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Original Paper

Cannabidiol injected into the bed nucleus of the stria terminalis reduces the expression of contextual fear conditioning via 5-HT_{1A} receptors

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Abstract

Systemic administration of cannabidiol (CBD) attenuates cardiovascular and behavioral changes induced by re-exposure to a context that had been previously paired with footshocks. Previous results from our group using cFos immunohistochemistry suggested that the bed nucleus of the stria terminalis (BNST) is involved in this effect. The mechanisms of CBD effects are still poorly understood, but could involve 5-HT_{1A} receptor activation. Thus, the present work investigated if CBD administration into the BNST would attenuate the expression of contextual fear conditioning and if this effect would involve the activation of 5-HT_{1A} receptors. Male Wistar rats with cannulae bilaterally implanted into the BNST were submitted to a 10 min conditioning session (six footshocks, 1.5 mA/3 s). Twenty-four hours later freezing and cardiovascular responses (mean arterial pressure and heart rate) to the conditioning box were measured for 10 min. CBD (15, 30 or 60 nmol) or vehicle was administered 10 min before the re-exposure to the aversive context. The second experiment was similar to the first one except that animals received microinjections of the 5-HT_{1A} receptor antagonist WAY100635 (0.37 nmol) 5 min before CBD (30 nmol) treatment. The results showed that CBD (30 and 60 nmol) treatment significantly reduced the freezing and attenuated the cardiovascular responses induced by re-exposure to the aversive context. Moreover, WAY100635 by itself did not change the cardiovascular and behavioral response to context, but blocked the CBD effects. These results suggest that CBD can act in the BNST to attenuate aversive conditioning responses and this effect seems to involve 5-HT_{1A} receptor-mediated neurotransmission.

Keywords

Anxiolytics, autonomic responses, cannabinoids, conditioned emotional response, freezing

Introduction

Cannabidiol (CBD) is a major component of *Cannabis sativa* which, unlike Δ^9-tetrahydrocannabinol (Δ^9-THC), is devoid of the typical psychotomimetic effects of cannabis (Zuardi, 2008). CBD systemic administration produces several pharmacological effects such as anticonvulsant (Carlini and Cunha, 1981; Cunha et al., 1980), neuroprotective (Hampson et al., 1998; Iuvone et al., 2009), antipsychotic (Zuardi et al., 2006) and anxiolytic (Crippa et al., 2004) effects. Regarding the latter, the anxiolytic properties of CBD have been demonstrated by several preclinical studies which employed different paradigms such as the Vogel conflict test (Moreira et al., 2006), the elevated plus maze (Guimarães et al., 1990; Onaivi et al., 1990) and the conditioned emotional response (Lemos et al., 2010; Resstel et al., 2006b). Similar to the data obtained in animal models, results from studies in healthy volunteers strongly suggested that CBD has an anxiolytic action (Crippa et al., 2004; Fusar-Poli et al., 2009; Zuardi et al., 1993) and also attenuated the anxiogenic effects induced by high doses of Δ^9-THC in humans (Karniol et al., 1974; Zuardi et al., 1982).

Concerning the conditioned emotional response, systemic administration of CBD attenuated the expression of contextual fear conditioning (Lemos et al., 2010; Resstel et al., 2006b). Conditioned fear to a context is produced by re-exposing the animal to an environment (context) that has been previously paired with an aversive or unpleasant stimulus such as an electrical footshock (Antoniadis and McDonald, 1999; Fanselow, 1980; Rudy et al., 2004). Animals submitted to this model show freezing behavior and autonomic changes such as an increase in mean arterial pressure (MAP) and heart rate (HR) (Blanchard and Blanchard, 1969; Carrive, 2000; Resstel et al., 2006b).

The mechanisms and brain structures involved in the anxiolytic effects of CBD are not entirely known, since this drug can produce multiple pharmacological effects by different mechanisms (Izzo et al., 2009). For example, CBD has a

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low affinity for cannabinoid receptors (Petitet et al., 1998; Thomas et al., 1998), but may enhance endocannabinoid-mediated actions through its ability to inhibit the hydrolysis and/or reuptake of endocannabinoid anandamide (Bisogno et al., 2001). Furthermore, CBD possesses agonistic properties at 5-HT_{1A} receptors (Russo et al., 2005) and recent results from our laboratory demonstrated its anxiolytic effects involve the activation of 5-HT_{1A} receptors (Campos and Guimarães, 2008; Resstel et al., 2009). It is unknown, however, if this mechanism is related to CBD effects on contextual fear conditioning.

Recently, our laboratory started to investigate the brain structures involved in the effects of CBD. This drug attenuated the increase in Fos protein expression, a marker of neuronal activation, induced by re-exposure to the aversively conditioned context in the bed nucleus of the stria terminalis (BNST) (Lemos et al., 2010), suggesting a possible involvement of this structure in CBD effects. Similarly, Beck and Fibiger (1995) reported that contextual fear conditioning activates the BNST, an effect attenuated by diazepam administered before the test session.

The BNST is a limbic structure associated with autonomic, neuroendocrine and behavioral functions (Casada and Dafny, 1991; Dunn, 1987; Dunn and Williams, 1995) that seems to be critically involved in the expression of anxiety-like responses (Davis et al., 2010; Walker et al., 2003). Reinforcing a possible role of this structure in contextual fear conditioning, recent findings from studies using lesions or reversible inactivation of the BNST showed attenuation of expression of conditioned emotional response to context (Resstel et al., 2008; Sullivan et al., 2004). Moreover, the BNST has also been proposed to participate in the cardiovascular changes observed in aversive situations such as restraint stress and contextual fear conditioning (Crestani et al., 2009; Resstel et al., 2008).

In light of the these findings, the aim of this study was to test the hypothesis that CBD injected into the BNST would attenuate behavioral and cardiovascular (MAP and HR) responses induced by contextual fear conditioning by, at least in part, activating 5-HT_{1A} receptors.

Materials and methods

Animals

The experiments were performed using male Wistar rats weighing 230–270 g. Animals were kept in the Animal Care Unit of the Department of Pharmacology, School of Medicine of Ribeirão Preto, University of São Paulo. Rats were housed individually 3 days before experimental manipulations (preconditioning, conditioning and testing) in plastic cages with free access to food and water under a 12 h light/dark cycle (lights on at 06:30 h). The institution's Animal Ethics Committee approved the housing conditions and experimental procedures (process number: 88/2009). Procedures were conducted in conformity with the Brazilian Society of Neuroscience and Behavior guidelines for the care and use of laboratory animals, which are in compliance with international laws and policy.

Stereotaxic surgery

Seven days before the experiment the rats were anesthetized with 2,2,2 tribromoethanol (250 mg kg^{-1} intraperitoneally; Sigma-Aldrich, St Louis, Missouri, USA) and fixed in a stereotaxic frame (Stoelting, Wood Dale, Illinois, USA). After scalp anesthesia with 2% lidocaine, the skull was surgically exposed and stainless steel guide cannulae (26 G) were implanted bilaterally into the BNST [anteroposterior = −0.4 mm from bregma; lateral = 4.0 mm from the medial suture; vertical = −5.5 mm from the skull with a lateral inclination of 23° (Paxinos and Watson, 1997)]. Cannulae were fixed to the skull with dental cement and a metal screw. An obturator inside the guide cannulae prevented obstruction.

Fear conditioning and testing

Preconditioning, conditioning and testing were carried out in 25 x 22 x 22 cm footshock chambers. The chambers had a grid floor composed of 18 stainless steel rods (2 mm in diameter), spaced 1.5 cm apart and wired to a shock generator (Automatic Reflex Conditioner, model 8572; Ugo Basile, Comerio, Varese, Italy). They were cleaned with 70% ethanol before and after use. Preconditioning started 7 days after the stereotaxic surgery and consisted of two 10-min-long pre-exposures (habituation) to the footshock chamber (one in the morning and one in the afternoon). No shock was given during the pre-exposures. In the conditioning shock session, performed 24 h after the first habituation session, animals were divided into two experimental groups: non-conditioned and conditioned. The non-conditioned group was exposed to the footshock chamber for 10 min but no shock was delivered. The conditioned group was submitted to a shock session consisting of six electrical 1.5 mA/3 s footshocks (Resstel et al., 2008) delivered at pseudorandom intervals (ranging from 20 s to 60 s). The animals were allowed to explore the chamber prior to shock delivery for 2 min, a procedure proposed as essential for context conditioning (Fanselow, 2000). After the conditioning session, the rats were anesthetized with 2,2,2 tribromoethanol (250 mg kg^{-1} intraperitoneally) and a catheter [a 4 cm segment of polyethylene (PE)-10 heat-bound to a 13 cm segment of PE-50; Clay Adams, Parsippany, New Jersey, USA] was implanted into the femoral artery for blood pressure and HR recording. The catheters were tunneled under the skin and exteriorized on the animal's dorsum.

Cardiovascular and behavioral responses evoked by re-exposure to aversively conditioned context were evaluated 24 h after conditioning. The test session consisted of a 10-min-long re-exposure to the footshock chamber without shock delivery. Animals were transferred from the animal room to the procedure room in their home cage. Cardiovascular recordings were initiated after a 60 min adaptation period. Since animals can associate cues of the environment with conditioning (Frank et al., 2004), the tests were performed in a different room than that used during the conditioning procedure to allow for baseline autonomic measures. MAP and HR were recorded using an HP-7754A amplifier (Hewlett Packard, Palo Alto, California, USA)

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connected to a signal acquisition board (Biopac M-100, Goleta, California, USA) and computer processed. Rats were tested one at a time. Five minutes after the last microinjection, a 5 min of baseline recording was performed and then the animals were placed at the center of the footshock chamber to record cardiovascular and behavioral responses evoked by the conditioned emotional response to context.

Behavioral responses were evaluated during the test by an experimenter blind to the experimental groups sat 45 cm away from the footshock chamber. Freezing was defined as the complete absence of movement, except that of the flanks related to respiration, while the animal assumed a characteristic tense posture (Fanselow, 1980). In addition to the duration of freezing, we also evaluated the animal's activity during the test by counting the number of crossings and rearings. A crossing was recorded when the animal crossed with the four paws from one to the other side of the cage.

Experimental design

Experiment 1: Effects of CBD microinjected into the BNST on contextual fear conditioning. The habituated and conditioned animals received bilateral injections of vehicle or CBD (15, 30 or 60 nmol; 200 nL per site; dose range used by Campos and Guimarães, 2008; Lemos et al., 2010) into the BNST 10 min before re-exposure to the conditioning box. For microinjections, we used a 33 G needle (Small Parts, Miami Lakes, Florida, USA) 1 mm longer than the guide cannulae, connected to a 10 μL syringe (7001 KH, Hamilton Co., Reno, Nevada, USA) through a PE-10 tube. The needles were carefully inserted into the guide cannulae, and the solutions were infused over a 30 s period at a rate of 400 nL min^{-1} with the help of an infusion pump (KD Scientific Inc., Holliston, Massachusetts, USA). The needles remained in place for an additional 45–60 s period to prevent reflux.

Experiment 2: Involvement of 5-HT_{1A} receptors in the effects of CBD injected into the BNST on contextual fear conditioning. Based on the results obtained in experiment 1, we chose the 30 nmol dose of CBD as the dose to be used in experiment 2. The protocol was similar to that of experiment 1, except that animals received intra-BNST injections of saline or the 5-HT_{1A} receptors antagonist, WAY100635 (0.37 nmol; 200 nL per site) followed 5 min later by a second injection of vehicle or CBD (30 nmol). Ten minutes after the last injection, the animals were placed at the center of the footshock chamber. The dose of WAY100635 was chosen based on previous studies using intracerebral injections (Campos and Guimarães, 2008; De Paula Soares and Zangrossi, 2004).

Drugs

CBD (kindly supplied by THC Pharm, Frankfurt am Main, Germany) was dissolved in grape seed oil (Campos and Guimarães, 2008; Lemos et al., 2010). The 5-HT_{1A} receptors antagonist N-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-N-(2-pyridinyl) cyclohexane carboxamide maleate (WAY100635, Sigma-Aldrich, St Louis, Missouri, USA) was dissolved in saline. The solutions were prepared immediately before the tests and protected from the light during the experimental sessions.

Histological procedure

At the end of the experiments the rats were anesthetized with urethane (1.25 g kg^{-1} intraperitoneally, Sigma-Aldrich, St Louis, Missouri, USA) and 200 nL of 1% Evans blue dye was bilaterally injected into the BNST as an injection site marker. The chest was surgically opened, the descending aorta occluded, the right atrium severed and the brain perfused with 10% formalin through the left ventricle. Brains were postfixed for 24 h at 4°C, and 40 mm sections were cut using a cryostat (CM-1900, Leica, Wetzlar, Germany). Serial brain sections were stained with 1% neutral red and injection sites determined using the rat brain atlas (Paxinos and Watson, 1997) as reference. As a histological control, the animals submitted to contextual fear conditioning that received CBD (30 nmol) outside the BNST were joined together in an additional group (OUT).

Statistical analysis

MAP and HR values were continuously recorded during the 5 min period before and the 10 min period after exposure to the footshock chamber. Data were expressed as means ± SEM of MAP or HR changes (respectively ΔMAP and ΔHR) sampled at 1 min intervals. Points sampled during the 5 min before exposure were used as the control baseline value. MAP and HR changes were analysed using a three-way ANOVA with condition (conditioned or non-conditioned) and treatment as the main independent factors, and time as a repeated measurement. When interactions between the factors were observed, groups (conditioned or non-conditioned) were compared using the Bonferroni's post hoc test. Freezing was expressed as a percentage of the test period. Freezing, crossings and rearings were expressed as means ± SEM and analysed using a two-way ANOVA with condition (conditioned or non-conditioned) and treatment as the two factors. A non-linear regression analysis was performed to investigate the dose-response curve obtained with CBD on freezing behavior. Animals receiving CBD (30 nmol) outside the BNST were compared to vehicle non-conditioned or conditioned groups by the Student's *t*-test. Experiment 2 was also analysed by two-way ANOVA using the first (WAY100635 or saline) and the second (CBD or vehicle) injections as main factors. When interaction between the factors was observed, specific one-way ANOVA followed by the Bonferroni's post hoc test was performed. Results of statistical tests with $P < 0.05$ were considered significant.

Results

Diagrammatic representations showing the bilateral injection sites in the BNST and a representative photomicrograph are shown in Figure 1.

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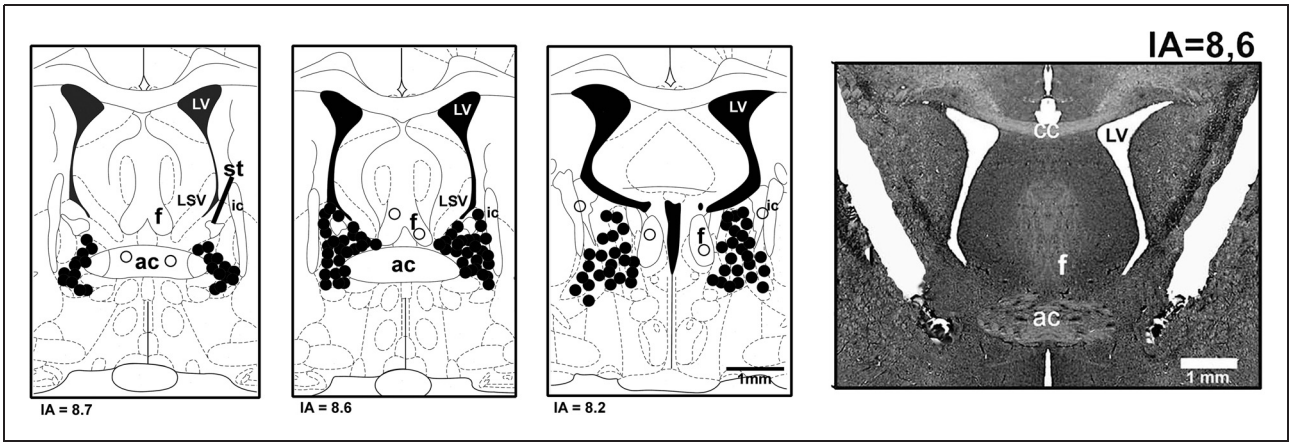

Figure 1. Photomicrograph of a coronal brain section from a representative rat showing bilateral microinjection sites in the BNST, and diagrammatic representation (based on the rat brain atlas of Paxinos G and Watson C (1997) The Rat Brain in Streretaxic Coordinates, 2nd edition with permission from Elsevier), indicating the injection sites into (black circles) and outside (white circles) the BNST of all the animals used in the experiments. AC: anterior commissure, BNST: bed nucleus of the stria terminalis, CC: corpus callosum, F: fornix, IA: interaural (coordinate), IC: internal capsule, LSV: lateral septal, ventral, LV: lateral ventricle, ST: stria terminalis.

Experiment 1: Effects of CBD microinjected into the BNST on contextual fear conditioning

Behavioral responses to fear conditioning. There were significant effects of condition ($F_{1,31} = 216.8$, $P < 0.001$), treatment ($F_{1,31} = 19.4$, $P < 0.001$) and interaction ($F_{3,31} = 20.7$, $P < 0.001$) on the percentage of freezing. Vehicle-treated rats which had received electrical footshocks (conditioned group) spent more time freezing during the re-exposure to context than the animals that did not receive shocks (non-conditioned group) (Figure 2A). CBD (30 and 60 nmol) injections significantly reduced ($F_{4,26} = 18.99$, $P < 0.0001$) freezing of conditioned animals ($n = 6$ per group) when compared with vehicle-treated conditioned animals ($n = 5$, Figure 2A). The drug (30 nmol) was unable to prevent the freezing response when injected into the structures surrounding the BNST ($t_6 = 5.73$, $P < 0.01$, Figure 2A). A non-linear regression analysis confirmed that CBD effects were dose-dependent, showing a significant association between drug doses and freezing attenuation ($r^2 = 0.75$, d.f. = 14, $P < 0.05$, Figure 2B). CBD (15, 30 or 60 nmol) injection into the BNST of non-conditioned rats did not produce any effect on freezing behavior ($n = 4$, $P > 0.05$, Figure 2A).

The evaluation of motor activity showed significant effects of condition ($F_{1,31} = 33.2$, $P < 0.001$), treatment ($F_{1,31} = 3.26$, $P < 0.05$) and interaction ($F_{3,31} = 5.54$, $P < 0.05$) on the number of crossings. Similar effects of condition ($F_{1,31} = 9.6$, $P < 0.01$), treatment ($F_{1,31} = 3.2$, $P < 0.05$) and interaction ($F_{3,31} = 4.46$, $P < 0.05$) were observed on the number of rearings. Rats treated with vehicle and 15 nmol CBD and pre-exposed to shocks showed a smaller number of crossings ($n = 4$–6 per group; $F_{2,12} = 31.5$, $P < 0.001$) and rearings ($n = 4$–6; $F_{2,12} = 9.34$, $P < 0.01$) than the non-conditioned vehicle group (Figure 3). Moreover, CBD (30 and 60 nmol) injections significantly increased the number of crossings ($F_{2,14} = 9.06$, $P < 0.01$) and rearings ($F_{2,14} = 7.71$, $P < 0.01$) of conditioned animals when compared to vehicle-treated conditioned animals (Figure 3).

Cardiovascular responses to fear conditioning. No differences were observed on basal levels of both MAP and HR among the groups recorded immediately before chamber re-exposure (Table 1).

The analyses of cardiovascular response during chamber re-exposition showed significant effects of condition (MAP: $F_{1,31} = 5.6$, $P < 0.01$; HR: $F_{1,31} = 24.01$, $P < 0.01$), treatment (MAP: $F_{3,31} = 4.41$, $P < 0.01$; HR: $F_{3,31} = 3.7$, $P < 0.01$) and time (MAP: $F_{14,434} = 114.2$, $P < 0.01$; HR: $F_{14,434} = 97.1$, $P < 0.01$). Conditioning versus time (MAP: $F_{14,434} = 4.6$, $P < 0.01$; HR: $F_{14,434} = 25$, $P < 0.01$), treatment versus time (MAP: $F_{42,434} = 2.7$, $P < 0.01$; HR: $F_{42,434} = 2.2$, $P < 0.01$) and conditioning versus treatment versus time interactions (MAP: $F_{42,434} = 2.3$, $P < 0.01$; HR: $F_{42,434} = 1.5$, $P < 0.05$) were also significant.

As shown in Figure 4, re-exposure to a context previously paired with footshocks induced a marked and sustained increase in HR and MAP during the 10 min test. In the non-conditioned groups, re-exposure to the context also increased HR and MAP. However, these increases were smaller than those observed in the conditioned groups (MAP: $F_{1,31} = 5.6$, $P < 0.01$; HR: $F_{1,31} = 24.01$, $P < 0.01$).

Similar to the behavioral responses, CBD (30 and 60 nmol) attenuated the increase in MAP and HR on conditioned groups (MAP: $F_{4,26} = 8.296$, $P < 0.001$; HR: $F_{4,26} = 5.387$, $P < 0.01$), but had no effect on non-conditioned animals (MAP: $F_{3,15} = 0.314$, $P > 0.05$; HR: $F_{3,15} = 0.517$, $P > 0.05$, Figure 4). No cardiovascular changes were observed when 30 nmol of CBD (Figure 4) was microinjected into the structures surrounding the BNST of conditioned animals before the test ($n = 4$, MAP: $P > 0.05$ and HR: $P > 0.05$ respectively).

Experiment 2: Involvement of 5-HT$_{1A}$ receptors on CBD effects

Behavioral responses to fear conditioning. Similar to experiment 1, 30 nmol of CBD reduced freezing of

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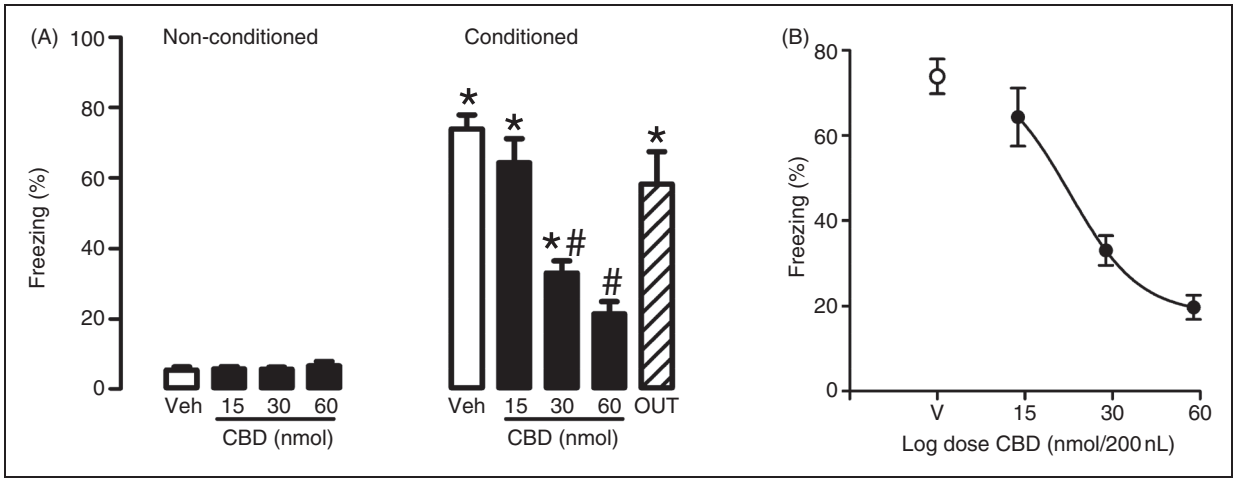

Figure 2. (A) Effects of bilateral microinjection of 200 nL of vehicle or CBD (15, 30 or 60 nmol) in non-conditioned ($n = 4$ per group) and conditioned ($n = 5$–6) animals on the percentage of time spent in freezing behavior. Conditioned animals receiving CBD (30 nmol) injections outside the BNST were joined in an additional group (OUT, $n = 4$). Columns represent the mean and bars the SEM, $^{*}P < 0.05$ compared to vehicle non-conditioned group and $^{\#}P < 0.05$ compared to vehicle conditioned group, Bonferroni's post hoc test. (B) Percentage of time spent in freezing behavior in response to the bilateral microinjection of vehicle (V, $n = 5$) or increasing doses of CBD ($n = 5$–6) in conditioned rats. Dose-effect curves were generated by non-linear regression analysis. Symbols represent the means ± SEM. BNST: bed nucleus of the stria terminalis, CBD: cannabidiol, Veh: vehicle.

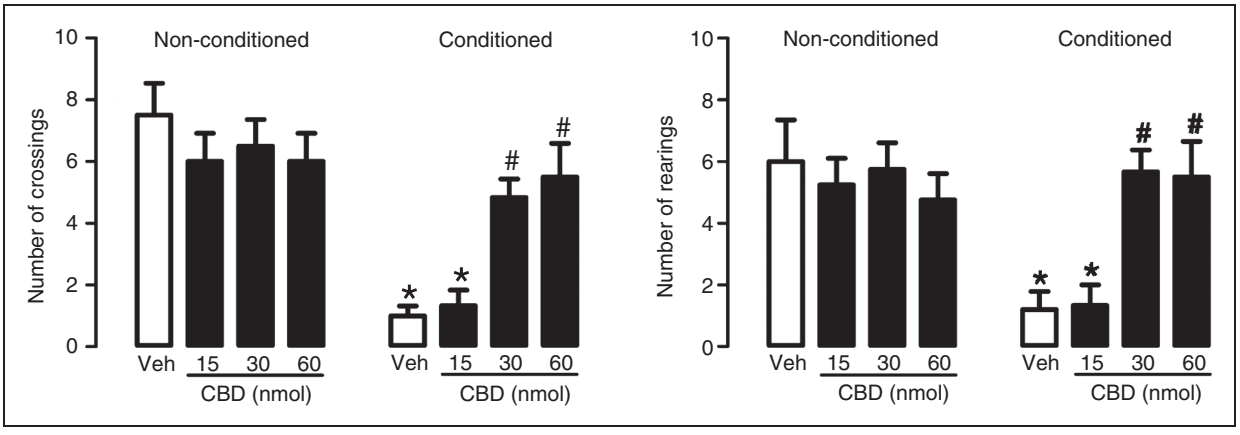

Figure 3. Effects of bilateral microinjection of 200 nL of vehicle or CBD (15, 30 or 60 nmol) on the number of crossings and rearings of non-conditioned ($n = 4$ per group) and conditioned ($n = 5$–6) rats. Columns represent the mean and bars the SEM, $^{*}P < 0.05$ compared to vehicle non-conditioned group and $^{\#}P < 0.05$ compared to vehicle conditioned group, Bonferroni's post hoc test. CBD: cannabidiol, Veh: vehicle.

conditioned animals ($n = 5$) as compared to control animals ($n = 5$) ($F_{3,20} = 11.73$, $P < 0.001$). No effect on freezing was found in animals treated only with WAY100635 ($n = 6$, $P > 0.05$), but the drug was able to reduce the effects of CBD ($n = 5$, $P > 0.05$, compared to control vehicle group, Figure 5A). Moreover, there was significant interaction between the first and second injections ($F_{1,17} = 12.73$, $P < 0.01$).

Cardiovascular responses to fear conditioning. WAY100635 did not affect the basal values of MAP ($F_{3,20} = 0.21$, $P > 0.05$) or HR ($F_{3,20} = 0.61$, $P > 0.05$) compared to 30 nmol of CBD and vehicle (Table 1). Confirming the results from experiment 1, 30 nmol of CBD attenuated the increase of the cardiovascular responses (MAP: $F_{3,20} = 6.948$, $P < 0.01$; HR: $F_{3,20} = 3.855$, $P < 0.05$) induced by the aversive context. WAY100635 did not affect cardiovascular responses by itself (MAP: $P > 0.05$; HR: $P > 0.05$) but was able to inhibit CBD effects on the cardiovascular responses (MAP: $P > 0.05$; HR: $P > 0.05$) observed during aversive context re-exposition (Figure 5B). Furthermore, significant interaction between the first and second injections was found only relative to MAP ($F_{1,17} = 7.01$, $P < 0.05$), however, there was tendency to HR ($F_{1,17} = 3.61$, $P = 0.07$).

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Table 1. Basal values of MAP and HR in non-conditioned and conditioned animals

Group		MAP (mmHg)	HR (bpm)
Non-conditioned			
Vehicle	$n = 4$	107 ± 5	369 ± 9
CBD 15 nmol	$n = 4$	98 ± 3	343 ± 12
CBD 30 nmol	$n = 4$	99 ± 2	360 ± 21
CBD 60 nmol	$n = 4$	104 ± 6	359 ± 12
		$F_{3,15} = 0.957$	$F_{3,15} = 0.576$
Conditioned			
Vehicle	$n = 5$	96 ± 4	351 ± 13
CBD 15 nmol	$n = 6$	99 ± 3	356 ± 19
CBD 30 nmol	$n = 6$	100 ± 5	368 ± 12
CBD 60 nmol	$n = 6$	97 ± 3	345 ± 8
OUT	$n = 4$	102 ± 3	359 ± 4
		$F_{4,26} = 0.337$	$F_{4,26} = 0.465$
Saline + Vehicle	$n = 5$	100 ± 3	365 ± 15
WAY100635 + Vehicle	$n = 6$	100 ± 3	345 ± 5
Saline + CBD	$n = 5$	101 ± 3	364 ± 13
WAY100635 + CBD	$n = 5$	102 ± 3	346 ± 21
		$F_{3,20} = 0.210$	$F_{3,20} = 0.618$

The values in the table represent the means ± SEM, one-way analysis of variance.
CBD: cannabidiol, HR: heart rate, MAP: mean arterial pressure.

Discussion

Confirming previous studies (Resstel et al., 2008; Zhang et al., 2004), conditioned animals exhibited significant cardiovascular changes (increases in HR and MAP) and behavioral responses (freezing, decreases in the number of crossings and rearings) after being re-exposed to a context previously paired with aversive footshocks. These effects were attenuated by intra-BNST injection of CBD. This compound did not induce any significant change in the basal levels of MAP and HR, which agrees with the reported lack of significant cardiovascular effects of this drug (McQueen et al., 2004; Resstel et al., 2006b; Resstel et al., 2009). It is unlikely, therefore, that the attenuation of the cardiovascular responses to aversive context depends on direct cardiovascular effects, but rather on an attenuation of the emotional response. In agreement with this proposal, acute administration of CBD has been shown to induce anxiolytic-like effects in several animal models (Guimarães et al., 1990; Moreira et al., 2006; Resstel et al., 2006b).

Although the extent of CBD diffusion from the injection site could not be precisely established, the lack of any effect when the drug was administered into nearby structures suggests that the effects were indeed mediated by drug action in the BNST. CBD effects could also be explained by

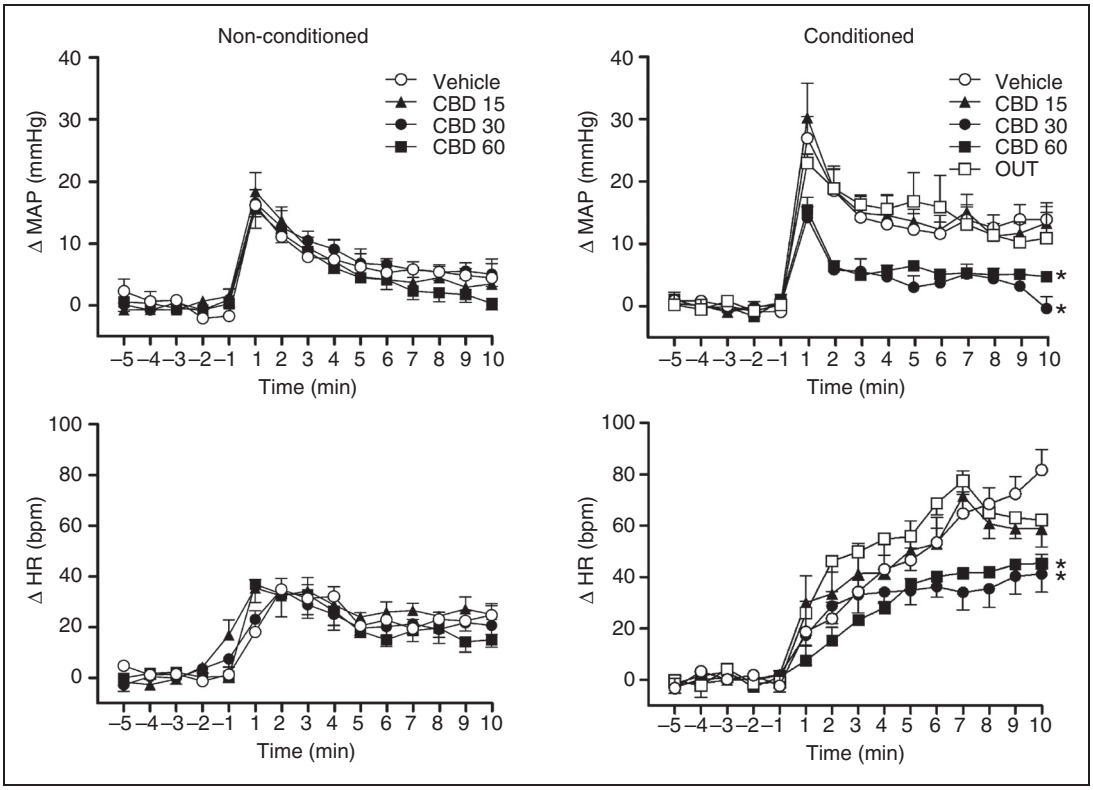

Figure 4. Time course of the effects of bilateral microinjection of 200 nL of vehicle ($n = 5$) or CBD (15, 30 or 60 nmol) on mean arterial pressure (ΔMAP) and heart rate (ΔHR) increases recorded in non-conditioned ($n = 4$ per group) and conditioned ($n = 5$–6) rats. Conditioned animals receiving CBD (30 nmol) injections outside the BNST were joined in an additional group (OUT, $n = 4$). Symbols represent the means and bars represent the SEM. The asterisk indicates significant treatment difference ($P < 0.05$, Bonferroni's post hoc test) over the whole footshock chamber exposure period compared to vehicle treated animals.
BNST: bed nucleus of the stria terminalis, CBD: cannabidiol, HR: heart rate, MAP: mean arterial pressure.

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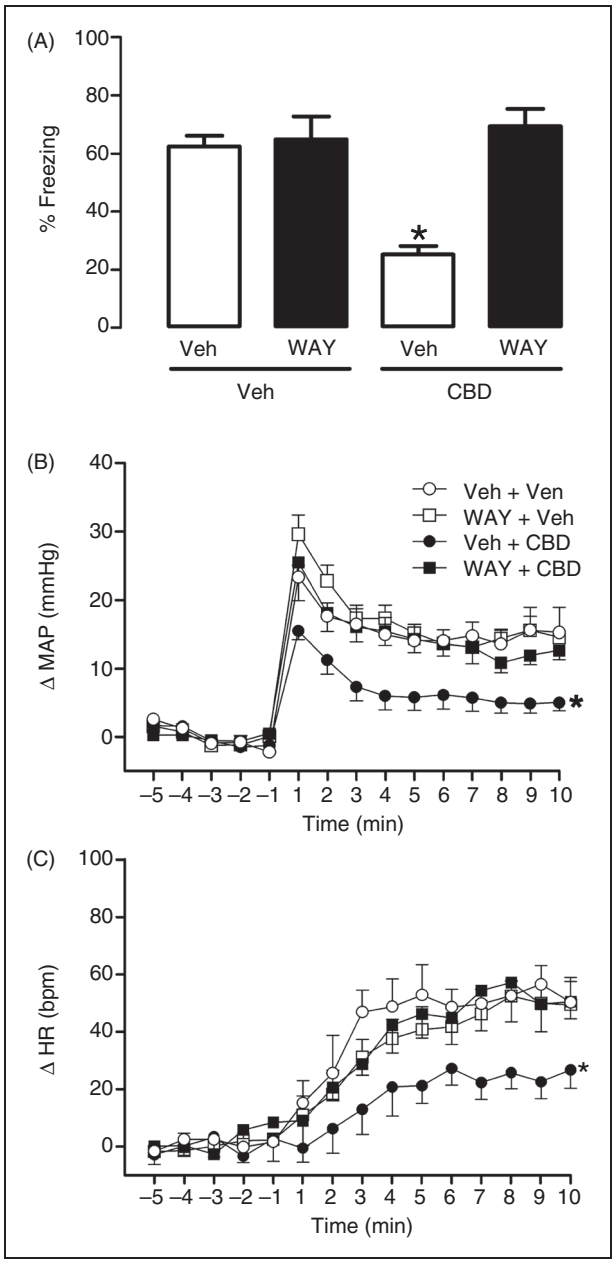

Figure 5. (A) Effect of bilateral pretreatment with saline (Veh, 200 nL) or WAY100635 (WAY, 0.37 nmol) followed by a second injection of grape seed oil (Veh) or CBD (30 nmol) on the percentage of freezing induced by re-exposure to the aversive context ($n = 5$–6). Data represent the mean ± SEM, $^*P < 0.05$ compared to other groups, Bonferroni's post hoc test. (B) Time course of the effects of bilateral pretreatment with saline (Veh, 200 nL) or WAY100635 (WAY, 0.37 nmol) followed by a second injection of grape seed oil (Veh) or CBD (30 nmol) on mean arterial pressure (ΔMAP) and heart rate (ΔHR) increase induced by re-exposure to the aversive context ($n = 5$–6). Symbols represent the mean and bars the SEM, $^*P < 0.05$, Bonferroni's post hoc test, over the whole footshock chamber exposure period compared to other groups. CBD: cannabidiol, HR: heart rate, MAP: mean arterial pressure, Veh: vehicle, WAY: WAY100635.

interference on memory recall. However, a low potential for interference with learning and memory has been reported for this drug (Fadda et al., 2004), making this possibility improbable.

Corroborating the present results, Lemos et al. (2010) found that systemic administration of CBD was able to reduce freezing behavior and activation of medial prefrontal cortex and BNST neurons induced by re-exposure to the aversively conditioned context. In that study, CBD injected into the prelimbic prefrontal cortex was also able to attenuate the conditioned aversive responses. Together, these observations and our results suggest that the BNST and the prelimbic cortex are involved in the effects of CBD on contextual fear conditioning when CBD is administered systemically.

Supporting a possible regulatory role in defensive responses, the BNST is proposed to be an important relay station linking important forebrain structures involved in conditioned emotional response to context such as the amygdala, hippocampus and medial prefrontal cortex (Dong et al., 2001a, 2001b; Davis et al., 1997a; Fanselow, 2000; Fendt and Fanselow, 1999; Massi et al., 2008; Milad and Quirk, 2002; Resstel et al., 2006a; Rudy et al., 2004; Vertes, 2006), to the hypothalamus and autonomic regulatory brainstem areas (Alheid, 2003; Davis et al., 1997b; Herman and Cullinan, 1997; Herman et al., 1994). In addition to autonomic responses, the BNST could also modulate behavior responses to aversive stimuli. Recent studies using lesions or pharmacological reversible inactivation showed that the BNST is important for the expression of aversive responses such as freezing, MAP and HR increases, as well as for increases in the release of corticosterone when the animals are exposed to the context in which they had previously received a footshock (Gray et al., 1993; Resstel et al., 2008; Sullivan et al., 2004). Another finding of the present study is that the effects of CBD microinjected into the BNST on the expression of contextual fear conditioning were blocked by pretreatment with WAY100635, a 5-HT_{1A} receptor antagonist, at a dose that did not produce any effect by itself. This finding agrees with recent studies showing that several CBD effects involve 5-HT_{1A} receptor-mediated neurotransmission. Antagonists to the 5-HT_{1A} receptor were able to prevent several effects induced by CBD, including antidepressive (Zanelati et al., 2010), anti-stress (Resstel et al., 2009) and anxiolytic effects after drug microinjection into the dorsolateral periaqueductal gray (Campos and Guimarães, 2008). In addition, Magen et al. (2010) showed that chronic treatment with CBD improves cognitive and motor impairments in an animal model of hepatic encephalopathy via 5-HT_{1A} receptor.

Brain 5-HT_{1A} receptors are located presynaptically in cell bodies in the raphe nuclei of the brainstem and postsynaptically predominantly in limbic structures including the BNST (Barnes and Sharp, 1999; Chalmers and Watson, 1991; Phelix et al., 1992; Verge et al., 1985). This receptor is thought to play an important role in the etiology of anxiety disorders (Akimova et al., 2009). 5-HT_{1A} receptor knockout mice display increased anxiety-related behavior (Heisler et al., 1998; Parks et al., 1998; Ramboz et al., 1998) and partial 5-HT_{1A} receptor agonists are anxiolytics (Barnes and Sharp, 1999; Rickels and Rynn, 2002).

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The BNST receives a reasonably dense innervation by serotonergic afferents from the caudal regions of the dorsal raphe nucleus (Commons et al., 2003; Lowry et al., 2008) and expresses several subtypes of serotonin receptors (Cornea-Hébert et al., 1999; Guo et al., 2009; Heidmann et al., 1998; Kia et al., 1996; Mengod et al., 1990; Pompeiano et al., 1994; To et al., 1995; Waeber and Moskowitz, 1995; Waeber et al., 1994; Wright et al., 1995; Xu and Pandey, 2000). However, single cell reverse transcriptase PCR (RT-PCR) data revealed that 5-HT_{1A} and 5-HT_7 receptors are the most prevalent receptor subtypes expressed in BNST neurons (Guo et al., 2009). Based on these pieces of evidence, Hammack et al. (2009) suggested that the BNST could be a critical structure for serotonergic modulation of anxiety-like behaviors.

Electrophysiological studies have demonstrated that the application of serotonin to BNST neurons elicits inhibitory responses mediated by the activation of postsynaptic 5-HT_{1A} receptors (Levita et al., 2004; Rainnie, 1999) and local infusion of a 5-HT_{1A} receptor agonist, 5-carboxamidotryptamine (5-CT), into the BNST attenuated the acoustic startle response in rats (Levita et al., 2004). Buspirone, a partial 5-HT_{1A} receptor agonist clinically used in the treatment of anxiety, blocks the effects of light-enhanced startle in rats and the BNST is described to be critically involved in this animal model of anxiety (Davis et al., 1997a; Davis et al., 2010; Walker and Davis, 1997; Walker et al., 2003). Together these results suggest that 5-HT_{1A} receptor-mediated inhibition of BNST neurons modulates anxiety-like behaviors. Although the present results indicate that CBD effects in the BNST depend on the activation of 5-HT_{1A} receptors, it is not possible to rule out the involvement of other pharmacological mechanisms. For example, intracerebroventricular administration of CBD has been shown to facilitate contextual fear conditioning extinction, an effect prevented by treatment with a cannabinoid-1 (CB1) receptor antagonist (Bitencourt et al., 2008). Despite having a low affinity for CB1 and cannabinoid-2 (CB2) receptors *in vitro* (Petitet et al., 1998; Thomas et al., 1998), CBD can facilitate endocannabinoid-mediated neurotransmission by decreasing anandamide hydrolysis or reuptake (Bisogno et al., 2001). CBD has also been found to activate transient receptor potential cation channel, subfamily V, member 1 (TRPV1) receptors (Bisogno et al., 2001), enhance adenosine signaling through inhibition of uptake (Carrier et al., 2006) and allosterically modulate 5-HT_3 (Yang et al., 2010) and μ- and δ-opioid receptors (Kathmann et al., 2006). The involvement of these mechanisms in the effects of CBD in the BNST remains to be tested. In conclusion, the results of the present study suggest that CBD can decrease the expression of contextual fear conditioning in part, at least, by activating 5-HT_{1A} receptors in the BNST.

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Conflicts of interest statement

The authors have no conflicts of interest to declare.

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