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Oral Cbd Administration: Assessing Bioavailability And Behavioral Outcomes In A Rodent Model

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ORAL CBD ADMINISTRATION: ASSESSING BIOAVAILABILITY AND BEHAVIORAL OUTCOMES IN A RODENT MODEL

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DEDICATION

This work is dedicated to my late mother Susan Skully, who passed before I could finalize my work. She was always my greatest support and only ever wanted the best for me. Having her in my corner drove me to achieve more than I ever thought possible. I intend to continue on that path, to continue to work hard day in and day out, to continue to make her proud of her son.

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Chapter 1: Introduction

1.1 Cannabidiol's Place in the Market

Cannabis produces both psychedelic and medicinal effects and has become one of the most widely used recreational drugs (M. Lee & Hancox, 2011). While these effects are unique in nature, they are generally attributed to two of the main cannabinoids: delta-9-tetrahydrocannabinol (THC) and cannabidiol (CBD). Despite widespread recreational and therapeutic popularity, cannabis remains a federally illicit drug in the U.S. However, regulations on isolated CBD have recently been loosened by Congress through labeling of the hemp-based derivative (<.03% THC concentration) as an agricultural commodity. Taking this hemp derived CBD out from under the schedule one classification of marijuana was introduced in the 2018 Farm Bill and has resulted in increased market presence of CBD. A wide variety of natural and synthetic CBD products now hold their fair share of the market: oral supplements (including pills, oils, and ingestible powders), topical solutions (ointments, body oils, and lotions), and various other options. However, inconsistencies in purity, quality, and CBD content or concentrations, highlight the need for more transparency in marketed CBD products. A once largely-ignored component of cannabis, CBD has recently entered the spotlight for its vast therapeutic potential without psychoactive effects. Hemp-derived CBD has been marketed as having a multitude of therapeutic properties, including anxiolysis, antinociception, anti-inflammation, and neuroprotection. There is a wide array of anecdotal support for such claims with minimal but expanding scientific backing in both humans (White, 2019) and rodents (Costa, Trovato, Comelli, Giagnoni, & Colleoni, 2007; Pagano et al., 2016; Zieba et al., 2019). Although there are many current studies that focus on the interaction of THC and CBD as found in different strains of cannabis, CBD's recent popularity spike as a safe and isolated supplement (Todd et al., 2017), guided research interests for this study.

1.2 Pharmacokinetics of CBD

First, it is essential to understand how CBD is processed in the body. Deiana et al. (2012) utilized both rat and mouse models to assess the concentration levels of varying cannabinoids, including CBD, in both blood plasma and in the brain. Oral or intraperitoneal (IP) administration of a single dose of 120 mg/kg CBD was analyzed over the course of 24 hours across six time points. For rats, Deiana et al. (2012) reported that CBD levels peaked at two hours after administration in plasma, had a half-life of approximately 4 hrs and was eliminated after 24 hours. CBD levels were shown to be substantially higher within the IP group compared to the oral dosing group, as CBD avoids first pass metabolism if given intraperitoneally. These findings were corroborated by Hložek et al. (2017), which utilized a 10 mg/kg dose of CBD and produced similar pharmacokinetic outcomes as it pertained to the absorption and elimination rate of CBD from the body. There was also an assessment of CBD in human plasma after administration through a nasal spray by Stott, White, Wright, Wilbraham, and Guy (2013), they administered CBD doses either as a single dose of 2, 4, or 8 sprays (2.5 mg CBD per spray) or repeatedly over 9 days. The single dose experiment resulted in CBD plasma concentration peaking after 2 hrs, almost clearing after 12 hrs, and was completely cleared from volunteer's plasma by the next day. The peak concentration of CBD within blood plasma also increased accordingly with the increase in the dose received by each volunteer. In the 9-day administration experiment though, there was some evidence of CBD accumulation with repeated dosing. After the last dose on the 9th day, CBD in blood plasma for 2 and 4 sprays a day still peaked at roughly the same max and at the same time. However, CBD concentration was not almost cleared by the 12th hour, and appeared to only be close to clearing at hour 24. When the CBD dose given was increased to 8 sprays a day, or 20mg of CBD, peak concentration in plasma increased and was not fully eliminated after 24 hrs. The

results from the repeated dosing experiment would suggest some potential for CBD accumulation in the body that increases with an increase in CBD dose administered.

The idea that there is change in the pharmacokinetic profile of CBD between repeated dosing compared to a single dose is further supported by a study by Taylor, Gidal, Blakey, Tayo, and Morrison (2018). The focus of the study addressed a single ascending, and a repeating administration model of highly purified CBD in human volunteers. When a CBD dose of 1500mg was given as a single dose, concentration in blood plasma peaked around 300ng/ml at 4hrs, and decreased drastically over 24 hrs. A direct comparison of the 1500mg single dose administration to a 1500mg repeated administration reveals a stark increase in CBD concertation in blood after the second same day administration (increased to 1400ng/ml); as well as after the final dose after 7 days of CBD administration CBD in blood plasma remained above 100ng/ml over the course of the following 72 hrs. Another pertinent finding was that the two same day administrations of 1500mg of CBD generated a larger peak concentration in plasma (1400ng/ml) then of a single dose at a larger quantity (6000mg for 800ng/ml plasma concertation). The findings of Taylor et al. (2018) would support the idea that CBD can accumulate in the body as a result of repeated exposure, changing the rate at which CBD is metabolized. Taylor et al. (2018) also evaluated the effects of food consumption on CBD concentration: CBD in blood plasma was shown to be elevated in volunteers who had eaten versus those who had fasted by a roughly 4x increase. This unique and counterintuitive effect of food consumption on CBD bioavailability would suggest that the ability for CBD to be metabolized may be influenced by its lipophilic nature. As a whole, CBD's absorption, metabolism, and elimination may be dependent on dose, dose schedule, as well as the eating behaviors of the subject.

Studies of both animals and humans indicate a bell-shaped dose-response curve of isolated CBD. Research has also conveyed the difficulty of interpreting rodent metabolism for human consumption, supporting the need for rigorous evaluation to ensure adequate translation from research to clinical applications (Alline Cristina Campos & Guimarães, 2009; Linares et al., 2019). The routes of administration in preclinical research largely consisted of injections, with limited focus on oral and topical administrations that are consistent with CBD products commonly found on the market today. CBD's pharmacological properties and pharmacokinetics appear to vary with different routes of administration as well as with the length of the dosing regimen. Inconsistencies in CBD's pharmacokinetic profile makes it difficult to evaluate the most appropriate CBD dosing for the production of therapeutic outcomes in the treatment of clinical conditions (Zhornitsky & Potvin, 2012), but open up several avenues of research interest.

1.3 Mechanisms of Action

The mechanisms of action underlying the physiological and biochemical activities of CBD are not very well understood. There may be a ubiquitous number of receptor binding sites, systems, and pathways leading to many various outcomes as shown in Figure 1 (Peres et al., 2018). CBD has a multitude of agonistic, antagonistic, and negative allosteric interactions with a plethora of different systems and receptor sites (Peres et al., 2018). A system commonly addressed for its interactions with CBD is the endocannabinoid system. Cannabinoids, such as THC, bind directly to endocannabinoid receptors known as type 1 and type 2 cannabinoid G-protein-coupled receptors (GPCRs), CB1 and CB2 (Kumar, 2018), in both the central (CNS) and peripheral nervous system (PNS). However, studies have shown that CBD possesses a low affinity for CB1 and CB2 receptors (Mcpartland, Glass, & Pertwee, 2007) and other studies revealed potential antagonistic effects of CBD at these receptor sites (Thomas et al., 2007). CBD has also been shown to behave as a non-

competitive negative allosteric modulator of the CB1 receptor, reducing the efficacy and potency of THC (Vučković, Srebro, Vujović, Vučetić, & Prostran, 2018). CB1 receptors are abundant throughout the CNS, particularly in nociception areas in the brain and spinal cord (Tsou, Brown, Sañudo-Peña, Mackie, & Walker, 1998). CB1 receptors are also present in the immune system, adipose tissue, liver, muscle, reproductive, kidney and lung cells (Herkenham, 1994). CB2 receptors are present mainly in the PNS and are common in lymph, immune, heart and liver cells (Corroon & Felice, 2019). CBD's lack of a direct interaction with these sites, only increases the need for improved understanding of its mechanism of action.

CBD also interacts directly with several other systems throughout the body that have led to its declared health benefits, including transient receptor potential vanilloid type 1 (TRPV1), serotonin (5-HT1) and dopamine systems (Jesus et al., 2019). Sharing significant overlap with the functions of the endocannabinoid system (Corroon & Felice, 2019), serotonin receptors are involved in various biological activities such as pain perception (Sommer, 2004), stress regulation (Haj-Dahmane & Shen, 2011), anxiety (Banchereau & Steinman, 1998) and sleep (Frazer & Hensler, 1999). CBD acts as an agonist at the 5-HT1a receptor in humans, and recent work suggests that the endocannabinoid and serotonergic systems interact to regulate stress response (Haj-Dahmane & Shen, 2011; Russo, Burnett, Hall, & Parker, 2005). The 5-HT1a receptor has also been implicated in anti-depressive like effects in an acute and chronic administration in the olfactory bulbectomy mouse model of depression (OBX) (Linge et al., 2016). There is still further work needed to assess how CBD's anxiolytic effects could be mediated by any or multiple receptors of either CB1R, 5-HT1a, or TRPV1 (Blessing, Steenkamp, Manzanares, & Marmar, 2015). CBD has also been shown to induce antipsychotic properties and reduce oxidative stress

through its interaction with the mesolimbic dopamine system (Pedrazzi, Issy, Gomes, Guimarães, & Del-Bel, 2015; Renard et al., 2016; Valvassori et al., 2011).

CBD is also a full agonist of TRPV1, (Iannotti et al., 2014), a non-selective transmembrane cation channel which is expressed mainly in sensory nerves. Stimulation of TRPV1 receptor channels is generally provoked by a variety of noxious stimuli such as heat, inflammation, etc. usually leading to sensory nerves interpreting pain and thermal hyperalgesia. This sensory nerve outcome coincides with the neurotrophic factors (NTFs) NGF and GDNF being increased in tissues under painful conditions, which have a sensitizing effect on sensory neurons via TRPV1 (Anand et al., 2020). TRPV1 receptor stimulation typically results in pain and neurogenic inflammation. Thus, CBD's agonistic effect at the receptor site may be expected to induce pain rather than inhibit it (Jia, McLeod, & Hey, 2005). However, studies have found that certain agonists of TRPV1, including CBD, inhibited pain expression (Starowicz, Cristino, & Di Marzo, 2008). One explanation of this paradoxical effect may be that CBD-induced chronic activation of a TRPV-1 receptor results in rapid desensitization. This rapid desensitization of TRPV1 could potentially be due to 2 synergistic pathways. One pathway could be through blocking cAMP which has a role in TRPV1 sensitization through phosphorylation via activation of protein kinase A. A second pathway could be through increasing the presence of Calcineurin which is responsible for protein dephosphorylation (Anand et al., 2020). In addition to apparent analgesic and anti-inflammatory properties, stimulation of TRPV1 receptors in the dorsal hippocampus of rodents has been shown to modulate anxiety-like behaviors (Hakimizadeh, Oryan, Hajizadeh moghaddam, Shamsizadeh, & Roohbakhsh, 2012).

CBD can also act via indirect pathways, which can lead to several downstream effects. For instance, CBD can contribute to increased levels of the endocannabinoid anandamide by inhibiting

anandamide's catabolic enzyme fatty acid amide hydrolase (FAAH). Inhibition of FAAH in turn can result in increased activation of CB1, CB2, and TRPV1 receptors by anandamide. CBD can also increase the activity of mitochondrial complexes, and of creatine kinase (Valvassori et al., 2013). Mitochondria are the primary source of adenosine triphosphate (ATP), while creatine kinase catalyzes the reversible transphosphorylation of creatine by ATP. Both Mitochondria and Creatine Kinase play significant roles in maintaining ATP homeostasis. In addition, CBD has been shown to activate the proliferator-activated receptor γ (PPARγ), a receptor primarily involved with regulating glucose metabolism, energy homeostasis, and lipid storage, along with downstream effects on inflammation (O'Sullivan, Sun, Bennett, Randall, & Kendall, 2009). PPARγ activation has been shown to mediate vasorelaxation, and adipogenesis (a process involved with sequestering lipids to appropriate areas to avoid lipotoxicity in critical organs). CBD activation of PPARγ could potentially lead to these anti-inflammatory outcomes and provide some neuroprotection from vascular complications (Alline C. Campos, Fogaça, Sonego, & Guimarães, 2016). CBD's pleiotropic nature and the lack of certainty on its various mechanisms of action supports the need for more extensive research on the subject.

1.4 Behavioral Outcomes of CBD Usage

1.4.1 CBD and Anxiety

In attempts to verify claims of therapeutic potential, several studies have provided support of CBD as an effective anxiolytic. 20mg/kg of CBD has been shown to reverse social withdrawal induced by low-dose THC (1mg/kg) in male Sprague-Dawley rats (Malone, Jongejan, & Taylor, 2009) as well as reduce anxiety-like behavior in mice and rats (D. L. Almeida & Devi, 2020; Zieba et al., 2019). At a dose of 5 to 10 mg/kg, CBD produces anxiolytic effects in rats which are blocked by 5-HT1a receptor antagonists (J. L. C. Lee, Bertoglio, Guimarães, & Stevenson, 2017). At higher

doses, CBD does not produce anxiolytic effects unless administered in combination with TRPV1 receptor antagonists, implicating CBD's activation of TRPV1 channels and suggesting higher doses may not be effective (Alline Cristina Campos & Guimarães, 2009). These results suggested that CBD's anxiolytic effects involve 5-HT1a and TRPV1 receptors with a clear dose-dependent effect. However, more research is needed to illuminate the dose-specific effect as well as the impact on anxiolytic behavior as it pertained to oral administration of CBD, which was aimed to be addressed with the current study.

1.4.2 CBD and Stress

Prior research that indicates agonistic properties of CBD for 5-HT1a receptors (Russo et al., 2005) coupled with evidence that the activation of these receptors may mediate stress adaptation (Joca, Ferreira, & Guimarães, 2007; Joca, Padovan, & Guimarães, 2003) suggested that CBD may attenuate the consequences of stress. Previous studies have assessed stress response in rodent models by utilizing acute restraint, a stress situation associated with the production of autonomic and endocrine responses as well as behavioral changes(Glaser & Kiecolt-Glaser, 2005). Resstel et al. (2009) assessed mean arterial pressure and heart rate following the application of 60 minutes of restraint stress on adult male rodents, half an hour after administering IP injections of CBD. Their results indicated that CBD decreased stress-induced cardiovascular responses at 10 and 20 mg/kg doses. Supporting these results, Granjeiro, Gomes, Guimarães, Corrêa, and Resstel (2011) performed intracisternal injections of 15, 30, and 60 nmol CBD into adult male rats and found that CBD attenuated both cardiovascular and behavioral responses induced by 60 mins of restraint stress at the 30 nmol dose. These outcomes indicated specific action of CBD upon stress-activated cardiovascular pathways. Furthermore, CBD has been shown to reduce resting blood pressure in humans, as well as various cardiovascular parameters in response to mental stress,

exercise stress, and cold stress among healthy volunteers (Jadoon, Tan, & O'Sullivan, 2017). Though past research indicated attenuating effects of CBD on stress in both rodents and humans, there is still a dearth of knowledge about the timing as well as the dose-specificity of these effects, which this research hoped to address.

1.4.3 CBD and Pain

Pain relief, one of CBD's most promising potential therapeutic properties, has sparked research interest in recent years. Most commonly, CBD has been shown to reduce pain-like responses in rodent models of osteoarthritis, and inflammatory pain (Costa et al., 2007; Philpott, O'Brien, & McDougall, 2017). In acute pain models in rodents, CBD acted as a potential therapeutic agent for incision pain (Genaro et al., 2017), and was effective in mitigating acute spinal cord disk inflammation and degeneration in rodents (Silveira et al., 2014). Furthermore, rodent models of Parkinson's disease displayed decreased sensitivity to thermal and mechanical pain stimuli after CBD administration, implicating an improved pain threshold (Crivelaro do Nascimento, Ferrari, Del Bel, Bortolanza, & Ferreira-Junior, 2020). Further, CBD appeared to alter pain perception in a dose-dependent manner, (Genaro et al., 2017) found that lower doses (5 nmol/0.25 μL) did not mitigate mechanical allodynia, while higher doses (10 to 40 nmol/0.25 μL) were effective in reducing pain. Many studies investigating CBD's effect on pain perception utilized injections to administer the drug (Genaro et al., 2017; Nascimentoa, , , Elaine Aparecida Del Bela, & 2020; Philpott et al., 2017; Silveira et al., 2014), while data on the efficacy of more commonly-utilized routes of CBD administration is needed to improve translational understanding.

1.4.4 CBD and Sleep

Although it is well established that THC increases sleep (Irwin Feinberg, Jones, Walker, Cavness, & Floyd, 1976; I. Feinberg, Jones, Walker, Cavness, & March, 1975; Pivik, Zarcone, Dement, & Hollister, 1972), studies on the effects of CBD on sleep have yielded inconsistent results. A study examining the cannabinoid concentration preference among 163 medicinal cannabis users suffering from sleep disturbances found that these users were more likely to report using strains of cannabis with significantly higher concentrations of CBD (Belendiuk, Babson, Vandrey, & Bonn-Miller, 2015). The first study to examine the effects of the systemic administration of CBD on sleep in rodents found increased duration of slow-wave sleep (SWS) and decreased wakefulness at a 40 mg/kg dose and decreased SWS latency at 20 mg/kg, with no significant changes indicated in the rapid eye movement (REM) phase of sleep (Monti, 1977). Another earlier study found that CBD improved sleep in human individuals who suffered from insomnia (Carlini & Cunha, 1981). More recent studies have found that CBD possesses alerting properties and acts as a wake-promoting agent in both humans and rodents (Nicholson, Turner, Stone, & Robson, 2004). Furthermore, Murillo-Rodríguez, Millán-Aldaco, Palomero-Rivero, Mechoulam, and Drucker-Colín (2006) found that CBD increases wakefulness in rats, causes no changes in SWS, and reduces the duration of REM sleep during a rodent's lights on cycle. This study also found an increase in c-Fos expression in the hypothalamus, and the dorsal raphe nucleus, both areas related to wakefulness. In a later study, similar results were shown for increased waking and reduced duration of REM sleep, as well as a significant reduction in SWS (Murillo-Rodríguez, Palomero-Rivero, Millán-Aldaco, Mechoulam, & Drucker-Colín, 2011). Chagas et al. (2013) intraperitoneally injected CBD into adult male rats in different dose groups and found an increase in REM sleep latency and a non-significant increase in SWS duration in rats treated with CBD 40

mg/kg in the light period of the day of administration, as well as an overall significant increase in total sleep percentage in rats injected with 10 and 40 mg/kg of CBD as compared to vehicle. Changes in the methodologies employed to evaluate sleep in recent years may be what accounted for discrepancies between older studies and more recent findings. While research on the effects of THC on sleep is robust, findings from both clinical and animal studies investigating the impact of CBD on sleep illustrated the need to further assess its effects, particularly at different doses.

1.5 Aims of the Current Research Model

The primary goals of this biphasic study were to a) establish a pharmacokinetic (PK) profile for a CBD compound in male rats, and b) investigate whether this CBD compound induced behavioral changes in a translational rodent model. The first aim of the study was to investigate plasma levels in male rodents after oral administration of the CBD product at a range of doses and time points to establish a better understanding of CBD's PK profile. The second aim of the study was to address the behavioral outcomes associated with this CBD product utilizing a rodent model to investigate pain response, sleep-like behavior, anxiety-like behavior, and stress response. This battery of behavioral testing was selected based on the prevalence of anecdotal support for CBD benefits. It was hypothesized that with higher dosing, a larger concentration of CBD would be present within the rodents' plasma. It is also anticipated that in the battery of behavioral assays, administration of CBD would yield significant changes in behavior: variation in sleep patterns, most likely longer periods of wakefulness coinciding with the more recent studies on CBD regarding sleep, delayed nociceptive response to a thermal pain stimulus, decreased anxiety-like behavior, and decreased cortisol levels in the presence of a stressor. Lastly it is believed that these behavioral outcomes would vary based on the CBD dose that was administered.

Chapter 2: Methods

2.1 Animals and Group Assignment

This research model used novice male Sprague Dawley rats (n=146) for all experiments (Charles Rivers, NY), with an arrival weight between 250-300g. Rodent age at beginning of oral gavage ranged from 65-100 days as a result of COVID-19 project delays. Animals were housed in standard laboratory cages in the DLAR with controlled temperature (72°F), constant humidity (55%), a 12h light/dark cycle (7a.m. lights on/7p.m.lights off) and *ad libitum* access to water and food. Animals were pair-housed with standard enrichment upon arrival to avoid any stressors due to single housing conditions. Rats were allowed to acclimate for a minimum of four days prior to being handled. Animals were then handled for 3 days to prepare rats for oral gavage. All experiments were conducted during the light phase of the day. All procedures were approved by the Wayne State University Institutional Animal Care and Use Committee (Protocol #19-03-1026) and care of subjects was in accordance with the guidelines of the National Institutes of Health and the Animal Care and Use Program within the National Institute on Drug Abuse Intramural Research Program.

Rats arrived in several batches (n=10-20 each) to stagger the experiment and keep the workload feasible. When the rats arrived, they were divided pseudo-randomly to the control or treatment groups using a random number generator (see Table 1). Animals were weighed each day and drug volume was adjusted accordingly. Fecal samples were also collected, and surface body temperature was measured from the lower abdomen on a daily basis.

The first cohort of rats (n=50) was used to establish the PK curve. These rodents received a 0, 5, 10, 20, or 40 mg/kg dose of CBD once daily (p.o.) for 10 days. Blood samples were collected at 1, 2 and 4hrs post administration to measure CBD concentration in plasma (see Fig. 1.1).

The second cohort of rats included only a vehicle (n=20) and 20mg/kg dose group (n=20) and these were used for behavioral testing. Half the rats underwent sleep and stress tests, and the other half was used for anxiety-like and pain behavior assessment.

A third cohort was used to study the same behaviors again (i.e. pain and sleep assessment, or anxiety and stress assessment), but this time the cohort included a 40mg/kg dose group (n=16 for 40mg/kg, n=6/group for vehicle and 20mg/kg resulting in a total of n=16 /dose group for cohort 2 and 3 together). This additional cohort was the result of initial errors in study design and improper running of dose groups against controls.

Primarily, a misunderstanding and miscommunication during project handoff from a previous student, resulted in a subprime study design. The outcome had the vehicle dose group performing the anxiety and stress tests while the 20mg/kg group was performing the sleep and pain test (and then vice versa for the next batch of rats), instead of some animals from both groups performing the same tests at the same time. The end result gave proper n-values per dose group, being that there were 10 rodents per dose group (0 and 20mg/kg) run through each behavioral test (pain and sleep 'arm' vs anxiety and stress 'arm' of the experiment), but not proper timing. Therefore, each dose group was not run appropriately against controls and this potentially introduces a bias with differences in personnel, environmental conditions, heritage of the rats, etc. Therefore, behavioral assessment was repeated with a smaller number of animals per group (n=6/group) with the proper study design and at the same time an additional dose group (40mg/kg, n=16/group) was added to the experiment.

Unfortunately, a campus wide shut down due to COVID-19 added another layer of complexity to this study as researchers were not able to run the additional cohort as planned. This resulted in a potential seasonal effect this time (Cohort 2 was run in November of 2019 and cohort

3 did not start until August of 2020) and in changes to some animals' age (first cohort 3 batch of rats were slightly older than the others (30 extra days of housing) due to limited lab access through the COVID shutdown) and changes to the personnel that performed the tests (reduced from a team of male and female students to just one male graduate student due to COVID-19 restrictions).

However, to account for most of these unfortunate circumstances by using sophisticated statistical models known as generalized estimating equations (GEE) that can account for these unintended differences between the groups and cohorts. Further, physiological measures showed only negligible differences between cohorts for body temperature, weight change, and corticosterone levels. For a more in-depth discussion of the potential impact of the study design and interruptions see Section 4.4 (strength and limitations) of the discussion.

2.2 **General Characteristic Collection and Assessment**

All rodents were individually transferred from their home cage to a triple beam balance (OHAUS, 700 series) at the beginning of each day to assess for body weight. Their weight was used as a generalized health check (ensuring no drastic drop in weight) as well as to provide needed information to ensure proper dosing for oral gavage CBD administration. Rodent body temperature was also recorded each day using an infrared laser thermometer (Etekcity, model Lasergrip 774). Body temperature was collected immediately following a rodent's weight measurement and preceding oral gavage. Temperature levels served as a secondary health check measure. Lastly, fecal samples were taken each day directly from each rodent and stored in the -20 freezer for later processing.

2.3 Drug Choice and Administration

The CBD product (Ellipse Analytics, Denver, USA) was a >99% pure CBD compound derived from U.S grown hemp. The CBD was extracted in Ellipse's labs (Denver, CO) using

ethanol and purified using a process of distillation and chromatography. In the lab, the CBD powder was dissolved in sesame oil under sterile conditions at four different concentrations (see below) and kept at 4 C until used. CBD was dissolved into sesame oil for final concentrations of 0 (vehicle control), 5, 10, 20, and 40 mg/ml. Solutions were administered at a volume of 1.0 ml/kg body weight. CBD was given in a single dose each day at 7:30 a.m. for 10 days for each rat using 18-gauge gavage needles (on the day of sleep assessment, rodents received their dose at 11am). All gavages were performed by the same trained experimenter.

2.4 Pharmacokinetic Profile Development

2.4.1 Blood Collection

Blood was collected using saphenous vein blood collection on the first, the fifth, and the tenth day at three different time points per day (Fig. 1.1). These time points were 1-, 2-, and 4-hours post drug administration, resulting in a total of 450 blood samples. Blood draws were taken from opposing hind legs with each draw. The hind leg area was cleaned with an 70% isopropyl alcohol prep pad and Petroleum jelly was applied to the shaved area to maintain a clean blood collection. The rats received a gentle poke to their saphenous vein using a 23-gauge needle and 0.2ml of blood was collected using EDTA-K collection vials. Bleeding was stopped immediately after collection using gauze and gentle pressure. The rat was then returned to its cage and monitored to ensure normal behavior. The same blood collection process was repeated for the 2hr and the 4hr blood draw time points. Once all blood was collected for that day, blood was spun down using a centrifuge using ThermoFisher Scientific's recommendation of 2,000 rpm for 15 minutes, which separated the plasma from the red blood cells. Plasma was then pipetted out into clean, sterile screw cap vials and stored in the -20° freezer until further processing.

2.4.2 Blood CBD Level Analyses

The blood plasma samples with the most available plasma (n=180) were selected from the total of 450 samples to reduce cost. Samples were given to the Wayne State Lumigen lab for analysis. They used the study's control samples (plasma from rodents that only received sesame oil), and spiked them to generate a standard curve, while 5 samples from each time point for each CBD dose were used for analysis. Plasma samples were analyzed for CBD levels using liquid chromatography-mass spectrometry by Wayne State University Lumigen laboratories. Samples were allowed to thaw on ice prior to being analyzed. Samples were then extracted using Agilent Captiva EMR lipid cartridges following the protocol provided by the manufacturer. Internal standards were added to each plasma sample volume before extraction. After extraction, the samples dried under nitrogen and then were brought up to the volume that was used for extraction in 0.1% formic acid and methanol. Next, samples were diluted by a factor of 3 with water, vortexed, and readied for analysis. Analysis took place using a Thermo TSQ Altis triple quadrupole mass spectrometer with EQuan Max Plus system. Samples were assessed in positive ion mode using heated electrospray ionization (H-ESI). The gradient mobile phases utilized were (A) 10 mM ammonium formate with 0.1% formic acid in LC-MS grade water and (B) 10 mM ammonium formate with 0.1% formic acid in LC-MS grade methanol. Compounds were separated using a Waters Acquity UPLC HSS C18 2.1 x 50 mm, 1.8 µm column. The gradient conditions proposed were as follows: gradient started at 60% B and increased from 0-3 minutes to 95% B, then was held at 95% B for 0.5 minutes. Columns were allotted to equilibrate from 3.5-4.25 minutes. Total run time consisted of 4.25 minutes, at a flow of 0.5 mL/min. Injection volume of the sample was 20 µL onto the column. The column oven was set to 40°C. Internal standards were extracted with each sample set and analyzed throughout the run.

2.5 Behavioral Assessment

Behavioral tests were conducted on the first, fifth and tenth day of CBD/vehicle administration respectively (see Figure 1.2 and 1.3). As described above, rats were assigned to either vehicle or 20 mg/kg CBD in a pseudo-random fashion for cohort 2, and in cohort 3 the 40mg/kg dose group was added (i.e., the behavioral tests were run with 40 rats from the 2nd cohort, and 56 from cohort 3 (unusual cohort separation due to COVID-19 shut down and associated Covid restriction). The tests either consisted of hot plate (day 1), sleep analysis (day 5), and hot plate again (day 10) for one set of animals (group 1)(cohort 2, n=20, cohort 3, n= 28), or elevated zero maze (day 1), restraint stress (day 5), and elevated zero maze again (day 10) for the second set of animals (group 2)(cohort 2, n=20 cohort 3, n= 28). Within each cohort, animals were staggered to ensure feasibility of behavioral tasks (limited by maximum space for sleep assessment allowing for 10 rats).

2.5.1 Hot Plate

Animals were first familiarized with the hot-plate at room temperature the day prior to testing to avoid novelty stress. On day 1, and again on day 10 of CBD/vehicle exposure, each rat in behavioral group 1 was then placed onto the hot-plate apparatus surrounded by a Plexiglas wall approximately 1 hour after the drug administration. The temperature on the digital hotplate (Fisher Scientific, model 11-100-49SH) was set to 55℃ before testing and confirmed from the center of the plate before each use using an infrared thermometer (54-56℃; Etekcity, model Lasergrip 774). On the test day, latency time to flinch, lick, and/or raise of their hind paws was recorded. These behaviors have been shown to be representative of a nociceptive response (Espejo & Mir, 1993; Minett et al., 2010). Rats were removed immediately after the first nociceptive behavior was observed. A maximum of 60 seconds was allotted to avoid tissue damage (i.e., if the rat did not

display any nociceptive behavior after 60-s on the hot plate, they were removed, and assessed for any paw scarring (though no scarring was found on any rat).

2.5.2 Sleep Analysis

On the 5th day of CBD/vehicle administration, each rodent pair were separated between their home cage, and a different cage with home cage bedding was introduced, and animals were monitored for their sleep behavior which allowed for greater visualization and appropriate tracking of rodents. Thirty minutes after drug administration, 10 rats were transported from the DLAR facilities to the lab. Only 10 cages could fit with the Noldus camera room, so staggered cohort start days ensured feasibility and kept the proper timeline for all rats. These rats were first allowed to habituate to the lab room for at least 30 minutes. The cages were positioned in a grid fashion in the recording room with the filter cage tops (but not the lids) removed. All larger enrichment objects were removed to improve visualization and food was moved inside of the cage. The water bottles remained positioned on top of the cage. Over the course of 6 hours (12 p.m.-6:00 p.m.), rats remained undisturbed while locomotor activity was recorded and coded for a variety of sleep behaviors. Sleep was defined as at least 40 s or more of immobility (Bains et al. 2018), and the parameters measured were as follows: frequency of entrance into a sleep state, latency to first onset of sleep, and cumulative duration of sleep in seconds and percentage (Ethovision, Ver. 13, Noldus).

2.5.3 Physical Restraint

Restraint stress is commonly employed to induce and investigate stress-associated behavioral, physiological, or biochemical changes within rodents (Alline C. Campos, Fogaca, Aguiar, & Guimaraes, 2013; Padovan & Guimarães, 2000). Rats were placed into a rodent-specific clear acrylic restraint container designed to restrict overall movement. The rodents were held in these tubes for 30 mins each. Blood was collected at 3 time points: Time-1 (baseline; before the

rat was placed into the restrainer tube), Time-2: (peak; immediately after the 30min stressor), and Time-3 (recovery; 90 min after being returned to their home cage). Blood was collected from the saphenous vein using the procedure detailed in phase one of blood collection. Maximum blood collection at any time was 200 ul. Once all blood was collected for that day, blood was spun down using the centrifuge at 2,000 rpm for 15 minutes to separate plasma from red blood cells. Plasma was then pipetted out into clean, sterile screw cap vials and stored in the -20° fridge until further processing.

2.5.5 Elevated Zero Maze (EZM)

The elevated zero maze consisted of two open areas and two closed (walled-in) areas and was elevated 78.7cm above the floor. The design of this maze in combination with comprehensive behavioral analysis allows for sensitive detection of anxiolytic drug action within rodents (Shepherd, Grewal, Fletcher, Bill, & Dourish, 1994). The EZM has been shown to be a better tool for studying anti-anxiety drug effects over alternative models, such as the elevated plus maze and mirror chamber (Kulkarni, Singh, & Bishnoi, 2007). Rats were placed individually into the entrance of a closed area and allowed to explore the maze for a 5-minute period. Total distance traveled, total time spent in each area, and entries into each area were tracked and scored from these sessions by the Ethovision Software (Ethovision, Ver. 13, Noldus). More time spent in open areas has been considered less anxious behavior.

2.5.6 Corticosterone Analysis

Plasma samples were analyzed using the DetectX® Corticosterone Immunoassay kit from Arbor Assays (Ann Arbor, MI, USA, #K014), designed to quantitatively measure corticosterone levels. Reactants and reagents were allowed to warm to room temperature for one hour before usage. Assay buffer concentrate was diluted with distilled water at a 1:5 ratio. To prepare each

sample, 5 µL of serum was added to pre-labeled tubes using a 10 uL pipette with clear (0-10 µL) pipette tips, utilizing a fresh tip for each sample. Dissociation Reagent (5 µL) was added into each sample tube and vortexed. Assay Buffer (495 µL) was added to each sample tube to create a 1:100 dilution of the sample.

Each sample and standard were vortexed again immediately before being transferred to the wells of the assay plate. Each standard and sample were run in duplicate and pipetted into two wells. Fifty µL of each sample or standard was pipetted into the corresponding wells. Fifty µL of Assay Buffer was pipetted into maximum control wells, and 75µL into non-specific binding control wells. Twenty-five µL of DetectX Corticosterone Conjugate was added to each well, followed by 25µL of DetectX Corticosterone Antibody. Non-specific binding control wells did not receive the antibody. The assay plate was covered with tape (as provided in the kit) and placed on a shaker at room temperature for one hour at 220 rpm.

The assay plate was aspirated and the wells were emptied. Each well was washed with 290µL of wash buffer and aspirated four times. One hundred µL of TMB Substrate was added to each well, and the plate was incubated at room temperature for 30 minutes. The Stop Solution was vortexed, and 50µL was added to each well. The assay plate was then inserted into the EPOCH Reader, measuring optical density at 450 nm.

2.6 Euthanasia

2.6.1 Standard CO2

Animals utilized in the behavioral assays were euthanized using standard CO2 procedure and then decapitated as secondary confirmation of death. Brains were removed and rapidly frozen on dry ice. Brains were then stored in the -80 °C freezer for future dissection and western blot analysis.

2.6.2 Perfusions

Animals utilized for blood collection were sacrificed using a single injection of an overdose of sodium pentobarbital (150mg/kg; Fatal Plus©). Once the animal reached a surgical plain, they were transcardially perfused using saline followed by 4% paraformaldehyde. Brains were removed and stored in 4% paraformaldehyde for 24h before being transferred into phosphate buffer containing 30% sucrose until further processing.

2.7 Statistical Analyses

All statistical tests were run using SPSS™ (version 27), and graphs were produced in Prism™ (GraphPad Software, San Diego, California USA, www.graphpad.com) either using ANOVA or generalized estimating equations (GEE). As the assumption of independence of errors was not met for the behavioral data because of the separate cohort situation discussed above, a standard ANOVA design was not a viable option to evaluate this data. An ANOVA model requires residual errors to be unique from all other predictors. However, in this experiment the difference in time, or the difference in cohort (2 vs 3) could potentially result in a form of unique variance or error that is specific or correlated to that cohort. To address these concerns, several steps were taken. An initial comparison of physiological measures (weight and temperature) between cohorts were compared using ANOVA. These physiological measures were recorded consistently across all animals regardless of group. Results from these physiological assessments from both behavioral groups for the control and 20mg/kg dose group (40mg/kg could not be included due to it being assessed in only one behavioral group) were analyzed using ANOVA to give insight on the error structure of a variable that was properly assessed against controls in all groups. There was no statistically significant difference between cohorts in weight or temperature, suggesting that group comparisons can be made using the pooled data set for behavioral analysis of the control and

20mg/kg dose group. This pooled data set then used an additional statistical analysis, generalized estimating equations (GEE), to account for the potential correlated error, and improve the interpretation of the data. GEE accounts for this error by correlating it by timepoint, or in this case cohort. The residual error is included or nested within the observation as apart of GEE, and is no longer required to be independent. This statistical measure goes above and beyond another consideration which was to control for error by using the cohort has a covariate, but this is also insufficient, since the variations in these error structures could have changed the overall measurement of individual observations for each variable.

Even though there were no significant differences between cohorts when it came to weigh and temperature, further analysis using repeated measures ANOVA was run to look at progressive changes that may have occurred by day or due to CBD dosing. Repeated measures ANOVA for assessing weight allowed for the parsing out some of the concerns that arose due to the set of rats that were slightly older than the others (30 extra days of housing) due to limited lab access through the COVID shutdown. Differences in temperature were also looked at using repeated measures ANOVA.

To investigate serum CBD levels, an omnibus ANOVA was run using a 5 dose groups (0, 5, 10, 20, and 40 mg/kg) x 3 blood draw time points (1, 2, and 4 hrs. post-administration) x 3 days (1, 5, and 10 days of administration) model. If significance was found, separate ANOVAs were run for each day to assess changes that occur on individual days.

Behavioral differences within cohort 3 were assessed using ANOVA to test the hypotheses surrounding pain outcomes, anxiety, stress, and sleep of the additional 40mg/kg dose group. However, to evaluate these hypotheses between cohorts, a GEE approach was used because it does not hold the same assumptions required of ANOVA design. GEE was evaluated using a linear

model, assuming normality and correcting for observations within cohorts as an exchangeable correlation matrix to make assessments of all behavioral outcomes between the 20mg/kg, and control dose group in cohort 2 and 3. The exchangeable correlation matrix is one of several options utilized to label how error can be correlated with the data. The Exchangeable matrix was chosen because there is no sequence (no repeated or meaningful time measurements) within this model, only two distinct time points, being the separation between the two distinct cohorts. Other matrix considerations within GEE can expand upon additional timepoints of measurement if needed. MANOVA analysis was used to assess behavioral assays with multiple recorded outcomes from cohort 3 as a standalone measure, to allow for assessment across all dose groups. Behavioral assays with only one variable measured were assessed using ANOVA. Anxiolytic behavioral data was assessed using a repeated-measures MANOVA to analyze total duration in open arms of the EZM, frequency of entrance into each area, and total distance traveled between dose groups from the third cohort on the first and tenth day of administration. This MANOVA consisted of a 3-level (Vehicle, 20mg/kg CBD, and 40mg/kg CBD) between-subjects factor of dose with a 2-level (day 1, day 10) within-subject factor of day of testing. A repeated measure ANOVA was utilized to assess stress response levels during the physical restraint assay. This was a 3-level (vehicle, 20, 40 mg/kg CBD) between-subject factor of dose, and 3-level within-subject factor of time of blood draw: Time-1 (baseline-immediately prior to restraint), Time-2 (peak-immediately following restraint), and Time-3 (recovery-1.5 hours after restraint is removed). Latency to a nociceptive response on the first and tenth day of administration between control, CBD 20, and 40 mg/kg, was assessed using a one-way repeated-measures ANOVA with the day of testing as the 2-level (day 1, day 10) within-subject's factor. To evaluate sleep outcomes, a MANOVA was used to assess differences between dose group in the parameters of frequency of entrance into a sleep state,

latency to first onset of sleep, and cumulative duration of sleep. For all experiments, an alpha level of $p<.05$ was used as the measure of statistical significance. For all experiments, univariate and multivariate outliers were removed using pairwise deletion if they exceeded the threshold of a z-score of 3.29 harmful to overall understanding of the analysis, or if rational indicates a potential error in the measurement. If Mauchly's test was found to be significant, and therefore violating the assumption of Sphericity, a Greenhouse-Geisser (epsilon < 0.75) or Huynh-Feldt (epsilon > 0.75) correction was applied to assuage the chance of inflation within the significance value estimated. However, if Levene's test was significant, $p<.05$ (tests the null hypothesis that error variances are equal across groups) then the output no longer meet the assumption of homogeneity of variance and the multivariate test statistic will be used. Box's M test will also be used, $p< .001$, to evaluate the assumption of homogeneity of variance-covariance matrices. When significant main differences were shown, univariate differences between all pairwise comparisons were addressed using Tukey's HSD post-hoc analysis. Tukey's HSD allowed for these comparisons to be made while controlling for type 1 error rate inflation. A supplemental table (Table 2) was included for measures of effect sizes across all behavioral assays.

Chapter 3: Results

3.1 General Characteristics

3.1.1 Weight Outcomes

Weight gain was recorded every day from initial gavage and converted to a percent change over the original day 1 weight, to account for initial weight differences amongst rodents (see Figure 2). A repeated measure ANOVA for cohort 2 (Figure 2.1), showed that there was no significant main effect of CBD [$F(1,38)=2.67$, $p=.11$]. A significant day effect was observed [Greenhouse-Geisser $F(2.21, 84.10)= 339.31$, $p<.001$] with all animals gaining weight across the timeframe of the experiment. A repeated measures ANOVA for cohort 3 (Figure 2.2) showed no significant effect of dose [$F(2,53)=.889$, $p=.417$]. However, there was a significant difference between days with all rats gaining weight throughout the course of the experiment [Greenhouse-Geisser $F (1.52,80.63)=84.61$, $p<.001$].

3.1.2 Temperature Outcomes

A repeated measures ANOVA of cohort 2, showed that there was no significant difference between dose groups [$F(1,38)= .004$, $p=.95$] on body temperature (see Figure 3.1). There was however a significant effect of day [Greenhouse-Geisser $F(5.33, 202.55)=8.62$, $p<.001$]. The rodent colony room had one cold day (69°F) but even removing this day did not change the significant outcome. Average rodent temperatures fluctuated between 30.6° and 31.8°C, with their temperatures dropping close 29.5°C on the cold room day.

A repeated measures ANOVA of cohort 3 (Figure 3.2), showed no significant difference between dose groups [$F(2,53)= .27$, $p=.77$]. There was a significant effect of day on body temperature [Huynh-Feldt $F (8.78,465.21)=2.69$, $p<.01$], with body temp fluctuating again between 30.5 and 31.8 on average across all 10 days.

3.2 Pharmacokinetics

Within the 5x3x3 ANOVA, five of the 180 blood sample measurements did not meet the threshold amount for appropriate measurement at the Lumigen lab, and three were removed as outliers based on the outlier criteria. First looking at the interactions, significance was found across all interactions; day*time*dose [$F(12,172)=1.99$, $p=.031$], time*dose [$F(6,172)=3.61$, $p=.002$], day*dose [$F(6,172)=2.40$, $p=.031$], and time*day [$F(4,172)=2.61$, $p=.039$]. There were significant main effects for dose of CBD in plasma, [$F(3,172)=104.512$, $p<.001$], and time, [$F(2,172)=32.359$, $p<.001$], but not by day [$F(2,172)=2.187$, $p=0.116$] (as such, days were collapsed for visual representation, see Figure 4). Tukey *post hoc* tests revealed that the 40mg/ml and a 20mg/ml dose resulted in significantly different CBD plasma concentrations as compared to all other dose groups ($p<.001$), with 5mg/ml of CBD showing no significant differences from 10mg/ml of CBD. Further, CBD concentrations at 4 hours after drug administration were significantly lower compared to hours 1 and 2 ($p<.001$) for all groups (see Figure 4).

3.3 Behavioral Outcomes

3.3.1 GEE modeling to compare 0mg/kg to 20mg/kg

Generalized Estimating equation (GEE) modeling was used across all behavioral assessments between cohorts for both the control and 20mg/kg CBD dose group. This statistical approach was used to control for differences resulting from running several cohorts and other COVID restriction-induced changes (i.e. number of researchers present during testing etc.).

3.3.1.1 Hot Plate Test

Two outliers were removed, 1 from the control cohort on day 10, and 1 from the 20mg/kg dose group, both for reaching max time. GEE assessment of Hot plate latency (s) showed no effect of dose on Day 1 [Wald $\chi^2=.77$, $p=.38$], however, there was an effect of dose on Day 10 [Wald

χ^2=4.83, p=.028]. B-weights showed that, on Day 10, the 20 mg/kg dose group had a 6.800 second increase in latency in reaction to the hot plate on average when compared to controls. Mean hot plate latency for 0 mg/kg on Day 10 was 17.73 seconds (95% Wald Confidence interval, 14.02-21.45) while mean hot plate latency for 20 mg/kg on Day 10 was 24.53 seconds, (95% Wald Confidence interval, 20.59-32.91) see Figure 5.

3.3.1.2 Sleep Assessment

Sleep assessment was run using Ethovision, Ver. 13, Noldus over a 6hr period (12pm-6pm) and measures included; distance traveled, frequency of entrance into a sleep state (40s of immobility), latency to first onset of sleep, and cumulative duration of sleep in seconds. GEE revealed no difference for distance traveled (cm) or entries into the state of inactivity during the sleep assessment between groups [Wald χ^2=.45, p=.50; see Figure 6.1. and Wald χ^2=2.57, p=.11; see Figure 6.2. respectively]. However, even though the number of entries into a sleep state were not significantly different, GEE revealed that cumulative time (s) spent in an inactive state was significantly different between dose groups [Wald χ^2=7.11, p=.008]; see Figure 6.3. B-weights showed that the 20mg/kg dose group spent 2923.69 seconds less on average in an inactive state than 0mg/kg. The mean cumulative duration spent in an inactive state for 0mg/kg and 20mg/kg dose groups were 11824.2 seconds (95% Wald Confidence interval, 10465.38-13183.02) and 8900.51 (95% Wald Confidence interval, 7234.51-10566.50) seconds, respectively. The time it took for them to show their first instance of sleep like behavior was not significantly different between dose groups [Wald χ^2=.068, p=.79] (Figure 6.4).

3.3.1.3 Elevated Zero Maze

The elevated Zero Maze was used as a measure of anxiety like behavior. This procedure was used on the 1st and 10th day of administration. GEE was used to analyze outcomes of open

arm entry, open arm duration, and distance traveled in centimeters separately on each day. Evaluation of distance traveled (cm) showed no significant effect of dose on day 1 [Wald χ^2=.21, p=.65]. However, on day 10, there was a significant difference between the dose groups [Wald χ^2=5.72, p=.017]. B-weights show that the control traveled on average 283.26 cm more in the EZM than the 20mg/kg dose group on Day 10. The average total distance traveled by 0mg/kg and 20mg/kg on Day 10 was 1428.44cm (95% Wald Confidence interval, 1266.94-1589.94) and 1145.18cm (95% Wald Confidence interval, 978.48-1311.88), respectively (Figure 7.1). When looking at cumulative time (s) spent in the open arm of the EZM, there was no significant effect of dose group on day 1 [Wald χ^2=1.41, p=.24]. There was a significant difference between dose groups on Day 10 [Wald χ^2=4.08, p=.043]. Based on b-weights, the control (0mg/kg) group spent on average 16.4 seconds longer in the open arm of the Elevated Zero Maze than the 20mg/kg dose group on Day 10. On average the control group spent 42.10 seconds in the open arm of the EZM (95% Wald Confidence interval, 31.01-53.18), while the 20mg/kg dose group spent 25.6975 seconds (95% Wald Confidence interval, 14.29-37.11) (Figure 7.2). Alongside time spent in each arm, the number of entries also showed some differences. Average number of entries into the open arm showed no significant differences between dose groups on Day 1 [Wald χ^2=.13, p=.72] but there was a significant difference between dose groups on Day 10 [Wald χ^2=5.22, p=.022]. B-weights show that 0mg/kg had a mean number of entries that were 3.06 more than the 20mg/kg dose group for the open arm on Day 10. On average, the 0mg/kg dose group entered the open arm of the EZM 7.88 times (95% Wald Confidence interval, 5.68-10.07) on Day 10, while the 20mg/kg dose group entered 4.81 times (95% Wald Confidence interval, 3.36-6.26), Figure 7.3.

3.3.1.4 Restraint Stress and Corticosterone Analysis

Using GEE analysis, corticosterone concentration in the plasma was statistically analyzed

to assess the mean differences at each of the three timepoints individually, see Figure 8. 1 outlier was removed from the control recovery time point due to exceeding the z-score threshold. Corticosterone levels immediately following restraint stress (i.e. stress response levels) were not statistically different across dose groups, but there was a trend towards significance [Wald χ^2=3.44, p=.064]. There was no dose effect for baseline or recovery measures [Wald χ^2=.21, p=.65] and [Wald χ^2=.000, p=.998] respectively.

3.3.2 Behavioral Cohort 3 Assessments

Cohort 3 assessment focused only on the second round of rats received for behavioral assessment. By focusing on just this cohort, it allowed for the evaluation of outcomes produced by the 40mg/kg dose group that were not able to address using GEE (no 40mg/kg dose group was present in the other cohort of behavioral assessment).

3.3.2.1 Hot Plate Test

A single outlier was removed from the control cohort on day 10 due to hot plate cooling, and a single outlier was removed from day 1 of the 40mg/kg dose group for exceeding the z-score threshold. Repeated measures ANOVA assessing paw withdrawal latency (s) by dose (Figure 9) revealed no significant differences between dose groups. [F(2,23)=1.90, p=.17]

3.3.2.2 Sleep Assessment

Sleep assessment was kept consistent, running Ethovision, Ver. 13, Noldus over a 6hr period (12pm-6pm) and measuring distance traveled, frequency of entrance into a sleep state (40s of immobility), latency to first onset of sleep, and cumulative duration of sleep in seconds. Distance traveled (cm) assessment using ANOVA displayed no significance between dose groups (Figure 10.1) [F(2,25)=.91, p=.42]. Number of inactive state entries showed no mean differences that were significantly different between dose groups (Figure 10.2) [F(2,25)=.894, p=.422]. The average

time it took each rat to reach its first inactive state (s) (Figure 10.3) did not differ significantly between dose groups [F(2,25)=0.10, p=0.91]. There were also no significant differences in effect of dose on a rodent's cumulative inactive duration within cohort 3 (Figure 10.4) [F(2,25)=1.32, p=.28].

3.3.2.3 Elevated Zero Maze

1 outlier was removed for exceeding the z-score threshold, a single 20mg/kg rodent on day 1. The Elevated Zero Maze was used on the 1st and 10th day of administration. A Repeated Measures ANOVA was used to analyze outcomes of open arm entry, open arm duration, and distance traveled in centimeters on each day by dose. As seen in Figure 11.1, total distance traveled (cm) was not significantly affected by dose received [F(2,25)=1.71, p=.20]. Figure 11.2 represents the total time (s) spent in the open arm of the EZM separated by dose, and there was no effect of dose shown within the Omnibus repeated measures ANOVA [F(2,25)=.44, p=.65]. 2 outliers were removed for exceeding the z-score threshold (freezing), a single 40mg/kg rodent on day 1, and a different one on day 10. Lastly, the number of entries into the open arm of the EZM by were not significantly different between dose groups (Figure 11.3) [F(2,25)=1.00, p=.38].

3.3.2.4 Restraint Stress and Corticosterone Analysis

A repeated measure ANOVA was run to assess the omnibus effect of dose on corticosterone levels across restraint stress time points, Figure 12. 1 outlier was removed from the control recovery time point due to exceeding the z-score threshold. There was a significant effect of dose group [F(2,24)=3.77, p<.05] with the Elisa plate as a covariate. Tukey Post Hoc testing revealed the significance of this effect was being driven by the differences between the control and the 40mg/kg dose group (P<.05). With no significance shown in the GEE for baseline or recovery, but a trend in corticosterone levels being shown for peak stress levels, A planned comparison

assessment of peak corticosterone levels immediately following restraint stress was run indicating that at this time point significance was reached between the control and 40mg/kg CBD [$F(1,19)=5.81$, $p<.05$].

Chapter 4: Discussion

This project sought out to provide further insight into the properties and benefits of CBD use. This experiment utilization of a rodent model for all of its varying assessments allowed for a more controlled environment, and clearer evaluation of the CBD product as a stand-alone drug. Further experimentation is needed, and clinical research still needs to be done to evaluate these outcomes more in humans, but the implications of this study provide beneficial information to the overall literature on CBD. Significant increases in blood CBD concentration were observed with increasing CBD dose, and elevated blood CBD concentrations were seen during the 1st and 2nd hr post-administration time points as compared to the 4th hour. Significant dose effects of CBD consumption were observed for pain sensitivity, sleep behavior, stress response, and overall sedation. There were no effects at any dose on rodent weight gain, or body temperature.

4.1 General Characteristic Development

4.1.1 Weight

Day was the only variable within this study that affected weight. This is to be expected, as the rats age and eat they gain weight. There was no effect of CBD on weight within the experiment. There was a small portion of rodents (8 rodents) that had a higher starting weight within the 3rd cohort due to a 30 day longer housing period during the COVID-19 shutdown. A similar outcome was found in a study by Osborne, Solowij, Babic, Huang, and Weston-Green (2017) that collected food and water intake, as well as weight. This study looked to assess CBD's effect within a rodent model of prenatal infection, and the male offspring (PN56) were injected twice daily with 10mg/kg of CBD for 3 weeks. There were no differences between groups when it came to weight, food intake, or water intake. The lack of observed changes in weight gain as a result of CBD treatment is in contrast to an earlier study by (Ignatowska-Jankowska, Jankowski, & Swiergiel, 2011) that

found weight gain was reduced over 14 days of 2.5 or 5mg/kg of CBD i.p. injections in Wistar rats. In a Meta-analysis presented by Pamplona, Da Silva, and Coan (2018), that looked to discuss the effects of CBD on treatment resistant Epilepsy in humans, weight gain and increased appetite were the only consistent "adverse" events that occurred across studies with the addition of CBD. Due to the limited assessment of CBD on weight change, and the conflicting reports in the literature, continued assessment of this and other physiological characteristics is needed.

4.1.2 Temperature

The literature varies on the topic of body temperature regulation, but overall, appears to agree that CBD does not have a noticeable effect on overall body temperature. A review of the safety and side effects of CBD, that reported across 132 studies of CBD using a variety of routes of administration (oral, i.p, i.v, intratumor, and intra-arterial) and doses (0-1500mg/kg/day) showed that CBD did not have an effect on physiological measures such as body temperature, heart rate, and blood pressure (Machado Bergamaschi, Helena Costa Queiroz, Waldo Zuardi, & Crippa, 2011). However, it has been reported that 30 minutes vapor inhalation of CBD at either 100 or 400 mg/ml induced dose dependent hypothermia in male Wistar rats, but not in females, and not through i.p. injection (Javadi-Paydar, Creehan, Kerr, & Taffe, 2019). No differences in thermoregulation or body temperature were observed as a result of CBD consumption in the present investigation; however, there were differences in body temperature by day. The difference in body temperature could be the result of varying in room temperature, slight variations in the position of the infrared laser on the belly of the rodent, battery strength of the thermometer, etc. Body temperature though does typically fluctuate within a small range, but measurements could still be improved. Instead of measuring surface body temperature from the belly using infrared laser, taking rectal thermometer measurements could improve consistency. Rectal readings would

need to be evaluated in the grand scope of the project however, because this procedure could be another source of stress.

4.2 Characteristics of CBD Bioavailability

This study supports the idea that orally administered CBD is eliminated over the course of 24 hrs. (Deiana et al., 2012). There were no day effects, suggesting that CBD did not buildup or remain within the bloodstream. These results agree with previous pharmacokinetic studies of CBD using rodent models (Deiana et al., 2012; Hložek et al., 2017). However, with typically lower levels of CBD bioavailability being present through oral administration, CBD injections tended to be a more common practice within the literature (Genaro et al., 2017; Nascimentoa et al., 2020; Philpott et al., 2017; Silveira et al., 2014). CBD has been shown to peak within the bloodstream between the first and second hour with the half-life being around 4 hours. While elimination in rodents seems to be the same no matter the route of administration, IP injections and inhalations both produce their peak concentrations more rapidly when compared to oral administration, with oral administration producing the highest concentrations in the brain (Deiana et al., 2012; Hložek et al., 2017). However, larger concentrations of CBD are eliminated in the same timeframe as smaller concentrations may indicate first order kinetics. The higher the concentration of a drug given, the more that is eliminated in a unit of time. A constant proportion of CBD appears to be eliminated over time, allowing different concentrations to all be eliminated over the course of 24 hrs. However, there have been findings in human studies surrounding CBD use that support the idea that CBD absorption and metabolism may be more complicated; that CBD concentration in the blood may be dependent on both the physiological status (fasted/full) and the length/type of the dosing period a subject is on (Stott et al., 2013; Taylor et al., 2018). The current understanding

though is that the pharmacokinetic profiles in both animals and humans show a bell-shaped dose-response curve of isolated CBD (Alline Cristina Campos & Guimarães, 2009; Linares et al., 2019).

The idea of the fasted vs. full effects on CBD absorption become more intriguing with the understanding that CBD along with other cannabinoids are also highly lipophilic, and are easily stored in fatty tissue. This fat tissue storage may require improved estimates of CBD within the body, above and beyond strict blood analysis. CBD being hepatically metabolized, by isozymes CYP2C19 and CYP3A4; producing hydroxylated 7-hydroxy cannabidiol (7-OH-CBD) which is then excreted primarily through fecal, this metabolite could provide greater insight through further investigation (Lucas, Galettis, & Schneider, 2018) and give rise to an interesting connection between the effects produced by CBD, and CBD's interactions within the gut. The interaction between CBD and the gut may be supported by oral CBD providing more beneficial outcomes for individuals in need of symptomatic relief. This current project helps address concerns of CBD bioavailability after oral administration, since primary routes of administration in preclinical research have consisted of injection. CBD is primarily available as an oral supplement, signifying that oral gavage and oral administration of CBD in rodent models provide an improved translational model of human consumption, while improving guidance on appropriate dosing for therapeutic outcomes (Zhornitsky & Potvin, 2012).

4.3 CBD and Behavior

4.3.1 Pain-like Behavior

In this current study rodents that received a moderate dose of CBD (20mg/kg) had an increased latency to the hot plate stimulus when compared to controls, mimicking a reduced pain-like response. The results produced from this project would suggest that there is potential for antinociceptive outcomes and pain relief as a result of CBD consumption. It has been shown that

10mg/kg CBD through i.p injection in a mice Parkinson's model decreased sensitivity to thermal and mechanical pain stimuli in an acute and chronic manner (Nascimentoa et al., 2020). CBD has also shown benefits in regards to acute pain models. CBD served as a therapeutic agent for incision pain (Genaro et al., 2017), and disk inflammation in rodents (Silveira et al., 2014). CBD can also alleviate chronic pain, including osteoarthritic, neuropathic and inflammatory pain in rodents (Costa et al., 2007; Philpott et al., 2017). The models of prolonged pain may be may be more translatable for the current research. One hypothesis on how CBD may be producing its anti-nociceptive properties is through its agonistic influence on TRPV1. CBD serving as an agonist, activates TRPV1, allowing for a greater influx of Ca^{2+} to enter into a nociceptive neuron. Incoming Ca2+ binds to and activates Calmodulin, which then binds to a calmodulin recognition region on Calcineurin. Calcineurin acts as a protein phosphatase removing the phosphate that is bound to TRPV1 deactivating and desensitizing the neuron over time. This general agonistic pathway is mapped out by Patapoutian, Tate, and Woolf (2009), and is displayed in Figure 14. This path could serve synergistically with CBD's inverse agonistic effect on G-protein-coupled receptor 12 (GPR12). Brown, Laun, and Song (2017) investigate CBD's pathway through GPR12, which would inhibit cyclic adenosine monophosphate (cAMP) reducing protein kinase a (PKA) activation, limiting the phosphorylation of TRPV1. Both pathways work to either remove phosphate from TRPV1 or keep phosphate from binding to it, leading to a reduction in the signal being generated by a noxious stimulus. Leading to reduced pain perception. Further evaluation in a chronic model may be more suitable to assess pain relief due to a potential delay in CBD release into the blood from fat storage. Even though further work needs to be done to better interpret the potential for dose dependent variations in pain perception on account of the 40mg/kg dose group assessment yielding nil results, there has been some literature that indicated that higher doses of

CBD improve upon these effects (Genaro et al., 2017). There is a hope to expand more on these studies to improve upon the evaluation of appropriate dosing and routes of administration for improved translation to a more generalizable population. CBD could provide an alternative to the effective, yet potentially harmful current standard for analgesia, opiates. Pain is such a universal experience, that CBD's potential analgesic response may be one of its most promising.

4.3.2 Sleep Response

The results indicate that rodents that received 20mg/kg of CBD were more awake and alert, as they spent less time in an inactive state. Looking strictly at the third behavioral cohort of rodents, there are similar patterns (though non-significant) in time spent in an inactive state for the 20mg/kg group as well. The mean time spent in an inactive state for those that received 40mg/kg was beginning to return to being more in line with that of the control rodents. Further studies would need to be run to ensure that this dose dependent effect is not an error caused by low power within the third cohort, but the implications of these outcomes would be beneficial in evaluating CBD's potential as a natural stimulant. Coinciding with recent literature that examine sleep, CBD served as a wake-promoting agent in both humans and rodents (Nicholson et al., 2004), it caused no changes in SWS, reduced the duration of REM sleep, and increased c-Fos expression in the hypothalamus and the dorsal raphe nucleus (Murillo-Rodríguez et al., 2006). However, earlier studies determined that systemic administration of CBD increased duration of slow-wave sleep (SWS) and decreased wakefulness at a 40 mg/kg dose (Monti, 1977) and increased total sleep percentage in rats injected with 10 and 40 mg/kg of CBD as a whole (Chagas et al., 2013). Although it was first postulated that these changes of interpretation of CBD sleep outcomes could have been due to changes in methodologies, expanded research should be done into potential biphasic and situational effects of CBD on sleep. The outcomes produced in this experiment along

with further research could go a long way into evaluating therapeutic dosing required for improved wakefulness (stimulation) at low doses, and also improved restfulness at high doses.

4.3.3 Anxiety-like Behavior

There is minimal support from these findings that support any anxiolytic behavior, but there were signs of reduced movement. There is literature that does support anxiolysis with CBD administration in regards to social withdrawal (Malone et al., 2009) and overall anxiety (V. Almeida et al., 2013; Zieba et al., 2019). The Elevated Zero Maze, though a viable test is easily susceptible to handling and learned effects. This assay could be improved by only running it on the 10th day of administration, eliminating the potential for learned behavior coinciding with greater dose induced outcomes. There are also concerns with the dosing that was used for the behavioral assessments. Though it produced interpretable results within other anxiety testing assays, some of these effects were only seen at lower doses such as 5 to 10 mg/kg (J. L. C. Lee et al., 2017). The higher concentrated doses may not produce anxiolytic effects unless administered in combination with a TRPV1 receptor antagonists (Alline Cristina Campos & Guimarães, 2009). This variance by dose concentration makes it that much more crucial to continue the investigation of CBD's dose dependent anxiolytic effects and the receptors involved such 5-HT1a and TRPV1 receptors.

4.3.4 Stress Attenuation

A non-significant decrease in corticosterone concentration is seen immediately following restraint stress in the 20mg/kg dose group when compared to controls, and there is a significant decrease in the 40mg/kg dose group when compared to controls. This change in corticosterone concentration would imply that as the dose of CBD administered increases, the level of corticosterone produced in response to stress decreases; providing support for a dose dependent

attenuation of the stress response. There was no effect at baseline, and no change once a rodent had recovered from the stressor, which might be due to relatively low levels of corticosterone at these time points in general. The absence of an effect at baseline or recovery falls in line with the idea that CBD attenuates the consequences of stress, providing its therapeutic benefit in the presence of a stressful event. CBD helps to moderate the response to a stressful event (Joca et al., 2007; Joca et al., 2003). CBD also decreased mean arterial pressure in response to restraint stress (Resstel et al., 2009). This research has begun to address the question of the timing behind CBD's effect on stress, but further research would be necessary to address issues of more broad instances of stress, stress adaptation, prolonged stress, and the receptors involved such as 5-HT1a receptors (Russo et al., 2005).

4.4 Strengths and Limitations

This study had several limitations and strengths. One event that resulted in several limitations was the COVID-19-induced shut down of the laboratory that happened half way through the study. This necessary change in campus activity stymied project pace, and resulted in a change of personnel involved with the project. This shut down resulted in the putting down of rodents before the end of their behavioral testing (data excluded from final results), aged one group of rodents by an additional 30 days, extended time between the second cohort and the third, and drastically reduced the team available to run varying assays. These changes could potentially have led to both seasonal and handling effects. The plan of additional rats also resulted in unbalanced cohorts, resulting in an assessment of the 40mg/kg dose group with reduced power when compared to the GEE assessment of the control and 20mg/kg dose groups. In the course of rodent work, keeping all extraneous variables as consistent as possible is critical in producing the most reliable results. The COVID interruption did hinder the researcher's ability to maintain this consistency,

but the results produced are still interpretable and meaningful. A follow up study would benefit from rebalancing out the number of rats per group on a tighter timeline to increase power for interpreting dose effects.

A review of seasonal effects on laboratory rodent behavior by Ferguson and Maier (2013) found minimal to nil results supporting a seasonal difference in rodent behaviors tied to activity, and learning; ambiguous results for anxiety and depression; and some support for seasonal differences in pain response. Considering that the initial behavioral cohort batches of rats were run only 3 weeks apart, the effect of season is probably minimal within theses batches, but differences in genetic heritage, environmental conditions ((temperature and humidity variations, and construction noises, which are well documented as uncontrollable contributors to error in rodent studies (Blaustein, 2011; Dallman et al., 1999; Toukh, Gordon, & Othman, 2014), building construction was occurring on a building in close proximity)) cannot be excluded.

A study run by Sorge et al. (2014) found that exposure to male, not female experimenters produced pain inhibition in male rats, which was associated with an increased stress response. Sorge et al. (2014) would suggest that the change in personnel in the current study may have had an impact on the outcomes. There is also variation from individual to individual in quality of handling; differences in handling have been associated with variations in cognition, stress, and emotional response across behavioral procedures (Chapillon, Patin, Roy, Vincent, & Caston, 2002; Gouveia & Hurst, 2013; Meijer, Sommer, Spruijt, Van Zutphen, & Baumans, 2007; Núñez, Ferré, Escorihuela, Tobeña, & Fernández-Teruel, 1996). Though experimenters tried their best to standardize handling procedures across all personnel, COVID restrictions reduced the overall team size, which may have contributed to cohort differences (this could be due to handling, or even the timing of the tests, as they become more complicated to complete with a smaller team).

These variations in behavioral outcomes as a result of external factors may have occurred within the scope of this project. An initial observation of the data from rodents that received 20mg/kg CBD and controls to address cohort differences found some differences in behavioral outcomes. An example would be that in hot plate testing, the original cohort showed a distinct increase in hot plate latency of the 20mg/kg from day 1 to day 10, while controls were consistent. In the additional behavioral cohort there was a slight increase from day 1 to day 10 in hot plate latency for both the controls and those rodents that received CBD. The reduction in sample size (10 to 6) in the cohorts may have some influence over these differences with an increased potential for variation.

Another improvement that could be made to this study would be to use an improved hotplate assay. The hotplate device used suffered from inconsistent heat stability, and significant edge cooling drop off. Another potential limitation that could be viewed is that the rodents reduced in their movement during the EZM task, and these outcomes could hold implications when it came to the sleep assessment as well. However, this effect most likely has minimal weight when it comes to the interpretation of this sleep study given that the time frames are vastly different (5 minutes vs 6 hours) giving very different scopes of the rodents' behavior. There may be a need to run these assessments on the same day though to check and ensure that these outcomes do not influence one another.

A strength of this study is in its design. The use of oral gavage provides an improved translational model over other forms of administration, due to most current CBD projects marketed to humans being oral supplements. This Improved translation is also accompanied by the benefit of the purity of the product; using a 99% pure CBD product allowed for greater assurance that any produced outcomes were a result of the drug itself. Overall project reliability and reproducibility

improve with a purer product. An additional strength of this study would be that its design developed a pharmacokinetic curve that directed the implementation of CBD administration for behavioral assessment of a novel CBD compound. There are minimal projects within the literature that both seek to address the Pharmacokinetics of a novel CBD compound, as well as interpret how the compound affects behavior. In addition to co-assessment of both pharmacokinetics and behavior, the volume of CBD in sesame oil was appropriately increased each day due to weight measurements being taken at the start of each day of administration. One last strength is that this project has already begun to expand with future collaborations to assess varying brain regions of interest, and well as gut microbiome outcomes.

4.5 Dose Dependent Effects

Looking at the pharmacokinetic cohort (#1) that was run with all dose groups included at the same time, increases in CBD dosage is correlated with CBD present in blood plasma, and that all dose levels followed a similar pattern. Therefore, it would be expected that there would be a dose dependent effect within the behavioral outcomes as well. A dose-dependent effect appears to hold true for a few of the behaviors. This research does have to contend with the understanding that there were no effects that occurred for day 1 behavioral testing, so CBD concentration in blood may not be the best measure to evaluate CBD's potential effect on behavior. Expanding on this day effect, the study showed that CBD concentrations were similar across the 10-day experiment and did not show any buildup in blood from day to day, with the behavioral effects occurring only on the 5^{th} or 10^{th} day. The presence of behavioral outcomes in spite of CBD clearing the bloodstream over 24 hrs, and not showing any buildup from day to day, may signify other processes being involved in CBD's produced outcome such as storage and tissue concentration. High levels of CBD storage in tissue may occur due to its highly lipophilic nature, as well its

potential to quickly crosses the Blood Brain Barrier (BBB) (Hložek et al., 2017). CBD seems to have a greater affinity to crossing the blood brain barrier that isn't fully understood (Aparicio-Blanco et al., 2019), but the process for CBD's crossing appears to be associated with transmembrane diffusion, a process highly influenced by a compounds molecular weight, and lipid solubility (Banks, 2009). However, even if CBD does cross using transmembrane diffusion, and with it being shown that oral administration of cannabinoids produced relatively high concentrations within the brain, 10mg/kg of CBD still clears the brain entirely by the 24th hr (Hložek et al., 2017). This outcome calls into question how CBD is having its effects, and requires further investigation. Understanding CBD's effects could be improved upon by assessing if higher dosing levels result in higher CBD concentration in the brain for longer periods of time; is it strictly CBD brain concentration that plays this determining role or what other physiological interactions with CBD administration could be involved in producing these behavioral changes.

Though integration from the output from the 40mg/kg dose group and 20mg/kg dose group isn't statistically viable due to limitations of the project, it is still meaningful to look at how their output can be interpreted together. Understanding how thee varying doses coincide together becomes one of the most important implication to address from this data analysis, replication between cohorts. In evaluating these outcomes between the behavioral doses (20mg/kg and 40mg/kg) the stress assessment is a good starting point. In looking at Figures 8 and Figure 12, one can begin to see the trend of how corticosterone levels are affected by increases in CBD administration. In Figure 8, a trend can be seen within the 20mg/kg dose group which begins to reduce corticosterone levels after a stressful event, then a continuation of this trend is shown with the 40mg/kg dose group reaching significance; having a significantly lower level of corticosterone present as a result of a stressor when compared to control. This outcome suggests a potential dose

dependent effect, were increases in CBD concentration administered is inversely related to corticosterone levels present after a stressful event. Further analyses are needed to address where these effects may plateau in relation to the concentration of CBD administered.

Next, an evaluation of dose dependent implications for pain assessment. In looking at the output comparing all controls to 20mg/kg dose group (Figure 5), a significant increase in hotplate latency for 20mg/kg dose group is shown on day 10 only. A similar pattern is shown in cohort 3 (Figure 9), but the difference is not significant. An absence of significant pain-like behavior findings may be the result of a smaller sample size, or just issues with the hotplate itself (issue with hotplate stability, and edge heat). When looking at how the 40mg/kg dose group behaved on the hotplate, on average rats had a non-significantly reduced latency time to hotplate response when compared to both control and the 20mg/kg dose group. However, looking at the average for all controls (n=16) the average is very similar to that of the 40mg/kg dose group. These averages would imply that a higher concentration of CBD would not produce an effect. This outcome is slightly puzzling, and not as commonly seen in the literature, but there is some support for it. Nascimentoa et al. (2020) showed a significant increase in hot plate latency for all CBD dose groups compared to control, but 100mg/kg was the only dose significantly different from the other two. It has also been showing using other pain inducing assays (cold stimulus intensity and mechanical stimulus) that the withdrawal latency of 30mg/kg CBD 30mg/kg dose group was the same as control, while 10 and 100mg/kg improved latency (Nascimentoa et al., 2020). The odds to have come across similar outcomes within the hot plate testing, where an improved response to a painful stimulus is shown, and that benefit is lost at a higher dose is slim. A continuation of this experiment into an even higher dose is needed and could be used to see if the same response occurs. Interpretation of the literature is also complicated due to the majority of CBD administration and

pain threshold testing being done in hyperalgesia rodent models, not from healthy rats. These implications will need to be kept in mind when running further assessments of pain.

Looking at the sleep output to address differences that arose with CBD dosing. Looking at figures 6 and 10, across all measures, excluding time spent in an inactive state, in all assessments there is no difference between control, 20mg/kg, or 40mg/kg dose group. As it relates to time spent in an inactive state, 20mg/kg reduces this time regardless of assessment (significant in combined assessment and trending when limited to just cohort 3). However, there is not a decrease in inactivity for the 40mg/kg group, it more closely resembles the control. The implications of these changes occurring at one dose and not the other would be that the 20mg/kg dose group would actually help keep them awake, while 40mg/kg either has no effect, or signifies the potential for this outcome to reverse at higher doses. A potential biphasic effect where low dose CBD can promote wakefulness, and high doses can promote restfulness. Increased wakefulness (Murillo-Rodríguez et al., 2011) and restfulness (Chagas et al., 2013) have both been proposed as potential outcomes of CBD use, but not within the same context or experiment. As Murillo-Rodríguez et al. (2011) looked into c-fos expression in the lateral hypothalamus as a measure of activation that would indicate wakefulness, assessing this same measure at varying dose levels could provide greater insight into this phenomena. Sleep assessments would have to be assessed further but there is potential as it relates to beneficial sleep outcomes.

Lastly, a brief discussion of dosing concerns in regards to the anxiolytic results. In this assessment of control against the 20mg/kg group (Figure 7) a significant reduction is seen across the board on day 10 as a result of CBD administration; less movement, less time in the open arm, and lower entries into the open arm. This pattern continues when looking at strictly cohort 3 (Figure 11). Although the outcomes in cohort 3 may not be significant, only on day ten is a

reduction in movement, open arm entries, and time spent in the open arm as a result of this CBD product seen, both at the 20 and 40mg/kg dose. There is also consistency in produced outcomes overall between this analysis of control against 20mg/kg and cohort 3 results across all days, even with reduced sample sizes. Interestingly, open arm time and entries appears similar between the 20 and 40 mg/kg dose group, while the 40mg/kg group appears to move even less then the 20mg/kg group. Lack of movement complicates further analysis of any potential anxiolytic properties, as that assessment relies on rodent movement. An improvement on this analysis going forward could be to run it only on the 10^{th} day of analysis, or by assessing anxiety using a less movement dependent assay such as novelty induced hypophagia (Dulawa & Hen, 2005). There is potential for any variations between cohorts to be the result of changes in season, handling, timing, and overall power, but these results are primarily similar across cohorts, and most outcomes do align with, and expand upon the literature reviewed .

4.6 Future Directions

Though this project has produced a variety of interesting and meaningful results, there are several future directions that would bolster understanding even more. In regards to the project overall, assessment of different age groups, routes of administration, cannabinoids, and sexes would add additional information to each of the currently produced outcomes and produce a more well-rounded understanding of this compound. Directing more stringent assessment towards weight and temperature in relation to rodent models of Diabetes or Aids, would allow for improved evaluation of CBD's effect on appetite and thermoregulation. This project is also already being expanded within the lab to make assessments of both the microbiome and the brain. These future studies will help improve upon understanding of CBD's potential mechanisms involved with the varying behavioral outcomes, and will help direct work towards improving health outcomes. Pain

sensitivity tests could be improved upon with the inclusion of an induced hyperalgesia group, allowing for improved assessment on varying effects caused different CBD doses. Sleep outcomes could be supplemented by introducing a sleep disturbance to the assay, allowing for an assessment of the potential of CBD to act as a sleep-inducing agent, not a wake inducing one. It would also be interesting in regards to anxiety like outcomes to delay the initial assessment, to parse out any potential learning effects, from the effects of the drug. There is also potential to assess a plateau or ceiling effect regarding stress response, by including much higher dose groups to assess if corticosterone continues to decrease with an increase in CBD dose. Also, CBD administration over a longer period of time, and potentially including multiple administrations may also improve understanding of how it is processed in the body. Finally, Due to CBD's fat-soluble nature and its rapid elimination through the bloodstream, the effects of CBD may linger longer than this project's current ability to detect it in blood, requiring an improved or different measures of CBD concentration in the body (being directly from tissue, fat, hair etc). This evaluation would allow for greater linkage between behavioral outcomes and CBD bioavailability, providing information to improve therapeutic dosing standards.

4.7 Conclusion

This was the first lab to use this novel hemp derived CBD compound from Ellipse Analytics (Denver, Colorado), to try and establish connections between CBD's bioavailability and the behavioral outcomes that accompany its consumption. This study found that CBD oral administration resulted in potential reduction of pain sensitivity, increased wakefulness over an extended period of time, reduced movement over a limited period of time, and reduced stress response, all relative to the dose administered. There was no observed effect of CBD consumption on weight or body temperature change, but further investigation into higher doses is needed. This

study also produced a few findings on the EZM in regards to reduced movement, making evaluation of anxiety like outcomes more problematic. Future projects intend to look at more extensive biological and neurological outcomes, such as microbiome impact, and TRPV1 expression, as a result of the consumption of CBD and other cannabinoids. This research intended to expand the understanding of CBD broadly to better inform the healthcare and economic practices that surround this product. The work here, along with future projects, would help improve upon standards for regulation and consumption within the human population.

Tables and Figures

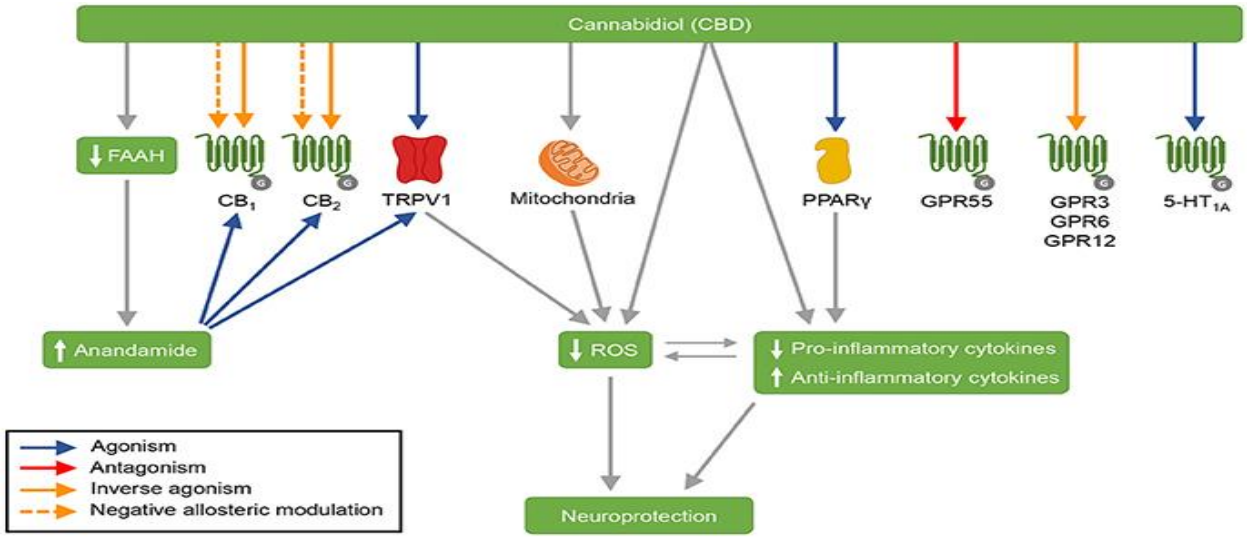
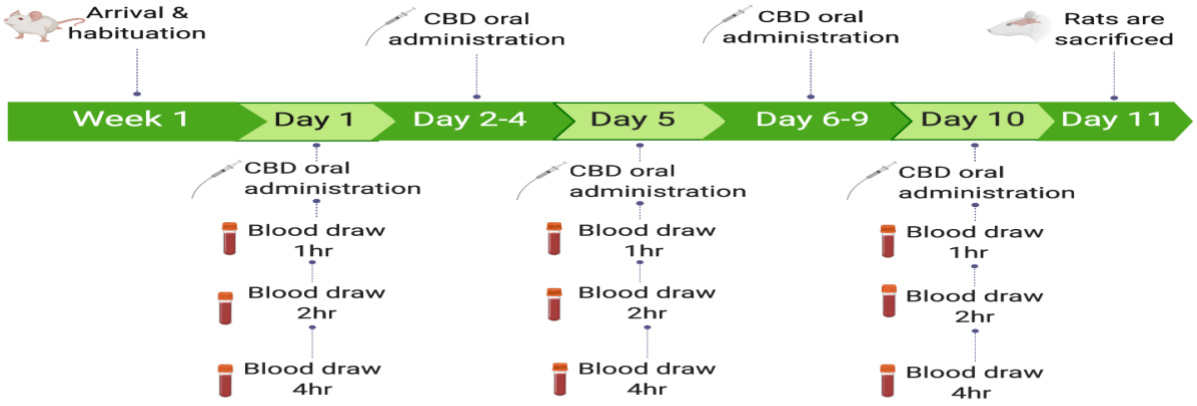
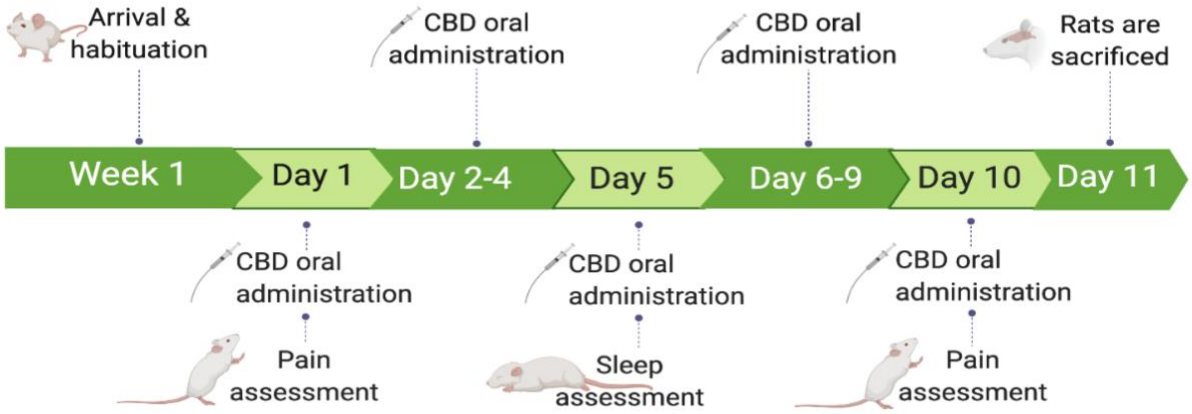


Figure 1. CBD sites of interaction , Peres et al. (2018). CBD serves as an inverse agonist and a negative allosteric modulator of CB1 and CB2 receptors. CBD agonistic behavior is shown at receptors TRPV1, PPARγ, and 5-HT1A, and antagonistic behavior is typical on receptors GPR55. Inverse agonism also occurs at GPR3, GPR6, and GPR12. *Grey arrows depict indirect interactions*

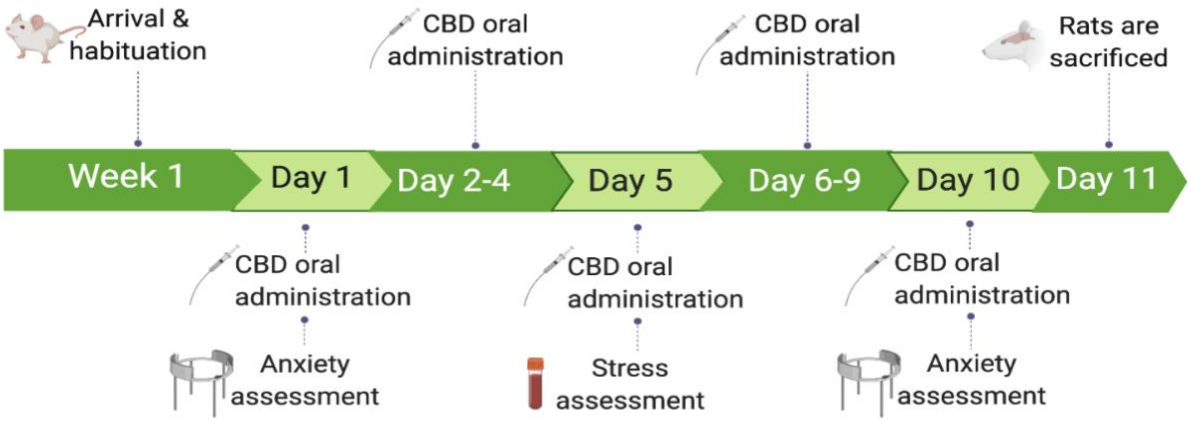
Figure 2. Timeline of experimental procedures (1) blood draws for pharmacokinetic assessment, (2) the pain and sleep behavioral arm, and (3) the anxiety and stress behavioral arm



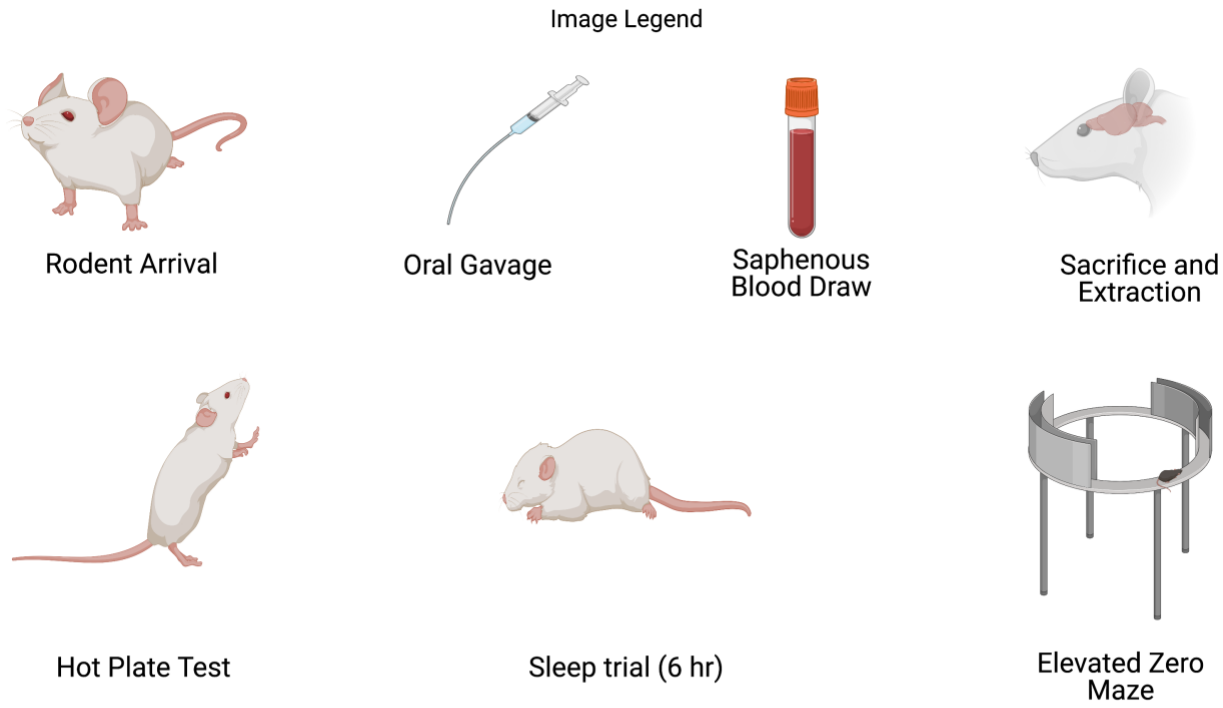
2.1 **Timeline representation of CBD administration and saphenous blood draws for bioavailability assessment** **Created with BioRender.com**



2.2 **Timeline representation of CBD administration and progression through the pain and sleep assays** **Created with BioRender.com**



2.3 **Timeline representation of CBD administration and progression through the anxiety and stress assays** **Created with BioRender.com**



Created with BioRender.com

Table 1: Sample size per experimental condition

Experimental Condition	**0mg/kg**	**5mg/kg**	**10mg/kg**	**20mg/kg**	**40mg/kg**	**N**
Cohort 1: Pharmacokinetic Assessment	10	10	10	10	10	**50**
Cohort 2 Pain & Sleep	10	-	-	10	-	**20**
Cohort 2 Anxiety & Stress	10	-	-	10	-	**20**
Cohort 3 Pain & Sleep	6	-	-	6	16	**28**
Cohort 3 Anxiety & Stress	6	-	-	6	16	**28**
Total Pain and Sleep	16	-	-	16	16	
Total Anxiety and Stress	16	-	-	16	16	
Total						**146**

Table 1 displays the number of rats used in each cohort and assessment. 10 rats from each dose group (0mg/kg, 5mg/kg, 10mg/kg, 20mg/kg, and 40mg/kg) were used in the pharmacokinetic assessment, totaling 50 rats. 10 rats from the control (sesame oil) and 10 rats from 20mg/kg dose groups were part of cohort 2 of the pain and sleep assessment totaling 20 rats. In cohort 2 of the anxiety and stress arm, 10 rats from both the control (sesame oil) and 20mg/kg dose groups were used, totaling 20 rats. Cohort 3 of the pain and sleep arm had 6 rats from both 0mg/kg and 20mg/kg dose groups in addition to 16 rats from 40mg/kg dose group, adding up to 28 rats. In cohort 3 of the anxiety and stress assessment, 28 rats were used, including 6 from 0mg/kg and 20mg/kg dose groups and 16 rats from 40mg/kg dose group.

Table 2: Effect Size Comparisons

Experimental Analysis	**(Cohorts 2+3)**		**(Cohort 3)**
	Fishers z		η_p^2
Value Assessed	Dose		Dose
Hot Plate (latency)	0.11 (1)	0.28 (10)	.14
Sleep (distance traveled)	0.084		.068
Sleep (# of inactive entries)	0.20		.067
Sleep (latency to first)	0.033		.008
Sleep (total inactive time)	0.35		.096
EZM (distance traveled)	0.057 (1)	0.31 (10)	.120
EZM (time in open arm)	0.15 (1)	0.26 (10)	.034
EZM (open arm entries)	0.045 (1)	0.29 (10)	.074
Restraint Stress	0.24		.239

Table 2 displays estimates of effect sizes from behavioral experimentation for CBD dose assessment. All Fisher's Z values were calculated using https://www.campbellcollaboration.org/escalc/html/EffectSizeCalculator-R5.php, and η_p^2 were produced in SPSS

Weight

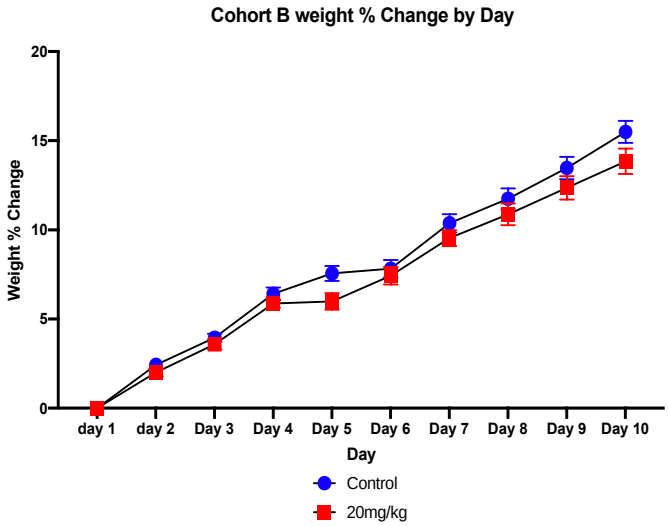


Figure 3.1 Weight percent change by day by dose group. Dose groups displayed are control (sesame oil, n=20), and 20mg/kg (n=20). There was no significant effect of dose [F(1,38)=2.669, P=.111]. However, as Mauchly's test reached significance of P<.001, a Greenhouse-Geisser correction was used, and it was shown as there was a significant difference between days with all rats gaining weight throughout the course of the experiment [F(2.210, 717.051)=427.827, P<.001] Data shown are means ± SEM.

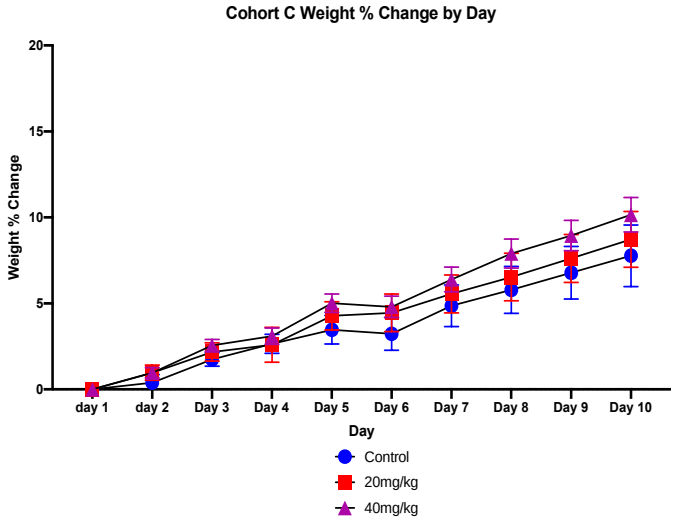


Figure 3.2 Weight percent change by day by dose group. Dose groups displayed are control (sesame oil, n=12), 20mg/kg (n=12), and 40mg/kg (n=32). There was no significant effect of dose [F(2,53)=.889, P=.417]. However, as Mauchly's test reached significance of P<.001, a Greenhouse-Geisser correction was used, and it was shown that there was a significant difference between days with all rats gaining weight throughout the course of the experiment [F(1.465,77.647)=86.699, P<.001] Data shown are means ± SEM.

Temperature

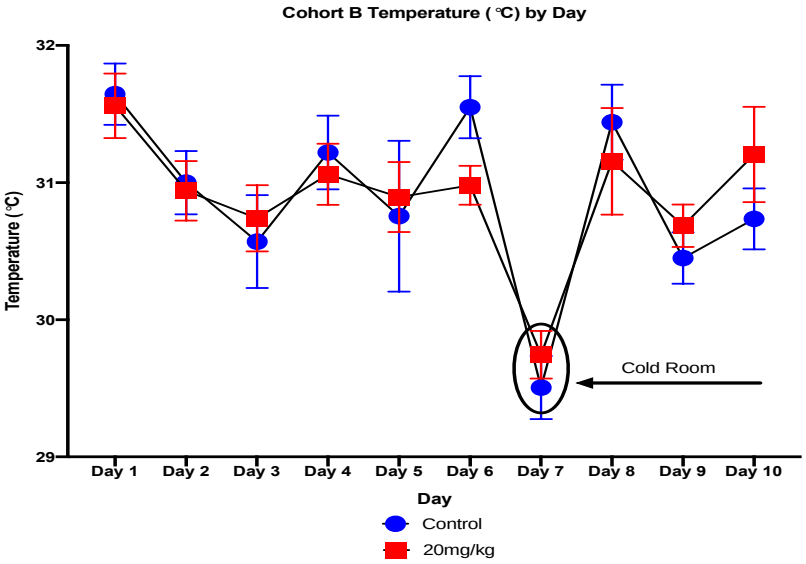


Figure 4.1 Means displayed represent temperature (ºC) by day by dose group. Dose groups displayed are control (sesame oil, n=20), and 20mg/kg (n=20). No significant difference between dose groups was experienced [F(1,38)= .004, P=.952]. Sphericity not assumed (Mauchly's test P<.001), a Greenhouse-Geisser correction was used, revealing a significant difference between days [F(5.330,467.971)=8.618, P<.001] Data shown are means ± SEM.

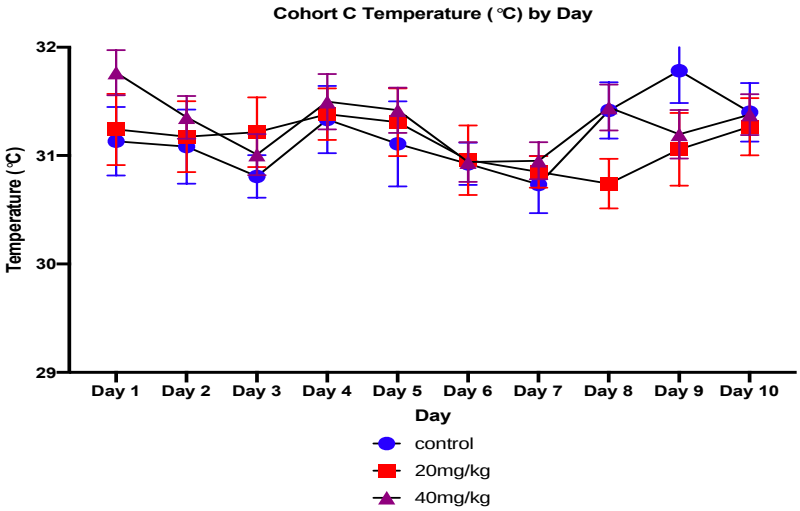


Figure 4.2 Means displayed represent temperature (ºC) by day by dose group. Dose groups displayed are control (sesame oil, n=12), 20mg/kg (n=12), and 40mg/kg (n=32). No significant difference between dose groups was experienced [F(2,53)= .266, P=.768]. Sphericity not assumed (Mauchly's test P<.001), a Greenhouse-Geisser correction was used, revealing a significant difference between days [F(7.213,315.800)=2.685, P<.01] Data shown are means ± SEM.

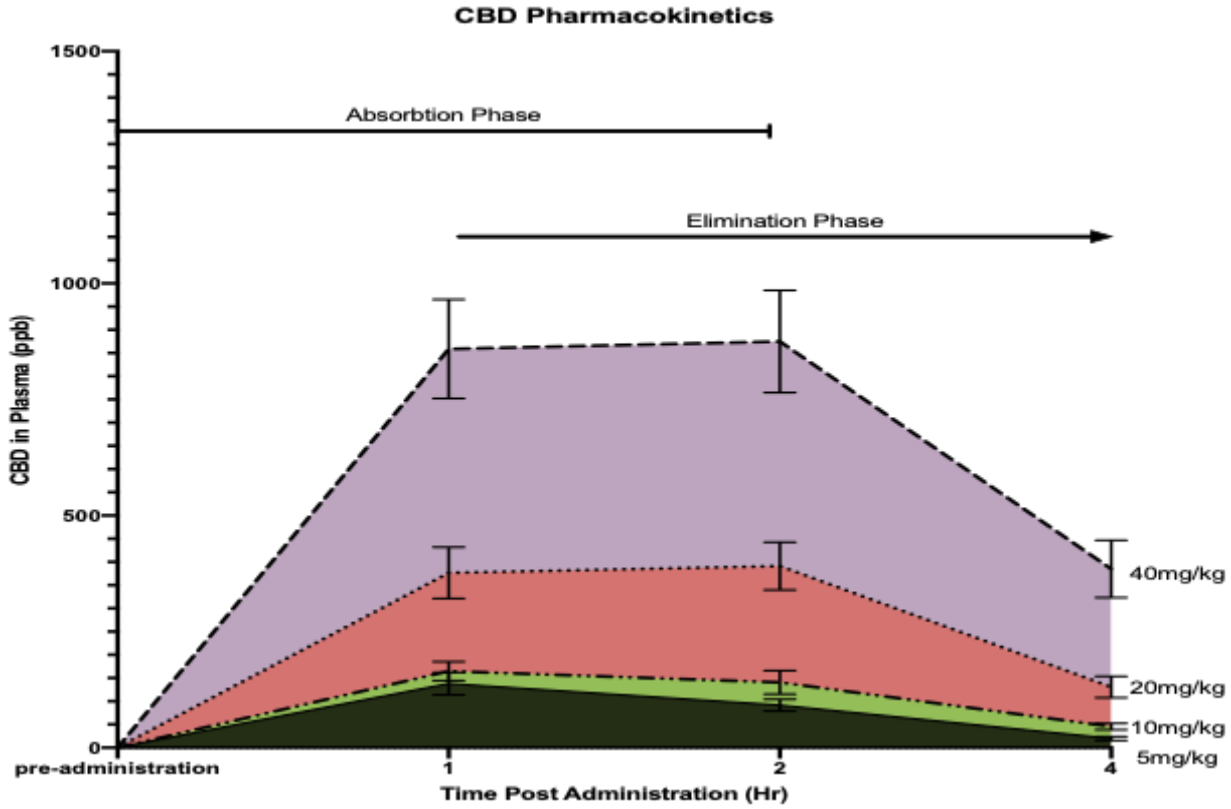


Figure 5. **There is a dose dependent increase in CBD bioavailability with plasma levels peaking between the 1st and 2nd hour post-administration, and significantly decreasing by the 4th hour across all groups.** Doses represented are 5mg/kg (n=10), 10mg/kg (n=10), 20mg/kg (n=10), and 40mg/kg (n=10). 5 of 180 blood sample measurements were removed as outliers. Effects were not shown to differ by day [$F(2,136)=2.187$, $P=0.116$]. CBD in plasma differed by both dose [$F(3,136)=104.512$, $P<.001$] and time [$F(2,136)=32.359$, $P<.001$]. Post Hoc testing revealed 40mg/kg was different from all other doses ($P<.001$). Further, CBD values at hour 4 differed significantly from hours 1 and 2 ($P<.001$). Data shown are means ± SEM.

0 and 20mg assessment [data run through SPSS (GEE), graphs generated in Prism]

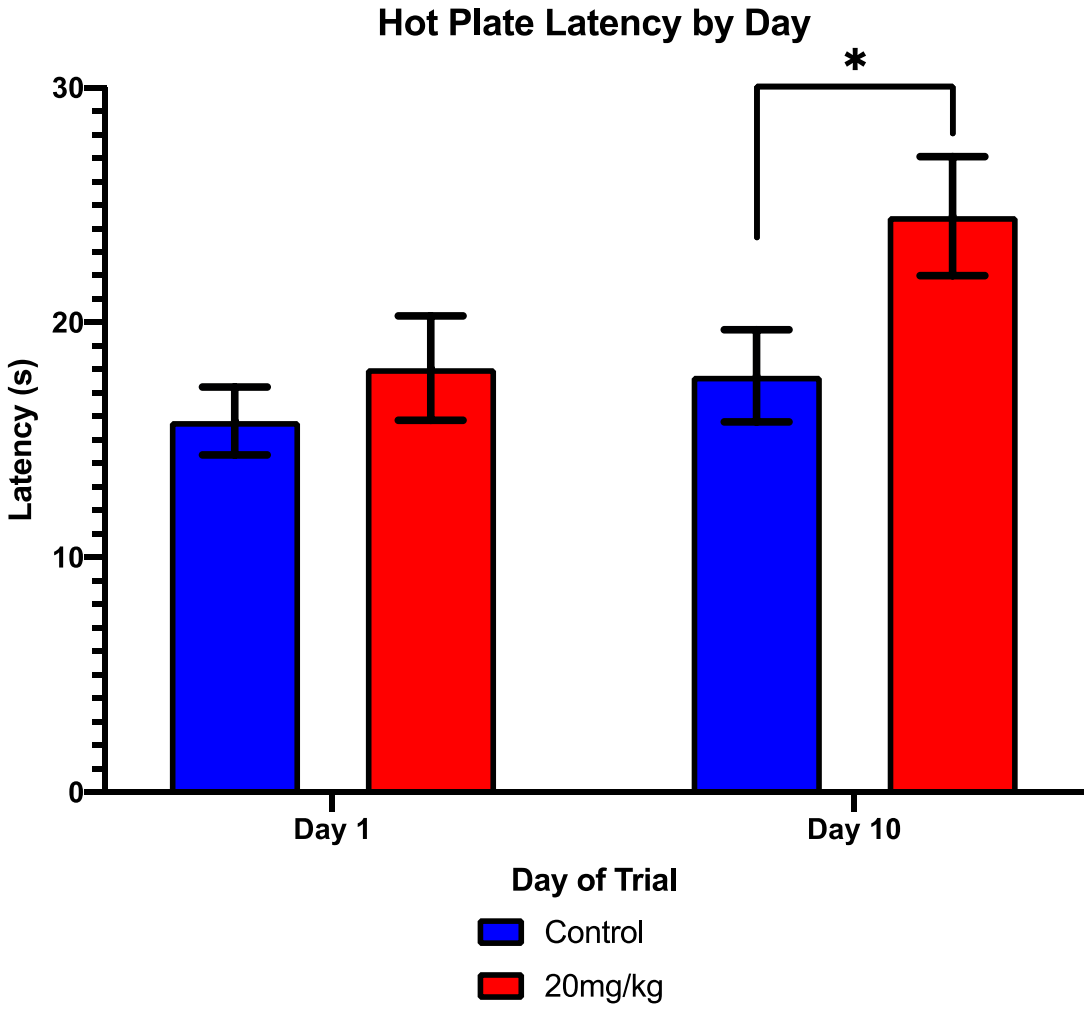


Figure 6. Hot plate latency (s) by day by dose group. Dose groups displayed are control (sesame oil, n=16) and 20mg/kg (n=16). Two outliers were removed 1, from the control cohort on day 10, and 1 from the 20mg/kg dose group, both for reaching max time. No effect of dose on Day 1 was experienced [Wald χ^2 =.766, P=.381]. However, there was an effect of dose on Day 10 [Wald χ^2=4.833, P=.028]. Beta weights showed that, on Day 10, the 20 mg/kg dose group had a 6.800 second increase on average in latency in reaction to the hot plate. Mean hot plate latency for 0 mg/kg on Day 10 was 17.733 seconds while mean hot plate latency for 20 mg/kg on Day 10 was 24.533 seconds. Data shown are means ± SEM.

Sleep Assessment

Figure 7.1. Means displayed represent distance traveled (cm) during the sleep assessment by dose group. Dose groups represented are control (sesame oil, n=16) and 20mg/kg (n=16). No significant difference between dose groups was experienced [Wald x^2=.446, P=.504]. Data shown are means ± SEM.

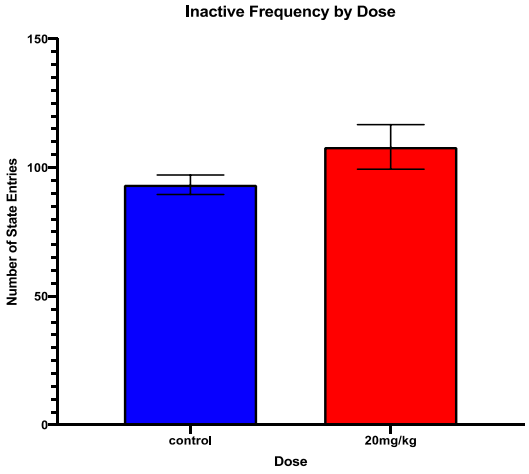


Figure 7.2. Means displayed represent the number of entries into the state of inactivity by dose group. Dose groups exhibited are control (sesame oil, n=16) and 20mg/kg (n=16). There was no effect of dose group evaluated [Wald x^2=2.569, P=.109]. Data shown are means ± SEM.

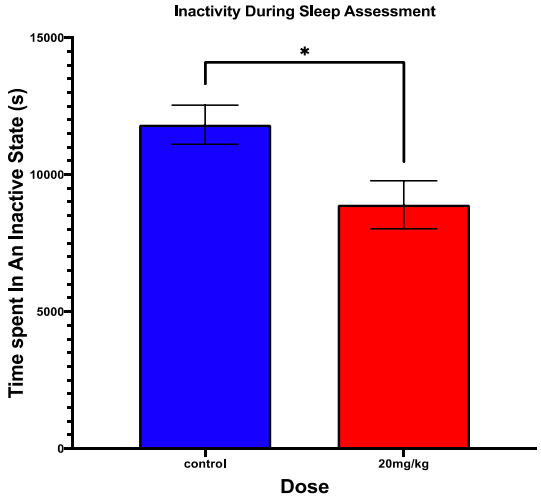


Figure 7.3. Means displayed represent the cumulative time (s) spent in an inactive state by dose group. Dose groups represented are control (sesame oil, n=16) and 20mg/kg (n=16). There was a significant difference between dose groups [Wald x^2=7.105, P=.008]. Beta weights showed that the 20mg/kg dose group spent 2923.693 seconds less in an inactive state than 0mg/kg on average. The mean cumulative duration spent in an inactive state for 0mg/kg and 20mg/kg dose groups were 11824.2 seconds and 8900.5075 seconds, respectively. Data shown are means ± SEM.

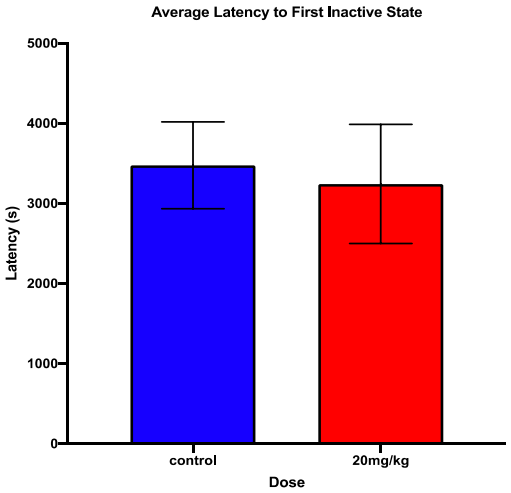


Figure 7.4. Means displayed represent the latency to first inactive state (s) by dose group. Dose groups displayed are control (sesame oil, n=16) and 20mg/kg (n=16). No significant difference between dose groups was shown [Wald x^2=.068, P=.794]. Data shown are means ± SEM.

Elevated Zero Maze Assessment

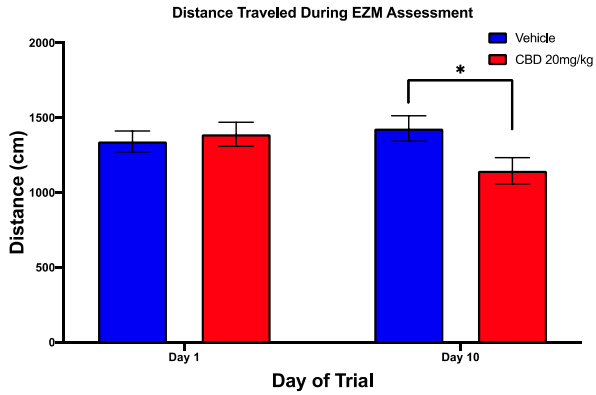


Figure 8.1. Graph means represent the distance traveled (cm) througout the Elevated Zero Maze by dose group by day. Dose groups are control (sesame oil, n=16) and 20mg/kg (n=16). On day 1, no significant effect of dose group on the total distance traveled was found [Wald x^2=.209, P=.647]. However, on day 10 there was a significant difference between the dose groups [Wald x^2=5.721, P=.017]. Beta weights show that the control (0mg/kg) dose group traveled on average 283.261 cm more in the EZM than the 20mg/kg dose group on Day 10. The average total distance traveled by 0mg/kg and 20mg/kg on Day 10 was 1428.4388 cm and 1145.1781 cm, respectively. Data shown are means ± SEM.

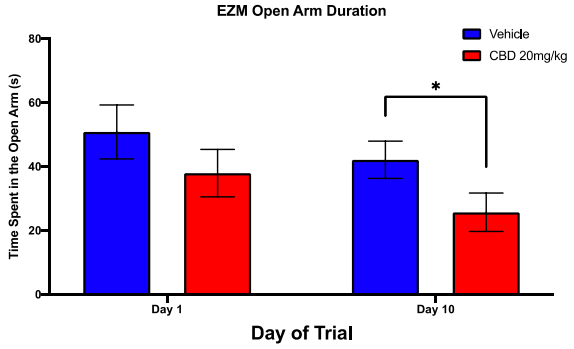


Figure 8.2. Graph means represent the cumulative time (s) spent in the open arm of the EZM by dose by day. Dose groups are control (sesame oil, (n=16) and 20mg/kg (n=16). Significance was not reached for an effect of dose group on Day 1 [Wald x^2=1.407, P=.236]. However, there was a significant difference between dose groups on Day 10 [Wald x^2=4.084, P=.043]. Beta weights signify that the control (0mg/kg) group spent on average 16.4 seconds longer in the open arm of the Elevated Zero Maze than the 20mg/kg dose group on Day 10. On average the control group spent 42.095 seconds in the open arm of the EZM, while the 20mg/kg dose group spent 25.6975 seconds. Data shown are means ± SEM.

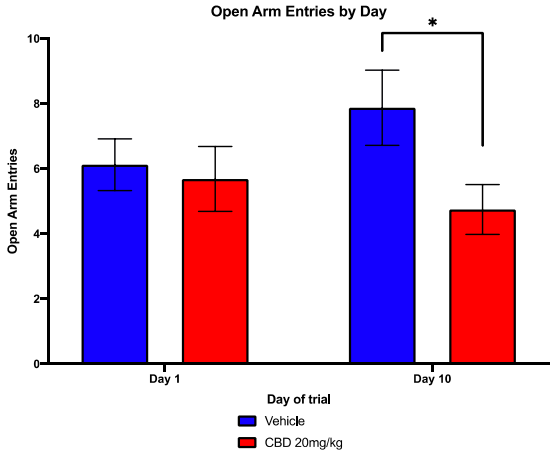


Figure 8.3. Graph means represent the average number of entries into the open arm of the EZM by dose group by day. Dose groups are control (sesame oil, n=16) and 20mg/kg (n=16). Dose group did not have a significnat effect on day 1 open arm entries [Wald x^2=.125, P=.723]. However, there was a significant difference between dose groups on Day 10 [Wald x^2=5.217, P=.022]. Beta weights show that 0mg/kg had a mean number of entries that were 3.062 more than the 20mg/kg dose group for the open arm on Day 10. On average, the 0mg/kg dose group entered the open arm of the EZM 7.88 times on Day 10, while the 20mg/kg dose group entered 4.81 times. Data shown are means ± SEM.

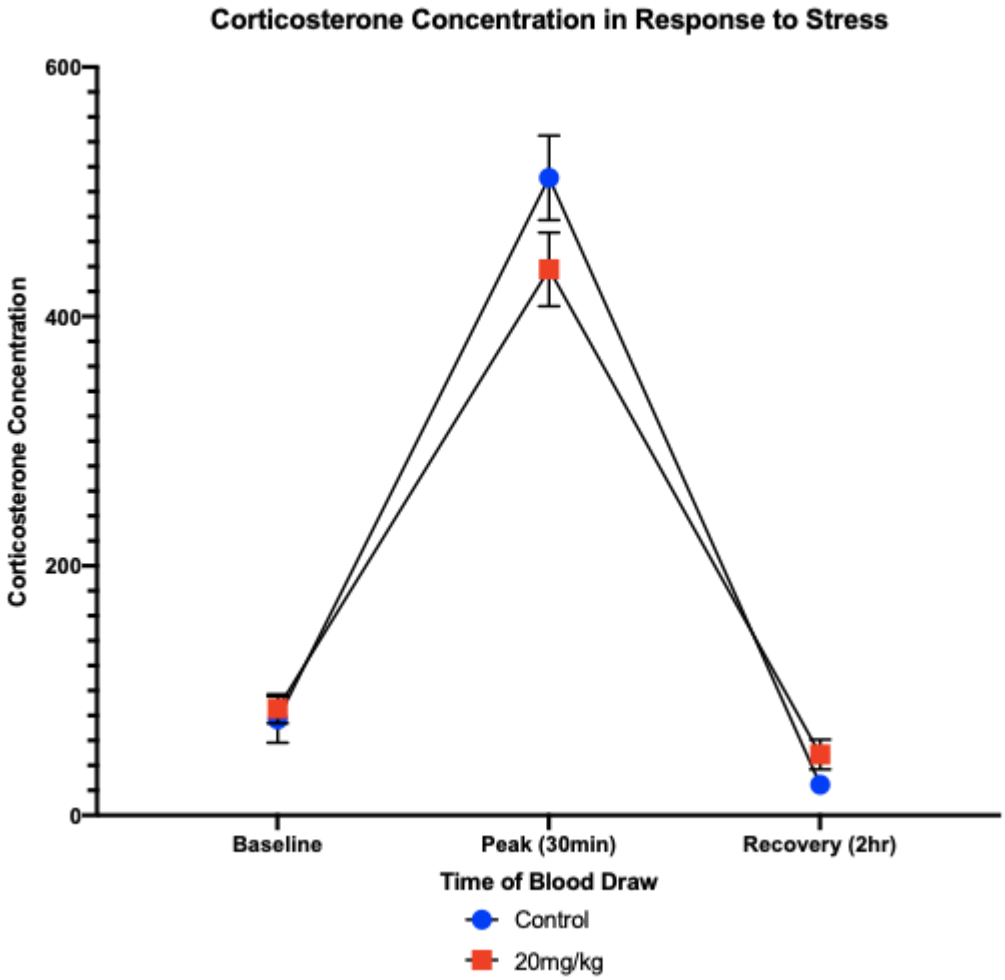


Figure 9. Means represent Corticosterone levels (ng/ml) by dose by time of blood draw. Dose groups shown are control (sesame oil, n=16) and 20mg/kg (n=16). 1 outlier was removed from the control recovery time point due to exceeding its z-score threshold. Time of blood draw is expressed by baseline (initial), peak (30 minutes of restraint stress), and recovery (2 hours from baseline). GEE analysis showed that statistical significance was not reached for baseline or recovery measures, [Wald χ^2=.208, P=.648] and [Wald χ^2=.000, P=.998] respectively. Corticosterone levels immediately following restraint stress did not reach, but trend towards, significance, with the 20mg/kg dose group appearing to have lower levels than control [Wald χ^2=3.442, P=.064]. Data shown are means ± SEM.

Cohort 3 (40mg/kg assessment) Figures

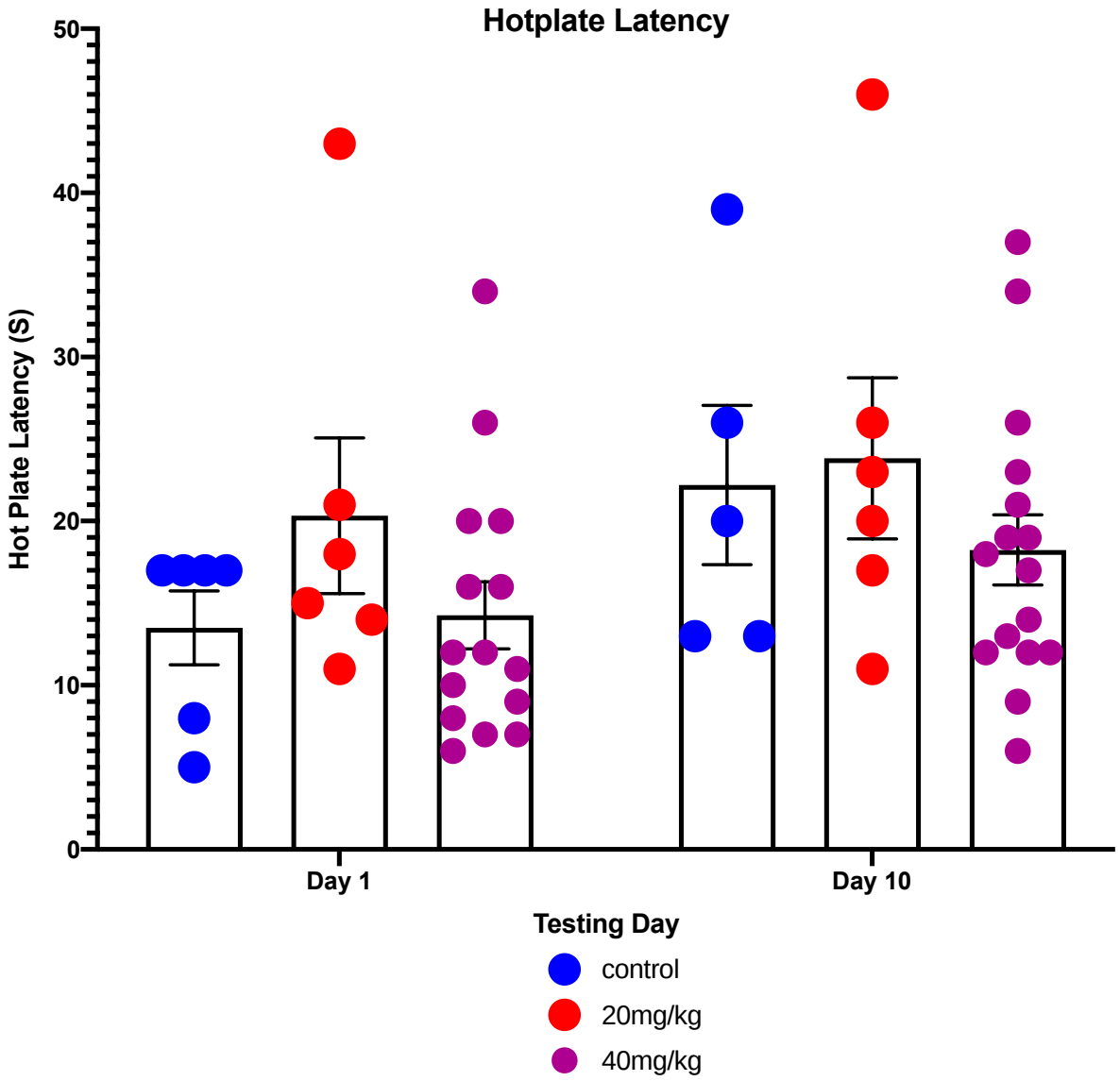


Figure 10. Means displayed represent hot plate latency (s) by dose group. Dose groups represented are control (sesame oil, n=6), 20mg/kg (n=6), and 40 mg/kg (n=16). A single outlier was removed from the control cohort on day 10 due to reaching max time (hot plate cooling), and a single outlier was removed from day 1 of the 40mg/kg dose group for exceeding the z-score threshold. A repeated measure ANOVA revealed no significant differences between dose groups. [F(2,23)=1.897, P=.173] Data shown are means ± SEM.

Sleep Assessment

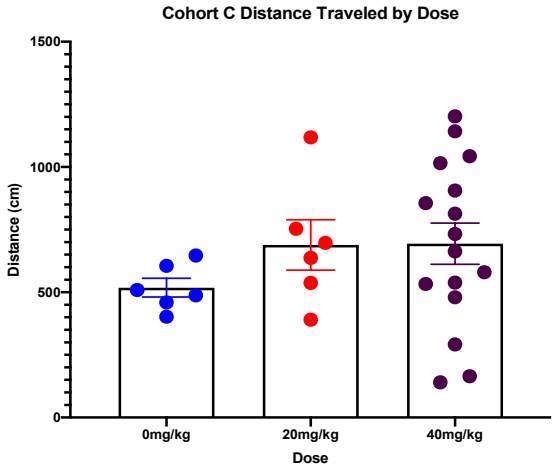


Figure 11.1. Means displayed represent the distance traveled (cm) by dose group. Dose groups represented are control (sesame oil, n=6), 20mg/kg (n=6), and 40 mg/kg (n=16). ANOVA displayed no significance between dose groups [F(2,255)=.906, P=.417]. Data shown are means ± SEM.

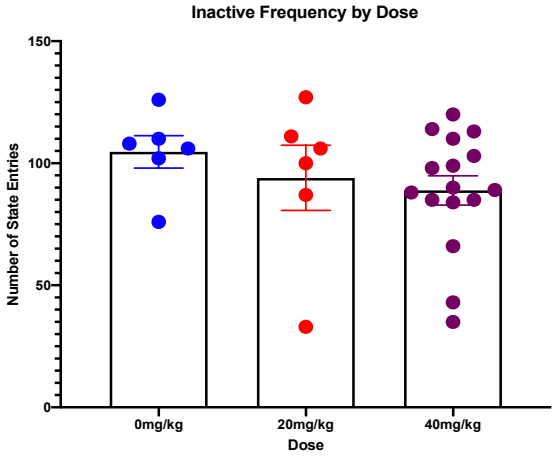


Figure 11.2. Means displayed are number of entries into state of inactivity by dose group. Dose groups are control (sesame oil, n=6), 20mg/kg (n=6), and 40 mg/kg (n=16). ANOVA revealed no significant differences between dose groups [F(2,25)=.894, P=.422]. Data shown are means ± SEM.

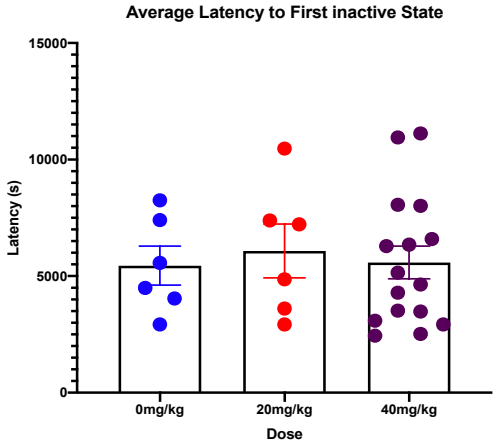


Figure 11.3. Means displayed represent the latency to first inactive state (s) by dose group. Dose groups represented are control (sesame oil, n=6), 20mg/kg (n=6), and 40 mg/kg (n=16). An ANOVA showed no significant differences between dose groups [F(2,25)=0.099, P=0.906]. Data shown are means ± SEM.
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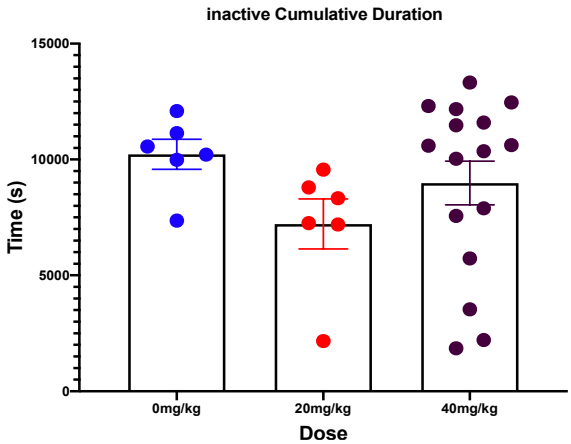

Figure 11.4. Means displayed represent the time (s) spent in an inactive state by dose group. Dose groups represented are control, (sesame oil, n=6), 20mg/kg (n=6), and 40 mg/kg (n=16). ANOVA revealed no significant effect of dose on inactivity cumulative duration [F(2,25)=1.323, P=.284]. Data shown are means ± SEM.

Elevated Zero Maze Assessment

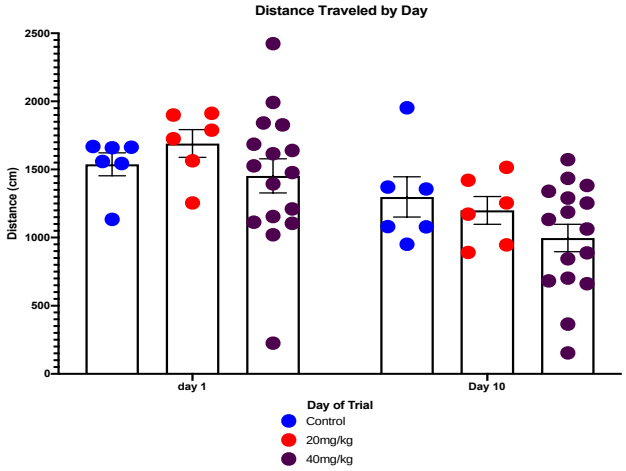

Figure 12.1. Means displayed represent the total distance traveled (cm) by dose group by day. Dose groups consisted of control (sesame oil, n=6), 20mg/kg (n=6), and 40 mg/kg (n=16). After being evaluated, there was no significant effect of dose related to the total distance traveled [F(2,25)=1.706, P=.202]. Data shown are means ± SEM.

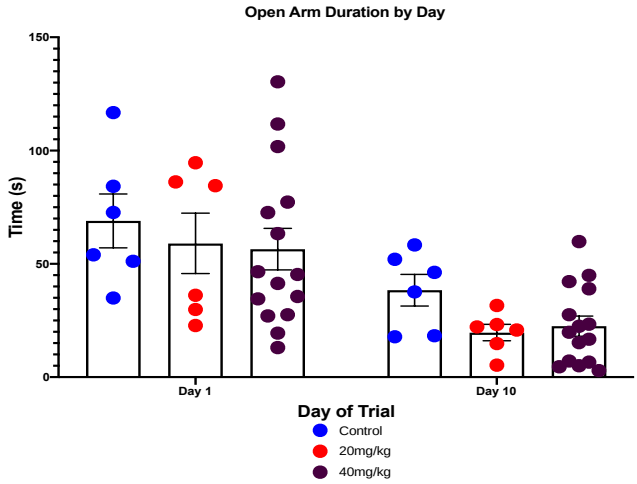

Figure 12.2. Means displayed represent the total time (s) spent in the open arm of the EZM by dose by day. Dose groups evaluated are control (sesame oil, n=6), 20mg/kg (n=6), and 40 mg/kg (n=16). 2 outliers were removed for exceeding our z-score threshold (freezing), a single 40mg/kg rodent on day 1, and a different one on day 10. Within the Omnibus repeated measures ANOVA. there was no significant effect of dose group [F(2,25)=.443, P=.647]. Data shown are means ± SEM.

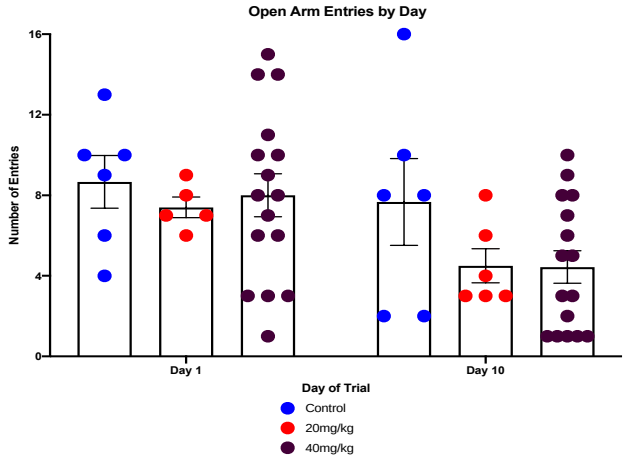

Figure 12.3. Means displayed represent the number of entries into the open arm of the EZM by dose group by day. Dose groups represented are control (sesame oil, n=6), 20mg/kg (n=6), and 40 mg/kg (n=16). 1 outlier was removed for exceeding our z-score threshold, a single 20mg/kg rodent on day 1. There were no significant differences between dose groups [F(2,25)=0.999, P=.383]. Data shown are means ± SEM.

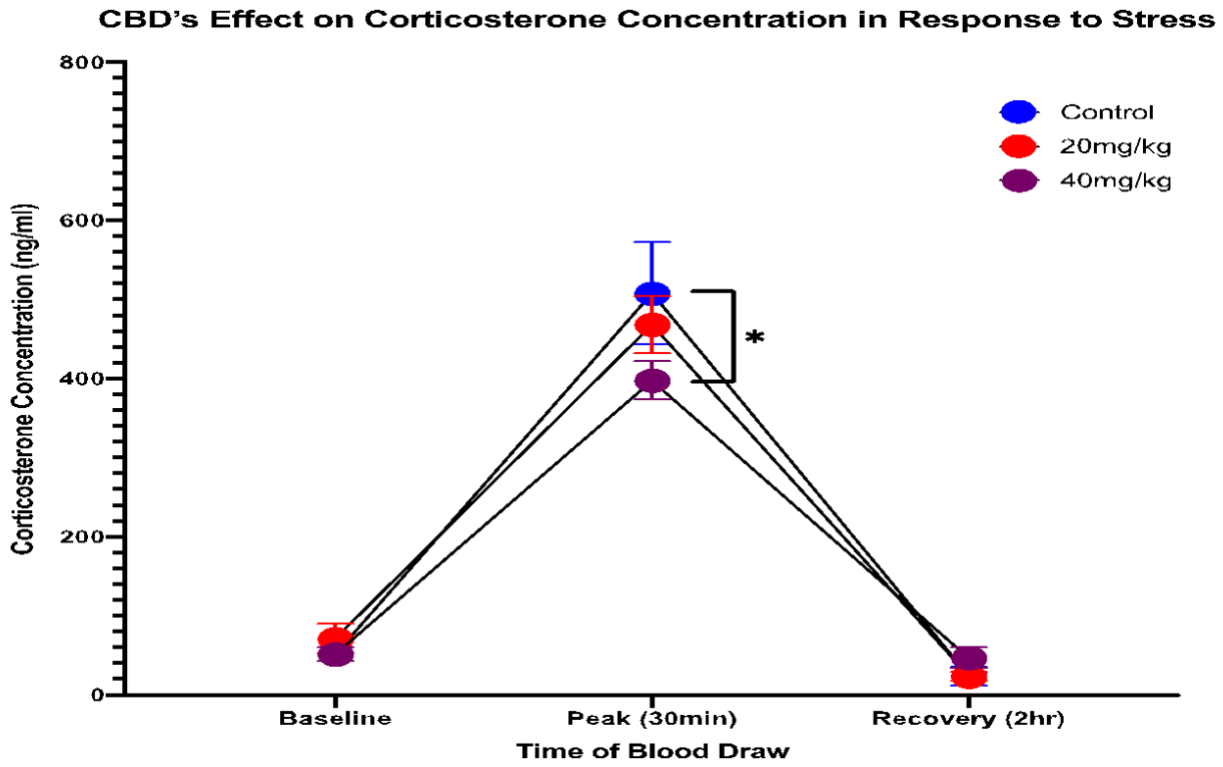

Figure 13. 40mg/kg dose of CBD lowered corticosterone levels in response to Stress.
Means represent Corticosterone levels (ng/ml) by dose by time of blood draw. Dose groups shown are sesame oil (control) (n=6), 20mg/kg (n=6), and 40mg/kg (n=16). 1 outlier was removed from the control recovery time point due to exceeding the z-score threshold. Time of blood draw was expressed by baseline (initial), peak (30 minutes of restraint stress), and recovery (2hrs from baseline). A repeated measure ANOVA displayed a significant effect of dose group [$F(2,24)=3.773$, $P<.05$] when using the Elisa plate as a covariate. Post Hoc testing revealed the significance between control and the 40mg/kg dose group ($P<.05$). Planned comparisons showed that there was only a significant difference occurring at peak stress levels between control and 40mg/kg CBD [$F(1,19)=5.805$, $P<.05$] Data shown are means ± SEM.

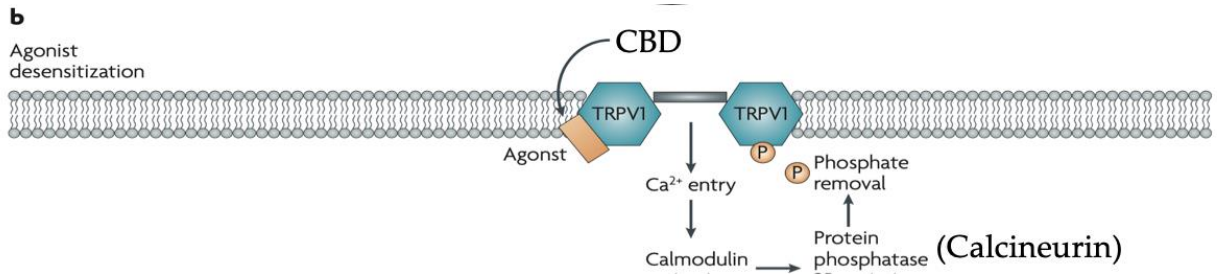

Figure 14. CBD adaptation of general agonistic behavior on the TRPV1 channel, depicted from Patapoutian et al. (2009).

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ABSTRACT

ORAL CBD ADMINISTRATION: ASSESSING BIOAVAILABILITY AND BEHAVIORAL OUTCOMES IN A RODENT MODEL

by

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August 2021

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There has been a recent surge in popularity for cannabidiol (CBD), a major non-psychotropic constituent of cannabis, due to numerous claims of potential therapeutic properties, which include, but are not limited to, anxiolytic, antinociceptive, and anti-inflammatory effects. However, as previous scientific literature on CBD's effectiveness in providing such therapeutic effects is limited, this project was aimed to evaluate the potential beneficial properties of a hemp derived 99% pure CBD compound provided from Ellipse Analytics (Denver, CO) in a rodent model. We analyzed the pharmacokinetics of this CBD product as well as the behavioral outcomes after acute and chronic administration. Pharmacokinetics of CBD were assessed by administering CBD to adult male rats (n=10/group) using oral gavage at 0, 5, 10, 20 or 40 mg/kg in sesame oil for 10 days while drawing blood 1hr, 2hr, and 4hrs post administration on the 1st, 5th and 10th day to measure plasma CBD levels. In a second and third cohort of rats (n=6-16/group), CBD was administered by oral gavage at a 0, 20, or 40 mg/kg in sesame oil over 10 days, with behavioral assays being run on the 1st, 5th, and 10th day to assess outcomes associated with pain response, sleep behavior, anxiety response, and stress levels. Our results showed a dose dependent increase in CBD bioavailability with plasma levels peaking between the 1st and 2nd hour post-

administration, and significantly decreasing by the 4th hour across all groups. There was a minimal effect of CBD on sleep, pain, and anxiety-like outcomes, however 40 mg/kg CBD significantly decreased corticosterone levels during restraint stress as compared to controls. These findings provide evidence for a potential therapeutic effect of CBD on hypothalamic pituitary axis function and thus stress regulation. Further analysis is necessary to assess the potential for dose dependent increases in overall therapeutic effectiveness with acute or prolonged exposures in males and females.

AUTOBIOGRAPHICAL STATEMENT

I am a doctoral student finishing his third year in the Behavioral and Cognitive Neuroscience program as a part of the Psychology department at Wayne State University (WSU). I arrived at WSU with a Bachelor's of Science in Neuroscience from Alma College where my studies focused on both the Biochemical and Psychological disciplines. My final two years at Alma honed in my research interests and prepared me for my time at WSU. As a first-generation college student of African American descent, I was graciously awarded the Dean's Diversity Fellowship at WSU, a program designed to attract new doctoral students with minority backgrounds. This award, along with the tutelage of my advisors Dr. Susanne Brummelte and Dr. Scott Bowen, has allowed me to design my graduate research path around the behavioral and neurochemical influences of cannabinoid use.

My research, though stymied through COVID-19 complications, has started to bear multiple opportunities that I am deeply grateful for. I have presented my research locally, (Graduate poster sessions, Drug and Alcohol Research Network, and Graduate School Research Symposium) as well as at international conferences (International Behavioral Neuroscience Society (IBNS), Society for Neuroscience (SFN)). I plan to use the opportunities that WSU has given me, such as research collaboration or my Barber Fellowship, to not only produce quality research, but to guide my career goals as well. While expanding upon my research portfolio during my time at Wayne State, I will be continuously looking forward to understanding new concepts and exploring new research relevant techniques. Though there is always more to learn, the support from those around me in what I have done, and what I hope to do, is unmatched. This support has set the foundation for me to develop into the research professional I desire to be, and I could not be more thankful.