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Cannabidiol disrupts the consolidation of specific and generalized fear memories via dorsal hippocampus CB_1 and CB_2 receptors

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ABSTRACT

Pharmacological interventions able to modulate a fear memory while it is consolidated could have therapeutic value in tempering those maladaptively overconsolidated. Animal and human studies have shown the intensity of unconditioned stimulus delivered during fear conditioning influences qualitative and quantitative aspects of the memory to be established. By varying the shock intensity used for contextual pairing in rats, here we induced specific and more generalized long-term fear memories to investigate whether, how and where in the brain the cannabidiol (CBD; 3.0–30 mg/kg i.p.) could impair their consolidation and related outcomes. When given immediately after their acquisition, it reduced respectively the conditioned fear expression, and fear generalization, ultrasonic vocalizations at 22-kHz and the relative resistance to extinction. CBD had no effects on short-term fear memory, and its delayed treatment no longer affected the consolidation process. As the dorsal hippocampus (DH) modulates fear memory specificity and generalization, and cannabinoid type-1 (CB_1) and type-2 (CB_2) receptors contribute to consolidation, we investigated their involvement in CBD effects. Both systemic and intra-DH treatment with the CB_1 receptor antagonist/inverse agonist AM251 or the CB_2 receptor antagonist/inverse agonist AM630 prevented the disrupting CBD effects on consolidation. Since the CBD effects on the endocannabinoid transmission are probably indirect, we investigated and demonstrated the FAAH inhibitor URB597 induced effects similar to those of CBD when given systemically or intra-DH. Altogether, the present results suggest the CBD disrupts the consolidation of different fear memories via anandamide-mediated activation of DH CB_1 and CB_2 receptors.

1. Introduction

Analyzing how to attenuate the behavioral outcomes related to a generalized aversive memory could have therapeutic value in mitigating abnormally overconsolidated memories, such as those underlying the posttraumatic stress disorder (PTSD), which occasionally are associated with fear generalization and/or extinction deficits (Milad et al., 2009; Anastasides et al., 2015; Morey et al., 2015; Yehuda et al., 2015, 2017; McGuire et al., 2016).

Accumulating evidence suggests the greater the number of shocks delivered, the more intense the fear memory generated (Baldi et al., 2004; Kroon and Carobrez, 2009; Gazarini et al., 2014). This positive correlation would be valid at least until the conditioned fear response achieves its asymptotic level (Baldi et al., 2004). Indeed, rats in which the context A was paired with three or five shocks presented a higher amount of freezing time than did those paired with a single shock when re-exposed to this context, even though this measure was comparable in the first two groups (Gazarini et al., 2014). Similar results have been reported using the olfactory fear conditioning (Kroon and Carobrez, 2009). The quality of a given memory should also be considered, for the reason that often the greater the number or the intensity of shocks delivered,

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the more generalized the fear memory induced (Baldi et al., 2004; Kaouane et al., 2012; Gazarini et al., 2014; Ghosh and Chattarji, 2015; Poulos et al., 2016; Dunsmoor et al., 2017). Of note, the fear memory established using higher shock intensities for contextual pairing is also frequently less prone to behavioral suppression by extinction (Gazarini et al., 2014; Finsterwald et al., 2015). Based on the above, (i) it would be appropriate to use criteria to support the claim of being inducing fear memories that differ in their qualitative and/or quantitative aspects, and (ii) it is possible to compare the effects of a given drug on the consolidation of different (e.g. specific *vs*. more generalized) fear memories by varying the features of the stressful event.

It has been reported that PTSD patients may present changes in cannabinoid transmission functioning, including lower circulating levels of anandamide (Hauer et al., 2013; Neumeister et al., 2013). Humans and mice with a genetic variation in the fatty acid amide hydrolase (FAAH), the primary catabolic enzyme for the anandamide, which reduces its activity and increases the brain anandamide levels, have displayed low anxiety and enhanced fear extinction (Dincheva et al., 2015). Based on these facts, increasing the brain availability of anandamide has been suggested as a new and potentially more effective therapeutic strategy for PTSD treatment (Hill and Gorzalka, 2009; Neumeister, 2013; Trezza and Campolongo, 2013). It is worth mentioning the cannabidiol (CBD) could be a candidate to achieve the goal of attenuating the fear memory formation or maintenance because it is able to augment the anandamide levels in the brain indirectly, probably through inhibition of the FAAH activity (Bisogno et al., 2001; De Petrocellis et al., 2011) and/or the fatty acid-binding proteins that mediate anandamide transport to FAAH (Elmes et al., 2015). Indeed, the CBD has already been shown to attenuate the aversive memory formation after being infused into the rat nucleus accumbens (Norris et al., 2016) or the prelimbic cortex (Rossignoli et al., 2017). Moreover, it has mitigated enduring contextual fear memories through extinction facilitation or reconsolidation disruption when given before or after their retrieval, respectively (Bitencourt et al., 2008; Stern et al., 2012; Do Monte et al., 2013; Gazarini et al., 2014; Stern et al., 2015; Song et al., 2016). However, is still unknown whether, how and where in the brain the CBD could interfere with the consolidation of specific and more generalized fear memories.

The first objective of the present study was to investigate this question in rats. The working hypothesis was the CBD would affect the consolidation of specific and more generalized fear memories when given immediately after their acquisition, reducing the conditioned fear expression in the first case, and preventing fear generalization and the relative resistance to extinction in the second case. A complementary analysis of CBD effects on consolidation of a more generalized fear memory was performed by investigating the number of 22-kHz ultrasonic vocalizations (USVs) emitted during re-exposure to the paired context and the exposure to a novel and unpaired context. Considering the dorsal hippocampus is associated with fear memory consolidation and generalization (Zelikowsky et al., 2014; Lynch et al., 2017), and cannabinoid type-1 (CB_1) and type-2 (CB_2) receptors contribute to consolidate either emotional or non-emotional memories (Clarke et al., 2008; De Oliveira Alvares et al., 2008; Wise et al., 2009; García-Gutiérrez et al., 2013; Li and Kim, 2016; Nasehi et al., 2017), the second objective was to investigate their involvement in the CBD-induced effects. The working hypothesis was the disrupting effects of CBD on consolidation would be associated with the activation of dorsal hippocampus CB_1 and CB_2 receptors. Finally, to support present results, we investigated and demonstrated the FAAH inhibitor URB597 induced effects similar to those of CBD on consolidation of a more generalized fear memory when given systemically or intra-dorsal hippocampus.

2. Material and methods

2.1. Subjects

A total of 277 male Wistar rats aged 13–15 weeks and weighing from 250 to 300 g were used in the present study. The animals were obtained from local breeding facilities, housed in groups of four in Plexiglas cages measuring 60 × 25 × 25 cm, kept in the animal facility under controlled temperature (23 ± 2 °C) and illumination (12 h cycle) conditions, and had free access to water and standard laboratory chow. All experimental procedures conducted here were approved by local Committee on the Care and Use of Laboratory Animals, in compliance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals (NIH publication No. 80-23, 1996) and the Brazilian legislation. In addition, all attempts were made to minimize the number of animals used and their suffering.

2.2. Drugs

CBD (THC-Pharma, Germany; 3.0–30 mg/kg), AM251 (Tocris, USA; 1.0 mg/kg or 0.5 nmol), AM630 (Tocris, USA; 0.3 mg/kg or 0.1 nmol) and URB597 (Tocris, USA; 0.5–1.0 mg/kg or 0.01 nmol) were dissolved in NaCl 0.9% containing 5.0% of polyoxyethylenesorbitan monooleate (Tween® 80, Sigma-Aldrich, USA). The selection of doses was based on previously published studies where CBD and URB597 interfered with expression or reconsolidation of contextual fear memories (Stern et al., 2012, 2015; Gazarini et al., 2014; Lisboa et al., 2015), and where AM251 and AM630 did not produce effect by itself, but prevented the behavioral effects of the CBD after systemic injection (Ignatowska-Jankowska et al., 2011; Stern et al., 2012). The interval of 30 min between drug pretreatment and treatment used to investigate the mechanism(s) of action of CBD was also based on previously published studies (Stern et al., 2012, 2015). All solutions were prepared immediately before use, and injected intraperitoneally in a volume of 1.0 ml/kg or into the dorsal hippocampus in a volume of 0.5 μl/side.

2.3. Stereotaxic surgery, drug infusion into the dorsal hippocampus, and histology

The animals were anesthetized with 1.0 ml/kg of a solution containing xylazine (10 mg/ml; Carlier, Brazil) and ketamine (100 mg/ml; Sespo, Brazil), associated with local anaesthesia (3.0% lidocaine with norepinephrine 1:50000; Dentsply, Brazil), and positioned in a stereotaxic frame. Two stainless steel guide cannulas (length = 9.0 mm, outer diameter = 0.8 mm) were implanted bilaterally aimed at the dorsal hippocampus CA1 region following the coordinates (AP = - 4.0 mm from Bregma, L = 2.5 from central suture, DV = 1.8 from the skull bone) from the rat brain atlas by Paxinos and Watson (2009) and fixed to the skull with acrylic resin and two stainless steel screws. To avoid occlusion, a stylet was introduced inside each guide cannula.

After ten days, the animals received a bilateral infusion with dental needles introduced through the guide cannulas until their tips were 1.5 mm below the cannula end. During 1 min, 0.5 μl/side of either vehicle or drug was injected using two 5.0-μl syringes connected to an infusion pump. A polyethylene catheter was interposed between the upper end of the dental needles and the syringes. The displacement of an air bubble inside the polyethylene was used to monitor drug flow. The needles were removed 30 s after the end of injections.

After the end of experiments involving intra-dorsal hippocampus treatment, animals were intraperitoneally anesthetized as already described. Evans Blue (0.5 μl/side) was injected through the guide cannulas to subsequent evaluation of the infusion site. The brain was removed and immersed in a 10% formalin solution. Brain slices (50 μm thick) were obtained in a vibratome and the site of injection was determined. Animals were included in the analysis when both sides of the dorsal hippocampus were tagged by Evans Blue (Fig. 3C).

2.4. Behavioral procedures and data collection

General procedures were conducted as previously described (Stern et al., 2012; da Silva et al., 2016). The behavioral testing was performed from 1 to 5 p.m. to minimize the possible circadian influence on learning and memory processing.

Contextual fear conditioning was performed in a rectangular chamber (35 × 20 × 30 cm), with aluminum sidewalls and a front wall and ceiling-door made of clear plexiglass acrylic, designated herein as Context A. Its grid floor, made of stainless steel bars, was connected to a circuit board and a shock generator (Insight, Brazil) to enable the delivery of controlled electrical shocks as detailed subsequently. A second chamber (30 × 30 × 30 cm), designated herein as Context B, was made of clear plexiglass acrylic and had a black lid to provide contextual cues as different as possible from those of the shock-paired Context A.

In all experiments, the animal was initially placed in Context A and allowed to explore it freely for 3 min, as a familiarization session, and returned to its home cage. This early exposure to the context to-be-conditioned has been reported to allow the acquisition of a better contextual representation (Fanselow et al., 2010).

On the next day, the animal was again placed in Context A for fear conditioning, during which it received, after an initial 30 s delay, the unconditioned stimulus (US, three electrical shocks for 3 s, with a 30 s inter-trial period). After the last US presentation, the animal remained in this chamber for another 30 s before returning to its home cage. The US intensity varied, being of 0.6 mA in experiments 1, 2 and 3, and of 0.8 mA in experiments 4, 5, 6, 8 and 10. The selection of the 0.6 mA was based on prior studies showing it does not induce generalized fear expression (Baldi et al., 2004; da Silva et al., 2016) while the 0.8 mA was selected based on pilot experiments showing it is able to induce generalized fear expression under our experimental conditions. In experiments 7 and 9, the US intensity was adjusted to 1.0 mA to achieve similar levels of freezing time in animals treated with vehicle either intra-dorsal hippocampus or intraperitoneally as previously reported (Stern et al., 2015; Vanvossen et al., 2017).

The assessment of treatment effects on consolidation of specific (0.6 mA) and more generalized (0.8 mA) fear memories was performed on the following days either by re-exposing the animal to the paired Context A for 3 min in the absence of US presentation (Test A_1) or by exposing the animal to the novel and unpaired Context B for 3 min (Test B_1). In experiments 1 and 4, Tests A_2 and B_2 were performed six days after Tests A_1 and B_1 because it was of interest to investigate the CBD effects at a more remote time point.

Freezing behavior, defined as the total absence of the body and head movements except for those associated with breathing (Blanchard and Blanchard, 1969), was recorded as an index of fear memory. The freezing time was quantified in seconds by a trained observer (inter- and intra-observer reliabilities ≥ 90%) blind to the experimental groups and expressed as the percentage of total session time.

A complementary assessment of CBD effects on consolidation of a more generalized fear memory (experiment 5) was performed by investigating the number of 22-kHz USVs. To this aim, an ultrasound gate condenser microphone CM16 (Avisoft Bioacoustics, Berlin, Germany), sensible to frequencies between 15 and 180 kHz, was mounted above the Contexts A and B and connected to a computer with the recording software Avisoft Recorder (version 2.95; Avisoft Bioacoustics). For USVs measurement, the Avisoft SASLab Pro software (version 4.34; Avisoft Bioacoustics) was used. A spectrogram from the animal's USVs recording was generated at a frequency resolution of 488 Hz and a time resolution of 0.512 ms for manual quantification (Wöhr et al., 2005; Yee et al., 2012). A 22 kHz USV was defined as a call between 21 and 30 kHz of frequency, separated from other calls by intervals longer than 320 ms (Van Der Poel and Miczek, 1991; Yee et al., 2012).

2.5. Experiments

2.5.1. Experiment 1: effects of CBD on consolidation of a specific fear memory

To investigate whether CBD given immediately after acquiring a specific contextual fear memory could interfere with its consolidation, 23 animals had Context A paired with three shocks of 0.6 mA and then were randomly allocated to three groups (n = 7–8/group) based on the systemic treatment (vehicle or CBD 3.0 or 10 mg/kg). All groups performed Test A_1, Test B_1, Test A_2 and Test B_2 on days 1, 2, 7 and 8, respectively.

2.5.2. Experiment 2: effects of CBD on short-term fear memory

To investigate whether 10 mg/kg of CBD given immediately after contextual fear conditioning could interfere with short-term memory, 14 animals had Context A paired with three shocks of 0.6 mA and then were randomly allocated to two groups (n = 7/group) based on the systemic treatment (vehicle or CBD). Both groups performed Test A_1 2 h later, as previously reported (Lunardi et al., 2017).

2.5.3. Experiment 3: effects of delayed CBD treatment on consolidation of a specific fear memory

To investigate whether 10 mg/kg of CBD given 6 h after acquiring a specific contextual fear memory could also interfere with its consolidation, 16 animals had Context A paired with three shocks of 0.6 mA and then were randomly allocated to two groups (n = 7–9/group) based on the systemic treatment (vehicle or CBD). Both groups performed Test A_1 on day 1.

2.5.4. Experiment 4: effects of CBD on consolidation of a more generalized fear memory

To investigate whether CBD given immediately after acquiring a more generalized fear memory could affect its consolidation, 41 animals had Context A paired with three shocks of 0.8 mA and then were randomly allocated to four groups (n = 9–11/group) based on the systemic treatment (vehicle or CBD 3.0, 10 or 30 mg/kg). All groups performed Tests A_1, B_1, A_2 and B_2 on days 1, 2, 7 and 8, respectively.

2.5.5. Experiment 5: effects of CBD given during the consolidation of a more generalized fear memory on 22-kHz USVs emitted throughout tests A_1 and B_1

To investigate whether 10 mg/kg of CBD given immediately after acquiring a more generalized fear memory could interfere with the emission of 22-kHz USVs, 25 animals had Context A paired with three shocks of 0.8 mA and then were randomly allocated to two groups (n = 12–13/group) based on the systemic treatment (vehicle or CBD). Both groups performed Tests A_1 and B_1 on days 1 and 2, respectively.

2.5.6. Experiment 6: effects of systemic CB_1 or CB_2 receptor antagonism on the more generalized fear memory consolidated under the CBD influence

To investigate how CBD interferes with consolidation, 52 animals had Context A paired with three shocks of 0.8 mA and then were randomly allocated to six groups (n = 8–10/group) based on the systemic pretreatment (vehicle, 1.0 mg/kg of the CB_1 receptor antagonist/inverse agonist AM251 or 0.3 mg/kg of the CB_2 receptor antagonist/inverse agonist AM630) given immediately after fear conditioning and the systemic treatment (vehicle or CBD 10 mg/kg) given 30 min later. All groups performed Tests A_1 and B_1 on days 1 and 2, respectively.

2.5.7. Experiment 7: effects of dorsal hippocampus CB_1 or CB_2 receptor antagonism on the more generalized fear memory consolidated under the CBD influence

To investigate whether activation of dorsal hippocampus CB_1 or CB_2 receptors could be involved in the CBD effects on consolidation of a more generalized fear memory, 45 animals had Context A paired with three shocks of 1.0 mA and then were randomly allocated to six groups (n = 7–8/group) based on local (vehicle, AM251 0.5 nmol or AM630 0.1 nmol) and systemic treatments (vehicle or 10 mg/kg of CBD) given immediately after fear conditioning. All groups performed Tests A_1 and B_1 on days 1 and 2, respectively.

2.5.8. Experiment 8: effects of systemic URB597 treatment during consolidation of a more generalized fear memory

To investigate whether URB597-induced inhibition of the FAAH activity immediately after acquiring a more generalized fear memory could interfere with its consolidation, 27 animals had Context A paired with three shocks of 0.8 mA and then were randomly allocated to three groups (n = 8–10/group) based on the systemic treatment (vehicle or URB597 0.5 or 1.0 mg/kg). All groups performed Tests A_1 and B_2 on days 1 and 2, respectively.

2.5.9. Experiment 9: effects of dorsal hippocampus URB597 infusion during consolidation of a more generalized fear memory

To investigate whether dorsal hippocampus FAAH inhibition immediately after acquiring a more generalized fear memory could also interfere with its consolidation, 18 animals had Context A paired with three shocks of 1.0 mA and then were randomly allocated to two groups (n = 8–10/group) based on the treatment (vehicle or URB597 0.01 nmol). Both groups performed Tests A_1 and B_2 on days 1 and 2, respectively.

2.5.10. Experiment 10: effects of CBD given during consolidation of a more generalized fear memory on its subsequent extinction

To investigate whether CBD given immediately after acquiring a more generalized fear memory could interfere with its subsequent behavioral suppression by extinction, 16 animals had Context A paired with three shocks of 0.8 mA and then were randomly allocated to two groups (n = 8/group) based on the systemic treatment (vehicle or CBD 10 mg/kg). On day 1, both groups underwent a session of extinction (Context A re-exposure for 15 min in the absence of US presentation), and performed the extinction test (Context A re-exposure for 5 min) and Test B_1 on days 2 and 3, respectively.

2.6. Statistical analysis

The results are expressed as mean ± standard error (SEM). After ensuring the assumptions of normality and homoscedasticity, the freezing times scored in Contexts A and B were subjected to separate one-way (independent factor: treatment), two-way (independent factors: pretreatment and treatment) or repeated-measures (independent factors: treatment and Context A or B re-exposures) analysis of variance (ANOVA). When ANOVA showed significant effects of the interaction between the two independent factors under study, results for the F tests of their main effects were omitted. The Newman-Keuls test was used for *post-hoc* multiple comparisons. When there were only two groups and no context re-exposure was performed, an unpaired Student's *t*-test was conducted. The statistical significance level was set at $P < 0.05$. We used Statistica 10 (StatSoft, EUA) for statistical analysis and GraphPad Prism 6 (GraphPad Prism, EUA) for graphing.

The *a priori* sample size determined by power analysis was of eight animals per group ($\alpha = 0.05$; $\beta = 0.8$ and standardized effect size or Cohen's d = 1.0). The group sizes were equal by design, but due to experimental losses (e.g. when treatment was infused outside the target brain region) or the violation of the predetermined exclusion criterion (fear conditioned animals were those spending at least 35% of freezing time during Test A_1), in a few cases they were slightly unequal. We have replaced the exclusions to attempt to keep the study balanced and to maintain its power.

3. Results

3.1. Experiment 1: CBD disrupted the consolidation of a specific contextual fear memory

The repeated-measures ANOVA showed significant effects of the treatment [$F_{(2,20)} = 4.8$; $P = 0.02$] and the Context A re-exposures [$F_{(1,20)} = 29.6$; $P = 0.0004$], but not the interaction of these factors [$F_{(2,20)} = 0.53$; $P = 0.60$], for freezing time expressed during Tests A_1 and A_2. As shown in Fig. 1A, in both cases the group treated with 10 mg/kg of CBD presented statistically less freezing time than did the controls, which expressed a comparable amount of freezing time during Tests A_1 and A_2.

The repeated-measures ANOVA showed no significant effects of the treatment [$F_{(2,20)} = 1.6$; $P = 0.22$], the Context B re-exposures [$F_{(1,20)} = 0.1$; $P = 0.80$] or the interaction of these factors [$F_{(2,20)} = 1.5$; $P = 0.24$], for freezing time expressed during Tests B_1 and B_2. As shown in Fig. 1A, all groups presented a comparable amount of freezing time in both cases.

3.2. Experiment 2: CBD did not interfere with short-term fear memory expression

One-way ANOVA showed no significant effects of the treatment for freezing time expressed during Test A_1 performed 2 h after fear conditioning [$F_{(1,12)} = 0.002$; $P = 0.97$]. As shown in Fig. 1B, both CBD and control groups presented a high but comparable amount of freezing time in this session.

3.3. Experiment 3: delayed CBD treatment had no effects on fear memory consolidation

Separate one-way ANOVAs showed no significant effects of the treatment for freezing time expressed during Test A_1 [$F_{(1,14)} = 0.56$; $P = 0.47$] or Test B_1 [$F_{(1,14)} = 0.02$; $P = 0.90$]. As shown in Fig. 1C, both CBD and control groups presented a comparable amount of freezing time in any case.

3.4. Experiment 4: CBD also disrupted the consolidation of a more generalized fear memory

The repeated-measures ANOVA showed significant effects of the Context A re-exposures [$F_{(1,37)} = 7.0$; $P = 0.01$], but not the treatment [$F_{(3,37)} = 0.45$; $P = 0.71$] or the interaction of these factors [$F_{(3,37)} = 0.45$; $P = 0.72$], for freezing time expressed during Tests A_1

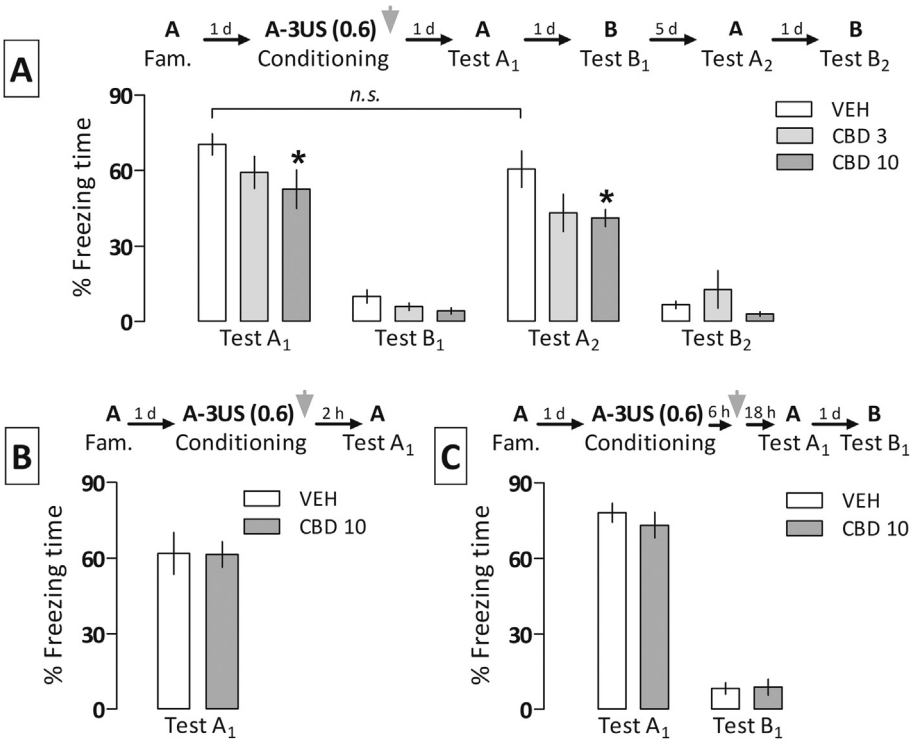

Fig. 1. **(A)** Effects of cannabidiol (CBD) on consolidation of a specific and long-term contextual fear memory (experiment 1). At the dose of 10 mg/kg, it reduced the freezing time during re-exposures to the paired Context A; (**B**) Effects of CBD (10 mg/kg) on short-term fear memory (experiment 2). It induced no changes in freezing time during Context A re-exposure; (**C**) Effects of delayed CBD (10 mg/kg) treatment on consolidation of a specific fear memory (experiment 3). When given 6 h after acquisition, it no longer reduced the freezing time during Context A re-exposure. The scheme above the graph represents the experimental design used in each case. Values are expressed as mean ± SEM (n = 7–9/group). The asterisk represents a significant difference ($P < 0.05$) from the respective control group.

and A_2. As shown in Fig. 2A, each one of the groups expressed a comparable amount of freezing time during these sessions.

The repeated-measures ANOVA showed significant effects of the treatment [$F_{(3,37)} = 12.1$; $P = 0.00001$] and the Context B re-exposures [$F_{(1,37)} = 5.5$; $P = 0.02$], but not the interaction of these factors [$F_{(3,37)} = 0.01$; $P = 0.99$], for freezing time expressed during Tests B_1 and B_2. As shown in Fig. 2A, in both cases the group treated with 10 or 30 mg/kg of CBD presented statistically less freezing time than did the controls, which expressed a comparable amount of freezing time during Tests B_1 and B_2.

To complement preceding analyses, the ratio of fear to Context B *vs.* Context A was calculated using the following formula: [(Freezing to Context B)/(Freezing to Context A + Freezing to Context B)].

The repeated-measures ANOVA showed significant effects of the treatment [$F_{(3,37)} = 6.1$; $P = 0.002$] and the session repetition [$F_{(1,37)} = 8.7$; $P = 0.005$], but not the interaction of these factors [$F_{(3,37)} = 0.42$; $P = 0.74$], for the ratio of fear to Context B *vs.* Context A during the first and the second sessions of exposure to these experimental contexts. As shown in Fig. 2B, in both cases, the group treated with 10 or 30 mg/kg of CBD presented a statistically lower ratio of fear to Context B *vs.* Context A when compared with controls.

To investigate whether the use of 0.6 and 0.8 mA for contextual pairing have induced different fear memories, the freezing time expressed by the control group from experiments 1 (0.6 mA) and 4 (0.8 mA) were compared.

The repeated-measures ANOVA showed no significant effects of the shock intensity [$F_{(1,14)} = 2,53$; $P = 0.13$], the Context A re-exposures [$F_{(1,14)} = 2.64$; $P = 0.13$] or the interaction of these factors [$F_{(1,14)} = 0.42$; $P = 0.55$], for freezing time expressed during Tests A_1 and A_2. As shown in Fig. 2C, both groups presented a high, but comparable, amount of freezing time in these sessions.

The repeated-measures ANOVA showed significant effects of the shock intensity [$F_{(1,14)} = 17.9$; $P = 0.001$], but not the Context B re-exposures [$F_{(1,14)} = 0.11$; $P = 0.75$] or the interaction of these factors [$F_{(1,14)} = 1.70$; $P = 0.22$], for freezing time expressed during Tests B_1 and B_2. As shown in Fig. 2C, in both cases the 0.8 mA group presented statistically more freezing time than did the 0.6 mA group.

3.5. Experiment 5: CBD given during consolidation of a more generalized fear memory attenuated the emission of 22-kHz USVs during tests A_1 and B_1

Separate unpaired Student's t tests showed significant effects of the treatment for 22-kHz USVs emitted during Test A_1 ($t_{23} = 2.3$; $P = 0.02$) and Test B_1 ($t_{23} = 2.5$; $P = 0.02$). As shown in Fig. 2D, the CBD group presented a statistically lower number of these calls relative to controls in both sessions. Moreover, as already shown in experiment 4 (Fig. 2A), these groups presented a similar amount of freezing time during Test A_1 ($t_{23} = 1.0$; $P = 0.31$), but CBD-treated animals presented statistically less freezing time than the controls during Tests B_1 ($t_{23} = 4.7$; $P = 0.0001$) (Fig. 2D).

3.6. Experiment 6: systemic antagonism of CB_1 or CB_2 receptors prevented the CBD effects on consolidation of a more generalized fear memory

Two-way ANOVA showed no significant effects of the pretreatment [$F_{(1,46)} = 1.6$; $P = 0.22$], the treatment [$F_{(1,46)} = 0.1$; $P = 0.79$] or the interaction of these factors [$F_{(2,46)} = 0.6$; $P = 0.54$], for freezing time expressed during Tests A_1. As shown in Fig. 3A, all groups presented a comparable amount of freezing time in this session.

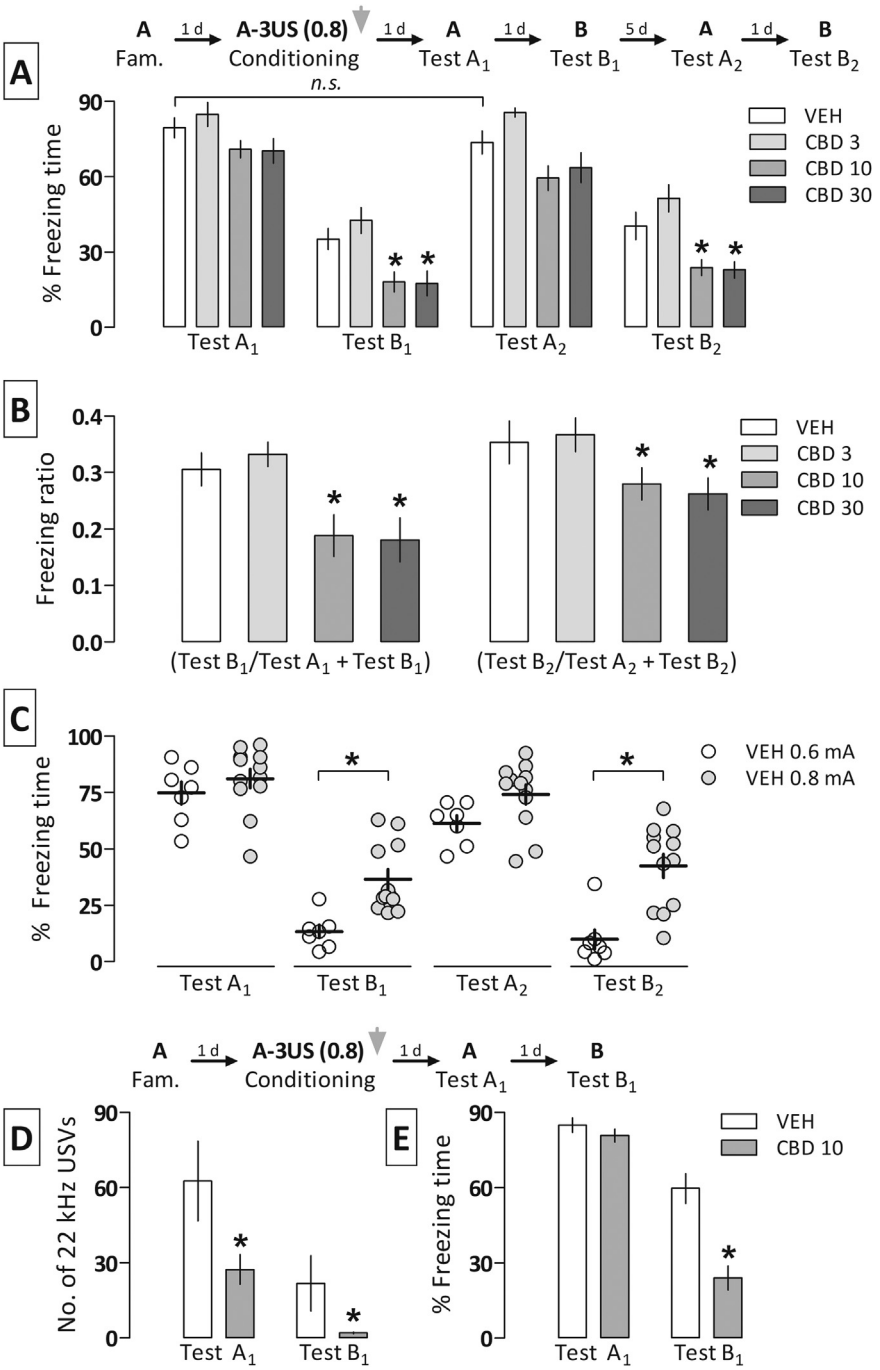

Fig. 2. **(A)** Effects of cannabidiol (CBD) on consolidation of a more generalized fear memory (experiment 4). At the doses of 10 or 30 mg/kg, it reduced the freezing time during exposures to the unpaired Context B; (**B**) Effects of CBD on the ratio of fear to Context B *vs.* Context A. At the doses of 10 or 30 mg/kg, it reduced this measure on the first and the second experimental sessions; (**C**) Comparison of freezing times expressed by the control group (VEH) from experiments 1 (0.6 mA) and 4 (0.8 mA). The 0.8 mA group presented more freezing time than did the 0.6 mA group; (**D, E**) Effects of CBD (10 mg/kg) given during the consolidation of a generalized fear memory on 22-kHz ultrasonic vocalizations (USVs) and freezing time emitted during Tests A_1 and B_1 (experiment 5). It reduced the number of 22-kHz USVs measure in both cases. As shown in experiment 4 (panel A), CBD reduced the freezing time during exposure to the Context B only. The scheme above the graph represents the experimental design used in each case. Values are expressed as mean ± SEM (n = 9–11/group for experiment 4; n = 12–13/group for experiment 5). The asterisk represents a significant difference ($P < 0.05$) from the respective control group.

Two-way ANOVA showed significant effects of the interaction between pretreatment and treatment factors for freezing time during Test B_1 [$F_{(2,46)} = 3.6$; $P = 0.03$]. As shown in Fig. 3A, animals treated with CBD presented statistically less freezing time than did the controls. The CBD effect, however, was no longer observed in animals pretreated with AM251 or AM630.

3.7. Experiment 7: dorsal hippocampus CB_1 or CB_2 receptor antagonism was sufficient to prevent the CBD effects on consolidation of a more generalized fear memory

Two-way ANOVA showed no significant effects of the pretreatment [$F_{(1,39)} = 1.6$; $P = 0.22$], the treatment [$F_{(1,39)} = 0.1$; $P = 0.79$] or the interaction of these factors [$F_{(2,39)} = 1.7$; $P = 0.18$], for freezing

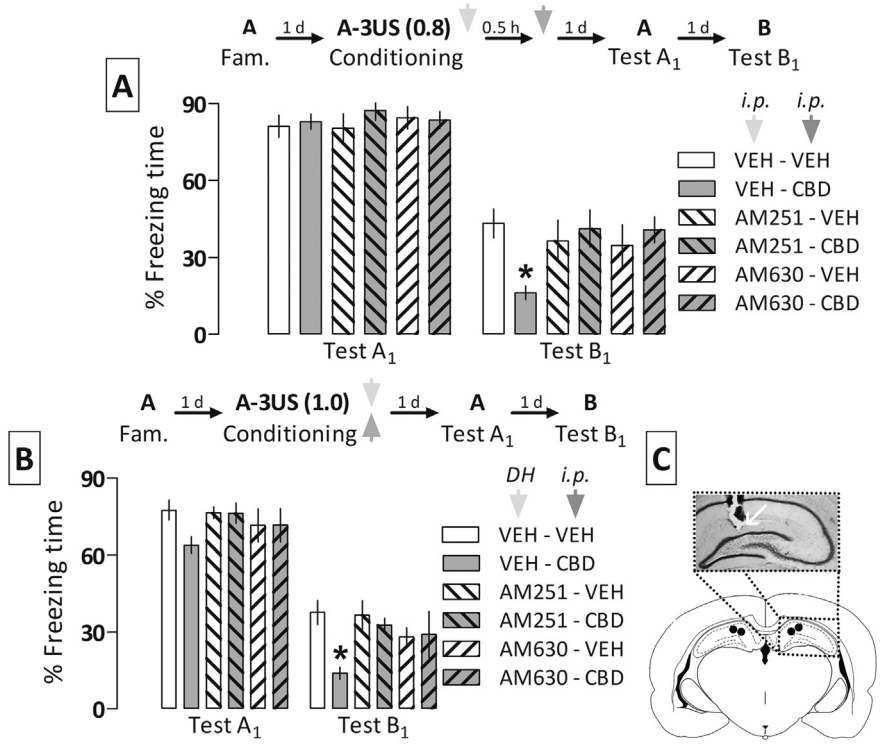

Fig. 3. **(A)** Effects of systemic pretreatment with the CB_1 receptor antagonist/inverse agonist AM251 (1.0 mg/kg) or the CB_2 receptor antagonist/inverse agonist AM630 (0.3 mg/kg) on the more generalized fear memory consolidated under the cannabidiol (CBD) influence (experiment 6). The freezing time reduction induced by CBD (10 mg/kg) in Test B_1 was prevented in both cases; (**B**) Effects of intra-dorsal hippocampus (DH) pretreatment with AM251 (0.5 nmol/side) or AM630 (0.1 nmol) on the more generalized fear memory consolidated under the CBD influence (experiment 7). In any case, the CBD-induced freezing time reduction during Test B_1 was prevented; (**C**) Representative infusion site and schematic diagram showing the placement of treatment infusion into the DH (*filled circles*). The scheme above the graph represents the experimental design used in each case. Values are expressed as mean ± SEM (n = 8–10/group for experiment 6; n = 7–8/group for experiment 7). The asterisk represents a significant difference ($P < 0.05$) from the respective control group.

time expressed during Tests A_1. As shown in Fig. 3B, all groups presented a comparable amount of freezing time in this session.

Two-way ANOVA showed significant effects of the interaction between pretreatment and treatment factors for freezing time during Test B_1 [$F_{(2,39)} = 3.3$; $P = 0.04$]. As shown in Fig. 3B, animals treated with CBD presented statistically less freezing time than did the controls. The CBD effect, however, was no longer observed in animals pretreated with AM251 or AM630.

3.8. Experiment 8: systemic URB597 treatment disrupted the consolidation of a more generalized fear memory

Separate one-way ANOVAs showed significant effects of the treatment for freezing time during Test B_1 [$F_{(2,24)} = 9.9$; $P = 0.001$], but not Test A_1 [$F_{(2,24)} = 2.5$; $P = 0.10$]. As shown in Fig. 4A, all groups presented a similar amount of freezing time during Test A_1, but animals treated with the highest dose of URB597 tested expressed statistically less freezing time than the controls during Test B_1.

3.9. Experiment 9: dorsal hippocampus infusion of URB597 was sufficient to disrupt the consolidation of a more generalized fear memory

Separate one-way ANOVAs showed significant effects of the treatment for freezing time during Test B_1 [$F_{(1,16)} = 5.3$; $P = 0.04$], but not Test A_1 [$F_{(1,16)} = 0.52$; $P = 0.48$]. As shown in Fig. 4B, all groups presented a similar amount of freezing time during Test A_1, but URB597-treated animals expressed statistically less freezing time than did the controls during Test B_1.

3.10. Experiment 10: CBD given during consolidation of a more generalized fear memory potentiated its extinction

The repeated-measures ANOVA showed significant effects of the interaction between treatment and time-bin factors for freezing time during the extinction session [$F_{(4,56)} = 5.2$; $P = 0.001$]. As shown in Fig. 5A, both vehicle and CBD groups expressed significantly less freezing from the third to the fifth 3-min session block when compared with the respective first block, but the extinction in CBD-treated animals was accelerated during the third and the fourth session blocks. Of note, the difference between groups remained when the extinction session was analyzed as a total ($t_{14} = 5.1$; $P = 0.04$).

CBD-treated animals also expressed significantly less freezing time than did controls during the extinction test ($t_{14} = 2.5$; $P = 0.03$) and Test B_1 ($t_{14} = 2.5$; $P = 0.02$) (Fig. 5B). As shown in Fig. 5C, the min-by-min analysis of Test B_1 data confirmed the CBD group expressed significantly less freezing time than did the controls, but the respective level of freezing time remained stable throughout the session.

4. Discussion

Animals administered with CBD (10 mg/kg) immediately after performing the session in which the Context A was paired with three shocks of 0.6 mA spent less time freezing when re-exposed to the paired context one and seven days later, showing it disrupted the consolidation of a specific and long-term fear memory. This result is in line with impaired aversive memory formation reported after infusing CBD into the nucleus accumbens (Norris et al., 2016)

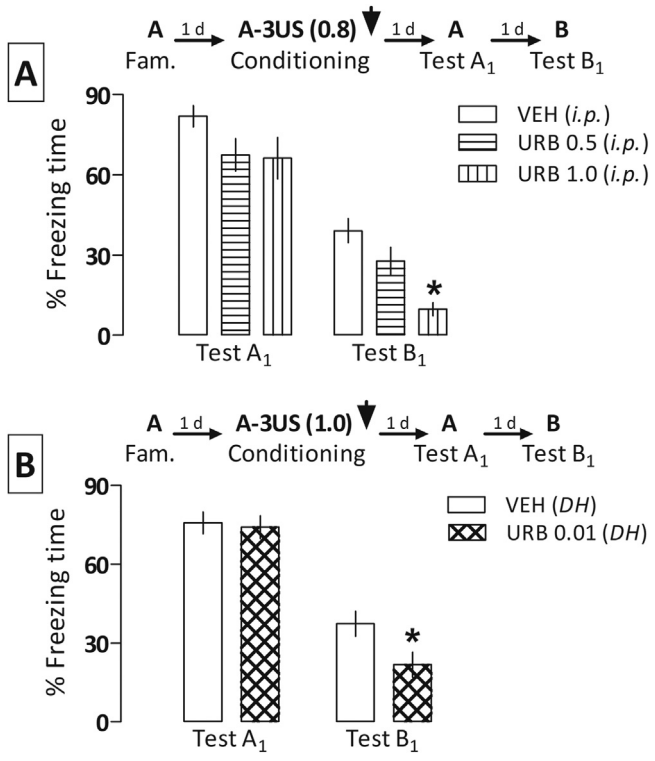

Fig. 4. (A) Effects of URB597 (URB) given systemically on consolidation of a more generalized fear memory (experiment 8). At the dose of 1.0 mg/kg, it reduced the freezing time during Context B exposure; **(B)** Effects of dorsal hippocampus infusion of URB (0.01 nmol/side) on consolidation of a more generalized fear memory (experiment 9). It also reduced the freezing time during Context B exposure. The scheme above the graph represents the experimental design used in each case. Values are expressed as mean ± SEM (n = 8–10/group for both experiments). The asterisk represents a significant difference ($P < 0.05$) from the respective control group.

or the prelimbic cortex (Rossignoli et al., 2017), and with mitigation of enduring contextual fear memories through extinction facilitation or reconsolidation disruption reported after treatment with CBD before or after their retrieval, respectively (Bitencourt et al., 2008; Stern et al., 2012; Do Monte et al., 2013; Gazarini et al., 2014; Stern et al., 2015; Song et al., 2016). It is also consistent with a previous study showing the dose of 10 mg/kg was the most effective one and the disrupting effects of the CBD on fear memory lasted over a week (Stern et al., 2012), which rule out the possibility that its anti-aversive effects (Lemos et al., 2010; Gomes et al., 2012; Jurkus et al., 2016) could explain the abovementioned results (the CBD half-life is of ≤8 h in rats; Deiana et al., 2012). Importantly, delayed CBD (10 mg/kg) treatment no longer affected the consolidation of a specific and long-term memory, indicating its action was restricted to a time window of ≤6 h. CBD also had no effects on short-term memory expression.

Previously published studies have reported the greater the number or the intensity of shocks delivered, the more generalized the fear memory induced (Baldi et al., 2004; Kaouane et al., 2012; Gazarini et al., 2014; Ghosh and Chattarji, 2015; Poulos et al., 2016; Dunsmoor et al., 2017). As a result, laboratory animals often present a significant amount of freezing time during the exposure to a novel and unpaired context. Here, increased freezing time was observed during Tests B_1 and B_2 when the vehicle-treated group subjected to the 0.8 mA shock intensity for contextual pairing was compared with that receiving 0.6 mA, which corroborates the induction of different (specific and more generalized) fear memories using experimental protocols that varied the shock intensity for fear conditioning. Of note, the level of generalized fear was equivalent to that previously reported using the same rat strain, sex and age (Baldi et al., 2004; Gazarini et al., 2013, 2014).

As the consolidation of a more generalized fear memory could underlie the development of a long-lasting inability to restrict fear to the paired context, the next step was to investigate whether CBD could influence this process. Indeed, animals treated with CBD (10 or 30 mg/kg) post-acquisition did not present a significant amount of freezing time during Test B_1 (day 2) and Test B_2 (day 8), showing it prevented the expression of generalized fear owing to its disrupting effects on consolidation. It is noteworthy that vehicle- and CBD-treated animals had a similar time of freezing during Test A_1. A possible explanation for this finding is that this behavioral response has achieved its asymptotic level in animals in which 0.8 mA was used fear contextual pairing. As a result, it is likely the CBD also decreased conditioned fear during re-exposure to the paired Context A, but it only caused an observable reduction in 22-kHz USVs because freezing data exhibited a ceiling effect and USV data did not. Moreover, perhaps the use of a longer test could allow the emergence of differences between groups during Test A_1. However, the freezing time expressed by CBD- and vehicle-treated animals remained similar for the first 6 min of the extinction session (Fig. 5A). Of note, the duration of the test used here has been similar to that regularly used by studies conducted with rats investigating the effects of drugs on contextual fear memory consolidation (Schafe et al., 1999; Ji et al., 2003; Gazarini et al., 2013; Heath et al., 2015; da Silva et al., 2016; Lunardi et al., 2017;

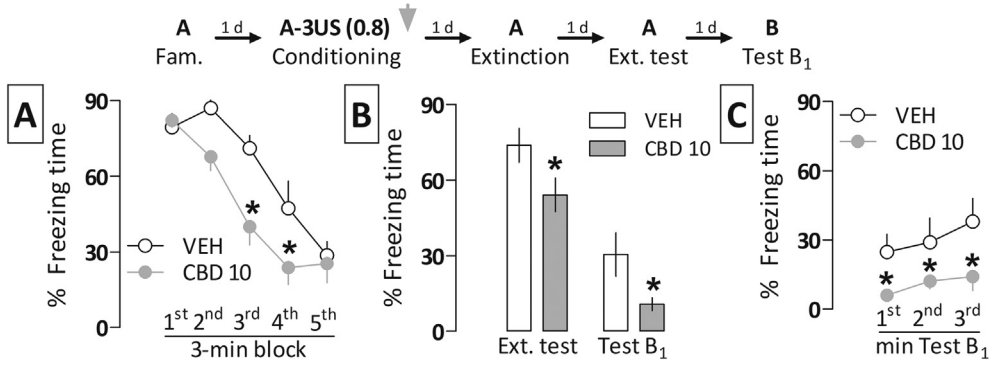

Fig. 5. Effects of CBD (10 mg/kg) given during consolidation of a more generalized fear memory on its subsequent extinction (experiment 10). (**A**) CBD rendered the memory more prone to extinction; (**B**) The freezing time reduction in the Extinction test or the Test B_1 was greater in CBD-treated animals; (**C**) Min-by-min analysis of freezing time in Test B_1 confirmed the difference between groups, but its respective level remained stable throughout the session. The scheme above graphs represents the experimental design used in this experiment. Values are expressed as mean ± SEM (n = 8/group). The asterisk represents a significant difference ($P < 0.05$) from the respective control group.

Vanvossen et al., 2017). Alternatively, CBD might no longer be effective in disrupting memory consolidation when stronger shock intensities are used during fear conditioning. The 22-kHz USVs data, however, do not support this possibility because the number of these calls was lower in CBD-treated animals during Test A_1. This result corroborates the CBD has disrupted the consolidation of a more generalized fear memory, and highlights the importance of assessing USVs at 22-kHz to detect drug-induced differences not observed by measuring visible (freezing) behavior (Yee et al., 2012).

The measurement of 22-kHz USVs during fear conditioning has also been validated as an index of anxiety (Borta et al., 2006). However, as the CBD-induced reduction in this measure was shown on Tests A_1 and B_1 performed respectively 24 and 48 h after its treatment, this effect was not directly attributable to the anti-aversive effects of the CBD. Rather, it would be the outcome of CBD-induced attenuating effects on consolidation. The exposure to an unfamiliar but neutral context has been shown to be insufficient to induce 22-kHz USVs (Wöhr et al., 2005). However, our results suggest that this could depend on the features of the conditioning protocol: vehicle-treated animals in which the Context A was paired with three shocks of 0.8 mA also emitted a considerable number of USVs at 22-kHz during Test B_1, a response that was virtually abolished in the CBD group. This result corroborates the CBD has attenuated the consolidation of a more generalized fear memory.

Both systemic and intra-dorsal hippocampus pretreatment with AM251 or AM630 prevented the attenuating effects of CBD on the consolidation of a more generalized fear memory and related generalized fear. These results are consistent with those showing the contribution of hippocampal CB_1 and CB_2 receptors to consolidate either emotional or non-emotional memories (Clarke et al., 2008; De Oliveira Alvares et al., 2008; Wise et al., 2009; García-Gutiérrez et al., 2013; Li and Kim, 2016; Nasehi et al., 2017). They are also in line with those relating the brain CB_1 receptors in CBD-induced effects on extinction and reconsolidation of contextual fear memories (Bitencourt et al., 2008; Stern et al., 2012, 2015; Do Monte et al., 2013; Song et al., 2016), and the dorsal hippocampus activity in contextual memory specificity and generalization (Wiltgen et al., 2010; Lynch et al., 2017). Of note, stressful experiences or specific brain insults have been reported to alter significantly the expression of hippocampal CB_2 receptors (Lopez-Rodriguez et al., 2015; Robertson et al., 2017). However, as the AM630 was given immediately after conditioning, it is unlikely the present results depend on a significant change in its level. The medial prefrontal cortex is another brain region in which activation of cannabinoid receptors has been associated with aversive memory consolidation (Kuhnert et al., 2013). Future studies could investigate whether they are also involved in the abovementioned CBD effects on memory consolidation.

Convergent evidence from *in vitro* and *in vivo* studies has suggested the effects of CBD on the cannabinoid transmission are indirect (Izzo et al., 2009; Campos et al., 2012; Leweke et al., 2012; Elmes et al., 2015). Indeed, it is able to inhibit the uptake and/or the degradation of the anandamide, an endogenous agonist of CB_1 and CB_2 receptors (Bisogno et al., 2001; Izzo et al., 2009). If the disrupting effects of CBD on consolidation of a more generalized fear memory are associated with increased bioavailability of the anandamide, one could anticipate that the selective inhibition of its catabolizing enzyme would induce a similar effect to that of CBD. That was the case when the FAAH inhibitor URB597 was given systemically or intra-dorsal hippocampus. These results agree with the impaired aversive memory consolidation reported after systemic or intra-dorsal hippocampus infusion of URB597, anandamide or other CB_1/CB_2 receptor agonists (Castellano et al., 1997; Murillo-Rodríguez et al., 1998; Maćkowiak et al., 2009; Busquets-Garcia et al., 2011; Segev and Akirav, 2011; Zarrindast et al., 2012; Kuhnert et al., 2013). However, the opposite effect was reported when this drug was given during the consolidation of an inhibitory avoidance memory in rats (Morena et al., 2014). Differences in the level of emotional arousal by the stressfulness of the experimental conditions, behavioral tasks and/or doses used may account for the conflicting data. Interestingly, the URB597-induced effects on aversive memory consolidation have also been associated with the activation of CB_1 and CB_2 receptors (Ratano et al., 2017).

Vehicle-treated animals in which 0.8 mA was used for contextual pairing presented a relative resistance to fear extinction. This result is in line with findings showing it is often associated with a generalized fear memory (Gazarini et al., 2014; Finsterwald et al., 2015). In contrast, consolidating a more generalized fear memory under the CBD influence prevented this behavioral outcome, showing this drug rendered it more prone to extinction. As this effect was observed 24 h after treatment with CBD, a time period in which the drug would have been almost completely eliminated (Deiana et al., 2012), it cannot be directly attributable to a reduced fear expression associated with the anti-aversive and/or the facilitating effects on extinction of this phytocannabinoid (Bitencourt et al., 2008; Do Monte et al., 2013; Song et al., 2016). Moreover, the present result is consistent with those showing that the CB_1/CB_2 receptor agonist WIN 55,212-2 prevented the stress-induced impairment of extinction when administered immediately after trauma exposure (Ganon-Elazar and Akirav, 2013). Consonant with laboratory animal studies, CBD has also facilitated fear memory extinction in healthy humans (Das et al., 2013). The latter effect was observed when it was given around the memory retrieval, which contrasts with the present study where the CBD was given immediately after acquisition. Although these results are not directly comparable, it would be interesting to investigate whether CBD given to humans in the trauma aftermath could make the related memory more susceptible to extinction.

In summary, the present findings provide evidence the CBD is able to attenuate the consolidation of specific and more generalized fear memories and related outcomes. This effect involves the activation of dorsal hippocampus CB_1 and CB_2 receptors, and is probably mediated by anandamide because the pharmacological inhibition of its degradation induced similar effects.

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Conflict of interest

All authors declare they have no conflict of interest.

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