The Effects of Cannabidiol and Serotonin on Anxiety-Like Behavior in Crayfish

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ABSTRACT

Cannabidiol (CBD) is a cannabinoid that is being used as a new treatment for many mental and physical medical conditions, including anxiety. Serotonin (5-HT) is a neurotransmitter that has been shown to have many roles, including stabilizing mood. Crayfish are invertebrate animals that have been used as a model system to study human diseases. Crayfish are well suited to study the effect of CBD on anxiety due to their documented endocannabinoid system and anxiety-like behavioral traits. In this study, the effects of CBD on anxiety-like behavior in crayfish were investigated using a Light-Dark maze as a behavioral analysis tool. At the doses used, CBD did not independently show any influence on either the Light-Dark preference or the total position moves made by crayfish. 5-HT showed quite variable results compared to the saline solution. 5-HT injection significantly decreased time spent in the dark for crayfish injected with 2µg/g of CBD and significantly decreased movement for crayfish injected with 4µg/g of CBD. The dichotomous behavior of the 5-HT injected crayfish requires more study to determine if underlying factors can explain the varying responses. The concentrations of CBD may also need to be increased to properly examine its role in reducing anxiety.

Introduction

Cannabis Sativa

The *Cannabis sativa* plant has been known to have medicinal qualities for thousands of years. Within the cannabis plant, there are unique compounds called cannabinoids; the most common cannabinoid varieties are THC (tetrahydrocannabinol) and CBD (cannabidiol), but there have been over one hundred different types identified (Hopp, et al. 2019). The term "marijuana" refers to a particular type of *Cannabis sativa* that has greater than 0.3 % of THC (Fetzner, 2021), otherwise the plant is called "hemp". Marijuana remains a regulated substance by the federal government, but hemp is exempt from those prohibitive regulations.

Cannabis is a newly emerging treatment for conditions impacting mental and physical health. The number of patients that are prescribed medicinal cannabis or individual cannabinoids is a growing statistic. The numerous effects that cannabis consumption has are still being researched (Hopp, et al. 2019). Ranging from pain management to anxiety to retention of information, cannabis can play a major role in the neurological systems of humans and animals. This is because cannabinoids can bind to receptors that have been identified in many species, CB1 and CB2. These receptors are part of an endogenous system known as the endocannabinoid system that is implicated in regulating processes such as metabolism and memory (An, et al. 2020). This is a burgeoning research field and a hot topic in many areas of medicine.

Effect of CBD on Anxiety and Stress

Cannabidiol, abbreviated as CBD, is a specific cannabinoid that has emerged as a new medical treatment for certain disorders, ranging from neurological to autoimmune disorders (Timmer, 2020). It has been shown that individuals suffering from anxiety-related disorders benefit from using CBD as it is associated with reduced heart rate and blood pressure, calming the body system and reducing overall stress levels (Blessing, et al. 2015). This is typically in lower and moderate doses, though, and higher doses can cause adverse effects and even worsen anxiety. Dosage is critical to the effectiveness of CBD, making research to clarify an appropriate dosage carry a high level of importance (Blessing, et al. 2015).

New research has additionally supported cannabidiol as a treatment for anxiety. In an article by Melas, et al., the authors discuss using animals, such as mice and rats, in a plus-shaped maze to observe their anxiety-like behavior after being administered CBD. After review of many articles examining the effects of CBD on behavior, it was concluded that "CBD is considered a promising new agent in the treatment of anxiety and mood disorders" (Melas, et al. 2021).

Crayfish Model System

Freshwater crayfish are invertebrates in the crustacean family, and astacology is the study of crayfish. Crayfish have adapted to many different environments and serve various purposes ranging from being a nutritional source of protein for humans and other animals to helping preserve balance in many ecosystems (Fetzner, 2021).

For over two decades, crayfish have been used as a successful model system in experiments (Hossain, et al. 2018). This is because they are known for having a wide range of behaviors that allow them to be studied for physiological, neurological, developmental, ethological, and other varieties of scientific research. They display a wide range of behaviors that often parallel those of humans (Hossain, et al. 2018).

Crayfish Behavior Relating to Stress and Anxiety

It has been suggested that crayfish mood can be analyzed by observing their behavior using a Light-Dark maze. It has been found that when crayfish are stressed, they prefer the darker areas and will avoid the lit areas (de Abreu, et al. 2020) (Hossain, et al. 2018). Crayfish naturally dwell in darker areas as darkness assists with predator evasion, and so it fits that only an unstressed crayfish would explore the lit areas.

In humans, serotonin (5-HT) was found to be a mood boosting hormone, to increase levels of happiness, and to regulate emotions (Bancos 2018). Fossat, et al. discovered that giving crayfish injections of 5-HT mimics the behavioral and biochemical responses caused by electrical shocks. With either 5-HT or electrical shocks, there is a decreased exploration of the maze, greater amount of time spent in the darker area of the maze, and an increase in blood glucose levels (Fossat, et al. 2014). Therefore, it is believed that 5-HT injections can be used to induce stress in crayfish.

Hypothesis

If crayfish are administered an appropriate dose of CBD, they will exhibit signs of decreased anxiety and stress after a 5-HT induction of an anxiety-like state. This will be assessed through behavioral analysis of their preference for lit or dark areas of a Light-Dark maze.

Methodology

The research was conducted in the physiology lab at the University of St. Francis in Joliet, Illinois. *Procambarus clarkii* were purchased from Carolina Biological Supply Company and housed two per tank in 10-gallon tanks. Each tank contained: 2 terra cotta clay pots to act as a shelter, pebbles on the bottom of the tank, a filter to clean the water, a bubbler for the water, and a lid on the top that kept the crayfish from escaping. Crayfish were fed shrimp pellets 2-3 times per week, and crayfish were housed for a minimum of five days before being used in the experiment.

There were eight experimental crayfish treatments, with six to eight crayfish receiving each treatment (Table 1). The experimental groups were: stress-induced (5-HT injected) or non-stressed (saline-injected), and each group of crayfish was treated with four different doses of CBD.

Before administration of the CBD and/or 5-HT, the crayfish were weighed (in grams) to determine the proper dosage. The CBD was administered in the aforementioned four different amounts of 0 µg/g, 2 µg/g, 4 µg/g, and in 6 µg/g. After CBD injection, stress-induced crayfish received 5 µg/g 5-HT. Unstressed crayfish received an injection of the delivery vehicle, which was a saline solution: Van Harrevelds Solution. The saline solution was made with 195 mM NaCl, 5 mM KCl, 13 mM CaCl2, 2 mM MgCl2, and 3 mM HEPES, pH 7.65 (Timmer, 2020). This solution was made prior to the experiment and used for the entire course of the experiment. All crayfish were injected dorsally between two body segments directly into the hemolymph.

Table 1. The Eight Different Groups of Crayfish.

Crayfish	Group	n	Cannabidiol Amount	5-HT Amount
Stress-induced	1	8	0 µg/g	5 µg/g
	2	8	2 µg/g	5 µg/g
	3	8	4 µg/g	5 µg/g
	4	6	6 µg/g	5 µg/g
Non-stressed	5	6	0 µg/g	Saline Solution
	6	6	2 µg/g	Saline Solution
	7	6	4 µg/g	Saline Solution
	8	6	6 µg/g	Saline Solution

The crayfish were put individually into the Light-Dark maze during their trial. The maze had four tunnels, two with light shining in them and two dark as shown in Figure 1, and the maze was submerged in water in a plastic pool. The length of each of the four tunnels was approximately twenty-five centimeters, and the width of each tunnel was approximately fifteen centimeters. The “middle” section of the maze was a square with fifteen centimeters per side. The floor of the maze had white pebbles on the bottom that were leveled out to create a uniform background for the crayfish throughout the maze.

After the appropriate injections, the crayfish was placed into the Light-Dark maze under an opaque box to acclimate for ten minutes in the middle of the maze where all four tunnels overlap. After 10 minutes, the vessel was lifted and removed, and the crayfish was free to explore the Light-Dark maze for 30 minutes. The crayfish was in the experiment tank for forty minutes total. Once in the tank, the researchers recorded the movement of the crayfish. The crayfish was recorded to be in either Light 1, Light 2, Middle, Dark 1, or Dark 2 positions (Figure 2). The researchers analyzed the movements without the aid of software.

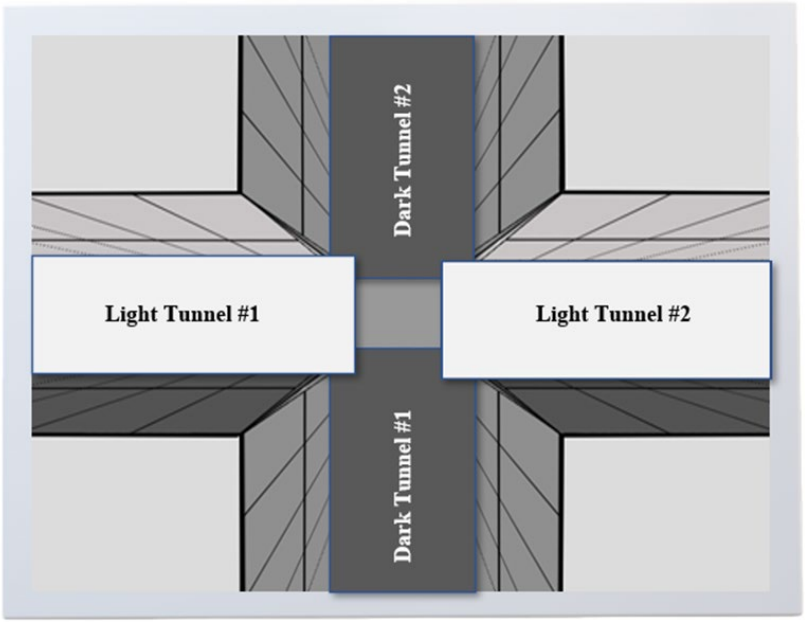

Figure 1. The Configuration of the Light-Dark Maze

Date:_____ Time:_____ Tank:_____
Weight:_____CBD:_____ 5HT:_____

Light 1	Light 2	Middle	Dark 1	Dark 2	Time

Figure 2. The Log of Crayfish Information Kept During Trials.

Results

In all instances, the crayfish spent more time in the dark than any other position within the maze. The average amount of time spent in the dark ranged from 1493 to1571 seconds out of the 1800 seconds spent in the maze (Figure 3). The lit area was the next most frequented area of the maze, although crayfish in each group spent, on average, less than 200 seconds there. The amount of CBD injected did not significantly impact the amount of time spent in the dark versus lit sections of the maze.

While all groups showed, on average, a decreased amount of time in the dark after 5-HT injection, this was only statistically significant for the 2μg CBD group (p-values of 0.10, 0.03, 0.07, and 0.36 for 0, 2, 4, and 6μg CBD respectively) (Figure 4). The 2μg group injected with 5-HT spent 443 seconds less in the dark, on average, than the 2μg group injected with saline. Just as shown in Figure 1 for the non-5-HT treated groups, the CBD injection did not appear to impact the time spent in the dark versus lit sections of the maze for the 5-HT treated groups.

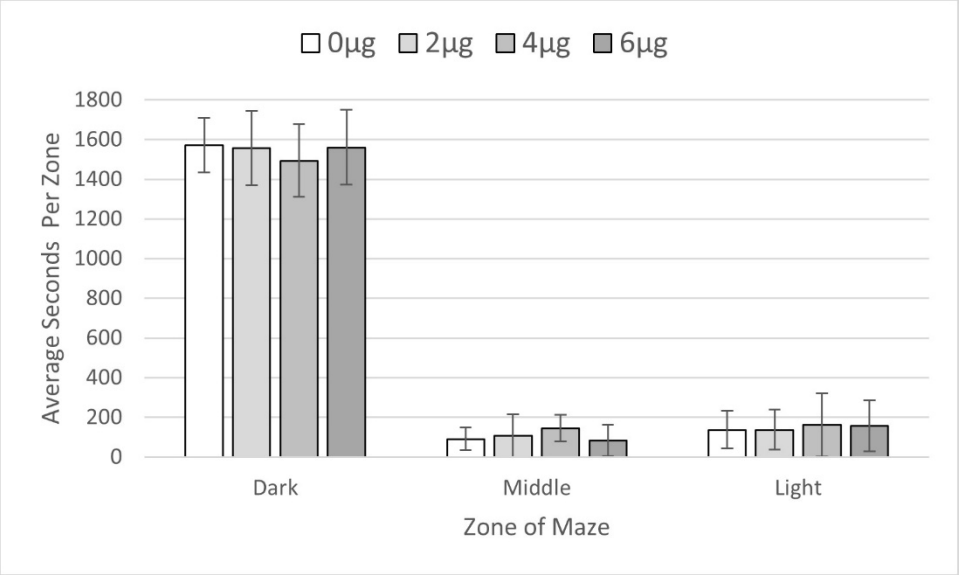

Figure 3. Average Time Spent in Each Area of the Maze After CBD Injection. Either saline or 2, 4, or 6 micrograms of CBD were injected into each crayfish (n=6 in each group). Each also received a second injection of saline. No significant differences were found amongst the concentrations using a one-way ANOVA comparing times in the dark.

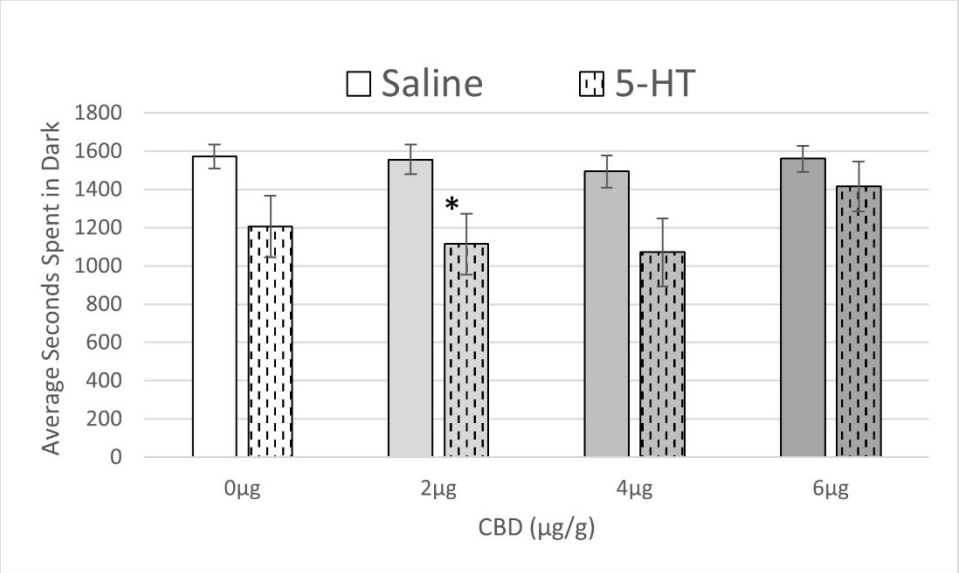

Figure 4. Average Time Spent in the Dark After CBD Injection and Either Saline or 5-HT Injection. Either saline or 2, 4, or 6 micrograms of CBD were injected into crayfish. Each also received a second injection of saline (n=6 in each group) or 5-HT (n=8, 8, 8, and 6). A two-tailed t-test was performed to compare saline vs. 5-HT for each CBD group (for 0, 2, 4, and 6µg CBD; p= 0.10, 0.03, 0.07, and 0.36 respectively)

When the total position changes were counted, there was great variability amongst the groups. When assessing the 5-HT treatment, it only appeared to have an effect when paired with the 4µg dose of CBD; the 5-HT treated group moved, on average, 7 times less than those not treated with 5-HT (Figure 5). CBD did not show any effect on the total position changes, which ranged from 7.9 to 15.7 moves within the 1800 seconds in the maze.

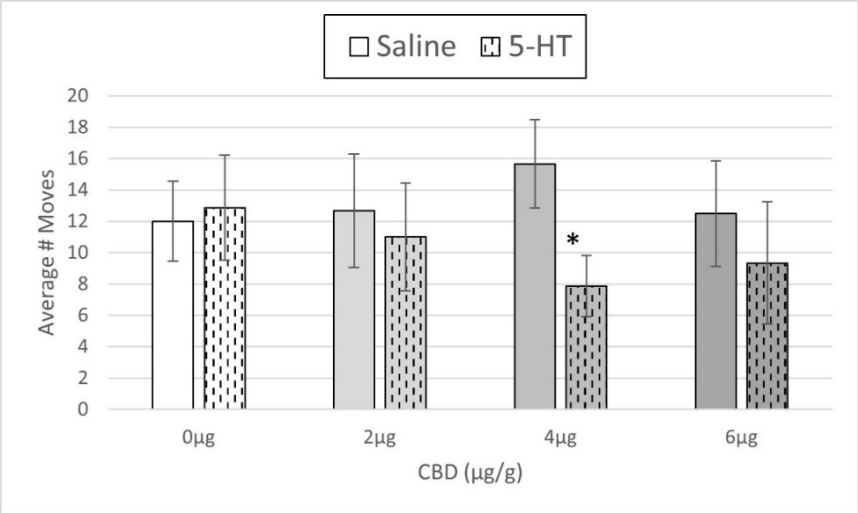

Figure 5. Average Moves After CBD Injection and Either Saline or 5-HT Injection. Either saline or 2, 4, or 6 micrograms of CBD were injected into crayfish. Each also received a second injection of saline (n=6 in each group) or 5-HT (n=8, 8, 8, and 6). A two-tailed t-test was performed to compare saline vs. 5-HT for each CBD group (for 0, 2, 4, and 6µg CBD; p=0.85, 0.75, 0.036, 0.55)

Discussion

This study investigated if crayfish could be used as a model to study CBD as an anxiolytic. Previous published research showed evidence to support that CBD would cause the crayfish to consistently spend a lot of time in the dark (Timmer, 2020). However, decreased anxiety would be demonstrated by an increase in travel into the lit areas of a Light-Dark maze. In this study, when crayfish were injected with CBD and a saline solution, there was no significant difference in darkness preference between any dosage groups (Figure 3). This is inconsistent with the hypothesis that CBD will reduce anxiety and increase boldness and travel throughout the maze.

The study aimed to utilize 5-HT as a stressor for crayfish, with the aim of using CBD to reduce this stress. Indeed, Fossat, et al. showed that 5-HT injections mimicked the stress response of electric shocks and caused crayfish to experience behavioral changes. However, contrary to the results of Fossat, et al., darkness preference in our study did not increase upon injection with 5-HT (Figure 4). In fact, among the crayfish injected with 2 µg/g CBD, those that also received 5-HT spent significantly less time in the dark compared to those injected with saline, which indicates that 5-HT prompted exploration and boldness.

Reisinger, et al. published research after the commencement of our study that suggests that 5-HT may increase boldness in crayfish rather than decrease it. This nuanced and variable effect in response to 5-HT is supported by our study because crayfish that received 5-HT showed consistent variability in Light-Dark preference compared to the non-5-HT groups, which had very consistent results with low variability. This variation could be due to factors that are involved with the function of 5-HT. Even in human studies, 5-HT seems to have variable effects (Gordon, 2004).

When comparing the total number of times a crayfish switched chambers in the maze, the crayfish administered 5-HT and 4 µg/g CBD had significantly less moves compared to the crayfish that received the saline solution and 4 µg/g CBD (Figure 5). This slightly conflicts with the data in Figure 4, which showed that 5-HT was linked to more time spent in the light (among the 2µg/g CBD group). More time spent in the light was assumed to be indicative of less anxiety and a resultant bold exploration. However, showing less overall movement when injected with 5-HT (among the 4µg/g CBD group) disrupts that assumption. It is possible that the effects of 5-HT injections vary when paired with 2µg/g and 4µg/g of CBD. The lower dose indicated less time spent in the dark and the higher dose indicated

less movement overall. The varying results could also be further evidence of a very nuanced effect of 5-HT injections that is unrelated to the CBD. In summation, CBD did not independently show any influence on either the Light-Dark preference or the total position moves made by crayfish. 5-HT showed quite variable results compared to the saline solution, and it did not confirm the work of Fossat et al. that it increases anxiety and a preference to hide in the dark. Our results did support a study by Reisinger et al. that indicates 5-HT may increase boldness. 5-HT injection significantly decreased time spent in the dark for crayfish injected with 2μg/g of CBD and significantly decreased movement for crayfish injected with 4μg/g of CBD.

The use of CBD as a treatment for mood disorders is still an important field of study (Melas, et al. 2021). The use of an invertebrate model, such as crayfish is still appealing; however, there is still much to be explored. A future direction for this field of study could include the use of more 5-HT dosages or to study single doses versus long-term exposure to tease out why 5-HT sometimes causes boldness and sometimes causes stressed/hiding behavior. Another direction for this project would be to use a different stressor than 5-HT and pair it with the same CBD doses to answer the original question about the effect of CBD on anxiety. Crayfish could also receive multiple CBD injections over a period to see the effects of CBD after it is continually stimulating the cannabinoid receptors. Lastly, the same experiment could be repeated using a different style maze, such as the Y maze or a fighting ring to observe fight results for anxiety vs. boldness. In addition, all of these experiment proposals, including the original experiment, could be repeated with a larger sample size in hopes of clarifying some ambiguous data by increasing statistical power.

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