatment Incubation Times, Concentrations, and Groups

For acute dosing, cells were incubated with drug for 5 minutes. Chronic dosing involved cell incubation with drug for 21 hours. Treatments were prepared by pipetting 5mL of SH-SY5Y basic culture medium into 5mL macrocentrifuge tubes (8 total). Then, appropriate concentrations of each drug were pipetted into the correct tube, determined by the replicate format. Drug stocks (1mM) of CBD, Quinpirole, and WIN 55, 212-2 were prepared by Dr. Middleton. Drug treatment concentrations can be found in **Table 1**. Treatment group organization for acute and chronic treatments, replicates 1-3 can be found in **Table 2-4**.

Table 1. Final treatment group concentrations from drug stocks

Drug	Stock Concentration	Final Volume	Final Concentration	Required Volume Used
CBD	1mM	5mL	5μM	25μL
Quinpirole	1mM	5mL	100nM	0.5μL
WIN 55, 212-2	1mM	5mL	10μM	50μL
Vehicle	70% EtOH	5mL	70% EtOH	50μL

Table 2. Replicate 1 format for acute and chronic treatment groups

Treatment Conditions	Vehicle	CBD	D2 Agonist (Quinpirole)	CB1 Agonist (WIN 55, 212-2)
1	X			
2				X
3		X		
4		X		X
5			X	
6			X	X
7		X	X	
8		X	X	X

Table 3. Replicate 2 format for acute and chronic treatment groups

Treatment Conditions	Vehicle	CBD	D2 Agonist (Quinpirole)	CB1 Agonist (WIN 55, 212-2)
1	X			
2		X		
3				X
4			X	
5		X		X
6		X	X	
7		X	X	X
8			X	X

Table 4. Replicate 3 format for acute and chronic treatment groups

Treatment Conditions	Vehicle	CBD	D2 Agonist (Quinpirole)	CB1 Agonist (WIN 55, 212-2)
1	X			
2			X	X
3		X	X	X
4		X		X
5			X	
6				X
7		X		
8		X	X	

2.3 Lysate Preparation, Measuring Protein Concentration, and Preparing Samples

2.3.1 Lysate Preparation for SH-SY5Y Cells

Lysate preparation was done either 5 minutes (acute) or 21 hours (chronic) after initial treatment incubation. Utilizing aseptic cell culture technique, treatments were removed and discarded. Then, cells were washed twice with 3mL cold PBS using rocking motions 3-4 times. Cells were placed on ice and lysed with 1mL of radioimmunoprecipitation assay buffer (RIPA buffer). The flask was rocked every minute while on ice for a total of 5 minutes. Cells were then harvested using a cell scraper, pipetted into a 1.5mL microcentrifuge tube, and placed in ice until all treated cells were collected. Once all 8 lysates were collected, they were centrifuged at 14 x g for 20 minutes in the “cold room” (Science Building 5203). After centrifugation, lysates were clarified by removing the viscous layer at the bottom of the microcentrifuge tube. After clarification, lysates were sonicated (QSONICA Q55 Sonicator) by 5 second pulses x 3. In-between sonicating each lysate, the sonicator was cleaned with 70% ethanol and distilled (DI) water.

2.3.2 Bradford Protein Assay and Preparing Samples

First, 2mL of Coomassie Brilliant Blue G-250 (Bradford Reagent) was pipetted into a 15mL conical centrifuge tube. Then, the Bradford Reagent was diluted using 8mL DI water and vortexed for approximately 20 seconds. A 96-well microplate was obtained and 199µL of diluted Bradford Reagent was pipetted into lanes A1, A2, A3, A4, A5, B1, B2, B3, B4, B5, C1, C2, C3, C4, and C5. Next, 1µL of prepared bovine serum albumin (BSA) standards (1mg, 2mg, 3mg, and 4mg) were pipetted into columns 2 (1mg), 3

(2mg), 4 (3mg), and 5 (4mg). The 96-well microplate was then read by Eastern Kentucky University's Epoch™ 2 Microplate Spectrophotometer. The results were then exported to Microsoft Excel where a series of calculations were computed. Columns 1-5 were averaged (only for rows A, B, and C) separately and used to create a scatterplot. A trendline and equation for the slope intercept ($y=mx + b$) was inserted. Only plates with an R^2 of 0.97 or above were used. If the R^2 was or above 0.97, then the plate was used to transpose standard concentrations and averages for treated protein samples. Furthermore, 199µL of diluted Bradford Reagent was pipetted in a similar fashion for lysates. The plate was re-run with new numbers for the BSA standards and lysates. A new scatterplot with updated numbers was computed, as well as its trendline, slope intercept equation, and R^2. For each lysate, the concentration of protein was calculated (**Equation 1**). Additionally, the amount of lysate required to get 40µg of protein per each sample was calculated (**Equation 2**). Lastly, the volume of 6X and 1X Laemmli buffer (**Equation 3 and 4**) was also calculated for each sample.

Equation 1. Concentration of protein

$$\frac{(\text{Sample Average} - \text{b})}{\text{m}}$$

Equation 2. Amount of lysate needed for 40µg of protein per well

$$\frac{40}{\text{Concentration of Protein}}$$

Equation 3. Amount of 6X Laemmli buffer to add

$$\frac{\text{Amount of Lysate Needed for 40µg of Protein Per Well}}{5}$$

Equation 4. Amount of 1X Laemmli buffer to add

Sample with Highest Volume − (Amount of Lysate Needed for 40µg of Protein Per Well + Amount of 6X Laemmli Buffer to Add)

2.4 Protein Detection, Probing for CB1 Cannabinoid Receptor, Probing for D2 Dopamine Receptor, and Probing for Beta-Tubulin Loading Control

2.4.1 Western Blot and Gel Electrophoresis Protocol

Protein samples for each treatment group were prepared after performing a Bradford Protein Assay and utilizing **equations 1**, **2**, **3**, and **4**. Samples for each treated group included 40µg of protein from lysate, 6X Laemmli buffer, and 1X Laemmli buffer (excluding the sample with the highest volume), which were pipetted in a 1.5 microcentrifuge tube on ice. Samples were then boiled on a dry bath multi heat block at 95°C for 5 minutes. Samples were loaded in a 10% SDS-PAGE gel according to replicate number (see **Table 2-4**). Each gel contained 5µL of a molecular weight marker (Precision Plus Protein™ Dual Color Standard) in lane 1. SDS-PAGE gels were run at 150 volts for 1 hour.

2.4.2 Western Blot Transfer Method

Protein transferring after gel electrophoresis was performed by using Dr. Cormier's Lab Trans-Blot® Turbo™ Transfer System. First, a Polyvinylidene difluoride (PVDF) membrane was submerged in Absolute Ethanol (200 proof) until clear. Then, two transfer stacks provided by the Trans-Blot® Turbo™ Transfer System and clear PVDF membrane were submerged in 1X transfer buffer for 3 minutes. Within the system's cassette, a blotting sandwich was assembled. Each blotting sandwich

consisted of a saturated top ion reservoir stack, SDS-PAGE gel with separated proteins, saturated blotting membrane (PVDF), and saturated bottom reservoir stack. A roller was used on each layer to minimize air trapped between layers. Next, the cassette was placed back in the machine and the correct protocol chosen. Each transfer took 7 minutes to complete.

2.4.3 Probing for CB1 Cannabinoid Receptor and D2 Dopamine Receptor

Once successful protein transferring was completed, the PVDF membrane was blocked in 7mL of 5% nonfat dried milk diluted in 0.1% PBS-tween (blocking buffer) for 1 hour. After blocking was finished, direct detection for the CB1 Cannabinoid and D2 Dopamine Receptors was accomplished by incubating in primary antibody (CB1 Cannabinoid Receptor primary antibody dilution: 1:200 and D2 Dopamine Receptor primary antibody dilution: 1:1000) overnight in the “cold room” (Science Building 5203) on a rocking platform shaker.

After overnight incubation with primary antibody, 4 washes were performed using 7mL of 0.1% PBS-tween for 10 minutes each on a rocking platform shaker at room temperature. Then, indirect detection for CB1 Cannabinoid and D2 Dopamine Receptors was done by incubating the membrane in 7mL of secondary antibody (dilution: 1:20000) for 1.5 hours at room temperature. Once incubation with secondary antibody was finished, 4 more washes occurred with 7mL 0.1% PBS-tween at 10 minutes each. Proteins were then ready to be visualized.

2.4.4 Imaging Protein Gels

Protein blots were viewed using the Cormier Lab's Bio-Rad ChemiDoc MP imaging system. Then, blots were analyzed using Image Lab Software. The signal accumulation mode for each protein blot was set at 2 seconds for the first image and 90 seconds for the last image at 75 images total. SuperSignal™ West Femto Maximum Sensitivity Substrate was used for low femtogram protein level detection by chemiluminescence.

2.4.5 Stripping and Reprobing Western Blot Membranes for β-tubulin

Western blots were stripped after CB1 and D2 Receptor detection for imaging of a protein loading control (β-tubulin). Protein blots were rocked on a rocking platform shaker in stripping buffer (Restore™ Western Blot Stripping Buffer) for 10 minutes. Next, protein blots underwent 3 "quick" washes by pipetting 7mL of 0.1% PBS-tween and rocking 2-3 times gently by hand. Once quick washes were finished, 2 washes with 0.1% PBS-tween for 10 minutes each were performed. Protein blots were then rocked in blocking buffer for 10 minutes for a total of two times. Afterwards, membranes were incubated in primary antibody (dilution: 1:200) overnight in the "cold room" (Science Building 5203) on a rocking platform shaker. The next day, 4 washes were performed using 7mL of 0.1% PBS-tween for 10 minutes each on a rocking platform shaker at room temperature. Then, indirect detection for β-tubulin was done by incubating the membrane in 7mL of secondary antibody (dilution: 1:12000) for 1.5 hours at room temperature. Once incubation with secondary was finished, 4 washes occurred with

7mL 0.1% PBS-tween at 10 minutes each. Proteins were then ready to be visualized by the same procedure explained in **2.4.4**.

2.5 Immunostaining and Microscopy of SH-SY5Y Cells

2.5.1 SH-SY5Y Cell Fixing and Immunostaining Procedure

Using aseptic cell culture technique inside a cell culture hood and sterilizing with 70% ethanol, old culture medium from a 75cm^2 vented flask of SH-SY5Y cells was removed. Next, cells were washed in 3mL of warm PBS. After washing cells, 2mL of Trypsin-EDTA was added to the flask and incubated for 2 minutes. Once incubation with Trypsin-EDTA was finished, the flask was gently agitated by tapping the sides. Approximately 8mL of new culture medium was added to the flask. Then, 100µL of cells were added to an 8-well slide containing a removable polystyrene media chamber. Eight-well slides were coated with 100µL of Poly-D-Lysine (PDL) the day before usage. Each well containing 100µL of cells were given an additional 200µL of warm culture medium and placed in a cell culture incubator overnight (37°C, 5% CO_2). Flasks of SH-SY5Y cells were brought to 80% confluency for immunostaining purposes.

The following day, each well was treated with drug. For acute dosing, 5-minute incubation times were used. For chronic dosing, a 21-hour incubation time was used. For each well, 500µL of treatment was added and incubated at 5 minutes or 21 hours. After incubation, drug treatments were pipetted out of each well and washed with 200µL of PBS two times. Cells were fixed using two methods. The first method utilized paraformaldehyde, while the second method utilized methanol. Once each well was washed, the PBS was removed and 200µL of 4% paraformaldehyde (PFA) was added

to fix cells. An additional 50µL of PFA was added to each well and incubated for 25 minutes at room temperature. After incubation, the PFA was removed from each well and washed with 250µL of PBS. The PBS was then removed from each well and replaced with 500µL of PBS to be stored at 4°C. When using methanol to fix cells, the 8-well slide was placed on ice after being washed with 200µL of PBS. Then, 300µL of ice-cold methanol was pipetted into each well and incubated for 20 minutes. Wells were washed 3 times for 5 minutes each with 300µL of PBS.

Once cells were fixed, the remaining PBS in each well was removed. Next, 200µL of permeabilization buffer (10% Normal Goat Serum and 0.1% PBS-triton) was added to each well and incubated for 1 hour. After 1 hour, the permeabilization buffer was removed and 200µL of primary antibody for CB1 Cannabinoid Receptor (dilution: 1:200) and D2 Dopamine Receptor (dilution: 1:1000) diluted with permeabilization buffer was added. Overnight incubation with primary antibody occurred in 4°C.

The next day, the primary antibody was removed from each well and 2 x 5-minute washes with 0.1% PBS-triton were done. In addition, 2 x 5-minute washes with straight PBS were completed before adding Alexa Fluor™ Plus 488 goat anti-rabbit IgG (H+L) for CB1 Cannabinoid Receptor and Alexa Fluor™ 568 goat anti-mouse IgG (γ1) for indirect detection of D2 Dopamine Receptor. A 1:1000 dilution was used for both Alexa Fluor 488 and 568. When using Alexa Fluor 488 and 568, all techniques were carried out in a dark room. The secondary antibodies were incubated for 1.5 hours before undergoing 4 x 5-minute washes with straight PBS. Once the last wash was accomplished and removed, the polystyrene media chamber was separated from the glass slide and discarded. Next, a drop of mounting media was placed on each well

(200µL total) and a glass coverslip was placed on top of the slide. Clear fingernail polish was used to coat the sides of the slide and coverslip. The slide was left overnight to dry in a dark room. Receptor activity was then visualized using Eastern Kentucky University's Zeiss LSM 800 Confocal Microscope System with Airyscan Detector.

2.5.2 Confocal Microscopy

Confocal images were collected using Zen 2.6 software. Following calibration, glass slides were loaded facing downward on the stage. Once the 5x and 10x objectives were focused, the 20x objective was used to collect sample images. To prevent photobleaching, the reflected light was turned off manually in-between collecting images. Each laser and detector were set to detect DAPI (blue at 405nm) and Alexa Fluor™ 568 (red at 568nm). Two tracks were used to measure each fluorophore, preventing overlap between the two colors. Scans were performed unidirectionally at a speed 1-2 below the objective maximum to maintain image quality. Optimum pixel size was generated using Zen software where 8-bit images were taken.

2.6 Measuring Cyclic Adenosine Monophosphate (cAMP) in SH-SY5Y Cells

Cyclic Adenosine Monophosphate (cAMP) Enzyme-Linked Immunosorbent Assay (ELISA) kits were purchased from ENZO Life Sciences. A 75cm^2 flask of SH-SY5Y cells were plated in 6-well cell culture plates and incubated overnight in the cell culture incubator (37°C and 5% CO_2). For acute drug treatment, cells were incubated for 5 minutes. For chronic drug treatment, cells were incubated for 21 hours. Once incubation times for treatments were completed, drug was removed, and cells were washed once with PBS. To induce cell lysis, cells were incubated with 1mL of 0.1M

hydrochloric (HCl) acid for 10 minutes at room temperature. Cell lysis was confirmed by visualization under a microscope (Nikon Inverted Routine Microscope ECLIPSE Ts2). Each well was scraped, and the cells were resuspended 4-5 times. Then, cells were transferred to a 1.5mL microcentrifuge tube and centrifuged at 800 x g for 15 minutes. The supernatant was removed and split between two 1.5mL microcentrifuge tubes (200µL was used for the assay procedure, while the remaining supernatant was frozen at -20°C).

All components included in the cAMP kit were brought to room temperature at least 30 minutes prior to running the assay. First, 5 glass test tubes were labeled “1”, “2”, “3”, “4”, and “5”. Next, cAMP standards were prepared as shown in **Figure 2**.

Standards were used within 60 minutes of preparation.

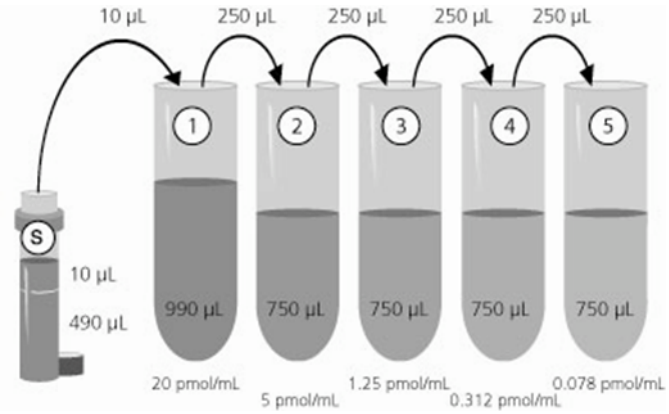

Figure 2. **How Standards Were Prepped**. Five glass test tubes were labeled 1-5 in chronological order. Glass test tube 1 contained 990µL of HCl, while tubes 2-5 contained 750µL of HCl. The first dilution started with 10µL of cAMP standard (S) into test tube 1. Test tube 1 was then vortexed for 5 seconds. Next, 250µL of standard 1 was pipetted into test tube 2 and vortexed for 5 seconds. This process was repeated for each test tube as seen in the figure above. Image from product manual for the cAMP ELISA kits, Enzo Life Sciences.

96-well microplates were used to conduct cAMP assays. Wells were coated in Goat-anti-Rabbit IgG antibody (**Figure 3**). Each plate had wells designated for “Blanks”,

“Nonspecific Binding” (NSB), “Total Activity” (TA), zero pmol/mL standard (Bo), and standard curve. All samples were run in duplicate. Since HCl was used for cell lysis and standard production, 50µL of neutralizing reagent was added to all wells except for TA and “blank” wells. Next, 100µL of HCl was pipetted into NSB and Bo wells. Following this, 100µL of each standard and samples were added to the appropriate wells. After samples were added to wells, 50µL of cAMP conjugated to alkaline phosphatase (Blue Conjugate) was added to each well, except blank and TA wells (**Figure 3**). Then, 50µL of rabbit polyclonal cAMP antibody (Yellow Solution) was added to each well except for blank, TA, and NSB wells (**Figure 3**). At this point, all wells appeared green in color except for NSB wells which appeared blue. In addition, blank and TA wells were clear. Microplates were sealed and incubated for 2 hours at room temperature on an orbital shaker (500rpm). Following the 2-hour incubation period, contents in the microplate were emptied and the wells were washed with wash buffer (Tris-buffered saline containing detergents) for a total of 3 times. After the last wash, the wash buffer was removed, and the microplate was firmly tapped upside down on paper towels to remove all remaining wash buffer. Blue conjugate (5µL) was added to TA wells. Next, 200µL of p-nitrophenyl phosphate (pNpp substrate) solution was added to all wells. Microplates were then incubated without shaking for 1 hour at room temperature. Lastly, 50µL of trisodium phosphate in water (stop solution) was added to all wells.

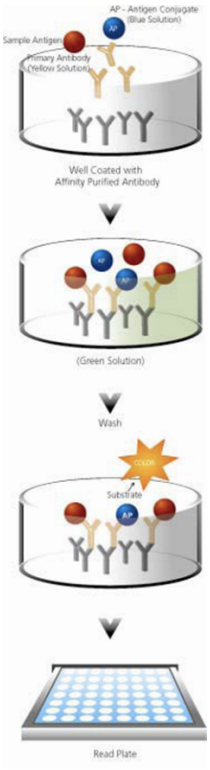

Figure 3. **96-Well Plate Organization and Sample Preparation.** A) Each well in a 96-well microplate were coated in Goat-anti-Rabbit IgG antibody. cAMP antibody and conjugate were added to all wells. B) The interaction between cAMP conjugate, primary antibody, and secondary antibody was seen by a green color change in solution in wells. C) Addition of pNpp solution (detection of phosphatase activity in association with cAMP) resulted in a yellow color change in wells. D) Microplates were then read on an Epoch™ 2 Microplate Spectrophotometer at a wavelength of 405nm. Image from product manual for the cAMP ELISA kits, Enzo Life Sciences.

The optical density of samples was read using Eastern Kentucky University's Epoch™ 2 Microplate Spectrophotometer at a wavelength of 405nm. Data was then transferred to Microsoft Excel where readings from duplicate samples were averaged. Maximum binding (Bo) is the maximum amount of cAMP conjugate the antibody can interact with. Non-specific binding (NSB) is the amount of binding of the conjugate to the well, in the absence of antibodies. A ratio was generated using the absorbance of the unknown samples divided by the maximum binding (%B/Bo). Average blank optical densities were subtracted from all samples and a standard curve of %B/Bo was

generated. Each plate was able to run n=2 acute treatments and n=2 chronic treatments. Two complete plates were used for a total of n=4 per treatment condition.

Chapter 3

Results

3.1 Western Blot Results

SH-SY5Y cells were treated by either acute (5-minute) or chronic (21-hour) incubation times with drug treatments (n=3). Each replicate maintained a different organization to account for pipetting error (**Tables 2-4**). Then, cells were lysed and prepared by performing a Bradford Protein Assay. Protein samples were boiled for 5 minutes at 95°C and loaded into a 10% SDS-PAGE gel, which was run at 150V for approximately 1 hour. Protein blots were probed directly for CB1 and D2 receptors and visualized using chemiluminescence. Representative Western blots analyzing CB1 protein levels during acute and chronic drug treatments can be found in **Figures 4** and **5**. Representative Western blots analyzing D2 protein levels during acute and chronic drug treatments can be found in **Figures 6** and **7**.

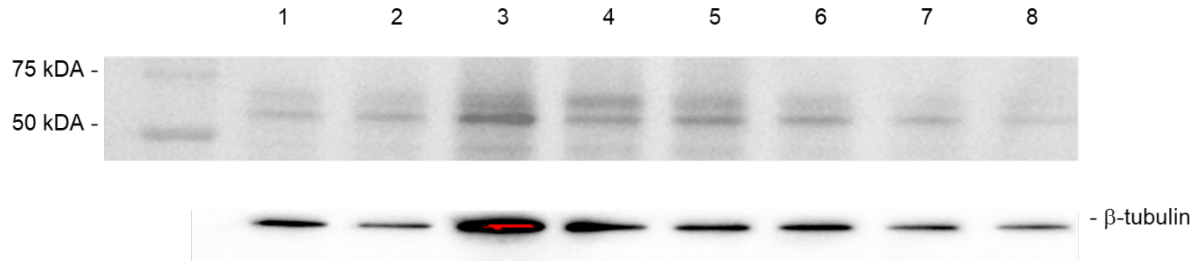

Figure 4. Representative CB1 receptor Western blot analyses demonstrating acute treatment of SH-SY5Y cells with WIN, CBD, and/ or QUIN. SH-SY5Y cells were treated for 5 minutes with drug treatments found in **Table 4**. Lysates were then used for Western blot analysis using an antibody specific for Cannabinoid Receptor CB1 (c-term). The protein blot was stripped and probed for the protein loading control β-tubulin (bottom panel). Protein bands specific to Cannabinoid Receptor CB1 (c-term) are found at ~60 kDA. Lane 1 = Vehicle, Lane 2 = WIN + QUIN, Lane 3 = WIN + QUIN + CBD, Lane 4 = WIN + CBD, Lane 5 = QUIN alone, Lane 6 = WIN, Lane 7 = CBD, Lane 8 = QUIN + CBD.

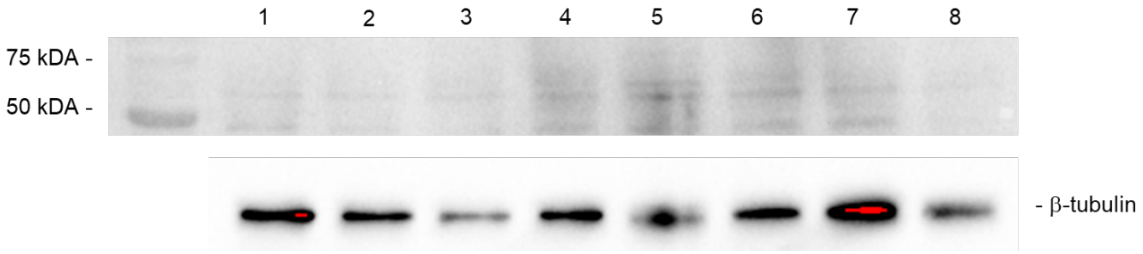

Figure 5. Representative CB1 receptor Western blot in SH-SY5Y cells chronically treated with WIN, QUIN, and/ or CBD. SH-SY5Y cells were treated for 21 hours with drug treatments found in **Table 4**. Lysates were then used for Western blot analysis using an antibody specific for Cannabinoid Receptor CB1 (c-term). The protein blot was stripped and probed for the protein loading control β-tubulin. Protein bands specific to Cannabinoid Receptor CB1 (c-term) are found at ~60 kDA. Lane 1 = Vehicle, Lane 2 = WIN + QUIN, Lane 3 = WIN + QUIN + CBD, Lane 4 = WIN + CBD, Lane 5 = QUIN alone, Lane 6 = WIN, Lane 7 = CBD, Lane 8 = QUIN + CBD.

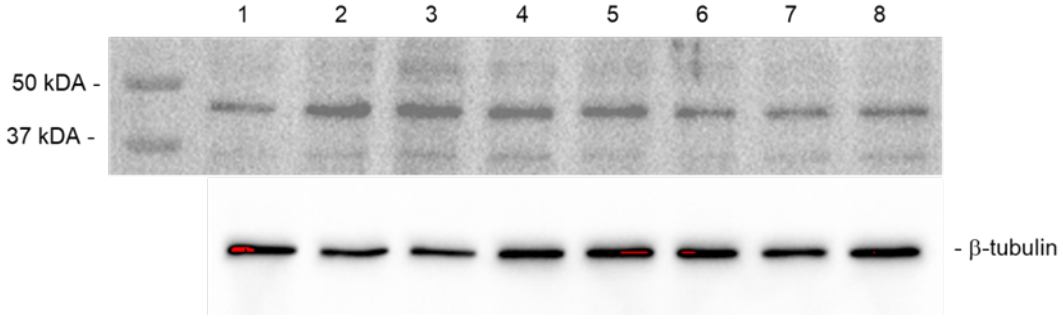

Figure 6. Representative D2 receptor Western blot in SH-SY5Y cells acutely treated with WIN, QUIN, and/ or CBD. SH-SY5Y cells were treated for 5 minutes with drug treatments found in **Table 3**. Lysates were then used for Western blot analysis using an antibody specific for Anti-Dopamine D2 Receptor. The protein blot was stripped and probed for the protein loading control β-tubulin (bottom panel). Protein bands specific to Anti-Dopamine D2 Receptor are generally found between 40-51 kDA. Lane 1 = Vehicle, Lane 2 = CBD, Lane 3 = WIN, Lane 4 = QUIN, Lane 5 = WIN + CBD, Lane 6 = CBD + QUIN, Lane 7 = WIN + QUIN + CBD, Lane 8 = WIN + QUIN.

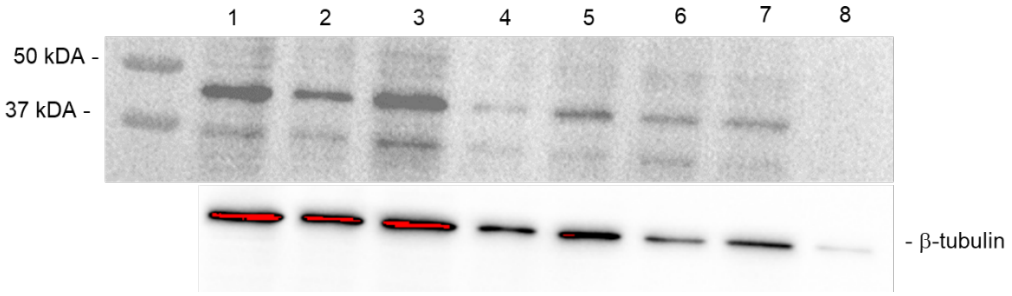

Figure 7. Representative D2 receptor Western blot in SH-SY5Y cells chronically treated with WIN, QUIN, and/ or CBD. SH-SY5Y cells were treated for 5 minutes with drug treatments found in **Table 2**. Lysates were then used for Western blot analysis using an antibody specific for Anti-Dopamine D2 Receptor. The protein blot was stripped and probed for the protein loading control β-tubulin (bottom panel). Protein bands specific to Anti-Dopamine D2 Receptor are generally found between 40-51 kDA. Lane 1 = Vehicle, Lane 2 = WIN, Lane 3 = CBD, Lane 4 = WIN + CBD, Lane 5 = QUIN, Lane 6 = WIN + QUIN, Lane 7 = CBD + QUIN, Lane 8 = WIN + QUIN + CBD.

3.2 Preliminary Confocal Image Data

SH-SY5Y cells were grown in 8-well slides containing a removable polystyrene media chamber and acutely (5-minute) treated with drug treatments found in **Table 3**. Then, cells were incubated in anti-dopamine D2 receptor antibody overnight in 4°C. Cells were fixed using methanol and tagged with Alexa Fluor™ 568. Additionally, cell nuclei were stained using DAPI. A representative confocal image showing dopamine D2 receptor localization can be found in **Figure 8**.

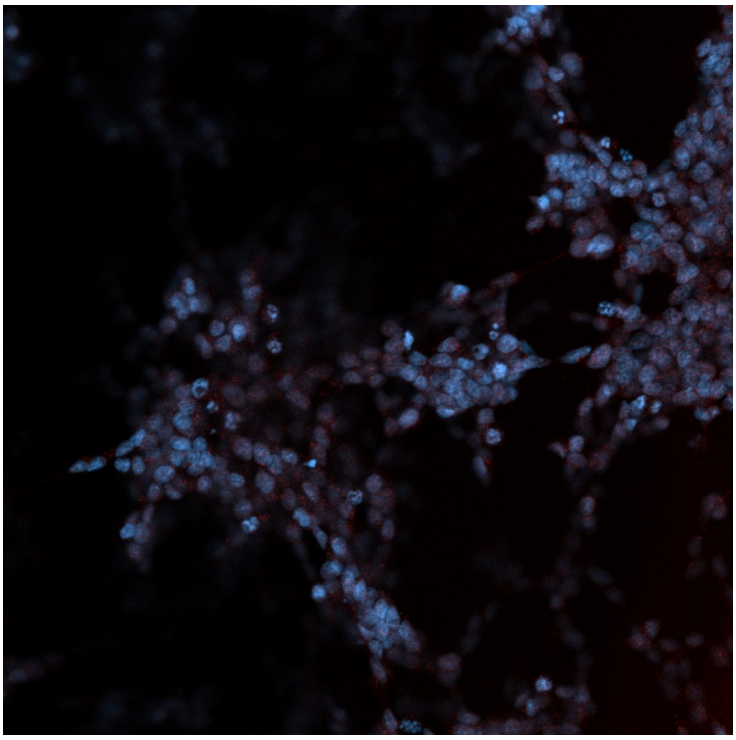

Figure 8. Representative confocal microscopy image of localization of dopamine D2 receptors in treated SH-SY5Y cells. SH-SY5Y cells were treated for 5 minutes with treatment groups found in **Table 3**. Cells were fixed using methanol and direct detection for D2 dopamine receptor occurred overnight in 4°C. Cells were tagged with Alexa Fluor™ 568 goat anti-mouse IgG (γ1) and nuclei stained with fluorescent DAPI to visualize nuclei. Dopamine D2 receptor localization is seen on the cell surface of cells treated with CBD and quinpirole.

3.3 Preliminary cAMP Level Data in Treated SH-SY5Y Cells

cAMP level expression in SH-SY5Y cells were measured using a cAMP complete ELISA kit purchased from Enzo Life Sciences. Next, optical densities of all samples were read using an Epoch™ 2 Microplate Spectrophotometer at a wavelength of 405nm. Data was then compiled into Microsoft Excel. A bar graph of all treatment groups for acute and chronic treated cells can be found in **Figure 9**.

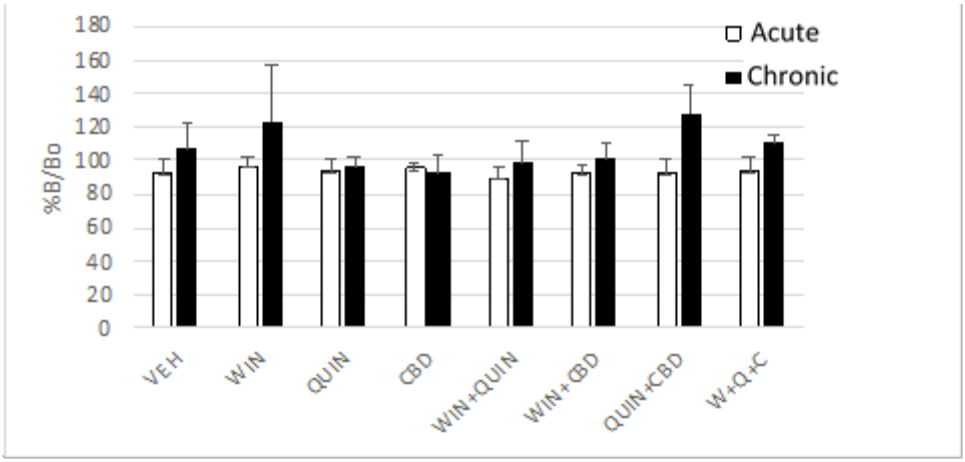

Figure 9. Effect of acute and chronic drug treatment on cAMP levels in SH-SY5Y cells. Data are expressed as % sample binding (B) divided by total binding (Bo). Acute treatment = white bars. Chronic treatment = black bars. Data are presented as mean ± SEM.

Chapter 4
Discussion, Conclusion, Future Research Directions

4.1 Western Blot Analysis for the Presence of Cannabinoid Receptor CB1 in Acute Treatment of SH-SY5Y Cells

Following the acute treatment paradigm described above, it appears that acute treatment with WIN alone or in combination with CBD and/ or QUIN does not have an impact on total CB1 receptor protein concentration (**Figure 4**). Differences in CB1 receptor band widths in **Figure 4** are likely due to variation in protein loading controls, as shown by variation in the β-tubulin bands. A decrease in protein concentration would indicate metabolism of a protein (23). A 5-minute treatment is unlikely to decrease protein concentration; however, it is possible that this treatment time could induce internalization of receptors (24).

4.2 Western Blot Analysis for the Presence of Cannabinoid Receptor CB1 in Chronic Treated SH-SY5Y Cells

Chronic treatment of SH-SY5Y cells appears to alter CB1 receptor band density in several of the treatment groups (**Figure 5**). Specifically, the cells treated with WIN + QUIN, WIN + CBD, WIN + CBD + QUIN, and QUIN + CBD appear to have a lower CB1 receptor protein concentration. However, it appears that the vehicle treatment may have a lower CB1 receptor concentration. Therefore, further analyses will need to be performed.

4.3 Western Blot Analysis for the Presence of Dopamine D2 Receptor in Acute Treated SH-SY5Y Cells

Following the acute treatment paradigm, D2 dopamine receptors are observed in SH-SY5Y cells (**Figure 6**). It appears that acute treatment does not alter D2 receptor total protein levels at any of the treatment conditions.

4.4 Western Blot Analysis for the Presence of Dopamine D2 Receptor in Chronic Treated SH-SY5Y Cells

D2 dopamine receptor levels following chronic treatment of the SH-SY5Y cells are shown in **Figure 7**. It appears that chronic treatment does not alter D2 receptor total protein levels at any of the treatment conditions. While it appears that WIN + CBD (lane 4) and WIN + QUIN + CBD (lane 8) have lower amounts of protein expression, the β-tubulin levels are also lower. Further data analysis across all Western blots will need to be performed to identify true differences in D2 levels.

4.5 Preliminary Data: Confocal Image Showing Dopamine Receptor D2 Localization in SH-SY5Y Cells

Confocal microscopy was performed to determine D2 dopamine receptor localization in SH-SY5Y cells. To date, one image has been obtained from SH-SY5Y cells acutely treated with CBD + QUIN (**Figure 8**). The image demonstrates that D2 receptors are present in the SH-SY5Y cells. After acute treatment with CBD + QUIN the D2 receptors appear to be localized primarily on the cell surface. Cells have been treated with the other drug treatments and fixed for confocal. Comparisons of treatment groups will be performed following imaging of all treatment groups.

4.6 Preliminary Data: cAMP Signaling in Acutely and Chronically Treated SH-SY5Y Cells

The effect of acute and chronic treatment conditions is shown in **Figure 9**. Data indicate that at all conditions, there is a robust production of cAMP in the SH-SY5Y cells with maximum cAMP production at all treatment conditions for both acute and chronic. Because CB1 and D2 receptors signal through Gi/Go G-proteins, a decrease in cAMP activity was expected for WIN and QUIN alone for both acute and chronic treatment conditions. However, neither condition inhibited cAMP (**Figure 9**). Additionally, previous studies have shown that a combination of WIN + QUIN stimulates cAMP; this was not observed in this experiment (25). It appears that QUIN and CBD may increase cAMP levels. However, for both plates, the cAMP levels for the acute and chronic treatment groups fell outside the range of the standard curve. Therefore, these data will need to be repeated and the samples will need to be diluted to fall into the proper range. Statistical analyses will then be used to compare treatment conditions.

4.7 Conclusion and Future Experiments

While performing Western blot analyses, multiple non-specific bands would appear on protein blots which could be due to several reasons. Post-translational modifications such as phosphorylation could have been one reason why non-specific bands were showing up (26). However, another reason for such bands could have been the presence of endogenous proteolytic and phospholytic enzymes sometimes released during cell lysis (27). To test this theory, a protease inhibitor was used during SH-SY5Y lysis preparation. By incorporating a protease inhibitor into the protocol, a cleaner blot was observed with less non-specific bands. Unfortunately, this was implemented

following lysate preparation for replicates 1-3, so it was not incorporated into the procedures used to obtain protein blots for this thesis. Future experiments will utilize this method to account for non-specificity. Additionally, timing issues due to faulty antibodies and shipping delays caused protein blots for dopamine D2 receptors in treated SH-SY5Y cells to be only preliminary data. Western blot analysis for D2 receptors was successful twice in non-treated SH-SY5Y cells. However, no visualization at the D2 receptor band was able to be seen in replicates 1-3 for acute and chronic treatments. To account for this issue, two different D2 antibodies were ordered. These antibodies did not work, which was confirmed by performing Western blot analyses using the same lysates and reagents for CB1 receptor. Western blots probing for CB1 were successful, showing that it was indeed a faulty D2 antibody leading to no visible protein bands. Additionally, immunostaining with Alexa Fluor™ 488 was not possible due to issues with shipping and production. Because of these reasons, looking for colocalization between CB1 and D2 receptors was not possible. Future explorations include, but are not limited to, incorporating protease inhibitors into lysate preparation, running Western blot analyses to probe for D2 dopamine receptors, and performing confocal microscopy to look for colocalization between CB1 and D2 receptors.

Further exploration into the potential of SH-SY5Y cells as a model for CB1 and D2 signaling is also necessary. It is important to determine that the appropriate G-proteins are present in these cells to confirm that drug action mimics effects seen in brain. Additionally, the SH-SY5Y cells develop neurites and differentiate. Treatments, like retinoic acid, can be added to the cells while they are growing to push them towards becoming more dopaminergic (28) (29). These studies could be repeated using these

types of treatments to further determine the impact of the dopamine system on both CB1 receptor function and CBD signaling.

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