Caffeine protects against memory loss induced by high and non-anxiolytic dose of cannabidiol in adult zebrafish (*Danio rerio*).

Luiza Reali Nazario, Régis Antonioli Junior, Katiucia Marques Capiotti, Jaime Eduardo Cecílio Hallak, Antonio Waldo Zuardi, José Alexandre S. Crippa, Carla Denise Bonan, Rosane Souza da Silva.

**Abstract**

Cannabidiol (CBD) has been investigated in a wide spectrum of clinical approaches due to its psychopharmacological properties. CBD has low affinity for cannabinoid neurotransceptors and agonistic properties to 5-HT receptors. An interaction between cannabinoid and purinergic receptor systems has been proposed. The purpose of this study is to evaluate CBD properties on memory behavioral and locomotor parameters and the effects of pre-treatment of adenosine receptor blockers on CBD impacts on memory using adult zebrafish. CBD (0.1, 0.5, 5, and 10 mg/kg) was tested in the avoidance inhibitory paradigm and anxiety task. We analyzed the effect of a long-term caffeine pre-treatment (~20 mg/L — four months). Also, acute block of adenosine receptors was performed in co-administration with CBD exposure in the memory assessment. CBD promoted an inverted U-shaped dose-response curve in the anxiety task, in the memory assessment, CBD in the dose of 5 mg/Kg promoted the strongest effects without interfering with social and aggressive behavior. Caffeine treatment was able to prevent CBD (5 mg/kg) effects on memory when CBD was given after the training session. CBD effects on memory were partially prevented by co-treatment with a specific A₁ receptor antagonist when given prior to or after the training session, while CBD effects after the training session were fully prevented by adenosine A₁ receptor antagonist. These results indicated that zebrafish have responses to CBD anxiolytic properties that are comparable to other animal models, and high doses changed memory retention in a way dependent on adenosine.

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**Keywords:**

Adenosine, Anxiety, Caffeine, Cannabidiol, Memory, Zebrafish

1. Introduction

Cannabidiol (CBD) and Delta-9-tetrahydrocannabinol (THC) are the main active compounds from *Cannabis sativa*. Much evidence suggests that CBD acts as an anticonvulsant, anxiolytic, antipsychotic, and anti-rheumatic (Carlini and Cunha, 1981; Hampson et al., 1998; Zuardi, 2008; Campos et al., 2013). The main proposed mechanisms of action underlying CBD properties are related to the 5HT1A receptors' activation and to the increase of endocannabinoid effects (Bisogno et al., 2001; Russo et al., 2005). It was shown that pre-treatment of rats with a 5HT1A antagonist blocked the anxiolytic-like effect promoted by injection of CBD into the bed nucleus of the *striatum terminalis* in two models of anxiety (Gomes et al., 2011). The second main mechanism of action of CBD is the activation of the endocannabinoid system, proposed by the facilitation of CBD on endocannabinoid-mediated neurotransmission as a consequence of the blockade of anandamide metabolism and uptake (Bisogno et al., 2001). However, several additional biological targets of CBD have been identified (reviewed by Das et al., 2013; Devinsky et al., 2014). At low concentrations, CBD is a blocker of the equilibrative nucleoside transporter (ENT), the orphan G-protein-coupled receptor GPR55, and the transient receptor potential of melastatin type 2 (TRPM2) channel. CBD also activates the α3 and α2 γlicine receptors and the transient receptor potential of ankyrin type 1 (TRPA1) channel, and has a bidirectional effect on intracellular calcium. At high concentrations, CBD activates the nuclear peroxisome proliferator-activated receptor-c and the transient receptor potential of vanilloid type 1 (TRPV1) and 2 (TRPV2) channels while also inhibiting cellular uptake and fatty acid amide hydrolase-catalyzed degradation.

Learning and memory appear to be affected by CBD through a mechanism including the endocannabinoid system (Marsicano and Laffanêtre, 2009; Campos and Guimarães, 2009; Campos et al., 2013). Endocannabinoids are released during stressful conditions, and CB1 receptor agonism impairs the acquisition of contextual fear (Marsicano et al., 2002; Hohmann and Suplita, 2006). Acute and chronic
administration of CBD and the use of different doses reveal a variety of responses, especially in avoidance tasks (Soares et al., 2010; Cassol-Jr et al., 2010; Barichello et al., 2012; Fagherazzi et al., 2012). In rodents, CBD has demonstrated therapeutic potential for specific cognitive impairments associated with Alzheimer’s disease, probably through enhancement of endocannabinoid-mediated actions (Cheng et al., 2014).

Those complementary mechanisms of action proposed to underlie CBD action could explain some controversial results, such as the indirect agonistic effect on adenosine receptors, especially A2 receptors, by increasing adenosine levels as a response to nucleoside transport inhibition (Carrier et al., 2006; Liou et al., 2008). Also, endocannabinoids have the same potential to block adenosine transporters as the known inhibitor of nucleoside transporters, diprydamole (Pandolfo et al., 2011). Another interaction proposed between adenosine and cannabinoids is the existence of A2AR-CB1 receptor heterodimerization, which has been demonstrated by means of bioluminescence resonance energy transfer (BRET) techniques (Carriba et al., 2007; Ferrê et al., 2010). However, CBD is 10 times less active on CB receptors than THC.

Despite the increased number of studies on the CBD mechanism of action from clinical and preclinical investigations, the therapeutic window for CBD is not defined. For this reason, we performed experiments with zebrafish—a model animal with well-described signaling pathways, allowing the translation of information to higher vertebrates by overcoming the intrinsic differences—in order to contribute to preclinical studies. We analyzed the effects of several doses of CBD on anxiety and memory. Considering that adenosine could have some effect on cannabinoid signaling, we analyzed the effect of long-term treatment with caffeine, a non-specific adenosine receptor antagonist. Caffeine is a known psychoactive drug with mnemonic effects credited to the non-specific antagonism of adenosine receptors (Fredholm et al., 1999; Cunha and Agostinho, 2010). We also analyzed the effect of acute specific block of adenosine receptors on CBD mnemonic properties in order to contribute to the study of a possible interaction between CBD and purinergic signaling.

2. Materials and methods

2.1. Drugs

Caffeine, ZM241385, and DPCPX were purchased from Sigma-Aldrich (USA). Tween 80 was purchased from Invitrogen (USA). CBD, approximately 99.9% pure, was kindly supplied by THC-Pharm, Frankfurt, Germany, and STI-Pharm, Brentwood, UK. All other reagents were of analytical grade.

2.2. Animals

Fertilized eggs were obtained from wild-type adult zebrafish (Danio rerio) (Tübingen background) from the sixth generation of our breeding stock held at the Pontifícia Universidade Católica do Rio Grande do Sul. Fertilized eggs were collected and kept in maintenance water (water from reverse osmosis reconstituted with marine salt, 0.4 parts per trillion) in an incubator at 28.5°C on a 14:10 light/dark cycle. After egg hatching, the animals were maintained in an aquarium (5 L; 27 × 17 × 12 cm [width × height × depth]) with maintenance water (300 mL/fish) containing water of the animals. The treatment was concomitant to CBD injection, 1 h prior to or after the training session on the memory analysis, as indicated below (Scheme A3 and 4). Control animals received 0.1 mg/kg of Tween 80 diluted in saline under the same conditions of the treated group.

2.3. Cannabidiol exposure

One week before the experiments, all the fishes were weighted to adjust the CBD dose into 10 μL per fish via intraperitoneal (i.p) injection. The CBD doses (0.1 0.5, 5.0, or 10 mg/kg) were freshly prepared on 2% of Tween 80 diluted in saline. The animals were anesthetized with tricaine (100 mg/L) and then treated in four separate groups: Tween/CBD 0.1, 0.5, 5.0, and 10 mg/kg. The CBD exposure was performed 1 h before the behavior and locomotor assessment and 1 h before or after the training within the memory analysis (Scheme A). The fish spent this time in constantly aired tanks. All experiments were carried out with two concomitant control groups: one receiving saline and the other receiving 2% of Tween 80 diluted in saline under the same conditions of the treated group.

2.4. Caffeine long-term pre-treatment

At three days post-fertilization, embryos were divided into two groups: the Control (CTRL) group (no caffeine added) and CAF group (with caffeine added). The CAF fishes started being treated with −20 mg/L of caffeine (19.4 mg/L) (Capiotti et al., 2011) dissolved in water. This treatment was extended over four months, after which the memory and anxiety experiments were performed (Scheme A1 and 2). To guarantee constancy on caffeine concentration, the caffeine solution was changed each of the three days, and animal density was adjusted over time.

2.5. Acute block of adenosine receptor

Four month-old animals were exposed to adenosine receptor antagonist (DPCPX 6 mg/L and ZM241385 6 mg/L) for 1 h in recipient containing maintenance water (300 mL/fish). Antagonists were dissolved in DMSO until achieving the designed doses in a final DMSO concentration of 1% in the water of the animals. The treatment was concomitant to CBD injection, 1 h prior to or after the training session on the memory analysis, as indicated below (Scheme A3 and 4). Control animals received DMSO 1% in the same condition of the animals treated with antagonist blockers.

2.6. Locomotor and behavioral assessment

A curve of CBD dose (0–10 mg/kg) was used to analyze the effects of CBD on the time spent in the top area of the aquarium as a measure of anxiety behavior (Egan et al., 2009) and locomotor parameters. The animals were individually placed in a single tank (30 × 15 × 10 cm; w × h × d) virtually divided into two horizontal lines (lower and upper zones). After 30 s of habituation, the behavior was recorded by a digital camera for 5 min for posterior analysis. The parameters, total distance (m), max speed (m/s), mean speed (m/s), absolute turn angle (degrees), and the time spent in the upper zone were registered.

Aggressive behavior and social interaction were analyzed after CBD (5 mg/kg) exposure. For aggressive behavior, the fishes were placed individually in a single tank (30 × 15 × 10 cm; w × h × d), with a mirror positioned in the back of the tank forming a 22.5° angle. The tank was divided into four equal sections from which the time spent in each zone was analyzed, with the time spent in the zone near the mirror being the sign of aggressive behavior. Additionally, the observation of aggressive behavior like biting, sprinting, and changes in color pattern was also registered. The fishes were habituated for 5 min, after which the video was recorded for 1 min (Gerlai et al., 2000). The last behavioral analysis was the social interaction in which three tanks (30 × 15 × 10 cm; w × h × d) were placed side by side, the far left being empty, the one in the middle with the test animals, and the far right with stimulus fish (Gerlai et al., 2000). After a 5 minute habituation, the social interaction was recorded for 10 min. The tank with the test fish was separated into two equal sessions, with the social
interaction indicator being the time spent in the side with the stimulus fish. All the data were analyzed by the Software ANY-maze (Stoelting Co., Wood Dale, USA).

2.7. Memory assessment task

A curve of CBD dose (0–10 mg/kg) was used to analyze the effect of CBD on the latency to cross chambers in an avoidance inhibitory task as a measure of memory retention (Blank et al., 2009). Also, the animals were evaluated for the ability of memory formation after prolonged caffeine exposure challenged by an acute CBD (5 mg/kg) administration before or after the training session (Scheme A). To address the results of specific adenosine receptors, we treated the animals with DPCPX or ZM241385, antagonists of A<sub>1</sub> and A<sub>2A</sub> adenosine receptors, respectively, concomitantly with CBD (5 mg/kg), 1 h before or after the training session. The protocol followed Blank et al. (2009). Briefly, the animals were individually trained and tested in a tank (18 cm × 9 cm × 7 cm; w × h × d) subdivided into white and dark chambers apart from a sliding wall. In each session, the animals were gently placed in the white tank compartment while the sliding wall was closed. After 1 min of habituation and orientation, the wall was raised, allowing the fish to cross to the dark side of the tank through a 1 cm high opening. In a training session, immediately after crossing to the dark compartment, the sliding partition was closed and a pulsed electric shock of 3 ± 0.2 V administered for 5 s, after which animals were removed from the apparatus. Twenty-four hours after training, the animals were submitted to a test session that repeated the training protocol, except that no shock was administered and the sliding wall was kept open, allowing the animals to freely explore the apparatus. The latency to enter the dark compartment was measured in all sessions and the test latency was used as an index of memory retention. In the set of experiments with long-term caffeine treatment, the control group was maintained for the same period in normal water and received an injection of Tween 80 2% as a vehicle of CBD.

For memory assessment, a T test was used to compare latency in training versus latency in the test session during the inhibitory avoidance task (comparison inside the groups), while One-Way ANOVA was used to compare the latency between groups. One-Way ANOVA was also used to compare the results from the CBD dose on anxiety and locomotion. Aggressive and social interaction between CAF, CBD, and control groups was analyzed by One-Way ANOVA. The T test was used to compare weights between the control and caffeine-treated animals. The significance levels were attributed at p < 0.05. The multiple comparisons of means were conducted when appropriated by Tukey's test. Data are expressed as mean ± standard error of mean.

3. Results

3.1. Cannabidiol effects

The locomotor parameters evaluated (total distance [TD], mean [MS] and maximum speed [MA], and absolute turn angle [ATA]) were not affected by CBD 0.1–10 mg/kg (Table A). In order to check if CBD could have some effect on anxiety in zebrafish, we evaluated the time spent in the upper zone of the aquarium as a marker of anxious behavior. CBD exhibited an inverted U-shaped dose–response curve on this parameter (Fig. A) (F[4,40] = 5.996; p = 0.0002). The control animals spent 25% of the total time in the upper zone, while the CBD-treated animals with 0.5 mg/kg spent 58% of the total time there. The lowest (0.1 mg/kg) and highest doses (5 and 10 mg/kg) did not alter the time in the upper zone in relation to the control group (Tween) (Fig. A). Control/saline did not differ from control/Tween (data not shown; p = 0.8824).

### Table A

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Ctrl/Tween</th>
<th>0.1 mg/kg</th>
<th>0.5 mg/kg</th>
<th>5 mg/kg</th>
<th>10 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total distance (m)</td>
<td>16.49 ± 2.706</td>
<td>20.12 ± 5.337</td>
<td>10.53 ± 1.450</td>
<td>12.33 ± 1.012</td>
<td>10.59 ± 0.8632</td>
</tr>
<tr>
<td>Mean speed (m/s)</td>
<td>0.0430 ± 0.00359</td>
<td>0.0610 ± 0.01344</td>
<td>0.03518 ± 0.00484</td>
<td>0.0411 ± 0.00333</td>
<td>0.0333 ± 0.00226</td>
</tr>
<tr>
<td>Maximum speed (m/s)</td>
<td>0.8137 ± 0.1442</td>
<td>0.9745 ± 0.2108</td>
<td>0.4763 ± 0.1113</td>
<td>0.8088 ± 0.1487</td>
<td>0.4500 ± 0.1086</td>
</tr>
<tr>
<td>Absolute turn angle (°)</td>
<td>38078 ± 4121</td>
<td>41050 ± 5789</td>
<td>29064 ± 3230</td>
<td>32556 ± 3725</td>
<td>28509 ± 3281</td>
</tr>
</tbody>
</table>
In the inhibitory avoidance paradigm, no differences in the time to cross chambers were detected among the groups during the training session (Fig. B1 and B2). The vehicle Tween 80 2% did not change latencies during the memory assessment when compared to the control/saline group (training and test latencies; \( p = 0.4598, p = 0.8936 \), respectively) (data not shown). As expected, the control/Tween animals increased their latencies to cross chambers during the test session, demonstrating preserved memory (Fig. B1 and B2). CBD-treated animals had their latencies to cross chambers during the test session altered in different ways according to the dose and period of exposure to CBD (pre-training: \( F[4;57] = 2.651; p = 0.0423 \); post-training: \( F[4;58] = 7.124; p = 0.0001 \)). When CBD was given prior to the training session, the lowest dose (0.1 mg/kg) did not affect memory retention (Fig. B1). The test latency in the animals treated with CBD 0.1 mg/kg was significantly higher than the training session latency when compared to the Tween group (Fig. B1). CBD 0.5 and 10 mg/kg, increased the test latency in relation to training latency, but did not reach similar results to the Tween group (Fig. B1). The dose of 5 mg/kg CBD given prior to the training session decreased the latency in the test session, affecting memory retention (Fig. B1). When CBD was given after the training session, the doses 0.1 and 10 mg/kg reduced the latency to cross chambers in the test session. This was not enough to be statistically similar to the latency in the training section, but enough to be statistically different from the Tween group (Fig. B2). CBD given at 0.5 mg/kg did not affect the memory parameter, showing similar latency to the Tween group to cross chambers in the test session. CBD given at 5 mg/kg affected the memory parameter, showing similar latency to cross chambers between the training and test sessions.

We selected one of the CBD doses, 5 mg/kg, to test the hypothesis of contribution of purinergic system on CBD effects. We performed these two kinds of behavioral assessment, aggressive and social behaviors, just on the selected dose since the rationale to do that was to evaluate the innate behavioral aspects of zebrafish could interfere with the effects detected on the inhibitory avoidance task. Aggressive behavior and social interaction were not affected by CBD 5 mg/kg (\( p = 0.2902; p = 0.6675 \), respectively).

### 3.2. Caffeine long-term treatment and cannabidiol

The long-term treatment with caffeine affects the body weight gain of zebrafish (\( p = 0.017 \)). The average weight of the control animals was 0.121 ± 0.006 g, while the average weight of the caffeine-treated animals was 0.099 ± 0.006 g at the end of the treatment (four months). Locomotor parameters were not affected by long-term treatment with caffeine (data not shown). The evaluation between groups indicated that during the training session all groups presented similar profiles of latencies to cross chambers (pre-training injected animals: \( F[3;40] = 1.138; p = 0.3454 \); post-training injected animals: \( F[3;45] = 1.367; p = 0.2649 \)), suggesting no effect of long-term caffeine exposure on exploratory behavior as confirmed by locomotor evaluation. Caffeine by itself did not alter memory retention, since the latency from the test session was not different from the Tween group (Fig. C1 and C2).

The previous long-term caffeine treatment was not able to alter CBD (5 mg/kg) effects on memory, when CBD was given prior to training in the inhibitory avoidance task, considering the small latency to cross chambers in the test session (CAF/CBD: training latency versus test latency \( p = 0.2049 \)) (Fig. C1). Long-term caffeine-treated animals exhibited a protective effect against the memory disruption promoted by CBD (5 mg/kg) when CBD was given after training in the inhibitory avoidance task, as seen by the increase of the latency period in the test session (CAF/CBD: training latency versus test latency \( p = 0.0003 \)) (Fig. C2).

### 3.3. Acute treatment with specific adenosine receptor antagonists and cannabidiol

The acute treatment with DPCPX and ZM241385 was accompanied by control/vehicle 1% DMSO. The control/DMSO/Tween had preserved memory as demonstrated by the increased latency to cross chambers in the test session in comparison to the training session in both sets of experiments (Fig. D1 and D2) (pre- and post-training sets of experiments; \( p < 0.001 \) and \( p < 0.0001 \)). The decrease in latency in the test session, which means the impairment on memory, exerted by CBD 5 mg/kg given before the training session was prevented by the co-administration of DPCPX, a specific antagonist of A1 adenosine receptors (Fig. D1). However, DPCPX effects were hard to interpret since DPCPX affected the latency to cross chambers in the training session (Fig. D1). When DPCPX was co-administered with CBD 5 mg/kg after the training session, the latency to cross chambers was increased and had similar results to the control animals (Fig. D2). This indicated that DPCPX per se affected the behavior during the training phase, which could affect the...
memory formation process. Also, DPCPX was able to prevent CBD effects on memory when CBD was given after the training session.

ZM241385, a specific A_{2A} adenosine receptor antagonist, did not affect the time to cross chambers during the training session (Fig. D1 and D2). When ZM241385 was co-administered with CBD 5 mg/kg before the training session, the animals increased their latency between the training and test sessions (p = 0.0026). When ZM241385 was co-administered with CBD 5 mg/kg after the training session, the animals increased their latency between the training and test sessions (p = 0.0168), but not enough to be similar to the control latency (Fig. D2).

4. Discussion

Behavioral effects of CBD of clinical interest have been well recognized, especially concerning anxiolytic properties (Campos and Guimarães, 2009). The anxiolytic effects show an inverted U-shaped dose–response curve in several models of anxiety protocols (Campos and Guimarães, 2009; Campos et al., 2013; Schier et al., 2012). Here, for the first time, we show that zebrafish also display anxiolytic responses to CBD in an inverted U-shaped dose–response curve. While the link between anxiety decrease and CBD use has been examined in several pre-clinical and clinical studies, the safety of the CBD doses is still uncertain. Despite the well-known issue about anxiolytic properties of low-intermediary CBD doses, several studies have supported the use of a higher CBD dose as a protective substance against memory deficits induced by THC, inflammatory illness, and other memory-disruptive conditions (Cassol-Jr et al., 2010; Soares et al., 2010; Barichello et al., 2012). On this basis, the use of CBD-rich strains of cannabis has been encouraged, mainly through other strategies of memory assessments that are not aversive (Morgan et al., 2010; Mechoulam and Parker, 2013; Wright et al., 2013).

In general, CBD appears as an innocuous drug in memory formation or as a potential protective drug against memory disruption in several protocols, while few studies are concerned about high CBD doses (Fadda et al., 2006; Hayakawa et al., 2008; Cassol-Jr et al., 2010; Long et al., 2010; Soares et al., 2010; Barichello et al., 2012). However, endocannabinoid signaling, especially through CB1 receptors, has been shown to cause impairment of memory acquisition in a contextual fear assessment and memory extinction (Pamplona et al., 2006; De Carvalho et al., 2014). In this study, we used a dose of CBD 10 times higher than an anxiolytic one, which caused memory impairment in an avoidance task apparatus used to assess memory formation in zebrafish. In zebrafish telencephalon, long-term potentiation (LTP) has been shown to depend on glutamatergic transmission (Nam et al., 2004). The importance of glutamatergic signaling during memory acquisition and consolidation has been demonstrated in non-aversive tests like the Y-maze memory task and inhibitory avoidance in zebrafish (Blank et al., 2009; Cognato et al., 2012). Endocannabinoids have been implicated to affect neurotransmission mediated by glutamate through pre- and post-synaptic mechanisms involving CB1 receptor activation (Gerde and Lovinger, 2001; Huang et al., 2001), while no information is available for specific CBD effects. On the other hand, CBD is able to reduce glutamate reuptake in striatal synaptosomes of rats just in the higher doses tested (30–100 μM) by Pandolfo et al. (2011). Additionally, glutamate neurotransmission could be enhanced by CBD inhibition of nucleoside transport, increasing adenosine extracellular levels and action on facilitative A_{2A} adenosine receptors, as demonstrated in previous works (Carrier et al., 2006). Here, we present a striking effect of CBD on memory, which could be a result of a different interaction between animal species and its response to high doses of CBD in the context of aversive memory, possible through exacerbated glutamatergic activation.
These results of CBD on latency to cross chambers could not be associated with a catalepsy-like effect, since regular locomotion was always seen when animals received this high of a dose of CBD. Also, the literature shows no catalepsy effects of CBD (Long et al., 2010). However, hypnotic effects of high doses of CBD and longer sleep in humans have already been registered, while alertness was described in rats exposed to CBD (Carlini and Cunha, 1981; Murillo-Rodríguez et al., 2006). Additionally, cannabis cigarettes, cannabis extract, and analogs of THC and derivatives have been indicated to relieve pain in non-cancer types of chronic pain. The analgesic effect could influence the sensitivity to the inhibitory avoidance task, but the pharmacological base seems to be related to CB receptor activation, which is a weak property of CBD, while THC seems to be responsible for the analgesic property (Bushlin et al., 2010; Ellis et al., 2009; Grotenhermen and Müller-Vahl, 2012).

Several methodological exposures to caffeine in animal and human studies contribute to the recognition of caffeine as a memory enhancer (reviewed by Cunha and Agostinho, 2010). In doses relevant to human consumption, caffeine is able to block $A_1$ and $A_{2A}$ adenosine receptors, with emphasis on the latter (Fredholm et al., 1999). However, caffeine has been proposed to have a role more related to a normalizer than an impairer of memory, based on its ability to prevent memory impairment induced by stress and chronic neuropathology (Cunha and Agostinho, 2010). This information appears to be more related to $A_{2A}$ adenosine receptor when CBD was given before training. DPCPX (6 mg/L) used in the index of memory retention, but not a full prevention of CBD effects. Meanwhile, more studies on caffeine and CBD interaction should be performed.

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References


