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THE EFFECTS OF FULL SPECTRUM HEMP OIL ON EXTINCTION OF STRESS ENHANCED FEAR LEARNING IN A RODENT MODEL OF PTSD

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ABSTRACT

There are only 2 FDA-approved treatments for Post-Traumatic Stress Disorder (PTSD) despite the overwhelming evidence of the need for safer and more effective treatments. For example, roughly 50% of treatment seeking patients with PTSD experience relief from conventional pharmaceutical medications and only one third experience full remission. The *Cannabis* plant is a promising novel treatment for PTSD for several reasons. The endocannabinoid system plays a role in stress, emotion, cognition, suicidal phenotypes, fear memory consolidation, retrieval and reconsolidation and extinction. Cannabidiol (CBD), one of the most widely studied phytocannabinoids found in the *Cannabis* plant, has both anxiolytic and antidepressant qualities. CBD blocks the CB1 receptor and activates the CB2 receptor which plays a significant role in the learning processes, from consolidation to extinction, and has been shown to activate serotonin (5HT)-1A and $GABA_A$ receptors thereby producing anxiolytic effects. Therefore, the present study examined the effects of full spectrum hemp oil on extinction of stress enhanced fear learning in a rodent model of PTSD. During trauma exposure, rats were assigned to either a control (no-shock) or trauma (shock) group. After the trauma exposure, rats were assigned to either a control (peanut butter) or hemp (hemp oil using a peanut butter vehicle) group and tested for rates of fear extinction.

The stress enhanced fear learning procedure produced robust freezing in the trauma group compared to controls. However, in the trauma group there were no significant differences in the rates of freezing when comparing the non-hemp and hemp animals during the 5 extinction trials. These results suggest that full spectrum hemp oil did not have a significant effect on the extinction of stress enhanced fear learning.

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1. Introduction

1.1 Background

Post-Traumatic Stress Disorder (PTSD) is a chronic psychopathology where sufferers experience debilitating fluctuations in emotions, mood, cognition and social abilities (Locci & Pinna, 2019). PTSD and other stress-related disorders were largely overlooked until the diagnostic criteria for these syndromes were included in the Diagnostic and Statistical Manual of Mental Disorders (DSM) III in 1980, after which research into the epidemiology of PTSD formally began (Kessler, 2000). Currently, the DSM-V-TR classifies PTSD as a trauma and stress related disorder characterized as having 4 main symptom clusters: Intrusion (nightmares) and disassociation (flashbacks), Avoidance (of the trauma or memory), Negative changes in mood or cognition, Hyperarousal and reactivity, including but not limited to a lack of concentration, sleep disturbances and irritability (American Psychiatric Association, 2017). These four symptom clusters are often associated with or driven by traumatic memories, with a hallmark feature of PTSD being an inability to extinguish memories associated with the traumatic event. Indeed, if memories hold emotional significance, they are often fully consolidated, easily retrieved and difficult to extinguish over time (Trezza & Campolongo 2013). While this learning process is believed to be a functional component of basic, mammalian threat avoidance systems it is usually experienced as aversive, resulting in maladaptive behaviors and psychological consequences in humans, including the development of PTSD (Trezza & Campolongo 2013; Vigil, 2009). These emotional memories and the resulting symptoms of PTSD can result from exposure to military combat, physical or sexual assault, natural disasters, car accidents, or other types of traumatic events (Kessler, 2000). PTSD occurs in the general population at the rate of 8-12%

but is higher in specific subpopulations; 15% of assault survivors, 19% of veterans, 45% of physically and/or sexually abused women and 50% of physically and/or sexually abused children develop PTSD (Bae et al., 2018; Dardis et al., 2018; Hoge et al., 2014; Kilpatrick et al., 2013; Locci & Pinna, 2019).

PTSD is associated with numerous health risks, both physical and mental in nature. People diagnosed with PTSD typically suffer from additional comorbid psychiatric disorders, with the most common being other anxiety disorders, mood disorders and substance use disorders (Kessler, 2000). There is also an increased risk of suicidal ideation, suicide attempts and suicide completion among people who suffer from PTSD when compared to the general population (Locci & Pinna, 2019; Pompili, et al., 2013). Indeed, there is a stronger association with suicide among patients with PTSD than with any other anxiety disorder and people with PTSD are 6 times more likely to attempt suicide than their non-PTSD counterparts (Kessler, 2000). Physical health problems are influenced both directly and indirectly by the cognitive and behavioral issues associated with PTSD (Pacella et al., 2013). Due to the long-term activation of stress pathways, PTSD sufferers experience decreased immune response, putting them at higher risk for sickness and infection. Additionally, PTSD patients are also less able to fight off contracted illness and thus seemingly mundane illnesses can have devastating and long-lasting effects on their general health (Pacella et al., 2013). PTSD increases the risk of fibromyalgia and other musculoskeletal pain, cardio, and respiratory problems, including but not limited to angina, heart disease, shortness of breath, and asthma, as well as gastrointestinal (GI) problems (Pacella et al., 2013).

People with PTSD also tend to experience higher rates of certain societal consequences, as compared to the general public, contributing to decreases in overall quality

of life. An estimated $3 billion in productivity is lost annually due to work impairment associated with PTSD (Ettner et al., 1997; Kessler, 2000; Kessler & Frank, 1997). Work impairment is generally defined as working less efficiently, or missing part or whole workdays (Ettner et al., 1997; Kessler, 2000; Kessler & Frank, 1997), with PTSD patients missing an average of 3.6 days a month from work (Ettner et al., 1997; Kessler, 2000; Kessler & Frank, 1997). Additionally, PTSD sufferers are often forced to work in lower-income jobs due to the inability to maintain higher paying, stress-related occupations (Jayakody et al., 1998; Kessler, 2000). Many people with PTSD fail to realize their potential in school, marriage, or employment (Kessler, 2000). For example, compared to the general population, patients with PTSD are 40% more likely to fail in high school or college, 30% more likely to have children during their teenage years (Kessler, 2000), 60% more likely to report marital instability and 150% higher risk for unemployment (Kessler, 2000). Collectively, these data highlight the negative psychological, physical and societal impact for individuals suffering from PTSD.

Currently, there are only 2 FDA-approved medications for treating PTSD, the selective serotonin reuptake inhibitors (SSRIs) Sertraline (Zoloft) and Paroxetine (Paxil) (Alexander, 2012; Jeffreys, 2009; Krystal et al., 2017). If Zoloft or Paxil are ineffective or have undesirable side effects, additional off label medications include SSRIs like Fluoxetine (Prozac), SNRIs like Venlafaxine (Effexor) and/or antiepileptic drugs like Topiramate (Topamax; Jeffreys, 2009). There are several novel targets for treating the symptomology and malfunctioned learning processes of PTSD. The most difficult learning process to treat is consolidation because PTSD is typically the result of unexpected trauma that was previously consolidated, leaving therapies to target symptomology, memory retrieval and

reconsolidation and extinction. Beta adrenergic antagonists or beta-blockers treat the symptomology of hyperarousal and have been shown to enhance fear extinction in patients with PTSD (Steckler & Risbrough, 2012). Glucocorticoid receptor agonists impair the retrieval and reconsolidation of fear memories; however only one is approved for clinical use and it is unsafe for unborn fetuses (Steckler & Risbrough, 2012). GABAergic agonists decrease the retrieval and reconsolidation of fear memories (Sartori & Singewald, 2019; Steckler & Risbrough, 2012), while N-methyl-D-aspartate receptor antagonists reduce fear memory retrieval and enhance fear extinction. However, due to their abuse potential and sometimes intense side effects these drugs are not recommended for daily or continued use (Sartori & Singewald, 2019; Steckler & Risbrough, 2012).

Unfortunately, only 38% of people with PTSD are in treatment, of which 22% are treated in the medical sector (i.e., psychiatrist, clinical psychologist, or other mental health professional) with the remaining 16% treated in the human services or self-help sector (Kessler, 2000). The 62% of people with PTSD who are not in treatment often deny that there is a problem, even when they reported significant debilitation from the disorder (Kessler, 2000). Unfortunately, SSRIs help only 50% of PTSD patients, and less than 30% of patients experience full remission even after years of treatment (Berger et al., 2009 Darves-Bornoz et al., 2008; Trezza & Campolongo, 2013). While antidepressants (SSRIs) may help with mood and anxiety in some patients with PTSD there is no approved treatment for the cognitive symptomology of PTSD (Trezza & Campolongo, 2013). Psychotherapy for PTSD includes cognitive behavioral therapy, and eye movement desensitization and reprocessing (Jeffreys, 2009). Various types of psychotherapies have been shown to be effective and, in some cases, even more effective when compared to SSRI's alone. 41-95% of PTSD patients

who receive psychotherapy experience full remission depending on the type of therapy received (Jonas et al., 2013; Watkins et al., 2018). PTSD extinction deficits reduce the effectiveness of psychotherapy; therefore, to improve PTSD treatment, future research that targets novel extinction-enhancing pharmacotherapies that can be used in conjunction with psychotherapies is desperately needed (Locci & Pinna, 2018; Shallcross et al., 2019).

1.2 Cannabis and the endocannabinoid system

Cannabis is a plant that contains over 100 cannabinoids that are non-psychoactive and non-addictive including the most well-known, cannabidiol (CBD, Rock et al., 2017; Russo, 2007). The infamous and main psychoactive compound in cannabis is Δ9 Tetrahydrocannabinol (THC, Rock et al., 2017). Little was known about the pharmacology of cannabis until scientists Dr. Raphael Mechoulam and Dr. Yechiel Gaoni discovered THC in 1964. This discovery subsequently led to the discovery of the endocannabinoid system in 1988, which has been characterized in both humans and animals (Locci & Pinna; Gaoni & Mechoulam 1964; Silver, 2019). The endocannabinoid system includes 2 cannabinoid receptors (CB1 and CB2), 2 main signaling endocannabinoids (Anandamide and 2-arachidonoyl-glycerol), and four enzymes that synthesize and degrade the endocannabinoids (fatty acid amide hydrolase, monoacylglycerol lipase and Alpha/beta domain hydrolases 6 & 12; Cravatt et al., 1996; Di Marzo et al., 2015; Karlsson et al., 1997; Locci & Pinna, 2019; Navia-Paldanius et al., 2012; Pompili et al., 2013; Trezza & Campolongo, 2013).

The two cannabinoid receptors (CB1 and CB2) are believed to be primarily responsible for the pharmacological actions of cannabis (Locci & Pinna, 2019; Pertwee, 2008). Most of cannabis' known properties are employed through the CB1 receptor, which is found in heavy concentrations in the brain, lungs, GI system, and other organs as well as the

muscular system, vascular system, immune system and bone marrow (Locci & Pinna, 2019; Pertwee, 2008). The CB1 receptor is the most abundant type of receptor in the mammalian brain (Locci & Pinna, 2019). Other pharmacological actions of cannabis occur at the CB2 receptor, which is found in heavy concentrations in the immune system, integumentary system, bone, pancreas and spleen (Locci & Pinna, 2019; Pertwee, 2008). CB1 receptors are heavily expressed in areas of the brain that regulate stress, emotions, learning, anxiety-like behaviors and fear learning, like the prefrontal cortex, hippocampus and amygdala (Locci & Pinna, 2019; McPartland et al., 2007; Viveros et al., 2005). Pharmacologically targeting or deleting both CB1 and CB2 receptors plays an incremental change in the development of a suicidal-like phenotype in rodent models (Locci & Pinna, 2019; Rodriguez-Arias et al., 2013; Rodriguez-Arias et al., 2015). Blocking or deleting CB1 receptors impairs the extinction of fear learning and induces an anxious-like phenotype in rodents (Locci & Pinna 2019; Marsicano et al., 2002). CB1 receptor agonists decrease the reconsolidation of fear memories while enhancing fear extinction in rodents (Steckler & Risbrough, 2012). Previous research suggested that CBD does not bind to the CB1 or CB2 receptors, however, a recent study reported that CBD not only binds to both receptors but that CBD's anxiolytic effects on anxiety in rodents directly involve actions at the CB1 and CB2 receptors that cause alterations in the expression of $GABA_{A,}$ glutamate, and noradrenaline (Austrich-Olivares, 2022; Locci & Pinna, 2019). Collectively, CB1 and CB2 receptors regulate stress, anxiety, cognition and learning processes; highlighting the important role the cannabinoid receptors may play in the treatment of PTSD (Austrich-Olivares, 2022; Locci & Pinna, 2019; McPartland et al., 2007; Viveros et al., 2005).

There are two main “endo” or endogenous signaling cannabinoids, Anandamide (AEA) and 2-arachidonoyl-glycerol (2-AG), that activate the CB1 and CB2 receptors (DiMarzo et al., 2005; Locci & Pinna, 2019; Pompili et al., 2013; Trezza & Campolongo, 2013). Endocannabinoids play a significant role in the maintenance of both physical and mental homeostasis due to their activation of CB1 and CB2 receptors located throughout the body (Hill & Gorzalka, 2009; Moriera & Wotjak, 2009; Parolaro et al., 2010; Ruehle et al., 2012; Sharma et al., 2021; Trezza & Campolongo, 2013). Endocannabinoids greatly affect emotional, behavioral, and cognitive processes due to their large concentration in the limbic system (Atsak et al., 2012; Campolongo et al., 2009a; Campolongo et al., 2012; Hill and Gorzalka, 2009; Riedel and Davies, 2005; Trezza & Campolongo, 2013). Increased levels of 2-AG decrease depression, stress and anxiety while, increased levels of AEA decreases anxiety and blocks the reconsolidation of fear memories (Lisboa et al, 2015; Patel et al., 2017; Steckler & Risbrough, 2012). Conversely, chronic stress decreases concentrations of AEA and 2-AG, which is directly linked to depression and PTSD (Hill and Gorzalka, 2009; Locci & Pinna, 2019; Papagianni & Stevenson, 2019). Endocannabinoids, as well as the administration of cannabinoids, also directly affect learning; specifically, consolidation, retrieval, reconsolidation and extinction in both humans and rodents (Atsak et al., 2012; Campolongo et al., 2009a; Campolongo et al., 2012; Marsicano & Lafenetre, 2009; Niyuhire et al., 2009; Trezza & Campolongo, 2013). Collectively, these data highlight the potential utility of targeting endocannabinoids for treating symptoms of PTSD.

There are four enzymes that degrade the two endocannabinoids, fatty acid amide hydrolase (FAAH), monoacylglycerol lipase (MAGL) and Alpha/beta domain hydrolases 6 & 12 (Cravett et al., 1996; Karlsson et al., 1997; Locci and Pinna, 2019; Navia-Paldanius et

al., 2012). FAAH is the enzyme that degrades AEA, and MAGL and Alpha/beta domain hydrolases 6 &12 are the enzymes that degrade 2AG (Cravett et al., 1996; Karlsson et al., 2997; Locci and Pinna, 2019; Navia-Paldanius et al., 2012). It has been well established that increased signaling of AEA and 2-AG through inhibition of FAAH and MAGL decreases anxiety in both human and rodent models (Lisboa et al., 2015; Patel et al., 2017). FAAH plays a role in anxiety through its regulation of AEA signaling; when FAAH is decreased, AEA is increased and reduces anxiety (Gunduz-Cinar et al., 2012; Hill et al., 2009; Locci & Pinna; 2019). Importantly, several FAAH inhibitors have been shown to reduce anxiety in humans (e.g., URB597) and facilitate fear extinction (e.g., AM3506) in rodents (Gunduz-Cinar et al., 2013; Hill & Gorzalka, 2009; Locci & Pinna; 2019). Together, these findings emphasize the role of the endocannabinoid system in the regulation of anxiety and fear learning, suggesting it is a promising therapeutic target to improve PTSD symptomology and facilitate the extinction of fear memories.

1.3 Cannabidiol

While several clinical and preclinical studies have reported that Cannabidiol (CBD) produces anxiolytic actions, the pharmacodynamic profile of CBD is diverse and the complete characterization of the underlying mechanisms are still unclear (Austrich-Olivares, 2022; Vigil et al., 2020). CBD produces agonistic and antagonistic effects at over 65 different cannabinoid and non-cannabinoid target sites (Austrich-Olivares, 2022; Vigil et al., 2020). CBD is a non-competitive negative allosteric modulator at the CB1 receptor and an inverse agonist at the CB2 receptor, an agonist at $5HT_{1A}$ receptors, and a positive allosteric modulator of $GABA_A$ receptors (Austrich-Olivares, 2022; Vigil et al., 2020). Importantly, animal and human studies suggest that some of the anxiolytic effects of CBD are due in part

to its activation of the $5HT_{1A}$ and $GABA_A$ receptors (Austrich-Olivares et al., 2022; Espejo-Porras et al., 2013; Fogaça et al, 2014; Linge et al., 2016; Patel et al., 2017; Rock et al., 2017; Zanelati et al., 2020). CBD also acts as an indirect endocannabinoid agonist by increasing levels of AEA and 2-AG via blocking reuptake and inhibiting their breakdown, thus stimulating the release of endocannabinoids (Vigil et al., 2020). CBD inhibits FAAH, preventing the breakdown of AEA and thus decreasing anxiety through increased AEA levels (Fogaça et al, 2018). CBD also inhibits the degradation of 2-AG by MAGL thus decreasing anxiety through increased 2-AG levels (Papagianni & Stevenson, 2019). CBD disrupts the retrieval and reconsolidation of fear memories and extinction of fear memories is potentiated by the administration of CBD in both human and rodent models (Locci & Pinna 2018; Papagianni & Stevenson, 2019). Thus, via its action at CB1, CB2, $5HT_{1A}$ and $GABA_A$ receptors, CBD may be a potential treatment for PTSD due to its antidepressant and anxiolytic properties as well as its effects on learning processes (Austrich-Olivares et al., 2022).

1.4 Cannabis and PTSD

The main reasons people report not seeking treatment for PTSD are expense/costs, thinking PTSD will heal itself over time and the desire to heal oneself (Kessler, 2000). Cannabis could possibly address each of these issues. Cannabis allows people to treat themselves and cannabis is less expensive than conventional pharmaceuticals; especially for those without access to health coverage. If you live in a state where it is legal to grow your own *Cannabis* plants, it decreases the cost even more and makes cannabis easily accessible. It is cheaper and easier to grow your own medication at home than it is to go to the doctor and have prescriptions filled, even more so for those in rural areas. Cannabis could also be

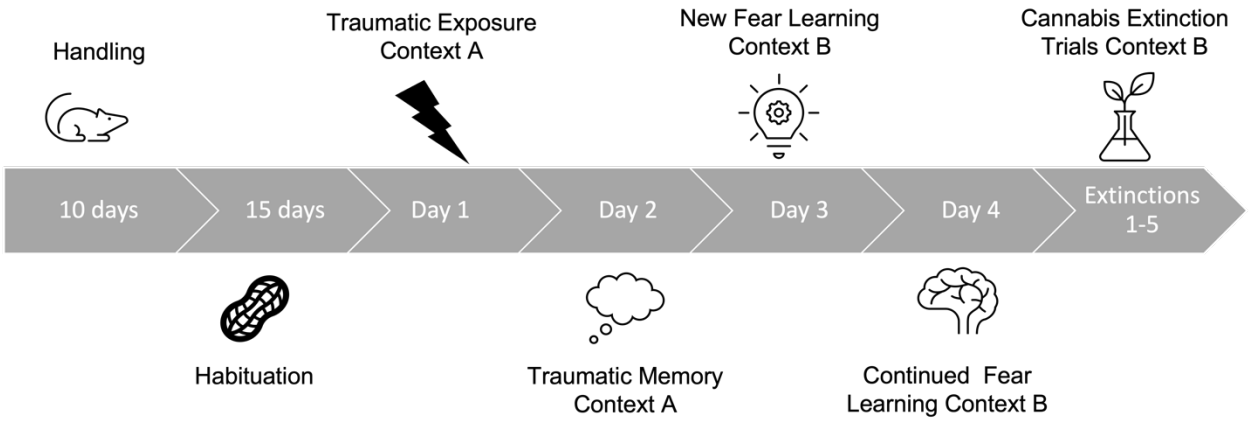
more effective in some cases; where conventional pharmaceuticals can take weeks before seeing an effect, a clinical study recording real-time cannabis use found a reduction in feelings of irritability/agitation and stress, and an increase in mood, nearly immediately following the use of cannabis (Stith et al., 2020; Stith et al., 2018; Vigil et al., 2022). There is a large and growing population that believes cannabis is safer and has fewer side effects than many classes of conventional pharmaceuticals; dry mouth and feeling foggy are the two most commonly reported adverse side effects of cannabis consumption (Li et al., 2019). Collectively, these data suggest that cannabis could be used to treat PTSD, with one remaining large unanswered question being, does cannabis facilitate extinction of PTSD associated memories.

While cannabis has shown potential in the treatment of several psychiatric disorders, it does not come without risk. The DSM-V-TR characterizes cannabis use disorder (CUD) as a "problematic pattern of cannabis use leading to clinically significant impairment or distress" (American Psychiatric Association, 2017). Some of the symptoms include but are not limited to a strong urge to use cannabis, spending too much time obtaining, using, or recovering from cannabis, social withdrawal, responsibility failures at school, work, and/or home, unsuccessful discontinuation of cannabis, tolerance, and withdrawal (American Psychiatric Association, 2017). People with PTSD are 3 times more likely to have CUD than those without PTSD and military veterans have an even higher rate of cannabis usage (Bonn-Miller et al., 2011; Trezza & Campolongo, 2013; Stewart et al., 1998). Further research is needed to fully elucidate whether this pattern of cannabis use is truly an addiction or if it more accurately reflects self-medication.

In summary, both human and rodent studies indicate that in general cannabis regulates emotional, cognitive and learning processes suggesting that hemp oil or CBD-concentrated *Cannabis* plant extract may be a promising novel treatment for PTSD (Austrich-Olivares et al., 2022; Espejo-Porras et al., 2013; Fogaça et al, 2014; Linge et al., 2016; Rock et al., 2017; Zanelati et al., 2020). Specifically, by its action at the CB1, CB2, $GABA_A$ and $5HT_{1A}$ receptors CBD can produce antidepressant and anxiolytic properties thus highlighting its potential as a novel treatment for PTSD. Therefore, in the present study, we will utilize the stress enhanced fear memory model to examine the effects of full spectrum hemp oil on extinction of fear memory in adult male rats. We hypothesize that the administration of full spectrum hemp oil to rats prior to extinction exposure will facilitate extinction of fear memory.

2. Materials and methods

FIGURE A: Timeline



The experimental design consists of 10 days of handling, 15 days of habituation, 1 day of trauma exposure, 1 day of traumatic memory testing, 1 day of new fear learning, 1 day of new fear memory testing and 5 extinction trials, 1 week apart.

2.1. Animals

Subjects were experimentally naïve male Long-Evans hooded rats (N=35, PND 110-111 at the time of trauma exposure). Rats were acquired from Envigo and all husbandry occurred in the Logan Hall Animal Research Facility in the Psychology Department at the University of New Mexico. Rats were pair housed in standard rat cages (21.6 x 45.7 x 17.8 cm) until 3 days prior to the trauma exposure when they were then single housed in standard home cages for the duration of the experiment. The colony room has a reverse 12:12-h light-dark cycle (with the lights turning off at 10 am) and is temperature controlled (21-24°C). Food and water were available *ad libitum* in the rat's home cages. Experimental and husbandry procedures followed the Guide for the Care and Use of Laboratory Animals and were adopted by the University of New Mexico's Institutional Animal Care and Use Committee (National Research Council, 2011). Prior to the beginning of the trauma exposure, rats were handled for 10 days for 60-90 seconds each day to adapt to experimenter handling (Rajvhandari, et al., 2018). After handling, rats were habituated for 15 days to peanut butter, which will be used as the vehicle for cannabis administration. Next, rats were randomly assigned to one of four groups: no-trauma/control, no-trauma/hemp, trauma/hemp and trauma/control. Upon completion of the experiment, rats were humanely euthanized using CO_2.

2.2. Drugs

Full-spectrum hemp oil was administered using a peanut butter vehicle. Briefly, the cannabis goes through an extraction procedure where the cannabinoids, terpenes, lipids, chlorophyll and wax compounds are stripped from mature hemp flower ("buds") using an

ethanol bath. Once all compounds of the plant are extracted from the plant and are in the alcohol solution, the solution is poured through a strainer to remove any plant material and is filtered until free of particles. Next, the alcohol is evaporated, leaving only the cannabis compounds in an ultra-concentrated form. This ultra-concentrate is then combined with medium-chain triglyceride (MCT) oil to increase its bioavailability, for its final retail form. Rats assigned to the two hemp groups received an ultralow dose, 1 μL or approximately .002mg/kg, of full-spectrum hemp oil (Fishbein-Kaminietsky et al., 2014) dissolved in a peanut butter (.152 g) vehicle; rats in the two control groups received the peanut vehicle. Peanut butter was chosen for the vehicle as it is high in fat and cannabis is fat soluble, thus increasing the bioavailability of the hemp oil.

2.3. Stress-Enhanced Fear Learning

The Stress-Enhanced Fear Learning model was adapted from an animal model of PTSD created by Rau et al. in 2005 in Dr. Michael Fanselow's lab at the University of California Los Angeles. Most rodent models studying PTSD use traditional fear conditioning, however, in most cases, these models are often too simple to capture all of the complexities and symptomology of PTSD. While no animal model can capture all of the human complexities associated with PTSD, this model captures 3 critical components. First, in this model, PTSD is caused by an un-cued, single traumatic event, realistic to the development of PTSD in humans (Long & Fanselow, 2011; Rajbhandari et al., 2018; Rau, et al., 2005). Second, this model affects new fear learning, shown by the exaggerated fear response to a new context, similar to symptomology seen in PTSD patients (Long & Fanselow, 2011; Rajbhandari et al., 2018; Rau, et al., 2005). Third, this model leads to lasting behavioral changes shown through the resistance to extinction (Rajbhandari et al., 2018). To our knowledge, no other research

has been conducted using this model to study the effects of cannabis on the extinction of stress-enhanced fear learning.

Stress-Enhanced Fear Learning took place in two distinct contexts (A & B) that differ in lighting, sound and odor. We also used specific methods such as distinct methods of transportation, odor, lighting, noise and flooring to differentiate the contexts to reduce fear generalization to the novel context (B). Each context consists of a larger sound attenuating chamber that houses a smaller fear-conditioning shock chamber (25.4 X 29.21 X 29.21 cm; Coulbourn Instruments LLC; Whitehall, PA) that differ in internal layout, floor pattern and scent (Rajbhandari et al., 2018). Context A consist of a while visible light and a fan at 60 dB for background noise (Long & Fanselow, 2011; Rau et al., 2005). The shock chamber inside context A consist of smooth steel rod flooring with 27-4mm diameter steel bars arranged vertically every .635 cm; 1% acetic acid was used to clean the box and underneath pan after each use and then 11% coconut extract was used to scent the pan underneath (Long & Fanselow, 2011; Rajbhandari et al., 2018; Rau, et al., 2005). Context B consist of red lighting and a white noise generator set at 60 dB for background noise (Long & Fanselow, 2011; Rajbhandari et al., 2018; Rau, et al., 2005). The shock chamber inside context B has 2 black plexiglass side walls at a 60° angle to create an A-frame and has heavily textured steel rod flooring with 18-6mm diameter steel bars arranged vertically every 1.27 cm (Long & Fanselow, 2011). 5% ammonium hydroxide was used to clean the box and underneath pan after each use and some of the cleaning solution was left in the pan underneath for scenting purposes (Rau, et al., 2005). The two contexts were further differentiated by modes of transportation. Rats were transported in their home cage on a cart, uncovered, to context A

and in a clean, empty cage, covered and transported by hand to context B (Rajbhandari et al., 2018).

Day one or traumatic exposure day consist of 2 different groups, trauma and no trauma. Trauma rats were placed into the shock box in context A and received 240 seconds of pre-exposure to the environment. After this pre-exposure period, rats received 15 1-second footshocks (1milliamp) randomly over a 90-minute period (Long & Fanselow, 2011; Rajbhandari et al., 2018; Rau, et al., 2005). Rats in the no-trauma group were placed in the shock box for the same amount of time but did not receive any footshocks (Long & Fanselow, 2011; Rajbhandari et al., 2018; Rau, et al., 2005). Rat behavior will not be recorded on day one.

On day 2, rats were placed into context A for 8 minutes without receiving any footshocks. Rat behavior was recorded to quantify freezing as the primary index of fear (Blanchard et al., 1969; Fanselow, 1980). Freezing in this context reflects the fear memory associated with the trauma.

On day 3, all rats were placed into context B for a 180-second baseline period (Rajbhandari et al., 2018). Next, all rats received a single 1-second footshock (1milliamp) and remained in the box for 30 seconds following the shock (Rajbhandari et al., 2018). Rat behavior was recorded on this day for later analyses of freezing.

On day 4, rats were placed into context B and behavior was recorded for 8 minutes (Rajbhandari et al., 2018). Freezing in context B reflects stress enhanced fear learning from context A.

Extinction began one week after day 4 and occurred once a week for 5 weeks. Rats were pretreated with their assigned drug 2 hours prior to being placed in context B for an 8 minute

extinction session to examine the effects of full spectrum hemp oil on the extinction of stress-enhanced fear-learning behavior (Deiana et al., 2011).

2.4. Data Analysis

Freezing is operationally defined as the lack of movement except for respiration (Fanselow, 1980). Percentage freezing was calculated by hand for day 2 and day 4 as well as both baseline and aftershock periods on day 3 and all 5 extinction trials. To score freezing by hand, an experimenter observed the animal throughout the period of interest and noted the percentage of time freezing vs time spent moving. Percent freezing was calculated using the formula [(A1-A2)/A1] *100 where A1 represents the total time spent in the context while A2 represents the time spent moving in the context. In order to verify that the SEFL model worked, separate independent samples t-test were run comparing trauma and no trauma groups on measures of percent freezing. First, we analyzed fear to the trauma context on day two, confirming the traumatic memory was still intact. Next, we analyzed the generalization of fear to the novel context during baseline on day 3 as well as exaggerated fear response after the shock on day 3. Lastly, we analyzed fear learning on day 4. In order to examine the effects of full spectrum hemp oil on rates of extinction, we used a repeated measures ANOVA to analyze freezing across the 5 extinction trials.

3. Results

Figure 1 indicates that rats in the trauma group froze significantly more in context A on day 2 compared to their non-trauma counterparts suggesting the memory of the traumatic event was intact ($t(1, 23.64) = 15.86$, $p<.001$, $g=-4.92$). Importantly, there was no significant difference between groups during the baseline period before the single shock on day 3 indicating that context A and B were sufficiently different and no fear generalization between

the two contexts occurred (see Figure 2; $t(1, 22) = 2.02$, $p>.05$, $g=-.63$). Figure 3 indicates that in the 30 seconds following the single 1-second 1-milliamp footshock, rats from the trauma group froze significantly more than their non-trauma counterparts ($t(1,33) = 6.82$, $p<.001$, $d=-2.32$), suggesting that previous exposure to the traumatic stressor creates an exaggerated fear response to the mild stressor. As shown in Figure 4, the trauma group froze significantly more than their non-trauma counterparts indicating that the exposure to the traumatic experience on day 1 enhanced fear learning to the mild stressor on day 4 ($t(1,33) = 7.88$, $p<.001$, $d=-2.68$).

The impact of full spectrum hemp oil on extinction over the course of the 5 extinction trials is shown in figure 5. The repeated measures ANOVA testing the effects of full spectrum hemp oil on extinction detected a significant main effect of extinction ($F(4,124) = 20.87$, $p<.001$) as well as an extinction by shock interaction ($F(4,124) = 18.48$, $p<.001$); however no significant interaction was found between extinction and hemp ($F(4,124) = .85$, $p>.05$) or the three way interaction between extinction, shock and hemp ($F(4, 124) = .28$, $p>.05$). This suggest that full spectrum hemp oil did not facilitate rates of extinction.

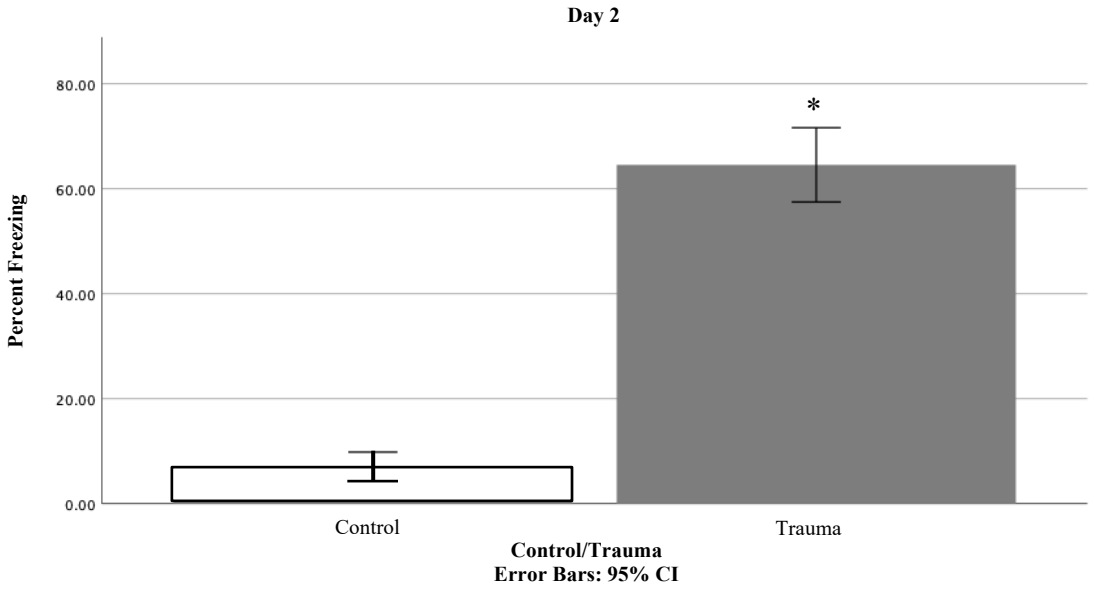


Figure 1: Freezing in context A on day 2. The trauma group froze significantly more than the control group suggesting the memory of the traumatic experience was still intact. Asterisk (*) represents a significant difference compared to controls (p<.001).

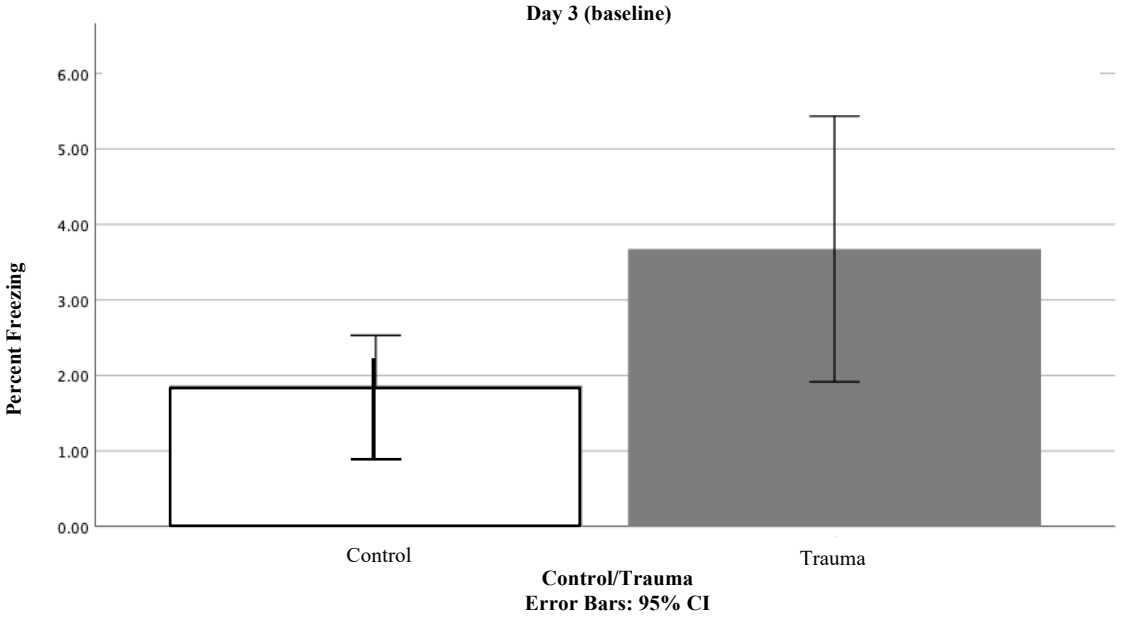


Figure 2: Freezing in context B on day 3 baseline. There was no significant difference between the two groups (p>.05), suggesting that there was no fear generalization between the two contexts.

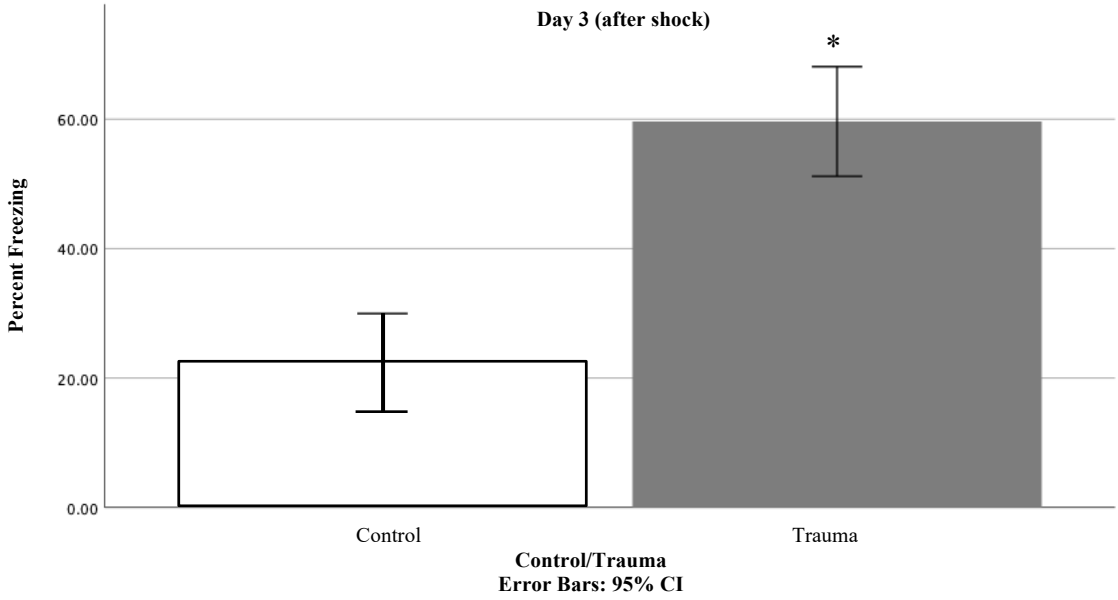


Figure 3: Freezing after the single shock on day 3. The trauma group froze significantly more than the control group immediately following the single shock on day 3 indicating an exaggerated fear response. Asterisk (*) represents a significant difference compared to controls ($p<.001$).

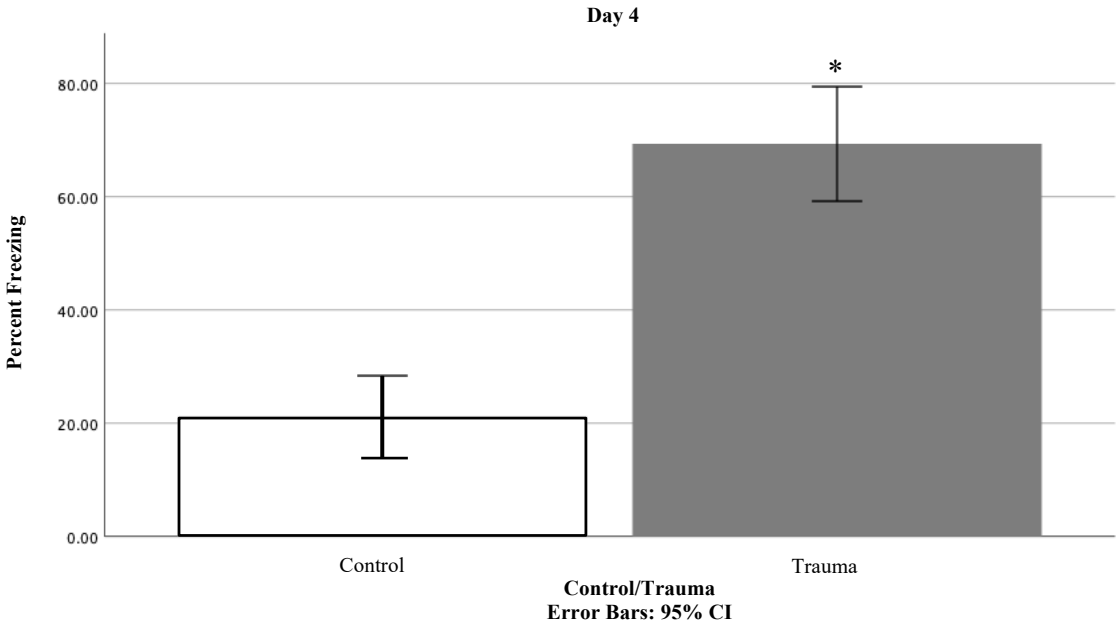


Figure 4: Freezing in context B on day 4. The trauma group froze significantly more than the control group indicating enhanced fear learning. Asterisk (*) represents a significant difference compared to controls ($p<.001$).

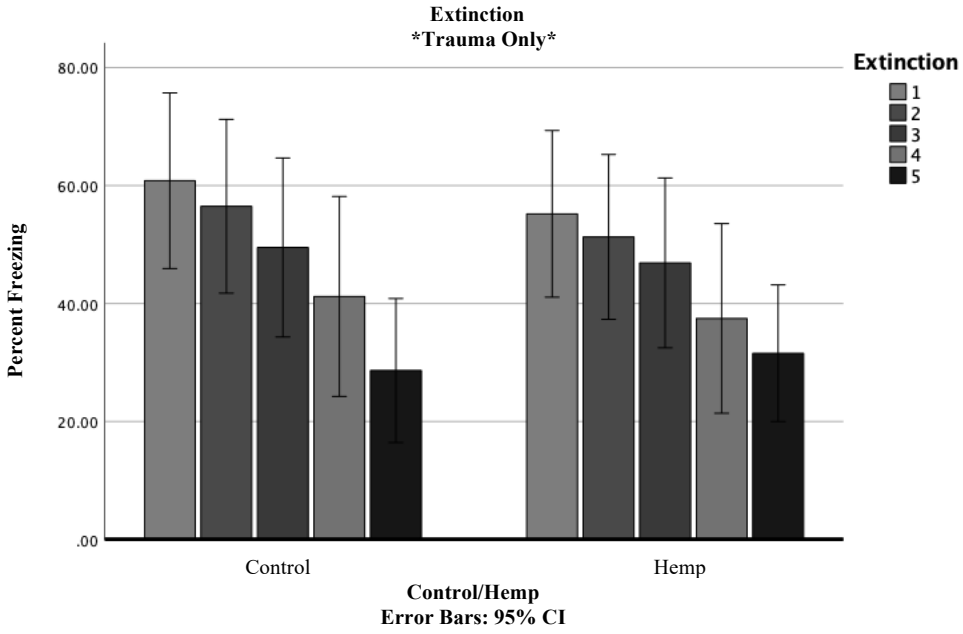


Figure 5: Freezing in context B over 5 extinction trials. There were no significant differences between the groups suggesting that full spectrum hemp oil did not facilitate extinction.

4. Discussion

Our results replicate previous reports that the SEFL model is a robust, rodent model of PTSD in male Long Evans rats (Long & Fanselow, 2011; Rajbhandari et al., 2018; Rau, et al., 2005). As expected, the trauma group showed higher levels of freezing when returned to the traumatic context on day 2 (Figure 1), indicating that the memory of the traumatic stressor is intact. Both groups showed minimal fear generalization to the novel context (B) during the baseline period on day 3 (Figure 2), suggesting that the enhanced freezing that

occurs in this context is due to the mild stressor interacting with the prior trauma and is not generalization from the trauma context. Rats exposed to the traumatic stressor showed increased freezing following the single shock on day 3 (Figure 3) as well as when they were returned to context B on day 4 (Figure 4), indicating a stress enhanced fear response or stress enhanced fear learning. It should be noted that this enhanced fear response is not due to generalization or increased fear expression as the traumatic stressor must come before the mild stressor to increase fear of the novel context (Rau et al, 2005). Collectively, these data and results from previous studies (Long & Fanselow, 2011; Rajbhandari et al., 2018; Rau, et al., 2005) indicate that the SEFL model produces reliable results and long-lasting enhanced fear learning that are consistent with the symptomology seen in PTSD patients, suggesting it is a valid rodent model of PTSD.

Contrary to our prediction, results from the present study indicate that full spectrum hemp oil (.002 mg/kg) did not facilitate extinction of stress enhanced fear learning (Figure 5) across the five extinction trials. One possible reason we failed to detect a significant effect is that we may have been using an insufficient dose. A study by Franzen et al. found an effect on fear memory retrieval and expression as well as anxiety using substantially higher doses (3 mg/kg and 10 mg/kg; Franzen et al., 2023) than the current dose. However, in an unpublished pilot study conducted in our lab using the lower dose of .002 mg/kg we found comparable results to Franzen et al.'s study on anxiety-like behavior. However, because the EPM and fear conditioning models reflect different underlying constructs (i.e., anxiety vs. fear), as well as the different procedures between the fear conditioning procedures in the two studies (i.e., stress enhanced fear learning vs. classic fear conditioning), a higher dose may be needed to reduce freezing in our model.

A second possible reason we did not detect effects of full spectrum hemp oil on the facilitation of extinction of stress enhanced fear learning might be that cannabis alters locomotor activity. CBD is thought to have little to no effects on locomotion except in certain cases where locomotor activity is altered, in these cases CBD is thought to balance out these deficits. For example, one study suggests that CBD increases locomotor activity after a spinal injury where other studies suggest that CBD decreases locomotor activity in cases of Tardive Dyskinesis as well as from cocaine-induced hyperactivity (Kajero et al., 2020; Kwiatkoski et al., 2011; Ledesma et al., 2021). CBD is also thought to decrease catalepsy and oral movements in patients with Parkinson's Disease (Calapai et al., 2022). Research on the effects of CBD on spontaneous locomotor activity is very mixed. One study concluded that in rodents, CBD does not affect locomotor activity in the rotarod test, however, it decreased locomotor activity in the open field test (Schleicher et al., 2019). This directly contradicts another study that reported CBD did not alter locomotor activity in the open field test (Viudez-Martínez et al., 2018). Other studies suggest that CBD does not affect locomotor activity in test like the Light/Dark test or the Elevated Plus-Maze however, they found that CBD did increase time freezing in both the Conditioned Emotional Response test as well as in Fear Conditioning models (Calapai et al., 2022; ElBatsh et al., 2011; Koo & Duman, 2009). This contradicts Franzen et al.'s study that found CBD decreased freezing in a standard contextual fear conditioning model (Franzen et al., 2023). In our study, the full spectrum hemp oil treated rats did not show increased time freezing when compared to their non-hemp oil counterparts however, the possible effect of CBD on freezing in Fear Conditioning models may be a reason why we found no effect of full spectrum hemp oil on the facilitation of extinction of stress enhanced fear learning.

A third possible reason we did not detect effects of full spectrum hemp oil on the facilitation of extinction on stress enhanced fear learning maybe due to what is known as the "entourage effect". The entourage effect is when all the 100 or more compounds in the cannabis plant are combined, including cannabinoids, flavonoids and terpenes, and are hypothesized to work together to produce the desired effects of cannabis (Koltai & Namdar, 2020). However, Federal laws limits the legal amount of THC in any type of *Cannabis* plant to equal to or less than 0.3% weight/dried flower (Wakshlag et al., 2020). Thus, in the present study we looked at is an opportunity to explore a full spectrum hemp oil containing less than 0.3% THC as there is a large population of people who like the health effects of cannabis without the psychoactive effects of THC and appreciate the federal legality of hemp oil. However, it may be possible that a higher percentage of THC maybe needed for the entourage effect to take effect in the facilitation of extinction of stress enhanced fear learning. Indeed, THC content may need to be higher because studies suggest it has a greater impact on memory as compared to CBD. A recent study looked at THC to CBD ratios and their effects on memory and found that cannabis use with higher THC content had the greatest effect on memory; therefore, it is possible that a higher THC content would better facilitate the extinction of stress enhanced fear learning (Curran et al., 2020). One concern about increasing the THC content is that it could possibly cloud the results as THC also has stronger effects on locomotion (Schramm-Sapyta et al., 2007).

Finally, we may have failed to detect an effect of full spectrum hemp oil on the facilitation of extinction as a result of the extinction protocol we employed. First, hemp oil may have enhanced extinction rates if they were run consecutively across days instead of weekly. Lastly, it is also possible that more than 5 extinction sessions, either run

consecutively or weekly, could be needed to for hemp oil to produce an effect on rates of extinction. Future studies are needed to address these possible explanations.

5. Conclusion

In summary, the present results indicate that full spectrum hemp oil did not facilitate the extinction of stress enhanced fear learning. Given that THC may have greater effects on memory, a broader THC:CBD ratio dose range should be investigated using this experimental design. Previous studies have reported conflicting results with CBD impacting locomotion while other studies report no change in locomotion (Calapai et al., 2022; ElBatsh et al., 2011; Koo & Duman, 2009; Schleicher et al., 2019; Viudez-Martínez et al., 2018). Research needs to be conducted to understand the relationship between CBD and locomotion as this could impact the present results.

While we found no effect of full spectrum hemp oil on extinction in males, Franzen et al., found an effect in females suggesting sex may play a significant role on how cannabis effects the extinction of fear memories. Thus, future research examining the effects of hemp oil on extinction of stress enhanced fear learning in female rats is needed.

Lastly, a promising direction of PTSD research could combine cannabis with other pharmacotherapies and psychotherapies. This approach might yield a more effective PTSD treatment as we know monotherapy is rarely successful in the treatment and remission of PTSD in patients. Future research should continue to investigate the link between the endocannabinoid system and its influence on the treatment of PTSD. Replication and extension of the present results using additional doses and or cannabis combinations are needed to fully clarify if cannabis represents a viable treatment for PTSD.

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