Melanopsin-Containing or Non-Melanopsin–Containing Retinal Ganglion Cells Response to Acute Ocular Hypertension With or Without Brain-Derived Neurotrophic Factor Neuroprotection

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Purpose. To analyze the responses of different retinal ganglion cell (RGC) types to acute ocular hypertension (AOH) and intravitreal administration of brain-derived neurotrophic factor (BDNF).

Methods. In adult albino rats, the anterior chamber of the left eye was cannulated with a needle connected to a saline container elevated 1½ meters above the eye for 75 minutes. Rats received 12 hours before a 5 µl intravitreal injection containing 5 µg BDNF in 1% albumin PBS or vehicle and were analyzed 3, 7, 14, or 45 days later. Both retinas were dissected as wholemounts and immunolabeled for melanopsin (to identify intrinsically photosensitive RGCs) or Brn3a (to identify all RGCs except melanopsin $^{+}$RGCs).

Results. During AOH there is ischemic damage and mechanical eye-globe deformation. Acute ocular hypertension results in a progressive loss of Brn3a^{+}RGCs in the vehicle-treated retinas (39%, 35%, 25%, and 13% of the original value, at 3, 7, 14, or 45 days, respectively), whereas BDNF increases their survival to 81%, 73%, 59%, or 57% at the same time periods. In vehicle-treated retinas, 37% or 39% of m^{+}RGCs survive at 14 or 45 days, respectively, whereas BDNF treatment increases their survival to 40% or 78% at the same time points.

Conclusions. Different types of RGCs respond differently to AOH because Brn3a^{+}RGCs die progressively, but m^{+}RGCs do not. After a transient downregulation of melanopsin expression, their number remains constant and their survival is proportionally higher than that of Brn3a^{+}RGCs. BDNF affords a permanent protection up to 45 days after AOH injury in both types of RGCs.

Keywords: rat retinal ganglion cells, acute ocular hypertension, intrinsically photosensitive RGCs, BDNF neuroprotection, melanopsin retinal ganglion cells, adult albino rats, experimental glaucoma, melanopsin, Brn3a, transient ischemia of the retina, long lasting neuroprotection, ultrasound Doppler

Glaucoma, the second leading cause of blindness in developed countries, courses with characteristic defects in the nerve fiber layer of the retina and in the optic disc, a progressive loss of RGCs, and functional deficits in the visual field that may end up in irreversible blindness.[1] Although a number of mechanisms have been potentially associated with the pathophysiology of glaucoma, including axonal compression,[2] retinal ischemia,[3] inflammation,[4–5] autoimmune mechanisms,[6] and myelination transition zone astrocyte dysfunction,[7] the main causes remain elusive.[8] The current thinking implies a lesion to the optic nerve (ON) axons somewhere near the optic nerve head with a consequent anterograde and retrograde degeneration that may have mechanical, ischemic, and inflammatory components.[9–11]

To study the effects of ON injury, a classic model consists of crush or transection of the intraorbital portion of the ON.[12–14] Recently, additional models of glaucoma have been studied in rodents to foster our knowledge of the pathophysiology of human glaucoma, and most of these show spontaneous[15] or induced chronic ocular hypertension by altering the draining system of the aqueous humor either by laser photocoagulation of the episcleral and perilimbal veins,[10–11,16–19] episcleral vein cauterization,[20] hypertonic saline injection into episcleral veins,[21] or injection of microbeads or viscoelastics into the anterior chamber.[22] Another experimental model to study glaucoma consists of inducing an acute ocular hypertension (AOH), a situation that may mimic acute angle-closure glaucoma but may also result in retinal ischemia and lead to ON damage and RGC loss.[23]

Adult rodent RGCs comprise many subtypes, each one with its own typical functional, morphologic, and projecting attributes, and this makes it challenging to characterize the

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response of particular RGC types to experimental retinal insults.[24] For the rat retina there are only two molecular markers that can make a clear distinction between the general population of RGCs, implied in image-forming functions, that are labeled with Brn3a antibodies and the intrinsically photosensitive RGCs, implied in nonimage forming functions, which can be largely identified with melanopsin antibodies (m^+RGCs).[25–29] Thus, using these modern tools we can study independently but in parallel the fate of these two populations after retinal injury and neuroprotection.[24] As such, the responses of the general population of RGCs and the population of m^+RGCs to transient ischemia of the retina has been recently studied.[30]

Retinal injuries may result in RGC loss, which may be prevented with a number of neuroprotective substances.[31–35] Among the RGC neuroprotective agents, the neurotrophin brain derived neurotrophic factor (BDNF) has been shown to be the most efficacious,[36–38] but its effects are only transient and thus result in delayed RGC death.[39–43] BDNF has also been shown to be neuroprotective for the general population of RGCs against acute[31,44–45] or chronic ocular hypertension,[17,46–50] but not for the population of m^+RGCs.[17] However, most of these studies were short-term studies, and whether BDNF intravitreal administration provides long-lasting protection of RGCs from AOH-induced retinal injury is unknown.

In this study, we propose to further investigate the responses of two different populations of RGCs—the general population of RGCs and the population of melanopsin RGCs—to acute transient ocular hypertension at short and long periods of time after the injury. Moreover, we investigate whether BNDF rescues these two populations of RGCs from AOH-induced degeneration and whether this protection is transient or long lasting.

Materials and Methods

Animal Handling

The study was approved by the Committee of Animal Care of the University of Murcia (Murcia, Spain). All experimental procedures were performed in accordance with the European Union Directive 2010/63/EU for animal experiments and the ARVO Statement for the Use of Animals in Opthalmic and Vision Research. Adult female Sprague-Dawley rats (200–250 g body weight) were fed ad libitum and kept in an environmentally controlled room with an alternating 12 hours light/12 hours dark cycle. Surgical procedures were carried out under deep anesthesia induced by an intraperitoneal injection of a mixture of xylazine (10 mg/kg Rompun; Bayer, Kiel, Germany) and ketamine (70 mg/kg, Imalgene, Merial Laboratorios S.A., Barcelona, Spain). Analgesia was provided by subcutaneous administration of buprenorphine (0.1 mg/kg). All efforts were made to minimize animal suffering. For euthanasia, rats were sedated with an intraperitoneal injection of sodium pentobarbital and then euthanized with an intraperitoneal overdose of sodium pentobarbital diluted 1:1 in saline (Dolethal, Vetoquinol; Especialidades Veterinarias, S.A., Madrid, Spain).

Intravitreal Injection

At 12 hours before the induction of AOH, 5 μg of BDNF diluted in 1% bovine serum albumin in PBS (Peprotech EC Ltd., London, UK) were intravitreally injected into the left eye following standard procedures in our lab (BDNF-treated group).[41,51] A matched group of rats were treated with the same volume (5 μL) of vehicle (vehicle-treated group).

Induction of AOH

This technique has already been described in detail previously.[23,52] In anesthetized rats, a 30-gauge infusion needle connected to a 500-ml bottle of sterile saline was placed into the anterior chamber of the left eye, taking care not to injure the lens or iris, and this did not cause alteration of the basal IOP levels. Elevation of the saline bottle above the level of the eye to a height of 150 mm above the rat resulted in an IOP elevation to 76 ± 3 mm Hg, which was maintained for a period of 75 minutes (Fig. 1A, Supplementary Fig. S1), while the animal was seated over a heating pad to maintain normal body temperature. This period of transient ischemia was chosen because previous studies have indicated that it produces irreversible damage of the RGC population.[53–55] In all rats, an interruption of retinal blood flow was examined by direct ophthalmoscopy of the eye fundus with the operating microscope (Spot OPMI 11, Carl Zeiss, Oberkochen, Germany; Supplementary Fig. S1). Shortly after instauration of the AOH, we observed transient cloudiness of the lens, but this reversed spontaneously a few minutes after reperfusion and did not preclude examination of the blood flow.[54] After 75 minutes, the infusion needle was removed from the anterior chamber, the eye fundus was examined (Supplementary Fig. S1), and an antibiotic ointment (Tobrex; Alcon S.A., Barcelona, Spain) was placed over the cornea to prevent desiccation while the animal recovered from anesthesia. Eyes without complete recovery of the retinal blood flow within the first few minutes after the 75 minutes of AOH were discarded. Only animals without complications such as retinal detachment, lens opacification, or corneal lesions were further used in the study. Animals were euthanized at 3, 7, 14, or 45 days after the AOH. Because injury to one eye may result in molecular and cellular changes in the fellow contralateral eye[4–5,35,39] we used retinas from intact animals as controls. The number of retinas used in each group is detailed in the Table.

IOP Measurement

In all animals, the intraocular pressure was measured in both eyes using a Tono Pen (Tono-Pen; Medtronic Co., Dublin, Ireland) following previously described methods.[17–19,56] The IOP was measured before induction of the AOH (baseline), approximately 1 minute after needle insertion, and then every 15 to 20 minutes until the end of the procedure (Supplementary Fig. S1). Afterward, the IOP was measured after removing the needle (approximately 1 minute and 10 minutes later), 3 days later, and at 45 days later in the groups of animals analyzed at this time point (Supplementary Fig. S1).

Ultrasound

In four animals per group (vehicle- or BDNF-treated), the anatomical and blood irrigation changes in the eye were monitored during the AOH procedure (before the needle insertion, during the 75 minutes of AOH, and after removal of the needle). This experiment was carried out using ultrasound imaging Doppler mode (Vevo3100 instrument; VisualSonics Toronto, Canada) equipped with the VisualSonics MX550D (22–55 MHz, mean frequency 40 MHz; resolution 40 μm). Images were taken on the horizontal plane and focused in the middle plane of the eye (through the optic nerve). In the resultant images, the length of the anterior chamber (from the cornea to the anterior limit of the lens), the length from the anterior limit of the lens to the optic nerve (vertical), and the main width of the eye globe (horizontal) were measured using the VisualSonics software.

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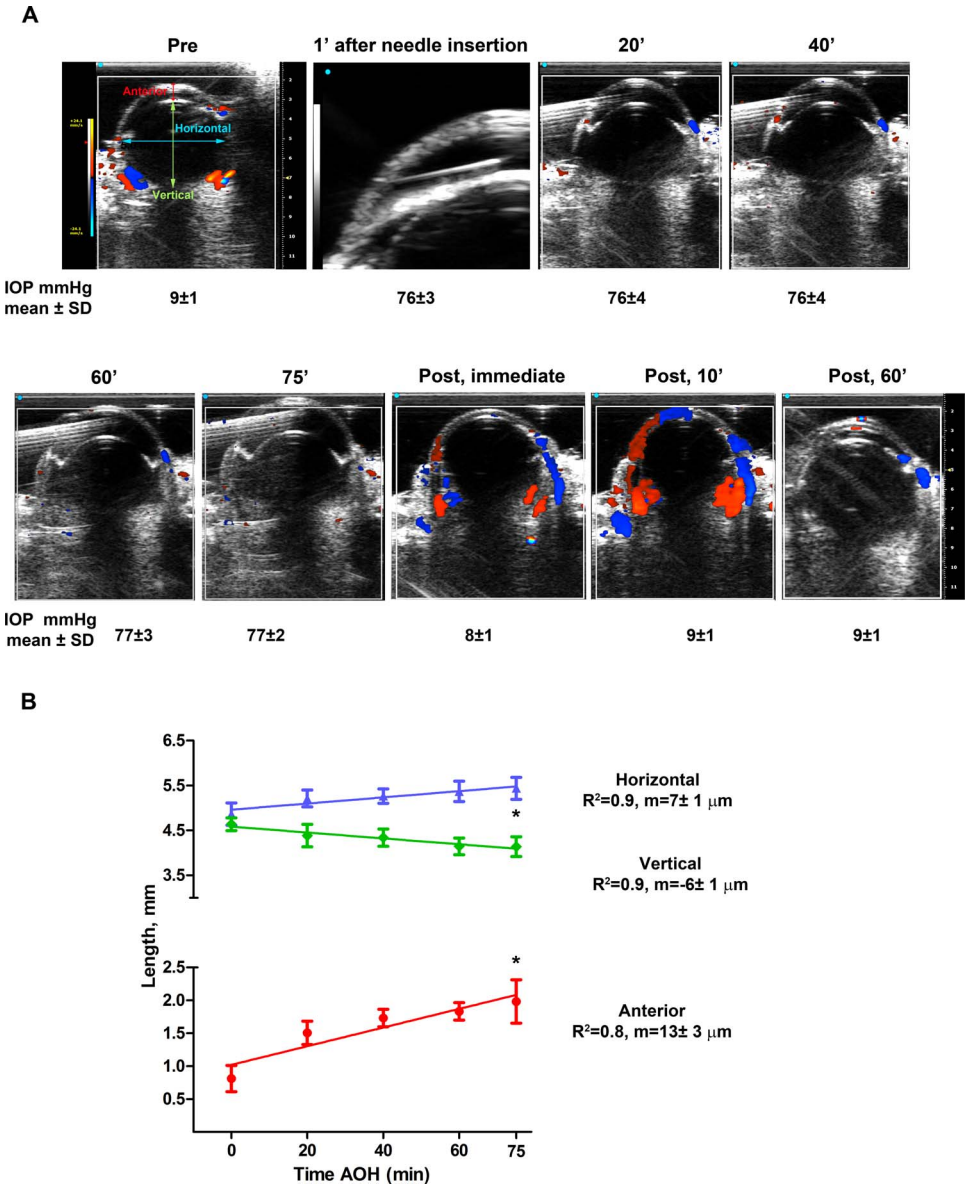


FIGURE 1. Acute intraocular hypertension produces a transient retinal ischemia and deforms the eye in its horizontal axis. (**A**) Ultrasound (black and white) and Doppler (*ultramarine blue and red spots*) images spanning the eye and taken before needle insertion (pre), at increasing times after saline infusion, and after needle removal. The first image (*left*) shows how measurements of the anterior chamber (from the cornea to the anterior limit of the lens; *red arrow*), the length from the anterior limit of the lens to the optic nerve (vertical axis; *light green*), and the main width of the eye globe (naso-temporal, horizontal axis; *light blue*) were taken. Images were taken on the horizontal plane with the nasal pole of the eye to the right of the image and the temporal pole to the left. (**B**) X–Y plot where time of AOH (X, min) has been plotted against the main length and/or width of the measurements (Y, mm). Values are mean ± SD. R^2, adjustment of the linear regression and m the slope of the line; m, negative for the vertical axis because it decreases as the time of acute ocular hypertension increases.

Retinal Dissection and Immunodetection

Unless otherwise stated, all reagents were from Sigma-Aldrich Química S.A. (Madrid, Spain). After euthanasia, all animals were perfused transcardially first with saline to remove the blood and then with 4% paraformaldehyde in 0.1 M phosphate to fix the tissue. All retinas were dissected as flattened whole-mounts and double immunofluorescence staining was performed with Brn3a (1:750; C-20 Santa Cruz Biotechnologies, Heidelberg, Germany) and melanopsin (1:500; PAI-780 Thermo Fisher Scientific, Madrid, Spain) antibodies. Immunodetection was carried out as previously described.[17] Briefly, retinas were permeated in PBS 0.5% Triton X-100 (Tx) by freezing them for 15 minutes at −70°C. Then they were thawed at room temperature, rinsed in new PBS-0.5%Tx, and incubated overnight at 4°C with the primary antibodies diluted in blocking buffer (PBS, 2% normal donkey serum, 2% Tx). Retinas were washed three times in PBS and incubated 2 hours at room temperature with a mixture of the secondary antibodies diluted each 1:500 in PBS-2% Tx (donkey anti-goat Alexa 594 and donkey anti-rabbit Alexa 488 Molecular Probes; Thermo Fisher Scientific). Finally, the retinas were thoroughly washed in PBS-0.5% Tx and, after a last rinse in PBS, they were mounted vitreal side up on subbed (cleaned and gelatine coated) slides and covered with antifading media (Vectashield mounting medium; Vector Laboratories, Palex Medical, Barcelona, Spain).

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Table. Total Number of Brn3a$^+$RGCs or m$^+$RGCS after AOH and BDNF Treatment

RGC Types and Groups	Intact, $n = 6$	Time After AOH Induction, Days* 3, $n = 9$	7, $n = 9$	14, $n = 9$	45, $n = 14$
Brn3a$^+$RGCs					
Intact					
Mean	78,211				
SD	450				
Vehicle†					
Mean		30,857†	27,734†	19,422†	10,157†‡
SD		9,640	14,220	14,249	3,853
BDNF					
Mean		63,691§	56,983§	45,887†§	44,294†§||
SD		8,721	14,977	19,431	11,198
m$^+$RGCs					
Intact					
Mean	2,247				
SD	72				
Vehicle					
Mean		461†	693†	838†||	871†
SD		222	307	241	258
BDNF					
Mean		631†	776†	910†||	1,747§¶
SD		188	196	218	420

Mean ± SD of the total number of Brn3a$^+$RGCs or melanopsin$^+$RGCs in intact retinas and in retinas analyzed at increasing survival intervals after induction of AOH and vehicle or BDNF administration. *n*, number of retinas per group.

* Pairwise multiple comparison procedures, Kruskal-Wallis followed by Dunn's post hoc test.

† Significant when compared with intact retinas ($P < 0.01$).

‡ Significant when compared with 7 days ($P < 0.001$).

§ Significant when compared with vehicle-treated retinas at the same time point ($P < 0.01$).

|| Significant when compared with 3 days ($P < 0.05$).

¶ Significant when compared with 14 days ($P < 0.001$).

Image Acquisition, Automated Quantification, and Topographical Maps

Brn3a and melanopsin signals were acquired under an epifluorescence microscope (Axioscop 2 Plus; Zeiss Mikroskopie, Jena, Germany) equipped with a computer-driven motorized stage (ProScan H128 Series; Prior Scientific Instruments, Cambridge, UK) controlled by Image-Pro Plus (IPP 5.1 for Windows; Media Cybernetics, Silver Spring, MD, USA) as described.[25,27] In brief, retinal multiframe acquisitions were photographed in a raster-scan pattern in which frames were captured side by side with no gap or overlap between them with a 10× objective. Single frames were focused manually before acquisition of the image. All frames from each retina (154 per retina) were then fed into the IPP image analysis program to tile them and reconstruct the retinal photomontages.

Brn3a$^+$RGCs were automatically quantified as reported.[26] m$^+$RGCs were dotted on the retinal photomontage, and their number was automatically quantified using the IPP software. Each count was exported to a spreadsheet (Microsoft Office Excel, 2003; Microsoft Corporation, Redmond, WA, USA) for statistical analysis.

The topographical distribution of Brn3a$^+$RGCs was obtained through quadrant analysis and visualized on isodensity maps as reported,[57] and the distribution of m$^+$RGCs was carried out using the next-neighbor algorithm.[25] Isodensity maps depict the density (number of cells/mm^2) with a color scale that goes from 0 RGCs/mm^2 (purple) to ≥3500 RGCs/mm^2 (red). In the neighbor maps, each dot represents a m$^+$RGC and its color indicates the number of m$^+$RGCs (neighbors) around it in a radius of 0.276 mm from 0 to 4 (purple) to a maximum of ≥36 to 40 (red) neighbors. All maps were performed using Sigmaplot (SigmaPlot 9.0 for Windows; Systat Software, Inc., Richmond, CA, USA).

Statistics

Graphs, regression analysis, and statistical comparisons (pairwise multiple comparison procedures, Kruskal-Wallis followed by Dunn's post hoc test) were performed with GraphPad Prism (GraphPad, San Diego, CA, USA).

Results

Anatomical and Irrigation Changes in the Eye During AOH

The increase of the intraocular pressure as well as the anatomical changes were comparable in vehicle- or BDNF-treated rats, thus data presented in Figure 1 are the averaged values obtained from both groups. Elevation of the saline reservoir to 150 cm above the eye resulted in an almost immediate increase of the IOP from 9 ± 1 to 76 ± 3 mm Hg, and this increase in IOP was maintained constant during the 75 minutes of the procedure. The acute increase of the IOP causes a transient ischemia of the entire retina, as evidenced by direct eye fundus examination. The removal of the infusion needle resulted in the rapid diminution of the IOP to basal values (Fig. 1A), which were maintained for up to 45 days after AOH, the longest time period examined in this study (Supplementary Fig. S1).

Besides an ischemic damage, there was also a mechanical deformation of the eye (Fig. 1A) along its nasotemporal axis. Based on the ultrasound images, the anterior chamber elongated in its vertical axis, increasing its length as the time of infusion increased. In addition, the eye became horizontally longer and vertically shorter (Fig. 1). The removal of the infusion needle caused a rebound effect, where the anterior chamber collapsed and the eye returned to its baseline width but elongated in the vertical axis. The anterior chamber and eye length recovered 1 hour later; that is, the eye was no longer deformed (Fig. 1).

Retinal Ganglion Cell Survival After AOH: Neuroprotection With BDNF

We analyzed in parallel Brn3a$^+$RGCs and m$^+$RGCs. Qualitative representative images from control or treated retinas analyzed at the earliest and latest time interval are shown in Figure 2. Quantitative data are shown in the Table (mean ± SD) and Figure 3A (% versus intact), and the topographical analysis is shown in Figures 3B through 3D.

Brn3a$^+$RGCs. In the vehicle-treated group, the loss of Brn3a$^+$RGCs was already significant at day 3 (40% of survival), and thereafter their numbers decreased progressively (35%, 30%, and 13% of the original RGCs survive at 7, 14, and 45 days, respectively). Intravitreal administration of BDNF protects this population at all times; RGC survival was significantly higher in the BDNF-treated than in the vehicle-treated group. Moreover, whereas in the vehicle-treated group Brn3a$^+$RGCs diminished progressively with time (by 45 days the surviving population had decreased to 13% of the original), in the BDNF-treated groups, RGC loss stopped at 14 days, and the number

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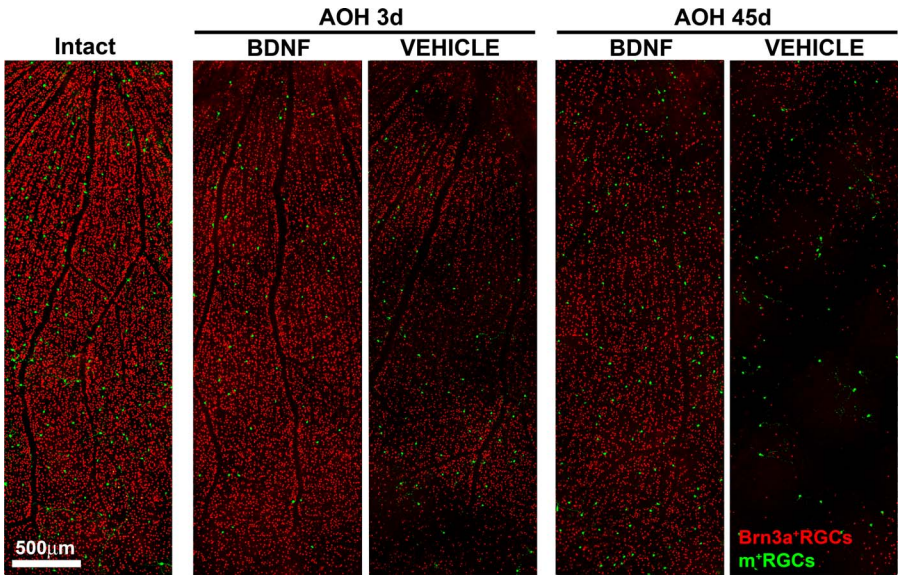


FIGURE 2. Effect of AOH and BDNF or vehicle treatment on RGCs. Images showing Brn3a$^+$RGCs and melanopsin$^+$RGCs in intact retinas and in retinas analyzed 3 or 45 days after AOH+BDNF or AOH+vehicle. These images have been taken from the areas squared in the maps shown in Figure 3.

of these neurons remained stable up to 45 days (57% survival). Thus, 45 days after AOH and BDNF treatment there were fourfold more Brn3a$^+$RGCs than after vehicle administration (Figs. 2, 3; Table).

m$^+$RGCs. At 3 days after AOH, the numbers of m$^+$RGCs appeared diminished to less than 30% of their original values, both in the vehicle- (21%) and BMD-treated (28%) groups. At increasing survival times, the numbers of m$^+$RGCs increased so that by 14 days the numbers of m$^+$RGCs represented approximately 40% of their original values, both in the vehicle- (37%) and BDNF-treated (40%) groups. Although these numbers were maintained at 45 days for the vehicle-treated retinas (39%), in the BDNF-treated retinas, there was an important increase to 78% of their original values, suggesting an important rescuing effect (Fig. 3; Table). Because this was a staggering result, we analyzed 5 additional animals that corroborated these findings (Table).

Topographical Analysis. The retinal distribution of Brn3a$^+$RGC loss was examined with isodensity maps that were constructed for each retina. The loss of Brn3a$^+$RGCs appeared diffuse over the retina, with some patchy areas of the retina lacking more Brn3a$^+$RGC than others, but without a characteristic pattern (Figs. 3B–D). The retinal distribution of m$^+$RGC loss was examined with neighbor maps that were constructed for each retina. As for the loss of Brn3a$^+$RGCs, m$^+$RGC loss appeared diffuse over the retina without a characteristic pattern, although the recovery of m$^+$RGCs tended to be more marked in the dorsal retina (Figs. 3B–D).

Comparison of Brn3a$^+$RGCs and m$^+$RGCs Survival After AOH

In the vehicle-treated groups, Brn3a$^+$RGCs survived in smaller proportions than m$^+$RGCs. By 45 days, the latest time point analyzed, approximately 13% of the original Brn3a$^+$RGCs survived, whereas 39% of the m$^+$RGCs were detected, indicating that m$^+$RGCs were clearly more resistant to AOH than the general population of RGCs. The same general observation applied for the BDNF-treated groups, but with an important rescue of m$^+$RGCs at 45 days, suggesting that BDNF also protected m$^+$RGCs from AOH-induced RGC loss. Because of the transient downregulation of melanopsin following retinal injury, which has been shown to occur during the first 15 days,[13,58] the neuroprotective effects of BDNF were clearly observed at the longest period of time examined after the insult.

DISCUSSION

In the present study, we investigated the fate of RGCs after AOH and intraocular administration of BDNF. Acute ocular hypertension was induced by the elevation of the intraocular pressure above systolic levels for 75 minutes, and the retinas were examined from 3 to 45 days after AOH. We used ultrasound Doppler of the eye to monitor ocular changes as well as modern tools to identify, image, and map two subpopulations of RGCs. One population of RGCs can be identified with Brn3a antibodies, which labels most RGCs except for the melanopsin$^+$RGCs and one half of the ipsilaterally projecting RGCs; in albino rats, this population amounts to approximately 80,000 RGCs, and this corresponds to approximately 97% of the entire population of RGCs.[13,27,58] Another population of RGCs can be identified with melanopsin antibodies, which identify most of the intrinsically photosensitive RGCs; in rats this population amounts to approximately 2,200 RGCs, and these correspond to 2.7% of the entire RGC population.[25–27] Our results indicate that (1) during AOH, there is transient ischemia and a mechanical deformation of the eye associated with the elevation of the IOP; (2) AOH results in progressive RGC loss, indeed Brn3a$^+$RGCs die a in a time-dependent manner; (3) BDNF rescues a substantial proportion of Brn3a$^+$RGCs from AOH-induced loss, and this neuroprotection appears to be long lasting; (4) following a transient downregulation of melanopsin expression, m$^+$RGCs appear more resistant than Brn3a$^+$RGCs to AOH-induced loss, and (5) m$^+$RGCs are also responsive to BDNF neuroprotection.

AOH Results in Retinal Ischemia and Mechanical Deformation of the Eye

The experimental design consisted of an elevation of the IOP to 75 mm Hg for a period of 75 minutes, an acute and transient

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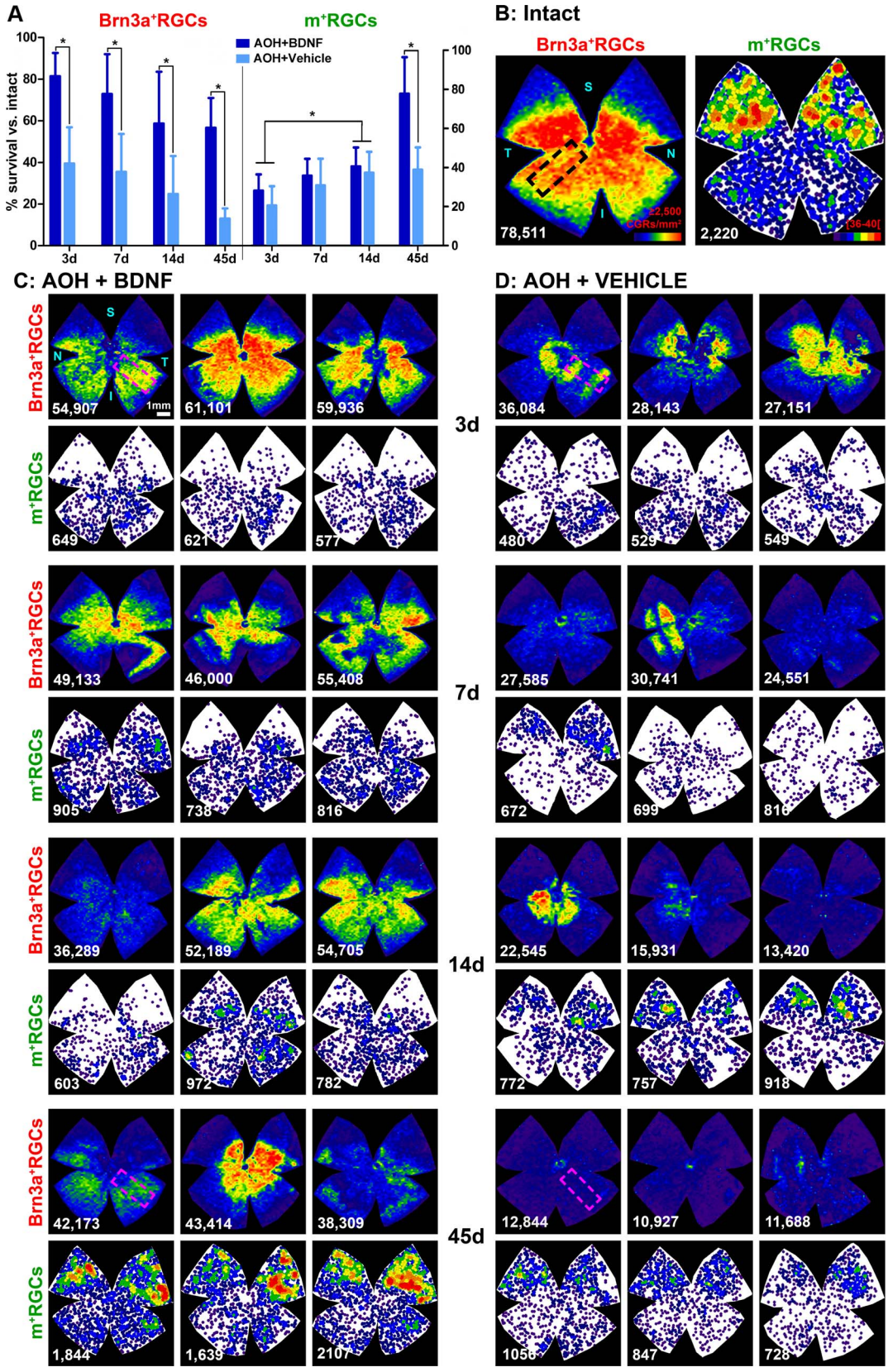


FIGURE 3. BDNF protects Brn3a+RGCs and melanopsin+RGCs after AOH. (**A**) Column graph showing the mean percent ± SD of surviving Brn3a+RGCs (*left*) and m+RGCs (*right*) versus intact retinas. *Significant difference ($P < 0.01$; for details see Table legend). (**B**) Isodensity (*left*) and neighbor map (*right*) showing the distribution of Brn3a+RGCs and m+RGCs, respectively, in the same retina from an intact animal. (**C–D**) Topographical loss of Brn3a+RGCs and m+RGCs shown at increasing survival intervals after AOH in the vehicle-treated (**C**) and BDNF-treated (**D**) groups. For each time point and treatment, three retinas are shown. *Squares* mark the areas where the magnifications in Figure 2 were taken. Isodensity map color scale (**B**, *left*): 0 RGCs/mm^2 (*purple*) to ≥3500 RGCs/mm^2 (*red*). Neighbor map color scale (**B**, *right*): 0 to 4 (*purple*) to ≥36 to 40 (*red*) neighbors in a radius of 0.276 mm. S, superior; N, nasal; I, inferior; T, temporal.

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increase of the intraocular pressure that is clearly above the limits of retinal tolerance[55,59–62] and has been previously shown to induce RGC loss.[53] This transient AOH may mimic acute angle-closure glaucoma and thus may be particularly relevant to further understand the pathophysiology underlying this disease.[55,59–60,62–65] Our ultrasound imaging Doppler studies document that AOH also results in mechanical deformation of the ocular globe, adding to the previous literature showing that IOP elevation results in deformation of the optic nerve head and peripapillary structures.[66] Such deformations may in turn play a significant role in the pathophysiology of RGC axonal damage following chronic[67–68] or acute[69] elevation of the IOP. Thus, AOH may result in a complex severe insult that leads to a (1) direct ischemic injury to the retina because it halts blood flow, (2) pressure-induced retinal damage because the IOP is increased several fold its basal value during a period of 75 minutes, and (3) deformation of the eye that may also affect retinal axons within the retina and at their exit of the eye through the optic nerve head.

AOH Induces Progressive Brn3a$^+$RGC Loss

A transient insult to the retina triggers a protracted degenerative event in the RGC population; for example, 75 minutes of acute elevation of the intraocular pressure induces retinal ischemia and ocular deformation that leads within the first week to the death of approximately 65% of the RGC population, and this is followed by a protracted additional loss of approximately 22% of the RGC population by day 45. Such a time-dependent loss of RGCs is in agreement with previous studies in adult rats in which retrograde labeling[32,53–54,70] or immunocytochemical[52,55] techniques were employed to identify and count RGCs following transient ischemia of the retina. The effects of AOH on the thickness of the retinal layers were not investigated in the present study, but previous studies following the transient ischemia of the retina induced by selective ligature of the ophthalmic vessels indicate an important decrease in retinal layer thickness with time[71] or even in the overall volume of the superficial layers of the contralateral superior colliculus, which receive the main input from the injured eye.[70] Alterations in the retrograde axoplasmic flow have been shown after transient elevation of the intraocular pressure,[46,63,72–74] transient ischemia,[75] or axotomy of the ON.[76–77] Moreover, transient ischemia of the retina has been shown to induce an important glial reaction over the entire primary visual pathway[52] as well as important retinal functional deficits as assessed with electroretinogram and anterograde axonal transport studies.[55,65,70–71]

Topography of Brn3a$^+$RGC and m$^+$RGC Loss

Isodensity map reconstructions of individual retinas revealed diffuse loss of Brn3a$^+$RGCs or m$^+$RGCs, with some areas showing fewer RGCs, but without a consistent pattern. The pattern of AOH-induced RGC loss differed from the typical sectorial loss observed after chronic ocular hypertension induced by laser photocoagulation of the episcleral and perilimbal veins.[10–11,17–19] The sectorial loss occurs plausibly by damage inflicted to bundles of axons near the optic nerve head where retinotopic arrangement is greatest.[10–11] The pattern of RGC loss observed after ocular hypertension (OHT) resembled more the patchy RGC loss observed after transient ischemia of the retina induced by selective ligature of the ophthalmic vessels (see Figs. 2 and 3 in Lafuente López-Herrera et al.[75]). Although AOH results in a severe complex insult to the retina, it is tempting to speculate that the geographical pattern of RGC loss observed in the present experiments may reflect a predominant ischemic nature of the insult inflicted to the retinas, and this would have important implications on possible mechanisms by which neuroprotectants result in long-lasting effects. Indeed, previous studies from this and other laboratories have shown long-lasting effects of neuroprotectants against retinal-ischemia induced RGC loss.[32,54,78–79]

Long-Lasting Neuroprotection of Brn3a$^+$RGCs With BDNF

Neuroprotective strategies aiming to block AOH-induced RGC loss may be helpful for neuronal survival and function preservation. Intraocular administration of BDNF afforded protection from AOH-induced RGC loss of Brn3a$^+$RGCs. These results are in agreement with previous studies that have investigated short-term survival of RGCs following transient ischemia of the retina induced by elevation of the IOP[31,44–45] or by selective ligature of the ophthalmic vessels.[31] Moreover, because the proportion of surviving RGCs was comparable at 15 and 45 days after AOH, we interpret these findings as an indication that BDNF halted the protracted loss of RGCs and thus resulted in long-lasting protection. These results differ from the neuroprotective response of RGCs when the insult consists of a complete crush or transection of the ON, because in those situations RGC loss continues and the prevention achieved with BDNF is only temporary.[36–38,42–43] The differences between these injury-induced models (AOH versus axotomy) may reside in the fact that AOH is a transient insult and axotomy is permanent, and thus the activated cascades of RGC loss upon which BDNF acts may be different.[38,45,80–81]

m$^+$RGCs Appear More Resilient Than Brn3a$^+$RGCs to AOH-Induced Loss

In the vehicle-treated group, shortly after AOH, the numbers of m$^+$RGCs were lower (20% in the vehicle group, 25% in the BDNF group) than those of Brn3a$^+$RGCs and recovered to greater values by 14 days after AOH. We interpret this diminution and the subsequent increase in m$^+$RGCs numbers as an indication of a transitory downregulation of the melanopsin protein shortly after AOH and its gradual recovery to their normal values by 14 days. A similar phenomenon of transient downregulation of melanopsin expression has been demonstrated during the first two weeks after optic nerve axotomy.[13,58,82] However, 45 days after AOH, the numbers of m$^+$RGCs in the vehicle group remain comparable with those found at 14 days (38% of the original population) and represent a much higher survival rate than that of the general population of Brn3a$^+$RGCs (13%). Thus, overall our results add up to the bulk of previous work reporting that m$^+$RGCs are more resilient to various types or retinal injuries,[17–18,58,83–87] including pressure-induced transient ischemia of the retina.[30] González Fleitas and colleagues[30] have documented in adult pigmented rats a special resilience of m$^+$RGC against transient elevation of the IOP during 45 minutes using a variety of morphologic, immunohistochemical, Western blot, and behavioral techniques. Our present results showing a 60% loss of m$^+$RGCs appears to be at odds with the study of González Fleitas and colleagues,[30] which shows no m$^+$RGC loss, but the following methodological differences may explain this discrepancy: (1) the transient ischemic interval inflicted to the retinas was longer in our study (75 against 40 minutes), and RGC damage depends on the length of the transient ischemic period with a threshold between 45 to 60 minutes[53]; (2) we increased the IOP to 75 mm Hg, which sufficed to halt retinal perfusion, whereas González Fleitas and colleagues[30] raised the IOP to 120 mm Hg; and (3) slight differences in the immunohistochemical methods (the primary antibody used as well as the

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incubation time). In control pigmented or albino rats, the total numbers of m^+RGCs that we obtain[25–27] are considerably larger than those reported by González Fleitas and colleagues[30] (2,200–2,300 vs. 1,300–1,400), suggesting a different threshold for the detection of melanopsin, which may result in different numbers. Overall, these methodological differences could justify the different total numbers of m^+RGCs identified in these two studies.

Neuroprotection of m^+RGCs With BDNF

In the m^+RGC subpopulation, BDNF administration also resulted in an important rescue. Indeed, in the retinas treated with BDNF, we observed at 45 days a substantial increase in their m^+RGCs number (77% of them were immunodetected). This is a somewhat surprising effect because in a previous study in which BDNF was administered shortly after laser-induced OHT, the m^+RGC subpopulation did not respond to BDNF. Possible explanations for these apparent discrepancies with our previous study[17] are the type of injury induced to the retina, which was different, and the fact that the survival intervals analyzed were much shorter (12 and 15 days), and thus melanopsin downregulation may not have fully recovered yet.

Conclusions

In summary, we show for the first time the responses of two distinct populations of RGCs to AOH and BDNF afforded neuroprotection. Although the general population of RGCs ($Brn3a^+$RGCs) involved in image-forming visual functions die progressively following AOH, the population of RGCs involved in nonimage forming visual functions (m^+RGCs) do not and are in fact more resilient to this kind of retinal injury. Both populations, however, are responsive to BDNF that shows long-lasting neuroprotective effects.

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