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Cannabidiol modulates contextual fear memory consolidation in animals with experimentally induced type-1 diabetes *mellitus*

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Original Article

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Abstract

Objectives: In view of the neuroprotective characteristic of cannabidiol (CBD) and its beneficial action on aversive memory in non-diabetic animals, we aimed to investigate in animals with experimentally induced type-1 diabetes mellitus (T1DM) whether CBD treatment would be able to impair the contextual fear memory consolidation, its generalisation and whether the effect would be lasting. We also investigated the CBD effect on anxiety-like responses. *Methods:* After T1DM induction, animals received single or more prolonged treatment with CBD and were submitted to the contextual fear conditioning test. As expression of activity-regulated cytoskeletal-associated (Arc) protein is necessary for memory consolidation, we evaluated its expression in the dorsal hippocampus (DH). For evaluating anxiety-related responses, animals were submitted to the elevated plus maze test (EPMT), in which the time and number of entries in the open arms were used as anxiety index. *Results:* A single injection of CBD impaired the contextual fear memory consolidation and its generalisation, which was evaluated by exposing the animal in a neutral context. This single injection was able to reduce the elevated expression of Arc in the DH from these animals. Interestingly, more prolonged treatment with CBD also impaired the persistence of context-conditioned fear memory and induced an anxiolytic-like effect, as the treated group spent more time in the open arms of the EPMT. *Conclusion:* CBD interferes with contextual fear memory and the dosage regimen of treatment seems to be important. Moreover, we cannot rule out the involvement of emotional aspects in these processes related to fear memory.

Significant outcomes

- CBD improves contextual fear memory performance, *i.e.*, impairs early and late aversive memory overexpressed in STZ animals.
- A single injection of CBD in induced T1DM animals impaired the generalisation of the fear memory, not being persistent this effect.
- The more prolonged treatment with CBD impaired the persistence of exacerbated conditioned fear memory and induced an anxiolytic-like effect.

Limitations

- The study was conducted only in male *Wistar* rats.
- These studies only used one animal model of T1DM and one test of anxiety. So, the results may not be valid for other models or tests.
- The electric foot shock sensitivity in both NGL and STZ groups was not evaluated.
- A group of T1DM animal models treated with insulin plus cannabidiol could give a more translational value.

Introduction

The study of psychiatric comorbidities associated with diabetes *mellitus* (DM), the most prevalent chronic metabolic disease in the population, is a major challenge. It is known that many of

these psychopathologies are underdiagnosed or not effectively treated, which causes a further aggravation of the DM *per se* and may also facilitate the emergence of other comorbidities related to the disease (American Diabetes Association, 2018). Among psychiatric comorbidities, we highlight one of interest in the present study, which is post-traumatic stress disorder (PTSD). There are reports that DM can precipitate the development of PTSD when the individual is exposed to trauma, as well as anxiety (Dixon *et al.*, 2020; Renna *et al.*, 2016; Santos et al., 2014). The opposite also seems to be true, as studies are pointing out that patients with PTSD or anxiety also present facilitation to develop DM (Chien and Lin, 2016; Miller-Archie *et al.*, 2014).

Although type-2 diabetes *mellitus* is the most prevalent in the population (WHO, 2021; IDF, 2020), in the last two decades there has been a great increase in studies involving the type-1 diabetes *mellitus* (T1DM) which presents a prevalence around of 10% (Mobasseri *et al.*, 2020; Gomes *et al.*, 2012; Maahs *et al.*, 2010). It is important to highlight that T1DM is not preventable, unlike type-2 DM for which there are recommendations for public policies and changes in lifestyle habits aiming at a reduction of its prevalence (WHO, 2021; American Diabetes Association, 2018; PAHO, 2012). Thus, more than political advances and changes in behaviour by society, in the current scenario, there is an urgent need to deepen preclinical and clinical studies related to T1DM to reach advances in the medical field and reduce the socioeconomic impacts related to the disease.

About preclinical studies, the evidence corroborates the clinical evidence, *i.e.* studies have shown in animals with experimentally induced T1DM an overconsolidation of the contextual fear memory and/or a difficulty in extinguishing it along with generalisation of this fear response when animals are submitted to a neutral context (de Lima Silva *et al.*, 2022; Ikeda *et al.*, 2021; Ribeiro *et al.*, 2020). Also, a more pronounced anxiety-like behaviour has been noted in these induced T1DM animals (de Lima Silva *et al.*, 2022; da Silva Dias *et al.*, 2016; de Morais *et al.*, 2014, 2016; Gambeta *et al.*, 2015). At this point, it is important to reinforce that changes in several brain areas have been implicated in this impaired regulation of emotions and fear-related memory, including the hippocampus. For example, preclinical findings have demonstrated in the hippocampus from experimental T1DM animals increased neuroinflammation and oxidative stress-related parameters along with dysregulation of the neurotransmitter systems, impaired synaptic plasticity, and neurogenesis (Wang *et al.*, 2022; Waltrick *et al.*, 2022; Redivo *et al.*, 2016; da Silva Dias *et al.*, 2016; Zhao *et al.*, 2016; de Morais *et al.*, 2014, 2016). In the same direction, clinical studies showed significant changes in this same brain area of diabetic individuals associated with cognitive decline (Hamed, 2017; Moheet *et al.*, 2015), being these changes also linked to an increased predisposition to anxiety and stress-related disorders, such as PTSD (Renna *et al.*, 2016; Bystritsky *et al.*, 2014).

In view of these damages in the hippocampus, as well as in other areas of the brain, and knowing that T1DM is a disease with an inflammatory character both peripherally and in the central nervous system, a neuroprotective pharmacological approach would be desirable and it has already been suggested (for a review, see Hamed, 2017). In that sense, it has been demonstrated that cannabidiol (CBD), the major pharmacologically active phytocannabinoid that is devoid of psychotomimetic effects (Silote *et al.*, 2019; Moreira and Guimarães, 2005; Mechoulam, 1970), present neuroprotective properties (for a review, see Yousaf *et al.*, 2022; Scuderi *et al.*, 2009).

Although no study has yet studied the effects of CBD on aversive memory using animal models of T1DM, there are reports demonstrating that CBD induces protective effects in animal models of T1DM on destructive insulitis (Weiss *et al.*, 2006) and early pancreatic inflammation (Lehmann *et al.*, 2016). Also, of importance to the present study, it was demonstrated in non-diabetic animals that CBD treatment was able to impair the contextual fear memory consolidation and its generalisation (Raymundi *et al.*, 2020; Stern *et al.*, 2017; Gazarini *et al.*, 2015).

Considering the above, the present study initially aimed to investigate in animals with T1DM induced by peripheral injection of streptozotocin (STZ) whether a single injection with CBD would be able to impair the consolidation of contextual fear memory, as well as its generalisation and whether the effect was lasting. Taking into account that activity-regulated cytoskeleton-associated (Arc) protein is a product of an immediate early gene necessary for memory consolidation (Gallo *et al.*, 2018; Korb and Finkbeiner, 2011; Plath *et al.*, 2006), we evaluated whether CBD would be able to change the expression of this protein in STZ animals in the dorsal hippocampus (DH). Based on the findings, the next set of experiments was carried out in STZ animals receiving a more prolonged treatment (1 week) with CBD to evaluate whether CBD would induce a more lasting effect on contextual fear memory and its generalisation, as well as on a more pronounced anxiety-like response.

Material and methods

Ethical statement

All experiments were conducted in accordance with the rules and legislation contained by the UFPR Animal Research Ethics Committee (CEUA/BIO-UFPR, number #1390) with the consistency of ethical principles of the National Council for Control of Animal Experimentation (CONCEA). All efforts were made to optimize the number of animals used and to reduce their stress and suffering.

Animals

Male *Wistar* rats (180–200 g, age 45 days) were supplied by the Central *vivarium* of the Biological Sciences Sector of the Federal University of Paraná (UFPR). A total number of 101 rats were used, and the number of animals used for every experiment was determined based *on priori* sample size calculations performed with G * Power programme. The animals were randomly allocated in groups of four in plexiglass cages and had free access to water and food and were kept under a 12-hour light/dark cycle (7:00 am to 7:00 pm) and controlled temperature (at 22 ± 1°C).

Drugs

Streptozotocin (STZ, 60 mg/kg, i.p., Santa Cruz Biotechnology Inc., USA), sodium citrate (Merck SA, Brazil) and CBD (10, 30, 60 mg/kg, i.p., 99.6% pure provided by BSPG-Pharm, Sandwich, United Kingdom). STZ was freshly dissolved in citrate buffer (10 mM, pH 4.5), whereas CBD was diluted in 2% Tween 80 and 98% saline. Control group received vehicle (VEH; 2% tween 80 and 98% saline). The doses were chosen based on the previous works (Chaves *et al.*, 2021; de Gregorio *et al.*, 2019; de Morais *et al.*, 2014, 2016, 2018; Stern *et al.*, 2017; Jesus *et al.*, 2019).

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Induction of T1DM

The experimental T1DM was induced by a single administration of STZ in rats that previously fasted for 12 hours. Hyperglycaemia was confirmed 3 days after STZ injection, by applying a small volume of peripheral blood collected from the animals' tails (5 µl) on test tapes impregnated with glucose oxidase (Accu-Check ActiveTM, Roche) and performed again at the end of behavioural tests. Animals with blood glucose equal to or greater than 250 mg/dL were considered experimentally induced T1DM (STZ animals) and kept in the experimental groups. In parallel, we used as a control group of the diabetic condition, the normoglycaemic (NGL) animals which received only citrate buffer (10 mM, pH 4.5, equivalent volume) (Chaves *et al.*, 2020, 2021; de Morais *et al.*, 2014, 2016).

Contextual fear conditioning (CFC) test

The apparatus used in the experiment consisted of a rectangular chamber (context A) with three steel sidewalls and a front and ceiling door made of plexiglass acrylic (26 × 31.5 × 21 cm; Insight, Ribeirão Preto, SP, Brazil). The bottom of the box consisted of small metal bars attached to a circuit board and a shock generator to enable the delivery of controlled electrical footshocks as detailed subsequently. For the generalisation test, to offer contextual cues as different as possible from context A, we used a second chamber (context B) that consisted of four sidewalls made of plexiglass transparent acrylic (30 × 30 × 30 cm) (Ribeiro *et al.*, 2020; de Lima Silva et al., 2022). Between each animal, the chamber was cleaned with a 20% alcohol solution. The CFC test obeyed the following steps (the 1st day being the day 26 after the confirmation of the diabetic condition):

1st day (day 26) – familiarisation

The animals were placed in context A to explore it for 3 min and returned to their home cage afterward.

2nd day (day 27) – CFC session

Context A becomes a conditioned stimulus (CS) with the presentation of the unconditioned aversive stimulus (US): The animals were placed in context A, and after 30 s, they received three electrical footshocks (US; 0.8 mA, lasting 3 s), with 30 s of intertrial intervals. The animals remained in this chamber for 30 s more before being returned to their home cage.

3rd day (day 28) – Test A1

The animals were placed in context A (CS) and remained there for 3 min without the presence of the US.

4th day (day 29) – Test B1 (generalization test)

The animals were placed in context B and remained there for 3 min.

10th day (day 35) – Test A2

After 7 days of the CFC, the animals were placed into context A and remained for 3 min without the presence of the US to investigate the persistence of the conditioned fear memory.

11th day (day 36) – Test B2

The animals were placed in context B for 3 min to investigate the persistence of the fear memory generalisation.

In all tests, the time the animal remained in freezing was quantified in seconds (s). The freezing behaviour of each animal was used as an aversive conditioning index. An animal was considered freezing when it presented a stereotyped position with complete immobility, except for breathing movements. The freezing time was expressed as a percentage (%) of the total session time. Freezing behaviour was measured by a trained observer blind to the treatments.

Elevated plus maze test (EPMT)

The potential anxiolytic-like effect of all treatments was assessed using the EPMT as described by Pellow *et al.* (1985). The apparatus was made of wood and was 50 cm from the floor, consisting of 4 arms, being 2 open and 2 closed and at the cross between the arms, and there was a central area of 10 cm^2 under incandescent light (40 W-60 Lx). Each animal was placed in the centre of the apparatus facing a closed arm and the session had a duration of 5 min. As anxiety index, we evaluated the open arm time and entries in these same arms in percentage (%): % of open arm entries (%OAE = 100 × open arm entries/total entries) and % of time spent on the open arms (%OAT = 100 × time spent on open arms/[time spent on open arms + time spent on closed arms]). As index of locomotor activity, the number of entries in the enclosed arms was quantified. Between each session, the elevated plus maze was cleaned with a 20% alcohol solution.

Western blotting for analysis of Arc expression

The DH was quickly removed and stored at −80°C. The DH from a group of non-STZ and STZ animals not submitted to the CFC session was used to record the basal expression of the Arc protein. For protein extraction, the tissues were homogenised in 0.6 ml of solubilisation buffer (10 mM EDTA, 100 mM Tris pH 7.5, 0.2% protease inhibitor cocktail [PROMEGA], and 1% Triton X-100). Insoluble material was removed by centrifugation (20 min, 10,000 rpm, 4 °C). The supernatant protein concentration was determined colorimetrically (Bradford Protein Assay, Bio-Rad). Tissue extracts (500 µl) were denatured in boiling water for 5 min in Laemmli buffer containing 200 mM of DTT. Protein extracts were separated by SDS-PAGE, transferred onto a nitrocellulose membrane (0.45 µm; BIO-RAD), blocked with basal solution (20-mM Tris pH 7.6, 137-mM NaCl, and 0.025% Tween® 20) containing 3% BSA (Sigma, USA) for 2 h, and then incubated with monoclonal primary antibody anti-Arc 1:500 (Santa Cruz Biotechnology Cat# sc-17839, RRID:AB_626696) overnight and secondary antibody anti-mouse 1:5,000 (Santa Cruz Biotechnology Cat# sc-516102, RRID:AB_2687626) for 1 h. For evaluation of protein loading, all membranes were stripped and reblotted with monoclonal primary anti-GAPDH antibody 1:500 (Santa Cruz Biotechnology Cat# sc-134237, RRID:AB_2212295). After incubation with the appropriate secondary antibody conjugated with Western ECL Substrate (Bio-Rad), membranes were developed by chemiluminescence. Quantitative analysis was performed by densitometry using Scion Image software (Scion Corporation, USA). The intensities were normalised to corresponding values for GAPDH expression (Arc value of the sample of interest * 100/GAPDH value of the sample of interest) and expressed with relative value to the basal expression (NGL group expression) (normalization value between Arc and GAPDH of the sample of interest * 100/mean of normalisation value of the NGL/VEH group) (Raymundi *et al.*, 2020).

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Experimental design

All experimental sessions were recorded using a Sony® action cam 4 K for posterior analysis and performed by experimenters (Y.C.C., A.M.R, and A.P.F.W.) blinded to the treatment conditions. In all experiments, as a control of the diabetic condition *per se*, we check the confirmation of the hyperglycaemia at the end of behavioural tests and also calculate the body weight gain, by subtracting the body weight taken on the last day of the experiment from that taken on day 0. All behavioural tests were performed in the afternoon period (12–18 p.m.) and the animals were randomly allocated to groups based on the treatments.

- Experiment 1: We aimed to evaluate in STZ animals the effects of a single injection of CBD on the consolidation of fear memory, its generalisation, and persistence. For that, five groups were formed: NGL animals treated with vehicle (VEH) - NGL/VEH ($n = 7$), and STZ groups treated with VEH or CBD: STZ/VEH ($n = 7$), STZ/CBD (10 mg/kg, i.p.; $n = 8$), STZ/CBD (30 mg/kg, i.p.; $n = 7$), and STZ/CBD (60 mg/kg, i.p.; $n = 6$). The animals were treated in the fourth week after hyperglycaemia confirmation (day 27) immediately after the CFC session and were submitted to the tests in the subsequent days, as already described above in detail.

- Experiment 2: We investigated the possible effects of the CBD given immediately after CFC on short-term fear memory, according to Stern *et al.* (2017). For that, three groups were performed: NGL/VEH ($n = 6$), and STZ groups treated with VEH ($n = 6$) or CBD (60 mg/kg, i.p; $n = 6$). The animals received a single injection of CBD immediately after the CFC session (day 27 after hyperglycaemia confirmation and 3 h later were submitted to test A1 followed by test B1, 30 min later).

- Experiment 3: This experiment was designed to investigate whether a single injection of CBD administered immediately after CFC (day 27 after hyperglycaemia confirmation) would interfere with the contextual fear memory consolidation by analysing the expression of Arc protein in the DH. Thus, the following four groups were performed: NGL animals (conditioned) treated with VEH ($n = 5$), STZ animals non-conditioned (naïve; $n = 6$), STZ animals (conditioned) treated with VEH ($n = 6$), or CBD (60 mg/kg, i.p.; $n = 4$)). The animals were euthanized by decapitation 120 min after treatment.

- Experiments 4 and 5: In this experiment, we studied whether a sub-chronic treatment with CBD would induce a better effect on the increased fear response related to CFC, its generalisation, and persistence. So, the first injection of CBD was administered immediately after CFC (day 27 after diabetic condition confirmation) and the subsequent injection with CBD was made on days 30 to 36 (with an interval of at least 7 h after tests A1 and B2 on days 35 and 36). All groups of animals – NGL animals treated with VEH ($n = 6$), STZ animals treated with VEH ($n = 7$), CBD (30 mg/kg, i.p.; $n = 7$), or CBD (60 mg/kg, i.p.; $n = 7$) – were submitted to the CFC protocol, as already described. Twenty-four hours after test B2 (day 36), given that an anxiolytic-like effect could alter the freezing behaviour in the chambers (contexts A and B), we evaluated the effects of the drug on the EPMT (day 37).

Statistical analysis

It is important to mention that two important exclusion criteria were previously determined. Thus, animals were excluded from the statistical analysis when: 1. at the end of each experiment the hyperglycaemia was totally reversed and, 2. based on the animal's welfare: when animals remained more isolated in their respective home cages (low interaction or activity), in addition to piloerection, diarrhoea, and exaggerated weight loss. In these cases, the number of animals per group was different in some (few) groups.

Shapiro–Wilk normality test was applied to ensure that the data met the criteria for performing parametric tests. Once the criteria were accepted, the results were expressed as mean ± 95% confidence interval (CI). Initially, we performed a two-way analysis of variance (ANOVA) considering as two factors the different groups and the tests (A1 and A2 or B1 and B2). Once there was no interaction between the two factors, we applied one-way ANOVA for each test separately (A1, A2, B1 or B2). The ANOVA was performed between all STZ groups treated with vehicles and drugs, considering the treatments (different treatment groups) as a single independent factor. Importantly, except for experiment 3, we performed Student's *t*-test between the NGL and STZ groups treated with VEH, in order to assess whether the experimental induction of T1DM actually occurred (on weight gain and glycaemia parameters) and whether the behavioural findings would be reproduced according to previous data (Ribeiro *et al.*, 2020; de Lima Silva *et al.*, 2022). In relation to experiment 3, one-way ANOVA was performed including all groups, being considered as one factor the different groups.

When appropriate, Newman–Keuls test was used for *post-hoc* analysis. The differences were considered statistically significant when $p \leq 0.05$. The data were analysed using Graph Pad Prism Software 7.0.

Results

Experiment 1: Effects of a single injection of CBD in STZ animals on the consolidation of fear memory, its generalisation, and persistence

As shown in Fig 1A, Student's *t*-test showed a difference between the NGL/VEH and STZ/VEH animals in the freezing time ([$t = 4.151$; $df = 12$; $p < 0.05$; test A1], [$t = 3.42$; $df = 12$; $p < 0.05$; test B1], [$t = 2.795$; $df = 12$; $p < 0.05$; test A2] and [$t = 3.209$; $df = 12$; $p < 0.05$; test B2]), *i.e.* STZ animals presented an increased freezing time. When all groups of STZ animals were analysed, one-way ANOVA showed that the treatment was able to change the freezing time only during tests A1 and B1 [$F(3, 24) = 3.32$; $p < 0.05$; test A1] and [$F(4, 24) = 3.11$; $p < 0.05$; test B1]. The Newman–Keuls *post-hoc* test showed CBD (60 mg/kg) decreased the freezing time in both contexts – conditioned and neutral ($p < 0.05$). In Fig. 1B, Student's *t*-test showed a difference between the NGL/VEH and STZ/VEH animals in the discrimination index A1/B1 [$t = 7.678$; $df = 12$; $p < 0.05$; tests A1/B1] and A2/B2 [$t = 5.054$; $df = 12$; $p < 0.05$; tests B1/B2]. When all groups of STZ animals were analysed, one-way ANOVA showed that the treatment was able to increase this index only in the A1/B1 [$F(3,24) = 2.94$; $p \leq 0.05$; tests A1/B1]. The Newman–Keuls *post-hoc* test showed that CBD (60 mg/kg) increased the discrimination index ($p < 0.05$).

Student's *t*-test showed a difference between NGL/VEH and STZ/VEH animals when blood glucose [$t = 14.22$; $df = 12$; $p < 0.05$] and weight gain [$t = 2.97$; $df = 12$; $p < 0.05$] were evaluated. When STZ groups were analysed, one-way ANOVA revealed that the treatment did not alter these parameters (see Table S1, supplementary material).

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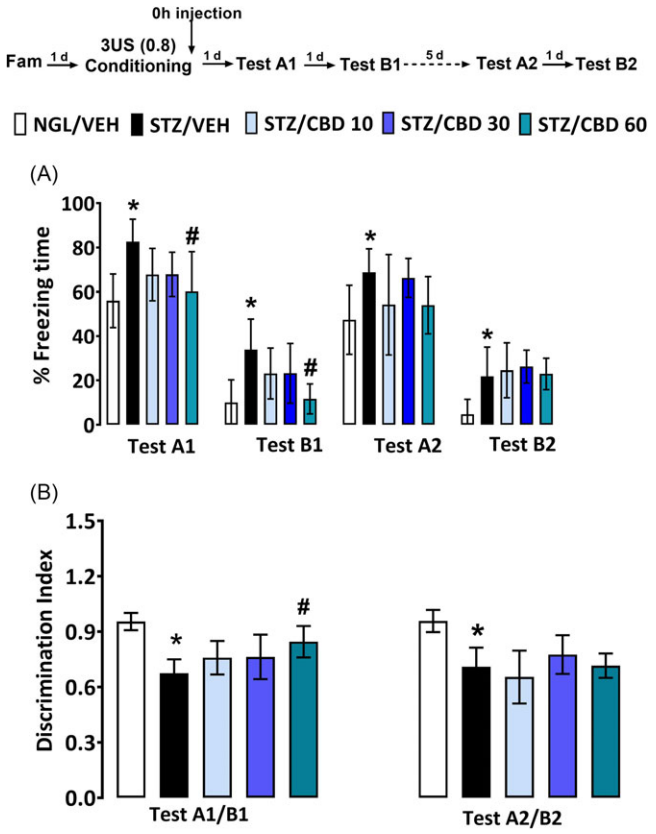

Fig. 1. Effect of a single injection with cannabidiol (CBD; 10, 30, 60 mg/kg, ip) or vehicle (VEH) immediately after the conditioning session – evaluation of the treatment on consolidation of the fear memory (test A1), generalisation (test B1), and persistence (test A2 and B2; panel A). Panel B represents the calculation of the discrimination index. Values were expressed as mean ± 95% CI ($n = 6$–7). *$p < 0.05$ when compared to NGL animals treated with VEH (NGL/VEH); #$p < 0.05$ when compared to STZ animals treated with VEH (STZ/VEH).

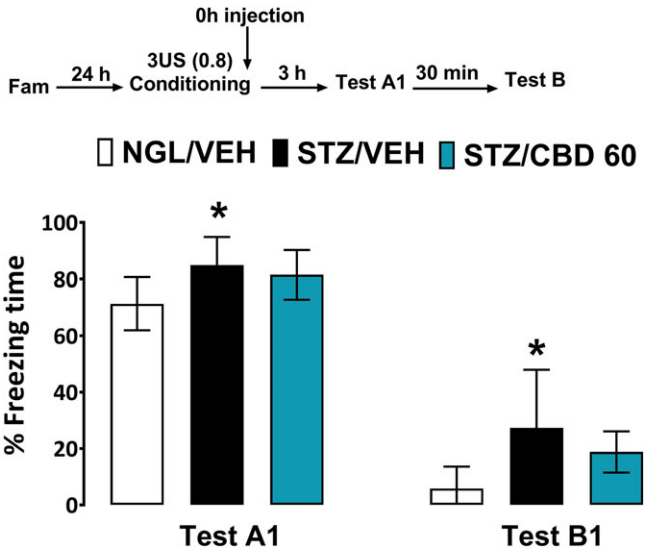

Fig. 2. Effect of a single injection with cannabidiol (CBD; 60 mg/kg, ip) or vehicle (VEH) on short-term fear memory – evaluation of the treatment on consolidation of the fear memory (test A1) and its generalisation (test B1). Values were expressed as mean ± 95% CI ($n = 5$–6). *$p < 0.05$ when compared to NGL animals treated with VEH (NGL/VEH); #$p < 0.05$ when compared to STZ animals treated with VEH (STZ/VEH).

Experiment 2: Effects of a single injection of CBD in STZ animals on short-term fear memory

As shown in Fig. 2, one-way ANOVA showed a significant difference between the groups: [F (2, 15) = 3.779; $p < 0.05$; test A1] and [F (2, 15) = 4.309; $p < 0.05$; test B1]. Multiple comparisons showed that STZ/VEH presented an increased freezing time compared to NGL/VEH ($p < 0.05$) and the treatment with CBD did not alter this parameter in STZ animals ($p > 0.05$) neither in A1 nor B1.

One-way ANOVA showed a difference between the groups when blood glucose [F (2, 15) = 128.8; $p < 0.05$] and weight gain [F (2, 15) = 19.91; $p < 0.05$] were evaluated. *Post-hoc* analysis showed a difference between all STZ groups with NGL/VEH ($p < 0.05$) (see Table S1, supplementary material).

Experiment 3: Effects of a single injection of CBD on the expression of Arc protein in the DH of STZ animals

As can be seen in Fig. 3, one-way ANOVA showed a difference between the groups [F (3, 17) = 5.62; $p < 0.05$]. Newman–Keuls *post-hoc* test showed a difference between all STZ groups with NGL/VEH ($p < 0.05$). Moreover, a decrease in the Arc expression into DH from all STZ animals ($p < 0.05$).

Experiment 4 and 5: Effects of sub-chronic treatment with CBD (30 or 60 mg/kg) in STZ animals on processes related to fear memory and anxiety-like behaviour

As shown in Fig. 4A, Student's *t*-test showed a difference between the NGL/VEH and STZ/VEH animals in the freezing time ([$t = 3.644$; df = 11; $p < 0.05$; test A1], B1 [$t = 10.75$; df = 11; $p < 0.05$; test B1], [$t = 6.26$; df = 11; $p < 0.05$; test A2], and [$t = 3.627$; df = 11; $p < 0.05$; test B2]), *i.e.* STZ/VEH animals spent more time in freezing behaviour. When all groups of STZ animals were analysed, one-way ANOVA showed that the treatment was able to change the freezing time during tests B1 and A2: [F (2,18) = 5.636; $p < 0.05$; test B1] and A2 [F (2,18) = 5.753; $p < 0.05$; test A2]. The Newman–Keuls *post-hoc* test showed that CBD (60 mg/kg) decreased the freezing time ($p < 0.05$) in the neutral context (test B1) and in the conditioned context (test A2). In Fig. 4B, Student's *t*-test showed a difference between the NGL/VEH and STZ/VEH animals in the discrimination index A1/B1 [$t = 10.23$; df = 11; $p < 0.05$; tests A1/B1] and A2/B2 [$t = 4.416$; df = 11; $p < 0.05$; tests A2/B2]. In that situation, STZ animals presented a reduction in this index, compared to NGL animals. When all groups of STZ animals were analysed, one-way ANOVA showed that the treatment was able to change the discrimination index A1/B1 [F (2,18) = 7.745; $p < 0.05$; tests A1/B1] and A2/B2 [F (2,18) = 5.98; $p < 0.05$; tests A2/B2]. The Newman–Keuls *post-hoc* test showed that STZ animals treated with CBD (60 mg/kg) increased the discrimination index in both cases, compared to STZ/VEH group ($p < 0.05$).

As observed in Fig. 5 (panels A, B and C), the Student's *t*-test showed a difference between the NGL/VEH and STZ/VEH animals in the time spent in the open arms [panel A: $t = 3.552$; df = 11; $p < 0.05$], the number of entries in the open arms [panel B: $t = 2.493$; df = 11; $p < 0.05$], and the number of total entries into the arms [panel C: $t = 4.437$; df = 11; $p < 0.05$]. When only the group of STZ animals was analysed, one-way ANOVA showed that the treatment was able to change the time spent in the open arms [panel A: F (2,18) = 6.358; $p < 0.05$], the number

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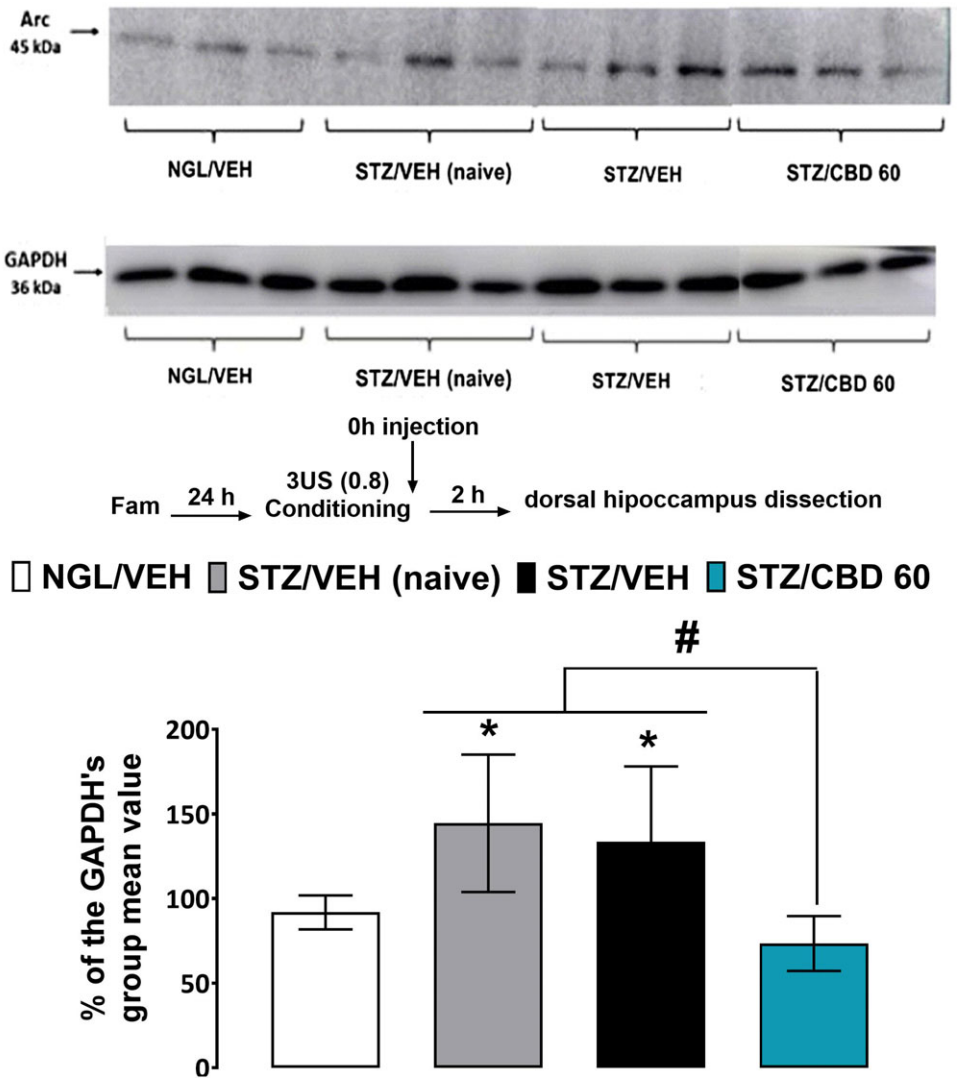

Fig. 3. Effect of a single injection with cannabidiol (CBD; 60 mg/kg, ip) or vehicle (VEH) immediately after the conditioning session on the expression of Arc protein in the dorsal hippocampus (DH). Values were expressed as mean ± 95% CI ($n = 6$-7). *$p < 0.05$ when compared to NGL animals treated with VEH (NGL/VEH).

of entries in the open arms [panel B: $F(2,18) = 4.86$; $p < 0.05$], and the number of total entries into open and closed arms [panel C: $F(2,18) = 7.07$; $p < 0.05$]. The Newman–Keuls *post-hoc* test showed that CBD increased the time spent in the open arms ($p < 0.05$) and the number of entries in the open arms ($p < 0.05$), indicative of an anxiolytic-like effect. Also, the treatment increased the number of total entries into open and closed arms, indicating an improvement in exploratory activity ($p < 0.05$).

Student's *t*-test showed a difference between NGL/VEH and STZ/VEH animals when blood glucose [$t = 13.59$; $df = 11$; $p < 0.05$] and weight gain [$t = 2.44$; $df = 11$; $p < 0.05$] were evaluated. When STZ groups were analysed, one-way ANOVA revealed that the treatment significantly altered weight gain [$F(2.31) = 3.453$; $p < 0.05$]. The Newman–Keuls *post-hoc* test showed that CBD (60 mg/kg) decreased the weight gain ($p < 0.05$) without changing the blood glucose (see Table S1, supplementary material).

Discussion

This study was originally designed to examine, for the first time, in STZ animals the effects of CBD on the consolidation and generalisation of contextual fear memory, as well as the persistence of effects.

Regarding STZ animals, these animals when submitted to the CFC test presented a greater expression of freezing behaviour compared to NGL animals in both contexts – the conditioned (test A1) and the neutral (test B1). This increase in the expression of conditioned fear and its generalisation was persistent as it was maintained after the animals were re-tested 1 week later (tests A2 and B2, Fig. 1A). This result shows a more pronounced conditioned fear memory response associated with a generalisation of this fear response corroborates previous evidence from our lab and others (Ribeiro *et al.*, 2020; de Lima Silva *et al.*, 2022; de Souza *et al.*, 2018; Ikeda *et al.*, 2015, 2021). Moreover, this fear memory seems to be quite resistant, as we previously demonstrated that these STZ animals presented this same pronounced freezing response even during and after an extinction training session (de Lima Silva *et al.*, 2022; Waltrick *et al.*, 2022; Ribeiro *et al.*, 2020; de Souza *et al.*, 2018; Gambeta *et al.*, 2015). Important to highlight that our data demonstrated for the first time the persistence of these effects, which were maintained up to 7 days after the first tests (tests A1 and B1). Thus, it is plausible to speculate that STZ animals present an overconsolidation of the fear memory. With that in mind, we designed a study to evaluate the expression of the Arc protein in DH.

It is known that the Arc protein is a product of an immediate early gene necessary for memory consolidation (Gallo *et al.*, 2018;

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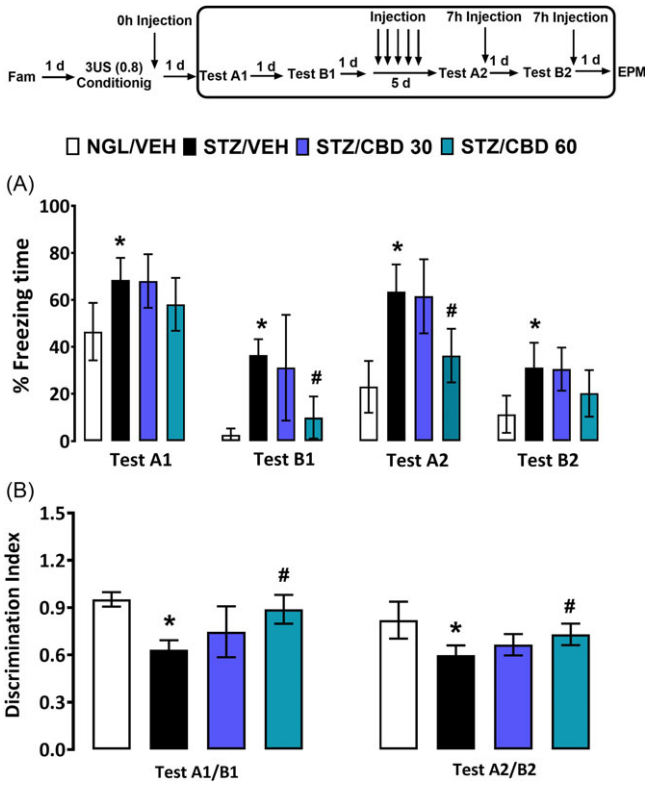

Fig. 4. Effect of sub-chronic (7 days between A1/B1 and A2/B2) treatment with cannabidiol (CBD; 30, 60 mg/kg, ip), or vehicle (VEH) – evaluation of the treatment on consolidation of the fear memory (test A1), generalisation (test B1), and persistence (Test A2 and B2; panel A). Panel B represents the calculation of the discrimination index. Values were expressed as mean ± 95% CI ($n = 6$–7). *$p < 0.05$ when compared to NGL animals treated with VEH (NGL/VEH); #$p < 0.05$ when compared to STZ animals treated with VEH (STZ/VEH).

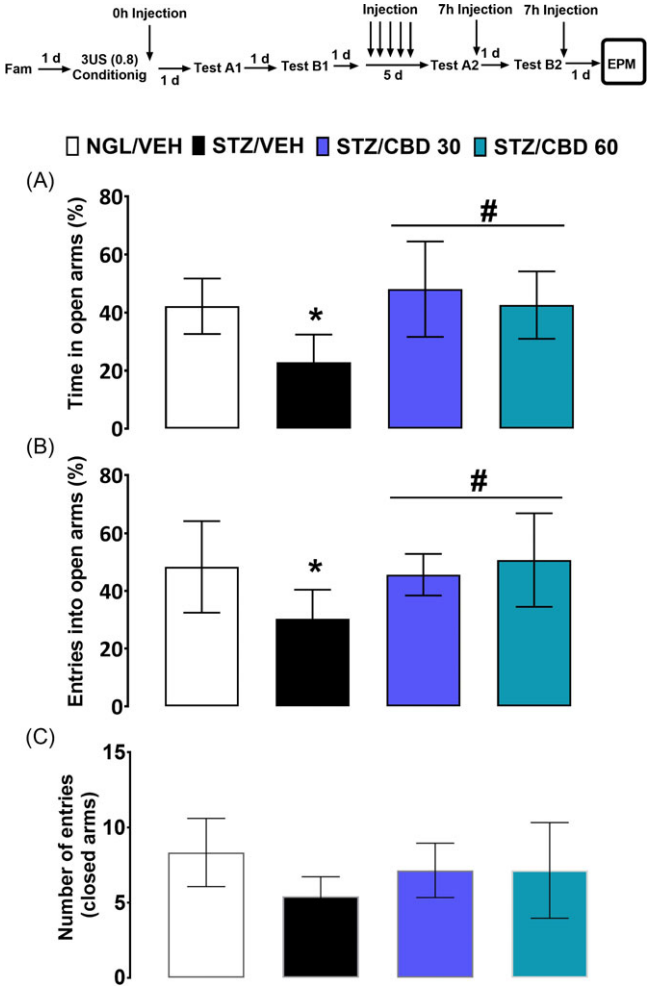

Fig. 5. Effect of sub-chronic (7 days between A1/B1 and A2/B2) treatment with cannabidiol (CBD; 30, 60 mg/kg, ip), or vehicle (VEH) on anxiety-like behaviour – evaluation of time in the open arms (%, panel A), entries into open arms (%, panel B), and total entries into arms (%, open + closed, panel C) of STZ or NGL animals submitted to EPMT. Values were expressed as mean ± 95% CI ($n = 6$–7). * = $p < 0.05$ when compared to NGL animals treated with VEH; # = $p < 0.05$ when compared to STZ animals treated with VEH.

Korb and Finkbeiner, 2011; Plath *et al.*, 2006). In addition, several studies show the relationship between increased Arc expression with activation/plasticity of brain areas associated with memory consolidation, like DH (Raymundi *et al.*, 2020; Gouty-Colomer *et al.*, 2016; Besnard *et al.*, 2014; Lonergan *et al.*, 2010), as well as its involvement in the persistence of aversive memory, mainly through the second wave of its expression commonly reported after 12 h of memory reactivation (da Silva *et al.*, 2020; Nakayama *et al.*, 2015). Our results demonstrated an increase in the expression of the Arc in STZ animals (naïve and conditioned), compared to NGL animals (conditioned), indicating a facilitation of synaptic plasticity. This finding is according to our premises that these animals present an overconsolidation of the fear memory (Fig. 3). Here, it is worth mentioning that considering that STZ animals present mechanical allodynia (Jesus *et al.*, 2019; Gasparin *et al.*, 2021) and that we did not assess the sensitivity specifically to footshock in these animals, we cannot rule out the interference from the neuropathic pain in the processing of the contextual fear memory consolidation. Furthermore, the increased Arc protein expression in the DH from these STZ animals (naive or treated with VEH) could also be a reflection of the interference of the neuropathic pain in the processing of contextual fear memory consolidation.

In this way, it is evident the importance of deepening the studies in the search for a better understanding of the complexity of the pathophysiology of the T1DM, as well as for a more effective treatment that can reverse or delaying in some way the emergence of these impairments in learning/memory processes.

In that sense, it has been demonstrated that CBD presents neuroprotective properties (for a review, see Yousaf *et al.*, 2022; Scuderi *et al.*, 2009) and also induces protective effects in animal models of T1DM on destructive insulitis (Weiss *et al.*, 2006) and early pancreatic inflammation (Lehmann *et al.*, 2016). Of importance to the present study, studies conducted with non-diabetic animals have shown beneficial evidence of CBD in dealing with the impairment in fear memory formation and in facilitating or restoring learning (Raymundi *et al.*, 2020; Uhernik *et al.*, 2018; Stern *et al.*, 2012, 2017). Thus, these studies demonstrated that CBD disrupts the consolidation and generalisation of this type of memory, in addition to facilitating its extinction memory formation. The authors propose that fear overgeneralisation is a process associated with the consolidation of memory and loss of memory specificity which indeed is resultant of a transference of the conditioned response to stimuli that perceptually differ from the original CS (Bergstrom, 2020). Also, even when other types of memory were studied, CBD demonstrated beneficial effects, such as improvement on cognition when animals were submitted to the social preference or recognition tests and novel object recognition

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task (Assareh *et al.*, 2020; Shallcross *et al.*, 2019; Song *et al.*, 2016; Cheng *et al.*, 2014a, 2014b; Das *et al.*, 2013; Bitencourt *et al.*, 2008).

Given that no study has investigated the effects of CBD specifically on contextual fear memory in STZ animals, as well as on other types of memories; in this study, we aimed to investigate the effect of CBD on contextual fear memory using an animal model of T1DM. We initiated the studies by performing a single administration of CBD. Our data show that a single injection of the highest dose of CBD immediately after a CFC session, which is within the fear memory consolidation window (Lamprecht & LeDoux, 2004; McGaugh, 2000), impaired the consolidation of the contextual fear memory. That is, CBD, in addition to decreasing the elevated expression of Arc in the DH of STZ rats (Fig. 3), decreased the freezing time of animals when exposed to the A1 and B1 tests. When analysed the discrimination index (Fig. 1B), we showed that CBD induced an increase in this index (A1/B1), which allows us to assume that a single injection of CBD may exert some biologically significant effect on improving learning in that diabetic condition. All these beneficial actions of a single injection of CBD on contextual fear memory consolidation, generalisation and Arc expression in the DH had already been demonstrated, but in non-diabetic animals (Raymundi et al., 2020) and with a lower dose (10 mg/kg) of CBD. As our data did not show any change of a single injection of CBD on short-term memory, which is impaired in these STZ animals (Fig. 2), we conclude that CBD may be acting preferentially in long-lasting memory processes.

Here, it is important to consider previous data from our group demonstrating that: (1). a single injection of CBD induced an antinociceptive effect over mechanical allodynia in STZ rats, which was transient peaking as early as 1 h post-injection, still apparent at 2 h and absent at the third hour, and that (2). the memory consolidation time window lasts 6 h (McGaugh, 2000). Thus, we can consider the hypothesis that emotional comfort related to a decrease in the neuropathic pain may have interfered in the processing of contextual fear memory consolidation. In fact, as our data demonstrated that the animal treated with a single injection of CBD decreased the fear response to the context in test A1, as well as the generalisation of this fear response (test B1), we cannot rule out this possibility. The same may be true for the findings related to the Arc expression in DH which was reduced after CBD injection. As the hippocampus was dissected 2 h after the injection of VEH or CBD, we are also unable to infer with certainty that the action of CBD under Arc expression reduction is independent of neuropathic pain relief.

Despite these beneficial effects observed during tests A1 and B1, our findings did not show persistent effects of CBD when STZ animals were re-tested 5 to 6 days later in tests A2 and B2, respectively (see Fig. 1A), unlike what was observed by Raymundi et al. (2020) in non-diabetic animals. This difference between non-diabetic and STZ animals after acute treatment with CBD is not surprising as several studies demonstrate that experimental T1DM induces encephalopathy in these animals characterised by increased oxidative stress and neuroinflammation, dysregulation of the neurotransmitter systems, impaired synaptic plasticity, and neurogenesis (Waltrick *et al.*, 2022: Wang *et al.*, 2022; Redivo *et al.*, 2016; da Silva Dias *et al.*, 2016; Zhao *et al.*, 2016; de Morais *et al.*, 2014, 2016). In addition, studies have already reported that the endogenous cannabinoid system of STZ animals seems to be dysregulated (Duarte *et al.*, 2007; de Morais *et al.*, 2016). For example, it was observed an elevated density of the cannabinoid type-1 receptor (CB1R) and also of CB1R binding sites in the hippocampus (Duarte *et al.*, 2007). Also, our group demonstrated an increase in the expression of CB1R in the hippocampus (de Morais *et al.*, 2016). Taken together, based on these complexities related to DM, a longer treatment would be necessary for a beneficial effect to occur.

In the next set of experiments, we performed a continued treatment with CBD in the week preceding the A2 and B2 tests. Although we did not observe a statistically significant effect on freezing time in the treated STZ animals during test A1, we confirmed in test B1 that treatment decreased the freezing time (Fig. 4A). Furthermore, as in Experiment 1, the discrimination index was increased (Fig. 4B). Interestingly, the continued or sub-chronic treatment with CBD, differently from acute treatment, at the highest dose decreased the freezing time of STZ animals when re-exposed to the same context 7 days after conditioning (test A2, Fig. 4A). Although there is a visual tendency, we did not observe a significant statistical effect during the B2 test, as observed previously when animals received only a single injection of CBD 1 week later. However, when we calculate the discrimination index, we can see more clearly that the sub-chronic treatment increased this parameter when the animals were re-exposed to the contexts, indicating that somehow CBD is acting in a beneficial way on the generalisation of fear memory and also on its persistence. In this case, the generalisation cannot be considered as a false memory because, what differentiates both is precisely the details of cues, which in false memory are necessary to replace the original memory and in generalisation, the new details do not influence the aversive response, because precisely the lowest similarity between the paired contexts and neutral cause the individual to express aversive behaviour, as seen mainly with hyperglycaemic animals (Bergstrom, 2020; Rovee-Collier and Gulya, 2000). The details that in this case could differentiate both contexts were not relevant for the animal to express the specificity of the fear memory, leading to this defensive behaviour, but that interfered with CBD. Considering that an anxiolytic-like effect has already been demonstrated in STZ animals after sub-chronic treatment with CBD (Chaves *et al.*, 2020, 2021), we cannot state that these effects are due to an exclusive improvement in the processing of the contextual fear memory.

To confirm whether the effects induced by sub-chronic treatment with CBD are related or not to emotional processes, this same sub-chronically treated STZ animals were subjected to the EPMT, 24 h after the B2 test (and at least 18 h after the last CBD treatment). Our data showed that both doses – 30 and 60 mg/kg – were able to reverse the anxious-like behaviour of these animals (Fig. 5). Thus, as previously demonstrated (Chaves *et al.*, 2020, 2021), our data confirm that CBD acts by relieving emotional behaviour such as anxiety. But it is noteworthy that the lowest dose of CBD was able to induce an anxiolytic-like effect, without inducing an improvement in the consolidation or generalisation of fear memory and its persistence in these animals. Thus, the anxiolytic-like effect induced by CBD is important, but it does not seem to be the only factor associated with the improvement induced by CBD on the discrimination between neutral and aversive contexts in these animals, because there was no improvement in these parameters with the lowest dose of CBD (30 mg/ kg).

Based on the above and considering data already published, it is evident the difference between non-diabetic animals and STZ animals in the processing of emotions, as well as in the effect of drugs. However, it is important to emphasise that, unlike what was observed in the present study, a few reports involving preclinical studies have already demonstrated a significant improvement in the memory performance when it comes to

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non-diabetic individuals treated with CBD (Raymundi *et al*., 2020; Coles *et al*., 2020; Osborne et al., 2017; Stern *et al*., 2012). As this is the first study conducted in experimentally induced T1DM animals involving CBD treatment and its effects on contextual fear memory performance, it is important that these data be confirmed.

Regarding to diabetic conditions, it is important to highlight that CBD, acute, or sub-chronic did not interfere with the hyperglycaemia of the STZ animals. But, during the sub-chronic treatment, CBD (at the highest dose) further reduced the weight gain, compared to VEH-treated STZ animals (see Table 1experiments 4/5 at the supplementary material). Here, it is important to highlight that several studies reinforce this animal model with STZ as a well-validated T1DM model, in which significant physiological changes occur, such as changes in the body weight, intake of solid foods, and water, in addition to changes in hormones, like a reduction of insulin which is greatly reduced in these animals (Smith and Gannon, 1991; Wu and Yan, 2015; Chaves *et al*., 2020). This worsening of the already reduced weight gain seen after CBD treatment could be a side effect of this dose or random data and need to be better checked in future experiments. The modulation of weight gain through the endocannabinoid system, mainly through the antagonism of cannabinoid receptors, has already been widely reported even in the clinic, with the drug rimonabant, which has already been approved but is currently no longer in circulation due to its induction of depression and anxiety (Le Foll *et al*., 2013). Despite the non-property of CBD to antagonise such receptors, studies focusing on the inverted U-shaped dose–response curve of this compound show that higher doses of CBD, when administered, can activate the transient receptor potential vanilloid subfamily 1 (TRPV1), the latter also participating in enhancing metabolism and energy expenditure, a possible explanation for this effect (Linares *et al*., 2019; Baskaran *et al*., 2017; Varghese *et al*., 2017; Zheng *et al*., 2017; Campos *et al*., 2012).

In conclusion, our findings demonstrate that CBD improves contextual fear memory performance, *i.e.*, impairs early and late aversive memory overexpressed in STZ animals. For that, the choice of the type of treatment, if acute or sub-chronic, seems to be important. Once the treatment induces an anxiolytic-like effect, the emotional process cannot be discarded on these effects. Finally, further studies need to be conducted in animals with DM induced experimentally to better understand and discriminate the action of CBD on emotional and pain-related behaviours and memory associated or not with fear.

Supplementary material. To view supplementary material for this article, please visit https://doi.org/10.1017/neu.2023.13

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Author contributors. Janaina Menezes Zanoveli designed the study conception. Material preparation and data collection were performed by Yane Costa Chaves, Ana Maria Raymundi, Ana Paula Farias Waltrick, and Cristina Aparecida Jark Stern. Statistical analysis was performed by Janaina Menezes Zanoveli. The first draft of the manuscript was written by Yane Costa Chaves, while the following versions with English revisions were made by Janaina Menezes Zanoveli and Joice Maria da Cunha. The CBD was donated by José Alexandre Crippa. All authors commented on previous versions of the manuscript and read and approved the final manuscript.

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Conflict of interest. José Alexandre Crippa reported receiving grants from the National Institute of Translational Science and Technology in Medicine and personal fees from the National Council for Scientific and Technological Development (CNPq 1A) during the conduct of the study, being a co-owner of a patent for fluorinated CBD compounds (licensed to Phytecs) and having a patent pending for a cannabinoid-containing oral pharmaceutical composition outside the submitted work. JAC is a consultant and/or has received speaker fees and/or sits on the advisory board and/or receives research funding from Janssen-Cilag, Torrent, GreenCare, PurMed Global, BioSynthesis Pharma Group (BSPG), and Prati-Donaduzzi. JAC reported receiving grants from the São Paulo Research Foundation FAPESP.

Ethical statement. All experiments were conducted in accordance with the rules and legislation contained by the UFPR Animal Research Ethics Committee (CEUA/BIO-UFPR, number #1390) with the consistency of ethical principles of the National Council for Control of Animal Experimentation (CONCEA). All efforts were made to optimize the number of animals used and to reduce their stress and suffering.

Animal welfare ethical. All authors confirm that the experimental procedures that contribute to the studies are in accordance with the ethical standards of the relevant national and institutional guides on the care and use of laboratory animals.

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