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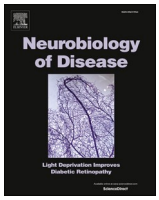

Cannabidiol inhibits microglia activation and mitigates neuronal damage induced by kainate in an in-vitro seizure model

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ABSTRACT

Background: Epilepsy is one of the most common brain disorder and, despite the possible use of several therapeutic options, many patients continue to have seizures for their entire lifespan and they need new therapeutic approaches. In the last years the interest on the non-psychoactive compounds present in *Cannabis sativa* has massively increased, and cannabidiol (CBD) has been shown to be effective in the treatment of different types of neurological disorders and neurodegenerative diseases such as epilepsy, ischemia, multiple sclerosis and Alzheimer's Disease.

Methods: We investigated the effects of the selected cannabinoids, Δ9-tetrahydrocannabinol (THC), CBD and cannabigerol (CBG) in rat organotypic hippocampal slices exposed to kainate, an in vitro seizure model. Cell death in the cornu Ammonis 3 (CA3) hippocampal subregion was quantified by propidium iodide fluorescence. Morphological analysis and tissue organization were examined by immunohistochemistry and confocal microscopy and microglia activation and polarization was evaluated using flow cytometry and morphology analysis.

Results: When present in the incubation medium, cannabidiol reduced dose-dependent CA3 injury induced by kainate. Conversely, incubation with THC exacerbated hippocampal damage. The neuroprotective effects of cannabidiol were blocked by TRPV1, TRPV2, 5-HT1A, and PPARγ antagonists. Confocal microscopy confirmed that CBD but not THC had a significant protective effect against neuronal damage and tissue disorganization caused by kainate. Cannabidiol incubation significantly block the microglia activation from the M0 to M1 phenotype observed in the kainate in-vitro seizure model, pushing toward a transition from M0 to M2.

Conclusions: Our results suggest that CBD mitigated neuronal damage induced by kainate and blocked the transition from the M0 to the M1 phenotype.

1. Introduction

Epilepsy is one of the most common brain disorders, affecting more that 50 million people of any age worldwide, with 80% of them living in low- and middle-income countries (Vergonjeanne et al., 2022), characterized by recurrent epileptic seizures caused by loss of neuronal excitation/inhibition balance toward overexcitation (Liu et al., 2020). Despite the possible use of many therapeutic options, including third-generation antiseizure medications, surgical and dietary interventions, 30% of the patients continue to have seizures for their entire lifespan (Denton et al., 2021; Zaccara and Schmidt, 2017). Furthermore, children with epilepsy are resistant to standard antiepileptic drugs (Brodie et al., 2013) and the pharmacological tools for the specific treatment are limited. In adult patients the most common form of epilepsy is temporal lobe epilepsy (TLE) (Engel, 1996) and the administration of antiseizure medications (ASMs) results in seizure freedom in about 65% (Chisholm, 2005). Thus, there is an urgent need to develop new medicines to control refractory epilepsy (Ji et al., 2021) in order to gain deeper knowledge on the cellular and molecular alterations induced in the brain during epileptogenesis.

In the last years, the medical interest on the possible use of cannabinoids in this pathology has risen, with particular focus on two main components of *Cannabis* Δ9-tetrahydrocannabinol (THC) and cannabidiol (CBD). While the implications of THC use in epilepsy are quite

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controversial (Kaczor et al., 2022), the interest of CBD use is high since CBD, unlike THC, has anticonvulsant properties in the absence of psychoactive effects and abuse potential (Devinsky et al., 2014; Head et al., 2020; Paolino et al., 2016). CBD was approved by Food and Drug Administration (FDA) in 2018 and by European Medicines Agency (EMA) in 2019 as add-on antiepileptic drug in 2-years old children with Dravet syndrome and Lennox-Gastaut syndrome (LGS). The first of a new class of anti-seizure medication (ASM), EPIDYOLEX® is produced by GW pharma and contains purified CBD (Lattanzi et al., 2021). A network meta-analysis reviewed the randomized controlled trial and open-label extension studies on LGS comparing ASMs with placebo or other ASMs, and concluded that the high-dose clobazam, CBD and rufinamide are the most efficacious and safest treatments in short and long-term outcomes (Devi et al., 2022). A meta-analysis of 670 patients showed significantly fewer severe adverse events and significantly higher proportion of participants achieving 50% or higher reduction in seizure frequency (71% vs 36%) in patients using CBD-predominant-*Cannabis* extracts vs purified CBD (Pamplona et al., 2018). Many studies indicate that CBD can modify the deleterious effects on BBB caused by inflammatory cytokines and may play a pivotal role in ameliorating BBB dysfunction and integrity consequent to neurological disorders (Calapai et al., 2020). To date, the neuroprotective effect of CBD in various neurological pathologies such as epilepsy is known, while its mechanism of action is not yet completely understood, since it has been demonstrated to activate multiple targets (Vitale et al., 2021).

Although neurons determine the excitation/inhibition balance, many studies reveal an increasingly important role of glia in modulating excitation/inhibition homeostasis, which is lost during epilepsy. (Liu et al., 2020). Microglia are the resident immune cells in the brain (Hiragi et al., 2018), and participate in the development, homeostasis, and damage response in the central nervous system (CNS) (Miron and Priller, 2020). During status epilepticus (SE) and other acute epileptogenic brain injuries, microglia are rapidly activated in brain regions of the seizure network. Activated microglia give a primary contribution to the expression of factors that can be either proinflammatory or anti-inflammatory, promoting the process of epileptogenesis, or, conversely, delaying epileptogenesis (Vezzani et al., 2011).

In a previous study, we have demonstrated that CBD, but not THC, consistently reduced post-ischemic neuronal damage and that the neuroprotective effect is dependent on Transient receptor potential cation channel subfamily V member 2 (TRPV2), serotonin 1A receptor (5-HT1A) or peroxisome proliferator-activated receptor gamma (PPARγ) receptors (Landucci et al., 2021b). Furthermore, it has been demonstrated that CBD had the ability to restrain inflammatory responses mediated by activated macrophages (Huang et al., 2019), and inflamed microglia cells (Barata et al., 2019; Dos-Santos-Pereira et al., 2020; Martín-Moreno et al., 2011; Mecha et al., 2013). CBD modulates a wide variety of microglial genes involved in the regulation of stress responses and inflammation (Juknat et al., 2012), and enhances microglial phagocytosis via transient receptor potential (TRP) channel activation (Yang et al., 2022). Based on all these data, in this study we examined the effects of selected active principles of *Cannabis* in rat organotypic hippocampal slices exposed to kainate, a widely accepted in-vitro seizure model (Gerace et al., 2017; Landucci and Bilia, 2019; Landucci et al., 2021a; Laurino et al., 2018). To explore the putative underlying mechanisms, we also investigated the dependence of their effects on different cannabinoids receptors, and we analysed neurodegeneration and microglia activation by immunohistochemistry as well as transition between microglia phenotypes by cytofluorimetric analysis.

2. Materials and methods

2.1. Animals

Male and female Wistar rat pups (7–9 days old) were purchased from Charles River (MI, Italy). Animals were housed at 23 ± 1 °C under a 12 h light–dark cycle (lights on at 07:00) and were fed a standard laboratory diet with ad libitum access to water.

The experimental procedures were conducted in accordance with the ARRIVE guidelines and were authorized by the Italian Ministry of Health. The ethical policy of the University of Florence complies with to the Directive 2010/63/EU of the European Parliament and to the Italian Regulation DL 26/2014 on the protection of animals used for scientific purposes. According to the law, all efforts were made to fulfil to the principle of 3 Rs.

2.2. Materials

AM251, AM630, capsazepine, cannabigerol (CBG) and cannabidiol (CBD) were purchased from Tocris Cookson (Bristol, UK). Tissue culture reagents were obtained from Gibco-BRL (San Giuliano Milanese, MI, Italy) and Sigma (St. Louis, MO, USA). Δ9-tetrahydrocannabinol (THC), tranilast (TNL) and WAY-100635 were purchased from Sigma (St. Louis, MO, USA). G3335 was purchased from Cayman (Ann Arbor, MI, USA). Authorisation by the Ministry of Health for the use of THC n. SP/94 issued on July 2021.

2.3. Preparation of rat organotypic hippocampal slice cultures

Organotypic hippocampal slice cultures were prepared as previously reported (Gerace et al., 2012).

The organotypic hippocampal cultures are an in vitro model widely used to study neurodegeneration (oxygen glucose deprivation, epileptogenics, hypothermia, ethanol), neurotoxicity (*n*-methyl-D-aspartate, and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid toxicity), as well as neuroinflammation and neuroprotection. Organotypic cultures allow several aspects of structural and synaptic organization of the original tissue to be preserved. Furthermore, another advantage of organotypic slices is that the mechanisms of neurodegeneration can be followed for several days after an insult, and treatments can be administered in chronic to demonstrate neuroprotection over time. Organotypic slices reduce the number of severe animal experiments contributing to the 3Rs (reduce, refine, replace).

Briefly, hippocampi were removed from the brains of 7–9 days old Wistar rat pups (Charles River, Milan, Italy), and transverse slices (420 μm) were prepared using a McIlwain tissue chopper and then transferred onto 30-mm-diameter semiporous membranes 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 incubator in atmosphere of humidified air and 5% CO_2 for 2 weeks. During the period of incubation, the slices become mature and ready for the experiments. The two weeks in culture are needed to guarantee that slices are not activated by endogenous release of Calcium or glutamate and that reactive astrogliosis is minimized. The slices were incubated for 24 h with 5 μM kainic acid (KA), in the presence or absence of cannabinoids. KA was dissolved in water at the concentration of 20 mM (Gerace et al., 2012) and then diluted in the medium. KA concentration and incubation time were chosen based on previous studies and induced a selective damage in the CA3 (Gerace et al., 2017; Holopainen et al., 2004; Landucci and Bilia, 2019; Landucci et al., 2021a; Laurino et al., 2018), whereas higher concentrations of KA (100 μ M) for a shorter period of time (60 min), induced a more diffuse and less selective damage (Gerace et al., 2012). The slices were incubated for 24 with THC (0.1–1 μM), CBD (0.1–10 μM) or CBG (0.1–10 μM) h alone or combination with 5 μM KA. The concentrations of cannabinoids were based on previous studies (Landucci et al., 2021b). Cell death was evaluated using the fluorescent dye propidium iodide (PI, 5 μg/mL) and fluorescence was viewed using an inverted fluorescence microscope (Olympus IX-50; Solent Scientific,

Segensworth, UK) equipped with a xenon-arc lamp, a low-power objective (4×) and a rhodamine filter. Images were digitized using a video image obtained by a CCD camera (Diagnostic Instruments Inc., Sterling Heights, MI, USA) controlled by software (InCyt Im1TM; Intracellular Imaging Inc., Cincinnati, OH, USA). Images were analysed using morphometric analysis software. Cellular death induced by KA in the CA3 hippocampal subfields was quantified by the image software (ImageJ; NIH, Bethesda, USA) detecting the optical density of PI fluorescence (the fluorescence measured in KA-exposed slices in the CA3 region was taken as 100%) (Landucci and Bilia, 2019, Landucci et al., 2021a; Laurino et al., 2018).

2.4. Fluorescence immunohistochemistry and confocal microscopy

At the end of treatments with KA alone or in presence of either THC (1 μM) or CBD (10 μM), the organotypic hippocampal slices were harvested and fixed overnight (O/N) in ice-cold paraformaldehyde (4% in phosphate buffered saline (PBS) buffer). The following day, slices were placed for at least 2 days in a sucrose solution (18% in PBS) and finally conserved in anti-freezing solution at −20 °C until the usage for immunohistochemistry. Immunohistochemistry was performed with the free floating method as previously reported (Lana et al., 2022; Landucci et al., 2022b; Landucci et al., 2022a).

First day: hippocampal slices were placed in a multiwell and incubated for 60 min with Blocking Buffer (BB) containing 10% Normal Goat Serum. The slices were then incubated overnight at 4 °C under slight agitation with a mouse anti-NeuN to immunostain neurons (1:400 in BB; Product Code #MAB377, Millipore, Billerica, MA, U.S.A.) and a rabbit anti-IBA1 to immunostain total microglia (1:300; Product Code #016–20,001, WAKO, Osaka, Japan).

Second day: slices were incubated for 2 h at room temperature in the dark with AlexaFluor 555 donkey anti-mouse (1:400 in BB; Product Code #A31570, Thermo Fisher Scientific, Waltham, MA, USA). Slices were then incubated for 2 h at room temperature in the dark with AlexaFluor 555 donkey anti mouse plus AlexaFluor 488 donkey anti-rabbit (1:400 in BB; Product Code #A21206, ThermoFisher Scientific). The slices were mounted onto gelatin-coated slides using Vectashield mounting medium with DAPI (Product Code #H-1200, Vectashield, Burlingame, CA, U.S.A.).

In the protocol for CytC and NeuN immunostaining, at the end of the first day the primary antibody used was a mouse anti-CytC (1:200 in BB; Product Code #556432, Becton and Dickinson, Franklin Lakes, NJ, United States). During the second day slices were incubated for 2 h at room temperature in the dark with AlexaFluor 555 donkey anti-mouse and then for 2 h at room temperature in the dark with a mouse anti-NeuN antibody conjugated with the fluorochrome AlexaFluor 488 (1:100 in BB; Product Code #MAB377X, Millipore). Slices were observed under a LEICA TCS SP5 confocal laser scanning microscope (Leica Microsystems CMS GmbH, Mannheim, Germany) equipped with 20×, 40× and 63× objective (z step of 1.2 μm, 0.6 μm or 0.3 μm respectively). Confocal scans were acquired keeping all parameters constant.

2.5. Quantitative analyses for immunohistochemical experiments

Image analyses were performed with ImageJ software (National Institute of Health, http://rsb.info.nih.gov/ij) on z-stacks projections of the region of interest, corresponding to area CA3.

We quantified the number of NeuN positive neurons with Shrunk Cytoplasm (SC neurons), defined as neurons with an elongated cytoplasm, in CA3 Stratum Pyramidalis (SP). In comparison to healthy CA3 neurons that have similar axes in the two directions (8–10 μm), SC neurons show an elliptical shape (long axis 15–20 μm, short axis 4–6 μm) with the long axis disposed perpendicularly to CA3 SP (see examples of SC morphology in Fig. 3). In addition, we evaluated the thickness of the CA3 Stratum Pyramidalis (SP) averaging 3 measurements evenly distributed throughout SP. On the same z-projection obtained from NeuN-IBA1 immunostaining, we evaluated both total and phagocytic microglia density in CA3 SP. A microglia cell was considered phagocytic if IBA1 was colocalized with NeuN immunostaining. All cell counts were expressed as density (cells/mm^2).

2.6. Isolation of microglia cells

At the end of 24 h of the treatment with KA in presence or absence of cannabinoids (1 μM THC or 10 μM CBD) (eight slices for sample), rat organotypic hippocampal slices were gently transferred to 3 mL of ice-cold PBS in C-Tubes (Miltenyi Biotec®, Bergisch Gladbach, Germany) and were triturate and homogenized using a gentleMACS Octo Dissociator (Miltenyi Biotec®) with an appropriate Heaters run program. After homogenization, the samples were filtered with pre-separation filters (MACS SmartStrainers 70 μM, Miltenyi Biotec®) to remove cell aggregates or large particles. The cells were subsequently centrifuged for 5 min at 1200 rpm and the pellet was dissolved in 150 μL of AutoMACS Buffer (Miltenyi Biotec®) according to the manufacturer's instructions.

2.7. Flow cytometry and morphology analysis

Cells isolated from rat organotypic hippocampal slices were incubated and stained with various appropriately diluted combinations of the following fluorochrome-conjugated antibodies: anti-CD68 PE-Vio 770 (130–103-365)(Van Velzen et al., 1997), anti-CD32 APC-Vio 770 (130–103-391)(Hubert et al., 2004), anti-CD11b/c APC (130–120-288) (Tamatani et al., 1991), anti-CD86 Vio Bright FITC (130–109-180) (Damoiseaux et al., 1998), anti-CD45 Vio Blue (130–125-229)(Mojcik et al., 1987), according to the manufacturer's instructions (all from Miltenyi Biotec®, Bergisch Gladbach, Germany). The stained cells were acquired using a MACSQuant Analyzer 10 Flow Cytometer (Miltenyi Biotec®), and the data were processed using Flowlogic (Miltenyi Biotec®).

2.8. Statistical analysis

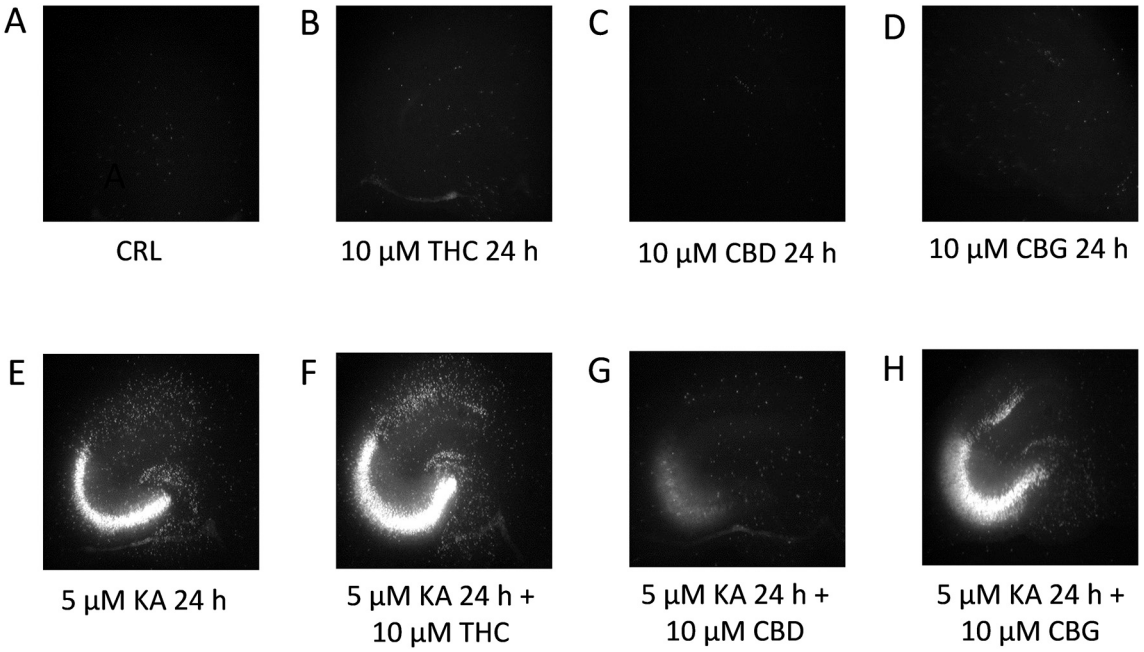
Data are presented as means ± SEM. The statistical significance of differences between PI fluorescence intensities and flow cytometry data were analysed using one-way ANOVA with a post hoc Dunnett and Tukey's *w*-test for multiple comparisons. Immunohistochemistry data were statistically analysed by one-way analysis of variance (ANOVA) followed by Newman-Keuls multiple comparison test. 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.

3. Results

3.1. Effects of cannabinoids on kainate neurotoxicity in organotypic hippocampal slices

In our previous studies we had observed that increasing concentrations and different times of exposure to THC and CBD did not induce damage in the CA1 regions of organotypic hippocampal slices, as evaluated by PI fluorescence (Landucci et al., 2022a; Landucci et al., 2021b). In this study we observed that the same drugs at the same concentrations did not induced damage in the CA3 area of slices (Fig. 1B, C, D and I). When the slices were incubated for 24 h with 5 μM KA, we observed a selective injury in the SP of CA3 area (Fig. 1B) (Landucci et al., 2021a).

Treatment with THC during the 24 h of kainate exposure significantly increased neuronal death in a dose-dependent manner in CA3 at the concentrations of 1 and 10 μM (Fig. 1F and J). On the contrary, cannabidiol reduced the neuronal damage and the effect was significant at the concentration of 10 μM (Fig. 1G and J). CBG had no effects at all the doses used (0.1, 1 and 10 μM) (Fig. 1H and J). In an in vitro model of



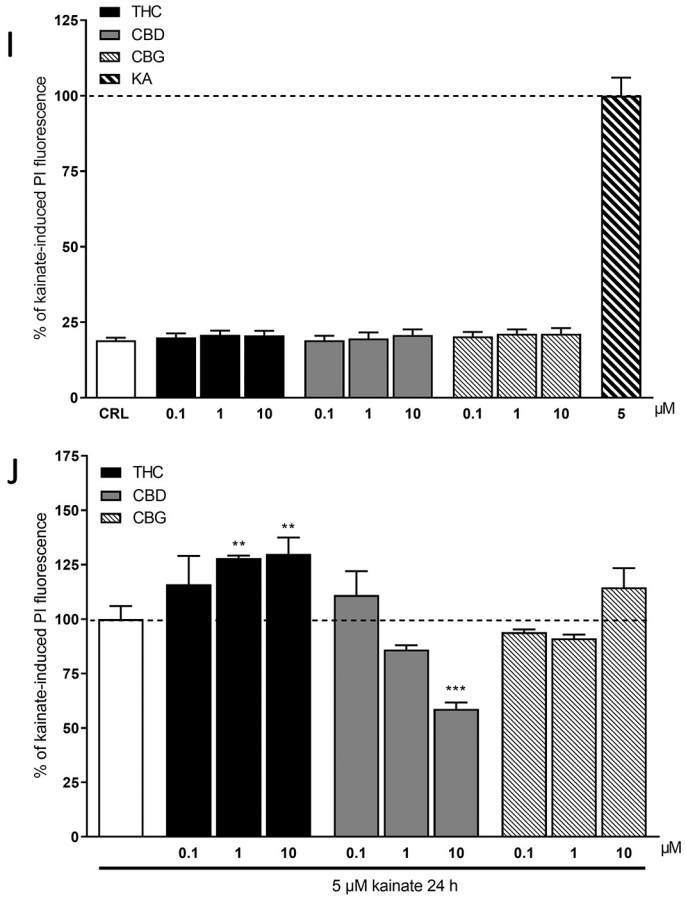


Fig. 1. Qualitative and quantitative analysis of the effects of selected cannabinoids in rat organotypic hippocampal slices in normal conditions or exposed to KA. (A): Hippocampal slice under normal conditions (background PI fluorescence), (B): slice exposed to 10 µM THC for 24 h, (C): slice exposed to 10 µM CBD for 24 h, (D): slice exposed to 10 µM CBG for 24 h, (E): slice exposed to 5 µM KA for 24 h displaying intense PI labeling in the CA3 subregion, (F): CA3 damage induced by kainate was exacerbated by the presence of 10 µM THC (G): CA3 damage induced by kainate was attenuated by the presence of 10 µM CBD (H): CA3 damage induced by kainate was not modified by the presence of 10 µM CBG. (I): cannabinoids alone didn't induce side effects. (J): THC, the psychoactive constituent of *Cannabis*, significantly exacerbated the neurotoxic effects induced by kainate at the concentration of 1 and 10 µM. CBD significantly attenuated CA3 injury with the maximal effect at 10 µM, and CBG had no effect. Bars represent the mean ± SEM of at least 5 experiments run in quadruplicate. ** $p < 0.01$ and *** $p < 0.001$ vs. kainate (one-way ANOVA plus Dunnett's test).

ischemia we previously observed a similar effect for THC and CBD, which depends on the activity of the compounds on different cannabinoid receptors (Landucci et al., 2021b). On the basis of these data, we used selective antagonists to determine whether the effects of THC and CBD were mediated by cannabinoid receptor type 1 (CB1), type 2 (CB2), Transient receptor potential cation channel subfamily V member 1 (TRPV1), TRPV2, 5-HT1A or PPARγ receptor (Fig. 2A and B).

Only the submaximal non active concentration of AM251 (10 nM), a CB1 antagonist, added to the incubation medium together with THC and kainate, prevented the toxic effects of 1 μM THC, but not by any of the other antagonists that were investigated (Fig. 2A). Furthermore, submaximal non active concentrations of the TRPV1 antagonist capsazepine (1 μM), or of the PPARγ antagonist G3335 (0.1 μM) prevented the CBD effect only in part. The TRPV2 antagonist TNL (50 μM) and the 5-HT1A antagonist (0.1 μM) significantly reverted the neuroprotective effect of CBD (Fig. 2B), but not the CB1 antagonist AM251(10 nM), the CB2 antagonist AM630 (1 μM).

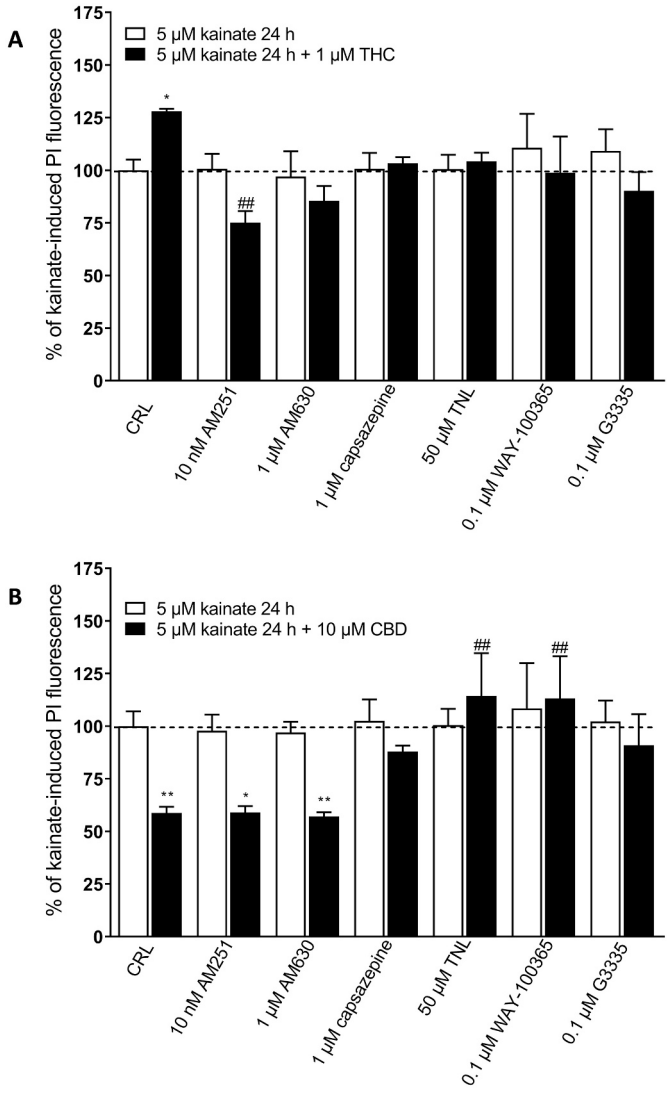


Fig. 2. The effects of THC and CBD on kainate toxicity in rat organotypic hippocampal slices depend on different receptors. (A): The neurotoxic effects of 1 μM THC on CA3 were completely prevented only by co-incubation with the CB1 receptor antagonists AM251. (B): On the other hand, the neuroprotective effect of 10 μM of CBD was completely prevented by co-incubation with the TRPV2 antagonist tranilast (TNL), or 5-HT1A antagonist WAY-100635. Partially by TRPV1 antagonist capsazepine and PPARγ antagonist G3335, but not by the CB1 receptor antagonists AM251, the CB2 antagonist AM630. Bars represent the mean ± SEM of at least 5 experiments run in quadruplicate. * $p < 0.05$, ** $p < 0.01$ vs. kainate; ## < 0.01vs. kainate + THC for (A); ## < 0.01vs. kainate + CBD for (B) (one-way ANOVA plus Tukey's w-test).

3.2. Immunohistochemical assessment of neuronal damage induced by kainate in CA3 region of organotypic hippocampal slices

In order to investigate the effect of cannabinoids alone on neuronal viability we incubated the slices for 24 h with CBD (10 μM) or THC (1 μM). Quantitative analyses of density of SC neurons in CA3 hippocampus demonstrated no significative differences both in THC alone treated slices (about +13% vs CRL) and in CBD alone treated slices (about −1% vs CRL). We assessed the effect of cannabinoids on neuronal viability in CA3 region using immunostaining for NeuN and IBA1 on the organotypic hippocampal slices exposed to 5 μM kainate for 24 h by immunofluorescence and confocal microscopy. The qualitative analyses of CA3 SP cytoarchitecture obtained with confocal microscopy images (Fig. 3A-D), showed that CA3 SP appeared remarkably thinner in KA and KA + THC (1 μM) slices in comparison to control slices.

However, images showed that in KA + CBD slices the thickness of CA3 was comparable to control slices. The measure of SP thickness (Fig. 3E) demonstrated that the CA3 of KA and KA + THC slices was significantly thinner than that of CRL slices, and the treatment with CBD (10 μM) significantly reverted this effect (Fig. 3E, One-way ANOVA followed by Newman-Keuls multiple comparison test, **P < 0.01 KA vs CRL, *P < 0.05 KA + THC vs CRL, #P < 0.05 KA + CBD vs KA).

Furthermore, in KA and KA + THC slices the morphology of neurons appeared distorted. Indeed, numerous neurons of CA3 SP had a shrunk cytoplasm (Fig. 3 B1, SC neurons, open arrows) in comparison to SP neurons from CRL slices, while in KA + CBD slices (Fig. 3 D1), the morphology of neurons was more similar to those of CRL (Fig. 3 A1-D1, enlargements of the framed areas in Fig. 3A-D). Quantitative analysis demonstrated a significant increase of the density of SC neurons in KA and KA + THC slices compared to CRL slices, while treatment with CBD reverted, significantly although not completely, this effect (Fig. 1F, One-way ANOVA followed by Newman-Keuls multiple comparison test, ***P < 0.001 KA vs CRL, ***P < 0.001 KA + THC vs CRL, ##P < 0.01 KA + CBD vs KA). To further investigate the kainate-induced damage in CA3 SP, we performed double labelling fluorescent immunohistochemistry for NeuN and CytC. In KA-treated slices, many SC neurons in SP displayed a diffuse cytoplasmic staining for CytC, a typical sign of the late phase of apoptosis (see 20× and 63× confocal microscopy images in Fig. 1 G-G1 and I-I2 respectively, open arrows). Healthy neurons in CRL slices showed no staining for CytC in the cytoplasm (Fig. 3H).

Quantitative analyses showed that CytC positive cells increased significantly by +363% in KA-treated slices and by +364% in KA + THC-treated slices. In KA + CBD slices, CytC positive cells had increased non significantly by about +20% vs CRL, and their density was significantly lower than in KA-treated slices (−47% vs KA) (One-way ANOVA followed by Newman-Keuls multiple comparison test, *P < 0.05 KA vs CRL, **P < 0.001 KA + THC vs CRL, n.s. KA + CBD vs CRL, #P < 0.05 vs KA). These data show that CBD protected CA3 SP neurons from apoptosis.

3.3. Quantitative and morphological modifications of microglia in CA3 region of organotypic hippocampal slices exposed to kainate

To evaluate the quantitative and morphological alterations of microglia on in CA3 of organotypic hippocampal slices exposed to KA in presence or absence of either CBD (10 μM) or THC (1 μM), we performed double fluorescence immunohistochemistry for NeuN and IBA1 (Fig. 4A-D) followed by confocal microscopy.

The qualitative analysis showed remarkable morphological alterations of microglia in KA and KA + THC slices compared to CRL slices, while in KA + CBD slices the morphology of microglia was comparable to CRL slices (IBA1 staining). Fig. 4 A2-D2 show more closely the alterations of microglia morphology in CA3 SP. In KA and KA + THC

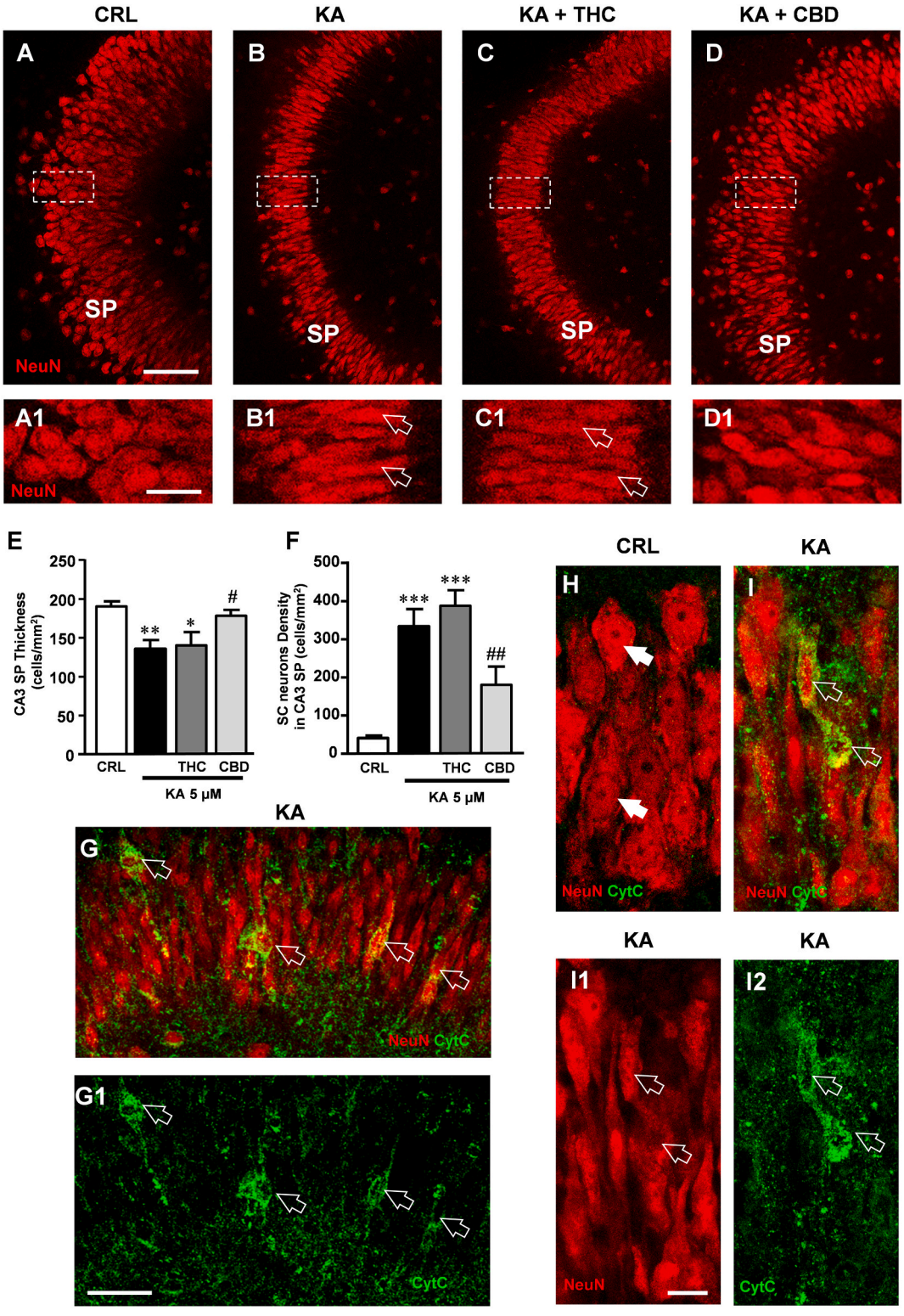


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Fig. 3. (A-D): Immunohistochemical assessment of neuronal damage in CA3 hippocampal area. (A): Representative confocal images of fluorescent immunostaining of NeuN positive neurons in area CA3 of CRL, (B): KA, (C): KA + THC, and (D): KA + CBD slices captured with a 20× objective. Scale bar: 100 μm. (A1-D1): Magnifications of the framed areas of the corresponding slice shown in A, B, C, D respectively. (A1): Image taken from the CRL slice showing healthy pyramidal neurons. (B1-C1): Images taken from the KA and KA + THC slice showing the presence of numerous neurons with elongated, shrunk cytoplasm (SC neurons, empty arrows). (D1): Image taken from the KA + CBD slice showing that the morphological alterations of neurons are less evident. Scale bar: 30 μm. (E): Quantitative analysis of CA3 SP thickness (CRL $n = 7$, KA n = 7, KA + THC $n = 6$, KA + CBD n = 7). Statistical analysis: One-way ANOVA $P < 0.01$; Newman-Keuls post hoc test $^{*}P < 0.05$ and $^{**}P < 0.01$ vs CRL, #P < 0.05 vs KA. (F): Quantitative analysis of the density of SC neurons in CA3 SP (CRL n = 7, KA $n = 6$, KA + THC n = 6, KA + CBD n = 7). Statistical analysis: One-way ANOVA $P < 0.0001$; Newman-Keuls post hoc test $^{***}P < 0.001$ vs CRL, ##P < 0.01 vs KA. Bars represent the mean ± SEM of n experiments. (G-G1): Representative confocal images of fluorescent immunostaining of neurons (NeuN, red) and Cytochrome C (CytC, green) in CA3 SP of a KA slice, captured with a 20× objective. Numerous SC neurons in CA3 SP showed diffuse immunostaining for CytC in the cytoplasm, typical of late phase apoptosis (empty arrows). Scale bar: 40 μm. (H, I-I2): Representative confocal images of immunostaining of neurons (NeuN, red) and Cytochrome C (CytC, green) in CA3 SP of (H): a CRL and (I-I2): a KA slice, captured with a 63× objective. In the CRL slice neurons are healthy, with a round cell body (white arrows, panel H). In the KA slice many SC neurons have diffuse cytoplasmatic CytC immunostaining, sign of apoptosis (empty arrows, panels I-I2). Scale bar: 10 μm. Doses of compounds: KA 5 μM, CBD 10 μM, and THC 1 μM in all experiments shown in the figure.

slices, the cytoplasmic projections of microglia were thicker and less numerous than in CRL slices and the cell body was larger and amoeboid, all patterns typical of microglia in reactive/activated state. In KA + CBD slices the morphology of microglia was comparable to that of CRL slices. Numerous phagocytic microglia cells were present in CA3 SP of KA and KA + THC slices, compared to CRL slices. Fig. 4 A3-D3 (NeuN+IBA1 staining) and Fig. 4 A4-D4 (NeuN staining) show that in KA and KA + THC slices several microglial cells had an amoeboid shape (Fig. 4 B3-C4, empty arrows) and were phagocytosing neurons of CA3 SP (Fig. 4 B4-C4, arrows). Treatment with CBD appeared to decrease this effect. The quantitative analysis of total microglia and phagocytic microglia in CA3 SP (Fig. 4E-F) demonstrated that although the density of total microglia did not change among the 4 experimental groups (Fig. 4E, One-way ANOVA, P > 0.05, not significant), the density of phagocytic microglia in CA3 SP was significantly higher in KA and KA + THC slices compared to CRL slices. Interestingly, the treatment with CBD significantly reverted this effect (Fig. 4F, One-way ANOVA followed by Newmann-Keuls multiple comparison test, ***P < 0.001 KA vs CRL and KA + THC vs CRL, #P < 0.05 KA + CBD vs KA).

3.4. CBD prevented the kainate-induced increase of M1 activated microglia

To further understand the involvement of microglia in the kainate epilepsy model, we defined the microglia phenotype using cyto-fluorimetric analysis. Rat organotypic hippocampal slices exposed to 5 μM kainate for 24 h showed a significant increase of M1 phenotype and a reduction of M2 compared to controls (Fig. 5D and E). Cells were first gated on the basis of their scatter properties, forward (FSC) and side scatter (SSC) show size and granularity of the cells, respectively. In the dot plots shown in Fig. 5A, cells were first gated for live cells on the basis of 7ADD expression. Live cells were gated for CD45 negative population, to exclude blood cells, CD11b and CD68 were used to identify the positive population of general microglia indicated as 7AAD+/CD45-/CD11b+/CD68+. This population were gated for CD86 expression to indicate M1 sub population and to identify M2 sub population cells were gated for CD32 expression.

Quantitative data shown in Fig. 5D revealed that co-incubation of the slices with 1 μM THC increased the percent of M1 activated microglia induced by kainate. Interestingly, 10 μM CBD significantly block the amount of M1 activated microglia induced by kainate, demonstrating a consistent prevention of the inflammatory phenotype induced by kainate.

4. Discussion

In the last years the interest toward medical *Cannabis* and cannabinoids for the treatment of acute neurodegenerative disorders has increased, particularly after the approval of cannabidiol (Epidiolex, Epydiolex) by the US Food and Drug Administration (FDA) in June 2018 and by the European Medicines Agency (EMA) in September 2019 as new therapies for Dravet syndrome and Lennox Gastaut. In the present study, we examined for the first time the effects of selected cannabinoids on rat organotypic hippocampal slices exposed to 5 μM kainate for 24 h (Landucci et al., 2021a), an *in- vitro* seizure model widely used in our laboratory as reported in material and methods paragraph. Our data showed that CBD attenuated the neuronal damage induced by kainate in CA3 SP. The neuroprotective effect of CBD was blocked by TRPV2 and 5-HT1A antagonists. On the contrary, THC exacerbated kainate-induced neurotoxicity, and this effect was prevented by CB1 receptor antagonists. In addition, CBD significantly decreased activation of microglia, in particular it can block the transition from the M0 to the M1 phenotype induced by KA, and the phagocytosis of damaged pyramidal neurons observed after exposure to KA in this in-vitro seizure model. Despite all the limitations of this in vitro model, our data point to the development of future CBD derived drugs for the treatment of seizures.

The role of cannabinoids in epilepsy is currently under investigation. CBD shows a better anticonvulsant profile, and it is largely devoid of the adverse psychoactive and addictive effects characteristic of THC (Perucca, 2017). CBD is well tolerated and the most common side effects were hyporexia, diarrhea, drowsiness and fatigue (Madan Cohen et al., 2021; Miller et al., 2020).THC and other CB1R agonists such as anandamide (AEA) and WIN55,212 have anticonvulsant properties, but they produce also undesirable side effects (e.g. intoxication) and can be proconvulsant at high doses (Citraro et al., 2013; Perescis et al., 2020; Rosenberg et al., 2017). Addition of low dose of Delta(9)-tetrahydrocannabinol to the culture medium attenuated the toxicity produced by high dose of kainate in cultured mouse spinal neurons (Abood et al., 2001). THC administered i.p. dramatically increases the presence and duration of spike-and-wave discharges (SWDs) in Genetic Absence Epilepsy Rats from Strasbourg (GAERS) (Roebuck et al., 2022). In our experiments, we observed that THC worsens the KA effect, whereas CBD resulted neuroprotective. Further experiments are needed to assess the worsens effect of THC on our model. The neuroprotective effects and safety of CBD on animal models of epilepsy and patient studies is well known (Lattanzi et al., 2018; Samanta, 2019). In our in-vitro seizure model, CBD exhibited a protective effect in a dose-dependent manner. In addition, we observed that CBG had no effects on the toxicity induced by kainate, data in accordance with Hill and colleagues who observed that both CBD and CBG are NaV channel blockers, but only CBD is efficacious against PTZ-induced seizures in the rat (Hill et al., 2014). CBD showed anticonvulsant activity by attenuating hyperactivity, clonic convulsions, and electroencephalogram rhythmic oscillations induced by kainate (Friedman and Wongvravit, 2018). In confirmation to what previously observed in a model of ischemia (Landucci et al., 2021b), in this in-vitro seizure model the effect of THC largely depended on the interaction with the CB1 receptor, while the pharmacological effect of CBD was more complex, probably for the ability of its versatile scaffold to interact with different molecular targets that are not restricted to the endocannabinoid system (Vitale et al., 2021). All these targets are involved in epilepsy; Bhaskaran and Smith demonstrated that TRPV1 expression was significantly higher in

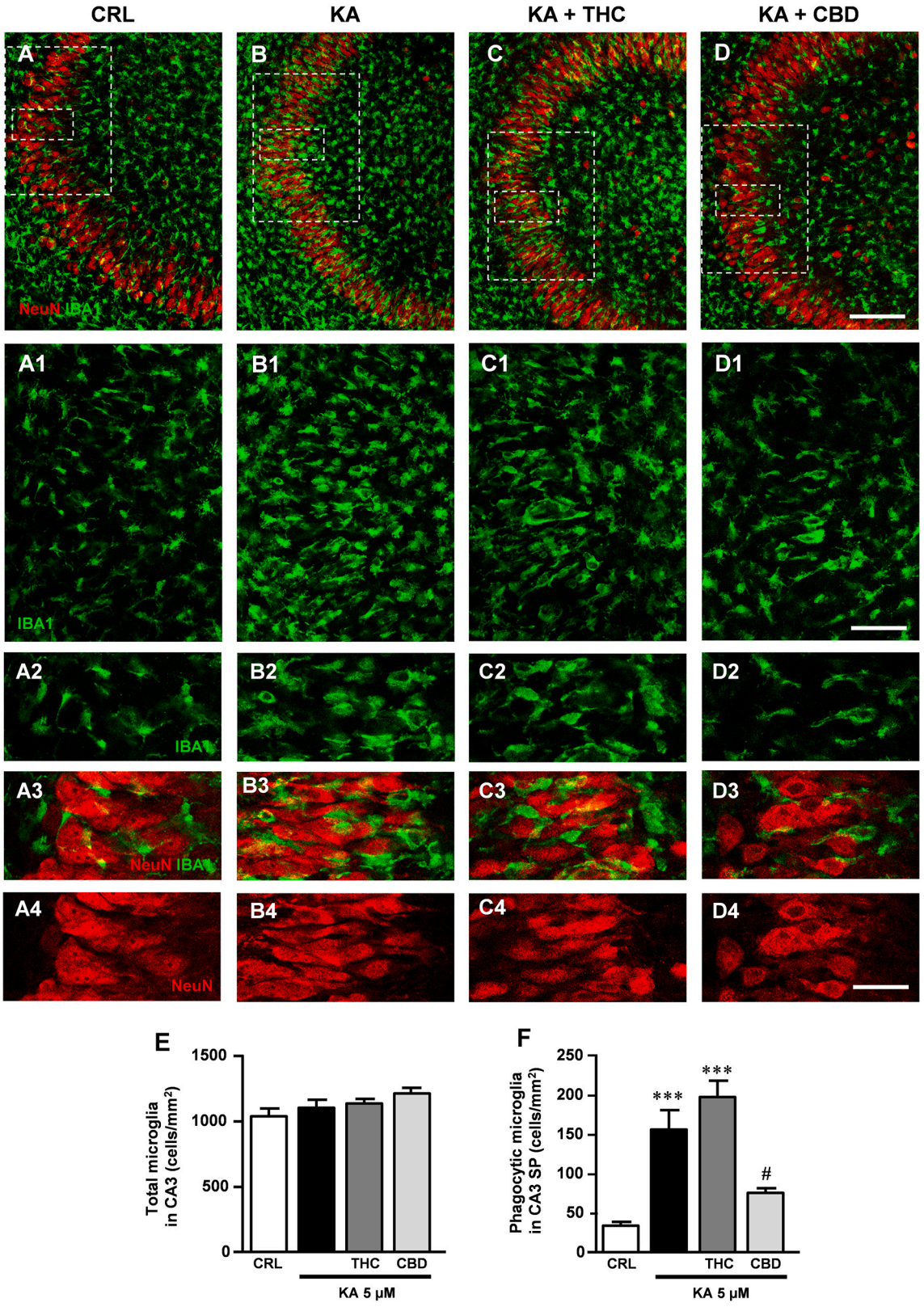

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Fig. 4. Evaluation of microglia quantitative and morphological modifications in CA3 hippocampal region of organotypic hippocampal slices. (A-D): Representative confocal images of fluorescent immunostaining of neurons (NeuN, red) and total microglia (IBA1, green) in CA3 of (A): CRL, (B): KA, (C): KA + THC, and (D): KA + CBD slices captured with a 20× objective. Scale bar: 80 μm. (A1-D1): Confocal acquisitions of the larger framed areas in A, B, C, D respectively (40× objective). (A1): In CRL slice microglia is predominantly in a resting condition. (B1): In KA and (C1): KA + THC slices microglia is reactive with amoeboid morphology, larger body and shorter and thicker branches. (D1): In KA + CBD slice microglia activation is less evident. Scale bar: 30 μm. (A2-D4): Confocal microscopy acquisition with a 63× objective of the smaller framed areas in A, B, C, D, respectively (. (A2-A4): In CRL slice microglia are in resting, non-phagocytic state. (B2-B4): In KA and (C2-C4): KA + THC slices, many microglia become reactive/amoeboid, and phagocytose pyramidal neurons (white arrows in B3-D3 point to phagocytic events). (D2-D4): CBD (KA + CBD slice), reverts this effect. Scale bar: 15 μm. (E): Quantitative analysis of microglia density in CA3 (CRL n = 7, KA *n* = 8, KA + THC n = 7, KA + CBD n = 7). Statistical analysis: One-way ANOVA $P > 0.05$ n.s. (F): Quantitative analysis of density of phagocytic microglia in CA3 SP (CRL n = 6, KA n = 6, KA + THC n = 6, KA + CBD n = 6). Statistical analysis: One-way ANOVA followed by Newman-Keuls post hoc test ***$P < 0.001$ vs CRL, #$P < 0.05$ vs KA. Bars represent the mean ± SEM of n experiments. Doses of compounds: KA 5 μM, CBD 10 μM, and THC 1 μM in all experiments shown in the figure. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the dentate gyrus of mice with Temporal lobe epilepsy (TLE) compared with control mice and also that anandamide, an endocannabinoid and endovanilloid endowed with an intrinsic agonist activity on TRPV1, enhanced glutamate release (Bhaskaran and Smith, 2010). TRPV2 has been directly associated with models of epileptic form activity and acute seizure (Günaydın et al., 2020). The 5-HT1AR density is associated with epilepsy duration in patients with TLE (Fonseca et al., 2017). The protective effects of Clock (circadian locomotor output cycles kaput) against KA-induced seizures was correlated with over expression of PPAR gamma (Wang et al., 2022). In our study we observed that the neuroprotective effects of CBD were prevented by pre-incubation with TRPV2 and 5-HT1A antagonists. PPARγ and TRPV1 antagonists reverted the CBD effect, but computational (Landucci et al., 2022b) and experimental evidences suggest that TRPV2 is among the preferential interactors of CBD and that this macromolecular partner can be directly involved in the observed neuroprotective effects.

In this in-vitro seizure model on organotypic hippocampal slices we observed for the first time that kainate induced remarkable alterations of CA3 cytoarchitecture, as demonstrated by the significant reduction of the thickness of SP and by the presence of many pyramidal neurons with altered morphological features, such as shrunk cytoplasm (SC neurons). We hypothesize that the decreased thickness of CA3 SP was caused by neuronal death. The increase of cytoplasmic CytC (Cerbai et al., 2012; Fusco et al., 2018; Lana et al., 2017b; Lana et al., 2017a; Lana et al., 2016; Lana et al., 2014; Suen et al., 2008; Ugolini et al., 2018) and other morphological characteristics of damaged neurons point toward apoptotic cell death, a phenomenon already described in a model of neurodegeneration caused by kainate (Dong et al., 2003) and previously observed by western blot in hippocampal slices (Landucci et al., 2021a). Indeed, cell shrinkage and volume decrease are typical signs of apoptotic cells (Bortner and Cidlowski, 2020). Furthermore, SC neurons have strong similarity with the morphology of apoptotic cells undergoing cellular disassembly mediated by microtubule spikes, a newly characterized process occurring during later stages of apoptosis (Atkin-Smith and Poon, 2017). Microtubule spikes induce cytoplasmic elongation that favours the formation and spread of the apoptotic bodies, and microtubule spikes give the cell an oblong shape that recalls SC neurons shape present in our slices. Indeed, we demonstrated that many SC neurons were undergoing apoptosis, since diffuse cytoplasmic immunostaining of CytC, a sign of late phase apoptosis (Cerbai et al., 2012; Fusco et al., 2018; Jiang and Wang, 2004; Kluck et al., 1997; Lana et al., 2017a, 2017b; Lana et al., 2016; Lana et al., 2014; Suen et al., 2008; Ugolini et al., 2018; Yang et al., 1997), was present in several CA3 SC pyramidal neurons. CBD significantly reduced the apoptotic/neurodegenerative effect caused by kainate, while THC did not ameliorate the kainate effect on neuronal degeneration and apoptosis. The literature is still inconsistent on the role of cannabinoids on apoptosis. Furthermore, recent studies found that CBD induces apoptosis in tumour cells via CB1, CB2 and the ion channel receptor TRPV1 (Morelli et al., 2014; Sreevalsan et al., 2011), while others found its apoptotic actions to be receptor independent (Shrivastava et al., 2011). On the other hand, in neurons and cardiomyocytes subjected to injury or stress CBD exerts a protective function through anti-apoptosis signal pathways (Ceprián et al., 2017; da Silva et al., 2018; Iuvone et al., 2004; Lin et al., 2020). The anti-apoptosis action in neurons and cardiomyocytes reduces the inflammatory response which may be mediated by the CB2 endocannabinoid system (Castillo et al., 2010; Mecha et al., 2012; Rajesh et al., 2010; Szaflarski and Bebin, 2014). Our data add new insight into the protective role of CBD on apoptotic-driven neurodegeneration caused by excitotoxicity such as in this in-vitro seizure model.

Studies that analysed the brains of patients with epilepsy and those from animal models of epilepsy have revealed that microglia are excessively activated, especially in the hippocampus, a brain region that exhibits major structural and functional changes in epilepsy (Andoh et al., 2020). Find-me and eat-me signals expressed and released by apoptotic neurons drive microglia toward their targets and bring them to the phagocytosis of dying neurons (Lana et al., 2021; Lana et al., 2019; Nosi et al., 2021). We did not find modifications in the number of microglia cells between the different treatments, while we observed that microglia were in reactive/activated state, with remarkable morphological alteration, round cell body, retracted branches and amoeboid shape. Furthermore, we found that in slices incubated with kainate many microglia cells phagocytosed damaged CA3 pyramidal neurons. The finding that in kainate-treated slices there was a strong increase of neuronal phagocytosis, supports our hypothesis that the alteration of CA3 cytoarchitecture, particularly the reduction of SP thickness, was due to neuronal death. Moreover, in support of these data, CBD, but not THC, prevented both neuronal degeneration, appearance of SC neurons and decreased CA3 thickness. It is well-known that under normal conditions, microglia not only perform their role in immune system but also react to threat of danger shifting among distinct phenotypes (Walker et al., 2014).

In neurological disorders, the states of microglia cells can shift from resting (M0) to the activated (M1) phenotype, able to release proinflammatory cytokines resulting in self-perpetuating injury to neuron (Dang et al., 2018), or to the neuroprotective (M2) phenotype, able to promote neuronal recovery (Hu et al., 2015). Consistent with this, in our study CBD decreased the M1 phenotype induced by KA and increased the M2 microglia phenotype, maybe promoting the transition from M1 to M2. CBD has the ability to restrain inflammatory responses mediated by activated microglia (Barata et al., 2019; Dos-Santos-Pereira et al., 2020; Martín-Moreno et al., 2011; Mecha et al., 2013), modulating many microglia genes involved in the regulation of stress responses and inflammation (Juknat et al., 2012). On the contrary, it has also been demonstrated (Zhao et al., 2018) that increased mTOR signaling causes morphofunctional changes in mouse microglia, including increased proliferation, amoeboid-like phenotype, and intense phagocytosis activity, without significant expression of pro-inflammatory cytokines. These noninflammatory changes lead to reduced synaptic density, microglia infiltration into hippocampal pyramidal layers, and mild neurodegeneration. Moreover, these mice develop severe early-onset spontaneous recurrent seizures (SRSs), revealing an epileptogenic mechanism independent of microglia inflammatory responses and suggesting that microglia can be an appropriate target for epilepsy prevention and or treatment.

On the base of reported data, it is possible to assert that CBD block

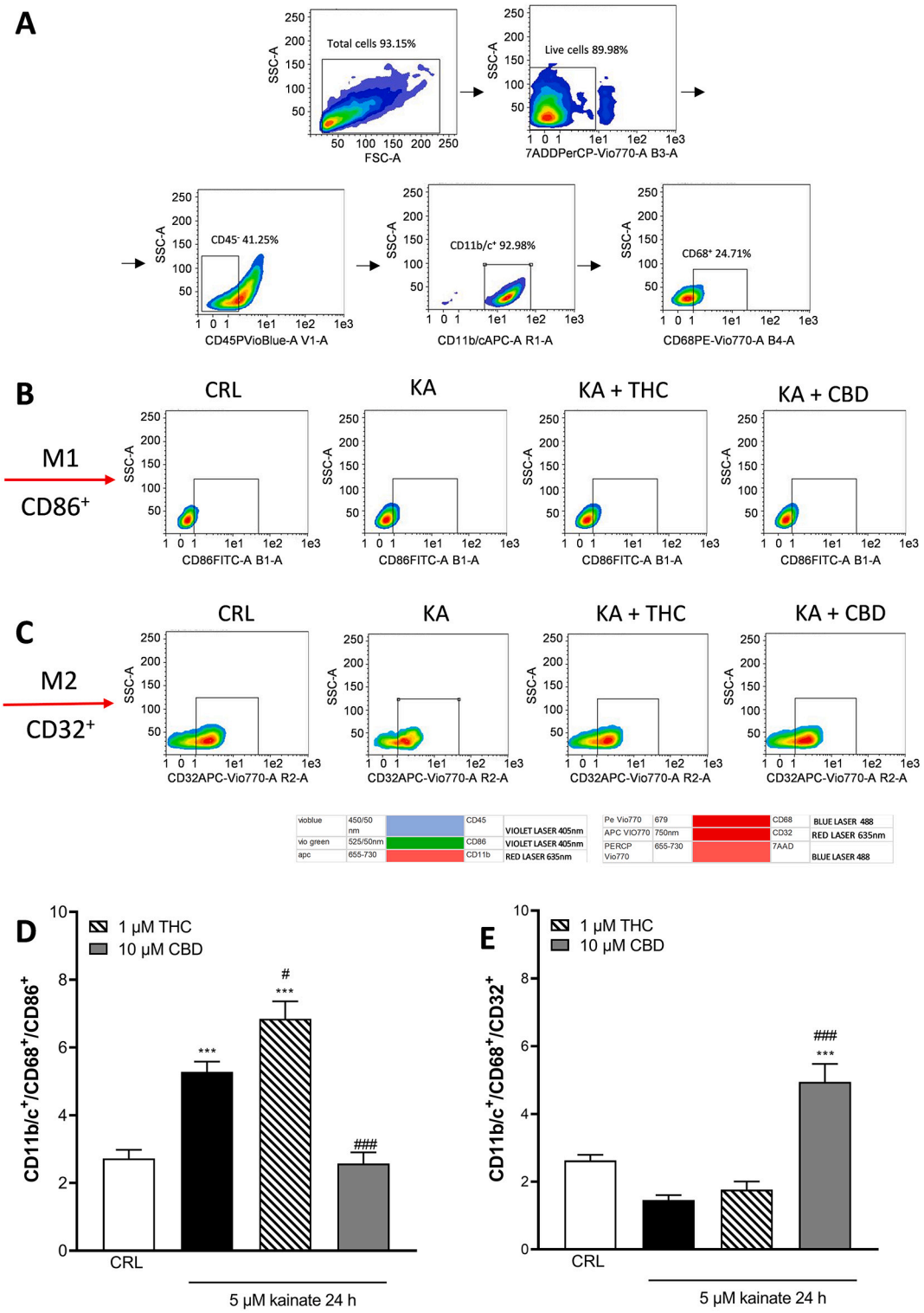

Fig. 5. Evaluation of M1 and M2 phenotype in CA3 hippocampal region of organotypic hippocampal slices by cytofluorimetric analysis. (A) Representative plot of viable microglia derived from hippocampal slices indicated as ADDP +/CD45-/CD11b+/CD68+ cells. (B) M1 phenotype indicated as ADDP +/CD45-/CD11b+/CD68+/CD86+ cells. (C) M2 phenotype indicated as ADDP +/CD45-/CD11b+/CD68+/CD32+ cells. (D) quantification of the percentage of M1 cells (E) quantification of M2 cells. Bars represent the mean ± SEM of at least 5 experiments. *** < 0.001 vs. CRL; # < 0.05vs. kainate; ### < 0.001vs. kainate (one-way ANOVA plus Tukey's w-test).

MO-M1 transition induced by kainate, pushing toward a M0/M2 transition, and the decrease of phagocytic microglia. Further experiments are needed to assess if proinflammatory or antiinflammatory cytokines are modified by CBD to drive the M1 to M2 transition and its neuroprotective effect in this in-vitro seizure model.

5. Conclusions

CBD has a safety profile and it is well tolerated in acute neurological diseases. Nevertheless, in vitro and in vivo preclinical studies are essential prior to new clinical trials on the use of CBD as neuroprotective

agent for different chronic pathologies. In this study, the neuroprotective effect of CBD was evaluated in an in vitro model of temporal lobe epilepsy. CBD was neuroprotective and its effect depended on TRPV2 and 5-HT1A receptors. Our research also demonstrated that the pattern of neurodegeneration found in CA3 of kainate treated slices was differently affected by CBD or THC treatment. CBD significantly reduced the kainate-induced increase of SC neurons and the phagocytic microglia in CA3 SP, while THC did not revert both kainate effect. Furthermore, CBD appeared to promote the morphofunctional transition from M0 to M2 microglia phenotype. These findings support the idea that CBD may became a valid and safe therapeutic intervention in the treatment of epilepsy.

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CRediT authorship contribution statement

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

Data availability

Data will be made available on request.

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