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ORIGINAL INVESTIGATION

Involvement of serotonin-mediated neurotransmission in the dorsal periaqueductal gray matter on cannabidiol chronic effects in panic-like responses in rats

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Abstract

Rationale Cannabidiol (CBD) is a non-psychotomimetic constituent of *Cannabis sativa* plant that promotes antianxiety and anti-panic effects in animal models after acute systemic or intra-dorsal periaqueductal gray (DPAG) administration. However, the effects of CBD repeated administration, and the possible mechanisms involved, in animal models of anxiety- and panic-related responses remain poorly understood.

Objective The present study evaluates the role of the serotonergic neurotransmission within the DPAG in the modulation of escape responses of rats chronically treated with CBD.

Methods Male Wistar rats received acute or repeated (5 mg/Kg/daily/21 days) administration of CBD and were submitted to the elevated T-maze (ETM). We also investigated if CBD effects on the ETM depend on facilitation of 5-HT1A-mediated neurotransmission in the DPAG. To this latter aim, we verified if these effects would be prevented by intra-DPAG injection of the 5-HT1A receptor antagonist WAY100635 (0.37 nmol/0.2 μL). Also, we verified, by in vivo microdialysis, if CBD chronic treatment increases serotonin (5-HT) release and, by quantitative polymerase chain reaction, if there are changes in 5HT-1A or 5HT-2C mRNA expression in DPAG.

Results The results showed that repeated but not acute peripheral administration of CBD decreases escape responses in the ETM, suggesting a panicolytic effect. This treatment did not change 5HT-1A or 5-HT-2C receptor mRNA expression nor modify serotonin extracellular concentrations in the DPAG. CBD effects were prevented by DPAG injection of the 5-HT1A receptor antagonist.

Conclusions Together, these findings suggest that repeated treatment with CBD induces anti-panic effects by acting on 5-HT1A receptors in DPAG.

Keywords Panic disorder · Cannabidiol · Serotonin · 5-HT1A receptors · Dorsal periaqueductal gray

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Introduction

Panic disorder (PD) is a chronic and disabling psychiatric disorder characterized by unexpected and recurrent panic attacks that affects about 5 % of people worldwide (American Psychiatry Association 2000). PD patients experience psychosocial impairment and a high risk of psychiatric comorbidities and suicide. Serotonin selective reuptake inhibitors (SSRI) are first-line compounds in the treatment of PD, although others drugs such as tricyclic antidepressants and high-potent benzodiazepines could also be used. However, despite these pharmacological options, less than half of the patients that suffer from PD shows completed and sustained remission of the symptoms (Roy-Byrne et al. 2006). The development of more effective drugs to treat this psychiatric condition, therefore, is clearly needed.

In the last two decades, attention has been given to the therapeutic potential of cannabinoids. In addition to the pharmacological effects of Δ^9-THC, a growing body of evidence suggests that non-psychotomimetic phytocannabinoids could be useful as therapeutic tools. The most promising of these compounds is cannabidiol (CBD), the major non-psychotomimetic constituent of *Cannabis sativa*. Different from the endogenous ligands anandamide and 2- arachidonylglycerol, which act as agonists of CB1/CB2 receptors (Devane et al. 1992; Mechoulam et al. 1995), CBD has a very low affinity for these receptors in vitro (Bisogno et al. 2001; Thomas et al. 2007). Even so, some studies suggest that CBD could act as an antagonist of CB1 receptors and an agonist of CB2 receptors (Thomas et al. 2007). It has also been proposed that CBD could facilitate endocannabinoid signaling by inhibiting the cellular uptake and enzymatic hydrolysis of endocannabinoids (Bisogno et al. 2001). Finally, CBD can also promote the blockade of adenosine uptake or act as an agonist of vanilloid (TRPV1) or 5-HT1A-serotonergic receptors (Bisogno et al. 2001; Campos and Guimarães 2008, 2009; Carrier et al. 2006; Russo et al. 2005; Soares Vde et al. 2010).

Preclinical studies show that systemically administered CBD induces anxiolytic-like effects in several animal models that have been associated with generalized anxiety disorder, such as the elevated plus maze (EPM), the Vogel conflict test and aversive conditioning (Guimarães et al. 1990; Moreira et al. 2009; Onaivi et al. 1990; Resstel et al. 2006). Reinforcing these findings, human studies suggested that the drug decreases generalized anxiety symptoms (Fusar-Poli et al. 2009; Zuardi et al. 1982, 1993). Moreover, there is evidence indicating that CBD could also be effective in the treatment of panic disorder (Zuardi et al. 1993). According to this proposal, we have recently demonstrated that direct injections of CBD into the dorsal portions of the periaqueductal gray matter (DPAG) decrease the escape response induced by local electrical stimulation by activating 5HT-1A receptors (Soares Vde et al. 2010). Since electrical stimulation of the DPAG has been proposed as a model of panic attack (Schenberg et al. 2001), this result suggests that, in addition of being anxiolytic, CBD could also have anti-panic properties.

The elevated T-maze (ETM) is an animal model proposed to generate, in the same animal, anxiety-(i.e., inhibitory avoidance) and fear-(i.e., escape) related defensive responses. Pharmacological validation has suggested that these responses are related in pathological terms to generalized anxiety and panic disorder symptoms, respectively (Graeff et al. 1993, 1998; Viana et al. 1994; Teixeira et al. 2000; Poltronieri et al. 2003). Moreover, similar to the results obtained with DPAG electrical stimulation, 5-HT1A agonists and chronic antidepressant (tricyclic and SSRI) treatment have also been shown to inhibit escape responses in this model (De Paula Soares and Zangrossi 2004; Poltronieri et al. 2003; Zanoveli et al. 2010).

Given that CBD intra-DPAG injections affect escape response (a behavioral measure that has been associated with panic attacks - Soares Vde et al. 2010) and since the behavioral effects of repeated CBD remain poorly investigated, the present study explored the effects and mechanism involved in the chronic administration of CBD in rats tested in the ETM. We also investigated if these effects were dependent on DPAG changes in serotonin-mediated neurotransmission. More specifically, we tested this hypothesis by using in vivo microdialysis approach to determine if repeated CBD treatment increases serotonin release in the DPAG. Also, in an independent experiment, the effect of CBD chronic administration on 5HT-1A and 5HT-2C mRNA expression in this brain structure was also assessed.

Materials and methods

Animals

Male Wistar rats (University of São Paulo, Campus Ribeirão Preto), weighing 250–300 g on the day of the surgery, were housed in groups of four to five per cage (50×60×22 cm) until surgery. After surgery, animals were housed in pairs in Plexiglas-walled cages (30×19×13 cm). Room temperature was maintained at 22±1 °C with lights on from 7 to 19 h. Food and water were freely available throughout the experiments. Independent groups of naive rats were used in all experiments except in experiment 4, where the receptor expression measurements were performed in the same animals of experiment 1. The experimental protocol was based on the ethics principles of the Brazilian Society of Neuroscience and Behaviour (SBNeC) and was approved by the local ethical committee (process 114/2007).

Drugs

The following drugs were used: CBD (5–20 mg/Kg/mL; kindly supplied by THC Pharma, Frankfurt, Germany), diazepam (1 mg/Kg/mL, Roche, Brazil), and fluoxetine hydrochloride (10 mg/Kg/mL, Lily, Brazil), suspended in polyoxyethylenesorbitan monooleate (Tween 80) 2 % and saline, WAY-100635 (0.37 nmol/0.2 μL, Sigma-Aldrich, USA) dissolved in saline. The doses were based on previous studies (Graeff et al. 1998; Guimarães et al. 1990; De Paula Soares and Zangrossi 2004, Zanoveli et al. 2010). All drugs were systemically injected at a volume of 1 mL/Kg. Other drugs used during surgical, perfusion, or microdialysis procedures are described below.

Apparatus

The ETM is made of wood and has three arms of equal dimensions (50 cm×12 cm). One arm, enclosed by 40-cm high walls, is perpendicular to two opposed open arms. To prevent falls, a 1-cm-high Plexiglas rim surrounds the open arms. The whole apparatus is elevated 50 cm above the floor. The open field test, used to control nonspecific effects on exploratory behavior, was performed in a wooden square arena (60 cm×60 cm), with 30-cm-high walls. The apparatus for the microdialysis and PCR studies are described below.

Procedures

Elevated T-maze and open field test

Three days before the ETM, test the rats were gently handled by the experimenter for 5 min. Twenty-four hours later, they were exposed to one of the open arms of the T-maze for 30 min. A wooden barrier mounted at the junction between the maze central area and the proximal end of the open arm isolated this arm from the rest of the maze. It has been shown that a prior forced exposure to one of the open arms of the maze decreases the latency to leave this arm on a later trial. This result has been attributed to habituation of behavioral responses to novelty (exploration, behavioral inhibition), which may interfere with one-way escape (Teixeira et al. 2000). Twenty-four hours after the preexposure to the open arm, animals were tested in the ETM and open field. The test in the ETM began by measuring inhibitory avoidance acquisition. Each animal was placed at the distal end of the enclosed arm of the elevated T-maze facing the intersection of the arms. The time taken by the rat to leave this arm with the four paws was recorded (baseline latency). The same measurement was repeated in two subsequent trials (avoidances 1 and 2) at 30-s intervals. Following avoidance training (30 s), each rat was placed at the end of the same previously experienced open arm and the latency to leave this arm with the four paws was recorded for three consecutive trials (escapes 1, 2, and 3) with 30-s intertrial intervals. A cut-off time of 300 s was established for the avoidance and escape latencies. Immediately after being tested in the ETM, each animal was placed in the center of the open field and the locomotor activity was evaluated for 5 min. The total distance traveled was analyzed by a video tracking system (Ethovision; Noldus, Holland).

Stereotaxic surgery

Rats were anesthetized with 2,2,2-tribromoethanol (250 mg/kg, i.p.) associated with local anesthesia (2 % lidocaine with a vasoconstrictor; Harvey, Brazil) and fixed in a stereotaxic frame (David Kopf, USA). A guide cannula made of stainless steel (12 mm long, 0.6 mm outer diameter, and 0.4 mm inner diameter) was implanted into the midbrain aimed at the DPAG following the coordinates of Paxinos and Watson's atlas (Paxinos and Watson 2007). Holding the incisor bar 2.5 mm below the interaural line, the cannula was introduced 1.9 mm lateral to lambda at an angle of 22° with the sagittal plane, until it was 3.2 mm below the surface of the skull. The guide cannulae were attached to the skull by means of acrylic resin and two stainless steel screws. A stylet with the same length of the guide cannula was introduced into the cannula to prevent obstruction. To prevent infections, all animals were injected (IM) with a 0.2-mL pentabiotic preparation (Pentabiotico Veterinário Pequeno Porte; Forte Dodge, Brazil) at the end of the surgery. In addition, fluxinin meglumine (Schering–Plough, Brazil; 2.5 mg/kg), a drug with analgesic, antipyretic, and anti-inflammatory properties, was administered subcutaneously for post-surgery analgesia.

Neurochemical study

Briefly, animals were submitted to the stereotaxic surgery procedure (as described above) for the implantation of a guide cannula for the dialysis probe (CMA/12; CMA/Microdialysis AB, Stockholm, Sweden). Five days later, the animals were placed in a microdialysis bowl (BAS, Lafayette, USA) and a probe (1-mm membrane: polyarylethersulfone cut-off: 20,000 Da; CMA/12; CMA/Microdialysis AB, Stockholm, Sweden) was inserted into the DPAG, perfused with Ringer's solution (in mM: 145.0 NaCl, 2.7 KCl, 1.2 CaCl2, and 1.0 MgCl2 (pH:7.4)) at a constant flow rate of 1.5 μL/min (microinjection pump; BAS). Following a 2-h equilibrium period, eleven dialysate samples (three during baseline and eight post-injection) were collected every 30 min into vials containing 5 μL perchloric acid solution (0.05 M). After baseline sample collection (considered 100 %), the animals were injected (i.p.) with CDB or vehicle, according to the experimental

design described below. Thereafter, samples of the dialysate were collected for 240 min.

Serotonin assay

The amount of 5-HT in the collected fractions was analyzed using HPLC coupled with electrochemical detection. The reverse column was a 150 mm×2 mm Nucleosil C-18 with 3 μm particle size (Phenomenex, USA). The HPLC consisted of a BAS Epsilon electrochemical detector with a glass-carbon electrode and a pump (PM-92e, BAS, USA). The potential was set at 650 mV (vs. Ag–AgCl reference electrode). The mobile phase containing 172 mM sodium dihydrogen phosphate monohydrate, 1 mM EDTA, 0.84 mM sodium octylsulfate, 15 mM sodium chloride, 0.125 % diethylamine, and 15 % methanol (adjusted at pH 3.5 with orthophosphoric acid) was filtered and pumped through the system at a flow rate of 120 μL/min. The injection volume was 50 μL. All reagents for the HPLC mobile phase were of analytical grade and were obtained from Merck (Germany) or Sigma Chemical Co. (USA). This set-up allowed the analysis of 5-HT and 5-HIAA levels in each dialysate sample in a run that lasted approximately 30 min. The HPLC limit of detection was 1.25 pg/50ul for 5-HT and 5-HIAA.

Quantitative polymerase chain reaction

Total RNA was extracted from frozen brain tissue samples using Trizol (Invitrogen, Carlsbad, CA, USA), and cDNA reaction was performed using 1 μg of Total RNA at High-capacity cDNA kit (Applied Biosystem, Foster City, CA, USA) according to the manufacturer's instructions. The relative level of mRNA expression of the serotonin type 1A receptor (5-HT1A) and the serotonin 2C receptor (5-HT2C) genes were evaluated in the StepOne real-time PCR system, using Applied Biosystems real-time master mix with SYBRGREEN® gene expression probes. Total RNA was normalized based on Ct values for GAPDH housekeeping gene. All reactions were duplicated, and fold change was calculated using $2^{-\Delta\Delta Ct}$ method.

Histology

After the experiments, animals were anesthetized with 25 % urethane (10 mL/kg, i.p.; Sigma, USA) and then perfused through the heart with saline followed by 10 % formalin solution. After removal of the brain, and following a minimum period of 2 days of immersion in a 10 % formalin solution, brain slices of 55 μm were obtained by means of a cryostat in order to localize the positions of the cannulae tips according to Paxinos and Watson's atlas (Paxinos and Watson 2007). Only data from rats having cannulae tips inside the DPAG (dorsomedial or dorsolateral columns) were included in the statistical analysis.

Experimental design

Experiment 1 – acute (experiment 1A) and repeated (experiment 1B) peripheral administration of CBD

In experiment 1A, rats (n=11–14) were tested in the ETM 30 min after acute injection of CBD (5, 10, or 20 mg/Kg), diazepam (1 mg/Kg/mL) or vehicle (tween 80: 2 % in saline). In experiment 1B, an independent group of rats (n=9–11) received daily injections of CBD (5 mg/Kg—effective dose of the experiment 1A), fluoxetine (10 mg/Kg), or saline during 21 days. Three hours after the last injection, animals were tested in the ETM (Zanoveli et al. 2007) and open field as described above.

Experiment 2 – Intra-DPAG injection of the 5-HT1A receptor antagonist WAY-100635 in animals submitted to repeated peripheral administration of CBD

On the 14th day of repeated treatment with CBD (5 mg/Kg/mL- 21 days) or vehicle, animals were submitted to stereotaxic surgery procedure. Seven days after surgery and 10 min before the last injection (Zanoveli et al. 2010) of CBD or vehicle, a needle (14 mm length/outside diameter 0.3 mm) was introduced through the guide cannula. A volume of 0.2 μL of WAY-100635 (0.37 nmol) or saline was injected for 120 s using a 10-μL microsyringe (Hamilton, USA) attached to a micro-infusion pump (KD Scientific, USA). The displacement of an air bubble inside the polyethylene catheter connecting the syringe to the intracerebral needle was used to monitor the microinjection. The needle was removed 60 s after the injection was finished. Forty minutes after intra-DPAG injection of WAY-100635 or saline, the animals (n=9–14) were tested in the ETM and open field tests as described before.

Experiment 3 – Analysis of 5-HT and 5-HIAA extracellular concentrations in DPAG dialysate of animals submitted to acute (experiment 3A) or repeated (experiment 3B) administration of CBD

In the experiment 3A, 5-HT and 5-HIAA extracellular concentrations in DPAG were analyzed after a single peripheral injection of CBD (5 mg/Kg/mL) or vehicle. In the experiment 3B, rats were injected daily (i.p.) with CBD (5 mg/Kg/mL) or vehicle during 21 days. Both experiments were conducted with an independent group of rats. Animals of the experiments 3A were submitted to

stereotaxic surgery (as described below) for the implantation of a cannula guide for the dialysis probe (CMA/12; CMA/Microdialysis AB, Stockholm, Sweden) 5 days before the measurement of 5-HT and 5-HIAA tissue concentrations. In experiment 3B, the guide cannula was implanted on the 16th day after the beginning of treatment with CBD or vehicle. The animals were placed in a microdialysis bowl, and after baseline sample collection (considered 100 %), the animals were injected (i.p.) with vehicle solution (acute control group, $n=5$)

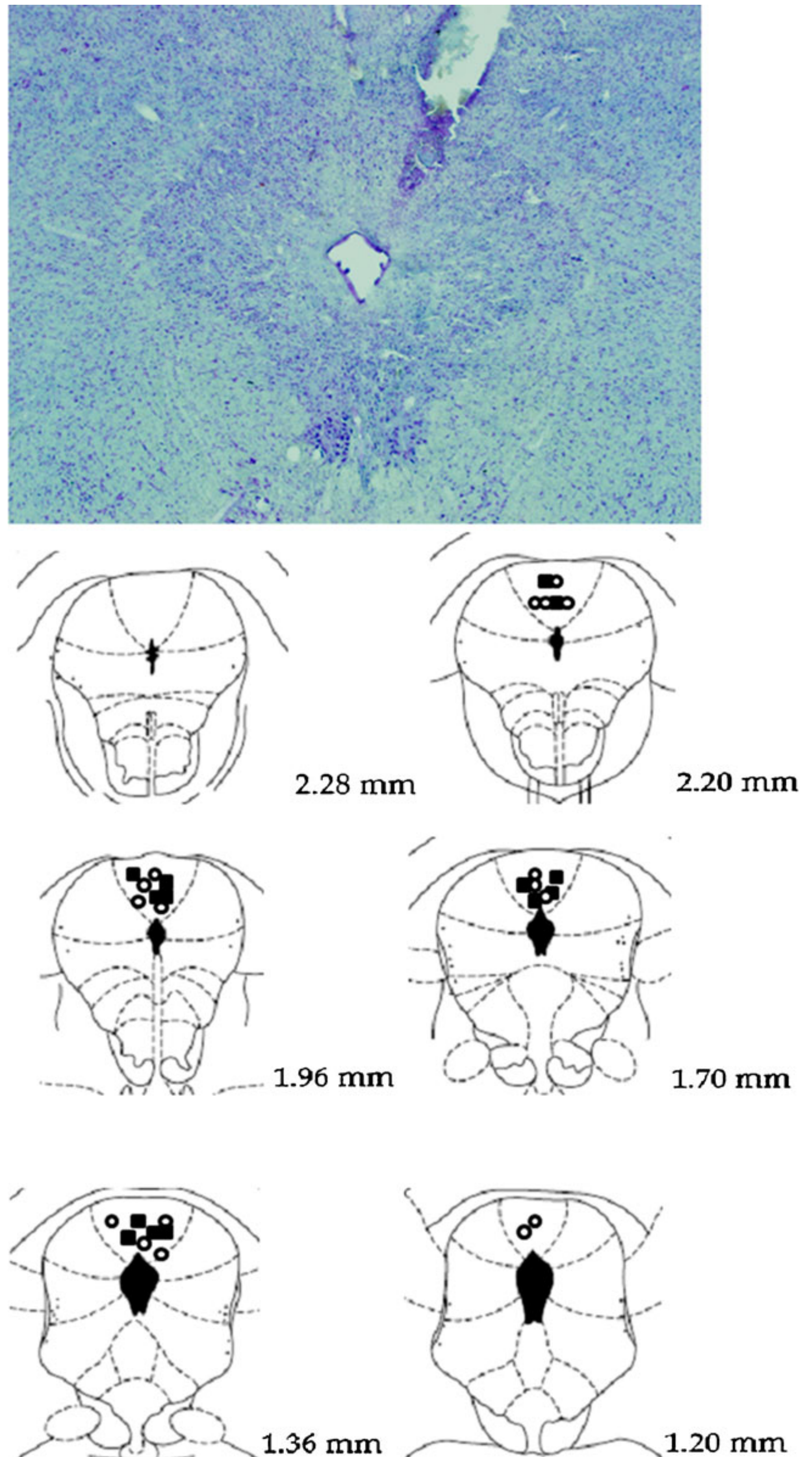

Fig. 1 Representative histological localization of injection sites (*open circle*) and microdialysis probe (*black squares*) that successfully reached the DPAG. Diagrams based on Paxinos and Watson's (1997) rat brain atlas

or CBD (acute treatment group, $n=6$). In experiment 3B, previously CBD-treated animals received one additional injection of CBD (21 days treatment group, $n=5$), and vehicle-treated animals (21 days treatment group, $n=4$) received one more injection of vehicle. Subsequently, samples of dialysate were collected for 240 min. 5-HT and 5-HIAA levels in the DPAG were measured by microdialysis, as previously reported in other studies of our research group (Zanoveli et al. 2009, 2010).

Experiment 4 – Analysis of 5-HT1A-mRNA and 5-HT2C-mRNA receptors expression in the DPAG following repeated administration of CBD or fluoxetine

One hour after the end of the behavioral procedures (4 h after the last administration of CBD), animals of experiment 1B were killed under deep urethane (Sigma-Aldrich, St. Louis, MO USA, 5 mL/kg, IP) anesthesia. The brains were removed and punch samples from the dorsal portions of periaqueductal gray matter were obtained. The brain tissues were processed and stored at −70 °C for posterior quantitative PCR analysis.

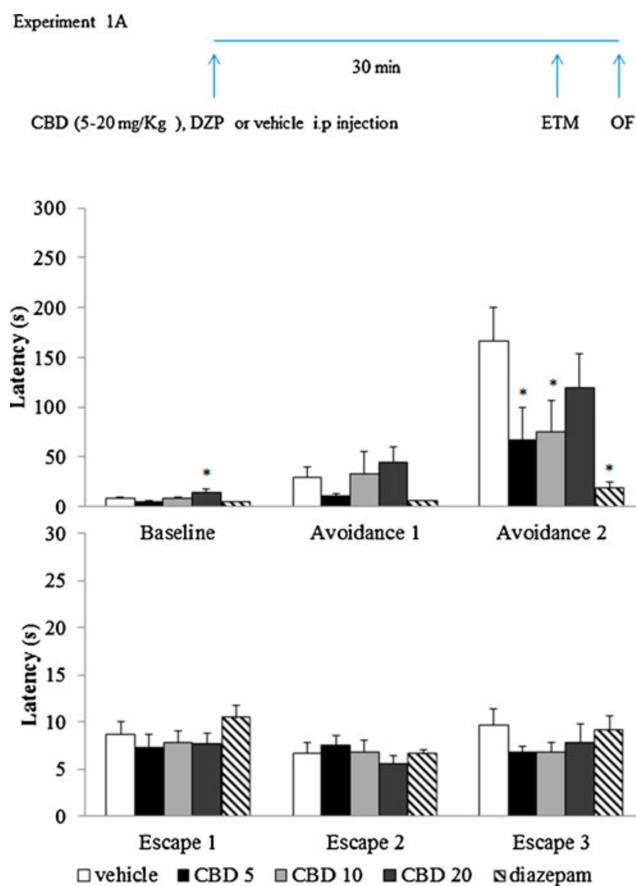

Fig. 2 Effects (mean ± SEM) of acute peripheral injection of CBD (5, 10, or 20 mg/Kg), diazepam (1 mg/Kg), or vehicle on inhibitory avoidance (*upper panel*) and escape (*lower panel*) latencies measured in the elevated T-maze. $n=11–14$; the *asterisk* indicates $p<0.05$ compared to the control group in the same trial (experiment 1A)

Statistical analysis

Repeated measures analysis of variance (ANOVA) was used to analyze avoidance and escape data obtained in the ETM, with drug treatment as the independent factor and trials as the repeated measure. When appropriate, post hoc comparisons were performed by Duncan's test. Locomotor activity and mRNA fold change (PCR) data were submitted to one-way ANOVA. Data from neurochemical study (microdialysis) were also analyzed by repeated measures analysis of variance. Post hoc comparisons were performed by Duncan's test. The significance level was set at $p<0.05$.

Results

A representative photomicrograph showing a typical injection site within the DPAG can be seen in Fig. 1.

Experiment 1A

Figure 2 (upper panel) shows that inhibitory avoidance was acquired during the test (trial effect: $F_{(2,118)}=27.79$; $p<0.05$), an effect that was inhibited by acute administration of either CBD (5 and 10 mg/Kg/mL) or diazepam (trial by treatment interaction: $F_{(8,118)}=2.60$; $p<0.05$, Duncan test). As shown in the lower panel of

Table 1 Distance traveled in the open field test by rats

Experiment	Group	Distance traveled (m, mean ± SEM)
1A	Vehicle	38.62±2.85
	CBD 5	36.62±2.38
	CBD 10	37.72±2.05
	CBD 20	37.49±2.51
	Diazepam	36.14±2.46
1B	Saline	43.19±5.20
	CBD 5	45.48±6.98
	Fluoxetine	45.13±5.75
2	Saline-Vehicle	45.03±5.76
	Saline-CBD	34.82±2.20
	WAY-vehicle	36.74±4.69
	WAY-CBD	40.24±4.23

Experiment 1A: acute peripheral administration of CBD (5, 10, or 20 mg/Kg/mL), diazepam, or vehicle; experiment 1B: chronic peripheral administration of CBD (5 mg/Kg/mL), fluoxetine, or vehicle; experiment 2: vehicle or WAY-100635 (0.37 nmol) injected intra-DPAG previously to CBD chronic peripheral administration (5 mg/Kg/mL). Data represented by means ± SEM; No significant difference was found

Fig. 2, acute administration of CBD or diazepam did not change one-way escape performance during the test. Locomotor activity, measured as the total distance traveled in the open field test, was not changed by CBD or diazepam (see Table 1).

Experiment 1B

Figure 3 (upper panel) indicates that inhibitory avoidance was acquired during the ETM inhibitory avoidance task (trial effect: $F_{(2,54)}=19.14$; $p<0.05$). There was a trend for an interaction between treatment and trial ($F_{(4,54)}=2.14$; $p=0.089$). No general treatment effect was found. As shown in the lower panel of Fig. 3, CBD chronic treatment impaired escape 2 performance, while fluoxetine impaired both escape 1 and escape 3 performance during the test (treatment effect: $F_{(2,27)}=4.01$; $p<0.05$, Duncan test). Total distance traveled in the open field was not changed by CBD or fluoxetine (see Table 1).

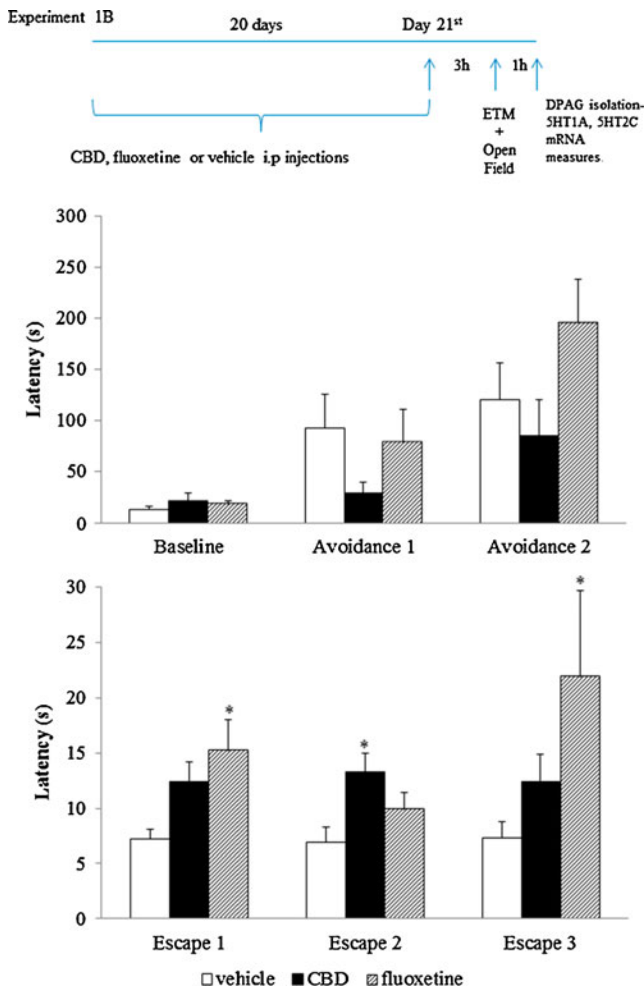

Fig. 3 Effects (mean ± SEM) of chronic peripheral injection of CBD (5 mg/Kg/day for 21 days), fluoxetine (10 mg/Kg/day for 21 days), or vehicle on inhibitory avoidance (*upper panel*) and escape (*lower panel*) latencies measured in the elevated T-maze (experiment 1B). n=9–11; the *asterisk* indicates $p<0.05$ compared to the control group in the same trial

Experiment 2

Similar to experiments 1A and 1B, inhibitory avoidance was acquired during the test (trial effect: $F_{(2,84)}=35.70$; $p<0.001$). Different from experiment 1B, however, chronic CBD treatment attenuated inhibitory avoidance acquisition (treatment effect, $F_{(3,42)}=4.10$, $p=0.011$; treatment×trial, $F_{(2,84)}=3.3$, $p=0.005$, Fig. 4, upper panel), an effect not modified by pretreatment with the 5-HT1A receptor antagonist WAY-100635 (Duncan, $p<0.01$). Chronic treatment with CBD also impaired escape performance in the ETM (Fig. 4, lower panel) in all trials (treatment effect: ($F_{(3,42)}=14.95$; Duncan test, $p<0.05$)), and this effect was blocked by a single intra-DPAG administration of WAY-100635. Locomotor

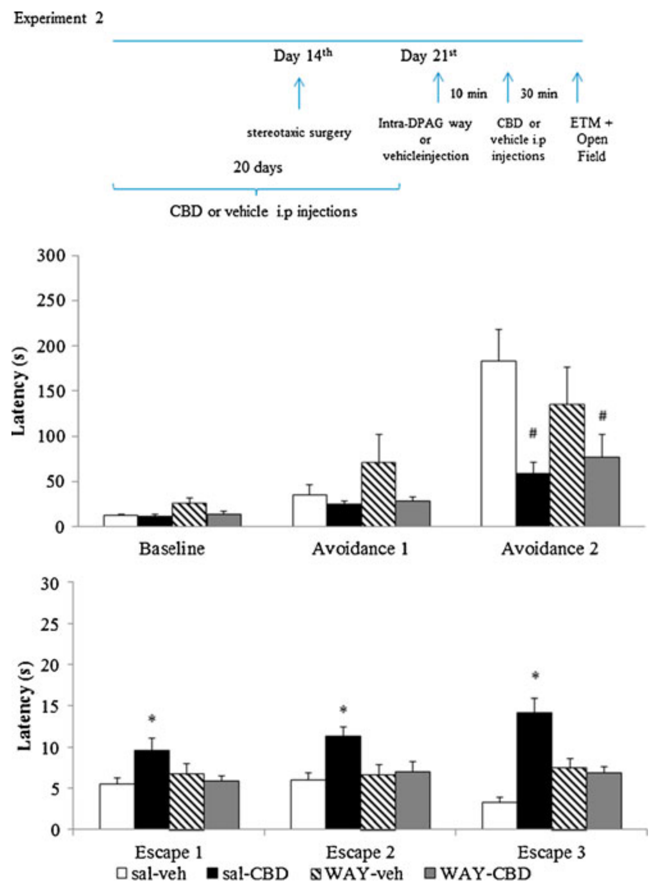

Fig. 4 Effects (mean ± SEM) of WAY-100635 (0.37 nmol) injected intra-DPAG on CBD chronic peripheral administration (5 mg/Kg/day for 21 days) effects on inhibitory avoidance (*upper panel*) and escape (*lower panel*) latencies measured in the elevated T-maze (experiment 2). n=9–14; the *number sign* indicates $p<0.05$ related to sal-veh group in the same trial and the *asterisk* indicates $p<0.05$ compared to all groups in the same trial

activity in the open field was not affected by treatments (see Table 1).

Experiment 3

As can be seen in Fig. 5, statistical analysis did not show any significant difference in 5-HT or 5-HIAA extracellular concentrations in the DPAG in rat submitted to either acute (experiment 3A) or chronic (experiment 3B) peripheral injections of CBD, when compared to control groups.

Experiment 4

As shown in the Fig. 6, repeated treatment with CBD or fluoxetine did not alter 5-HT1A (upper panel) or 5-HT2C mRNA expression (lower panel) when compared with the vehicle group.

Discussion

Our results showed that, similar to the benzodiazepine diazepam, single i.p. CBD injections impaired inhibitory avoidance acquisition without changing escape responses in the ETM. However, like the SSRI fluoxetine, CBD repeated treatment inhibited the escape response evoked in the ETM. Even considering that there is a graphic trend, chronic CBD administration failed to decrease inhibitory avoidance in experiment 1B. In experiment 2, however, the same treatment was able to impair inhibitory avoidance, indicating an anxiolytic. No drug treatment affected the total distance traveled in the open field test, suggesting that the results observed in the ETM were not due to nonspecific alterations in motor function.

Our results suggested that CBD anti-panic effect relies on direct activation of 5-HT1A receptors located in the DPAG since it was blocked by intra-DPAG injections of WAY-

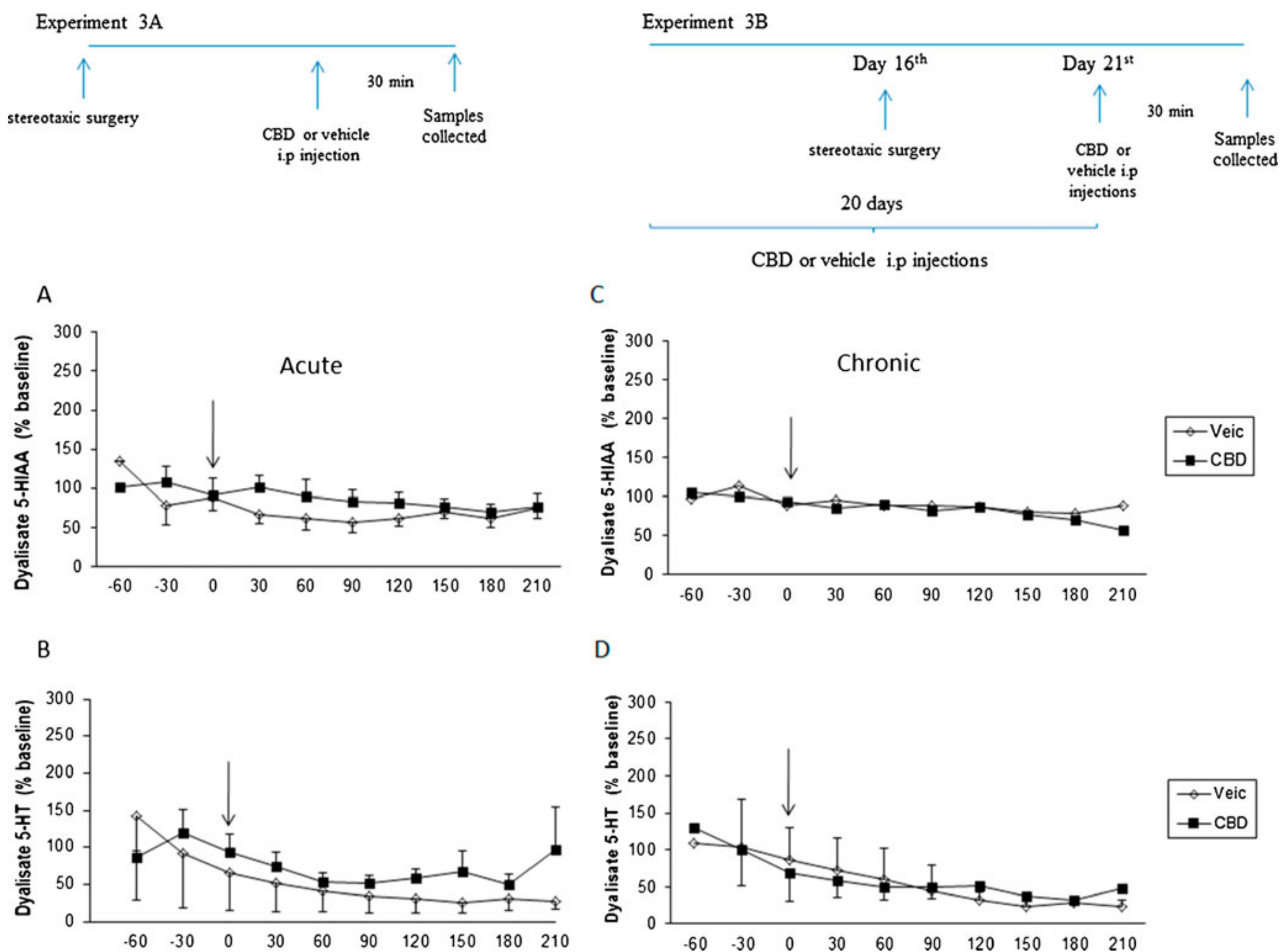

Fig. 5 Effects (mean ± SEM) of acute (5 mg/kg, A and B- experiment 3A) or chronic (5 mg/kg/day for 21 days, C and D-(experiment 3B)) treatment (i.p.) of cannabidiol or vehicle on extracellular levels of 5-HT or 5-HIAA in the dorsal periaqueductal gray matter dialysates. The *arrows* indicate the injection of cannabidiol (5 mg/kg) or vehicle. Data are expressed as percentage of baseline levels (average of three samples before last systemic injection; n=5 (vehicle) and 6 (CBD-acute) and n=4 (vehicle) and 5 (CBD-chronic))

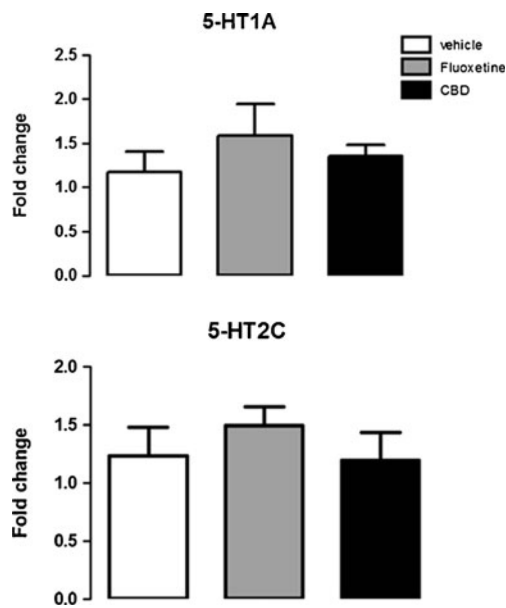

Fig. 6 Cannabidiol chronic effects were not associated with changes in DPAG fold change ($2^{-\Delta/\Delta ct}$) mRNA for 5HT-1A and 5HT-2C receptors (experiment 4). Data represent the means ± SEM of six animals

100635, a 5-HT1A receptor antagonist. CBD affects escape responses, but not inhibitory avoidance. Because CBD affected escape responses only after repeated treatment, we hypothesized that, similar to antidepressants, CBD could be acting by inhibiting 5-HT reuptake (Hershkowitz and Szechtman 1979). Despite our expectation, the microdialysis results suggested that, at least in the DPAG, CBD effects are not mediated by increased 5-HT concentrations in the synaptic cleft. A possible limitation of these results is that the four baseline samples were collected following a short period (2.5 h) after the placement of the probes, while other studies also report overnight probe placement before baseline samples collection. A similar interval, however, has been used by other groups with positive results (De Souza Silva et al. 2009; Karrenbauer et al. 2011; Zanoveli et al. 2010).

After repeated CBD treatment, 5-HT1A and 5-HT2C genes were not up- or downregulated in DPAG. Interestingly, several studies in the literature suggest that, similar to our results, antidepressant chronic treatment does not modify serotonin-1A receptor expression in brain areas related to the control of emotional states such as raphe nuclei (Castro et al. 2003, 2008, Moulin-Sallanon et al. 2009).

In the ETM test, the anxiolytic agents diazepam (benzodiazepine), buspirone, and ipsapirone (5-HT1A agonists) as well as ritanserin (5-HT2 antagonist) selectively impaired inhibitory avoidance while leaving one-way escape unchanged. Thus, impairment of inhibitory avoidance acquisition has been proposed to indicate anxiolytic-like effects (Graeff et al. 1993; Viana et al. 1994; Zangrossi and Graeff 1997; Teixeira et al. 2000; Zanoveli et al. 2003; Poltronieri et al. 2003). Our results, therefore, are in agreement with those previously found with CBD acute injections in other animal models such as the EPM and Vogel conflict tests (Guimarães et al. 1990; Moreira et al. 2006). Regarding the chronic treatment findings, it is of note that CBD anxiolytic effect was only significant in experiment 2, but not in experiment 1B. This discrepancy may conceivably reflect the difference in the time interval between the last drug injection and behavioral testing followed in these two studies. This time interval was 3 h in experiment 1B, while in experiment 2, it was 40 min. The interval of the former study was chosen based on results obtained in our previous work with fluoxetine using a similar protocol (Zanoveli et al. 2010) and in pharmacokinetics results showing that, in rats, the maximum brain concentration of CBD is reached only 60 min after i.p. administration, with an apparent elimination half-life of 289 min (Deiana et al. 2011). This would assure brain levels of CBD around 60–70 % of the maximum reached. In experiment 2, we now again used the same interval employed by Zanoveli et al. (2010) to compare the behavioral and neurochemical effects of CBD. In this case, therefore, the observed anxiolytic-like effect could be reflecting the acute, instead of chronic, effect of CBD.

It should also be noticed that the latencies to reach the open arms of vehicle-treated animals in experiment 1B were smaller than in all other experiments, which could have prevented a significant effect of CBD. Even so, results from experiment 2 suggest that the anxiolytic effects of acute CBD administration do not show tolerance after repeated CBD administration. However, different from the acute anxiolytic effects observed after CBD injection into the DPAG and bed nucleus stria terminalis (Campos and Guimarães 2008; Gomes et al. 2011), the anxiolytic effects of repeated CBD do not seem to depend on activation of 5-HT1A receptors in the DPAG. Contrasting with these results, Elbatsh and colleagues (2012) have recently shown that repeated CBD treatment enhances contextual fear conditioning, a result suggesting anxiogenic effects of CBD repeated treatment. However, in this study, the animals were conditioned under the drug effect, precluding a conclusion whether CBD was influencing learning/memory mechanisms or the expression of fear conditioning.

Repeated CBD treatment increased the latency for escape responses, an effect similar to those observed after chronic treatment with classical anti-panic drugs, such as SSRIs and imipramine (Pinheiro et al. 2008; Teixeira et al. 2000; Zanoveli et al. 2010). Reinforcing the proposal of CBD panicolytic-like effect, we have previously shown that direct

injections of this drug into the DPAG also inhibit escape responses generated by electrical stimulation of this brain area (Soares Vde et al. 2010).

Preclinical and clinical studies have established a link between 5-HT and PD. Moreover, in the last three decades, a body of evidence has pointed to the periaqueductal gray matter as a converging link between 5-HT and panic. The PAG is a midbrain structure that, among other functions, integrates defensive behavior. Increasing 5-HT release by electrically or chemically stimulating the dorsal raphe nucleus (DRN) causes an inhibitory effect on escape reactions induced by electrical/chemical stimulation of the DPAG (Kiser et al. 1980; Pobbe and Zangrossi 2005). Similar effects are observed after direct intra-DPAG injections of the serotonin type 1A receptor agonist 8-OH-DPAT, implicating these receptors in the modulation of defensive reactions in this area (De Paula Soares and Zangrossi 2004; Nogueira and Graeff 1995; Zanoveli et al. 2003).

Russo and colleagues (2005) were the first to suggest that CBD could act as a 5-HT1A receptor agonist. They observed that CBD, at micromolar range, was able to displace a 5-HT1A receptor agonist from cloned human 5-HT1A receptors expressed in Chinese hamster ovary cultured cells. The involvement of 5-HT1A receptors in the effects of CBD was also supported by in vivo experiments (Campos and Guimarães 2008; Gomes et al. 2011; Mishima et al. 2005). For instance, the 5-HT1A receptors antagonist WAY-100635 prevented the anxiolytic and panicolytic-like effects of intra-DPAG injections of CBD in rats tested respectively in the elevated plus maze and DPAG-electrically induced escape responses models (Campos and Guimarães 2008; Soares Vde et al. 2010). Previous administration of CB1 or TRPV1 receptor antagonists failed to block these effects (Campos and Guimarães 2008) suggesting that, even if these receptors have been related to some in vitro and in vivo effects of CBD (Bisogno et al. 2001; Campos and Guimarães 2009; Casarotto et al. 2010), they are not mediating the acute anxiolytic effects of CBD in the DPAG.

The involvement of 5-HT1A receptors on CBD effects, however, is still not completely clear. Recent studies suggested that, despite the ability of CBD to mimic the action of 8-OH-DPAT, the drug does not act as a direct agonist on 5-HT1A receptors in brainstem preparations (Rock et al. 2011). Rock and colleagues (2011) suggested that CBD could facilitate 5-HT1A-mediated neurotransmission by targeting 5-HT1A receptors allosteric sites. Also, CBD can enhance the ability of a 5-HT1A agonist to stimulate [35S] GTPγS binding (Rock et al. 2011), a mechanism that could help to explain our results. Another possibility is modifications in intracellular signaling transduction pathways such as adenylate cyclase and protein kinase A. Supporting this proposal, Mato and colleagues (2010) suggested that, after fluoxetine chronic treatment, the mechanism behind the inhibition of adenylyl cyclase in the prefrontal cortex depends on an interaction between CB1 and 5-HT1A receptors.

Although these possibilities remain to be tested, they are compatible with the present results suggesting that, at least in the DPAG, CBD repeated treatment decreases escape responses by facilitating local 5-HT1A-mediated neurotransmission. This facilitation does not seem to involve increased serotonin release or 5-HT1A gene expression.

In addition to 5-HT1A, changes in endocannabinoid-mediated neurotransmission could also be involved in the effects of CBD chronic administration. Cannabinoids can modulate not only serotonergic neurotransmission but also serotonin subtypes 1A and 2A/2C receptors expression in the brain (Bambico et al. 2010, Cassano et al. 2011). Genetic deletion of the eCB/endovanilloid degradation enzyme *fatty acid amide hydrolase* (FAAH) increases the firing of serotonergic neurons located in dorsal raphe nucleus. As a consequence, serotonin release is increased in limbic areas such as prefrontal cortex (Bambico et al. 2009). As mentioned before, CBD can also facilitate eCB-mediated neurotransmission by inhibiting FAAH activity (Bisogno et al. 2001), a mechanism that could also contribute to its effects. The involvement of these mechanisms on CBD effects after repeated treatment remains to be investigated.

In conclusion, the present results suggest that repeated administration of CBD induces anti-panic effects by facilitating 5-HT1A-mediated neurotransmission in the DPAG. This facilitation does not seem to involve a local increase in serotonin release or 5-HT1A mRNA expression.

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Conflict of interest Authors do not report any conflict of interest.

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