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RESEARCH PAPER

A time-dependent contribution of hippocampal CB_1, CB_2 and PPARγ receptors to cannabidiol-induced disruption of fear memory consolidation

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Background and Purpose: In preclinical studies, cannabidiol (CBD) mitigates fear memories by facilitating their extinction or interfering with their generalization and reconsolidation. The brain regions and mechanisms underlying these effects, and their temporal window, are still poorly understood. Here, we have investigated related questions in the dorsal hippocampus (DH) during contextual fear consolidation.

Experimental Approach: Adult male Wistar rats received CBD (10–30 pmol) intra-DH immediately, 1 or 3 hr after fear conditioning. Effects of CBD on consolidation were inferred behaviourally and by analysing expression of the activity-regulated, cytoskeleton-associated (Arc) protein. The contribution of anandamide, CB_1, CB_2, $5\text{-}HT_{1A}$, A_{2A}, and PPARγ receptors was also assessed.

Key Results: CBD impaired memory consolidation when given immediately or 1 hr after fear conditioning, but not after 3 hr. Expression of Arc protein in DH was reduced by systemic CBD treatment in both cases. Immediately after fear conditioning, CBD effects were abolished by CB_1 or CB_2 receptor blockade, partly reduced by $5\text{-}HT_{1A}$ or A_{2A} antagonism, and remained unchanged after antagonism of PPARγ receptors. One hour after fear conditioning, CBD effects were prevented only by PPARγ receptor antagonism. Also, inhibition of fatty acid amide hydrolase by URB597, impaired memory consolidation when infused immediately, but not 1 hr after fear conditioning.

Conclusions and Implications: CBD disrupts memory consolidation up to 1 hr after fear conditioning, allowing an extended window of opportunity to mitigate aversive memories after their acquisition. Our results suggest time-dependent participation of anandamide, CB_1, CB_2 and PPARγ receptors in the DH, during this process.

1 | INTRODUCTION

Classically, the memory consolidation time-window lasts 6 hr (McGaugh, 2000), a period in which cellular and molecular events occur to stabilize and store a newly acquired long-term memory (Lamprecht & LeDoux, 2004; McGaugh, 2000). Most studies investigating the effects of pharmacological interventions on memory consolidation are conducted at very short intervals after the acquisition, that is, 0–10 min (Lunardi et al., 2017; Schmidt et al., 2017). Few have explored longer intervals of the consolidation time-window. In general, they have reported that either 1 hr (Igaz, Vianna, Medina, &

Abbreviations: Arc, activity-regulated cytoskeleton-associated protein; CBD, cannabidiol; DH, dorsal hippocampus; FAAH, fatty acid amide hydrolase.

Izquierdo, 2002) or 3 hr (Gafford, Parsons, & Helmstetter, 2005) after acquisition, the memory is less susceptible to interference. In any case, the mechanisms underlying the memory consolidation over its time-window are still poorly understood. The study of these mechanisms is relevant because several psychiatric disorders, such as post-traumatic stress disorder, seem to involve abnormal memory consolidation of the aversive events (Ehlers, Hackmann, & Michael, 2004).

The systemic administration of **cannabidiol** (CBD), the major compound from *Cannabis* sp. devoid of psychotomimetic effects, immediately after memory acquisition, has been reported to impair the contextual fear memory generalization, an effect depending on the activation of **CB_1** and **CB_2** receptors located in the dorsal hippocampus (DH; Stern et al., 2017). It has been proposed that fear overgeneralization, a feature of post-traumatic stress disorder, is associated with memory consolidation (Gazarini, Stern, Piornedo, Takahashi, & Bertoglio, 2015; Stern et al., 2017). Several studies support the involvement of the DH in fear memory processing, especially during the consolidation phase (Izquierdo & Medina, 1997). However, it is unknown whether, when, and for how long, CBD administered into the DH would impair the memory consolidation process.

Various mechanisms have been associated with the effects of CBD (Campos et al., 2017). For example, the cannabinoid CB_1 and CB_2, and **5-HT_{1A}** receptors have been involved in the effects of CBD on fear memory and anxiety-related responses, respectively (Bitencourt, Pamplona, & Takahashi, 2008; Lee, Bertoglio, Guimarães, & Stevenson, 2017; Stern, Gazarini, Takahashi, Guimarães, & Bertoglio, 2012). Moreover, the adenosine **A_{2A}** receptor and the **PPARγ** receptor, a member of a family of nuclear receptors, have been associated with CBD-induced neuronal plasticity and learning and memory changes in animal models of neurodegenerative diseases (Esposito et al., 2011; Jahrling, Hernandez, Denner, & Dineley, 2014; Simões et al., 2016). All these receptors are expressed in the DH (Moreno, Farioli-Vecchioli, & Cerù, 2004). However, their relative contributions to CBD-induced effects on fear memory consolidation have not yet been explored.

The main objective of this study was to investigate the effects of CBD administered intra-DH immediately, 1 hr, or 3 hr after a contextual fear conditioning protocol. We observed that CBD impairs memory consolidation when given immediately or 1 hr after memory acquisition. This effect is accompanied by a reduction in the expression of activity-regulated cytoskeleton-associated protein (Arc), a product of an immediate early gene necessary for memory consolidation (Besnard, Laroche, & Caboche, 2014), in the DH. We also investigated the involvement of CB_1, CB_2, 5-HT_{1A}, A_{2A}, and PPARγ receptors in the CBD-induced impairments of memory consolidation. the effects of CBD on memory consolidation depended on the activation of CB_1 and CB_2, but not 5-HT_{1A}, A_{2A}, or PPARγ, receptors in the DH immediately after fear conditioning. In contrast, when given 1 hr after memory acquisition, the effects of CBD were mediated by activation of PPARγ receptors in the DH. Selective inhibition of fatty acid amide hydrolase (**FAAH**) by **URB597** impaired memory consolidation when infused immediately, but not 1 hr, after fear conditioning.

What is already known

- Cannabidiol given immediately after fear conditioning impaired memory generalization.
- Effects of cannabidiol on fear generalization are mediated by CB_1 and CB_2 receptors.

What does this study add

- Cannabidiol effects on memory consolidation are associated with reduced Arc expression in the dorsal hippocampus.
- Cannabidiol effects on memory consolidation rely on different mechanisms of action across the consolidation time-window.

What is the clinical significance

- There is an extended time-window to mitigate fearful memories after their acquisition with cannabidiol.
- The PPARγ receptor could be investigated as a new target to mitigate aversive memories

2 | METHODS

2.1 | Animals

All animal care and experimental procedures were approved by the local Committee on the Care and Use of Laboratory Animals (authorization number 1048). Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny et al., 2010) and with the recommendations made by the *British Journal of Pharmacology*. Three-month-old male Wistar rats (270-320 g) were obtained from the local breeding facilities. The animals were housed in groups of five in Plexiglas cages measuring 60 × 25 × 25 cm. They were kept in the animal facility under controlled temperature (22 ± 2 °C) and illumination (12-hr cycle, lights on at 7 a.m.) conditions and had free access to water and standard laboratory chow.

2.2 | Drugs

CBD (Phytoplant, Spain; 10–30 pmol or 10 $mg \cdot kg^{-1}$), **AM251** (antagonist of CB_1 receptors; Tocris, USA; 0.5 nmol), **AM630** (antagonist of CB_2 receptors; Tocris, USA; 0.1 nmol), **ZM241385** (antagonist of A_{2A} receptors; Sigma, USA; 10 nM), and URB597 (FAAH inhibitor; Tocris, USA; 0.1 μg) were dissolved in 0.9% NaCl containing 5.0% Tween® 80 (Sigma-Aldrich, USA). **WAY100635** (antagonist of 5-HT_{1A} receptors; Sigma-Aldrich, USA; 0.14 nmol) was dissolved in 0.9% NaCl.

GW9662 (antagonist of PPARγ receptors; Sigma, USA; 32 pmol) was dissolved 0.9% NaCl containing 5.0% DMSO. The dose selection was based on pilot experiments or previously published studies (Dall'Igna, Porciúncula, Souza, Cunha, & Lara, 2003; De Paula Soares & Zangrossi, 2004; Denner et al., 2012; Segev et al., 2018; Stern et al., 2012, 2017). All solutions were prepared immediately before use and injected into the DH in a volume of 0.5 μl per side or i.p. in a volume of 1.0 ml·kg^{-1}. In all experiments, the vehicle solution of the control group was 0.9% NaCl containing 5.0% Tween® 80.

2.3 | Stereotaxic surgery and drug infusion

The rats were anaesthetized with 1.0 ml·kg^{-1} of xylazine (10 mg·ml^{-1}; Carlier, Brazil) and ketamine (100 mg·ml^{-1}; Sespo, Brazil), together with local anaesthesia (3% lidocaine with noradrenaline 1:50,000; Dentsply, Brazil) given i.p., and positioned in the stereotaxic frame. Two stainless steel guide cannulas (length = 9.0 mm and outer diameter = 0.7 mm) were implanted bilaterally aimed at the CA1 region of the DH following the coordinates (AP = −3.8 mm from bregma, L = ±2.5 mm from the central suture, and DV = −1.8 mm from the skull bone) from the rat brain atlas by Paxinos and Watson (2009). They were fixed to the skull with acrylic resin and two stainless steel screws. To prevent occlusion, a stylet was introduced inside each guide cannula. At the end of the surgery, the animals received 0.4 ml of ibuprofen orally (20 mg·ml^{-1}, Natulab, Brazil).

After 10 days, the animals received a bilateral infusion with dental needles (outer diameter = 0.3 mm) introduced through the guide cannulas until their tips were 1.5 mm below the cannula ends. During 1 min, either the vehicle or drug was injected using two microsyringes connected to an infusion pump (Insight, Brazil). A polyethylene catheter was interposed between the upper end of the dental needles and the microsyringes. The displacement of an air bubble inside the polyethylene was used to monitor the drug flow. The needles were removed 30 s after the end of injections. In cases receiving pretreatment, the second infusion was performed 10 min after the first one.

After the end of each experiment, the rats were anaesthetized, as described above and Evans Blue (0.5 μl per side) was injected through the guide cannulas for subsequent evaluation of the drug infusion site. The brains were removed and immersed in a 10% formalin solution. Brain slices (100 μm thick) were obtained in a vibratome (Leica, Germany), and the injection site was determined. Animals were only included in the analysis when both sides of the DH were tagged by the Evans Blue (Figure 1).

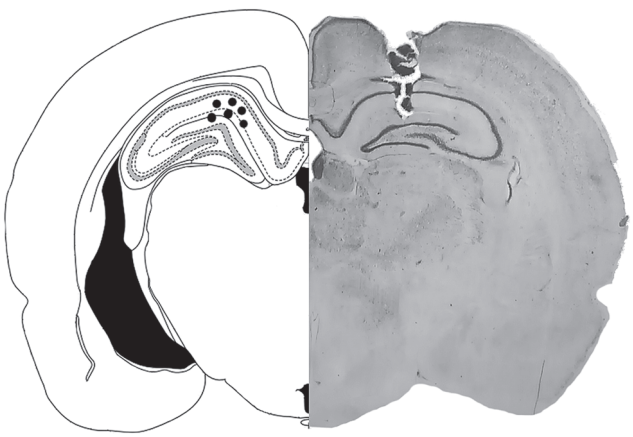

FIGURE 1 Representative infusion site (right) and schematic diagram (left) adapted from Paxinos and Watson (2009; Bregma—3.8 mm) showing the placement of drug infusions into the dorsal hippocampus (filled circles)

2.4 | Behavioural procedures, experiments, and data collection

General procedures were conducted as previously described (Stern et al., 2017). The behavioural testing was conducted under 70 lux, from 1 to 5 p.m. to minimize possible circadian effects on learning and memory processing.

Fear conditioning was performed in a rectangular chamber (Context A; 35 × 20 × 30 cm), with aluminium sidewalls and a front wall and ceiling-door made of clear plexiglass acrylic. 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. In all experiments, the animals were placed in Context A and allowed to freely explore it for 3 min, as an initial familiarization session. They were then returned to their home cage.

On the next day, each animal was again placed in Context A for fear conditioning, during which it received, after an initial 30-s delay, the unconditioned stimulus (three electrical footshocks of 0.8 or 1.0 mA, for 3 s, with a 30-s inter-trial period). The animal remained in this chamber for another 30 s before returning to its home cage. The animals of each cage were randomly allocated in the groups so that each cage had animals of different groups. The use of two different intensities was based on a prior study (Stern et al., 2017) showing that animals subjected to stereotaxic surgery require higher footshock intensity to present freezing time values similar to those without stereotaxic surgery.

In Experiment 1A, to investigate whether intra-DH CBD could interfere with the contextual fear memory consolidation, 21 animals were randomly allocated to three groups (n = 7 rats each group) based on the treatment (vehicle, CBD 10 or 30 pmol) given immediately after pairing the Context A with three footshocks.

In Experiment 1B, to investigate whether CBD could interfere with Arc protein expression in the DH, 18 contextually fear-conditioned animals were randomly allocated to two groups based on the systemic treatment VEH (n = 8) or CBD 10 mg·kg^{-1} (n = 10) given immediately after Context A-footshock pairing. An additional group (n = 8) of naive, non-conditioned animals was used to assess the basal Arc expression in the DH. The animals were killed immediately after

being removed from the vivarium (naive group) or 90 min after treatment (VEH and CBD groups).

In Experiment 2A, to investigate whether CBD could still affect the contextual fear memory consolidation when administered into the DH at a later time point of the consolidation time-window, 20 animals were randomly allocated to two groups based on the treatment VEH (n = 11) or CBD 30 pmol (n = 9) given 1 hr after Context A-footshock pairing.

In Experiment 2B, to investigate whether CBD could still reduce Arc protein expression in the DH when given at a later time point of the consolidation time-window, 20 contextually fear-conditioned animals were randomly allocated to two groups based on the systemic treatment VEH (n = 9) or CBD 10 mg·kg^{-1} (n = 11) given 1 hr after Context A-footshock pairing. An additional group (n = 7) of naive, non-conditioned animals was used to assess the basal DH Arc expression. Animals were killed immediately after being removed from the vivarium (naive group) or 90 min after treatment (VEH and CBD groups), that is, 150 min after the conditioning procedure.

In Experiment 3, to investigate whether CBD could still affect contextual fear memory consolidation when injected into the DH at a later time point of the consolidation time-window, 14 animals were randomly allocated to two groups (n = 7 rats each group) based on the treatment (vehicle or CBD 30 pmol) given 3 hr after Context A-footshock pairing.

In Experiment 4, to investigate the receptors involved in the impairment of memory consolidation when CBD was injected into the DH immediately after contextual fear conditioning, 111 animals were randomly allocated to 12 groups based on the pretreatment with vehicle, AM251 (0.5 nmol), AM630 (0.1 nmol), WAY100635 (0.14 nmol), ZM241385 (10 nM), or GW9662 (32 pmol) and the treatment (VEH or 30 pmol of CBD). Pretreatment and treatment were given immediately after Context A-footshock pairing and 10 min later, respectively. The group sample sizes (n) were as follows: VEH-VEH = 9; VEH-CBD = 11; AM251-VEH = 10; AM251-CBD = 8; AM630-VEH = 9; AM630-CBD = 9; WAY-VEH = 10; WAY-CBD = 10; ZM-VEH = 7; ZM-CBD = 8; GW-VEH = 10; and GW-CBD = 10.

In Experiment 5, to investigate the receptors involved in the impairment of memory consolidation when CBD was injected into the DH 1 hr after contextual fear conditioning, 104 animals were randomly allocated to 12 groups based on the pretreatment with vehicle, AM251 (0.5 nmol), AM630 (0.1 nmol), WAY100635 (0.14 nmol), ZM241385 (10 nM), or GW9662 (32 pmol) and the treatment (VEH or 30 pmol of CBD). Pretreatment and treatment administrations were performed 1 hr after Context A-footshock pairing and 10 min later, respectively. The group sample sizes (n) were as follows: VEH-VEH = 8; VEH-CBD = 8; AM251-VEH = 8; AM251-CBD = 10; AM630-VEH = 7; AM630-CBD = 9; WAY-VEH = 8; WAY-CBD = 9; ZM-VEH = 7; ZM-CBD = 9; GW-VEH = 10; and GW-CBD = 11.

In Experiment 6A, to investigate whether URB597 given into the DH would interfere with contextual fear memory consolidation, 19 animals were randomly allocated to two groups based on the treatment VEH (n = 9) or URB597 0.1 μg (n = 10) given immediately after Context A-footshock pairing.

In Experiment 6B, to investigate whether URB597 could affect the contextual fear memory consolidation when injected into the DH 1 hr later, 19 animals were randomly allocated to two groups (VEH[n = 9] or URB597 0.1 μg [n = 10]). The drug injections were performed 1 hr after Context A-footshock pairing.

In the behavioural experiments, the assessment of treatment effects on fear memory consolidation was performed on the following day by re-exposing the animals to the conditioning Context A for 3 min in the absence of the unconditioned stimulus (Test 1). To investigate whether the effects of CBD are long-lasting, in some cases, the animals were again re-exposed to the conditioning Context A (Test 2) one week after Test 1.

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

2.5 | Evaluation of Arc expression by western blotting

The antibody-based procedures used in this study comply with the recommendations made by the *British Journal of Pharmacology*. The DH was quickly removed and stored at −80 °C. The DH from a group of naive animals was used to record the basal expression of the Arc protein. For protein extraction, the tissues were homogenized in 0.6 ml of solubilization buffer (10-mM EDTA, 100-mM Tris pH 7.5, 0.2% protease inhibitor cocktail [PROMEGA], and 1% Triton X-100). Insoluble material was removed by centrifugation (20 min, 6,613 *g*, 4 °C). The supernatant protein concentration was determined colorimetrically (Bradford Protein Assay, Bio-Rad). Tissue extracts (500 μl) were denatured in boiling water for 5 min in Laemmli buffer containing 200 mM of DTT. Protein extracts were separated by SDS-PAGE, transferred onto a nitrocellulose membrane (0.45 μm; BIO-RAD), blocked with basal solution (20-mM Tris pH 7.6, 137-mM NaCl, and 0.025% Tween® 20) containing 3% BSA (Sigma, USA) for 2 hr, and then incubated with monoclonal primary antibody anti-Arc 1:500 (Santa Cruz Biotechnology Cat# sc-17839, RRID:AB_626696) overnight and secondary antibody anti-mouse 1:5,000 (Santa Cruz Biotechnology Cat# sc-516102, RRID:AB_2687626) for 1 hr. For evaluation of protein loading, all membranes were stripped and reblotted with monoclonal primary anti-α-tubulin antibody 1:1,000 (Santa Cruz Biotechnology Cat# sc-134237, RRID:AB_2212295). The membranes were stripped because the MW of Arc and α-tubulin are 45 and 55 kDa, respectively. Therefore, separate assays were performed for each protein, and consequently, they were individually represented in the Figures 2b and 3b.

After incubation with the appropriate secondary antibody conjugated with Western ECL Substrate (Bio-Rad), membranes were developed by chemiluminescence. Quantitative analysis was performed by densitometry using Scion Image software (Scion Corporation, USA).

The intensities were normalized to corresponding values for α-tubulin expression and expressed with relative value to the basal expression (naive group expression), according to Alexander et al. (2018).

2.6 | Data and statistical analysis

The data and statistical analysis comply with the recommendations of the *British Journal of Pharmacology* on experimental design and analysis in pharmacology (Curtis et al., 2018). The results are expressed as mean ± SEM. After testing for data normality, the freezing times scored from behavioural experiments and the Arc/α-tubulin relation from western blotting were subjected to separate one-way, two-way, or repeated-measures ANOVA. When ANOVA revealed a significant interaction between independent variables, the *F* values from the main effects were omitted. The Newman–Keuls test was used for post hoc comparisons only when *F* values achieved statistical significance ($P < .05$). When there were only two groups and no context re-exposure was performed (URB597 experiment), an unpaired Student's *t* test was used. The effect size was calculated using the formula for Hedges' *g* to reflect the mean differences between the two groups ($n \leq 20$ per group) that could be dissimilar in size. A $g \geq 0.8$ was considered a large effect size (Ellis, 2010). The statistical significance level was set at $P < .05$. For statistical analysis, Statistic 12 (StatSoft, EUA) was used, and GraphPad Prism 8 (GraphPad Prism, EUA) was used for graphing.

The a priori sample size determined by power analysis was of eight animals per group ($\alpha = .05$; $\beta = .20$; 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), in a few cases, they were unequal. We have replaced the exclusions to attempt to keep the study balanced and to maintain its power.

2.7 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding et al., 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20 (Alexander et al., 2019; Alexander et al., 2019; Alexander et al., 2019).

3 | RESULTS

3.1 | CBD impairs memory consolidation and reduces Arc expression in the DH when administered immediately after contextual fear conditioning

In Experiment 1A, the one-way repeated-measures ANOVA showed significant effects of the treatment and the Context A re-exposure, but not the interaction between these factors. As shown in Figure 2a, the group treated with 30 pmol of CBD presented for less freezing times than the controls during Test 1 and Test 2, indicating a persistent memory consolidation impairment. The group treated with 10 pmol of CBD and the controls had similar values.

In Experiment 1B, the one-way ANOVA showed significant effects of the experimental group for Arc expression. As shown in Figure 2b, CBD-treated animals presented lower values than the controls which did not differ from the naive group.

3.2 | CBD also impairs memory consolidation and reduces Arc expression in the DH when administered 1 hr after contextual fear conditioning

In Experiment 2A, the one-way repeated-measures ANOVA showed significant effects of the treatment, but not the Context A re-exposure or the interaction between these factors. As shown in Figure 3a, the CBD-treated group presented for less freezing times than the controls during Test 1 and Test 2, indicating a persistent memory consolidation impairment.

In Experiment 2B, the one-way ANOVA showed significant effects of the experimental group for Arc expression. As shown in Figure 3b, the VEH group presented higher values than the naive and the CBD-treated animals. The naive and CBD groups had similar values.

3.3 | CBD no longer impairs memory consolidation when administered into the DH 3 hr after contextual fear conditioning

In Experiment 3, the one-way repeated-measures ANOVA showed no significant effects of the treatment, the Context A re-exposure or the interaction between these factors. As shown in Table 1, CBD-treated animals presented freezing times similar to the controls, during Tests 1 and 2, indicating no CBD-induced changes in memory consolidation.

3.4 | The memory consolidation impairing effects of CBD injected immediately after fear conditioning depends on the activation of CB_1 and CB_2 receptors

In Experiment 4, the two-way ANOVA showed significant effects of the interaction between pretreatment and treatment for time spent freezing during the test. As shown in Figure 4, the VEH-CBD and GW9662-CBD groups presented for less freezing times than their respective controls. In contrast, both the AM251-CBD and AM630-CBD groups had similar values relative to their respective control group. The AM251-CBD and AM630-CBD groups presented for higher freezing times than the VEH-CBD group, which had values similar to the WAY100635-CBD, ZM241385-CBD, and GW9662-CBD groups. Altogether, the impairment of memory consolidation when CBD was infused intra-DH immediately after fear conditioning was mediated mostly by activation of CB_1 and CB_2 receptors.

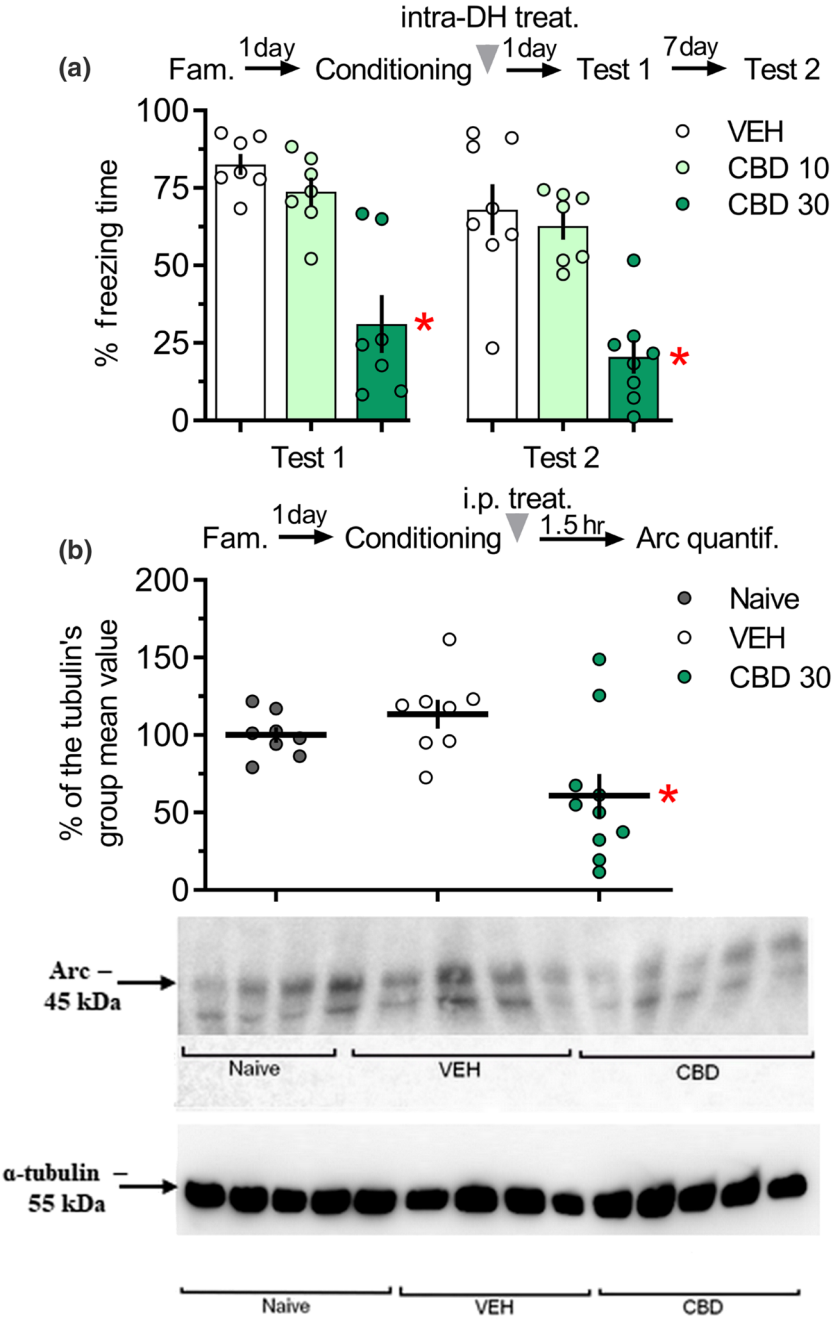

FIGURE 2 CBD infused into the DH immediately after fear conditioning impaired fear memory consolidation and the systemic administration of CBD reduced Arc expression in the DH. (a) The group that received CBD (30 pmol in 0.5 μl per side) into the DH, immediately after conditioning, presented for less freezing times than the control group, during Tests 1 and 2. *P < .05, significantly different from vehicle group in the same session. n = 7 rats in each group. (b) Ninety minutes after fear conditioning, there was a reduction in Arc expression of CBD-treated rats, compared with controls. Data are presented as individual units and mean ± SEM; naive = 8 rats per group; vehicle = 8 rats per group; CBD = 10 rats per group. *P < .05, significantly different from vehicle group. The arrowhead indicates the time of drug injection after fear conditioning. Arc, activity-regulated cytoskeleton-associated protein; CBD, cannabidiol; DH, dorsal hippocampus

3.5 | The impairment of memory consolidation by CBD injected 1 hr after fear conditioning was mediated by activation of PPARγ receptors

In Experiment 5, the two-way ANOVA showed significant effects of the interaction between pretreatment and treatment, for freezing during the test. As shown in Figure 5, the VEH-CBD group presented for less freezing times than the VEH-VEH group. The significant CBD-induced reduction in freezing time was similarly observed in animals pretreated with AM251, AM630, WAY100635, or ZM241385. In contrast, the GW9662-VEH and GW9662-CBD groups had similar values. The GW9662-CBD group presented for more freezing times than the VEH-CBD group which had values similar to the AM251-CBD, AM630-CBD, WAY100635-CBD, and ZM241385-CBD groups. Altogether, the impairment of memory consolidation when CBD was infused intra-DH, 1 hr after fear conditioning, depended mostly on PPARγ receptor activation.

3.6 | URB597 impairs memory consolidation when administered in the DH immediately after contextual fear conditioning, but not 1 hr later

In Experiment 6A, the unpaired Student's t test showed significant effects of the treatment during the test. As shown in Figure 6a, the URB597-treated group presented less freezing time than the controls, indicating impairment of memory consolidation.

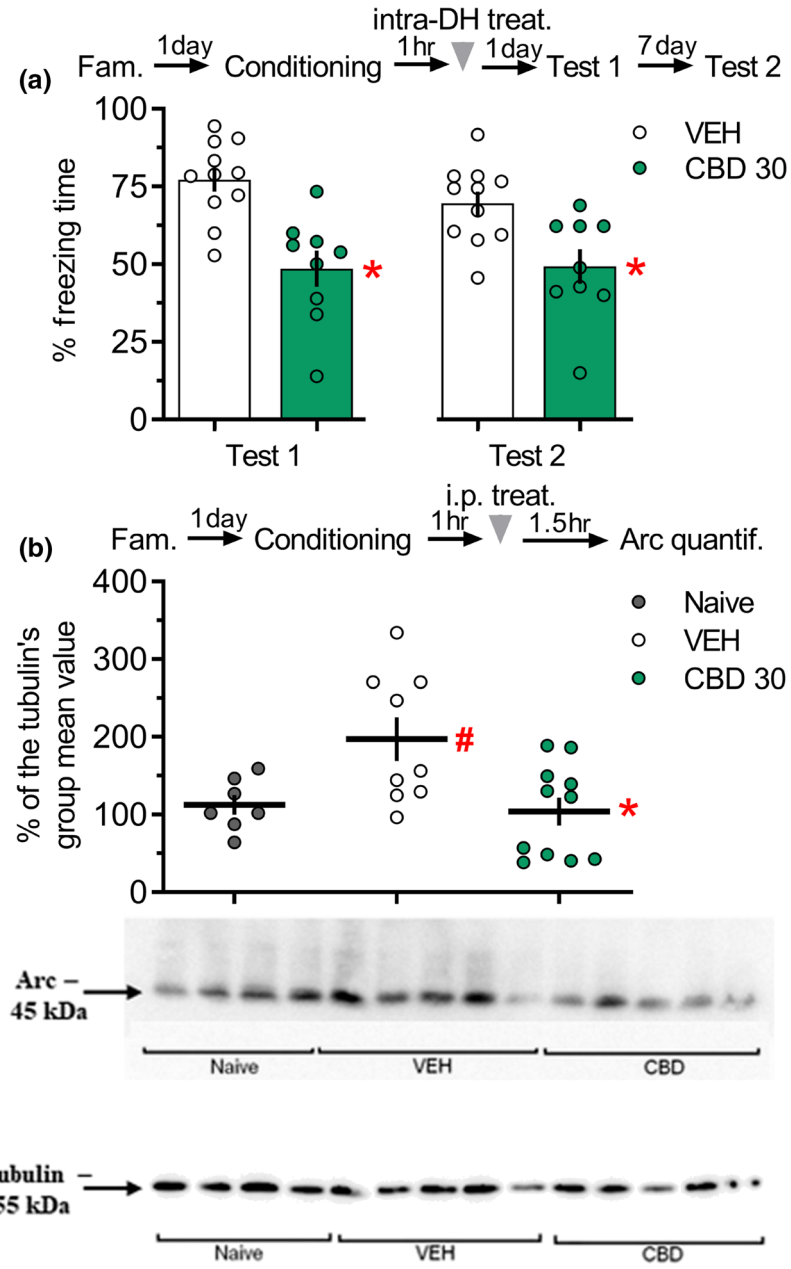

FIGURE 3 CBD infused into the DH 1 hr after fear conditioning impaired fear memory consolidation and the systemic administration of CBD reduced Arc expression in the DH. (a) The group that received CBD (30 pmol in 0.5 μl per side) into the DH, 1 hr after fear conditioning presented less freezing time than control during Tests 1 and 2. *P < .05, significantly different from vehicle group in the same session. Vehicle = 11 rats per group; CBD = 9 rats per group. (b) One hundred fifty minutes after fear conditioning, there was an increase in Arc expression of vehicle-treated rats when compared to basal. CBD reduced this expression when administered 1 hr after fear conditioning. Data are presented as individual units and mean ± SEM;.naive = 7 rats per group; vehicle = 9 rats per group; CBD = 11 rats per group. *P < .05, significantly different from vehicle group. #P < .05, significantly different from naive group (baseline). The arrowhead indicates the time of drug injection after fear conditioning. Arc, activity-regulated cytoskeleton-associated protein; CBD, cannabidiol; DH, dorsal hippocampus

TABLE 1 Infusing CBD (30 pmol) into the dorsal hippocampus 3 hr after contextual fear conditioning induced no changes in memory consolidation

Session group	Test 1	Test 2
VEH	73.3 ± 4.9	55.4 ± 7.4
CBD	66.2 ± 6.9	62.22 ± 6.0

Note. The CBD group presented freezing times similar to those in the VEH group during Tests 1 and 2 performed 1 and 7 days later, respectively. Values are expressed as mean ± SEM of the % freezing time (n = 7 per group). CBD, cannabidiol.

In Experiment 6B, the unpaired Student's t test showed no significant effects of the treatment during the test. As shown in Figure 6b, the URB597-treated group presented freezing times, similar to those in the controls, indicating no changes in memory consolidation.

4 | DISCUSSION

Infusing CBD (30 pmol) into the DH immediately after contextual fear conditioning reduced freezing times in animals exposed to the paired Context A, 1 day (Test 1) and 7 days (Test 2) later, indicating a persistent memory consolidation impairment. This result is in line with results reported after systemic administration of CBD (Stern et al., 2017). However, when infused into the prelimbic cortex immediately after contextual fear conditioning, CBD did not change memory consolidation (Rossignoli et al., 2017). Altogether, it seems that CBD acts primarily in the DH to interfere with the consolidation process at its early stage.

CBD (30 pmol) injection into the DH 1 hr after contextual fear conditioning reduced freezing during Tests 1 and 2, also indicating a persistent memory consolidation impairment. However, when given

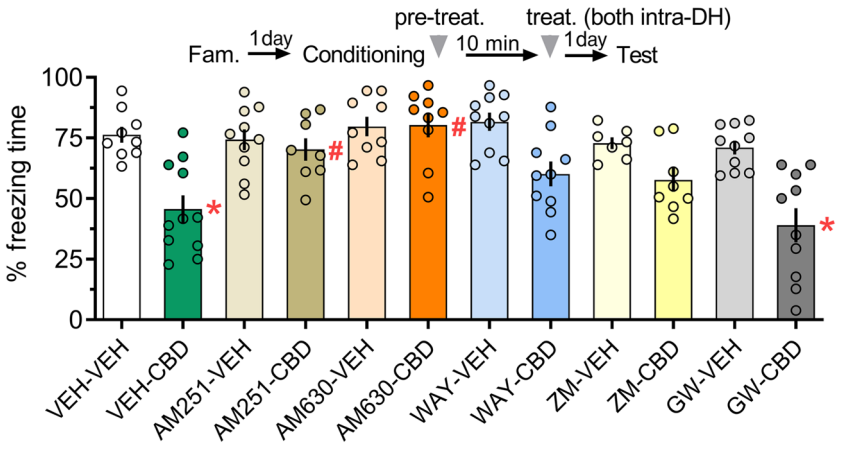

FIGURE 4 The impairment of memory consolidation induced by infusing CBD (30 pmol) into the DH immediately after fear conditioning was mediated by activation of CB_1 and CB_2 receptors. The CBD-treated group presented for less freezing times than controls during Test 1. This effect was prevented by pretreatment with the CB_1 receptor antagonist/inverse agonist AM251 (0.5 nmol) or the CB_2 receptor antagonist/inverse agonist AM630 (0.1 nmol). Pretreatment with the 5-HT_{1A} receptor antagonist WAY100635 (WAY; 0.14 nmol) or the A_{2A} receptor antagonist ZM241385 (ZM; 10 nM) partly reduced the effects of CBD. The PPARγ antagonist GW6992 (GW; 32 pmol) did not affect the CBD-induced changes. The arrowhead indicates the time of drug injection after fear conditioning. Data are presented as individual units and mean ± SEM; VEH-VEH = 9 rats per group; VEH-CBD = 11 rats per group; AM251-VEH = 10 rats per group; AM251-CBD = 8 rats per group; AM630-VEH = 9 rats per group; AM630-CBD = 9 rats per group; WAY-VEH = 10 rats per group; WAY-CBD = 10 rats per group; ZM-VEH = 7 rats per group; ZM-CBD = 8 rats per group; GW-VEH = 9 rats per group; and GW-CBD = 11 rats per group. $^{*}P < .05$, significantly different from the control group. $^{\#}P < .05$, significantly different from the VEH-CBD group. CBD, cannabidiol; DH, dorsal hippocampus

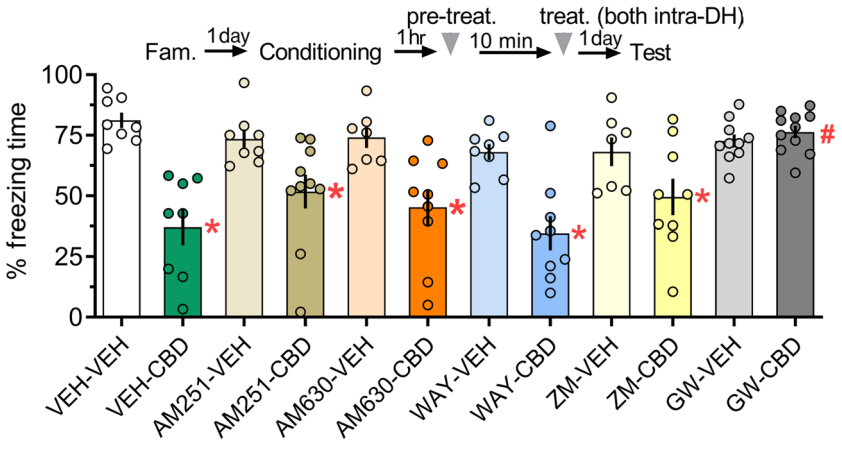

FIGURE 5 The impairment of memory consolidation induced by infusing CBD (30 pmol) into the DH 1 hr after fear conditioning was mediated by the activation of PPARγ receptors. The CBD-treated group presented shorter freezing times than controls during Test 1. This effect was prevented by pretreatment with the PPARγ antagonist GW6992 (GW; 32 pmol). Pretreatment with the CB_1 receptor antagonist/inverse agonist AM251 (0.5 nmol), the CB_2 receptor antagonist/inverse agonist AM630 (0.1 nmol), the 5-HT_{1A} receptor antagonist WAY100635 (WAY; 0.14 nmol), or the A_{2A} receptor antagonist ZM241385 (ZM; 10 nM) did not affect the CBD-induced changes in memory consolidation. The arrowhead indicates the time of drug injection 1 hr after fear conditioning. Data are presented as individual units and mean ± SEM; VEH-VEH = 8 rats per group; VEH-CBD = 8 rats per group; AM251-VEH = 8 rats per group; AM251-CBD = 10 rats per group; AM630-VEH = 7 rats per group; AM630-CBD = 9 rats per group; WAY-VEH = 8 rats per group; WAY-CBD = 9 rats per group; ZM-VEH = 7 rats per group; ZM-CBD = 9 rats per group; GW-VEH = 10 rats per group; and GW-CBD = 11 rats per group. $^{*}P < .05$, significantly different from the respective control group. $^{\#}P < .05$, significantly different from the VEH-CBD group. CBD, cannabidiol; DH, dorsal hippocampus

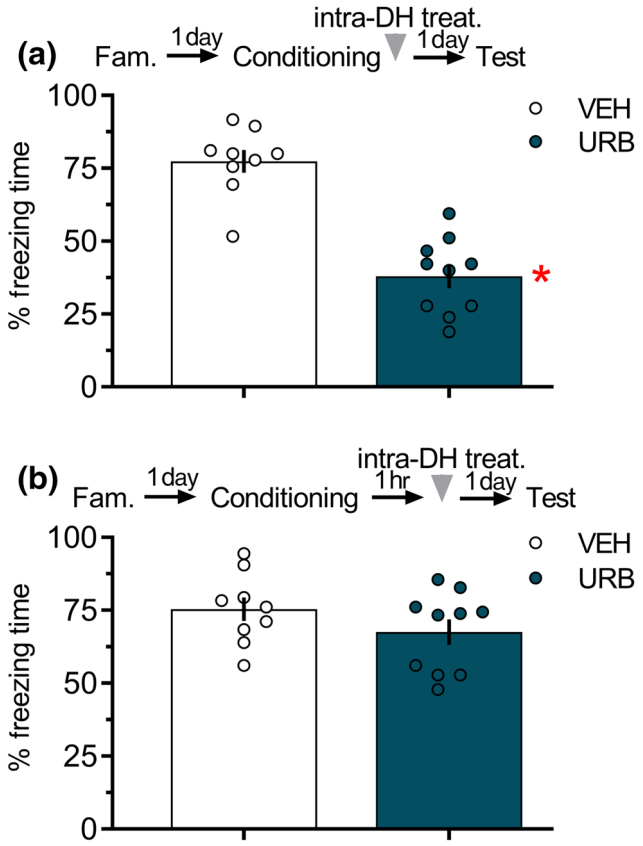

FIGURE 6 Inhibition of fatty acid amide hydrolase by URB597 infusion into the DH immediately, but not 1 hr, after fear conditioning impaired fear memory. (a) The group that received URB597 (0.1 μg in 0.5 μl per side) into the DH immediately after conditioning presented shorter freezing times than control during Test 1. The arrowhead indicates the time of drug injection after fear conditioning. Data are presented as individual units and mean ± SEM; VEH = 9 rats per group and URB = 10 rats per group. (b) The group that received URB597 (0.1 μg in 0.5 μl per side) into the DH 1 hr after conditioning did not differ from the controls. The arrowhead indicates the time of drug injection after fear conditioning. Data are presented as individual units and mean ± SEM; VEH = 9 rats per group and URB = 10 rats per group. * $P < .05$, significantly different from vehicle group in the same session. DH, dorsal hippocampus

3 hr later, the CBD no longer affected the consolidation process. Of note, the more intense the fear conditioning protocol, the narrower the consolidation time-window will be in the DH. Thus, using an intense fear conditioning protocol, the consolidation time-window would be less than 6 hr, remaining susceptible to experimental intervention for only 3 hr (Casagrande et al., 2018). A strong fear conditioning stimulus was adopted in the present work. This situation, therefore, could have shortened the consolidation time-window, explaining the lack of the effects of CBD when given 3 hr after fear conditioning. At later time intervals, other brain areas than the DH might be involved in the impairment of memory consolidation by CBD. Indeed, infusing CBD into the prelimbic cortex 5 hr after acquisition has been reported to impair the consolidation of contextual memory (Rossignoli et al., 2017).

Immediate early genes such as Arc have been studied as markers of activation and plasticity in brain areas associated with memory consolidation, including the DH (Besnard et al., 2014; Lee, Everitt, & Thomas, 2004). Systemic administration of CBD either immediately or 1 hr after contextual fear conditioning reduced the levels of Arc protein expression in the DH. These findings suggest that the effects of CBD on memory consolidation involve changes in synaptic plasticity in the DH. Corroborating this proposal, the anxiolytic-like effect of chronic treatment with CBD in stressed animals was associated with dendritic remodelling and increased neurogenesis in the hippocampus (Fogaça, Campos, Coelho, Duman, & Guimarães, 2018). However, further studies are needed to investigate if the reduced Arc expression in the DH and the decrease in contextually conditioned freezing observed after CBD administration are causally related.

To investigate how CBD impaired fear memory consolidation when given immediately and 1 hr after fear conditioning, we used selective antagonists of various receptors that have been associated with the effects of CBD (Campos et al., 2017). Pretreatment with AM251 or AM630 immediately after fear conditioning totally prevented the CBD effect on memory consolidation. These results agree with earlier findings showing that either systemic or intra-DH antagonism of **CB_1** or **CB_2** receptors prevents the systemic effects of CBD on memory generalization (Stern et al., 2017). Besides, they are in line not only with the results showing the contribution of CB_1 and/or CB_2 receptors to consolidating aversive (Shoshan & Akirav, 2017) and non-aversive memories (Clarke et al., 2008) but also with those showing that CBD-induced facilitation in extinction learning and impairment of fear memory reconsolidation involves the activation of CB_1 receptors (Bitencourt et al., 2008; Stern et al., 2012, 2017). It is worth mentioning that CBD is not a direct agonist of **CB_1** or **CB_2** receptors but can inhibit the metabolism of the endocannabinoid anandamide by the enzyme FAAH (Bisogno et al., 2001). The interference by CBD on memory consolidation reported here, therefore, could be indirectly mediated by the endogenous **CB_1** and **CB_2** receptor agonist, anandamide (Gonsiorek et al., 2000). The affinity of anandamide for CB_1 receptors is higher than for CB_2 receptors, where it acts as a partial agonist with low intrinsic activity (Gonsiorek et al., 2000). Of note, infusing the FAAH inhibitor URB597 into the DH immediately after fear conditioning also induced a memory consolidation impairment, which would support the participation of anandamide, at the early phase of this process (Busquets-Garcia et al., 2011; Stern et al., 2017).

Neither **CB_1** nor **CB_2** receptor blockade prevented CBD-induced memory consolidation disruption when the drug was administered 1 hr after fear conditioning. This result could be explained by the fact that the anandamide level reaches its peak in the DH as early as 10 min after a high-intensity shock (Morena et al., 2014). Moreover, the anandamide effects are short-lived due to rapid hydrolysis or reuptake into the synaptic terminals (Giuffrida, Beltramo, & Piomelli, 2001). In agreement with this observation, infusing URB597 into the DH 1 hr after fear conditioning no longer changed memory consolidation, suggesting that anandamide was not involved with memory consolidation at this stage.

Pretreatment with WAY100635 partly prevented the effects of CBD on memory consolidation when given immediately, but not at 1 hr, after fear conditioning. The dose of WAY100635 used in our experiments was based on reports showing that it could block the facilitatory effect on inhibitory avoidance of the 5-HT_{1A} receptor agonist **8-HO-DPAT** in rats (De Paula Soares & Zangrossi, 2004) and was above its *Ki*. However, it cannot be ruled out that a higher dose of WAY100635 would completely prevent the effects of CBD on consolidation when given immediately after fear conditioning. There is evidence of active trafficking and recycling of 5-HT_{1A} receptors in the DH after contextual fear conditioning (Sase, Stork, Lubec, & Li, 2015). However, 5-HT_{1A} receptors are believed to play a more significant role in memory acquisition (Homberg, 2012). Indeed, animals with decreased 5-HT neurotransmission present deficits in memory acquisition in the object recognition task. However, when 8-OH-DPAT or the antagonist WAY100635 was administered into the DH immediately after an auditory fear conditioning, no differences in memory consolidation were observed (Stiedl, Misane, Spiess, & Ogren, 2000). Corroborating the proposed role of 5-HT_{1A} receptors in memory acquisition, the blockade of these receptors in the nucleus accumbens prevents impairment of the acquisition of olfactory fear memory by CBD (Norris et al., 2016). Of note, CBD-induced changes in contextual fear memory reconsolidation and extinction have also not been prevented by 5-HT_{1A} antagonism (Do Monte, Souza, Bitencourt, Kroon, & Takahashi, 2013; Stern et al., 2012). In contrast, activation of 5-HT_{1A} receptors mediated the anxiolytic-like effects of CBD following acute treatment (Fogaça, Reis, Campos, & Guimarães, 2014). Intriguingly, in chronically stressed animals, the anxiolytic-like effects of CBD were not associated with 5-HT_{1A}, but rather with the **CB_1** and **CB_2** receptors (Fogaça et al., 2018). Altogether, these findings indicate that multiple mechanisms could be involved with the effects of CBD on anxiety and learning and memory processes.

Pretreatment with ZM241385 also partly prevented the effects of CBD when infused immediately, but not 1 hr, after fear conditioning. The concentration of ZM241385 we used showed neuroprotective effects in previous work (Dall'Igna et al., 2003) and was in the *Ki* range of the drug. However, it is possible that a higher concentration would have totally prevented the CBD-induced impairments in memory consolidation when given immediately after fear conditioning. CBD inhibits adenosine reuptake (Carrier, Auchampach, & Hillard, 2006), acting indirectly at adenosine receptors. There is evidence showing that the antagonism of A_{2A} receptors blocks the effects of CBD in animal models of hypoxic-ischaemia and multiple sclerosis (Castillo, Tolón, Fernández-Ruiz, Romero, & Martinez-Orgado, 2010; Mecha et al., 2013). In the object recognition task, administration of CBD, 10 min after training, impaired memory consolidation only in the presence of a CB_1 receptor antagonist, and this effect was prevented by antagonism of A_{2A} receptors (Aso, Fernández-dueñas, López-cano, Taura, & Watanabe, 2019). This finding might be explained by the existence of A_{2A} and CB_1 receptor heteromers (Ferré et al., 2010). Importantly, unlike the present study, in the work by Aso et al. (2019), all the drugs were systemically injected, and the antagonists were administered before memory acquisition. Consequently, interference with this memory phase cannot be excludedt. The partial involvement of A_{2A} receptors in the CBD-induced effects observed here, therefore, might be explained by differences in the protocols adopted and the administration routes. Moreover, the role of A_{2A} receptors in modulating fear memory seems to depend on the brain area studied. For instance, both genetic and pharmacological inhibition of A_{2A} receptors in the basolateral amygdala impaired the acquisition and retrieval of contextual fear memory in mice (Simões et al., 2016). Deletion of striatal A_{2A} receptors increased contextual and tone fear conditioning. However, when the deletion was extended to the forebrain, only the tone fear conditioning was attenuated. When hippocampal A_{2A} receptors were deleted, there was an impairment in contextual fear conditioning (Wei et al., 2014). Although these findings do suggest a role for A_{2A} receptors in fear conditioning, their relative contribution in different memory phases remain unknown. Moreover, acquisition and consolidation mechanisms might overlap at very short intervals after learning, in the object recognition test. It is not known if the same happens in fear conditioning (Akkerman, Blokland, & Prickaerts, 2015). Thus, we cannot exclude the possibility that the partial reversal of the effects of CBD, induced by 5-HT_{1A} or A_{2A} blockade immediately after fear conditioning, might be mediated by residual participation of these receptors in the acquisition.

PPARγ blockade did not induce any effect by itself and did not prevent the CBD-impairing effects on memory consolidation when administered immediately after fear conditioning. However, GW9662 totally prevented the effects of CBD on consolidation, when administered 1 hr after fear conditioning. The involvement of PPARγ receptors in the effects of CBD has been described in animal models involving neuroinflammation. CBD reduced the neuroinflammation and promoted neurogenesis in animal models of Alzheimer's disease through PPARγ activation (Esposito et al., 2011; Fernández-Ruiz et al., 2013). The impairing effect of **amyloid β1-42** on LTP in hippocampal slices was prevented by CBD infusion, an effect mediated by PPARγ, but not by CB_1, 5-HT_{1A}, or A_{2A} receptors (Hughes & Herron, 2018). Moreover, the protective effects of CBD in **haloperidol**-induced dyskinesia and LPS-stimulated microglial activation were also mediated through PPARγ receptors (Sonego et al., 2018). Of potential relevance to this discussion are the results suggesting the involvement of the immune system and microglial activation in Pavlovian fear memory acquisition, consolidation, and extinction (Adamsky et al., 2018; Young et al., 2018). Activation of PPARγ receptors promotes the inhibition of the NF-κB signalling pathway, which mediates pro-inflammatory mechanisms and the late phase of LTP (Ryan et al., 2012). Therefore, it is possible to suggest that 1 hr after fear conditioning, there is an engagement of inflammatory-related mechanisms in memory consolidation. Further, the PPARγ receptor is activated by endocannabinoids such as anandamide. Activation of membrane CB_1 receptors triggers an intracellular cascade that activates the PPARγ receptors (Pistis & O'Sullivan, 2017). Therefore, a temporal shift in the mechanism observed here might depend on the initial activation of the endocannabinoid system. However, FAAH inhibition 1 hr after fear conditioning failed to impair memory consolidation. As CBD can be transported intracellularly by fatty acid-binding proteins (Elmes et al.,

2015), it is possible that the memory effects observed when the drug was administered 1 hr after fear conditioning depend on the direct activation of PPARγ receptors. Further studies are necessary to examine this possibility.

In summary, the present results show that multiple mechanisms mediate the interference by CBD with memory consolidation in the DH. The recruitment of these mechanisms is time-dependent, involving an initial activation of CB_1 and CB_2 receptors, followed by a later engagement of PPARγ receptors. These findings bring new insights on how to mitigate fearful memories, highlighting the importance of exploiting the consolidation time-window.

AUTHOR CONTRIBUTIONS

A.M.R. and T.R.S. conducted the experiments. All authors analysed the data, elaborated on the work design, interpreted the results, and wrote the paper.

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CONFLICT OF INTEREST

All authors declare no conflict of interest.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for **Design & Analysis**, **Immunoblotting and Immunochemistry**, and **Animal Experimentation**, and as recommended by funding agencies, publishers and other organizations engaged with supporting research.

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