Cannabidiol confers neuroprotection in rats in a model of transient global cerebral ischemia: impact of hippocampal synaptic neuroplasticity

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Abstract

Evidence for the clinical use of neuroprotective drugs for the treatment of cerebral ischemia (CI) is still greatly limited. Spatial/temporal disorientation and cognitive dysfunction are among the most prominent long-term sequelae of CI. Cannabidiol (CBD) is a non-psychotomimetic constituent of *Cannabis sativa* that exerts neuroprotective effects against experimental CI. The present study investigated possible neuroprotective mechanisms of action of CBD on spatial memory impairments that are caused by transient global cerebral ischemia (TGCI) in rats. Hippocampal synaptic plasticity is a fundamental mechanism of learning and memory. Thus, we also evaluated the impact of CBD on neuroplastic changes in the hippocampus after TGCI. Wistar rats were trained to learn an eight-arm aversive radial maze (AvRM) task and underwent either sham or TGCI surgery. The animals received vehicle or 10 mg/kg CBD (i.p.) 30 min before surgery, 3 h after surgery, and then once daily for 14 days. On days 7 and 14, we performed a retention memory test. Another group of rats that received the same pharmacological treatment was tested in the object location test (OLT). Brains were removed and processed to assess neuronal degeneration, synaptic protein levels, and dendritic remodeling in the hippocampus. Cannabidiol treatment attenuated ischemia-induced memory deficits. In rats that were subjected to TGCI, CBD attenuated hippocampal CA1 neurodegeneration and increased brain-derived neurotrophic factor levels. Additionally, CBD protected neurons against the deleterious effects of TGCI on dendritic spine number and the length of dendritic arborization. These results suggest that the neuroprotective effects of CBD against TGCI-induced memory impairments involve changes in synaptic plasticity in the hippocampus.

1. Introduction

Transient global cerebral ischemia (TGCI) is a devastating outcome of reversible cardiac arrest and other clinical conditions, such as severe cardiac arrhythmias, respiratory arrest, gas poisoning, hypotensive shock, and perinatal asphyxia, which may result in hypoxic/ischemic brain damage [1, 2]. A few minutes of global cerebral ischemia (GCI) can produce extensive neuronal damage and impact synaptic plasticity in vulnerable areas of the brain [3, 4]. Hippocampal pyramidal neurons in the cornu ammonis (CA), especially in the CA1 subfield, are dramatically affected by TGCI in both humans [5, 6] and experimental animals [7, 8]. Hippocampal damage is associated with long-term sequelae of GCI, such as cognitive impairments, spatial/temporal disorientation, and deficits in learning, memory, and attention [9]. Despite intense research efforts, no safe and effective pharmacological therapy has yet been discovered that can treat cognitive impairments that are caused by cerebral ischemic insult [10, 11].

Cannabidiol (CBD) is a major non-psychotomimetic phytochemical that is present in the *Cannabis sativa* plant and has emerged as a potential treatment for several clinical conditions, many of which are characterized by alterations of memory processing. For example, CBD treatment improved memory deficits in patients with treatment-resistant epilepsy [12–14]. In preclinical models, CBD improved cognition in animal models of schizophrenia, Alzheimer's disease, meningitis, cerebral malaria, and hepatic encephalopathy (for review, see [15]). However, only a few studies have investigated the effects of CBD on cognitive impairments in GCI. Pazos et al. (2012) investigated the effects of CBD on cognition in

newborn rats that were subjected to hypoxia/ischemia (HI)-induced brain injury. A single injection of CBD (1 mg/kg, s.c.) 10 min after the HI insult led to long-lasting neuroprotection, reflected by better performance in the novel object recognition test 30 days after the insult [16]. In mice with global GCI that was induced by bilateral common carotid artery occlusion (BCCAO), daily CBD treatment (3–30 mg/kg) for 14 days improved spatial memory in the Morris water maze [17]. Mice that were subjected to BCCAO and received short-term CBD treatment (10 mg/kg, 30 min before and 24, 48, and 72 h after BCCAO) performed better than sham controls in spatial memory tests (i.e., Y-maze test and object location test [OLT]) [18].

The beneficial effects of CBD on spatial memory performance in ischemic mice have been associated with decreases in hippocampal neuronal death and markers of neuroinflammation and increases in hippocampal levels of trophic factors and proteins that are involved in synaptic plasticity [17, 18]. Fogaça et al. (2018) reported that CBD induced anxiolytic-like responses in chronic stressed mice by stimulating hippocampal neurogenesis and dendritic remodeling [19]. Remaining unknown, however, is whether CBD promotes such plastic changes in the hippocampus in rodents with GCI.

In the present study, we used a four-vessel occlusion (4-VO) model of TGCI in rats to investigate the impact of long-term treatment with CBD on ischemia-induced memory impairments and neuroplastic changes in the hippocampus. We first evaluated the effects of CBD treatment on spatial memory performance in ischemic rats in the aversive radial maze (AvRM) and OLT. We then evaluated the effects of CBD on the expression of key proteins that are involved in synaptic plasticity, including brain-derived neurotrophic factor (BDNF), synaptophysin (SYN), and postsynaptic density protein-95 (PSD-95). Dendritic remodeling in the hippocampus was investigated in CBD-treated TGCI rats using Golgi-Cox staining.

2. Materials And Methods

2.1. Ethics statement

This study was performed at the State University of Maringá in strict accordance with Brazilian College of Animal Experimentation (COBEA) recommendations. The animal experiments were approved by the local Ethics Committee on Animal Experimentation of the State University of Maringá (animal license no. CEUA 1555230316).

2.2. Animals

A total of 145 young adult male Wistar rats (3–4 months old) were acquired from the local vivarium of the State University of Maringá (Paraná, Brazil). The animals were housed in groups of three animals per cage in a temperature-controlled room (22°C ± 1°C) with a 12 h/12 h light/dark cycle (lights on at 7:00 AM, lights off at 7:00 PM). The animals had free access to tap water and a standard commercial chow diet (Nutrilab-CR1; Nuvital Nutrients, Curitiba, PR, Brazil) during the experiments. The local Ethics Committee on Animal Experimentation of the State University of Maringá approved the experimental

procedures in accordance with the guidelines of the U.S. National Institutes of Health and Brazilian College for Animal Experimentation (CEUA no. 1555230316). All efforts were made to minimize the number of animals used and reduce their suffering.

2.3. Drugs

Cannabidiol (THC Pharma, Frankfurt, Germany) was dissolved in 2% Tween 80 (Synth, Maringá, Brazil) in sterile isotonic saline (vehicle). The 10 mg/kg dose of CBD was based on previous studies that reported a neuroprotective effect of CBD against CI in rodents [18, 20].

2.4. Transient global cerebral ischemia

Transient global cerebral ischemia was induced using the 4-VO model [21] with modifications [22]. The animals were fist anesthetized in a chamber that contained halothane (Isoforine, Cristália, SP, Brazil), and the dorsal and ventral regions of the neck were trichotomized. The rats were then fixed in a stereotaxic frame, and a halothane/oxygen mixture was delivered (0.5 L/min) through a face mask that was affixed to the rat's snout. After bilateral exposure of the alar foramen of the first cervical vertebrae, the vertebral arteries were permanently electrocoagulated (unipolar current, 3–4 mA). The common carotid arteries were exposed through an incision on the ventral neck and loosely tied with silk thread. Four to 5 h later, when the animals completely recovered from anesthesia, the silk thread was carefully tightened for 15 min. During this time, such signs as loss of the righting reflex, mydriasis, tonic stretching of the paws, and the absence of responses to touch were considered indicative of effective ischemia. After reperfusion, the animals were maintained in a warming box (37°C ± 1°C) for 1 h to avoid ischemia-induced cerebral hypothermia [23]. Sham-operated animals were subjected to the same surgical procedures without occlusion of the vertebral or carotid arteries.

2.5. Experimental design

2.5.1. Experiment 1

In Experiment 1 (Fig. 1), naive rats were trained to learn the eight-arm AvRM task (see details below). After 10 days of training, the rats underwent sham or TGCI surgery. Vehicle or 10 mg/kg CBD was administered (i.p.) 30 min before reperfusion, 3 h after reperfusion, and then daily for 14 days. On days 7 and 14 of reperfusion, retention memory trials (RMTs) were conducted to assess retrograde memory performance. The animals were then euthanized under deep anesthesia, and their brains were processed for immunohistochemistry and Western blot. Neuronal nuclei (NeuN), SYN, PSD-95, and BDNF protein levels were measured in the hippocampus.

2.5.2. Experiment 2

In Experiment 2 (Fig. 1), TGCI rats were treated with vehicle or 10 mg/kg CBD following the same administration protocol as in Experiment 1. On days 7, 13, and 14 postischemia, the animals were evaluated for locomotor behavior in the open field (OF) test and memory in the OLT (see below). One day

after the OLT, the animals were euthanized under deep anesthesia, and brains were collected and assayed for Golgi-Cox staining.

2.6. Behavioral tests

2.6.1. Eight-arm aversive radial maze

The AvRM consisted of a central, polygonal platform with eight arms that radiated outward from alternate sides (Fig. 2A). Aversive illumination was provided by spotlights that were positioned 1 m above the maze. An opening at the end of each arm provided access to a dark wooden box, which served as a shelter for the animals. Only one arm contained the true goal box (closed-end box). In the remaining arms, the boxes were open-ended (i.e., false goal box). Additional details about the AvRM apparatus can be found elsewhere [24].

The test was divided into two phases. In the training phase, naive rats were placed in the apparatus for 10 days to learn the goal box's spatial location, with one session/day and three trials/session. The animals were placed individually in the center of the arena. Thirty seconds later, the guillotine doors were opened simultaneously, thereby allowing the animal to explore the entire maze. When the animal entered an arm that contained the false goal box, the remaining arm doors were closed. After returning to the central arena, the animal was again confined there for another 10 s and then released to explore the arms. A trial ended when the rat found the true goal box or a cut-off time of 4 min elapsed. If the goal box was not found within that time, then the rat was led by the experimenter to the arm that contained the true goal box and gently guided to enter it. The rat remained in the goal box for 1 min and then was returned to its home cage. After each trial, the maze was cleaned and randomly rotated on its central axis, and the goal box was moved randomly to any other arm, but its spatial position remained unchanged relative to extra-maze cues. Preoperative learning performance was estimated by the following parameters: (*i*) latency to find the goal box, (*ii*) number of reference memory errors, and (*iii*) number of working memory errors. During each trial, a reference memory error was counted every time the rat first visited an arm that contained a false goal box. If it returned to an arm that had been previously visited, then a working memory error was recorded. After 10 days of training, the rats were subjected to TGCI and allowed to recover from surgery for 1 week. Retrograde memory performance was then assessed on day 7 (RMT1) and day 14 (RMT2) post-ischemia according to the same procedure that was used during the learning phase. All of the behavioral tests were conducted in a temperature-controlled (23ºC) and sound-attenuated room.

2.6.2. Open-field test

Locomotor activity was evaluated in the OF, which consisted of a wooden square box (70 cm · 70 cm) with 40 cm high walls. The rats were individually placed in the central area of the OF and allowed to freely explore the arena for 10 min. After each session, the OF was cleaned with 70% ethanol and water and then dried. The distance traveled (in meters) was evaluated using a contrast-sensitive video tracking system (ANYmaze, Stoelting, Wood Dale, IL, USA).

2.6.3. Object location test

The OLT is a one-trial test that measures the recognition memory of an object's location [25]. The apparatus consisted of a circular arena (83 cm diameter, 40 cm high wall) where the rats were exposed to two identical objects (i.e., glass bottles; 9 cm maximum diameter, 22 cm height) that were filled with water and sand so that the rat could not move them. On day 1 (i.e., day 13 post-ischemia), both objects were positioned equidistant (10 cm) from the arena wall. The animal was placed in the apparatus and allowed to explore it for 3 min. This process, considered the training session, was repeated five times at 15 min intervals. On day 2 (i.e., day 14 post-ischemia), a retraining (r-T) session was performed, in which the animal was placed in the arena for 3 min with the objects in the same positions as the previous day. After the r-T session, the animal was returned to its home cage, and the location of one of the objects was moved diagonally relative to the other object. After 15 min, the rat was placed again in the OF arena for the test session. The time spent exploring the two objects during the r-T and test sessions was manually recorded.

Another parameter, the discrimination index (D2), was also analyzed and calculated as the following: D2 = (exploration time in the novel location – exploration time in the familiar location) / (exploration time in the novel location + exploration time in the familiar location). Exploratory behavior was considered when the rat directed its nose toward the object at a distance of ≤ 1 cm and/or touched the object with its nose. Animals that explored the objects for less than 5 s were excluded from further analysis. Between sessions, the objects and arena were cleaned with 70% ethanol and water.

2.7. Biochemical and histological analyses

2.7.1. Immunohistochemistry

On day 15 post-ischemia, one group of animals was deeply anesthetized with sodium thiopental (75 mg/kg, Thiopentax, Cristália, SP, Brazil) and transcardially perfused with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde. Brains were removed and postfixed with the same fixative solution for 24 h and then cryoprotected by immersion in 30% sucrose for 72 h. The brains were frozen and sliced in a cryostat (Leica CM1860 UV, Wetzlar, HE, Germany) into 30 μm coronal sections (-2.30 to -4.52 mm from bregma) that encompassed dorsal portions of the hippocampus [26]. The brain slices were collected in replicates in tubes that contained antifreeze solution (15% sucrose and 30% ethylene glycol in PBS) and stored at -24°C until further processing.

NeuN immunohistochemistry was used to identify mature neurons. Free-floating sections were first washed with PBST (0.1 M PBS [pH 7.4] plus 0.3% Triton X-100) and then incubated with citrate solution at 50ºC for 30 min. Endogenous peroxidase activity was blocked by incubating the slices in 1% H_2O_2 in PBS for 30 min. After rinsing in PBST, the sections were blocked with 2% bovine serum albumin in PBST for 60 min. After three washes in PBST, the sections were incubated with rabbit polyclonal anti-NeuN antibody (1:500, catalog no. ab177487, Abcam, Cambridge, MA, USA) in PBST overnight at 4°C. The sections were incubated with specific biotinylated secondary antibody (1:500, Santa Cruz Biotechnology,

Santa Cruz, CA, USA) for 90 min and then in ABC solution (Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, CA, USA) for 2 h at room temperature. The colorimetric peroxidase reaction was performed using 3,3'-diaminobenzidine (DAB; Sigma-Aldrich, St. Louis, MO, USA) and 0.05% H_2O_2. $NiCl_2$ was added to the DAB solution to increase staining contrast. After the immunolabeling procedures, the sections were mounted on gelatin-coated slides and coverslipped with Permount mounting medium.

Quantification was performed by an experimenter who was blind to the experimental groups. The analysis was performed using an Olympus BX41 microscope (Olympus, Tokyo, Japan) coupled to a color high-performance device camera (QColor3, Ontario, Canada) with 10·, 20·, and 40· objectives for the dentate gyrus (DG) and CA3/CA1 areas of the hippocampus, respectively. The lighting conditions and magnifications were maintained constant during image capture to avoid signal saturation. ImageJ software (National Institutes of Health, Bethesda, MD, USA) was used to calculate the integrated optical density (IOD) when appropriate. Immunoreactive cells were quantified in prefixed digital microscopic areas of the CA1 and CA3, encompassing the entire region of interest. The DG, including the subgranular zone and granular cell layer, was measured in each section. Ten to 12 brain sections were evaluated per animal. For the IOD measurements, the selected images were converted to 16-bit image grayscale, and the background was subtracted. The threshold for a positive signal was predefined, and the IOD was calculated. The results are expressed as the mean IOD/area ± standard error of the mean (SEM).

2.7.2. Western blot

BNDF, SYN, and PSD-95 protein levels were determined in the hippocampus. The animals were deeply anesthetized with isoflurane/oxygen (Isoforine, Cristália, SP, Brazil) and decapitated. Brains were removed, and the whole hippocampus was carefully isolated. Tissues were macerated for protein extraction with lysis buffer (150 mM NaCl, 50 mM Tris-HCl [pH 7.5], 5 mM NaF, 1 mM Na_3VO_4, 2.5 mM $Na_2H_2O_7P_2$, and 1% Triton-X-100) and protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). The samples were centrifuged at 12000 rotations per minute (rpm) for 15 min at 4ºC, and the supernatant was preserved. For the experiments that were performed with the synaptosome fraction (synaptic proteins), a group of animals had their hippocampus homogenized in a solution that contained 0.32 M sucrose, 20 mM HEPES, 1 mM ethylenediaminetetraacetic acid (EDTA), 5 mM NaF, 1 mM Na_3VO_4, and protease inhibitor cocktail. The homogenate was centrifuged at 2800 rpm for 10 min at 4ºC, and the supernatant was discarded. Pellets that contained synaptosomes were resuspended in lysis buffer (150 mM NaCl, 50 mM Tris-HCl [pH 7.4], 1% Triton-X-100, 0.1% sodium dodecyl sulfate, 2 mM EDTA, 1 mM Na_3VO_4, and 5 mM NaF). Total proteins were quantified using the Bradford method. Homogenates were exposed to 4–12% NuPAGE Bis-Tris gel (Invitrogen, Carlsbad, CA, USA) for protein separation and transferred to a nitrocellulose membrane (GE Healthcare, Chicago, IL, USA). The membranes were blocked with 5% nonfat milk in TBST (Tris-buffered saline [pH 7.6] plus 0.1% Triton X-100) and incubated overnight at 4ºC with primary antibodies at the following dilutions: anti-PSD-95 (1:1000; Cell Signaling Technology, Danvers, MA, USA), anti-synaptophysin (1:1000; Cell Signaling Technology, Danvers, MA, USA), anti-BDNF (1:400; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-GAPDH (1:5000; Abcam), and anti-β-actin (1:10000; Cell Signaling Technology, Danvers, MA, USA). The membranes were then

incubated for 2 h with specific secondary antibodies (anti-rabbit or anti-mouse; 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and revealed using Novex ECL chemiluminescent reagent (Invitrogen, Carlsbad, CA, USA). The density of preserved and unsaturated bands were quantified using Image Studio Lite 5.2 software (LI-COR Biosciences, Lincoln, NE, USA). The results are expressed as percentages (± SEM) relative to sham-operated rats that were treated with vehicle.

2.7.3. Golgi-Cox staining

Golgi-Cox staining was based on previous studies [22] with some modifications. Under deep anesthesia (75 mg/kg, Thiopentax, Cristália, SP, Brazil), the rats' brains were perfused by transcardiac perfusion with PBS. The brains were maintained in Golgi solution for 24 h at 37ºC [27] and then transferred to a new Golgi solution where they remained for 19 days in the dark. After this period, the brains were immersed in a 30% sucrose/saline solution for at least 6 days. Afterward, the brains were rapidly frozen in isopentane and dry ice and cut into 100 µm thick sections in a cryostat (Leica CM1860 UV, Wetzlar, HE, Germany) to obtain slices of the hippocampus. The sections were then transferred to gelatinized slides. After drying for 2 days at room temperature, the sections were stained with the FD Rapid GolgiStain kit (FD NeuroTechnologies, Columbia, MD, USA). The mounted tissue was dehydrated in successive alcohol baths, followed by xylene baths. Finally, the sections were covered with Permount and coverslipped.

Neurons that were impregnated with Golgi-Cox in the DG, CA1, and CA3 of the hippocampus were chosen based on the following criteria: (*i*) relatively isolated neurons, (*ii*) defined soma, (*iii*) whole-cell impregnation, and (*iv*) well distinguished dendritic arborization. Five to six neurons/animal were analyzed using a DM2500 M polarizing microscope (Leica Microsystem, Wetzlar, HE, Germany) with 20· and 100· objectives. The length of apical and basal dendrites, number of branches, and number of dendritic spines were recorded. Neuron images were captured, and dendritic arborization was traced using 3D Simple Neurite Tracer FIJI 3.1.3. software (National Institutes of Health, Bethesda, MD, USA). The number of dendritic spines was counted manually in a 10 µm linear length of an apical and basal tertiary branch [19]. The data are expressed as mean ± SEM.

2.8. Statistical analysis

Statistica 8.0 software (StatSoft, Palo Alto, CA, USA) and Prism 8 software (GraphPad, San Diego, CA, USA) were used for the statistical analysis. Data were examined for assumptions of a normal distribution using the D'Agostino and Pearson omnibus test and tested for homoscedasticity using Levene's test. The data met the normality and homoscedasticity criteria. The behavioral data were analyzed using Student's *t*-test and one- or two-way analysis of variance (ANOVA) as appropriate, followed by the Duncan multiple-comparison *post hoc* test. In the two-way repeated-measures ANOVA, group was the between-subjects factor, and trial (test day) was the within-subjects factor. In the OLT, functional spatial memory within groups (i.e., a D2 value that differed significantly from zero) was analyzed with a two-way one-sample *t*-test [28].

For the molecular data, the generalized linear model with a gamma distribution was used for continuous data (i.e., IOD for NeuN immunoreactivity, Western blots, and dendritic length based on Golgi-Cox

staining). One-way ANOVA followed by Duncan's *post hoc* test was used for the number of branches and number of dendritic spines based on Golgi-Cox staining. Data are expressed as mean ± SEM of biological replicates (rats). Values of $p \leq 0.05$ were considered statistically significant.

3. Results

3.1. Behavioral tests

3.1.1. Cannabidiol prevented the amnestic effect of ischemia

Figure 2B shows learning performance curves in the AvRM before ischemia. Intact rats that were allocated to each treatment condition learned the task very well and similarly, indicated by a highly significant main effect of time for all three parameters ($F_{9,324}$ = 7.58–35.34, $p < 0.0001$) but no between-group differences ($F_{2,36}$ = 0.03–0.12, $p > 0.05$).

The postoperative retrograde memory performance in each group is shown in Fig. 2C. The two-way ANOVA revealed a main effect of group for all parameters (latency: $F_{2,36}$ = 22.91, $p < 0.0001$; reference memory errors: $F_{2,36}$ = 16.60, $p < 0.0001$; working memory errors: $F_{2,36}$ = 10.10, $p < 0.001$; Fig. 2C). A main effect of trial was not detected for any of the three parameters ($F_{1,36}$ = 0.74–1.62, $p > 0.05$), although memory performance appeared to improve from RMT1 to RMT2 in the TGCI + vehicle group. A significant group · trial interaction was found for latency ($F_{2,36}$ = 4.58, $p < 0.05$), reference memory errors ($F_{2,36}$ = 6.04, $p < 0.01$), and working memory errors ($F_{2,36}$ = 3.08, $p = 0.05$). Compared with the sham + vehicle group, the TGCI + vehicle group had a longer latency and committed more reference and working memory errors during RMT1 (Duncan's test, $p < 0.0001$; Fig. 2C). Similar outcomes for the sham + vehicle *vs*. TGCI + vehicle groups were found for latency and reference memory errors in RMT2 (Duncan's test, $p < 0.001$–0.01; Fig. 2C). These results indicate that TGCI caused persistent retrograde amnesia, despite some degree of improvement. The amnestic effect of ischemia was prevented by CBD treatment. The longitudinal analysis (Fig. 2C) indicated that both the latency and number of errors significantly decreased in the TGCI + CDB group compared with the TGCI + vehicle group ($p < 0.0001$-0.01). In the sham-operated group, memory performance did not differ between the pre- and postischemic phases, indicating the endurance of retrograde memory in the AvRM task.

3.1.2. Cannabidiol alleviated TGCI-induced spatial memory impairments without affecting general motor activity

No differences were found in the distance traveled in the OF ($F_{2,46}$ = 0.31, $p > 0.05$), demonstrating that CBD treatment and the TGCI procedure did not affect general locomotor activity (Fig. 3B).

Figure 3C and D show the effects of ischemia on memory in the OLT. During r-T, the objects were in a familiar location, and all groups similarly explored both objects (data not shown). During the test session

(Fig. 3C), the sham + vehicle group exhibited greater exploration of the object in the new location (O2; Student's *t*-test, $t_{32} = 3.43$, $p < 0.01$) compared with the object in the familiar location (O1), indicating the rats' ability to discriminate between the familiar and unfamiliar locations (i.e., spatial memory performance). This discrimination ability was lost in the TGCI + vehicle group (Student's *t*-test, $t_{34} = 0.35$, $p > 0.05$), indicating that they did not retain memory of the familiar location that was learned during training. This effect of TGCI was alleviated by CBD treatment (Student's *t*-test, $t_{26} = 2.60$, $p < 0.05$).

The effects of ischemia and CBD on spatial memory in the OLT were also indicated by the discrimination index (D2; Fig. 3D). The one-way ANOVA revealed significant differences in D2 among groups ($F_{2,46} = 4.67$, $p < 0.05$). Duncan's *post hoc* analyses revealed that the TGCI + vehicle group had a lower, negative D2 score than the sham + vehicle group ($p < 0.05$). The TGCI + CBD group exhibited a significant decrease in spatial memory impairment to sham levels compared with the TGCI + vehicle group ($p = 0.05$). No difference in spatial memory was observed between sham + vehicle and TGCI + CBD groups (p □ 0.05). Object location memory is also reflected by D2 relative to zero, where zero indicates no memory (Fig. 3D). The sham + vehicle group had a positive D2 score relative to zero (Student's *t*-test, $t_{29} = 3.21$, $p < 0.01$), indicating the presence of spatial memory capacity. The TGCI + vehicle group did not present a statistically significant D2 score relative to zero (Student's *t*-test, $t_{30} = 0.50$, $p > 0.05$), indicating memory impairment that was caused by ischemia. The TGCI + CBD group had a D2 score that was significantly different from zero (Student's *t*-test, $t_{26} = 2.87$, $p < 0.01$), indicating that CBD prevented TGCI-induced spatial memory deficits when the animals were treated with CBD before ischemia.

3.2. Biochemical and histological analyses

3.2.1. Cannabidiol attenuated TGCI-induced neurodegeneration

TGCI-induced neurodegeneration was assessed by analyzing NeuN immunoreactivity in the DG, CA1, and CA3 of the hippocampus. As shown in Fig. 4B, a significant difference in NeuN immunoreactivity in the CA1 was found among groups ($\chi^2 = 7.07$, $p < 0.05$). The loss of hippocampal pyramidal neurons was detected in the TGCI + vehicle group compared with the sham + vehicle group ($p < 0.01$), and CBD treatment attenuated this neurodegeneration ($p = 0.07$). No differences in NeuN immunoreactivity were found in the CA3 ($\chi^2 = 4.09$, $p > 0.05$). Significant differences in NeuN immunoreactivity were found in the DG among groups ($\chi^2 = 7.33$, $p < 0.05$). Cannabidiol increased NeuN immunoreactivity in the TGCI + CBD group compared with the sham + vehicle and TGCI + vehicle groups ($p < 0.01–0.05$).

3.2.2. Cannabidiol changed neuroplasticity markers in the hippocampus of rats that were subjected to TGCI

Figure 5 shows the influence of CBD on the impact of ischemia on synaptic protein levels in the hippocampus. Significant differences in BDNF protein levels were observed among groups ($\chi^2 = 11.93$, $p < 0.01$; Fig. 5A). No differences in BDNF levels were found between the TGCI + vehicle and sham + vehicle

groups ($p > 0.05$). BDNF expression increased in the TGCI + CBD group compared with the sham + vehicle and TGCI + vehicle groups ($p < 0.01–0.05$).

Significant differences in SYN protein levels were found among groups ($\chi^2 = 5.61$, $p = 0.05$; Fig. 5B). A decrease in SYN levels was detected in the TGCI + vehicle group compared with the sham + vehicle group ($p = 0.05$). No differences in SYN levels were found between the TGCI + CBD and sham + vehicle groups ($p > 0.05$).

Significant differences in PSD-95 levels were found among groups ($\chi^2 = 7.60$, $p < 0.05$; Fig. 5C). A decrease in PSD-95 levels was found in the TGCI + vehicle group compared with the sham + vehicle group ($p < 0.001$). No difference in PSD-95 levels was found between TGCI + CBD and sham + vehicle groups ($p > 0.05$).

3.2.3. Cannabidiol prevented ischemia-induced dendritic spine loss

As shown in Fig. 6B, ischemia affected the length of dendrites of granular neurons in the DG ($\chi^2 = 10.45$, $p < 0.01$; Fig. 6B). Interestingly, however, the total dendritic length increased in the TGCI + vehicle group compared with the sham + vehicle group ($p < 0.05$) but significantly decreased in the TGCI + CBD group compared with the TGCI + vehicle group ($p < 0.001$). No difference in the number of branches was found among groups ($F_{2,12} = 0.38$, $p > 0.05$; Fig. 6B). The one-way ANOVA revealed significant differences in the number of dendritic spines in the DG among groups ($F_{2,14} = 20.12$, $p < 0.0001$). Duncan's *post hoc* test showed that the TGCI + vehicle group exhibited a decrease in the number of dendritic spines compared with the sham + vehicle group ($p < 0.001$). Cannabidiol treatment alleviated this effect compared with the TGCI + vehicle group ($p < 0.05$).

The one-way ANOVA also revealed significant differences in apical, basal, and the total number of dendritic spines in the CA1 subfield among groups ($F_{2,14} = 5.84–18.02$, $p < 0.001–0.01$; Fig. 7A). Further analysis revealed that the number of dendritic spines decreased in the TGCI + vehicle group compared with the sham + vehicle group ($p < 0.001–0.05$). Cannabidiol reversed this ischemia-induced dendritic spine loss in both apical and basal dendritic branches compared with the TGCI + vehicle group ($p < 0.001–0.05$). No differences in dendritic length ($\chi^2 = 0.44–0.93$, $p > 0.05$) or the number of branches ($F_{2,12} = 0.12–0.31$, $p > 0.05$) were found among groups (Fig. 7A).

As shown in Fig. 7C, ischemia did not alter the dendritic length of pyramidal neurons in the CA3 subfield. However, ischemic rats that were treated with CBD exhibited a significant increase in apical dendritic length compared with the sham + vehicle and TGCI + vehicle groups ($p < 0.01–0.05$). The one-way ANOVA revealed significant differences in apical, basal, and the total number of branches ($F_{2,12} = 2.81–3.67$, $p < 0.05$). Ischemic mice that were treated with CBD exhibited a significant increase in the number of apical branches compared with the TGCI + vehicle group ($p = 0.05$). A significant decrease in the number of basal branches was found in the TGCI + vehicle group compared with the sham + vehicle group ($p < 0.05$).

Ischemia-induced dendritic spine loss in the CA1 subfield was also observed in apical branches of pyramidal neurons in the CA3 subfield ($F_{2,12}$ = 4.05, p < 0.05; Fig. 7C). The TGCI + vehicle group exhibited a significant decrease in the number of apical dendritic spines compared with the sham + vehicle group (p < 0.05). The one-way ANOVA revealed differences in the number of basal dendritic spines among groups ($F_{2,14}$ = 4.32, p < 0.05). No significant difference in the number of basal dendritic spines was observed between the TGCI + vehicle and sham + vehicle groups (p < 0. 05), whereas the TGCI + CBD group exhibited an increase in the number of basal dendritic spines compared with the other groups (p < 0.05).

4. Discussion

In the present study, we found that repeated CBD treatment prevented memory impairments and decreased hippocampal CA1 neurodegeneration that were caused by ischemia. Cannabidiol also increased hippocampal BDNF levels, attenuated the TGCI-induced decreases in SYN and PSD-95 levels, and elevated dendritic spine number and arborization in the hippocampus in ischemic animals.

The effects of TGCI on spatial memory performance have been well documented [29–32]. Studies from our group showed deleterious effects of TGCI on retrograde memory in rats in the AvRM up to 39 days after the ischemic insult [33–35]. In the present study, memory impairments were detected up to 14 days after TGCI in rats in the AvRM and OLT. Cannabidiol attenuated these effects of TGCI, reflected by decreases in latency and the number of errors in the AvRM, indicating improvements in spatial memory performance. Cannabidiol also ameliorated memory deficits in the AvRM paradigm in middle-aged diabetic rats that underwent chronic cerebral hypoperfusion [36]. Spatial memory recovery in the Y-maze, OLT, and Morris water maze was also observed in BCCAO mice that were treated with CBD [17, 18]. The beneficial effects of CBD on memory function may extend to other conditions that are associated with deficits in memory processing. For example, single or repeated (14 days) injections of CBD (10 mg/kg, i.p.) ameliorated memory function in the object recognition test in rats that were subjected to iron overload [37]. Moreover, accumulating evidence indicates that CBD reduces learned fear in paradigms that are translationally relevant to phobias and posttraumatic stress disorder [38].

Selective hippocampal CA1 damage is known to impair hippocampus-dependent memory, such as spatial learning and memory performance [29, 39–41]. In the present study, we found the significant loss of CA1 neurons (detected by NeuN immunohistochemistry) in TGCI animals, which paralleled memory impairments in those animals in the AvRM. Cannabidiol treatment attenuated hippocampal CA1 neuronal loss that was induced by TGCI in rats. These results extend previous studies that reported histological neuroprotection in the CA1 subfield after CBD administration in mice [18] and gerbils [42] that were subjected to TGCI.

The extent to which the modest reduction of CA1 pyramidal loss that was elicited by CBD treatment contributes to memory preservation (or recovery) is uncertain. Fish oil treatment was reported to restore memory loss that was caused by TGCI without rescuing hippocampal CA1 pyramidal cells [24, 43–45].

Alterations at the electrophysiological, synaptic, and subcellular levels and morphological changes that extend beyond certain structures can result in the dysfunction of complex behaviors and their recovery [46]. Hippocampal damage contributes to ischemia-induced cognitive deficits, and the extent of such deficits depends on whether such damage is intra- or extra-hippocampal, the task that is applied to assess such deficits, and the specific memory process that is measured [47]. Interestingly, TGCI rats that were treated with CBD exhibited an increase in NeuN immunoreactivity in the DG of the hippocampus compared with respective controls. However, the implications of this finding are unclear. Neurogenesis in the DG has been shown to reflect a compensatory mechanism that is triggered by TGCI in rats [48] and mice [49]. Moreover, CBD increases doublecortin expression, a marker of newborn neurons, in the DG in ischemic mice. The increase in NeuN immunoreactivity in the DG may reflect hippocampal neurogenesis, but further studies are necessary to confirm this possibility.

Several possible mechanisms may underlie the neuroprotective effects of CBD in GCI, such as CBD-induced neurogenesis, the rescue of CA1 neuronal death, and improvements in synaptic plasticity [18, 44, 50–51]. BDNF is a neurotrophin that regulates activity-dependent synaptic plasticity and contributes to learning and memory processes [52]. Favorable effects of BDNF on functional recovery and neuroplasticity after CI have been reported [28, 53–55]. In the present study, hippocampal BDNF levels did not significantly decrease 14 days after TGCI. However, CBD treatment increased BDNF in the hippocampus, suggesting that this effect may be related to functional recovery in ischemic rats. Indeed, evidence indicates that CBD interacts with serotonin 5-hydroxytryptamine-1A (5-HT_{1A}) receptors, which may positively regulate BDNF levels. Mishima et al. (2005) reported that CBD reduced the infarct size in rats with middle cerebral artery occlusion, a model of focal cerebral ischemia. The effect of CBD was inhibited by the 5-HT_{1A} receptor antagonist WAY100135 [56]. Notably, the direct activation of postsynaptic 5-HT_{1A} receptors in the hippocampus resulted in higher BDNF levels and an increase in neuroplasticity in mice with BCCAO [55]. Moreover, the antidepressant-like effects of CBD have been associated with higher levels of SYN and PSD-95 in the medial prefrontal cortex and higher levels of BDNF in the prefrontal cortex and hippocampus. An intracerebroventricular injection of the TrkB receptor antagonist K252a and mammalian/mechanistic target of rapamycin inhibitor rapamycin abolished the antidepressant-like effects of CBD. The positive behavioral effects of CBD were related to plastic changes through activation of the BDNF-TrkB signaling pathway [57].

Both SYN and PSD-95 are the main proteins that participate in structural synaptic plasticity [58]. Synapsin is a marker of presynapse development and activity [59], and PSD-95 is an essential factor for synaptic plasticity and postsynaptic membrane stabilization [60]. Experimental evidence indicates that SYN and PSD-95 levels markedly decrease in the hippocampus after CI [61–63]. In the present study, TGCI significantly decreased hippocampal levels of SYN and PSD-95. However, CBD at the tested dose (10 mg/kg) only slightly attenuated these effects of TGCI. Supporting our data, Sales et al. (2019) did not observe any difference in SYN or PSD-95 levels in the hippocampus in healthy mice that were treated with 10 mg/kg CBD for 7 days [57]. In contrast, treatment with 10 mg/kg CBD for 14 consecutive days reversed iron-induced reductions of hippocampal SYN levels in rats [64]. Moreover, CBD reversed the

decrease in hippocampal PSD-95 levels in mice that were exposed to a model of chronic unpredictable stress [19].

Under some conditions, modifications of the structure of dendritic spines are strongly associated with synaptic plasticity, which is critical for cognitive flexibility [65]. Cerebral ischemia can cause poor spatial memory performance as a result of hippocampal damage and a decrease in synaptic function [66, 67]. In the present study, TGCI significantly decreased the number of dendritic spines in neurons that survived the ischemic insult in the DG (granular neurons), CA1, and CA3. These effects of TGCI on dendritic deterioration were prevented by CBD treatment. Various pharmacological interventions can stimulate neuroplastic changes. For example, acute melatonin administration attenuated dendritic spine loss in the hippocampus in TGCI rats [50]. Treatment with progesterone resulted in a similar recovery of dendritic spines and mitigated ischemia-induced learning and memory deficits [68]. Acetyl-L-carnitine treatment before and after 2-vessel occlusion (i.e., a model of ischemia) prevented ischemia-induced dendritic spine loss in the hippocampus, paralleled by the normalization of long-term potentiation in the hippocampus [69]. The biased 5-HT_{1A} receptor agonist NLX-101 was recently reported to reverse dendritic spine loss in mice that were subjected to BCCAO [55]. The ability of CBD to increase hippocampal BDNF levels and reverse or prevent dendritic spine loss in the hippocampus suggests its influence on neuroplasticity and consequently cognitive recovery (or preservation) under conditions of GCI.

One limitation of the present study was that we did not investigate the pharmacological mechanism of action of CBD in rats with TGCI. We recently demonstrated the involvement of cannabinoid CB_1, CB_2, 5-$HT_{1A,}$ and peroxisome proliferator-activated receptor-γ (PPAR-γ) receptors in functional recovery that is elicited by CBD in BCCAO mice [70]. Whether similar mechanisms are engaged in TGCI rats remains to be determined. The pharmacological profile of CBD is complex. It has potent antiinflammatory and antioxidant properties [71, 72] and exerts its actions throughout both the endocannabinoid system and other neurotransmitter systems. Treatment with CBD increases anandamide levels, in turn further activating cannabinoid CB_1 receptors [73, 74]. Cannabidiol has been reported to act as a CB_1/CB_2 receptor inverse agonist [75]. Other effects of CBD have been reported to be mediated by PPAR-γ [76] and G-protein-coupled receptors [77]. Moreover, CBD was reported to increase adenosine A_{2A} receptor signaling [78] and modulate energy metabolism through elevations of the mitochondrial complex and creatine kinase activity [79].

Overall, the present findings suggest that the beneficial effects of CBD on spatial memory recovery in TGCI rats at least partially occur through molecular mechanisms that underlie synaptic plasticity and dendritic remodeling, suggesting that CBD may be useful for functional improvement after CI.

Declarations

Ethics approval and consent to participate

This study was carried out at the State University of Maringá in strict accordance with the Brazilian College of Animal Experimentation (COBEA) recommendations. Animal experiments were approved by the local Ethics Committee on Animal Experimentation of the State University of Maringá (animal license number: CEUA 1555230316).

Consent to participate and Consent for publication

All of the co-authors approved the final version of the manuscript and agreed to submit it to Molecular Neurobiology

Availability of data and material

Experimental data will be made available under reasonable request.

Competing interests

FSG is a co-inventor (Mechoulam R, JC, Guimaraes FS, AZ, JH, Breuer A) of the patent “Fluorinated CBD compounds, compositions and uses thereof. Pub. No.: WO/2014/108899. International Application No.: PCT/IL2014/050023” Def. US no. Reg. 62193296; 29/07/2015; INPI on 19/08/2015 (BR1120150164927). The University of São Paulo has licensed the patent to Phytecs Pharm (USP Resolution No. 15.1.130002.1.1). The University of São Paulo has an agreement with Prati-Donaduzzi (Toledo, Brazil) to “develop a pharmaceutical product containing synthetic cannabidiol and prove its safety and therapeutic efficacy in the treatment of epilepsy, schizophrenia, Parkinson’s disease, and anxiety disorders”.

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Author’s contributions

EM and RMWO conceived and designed the experiments with inputs from ACC. EM performed behavioral tests, western blot and Golgi analysis. She wrote the first draft of the manuscript. EM and JMB conducted the animals’ surgeries. BAM performed the immunohistochemistry. MAM performed data analysis. FSG and HM helped with statistical analysis and data interpretation. All authors read and approved the final manuscript.

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Compliance with Ethical Standards

Conflict of interest

There is no conflict of interest concerning the present study.

Research involving human participants and/and or animals

The local Ethics Committee on Animal Experimentation of the State University of Maringá approved the experimental procedures in accordance with the guidelines of the U.S. National Institutes of Health and Brazilian College for Animal Experimentation (animal license number: CEUA 1555230316).

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Figures

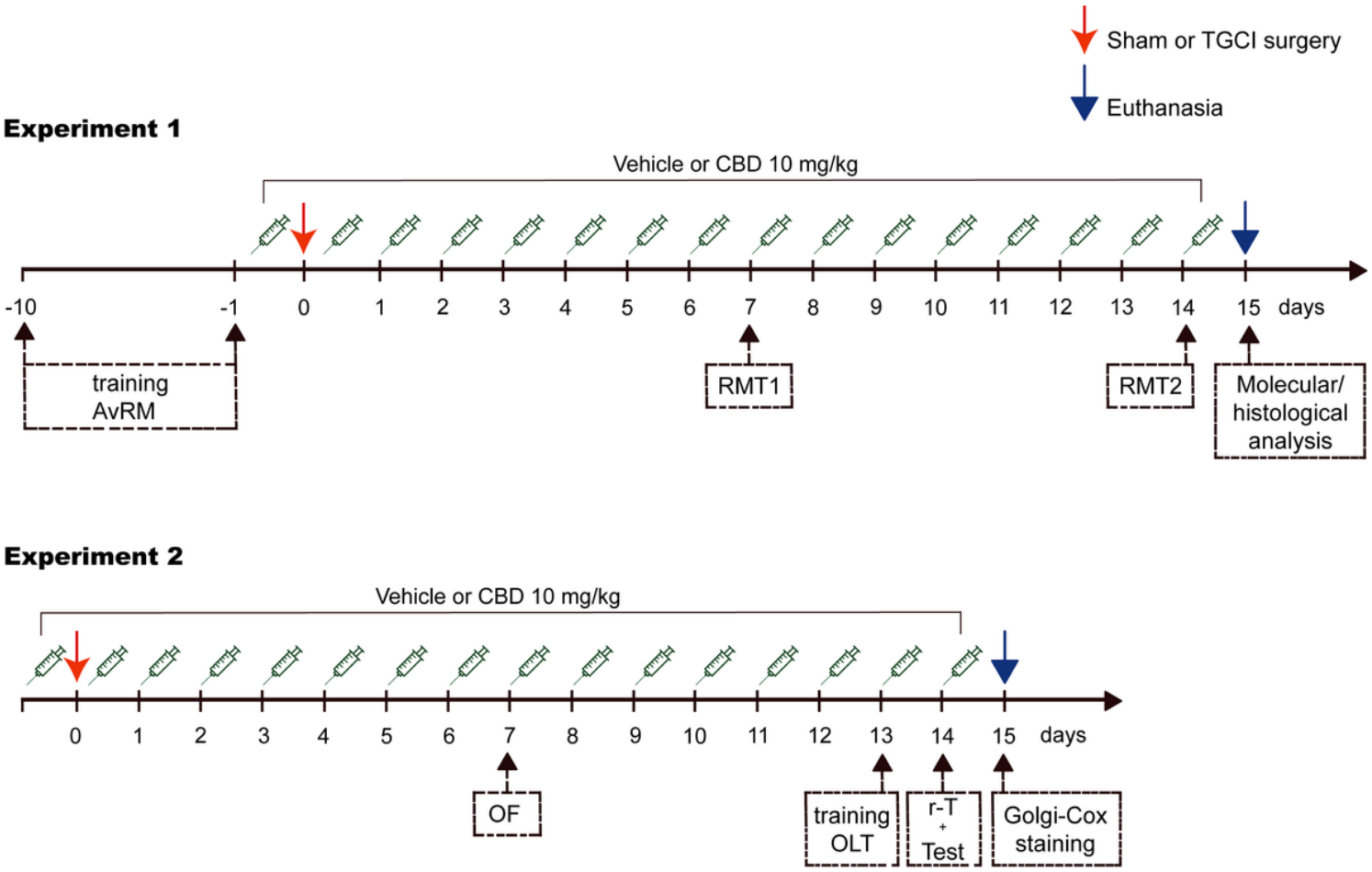

Figure 1

Experimental design. In Experiment 1, intact rats were trained for 10 days in the 8-arms aversive radial maze (AvRM) to find the goal box location, and then they were subjected to sham or transient global cerebral ischemia (TGCI) procedure. Vehicle or CBD 10 mg/kg (i.p.) were administered 30 min before, 3 h after surgery, and daily for 14 days once a day. On the 7th and 14th days after reperfusion, the retention memory trials (RMT) in the AvRM were carried out. One day after behavioral testing (day 15) the animals were euthanized and their brains were freshly collected for molecular and histological analysis. In Experiment 2, rats underwent TGCI and received vehicle or CBD treatment as described above. Seven days after TGCI the animals were evaluated in the open field test (OF) and 13 and 14 days after brain

ischemia they were subject to the object location test (OLT). One day after behavioral testing, the animals had their brains removed and assayed for Golgi-Cox staining.

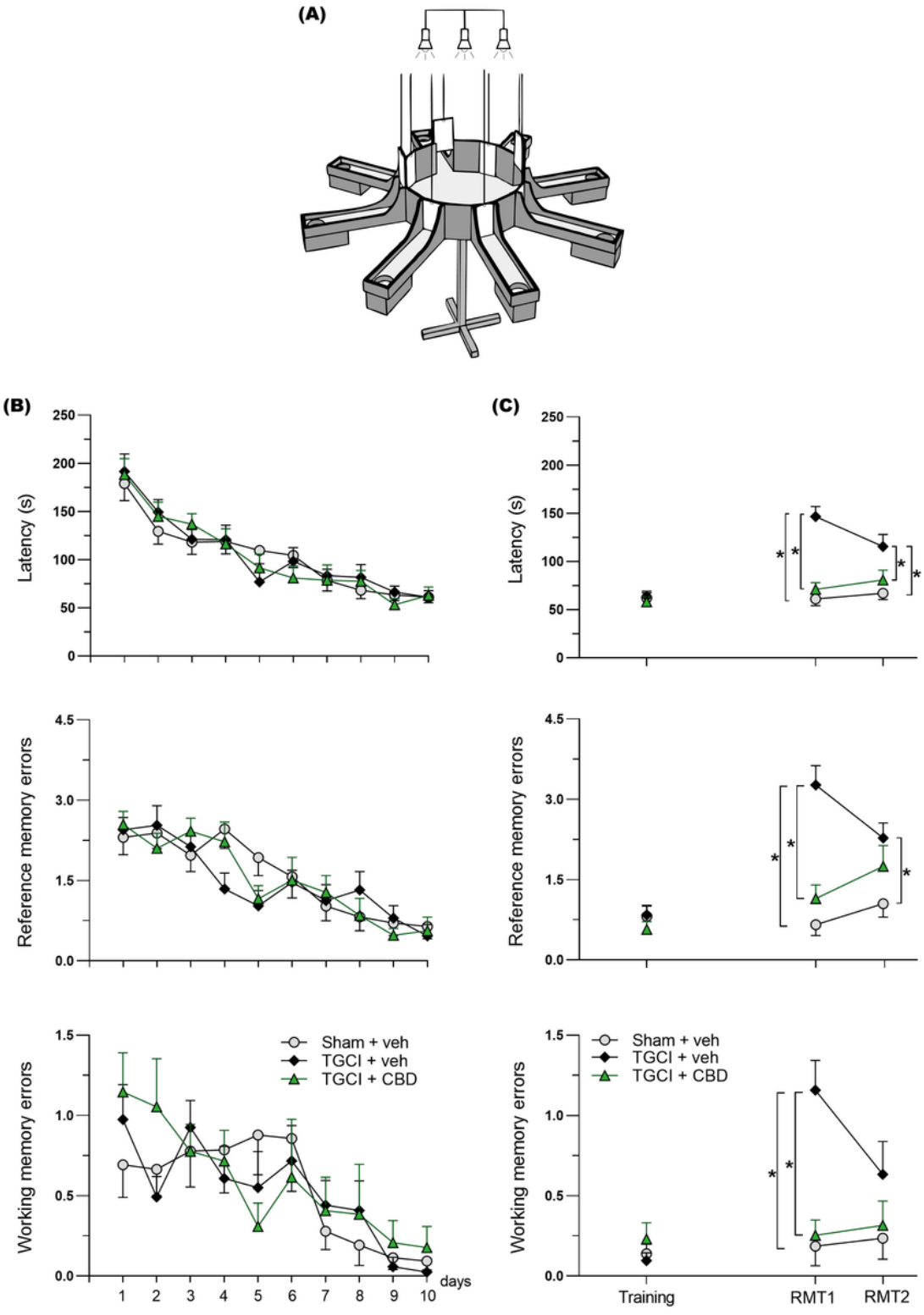

Figure 2

Cannabidiol prevented the amnestic effect of TGCI. (A) Schematic representation of the 8-arms aversive radial maze (AvRM). (B) Preoperative learning (acquisition) performance over 10 days of training in the AvRM, as expressed by the parameters latency (s), reference memory errors (number), and working

memory errors (number). (C) Temporal distribution of memory performance measured in each retention trial (RMT1 and RMT2). Preoperative performance (training) is expressed as the mean of the last 2 days of training. The bars represent the means ± SEM (n=12-14/group).

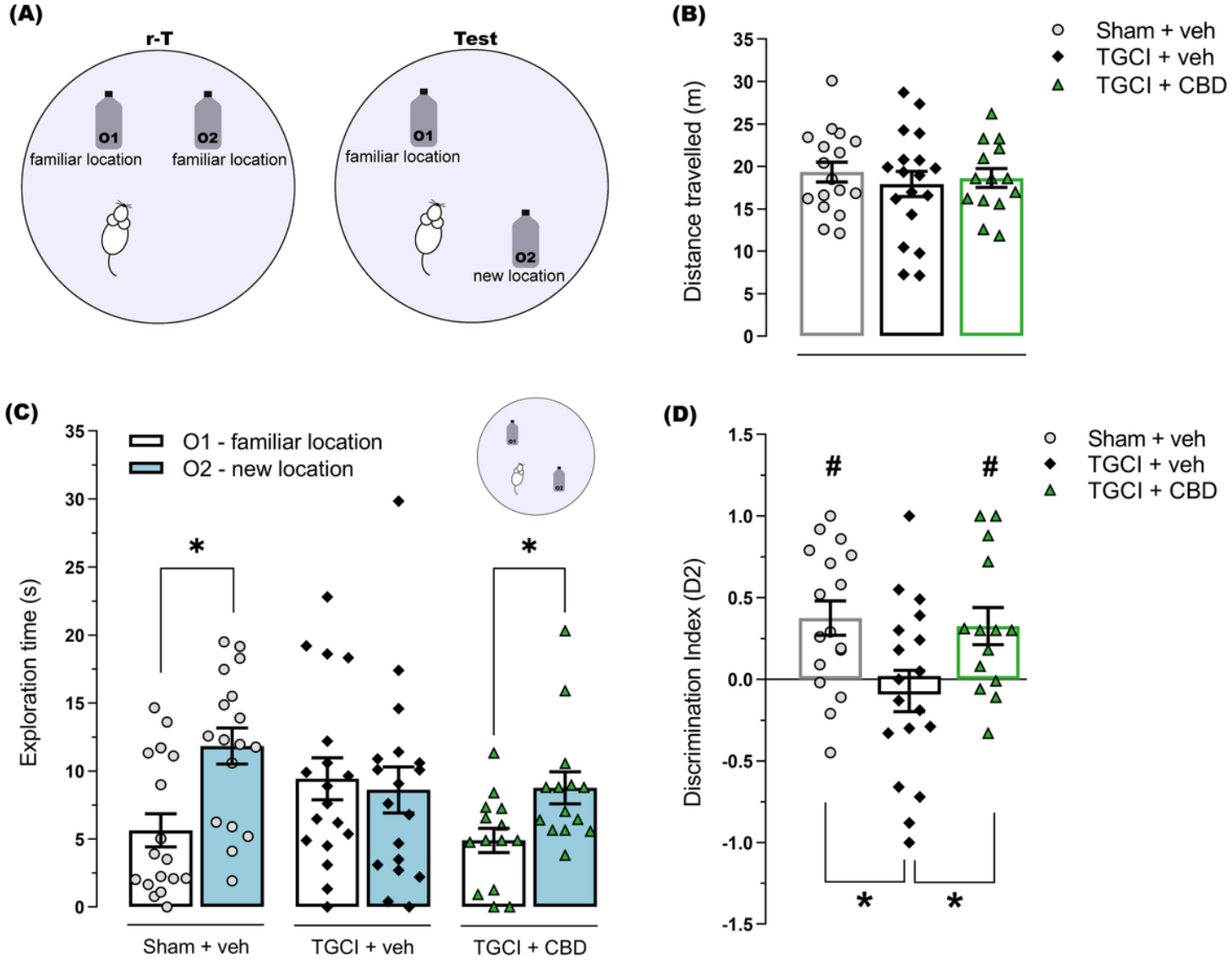

Figure 3

Cannabidiol alleviated TGCI-induced spatial memory impairments without affecting general locomotor activity. (A) Schematic representation of the identical objects (O1 and O2) located in the open field during retraining session (r-T) in a familiar position and during the Test session in which the O2 was moved diagonally relative to O1. (B) Locomotor activity was evaluated 7 days post-ischemia by measuring the distance traveled (m) in the open field (OF). (C) On the 13th day after ischemia the animals were trained in the OLT and afterward (day 14) re-exposed to the arena for the r-T and Test. Time spent exploring the two identical objects zone during the Test session was recorded. (D) The discrimination index in the OLT D2 was calculated as the following: D2 = (exploration time in the novel location – exploration time in the familiar location) / (exploration time in the novel location + exploration time in the familiar location). All

values represent means ± SEM of the number of the animals indicated in the figure (n=14-18/group). #p < 0.05 compared to zero (i.e., no memory in the OLT).

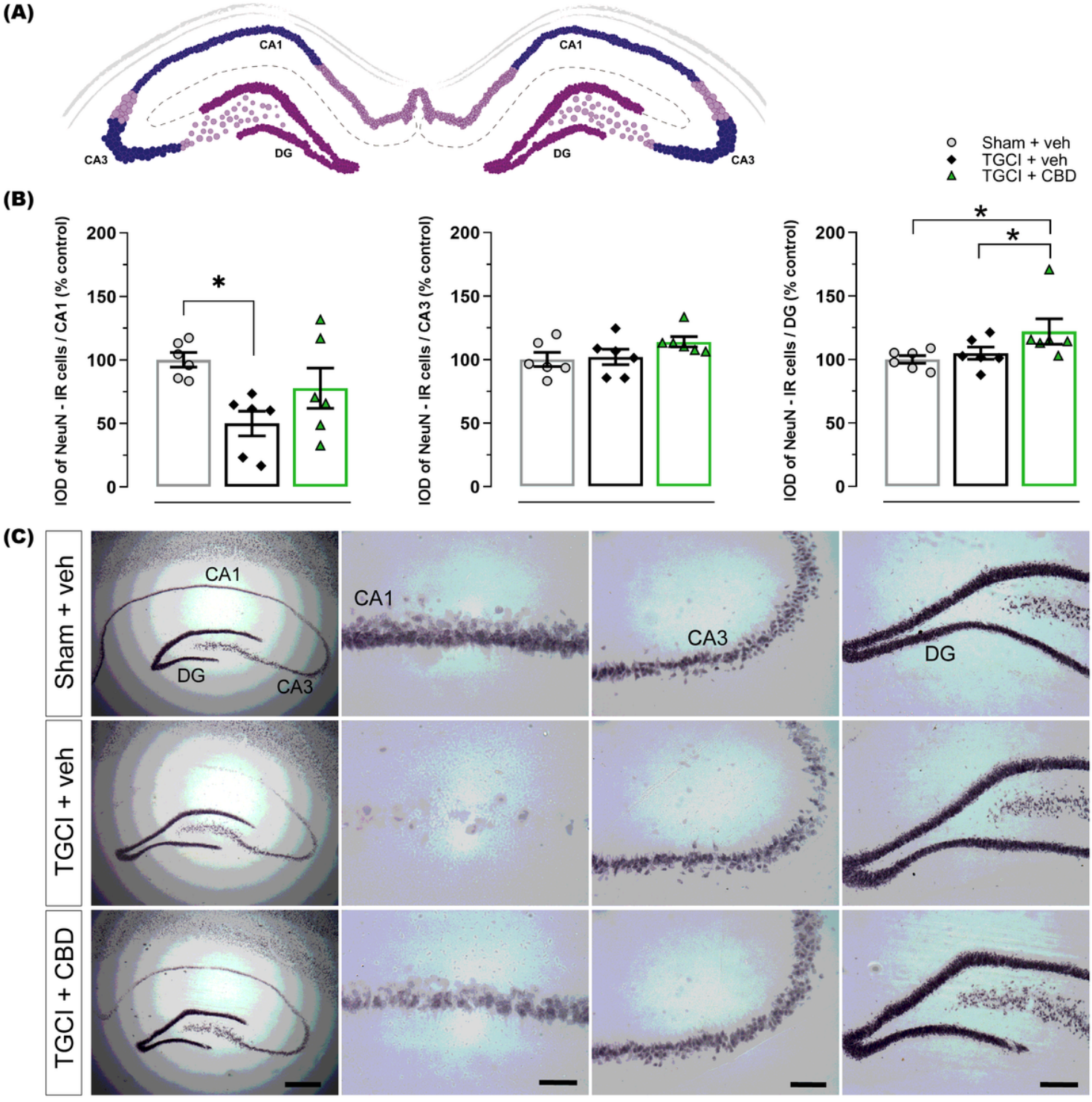

Figure 4

Cannabidiol attenuated TGCI-induced hippocampal neurodegeneration. (A) Representative diagram illustrating a coronal brain section at the intermediate level of the hippocampus showing the DG, CA1, and CA3 subfield where the analysis for NeuN-IR was performed. (B) Integrated optical density (IOD) of NeuN-positive neurons in the different hippocampal areas. (C) Representative photomicrographs of

NeuN-IR cells in the CA1 and CA3 subfield and DG in the different experimental groups, using 40x, 20x, and 10x objectives, respectively. All values represent means ± SEM of the number of the animals indicated in the figure (n=6/group). Scale bars: 500 µm (hippocampal formation); 50 µm (CA1); 100 µm (CA3); 200 µm (DG).

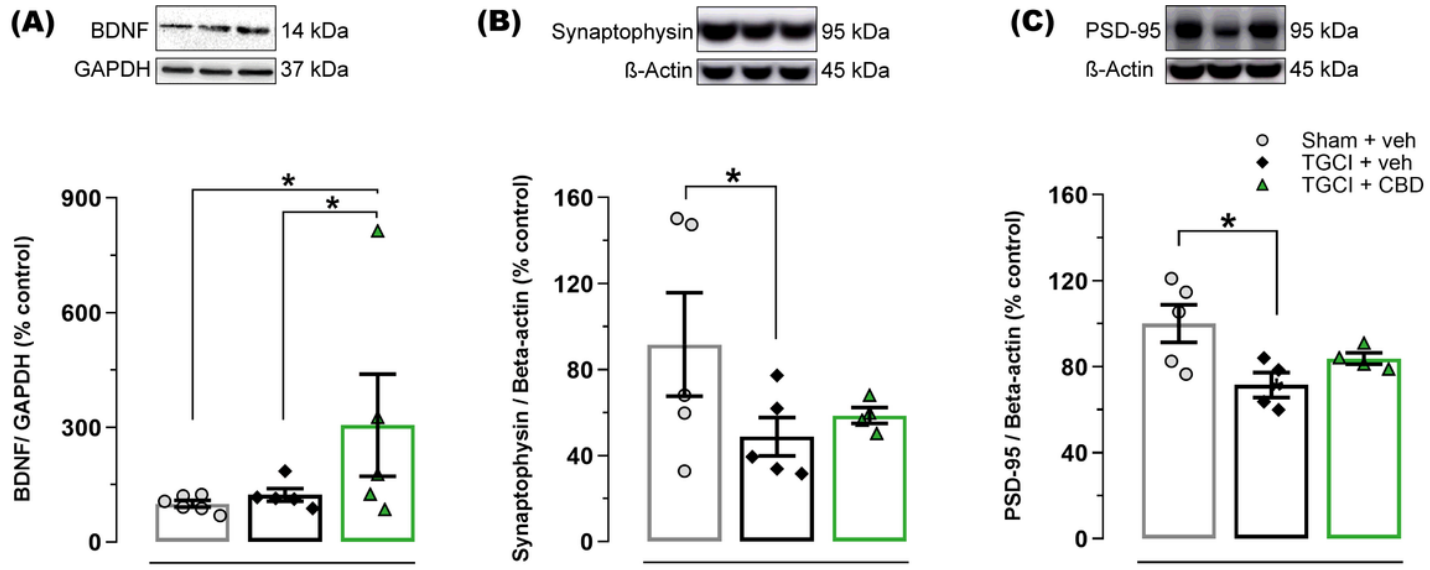

Figure 5

Cannabidiol changed neuroplasticity markers in the hippocampus of rats that were subjected to TGCI. (A-C) Protein levels of BDNF, synaptophysin, and PSD-95 in the hippocampus of sham or ischemic rats evaluated by Western Blot. All values represent means ± SEM of the number of the animals indicated in the figure (n=4-6/group).

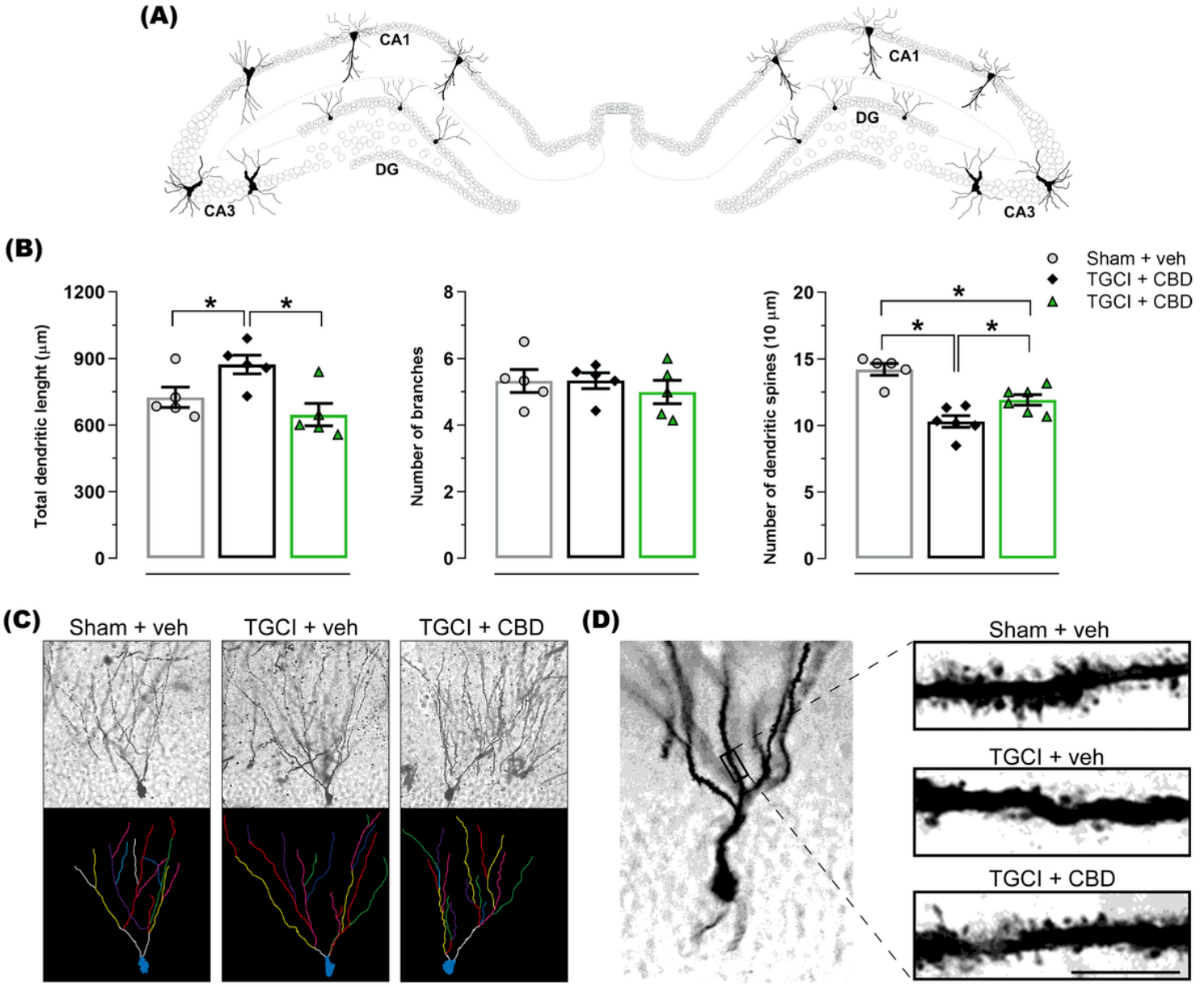

Figure 6

Cannabidiol ischemia-induced dendritic spine loss in hippocampal granular neurons. (A) Representative diagram illustrating a coronal brain section at the intermediate level of the hippocampus showing the DG, CA1, and CA3 subfield where the analysis for Golgi-cox was performed. (B) Total dendritic length, number of branches, and dendritic spines of granular neurons among the groups. (C) Representative photomicrography of stained neurons in the hippocampal DG and respective reconstructions. (D) Representative photomicrography of dendritic spines in the different experimental groups. All values represent means ± SEM of the number of the animals indicated in the figure (n= 5-6 mice/group).

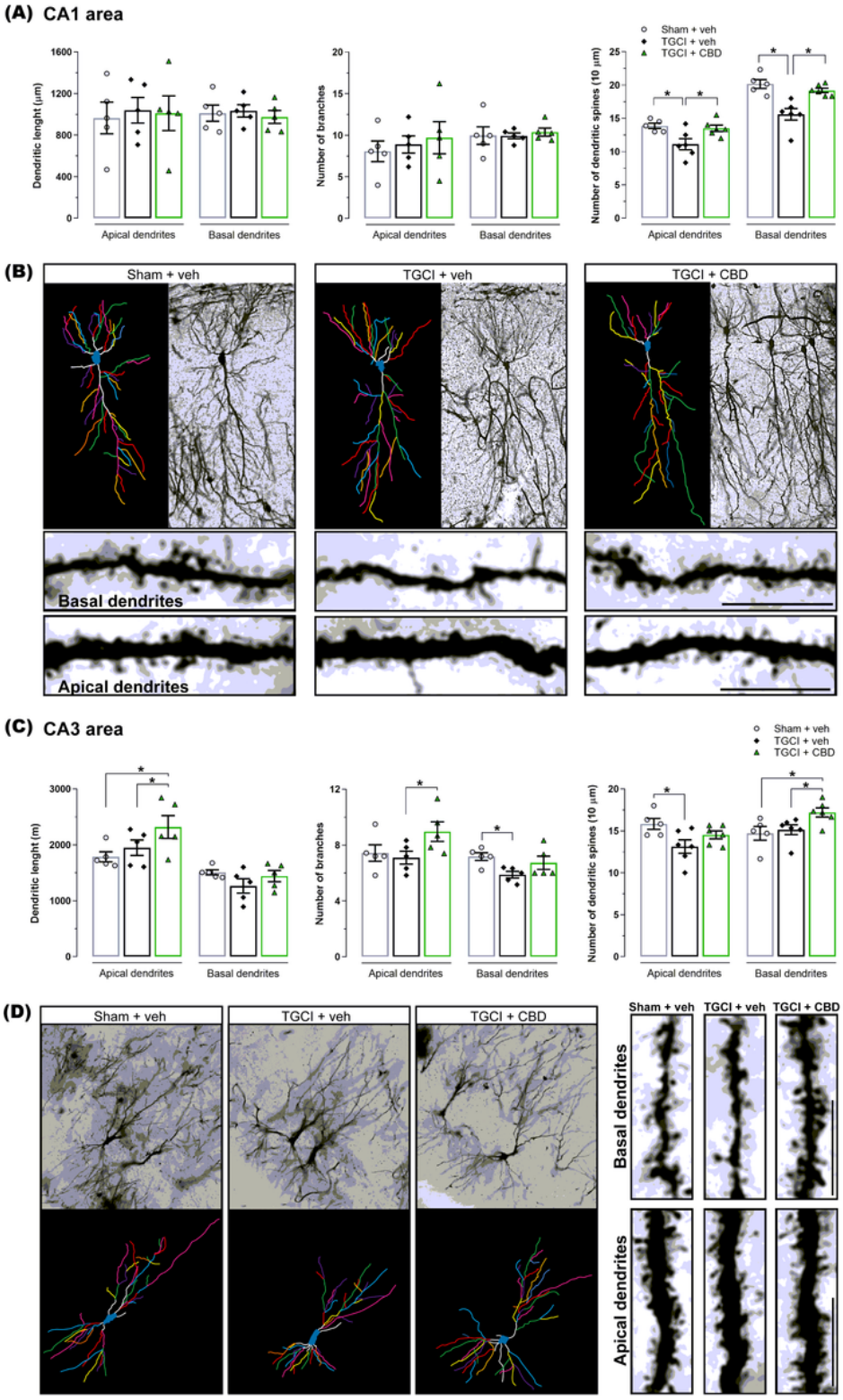

Figure 7

Cannabidiol protected pyramidal neurons against dendritic spines loss caused by TGCI. (A) Total dendritic length, number of branches, and dendritic spines of pyramidal neurons of CA1 subfield among the groups. (B) Representative photomicrography of stained neurons in the hippocampal CA1 area and basal/apical dendritic spines among the groups. (C) Total dendritic length, number of branche,s and dendritic spines of pyramidal neurons of CA3 subfield. (D) Representative photomicrography of stained

neurons in the hippocampal CA3 area and basal/apical dendritic spines in the different experimental groups. All values represent means ± SEM of the number of the animals indicated in the figure (n= 5-6 mice/group).