Cannabidiol in the dorsal hippocampus attenuates emotional and cognitive impairments related to neuropathic pain: role of prelimbic neocortex-hippocampal connections

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Research Article

Keywords: neuropathic pain, depression, cognition impairment, cannabidiol, prelimbic cortex, hippocampus

Posted Date: April 6th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-2781595/v1

Abstract

Background and Purpose

Chronic neuropathic pain (NP) is commonly associated with cognitive and emotional impairments. Cannabidiol (CBD) presents a broad spectrum of action with a potential analgesic effect. This work investigates the CBD effect on comorbidity between chronic NP, depression, and memory impairment.

Experimental Approach

The connection between the neocortex and the hippocampus was investigated with biotinylated dextran amine (BDA) deposits in the prelimbic cortex (PrL). Wistar rats were submitted to chronic constriction injury (CCI) of the sciatic nerve and CA_1-treatment with CBD (15, 30, 60 nmol).

Key Results

BDA-labeled were found in CA_1 and dentate gyrus. CCI-induced mechanical and cold allodynia increased c-Fos protein expression in the PrL and CA_1. The number of astrocytes in PrL and CA_1 increased, and the number of neuroblasts decreased in CA_1. The CCI animals showed increasing depressive-like behaviors, such as memory impairment. CBD (60 nmol) treatment decreased mechanical and cold allodynia, attenuated depressive-associated behaviors, and improved memory performance. Cobalt chloride ($CoCl_2$: 1 nM), WAY-100635 (0.37 nmol), and AM251 (100 nmol) intra-PrL reversed the CBD (60 nmol) effect intra-CA_1, both in nociceptive, cognitive, and depressive behaviors.

Conclusion

CBD represents a promising therapeutic perspective in the pharmacological treatment of chronic NP and associated comorbidities such as depression and memory impairments. The CBD effects possibly recruit the CA_1-PrL pathway, inducing neuroplasticity. CBD acute treatment into the PrL cortex produces functional, molecular, and morphological improvements.

1. Introduction

Pain is a condition related to suffering and disability, and the experience can influence emotional, mood, and cognitive aspects. When the injury is associated with the somatosensory system, the pain resulting from this process is defined as neuropathic pain (NP) (Scholz et al., 2019). Chronic NP results from changes in the central and peripheral nervous systems, including cortical and maladaptive neuroplasticity, increasing the primary nociceptive activity and recruiting new cortical areas, such as the rostromedial aspects of the frontal lobe (prefrontal cortex). Then, it can cause the alteration of endogenous pain modulation, neurochemical change, and structural changes in the cortex (Medeiros et al., 2019; Sator-Katzenschlager, 2014).

The medial prefrontal cortex (mPFC) is crucial in the affective and sensory components inherent in the nociceptive phenomenon. Changes in this neocortex, as well as the hippocampus, have been reported in many chronic pain conditions and related comorbidities such as anxiety, depression, and cognition (Eagle, Mazei-Robison, & Robison, 2016; Krishnan et al., 1985; Malvestio et al., 2021; Massart et al., 2016; Medeiros, dos Santos, et al., 2020; Barkus et al., 2010). Abnormal cytokinin expression, long-lasting potential deficits, and compromised neurogenesis process are observed (Al-Amin et al., 2011; Ren et al., 2011; Terada et al., 2008). This decline in neurogenesis has been associated with behavioral disorders such as anxiety, depression, and stress (Dranovsky & Hen, 2006; Revest et al., 2009; Sahay & Hen, 2007).

These two cerebral regions have a reciprocal connection, with the mPFC being an intermediary link between the hippocampus and neocortical association areas (Thierry, Giovanni, Dégénétais, & Glowinski, 2000). Changes in the mPFC-dorsal hippocampus (dH) neuronal activity resulting from an injury added to astroglial changes may amplify the chronic pain process. In fact, several neuronal and glial mechanisms can contribute to the chronicity of pain-signalling molecules involved in the activation of astrocytes are also associated with chronic pain (Koyama, 2014).

Cannabidiol (CBD), in turn, is an essential tool for treating symptoms associated with pain and comorbidities with emotional and cognitive changes. Several studies indicate that CBD treatment decreases rodents' nociceptive experience (Mlost et al., 2020). CBD's mechanisms are not fully understood, with a broad spectrum of actions, including G protein-coupled receptors, ionotropic receptors, and transporters recruitment (Mlost et al., 2020).

Malvestio and colleagues (2021) showed that an adapted CCI procedure evokes depression-associated behaviors, and PrL was evidenced to play a role in both chronic NP and depression-like behaviors. In this model, intra-PrL treatment with CBD causes antidepressant and analgesic effects (Malvestio et al., 2021).

The role of $5HT_{1A}$-serotonergic and CB_1-endocannabinoid receptors in PrL underlying the effect of CBD was also highlighted in this work. In this sense, we investigated morphophysiological changes resulting from CCI-induced neuropathy and the role of mPFC-dH connectivity in comorbid responses related to depression and cognitive deficits in animals with Chronic NP. The effect of CBD on this condition and its possible mechanisms of action were also investigated.

2. Material And Methods

2.1 Animals

This investigation used male Wistar rats (*Rattus norvegicus*, Rodentia, Muridae) from the University of São Paulo animal care facility at Ribeirão Preto Campus. The rodents were housed in a room with a temperature (of 22 ± 1°C), and a light-dark cycle (lights on 07:00–19:00) was controlled. The manipulation and experiments follow the recommendations of the Committee of Ethics in Animal Experimentation (CETEA) of the Ribeirão Preto Medical School of the University of São Paulo (FMRP-USP) (Process: 215/2019), which are in accordance with the Animal Research Ethics guidelines adopted by the National Council for Animal Experimentation Control (CONCEA) and with the International Association for the Study of Pain (IASP) guidelines for pain research on animals (Zimmermann, 1983). In all experiments (Fig. 1), the animals were habituated to the room for one hour before the start.

2.2 Neuroanatomical study

The connective anatomy between the dH-PrL neocortex and the dorsal hippocampus was investigated with biotinylated dextran amine (BDA; molecular weight 3.000; Molecular Probes Inc., Eugene, Oregon, USA) neural tract tracer deposits in PrL. The rodents (independent groups; n = 6) were anesthetized with intramuscular (IM) administration of 0.1 ml of ketamine at 92 mg/kg (União Química Farmacêutica Nacional, Pouso Alegre, Minas Gerais, Brazil), for 0.2 ml of 9.2 mg/kg xylazine (Hertape/Calier, Juatuba, Minas Gerais, Brazil) solution. After that procedure, the animals were fixed in the stereotaxic apparatus (Insight, Ribeirão Preto, São Paulo, Brazil) for microinjection of the non-fluorescent BDA in the PrL division of the mPFC, according to the following coordinates: AP = 3.2mm, ML = 0,.2mm, and DV = 2.2mm. The neural tract tracer was injected intracerebrally, directly into the structure studied, using a system consisting of a gingival needle (30 G) coupled to a polyethylene thread which, in turn, was coupled to a 10µL syringe (Hamilton, Reno, Nevada, USA) powered by an infusion pump (Stoelting, Kiel, Wisconsin, USA) with a flow of 0.2 µL/min. The incision was then sutured, and after 7 days, the animals were perfused and followed by histochemical staining procedures.

BDA neural tract tracer labeling was visualized using the avidin-biotin method (ABC standard Elite kit; Vector Laboratory, Burlingame, California, USA) with nickel-enhanced 3–3'-diaminobenzidine (DAB; Sigma/Aldrich, Saint Louis, Missouri, USA) peroxidase reaction. Sections were thoroughly washed in 0.1 M phosphate buffer (pH 7.4) after incubation, mounted on gelatine-coated glass slides, counterstained using the Nissl cresyl violet method, and viewed under a photomicroscope (AxioImager Z1, Zeiss, Oberkochen, Germany).

2.3 Drugs

Cannabidiol (CBD; (~ 99.9% pure, FarmaUSA, Volta Redonda, RJ, Brazil) dissolved in grape seed oil at 25, 50, and 100 nmol, and the CB_1 receptor antagonist N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (AM251; Tocris Bioscience, 147 Bristol, UK) at 100 pmol diluted in 10% DMSO (Medeiros et al., 2021; Malvestio et al., 2021). The WAY-100635 (0.37 nmol) selective $5HT_{1A}$ receptor antagonist (Sigma/Aldrich, USA) was dissolved in physiological saline (NaCl 0.9%). The doses of CoCl2 and CBD were based on previous studies (CoCl2: Crestani et al., 2006; de Freitas et al., 2014a, 2014b, 2016d, and CBD: Sonego et al., 2016). The dose of AM251 and WAY-100635 also were based on previous studies (Medeiros et al., 2016 and Roncon et al., 2017, respectively).

The compounds used were injected directly in either the PrL or the dH, using a system consisting of a gingival needle (30 G) coupled to a polyethylene thread which, in turn, will be coupled to a 10µL Hamilton syringe powered by an infusion pump, with a flow of 0.2 µL/min. CBD was used at 15, 30, and 60 nmol/200nL, microinjected in the CA_1 region of the dH or vehicle (grape oil). For the investigation of CBD effects on the DH-PrL pathway, the 5-hydroytrytamine 1A ($5\text{-}HT_{1A}$) receptor selective antagonist WAY-100635 (0.37 nmol), the cannabinoid type 1 (CB_1) receptor antagonist/inverse agonist AM251 (100 nmol), and the synaptic blocker $CoCl_2$ (1nM) or vehicle were administered either in the PrL cortex or in the dH with 5-min between each treatment as follow: vehicle (sham)-PrL + vehicle dH; vehicle (CCI)-PrL + vehicle dH; vehicle (CCI)-PrL + CBD 15 nmol-dH; vehicle (CCI)-PrL + CBD 30 nmol-dH; vehicle (CCI)-PrL + CBD 60 nmol-dH; vehicle (sham)-PrL + CBD 60 nmol-dH; $CoCl_2$ 1nM (CCI)-PrL + vehicle-dH; $CoCl_2$ 1nM (CCI)-PrL + CBD 60 nmol-dH; WAY-100635 0.37 nmol (CCI)-PrL + vehicle-DH; WAY-100635 0.37 nmol (CCI)-PrL + CBD 60 nmol-dH; AM251 100 nmol (CCI)-PrL + vehicle-dH; AM251 100 nmol (CCI)-PrL + CBD 60 nmol-dH.

2.4 Induction of peripheral mononeuropathy

For induction of experimental neuropathy, the model of chronic constriction injury (CCI) of the nervus ischiadicus (sciatic nerve) developed by Bennett and Xie (1988) (Bennett & Xie, 1988) adapted and adapted for a single ligature by Medeiros and her colleagues (Medeiros et al., 2019, 2020, 2021). IM-administration anesthetized the animals with a solution of 0.1 ml of ketamine at 92 mg/kg to 0.2 ml of xylazine at 9.2 mg/kg. Trichotomy of the right paw was performed, followed by a 15-mm longitudinal incision at the height of the thigh, dorsolateral region, at the level of the trochanter/femur, in the right hind limb. After the exposure of the sciatic nerve, a ligature was performed with the 4 – 0 Cat-gut wire in its half. The CCI animals were submitted to a single tie around the nerve, while the control (sham) group was submitted to all surgical procedures except for the peripheral nerve constriction. Finally, the skin incisions were sutured with 5 – 0 mononylon thread.

2.5 Stereotaxic surgery

All animals underwent stereotaxic surgery to implant a guide-cannula in the CA_1 region of the dH, according to the following stereotaxic coordinates: AP = -3.6 mm, ML = + 2.8 mm, and DV = -2.0 mm from Paxinos & Watson's rat brain in stereotaxic coordinates (Paxinos & Watson, 2007). Another cannula was implanted in the PrL cortex under the following coordinates: AP = 3.2 mm, ML = + 0.5 mm, and DV = -2.2 mm. The animals were anesthetized by IM administration of 0.1 ml of ketamine at 92 mg/kg to 0.2 ml of xylazine at 9.2 mg/kg. The anesthetized animals were placed in the stereotaxic area, and the skull was exposed to view the bregma region, from which the structure of interest was located. The skull was then trepanned, the guide cannula positioned accordingly, and fixed to the bone with acrylic resin and two stainless-steel screws. Each guide cannula was sealed with stainless steel wire to avoid blockage.

Each rodent received an intramuscular injection of penicillin G-benzathine (120 UI; 0.2 ml) and the analgesic and anti-inflammatory drug flunixin meglumine (2.5 mg/kg). Afterward, the rats were allowed one week to recover from the surgical procedure.

2.6 von Frey (VF) test

The animals are positioned on a platform that gives access to the plantar surface of the paws for the VF test. Then, different nylon monofilaments of various thicknesses that exert different degrees of force are applied to the hind paws. In this way, it is possible to assess the magnitude of force necessary to evoke the paw withdrawal behavior (Chaplan et al., 1994).

2.7 Acetone cold (AC) test

The cold acetone test (AC) was used to analyze mechanical allodynia. The rodents were positioned on the same platform used in the VF test, and after habituation, 0.5 mL of 100% acetone was administered on the plantar surface of the hind paw. Nociception recording was performed by a score that consists of 3 classes of different reactions: 0 - no movement; 1 - a quick and sudden movement of the paw; 2 - repeated lifting movement of the paw; 3 - paw movement followed by licking (Flatters & Bennett, 2004). If the animal shows any behavior in 20 seconds, the sum of the behaviors outlined in 40 seconds, repeated three times, corresponds to the nociceptive measure of the test for another 20s. If the score in the 20s is 0, no more 20s are needed.

2.8 Forced swim test (FST)

The FST assesses depressive-like behavior related to hopelessness. The animals were placed for 15 minutes in a vertical cylinder-shaped chamber filled with water in the pre-test session. The water had a 25°C temperature and a depth of 20 cm. After 24 hours, the rodents were placed in the same cylinder for a 5-min test. The sessions were recorded with a video camera (Sony Handycam, HDR-SR10, Osaki, Shinagawa-ku, Tokyo, Japan) in a second moment using the XPLORAT software to analyze the number of behavioral events and duration of immobility. Immobility represents passive coping, including floating behavior (the rat floating in the water without straining and making only movements with the tail and paws necessary to keep its head above the water surface) and freezing behavior (operationally defined as the absence of the body movements, except those for breathing, keeping the head above the water surface) (Malvestio et al., 2021).

2.9 Sucrose spray test (SPT)

The SPT was used to assess other components of depressive-like behavior, such as apathy and anhedonia. SPT is based on the frequency and timing of grooming behavior after spraying a 10% sucrose solution upon the back of the rodent. Hygiene sessions were recorded, including muzzle care (movements along the muzzle), head washing (semi-circular movements on top of the head and behind the ears), and body hygiene for 5 min (Kalueff & Tuohimaa, 2004).

2.10 Y-maze test (YMT)

The YMT assesses the cognitive component (working memory) of the brain function. YMT, in turn, consists of three closed arms converging on an equilateral triangular center. At the beginning of the experiment, the animal was positioned in the center of the apparatus. The number of spontaneous alternations (defined as many successive entries of triplets in each of the three arms without repeated entry) was monitored in a 5-min test session. The percentage of alternation was then calculated as the ratio of the number of alternations / (total number of entries in arm − 2) (Medeiros et al., 2020).

2.11 Object Recognition Test (ORT)

The ORT was used to assess cognitive impairment associated with chronic neuropathic pain. This behavioral test is based on the spontaneous exploratory behavior of the rodent, making it possible to access working and episodic memories. The test consists of three steps: habituation, familiarization (acquisition), and choice (evocation). The animals are placed in a circular acrylic apparatus (60 cm in diameter) following the following protocol: first day, habituation for 20 min; on the second day, habituation for 10 min, followed by exposure of the animal to the apparatus with identical objects A and A' for 5 min, the animal is removed for 20 min and then re-exposure of the animal to the apparatus with objects A and B for 5 min; on the third day, exposure of the animal to object A and C for 5 min (Barbieri et al., 2016). The ORT procedures were filmed and recorded by a digital camera, and only behaviors such as sniffing and touching with the forepaws defined the exploration. The data were presented in an index, resulting from the formula: object A minus the new object, divided by their sum (Barbieri et al., 2016).

2.12 Open Field Test (OFT)

The animals were submitted to an open field test to assess their motor behaviour. In the OFT, the animal was positioned on a surface surrounded by acrylic measuring 60 cm in diameter and with its floor divided into quadrants, separating the center and edge. The number of crossings and surveys were evaluated for 5 min (Malvestio et al., 2021).

2.13 Histology

The animals were deeply anesthetized with ketamine (92 mg/kg, IP) and xylazine (9.2 mg/kg, IP) and perfused through the left cardiac ventricle with physiological saline followed by 4% paraformaldehyde (PFA, Sigma) dissolved in 0.1 M phosphate buffer (pH 7.4). The encephalon was removed, post-fixed in PFA for 4 h, and then transferred to 10% and 20% sucrose dissolved in 0.1 M sodium phosphate buffer, ph 7.3, at 4°C for at least 12h in each solution. The nervous tissue was immersed in 2-methylbutane (Sigma), frozen in dry ice (30 s), embedded in Tissue Tek, sectioned (20 mm thickness) using a cryostat (CM 1950, Leica, Wetzlar, Germany) at 20 ºC, and mounted on glass slides coated with chrome alum gelatine. The histological sections were made from the cerebral region (PrL and dH), where the cannula was inserted for injection site analysis according to Paxinos and Watson's atlas (Paxinos and Watson, 2007).

2.14 Immunofluorescence

For all immunofluorescence staining performed in this work, eight slides were obtained from each brain containing serial sections with a thickness of 20 µm in the cryostat (CM 1950 Leica, Wetzlar, Germany). The slides were washed with buffer (TBS 0.1M) in each marking and then incubated in glycine (0.2M). After washing, the slides were incubated in 2% BSA to block nonspecific binding. After nonspecific epitope blockade, the primary antibody was applied (overnight), and, after washing, the secondary antibody. The slides were washed and covered with a Prolong coverslip with 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI) (Life Technologies, Camarillo, CA, USA). The slides were then observed under a microscope (AxioImager Z1, Zeiss, Oberkochen, Germany) and photographed using the ZEISS ZEN lite program for qualitative and quantitative analysis at magnifications of 10, 20, and 40x. Positively labeled cells were counted with the help of the ImageJ software (National Institutes of Health, Bethesda, Maryland, USA). For the quantitative analysis of the material, five fields were photographed at 20x magnification in each area, and the somatory of labeled nuclei found in these histological fields was adopted as a parameter.

c-FOS protein

For evaluating neuronal activity in response to the treatment with CBD, material collection and analysis of c-FOS protein labeling were performed. For this, the experimental procedure described above was followed. The animals underwent CCI surgery and, after 14 days, were submitted to a stereotaxic surgery for implantation of a guide cannula in the DH. After 7 days of recovery from this last surgery, the animals were treated with 60 nmol CBD or vehicle and then submitted to the von Frey and acetone nociceptive tests. After 2 hours of treatment, the animals were then anesthetized and perfused. As described above, each animal's brain was removed and processed to obtain frozen sections. Sections were incubated overnight with the primary anti-c-FOS protein antibody (1:200, Santa Cruz Biotechnology, Inc., Santa Cruz, California, USA) and with the secondary Alexa Fluor 594-labeled antibody (1:400, Santa Cruz Biotechnology, Inc, Santa Cruz, California, USA).

GFAP and NeuN immunolabeling

Glia cells play a crucial role in pain genesis. Astrocytes are cells that play an essential role in pain-related neuroplastic processes. In this sense, immunofluorescence labeling of these cells was performed in the PrL, dH and CA_1 areas. In this study, the glial acidic fibrillar protein (GFAP) found in astrocyte cells was labeled with the primary anti-GFAP antibody (1:500; Santa Cruz Biotechnology, Inc, Santa Cruz, California, USA), followed by the Alexa Fluor 594-labeled secondary antibody (1:1000; Invitrogen, Carlsbad, California, USA). In the same material, the neuronal nuclei protein (NeuN) was labeled in the nucleus, and neuronal perikarya (Gusel'nikova; Korzhevskiy, 2015) was identified to confirm the difference between neuronal and glial cells. The anti-NeuN primary antibody (1:200; Abcam, Cambridge, UK) and the Alexa Fluor 488-labeled secondary antibody (1:400; Invitrogen, Carlsbad, California, USA) were used. Finally, the slides were covered with a Prolong coverslip with 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI) (Life Technologies, Camarillo, California, USA).

Doublecortin protein (DCX)

The neuroplasticity was studied from the immunofluorescence labeling of the DCX. The previously described experimental immunofluorescence procedure was performed for neurogenesis evaluation in neuropathic animals and this response to CBD treatment. The animals underwent CCI surgery and, after 14 days, stereotaxic surgery to implement the guide cannula in the DH. After 7 days of recovery from this last surgery, the animals were treated with 60 nmol CBD or vehicle and then performed the von Frey and acetone tests. After 1 hour of treatment, the animals were then anesthetized and perfused. Each animal's brain was removed and processed to obtain the frozen sections described above. Sections were incubated in DCX primary antibody (1:1000; Abcam, Cambridge, UK) overnight and AlexaFluor 680-labeled secondary antibody (1:2000; Abcam, Cambridge, UK).

2.15 Data analysis

Data were expressed as mean ± standard error of the mean in behavioral studies of mechanical allodynia. Data related to mechanical allodynia and cold sensitivity were submitted to repeated measure split-plot ANOVA followed, when appropriate, by Tukey's post hoc test. The procedure (microinjection of different drugs) was considered an independent factor, and the von Frey threshold measurements, taken at different times, were considered repeated measurements. Behaviors related to depression and memory evoked by rodents with neuropathy were analyzed using the one-way analysis of variance, followed by Tukey's post hoc test. Data related to morphological approaches were submitted to the one-way ANOVA, followed by Tukey's post hoc test. Analyzes and graphs were obtained using the Graph Pad Prism 8 program.

3. Results

Neuroanatomical study

Microinjections of non-fluorescent BDA in the cerebral cortex were restricted to the PrL cortex, as shown in Fig. 2A. BDA-labeled cell bodies and varicosities were found in the pyramidal layer of the CA_1 region of DH (Fig. 2B). Cell bodies and fibers in the CA_1 are also labeled (Fig. 2C), indicating reciprocate connection between the PrL cortex and CA_1.

Furthermore, we found BDA-labeled neurons in the molecular and polymorphic regions of the dentate gyrus (DG) (Fig. 2D) and CA_2 molecular and pyramidal layers (Fig. 2E) of DH. Both structures are involved in elaborating emotions and processing chronic pain's sensory, cognitive, and emotional aspects.

Antinociceptive effect of CBD on CA $_1$ of animals with chronic neuropathic pain

Only animals whose microinjection sites corresponded to the area of interest were considered for statistical analysis. The microinjection sites are represented in Fig. 3.

Considering the von Frey test, according to a repeated measure two-way ANOVA, there were significant effects of treatment ($F_{5.40} = 70.059$; $p < 0.01$), of time ($F_{3.080, 123.2} = 7.445$; $p < 0.01$), and of treatment versus time interaction ($F_{30, 240} = 13.54$; $p < 0.01$). The mechanical allodynia thresholds of the CCI animals treated with vehicle in CA_1 were significantly higher than the sham group treated with intra-CA_1 vehicle (Tukey post-test; $p < 0.01$). After treatment with CBD in a dose of 60 nmol, there was an increase in the mechanical allodynia threshold compared to the vehicle-treated CCI group from 5 to 30 min (Tukey's post hoc test; $p < 0.001$). However, at 15 and 30 nmol concentrations, CBD did not change the threshold when compared to the vehicle group (CCI) (post hoc Tukey test; $p > 0.001$). The sham group treated with CBD 60 nmol intra-CA_1 showed no significant difference compared to those treated with vehicles in the same region. These data are shown in Fig. 4A.

Regarding the cold acetone test, according to a repeated measure two-way ANOVA, there were significant effects of treatment ($F_{5,34} = 33.38$; $p < 0.001$), of time ($F_{3.721, 126.5} = 37.94$; $p < 0.01$), and of treatment versus time interactions ($F_{30, 204} = 7.904$; $p < 0.001$). Cold allodynia thresholds increased in CCI animals treated with the intra-CA_1 vehicle compared to the sham group also treated with the intra-CA_1 vehicle (Tukey's post hoc test; $p < 0.01$). In addition, it is possible to identify an increase in the cold allodynia threshold after treatment with CBD at 60 nmol, but not at 15 or 30 nmol, administered in CA_1, from 0 to 60 min (Tukey's post hoc test; $p < 0.001$). These data are shown in Fig. 4B.

Pharmacological interaction with pretreatment in PrL cortex followed by microinjection of CBD intra-CA $_1$

Only animals whose microinjection sites corresponded to the area of interest (CA_1 and PrL) were considered for statistical analysis. According to a repeated measure two-way ANOVA, considering the von Frey test, there were significant effects of treatment ($F_{8,58} = 190.4$; $p < 0.01$), of time ($F_{2.733, 158.5} = 40.68$; $p < 0.01$), and of treatment versus time interactions ($F_{48, 348} = 10.54$ $p < 0.01$). The interaction between time and treatment factors was significant. The treatment had a significant effect, as well as the treatment time. CCI animals treated with vehicle (PrL) + CBD (CA_1) had the mechanical allodynia threshold increased compared to the CCI group treated with vehicle (PrL) + vehicle (CA_1) (Tukey's post hoc test; $p < 0.05$). PrL cortex pretreatment with the synapse blocker cobalt chloride at 1nM + CBD (CA_1) reduced the mechanical allodynia threshold compared to the group treated with vehicle (PrL) + CBD (CA_1) (Tukey's post hoc test; $p < 0.01$). In addition, PrL pretreatment with either serotoninergic (WAY-100635; 0.37 nmol) or endocannabinoid (AM251; 100 nmol) antagonists + CBD (CA_1) attenuated the mechanical allodynia threshold in relation to the vehicle (PrL) + CBD (CA_1)-treated group (Tukey's post hoc test; $p < 0.01$). Treatment in PrL with either $CoCl_2$, WAY-100635, or AM251 plus vehicle (CA_1) showed no difference in relation to the vehicle (PrL) + vehicle (CA_1)-treated group (Tukey's post hoc test; $p > 0.05$). These data are shown in Fig. 4C.

Regarding the acetone test, according to a repeated measure two-way ANOVA, there were significant effects of treatment (F8.49 = 19.16; $p < 0.001$), of time ($F_{4.159, 203} = 106.4$; $p < 0.01$) and of treatment versus time interaction ($F_{48, 294} = 7.176$; $p < 0.001$). CCI animals treated with vehicle (PrL) + CBD (CA_1) had the mechanical allodynia threshold increased compared to the CCI group treated with vehicle (PrL) + vehicle (CA1) (Tukey's post hoc test; $p < 0.05$). Pretreatment of PrL with $CoCl_2$ (1nM) + CBD (CA_1) treatment reduced the mechanical allodynia threshold compared to the group treated with vehicle (PrL) + CBD (CA_1) (Tukey's post hoc test; $p < 0.01$). In addition, PrL cortex pretreatment with either a 5_HT_{1A} receptor antagonist (WAY-100635; 0.37 nmol) or a CB1 cannabinoid receptor antagonist (AM251; 100 nmol) + intra-hipocampal (CA_1) treatment with CBD attenuated the mechanical allodynia threshold in relation to the vehicle (PrL) + CBD (CA_1) -treated group (Tukey's post hoc test; $p < 0.01$). Pretreatment of the PrL with either $CoCl_2$, WAY-100635, or AM251 plus dH (CA1) treatment with vehicle showed no difference in relation to the vehicle (PrL) + vehicle (CA_1)-treated group (Tukey's post hoc test; $p > 0.05$). These data are shown in Fig. 4D.

CBD effect on the comorbidity between chronic neuropathic pain and depression

In addition to evaluating the nociceptive responses, depressive-like behaviors of CCI animals were evaluated. Considering the sucrose spray test, measuring apathy and anhedonia behaviors, according to a one-way analysis of the variance, the latency to start self-cleaning was significantly different between groups ($F_{8, 72} = 7.440$; $p < 0.01$) (Fig. 5A). In addition, the time of self-cleaning animals in the sucrose spray test was significant (F8, 69 = 36.43; $p < 0$ .01) (Fig. 5B), as well as the frequency ($F_{8, 74} = 15.12$; $p < 0.01$). In addition, animals that underwent CCI surgery and were treated with vehicle in CA_1 showed a reduction (Tukey's post hoc test; $p < 0.001$) in self-cleaning behavior, characterizing depressive-like behavior associated with chronic neuropathic pain. This effect was attenuated after CA_1 treatment with CDB at the highest dose (60 nmol), which increased the number of episodes and duration of self-cleaning behavior, as well as caused a reduction in latency to start this behavioral response (Tukey's post hoc test; $p < 0.001$). Either $CoCl_2$, WAY-100635, or AM251 microinjections in the PrL cortex, followed by vehicle microinjection in the dH, attenuated the number and duration of grooming behaviors (Tukey's post hoc test; $p < 0.001$), as well as increased the latency of grooming responses (Tukey's post hoc test; $p < 0.001$) in comparison to the vehicle (PrL) + CBD (CA_1)-treated group. Microinjection of $CoCl_2$ in PrL followed by microinjection of CBD at 60 nmol in the dH increased the duration of grooming displayed by CCI animals, in comparison to the $CoCl_2$ (PrL) + Vehicle (CA_1)-treated group (Tukey's post hoc test; $p < 0.001$), and reduced the latency of grooming (Tukey's post hoc test; $p < 0.001$). Pretreatment of the PrL with either $CoCl_2$, WAY-100635, or AM251 plus dH (CA_1) treatment with vehicle showed no difference in relation to the vehicle (PrL) + vehicle (CA_1)-treated group (Tukey's post hoc test; $p > 0.05$). These data are shown in Fig. 5C.

Concerning immobility behaviors displayed in FST, both the frequency ($F_{8, 74} = 15.12$, $p < 0.001$) (Fig. 5D) and duration ($F_{8, 69} = 36.43$; $p < 0.001$) were significantly different between groups (Fig. 5E). Animals submitted to CCI surgery and treated with vehicle in CA_1 presented a significant increase in both number (Tukey's post hoc test; $p < 0.01$) and duration (Tukey's post hoc test; $p < 0.01$) of immobility. This chronic NP-related depressive-like behavior was significantly attenuated after CDB in a dose of 60 nmol microinjections in CA_1nmol, which decreased the frequency (Tukey's post hoc test; $p < 0.01$) and duration (Tukey's post hoc test; $p < 0.01$) of immobility behavior. Either $CoCl_2$, WAY-100635, or AM251 antagonists microinjected in the PrL of CCI animals significantly increased the duration of immobility behavior (Tukey's post hoc test; $p < 0.01$), but not the frequency (Tukey's post hoc test; $p > 0.05$) in

comparison to the vehicle (PrL) + CBD (CA_1)-treated group. Pretreatment of the PrL with either $CoCl_2$, WAY-100635, or AM251 plus dH (CA_1) treatment with vehicle showed no significant difference in relation to the vehicle (PrL) + vehicle (CA_1)-treated group (Tukey's post hoc test; $p > 0.05$).

To assess locomotor behavior, the OFT was used. According to the one-way analysis of the variance, there was a significant difference in locomotion (lifting and crossing) displayed by the animals ($F_{4, 19} = 9.935$; $p < 0.05$) (Fig. 5F). The CCI surgery does not alter the locomotion under CBD treatment compared to the control group (Tukey's post hoc test; $p > 0.05$). However, the locomotion of animals treated with $CoCl_2$ in PrL and vehicle in dH was significantly higher than that displayed by rats that received microinjection of the vehicle in both regions (Tukey's post hoc test; $p < 0.05$).

Effect of CBD on comorbidity between neuropathic pain and cognition impairment

Concerning the object recognition task, when the animals were exposed to objects A and B (new objects), there was no difference between the sham and vehicle-treated groups ($t = 0.9528$, $df = 14$; $p > 0.05$) (Fig. 6A). The treatment was carried out before the third stage, before exposure to objects A and C. According to the one-way analysis of the variance, the percentage of recognition was significantly different between groups ($F_{8,84} = 9.882$; $p < 0.01$) concerning the recognition of object C (third phase of the test) (Fig. 6B). The CCI animals equally explored the old object (A) and the new one (C). The index of CCI animals treated with vehicle was significantly lower than that displayed by sham animals also treated with vehicle (Tukey's post hoc test; $p < 0.01$). PrL treatment with CBD was not shown to significantly attenuate that cognitive performance (Tukey's post hoc test; $p > 0.05$). Rats submitted to PrL pretreatment with either $CoCl_2$, WAY-100635, or AM251 followed by CBD microinjections in CA_1 maintained the negative index, with no significant difference in relation to the vehicle (PrL) + CBD (CA_1)-treated group. Concerning the CCI groups pretreated with either $CoCl_2$, WAY-100635, or AM251 in PrL plus vehicle in CA_1, there were no significant differences as compared to vehicle (PrL) + vehicle (CA_1)-treated CCI group.

Concerning the performance of the laboratory animals in the Y-maze, according to the one-way analysis of the variance, the spontaneous alternation in the Y-maze test was significantly different between groups ($F_{8, 54} = 7551$; $p < 0.001$) (Fig. 6C). Animals that underwent CCI surgery and were treated with vehicle in CA_1 showed a decrease in spontaneous changes (Tukey's post hoc test $p < 0.001$). This effect was attenuated by treatment of the CA1 with CDB at the highest dose (60 nmol), which caused a significant increase in the percentage of spontaneous alternation (Tukey's post hoc test; $p < 0.001$). Microinjections of either $CoCl_2$, WAY-100635, or AM251 in the PrL cortex, followed by microinjection of vehicle in the DH, reduced the spontaneous alternation compared to the vehicle in PrL + CBD (60 nmol) in the CA_1-treated group (Tukey's post hoc test; $p < 0.001$). The PrL pretreatment with these 5-HT_{1A} and CB_1 receptors selective antagonists blocked the effect of intra-DH treatment with CBD 60nmol (Tukey's post hoc test; $p < 0.001$), rather than the blockade of PrL synapses performed with $CoCl_2$ on the spontaneous % change during Y-maze test(Tukey's post hoc test; $p > 0.05$).

Effect of CBD on neuronal activation recorded by c-Fos protein immunolabeling

Concerning neural activation, according to the one-way analysis of the variance, there was a significant difference between the groups regarding the number of c-Fos protein immunolabeled cells in CA_1 ($F_{3, 16} = 41.18$; $p < 0.01$) (Fig. 7A-F). There was a significant increase in the number of c-Fos protein-labeled cells in CA_1 of CCI animals treated with vehicle compared to the sham group treated with both vehicle and CBD at 60 nmol (Tukey's post hoc test, $p < 0 .01$). There was no difference between sham groups treated with vehicle or CBD at 60 nmol. Treating neuropathic animals with CBD intra-HD in a dose of 60 nmol increased the number of c-Fos protein-labeled cells in CA_1 (Tukey's post hoc test; $p < 0.01$).

Concerning the cortical activation, according to the one-way analysis of the variance, there was a significant difference between the groups regarding the number of cells immunolabeled for *c-Fos* protein in PrL ($F_{3, 16} = 5.85$; $p < 0.01$) (Fig. 7G-L). It was observed that the sham groups treated with vehicle and sham-treated with CBD at 60 nmol showed a significant increase in the number of labeled cells compared to the naïve group (Tukey's post hoc test; $p < 0.01$). There was no significant difference in sham groups treated with either vehicle or CBD at 60 nmol. Animals that underwent CCI surgery and were treated with vehicle showed a significant increase in c-Fos-labeled neurons compared to the vehicle-treated sham group (Tukey's post hoc test; $p < 0.01$). Acute treatment with CBD in a dose of 60 nmol in CA_1 reduced the number of c-Fos-labeled cells in the PrL of neuropathic animals after one hour compared to the vehicle-treated CCI group (Tukey's post hoc test; $p < 0.01$).

Effect of CBD on the number of astrocytes

According to the one-way analysis of variance, there was a significant difference between the groups regarding GFAP-labeled cells in both CA_1 ($F_{3, 17} = 24.47$; $p < 0.01$) (Fig. 8A-E) and PrL ($F_{3, 19} = 12.26$; $p < 0.01$) (Fig. 8F-J). It was possible to observe an increase in the number of astrocytes in CCI animals compared to the sham group when both were treated with vehicle (Tukey's post hoc test; $p < 0.01$). The acute treatment with CBD in the CA_1 region of dH significantly reduced the number of GFAP-labeled cells in this brain region (Tukey's post hoc test; $p < 0.01$). There were no significant differences between the vehicle-treated and sham groups treated with CBD at 60 nmol.

Regarding the PrL division of the mPFC, there was a significant reduction of GFAP-labeled cell number in the CCI animals treated with vehicle compared to the sham group treated with vehicle (Tukey's post hoc test; $p < 0.01$). Treatment with CBD significantly increased the number of astrocytes in CCI animals (Tukey's post hoc test; $p < 0.01$), but there was no difference between the sham groups.

Effect of CBD on the neurogenic doublecortin (DCX)-labeled cells

Our observations confirm the presence of immunoreactive DCX cells with morphological characteristics of migrating neuroblasts in CA_1 but not in the PrL cortex.

In the CA_1 region, there was a significant difference in the number of DCX-labeled perikarya between the groups ($F_{3, 10} = 73.81$; $p < 0.01$) (Fig. 9). CCI surgery significantly reduced active neurogenesis compared to the sham group (Tukey's post hoc test; $p < 0.01$). However, there was no significant difference between CCI rats treated with CBD in a dose of 60 nmol in the CA_1 hippocampal region and the CCI rats treated with vehicle (Tukey's post hoc test; $p > 0.05$).

4. Discussion

The present work showed that the CBD intra-CA_1 region of the dH caused an antinociceptive effect in animals with chronic NP submitted to CCI. In addition, CBD attenuated depressive-like behaviors and cognitive impairments associated with chronic NP. The observed effect depends on the PrL cortex activation, specifically recruiting the 5-HT_{1A}-serotoninergic and the CB_1-endocannabinoid receptors.

As demonstrated here, CCI surgery reduced nociceptive thresholds related to temperature (cold) and touch (mechanical) in rodents after 21 days, characterizing the triggering of chronic neuropathy resulting from peripheral and central nervous system alterations. We also showed that microinjection of CBD into the CA_1 increased nociceptive thresholds related to mechanical allodynia and cold in animals undergoing CCI surgery after 21 days. In fact, the hippocampus is a critical limbic structure in the motivational dimension of pain (Melzack and Casey, 1968), and pharmacological manipulations in this region reinforce the importance of CA_1 in the nociceptive pathway (He et al., 2019; Kooshki et al., 2018; Zakeri et al., 2020).

This effect reflects extensive connections between the hippocampus and other areas involved in cognitive, limbic (Almada et al., 2015), and pain processing (De Freitas et al., 2013) functions. Among them, the mPFC is highlighted, whose exacerbated activity is directly related to pain reports (Apkarian, Baliki, & Geha, 2009; Medeiros et al., 2019). The nonspecific blockade of synapses in PrL with cobalt chloride ($CoCl_2$) inactivated several pathways, such as opioid, GABAergic, cholinergic, noradrenergic, and even serotonergic systems may have been blocked (Cordeiro Matos et al., 2018; Thompson & Neugebauer, 2019). When nonspecific blockade of the PrL cortex synapses was performed with $CoCl_2$, the effect of CBD intra-CA_1 on pain was significantly impaired, corroborating the hypothesis that the effect of CBD depends on the activation of the PrL-CA_1 pathway, which is reciprocal, as we demonstrated here.

The PrL division receives afferent inputs from the hippocampus and the pain modulatory system structures, such as the dorsal raphe nucleus. That nucleus is the brainstem's primary source of 5-HT_{1A} serotoninergic heteroreceptors (Garcia-Garcia et al., 2014). In addition, it was shown that the suppression of 5-HT_{1A} heteroreceptors in mPFC results in depression-associated behaviors (Garcia-Garcia et al., 2017). These data were corroborated by our findings considering both the nociceptive and the behavioral analyses.

A similar result was obtained from the microinjection of the CB_1 cannabinoid receptor antagonist/inverse agonist AM251 in the PrL division of mPFC and the vehicle infusion in the CA_1. In fact, no significant change in mechanical or cold allodynia thresholds was observed. Medeiros et al. (2020) observed that at the same concentration, AM251 has a hyperalgesic effect, which was not possible to observe in our results since the baseline threshold and the vehicle (PrL) + vehicle (CA_1) group results were low.

The $5HT_{1A}$ receptor selective antagonist WAY-100635 and the CB_1 receptor antagonist/inverse agonist AM251 (microinjected into the PrL caused a blockade of CBD intra-CA_1 effect during the von Frey and acetone tests, showing the crucial involvement of the glutamatergic pathway between CA_1-PrL and serotonin in this effect.

However, in the acetone test, microinjection of AM251 in PrL followed by treatment of the CA_1 with CBD, from 30 min onwards, it is possible to observe the analgesic effect of intra-CA_1 CBD treatment, which does not occur with the $5HT_{1A}$ receptor blockade in PrL. In this context, we observed that the response to mechanical stimulation and the response to cold use different pathways, reflecting different pharmacological mechanisms of CBD.

When CBD is microinjected in the CA_1, the long-term potentiation (LTP) of the PrL/CA_1 pathway, which is low in individuals with pain, is favored. In CA_1, CB_1 receptors may be located on glutamatergic neurons, and thus, activation of the CB1 receptor may regulate LTP induction in CA_1. These mechanisms may also play a role in preventing excitotoxicity (Takahashi & Castillo, 2006). However, its location may be in a subpopulation of γ-aminobutyric acid (GABA)ergic interneurons. In this case, the blockade of postsynaptic inhibition usually promotes LTP at excitatory synapses (Wilson & Nicoll, 2002).

Moreover, the antinociceptive effect of CBD may be specified via cortical serotoninergic activation. The activation of $5HT_{1A}$ receptors possibly exerts tonic inhibition on the hyperexcitability of the PrL division of the mPFC, specifically related to the maintenance of chronic and neuropathic pain (Medeiros et al., 2019). In this context, the CBD was microinjected in the CA_1, and the serotoninergic availability in PrL increased. Thus, analgesia is elicited since the importance of $5HT_{1A}$ receptors in controlling the excitability of pyramidal neurons in the PrL cortex has already been demonstrated (Santana & Artigas, 2017).

Here, we study the acute effects of CBD intra-CA_1. In this context, studies show that the effect of acutely administered antidepressants is related to increased levels of brain-derived neurotrophic factor (BDNF) and synaptogenesis in mPFC and increased neurogenesis in the hippocampus (Silote et al., 2019). The BDNF levels are related to an intracellular cascade activated by a G protein-coupled receptor, such as a serotonergic or endocannabinoid receptor. Those cannabidiol targets may mediate the observed effect.

The hypothesis that treatment with CBD intra-DH promotes an increase in hippocampal activity and favors LTP via attenuation of the GABAergic system by activating CB_1 receptors present in the presynaptic terminal of these interneurons is supported by data obtained from c-Fos protein labeling in limbic structures presently investigated. We observed that CCI surgery is related to increased c-Fos protein-labeled cells in both PrL and CA_1. Similar results were found in animal models of inflammatory and neuropathic pain due to partial nerve ligation and postoperative pain.

Furthermore, electrophysiological studies showed increased excitatory activity in PrL of neuropathic animals and decreased inhibitory activity measured by in vivo electrophysiological recording (Medeiros et al., 2020). In this area, after intra-CA_1 CBD microinjection, a reduction in the number of c-Fos-labeled cells is observed, which corroborates our hypothesis that prelimbic hyperactivity facilitates pain. After treatment with CBD intra-CA_1, there is an increase in c-Fos-immunolabeled cells, indicating increased activity in this area. In fact, increased excitability of the dorsal hippocampus, either pharmacologically or by chemogenetics, can alleviate neuropathic pain behaviors (Xuhong et al., 2018).

Finally, the possible participation of astrocytes in the starting and maintenance of neuropathy and the effect of CBD intra-CA_1 were also evaluated. It is known that glial cells play an essential role in chronic pain. GFAP staining density is dependent on NMDA receptor activity, which, at least in part, contributes to the increase in GFAP observed after peripheral nerve injury (Garrison et al., 1994). The imbalance between glutamate and GABA due to the activation of astrocytes in these regions may be an underlying mechanism for chronic pain (Li et al., 2019).

In addition, activated gliocytes and increased proinflammatory cytokines in the hippocampus may be involved in the development of hyperalgesia after nerve damage signaled by serotonergic system receptors (Zhu et al., 2009). However, when microinjected in the CA_1, CBD can alleviate neuroinflammation and consequently reduce the activation of astrocytes.

In this context, our findings corroborate this point of view. In chronic pain, maladaptive plasticity related to the increase in PrL and hippocampal excitability reduced the communication of this pathway. The increase in the number of astrocytes in both regions favors this picture. In this way, we observe sensory, emotional and cognitive significant changes. The microinjection of CBD in the CA_1 evokes the LTP in this hippocampal region, attenuating nociceptive behaviors and comorbidities.

As an integrated circuit, the mPFC, dorsal hippocampus, and other areas that integrate the limbic system networks are involved in mood regulation and participate in learning processes and contextual memory. Chronic pain alters these connectivities, resulting in compromised limbic activity, which leads to neuroendocrine dysregulation and hyperactivity of some brain regions (Maletic et al., 2007). Morphological neuroplasticity, gliocytes proliferation, gene expression, significant molecular changes, and functional alterations can be seen in pain and comorbidities. We show that acute treatment with CBD can alleviate these symptoms, showing new treatment perspectives for chronic pain and neuropsychiatric comorbidities.

The present study observed that intra-CA_1 CBD has an antinociceptive effect on the response of mechanical allodynia and cold allodynia in animals with 21 days of CCI-induced neuropathy. In addition, CBD microinjections in CA_1 of CCI animals improve performance on behavioral tests decreasing depression and cognitive impairments. The role of CB_1 endocannabinoid and 5-HT_{1A} serotoninergic receptors of PrL/mPFC on cognitive and emotional aspects in animals with chronic NP was also demonstrated. Nevertheless, it has been shown that CCI-induced neuroanatomical changes, such as neuronal activation and the presence of astrocytes, can be reversed with the acute treatment of CA_1 with CBD in animals with chronic neuropathic pain.

Declarations

Author's contributions

A. C. Medeiros and P. de Medeiros performed the experiments, analyzed and interpreted the data, designed the figures, and wrote the manuscript. G. R. Pigatto performed neuromolecular experiments. N. C. Coimbra supervised the morphological experiments, analyzed data, and revised the final manuscript. R. L. de Freitas designed the experiments, analyzed and interpreted the data, wrote the manuscript, and approved the final manuscript. All authors have approved the final version of the manuscript.

Acknowledgments

Special thanks to Mr. Daoud Hibrahim Elias Filho, Mr. Paulo Castilho, Ms. Ariane Santana, and Ms. Maria Rossato for their expert technical assistance.

Funding

This research was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) (Research Grant 2013/12916-0 and Multi-user Equipment Grant 2014/11869-0). R.L. de Freitas was supported by FAPESP (researcher fellowship 2014/07902-2). A.C. Medeiros was supported by FAPESP (Scientific Initiation Scholarship 2017-12116-4). FAPESP also supported P. Medeiros (Sc.D. fellowship grant 2012/25167-2; post-doctoral fellowship grant 2017/13560-5). Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) supported Priscila Medeiros (post-doctoral fellowship grant 150806/2021-3). NC Coimbra is a researcher from CNPq (PQ_{1A} grants 301905/2010-0 and 301341/2015-0; PQ_2 grant 302605/2021-5). Neither of these funding sources had any role in the study design, collection, analysis, and interpretation of the data, report writing, or decision to submit the paper for publication.

Conflict of Interest Statement

The authors declare no conflicts of interest concerning the work presented herein.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Figures

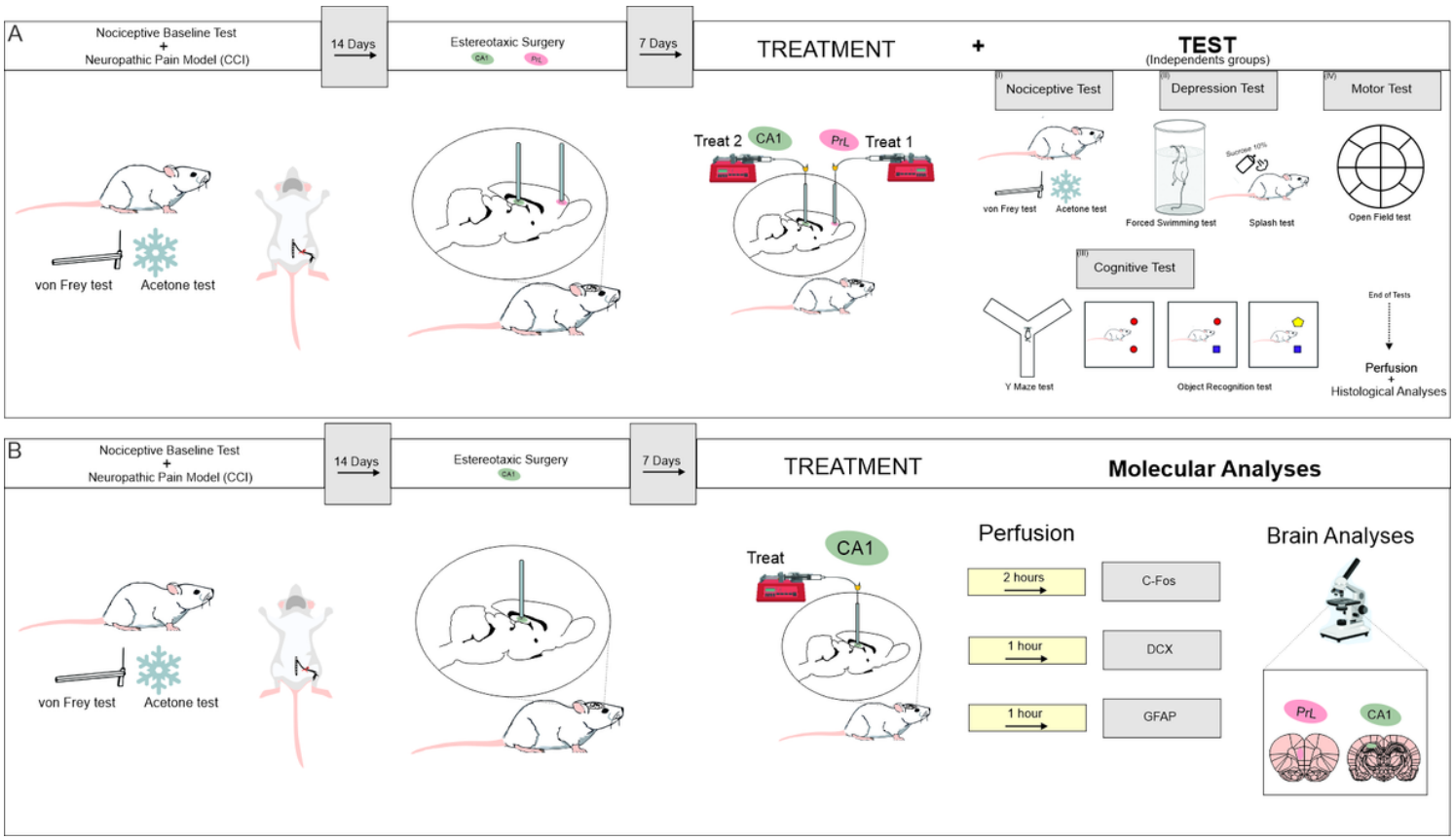

Figure 1

Representative scheme of the experimental design.

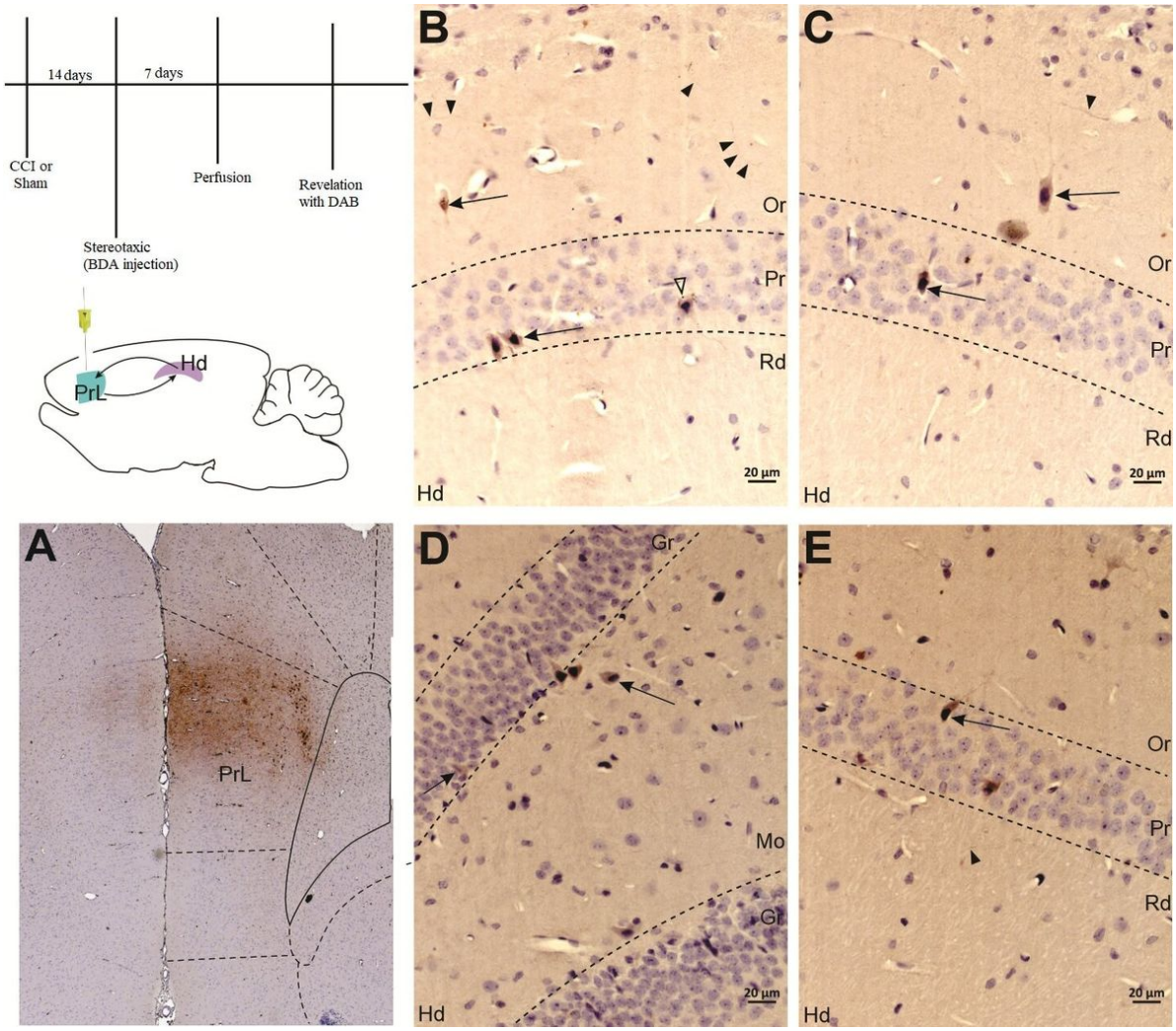

Figure 2

Photomicrographs of transverse sections of medial prefrontal cortex and dorsal hippocampus of *Rattus norvegicus*. (A) Wistar rat forebrain in a transverse section shows a representative BDA microinjection site in PrL. Photomicrograph of a representative coronal section of CA_1 (B and C), dentate gyrus (D), and CA2 (E), showing BDA-labeled terminal buttons (black dots), axonal fibers (black arrowheads), and cell bodies (arrow). (D) Photomicrograph of a representative dentate gyrus coronal section shows BDA-labeled axonal fibers (black arrowheads) and cell bodies (arrow).

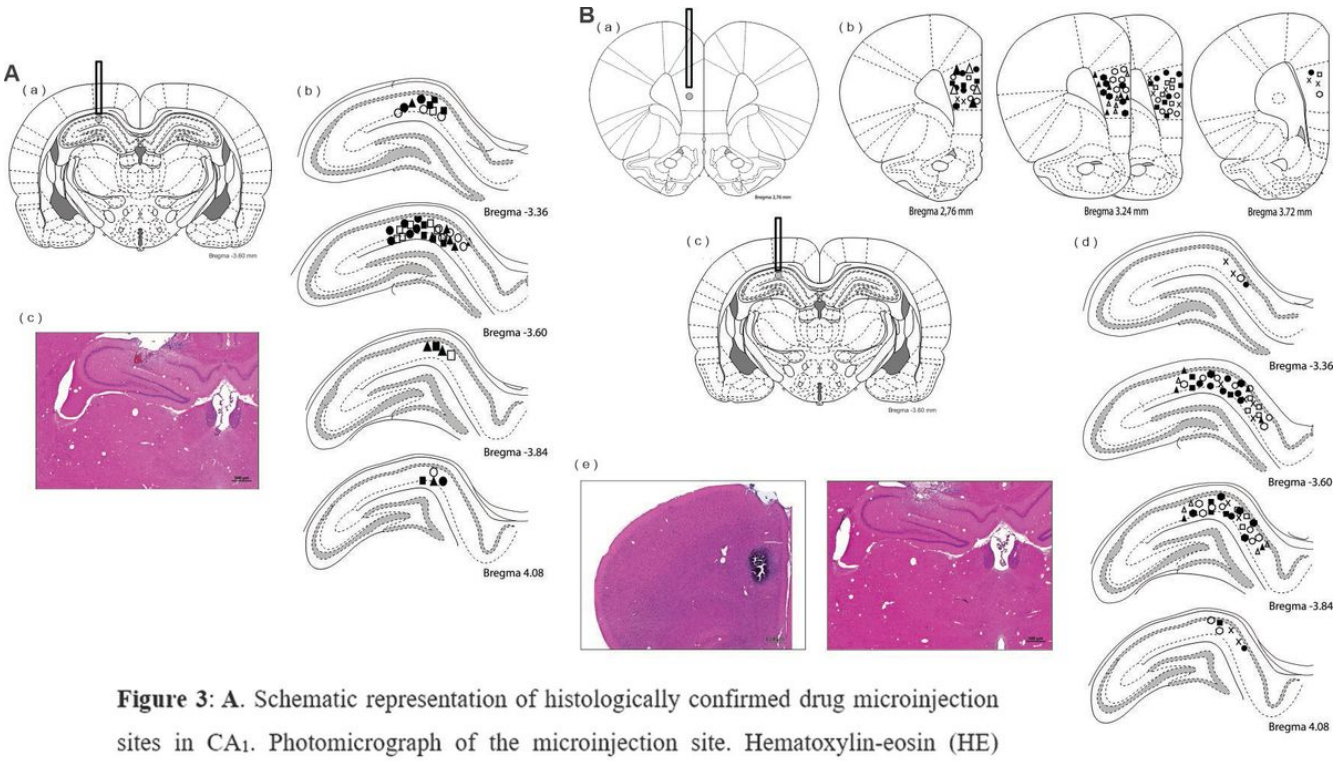

Figure 3: **A**. Schematic representation of histologically confirmed drug microinjection sites in CA_1. Photomicrograph of the microinjection site. Hematoxylin-eosin (HE) staining. Representation of microinjection sites in CA_1 according to the following experimental groups: (○) vehicle (CCI), (□) vehicle (sham), (▲) CBD 15 nmol, (■) CBD 30 nmol, (●) CBD 60 nmol stereotaxic atlas of Paxinos and Watson (2017). **D**. A. Schematic representation of drug microinjection sites in CA_1: (x) vehicle (CCI), (●) CBD 60 nmol, (⬡) Vehicle+$CoCl_2$, (□) WAY100635+Vehicle, (Δ) AM251 +Vehicle, (⬣) $CoCl_2$+ CBD, (■) WAY-100635+CBD, (▲) CBD+AM251.

Figure 3

See image above for figure legend.

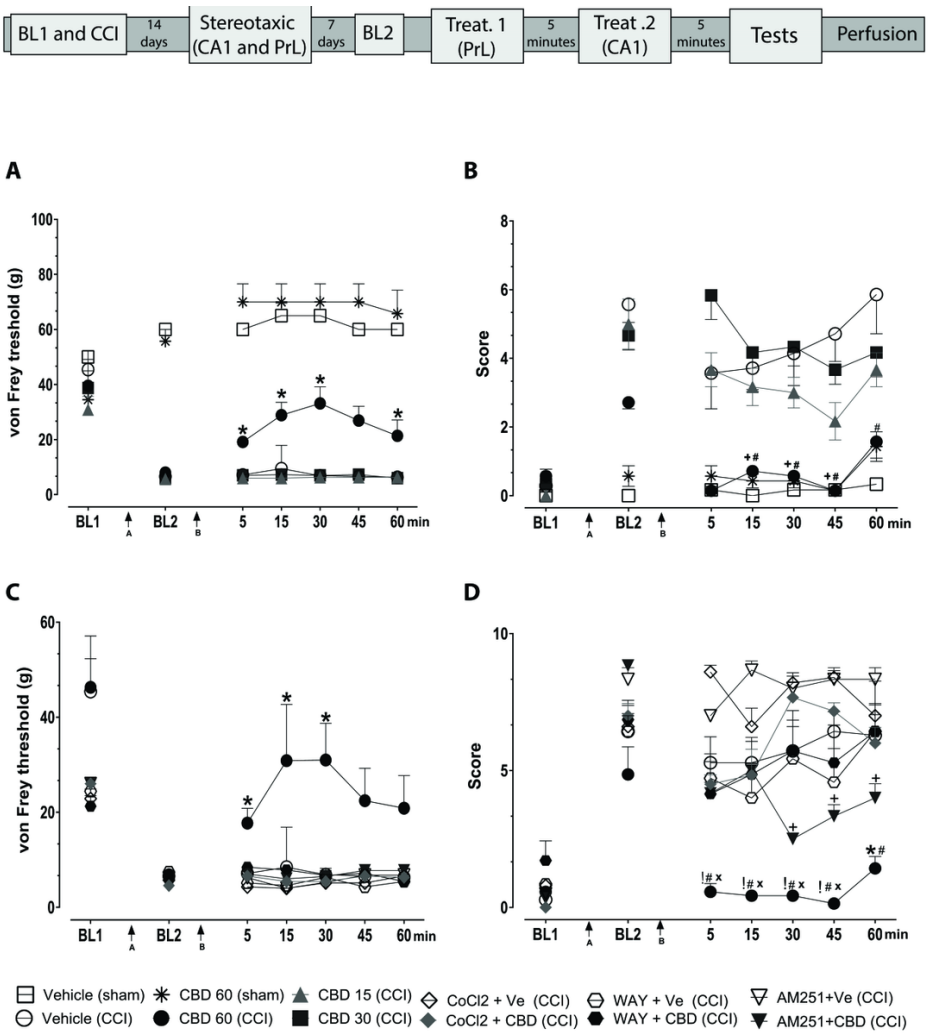

Figure 4

Representation of the effect of intra-CA_1 microinjections of CBD at 15, 30, and 60 nmol or vehicle (A and B) and PrL treatments with $CoCl_2$ (1nM), WAY-100635 (0.37 nmol), AM251 (100 nmol) or vehicle followed by intra-CA_1 microinjections of CBD 60 nmol or vehicle (C and D) of rats with chronic neuropathic pain induced by chronic sciatic nerve constriction (CCI) submitted to the von Frey and acetone tests. Data are represented as the mean and standard error of the mean. *Significant difference in relation to other groups; + significant difference from the vehicle-treated group; # significant difference in relation to the group treated with CBD at 30 nmol, according to Tukey's post-test (*$p<0.05$). BL1: baseline recorded before procedures; Arrow A: CCI or Sham surgery; BL2: new baseline recorded after 21-day CCI; Arrow B: drug microinjection (n=9). ! significant difference in relation to groups treated with $CoCl_2$ in PrL; # significant difference in relation to groups treated with WAY-100635 in PrL; x significant difference in relation to groups treated with AM251 in PrL; + significant difference compared to the AM251 + Vehicle (CCI) group.

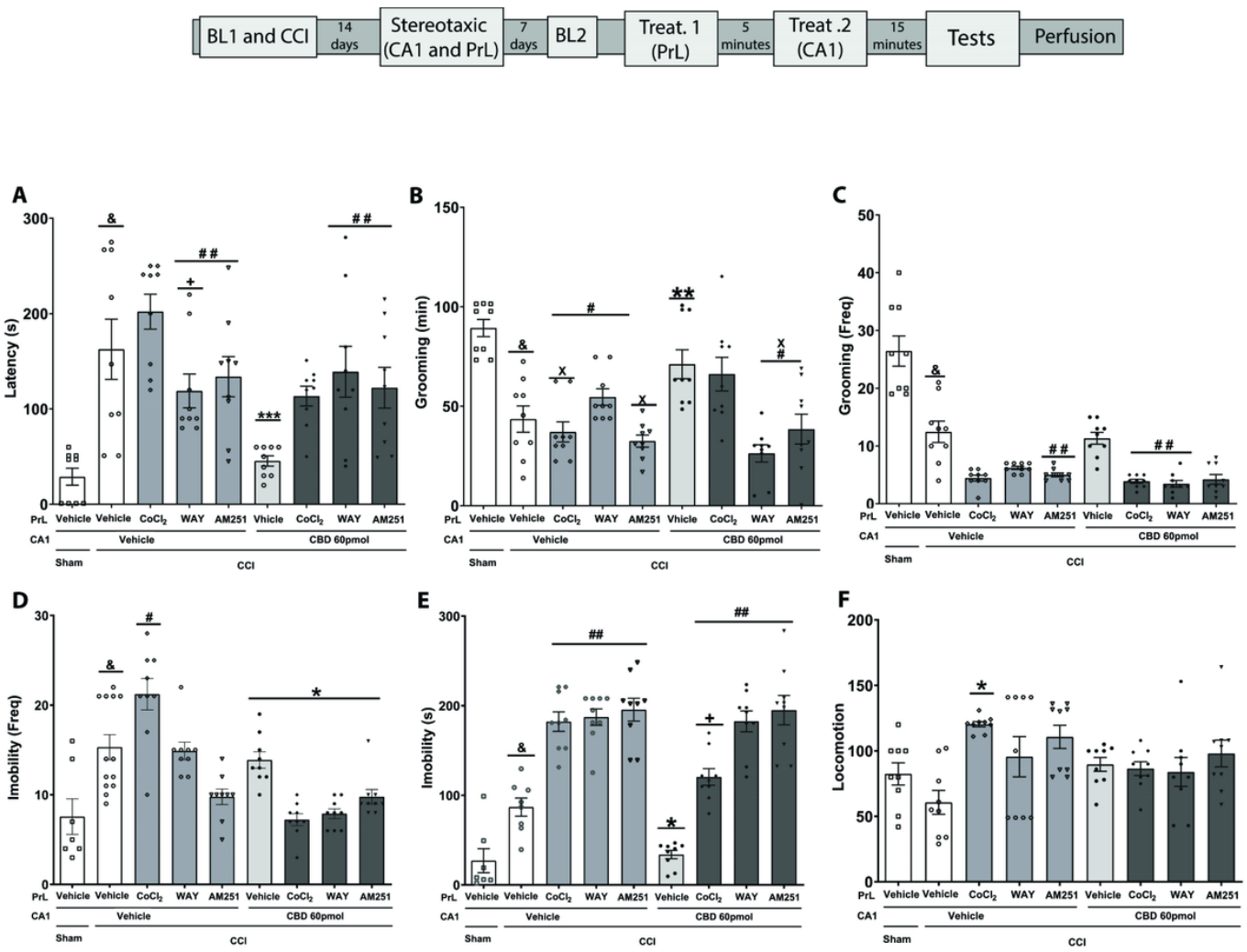

Figure 5

Representation of the effect of intra-DH microinjections of either 60 nmol CBD or vehicle and intra-PrL of either $CoCl_2$, WAY-100635, AM251, or vehicle in Wistar rats with chronic neuropathic pain induced by chronic sciatic nerve constriction on the behavior of self-cleaning, latency (A), time (B) and frequency (C) in the sucrose spray test. Graphic representation of the effect of intra-DH microinjections of either CBD at 60 nmol or vehicle and intra-PrL of either $CoCl_2$, WAY-100635, AM251, or vehicle in Wistar rats with chronic neuropathic pain induced by chronic sciatic nerve constriction. The effect over time (D) and frequency (E) of the immobility behavior in the forced swim test. Effect on total locomotion in the open field test (F). Data were represented as the mean and standard error of the mean. & Significant difference compared to the Sham (Sh) group; *Significant difference in relation to the vehicle group (Ve); # Significant difference from the CBD-treated group; + Significant difference compared to the $CoCl_2$+Vehicle group (*p<0.05; **p<0.01), according to Tukey's post-test; (n=9).

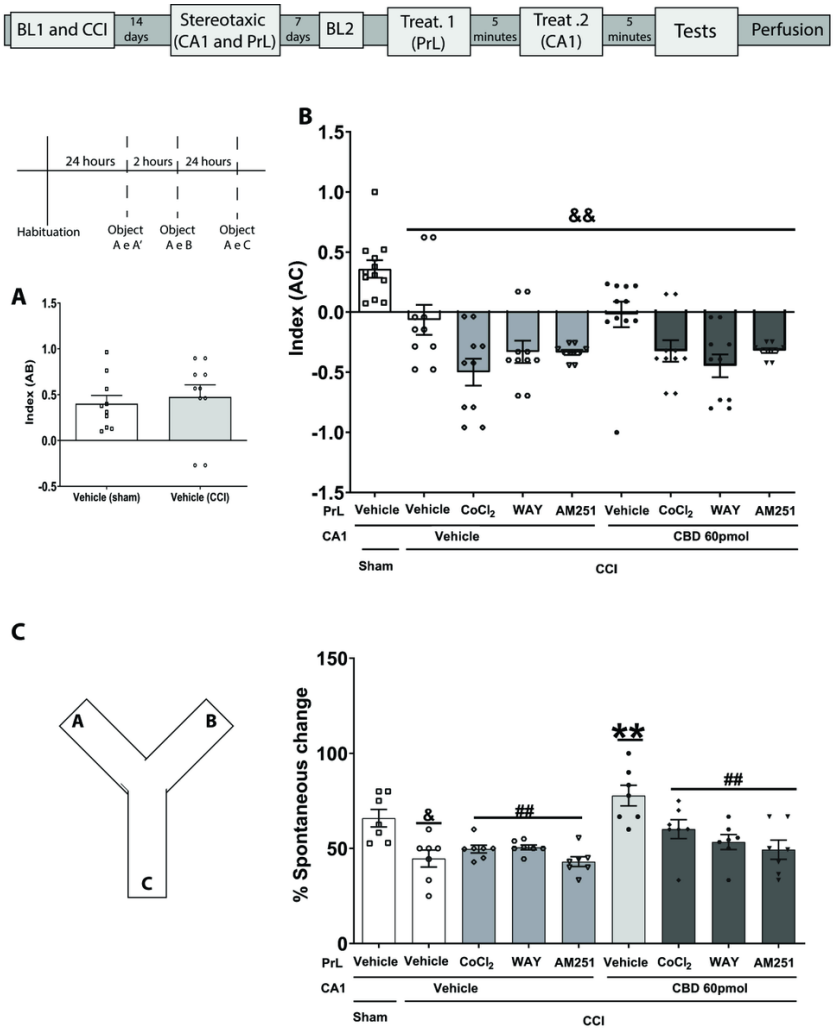

Figure 6

Representation of the effect of intra-DH microinjections of either CBD at 60 nmol or vehicle and intra-PrL of either $CoCl_2$, WAY-100635, AM251, or vehicle in Wistar rats with chronic neuropathic pain induced by chronic constriction of the sciatic nerve on the task of object recognition (**A-B**). **C**. Graph represented the effect of intra-DH microinjections of either CBD at 60 nmol or vehicle and intra-PrL of either $CoCl_2$, WAY-100635, AM251, or vehicle in Wistar rats with chronic neuropathic pain induced by chronic sciatic nerve constriction on spontaneous alternations in the Y-maze test. Data were represented as the mean and standard error of the mean. & Significant difference compared to the Sham (Sh) group; *Significant difference in relation to the vehicle-treated group (Ve); # significant difference in relation to CBD, according to Tukey's post-test (*p<0.05); (n=9).

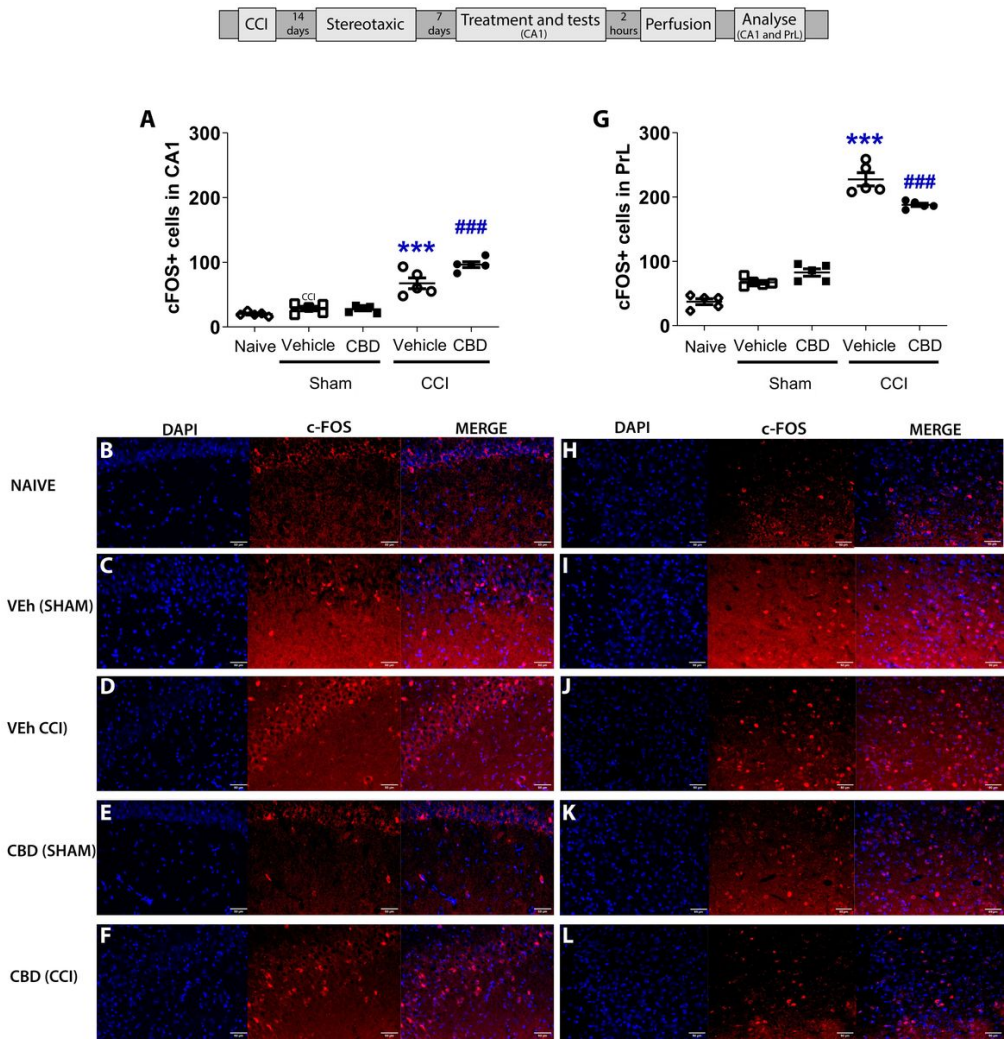

Figure 7

CBD at 60 nmol Effect on the number of c-Fos protein-labeled neurons in CA_1 and PrL. Graph representing the number of c-Fos protein-labeled cells in CA_1 (A) and representative figures showing c-Fos protein-labeled cells in CA_1 (B-F). Graph representing the number of c-Fos protein-labeled cells in PrL (G) and representative figures from c-Fos protein-labeled cells in PrL (H-L). Data were represented as the mean and standard error of the mean. *Significant difference from the sham group; # significant difference in relation to the vehicle-treated group, according to Tukey's post-test (*$p<0.05$); (n=5).

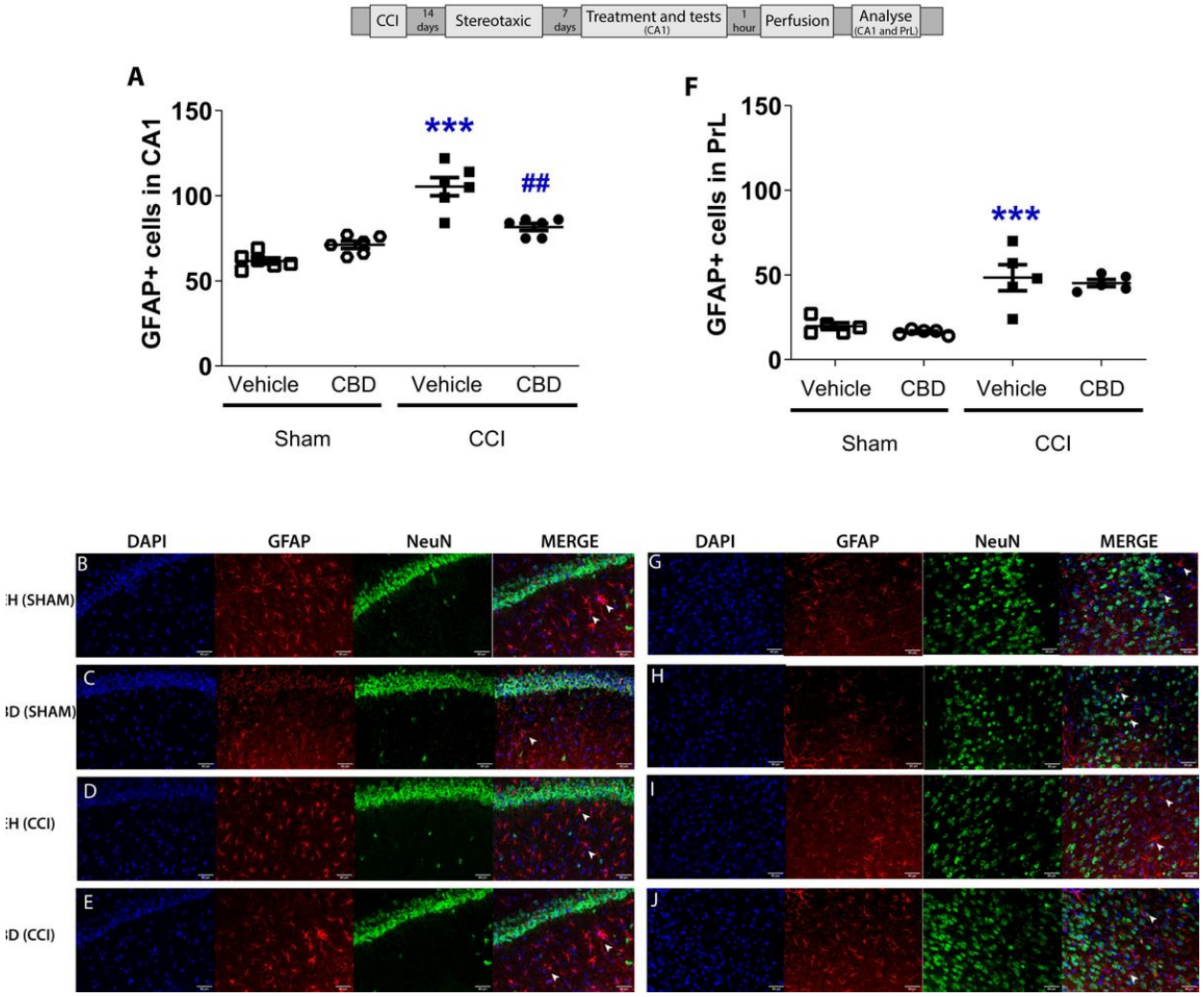

Figure 8

The effect of CBD at 60 nmol on the number of astrocytes in CA1 and PrL from the GFAP-labeling approach. Graph representing the number of GFAP-labeled cells in CA_1 (A) and representative figures from GFAP-labeled cells in CA_1 (B-E). Graph representing the number of GFAP-labeled cells in PrL (F) and representative figures from GFAP-labeled cells in PrL (G-J). The mean and standard error of the mean represented PrL data. *Significant difference from the sham group; # difference in relation to the vehicle-treated group, according to Tukey's post-test (*$p<0.05$); (n=5).

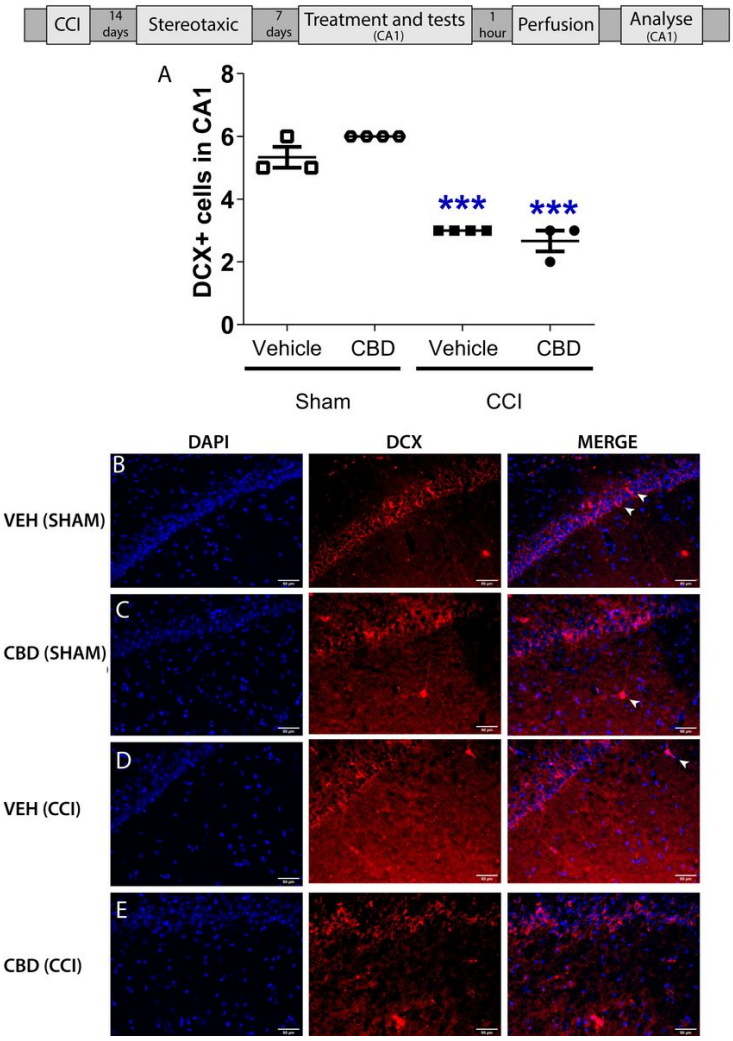

Figure 9

Effect of CBD at 60 nmol on the number of DCX-labeled cells in CA_1 (A) and representative figures from DCX-labeled in CA_1 (B-E). Data were represented as the mean and standard error of the mean. *Significant difference from the sham group; # difference in relation to the vehicle-treated group, according to Tukey's post-test (*$p<0.05$); (n=5).

Supplementary Files

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