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Endogenous and synthetic cannabinoids induce the downregulation of cannabinoid CB1 receptor in retina

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ABSTRACT

Endogenous and synthetic cannabinoids have been shown to provide neuroprotection to retinal neurons in acute animal models of retinopathy. Chronic exposure to cannabinoid receptor (CB1R) agonists has been reported to induce downregulation of the CB1R in brain and behavioral tolerance. The aim of this study was to investigate the effect of subchronic/chronic cannabinoid administration on CB1R downregulation in normal rat retina, its downstream prosurvival signaling and subsequent effect on retinal neuroprotection against AMPA excitotoxicity. Sprague-Dawley rats were administered intraperitoneally with vehicle (Control), the endogenous N-arachidonoyl ethanolamine (AEA), and the synthetic cannabinoids R-(+)-Methanandamide (MethAEA) and HU-210 daily (25, 50, 100 μg/kg) for four or fourteen days (4d/14d, subchronic/chronic administration, respectively). HU-210 was also administered acutely as follows, vehicle injection for 13 days and a single dose of HU-210 on the 14th day. Immunohistochemistry studies and Western blot analysis were employed to assess CB1R expression in control and AMPA treated retinas and cannabinoid induced changes in Akt and ERK1/2 phosphorylation (ph). Real time PCR was employed to examine the effect of MethAEA (50 mg/kg,4d) on CB1R mRNA expression. AEA, MethAEA and HU-210 attenuated CB1R expression in a dose-dependent manner (25, 50, 100 μg/kg), after subchronic and chronic administration. No effect was observed at the lower dose of 25 μg/kg. MethAEA (50 mg/kg,4d) attenuated CB1R mRNA expression. AM251 (CB1 antagonist/inverse agonist, 0.5 mg/kg,4d), administered prior to HU-210 (50 μg/kg,4d) inhibited CB1R downregulation. Chronic/subchronic treatments (50 μg/kg) of HU-210 and MethAEA reduced levels of ph-Akt and ph-Akt/ph-ERK1/2, respectively. AEA had no effect on ph-Akt nor ph-ERK1/2. All three cannabinoids (50 μg/kg,4d) failed to protect brain nitric oxide synthetase (bNOS) expressing amacrine cells against AMPA excitotoxicity, in agreement with the downregulation of CB1 receptor. At the lower doses of 12.5 and 25 μg/kg, HU-210 protected bNOS-expressing amacrine cells. This study provides novel information regarding agonist-induced CB1R downregulation in rat retina after subchronic/chronic cannabinoid treatment, and its effect on downstream prosurvival signaling and neuroprotection.

1. Introduction

The endocannabinoid system (ECS) has been investigated as a new therapeutic target in the treatment of CNS neurodegenerative diseases (Aymerich et al., 2018; Di Marzo, 2018). ECS is comprised of the endogenous cannabinoids N-arachidonoylethanolamine (anandamide, AEA) (Devane et al., 1992) and 2-arachidonoylglycerol (2-AG) (Mechoulam et al., 1995; Sugiura et al., 1995), the cannabinoid receptors [CB1R (Matsuda et al., 1990) and CB2R (Munro et al., 1993)], as well as the enzymes responsible for the synthesis and metabolism of AEA and 2-AG (De Petrocellis et al., 2004).

The two most well studied endocannabinoids, AEA and 2-AG, are found in brain, but also in mammalian and human retina (Bisogno et al., 1999; Straiker et al., 1999; Chen et al., 2005). AEA and 2-AG levels were found to vary in different ocular tissues, in normal human eyes and eyes from glaucomatous, diabetic or age related macular degeneration (AMD) patients. These findings suggested that endocannabinoids may play differential roles in eye function in different ocular diseases (Chen et al., 2005; Matias et al., 2006). ECS is functional in the retina (Nucci et al., 2007) and has become an important therapeutic target for the treatment of ocular disease (Yazulla, 2008; Cairns et al., 2016; Kokona et al., 2016). Cannabinoids have been shown to be efficacious neuroprotectants in various models of retinopathy. Δ^9-Tetrahydrocannabinol (THC) and cannabidiol protected retinal ganglion

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Abbreviations

AMPA	(RS)-a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid hydrobromide
AEA	anandamide, N-arachidonoyl ethanolamine
2-AG	2-arachidonoyl glycerol
bNOS	brain nitric oxide synthetase
CB1R	cannabinoid receptor type-1
ChAT	choline acetyl-transferase
ECS	endocannabinoid system
ERK	extracellular signal-regulated kinases
FAAH	fatty acid amide hydrolase
GCL	ganglion cell layer
INL	inner nuclear layer
IPL	inner plexiform layer
IR	immunoreactivity
MethAEA	methanandamide, R-(+)-methanandamide
PI3K	phosphoinositide 3-kinase
SDS-PAGE	sodium dodecyl Sulphate - polyacrylamide gel electrophoresis
THC	Δ^9-Tetrahydrocannabinol

cells against NMDA excitotoxicity (El-Remessy et al., 2003), the synthetic cannabinoids R-(+)-methanandamide (MethAEA) (Nucci et al., 2007) and *R*-(+)-WIN55,212-2 (Pinar-Sueiro et al., 2013) protected ganglion cells against ischemia-reperfusion. We have previously reported that AEA, MethAEA and HU-210 protected amacrine and horizontal cells against AMPA excitotoxicity (Kokona and Thermos, 2015). In all of the above paradigms, the neuroprotection of retinal neurons was mediated via the activation of the CB1R. These reports suggest that CB1R activation by acute administration of endo- and synthetic cannabinoids is efficacious in protecting the retina from toxic insults, such as excitotoxicity and oxidative stress that lead to cell death.

However, in different retinal disease models, CB1R activation was shown to be implicated in oxidative stress and cell death (Rajesh et al., 2010). These findings have suggested that inhibition of the CB1R may provide neuroprotection in different paradigms. In STZ induced diabetic mice, inhibition of CB1R was shown to reduce albuminuria and be beneficial in diabetic nephropathy (Barutta et al., 2010, 2017). In the ocular system, inhibition of CB1R protected retinal pigment epithelial cells from oxidative injury and cell death (Wei et al., 2013). Most recently, it was shown that CB1R inhibition by SR141716A reversed photoreceptor loss, glial activation and bipolar cell dendrite shrinkage in the N-methyl-N-nitrosourea mouse model of photoreceptor and bipolar cell degeneration (Chen et al., 2018).

As mentioned previously, CB1R agonists protected retinal neurons in acute models of excitotoxicity and ischemia-reperfusion. However, as early as the late seventies, tolerance development was observed after repeated administration of cannabinoids (CB1R agonists) in humans (Jones et al., 1976, 1981; Hollister, 1986). Many animal studies focused on the effect of repeated administration of Δ^9 -THC and other cannabinoids on the desensitization, downregulation of the CB1 receptor, and subsequent effects on G proteins/cAMP signaling cascade in order to ascertain the molecular mechanisms underlying the behavioral tolerance and cross-tolerance induced by different cannabinoids (Pertwee et al., 1993; Romero et al., 1995, 1999; Fan et al., 1996; Sim et al., 1996; Rubino et al., 1998; Breivogel et al., 1999; Sim-Selley and Martin, 2002; Dalton et al., 2009). Tolerance to cannabinoid-mediated behaviors varied with the nature of the cannabinoid examined, and was the result of a region specific downregulation and/or desensitization of the CB1R.

To the best of our knowledge, there are no reports in the literature to date suggesting the downregulation of the CB1R in the retina, as a result of chronic treatment of synthetic or endocannabinoids. In this study, we addressed this issue and investigated the ability of HU-210, AEA and MethAEA to induce downregulation of the CB1R in rat retina, when administered systemically (intraperitoneally, i.p.) to control animals under subchronic and chronic conditions. In addition, we examined the effect of these treatments on CB1R's downstream pro-survival signaling pathways and neuroprotection to the retina, using the in vivo model of AMPA excitotoxicity.

2. Materials & methods

2.1. Animals

Adult male and female Sprague-Dawley (200–300 g) rats were employed in all studies in accordance with the ARRIVE guidelines and in compliance with the EU Directive 2010/63/EU for animal experiments and with Greek National (Animal Act., P.D. 56/2013) legislation. Animals were housed 2 to 3 according to their sex and maintained on a 12 h light/dark cycle with free access to food and water. Euthanization was performed with diethyl ether inhalation. All protocols were approved by the Animal Care Committee assigned by the local Veterinarian Authorities (Project authorization: 109015/30-05-2017).

2.2. Drugs & treatment

The endogenous cannabinoid AEA (kindly offered by Prof. A. Makriyiannis), the synthetic cannabinoids MethAEA (Cayman, Michigan, USA) and HU-210 (Tocris, Bristol, UK) were dissolved in a vehicle solution of absolute ethanol/water for injection (EtOH/WFI). The rats were injected intraperitoneally (i.p.) with AEA, MethAEA or HU-210 (25, 50 or 100 μg/kg), daily for 4 days (4d, subchronic treatment) and for 14 days (14d, chronic treatment). In order to study the acute effects of HU-210, the rats were injected 13 days with vehicle and then received a single dose of HU-210 on the 14th day. Control rats were injected with vehicle (Dalton et al., 2009). AM251 (CB1 antagonist/inverse agonist) was administered (0.5 mg/kg, i.p, 4d) to Sprague-Dawley rats alone or prior to the administration of HU-210 (50 μg/kg, i.p, 4d) (Dalton et al., 2009).

2.3. AMPA-induced model of excitotoxicity and treatment

The AMPA excitotoxicity model was employed subsequent to the elucidation of the reduced expression of the CB1R after the subchronic treatment with HU-210, AEA and MethAEA. The neuroprotective properties of these cannabinoids against AMPA excitotoxicity were examined only after subchronic treatment, since both (subchronic/chronic) treatments induced CB1R downregulation, with no statistically significant differences between them.

Rat eyes received 5 μl PBS (50 mM PBS pH 7.4) or 5 μl AMPA (42 nmol per eye, diluted in 50 mM PBS; Tocris, Bristol, UK) intravitreally (Kiagiadaki and Thermos, 2008). Twenty four hours (24 h) after the intravitreal injections, rats were administered HU-210 (12.5, 25, 50 μg/kg, i.p.) or AEA (50 μg/kg, i.p.) or MethAEA (50 μg/kg, i.p.), daily for 4 days.

2.4. Immunohistochemical studies

2.4.1. Tissue preparation

Rats were euthanized and the eyes were removed to collect eyecups (posterior part of the eye) or retinas for immunohistochemical or Western blot studies, respectively, 24 h after the last intraperitoneal

injection. Eyecups were fixed, cryoprotected, frozen and sectioned according to Kokona and Thermos (2015). Vertical sections (10 mm width each) of the eyecups were spread into 4 gelatin covered slides (6 sections per slide).

2.4.2. Immunohistochemistry and colocalization studies

Immunohistochemical studies were performed to assess the CB1R immunoreactivity (CB1R-IR) and retinal cell loss/neuroprotection, as described in Kiagiadaki and Thermos (2008). Primary antibodies against the CB1R [rabbit polyclonal IgG, 1:300; Abcam (Cat.#:ab23703/Lot.#:GR241820-6 and Cat.#: ab23703/Lot.#:GR241820-19) Cambridge, UK] and the retinal amacrine cell marker, brain nitric oxide synthase (bNOS, rabbit polyclonal, 1:2000; Sigma, St. Louis, MO) were employed. CF543 goat anti-rabbit IgG (H+L) (1:1000; Biotium, Fremont, CA) secondary antibody was used for both CB1R-IR and bNOS-IR quantification and the slides were cover slipped with mounting medium with DAPI (4′,6-diamidino-2-phenylindole) (Biotium, Fremont, CA). Negative controls were obtained following the same protocol and omitting the primary antibody.

Colocalization studies were performed in order to identify the presence of CB1R in retinal ganglion cells, using antibodies against CB1R [rabbit polyclonal IgG, 1:750; Millipore (Cat.#: 209550/Lot.#: 2967819) Burlington, MA and a ganglion cell marker, β-tubulin (neuronal class III, mouse monoclonal IgG2a, 1:1000; Santa Cruz Biotechnology, Dallas, TX). Sections were incubated with the primary anti-CB1R antibody as above, followed by the incubation with the anti-rabbit secondary antibody and subsequently treated overnight with the anti-β-tubulin antibody, as above. Finally, the sections were incubated with the second secondary antibody, CF488A goat anti-mouse IgG (H+L) (1:400; Biotium, Fremont, CA, USA) and cover slipped with mounting medium.

2.5. Quantification studies

Light microscopy images were obtained using a fluorescence microscope Leica DMLB (HCX PL Fluotar, 40x/0.70 or 20x/0.50 lens; Leica Microsystems, Germany) with a Leica DC 300 F camera. Light adjustments (exposure time and gain) of immunofluorescence were set using the Leica software before acquiring images and remained fixed till completion.

CB1R-IR: two images were taken from 3 slices (2 images/slice) of each retina near the optic nerve head (central retina), containing the outer plexiform (OPL), inner nuclear (INL), inner plexiform (IPL) and ganglion cell (GCL) layers. Quantitative analysis of CB1R immunofluorescence was performed using unedited images with ImageJ 1.44 software. The GCL was delineated for quantification of control retinas. The area containing the INL, IPL and GCL was delineated for quantification of control and AMPA treated retinas in the AMPA excitotoxicity model (ImageJ 1.44 software). The mean gray value for the CB1R-IR was calculated in each image. The mean of six values (2 images/slice x 3 slices) was used for each retina (n = 1).

bNOS-IR: positive amacrine cells were manually counted along the entire retinal tissue, in the INL (amacrine cells) and GCL (displaced amacrine cells) in 3 slices of each retina. Quantification studies were carried out in a minimum of 3 retinas for each treatment.

Adobe Photoshop ver. 7.0 software (Adobe Systems, San Jose, CA) was used to a) crop the images for both CB1R-IR and bNOS+cells and b) adjust brightness and contrast in the images of bNOS-IR. bNOS-positive cells were counted manually prior to any adjustment.

2.6. Western blot studies

Retinas were homogenized and sonicated in lysis buffer, containing 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP40, 0.1% DOC, 0.1 mM PMSF and a protease and phosphatase inhibitor cocktail (Sigma, St. Louis, MO & Thermo Sci., Waltham, MA, respectively). Lysates were prepared for SDS-PAGE (12,5% of acrylamide) according to Ibán-Arias et al. (2018) and blotted onto a nitrocellulose membrane (Macherey-Nagel, Düren, Germany). Blots were incubated with specific antibodies against CB1R ([rabbit polyclonal, 1:200; Abcam (Cat.No:ab23703, Lot.#:GR241820-6), Cambridge, UK, or rabbit polyclonal, 1:1000; Millipore (Cat.#: 209550, Lot.#: 2967819), Burlington, MA), phospho-Akt (1:1000, rabbit monoclonal) and phosphor-ERK1/2 (1:1000, rabbit monoclonal). Blots were stripped and incubated with the antibody against total-Akt (1:1000, rabbit monoclonal) or total-ERK1/2 (1:1000), respectively. To normalize protein content in lysates, blots were stripped and incubated with an antibody against GAPDH (1:1000, rabbit monoclonal). All antibodies above, except those against CB1R, were purchased from Cell Signaling, Danvers, MA. Blots were subsequently incubated with the peroxidase-conjugated (HRP) secondary antibody HRP-goat anti-rabbit IgG (1:5000; Invitrogen, Waltham, MA) and proteins were visualized using LumiSensor Chemiluminescent HRP Substrate kit (Genscript, Piscataway, NJ). Quantification of the optical density of the bands was performed using ImageJ 1.44 software.

2.7. Real time PCR

Retinal samples obtained from rats treated with MethAEA (50 μg/kg, subchronic, i.p.) were employed for total RNA isolation using the NucleoSpin RNA kit (Macherey-Nagel, Duren, Germany) and cDNA

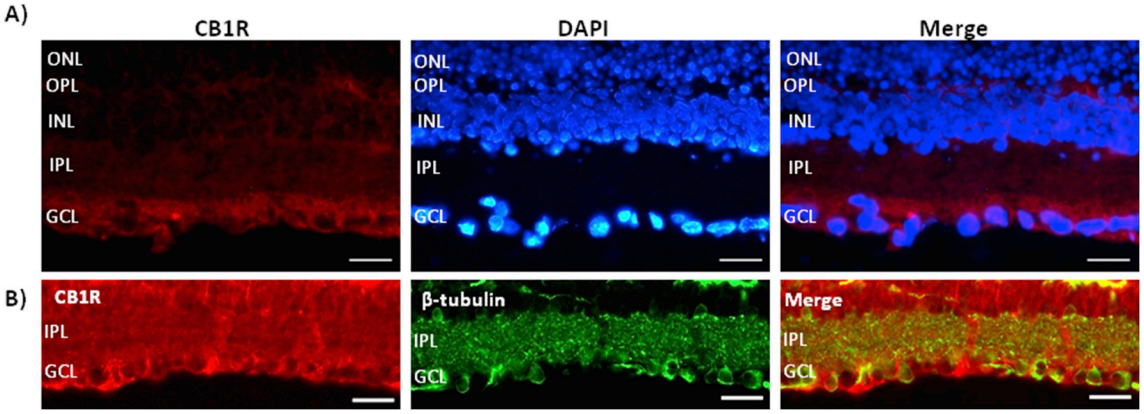

Fig. 1. Immunohistochemical studies showing the localization of the CB1R immunoreactivity (CB1R-IR) in adult rat retina **A.** Double stainining with DAPI and anti-CB1R showed that CB1R is expressed mostly in the GCL and less in the IPL, INL and OPL. x40 magnification, scale bar: 50 μm **B.** Colocalization studies of CB1R with β-tubulin III, a marker of ganglion cells. CB1 receptor is colocalized with β-tubulin immunoreactivity in ganglion cells, as well as in their processes in the IPL. x20 magnification, scale bar: 50 μm. OPL: outer plexiform layer, INL: inner nuclear layer, IPL: inner plexiform layer, GCL: ganglion cell layer.

synthesis was performed from total RNA using the PrimeScript 1st strand cDNA synthesis kit (Takara Bio Inc, Seta, Otsu, Shiga, Japan, Cat.No:6110), according to the instructions of the manufacturers. Real-time PCR analysis was performed using a StepOnePlus real-time PCR system (Applied Biosystems, Life Technologies, Thermo Fisher Scientific Inc.,Waltham, MA and expression levels of CB1 receptor mRNA were examined using the KAPA SYBR FAST qPCR Master Mix (2X) Universal kit (Kapa Biosystems, Wilmington, MA, Cat. No: KK4602) in a 15 μl reaction containing 7.5 μl KAPA SYBR FAST qPCR Master Mix (2X), 1.5 μl forward primer (10 pmol/μl), 1.5 μl reverse primer (10 pmol/μl), 0.3 μl ROX High Reference Dye (50X) and 4.2 μl template cDNA (20 ng). Real-time PCR conditions were as follows: 95°C for 20 s, followed by 40 cycles of 95°C for 3 s, 58°C for 30 s, 95°C for 15 s, and finally 60°C for 1 min and 95°C for 15 s. GAPDH was used as an internal control to which the relative expression of CB1 mRNA was normalized. The following primers were used:

CB1 Forward, 5-CATCATCATCCACACGTCAG 3; CB1 Reverse,5 ATGCTGTTGT CTAGAGGCTG-3; GAPDH forward, 5-GGTCGGTGTGAA CGGATTTG-3, GAPDH Reverse, 5-GTGAGCCCCAGCCTTCTCCAT-3 (Kokona and Thermos, 2015). In order to quantify the relative expression of CB1 mRNA, a relative standard curve was performed. Each sample was run in triplicate.

2.8. Statistical analysis

Data were expressed as percentage of control (100%) and analyzed using one or two-way analyses of variance (ANOVA) followed by Newman-Keuls and Bonferroni post hoc analysis, respectively, and two-tailed unpaired *t*-test, using GraphPad Prism 5.0 (GraphPad Software, Inc, San Diego, CA). Differences between the groups were considered as statistically significant when $p < 0.05$. Data were plotted as the Mean ± S.E.M (Standard Error of the Mean).

3. Results

3.1. Localization of CB1 receptor in the retina

Double labeling with the nuclear marker DAPI showed that in control rat retina CB1R-IR was localized in GCL and less in IPL, INL and OPL. No staining was observed in the ONL (Fig. 1A). CB1R-IR was co-localized with the ganglion cell marker β-tubulin III in ganglion cell somata and processes in the IPL (Fig. 1B). Staining with the secondary antibody (CF543 goat anti-rabbit IgG) alone, led to the lack of immunoreactivity in the control tissue (data not shown).

3.2. Time-dependent and dose-dependent effects of HU-210, AEA and MethAEA on CB1 receptor expression in rat retina – immunohistochemical studies

HU-210 was administered in different doses (25, 50, 100 μg/kg, i.p.), acutely, subchronically (4d) and chronically (14d). Representative photomicrographs are shown in Fig. 2A. HU-210 administered at the lowest dose (25 μg/kg) did not provide any statistically significant decrease of CB1R-IR, after acute, subchronic or chronic administration ($p > 0.05$ compared to control) (Fig. 2B). HU-210 (50 μg/kg) induced a statistically significant attenuation of CB1R-IR, approximately 43% and 67% ($^{***}p < 0.001$, compared to control) after subchronic and chronic treatments, respectively. No effect was observed when it was administered acutely ($p > 0.05$) (Fig. 2B). A statistically significant difference was also found between the acute and subchronic or chronic treated

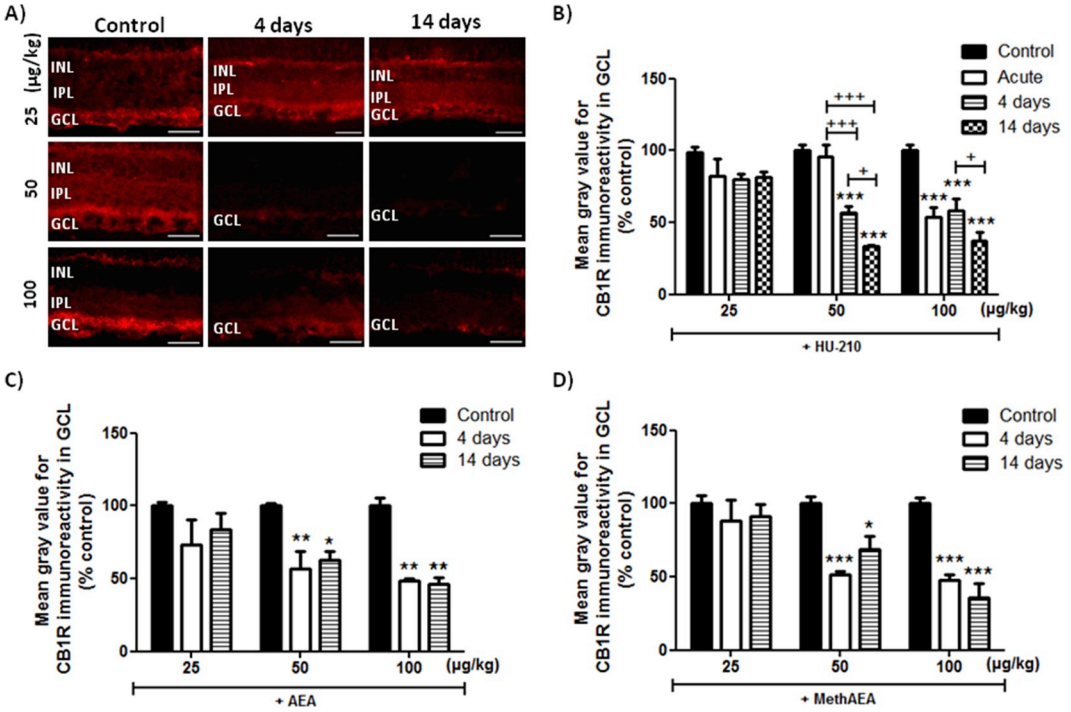

Fig. 2. Effect of HU-210, AEA and MethAEA on CB1 receptor immunoreactivity (CB1R-IR) in rat retina. A. Representative images of CB1R-IR in control and HU-210 treated retinas. B. HU-210 on for CB1R-IR HU-210 (25 μg/kg, i.p.) did not affect the CB1R-IR in the ganglion cell layer (GCL) after acute, subchronic or chronic treatment ($p > 0.05$, compared to control). HU-210 (50 μg/kg, i.p.) reduced CB1R-IR after subchronic and chronic administration. The highest dose of HU-210 (100 μg/kg) induced a statistically significant reduction after acute, subchronic and chronic treatment. C. AEA (50 or 100 μg/kg, i.p.) reduced CB1R-IR after subchronic or chronic administration. No effect was observed at the dose of 25 μg/kg (i.p.) ($p > 0.05$ compared to control). D. MethAEA (50 or 100 μg/kg) induced a statistically significant reduction of CB1R-IR. No effect was observed at the dose of 25 μg/kg (i.p) ($p > 0.05$, compared to control). Data are presented as Mean ± S.E.M, n = 3–6 retinas/group. $^{***}p < 0.001$, compared to control; $^{+}p < 0.05$, $^{+++}p < 0.001$, compared to different cannabinoid treatments; two-way ANOVA, followed by Bonferroni's post hoc analysis. OPL: outer plexiform layer, INL: inner nuclear layer, IPL: inner plexiform layer, GCL: ganglion cell layer. x40 magnification, Scale bar: 50 μm.

groups ($^{+++}p < 0.001$), and between subchronic and chronic treatment ($^{+}p < 0.05$). HU-210 (100 μg/kg) induced a statistically significant reduction in CB1R-IR, approximately 46%, 42% and 63% reduction ($^{***}p < 0.001$ compared to control) after acute, subchronic and chronic treatments, respectively (Fig. 2B) and a statistical significance was found between subchronic and chronic treated groups ($^{+}p < 0.05$).

The quantitative immunohistochemical data of CB1R-IR (mean gray area per section) used for the normalization of the HU-210 data as percentage of control (% control, Fig. 2B) are shown in Table 1.

Both AEA and MethAEA produced similar effects as HU210 on CB1R expression in retinas of control animals and after subchronic and chronic administration. AEA and MethAEA (25 μg/kg, i.p) had no effect ($p > 0.05$ compared to control) on CB1R-IR after subchronic and chronic administration, respectively (Fig. 2C and D). AEA led to a decrease of CB1R expression when administered subchronically (50 and 100 μg/kg; 43% and 52%, respectively, $^{**}p < 0.01$ compared to control) and chronically (50 and 100 μg/kg; 38% and 54%, $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to control, respectively) (Fig. 2C). A similar response was observed with MethAEA [subchronic (50 and 100 μg/kg; 48% and 52%, $^{***}p < 0.001$ compared to control, respectively); chronic administration (50 and 100 μg/kg; 31% and 64%, $^{*}p < 0.05$, and $^{***}p < 0.01$ compared to control, respectively] (Fig. 2D).

3.3. Subchronic and chronic effects of AEA, MethAEA, HU-210, on CB1 receptor expression and on its downstream signaling - western blot analysis

Western blot analysis was initially performed in order to examine the CB1R protein levels in retinal homogenates after subchronic and chronic administration of HU-210, AEA, MethAEA, (50 μg/kg, i.p). The CB1R was detected as a protein with an approximate 60kD molecular mass. Quantification data of Western blot analysis showing CB1R/GAPDH ratio and representative blots are presented in Figs. 3 and 4. HU-210 (50 μg/kg, i.p) led to a significant attenuation of the CB1R protein expression after subchronic ($^{*}p < 0.05$, Fig. 3A) or chronic treatment ($^{**}p < 0.01$, Fig. 3B), compared to control. Similarly, the highest dose of HU-210 (100 μg/kg) reduced the CB1R protein expression after subchronic ($^{**}p < 0.01$) or chronic treatment ($^{*}p < 0.05$), compared to control (data not shown). AEA and MethAEA (50 μg/kg, 4d, $^{**}p < 0.01$, $^{*}p < 0.05$, respectively) reduced CB1R expression compared to control (Fig. 4A and B).

Real time PCR analysis was also performed to assess the effect of the subchronic treatment of one of the three cannabinoids employed in the study, namely MethAEA, on CB1R mRNA expression. A statistically significant reduction in the CB1R/GAPDH ratio was observed (Fig. 4C).

In order to examine whether the reduction of CB1R expression/down-regulation affects the PI3K/Akt signaling pathway, Western blot analysis was performed using antibodies against the phosphorylated or total isoform of Akt protein. As shown in Fig. 3C, chronic administration of HU-210 (50 μg/kg) attenuated significantly the phospho/total Akt ratio ($^{***}p < 0.001$), as was observed at the dose of 100 μg/kg (data not shown). Similarly, the same effect was observed after subchronic administration of HU-210 (50 and 100 μg/kg, $^{*}p < 0.05$, compared to control, data not shown).

AEA (50 μg/kg, i.p., 4d) had no effect on the phospho/total Akt ($p > 0.05$, compared to control), nor the phospho/total ERK1/2 ratio ($p > 0.05$, compared to control, Fig. 5A and B). However, MethAEA (50 μg/kg, i.p., 4d) reduced both phospho/total Akt ratio ($^{**}p < 0.01$ compared to control) and phospho/total ERK ratio ($^{*}p < 0.05$, compared to control, Fig. 5C and D). In these experiments, we employed only the 50 μg/kg dose of AEA and MethAEA taking into consideration the immunohistochemical data (Fig. 2) that showed no statistical significant difference in CB1R expression between the dose 50 μg/kg and 100 μg/kg dose or between subchronic and chronic treatments.

3.4. Effect of AM251 on the HU-210 induced CB1R downregulation in the subchronic model

The CB1R antagonist/inverse agonist, AM251 (0.5 mg/kg, i.p., 4d), administered daily prior to the administration of HU210 (50 μg/kg, i.p., 4d), reversed the HU210-dependent downregulation of the CB1R ($^{###}p < 0.001$). AM251 (0.5 mg/kg, i.p, 4d) administered alone increased CB1R immunoreactivity ($^{*}p < 0.05$), compared to control, suggesting an upregulation of the receptor (Fig. 6A). Western blot analysis data are in agreement with the AM251 induced reversal of HU-210 (Fig. 6B). However, AM251 administered alone did not lead to a statistical significant increase in CB1R expression ($p > 0.05$), in contrast to what was observed in the immunohistochemical studies.

3.5. Effect of HU-210, AEA and MethAEA subchronic administration on CB1R expression and on the neuroprotection of retinal neurons in the model of AMPA excitotoxicity

Intravitreal administration of AMPA attenuated approximately 84% of bNOS-IR, ($^{***}p < 0.001$, compared to control, Fig. 7A, C), but had no effect on CB1R-IR ($p > 0.05$, compared to control, Fig. 7B,D). HU-210 (50 μg/kg, i.p., 4d), did not protect the bNOS expressing amacrine cells against AMPA excitotoxicity ($^{***}p < 0.001$, compared to control; $p > 0.05$, compared to AMPA, Fig. 7C). A significant attenuation of CB1R-IR was observed ($^{**}p < 0.01$, compared to control, $^{##}p < 0.01$, compared to AMPA Fig. 7D). HU-210 administered at the low doses of 12.5 μg/kg and 25 μg/kg reversed the AMPA induced attenuation of the number of bNOS-positive amacrine cells ($^{###}p < 0.001$, compared to AMPA, Fig. 7C). This neuroprotective effect is in agreement with its inability to attenuate CB1R expression at these low doses 12.5 μg/kg and 25 μg/kg, ($p > 0.05$, compared to control and AMPA, Fig. 7D).

AEA and MethAEA (50 μg/kg, i.p., 4d) also induced downregulation of the CB1R. Representative images of bNOS-IR and CB1R-IR are shown in Fig. 8A and B, respectively. An AMPA-induced reduction in the number of bNOS-positive amacrine cells was observed ($^{***}p < 0.001$, (Fig. 8C), but CB1R-IR remained unaffected ($p > 0.05$ compared to control, Fig. 8D). AEA and MethAEA (50 μg/kg) induced a statistically significant reduction of CB1R-IR ($^{**}p < 0.01$, compared to control, Fig. 8D). No reversal of bNOS-IR ($p > 0.05$, compared to AMPA, Fig. 8C) was observed.

4. Discussion

In this study, we show for the first time that subchronic and chronic administration of AEA, its synthetic stable analog methAEA (Abadji et al., 1994) and HU-210 induce a dose-dependent downregulation of the CB1R. This led to the loss of retinal neuroprotection that has been

Table 1
Effect of dose and treatment of HU-210 on CB1 receptor expression in rat retina.

HU-210 Dose (μg/kg,i.p)	Treatment	Mean gray area/section (Mean ± SEM)	Number of samples (n)
	Control	34.51 ± 4.58	10
25	Acute	36.18 ± 9.00	5
	4 days	30.93 ± 6.93	5
	14 days	39.80 ± 2.00	5
	Control	28.90 ± 4.28	10
50	Acute	37.33 ± 5.05	6
	4 days	11.92 ± 1.97	6
	14 days	8.47 ± 1.10	6
	Control	40.84 ± 4.91	7
100	Acute	23.53 ± 2.64	6
	4 days	18.38 ± 2.64	6
	14 days	16.39 ± 1.77	6

Mean values ± standard error of mean (S.E.M.) gray area/section for CB1R expression of control and HU-210 treated groups.

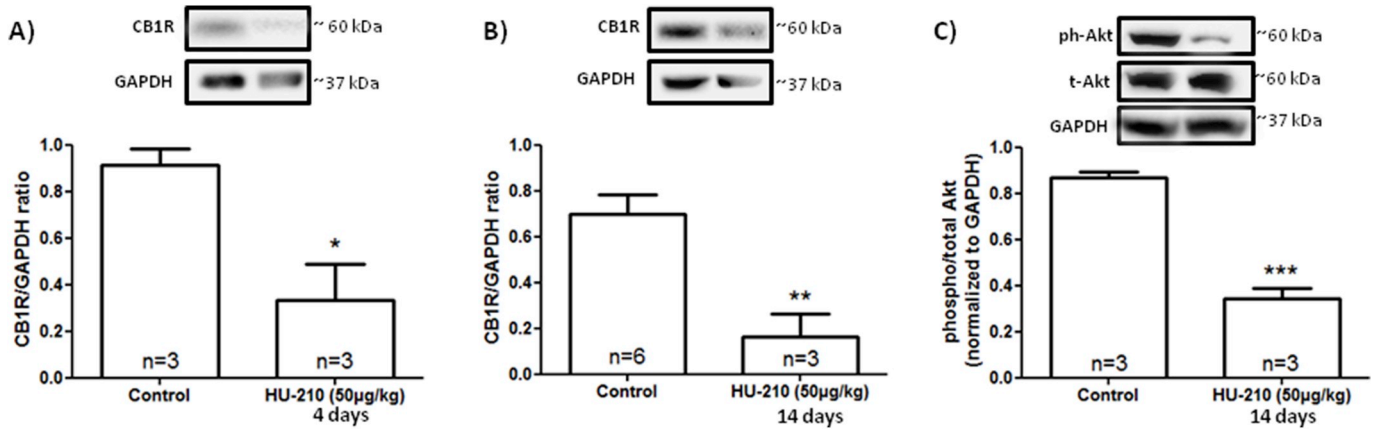

Fig. 3. Effect of HU-210 on CB1R protein and on Akt phosphorylation in rat retina: Western blot analysis. A. HU-210 (50 μg/kg, i.p., 4d) decreased the expression of CB1R. B. Chronic HU-210 (50 μg/kg, i.p., 14d) attenuated CB1 receptor expression. C. HU-210 (50 μg/kg, i.p., 14d) administration decreased significantly the phosphorylation of Akt protein. Data are presented as Mean ± S.E.M, n = 3–6 retinas/group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to control; two-tailed unpaired *t*-test. 4d: subchronic treatment, 14d: chronic treatment.

observed after acute cannabinoid administration in various experimental retinal disease models (El-Remessy et al., 2003; Nucci et al., 2007; Pinar-Sueiro et al., 2013; Kokona and Thermos, 2015).

Retinal diseases, such as glaucoma and diabetic retinopathy, are chronic ocular diseases. In order to assess the therapeutic potential of endocannabinoids and synthetic cannabinoids in chronic retinopathies, it is imperative to investigate their neuroprotective properties when administered chronically in experimental animal models. To this end, in the present study, we employed the subchronic and chronic administration protocols of Dalton et al. (2009) and observed that HU-210 induced an attenuation of CB1R expression in rat retina, in a dose-dependent manner. HU-210 administered at the doses of 50 and 100 μg/kg, induced a statistically significant decrease in CB1R expression under subchronic and chronic conditions, whereas the dose of 25 μg/kg had no effect. The highest dose of 100 μg/kg also induced a statistically significant decrease in CB1R expression under acute administration. These results are in total agreement with the findings of Dalton et al. (2009) who reported a dose-dependent decrease in CB1R density in several rat brain regions after acute, subchronic and chronic HU-210 treatments at the same doses. The results from the present study in the retina are also in agreement with the study of Hsieh et al. (1999) that showed HU-210 at low nanomolar concentrations (0.1 nM) to lead to rapid internalization of the CB1R stably expressed in AtT-20 cells.

The findings mentioned above were obtained by immunohistochemical studies, employing polyclonal antibodies against the CB1 receptor. In our control rat retinas, CB1R was expressed in the GCL, IPL, INL, and OPL, in agreement with other investigations (Straiker et al., 1999; Zabouri et al., 2011). Maccarone et al. (2016) reported a robust CB1R staining in OPL and IPL in rat retina. In the Zabouri et al. study, the CB1R expression in rat retina was examined during development (P1 until adulthood) and found to have a nuclear expression in ganglion cell somata. while from P1 till P15 there was a gradient increase in immunofluorescence in ganglion cell axons. In the INL a nuclear stain was observed (P1 till P21) which weakened and became more sparse from P21 till adulthood. In the present study, CB1R staining was highest in ganglion cells, as ascertained by the colocalization of the CB1R-IR with β-tubulin III–IR in ganglion cell somata and processes in the IPL (Fig. 1B). β-tubulin III was reported to be expressed in ganglion cell somata (De Lima et al., 2016).

Therefore, for our purposes, we chose to quantify the CB1R staining only in the GCL in control retinas of our experimental paradigms. Both treatments (subchronic and chronic) led to CB1R downregulation. This was substantiated by both immunohistochemical and Western blot analysis. The three commercially available CB1R antibodies [two Abcam (different batches) and one Millipore] employed in the present study provided reproducible and credible CB1R immunoreactivity data.

Real time PCR data showed that subchronic treatment with MethAEA (50 μg/kg, i.p.) also led to an attenuation of CB1R mRNA. These data are in agreement with the immunohistochemistry and Western blot analysis data.

Activation of the CB1R leads to the regulation of several intracellular transduction pathways. The first transduction pathway

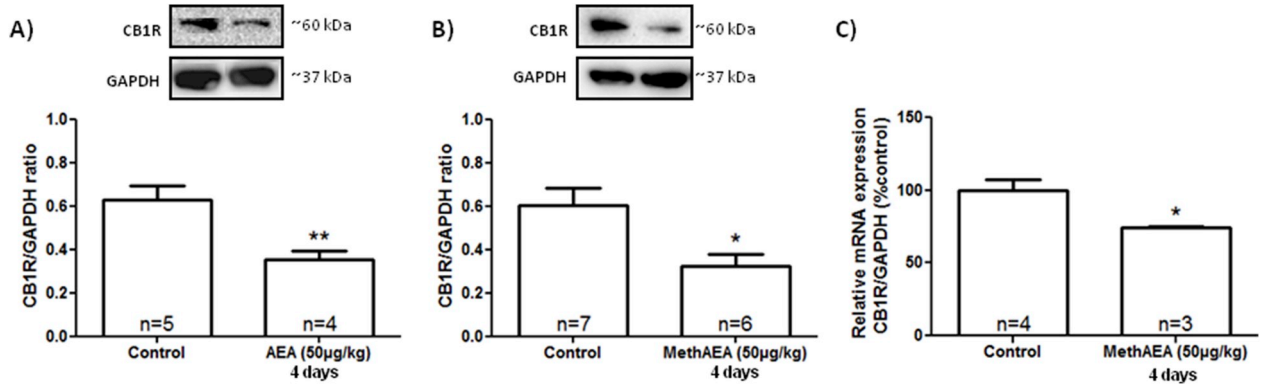

Fig. 4. Effect of AEA and MethAEA on CB1R protein expression and effect of MethAEA on CB1 mRNA expression. A. AEA and B. MethAEA (50 μg/kg, i.p., 4d) reduced CB1 receptor expression in rat retina. C. MethAEA (50 μg/kg, i.p, 4d) attenuated CB1R mRNA expression in rat retina as shown by the CB1R/GAPDH ratio. Data are presented as Mean ± S.E.M, n = 3–7 retinas/group. $^{*}p < 0.05$, $^{**}p < 0.01$ compared to control; two-tailed unpaired *t*-test. 4d: subchronic treatment.

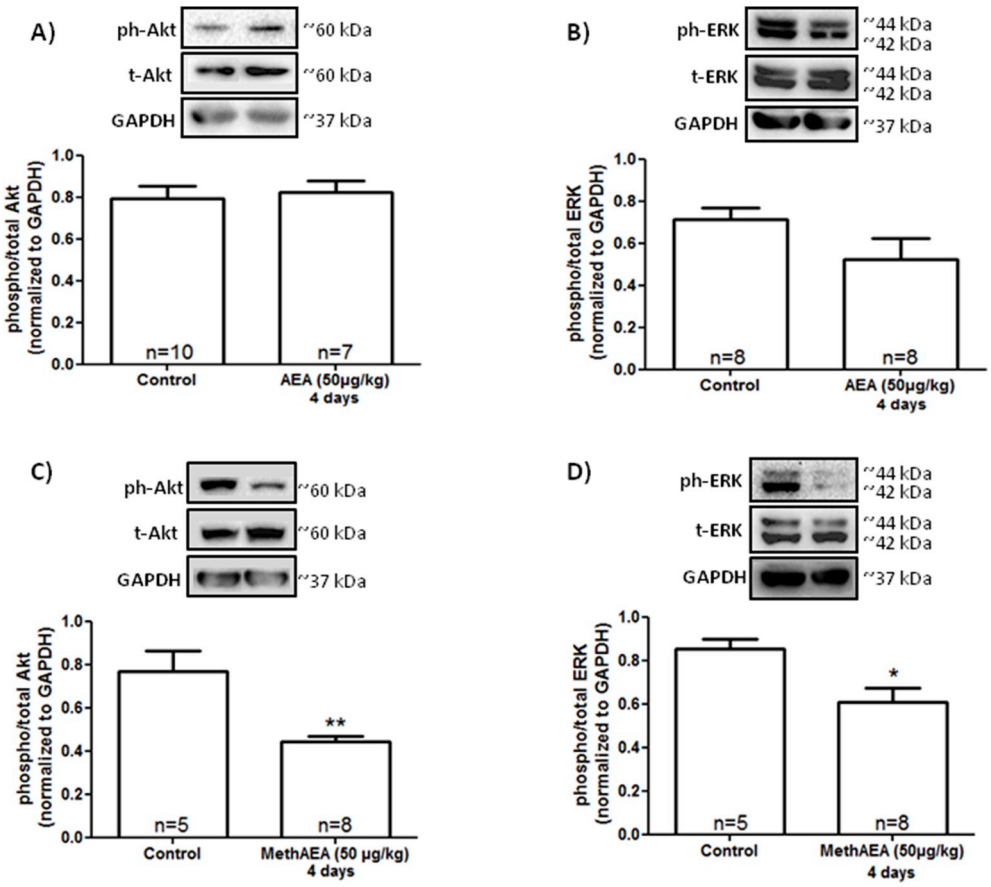

Fig. 5. Effect of AEA and MethAEA on Akt and ERK1/2 in rat retina, and effect of MethAEA. AEA (50 µg/kg, i.p., 4d) did not alter the phosphorylation levels of (A) Akt or (B) ERK1/2. MethAEA (50 µg/kg, i.p., 4d) reduced significantly both (C) Akt and (D) ERK1/2 phosphorylation. Data are presented as Mean ± S.E.M, n = 3–7 retinas/group. $^{*}p < 0.05$, $^{**}p < 0.01$ compared to control; two-tailed unpaired *t*-test.

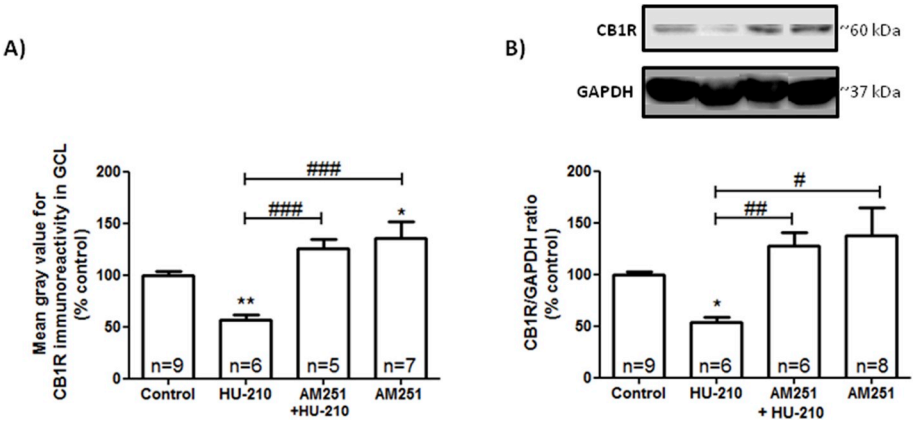

Fig. 6. Effect of subchronic administration of AM251 (CB1 antagonist/inverse agonist) on HU-210 subchronic induced effect on CB1 receptor in rat retina. A. AM251 (0.5 mg/kg, i.p., 4d) prior to HU-210 treatment reversed the HU-210 (50 µg/kg, i.p., 4d) induced a decrease in CB1 receptor immunoreactivity in the GCL. AM251 (0.5 mg/kg, i.p., 4d) administered alone increased CB1 receptor immunoreactivity. B. AM251 (0.5 mg/kg, i.p., 4 days) prior to HU-210 treatment reversed the HU-210 (50 µg/kg, i.p., 4d) induced decrease in the expression of CB1R. AM251 (0.5 mg/kg, i.p.,4d) administered alone had no effect on CB1 receptor expression ($p > 0.05$ compared to control). Data are presented as Mean ± S.E.M, n = 5–9/group. $^{*}p < 0.05$, $^{**}p < 0.01$ compared to control. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ comparison between treatments; one-way ANOVA followed by Newman-Keuls post hoc analysis.

reported was the coupling of the CB1R to Gi and subsequent inhibition of adenylyl cyclase (Howlett et al., 1986, 2002). Activation of the CB1R also leads to the regulation of prosurvival intracellular kinases, such as ERK1/2 kinases (Bouaboula et al., 1995; Wartmann et al., 1995) and PI3K/Akt (Gomez del Pulgar et al., 2000; Ozaita et al., 2007). Cannabinoids were shown to increase or decrease cAMP production via the βγ subunits of Gi depending on the different isoforms of adenylate cyclase present in CHO cells (Rhee et al., 1998). CB1R regulation of ERK1/2, with the intermediate involvement of PI3K, has been shown to be mediated via the βγ subunits of $G_{i/o}$ (Galve-Roperh et al., 2002; Davis et al., 2003). CB1R induced regulation of prosurvival intracellular kinases may be involved in the cannabinoid induced tolerance and dependence in different brain regions (Rubino et al., 2005; Tonini et al., 2006).

In the present study we showed that chronic administration of HU-210 resulted in the attenuation of Akt phosphorylation, in agreement with the downregulation of the CB1 receptor in ganglion cells. Previous data from our laboratory showed that acute administration of HU-210 resulted in a CB1-induced increase in the phosphorylation of Akt protein in the retina, leading to its neuroprotection from AMPA excitotoxicity. HU-210 was also shown to afford prosurvival effects in U373 MG human astrocytoma cells, mediated by both PI3K and ERK signaling pathways (Galve-Roperh et al., 2002).

Subchronic treatment of MethAEA, similarly to what was observed with HU-210, resulted in the downregulation of the CB1R. Hsieh et al. (1999) reported that MethAEA was less efficacious than HU-210 in inducing internalization of the CB1R stably expressed in AtT-20 cells, since MethAEA internalization was observed only at the high

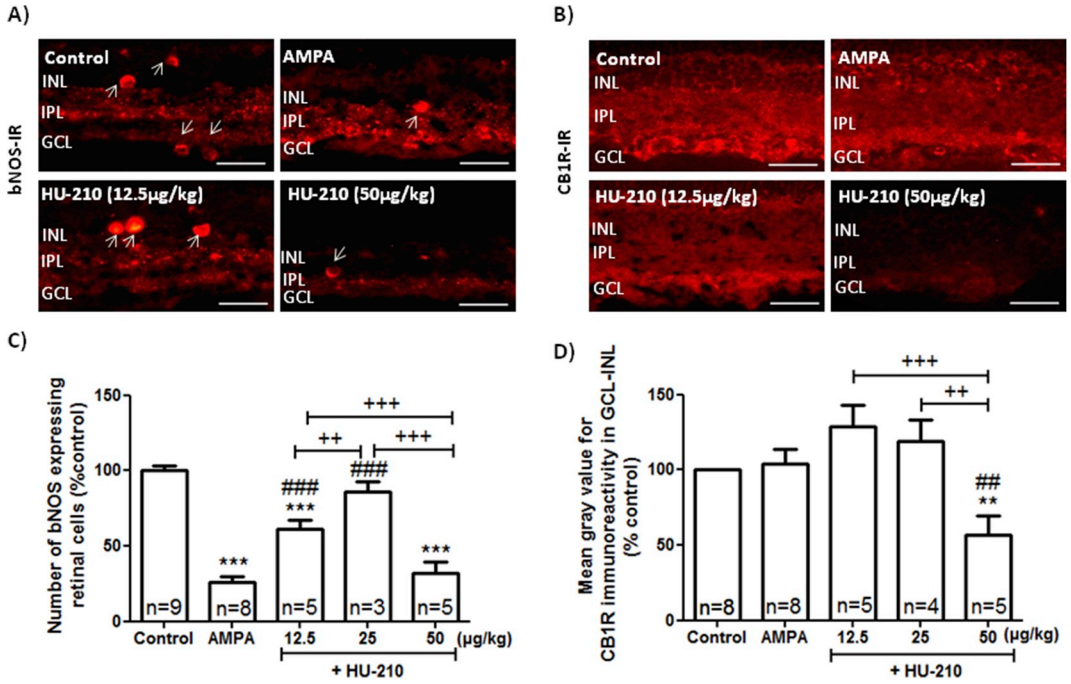

Fig. 7. Effect of HU-210 subchronic administration on retinal neuroprotection against AMPA excitotoxicity. Representative images of (A) bNOS- and (B) CB1R-IR. C. HU-210 (12.5, 25 μg/kg, i.p., 4d) reversed the AMPA induced reduction of bNOS-IR. No neuroprotection was observed with HU-210 (50 μg/kg, i.p., 4d). D. AMPA or HU-210 (12.5, 25 μg/kg, i.p, 4d) did not alter CB1R-IR ($p > 0.05$, compared to control). HU-210 (50 μg/kg, i.p, 4d) decreased CB1R-IR. Data are presented as Mean ± S.E.M, n = 3–9/group. $^{*}p < 0.05$, $^{**}p < 0.01$, compared to control; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$,comparison between treatments; one-way ANOVA followed by Newman-Keuls post hoc analysis. INL: inner nuclear layer, IPL: inner plexiform layer, GCL: ganglion cell layer. 4d: subchronic administration. x40 magnification, scale bar: 50 μm.

concentration of 1μM. Our in vivo results are in agreement with Romero et al. (1999), who reported that chronic administration of methAEA led to an attenuation of cannabinoid receptor binding and mRNA expression. In the same study it was also shown that chronic administration of MethAEA attenuated the WIN-55,212-2-stimulated [^{35}S]-GTPγS binding in brain areas. We report in the present study that subchronic administration of MethAEA resulted in a statistically significant reduction in the phosphorylation levels of Akt and ERK1/2, in correlation with the downregulation of the CB1 receptor in ganglion cell. The CB1R regulation of Akt and ERK1/2 may be mediated via the βγ subunits of Gi/o protein activation (Galve-Roperh et al., 2002; Davis et al., 2003, Dalton and Howllet, 2012).

To our surprise, AEA subchronic administration also resulted in the downregulation of the CB1R. Previous reports had suggested that subchronic/chronic administration of AEA produced differential adaptive changes in brain areas. In an earlier study in brain, it was shown that acute and chronic exposure to AEA led to differential effects in Bmax and Kd of [^{3}H]CP-55,940 binding in different brain areas, none of which was the attenuation of receptor density (Romero et al., 1995). Rubino et al. (2000) reported that chronic administration of AEA

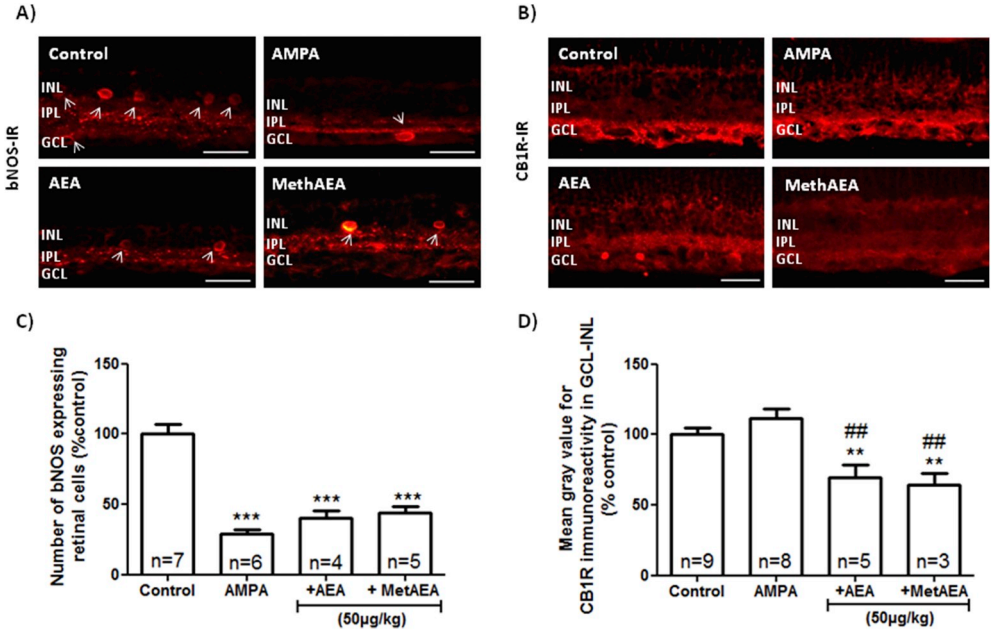

Fig. 8. Effect of AEA and MethAEA subchronic administration on retinal neuroprotection against AMPA excitotoxicity. Representative images of (A) bNOS- and (B) CB1R immunoreactivity.. C. AEA or MetAEA (50 μg/kg, i.p.,4d) failed to protect amacrine cells against AMPA excitotoxicity. D.Intravitreal AMPA administration had no effect on CB1R-IR ($p > 0.05$, compared to control). AEA or MethAEA (50 μg/kg, i.p, 4d) decreased significantly CB1R-IR Data are presented as Mean ± S.E.M, n = 3–9/group. $^{**}p < 0.01$, $^{***}p < 0.001$, compared to control; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, compared to AMPA; one-way ANOVA followed by Newman-Keuls post hoc analysis. INL: inner nuclear layer, IPL: inner plexiform layer, GCL: ganglion cell layer. 4d: subchronic treatment. x40 magnification, scale bar: 50 μm.

reduced agonist stimulated [^{35}S]-GTPγS in brain areas, yet downregulation of the receptor was not observed. Also, AEA chronic treatment had no effect on cAMP levels or in protein kinase A activity. The authors concluded that the observed behavioral tolerance to AEA may be due to the desensitization of the receptor but not to its downregulation. Falenski et al. (2010) compared Δ^9-THC and AEA induced tolerance and cross-tolerance after subchronic administration in mice lacking the AEA metabolic enzyme fatty acid amide hydrolase (FAAH$^{-/-}$ mice). AEA, in contrast to the effects of Δ^9-THC, produced a small non significant attenuation of CB1R expression and WIN55,212-2-stimulated [^{35}S]GTPγS binding in different brain areas, suggesting that subchronic administration of AEA leads to less adaptive changes, namely downregulation and desensitization of the CB1R.

Subchronic treatment of AEA attenuated CB1R expression but had no effect on either Akt nor ERK1/2 phosphorylation in rat retina. Rubino et al. (2004) reported that Δ^9-THC administration induced activation of ERK1/2 phosphorylation in caudate putamen and cerebellum that was still enhanced after chronic treatment in caudate putamen but decreased to control levels in the cerebellum. AEA administered acutely increased ERK1/2 phosphorylation (Kokona and Thermos, 2015) but had no effect when administered subchronically compared to control (present study).

The antagonist/reverse agonist, AM251, administered for four days prior to HU-210 (50 μg/kg, i.p.,4d) inhibited the HU-210 mediated downregulation of the CB1R, similarly to the effect of SR141716A (CB1 antagonist) on WIN55,212-2 (100 nM, 15 min, 37 °C) induced internalization of the CB1R, in AtT-20 cells stably expressing CB1 receptors (Hsieh et al., 1999). AM251, administered alone subchronically, led to an increase in the expression or upregulation of the CB1R in retinal ganglion cells. This finding is in agreement with the upregulation of GPCRs, observed after chronic administration of receptor antagonists (e.g antipsychotics and supersensitivity/upregulation of dopamine D2 receptors). This finding was not reproduced in the Western blot analysis using whole retina samples. However, upregulation of the CB1R after chronic administration of its antagonists should be further studied. It has been reported that inhibition of CB1R protected retinal pigment epithelial cells from oxidative injury and cell death (Wei et al., 2013). Most recently, it was shown that CB1R inhibition by SR141716A reversed photoreceptor loss, glial activation and bipolar cell dendrite shrinkage in the N-methyl-N-nitrosourea mouse model of photoreceptor and bipolar cell degeneration (Chen et al., 2018).

To assess the neuroprotective effects of the three cannabinoids in our subchronic paradigm, we employed the in vivo model of AMPA excitotoxicity, a model previously used to assess the neuroprotective properties of these agents after acute administration (Kokona and Thermos, 2015). HU-210, AEA and MethAEA (50 μg/kg, i.p. 4d) did not protect the retina against AMPA excitotoxicity, as shown by their inability to reverse the AMPA induced attenuation of bNOS expressing amacrine cells. AMPA alone had no effect on CB1R expression. However, CB1R-IR was greatly reduced by all three cannabinoids in the presence of AMPA. In contrast, HU-210 administered at the lower doses of 12.5 and 25 μg/kg (i.p., 4d) had no effect on CB1 receptor expression, and reversed in a dose dependent manner the AMPA induced reduction of bNOS-IR.

Our results demonstrate for the first time that the CB1R in rat retina undergoes a dose-dependent downregulation after subchronic or chronic administration of synthetic cannabinoids (HU-210 and MethAEA) and the endocannabinoid AEA. This led to the attenuation of CB1R induced phosphorylation of the prosurvival downstream signaling pathways Akt and ERK1/2 (HU-210 and MethAEA) and subsequent loss of the neuroprotective actions of the three cannabinoids against retinal toxicity. Therefore, one may suggest that the therapeutic potential of these CB1R agonists in the treatment of chronic retinal disease is minimal. In order to fully conclude on this tenet additional studies should be performed, namely assessment of the downregulation and trafficking kinetics of the CB1R after chronic treatment in cultures of retinal ganglion cells.

However, the upregulation of the CB1R receptor in the retina observed after chronic administration of CB1R antagonists in this study, along with findings by Wei et al. (2013) and Chen et al. (2018) suggest a new role for these agents and should be further investigated. Antagonism of the CB1R may prove to be a more efficacious therapeutic for neurodegenerative retinopathies. Endocannabinoid metabolic enzyme [FAAH and monoacylglycerol lipase (MAGL)] inhibitors are also considered putative therapeutic targets for retinal disease. Further studies are essential in order to identify the best target within the ECS that will provide efficacious therapeutics for the different pathophysiological components of retinal neurodegenerative diseases.

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Declaration of interest

None.

Contributors

Sofia Papadogkonaki performed experiments, analyzed and interpreted data and contributed to the drafting and revision of the manuscript. Kostas Theodorakis performed experiments, analyzed data, and contributed to the revision of the manuscript, Kyriaki Thermos conceived and designed experiments, analyzed and interpreted data, wrote and revised the manuscript and supervised the project.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.exer.2019.107694.

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