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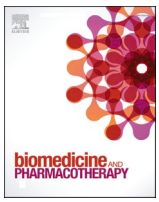

Prolonged incubation with Δ^9-tetrahydrocannabinol but not with cannabidiol induces synaptic alterations and mitochondrial impairment in immature and mature rat organotypic hippocampal slices

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ABSTRACT

Cannabis derivatives are among the most widely used psychoactive substances in the world, which leads to growing medical concerns regarding its chronic use and abuse especially among adolescents. Exposure to THC during formative years produces long-term behavioral alterations that share similarities with symptoms of psychiatric and neurodevelopmental disorders. In this study, we have analyzed the functional and molecular mechanisms that might underlie these alterations. Rat organotypic hippocampal slices were cultured for 2 days (immature) or 10 days (mature) *in vitro* and then exposed for 7 days to THC (1 μM) or CBD (1 μM). At the end of the treatment, slices were analyzed by Western blotting, electrophysiological recordings, RT-PCR, and fluorescence microscopy to explore the molecular and functional changes in the hippocampus. A prolonged (7-day) exposure to THC reduced the expression levels of pre- (synaptophysin, vGlut1) and post-synaptic (PSD95) proteins in both immature and mature slices, whereas CBD significantly increased the expression levels of PSD95 only in immature slices. In addition, THC significantly reduced the passive properties and the intrinsic excitability of membranes and increased sEPSCs in CA1 pyramidal cells of immature but not mature slices. Exposure to both cannabinoids impaired mitochondrial function as detected by the reduction of mRNA expression levels of mitobiogenesis genes such as VDAC1, UCP2, and TFAM. Finally, THC but not CBD caused tissue disorganization and morphological modifications in CA1 pyramidal neurons, astrocytes and microglia in both immature and mature slices. These results are helpful to explain the specific vulnerability of adolescent brain to the effects of psychotropic cannabinoids.

1. Introduction

Cannabinoids are among the most used drugs of abuse worldwide, with an estimated 219 million users in 2021, and the third most common psychoactive substances after alcohol and nicotine [1,2]. In Europe, 7.7 per cent of adults (15–64-year old) are regular users, and 15.4 per cent of these are young adults (15–34-year-old) [3]. Adolescents tend to have a particularly low perception of the risk associated with the heavy use of *Cannabis* [4,5]. The percentage of Δ^9-tetrahydrocannabinol (THC) in *Cannabis* preparations has increased fourfold in the United States between 1995 and 2018 and almost doubled in Europe between 2002 and 2018 [5,6]. The use of marijuana usually begins during adolescence [4,

Abbreviations: CBD, cannabidiol; GFAP, glial fibrillary acid protein; GLT1, glutamate transporter 1; GluA1, glutamate receptor ionotropic, AMPA 1; GluA2, glutamate receptor ionotropic, AMPA 2; IBA1, ionized calcium-binding adapter molecule 1; NeuN, neuronal nuclear protein; NRF-1, nuclear respiratory factor 1; PGC-1α, peroxisome proliferator-activated receptor-gamma coactivator α; SPSC, spontaneous post-synaptic current; PSD95, post-synaptic density protein 95; SEPSCs, spontaneous excitatory post-synaptic currents; TFAM, mitochondrial transcription factor A; THC, Δ^9-tetrahydrocannabinol; UCP2, uncoupling protein 2; VDAC1, voltage-dependent anion channel 1; VGlut1, vesicular glutamate transporter 1.

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7], it has been demonstrated that an onset of consumption before 16 years of age increases the risk of developing a psychiatric disorder, including *Cannabis* use disorder, later in adulthood [8–11]. The vulnerability of adolescents to drugs of abuse [1] has been ascribed to the fact that their cortical areas responsible for decision-making are still developing [12,13] and during this period neuronal maturation and rearrangement processes such as myelination, synaptic pruning and dendritic plasticity are known to occur [14]. In particular, the final maturational processes of neurotransmitter systems such as the glutamatergic, the dopaminergic, and the endogenous cannabinoid system are reached during this crucial period [15,16].

THC and cannabidiol (CBD) exhibit differential effects on the central nervous system: whereas THC is known to be responsible for the psychoactive effects of *Cannabis* and therefore has a high abuse liability, CBD is considered relatively "safe" because it has no psychotropic effects and in addition possesses the ability to counteract the effects induced by THC [17]. Although in a recent review Rodrigues and colleagues explores the neurodevelopmental and synaptic toxicity of THC and CBD in animals and humans (spanning fetal, adolescent and young adult stage) and it challenges the prevailing notion that CBD counteracts the effects of THC in the human body [18]. In younger people, chronic exposure to THC produces long-lasting neuropsychological deficits [19] and long-term behavioral alterations that share similarities with certain symptoms of psychiatric and neurodevelopmental disorders [20] which is not or less pronounced in adults [21,22]. Preclinical studies in juvenile animals have demonstrated that prolonged exposure to THC induces a long-lasting dysregulation in various hippocampal neurotransmitter signaling pathways and protein expression patterns [23,24], both in glutamatergic neurons and in glial cells [25], whereas CBD mitigates the long-term behavioral alterations induced by THC chronic exposure in adolescent female rats [26]. CBD displays anti-seizure abilities in the absence of psychoactive features and abuse potential [27]: both the Food and Drug Administration and the European Medicines Agency have approved Epidiolex® as an add-on antiepileptic drug in two severe forms of childhood epilepsy, the Lennox-Gastaut and the Dravet syndromes [28,29].

In order to contribute to the comprehension of the neurobehavioral alterations induced by chronic abuse of cannabinoids, we herein investigate the effects of a prolonged treatment (7 days) with THC and CBD at the synaptic, mitochondrial and cellular level in rat organotypic hippocampal slices cultured for 2 days (immature) or 10 days (mature) *in vitro*.

2. Materials and methods

2.1. Animals

Male and female Wistar rat pups of 7–9 days of age were used (Charles River, MI, Italy). Animals, housed at 23 ± 1 °C under a 12 h light–dark cycle (lights on at 07:00), were fed a standard laboratory diet with ad libitum access to water. All experimental procedures were carried out in accordance with the Italian regulations for the care and use of laboratory animals (EU Directive 2010/63/EU), with the Italian Regulation DL 26/2014 "protection of animals used for scientific purposes" and were approved by appropriate institutional and state authorities of University of Florence (17E9C.N.GS0/2021). In compliance with Italian law and EU directives, all efforts were made to minimize the number of animals used and suffering according to the principle of 3Rs.

2.2. Materials

Cannabidiol (CBD) was purchased from Tocris Cookson (Bristol, UK). The medium for tissue cultures were purchased from Gibco-BRL (San Giuliano Milanese, MI, Italy), Δ^9-tetrahydrocannabinol (THC) were purchased from Sigma (St. Louis, MO, USA). Authorisation by the Ministry of Health for the use of THC n. SP/94 issued in July 2021.

2.3. Preparation of rat organotypic hippocampal slice cultures

Organotypic hippocampal slice cultures are an *in vitro* model used for anatomical, molecular, and electrophysiological studies in which several aspects of structural and synaptic organization of the original tissue are preserved [30,31]. Hippocampal slices exhibit increasing protein and receptor expression, increasing electrical activity, during maturation *in vitro* mimicking what occurs *in vivo* animals [32]. Organotypic hippocampal slice cultures were prepared as previously reported [32]. Briefly, hippocampi of 7–9 days old Wistar rat pups were removed from the brains, and transverse slices (420 μm) were prepared using a McIlwain tissue chopper. After microscope selection the slices were transferred onto 30 mm diameter semiporous membrane inserts (Millicell-CM PICM03050; Millipore, Milan, Italy; 4 slices per insert), which were placed in 6-well tissue culture plates containing 1.2 mL medium per well. The culture medium compound of 50 % Eagle's minimal essential medium, 25 % heat-inactivated horse serum, 25 % Hanks' balanced salt solution, 5 mg/mL glucose, 2 mM L-glutamine, and 3.75 mg/mL amphotericin B. Slices were maintained at 37 °C in an incubator in atmosphere of humidified air and 5 % CO_2 for 2 weeks.

2.4. Prolonged cannabinoids exposure in organotypic hippocampal slices

Rat organotypic hippocampal slice cultures were exposed for 7 days (simil-chronic) to 1 μM CBD or 1 μM THC after 2 days (immature) or 10 days (mature) of culture *in vitro*. The medium was changed every day, adding CBD or THC to the fresh culture medium. CBD was dissolved in dimethyl sulfoxide (50 mM), THC was dissolved in methanol (1 mg / mL) and both were stored at −20 °C. For the experiments, they were diluted in cell culture medium.

2.5. Western blot analysis

Western blotting was conducted as previously reported [33]. Cultured slices were washed with cold 0,01 M phosphate-buffered saline, and 4 slices/sample were gently transferred and dissolved in 1 % sodium dodecyl sulfate. The total protein levels were quantified by bicinchoninic acid Protein Assay. Lysates (20 μg/lane of protein) were resolved by electrophoresis on a 4–20 % sodium dodecyl sulfate-polyacrylamide gel (Bio-Rad Laboratories, Hercules, CA, USA) and transferred onto nitrocellulose membranes using the Trans-Blot TURBO System (Bio-Rad, Hercules, CA). After blocking 5 min with EveryBlot Blocking Buffer (Bio-Rad Laboratories, Hercules, CA, USA), the blots were incubated overnight at 4 °C with the monoclonal-mouse antibody postsynaptic density protein 95 (PSD95) (Cell Signaling Technology, Beverly, MA, USA), Gephyrin (#147 111; RRID: AB_887719; Synaptic System, Goettingen, DE) with the polyclonal rabbit antibody Synaptophysin (Abcam, Cambridge, MA, USA), vesicular glutamate transporter 1 (vGlut1), glutamate receptor ionotropic AMPA1 (GluA1), glutamate transporter type 1 (GLT1) and polyclonal goat glutamate receptor ionotropic AMPA2 (GluA2) antibodies (Sigma-Merk, Darmstadt, Germany) all diluted 1:1000 in tris-buffered saline-tween containing 5 % non-fat dry milk. β-actin was used as a loading control (monoclonal mouse antibody purchased from Sigma-Merck). Immunodetection was performed with horseradish peroxidase-conjugated secondary antibodies (1:3000 anti-mouse, anti-rabbit or anti-goat IgG from donkey, Sigma-Merck) in tris-buffered saline-tween -T containing 5 % non-fat dry milk. Membranes were washed and then reactive bands were detected using chemiluminescence (ECL plus; Bio-Rad Laboratories, Hercules, CA, USA). The quantitative analysis was performed using the Quantity One software (Bio-Rad, Hercules, CA, USA). Results are presented as the mean ± SEM of different gels and expressed as arbitrary units, which depict the ratio between levels of target protein expression and β-actin normalized to basal levels.

Table 1
Antibodies used for immunohistochemistry. All primary and secondary antibodies are diluted in the Blocking buffer solution.

Antibodies used for immunohistochemistry							
Target	**Antigen**	**Supplier**	**Catalog #**	**Antibody**	**Host**	**Usage**	**Dilution**
Neuron	NeuN	Millipore, Billerica, MA, USA	MAB377	Monoclonal	Ms	Primary	1:400
Total microglia	Iba1	Wako, Osaka, JP	016–20001	Polyclonal	Rb	Primary	1:300
Total microglia	Iba1	Wako	011–27991	Polyclonal	Gt	Primary	1:200
Astrocyte	GFAP	Millipore	MAB3402X	Monoclonal	Ms	Primary Alexa Fluor 488 conjugated	1:500
Mouse FC	Mouse FC	Thermo Fisher	A31570	Polyclonal	Dn	Secondary Alexa Fluor 555	1:400
Rabbit FC	Rabbit FC	Thermo Fisher	A31577	Polyclonal	Gt	Secondary Alexa Fluor 635	1:400
Rabbit FC	Rabbit FC	Thermo Fisher	A21206	Polyclonal	Dn	Secondary Alexa Fluor 488	1:400
Goat FC	Goat FC	Thermo Fisher	A21082	Polyclonal	Dn	Secondary Alexa Fluor 635	1:400

2.6. Electrophysiological recordings

Electrophysiological recordings were performed on CA1 pyramidal neurons from both immature and mature organotypic hippocampal slices under control conditions or following simil-chronic treatment with CBD or THC. Slices were individually removed from the culture insert and placed in a flow chamber under an inverted microscope objective (Nikon Eclipse E600FN) equipped for infrared video microscopy. Slice was continuously perfused with warm (34–35°C) artificial cerebrospinal fluid composed of (in mM): NaCl (130), KCl (3.5), NaH_2PO_4 (1.25), $NaHCO_3$ (25), glucose (10), $CaCl_2$ (2) and $MgSO_4$ (1), and saturated with a 95 % O_2 + 5 % CO_2 gas mixture. Whole-cell pipettes, pulled from thin-walled borosilicate capillaries (Harvard Apparatus) using a vertical puller (Narishige PP830, Narishige International Limited), were back-filled with an intracellular solution composed of (in mM): K^+ gluconate (120), KCl (15), HEPES (10), EGTA (1), $MgCl_2$ (2), Na_2 phosphocreatine (5), NaGTP (0.3), MgATP (4), pH 7.3–7.4, 295–305 mOsm and resulting in a bath resistance of 3–5 MΩ. After achieving a Giga-Ohm seal, a whole-cell configuration was established by rupturing the neuronal membrane. Passive properties of CA1 neurons under the three experimental conditions (control, CBD and THC) were evaluated by measuring membrane capacitance, membrane resistance, and resting membrane potential. To study overall intrinsic excitability, CA1 neurons were stimulated with increasing depolarizing current steps (20 pA, 500 ms step) to measure the number and threshold of evoked action potentials. For spontaneous excitatory post synaptic currents (sEPSCs), recordings were performed in voltage-clamp with V_{HOLD}= -70 mV. Recordings were conducted in gap-free mode, electrical signals were sampled at 10 kHz and low-pass filtered at 3 kHz using an Axon Multiclamp 700B (Molecular Devices, Sunnyvale, CA, USA). Holding current traces were analyzed off-line with pCLAMP10 (Molecular Devices, Sunnyvale, CA, USA) to compute frequency and amplitude of sEPCS.

2.7. cDNA synthesis and real-time PCR

Total ribonucleic acid from organotypic hippocampal slices (4 slices/sample) was isolated using Trizol Reagent (Life Technologies). After quantification, one μg of RNA was retrotranscribed using iScript (Bio-Rad, Milan, Italy). Real-Time polymerase chain reaction (PCR) was performed using Rotor-Gene 3000 Instrument (Qiagen, Milan, Italy) and was carried out by means of SsoAdvanced TM Universal SYBR® Green Supermix (Bio-Rad, Milan, Italy) according to manual instruction. Ribosomal 18S RNA was used as the normalizer. Quantitative PCR was performed using the following procedure: 95 °C for 30 s, 95 °C for 5 s and 60 °C for 15 s for 45 cycles [34]. The following primers were used: peroxisome proliferator-activated receptor-gamma coactivator α (PGC-1α) forward 5'- CCAGCCTCTTTGCCCAGATCTTCC −3', and reverse 5'- GTGAGGACCGCTAGCAAGTTTGCC −3', uncoupling protein 2 (UCP2) forward 5'- CTTTCCCTCTAGACACCGCCAAAGTC −3' and reverse 5'- CAATGCGGACGGAGGCAAAGCTC −3', nuclear respiratory factor 1 (NRF-1) forward 5'- CTGCTGCAGGTCCTGTGGGAATG −3' and reverse 5'- TGGAGGGTGAGATGCAGAGAACAATG −3', mitochondrial transcription factor A (TFAM) forward 5'-CCAGGAGGCTAAGGATGAGTCAGCT −3' and reverse 5'- GCTTCACACTGCGACGGATGAGATC −3', voltage-dependent anion channel 1 (VDAC1) forward 5'- CTGAGATCACCGTGGAAGACCAGC −3' and reverse 5'- CCAACCCTCATAGCCAAGCACCAG −3' and 18S forward 5'-GGCGGCTTTGGTGACTCTAGATAACC −3' and reverse 5'-CCTGCTGCCTTCCTTGGATGTGG −3'. Primers were purchased from Integrated DNA Technologies (Iowa, USA).

2.8. Fluorescence immunohistochemistry and quantitative analysis

2.8.1. Immunohistochemistry

At the end of the treatments, slices were fixed in ice-cold paraformaldehyde (4 % in PBS buffer) for 1 day and then cryoprotected with a sucrose solution (18 % in PBS) for at least 2 days. Slices were stored in the −20 ° C refrigerator in anti-freezing solution (30 % ethylene-glycol, 30 % glycerol, 40 % PBS) till immunohistochemistry. Immunohistochemistry was performed with the free-floating method [35] using the antibodies specified in Table 1.

2.8.2. Triple labelling fluorescence immunohistochemistry for NeuN, GFAP, IBA1

Day 1: Organotypic hippocampal slices after 3 washes were blocked for 60 min with blocking buffer containing 10 % normal goat serum (Code: S-1000, Vectashield, Burlingame, CA, USA) and then were incubated overnight at 4 °C with a mouse anti-Neuronal Nuclei (NeuN) antibody and a rabbit anti-ionized calcium-binding adapter molecule 1 (IBA1) antibody.

Day 2: After washings, slices were incubated for 2 h at room temperature with AlexaFluor 635 goat anti-rabbit IgG secondary antibody and then were incubated for 2 h with Alexa Fluor 555 donkey anti mouse IgG plus AlexaFluor 635 goat anti-rabbit IgG. Astrocytes were immunostained using a mouse anti glial fibrillary acid protein (GFAP) primary antibody conjugated with AlexaFluor 488, for 2 h at room temperature. Slices were mounted onto gelatin-coated slides using Vectashield mounting medium with 4',6-diamino-2-phenylindole (Code #H-1200, Vectashield, Burlingame, CA, USA).

2.8.3. Microscopy techniques, qualitative and quantitative analysis

Confocal microscopy acquisitions were performed in the regions of interest stratum pyramidalis (SP) and stratum radiatum (SR) of CA1 to acquire immunofluorescence signals. Slices were observed under a LEICA TCS SP8 confocal laser scanning microscope (Leica Microsystems CMS GmbH) equipped with a 20X objective. The parameters of acquisition were maintained constant: frame dimension 1024 × 1024 pixels, frequency of acquisition 200 Hz, z step of 1.2 μm (20X objective) Qualitative analyses were performed on representative images of the different immunostaining. Quantitative analyses (see below) were performed in CA1 SP or SR on confocal microscopy z-projections of 10 consecutive z scans (20X objective, z step 1,2 μm, total thickness 12 μm). Phagocytic microglia were defined as $IBA1^+$ amoeboid microglia characterized by a total or partial spatial colocalization with a $NeuN^+$ neuron [36]. We measured astrocytes, total microglia and phagocytic microglia density (cells/mm2). To characterize the alteration of organization of

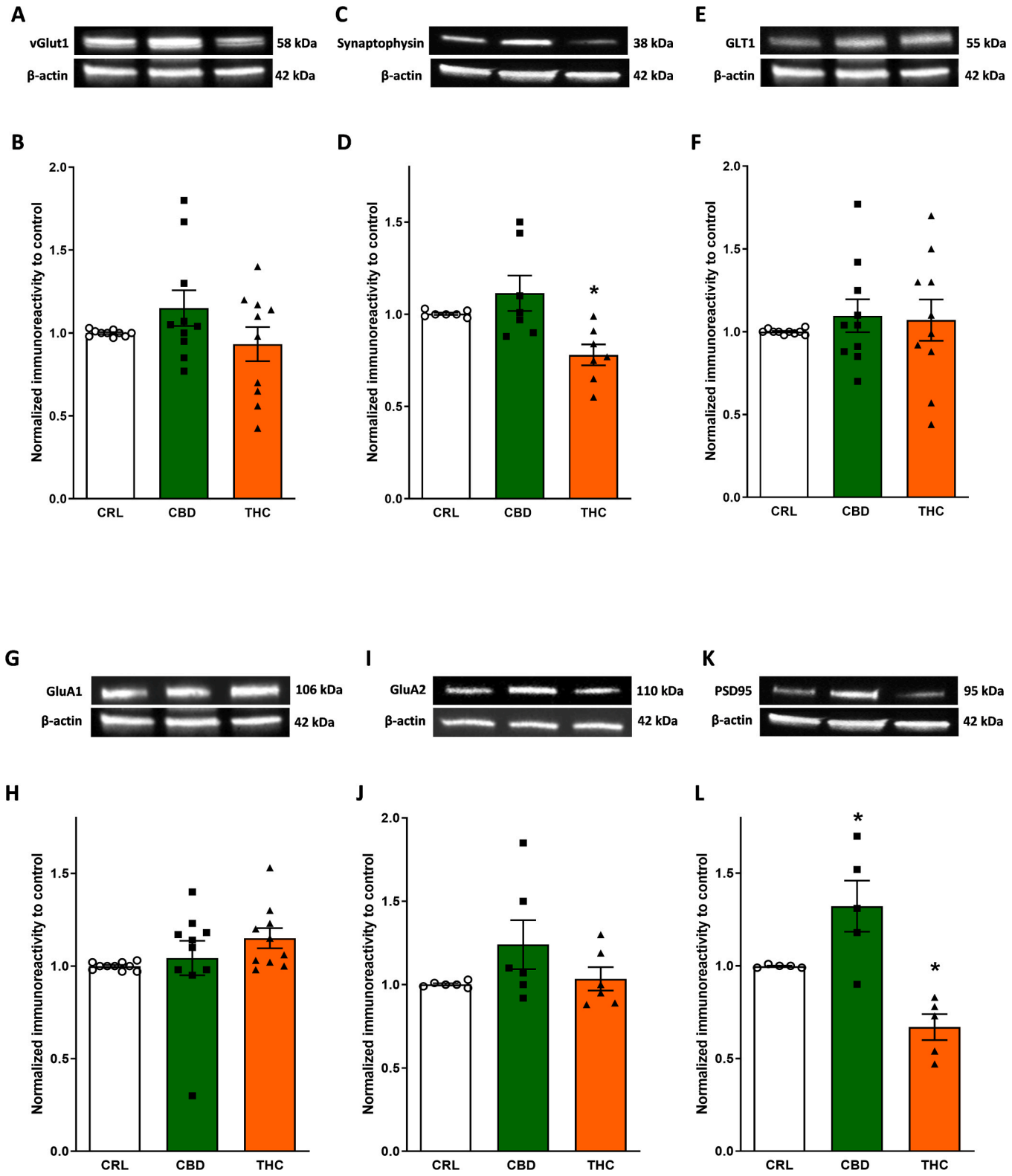


Fig. 1. Effects of prolonged treatment for 7 days with CBD or THC on the expression levels of pre- and post-synaptic proteins in immature rat organotypic hippocampal slices. Illustrative blots using antibodies directed against vGluT1 (A), synaptophysin (C), GLT1 (E), GluA1 (G), GluA2 (I), PSD95 (K) and β-actin. (B-D-F-H-J-L). Quantitative analysis of immunoreactive bands shows that simil-chronic treatment with 1 μM CBD induced a significant increase of PSD95 (L) whereas 1 μM THC induced a significant reduction of Synaptophysin (D) and PSD95 (L). Bars represent the mean ± SEM of at least 5 experiments. * $p < 0.05$ vs. CRL (ANOVA + Dunnett's w-Test).

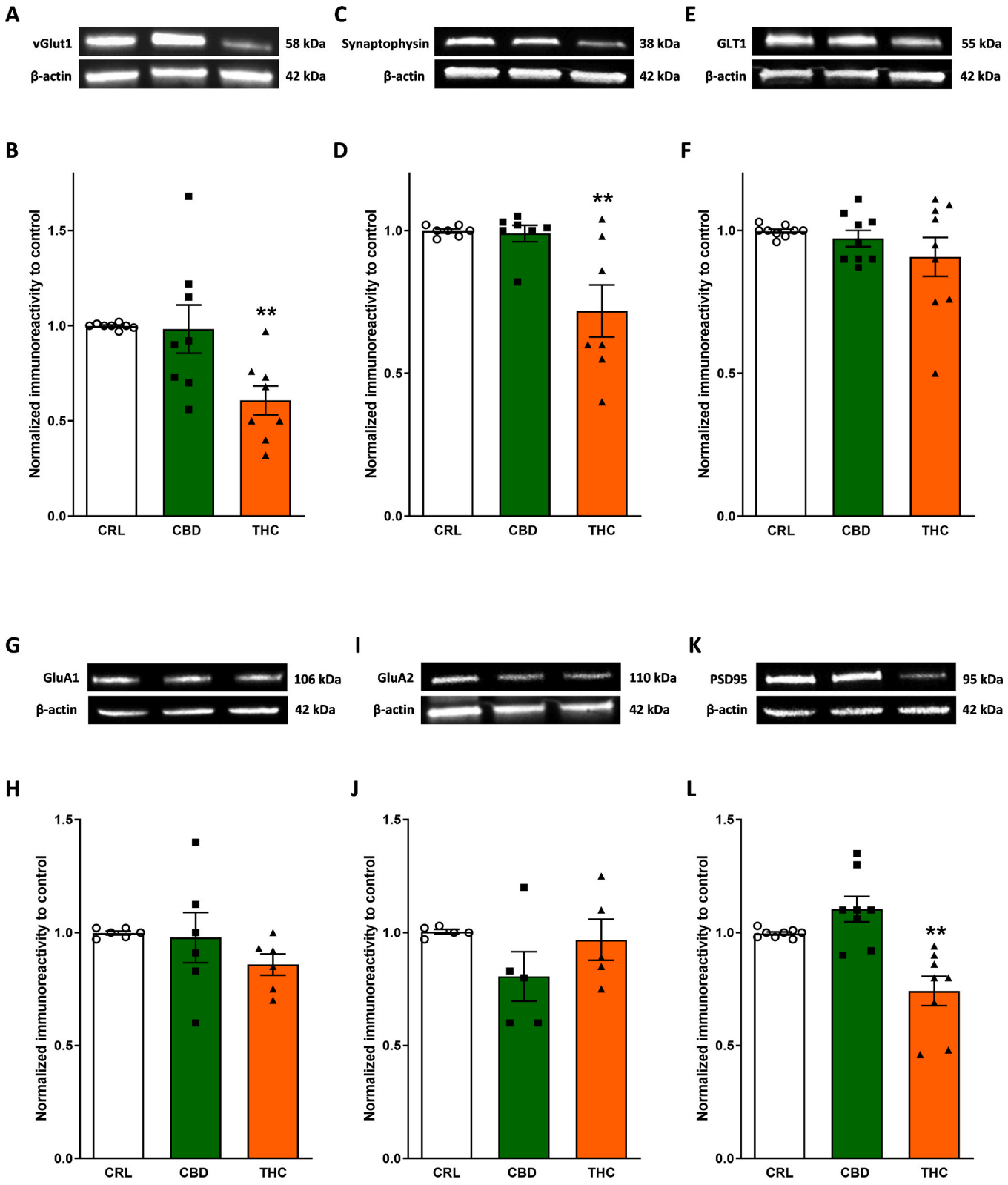


Fig. 2. Effects of prolonged treatment for 7 days with CBD or THC on the expression levels of pre- and post-synaptic proteins in mature rat organotypic hippocampal slices. Illustrative blots using antibodies directed against vGluT1 (A), synaptophysin (C), GLT1 (E), GluA1 (G), GluA2 (I), PSD95 (K) and β-actin. (B-D-F-H-J-L). Quantitative analysis of immunoreactive bands shows that chronic treatment with 1 μM THC induced a significant reduction of vGlut1 (B), Synaptophysin (D) and PSD95 (L). Bars represent the mean ± SEM of at least 5 experiments. ** $p < 0.01$ vs. CRL (ANOVA + Dunnett's w-Test).

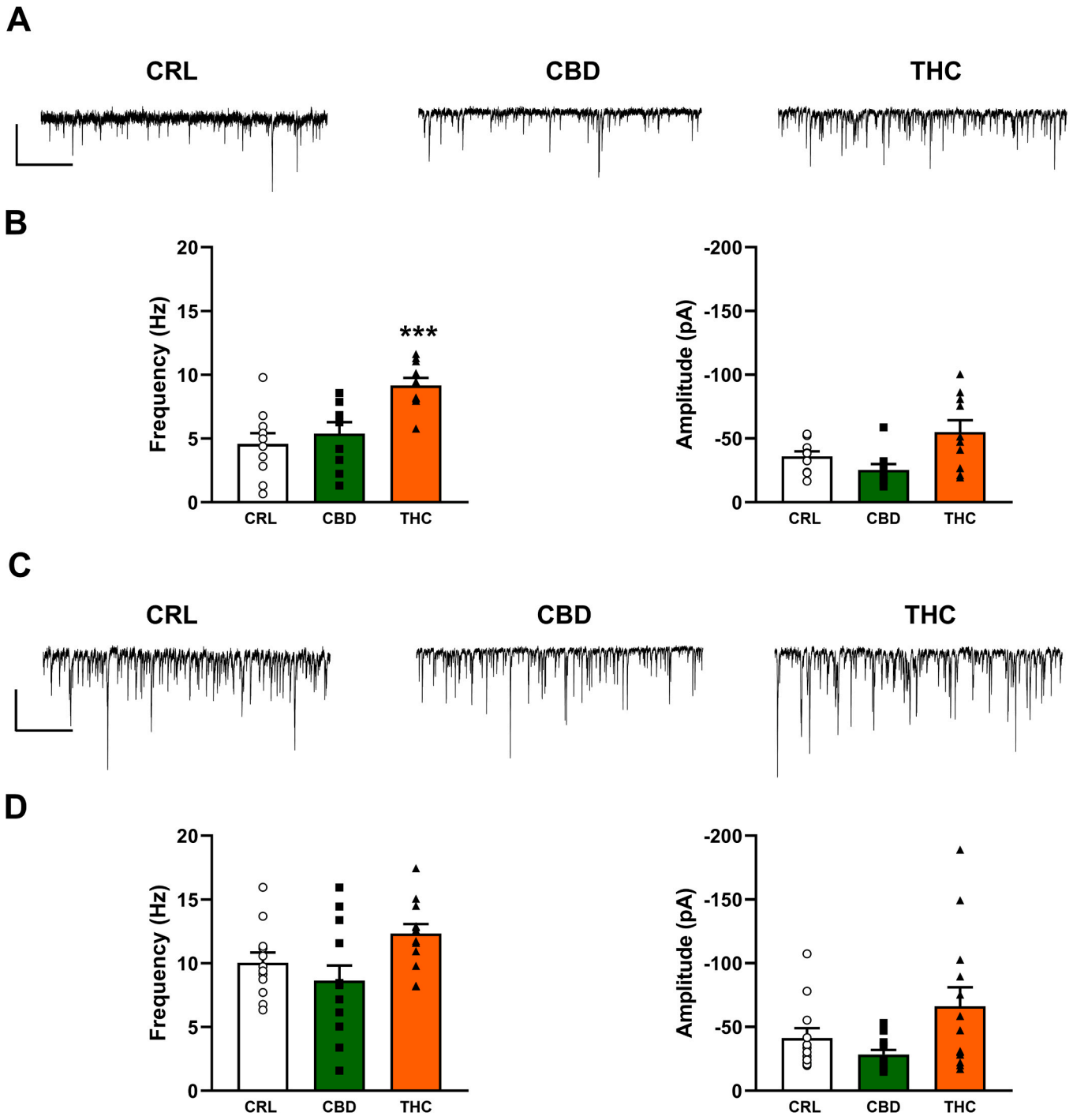


Fig. 3. Effects of prolonged treatment for 7 days with CBD or THC on intrinsic excitability and excitatory network activity. Representative voltage-clamp recordings of sEPSCs in the three different conditions. Scale bar: 50 pA, 2 sec. (A). THC induces a significant increase in frequency and a trend to increase in amplitude of sEPSCs in immature slices (B). While not affecting sEPSC frequency, exposure to CBD causes a trend to decrease in sEPSC peak amplitude (B). Chronic treatment with THC and CBD replicates the trend observed at earlier stages of maturation (D). Bars represent the mean ± SEM of 8–10 neurons (dots) from 4 to 8 different slices and 3–5 experiments.

the CA1 pyramidal cell layer, we also evaluated the thickness of the CA1 stratum pyramidalis (expressed in μm): in each NeuN z-projection image 3 measurements evenly distributed throughout SP were taken and averaged [35]. GFAP and IBA1 expression were detected using the threshold tool of Image J (National Institute of Health, http://rsb.info.nih.gov/ij), calculating the percentage of positive pixels above a threshold level in each confocal microscopy z-projections [37]. To characterize astrocyte morphological alterations, we performed the analysis of astrocytes branches length in accordance with our previous study [37]. Four principal branches of 10 different astrocytes evenly distributed in hippocampal CA1 SP or SR, were measured using Image J measure tool and data were averaged.

2.9. Data elaboration and statistical analysis

Data are presented as means ± SEM. Individual data points are shown as dots. The statistical significance of differences between protein assays, electrophysiological data, gene expression and

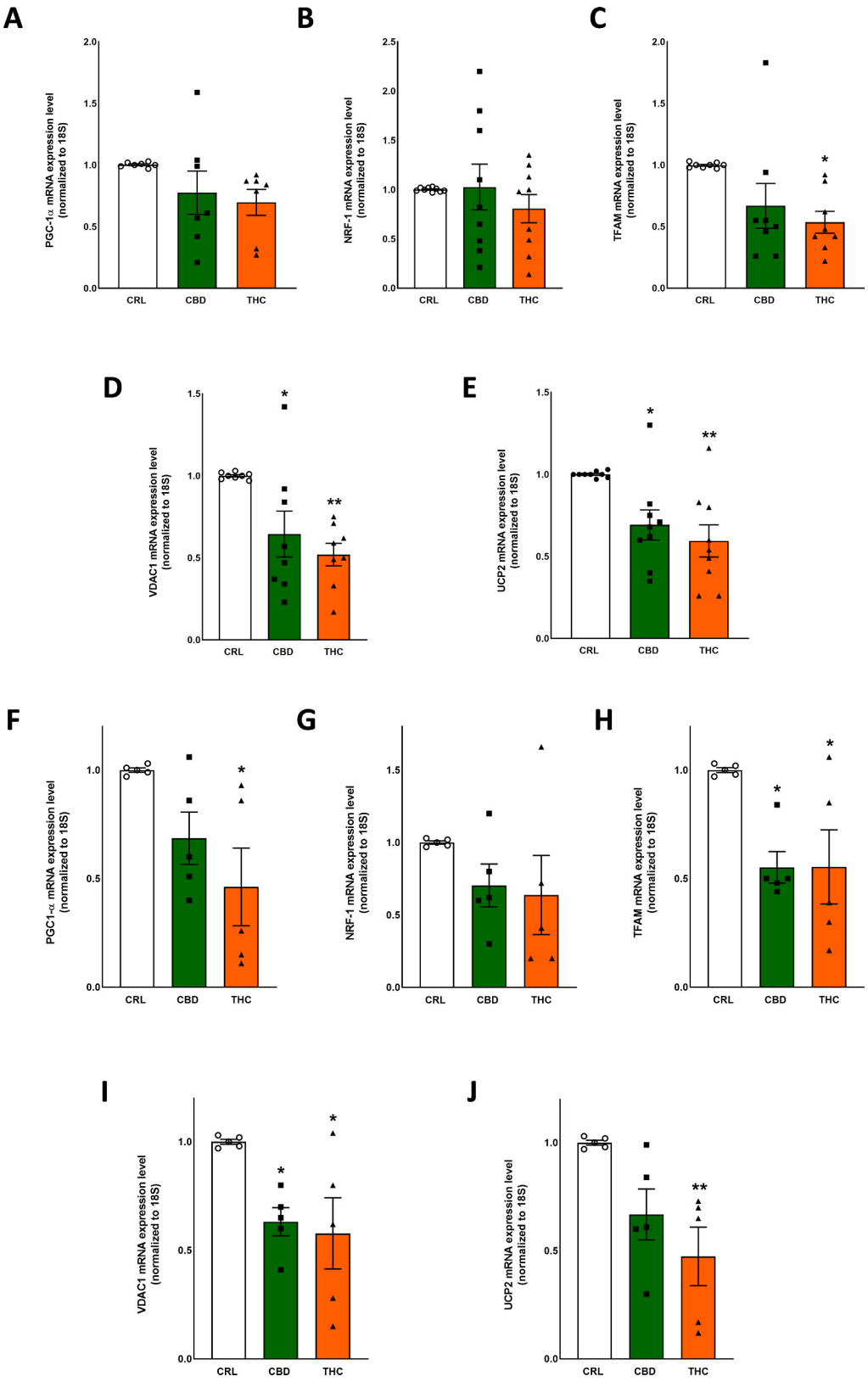


Fig. 4. Effect of prolonged treatment for 7 days with CBD or THC on the expression levels of mitochondrial markers involved in the mitobiogenesis process in immature and mature rat organotypic hippocampal slices. mRNA expression levels of PGC1-α (A, F), NRF-1 (B, G) and TFAM (C, H), VDAC1 (D, I), UCP2 (E, J). Similchronic treatment with 1 μM CBD and 1 μM THC significantly decreased the mRNA expression of VDAC1 (D) and UCP2 (E) and only THC significantly decreased the mRNA expression of TFAM (C) in immature slices. In mature slices, treatment with CBD and THC significantly decreased the mRNA expression of TFAM (H) and VDAC1 (I) and only THC significantly decreased the mRNA levels of PGC1-α (F) and UCP2 (J). Bars represent the mean ± SEM of at least 5 experiments. * $p < 0.05$ and * * $p < 0.01$ vs. CRL (ANOVA + Dunnett's w-Test).

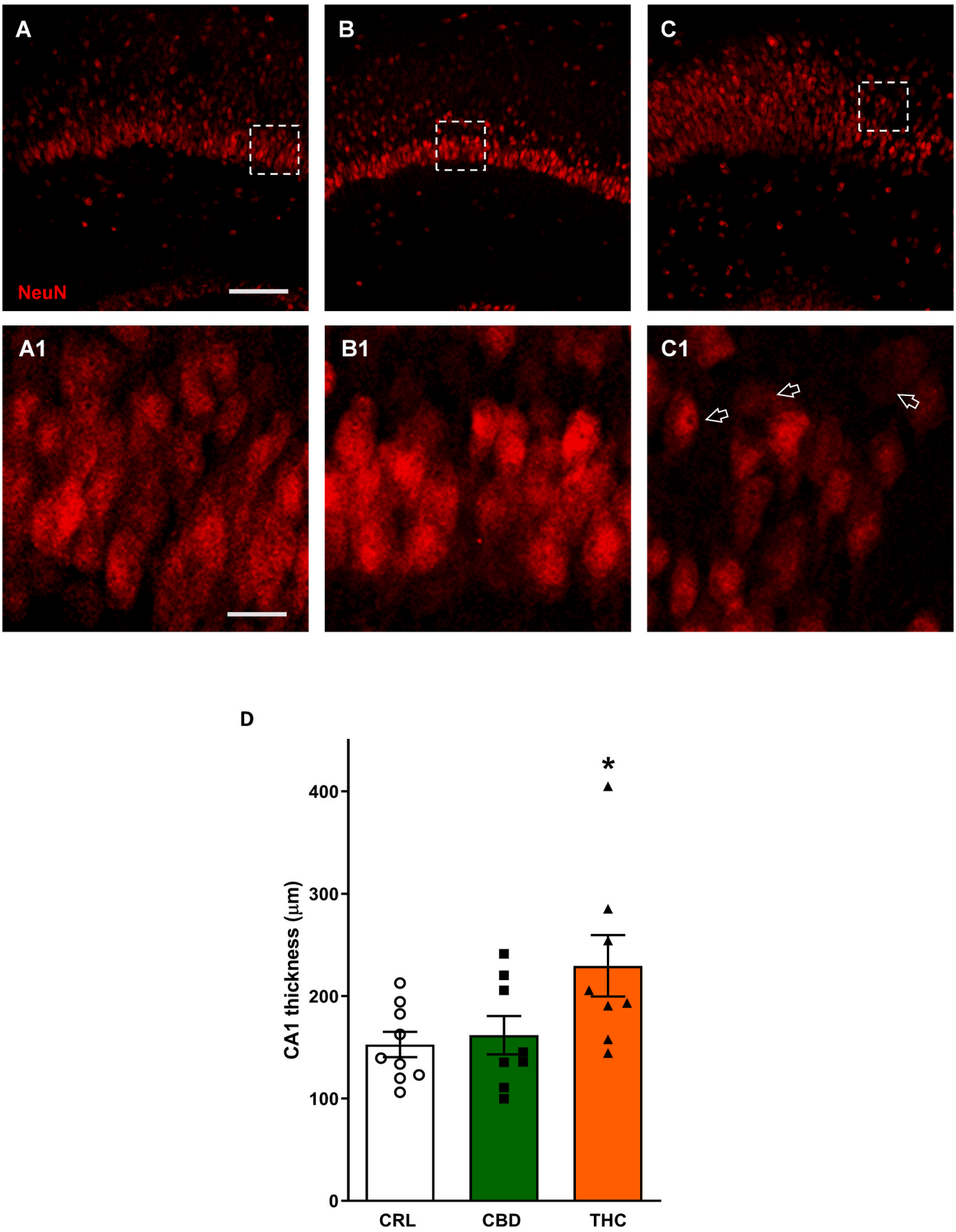


Fig. 5. Effects of prolonged treatment for 7 days with CBD or THC on neuronal viability in immature organotypic hippocampal slices. (A-C) Representative confocal images of neurons (NeuN-positive, red) in CA1 SP and SR of CRL (A), slice treated with CBD (B) or THC (C), captured with a 20X objective. A-C scale bar: 150 μm. (A1-C1) Magnification of framed areas in panels (A-C): CRL (A1), CBD (B1) and THC (C1). Scale bar: 20 μm. (D) Quantitative analysis of morphological alterations in CA1: thickness of CA1 SP. Bars represent the mean ± SEM of 8–10 samples for each group. * $p < 0.05$ vs. CRL (ANOVA + Dunnett's w-Test).

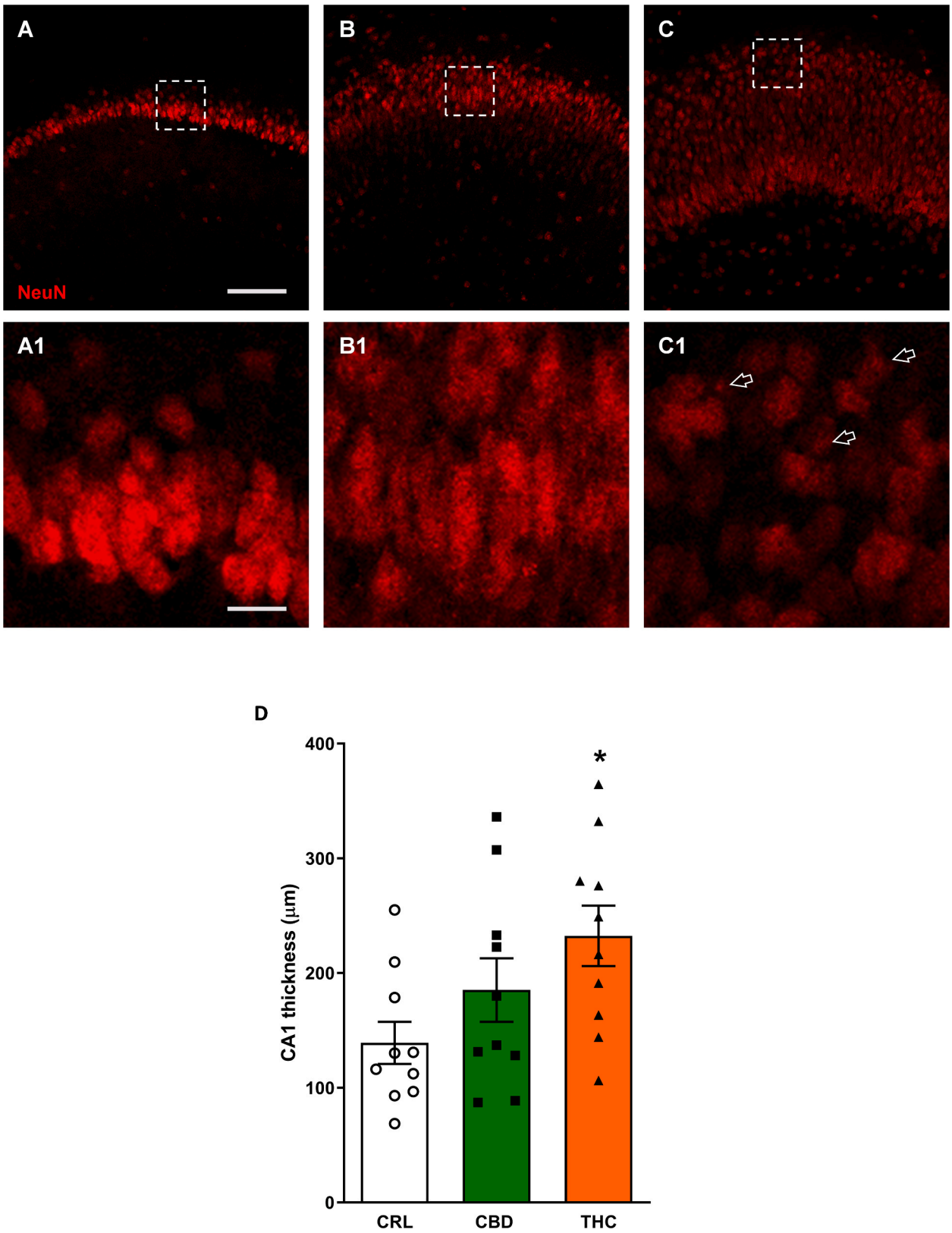


Fig. 6. Effects of chronic treatment for 7 days with 1 μM of CBD or 1 μM of THC on CA1 pyramidal neurons viability in mature organotypic hippocampal slices. (A-C) Representative confocal images of neurons (NeuN-positive, red) in CA1 SP and SR of CRL (A), slice treated with CBD (B) or THC (C), captured with a 20X objective. A-C scale bar: 150 μm. (A1-C1) Magnification of framed areas in panels (A-C): CRL (A1), CBD (B1) and THC (C1). Scale bar: 20 μm. (D) Quantitative analysis of morphological alterations in CA1: thickness of CA1 SP. Bars represent the mean ± SEM of 10 samples for each group. * $p < 0.05$ vs. CRL (ANOVA + Dunnett's w-Test).

immunohistochemistry data were assessed using one-way ANOVA with a post hoc Dunnett for multiple comparisons. All statistical calculations were performed using GRAPH-PAD PRISM v. 8 for Windows (GraphPad Software, San Diego, CA, USA). A probability value (P) of < 0.05 was considered significant. For simplicity, experimental results are described qualitatively in the main text, whereas experimental and statistical details, including sample size, statistical test, p-value, main effects are reported in Figure legends or in Supplementary Tables.

3. Results

3.1. Prolonged treatment with cannabinoids induces synaptic protein alterations in immature and mature organotypic hippocampal slices

To explore the mechanisms underlying the synaptic alterations induced by chronic cannabinoid use, we exposed immature (2-days in vitro) and mature (10-days in vitro) rat organotypic hippocampal slices for 7 days to 1 μM CBD or 1 μM THC and then analyzed the levels of the pre-synaptic proteins vGlut1, synaptophysin and GLT1 and those of the post-synaptic proteins Gephyrin, GluA1, GluA2 and PSD95 by Western blot. As shown in Figs. 1 and 2, a simil-chronic treatment with cannabinoids modified the levels of the pre- and post-synaptic proteins as follows: in immature slices CBD induced a significant increase of PSD95 (Fig. 1K and L) (CRL: 0.99 ± 0.0037, CBD: 1.32 ± 0.1379, p = 0.0448), whereas THC significantly reduced the levels of synaptophysin (Fig. 1C and D) (CRL: 1 ± 0.0064, THC: 0.78 ± 0.0567, p = 0.0459) and PSD95 (Fig. 1K and L) (CRL: 0.99 ± 0.0037, THC: 0.67 ± 0.0700, p = 0.423). In mature slices prolonged exposure to CBD had no effect, while THC significantly reduced vGlut1 (Fig. 2A and B) (CRL: 0.99 ± 0.0051, THC: 0.6075 ± 0.0760, p = 0.0075) synaptophysin (Fig. 2C and D) (CRL: 0.99 ± 0.0070, THC: 0.7186 ± 0.0912, p = 0.0042) and PSD95 (Fig. 2K and L) (CRL: 0.99 ± 0.0067, THC: 0.74 ± 0.0648, p = 0.0030). We didn't observe changes in Gephyrin protein levels in both immature and mature slices (Fig. S3).

3.2. THC increases the frequency of sEPSCs in immature organotypic hippocampal slices

Whole-cell patch clamp recordings from CA1 pyramidal neurons were obtained from immature and mature organotypic hippocampal slices at the end of the 7-day treatment with CBD and THC. Under control conditions, measurements of intrinsic somatic properties reveal a reduction of membrane resistance as an effect of maturation *in vitro*, consistent with an expected overall enrichment in ion channels, whereas membrane capacitance, resting potential or spike threshold were not affected (Fig. S1). The simil-chronic CBD exposure had no effect on any of the analyzed properties, regardless of the maturation stage. In contrast, THC caused a significant reduction of membrane capacitance, resting potential and spike threshold in immature (Fig. S1A) (*Membrane Capacitance*, CRL: 126.8 ± 6.445 pF, THC: 86.25 ± 4.833 pF, $p = 0.0011$; *Resting potential*, CRL: −67.63 ± 2.857 mV, THC: −52.37 ± 2.401 mV, $p = 0.0008$; *Spike threshold*, CRL: −46.85 ± 2.939 mV, THC: −33.82 ± 2.540 mV, $p = 0.0044$), but not in mature slices (Fig. S1B). Intrinsic electrical properties showed only partial consistency with somatic excitability, as expressed by the number of action potentials elicited by depolarizing stimuli of increasing amplitude observed under all treatment conditions (Fig. S2). In this case, we found no effect of the treatments in immature slices (Figs. S2A and B), as opposed to a significant reduction of excitability in matures slices following both CBD and THC exposure (Figs. S2C and D), in a physiological range of depolarizing stimuli (+ 60 to +90 pA) (Step +70 pA, CRL: 1.93 ± 0.4727, CBD: 0.40 ± 0.2895, THC: 0.60 ± 0.3352; CBD vs CRL, p = 0.0546; THC vs CRL, p = 0.0204; Step +90 pA, CRL: 3.53 ± 0.5151, CBD: 1.40 ± 0.5416, THC: 1.47 ± 0.5333; CBD vs CRL, p = 0.0177, THC vs CRL, p = 0.0151).

We then recorded sEPSCs as an index of local network activity. Under our recording conditions, inward currents were mostly mediated by AMPA-type glutamate receptors because complete synaptic silencing was observed when we applied 10 μM of the selective AMPA blocker NBQX at the end of the recording (data not shown, effect already described in Gerace et al., 2019 [38]). As previously reported, the overall strength of the network excitatory tone increases during normal maturation *in vitro* [32], as indicated by a twofold increase in sEPSCs frequency in mature slices (Fig. 3). Following CBD treatment, no alteration in the frequency or amplitude of sEPSCs was detected as compared to controls at both maturation stages (Fig. 3). In contrast, THC promoted the expression of a hyperexcitable synaptic phenotype as indicated by the increase in both sEPSCs frequency and amplitude, although statistical significance is achieved only for sEPSCs frequency in immature slices (Fig. 3A and B) (Frequency - Immature: CRL: 4.57 ± 0.8495 Hz, THC: 9.17 ± 0.5911 Hz, p = 0.0005; Mature: CRL: 10.05 ± 0.7991 Hz, THC: 12.33 ± 0.7453 Hz, p = 0.1669; Amplitude - Immature: CRL: −36.11 ± 3.853 pA, THC: −55.17 ± 9.199 pA, p = 0.0789; Mature: CRL: −41.42 ± 7.727 pA, THC: −66.32 ± 14.94 pF, p = 0.1643). Taken together, our slice recordings suggest that simil-chronic treatment with THC had a larger impact on the maturation of the electrophysiological phenotype of organotypic hippocampal slices as compared to both the control and CBD.

3.3. Prolonged treatment with cannabinoids induces impairment of mitochondrial marker in immature and mature organotypic hippocampal slices

As repeatedly reported in the scientific literature, chronic use and abuse of cannabinoids are associated with a consistent impairment of mitochondrial bioenergetics [39]. In this study, we analyzed, by using a RT-PCR assay, the expression levels of a number of mitochondrial markers involved in the mitobiogenesis process in immature and mature hippocampal slices. In immature organotypic hippocampal slices, the prolonged exposure to CBD and THC significantly decreased the mRNA expression levels of VDAC1 (Fig. 4D) (CRL: 1 ± 0.0070, CBD: 0.64 ± 0.1396, THC: 0.52 ± 0.0686; p = 0.0204 CBD vs CRL, p = 0.0021 THC vs CRL) and UCP2 (Fig. 4E) (CRL: 1 ± 0.0060, CBD: 0.69 ± 0.0917, THC: 0.59 ± 0.0978; p = 0.0183 CBD vs CRL, p = 0.0022 THC vs CRL), while only the treatment with THC significantly decreased the mRNA expression levels of TFAM (Fig. 4C) (CRL: 0.99 ± 0.0072, THC: 0.53 ± 0.0884, p = 0.0201). In mature organotypic hippocampal slices, the prolonged treatment with both CBD and THC significantly decreased the mRNA expression levels of TFAM (Fig. 4H) (CRL: 1 ± 0.0114, CBD: 0.55 ± 0.0728, THC: 0.55 ± 0.1707; p = 0.0222 CBD vs CRL, p = 0.0227 THC vs CRL), VDAC1 (Fig. 4I) (CRL: 1 ± 0.0114, CBD: 0.6320 ± 0.0646, THC: 0.57 ± 0.1638; p = 0.0456 CBD vs CRL, p = 0.0231 THC vs CRL), whereas only THC was able to significantly reduce the mRNA expression levels of PGC1-α (Fig. 4F) (CRL: 1 ± 0.0100, THC: 0.46 ± 0.1788, p = 0.0184) and UCP2 (Fig. 4J) (CRL: 1 ± 0.0114, THC: 0.47 ± 0.1352, p = 0.0069).

3.3.1. Prolonged treatment with THC disrupts CA1 cytoarchitecture in immature and mature organotypic hippocampal slices

In order to assess the CA1 cytoarchitecture of immature and mature organotypic hippocampal slices following a 7-day prolonged treatment with 1 μM CBD or 1 μM THC, we used immunostaining with NeuN followed by confocal microscopy. The qualitative analysis of NeuN-positive CA1 pyramidal neurons of immature (Fig. 5) and mature (Fig. 6) organotypic hippocampal slices shows that the simil-chronic treatment with THC caused an enlargement of the layering of CA1 stratum pyramidalis and morphological modifications in CA1 pyramidal neurons (pointed by arrows in Figs. 5 and 6). The thickness of the stratum pyramidalis following THC treatment increased significantly by about 50 % (Fig. 5D) (CRL: 152.7 ± 12.37, THC: 229.5 ± 29.98, $p = 0.0302$), and a similar effect was observed in mature slices (Fig. 6D) (CRL: 139.1 ± 18.33, THC: 232.3 ± 26.31, $p = 0.0222$). CBD, on the contrary, did not alter

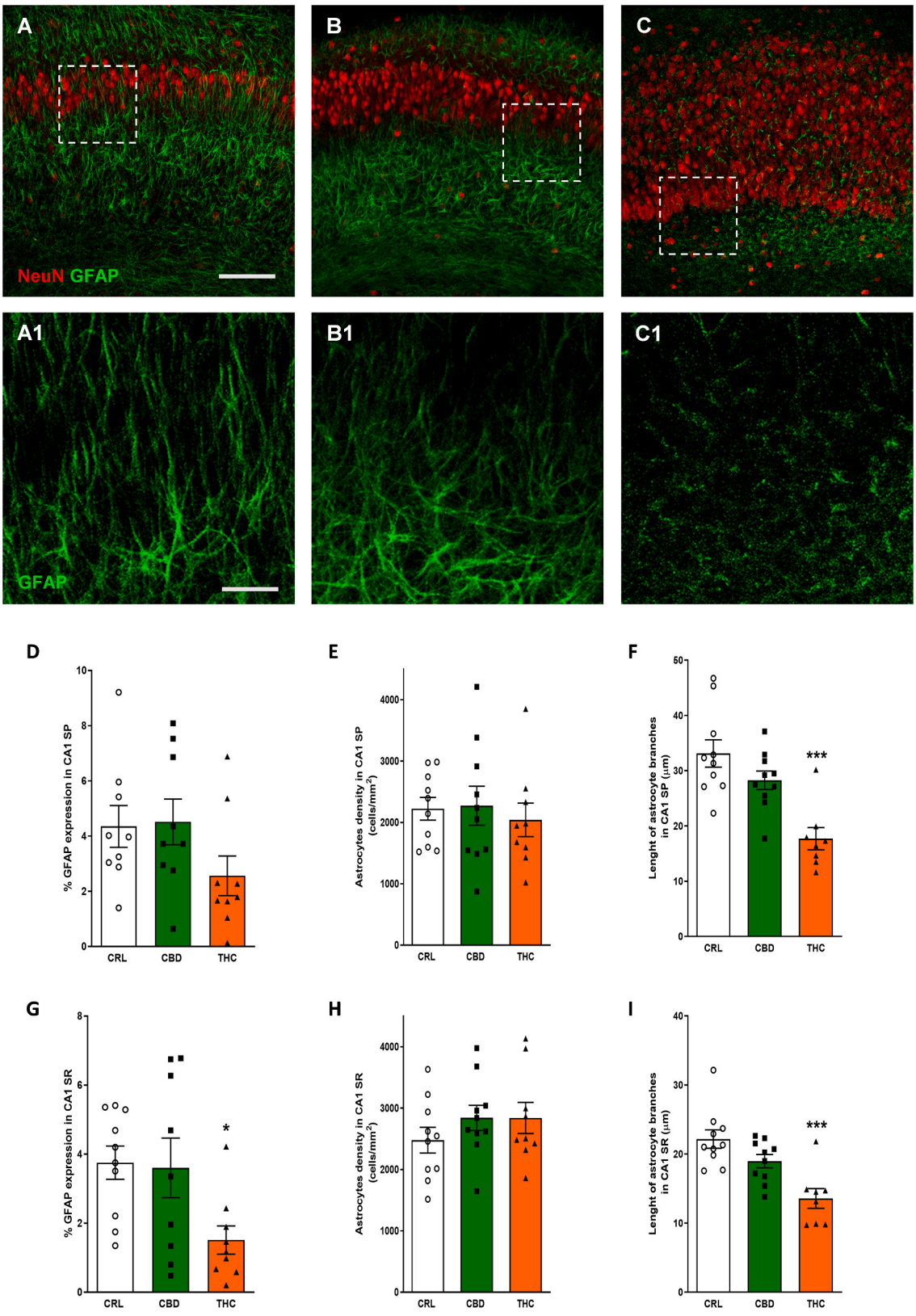


Fig. 7. Effects of prolonged treatment for 7 days with CBD or THC on astrocytes in immature organotypic hippocampal slices. (A-C) Representative confocal images of astrocytes (GFAP-positive, green) and neurons (NeuN-positive, red) immunostaining in CA1 SP and SR of CRL (A), slice treated with CBD (B) or THC (C), captured with a 20X objective. Scale bar: 150 μm. (A1-C1) Magnification of areas framed in panels (A-C) of astrocytes. Scale bar: 40 μm. (D) Quantitative analysis of GFAP expression in CA1 SP. (E) Quantitative analysis of astrocytes density in CA1 SP. (F) Quantitative analysis of astrocytes branches length in CA1 SP. (G) Quantitative analysis of GFAP expression in CA1 SR. (H) Quantitative analysis of astrocytes density in CA1 SP. (G) Quantitative analysis of astrocytes density in CA1 SR. (I) Quantitative analysis of astrocytes branches length in CA1 SR. Bars represent the mean ± SEM of 8–10 different slices. * $p < 0.05$ and * ** $p < 0.001$ vs. CRL (ANOVA + Dunnett's w-Test).

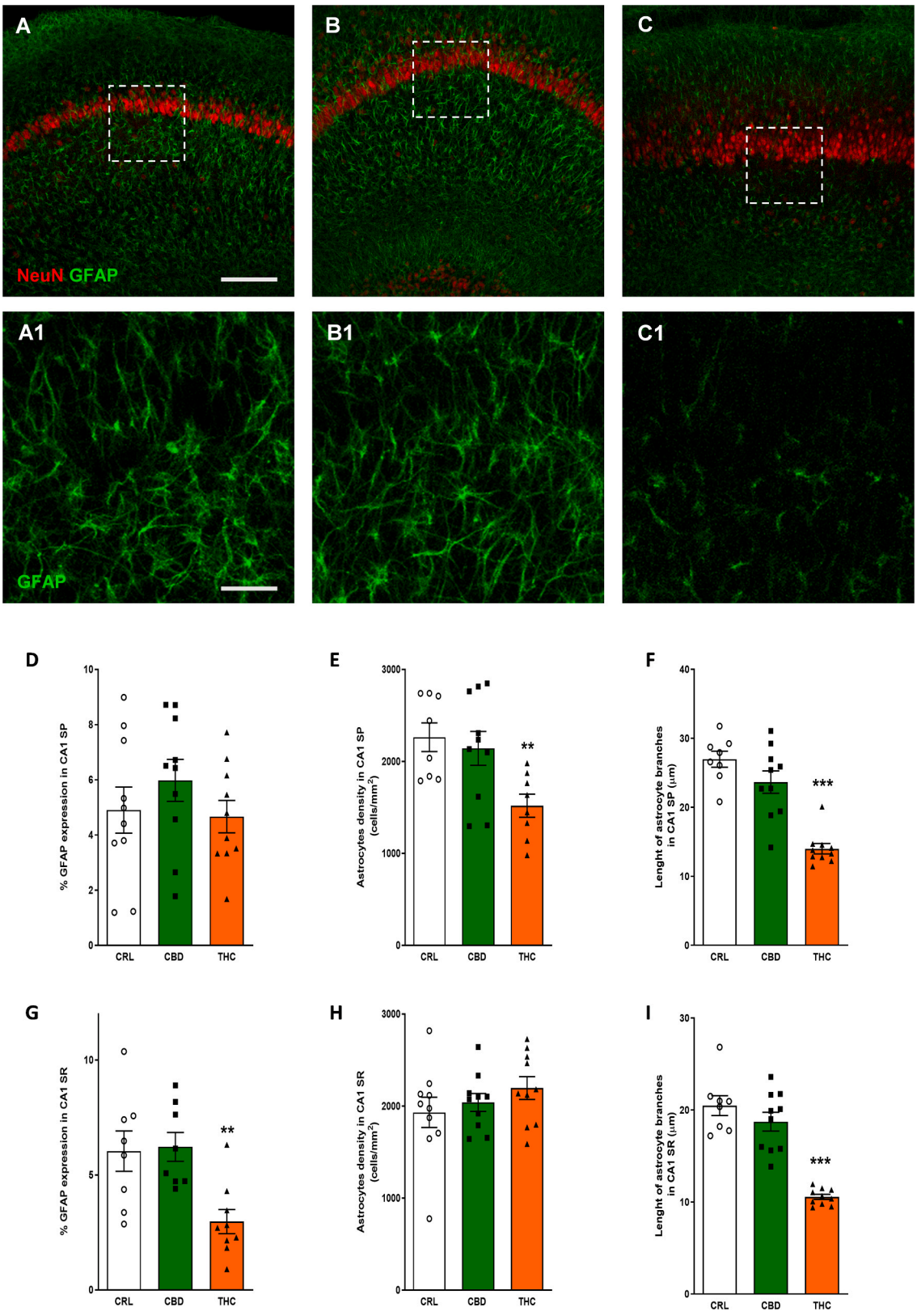
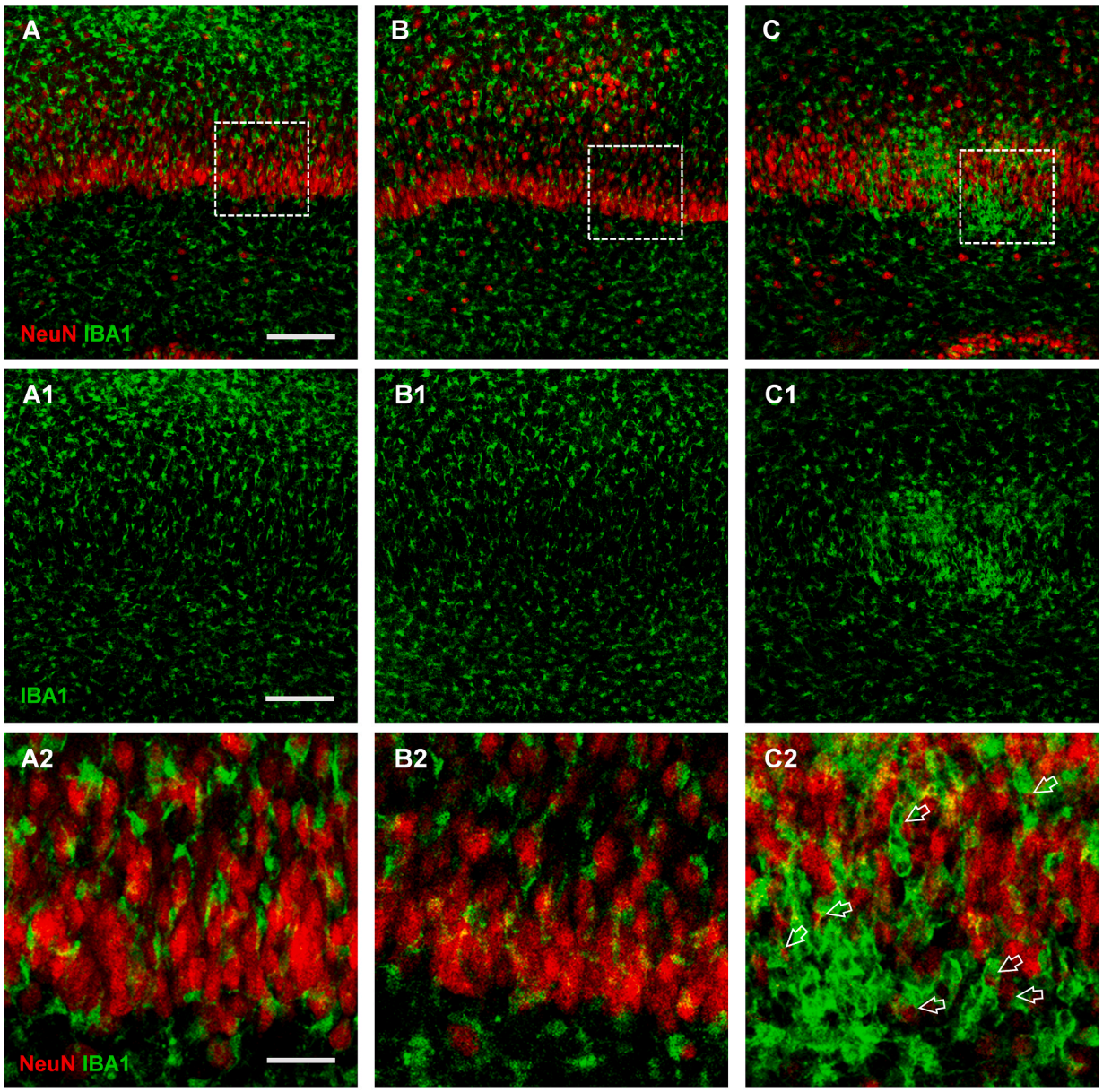
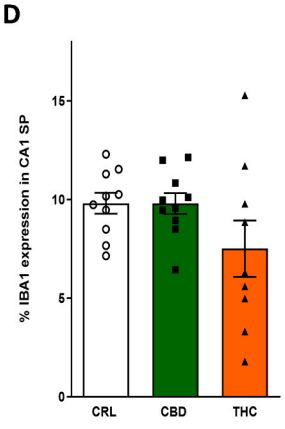
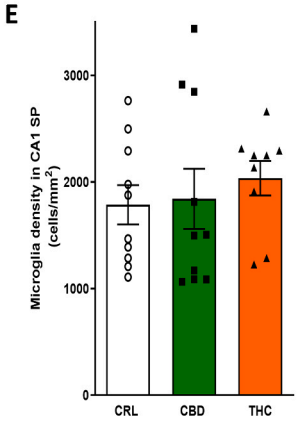


Fig. 8. Effects of prolonged treatment for 7 days with CBD or THC on astrocytes in mature organotypic hippocampal slices. (A-C) Representative confocal images of astrocytes (GFAP-positive, green) and neurons (NeuN-positive, red) immunostaining in CA1 SP and SR of a mature control slice (A), a mature slice treated with CBD (B), and a mature slice treated with THC (C) captured with a 20x objective. Scale bar: 150 μm. (A1-C1) Magnification of areas framed in panels (A-C) of astrocytes in CRL (A1), CBD (B1) and THC (C1). Scale bar: 40 μm. (D) (E) Quantitative analysis of astrocytes density in CA1 SP. (F) Quantitative analysis of astrocytes branches length in CA1 SP. (G) Quantitative analysis of GFAP expression in CA1 SR. (H) Quantitative analysis of astrocytes density in CA1 SP. (G) Quantitative analysis of astrocytes density in CA1 SR. (I) Quantitative analysis of astrocytes branches length in CA1 SR. Bars represent the mean ± SEM of 8–10 different slices. * * $p < 0.01$ and * ** $p < 0.001$ vs. CRL (ANOVA + Dunnett's w-Test).







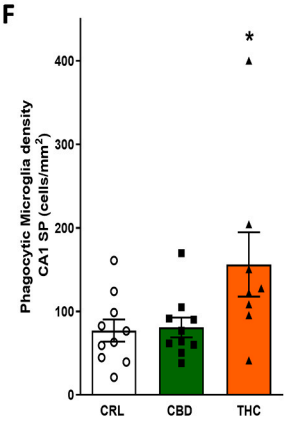


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Fig. 9. Effect of prolonged treatment for 7 days with CBD or THC on microglial cells activation and on the presence of phagocytic microglia in immature organotypic hippocampal slices. (A-C) Representative confocal images of microglia (IBA1-positive, green) and neurons (NeuN-positive, red) immunostaining in CA1 SP and SR of an immature control slice (A), an immature slice treated with CBD (B), and an immature slice treated with THC (C), captured with a 20x objective. Scale bar: 150 μm. (A1-C1) IBA1 immunostaining (green) of images in panels A-C was represented alone to better appreciate microglia activation in CRL (A1), CBD (B1) and THC (C1). Scale bar: 150 μm. IBA1 immunostaining (green) of images in panels A-C was represented alone to better appreciate microglia activation in CRL (A1), CBD (B1) and THC (C1). Scale bar: 150 μm. (A2-C2) Magnification of framed areas in panels (A-C): CRL (A2), CBD (B2) and THC (C2). Scale bar: 40 μm. C2: Image taken from the THC slice in C shows clearly that there was an increase in the number of phagocytic cells after simil-chronic treatment with THC in CA1 (pointed by arrows). (D) Quantitative analysis of IBA1 expression in CA1 SP. (E) Quantitative analysis of microglia density in CA1 SP. (F) Quantitative analysis of phagocytic microglia density in CA1 SP. Bars represent the mean ± SEM of 8–10 different slices. * $p < 0.05$ vs. CRL (ANOVA + Dunnett's w-Test).

the thickness of CA1 stratum pyramidalis neither in immature nor in mature slices (Figs. 5D and 6D).

3.3.2. Prolonged treatment with THC induces morphological alterations of astrocytes in the CA1 area of immature and mature organotypic hippocampal slices

In a previous study [37], we observed significant morphological alterations of astrocyte processes in mature slices exposed to THC for 72 h. In this study we observed that, in a similar manner, a 7-day THC exposure modified the morphology of astrocytes in the CA1 area of immature (Fig. 5C) and mature (Fig. 6C) hippocampal slices. Qualitative analysis of GFAP immunostaining showed that in THC slices astrocytes were smaller in both the stratum pyramidalis and stratum radiatum, with shorter and thinner branches (Fig. 7C-C1 and Fig. 8C-C1).The quantitative analyses confirmed that astrocytes branches length were significantly reduced (Fig. 7F,I and Fig. 8F,I) (CRL: 33.12. ± 2.481, THC: 17.68 ± 2.012, $p < 0.0001$; CRL: 22.16 ± 1.333, THC: 13.56 ± 1.433, $p = 0.0001$; CRL: 26.97 ± 1.171, THC: 13.98 ± 0.7536, $p < 0.0001$; CRL: 20.47 ± 1.079, THC: 110.56 ± 0.2779, $p < 0.0001$) GFAP expression was particularly less intense as compared to control slices in the stratum radiatum (Figs. 7G and 8G) (CRL: 3.75 ± 0.4830, THC: 1.511 ± 0.4086, $p = 0.0277$; CRL: 6.03 ± 0.8779, THC: 2.97 ± 0.5270, $p = 0.0077$). CBD did not alter the morphology of astrocytes and did not cause any statistically significant difference in GFAP expression in both immature and mature slices.

3.3.3. Prolonged treatment with THC induces morphological alterations of the microglia in the CA1 area of immature and mature organotypic hippocampal slices

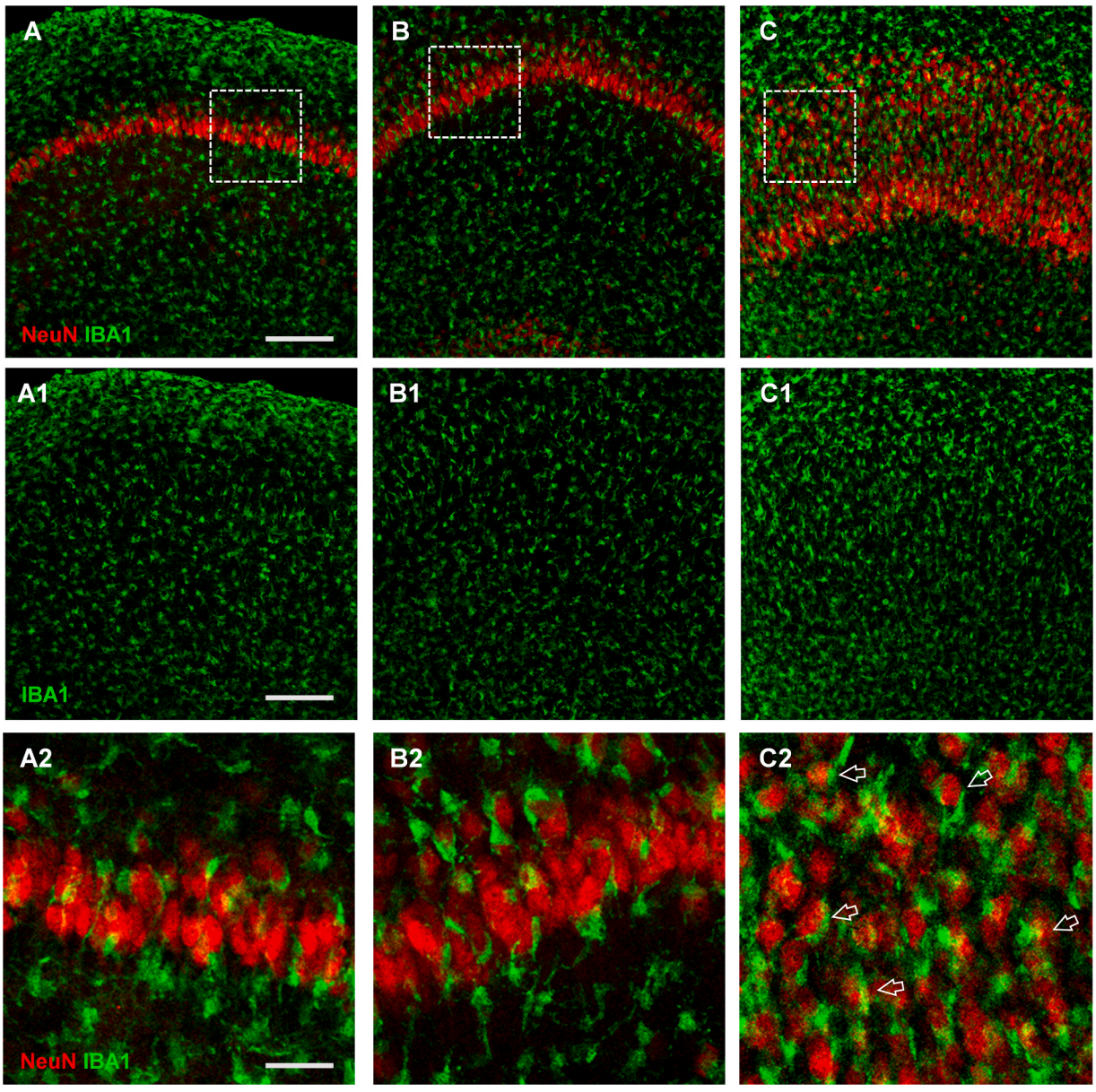
The effect of prolonged treatment with cannabinoids was also evaluated on microglial cells in the CA1 area of immature and mature hippocampal slices. Figs. 9 and 10 show that 7-day exposure to CBD or THC were unable to modify the expression of IBA1 nor the density of microglia in the CA1 stratum pyramidalis of immature or mature slices. However, in both immature and mature slices, the 7-day treatment with THC induced a morphological transformation of microglial cells towards the amoeboid, phagocytic phenotype, which suggests an activation of the microglia in response to cannabinoid injury (Figs. 9C2 and 10C2, phagocytic microglia are pointed by arrows). Quantitative analysis confirmed that THC but not CBD produced a significant increase in the density of phagocytic microglial cells in the CA1 of immature and mature slices (Figs. 9F and 10F) (CRL: 76.96 ± 13.28, THC: 156.2 ± 38.52, $p = 0.0360$; CRL: 146.4 ± 17.38, THC: 220.9 ± 17.41, $p = 0.0314$).

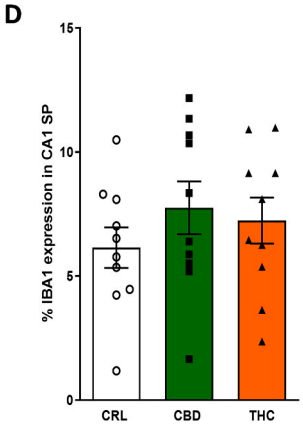
4. Discussion

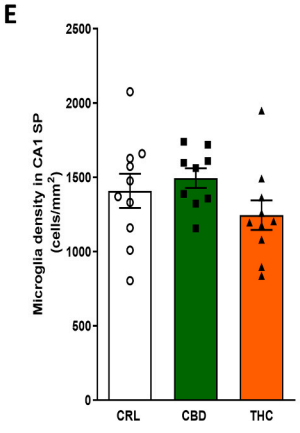
The long-term abuse of Cannabis derivatives leads to addiction, impaired cognitive function [40], memory deficits [41], and the development of hallucinations and psychosis [42,43], especially during the formative years of adolescence [44]. The behavioral alterations produced by the exposure to cannabinoids in animal models are known to be the result of neuronal morphological modifications in the prefrontal cortex and hippocampus [23,24,45,46], and impairment of GABAergic and glutamatergic synaptic transmission [47–50]. In this study, we use rat organotypic hippocampal slices cultured for 2 days or 10 days *in vitro*, in order to reproduce the maturation of either adolescent immature or mature brain, exposed to a prolonged (7-day) incubation to CBD or THC in order to mimic chronic exposure in humans. In this hippocampal experimental model, we analyze the expression of pre- and post-synaptic proteins by Western blot, glutamatergic synaptic transmission in the CA1 region by electrophysiology, the modifications of mitochondrial biogenesis markers by RT-PCR and the qualitative and quantitative morphology of CA1 neuronal populations by fluorescence microscopy.

Our experiments show that CBD evokes a significant increase of PSD95 while THC induces a significant reduction in the levels of the presynaptic proteins synaptophysin and PSD95 in immature hippocampal slices. In mature slices, THC induces a significant reduction of the presynaptic proteins vGlut1 and synaptophysin and of the post-synaptic protein PSD95. These data are in agreement with those observed by Rubino and colleagues [45], who showed that PSD95 decreased and spatial memory was impaired in rats treated for 10 days with THC. Also, chronic exposure to CP55,940, altered both the morphology of pyramidal neurons and the expression of PSD95 protein in the prefrontal cortex and induced plasticity changes in the hippocampus–cortex network of adult rats [51]. Increased expression of synaptophysin and PSD95 in the medial prefrontal cortex and elevated brain-derived neurotrophic factor levels in both cortex and hippocampus after a single injection of CBD are observed in Swiss mice and in Flinders Sensitive and Flinders Resistant Line rats, which translates into acute antidepressant effects [52]. The increase in PSD95 is proposed as one of the possible mechanisms of action for CBD anti-stress effects [53]. Cannabinoid exposure induces enhanced PFC expression of the scaffolding protein PSD95, and this was paralleled by increased expression of GluN1 [54], GluN2A/B, and GluA1 subunits [55].

Changes in synaptic transmission can be associated with mitochondrial dysfunction and may cause cognitive impairment in neurodegenerative diseases [56,57]. In this study, we observe that a simil-chronic treatment for 7 days with CBD and THC induces an impairment of mitochondrial biogenesis genes both in immature and mature organotypic hippocampal slices, namely a significant reduction in VDAC1 and UCP2, while THC but not CBD reduces the expression levels of TFAM in immature slices. In mature cultures, prolonged treatment with both CBD and THC significantly decreases the mRNA expression levels of TFAM, VDAC1, whereas only THC is able to significantly reduce the mRNA levels of PGC1-α and UCP2. The literature is somewhat in contrast regarding the effects of CBD on mitochondrial activity: the "dark side" claims that CBD reduces oxygen consumption, and mitochondrial complex activity [58,59], and disrupts mitochondrial calcium homeostasis [60], the "bright side" reports an increased mitochondrial complex activity in the rat hippocampus [61], in isolated mitochondria from mouse cardiac tissue [61,62], and in a preclinical model of Leigh syndrome [63] after administering CBD. Many studies are in accordance with our data on the effects of THC: for example, in a pulmonary transformed cell line [64] and in pig brain mitochondria [58] THC disrupts mitochondrial respiration. This effect is likely to be mediated by mitochondrial CB1 receptor activation [65] or through indirect modulation of the mitochondrial permeability transition pore [64]. Elevated UCP2 in neurons leads to increased mitochondrial proliferation and greater ATP production with dynamic fluctuations in neuronal activity [66], whereas in the absence of UCP2 neurons are less plastic in response







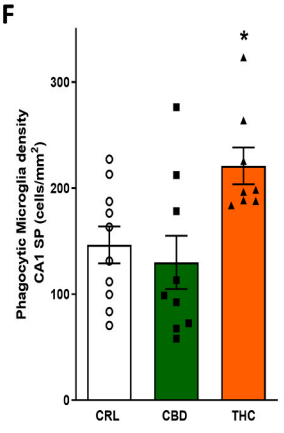


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Fig. 10. Effect of prolonged treatment for 7 days with CBD or THC on microglial cells activation and on the presence of phagocytic microglia formation in mature organotypic hippocampal slices. (A-C) Representative confocal images of microglia (IBA1 positive, green) and neurons (NeuN-positive, red) immunostaining in CA1 SP and SR of a mature control slice (A), a mature slice treated with CBD (B), and a mature slice treated with THC (C), captured with a 20x objective. Scale bar: 150 μm. (A1-C1) IBA1 immunostaining (green) of images in panels A-C was represented alone to better appreciate microglia activation in CRL (A1), CBD (B1) and THC (C1). Scale bar: 150 μm. (A2-C2) Magnification of framed areas in panels (A-C): CRL (A2), CBD (B2) and THC (C2). Scale bar: 40 μm. C2: Image taken from the THC slice in C shows clearly that there was an increase in the number of phagocytic cells after simil-chronic treatment with 1 μM THC in CA1 (pointed by arrows). (D) Quantitative analysis of IBA1 expression in CA1 SP. (E) Quantitative analysis of microglia density in CA1 SP. (F) Quantitative analysis of phagocytic microglia density in CA1 SP. Bars represent the mean ± SEM of 8–10 different slices. * $p < 0.05$ vs. CRL (ANOVA + Dunnett's w-Test).

to stress [66]. Therefore, a reduction of UCP2 levels in both models with THC but not CBD may be correlated with the synaptic alterations that we observe. On the other hand, the alterations of UCP2 levels treated with CBD that we observe in our study in immature slices do not translate into synaptic or electrophysiological alterations. Interestingly, it has been previously observed that a reduction in mitochondrial gene expression associated with an increase in synaptic modulation proteins may play a role in CBD ability to reduce seizure frequency in both human and animal models [67–69].

Our immunohistochemistry experiments show that a simil-chronic treatment for 7 days with THC but not CBD in both immature and mature slices produces an enlargement of the layering of CA1 stratum pyramidalis, an alteration of astrocyte morphology and GFAP expression, and an increase of phagocytic microglia density. Since cell-cell and cell-matrix adhesions are essential for tissue architecture and homeostasis [70] it appears that the treatment with THC may alter the neuronal cytoskeleton organization and also the physiological neuron-neuron and neuron-matrix adhesion pattern. Our findings are in accordance with other studies [51,71,72] in which the authors showed that the treatment with CB1 receptor agonists is able to alter neuronal cytoskeleton organization in the hippocampus and with a previous study of our lab [37] in which we demonstrated that a 3-day sub chronic treatment with THC compromises CA1 cytoarchitecture inducing a similar enlargement of CA1.

Our findings on the alteration of astrocyte morphology are in accordance with other studies, in other regions of the brain, by Suarez and colleagues [73,74] showing that pre- and perinatal THC exposure interferes with astrocytic maturation by disrupting normal cytoskeletal formation and causing long-lasting changes in GFAP expression. The loss of distal branches caused by THC exposure, resulting in reduced astrocytic syncytium and decreased coverage of synapses, could be a key factor in decreasing synaptic connectivity and neuronal homeostasis [75,76]. As for the increase in phagocytic microglia density induced by THC, it could be secondary to the neuronal alterations in CA1 stratum pyramidalis. Microglia expresses many of the components required for functional cannabinoid signaling and, conversely, cannabinoids control microglia immune-related responses in the initiation and propagation of neuroinflammation [77,78]. The shift of microglia from a resting state to a phagocytic state may be triggered by THC agonism on CB2 receptors, which are known to alter the balance among homeostatic, neuroprotective and neuroinflammatory gene expression patterns in microglial cells [79]. All these data are important in the context of translational relevance especially in view of human CB1R is 50 times more sensitive to THC than rat CB1R [80].

In conclusion, our results may be relevant to understand the effects that chronic cannabinoid consumption may cause in the CA1 hippocampus, a region involved in memory and learning mechanisms and point out that each THC and CBD have different effects. Moreover, we show that the use of THC in immature brain tissue produces alterations that may be relevant for the dysfunctions that THC causes in adolescents.

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CRediT authorship contribution statement

Costanza Mazzantini: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Maria Grazia Giovannini:** Writing – review & editing. **Alessio Masi:** Writing – original draft, Visualization. **Daniele Lana:** Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Lorenzo Curti:** Methodology, Formal analysis, Data curation. **Elisa Landucci:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Domenico E. Pellegrini-Giampietro:** Writing – review & editing, Visualization, Resources, Funding acquisition. **Giada Magni:** Methodology.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2024.117797.

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