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Research report

Cannabidiol modulates chronic neuropathic pain aversion behavior by attenuation of neuroinflammation markers and neuronal activity in the corticolimbic circuit in male *Wistar* rats

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ABSTRACT

Chronic neuropathic pain (CNP) is a vast world health problem often associated with the somatosensory domain. This conceptualization is problematic because, unlike most other sensations that are usually affectively neutral and may present emotional, affective, and cognitive impairments. Neuronal circuits that modulate pain can increase or decrease painful sensitivity based on several factors, including context and expectation. The objective of this study was to evaluate whether subchronic treatment with Cannabidiol (CBD; 0.3, 3, and 10 mg/kg intraperitoneal route - i.p., once a day for 3 days) could promote pain-conditioned reversal, in the conditioned place preference (CPP) test, in male *Wistar* rats submitted to chronic constriction injury (CCI) of the sciatic nerve. Then, we evaluated the expression of astrocytes and microglia in animals treated with CBD through the immunofluorescence technique. Our results demonstrated that CBD promoted the reversal of CPP at 3 and 10 mg/kg. In CCI animals, CBD was able to attenuate the increase in neuronal hyperactivity, measured by FosB protein expression, in the regions of the corticolimbic circuit: anterior cingulate cortex (ACC), complex basolateral amygdala (BLA), granular layer of the dentate gyrus (GrDG), and dorsal hippocampus (DH) - adjacent to subiculum (CA1). CBD also prevented the increased expression of GFAP and IBA-1 in CCI animals. We concluded that CBD effects on CNP are linked to the modulation of the aversive component of pain. These effects decrease chronic neuronal activation and inflammatory markers in regions of the corticolimbic circuit.

1. Introduction

Pain is a huge world health problem and, if it persists and becomes chronic, can drastically reduce the quality of life, which is usually associated with decreased immune function, impaired cognitive function, and inadequate responses to stress, among other long-term deleterious effects [1]. In addition, chronic neuropathic pain (CNP) is a complex multidimensional subjective experience that comprises sensory/discriminatory, affective/motivational, and cognitive components [2–4].

CNP is often associated with the somatosensory domain, but this conceptualization is problematic because, unlike most other sensations that are usually affectively neutral, the pain has its aversive components [2]. Recently several studies have investigated the perceptive-discriminative, emotive-affective, and cognitive aspects of CNP in different behavioral tests [5–9]. Emotions are mainly processed by afferent brain areas like the thalamus, amygdala (AMY), and anterior cingulate cortex (ACC) [10,11]. This cortical region connects to the mesolimbic evaluation/decision circuit, which integrates multiple information and selects the behavioral response that offers the most

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significant benefit to the organism [4,12–14].

The overlap of neural circuits for pain and reward extends at anatomically and functionally interconnected levels, as human neuroimaging studies have identified several brain regions implicated in pain perception and reward processing, including ACC and AMY [11,15,16]. The corticolimbic system, including AMY, hippocampus, and ACC, is a key circuit associated with persistent pain, anxiety, learning, and aversion modulation [17]. The ACC has been previously implicated in encoding the aversive characteristics of pain [18–21], while the AMY plays an essential role in the emotional-affective aspects of behaviors, including addiction, anxiety- and depressive-like behaviors, and pain modulation [4,22].

Different studies in models of chronic pain demonstrate promising results using cannabidiol (CBD) treatments [23–26]. CBD is a phytocannabinoid and an exogenous endocannabinoid system modulator [27–29]. Current literature points out that CBD is a well-tolerated and safe natural compound that exerts analgesic effects, decreasing hyperalgesia and mechanical/thermal allodynia in several animal models of pain and patients [30]. The mechanism of action of CBD is complex, involving several systems, including its activity on the endocannabinoid (CB1 and CB2 receptors), the endovanilloid system (TRPV1 receptor), and the serotonergic system (5-HT1A receptor), and among others [23,31,32]. Its use is a promising strategy to overcome the lack of effectiveness of conventional treatments for chronic pain. In this scenario, CBD can be an option to modulate sensory and emotional aspects in treating chronic pain [9,30,33].

Therefore, in the present study, we aimed to investigate whether systemic treatment with different CBD doses could modulate the affective-motivational and aversive components of chronic pain in male *Wistar* rats submitted to the chronic constriction injury (CCI) of the sciatic nerve and then to the conditioned place preference (CPP) test [34] and open field test (OFT, we analyzed locomotion parameters - number of crossings-, and anxiety measures - the percentage of time spent in the center of the apparatus, grooming time, immobility and rearing, [35]). CBD curve dose-response was performed regarding pain aversion, and as far as we know, it is an unprecedented study in the literature using the CCI model and the CPP paradigm for measuring component affective of chronic neuropathic pain. As this is an initial study, we chose to standardize these experiments with male rats to reduce the variables involved in the study of pain sensitivity in females (such as the estrous cycle and its hormonal fluctuation [36–40].

Under pathological conditions, astrocytes participate in the cellular response to damage known as reactive gliosis, along with microglia and other cell types [41]. After severe activation, astrocytes secrete various neurotoxic substances and express the protein GFAP, a marker protein for astrogliosis [42–44]. Another related issue in exploring the phases of microglial activation is the reliance on Iba-1 immunoreactivity to report its activation status [45]. Reactive astrogliosis becomes stable over time with glial scar formation [46]. Moreover, microglial reactivity is essential for the onset of hypersensitivity induced by peripheral nerve injury [47,48]; it is also involved in the long-term maintenance of neuropathic pain [49]. Also, astrocytes are responsible for the chronicity of hypersensitivity following peripheral nerve injury [47,50,51]. Nonetheless, supraspinal microglial and astrocytic reactivity and the possible CBD modulation of these components in chronic pain are still unclear.

Therefore, we evaluated the microglial (IBA-1) and astrocytic (GFAP) reactivity in the CCI model and how CBD treatment affected their reactivity. Besides, we also aim to evaluate chronic neuronal hyperactivity (FosB+ neurons). The FosB protein is currently viewed as a 'molecular switch' for repeated stimuli that gradually converts acute responses into relatively stable adaptations that underlie long-term neural and behavioral plasticity [52,53]. It has already been demonstrated that the CCI model is associated with increased expression of the FosB protein in the mPFC in animals with pain. Additionally, we aimed to investigate neuroplastic changes in regions of interest from the corticolimbic circuit (ACC, complex basolateral amygdala (BLA), granular dentate gyrus (GrDG), and dorsal hippocampus (DH) - adjacent to the subiculum (CA1).

2. Material and methods

2.1. Animals

Experiments were performed in 56 male *Wistar* rats (± 250 g and eight weeks old at the beginning of the experiments) obtained from the animal facility of the University of São Paulo (Ribeirão Preto, SP, Brazil). All animals were kept in home cages (4 rats/cage) of 35 × 19 x 25 cm polypropylene lined with shavings in an environment with controlled temperature (24ºC ± 1ºC), water, and food ad libitum. The same researcher conducted the behavioral evaluation; the groups were randomly organized. The researcher was blind to the treatment and the condition through a system of colors and numbers on the animals' tails (individual code). Experimental protocols were carried out in compliance with the recommendations of the Conselho Nacional de Controle de Experimentaç ão Animal – Ministério da Ciência e Tecnologia, Brazil, and received the approval of the Committee of Ethics in Animal Use of the University of São Paulo Campus Ribeirão Preto, protocol number 2018.1.103.58.5. All care was made to minimize animal suffering and to use as few animals as possible.

2.2. Experimental design

Animals were divided into cohorts in the behavioral tests to respect the CBD peak of effect; therefore, all tests were performed following the same criterion. The von Frey, acetone, and hot plate tests were performed between 9 am. and 1 pm., with 10 min of habituation and 10 min intervals between tests. A baseline test session for every animal was performed before surgery (day zero) and eighteen days after the surgery. The OFT test was carried out between 11 am. and 1 pm., with 60 min of habituation in the room before the test. Light intensity was measured in the center of the apparatus (60 lux), and the tests were recorded and stored for further analysis. After every test session, the apparatus was cleaned with 20% ethanol. See the experimental design in Fig. 1.

2.3. Assessment of locomotor activity and motor balance

To exclude the possibility that CCI surgery altered the CCP test nonspecifically (since this test is based on locomotion of the animals), rats were submitted to 1 training session on an accelerated rotarod - speed from 4 to 20 rpm (Initial speed is set to 4 rpm, acceleration rate to 20 rpm/min), maximum speed is 40 rpm [54,55]. The session lasted 12 min and was divided into three attempts on the apparatus and a 3 min rest interval between each attempt. CCI surgery was performed one 1 day after training. Fifteen days later, the rats were placed on a rotarod (speed 4–20 rpm), completing two sessions of 2 min and 30 s with 3 min of rest between sessions. We evaluated the latency to fall and the total time the animal remained in the test.

2.4. Mechanical and thermal (cold and heat) sensitivities assessment

The mechanical sensitivity was assessed by increasing pressure in the paw of the animals (von Frey electronic, Insight Instruments, Ribeirão Preto, São Paulo, Brazil), applied by an electronic analgesimeter [30,56]. On the first experimental day, before the CCI surgery, all animals were placed in the test box for 10 min (habituation period). The baseline evaluation was performed using a von Frey model digital analgesimeter. After this time, increasing progressive forces from the filament of an electronic von Frey analgesimeter were applied to the hind paw plantar surface until the paw was withdrawn. The mechanical withdrawal threshold was assessed in both the contralateral and ipsilateral paws. The mechanical paw threshold (in grams) was calculated as the mean of

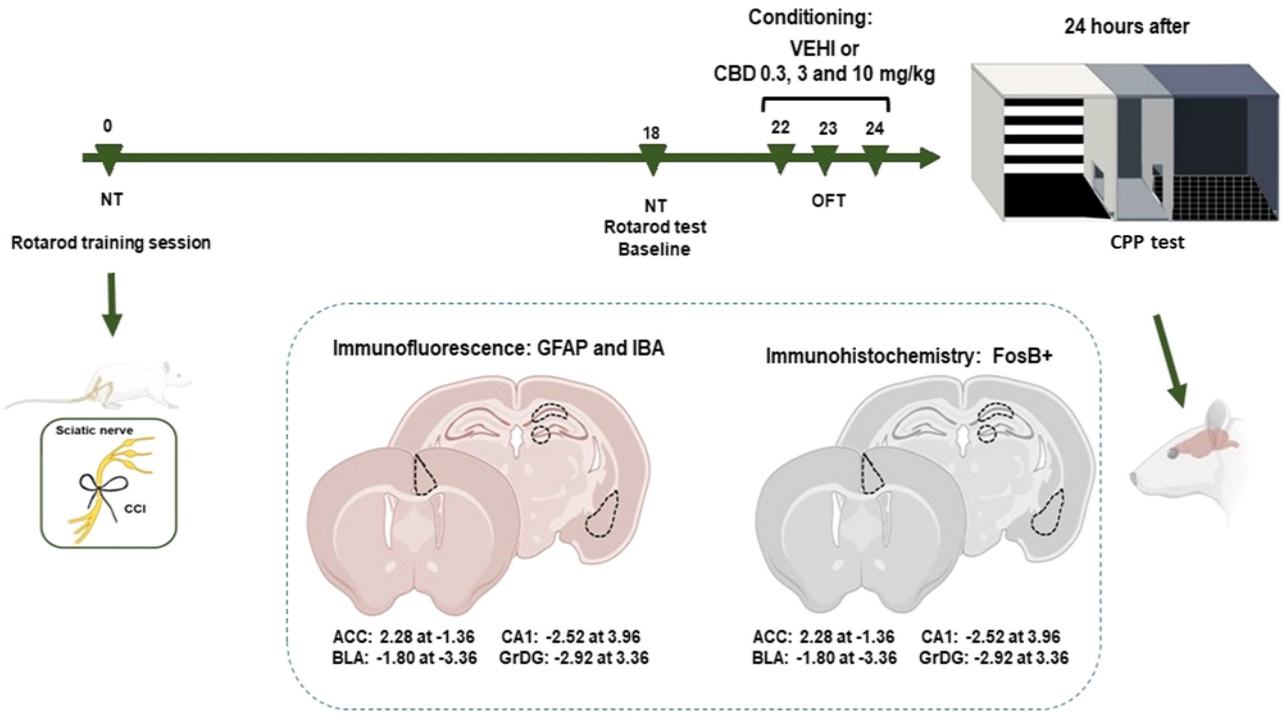

Fig. 1. Experimental design. Animals were submitted to rotarod training sessions and the measurement of NT baselines (Acetone, von Frey, and Hot plate). Then, animals underwent CCI or SHAM surgery. NT has performed baseline before surgery (zero-day) and 18th day after surgery. On the 18th day, the rotarod test and the CPP baseline session were performed. The OFT was performed on the 23rd day, 4 h after treatment. Animals were conditioned in the CPP between the 22nd and 24th days, and the test session was performed on the 25th day. Tissue was collected for immunohistochemistry measurement. Abbreviations: NT - nociception test, CPP - conditioned preference place, CCI - chronic constriction injury, VEHI - vehicle, CBD - cannabidiol, LD – lidocaine, OFT - open field, BLA - basolateral complex amygdala, CA1 - adjacent to subiculum -, ACC - anterior cingulate cortex, GrDG - granular layer of the dentate gyrus, AMY – amygdala, DH – the dorsal hippocampus.

three values obtained in each session [57].

To evaluate the threshold to cold the acetone's plantar installation test was performed, which measures the cold allodynia induced by CCI surgery [30,57]. Animals were placed in acrylic boxes (5 mm^2 floors) and 100 µl of acetone were instilled in the animal's left and right rear paws with an insulin syringe at approximately 5 mm from the paw through the mesh of the observation box. The behavior was evaluated for 1 min using the following score: 0 (no stimulus-response), 1 (quick withdrawal or paw movement), 2 (repeated paw movement), and 3 (repeated movements of the hind paw and licking the paw) [57,58]. The nocifensive response was considered the score to a cold stimulus, and the increase or reduction of the values found was interpreted as allodynia or cold hypoalgesia, respectively.

To assess latency to warm stimulus, the hot plate test was used. The hot plate test evaluates the time the animals remain on a heated metal surface (50 ± 1 °C) until they react to the thermal stimulus [57,58]. Animals are placed on the heated plate, and the response to thermal stimulation (jump, withdrawal, or licking of the hind or front legs) was recorded. A cut-off time of 30 s was adopted to avoid possible injuries to the animal's paw exposure to a long-term harmful thermal stimulus [57].

2.5. CCI model

The peripheral neuropathy was induced by one loose ligation of the right sciatic nerve, according to a method previously described [30,56,59]. The rats were anesthetized with 10% ketamine hydrochloride (75 mg/kg, Cetamin, SYNTEC, Barueri - SP - Brazil) and 2% xylazine hydrochloride (10 mg/kg, Xilazin, SYNTEC, Barueri - SP - Brazil) administered intramuscularly. Afterward, the animals were placed on a surgical table (dorsal position), a trichotomy was performed on the internal regions of their hindlimbs, and the area was disinfected with iodopovidone (Rioquímica, São José do Rio Preto, SP- Brazil). The constriction was performed using a 4–0 chromed Catgut (Bioline, Anapolis – GO/Brazil) suture and transfixed with the same wire using a 3/8 mini-needle. The sciatic nerve was transfixed in 3/4 of its diameter and constricted in one place. The false-operated control group (SHAM) did not expose the sciatic nerve. Finally, the epithelial tissue was sutured with 2.0 silk threads. The surgeries were performed between 9 am. and 3 pm., and 70% ethanol was used to clean the table, and the GermeRio (Rioquímica, São José do Rio Preto, SP- Brazil) was used for disinfecting the surgical instrumentation used for each surgery.

2.6. Treatment with CBD

CBD (99.6% purity; BSPG-Pharm, Sandwich, UK) was diluted in vehicle solution (VEHI, 98% saline solution, 2% Tween 80) [60]. Each administration was performed 60 min before the nociceptive tests based on CBD's plasma absorption time [61]. In the present study, was used subchronic treatment i.p., once a day for 3 days [30]; and eight experimental groups (n = 7/group) were performed: four SHAM groups (SHAM-VEIH, SHAM- CBD 0.3 mg/kg, SHAM- CBD 3 mg/kg, and SHAM- CBD 10 mg/kg) and four CCI groups (CCI-VEIH, CCI- CBD 0.3 mg/kg, CCI- CBD 3 mg/kg, and CCI- CBD 10 mg/kg).

2.7. CPP test

CPP assumes that pain relief is rewarding and having the ability to measure the affective component of pain, and pain relief is a major advantage of using CPP to study pain [9,34,62,63]. Another important advantage of the CPP is that it measures the non-evoked component of chronic pain, often referred to as the spontaneous or continuous component of pain [64]. It was conducted using a customized 3-chamber preference box consisting of a pair of chambers with distinct sensory

cues and an intermediate neutral chamber. To carry out this protocol, animals were habituated to the conditioned place preference on the 17th day of the study. During habituation, rats can freely move between the three chambers for 30 min. On the 18th day, we performed a baseline of preconditioning preference in which rats could move freely between the three chambers for 15 min, and the time spent in each chamber was recorded to determine each rat's preferred location. The animals were monitored in the CPP box by video recordings, and the time spent in each chamber was measured by a researcher blind to the experimental condition. Rats that spent > 720 s or < 180 s in any of the chambers will be excluded from the study to avoid biased results (in the study, no animal was included in this criterion).

Conditioning sessions took place over three days (on the 22nd, 23rd, and 24th). Two sessions were performed each day (30 min each), with an interval of 4 h between each. To evaluate CBD effects, rats received one i.p. administration of CBD or VEHI per day (according to the different doses and experimental group used in this study). One hour later, each animal received an injection of 0.9% saline solution (NaCl, 0.3 mL) in the right popliteal fossa and was immediately placed in the preferred chamber during the preconditioning session; 4 h later, the animal received an injection of 4% lidocaine (LD) volume of the 0.3 mL in the popliteal fossa and was placed in the opposite chamber. The order of treatment was randomized for each rat on every experimental day. The CPP test was performed one day after the conditioning phase (on the 25th). For the CPP test session, rats did not receive any drug treatment and could freely move between the three chambers for 15 min.

The time spent in each chamber was recorded to determine every animal's preferred chamber, and the number of entries into the chamber paired with CBD. Difference scores were calculated by subtracting the time spent in the CBD-paired chamber during the preconditioning session (18th) from the time spent in the same chamber during the post-conditioning test session (25th). The CPP is represented by an increased postconditioning time in the CBD, LD, or VEHI paired chamber compared to the preconditioning time [65]. The pairing chamber's first entry latency was also used to indicate preference and rewarding effect (analgesia) of CBD, LD, or VEHI [66].

2.8. Assessment of locomotor activity and anxiogenic-like behaviors

The locomotion pattern and anxious behavior were evaluated in the OFT on the 23rd experimental day before 4 h of the treatment with CBD. Animals were placed in the center of the apparatus, and the exploratory behavior was recorded for 5 min for behavioral analysis [35]. The time spent in the center and on the border of the apparatus was measured, and the number of crossings between the quadrants was counted to investigate possible changes in the locomotor activity and possible sedative effects [35], which may interfere with evaluating the nociceptive tests since the indexes of evaluation depend on motor responses. Complementary measures in the OFT were done, such as frequency and time of rearing, grooming, and freezing.

2.9. Histological processing

At the end of the protocol, animals were euthanized under anesthesia with 10% ketamine hydrochloride (225 mg/kg) and 2% xylazine hydrochloride (30 mg/kg) and submitted to intracardiac perfusion with 0.01 M phosphate-buffered-saline (PBS) at 20 ∘C, followed by Somogyi's solution (8% paraformaldehyde in 0.1 M phosphate-buffered + 4 mL of 25% glutaraldehyde + 300 mL of picric). After perfusion, brains were removed and kept in Somojyi solution for 6 h for tissue post-fixation. Afterward, tissue was cryoprotected in a 30% sucrose solution until it sank, and brains were frozen in dry ice and isopentane and preserved at −80 °C. Coronal sections (40 μm) from ACC, BLA, GrDG, and CA1 - DH [67] were obtained for immunohistochemical analysis, and floating sections were stored in an anti-freezing solution (50% PBS, 30% ethylene glycol, 20% glycerol).

2.10. Immunohistochemistry for FosB+ neurons

As previously described [68], tissues were washed in PBS (5x, 5 min each) and permeabilized with Triton X-100 0.3% v/v (20 min). Next, endogenous peroxidases were blocked in a 2% H_2O_2 solution for 30 min; then, tissues were incubated in a bovine serum albumin blocking solution (2% BSA and 0.05% Triton X-100) for 2 h. For FosB protein, the polyclonal primary antibody produced in rabbits (1:1000; sc-48, lot. 1809, Santa Cruz Biotechnology) was diluted in bovine serum albumin-blocking solution (2% BSA, Amresco) and incubated overnight (~ 18 h). Afterward, tissues were washed in PBS (5x) and incubated for 2 h in a biotinylated secondary antibody produced in goat anti-rabbit IgG (1:1000; BA-1000, lot. Zb0318, Vector), diluted in a 2% BSA solution. Tissues were then incubated in avidin-biotin-peroxidase complex (1:800, VectaStain ABC kit, Vector) and washed in PBS and Tris-HCl (0.05 M; pH 7.6). Immunoreactive sites were visualized using a kit with 3,3'-diaminobenzidine (DBA) peroxidase (HRP) with nickel (SK-4100, Vector). Nickel intensified the DBA labeling to avoid analysis errors associated with small neurons or neurons with low immunoreactivity. Negative controls were performed in sections incubated without primary antibodies, and immunoreactivity was absent. The sections were mounted on glass slides and coverslips with Permount (Sigma, USA).

2.11. Immunofluorescence for GFAP and IBA-1

For immunoreaction, tissues were washed in PBS and pre-incubated for 1 h in 10% normal goat serum (NDS, Jackson Immunoresearch Laboratories, West Grove, PA, USA) diluted in PBS, pH 7.4 containing 0.3% Triton X-100% and 0.05% sodium azide (Sigma). Then, sections were incubated for 1 day in a mixture of primary antibodies for IBA-1 (Millipore, MABN92, lot. 3068442) and GFAP (Abcam ab190288, lot. GR3359366–7) diluted in normal goat serum (1:1000, each one). On the second day, after five washes in PBS, tissue was incubated for 2 h in the Alexa Fluor 546 goat anti-rabbit antibody (AB_2534093, Thermo Fisher Scientific, lot. 2387450) and anti-mouse IgG Rhodamine B (AP192R, lot. 2189681) diluted (1:800 and 1:400, respectively) in normal goat serum. Then, the tissue was washed five times on PBS. Negative control sections were incubated in the same way as described but without the primary antibody. Slides were mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA) and stored in a refrigerator.

2.12. Image analysis

Immunofluorescence images were captured in a fluorescence microscope with appropriate filters (Olympus BX61VS). As previously described, immunoreactive signals from GFAF and IBA-1 were analyzed in 400 × magnification [30]. The Integrated Optical Density (IOD) method was used to analyze the immunofluorescence images. Six slices per animal were analyzed for each structure of interest, with a sample of 6 randomly selected animals per group. The intensity was analyzed using the software ImageJ (https://imagej.nih.gov/ij/), and the mean value of the integrated density (the product of area and the average gray value) was calculated using the average value of 3 regions of interest (ROI) selected randomly within each structure of each animal. In smaller areas, CA1 and GrDG, 3 ROIs of 2500 μm^2 were used, while in BLA and ACC, 3 ROIs of 10,000 μm^2 were used. FosB immunostaining was analyzed in 400 × magnification, as previously described [45]. Three slices per animal were analyzed for each region of interest, with a sample of 6 randomly selected animals per group. To analyze the number of FosB+ neurons, the manual counting method was used by a blind researcher. For each animal, we calculated the mean of the total FosB+ neurons detected in 3 ROIs (10.000 mm^2 each) randomly selected within each structure of interest.

2.13. Statistical analysis

The Shapiro-Wilk test was used to test data normality. Data from categorical scales were analyzed using nonparametric tests, while data from continuous scales were analyzed using parametric tests if they passed on the normality test. Data from nociception (von Frey and hot plate) and rotarod test were analyzed by unpaired t-test (SHAM vs. CCI). The nociception test for cold (acetone) was analyzed by Mann-Whitney U-test (nonparametric test; SHAM vs. CCI). Parametric data from the OFT, CCP test, immunofluorescence for GFAP and IBA-1 expression, immunohistochemistry for FosB+ expression was analyzed by two-way ANOVA (treatment: CBD vs. VEHI and condition: CCI vs. SHAM), followed by Tukey post hoc multiple comparisons test. Parametric data are represented as mean ± standard error mean (SEM). Nonparametric data are represented as a median ± 95% confidence interval (CI). The significance level was set at $P < 0.05$ for all analyses. Figures and statistics were prepared using Prism software (version 8.0, GraphPad Software).

3. Results

3.1. Assessment of nociception in the sciatic nerve constriction model

In SHAM and CCI rats, somatic sensitivity was examined, measuring the mechanical and thermal thresholds before surgery (baseline) and on the18th day (Fig. 2). CCI surgery reduced the mechanical threshold in the von Frey test on the 18th day (Student t-test). In SHAM animals, the mechanical threshold was not modified throughout the protocol (Fig. 2A). The statistical analysis of the mechanical threshold for the

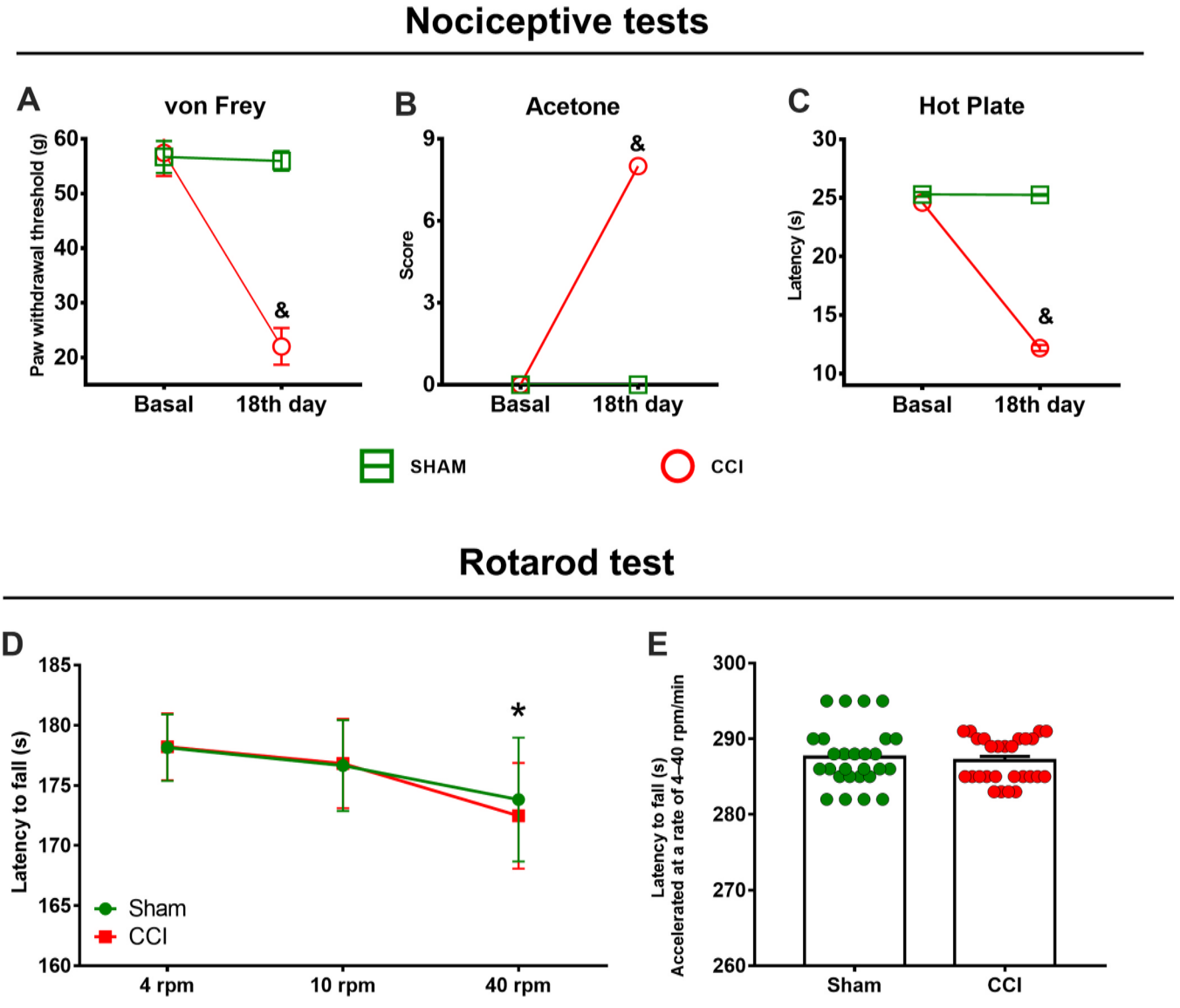

Fig. 2. Assessment of mechanical withdrawal threshold and thermal allodynia, induced by CCI. Mechanical sensitivity was assessed by the von Frey test (A), and thermal sensitivity was assessed by the acetone (B) and hot plate (C) tests. Mechanical or thermal thresholds were assessed at baseline, after CCI or SHAM surgery (18th). (D) Latency of time that the animals remained on the rod in the training sessions at three different rotation speeds (4, 10, and 40 rpm). (E) Test permanence time in the range of rod rotation speeds with smooth acceleration from 0 to 40 rpm in 2 sessions of 2 min and 30 s on the test day in a single attempt per animal. Data from nociception (von Frey and hot plate) and rotarod test were analyzed by unpaired t-test (SHAM vs. CCI). The nociception test for cold (acetone) was analyzed by Mann-Whitney U-test (nonparametric test; SHAM vs. CCI). The rotarod test (training sessions and test session) was analyzed by two-way ANOVA (condition: CCI vs. SHAM and velocity (4 vs. 10 vs. 40 rpm), followed by the Tukey post hoc multiple comparisons test. The latency to fall in the accelerated test session, at a rate of 4–40 rpm/min, was analyzed for the Mann-Whitney test. Parametric data are represented as mean ± standard error mean (SEM). Nonparametric data are represented as median ± 95% confidence interval (CI). N = 28 for each experimental group. & $P < 0.05$ Tukey test against its respective baseline. Abbreviations: rpm - rotations per minute.

SHAM and CCI groups showed a difference in the surgical condition ($P < 0.0001$).

For the analysis of the thermal threshold, two different tests were used, the acetone test to assess the sensitivity to cold and the hot plate test to assess the sensitivity to heat. In the CCI group, acetone (Mann-Whitney test) and hot plate (student t-test) tests showed differences in surgical status on the 18th day ($P < 0.0001$, Fig. 2B and C). In the SHAM group, the score or latency did not change over the experiments in both tests.

3.2. Assessment of locomotor activity and motor balance in animals with chronic pain

The rotarod test was performed to assess the effects of CCI surgery on the forced motor activity and balance of animals compared to SHAM animals (Fig. 2D-E). Two-way ANOVA showed the difference in latency to fall between the CCI and SHAM groups in the training sessions and in the 5-minute test session in factor velocity ($F_{2, 108} = 22.4{,}1$ $P < 0.0001$) but not in the conditions SHAM or CCI ($F_{1, 54} = 0.5166$, $P = 0.4754$). The posthoc test demonstrates the difference in the velocity at 40 rpm ($P < 0.05$) compared to lower velocities, regardless of the SHAM or CCI surgical condition. To analyze the latency to fall in the accelerated test session at a rate of 4–40 rpm/min, the Mann-Whitney test did not show significant differences ($P = 0.9246$, Fig. 2E).

3.3. Cannabidiol effects in the pain aversion test: a potential modulator of the affective-motivational behavior of neuropathic pain

The CPP measures modulation of the affective-motivational component of pain (Fig. 3). Two-way ANOVA showed differences in the condition factor (CCI or SHAM, $F_{1, 48} = 69.84$, $P < 0.0001$), in the treatment factor (CBD or VEHI, $F_{3, 48} = 14.46$, $P < 0.0001$), and the interaction between condition vs. treatment ($F_{3, 48} = 12.02$, $P < 0.0001$). The posthoc test demonstrated that all SHAM groups, treated or not with CBD, showed no significant difference from their control groups. The groups CCI treated with CBD 3 and 10 mg/kg showed significant differences ($P < 0.05$, Fig. 3A) compared to the control CCI-VEHI group. In relation to conditioning with LD, no significant difference was detected in the condition ($F_{1, 48} = 0.0021$, $P = 0.9629$) and treatment ($F_{3, 48} = 0.7482$, $P = 0.5288$).

In addition, when we analyzed the latencies to the first entry in the context that was paired with the treatment, ANOVA showed significant differences in the condition factor ($F_{1, 60} = 22.41$, $P < 0.0001$), treatment factor ($F_{4, 60} = 23.75$, $P < 0.0001$), and also in the interaction treatment vs. condition ($F_{4, 60} = 24.2$, $P < 0.0001$). Post hoc test showed that CCI-VEHI is different from SHAM-VEHI ($P < 0.05$, Fig. 3B) and the CCI groups treated with CBD (0.3, 3, and 10 mg/kg, $P < 0.05$). CCI-LD is different from all groups ($P < 0.05$).

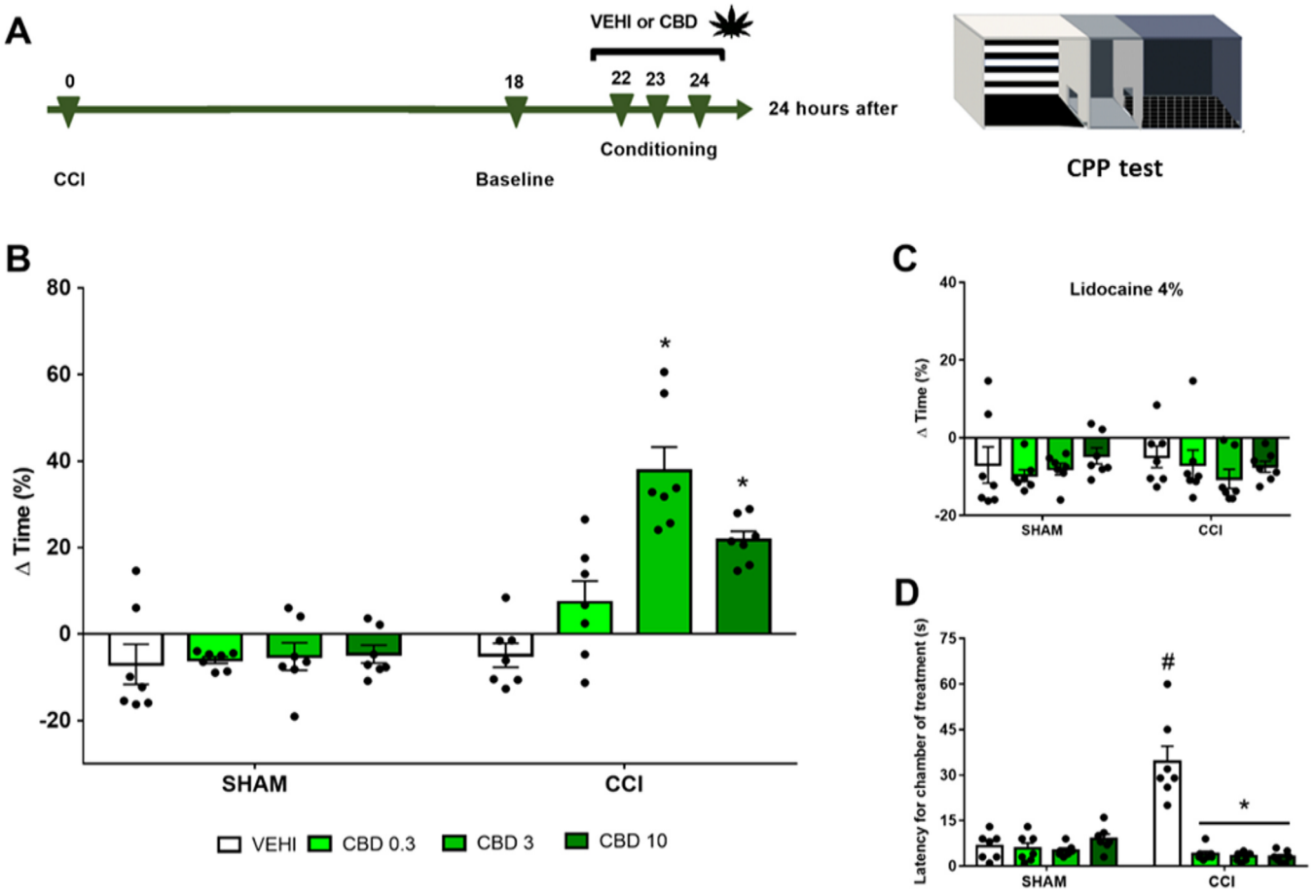

Fig. 3. Subchronic CBD treatment reversed the preferred place of animals with CCI-induced chronic neuropathic pain assessed in the CPP test. (A) CPP test protocol. (B) CPP test preference for CBD treatment. (C) CPP test preference for Lidocaine 4% treatment. (D) latency to the first entry into the conditioned compartment with its respective treatment. Two-way ANOVA: *$P < 0.0001$ Tukey test compared to CCI-VEHI; #$P < 0.001$ Tukey test compared CCI - VEHI with SHAM - VEHI. Data represent mean ± standard errors. N = 7/group. Abbreviation: Δ represents the threshold in the test – the baseline, CPP - conditioned preference pain aversion, CCI - chronic constriction injury, VEHI - vehicle, CBD – cannabidiol, LD – lidocaine 4%.

3.4. Assessment of motor activity and anxiety-like behaviors

The OFT was performed to assess the effects of CBD on emotional behavior and locomotor activity 4 h after the second CBD administration (Fig. 4). Two-way ANOVA showed that there are significant differences in the % of time spent in the center of the open field, in the factor treatment ($F_{3, 48} = 5.932$, $P = 0.0016$), surgical condition factor ($F_{1, 48} = 8.075$, $P = 0.0066$), and interaction factor ($F_{3, 48} = 2.931$, $P = 0.0429$). The number of crossings in the open field (Fig. 4B) did not change in terms of condition (SHAM or CCI, $F_{1, 48} = 3.408$, $P = 0.0711$) or treatment (VEHI or CBD, $F_{3, 48} = 1.28$, $P = 0.2919$).

We also assessed the complementary behaviors of the OFT. Two-way ANOVA showed significant differences in the immobility time in the treatment factor ($F_{3, 48} = 3.743$, $P = 0.0170$) and interaction between

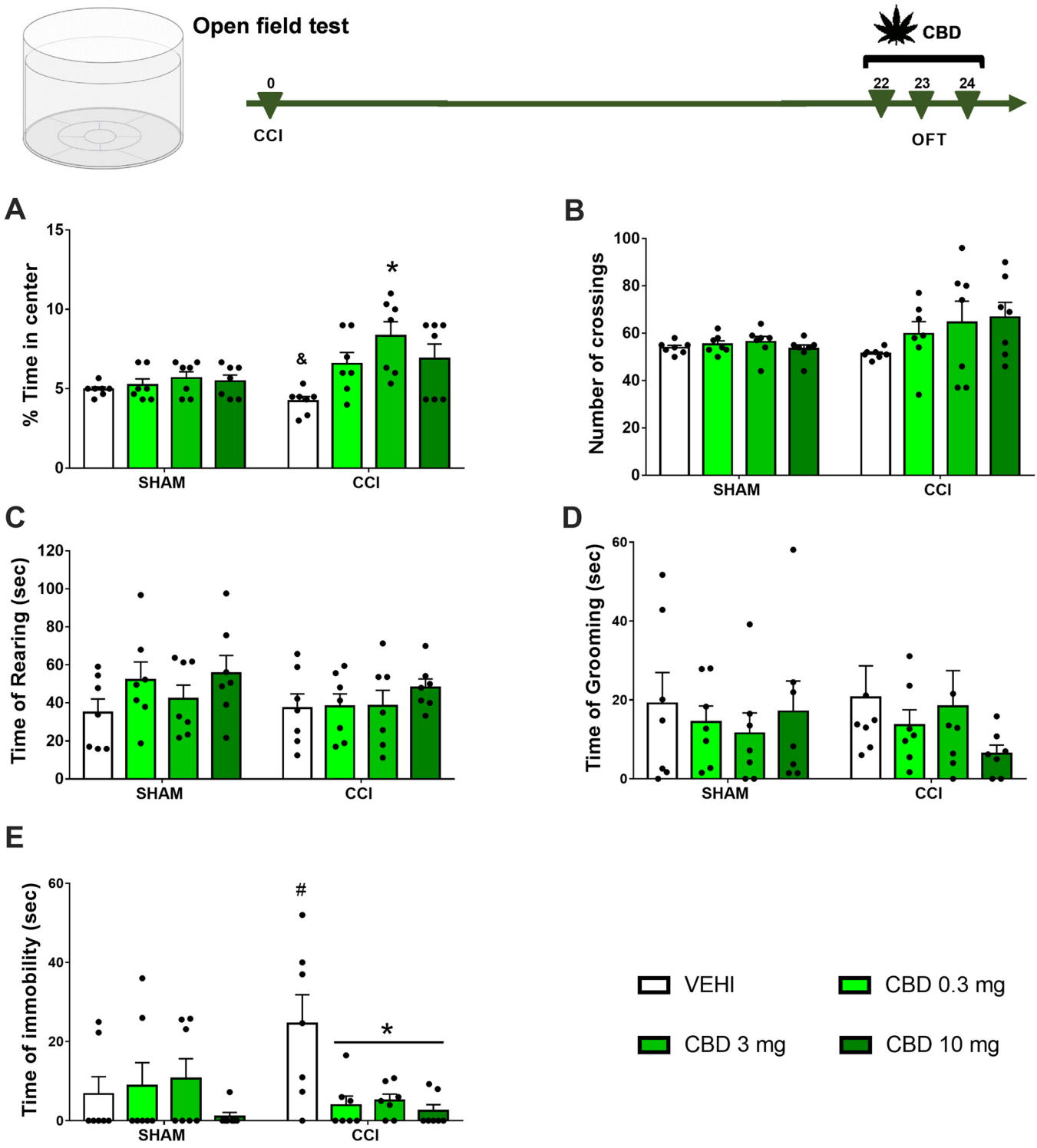

Fig. 4. CBD-induced anxiolytic-like effects in CCI rats in the OFT. (A) Percentage (%) of time spent in the center, (B) the total number of crossings, (C) Time of rearing, (D) Time of grooming, (E) Time of immobility. The OFT was performed for 5 min, 23 days after CCI or SHAM surgery, and 4 h after the second VEHI or CBD (0.3, 3, 10 mg/kg/day, ip.) treatment. Data represent mean ± standard errors. Two-way ANOVA: * $P < 0.05$ Tukey's test compared to CCI-VEHI or CCI-CBD. # $P < 0.05$ compared to SHAM-VEHI or CCI-VEHI. N = 7/group. Abbreviations: CCI - chronic constriction injury, VEHI - vehicle, CBD - cannabidiol, OFT - open field.

treatment and condition ($F_{3, 48} = 3.311$, $P = 0.0277$). Post hoc analysis showed that the CCI groups treated with CBD (0.3, 3, and 10 mg/kg) were different when compared to the control group ($P < 0.05$, Fig. 4E). Finally, regarding the time of rearing and time of grooming, we observed that there was no significant difference in treatment (respectively, $P = 0.2060$ and $P = 0.6387$) or condition (respectively, $P = 0.2956$ and $P = 0.8674$).

3.5. Immunohistochemistry for FosB+ neurons

We assessed chronic neuronal hyperactivity by measuring the number of FosB+ neurons in the ACC (Fig. 5B), BLA (Fig. 5C), GrDG (Fig. 5D), and CA1 (Fig. 5E). Tissue was collected on the 25th experimental day.

Considering the immunoreactivity for FosB protein, in the ACC, two-way ANOVA (Table 1) showed a difference in the condition ($P < 0.0001$) and treatment factors ($P = 0.0021$) and in the interaction between factors ($P < 0.0001$) (Table 1). Tukey's post-test revealed that the CCI group treated with CBD 3 mg/kg is different from the CCI-VEHI group, and the SHAM group treated with CBD 3 mg/kg ($P < 0.05$, Fig. 5B).

Regarding the BLA, the statistical analysis (Table 1) showed a significant difference in the condition ($P = 0.0003$), treatment ($P = 0.0021$), and the interaction between factors ($P = 0.0011$) (Table 1). Tukey's post-test revealed that the CCI group treated with CBD 3 mg/kg differs from the CCI-VEHI and SHAM groups treated with CBD 3 mg/kg ($P < 0.05$, Fig. 5B).

For the DH, in the GrDG region, the statistical analysis (Table 1) showed a difference in the condition ($P = 0.0003$), treatment ($P < 0.0001$), and the interaction condition vs. treatment ($P < 0.0001$) (Table 1), Tukey's post-test revealed that the CCI group treated with CBD 3 mg/kg is different from the CCI-VEHI and the SHAM group treated with CBD 3 mg/kg ($P < 0.05$, Fig. 5D).

Considering the immunoreactivity for FosB protein in CA1, Two-way ANOVA (Table 1) showed a difference in the condition ($P < 0.0001$), treatment ($P < 0.0001$), and in the interaction condition vs. treatment ($P < 0.0001$) (Table S1). Tukey's post-test revealed that the CCI group treated with CBD 3 mg/kg differs from the CCI-VEHI group and the SHAM group treated with CBD 3 mg/kg ($P < 0.05$, Fig. 5E).

3.6. Immunoreactivity assessment of GFAP

We evaluated GFAP expression in the ACC (Fig. 6B), BLA (Fig. 6C), GrDG (Fig. 6D), and CA1 (Fig. 6E) regions in CCI and SHAM rats after subchronic treatment (for 3 days, starting on the 22nd experimental day) with CBD at a dose of 3 mg/kg or VEHI.

Considering the immunofluorescence of GFAP in the ACC, two-way ANOVA (Table 1) showed a difference in the condition ($P < 0.0001$), treatment ($P < 0.001$), and the interaction condition vs. treatment ($P < 0.0001$) (Table 1). Tukey's post-test revealed that the CCI group treated with CBD 3 mg/kg is different from the CCI-VEHI group as well as from the SHAM group treated with CBD 3 mg/kg ($P < 0.05$, Fig. 6B). Also, the CCI group treated with VEHI is different from the SHAM VEHI group ($P < 0.05$, Fig. 6B).

Regarding the BLA, the statistical analysis (Table 1) showed a significant difference in the condition ($P = 0.0063$), treatment ($P = 0.0031$), and the interaction condition vs. treatment ($P = 0.0001$) (Table 1). Tukey's post-test revealed that the CCI group treated with CBD 3 mg/kg is different from the CCI-VEHI group and the SHAM group treated with CBD 3 mg/kg ($P < 0.05$, Fig. 6C). Additionally, the CCI group treated with VEHI is different from the SHAM VEHI group ($P < 0.05$, Fig. 6C).

Specifically, for the DrDG region, two-way ANOVA (Table 1) showed no difference in the condition factor ($P = 0.3371$) but showed significant differences in the treatment ($P < 0.0001$) and the interaction condition vs. treatment ($P < 0.0001$) (Table 1). Tukey's post-test revealed that the CCI group treated with CBD 3 mg/kg is different from the CCI-VEHI group, as well as from the SHAM group treated with CBD 3 mg/kg ($P < 0.05$, Fig. 6D). Additionally, the CCI group treated with VEHI is different from the SHAM VEHI group ($P < 0.05$, Fig. 6D).

Finally, in CA1, the statistical analysis (Table 1) showed a significant difference in the condition ($P < 0.0001$), treatment ($P = 0.0464$), and the interaction condition vs. treatment ($P < 0.001$) (Table 1). Tukey's post-test revealed that the CCI group treated with CBD 3 mg/kg is different from the CCI-VEHI group, as well as from the SHAM group treated with CBD 3 mg/kg ($P < 0.05$, Fig. 6E). Additionally, the CCI group treated with VEHI is different from the SHAM VEHI group ($P < 0.05$, Fig. 6E).

3.7. Immunoreactivity assessment of IBA-1

Similarly, we evaluated the IBA-1 expression in the ACC (Fig. 7B), BLA (Fig. 7C), GrDG (Fig. 7D), and CA1 (Fig. 7E) regions in CCI and SHAM rats after subchronic treatment (for 3 days starting on the 22nd experimental day) with CBD 3 mg/kg or VEHI.

Considering the immunofluorescence of IBA-1 in the ACC, two-way ANOVA (Table 1) showed a difference in the condition ($P < 0.0001$), treatment ($P < 0.001$), and the interaction condition vs. treatment ($P < 0.0057$) (Table 1). Tukey's post-test revealed that the CCI group treated with CBD 3 mg/kg differs from the CCI-VEHI group and the SHAM group treated with CBD 3 mg/kg ($P < 0.05$, Fig. 7B). Also, the CCI group treated with VEHI differs from the SHAM VEHI group ($P < 0.05$, Fig. 7B).

Regarding the BLA, statistical analysis (Table 1) showed a significant difference in the condition factor ($P = 0.0614$), treatment ($P = 0.0116$), and the interaction condition vs. treatment ($P = 0.0019$) (Table 1). Tukey's post-test revealed that the CCI group treated with CBD 3 mg/kg differs from the CCI-VEHI group and the SHAM group treated with CBD 3 mg/kg ($P < 0.05$, Fig. 7C). Additionally, the CCI-VEHI group differs from the SHAM VEHI group ($P < 0.05$, Fig. 7B).

Regarding the GrDG region, two-way ANOVA (Table 1) showed a difference in the condition ($P = 0.0183$) and the treatment factors ($P = 0.0200$) but not in the interaction condition vs. treatment ($P = 0.9522$) (Table 1). Tukey's post-test revealed that the CCI group treated with CBD 3 mg/kg differs from the CCI-VEHI group and SHAM group treated with CBD 3 mg/kg ($P < 0.05$, Fig. 7E). Also, the CCI group treated with VEHI differs from the SHAM VEHI group ($P < 0.05$, Fig. 7E).

Specifically, in the CA1 region, statistical analysis (Table 1) showed a difference in the condition ($P < 0.0001$), treatment ($P < 0.0001$), and the interaction condition vs. treatment ($P < 0.0001$) (Table 1). Tukey's post-test revealed that the CCI group treated with CBD 3 mg/kg differs from the CCI-VEHI group and SHAM group treated with CBD 3 mg/kg ($P < 0.05$, Fig. 7E). Finally, the CCI group treated with VEHI differs from the SHAM VEHI group ($P < 0.05$, Fig. 7E).

4. Discussion

Results of the present study show that subchronic (once a day for 3 days) CBD treatment reversed CPP in the pain-aversion test, the anxiogenic effect of the CCI model, and the number of FosB+ (ACC, BLA, and CA1 of the dorsal hippocampus). Furthermore, we observed that CBD treatment reversed the promoted increase in GFAP and IBA-1 expression caused by the CCI model, respectively, in the regions of ACC, BLA, DrDG, and CA1 of DH and ACC, BLA, and CA1 of the DH.

We observed that the subchronic treatment with CBD (3 and 10 mg/kg) reversed the CCP in the CPP in CCI animals. Thus, the positive reinforcement produced by the continuous relief of pain that the CCI was induced by systemic treatment with CBD. CPP for pain relief was also demonstrated after sciatic nerve axotomy [69], confirming the presence of an aversive state that likely reflects "spontaneous" neuropathic pain and providing an important control that eliminated concerns of pain

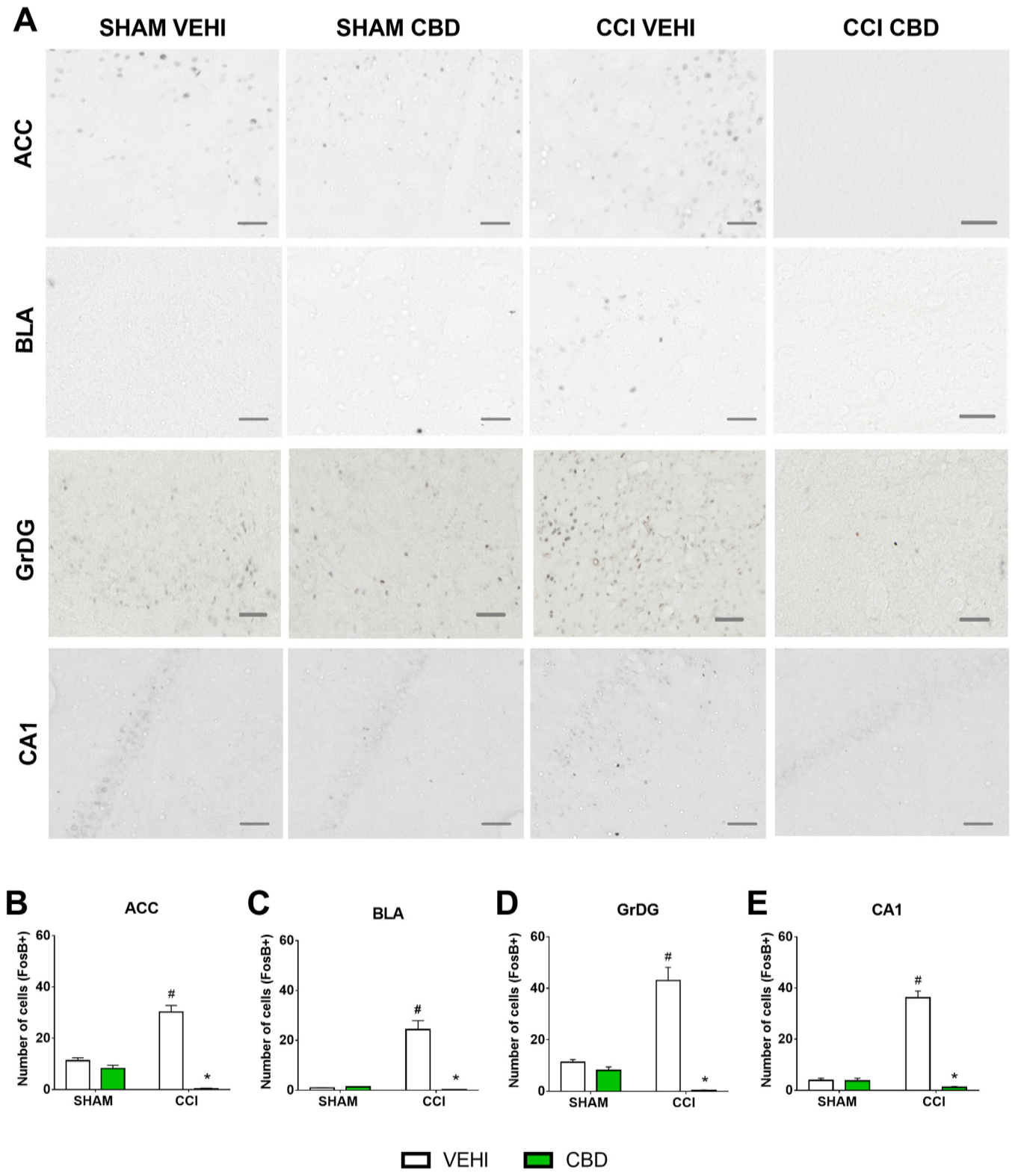

Fig. 5. Effects of CBD on chronic neuronal hyperactivity. (A) Immunostaining for FosB+ neurons, represented by dark dots, in the ACC, BLA, GrDG, and CA1 of the dorsal hippocampus. (B, C, D, E) Subchronic treatment (three days from the 22nd experimental day) with CBD 3 mg/kg reduced the number of FosB+ neurons observed in CCI animals compared to CCI-VEHI treatment and SHAM groups. Two-way ANOVA: * $P < 0.05$ Tukey's test compared to CCI-VEHI or CCI-CBD. # $P < 0.05$ compared to SHAM-VEHI or CCI-CBD. N = 6/group. Scale bars = 200 μm. Abbreviations: CCI - chronic constriction injury, VEHI - vehicle, CBD - cannabidiol, BLA - the basolateral complex amygdala, CA1 - adjacent to subiculum, ACC - anterior cingulate cortex, GrDG - granular layer of the dentate gyrus, DH – the dorsal hippocampus.

Table 1
Results were obtained from the original immunohistochemistry data for FosB+ neurons and Immunofluorescence for GFAP and IBA-1. Data were submitted to two-way ANOVA, followed by Tukey's test to compare experimental groups. The significance level was set at $P < 0.05$ for all analyses.

FosB+			
Region	Condition factor: (SHAM vs. CCI)	Treatment factor (VEHI vs. CBD)	Interaction (condition vs. treatment)
ACC	$F_{1, 20} = 13.07\ P = 0.0017$	$F_{1, 20} = 113.9\ P < 0.0001$	$F_{1, 20} = 75.53\ P < 0.0001$
BLA	$F_{1, 20} = 10.64\ P = 0.0021$	$F_{1, 20} = 12.22\ P = 0.0011$	$F_{1, 20} = 14.96\ P = 0.0003$
DH			
CA1	$F_{1, 20} = 100.4\ P < 0.0001$	$F_{1, 20} = 139.6\ P < 0.0001$	$F_{1, 20} = 136.9\ P < 0.0001$
GrDG	$F_{1, 20} = 19.36\ P = 0.0003$	$F_{1, 20} = 70.64\ P < 0.0001$	$F_{1, 20} = 52.99\ P < 0.0001$
GFAP			
Region	Condition factor (SHAM vs. CCI)	Treatment factor (VEHI vs. CBD)	Interaction (condition vs. treatment)
ACC	$F_{1, 36} = 31.15\ P < 0.0001$	$F_{1, 36} = 34.8\ P < 0.0001$	$F_{1, 36} = 20.64\ P < 0.0001$
BLA	$F_{1, 31} = 8.60\ P = 0.0063$	$F_{1, 31} = 10.32\ P = 0.0031$	$F_{1, 31} = 19.38\ P = 0.0001$
DH			
CA1	$F_{1, 33} = 4.326\ P = 0.0454$	$F_{1, 33} = 41.61\ P < 0.0001$	$F_{1, 33} = 41.88\ P < 0.0001$
GrDG	$F_{1, 29} = 0.9528\ P = 0.3371$	$F_{1, 29} = 33.11\ P < 0.0001$	$F_{1, 29} = 37.54\ P < 0.0001$
IBA-1			
Region	Condition factor (SHAM vs. CCI)	Treatment factor (VEHI vs. CBD)	Interaction (condition vs. treatment)
ACC	$F_{1, 36} = 23.09\ P < 0.0001$	$F_{1, 36} = 32.1\ P < 0.0001$	$F_{1, 36} = 8.66\ P = 0.0057$
BLA	$F_{1, 29} = 3.78\ P = 0.0614$	$F_{1, 29} = 7.27\ P = 0.0116$	$F_{1, 29} = 11.63\ P = 0.0019$
DH			
CA1	$F_{1, 35} = 20.48\ P < 0.0001$	$F_{1, 35} = 60.54\ P < 0.0001$	$F_{1, 35} = 60.36\ P < 0.0001$
GrDG	$F_{1, 35} = 6.12\ P = 0.0183$	$F_{1, 35} = 5.93\ P = 0.0200$	$F_{1, 35} = 0.003\ P = 0.9522$

resulting from tactile stimulation during ambulation within the test device [70]. In addition, our group has already demonstrated the analgesic effect of CBD at low doses in a subchronic treatment in a CCI model [30], and the literature demonstrates a lot of evidence of the analgesic effects of CBD in other pain models [23]. Thus, chronic pain can be seen as an extinction-resistant emotional learning state [12]. Finally, it is essential to note that chronic pain, as a strong stressor, is known to cause comorbidity to negative emotion disorders, including anxiety, stress, and depression in CNP patients [13,71–74].

Furthermore, our results reinforce that chronic pain can favor discriminative learning and easily fit into a learning structure by reinforcing aversive stimuli [9,62,63]. It is also important to note that the local anesthetic (lidocaine) could have acted as an inductor of leg paralysis and could work as positive punishment, supporting the excitatory-inhibitory opposite relationship between rewards and punishments [9]. In this case, in addition to CBD not promoting motor paralysis, assessed in the rotarod and OFT, it can reduce the aversive state generated by the pain of the CCI model. Nevertheless, CBD (3 and 10 mg/kg) did not evoke CPP in SHAM rats, supporting previous results from other pain models that did not produce CPP in control rodents [9,75,76]. These results suggest that these doses of CBD are unrewarding in SHAM rats and become rewarding in CCI rats due to pain relief and reduced pain aversion.

A limitation of our study was not using females. Recent literature already discusses the effects of CBD and different pain perceptions in males and females. However, the literature is still very divergent; for example, in a study using the model of persistent pain by intraplantar injection of complete Freund's adjuvant, the effects of CBD (0.0–10 mg/kg, i.p) on pain and inflammation were minimal, and few sex differences in antinociception or immune modulation were observed [61]. However, in a formalin test model, CBD (10 mg/kg, i.p.) provoked antinociception in phase I but not in phase II in male mice; however, in female mice, CBD did not show a significant antinociceptive effect at any stage of this test [77]. Therefore, future studies focusing on female rats and the different phases of the estrous cycle will undoubtedly expand the knowledge of the emotional modulation of CBD in chronic pain.

Our previous data indicate that CCI surgery can promote anxiety-like behaviors in rats [30] The results from the present study corroborate this finding in the OFT. Also, we found significant differences between the CCI animals treated with CBD 3 mg/kg even 4 h after administration; animals showed anxiolytic-like behaviors (increased time spent in the center of the apparatus and immobility time) when compared to their controls. Interestingly, despite the lack of anxiolytic-like effects with CBD 0.3 and 10 mg/kg, these doses showed effects on the time of immobility when compared to the CCI-VEHI control. These results agree with our previous data and another studies about CBD anxiolytic-like effects in the CCI model of CNP [30,33] and are supported by CBD half-life in rats [60].

Our results demonstrate that the CCI model promotes an increase in chronic neuronal hyperactivity, which was detected by the increased number of FosB+ neurons in the ACC, BLA, and CA1 of the HD. This increase in FosB expression was reversed and prevented in these regions by subchronic treatment with CBD at a dose of 3 mg/kg. The gradual accumulation of FosB protein strengthens the formation and maintenance of habitual memories and compulsive behaviors, perhaps by enhancing the effectiveness of neuronal circuits [53]. Our findings demonstrate the decrease of the FosB immunoreactivity in these regions of the corticolimbic circuit in animals treated with CBD; these data will help to elucidate the role of neuronal circuits in the mechanism of chronic pain modulation. In addition, FosB gene products contribute to the excitotoxic activation of microglia [78], and interestingly, changes in microglia expression were also detected in the present study.

Although several brain structures contribute to pain and emotion processing, CNP studies show that the ACC is consistently activated and involved in pain's affective dimension [79–81]. It should be noted that, given its rich functional connectivity, the ACC is likely to be important in a complex network of brain structures that regulate the increase in pain aversion [14,21,82], and it is a critical region for nociceptive perception and pain-related anxiety [83–86]. These findings corroborate our data regarding the increased expression of FosB+ neurons in the ACC, which might explain the anxiogenic-like behaviors in OFT in CCI-VEHI animals.

Additionally, ACC neurons project and receive input from various regions important for pain processing, such as AMY [87–89]. Direct neuronal projections from the ACC to the BLA play a critical role in modulating emotional functions, such as learning, fear, and pain aversion [90,91]. The AMY receives multisensory information from cortical regions through the BLA and sends direct projections of nociceptive input to the parabrachial nuclei (PB) [92,93], which is directly involved with pain chronicity. Through its bidirectional connections with the BLA, the PB can modulate negative emotions related to pain and fear, especially those involved with the neural circuit that processes the affective-motivational component of pain [94] and positive emotional responses, such as reward [13]. These results, therefore, corroborate our data of increased neuronal excitability in the BLA of CCI-VEHI animals.

Astrocytes, like microglia, produce cytokines and chemokines, resulting in neuroinflammation that contributes to nociceptive signaling [95]. Consequently, activated microglia have been reported to be involved in shaping the neuroplasticity underlying chronic pain [96] and pain-associated affective disorders [97]. Consequently, reactive astrocytes in cortical regions associated with emotion regulation are involved in CNP and induced emotional dysfunction [72]. Astrocytic reactivity has been observed in the medial prefrontal cortex, primary somatosensory cortex, ACC, AMY, thalamus, and hippocampus in

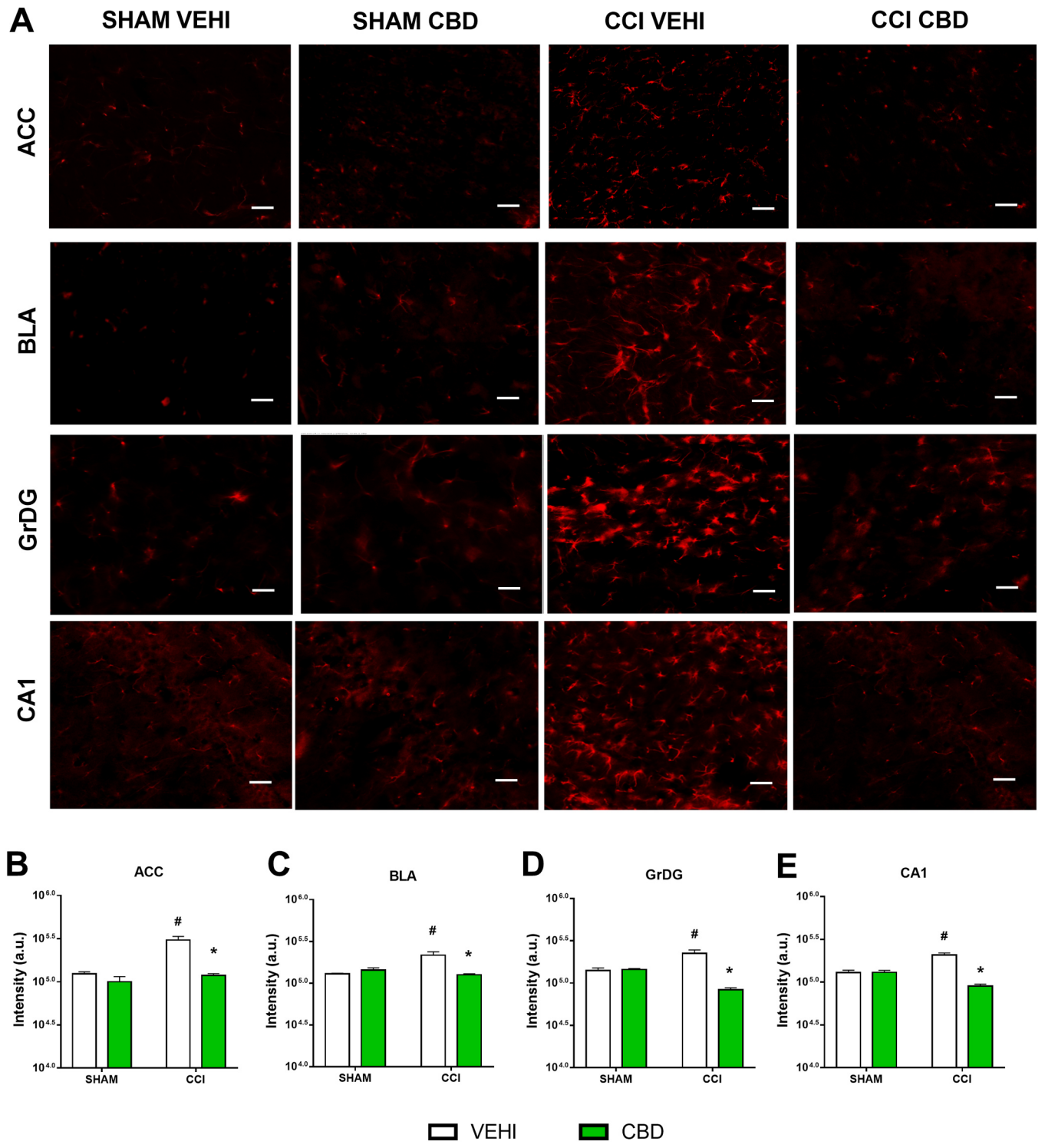

Fig. 6. Immunofluorescence for GFAP. (A) Representative images of GFAP expression (red) in the ACC, BLA, CA1, and GrDG of the dorsal hippocampus. (B, C, D, and E) Subchronic treatment (3 days starting on the 22nd experimental day) with CBD (3 mg/kg) reduced GFAP expression in CCI animals compared to VEHI treatment and SHAM groups. Two-way ANOVA: * $P < 0.05$ Tukey test compared to CCI-VEHI or CCI-CBD. # $P < 0.05$ compared to SHAM-VEHI or CCI-VEHI. N = 4–6/group. Scale bars of the ACC= 100 μm; BLA, GrDG and CA1 = 50 μm. Abbreviations: CCI - chronic constriction injury, VEHI - vehicle, CBD - cannabidiol, BLA - basolateral complex amygdala, CA1 - adjacent to subiculum, ACC - anterior cingulate cortex, GrDG - granular layer of the dentate gyrus, DH – dorsal hippocampus.

various peripheral models of chronic pain [98,99].

Our results demonstrate that the CCI model promoted increased GFAP expression in the ACC, BLA, GrDG, and CA1. Such an increase was prevented by subchronic treatment with CBD 3 mg/kg. The CCI model also promoted increased IBA-1 expression in the ACC, BLA, and CA1; this increase was reversed by subchronic treatment with CBD 3 mg/kg. During pain development, microglia responses are typically early and transient, whereas astrocyte activation is later and lasts longer than microglia [72,100]. Although our astrocyte data agrees with previous data, we also demonstrated long-term changes in microglia, indicating an important role in long-lasting responses to pain and its associated anxiety. These data reinforce the neuroplastic alterations that CNP

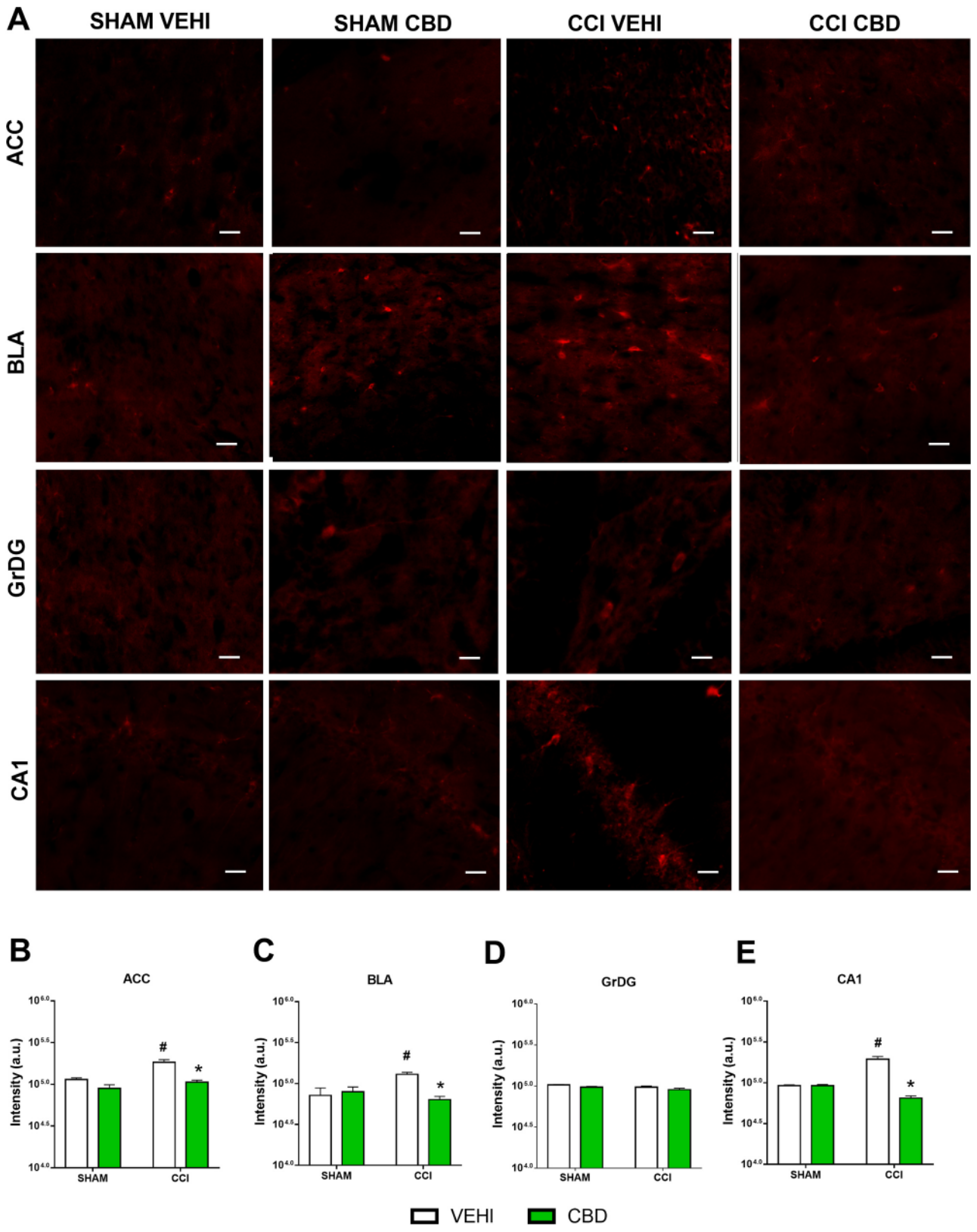

Fig. 7. Immunofluorescence staining for IBA-1 expression. (A) Representative images of IBA-1 expression (red) in the anterior cingulate cortex (ACC), basolateral amygdala (BLA) and dorsal hippocampus CA1 (CA1), and dentate gyrus (GrDG). (B, C, and E) Subchronic treatment (three days from the 21st experimental day) with cannabidiol (CBD, 3 mg/kg) reduced IBA-1 expression that was observed in CCI animals compared to CBD treatment and sham surgery (SHAM). Two-way ANOVA: * $P < 0.05$ Tukey test compared to CCI-VEHI or CCI-CBD. # $P < 0.05$ compared to SHAM-VEHI or CCI-VEHI. N = 4–6 per group. Scale bars of the ACC = 100 μm; BLA, GrDG and CA1 = 50 μm. Abbreviations: CCI - chronic constriction injury, VEHI - vehicle, CBD - cannabidiol, BLA - the basolateral complex amygdala, CA1 - adjacent to subiculum, ACC - anterior cingulate cortex, GrDG - granular layer of the dentate gyrus.

promotes in corticolimbic circuit structures such as the ACC, BLA, and DH.

Furthermore, cortical astrocytes also play an important role in CNP. Studies have shown that activated astrocytes in brain regions related to emotion regulation (ACC and DH) are associated with emotional dysfunction in CNP states [101,102], evidenced by behavioral and immunohistochemical results from the present study. Besides, studies using distinct CNP models have shown that GFAP expression in the ACC increases under CNP [103]. It is involved in regulating the experience of pain and the negative emotions associated with pain [99]. It is noteworthy that astrocytes play a crucial role in regulating the balance between GABA and glutamate, and an imbalance between these neurotransmitters may be the underlying mechanism of CNP [72]. Additionally, the number of GFAP-positive hippocampal astrocytes increased in rats submitted sciatic nerve transection compared to sham-operated rats [104], corroborating our findings in the present study. Furthermore, the expression of activated astrocytes in the hippocampus may indicate their role in persistent pain and mood disorders [105–107], which might explain our results of increased GFAP and FosB+ neurons in the same region.

CBD produced neuroprotection [108] and reduced glial reaction [109]. Here, we demonstrate that CBD treatment attenuates the activation of microglial cells in CNP. CBD has already been shown to inhibit the inflammatory responses of astrocytes and microglia stimulated with lipopolysaccharide [110]. Other studies suggested that CBD may inhibit pro-inflammatory signaling networks in astrocytes and microglial cells [111,112]. Additionally, products from FosB transcription may also contribute to excitotoxic activity displayed by microglia [78], which may help to explain the relationship between microglia activation and increased neuronal activity (FosB+ neurons) in the CCI-VEHI group.

We conclude that CBD treatment (3 mg/kg) modulates changes in reward/motivation and learning circuits that can prevent the emotional comorbidity disturbances often observed in patients with chronic pain, such as anxiety. And that the reduction of chronic neuronal hyperactivation and a reversal of the expression of microglia and activated astrocytes demonstrate that CBD may act in brain substrates that contribute to CNP suppression and modulation, such as those brain sites involved with the corticolimbic circuit. Also, motivated behavior and the neural circuits that mediate aversion and reward are highly conserved across species [2]; translation of these findings may help to better comprehend brain alterations during chronic pain and may benefit the development of therapies for CNP in humans.

CRediT authorship contribution statement

GKS-C and CRAL-P designed the study. GKS-C performed behavioral and immunohistochemical experiments, analyzed the data, prepared figures and tables, and wrote the draft manuscript. WL-L analyzed the behavior and immunohistochemical data. EOP analyzed data. CRAL-P managed the project and supervision. CRAL-P, JECH, AWZ, NG-C and JASC provided critical reviews of the manuscript and obtained funding. All authors read, reviewed, and approved the final manuscript.

Declaration of Competing Interest

JAC 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 1 A) during the conduct of the study, being a co-owner of a patent for fluorinated cannabidiol 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. JECH 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 1 A) during the conduct of the study, being a co-owner of a patent for fluorinated cannabidiol compounds (licensed to Phytecs) and having a patent pending for a cannabinoid-containing oral pharmaceutical composition outside the submitted work. JECH reported receiving grants from the São Paulo Research Foundation FAPESP.

Data availability

The datasets supporting the conclusions of the study are available to any qualified researcher on reasonable request.

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