Adrenergic-melatonin heteroreceptor complexes are key in controlling ion homeostasis and intraocular eye pressure and their disruption contributes to hypertensive glaucoma.

Hanan Awad Alkozi[1*], Gemma Navarro[2,3*], David Aguinaga[3,4], Irene Reyes-Resina[3,4], Juan Sanchez-Naves[5], Maria J Perez de Lara[1], Rafael Franco[3,5 #], Jesus Pintor[1#]

Affiliations:

1. Department of Biochemistry and Molecular Biology, Faculty of Optics and Optometry, University Complutense of Madrid, Madrid, Spain.

2. Centro de Investigación en Red, Enfermedades Neurodegenerativas (CiberNed), Instituto de Salud Carlos III. Sinesio Delgado 6. 28029 Madrid. Spain.

3. Department of Biochemistry and Physiology, School of Pharmacy and Food Sciences. Universitat de Barcelona. Avda Juan XXIII, 27. 08027 Barcelona. Spain

4. Department of Biochemistry and Molecular Biomedicine, School of Biology, Universitat de Barcelona. Diagonal 643. 08028 Barcelona. Barcelona, Spain

5. Department of Ophthalmology, Balearic Islands Institute of Ophthalmology, Palma de Mallorca, Spain.

* Equal contribution

 # Equal contribution

Corresponding authors:

- Jesús Pintor (e-mail: jpintor@ucm.es); Department of Biochemistry, Faculty of Optometry, Complutense University of Madrid.

- Rafael Franco (e-mail: rfranco123@gmail.com; rfranco@ub.edu); Department of Biochemistry and Molecular Biomedicine, School of Biology, Universitat de Barcelona. Diagonal 643. 08028 Barcelona. Barcelona, Spain. Tel +344021208.

Short title

Receptor disassembly alters eye calcium handling

Abstract

Melatonin regulates intraocular pressure (IOP) whose increase leads to glaucoma and eye nerve degeneration. Aiming at elucidating the role of melatonin receptors in humour production and IOP maintenance, we here demonstrate that glaucoma correlates with disassembly of α_1-adrenergic/melatonin receptor functional units in cells producing the aqueous humour. Remarkably, α_1-adrenoceptor-containing complexes do not coupled to the cognate Gq protein and, hence, phenylephrine activation of these receptors does not lead to Ca^{2+} mobilization. Functional complexes are significantly decreased in models of glaucoma and, more importantly, in human samples of glaucoma patients (GP). In such glaucomatous conditions phenylephrine produces, via α_1-adrenoceptor activation, an increase in cytoplasmic $[Ca^{2+}]$ that is detrimental in glaucoma. The results led to hypothesize that using melatonin, a hypotensive agent, plus blockade of α_1-adrenergic receptors may normalize pressure in glaucoma. Remarkably, co-instillation of melatonin and prazosin, a α_1-adrenergic receptor antagonist, results in long-term decreases in IOP in a well-established animal model of glaucoma. The findings are instrumental to understand the physiological function of melatonin in the eye and its potential to address eye pathologies by targeting melatonin receptors and their complexes.

Key words: α_1-adrenergic, Adrenergic, Melatonin, Receptor heteromer, Glaucoma, Ciliary body

1. Introduction

Glaucoma, a pathology characterized by visual field loss, is associated with optic nerve damage (Casson, Chidlow, Wood, Crowston, & Goldberg, 2012). The main risk factors are *inter alia* aging, genetic conditions and intraocular pressure (IOP) (Quigley, 2011). Besides being the second cause of blindness in the World, 61 million people suffer from glaucoma and by 2020 the number may approach 80 million (Quigley & Broman, 2006).

Normotensive IOP in adults is approximately 16 mmHg. Ocular hypertension is diagnosed when the value exceeds 21 mmHg (Li, Huang, & Zhang, 2018). Persistent ocular hypertension results in damage of the optic disc, causing degeneration of ganglion cells. The relationship between ocular hypertension and glaucomatous pathology is a well-known phenomenon (Sihota, Angmo, Ramaswamy, & Dada, 2018). However, despite the current therapeutic arsenal to decrease IOP, ocular hypertension is still the most important risk factor for optic nerve degeneration (Rossetti et al., 2015; Tamm, Braunger, & Fuchshofer, 2015).

Parasympathomimetics, adrenergic receptor antagonists, carbonic anhydrase inhibitors and prostaglandins reduce ocular hypertension (Hommer, 2010; D. A. Lee & Higginbotham, 2005) although may present notorious side effects (Beckers, Schouten, Webers, van der Valk, & Hendrikse, 2008). Better and safer interventions include drug co-administration as a way to reduce doses and side effects (Polo, Larrosa, Gomez, Pablo, & Honrubia, 2001; Sakai et al., 2005).

Anti-glaucomatous compounds reduce aqueous humour formation by the ciliary body thus leading to decreased IOP. In the healthy eye, the control of aqueous humour production is tightly controlled by hormones/neuromodulators, and aging leads to imbalance and increased hydrostatic pressure (Delamere, 2005). The role of adrenoceptors expressed in the ciliary body seems to be opposite as either α-adrenergic agonists or ß-adrenergic antagonists may decrease IOP (Kiuchi, Yoshitomi, & Gregory, 1992; Murray & Leopold, 1985; Naito, Izumi, Karita, & Tamai, 2001). Studies in different animal models indicated that epinephrine, i.e. the endogenous agonist, produces a reduction in IOP, which can be blocked by prazosin, a selective α_1-adrenoceptor antagonist (Funk, Wagner, & Rohen, 1992; P. F. Lee, 1958; Moroi, Hao, Inoue-Matsuhisa, Pozdnyakov, & Sitaramayya, 2000). Interest in melatonin is emerging in ocular diseases as it modulates aqueous humour production in the ciliary body. Thus, this indoleamine has potential in the treatment of ocular hypertension likely by acting via specific receptors: MT_1 and MT_2 (Crooke, Colligris, & Pintor, 2012; Mediero, Alarma-Estrany, & Pintor, 2009). The aim of this paper was to decipher in the eye the interplay of melatonin and epinephrine.

2. Material and methods

2.1 Human eye postmortem samples

Three eyes of normal subjects and two of glaucoma patients (GP) came from donations managed by the Balearic Islands tissue bank Foundation. After fixation with 4% paraformaldehyde, frontal sections (10 µm thick) were collected and stored at -20 º until use.

2.2 Animals and intraocular pressure measurements

Experiments were performed using female C57BL/6J (n=5) (control) and DBA/2J (n=5) (glaucoma model) mice delivered by Charles River Lab. Institutional and regional Ethic Committees approved all procedures that included ARVO Statement for Ophthalmic and Vision Research. Mice were anesthetized by inhalation of isoflurane and IOP determined as previously described (Martinez-Aguila, Fonseca, Perez de Lara, & Pintor, 2016).

2.3 Cells, fusion proteins and expression vectors

The human non-pigmented ciliary epithelial cell line, 59HCE, was kindly supplied by Dr. Coca-Prados (Yale University) and grown in 10% high-glucose Dulbecco's-modified Eagle's medium (Gibco/Invitrogen, Carlsbad, CA). HEK-293T cells were grown in 5% FBS DMEM (Gibco/Invitrogen, Carlsbad, CA) as previously described (Martinez-Pinilla et al., 2017; Navarro et al., 2018).

The human cDNAs for the MT_1, MT_2 and α_{1A} receptors cloned in pcDNA3.1 were amplified without their stop codons using sense and antisense primers harboring either unique Hind III and BamH1 sites (MT_1, MT_2) or EcoRI and BamHI sites (α_{1A}). The fragments were then subcloned to be in frame with a peYFP (YFP) and a pGFP^2 (GFP^2) placed on the C-terminal end of the receptor.

2.4 Transient transfection and sample preparation for energy-transfer experiments

HEK-293T cells growing in 6-well dishes were transiently transfected with the cDNA encoding for each protein/fusion proteins. Cells were incubated in serum-free medium (4h) with the corresponding cDNA, with polyethylenimine (PEI, 5.47 mM in nitrogen residues) and 150 mM NaCl. The rest of procedure was performed as described elsewhere for FRET, BRET and fluorescence determinations (Hinz et al., 2018; Martinez-Pinilla et al., 2017; Navarro et al., 2018).

2.5 cAMP, calcium determination, ß-arrestin recruitment and label-free dynamic mass redistribution (DMR) assays

For cAMP determination 59HCE cells or HEK-293T cells were detached and re-suspended in medium containing 50 µM zardaverine (2,500 cells/well), pretreated (15 min) with the indicated molecule(s) and stimulated with agonists (15 min) before addition of 0.5 µM forskolin or vehicle. For [Ca^{2+}] determination cells were transfected with the cDNA encoding for the GCaMP6 calcium sensor (3 µg cDNA) using lipofectamine 2000 (Thermo Fisher Scientific). 48 hours after transfection, cells (150,000 cells/well in 96-well black, clear bottom plates) were incubated with Mg^{2+}-free Locke's buffer pH 7.4 supplemented with 10 µM glycine and receptor ligands were added as indicated. To determine arrestin recruitment, BRET experiments were performed in HEK-293T cells 48 h after transfection with the cDNA corresponding to β-arrestin-2-Rluc (1 µg) and α_{1A}-YFP (1,5 µg), or to β-arrestin-2-Rluc (1 µg) and MT_1-YFP (1,5 µg), or to MT_2-YFP (1,5 µg) alone or with α_{1A} (0.05 to 0,5 µg). Further details on procedures are elsewhere described (Martinez-Pinilla et al., 2017; Navarro et al., 2018).

2.6 Immunofluorescence and in situ proximity ligation (PLA) assays

Frozen sections from healthy and glaucoma subjects were rinsed in phosphate buffer saline (PBS) 1X and permeabilized with PBS-0.05% Tx-100 solution for 30 min. After blocking, antibodies raised against MT_1 (1:200, Santa Cruz sc-13179), MT_2 (1:1,000, ABIN122307, antibodies-online) and α_1-adrenergic (1:500, Abcam ab3462) receptors were used. The rest of the protocol was similar to that elsewhere described using *ad hoc* secondary regents. PLA allows the *ex vivo* detection of molecular interactions between two endogenous proteins. PLA probes were obtained by linkage of primary anti-MT_1 or MT_2 receptor antibodies to PLUS oligonucleotide (DUO92009, Sigma, St. Louis, USA) and the α_{1A}-adrenergic antibody to MINUS oligonucleotide (DUO92010, Sigma, St. Louis, USA). Samples were analyzed using confocal microscope (Zeiss LSM 5, Jena, Germany) at 40X magnification. The rest of the protocol was performed as described elsewhere, red spots were counted in each of the ROIs obtained in the nuclei images and data analysis was performed using specific PLA software (Franco et al., 2018; Martinez-Pinilla et al., 2017; Navarro et al., 2018).

3 RESULTS

3.1 Melatonin and α_{1A}-adrenergic receptors in 59HCE cells are uncoupled from their cognate G proteins

Melatonin acts via MT$_1$ (MT$_1$R) and MT$_2$ (MT$_2$R) receptors, which control Cl$^-$ efflux from ciliary body epithelial cells (Huete-Toral, Crooke, Martinez-Aguila, & Pintor, 2015). Interestingly, prazosin blocks the effect of melatonin on reducing IOP. As it is a α_{1A}-adrenoceptor (α_{1A}-AR) antagonist (Dubocovich, 1995; Huete-Toral et al., 2015; Pintor, Pelaez, Hoyle, & Peral, 2003) we hypothesized that melatonin and α_{1A}-adrenergic receptors (α_{1A}AR) could interact.

Cognate heterotrimeric G proteins are G$_i$ for melatonin receptors, and G$_{q/11}$ for α_{1A}-AR (www.guidetopharmacology.org). We then measured, in 59HCE ciliary body epithelial cells, cAMP levels and dynamic mass redistribution (DMR). The effect of MT$_1$ or MT$_2$ receptor agonists did not result in decreasing forskolin-induced cytosolic [cAMP] (Fig. 1A-B). When cells were challenged with the α_{1A}AR agonist phenylephrine, a remarkable elevation of cytosolic forskolin-induced cAMP was achieved. The antagonist prazosin abolished the cAMP elevation produced by phenylephrine. We also assayed the canonical coupling of α_{1A}AR to heterotrimeric G$_q$ proteins. We however did not find any effect on Ca^{2+} levels in 59HCE cells treated with phenylephrine (Fig. 1C). These results demonstrate that human ciliary epithelial cells express melatonin MT$_1$ and MT$_2$, and α_{1A}-adrenergic receptors not coupled to cognate G proteins. Adrenoceptor mediated increases in cAMP reflect a Gs coupling able to overcome the effect of forskolin. It should be noted that the concentration of forskolin chosen for the assays (500 nM) is optimal as it allows detection of both Gi- and Gs-coupling.

To confirm atypical G protein coupling, similar assays were performed in the presence toxins that disrupt G$_s$- or G$_i$-mediated signaling. Results show that cholera but not pertussis toxin inhibited the action of agonists, unequivocally indicating G$_s$ involvement (Fig. 1D, 1E). Interestingly, the presence of the α_{1A}-adrenergic antagonist, prazosin, abolished any effect of melatonergic agonists. Further evidence of cross-antagonism, a well-accepted heteromer print (Franco, Martinez-Pinilla, Lanciego, & Navarro, 2016), was underscored using a label-free technique consisting of detecting cell dynamic mass redistribution (DMR). The signal induced by any agonist was relatively high, and the combination of phenylephrine and either melatonin (Fig. 1F) or IIK7 (Fig. 1G), provided a more robust DMR response. As in cAMP determination assays, a cross-antagonism between α_{1A}AR and melatonin receptors was detected. When the experiments were carried out in the presence of cholera and pertussis toxins, responses were again abolished by cholera toxin (Fig. 1H-I). Taken together, these results suggest that the crosstalk between melatonergic and α_{1A}-adrenergic receptors are solely due to coupling to G$_s$.

3.2 Atypical signaling in ciliary cells is due to α_{1A}-adrenergic/melatonin receptor complexes

To identify potential direct interactions, a FRET biophysical approach was used (Fig. 2A). A saturable FRET curve was obtained in HEK-293T cells transfected with constant [cDNA] for α_{1A}-ARGFP[2] and increasing [cDNA] for MT$_1$R-YFP (FRET$_{max}$ 49 mFU and FRET$_{50}$ 44) (Fig. 2B). Similar experiments using increasing amounts of [cDNA] of MT$_2$R-YFP also provided a saturable FRET curve with FRET$_{max}$ and FRET$_{50}$ values of, respectively, 168 mFU and 128 (Fig. 2C). Thus, both melatonin receptors may form heteromers with α_{1A}AR (α_{1A}-MT$_1$Hets and α_{1A}-MT$_2$Hets).

Receptor functionality was first addressed in single transfected cells. Upon α_{1A}AR activation a robust increase in cytosolic [Ca^{2+}] detected by the fluorescence due to calcium-bound to an engineered GCaMP6 calmodulin sensor was obtained (Fig. 2D). The agonist effect was inhibited

by prazosin, but not by the melatonin receptor antagonists, luzindole or 4-PPDOT. Neither MT_1 nor MT_2 receptors were coupled to $G_{q/11}$ in single-transfected cells (Fig. 2E-F). Experiments of determination of forskolin-induced cAMP levels showed a G_i-protein-coupling (Fig. 2G-I). The results confirm that, when expressed individually, receptors couple to their cognate G proteins. Activation of receptors in single transfected cells also provided significant DMR read-outs (Fig. 2J-L). Finally, BRET assays performed using β-arrestin-2-Rluc and receptors fused to YFP showed recruitment to either α_{1A}, MT_1 or MT_2 receptors when selective agonists were used (Fig. 2M-O).

G protein coupling was completely different in co-transfected cells (Fig. 3A-B). On the one hand, phenylephrine did not produce Ca^{2+} responses in cells expressing either α_{1A}-MT_1Hets or α_{1A}-MT_2Hets (Fig. 3C-D). cAMP determination data in co-transfected cells were similar to the results obtained in 59HCE cells; agonists did not decrease forskolin-induced cAMP levels in cells expressing α_{1A}-MT_1Hets or α_{1A}-MT_2Hets. Also, cross-antagonism reappeared in cotransfected cells. Analogies between 59HCE and cotransfected HEK-293T cells were further found in experiments with toxins; the combined effect of agonists was blocked by cholera but not by pertussis toxin (Fig. 3E-F). Also, matching results in 59HCE cells, a cross-antagonism and a potentiation of the label-free DMR signal were found upon co-activation, and cholera toxin did block the effect (Fig. 3G-J). These results indicate a lack of productive coupling of receptor heteromers with $G_{q/11}$ or G_i. Cross-modulation was found in β-arrestin-2 recruitment but with a particular feature, namely both α_{1A}-receptor activation and activation of MT_1 melatonin receptors recruited β-arrestin to the α_{1A}-receptor, co-activation resulting in a stronger signal (Fig. 3K). Antagonists of the two receptors abolished recruitment induced by agonists (cross-antagonism was, therefore, found). Similar results were obtained in cells co-expressing α_{1A}-MT_2 heteroreceptor complexes using *ad hoc* agonists/antagonists (Fig. 3L).

3.3 The glaucomatous eye has reduced heteroreceptor complex expression and altered signaling

First of all, identification of α_{1A}-MT_1Hets and α_{1A}-MT_2Hets was achieved in healthy and glaucomatous conditions. The *in situ* proximity ligation (PLA) technique is instrumental to detect receptor-receptor interactions in a native system. Red clusters coming from PLA assays proved the occurrence of both α_{1A}-AR/MT_1R and α_{1A}-AR/MT_2R complexes in 59HCE cells. The analysis of the PLA labeling provided values of 65±10 dots/nucleus in the case of the α_{1A}-MT_1Hets and 73±8 dots/nucleus in the case of the MT_2/α_1 heteromer. The percentage of cells that presented positive PLA was 56±4 for α_{1A}-MT_1Hets and 57±5 for α_{1A}-MT_2Hets (n=150).

Does eye hypertension correlate with altered expression of melatonin-adrenoceptor complexes? We approached this question using cells subjected to stimulation of the transient receptor potential vanilloid 4 (TRPV4) channel. As previously reported (H. A. Alkozi, Perez de Lara, Sanchez-Naves, & Pintor, 2017; H. A. Alkozi & Pintor, 2015), activation of the channel mimics the ion fluxes that drive the increase in hydrostatic pressure that occurs in the hypertensive/glaucomatous eye. The application to 59HCE cells of the TRPV4 agonist, GSK1016790A, modified the PLA signal in a dose-dependent manner. As shown in Fig. 4A-B, the higher the concentration of GSK1016790A, the lower the PLA signal. Therefore, a reduction in the expression α_{1A}-MT_1Hets or α_{1A}-MT_2Hets happens when a glaucomatous condition is reproduced in a preclinical model (Fig. 4C).

Heteromer expression was assayed in unique postmortem samples obtained from GP and age-matched controls. In the eye from healthy donors, the presence of melatonin MT_1, MT_2 and α_{1A}-adrenergic receptors was confirmed by immunoreactivity across the ciliary body (Fig. 4D-F). A strong labeling for the MT_1R was present in non-pigmented epithelial cells, while the labeling for

the MT_2R was observed in the basal membrane of the non-pigmented epithelium (Fig. 4D-E). Melatonin receptors were not found in the stroma. Concerning the α_{1A}-adrenergic receptor, a positive labeling was observed in the pigmented and non-pigmented epithelial cells as well as in the stroma (Fig. 4F). Similar immunohistochemical studies carried out in samples obtained from GP showed altered receptor expression (Fig. 4G-I). The expression of the α_1-adrenergic receptor showed a trend (to increase) that was not statistically significant (Fig. 4J). In sharp contrast, immunoreactivity for melatonin receptors was markedly reduced, 81±15 and 54±13% for, respectively, MT_1 and MT_2 receptors. For MT_1/α_{1A} and MT_2/α_{1A} receptor pairs, a marked PLA positive labeling was observed in control samples (Fig. 4K and 4M). Remarkably, when PLA was performed in samples from GP, the reduction of heteromers was 88% in the case of the MT_1/α_{1A} and 90 % in the case of the MT_2/α_{1A} heteromers (Fig. 4L, 4N and 4O). Once more, these results match with the results obtained in 59HCE cells treated with a TRPV4 activator to mimic a glaucoma-like condition.

3.4 A novel therapeutic approach to combat glaucoma

In GP eyes and in the eye of glaucomatous models, the coupling of α_{1A}-adrenoceptors to G_q and the subsequent Ca^{2+} signaling seems detrimental and markedly contributing to the disease. Accordingly, an antagonist of α_{1A}-AR would be beneficial as a blocker of calcium production and of Ca^{2+}-regulated chloride channels (Fleischhauer, Mitchell, Peterson-Yantorno, Coca-Prados, & Civan, 2001). To test the hypothesis we moved to a well-established murine model of glaucoma (Perez de Lara et al., 2014). 3-month-old DBA/2J mice have normotensive eyes and physiological levels of melatonin. Retinal electrophysiology parameters were undistinguishable from those in the control mouse (C57BL/6 background) (Fig. 5A-C), however prazosin antagonized hypotensive effect of melatonin (Fig. 5D). 9-month-old mice display a full glaucoma-like pathology (Fig. 5A-C). At 9 months of age, and despite elevated levels of melatonin (Fig. 5B), the glaucomatous eye of DBA/2J mice was sensitive to the hypotensive effect of exogenously added melatonin, which reduced the IOP from 16.6±0.6 to 11.8±0.4 mmHg. Remarkably, prazosin, enhanced melatonin hypotensive action (to 9.0 ± 0.5 mmHg) instead of antagonizing it (Fig. 5F). Moreover, the effect of prazosin lasted more than 6 hours, thus indicating a very appropriate therapeutic time window. These results open a new and easy-to-implement anti-glaucoma treatment, consisting of melatonin/prazosin co-administration, with few expectable side effects.

8

4. Discussion

Current therapy of GP addresses symptoms by interventions neither efficacious in all patients nor absent of adverse events. Prostaglandin analogues have been approved for GP because they reduce intraocular pressure but by unknown mechanisms (Lanza et al., 2018; Sanford, 2014; Weinreb et al., 2015). Blockers of ß-adrenoceptors or drugs able to engage α-adrenoceptors are in the portfolio; however, the rationale behind the hypotensive action of to ß-blockers and α-receptor agonists is unknown.

Melatonin, has become popular for potential properties for (among other) sleep induction and it is even on-line available. Already in 1993 (Serino, d'Istria, & Monteleone, 1993) described the production of melatonin in the pineal gland and in the retina by methylation of N-acetyl serotonin (Hardeland, 2010). Interestingly, melatonin decreases IOP but the significant increase in the aqueous humour of GP is not enough to normalize IOP (Alarma-Estrany, Crooke, Peral, & Pintor, 2007; H. Alkozi et al., 2017). Characterization of melatonin receptors has not been completed due to atypical pharmacological and non-fulfilled suspicions of a third receptor. We reasoned that atypical data could be due to interactions with other GPCRs as receptor heteromers display particular properties (Ferre et al., 2009). Here we selected α_{1A}-AR because its activation leads to increases in cytosolic $[Ca^{2+}]$ that in turn control ion fluxes in the ciliary body. We here report that MT_1 and MT_2 receptors may directly interact with α_{1A}-ARs.

The role of epinephrine in the physiology of the eye is known since 1970 (Drance & Ross, 1970; Zalta, Shock, Stone, & Petursson, 1983). Interestingly, the lack of coupling of receptors for epinephrine to $G_{q/11}$ in the ciliary body (Figure 1), both intriguing and relevant, is due to α_{1A}-Rs forming heteromers with melatonin receptors. Remarkably, the glaucomatous eye expresses few functional units and the signaling mediated by Gq-coupled α_{1A}-ARs negatively impacts on IOP.

Our results predicted that the effect of melatonin would be enhanced by blockade of G_q-coupled α_{1A}-adrenergic receptors by prazosin. Indeed, combination of the two compounds led to normalize IOP in a well-established model of glaucoma and the effect was not transient but lasted for 6 hours. The long lasting experience with prazosin as blood pressure lowering agent indicates that it is very safe and does not display the serious side effects provoked by other α-adrenergic receptor antagonists (Brogden, Heel, Speight, & Avery, 1977).

In summary, we have discovered a trend in glaucoma consisting of the disruption of complexes formed by adrenergic and melatonin receptors. Such trend found in animal models of the disease and in samples from human eye (from GP and age-matched controls) makes ineffective the huge 3.2-fold increase in the concentration of melatonin in the glaucomatous eye. The physiological functional unit is coupled to a G_s protein whereas the disassembly leads to α_{1A}-adrenergic receptors coupled to G_q and to melatonin receptors coupled to G_i. Increases in calcium via G_q and decreases of cAMP via G_i establish a vicious circle that negatively impacts on the ion channels controlling intraocular pressure. The mechanism consisting of allosteric interaction and shift of G protein coupling is quite noteworthy and explains one early finding in the laboratory that is included in the paper, namely the lack of calcium ion mobilization by phenylephrine in cells expressing α_{1A}-adrenoceptors (Fig. 1C). Together, our findings provide a better understanding of the ciliary body physiology, completing a preclinical translational research addressed to combat glaucoma. Remarkably, a therapeutic strategy resulting from combining melatonin, sold as a supplement and lacking collateral effects even at high doses in the eye (Rosenstein et al., 2010; Sanchez-Barcelo, Mediavilla, Tan, & Reiter, 2010), and prazosin, approved for the therapy of blood hypertension (Brogden et al., 1977; Mallorga, Buisson, & Sugrue, 1988; Singleton et al., 1989; Torvik & Madsbu, 1986), could readily enter into clinical trials to assay for safety and efficacy in humans.

AUTHOR'S CONTRIBUTION

HAA and GN participated in the design and performance of many of the experiments and analyzed the results; it is considered that their contribution was similar. JSN was instrumental in obtaining and providing human samples for this study. MJPL performed IOP measurements. DA participated in immunohistochemistry and PLA assays. RF and JP designed and supervised the work; it is considered that their contribution was similar. HAA, GN, RF and JP actively participated in writing and editing. All authors have edited the paper and have received a copy of the final version.

CONFLICT OF INTEREST

The authors report no conflict of interest.

References

AbdAlla, S., Lother, H., el Massiery, A., & Quitterer, U. (2001). Increased AT(1) receptor heterodimers in preeclampsia mediate enhanced angiotensin II responsiveness. *Nat Med, 7*(9), 1003-1009. doi:10.1038/nm0901-1003

Alarma-Estrany, P., Crooke, A., Peral, A., & Pintor, J. (2007). Requirement of intact sympathetic transmission for the ocular hypotensive effects of melatonin and 5-MCA-NAT. *Auton Neurosci, 137*(1-2), 63-66. doi:10.1016/j.autneu.2007.07.004

Alkozi, H., Sanchez-Naves, J., de Lara, M. J., Carracedo, G., Fonseca, B., Martinez-Aguila, A., & Pintor, J. (2016). Elevated intraocular pressure increases melatonin levels in the aqueous humour. *Acta Ophthalmol*. doi:10.1111/aos.13253

Alkozi, H., Sanchez-Naves, J., de Lara, M. J., Carracedo, G., Fonseca, B., Martinez-Aguila, A., & Pintor, J. (2017). Elevated intraocular pressure increases melatonin levels in the aqueous humour. *Acta Ophthalmol, 95*(3), e185-e189. doi:10.1111/aos.13253

Alkozi, H. A., Perez de Lara, M. J., Sanchez-Naves, J., & Pintor, J. (2017). TRPV4 Stimulation Induced Melatonin Secretion by Increasing Arylalkymine N-acetyltransferase (AANAT) Protein Level. *Int J Mol Sci, 18*(4). doi:10.3390/ijms18040746

Alkozi, H. A., & Pintor, J. (2015). TRPV4 activation triggers the release of melatonin from human non-pigmented ciliary epithelial cells. *Exp Eye Res, 136*, 34-37. doi:10.1016/j.exer.2015.04.019

Beckers, H. J., Schouten, J. S., Webers, C. A., van der Valk, R., & Hendrikse, F. (2008). Side effects of commonly used glaucoma medications: comparison of tolerability, chance of discontinuation, and patient satisfaction. *Graefes Arch Clin Exp Ophthalmol, 246*(10), 1485-1490. doi:10.1007/s00417-008-0875-7

Brogden, R. N., Heel, R. C., Speight, T. M., & Avery, G. S. (1977). Prazosin: a review of its pharmacological properties and therapeutic efficacy in hypertension. *Drugs, 14*(3), 163-197. doi:10.2165/00003495-197714030-00002

Casson, R. J., Chidlow, G., Wood, J. P., Crowston, J. G., & Goldberg, I. (2012). Definition of glaucoma: clinical and experimental concepts. *Clin Exp Ophthalmol, 40*(4), 341-349. doi:10.1111/j.1442-9071.2012.02773.x

Chung, K. Y., Rasmussen, S. G., Liu, T., Li, S., DeVree, B. T., Chae, P. S., . . . Sunahara, R. K. (2011). Conformational changes in the G protein Gs induced by the beta2 adrenergic receptor. *Nature, 477*(7366), 611-615. doi:10.1038/nature10488

Cordomi, A., Navarro, G., Aymerich, M. S., & Franco, R. (2015). Structures for G-Protein-Coupled Receptor Tetramers in Complex with G Proteins. *Trends Biochem Sci, 40*(10), 548-551. doi:10.1016/j.tibs.2015.07.007

Crooke, A., Colligris, B., & Pintor, J. (2012). Update in glaucoma medicinal chemistry: emerging evidence for the importance of melatonin analogues. *Curr Med Chem, 19*(21), 3508-3522.

Delamere, N. A. (2005). Ciliary Body and Ciliary Epithelium. *Adv Organ Biol, 10*, 127-148. doi:10.1016/S1569-2590(05)10005-6

Drance, S. M., & Ross, R. A. (1970). The ocular effects of epinephrine. *Surv Ophthalmol, 14*(4), 330-335.

Dubocovich, M. L. (1995). Melatonin receptors: are there multiple subtypes? *Trends Pharmacol Sci, 16*(2), 50-56.

Ferre, S., Baler, R., Bouvier, M., Caron, M. G., Devi, L. A., Durroux, T., . . . Franco, R. (2009). Building a new conceptual framework for receptor heteromers. *Nat Chem Biol, 5*(3), 131-134. doi:10.1038/nchembio0309-131

Fleischhauer, J. C., Mitchell, C. H., Peterson-Yantorno, K., Coca-Prados, M., & Civan, M. M. (2001). PGE(2), Ca(2+), and cAMP mediate ATP activation of Cl(-) channels in pigmented ciliary epithelial cells. *Am J Physiol Cell Physiol, 281*(5), C1614-1623. doi:10.1152/ajpcell.2001.281.5.C1614

Franco, R., Aguinaga, D., Reyes, I., Canela, E. I., Lillo, J., Tarutani, A., . . . Navarro, G. (2018). N-Methyl-D-Aspartate Receptor Link to the MAP Kinase Pathway in Cortical and Hippocampal Neurons and Microglia Is Dependent on Calcium Sensors and Is Blocked by alpha-Synuclein, Tau, and Phospho-Tau in Non-transgenic and Transgenic APPSw,Ind Mice. *Front Mol Neurosci, 11*, 273. doi:10.3389/fnmol.2018.00273

Franco, R., Martinez-Pinilla, E., Lanciego, J. L., & Navarro, G. (2016). Basic Pharmacological and Structural Evidence for Class A G-Protein-Coupled Receptor Heteromerization. *Front Pharmacol, 7*, 76. doi:10.3389/fphar.2016.00076

Funk, R. H., Wagner, W., & Rohen, J. W. (1992). The effect of epinephrine on ciliary process vasculature and IOP studied by intraocular microendoscopy in the albino rabbit. *Curr Eye Res, 11*(2), 161-173.

Hardeland, R. (2010). Melatonin metabolism in the central nervous system. *Curr Neuropharmacol, 8*(3), 168-181. doi:10.2174/157015910792246244

Hommer, A. (2010). A review of preserved and preservative-free prostaglandin analogues for the treatment of open-angle glaucoma and ocular hypertension. *Drugs Today (Barc), 46*(6), 409-416. doi:10.1358/dot.2010.46.6.1482107

Huete-Toral, F., Crooke, A., Martinez-Aguila, A., & Pintor, J. (2015). Melatonin receptors trigger cAMP production and inhibit chloride movements in nonpigmented ciliary epithelial cells. *J Pharmacol Exp Ther, 352*(1), 119-128. doi:10.1124/jpet.114.218263

Ismail, S. A., & Mowafi, H. A. (2009). Melatonin provides anxiolysis, enhances analgesia, decreases intraocular pressure, and promotes better operating conditions during cataract surgery under topical anesthesia. *Anesth Analg, 108*(4), 1146-1151. doi:10.1213/ane.0b013e3181907ebe

Kiuchi, Y., Yoshitomi, T., & Gregory, D. S. (1992). Do alpha-adrenergic receptors participate in control of the circadian rhythm of IOP? *Invest Ophthalmol Vis Sci, 33*(11), 3186-3194.

Lanza, M., Rinaldi, M., Carnevale, U. A. G., di Staso, S., Sconocchia, M. B., & Costagliola, C. (2018). Analysis of differences in intraocular pressure evaluation performed with contact and non-contact devices. *BMC Ophthalmol, 18*(1), 233. doi:10.1186/s12886-018-0900-5

Lee, D. A., & Higginbotham, E. J. (2005). Glaucoma and its treatment: a review. *Am J Health Syst Pharm, 62*(7), 691-699.

Lee, P. F. (1958). The influence of epinephrine and phenylephrine on intraocular pressure. *AMA Arch Ophthalmol, 60*(5), 863-867.

Leonardi, A., Bucolo, C., Drago, F., Salomone, S., & Pignatello, R. (2015). Cationic solid lipid nanoparticles enhance ocular hypotensive effect of melatonin in rabbit. *Int J Pharm, 478*(1), 180-186. doi:10.1016/j.ijpharm.2014.11.032

Li, F., Huang, W., & Zhang, X. (2018). Efficacy and safety of different regimens for primary open-angle glaucoma or ocular hypertension: a systematic review and network meta-analysis. *Acta Ophthalmol, 96*(3), e277-e284. doi:10.1111/aos.13568

Mallorga, P., Buisson, S., & Sugrue, M. F. (1988). Alpha 1-adrenoceptors in the albino rabbit ciliary process. *J Ocul Pharmacol, 4*(3), 203-214.

Martinez-Aguila, A., Fonseca, B., Bergua, A., & Pintor, J. (2013). Melatonin analogue agomelatine reduces rabbit's intraocular pressure in normotensive and hypertensive conditions. *Eur J Pharmacol, 701*(1-3), 213-217. doi:10.1016/j.ejphar.2012.12.009

Martinez-Aguila, A., Fonseca, B., Perez de Lara, M. J., & Pintor, J. (2016). Effect of Melatonin and 5-Methoxycarbonylamino-N-Acetyltryptamine on the Intraocular Pressure of Normal and Glaucomatous Mice. *J Pharmacol Exp Ther, 357*(2), 293-299. doi:10.1124/jpet.115.231456

Martinez-Pinilla, E., Varani, K., Reyes-Resina, I., Angelats, E., Vincenzi, F., Ferreiro-Vera, C., . . . Franco, R. (2017). Binding and Signaling Studies Disclose a Potential Allosteric Site for Cannabidiol in Cannabinoid CB2 Receptors. *Front Pharmacol, 8*, 744. doi:10.3389/fphar.2017.00744

Mediero, A., Alarma-Estrany, P., & Pintor, J. (2009). New treatments for ocular hypertension. *Auton Neurosci, 147*(1-2), 14-19. doi:10.1016/j.autneu.2008.12.009

Moroi, S. E., Hao, Y., Inoue-Matsuhisa, E., Pozdnyakov, N., & Sitaramayya, A. (2000). Cell signaling in bovine ciliary epithelial organ culture. *J Ocul Pharmacol Ther, 16*(1), 65-74. doi:10.1089/jop.2000.16.65

Murray, D. L., & Leopold, I. H. (1985). Alpha-adrenergic receptors in rabbit eyes. *J Ocul Pharmacol, 1*(1), 3-18.

Naito, A., Izumi, H., Karita, K., & Tamai, M. (2001). Effects of a beta-adrenergic blocking agent timolol on intra ocular pressure responses induced by stimulation of cervical sympathetic nerve in the cat. *Tohoku J Exp Med, 195*(4), 219-225.

Navarro, G., Reyes-Resina, I., Rivas-Santisteban, R., Sanchez de Medina, V., Morales, P., Casano, S., . . . Franco, R. (2018). Cannabidiol skews biased agonism at cannabinoid CB1 and CB2 receptors with smaller effect in CB1-CB2 heteroreceptor complexes. *Biochem Pharmacol, 157*, 148-158. doi:10.1016/j.bcp.2018.08.046

Perez de Lara, M. J., Santano, C., Guzman-Aranguez, A., Valiente-Soriano, F. J., Aviles-Trigueros, M., Vidal-Sanz, M., . . . Pintor, J. (2014). Assessment of inner retina dysfunction and progressive ganglion cell loss in a mouse model of glaucoma. *Exp Eye Res, 122*, 40-49. doi:10.1016/j.exer.2014.02.022

Pintor, J., Pelaez, T., Hoyle, C. H., & Peral, A. (2003). Ocular hypotensive effects of melatonin receptor agonists in the rabbit: further evidence for an MT3 receptor. *Br J Pharmacol, 138*(5), 831-836. doi:10.1038/sj.bjp.0705118

Polo, V., Larrosa, J. M., Gomez, M. L., Pablo, L., & Honrubia, F. M. (2001). Latanoprost versus combined therapy with timolol plus dorzolamide: IOP-lowering effect in open-angle glaucoma. *Acta Ophthalmol Scand, 79*(1), 6-9.

Quigley, H. A. (2011). Glaucoma. *Lancet, 377*(9774), 1367-1377. doi:10.1016/S0140-6736(10)61423-7

Quigley, H. A., & Broman, A. T. (2006). The number of people with glaucoma worldwide in 2010 and 2020. *Br J Ophthalmol, 90*(3), 262-267. doi:10.1136/bjo.2005.081224

Rasmussen, S. G., DeVree, B. T., Zou, Y., Kruse, A. C., Chung, K. Y., Kobilka, T. S., . . . Kobilka, B. K. (2011). Crystal structure of the beta2 adrenergic receptor-Gs protein complex. *Nature, 477*(7366), 549-555. doi:10.1038/nature10361

Rosenstein, R. E., Pandi-Perumal, S. R., Srinivasan, V., Spence, D. W., Brown, G. M., & Cardinali, D. P. (2010). Melatonin as a therapeutic tool in ophthalmology: implications for glaucoma and uveitis. *J Pineal Res, 49*(1), 1-13. doi:10.1111/j.1600-079X.2010.00764.x

Rossetti, L., Digiuni, M., Montesano, G., Centofanti, M., Fea, A. M., Iester, M., . . . Fogagnolo, P. (2015). Blindness and Glaucoma: A Multicenter Data Review from 7 Academic Eye Clinics. *PLoS One, 10*(8), e0136632. doi:10.1371/journal.pone.0136632

Sakai, H., Shinjyo, S., Nakamura, Y., Nakamura, Y., Ishikawa, S., & Sawaguchi, S. (2005). Comparison of latanoprost monotherapy and combined therapy of 0.5% timolol and 1% dorzolamide in chronic primary angle-closure glaucoma (CACG) in Japanese patients. *J Ocul Pharmacol Ther, 21*(6), 483-489. doi:10.1089/jop.2005.21.483

Sanchez-Barcelo, E. J., Mediavilla, M. D., Tan, D. X., & Reiter, R. J. (2010). Clinical uses of melatonin: evaluation of human trials. *Curr Med Chem, 17*(19), 2070-2095.

Sanford, M. (2014). Preservative-free latanoprost eye drops in patients with primary open-angle glaucoma/ocular hypertension. *Clin Drug Investig, 34*(7), 521-528. doi:10.1007/s40261-014-0203-4

Serino, I., d'Istria, M., & Monteleone, P. (1993). A comparative study of melatonin production in the retina, pineal gland and harderian gland of Bufo viridis and Rana esculenta. *Comp Biochem Physiol C, 106*(1), 189-193.

Sihota, R., Angmo, D., Ramaswamy, D., & Dada, T. (2018). Simplifying "target" intraocular pressure for different stages of primary open-angle glaucoma and primary angle-closure glaucoma. *Indian J Ophthalmol, 66*(4), 495-505. doi:10.4103/ijo.IJO_1130_17

Singleton, W., Dix, R. K., Monsen, L., Moisey, D., Levenstein, M., Bottiglieri, D. F., & Silver, L. (1989). Efficacy and safety of Minipress XL, a new once-a-day formulation of prazosin. *Am J Med, 87*(2A), 45S-52S.

Tamm, E. R., Braunger, B. M., & Fuchshofer, R. (2015). Intraocular Pressure and the Mechanisms Involved in Resistance of the Aqueous Humor Flow in the Trabecular Meshwork Outflow Pathways. *Prog Mol Biol Transl Sci, 134*, 301-314. doi:10.1016/bs.pmbts.2015.06.007

Torvik, D., & Madsbu, H. P. (1986). Multicentre 12-week double-blind comparison of doxazosin, prazosin and placebo in patients with mild to moderate essential hypertension. *Br J Clin Pharmacol, 21 Suppl 1*, 69S-75S.

Weinreb, R. N., Ong, T., Scassellati Sforzolini, B., Vittitow, J. L., Singh, K., Kaufman, P. L., & group, V. s. (2015). A randomised, controlled comparison of latanoprostene bunod and latanoprost 0.005% in the treatment of ocular hypertension and open angle glaucoma: the VOYAGER study. *Br J Ophthalmol, 99*(6), 738-745. doi:10.1136/bjophthalmol-2014-305908

Westfield, G. H., Rasmussen, S. G., Su, M., Dutta, S., DeVree, B. T., Chung, K. Y., . . . Skiniotis, G. (2011). Structural flexibility of the G alpha s alpha-helical domain in the beta2-adrenoceptor Gs complex. *Proc Natl Acad Sci U S A, 108*(38), 16086-16091. doi:10.1073/pnas.1113645108

Zalta, A. H., Shock, J. P., Stone, J. E., & Petursson, G. J. (1983). Topical timolol and epinephrine: biochemical actions and interactions in the rabbit eye in vivo. *Ann Ophthalmol, 15*(2), 141-149.

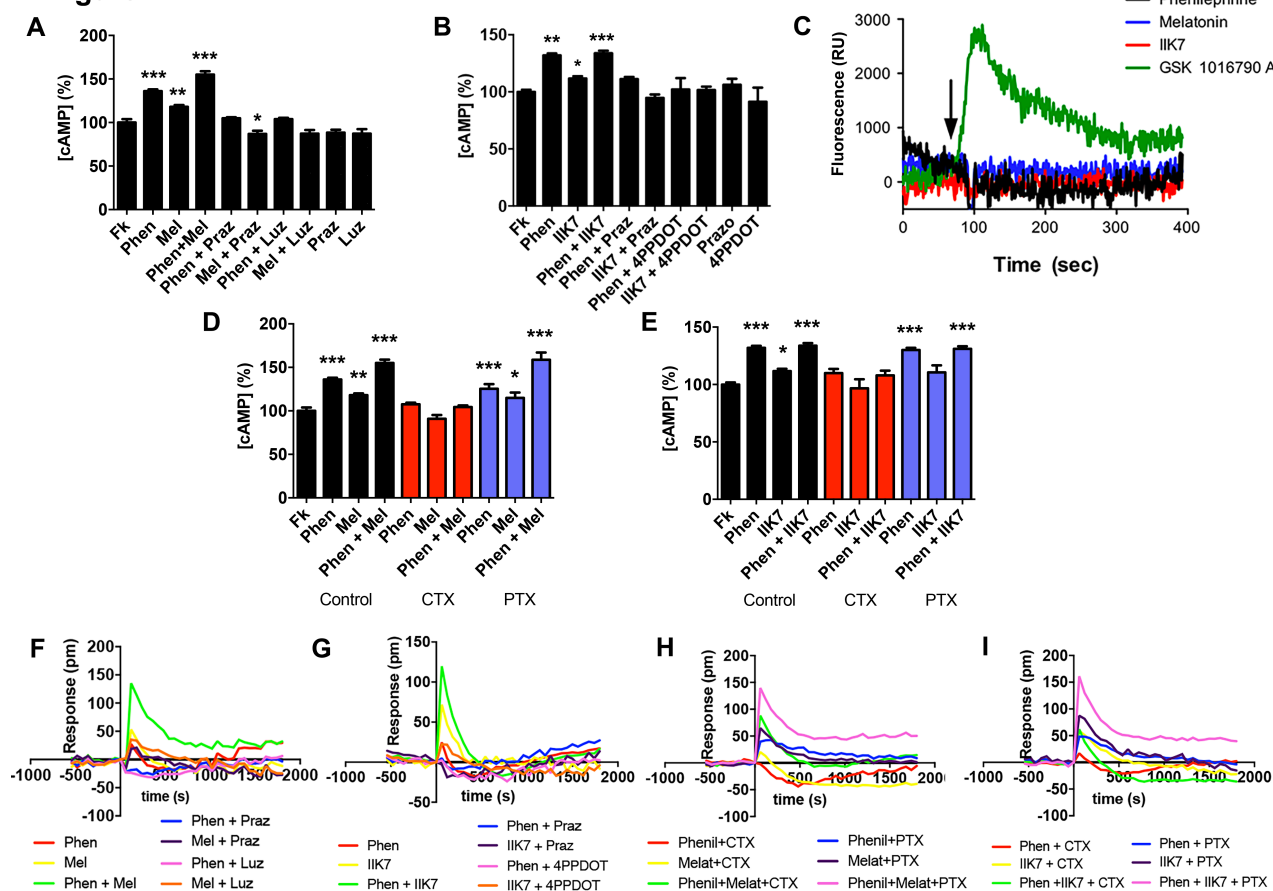**13**

Figures

Figure 1

Figure 1. Effect of melatonin receptor agonists and of phenylephrine in human 59HCE cells.
Panels A, B, D and E: Effect of ligands (single or combined treatment) on 0.5 μM forskolin-induced cAMP levels in the absence (A-B) or presence (D-E) of 10 ng/mL pertussis (overnight) or 100 ng/mL cholera (2 h) toxins. Data are given in percentage (100% represents the forskolin effect); they are the mean ± SEM (n=12, each in triplicates). One-way ANOVA followed by Bonferroni's multiple comparison *post-hoc* test were used for statistical analysis. *p<0.05; **p<0.01; ***p<0.001. Panel C: Time course of cytosolic calcium levels induced by receptor agonists or by GSK-1016790A, an agonist of TRPV4 channels used for positive control. Panels F-I: Effect of ligands (single or combined treatment) on dynamic mass redistribution (DMR) in the absence (F-G) or presence (H-I) of pertussis or cholera toxins. Concentrations in the assays were: 100 nM phenylephrine, 1 μM melatonin, 100 nM IIK7, 1 μM prazosin, 1 μM luzindole and 1 μM 4PPDOT.
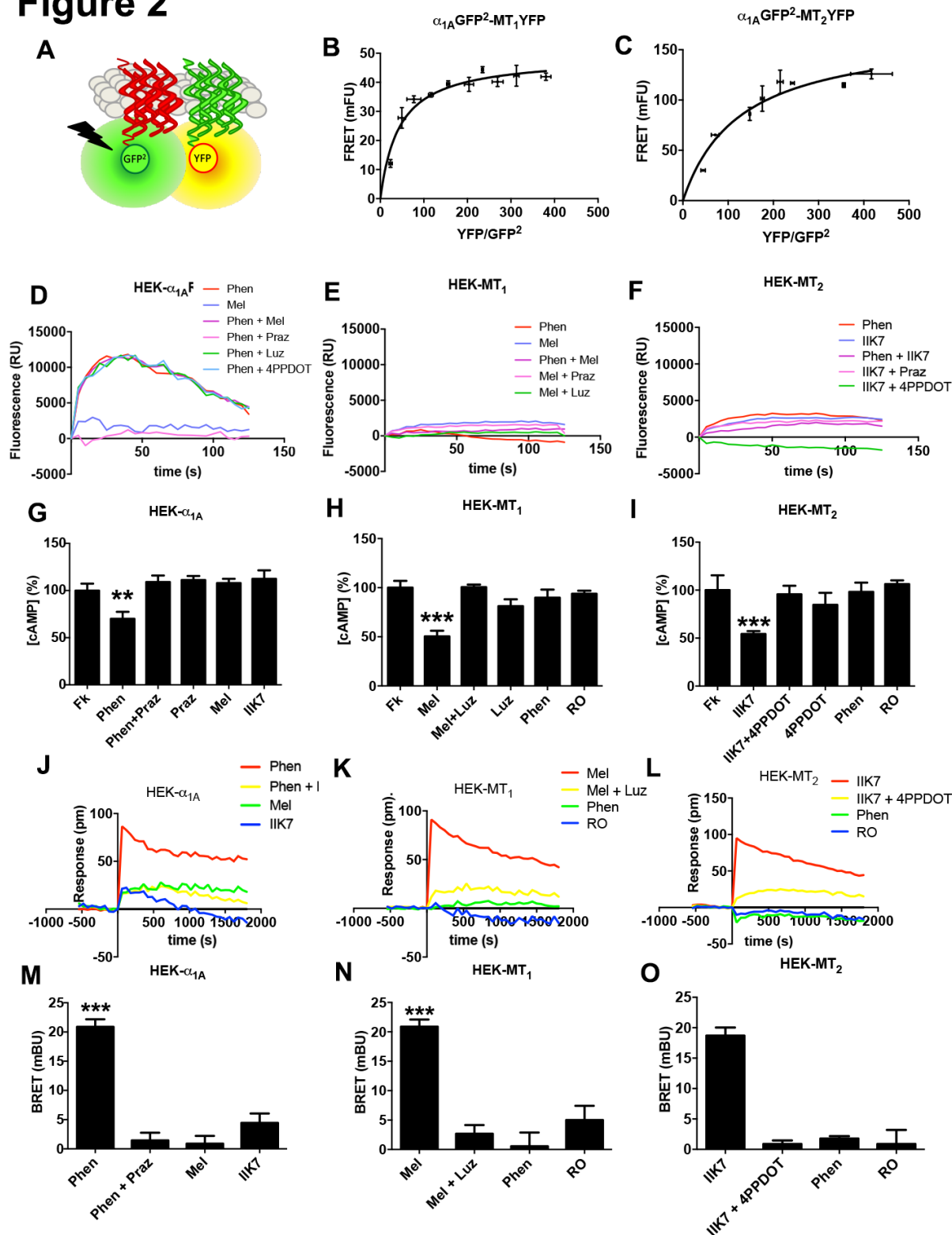
Figure 2

Figure 2. Dimerization of α_1-adrenergic and MT$_1$ or MT$_2$ receptors and signaling via adrenergic or melatonin receptors in a heterologous expression system. Panels A-C: Scheme of the FRET assays (A) and results of experiments performed in HEK-293T cells transfected with a fixed amount of cDNA for the α_1-adrenergic-GFP^2 fusion protein and increasing amounts of cDNA for either MT$_1$-YFP (B) or MT$_2$-YFP (C). Energy transfer data are given in milliFRET units (mFU). The remaining experiments were performed in HEK-293T cells transfected with cDNAs for α_1-adrenergic, MT$_1$ or MT$_2$ receptors. Panels D-F: Time course of cytosolic calcium

15

levels induced by receptor agonists (single or combined treatment; when indicated, a preincubation with antagonists was performed). Panels G-I: Effect of receptor ligands (single or combined treatment) on 0.5 µM forskolin-induced cAMP levels. Data are given in percentage (100% represents the forskolin effect); they are the mean ± SEM (n=12, each in triplicates). For negative control, a selective agonist for dopaminergic D_4 receptor, RO-105824, was used. One-way ANOVA followed by Bonferroni's multiple comparison *post-hoc* test were used for statistical analysis. **p<0.01; ***p<0.001. Panels J-L: Effect of ligands (single or combined treatment) on dynamic mass redistribution (DMR). Panels M-O: BRET-based measurements of the effect of receptor agonists (single or combined treatment) on ß-arrestin 2-Rluc recruitment to every receptor-YFP fusion protein. Data are given in milliBRET Units (mBU); they are the mean ± SEM (n=10, each in triplicates). One-way ANOVA followed by Bonferroni's multiple comparison *post-hoc* test were used for statistical analysis. ***p<0.001. Concentrations in the assays in panels D to O were: 100 nM phenylephrine, 1 µM melatonin, 100 nM IIK7, 1 µM prazosin, 1 µM luzindole, 1 µM 4PPDOT and 100 nM RO-105824.
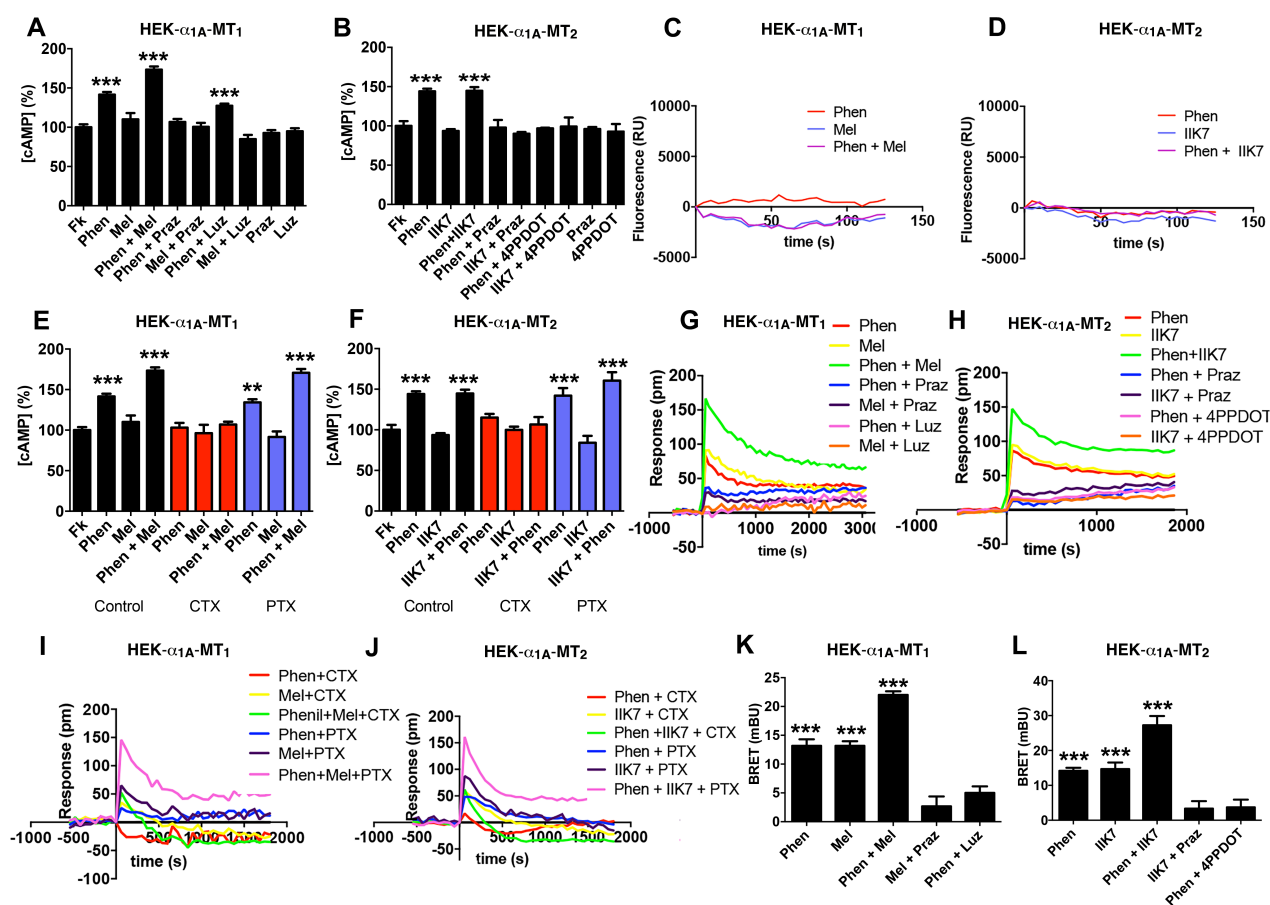
Figure 3

Figure 3. Signaling via adrenergic/melatonin receptor functional units in an heterologous expression system. Experiments were performed in HEK-293T cells transfected with cDNAs for full-length α_{1A}-adrenergic and MT_1 or MT_2 receptors. Panels A, B, E and F: Effect of ligands (single or combined treatment) on 0.5 µM forskolin-induced cAMP levels in the absence (A-B) or presence (E-F) of 10 ng/mL pertussis (overnight) or 100 ng/mL cholera (2 h) toxins. Data are given in percentage (100% represents the forskolin effect); they are the mean ± SEM (n=12, each in triplicates). One-way ANOVA followed by Bonferroni's multiple comparison *post-hoc* test were used for statistical analysis. **$p<0.01$; ***$p<0.001$. Panels C-D: Lack of effect of receptor agonist in cytosolic calcium levels. The agonist of TRPV4 channel, GSK-1016790A, did produce effect (data not shown). Panels G-J: Effect of ligands (single or combined treatment) on dynamic mass redistribution (DMR) in the absence (G-H) or presence (I-J) of pertussis or cholera toxins. Panels K-L: Effect of receptor agonists (single or combined treatment) on ß-arrestin 2 recruitment to MT_1-YFP (K) or to MT_2-YFP (L). Data are given in mBU and are the mean ± SEM (n=10, each in triplicates). One-way ANOVA followed by Bonferroni's multiple comparison *post-hoc* test were used for statistical analysis. ***$p<0.001$. Concentrations in the assays were: 100 nM phenylephrine, 1 µM melatonin, 100 nM IIK7, 1 µM prazosin, 1 µM luzindole and 1 µM 4PPDOT.
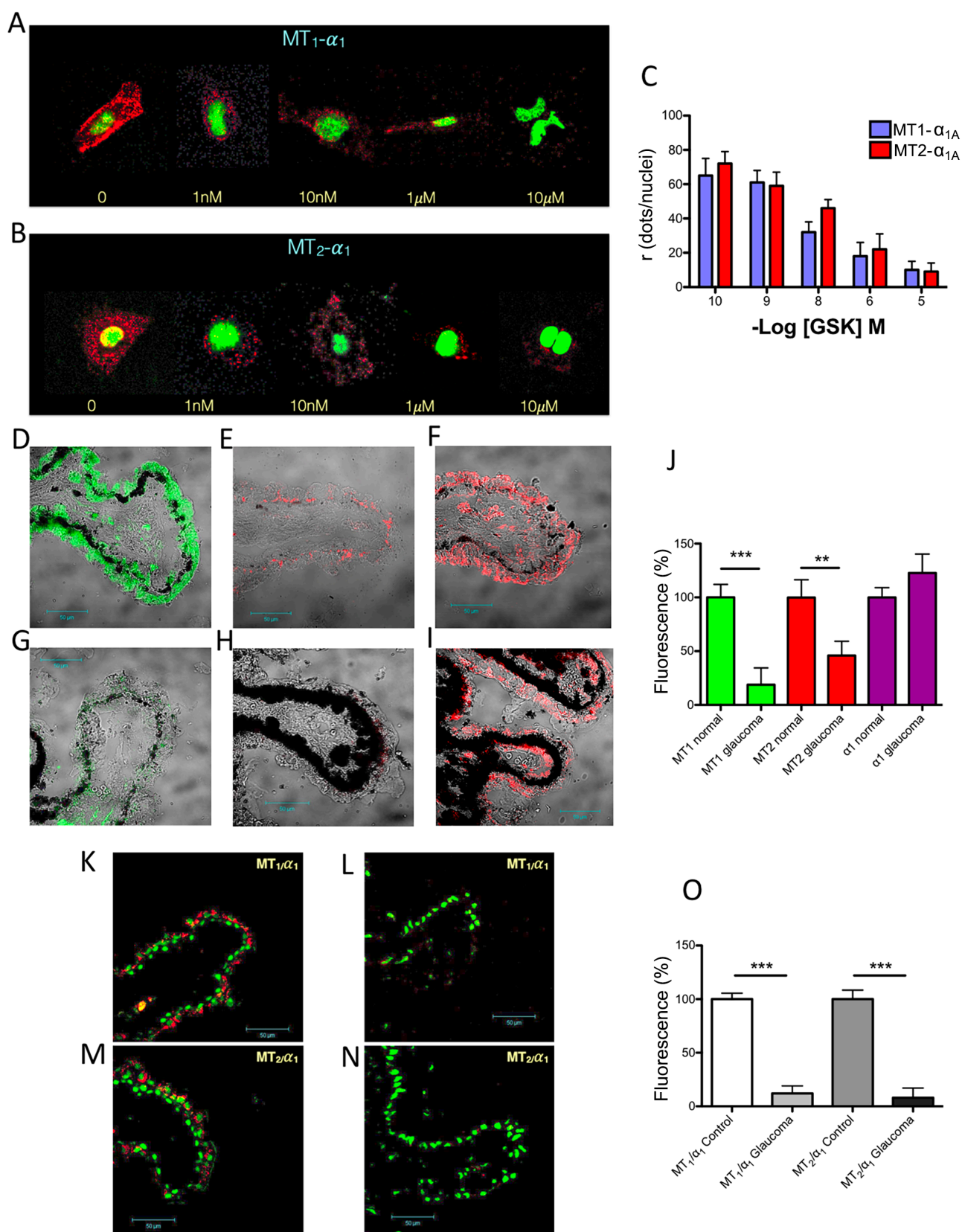
17

Figure 4. Identification of functional units in human 59HCE cells and in samples from normotensive and hypertensive human eyes. Panels A-B: Determination by PLA of α_1-adrenergic and either MT_1 (A) or MT_2 (B) receptor complexes in 59HCE non-pigmented ciliary body epithelial cells. Panel C: Quantification of clusters for α_1-adrenergic and either MT_1 (blue) or MT_2 (red) receptors. Data are the mean ± SEM (n=5). *p<0.005, using Student's t-test. Values for

negative control were, respectively, 5±1 and 2±1 dots/nucleus). Panels D-I. Immunolocalization of MT$_1$ (D), MT$_2$ (E) or α_1-adrenergic (F) receptors in the ciliary body of healthy controls. Immunolocalization of MT$_1$ (G), MT$_2$ (H) or α_1-adrenergic (I) receptors in the ciliary body of GP. Quantitation (I) of results comparing patients and controls using fluorescence values taken in identical experimental conditions. Data, given in percentage (100% given to values from controls) are the mean ± SEM (n=4). **p<0.01 and ***p<0.001, using Student's t-test. Panels K-O: Determination by PLA of α_1-adrenergic and either MT$_1$ (K) of MT$_2$ (M) receptor complexes in the ciliary body of age-matched IOP normotensive individuals. Determination by PLA of α_1-adrenergic and either MT$_1$ (L) of MT$_2$ (N) receptor complexes in the ciliary body of GP. Quantitation (O) of clusters comparing data in GP and controls using fluorescence values taken in identical experimental conditions. Data, given in percentage (100% given to values from controls) are the mean ± SEM, n=4 (***p<0.001, using Student's t-test).
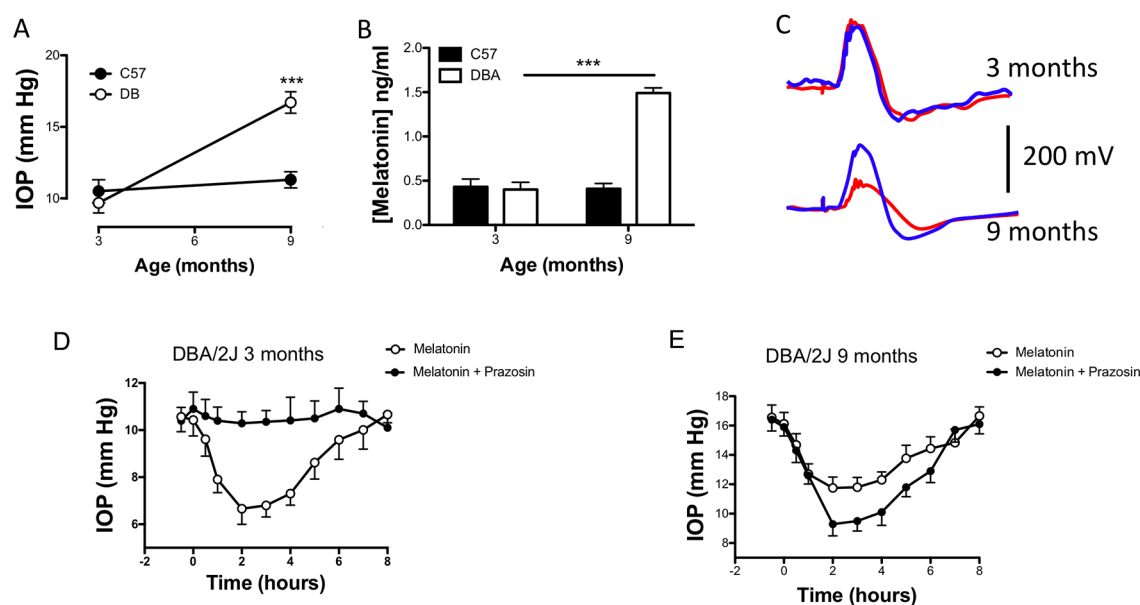

Figure 5. IOP-lowering intervention in the DBA/2J mice**.** Panel A: Intraocular pressure (IOP) values of control C57BL/6J and DBA/2J mice at 3 and 9 months of age. Data are the mean ± SEM (n=12). ***p<0.001 (Wilcoxon's test for paired samples). Panel B: Melatonin concentrations in the aqueous humour of C57BL/6J and DBA/2J mice at 3 and 9 months of age. Data are the mean ± SEM (n=6). ***p<0.001 (Wilcoxon's test for paired samples). Panel C: Electroretinogram in C57BL/6J (blue) and DBA/2J (red) mice. Positive scotopic threshold response (pSTR) amplitude was significantly reduced between 3 and 9 months of age (p<0.00001). Panels D-E: Time course of the effect on IOP in 3 (D) or 9 (E) month old DBA/2J mice after the instillation of melatonin ± prazosin. Data are the mean ± SEM (n=5). **p<0.01 (two-way ANOVA followed by *post-hoc* Tukey's test).