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Adrenergic-melatonin receptor complexes control ion homeostasis and intraocular pressure and their disruption contributes to hypertensive glaucoma.

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Short title

Receptor complex disassembly alters eye cell calcium handling

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/bph.14971

Abstract

Background and Purpose

Often, glaucoma courses with elevated eye hydrostatic pression, which is regulated by endogenous melatonin. Via α_1-adrenoceptor activation, phenylephrine increases cytoplasmic [Ca^{2+}] that is detrimental in glaucoma. The aims of the paper were i) elucidating the role of melatonin receptors in humour production and intraocular pressure (IOP) maintenance and ii) identifying glaucoma relevant melatonin-adrenergic interactions.

Experimental approach

Biophysical and proximity ligation assays were performed to identify interactions in heterologous systems, in cell lines and in human eyes. $G_s/G_i/G_q$ signaling was investigated in heterologous systems and cells producing the aqueous humour. IOP was determined in a mice model of glaucoma. Retinography and topically pharmacological treatment were performed in control and in glaucomatous mice.

Key Results

α_1-adrenergic and melatonin receptors form functional complexes in which the C-terminal tail of the adrenergic receptor play a relevant role. Remarkably, activation of α_1-adrenoceptors in the complex did not lead to cytosolic Ca^{2+} increases, suggesting G_s instead of G_q coupling. The number of complexes significantly decreased in models of glaucoma and, more importantly, in human samples of glaucoma patients. The results led to hypothesize that melatonin, a hypotensive agent, plus blockade of α_1-adrenergic receptors could normalize pressure in glaucoma. Remarkably, co-instillation of melatonin and prazosin, an α_1-adrenergic receptor antagonist, resulted in long-term decreases in IOP in a well-established animal model of glaucoma.

Conclusions & Implications

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

Abbreviations:

4PPDOT: cis-4-Phenyl-2-propionamidotetralin (antagonist of MT_2R)
α_{1A}-MT_1Hets: α_{1A}-MT_1 receptor heteromers
AR: Adrenoceptor
BRET: Bioluminescence Resonance Energy Transfer
FRET: Fluorescence Resonance Energy Transfer
GFP^2: Green fluorescence protein 2
GP: Glaucoma patients
IIK7: N-[2-(2-Methoxy-6H-isoindolo[2,1-a]indol-11-yl)ethyl]butanamide (MT_2R selective agonist)
IOP: Intraocular pressure
MT_1R: Melatonin MT_1 receptor
MT_2R: Melatonin MT_2 receptor
Rluc: *Renilla* luciferase
YFP: Yellow fluorescence protein

1. Introduction

Glaucoma, a pathology characterized by visual field loss, is associated with optic nerve damage (Casson et al., 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 and Broman, 2006).

Normotensive IOP in adults is approximately 16 mmHg. Ocular hypertension is diagnosed when the value exceeds 21 mmHg (Li et al., 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 et al., 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 et al., 2015).

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 (Mittag et al., 1985; Kiuchi et al., 1992; Naito et al., 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 (Lee, 1958; Funk et al., 1992; Moroi et al., 2000). Both epinephrine and prazosin bind the adrenergic receptor α_1, a G-protein-coupled receptor (GPCR) whose cognate heterotrimeric protein is Gq (Alexander et al., 2017, 2019).

Anti-glaucomatous compounds reduce aqueous humour formation by the ciliary body thus leading to a decrease in hydrostatic pressure. To combat elevated IOP, parasympathomimetics, adrenergic receptor antagonists, carbonic anhydrase inhibitors and/or prostaglandins are prescribed (Lee and Higginbotham, 2005; Hommer, 2010), although they may present notorious side effects (Beckers et al., 2008). Better and safer interventions include drug co-administration as a way to reduce doses and side effects (Polo et al., 2001).

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 (Mediero et al., 2009; Crooke et al., 2012) that also belong to the superfamily of GPCRs and whose cognate protein is Gi (Alexander et al., 2017, 2019). Accordingly, activation of MT_1 and MT_2 receptors engage Gi that in turn inhibits adenylyl cyclase and leads to decreasing cAMP levels and deactivation of protein kinase A (PKA) (Vanecek, 1998). Interestingly, melatonin receptors were reported to couple to different heterotrimeric G proteins and even a third melatonin receptor was conjectured. The exact reasons for pleiotropic signaling are not known, and the hypothesized third receptor is not a melatonin receptor but the enzyme quinone reductase 2 (Tsitn et al., 1996; Pintor et al., 2001; Mailliet et al., 2004; Shiu et al., 2010; Emet et al., 2016).

It is known that GPCRs form dimers/oligomers whose functionality is different from of that of individual receptors. One of the most relevant discoveries related to GPCR heteromerization is their ability to couple to more than one G protein and/or a shift in G-protein-coupling (Franco et al., 2018). The first report on G protein coupling shift was probably that reported for dopamine D_1/D_2 receptors heteromers; whereas D_1 couples to G_s and D_2 to G_i, the heteromer couples to G_q (Rashid et al., 2007; Hasbi et al., 2009; Verma et al., 2010). Heteromers may consist of receptors for the same endogenous ligand or of receptors for different endogenous ligands. Heteromerization of the two melatonin receptors was already reported in 2013 as a mechanism to control photoreceptor function (Baba et al., 2013). The aims of this paper were i) to assess whether adrenergic receptors, which are important in the control of IOP, may form heteromers with melatonin receptors and ii) decipher whether those heteromers may be involved in the interplay between melatonin and epinephrine on IOP effects in both the healthy and the glaucomatous eye.

2. Material and methods

To the best of our knowledge this manuscript adheres to BJP instructions to authors and to the guidelines elsewhere detailed ((Curtis et al., 2018)). Studies were designed to generate groups of equal size, using randomization and blinded analysis. Immunological based assays were conducted in line with BJP guidelines detailed in (Alexander et al., 2018).

2.1 Reagents

The MT_1 receptor antagonist luzindole and the MT_2 receptor agonist N-[2-(2-methoxy-6H-isoindolo[2,1-a]indol-11-yl)ethyl]butanamide (IIK7) were purchased from SigmaAldrich (St Louis, MO). The α_{1A} receptor agonist phenylephrine hydrochloride and antagonist prazosin

hydrochloride, the MT_1 receptor agonist melatonin, the MT_2 receptor antagonist, cis-4-Phenyl-2-propionamidotetralin (4PPDOT), and forskolin were purchased from Tocris Bioscience (Bristol, UK).

2.2 Human eye postmortem samples

Three eyes of healthy eye normotensive 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 following the sagittal axis) were collected and stored at -20º until use.

2.3 Animals and intraocular pressure measurements

Experiments of IOP measurements were performed using female C57BL/6J [RRID:IMSR_JAX:000664] n=12) (control) and DBA/2J [RRID:IMSR_JAX:000671] (n=12) (glaucoma model) mice of different ages delivered by Charles River Lab. Institutional and regional Ethic Committees approved all procedures that included ARVO Statement for Ophthalmic and Vision Research and all experiments took into account ARRIVE guidelines. Mice were anesthetized by inhalation of isoflurane and IOP determined as previously described (Martínez-Águila et al., 2016).

2.4 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 5% heat-inactivated fetal calf bovine serum (FBS) high-glucose Dulbecco's-modified Eagle's medium (Gibco/Invitrogen, Carlsbad, CA). HEK-293T cells [RRID:CVCL_0063] were grown in 5% FBS-DMEM (Gibco/Invitrogen, Carlsbad, CA) as previously described (Martínez-Pinilla et al., 2017; Navarro et al., 2018).

The human cDNAs for the MT_1, MT_2 and α_{1A} receptors (full-length or lacking its C-terminal domain) cloned in pcDNA3.1 were amplified without their stop codons using sense and antisense primers harboring either unique Hind III and BamH1 sites for MT_1 and MT_2 receptors or EcoRI and BamHI sites for α_{1A} receptor. The fragments were then subcloned to be in frame with a (p*RLuc*-N1; PerkinElmer, Wellesley, MA), (pEYFP-N1; Clontech, Heidelberg, Germany) or (pGFP^2-N3; Clontech, Heidelberg, Germany) placed on the C-terminal end of the receptor to generate MT_1YFP, MT_{1mut}YFP, MT_2YFP, MT_2mutYFP, α_{1A}GFP^2, α_{1A}YFP and α_{1Amut}GFP^2.

2.5 Transient transfection and sample preparation

Cells were transiently transfected with the cDNA encoding for each protein/fusion proteins and the PEI (PolyEthylenImine, SigmaAldrich, St. Louis, MO, USA) method as previously described (Navarro et al., 2018). Cells were incubated for 4h with the corresponding cDNA mixed with polyethyleneimine (PEI, 5.47 mM in nitrogen residues) and 150 mM NaCl in serum-free medium. Finally, medium was exchanged by supplemented DMEM medium and maintained for 48 h in a humid atmosphere of 5% CO_2 at 37ºC.

2.6 FRET assays

HEK-293T cells were transiently co-transfected with the plasmid cDNA corresponding to $\alpha_{1A}GFP^2$ or $\alpha_{1Amut}GFP^2$ (donor) and MT_1YFP, $MT_{1mut}YFP$, MT_2YFP or $MT_{2mut}YFP$ (acceptor) proteins using a ratio of donor to acceptor specified in figure legends. Cell suspension (20 µg of protein) was distributed into 96-well microplates (black plates with a transparent bottom) and was read in a Fluostar Optima Fluorimeter (BMG Labtechnologies, Offenburg, Germany) equipped with a high-energy xenon flash lamp, using a 10 nm bandwidth excitation filter at 400 nm (393–403 nm), and 10 nm bandwidth emission filters corresponding to 506–515 nm filter (Ch 1), 527–536 nm filter (Ch 2). Gain settings were identical for all experiments to keep the relative contribution of the fluorophores to the detection channels constant for spectral un-mixing. The contribution of the GFP variants, GFP^2 and YFP proteins alone, to the two detection channels (spectral signature), was measured in experiments with cells expressing only one of these proteins and normalized to the sum of the signal obtained in the two detection channels. FRET quantification was performed as described elsewhere (Navarro et al., 2010).

2.7 Intracellular Calcium Release

Cells were co-transfected with the cDNA for the indicated receptors and 1 µg of GCaMP6 calcium sensor (Chen et al., 2013) using the PEI method. 24h after transfection, cells were suspended in Mg^{+2}-free Locke's buffer (pH 7.4) (154 mM NaCl, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 2.3 mM $CaCl_2$, 5.6 mM glucose and 5 mM HEPES) supplemented with 10 µM glycine. 150,000 cells/well were placed in 96-well black, clear-bottom microtiter plates, treated with receptor antagonists (10 min), and subsequently treated with receptor agonists. The fluorescence emission intensity of GCaMP6 was recorded 1 min after agonists addition at 515 nm upon excitation at 488 nm on the EnSpire® multimode plate reader for 150 s every 5 s and 100 flashes/well.

2.8 cAMP determinations

Two hours before initiating the experiment, transfected HEK-293T cells or 59HCE cells medium was replaced by serum-starved DMEM medium. Then, cells were detached and resuspended in growing medium containing 50 µM zardaverine. Cells were plated in 384-well microplates (2,500 cells/well), pretreated (15 min) with the corresponding antagonists -or vehicle- and stimulated with agonists (15 min) before adding 0.5 µM forskolin or vehicle (15 min). Readings were performed after 1 h incubation at 25ºC. Homogeneous time-resolved fluorescence energy transfer (HTRF) measures were performed using the Lance Ultra cAMP kit (PerkinElmer, Waltham, MA, USA). Fluorescence at 665 nm was analyzed on a PHERAstar Flagship microplate reader equipped with an HTRF optical module (BMG Lab technologies, Offenburg, Germany).

2.9 Dynamic mass redistribution (DMR) assays

Cell mass redistribution induced upon receptor activation was detected by illuminating with polychromatic light the underside of a biosensor and measuring the changes in the wavelength of the reflected monochromatic light that is a sensitive function of the index of refraction. The magnitude of this wavelength shift (in picometers) is directly proportional to the amount of DMR. Transfected HEK-293T cells or human 59HCE cells were seeded in 384-well sensor microplates to obtain 70-80% confluent monolayers constituted by approximately 10,000 cells per well. Previous to the assay, cells were washed twice with assay buffer (HBSS with 20 mM HEPES, pH 7.15) and incubated (2 h) with assay-buffer containing 0.1% DMSO (24°C, 30 µl/well). Hereafter, the sensor plate was scanned and a baseline optical signature was recorded for 10 min before adding 10 µl of the specific antagonists for 30 min followed by the addition of 10 µl of specific agonists; all test compounds were dissolved in assay buffer. Then, DMR responses were monitored for at least 5,000 s in an EnSpire® Multimode Plate Reader (PerkinElmer, Waltham, MA, USA). Results were analyzed using EnSpire workstation Software v 4.10.

2.10 β-arrestin 2 recruitment assay

β-arrestin recruitment was determined as previously described (Hinz et al., 2018; Navarro et al., 2018). Briefly, BRET experiments were performed in transfected HEK-293T cells 48h after transfection with the cDNA corresponding to the indicated receptors fused to the YFP protein and 1 µg cDNA corresponding to β-arrestin 2Rluc. 48 h after transfection cells were adjusted to 20 µg of protein using a Bradford assay kit (Bio-Rad, Munich, Germany) using bovine serum

albumin for standardization. To quantify protein-YFP expression, fluorescence was read in a Mithras LB 940 multimode microplate reader (Berthold Technologies, Bad Wilbad, Germany) which allows the integration of the signals detected in the short-wavelength filter at 485 nm and the long-wavelength filter at 530 nm. Cells were distributed in 96-well microplates (Corning 3600, white plates with white bottom, SigmaAldrich) BRET readings corresponding to energy transfer between β-arrestin 2Rluc and receptor-YFP were collected 1 min after addition of 5 µM coelenterazine h (Molecular Probes, Eugene, OR). To quantify protein-Rluc expression, luminescence readings were done 10 min after 5 µM coelenterazine h addition. Net BRET is defined as [(long-wavelength emission)/(short-wavelength emission)]-C_f, where C_f corresponds to [(long-wavelength emission)/(short-wavelength emission)] for the donor construct expressed alone in the same experiment. BRET is expressed as milli BRET, mBU (net BRET x 1,000) units.

2.11 Immunofluorescence and in situ proximity ligation (PLA) assays

Frozen eye 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, Dallas, US. Cat# sc-13179) [RRID: AB_677236], MT_2 (1:1,000, Antibodies-online GmbH, Aachen, Germany. Cat# ABIN122307) and α_1-adrenergic (1:500, Abcam Cat# ab3462) [RRID: AB_2224924] 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, SigmaAldrich, St. Louis, USA) and the α_{1A}-adrenergic antibody to MINUS oligonucleotide (DUO92010, SigmaAldrich, 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 (Navarro et al., 2018).

2.12 Immunocytochemistry

HEK-293T cells seeded in coverslips were transfected with α_{1A}Rluc, MT_1YFP, MT_2YFP, α_{1A}Rluc and MT_1YFP or α_{1A}Rluc and MT_2YFP (0.5 µg cDNA each), fixed in 4% paraformaldehyde for 15 min and washed twice with PBS containing 20mM glycine before permeabilization with PBS-glycine containing 0.2% Triton X-100 (5 min incubation). Cells were

treated for 1 h with PBS containing 1% bovine serum albumin. HEK-293T cells were labeled with mouse anti-Rluc antibody (1/100; Millipore, Darmstadt, Germany) and subsequently treated with Cy3 anti-mouse (1/200; Jackson ImmunoResearch (red)) IgG (1 h each). Nuclei were stained with Hoechst (1/100; SigmaAldrich). Samples were washed several times and mounted with 30% Mowiol (Calbiochem). Samples were observed in a Leica SP2 confocal microscope (Leica Microsystems).

2.13 Data analysis

With the exception of experiments with samples from human eyes (see 2.2), all data were obtained from at least five independent experiments and are expressed as the mean ± SEM. Two-group comparisons were made by the Student's t test and multiple comparisons were analyzed by one-way ANOVA followed by Bonferronis's post-hoc test or two-way ANOVA followed by Tukey's post-hoc test. The normality of populations and homogeneity of variances were tested prior to ANOVA. Post-hoc tests were run only if F achieved $P<0.05$ and there was no significant variance inhomogeneity. Statistical analysis was undertaken only when each group size was at least n=5, n being the number of independent variables (technical replicates were not treated as independent variables). Unequal group sizes were due to i) different sources depending on the wide variety of experimental approaches, ii) need of increasing the n value for data reliability in some of the assays, iii) animal availability and/or iv) economy of resources to fulfill the 3Rs' (Replacement, Reduction and Refinement) rule in experimentation with animals. Differences were considered significant when $P \leq 0.05$. Statistical analyses were carried out with GraphPad Prism software version 5 (San Diego, CA, USA) [RRID:SCR_002798]. Outliers tests were not used; all data points (mean of replicates) were used for analysis.

2. 14 Nomenclature of Targets and Ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding et al., 2018) and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander et al., 2017, 2019).

3 RESULTS

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

Melatonin acts via MT_1 (MT_1R) and MT_2 (MT_2R) receptors, which control Cl^- efflux from ciliary body epithelial cells (Huete-Toral et al., 2015). Interestingly, a α_{1A}-adrenoceptor (α_{1A}-AR) antagonist, prazosin, blocks the effect of melatonin on reducing IOP (Dubocovich, 1995; Pintor et al., 2003; Huete-Toral et al., 2015). Hence 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) (Alexander et al., 2017, 2019). However, it has also been reported that α_{1A}-AR may couple to G_s (Martin, RM et al., 2018). We then measured, in 59HCE ciliary body epithelial cells, cAMP levels and dynamic mass redistribution (DMR) upon receptor activation. The effect of MT_1 or MT_2 receptor agonists (melatonin and IIK7, respectively) did not result in any decrease in forskolin-induced cytosolic [cAMP] (Fig. 1A-B). By contrast, MT_1 and MT_2 receptor agonists induced a significant increase in forskolin-induced cytosolic [cAMP] (Fig 1A-B). When cells were challenged with the α_{1A}AR agonist, phenylephrine, also a remarkable elevation of forskolin-induced cytosolic [cAMP] was achieved. The adrenoceptor-mediated increase in cAMP reflects a G_s coupling able to overcome the forskolin-induced cAMP levels. The effect was specific as the antagonist, prazosin, abolished it. We also investigated the canonical coupling of α_{1A}AR to G_q but failing to 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 that are not functionally coupled to their cognate G proteins. It should be noted that the concentration of forskolin chosen for the assays (500 nM) is submaximal thus allowing detection of either G_i- or G_s-coupling. Also, when MT_1 and MT_2 agonists were analyzed in 59HCE cells in the absence of forskolin, a significant increase in [cAMP] (over the basal level) was obtained (data not shown).

To confirm atypical G protein coupling, similar assays were performed in the presence of cholera toxin (CTX), which alters G_s-mediated signaling or pertussis toxin (PTX), which alters 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). Neither cholera nor pertussis toxins by themselves produced any effect (data not shown). Interestingly, the presence of the α_{1A}-adrenergic antagonist, prazosin, abolished the effect of melatonergic agonists (melatonin on MT_1R and IIK7 on MT_2R). Reciprocally, the MT_1R antagonist, luzindole, and the selective MT_2R antagonist, 4PPDOT, blocked α_{1A}-adrenergic-mediated signaling. Cross-antagonism, i.e. the agonist of one receptor in the heteromer is blocked by the antagonist of the partner receptor in the same complex, is an accepted heteromer print (Franco et al., 2016). Further evidence of cross-antagonism, was underscored using a label-free technique consisting of

detecting cell dynamic mass redistribution (DMR). Apart from the cross-antagonism, the signal induced by any agonist was relatively high, and the combination of phenylephrine and either a MT_1R agonist, melatonin (Fig. 1F), or a MT_2R selective agonist, IIK7 (Fig. 1G), provided a more robust DMR response. When the experiments were carried out in the presence of cholera and pertussis toxins, responses were only 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 direct interactions and G_s coupling of resulting complexes.

3.2 Atypical signaling in ciliary cells is due to α_{1A}-adrenergic/melatonin receptor complexes. Biophysical and signaling assays in a heterologous expression system.

To identify potential direct interactions, a FRET biophysical approach was used in HEK-293 cells (Fig. 2A). A saturable FRET curve was obtained in cells transfected with a constant [cDNA] for α_{1A}-GFP[2] and increasing [cDNA] for MT_1R-YFP ($FRET_{max}$ 49 mFU and $FRET_{50}$ 44) (Fig. 2B). Similar experiments using increasing amounts of [cDNA] of MT_2R-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 $\alpha_{1A}AR$ (α_{1A}-MT_1Hets and α_{1A}-MT_2Hets). Receptor expression and function at the plasma membrane level and colocalization between α_{1A} and melatonin receptors was confirmed by immunocytochemistry (Supplementary Fig. S1).

To know how heteromer formation is affecting signal transduction evens, functionality was first addressed in single-transfected HEK-293T cells. Upon $\alpha_{1A}AR$ 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 4PPDOT. In single-transfected cells, either MT_1 or MT_2 receptors were not coupled to $G_{q/11}$ (Fig. 2E-F) but to G_i, as their activation led to decreases in forskolin-induced cAMP levels (Fig. 2H-I). The results show that, indeed, when expressed individually, receptors couple to their cognate G proteins. However, $\alpha_{1A}AR$ activation induced a significant decrease in forskolin-induced cAMP levels. This result demonstrates that $\alpha_{1A}AR$ can couple both $G_{q/11}$ and G_i proteins when individually expressed in a heterologous system (Fig. 2G). 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).

Remarkably, 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 was identified in co-transfected cells, as melatonin receptor antagonists blocked phenylephrine-induced signal and the α_{1A}R antagonist prazosin completely abolished the MT_1R signal and slightly decreased the MT_2R signal (Fig. 3A-B). Oddly, there was as lack of Ca^{2+} responses when phenylephrine was added to cells expressing either α_{1A}-MT_1Hets or α_{1A}-MT_2Hets (Fig. 3C-D). Analogies between 59HCE and co-transfected HEK-293T cells were further found in experiments with toxins; the combined effect of agonists was blocked by cholera but not pertussis toxin (Fig. 3E-F). Also, matching results in 59HCE cells, a potentiation of the label-free DMR signal was found upon co-activation, cholera toxin did block the effect and cross-antagonism was detected (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 Structural insights into the mechanism underlying the atypical signaling due to receptor complex formation

Since results obtained in HEK-293T cells were virtually identical to those obtained in 59HCE cells, we assumed that heteromers functionally coupled to G_s occur in the 59HCE cell. Looking at the primary structure and membrane topological domains, we hypothesized that intracellular domains could be responsible of such differential G protein coupling. On the one hand, the area projected by a G protein on the membrane plane almost doubles that of a GPCR. On the other hand, the “clam-shell like” opening of the small globular domain in G_α subunits, which occurs in every GTP/GDP exchange, has been structurally elucidated (Chung et al., 2011; Westfield et al., 2011). With this information and considering the structural possibilities for G protein-receptor complexes described elsewhere (Cordomí et al., 2015), we noticed that the long C-terminal domain of the α_{1A}-adrenoceptor could lead to steric hindrance in the context of a complex formed by at least 2 receptors and G proteins (Rasmussen et al., 2011). It is predicted that the human α_{1A}-adrenergic receptor C-terminal domain contains 137 amino acids (uniprot: P35348), and that human MT_1 and MT_2 receptors have much shorter C-terminal ends (uniprot: P48039 and P49286, respectively). For the three receptors, cDNAs for fusion proteins

lacking most of the C terminal cytoplasmic domain were obtained. Energy transfer assays showed interaction of α_{1A}-AR with truncated melatonin receptors and of truncated α_{1A}-AR with melatonin receptors (Fig. 4A-D). Truncated receptors, when expressed individually, were functional (Fig. 4E). Data from controls performed in cells co-expressing combination of truncated and full-length receptors are shown in Supplementary Fig. S2A-D. Functional assays provided clues on the atypical signaling mediated by heteromers. Phenylephrine did not mobilize Ca^{2+} in cells co-expressing α_{1A}-AR and any of the truncated melatonin receptors (Fig. 4F-G). In contrast, activation of the truncated α_{1A}-AR in the heteromeric contexts, led to increases in cytosolic [Ca^{2+}] (Fig. 4H-I). In cAMP determination assays, we observed that truncated melatonin receptors interacting with full-length α_{1A}-ARs were still coupled to G_s (Fig. 4J-K). In summary, the C-terminal tail of the α_{1A}-AR plays a relevant role in the functionality of the heteroreceptor functional units.

3.4 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 (Alkozi and Pintor, 2015; Alkozi et al., 2017b), 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. 5A-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. 5C).

Exploratory experiments on heteromer expression were performed in postmortem samples obtained from glaucoma patients and age-matched controls. Due to the limited availability of

these unique samples that in part reflects ethical issues, we had access to 3 control (healthy) and 2 glaucomatous eyes. Accordingly, statistical analysis could not be performed on the results that are described below. 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. 5D-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. 5D-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. 5F). Similar immunohistochemical studies carried out in samples obtained from glaucoma patients showed altered receptor expression (Fig. 5G-I). The expression of the α_1-adrenergic receptor showed a trend to increase (Fig. 5J). 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. 5K and 5M). Remarkably, when PLA was performed in samples from glaucoma patients, 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. 5L, 5N and 5O). These results obtained in human samples match the results obtained in 59HCE cells treated with a TRPV4 activator to mimic a glaucoma-like condition.

3.5 A novel therapeutic approach to combat glaucoma

The above described results show that the coupling of α_{1A}-adrenoceptors to G_q and the subsequent Ca^{2+} signaling seems detrimental and markedly contributing to intraocular hypertension. Accordingly, an antagonist of α_{1A}-AR would be beneficial as a blocker of calcium production and of Ca^{2+}-regulated chloride channels (Fleischhauer et al., 2001). To test the hypothesis we moved to a well-established murine model of glaucoma (Pérez 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. 6A-C), however prazosin antagonized the hypotensive effect of melatonin (Fig. 6D). 9-month-old mice display a full glaucoma-like pathology (Fig. 6A-C). At 9 months of age, and despite elevated levels of melatonin (Fig. 6B), 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. 6F). Indeed, it was consistently found that prazosin did revert the effect of melatonin in control mice while it significantly enhanced it in the 12-month-old DBA/2J mouse

(Supplementary Fig. S2 E-H). 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.

4. Discussion

Current therapy of glaucoma addresses symptoms by interventions neither efficacious in all patients nor absent of adverse events. Prostaglandin analogues have been approved for use by glaucoma patients because they reduce IOP but by unknown mechanisms (Sanford, 2014; Weinreb et al., 2015; Lanza et al., 2018). 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 and is even on-line available. Its main potential is in sleep induction although other properties are been suggested. Already in 1993 (Serino et al., 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 glaucoma patients is not enough to normalize IOP (Alarma-Estrany et al., 2007) (Alkozi et al., 2017a). A complete characterization of melatonin receptors has not been completed due to atypical pharmacological and non-fulfilled suspicions of a third receptor. As receptor heteromers display particular properties, we reasoned that atypical data, including pleiotropic signaling, could be due to interactions with other GPCRs (Ferré 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. From our data the first relevant finding is that MT_1 and MT_2 receptors may directly interact with the α_{1A}-AR.

The role of epinephrine in the physiology of the eye is known since 1970 (Drance and Ross, 1970; Zalta et al., 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 G_q-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 et al., 1977).

Our results provide an explanation for the pleiotropic signaling attributed to melatonin receptors and to the reported differential coupling to G proteins depending on the experimental system (Tsitn et al., 1996). It should be noted that MT_1 and MT_2 receptor heteromers still couple to Gi (Ayoub et al., 2004) and, therefore, the effects that are mediated by this heteromer are not yet elucidated from a molecular point of view. In the interaction reported in this paper melatonin is not by itself increasing the concentration of intracellular calcium while the compound, via MT_1R, is able to do mobilize Ca^{2+} and increase phosphatidyl inositol hydrolysis in intestinal smooth muscle cells (Ahmed et al., 2013) Whether this atypical seemingly direct G_q coupling is due to heteromerization or to an alternative mechanism is, at present, unknown. From a mechanistic point of view, the C-terminal domain of the α_{1A}-adrenergic receptor is relevant for the interaction and for the shift of heterotrimeric G protein in the adrenergic-melatonin heteroreceptor complexes. In fact, the lack of the C tail favours G_q coupling a while it likely intermingles with partner melatonin receptors to impede G_q coupling. One few melatonin-receptor-containing melatonin receptors, is that formed by GPR50, an orphan GPCR with a long C terminal domain, and AT_1R. Interestingly, in such heteromer there is a negative cross-talk that is abolished by removing the C-.terminal end of GPR50 (Lique Levoye et al., 2006).Therefore it seems that melatonin receptors interacting with GPCRs with long C-terminal domains could lead to a shift in G protein coupling. To confirm this prediction more experimental work is needed.

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 glaucoma patients 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 IOP. 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; Sánchez-Barceló et al., 2010), and prazosin,

approved for the therapy of blood hypertension (Brogden et al., 1977; Mallorga et al., 1988) (Torvik and Madsbu, 1986; Singleton et al., 1989), could readily enter into clinical trials to assay for safety and efficacy in humans.

AUTHOR'S CONTRIBUTION

HAA, GN and IRR 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 and IRR 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 declare no conflict of interest.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design & Analysis, Immunoblotting and Immunochemistry, and Animal Experimentation, and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

Acknowledgements

This work was supported by grants from Spanish *Ministerio de Economía y Competitividad* (MINECO) Ref. [SAF2013-44416-R], [SAF2016-77084-R] and [BFU2015-64405-R], and from Spanish *Ministerio de Sanidad* Ref. RETICS [RD12/0034/0003] and [RD16/ 0008/0017].

Hanan A. Alkozi is a fellowship holder of Saudi Arabia government. MINECO grants may include EU FEDER funds.

The “NBM” Molecular Neurobiology laboratory of the University of Barcelona is considered of excellence (“*grup consolidat*”) by the regional Catalonian Government, which neither provides funds to the consolidated laboratory nor to perform the research here reported.

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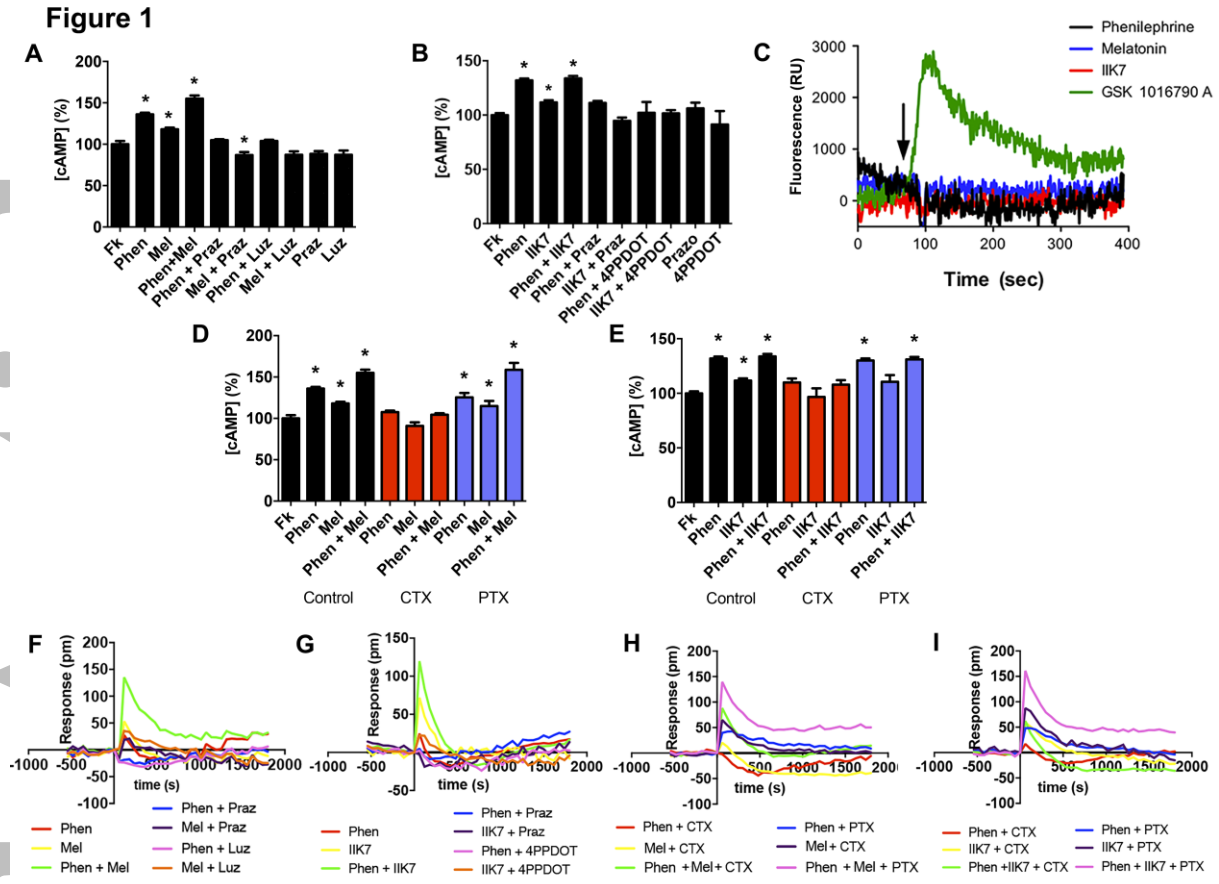


Figure 1. Effect of melatonin receptor agonists and of phenylephrine in human 59HCE cells. Panels A, B, D and E: Effect of ligands (single or combined treatment) on 0.5 µM forskolin-induced cAMP levels in the absence (A-B) or presence (D-E) of 10 ng/mL pertussis (overnight) or 100 ng/mL cholera (2 h) 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. 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.

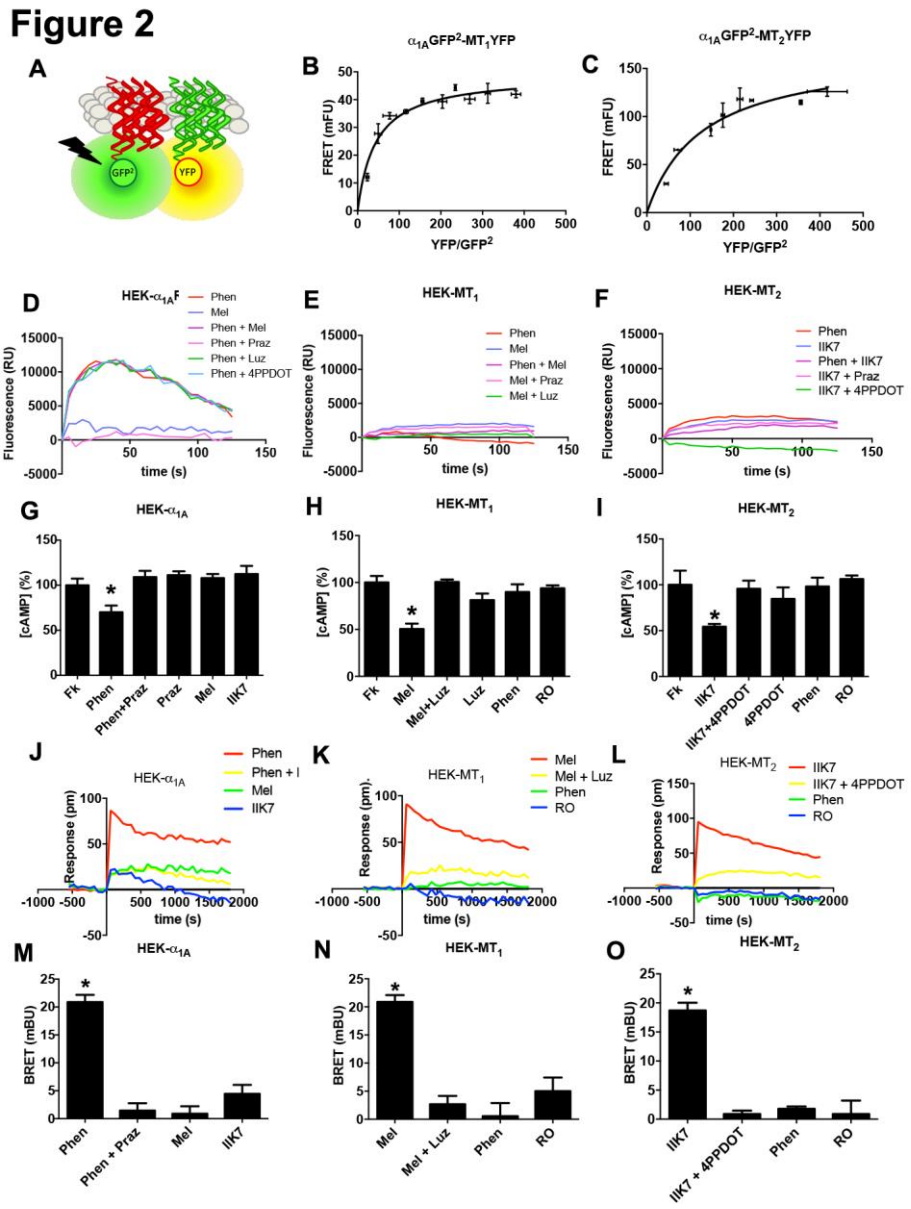


Figure 2. Dimerization of α_1-adrenergic and MT_1 or MT_2 receptors and signaling via adrenergic or via melatonin receptors in single transfected HEK-293T cells. Panels A-C: Scheme of the FRET assays (A) and results of experiments performed in HEK-293T cells transfected with a fixed amount of cDNA for the α_1-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 either α_1-adrenergic, MT_1 or MT_2 receptors. Panels D-F: Time course of cytosolic calcium 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<0.05. 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<0.05. 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

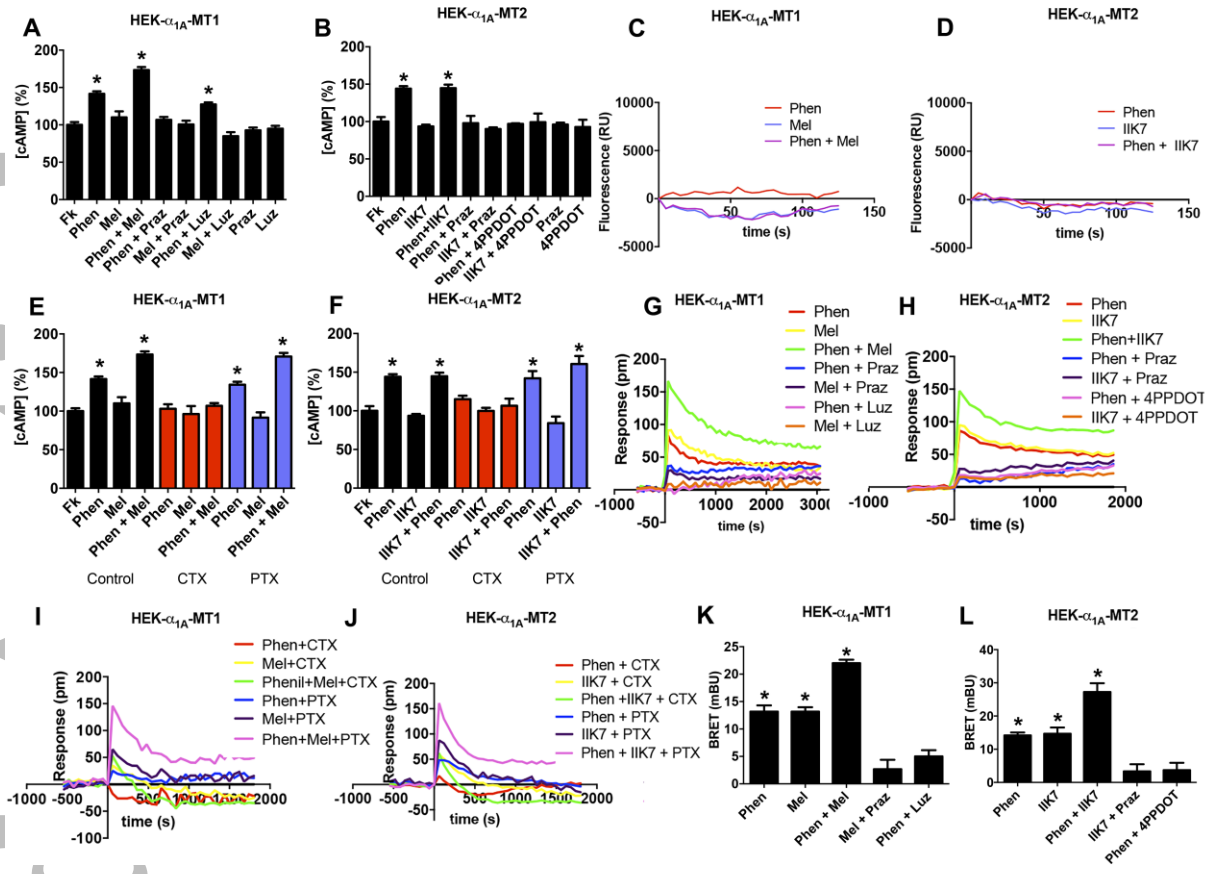


Figure 3. Signaling via adrenergic/melatonin receptor functional units in a heterologous expression system. Experiments were performed in HEK-293T cells transfected with cDNAs for full-length α_{1A}-adrenergic and either MT_1 or MT_2 receptors. Panels A, B, E and F: Effect of ligands (single or combined 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<0.05. Panels C-D: Lack of effect of receptor agonists in cytosolic calcium levels. The agonist of TRPV4 channel, GSK-1016790A, did produce an 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<0.05. 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 4

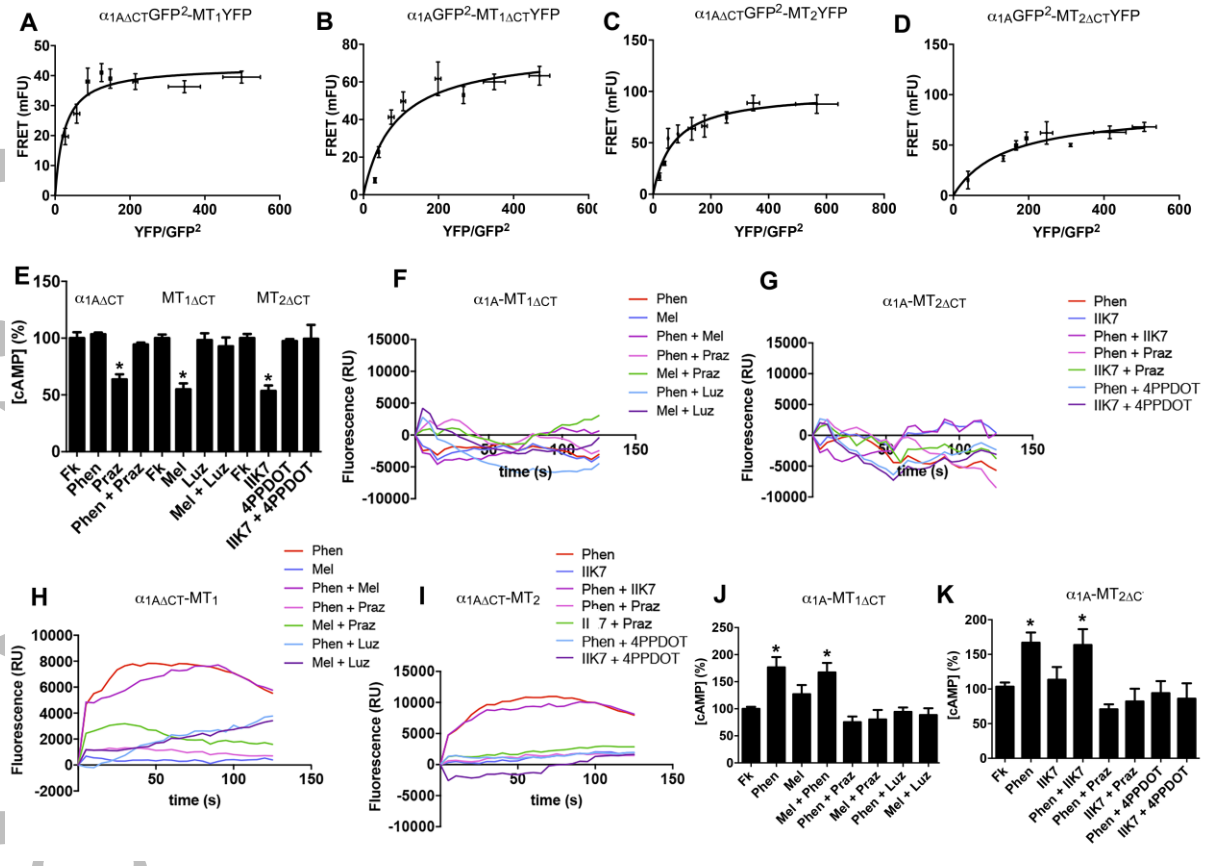


Figure 4. Dimerization of truncated versions of α_1-adrenergic MT_1 or MT_2 receptor and signaling via receptor heteromers containing truncated receptors. Experiments were performed in HEK-293T cells expressing the receptors indicated in the heading of each of the images. Panels A-B: FRET assays were performed in HEK-293T cells transfected with a fixed amount of cDNA for full-length α_1-GFP^2 or the truncated α_1-GFP^2 fusion proteins and increasing amounts of cDNA for, respectively full-length MT_1-YFP or truncated MT_1-YFP. Panels C-D: FRET assays were performed in HEK-293T cells transfected with a fixed amount of cDNA for the truncated α_1-GFP^2 or α_1GFP^2 fusion proteins and increasing amounts of cDNA for, respectively MT_2-YFP or truncated MT_2-YFP. Energy transfer data in panels A to D, are given in milliFRET units (mFU). Parameters were: $FRET_{max}$ =43±3 mFU; $FRET_{50}$ =25±5 (A), $FRET_{max}$ =77±9 mFU; $FRET_{50}$ =85±11 (B), $FRET_{max}$ =100±7 mFU; $FRET_{50}$ =70±8 (C) and $FRET_{max}$ =85±11 mFU; $FRET_{50}$ =137±18 (D). Panels E, J and K: Effect of ligands (single or combined treatment) of 0.5 µM forskolin-induced cAMP levels in cells expressing truncated α_1-adrenergic, truncated MT_1 or truncated MT_2 receptors (E) and in cells co-expressing α_1-adrenergic and truncated MT_1 (J) or MT_2 (K) receptors. 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. Panels F-I: Time course of cytosolic calcium level induced by receptor agonists (single or combined treatment) in cells co-expressing α_1-adrenergic and truncated MT_1 (F), α_1-adrenergic and truncated MT_2 (G),truncated α_1-adrenergic and MT_1 (H) or truncated α_1-adrenergic and MT_2 (I) receptors. 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 5

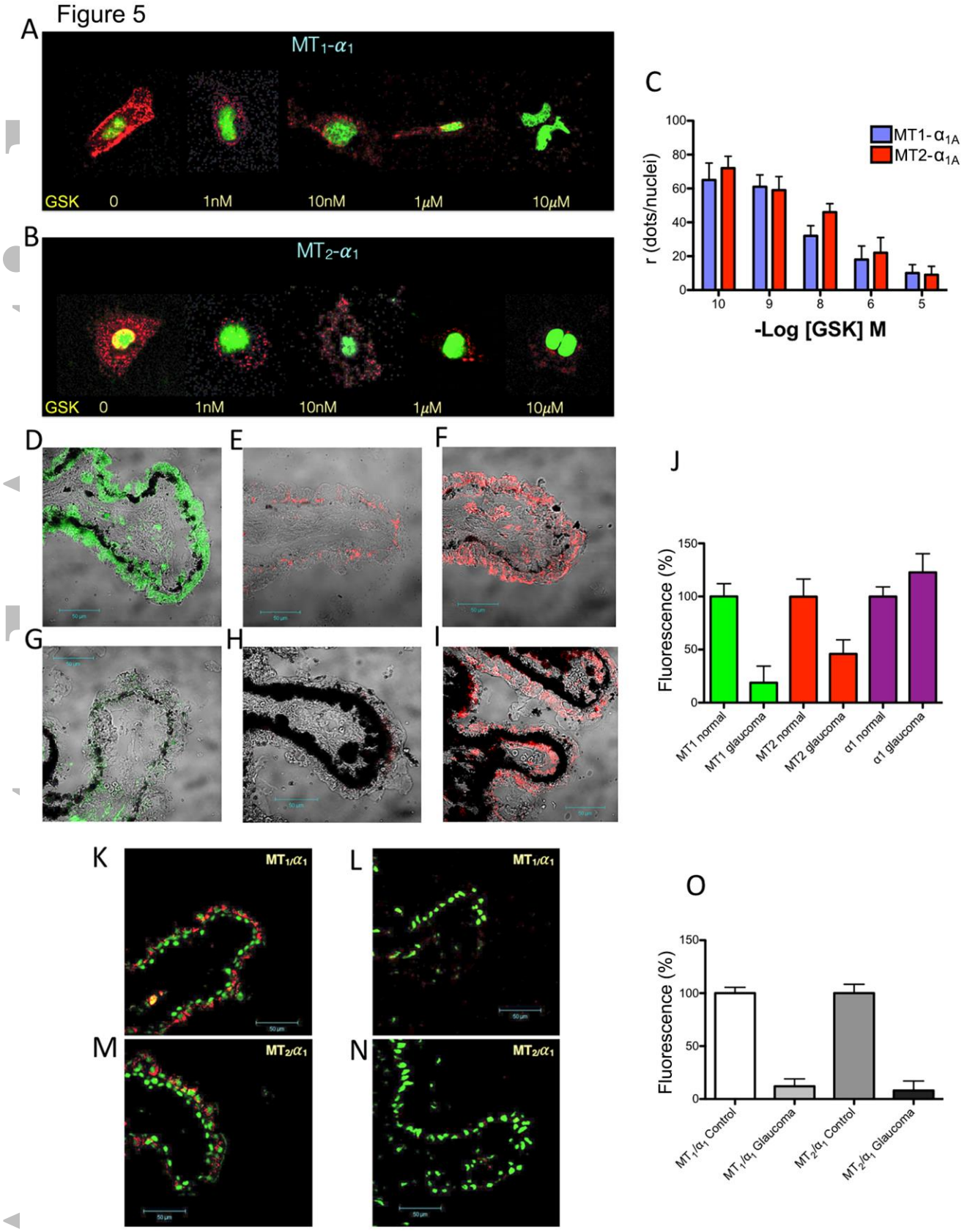


Figure 5. Identification of functional units in human 59HCE cells and in samples from normotensive and hypertensive human eyes. Panels A-B: Determination by PLA of α_1-adrenergic and either MT_1 (A) or MT_2 (B) receptor complexes in 59HCE non-pigmented ciliary body epithelial cells treated with increasing concentrations of the TRPV4 agonist, GSK1016790A. 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<0.05, 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 glaucoma patients. Quantitation (J) 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=5). Statistical analysis could not be performed as 3 was the number of healthy eyes and 2 the number of glaucomatous eyes; it should be noted that the within-groups results were similar. 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 glaucoma patients. Quantitation (O) of clusters comparing data in glaucoma patients and age-matched controls using fluorescence values taken in identical experimental conditions. Data, given in percentage (100% given to values from controls) are the mean ± SEM. Statistical analysis could not be performed as 3 was the number of healthy eyes and 2 the number of glaucomatous eyes; it should be noted that the within-groups results were similar.

Figure 6

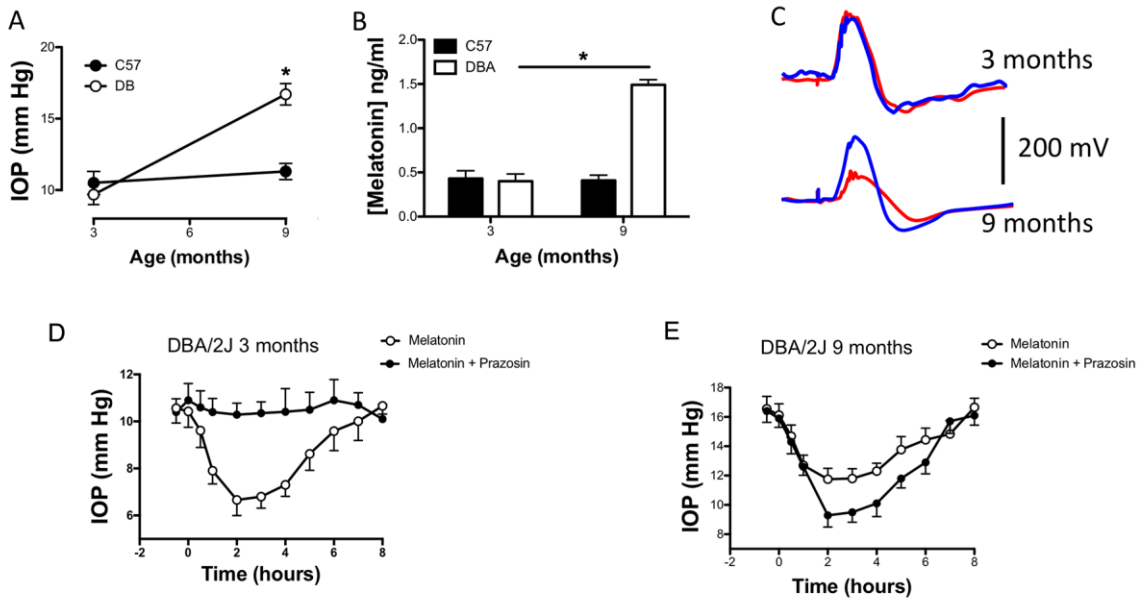
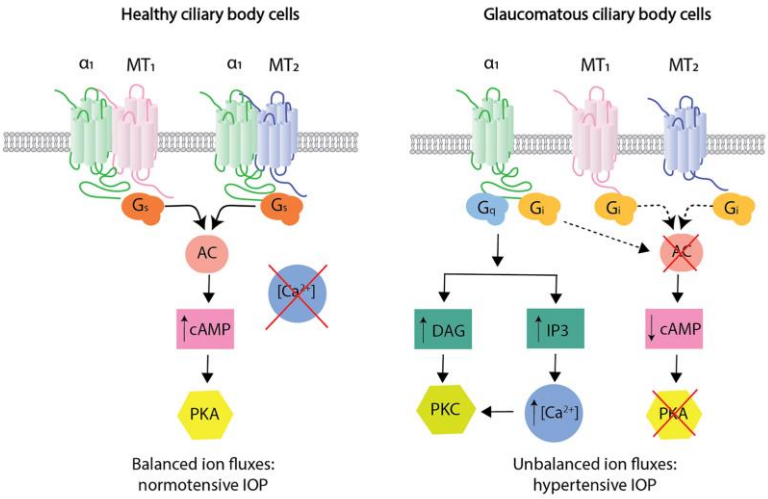


Figure 6. IOP-lowering intervention in the DBA/2J mice**.** Panel A: Intraocular pressure (IOP) values of control C57BL/6J and DBA/2J mice at 3 and 9 months of age. Data are the mean ± SEM (n=12). *P<0.05 (Wilcoxon's test for paired samples). Panel B: Melatonin concentrations in the aqueous humour of C57BL/6J and DBA/2J mice at 3 and 9 months of age. Data are the mean ± SEM (n=6). *P<0.05 (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<0.05). 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).

How do adrenergic-melatonin receptor complexes control intra-ocular pressure?



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