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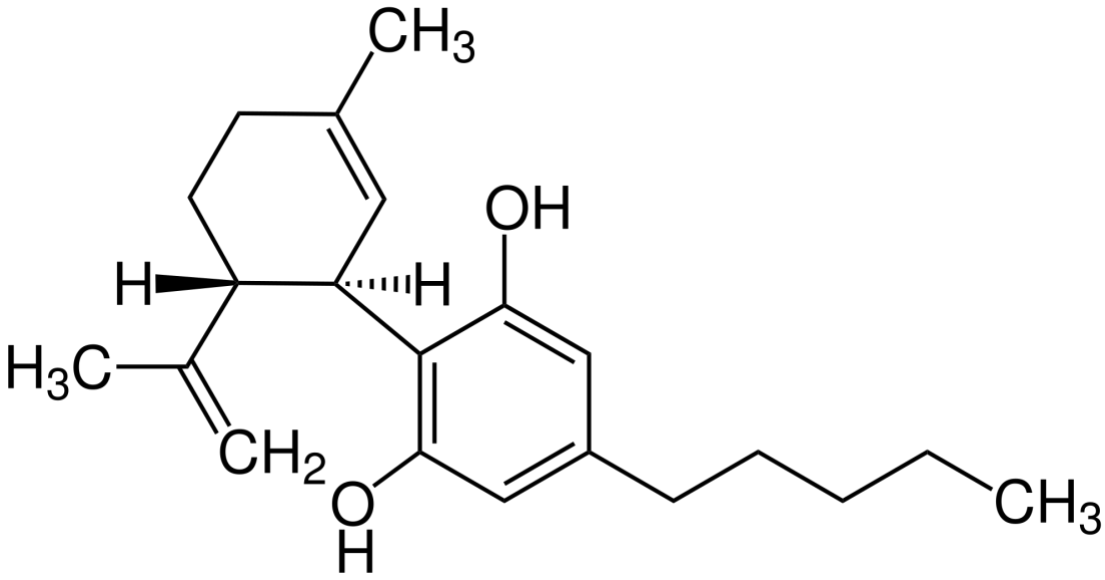
The Effect of Cannabidiol on Cardiac Responses to Fear Conditioning, Mental Sedation and State-dependent Learning



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Abstract

Impaired extinction is thought to be an important component in persistence of anxiety disorders. Ideal treatments for anxiety disorders should thus focus on both relieving symptoms as well as aiding in fear extinction, without having adverse side effects. Cannabidiol (CBD), a non-psychoactive constituent of *Cannabis Sativa*, has sparked interest, because of its putative anxiolytic properties and its potential effect on fear extinction, while exerting few side effects. The current study used a VR fear conditioning paradigm to measure conditioned fear responses in three phases: extinction, retention and reinstatement. Physiological fear was measured using HR responses to startle probe stimuli. Subjects received a capsule with either 300 mg of CBD or a placebo. No significant main effects of drug or significant interactions between drug and cue/context were found. This indicates that CBD did not affect fear extinction, retention and reinstatement. Furthermore, performance on the CPT-AX was tested to determine whether CBD exerted mental sedation, a side effect found by Crippa et al. (2004). No significant interactions were found, indicating that, in the current dose, CBD does not cause mental sedation. Performance of the 15-WT was also tested, to determine whether CBD induces state-dependent-learning. No significant interactions were found, indicating that CBD in the current dose does not induce state-dependent learning.

Introduction

Fear learning serves as an adaptive mechanism to enable organisms to avoid harm. When cues that previously predicted danger are no longer followed by an aversive event, the fear associated with the cue is normally extinguished (Hofmann, 2008). When extinction is impaired however, persistent and disproportional fear may ensue (Das, Kamboj, Ramadas, Curran, & Morgan, 2013). Impaired extinction is thought to be an important component in persistence of anxiety disorders (Das et al., 2013). Anxiety disorders are amongst the most prevalent classes of mental disorders, with estimates of lifetime prevalence up to 28% (Hofmann & Smits, 2008). A meta-analysis by Duits et al. (2015) found increases in both acquisition of fear learning and fear responses during extinction in anxiety patients when compared to healthy controls. A review by Graham & Milad (2011) also stresses the importance of fear extinction in anxiety disorders. Vervliet, Craske, & Hermans (2013) state that treatments should focus on the long-term retrieval of fear extinction, to prevent return of fear. Different mechanisms can lead to the return of fear, which in clinical terms is called relapse (Vervliet et al., 2013). One of these processes is called spontaneous recovery. In spontaneous recovery, fears spontaneously re-emerge after someone has not come into contact with the threat-associated stimulus for a while (Vervliet et al., 2013). Re-emergence of fear can also be called retention of fear: the amount of fear that transfers from the moment when fear is learned to a later time. Fear can also return when the aversive events that are normally predicted by the cue occur on their own, causing previously extinguished fear to return. This process is called reinstatement of fear (Vervliet et al., 2013). The current research will be looking at both fear extinction, retention of fear and reinstatement of fear.

Common pharmacological treatments prescribed for anxiety disorders are selective serotonin reuptake inhibitors (SSRIs), serotonin-noradrenaline reuptake inhibitors (SNRIs) and the calcium channel modulator pregabalin (Bandelow et al., 2008). Tricyclic antidepressants (TCAs) can also be effective for some disorders, but may have more side effects than previously mentioned medications (Bandelow et al., 2008). The mentioned medications are often accompanied by multiple short-term side effects, like headache, nausea, increased nervousness and drowsiness. Long-term side effects, such as sexual dysfunction, weight gain and persistent disturbed sleep may be even more concerning (Bandelow et al., 2008). The abovementioned drugs focus mostly on alleviating the symptoms of anxiety disorders. Most behavioural therapies, however, focus on decreasing the learned fear response (Lissek et al., 2005). Common therapies include cognitive behaviour therapy, counselling and problem solving therapy (Cape, Whittington, Buszewicz, Wallace, & Underwood, 2010). Ideal

treatments should thus focus on both relieving symptoms as well as aiding in fear extinction, without having adverse side effects.

Cannabidiol (CBD), a non-psychoactive constituent of *Cannabis Sativa*, has sparked interest, because of its putative anxiolytic properties and its potential effect on fear extinction (Crippa et al., 2004; Das et al., 2013). CBD has been found to have anxiolytic properties during stressful situations, such as a public speaking (SPS) test (Zuardi, Cosme, Graeff, & Guimarães, 1993). A recent follow-up study found that a dose of 300 mg was the most effective at reducing subjective anxiety during a similar public speaking test, compared to 100 mg and 900 mg (Zuardi et al., 2017). CBD has also been found to decrease the anxiety caused by ingesting Δ9-THC (Zuardi, Shirakawa, Finkelfarb, & Karniol, 1982). The anxiolytic effect of CBD has been studied more widely in animal studies. CBD administration in animals has been found to cause anxiolytic responses in emotional conditioning paradigms (Zuardi & Karniol, 1983; Musty et al., 1983) and the elevated plus-maze test (Guimarães, Chiaretti, Graeff, & Zuardi, 1990; Onaivi, Green, & Martin, 1990). Another benefit of CBD is that it has relatively few side effects (Bergamaschi, Queiroz, Zuardi, & Crippa, 2011). Crippa et al. (2004) found that CBD may cause mental sedation in normal subjects. Mental sedation was quantified using one of four factors on the Visual Analogue Mood Scale (VAMS), by Norris (1971). Another possible disadvantage of CBD is the fact that it could induce state-dependent learning. This means that improved extinction under the influence of CBD would not transfer to a later undrugged state. Evidence of state-dependent fear extinction has been found in animals for other anxiolytic drugs, such as diazepam (Bouton, Kenney, & Rosengard, 1990), and amobarbital (Barry, Etheredge, & Miller, 1965).

To measure drug effects on fear learning, it is important that fear is conditioned and objectively measured. Conditioned fear can be measured in multiple ways in a laboratory setting. Commonly used methods include the skin conductance response (SCR), the eyeblink startle and heart rate (Lissek et al., 2005). In conditioning experiments, it is important to differentiate between cued and contextual fear conditioning, since cue and context predict threat in a different way. In cued conditioning, a neutral stimulus (CS) is followed by an aversive stimulus (US), such as a shock. After multiple paired presentations, the CS will elicit responses similar to those elicited by US (Phillips & LeDoux, 1992). In contrast to cued CSs, contextual CSs are continually present and only predict whether cued CSs may be followed by a US. The context alone however, though associated with the US, cannot predict its time of onset (Phillips & LeDoux, 1992). An animal study found that lesions to the amygdala disrupted acquisition of a cued CS-US relation, while lesions to the hypothalamus disrupted contextual

conditioning (Phillips & LeDoux, 1992). This implies that cued and contextual conditioning are in fact two separate types of conditioning, processed by individual brain areas.

The current study will measure conditioned fear in the extinction phase as well as the retention and reinstatement phase by studying HR responses to startle stimuli. Startle probes were originally used to measure the eyeblink startle reflex, described in a different study. These stimuli were deemed appropriate for the analysis of conditioned HR responses as well, see methods for a more detailed description of this choice. HR changes have been widely used to measure conditioned fear responses (Lonsdorf et al., 2017). Both conditioned HR acceleration and deceleration have been observed, depending on the stimulus (Lonsdorf et al., 2017). Deceleration seems to reflect an orienting response to the stimulus, whereas acceleration indicates a defensive response and therefore learned fear (Hamm, Greenwald, Bradley, & Lang, 1993). However, individuals seem to differ in their HR responses to the same stimuli, with some showing acceleration and some showing deceleration to the same stimulus (Hodes, Cook & Lang, 1985). The current study looked at both accelerative and decelerative HR responses for this reason. Several factors need to be kept in mind when analysing HR to measure conditioned fear.

One of these factors is the type of (conditioned) stimulus that elicits the cardiac response. Previous studies have analysed HR responses to different types of conditioned stimuli. Moratti & Keil (2005) found that participants expressing different HR responses also differ in their neuromagnetic response patterns to CS+ and CS- stimuli. Whereas this study measured conditioned fear responses, it used startle sounds as UCS and neutral visual stimuli CS+ and CS-. Peri, Ben-Shakhar, Orr, & Shalev (1999) also used startle probes as UCS and neutral visual stimuli as CS+/CS- and found increased HR responses to CS+ stimuli during acquisition and extinction in PTSD patients compared to healthy controls. Block, Sersen, & Wortis (2018) used a car horn as an aversive auditory UCS to elicit conditioned cardiac responses to neutral CS+ and CS- in childeren. Other studies have also looked at HR responses to different unconditioned stimuli. A study investigating stress responses using the Cold Pressor Test, looked at cardiac responses to startle sounds (Deuter et al., 2012). A study researching cardiac reactions to startle stimuli found that startle stimuli caused acceleration in both PTSD patients and controls (Jovanovic, Norrholm, Sakoman, Esterajher, & Kozarić-Kovačić, 2009). Vossel & Zimmer (1992) found that stimulus intensity, but not rise time, influenced HR, by measuring HR responses to unconditioned startle stimuli. Elsesser, Sartory, & Tackenberg (2004) found that recent trauma victims and PTSD patients showed increased HR responses to visual trauma-related material.

The interval at which changes in HR can occur is another factor to consider when analysing HR. Various durations have been used to determine both baseline and post-stimulus HR. Moratti & Keil (2005) calculated HR for 4000 ms in 500 ms steps and subtracted a 2000ms baseline, to measure responses to both conditioned and unconditioned stimuli. Peri, Ben-Shakhar, Orr, & Shalev (1999) calculated HR acceleration by subtracting the average HR 2 seconds before the CS+/CS- onset from the highest value between 1 and 4 seconds after stimulus onset. Block, Sersen, & Wortis (2018) used only the shortest post-stimulus IBI within five seconds to determine cardiac acceleration, without comparing the IBI to baseline levels. Deuter et al. (2012) found increased HR 4 to 6 seconds after startle probe onset, compared to a -2 to 0 second baseline. Jovanovic et al. (2009) determined cardiac responses by averaging the IBI change from the 1 second pre-stimulus baseline to the first 3 seconds after CS+/CS- onset. The study by Vossel & Zimmer (1992) determined HR by looking at the largest acceleration or deceleration that occurred within 4 seconds of the startle probe presentation. Elsesser, Sartory, & Tackenberg (2004) determined cardiac reponses by comparing HR for 6000 ms after stimulus onset to a 1000ms prestimulus baseline. In their 1993 study, Zuardi et al. found no effect of CBD on HR. A metastudy on safety and side effects of CBD also found that CBD does not affect HR (Bergamaschi et al., 2011).

The primary aim of the current study is to investigate the effect of oral administration of 300 mg CBD on fear extinction, fear retention and reinstatement of fear, quantified using heart rate change in response to startle probes. The expectation is that increased fear will lead to increased HR acceleration, based on previous HR studies. The primary hypothesis is that CBD will cause improved fear extinction and less fear retention and return of fear when compared to placebo. The fear conditioning procedure will be done by using an adapted version of the Virtual Reality Task developed by Baas, van Ooijen, Goudriaan, & Kenemans (2008) and used in many other studies. The original version of this task was only used to measure fear acquisition. Secondary goals are to find out whether CBD induces mental sedation, as found by Crippa et al. (2004), and state-dependent learning, which has been observed in animal studies using other anxiolytic drugs (Barry et al., 1965; Bouton et al., 1990). Secondary hypotheses are that CBD will cause increased mental sedation and increased state-dependent learning, when compared to placebo. Mental sedation will be measured using the CPT-AX. State-dependent learning will be measured using the 15-word test (15-WT).

Methods

Participants

Thirty-four volunteers (22 female; mean age: 22.6 years [range: 19 - 30 years]) took part in the experiment after giving written informed consent. The study was approved by the medical-ethical review committee of the University Medical Hospital Utrecht. All participants spoke Dutch and were recruited via flyers and posters at Utrecht University, through Sona Systems Research Participation-system and by using various social media groups. Participants received €8/h as compensation. Subjects were obliged to meet the following inclusion criteria: Be between 18-30 years of age, be physically and mentally healthy, be able to make individual decisions about willing to participate and have normal or corrected eyesight and normal hearing. Subjects were not included when they previously participated in a fear conditioning study with distinct acquisition and extinction phases, suffered from car sickness, had a history of suffering an Axis I or II mental disorder, epilepsy, heart complications, drug dependence, frequent cannabis use, negative cannabis reactions, or a history of psychosis in the family. Subjects could only be included if they had not used any psychoactive drugs four weeks prior to the first testing day. Pregnant and lactating women were also excluded.

Participants were asked to not consume any drugs in between the study days and were tested for amphetamines, cocaine, methamphetamines, opiates / morphine, THC and XTC at the beginning of the first and second testing day. They were also asked to consume no alcohol and sleep regularly before and after the testing days.

Tasks and stimuli

Virtual reality task

All stimuli used in the Virtual Reality (VR) task were presented using the software Presentation 18.1 (Neurobehavioral Systems inc.). One block lasted 5 minutes and 25 seconds, in which multiple virtual surroundings were visited. The virtual surroundings used in the VR task were a house in a suburban neighbourhood and an apartment in a down-town area. The association of the conditioned context was counterbalanced across subjects. Each block, one of the contexts was visited once for 90 seconds and the other context was visited twice for 30 and 70 seconds. When transitioning between contexts, participants moved through street scenes and a metro sequence. The order of which contexts were visited was counterbalanced across participants. The cue that indicated whether shocks would follow was an increase in saturation and illumination, accompanied by a yellow colour filter, defined as the ‘light on’ condition. Participants were thus conditioned to both a cue (light on, light off) and a context (CTX; house,

apartment). The light on cue was presented in both contexts. In only one of the contexts the light on cue would have a chance of being followed by a shock. This resulted in four unique conditions per participant: light-on/CXT+, light-off/CTX+, light-on/CTX- and light-off/CTX. The surroundings in which shocks were administered were counterbalanced between participants. Six startle probes were presented in both contexts, three of which with the light on and three with the light off. In this way physiological reactions to both the cue and the context could be compared. Startle probes were presented 5-6 seconds after the light-on cue. In the light-off condition, startle probes were presented 10-12 seconds after entering the context or 6-12 seconds after the light went off.

The VR task consisted of three parts, spread out over three days. During the first phase (acquisition) shocks were administered in the light-on/CXT+ condition. The acquisition phase consisted of 7 blocks where shocks were administered, so that participants would learn the association between cue/context and the US. To make sure each participant consciously learned the CS-US association, an instruction was giving after four experimental blocks. Subjects were instructed that the shocks would only be imposed in the shock context when the light was on. After the instruction, three more experimental blocks were presented. The second phase (extinction) happened the day after the first. This phase contained no shocks, so that participants would learn that the presentation of the cue within the CXT+ would no longer result in receiving shocks. CBD was administered during this phase to aid the extinction of fear. The third phase (retention/reinstatement) happened 7-14 days after the second day. This phase contained no shocks during blocks. The retention of fear was measured during the first 4 blocks. However, following the procedure recommended by Lonsdorf et al. (2017), 4 shocks were presented after 4 blocks while subjects viewed a black screen with a fixation cross, so that the following 4 blocks could measure the effect of reinstatement.

Startle probes consisted of 50-ms 105-dB white noises with instantaneous rise time, presented through Sennheiser HD202 headphones. Shock amperage (mean: 1.8 mA) was determined by a shock work-up at the beginning of the first test session. Participants would press a mouse button to receive a shock after three seconds, after which they rated the shock's strength on a Likert scale (1-5; 0.5 step interval). A rating below than 4 would increase the next shock's strength, while a rating above 4 would decrease it. A rating of 4 would not change the shock strength. Very high or low ratings would change the shock strength more drastically than ratings close to 4. This algorithm aimed to set a final shock strength which all participants rated 4 out of 5 on a Visual Analogue Scale of unpleasantness. This final shock strength was used for the follow-up VR task. Shocks were administered through electrodes placed on the medial

nerve on the opposite hand to the hand that controlled the mouse. In between videos, several subjective questions, such as Visual Analogue Scales and control questions were presented. These questionnaires are not part of the current paper.

CPT-AX

Stimuli were presented using the software Neurobehavioral Systems Presentation 16.1. Stimuli were letters presented in black, in the centre of a grey screen for 150 ms, with a 1400-1600 ms inter stimulus interval. Participants were asked to press either the "Z" or "/" key when an A was followed by an X or Y, respectively. Distractor stimuli consisted of letters C, D, E, F, G, H, J and L. The valid cue A was followed by an X or Y 50% of the time. The other 50% of the time, the invalid cue A was followed by one of the distractor stimuli. One testing block consisted of 300 trials, with a one-minute pause after 150 trials. Valid cues were only presented if the last cue was preceded by at least two distractor stimuli. The CPT-AX was presented two times during the second day. At the first presentation of the CPT-AX at baseline, a practice block of 100 trials preceded the testing blocks to get the subjects acquainted with the task. The second presentation (after drug ingestion) was not preceded by a practice block. See Figure 1 for a timeline of the CPT-AX presentation.

15-WT

Subjects were asked to remember two sets of words from the Dutch 15-WT on session 2 of the experiment. Fifteen words were presented in fixed order for 1667 ms in white text on a black background. Participants were instructed to pay attention and after each presentation, subjects were asked to recall as many words as possible. Correct words were recorded manually by the researcher. Two different sets of the 15-WT were presented during the second session, to measure learning in both drug and non-drug state. The first set was thus presented at baseline and the second set was presented after drug ingestion. Each set of words was presented five times. The order of sets was counterbalanced across participants.

At the end of the second session, participants were asked to reproduce as many words as possible from both versions of the 15-WT. Subjects thus reproduced words from both non-drug state as well as CBD / placebo state, while being in a CBD / placebo state.

In the recognition task, subjects were asked to respond with left or right keypress whether they recognised a stimulus from the previous week or whether it was a word that they had not seen before, respectively. Stimuli were again presented in white text on a black background. The next stimulus was presented after the subject responded with the left or right

keypress. No time-limit for responses was present. The order of stimuli was randomised for the recognition task. For the recognition task that was administered on test day 3 the aim was to test retention of words from both non-drug state as well as CBD / placebo state, while being in a non-drug state. See Figure 1 for a timeline of the 15-WT presentation.

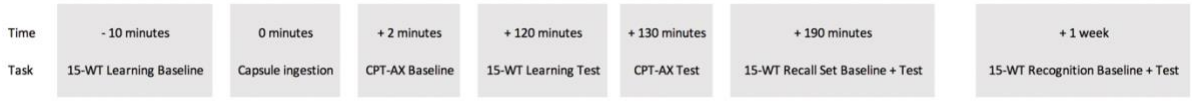

Figure 1. Timeline of CPT-AX and 15-WT presentation.

Subjective reports

Multiple subjective questionnaires were presented during the experiment. A subset of questions from the Visual Analogue Mood Scale (VAMS) translated in Dutch (CHDR, nd) (original questionnaire by Bond & Lader (1974)) was presented to measure mental sedation and calming effects caused by CBD. The Subjective Effects Scale (SES) was presented five times during the experiment to measure subjective mood at different moments after capsule administration. These questionnaires were not part of the current paper.

Drugs

CBD and placebo lactose were produced by THC Pharm Germany, and blinding labels were provided by Ace Pharmaceuticals. Participants were given a capsule containing either 300 mg CBD or 300 mg lactose (placebo). To ensure equal absorption of CBD for all participants, they were given a standardised breakfast to eat 2 hours prior to capsule administration. To maintain drug blinding, drugs were encased in a small plastic container reading only the participant number. Randomisation of drug and placebo cases was done by a separate researcher.

Procedure

Day 1

After giving informed consent and scoring negative on a drug screening, participants proceeded to fill out a questionnaire which is not part of the current paper. Next, electrodes recording eyeblink startle, skin conductance and electro-cardiography (ECG) were applied, as well as electrodes which could administer shocks. Shock amperage for each participant was first determined by running a shock work-up. The experiment started with a startle habituation

phase of four minutes, during which participants could habituate to the startle sounds. The habituation phase consisted of twelve startle probes. This was followed by a habituation video of the virtual environment where no shocks were administered, so that the participants could be acquainted with the virtual surroundings. Before proceeding with the experimental blocks, participants were instructed to pay attention, so they could try to predict in which context shocks would be administered.

Day 2

The second day started the morning after the first day. It began with a drug and pregnancy screening, after which participants filled out a questionnaire which is not part of the current paper. Next, participants were presented the baseline learning set of the 15-WT, Participants were subsequently given the capsule containing either 300 mg CBD or 300 mg lactose (placebo). Baseline CPT-AX was presented for the first time straight after ingestion of the capsule, to test baseline performance on mental sedation. To ensure optimal CBD-blood levels during the VR-task, participants waited for 90 minutes in a waiting room, after which they returned to the testing room. Upon returning to the testing room, 120 minutes after ingestion of the capsule, participants were subjected to the test set of the 15-WT, tested in the same manner as the first version. They also performed the test CPT-AX task, 130 minutes after capsule ingestion. Next, all recording and shock electrodes were applied, and the VR-task started, 150 minutes after ingestion of the capsule, when CBD is at its highest blood level. Participants viewed four extinction blocks, in which no shocks were given. See the section on the VR-task for a more detailed description of the VR-task. After the VR-task ended and all electrodes were removed, participants were instructed to recall as many words as possible from both versions of the 15-WT, at 190 minutes after ingestion of the capsule. The session ended with a second questionnaire, which is not part of the current paper.

The SES, a questionnaire not part of the current paper, was presented five times during the second day; before ingestion of the capsule, 60 minutes after ingestion, 120 minutes after ingestion, before the experiment (145 minutes after ingestion) and 5 minutes after the experiments. The VAMS was presented twice; right before ingestion and 145 minutes after ingestion.

Day 3

The third day was approximately one week after the second testing day. During this day subjects began by filling out a questionnaire, which is not part of the current paper. Next, the recognition task for the 15-WT was presented. The recording/shock electrodes were applied next, after which the VR-task was started. Participants viewed four blocks of videos without shocks to measure whether spontaneous recovery of fear was present. Subjects were subsequently given four reinstatement shocks while looking at a fixation cross. Subjects were presented four more blocks of videos without shocks, to measure the return of fear after reinstatement.

Analysis

VR-task

Data analysis for the VR-task was performed on the average heart rate responses to startle probes. HR responses to CSs could not be analysed because more than half of the CSs were followed by startle probes. This led to inconsistent HR responses, since some responses were based solely on the CS, while others were based on both the CS and the startle probe. Analysing only the CSs that were not followed by startle probes was not possible either, since too little trials would be left to determine a noise-free signal. The average peak acceleration and deceleration values were averaged across two blocks, to increase signal to noise ratio. This was done for both the shock and safe context, and for both the light-on and light-off condition separately. Each of the four unique conditions (light-on/CXT+, light-off/CTX+, light-on/CTX- and light-off/CTX) contained three startle probes per block. One data point thus consisted of the average of six trials. Analysis was only performed on data of the second and third day. This was done because on the first day startles in the shock context were sometimes followed by shocks if the light cue was present. The aversity of the shock could have a strong influence on HR, thus no analysis was performed on any of the acquisition startles.

The data of the second and third day were divided in three phases: extinction, spontaneous recovery and reinstatement. For extinction, analysis was performed on the final two blocks of the second day, to determine the maximal effect of extinction. For spontaneous recovery, analysis was performed only on the first two blocks of the third day, instead of all four, to minimise the effect of extinction that happened during the third day. For the same reason as with extinction, spontaneous recovery could not be determined relative to acquisition. For reinstatement, the first two blocks after the reinstatement shocks were analysed, to determine the effect of the reinstatement shocks. Reinstatement was determined in another way

by subtracting the two blocks before the reinstatement shocks from the two blocks immediately after the reinstatement shocks. A similar method was employed by Heitland et al. (2012), who calculated extinction by subtracting average extinction responses from average acquisition responses. Extinction could not be calculated this way in the current study, due to the shocks in the acquisition phase influencing the HR response.

Both the general fear effect and the effect of contextual fear were analysed. General fear was analysed by comparing the light-on/CXT+ condition with the light-off/CXT- condition, following (Heitland et al., 2012). This comparison should show the greatest effect of conditioning, since both the context and cue that predict the shock are compared to the context that does not predict the shock, with no cue present. Contextual fear was analysed by comparing the light-off/CXT+ condition with the light-off/CXT- condition. This analysis compared both contexts, without considering the effect of cued fear, caused by the light.

Primary data processing was done using BrainVision Analyser 2.1. Segments were extracted for all startle probes in each of the unique conditions. Each extracted segment consisted of all R-R-intervals starting 2 seconds before to 8 seconds after the startle probe. A 15 Hz low-pass filter and a 15 Hz high-pass filter were applied separately to eliminate noise from the signal. The peaks of the R-waves were determined by using a 150 µA threshold. Each segment was checked and corrected for artefacts manually. This was done by removing false peaks, such as movement artefacts or electrode movements, from the segments. Inter-beat intervals (IBIs) were calculated for each trial using MATLAB. The measurement interval was divided in 500ms steps (bins), and each IBI was linked to the closest bin. Subsequently, IBIs were converted into BPM. To eliminate some noise from the signal, no single values were used for baseline and peak HR, but 2 second averages were used, following Deuter et al. (2012). Preliminary analysis of HR responses showed the strongest accelerative peaks between 2000 and 3000 milliseconds after stimulus onset, see Figure 2. For this reason, HR acceleration or deceleration was determined by subtracting the average HR during a 2 second pre-stimulus interval from the average HR at 1.5-3.5 seconds after the stimulus onset, similarly to Deuter et al. (2012), who instead used the 4-6 second post-stimulus window to determine peak HR in relation to the same 2 second pre-stimulus baseline.

Using IBM SPSS 23, a repeated measures ANOVA was performed separately for each of the phases; extinction, spontaneous recovery and reinstatement (which was calculated in two ways). Separate ANOVAs were done for both the general fear effect (light-on/CTX+ vs light-off/CTX-) and the contextual effect (light-off/CXT+ vs light-off/CXT-). This resulted in eight

different ANOVAs. The CBD or placebo group was added as the between-subject factor "Group" in each ANOVA.

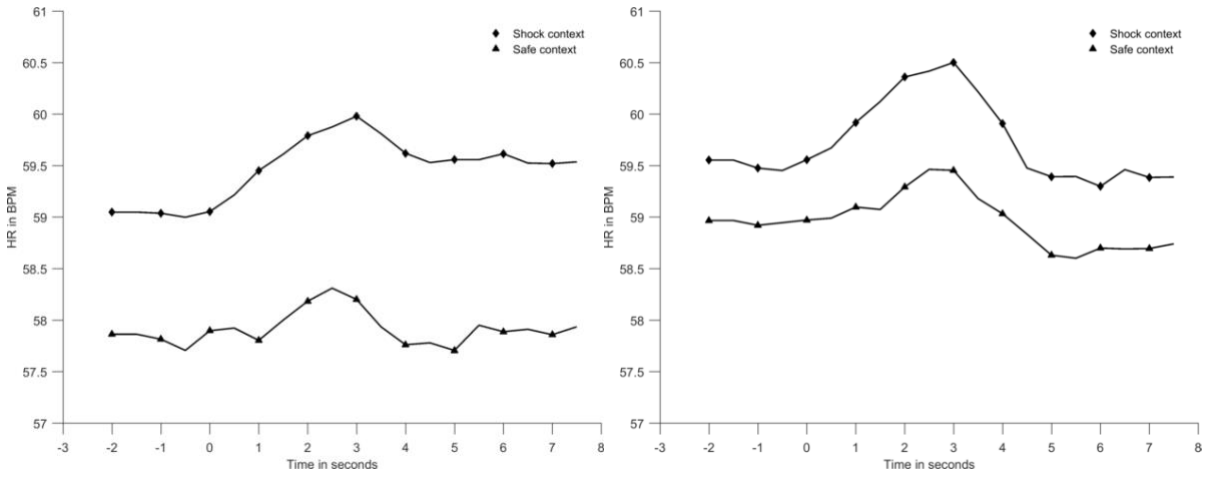

Figure 2. Average HR responses to startle probes, presented at t=0, with the light on (left) and the light off (right) in both safe and shock context.

CPT-AX

The average and standard deviation of the reaction time, the percentage correct responses and the percentage omissions were calculated separately for both testing sessions, for the average of X and Y stimuli. A separate repeated measures ANOVA was performed (IBM SPSS 23), for each of the different measurements. The measurement time (baseline/test measurement) was used as the within-subject factor. CBD or placebo group was added as the between-subject factor "Group".

15-WT

To test for state dependent-learning, a repeated measures ANOVA was performed (IBM SPSS 23) using the time of measurement (recall at the fifth learning trial vs delayed recall) and the time of learning (baseline vs test) as within-subject factors. CBD or placebo group was added as the between-subject factor "Group". If state-dependent learning were present, the CBD group would remember relatively fewer words on the delayed recall task than on the fifth learning trial, for the words that were learned at baseline compared to the words learned at test measurement under CBD. This analysis tested whether learning in a non-drug state would lead to difficulties while recalling under CBD, compared to both learning and recalling under CBD.

To test whether learning under CBD led to difficulties in recognition in a non-drug state, a repeated measures ANOVA was performed for the performance on the recognition task, using time of learning (baseline vs test) as the within-subject factor. Performance on the fifth trial, averaged between baseline and test learning session, was added as a covariate, to control for learning performance. CBD or placebo group was added as the between-subject factor "Group".

Results

VR-task

Each of the phases of the VR-task was analysed using a separate ANOVA, using context/cue as the within-subject variable and drug condition as the between-subject variable. HR responses were calculated by subtracting the average of the -2 to 0 second baseline from the average HR at 1.5-3.5 seconds post stimulus. This resulted in average acceleration or deceleration values for each condition during each block. The analysis was performed for both general fear (light-on/CXT+ vs light-off/CXT-) as well as contextual fear (light-off/CXT+ vs light-off/CXT-). The effects of reinstatement on HR responses were also calculated as a difference score between the two blocks after and the two blocks before the reinstatement shocks, to determine the effect of the reinstatement shocks.

General fear / context specific fear HR-responses

None of the comparisons of general fear in the extinction, retention and reinstatement phases led to significant differences being found between the light-on/CXT+ and light-off/CXT- condition. None of the comparisons of contextual fear in the extinction, retention and reinstatement phases led to significant differences being found between the light-off/CXT+ and light-off/CXT- condition. See Figure 3 for the average HR change for each of the blocks.

When reinstatement was calculated as the difference between the two blocks post- and pre-reinstatement shocks, a significant main effect of summed contextual and cued fear (light-on/CXT+ vs light-off/CXT-) was found on HR, $F(1,28) = 5.235$, $p = 0.030$, $r = 0.158$. The reinstatement shocks caused a decrease in HR responses from the blocks before to the blocks after reinstatement shocks in the light-on/CXT+ condition (M = -0.8090, SD = 2.47714), whereas the light-off/CXT- condition (M = 0.6290, SD = 2.12448) saw increased HR responses from the blocks before to the blocks after the reinstatement shocks. A significant main effect of contextual fear (light-off/CXT+ vs light-off/CXT-) was also found using this calculation of reinstatement, $F(1,28) = 5.059$, $p = 0.033$, $r = 0.153$. A similar pattern was found as for the general fear effect. The reinstatement shocks caused a decrease in HR responses from the blocks before to the blocks after reinstatement shocks in the light-off/CXT+ condition (M = -.6681, SD = 2.52175) whereas the light-off/CXT- condition (M = 0.6290, SD = 2.12448) saw increased HR responses from the blocks before to the blocks after the reinstatement shocks.

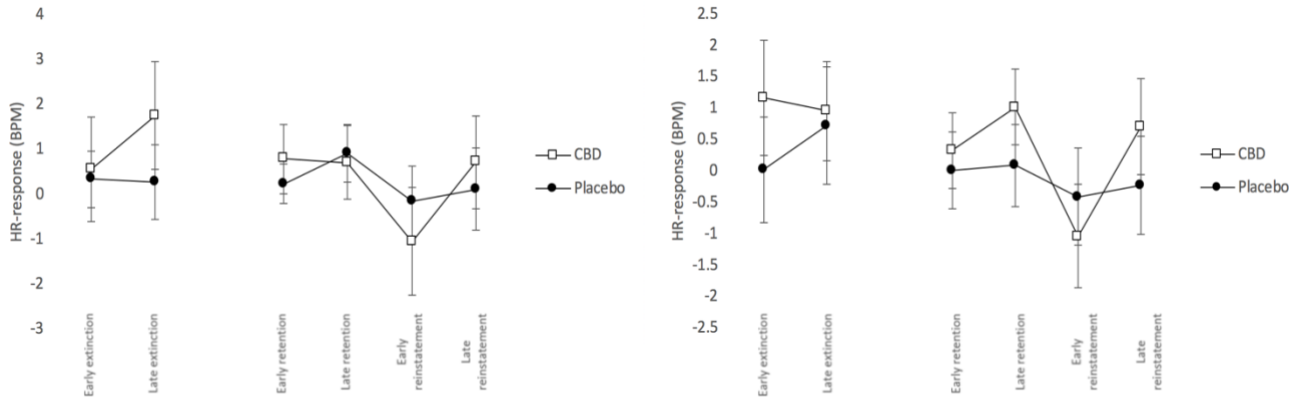

Figure 3. The general fear effect (left) and the contextual fear effect (right). The general fear figure displays the difference in BPM between light-on/CXT+ and light-off/CXT- for each of the blocks. The contextual fear figure displays the difference in BPM between light-off/CXT+ and light-off/CXT- for each of the blocks. Error bars reflect SEM.

Drug effects

None of the phases showed a main effect of drug or an interaction between drug and condition on general fear or contextual fear, see table 1.

Table 1

Main effects of drug and interactions between drug and condition on general and contextual fear in the different phases of the experiment.

Phase	Fear effect	Comparison	*F*	*p*
Extinction	General fear	Drug	1.134	0.296
		Drug * condition	1.317	0.261
	Contextual fear	Drug	2.284	0.142
		Drug * condition	0.81	0.779
Retention	General fear	Drug	3.339	0.078
		Drug * condition	0.437	0.514
	Contextual fear	Drug	2.800	0.105
		Drug * condition	0.157	0.695
Reinstatement	General fear	Drug	0.017	0.896
		Drug * condition	0.482	0.493
	Contextual fear	Drug	0.029	0.866
		Drug * condition	0.297	0.590
Reinstatement (pre-reinstatement – post-reinstatement)	General fear	Drug	0.280	0.601
		Drug * condition	0.322	0.575
	Contextual fear	Drug	0.018	0.866
		Drug * condition	1.836	0.186

An exploratory analysis looking at the effect of cued fear (light-on/CXT+ vs light-off/CXT+) found a significant difference between CBD (M = 1.487, SD = 2.095) and placebo (M = 0.263, SD = 1.578) in the retention phase $F(1,28) = 6.552$, $p = 0.015$, r = 0.192.

CPT-AX

Analysis of the CPT-AX data only looked at interaction effects between time of measurement (baseline vs test) and drug group (CBD vs placebo) to determine the presence of mental sedation caused by CBD. Main effects of drug were not analysed, since this would only indicate a pre-existing group difference, not caused by CBD. None of the interactions between time of measurement and drug condition were significant, $F(1,32) < 2$, $p > 0.1$.
Descriptive plots of the various CPT-AX measurements can be found in Figure 4.

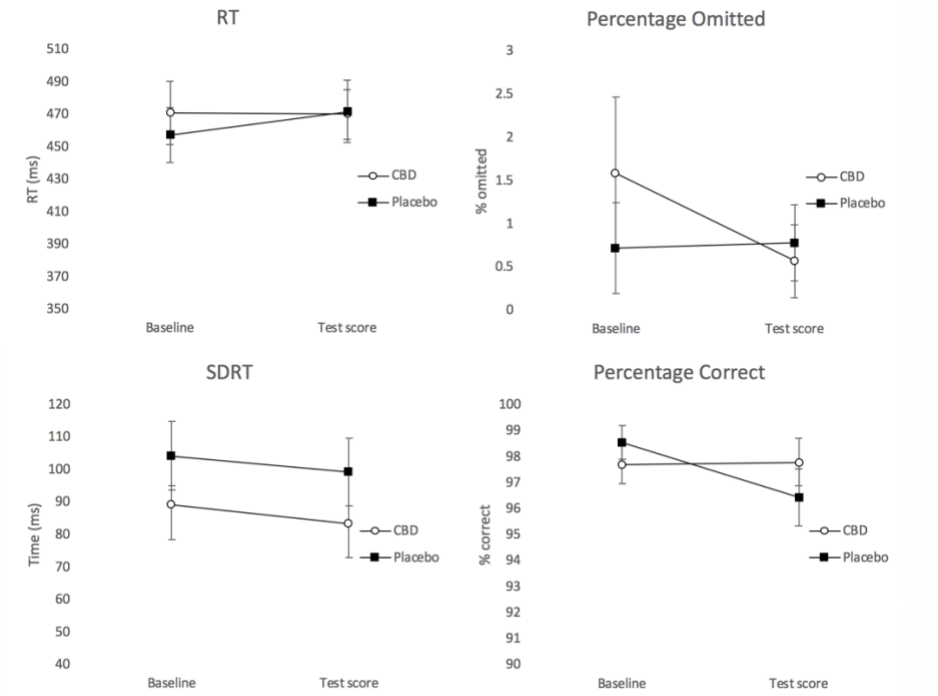

Figure 4. Different CPT-AX measurements; Reaction Time, Reaction Time Standard Deviation, Percentage omitted and percentage correct. Error bars reflect SEM.

15-WT

The first analysis of the 15-WT looked at the interaction between time of measurement (recall at learning vs delayed recall), time of learning (baseline vs test) and drug group (CBD vs placebo) to determine whether CBD induced state-dependent learning. This analysis tested whether learning in a non-drug state would lead to difficulties while recalling under CBD, compared to both learning and recalling under CBD and to both learning and recalling under placebo. Main effects of drug were not analysed, since this would only indicate a pre-existing group difference, not caused by CBD. No significant interaction between time of learning, time of recall and drug group was found, $F = 0.129$, $p = 0.721$. Descriptive plots of the 15-WT data can be found in Figure 5.

The second analysis of the 15-WT used the number of words remembered at the recognition test as dependent variable, comparing learning before and after ingestion for both drug groups. This analysis looked at the interaction between time of learning (baseline vs test) and drug group (CBD vs placebo), while controlling for average learning performance in both learning conditions. This analysis meant to determine whether learning under CBD led to difficulties in recognition in a non-drug state. First, the assumption that there was no effect of drug on the test learning session was tested using an independent samples t-test. This analysis found no significant difference between drug groups in the number of words recalled at the fifth learning trial, $t(32) = -0.185$, $p = 0.855$. The aim was to now insert this score as a covariate for the test of how well both drug groups recognized the words of both lists at recognition. However, the assumption of homogeneous regression slopes of learning performance and recognition performance between drug groups was not met, $F(1,28) = 4.625$, $p = 0.040$. Upon determining the violation of this assumption, the second analysis was discontinued.

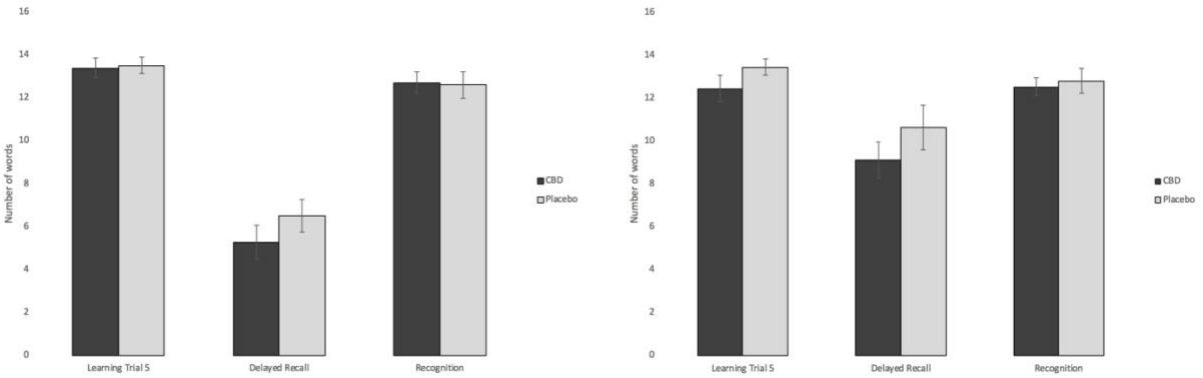

Figure 5. Number of words remembered at the fifth learning trial, delayed recall and recognition for both baseline (left) and test (right) learning sets. Error bars reflect SEM.

Discussion

The primary aim of the current study was to investigate the effect of CBD on fear extinction, fear retention and return of fear. HR change in response to startle probes was used as the physiological measure to determine fear responses. The hypothesis was that CBD would cause improved fear extinction and less fear retention and return of fear, when compared to placebo. Subjects were conditioned to fear a certain environment, which in combination with a light-on cue, predicted an electrical shock. In the second phase subjects underwent an extinction procedure, where the environment no longer predicted shocks. In the third phase, no shocks were given during the VR videos, but several shocks were administered halfway through the phase, in order to reinstate fear. Secondary goals were to find out whether CBD causes mental sedation and state-dependent learning.

The analysis of general fear (light-on/CXT+ vs light-off/CXT-) showed no significant main drug effects or interactions. In the extinction phase however, the CBD group saw an increase in acceleration in the light-on/CXT+ condition at trend level, compared to the light-off/CXT- condition, while this difference was not present for the placebo group. In the retention phase, the effect of drug was close to being significant, indicating that subjects in the CBD condition might have had slightly more acceleration in response to any of the startle probes than subjects in the placebo condition. The trend of CBD causing acceleration was in fact the opposite to what was expected. These results suggest that CBD might not reduce fear that is reflected by HR acceleration. The analysis of general fear did show a significant main effect of summed context and cue when the reinstatement phase was calculated as the difference between the two blocks after the reinstatement shocks and the two blocks before the reinstatement shocks. No significant main effect of summed context and cue was found when only the first two blocks after the reinstatement shocks were analysed. This implies that using differences scores of blocks to reveal effects that occur from phase to phase rather than within a phase, may be a more effective way to determine effects of fear conditioning than using only the scores in a block that are most relevant to that phase. In this case, the blocks after the reinstatement shocks likely only measured general fear after reinstatement, while the difference score likely measured the effect of the reinstatement shocks.

The analysis of contextual fear (light-off/CXT+ vs light-off/CXT-) showed no significant main drug effects or interactions. In the retention phase, the main effect of drug was close to trend level, similar to what was found in the analysis of general fear. This suggests that participants in the CBD condition may have had slightly more acceleration in response to any of the startle probes in both contexts than subjects in the placebo condition. Like in the analysis

of general fear, this direction was the opposite to what was expected. The analysis of contextual fear did show a significant main effect of context (light-off/CXT+ vs light-off/ CXT-) when the reinstatement phase was calculated as the difference between the two blocks after the reinstatement shocks and the two blocks before the reinstatement shocks. The main effect of context was not present when only the first two blocks after the reinstatement shocks were analysed. Again, using difference scores might be a more reliable way to test for conditioning effects than using only certain blocks within a phase.

The use of difference scores has been used before in similar studies for the startle reflex (Heitland et al., 2012). Unlike HR responses, startle magnitudes are usually calculated as positive values, whereas HR responses can reflect both acceleration and deceleration. It is therefore more difficult to determine whether the difference between two blocks reflect more acceleration or less deceleration, which would both result in positive values. Difference scores for the extinction and retention phase are normally calculated by subtracting these from the last blocks of the acquisition phase (Heitland et al., 2012). This was not possible in the current study, since acquisition phase responses could not be analysed. Startles in the acquisition phase were frequently followed by shocks which would likely have a strong effect on HR (Lonsdorf et al., 2017). Too little startles would be left to calculate a reliable average.

An exploratory analysis of cued fear (light-on/CXT+ vs light-off/CXT+) showed a significant main effect of drug in the retention phase. This difference in HR responses between drug groups was found in both the cued and uncued startles in the shock context, implying a difference between drug groups at the start of the retention phase. Whether this difference was caused by an inherent group difference or by the administration of CBD during extinction remains unclear, since retention data could not be compared to acquisition data. Furthermore, the analysis of cued fear is ambiguous, since both cued and uncued startles occur within the shock context. Therefore, the effect of context cannot be removed from this analysis. In conclusion, it is hard to draw conclusions on this finding, for which reason the analysis of cued fear was not included in the original planned comparisons.

A limitation of the VR-task was the use of startle as the onset stimulus for conditioned HR responses. The startle stimulus was the only stimulus which could be used to measure HR responses in all contexts which would not be influenced by other stimuli interfering, such as the shock or cue onset. HR responses to startle stimuli have been studied before (Deuter et al., 2012; Jovanovic et al., 2009), but these studies did not use a fear conditioning paradigm. Other studies have used startle probes as UCS, but used HR responses to neutral visual CS+/CS- stimuli (Moratti & Keil, 2005; Peri et al., 1999). In conclusion, future experiments should focus

on having only one type of stimulus within the HR response window. The current study had three types of stimuli: Shocks, startles and light-on cue presentations. The only stimulus for which HR could be reliably analysed was the startle probe in the second and third session, since it was not followed by any of the other stimuli. In the current study, startle probes were originally used to analyse the startle reflex, described in a different study. An experiment using distinct CS+ and CS- presentations that were not directly followed by other stimuli might be able to measure conditioned HR responses in a more consistent manner. Furthermore, with no shocks directly following CS+ presentations, reliable acquisition data could be calculated without interference of shocks.

No significant interactions between time of measurement and drug group were found for any of the CPT-AX measurements. This suggests that CBD does not cause mental sedation, as measured by the CPT-AX. A possible explanation for not finding any effect of CBD on CPT-AX performance could be that CPT-AX does not adequately measure the construct of mental sedation as measured by Crippa et al. (2004). Multiple differences in study design could be the cause for the difference in findings. Crippa et al. (2004) quantified mental sedation using one of four factors on the Visual Analogue Mood Scale (VAMS), by Norris (1971). It is possible that CPT-AX performance does not adequately compare to behaviour on a self-report questionnaire. Crippa et al. (2004) also used a dose of 400 mg CBD instead of 300mg. They presented the VAMS at different timings (60 and 75 minutes after ingestion), compared to 130 minutes after ingestion. If CBD in fact does not cause mental sedation, as found by Crippa et al. (2004), this would make it more suitable as a future medication for anxiety disorders. This is especially true because current anxiolytics come with a host of side effects, including mental sedation (Bandelow et al., 2008).

The analysis of the 15-WT data revealed no significant interactions between time of measurement, time of learning and drug group. These results suggest that CBD does not induce state-dependent learning, as measured by the 15-WT and in this particular dose. If CBD does not induce state-dependent learning, this would be advantageous if it were to be used as a medication for anxiety disorders. This would mean that extinction learned while being under the influence of CBD would transfer to a later undrugged state, without impairment of state-dependent learning. Nonetheless, the current study did have some limitations when measuring state-dependency. The fact that delayed recall took place at different times from the different learning sessions created a recency bias in delayed recall performance towards the words that were learned after capsule ingestion, see Figure 5. This bias was not present for the recognition task however, likely because too much time had elapsed since the learning sessions. These

findings might have been cause for the lack of findings concerning state-dependent learning. Future studies on the effect of CBD on state-dependent memory should consider these effects of timing in their design.

A general limitation of the current study was the oral administration of CBD capsules of the same dose, which may have led to varying CBD-blood levels. With varying weights and bodyfat percentages across participants of both genders, it was hard to control for the timing at which CBD reached its peak blood-level. To control this as much as possible, subjects were given a standardised breakfast, which they were instructed to consume 2 hours before capsule administration. Nonetheless, we were unable to check CBD-blood levels by means of saliva or blood testing. Furthermore, the dose that was used was relatively low, which may have led to a weaker anxiolytic effect than expected. This dosage was chosen based on a study by Zuardi et al. (2017), who determined 300 mg to be the most effective dose at reducing anxiety caused by a public speaking test.

Perhaps the greatest cause of the lack of significant effects could be the number of participants which were included in the current study. The study originally aimed to include 56 participants, but due to time restrictions only 34 participants could be included. Some of the data indicate that there may be an effect of CBD on fear conditioning, but these effects were not significant. Descriptive data show high standard deviations for most of the HR data, which could be due to the low number of participants.

In conclusion, the hypothesis that CBD would cause improved fear extinction and less fear retention and return of fear, when compared to placebo, is not supported by the data. The fact that no results similar to those in other studies were found, can be explained by the onset stimuli which were used for the HR responses and the way in which these HR responses were calculated. The hypothesis that CBD induces mental sedation and state-dependent learning is not supported by the data. An alternative explanation for the lack of significant results for both fear conditioning, mental sedation and state-dependency would be the low number of participants which has thus far been included in the current study, resulting in high variance in the data. With the intended sample size, expected effects of CBD may still occur.

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