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Facilitation of contextual fear memory extinction and anti-anxiogenic effects of AM404 and cannabidiol in conditioned rats

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Abstract

The present study investigated the central effects of the eCB uptake/metabolism inhibitor AM404 and the phytocannabinoid cannabidiol (CBD) on the extinction of contextual fear memories in rats. Rats were conditioned and 24 h later subjected to three consecutive 9-min non-reinforced exposures to the conditioning context (extinction sessions, 24 h intervals). AM404 or CBD was injected i.c.v. 5 min before each extinction session and a 3-min drug-free test of contextual memory was performed 24 h after the last extinction session. AM404 (1.0 μg/μl, i.c.v.) and CBD (2.0 μg/μl, i.c.v.) facilitated extinction of contextual fear memory, with persistent effects. These responses were antagonized by the CB1-selective antagonist SR141716A (0.2 mg/kg, i.p.), but not by the TRPV1-selective antagonist capsazepine (5.0 μg/μl, i.c.v.). The effect of the anxiolytic drug Diazepam (DZP) on the extinction of contextual fear memory was also investigated. In contrast with the CBD and AM404 results, DZP induced a general reduction in the expression of conditioned freezing. Both AM404 and CBD induced anti-anxiogenic effect in the fear-potentiated plus-maze test, whereas DZP was anxiolytic in conditioned and unconditioned rats. In conclusion, CBD, a non-psychoactive phytocannabinoid could be an interesting pharmacological approach to reduce the anxiogenic effects of stress and promote the extinction of fear memories.

1. Introduction

Cannabinoid compounds were first identified in extracts of the plant *Cannabis sativa*, which contains at least 66 compounds of this class. Among these compounds, Δ^9-tetrahydrocannabinol (Δ^9-THC) is the most widely studied and is considered to be responsible for the majority of Cannabis psychoactive effects

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(reviewed in Pertwee, 2006). The chemical isolation of Δ^9-THC and of other major cannabinoid compounds such as cannabinol and cannabidiol (CBD) was promptly followed by its synthesis, which boosted the researches in the cannabinoid field (Gaoni and Mechoulam, 1964); for a historical review, see (Mechoulam and Hanus, 2000). Although Δ^9-THC was readily recognized to be psychoactive, CBD has been considered to be a non-psychoactive cannabinoid (Bisogno et al., 2001; Di Marzo and Petrocellis, 2006; Mechoulam et al., 2002; Mechoulam et al., 1970). However, there is compelling experimental evidence suggesting that CBD induce effects in the central nervous system, being anxiolytic (Crippa et al., 2004) and antipsychotic (Zuardi et al., 1995) in humans and anxiolytic (Moreira et al., 2006; Resstel et al., 2006) and anticonvulsivant (Carlini et al., 1973; Consroe and Wolkin, 1977; Izquierdo et al., 1973) in laboratory animals. The pharmacology of CBD is not completely understood and several mechanisms of action have been proposed, including diffuse targets on the endocannabinoid (eCB) system (Bisogno et al., 2001), enhancement of adenosinergic signaling (Carrier et al., 2006), agonism of $5HT_{1A}$ serotoninergic receptors (Mishima et al., 2005) and $TRPV_1$ vanilloid receptors (Bisogno et al., 2001) (for recent reviews about the CBD pharmacology, see Mechoulam et al., 2007; Pertwee, 2008). Within the eCB system, CBD weakly binds to CB_1 and CB_2 receptors and inhibits the uptake and hydrolysis of anandamide, an endocannabinoid ligand (Bisogno et al., 2001). The pharmacological profile of CBD somewhat resembles that of AM404, a synthetic drug known to inhibit anandamide uptake/metabolism (Beltramo et al., 1997; De Petrocellis et al., 2000; Fegley et al., 2004; Fowler et al., 2004; Jarrahian et al., 2000) and to activate $TRPV_1$ receptors (De Petrocellis et al., 2000; Zygmunt et al., 2000). Some of the in vivo effects of AM404 seem to involve enhanced anandamide levels and therefore indirect activation of CB_1 cannabinoid receptors (Beltramo et al., 2000; Bortolato et al., 2006; Freund et al., 2003).

The eCB system is important for a number of physiological brain functions and there is an emerging interest in eCB-mediated modulation of emotionality (Kathuria et al., 2003; Viveros et al., 2005). Most cannabinoid effects in the brain occur through activation of CB_1 receptors, which are densely expressed in regions known to play an important role in anxiety and aversive learning, including amygdala and hippocampus (Freund et al., 2003; Herkenham et al., 1990), where eCB-related enzymes, such as FAAH are also found (Egertova et al., 2003). Therefore, not only exogenous cannabinoids can influence anxiety, but also enhancement of eCB neurotransmission modulates it, inducing anxiolytic-like effects (Patel and Hillard, 2006). Since the finding that CB_1 receptors play a pivotal role in extinction of conditioned fear (Marsicano et al., 2002), intense efforts have been made to further understand how the eCB system modulates aversive memories extinction and its possible consequences for anxiety pharmacotherapy. Given the similarities between extinction procedures and exposure-based psychotherapy used for the treatment of fear disorders in humans (Myers and Davis, 2007), it is believed that the eCB system represents a novel pharmacological target for anxiety disorders related to inappropriate retention of aversive memories (Chhatwal et al., 2005; Marsicano et al., 2002). So far, there is one report that the inhibition of eCB uptake/metabolism facilitates extinction of tone-cued fear-potentiated startle (Chhatwal et al., 2005), but it remains to be determined if this is true for other behavioral tasks.

In contextual fear conditioning, aversive memories are studied by exposing the animal to a context (e.g., conditioning chamber) where an aversive stimulus (normally a mild foot shock) is delivered (Rudy et al., 2004). Re-exposure to the same context induces conditioned fear responses, such as freezing behavior, defined by the absence of movements except for those necessary for breathing (Blanchard and Blanchard, 1969). Extinction of contextual fear memory is elicited with repeated or prolonged non-reinforced exposures to the context, which tends to decrease the conditioned fear responses (Pavlov, 1927); for a recent view, see (Myers and Davis, 2007). In a broader sense, memory extinction may reflect behavioral flexibility and adaptation to environmental changes (Hill et al., 2006; Kamprath et al., 2006). Bearing in mind that eCBs are released in specific brain areas during fear memory extinction (Marsicano et al., 2002), the aim of the present study was to investigate the effects of i.c.v. injection of the eCB metabolism/uptake inhibitor, AM404, and the phytocannabinoid, CBD, on the extinction of contextual conditioned fear in rats. Experiments of pharmacological antagonism were performed using SR141716A, a selective CB_1 antagonist, and capsazepine, an antagonist of $TRPV_1$ vanilloid receptors. The elevated plus-maze (EPM) test was used to investigate whether selected doses of CBD and AM404 induce anxiolytic-like effect in naive and/or conditioned rats.

2. Experimental procedures

2.1. Animals

Male adult Wistar rats (3 months old) bred and reared at the animal facility of our department were used. The animals were kept in collective plastic cages (4–5 rats/cage) with food and water available ad libitum. The animals were maintained in a room at a controlled temperature (23 ± 2 °C) under a 12:12-h light/dark cycle (lights on at 7:00 A.M.). Each behavioral test was conducted during the light phase of the cycle (9:00 A.M.–5:00 P.M.) using independent experimental groups consisting of 7–16 animals per group. All experimental procedures were performed according to the Principles of Laboratory Animal Care of the NIH.

2.2. Drugs

AM404, an inhibitor of anandamide uptake (Tocris, USA), (−) CBD, a major constituent of Cannabis sativa (Tocris), capsazepine (CPZ), a $TRPV_1$ vanilloid receptor antagonist (Tocris), SR141716A (SR), a CB_1 cannabinoid receptor antagonist (Sanofi-Aventis, France), and diazepam (DZP), a positive allosteric modulator of $GABA_A$ receptor (Sanofi Winthrop, Brazil), were used. For i.c.v. injections, all drugs were stored in DMSO stock solutions (50 mM) and freshly diluted in 0.1 M PBS, pH = 7.4, yielding a final concentration of 10% DMSO. For i.p. injections, a similar procedure was used, but 0.1% Tween 80 was added to the final solution. The respective vehicle was used as control for i.c.v. and i.p. injections. AM404, CBD and DZP were injected i.c.v. 5 min before the behavioral tests. CPZ was injected i.c.v. 5 min

before AM404, CBD or control. SR was injected intraperitoneally (i.p.) 20 min before AM404, CBD or control. Drug doses were selected based on previous reports and pilot studies (Chhatwal et al., 2005; Murillo-Rodriguez et al., 2006; Pamplona et al., 2006).

2.3. Stereotaxic surgery

The rats were deeply anesthetized with a 1:1 mixture of ketamine (75 mg/kg) and xylazine (15 mg/kg) and placed in a stereotaxic apparatus (Kopf, model 957), with bregma and lambda being kept on the same horizontal plane. A hole was drilled into the skull and a stainless steel guide cannula (23 gauge, 10 mm long) was lowered aiming at the right lateral ventricle. The following stereotaxic coordinates were used: LL = −1.6 mm; DV = −3.6, AP = −0.8 mm from bregma according to the rat brain atlas (Paxinos and Watson, 2002). Two screws were implanted into the skull and fixed with dental acrylic. A 30-gauge stainless steel stylet was placed into the guide cannula to prevent entry of foreign materials. The experimental procedure started 5–7 days after surgery.

2.4. Infusion procedure

An injector (30 gauge, 11 mm long) was fitted into the guide cannula and i.c.v. infusions were made using 10 μl microsyringes (Hamilton, USA) attached to the injector with a polyethylene tube (PE10). Drugs were injected with an automatic infusion pump (Insight, Brazil), at a rate of 2 μl/min and total injection volume of 1 μl. The injector was left in place for additional 30 s after drug injection.

2.5. Behavioral procedures

2.5.1. Contextual fear conditioning

The conditioning chamber consisted of a modified shuttle box (Automatic Reflex Conditioner model 7531, Ugo Basile, Italy) made of gray opaque Plexiglas. One of the compartments (22 × 22 × 25 cm) of the chamber was used for contextual fear conditioning. The experiments were carried out in a sound-attenuated room under low-intensity light (10 lx). Conditioning procedures have been previously described in Pamplona et al. (2006). For contextual fear conditioning, rats were placed in the conditioning chamber for 3 min, received a 1-s electric foot shock (1.5 mA) and were kept for an additional minute in the chamber before being returned to their home cages. Freezing, defined as complete immobility of the animal in a stereotyped crouching position, except for movements necessary for breathing, was used as a memory index during the subsequent non-reinforced re-exposures to the context (Blanchard and Blanchard, 1969; Fanselow, 1980). Freezing time was recorded with stopwatches by an experienced observer who was unaware of the treatment conditions. The same observer recorded freezing in all experiments to avoid individual variability and to obtain more reliable results.

2.5.1.1. Experiment 1. Effects of i.c.v. administration of AM404 and CBD on the extinction of contextual fear memory. Successive long exposures to the conditioning chamber were used to test the effects of AM404 or CBD on the extinction of contextual fear memory. Twenty-four hours after contextual fear conditioning, the animals were exposed to the conditioning chamber and freezing behavior was evaluated for 9 min. This procedure was performed three times at 24-h intervals to induce extinction of contextual fear memory. The animals were treated with AM404 (0.2, 1.0 or 2.0 μg/μl, i.c.v.), CBD (0.2, 1.0 or 2.0 μg/μl, i.c.v.) or control solution 5 min before each extinction session. A drug-free test of contextual fear memory (3 min) was performed 24 h after the last extinction session to investigate whether drug effects on fear memory extinction were acute (i.e., drug-dependent) or induced persistent effects.

2.5.1.2. Experiment 2. Role of CB_1 cannabinoid receptors in the facilitation of conditioned fear extinction by AM404 and CBD. This experiment was performed to investigate whether the effects of AM404 and CBD on the extinction of contextual fear memory are related to the activation of CB_1 cannabinoid receptors. The procedure was the same as in Experiment 1, except that the CB_1 receptor antagonist SR141716A (0.2 mg/kg, i.p.) or control solution was administered 20 min before i.c.v. injection of AM404 (1.0 μg/μl, i.c.v.) or CBD (2.0 μg/μl, i.c.v.).

2.5.1.3. Experiment 3. Role of $TRPV_1$ vanilloid receptors in the facilitation of conditioned fear extinction by AM404 and CBD. This experiment was performed to investigate whether the effects of AM404 and CBD on the extinction of contextual fear memory are related to the activation of $TRPV_1$ vanilloid receptors. The procedure was the same as in Experiment 1, except that the $TRPV_1$ receptor antagonist CPZ (5.0 μg/μl, i.c.v.) or control solution was administered 5 min before i.c.v. injection of AM404 (1.0 μg/μl, i.c.v.) or CBD (2.0 μg/μl, i.c.v.). The selected dose of CPZ was based on a pilot study carried in our laboratory, where the same dose and route of administration (5.0 μg/μl, i.c.v.) of CPZ was able to fully antagonize the analgesic effects of TRPV1 agonist capsaicin (2.0 μg/μl, i.c.v.) in the hot-plate test.

2.5.1.4. Experiment 4. Effects of i.c.v. administration of DZP on the extinction of contextual fear memory. This experiment aimed to investigate the effects of one standard anxiolytic drug on the extinction of contextual fear memory. Therefore, we tested the effects of DZP, a classical benzodiazepine anxiolytic, in a procedure similar to that of Experiment 1, except that the animals were injected with DZP (2.85 μg/μl, i.c.v.) or control solution 5 min before each extinction session.

2.5.2. Elevated plus-maze

The EPM test was used on the basis of its ability to detect both anxiolytic- and anxiogenic-like drug effects in rats (Pellow et al., 1985). The apparatus was made of wood covered with impermeable black Formica, consisted of four arms (50 cm long, 10 cm wide), and was placed 52 cm above the floor. Two opposite arms were surrounded by walls (10 cm high, closed arms) and the other two were devoid of enclosing walls (open arms). The experiments were conducted in a sound-attenuated room under low-intensity light (10 lx). Each animal was placed in the central area of the maze facing an open arm and its behavior was observed and manually recorded for 5 min by an experienced observer who

was unaware of the treatment condition. Arm entries were recorded when the rat placed all four paws into an arm. The % open arm entries (number of open arm entries/total arm entries) and the % open arm time (time spent in open arms/total arm time) were used as indices of anxiety-like behavior in this task and the number of closed arm entries was used as an index of locomotor activity (Cruz et al., 1994).

2.5.2.1. Experiment 5. Effects of AM404,CBD and DZP in the fear-potentiated EPM test. The fear-potentiated EPM test was conducted as a modification of the procedure previously reported (Mechiel Korte and De Boer, 2003). Rats were submitted to contextual fear conditioning as in Experiment 1 and re-exposed to the conditioning chamber 24 h later. Five days after context re-exposure, the animals were tested in the EPM. The 5-day interval between context re-exposure and EPM test has been defined in a pilot study, in which delay periods shorter than 24 h induced decreased EPM exploration, which would bias the interpretation of results. An unconditioned group consisting of test naive rats was used as control. Animals of the two groups were injected with AM404 (1.0 μg/μl, i.c.v.), CBD (2.0 μg/μl, i.c.v.), DZP (2.85 μg/μl, i.c.v.) or control solution 5 min before the fear-potentiated EPM test.

2.6. Verification of the injection site

After the experiments, all animals were deeply anesthetized with chloral hydrate (400 mg/kg, i.p.) and perfused transcardially with 4% formaldehyde solution in 0.1 M PBS, pH = 7.4. Shortly after perfusion, 1 μl of Evans Blue dye (0.1%) was injected through the guide cannula. The brain was removed and cut along the coronal plane. The injection site was confirmed by the presence of dye in the ventricular system. Only animals showing accurate cannula placement were included in the statistical analysis.

2.7. Statistical analysis

All values are expressed as mean ± S.E.M. The results of the extinction experiments were analyzed statistically by two- or three-way ANOVA with pretreatment, treatment and extinction sessions as independent variables. The results of the drug-free test were analyzed by one- or two-way ANOVA using pretreatment and treatment as independent variables. The results of the elevated EPM test were analyzed by two-way ANOVA with treatment and condition (conditioned vs unconditioned) as independent variables. Following significant ANOVAs, post hoc comparisons were performed using LSD test. The accepted level of significance was $p \leq 0.05$. All tests were performed using the Statistica® 6.0 software package (StatSoft, USA).

3. Results

3.1. Experiment 1

Effects of i.c.v. administration of AM404 or CBD on the extinction of contextual fear memory. The timeline of the behavioral procedures of Experiment 1 is shown in Fig. 1A. The effects of AM404 (0.2, 1.0 or 2.0 μg/μl, i.c.v.) on the extinction of contextual fear memory are shown in Fig. 1B and D. Two-way ANOVA revealed significant effects of treatment [$F(3,141) = 3.94$, $p < 0.01$] and sessions [$F(2,141) = 12.78$, $p < 0.01$], but there was no effect of treatment × session interaction [$F(6,141) = 0.25$, $p = 0.95$]. Post hoc comparisons indicated that the 3-day extinction protocol decreased % freezing time across successive exposures of the control group to the conditioning chamber ($p < 0.05$, 3rd session compared to the 1st). The group treated with the intermediate dose of AM404 (1.0 μg/μl, i.c.v.) underwent partial extinction already in the 2nd session ($p < 0.05$ compared to the 1st) and exhibited decreased % freezing time during the 2nd and 3rd trials compared to the control group ($p < 0.05$), suggesting a facilitative effect of AM404 on the extinction of contextual fear memory. One-way ANOVA applied to the results of the drug-free test revealed no effect of treatment [$F(3,47) = 0.83$, $p = 0.48$]. However, the group treated with AM404 (1.0 μg/μl, i.c.v.) presented a trend towards reduced % freezing time compared to control in the drug-free test ($t = 1.76$, $p = 0.08$).

The effects of CBD (0.2, 1.0 or 2.0 μg/μl, i.c.v.) on the extinction of contextual fear memory are shown in Fig. 1C and E. Two-way ANOVA revealed significant effects of treatment [$F(3,126) = 3.74$, $p \leq 0.01$] and sessions [$F(2,126) = 19.18$, $p < 0.01$], but not of treatment × session interaction [$F(6,126) = 0.59$, $p = 0.73$]. Post hoc comparisons indicated that the group treated with the highest dose of CBD tested (2.0 μg/μl, i.c.v.) underwent partial extinction already in the 2nd session ($p < 0.05$, compared to the 1st) and exhibited decreased % freezing time during the 2nd and 3rd sessions compared to the control group ($p < 0.05$), suggesting a facilitative effect of CBD on the extinction of contextual fear memory. One-way ANOVA applied to the results of the drug-free test revealed a trend towards an effect of treatment [$F(3,42) = 2.33$, $p = 0.08$]. The group treated with CBD (2.0 μg/μl, i.c.v.) presented reduced % freezing time compared to control in the drug-free test ($t = 2.66$, $p < 0.05$).

3.2. Experiment 2

Role of CB_1 cannabinoid receptors in the facilitation of conditioned fear extinction by AM404 and CBD. The timeline of the behavioral procedures of Experiment 2 is shown in Fig. 2A. The effects of pre-administration of SR (0.2 mg/kg, i.p.) 20 min before AM404 (1.0 μg/μl, i.c.v.) on the extinction of contextual fear memory are shown in Fig. 2B and D. Three-way ANOVA revealed significant effects of treatment [$F(1,117) = 5.61$, $p < 0.05$], sessions [$F(2,117) = 17.27$, $p < 0.001$] and pretreatment × treatment interaction [$F(1,117) = 4.84$, $p < 0.05$]. Treatment with AM404 (1.0 μg/μl, i.c.v.) facilitated the extinction of contextual fear memory, reproducing the results of Experiment 1. Moreover, a per se ineffective dose of SR (0.2 mg/kg, i.p.) antagonized the facilitative effect of AM404 (1.0 μg/μl, i.c.v.) on the extinction of fear memory ($p < 0.05$, 2nd and 3rd sessions compared to the AM404-treated group), suggesting that it was related to the activation of CB_1 cannabinoid receptors. Two-way ANOVA applied to the results of the drug-free test revealed effects of treatment [$F(1,39) = 4.52$, $p < 0.05$] and pretreatment × treatment interaction [$F(1,39) = 5.03$, $p < 0.05$]. The group treated with AM404 (1 μg/μl, i.c.v.) presented reduced % freezing time compared to control ($p < 0.05$), confirming the trend observed in Experiment 1. Moreover, SR partially antagonized

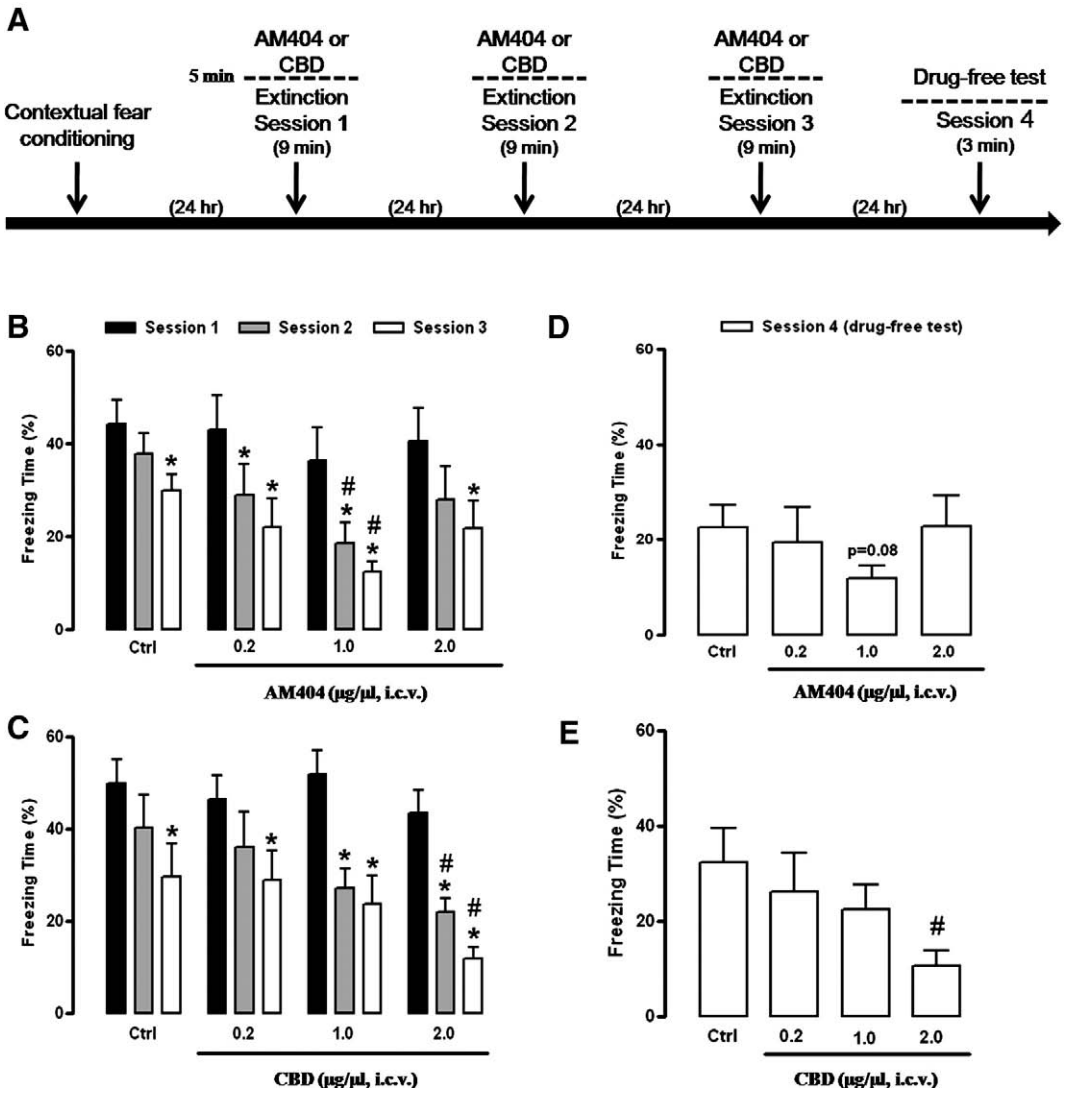

Figure 1 Effects of i.c.v. administration of AM404 (0.2, 1.0, 2.0 µg/µl) or cannabidiol (CBD; 0.2, 1.0, 2.0 µg/µl) on the extinction of contextual fear memory in rats. (A) Timeline of the behavioral procedures of Experiment 1. (B,C) Mean ± S.E.M. percent freezing time expressed by rats treated with AM404 or CBD and subjected to three 9-min exposures to the conditioning chamber at 24-h intervals (each bar represents the data of one session). (D, E) The same groups of rats during a single 3-min exposure to the conditioning chamber in a drug-free state 24 h after the last extinction session. *$p<0.05$ compared to the first session of the respective group. #$p<0.05$ compared to the respective session of the control (Ctrl) group (LSD post hoc test). (Ctrl $n=16$, AM404 0.2 $n=11$, AM404 1.0 $n=12$, and AM404 2.0 $n=12$) (Ctrl $n=13$, CBD 0.2 $n=10$, CBD 1.0 $n=11$, CBD 2.0 $n=12$).

the effect of AM404 administration ($p<0.05$) at a per se ineffective dose.

The effects of pre-administration of SR (0.2 mg/kg, i.p.) 20 min before CBD (2.0 µg/µl, i.c.v.) on the extinction of contextual fear memory are shown in Fig. 2C and E. Three-way ANOVA revealed significant effects of treatment [$F(1,105)=7.66$, $p<0.01$], sessions [$F(2,105)=16.32$, $p<0.001$] and pretreatment × treatment interaction [$F(1,105)=3.87$, $p<0.05$]. Treatment with CBD (2.0 µg/µl, i.c.v.) facilitated the extinction of contextual fear memory, reproducing the results of Experiment 1. Moreover, a per se ineffective dose of SR (0.2 mg/kg, i.p.) antagonized the facilitative effect of CBD (2.0 µg/µl, i.c.v.) on the extinction of fear memory ($p<0.05$, 2nd and 3rd sessions compared to the CBD-treated group), suggesting that it was related to the activation of CB_1 cannabinoid receptors. Two-way ANOVA applied to the results of the drug-free test revealed an effect of pretreatment × treatment interaction [$F(1,35)=4.72$, $p<0.05$]. The group treated with CBD (2 µg/µl, i.c.v.) presented reduced % freezing time compared to control ($p<0.05$), confirming the results of Experiment 1. This effect was fully antagonized by SR ($p<0.05$).

3.3. Experiment 3

Role of $TRPV_1$ vanilloid receptors in the facilitation of conditioned fear extinction by AM404 and CBD. The timeline of the behavioral procedures of Experiment 3 is shown in Fig. 3A. The effects of pre-administration of CPZ (5.0 µg/µl, i.c.v.) 5 min

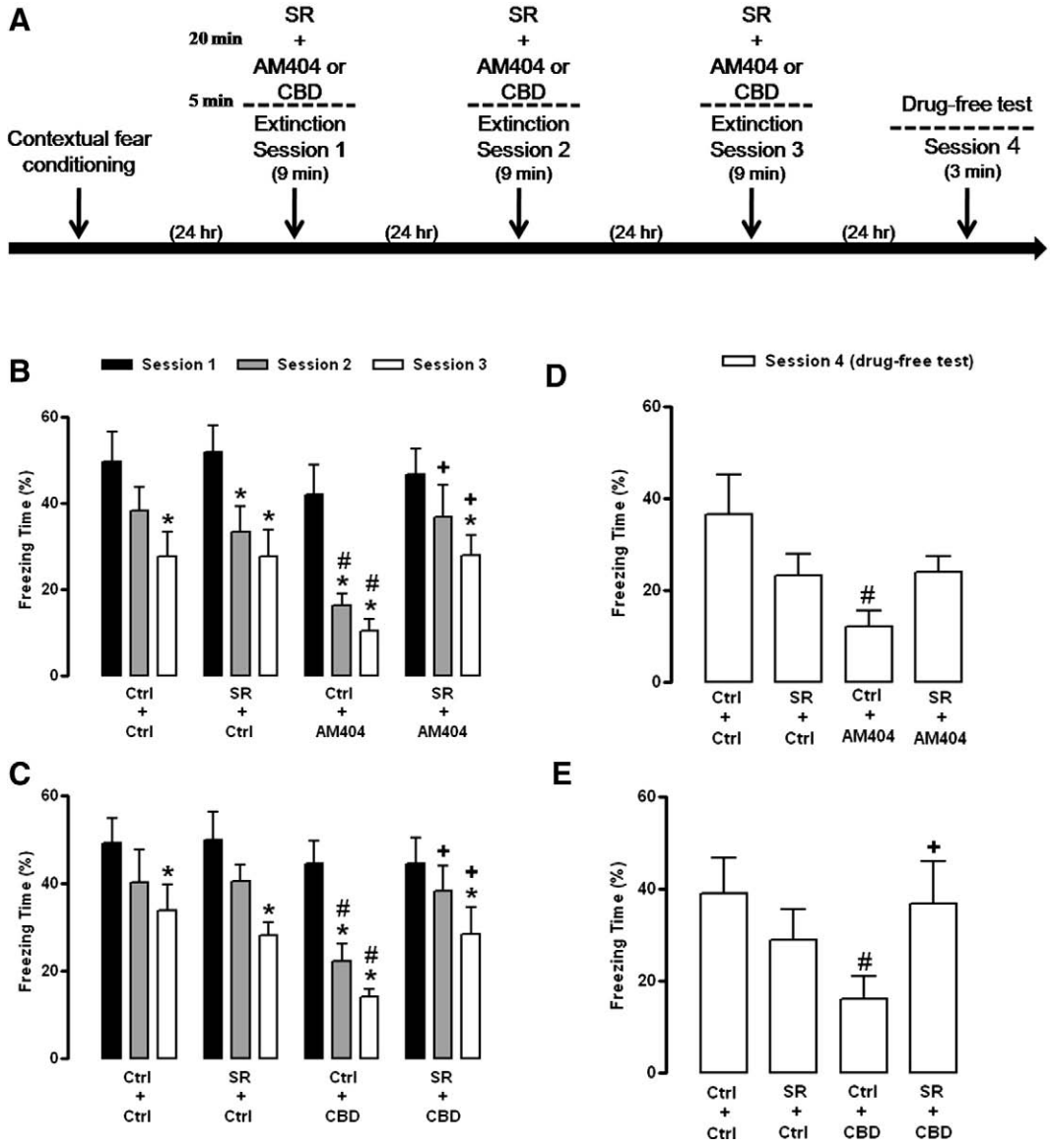

Figure 2 Effects of the CB_1-selective antagonist SR141716A (SR; 0.2 mg/kg, i.p.) on the facilitation of contextual fear memory extinction induced by AM404 (1.0 μg/μl, i.c.v.) or cannabidiol (CBD; 2.0 μg/μl, i.c.v.). (A) Timeline of the behavioral procedures of Experiment 2. (B,C) Mean ± S.E.M. percent freezing time expressed by rats pretreated with SR, treated with AM404 or CBD and subjected to three 9-min exposures to the conditioning chamber at 24-h intervals (each bar represents the data of one session). (D, E) The same groups of rats during a single 3-min exposure to the conditioning chamber in a drug-free state 24 h after the last extinction session. *$p<0.05$ compared to the first session of the respective group. #$p<0.05$ compared to the respective session of the control (Ctrl+Ctrl) group. +$p<0.05$ compared to the respective session of the Ctrl+AM404 or Ctrl+CBD group (LSD post hoc test). (Ctrl+Ctrl $n=11$, SR+Ctrl $n=12$, Ctrl+AM404 $n=9$ and SR+AM404 $n=11$) (Ctrl+Ctrl $n=9$, SR+Ctrl $n=10$, Ctrl+CBD $n=11$, SR+CBD $n=9$).

before AM404 (1.0 μg/μl, i.c.v.) on the extinction of contextual fear memory are shown in Fig. 3B and D. Three-way ANOVA revealed significant effects of treatment [$F(1,90)=16.97$, $p<0.001$], sessions [$F(2,90)=39.22$, $p<0.001$] and treatment × session interaction [$F(2,90)=7.02$, $p<0.01$]. Treatment with AM404 (1.0 μg/μl, i.c.v.) facilitated the extinction of contextual fear memory. A per se ineffective dose of CPZ (5.0 μg/μl, i.c.v.) did not antagonize the facilitative effect of AM404 (1.0 μg/μl, i.c.v.) on fear memory extinction, suggesting that it was not related to the activation of $TRPV_1$ vanilloid receptors. Two-way ANOVA applied to the results of the drug-free test revealed an effect of treatment [$F(1,30)=14.23$, $p<0.001$]. The group treated with AM404 (1 μg/μl, i.c.v.) presented reduced % freezing time compared to control ($p<0.05$), and CPZ did not antagonize the effect of AM404.

The effects of pre-administration of CPZ (5.0 μg/μl, i.c.v.) 5 min before CBD (2.0 μg/μl, i.c.v.) on the extinction of contextual fear memory are shown in Fig. 3C and E. Three-way ANOVA revealed significant effects of treatment [$F(1,99)=23.00$, $p<0.001$], sessions [$F(2,99)=30.01$, $p<0.001$] and treatment × session interaction [$F(2,99)=3.56$, $p<0.05$]. Treatment with CBD (2.0 μg/μl, i.c.v.) facilitated the extinction of contextual

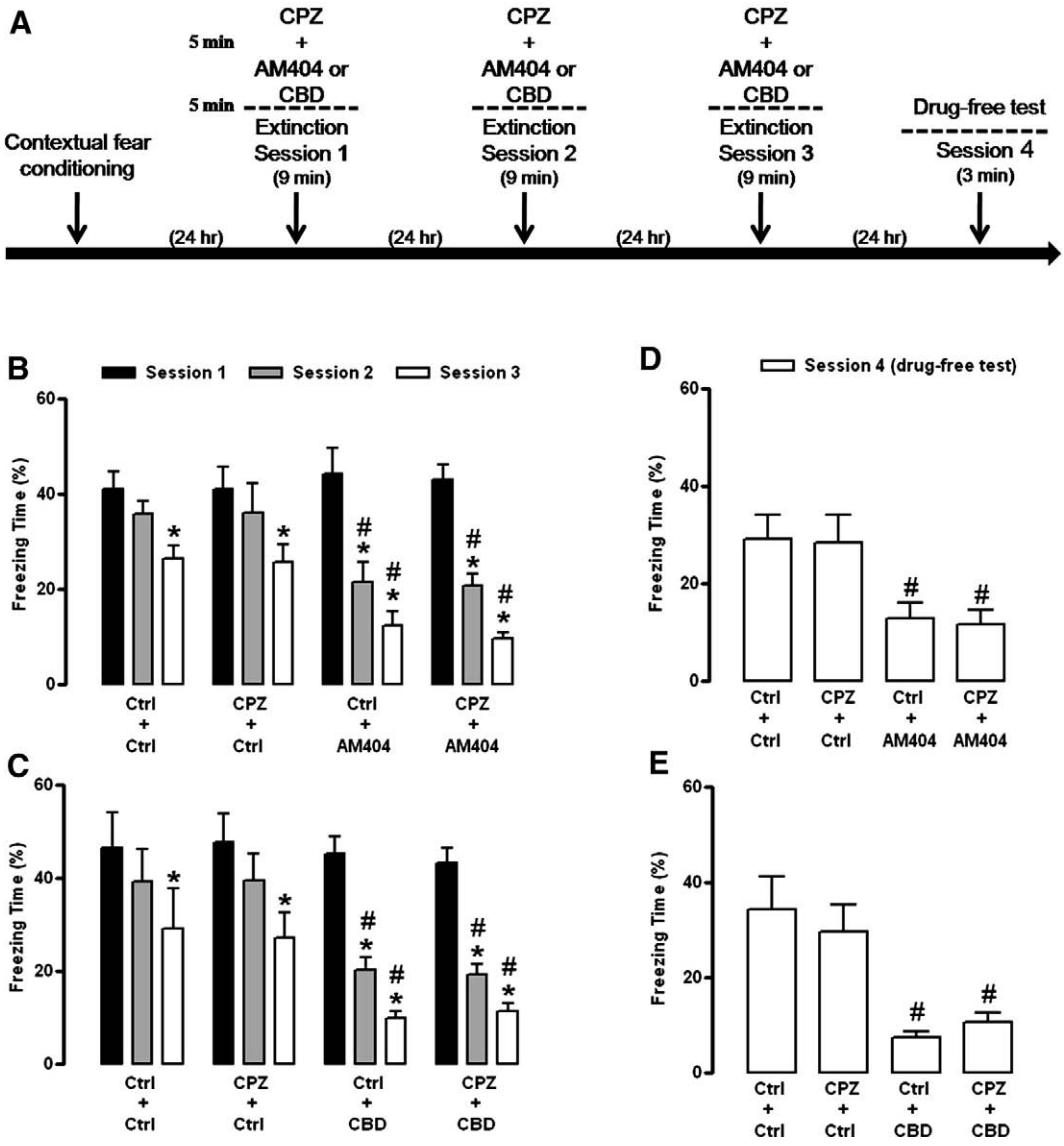

Figure 3 Effects of the $TRPV_1$-selective antagonist capsazepine (CPZ; 5.0 μg/μl, i.c.v.) on the facilitation of contextual fear memory extinction induced by AM404 (1.0 μg/μl, i.c.v.) or cannabidiol (CBD; 2.0 μg/μl, i.c.v.). (A) Timeline of the behavioral procedures of Experiment 3. (B,C) Mean ± S.E.M. percent freezing time expressed by rats pretreated with CPZ, treated with AM404 or CBD and subjected to three 9-min exposures to the conditioning chamber at 24-h intervals (each bar represents the data of one session). (D,E) The same groups of rats during a single 3-min exposure to the conditioning chamber in a drug-free state 24 h after the last extinction session. * $p<0.05$ compared to the first session of the respective group. #$p<0.05$ compared to the respective session of the control (Ctrl + Ctrl) group (LSD post hoc test). (Ctrl + Ctrl $n=8$, CPZ + Ctrl $n=9$, Ctrl + AM404 $n=8$ and CPZ + AM404 $n=9$) (Ctrl + Ctrl $n=8$, CPZ + Ctrl $n=9$, Ctrl + CBD $n=10$, CPZ + CBD $n=10$).

fear memory, and this effect was not antagonized by CPZ (5.0 μg/μl, i.c.v.), suggesting that it was not related to the activation of $TRPV_1$ vanilloid receptors. Two-way ANOVA applied to the results of the drug-free test revealed an effect of treatment [$F(1,33)=27.80$, $p<0.001$]. The group treated with CBD (2 μg/μl, i.c.v.) presented reduced % freezing time compared to control ($p<0.05$), and CPZ did not antagonize the effect of CBD.

3.4. Experiment 4

Effects of i.c.v. administration of DZP on the extinction of contextual fear memory.

The timeline of the behavioral procedures of Experiment 4 is shown in Fig. 4A. The effects of DZP (2.85 μg/μl, i.c.v.) on the extinction of contextual fear memory are shown in Fig. 4B and C. Two-way ANOVA revealed significant effects of treatment [$F(1,36)=18.61$, $p<0.001$] and sessions [$F(2,36)=3.80$, $p<0.05$], but not of treatment × session interaction [$F(2,36)=1.71$, $p<0.19$]. Post hoc comparisons indicated that the DZP-treated group presented reduced % freezing time during the 1st and 2nd sessions compared to the control group ($p<0.05$). There was no apparent fear memory extinction in DZP group, but a general reduction in the expression of conditioned freezing behavior. One-way ANOVA analysis of the results of

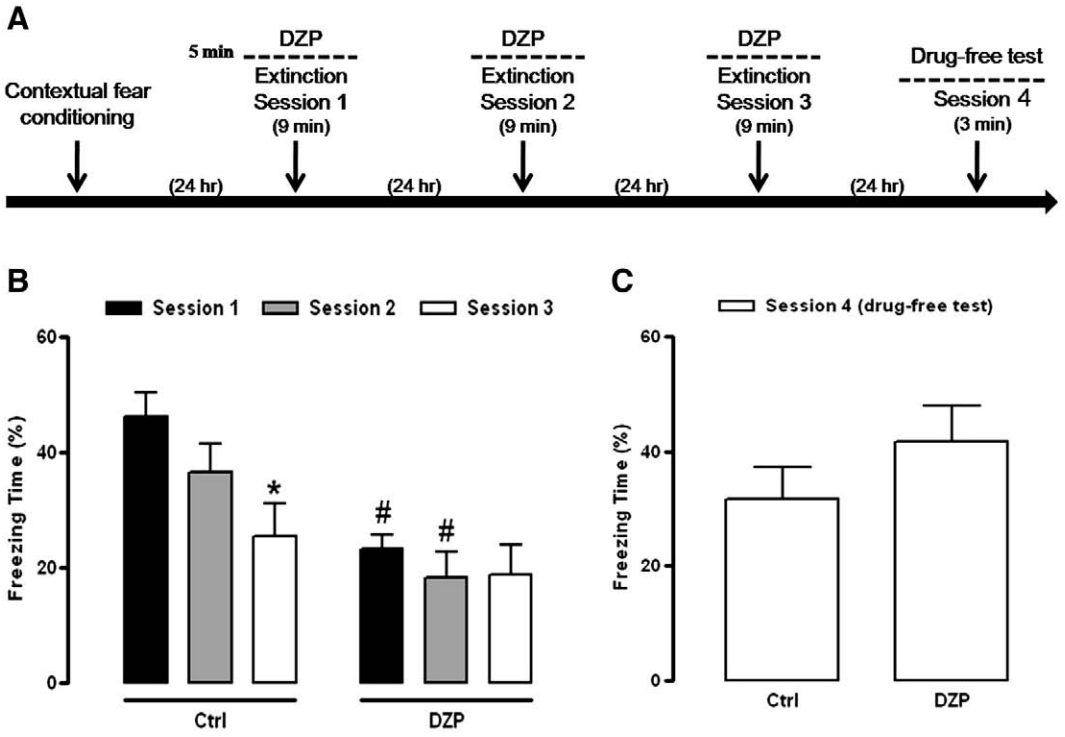

Figure 4 Effects of i.c.v. administration of diazepam (DZP; 2.85 μg/μl) on the extinction of contextual fear memory in rats. (A) Timeline of the behavioral procedures of Experiment 4. (B) Mean ± S.E.M. percent freezing time expressed by rats treated with DZP and subjected to three 9-min exposures to the conditioning chamber at 24-h intervals (each bar represents the data of one session). (C) The same groups of rats during a single 3-min exposure to the conditioning chamber in a drug-free state 24 h after the last extinction session. *$p<0.05$ compared to the first session of the respective group. # $p<0.05$ compared to the respective session of the control (Ctrl) group (LSD post hoc test). (Ctrl $n=7$, DZP $n=7$).

the drug-free test revealed no difference between groups [$F(1,12)=1.46$, $p=0.25$].

3.5. Experiment 5

Effects of AM404, CBD and DZP in the fear-potentiated EPM test. The effects of the selected doses of AM404 (1.0 μg/μl, i.c.v.), CBD (2.0 μg/μl, i.c.v.) and DZP (2.85 μg/μl, i.c.v.) in the fear-potentiated EPM test are shown in Table 1. Two-way ANOVA for the % open arm time revealed a significant effect of condition [$F(1,42)=5.74$, $p<0.05$] and treatment × condition interaction [$F(2,42)=3.50$, $p<0.05$]. The conditioned group exhibited reduced % open arm time compared to the unconditioned control group ($p<0.05$), suggesting an anxiogenic-like effect of the fear conditioning procedure. The groups treated with AM404 (1.0 μg/μl, i.c.v.) and CBD (2.0 μg/μl, i.c.v.) showed increased % open arm time compared to conditioned control animals ($p<0.05$). Treatment with AM404 or CBD did not affect % open arm time in the unconditioned groups. Two-way ANOVA for the effects of the positive control diazepam (2.85 μg/μl, i.c.v.) revealed an overall effect of treatment [$F(1,31)=11.38$, $p<0.01$] on % open arm time. Further comparison indicated that diazepam (2.85 μg/μl, i.c.v.) increased the % open arm time in both conditioned and unconditioned groups ($p<0.05$).

Two-way ANOVA for the percentage of open arm entries revealed significant effects of treatment [$F(2,42)=5.20$,

Table 1 Effects of AM404 (1.0 μg/μl, i.c.v.) and cannabidiol (2.0 μg/μl, i.c.v.) in the fear-potentiated plus-maze test

	Treatment	% Open arm time	% Open arm entries	Closed arm entries	*N*
Unconditioned	Control	34.03 ± 5.81	17.41 ± 4.08	6.50 ± 1.07	8
	AM404	25.48 ± 6.57	14.08 ± 4.04	6.56 ± 0.91	9
	CBD	43.47 ± 5.15	20.33 ± 4.12	5.12 ± 0.64	8
	DZP	52.34 ± 2.87*	30.14 ± 4.64*	7.00 ± 0.69	9
Conditioned	Control	4.90 ± 3.22#	2.14 ± 1.74#	6.57 ± 1.21	7
	AM404	32.43 ± 6.41*	10.78 ± 3.45*	5.25 ± 0.96	8
	CBD	32.60 ± 5.23*	11.17 ± 3.13*	6.12 ± 1.23	8
	DZP	48.82 ± 6.41*	30.42 ± 8.05*	5.36 ± 0.72	11

CBD, cannabidiol; DZP, diazepam.
*$p<0.05$ compared to the control group of the respective condition. #$p<0.05$ compared to the unconditioned control group.

$p<0.01$], condition [$F(1,42)=5.65$, $p<0.05$] and treatment × condition interaction [$F(2,42)=5.04$, $p<0.05$]. The conditioned group exhibited reduced % open arm entries compared to the unconditioned control group ($p<0.05$). Treatment with AM404 (1.0 μg/μl, i.c.v.) or CBD (2.0 μg/μl, i.c.v.) increased the percentage of open arm entries in conditioned animals ($p<0.05$), without affecting the percentage of open arm entries in unconditioned animals. Two-way ANOVA for the effects of the positive control diazepam (2.85 μg/μl, i.c.v.) revealed significant effects of treatment [$F(1,31)=34.35$, $p<0.001$], condition [$F(1,31)=9.46$, $p<0.01$] and treatment × condition interaction [$F(1,31)=5.81$, $p<0.05$] on the percentage of open arm entries. Further comparison indicated that diazepam (2.85 μg/μl, i.c.v.) increased the percentage of open arm entries in both the conditioned and unconditioned groups ($p<0.05$). There were no significant effects of treatment or condition on the number of closed arm entries.

4. Discussion

The present study demonstrates that the eCB uptake/metabolism inhibitor, AM404, and the relatively unheralded phytocannabinoid, CBD, facilitate the extinction of contextual fear memory in rats. These responses were antagonized by the CB_1-selective antagonist SR141716A, but not by the $TRPV_1$-selective antagonist CPZ, thus suggesting the involvement of CB_1 cannabinoid receptors in the facilitation of extinction by these drugs. Moreover, animals treated with either AM404 or CBD during the extinction sessions presented reduced fear response to context exposure in a drug-free test performed 24 h after the last extinction session, suggesting persistent effects. Notably, the anti-anxiogenic effect of CBD and AM404 in conditioned rats might have contributed to the facilitation of fear extinction.

The facilitation of fear memory extinction by AM404 and CBD suggests a role for the eCB system in the regulation of emotional states elicited by fear memory retrieval. These findings extend those of a recent study showing that the potentiation of eCB transmission by AM404 facilitates extinction of fear-potentiated startle in rats via CB_1 cannabinoid receptors (Chhatwal et al., 2005). Additionally, our results support the role of CB_1 cannabinoid receptors, but not of $TRPV_1$ vanilloid receptors, in the extinction of conditioned fear in rats, which is in line with previous reports on the role of the eCB system in the extinction of conditioned fear (Kamprath et al., 2006; Marsicano et al., 2002; Pamplona et al., 2006; Suzuki et al., 2004), inhibitory avoidance (Niyuhire et al., 2007), water maze spatial reference task (Pamplona et al., 2006; Varvel et al., 2005; Varvel and Lichtman, 2002), but not in the extinction of operant conditioning tasks (Holter et al., 2005; Niyuhire et al., 2007).

Interestingly, the facilitation of short-term extinction observed after administration of a low dose of the cannabinoid agonist WIN55212-2 in the same behavioral protocol (Pamplona et al., 2006) was not found after AM404 or CBD administration, suggesting that these two drugs might reach CB_1 receptors in an indirect way. Although it is already known that AM404 enhances eCB levels in vivo (Bortolato et al., 2006), whether or not CBD also share this property remains to be demonstrated. Another possible pharmacological explanation for the present effects of AM404 and CBD could be an interaction with $TRPV_1$ vanilloid receptors (Bisogno et al., 2001), especially because even an increase in eCB ligands such as anandamide can activate these receptors (Rawls et al., 2006; Zygmunt et al., 1999). However, this explanation seems unlikely because the $TRPV_1$ receptor antagonist, CPZ, was unable to antagonize the effects of AM404 and CBD on the extinction of conditioned fear, in a dose high enough to antagonize the analgesic effects of the $TRPV_1$ agonist, capsaicin (unpublished results). In this context, it is noteworthy that Rubino et al. (2008) found that the same dose (5 μg) of CPZ given directly into the prefrontal cortex blocked the $TRPV_1$ receptors but was ineffective at altering anxiety behavior in rats. Nevertheless, the role of $TRPV_1$ receptors in conditioned fear is just beginning to be elucidated, and some important implications of the vanilloid system in mnemonic functions might be revealed (Marsch et al., 2007). Importantly, administration of DZP led to a consistent reduction in the % of freezing time during extinction training; but contrary to AM404 and CBD, this effect was not observed in the drug-free test performed after 1 day of drug washout. These results may suggest that the effects of diazepam actually reflect state-dependency (Bouton et al., 1990) or that generalized anxiolysis might not be an effective mechanism for long-term facilitation of extinction, but for acute decrease in the expression of defensive behaviors upon context exposure (Pain et al., 2002).

Therefore, a possible anxiolytic-like effect of selected doses of AM404 and CBD was investigated using the fear-potentiated EPM test. Interestingly, both drugs failed to affect behavior of naive rats, but reversed the anxiogenic-like state of rats previously submitted to the fear conditioning procedure. In contrast, diazepam exerted anxiolytic-like effects in both naive and conditioned rats. It is noteworthy that the eCB modulation of emotionality highly depends on the aversiveness of the experience, as CB_1-knock out mice behave like control animals when tested under low-light condition in the EPM, but display anxiogenic-like behavior when tested under high illumination, known to be aversive for rodents (Haller et al., 2004). Therefore, this specific anxiolytic effect of CBD and AM404 in conditioned animals may suggest that these compounds exert distinct effects depending on the emotional state of the individual, thus representing an original class of anxiolytic drugs (Patel et al., 2005). This hypothesis is supported by the observation of oscillations in eCB tonus during stressful situations (Hohmann et al., 2005; Patel et al., 2004). Additionally, there is a previous report showing anxiolytic effects of CBD in conditioned freezing using a stronger fear conditioning protocol (Resstel et al., 2006). In this study, a conditioning protocol consisting of 6 randomly-delivered 2.5 mA footshocks was employed and freezing behavior and cardiovascular responses were registered during a 10-min re-exposure to the conditioning chamber (Resstel et al., 2006). The authors were able to show anxiolytic effect of systemic DZP and CBD in both behavioral and physiological parameters, in partial disagreement with the present effects of CBD, as we did not observe any effects of CBD in the first context re-exposure. One likely explanation for the difference between these two findings might be that their protocol induces a more intense anxiogenic state in the animals and that the effects of CBD in that protocol may reflect the aforementioned interaction of the eCB system with stress. This explanation would make sense in face of our fear-potentiated EPM results showing anxiolytic effect of CBD in conditioned animals. Nevertheless, there are also previous reports of anxiolytic effects of systemically administered CBD (Guimaraes et al., 1990) and AM404 (Patel

and Hillard, 2006) in naive rats. It can still be possible that differences in EPM illumination (therefore in the test aversiveness) or other factors may explain this discrepancy. Finally, another important difference between the present study and that by Resstel et al. is the administration route (i.c.v. vs i.p.) employed for CBD and AM404 administration.

Taken together, our results complement other lines of evidence suggesting a role of the eCB system in the modulation of emotional states and emphasize that enhancement of eCB levels by uptake inhibitors might represent an interesting pharmacological approach to reduce the anxiogenic effects of stress and promote the extinction of fear memories. Moreover, since CBD is an abundant phytocannabinoid and has already demonstrated its efficacy and safety in humans (Crippa et al., 2004; Zuardi et al., 1995), we emphasize the importance of considering this drug in future studies aiming to evaluate the usefulness of cannabinoids as adjuvants in exposure-based psychotherapies for anxiety disorders related to inappropriate retention of aversive memories.

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Contributors

RMB performed all the experiments and statistical analysis. FAP wrote the protocol and collaborated with the experiments. RNT, RMB and FAP performed the analysis of the data, managed the literature searches and the final manuscript. All authors have approved the final manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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