Changes in melatonin receptor expression in a murine model of glaucoma

Alejandro Martínez-Águila,[1] Begoña Fonseca,[1] María J. Pérez de Lara,[1] M. Teresa Miras-Portugal,[2] Rosa Gómez-Villafuertes,[2] Gonzalo Carracedo,[3] Jesús Pintor[1]

[1]Department of Biochemistry and Molecular Biology, Faculty of Optics and Optometry, Complutense University of Madrid, Madrid, Spain; [2]Department of Biochemistry and Molecular Biology, Faculty of Veterinary, Complutense University of Madrid, Madrid, Spain; [3]Department of Optometry and Vision, Faculty of Optics and Optometry, Complutense University of Madrid, Madrid, Spain

Purpose: The objective of this study was to evaluate the changes in the melatoninergic receptors of DBA/2J and C57BL/6J mice with the development of glaucoma. DBA/2J mice are widely used to study the physiopathology of glaucoma due to the similarities of their eyes to human eyes and the resulting similarity in the development of their pathology. In addition, melatoninergic receptors are known for their control of intraocular pressure (IOP), reducing the production of aqueous humor; however, little is known about their relationship with the development of this pathology.
Methods: mRNA expression of MT_1, MT_2, and GPR50 melatoninergic receptors was performed with quantitative real-time PCR. In addition, receptor expression was performed with immunohistochemical techniques on the ciliary processes. To further investigate the effect of melatonin and its analog 5-methoxycarbonylamino-N-acetyltryptamine (5-MCA-NAT) on IOP, animals were instilled with these compounds and the corresponding melatoninergic antagonists to assess their effect on IOP.
Results: All melatoninergic receptor expression decayed with the development of the glaucomatous pathology in the DBA/2J mice, and was especially visible for the MT_2 receptor. However, receptor expression was consistent in the C57BL/6J control mice across all ages investigated. Furthermore, IOP blockage was stronger with 4PPDOT (MT_2 antagonist) only in the DBA/2J mice which suggests a correlation of this receptor with the development of the glaucomatous pathology in DBA/2J animals.
Conclusions: Melatonin receptor expression decays with the development of the glaucomatous pathology. This implies that the physiologic hypotensive effect of endogenous melatonin reducing IOP is not possible. A solution for such changes in receptor expression is the exogenous application of melatonin or any of its analogs that permit the activation of the remaining melatonin receptors.

Elevated intraocular pressure (IOP) is one of the main factors contributing to glaucoma pathology. Elevation of IOP is mainly due to resistance in aqueous humor outflow in the trabecular meshwork and the uveoscleral pathway [1], after being produced in the ciliary body [2]. Most models displaying ocular hypertension and glaucoma require physical, chemical, or surgical animal modifications to transform the models from normotensive into hypertensive [3,4]. Nonetheless, for several years, a mouse strain classified as DBA/2J has been used as the model for glaucoma because this model develops the pathology spontaneously. These animals present several mutations in the genes *Tyrp1* and *Gpnmb*, which encode for tyrosinase-related and glycosylated transmembrane proteins, respectively. Consequently, these mice present iris pigment dispersion, iris transillumination as well as iris atrophy, and anterior synechia [5]. The pigment dispersion likely is responsible for the blockade of aqueous outflow, which generates ocular hypertension at 9 months of age. This rise in IOP is accompanied by the canonical symptoms of glaucoma-related disease, including the death of retinal ganglion cells (RGCs), optic nerve atrophy and cupping, and visual deficits [6]. Altogether, these symptoms lead to progressive optic neuropathy characterized by functional and structural impairment of ocular tissues that may result in vision loss [7].

Many new compounds, apart from those already commercially available, have appeared attempting to provide effective treatments to stop ocular hypertension and glaucoma progression [8]. One of the emerging compounds is melatonin and its analogs, which clearly present ocular hypotensive effects, as well as interesting neuroprotective actions [9,10]. Melatonin is present naturally in the aqueous humor and may regulate IOP physiologically [11]. Melatonin and some of its analogs, such as 5-methoxycarbonylamino-N-acetyltryptamine (5-MCA-NAT), when topically applied, can produce a marked reduction in IOP [12-14,15]. The action

Correspondence to: Alejandro Martínez Águila, Dpto de Bioquímica y Biología Molecular, Facultad de Óptica y Optometría, Universidad Complutense de Madrid, Madrid, Spain. Phone: +0034 913946884; FAX: +0034 913946885; email: amaguila@ucm.es

of melatoninergic agents is mediated by membrane proteins termed MT_1 and MT_2 and the putative MT_3 receptors [14]. In addition, the GPR50 protein has been identified as a possible melatonin receptor due to its homology with MT_1 and MT_2 receptors [16], although this protein has not yet been proved to be activated by melatonin. The presence of such receptors is widespread in the eye, and in particular, MT_1 and MT_2, are present in the ciliary body, the tissue where aqueous humor is produced [17].

Experiments preformed in normotensive rabbits have demonstrated that melatonin and 5-MCA-NAT can reduce the chloride and water outflow the from ciliary body epithelial cells, this being the reason for the observed decrease in IOP when those melatoninergic agents are applied [18]. We also found that melatonin analogs could reduce IOP in the DBA/2J mouse glaucoma model. This is particularly interesting because the hypotensive effect is especially relevant when the animal has fully developed the pathology, which occurs from 9 months of age. Moreover, when treatment with the analog 5-MCA-NAT is performed chronically, the typical rise in IOP observed from 9 months of age does not progress, and IOP remains consistent [19].

Little is known about why IOP rises when the glaucomatous pathology is established. It could be that among other factors, the endogenous melatonin present in the aqueous humor was unable to produce the physiologic control of IOP due to a change in the presence of melatonin receptors. To investigate this possibility, in the present study we measured changes in the expression of melatonin receptors according to time intervals in control (C57BL/6J) and DBA/2J mice, observing a decrease in melatonin receptors in the glaucomatous mice but not in the control mice.

METHODS

Animals: Experiments were performed on adult female C57BL/6J (control animals) and DBA/2J (glaucomatous animals) mice obtained from the European distributor of Jackson Laboratories Mice (Charles River Laboratories, Saint-Germain-Nuelles, France). All animal maintenance and experimental procedures followed Spanish and European guidelines for animal care in the laboratory and animal research (Guide for the Care and Use of Laboratory Animals) and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Mice were housed (one to four mice per cage) in temperature- and light-controlled rooms maintained according to a 12 h:12 h light-dark cycle; all animals were fed ad libitum. The DBA/2J and C57BL/6J mice were examined at 3, 6, 9, and 12 months of age. The

study was approved by the Animal Experimental Committee of the Universidad Complutense de Madrid and Comunidad de Madrid, reference 45/057949.9/16.

Study design: A total of 48 animals of each strain were used for the study. Among them, 24 were used for PCR (six animals at each time point: 3, 6, 9, and 12 months), 12 for immunofluorescence (six animals at 3 and 12 months), and 12 for IOP measurements.

RNA isolation and RT–PCR: Total RNA from isolated ciliary bodies and irises of the DBA/2J (n = 24) and C57BL/6 (n = 24) mice was extracted using the SpeedTools total RNA extraction kit (Biotools, Madrid, Spain), following the manufacturer's instructions. After digestion with TURBO DNase (Ambion, Austin, TX), total RNA was quantified and reverse-transcribed using M-MLV reverse transcriptase, 6 µg of random primers, and 350 µM deoxynucleotides (dNTPs; Invitrogen, San Francisco, CA). Due to the small size of the tissues, and to improve the sensitivity of the PCR analysis, preamplification reactions were performed using DNA AmpliTools Master Mix (Biotools), 5 µl of the reverse transcriptases (RT) product, and specific commercial oligonucleotide primers for mouse MT_1, MT_2, and GPR50 receptors (Applied Biosystems). Non-template control was amplified to check for contamination during the procedure. Preamplification reactions were performed on a 2720 Thermal Cycler (Applied Biosystems) with the following program: the initial denaturation step at 94 °C for 5 min followed by 14 cycles of amplification (94 °C for 30 s; 60 °C for 30 s; and 72 °C for 30 s). Then, 5 µl of the preamplified product diluted 1:5 in water were used for the subsequent PCR assay. The PCR reactions were identical to the preamplification reactions, with the exception of the number of cycles, which was 40 in the last case. The amplified PCR products were electrophoresed on a 2% agarose gel and visualized with SYBR® Safe DNA gel stain (Invitrogen). The DNA ladders used were GeneRuler 1 kb and 100 bp (Thermo Scientific).

Quantitative real-time PCR: Following preamplification, 5 µl of each product diluted 1:5 in water were used for the subsequent quantitative real-time PCR assay (qPCR). qPCR reactions were performed using LuminoCt® qPCR ReadyMix (Sigma-Aldrich), 5 µl of the RT product, and specific commercial oligonucleotide primers and TaqMan MGB probes for the mouse MT_1, MT_2, and GPR50 receptors, as well as for glyceraldehyde-3-phosphate dehydrogenase (GAPDH; all from Applied Biosystems). Fast thermal cycling was performed using a StepOnePlus® Real-Time System (Applied Biosystems, Foster City, CA) as follows: denaturation, one cycle of 95 °C for 20 s, followed by 40 cycles each of 95 °C for 1 s and 60 °C for 20 s. The results

were normalized as indicated by parallel amplification of the GAPDH housekeeping gene.

Immunohistochemistry: C57BL/6J and DBA/2J mice at 3 and 12 months of age (n = 6 animals for each group) were euthanized with an intraperitoneal injection of 20% sodium pentobarbital (Dolethal, Vetoquinol®; Especialidades Veterinarias, S.A., Alcobendas, Madrid, Spain) and perfused pericardially with PBS (1X; 137 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.3) followed by a solution of 4% paraformaldehyde in PBS 0.1 M, pH 7.4 at 4 °C. The eyes were enucleated and dissected with curved forceps and sterile scissors. The anterior pole was immersed in paraformaldehyde (PFA) fixative solution for 1 h at 4 °C, washed in PBS, rinsed in 11% sucrose solution for 1 h, and placed in 33% sucrose solution overnight at 4 °C as the cryoprotection procedure. Finally, the structures were embedded in tissue medium freezing medium (Tissue-Tek© OCT, Sakura Finetek, Torrance, CA) using liquid dinitrogen. Vertical sections (10 µm thick) were cut on a cryostat (Microm, Walldorf, Germany) and collected on poly-L lysine-coated slides and stored at −20 °C until use.

The frozen sections were rinsed in PBS 1X and permeabilized with PBS containing 0.25% Triton X-100 (TX-100) for 30 min. To avoid non-specific staining, the sections were incubated with blocking solution containing 10% normal donkey serum (NDS; Jackson ImmunoResearch, West Grove, PA) and 0.1% TX-100 in PBS for 1 h at room temperature. Then, the following primary antibodies diluted in PBS containing 0.1% TX-100 were incubated at 4 °C overnight: goat anti-MT_1 (Santa Cruz Biotechnologies, Inc. Santa Cruz, CA; sc-15204, 1:100), goat anti-MT_2 (Alomone Labs, Jerusalem, Israel; APR-010, 1:100), and goat anti-GPR50 (Santa Cruz Biotechnologies; sc-15215, 1:75). The tissue sections were then washed in PBS containing 0.1% TX-100 and incubated with the secondary antibody donkey anti-rabbit Alexa Fluor 488 immunoglobulin G (IgG; H+L; Jackson ImmunoResearch) diluted 1:200 in PBS containing 0.1% TX-0.1% for 1 h in darkness at room temperature. The nuclei were stained with propidium iodide (red, Sigma-Aldrich, St. Louis, MO) diluted 1:500 in PBS for 10 min. Finally, the sections were rinsed and mounted in Vectashield (Vector Laboratories, Palex Medical, Barcelona, Spain) and coverslipped. For the analysis, images were acquired using a laser-scanning microscope (Zeiss LSM 5, Jena, Germany) at 40X magnification and exported as .tiff layers for further analysis.

IOP measurements: Melatonin (Sigma) and 5-MCA-NAT (Tocris, Bristol, UK) were formulated in isotonic saline containing 1% dimethyl sulfoxide (DMSO; Sigma, Darmstadt, Germany) and tested at 100 µM. The substances were

applied in drops to the cornea at a fixed volume of 2 µl in both eyes. The control animals received the same volume of saline + 1% DMSO. IOP was measured with a TonoLab® non-invasive rebound tonometer supplied by Tiolat Oy (Vantaa, Finland). To avoid the effect of the circadian rhythm, the IOP was always tested at the same time of the day. Six consecutive measurements were taken for each reading, and three readings were obtained for each eye.

To study the effect of melatonin and 5-MCA-NAT, two IOP measurements were taken before they were instilled, and once every hour for 6 consecutive h. Luzindole (a non-selective melatonin antagonist), 4-phenyl-2- propionamidotetralin (4PPDOT, an MT_2 melatonin antagonist), and prazosin (a MT_3 melatonin antagonist) were used as antagonists of the melatonin receptors, all tested at 100 µM. To study the effect of the different antagonists tested, they were instilled at a volume of 2 µl, 30 min before the agonist, at a concentration of 100 µM. IOP was measured in the same fashion as previously described [19].

Statistical analysis: All data are presented as the mean ± standard error of the mean (SEM). Statistical differences were calculated using one-way analysis of variance (ANOVA) test with Dunnett's multiple comparisons test. Plotting and fitting were performed with GraphPad Prism 6 computer program (GraphPad Software, San Diego, CA).

RESULTS

Expression of melatonin receptors in murine ciliary processes: We performed RT–PCR experiments demonstrating that MT_1, MT_2, and GPR50 transcripts are expressed in the ciliary processes of C57BL/6J and DBA/2J mice. These results indicated that the expression levels of melatonin receptors were presented in the control and glaucomatous animals. Taking into account that the intensity appeared different between the two mice strains, we analyzed the expression of the MT_1, MT_2, and GPR50 receptors in the ciliary processes of the C57BL/6J and DBA/2J mice at different ages with qPCR and immunohistochemistry to check whether there was any change.

Expression of the melatonin MT_1 receptor in the ciliary body: The qPCR results for the non-glaucomatous mouse (C57BL/6J) ciliary body revealed that the expression of the MT_1 mRNA remained consistent at 3, 6, 9, and 12 months of age (Figure 1A, black bars). Interestingly, when the same type of analysis was performed in the glaucomatous DBA/2J mice, the MT_1 transcript level was 2.2-fold greater in the 3-month-old DBA/2J mice compared to the control C57BL/6J at the same age (p<0.001), decreasing with the progression of the pathology during the following months (Figure 1A,

orange bars). The reduction was the highest at 12 months of age which is 1.7-fold smaller than in the 3-month-old DBA/2J mice.

Immunohistochemistry demonstrated the presence of the MT_1 receptor in the non-pigmented epithelium of the ciliary processes of the C57BL/6J and DBA/2J animals. The fluorescence was very intense in the 3-month-old DBA/2J mice compared to the 12-month-old animals (Figure 1B), which seems to indicate that there is also a reduction at the protein level with the development of the pathology.

Expression of the melatonin MT_2 receptor in the ciliary body: The expression pattern of the MT_2 receptor was very similar to that observed for the MT_1 melatonin receptor. As shown in Figure 2A, the MT_2 receptor transcript levels remained constant in the C57BL/6J mice (black bars), but an age-dependent decrease in the MT_2 receptor mRNA was observed in the DBA/2J mice (blue bars). This reduction was significant from 6-month-old animals in advance, as the maximum expression at 3 months of age, before the pathology starts, and the minimal at 12 months was 8.9-fold lower.

Immunohistochemistry confirmed that the MT_2 receptor was also located in the non-pigmented epithelium of the ciliary body in both mouse strains. Moreover, the fluorescence of the MT_2 receptor was statistically significantly higher in the 3-month-old DBA/2J animals compared to the older ones and compared to the young C57BL/6 mice (Figure 2B).

Expression of the GPR50 receptor in the ciliary body: The expression levels of the GPR50 transcript were compared between the control and glaucomatous mice in the same way as the other melatonin receptors described above. In the C57BL/6J mice, the mRNA expression of GPR50 remained consistent during the adult life of the animals (Figure 3A, black bars). Additionally, expression of GPR50 in the DBA/2J mice was fourfold higher than that in the C57BL/6J animals at 3 months old ($p<0.001$), but decreased to control values in older mice (Figure 3A, purple bars), with the minimum expression 2.5-fold smaller when the mice were 12 months old.

Immunohistochemistry revealed that the GPR50 receptor is widely distributed over all in the non-pigmented epithelium of the ciliary processes of the eye in the C57BL/6 and DBA/2J mice. As observed, the fluorescence of the GPR50 receptor was also enhanced in the 3-month-old DBA/2J animals compared to the 12-month-old mice and to the 3-month-old C57BL/6 mice (Figure 3B).

Activation of melatonin receptors decreases IOP in C57BL/6J and DBA/2J mouse strains: The action of melatonin and its analog 5-MCA-NAT was tested on the C57BL/6J and glaucomatous DBA/2J aged mice. As can be observed in Figure 4A (left), in non-glaucomatous animals, melatonin produced a reduction in IOP of 18.7±3.70% when compared to the control mice (vehicle; n = 6, *$p<0.05$ versus control; one-way ANOVA with Dunnett's multiple comparisons test). The application of melatonin receptor antagonists, such as luzindole (non-selective MT_1/MT_2), 4-PPDOT (MT_2), and prazosin (MT_3 and α1-adrenergic antagonist), reversed the hypotensive effect of melatonin suggesting that melatonin acts on the different receptors present in the ciliary body. To discard the involvement of alpha-adrenergic receptors due to the use of prazosin, the selective α1-adrenergic antagonist corynanthine was tested. As shown in Figure 4A, this compound did not modify IOP statistically significantly when melatonin was present.

When melatonin was topically applied in the glaucomatous DBA/2J mice, a strong reduction in IOP was observed (36.2±5.90%, n = 6, **$p<0.01$ versus control; one-way ANOVA with Dunnett's multiple comparisons test; Figure 4A, right). When the same antagonists for melatonin receptors were applied in the DBA/2J mice, only luzindole and 4-PPDOT were able to antagonize the hypotensive effect of melatonin. Prazosin produced some reversion, but the value was not statistically significant. In the C57BL/6J mice, corynanthine did not produce any change in the IOP.

When the melatonin analog 5-MCA-NAT was used in non-glaucomatous mice, it was possible to observe a reduction of 20.4±2.80% in IOP when compared to the control mice (Figure 4B, left; n = 6, **$p<0.01$ versus control; one-way ANOVA with Dunnett's multiple comparisons test). The application of the antagonists luzindole, 4-PPDOT, and Prazosin completely reversed the 5-MCA-NAT effect.

In the DBA/2J mice, the effect of 5-MCA-NAT diminishing IOP was also observed (32.5±4.30% n = 6, **$p<0.01$ versus control; one-way ANOVA with Dunnett's multiple comparisons test). Interestingly, when the three melatonin receptor antagonists were assayed, only the selective MT_2 antagonist 4-PPDOT was able to statistically significantly reverse the effect of 5-MCA-NAT (27.71±11.70%, n = 6, *$p<0.05$ versus control; one-way ANOVA with Dunnett's multiple comparisons test). The other two agonists showed a trend, but the differences were not statistically significant (Figure 4B, right).

DISCUSSION

The present study showed changes in the melatonin receptors MT_1 and MT_2 and the protein GPR50 expression in the ciliary body of a model of glaucoma, observed in the DBA/2J
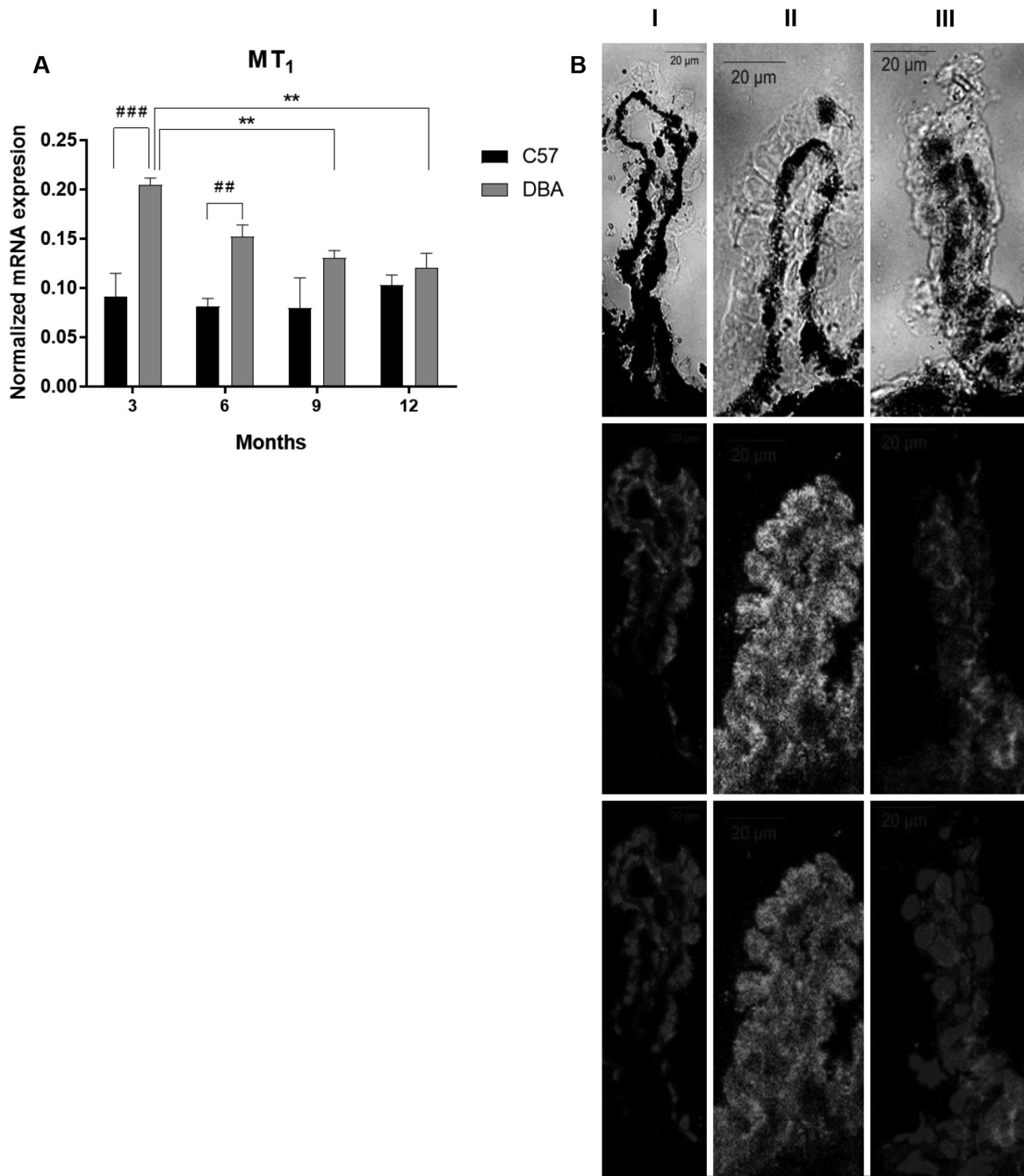
Figure 1. Temporal pattern of MT$_1$ mRNA expression and cellular distribution of the MT$_1$ receptor (in ciliary processes of C57BL/6J versus DBA/2J mice). **A**: Total RNA from ciliary processes of either control (C57BL/6J) or glaucomatous (DBA/2J) animals of 3, 6, 9, or 12 months of age was extracted, and MT$_1$ mRNA was quantified with quantitative real-time PCR (qPCR) as described in the Methods section. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control for the differences in the cDNA input. Results are the mean ± standard error of the mean (SEM) of 24 animals of each strain (*p<0.05, **p<0.01 versus the same mouse strain; #p<0.05, ###p<0.001 versus a different mouse strain; one-way analysis of variance (ANOVA) with Dunnett's multiple comparisons test). **B**: Immunofluorescence images of ciliary processes from 3-month-old C57BL/6J (I), 3-month-old DBA/2J (II), and 12-month-old DBA/2J (III) mice labeled with antibodies against the MT$_1$ receptor (green). Nuclei were counterstained with propidium iodide (red). Phase-contrast and confocal images show that the MT$_1$ receptor is mainly located in the non-pigmented epithelium of the ciliary processes, and expression of the receptor is increased in the DBA/2J versus C57BL/6J mice at the age of 3 months. Scale bar = 20 μm.
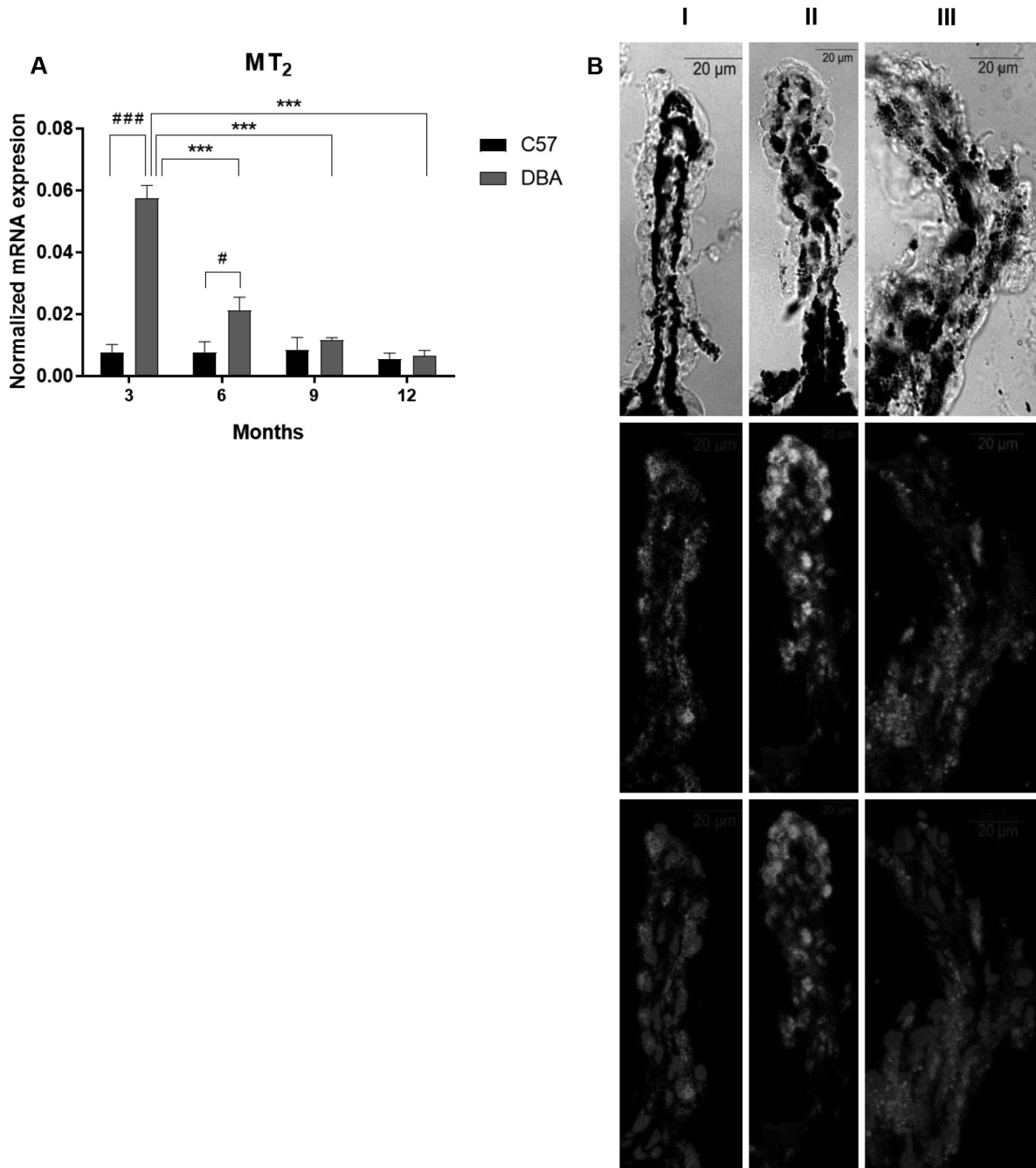
Figure 2. Temporal pattern of MT_2 mRNA expression and cellular distribution of MT_2 receptor in ciliary processes of C57BL/6J versus DBA/2J mice. **A**: Total RNA from the ciliary processes of either control (C57BL/6J) or glaucomatous (DBA/2J) animals at 3, 6, 9, or 12 months of age was extracted, and MT2 mRNA was quantified with quantitative real-time PCR (qPCR) as described in the Methods section. Values were normalized to the content of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcript. Results are the mean ± standard error of the mean (SEM) of 24 animals of each strain (***$p<0.001$ versus the same mouse strain; #$p<0.05$, ###$p<0.001$ versus a different mouse strain; one-way analysis of variance (ANOVA) with Dunnett's multiple comparisons test). **B**: Immunofluorescence images of ciliary processes from 3-month-old C57BL/6J (I), 3-month-old DBA/2J (II), and 12-month-old DBA/2J (III) mice labeled with antibodies against the MT_2 receptor (green). Nuclei were counterstained with propidium iodide (red). Phase-contrast and confocal images show that the MT_2 receptor is mainly located in the non-pigmented epithelium of the ciliary processes, and expression of the receptor is strongly increased in the 3-month-old DBA/2J mice compared to the older mice and to the 3-month-old C57BL/6J mice. Scale bar = 20 μm.
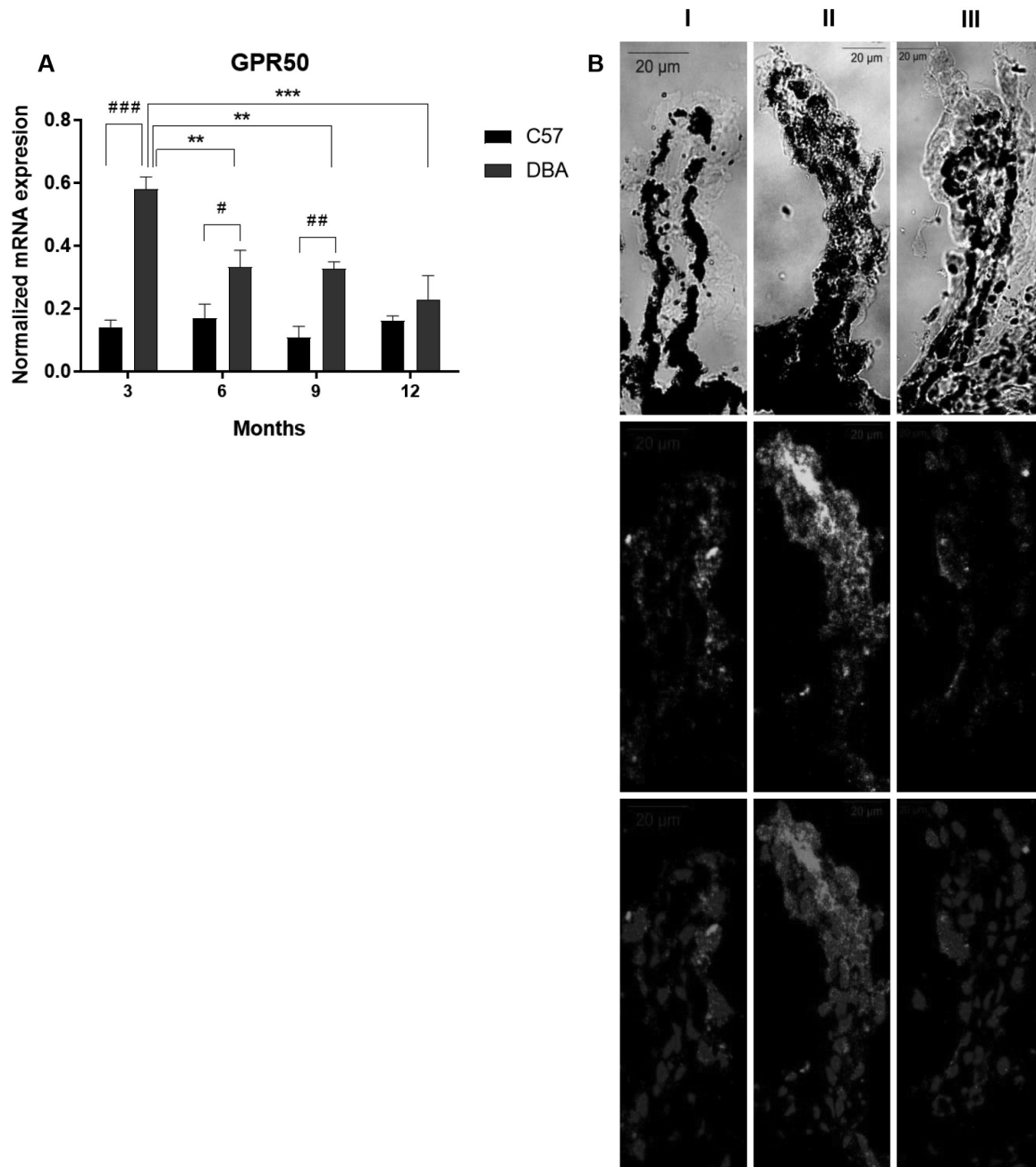
Figure 3. Temporal pattern of GPR50 mRNA expression and cellular distribution of GPR50 receptor in ciliary processes of C57BL/6J versus DBA/2J mice. **A**: Total RNA from the ciliary processes of either control (C57BL/6J) or glaucomatous (DBA/2J) animals at 3, 6, 9, or 12 months of age was extracted, and GPR50 mRNA was quantified with quantitative real-time PCR (qPCR) as described in the Methods section. Values were normalized to the content of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcript. Results are the mean ± standard error of the mean (SEM) of 24 animals of each strain (*p<0.05, ***p<0.001 versus the same mouse strain; #p<0.05, ###p<0.001 versus a different mouse strain; one-way analysis of variance (ANOVA) with Dunnett's multiple comparisons test). **B**: Immunofluorescence images of the ciliary processes from 3-month-old C57BL/6J (I), 3-month-old DBA/2J (II), and 12-month-old DBA/2J (III) mice labeled with antibodies against the GPR50 receptor (green). Nuclei were counterstained with propidium iodide (red). Phase-contrast and confocal images show that GPR50 immunostaining is mainly located in the non-pigmented epithelium of the ciliary processes, and enhanced in the DBA/2J mice versus the C57BL/6J mice at the age of 3 months. Scale bar = 20 μm.

mouse. Compared with the control animals (the C57BL/6J mouse strain), the DBA/2J mice presented a clear reduction in the mRNA expression of all the receptors investigated, which decreased concomitantly with the advance of the pathology.

The importance of melatonin receptors in the ciliary body is related to their role in control of the homeostasis of aqueous humor. The presence of the melatonin MT_1 and MT_2 receptors and the putative MT_3 receptors in the ciliary body has been described in various models [14,20-22], including those involved in homeostasis of aqueous humor. Melatonin in aqueous humor must be present for their activation. In this regard, melatonin's presence in this humor has been known since the mid-1990s [17,23]. Several research groups have examined the levels of melatonin in this ocular fluid [11,24], but only recently has it been possible to demonstrate that in the case of elevated IOP, melatonin concentrations are also high. Changes related to ocular hypertension have been observed in humans and in DBA/2J mice, where the levels of melatonin in aqueous humor present a fourfold increase [25]. This increase in melatonin concentration together with the elevation in IOP indicates a kind of uncoupling between endogenous melatonin concentrations (nM) and the receptors, as it has been demonstrated that the application of exogenous melatonin concentrations (µM) can reduce IOP in normotensive and hypertensive models, including the model used in this study [19,22]. In ocular hypertensive situations, it was

recently demonstrated that a TRPV4 channel is in control of melatonin synthesis in the ciliary body [26]. This channel acts as a pressure sensor that can stimulate expression of the key enzyme in melatonin synthesis, arylalkymine N-acetyl-transferase (AANAT) [27], as well as its phosphorylation, both mechanisms promoting the synthesis of melatonin in the ciliary body [28].

The present results offer an explanation for why the elevated melatonin levels in the IOP of glaucomatous mice remain high. In this instance, we demonstrated a reduction in the expression of mRNA melatonin receptors in the ciliary body that may explain why although the concentrations in the aqueous humor of this substance are increased, there is not enough to reduce the IOP. The experiments performed in the DBA/2J model applying melatonin at micromolar concentrations were capable of producing short- and long-term effects on IOP. However, melatonin was able to produce a reduction of about 20% in IOP detectable for a few hours. This effect was more intense in the melatonin analog 5-MCA-NAT, and this compound was able to stop the elevation of IOP when applied chronically for 3 months [19]. These two facts indicate that the receptors are still active, but higher concentrations of melatonin are needed to produce the hypotensive effect.

Although protein expression detected with immuno-fluorescence seems to be decreased in DBA/2J mice with the development of the pathology, it could not necessarily have
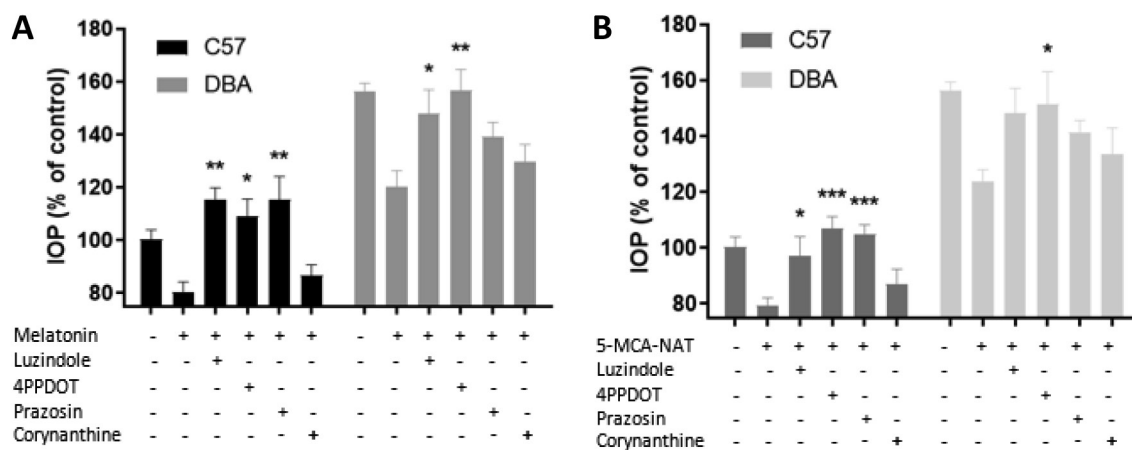

Figure 4. Effect of melatonin receptor antagonists on IOP reduction mediated by melatonin and 5-MCA-NAT in C57BL/6J (dark bars) versus DBA/2J (light bars) mice at 9 months old. Two intraocular pressure (IOP) measurements were taken before a single dose of either vehicle or 100 µM melatonin (**A**) or 5-MCA-NAT (**B**) was applied, and 3 h later, the IOP was measured again. When indicated, melatonin antagonists, all tested at 100 µM, were instilled 30 min before the agonist. Luzindole is a non-selective melatonin antagonist; 4-phenyl-2- propionami-dotetralin (4PPDOT) is a MT2 melatonin antagonist, prazosin is a MT3 melatonin antagonist/alpha blocker, and corynanthine is an alpha blocker. Data are the mean ± standard error of the mean (SEM) of four mice (*p<0.05, **p<0.01, ***p<0.001 versus agonist compound of each strain; one-way analysis of variance (ANOVA) with Dunnett's multiple comparisons test). One hundred percent were calculated as the values of the control C57BL/6J mice.

the same difference found at the mRNA level. Possible non-specific staining could not be discarded [29] with the present results and should be confirmed in future experiments to ensure that the strong decrease at the mRNA level translates into protein expression.

Among the three proteins under investigation, the melatonin MT_2 receptor seems to be the most affected by the reduction in the receptor's mRNA expression levels. This may explain in part why melatonin is unable to reduce IOP. Nevertheless, the melatonin MT_1 receptor changes should also be considered. Experiments performed on MT_1 knockout (KO) mice showed clear elevations in IOP [30], with severe changes in retinal ganglion cell survival [31]. Moreover, it is important to bear in mind that the GPR50 orphan receptor, which shares 45% of homology with melatonin receptors, has a close relation with melatonin receptors. Although the GPR50 receptor does not seem to be a melatonin receptor, being more related to energy metabolism [32], this receptor can form heterodimers with MT_1 and MT_2 receptors [33]. As a result of such dimerization, the activity of the MT_1 melatonin receptor gets inhibited, while the dimer with the MT_2 receptor does not affect its activity [34]. As in the present study the expression of mRNA GPR50 decreased with the development of the pathology, and considering previous comments about heteromerization, the melatonin MT_1 receptor would be disadvantaged in its total effect on IOP, by the reduction of the expression of the receptor and by the possible dimerization with the GPR50 protein.

In conclusion, expression of melatonin mRNA receptors decays with the development of the glaucomatous pathology. This implies that the physiologic hypotensive effect of endogenous melatonin reducing IOP is not possible. A solution for such changes in receptor expression is the exogenous application of melatonin or any of its analogs to permit the activation of the remaining melatonin receptors.

ACKNOWLEDGMENTS

REFERENCES

1. Tomarev SI. Eyeing a new route along an old pathway. Nat Med 2001; 7:294-295. [PMID: 11231625].

2. Morrison JC, Pollack IP. Anatomy and physiology of aqueous humor outflow. In: editor MJC editor and PIP, editor. Glaucoma: Science and Practice. New York: Thieme; 2003.

3. Ishikawa M, Yoshitomi T, Zorumski CF, Izumi Y. Experimentally Induced Mammalian Models of Glaucoma. BioMed Res Int 2015; 2015:281214-[PMID: 26064891].

4. Overby DR, Clark AF. Animal models of glucocorticoid-induced glaucoma. Exp Eye Res 2015; 141:15-22. [PMID: 26051991].

5. Anderson MG, Smith RS, Hawes NL, Zabaleta A, Chang B, Wiggs JL, Jhon SWL. Mutations in genes encoding melanosomal proteins cause pigmentary glaucoma in DBA/2J mice. Nat Genet 2002; 30:81-5. [PMID: 11743578].

6. Libby RT, Anderson MG, Pang IH, Robinson ZH, Savinova OV, Cosma IM, Snow A, Wilson LA, Smith RS, Clark AF, John SWM. Inherited glaucoma in DBA/2J mice: Pertinent disease features for studying the neurodegeneration. Vis Neurosci 2005; 22:637-48. [PMID: 16332275].

7. Davson H. The Aqueous Humour and the Intraocular Pressure. In: Physiology of the Eye. London: Macmillan Education UK; 1990. p. 3–95.

8. Mediero A, Alarma-Estrany P, Pintor J. New treatments for ocular hypertension. Auton Neurosci Basic Clin. 2009; 147:14-9. [PMID: 19176290].

9. Rowland JM, Potter DE, Reiter RJ. Circadian rhythm in intraocular pressure: A rabbit model. Curr Eye Res 1981; 1:169-73. [PMID: 7297102].

10. Agorastos A, Huber CG. The role of melatonin in glaucoma: Implications concerning pathophysiological relevance and therapeutic potential. J Pineal Res 2011; 50:1-7. [PMID: 21073517].

11. Martin XD, Malina HZ, Brennan MC, Hendrickson PH, Lichter PR. The ciliary body–the third organ found to synthesize indoleamines in humans. Eur J Ophthalmol 1992; 2:67-72. [PMID: 1379862].

12. Martínez-Águila A, Fonseca B, Bergua A, Pintor J. Melatonin analogue agomelatine reduces rabbit's intraocular pressure in normotensive and hypertensive conditions. Eur J Pharmacol 2013; 701:213-7. [PMID: 23270715].

13. Pescosolido N, Gatto V, Stefanucci A, Rusciano D. Oral treatment with the melatonin agonist agomelatine lowers the intraocular pressure of glaucoma patients. Ophthalmic Physiol Opt 2015; 35:201-5. [PMID: 25600174].

14. Serle JB, Wang RF, Peterson WM, Plourde R, Yerxa BR. Effect of 5-MCA-NAT, a putative melatonin MT3 receptor agonist, on intraocular pressure in glaucomatous monkey eyes. J Glaucoma 2004; 13:385-8. [PMID: 15354076].

15. Crooke A, Huete-Toral F, Martínez-Águila A, Martín-Gil A, Pintor J. Melatonin and its analog 5-methoxycarbonylamino-N-acetyltryptamine potentiate adrenergic receptor-mediated ocular hypotensive effects in rabbits: Significance for combination therapy in glaucoma. J Pharmacol Exp Ther 2013; 346:138-45. [PMID: 23591996].

16. Gubitz AK, Reppert SM. Assignment of the melatonin-related receptor to human chromosome X (GPR50) and mouse chromosome X (Gpr50). Genomics 1999; 55:248-51. [PMID: 9933574].

17. Osborne NN, Chidlow G. The presence of functional melatonin receptors in the iris-ciliary processes of the rabbit eye. Exp Eye Res 1994; 59:3-9. [PMID: 7835395].

18. Huete-Toral F, Crooke A, Martínez-Águila A, Pintor J. Melatonin receptors trigger cAMP production and inhibit chloride movements in nonpigmented ciliary epithelial cells. J Pharmacol Exp Ther 2015; 352:119-28. [PMID: 25344385].

19. Martínez-Águila A, Fonseca B, Pérez De Lara MJ, Pintor J. Effect of Melatonin and 5-Methoxycarbonylamino-N-Acetyltryptamine on the Intraocular Pressure of Normal and Glaucomatous Mice. J Pharmacol Exp Ther 2016; 357:293-9. [PMID: 26941171].

20. Alarma-Estrany P, Pintor J. Melatonin receptors in the eye: Location, second messengers and role in ocular physiology. Pharmacol Ther 2007; 113:507-22. [PMID: 17229466].

21. Wiechmann AF, Summers JA. Circadian rhythms in the eye: The physiological significance of melatonin receptors in ocular tissues Prog Retin Eye Res 2008; 27:137-60. [PMID: 18316227].

22. Pintor J, Peláez T, Hoyle CHV, Peral A. Ocular hypotensive effects of melatonin receptor agonists in the rabbit: Further evidence for an MT 3 receptor. Br J Pharmacol 2003; 138:831-6. [PMID: 12642384].

23. Osborne NN. Serotonin and melatonin in the iris/ciliary processes and their involvement in intraocular pressure. Acta Neurobiol Exp (Warsz) 1994; 54:Suppl57-64. [PMID: 7801793].

24. Chiquet C, Claustrat B, Thuret G, Brun J, Cooper HM, Denis P. Melatonin Concentrations in Aqueous Humor of Glaucoma Patients. Am J Ophthalmol 2006; 142:325-327. [PMID: 16876520].

25. Alkozi H, Sánchez-Naves J, Pérez de Lara MJ, Carracedo G, Fonseca B, Martinez-Aguila A, Pintor J. Elevated intraocular pressure increases melatonin levels in the aqueous humour. Acta Ophthalmol 2017; 95:e185-9. [PMID: 27595784].

26. Alkozi HA, Pintor J. TRPV4 activation triggers the release of melatonin from human non-pigmented ciliary epithelial cells. Exp Eye Res 2015; 136:34-7. [PMID: 25933715].

27. Alkozi HA, de Lara MJP, Sánchez-Naves J, Pintor J. TRPV4 stimulation induced melatonin secretion by increasing arylalkymine N-acetyltransferase (AANAT) protein level. Int J Mol Sci 2017; 18:746-757-[PMID: 28368307].

28. Alkozi HA, Perez de Lara MJ, Pintor J. Melatonin synthesis in the human ciliary body triggered by TRPV4 activation: Involvement of AANAT phosphorylation. Exp Eye Res 2017; 162:1-8. [PMID: 28655604].

29. Shakes DC, Miller DM, Nonet ML. Immunofluorescence Microscopy. In: Methods in Cell Biology. Academic Press Inc.; 2012. p. 35–66.

30. Alcantara-Contreras S, Baba K, Tosini G. Removal of melatonin receptor type 1 increases intraocular pressure and retinal ganglion cells death in the mouse. Neurosci Lett 2011; 494:61-4. [PMID: 21362461].

31. Tosini G, Boatright JH. Is the melatonin receptor type 1 involved in the pathogenesis of glaucoma? J Glaucoma 2013; 22:49-50. [PMID: 23733129].

32. Ivanova EA, Bechtold DA, Dupré SM, Brennand J, Barrett P, Luckman SM, Loudon ASI. Altered metabolism in the melatonin-related receptor (GPR50) knockout mouse. Am J Physiol Endocrinol Metab 2008; 294:E176-82. [PMID: 17957037].

33. Levoye A, Dam J, Ayoub MA, Guillaume JL, Couturier C, Delagrange P, Jockers R. The orphan GPR50 receptor specifically inhibits MT1 melatonin receptor function through heterodimerization. EMBO J 2006; 25:3012-23. [PMID: 16778767].

34. Jockers R, Maurice P, Boutin JA, Delagrange P. Melatonin receptors, heterodimerization, signal transduction and binding sites: What's new? Vol. 154 Br J Pharmacol 2008; 154:1182-95. [PMID: 18493248].