Learning and memory is modulated by cannabidiol when administered during trace fear-conditioning

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ABSTRACT

Cannabidiol (CBD) is thought to have therapeutic potential for treating psychiatric conditions that affect cognitive aspects of learning and memory, including anxiety and post-traumatic stress disorder (PTSD). Studies have shown that CBD enhances extinction of fear memory when given after conditioning. This led us to hypothesize that CBD, if administered prior to fear conditioning, might modulate cognitive learning and memory processes in additional ways that would further guide its potential use for treating PTSD. Therefore, we designed a study to investigate effects of CBD on fear learning and memory when administered to mice prior to administering a trace fear conditioning protocol which imposes cognitive demands on the learning and memory process. We show that CBD-treated animals had increased levels of freezing during conditioning, enhanced generalized fear, inhibited cue-dependent memory extinction, slightly increased levels of freezing during an auditory-cued memory test, and increased contextual fear memory. Because synaptic plasticity is the fundamental mechanism of learning and memory, we also evaluated the impact of CBD on trace conditioning-dependent dendritic spine plasticity which occurred in the dorsal lateral amygdala and CA1 region of the ventral hippocampus. We showed that CBD mildly enhanced spine densities independent of conditioning, and inhibited conditioning-dependent spine increases in the hippocampi, but not the amygdala of fear conditioned animals. Overall, the memory-modulating effects of a single pre-conditioning dose of CBD, which we show here, demonstrate the need to more fully characterize its basic effects on memory, suggest caution when using it clinically as an anxiolytic, and point to a need for more research into its potential as a therapeutic for treating memory-loss disorders.

1. Introduction

Estimated at around 10%, the world-wide lifetime prevalence of post-traumatic stress disorder (PTSD) is very high (Atwoli, Stein, Koenen, & McLaughlind, 2015). However, currently available pharmacotherapy is limited to selective serotonin reuptake inhibitors (SSRIs), tricyclic antidepressants, and Monamine Oxidase inhibitors, all of which elevate neurotransmitter levels, provide only small benefits, and can produce dangerous side effects (Hoskins et al., 2015; Jeffreys, Capehart, & Friedman, 2012). This makes discovery of new therapeutics for PTSD and other anxiety disorders very important. In an attempt to address this deficiency, prescription of medical marijuana for treating stress disorders, including PTSD, has become legalized in about half of the states in the United States of America. However, major cannabinoids in marijuana include Tetrahydrocannabinol (THC), Cannabidiol (CBD), and also an entourage of over 100 compounds which have been poorly evaluated for their neuropsychiatric value (Turna, Patterson, & Van Ameringen, 2017; Mechoulam, 2016; Aizpurua-Olaizola et al.,

2016; O'Neil et al., 2017; Rong et al., 2017). In addition, the use of THC for treating neuropsychiatric disorders is of concern due to its ability to induce psychosis (reviewed in Wilkinson, Radhakrishnan, & D'Souza, 2014), and variables in genetics, delivery systems, and dosing, make assessing positive and negative effects of medicinal marijuana difficult.

CBD, in contrast, is a major cannabinoid which is not thought to be psychotropic and exerts certain anxiolytic properties that suggest its potential as a therapeutic for treating psychiatric conditions including anxiety disorders and PTSD (Mechoulam & Hanus, 2002; Mechoulam, Peters, Murillo-Rodriguez, & Hanus, 2007; Iuvone, Esposito, De Filippis, Scuderi, & Steardo, 2009; Campos, Moreira, Gomes, Del Bel, & Guimarães, 2012; Jurkus et al., 2016). However, we need a better understanding of CBD's basic effects, as variability in animal models and study designs have given divergent results, in some cases suggesting it could have anxiogenic properties (Marinho, Vila-Verde, Fogaça, & Guimarães, 2015; Lemos, Resstel, & Guimarães, 2010). Pavlovian fear conditioning studies have demonstrated extinction memory enhancing effects of CBD when administered 24 h after contextual fear

conditioning in rats, and protective effects against THC-dependent memory-impairment in humans (Bitencourt, Pamplona, & Takahashi, 2008; Do Monte, Souza, Bitencourt, Kroon, & Takahashi, 2013; Morgan, Schafer, Freeman, & Curran, 2010; Song, Stevenson, Guimaraes, & Lee, 2016). While this suggests the potential for CBD as an agent that might augment exposure therapy when treating anxiety disorders such as PTSD, we wondered whether CBD might also enhance the formation of *de novo* fear memories if it were administered prior to memory acquisition. If true, this would suggest a counter indication for CBD when used in a setting where harmful new fear-memories could be acquired.

To test this possibility, we designed a behavioral study to evaluate the effect of CBD on fear learning and memory in mice using a single dose given just prior to conditioning. Fear conditioning is a widely used pre-clinical tool for investigating the neurobiology and pharmacology of learning and memory, anxiety, trauma-based disorders, and other psychiatric disorders including PTSD (Fendt & Fanselow, 1999; Jacobs, Cushman, & Fanselow, 2010; Campos et al., 2012). Because many such anxiety disorders have a strong cognitive component in both rodents and humans (Zoladz & Diamond, 2016; Bangasser & Kawasumi, 2015), we used a trace-conditioning protocol, which requires cognitive engagement for memory formation in both humans and rodents (Knight, Nguyen, & Bandettini, 2006; Sanders et al., 2009). With this and other forms of Pavlovian fear conditioning, a fear memory is generated by pairing a neutral conditioning stimulus (CS) with a noxious unconditional stimulus (US). Subjects rapidly learn the relationship between the two stimuli so that, 24 h after conditioning, an autonomic response to the CS-alone can be measured to index the strength of the memory. With traditional delay-conditioning, the CS and US co-terminate, and processing primarily involves the amygdala to drive the autonomic output (Raybuck & Lattal, 2011). With trace-conditioning, however, a short temporal gap is placed between the CS and US, and this increases the cognitive demands of the memory process and requires additional processing by the hippocampus and prefrontal cortex (Beylin et al., 2001). We are unaware of any published research to date that shows the effects of CBD on the acquisition and retention of memories using trace-fear conditioning to model anxiety disorders with a cognitive component.

In addition, since synaptic plasticity is essential in the mechanism of learning and memory (for review see Mapelli, Pagani, Garrido, & D'Angelo, 2015; Park, Jung, & Eun, 2014), we evaluated the effect of trace fear conditioning and CBD on dendritic spine plasticity in the CA1 region of the ventral hippocampus and dorsal lateral amygdala of brain slices from the mice used in our behavioral study (LeDoux, Cicchetti, Xagoraris, & Romanski, 1990; Maren & Fanselow, 1996; Chowdhury, Quinn, & Fanselow, 2005; Runyan, Moore, & Dash, 2004; Han et al., 2003). These brain regions are strongly involved in processing fear memories evoked by the trace-conditioning, with acquisition of trace fear-conditioning being critically dependent on the ventral hippocampus (Raybuck & Lattal, 2011; Yoon & Otto, 2007). This allowed us to investigate whether pre-acquisition administration of CBD directly affects synaptic plasticity in brain regions which support this form of memory, or whether its mechanism of action exclusively involves some other pathway (e.g. modulation of sensory receptors or other intrinsic properties of cells involved in memory processing instead of synaptic plasticity). The results of our study help answer the question of whether CBD might enhance the acquisition of new fear memories, and support the need for further research into suggested clinical paradigms involving CBD for treating disorders of learning and memory, like PTSD, anxiety disorders, and also diseases involving memory loss such as Alzheimer's disease.

2. Materials and methods

2.1. Subjects

All experimental procedures were carried out in accordance with approved Colorado State University-Pueblo Institutional Animal Care and Use Committee protocols and guidelines. Twenty day old C57BL/6 male mice (Charles River Laboratories) were housed in groups of four under a 12 h dark-light cycle and given *ad libitum* food and water. Mice were weighed 24 h after arrival and weights were distributed across experimental groups to ensure similar group averages. All mice were allowed to acclimate for at least 3.5 days prior to the start of experimentation.

2.2. Pharmacological treatment

All solutions were prepared immediately before use. Cannabidiol (CBD) was dissolved in 2% ethanol, 2% Tween-80, and 0.9% saline, and the vehicle control solution consisted of 2% ethanol, 2% Tween-80, and 0.9% saline. CBD and vehicle control solutions were administered intraperitoneally, 30 min prior to fear conditioning, with CBD at 10 mg/kg and an equivalent volume administered for vehicle controls.

2.3. Apparatus

All experiments were performed using an automated, computerized fear conditioning system (Coulbourn Instruments). The system consisted of two 8.5 × 9 × 10 in. Plexiglas chambers, each equipped with a top-mounted 60 frame per second USB camera, and a LED house light module mounted on the side wall. Foot shocks were delivered through a removable floor grid by a shocker-scrambler unit that was controlled by FreezeFrame software (Actimetrics). Auditory cues were delivered by a computer-controlled loudspeaker situated on the side of the conditioning chamber. Each chamber was placed inside a sound-attenuating isolation cubicle which was equipped with a ventilation fan that produced 60 dB white noise. The freezing response of mice was captured on digital video during both the training and testing trials and analyzed using motion detection software (Actimetrics). For more information on how the data was analyzed see Section 2.6.

2.4. Trace fear conditioning

All fear conditioning procedures were conducted in a dedicated conditioning room. Animals were transported from their home cages to the conditioning room using a transfer cage, the floor of which was covered either with wood shavings or shredded paper. The conditioning chamber was altered between two different contextual configurations depending upon whether it was used for training or memory testing. Context A consisted of a grid shock floor, a white back-wall, and the chamber was cleaned using 70% ethanol each time a new mouse was introduced to the chamber. When the chamber was in this configuration, the transfer cage was filled with wooden shavings and the mice were transported to the testing room via route one.

A different configuration, defined as context B, was used for memory testing (described below in Section 2.4.4), and consisted of a perforated stainless-steel non-shock floor which was different in appearance and texture from the shock floor, and the back of the chamber was made black in color. Also in context B, 409 Lemon Fresh Multi-Surface Cleaner was used to clean the chamber between sessions, and vanilla extract was placed in a weigh boat under the waste collection pan of the chamber to provide a unique odor. In context B, the transfer cage was filled with shredded paper and mice were transported to the testing room via route two.

2.4.1. Stimuli

The conditioning stimulus (CS) was, in all cases, an audible 85 dB, 7 kHz tone of 30 s in duration, and the unconditional stimulus (US) was, in all cases, a one second long 0.5 mA footstock. Both stimuli were computer controlled and delivered by the fear conditioning system described above.

2.4.2. Day 1: Habituation

On day one of experimentation, all mice were divided into 3 conditioning groups labeled: paired-conditioned (N = 24 mice), unpaired-conditioned (N = 24 mice), and non-conditioned (N = 24 mice). All mice, regardless of group, were individually placed in a fear conditioning chamber, configured in context A, and habituated for 20 min before being returned to their home cages. During habituation, mice in the unpaired group received seven 30 s presentations of the conditioning stimulus (CS). Non-conditioned and paired-conditioned mice did not receive tone presentations during the habituation period; however, these mice were habituated for the same duration of time as the unpaired group.

2.4.3. Day 2: Trace fear conditioning

All fear-conditioning was completed in context A, 24 h after habituation, on the second day of experimentation. Thirty minutes prior to conditioning, each of the three conditioning groups was further divided in half and mice in each half received an intraperitoneal injection of either vehicle, or CBD. This resulted in a final total of six experimental groups containing 12 animals per group. Paired-conditioning consisted of a two minute baseline period followed by seven 30 s long presentations of the CS, each paired with an unconditional stimulus (US). A trace interval of 17 s was placed between presentations of the CS and US, and the seven CS-US pairs were separated by an inter-trial interval (ITI) of 2 min. Animals in the unpaired-group received seven presentations of the US at pseudo-random intervals. Non-conditioned animals received seven presentations of the CS with a 2 min ITI. All animals were exposed to the conditioning chamber for the same overall duration, regardless of group.

2.4.4. Day 3: Memory testing

Fear memory was evaluated 24 h after conditioning, on the third day of experimentation, in context B (a description of context B is described above in Section 2.4). For the cued memory test, freezing was measured during a three minute baseline period, during three 30 s presentations of the CS which were each separated by a 60 s ITI, and during the ITI. Contextual memory was tested four hours later by returning the mice to context A for five minutes and measuring the percent time freezing over the entire period.

2.5. Delay conditioning

To serve as a control for the spine plasticity experiments, a separate set of mice were fear conditioned using a delay paradigm. These mice underwent the exact same habituation, conditioning, and testing protocols as mice that underwent the trace paradigm (described above), except that the CS and US co-terminated. Also, the ITI between the CS-US presentations was increased to 138 s to account for the trace interval.

2.6. Data analysis

The freezing response of mice was captured at 60 frames per second with a digital USB video camera mounted at the top of the chamber and was analyzed during both the training and testing trials using motion detection software (Actimetrics) to generate a motion index. The motion index was binned into 10 s intervals which were averaged during various epochs, e.g. baseline, tone, and ITI, as reported in the text. Statistical analysis was completed using one-way repeated measures ANOVA between treatments, animals, or time when appropriate. Additionally, a non-paramentric Kolmogorov-Smirnov test and general *t*-test were used as described in the text.

2.7. Spine histology

Tissue was processed using a modified Glaser and Van der Loos'

Golgi stain (Glaser & Van der Loos, 1981; Martin & Wellman, 2011). Briefly, immediately following the memory tests mice were decapitated (IACUC approved), and their brains were rapidly removed and immersed in a Golgi-Cox solution composed of four parts 5% potassium chromate, five parts 5% mercuric chloride, and five parts 5% potassium chromate. The stain was replaced three times a week. After two weeks, brains were sliced horizontally into 250 μm thick sections. Slices were sequentially incubated in 18% ammonia, Kodak Dektol, and Kodak Rapid fix and rinsed in deionized water between each incubation. Slices were then dehydrated in a graded ethanol series, cleared with xylene, and mounted with Permount. Microscope slides were coded so the experimenter was blind to the treatment group during scoring.

2.8. Spine analysis

Spiny neurons with a pyramidal cell body were chosen for analysis in both the ventral hippocampal CA1 and the dorsal lateral amygdala (LA) regions of stained brain slices. Cells were chosen based on the anatomical location of the soma, relative isolation from other stained neurons, and integrity of the dendritic arbor. Only secondary or tertiary dendritic branches were scored, and spine density was calculated in four or five segments per neuron of 10–75 μm in length. Spines were defined as clear protrusions from the dendritic branch of at least 0.3 μm in length. Four pyramidal neurons were selected for analysis per brain and six brains were used for each experimental group (n = 96–120 segments per experimental group).

Images were taken with a Leica DM1000 widefield microscope at 100X and multiple focal planes were acquired of each neuron imaged using Q-Capture Pro 7. Digital image stacks were compressed and enhanced using the compression and edge-detect features of MetaMorph Imaging software (version 7.7.8.0). Two tailed *t*-tests were used to compare spine densities as reported in the results Section 3.5.

3. Results

3.1. CBD treated animals showed an increased level of freezing during fear conditioning

Trace fear conditioning was administered to juvenile male C57Bl/6 mice using an automated fear conditioning chamber. Animals in all experimental groups were habituated by a 20 min exposure to context A. The day following habituation and 30 min prior to fear conditioning, the mice in each experimental group were injected intraperitoneally with either CBD (10 mg/kg) or vehicle. We chose this dose of CBD because it has been well-documented to be an effective dose in fear conditioning studies (Resstel, Joca, Moreira, Corrêa, & Guimarães, 2006; Song et al., 2016; Stern, Gazarini, Takahashi, Guimarães, & Bertoglio, 2012; Gazarini, Stern, Piornedo, Takahashi, & Bertoglio, 2014; ElBatsh, Assareh, Marsden, & Kendall, 2012; Jurkus et al., 2016; Lemos et al., 2010). The CS in these experiments always consisted of an auditory tone, and the US always consisted of a brief foot shock (see materials and methods for details). Experimental groups included a paired-conditioned group, in which an associative memory was induced by administering seven paired CS-US trials, where the CS and US were separated by a 17 s trace interval and each trial was separated by a 2 min inter-trial interval (ITI). An unpaired-conditioned group was also included as a necessary control for assessing the quality of the associative memory induced by the paired protocol (see Smith, Gallagher, & Stanton, 2007). In this control the mice received seven CS trials during habituation and seven US trials on the following day during conditioning. In a third, non-conditioned group, mice were presented seven CS trials to show the level of freezing not attributable to the US.

Fig. 1A shows the time courses of the average percent of time the mice spent freezing during fear conditioning for each of the training and treatment groups. As expected, freezing increased during the seven successive tones for both the paired and unpaired conditioning groups,
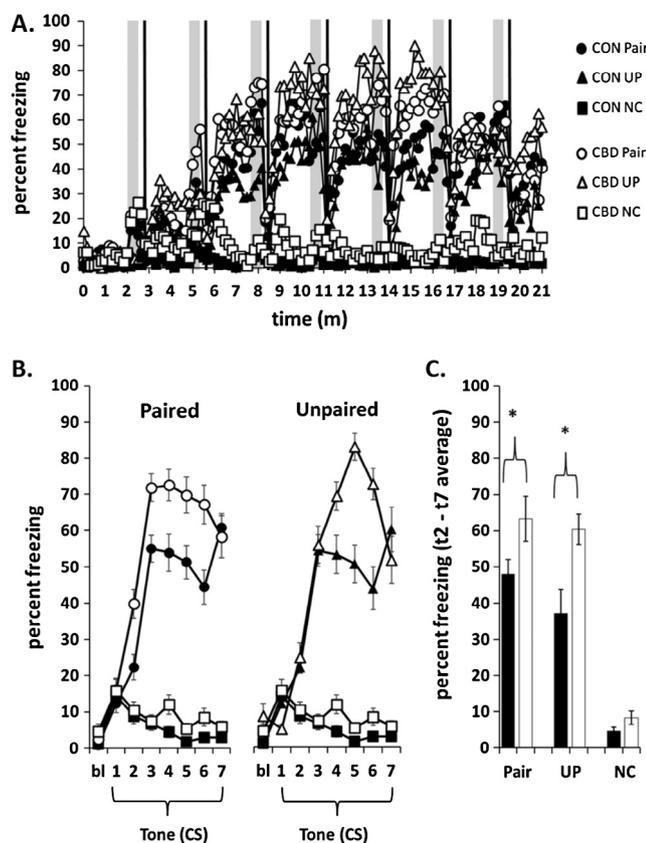
Fig. 1. CBD treated animals showed an increased level of freezing during training. A. Time course showing the average percent freezing for each group during the training sessions. Gray shading indicates the period when tones (CS) were administered (paired and non-conditioned groups only), and dark vertical lines show the timing of the shocks (US). N = 12 mice for each experimental group B. On the left is a plot showing the freezing levels averaged among animals in the paired (circles) and non-conditioned groups (squares), for each of the treatments (vehicle = black, CBD = white). On the right is a plot showing the freezing levels averaged among animals in the unpaired (triangles) and non-conditioned groups (squares), for each of the treatments (vehicle = black, CBD = white). C. Graphs showing the percent freezing averaged across CS presentations two through seven for each conditioning group (black = vehicle and white = CBD). Brackets indicate the statistical comparisons reported in the text.

with animals being very ambulatory during the presentation of each US and freezing more during the ITIs. Non-conditioned animals, on the other hand, showed very low levels of freezing throughout the training session.

In general, it appeared that CBD-treated animals in each of the three conditioning groups froze more than did vehicle controls. To evaluate this more clearly, we averaged the percent freezing for each treatment group over specific periods, including the baseline and each of the seven CS presentations, and showed the averages in separate plots for the paired and unpaired conditioning groups. The freezing responses of non-conditioned animals were also included on these plots for comparison (Fig. 1B). In both conditioning groups, increased freezing was apparent with CBD after the baseline and first CS. Therefore, we took the average percent freezing combined across tones 2 through 7 (tone one period was not included since the US had not been presented prior to it), and compared the effects of CBD to vehicle treatment within each conditioning group statistically (Fig. 1C). A general *t*-test revealed that the average percent freezing for the paired group was increased by CBD (p = 0.05). Also, the *t*-test revealed that freezing in the unpaired conditioning group was increased (p = 0.007). No significant difference was found between the non-conditioned control and CBD-treated groups with overall freezing levels below 9% in each group. Thus, CBD treated animals were more responsive to the US, since they froze significantly more than vehicle-treated controls during conditioning.
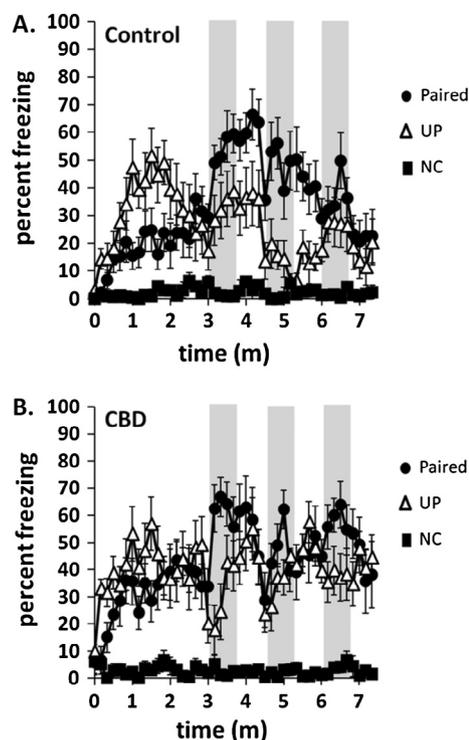

Fig. 2. Trace fear-conditioning produced elevated freezing in both vehicle and CBD treatment groups. Time course of the average percent freezing ± sem during the memory test for each training group exposed to vehicle (A) or CBD (B). In both panels, presentations of the CS are indicated by gray shading. Black circles show results with paired conditioning, white triangles show results with unpaired conditioning (UP), and black squares show results from non-conditioned animals (NC). N = 12 mice for each of the six experimental groups.

3.2. Trace-conditioning induced an associative fear memory in male mice

Twenty-four hours after conditioning, all mice underwent memory testing using a modified conditioning chamber configured for context B. In the memory test, the CS was presented three times with a 60 s ITI, and the level of freezing was measured and recorded using automated motion detection software to provide an index of memory. Mice trained using the paired and unpaired conditioning protocols showed elevated levels of baseline and tone-dependent freezing. The average freezing level across the entire memory test for the vehicle-treated paired-conditioned group was 33.6 ± 2.6% compared to 26.4 ± 1.8% for the vehicle-treated unpaired-conditioned group (Fig. 2A). The freezing level for the CBD-treated paired-conditioned group was 42.6 ± 2.2% and 39.7 ± 1.5% for the CBD-treated unpaired-conditioned group (Fig. 2B).

To further evaluate the associative memory induced by conditioning, we compared results between the paired and unpaired conditioning groups within each treatment group using a one-way between subjects repeated measures ANOVA. There was a significant effect of pairing on freezing for controls, $[F(1, 11) = 3.22, p = 0.03]$ (Fig. 2A), and CBD-treated animals $[F(1, 11) = 2.77, p = 0.05]$ (Fig. 2B). In contrast, non-conditioned animals showed very low levels of freezing throughout the memory test, revealing that freezing was mostly dependent upon the US, with all other context variables producing only a very small response. Overall, these results indicate that trace fear-conditioning was effective in producing a CS-US associative memory in both treatment groups. Therefore, we went on to evaluate the effects of CBD on this memory.

3.3. Generalized fear and resistance to extinction of auditory-cued memory were increased by CBD

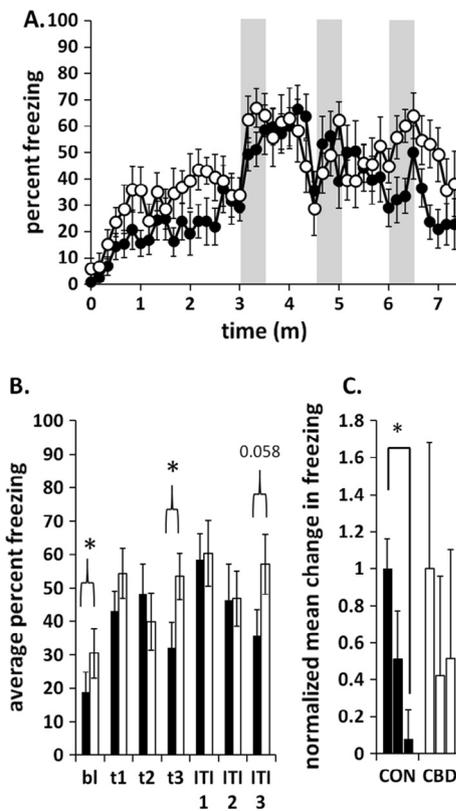During a memory test to evaluate the fear memory produced by

Fig. 3. Animals treated with CBD just prior to paired conditioning had modulated fear memory. A. Time course of the average percent freezing ± sem during the memory test for vehicle (closed circles, n = 12) and CBD-treated animals (open circles, n = 12) that had received paired conditioning. Presentations of the CS are indicated by gray shading. B. Bar graph showing the average percent freezing during specific epochs of the memory test in panel A, for vehicle (black bars), and CBD-treated animals (white bars). Total baseline freezing is indicated by bl, t1–t3 indicate freezing during the presentation of tones, ITI1–ITI3 indicate freezing during the 30 s following each tone. Brackets indicate the statistical comparisons reported in the text. C. Graph showing the normalized mean change in freezing during the 30 s period following each of the three tones of the memory test. The bracket indicates the statistical comparison between the first and third tone reported in the text.

trace-conditioning, freezing behavior among trace-conditioned mice in both treatment groups increased throughout a three minute baseline period (Fig. 3A). One-way repeated measures ANOVA comparing freezing levels of CBD to vehicle-treated mice averaged over the entire baseline showed that CBD treated mice froze significantly more during this period [F(1, 11) = 11.5, p = 0.006] (Fig. 3B). The increased freezing among CBD-treated mice during the baseline period in a novel context suggested that CBD-treatment increased the expression of generalized fear.

Following the baseline period, the CS was presented three times to evaluate the tone-associated memory produced by trace-conditioning. With both treatment groups freezing rose sharply during the presentation of each tone. When freezing during the three tone intervals was combined and averaged, CBD-treated mice froze 49 ± 8% and vehicle controls froze 41 ± 7% of the time, however, the difference was not statistically significant. Freezing levels for both groups also remained high during the initial period following the tone-offset, and then decreased in the second half of the ITI. This pattern during the tone-offset would be predicted for mice anticipating the US after a trace interval, therefore, we evaluated freezing levels only during the first half of the ITIs. When averaged across these three periods, freezing also trended higher with CBD treatment, but again the difference was not statistically significant (CBD 55 ± 9% and control 47 ± 9%). To evaluate these results more closely, we went on to compare freezing levels between treatment groups for each tone and ITI individually. In this

analysis, the means were higher with CBD treatment for all epochs except during the second tone, in which CBD treated animals froze less that controls. A statistically significant difference between treatment groups was found only during the presentation of the third tone [F(1, 11) = 11.5, p = 0.004], and, we noted a strong trend to increased freezing with CBD during the first half of the third ITI [F(1, 11) = 11.5, p = 0.058] (Fig. 3B). Overall, these results show CBD produced a mild trend to increased freezing during the presentation of tones and during the first half of the ITIs.

Because individual animals sometimes froze less during successive tone presentations, we were also interested in characterizing differences between treatment groups in any extinction learning that might have occurred during the memory test. Therefore, we computed the normalized mean change in freezing as previously described (Huerta, Sun, Wilson, & Tonegawa, 2000). To normalize the data, we calculated the average freezing level for each of the three tones for each group, and the freezing level for each animal in their respective groups was taken as a percentage of the group average. This calculation allowed us to compare changes in freezing during progressive presentations of tones across treatment groups, and revealed a strong extinction profile by the third tone in vehicle-treated controls. In contrast, CBD-treated animals had no clear trend toward reduced freezing with successive tones. A non-parametric Kolmogorov-Smirnov test comparing the first and third tones gave p = 0.009 in controls and p = 0.1 in CBD treated mice (Fig. 3C). This result suggests that, compared to vehicle-treated controls, the memory in CBD-treated animals was more resistant to extinction.

3.4. Contextual freezing was enhanced in CBD treated mice

The enhanced freezing we observed with control and CBD-treated animals during the baseline period of the cued memory test (Fig. 3) is a measure of the generalized fear response to unavoidable contextual cues in fear conditioning (Radulovic, Kammermeier, & Spiess, 1998; Huerta et al., 2000). Since CBD treated animals froze more during the baseline in context B, we were interested in comparing contextual freezing between treatment groups when the animals were placed in the original training chamber configured for context A. Therefore, 4 h after the initial cued memory test, mice were returned to context A and freezing was recorded over a five minute period. In general, CBD-treated animals appeared to freeze more than vehicle controls (Fig. 4A); however, the freezing level of mice in both groups declined after the
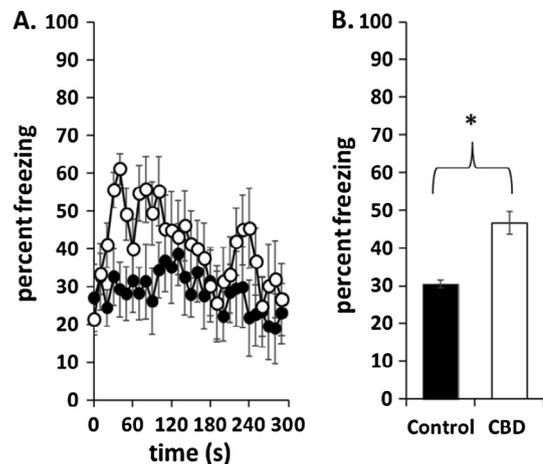

Fig. 4. Context-dependent freezing was enhanced by CBD. A. Time course showing the average percent freezing ± sem in context A over a 5 min period. The average percent time freezing among animals in the vehicle treated groups are shown as closed symbols, with results from CBD treated groups shown as open symbols. B. Bar graphs showing the average percent freezing for vehicle (black) and CBD treated animals (white). The bracket indicates the statistical comparisons reported in the text. N = 12 mice per group.

first few minutes to a common point by the end of the test. This suggested that extinction may have occurred. Therefore, we averaged the percent freezing across only the first 120 s of the contextual memory test and compared treatment groups. One-way repeated measures ANOVA showed a statistically significant difference between vehicle- and CBD-treated animals with [F(1, 11) = 5.1, p = 0.044] when animals were taken as the repeated variable, and [F(1, 12) = 50.5, p < 0.001] when time was the repeated variable (Fig. 4B). Taken together, and consistent with previous findings (ElBatsh et al., 2012), these results show that contextual memory was enhanced with CBD. Combined, the context A and B memory testing data supports the conclusions that acute administration of CBD prior to conditioning did not disrupt the CS-US associative memory, increased generalized and context fear, made an associative memory that was harder to extinguish, and produced a mild trend to enhanced freezing during the presentation of tones and intertrial intervals.

3.5. Synaptic plasticity in the amygdala and hippocampus was slightly altered by CBD

Because of the importance of synaptic plasticity as a fundamental mechanism that supports learning and memory, we next evaluated changes in the density of dendritic spines in the dorsal lateral amygdala and CA1 region of the ventral hippocampi of the trace-fear conditioned mice from our studies. These brain regions are particularly important for processing fear memories that are evoked by trace-conditioning (Raybuck & Lattal, 2011). Spine density measurements have been used to show effects of conditioning and treatments on synaptic plasticity in anatomically-defined brain regions, and how plasticity in the regions correlate with behavioral outcomes (Moser, Trommald, & Andersen, 1994; Misane et al., 2005; Raybuck & Lattal, 2011). In these studies, for the trace-conditioned and non-conditioned groups, we decapitated the mice immediately following memory testing, rapidly extracted their brains, and stained neurons *in situ* using a modified Golgi staining method (Glaser & Van der Loos, 1981). Digital images of brain slices showing the stained neurons in the hippocampus and amygdala were further processed to enhance the appearance of dendritic spines, which were then quantified in secondary and tertiary dendritic branches of spiny pyramidal cells as shown in Fig. 5A and B (see methods 2.7 and 2.8).

Spine densities of non-conditioned vehicle-controls were statistically significantly lower than those in trace fear-conditioned vehicle-control animals with two tailed t-tests in both the lateral amygdala (p = 0.03) and hippocampus (p < 0.001) (Fig. 5C and D). In contrast, spine density was increased only in the lateral amygdala (p < 0.001), but not the hippocampus (p = 0.87), of animals that received delay fear conditioning as a control (data not shown). This is consistent with previous studies establishing a role for amygdala and hippocampal plasticity in fear-learning evoked by trace-conditioning (McEchron, Bouwmeester, Tseng, Weiss, & Disterhoft, 1998; Misane et al., 2005), and others showing that the amygdala alone is sufficient to support delay conditioning (Raybuck & Lattal, 2011). Therefore, this control confirmed the anatomical specificity of our spine density measurements. Similar to controls, spine density was increased in the lateral amygdala and hippocampi of CBD-treated mice undergoing trace conditioning, however, statistical significance was only achieved in the lateral amygdala (p = 0.004) and not the hippocampus (p = 0.194) of the CBD-treated mice. Also, the amygdala of conditioned animals that had received CBD had somewhat higher spine densities than vehicle-treated conditioned animals, however, this trend was reversed in the hippocampus. Interestingly, there was also a trend toward increased spine densities when comparing CBD to vehicle-treated animals in the non-conditioned groups for both the amygdala and hippocampus, suggesting that CBD alone might exert small effects on spine density. We calculated that this CBD-dependent increase, on average, added one spine per 20 μm of dendritic length in the lateral amygdala and smaller
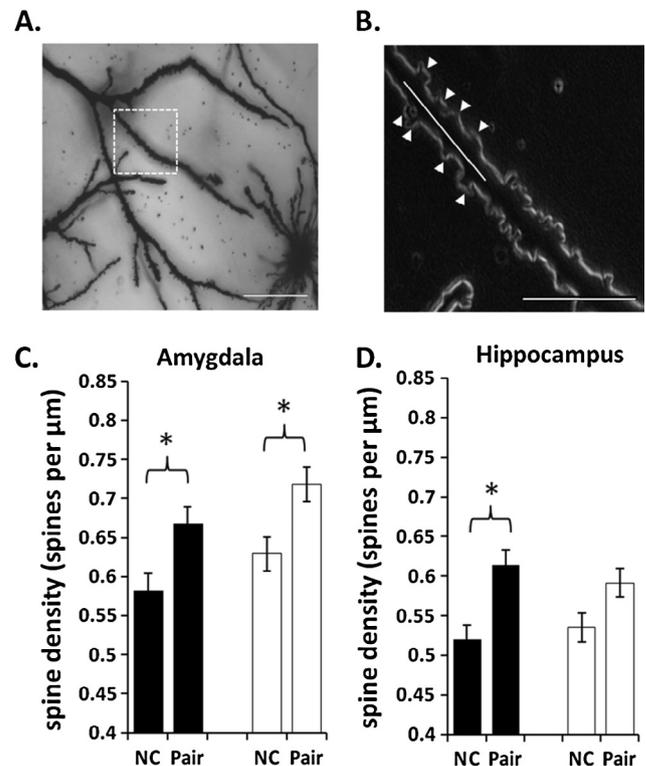

Fig. 5. Fear conditioning-dependent changes in dendritic spine densities were modulated by CBD. A. Example image showing segments in the dendritic tree of a Golgi stained hippocampal neuron. The boxed area is enlarged in panel B. Scale bar = 30 μm, B. Processed high power image of the boxed dendritic segment highlighted in A, with arrows showing examples of spines that were counted in a 10 μm segment (line). Scale bar = 10 μm. C. Average spine densities ± sem in the lateral amygdala. Brackets indicate the statistical comparisons reported in the text. D. Average spine densities ± sem in the hippocampal CA1 field. Brackets indicate the statistical comparisons reported in the text. For both C and D, black bars show results from vehicle-treated controls and white bars show results from CBD-treated animals, NC = non-conditioned and pair = paired conditioning groups.

numbers to hippocampal cells. Overall, these results show CBD produced subtle increases in spine densities which were independent of trace fear conditioning, and differential effects on conditioning-dependent plasticity in the hippocampus and amygdala, with plasticity in the hippocampus being reduced in CBD treated animals.

4. Discussion and conclusions

We used trace fear-conditioning because of its strong cognitive requirement which makes it an excellent model for disorders of learning and memory, such as PTSD, which involve cognitive deficits in learning and memory (Fendt & Fanselow, 1999; Jacobs et al., 2010; Campos et al., 2012; Orsini & Maren, 2012; Beylin et al., 2001). As a first major finding, we showed a significantly enhanced freezing response in CBD-treated mice during conditioning, when the drug was present systemically. This result suggests that CBD somehow increased the perceived shock intensity and/or downstream processing in the central nervous system to drive enhanced autonomically-driven freezing. The mechanism for this would necessarily involve either a CBD-dependent enhancement of sensory neuron function, modified sensory processing in the central nervous system, or changes to motor output. While evaluating whether CBD directly targeted sensory receptor cells was beyond the scope of the study, our results do not rule out the possibility. Interestingly, CBD has been shown to modulate the activity of cultured sensory neurons by activating transient receptor potential channels (Qin et al., 2008). Thus, as a potential mechanism of action in our studies, CBD could have conceivably affected sensory neurons

ultimately leading to modulated inputs to brain regions such as the hippocampus and amygdala which process autonomic motor output. On the other hand, our dendritic spine data clearly demonstrated that CBD at some point produced measurable effects on synaptic plasticity in the hippocampus and amygdala, both of which are major brain centers for processing auditory and nociceptive sensory information during trace conditioning (see Raybuck & Lattal, 2011). Thus, CBD could conceivably have directly targeted these regions to affect the central processing required to drive the autonomic freezing response during training. The enhanced freezing could also suggest CBD had a paralytic or sedative effect, however, treated mice were more responsive to the US during both paired and unpaired training, while non-conditioned mice showed similar low levels of freezing regardless of treatment group. This clearly showed that CBD did not act by simply targeting general motor function, and that it was not acting as a sedative.

As a second major finding, the enhanced baseline freezing among CBD-treated animals in the non-conditioned context B during memory testing, 24 h after conditioning, shows that CBD produced an enhanced level of generalized fear. Increased generalization of fear is a symptom of anxiety disorders (Ghosh & Chattarji, 2015). This might also suggest that CBD disrupted memory specificity rather than directly targeting the associative memory, which showed a mild trend to increased freezing with CBD-treatment in our studies (Fig. 3A and B). The greater resistance to cue-dependent memory extinction is also very important, since enhanced extinction with CBD, not resistance to it, would be the goal of combining CBD with exposure therapy. A number of studies have suggested this application for CBD by showing enhanced extinction memory when CBD was administered just prior to extinction training, 24 after the initial memory was acquired (Das et al., 2013; Bitencourt et al., 2008; Do Monte et al., 2013; Song et al., 2016). Ours, on the other hand, is the first study to investigate the impact of CBD when given prior to trace fear-conditioning. Since memory testing was conducted after the period required for CBD to be mostly metabolized (Deiana et al., 2012), direct effects of CBD on the memory recall process were likely excluded from the mechanism, and instead CBD probably acted during the memory acquisition and/or earlier consolidation phases. Therefore, these results combined suggest CBD could potentially be anxiogenic for anxiety disorders like PTSD, and counterproductive for augmenting exposure therapy, if given in a context where new fear memories might be formed.

Third, the elevated freezing during the 2 min baseline in context A with CBD suggests a genuine increase in context-evoked fear using our conditioning and drug-administration protocols. To date, the majority of fear conditioning studies with CBD have focused on contextual and extinction learning and memory, and have shown divergent effects depending on the animal strain, injection route, dose timing, conditioning protocol, and concentration (Jurkus et al., 2016). For instance, systemic CBD may enhance extinction of contextual fear memories, may block reconsolidation, (Stern et al., 2012), and has opposite effects on contextual fear memory depending upon the location of microinjection and strength of conditioning (Lemos et al., 2010; Song et al., 2016). There are only two studies where CBD was administered before acquisition of contextual fear memory, both conducted with rats (ElBatsh et al., 2012; Levin et al., 2012). Interestingly, CBD was anxiogenic in one study and anxiolytic in the other, with major differences being the duration of treatment and strain of rat used. Our experimental approach is most similar to Levin et al., however, the results were contradictory, possibly due to differences in type and age of rodent, and in conditioning paradigms (i.e. trace vs contextual conditioning). Therefore, more research is needed to more fully characterize the effects of CBD on various specific aspects of learning and memory before it can efficaciously be used in the clinic for disorders such as PTSD, since, given the constellation of fear-memory enhancing effects that we noted in our study, it would appear that CBD could potentially increase the formation of harmful new fear memories.

The final finding of our study is that CBD mostly had mild, statistically insignificant effects on the changes in dendritic spine densities which accompany fear learning in pyramidal neurons of the dorsal lateral amygdala and ventral hippocampal CA1 regions. Therefore, synaptic plasticity did not at first glance appear to be overtly impacted at the gross histological level by CBD. However, the statically significant increase in spine density in the hippocampus that accompanied fear conditioning among controls was not present with CBD; thus, the drug in fact did have an important impact on conditioning-dependent synaptic plasticity in this brain region which was statistically demonstrable. It is important to consider the possibility that the spine changes accompanying conditioning may have been due to shock exposure alone, and not the associative learning shown by the behavioral component of this study. We did not include an unpaired group in our spine analysis in an attempt to resolve this. Rather, we were simply interested in evaluating whether there were CBD-dependent changes in the morphological plasticity that accompanied trace-conditioning. Interestingly, increases in context-dependent memory such as we observed with CBD are often thought to involve increased hippocampal function (Jacobs, et al., 2010; Anagnostaras, Gale, & Fanselow, 2001; Chen, Kim, Thompson, & Tonegawa, 1996); however, our observed decrease in conditioning-dependent plasticity with CBD in this region suggests a more complex mechanism. It is tempting to speculate, because spine densities were somewhat increased in the amygdala of CBD-treated and conditioned mice but simultaneously decreased in the hippocampus, that the drug may have worked to increase the role of the amygdala, relative to the hippocampus. Such a shift away from hippocampal and toward the amygdalar-dependent processing has been previously characterized to represent a shift to a more reflexive, less cognitive, memory which can be triggered by changes in mineralocorticoid signaling (Vogel et al., 2015). Interestingly, CBD can interfere with cortisol secretion in humans, suggesting a direction for future studies (Zuardi, Guimarães, & Moreira, 1993). It is also interesting to consider the possibility that the small increase in spine densities that we measured with CBD among non-conditioned animals might also be meaningful, since (assuming 1000 synapses per pyramidal cell) the addition of a single spine per 20 μm of dendrite would represent up to an 8% increase in synaptic count among pyramidal cells in the amygdala, and up to a 3% increase in the hippocampal neurons we measured. This could be functionally important if it relates to a change in circuitry that underlies the processing and storage of fear learning and memory. This interesting possibility could be better elucidated by detailed molecular-level histology and electrophysiological analysis in the future. To the best of our knowledge this is the first report demonstrating that as part of its mechanism of action, CBD can directly affect synaptic plasticity in the brain regions that support memory formation following trace-fear conditioning.

In conclusion, our combined context A and B memory testing data support that acute administration of CBD prior to trace fear-conditioning enhances generalized and context fear, and makes an associative memory that is harder to extinguish. This is congruent with previously reported extinction memory-enhancing effects of CBD, and extends those findings to include effects on the acquisition of new fear memories which involve cognitive processing by the hippocampus and amygdala following trace conditioning. This suggests a potential contraindication for CBD as a therapeutic for anxiety disorders if given in an inappropriate clinical context, and also suggests a need to better evaluate the possible benefit of CBD for treating memory-loss disorders presenting cognitive memory deficits, such as Alzheimer's disease (AD). This latter idea is supported in reports showing improvements with CBD in a social object recognition deficit present in an AD transgenic mouse (Cheng, Low, Logge, Garner, & Karl, 2014; Cheng, Spiro, Jenner, Garner, & Karl, 2014). Future studies to better evaluate the effect of CBD on sensory neuron function, its interaction with stress hormone signaling during memory formation in the brain, and a closer evaluation of how CBD affects synaptic plasticity are also suggested by our results. Additionally, studies to evaluate effects of CBD on memory

acquisition using study designs that more closely model human chronic use patterns of CBD use, and studies including females are needed, especially given that PTSD is more prevalent in women (Glover, Jovanovic, & Norrholm, 2015; Jasnow, Schulkin, & Pfaff, 2006; Wiltgen, Sanders, Behne, & Fanselow, 2001).

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