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Cannabinoid Conundrum: A Study of Anti-Epileptic Efficacy and Drug Policy

Kennedy Dickson
University of Mississippi

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CANNABINOID CONUNDRUM:
A STUDY OF ANTI-EPILEPTIC EFFICACY AND DRUG POLICY

By:
Kennedy Dickson

A thesis submitted to the faculty of the University of Mississippi in partial fulfillment of
the requirements of the Sally McDonnell Barksdale Honors College

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Approved by

_____
Advisor: Dr. Kristine Willett

_____
Reader: Dr. Erin Holmes

_____
Reader: Dr. Nicole Ashpole

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ABSTRACT
KENNEDY DICKSON: Cannabinoid Conundrum: A Study of Anti-Epileptic Efficacy
and Drug Policy

Cannabis is the most commonly used, cultivated, and trafficked illicit drug
worldwide. The use and acceptance of marijuana is evolving rapidly, as indicated by the
volume of new State cannabis legislation across the U.S. Many of the changes in state
laws have occurred without significant input from medical or scientific communities.
Additionally, marijuana policy in the US is convoluted with significant inconsistencies
between state and federal law. The status of marijuana as a Schedule I drug under the
Controlled Substance Act creates numerous restrictions and issues that impact the
industry as a whole. The most promising development has been the 2018 Food and Drug
Administration approval of the first ever marijuana-derived drug, Epidiolex (cannabidiol
or CBD). This drug is now indicated for the treatment of the pharmaco-resistant forms of
epilepsy, Dravet and Lennox-Gaustaut syndromes. Further clinical development is
necessary in order to substantiate marijuana's therapeutic status. Moreover, scientific
research needs to be a key factor in the creation of new marijuana policy. In an effort to
conduct this research, and to explore the anti-epileptic efficacy of CBD, this study
utilized a zebrafish model of Dravet Syndrome. About 80% of Dravet Syndrome patients
carry a mutation in the voltage-gated sodium channel Nav1.1 (*scn1a*). *scn1a* mutant
zebrafish underwent both acute and subchronic exposures to various concentrations of
CBD. CBD was found to significantly decrease seizure activity within the acute
exposure. To provide context and relevancy to this research, the complicated legal status
of marijuana is discussed, and potential reform options are provided as advocacy for
policy change.

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LIST OF ABBREVIATIONS

AED   Anti-Epileptic Drug

bdnf   Brain-Derived Neurotrophic Factor

CBD   Cannabidiol

cnr1   Cannabinoid Receptor 1

cnr2   Cannabinoid Receptor 2

CNS   Central Nervous System

CSA   Controlled Substance Act

CYP450   Cytochrome 450

DEA   Drug Enforcement Agency

dpf   Days Post Fertilization

DS   Dravet Syndrome

EPA   Environmental Protection Agency

FD&C   Federal Food, Drug, and Cosmetic Act

FDA   Food and Drug Administration

hpf   Hours Post Fertilization

IND   Investigational New Drug

LOA   Letter of Authorization

LSD   Lysergic Acid Diethylamide

NIDA   National Institute of Drug Abuse

NIH   National Institute of Health

PCR   Polymerase Chain Reaction

pparγ   Peroxisome Proliferator Activated Receptor

PTZ   Pentylentetrazole

SMEI   Severe Myoclonic Epilepsy of Infancy

SSRI's   Selective Serotonin Reuptake Inhibitors

SUDEP   Sudden Unexpected Death in Epilepsy

THC Δ^9-tetrahydrocannabinol

TLE   Temporal Lobe Epilepsy

1. INTRODUCTION

1.1 Cannabinoids as Therapeutics

For thousands of years, the chemical derivatives obtained from the *Cannabis sativa* plant have been used for medicinal and recreational purposes. Cannabinoids are lipophilic ligands for specific cell-surface receptors. This class of molecules are divided into three main categories: phytocannabinoids, endocannabinoids, and synthetic cannabinoids. Phytocannabinoids are cannabinoids directly obtained from the cannabis plant, and are the primary focus of this study. Phytocannabinoids consist of over 100 naturally occurring compounds found in the cannabis plant (Pertwee, 2006). The best characterized and most abundant phytocannabinoids are Δ^9-tetrahydrocannabinol (THC) and cannabidiol (CBD). THC and CBD are structural isomers and both interact with cannabinoid receptors throughout the body, but produce disparate effects. The biosynthesis of THC and CBD in cannabis follows a very similar pathway. The main difference in the structures, as shown in **Figure 1,** between these two molecules is that where THC contains a cyclic ring, CBD contains a hydroxyl group.
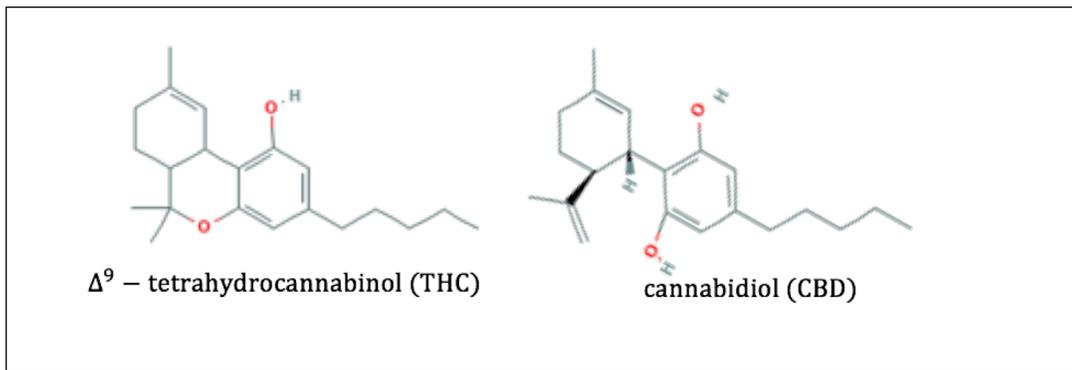
Figure 1: Structures of THC and CBD
(National Center for Biotechnology Information, 2019)

THC, the main psychoactive component of marijuana, imitates the effects of the

neurotransmitter anadamide, which is responsible for pain perception and sleep/appetite

regulation (Koubeissi, 2017). CBD on the other hand, is not psychoactive and has been

effective in reducing psychosis related to anxiety, inflammation, nausea, and seizures

(Koubeissi, 2017).

The current therapeutic areas best associated with cannabinoid treatments are

palliative care, epilepsy, appetite disorders, multiple sclerosis, and glaucoma (Pertwee,

2016). Potential future medicinal applications include cancer and neurological disorders

like dementia and Parkinson's disease.

1.1.1   Cannabis and Epilepsy

Cannabis use and epilepsy dates back to ancient times. Ancient Sumerian and

Akkadian tablets reference the use of a medicinal plant that is most likely cannabis for

several ailments including "nocturnal convulsions" around 1800 BCE as reviewed in

Friedman & Sirven, 2017. In the early 19th century, medicinal cannabis was introduced

into western medicine by the studies of William O'Shaughnessy. Through his published

case reports, he referenced the success of the use of "Indian hemp" in the treatment of

"severe infantile convulsions" (Friedman & Sirven, 2017). By the early 20th century however, the use of cannabis for disease treatment began to fall out of favor as Western medicine started to focus on synthetic isolated chemical entities for various pharmacotherapies. This growing disinterest was eventually compounded by the international prohibition of cannabis – thus relegating the evidence for cannabis as an anticonvulsant to the realm of anecdotal claims and small clinical studies (Friedman & Sirven, 2017).

In the mid 20th century, the molecular structures of THC and CBD were elucidated, which allowed for further investigation of structure-function relationships and pharmacological potential (Mechoulam & Shvo, 1963), (Ganoi & Mechoulam, 1964), and (Pertwee, 1973). Widespread recreational use in the 1960s and 1970s allowed for clinicians to report on smoked cannabis effects on seizures (Friedman & Sirven, 2017). The interest in the understanding of the therapeutic potential of cannabinoids as a treatment for epilepsy experienced a resurgence in the early 1990s with the discovery of the cannabinoid receptor (Pertwee, 2007).

The 2018 Food and Drug Administration (FDA) approval and Drug Enforcement Agency (DEA) scheduling of Epidiolex brought to market the first all CBD-based drug in the US. Through several controlled clinical trials, Epidiolex was able to meet rigorous criteria for FDA approval. In the Phase 3 clinical trials, Epidiolex significantly reduced seizure frequency compared to placebo in highly treatment-resistant patients (Thiele et al., 2018). Between April 2015 and October 2015, 171 patients in this clinical trial were randomly assigned to receive CBD (n=86) or placebo (n=85). CBD was found to be efficacious for the patients with drop seizures associated with Lennox-Gastaut syndrome

and was generally well tolerated (Thiele et al., 2018). With the FDA approval of Epidiolex for the treatment of pharmaco-resistant forms of epilepsy, it opens the door to future potential of more cannabinoid-based epilepsy drugs.

1.1.2 Current Indications

There are several other cannabinoid therapeutic drugs currently on the market. Dronabinol, sold as Marinol and Syndros, is synthetic THC and indicated for treatment of nausea and vomiting caused by cancer chemotherapy. It is also used as an appetite stimulant for HIV patients. Nabiximols, sold as Sativex, is a mixture of both synthetic THC and CBD. Sativex is a mouth spray indicated to alleviate symptoms of multiple sclerosis, and is only available in the U.K. As mentioned above, Epidiolex, made of CDB derived from the marijuana plant, is indicated for the treatment of seizures associated with Dravet and Lennox-Gastaut syndromes.

1.2 Epilepsy

Epilepsy is one of the most common neurological disorders, and it affects over 50 million people of all ages worldwide (World Health Organization, 2019). The hallmark of this disease is recurrent, unprovoked seizures that vary in type, frequency, and duration. A seizure is an abnormal burst of electrical discharges that disrupts the normal electrical function of the brain. Common causes of epilepsy arise from genetics, abnormal brain development, metabolic disorders, head trauma, brain tumors, and stroke. Although many common causes are known, epilepsy is considered to be idiopathic. In about half of the patient cases, no clear underlying cause is found (Gawala, 2016).

Current treatment methods include anti-epileptic drugs (AEDs), surgery, neurostimulation, and dietary therapies. Pharmaceutical AEDs are the mainstay of treatment for most people with epilepsy (Novak, 2017). Common side effects from AEDs include fatigue, dizziness, weight gain, loss of coordination, and memory/thinking problems. Several AEDs have also been associated with more serious side effects such as depression, suicidal thoughts or actions, and organ inflammation. Despite considerable progress in drug research and carefully optimized AED treatment, approximately 30-40% of patients are nonresponsive to these treatments (Sorenson & Kokia, 2013). Drug resistance is a particular problem in many patients with Dravet Syndrome (DS) and temporal lobe epilepsy (TLE). For this reason, development of new and effective AEDs is crucial.

1.2.1 Epilepsy Genes Investigated in this Study

It is believed that alterations in gene expression are necessary to drive the development of epilepsy. Seizures themselves and differences in the expression levels of some genes can also reflect abnormal functioning of epileptic tissue (Lukasiuk & Pitkanen, 2004). The genes analyzed in this study were *c-fos, bdnf, cnr1, pparγ,* and *18s* as a reference gene. The expression of *c-fos* in the brain is hypothetically indicative of seizure activity, so if seizures increase, *c-fos* expression should as well (Baraban et al., 2005). Brain-derived neurotrophic factor, or *bdnf*, is another gene associated with the onset and progression of epilepsy. Various studies have shown that *bdnf* increases neuronal excitability and is localized and up-regulated in areas implicated in epileptogenesis (Binder et al., 2001), (Iughetti et al., 2018), and (Murray et al., 2000).

The biological effects of cannabinoids are mediated by two members of the G-protein receptor family, cannabinoid receptors 1 and 2 (*cnr1* and *cnr2*). *cnr1* is the most common subtype and is located in the central and peripheral nervous systems. CBD is a neutral antagonist of the *cnr1* receptors, with a Ki= 4900 nM in humans (Bow & Rimoldi, 2016). Because this Ki value for CBD at *cnr1* is high, it indicates a low affinity for this receptor. Cannabinoids activate and target different isoforms of the peroxisome proliferator activated receptors (*PPARs*). Activation of all the variants of the PPAR genes mediates some of the analgesic, neuroprotective, neuronal function modulation, and anti-inflammatory effects of some cannabinoids, often in conjunction with activation of other traditional target sites of action such as *cnr1* and *cnr2* (O'Sullivan, 2016). The isoform *pparγ* (peroxisome proliferator activated receptor gamma) is activated by CBD. 18S ribosomal RNA is a control used in polymerase chain reaction (PCR) analyses because of its invariant expression in tissues, cells, and experimental treatments (Valente et al., 2009).

1.3 Dravet Syndrome

Dravet Syndrome, also known as Severe Myoclonic Epilepsy of Infancy (SMEI), is a rare and complex genetic disease. Approximately 70-80% of DS cases are caused by a heterozygous loss-of-function mutation in the *scn1a* gene (Shmuley et al, 2016). The *scn1a* gene belongs to a family of genes which are involved in the production of sodium ion channels. Mutations of ion channel genes play a major role in the pathogenesis of epilepsy, because these channels are in part responsible for controlling electrical excitability within cells.

DS patients typically begin experiencing seizures in the first year of life, with frequent fever-related (febrile) seizures. Often as the disease progresses, other types of seizures including myoclonus, absences, and complex partial seizures typically occur. Intellectual developmental issues arise around age two, as affected individuals often have lack of coordination, poor language development, and hyperactivity (NINDS, 2018). DS is associated with a significant premature mortality, which is greater in comparison to the general population of epilepsy patients. Estimates of mortality range from 15-20% before adulthood, with most premature deaths occurring before 10 years of age (Cooper, 2016). The major feature of DS remains in the great risk of lethality due to high incidence of sudden unexpected death in epilepsy (SUDEP).

Current treatment options are limited because of the drug resistant nature of DS. Traditionally, treatment of DS requires a combination of medications to treat the multiple types of seizures experienced by patients. These drug combinations often lacked in efficacy and caused severe side effects, including: liver damage, pancreatitis, and low blood platelet count. Furthermore, not all AEDs can be prescribed for children, which makes the management of this disease even more difficult (NORD, 2019). In June of 2018, Epidiolex, a cannabinoid-based drug, was approved by the FDA for the treatment of DS. Further research and clinical development with cannabinoid-based drugs is essential for the future of rare epilepsy disease treatment.

1.4 Zebrafish as an Epilepsy Model

Mammalian models, such as rodents, have traditionally been used for modeling human diseases due to the homology between mammalian genomes, anatomy, and

overall cell biology. Despite the advantages, mammalian models are expensive to maintain, more difficult to modify genetically, and have difficulty in providing specific targeted treatments.

Traditionally, the discovery and development of new AEDs has relied on preclinical testing in acute seizure models using otherwise healthy animals (Griffin et al. 2016). In these epilepsy studies, rodents are involved in maximal electroshock, which models generalized types of seizures, with the help of a convulsant agent such as pentylentetrazole (PTZ). This model is limited because it does not accurately reflect spontaneous recurring events as observed in human epilepsy. Moreover, when a clinically successful AED is identified, these drugs offer a broad-spectrum suppression against a range of different seizure types (Griffin et al. 2016). Epilepsy is a genetically complex disease associated with a wide variety of proteins within cells, such as ion channels, neurotransmitters, and trafficking proteins. Different types of epilepsy require very different treatment methods. Thus, AEDs with broad-spectrum applicability may not be particularly helpful in relieving symptoms of specific patient groups. There is a need for a genetically relevant in vivo model for the identification of disease-specific AEDs (Griffin et al., 2016).

Rodent models of DS are available, and are widely utilized due to the high conservation of their genome with humans. In addition, rodent models have been successfully used to validate drug targets, and to determine efficacious and safe dosage schemes for combination treatments in humans (Vandamme, 2014). However, rodent testing is not high-throughput, requires labor-intensive monitoring, and is extremely

sensitive to the laboratory environment. Factors including husbandry, food, social environment can change developmental and physiological processes (Vandamme, 2014).

Researchers have begun to look at lower organisms as useful species for the initial screening of new AEDs and genetic mutations related to epilepsy (Stewart et al., 2011). The utility of zebrafish as a model for epilepsy research is growing rapidly because of several key advantages. Zebrafish have the ability to display seizure-like behavioral and neurophysiological responses by various pharmacological and genetic manipulations (Stewart et al. 2011). Overall, external fertilization, rapid development, and high fecundity make zebrafish an ideal animal model. The optical clarity of embryos and larvae also allow for easy visualization of developmental processes (Hoo et al., 2016).

1.4.1   *scn1a* Zebrafish

Zebrafish have a fully characterized genome and display significant physiological homology to mammals (Stewart et al., 2011). Approximately 84% of genes known to be associated with human diseases have a zebrafish counterpart identified. Therefore, zebrafish are considered a valuable resource in determining how genetic mutations affect neuronal activity and central nervous system (CNS) development (Griffin et al., 2016). One of the best characterized zebrafish epilepsy models is for DS.

The zebrafish used in this study have a single point mutation of the *scn1a* gene. This mutation causes epileptic seizures accompanied by behavioral changes. Homozygous mutants of *scn1a* have significant phenotypic similarity to humans with DS, including spontaneously occurring seizures, resistance to many available AEDs, and early fatality (Baraban et al., 2013). The impacts on the pattern and swimming speed of both mutant larvae and adults can be readily quantified (Cunliffe, 2016).

1.5 Legality Issues

Marijuana policy in the U.S. is inconsistent with significant conflicts between state and federal law. The status of cannabis as a Schedule 1 drug under the Controlled Substance Act (CSA) creates numerous restrictions which ultimately impact the medical marijuana patient's health and wellbeing. The attitudes and cultural norms surrounding marijuana are shifting in a positive direction as shown by the rapidly evolving cannabis policy on the state level within the U.S. A majority of states have passed laws that broadly legalize marijuana in some form. Significant controversies remain regarding the legal, ethical, and societal implications of cannabis use, which are ultimately augmented by restricted clinical research for therapeutic indications, and overall safety/efficacy regulation by the federal government. Marijuana policy in the U.S. must evolve to protect the individuals involved in the cannabis industry. I will describe the central issues regarding marijuana legality in the U.S., provide potential legislative solutions, and pose several core questions that must be answered before significant policy changes occur at the federal level.

1.6 Study Goals

The four main goals of this study are:

1. Determine the anti-epileptic efficacy of cannabinoid treatment in a zebrafish model of Dravet syndrome.

2. Evaluate gene expression associated with CBD exposure through the analysis of several epilepsy and cannabinoid-related genes.

3. Provide context and relevancy through the discussion of the complicated legal status of marijuana in the United States.

4. Emphasize and explain the importance of scientific research in marijuana policy for the overall advocacy of legislative change.

2. MATERIALS AND METHODS

2.1 Zebrafish Husbandry

$scn1a^{+/-}$ fish were obtained from Dr. Peter DeWitte at the University of Leuven. All fish were raised under the approved IACUC protocol. Fish were kept in Aquatic Habitats ZF0601 Zebrafish Stand-Alone System (Aquatic Habitats, Apopka FL) with zebrafish water (pH 7.0-7.6, 340 parts per million (ppm), Instant Ocean, Cincinnati OH) in a climate 25-28°C, 14 hours of light and 10 hours of dark controlled room. Fish were fed twice daily with Gemma Micro food (Skretting Nutreo Company, Westbrook, ME). Sexually mature and healthy fish without any signs of deformities or disease were selected as breeders.

For egg collection, the heterozygous $scn1a^{+/-}$ fish were transferred to breeding tanks, with a 1:1 ratio of males to females, the night prior to collection day. Fish lay their eggs when the light turns on, and eggs were collected an hour later. All eggs that fell through the protective grate at the bottom of the breeding tank were collected by pouring water from the breeding tanks through a small sieve. Eggs were cleaned, transferred to a petri dish and raised in embryo water (pH 7.5, 60 ppm Instant ocean, 14:10 light dark cycle) in a 28°C incubator. Unfertilized/dead eggs and debris were removed daily using a transfer pipette. For the acute exposure, exposure to control or CBD in both $scn1a^{-/-}$ homozygous and $scn1a^{+/-}$ heterozygous larvae began at 5 days post fertilization (dpf) as described in section 2.2. For the subchronic exposures, exposure to control or CBD in both $scn1a^{-/-}$ homozygous and $scn1a^{+/-}$ heterozygous larvae began at 3 or 5 dpf depending

on the trial and is described in Section 2.3. Homozygous $scn1a^{-/-}$ larvae have characteristic phenotypes to easily distinguish them from heterozygous $scn1a+/-$ fish such as hyperpigmentation, non-inflated swim bladders, slight body curvature, and spontaneous seizures begin at 3 dpf and increase as the fish ages. All scn1a$^{-/-}$ fish used in this study had non-inflated swim bladders, and all scn1a$^{+/-}$ had inflated swim bladders.

2.2 Acute Exposure

At 120 hpf, both $scn1a^{-/-}$ and $scn1a^{+/-}$ larvae, without any deformities, were transferred to a 96-well plate, (1 larva per well). Dosing water (control (0.05% DMSO) and CBD (0.075, 0.18, or 0.30 mg/L; 0.24, 0.57, or 0.95 µM) was added to each well (150 μL). Plates were lightly covered and placed in a 28°C incubator. Following 24 hours of exposure, larvae were screened for deformities including body axis, pericardial edema, yolk sac edema, and lack of touch response. Behavior was measured following the deformity screening.

2.3 Subchronic Exposures

2.3.1   Trial 1

At 3 dpf, both $scn1a^{-/-}$ and $scn1a^{+/-}$ larvae, without any deformities, were transferred to a 24-well plate (1 larva/well). Dosing water (control 0.05% DMSO) or CBD (0.18 mg/L; 0.57 µM) was added to each well (2 mL/well). Plates were lightly covered and placed in a 28°C incubator. The water in the plates was changed daily and redosed to simulate a continuous exposure schedule. Larvae were screened for deformities including body axis, pericardial edema, yolk sac edema, and lack of touch

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response each day. Behavior was also measured daily. Beginning at 8 dpf, larvae were fed Gemma Micro food daily prior to the water change but following behavior screening. The exposure ended at 10 dpf.

2.3.2 Trial 2

Trial 2 was conducted in a similar manner to Trial 1, but the larvae were older and they were treated with a lower concentration of CBD. At 5 dpf, both $scn1a^{-/-}$ and $scn1a^{+/-}$ larvae, without any deformities, were transferred to a 24-well plate (1 larva/well). Dosing water (control (0.05% DMSO) or CBD (0.075 mg/L; 0.24 μM)) was added to each well (2 mL/well). Water was changed daily to simulate a continuous exposure schedule. Plates were lightly covered and placed in a 28°C incubator. Larvae were screened for deformities including body axis, pericardial edema, yolk sac edema, and lack of touch response each day. Behavior was also measured daily. Beginning at 8 dpf, the larvae were fed with Gemma micro food daily prior to the water change but following behavior screening. The exposure ended at 10 dpf.

2.3.3 Trial 3

At 5 dpf, both $scn1a^{-/-}$ and $scn1a^{+/-}$ larvae, without any deformities, were transferred to scintillation vials (5 vials per treatment, 5 fish per vial). Dosing water (control 0.05% DMSO or CBD (0.18 mg/L; 0.24 μM)) was added to each vial. Water was changed daily. Scintillation vials were placed in Styrofoam containers to keep them propped up, lightly covered, and placed in a 28°C incubator. Larvae were transferred using transfer pipettes to a 96-well plate, 1 larva per well, for behavioral analysis every other day beginning at 6 dpf. After behavioral analysis was completed, larvae were

transferred back to their respective vials. Larvae were fed Gemma micro food at 8 dpf, and water was changed and redosed. The exposure ended at 9 dpf.

2.3.4   Gas Chromatography Confirmation

To confirm exposure concentrations of CBD, water concentrations were verified using gas chromatography/mass spectrometry. For each trial, 2 mL of dosed and control water were collected for extraction. The protocol as previously described in (Carty et al., 2018) was followed for further processing and chromatographic analysis of the water samples. A total of two samples per treatment were analyzed.

2.4 Behavioral Screening

For the subchronic exposures, on days 6-10 post fertilization, larvae were placed in the Viewpoint Zebrabox, allowed to acclimate for 5 minutes, and movements were recorded for 45 minutes with 100% light. For the acute exposures, behavioral screening was done at 6 dpf as described for the subchronic exposure. Behavioral screening procedures were conducted prior to feeding and water changes.

The Viewpoint Zebrabox tracks larval movements in 15 minute intervals. The Zebrabox software generates an Excel spreadsheet that describes the duration each larvae spends in the inactive (0-5 mm/sec), small (5-9 mm/sec), and large movement (>9 mm/sec) categories during each interval. Large activity movement is indicative of seizure activity, and accordingly was used as the data to assess the antiepileptic efficacy of CBD. Data is presented as the first 15-minute interval of the behavioral screen. The first 15-minute interval displayed the same statistical significance as the entire 45-minute screen.

2.5 RNA Extraction, Reverse Transcription, PCR Amplification

To determine changes in gene expression caused by CBD, mRNA expression of *c-fos, bdnf, cnr1,* and pparγ was measured using qPCR. Following the final behavioral screening for each trial, larvae were euthanized with buffered MS-222, pooled into tubes (4-8 fish per replicate) containing RNAlater and sotred at -80°C until RNA extraction was conducted. RNA was isolated using TRIzol (Invitrogen #A33251; Waltham Massachusetts), RNAse Free DNase set (Qiagen #79254; Walencia California) and RNAeasy mini kit (Qiagen #74004) according to the manufacturer's protocol. Extracted RNA was then quantified and assessed using a NanoDrop 2000 (Thermofisher Scientific, Waltham Massachusetts) for an acceptable 260:280 ratio. The ratio of absorbance at 260 nm and 280 nm is used to assess the purity of DNA and RNA. A ratio of 2.0 is generally accepted as "pure" RNA (Thermo Scientific, 2009).

The purified RNA was reverse transcribed to cDNA using TaqMan Reverse Transcription reagents (Applied Biosystems). The abundance of each gene's expression was normalized to 18S reference gene expression and quantified using qPCR with SYBR Green in a GeneAmp 7500 Sequence Detection System (Applied Biosystems).

2.6 Statistics

Behavioral results were analyzed using GraphPad Prism 5.0 (La Jolla, CA) and presented as mean $\pm$ standard error of mean. Data sets were first analyzed by the Kolmogorov-Smirnov test to determine if they were normally distributed. For the acute exposure, data was not normally distributed, and therefore, Kruskal-Wallis followed by Dunn's post hoc test was used. Statistical significance was accepted $p \leq 0.05$ for all tests.

For the subchronic exposure we would have used two-way ANOVA for further statistical analysis of significance, but since some of the fish died before the final screen, we could not use this test. Therefore, as is, statistical analysis has not been performed for the subchronic exposures.

Gene expression data was analyzed with the method detailed in (Livak & Schmittgen, 2002). Threshold values were averaged across each plate to account for variability among plates, and Ct values were normalized to the 18s reference gene. Data is presented as the fold change in comparison to control. Data was analyzed with an unpaired t-test after verifying data was normally distributed with the Kolmogorov-Smirnov test. Statistical significance was accepted $p \leq 0.05$ for all tests.

3. RESULTS

Two different length exposures to CBD were studied. The acute exposure of 24 hours was conducted to determine an efficacious concentration of CBD that significantly decreased large duration activity of the *scn1a* zebrafish. Subsequently, to expand on the findings in the acute exposure, three trials of subchronic exposures were performed to mimic a patient's daily exposure to a CBD-based medicine. In order to explore the mechanisms by which CBD exposure affected the behavior of the zebrafish, gene expression of several epilepsy and cannabinoid-related genes was analyzed.

3.1 Acute Exposure

In the acute exposure, $scn1a^{-/-}$ and $scn1a^{+/-}$ larvae were exposed to control or 0.075, 0.18, or 0.3 mg/L CBD from 5-6 dpf in 96-well plates. Duration of large activity is shown in **Figure 2** for both homozygous and heterozygous mutant zebrafish. The results shown are from a 15-minute segment of the behavioral analysis. One concentration of CBD (0.18 mg/L) significantly decreased the duration of large movement for homozygous mutants when compared to control by 32%. None of the CBD concentrations tested showed significant decrease in duration of large movement for the heterozygous mutants compared to control. No significant deformities, such as yolk edema or body axis, were noted following CBD treatment.
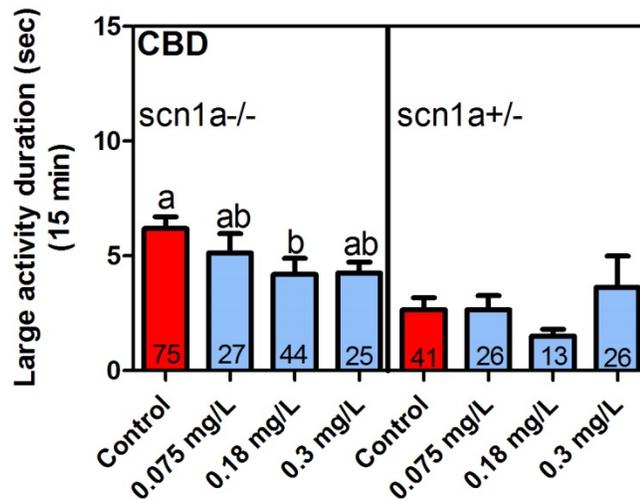
Figure 2: *scn1a$^{-/-}$* **and** *scn1a$^{+/-}$* **acute exposure seizure activity.** Zebrafish larval behavior was analyzed using the Viewpoint Zebrabox (45-minute total recording with 100% light) to record duration of large activity. Behavioral analysis was conducted at 6 dpf, following exposure to CBD (0.075, 0.18, and 0.3 mg/L) to determine if CBD displayed antiepileptic properties. *scn1a$^{-/-}$* data was analyzed with Kruskal-Wallis test followed by Dunn's posthoc test because it was not normally distributed. The number in each bar represents n per treatment; bars with different letters are significantly different (p≤0.05).

3.2 Subchronic Exposures

Because 0.18 mg/L CBD significantly decreased large activity in the acute exposure, we wanted to determine if a continuous exposure of CBD would further reduce the duration of large activity, and to establish whether or not that concentration would be toxic to the larvae. Therefore, we completed three subchronic trials to address these questions. In Trial 1 of the subchronic exposure, *scn1a$^{-/-}$* and *scn1a$^{+/-}$* larvae were exposed to control or 0.18 mg/L CBD from 3-10 dpf in a 24-well plate. Daily large activity duration for *scn1a$^{-/-}$* homozygous fish are shown in **Figure 3a.** The CBD-exposed homozygous mutants showed a sharp decrease in movement from 4 to 5 dpf, then an increase in movement from 6 to 7 dpf which then decreases from 8-10 dpf. Daily large

activity duration for *scn1a*$^{+/-}$ heterozygous fish are shown in **Figure 3b.** The CBD-exposed and control heterozygous mutants showed an increasing range of movement throughout the exposure period from 4 to 8 dpf, then began to decrease until the exposure ended at 10 dpf. On days 9-10 post fertilization, larvae exposed to 0.18 mg/L CBD began having deformities such as curvatures in body axis, enlarged eyes, lethargy, and death. Another complication that was encountered during this trial is that it was difficult identify homozygous fish at 3 dpf (hyperpigmentation was not as apparent as it is at 5 dpf). Since some of the fish died mid-trial, we did not perform statistical analysis on this data, to determine if any of the days showed a significant difference in duration of large activity.

Figure 3: *scn1a*$^{-/-}$ (a) and *scn1a*$^{+/-}$ (b) subchronic exposure Trial 1 seizure activity. Zebrafish larval behavior was analyzed using the Viewpoint Zebrabox (45-minute recording with 100% light) to record duration of large activity. Behavioral analysis was recorded each day from 4 dpf to 10 dpf, following their continuous exposure to CBD (0.18 mg/L) to determine if CBD displayed antiepileptic properties. Data presented is within the first 15 minutes of behavioral screening. Further statistical analysis was not performed to determine significance in duration of large activity because some of the fish died mid-exposure.

Given that larvae exhibited deformities in Trial 1 following exposure to 0.18 mg/L CBD, and homozygous larvae were difficult to identify at 3 dpf, in Trial 2 $scn1a^{-/-}$ and $scn1a^{+/-}$ larvae were exposed to control or 0.075 mg/L CBD from 5-10 dpf in a 24-well plate. Daily large activity duration for $scn1a^{-/-}$ fish is shown in **Figure 4a.** The homozygous fish showed very similar trends in movement for both control and 0.075 mg/L CBD, and did not change from 6-10 dpf. Daily large activity duration for $scn1a^{+/-}$ fish are shown in **Figure 4b.** The heterozygous mutants showed a similar pattern of erratic increases and decreases in movement to that in Trial 1. At 8 dpf, 0.075 mg/L CBD showed about two times as much large activity as control. At 9 to 10 dpf, deformities such as curvatures in body axis, enlarged eyes, lethargy and death.
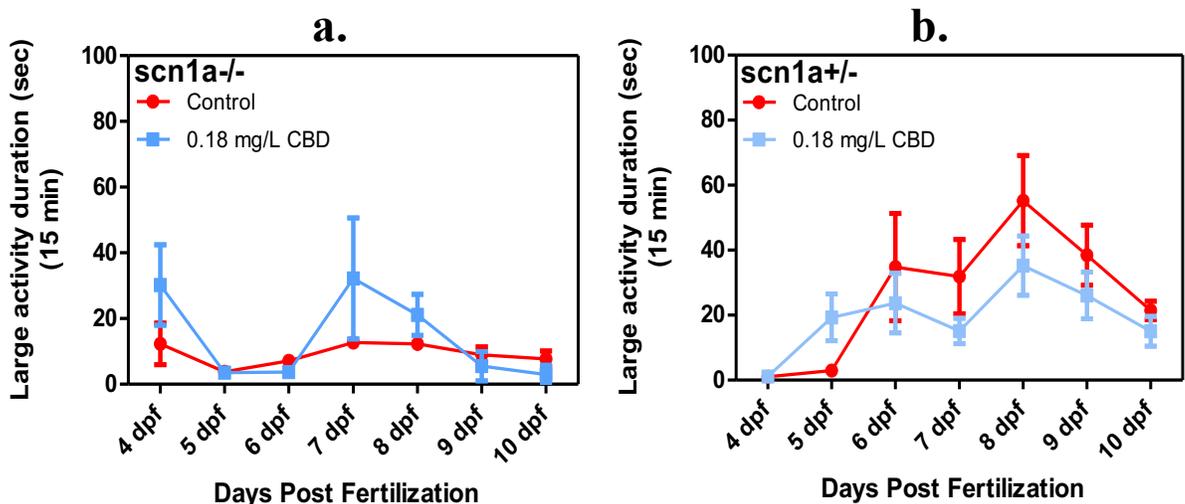

Figure 4: $scn1a^{-/-}$ **(a) and** $scn1a^{+/-}$ **(b) subchronic exposure Trial 2 seizure activity.**
Zebrafish larval behavior was analyzed using the Viewpoint Zebrabox (45-minute recording with 100% light) to record duration of large activity. Behavioral analysis was recorded daily from 6 to 10 dpf, following their continuous exposure to CBD (0.075 mg/L) to determine if CBD displayed antiepileptic properties. Further statistical analysis was not performed to determine significance in duration of large activity because some of the fish died mid-exposure.

Due to the continued to observation of deformities and death in Trial 2 even with

a reduced CBD concentration, in Trial 3 we exposed *scn1a*^{-/-} and *scn1a*^{+/-} larvae to

control or 0.18 mg/L CBD (as in Trial 1) from 5-10 dpf, in scintillation vials for the fish

to have more space to grow. Behavior was screened at 6 and 8 dpf after fish were

transferred to a 96-well plate. Large activity duration for *scn1a*^{-/-} fish at 6 and 8 dpf are

shown in **Figure 5a.** Both control and 0.18 mg/L CBD treatments had very little activity

in the homozygous fish at 6 and 8 dpf. Large activity duration for *scn1a*^{+/-} fish are shown

in **Figure 5b.** As with the homozygous fish, the heterozygous fish also had very little

large activity. Trial 3 ended prematurely at 9 dpf instead of 10 dpf. At 9 dpf, there was

100% mortality in both treatments probably due to the stress of moving them back and
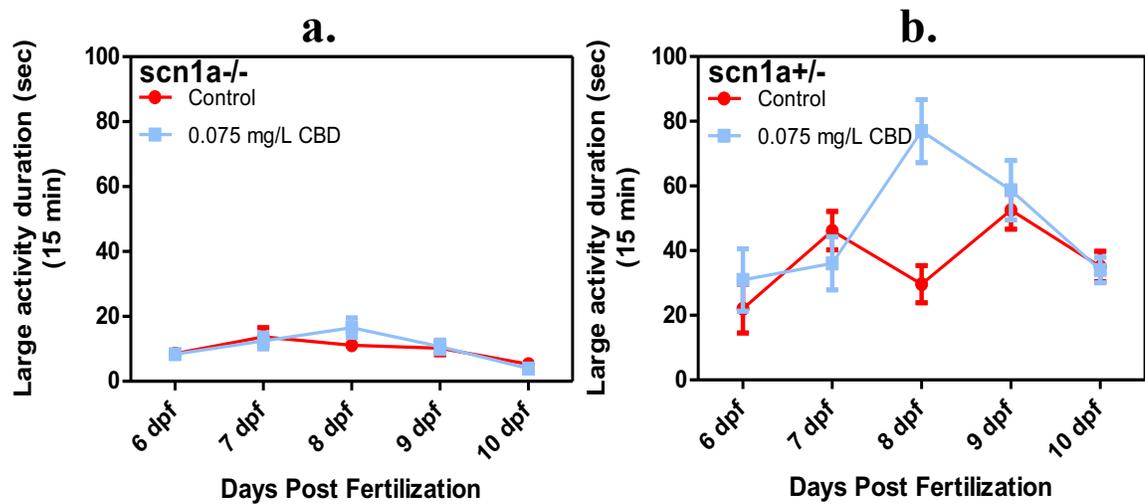
forth from vials to plates.
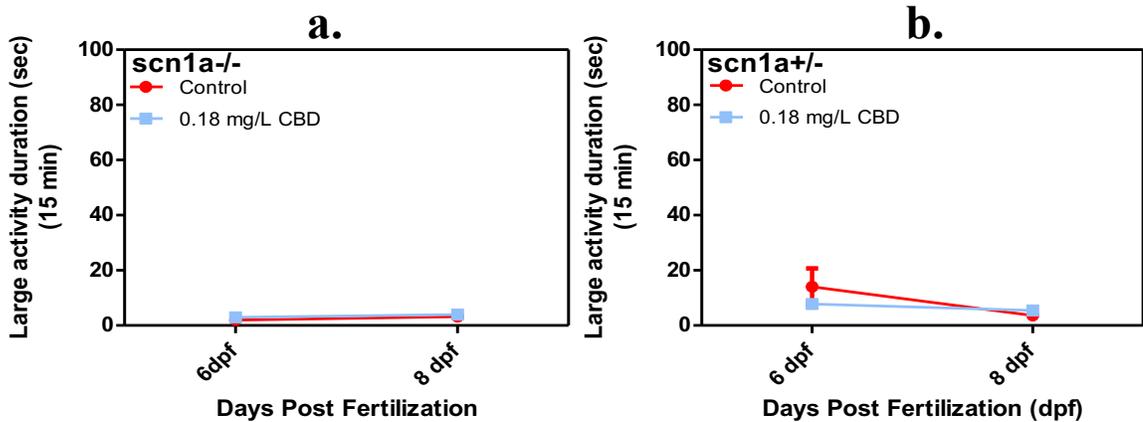
Figure 5: *scn1a*^{-/-} (a) and *scn1a*^{+/-} (b) subchronic exposure Trial 3 seizure activity. Zebrafish larval behavior was analyzed using the Viewpoint Zebrabox (45-minute recording with 100% light) to record duration of large activity. Behavioral analysis was recorded at 6 and 8 dpf, following continuous exposure to CBD (0.18 mg/L) to determine if CBD displayed antiepileptic properties. Further statistical analysis was not performed to determine significance in duration of large activity because of the premature death of 100% of the fish at 9 dpf.

Gas chromatography mass spectrometry was used to confirm the cannabinoid water concentrations for each trial. Calculations for average and standard deviation were calculated by constructing a standard calibration curve for 0.016, 0.08, 0.4, 2, and 10 mg/L for CBD. **Table 1** depicts the comparison between nominal concentrations and actual concentrations of each sample. Actual average concentrations of CBD were 100% higher for 0.075 mg/L CBD and 61% higher for 0.18 mg/L CBD.

Table 1: Gas Chromatography/Mass Spectrometry Confirmation of Cannabinoid Water Concentrations

Nominal (mg/L)	Average (mg/L)	Standard Deviation
0.075 CBD	0.15	0.004
0.18 CBD	0.29	1.326

3.3 Gene Expression following acute exposure to CBD

Expression of the genes *c-fos, bdnf, cnr1,* and *pparγ* following control or 0.18 mg/L CBD exposure from 5-6 dpf was determined by qPCR. The results shown in **Figure 6** are expressed as the fold change in comparison to control. Only the 0.18 mg/L CBD samples were analyzed for gene expression because that concentration was the only one that caused significant decreases in duration of large activity during the acute exposure. Statistical significance is reported as $p \leq 0.05$. *c-fos* is the only gene that did not show a significant change in expression with 0.18 mg/L CBD when compared to control, it had p-values of 0.08 and 0.28 for the homozygous and heterozygous mutants, respectively. *bdnf, cnr-1,* and *pparγ* each showed a significant increase in expression for the homozygous mutants when compared to control.

a. c-fos

b. bdnf

c. cnr1

d. ppary

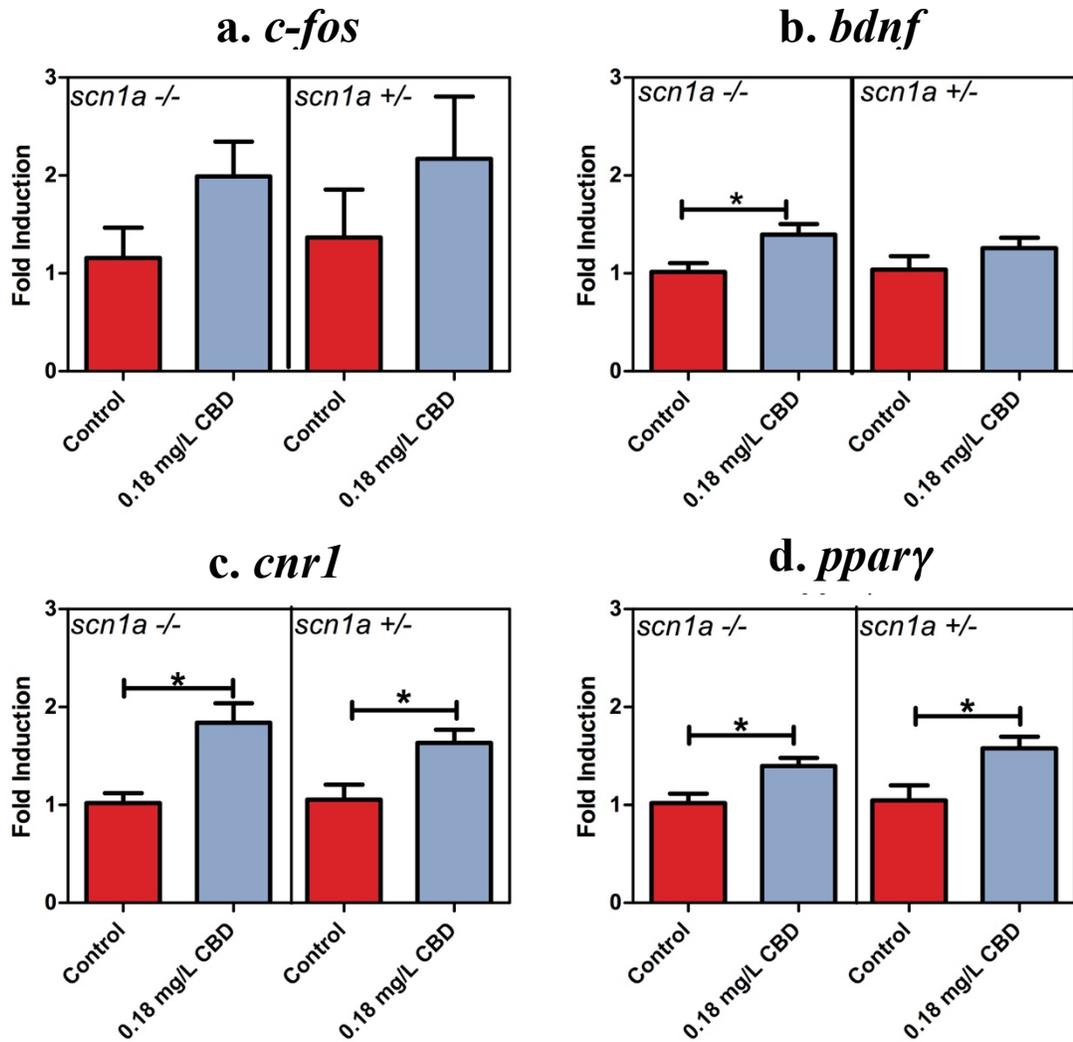**Figure 6:** *Gene Expression of a) c-fos, b) bdnf, c) cnr1, and d) ppary.* Gene expression was determined with qPCR following exposure to control and 0.18 mg/L CBD from 5-6 dpf. The data is presented as the fold change compared to control. Data was analyzed with an unpaired t-test. Statistical significance was accepted $p \leq 0.05$ for all tests (n=5 replicates/treatment; 6-8 fish/replicate).

4. DISCUSSION

The two goals of this portion of the study were to determine the anti-epileptic efficacy of cannabinoid treatment in a zebrafish model of Dravet Syndrome and to evaluate gene expression associated with CBD exposure through the analysis of several epilepsy and cannabinoid-related genes. DS is a highly AED-resistant form of epilepsy with very limited available treatment options. CBD was of interest in this research because of the 2018 FDA approval of Epidiolex (a CBD-based drug), as it is the first FDA-approved drug that contains a purified drug substance from marijuana. Epidiolex is also the first drug to be indicated directly for the treatment of DS. Further research on the efficacy of this compound and clinical development is essential for the future of the treatment of this disease and other rare types of epilepsy.

4.1 Acute Exposure

For the acute exposure, the three different concentrations of CBD used were: 0.075, 0.18, and 0.3 mg/L. Exposure began on 5 dpf and behavior was recorded at 6 dpf. The homozygous group showed a significant decrease in duration of large activity at 0.18 mg/L CBD when compared to control. While not significant, CBD at 0.075 and 0.3 mg/L did show a decrease in the duration of large activity. The heterozygous mutants did not show significant decreases in duration of large activity at any of the three CBD concentrations. The significant decrease in duration of large activity of CBD at 0.18 mg/L for homozygous mutants indicates this compound's efficacy in treatment of DS.

Similar acute CBD exposure studies confirm these results (Jensen et al., 2018) (Achenbach et al., 2018) (Devinsky et al., 2014).

4.2 Subchronic Exposures

To expand on the results seen in the acute exposure, the subchronic exposure was designed to mimic a patient's daily exposure to a CBD-based medicine. The goal was to determine if CBD decreased and controlled the zebrafish larval seizure activity from 3 to 10 dpf. The first trial began at 3 dpf because that is when *scn1a* homozygous mutants begin to show spontaneous seizure activity (Griffin et al., 2018).

Trial 1 produced varied results for the homozygous mutants. Within the CBD exposure, there was a decrease in duration of large activity from 4 to 5 and 6 dpf, but then an increase at 7 dpf. From 7 to 10 dpf there were incremental decreases in overall duration of large activity. The heterozygous mutants showed gradual increases in duration of large activity from 4 to 6 dpf, a decrease at 7 dpf, followed by another increase at 8 dpf. Finally, at 9 and 10 dpf, there was a marked decrease in duration of large activity. In response to the malformations/death seen in Trial 1 with 0.18 mg/L CBD, the concentration was reduced to 0.075 mg/L in Trial 2, to determine if the concentration of CBD impacted the larval zebrafish seizure activity. There was less movement overall for homozygous mutants in both CBD and control in Trial 2 compared to Trial 1. Similar sporadic results to behavior in Trial 1 were observed for heterozygous mutants.

For Trial 3, a different approach was used to determine if the 24-well plates used in trials 1 and 2 had an effect on the larvae's behavior and survival. To address this,

zebrafish were placed in scintillation vials which would allow them to have more space to swim and grow. The exposure was also started at 5 dpf (similar to Trial 2), as opposed to 3 dpf as in Trial 1. The zebrafish were transferred from their respective vials to well-plates for behavioral analysis, then back to the vials for feeding/dosing. To avoid stressing the zebrafish with movement every day, behavioral analysis was planned to be done every other day. There was a decrease in duration of large activity from 6 to 8 dpf for the homozygous group. In the heterozygous mutants, there was almost no change in duration of large activity between 6 and 8 dpf. At 9 dpf, 100% mortality in both control and CBD treatment occurred, likely due to stress of transferring the fish to and from the well plates, and therefore, the trial ended at 9 dpf.

In a generalized study of zebrafish larval activity, there were significant variations in duration and speed of movement from 5 to 7 dpf, with the emergence of spontaneous swimming at 5 dpf (Ingebreston & Masino, 2013). Although the fish used in our study are epileptic-mutants, this observation could help explain a wide variability of movement that is innate to zebrafish larvae. These findings could be especially useful in describing the increase in activity from 4 to 5 dpf in Trial 1. Zhang et al. 2015 assessed *scn1a* larvae locomotor activity from 3 dpf to 7dpf, and saw increased total movement compared to control larvae, being most pronounced from 4 to 5 dpf. They also noted that the total movement of non-inflated swim bladder control larvae was lower in comparison to control larvae with inflated swim bladders. In this study, hyperactive behavior of the scn1a mutants was not due to inflated swim bladder deficiency, but due to abnormal brain activity (Zhang et al., 2015). All of the homozygous fish in our study all had non-inflated swim bladders, while all of the heterozygous fish had inflated swim bladders. In all three

trials of the subchronic exposures, the heterozygous mutants both CBD-exposed and control, displayed an increased duration of large activity.

A similar locomotor behavioral study saw both maturational and experiential effects of zebrafish larvae, when contained in well-plates (Colwill & Creton, 2011). In our study, zebrafish larvae were observed in individual wells of a 12-well plate for 4, 5, 6 and 7 dpf. Colwill and Creton explain that the small testing arenas (well-plates) used may have limited the opportunity for exploratory behavior in older larvae, especially when coupled with their extensive exposure and habituation to the wells. The well-plates used in our study were 24 well-plates, an even smaller testing arena than in the Colwill and Creton study, which in turn may have affected development of the zebrafish, and consequently their behavior. In future studies, the scintillation vial method, as used in Trial 3, should be tested to determine if prolonged habituation in well-plates has a significant impact on behavior.

Another explanation for the observed sporadic behavior is that the effects may be due to interactions between unaltered CBD and the zebrafish, as it is known that CBD degrades into THC in gastric fluid (Jensen et al., 2018). Therefore, to some extent CBD was converted to THC in the zebrafish stomach. Consequently, THC could have bioaccumulated in the zebrafish as the subchronic exposure continued. This could have confounded the results with the observed variation of swim activity. Achenbach et al. 2018 described that in a short term exposure, both THC and CBD bioaccumulate in zebrafish larvae. Carty et al. 2018 saw that even when CBD was used at low concentrations it tended to bioacumulate in zebrafish tissue. In future subchronic exposure experiments, larval concentrations of THC and CBD should be calculated along

with metabolic information, to determine if the rate of bioaccumulation to metabolism of these compounds is significant.

An additional factor in the subchronic exposures was that beginning at 8 dpf, the zebrafish were fed Gemma Micro Food. Fish were fed after behavioral analysis was completed with the Viewpoint Zebrabox. Zebrafish were left to eat for about 15 minutes before all food was removed from the well or scintillation vial and water was changed and redosed. It is possible that the fish were overfed, which caused an increase in nitrate level in the water and the fish itself. Increased nitrate levels in zebrafish and their environment has been observed to adversely affect their viability (Avdesh et al., 2012).

4.3 Gene Expression

Gene expression analysis was performed on control and 0.18 mg/L CBD exposed zebrafish in the acute exposure, as this was the only concentration that significantly reduced duration of large activity. The four genes analyzed in this study were *c-fos, bdnf, cnr1,* and *pparγ.* The expression of *c-fos* in the brain is hypothetically indicative of seizure activity, so if seizures increase, *c-fos* expression should as well (Baraban et al., 2005). However, there was an insignificant increase in *c-fos* expression for both homozygous and heterozygous mutants. This is surprising because with a significant decrease in duration of large activity for homozygous mutants, we expected to see a decrease in *c-fos* expression when compared to control because of CBD's anti-convulsant indications. Carty et al. 2018 saw similar results in that CBD caused a dose-dependent upregulation of *c-fos* in a manner that was inconsistent with decreased behavioral

activity. They hypothesized that this outcome may have been due to a neurotoxic event which affected molecular signaling and behavioral phenotypes.

bdnf expression is increased in animal models and humans with epilepsy (Iughetti et al., 2018). There was a significant increase in *bdnf* expression in the CBD-exposed homozygous mutants when compared to control. Several studies have shown that CBD increases *bdnf* expression in certain parts of the brain like the hippocampus and the medial prefrontal cortex (Sales et al., 2018), (Campos et al. 2015), and (Valvassori et al., 2009). Expression of the *bdnf* gene induces the production of the BDNF protein. This protein binds to its cognate receptor and promotes neuronal survival (NCBI, 2019). In relating this information to epilepsy, when CBD stimulates the expression of *bdnf* and subsequently the production of the BDNF protein, it could potentially play a role to help repair damaged neurons and assist in neurogenesis (Valvassori, et al. 2009). CBD induced altered neurogenesis by *bdnf* could be seen as an attempt for the brain to repair damaged neurons from seizure activity. Because BDNF can cross the blood-brain barrier, it is reasonable to assume that blood BDNF gene expression and protein levels correlate with one and other. Cattaneo et al. 2016 found a positive correlation between serum BDNF and mRNA levels in rats during neurodevelopment. To put it into perspective of our study, although we did not measure protein BDNF levels, the significant increase in *bdnf* expression could be an appropriate representation for the gene's potential downstream neurogenic effects.

cnr1 and *pparγ* are cannabinoid-related genes. There was a significant increase in *cnr1* expression in both homozygous and heterozygous mutants. CBD is known to be a neutral antagonist of the *cnr1* gene (Pertwee, 2008). Because CBD has low binding

affinity for *cnr1*, it is hypothesized that CBD can indirectly affect receptor activity, through channels like the TRPV family and PPAR's (McPartland et al., 2015). CBD has a K_i value of 4900 nM for *cnr1*, which is reflective of its binding affinity. If a K_i value is relatively much higher in comparison to a drug concentration that a patient is typically exposed to, than it has weak affinity for the enzyme/receptor (Busti, 2015). This K_i value corresponds to a CBD concentration of 1.54 mg/L, which is a very high concentration for zebrafish. Ahmed et al. 2018 saw that in the context of a 3 day subchronic exposure, increasing concentrations of CBD from 1-4 mg/L, showed a related increase in incidence of zebrafish larval deformities and mortality. This implies that concentrations of CBD greater than 1 mg/L are toxic to zebrafish, as they would not normally be exposed to concentrations that high. Thus, CBD has a weak affinity for *cnr1*.

There was a significant increase in *pparγ* expression in both homozyogous and heterozygous mutants. *pparγ* is a non-cannabinoid receptor that is activated by CBD (O'Sullivan, 2016). CBD promotes *PPAR* activity by inhibiting fatty acid amide hydrolase, which is a metabolic enzyme that breaks down endogenous fatty acid compounds known as N-acylethanolamides (Kaczocha et al., 2012). This family of fatty acid molecules includes anandamide, an endocannabinoid that binds directly to *cnr1*. O' Sullivan, 2016 found that the activation of *PPAR* genes mediates some of the analgesic, anti-convulsant, and neuroprotective effects of some cannabinoids, often in conjunction with the activation of *cnr1* and *cnr2*. Thus, the upregulation of *pparγ*, could have indirectly upregulated *cnr1*. In future studies, the relationship between *cnr1* and *pparγ* in their relation to anti-convulsant effects should be explored further.

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Results varied in the subchronic trial, due to outstanding factors like limited space for growth/movement in well-plates for extended periods of time, potential bioaccumulation of CBD or THC in zebrafish, and feeding issues. In future exposures, these factors should be accounted for and adjusted to ensure coherent results. The failure of these trials to demonstrate consistent decreases in duration of large activity should not discount the efficacy of CBD in treatment of DS. Success in the acute exposure of CBD should encourage further research and development in this area of pharmaceutics. Results garnered from the gene expression portion of this study should encourage future exploration into the complex interactions of both epilepsy and cannabinoid genes. Research that elucidates the mechanisms of action of these compounds is essential for the creation of new and effective cannabinoid based AEDs.

5. DISSCUSION ON OF MARIJUANA LEGALITY IN THE U.S.

5.1 History

Cannabis is a botanical product with medicinal origins dating back to ancient times. In the 19th and early 20th centuries, cannabis was widely used throughout the United States as a medicinal drug and could easily be purchased in pharmacies and general stores. In 1850, it was described in the *United States Pharmacopedia* for the first time as "Extractum Cannabis." Cannabis was listed as a treatment for various conditions like neuralgia, tetanus, cholera, opiate addiction, and convulsive disorders (Bridgeman & Abazia, 2017). Federal restriction on cannabis use/sale first occurred with the passage of the Marihuana Tax Act in 1937. This act imposed registration requirements and a tax on growers, sellers, and buyers of marijuana. It did not outright prohibit marijuana, but its effect was very similar. Prescriptions of the drug greatly decreased after passage of the act because doctors generally concluded that it was easier to not prescribe marijuana than to contend with the extra work imposed by this law (Pacula, 2002). Subsequent to the act of 1937, cannabis was dropped from the *United States Pharmacopedia* in 1942, which caused the drug to lose its remaining therapeutic legitimacy.

In 1970, Congress passed the Controlled Substance Act (CSA) which established a single system of control for both narcotic and psychotropic drugs for the first time in US history (Drug Enforcement Agency, 2019). The extent of control exercised by the Drug Enforcement Agency (DEA) is determined by a substance's classification in one of five schedules for controlled substances. Marijuana was and still is classified as a Schedule I

substance. The criteria for a substance to be designated as Schedule I, is no currently accepted medical use in the United States, high potential for abuse, and lack of accepted safety for use of the drug or other substance under medical supervision (Mead, 2017). The reasoning behind this classification was mainly due to lack of solid research about the plant and the active substances contained within it.

In 1996, California became the first state to permit legal access to and use of botanical cannabis for medical purposes under physical supervision in accordance to the Compassionate Use Act (Bridgeman & Abazia, 2017). In 2001, the Rohrabacher-Farr amendment prohibited the Department of Justice to use federal funds to supersede State law in those states that have legalized the use of medical marijuana (US Congress, 2019). As of April 2019, 33 states and the District of Columbia currently have passed laws broadly legalizing marijuana in some form. The District of Columbia and 10 states have legalized marijuana for recreational use.

The most recent legislative progress in the realm of marijuana was with the passage of The Hemp Farming Act of 2018. This law removed hemp, a less potent cultivar of marijuana, from the list of controlled substances. The 2018 Hemp Bill defines hemp as all parts of the *Cannabis sativa* plant that do not exceed 0.3% THC by dry weight, including "derivatives," "extracts," and "cannabinoids" (Corron and Kight, 2019). Prior to the passage of this bill, cultivated hemp was only federally lawful under certain state-sanctioned pilot programs.

5.2 Central Issues

As a Schedule I controlled substance, controversies surrounding legal, ethical, and societal implications associated with the use of marijuana are compounded by its adverse health effects, limited clinical data for therapeutic indications, and safe administration/ packaging/ dispensing regulation. The fragmented transition of marijuana from a vilified substance, to one with legitimate therapeutic merit has been convoluted and controversial.

Cannabis is the most commonly cultivated, trafficked, and abused drug worldwide, with an annual usage by approximately 147 million individuals, which equates to 2.5% of the global population (World Health Organization, 2016). The social attitudes and cultural norms surrounding marijuana use are shifting in a positive direction, as shown by the rapidly evolving cannabis policy at the state level within the U.S., state cannabis laws are widespread and highly variable – which leads to some ambiguity and concern. As state legal restrictions have eased, marijuana use has increased. In states where it is legal, sales topped $8 billion in 2017, and they are projected to grow to $24 billion by 2025 (Haffajee et al., 2018). State marijuana legalization and industry growth show no signs of slowing.

My goal of this paper is to outline the central issues within marijuana legality, to provide potential legislative solutions, and to pose several core questions that must be answered before significant changes occur at the federal level. The central issues regarding marijuana legality include: convoluted state and federal law, adverse health effects of cannabis use, research restrictions that produce knowledge gaps, and inconsistency with FDA and EPA regulations.

In order to resolve the conflict, it is imperative to stress the importance of the role of science in this policy debate. The changes in state laws have occurred largely without significant input from the medical, scientific, or policy research communities (Weiss et al., 2017). Updating marijuana policy on the federal level is a desirable goal, but we must seek to minimize any adverse consequences in the form of social and public health costs. Scientific research must be at the heart of all legislative decisions.

5.2.1 Convoluted Law

Federal and state laws regarding the medical use of cannabis and cannabinoids are in conflict and have led to severe confusion among patients and healthcare providers. As stated, marijuana and its cannabinoid derivatives are classified as Schedule I drugs that have no currently accepted medical use, high potential for abuse, and lack of accepted safety for use of the drug or other substance under medical supervision. This places marijuana on the same level as drugs like mescaline, psilocybin, heroin, and lysergic acid diethylamide (LSD). Schedule I substances cannot be prescribed, only "recommended" as treatment by a health care provider. In contrast, state laws are commonly divided into four groups: medical use, High-CBD/Low-THC only, (de)criminalization, and recreational legalization for adults 21 years old and up.

The cannabis plant contains over 100 individual cannabinoids, most abundantly: THC and CBD. There are no standardized definitions of "medical marijuana" and "high-CBD" or "low-THC" products as mainstream media commonly uses these terms interchangeably (Mead, 2017). The term, "medical marijuana" does not explicitly refer to a special strain of cannabis, mode of preparation, or dosage method. "Medical marijuana" products contain a wide range of cannabinoids with varying concentrations of active

ingredients. Overall, there is a lack of common descriptions for "medical marijuana" or even "CBD-access only" laws, which vary significantly from state to state. Some laws decriminalize possession by qualified patients or their caregivers, while others authorize full panoply of manufacturing and distribution/retail sales (Mead, 2017).

CBD is considered the non-psychoactive component of marijuana and has become the center of the legality confusion, especially after the FDA approval of Epidiolex (CBD-based epilepsy drug). In September 2018, the DEA scheduled Epidiolex and any future drug products containing CBD derived from marijuana with no more than 0.1% THC in Schedule V of the CSA (Corroon and Kight, 2019). This is a huge stepping-stone in the journey of cannabis legalization. A Schedule V substance is considered to have a low potential for abuse and consists of primarily limited quantities of certain narcotics (Drug Enforcement Agency, 2019).

Despite the approval of Epidiolex and growing popularity of CBD, its regulatory status remains convoluted. The source of CBD is critically important in determining its legal status (Corroon and Kight, 2019). The most common source is the plant *Cannabis sativa*, which encompasses both cannabis and hemp. While they are the same chemical compound, marijuana (cannabis)-derived CBD and hemp-derived CBD each have their own unique regulatory status and legal implications. There are various methods for differentiating marijuana and hemp – i.e. genotype, phenotype, etc. From a regulatory standpoint, the differences between the two is in their respective concentrations of THC. Hemp is legally defined as a cultivar of *Cannabis sativa* with low concentrations of THC, which cannot exceed 0.3% (Corroon and Kight, 2019). Despite clear differences in traits, marijuana and hemp appear to readily interbreed making it difficult to differentiate the

species (Sandler et al., 2018). CBD from marijuana is still considered a Schedule I controlled substance. While the scheduling of Epidiolex as a Schedule V substance greatly increased the access to the drug, it did not change the regulatory status of CBD itself.

To add another layer of complexity, the approval of the 2018 Hemp Farming Act removed hemp from the list of controlled substances. The bill redefined hemp as all parts of the *Cannabis sativa* plant that do not exceed 0.3% THC by dry weight – including derivatives, extracts, and cannabinoids (Corroon and Kight, 2019). Thus, the bill explicitly removed hemp-derived CBD from regulation under the CSA. In addition to domestically cultivated hemp, CBD may also be legal if it is derived from "non-psychoactive hemp" imported into the US from Canada and Europe. Hemp-derived CBD products can currently be purchased both online and over-the-counter throughout the country, as if they were dietary supplements. Marijuana-derived CBD products can only be purchased by qualifying patients with state medical marijuana laws (Corroon and Kight, 2019).

To further complicate regulation issues, with the approval of Epidiolex, the FDA ruled that any CBD product cannot be included or listed as a dietary supplement. This ruling now brings a level of uncertainty to the future of online or over-the-counter sales of CBD products. The FDA defines a dietary supplement as a product taken by mouth that contains a "dietary ingredient," which may include vitamins, minerals, amino acids, and herbs (Food and Drug Administration, 2019). According to the Federal Food, Drug, and Cosmetic Act (FD&C Act), if a substance (such as CBD) is an active ingredient in an approved drug, then products containing that substance fall outside the definition of a

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dietary supplement. Thus, CBD products cannot be marketed, labeled, or produced as containing CBD.

Regardless of rulings that have provided greater access to CBD, marijuana and marijuana-derived CBD is still considered to be illegal on the federal level under the CSA. The removal of hemp from the controlled substance list is very encouraging progress for the future of marijuana legality as a whole. In October of 2009, the Obama Administration sent a memo to federal prosecutors encouraging them not to prosecute people who distribute marijuana for medical purposes in accordance with state law (National Conference of State Legislators, 2019). This guidance lead to the approval of the 2013 Cole Memorandum, which deprioritized marijuana prosecutions in states where use was legal. The Rohrabacher-Farr amendment adopted by Congress in 2014, prohibits the use of federal funds to prosecute medical marijuana activities. This amendment must be renewed each year, and was most recently renewed through September 2019. More recently, in January of 2018, The Cole Memorandum which allows federal prosecutors to decide how to prioritize enforcement of federal marijuana policy, was revoked by Attorney General Sessions by the issuance of a Marijuana Enforcement Memorandum (Haffajeee et al., 2018). Sessions noted that the purpose of his memorandum was to "direct all U.S. attorneys to use previously established prosecutorial principles that provide them all necessary tools to disrupt criminal organizations, tackle the drug crisis, and thwart violent crime" (Department of Justice, 2018). The most significant policy decisions now relate to how and when the federal government will update marijuana legislation to create a comprehensive, safe, and effective system.

5.2.2 Adverse Effects of Cannabis Use

Most of the knowledge regarding the adverse effects of medical cannabis comes from the limited clinical trial data and anecdotal studies of recreational users of marijuana. The effects associated with acute use are well known: relaxation, appetite stimulation, heightened sensation, increased heart rate, impairment of short-term learning/memory, and possible paranoia or psychosis (Weiss et al., 2017). Chronic use of cannabis, especially in individuals who begin using at a young age, has lead to altered brain development, cognitive impairment, chronic bronchitis, and increased risk of psychosis health disorders, like schizophrenia and depression (Weiss et al., 2017). Vascular conditions, including heart attack and stroke have also been associated with cannabis use (Bridgeman & Abazia, 2017).

Understanding of the consequences of chronic cannabis use with regard to their permanence and causality is inadequate and inconsistent. This is largely due to cannabis and its constituents continued Schedule I status and preclusion of randomized controlled exposures (for ethical reasons). Controlled exposures to the drug could possibly rule out pre-existing differences, and the common use of multiple substances (i.e. tobacco and alcohol) at the same time as cannabis, especially in adolescent users (Weiss, 2017).

Compounding the debate, metabolic and pharmacokinetic interactions exist between medical cannabis and other pharmaceuticals. Cytochrome 450 (CYP450) isoenzymes 2C9/3A4 and 2C19/3A4, are responsible for the metabolism of THC and CBD, respectively (Colby, 2018). Products that contain both THC and CBD will have drug interactions with all three enzymes. On a broader scale, the CYP450's constitute the major enzyme family capable of metabolizing most drugs (Zanger & Schwab, 2013).

THC is a CYP1A2 inducer; so theoretically, THC can decrease serum concentrations of clozapine, duloxetine, naproxen, and haloperidol because their metabolic breakdown is CYP1A2 mediated (Flockhart 2007). These drugs are from various classes including antidepressants, antipsychotics, anti-inflammatories, and sedatives. CBD is a potent inhibitor of CYP3A4 and CYP2D6. CYP3A4 metabolizes about a quarter of all drugs, therefore, CBD may increase serum concentrations of benzodiazepines, antihistamines, and some statins (District of Columbia Department of Health, 2019). CYP2D6 metabolizes many antidepressants, so CBD may also increase serum concentrations of selective serotonin reuptake inhibitors (SSRI's) and antipsychotics. It is imperative for patients seeking medical marijuana treatment to consult with their health care provider to learn about and avoid potentially adverse drug interactions.

Truly chronic studies with CBD are still scarce, especially toxicological evaluations of genotoxicity and effects on hormones (Iffland & Grotenhermen, 2017). Therefore, more toxicological studies that explore CBD side effects after chronic administration must be conducted. This research is crucial because currently, the majority of patients being prescribed CBD, in the form of Epidiolex, are children under 10 years of age. In a 2017 review of CBD clinical studies, the most common side effects reported were elevated liver enzymes, tiredness, diarrhea, and changes of appetite/weight (Iffland & Grotenherman, 2017). In comparison with other prescription drugs studied in these trials, CBD had a better side effect profile. Nonetheless, much more research is needed in large scale human trials to determine CBD's toxicological safety/efficacy.

5.2.3 Research Restrictions and Knowledge Gaps

The Schedule I listing of cannabis according to the CSA has led to difficulties in access for research purposes. Researchers conducting clinical research on biological products such as cannabis must submit an investigational new drug (IND) application to the FDA (National Academies of Sciences, 2017). Next, the investigator must obtain an administrative letter of authorization (LOA) from the National Institute of Drug Abuse (NIDA). The LOA describes the investigators' facilities and the specifics about the desired cannabis product they desire to obtain. To safeguard against the acquisition of cannabis or cannabinoids for non-research purposes, investigators must also apply for a DEA registration and site licensure before conducting any studies involving cannabis or cannabinoid constituents (National Academies of Sciences, 2017). Finally, the investigator must submit the IND and the LOA to the FDA and the DEA for further review and approval.

Currently, investigators interested in conducting research on cannabis must obtain that cannabis through NIDA. Historically, NIDA has only contracted with the University of Mississippi to cultivate different varieties of research-grade cannabis with various THC:CBD ratios (Mead, 2017). However, the DEA announced in 2017 that it will register additional sources of cannabis cultivated for research on the development of FDA-approved products. Since this announcement, however, no other institution has been authorized/contracted by NIDA to cultivate cannabis.

Drugs that fall under Schedule II-V are subject to less stringent rules. FDA-approved products that contain a Schedule II-V substance may be prescribed and dispensed within a clinical practice. While Schedule I substances cannot be legally

prescribed by a physician, only "certified" or "recommended." Additionally, a Schedule I substance cannot be dispensed outside of a research program, so patients must obtain cannabis products from a dispensary, not directly from their health care provider or a pharmacy. Physicians who hold Schedule II-V prescriber registrations, may conduct research on a Schedule II-V substance lawfully. They do not need to seek further DEA or state controlled drug agency approval, and they can obtain the substances from a wide number of registered manufacturers.

Funding for cannabis research is another restrictive process. Without adequate financial support, cannabis research will be unable to inform health care or public health practice, or to keep pace with changes in cannabis policy and patterns of cannabis use (National Academy of Sciences, 2017). The National Institute of Health (NIH) is responsible for funding research across a number of health domains, and NIDA is a member institute of NIH. In the fiscal year of 2017, NIH spent almost $140 million on cannabis research (NIH, 2018). In 2017, studies supported by NIDA accounted for 60% of all NIH spending on cannabinoid research (National Academy of Sciences, 2017). There has been a push recently for more experimental therapeutic research with cannabis for a range of conditions including: cardiovascular disease, obesity, and Alzheimer's disease. These conditions are usually handled by other branches and institutes of NIH. It is unrealistic to expect NIDA to have the resources or interest to fund a broader therapeutic research agenda for cannabinoid products. If the legal status of cannabis were to change to allow for broader research access, this will assuredly have an impact on treatments and conditions studied by institutes other than NIDA.

Due to numerous research and funding restrictions, there are inherent knowledge gaps associated with cannabis use that must be addressed. There is insufficient high quality data regarding the efficacy, dose-dependent curve, drug interactions, expected adverse effects, and safety of commercially available medical cannabis products (Sagy et al., 2018). There is a further lack of sufficient knowledge regarding the exact content and purity of various medical cannabis derivatives. These gaps impair physicians' and patients' ability to reach a fully informed decision regarding the recommendation and use of medical cannabis as a pharmaceutical, because many issues of the substance's pharmacokinetics are still unclear. There are no clear guidelines of when to "recommend" medical cannabis for a patient. The vague indications and relatively high availability of the product, may lead to over-use and misuse by patients (Sagy et al., 2018).

5.2.4 FDA/EPA Regulation Inconsistency

As a Schedule I substance, cannabis is effectively barred from obtaining further regulatory policies in terms of differentiation of application, pesticide regulation, and product safety development. Inconsistency within the two regulatory agencies of the FDA and the Environmental Protection Agency (EPA) has led to further confusion and risks associated with the medical cannabis industry as a whole.

Inconsistent regulation by the FDA is disconcerting given the widespread and ever-growing use of cannabis products all over the country. The FDA exercises control over approved cannabis drugs like Epidiolex and Marinol, but it does not regulate most of the medical marijuana products sold online or in dispensary stores. The role of the FDA in the drug approval and review process is designed to ensure that new medicines, including those derived from botanicals, are appropriately evaluated for safety, effectiveness, and

are cultivated/ manufactured under safe conditions for human consumption (Weiss et al., 2017).

Currently, many patients are using cannabis products or extracts that: 1) have not undergone rigorous clinical trials, 2) are not regulated for consistency or quality, and 3) are indicated for medical conditions without a sufficient evidence base for supporting their claimed effectiveness. Without the FDA offering a comprehensive and universal regulation plan for medical marijuana products, state governments are left to make decisions for themselves. Irregularity in marijuana regulation from state to state can allow for inappropriate marketing, formulation, and packaging practices to persist – making THC/CBD content across samples unpredictable and potentially dangerous (Haffajee et al., 2019).

Independent research, separate from the FDA, has confirmed that the CBD content in almost 70% of products available online could be mislabeled (where 43% of products were under-labeled and 26% over-labeled for actual CBD concentrations) (Corroon & Kight, 2018). In another study conducted by the FDA in 2016, the results showed that most of the online marijuana products contained little-to-no CBD, and other products contained much higher levels of THC than listed on the label (Mead, 2017). Without FDA approval and regulation, health care providers and patients are left with a lack of knowledge about the efficacy, dosing, adverse effects, and accessibility to safe marijuana products. If all marijuana products were subjected to FDA approval, access to such products would be hindered initially, while intensive efficacy and safety research is conducted. FDA regulation would ultimately foster a complete and robust system for the improvement of product safety and consistency within the medical cannabis industry.

The EPA has oversight of pesticide registration, safe use, and enforcement over botanical products. Unfortunately, there is limited information available about cannabis pests, and there are no pesticides specifically labeled for marijuana cultivation. The status as a Schedule I compound directly impacts whether or not conventional pesticides can be legally used to manage pests associated with cannabis. The EPA does not allow registration of pesticides on cannabis, because federal law categorizes the plants as illegal (Sandler et al., 2019). Without this registration, conventional pesticides cannot be used legally for marijuana cultivation in the US.

Another role of the EPA is to establish pesticide tolerance levels for crops and botanical products. The EPA sets a pesticide tolerance which is a maximum residue level acceptable for a specific crop. The pesticide tolerance information is required before the EPA can officially register pesticides for crops. Consequently, as long as cannabis remains a Schedule I drug, the EPA cannot recognize it as a legal crop, thereby preventing the establishment of pesticide tolerances.

Cannabis growers have an economic incentive to improve the quantity and quality of their crops through the use of registered pesticides available for other agricultural crops (Sandler et al., 2019). Cannabis crops are agronomic and have similar pests to other greenhouse crops. However, pesticides used on other EPA regulated crops cannot be legally used on cannabis. Under federal and state laws, using a pesticide on a crop that is not listed on a product's label is considered illegal; which subjects the grower to crop confiscation, fines, and imprisonment (Sandler et al., 2019).

The EPA has failed to examine potential health effects of pesticide compounds on cannabis by not offering a standardized risk assessment at the federal level. This makes it

difficult to determine how serious the exposure to certain pesticides may be to potential consumers. A 2013 study found that 69.5% of tested common pesticides, like bifenthrin, diazinon, and permethrin, were found remaining in cannabis smoke condensate (Sullivan et al., 2013). Pesticide residues in cannabis could be substantial and thus pose significant toxicological risks.

It is an unfortunate irony that a Schedule I drug has been legalized in some states prior to the pesticides potentially needed to produce and protect the substance. This gives the appearance that pesticides are more austerely regulated in the US than a Schedule I drug. It is imperative for the federal government to establish overall guidelines regarding pesticide legislation and to implement a program for the enforcement of cannabis pesticides.

5.3 Potential Marijuana Reform Options

The present situation of conflicting federal and state marijuana laws is suboptimal and will begin to adversely affect consumers if changes are not made. The absence of a sensible, stable federal marijuana policy affects the safety of marijuana products and physicians' comfort in recommending or prescribing them (Haffaje et al., 2018). Federal regulation that accommodates, reinforces, and standardizes state marijuana policy would result in a safer, more reliable, and more accessible supply of cannabis products. It is no longer a matter of *whether* marijuana laws will change, but *how* and *when* they will change. This section will outline several federal marijuana reform proposals, and pros and cons for each are provided.

5.3.1 Federal Exemptions Following State Compliance

One type of federal reform proposal would be to create exemptions for state-legal marijuana activity from federal prosecution. Meaning, that federal marijuana laws simply do not apply to state-compliant activity, potentially requiring the government to prove noncompliance with state law its main objective for enforcement (Kriet, 2015). Unlike current legislation, including the Rohrabacher Farr Amendment, which must be approved every year, this type of policy would provide marijuana users, growers, physicians, etc. with more than temporary protection. Potential federal exemptions would unquestionably apply to and protect any conduct that takes place while they were enacted, and even if they were repealed later.

The flaw in this reform proposal is the inevitability of what constitutes "compliance" with state law. For example, a seller who failed to abide by their state's regulations for packaging or manufacturing could thereby be open to a federal drug prosecution. How will the federal government measure and gauge state compliance? This type of reform policy also does not explicitly address the status of marijuana for federally-funded research. Under this legislation, marijuana would still be considered a Schedule I substance, and would still be subjected to those research restrictions. In the end, there would be marijuana policies enacted that still do not have the fundamental science backing to ensure safety for all involved.

5.3.2 Rescheduling of Marijuana

The second type of reform option would be to reschedule marijuana and all of its derivatives. In doing so, marijuana would become legal for medicinal purposes, but would still be a regulated substance. There is considerable evidence in support of

marijuana's therapeutic benefits in reducing chronic pain, nausea, spasms, and epileptic episodes. Accordingly, there is a compelling argument that marijuana would be more appropriately designated as a Schedule II or III drug. Schedule II substances are defined as drugs with a high potential for abuse, with use potentially leading to severe psychological and physical dependence. Schedule III substances are defined as drugs with a moderate to low potential for physical and psychological dependence, which drug abuse potential less than Schedule I or II, but more than Schedule IV (DEA, 2019). Most importantly, Schedule II or III substances have accepted medical benefits and uses.

Rescheduling would facilitate further study of products for FDA approval, but would not automatically change the severity of penalties for marijuana crimes, to ensure that this substance is only used for legitimate medical and scientific purposes (Haffajee et al., 2018). However, there are several concerns associated with this reform possibility. One issue relates to how recreational users, in states which allow recreational use of the drug, would proceed with a new federal distinction of marijuana. Would recreational users still be subject to federal prosecution? Or could their access to marijuana be restricted all together? Another concern arises with accessibility of marijuana products. Rescheduling would subject all marijuana products to FDA approval, which could hinder access initially, but ultimately foster a robust system for regulation and research (Hajaffee et al., 2018). FDA oversight of marketing, packaging, and manufacturing, would improve product safety, consistency, and even efficacy.

5.3.4 Removal of Marijuana from the CSA

Finally, the most straightforward solution would be to completely remove marijuana from the CSA all together. This would effectively eliminate the conflict

between state and federal law. The federal government could conceivably retain the federal prohibition in states that want it, while simultaneously regulating marijuana in states that opt to legalize it. Marijuana could be regulated in a similar fashion to how alcohol is regulated in the US, and be enforced under the Bureau of Alcohol, Tobacco, Firearms, and Explosives. This approach could entail varying degrees of federal regulation within the marijuana market. In addition, this dramatic change could also come with FDA oversight of marijuana products, which would effectively regulate their manufacturing to ensure the product's efficacy and safety, and benefit the entire industry. Removal of marijuana from the CSA would allow for widespread availability for research purposes. Regardless of the level of restrictiveness of a potential federal marijuana regimen, this approach would successfully resolve any state and federal conflict. Replacement of federal prohibition with regulation would leave states free to decide to legalize marijuana on their own terms.

Legalization opponents have cited a range of concerns, chief among them is the possibility of a large-scale commercial marijuana industry (Kriet, 2015). Some opponents argue that legalization would in effect become like the tobacco industry during the mid-late 20th century. "Big Marijuana," as some refer to it, would invest heavily in promoting and advertising marijuana, which would create addicts and target youth (Kriet, 2015). However, these claims are unfounded because federal regulation of the marijuana industry would allow for federal control. Federal regulation could strictly limit the amount of marijuana a licensed grower could produce/sell annually, all related packaging/advertising, and even place restrictions on the amount a consumer could purchase in a given time period.

5.4 Future of Marijuana Policy

Removal of marijuana from the CSA poses the greatest advantages for the industry as a whole. However, the conundrum in this situation is timing. Timing of legislative changes will be crucial in the creation and enforcement of drug policy that is comprehensive and scientifically sound. As it stands, in order to address some of the central issues surrounding the marijuana industry, the drug needs to be federally legalized. However, in order to federally legalize the drug, the central issues must be addressed first.

The first and most vital step in the federal legalization process needs to be less restrictive research opportunities for marijuana. Research must be opened to a larger community of scientists in order to address the current knowledge gaps associated with its use. Once those questions are answered, the industry would be in a better position to defend and verify the therapeutic value of marijuana. Subsequently, the comprehensive and robust research will allow for the creation of effective marijuana policy by scientists and legislators, to ensure safety and stability.

The legal status of marijuana is complex and constantly evolving. Moreover, the inevitable policy changes will be guided by multiple competing interests. It is unlikely that any short term solutions will become the universal formula for the future of marijuana legality in the United States, as it is abundantly clear we do not have all the answers we need. Key questions for scientists, policy researchers, and decision makers, to focus efforts as different paths for the future of marijuana legality are explored include:

- What policies need to be pursued to speed up the research needed to fully exploit the therapeutic potential of marijuana? What specific medical conditions need to be focused?

- What will be the effects suffered by chronic users of marijuana and how might they be alleviated?

- How should strain, potency, indications, and routes of administration be regulated and monitored?

- How will the FDA and the EPA go about creating robust cannabis product manufacturing, packaging, and safety testing regulations?

- How would a comprehensive list of all potential drug interactions of marijuana and other substances be determined?

- Who will be the governing authority that sets all standards and regulations associated with the marijuana industry?

- What will the standards for widespread marijuana usage be? An age requirement to protect susceptible children from using the drug? Limitations for the amount of marijuana one can buy in a certain time period?

- Finally, how much will policy makers rely on scientific evidence in creation of new marijuana policy? Scientific involvement should be a requirement for any proposed cannabis legislation, but to what extent and form?

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