Abstract

Experimental studies demonstrated that saffron (Crocus sativus) given as a dietary supplement counteracts the effects of bright continuous light (BCL) exposure in the albino rat retina, preserving both morphology and function and probably acting as a regulator of programmed cell death [1]. The purpose of this study was to ascertain whether the neuroprotective effect of saffron on rat retina exposed to BCL is associated with a modulation of the endocannabinoid system (ECS). To this aim, we used eight experimental groups of Sprague-Dawley rats, of which six were exposed to BCL for 24 hours. Following retinal function evaluation, retinas were quickly removed for biochemical and morphological analyses. Rats were either saffron-prefed or intravitreally injected with selective type-1 (CB$_1$) or type-2 (CB$_2$) cannabinoid receptor antagonists before BCL. Prefeeding and intravitreally injections were combined in two experimental groups before BCL. BCL exposure led to enhanced gene and protein expression of retinal CB$_1$ and CB$_2$ without affecting the other ECS elements. This effect of BCL on CB$_1$ and CB$_2$ was reversed by saffron treatment. Selective CB$_1$ and CB$_2$ antagonists reduced photoreceptor death, preserved morphology and visual function of retina, and mitigated the outer nuclear layer (ONL) damage due to BCL. Of interest, CB$_2$-dependent neuroprotection was more pronounced than that conferred by CB$_1$. These data suggest that BCL modulates only distinct ECS elements like CB$_1$ and CB$_2$, and that saffron and cannabinoid receptors could share the same mechanism in order to afford retinal protection.

Introduction

Progressive degenerative diseases of retina, including age-related macular degeneration (AMD), diabetic retinopathy, retinitis pigmentosa, uveitis, retinal detachment as well as eye cancers (ocular melanoma and retinoblastoma), represent a large group of conditions that
affect visual function in young and old people [2,3]. Photoreceptors are the major targets of many of these retinal diseases [4], and a sequence of events has been shown to lead to their malfunction and eventually to death, [5]. Recently, it has been shown that photoreceptor death can be reduced in several animal models of neurodegeneration, by using both neuroprotectants [6] and antioxidants [7], and remarkably saffron (see for ref [8]). Saffron (Crocus sativus) is a well-known spice largely used in traditional medicine [9,10]. Its efficacy in slowing down retinal degenerative processes in rats exposed to high intensity light has been recently documented [1]. In particular, orally administered saffron partially preserved both morphology and function in light damaged retina [1]. A pilot clinical trial conducted on AMD patients provided the first evidence of successful saffron treatment in therapy [11], the positive effects being maintained in time [12] and in patients carrying genetic mutation [13]. Multiple actions of saffron have been suggested, including modulation of gene expression in animal models of retinal degeneration [14]. The latter process activates rather complex pathways, with many receptors and diffusible molecules playing pivotal roles in disease progression and activation of neuroprotective mechanisms [15,16]. All of them represent, indeed, potential targets of neuroprotectants. In the last few years, a new family of lipid mediators, called endocannabinoids (eCBs), received attention as possible activators of retina protection mechanisms in an animal model of ganglion cell death induced by high-intraocular pressure [17]. eCBs, such as N-arachidonoylthanolamine (anandamide, AEA) and 2-arachidonoylglycerol (2-AG), represent major neurotransmitters/neuromodulators in neural cells [18]. They bind to type-1 (CB1) and type-2 (CB2) cannabinoid receptors [19] and to the transient receptor potential vanilloid type 1 (TRPV1) channels [20,21]. Several enzymes are involved in eCB metabolism: AEA is synthesized mainly by N-acyl-phosphatidylethanolamines-specific phospholipase D (NAPE-PLD), and is degraded by fatty acid amide hydrolase (FAAH); 2-AG is mainly synthesized by a sn-1-specific diacylglycerol lipase (DAGL) and is degraded by a specific monoacylglycerol lipase (MAGL) [22–24]. Altogether eCBs, their target receptors and metabolic enzymes form the so-called endocannabinoid system (ECS) [25]. Incidentally, rat retina has been already shown to possess several components of a functional ECS [17].

Against this background, here we sought to investigate whether bright continuous light (BCL) could modulate ECS in the rat retina, and whether saffron treatment could exert a neuroprotective effect by involving distinct elements of this signaling system.

Materials and Methods

All experiments were conducted in accordance with ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, authorization number 83/96-A of 29/11/1996 by the Ministry of Health, and were approved by the local Ethical Committee of University of L’Aquila. Animals were born and reared in dim cyclic light conditions (12 hours light, 12 hours dark) with an ambient light level of approximately 5 lux [1].

Data reported in this study were obtained from experiments carried out on 78 Sprague Dawley (SD) rats 3 months old, divided in eight groups as described below (in brackets are the same abbreviations used in the Results section):

1. Group: control SD rats (control), (n = 6);
2. Group: SD rats treated with stigmas of saffron for two weeks (saffron);
3. Group: SD rats exposed to BCL, 1000 lux for 24 h (light damage, LD);
4. Group: SD rats treated with stigmas of saffron for two weeks and exposed to BCL (saffron+LD);
5. Group: SD rats injected intravitreally with the selective CB1 antagonist SR141716A [26] in the right eye (SR1+LD) and with saline in the left eye, and then exposed to BCL;

6. Group: SD rats injected intravitreally with the selective CB2 antagonist SR144528 [26] in the right eye (SR2+LD) and with saline in the left eye, and then exposed to BCL;

7. Group: SD rats pretreated with saffron for two weeks, injected with SR141716A in the right eye and then exposed to BCL (saffron+SR1+LD);

8. Group: SD rats pretreated with saffron for two weeks, injected with SR144528 in the right eye and then exposed to BCL (saffron+SR2+LD).

For each experimental group 12 animals were used; 6 were sacrificed immediately after LD, while the other half (n = 6) seven days after light exposure.

Diet supplementation
Albino rats were fed daily with a water suspension of 5 mg/kg stigmas as reported [1]. To avoid to use saffrons coming from different cultivars that may present different composition of the extract, in this paper we used only saffron derived from Hortus Novus (L’Aquila, Italy), whose chemical characteristics has been analytically determined (see for ref [27]).

Light exposure
Animals were placed in individual plexiglass cages with food available on the floor, water in plastic bottles and dark adapted overnight. At 9 am they were exposed to BCL (1000 lux) for 24 hours, as reported [1]. For each experimental group, half of animals were immediately euthanized after LD, and the other half were allowed to recover for one week after BCL exposure. Retinas for biochemical analysis were immediately removed and frozen at –80°C.

Intravitreal injections
Immediately before BCL, rats were anaesthetized by intraperitoneal injection of ketamine/xylazine (10 mg/100g – 1.2 mg/100g), were placed on the stereotactic microscope and a drop of local anesthetic (novocaine) was administered to each eye. 0.1 μM of SR141716A or SR144528 in 2 μl 0.9% NaCl were injected intravitreally in the right eye (of groups 5–8) using an Hamilton syringe with fixed needle (SYR 10 μl., ga 26s/51mm). Left eye was injected with 2 μl 0.9% NaCl alone, as a control. After the procedure the animals were located in post-operative cages and monitored until complete awakening. The binocular injection allowed the halving of the number of animals undergone to surgery since we did not included the control group (saline).

Quantitative RT-PCR analysis
RNA was extracted from rat retinas by using the RNeasy extraction kit (Qiagen, Crawley, UK), as suggested by the manufacturer. Quantitative real time reverse transcriptase-polymerase chain reaction (qRT-PCR) assays were performed using the SuperScript III Platinum Two-Step qRT-PCR Kit (Invitrogen, Carlsbad, CA, USA). One μg total RNA was used to produce cDNA with 10 U/μL SuperScript III reverse transcriptase, in the presence of 2 U/μL RNase-OUT, 1.25 μM oligo(dT)20, 1.25 ng/μL random hexamers, 5mM MgCl2, 0.5 mM dNTP mix and DEPC-treated water. The reaction was performed by using the following qRT-PCR program: 25°C for 10 min, 42°C for 50 min, 85°C for 5 min; then, after addition of 0.1 U/μL of E. coli RNase H, the product was incubated at 37°C for 20 min. Target transcripts were amplified
using an ABI PRISM 7700 sequence detector system (Applied Biosystems, Foster City, CA),
with the following primers: NAPE-PLD F, 5’-TGTCGGGTTCAAGAGAGC-3’,
NAPE-PLD R, 5’-ACCATCGGCTCGTCC-3’; DAGL F 5’-ATCTCTCTGCTCC TG-3’, DAGL R 5’-TTTGGCCGTGCTTGTTCCG-3’; FAAH F 5’-ATGGAAGAGGAGC
TGACATGC-3’, MAGL R 5’-ATGCGATCAGGTGATGGC-3’; CB1 F, 5’-TTCCACCGTA
AAGACAGCC-3’, CB1 R, 5’-TCCACCATCGGCAAAAGGC-3’; CB2 F, 5’-TTGAGGCAT
ACTATGCTGTC-3’, CB2 R, 5’-TGCTTCTCAGGAGCATACC-3’; TRPV1 F 5’-ATT
GAACGGCGGAACATGCAG-3’, TRPV1 R 5’-ATCTCTCCGCTCAGCG-3’; β-Actin F,
5’-ATGGAAGTCCTCCAGAGC-3’, β-Actin R 5’-TAGAGCTTTCAGGCATAGCG-3’;
DAGL F 5’-ATTCTCTCCTTCCTCTTG-3’, DAGL R 5’-ATTTGGGCTTGGTGCTTG-3’;
FAAH F 5’-ATGGAAGTCCTCCAGAGC-3’, FAAH R 5’-TAGAGCTTTCAGGCATAGCG-3’;
MAGL F 5’-ATGTTGAAGAGGTGGACATGC-3’, MAGL R 5’-ATGCAGATTCCGGA
TTGGC-3’; CB1 F, 5’-TTCCACCGTAAAGACAGCCC-3’, CB1 R, 5’-TCCACCATCG
GCAAAAGGC-3’; CB2 F, 5’-TTGAGGCATACCTCCAGAGC-3’, CB2 R, 5’-TGCTTC
TCCAGGAGCATACC-3’; TRPV1 F 5’-ATTGAACGGCGGAACATGCAG-3’, TRPV1 R
5’-ATCTCTCCAGCTCAGCG-3’; β-Actin F, 5’-ATGGTTCAGAATGATGATG-3’, β-Actin R,
5’-AAGGTTCAGAACATGATGTTG-3’. Differences in threshold cycle (Ct) number were
used to quantify the relative amount of PCR target in each tube. Relative expression of
different gene transcripts was calculated by the ΔΔCt method, and was converted to
relative expression ratio (2−ΔΔCt) for statistical analysis. β-Actin was used as
housekeeping gene for quantification [28].

Analysis of protein expression
Retinal lysates were obtained by sample homogenization in ice-cold lysis buffer (10 mM
EDTA, 50 mM Tris-HCl (pH 7.4), 150 mM sodium chloride, 1% Triton-X-100, 2 mM
phenylmethylsulfonylfluoride, 2 mM sodium orthovanadate, 10 mg ml−1 leupeptin, and 2
mg ml−1 aprotinin), and the amount of proteins was determined by the Bio-Rad Protein
assay (Bio-Rad Laboratories, Hemel Hempstead, UK). Equal amounts of total extracts (30
μg of protein) were electrophoresed on 10% acrylamide gels and transferred to polyvinylidene
fluoride membranes (Amersham Biosciences, Piscataway, NJ, USA). Membranes were
saturated with a solution of 5% nonfat dry milk, then were incubated with anti-NAPE-PLD
(1:100) (Cayman Chemicals, Ann Arbor, MI, USA; item n. 1035), anti-FAAH (1:500),
anti-DAGL (1:1000) (Santa Cruz Biotechnology Inc., Santa Cruz, CA; sc-26427, sc-133307), anti-MAGL
(1:200) anti-CB1 (1:250), anti-CB2 (1:250) (Cayman Chemicals, Ann Arbor, MI, USA, item n. 10035, n. 10006590, n. 101550), anti-TRPV1
(1:200) antibodies or with anti-β-actin (1:1000) antibody (Santa Cruz Biotechnology Inc.,
Santa Cruz, CA, sc-12498. Sc-1616). Then, they were incubated with specific horseradish
peroxidase-conjugated (HRP) secondary antibodies diluted 1:2000 (Santa Cruz Biotechnology Inc.,
Santa Cruz, CA, USA). Detection was performed by using the West Dura Chemiluminescence
System (Pierce, Rockford, IL, USA), and the intensity of the immunoreactive bands was
quantified by densitometric analysis through the ImageJ software (NIH, Bethesda, MD, USA).
The specificity of each antibody used was tested in rat as already reported [17,29,30].

In some experiments, protein expression of CB1 and CB2 was also determined by enzyme
linked immunosorvent assay (ELISA), as reported [31]. Briefly, wells were coated with retinal
lysates (20 μg/well) and were incubated for 1 h at room temperature with anti-CB1 or anti-CB2
polyclonal antibodies at the same dilutions used in Western blotting analysis. After rinsing
three times with 5% BSA/PBS-Tween 20, 100 μl of HRP-conjugated secondary antibody
diluted 1:5000 was added and the ELISA plate was further incubated for 30 min at room
temperature. HRP enzymatic activity was determined by the addition of 100 μL/well of
tetramethylbenzidine (TMB) containing H2O2 (0.002%), and the absorbance was read on a
Multiskan ELISA Microplate Reader (ThermoLabsystems, Bevery, MA, USA) at 450 nm.
Results were expressed as a percentage of the control (100%).

Morphology and immunohistochemistry
Animals were sacrificed immediately after LD, the eyes were enucleated, fixed, embedded,
cryosectioned and immunostained. Sections were labelled for apoptotic cell death using the
terminal deoxynucleotidyltransferase d-UTP nick end labeling (TUNEL) technique following protocols, as previously described [32]. Counts of TUNEL+ (apoptotic) cells in the outer nuclear layer ONL were made using a calibrated 20 x objective. Each section was scanned from the superior to inferior edge, and the number of TUNEL+ cells was recorded for each 400 μm length of the section. The total number of TUNEL+ cells for each experimental group was normalized respect to LD group.

Retinal sections were also immunolabeled for CB1 and CB2, removing non-specific binding with 0.75% horse serum. Sections were incubated with rabbit anti-CB1 (overnight at 4˚C) or anti-CB2 (3 days at 4˚C) polyclonal antibodies diluted 1:200. For immunohistochemistry of CB2 a different antibody was used abcam ab3561), compared to Western Blotting analysis, because it is designed for use with frozen tissues sections. Secondary antibody was anti-rabbit IgG conjugated to fluorescent dye (Alexa Fluor 594 or 488; Life Technology) diluted 1:200 and incubated at 37˚C for 2 hours. At the end of the procedure the images were taken by confocal microscope (Nikon 80i), as reported [1].

To evaluate the entity of the damage in the superior retina, the extension of the “hot spot” was measured. This analysis was performed in retinal sections one week after BCL. Sections were labelled with the DNA-specific dye bisbenzimide (Calbiochem, La Jolla, CA), by incubating them for 2 min in a 1:10,000 solution in 0.1 M PBS. Images were taken by confocal microscope (Nikon 80i).

**Electrophysiologic recordings**

To evaluate visual function, electroretinogram (fERG) in response to flashes of increasing luminance was recorded one week after BCL. Albino rats were dark adapted for a 12 hour period overnight and electroretinograms were recorded in a completely darkened room [33]. Briefly, animals were anaesthetized by an intraperitoneal injection of Ketamine/Xylazine (10 mg/100g–1.2 mg/100g) and mounted in a stereotaxic apparatus and positioned inside the opening of the Ganzfeld dome (Biomedica Mangoni, Pisa, Italy). The body temperature was maintained at 37.5˚C with a heating pad controlled by a rectal temperature probe. Corneas were anesthetized with a drop of novocaine, and pupils were dilated with 1% tropicamide. This electronic flash unit generated flashes of a range of intensities from 0.001–100 cd/m². Responses were recorded over 300 ms plus 25 ms of pre-trial baseline, amplified differentially, bandpass filtered at 0.3 to 300 Hz, digitized at 0.25- to 0.3-ms intervals by a LabVIEW 8.2 personal computer interface (National Instruments, Milan, Italy). The amplitude of the b-wave was measured from the most negative point of the average trace to the highest positive point. At the end of the recording session, animals were sacrificed, the eyes removed and retinas were used for retinal histology.

**Statistical Analysis**

Data are reported as means ± S.E.M of at least six independent experiments, each performed in duplicate. Data were analysed by the Prism 5 program (GraphPad Software, La Jolla, CA), using one-way analysis of variance (ANOVA) followed by Tukey test or Bonferroni *post hoc* analysis. A level of p<0.05 was considered statistically significant.

**Results**

**Effect of BCL on expression of ECS genes and proteins**

In the first set of experiments, the effects of retinal damage induced by exposure to BCL were assayed on ECS expression, by means of qRT-PCR and Western blotting analyses. These
procedures were carried out on 12 animals belonging to 1˚ and 3˚ group (control and LD without recovery). The results of qRT-PCR experiments on gene expression of the main components of ECS in retina from LD rat are shown in Fig 1. Only CB$_1$ and CB$_2$ mRNA levels increased in the retinas of LD rats with respect to controls, by ~3-fold and ~4-fold respectively. Instead, none of the other ECS elements tested (i.e., NAPE-PLD, DAGL, FAAH, MAGL and TRPV1) was affected (Fig 1).

In keeping with these mRNA data, Western blotting (Fig 2) showed a significant increase ($p<0.05$) only in the expression of CB$_1$ and CB$_2$ proteins in LD rats with respect to controls.

Effect of saffron on BCL-induced expression of CB$_1$ and CB$_2$

In order to investigate the involvement of CB$_1$ and CB$_2$ in the neuroprotective effect of saffron against BCL, further analyses were performed in retinas from rats prefed with saffron. Interestingly, after two weeks of treatment with saffron stigmas and after exposure to BCL (without recovery), mRNA levels of CB$_1$ and CB$_2$ decreased significantly ($p<0.001$ for CB$_1$; $p<0.0001$ for CB$_2$), and returned to controls (Fig 3).

Similarly, at protein level a significant reduction of CB$_1$ and CB$_2$ ($p<0.01$) expression was observed following saffron treatment (saffron+LD) with respect to LD group (Fig 4). Additional ELISA assays (Table 1) confirmed the increase of CB$_1$ and CB$_2$ ($p<0.001$ for CB$_1$; $p<0.01$ for CB$_2$) protein expression in LD rats compared to controls, as well as their reduction upon saffron treatment (saffron+LD). Instead, no statistically significant differences were found between saffron and control groups (data not shown). In particular, the effect of saffron on CB$_2$ transcription and translation was larger than that on CB$_1$, suggesting a stronger engagement of this receptor in retinal protection by saffron. These analysis were performed on 18 animals (groups 1˚, 3˚ and 4˚).
Localization of CB\textsubscript{1} and CB\textsubscript{2}

In order to confirm the modulation of CB\textsubscript{1} and CB\textsubscript{2} by saffron treatment, their retinal localization was evaluated through immunohistochemistry technique in 24 animals (1\textdegree, 2\textdegree, 3\textdegree and 4\textdegree group) (Figs 5 and 6). Our data demonstrate that CB\textsubscript{1} was localized in both outer and inner plexiform layers (OPL and IPL) of all experimental groups. In particular, axon terminals of bipolar cells were labelled in the IPL of controls (Fig 5), in keeping with a previous report [34]. After exposure to BCL (without recovery), CB\textsubscript{1} immunoreactivity increased without any change in receptor localization (LD group in Fig 5). Saffron treatment reduced CB\textsubscript{1} expression after exposure to LD (saffron+LD group). The immunofluorescence intensity of CB\textsubscript{1} in saffron group appears reduced respect to control, although quantitative analysis of protein level did not show any statistical difference between the two groups (Fig 5). Similar effect was observed.
Fig 3. CB₁ and CB₂ mRNA levels following saffron treatment and BCL exposure. qRT-PCR analysis of CB₁ and CB₂ in the retinas from untreated controls, rats exposed to bright continuous light alone (LD), or in combination with saffron (saffron + LD). Data were expressed as means ± SEM (n = 6), and were analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni post hoc analysis. ***p<0.001, ****p<0.0001 vs control, ###p<0.001, ####p<0.0001 vs LD.

doi:10.1371/journal.pone.0166827.g003
also for CB$_2$ immunolocalization. Indeed, in all experimental groups CB$_2$ was mainly localized in the inner segment of photoreceptors and also in the inner retina (inner nuclear layer and ganglion cell layer) (Fig 6), extending previous studies [35]. After exposure to BCL (LD group in Fig 6), CB$_2$ immunoreactivity increased in the inner retinal layers and was reduced by saffron treatment (saffron and saffron+LD groups in Fig 6).
Quantitative analysis of apoptotic cells

It has already demonstrated that exposure to BCL (24 hours at 1000 lux) induces photoreceptor death; the maximum level of apoptotic cells is evident immediately after damaging light particularly in the superior retina, an area known as “hot spot” [36,37,8]. This area is represented in Fig 7A where dying photoreceptors (red dots) are evident in the ONL. In Fig 7B we report the normalized TUNEL+ (apoptotic) cells, showing a highly significant reduction of apoptotic cells in all experimental groups respect to LD group. Saffron treatment significantly reduced the number of dying neurons in line with previous data [1]. Interestingly, intravitreal pre-treatment with CB\(_1\) (SR1+LD group) or CB\(_2\) (SR2+LD group) antagonists also reduced neuronal death, though in a slightly less efficient manner than saffron. Supplementation with the latter substance reduced further the number of TUNEL+ cells only in rats pre-treated with CB\(_1\) antagonist (saffron+SR1+LD group), suggesting that saffron and CB\(_1\) may share the same transduction pathways. This conclusion was supported by the ELISA assay (Table 1).

Retinal function

Retinal function was evaluated one week after exposure to BCL in all experimental groups, by recording fERG under dark-adapted conditions (Fig 8). The amplitude of the b-wave was strongly reduced by LD, an effect counteracted by saffron treatment (saffron+LD group), as already reported [1]. Moreover b-wave amplitude, recorded in rats intravitreally pre-treated with SR1 or SR2 with or without saffron supplementation, was improved respect to that of LD group (Fig 8A and 8B). Indeed, Fig 8A shows that b-wave amplitude recorded after inactivation of CB\(_1\) (SR1+LD group) was super imposable on that of the saffron + LD group, yet only at a low intensity light stimulus (0.001–10 cd/m\(^2\)). At higher intensity (100 cd/m\(^2\)), the reduction of fERG response was still significant, but the response of the SR1 + LD group differed from that of the saffron + LD group (Fig 8A). Co-administration of saffron and SR141716A (saffron+SR1+LD group) did not further improve b-wave amplitude (Fig 8A). The same analysis of fERG response was performed after inactivation of CB\(_2\) by SR144528 (SR2+LD group) with or without saffron treatment (Fig 8B). Upon SR2 treatment, b-wave amplitude increased compared to LD alone, especially at high intensity light flashes (100 cd/m\(^2\)); (Fig 8B). A combination of saffron and SR2 (saffron+SR2+LD group) yielded a fERG response super imposable on that of the saffron + LD group (at least up to 100 cd/ m\(^2\) Fig 8B).

Morphological analysis

The superior retina, from dorsal edge to optic nerve, was analyzed in order to evaluate the extension of degeneration induced by light exposure under all experimental conditions (Fig 9).
Representative images of nuclear staining with bisbenzimide showed a specific dorsal area of ONL, called “hot spot” (Fig 9A). Light exposure induced a maximal damage to this region (Fig 9A), in keeping with a previous report [38]. Comparing ONL morphology across all groups, photoreceptor layer appeared well-preserved compared to the LD group; in particular, retinal morphology of the saffron group was very close to that of controls (Fig 9A). To better assess the rate of photoreceptor survival, the extension of the hot spot area was measured with respect to the entire length of the superior retina (Fig 9B). The least damaged area was observed in the saffron + LD group, but also SR141716A and SR144528 decreased hot spot damage, suggesting that selective blockage of CB₁ and CB₂ can protect photoreceptors against environmental stress (Fig 9B). Again, saffron in combination with CB₁ (saffron+SR1+LD group) or CB₂ antagonists (saffron+SR2+LD group) did not induce any additional protective effect (Fig 9B).
Regarding the intravitreal injections in both eyes (vehicle and antagonist respectively), even though a cross-talk was present between both eyes, the different effect between the two allowed us to conclude that antagonist administration protects neuroretina independently of the mechanical damage (that was the same in both eyes). In addition, results obtained after saline injections have demonstrated the absence of a systemic effect of the antagonists (SR1, SR2). Incidentally, we chose to skip data of the contralateral retinae in the plots, because they did not add any further information.

**Discussion**

The presence of ECS in retina is well-documented in numerous species from fishes to primates [15,35,39,40] although it has been recently pointed out that “the biological functions of eCBs,
TRPV1 and their interactions across retinal circuits remain almost entirely unknown” [41].

The evidence for 2-AG metabolic enzymes in rat retina is demonstrated here for the first time at the gene level, extending previous data on localization of DAGL and MAGL proteins during postnatal development of the rat retina [42]. Present data also extend previous evidence on the neuroprotective role of saffron in retinal damage [1], and suggest an unprecedented engagement of eCB signaling in this process. Indeed, eCBs have been reported to regulate photoreception and neurotransmission in the retina, and to have effects on intraocular pressure and ocular blood vessels [15,17,41,43] as well as neuroprotective effects against retinal neurotoxicity [15,44]. In line with this, in a previous study we demonstrated that anandamide is neuroprotective against retinal ganglion cell death induced by high intraocular pressure, via a CB1-dependent pathway [17]. Here, we provide the first evidence that BCL selectively affects ECS...
gene and protein expression in retina, where only CB\(_1\) and CB\(_2\) levels were increased. Instead, the other major components of retinal ECS were not modulated by BCL, including TRPV1 that plays a role in retinal death induced during IOP-related disease [17,41]. Apparently, saffron per se has little effect on retinal gene expression, but when administered before LD it does modulate the large changes in gene expression induced by this treatment [14]. In keeping with this notion, here we demonstrate that saffron down-regulates gene and protein expression of CB\(_1\) and CB\(_2\) in an animal model of retinal degeneration induced by light exposure. Consistently, we document that selective blockage of both CB\(_1\) and CB\(_2\) is able to reduce LD-induced photoreceptor death, thus preserving morphology and visual function, suggesting that these receptors are involved in neurodegenerative processes and are negatively modulated by saffron. Interestingly, literature data indicate that CB\(_2\) might be implicated in rod and cone sensitivity and light adaptation (see for ref. [35]). Consistently, our data suggest a major involvement of CB\(_2\) compared to CB\(_1\) in protecting photoreceptors from LD. Altogether our results indicate the possibility that the neuroprotective effects of saffron might impinge upon CB\(_1\)/CB\(_2\) –dependent signal transduction pathways. Here, we found an increased amplitude in the b-wave of rats with retinal damage treated with CB\(_1\)/CB\(_2\) antagonists but interestingly the b-wave amplitude in animal prefer with saffron or double treated with saffron and CB\(_1\) or CB\(_2\) antagonists are quite similar. The major difference happens to be at high luminance and always in favour of saffron only. In addition, accumulating evidence shows that CB\(_1\)/CB\(_2\) levels are elevated in pathological retinal conditions sometime associated to oxidative stress [15,45]. Also in this study we observed such an increase after retinal damage induced by exposure to BCL, that often results in retina degeneration. Saffron is also endowed with a potent antioxidant activity, that has been attributed primarily to its crocins constituent (see for ref [8]). In line with this, reduction of inflammation due to the downregulation of chemokine CCL2 by saffron [14], and remarkably also by CB\(_1\)/CB\(_2\) antagonists [46], could be a common pattern of response against retinal damage. Indeed, when the retina is damaged by bright light, a variety of pathways are activated, including an upregulation of Chemokine (C-C motif) Ligand 2 (CCL2) which recruits macrophages to scavenge retinal debris. Unsurprisingly, homozygous deletion of CCL2 results in a mouse phenotype similar to human AMD [47]. In general, CCL2 is implicated in inflammatory cell migration into inflamed tissues and nociception, processes that have been both related also to CB\(_2\)-dependent signaling [48–50]. In this context, it should be recalled that CB\(_2\) plays a key-role in chemokine production and release by immune cells, i.e. microglia, thus regulating several inflammatory processes [51,52]. Retinal neuro-inflammation is strictly related to the activation of microglia (see for ref [53]), that in physiological condition maintain homeostasis in the retina also controlling synaptic activity in a continuous cross-talk with other retinal neurons. It can be suggested a direct control on CB receptors widely expressed across the retina and whose activation regulates calcium and potassium current. In conclusion, it might be that one of the neuroprotective pathways activated by saffron includes the activation of endocannabinoid system. Detailed analysis of a variety of possible neuroprotective ways of action is under investigation. Overall, topical CB\(_1\) and CB\(_2\) antagonists, in combination with saffron supplement in the diet, might be a potential novel treatment to cope with retinal neurodegenerative processes.
We wish to thank Dr. Maria Maggi (Hortus Novus s.r.l. and Laboratory of Analytical Chemistry, University of L’Aquila) for performing chemical analysis of saffron.

Fig 9. Bisbenzimide labelling and quantitative analysis of hot spot extension. Panel A: Representative images labelled with a nuclear staining (bisbenzimide) in a dorsal position one millimeter from optic disc (“hot spot”) in LD, saffron+LD, SR1+LD, saffron+SR1+LD, SR2+LD, saffron+SR1+LD, one week after light damage (LD). Scale bar, 50 μm. Panel B: Ratio of hot spot/superior retinal length. Data were expressed as means ± SEM (n = 6). *** p<0.001 vs LD.

doi:10.1371/journal.pone.0166827.g009

Acknowledgments

We wish to thank Dr. Maria Maggi (Hortus Novus s.r.l. and Laboratory of Analytical Chemistry, University of L’Aquila) for performing chemical analysis of saffron.
Author Contributions
Conceptualization: SB MM.
Data curation: SDM.
Formal analysis: DZ MDT.
Funding acquisition: SB MM.
Investigation: RM CR NB DZ SDM MDT.
Methodology: RM CR.
Project administration: RM CR.
Resources: DZ MDT.
Software: SDM.
Supervision: SB MM.
Validation: RM CR NB.
Visualization: DZ RM CR NB.
Writing – original draft: CR RM.
Writing – review & editing: SB MM.

References
1. Maccarone R, Di Marco S, Bisti S. Saffron supplement maintains morphology and function after exposure to damaging light in mammalian retina. Invest Ophthalmol Vis Sci. 2008; 49: 1254–61. doi: 10.1167/iovs.07-0438 PMID: 18326756
2. Marigo V. Programmed cell death in retinal degeneration: targeting apoptosis in photoreceptors as potential therapy for retinal degeneration. Cell Cycle. 2007; 6: 652–5. Available: http://www.ncbi.nlm.nih.gov/pubmed/17374995 doi: 10.4161/cc.6.6.4029 PMID: 17374995
3. Murakami Y, Notomi S, Hisatomi T, Nakazawa T, Ishibashi T, Miller JW, et al. Photoreceptor cell death and rescue in retinal detachment and degenerations. Prog Retin Eye Res. 2013; 37: 114–40. doi: 10.1016/j.preteyeres.2013.08.001 PMID: 23994436
4. Wright AF, Chakarova CF, Abd El-Aziz MM, Bhattacharya SS. Photoreceptor degeneration: genetic and mechanistic dissection of a complex trait. Nat Rev Genet. 2010; 11: 273–84. doi: 10.1038/nrg2717 PMID: 20212494
5. Sancho-Peluz J, Arango-Gonzalez B, Kustermann S, Romero FJ, van Veen T, Zrenner E, et al. Photoreceptor cell death mechanisms in inherited retinal degeneration. Mol Neurobiol. 2008; 38: 253–69. doi: 10.1007/s12035-008-8045-9 PMID: 18982459
6. Strettoi E, Gargini C, Novelli E, Sala G, Piano I, Gasco P, et al. Inhibition of ceramide biosynthesis preserves photoreceptor structure and function in a mouse model of retinitis pigmentosa. Proc Natl Acad Sci U S A. 2010; 107: 18706–11. doi: 10.1073/pnas.1007644107 PMID: 20937879
7. Komeima K, Rogers BS, Campochiaro PA. Antioxidants slow photoreceptor cell death in mouse models of retinitis pigmentosa. J Cell Physiol. 2007; 213: 809–15. doi: 10.1002/jcp.21152 PMID: 17520694
8. Bisti S, Maccarone R, Falsini B. Saffron and retina: neuroprotection and pharmacokinetics. Vis Neurosci. 2014; 31: 355–61. doi: 10.1017/S0952523814000108 PMID: 24819927
9. Abdullaev FI. Biological effects of saffron. Biofactors. 1993; 4: 83–6. Available: http://www.ncbi.nlm.nih.gov/pubmed/8347278 PMID: 8347278
10. Soeda S, Ochiai T, Paopong L, Tanaka H, Shoyama Y, Shimeno H. Crocin suppresses tumor necrosis factor-alpha-induced cell death of neurally differentiated PC-12 cells. Life Sci. 2001; 69: 2887–98. Available: http://www.ncbi.nlm.nih.gov/pubmed/11720092 PMID: 11720092
11. Falsini B, Piccardi M, Minnella A, Savastano C, Capoluongo E, Fadda A, et al. Influence of saffron supplementation on retinal flicker sensitivity in early age-related macular degeneration. Invest Ophthalmol Vis Sci. 2010; 51: 6119–24. doi: 10.1167/iovs.09-4995 PMID: 20686744
12. Piccardi M, Marangoni D, Minnella AM, Savastano MC, Valentini P, Ambrosio L, et al. A longitudinal follow-up study of saffron supplementation in early age-related macular degeneration: sustained benefits to central retinal function. Evid Based Complement Alternat Med. 2012; 2012: 429124. doi: 10.1155/ 2012/429124 PMID: 22852021

13. Marangoni D, Falsini B, Piccardi M, Ambrosio L, Minnella AM, Savastano MC, et al. Functional effect of Saffron supplementation and risk genotypes in early age-related macular degeneration: a preliminary report. J Transl Med. 2013; 11: 228. doi: 10.1186/1479-5876-11-228 PMID: 24067115

14. Natoli R, Zhu Y, Valter K, Bisti S, Eells J, Stone J. Gene and noncoding RNA regulation underlying photoreceptor protection: microarray study of dietary antioxidant saffron and photobiomodulation in rat retina. Mol Vis. 2010; 16: 1801–22. Available: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid= 2932490&tool=pmcentrez&rendertype=abstract PMID: 20844572

15. Yazulla S. Endocannabinoids in the retina: from marijuana to neuroprotection. Prog Retin Eye Res. 2008; 27: 501–26. doi: 10.1016/j.preteyeres.2008.07.002 PMID: 18725316

16. Unsicker K. Neurotrophic molecules in the treatment of neurodegenerative disease with focus on the retina: status and perspectives. Cell Tissue Res. 2013; 353: 205–18. doi: 10.1007/s00441-013- 1585-y PMID: 23463189

17. Nucci C, Gasperi V, Tartaglione R, Cerulli A, Terrinoni A, Bari M, et al. Involvement of the endocannabinoid system in retinal damage after high intraocular pressure-induced ischemia in rats. Invest Ophthal mol Vis Sci. 2007; 48: 2997–3004. doi: 10.1167/ iovs.06-1355 PMID: 17591864

18. Fezza F, Bari M, Florio R, Talamonti E, Feole M, Maccarrone M. Endocannabinoids, related compounds and their metabolic routes. Molecules. 2010; 19: 17078–106. doi: 10.3390/ molecules19117078 PMID: 25347455

19. Pertwee RG, Howlett AC, Abood ME, Alexander SPH, Di Marzo V, Elphick MR, et al. International Union of Basic and Clinical Pharmacology. LXXIX. Cannabinoid receptor s and their ligands: beyond CB₁ and CB₂. Pharmacol Rev. 2010; 62: 588–631. doi: 10.1124/pr.110.003004 PMID: 21079038

20. Di Marzo V. Endocannabinoids as regulators of transient receptor potential (TRP) channels: A further opportunity to develop new endocannabinoid-based therapeutic drugs. Curr Med Chem. 2010; 17: 1430–49. Available: http://www.ncbi.nlm.nih.gov/pubmed/20166923 PMID: 20166923

21. Starowicz K, Makuch W, Korostynski M, Malek N, Slezak M, Zychowska M, et al. Full inhibition of spinal FAAH leads to TRPV1-mediated analgesic effects in neuropathic rats and possible lipoxygenase-mediated remodeling of anandamide metabolism. PLoS One. 2013; 8: e60040. doi: 10.1371/journal.pone.0060040 PMID: 23573230

22. Ahn K, McKinney MK, Cravatt BF. Enzymatic pathways that regulate endocannabinoid signaling in the nervous system. Chem Rev. 2008; 108: 1687–707. doi: 10.1021/cr0782067 PMID: 18429637

23. Di Marzo V. Endocannabinoids: synthesis and degradation. Rev Physiol Biochem Pharmacol. 2008; 160: 1–24. doi: 10.1007/112_0505 PMID: 18481028

24. Ueda N, Tsuboi K, Uyama T, Ohnishi T. Biosynthesis and degradation of the endocannabinoid 2-arachidonoylglycerol. Biofactors. 37: 1–7. doi: 10.1002/biof.131 PMID: 21328621

25. Maccarrone M, Guzmán M, Mackie K, Doherty P, Harkany T. Programming of neural cells by (endo) cannabinoids: from physiological rules to emerging therapies. Nat Rev Neurosci. 2014; 15: 786–801. doi: 10.1038/nrn3846 PMID: 25409697

26. Pertwee RG. Receptors and channels targeted by synthetic cannabinoid receptor agonists and antagonists. Curr Med Chem. 2010; 17: 1360–81. Available: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3013229&tool=pmcentrez&rendertype=abstract PMID: 20166927

27. Corso L, Cavallero A, Baroni D, Garbati P, Prestipino G, Bisti S, et al. Saffron reduces ATP-induced retinal cytotoxicity by targeting P2X7 receptors. Purinergic Signal. 2016; 12: 161–74. doi: 10.1007/s11302- 015-9490-3 PMID: 26739703

28. Pucci M, Pasquarisi N, Battista N, Di Tommaso M, Rapino C, Fezza F, et al. Endocannabinoids stimulate human melanogenesis via type-1 cannabinoid receptor. J Biol Chem. 2012; 287: 15466–78. doi: 10.1074/jbc.M111.314880 PMID: 22431736

29. Viscomi MT, Oddi S, Latini L, Pasquarisi N, Fiorenzano F, Bernardi G, et al. Selective CB2 receptor agonist protects central neurons from remote axotomy-induced apoptosis through the PI3K/Akt pathway. J Neurosci. 2009; 29: 4564–70. doi: 10.1523/JNEUROSCI.0786-09.2009 PMID: 19357281

30. Latini L, Bisicchia E, Sasso V, Chiurchiu V, Cavallucci V, Molinari M, et al. Cannabinoid CB2 receptor (CB2R) stimulation delays rubrospinal mitochondrial-dependent degeneration and improves functional recovery after spinal cord hemisection by ERK1/2 inactivation. Cell Death Dis. 2014; 5: e1404. doi: 10.1038/cddis.2014.364 PMID: 25188514
31. Gasperi V, Fezza F, Pasquariello N, Bari M, Oddi S, Agrò AF, et al. Endocannabinoids in adipocytes during differentiation and their role in glucose uptake. Cell Mol Life Sci. 2007; 64: 219–29. doi: 10.1007/s00018-006-4645-4 PMID: 17187172

32. Maslinski J, Valter K, Egensperger R, Holländer H, Stone J. Tissue oxygen during a critical developmental period controls the death and survival of photoreceptors. Invest Ophthalmol Vis Sci. 1997; 38: 1667–77. Available: http://www.ncbi.nlm.nih.gov/pubmed/9286255 PMID: 9286255

33. Gargini C, Bisti S, Demontis GC, Valter K, Stone J, Cervetto L. Electoretinogram changes associated with retinal upregulation of trophic factors: observations following optic nerve section. Neuroscience. 2004; 126: 775–83. doi: 10.1016/j.neuroscience.2004.04.028 PMID: 15183525

34. Yazulla S, Studholme KM, McIntosh HH, Deutsch DG. Immunocytochemical localization of cannabinoid CB1 receptor and fatty acid amide hydrolase in rat retina. J Comp Neurol. 1999; 415: 80–90. Available: http://www.ncbi.nlm.nih.gov/pubmed/10540359 PMID: 10540359

35. Bouchard J-F, Casanova C, Cécyre B, Redmond WJ. Expression and Function of the Endocannabinoid System in the Retina and the Visual Brain. Neural Plast. 2016; 2016: 9247057. doi: 10.1155/2016/9247057 PMID: 26839718

36. Stone J, Maslinski J, Valter-Kocsi K, Mervin K, Bowers F, Chu Y, et al. Mechanisms of photoreceptor death and survival in mammalian retina. Prog Retin Eye Res. 1999; 18: 689–735. Available: http://www.ncbi.nlm.nih.gov/pubmed/10530749 PMID: 10530749

37. Rutar M, Provis JM, Valter K. Brief exposure to damaging light causes focal recruitment of macrophages, and long-term destabilization of photoreceptors in the albino rat retina. Curr Eye Res. 2010; 35: 631–43. doi: 10.3109/02713681003682925 PMID: 20597649

38. Wenzel A, Grimm C, Samardzija M, Remé CE. Molecular mechanisms of light-induced photoreceptor apoptosis and neuroprotection for retinal degeneration. Prog Retin Eye Res. 2005; 24: 275–306. doi: 10.1016/j.preteyeres.2004.08.002 PMID: 15610977

39. Lograno MD, Romano MR. Cannabinoid agonists induce contractile responses through Gi/o-dependent activation of phospholipase C in the bovine ciliary muscle. Eur J Pharmacol. 2004; 494: 55–62. doi: 10.1016/j.ejphar.2004.04.039 PMID: 15194451

40. Urquhart P, Wang J, Woodward DF, Nicolaou A. Identification of prostamides, fatty acyl ethanolamines, and their biosynthetic precursors in rabbit cornea. J Lipid Res. 2015; 56: 1419–33. doi: 10.1194/jlr.M055772 PMID: 26031663

41. Ryskamp DA, Redmon S, Jo AO, Krížaj D. TRPV1 and Endocannabinoids: Emerging Molecular Signals that Modulate Mammalian Vision. Cells. 2014; 3: 914–38. doi: 10.3390/cells3030914 PMID: 25222270

42. Cécyre B, Monette M, Beudjekian L, Casanova C, Bouchard J-F. Localization of diacylglycerol lipase alpha and monoacylglycerol lipase during postnatal development of the rat retina. Front Neuroanat. 2014; 8: 150. doi: 10.3389/frnana.2014.00150 PMID: 25565975

43. Cairns EA, Baldridge WH, Kelly MEM. The Endocannabinoid System as a Therapeutic Target in Glaucoma. Neural Plast. 2016; 2016: 9364091. doi: 10.1155/2016/9364091 PMID: 26881140

44. El-Remessy AB, Khalil IE, Matragoon S, Abou-Mohamed G, Tsai N-J, Roon P, et al. Neuroprotective effect of (-)Δ9-tetrahydrocannabinol and cannabidiol in N-methyl-D-aspartate-induced retinal neurotoxicity: involvement of peroxynitrite. Am J Pathol. 2003; 163: 1997–2008. Available: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1892413&tool=pmcentrez&rendertype=abstract PMID: 14578199

45. Wei Y, Wang X, Zhao F, Zhao P-Q, Kang X-L. Cannabinoid receptor 1 blockade protects human retinal pigment epithelial cells from oxidative injury. Mol Vis. 2013; 19: 357–66. Available: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3580988&tool=pmcentrez&rendertype=abstract PMID: 23441106

46. Guabiraba R, Russo RC, Coelho AM, Ferreira MAND, Lopes GAO, Gomes AKC, et al. Blockade of cannabinoid receptors reduces inflammation, leukocyte accumulation and neovascularization in a model of sponge-induced inflammatory angiogenesis. Inflamm Res. 2013; 62: 811–21. doi: 10.1007/s00011-013-0638-8 PMID: 23722450

47. Chan C-C, Ross RJ, Shen D, Ding X, Majumdar Z, Bojanowski CM, et al. Ccl2/Cx3cr1-deficient mice: an animal model for age-related macular degeneration. Ophthalmic Res. 2008; 40: 124–8. doi: 10.1159/000119862 PMID: 18421225

48. Miller AM, Stella N. CB2 receptor-mediated migration of immune cells: it can go either way. Br J Pharmacol. 2008; 153: 299–306. doi: 10.1038/sj.bjp.0707523 PMID: 17982478

49. Racz I, Nadal X, Alterink J, Baños JE, Rehnel J, Martin M, et al. Crucial role of CB(2) cannabinoid receptor in the regulation of central immune responses during neuropathic pain. J Neurosci. 2008; 28: 12125–35. doi: 10.1523/JNEUROSCI.3400-08.2008 PMID: 19005077
50. Adhikary S, Li H, Heller J, Skarica M, Zhang M, Ganea D, et al. Modulation of inflammatory responses by a cannabinoid-2-selective agonist after spinal cord injury. J Neurotrauma. 2011; 28: 2417–27. doi: 10.1089/neu.2011.1853 PMID: 21970496

51. Kishimoto S, Kobayashi Y, Oka S, Gokoh M, Waku K, Sugiura T. 2-Arachidonylglycerol, an endogenous cannabinoid receptor ligand, induces accelerated production of chemokines in HL-60 cells. J Biochem. 2004; 135: 517–24. Available: http://www.ncbi.nlm.nih.gov/pubmed/15115777 PMID: 15115777

52. Chiurchiu V, Leuti A, Maccarrone M. Cannabinoid Signaling and Neuroinflammatory Diseases: A Melt-ing pot for the Regulation of Brain Immune Responses. J Neuroimmune Pharmacol. 2015; 10: 268–80. doi: 10.1007/s11481-015-9584-2 PMID: 25601726

53. Karlstetter M, Lippe E, Walczak Y, Moehle C, Aslanidis A, Mirza M, et al. Curcumin is a potent modulator of microglial gene expression and migration. J Neuroinflammation. 2011; 8: 125. doi: 10.1186/1742-2094-8-125 PMID: 21958395