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Cannabinoids Induce Cell Death and Promote P2X7 Receptor Signaling in Retinal Glial Progenitors in Culture

Hércules Rezende Freitas[1,2] · Alinny Rosendo Isaac[1] · Thayane Martins Silva[3] · Geyzzara Oliveira Ferreira Diniz[4] · Yara dos Santos Dabdab[4] · Eduardo Cosendey Bockmann[5] · Marília Zaluar Passos Guimarães[6] · Karin da Costa Calaza[5] · Fernando Garcia de Mello[1] · Ana Lucia Marques Ventura[3] · Ricardo Augusto de Melo Reis[1] · Guilherme Rapozeiro França[4]

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Abstract

Development of progenitors in the embryonic retina is modulated by signaling molecules, and cannabinoid receptors are highly expressed in the early developing retina. Here, we investigated whether the CB1/CB2 receptor agonist WIN 5212-2 (WIN) modulated the proliferation, viability, and calcium responses in chick embryo retinal progenitors in culture. A decline in [^{3}H]-thymidine incorporation was observed when cultures were incubated with 0.5–1.0 μM WIN, an effect that was mimicked by URB602 and URB597, inhibitors of the monoacylglycerol lipase and fatty acid amide hydrolase, respectively. A reduction in the number of proliferating cell nuclear antigen-positive nuclei was also noticed in WIN-treated cultures, suggesting that activation of cannabinoid receptors decreases the proliferation of cultured retinal progenitors. WIN (0.5–5.0 μM), but not capsaicin, decreased retinal cell viability, an effect that was blocked by CB1 and CB2 receptor antagonists and by the P2X7 receptor antagonist A438079, implicating this nucleotide receptor in the cannabinoid-mediated cell death. Treatment with WIN also induced an increase in mitochondrial superoxide and P2X7 receptor-mediated uptake of sulforhodamine B in the cultured cells. While a high proportion of cultured cells responded to glutamate, GABA, and 50 mM KCl with intracellular calcium shifts, very few cells responded to the activation of P2X7 receptors by ATP. Noteworthy, while decreasing the number of cells responding to glutamate, GABA, and KCl, treatment of the cultures with WIN induced a significant increase in the number of cells responding to 1 mM ATP, suggesting that activation of cannabinoid receptors primes P2X7 receptor calcium signaling in retinal progenitors in culture.

Keywords ATP · Endocannabinoid · Retina · P2X7 receptor · Müller glia · Calcium signaling

✉ Guilherme Rapozeiro França
guilherme.franca@unirio.br

[1] Laboratory of Neurochemistry, Institute of Biophysics Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brazil

[2] School of Health Sciences, Centro Universitário IBMR, Rio de Janeiro, RJ, Brazil

[3] Laboratory of Neurochemistry, Institute of Biology, Universidade Federal Fluminense, Niterói, RJ, Brazil

[4] Biomedical Institute, Universidade Federal do Estado do Rio de Janeiro, Rua Frei Caneca, 94, Rio de Janeiro, RJ 20211-040, Brazil

[5] Laboratory of Retina Neurobiology, Institute of Biology, Universidade Federal Fluminense, Niterói, RJ, Brazil

[6] Laboratory of Molecular Pharmacology, Institute of Biomedical Sciences, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brazil

Introduction

The vertebrate embryonic retina is composed of multipotent progenitors in the neuroblastic layer where pre-specified precursors generate diverse cell types. Clonal analysis of the lineages of retinal cells using retroviral infection or cell-impermeant high molecular weight fluorescent tracing molecules revealed that a single progenitor can give rise to many labeled descendants that have the potential to generate all the different types of neurons and Müller glia [1–4]. Intrinsic (transcriptional factors) and extrinsic (growth factors, transmitters, or second messengers) factors modify the rate of cell proliferation, differentiation, maturation, or death, accelerating or slowing the appearance of retinal cells and changing the final cellular composition of the retina [4–6].

In the chick retina, cyclic AMP is a well-known differentiating factor, synthesized through adenylate cyclase upon

activation by different transmitters, like dopamine, adenosine, or pituitary adenylate cyclase–activating polypeptide (PACAP). Activation of their G protein–coupled receptors (GPCRs) increases the levels of this second messenger [7, 8]. Indeed, PACAP acts as a determinant of the tyrosine hydroxylase (TH) phenotype of dopaminergic cells during retinal development [9]. In addition, cAMP induced by forskolin or dopamine accelerates the differentiation of dopaminergic retinal cells in the chick retina [10].

Another group of receptors known to modulate cAMP levels is cannabinoid receptors, a GPCR highly expressed in the central nervous system (CNS), having at least two different members known as CB1 and CB2. Both are present in the vertebrate retina, in neurons as well as Müller glia [11–13]. However, the CB1 expression is found mainly in neurons in different retinal layers such as horizontal, amacrine, and ganglion cells [11, 12] while CB2 receptors are found in both neurons and glial cells [13, 14]. Activation of CB1/CB2 receptors by the nonselective receptor agonist WIN 5212-2 (WIN) leads to a decrease in the levels of cAMP in chick retinal cells and in the number of glial cells that respond to ATP with Ca^{2+} increase. WIN also inhibits [^{3}H]-GABA release induced by KCl or L-aspartate [14].

Single-cell calcium imaging (SCCI) was used to functionally evaluate cells emerging from differentiated retinal neurosphere [15]. Activation of retinal progenitors by KCl or α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate (AMPA) stimulated Ca^{2+} transients in microtubule-associated protein 2 (MAP-2)–positive neurons while ATP increased Ca^{2+} influx in glial fibrillary acidic protein (GFAP) cells. Also, a major purinergic receptor in Müller glia was identified as the P2X7 receptor (P2X7R) [16], using SCCI, electrophysiology, and propidium iodide uptake to indicate the contribution of this purinergic receptor in calcium transients and pore formation.

Nucleotide receptors control retinal cell proliferation and death. In mice, ATP-induced late progenitor cell proliferation depends on P2Y1 receptor activation and involves the regulation of cyclin D1 and p27 Kip1 levels [17]. In the postnatal rat retina, activation of P2Y1 receptors regulates the transition from G1 to S phase of the cell cycle [18], and P2Y12 receptors are required for the successful exit from the cell cycle [19]. In the chick retina, while UTP induces the proliferation of ganglion, amacrine, photoreceptor, and horizontal cell precursors through activation of P2Y2/4 receptors [20], ADP induces the proliferation of bipolar and Müller glial cell precursors through P2Y1/13 receptors, PLC, MAPK, CREB, and PI3K/Akt pathways [21–25]. In addition to cell proliferation, nucleotides also control cell death in the chicken retina. Anccasi and colleagues [26] revealed that ATP, in millimolar concentrations, promotes the death of retinal progenitors, through a mechanism dependent on P2X7R and ionotropic glutamate receptors.

Since cannabinoid receptors are highly expressed at early embryonic retina and act as regulators during development [12, 13, 27] and nucleotide P2 receptors modulate retinogenesis, we asked if cannabinoids and nucleotides could cooperate in the modulation of death, proliferation, and differentiation of embryonic retinal cells.

Methods

Embryonic Retinal Cultures

Fertilized white Leghorn eggs (*Gallus gallus domesticus*) were purchased from a local hatchery. Experiments were approved and carried out in accordance with the guidelines of the Institutional Animal Care and Use Committee of the Federal University of Rio de Janeiro (permit number IBCCF-035); the Ethics Committee for Animal Research of the CEPA/PROPPi, Fluminense Federal University (protocol number 197/2012); and the guidelines of the Brazilian Society for Neuroscience and Behavior (SBNeC). The embryos were staged according to Hamburger and Hamilton [28] and sacrificed by decapitation on embryonic day 7 (E7). Eyes were removed, and retinas were dissected out in a Ca^{2+}- and Mg^{2+}-free Hank's (CMF) solution. Trypsin, at a final concentration of 0.1%, was then added, and the suspension was incubated at 37 °C for 15–20 min. Followed by removal of the trypsin solution, retinas were resuspended in MEM containing 5% fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin. The tissues were mechanically dissociated by successive aspirations of the medium. Cells were seeded on culture dishes at a density of 3×10^6 cells/dish (3.1×10^3 cells/mm^2) and incubated at 37 °C for the indicated periods of time (i.e., 2 days and 7 days in vitro, E7C2 and E7C7, respectively), in humidified atmosphere of 95% air/5% CO_2, essentially as previously described [29] and recently reproduced in [30, 31]. Treatment with selected drugs occurred after 1 day in vitro (E7C1). In this study, we used around 20 fertilized white Leghorn chicken eggs (40 retinae).

Immunofluorescence Assay for Phenotype Discrimination

Retinal cells in culture were fixed in 4% PFA, for 30 min at room temperature, and then washed twice with phosphate-buffered saline (PBS), for 5 min. Cultures were permeabilized, and nonspecific binding sites were blocked with 3% bovine serum albumin (BSA) dissolved in PBS/0.25% Triton X-100 for 30 min at room temperature. Retinal cells were subsequently incubated overnight at 4 °C with the following primary antibodies, all of which were prepared in PBS containing 0.1% Triton X-100 and 0.3% BSA: rabbit anti-Tuj-1 (1:200; Abcam), mouse anti-Tuj-1 (1:200; Sigma), mouse anti-2M6

(1:300), rabbit anti-CB1 (1:300; Calbiochem), rabbit anti-CB2 (1:300; Abcam), and mouse anti-nestin (1:300; BD Biosciences). Thereafter, the coverslips were rinsed in PBS and incubated for 2 h at room temperature with the appropriate secondary antibodies, Alexa Fluor® 488 donkey anti-mouse and Alexa Fluor® 594 donkey anti-rabbit (1:500; Thermo Fisher Scientific). After an additional rinse in PBS, retinal cell nuclei were stained with DAPI (1 μg/mL in PBS containing 0.25% BSA), for 2 min at room temperature. Finally, the preparations were mounted using Dako-Cytomation fluorescent medium.

Western Blot

E7C2 cultures were washed twice in PBS (10 min each), homogenized, and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described before [32]. Protein concentration was estimated using the Bradford method [33]. Samples were diluted in buffer (10% glycerol (*v*/*v*), 1% β-mercaptoethanol, 3% SDS, and 62.5 mM Tris base) and boiled for 5 min. Approximately 45 μg of protein from each sample was electrophoresed in 10% SDS-PAGE and transferred to PVDF membranes (Millipore). Membranes were washed with Tween 20 Tris-buffered saline (TTBS) and blocked for 1.5 h with 5% nonfat milk in TTBS. Membranes were incubated with different antibodies: anti-CB1 (1:1000 in TTBS; Sigma) or anti-CB2 (1:1000 in TTBS; Proteimax) overnight at 4 °C. Membranes were rinsed in TTBS and incubated with the anti-rabbit peroxidase–conjugated secondary antibody (1:2000 in TTBS; Sigma-Aldrich) for 2 h at room temperature. Following three washes in TTBS (10 min each), labeling was detected with an ECL kit (Amersham). Blots were re-probed with anti-actin antibody (1:20,000 in TTBS; Sigma-Aldrich) for 1 h at room temperature, rinsed in TTBS, and incubated with anti-mouse peroxidase–conjugated secondary antibody for 45 min at room temperature. Following three TTBS washes (10 min each), the labeling was detected with the ECL kit. Band intensities were analyzed using Quantity One 4–6 software (Bio-Rad Laboratories, Inc.).

Incorporation of [^{3}H]-Thymidine

Retinal cell cultures at E7C1 containing 4×10^6 cells/35-mm culture dishes were stimulated for 24 h with increasing concentrations of URB602 (1 μM, 10 μM, 50 μM, and 100 μM), URB597 (0.01 μM, 0.1 μM, and 1.0 μM), or WIN (0.01 μM, 0.1 μM, 0.5 μM, and 1.0 μM). Retinal cells were then incubated with 0.25 μCi [^{3}H]-thymidine for 60 min, at 37 °C. Cultures were washed four times with MEM buffered with 25 mM HEPES (pH 7.4), and the cells were dissolved with 0.4 N NaOH. After dilution of the samples with 3 mL H_2O, 50% trichloroacetic acid (TCA) was added and the mixtures were incubated, at 4 °C, for at least 30 min. Samples were filtered through Whatman GF/B glass fiber filters and washed three times with 5% TCA. Filters were dried, and the radioactivity was determined by scintillation spectroscopy.

Immunofluorescence of Proliferating Cell Nuclear Antigen

Retinal cell cultures at E7C1 containing 4×10^6 cells/coverslip were stimulated for 24 h with 0.5 μM WIN, washed with PBS, and fixed for 15 min in 0.16 M phosphate buffer (pH 7.6) with 4% paraformaldehyde. After three washes of 5 min with PBS (pH 7.6), cells were permeabilized with 0.25% Triton X-100 for 30 min. Nonspecific sites were blocked by incubating cells for 60 min in PBS/Triton X-100 containing 0.1% normal goat serum and 5% BSA. Cells were incubated overnight at 4 °C with anti-proliferating cell nuclear antigen (PCNA) primary antibodies at a dilution of 1:1000. Cultures were washed and incubated with Alexa Fluor® secondary antibody (1:200) for 2 h at room temperature. Nuclei were counterstained with DAPI, and cells were examined and photographed on a Nikon TE 2000-U fluorescence microscope. A rate of $PCNA^+/DAPI^+$ cells was determined to estimate cell proliferation.

Live and Dead Assay

A fluorescence-based viability assay was performed exactly as suggested by the manufacturer (live-dead assay; Invitrogen, Carlsbad, CA) and as previously performed [30, 31]. Quantification of live and dead cells was carried out using the "cell counter" plugin from ImageJ (image processing and analysis in Java; NIH, Bethesda, MD) software.

MitoSOX Assay

A fluorogenic dye assay, targeted to mitochondria in live cells, was performed exactly as suggested by the manufacturer (MitoSOX Red Mitochondrial Superoxide Indicator; Thermo Fisher). Oxidation of MitoSOX Red reagent by superoxide produces red fluorescence. Quantification of fluorescence intensity and subtraction of background values were performed using the "measure" tool from ImageJ (image processing and analysis in Java; NIH, Bethesda, MD) software.

CM-H2DCFDA Oxidation Assay

Intracellular reactive oxygen species (ROS) accumulation was evaluated in live cells using a chloromethyl derivative of H2DCFDA, CM-H2DCFDA (Molecular Probes), which is useful as an indicator for ROS in cells, as previously reported [34]. E7 cells were plated on 96-well (black, optically clear bottom) plates, treated from E7C1 to E7C2 (overnight) with

0.5 μM and 1 μM WIN. E7C2 cells were incubated with 5 μM CM-H2DCFDA in PBS for 30 min at 37 °C in the dark. Cells were washed three times with Krebs solution, and a WIN-absent group of cells was exposed to 0.5 mM H_2O_2 for 30 min. Readings were carried out at 485 nm/535 nm (excitation/emission, 1 s/well) using a VICTOR X Multilabel Plate Reader (PerkinElmer, Inc.).

Single-Cell Calcium Imaging

Free intracellular calcium levels ($[Ca^{2+}]_i$) were measured in avian retinal cells in culture [15, 30]. Briefly, E7C2 or E7C7 cells in culture were loaded for 40 min with 5 μM fura-2/AM (Molecular Probes), 0.1% fatty acid-free BSA, and 0.02% Pluronic F-127 (Molecular Probes) in Krebs solution (132 mM NaCl, 4 mM KCl, 1.4 mM $MgCl_2$, 2.5 mM $CaCl_2$, 6 mM glucose, 10 mM HEPES, pH 7.4), in an incubator with 5% CO_2 and 95% atmospheric air at 37 °C. After a 10-min post-loading period at room temperature in Krebs solution, to obtain a complete hydrolysis of the probe, a 15-mm coverslip (Marienbad, Germany) with the cells was mounted on a chamber in a PH3 platform (Warner Instruments, Hamden, CT) on the stage of an inverted fluorescence microscope (Axiovert 200; Carl Zeiss). Cells were continuously perfused with Krebs solution and stimulated with different drugs (i.e., 1 mM glutamate, 1 mM GABA, 50 mM KCl, and 0.01 mM, 0.1 mM, 0.5 mM, and 1.0 mM ATP). Solutions were added to the cells by a fast-transition system that allowed drugs to reach the cells in less than 8 s. The variations in $[Ca^{2+}]_i$ were evaluated by quantifying the ratio of the fluorescence emitted at 510 nm following alternate excitation (750 ms) at 340 nm and 380 nm, using a Lambda DG-4 apparatus (Sutter Instrument, Novato, CA) and a 510-nm long-pass filter (Carl Zeiss) before fluorescence acquisition with a × 40 objective and a CoolSNAP digital camera (Roper Scientific, Trenton, NJ). Acquired values were processed using MetaFluor software (Universal Imaging Corp., West Chester, PA). Values for fura-2 fluorescence ratio (F340/380) were calculated based on a cutoff of 10% increase in the $[Ca^{2+}]_i$ level induced by the stimulus.

Sulforhodamine B Uptake Assay

Retinal cell cultures (E7C1) were treated with 0.5 μM WIN for 24 h, pre-incubated for 5 min at 37 °C in Hank's salt solution without Mg^{2+}, and then exposed to 3 mM sulforhodamine B in the presence of 3 mM ATP and/or 10 nM A438079 for 10–15 min at 37 °C. Cultures were washed twice with Hank's solution and immediately observed and photographed on a Nikon TE 2000-U fluorescence microscope using a B-2E/C filter block for TRICT. Labeled cells were counted in five photomicrographs of random fields from the cultures.

Data Analysis

Statistical analysis was performed (Prism 6) using Student's *t* test with Welch's correction. Significance was set at $p < 0.05$, and significance levels are indicated in each dataset. We also performed the multiple comparisons Bonferroni test using analysis of variance (ANOVA) in each dataset. Unless specified otherwise, the results are expressed as the mean ± standard deviation (SD) of at least three separate experiments performed in triplicate.

Results

Cannabinoids Decrease Cell Proliferation in Retinal Nestin–Positive Cell Cultures

Embryonic chick retinal cell cultures at E7C2 express nestin-positive (+) progenitors (Fig. 1a, b), Tuj-1^+ neurons (red), and $2M6^+$ glial cells (green), as shown in Fig. 1c–e. As previously described, both neurons and glial cells express CB1 as well as CB2 receptors [14]. Here, E7C2 cultures expressed similar levels of CB1/CB2 receptors (Fig. 1f, g), and Tuj-1^+, Tuj-1^-, and $2M6^+$ cells expressed both cannabinoid receptors, confirmed by immunofluorescence in the present study (Fig. 2a–l). Additionally, nearly all nestin$^+$ progenitor cells displayed CB1 and CB2 labeling (Fig. 2m–p).

CB1 and CB2 cannabinoid receptors are involved in cell differentiation [35, 36] and neurogenesis [37, 38]. Aiming to evaluate whether WIN could modify proliferation in developing chick retinal cells, progenitors were assayed in E7C2 cultures through [^{3}H]-thymidine incorporation. Cells were incubated with increasing concentrations of WIN (0.01 μM, 0.1 μM, 0.5 μM, 1.0 μM, and 5.0 μM) for 24 h. A progressive decline in [^{3}H]-thymidine incorporation is observed in the presence of 0.5–1.0 μM WIN (Fig. 3a). A similar decrease was noticed in the presence of 50–100 μM URB602, a monoacylglycerol lipase (MAGL) inhibitor, which increases the levels of 2-arachidonoylglycerol (2-AG), a full agonist of endocannabinoid CB1 receptor (Fig. 3b). In addition, 1 μM URB597 which inhibits fatty acid amide hydrolase (FAAH), the primary degradation enzyme for anandamide (AEA), also decreased cell proliferation (Fig. 3c). Therefore, our data suggest that activation of retinal cannabinoid receptors by WIN and endocannabinoids decreases retinal cell proliferation in culture.

To confirm the involvement of the cannabinoid system in chick retinal cell proliferation, a second method was performed. The antibody anti-PCNA and DAPI were used to label, respectively, progenitor cell nuclei and all cell nuclei present in the retinal cell culture. As shown in Fig. 3, a significant decrease of ~ 21% in the proportion of PCNA-positive nuclei was clearly noticed in 0.5 μM WIN-treated cultures

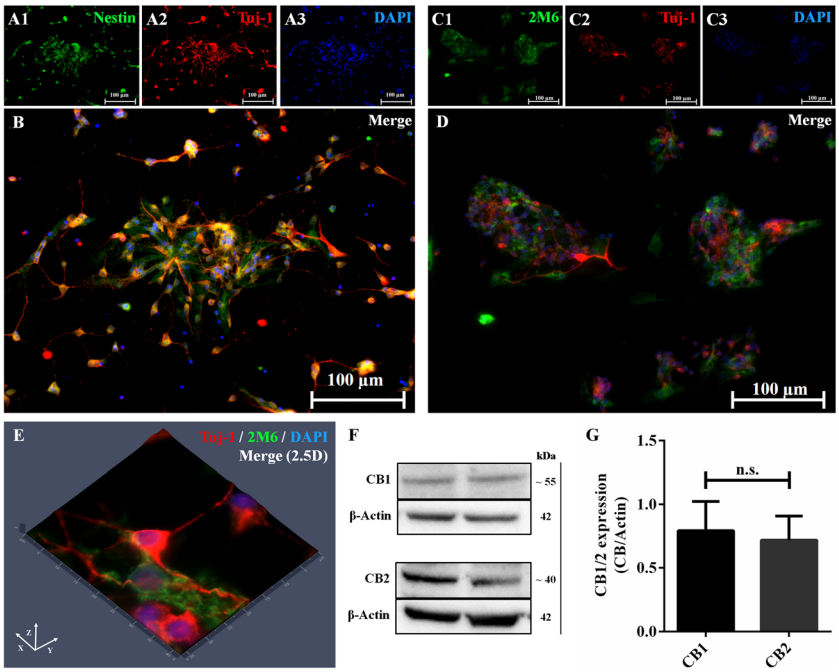

Fig. 1 Immunofluorescence assay and western blot for phenotypic discrimination in chick embryonic (E7C2) retinal cell cultures. Labeling for anti-nestin (green) (**a** (1)), anti-Tuj-1 (red) (**a** (2)), DAPI (blue) (**a** (3)), and the corresponding merged figure (**b**). Staining with anti-2M6 (green) (**c** (1)), anti-Tuj-1 (red) (**c** (2)), DAPI (blue) (**c** (3)), and the corresponding merged figure (**d**). **e** A ROI section at × 63 augment is shown as a 2.5D projection (pseudo 3D) showing cells labeled for Tuj1 (red) or 2M6 (green). **f** Representative immunoblots of CB1 and CB2 receptors from mixed neuron-glia cultures at stage E7C2. CB1- and CB2-specific bands appear at 55 kDa and 40 kDa, respectively. **g** Quantitative analysis of blot bands shows equivalent CB (1 or 2)/actin density ratios (n.s. nonsignificant). $N = 3$ for each experiment. Scale bars = 100 µm

(Fig. 3d–j), suggesting that WIN decreases the number of proliferating progenitors in the cultures.

WIN Induces Concentration-Dependent Cell Death

The decrease in the number of $PCNA^+$ cells could be due to the death of retinal progenitors in the WIN-treated cultures. Using a live-dead assay, we observed a progressive decrease in retinal cell viability with increasing concentrations of WIN (0.5–5.0 µM) (Fig. 4a–e). At 1 µM, a decrease of ~ 41% in the number of living cells was detected. Moreover, the addition of 1 µM AM-251 or 1 µM AM-630, two selective antagonists for CB1 and CB2 receptors, respectively, prevented the decrease in cell viability induced by WIN (Fig. 4f). These data suggest that both CB1 and CB2 receptors are involved in retinal cell death in culture. To exclude the involvement of transient receptor potential vanilloid (TRPV) receptor, also a target of cannabinoids, the effect of capsaicin was evaluated. As shown in Fig. 4g, capsaicin had no effect in cell viability in the retinal cultures. To evaluate a cell type that dies after cannabinoid treatment, retinal cultures at E7C1 were treated with 0.5 µM and 1.0 µM WIN for 24 h, cell preparations were submitted to western blot assay, and membranes were incubated with anti-2M6 and TUJ-1 antisera. No significant decrease in 2M6 or beta-tubulin III expression was observed in cultures treated with 0.5 µM WIN. However, a ~ 52% decrease in 2M6 expression in cultures treated with 1.0 µM WIN was detected (Fig. 4h–j).

ROS are produced in both cell mitochondria and cytoplasm. Mitochondrial superoxide and cytoplasmic ROS were measured using MitoSOX Red and H2DCFDA, respectively. Cells treated with 0.5–1 µM WIN show a consistent increase of ~ 66% in mitochondrial superoxide content (Fig. 5a–e). However, no significant oxidation of H2DCFDA (cytoplasmic ROS) was detected in cultures under WIN treatment (Fig. 5f). As expected, H_2O_2 increased the content of mitochondrial superoxide and cytoplasmic ROS.

WIN Primes P2X7 Receptors, Increasing Calcium Shifts, Fluorescent Dye Uptake, and Cell Death

In retinal cell cultures, the $GABA_A$ agonist muscimol is able to induce intracellular calcium increases in retinal progenitors, while KCl and the glutamate receptor agonist AMPA stimulate Ca^{2+} transients in MAP-2–positive neurons [15]. Moreover, ATP increases Ca^{2+} influx in GFAP-positive cells. The effect of these agents on the intracellular levels of Ca^{2+} of retinal progenitors from cultures at E7C2 (Fig. 6a, b) is shown in Fig. 6. While 1.0 mM glutamate or 50 mM KCl stimulated Ca^{2+} shifts in approximately 80% of cells, 1 mM GABA increased $[Ca^{2+}]_i$ in approximately 50% of the cultured retinal

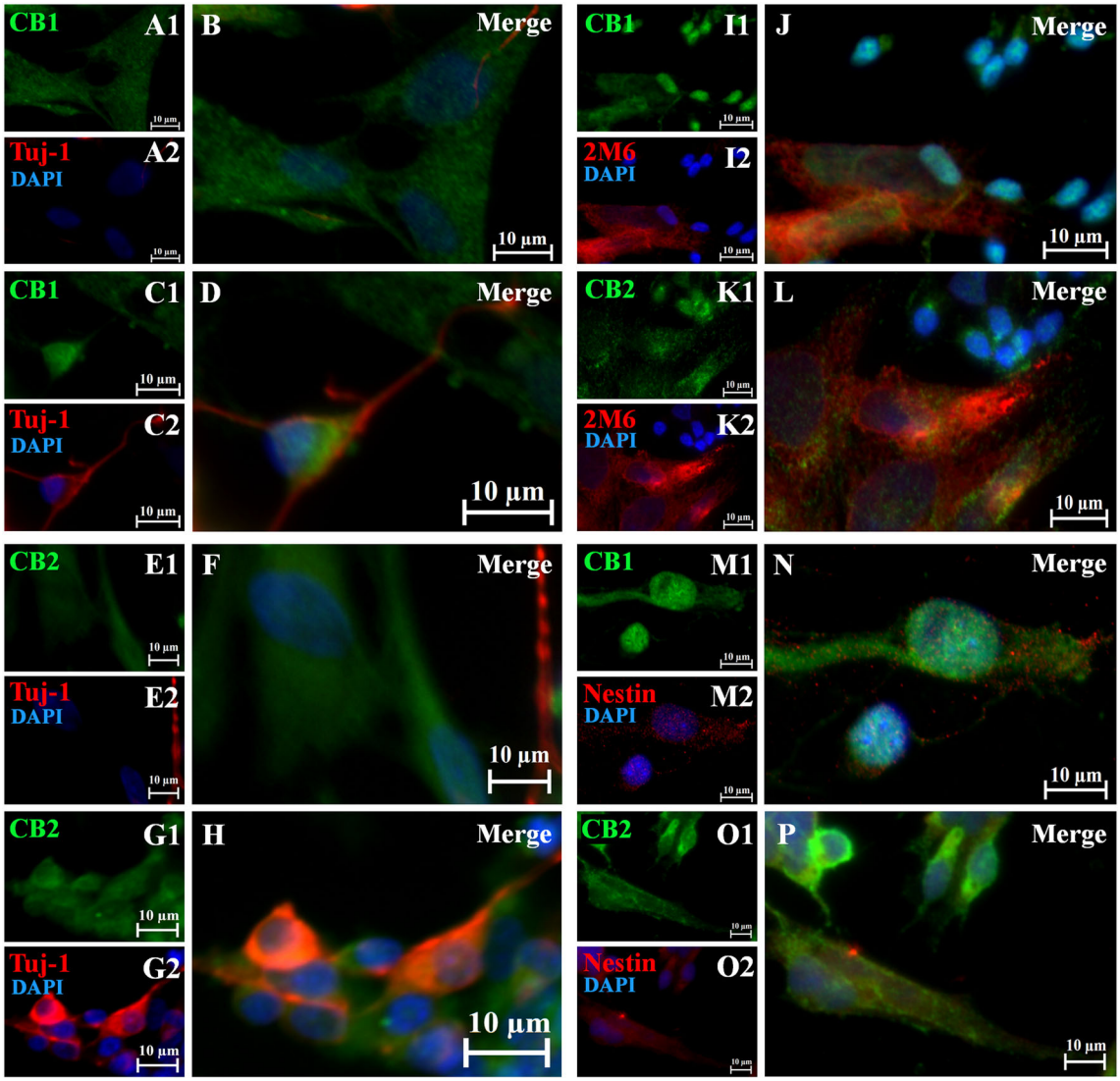

Fig. 2 Immunofluorescence assay for CB1 and CB2 cannabinoid receptors in chick embryonic (E7C2) retinal cell cultures. Double labeling experiments with anti-CB1 (green) (**a** (1), **c** (1)) and anti-Tuj-1 (red) (**a** (2), **c** (2)) are shown in individual or merged panels (**b**, **d**). Similarly, anti-CB2 (green) (**e** (1), **g** (1)) and anti-Tuj-1 (red) (**e** (2), **g** (2)) are represented in individual (**e**, **g**) or merged panels (**f**, **h**). Double staining for anti-CB1 (**i** (1), **k** (1)) and anti-2M6 (**i** (2), **k** (2)) and the corresponding merged images (**j**, **l**). Also, cells were labeled for anti-CB1 (**m** (1)) and anti-nestin (**m** (2)) or anti-CB2 (**o** (1)) and anti-nestin (**o** (2)), with the respective merged figures (**n**, **p**). $N = 3$ for each experiment. Scale bars = 10 µm

cells (Fig. 6c, right panel). No significant increase in $[Ca^{2+}]_i$ levels was observed when cultures at E7C2 were incubated with 0.01–1.0 mM ATP (Fig. 6c, d).

When retinal cell cultures were treated with 0.5 µM (Fig. 7a) or 1.0 µM (Fig. 7b) WIN for 24 h, a different pattern of calcium responses was observed. An increase in the number of responsive cells to calcium shifts induced by 1.0 mM ATP was observed (from 2 to 30%) in the cultures treated with both WIN concentrations (Fig. 7c). Interestingly, a decrease in the number of glutamate-, GABA-, and KCl-responsive cells was detected (Fig. 7a, b). While no responsive cells to glutamate and GABA were observed, only approximately 3% of the cells remained responsive to KCl in WIN-treated cultures.

The P2X7 receptor is a major purinergic receptor expressed in the chick Müller glia [16] responsible for inducing cell death of developing avian retinal neurons in culture [26]. As indicated in Fig. 7, cells under WIN treatment show a different pattern of $[Ca^{2+}]_i$ response to ATP. Aiming to identify the receptor involved in this response, WIN-treated (0.5 µM) cultures were stimulated with 1 mM ATP in the presence of a selective P2X7 receptor antagonist, A438079. As indicated, a strong decrease in $[Ca^{2+}]_i$ was observed (Fig. 8a, b). Also, ATP-responsive cells without WIN treatment showed no change in calcium response in the presence of the antagonist. Cells previously treated with WIN, however, showed a significant decrease in $[Ca^{2+}]_i$ when the antagonist was co-

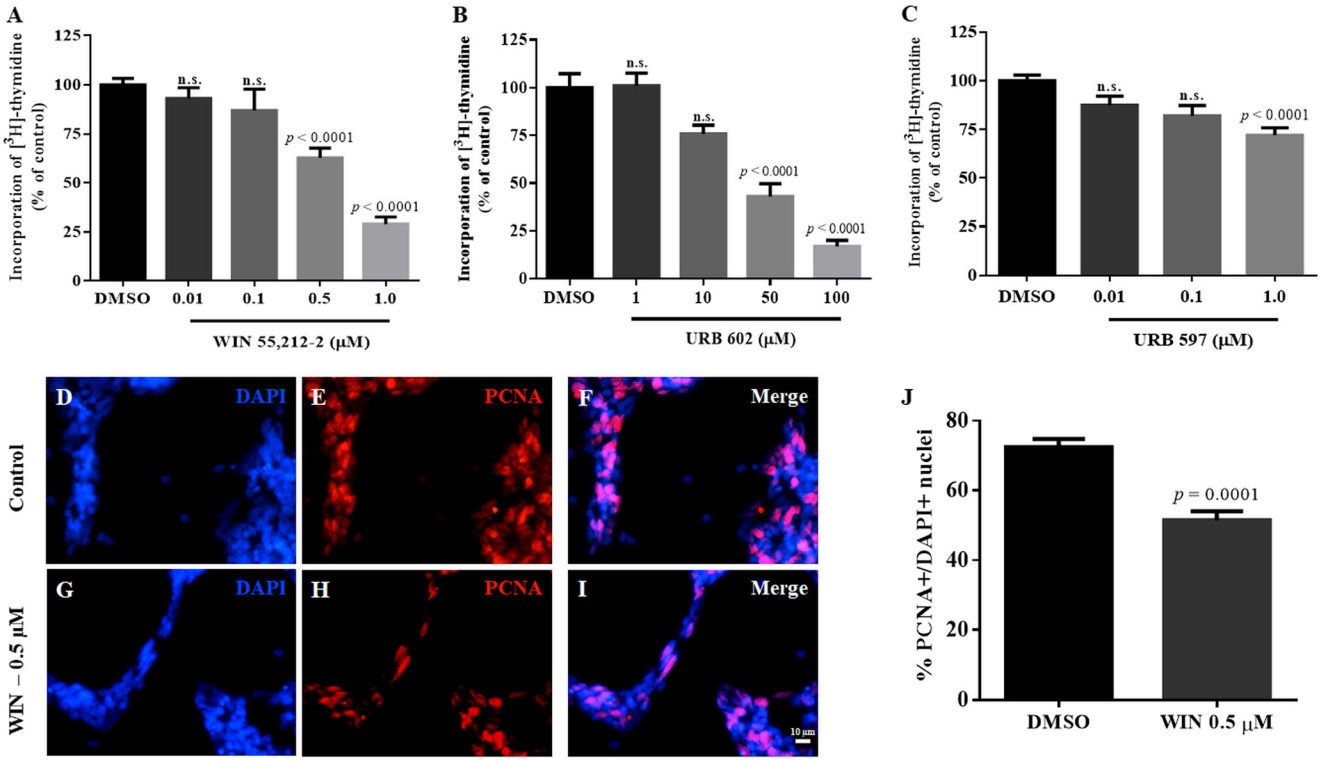

Fig. 3 Incorporation of [^{3}H]-thymidine in retinal cell cultures treated with MAGL/FAAH inhibitors or WIN 55,212-2 and PCNA/DAPI proliferation assay after WIN 55,212-2 exposure. Graphs representing [^{3}H]-thymidine incorporation of E7C1 retinal cell cultures after exposure with increasing concentrations of **a** WIN 55,212-2 (0.01 μM, 0.1 μM, 0.5 μM, and 1.0 μM; $n = 4$), **b** URB 602 (1 μM, 10 μM, 50 μM, and 100 μM; $n = 5$), or **c** URB 597 (0.01 μM, 0.1 μM, and 1.0 μM; $n = 6$). Significance level is shown in each dataset (n.s. nonsignificant) in comparison to control (DMSO). Representative photomicrographs of **d**–**f** DMSO and **g**–**i** 0.5 μM WIN-treated retinal cells labeled for PCNA (red) and DAPI (blue). **j** Ratio of PCNA$^+$/DAPI$^+$ nuclei in retinal cell cultures treated with 0.5 μM WIN. Significance level for $n = 3$ is shown in **j**. Scale bar = 10 μm

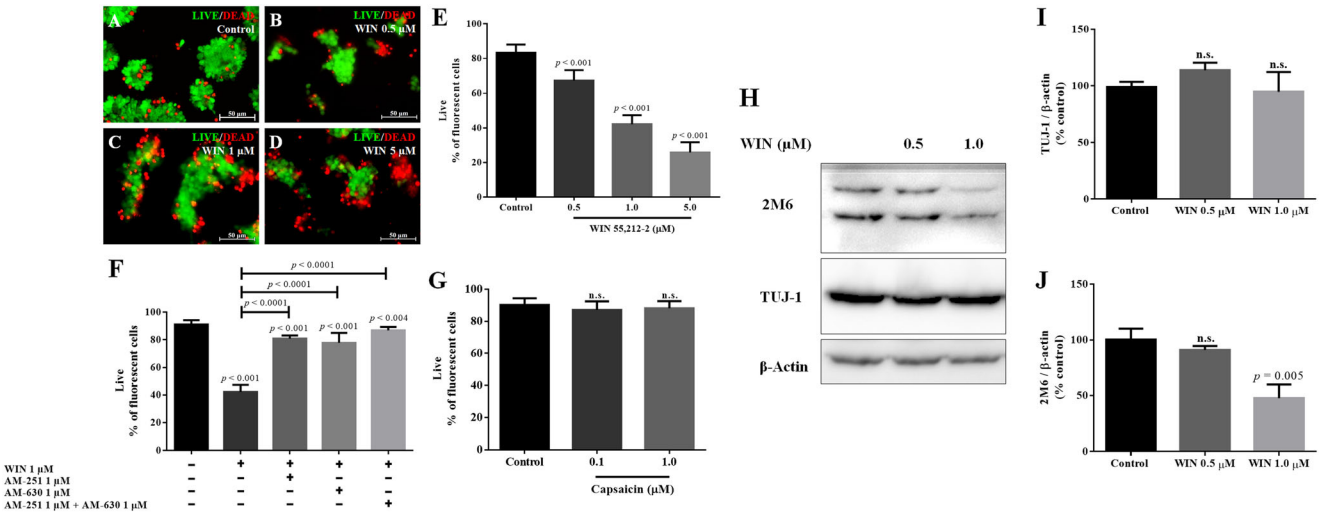

Fig. 4 Live and dead assay in chick embryonic (E7C2) retinal cell cultures under WIN 55,212-2, CB antagonists, and capsaicin treatments. Live (green) and dead (red) E7C2 retinal cells in culture under **a** control, **b** 0.5 μM WIN, **c** 1.0 μM WIN, or **d** 5.0 μM WIN overnight treatment. **e** Quantification of live cells (%) under control and WIN treatments. **g** Quantification of total (live$^+$ dead) cells in the field (× 40, the percentage of control) under each condition. Significance level ($n = 4$) is shown in **e**. Scale bar = 50 μm. Live and dead assay for **f** 1.0 μM WIN treatment in the presence of cannabinoid receptor antagonists AM-251 (1.0 μM), AM-630 (1.0 μM), or AM-251 + AM-630 (both 1.0 μM). **g** Live and dead assay for 0.1 μM and 1.0 μM capsaicin treatments. **h** Representative immunoblots of 2M6 (40 kDa and 46 kDa) and Tuj-1 (55 kDa) from E7C2 cultures treated with 0.5 μM WIN and 1.0 μM WIN. Quantitative analysis is of blot bands for **i** Tuj-1/β-actin and **j** 2M6/β-actin density ratios. Significance level is shown in each dataset (n.s. nonsignificant). $N = 3$ for each experiment. Scale bar = 50 μm

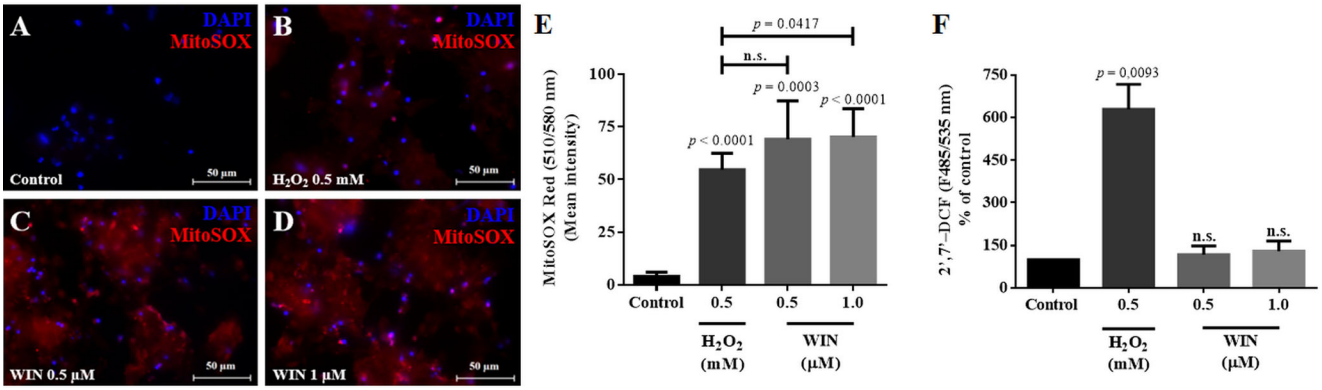

Fig. 5 MitoSOX and CM-H2DCFDA oxidation assays in chick embryonic (E7C2) retinal cell cultures. DAPI (blue) and MitoSOX (red) merged images of retinal cultured cells under **a** control, **b** 0.5 mM H_2O_2 (30 min), **c** 0.5 μM WIN, and **d** 1.0 μM WIN (overnight) conditions. **e** Quantification of fluorescence intensity for each group. **f** Graph showing quantification of the CM-H_2DCFDA oxidation assay for control, 0.5 mM H_2O_2, and 0.5 μM and 1 μM WIN treatments. Significance level (n.s. nonsignificant) is shown in **e**, **f**. $N = 3$ for each experiment. Scale bar = 50 μm

incubated with ATP, as indicated by the ratio between ATP and ATP + A 438079 responses (Fig. 8c).

Interestingly, we detected the presence of a selective band at 65 kDa for the P2X7 receptor in E7C2 cultures (Fig. 9a, lane 1) that was totally blocked by the control peptide antigen (Fig. 9a, lane 2); this result was corroborated by double labeling for the Müller glia marker 2M6 and the receptor P2X7 (Fig. 9b–d). To investigate whether the P2X7 receptor was involved in a WIN-mediated decrease in cell viability, increasing concentrations (0.1–1.0 μM) of the selective P2X7 receptor antagonist A438079 were added in retinal cultures at E7C1. As shown in Fig. 9e, 100 nM A438079 completely blocked cell death induced by 1 μM WIN. However, no effect of 100 nM A438079 was observed in the decrease of [^{3}H]-thymidine incorporation promoted by 0.5 μM WIN in retinal progenitors (Fig. 9f).

It is well known that activation of P2X7 receptor induces the uptake of high molecular weight fluorescent dyes in several cell types. Incubation of the retinal cultures with 0.5 μM WIN for 24 h induced an increase of twofold in the uptake of sulforhodamine B (SB) by cultured cells. A similar increase was observed with 3 mM ATP for 10 min. Incubation of 0.5 μM WIN treated for 24-h cultures with 3 mM ATP for 10 min resulted in a higher increase of fourfold in the uptake of the fluorescent dye. All increased responses were inhibited by A438079, indicating that activation of P2X7 receptor mediates SB uptake in the retinal cultures (Fig. 9g–k).

Discussion

Cannabinoids and nucleotides can control the balance between cell death and survival, proliferation, and differentiation in the brain, although their effects can vary noticeably according to the cannabinoid or nucleotide type [39–43] as well as the concentration and duration of treatment [44]. Here, we show that cannabinoids and P2X7 nucleotide receptor can cooperate to control cell death of progenitor retinal cells in culture, which may affect differentiation and phenotype change in the mature retina.

The present study shows that mixed cultures containing neurons (Tuj1$^+$ cells) and Müller glia (2M6$^+$ cells) from retinas of 7-day-old chick embryos were positive for nestin, CB1, and CB2 receptors. Furthermore, no difference between CB1 and CB2 receptor expression levels in the cultures was found. During the development of the chick retina, CB1 receptor RNA transcripts and protein are detected in ganglion cells as early as embryonic days 3 and 4, respectively [12, 45]. Both CB1 and CB2 receptors are detected in the chick retina since the early embryonic stages of E5 up to the more differentiated, post-hatching period of PE7 [27]. Since the model used in the present study consists mainly of late retinal progenitors, our results confirm the presence of CB1/CB2 cannabinoid receptors in this population and suggest the involvement of the endocannabinoid signaling in the development or fate of late retinal progenitors.

Neural progenitors synthesize and release endocannabinoids, mainly AEA and 2-AG, that induce their proliferation via CB1 receptors [46]. In addition, progenitors obtained from embryonic mice cortices express CB2 receptors that induce cell proliferation [47]. In contrast, cannabinoids can inhibit proliferation of BEL7402 hepatocellular carcinoma cells [48], T cells [49], as well as human glioblastoma cells [50]. The present study reveals that the cannabinoid agonist WIN-55,212-2, as well as the inhibitors of the hydrolases FAAH and MAGL (URB597 and URB602, respectively), reduced significantly the incorporation of [^{3}H]-thymidine in the retinal cultures. In addition, WIN reduced the number of PCNA$^+$ cells in culture, suggesting that activation of cannabinoid receptors inhibits the proliferation of retinal progenitors in culture. The decrease in [^{3}H]-thymidine incorporation observed with the MAGL inhibitor URB602 was clearly more

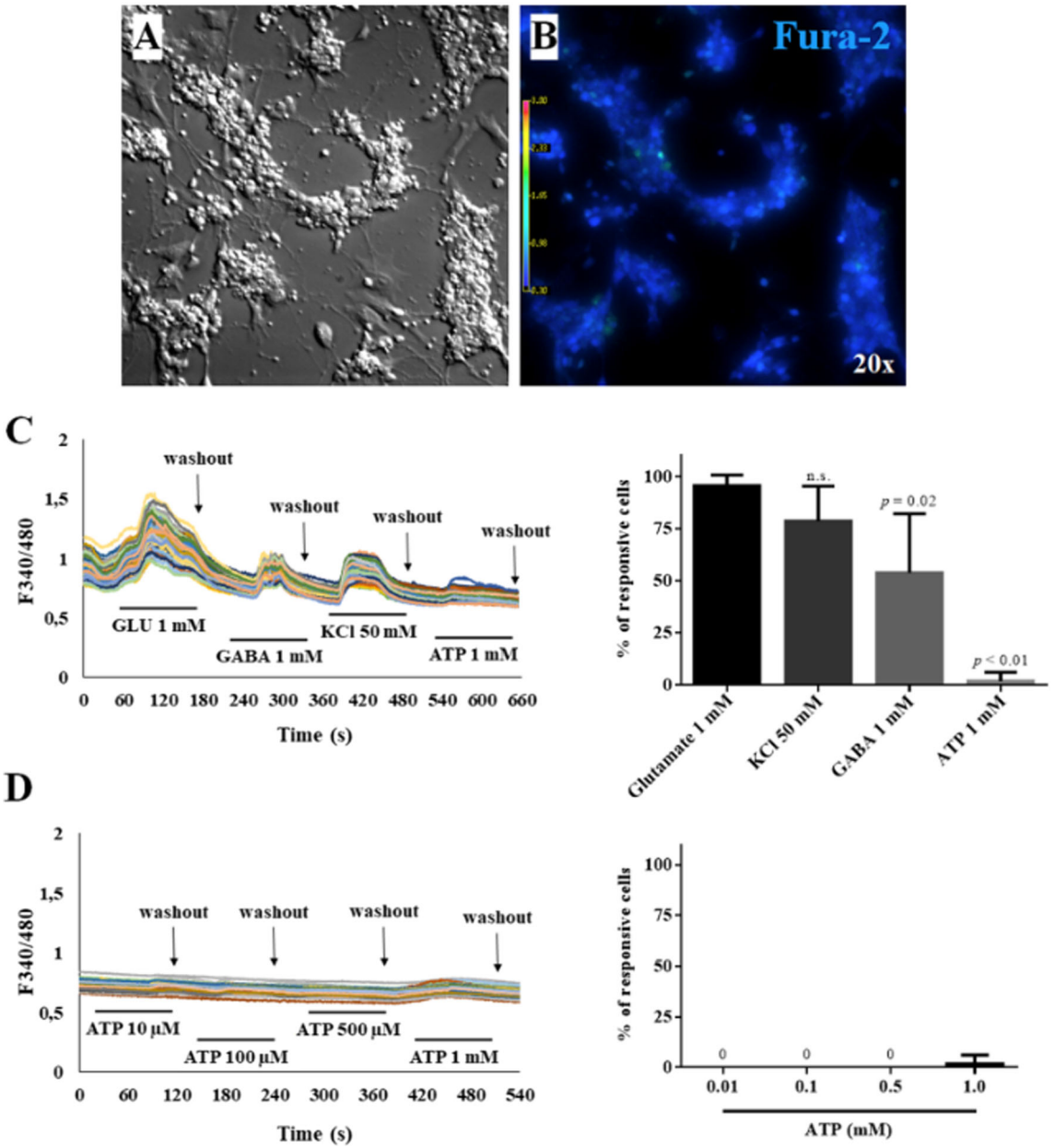

Fig. 6 Calcium shifts induced by several agonists in E7C2 chick embryonic retinal cells. **a** Mixed retinal cells in culture prepared from embryonic avian retina showing a typical flat appearance. **b** The same microscope field is shown under fura-2 fluorescence in single-cell calcium imaging experiments. **c** Representative cell-response curve (left panel) for 1.0 mM glutamate (GLU), 1.0 mM GABA, 50 mM KCl, or 1.0 mM ATP. **d** Cell-response curve (left panel) for 0.01 mM, 0.1 mM, 0.5 mM, or 1.0 mM ATP. Right panels in **c**, **d** show quantification (% of responsive cells) for each stimulus. Significance level (n.s. nonsignificant) is indicated in **c**, **d** graphs. Cells were continuously washed with the buffer solution for 60 s in between stimuli, as indicated by the arrows in **c**, **d**. $N = 3$ for each experiment

pronounced than the effect obtained with the FAAH inhibitor URB597. 2-AG is a major endocannabinoid found in the retina of several vertebrates [13], including the developing avian retina. Since expression of MAGL that hydrolyses 2-AG is detected at early stages of retinal development [27], it is reasonable to suggest that 2-AG is the endocannabinoid responsible for the inhibition of cell proliferation in chick embryonic retinal cultures. It is interesting to notice that millimolar ATP increases the production of 2-AG in cultured mouse astrocytes via activation of P2X7 receptors, and sustained rise in intracellular calcium [51]. Since MAGL is the major hydrolyzing enzyme for 2-AG and is mainly localized in glial cells, it is possible that its blockade increases ATP-induced 2-AG production [51]. In addition, cultured microglia activated by ATP also induces 2-AG production in a phosphatidylinositol-specific phospholipase C and DG lipase-dependent manner [52]. As 2-AG is at least 2 orders of magnitude more abundant in the brain compared to anandamide (reviewed in [53]) and it

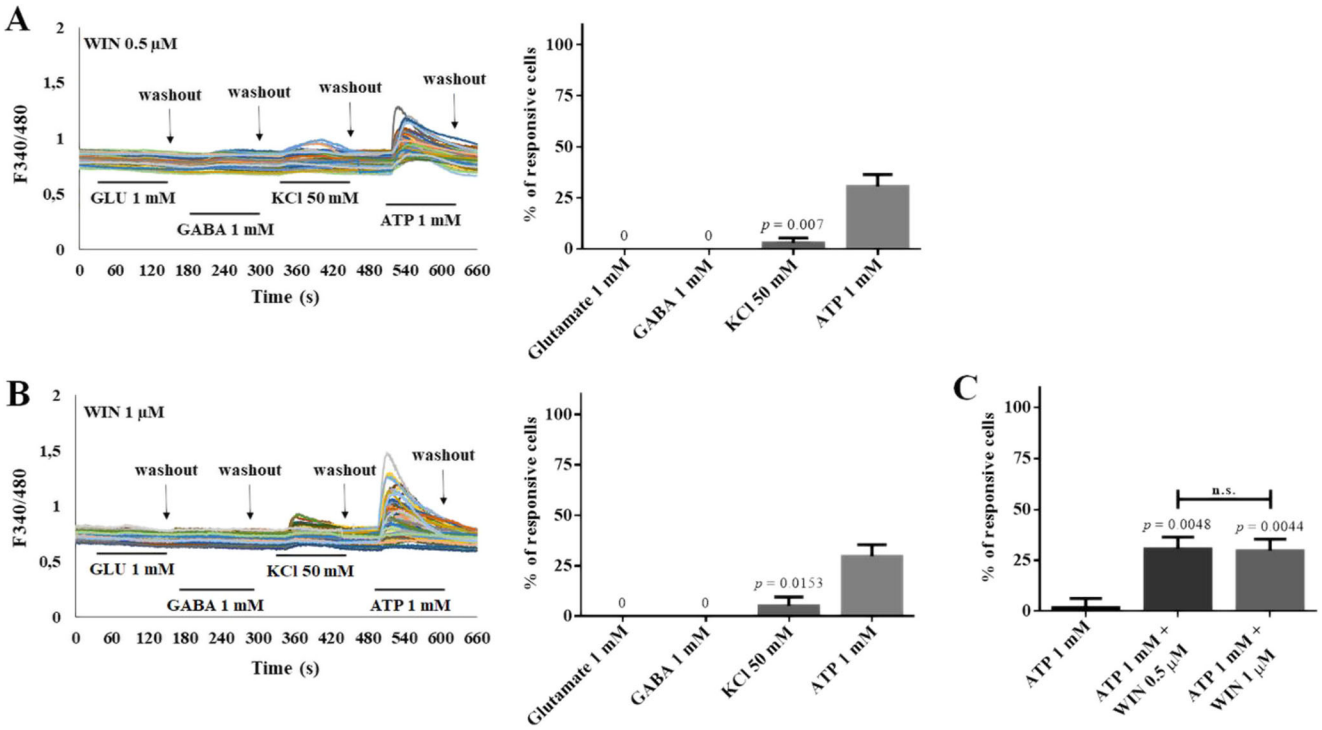

Fig. 7 Calcium shifts induced by several agonists in E7C2 chick embryonic retinal cells after WIN 55,212-2 treatment. Cell-response curve for 1.0 mM glutamate (GLU), 1.0 mM GABA, 50 mM KCl, or 1.0 mM ATP after 0.5 μM WIN (**a**) or 1.0 μM WIN (**b**) overnight treatment. Right panels in **a**, **b** show quantification (% of responsive cells) for each stimulus. **c** Percentage of cells responsive to 1.0 mM ATP in control or WIN-treated (0.5 μM or 1.0 μM) cultures. Significance level is shown in each dataset (n.s. nonsignificant). Cells were continuously washed with the buffer solution for 60 s in between stimuli, as indicated by the arrows in **a**, **b**. $N = 3$ for each experiment

is also more efficiently acting at both CB1 and CB2 receptors, our data suggest that a P2X7-2-AG loop is an important target for the control of differentiation and death of retinal progenitors.

Abundant evidence shows that exogenous cannabinoids are involved in cell survival responses [50, 54]. In pathological conditions, CB1 receptor can be neuroprotective [55, 56]. In contrast, during retinal development, our present results show that WIN, in a dose-dependent manner, induced a significant decrease in the number of living retinal cells in cultures at E7C2, a decrease that could be prevented by a CB1 (AM251) or CB2 (AM630) receptor antagonist. Since capsaicin did not affect the survival of the cultured retinal cells, our results strongly suggest that activation of CB1 or CB2, but not TRPV, receptors induces the death of developing retinal cells in culture. Accordingly, the synthetic cannabinoid CP-55,940 induces cell toxicity in the NG-108-15 cell line [57] and cell apoptosis in mouse forebrain cultures [58], an effect that is blocked by the CB1 receptor antagonist AM251. The hypothesis that activation of CB1 and CB2 receptors induces retinal cell death is also reinforced by the observation that WIN increases mitochondrial stress in chick embryonic retinal

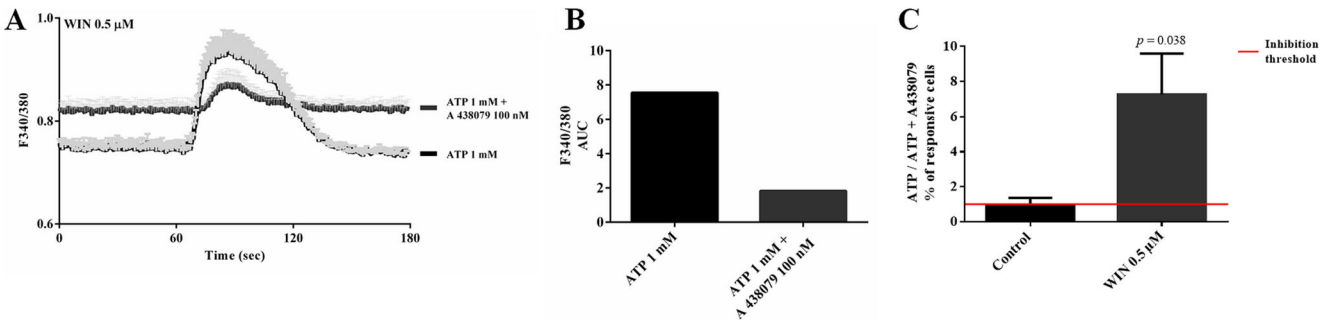

Fig. 8 Response of WIN-treated E7C2 retinal cells to ATP and a selective P2X7 receptor antagonist. Calcium shifts in **a** WIN-treated cells (0.5 μM) in the presence of 1 mM ATP or 1 mM ATP + 100 nM A438079 (selective P2X7R antagonist). **b** Quantification of the area under the curve (AUC) representative of **a** is shown in the middle panel. **c** Ratio of ATP/ATP + A438079 cell responsivity. A red line indicates the threshold (ATP/ATP + A438079 ratio > 1) for antagonistic activity of A438079 in control and WIN-treated cultures. Significance level is shown in each dataset (n.s. nonsignificant). $N = 3$ for each experiment

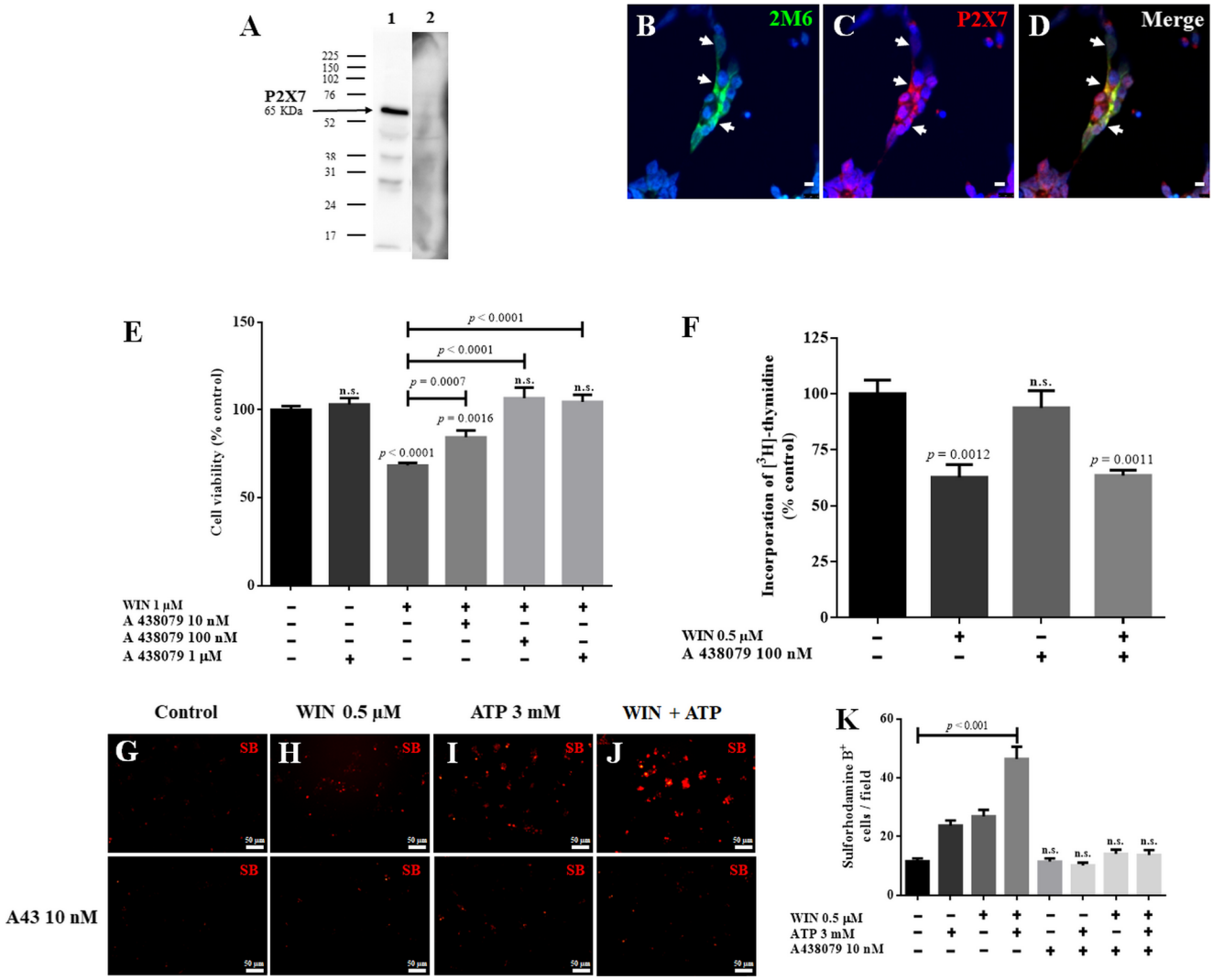

Fig. 9 Cell viability, proliferation, and sulforhodamine B (SB) uptake assay under WIN 55,212-2 treatment in the presence of a selective P2X7R antagonist. Representative immunoblot of the P2X7 receptor (**a**, lane 1), detected at 65 kDa, and anti-P2X7 receptor antibody, pre-incubated with the control peptide antigen (lane 2). Double labeling with **b** anti-2M6 and **c** anti-P2X7 from E7C2 cultures; the corresponding merged panel is shown in **d**. E7C2 cell viability loss under **e** 1.0 μM WIN challenged with 0.01 μM, 0.1 μM, or 1.0 μM A438079 cotreatments. **f** [^{3}H]-Thymidine incorporation under 0.5 μM WIN treatment and 0.1 μM A438079 cotreatment. **g**–**j** Photomicrographs of SB incorporation (red) in retinal cell culture after **g** control, **h** 0.5 μM WIN, **i** 3 mM ATP (10 min), and **j** 0.5 μM WIN + 3 mM ATP treatment in the absence (upper panels) or presence (bottom panels) of a selective P2X7R inhibitor (10 nM A438079). WIN was added overnight (for 24 h) from E7C1 (embryonic day 7 1 day in vitro) to E7C2. "WIN + ATP" indicates that cells treated overnight with WIN were incubated with ATP for 10 min prior to the experiment. **k** Quantification of the number of SB$^+$ cells per field under treatment conditions. Significance level (n.s. nonsignificant) is shown in **e**, **f**, and **k**. $N = 3$ for **a**–**f**, $N = 4$ for **k**. Scale bar in **b**–**d** = 10 μm. Scale bar in **g**–**j** = 50 μm

cultures. In good agreement, CB1 receptors were identified in neuronal mitochondria [59] and WIN decreases mitochondrial respiration in a dose-dependent manner in crude mitochondrial preparations from the pig brain cortex [60, 61].

Our data raise the possibility of CB1-CB2 receptor heterodimers involved in cell proliferation and cell death in the avian retina. In the last decade, heteromers associated to cannabinoid receptors have been reported in diverse models, such as CB1-5HT2A (serotonin) in olfactory-ensheathing cells [62], CXCR4-CB2 (chemokine) heteromers in human breast and prostate cancer cells [63], and CB1-OX1 (orexin) [64], mu-opioid receptor (muOR)-CB1 [65], or CB1-D2R (dopamine) [66] in cell lines or even expressed in vivo [67], but not in the retina, to our knowledge. Further studies should reveal their location and compartmentalization, as both CB1 and CB2 receptors are found in neurons and glial cells in the retina [12, 14, 27, 68].

As cannabinoids, nucleotides are increasingly associated with the development of the CNS, where they seem to trigger multiple events by activating both ionotropic P2X and G

protein–coupled P2Y receptors. While P2X7Rs are frequently associated with fast synaptic plasticity and transmission [69], inflammation, cell death, and pain [39, 70], P2Y receptors largely trigger trophic signaling required for cell proliferation, survival, and differentiation [71]. In the present study, we show that WIN-mediated retinal cell death is completely prevented by A438079, a highly selective P2X7R antagonist. Interestingly, no effect of the P2X7R antagonist was observed on the WIN-mediated decrease in [^{3}H]-thymidine incorporation in the cultures, suggesting that P2X7R is involved in the death of cultured retinal cells promoted by cannabinoids but not in the cannabinoid-dependent inhibition of retinal cell proliferation. Accordingly, activation of P2X7R induces the death of specific neuronal populations in the adult and developing mammalian retina [26, 72–74]. Here, we found that 1 μM WIN significantly decreases 2M6 content and abolish calcium responses from cell progenitors and neurons. Therefore, even though 1 μM WIN-mediated retinal cell death involves CB1/CB2 and P2X7 receptors, the cell population target is probably glial progenitors, but not neurons. Furthermore, the augment in calcium responses by ATP stimuli together with the increase in the number of cells that taken up the fluorescent dye SB, both when the cultures are treated with WIN for 24 h, suggests that the remaining glial cells underwent an increase in P2X7 content/response.

Reversible pore formation is a classical event associated with P2X7R activation, and it can be studied by the uptake of fluorescent dyes [75–77]. ATP is known to induce fluorescent dye uptake in cortical astrocytes [78] and Müller glial cells [16] through a phenomenon described as pore dilation. One intriguing finding observed in the present study is the increase in the number of cells that take up the fluorescent dye SB when the cultures are treated with WIN for 24 h. A similar number of labeled cells is observed when cultures are incubated with 3 mM ATP for 10 min, but the number of SB$^+$ cells increases further when WIN-pre-treated cultures are stimulated with the nucleotide. Since WIN-mediated uptake of fluorescent dye in the retinal cultures is completely blocked by a P2X7R antagonist, our results suggest that cannabinoid-mediated dye uptake involves the activation of P2X7R.

In this study, we show that prolonged treatment of the retinal cultures with WIN completely inhibited calcium currents induced by glutamate. Cannabinoids are involved in glutamatergic hypofunction, apparently through several mechanisms [79]. Although the majority of evidence points to a cannabinoid-mediated regulation of glutamate uptake and release [80, 81], cannabinoids also promote glutamate hypofunction directly, by forming a molecular complex between the C-terminus of the CB1R and the NR1 subunit of the NMDAR, causing a decrease in calcium currents and internalization of the NMDAR [82]. This possibility is in accordance with the findings that 1 μM WIN treatment did not alter Tuj-1 content, raising the possibility that cannabinoid/P2X7 receptors could modulate neuronal maturation response to glutamate in chick retinal cell cultures.

The present results also show that treatment with WIN prevents GABA-induced calcium shifts in the embryonic retinal cultures. One possibility would be that WIN induced the death of neuronal progenitors, eliminating not only cells that respond with GABA-induced calcium shifts but also KCl- and glutamate-responsive neurons. However, Tuj-1 content is not altered by 1.0 μM WIN treatment. On the other hand, the reduction of 2M6 content after 1 μM WIN exposure suggests that the cell death occurs preferentially from glial progenitor population.

Finally, our present results show that in early developing cultures, most cells are responsive to glutamate, KCl, and, to a less extent, GABA, but not to ATP. Thus, one intriguing finding of the present study is the increase in the number of ATP-responsive cells after treatment of the cultures with WIN, concomitant with the loss of GABA, glutamate, and KCl calcium responses. In retinal cultures at the developmental stage of E7C1, incorporation of [^{3}H]-thymidine occurs mostly in late-developing glial progenitors [23]. Since the present study shows that activation of cannabinoid receptors decreases the incorporation of [^{3}H]-thymidine in cultures at the same stage of development, the increase in the number of cells that responds to ATP with intracellular calcium increases may reflect a differentiation process of glial progenitors. Alternatively, since ATP-mediated calcium shifts are blocked by a P2X7R antagonist, the increase in the number of ATP-responsive cells could reflect only an increase in the expression of P2X7 receptors in glial cells with low expression of this receptor that cannot be detected in the calcium imaging experiments. To discriminate between these possibilities certainly will help clarify the contribution of cannabinoid receptors to the development and function of retinal glia.

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Author Contributions HRF and ALMV: conception and design, provision of the study material, collection and assembly of the data, data analysis and interpretation, manuscript writing, and final approval of the manuscript.

AIR, TMS, ECB, GOD, YSD, and MPG: collection and assembly of the data, and data analysis and interpretation.

FGM, RMR, GRF, and KCC: conception and design, provision of the study material, assembly of the data, data analysis and interpretation, manuscript writing, final approval of manuscript, financial support, and administrative support.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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