



Neuroprotective effects of topical CB1 agonist WIN 55212-2 on retinal ganglion cells after acute rise in intraocular pressure induced ischemia in rat

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ABSTRACT

Neuroprotection in retinal experimental work consists primarily of preventing retinal ganglion cell (RGC) loss after exposure to a hostile event. We have studied the neuroprotective effect on RGCs in an ischemia-reperfusion model by activation of the cannabinoid receptor CB1 using topical application of WIN 55212-2. Intraocular pressure (IOP) was increased by continuous infusion of phosphate buffer saline (PBS) into the anterior chamber of the eye. Mean intraocular pressure was increased up to 88.5 ± 0.29 mm Hg (control normal IOP 15.1 ± 0.25 mm Hg), for 35 min. Animals were distributed in 3 groups. Left eyes underwent acute rise in intraocular pressure. First group was treated with topical Tocrisolve™ 100 in both eyes. Second group was treated with 1% solution of CB1 agonist WIN 55212-2 in both eyes. Third group was treated with WIN 55212-2 1% and CB1 antagonist AM 251 1% solutions in both eyes. Subsequently, RGCs were immunolabeled with Brn3a and automated quantification of retinal mosaics of RGCs were performed. The ischemic damage led to a mean loss in RGC density of 12.33%. After topic administration of WIN 55212-2, mean loss of RGCs was of 2.45%. Co-treatment with CB1 antagonist AM 251 abolished almost completely the neuroprotective effect of WIN 55212-2. Topic 1% WIN 55212-2 showed a neuroprotective effect on RGC degeneration after ischemia-reperfusion without pre-activation of CB1 receptors.

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Neuroprotection consists of preventing the death of damaged neurons and the degeneration of those cells that had undergone the hostile environment created by an initial insult. By definition, in ocular neuroprotection, the target neurons should be located in the visual pathway, including retinal ganglion cells (RGCs) (Wheeler et al., 2001). Ocular neuroprotection has been investigated for various diseases affecting RGCs and the optic nerve, such as Leber's optic neuropathy (Johns and Colby, 2002), traumatic/compressive optic neuropathy (Ben Simon et al., 2006), toxic–metabolic optic neuropathies (Pinar-Sueiro et al., 2010), ischemic (Wilhelm et al.,

2006), inflammatory (Bessero and Clarke, 2010), demyelinating neuropathies (Croxford et al., 2008), diabetic retinopathy (Verma, 1993), and glaucoma (Nucci et al., 2007). All these neuropathies have several common degeneration pathways such as glial activation, oxidative stress, and excitotoxicity (Yuan and Neufeld, 2000; Seki and Lipton, 2008; Hare and Wheeler, 2009). Except for glaucoma, no treatment has been proven to be sufficiently effective as to establish a gold standard. For these reasons, efforts are consistently being directed toward the development of new drugs to prevent RGC degeneration from diverse optic neuropathies.

Cannabinoid receptors (CB) were classified as G protein coupled receptors with high affinity for the agonist tetrahydrocannabinol. Two types of CB receptors have been described, CB1 and CB2 (Howlett, 2002) and both have been found in retina (Cabral et al., 2008).

WIN 55212-2 is a synthetic aminoalkylindole that mainly binds to CB1 cannabinoid receptor (Chien et al., 2003). Cannabinoid agonists were first investigated as a possible treatment for glaucoma

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due to their efficacy to decrease intraocular pressure (Hepler and Frank, 1971; Porcella et al., 2001). Progress in the role of cannabinoids in providing neuroprotection in traumatic, ischemic, inflammatory and neurotoxic damage to neurons has been made (Van der Stelt and Di Marzo, 2005; de Lago and Fernández-Ruíz, 2007; Mechoulam and Shohami, 2007). Other studies have shown that CB1 agonists (THC and cannabidiol) protect RGCs from glutamate-induced excitotoxicity (El Remessy et al., 2003; Opere et al., 2006), and secondary degeneration in an experimental model of glaucoma in rat (Crandall et al., 2007; Nucci et al., 2007). Several cannabinoid agonists have been shown to act as non-competitive NMDA antagonist (Feigenbaum et al., 1989) thereby providing neuroprotection for RGCs (Yoles et al., 1996). However the direct neuroprotective effect on RGCs of a topic CB1 agonist (without preconditioning) has not been described in an ischemia-reperfusion animal model. The aim of the present study was to investigate the neuroprotective effect on RGCs of topical application of 1% solution of CB1 agonist WIN 55212-2.

27 female Sprague–Dawley rats, weighing 250–300 g were used. Animals were housed with ad libitum access to food and water, in a room with a 12:12 h light: dark cycles at 21 °C. All experimentation adhered to the ARVO Statement for the Use of Animal in Ophthalmic and Vision Research.

The rats were placed under deep anesthesia with intramuscular injection of ketamine and xylazine (66.7 mg/kg and 6.7 mg/kg body weight, respectively). Ischemia was induced by blocking off blood supply to the retina from the retinal artery by increasing IOP. Increased pressure was achieved by infusion of 100 μ l of phosphate buffer saline (PBS) into the anterior chamber for 35 min through a 32-gauge needle. The needle was attached to Tygon tubing (Hamilton) linked to a syringe pump (Panlab, Kd Scientific) with a flow rate of 2.9 μ l/min. Retinal ischemia was confirmed by observing bleaching of the retina. IOP was monitored every 5 min and the absence of retinal perfusion was maintained. Increased IOP was

maintained for 35 min and then infusion was removed allowing the eye to reperfuse. Contralateral right eyes were treated by inserting a 32-gauge needle into the anterior chamber of the eye through the cornea without the infusion and it served as control. Eyes undergoing continuous infusion reached increased intraocular pressure up to 88.5 ± 0.29 mm Hg ($n = 11$) with respect to their control right eyes (mean basal IOP of 15.1 ± 0.25 mm Hg).

Eye drops were applied in both eyes once daily at 9:00 h a.m. 48 h after the ischemia, rats were sacrificed by decapitation and eyes were enucleated for the posterior retinal fixation, immunolabeling and RGC quantification.

WIN 55212-2 mesylate salt (Cat. No. 1038, Tocris, Madrid, Spain) and AM-251 (Cat. No. 1117, Tocris, Madrid, Spain) were dissolved in Tocrisolve™ 100 (Cat. No. 1684, Tocris, Madrid, Spain) solution. Final solutions consisted of 1% WIN 55212-2; and 1% AM 251, and were kept in borosilicate glass tubes (Sigma–Aldrich, Madrid, Spain).

Animals were distributed in 3 groups. Left eyes underwent acute rise in intraocular pressure for 35 min. First group ($n = 11$) was treated with topical Tocrisolve™ 100 (20 μ l) in both eyes. Second group ($n = 8$) was treated with 1% WIN 55212-2 solution (20 μ l) in both eyes. Third group ($n = 8$) was treated with 1% WIN 55212-2 and 1% AM 251 solution (20 μ l) in both eyes. Both eyes in each animal were subjected to the same treatment, for providing the best pair group for statistics and avoiding complications due to crossover of drug into the contralateral eye.

Topical treatments of Tocrisolve™ 100, 1% WIN 55212 and 1% AM 251 were applied immediately following ischemia/reperfusion. Treatments were repeated once daily, at the same time of the day (9 a.m.) for the following 48 h. IOP was measured using electronic indentation tonometry (TonoLab, Icare®LAB; Icare, Finland). Increased IOP was monitored every 5 min under deep anesthesia during ischemia induction, and, afterward, it was measured in awake animals every 24 h, at 8:00 h a.m. daily to avoid natural IOP

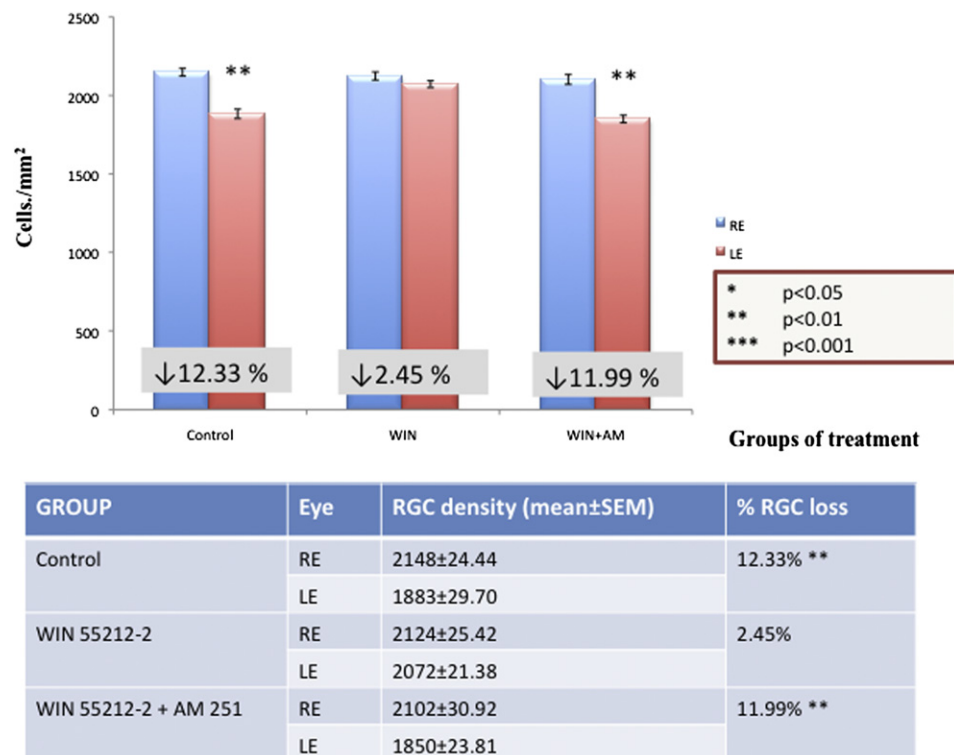


Fig. 1. Retinal ganglion cell densities in different treatment groups. Abbreviations: RE: Right eye; LE: Left eye; WIN 55212-2: Group treated with topic 1% WIN 55212-2; WIN 55212-2 + AM 251: Group treated simultaneously with 1% WIN 55212-2 and 1% AM 251; Control: Control group, treated with Tocrisolve™ as placebo; SEM: Standard error of the mean.

circadian changes, following the method described by Urcola et al. (2006). Measurements of IOP were repeated five times for each eye and were accepted if the confidence interval was greater than or equal to 95%. The mean values of the IOP measurements were averaged, and results were expressed as mean IOP \pm SEM.

48 h after initiating topical treatments, animals were deeply anesthetized using inhalational isoflurane and were sacrificed by decapitation. Eyes were isolated, vitreous removed and the retina was carefully extracted. Postfixation of the retina in 4% Paraformaldehyde for 4 h was performed on a filter paper (Millipore, Madrid). The flat fixed retinas were washed in PBS and incubated for 24 h at 4 °C with 1% PBS-Triton-X-100-BSA. Samples were labeled with anti-Brn-3a Goat polyclonal antibody in a dilution 1:1000, with gentle shaking for 48 h at 4 °C (C-20; sc-31984; Santa Cruz Biotechnology, Heidelberg). Retinas were washed with PBS. Samples were incubated in the secondary antibody Alexa 568 Donkey anti-goat Texas Red in a dilution 1:500 for 5 h at room temperature (Invitrogen, Madrid). Retinas were, then, washed with PBS. The retinas were mounted in PBS/glycerol (1:1) and examined under an Epifluorescence microscope (Imagen.M1, AX10, Carl Zeiss, Jene, Germany).

Software employed for RGC counting and for Mosaic definition was AxioVision 4.7.2.0. (Axio Imager M1; GMBH, Carl Zeiss, Jene, Germany) Texas-red filter, 64 HE mPlum was used with 20 \times objective. The area of the mosaic was defined by overlapping pictures obtained automatically by the microscope of a defined area of the retina; the total flat mounted retinas were measured for each eye. Once the mosaic was defined, the contour of the retina was measured and the surface retinal area was calculated (μm^2). The number of the RGCs in each retinal area was compared for the experimental (left) and control (right) eyes.

The density of RGCs in different groups was described as the mean and standard deviation. Comparison of different RGC densities in different groups was done by the non-parametric Kruskal–Wallis test. For the individualized comparison between groups, the Mann–Whitney *U* test was employed. Comparison among related groups (each treated group with its control) was carried out using the non-parametric Wilcoxon test. Statistical analysis was performed using SPSS software v 18.0 (SPSS Sciences, Chicago, IL). The minimum value of significant differences was defined as $p < 0.05$.

Mean RGC density in group 1 for the control right eyes was 2148 ± 24.44 cells/ mm^2 ($n = 11$) ($p < 0.001$). The mean density of RGCs in left (experimental) eyes that underwent transient ischemia was 1883 ± 29.70 cells/ mm^2 ($n = 11$). The ischemic damage triggered a mean loss in RGC density of 12.33% (Figs. 1 and 2). Statistically significant differences were observed among groups using Kruskal–Wallis test ($p < 0.001$).

Mean RGC density in right eyes of those rats treated with WIN 55212-2 was 2124 ± 25.42 cells/ mm^2 , and in left eyes 2072 ± 21.38 cells/ mm^2 , with a mean decrease of 2.45% RGCs.

Mean RGC density in control right eyes that had combined treatment of 1% WIN 55212-2 and AM 251 was of 2102 ± 30.92 cells/ mm^2 . In left experimental eyes, RGC density was 1850 ± 23.81 cells/ mm^2 , with a mean decrease in RGC density of 11.99%, quite similar to that obtained after transient ischemia without treatment (Figs. 1 and 2).

This ischemia/reperfusion model as described above was a well-controlled transient model of acute ischemia for the retina. Brn-3a labeling facilitated the quantification of remaining RGCs. This labeling is not dependent on retrograde transport of labeled material to the RGCs, that may have suffered a functional breakdown or, a mechanical disorder following ischemic damage to the retina (Sánchez-Migallón et al., 2011). Since ischemia/reperfusion were induced only in the left eyes, it can be assumed that the loss of RGCs was triggered primarily by the initial insult. In the retinal ischemia-reperfusion injuries, retinal degeneration shares many characteristics of retinitis pigmentosa, retinal detachment, age-

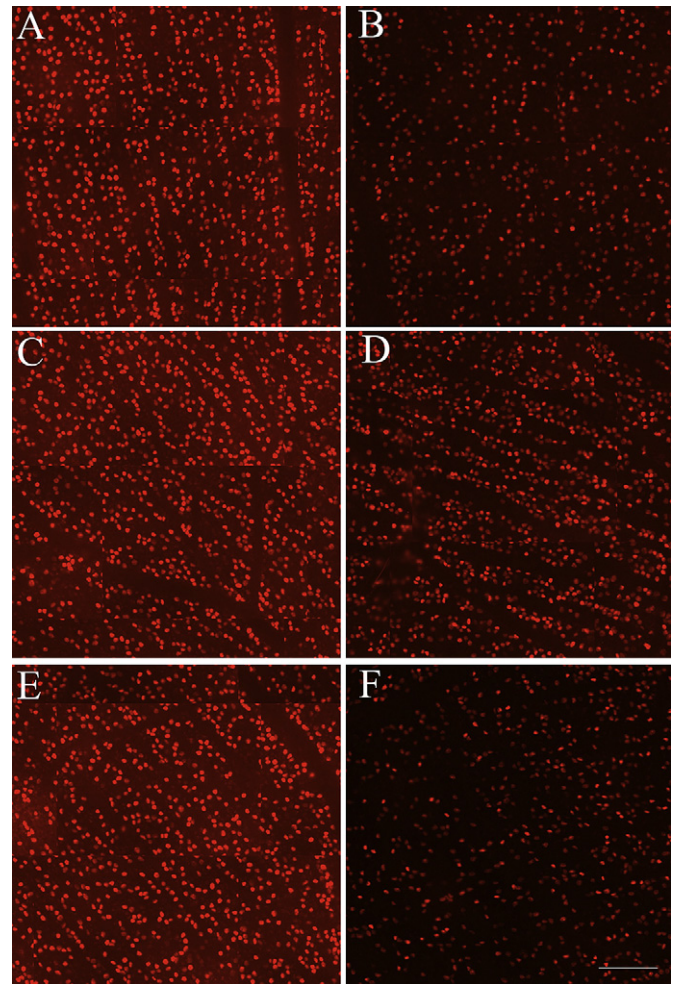


Fig. 2. Brn-3a labeled retinal ganglion cell densities in different groups of treatment. Labeled cells in control eyes after topical treatment with placebo (Tocrisolve™) (A), WIN 55212-2 (C), and WIN 55212-2 and AM 251 (E). RGC densities in eyes that underwent ischemia-reperfusion, receiving treatment with placebo (B), WIN 55212-2 (D), and WIN 55212-2 and AM 251 (F). RGC density was statistically smaller after retinal ischemia induction (B) with respect to that of control retina (A). Application of topical WIN 55212-2 prevented RGC loss (C) when compared to control eyes treated with Tocrisolve (A). Eyes treated with both AM 251 and WIN 55212-2 (F) showed similar RGC loss as those treated with placebo (B). Scale bar, 100 μm .

related macular degeneration, and glaucoma, and the retinal neuronal cells die via apoptosis. As it was previously demonstrated for transient cerebral ischemia (Rosenbaum et al., 1998), apoptotic neuronal cell death has also been described in the inner retina as a consequence of transient retinal ischemia (Rosenbaum et al., 1998); however, the reduction in retinal thickness and cell density has been shown to be less pronounced in the inner nuclear layer than in the outer nuclear layer. A significant proportion of inner retinal neurons are more resistant to ischemic insult as induced by ischemia-reperfusion and are more amenable to possible neuroprotective therapies. These therapies should be applied early, as cell death in the inner retina occurs more transiently and rapidly, when compared to outer nuclear layer, where it occurs in a delayed and prolonged fashion (Ju et al., 2000).

The present study showed that loss of RGC occurred as consequence of the increased IOP 48 h after undergoing ischemic damage and topical 1% WIN 55212-2 used as treatment without preconditioning had a neuroprotective effect on RGCs by activating CB1 receptors. Its effect on RGC survival could be isolated from its well-known hypotensive effect, as basal intraocular pressure was

not an essential factor to avoid progression of RGC degeneration. An intense CB1 labeling has been described in the inner and outer plexiform layers (IPL and OPL) of the retina, as well as the retinal ganglion cell layer (GCL) and nerve fiber layer (NFL) (Straiker et al., 1999). Due to strong CB1 immunohistochemical labeling in the inner retina, CB1 agonists like WIN 55212-2 may exert an important role to inhibit RGC apoptosis after intense transient insults of the retina by activation or up regulation of CB1 receptors. In rat retina, ischemia-reperfusion insult has been associated to an enhanced fatty acid amide hydrolase (FAAH) activity and protein expression, paralleled by a significant decrease in endogenous anandamide (AEA) tone. Consequently, endocannabinoid inhibited tone seems to play an important role in retinal cell loss (Nucci et al., 2007), and the potent agonism played by WIN 55212-2 of the remaining CB1 receptors may counteract the damage and enhance survival of the RGCs. Moreover, CB1 agonism may play a beneficial role in ischemic damage of RGCs by other means.

As previously described in models of central nervous system ischemia, WIN 55212-2 provide robust neuroprotection by acting on glutamatergic excitotoxicity, TNF- α release, and iNOS expression. Although these beneficial effects have been attributed to activation of both CB1 and CB2 receptors (Fernández-López et al., 2006), our results indicated that main neuroprotective effect in the inner retina occurred through activation of CB1 receptors. Moreover, recent published data showed that cannabinoid receptor CB1 agonists, and not CB2 agonists, may benefit RGCs from ischemic damage of the retina, by reducing K⁺-induced [3H]D-aspartate release (Opere et al., 2006). Our findings, describing CB1 receptor-mediated RGC survival, and other data (e.g. Sánchez-Migallón et al., 2011; Ju et al., 2000), give credence to study the potential neuroprotective effects of CB1 agonist when dealing with ischemic insults to the retina.

While CB1 agonist WIN 55212-2 has been proven efficient in preventing inner retinal degeneration following retinal acute-ischemia, further studies must establish the role of CB1 agonists during the secondary degeneration after acute ischemia, and events related to the apoptotic cascade (Agudo et al., 2009), inflammation and macrophage activation. Understanding these processes will facilitate the search for potential neuroprotective therapies.

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