Effects of Cannabidiol and Δ^9-Tetrahydrocannabinol in The Elevated Plus Maze and Forced Swim Tests

by

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Abstract

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The effects of THC, CBD and a combination of CBD and THC (15:1) were tested in mice models of anxiety (elevated plus maze EPM) and depression (forced swim test FST). Adult, male CD1 mice were injected intraperitoneally (I.P.) with CBD (0-120mg/kg), THC (0-8mg/), CBD + THC (up to 120+8mg/kg), diazepam (0, 2.5mg/kg) or imipramine (0, 30mg/kg). EPM ran for 5mins, FST for 6mins, and open field test for 10mins. CBD was injected 30 or 60mins prior to testing and THC was injected 30mins prior. CBD decreased immobility time in FST. THC increased time spent in the open arms in EPM, and decreased immobility time in FST. The combination of CBD and THC was better than CBD or THC alone in FST at 30mins. CBD did not show anxiolytic effects but showed anti-depressant effects. THC had both anxiolytic and antidepressant effects at some doses. CBD and THC combined might be useful in patients with anxiety and depression.

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Preface

This present study work was part of a larger research program designed to investigate the effects of cannabinoids on epilepsy and its comorbidities. Anxiety and depression are among the most common and debilitating comorbidities of epilepsy. Although seizures and epilepsy are not the primary focus of this thesis, this research was conceived in the context of epilepsy. Epilepsy, therefore, will be discussed briefly in the Introduction section of this thesis, as well as anxiety and depression and the animal models for anxiety and depression.

This project was somewhat hampered because Health Canada stopped issuing licenses for the laboratory use of cannabinoids in October, 2019. For a period of six months no new licenses were issued. The present thesis was therefore completed using the supply of cannabinoids we had on hand, but our supply was exhausted before complete dose-response curves could be completed in some studies. Some curves have only two points, and some N's are smaller than desirable. A new license has been obtained, and these studies will be completed before the work is published.

List of Abbreviations

2-AG: 2-arachidonoylglycerol

5-HT1A: 5-hydroxytryptophan type 1A

AED: Anti-Epileptic Drug

ANOVA: Analysis of Variance

CBD: Cannabidiol

CMS: Chronic Mild Stress

CTLS: Cannabis Tracking and Licensing System

DBS: Deep Brain Stimulation

DSM: Diagnostic and Statistical Manual of Mental Disorders

ECT: Electro-Convulsive Therapy

EPM: Elevated Plus Maze

FAAH: Fatty Acid Amide Hydrolase

FAS: Focal Awareness Seizures

FIAS: Focal Impaired Awareness Seizures

FST: Forced Swim Test

GAD: Generalized Anxiety Disorder

GPR55: G-protein-coupled receptor 55

HPA axis: Hypothalamus-Pituitary-Adrenal Gland axis

ILAE: International League Against Epilepsy

I.P.: Intraperitoneally

LHPP: phosphorlysine phosphohistidine inorganic pyrophosphate phosphatase gene

LHT: Learned Helplessness Test

MAGL: Monoacylglycerol Lipase

MAOI: Monoamine Oxidase Inhibitor

MDD: Major Depressive Disorder

OF: Open Field

SIRT1: sirtuin1 gene

SNRI: serotonin–noradrenaline reuptake inhibitors

SSRI: Selective Serotonin Reuptake Inhibitor

TCA: Tricyclic Antidepressant

THC: Tetrahydrocannabinol

TMS: Transcranial Magnetic Stimulation

TRD: Treatment-Resistant Depression

TRPV1-3: transient receptor potential cation channel, subfamily V, members 1-3

TST: Tail Suspension Test

WHO: World Health Organization

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Chapter 1

1 « General Introduction »

1.1 « Epilepsy and Seizures»

"Epilepsy" is a neurological disorder characterized by recurrent (two or more) and spontaneous seizures (Cervenka & Kaplan, 2016).

"Seizures" are periods of self-sustained hyperactivity in the central nervous system. They involve periods of rapid and synchronized firing in cerebral neurons (Burnham, 1998).

A number of different types of seizures exist, as classified by the International League Against Epilepsy (ILAE)

1.1.1 Classifications of Seizures

The ILAE classifies seizures into three large categories: 1) generalized onset seizures, where onset involves both hemispheres; 2) focal onset seizures, where seizures start in one part of the brain; and 3) unknown onset seizures (Fisher et al., 2017).

Generalized-Onset Seizures The generalized onset seizures, which are sub-classified as "motor" or "non-motor", encompass more than ten different seizure types (Fisher et al., 2017). Most commonly seen are tonic-clonic seizures (motor) and absence seizures (non-motor) (Fisher et al., 2017).

Tonic-clonic seizures are the major convulsive seizures that most people think of when they think of epilepsy. They can occur at any age. During tonic-clonic attacks, patients first lose

consciousness and then their whole bodies stiffen (tonus), and subsequently exhibit rhythmic jerking and shaking (clonus) (Carlson, 2013c). Tonic-clonic seizures typically last for 1 to 3 minutes. After a tonic-clonic seizure, patients are briefly comatose, and then usually feel tired. Their full consciousness will slowly return, sometimes taking up to 48 hours. They have no subsequent memory for the period of the attack (Burnham, 2006).

Absence seizures are non-convulsive seizures that usually occur in children. Symptoms include a brief loss of consciousness during which patients usually stop all activity and stare blankly. This type of seizure usually lasts for less than 30 seconds (Burnham, 1998). After an absence seizure, patients will re-start their previous activities, and may be unaware that an attack has occurred. They have no subsequent memory for the period of the attack (Burnham, 2006). These mild seizures sometimes go unnoticed and undiagnosed, and are mistaken for "day dreaming" (Carlson, 2013c). Around 70% of children who have absence seizures will outgrow them by the age of 18 (Kiriakopoulos & Shafer, 2017a)

Focal-Onset Seizures Focal onset seizures – which originate in one part of one hemisphere – are divided into seizures where awareness is retained ("focal awareness seizures", "FAS") and seizures with impaired awareness ("focal impaired awareness seizures", "FIAS") (Fisher et al., 2017).

Focal-onset seizures need to be discussed in detail, since they often arise from the temporal lobe and are particularly associated with comorbidities like anxiety and depression (Filho, Mazetto, da Silva, Caboclo, & Yacubian, 2011).

FAS were previously called "simple partial" seizures. They may arise from many different parts of the forebrain. Symptoms of the seizures depend on the affected brain areas.

When focal “spiking” is in the occipital lobes, for instance, patients will experience visual distortions, whereas “spiking” in the olfactory and gustatory cortices produces a strange smell or taste. Spiking in the amygdala is associated with a feeling of fear (Burnham, 1998). Patients are usually fully conscious and awake during a FAS and are able to respond to outside stimuli (Burnham, 1998). These episodes usually last for less than 2 minutes, and patients will remember what happened during the period of the seizure (Kiriakopoulos & Shafer, 2017b).

FIAS were formerly known as “complex partial” seizures. They usually arise from the temporal lobe or the frontal lobe. During a FIAS, the patient appears to be conscious, but is out of contact with his/her environment (Kiriakopoulos & Shafer, 2017b). He/she is often immobile at first – with a blank stare - but may later show repetitive, involuntary movements - such as chewing or picking at the clothes. These are called “automatisms” (Burnham, 1998). FIAS may be preceded by an “aura”, which is actually an FAS and usually of temporal lobe origin.

FIAS (and FAS) may also spread to the rest of the brain to produce “focal to bilateral tonic-clonic seizures” - which is the term that has replaced the term “secondarily generalized seizures”. More than 30% of patients who have focal epilepsies will also have focal to bilateral tonic-clonic seizures (Fisher et al., 2017).

1.1.2 Etiology and Risk Factors for Epilepsy.

Traditional Terminology There have traditionally been considered to be three types of etiology for epilepsy: idiopathic, symptomatic and cryptogenic.

The term “idiopathic” has traditionally been used for epilepsies where the brain shows no clear anatomical or metabolic abnormality. They have an assumed genetic cause, either single-gene or polygenic (Shorvon, 2011).

The term “symptomatic” has been used for epilepsies where the brain shows well-defined neuroanatomic or neuropathologic abnormalities, either structural or metabolic (Shorvon, 2011). FAS and FIAS of temporal-lobe origin frequently have symptomatic causes (Panayiotopoulos, 2005)

“Cryptogenic” epilepsies have been those that are presumed to be symptomatic, but where the underlying causes have not been identified. Although the number of epilepsies in this category is decreasing, it still accounts for 40% of adult-onset epilepsies (Shorvon, 2011).

New Terminology In 2010, an ILAE report suggested that the terms “idiopathic”, “symptomatic” and “cryptogenic” should be replaced by the terms “genic”, “structural/metabolic” and “unknown cause” (Berg et al., 2010). This has stirred debate among researchers concerning its usefulness, since there doesn’t seem to be a meaningful change to the underlying concepts (Shorvon, 2011).

1.1.3 Incidence and Prevalence of Epilepsy

Epilepsy is the second most common neurological disorder following stroke (Carlson, 2013c). Two epidemiological indices are usually discussed in relationship to epilepsy: point prevalence and life-time prevalence.

“Point prevalence” is the number of people that have a given disorder at any particular time. Active epilepsies are found in 4 to 10 in 1000 persons in developed countries, and 6 to 10 in 1000 persons in developing countries (Sander, 2003).

"Life-time prevalence" is the number of people that will have a given disorder some time during their lives. The life-time prevalence of epilepsy is estimated to be about 5% (Sander, 2003).

1.1.4 Treatment for Epilepsy

Epilepsies can be treated by anti-epileptic drugs (AEDs), surgery, neurostimulation or dietary therapies. The use of AEDs is the current front-line treatment for epilepsy.

AEDs Currently there are over twenty AEDs on the market, plus a handful of agents in clinical trials. The mechanisms of action are generally to block neural excitation or enhance neural inhibition (Devinsky et al., 2018). Unfortunately, only about 70% of seizures can be controlled with one or two first-line AEDs. The remaining 30% of patients are said to have drug resistant or "intractable" epilepsies (Devinsky et al., 2018).

Surgery Surgery is recommended for patients with intractable focal-onset epilepsies. The most common epilepsy surgery is anterior temporal lobectomy (Devinsky et al., 2018). About 60-70% of patients will achieve seizure remission after anterior temporal lobectomy (Cervenka & Kaplan, 2016).

Diet Therapy Diet therapy is recommended for intractable patients with generalized-onset epilepsies. Over eighty percent of patients respond, at least in part, to diet therapies. These therapies include the classic ketogenic diet, the modified Atkins diet and the low glycemic index treatment (Devinsky et al., 2018).

Neurostimulation When other therapies have failed, neurostimulation may be tried. Currently, vagus nerve stimulation is the most common stimulation therapy, although direct stimulation of the brain is also possible (Cervenka & Kaplan, 2016).

Therapy and Comorbidities Unfortunately, none of the present therapies is effective at treating the comorbidities of epilepsy. AEDs, the current front-line therapy, may actually make the comorbidities worse (Nadkarni & Devinsky, 2005).

1.1.5 The Comorbidities of Epilepsy

Patients suffering from epilepsy also suffer from a number of associated disorders, called the "comorbidities of epilepsy". These may be physical, cognitive or psychiatric (Seidenberg, Pulsipher, & Hermann, 2009).

Psychiatric comorbidities, such as depression and anxiety, are some of the most common comorbidities, and are particularly common in people with FIAS (complex partial seizures) arising from the temporal lobes (Seidenberg et al., 2009). Ten to 25% of temporal-lobe epileptic patients suffer from anxiety, and 20-55% suffer from depression (Jackson and Turkington, 2005). It has been reported by both the patients and their care givers that anxiety and depression are major contributors to the low quality of life seen in people with epilepsy (Jackson & Turkington, 2005).

The present thesis is focused on the question of whether the cannabinoids – which are now being used to control epilepsy - might also be useful in treating these two common comorbidities of epilepsy. The cannabinoids will be discussed in the next section (1.2), and then anxiety and depression will be discussed in detail in the two sections that follow (1.3 and 1.4).

1.2 « Cannabinoids and the Endocannabinoid System»

The chemical compounds isolated from cannabis are collectively called the "cannabinoids". Tetrahydrocannabinol (THC) and cannabidiol (CBD) are two of the most abundant cannabinoids (Tambaro & Bortolato, 2012).

The use of cannabis as a medicine dates back five thousand years to ancient China (Aggarwal et al., 2009). Due to its psychoactive properties, cannabis has also been a popular recreational drug in many parts of the world (Latimer & Zur, 2010).

Cannabinoid Receptors

There are two receptors in the endocannabinoid system: CB1 and CB2. Both receptors are G-protein coupled (Howlett, Qualy, & Khachatrian, 1986). CB1 is the most abundant receptor in the brain, modulating the release of neurotransmitters in many different transmitter systems (Herkenham et al., 1990) and (Freund, Katona, & Piomelli, 2003). CB2 receptors are primarily expressed in the peripheral immune system, and are not found in large quantities in the brain (Walter & Stella, 2004). CB1 receptors are primarily on the pre-synaptic neurons. Upon activation, CB1 receptors inhibit other neurotransmitters' release from the pre-synaptic neurons. This is one of the major brain retrograde feedback systems (Castillo, Younts, Chávez, & Hashimotodani, 2012).

Endocannabinoids

The endogenous ligands for the endocannabinoid system are called "endocannabinoids". There are two endocannabinoids: 2-arachidonoylglycerol (2-AG) (Devane et al., 1992) and N-arachidonoyl-ethanolamine (commonly known as "anandamide") (Mechoulam et al., 1995).

Anandamide is a partial agonist at both CB1 and CB2, whereas 2-AG is a full agonist at both receptors (Tambaro & Bortolato, 2012).

Anandamide is degraded by the membrane enzyme fatty acid amide hydrolase (FAAH) (Hillard, Wilkison, Edgemond, & Campbell, 1995), while 2-AG is degraded by the cytosolic serine hydrolase monoacylglycerol lipase (MAGL) (Dinh, Freund, & Piomelli, 2002).

CBD and THC

THC is the major psychoactive and psychotoxic component found in cannabis. THC can cause changes in mood, cognitive perception and appetite, as well as producing analgesia and many other effects. Its pharmacological effects are mediated through the two cannabinoid receptors: CB1 and CB2. THC is a partial agonist at both of these receptors (Pertwee, 2008).

CBD, on the other hand, is only psychoactive but not psychotoxic. CBD has been reported to have many therapeutic effects, including anti-inflammatory, analgesia, anxiolytic, antidepressant and anti-seizure effects (National Academies of Sciences et al., 2017). CBD does not appear to act via CB1 or CB2 receptors, since it binds to CB1 and CB2 with a very low affinity (Pertwee, 1999). CBD does, however, interact with a wide variety of other receptors, including: 1) G-protein-coupled receptor 55 (GPR55), 2) transient receptor potential cation channel, subfamily V, members 1-3 (TRPV1-3), and 3) 5-hydroxytryptophan type 1A (5-HT1A) (Friedman & Devinsky, 2015).

CBD and THC can appear in varying ratios in different breeds of cannabis. In the present study, a ratio of 15:1 is used because it is commercially available through our industry partner MedReleaf/Aurora, in the preparation named “Avidekel”.

1.3 « Anxiety »

As noted above, the present thesis will investigate the effects of CBD and THC in anxiety and depression.

"Anxiety" is a state characterized by worry and fear. When the worry and fear are excessive and unrealistic, the anxiety is considered to be part of an "anxiety disorder" (Carlson, 2013a).

The anxiety disorders are a group of psychiatric disorders characterized by the occurrence of anxiety as a major symptom. Anxiety disorders lead to the avoidance of anxiety-provoking situations and can greatly curtail the lives of affected individuals. (Craske et al., 2017).

The anxiety disorders include: 1) separation anxiety disorder (fear of being separated from attachment figures); 2) specific phobias (e.g. fear of animal, height, needles or airplanes); 3) agoraphobia (fear of open spaces and not being to get help in a panic attack situation); 4) selective mutism (inability to speak); 5) social anxiety disorder (fear of social interactions); 6) panic disorder (recurrent and unpredictable panic attacks) and 7) generalized anxiety disorder (GAD, fear of various uncontrollable situations) (Craske et al., 2017).

Post-traumatic stress disorder and obsessive-compulsive disorder were previously categorized as anxiety disorders. They have now been removed from the group of anxiety disorders in the fifth edition of Diagnostic and Statistical Manual of Mental Disorders (DSM-5) (American Psychiatric Association, 2013), however. They are currently described in an adjacent chapter (American Psychiatric Association, 2013).

1.3.1 Diagnostic Criteria

According to the DSM-5 (American Psychiatric Association, 2013) and the International Classification of Diseases, 10th edition (ICD-10) (World Health Organization, 2010), anxiety disorders should be diagnosed on the basis of a set of criteria that are detailed and specific. For example, panic disorder is diagnosed when the individual experiences recurrent unpredictable panic attacks and shows the fear of having more panic attacks for more than a month. The diagnostic criteria for other types of anxiety can be found in a review by Craske et al. (2017)

In clinical settings, however, the criteria are not followed closely by physicians (Vermani, Marcus, & Katzman, 2011). As a result, the misdiagnosis of anxiety disorders is fairly common. A study conducted in seven primary care clinics across three Canadian provinces showed that the misdiagnosis rate for panic disorder is 85.8%, for GAD is 71.0%, and as high as 97.8% for social anxiety disorder (Vermani et al., 2011).

One of the reasons for missed diagnoses is that anxiety disorders are generally taken less seriously than depression or psychosis (Wittchen et al., 2002). Also, some patients complain of somatic discomfort rather than anxiety causing confusion for physicians (Wittchen et al., 2002). Finally, the social stigma associated with "mental disorders" prevents some patients from even seeking professional help (Hasan & Musleh, 2018).

1.3.2 Epidemiology and Risk Factors

Prevalence The life-time prevalence of anxiety disorders varies in different countries. It is lowest in China at 4.8% and highest in the U.S. at 31%, according to the World Mental Health Survey conducted by World Health Organization (Kessler et al., 2007).

The twelve-month prevalence for anxiety disorders in European countries is estimated to be around 14% (Wittchen et al., 2011), although these figures were based on DSM-4 criteria. They would be lower with obsessive-compulsive disorder and post-traumatic stress disorder excluded.

Risk Factors Female sex, family history and negative early-childhood experiences are some of the major risk factors for anxiety disorders: **1) Female Sex** According to an American national survey study, the life-time prevalence and 12-month prevalence of anxiety disorders are 1.7 and 1.79 times higher in women than men (McLean, Asnaani, Litz, & Hofmann, 2011). **2) Family History** Based on a study with 2427 subjects, if one parent is diagnosed with an anxiety disorder, the odds ratio that a child will be affected are 1.6. The odds ratio increases to 2.1 if both parents have an anxiety disorder (Lieb, Isensee, Höfler, Pfister, & Wittchen, 2002). **3) Childhood Trauma** Long-term childhood adversities and severe life events can predispose individuals to anxiety later in life (Allen, Rapee, & Sandberg, 2008). This will be discussed in more detail in section 1.2.3.

In addition, as mentioned above, temporal-lobe epilepsy is a major risk factor for anxiety. Ten to 25% of temporal-lobe epileptic patients suffer from serious anxiety (Jackson and Turkington, 2005)

1.3.3 The Etiology of Anxiety

Genetics, environmental and abnormal brain function have all been indicated in the etiology of anxiety. To a certain extent, these overlap the risk factors.

Genetics The heritability of anxiety disorders is estimated to be about 30-50% - which is why parental anxiety is a risk factor (Hettema, Neale, & Kendler, 2001). The inheritance of

anxiety is polygenic and multifactorial. No single “anxiety gene” has been identified. Genome-wide association studies, however, have identified certain genes that are highly correlated with anxiety: 1) CRHR1 encoding corticotropin releasing factor receptor 1 (Weber et al., 2016), 2) COMT encoding catechol-O-methyltransferase (Howe et al., 2016), 3) FKBP5 encoding a glucocorticoid receptor modulating molecule (Arloth et al., 2015), and 4) FAAH encoding endocannabinoids degrading enzyme (Gunduz-Cinar et al., 2013).

Environment Environmental factors also contribute to anxiety. Although environmental factors and genetic factors often interact (Kendler, Thornton, & Prescott, 2001), twin studies have shown that environmental factors lead to anxiety even when genetics are controlled for (Eley et al., 2015).

Among the environmental factors that contribute to anxiety, parenting style plays a role. Parents who have a pessimistic world view and an over-expectation of negative events tend to produce anxious children - as do parents who are over controlling (Mathews, Mackintosh, & Fulcher, 1997) (L. Murray, Creswell, & Cooper, 2009).

Also, as mentioned above, childhood trauma can also paly an important role. Physical and sexual abuse at a young age significantly correlate with later anxiety disorders (Hudson, 2005).

Brain Function Certain brain structures seem to be overactive in anxiety, perhaps as the result of genetic or environmental factors. The majority of research on the brain function in anxiety patients converges on the amygdala. The amygdala is involved in fear, fear learning, decision making and the processing of negative emotions (Bzdok, Laird, Zilles, Fox, & Eickhoff,

2013). Imaging studies have detected increased activity in the amygdala of patients with anxiety disorders (Pfleiderer et al., 2007).

Other brain regions may also play a role. Regions associated with anxiety include the cingulate and the prefrontal cortex and insular cortices. For instance, the anterior cingulate and right orbitofrontal cortex of a patient having a panic attack have been found to have decreased blood flow (Fischer, Andersson, Furmark, & Fredrikson, 1998), whereas increased insular activity has been observed in anxious college students (Stein, Simmons, Feinstein, & Paulus, 2007).

1.3.4 The Treatment of Anxiety

Psychotherapy and pharmacotherapy are two main treatment options currently available for anxiety.

Psychotherapy There are many variations to psychotherapy: 1) cognitive-behavioral therapy, 2) mindfulness and acceptance-based approaches, and 3) interpersonal therapy.

Cognitive-behavioral therapy encompasses a wide variety of techniques. Patients are taught to self-monitor thoughts and emotions (psychoeducation), identify unhealthy thought processes and replace them with evidence-based thoughts (cognitive re-constructing), and expose themselves to feared stimuli (desensitization) (Craske et al., 2017). Cognitive-behavioral therapy is the most widely accepted and most effective psychotherapy, with a response rate of 45-55% (Loerinc et al., 2015). The treatment is usually once a week for 10-20 weeks.

The mindfulness and acceptance-based approaches use meditation and breathing techniques to help patients accept certain negative or anxious experiences and feelings (Craske et

al., 2017). There is much less evidence supporting the effectiveness of the mindfulness and acceptance-based approaches due to the low quality of published research, although it is gaining more popularity (Craske et al., 2017).

Interpersonal therapies are short-term treatments focusing on interpersonal relationships. They involve understanding how unconscious thoughts can manifest as current feelings (Craske et al., 2017). Compared to cognitive-behavioral therapy, interpersonal therapies are less effective (Cuijpers, Donker, Weissman, Ravitz, & Cristea, 2016).

Pharmacotherapy Anti-depressants and benzodiazepines are commonly used to treat anxiety.

The drugs initially developed as antidepressants are now also the first-line treatment for anxiety. The selective serotonin reuptake inhibitors and the serotonin–noradrenaline reuptake inhibitors are now the major drugs used in the treatment of anxiety (Carlson, 2013a). Despite the wide-spread use of these serotonergic agents, the side-effects associated with them are sometimes concerning. Gastrointestinal discomfort, nausea, dizziness, headaches and insomnia can all appear, for instance, and can be mis-perceived as anxiety symptoms. Weight gain and sexual dysfunction may also occur (Cassano & Fava, 2004). Because of these serious side-effects, patients are usually titrated up from a lower dose to allow acclimatization to the drugs' adverse effects modulation and acclimation.

Benzodiazepines are now considered the second- or third-line treatment for anxiety (Ravindran & Stein, 2010). They are mostly used for emergency situations, as they have a more rapid onset of action than the antidepressants (Carlson, 2013a). They are not generally used for

long-term treatment, however, because of their sedating side effects, tolerance and abuse profile (Carlson, 2013a).

Because of the limited pharmacotherapy options currently available, new treatments are being sought. Atypical antipsychotics have been indicated in the treatment of anxiety disorders, however, the quality of evidence supporting their use is generally low (Kreys & Phan, 2015). D-cycloserine, a partial NMDA receptor agonist, has sometimes been reported to facilitate cognitive-behavioral therapy, but the evidence is currently conflicting (Ressler et al., 2004) (Ori et al., 2015).

As mentioned above, CBD has also been reported to have anxiolytic effects. If these effects could be substantiated, CBD might be developed as a new therapeutic for the treatment of anxiety.

. Development of new anxiolytics is done using animal models of anxiety.

1.3.5 Behavioral Animal Models of Anxiety

Commonly used behavioral animal tests of anxiety can be grouped into two categories: 1) "conditioned", where the aversive stimulus is learned, and 2) "unconditioned", where the aversive stimulus is innate (Handley & Mithani, 1984). Compared to the conditioned tests, the unconditioned tests seem to better model human anxiety (Bourin, Petit-Demoulière, Dhonnchadha, & Hascöet, 2007).

The open field (OF) test, the light/dark box (L/D box) test and the elevated plus maze (EPM) test are the three of the most commonly used unconditioned animal tests of anxiety.

The Open Field (OF) The OF test involves putting animals in an open arena where they are allowed to explore freely for a period of time (Hall & Ballachey, 1932). Locomotion is measured. The amount of time the animals spend in the center of the arena versus in the periphery may also be is measured, and sometimes urination and defecation are recorded (Hall, 1934). Anxiolytics cause rodents to spend more time in the center of the arena (Bourin et al., 2007).

The OF test is easy to perform and easy to score, however, it lacks standardization among different labs. The size and shape of the OF arena, the time being recorded, and the light levels can all vary depending on the lab set up. This makes inter-laboratory replications difficult.

When only locomotory activity is measured, the OF can be used to test the general level of activity in animals. In this usage, it is often paired with the forced swim test, a test of depression-like behavior (below).

The Light/Dark Box (L/D box) The L/D box involves putting animals in two inter-connected boxes where they are allowed to roam freely (Crawley & Goodwin, 1980). One of the boxes is white and well-lit, while the other is black and dimly-lit (Bourin & Hascoët, 2003). Anxious rodents tend to stay in the dark box. When treated with anxiolytics, they spend more time in the lighted box (Bourin & Hascoët, 2003). This test is fairly commonly used, but different ages and strains produce differences that cause variability from experiment to experiment (Hascoët, Colombel, & Bourin, 1999).

The Elevated Plus Maze (EPM) The EPM involves putting animals in a "maze" consisting of two elevated arms crossed at a ninety-degree angle (Sharon Pellow & File, 1986). One of the arms is "open" (with no walls), while the other is "closed" (with walls). Anxious rodents normally spend more time in the closed arms. When they are treated with anxiolytics,

they spend more time in the open arms (Lister, 1987). The percent time spent in open arms and number of entries into the open arms are scored – the former being more sensitive to drug effects (Pellow et al., 1985). The number of closed arm entries is sometimes used as a measure for general locomotor activity level in the EPM (Lister, 1987). Age, gender and strain of animals (Griebel et al., 2000), housing conditions (Rodgers & Shepherd, 1993) and illumination (Jones & King, 2001) can all produce variability in the results. Despite these sources of variability, the EPM is the most commonly used animal behavioral tests for anxiety.

Because of the abundance of previous literature using the EPM, we have selected the EPM as the behavioral test to be used in the present study.

1.3.6 Past Studies of the Cannabinoids on Anxiety

It has been reported by both recreational users and scientists that cannabis can affect anxiety levels (Tambaro & Bortolato, 2012). Whether the effects are anxiolytic or anxiogenic is still under debate, and it is possible that both anxiolytic and anxiogenic effects occur.

In this section, both human and animal studies of the effects of CBD and THC on anxiety will be reviewed. The popular belief – supported by some researchers - is that THC is both anxiolytic and anxiogenic, while CBD is anxiolytic.

Human Studies

THC Cannabis is a recreational drug, used partly for its anxiolytic effects (Tambaro & Bortolato, 2012). Some studies have also reported THC to be anxiogenic, however. Orally administered THC from 0.5mg/kg to 30mg/kg, for instance, has been reported to increase anxiety as rated through questionnaires or simply self-reports (Karniol, Shirakawa, Kasinski, Pfeferman,

& Carlini, 1974) (Peters, Lewis, Dustman, Straight, & Beck, 1976) (Zuardi, Shirakawa, Finkelfarb, & Karniol, 1982) (Fusar-Poli et al., 2009). Intravenous THC administered at doses up to 5mg/kg has also showed anxiogenic effects as measured by anxiety scales (D'Souza et al., 2004). Not all reports have found anxiogenic effects, however. Some studies have shown no significant effects of THC or nabilone (an orally administered synthetic cannabinoid) on anxiety (Glass, Uhlenhuth, Hartel, Schuster, & Fischman, 1980) (Ilan, Gevins, Coleman, ElSohly, & de Wit, 2005).

CBD Currently, CBD has been reported consistently to decrease anxiety in human studies, or at least the anxiety caused by THC. Karniol et al. (1974) showed that orally administered CBD at 15, 30 and 60mg/kg alleviated the anxiety induced by THC in forty healthy volunteers. Similarly, four other studies have reported anxiolytic effects in volunteers following orally administered CBD (Zuardi et al., 1982) (Zuardi, Cosme, Graeff, & Guimarães, 1993) (Crippa et al., 2009) (Fusar-Poli et al., 2009).

Quality of Research It is worthwhile to note that many of the studies of THC and CBD mentioned above were conducted with a small subject number and were done decades ago. Therefore, the effects observed in these clinical studies should be interpreted carefully.

Animal Studies

There are a number of studies looking at the effects of cannabinoids on anxiety. This section will focus CBD's and effects on anxiety-like behaviors in rodents.

THC The studies of THC in animals have produced conflicting results. Three studies have showed anxiolytic effects of THC injected I.P. in rodents. (Braida, Limonta, Malabarba, Zani, & Sala, 2007) (Mayer, Matar, Kaplan, Zohar, & Cohen, 2014) (Schiavon, Bonato, Milani,

Guimarães, & Weffort de Oliveira, 2016). Two other studies, however, have showed anxiogenic effects of THC (Schramm-Sapyta et al., 2007) (Stopponi et al., 2014).

To a certain extent, these differing findings may reflect differences in dosing. THC appears to have an inverted U dose-response curve and may produce different effects at different doses. In addition, the differing findings may reflect the state of stress in the subjects. Fokos and Panagis have shown that the effects of THC on anxiety depend on whether or not the subjects are pre-stressed (Fokos & Panagis, 2010). They have found that THC is anxiolytic in non-stressed mice at 0.5 and 1mg/kg (I.P.), but anxiogenic at 0.5mg/kg in stressed mice. Even in stressed mice, THC is anxiolytic at the higher dose of 1mg/kg (I.P.)

CBD Many studies have demonstrated anxiolytic effects of CBD (Guimarães, Chiaretti, Graeff, & Zuardi, 1990) (Onaivi, Green, & Martin, 1990) (Resstel et al., 2009) (Campos, Ferreira, & Guimarães, 2012) (Schiavon et al., 2016). No studies have shown anxiogenic effects, but one study (Stern et al., 2015) has failed to show anxiolytic effects at doses similar to those used in the studies with positive results.

THC and CBD No previous study has investigated the effects of CBD and THC in combination.

1.3.7 Past Studies of the Effects of CBD and THC on the Elevated Plus Maze (EPM)

A number of the past studies on the effects of CBD and THC on anxiety have used the Elevated Plus Maze (EPM) - the behavioral test used in the present experiments. These are listed in Table 1.1, which includes the vehicle used to dissolve CBD and/or THC, the doses tested, the injection-test intervals, the animal strains and a brief summary of key results.

Table 1.1: A summary of rodent studies investigating the effects of CBD and THC in the EPM. Vehicle used to dissolve CBD and/or THC, the doses tested, the injection-test intervals, the animal strains and a brief summary of key results are provided.

Publication Year	First Author	Drug Intervention	Vehicle	Doses Tested	Injection-Test Interval	Animal Strain	Results
1990	Guimaraes	CBD	10% proplyene glycol - 1% Tween 80 - saline, Sofia 1971	2.5; 5; 10; 20	60mins	Wistar rats	5mg/kg peak effect, 20mg/kg no more
1990	Onaivi	CBs	saline:ethanol:emulphor (1:1:18).	THC 0.3, 1, 3, 5, 10mg/kg for rats and 1, 10, 20mg/kg for mice; CBD 0.01, 0.1, 0.5, 1, 2.5, 5, 10, 50, 100	30mins	Male Sprague Dawley Rats and ICR Mice	THC (anxiogenic at 1, 3, 5, 10 for rats; anxiogenic at 10, 20 for mice) CBD (anxiolytic from 0.5-50 but not 100)
2006	Braida	THC	saline, cremophor, ethanol, (18:1:1)	0.015, 0.075, 0.75 mg/kg	30mins	SD rats	anxiolytic at 0.075, 0.75
2007	Sapyta	THC	saline, emulphor, ethanol, (18:1:1)	0.5, 2.5mg/kg	30mins	CD rats-teen/adults	anxiogenic at 0.5 2.5 for adults, anxiogenic at 2.5 for teens
2010	Fokos	THC	Saline, DMSO, Cremophor (18:1:1)	0.5, 1mg/kg	30mins	SD rats	for non-stressed rats: anxiolytic at 0.5 and 1; for stressed rats: anxiogenic at 0.5 and anxiolytic at 1
2014	Mayer	THC	saline, cremophor, ethanol, (18:1:1)	1, 5, 10mg/kg	1h 6h 24h 7d after injection	SD rats	anxiolytic: In the short term (1–6 h), 5 mg/kg is anxiolytic. In the longer-term (7 days), no effect in attenuating PTSD-like behavioral stress responses, or freezing response to trauma cue.
2016	Schiavon	CBD	1% Tween 80 and 99% of saline	3, 10, 30 mg/kg	60mins	Swiss mice	Single CBD 3 mg/kg administration resulted in anxiolytic-like, Single CBD3 and CBD10 antidepressant-like

1.4 « Depression »

Major Depression Disorder (MDD), commonly referred to as “depression”, is associated with negative mood, anhedonia, and changes in appetite, sleep and energy. It belongs to a group of disorders called the “mood disorders”, which include bipolar disorder (alternating episodes of mania and depression), dysthymia (chronic depressed mood that doesn’t meet the criteria for MDD), seasonal affective disorder (mood affected by seasonal changes), and post-partum depression (mood changes after childbirth).

1.4.1 Criteria for Diagnosis

DSM-5 Criteria According to DSM-5 (Table 1.1), an individual is diagnosed with MDD if they show at least five depressive symptoms during a 2-week period, at least one of which is depressed mood or loss of interest. Depressive symptoms include: 1) depressed mood; 2) diminished interest in pleasurable things (i.e. anhedonia); 3) weight or appetite changes; 4) insomnia or hypersomnia; 5) psychomotor agitation or retardation; 6) fatigue; 7) feelings of self-worthlessness or excessive/inappropriate guilt; 8) diminished ability to think or concentrate; 9) recurrent thoughts of death or suicidal ideation; 10) making a suicide attempt or a specific plan for committing suicide (American Psychiatric Association, 2013).

Table 1.2: Diagnostic criteria of Major Depressive Disorder according to DSM-5. This table was adapted from Otte et al., 2016.

• An individual will show five (or more) of the following symptoms, which should be present during the same 2-week period nearly every day and should represent a change from previous functioning:
○ Depressed mood* ○ Markedly diminished interest or pleasure in all, or almost all, activities* ○ Considerable weight loss when not dieting, weight gain, or decrease or increase in appetite ○ Insomnia or hypersomnia ○ Psychomotor agitation or retardation ○ Fatigue or loss of energy ○ Feelings of worthlessness, or excessive or inappropriate guilt, which might be delusional; that is, not merely self-reproach or guilt about being sick ○ Diminished ability to think or concentrate, or indecisiveness ○ Recurrent thoughts of death (not just fear of dying), recurrent suicidal ideation without a specific plan; the individual has made a suicide attempt or a specific plan for committing suicide
• The symptoms cause clinically significant distress or impairment in social, occupational or other important areas of functioning
• The episode is not attributable to the physiological effects of a substance or to another medical condition
• The occurrence of the episode is not better explained by schizoaffective disorder, schizophrenia, schizophreniform disorder, delusional disorder or other psychotic disorders
• The individual has never had a manic episode or a hypomanic episode
*Depressed mood and/or diminished interest or pleasure must be evident for a diagnosis.

Mild, Moderate and Severe When depression is diagnosed, clinicians typically rate patients' MDD as "mild", "moderate" or "severe" based on the severity of symptoms. "Mild" depression is diagnosed in patients who have five of the symptoms required for diagnosis, plus a few extra, and little functional impairment. "Severe" MDD is diagnosed when patients have most of the symptoms listed in table 1.1 and when functional impairment is markedly obvious. "Moderate" MDD is somewhere between mild and severe (National Institute for Health and Care Excellence, 2018). This rating of severity is useful when clinicians decide on the treatment plan for patients, which will be discussed in section 1.4.4 below.

Treatment-Resistant Depression (TRD) Thirty to sixty percent of MDD patients do not respond to at least two antidepressant, and these patients are considered to have "treatment resistant depression" or "TRD" (Fava & Davidson, 1996).

1.4.2 Epidemiology

Prevalence According to the World Health Organization (WHO), depression is the fourth leading cause of disability worldwide (C. J. Murray & Lopez, 1996).

Because of the constant changes in diagnostic criteria, there are discrepancies in the results of epidemiological studies done on MDD. One of the most reliable and referenced studies was conducted by the WHO World Mental Health (Bromet et al., 2011). DSM-4 criteria of MDD was used and almost 90,000 subjects from 18 countries were surveyed. The average 12-month prevalence of MDD was 5.5% in the ten high-income countries and 5.9% in the eight low- to middle-income countries. The average lifetime prevalence was 14.6% and 11.1%, respectively, in high-income countries and low- to middle-income countries (Bromet et al., 2011). (See Figure 1.1 for incidence of MDD broken down by country).

Demographic Correlations: Gender, Age, Marital Status **Gender** In terms of gender, women are twice as likely to be diagnosed with MDD as men (Bromet et al., 2011). **Age** The correlation between age group and the risk of developing MDD is inconsistent among different countries. It is significantly stronger in high-income countries than low- to middle-income countries. In these countries, the age group of 18-34 has the highest odds of developing MDD (Bromet et al., 2011). **Status** Marital status correlates significantly with risk of MDD if there is separation or divorce. Being separated or divorced is associated with a higher risk of MDD (Bromet et al., 2011).

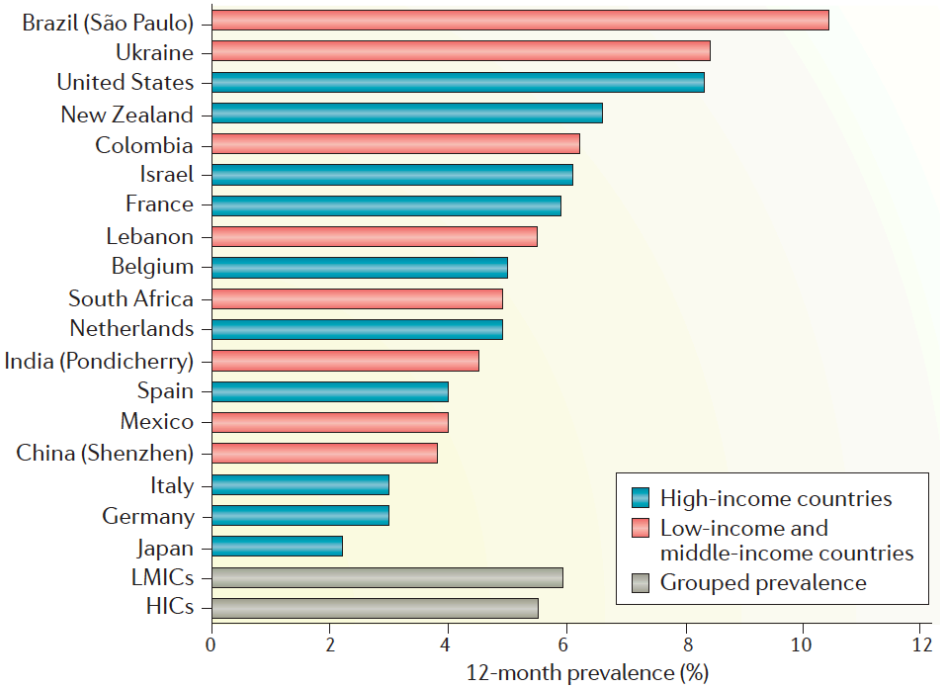


Figure 1.1 Twelve-month prevalence of Major Depressive Disorder in 18 countries. Data were extracted from (Bromet et al., 2011). This figure was taken directly from (Otte et al., 2016)

1.4.3 Pathophysiology of Depression – Risk Factors and Theories

There are many proposed theories related to MDD, including theories related to genetics, to environmental factors, and to neuroendocrinology, inflammation, monoamines, and structural and functional brain alterations (Otte et al., 2016). A few of these will be considered here.

Genetics: A Risk Factor

Genetics has been estimated to contribute to 30-40% of MDD, whereas environmental factors contribute to the other 60-70% (Sullivan, Neale, & Kendler, 2000).

Genetically, depression appears to be very polymorphic in causality, with many different genes in play. Despite extensive research to find genetic loci that can definitely be linked to MDD, current research has not yet yielded consistent or replicable conclusions. For instance, two genome-wide association studies with large sample sizes have reported: 1) 15 significant genetic loci in people of European descent (Hyde et al., 2016), plus 2) nine neuroticism-associated loci in the UK population (Smith et al., 2016). The Hyde el al. study, however, selected subjects based on self-reported depression, and Smith et al. have acknowledged that their significant genes are not replicated in other databases.

A widely discussed study by Caspi et al. (2003) has suggested that a polymorphism in the gene for the promotor region of the serotonin transporter could affect the risk that stressful life events would produce depression. A subsequent meta-analysis (Risch et al., 2009), however, found no link between abnormalities in the serotonin transporter and the risk of depression.

Overall, there is a great inconsistency in the current genetic research. Genetic underlying causes for depression, however, are still being actively sought for.

Environmental Risk Factors

As noted above, environmental factors are thought to contribute to about 60-70% of MDD (Sullivan et al., 2000). Research on environmental factors for depression can be grouped into two categories: 1) research on stressful life events in adulthood, and 2) research on early childhood trauma.

Stressful Events Stressful life events that predispose adults to depression differ with gender. Women are more affected by interpersonal stressful events, such as housing problems, difficult relationships, deaths among friends and relatives, etc. Men are more affected by legal and work-related events, such as job loss and robbery (Kendler et al., 2001).

Childhood Trauma Childhood trauma has clearly been identified as a risk factor for depression (Li, D'Arcy, & Meng, 2016). The top three childhood traumas linked to depression are physical abuse, sexual abuse, and neglect. Childhood trauma may be related to the hypothalamus-pituitary-adrenal axis (HPA axis) theory of MDD, which is explained in the next section.

The HPA Axis Theory

The HPA axis theory links depression to an overactive hypothalamic-pituitary-adrenal (HPA) axis. The hypothalamus secretes corticotropin releasing hormone (CRH), which stimulates the secretion of adrenocorticotropic hormone (ACTH) from the pituitary. ACTH then circulates in the blood stream to reach the cortex of the adrenal gland, where cortisol is released as a result.

A study by Meaney and colleagues has shown that childhood trauma produces a sustained increase in the levels of CRH, which would make the HPA axis overactive (Meaney, 2001). Other studies have shown a correlation between a hyperactive HPA axis and the risk of MDD. One meta-analysis, containing 2406 subjects, showed increased salivary cortisol levels in patients with MDD. (Knorr, Vinberg, Kessing, & Wetterslev, 2010). Similarly, Stetler & Miller (2011) have found that both cortisol and ACTH levels were higher in depressed individuals.

The Neuroinflammation Theory

A second theory links MDD to neural inflammation. Inflammation is mediated by pro-inflammatory cytokines, such as interferon-alpha, interleukin-1, interleukin-6, and tumor necrosis factor-alpha. The symptoms of systemic inflammation – fatigue, anhedonia and psychomotor impairment – overlap with the symptoms of MDD (Hasler, 2010). A meta-analysis has, in fact, showed increased levels of interleukin-6 and c-reactive protein (a biomarker for neuroinflammation) in depressed patients (Haapakoski, Mathieu, Ebmeier, Alenius, & Kivimäki, 2015). Another meta-analysis found that MDD patients had higher concentrations of tumor necrosis factor-alpha and interleukin-6 (Dowlati et al., 2010). Finally, a nation-wide, population-based, prospective cohort study with 3.56 million participants has showed that patients with prior exposure to severe infections and patients with autoimmune disease patients had a 62% and 45% higher risk, respectively, of developing mood disorders at a later time (Benros et al., 2013). Neuroinflammation, therefore, is one of the major foci for current depression research.

The Monoamine Theory

The monoamine theory is one of the classic theories of depression. The monoamines include serotonin, norepinephrine and dopamine. The association between monoamines and

MDD was first made when it was found that some antihypertensive drugs that reduce monoamine levels induced MDD (Schildkraut, 1965). Later, it was found that the therapeutic drugs that treated MDD – the monoamine oxidase inhibitors (MAOI) and the tricyclic antidepressants (TCA) – raised monoamines. These findings led to the monoamine or "biogenic amine" theory – one of the oldest theories of depression (Delgado, 2000).

Efforts have been made to attribute depression to both abnormal catecholamine receptors and to cerebral insufficiency of monoamines as potential mechanisms. Despite continuing research, however, there are problems with the biogenic amine theory. One of the major problems is that monoamine-based antidepressants do not elicit therapeutic effects until after several weeks of treatment, whereas MAOIs and TCAs elevate monoamine levels within hours of administration (Tylee & Walters, 2007).

The Hippocampal Theory

This theory links MDD to a decrease in hippocampal volume. Research has showed that depressed patients have smaller hippocampal volumes than healthy controls. (Kempton et al., 2011). Hippocampal loss is seen in other disorders as well, such as obsessive-compulsive disorder, anxiety, and bipolar disorder, but the loss in hippocampal volume is greater in MDD than in other psychiatric diseases (Goodkind et al., 2015). High levels of cortisol, found in MDD, suppress hippocampal neurogenesis, which may explain the loss of hippocampal volume, and several studies have shown that the administration of antidepressants can increase neurogenesis both in animals and in vitro (Malberg, Eisch, Nestler, & Duman, 2000) (Santarelli et al., 2003).

Conclusions Although many theories of MDD exist - each with some supporting data - no single theory yet explains all of the aspects of MDD (Otte et al., 2016). This is still a field that requires more research.

1.4.4 Treatment

Current treatment options for MDD include psychotherapy, pharmacotherapy, electroconvulsive shock, transcranial magnetic stimulation and deep brain stimulation. Psychotherapy and pharmacotherapy are the front-line treatment options, whereas the rest are considered only when patients have treatment-resistant depression.

There is a general consensus that mild MDDs should be treated with psychotherapy alone first, whereas moderate to severe MDD should be treated with a combination of psychotherapy and pharmacotherapy (Otte et al., 2010).

Psychotherapy There are many different types of psychotherapy: 1) cognitive-behavioral; 2) behavioral-activation; 3) psychodynamic; 4) problem-solving; 5) interpersonal; 6) mindfulness-based (Otte et al., 2016). These have been discussed above in the context of anxiety.

Many theorists believe that psychotherapy is as effective as pharmacotherapy, particularly for mild MDD. One meta-analysis comparing 794 patients receiving cognitive-behavioral therapy and 906 patients on antidepressants found that there was no significant difference between the two in terms of treatment response (Weitz et al., 2015).

Pharmacotherapy The majority of the current antidepressants targets the monoamine neurotransmitter system, and especially serotonin and norepinephrine. The selective serotonin reuptake inhibitors (SSRIs), as their names indicate, block serotonin transporters and increase

serotonin levels in the synaptic cleft. Serotonin-norepinephrine reuptake inhibitors (SNRIs) block both serotonin and norepinephrine transporters (Carlson, 2013b).

The previously mentioned MAOIs and TCAs are still being used in clinics, but the main first-line options are SSRIs and SNRIs. Compared to the MAOIs and TCAs, the SSRIs and SNRIs are much safer, lacking anticholinergic and cardiac side effects (Otte et al., 2016). The older drugs are tried when the SSRIs and SNRIs have failed to be effective (Carlson, 2013b).

Despite their advantages, the SSRIs and SNRIs still have many drawbacks. As noted above they cause acute and long-term side effects, such as nausea, insomnia, headache, dizziness, weight gain and sexual dysfunction (Cassano & Fava, 2004). Moreover, the response rate is still only moderate (50%), with a relatively long delay of therapeutic effects (Cipriani et al., 2009).

Because pharmacotherapy is one of the major treatment options for MDD, and because it is less than ideal, novel agents with different physiological targets are currently being explored vigorously. CBD and THC will be tested in the present study, using animal models of depression-like behavior.

When psychotherapy and pharmacotherapy are ineffective, electroconvulsive therapy, deep brain stimulation and transcranial magnetic stimulation may be tried. These are covered in Appendix 1.

1.4.5 Behavioral Animal Tests of Depression

Preclinical studies, done in animals, are a first step towards human trials for new therapeutics. Common behavioral animal models for depression include the learned helplessness test, the chronic mild stress procedure, the tail-suspension test and the forced swim test.

The Learned Helplessness Test (LHT) The learned helplessness test is based on a serendipitous discovery. When Richard L. Solomon was studying Pavlovian conditioning, he found that uncontrollable and unpredictable shocks produced unexpected and unproductive behavioral changes in animals (Yan, Cao, Das, Zhu, & Gao, 2010). Overmier and Seligman built on this discovery to evolve the LHT (Overmier & Seligman, 1967). The LHT involves exposing animals to repeated inescapable foot shocks and tests for how long it takes for the animal to cease attempts to escape, even when escape is possible. The LHT, like human depression, causes a loss of weight and appetite, as well as decreased aggression and locomotion (Willner, 1984). It is also responsive to antidepressants medications (Sherman, Sacquitne, & Petty, 1982).

The Chronic Mild Stress (CMS) Procedure Chronic mild stress (CMS) is a paradigm in which animals are subjected to unpredictable, though mild, stressful events such as food and water deprivation, changed cage mates, changing temperatures, etc. – typically for three weeks or so. Animals subjected to CMS fail to respond to bright lights or loud noises, which would cause increased locomotion in normal animals (Willner, 1984). This lack of responsiveness somewhat resembles the lack of responsiveness seen in depressive patients. The CMS was first drug-validated by Katz and colleagues (Katz, Roth, & Schmaltz, 1981) and further developed by Paul Willner et al. (Willner, Towell, Sampson, Sophokleous, & Muscat, 1987), who showed that the hypo-responsiveness is reversed by antidepressants (Willner, 1984). Although drug valid, the test is labor intensive and takes a long time to do.

The Tail-Suspension Test (TST) The tail-suspension test (TST) involves suspending rodents by their tails for 6mins and measuring the time spent being immobile or struggling. It was first introduced in 1985 and gained popularity due to its simplicity and ease of scoring (Steru, Chermat, Thierry, & Simon, 1985). It can detect antidepressants with high reliability – as they increase struggling - making it a good predictive test.

The Forced Swim Test (FST) The FST somewhat resembles the TST. It was developed in 1977 by Porsolt (Porsolt, Bertin, & Jalfre, 1977). It involves putting rats or mice into a cylinder of water where escape is impossible. Active escape attempts and immobility are scored. Active escaping involves swimming (actively crossing the surface of the water) and climbing (using forepaws as an attempt to climb up the wall of the cylinder). Immobility occurs when the animal ceases escape attempts and float in an immobile fashion. The antidepressants increase escape attempts.

Originally FST was not able to detect the SSRI, since only active struggling and immobility were measured. Later the test was modified by Detke and colleagues (Detke & Lucki, 1995) to separate swimming and climbing as two different struggling activities. SSRIs promote swimming, whereas TCAs promote climbing.

The FST in rats requires two test days. On day 1, the rats are put into the cylinder for 15mins in order to teach them that it is an inescapable situation. On day 2, the rats are put into the cylinder again for 5mins while the different behaviors are scored (Porsolt et al., 1977).

For mice, only one test of 6mins is required. The first two minutes seem to represent the acclimation (learning) period, and behavioral scoring is done during the last four minutes (Slattery & Cryan, 2012)

Water temperature, animal weight, and water height can all significantly affect the results of the test (Slattery & Cryan, 2012). Therefore, experimental procedures have to be kept consistent for cross-comparisons of data.

For the purpose of the present experiments, the FST was chosen because it is predictive, practical and inexpensive.

1.4.6 Past Studies of the Cannabinoids on Depression

The antidepressant effects of the cannabinoids have been studied both in humans and in animal models.

Human Studies

So far, there have been no randomized control trials looking at the effects of CBD and/or THC on depression. Most human research has consisted of correlational studies, correlating cannabis use with depression. One systematic review found a correlation between heavy cannabis users and depression. Causal relationships could not be established, however (Degenhardt, Hall, & Lynskey, 2003).

In addition, there have been studies trying to find abnormalities in the endocannabinoid systems in depressed people One study of alcoholic suicide victims found that the endocannabinoid system in these subjects was hyperactive, suggesting the elevated endocannabinoid system as a potential target for future drug development (Vinod et al., 2005). In contrast, another study found decreased serum levels of anandamide and 2-arachidonoylglycerol, two endogenous cannabinoids, in patients with major depressive disorder (M. N. Hill, Miller, Ho, Gorzalka, & Hillard, 2008). Further research is indicated.

Animal Studies

There have been a number of studies looking at the effects of cannabinoids on depression-like behavior in animals. The present discussion will focus on studies examining CBD's and THC's effects on depression in rodent models.

THC THC has showed varying effects on depression-like behavior. Some studies have reported THC as depression-inducing. One study, for instance, found that female rats receiving THC chronically showed increased immobility time in the FST and anhedonia-like behaviors, whereas male rats only showed anhedonia-like behaviors (Rubino et al., 2008). Another study found significantly increased immobility time in male mice in the FST when THC was injected I.P. at 2 and 6 mg/kg (Egashira et al., 2008). Zamberletti et al. showed that THC significantly increase immobility time in the FST, but only in male animals that had experienced early-life stressors (Zamberletti et al., 2012).

In contrast, other studies have showed anti-depressant effects of THC. Bambico et al., for instance, found that repeated I.P. injections of THC significantly reduced immobility time in the rats in the FST (Bambico, Hattan, Garant, & Gobbi, 2012). Haring et al. also found that 0.5 and 1mg/kg of THC injected I.P. in mice could reduce immobility time in the FST (Häring, Grieb, Monory, Lutz, & Moreira, 2013).

CBD Four behavioral studies looking at the effects of CBD on depression in rodents have been published. All of them seem to agree that CBD has anti-depressive effects. Zanaletti et al. and Reus et al. both found anti-depressant effects of CBD at 30mg/kg injected I.P. Tests were done at 60min and 30min after injection (Zanelati, Biojone, Moreira, Guimarães, & Joca, 2010), (Réus et al., 2011). El-Alfy et al. found similar effects at 200mg/kg (El-Alfy et al., 2010).

Sartim et al. found anti-depressive effects of CBD when it was injected directly into the brain (Sartim, Guimarães, & Joca, 2016).

CBD+THC Previous to the present study, combinations of CBD and THC had not been studied in the context of depression.

1.4.7 Past Studies of the Effects of CBD and THC on Depression in The Forced Swim Test

Several of the past animal studies on the effects of CBD and THC have used the Forced Swim Test (FST) - the behavioral test used in the present experiments. These are listed in Table 1.3, which includes the vehicle used to dissolve CBD and/or THC, the doses tested, the injection-test intervals, the animal strains and a brief summary of key results.

Table 1.3: A summary of rodent studies investigating the effects of CBD and THC in the FST. Vehicle used to dissolve CBD and/or THC, the doses tested, the injection-test intervals, the animal strains and a brief summary of key results are provided.

Publication Year	First Author	Drug Intervention	Vehicle	Doses Tested	Injection-Test Interval	Animal Strains	Results
2016	Schiavon	CBD	1% Tween 80 and 99% of saline	3, 10, 30 mg/kg	60mins	Swiss mice	Single CBD 3 mg/kg administration resulted in anxiolytic-like, Single CBD3 and CBD10 antidepressant-like
2008	Egashira	THC	1% Tween 80 and 99% of saline	1, 2, 6mg/kg	60mins	ddY mice	2 and 6 mg/kg are pro-depression
2010	Zanelati	CBD	2% Tween 80 and 98% of saline	3, 10, 30, 100	30mins	Swiss mice	antidepressant effective at 30mg/kg only
2010	El-Alfy	CBD&THC	saline, cremophor, ethanol, (18:1:1)	THC: 1.25, 2.5, and 5 mg/kg CBD 20, 100, 200mg/kg	30mins	Swiss mice	significant antidepressant at 2.5 mg/kg in the FST, CBD anti-depressant at 200mg/kg only
2011	Reus	CBD	2% Tween 80 and 98% of saline	15, 30, 60 mg/kg	60mins	Wistar rats	30mg/k reduced immobility time
2012	Bambico	THC	saline, Tween 80, polyethylene glycol, (18:1:1)	1mg/kg	45mins	SD rats	Repeated (5 days), but not single (acute) intraperitoneal treatment with delta-9-THC (1 mg/kg) exerts antidepressant-like properties in the rat forced swim test (FST).
2012	Haring	THC	0.9 w/v % NaCl solution containing 0.5 vol % ethanol and 2.5 vol % Tween-80.	0.1 or 0.5 mg/kg	30mins	C57 mice	0.1 and 0.5 mg/kg are anti-depression

1.5 « Objectives and Hypotheses »

Objectives: The present study was designed to test the effects of CBD, THC and a combination of CBD and THC at 15:1 ratio in animal models of anxiety and depression, the EPM and the FST. A dose-response paradigm was used.

Hypothesis: Based on previous studies, we hypothesized that CBD would be anxiolytic and anti-depressive. We further hypothesized that THC would be both anxiogenic and anxiolytic depending on the dose. Based on other studies in our laboratory (Fallah, personal communication), we hypothesized that the combination of CBD and THC at a 15:1 ratio might left-shift the dose-response curve for CBD, making it effective at a lower dose.

Chapter 2

2 « General Methods »

The protocol for this study was approved by the Animal Care Committee of the Faculty of Medicine at University of Toronto. The experimental procedures adhered to the guidelines of the Canadian Council on Animal Care.

2.1 Subjects

Adult, male CD1 mice were ordered from Charles River (Quebec, Canada) and were housed in groups of 2-4 in 20x30x16cm, transparent plastic cages with corn-cob bedding. Red polycarbonate safe harbors, chewing toys and crinkle papers were provided as enrichments. Mice were provided with food (2918 Teklad Global 18% Protein Rodent Diet, Envigo) and water ad libitum. The temperature of the vivarium was maintained at 21 degrees Celsius and it had a 12-h light/dark cycle (lights on at 7am).

2.2 Drugs

Diazepam was obtained in injectable form from the Division of Comparative Medicine. Imipramine was obtained from Toronto Research Chemicals in powder form and dissolved in physiological saline. CBD and THC were donated by MedReleaf (Markham, Ontario). Full information about dosage, vehicle and administration appears in Chapters 3 and 4.

2.3 Elevated Plus Maze

The maze and the maze procedures are described in Chapter 3.

2.4 Forced Swim Test

The apparatus and forced swim procedures are described in Chapter 4.

2.5 Open Field Test

The open field and open field procedures are described in Chapter 4.

2.6 Drug Testing Paradigm

The EPM, FST and OF were performed following I.P. injections of increasing doses of CBD, THC, or a combination of CBD and THC at 15:1 ratio. Details of procedures are described in Chapter 3 and Chapter 4.

2.7 Statistical Analysis

One-way analyses of variances (ANOVAs) were conducted when the data included more than two groups. These were followed by post-hoc Dunnett's multiple comparison tests when appropriate. Un-paired t-tests were conducted on data involving two groups.

Chapter 3

3 « The effects of CBD and THC in mice in the elevated plus maze: Experiment 1 »

3.1 Rationale

As discussed in the General Introduction, past studies in animals have suggested that CBD is “anxiolytic” in the Elevated Plus Maze (EPM), and that THC is both “anxiolytic” and “anxiogenic”. There has been little agreement, however, as to what doses are effective, and there has, as yet, been no test of CBD and THC in combination.

Experiment 1 consisted of dose-response studies of CBD alone, THC alone, and CBD and THC in a 15:1 combination in mice in the EPM.

Initially, CBD was injected 60mins before the EPM and THC was injected 30mins before the EPM, based on past literature (Schiavon et al., 2016). These studies are described below as “Effects of Cannabidiol (CBD) at 60 minutes” and “Effects of Tetrahydrocannabinol (THC) at 30 minutes”.

Subsequently, however, since CBD had had no effect in the EPM at 60mins, we began a re-test of CBD with a 30-mins injection-test interval. A previous study had found effects at this interval (Onaivi et al., 1990). These data are described below as “Effects of Cannabidiol (CBD) at 30 minutes”. Due to the licensing problems, however, only two doses could be tested at 30 minutes.

Different vehicle mixtures were also tested (see Appendix 2 Figure 3.6 below).

3.2 Subjects

A total of 341 male CD1 mice were used in the EPM study. All mice were ordered from Charles River (Quebec, Canada) at a weight of 25-30g. They were given one-week (minimum) of acclimation to the animal housing facility at University of Toronto Medical Science Building before the start of EPM testing, during which time they had free access to food and water. (Mice generally weighed of 30-35g at the time of EPM testing.) Each mouse was used only once, being injected with one dose of drug or vehicle, tested, and then euthanized in a CO_2 chamber followed by cervical dislocation.

3.3 The Elevated Plus Maze Test

The maze consisted of two “closed” and two “open” arms crossed at a ninety-degree angle and elevated 38.5cm above the table top that supported the maze. The length of each arm was 30cm, and the width was 5cm. The walls of the “closed” arms were 15cm high. The apparatus was situated in dim light, where the open arms were illuminated to 12-14 lux and closed arms were at 5-6 lux.

Mice were put into the center of the maze at appropriate times following drug injections and roaming activity was tracked for 5mins by the Viewer Software (Bio-Observe Inc.). The background of the maze was white. Therefore, to create a color contrast measurable by the software, the white CD1 mice were painted black from head to base of the tail with a non-toxic Sharpie marker just before the test. Pre-empt was used to clean the apparatus after each trial.

3.4 Drugs

Diazepam (DZP, Positive Control) Diazepam was obtained from the Division of Comparative Medicine pre-dissolved in a liquid injectable form (Sandoz Canada Inc.). The DZP premixed stock solution (5mg/ml) was injected intraperitoneally (I.P.) directly without dilution. Only one dose was tested, 2 mg/kg, an effective dose based on past literature (below). The vehicle was made up of propylene glycol 40%, anhydrous ethyl alcohol 10%, benzoic acid 4.25%, benzyl alcohol 1.5%, and 44.25% water with sodium hydroxide to adjust pH. The volume of the vehicle injection (0 dose) was matched to the volume of the DZP injections.

Cannabidiol (CBD) and Tetrahydrocannabinol (THC) CBD and THC stock solutions were provided by MedReleaf at a pre-mixed concentration of 400mg/ml for CBD and of 100mg/ml for THC. Stock solutions of both CBD and THC were dissolved in 95% ethanol:Cremophor EL at 1:1 ratio. On the morning of each experiment, sterile physiological saline was added to the stock solutions to make solutions with a concentration of 2mg/ml for CBD solution or 0.32mg/ml for THC. These were then injected I.P. in different volumes to provide the doses employed. The volume of the 0 dose injection (vehicle control) was matched to the volume of the largest drug dose.

Different Vehicles The procedure outlined above resulted in vehicles of 1:1:398 (95% ethanol:Cremophor EL:sterile saline) for CBD and 1:1:623 (95% ethanol:Cremophor EL:sterile saline) for THC. Because previous studies had usually used 95% ethanol:cremophor:sterile saline at a 1:1:18 ratio as the vehicle for CBD and THC, we later added a trial with a 1:1:18 combination to compare the effects of vehicles of different ratios (Appendix 2 Figure 3.6).

3.5 Doses and Injection-Test Intervals

Drug Dose Subjects were injected with: 1) CBD (0-96mg/kg), 2) THC (0-6.4mg/kg), 3) CBD and THC at 15:1 ratio (0+0 to 96+6.4mg/kg), 4) DZP (0, 2mg/kg) or 5) vehicle: 95% ethanol, Cremophor EL and sterile saline.

The doses selected for CBD and THC are based on previous studies (Onaivi, Green, & Martin, 1990) (Schiavon et al., 2016). The selection of 2mg/kg for DZP was based on (Guimarães et al., 1990) and (Sharon Pellow & File, 1986).

Injection-Test Intervals Initially, CBD, at varying doses, was injected I.P. 60mins before the EPM test, whereas were THC and DZP were injected I.P. 30mins before the EPM test. These injection-test intervals were chosen based on previous studies conducted by Guimarães et al. (1990) and Schiavon et al. (2016). In two subsequent experiments (Figure 3.5 and Figure 3.6 below), CBD was injected at 30mins before the EPM test. This injection-test interval was chosen based on the study published by (Onaivi et al., 1990)

Data Analysis The percentage of time spent in the open arms was calculated as: time spent in open arms/(time spent in closed arms + time spent in open arms) x100%. Total closed arm entries were calculated as the total number of entries into the two closed arms. An entry is defined as a full body – head to tail – entry into the arms. Data are presented as mean ±SEM.

3.6 Results

Effects of Diazepam (DZP) Figure 3.1 shows the effect of DZP injected at 2mg/kg I.P. 30mins before testing (positive control). As indicated, DZP produced a clear increase in the percent time the subjects spent in the open arms, as compared to vehicle. An unpaired t-test

showed that the increase was highly significant (p=0.0004). Thus, diazepam at 2mg/kg produced a significant anxiolytic effect in the EPM.

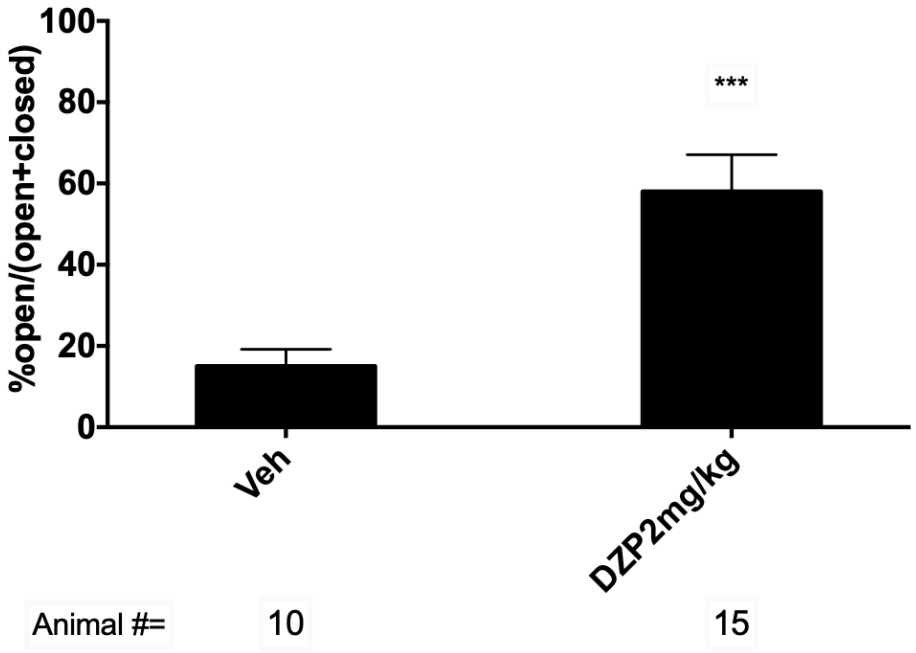


Figure 3.1 The effects of diazepam (DZP) 2mg/kg on percent time spent in open arms when DZP was injected I.P. 30mins before the test. Animal numbers for each dose are shown below the x-axis. *** $p<0.0002$ compared to vehicle control. Data are presented as mean ±SEM.

Effects of Cannabidiol (CBD) at 60 minutes Figure 3.2 shows the effects of increasing doses of CBD on percent time spent in the open arms of the EPM. CBD was injected I.P. 60mins before the mice were put into the EPM. As indicated, there were no obvious increases in open arm time at any of the CBD doses. The application of a one-way analysis of variance (ANOVA) showed no significant differences among this group of means (F=0.7371, p=0.6215). Thus, CBD at 60mins did not produce any effect in the EPM.

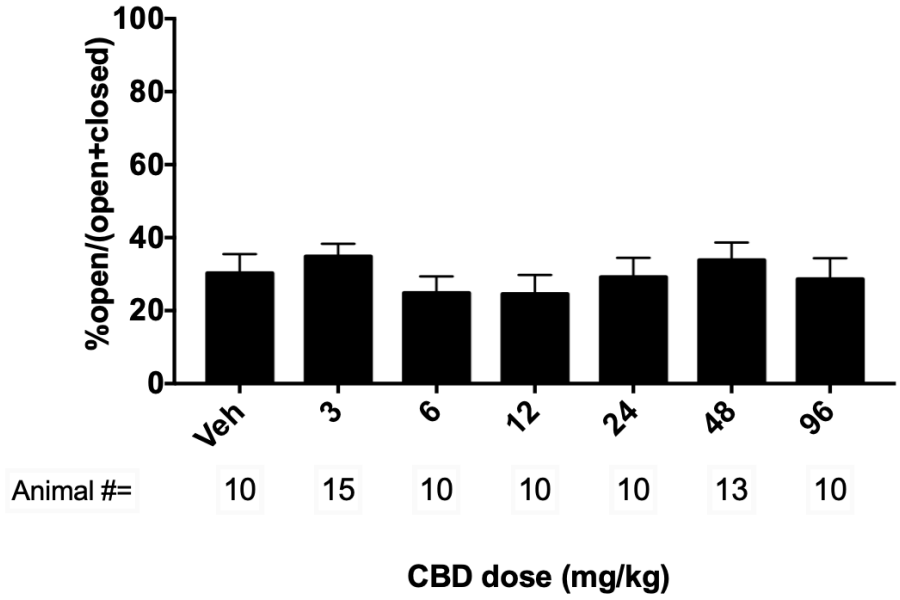


Figure 3.2 The effects of increasing doses of CBD on percent time spent in open arms when CBD was injected I.P. 60mins before the test. Animal numbers for each dose are shown below the x-axis. Data are presented as mean ±SEM.

Effects of Cannabidiol (CBD) at 30 minutes As noted above, since CBD had no effects at 60mins, it was re-tested at 30mins. Figure 3.3 shows the effects of two doses of CBD (12mg/kg and 48mg/kg) on percent time spent in the open arms of the EPM 30mins after injection. These two doses tested at this injection-test interval had been reported to have significant effects in a previous publication (Onaivi et al., 1990).

As indicated by Figure 3.5, both doses of CBD slightly increased the time spent in the open arms. The application of a one-way ANOVA, however, showed no significant differences among this group of means ($F=0.8192$, $p=0.4514$). Thus, CBD showed no significant anxiolytic effects at 30mins in the EPM. Future experiments with higher doses will be done.

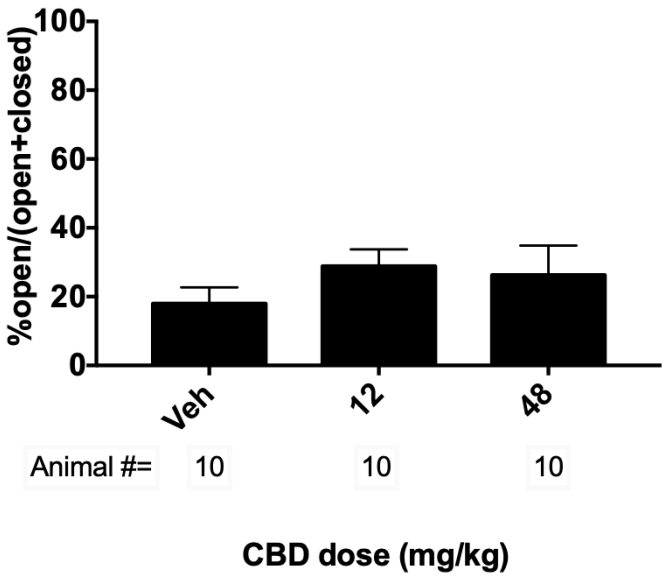


Figure 3.3 The effects of increasing doses of CBD on percent time spent in open arms when CBD was injected I.P. 30mins before the test. Animal numbers for each dose are shown below the x-axis. Data are presented as mean ±SEM

Effects of Tetrahydrocannabidiol (THC) at 30 minutes Figure 3.4 shows the effects of increasing doses of THC on percent time spent in the open arms. THC was injected 30mins before the mice were put into the EPM. An increase in the percent time spent in open arms was observed at the two highest doses: 3.2 and 6.4 mg/kg. The application of a one-way ANOVA indicated that there were significant differences among this group of means ($F = 6.313$, $p<0.0001$), and post-hoc Dunnett's multiple comparison tests showed that the open arm time in the two high-dose groups was significantly different from the open arm time in the vehicle group ($p=0.0213$ for 3.2mg/kg, $p=0.0004$ for 6.4mg/kg). Thus, THC produced anxiolytic effects at higher doses in the EPM.

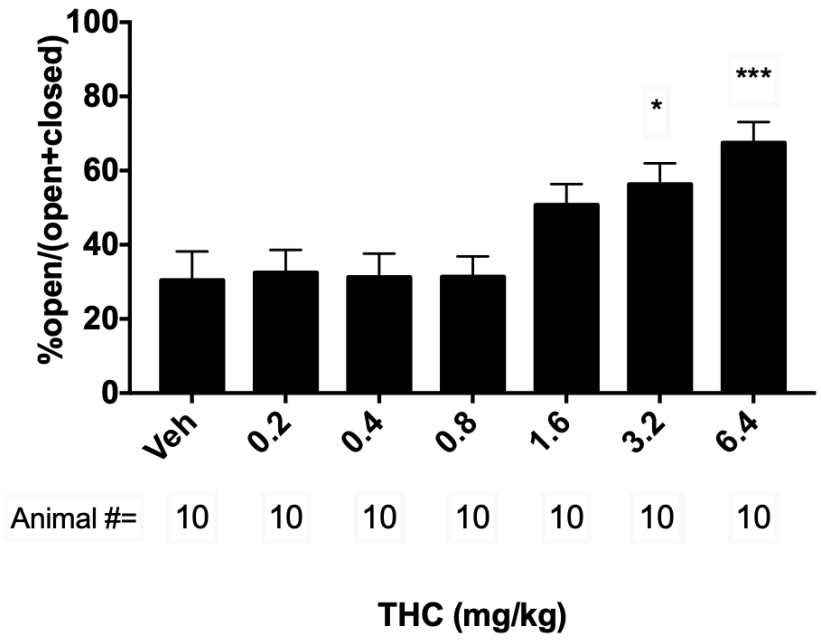


Figure 3.4 The effects of increasing doses of THC on percent time spent in open arms when THC was injected I.P. 30mins before the test. Animal numbers for each dose are shown below the x-axis. * $p<0.0332$, *** $p<0.0002$ compared to vehicle control. Data are presented as mean ±SEM.

Effects of Combined CBD and THC (15:1) Figure 3.5 shows the effects of increasing doses of CBD and THC in combination (15:1 ratio) on percent time spent in the open arms. CBD and THC were injected 60mins and 30mins, respectively, before mice were put into the EPM. An increase in the percent time spent in open arms was observed at the two highest CBD/THC doses: 48mg/kg CBD plus 3.2 mg/kg THC, and 96mg/kg CBD plus 6.4 mg/kg THC. A one-way ANOVA indicated that there were significant differences among this group of means ($F = 8.362$, $p<0.0001$), and post-hoc Dunnet's multiple comparison tests showed that the open arm time in the two high-dose groups was significantly different from the open arm time in the vehicle group ($p=0.0282$ for 48mg/kg CBD plus 3.2 mg/kg THC; $p<0.0001$ for 96mg/kg CBD plus 6.4 mg/kg THC). Thus, the combination of CBD and THC at a 15:1 ratio was anxiolytic at higher doses.

The effects, however, were not much different from THC alone.

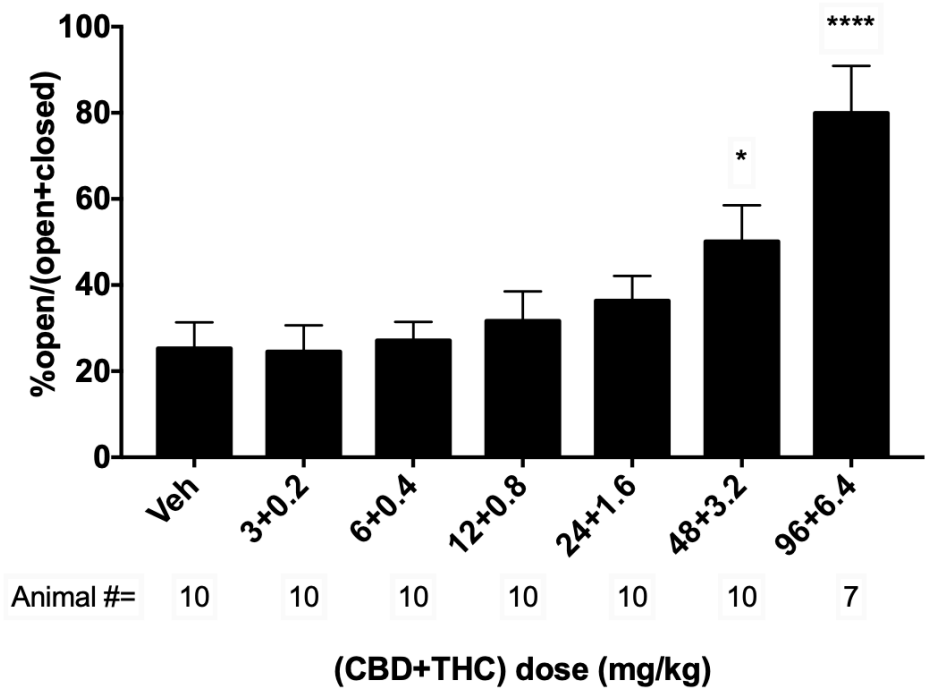


Figure 3.5 The effects of increasing doses of CBD and THC combination at 15:1 ratio on percent time spent in open arms when CBD and THC were injected I.P. 60mins and 30mins before testing, respectively. Animal numbers for each dose are shown below the x-axis. * $p<0.0332$, **** $p<0.0001$ compared to vehicle control. Data are presented as mean ±SEM

Toxicity/Level of Activity Toxicity – as in ataxia - was not measured in Experiment 1. It was not expected and was not seen at these doses.

Cannabinoids, however, are known to affect locomotion and activity levels. In the EPM, the number of closed arm entries gives an index of locomotor activity. The number of closed arm entries for CBD and THC are illustrated in Appendix 5. No change of locomotor activity was observed at doses that showed significant percent open arm changes in the EPM.

Locomotion in the OF test was measured in Experiment 2 (Chapter 4) and will be discussed there.

Effects of Different Ratios of Vehicle As noted above, we used a vehicle of 95% ethanol:cremophor:sterile saline at 1:1:398 for CBD, and 95% ethanol:cremophor:sterile saline at 1:1:623 for THC consistently. Because previous studies had used vehicles at a 1:1:18 ratio, we later added trials of CBD and THC with a 1:1:18 vehicle. Both CBD and THC were injected 30mins before the EPM. The data are presented in Appendix 2 (Figure 3.6). There was no significant difference related to vehicle.

3.7 Discussion

Effects of the Positive Control DZP

DZP 2mg/kg injected I.P. significantly increased the percent time spent in open arms. There was no observed effect on closed arm entries. Thus, we observed a clear anxiolytic effect of DZP at 2mg/kg. These data are in agreement with the effects of 2mg/kg DZP reported in the previous paper by Pellow et al. (Sharon Pellow & File, 1986), and later confirmed in multiple animal studies (e.g., Guimarães et al., 1990). These “positive control” data confirm that the EPM test – in our hands – can detect anxiolytic drugs.

Effects of CBD

In our study, we found that CBD on its own did not affect open arm times whether it was injected 60mins or 30mins before the EPM.

Our data do not agree with some of the previous publications in this field. Schiavon et al., for instance, previously tested CBD at 3, 10, and 30mg/kg at 60mins in the EPM. They reported

significant anxiolytic effects, but only at the dose of 3mg/kg (Schiavon et al., 2016). Their observed effect, however, was small, and was only significant in terms of percent open arm entries, not in percent time spent in the open arms. Actually, Schiavon's data do not differ greatly from ours. We also saw a slight trend toward increased open arm time at 3 mg/kg, but it did not reach significance.

Our data also contrast with those of Onaivi et al. (1990). Onaivi et al. tested CBD from 0.01-100mg/kg at 30mins in the EPM. They found significant anxiolytic effects from 0.5-50mg/kg. We found non-significant trends at two of their doses, but no significant effects. As noted above, a number of factors can influence the results obtained in the EPM. Future experiments might explore the differences between the two studies. In our experiments, we have not seen anxiolytic effects of CBD, but they might appear at other levels of illumination, or in differently stressed subjects.

Effects of THC

We found that THC, injected 30mins before the test, had anxiolytic effects in the EPM at the two highest doses: 3.2mg/kg and 6.4 mg/kg.

The present study seems to be the first that has tested THC effects in the EPM in mice. The four previous rat studies, however - listed in Table 1.1 in Chapter 1 Introduction - showed that THC is anxiolytic (although see Schramm-Sapyta el al. (2007). Thus, our data are in general agreement with the majority of the previous rodent literature.

Effects of the Combination

The present study is the first study to look at CBD and THC in combination in the EPM. We found that the effects of the combination of CBD and THC at a 15:1 ratio were very similar to the effects of THC injected alone. (Figure 3.7 (below) presents the curve for THC alone and the curve for the combination, plotted in the same graph.) We speculate that the anxiolytic effects seen are simply produced by the THC, and that CBD neither enhances nor diminishes them.

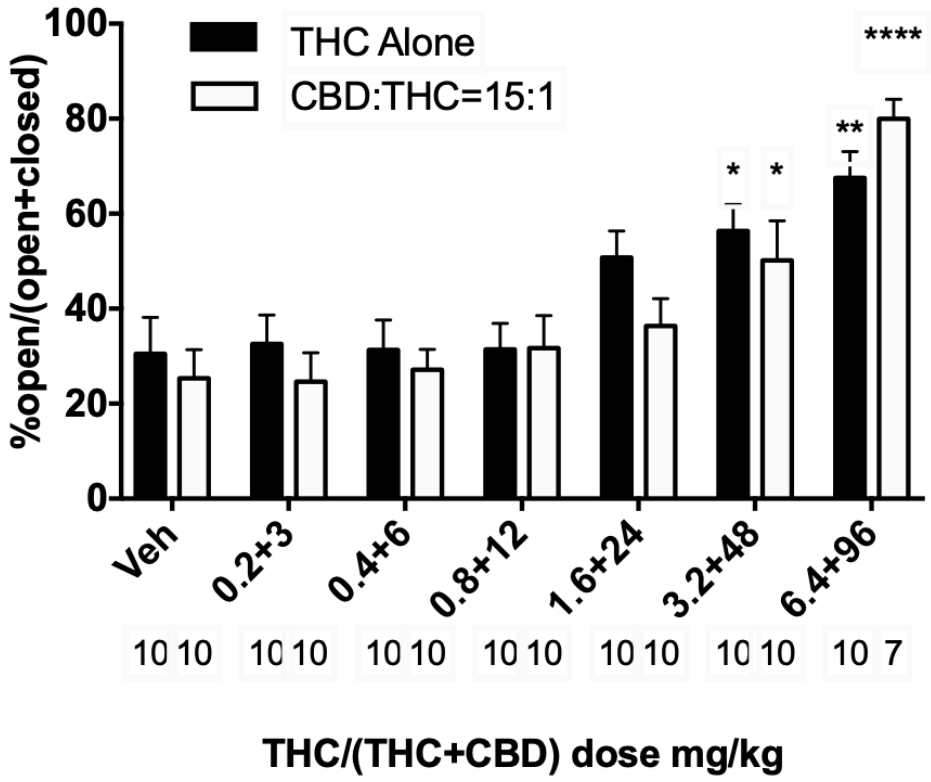


Figure 3.7 The effects of increasing doses of CBD and THC combination at 15:1 ratio and THC alone on percent time spent in open arms when CBD and THC were injected I.P. 60mins and 30mins before testing, respectively. Animal numbers for each dose are shown below the x-axis. * $p<0.0332$, **** $p<0.0001$ compared to vehicle control. Data are presented as mean ±SEM

Effects of Injection-Test Interval

The effects of CBD did not differ between injection-test intervals 60mins and 30mins in our study (Figures 3.2 and 3.3). This differs from our findings in the forced swim test, which will be presented in Chapter 4.

Effects of THC on Locomotion

We found that one low dose of THC (0.8 mg/kg) increased closed arm entries in the EPM (Appendix 5), suggesting an increase in locomotion. Increased locomotion has been previously reported with low doses of THC (Davis, Moreton, King, & Pace, 1972).

An increase in locomotion in the EPM might non-specifically increase open arm time and appear to be anxiolytic. It should be noted, however, that the dose of THC that increased locomotion was not a dose at which increased open arm time was seen.

Conclusions

We did not observe any effects of CBD on anxiety. THC, however, appears to be anxiolytic at doses that are not toxic, at least as judged by closed arm entries (3.2mg/kg and 6.4mg/kg). The combination of CBD and THC at a 15:1 ratio seems to be anxiolytic as well, probably due to the effects of the THC.

The 15:1 combination is currently being tested as an anticonvulsant in patients with uncontrolled seizures. It appears to be working (Burnham, personal communication). Clinically, the 15:1 combination might be particularly useful for epilepsy patients who are also suffering from anxiety.

Chapter 4

4 « The effects of CBD and THC in mice in the forced swim test and the open field test: Experiment 2 »

4.1 Rationale

As discussed in the General Introduction, past studies in animals have suggested that CBD is “antidepressant” in the forced swim test (FST), and that THC is both “antidepressant” and “pro-depressant” (Table 1.3 in the introduction). There has been little agreement, however, as to what doses are effective, and there has, as yet, been no test of CBD and THC in combination.

Experiment 2 consisted of dose-response studies of CBD alone, THC alone, and CBD and THC in a 15:1 combination in mice in the forced swim test (FST, a depression model) and the open field test (OF, an activity model), which is usually done in combination with the FST.

Initially, CBD was injected 60mins before the FST and THC was injected 30mins before the FST, based on past literature (Schiavon et al., 2016). These are described below as “Effects of Cannabidiol (CBD) at 60 minutes” and “Effects of Tetrahydrocannabinol (THC) at 30 minutes”.

Since CBD had very little effect in the FST at 60mins, we began a re-test of CBD with a 30-mins-injection-test interval. These data are described below as “Effects of Cannabidiol (CBD) at 30 minutes”. Positive results were obtained, but – due to the licensing problems – only two doses could be tested.

Similar to Experiment 1, different vehicle mixtures were also tested (see Appendices 3 and 4).

4.2 Subjects

A total of 184 male CD1 mice were used in the FST study, and 198 mice were used in the OF. The animal housing conditions, food and water supplies, body weight and method of euthanization are described in Chapter 2.

4.3 Drugs and Drug Mixing

Imipramine (IMP, positive control) IMP was obtained from Toronto Research Chemicals in powder form and dissolved in physiological saline to achieve a concentration of 2mg/ml.

Cannabidiol (CBD) and Tetrahydrocannabinol (THC) The preparations of CBD and THC are similar to those described in Chapter 3.

On the morning of each experiment, sterile physiological saline was added to the stock solutions to make solutions of 10mg/ml for CBD solution and of 0.32mg/ml for THC. These constant concentrations were then injected I.P. at different volumes to provide the various doses employed in the experiments. The volume of the 0 dose injection (vehicle control) was matched to the volume of the largest drug dose.

Different Vehicles The procedure, as outlined above resulted in vehicles of 1:1:78 (95% ethanol:Cremophor EL:sterile saline) for CBD and of 1:1:623 (95% ethanol:Cremophor EL:sterile saline) for THC. Because previous studies had used vehicles at a 1:1:18 ratio of 95%

ethanol:cremophor:sterile saline, we later added trials of CBD and THC with a 1:1:18 vehicle (Appendices 3&4 Figures 4.11 and 4.12).

4.4 Doses and Procedures for Dosing

Drug Dose Subjects were injected with: 1) CBD (0-240mg/kg), 2) THC (0-8mg/kg), 3) CBD and THC at 15:1 ratio (0+0 to 60+4mg/kg), 4) IMP (0, 30mg/kg) or 5) vehicle (95% ethanol, Cremophor EL and sterile saline). These doses were based on doses previously reported in the literature (Table 1.3 in the Introduction).

Injection-Test Intervals Initially, CBD was injected I.P. 60mins before the FST or OF tests, whereas THC and IMP were injected I.P. 30mins before the FST or OF tests. These injection-test intervals were based on injection-test intervals previously reported in the literature (Table 1.3 in the Introduction).

In four subsequent experiments – "Re-test of CBD at 30 Minutes" (Figures 4.7-4.10 below) - CBD was injected alone or in combination, 30mins before the FST or OF tests. The 30mins injection-test interval was tried because effects had not been seen at 60mins. The 30mins injection-test interval had been used in previous positive experiments by Zanelati et al. (Zanelati et al., 2010). Only two doses could be tested due to licensing problems. Further doses will be tested in future experiments.

Data Analysis For the FST data, the percentage of time mice spent being immobile, swimming or climbing was calculated as: "time spent being immobile", "swimming" or "climbing" / 4 mins x100%. For the OF data, the total distance traveled in the 10 mins trial in centimeters are presented. All data are presented as mean±SEM.

4.5 Procedure for the Forced Swim Test (FST)

Procedure One the day of the FST, following their I.P. injections, each mouse was placed in a transparent cylinder (25 cm high x 15 cm wide) filled with tap water to a level of 15 cm and at a temperature of 25-26 ° C for 6mins. Behavior was scored during the last 4mins of the trial. Mice were then removed from the water, dried with paper towels and placed in a new cage under a heat lamp until they were completely dry. The water was changed, and cylinder cleaned with Pre-empt between trials.

Data Analysis During the test, mice were recorded using a camera mounted on the side of the cylinder. The total duration of immobility (minimal movements to keep afloat), swimming (actively moving forepaws and hindpaws across the surface of the water) and climbing (actively scratching forepaws against the wall of the cylinder in and out of the water) were measured and scored during the last 4mins using software developed on Labview platform by Dr. Brian Scott.

4.6 Procedure for the Open Field Test (OF)

Procedure For the open field test (OF), following their I.P. injections, mice were placed in a square open field arena (40 cm in diameter with a 50 cm high plexiglass wall) where their exploratory activity was tracked for 10mins. Pre-empt was used to clean the apparatus after each trial.

Data Analysis The behavior was analyzed using Fusion v4 Software (Omnitech Electronics, Inc.). This software detects the position of the animal in the open field arena and calculates the total distance moved in centimeters.

4.7 Results

Effects of the Positive Control Imipramine: FST Figure 4.1 shows the effects of IMP (30mg/kg) and its vehicle control injected I.P. 30mins before testing in the FST. IMP decreased immobility time and increased swimming and climbing time. These changes were significant, as compared to the vehicle control (sterile physiological saline) (un-paired t-test, p=0.0024 for immobility, p=0.0243 for swimming, p=0.0011 for climbing). Thus, IMP caused a significant decrease in "depression-like behavior" in the FST. This shows that, in our hands, the FST was able to detect the effects of a known antidepressant.

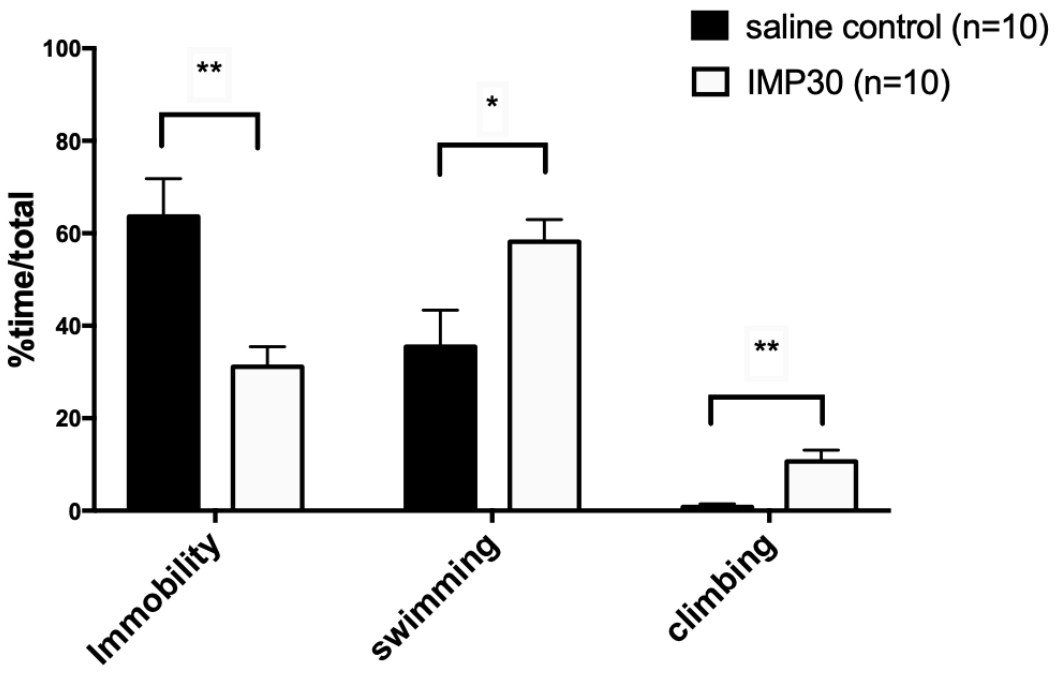


Figure 4.1 Effects of IMP 30mg/kg on immobility, swimming and climbing in the FST compared to vehicle control (sterile physiological saline). IMP and saline were injected I.P. 30mins before the FST. Each group contained 10 animals. Data are presented as mean±SEM.

Effects of the Positive Control Imipramine: OF Test Figure 4.2 shows the effects of I.P. IMP, and its vehicle control (sterile physiological saline) injected at 30mg/kg on locomotion in the OF test. Data were recorded from 30mins to 40mins post-injection. As indicated, there was little difference in locomotion between the IMP group and the vehicle control group on total distance traveled. An un-paired t-test showed no significant difference between the saline control group and the IMP30 group ($p=0.7458$). Thus, IMP had no significant effects on activity levels in the OF Test. This shows that the effects of IMP observed in the were not caused by non-specific effects on locomotion.

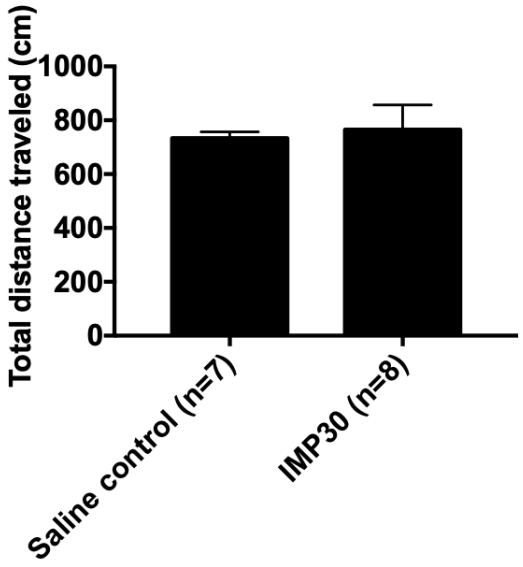


Figure 4.2 Effects of IMP 30mg/kg and sterile physiological saline on locomotion in the 10mins OF test. IMP and saline were injected I.P. 30mins before the OF test. IMP group contained 8 animals and the saline group contained 7 animals. Data are presented as mean±SEM.

Effects of Cannabidiol (CBD) at 60 minutes: FST Figure 4.3 shows the effects of increasing doses of CBD, from 15mg/kg to 240mg/kg, in the FST test. CBD was injected I.P. 60mins before the FST. As indicated, there is a "trend" towards an inverted U-shaped dose-

response curve for swimming, and a "trend" for U-shaped dose-response curve for immobility. Animals displayed little climbing activity at any CBD dose. One-way ANOVAs (one for each behavioral effect), however, showed no significance for percent time spent swimming ($F=0.9597$, $p=0.4533$), climbing ($F=0.3998$, $p=0.8462$) or immobility ($F=1.136$, $p=0.3564$). Thus, CBD did not have any significant effects on the FST at 60mins.

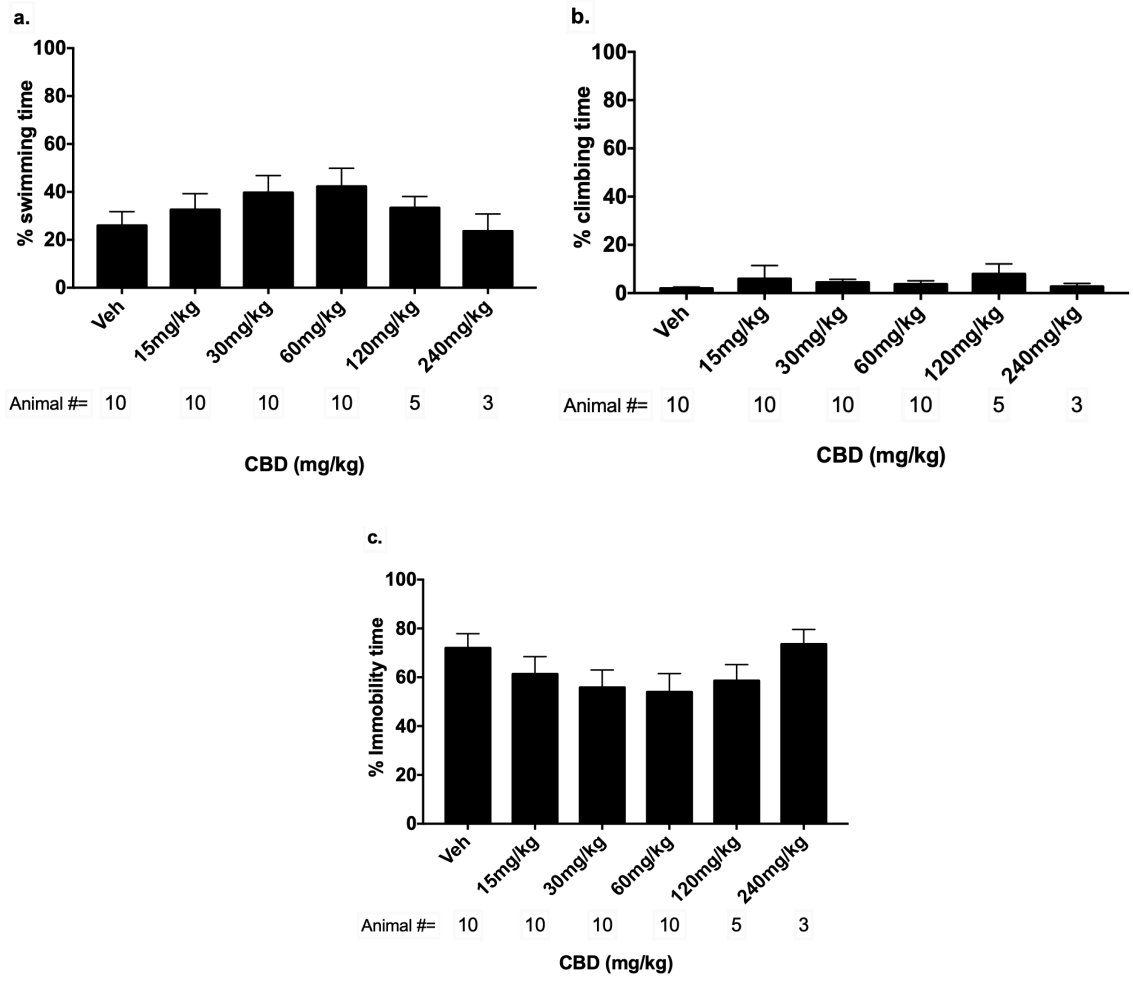


Figure 4.3 Effects of increasing doses of CBD (0-240mg/kg) on % swimming (a), climbing (b) and immobility (c) time in FST. CBD were injected I.P. 60mins before FST. Animal numbers for each dose are shown below the x-axis. Data are presented as mean±SEM.

Effects of Cannabidiol (CBD): OF Test at 60 Minutes Figure 4.4 shows the effects of increasing doses of CBD from 15mg/kg to 120mg/kg on locomotion in the OF test between 60mins and 70mins post-CBD injection. As indicated, CBD at 120mg/kg decreased total distance traveled between 60mins and 70mins post-injection in the OF test. A one-way ANOVA ($F=2.786$, $p=0.0425$) and post-hoc Dunnett's multiple comparison tests showed that there was a significant decrease in locomotion at CBD 120mg/kg ($p=0.0108$), as compared to vehicle. Thus, CBD decreased locomotion at the high dose of 120mg/kg. (240 could not be tested due to licensing problems.) This would have to be taken into consideration if 120 mg/kg had produced significant results in the FST, but 120 mg/kg didn't produce significant results in the FST.

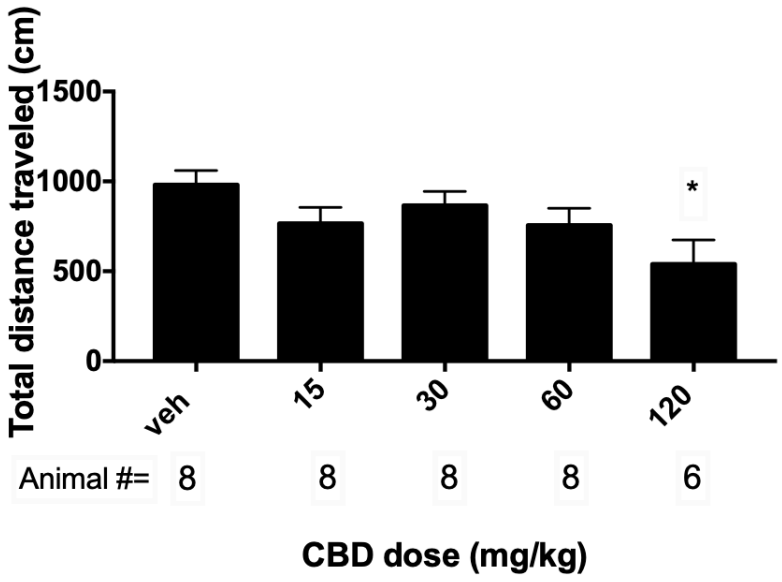
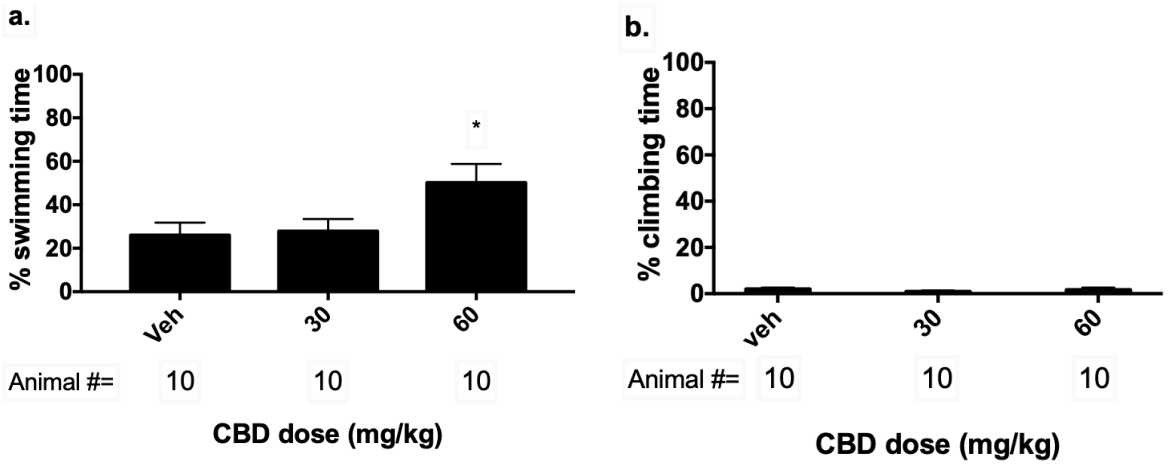


Figure 4.4 Effects of increasing doses of CBD (0-120mg/kg) on locomotion tested in 10mins of the OF test. CBD were injected I.P. 60mins before the OF test. Animal numbers for each dose are shown below the x-axis. Data are presented as mean±SEM. * $p<0.0332$ as compared to vehicle control.

Effects of Cannabidiol (CBD) at 30 minutes: FST Since past experiments had shown an effect of CBD in the FST 30mins after injection (Zanelati et al., 2010), two of the doses tested

at 60mins were retested at 30mins. Figure 4.5 shows the effects of CBD at 30mg/kg and 60mg/kg in the FST. CBD was injected I.P. 30mins before the FST.

As indicated, CBD at 30mg/kg was little different from vehicle, but CBD at 60mg/kg increased swimming and decreased immobility. The application of a one-way ANOVA suggested that there were significant differences among the means for swimming ($F=3.949$, $p=0.0313$) and immobility ($F=3.715$, $p=0.0376$), but not for climbing ($F=0.5789$, $p=0.5673$). Post-hoc Dunnett's multiple comparison tests showed that 60mg/kg CBD significantly increased swimming time ($p=0.0328$), and significantly decreased immobility time ($p=0.043$). Thus, a reduction in depression-like behavior was seen in the FST at 30mins post-injection that had not been seen at 60mins post-injection.



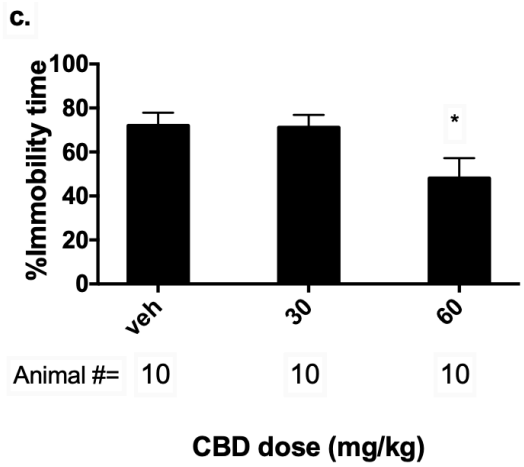


Figure 4.5 Effects of increasing doses of CBD (0, 30, 60mg/kg) on % swimming (a), climbing (b) and immobility (c) time in the FST. CBD were injected I.P. 30mins before the FST. Animal numbers for each dose are shown below the x-axis. Data are presented as mean±SEM. * $p<0.0332$ as compared to vehicle control.

Effects of Cannabidiol (CBD) at 30 minutes: OF test Figure 4.6 shows the effects of CBD at 30 and 60mg/kg on locomotion in the OF test between 30mins and 40mins post injection. As indicated, all of the means were very similar, with no apparent differences between the vehicle and CBD groups. A one-way ANOVA suggested that there were no significant differences among this group of means ($F=0.5103$, $p=0.6076$). Thus, CBD decreased depression-like behavior in the FST at doses that did not affect locomotion in the OF test.

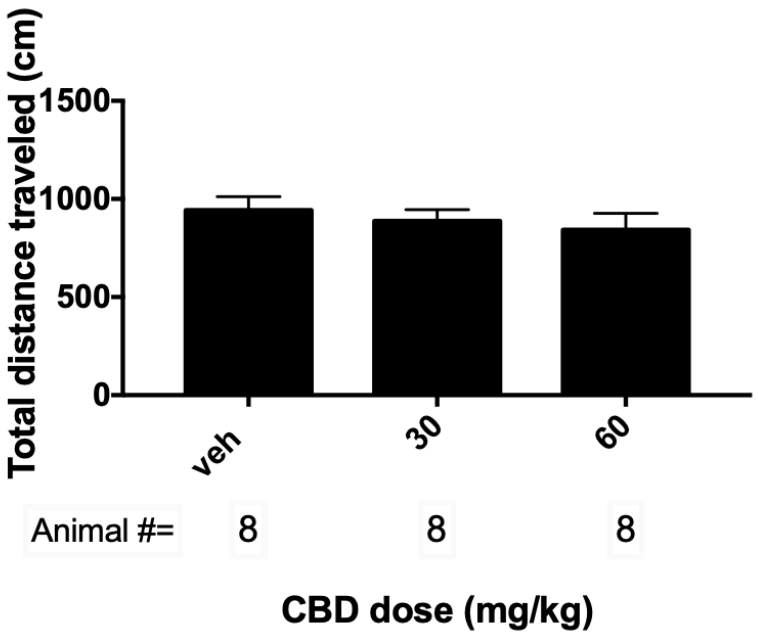


Figure 4.6 Effects of increasing doses of CBD (0, 30, 60mg/kg) on locomotion tested in 10mins of the OF test. CBD were injected I.P. 30mins before the OF test. Animal numbers for each dose are shown below the x-axis. Data are presented as mean±SEM.

Effects of Tetrahydrocannabinol: FST Figure 4.7 shows the effects of increasing doses of THC from 0.5mg/kg to 8mg/kg in the FST. THC was injected I.P. 30mins before the FST. As indicated, 2 mg/kg of THC was associated with an increase in swimming time and a decrease in immobility. (There was no apparent effect on climbing.) There were no obvious effects of any of the other doses on any type of behavior. The application of a one-way ANOVAs showed that there were significant differences among the means for swimming (F=02.399, p=0.0485), but not for climbing (F=0.8337, p=0.5314) or immobility (F=2.036, p=0.0875). Post-hoc Dunnett's multiple comparison tests, done on percent time spent swimming, showed that 2mg/kg THC significantly increased swimming time, as compared to vehicle (p=0.024). Thus, one dose of THC decreased one type of depression-like behavior in the FST, but the other doses did not.

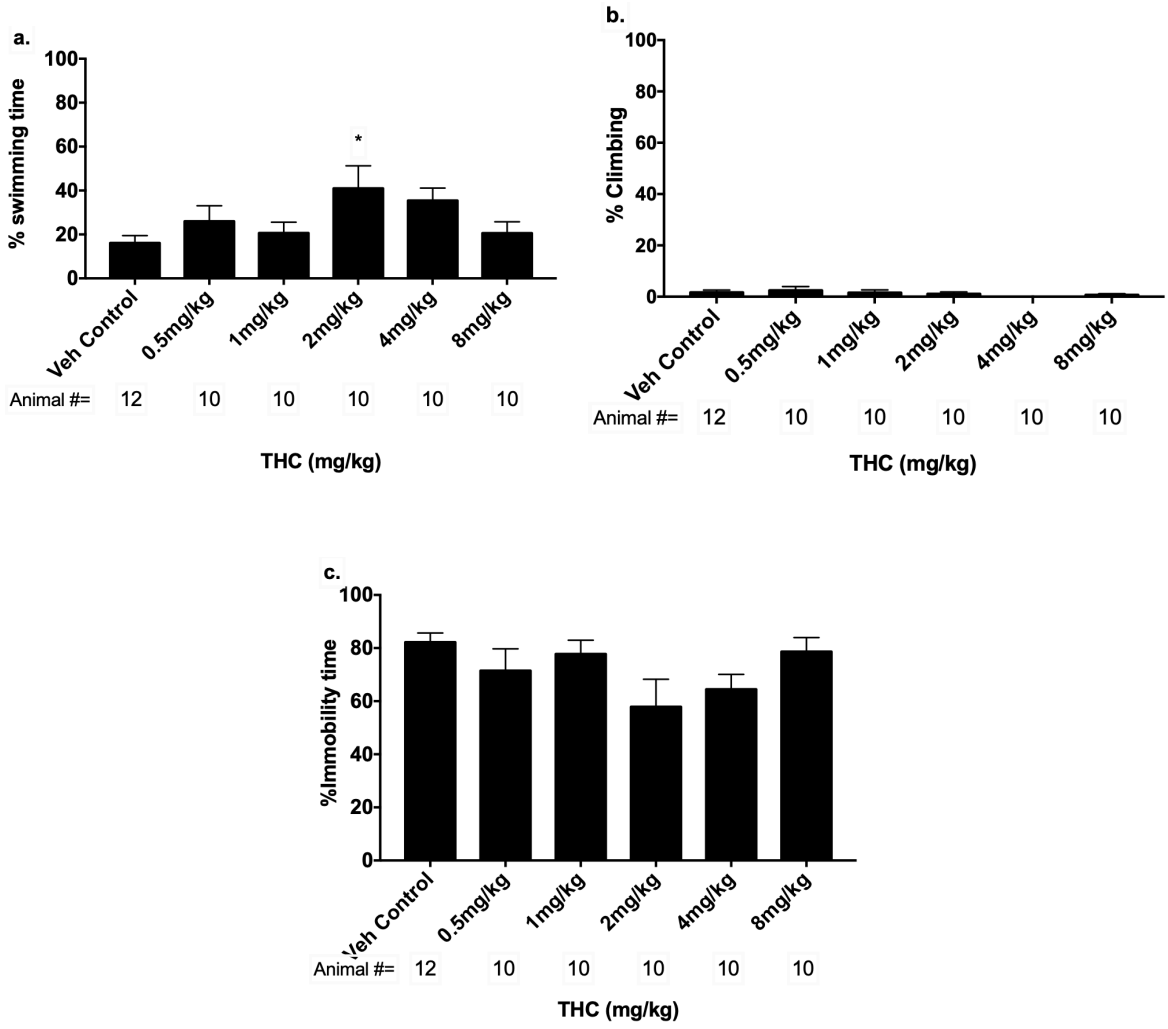


Figure 4.7 Effects of increasing doses of THC (0-8mg/kg) on % swimming (a), climbing (b) and immobility (c) time in the FST. THC were injected I.P. 30mins before the FST. Animal numbers for each dose are shown below the x-axis. Data are presented as mean±SEM. * $p<0.0332$ as compared to vehicle control.

Effects of Tetrahydrocannabinol: OF Test Figure 4.8 shows the effects of increasing doses of THC from 0.5mg/kg to 8mg/kg on locomotion in the OF test. Effects were measured between 30mins and 40mins post injection as "total distance traveled". As indicated, there was a slight increase in total distance traveled from 0.5 to 2.0mg/kg, and then a decrease at higher doses, suggesting an inverted U-shaped dose-response curve. A one-way ANOVA suggested that

there were significant differences among this set of means (F=3.024, p=0.0192), but post-hoc Dunnett's multiple comparison tests did not detect significance between any two pairs. Thus, THC did not significantly affect locomotion in the OF. A non-significant "trend" towards increased activity was seen at the dose that produced an increase in swimming in the FST, however.

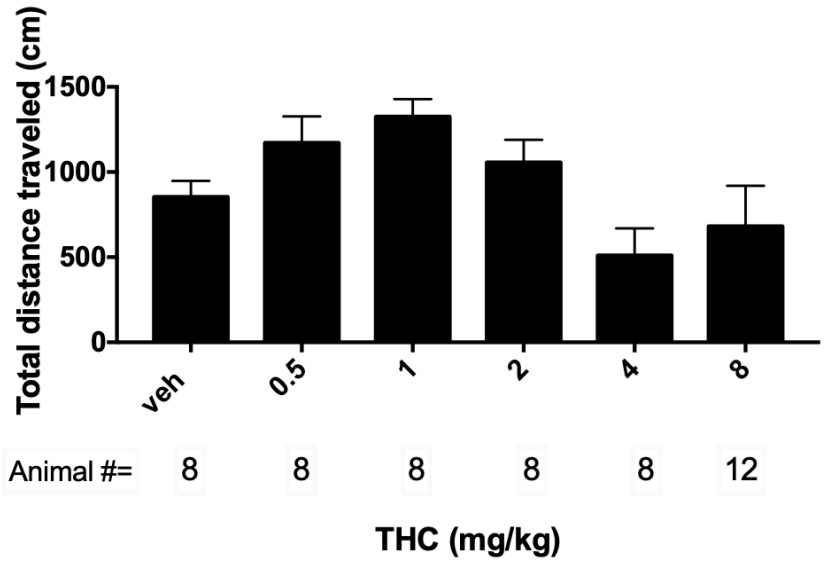


Figure 4.8 Effects of increasing doses of THC (0-8mg/kg) on locomotion tested in 10mins of the OF test. THC was injected I.P. 30mins before the OF test. Animal numbers for each dose are shown below the x-axis. Data are presented as mean±SEM.

Effects of Combined CBD and THC (15:1) at 30 Minutes: FST The effects of CBD and THC in a 15:1 combination were tested at 30mins post-injection. This is the time at which CBD had significant effects in the FST. Two doses of CBD previously tested at 30mins were re-tested in the presence of THC. Further doses could not be tested due to licensing problems.

Figure 4.9 shows the effects of CBD and THC combined at a ratio of 15:1 in the FST. Both CBD and THC were injected I.P. 30mins before the FST. The two dose combinations of CBD and THC that were tested were: 30+2 and 60+4mg/kg.

As indicated, both 30+2 and 60+4mg/kg increased swimming and decreased immobility in the FST. The effects with 30+2 were larger than the effects at 60+4mg/kg. Neither combination had much effect on climbing.

Application of a one-way ANOVA suggested that there were significant effects among the means for swimming ($F=5.095$, $p=0.0157$) and immobility ($F=4.901$, $p=0.0179$), but not for climbing ($F=3.081$, $p=0.0671$). Post-hoc Dunnett's multiple comparison tests showed that the CBD 30mg/kg and THC 2mg/kg combination significantly increased swimming time ($p=0.0089$) and decreased immobility time ($p=0.0099$). The "trend" seen with the 60 +4 combination was not significant ($p =0.0629$ for swimming; $p=0.0817$ for immobility), although the increase in swimming was as large as the significant increase previously seen with CBD alone (Figure 4.5). Thus, the addition of THC to CBD caused a left-shift in the dose-response curve, making a lower dose of CBD become effective.

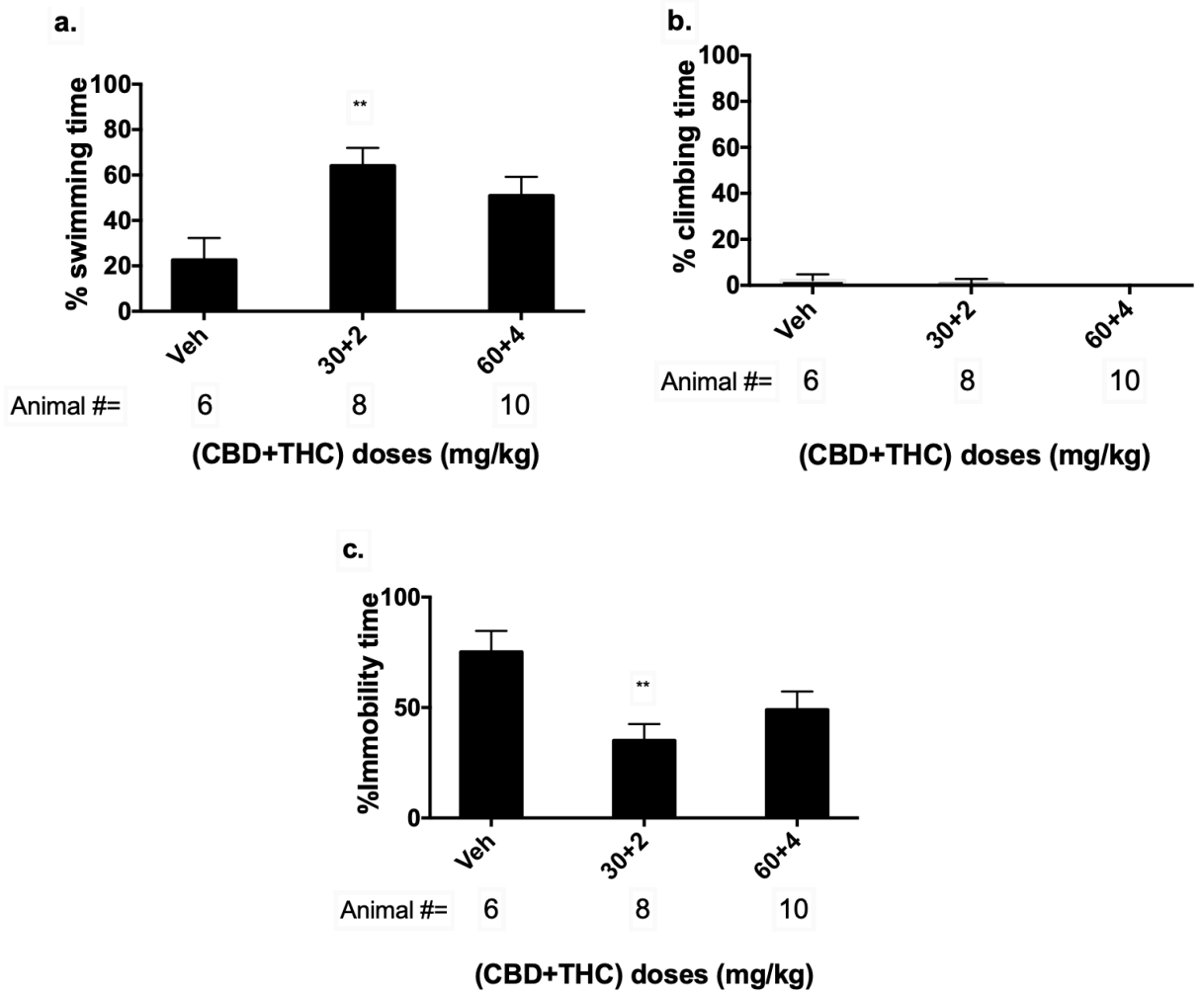


Figure 4.9 Effects of two doses of CBD and THC in combination at a 15:1 ratio (30+2 and 60+4mg/kg) on % swimming (a), climbing (b) and immobility (c) time in the FST. Both CBD and THC were injected I.P. 30mins before the FST. Animal numbers for each dose are shown below the x-axis. Data are presented as mean±SEM. ** $p<0.0021$ as compared to vehicle control.

Effects of Combined CBD and THC (15:1) at 30 Minutes: OF Test Figure 4.10 shows the effects of two doses of CBD and THC in combination at a 15:1 ratio (30+2 and 60+4mg/kg) on total locomotion in the OF test between 30mins and 40mins post-drug-injections. As indicated, the effects of CBD and THC in combination were not much different from the effect of the vehicle, although the mean of the 30+2 combination was slightly higher. A one-way ANOVA did not detect any significant differences in this group of means ($F=1.637$, $p=0.2184$).

Thus, the effect of the 30+2 combination in the FST cannot be attributed to a non-specific change in the baseline level of activity.

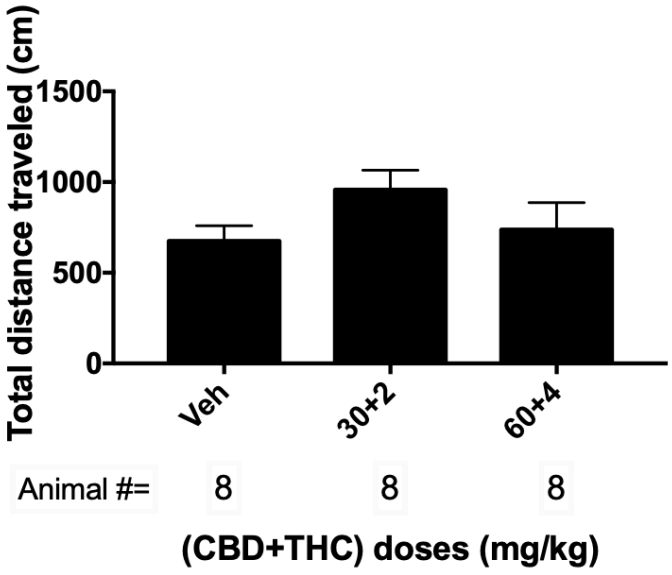


Figure 4.10 Effects of CBD and THC combination at 15:1 ratio (30+2 and 60+4mg/kg) on total locomotion tested in 10mins of the OF test. CBD and THC were both injected I.P. 30mins before the OF test. Animal numbers for each dose are shown below the x-axis. Data are presented as mean±SEM.

Effects of Different Ratios of Vehicle We used vehicle of 95% ethanol:cremophor:sterile saline at 1:1:78 for CBD, and 95% ethanol:cremophor:sterile saline at 1:1:623 for THC. Because previous studies had used vehicles at a 1:1:18 ratio, we later added trials of CBD and THC with a 1:1:18 vehicle (Appendices 3&4 Figures 4.11 and 4.12). Both CBD and THC were injected 30mins before the tests. There was no significant difference related to vehicle.

4.8 Discussion

Drug Toxicities/Effects of Locomotion of IMP, CBD and THC

The OF tests were performed throughout this chapter to evaluate toxicity. Only CBD injected at 120mg/kg significantly decreased locomotion. IMP at 30mg/kg, THC from 0.5-8mg/kg did not alter locomotion activities significantly.

The Effects of the Positive Control IMP

We found that IMP 30mg/kg injected I.P. caused a significant decrease in immobility time and an increase in swimming and climbing time (Figure 4.1). IMP at the same dose did not alter the locomotor activities of the mice (Figure 4.2).

These data are in agreement with the original paper that first described the effects of IMP in the FST. In that paper, IMP was also shown to decrease immobility time in the FST at 30mg/kg (Porsolt et al., 1977). Since that time, IMP 30mg/kg has since been used in many other studies as a positive control for the FST with similar results (Zanelati et al., 2010) (Réus et al., 2011) (Häring et al., 2013).

These positive control data simply show that, in our hands, the FST was able to detect the effects of a known antidepressant.

The Effects of CBD at 60 Minutes in the FST and OF

Testing at 60mins, we found no significant effect of CBD in the FST. The data did appear to show a "trend" toward effectiveness with a peak effect at around 60 mg/kg (Figure 4.3), but the overall ANOVA was not significant.

In the OF test, CBD significantly decreased locomotion at 120mg/kg (Figure 4.4) (240 mg/kg could not be tested due to the licensing problems.) This suggests that at 120 mg/kg and above, CBD decreases motor activity, which may explain the "trend" towards an increase in immobility seen in the FST at 120 mg/kg.

There have been no previous experiments done on mice in the FST at 60mins, and therefore no comparisons to previous literature can be made.

The Effects of CBD at 30 Minutes in the FST and OF

Tested at 30mins, CBD caused a significant decrease in immobility time at 60mg/kg in the FST (Figure 4.5), suggesting an "antidepressant" effect. In the OF test, CBD did not significantly alter locomotor activity of the mice at either 30 or 60 mg/kg (Figure 4.6).

These studies are in agreement with the past report of Zenaleti et al. who also found anti-depressant effects of CBD tested at 30mins. (Zanelati et al., 2010).

Based On the pharmacokinetic data, it seems puzzling that significant effects were seen at 30mins but not at 60mins. Pharmacokinetic data suggest that blood levels should be higher at 60mins than at 30mins (Deiana et al., 2012). This paradox might be explained if CBD has an inverted U dose-response curve in the FST, which would mean that higher concentrations would be less effective. Several past studies do suggest that CBD does have an inverted U dose-response curve. Schiavon et al. (2016) found CBD to be "antidepressant" at 3 and 10mg/kg but not at 30mg/kg in the TST. Réus et al. (2011) also observed CBD's "antidepressant" effects at 30mg/kg but not at 60mg/kg in the rats.

The Effects of THC at 30 Minutes in the FST and OF

We found a significant increase in swimming time when THC was injected I.P. at 2mg/kg, but not at other doses (Figure 4.7). There was a trend toward decreased immobility at this dose, but it didn't reach significance.

THC did not significantly alter locomotor activities of the animals at the doses tested (Figure 4.8), but there was a "trend" toward increased activity at the lower THC doses which disappeared at higher doses. Past studies have indicated that low-dose THC can increase activity (Davis et al., 1972). The effects of THC in the FST at 2 mg/kg should possibly be viewed in this light.

The previous literature on THC's effects on depression is rather contradictory. Egashira et al. (2008) found that 2 and 6mg/kg of THC prolonged the immobility time in the mice FST – suggesting pro-depressant effects. Häring et al. (2012), to the contrary "antidepressant" effects of THC at 0.1 and 0.5mg/kg in the FST, and Bambico and colleagues (2012) found that THC at 1mg/kg did not have any effect. It could be that THC causes changes in FST behavior only at a very limited range of doses.

The Effects of CBD+THC at 30 Minutes in the FST and OF

The present study was the first to study the effects of CBD and THC in combination in the FST. CBD at 30mg/kg and THC at 2mg/kg (CBD30/THC2) significantly decreased immobility time in the FST (Figure 4.9). There was a "trend" for decreased immobility time for CBD 60mg/kg and THC 4mg/kg (CBD60/THC4), but it was not significant - although it was nearly as large as the change seen with CBD at 30mg/kg and THC at 2mg/kg. The loss of significance at CBD60/THC4 is likely due to a low animal number.

In the OF, neither CBD30/THC2 nor CBD60/THC4 significantly affected the locomotor activities of the mice (Figure 4.10).

Comparing CBD alone (Figure 4.5) to CBD in the presence of THC (Figure 4.9), it looks as if THC has left-shifted the CBD dose-response curve, but it will take more data before ED50s can be calculated and a proper comparison made. Now that the CTLS licence has been approved, more animal numbers will be added in future experiments.

Effects on Locomotion

No significant effects on locomotion in the OF were seen for either drug tested alone, except for the decrease caused by CBD at 120 mg/kg. Hypo-locomotion has been reported in the past for THC at higher doses (Davis et al., 1972), however, and it was, perhaps, beginning to be seen at the higher doses of THC alone in the present experiment.

No hypo-locomotion was seen, however, with the CBD+THC combinations.

Conclusions

From the present 30-minute data, it appears that CBD is “anti-depressive” at 60mg/kg and that THC may be “anti-depressive” at 2mg/kg. The most interesting finding, however, is that the effects of CBD seem to be enhanced by adding a small amount of THC. A similar effect has been seen when CBD and THC are used together to suppress seizures (Fallah, personal communication).

Clinically, these data suggest that it would be worthwhile to test the CBD+THC mixture in patients with MDD. The combination might also be very helpful in epileptic patients suffering from depression as a comorbidity.

Chapter 5

5 « General Discussion »

The present study was designed to study the effects of CBD and THC, alone and in combination, in mouse models of anxiety and depression. CBD did not show anxiolytic effects but did show anti-depressive effects in our study. THC, however, showed both anxiolytic and antidepressant effects at some doses. Synergy between CBD and THC was seen in the antidepressant effects, but not in the anxiolytic effects.

The present section will discuss: 1) possible mechanisms of the anxiolytic effects, 2) possible mechanisms of the antidepressant effects, 3) possible mechanisms of synergy, 4) clinical significance, 5) limitations of the study, and 6) future experiments.

5.1 Possible Mechanisms of Anxiolytic effects

5.1.1 Anxiolytic Effects of CBD

The present study did not observe any effects of CBD on “anxiety” in the EPM test. Two past studies, however, have claimed to have found anxiolytic effects of CBD in this model (Schiavon et al., 2016; Onaivi et al., 1990). This discrepancy in results may be due to a variety of different experimental factors. (Please see Chapter 3 Section 3.7 for detailed discussion.) Future studies will be required to resolve these differences. It is possible, however, that future studies may, in fact, disclose anxiolytic effects in the EPM. Our initial studies did not disclose antidepressant effects of CBD, but they were found in our follow-up studies.

Two major hypotheses have been proposed to explain the anxiolytic effects of CBD previously reported: 1) actions mediated through the adenosine system, and 2) actions mediated through the 5-hydroxytryptamine 1A (5-HT1A) receptor.

Adenosine Adenosine receptors are widespread in the central nervous system, where they work to modulate other neurotransmitters, and have a net neuron-inhibitory effect (Sebastião & Ribeiro, 2009). They also produce sedative effects, and their antagonists, like caffeine, are stimulants (Dunwiddie & Masino, 2001).

CBD has been shown to inhibit the uptake of adenosine, and thereby increase adenosine signaling in the brain (Carrier, Auchampach, & Hillard, 2006). Increased adenosine signaling has been shown to be anxiolytic in a randomized control trial (Koran, Aboujaoude, & Gamel, 2009). This is one mechanism by which CBD could have anxiolytic effects. It should be noted, however, that CBD does not seem to be associated with sedation, at least at lower therapeutic doses.

5-HT1A Receptors The 5-HT1A receptor is a pharmacological target for the therapy of anxiety disorders. Buspirone, for instance is a 5-H1A partial agonist (Roncon et al., 2013).

CBD has been proposed as a positive allosteric modulator for the 5-HT1A receptor (Patel, Hill, Cheer, Wotjak, & Holmes, 2017) (Russo, Burnett, Hall, & Parker, 2005). The anxiolytic effects that are reported for CBD are said to attenuated by 5-HT1A receptor antagonists (Marinho, Vila-Verde, Fogaça, & Guimarães, 2015) (Fogaça, de Campos, Coelho, Duman, & Guimarães, n.d.) (Gomes et al., 2013). Thus, this is a second mechanism by which CBD could have anxiolytic effects.

Other Receptors/The Need for More Research Other receptors might also be candidates. For example, CBD is an agonist at the transient receptor potential cation channel subfamily V member 1 (TRPV1) receptor. The TRPV1 initiates burning pain in peripheral nerves, and is also found in the brain where it has shown to modulate fear and anxiety (Patel et al., 2017).

None of the hypotheses, however, have been supported by an abundance of evidence. Further research is needed to determine whether CBD actually has anxiolytic effects, and what the mechanism of these might be, if they are found.

5.1.2 Anxiolytic Effects of THC

The present study observed dose-dependent anxiolytic effects of THC in the EPM test at the two highest doses. The main mechanism through which THC exerts its anxiolytic effects is believed to be via the CB1 receptor. THC is a partial agonist for the CB1 receptor (Pertwee, 2008).

Evidence relating the CB1 receptor to anxiety is abundant. For example, administration of a CB1 receptor agonist produces anxiolytic-like effects, which are abolished by the administration of CB1 receptor antagonists (Haller, Varga, Ledent, & Freund, 2004).

However, many human and animal studies have also shown THC to be anxiogenic. THC's anxiogenic effects may occur because it is a partial agonist for the CB1 receptor, functioning as an antagonist by competing with full agonists like 2AG.

Thus, THC's effects may depend on the endogenous levels of endocannabinoids. In the present study, for example, Swiss mice may have a less active endocannabinoid system, making THC work like an agonist at CB1 receptor, producing anxiolytic effects.

5.2 Possible Mechanisms of Anti-Depressant effects

5.2.1 CBD's Antidepressant Effects

The present study found CBD to be "antidepressant" when tested 30mins post-injection. Two different receptors have been proposed to explain CBD's antidepressant effects: 5-HT1A receptors and TRPV1 receptors. These are two of the receptors also proposed to explain CBD's effects on anxiety, which often goes along with depression.

5-HT1A As noted above, agonism of the 5-HT1A receptor has been proposed as a mechanism for CBD's anxiolytic effects. It has also been proposed as a mechanism for CBD's antidepressant effects. Studies that have reported that CBD has antidepressant effects in animals have also shown that 5-HT1A receptor antagonists attenuate these effects (Zanelati et al., 2010)(Sartim et al., 2016)(Linge et al., 2016)(Resstel et al., 2009).

TRPV1 As also noted above, CBD is an agonist at TRPV1 receptor (Bisogno et al., 2001). The mechanism of by which TRPV1 agonism could be antidepressant, however, remains unclear. The TRPV1 agonist capsaicin has been reported to produce antidepressant effects in animals (Hayase, 2011). Another study, however, has shown that desensitization of the TRPV1 receptor leads to depression-like behaviors in mice (Abdelhamid, Kovács, Nunez, & Larson, 2014). Further study is needed to determine whether it is TRPV1 agonism or desensitization (antagonism) that produces antidepressant effects. One study has argued that TRPV1 agonism

produces antidepressant effects by affecting levels of 5-HT1A, GABA and NMDA receptors in the brain (You et al., 2012).

5.2.2 THC's Anti-Depressant Effects

The present study found that that THC showed anti-depressive effects at one dose: 2mg/kg. It has been hypothesized that THC's effects on depression are mediated through CB1 receptors and 5-HT1A receptors.

CB1 Studies have shown that stimulation of the CB1 receptor produces antidepressant effects. These studies have involved: 1) direct agonists (Rutkowska & Jachimczuk, 2004), 2) blockers of reuptake endocannabinoid uptake (Matthew N. Hill & Gorzalka, 2005) and 3) inhibition of the breakdown of endocannabinoids (Gobbi et al., 2005). It has been shown that direct activation of the CB1 receptor leads to a decrease in immobility in the FST in rats, which is blocked by pre-treatment with CB1 antagonist (Matthew N. Hill & Gorzalka, 2005).

5-HT1A The 5-HT1A receptor has also been proposed as a mediator of 5HT's anti-depressant effects, although the evidence is here is not strong. Bambico et al. Have reported that repeated injections of THC - but not a single injection - enhanced 5-HT1A receptor activity in the hippocampus (Bambico et al., 2012). Zavitsanou et al. have also found that repeated injections of CB1 agonist increased 5HT1A binding and mRNA expression in the hippocampus (Zavitsanou, Wang, Dalton, & Nguyen, 2010). This mechanism, however, still requires further investigation. There isn't much direct evidence tying THC's anti-depressive effects to 5-HT1A.

5.2.3 Possible Mechanisms of Synergy

In the present study, we saw no interactions between CBD and FST in the EPM, but we observed a synergistic effect of CBD and THC in the FST at 30 minutes. Similar synergistic effects have been seen in other studies of CBD and THC in our laboratory (Fallah, personal communication).

Our studies are some of the first to test CBD and THC in combination, and there have been no studies looking into possible mechanisms of these synergistic interactions. Some speculation is possible, however.

Synergy sometime occurs when different drugs bind to different sites on the same drug receptor. An example would be alcohol and the benzodiazepines. Supra-addictive effects occur when alcohol and diazepam were administered together, sometimes leading to fatal overdoses (van Steveninck et al., 1993). Alcohol and diazepam both bind to sites on the GABA-A related chloride channel, but the sites are different (Mihic et al., 1997) (Sigel & Buhr, 1997). Thus, synergy occurs.

CBD and THC have both been shown to bind to 5-HT1A receptors. If the sites of binding are different, this might be an explanation for the antidepressant synergy observed in the present study. It must be noted, however, that the anxiolytic effects of CBD and THC are also thought to be mediated by binding to the 5-HT1A receptor, and no synergy was seen in our study in the EPM.

It should also be noted that the antagonism between CBD and THC, reported in some studies (Onaivi et al., 1990), was not seen in the present study.

Future studies will be required to investigate the synergistic effects of CBD and THC.

Clinical significance of THC and CBD's effects on Anxiety

Experiment 1 in Chapter 3 was designed to evaluate the effects of CBD, THC, and a combination of CBD and THC at a 15:1 ratio on anxiety.

CBD We did not find significant effects of CBD on anxiety, although other studies have done so. Due to its lack of psycho-toxicity, CBD would be ideal as an anxiolytic. Future studies might be conducted to further investigate CBD's possible effects on anxiety.

THC We found THC to be anxiolytic at two doses: 3.2 and 6.4 mg/kg. THC is psycho-toxic at high doses, but the anxiolytic doses used in the present study were doses that did not cause significant hypo-locomotion in the OF - suggesting that they were not seriously psycho-toxic (Chapter 4). Low doses of THC, therefore, might be helpful for patients with anxiety disorders, or for patients with seizures and anxiety. Clinical trials might be done to test these possibilities. A potential drawback for THC, of course, is that it can be anxiogenic as well (See Chapter 1 Section 1.2.6).

CBD and THC CBD and THC combined at a ratio of 15:1 had similar effects to THC alone. There was neither synergy nor antagonism. Commercially available preparations of cannabinoids often contain a mixture of CBD and THC. Our data suggest that CBD would not antagonize the anxiolytic benefits of THC if they were given in combination.

5.3 Clinical significance of THC and CBD's effects on Depression

Experiment 2 in Chapter 4 was designed to evaluate the effects of CBD, THC and a combination of CBD and THC at a 15:1 ratio on depression.

CBD We found CBD alone to be "antidepressant" at 60mg/kg. This was a dose that did not cause hypo-locomotion. CBD has also been previously observed to relieve depression in both human and rodent studies. CBD, therefore, might be helpful for patients with anxiety disorders, or for patients with seizures and depression. Clinical trials might be done to test these possibilities.

THC We found one dose of THC to be anti-depressive at 2mg/kg, but this effect was lost at higher doses. Previous literature has shown contradictory results regarding the antidepressant effects of THC. It could be that THC is only anti-depressive at a very limited range of doses. Clinically, it might also be that THC would be most effective in combination with CBD, as discussed below.

CBD and THC A synergistic effect of CBD and THC was seen in Experiment 2. The combination seemed to left-shift the curve for CBD, making an ineffective dose of CBD effective with the addition of a small (ineffective) dose of THC. Clinically, this might mean that lower doses of CBD could be used in depression if small and non-toxic amounts of THC were added. Thus, combinations of CBD and THC might be useful for depression and in epilepsy with depression. Clinical trials should be done to confirm these effects in humans.

5.4 Limitations of the Present Study

Limited Doses Tested

Due to a delay in the Cannabis Tracking and Licensing System (CTLS), we did not have enough CBD to carry out complete dose-response curve at 30mins. As a result, only two doses were tested. These two doses were 12 and 48mg/kg in the EPM study, and 30 and 60mg/kg in the FST study. Future studies should involve the whole range of doses.

Small Animal Numbers

Similarly - due to the CTLS licensing delay - we have an average animal number of only 10 per dose for the dose-response curves - and some points have as few as 4 animals. Previous studies generally have had animal numbers between 10-15, as CD1 mice are outbred mice and show a good deal of variability.

There were a several cases in the present study where effects just missed statistical significance. Future studies should involve larger numbers of subjects.

Male-Only Subjects

Only male subjects were used in the present study. Due to the confounding factors such as hormonal cycles, female mice were not tested. Previous studies have shown that male and female animals could respond differently to drugs. For example, one study showed that male Sprague-Dawley rats are more sensitive to the anxiolytic and anti-depressive effects of THC (Silva, Black, Michaelides, Hurd, & Dow-Edwards, 2016). Human studies have also shown sex differences with respect to sensitivity to cannabinoids (Craft, Marusich, & Wiley, 2013). Future studies should involve both male and female subjects.

5.5 Future Studies

Full Dose-Response Curve for CBD at 30mins, Larger Animal Number

As noted above, future studies should involve full dose-response curves for CBD and CBD+THC at 30 minutes. The animal number should also be increased in most studies.

Antagonist Studies to Investigate the Mechanisms of CBD and THC's Anxiolytic and Antidepressant Effects.

The mechanisms by which CBD and THC elicit their anxiolytic and antidepressant effects are still unclear. Future studies could involve antagonists of the various receptors thought to be involved (CB1, TRPV1, 5-HT1A, and adenosine receptors). If the anxiolytic and antidepressant effects are actually mediated by these receptors, they should disappear in the presence of receptor antagonists.

5.6 Conclusions

The present studies suggest that THC or THC+CBD might be useful in the treatment of anxiety or epilepsy with anxiety, and that CBD or CBD+THC might be useful in the treatment of depression or epilepsy with depression.

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Appendix 1 Other Treatment Options for Depression

Electroconvulsive Therapy (ECT) ECT is performed with applying electrical shock through scalp electrodes to patients, with a prior administration of a muscle paralysis agent to prevent motor injuries. The electrodes are usually put on the non-speech hemisphere to avoid speech damage (Carlson, 2013b). A meta-analysis showed that ECT could be more effective than pharmacotherapy in a short term treatment (UK ECT Review Group, 2003). It produces rapid results typically within a few days; however, relapse is slightly more common and sooner with ECT compared to pharmacotherapy (Kellner et al., 2006). Cognition and memories are also affected by ECT, which is one of the major setbacks (Carlson, 2013b). Because of its effectiveness, rapid results yet serious cognitive side effects, ECT is usually only used for TRD patients and those who are highly suicidal.

Deep Brain Stimulation (DBS) DBS was originally used to treat Parkinson's disease, but was later found to be effective in MDD as well (Mayberg et al., 2005). It is the procedure of implanting electrodes and providing stimulation into the subgenual anterior cingulate cortex (Carlson, 2013b). It is the most invasive among all different treatments for depression. There are many risks associated with DBS: hemorrhage, seizure and infection from surgery procedures and lowered mood, fear and anxiety from the stimulations (Fitzgerald, 2013). The response rates for DBS in TRD patients after one, two or three years' of implantation are 62.5%, 46.2% and 75%, respectively (Kennedy et al., 2011). Because of its highly invasiveness and relatively more serious risks, it is only reserved for treatment of very difficult-to-treat TRD (Fitzgerald, 2013).

Transcranial Magnetic Stimulation (TMS) TMS passes electricity through coiled wires on patients scalp, which produces a magnetic field that penetrates to 1.5-2.5cm below the scalp (Otte et al., 2016). The targeted brain region for TMS is typically in the prefrontal cortex, where activity is low in MDD patients (Murray, Wise, & Drevets, 2011). TMS is generally well tolerated with occasional transient headache after the procedure, and very low seizure risks (Machii, Cohen, Ramos-Estebanez, & Pascual-Leone, 2006). It is believed that TMS is less responsive than ECT (Ren et al., 2014); however, if patients do not require immediate relief of depressive symptoms, TMS is usually indicated rather than ECT due to its lower risk profile.

Appendix 2 Effects of CBD in Different Vehicle Ratios in the EPM

Figure 3.6 shows that the effects of CBD at 48mg/kg does not differ between the vehicle ratio of 1:1:398 (95% ethanol:Cremophor EL:sterile saline) and 1:1:18. An un-paired t-test showed that there is no significant difference (p=0.7027).

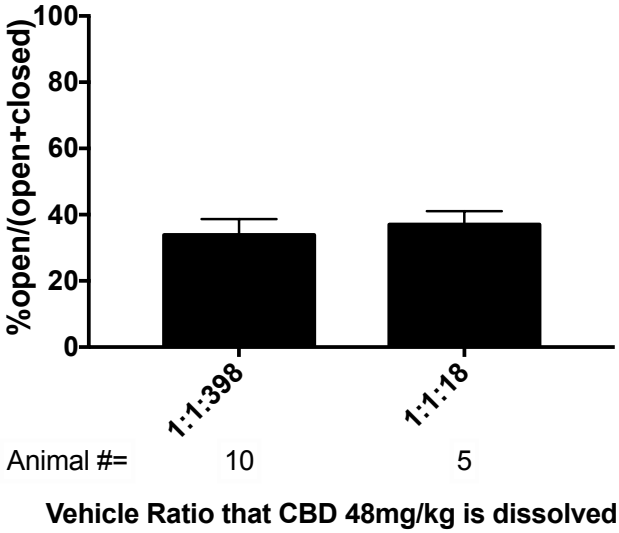

Figure 3.7 The effects of CBD 48mg/kg dissolved in 1:1:398 and 1:1:18 vehicle ratios (95% ethanol:Cremophor EL:sterile saline) on percent time spent in open arms when CBD was injected i.p. 30mins before the test. Animal numbers for each dose are shown below the x-axis. Data are presented as mean ±SEM

Appendix 3 Effects of CBD in Different Vehicle Ratios in the FST

Figure 4.11 compares CBD 60mg/kg dissolved in different ratios of vehicle (95% ethanol:Cremophor EL:sterile saline), either 1:1:78 or 1:1:18 in FST. CBD were injected I.P. 30mins before FST. Unpaired t-test did not detect any significant difference between the two vehicles (p=0.1794 for swimming, p=0.662 for climbing, p=0.2183 for immobility).

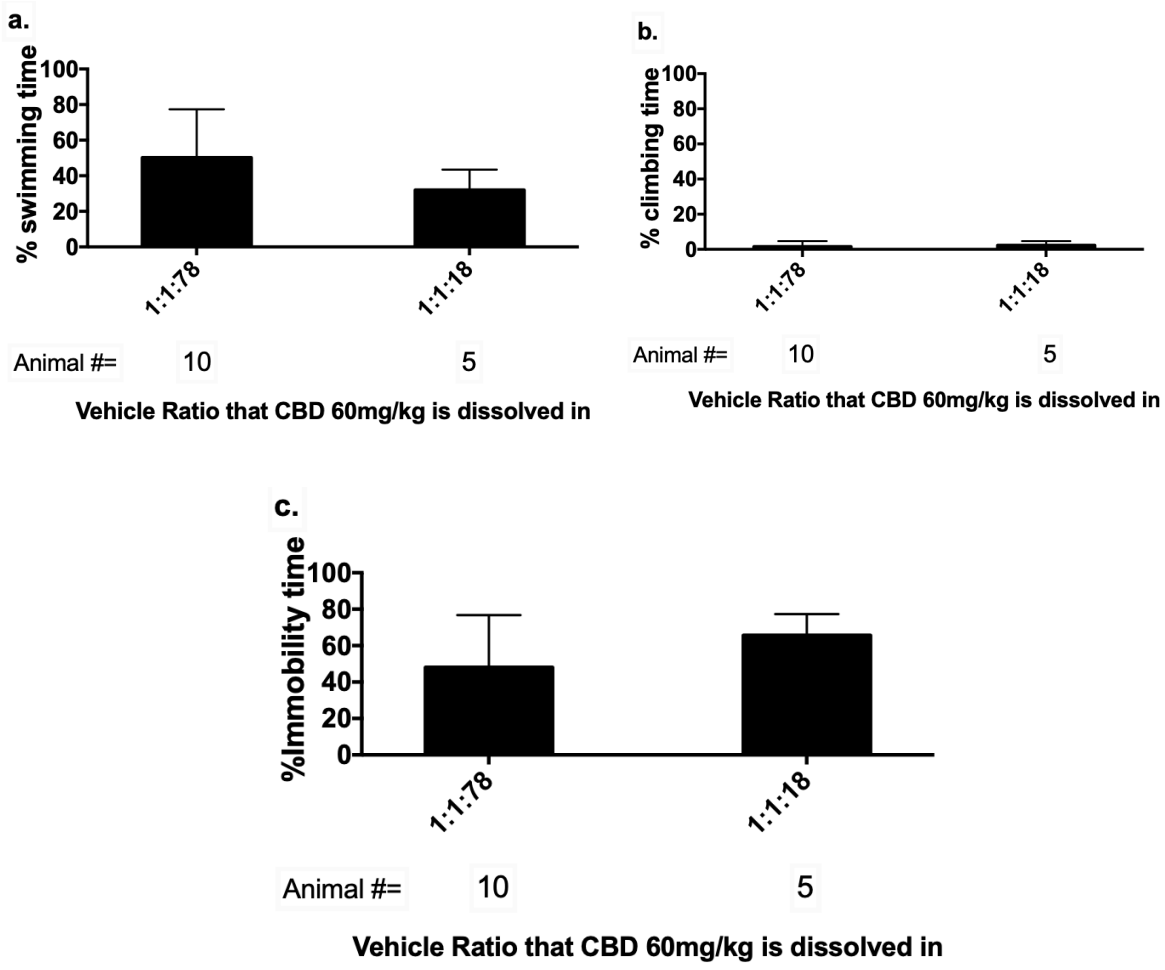

Figure 4.11 Comparison between CBD 60mg/kg dissolved in 1:1:78 and 1:1:18 vehicle (95% ethanol:Cremophor EL:sterile saline) on % swimming (a), climbing (b) and immobility (c) time in FST. CBD was injected I.P. 30mins before FST. Animal numbers for each dose are shown below the x-axis. Data are presented as mean±SD.

Appendix 4 Effects of THC in Different Vehicle Ratios in the OF test

Figure 4.12 compares THC 4mg/kg dissolved in different ratios of vehicle (95% ethanol:Cremophor EL:sterile saline), either 1:1:623 or 1:1:18 in the OF test. THC were injected I.P. 30mins before OF. Unpaired t-test did not detect any significant difference between the two vehicles (p=0.1865).

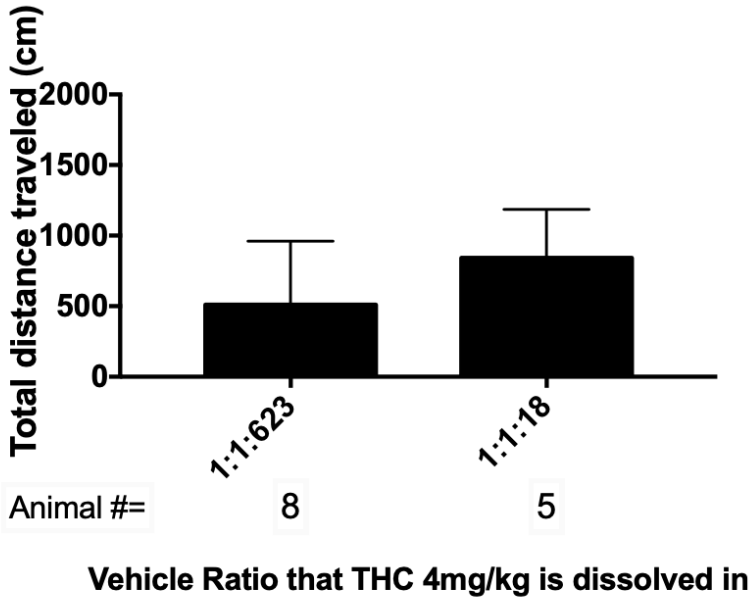

Figure 4.12 Comparison between THC 4mg/kg dissolved in 1:1:623 and 1:1:18 vehicle (95% ethanol:Cremophor EL:sterile saline) on locomotion in OF. THC was injected I.P. 30mins before OF. Animal numbers for each dose are shown below the x-axis. Data are presented as mean±SD.

Appendix 5 Effects of DZP, CBD and THC on closed arm entries in the EPM

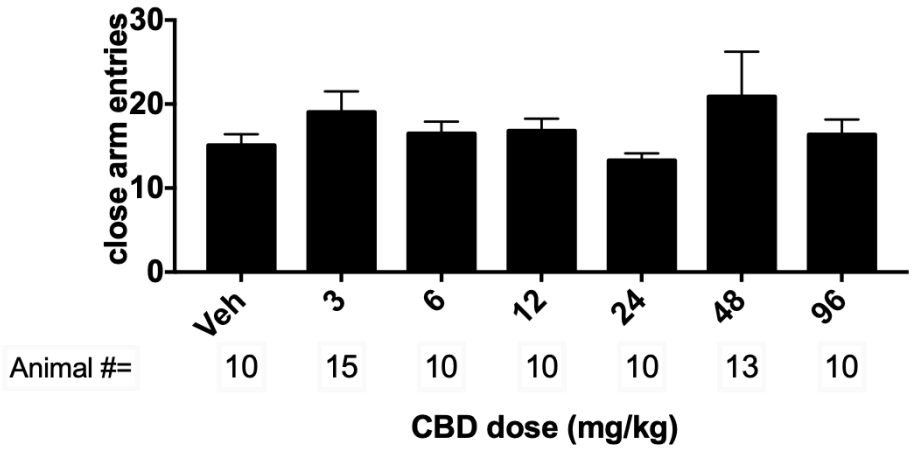

Figure 3.8 The effects of increasing doses of cannabidiol (CBD) on closed arm entries in the EPM when CBD was injected I.P. 60mins before the test. Animal numbers for each dose are shown below the x-axis. Data are presented as mean ±SEM. A one-way ANOVA showed no significant differences among groups.

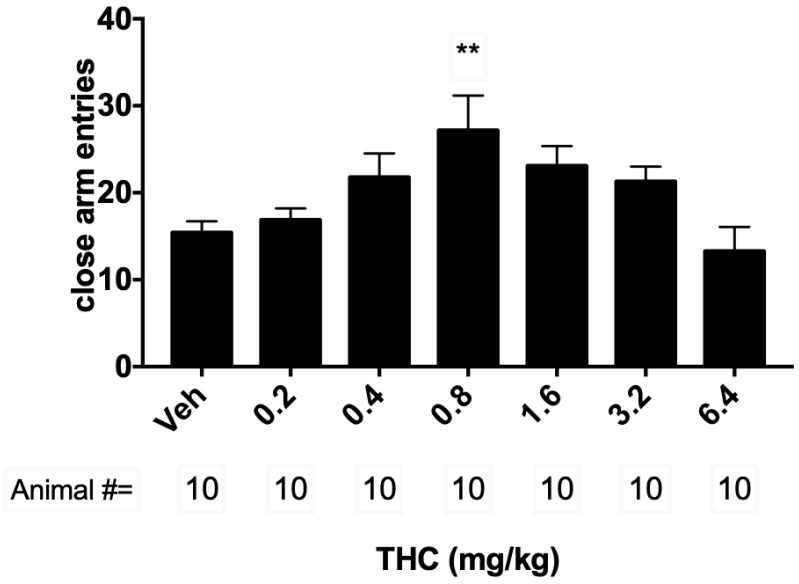

Figure 3.9 The effects of increasing doses of tetrahydrocannabinol (THC) on closed arm entries in the EPM when THC was injected I.P. 30mins before the test. Animal numbers for each dose are shown below the x-axis. Data are presented as mean ±SEM. A one-way ANOVA and post-hoc Dunnett's multiple comparison tests were done as statistical analysis. **p=0.0089

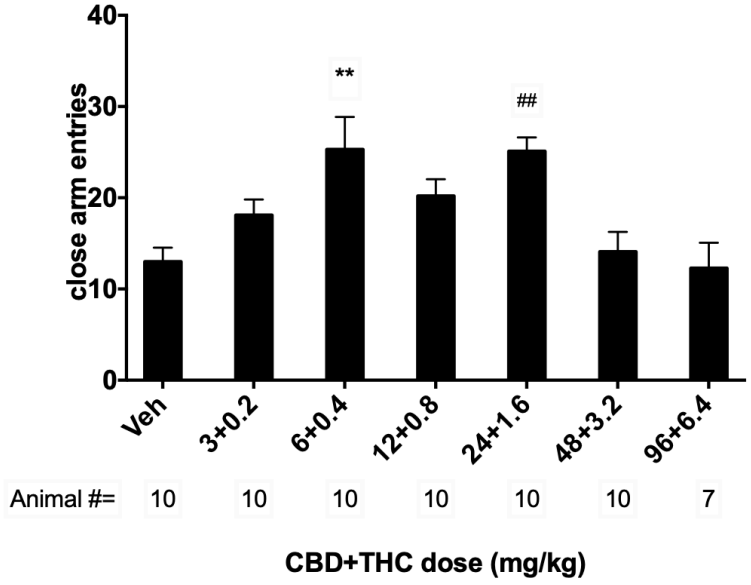

Figure 3.10 The effects of increasing doses of cannabidiol (CBD) and tetrahydrocannabinol (THC) combination at a 15:1 ratio on closed arm entries in the EPM when CBD was injected I.P. 60mins and THC was injected I.P. 30mins before the test. Animal numbers for each dose are shown below the x-axis. Data are presented as mean ±SEM. A one-way ANOVA and post-hoc Dunnett's multiple comparison tests were done as statistical analysis. **p=0.0011, $^{\#\#}$p=0.0013.

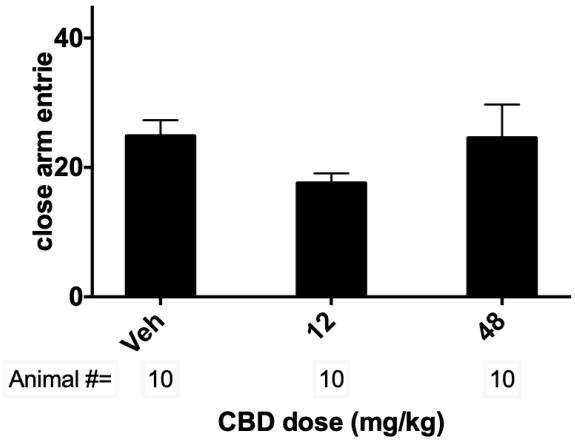

Figure 3.11 The effects of increasing doses of cannabidiol (CBD) on closed arm entries in the EPM when CBD was injected I.P. 30mins before the test. Animal numbers for each dose are shown below the x-axis. Data are presented as mean ±SEM. A one-way ANOVA showed no significant differences among groups.