Neuroscience 350 (2017) 85–93

SELECTIVE POST-TRAINING TIME WINDOW FOR MEMORY CONSOLIDATION INTERFERENCE OF CANNABIDIOL INTO THE PREFRONTAL CORTEX: REDUCED DOPAMINERGIC MODULATION AND IMMEDIATE GENE EXPRESSION IN LIMBIC CIRCUITS

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Abstract—The prefrontal cortex (PFC), amygdala and hippocampus display a coordinated activity during acquisition of associative fear memories. Evidence indicates that PFC engagement in aversive memory formation does not progress linearly as previously thought. Instead, it seems to be recruited at specific time windows after memory acquisition, which has implications for the treatment of post-traumatic stress disorders. Cannabidiol (CBD), the major non-psychotomimetic phytocannabinoid of the *Cannabis sativa* plant, is known to modulate contextual fear memory acquisition in rodents. However, it is still not clear how CBD interferes with PFC-dependent processes during post-training memory consolidation. Here, we tested whether intra-PFC infusions of CBD immediately after or 5 h following contextual fear conditioning was able to interfere with memory consolidation. Neurochemical and cellular correlates of the CBD treatment were evaluated by the quantification of extracellular levels of dopamine (DA), serotonin, and their metabolites in the PFC and by measuring the cellular expression of activity-dependent transcription factors in cortical and limbic regions. Our results indicate that bilateral intra-PFC CBD infusion impaired contextual fear memory consolidation when applied 5 h after conditioning, but had no effect when applied immediately after it. This effect was associated with a reduction in DA turnover in the PFC following retrieval 5 days after training. We also observed that post-conditioning infusion of CBD reduced c-fos and zif-268 protein expression in the hippocampus, PFC, and thalamus. Our findings support that CBD interferes with contextual fear memory consolidation by reducing PFC influence on cortico-limbic circuits.

Key words: cannabidiol, contextual fear memory, medial prefrontal cortex, dopamine, C-fos.

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Abbreviations: 5-HIAA, 5-hydroxyindole-3-acetic acid; AMPA, aminohydroxymethyl isoxazole propionate receptor; ANOVA, analysis of variance; BLA, basolateral nucleus of the amygdala; CA, Ammon's horn of the hippocampus; CB_1, cannabinoid subtype 1 receptor; CB_2, cannabinoid subtype 2 receptor; CBD, cannabidiol; DA, dopamine; DG, granular layer of the dentate gyrus; DOPAC, 3,4-dihydroxyphenylacetic acid; EDTA, ethylenediaminetetraacetic acid; $GABA_A$, γ-aminobutyrate type A receptor; HPLCD-ED, high-performance liquid chromatography with electrochemical detection; MD, mediodorsal thalamic nucleus; mPFC, medial prefrontal cortex; NMDA, glutamate N-methyl-D-aspartate receptor; PBS, phosphate-buffered saline; PFA, paraformaldehyde; PFC, prefrontal cortex; PL, prelimbic area of the prefrontal cortex; PVN, paraventricular thalamic nucleus; Re, reuniens thalamic nucleus; SEM, standard error of mean; TRPV1, vanilloid subtype 1 receptor; VEH, vehicle.

http://dx.doi.org/10.1016/j.neuroscience.2017.03.019
0306-4522/

INTRODUCTION

Long-term memory consolidation of emotional events is essential for the organism survival. Impairment of its mechanisms is thought to be associated with maladaptive retrieval of traumatic events present in some psychiatric conditions, such as post-traumatic stress disorder (Parsons and Ressler, 2013). The encoding of emotional events is known to require the coordination of activity in limbic, thalamic and prefrontal cortical (PFC) circuits (Tayler and Wiltgen, 2013; Tovote et al., 2015). It has been demonstrated that the consolidation of some forms of memories can be modulated at distinct post-training time-windows (Dudai et al., 2015). For

instance, pharmacological manipulations of the PFC of rats at 3–6 h after conditioning are more effective in disrupting associative fear memory consolidation than treatments performed immediately after conditioning (Souza et al., 2000; Izquierdo et al., 2006, 2007; Gonzalez et al., 2014). A possible mechanism involves the synthesis of new proteins required for strengthening synaptic connections in fear-related circuits. In addition, microstructural changes (Sandkühler and Lee, 2013) and activity-dependent gene expression have particular time-courses during memory consolidation (Bero et al., 2014; Aceti et al., 2015). These findings suggest that post-learning "sensitive" periods are windows of opportunity during which traumatic memories can be manipulated.

Cannabidiol (CBD) is the major non-psychotomimetic phytocannabinoid component of the *Cannabis sativa* plant (Mechoulam and Hanuš, 2002). Despite its broad pharmacological action, CBD has been considered a potential therapeutic agent for treating some neurological and psychiatric disorders (Izzo et al., 2009; Devinsky et al., 2014) (Pertwee, 2008; Campos et al., 2012). CBD is known to act on multiple molecular targets and regulate dopaminergic and serotoninergic systems (Murillo-Rodríguez et al., 2011; Fogaça et al., 2014). In particular, it is known to regulate DA release in limbic structures and serotonin subtype 1A receptor (5-HT1_A)-mediated neurotransmission in PFC (Murillo-Rodríguez et al., 2011; Fogaça et al., 2014).

Behavioral pharmacology experiments have shown that CBD can modulate the acquisition and extinction of a contextual fear conditioning (Resstel et al., 2006; Bitencourt et al., 2008). Direct infusion of CBD into the PFC prior to conditioning is sufficient to disrupt associative fear memory in rats (Lemos et al., 2010; Do Monte et al., 2013). However, it remains to be elucidated whether CBD can influence PFC-dependent processing between the period of 3–6 h of consolidation phase. Here, we tested whether intra-PFC infusion of CBD immediately after or 5 h following contextual fear conditioning was able to interfere with the formation of an aversive memory. In order to explore the possible mechanisms associated with the intra-PFC CBD infusion, we also evaluated the extracellular levels of monoamines and their metabolites in the PFC and the cellular expression of activity-dependent proteins c-fos and zif-268 in relevant brain regions. We hypothesized that intra-PFC CBD infusion differentially impacts associative memory consolidation depending on the selected post-conditioning temporal window.

EXPERIMENTAL PROCEDURES

Animals

We used seventy-three adult male Wistar rats (250–400 g) housed in standard rodent cages (2–3 rats/cage). Animals were maintained at 25 ± 2 °C temperature and a 12-h light/dark cycle with lights on at 07:00 h. During all experiments, food and water were freely available. Each behavioral test was conducted during the light phase using independent experimental groups consisting of 9–19 rats per group. All procedures were performed according to the Brazilian College of Animal Experimentation (COBEA) guidelines for animal research, affiliated with the International Council for Laboratory Animal Science (ICLAS). Experiments were approved by the Ethics Commission at the University of São Paulo and performed to minimize animal suffering.

Stereotaxic surgery

Rats were anesthetized with ketamine (100 mg/kg i.p.) and xylazine (15 mg/kg i.p.) and head-fixed in a stereotaxic frame (Insight, Brazil). Body temperature was maintained at 37 ± 0.5 °C by using a heating pad, and the level of anesthesia was verified by the tail pinch reflex. In brief, the skull was exposed, cleaned and two stainless steel guide cannulae (23 gauge, length 12 mm) were implanted bilaterally 1 mm above the prelimbic region of the medial PFC (mPFC; AP = +3.0 mm; ML = ±0.5 mm; DV = −2.3 mm), according to the rat brain atlas (Paxinos and Watson, 2007). Two micro screws were inserted in the skull and fixed with dental acrylic. Behavioral experiments started three days after surgery.

Drugs

CBD (THC-Pharm, Frankfurt, Germany) was dissolved in grape seed oil for intra-PFC microinjections. For microinjections, we used a 33-G needle 1 mm longer than the guide cannula, aiming at the −3.3 mm DV coordinate of the prelimbic region. The solutions were prepared immediately before the tests and were protected from the light during the experimental session. The dose of CBD was chosen based on previous reports and on pilot studies in our laboratory showing its effect on fear memory (Bitencourt et al., 2008; Campos and Guimarães, 2008; Lemos et al., 2010; Do Monte et al., 2013).

Behavioral procedures

All behavioral procedures were performed in a conditioning chamber made of a metal floor with 18 bars (2 mm diameter; spaced 1 cm) and acrylic walls (23 × 23 × 24 cm). The floor was connected to a software-controlled scrambler shock generator (Insight, Brazil). The apparatus was cleaned with 30% ethanol and water between each trial. For contextual fear conditioning, rats were placed in the conditioning chamber for habituation followed by the conditioning session. Habituation consisted of a 4 min pre-exposure to the conditioning chamber. During conditioning, animals were exposed to five electrical footshocks (1.0 mA/2 s), 75 s apart. Immediately (0 h) or 5 h after training, animals received bilateral intra-PFC microinjections of CBD (0.2 μl/hemisphere; [CBD] = 2 μg/μl; flow = 0.1 μl//min) or VEH (0.2 μl/hemisphere; grape seed oil). Two retrieval sessions were performed: one at 24 h and another 5 days after conditioning. They consisted of re-exposing the animals to the same context where they were shocked for 8 min, but with no acoustic stimulus or footshock. Freezing

behavior was used as an index of fear memory during subsequent non-reinforced re-exposure to the context. It was defined as a behavioral arrest with immobility of the animal in a stereotyped position, except for movements necessary for breathing (Fanselow, 1976). Freezing behavior was recorded with a video camera and further quantified in blocks of 15 s by an experimenter blinded to the experimental condition.

High-performance liquid chromatography with electrochemical detection (HPLC-ED)

Dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC), serotonin (5-HT), and 5-hydroxyindole-3-acetic acid (5-HIAA) concentrations were measured in tissue samples of PFC by HPLC-ED as previously described (Lopes Aguiar et al., 2008). After all behavioral procedures, two groups of animals (VEH and CBD group) were prepared for monoamine quantification. Briefly, rats were killed by decapitation under CO_2 anesthesia and the brains were removed, immediately frozen on dry ice and stored at −70 °C. Using a cryostat at −10 °C, microdissections of the mPFC were performed bilaterally by the punch method accordingly to the rat brain atlas (Palkovits, 1973; Paxinos and Watson, 2007). The microdissections were homogenized in a solution of 0.2 M perchloric acid containing 8 ng/mL of 3,4-dihydroxybenzylamine, as internal standard. The homogenates were centrifuged for 20 min at 12,000 g. The supernatant was taken for monoamine analysis and the pellets were used for determination of protein content (Bradford, 1976). Separation was performed on a 250 × 4 mm C18 column (Purospher Star, 5 μm, Merck), preceded by a 4 × 4 mm C18 guard column. The mobile phase consisted of 100 mM sodium dihydrogen phosphate (pH 3.5), 10 mM sodium chloride, 0.1 mM EDTA, 0.20 mM sodium 1-octanesulfonic acid, and 15% methanol. The pump flow rate was 0.6 mL/min and the electrochemical potential was set to 600 mV versus *in situ* Ag/AgCl reference electrode. Chromatography data were plotted with Class-VP software (Shimadzu, Kyoto, Japan). Quantification was performed using the internal standard method based on the area under the peak. All samples from the experiment were measured in the same assay. The intra-assay coefficient of variation was less than 5% for all measured compounds. The neurotransmitter levels (DA and 5-HT) were considered to reflect neurotransmitter stocks in synaptic vesicles. DOPAC and 5-HIAA levels reflected the release of dopamine and 5-HT, respectively DOPAC/dopamine and 5-HIAA/5-HT ratios were taken as an index of neurotransmitter turnover.

Immunohistochemistry

To investigate the effects of CBD on neuronal activity in limbic–cortical sites, we evaluated the expression of the immediate-early genes c-fos and zif-268. A separate group of animals subjected to the same behavioral procedures as described were anesthetized with urethane (1.5 g/kg ip 0.15 M NaCl) two hours after microinjection intra-PFC to perfusion. Transcardial perfusion was carried out with 200 mL phosphate-buffered saline (PBS) at 25 °C, followed by 400 mL of the fixative solution consisting in paraformaldehyde (PFA) 4% in phosphate buffer (PB) with pH 7.4 at 4 °C. Brains were removed from the skull, post-fixed in PFA for 4 h/4 °C and immersed in 70% ethanol for one day, followed by paraffin embedding.

Immunohistochemistry was performed in 8-μm-thick coronal brain sections, following published protocol (Peixoto-Santos et al., 2012). In order to avoid cannula lesion and secondary tissue damage, we only used sections collected posterior to the cannula track with no signs of scars and gliosis. The sections were submitted to endogenous peroxidase block, antigenic microwave retrieval with citrate buffer (10 mM, pH 6.0), and overnight incubation with the primary polyclonal rabbit anti-c-fos (dilution 1:50 in skim milk blocking buffer, cod. sc-52, Santa Cruz Biotechnology), and anti-zif-268 (dilution 1:100, cod sc-189, Santa Cruz Biotechnology). Primary antibody visualization was performed using avidin–biotin-peroxidase complex (Vectastain Elite ABC kit, cod PK6100, Vector) and 3,3′-diaminobenzidine tetrahydrochloride as cromogen (DAB, cod 34001, Pierce Biotechnology, Waltham, Massachusetts, USA).

Micrographs from the regions of interest, delineated according to the Paxinos Atlas (Paxinos and Watson, 2007), were collected with an AxioCamMR5 attached to AxioImager M1 microscope. All images were obtained with 200× magnification under constant illumination (3 V, 60 ms exposure). The semi-quantitative analysis was performed by threshold tool with ImageJ 1.48 software (National Institutes of Health, USA), following published protocols (Kandratavicius et al., 2013; Wolf et al., 2016). Regions of interest comprised the prelimbic cortex (PL), basolateral nucleus of the amygdala (BLA), mediodorsal thalamic nucleus (MD), paraventricular thalamic nucleus (PVN), reuniens thalamic nucleus (Re), perirhinal cortex (PRh), entorhinal cortex (Ent), ectorhinal cortex (Ect), granular cell layer of hippocampus (DG), and pyramidal layer of hippocampus subfields (CA4, CA3, CA2, and CA1). Results are shown as a percentage of immunopositive area in total area evaluated.

Cannulae placement histology

All animals, except those subjected to the brain microdissection protocol, were subjected to histological examination. After the behavioral tests, all animals were decapitated under CO_2 anesthesia and were transcardiacally perfused with 100 mL of NaCl 0.15 M followed by 250 mL of 4% formaldehyde in 0.1 M PBS, pH 7.4. Brains were removed, post-fixed in the formaldehyde solution for 14 h at 4 °C and cryoprotected for 48 h in 20% sucrose solution. After freezing in dry ice-chilled isopentane, brains were cut along the coronal plane in 30 μm slices, mounted on gelatinized slides and processed for cresyl violet staining. Injection sites were determined after analysis of the slides with a bright field microscope (BX-60 Olympus, Center Vally, PA, USA). Only animals showing accurate cannula placement were included in the statistical analysis.

Statistical analysis

Behavioral data were analyzed by a two-way analysis of variance (ANOVA) for repeated measures (Two-way ANOVA RM). Post-hoc comparisons were performed using the Bonferroni test. Neurotransmitter and metabolite levels were evaluated by Student's *t*-test. Non-Gaussian distribution variables were tested using Wilcoxon Rank-Sum Test (Rank-Sum Test). All results are expressed as mean ± SEM (standard error of mean) and statistical significance defined as $p < 0.05$.

RESULTS

mPFC CBD injection 5 h after conditioning affects emotional memory formation

To evaluate whether CBD interferes with PFC-dependent processes important for emotional memory formation in a time-dependent manner, we bilaterally infused CBD intra-PFC immediately (0 h) and 5 h after training (Fig. 1A, B). As shown in Fig. 1C, the freezing rate decreased over time independently of the group [−30.4% in 24 h test, −40.6% in 5 d test; $F(2,36) = 25.024$; $p < 0.001$; Two-way ANOVA-RM]. Besides, CBD injection immediately (0 h) after conditioning did not influence consolidation of contextual fear memory measured by freezing rates at 24 h and 5 d tests [$F(1,36) = 0.278$; $p > 0.05$; Two-way ANOVA-RM). As mentioned in the Introduction section, the PFC seems to be more engaged in contextual fear memory consolidation during a window of opportunity from 3 to 6 h after conditioning (Izquierdo et al., 2007). We observed that animals submitted to CBD infusion into the PFC 5 h after training displayed lower rates of freezing behavior in the 5-d test when compared to the control group (−12%; $F(2,66) = 3.328$; $p < 0.05$; treatment-time interaction; $p < 0.001$; Bonferroni test; Fig. 1D).

mPFC CBD injection 5 h after conditioning decreases cortical dopamine release

Here, we measured DA and serotonin release following memory retrieval 5 days after training. We analyzed the monoamines and their metabolites in PFC samples using HPLC-ED fractionation (Fig. 2A). We observed that CBD significantly decreased the DOPAC/DA ratio when compared to the control group (−38%; $p < 0.05$; *t* test; Fig. 2B). 5-HT and 5-HIAA levels were not affected by CBD treatment ($p > 0.05$; Student's *t*-test; Fig. 2C).

mPFC CBD 5 h after conditioning reduces genes expression in cortico-limbic circuits

To test whether bilateral CBD injection into the PFC was able to disrupt activity-related gene expression in extra-cortical regions, we examined c-fos and zif-268 in areas relevant for memory consolidation. Perfusion and brain processing were performed two hours after intra-PFC drug administration (Fig. 3A). Immunohistochemistry analyses showed that CBD decreased c-fos-immunoreactivity in the PL of the mPFC, midline thalamic structures and hippocampal regions ($p < 0.05$; *t* test; Fig. 3B). Consistently, CBD also decreased zif-268-immunoreactivity in the midline thalamus and hippocampal structures ($p < 0.05$; Student's *t*-test; Fig. 3C).

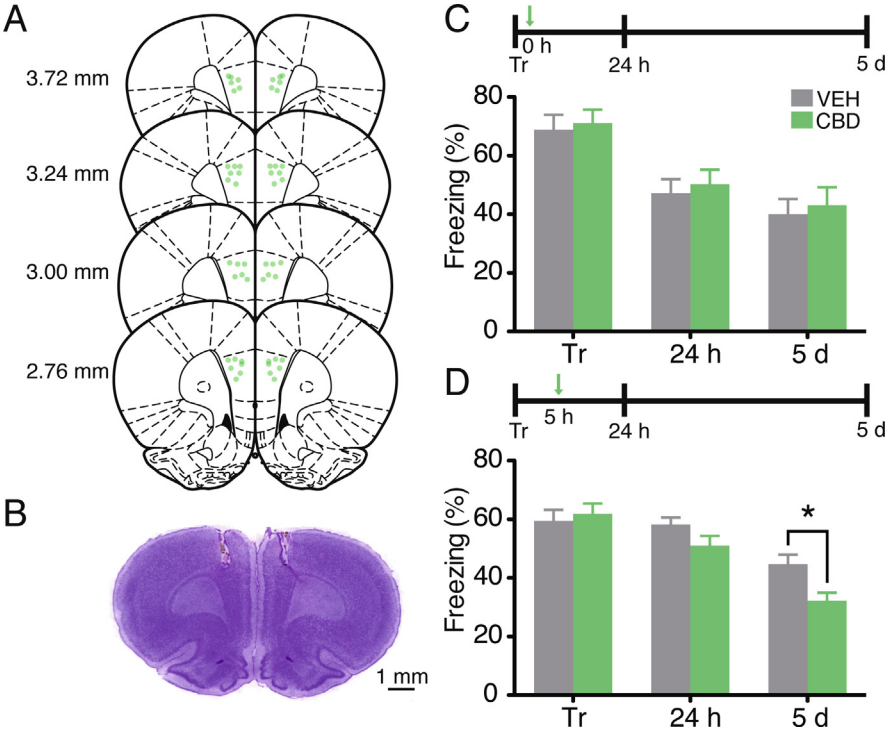

Fig. 1. Intra-PFC CBD infusion 5 h after training impairs consolidation of remote contextual aversive memory. (A) Diagrams illustrating representative injection sites. Animals were included in the analyses contingent upon correct cannula placement above the PL. (B) Typical tracts from bilateral cannula implantation, as shown by a representative cresyl-stained coronal section. (C) CBD infusion 0 h after conditioning does not change freezing on 24 h and 5-d retrieval tests ($p > 0.05$; VEH $n = 10$, CBD $n = 10$). (D) CBD infusion 5 h after conditioning reduces freezing on 5 d retrieval test (−12%, $p < 0.01$; VEC $n = 16$, CBD $n = 19$). All data are presented as mean ± SEM. $^{*}p < 0.01$ Two-way ANOVA RM, post hoc Bonferroni. Tr, conditioning.

DISCUSSION

Our findings indicate that intra-PFC CBD administration disrupts contextual fear memory consolidation when infused 5 h after training. This effect is associated with (1) decreased dopaminergic release in the PFC at retrieval 5 days after training and (2) decreased c-fos and zif-268 protein expression in the prelimbic cortex, and a subset of PFC projection targets, such as the midline thalamus and hippocampus.

Besides its role in working memory, the PFC is important for aversive learning and emotional memory expression (Rozeske et al.,

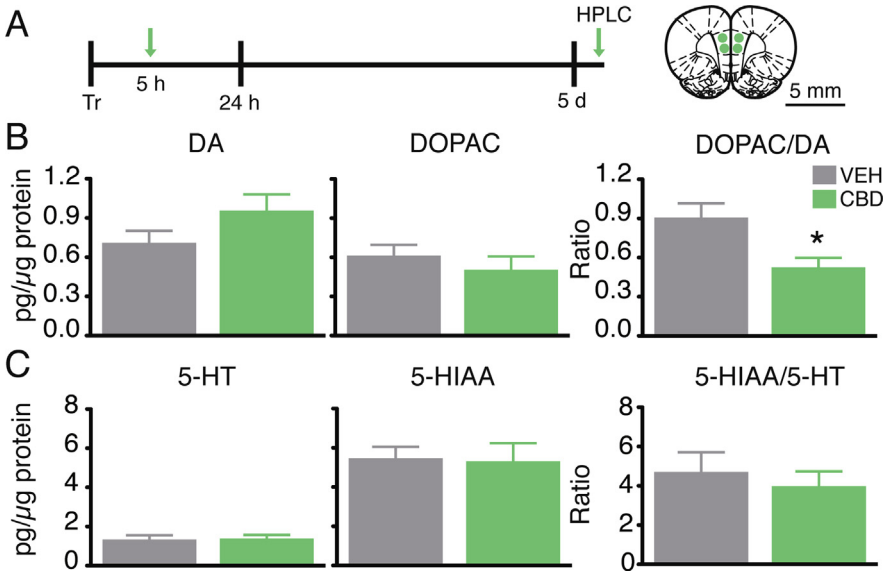

Fig. 2. Intra-PFC CBD infusion 5 h after training reduces dopamine release in mPFC. (A) Experimental paradigm for quantification of monoamines. (B) Schematic representation of the mPFC sites from which the samples were collected through micro-dissection. (C) CBD infusion 5 h after conditioning decreases DOPAC/DA ratio in the mPFC (−42%; $p < 0.05$; VEH $n = 5$, CBD $n = 6$). (D) CBD infusion 5 h after training does not change the level of 5-HT and 5-HIAA, or the 5-HIAA/5-HT ratio ($p > 0.05$; VEH $n = 5$, CBD $n = 5$). Data are shown as mean ± SEM. $^{*}p < 0.05$ compared with control, *t*-test. Tr, conditioning.

2015; Giustino and Maren, 2015). Inactivation of the prelimbic and infralimbic subfields reduces fear expression to context and memory extinction, respectively (Corcoran and Quirk, 2007; Sierra-Mercado et al., 2011). In fact, it has been postulated that memory consolidation involves a time-dependent reorganization of activity in the PFC and associated limbic structures (hippocampus, amygdala and entorhinal cortex) (Preston and Eichenbaum, 2013; Izquierdo et al., 2016). In support to this, intra-PFC infusion of dopamine D1, amino–hydroxymethyl-isoxazole propionate (AMPA) and glutamate N-methyl-D-aspartate (NMDA) receptor antagonists or infusion of γ-amino-butyrate type A ($GABA_A$) agonist were shown to interfere with aversive memory consolidation at distinct time windows. This effect was significant when applied at different time-points between 1.5 and 12 h after fear conditioning (Souza et al., 2000; Izquierdo et al., 2007; Gonzalez et al., 2014). In particular, blockade of D1 receptors in the medial PFC 6 h after training disrupts the long-term retrieval of a step-down inhibitory avoidance memory (Gonzalez et al., 2014).

Moreover, contextual fear memory consolidation can elicit early transcriptional, structural, and functional remodeling of PFC cells few hours after conditioning (Vetere et al., 2011; Bero et al., 2014). These observations are consistent with the demonstration that PFC neurons are reactivated during encoding of associative memories (Lesburguères et al., 2011). This process is thought to be driven by the hippocampus, leading to a gradual consolidation of contextual fear memories (Laroche and Davis, 2000; Restivo et al., 2009). Our findings show that intra-PFC CBD infusion at a particular time window after training (5 h) disrupts the formation of long-term emotional memory 5 days later. In accordance with its amnesic effects, it has been demonstrated that CBD infusions before training impairs long-term aversive memory acquisition (Resstel et al., 2006; Lemos et al., 2010); facilitation of aversive memory extinction before retrieval test (Bitencourt et al., 2008); and disruption of aversive memory reconsolidation immediately after retrieval test (Stern et al., 2012). Recently, it has been reported that CBD can modulate DA release and decrease the population activity of mesolimbic neurons (Ren et al., 2009; Murillo-Rodríguez et al., 2011, 2014). PFC dopaminergic neurotransmission is also essential for long-term storage of contextual fear memories (Espejo, 2003), which is supported by studies showing that reduced levels of DA release during contextual fear memory acquisition are associated with reduced freezing behavior (Pezze and Feldon, 2004; Ikegami et al., 2014). In response to fear expression, PFC neurons display periods of burst firing (Burgos-robles et al., 2007) that are associated with increased dopaminergic neuronal activity (Lodge, 2011). In addition, dopamine receptor activation in the hippocampus and PFC plays an important role in the long-term consolidation of fear memories (Izquierdo et al., 2006, 2007; Rossato et al., 2009). In fact, intra-CA1 infusion of D1 receptor antagonists 3 h or 6 h post-training has been shown to decrease step-down inhibitory avoidance latency (i.e. aversive memory expression) during memory retrieval (Bevilaqua et al., 1997). Similarly, intra-PFC infusions of D1 receptor antagonists 3 h after conditioning disrupt the consolidation of fear memory in the same task (Izquierdo et al., 2007). Consistent with these studies, our results indicate that intra-PFC infusions of CBD impaired memory consolidation and the expression of aversive memory at a retrieval session 5 days after training. Interestingly, such poor performance correlated with a decrease in dopamine release in the PFC. Although we did not measure dopamine levels following CBD administration, we hypothesize that the acute inhibitory action of CBD following training may have produced long-lasting effects on the PFC local circuitry with implications to memory performance and dopamine release, through its projections to the ventral tegmental area. In fact, the reduced neuronal activation of the prelimbic region during retrieval could be the result of a diminished feedback from brainstem dopaminergic neurons due to PFC inhibition (Karreman and Moghaddam, 1996).

Although it is well documented that CBD facilitates fear memory extinction (Bitencourt et al., 2008; Do Monte et al., 2013), its effects on a network scale, measured by activity-dependent gene expression in multiple limbic structures during aversive memory consolidation, are still unknown (Izzo et al., 2009). Here, we observed that intra-PFC CBD infusions produced similar patterns

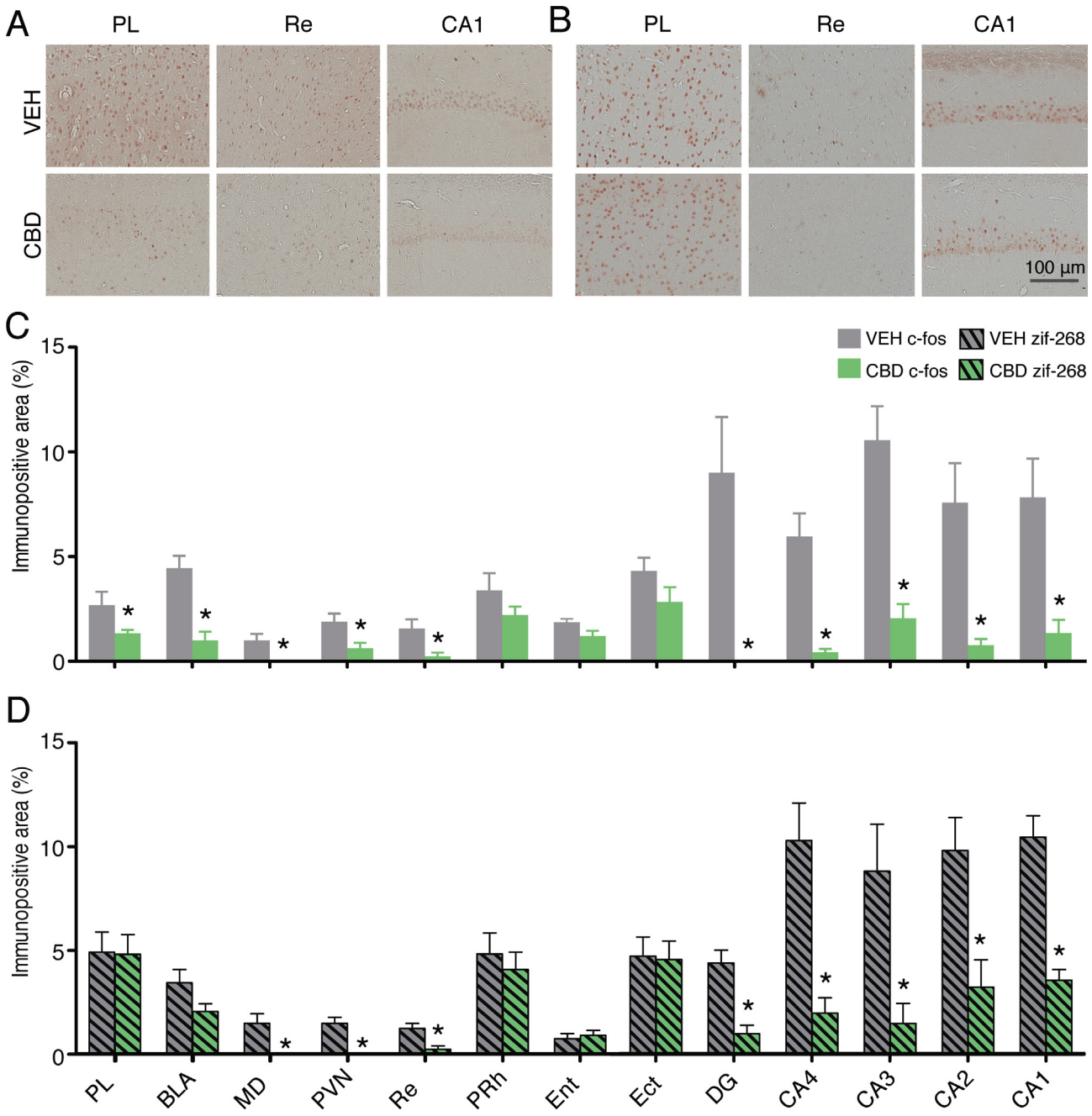

Fig. 3. Intra-PFC CBD infusion 5 h after conditioning reduces c-fos and zif-268 expression in limbic structures. (A) Experimental paradigm for immunohistochemical quantification of c-fos and zif-268. (B) CBD infusion 5 h after conditioning reduces the immunopositive area for *c-fos* on PL (−50%), BLA (−78%); MD (−100%), PVN (−70%), Re (−83%), DG (−100%), CA4 (−93%), CA3 (−81%), CA2 (−90%) and CA1 (−83%) (VEH n = 7–9, CBD n = 7–9). **(C)** CBD 5 h after conditioning reduces immunopositive area for *zif-268* on MD (−100%), PVN (−100%), Re (−80%), DG (−77%), CA4 (−81%), CA3 (−83%), CA2 (−67%) and CA1 (−66%) (VEH n = 4–7, CBD n = 4–8). Data are shown as mean ± SEM. $^{*}p < 0.05$ compared to control, Student's t-test. Tr, conditioning; PL, prelimbic cortex; MD, mediodorsal thalamic nucleus; PVN, paraventricular thalamic nucleus; Re, reuniens thalamic nucleus; PRh, perirhinal cortex; Ent, entorhinal cortex; Ect, ectorhinal cortex and DG, granular cell layer of hippocampus.

of c-fos and zif-268 gene expression in mPFC targets such as the hippocampus, midline thalamus, amygdala and rhinal cortices. Although c-fos and zif-268 represent activity-dependent immediately early genes with different activation sensitivities, their expression levels were similarly reduced in all limbic areas analyzed with the exception of rhinal (entorhinal, perirhinal and ectorhinal) cortices. In the BLA and PL, however, zif-268 levels did not change after CBD administration, possibly due to the low signal-to-noise ratio for zif-268 as a result of its high basal levels in the rodent brain. The fact that activity-dependent gene expression in the rhinal cortices was not altered after intra-PFC CBD infusion argues against a generalized inhibitory effect of CBD on PFC

targets. Selective decrease of neuronal activity induced by CBD is supported by previous *in vitro* and *in vivo* studies. *In vitro* application of CBD differentially reduces burst amplitude and frequency of local field potentials in slices of epileptic hippocampus, subfields CA1, CA3 and DG (Jones et al., 2010). Furthermore, systemic injection of CBD in rodents promotes restricted patterns of brain activation. It increases c-fos expression in the nucleus accumbens, but not in the striatum, as well as decreases c-fos expression in the mPFC (Guimarães et al., 2004; Lemos et al., 2010). An important experimental aspect of our study is that we used bilateral CBD infusions into the mPFC in a very small volume (0.2 μL/hemisphere; 2.0 μg/μL). So, we can confidently assert that its effects were mediated by its direct action on the PFC activity and not due to widespread brain diffusion of the drug.

Considering that the activity-dependent expression of zif-268 is associated with the consolidation of long-lasting memories (Frankland et al., 2004; Goshen et al., 2011), it is interesting to notice that reduced c-fos and zif-268 expression in response to intra-PFC CBD injection were not restricted to the PFC or generalized, but were found in some of the PFC target regions. This implicates the inhibition of neuronal activity in some cortical targets in the impairment of long-term aversive memory. Neuronal tracing studies have shown that the PFC reciprocally projects to the BLA (Hübner et al., 2014) and midline thalamus (Vertes and Hoover, 2008; Varela et al., 2014; Rozeske et al., 2015). Besides, the PFC can indirectly modulate hippocampus activity through its efferences to the nucleus reuniens of the midline thalamus, which directly projects to CA1 (Varela et al., 2014). CA1, in turn, sends excitatory monosynaptic projections back to the PFC (Jay and Witter, 1991; Laroche and Davis, 2000; Gabbott et al., 2002), closing the thalamus–hippocampus–prefrontal cortex loop (Cenquizca and Swanson, 2007). Interestingly, direct inactivation of nucleus reuniens is sufficient to disrupt the expression of contextual fear memory (Xu and Südhof, 2013) and, lesions in this region reduce dendritic branching in the PFC and hippocampus (Torres-Garcia et al., 2012). Therefore, we postulate that CBD modulation of PFC activity 5 h post-training interferes with contextual fear memory formation by reducing its influence on thalamo-limbic circuits.

CONCLUSIONS

In summary, the present study shows that CBD into the PFC interferes on memory consolidation in a selective post-training time window. This effect is associated with reduced dopaminergic modulation in PFC and reduced immediately-gene expression in thalamic-limbic circuits.

CONFLICTS OF INTEREST

J.E.C.H., A.W.Z., and J.A.C are co-inventors (Mechoulam R., Crippa J.A., Guimaraes F.S., Zuardi, A.W, Hallack J. E.C., Breuer A.) of the patent "Fluorinated CBD compounds, compositions and uses thereof. Pub. No.: WO/2014/108899. International Application No.: PCT/IL2014/050023"; Def. US No. Reg. 62193296; 29/07/2015; INPI em 19/08/2015 (BR1120150164927). University of São Paulo licensed it to Phytecs Pharm (Resolução USP No. 15.1.130002.1.1). University of São Paulo has an agreement with Prati-Donaduzzi (Toledo, Brazil): "Desenvolvimento de um produto farmacêutico contendo canabidiol sintético e comprovação de sua segurança e eficácia terapêutica na epilepsia, esquizofrenia, doença de Parkinson e transtornos de ansiedade". J.A.C. and J.E.C.H. received a travel support from BSPG-Pharm. All the other authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

AUTHOR CONTRIBUTIONS

Designed the experiments: MTR, CLA, RNRP.
Performed the experiments: MTR, RNR.
Contributed with data collection: RADVS, LK, JEPS, RES.
Analyzed the data: MTR, CLA, RNR, LSBJ, LK, JEPS, RNRP.
Edited the figures: MTR, CLA.
Wrote the paper: MTR, CLA, RNR, RADVS, LSBJ, LK, JEPS, JAC, JECH, AWZ, RES, JAF, JPL, RNRP.
Formatted/submitted the paper: RNRP.
Institutional support: JAC, JECH, AWZ, JAF, JPL.
All authors have approved the final manuscript.

Acknowledgments—We would like to thank Renata Caldo Scandiuzzi, Ruither de Oliveira Gomes Carolino and Antônio Renato Meirelles e Silva for their excellent technical support. We also thank THC-Pharm (Frankfurt, Germany), STI-Pharm (Brentwood, UK) and BSPG-Pharm (Sandwich, UK) for kindly supply our studies of CBD at no cost. This study was financed by the Brazilian National Council for Scientific and Technological Development (CNPq) (grants: 119628/2009-0, 476250/2013-7, 466995/2014-8, 466805/2014-4, 423977/2016-4) and São Paulo Research Foundation (FAPESP) (grant: 2012/23918-0, 2014/09772-9, 2015/20840-9, 2016/17882-4).

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(Received 5 September 2016, Accepted 15 March 2017)
(Available online 24 March 2017)