UNIVERSIDAD COMPLUTENSE DE MADRID
FACULTAD DE ÓPTICA Y OPTOMETRÍA
DEPARTAMENTO DE BIOQUÍMICA Y BIOLOGÍA MOLECULAR IV

TESIS DOCTORAL

Regulatory mechanisms of melatonin synthesis in the control of intraocular pressure

Mecanismos de regulación de la síntesis de la melatonina para el control de la presión intraocular

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

Hanan Awad Alkozi

DIRECTORES

Jesús Pintor Just
María Jesús Pérez de Lara

Madrid, 2018

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Autor
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María Jesús Perez de Lara

Madrid, 2017

PROF. JESÚS PINTOR JUST, CATEDRÁTICO DEL DPTO. DE BIOQUÍMICA Y BIOLOGÍA MOLECULAR IV DE LA FACULTAD DE VETERINARIA DE LA UNIVERSIDAD COMPLUTENSE DE MADRID,

Drª. MARÍA JESÚS PEREZ DE LARA, INVISTIGADORA EN OCUPHARM DIAGNOSTICS,

CERTIFICAN QUE:

Dª. Hanan Awad Alkozi, Master en Óptica, Optometría y Visión por la Universidad Complutense de Madrid, ha realizado en el Dpto. de Bioquímica y Biología Molecular IV de la Facultad de Óptica, Optometría y Visión de la UCM, bajo la dirección del que suscribe, el trabajo titulado "*MECANISMOS DE REGULACIÓN DE LA SÍNTESIS DE LA MELATONINA PARA EL CONTROL DE LA PRESIÓN INTRAOCULAR*" como tesis Doctoral cumpliendo las condiciones exigidas para optar al grado de Doctor por la Universdad Complutense de Madrid.

Madrid, a 27 de junio de 2017

Los Directores,

Dr. Jesús Pintor Just Drª. María J. Perez de Lara

To my off-the-field heroes, the people who gave me the values to live by and who inspired me with their hard work and selfless dedication to their family, to my mom, Rowaidah, and my dad, Awad.

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Thanks to all these people I faced during this journey. First of all, thanks to my supervisor, Suso, a very young soul, full of life, active, very positive and creative! you encouraged me to think, analyse, and speak up to myself. Without your support, I wouldn't be the person I am right now. Words cannot express how deeply I am grateful to you. You opened new unexpected windows for me that I didn't even know it existed. You are always open for new ideas, allowing me to grow as a scientist and as a person. I am deeply thankful for your care and respect. I thank you million times to the moon and back!

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Here, the journey started, quite hard. I came back from home just to take my mind off the situation. As long as I was investigating, performing experiments, discovering new areas I was absolutely ignorant of, I started to fully fall in love with science. Science made my life meaningful, joyful, and opened my eyes to a whole new world. Thanks to science!

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بنيتي الغالية : كما استفتحت يومي بالنظر إلى رسالتك المملوءة بالمشاعر الجياشة بالحب الصادق، النابضة بالعواطف المخلصة، أردت من رسالتي هذه أن تكون أول ما يعانق نظراتك في هذا الصباح الذي أرجو أن يكون سعيدا جميلا. كلمات التهنئة أقل حجما واتساعا مما يجيش في صدري من تعبير يعكس الفرحة التي أنستني المرض الذي تعرفين، والهموم التي لا تعرفين، أنستني ضغوط العمل وأشجان البحوث، وزرعت أمام ناظري خميلة حب طرزتها الأزاهير الفواحة الملونة بالطبيعة الخلابة، المطعمة بحلاوة الحب العميق الصادر من قلب مخلص، بنيتي : الكلمات تعجز عن حمل مشاعري تجاه إنجازك، وأمام تطلعاتك وآملك، فلا تسألي عاجزا أن يفوه بأكثر من طاقته ، واهنئي بحب لم تلونه أصباغ زائلة أو ألوان خادعة ، ورددي معي :

(إني أحبك) ووجهي ضمير الخطاب نحو من يردد كل حين : (إني أحبك) :

أبوك :

أ.د.عوض بن حمد القوزي

9/10/2013

والدي العزيز: إني أحبك، وإيماني بأنك معي، تسمعني وتشعر بي، هذا الإيمان هو المحرك وهو المشجع لي لأكمل هذا الطريق الذي بدأت.

أتمنى وجودك، هقاتك، وابتسامتك الفخورة بأبنائك!

أمي الغالية، أغلى الباقين على وجه الأرض:

رسالتي هذه، علمي، ومعرفتي، لم تكن لولا وجودك، إيمانك، وثقتك بقدرتي على إكمال هذا الطريق.

INDEX

Abbreviation

5-HT: 5 hydroxy-tryptamine

AANAT: Arylalkymine *N*-acetyltransfrerase

AC: Adenylate cyclase

AH: Aqueous Humor

AH: Aqueous humour

ARVO: The association for Research in vision and Ophthalmology

ATP: Adenisine triphosphate

BRET: bioluminescence resonance energy transfer

CA: Carbonic Anhydrase

Ca^{2+}: Calcium Ion

CaM: Calcium Calmodulin

cAMP:cyclic adenosine mono- phosphate

Cl^-: Chloride

CO_2: Carbon dioxide

CREB: cAMP-responsive element binding

DAG: diacylglycerol

DG: diacylglycerol

DMEM: Dullbecco's modified Eagle's medium

DMR: Dynamic mas redistribution

DMSO: Dimethyl Sulfoxide

DNA: Deoxyribonucleic acid

ECL: Enhanced chemiluminescence

ECM: Extracellular matrix

ER: Endoplasmic Reticulum

FDA: Food and Drug Administration

FISH: Fluorescence in situ hybridization

FITC: fluorescein isothiocyanate

FRET: fluorescence resonance energy transfer

GABA: Gamma-Amino Butyric Acid

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GC: guanylate cyclase

GPCR: G protein-coupled receptor family

H_2O_2: Hydrogen peroxide

HCIO: Hypochlorous acid

HEK: Human Embryonic Kidney

HIOMT: Hydroxyindole-*O*-methyltransferase

HPLC: High-pressure liquid chromatography

IOP: Intraocular pressure

IP_3: inositol 1,4,5-triphosphate

ipRGC: Intrinsically photosensitive retinal ganglion cells

K^+: Potassium Ion

K_m: Michaelis constant

LED: Light Emitting Diode

LGN: Lateral geniculate nucleus

mRNA: Messenger RiboNucleic Acid

$MT_{1\text{-}2}$: Melatonin receptor 1, 2

MT_3: Putative melatonin receptor 3

Na^{2+}: Sodium Ion

NAS: N-acetylserotonin

NDS: Normal donkey serum

NE: Norepinephrine

NINDS: National institute of Neurological Disorders and Stroke

NO: Nitric Oxide

NPE: Non-pigmented Epithelium

OAG: Open Angle Glaucoma

$ONOO^-$: Peroxynitrite

OPN: Olivary pretectal nucleus

PBS: Phosphate-buffered saline

PCR: Plymerase Chain Reaction

PEI: PolyEthylenImine

PI: phosphatidylinositol

$PI(4,5)P_2$: Phosphatidylinositol 4,5-bisphosphate

PKA: Protein kinase A

PKC: Protein kinase C

PLA: proximity ligation assay

PLC: Phospholipase C

PMSF: Phenyl methanesulfonyl fluoride

PP: Protein phosphotase

PVN: Paraventricular nucleus

QR2: Quinone reductase 2 enzyme

RGC: Retinal ganglion cells

RIPA: radioimmunoprecipitation assay

Rluc: Renilla luciferase

ROI: Regions of interests

ROS: Reactive Oxygen Species

RPE: Retinal Pigment Epithelium

RR: Ruthenium Red

RV: Regulatory volume

RV: Regulatory Volume

SCG: Superior cervical ganglia

SCN: Suprachiasmatic nucleus

SDS-PAGE: dodecyl sulfate-polyacrylamide gel electrophoresis

SFKs: Src family tyrosin kinases

siRNA: Small interfering RNA

TBS: Tris Buffer Saline

TM: Trabecular meshwork

TRP: Transient Receptor Potential

TRP: Transient Receptor Potential Ion Channels

UV: Ultraviolet

V_{max}: Maximal velocity

WHO: World Health Organization

YFP: Yellow Fluorescent Proteins

SUMMARY
RESUMEN

SUMMARY

Ocular hypertension, although not considered a disease itself, it leads to glaucoma which eventually cause irreversible vision loss. Consequently, glaucoma therapeutic approach is to lower intraocular pressure (IOP). Nonetheless, anti-glaucoma drugs come often with several side effects that can lead to treatment withdrawal in some cases. In this context, melatonin and its analogs emerges as potential complimentary/alternative treatment, offering the advantage of hypotensive and antioxidant properties.

The present PhD thesis aim to investigate endogenous melatonin content in the aqueous humor and describes mechanisms involved in detecting increased IOP, along with a focus on the possibility of melatonin receptors to interact with different receptors with the intention to find a more effective combined therapeutical approach. Finally, to study the attribution of the crystalline lens to the aqueous humor melatonin content and to discover the regulation of its synthesis in this ocular structure.

Results showed a correlation between melatonin levels in the aqueous humor and IOP. Melatonin levels were found higher in patients with elevated IOP. Using a glaucomatous animal model (DBA/2J) it was possible to corroborate our findings. In vitro assays pointed to the participation of the TRPV4 channel, which is sensitive to pressure, among other stimuli. Activation of the mentioned channel in human immortalised non-pigmented ciliary body epithelial cells resulted in an increment of melatonin secretion through the increase of aralkylamine N-acetyltransferase (AANAT) expression, the first enzyme in melatonin synthesis. In addition, short term assays showed that the TRPV4 activation leaded to AANAT phosphorylation through a cascade of intracellular events that

involves the participation of calmodulin and calcium-calmodulin dependent protein kinase II. This phosphorylation activates AANAT and therefore melatonin synthesis is stimulated.

From the other hand, finding increased levels in ocular hypertensive cases has raised some questions, such as the reason behind the inability of endogenous melatonin to lower IOP, suggesting some alteration in melatonin receptors along with the development of glaucoma. Our results confirmed a decline in melatonin receptors expression when glaucoma is developed. More importantly, we introduced the concept of receptor heteromerization, using different techniques such as the technology of bioluminescence resonance energy transfer (BRET), we confirmed that heteromerization exists between both melatonin receptors and α_{1A}-adrenergic receptors. Different assays were done to study the intracellular mechanism resulting from the activation of both heteromers showing that they are functional, and they present a cross antagonism in the case of healthy subjects. On the contrary, in vivo studies showed that melatonin instillation after blocking α_{1A}-adrenergic receptor with prazosin was more effective in lowering IOP than melatonin alone. Findings offers an effective combined therapy for glaucoma.

Finally, the discovery of melanopsin presence in the human crystalline lens was proved using immunolabelling in human lens tissue and crystalline epithelial cells, as well as by western blot. Such findings lead to the study of melatonin regulation in the crystalline lens, results showed an increment of AANAT, and its activity to produce melatonin under darkness condition.

Melatonin release to the aqueous humor from different structures showed clearly its importance in regulating IOP, thus, suggesting a possible mechanism to control pathologies such as glaucoma.

RESUMEN

La hipertensión ocular, aunque no se considera una enfermedad en sí, es un factor de riesgo para desarrollar glaucoma, que eventualmente causa pérdida irreversible de la visión. En consecuencia, el enfoque terapéutico más habitual en el glaucoma es reducir la presión intraocular (PIO). No obstante, los fármacos anti-glaucomatosos presentan varios efectos secundarios que pueden conducir a la retirada del tratamiento en algunos casos. En este contexto, la melatonina y sus análogos emergen como un tratamiento complementario/ alternativo, ofreciendo la ventaja de poseer propiedades hipotensoras y antioxidantes.

La presente tesis doctoral tiene como objetivo investigar el contenido de la melatonina endógena en el humor acuoso y describir los mecanismos implicados en la detección de los cambios en la IOP. Igualmente se estudia la capacidad de los receptores de melatonina para interactuar con otros receptores con la intención de encontrar un enfoque terapéutico combinado más eficaz. Finalmente, esta tesis también estudia la contribución del cristalinoen su aporte de melatonina al humor acuoso.

Los resultados han mostrado una correlación entre los niveles de melatonina en el humor acuoso y la PIO. Los niveles de melatonina se encontraron más altos en pacientes con PIO elevada. El uso de modelo animal glaucomatoso (DBA/2J) corroboró nuestros hallazgos de una manera consistente. Por otro lado, los ensayos in vitro señalaron la participación del canal TRPV4, proteína que es sensible a la presión,como principal activador de la sintesis de la melatonina. De este modo, la estimulación de este canal en células epiteliales del cuerpo ciliar no pigmentadas inmortalizadas humanas dio como resultado un incremento de la secreción de melatonina a través de un aumento de la expresion de la Aralquilamina N-acetiltransferasa(AANAT), la primera enzima en la

síntesis de melatonina. Además, los ensayos a corto plazo mostraron que la activación de TRPV4 induce a la fosforilación de AANAT, y su activación, a través de una cascada de eventos intracelulares implica la participación de la calmodulina y la calmodulina quinasa II dependiente de calcio-calmodulina.

Por otra parte, encontrar niveles aumentados en los casos de hipertensión ocular ha planteado algunas incógnitas. El motivo detrás de la incapacidad de la melatonina endógena para bajar la PIO, sugiere una cierta alteración en los receptores de melatonina paralelo al desarrollo de glaucoma. Nuestros resultados confirmaron una disminución en la expresión de receptores de melatonina cuando se desarrolla glaucoma. Más importante aún, introdujimos el concepto de heteromerización de receptores, utilizando diferentes técnicas como la tecnología de transferencia de energía de resonancia de bioluminiscencia (BRET), y confirmamos que la heteromerización entre los receptores de melatonina y el receptor α_{1A}-adrenérgico exsite. Se realizaron diferentes ensayos para estudiar el mecanismo intracelular resultante de la activación de ambos heterómeros mostrando que son funcionales, y presentando un antagonismo cruzado en el caso de sujetos sanos. Sin embargo, los estudios in vivo demostraron que la instilación de melatonina después de bloquear el receptor α_{1A}-adrenérgico con prazosin era más eficaz en la reducción de la PIO que la melatonina sola. Este hecho sugiere la posibilidad de una nueva terapia combinada eficaz para el glaucoma.

Finalmente, el descubrimiento de la presencia de melanopsina en el cristalino fue demostrado usando inmunomarcaje en tejido humano y células epiteliales del cristalino, así como mediante inmunotransferencia. Estos hallazgos conducen al estudio de la regulación de la melatonina en el cristalino, los resultados mostraron un incremento de

AANAT, y un aumento en su actividad para producir melatonina bajo condiciones de oscuridad.

La liberación de melatonina al humor acuoso de diferentes estructuras mostró claramente su importancia en la participación en la regulación de la PIO, sugiriendo un posible mecanismo de control en patologías como el glaucoma.

Chapter I: General aspects

“Wisdom begins in wonder”

Socrates

The eye: complexity makes our window to the outside world

The eye is the organ of photo-reception, a process of transforming the light energy of the environment by producing changes in specialized nerve cells in the retina, leading to nerve action potentials reaching the brain through the optic nerve, where the information is processed and consciously appreciated as vision. This is the main physiological function of the eye.

The eye is a round sphere organ 2.5 cm in diameter and formed by two segments of different size and curvature, the anterior segment, the cornea; which is smaller and more convex, and the posterior segment with a greater radius of curvature; the sclera. The anterior segment of the eye is divided in turn, forming two internal cavities, the anterior and the posterior chamber, while the spherical shape of the posterior segment forms the vitreous cavity (Fig. 1.1) (John V. Forrester, 2015).

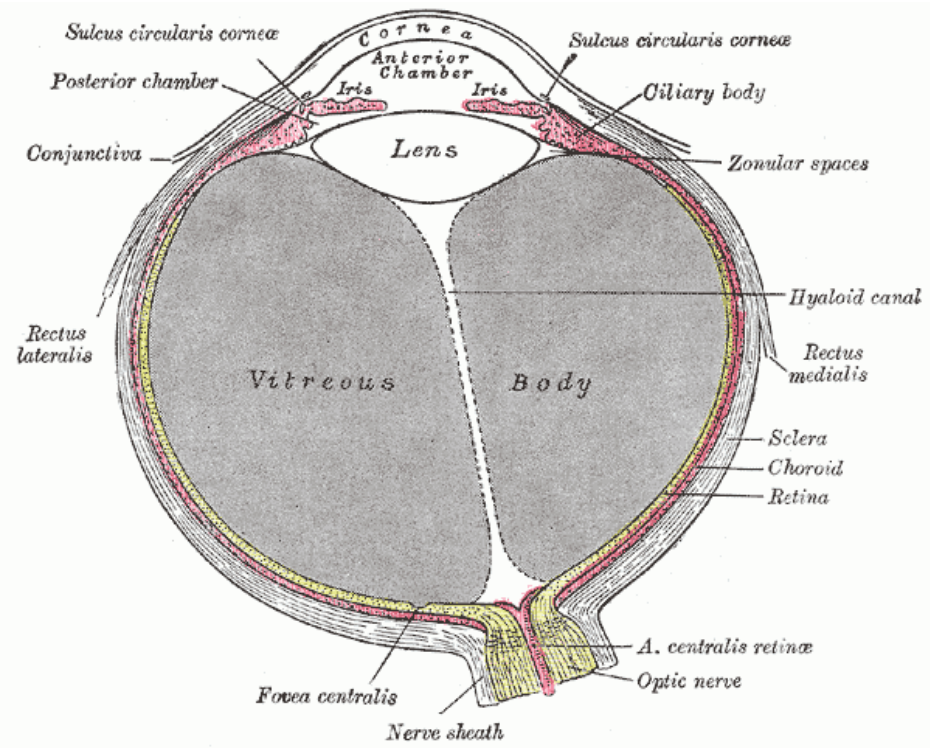

Figure 1.1. Schematic diagram of the human eye in horizontal section showing the major components and the arrangement of ocular structures (Image taken from Henry Gray, 1918).

It is made up of three basic layers or coats, often known as tunics. These are the fibrous (corneoscleral) coat, the vascular coat (composed of choroid, ciliary body and iris), and the neural layer (retina). The coats surround the contents, namely the lens and the transparent media (aqueous humor and vitreous body).

The cornea and the sclera envelop the eye in a way which forms protection of the ocular structures, as well as being an important structural support for the attachment of extraocular muscles. The cornea, is covered from the outside with the tear film, and it is responsible for most of the refraction of the eye. Its transparency is the most important property for vision and it is achieved in several ways; its avascularity; the regularity and smoothness of the covering epithelium; and the regular arrangement of the extracellular and cellular components in the stroma, which is dependent on the state of hydration, metabolism and nutrition of the stromal elements (John V. Forrester, 2015).

The uveal tract, the middle vascular pigmented layer of the eye, consists of three components, the iris, ciliary body, and the choroid. All are connected and have an initial opening in the pupil, and latterly at the optic nerve canal. The iris is a thin, pigmented, contractile disk which separates the anterior and poster chambers through the pupil opening, and is surrounded by the aqueous humor. The size of the pupil regulates the amount of light entering the eye and is dependent on the state of contraction of the intrinsic pupillary muscles, the dilator and sphincter pupillae (Davis-Silberman and Ashery-Padan, 2008). The ciliary body is a wide ring of tissues that extends from the scleral spur anteriorly to the ora serrata posteriorly. It is divided into an anterior zone called pars plicata (corona viliaris) and posterior, pars plana zone and it performs several functions such as accommodation, aqueous humor production and the lens zonule production. The

last ocular structure of the middle ocular layer is the choroid, which is the posterior part of the vascular coat of the eye. It is a thin, highly pigmented, vascular, loose connective tissue situated between the sclera and the retina, whose principal function is to nourish the outer layers of the retina (Nickla and Wallman, 2010). It also acts as a conduit for vessels travelling to other parts of the eye. Furthermore, absorption of light by choroidal pigment prevents unwanted light from reflecting back through the retina, something which occurs in some nocturnal species that possess a tapetum (Banerjee et al., 1992; Chinnery et al., 2017).

The third and innermost coat of the eye is the retina and the retinal pigment epithelium. The retina is responsible for converting the images from the external world into neuronal impulses which are transmitted to the brain for analysis. it consists of the inner neurosensory retina and the outer simple epithelium, the retinal pigment epithelium (RPE). The neural retina is firmly attached only at its anterior termination, the ora serrata, and at the margins of the optic nerve head (Martinez-Morales et al., 2004).

The three tunics that make up the structure of the eyeball are arranged in such a way that within the eye delimit different cavities, we can find the lens, vitreous humour and aqueous humour (Fig. 1.1).

The crystalline lens

The crystalline lens is located in the anterior segment of the eye, historically, it was reported that ancient Greco-Roman authors believed that the lens was located in the exact centre of the eye, however, studies showed that the centrality of the lens was an influential and enduring notion which seems to have originated with the ninth-century Arabic authors

in Mesopotamia, since ancient scientist used to dissect the eye and earlier photos showed the right location of the lens (Leffler et al., 2016).

The lens is a biconvex avascular and transparent structure that is located between the iris and the vitreous humor. It is the second main structure in the refractive function of the eyeball after the cornea. Its main characteristic is its elasticity, being able to modify its curvature during the process of accommodation, like a lens in a camera, the basic function of the eye lens is to transmit and focus light onto the retina (Fig. 1.2) (Hejtmancik and Shiels, 2015).

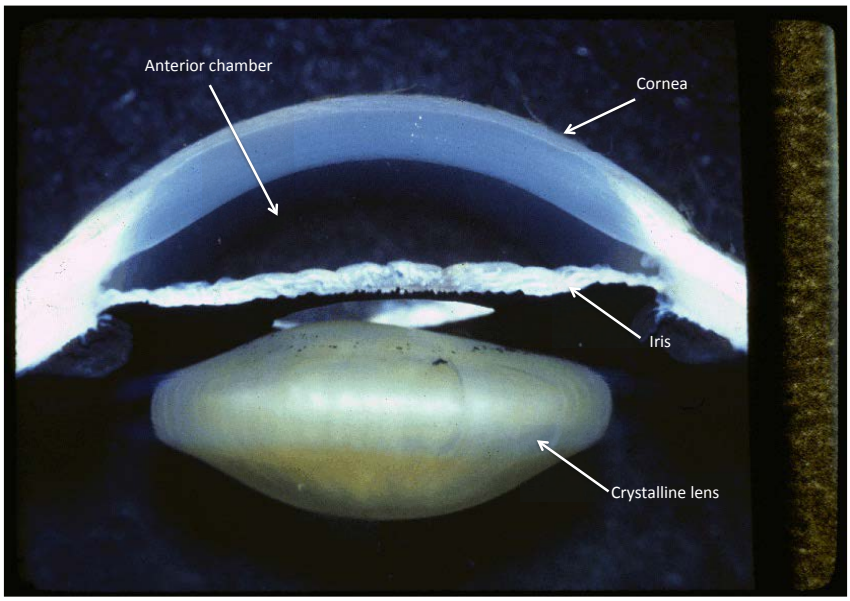

Figure 1.2. Image of the anterior part of the eye showing the location of the crystalline lens (Image modified from David G. Cogan opthalmic collection, National Eye Institue).

Structure and cells of the crystalline lens

The lens comprises three parts: the capsule; lens epithelium; and the lens fibers. The lens capsule completely envelops the lens and acts as a barrier to diffusion and contributes to shaping the lens during accommodation. It is a thickened, smooth, collagenous membrane which is composed mainly of type IV and XVIII collagens, laminin, entactin, perlecan, heparin sulfate proteoglycan, and fibronectin (Cammarata et

al., 1986; Parmigiani and McAvoy, 1984). The lens capsule is first detectable at 5–6 weeks of gestation in humans and is produced continually throughout life firstly by the cuboidal epithelium and then more slowly by the fiber cells (Fisher and Pettet, 1972).

Lens epithelium is restricted to the anterior surface of the lens, where the mitotic division in the lens occurs. The anterior epithelial cells are connected by gap-junctions, allowing the passing of low-molecular weight metabolites and ions. They present few or no tight junctions that would make the extracellular spaces impermeable to these molecules. The anterior epithelium is also rich in organelles and contains large amounts of cytoskeletal proteins, such as microtubules, spectrin, α-actinin, actin, myosin, and vimentin, presumably to help stabilize the cell structures during accommodation (Fig. 1.3).

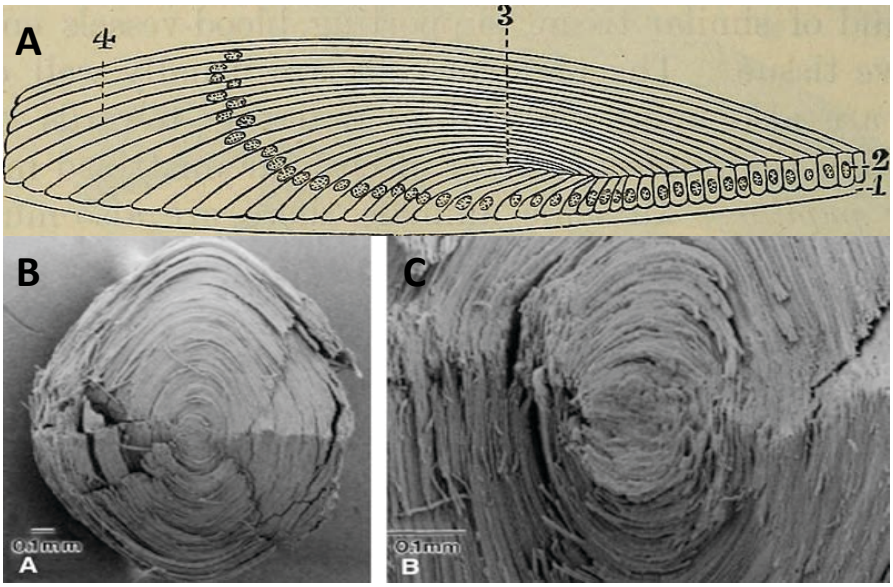

Figure 1.3. A: Meridional section through the lens; 1. lens capsule; 2. lens epithelium; 3. transition of the epithelium into the fibers; 4. lens fibers (Image taken from the handbook of physiology, 1890). B and C: Scanning electron micrograph illustration the tightly packed mass of fiber cells in the human lens (Image taken from Al-Ghoul KJ, Nordgren RK et al, 2001).

The cells become more columnar at the equator. As they elongate, the apical portion comes to lie deeper than other, more superficially positioned, lens cells. These elongated lens cells are known as lens ‘fibers’. The cell nucleus and cell body sink deeper

into the lens as further cells are laid down externally. Mitotic activity is maximal in the pre-equatorial and equatorial lens epithelium, known as the germinative zone, where the mitotic division in the lens occurs (Goodenough et al., 1980).

At birth, the lens weight is about 65 mg, at the age of 10 the human lens weight increases to about 160 mg and it continues growing substantially slower so that it weighs about 250 mg by the age of 90 (Augusteyn, 2007). Of the total mass of the lens, 60% is made up of proteins, much higher than almost any other tissue (Wistow and Piatigorsky, 1988). The lens is surrounded by a collagenous capsule which acts as a barrier to diffusion and contributes to shaping the lens during accommodation (Atchison, 1995; Fisher and Pettet, 1972).

The crystalline lens is composed of a single cell type which follows a developmental pattern, starting as a member of the germinative zone in the single layer of anterior epithelial cells overlying the fiber cell mess. These epithelial cells migrate laterally towards the equator, where they elongate and invert to form secondary fibers. To increase light transmission, organelles such as mitochondria, Golgi bodies, and both rough and smooth endoplasmic reticulum (ER) are degraded in the differentiating lens fiber cells so that they are absent from the nuclear fiber cells (Hejtmancik and Shiels, 2015). As the cells elongate newer cortical fiber cells are layered over them so that they are moved toward the lens nucleus, stretching anteriorly from the cuboidal epithelial cells posteriorly to the posterior capsule. Transcriptional control plays a significant role in the differential synthesis of lens crystallins (Piatigorsky, 1987). The distribution of β-crystallin mRNAs in chickens and the β- and γ-crystallin proteins and mRNAs in rats provides examples of the

spatial and temporal control of crystallin gene expression during lens development (Clark and Huang, 1996; Hejtmancik et al., 1985; Muchowski et al., 1997).

Transparency and aging

The main optical function of the lens is to transmit light to the retina through the cornea. It fine-tunes the focusing of light to the retina, and by changing shape, it adjusts focal distance (accommodation) (Pasta et al., 2004).

The human lens is colorless at birth; its transparency is due to its highly organized structure. However, during the earlier part of the sixth decade the color intensifies, and this is primarily confined to the nucleus, causing the effect on color perception (Petrash, 2013), probably due to the production of 3-hydroxykynurenine and other metabolites of tryptophan that filter UV light (Regini et al., 2007). Since the change is gradual, it generally goes unnoticed (Pescosolido et al., 2016). The limit of visual perception is about 720 nm, the lens transmits light of wavelength up to 1200 nm efficiently, and a very little light below 390 nm. The lens absorption makes blue objects seem dull and grey unless they are very bright blues whereas green (made from mixing blue with yellow) appears yellow (Fig. 1.4) (Hejtmancik and Shiels, 2015).

As discussed previously, the architecture and cellular contents of the lens are critical for its transparency. The transparency and high-refractive index of cells in the lens result from the tight packing of their proteins, providing a constant refractive index over distances approximating the wavelength of the transmitted light. For several reasons, such as ageing or diabetes, the proteins in the lens aggregate resulting in the clouding of the lens and the formation of cataracts. As the light cannot pass clearly through the lens, there is some loss of vision. Since new cells cover the outside of lens, the other cells are

compacted into the centre of the lens, resulting in the cataract. The cataract ultimately results in the loss of vision in people over the age of 40 years. The most recent estimates from World Health Organization (WHO) reveal that 47.8% of global blindness is due to cataracts, making this pathology one of the top causes of reversible blindness (Cook et al., 1994; Croft et al., 2001; Kaur et al., 2017).

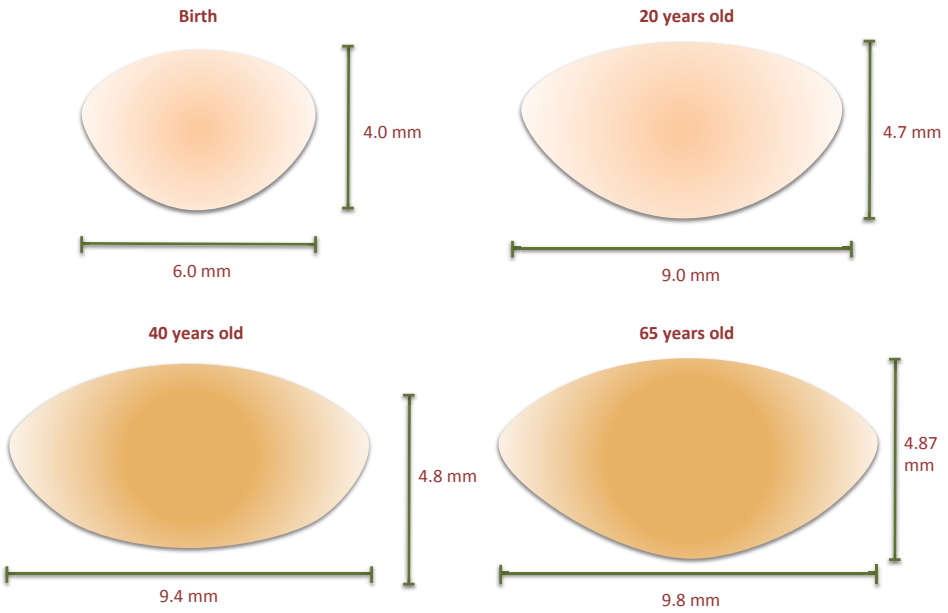

Figure 1.4. A series of scaled changes in lens equatorial and polar dimensions with ageing.

The ciliary body

The ciliary body is a complex, highly specialized tissue that comprises several cell types. It is hidden behind the iris, which makes it impossible to visualize simply when someone looks at an eye. It is a wide ring of tissue that extends from the scleral spur anteriorly to the anterior border of the retina (ora serrata) posteriorly. It is divided into two zones, an anterior pars plicata (corona ciliares) and a posterior pars plana. On cross-section, it have a shape of a triangle, and its base faces the anterior chamber and the apex blends posteriorly with the vascular choroid. The surface of the ciliary body (pars plicata) is elaborated into a series of ridges and consists of 70 radially arranged folds known as

ciliary processes, each is approximately 2 mm in size. The pars plana is an approximately 4 mm wide zone stretching from the posterior limits of the ciliary processes to the *ora serrata*, the sharp serrated or dentate junction where non-pigmented ciliary epithelium undergoes a sharp transition to become the neural retina (Fig. 1.5) (Delamere, 2006).

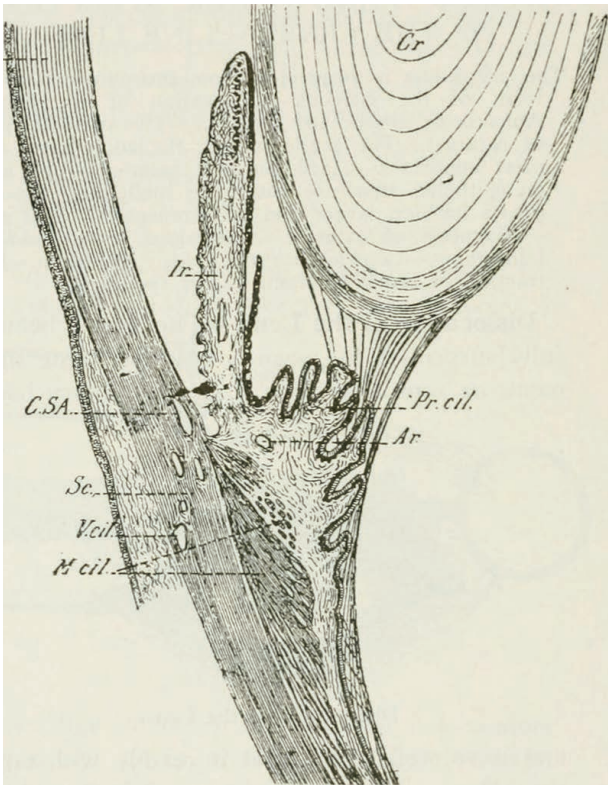

Figure 1.5. The ciliary body location (taken from The Commoner diseases of the eye, how to detect and how to treat them, Casey Albert Wood ,1904).

The ciliary body can be divided histologically into the ciliary epithelium, ciliary body stroma and ciliary muscle, and its function can be divided into accommodation, aqueous humor production and production of the lens zonules, vitreal glycosaminoglycans and vitreal collagen. The base of the ciliary body is home to the ciliary muscle, the contraction of which causes the lens to assume a more rounded shape. This is because the lens is suspended by fine ligaments, called zonules that attach to the ciliary body. When the ciliary muscle contracts, the anchoring point of the zonules moves slightly inward, relaxing tension on the zonules, and the natural elasticity of the lens causes it to take on a

more spherical shape. This is how our focus shifts. It is the process of accommodation (Croft and Kaufman, 2006). When the ciliary muscle relaxes, there is a slight outward motion that tightens the zonules and flattens the lens (Croft and Kaufman, 2006).

The ciliary processes

The ciliary processes have a radial orientation with each ridge pointing toward the pupil. Each of the 70-80 processes is densely vascularized and covered by an epithelial lining of highly active, secretory cells to comply with the function of the ciliary processes; the aqueous humour production (Tamm and Lutjen-Drecoll, 1996). The covering epithelial layers is, in fact, a bilayer made up of two different epithelial cell types, the cell layer farthest from the anterior of the eyeball is developmentally related to the retinal pigment epithelium. It is named the pigmented epithelium (PE) and it is composed of cuboid cells with numerous melanosomes, and a small number of mitochondria and rough endoplasmic reticulum.This layer is separated from the stroma by Bruch's membrane which contains collagen and elastic fibers. The other half of the ciliary epithelium bilayer, the non-pigmented layer (NPE), is developmentally related to the neural retina. Unlike the EP cells, the NPE cells do not present melanin, however the amount of mitochondria and rough endoplasmic reticulum is much higher in these cells. This increase is even greater in the anterior cells of the pars plana, which is indicative of a higher metabolic rate (Delamere, 2006; Do and Civan, 2004).

The NPE is also composed of cuboid cells. These cells present a remarkably large number of invaginations and folds on that part of their surface. The basement membrane faces the aqueous humor. In addition to the existence of a basement membrane, there is an internal limiting membrane. This invagination of what is the basolateral membrane of the

non-pigmented ciliary epithelium, appears to be another anatomical feature, providing the ciliary body with an enormous surface area available for fluid secretion. There are other side interdigitations, close to which, the existence of enzymes can be detected: ATPase Na^{+}-K^{+} (potassium sodium pump) and carbonic anhydrase (CA), whose presence is also observed in the apical area (Civan and Macknight, 2004).

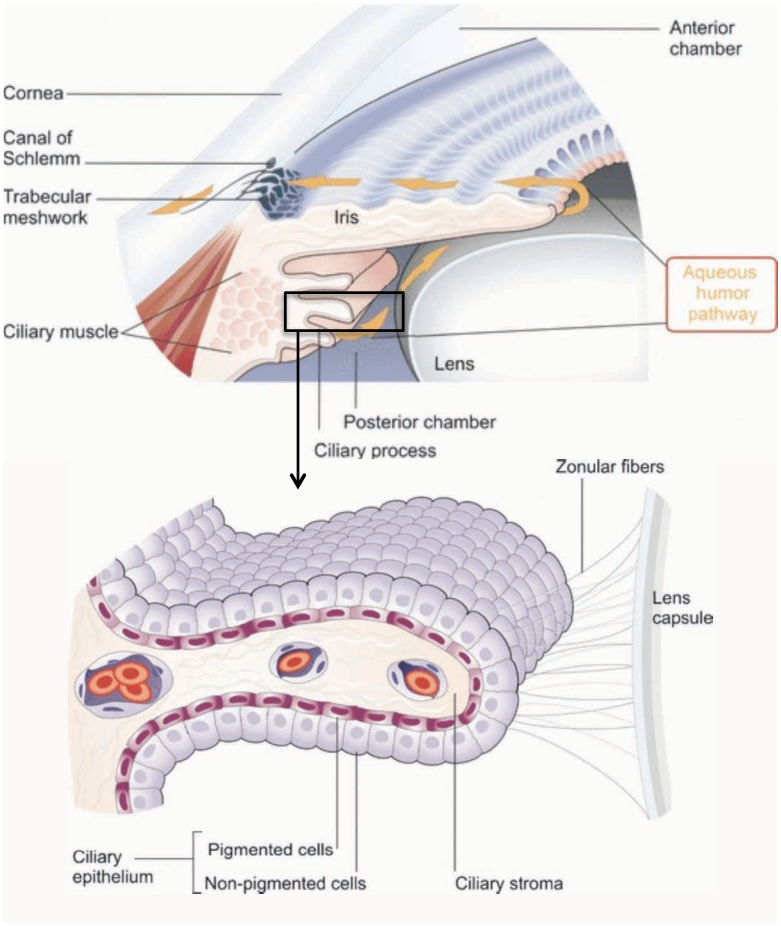

Figure 1.6 Schematic representation of a sagittal section of the eye, a focus on the bilayer of the ciliary epithelium, which constitutes the blood-aqueous barrier.

The union of the two epithelial layers is established by desmosomes. These bonds are permeable, and allow the transit of water and ions between the two cell types. There are also gap junctions that regulate the intercellular transport of small molecules by opening channels with diameters of approximately 1.5 nm.

The ciliary processes, through the specific transport of substances, are responsible for the formation of aqueous humor. This transparent fluid is essential for the maintenance of ocular structures and their nutrition (Freddo, 2013; Kaufman, 2011).

Aqueous Humour

The aqueous humour is a clear transparent fluid which bathes the anterior and posterior chambers of the eye. It is similar to blood plasma, composed mainly of water, electrolytes and substances of low molecular weight. Its ionic composition varies between different species (Adler, 1933; Civan and Macknight, 2004; Davson et al., 1936).

Some intraocular structures such as the iris, lens, and corneal endothelium contribute to the final composition of AH, however the formation of AH occurs mainly as an active process of secretion in the ciliary processes. After its secretion in the ciliary processes towards the posterior chamber, the aqueous humor circulates through the pupil towards the anterior chamber, where it drains (Bill, 1966b). The circulation of the aqueous humor from the posterior to the anterior chamber is directly related to its functions, such as providing oxygen and nutrients to the cornea, lens and trabecular meshwork, as well as removing waste substances from cellular metabolism, it also maintains a certain volume, giving the eye a certain intraocular pressure (IOP), which is essential for the correct functioning of the visual system, and it transports ascorbic acid to the anterior segment, where it acts as an antioxidant removing free radicals, and facilitates the immune response at the local level during an inflammatory process (Krupin et al., 1986).

In humans, the total aqueous humor volume is about 250 µl and it is produced at around 2-4 µl/minute (Toris et al., 1999).

Aqueous humor formation and drainage

The formation of the aqueous humour is a complex process which requires three steps to be carried out. First, the blood flow must reach the ciliary processes in the anterior uvea. From the plasma from these capillaries, an ultrafiltrate is generated towards the stroma, within the interstitial spaces between the vessels and the ciliary epithelium. The solutes of the ultrafiltrate cross the ciliary epithelium into the posterior chamber, and finally, they are accompanied by a passive flow of water following an osmotic gradient. The movement of solutes through the ciliary epithelium can be carried out through three interdependent physiological mechanisms, first, diffusion which is defined as the passive movement of solutes through the ciliary epithelium in response to a concentration gradient. In addition, substances with a high liposolubility coefficient are those that pass through the cell membrane more easily. Second is the ultrafiltration, which is the passive movement of water and water soluble substances through cell membranes as a result of hydrostatic and osmotic pressures and is produced as a result of the difference in pressures between the capillary blood pressure of the ciliary processes and the intraocular pressure. It represents the flow of most of the blood plasma through the endothelium of the fenestrated capillaries into the ciliary stroma. Finally, the active secretion which involves the movement of solutes through the ciliary epithelium against a gradient with energy consumption, and is the major mechanism in the aqueous humor formation. Aqueous humor formation through the active transport mechanism has been reported to be greater than by ultrafiltration and/ or diffusion, and is considered to be responsible for 80% of aqueous humor formation under normal conditions (Fig. 1.7) (Brubaker, 1991; Cole, 1977; To et al., 2002).

After the formation of the aqueous humor by the ciliary processes and its secretion to the posterior chamber, it passes through the iris and passes into the anterior chamber,

from where it will be drained to the level of the iridocorneal angle by some escape routes. Two main drainage routs exist inside the venous circulation: the conventional one through the trabecular meshwork, and the non-conventional route which is the uveoscleral pathway.

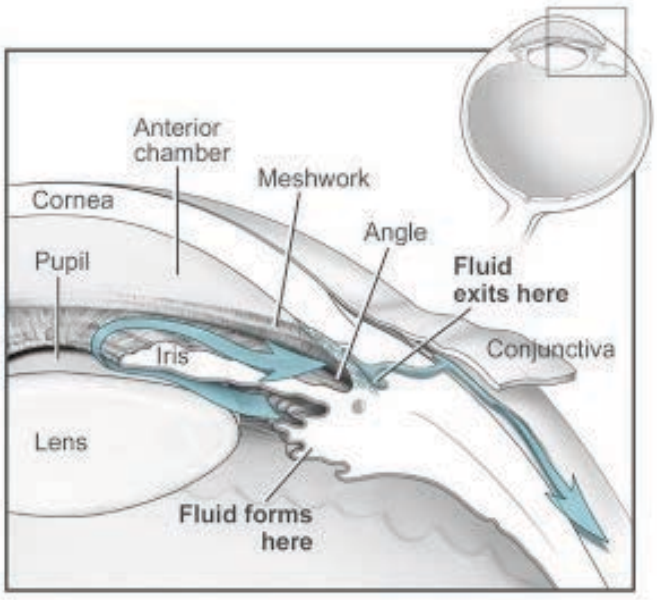

Figure 1.7. Aqueous humor pathway. The aqueous homor formation takes palce in the ciliary processes and it drain by the pathways describes in the test (photo taken for the National Eye Institute).

1- Trabecular pathway:

The trabecular meshwork is the main pathway through which the aqueous humour is drained. Approximately 80-90% of the aqueous humour is evacuated through the trabecular pathway. For its drainage, the AH crosses different layers that constitute the trabecular meshwork: uveoscleral, corneoescleral and cribiform, until reaching the Schlemm canal. From here, the AH reaches the collecting channels, whereby it is drained into the intraclass and episcleral veins (Bill, 1966a; Weinreb et al., 2002).

The transcellular pathway is the main way in which the aqueous humor is transferred into the canal. The particularity of the structures of the transcellular pathway is

its regulation by means of pressure mechanisms. Thus, an increase in IOP results in an increase in the number of these structures; Almost disappearing when it decreases (Bill, 1966a). Thus, in a range of normal pressures, the increase in IOP will be accompanied by a linear increase in HA evacuation; However, after a certain pressure limit, the drainage decreases due to the compression of the trabecular meshwork and the canal, which increases resistance to fluid passage (Brubaker, 1975, 1991) .

2- Uveoscleral pathway

It is considered a minor pathway for the aqueous humor outflow, with approximately 10-20% drainage rate in humans. The lack of an epithelial barrier between the anterior chamber and the ciliary muscle favors the entry of AH into the ciliary body from the iridiocorneal angle. The fluid flows freely between the muscle bundles and enters the suprachoroid and supraciliar spaces, from where the sclera can be reached. The uveoscleral pathway is relatively insensitive to pressure increases (Pederson and Toris, 1987; Suguro et al., 1985).

Intraocular pressure (IOP) is the force that supports intraocular tissues. It is maintained within stable values due to a complex and dynamic balance between the constant production of the aqueous humor and its drainage. Small variations in either direction cause changes in IOP, the main factors that affect IOP are circadian rhythms, episcleral vein pressure, the ratio between production and drainage of aqueous fumes, and the influence of hormones and nerves (cranial nerves V and VII). As for circadian rhythms, the IOP undergoes small variations throughout the day, being maximal first thing in the morning and reaching its minimum values at night. In humans, the rate of HA formation

was 2.61 µl / min during daytime and 1.08 µl / min during nights (McCannel et al., 1992). In terms of IOP , healthy human eyes presents a diurnal fluctuation which varies between 3-5 mm Hg, being significantly greater in glaucomatous eyes (Asrani et al., 2000).

The IOP is considered to be elevated when it exceeds the values corresponding to the 97.5 percentile of the population, which in humans is usually established in values greater than 21 mm Hg. However, it is not considered a pathological process per se, although it constitutes an important risk factor in the development of certain ocular pathologies such as glaucoma (Casson et al., 2012a).

Melatonin: a great discovery reveals a multitasking molecule

Melatonin is an indoleamine (N-acetyl-5-methoxytryptamine) first discovered and described in the pineal gland by the dermatologist Aaron Lerner (Lerner et al., 1960). It derives from an important neurotransmitter called serotonin and it is mainly known to be responsible for regulating the circadian rhythm; one of the most relevant characteristics of melatonin is its variable synthesis throughout the day. Melatonin synthesis is increased during the night and reduced at day time (Pevet, 2002, 2014; Tosini and Fukuhara, 2003; Zawilska et al., 2002). Since the discovery of melatonin, a diversity of functions for melatonin has been defined. One of the initial uses of melatonin in humans was to minimize the circadian disruption resulting from jet lag (Arendt, 1988). Endogenous nocturnal peak of melatonin is followed by a drop in body temperature, and a reduction in both systolic and diastolic blood pressure (Simko and Pechanova, 2009). On the other hand, bright light exposure at night, which inhibits melatonin rise, leads to an elevation of body temperature, higher level of performance, reduced sleepiness and an absence of blood pressure reduction. Exogenous melatonin intake promotes sleepiness among a series of proven body reactions enhancing the opening of the sleep gate (Krauchi et al., 2006; Nishiyama et al., 1997).

Melatonin have several functions apart from transducing photoperiod information. One of these functions is regulating seasonal reproductive activity in photoperiodic animals. Changes in the melatonin cycle, according to variations of ambient photoperiod duration, for instance, long nights during the winter, compared to shorter nights thus less melatonin during summer period, function as a calendar rather than just a clock providing the internal organs information about the time of year. Melatonin message is a signal

which allows seasonal breeders to anticipate the changing seasons and make the necessary adjustments in advance of the actual breeding period (Arendt et al., 1995; Brainard et al., 1982; Gupta and Haldar, 2014; Li and Zhou, 2015).

Significant evidence points towards the role of melatonin in suppressing carcinogenesis. Many tumours develop following damage to nuclear DNA when it goes unrepaired, as injuries of the DNA are frequently a result of free radicals. Since melatonin neutralizes free radicals, it protects DNA from the oxidative damage, besides melatonin may help in repairing the already damaged DNA. Melatonin can limit tumour progression by acting as anti proliferation, anti metastasis, proapoptotic, and prodifferentiation substance (Assayed and Abd El-Aty, 2009; Cerutti et al., 1994; Karbownik et al., 2000a; Karbownik et al., 2000b; Mao et al., 2016; Sliwinski et al., 2007). Currently, light at night is being examined as a causative factor for the elevated breast cancer in women and prostate cancer in men, giving the high incidence of breast and prostate cancer in human population and that light exposure after darkness onset alters biological rhythmicity and supresses melatonin (Fig. 1.7) (Karasek and Pawlikowski, 1999; Reiter et al., 2007a; Stevens, 2005, 2009a, b; Zamfir Chiru et al., 2014).

Melatonin ability to quench the devastatingly reactive and toxic -OH was first proven (Poeggeler et al., 1993; Tan et al., 1993) and then followed by the confirmation that melatonin is able to detoxify different damaging oxidizing agents like O_2, H_2O_2, $ONOO^-$, and HCIO (Reiter et al., 2008; Reiter et al., 2003; Reiter et al., 2007b). Melatonin is considered one of the best FDA-approved drugs as part of the Neurodegeneration Drug Sreening Consortium of the National Institute of Neurological Disorders and Stoke (NINDS) because of its ability to reduce cytochrome c discharge from mitochondria,

beside its capacity to easily cross the blood-brain barrier and its virtual absence of toxicity (Wang, 2009; Wang et al., 2013).Melatonin is of special importance in the ocular system.

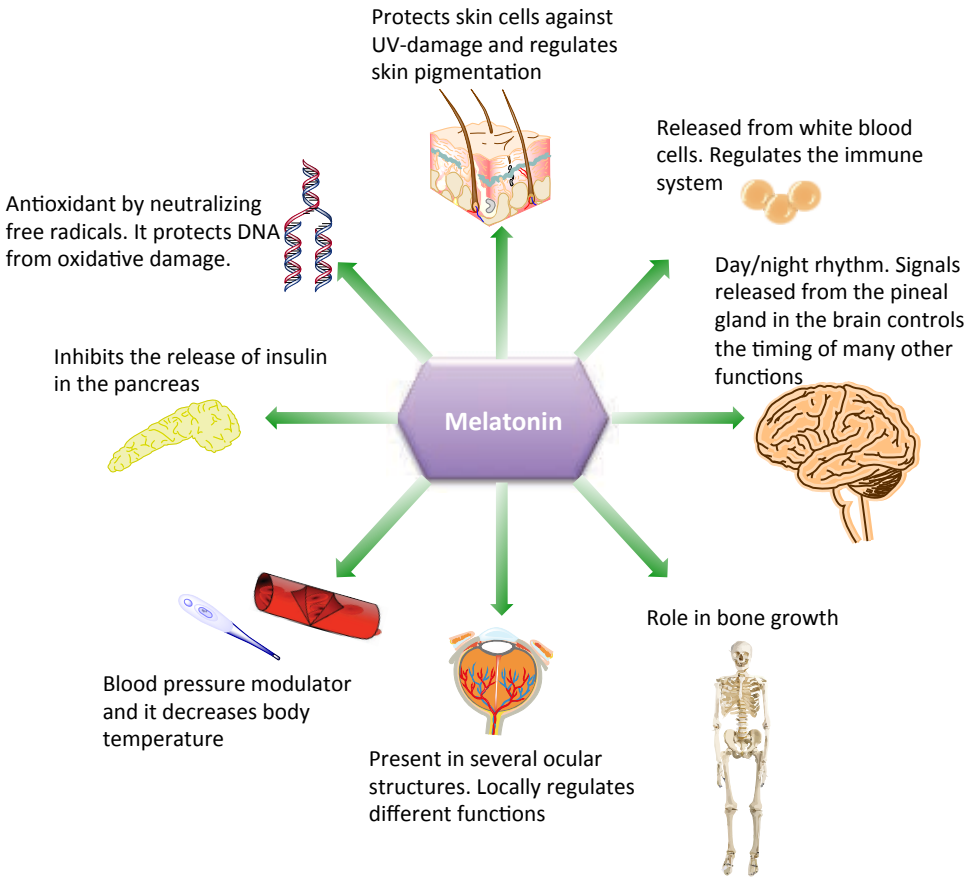

Figure 1.8. Some of the numerous actions of melatonin in mammals from pineal and extra-pineal sources.

The eye is the window of our body to the outside world, informing us of the time of the day in order to regulate melatonin, starting from the retina. Independently of regulating light/dark cycles starting from the eye, melatonin contribute in numerous functions within several ocular structures. The cornea is the most superficial part of the eye and therefore it is likely to suffer accidents like foreign body entrance or inadequate contact lens wearing among others. Corneal health and transparency is of great importance to maintaining clear vision. Melatonin accelerate corneal wound healing, and it has been possible to demonstrate that the effect of melatonin is to increase the rate of cell migration rather than

mitosis (Crooke et al., 2015). And last but not least, melatonin acts as an antioxidant protecting ocular structures against free radicals. It have been proved to reduce intraocular pressure by decreasing the rate of aqueous humour secretion by the non-pigmented ciliary epithelium, resulting in a modulation of IOP (Fig. 1.8) (Wiechmann and Wirsig-Wiechmann, 2001).

Melatonin synthesis

Melatonin is synthesized primarily from tryptophan which hydroxylates to 5-hydroxytryptophan, this amino acid is then decarboxylated by I-aromatic amino acid decarboxylase to form the biogenic amine serotonin (Axelrod, 1974). 5-25% of produced serotonin is then converted to N-acetylserotonin (NAS) through acetylation, which has been discovered to be involved in melatonin biosynthesis by the enzyme called arylalkymine *N*-acetyltransfrerase (AANAT). Finally, to convert NAS to melatonin, an enzyme called hydroxyindole-*O*-methyltransferase (HIOMT) is responsible for the *O*-methylation step of NAS (Fig. 1.9) (Axelrod and Weissbach, 1961).

At night, an increment of melatonin synthesis is stimulated by electrical signals originated from neurons present in the suprachaismatic nucleus (SCN). These neurons receive inputs from the eyes and send inputs through the paraventricular nucleus (PVN) and then the spinal cord to the superior cervical ganglia (SCG) of the sympathetic nervous system, the fibres terminating adjacent to pinealocytes (Moore and Klein, 1974).

Norepinephrine (NE) is the neurotransmitter at the postganglionic sympathetic nerve terminal. It binds to α- and β- adrenergic receptors on the pinealocyte membrane and triggers a numerous of intracellular signalling.

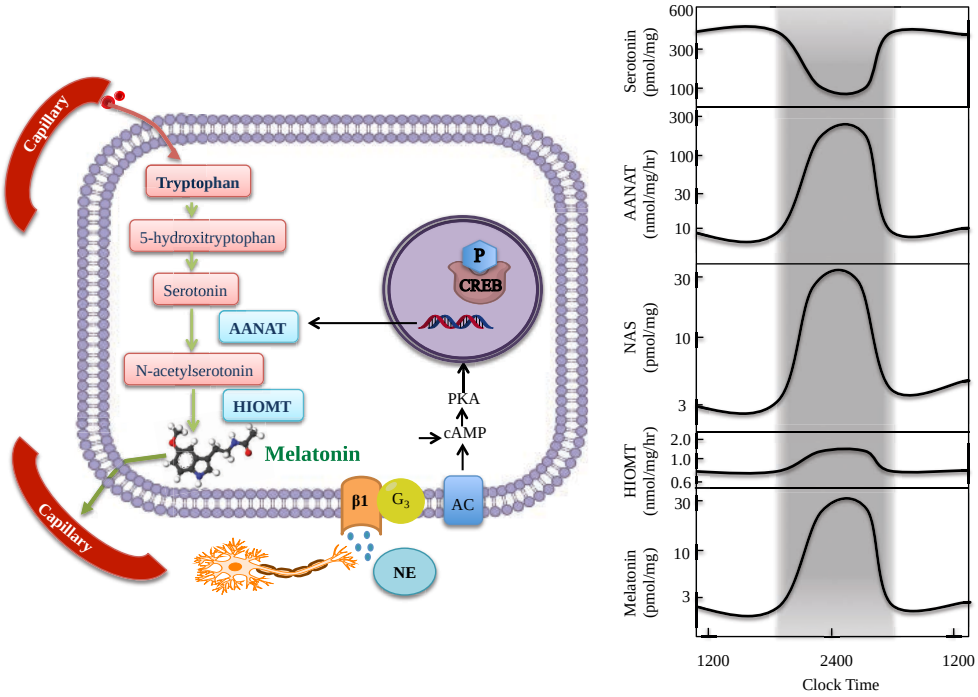

Figure 1.9. Diagramatic respresentation of melatonin synthesis in the pineal gland (on the left). On the rigth, a demonstration of daily rhythm in pineal indoles. Serotonin levels are hight during the day and decrease at night (dark panel) because of an increase in AANAT protein and activity. AANAT transfers an acetyl group from acetyl CoA to 5-HT. The increase in AANAT activity results in an increase in the intracellular concentration of N-acetylserotonin which is converted to melatonin by hydroxyindole-O-methyltransferase (modified after Klein,1974). AANAT, arylalkylamine *N*-acetyltransferase; AC, adenylate cyclase; HIOMT, hydroxyindole-*O*-methyltransferase; cAMP, cyclic adenosine monophosphate; CREB, cAMP response element-binding protein;NAS, N-acetyl 5-methoxytryptamine; NE, norepinephrine; PKA, protein kinase A.

When β- receptors are activated it stimulates guanine nucleotide binding protien (G-protein) which activates adenylate cyclase. ATP is hydrolyzed and intracellular cAMP levels rise, which enhance cAMP dependent protein kinase. This activates mRNA expression to synthesize AANAT or boost its activity. In parallel with the increment of cAMP levels and AANAT activity at night, there is a peak activity of phosphodiesterase which hydrolyses cAMP (Klein et al., 1997; Klein et al., 1992; Minneman and Iversen, 1976).

Stimulation of α- receptors activate a G-protein-linked to phosphotidylinositol cascade process involving phospholipase C (PLC), diacylglycerol (DG), and protein kinase

C (PKC). PKC in turn activates adenylate cyclase resulting in the enhancement of AANAT activity and melatonin synthesis (Fig. 1.8) (Ho et al., 1988).

Arylalkymine *N*-acetyltransferase, also known as serotonin N-acetyltransferase. It catalyzes the transfer of acetyl group from acetyl-CoA to serotonin resulting in the synthesis of NAS. This is the rate limiting step in melatonin production, with an activity in rat pineal gland showing a 24 hours rhythm with a nocturnal peak of 20- to 100 fold greater than daytime levels, which explain the higher melatonin levels during night (Reiter, 1993).

Hydroxyindole-*O*-methyltransferase is a cytosolic protein which constitutes 2 to 4% of total soluble proteins in the pineal gland. This enzyme catalyses the *O*-methylation of NAS by S-adenosyl methionine to form melatonin. In mammals, pineal HIOMT activity is high and appears to exhibit no diurnal variation (Sugden, 1989).

AANAT: the key enzyme in melatonin regulation ¨THE TIMEZYME¨

Briefly after Aaron Lerner reported melatonin isolation, Weissbach reported studies describing an enzyme in pineal extracts which transfers a methyl group from S-adenosylmethionine to N-acetylserotonin, and he described the presence of an enzyme in rat liver and beef pineal gland which could acetylate serotonin in the presence of acetyl-CoA-generating system. Since beef pineal extracts also contained hydroxyindole-O-methyltransferase, it was possible to show the conversion of serotonin to melatonin as a process performed in two steps (Weissbach et al., 1960).

Arylalkylamine N-acetyltransferase, AA-NAT, HGMW-approved symbol AANAT; EC 2.3.1.87. is of special interest because of its daily fluctuations indicating an important

role, which is the molecular interface between the clock-driven regulatory systems and melatonin production (Fig. 1.9) (Klein, 1985; Klein et al., 1992).

AANAT localization and Characteristics

The portion of AANAT gene encoding the mRNA transcript is approximately 2.5 kb, 1.9 kb, 3.8 kb in human, sheep, and chicken respectively (Coon et al., 1996; Klein et al., 1996) .

The human AANAT gene is localized at 17q25. Studies done by Coon,1996 suggested that AANAT have a single locus in the human genome using several techniques like PCR of a somatic cell hybrid panel or Fluorescence in situ hybridization (FISH) in addition to genomic Southern blots. In addition, it does not appear to be linked to any known genetic disease (Coon et al., 1996). The AANAT sequence contains conserved putative acetyl CoA binding motifs at $AANAT_{121-140}$ and $AANAT_{165-177}$ (Tercero et al., 1992). Motifs A and B, which are located between these sites, may play a role in acetyl-CoA binding or acetyl group transfer. The term motif A\B refers to two conserved sequences that identify family members, this is indicated by evidence that all proteins with these motifs are acetyltransferases, that point mutations in these motifs block activity (Namboodiri et al., 1980b; Tercero et al., 1992) and that similar structures are involved in nucleotide binding (Schulz et al., 1990).

A conserved histidine-rich region which includes motif A, within $AANAT_{112-146}$. This region contains approximately 50% of the histidine although the sequence represents 15% of total AANAT. Some of these histidine could function as a catalytic site based on the ability of the imidazole group to catalyze acetylation of indolamines (Klein et al., 1976). Finally, the six conserved cysteines in mammalian AANAT can play a role in

protein thiol:disulfate exchange inactivation of the enzyme (Namboodiri et al., 1981; Namboodiri et al., 1980a).

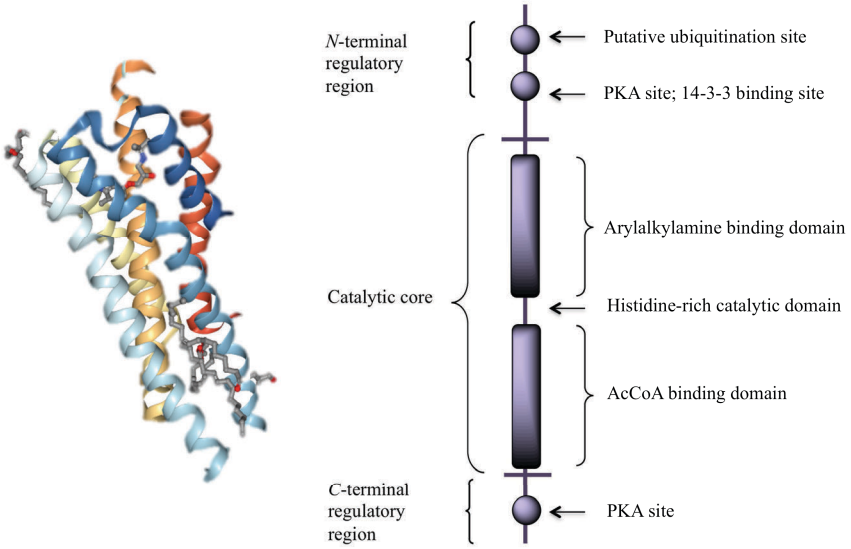

Figure 1.10. Structure of arylalkylamine N-acetyltransferase (AANAT) Vertebrate AANATs are organized into a central catalytic core, which alone has enzymatic activity, and flanking regulatory regions, each of which has a PKA site. Phosphorylation of the *N*-terminal PKA site converts the 6-residue sequence containing the PKA site into a 14-3-3 binding motif: *RRHT*LPAN → *RRHpT*LP. The arylalkylamine-binding domain characterizes the AANAT family. The AcCoA binding domain is similar to that found in the acetyltransferase superfamily identified as the 'Motif A/B' superfamily, reflecting two highly conserved motifs in the AcCoA binding domain, or, as the 'GNAT' superfamily, reflecting membership of an important enzyme, GCN5. The structure and function of this superfamily are reviewed in detail in Dyda et al. (2000).

Interestingly, 100% conservation is kept among the species examined (human, rat, sheep) in peptide sequences $AANAT_{48\text{-}59}$, $AANAT_{72\text{-}85}$, and $AANAT_{90\text{-}100}$. these sites might be involved in binding arylalkylamine substrates (Klein et al., 1996).

Human AANAT deduced amino acid sequence defines a 23.2 kDa protein (Coon et al., 1995). The specificity of the substrate, K_m values and stability of expressed and of partially purified ovine AANAT are similar. The degree of similarity to humans is the following: monkey (97%), sheep (84%), rat (90%), and chicken (76%). Closer observation of the sequence revealed similarity not uniformed, with the lowest level of conservation at

the extreme N-terminal portion of the molecule, and the remarkably conserved regions are found within a core that extends from the N-terminal PKA site to the end of motif A (Fig. 1.10). Interestingly, within that region a 81% conservation is found among all available sequences. Furthermore, closer examination reveals a number of highly conserved characteristic features, including a number of stretches of 10 or more (example: $AANAT_{48-59}$, $AANAT_{72-85}$, and $AANAT_{90-100}$) which is 100% identical amino acids in human, rats, and sheep, these sites might be involved in binding arylalkylamine substrates (Klein et al., 1997; Klein et al., 1996).

Conserved putative protein kinase A/protease regulatory regions are present at the C- and N-termini (RRXT/S). Moreover, a conserved protease cleavage site (RR) is present in the human sequence at amino acid 154, as well as conserved putative casein kinase phosphorylation site and a single putative protein kinase C phosphorylation site are present in human AANAT (Coon et al., 1996). These phosphorylation sites are of a special interest since cAMP is known to regulate the activity of the enzyme (Klein et al., 1992); a drop in cAMP is thought to be the signal for the rapid turn-off in activity (Klein, 1978). cAMP acts on AANAT in several ways, most commonly is the prevention of proteosomal proteolysis (Gastel et al., 1998). AANAT protein binds to a member of an unfamiliar proteins called 14-3-3 in which they form dimers. Studies showed that the N-terminal PKA site is embedded within a sequence that becomes a consensus 14-3-3 binding motif upon phosphorylation (RRHTLPANRRHpT LPAN ; where pT is phosphothreonine) (Muslin et al., 1996; Petosa et al., 1998). A complex formation is triggered at night by cAMP-dependent phosphorylation of the enzyme resulting in activation and protection against proteolysis. This complex is formed only if AANAT is phosphorylated and it is reversed and prevented by a 14 amino acid phosphopeptide that includes the N-terminal

PKA-14-3-3 binding motif of AANAT, which, in turn, binds to an amphiphathic groove of 14-3-3. The 14-3-3 binding restricts the flexibility of loop 1 and stabilize binding pockets, leading to a decrease of the K_m and, as a consequence, a reduction of V_{max}, for arylalkymines by 10 folds. This can be especially good for the cells since they can maintain the enzyme, thereby helping melatonin production when serotonin levels are low, like the case at night when acetylation lowers total serotonin levels as much as 95% (Fig. 1.9).

The regulation of AANAT protein by cAMP is a balance between destruction and protection. When cAMP is absent, AANAT does not bind to 14-3-3, therefore it is rapidly destroyed by proteasomal proteolysis. cAMP shifts the balance by directing phosphorylation of AANAT (pAANAT), which triggers binding to 14-3-3, thus protected. However, this binding is reversible by the drop of cAMP levels, because existing molecules of pAANT disassociate from the complex and re-association is blocked by dephosphorylation by protein phosphatase (PP), AANAT is then destroyed (Klein et al., 2003).

Specificity is characterized by high selectivity for some arylalkylamine; like serotonin, tryptamine, methoxytryptamine and mescaline, but not for all, as in the cases of , histamine, norepinephrine and dopamine. The selectivity of the enzyme is further emphasized by the high activity towards tryamine and very low activity toward 5-hydroxytyramine (dopamine). This selectivity, together with the number of potential arylalkylamine substrates in vertebrates, limits the physiological function of AANAT to serotonin acetylation (Klein et al., 1996).

Studies first done in rats indicated that the nocturnal increase in enzyme activity is controlled by the 24-hour clock in the suprachiasmatic nucleus (SCN), that is linked to the pineal gland by a neural circuit which is activated at night, resulting in the release of norepinephrine from sympathetic nerves in the pineal gland which finally elevate cAMP levels which increase serotonin N-acetylation through a mechanism which requires membrane hyperpolarization, protein synthesis, and N-acetyling enzyme stabilization (Namboodiri et al., 1985; Namboodiri et al., 1983).

Hydroxyindole-O-Methyl Transferase (HIOMT) :last step in melatonin synthesis

The final step in melatonin synthesis the conversion of N-acetylserotonin to melatonin, is catalyzed by the enzyme Hydoxyindole-*O*-methyltransferase (HIOMT).

Hydroxyindole-*O*-methyltransferase (HIOMT, EC 2.1.1.4.) is a cytosolic protein which constitutes 2 to 4% of the total soluble proteins in the pineal gland. This enzyme catalyses the *O*-methylation of NAS by S-adenosyl methionine to form melatonin. it was isolated and purified from bovine pineal gland and it appears to be formed of a dimer of two 39-kDa subunits. Nevertheless, human isolated HIMOT revealed a deduced amino acid sequence of 373 residue with a predicted pI of 5.7 and size of 41.6 kDa. Studies using western blot and immunodetection analysis confirmed the mass of human HIOMT to be about 42 kDA, which is larger than bovine HIOMT. Avian HIOMT has extra amino acids after residue 9 compared to the one of mammalian. The deduced amino acid sequences of bovine and avian HIOMT are 70% and 57% identical to the human HIOMT (Donohue et al., 1993; Donohue et al., 1992).

Computer analysis of human HIOMT sequence indicates several potential sites for secondary modification. two sites are conserved among the three sequences: the casein kinase II phosphorylation site at Ser-149 and the myristoylation site at Gly-216. In addition, 7 cysteines are conserved and it is possible that these form disulfide bonds or participate in protein thiol:disulfide exchange.

Analysis of a sample of 14 human individuals pineal gland homogenates indicated that all contained HIOMT with evident variability between individuals (As melatonin levels varies remakably between individuals).

When the question of the photoneural regulation of melatonin synthesis in the pineal gland was first examined, emphasis was placed on this enzyme, thinking that it was ¨the¨ key enzyme in melatonin synthesis because of its narrow specificity and limited distribution. However, this view started to change when it was realized that HIOMT did not have a large daily rhythm while pineal serotonin and melatonin did. In addition, it became clear that adrenergic agonists could stimulate melatonin production without changing HIOMT activity. Altogether, this made the focus shift towards acetylation as the key regulating step in melatonin pathway (Sugden, 1989).

Melatonin biosynthesis in extrapineal tissues

For several years after melatonin was discovered, it was considered to be exclusively produced by the pineal gland and only related to the control of the circadian and circannual rhythms. Afterwards, the presence of melatonin-related enzymes was subsequently discovered in the retina and the cerebellum (Bubenik et al., 1974). This was followed by its identification in many other peripheral tissues and organs (Reiter et al., 1983; Stefulj et al., 2001).

After melatonin synthesis was detected in the retina, Vlahakes and Wurtman discovered the presence of melatonin synthesizing enzymes in the rat harderian gland. Moreover, studies on pinealectomized rats were done and melatonin presence was found in the retina, cerebellum, and the harderian gland The expression of AANAT and HIOMT using more specific molecular technology revealed its presence in various tissues and organs including the retina (Olcese and Moller, 1989), iris, ciliary body (Aimoto et al., 1985), crystalline lens (Abe et al., 2000; Quay, 1984), Harderian gland (Djeridane et al., 1998) and the lacrimal gland (Mhatre et al., 1988), spleen, heart, skeletal muscle, liver, stomach, gut, placenta, testes, ovaries, cerebral cortex and striatum (Sanchez-Hidalgo et al., 2009).

Focusing on the eye, the retina was the first identified structure where melatonin is synthesized extrapineally after discovering the expression of AANAT and HIOMT in several retinal layers. First, it was thought that melatonin was synthesized in the photoreceptors, in the outer nuclear layer of the retina. However, different studies suggested that the photoreceptors are not the only place in the retina which produces melatonin. AANAT levels have been detected in the inner nuclear layer containing cell bodies of amacrine, horizontal, bipolar, and muller cells and in the ganglion cell layers as well (Coon et al., 2002; Liu et al., 2004). In the ciliary epithelium of the ciliary body, reports have indicated that these cells rhythmically synthesize melatonin and secrete it to the aqueous humour (Martin et al., 1988; Martin et al., 1992). AANAT and HIOMT activity have been detected in the human ciliary body (Martin et al., 1992). Specifically, the non-pigmented ciliary epithelium ,which is the one facing the aqueous humour, is the main area responsible for producing melatonin (Brubaker, 1991; Cole, 1977; Edelman et al., 1994; Rowland et al., 1986; Smith and Gregory, 1989). AANAT and HIOMT activity

have been also detected in the rat and rabbit lens. While AANAT activity showed a rhythmic behavior with peak levels during the night, HIOMT activity did not appear to fluctuate (Abe et al., 1999; Abe et al., 2000). Immunohistochemistry studies revealed that AANAT is localized in the lens cortical fiber cells.

Variation of melatonin synthesis in a day-night manner does not necessarily occur in all extrapineal synthesizing tissues, suggesting different signalling pathways from the photoperiod regulation of melatonin production. Furthermore, many extrapineal tissues have higher melatonin concentration than plasma throughout the 24 hours, and the intracellular melatonin from these tissues is not generally released into the circulation. All these observations suggest that extrapineal melatonin acts locally, possibly protecting cells from oxidative and inflammatory damage (Venegas et al., 2012). However, there are several exceptions to this assumption, for example, oral overload with tryptophan in rats and chicks induced a dose-dependent increase of melatonin in serum. Melatonin production was related to the enterochromaffin cells of the gastrointestinal tract (Huether et al., 1992). In other conditions, peripherally generated melatonin seem not to enter the circulation.

Melatonin degradation

Unlike melatonin synthesis, melatonin metabolism is less thoroughly understood. It was believed for several decades that melatonin had only one metabolite, 6-hydroxymelatonin, which is now considered the classical hepatic way of degradation (Facciola et al., 2001). However, melatonin metabolism is a highly complex process and 6-hydroxymelatonin is only one of its metabolites. Melatonin is metabolized by either an enzymatic process, a pseudoenzymatic process or via its interaction with ROS and NOS.

In the classical melatonin metabolism pathway, the liver CYP P450 enzymes CYP1A1, CYP1A2, and CYP1B1 metabolize melatonin to 6-hydroxymelatonin, which is then conjugated with either sulfate or glucuronide and secreted in the urine (Arendt, 1988; Facciola et al., 2001; Ma et al., 2005).

The alternative indolic pathway involves melatonin being metabolized to 5-methoxytryptamine by melatonin deacetylase (Grace et al., 1991; Rogawski et al., 1979; Semak et al., 2005). 5-Methoxytryptamine is then converted by monoamine oxidase to 5-methoxyindoleacetaldehyde, which is further enzymatically metabolized to either 5-MIAA or 5-MTOL by aldehyde dehydrogenase and alcohol dehydrogenase, respectively. Recent studies performed on the liver have indicated, however, that both mitochondrial and microsomal cytochrome P450s metabolize melatonin predominantly by 6-hydroxylation, with *O*-demethylation representing a minor metabolic pathway (Semak et al., 2008).

In the kynuric pathway, cleavage of the pyrrole ring of melatonin by indoleamine 2,3- dioxygenase yields AFMK, which is further metabolized by arylamine formamidase to form *N*1-acetyl-5-methoxykynuramine (AMK) (Hardeland et al., 1993; Hirata et al., 1974). The kynurenic acid degradation pathway can also be executed by other enzymes, including peroxidase, and myeloperoxidase (Slominski et al., 2008). The free radical species have also been shown to be involved in a non-enzymatic kynurenic pathway (Tan et al., 2000; Tan et al., 2007). The additional route of melatonin degradation in mitochondria to AFMK by cytochrome *C* was also described (Semak et al., 2005).

Melatonin Receptors

Melatonin shows its effects by several mechanisms in mammals. First, by binding to melatonin receptors in plasma membrane, or to orphan nuclear membrane. Another

mechanism is biding to intracellular proteins such as calmodulin, calreticulin and tubulin. Finally comes the antioxidant effect (Pandi-Perumal et al., 2008).

In mammals, melatonin receptors are found in many body structures and tissues, such as the brain, retina, cardiovascular system, skin, immune system cells, kidney, platelets among many other sites. Melatonin receptors MT_1, MT_2 have been cloned and characterized, while the putative *MT_3* was pharmacologically characterized, and in some animal models it has been suggested that it is the quinone reductase 2 enzyme (QR2) which plays a role in preventing oxidative stress and presenting features of melatonin receptors. Apart from the three receptors, a melatonin related orphan receptor has been found in mammals, GPR50, which surprisingly does not bind melatonin (Li et al., 2013). Finally, a different orphan nuclear hormone receptor RZR/ROR α has also been described (Pozo et al., 2004; Rafii-El-Idrissi et al., 1998).

Nuclear receptor

Melatonin is a ligand for a retinoid related orphan nuclear hormone receptor (RZR/ ROR α; human gene ID: 6095). theses receptors include three subtypes (a, b, d) and four splicing variants of the a-subtype (Becker-Andre et al., 1993). The structure of nuclear receptors consist of an N-terminal domain, a DNA binding domain that contains a zinc double finger, a hinge region, and a ligand binding domain included in the C-terminal (Lardone et al., 2009; Smirnov, 2001). Little is known about the interaction between melatonin and these nuclear receptors, and whether they interact directly has been a source of controversy. Some studies theorized that melatonin interacts with the nuclear receptors of the retinoic acid family of orphan receptors (Becker-Andre et al., 1993; Carlberg and

Wiesenberg, 1995). However, others propose that melatonin indirectly regulates nuclear receptors through MT_1 membrane receptor (Ram et al., 2002).

Membrane receptors

Most of melatonin actions are mediated by its membrane receptors. Melatonin receptors are members of the 7-transmembrane G protein-coupled receptor family (GPCRs). Both MT_1 and MT_2 receptor subtype belong to the class A of rhodopsin-like GPCRs (www.gpcr.org/7tm) and contains 7 hydrophobic transmembrane helices.

The MT_1 receptor is formed by 350 amino acids, and it has a calculated molecular weight of 39 kDa. The chromosome location for MT_1 receptor is 4q35-1(Navajas et al., 1996). The MT_2 receptor is formed by 362 amino acids with a 40 kDA molecular weight and it is located in 11q21-22 (Reppert et al., 1995). Both receptors share 60% homology.

The existence of 3 extracellular loops alternating with 3 intracellular loops to link the 7-transmembrane regions suggests the presence of potential sites for glycosylation and phosphorylation. The MT_1 melatonin receptor has 2 potential glycosylation sites in the N-terminal region and it may exist in more than one glycosylated form, while MT_2 receptor has one potential glycosylation site in the N-terminal region (Navajas et al., 1996; Reppert et al., 1996b).

An orphan receptor called the melatonin related receptor GPR50 is encoded by a gene located on the X chromosome (Xq28) and it has 45% amino acid homology to melatonin receptors (Dufourny et al., 2008). Despite this homology, melatonin does not bind to GPR50 and the identity of its real ligand is unclear (Reppert et al., 1996a). However, GPR50 has been shown to heterodimerize with both MT_1 and MT_2, but it only

interferes with MT_1; deletion of the large C-terminal tail of GPR50 abolishes the inhibitory effect of GPR50 on MT_1 without affecting heterdimerization (Li et al., 2013).

Receptors heteromerization: A new era involving melatonin receptors

G protein-coupled receptors (GPCRs) are the largest class of cell surface receptors in mammalian genome. Approximately 1000 genes thought to encode GPCRs in humans, around 300 to 400 of these genes mediate the effects of endogenous ligands, with the remainder being sensory receptors. Almost half of all current prescription drugs act through GPCRs. All these facts taken together point towards the importance this class of receptors has from a pharmacological point of view (Drews, 1996; Prinster et al., 2005).

GPCRs have traditionally been thought to act as monomers. iInterestingly, a new era emerged in 1979-1980 when Luigi F. Agnati and Kjell Fuxe hypothesized that an inter-membrane interaction between neuropeptide and monoamine receptors could occur. The first observations were published in 1980, showing that substance P could modulate the high-affinity serotonin binding sites in spinal cord membrane preparations using biochemical binding techniques (Agnati et al., 1980). This idea has been challenged over the past few years by accumulating pharmacological and biochemical data, all indicating that the primary functional GPCR signaling unit may actually consist of dimers or oligomers of receptors (Angers et al., 2002; George et al., 2002; Jordan and Devi, 1999; Marshall, 2001). Although the idea did not gain wide acceptance until more than a decade later, early evidence for GPCR dimerization came from unexplained cooperativity observed in ligand binding assays and unexpectedly large estimates of the size of receptor complexes on gel filtration columns (Agnati et al., 2003; Angers et al., 2002).

Multimerization of GPCRs was originally based on studies investigating the possibility of certain GPCRs to dimerize with themselves (homodimerization). Currently, there is a growing list of receptors that have been found to associate with other receptors (heterodimerization). The reports of heterodimerization suggest a potential level of receptor complexity that could explain previously unexpected pharmacological profiles. In addition to fundamentally changing our views on the structure and activation processes of GPCRs, the concept of homo- and heterodimerization could have dramatic impacts on drug development and screening (Prinster et al., 2005).

With the challenge of such a discovery, a concern in the field that GPCRs heterodimerization might be an artifact of receptor over-expression and/or a result of the techniques used to study receptor associations has been addressed. This issue appeared clearly since studies are normally carried out in heterologous cells, nevertheless, new techniques and approaches are being tested. To best address this issue we need to consider the evidence that these receptor-receptor interactions are specific versus non-specific and that they are functionally important versus physiologically irrelevant (Prinster et al., 2005). A number of new approaches has made it possible to obtain credible evidence of homomers of many types of GPCRs, such as complimentary chimeras, co-immunoprecipitation with differentially epitope-tagged receptors, the use of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), often in combination with covalent cross- linking, and biophysical methods, namely bioluminescence resonance energy transfer (BRET) and fluorescence resonance energy transfer (FRET), and finally, a novel in situ proximity ligation assays (PLA)(Agnati et al., 2003; Soderberg et al., 2006).

Concerning melatonin receptors, a study was conducted to investigate the possible interaction between MT_1 and MT_2 melatonin receptors. An experiment to investigate the

existence of constitutive homo and hetero-oligomerization among MT_1 and MT_2 was carried out in HEK 293 cells expressing physiological levels of both receptors using BRET approach among other techniques. The putative oligomerization of MTs receptors was assessed using co-oimmunoprecipitation assays, the results indicating that both MT_1 and MT_2 homo-oligomers can be formed. Also, western blot analysis of membrane derived from HEK 293 cells expressing tagged melatonin receptors revealed three groups of immunoreactive bands with a similar migration pattern, suggesting the presence of monomeric, dimeric, and higher oligomeric states for other GPCR (Ayoub et al., 2002).

Investigation showed that MT_1 and MT_2 are predominantly expressed as dimers in living cells, such results are consistent with the assumption that they represent functional signaling units. Thus, although receptor dimerization is not modulated by ligand binding, dimers may still be necessary for biological function (Ayoub et al., 2002).

Melatonin receptors, as other GPCR receptors, can interact with different types of receptors. Recent studies showed that both MT_1 and MT_2 receptors and serotonin receptor 5-HT_{2C} form heteromers. Within both heteromers, melatonin showed the ability to activate different cellular cascades, the former Gi/cAMP in the case of MT_2 homomer, as well as Gq/PLC pathway by transactivation of the 5-HT_{2C} promoter. This transactivation was unidirectional and it was not observed for the MT_2 protomer upon 5-HT stimulation.
Whereas melatonin activates both pathways, other ligands have a more restricted profile using either the direct activation or transactivation mode. Interestingly, the clinically active antidepressant agomelatine shows functional properties on MT_2/5-HT_{2C} heteromers that are biased toward the Gi /cAMP pathway and thus distinct from those of melatonin- and 5-HT_{2C}-specific antagonists (Kamal et al., 2015).

Heteromerization behavior of GPCR class of receptors, in this case melatonin receptors, could be an important therapeutic target for several pathologies, specially because of melatonin's diverse functions in several body organs. Many questions and observations are still to be addressed on this regard.

Signalling pathways

A well established classical intracellular pathway for MT_1 and MT_2 receptors has been canonically accepted . In the classical way, MT_1 and MT_2 stimulation would lead to adenylate cyclase (AC) inactivation in a Gαi subunit process of the G protein coupled to the membrane receptor (von Gall et al., 2000). When AC is inactivated, it would lead to a decrease in intracellular cAMP concentration, and finally a decrease in activated protein kinase A (PKA). The action of these receptors has been shown to inhibit forskolin-induced cAMP formation (Vanecek, 1998).

However, depending on the tissue, organ or species studied, the MT_1 and MT_2 receptors are capable of activating very different signaling cascades. In the HEK293 cell line the MT_1 receptor has been implicated in the activation of the phospholipase C-α (PLC-α) (Brydon et al., 1999); While MT_2 receptor, in addition to classical inhibition of AC, has been described in the inhibition processes of the enzyme guanylate cyclase (GC) (Petit et al., 1998).

The plasticity in signal transduction demonstrated by melatonin receptors is at least partially related to their ability to associate with different types of G proteins. It is known that the cloned melatonin receptors are capable of binding G_i proteins to subtypes of 1 -3, G_z and possibly G_s (Tslm et al., 1996). In fact, G_s activation has recently been confirmed for the MT_1 receptor in human prostate epithelial cells. Signal transduction of receptor-

mediated antiproliferative action of melatonin on human prostate epithelial cells involves dual activation of Gα(s) and Gα(q) proteins (Shiu et al., 2010). Similarly, MT_1 and MT_2, have demonstrated the ability to associate to G_{16} protein in transfected COS-7 cells (Lai et al., 2002; Mody et al., 2000). Activation of phospholipase C (PLC) by melatonin stimulation also suggested a possible association of these receptors with G_q, G_{11} or G_{14} protein (New et al., 2003). Activation of G_q has been demonstrated for the MT_1 receptor in gastrointestinal smooth muscle, associated with PLC activation and increased intracellular Ca^{2+} and phosphatidylinositol (PI) hydrolysis (Ahmed et al., 2013). Similarly the MT_2 receptor is able to activate PKC_ζ in a process associated with the activation of G_q in mesenchymal cells (Lee et al., 2014).

Signalling pathway through adenylate cyclase

Adenylate cyclase is an enzyme, belonging to the group of lyases, which catalyzes, from ATP, the synthesis of cAMP. It acts as a second messenger in multiple intracellular pathways, the most studied being the activation of PKA.

Ten different isoenzymes, called type 1 to 10, have been described, differing mainly in their sensitivity to the different types of G_α, $G_{\beta\gamma}$ and CaM subunits (New et al., 2003).

Mediation of pertussis toxin-sensitive G protein, and its action on the accumulation of intracellular cAMP, induced by melatonin receptors, was already described by Carlson et al in 1989 (Carlson et al., 1989). Inactivation of this pathway by pertussis toxin clearly indicated the involvement of G_i protein as a mediator in transduction. In 1995, the coupling of Mel1c to G_i protein, and the direct action of the $G_{\alpha i}$ subunit on adenylate cyclase and the consequent decrease in cAMP (Yung et al., 1995) was demonstrated.

However, this is not the only way in which melatonin receptors modulate AC action. This is due to the large number of different isozymes and the ability of melatonin receptors to be associated with different types of G protein. The $G_{\alpha i}$ and $G_{\alpha z}$ subunits are able to inhibit type 1 AC to 6, whereas G_o has not been shown to inhibit ACs types 2,4,5 and 6 (Lee et al., 2014). The $G_{\beta\gamma}$ subunits are capable of inhibiting type 1 AC; However, they act as activators of type-2 and 6-type ACs when they have been pre-activated by Gαs or phosphorylated by PKC. This phenomenon has been observed in the model of renal embryonic cells transfected with Mel1c receptor and overexpression of type 2 AC. In this model melatonin was able to increase the accumulation of intracellular cAMP (Yung et al., 1995) through a melatonin receptor coupled to G_i and G_z, via Gβγ subunit.

This model could explain apparent contradictions such as the potentiation that produces melatonin in the accumulation of cAMP-induced vasoactive intestinal peptide in human leukocytes (Lopez-Gonzalez et al., 1992). Independently of the aforementioned mechanisms, CaM is capable of inhibiting AC activation type 5 and 6 (Taussig and Gilman, 1995). Thus, melatonin by direct action on CaM, preventing the binding of Ca^{2+} to calmodulin, would be able to deactivate the inhibition on these two types of AC, and thus increase the intracellular concentrations of cAMP. In contrast, type 1 and 2 CAs are activated by CaM (Taussig and Gilman, 1995); So that inhibition of it, would result in a decrease in cAMP levels (Fig. 1.11).

Another possible pathway for the regulation of CA involves its activation by phosphorylation mediated by PKC. Type 1-8 CAs present this type of regulation (Taussig and Gilman, 1995). As previously discussed, melatonin receptors may activate this signaling pathway by different mechanisms. Activation of PKC and phosphorylation of

type 1 AC to 8 would therefore promote increased intracellular cAMP concentrations (New et al., 2003).

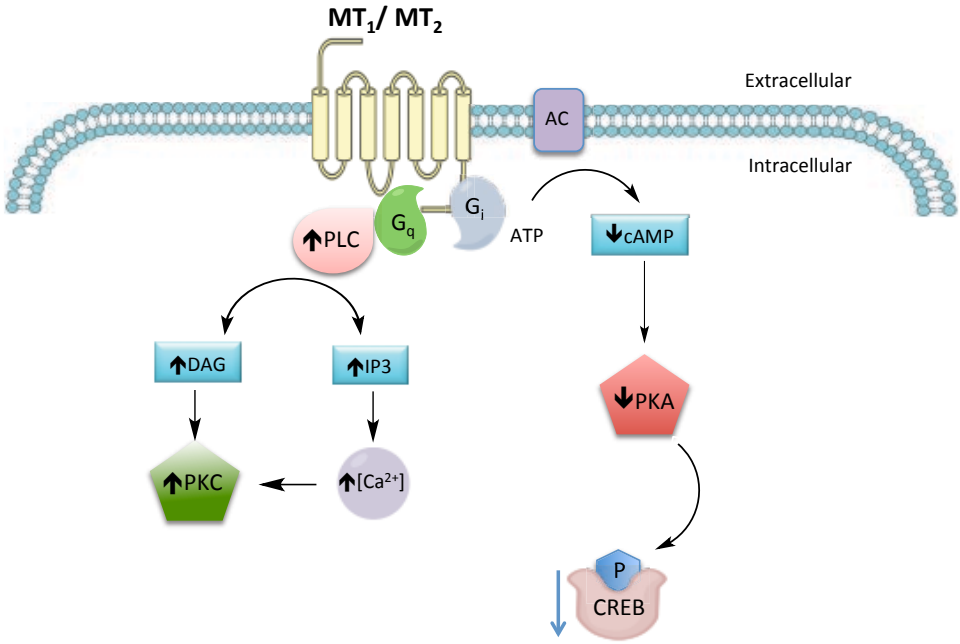

Figure 1.11. Scheme showing membrane signalling by melatonin receptors MT_1 and MT_2, both G-protein coupled receptors. On the right is shown the interaction of G_i with adenyl cyclase (AC) to decrease cAMP levels and therefore cyclic AMP-dependent protein kinase activity (PKA). On the left is shown the interaction of G_q with phospholipase C (PLC) leading to the cleavage of phosphatidyl inositol diphosphate (PIP2) into inositol triphosphate (IP3) and diacylglycerol (DAG). These second messengers stimulate increased intracellular Ca2+ and protein kinase C (PKC), respectively. The downstream effects of these events at the membrane vary with cell type.

Therefore, the final response triggered by melatonin and its receptors, will depend on the different combinations between G proteins, activated signalling pathway and the majority presence of the different isoenzymes belonging to that pathway present in each cell type.

This variety in the transduction of different intracellular signals makes it difficult to study the melatonin receptors, even more so, when the same receptors besides regulating different second messengers can regulate the same transduction pathway positively or

negatively, according to the conditions environmental or cell type studied (New et al., 2003).

PLC / PKC signaling pathway

PLCβ is an enzyme involved in the metabolism of phosphatidylinositols (PI) and intracellular signaling measured by Ca^{2+}. Activation of this enzyme has been associated with different G protein subunits: $G_{\alpha q}$, $G_{\alpha 11}$, $G_{\alpha 14}$, $G_{\alpha 15}$, $G_{\alpha 16}$, and $G_{\beta\gamma}$ subunits (Rebecchi and Pentyala, 2000). There is extensive literature demonstrating the activation of this pathway mediated by melatonin, in various cell models (Benitez-King et al., 2001; Eison and Mullins, 1993; Mullins et al., 1997; Popova and Dubocovich, 1995). As we have already mentioned, recent work in this field has shown that the interaction between melatonin receptors and PLC is mediated by the G_q protein (Ahmed et al., 2013). This pathway of activation binds the already reported mediated by G_{16}. However, these are not the only mechanisms that enable PLC / PKC activation. Prior to the discovery of the association of melatonin receptors with G_q protein, an alternative mechanism mediated by $G_{\beta\gamma}$ subunits was postulated (New et al., 2003). There are multiple examples of activation of this pathway mediated by $G_{\beta\gamma}$ subunits of G_i protein-associated receptors. In fact, there are systems in which the activation of the PLC/PKC pathway by melatonin receptors is abolished by the action of the pertussis toxin, which directly implies the intermediation of a G_i protein (MacKenzie et al., 2002; McArthur et al., 1997). In the same way, an effect described in the literature is the potentiation of activation of the PLC, pre-activated by G_q, mediated by G_i protein (Chan et al., 2000). This effect has been observed in HEK-293 cells in which a potentiation of PLC activation has been observed when MT_1 receptors and P2Y receptors coupled to G_q protein are stimulated simultaneously (Roka et al., 1999).

Transient Receptor Potential Ion Channels (TRP)

TRPs are a super-family of non-selective cation channels which play critical roles in the responses to various environmental changes and stimuli. TRP channels derive their name from a Drosophila *trp* mutant that was unable to respond to repeated or constant bright light stimulation (Cosens and Manning, 1969). Twenty years later, molecular cloning and functional analysis led to the discovery that the defect lies in a gene encoding for a cation channel known as TRP in Drosophila (Montell and Rubin, 1989). Up to date, 28 mammalian TRP channels are known, divided into 6 subfamilies: canonical (TRPC), vanilloid (TRPV), melastatin (TRPM), mucolipin (TRPML), polycystic (TRPP) and ankyrin (TRPA).

TRP channels are tetrameric proteins with subunits having six transmembrane domains, with varying sizes of cytoplasmic C- and N- terminal tails. Depending on the family branch, the cytoplasmic amino-terminal domain contains different numbers of ankyrin repeats, ranging from zero to twenty nine, which have been seen to be involved in a range of interactions (Gaudet, 2008). The majority of functionally characterized TRP channels are permeable to sodium, chloride, potassium and calcium, but each of them has unique characteristics for ion permeability, conductance, voltage dependence and gating (Ramsey et al., 2006).

Many TRPs are sensitive to physical stimuli like temperature, pH, shear stress and other mechanical forces such as stretch and hypoosmolarity. Furthermore, many TRPs are ligand gated and voltage sensitive. For example, in high temperatures greater than 42^0 C and caspaisin (the spicy component of chilli peper), the channel TRPV1 is opened.

Meanwhile, TRPM8 is opened by low temperatures, below 23^0 C, and by cooling agent like menthol and icillin (Caterina et al., 1997; Peier et al., 2002a).

TRP channels are widely distributed through the body, expressed in a vast number of different cell types. They are specially abundant in sensory receptor cells, and play a critical role in vision, hearing, taste, pain and temperature sensation.

TRP channels mediate the transmembrane flux of cations down their electrochemical gradients, thereby raising intracellular Ca^{2+} and Na^{2+} concentrations and depolarizing the cell. Transmembrane voltage changes underlie neuronal action potential propagation and muscle contraction, and direct the driving force for calcium entry through plasma membrane channels in non-excitable cells, as well as controlling the gating of voltage-dependent Ca^{2+}, K^+, and Cl^- channels. TRPs are well positioned to regulate $[Ca^{2+}]_i$, $[Na^{2+}]_i$, and V_m in both excitable and non-excitable cells (Ramsey et al., 2006).

TRPV4

The TRPV4 cation channel (GenBank accession number NM_022017) was first described in 2000, by cloning cDNAs with homology to the conserved coding regions of mammalian TRPV1 and TRPV2 and Osm-9, the osmosensor from *C. elegans* (Liedtke, 2007) and was identified as the possible sensor for osmolarity in the kidney, liver and heart (Strotmann et al., 2003).

TRPV4 gene is located in the long (q) arm of chromosome 12 at position 24.1, being located from the base pair 109,783,086 to base pair 109,833,406 on chromosome 12 as commented. The TRPV4 protein is known to compose 871 amino acid residue and normally assembled as a homotetramer. Each TRPV4 monomer has six transmembrane helices (S1-S6) stretching across the lipid bilayer, together with the re-entering loop

known as the pore loop, found between S5 and S6. The transmembrane region is found to be similar to that of TRPV1. NH_2 and COOH termini are localized in the cytoplasm (Fig. 1.12) (Stewart et al., 2010). Heterotetramers have been also reported with TRPC1 and TRPP2, as well as heteromeric TRPV4-TRPC1-TRPP2" complexes (Du et al., 2014).

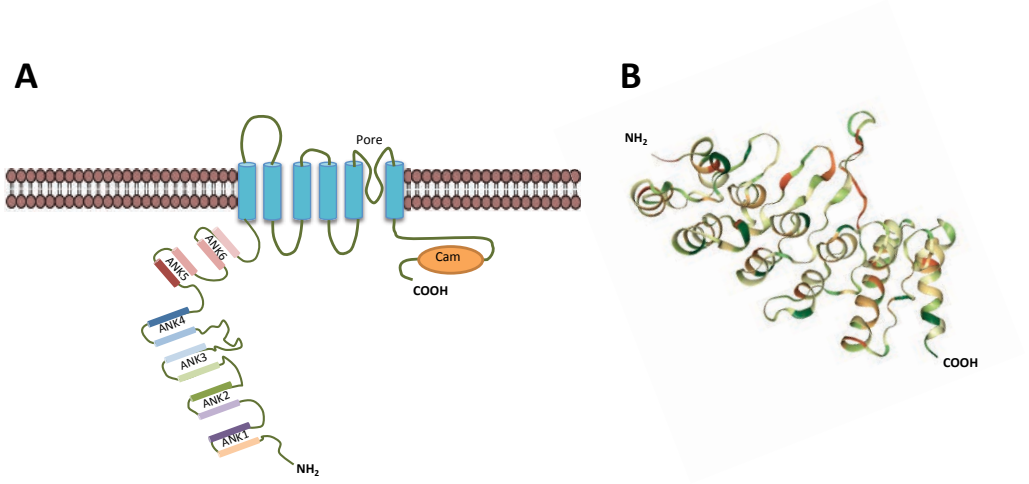

Figure 1.12. The TRPV4 protein is composed of a cytosolic N-terminal region and six transmembrane domains, including the pore region and an intracellular C-terminal tail. The N-terminal region contains the ankyrin repeat domain, which consists of six ankyrin repeats

TRPV4 is a polymodal channel which can be activated either by sensitization or opening by intracellular signalling. Besides its former exclusively known function as osmoregulator,it is also sensitive to moderate heating ranging between 25–34 °C (Guler et al., 2002; Watanabe et al., 2002). Studies in mice knock out ($TRPV4^{-/-}$) exhibited delayed tail withdrawal to moderately hot temperatures and a strong preference for warmer floor temperatures, whereas wild type mice did not discriminate between 30 and 34 °C (Lee et al., 2006).

This channel is also sensitive to changes in the pH. Reports have revealed the TRPV4 channel opening by low pH or citrate. Mechanical stress has also been described, both in vivo and in a cellular level, by extracellular osmotic pressure. Moreover, TRPV4 is capable of being gated in response to inflammation, since it increases interstitial fluid

volume and tonicity, which create abnormal mechanical pressure on the plasma membranes of the cells (Alenmyr et al., 2014).

Mechanistically speaking, the TRPV4 is a Ca^{2+} permeable non-selective cation channel that exhibits both inward and outward transition. Both spontaneous and agonist-induced TRPV4 currents are substantially diminished without the presence of extracellular Ca^{2+}. The rate and extent of channel activation is increased by Ca^{2+} entry during agonist-induced TRPV4 activation. Ca^{2+} selectivity of TRPV4 pore was found to be influenced by two aspartate residues, Asp672 and Asp682. When both aspartates are neutralized, Ca^{2+} permeability decreases to a much higher extent than the reduction in channel rectification. While ,when either aspartate is neutralized to alanine, there is a small decrease both in the relative permeability for divalent cations and in the extent of outward rectification (Voets et al., 2002).

The modulation of the TRPV4 channel can occur by different mechanisms. For instance, studies suggest that the COOH terminus CaM-binding domain is a pre-requisite for Ca^{2+} induced potentiation of TRPV4 in certain systems. Nevertheless, NH_2 terminal ARDs of TRPV4 possess CaM-binding site as well and it may function as Ca^{2+} induced modulator for TRPV4 in other systems (Strotmann et al., 2003; Strotmann et al., 2010).

Phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$), a minor phospholipid component of cell membranes, is important for TRPV4 proper functioning. $PI(4,5)P_2$ binds and rearranges the cytosolic tails of TRPV4 to enable hypotonic and heat stimuli to activate the channel (Garcia-Elias et al., 2013).

The ankyrin repeat domain found in TRPV1,TRPV3 and TRPV4 possesses a ligand binding site for ATP and calmodulin among other ligands. ATP and CaM function

through this multiligand site to mold the varying sensitivity and adaptation profiles of these several ion channels. Both function interactively to determine receptor sensitivity to Ca^{2+} concentration changes (Phelps et al., 2010).

High viscous loading and hypotonicity stimuli activates TRPV4 through phospholipase A_2(PLA_2) activation and the subsequent production of the arachidonic acid metabolites, epoxyeicosatrienoic acid (EET). Under conditions of low PLA_2 activation, both activators also use extracellular ATP-mediated activation of phospholipase C (PLC)-inositol trisphosphate (IP(3)) signaling to support TRPV4 gating (Fernandes et al., 2008). The IP_3 receptor engages in a physical interaction with TRPV4 sensitizing this channel to stimulation by mechanical and osmotic stimuli although IP_3 alone does not activate TRPV4. Threfore, binding of inositol 1,4,5-triphosphate receptor type 3 (IP_3R3) to TRPV4 is essential for IP_3 effected sensitization of TRPV4 (Garcia-Elias et al., 2014).

TRPV4 can also become sensitized through both PKC and PKA pathways. Treatment with phorpol 12-myristate 13-acetate (PMA), TRPV4 is phosphorylated on Ser824 activating PKC. Ser824 is also phosphorylated after forskolin activates PKA pathway. phorphorylation in this site by the insertion of aspartic acid in lieu of Ser824 increases Ca^{2+} influx in both resting and stimulated cells (Peng et al., 2010).

TRPV4 in the eye

TRPV4 expression is found in several ocular structures and cell types. Functional TRPV4 expression is found in neurons of the trigeminal ganglion nerve ending in the inner walls of the anterior chamber of the rat (Meng et al., 2015a) , as well as in retinal ganglion cells, in Muller glial cells, at the optic nerve head in the mouse (Ryskamp et al., 2011) and the retinal pigmented epithelium of human fetal cultured cells (Zhao PY,2015). TRPV4 is

also found in human corneal endothelial cells and at the cell membrane surface and the cytosol of human epithelial corneal cells (Mergler et al., 2011a; Pan et al., 2008). Moreover, it is present in the trabecular meshwork and the epithelium of porcine lens (Luo et al., 2014; Shahidullah and Delamere, 2014).

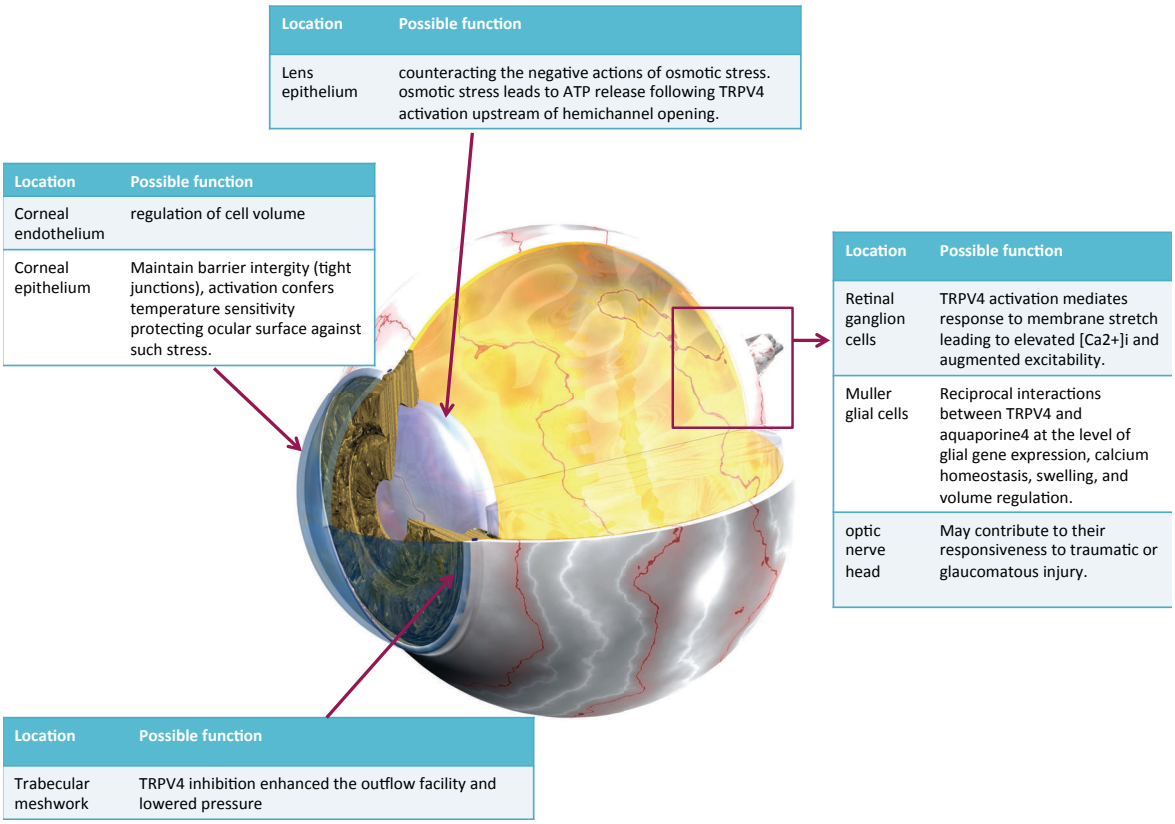

Figure 1.13. Representative scheme of the distribution of TRPV4 in the eyeball and its importance in each structure.

Studies have been made in the search for mechanosensitive channels in the trigeminal ganglion nerve ending in the inner walls of rat anterior chamber. The study took place in order to better understand the modulation of intraocular pressure (IOP). Given that IOP is regulated, in part, through a neuronal feedback mechanism, the involvement of the trigeminal nerve as the primary afferent nerve in neural regulation of IOP seemed to be plausible (Lele and Grimes, 1960; Perkins, 1957; Zuazo et al., 1986). Using the whole cell patch-clamp technique, together with neuronal labelling, TRPV4 determined the activity

and localized its presence by immunofluorescence (Meng et al., 2015b). Investigation must continue for better understanding of the role of TRPV4 in the trigeminal ganglion nerve ending in regulating IOP.

Based on the idea of better understanding the regulation of IOP from different perspectives, anatomical, molecular and physiological evidences of TRPV4 participation in the transduction of hypotonic stimuli and its contribution to Ca^{2+} dependent intracellular signalling and membrane excitability have been shown in the mouse retina. Localization of TRPV4 was determined in retinal ganglion cells (RGC) somata and axons. Cell stretch induced by hypotonic swelling caused a marked increase in [Ca2+]i of RGCs. Because the remaining membrane potential of RGCs is within the operational range for inactivating and persistent Na^{+} conductances (~ −60 mV) (Hayashida and Ishida, 2004; Kim and Rieke, 2003; Margolis et al., 2010), TRPV4 agonists, 4α-PDD and GSK1016790A elevated intracellular calcium concentration and cause a >100-fold increase in the frequency of spontaneous RGC spiking. Furthermore, these agonists showed similar characteristics to those observed in hypotonicity conditions inducing $[Ca^{2+}]_i$ increases in RGCs and initiating cell death pathways in RGCs. These results seem to suggest that retinal output neurones are capable of transducing mechanical, thermal, and/or osmotic stimuli (Ryskamp et al., 2016; Ryskamp et al., 2011).

TRPV4 has been described in endothelial and corneal epithelial cells (Fian et al., 2007; Hartmannsgruber et al., 2007; Kohler and Hoyer, 2007; Pan et al., 2008; Yao and Garland, 2005). Thermo- and osmo-sensitive TRPV4 channel activity was characterized in the corneal endothelial cells. First by detection of mRNA expression of this channel, and then by several functional assays as planer patch-clamp recordings and fluorimetric measurements along with its response to described TRPV4 chemical agonists such as

GSK1016790A, which resulted in a significant increase of intracellular Ca^{2+} concentration, which diminished after the application of Ruthenium-red (TRPV antagonist). Also, the determination of the increment of Ca^{2+} influx via TRPV4 in endothelial cells was studied by using different hypotonic challenges, resulting in a significantly increased fluorescence ratio corresponding to intracellular Ca^{2+} inflow. These findings are of great relevance because corneal endothelial cells can suffer several processes in which TRPV4 can be activated in vivo, for example, membrane strech resulting for an elevation of IOP, cytokine-induced depletion of intracellular Ca^{2+} stores resulting in the release of calcium influx mediators which activates TRPV4, or an increase of IOP sufficient enough to induce endothelial injury and anandamide release, which sensitizes activation of TRPV4 (Mergler et al., 2011b).

Corneal epithelial integrity is required to maintain tissue transparency and deturgescence (the mechanism in which the corneal stroma remains relatively dehydrated). This outermost layer, facing the tear film, provides a barrier function to protect the cornea from noxious insults. Fluctuations in tear film osmolarity can occur in daily living and challenge corneal epithelial barrier function by inducing acute epithelial cell volume changes. Nevertheless, under physiological conditions, corneal epithelial cells withstand such stresses by mounting regulatory volume (RV) responses (Capo-Aponte et al., 2005). During exposure to a hypertonic challenge, these cells initially shrink, which is rapidly compensated by activation of a RV increase (RVI) response. Ca^{2+} signaling is critical to induce regulatory volume decrease (RVD) during exposure to a hypotonic challenges in the cornea. TRPV4 expression was detected in human corneal epithelial cells as well as in intact human cornea, the expression proved to be functional since either TRPV4 agonist, 4α-PDD, or exposure to a hypotonic medium mediated Ca^{2+} transients, suggesting the

importance of TRPV4 in maintaining corneal epithelial cells barrier integrity during the exposure to hypotonic challenges (Mergler et al., 2011b; Pan et al., 2008).

Another ocular tissue with an important role in maintaining the intraaocular pressure balance through the aqueous humor outflow is the trabecular meshwork (TM), which also, expresses TRPV4 channel. Studies indicated that elevated IOP mechanically strains the trabecular meshwork by stretching extracellular matrix (ECM) proteins, resulting in Ca^{2+} homeostasis perturbation and restructuring the architecture of the ECM/ cytoskeleton . In biomimetic TM scaffolds using human TM cells, TRPV4 agonists evoked TRPV4 dependent cation/Ca^{2+} influx producing thickening of F-actin stress fibers and reinforcing focal adhesion contacts. On the contrary, TRPV4 inhibition enhanced the outflow facility and lowered perfusate pressure. In vivo assays using a glaucomatous mice model, intraocular injection and topical application of TRPV4 antagonists lowered IOP, altogether indicating the critical role of TRPV4 channel as mechanosensitive and Ca^{2+} signalling machinery within the TM (Ryskamp et al., 2016).

TRPV4 is also expressed in the porcine lens epithelium and functions counteracting the negative actions of osmotic stress. Studies suggested that TRPV4 is activated when the lens goes through osmotic or damage-induced swelling, this permits Ca^{2+} entry and triggers a chain of events that open hemichannels (formed from connexin proteins) which allows ATP release and subsequently activates purinergic receptors in the lens epithelium and certain Src family tyrosine kinases (SFKs). This stimulates Na, K-ATPase activity which allows the lens to improve its regulation of ion concentrations in the fiber mass, this effect being blocked by TRPV4 antagonists proving the involvement of this channel in ATP release (Mandal et al., 2015; Shahidullah et al., 2015).

In view of all the studies and mentioned observations, TRPV4 can be legitimately considered a multi-micromachine, capable of sensing diverse stimulti and converting them to Ca^{2+} signals in the various tissues.

Melanopsin: A novel photopigment for ancient functions

Many years ago, a scientist, named Clyde Keeler, observed light response in mice with significant rod and cone dystrophy who were still able to generate a number of visual reflexes, including constriction of their pupils in response to light and photoentrainment (Keeler, 1928; Keeler et al., 1928) . These processes disappeared, however, when animals have their eyes removed, strongly supporting Keeler's prediction of a novel ocular photoreceptor (Klein and Weller, 1970). In 1998, Provencio and colleagues identified melanopsin in photosensitive dermal melanophores of *Xenopus laevis*, which was responsible for the redistribution of skin pigment in direct response to light. Finally, they identified human melanopsin exclusively present in the inner retina which was different from the classically known photoreceptors (rods and cones) (Provencio et al., 2000; Rollag et al., 2003).

In the mammalian eye, the image forming pathway uses spatial light dependent information to transmit information from rod and cone receptors through an intermediate retinal network of bipolar, amacrine, and horizontal cells which convey light responses through conventional retinal ganglion cells (RGCs) to the lateral geniculate nucleus (LGN) which contributes to conscious vision. The non image-forming pathway, is an ancestral pathway that enabled species to sense light levels as a key for organism response and survival, classically thought to inform unconscious vision that photoentrains the circadian cycle. Melanopsin was shown to be an active photopigment expressed in a small subclass of RGCs which are intrinsically photosensitive ganglion cells (ipRGC) projecting to the suprachiasmatic nucleus (SCN) of the brain (Czeisler, 1995). Nevertheless, melanopsin containing RGCs project directly onto the olivary pretectal nucleus (OPN) as well. This

may explain the persistence of pupillary light reflex in rodless and coneless mice (Lucas et al., 2003) since the OPN is a crucial node in the pupil reflex circuit linking the retina to the parasympathetic innervation of the iris. More recent studies proved that ipRGCs also project onto the visual centres of the lateral geniculate nucleus (LGN), therefore, the ability of rodless, coneless mice to perform pattern discrimination suggested that ipRGCs contribute to image-forming vision (Ecker et al., 2010; Hicks, 2011). The importance in contrast detection was also demonstrated when mice lacking melanopsin showed decreased contrast sensitivity (Schmidt et al., 2014).

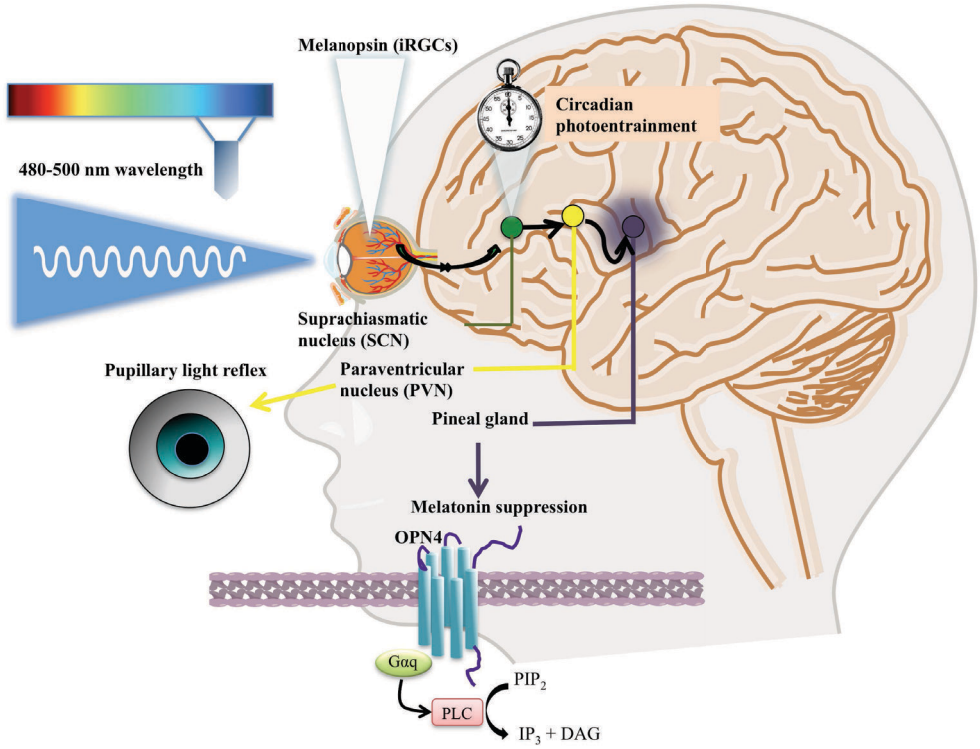

Figure 1.14. Scheme of melanopsin pathway through retinal iRGCs to the suprachiasmatic nucleus for photoentrainment, reaching the paraventricular nucleus where it participate in pupillary light reflex. Finally signals reachs the pineal gland where melatonin synthesis is inhibited/activated by the intracellular pathway shown at the bottom of the photo.

The human melanopsin gene (OPN4) consists of 10 exons and is mapped to chromosome 10q22. The location and structure of the melanopsin gene is totally different

from the rest of human opsins, which typically have four to seven exons. Melanopsin is a 534-amino acid membrane integral protein with 7 trans-membrane domains. The amino-terminus end is found at the extracellular region, while the carboxy-terminus is in the cytoplasm. It has 3 extracellular loops, with the latter two loops containing cysteine residues (Cys-100 and Cys-178) which stabilize the tertiary structure by means of disulfur bonds. Melanopsin protein also has 3 loops in the cytoplasm where different amino acids are candidates as intracellular phosphorylation sites. Melanopsin has an insertion (Asn-225 to Gly-233) in the third cytoplasmic loop. This third cytoplasmic loop determines the family of G protein that is activated by GPCRs. At the seventh trans-membrane domain, interaction occurs with the chromophore retinaldehyde. The most striking feature that distinguishes melanopsin from different human opsin is the greater homology to invertebrate opsins than those of vertebrates. The typical acidic Schiff´s base ,which is typical in vertebrates, is alternated by an aromatic residue (Tyr-103) that is typical in the invertebrate opsins.

Melanopsin phototransduction

Visual phototransduction is the mechanism by means of which photons are absorbed by retinal photoreceptors to transduce them into a nerve impulse that the brain is able to interpret. Most animals have photoreceptors of one sort or another to detect food source, mate, predator, orientation, or simply the light/dark cycle dictated by the sun. Photoreceptors can be of two distinct morphologies: ciliary and rhabdomeric, both cell types enlarge the membraneous surface to storage photopigment. In particular rhabdomeric photoreceptors do so by folding the apical cell surface, while the ciliary cells fold the ciliary membrane (Eakin, 1982; Yau and Hardie, 2009). Physiologically, the ciliary rods

and cones hyperpolarize to light, whereas rhabdomeric photoreceptors depolarize to light. However, light response polarity is not an absolute distinguishing feature between both types of photoreceptors. Uniformly, these photoreceptors sense light using a visual pigment composed of vitamin A-based chromophore and a seven-transmembrane-helix apoprotein, opsin. These pigments are prototypical G protein-coupled receptors (GPCRs). Studies involving electrophysiological and pharmacological approaches with ipRGCs strongly suggest that the biochemical cascade of phototransduction pathway is similar to that of the invertebrate rhabdomeric photoreceptors acting through Gq protein coupled opsin (Contin et al., 2010; Qiu and Goz, 2010; Qiu et al., 2005).

Melanopsin shows a maximal absorbance at 460-480 nm, which corresponds to blue light. After light stimulation, the photopigment of ipRGC triggers signalling, presumably, through Gq/11-class G-superfamily, which in turn activates a phospholipase C (PLC), provoking the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) in the membrane generating inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG) and the later increase in cytoplasmic Ca^{2+} and ultimately causing membrane depolarization. Furthermore, the participation of TRP and TRPL channels, the Ca^{2+}-permeable light-sensitive channels, made clear since treatments with a Ca^{2+} chelator or a TRP channel blocker were able to reduce the light effect. Treating chicken primary RGCs cultures with PLC inhibitors abolished the light-suppressive effect on 3H-melatonin synthesis. These findings demonstrated the chemical components of the phototransduction cascade operating in vertebrate embryonic RGCs is involving a Gq-protein (Contin et al., 2010; Graham and Wong, 1995; Graham et al., 2008; Isoldi et al., 2005; Koyanagi et al., 2005; Koyanagi et al., 2015; Valdez et al., 2009)

Melanopsin life cycle: a promising potential

From an evolutionary point of view, the melanopsin system is older than the rod and cone systems. Given its ancient origin, plus its key role in light dependent survival, it is worthwhile mentioning that melanopsin is expressed pre-natally and that ipRGCs are the first developing photosensitive cells in the mammalian retina (Sekaran et al., 2005). Moreover, studies done in mice proved that they are capable of light detection embryonically (Rao et al., 2013).

At the same time ipRGCs are the first photoreceptors in development, they have a longer period of proliferation and may be some of the last retinal neurones to die in the course of an organism's lifetime. They are considered atypical central nervous system neurons, acting both as photoreceptors responding directly to environmental stimuli, as well as standard neurons integrating synaptic input and generating action potentials (Pickard et al., 2009; Pickard and Sollars, 2012; Rollag et al., 2003). Many studies have raised awareness towards these cells being resistant or less vulnerable to damage and disease compared to conventional RGCs. For instance, one of the leading causes of vision loss is glaucoma, which is associated to elevated intraocular pressure, resulting in optic nerve damage and RGCs loss. Studies in some rodent glaucoma model examined the sparing of ipRGCs, suggesting that IOP threshold to damage these cells is much higher than it is in conventional RGCs (Zhang et al., 2013). Different studies indicate melanopsin resistance to damage in optic nerve damage in inherited optic neuropathy, as well as studies indicating that these cells may be resistant to glutamate-induced excitotoxicity (DeParis et al., 2012; Fujishiro et al., 2014; Moura et al., 2013; Muller et al., 2010). Little is known about the cellular and molecular mechanisms that provide neuroprotection to

these RGCs, and more studies are needed for a better understanding of these mechanisms, which may lead to valuable insights for designing strategies to diminish vision loss following ocular diseases.

Hypothesis and Objectives

Hypothesis:

Melatonin have been a molecule of interest in our group of investigation for the last decade, with a special attention towards this molecules' potential to lower intraocular pressure. Such a molecule presence was already described in several ocular structures including the ciliary body, where the aqueous humor is formed. However, the composition of the aqueous humor is also due to its contact with other structures like the cornea and the crystalline lens. The focus has been over the effect of exogenous melatonin application on intraocular pressure, and results showed that melatonin administration have the ability to decrease IOP in heathy and glaucomatous models. Results are supported by different groups and with different experimental models, including humans.

Questions raised regarding the endogenous status of melatonin synthesis in the healthy and diseased subjects. The mechanisms regulating the changes in melatonin synthesis in the aqueous humor, independently of the circadian rhythm, is a matter of interest since it can be related to the changes in IOP this being a main risk factor for developing glaucoma disease.

Our hypothesis is that melatonin levels in the aqueous humor of normal and hypertensive patients are different. These possible variations will rely on the presence of a "sensor" that may connect intraocular pressure with the synthesis of melatonin, probably by modifying the activity of those enzymes that synthesize melatonin, such as the key enzyme AANAT. Moreover, we consider that those possible changes in the aqueous humor melatonin levels may influence melatonin receptors present in the ciliary body. In this sense and since it has been observed unusual pharmacological behaviors (i.e. melatonin

effect can be blocked by the adrenergic antagonist prazosin), it might be the case that receptor-receptor interactions between melatonin and adrenergic receptors may occur.

Finally, we believe that melatonin detected in the aqueous humor is not only produced by the ciliary body but also by the crystalline lens. Our hypothesis is that the crystalline lens contains a light sensor which regulates melatonin synthesis, and that melatonin coming from the crystalline is an important participant in the physiology of the aqueous humor.

Objectives:

1. **To study the correlation between the increase in IOP and the increase in the levels of melatonin in the aqueous humor:** Melatonin levels in the aqueous humor is of great importance because this molecule is known to lower IOP, however, a correlation between endogenous melatonin and IOP need to be addressed.

2. **To study the participation of the TRPV4 channel in regulating melatonin synthesis in the ciliary body:** TRPV4 channel is known as an osmo- mechano- sensor. The present Thesis investigates its role in the ciliary body in regulating melatonin synthesis and the possible impact on intraocular pressure.

3. **To describe the cellular signalling mechanisms of AANAT regulation:** The changes in the expression as well as the modulation by phosphorylation of the key enzyme in synthesizing melatonin (AANAT) will be studied in clear connection with the activation of a TRPV4, measuring the changes in the protein AANAT as well a the changes in melatonin synthesis.

4. **To investigate the possible receptor heteromerization between melatonin receptors and different receptors:** To study the interaction between melatonin receptors and mostly α_{1A}-adrenergic receptor and to investigate wether such an interaction is functional, as well as to interpret the meaning of such a phenomena regulating IOP by means of "in vivo" assays.
5. **To identify the presence of melanopsin in the crystalline lens and its role in regulating melatonin synthesis:** To investigate the response of human crystalline lens epithelial cells to light and darkness, and to describe melanopsin presence in the studied cells as well as corroborating its presence in human crystalline tissue. In addition to study the mechanisms underlying melatonin synthesis controlled by melanospin.

Chapter II: Melatonin, Intraocular pressure, and TRPV4: Clear dots to connect.

Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less.

Marie Curie

Introduction

Melatonin was classically considered to be exclusively produced by the pineal gland (Arendt, 2007). However, this indolamine is synthesized and present in many ocular structures, although its implications in ocular physiology is still under investigation (Adachi et al., 1998; Agorastos and Huber, 2011; Alarma-Estrany and Pintor, 2007) . For instance, discovering its presence in the aqueous humour (AH) and knowing the importance of the AH in regulating the intraocular pressure, attracted the attention of melatonin's role in the aqueous humour dynamics and formation (Clarke and Osborne, 1994). Melatonin levels are normally high during the night, while the intraocular pressure is decreased (Wurtman et al., 1963). Melatonin is, therefore, considered to regulate intraocular pressure (IOP) (al-Qassab et al., 1988; Belforte et al., 2010; Chiou and McLaughlin, 1984; Pintor et al., 2001). This connection between IOP and melatonin was demonstrated on human patients who were treated orally with melatonin before undergoing cataract surgery to lower their IOP (Ismail and Mowafi, 2009). Moreover, Melatonin and its analogues were suggested as a treatment for elevated IOP and glaucoma(Lundmark et al., 2007; Mediero et al., 2009). Recent studies on animal models of elevated IOP showed a significant reduction of IOP after melatonin treatment (Martinez-Aguila et al., 2013), as well as more recent studies on human glaucoma patients, supporting and potentiating the results obtained on animal models (Pescosolido et al., 2015). Melatonin mechanism of action to reduce IOP is mediated by its membrane receptors MT_1, MT_2 and MT_3 which are present in the ciliary body among different ocular structures (Alarma-Estrany and Pintor, 2007). Melatonin activates MT_3 receptor, which stimulates adenylate cyclase with concomitant activation of PKA. This activation produces

an inhibition of chloride efflux (Huete-Toral et al., 2015), the main factor responsible for the movement of water from the ciliary epithelium to the posterior chamber (Civan, 1998). Consequently, the reduction of chloride efflux causes a reduction in the formation of AH (Civan and Macknight, 2004).

Little is known either about the mechanism or the substances which may stimulate the endogenous melatonin's presence in the aqueous humour. A channel which is sensitive to physical and chemical stimuli could be a possible candidate. The TRPV4 is a membrane protein that belongs to the transient receptors, a non-selective cation channel permeable to Ca^{2+} (Garcia-Elias et al., 2014).

The connection between IOP and melatonin seems logical. However, insufficient studies have been made to measure melatonin levels in the aqueous humour to establish a relationship between both areas of a great interest. Some groups have obtained non-conclusive results, due to variability, indicating that melatonin concentrations can vary from pg/mL to the ng/mL range (Chiquet et al., 2006; Martin et al., 1992). In order to understand this natural phenomena, the present chapter is dedicated primarily to comparing melatonin levels in the aqueous humour of ocular normotensive patients and those of elevated IOP. This study is completed by measuring the same in a well established glaucoma animal model before and after developing an increment in IOP and a further comparison to normotensive mice. Finally, the TRPV4 channel activation emerges as a possible candidate, explaining the mechanism by which melatonin is produced from non-pigmented ciliary body epithelium cells. Therefore, this chapter ends with the demonstration of the presence of this channel and its ability to stimulate melatonin synthesis.

Materials and methods

“In vivo” Assays

Subject recruitment and aqueous humour collection

Sample collection was made possible by the cooperation of Dr. Juan Sanchez Naves, an ophthalmologist practicing in the Instituto Balear de Oftalmología, Palma de Mallorca, Spain. Aqueous humour samples were brought to our lab for analysis (see below). The study protocol was approved by the clinical research ethics committee of Instituto Balear de Oftalmología, Palma de Mallorca, Spain. Signed informed consent to the aqueous humour contribution was obtained from all patients in accordance with the Declaration of Helsinki.

37 aqueous humour samples of patients who underwent cataract surgery were collected. Samples were divided according IOP measurements considering IOP > 21 mm Hg as hypertensive patients (Tavares et al., 2006) and IOP below 21 mmHg were considered normotensive. A random selection of hypertensive patients (n=14) and normotensive (n=23) took place (Table.1).

Table 2.1. Demographic characteristic of patients participating in the study

Parameter	Total	**< 21 mmHG**	**> 21 mmHG**
Patients	37	23	14
Mean age (years)± SD	63.41± 13.12	62.13 ± 13.97	66.36 ± 10.81
Age range (years)	[24, 84]	[35, 82]	[35, 84]
Gender (male/female)	[21,16]	[15, 8]	[6, 8]
IOP presurgery (mmHg)	17.70 ± 3.80	15.48 ± 0.48	22.25 ± 0.32

Patients with a history of primary acute angle-closure attack, chronic angle-closure glaucoma, any treatment for glaucoma or active inflammatory ocular disease were

excluded from the experiment. Glaucoma was defined as high intraocular pressure associated with optic nerve glaucomatous characteristics, consistent with the visual field defect. The IOP measurements were taken before the surgery by means of a Perkins tonometer after the instillation of 1 drop of double anaesthetic Colircusí® which contains tetracaine 0.1% and oxybuprocaine 0.4% (Colircusí, Alcon Cusí SA, Barcelona). This value should be representative of at least three other previous visits with non-statistically different IOP values from those presented in the manuscript. Patients with variations among visits were also not considered in this study.

Samples were collected during the first step of the cataract surgery using anaesthetic drops (lidocaine 2%). Posterior to standard clear corneal microinsicion (mice 2.2 mm), aqueous humour was aspirated using a 30-gauge Rycrof cannula on a tuberculin syringe (1ml). Aqueous humour volumes ranged between 0.1 to 0.2 ml, and immediately transferred to an eppendorf and stored at -20ºC protected from light until the chromatographic analysis were performed (Castany et al., 2011).

Glaucomatous mice model

Aqueous humours were collected from female C57BL/6J (control, n=6) and DBA/2J (glaucomatous, n=6) mice obtained from the European distributor of Jackson Laboratories Mice (Charles Rivers Laboratories). All animal maintenance and experimental procedures followed Spanish and European guidelines for animal care in the laboratory and animal research (Guide for the Care and Use of Laboratory Animals) and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Mice were housed (1-4 mice per cage) in temperature and light-controlled rooms maintained

according to a 12-hour light/dark cycle; all animals were fed ad libitum. DBA/2J and C57BL/6J mice were studied at 3 and 12 months of age.

Aqueous humour collection was performed after anesthetizing the mice with an intraperitoneal (i.p.) injection of a mixture of ketamine (95 mg/kg, Imalgene 1000, Merial, Barcelona, Spain) and xylazine (5 mg/kg, Rompún), Bayer, S.A., Barcelona, Spain). Aqueous humour was processed in the same way as with the human samples (for more details, see HPLC). Just before aqueous humor collection, IOP was measured by means of a TonoVet® contact tonometer supplied by Tiolat Oy (Finland). The application of this tonometer does not require the use of any anaesthetic.

"In vitro" Assays

Cells

Non-pigmented ciliary epithelial cells (59HCE), a human immortalized cell line was kindly supplied by Dr. Coca-Prados (Yale University, USA). Cells were grown in high glucose Dulbecco's modified Eagle's medium (Gibco/Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO) and 0.05 mg/ml penicillin/streptomycin (Gibco/Invitrogen) at 37°C in humidified atmosphere 5% CO_2–95% air. After the culture reached the confluence, cells were detached with 0.25% trypsin and seeded into 6 well plates and/or to 4 well chamber slides respectively. All the experiments were performed using cells comprised numbers 10-15 passages to assure assays reproducibility.

Immunocytochemistry Studies

Staining was performed to evaluate location of the TRPV4 in the cells, cells were washed with PBS1X three times for 5 minutes each before permeabilization. Cell membranes were permeabilized with a solution of Triton X-1000- 0.05% - PBS for 30 minutes, followed by blocking. In order to remove the joints, the sections were incubated in a PBS 1X-Triton X-100 0.1% solution, and normal serum from the animal from which the secondary antibody comes, in this case 10% normal donkey serum (NDS, Jackson Immunoresearch, West Grove, PA) was used and sections were incubated for 1 hour at room temperature. Sections were incubated in 0.1% Triton X-100 and 1% normal donkey serum together with the primary antibody TRPV4 primary antibody (Santa Cruz Biotechnology) with a dilution of 1:50 overnight at 4˚C.

The following day, in order to remove the excess of the primary antibody, three washes with PBS1X-Triton X-100-0.1% were performed for 5 minutes each. Followed by the secondary antibody; donkey anti-immunoglobulin IgG goat antibody conjugated with fluorescein isothiocyanate (FITC; green, Jackson ImmunoResearch, West Grove, PA, USA) at 1:100 dilution in PBS-0.1% Tx-100 for 1 hour in a dark chamber at a room temperature. Finally, sections were rinsed and mounted in Vectashield (Vector Laboratories, Palex Medical, Barcelona, Spain) and coverslipped. The samples were examined under a confocal microscope (Zeiss LSM 5, Jena, Germany).

TRPV4 Experiments

Cells were plated in multiwells at a density of $1.2x10^6$ cells and challenged with the TRPV4 agonist GSK1016790A (GSK) at the following concentrations: 1nM, 10nM,

100nM, 1µM, and 10µM. The cells were stimulated for 24 h and afterwards, the medium was collected and processed for melatonin analysis.

For antagonist analysis, the compounds ruthenium red (RR) and RN-1734 (RN) (Vincent et al., 2009), were assayed at concentrations of 1 nM and 10 nM respectively, either alone or 30 min before the application of the TRPV4 agonist GSK1016790A.

High performance liquid chromatography (HPLC) analysis

High performance liquid chromatography is a technique that allows the separation of the components of a mixture of substances depending on the different chemical interactions of the same by a mobile phase subjected to high pressure and a stationary phase (chromatographic column). The high-performance liquid chromatography (HPLC) equipment used for the determination of melatonin levels consisted of an isocratic pump, a Rheodyne ® manual injection valve (with an external loop of 10-250 µl) and a detector UV / VIS. The column employed was a Kromaphase C18 ion-pair reverse phase column (5 µm particle size, 25 cm in length, 4 mm internal diameter) (Scharlau, Madrid, Spain). a 1515 Isocratic HPLC pump, a 2487 dual absorbance detector, and a Reodyne injector, all managed by the software Breeze from Waters (Milford, MA). The system was equilibrated overnight with 40% methanol, 60% H_2O. Measurements were performed at a flow rate of 0.8 ml/min fixing the detector at a wavelength of 244 nm.

After the experiments, the collected aqueous humour and mediums were maintained in ice for 5 min. The tubes were heated in a 98◦ C bath for 2 min and transferred to ice for 10 min for precipitation. To pellet the proteins, we centrifuged the tubes at 22,000g for 10 min at 4◦ C. For manual injection of the samples into the chromatographic system Hamilton, syringes were used. Quantification of melatonin was

performed by comparing the samples with external standards provided by Sigma (St Louis, MO. USA).

Statistical analysis

The presented data were analyzed using the statistical software SPSS 22.0 (SPSS, Inc., Chicago, IL). Normal distribution of melatonin concentration was assessed by the Shapiro-Wilk normality test, resulting in no normality due to the heterogeneity of the patients. Then, its values were presented in the median and quartiles (first quartile; third quartile) of the experiments performed. The rest of variables are shown as means ± SD. Therefore, differences between before surgery and after surgery were estimated by the non-parametric test of Wilcoxon for paired samples. In the animal experiment, the differences were also evaluated by Wilcoxon for paired samples, comparing between ages of 3 and 12 months. For correlations, Pearson bivariate regression was used. $P<0.05$ was considered statistically significant.

GraphPad Prism (GraphPad Software Inc., San Diego, CA) was used to obtain the plots and to fit nonlinear regression curves in order to obtain the pD_2 value (EC_{50}). Statistical significance was calculated by ANOVA test, when needed.

Results

"In vivo" Assays

Melatonin levels in hypertensive patients' aqueous humour

All patients who were included; either not being diagnosed for, or treated for, glaucoma were divided in two groups. All patients with IOP values below 21 mm Hg were considered normotensive, and the hypertensive group was formed by those with IOP above 21 mm Hg. Demgraphic details of recruited patients is shown in table 2.1.

After collecting and processing aqueous humour samples, chromatography analysis clearly showed that patients exhibiting elevated IOP presented concomitantly higher concentrations of melatonin (figure 2.1, left panel), while normotensive patients (IOP below 21 mm Hg) showed values of 14.62 (5.38; 37.99) ng/ml (n=23), hypertensive patients presented melatonin concentrations of 46.63 (10.28; 167.28) ng/ml (n=14) these values being statistically significant ($p<0.002$). Furthermore, a significant correlation was found between melatonin concentration and IOP pre-surgery ($p= 0.03$), the Pearson's correlation coefficient being 0.441 (figure 2.2).

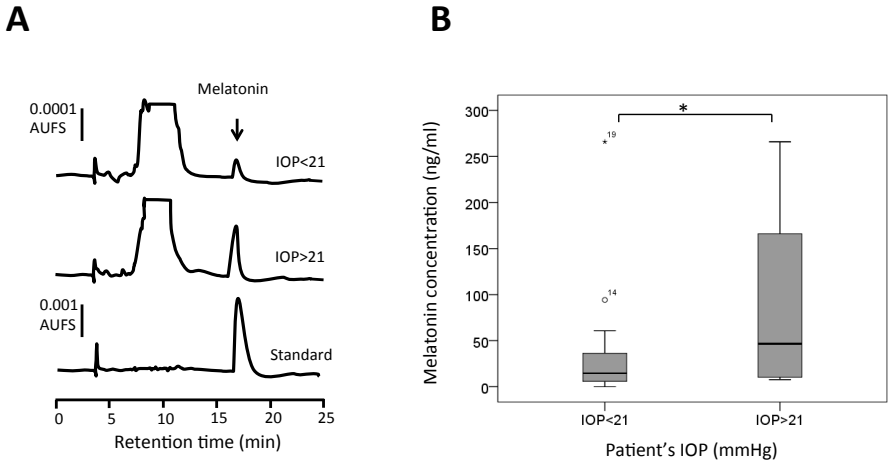

Figure 2.1. Presence of melatonin in the aqueous humour. On the left, representative HPLC elution profiles of human aqueous humour samples of patients with IOP above 21

mm Hg and below 21 mm Hg. On the right, bar plot showing the concentrations of melatonin in the two populations of patients. *p<0.039 (Wilcoxon for paired samples).

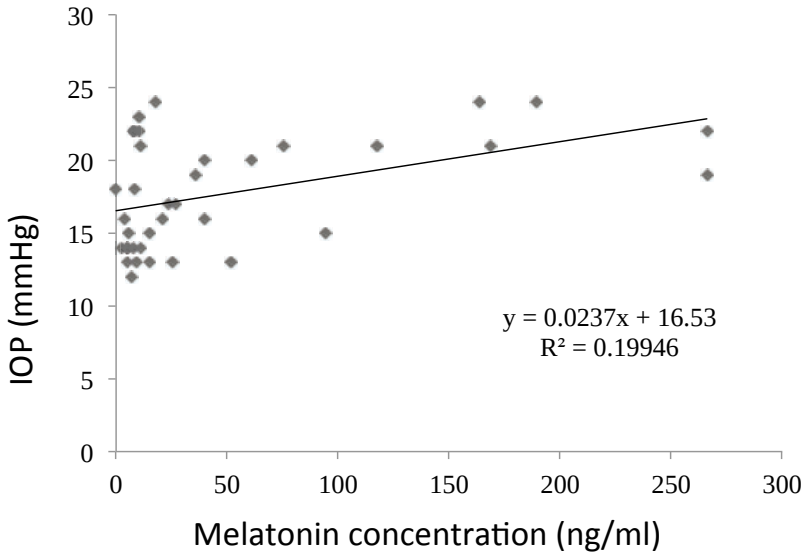

Figure 2.2. Correlation between melatonin concentration (ng/ml) in aqueous humour of patients who underwent cataract surgery and the IOP before the surgery. Pearson correlation was statistically significant (p= 0.025; r = 0.441).

Melatonin levels in control and glaucomatous mice's aqueous humour

In parallel to the analysis of melatonin levels in the aqueous humour of human patients, melatonin concentration was determined in mice as well. The study took place using a glaucomatous mice model (DBA2/J) and a normal mice strain (C57BL/6J). The IOP values measured in the DBA2/J were 15.05 ± 0.87 mmHg at 3 months of age, and then raised to 30.41 ± 1.35 mmHg when they reached 12 months of age. IOP values obtained from the (C57BL/6J) mice were 11.86 ± 0.69 mmHg at 3 months and 11.13 ± 1.21 mmHg at 12 months of age.

HPLC analysis to determine melatonin levels in the two animal groups at both ages showed statistically significant differences in the DBA2/J, before and after developing glaucoma. Melatonin values have changed from 0.37 (0.34; 0.59) ng/ml to 1.55 (0.94;

1.88) ng/ml at 3 and 12 months of age, respectively. Melatonin levels rose when the glaucoma model developed the pathology (n= 6, p<0.001) (Fig. 2.3).

However, the aqueous humour obtained from the control mice (C57BL/6J) at the same ages as the DBA2/J model did not show significant differences. Results showed melatonin levels of 0.47 (0.42; 0.53) ng/ml and 0.42 (0.32; 0.56) ng/ml at 3 and 12 months, respectively (n= 6, p = 0.418).

Melatonin concentration in the aqueous humour and IOP revealed a strong correlation in mice the Pearson's correlation coefficient being 0.851 (p < 0.001) for all the animals under study (Figure 2.3).

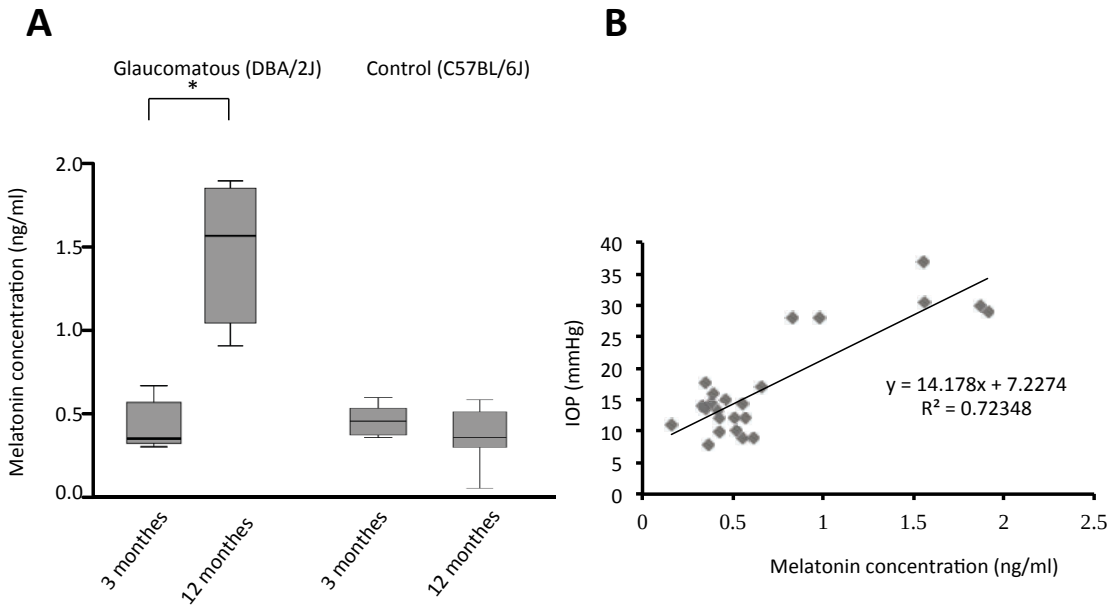

Figure 2.3. Melatonin concentrations in the aqueous humour of control (normotensive) and glaucomatous mice. One left panel, The DBA glaucomatous mice presented a marked increase in their aqueous humour melatonin concentrations when comparing 3 months of age, before the pathology starts and at 12 month of age, when the glaucomatous pathology has been fully established and the pressure in maximal. On the contrary, control mice, C57, did not change their melatonin concentrations with ageing. * p<0.001 (Wilcoxon for paired samples). Pearson correlation between melatonin concentration (ng/ml) in aqueous humour of glaucomatous (DBA2/J) and non-glaucomatous (C57) mice strains and the IOP measured in all times of experiment was statistically significant (p< 0.001; r = 0.851) (right panel).

"In vitro" Assays

TRPV4 presence in non-pigmented ciliary epithelial cells

Immunofluorescence experiments took place to confirm the presence of TRPV4 channel in the human non-pigmented ciliary epithelial cells. As can be observed in Fig. 2.4., a positive labelling of TRPV4 can be seen mostly in the cytoplasm membrane of the cells.

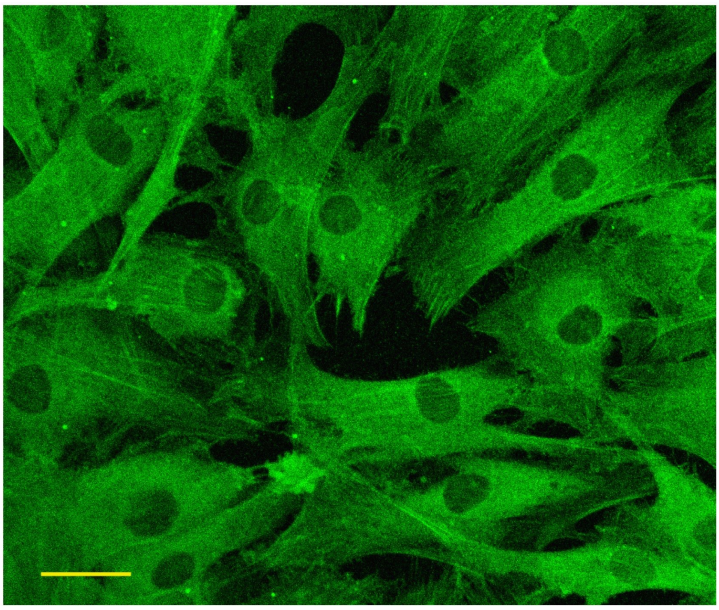

Figure.2.4. presence of TRPV4 in human non-pigmented ciliary epithelial cells (observed in green). The bar represents 20 μm

Extracellular melatonin level changes depending on TRPV4 channel status

Melatonin concentration was measured from the supernatants of human non-pigmented ciliary body epithelial cells by means of HPLC analysis. The concentration of melatonin obtained from a control culture (untreated cells) was 8.5 ± 1.6 nM/well of cells (n=12) (equivalent to 1.9 ng/ml). Whereas, activating the TRPV4 channel by using a

specific agonist GSK1016790A at a dose of 10 nM resulted in an increment of melatonin levels 23.3 ± 2.1 nM/well of cells (n=12) (equivalent to 5.4 ng/ml) (Fig. 2.5).

Antagonists were applied in different experiments to confirm that the effect shown is caused by TRPV4 channel, the selective antagonist RN1734 prevented the increase of melatonin release from the cells when applying GSK1016790A. A less selective antagonist, Ruthenium red was also able to suppress the agonist-induced effect over melatonin levels (Fig. 2.5.).

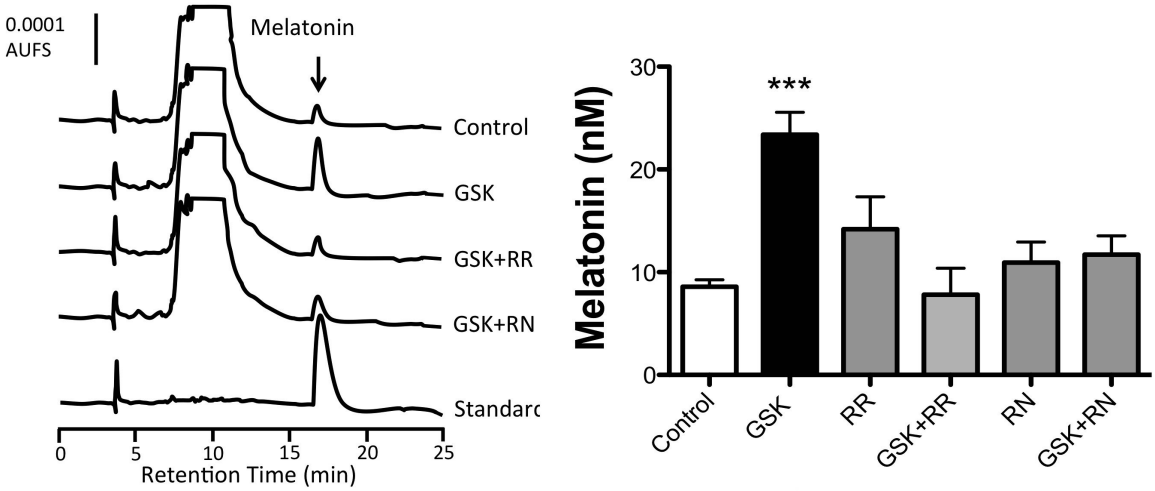

Figure.2.5. Representative HPLC chromatograms and retention time for melatonin in the supernatant of human non-pigmented epithelial cells under normal conditions compared to the addition of TRPV4 agonist (GSK) and antagonists (RR, RN) as described in methods. C.- Quantification of the experiments described in A. The values represent the mean ± SD of 12 independent experiments (***$p < 0.001$ vs control).

Dose-Response assays of GSK1016790A

TRPV4 agonist GSK1016790A was then evaluated at a wide range of concentrations in order to obtain the corresponding concentration-response curve. Concentrations of GSK ranging from 10-10 to 10-5 M, permitted to observe a sigmoid plot

from which a pD2 value of 8.5 ± 0.1 was obtained (EC50 of 3.0 nM). Melatonin content in the extracellular medium reached the maximum when cells were challenged with the TRPV4 agonist at 10nM concentration (n=7, figure 2.6.).

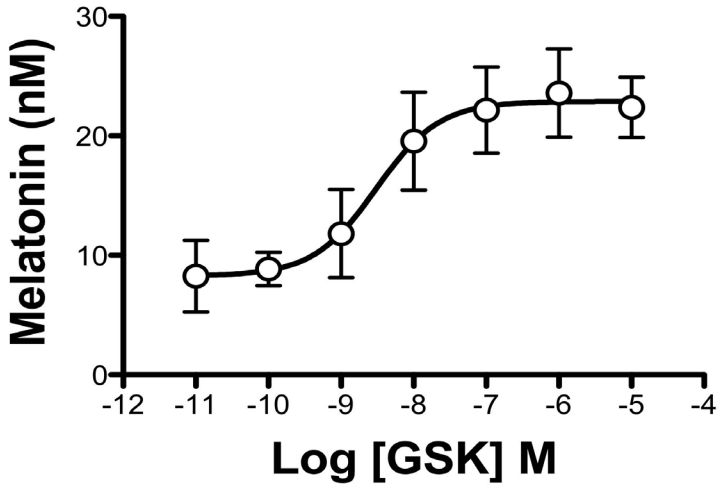

Figure 2.6. Concentration-response analysis of GSK1016790A and possible role of the TRPV4 inducing the release of melatonin and how it may control the aqueous humour formation. Concentration-response curve for melatonin production by using the TRPV4 agonist GSK in different concentrations (see methods). The values are the mean ± SD of 7 independent experiments.

Discussion

The present work describes the changes of melatonin levels observed under elevated pressure. It shows the differences in melatonin concentration among patients with normal and elevated IOP and it correlates melatonin values with IOP measurements using a glaucomatous animal model (DBA/2J). Furthermore, studies took place using a human immortalized cell line of non-pigmented ciliary body epithelium in order to find a possible explanation for these differences in melatonin levels.

Findings pointed towards an increment of almost 3-fold of melatonin levels in patients with elevated IOP, compared to those patients who had normal IOP. Similar behaviour was observed in the glaucomatous mice model, when animals reached 12 months of age when they fully developed glaucoma pathology and their IOP were high.

On a cellular level, when the cells which produce the aqueous humour, the non-pigmented ciliary body cells, were tested, results showed that they produce melatonin as well, and comparable findings were observed when TRPV4 channel was activated using an agonist. Melatonin levels increased.

The use of cellular model to mimic the natural finding in the human aqueous humour can be very useful to understand the mechanism of action. Nevertheless, using an animal model such as the DBA/2J, can be very useful to understand the changes in melatonin levels in the aqueous humour before and during the pathology after it is fully developed. This is very valuable, given the difficulties of collecting aqueous humour samples from patients in such a way.

The observations in the present work clearly showed the highest increment of melatonin levels in the aqueous humour in hypertensive patients, compared to healthy subjects, this increment being higher than the one observed in other fluids such as the

tears. Human tears contain melatonin and follow a circadian manner, but the changes of melatonin levels are about 1.6-fold higher during the night compared to the day (Carracedo et al., 2016). This clearly indicates that melatonin concentration in the hypertensive patients, as well as in mice, is not only governed by circadian aspects, but it may also be related to the fact of the abnormally elevated intraocular pressure.

The connection between melatonin and the intraocular pressure may be due to the existence of a pressure sensor, the TRPV4 channel, which, when activated, triggers the release of melatonin in the aqueous humour. The EC50 value obtained in non-pigmented ciliary body epithelium (3 nM) compared to different human cells was very similar to that obtained by Thorneloe and co-workers (Thorneloe et al., 2008) in the search of new agonists which induces urinary bladder contraction and hyperactivity, using TRPV4 knock out mice model together with TRPV4-expressing human embryonic kidney (HEK) cells. They showed a dose response behaviour of the agonist GSK1016790A, close to the one found in the current work. The values obtained from the cellular model used in this study were one order of magnitude lower than those obtained in the cerebrospinal fluid barrier protection carried out by TRPV4 (EC50 34 nM) (Narita et al., 2015), and also lower than the values found when measuring the human bronchus constriction (EC50 10 nM) (McAlexander et al., 2014).

TRPV4 channel activation produces changes in the extracellular levels of melatonin on a continuous stimulation basis. In this sense, glaucoma patients suffer the permanent rise in IOP before starting any treatment or those who are not using an effective treatment to lower IOP. Therefore, changes in the composition of the aqueous humour of patients presenting ocular hypertension could be a natural process to reduce IOP, given that

they showed higher levels of melatonin, and studies confirmed that melatonin has the ability to decrease IOP (Crooke et al., 2012).

The fact that melatonin lowers IOP, together with the findings in the present work, causes challenging questions to arise, why do patients with elevated IOP have more melatonin? And why is melatonin not working as expected, in decreasing IOP in this case? To be able to fully understand and answer the role of melatonin in pathologies presenting high IOP, further studies are necessary. However, some hypothesis could be made. In order for melatonin to work properly, its receptors should be functional, and this could not be happening in glaucoma patients. The existence, of age-related and region-specific changes in the expression of the melatonin receptor subtypes has already been described in animal models (Guo et al., 2015). Different studies showed that mice lacking melatonin receptor type 1 presented elevated IOP, these new studies suggest that melatonin signalling dysfunction can be a possible risk factor in the pathogenesis of glaucoma (Alcantara-Contreras et al., 2011; Tosini et al., 2013). This could be the case of some patients and therefore, increased melatonin levels are not enough to reduce IOP as one could expect.

Melatonin has many functions, besides regulating the circadian rhythm, it is a powerful free-radical scavenger and wide-spectrum antioxidant. This is a fundamental role, as glaucoma pathology presents an oxidative damage (Sacca and Izzotti, 2014). Therefore, melatonin increment could be also reflecting a mechanism of protection from the oxidative stress carried out in the eye to protect high efficient tissues as the ciliary body and the trabecular meshwork which are essential to control IOP (Sacca et al., 2016a, b). Moreover, if similar changes regarding melatonin levels were occurring in the retina, this molecule would help to ameliorate the glaucomatous pathology by acting on retinal glutamate clearance, GABA concentrations, NO synthesis, and retinal redox status

(Belforte et al., 2010). More experiments should be carried out to fully understand and confirm all possible explanations.

Future investigation may cover several aspects of the role of melatonin in glaucoma pathology. It would be worthwhile to study the effect of glaucoma current treatments on the aqueous humour melatonin levels. It is possible that it could modify the aqueous humour melatonin concentrations, as happens with other aqueous humour components in experimental animal models (Reyes et al., 1998). Furthermore, understanding the connection between melatonin levels and anti-glaucomtous treatments could be very useful to clarify the best moment of the day to apply medication, since melatonin follows a circadian rhythm as well as IOP (Bron, 2004; Santos et al., 2009).

Regarding the possible mechanism of TRPV4 activation when IOP is increased, although a response to hydrostatic pressure might be possible, it is worth taking into consideration that the mechanical distortion caused in some way by the elevation of IOP could be the main reason for TRPV4 activation, as previously suggested in other models (Garcia-Elias et al., 2014). Melatonin, acting in an autocrine way through its existent receptors in the ciliary body (Alarma-Estrany et al., 2008) could reduce chloride production as recently described (Huete-Toral et al., 2015).

To summarise then, TRPV4 channel stimulation is able to induce melatonin in human ciliary non-pigmented epithelium cells and this can explain the elevated melatonin concentration in the aqueous humour of patients with high IOP compared to normotensive patients. The lack of effect of melatonin in the aqueous humour to lower IOP could be due to an imbalance between melatonin release and the receptors that should reduce IOP.

Chapter III: TRPV4 activation effect on aralkylamine N-acetyltransferase (AANAT) and its phosphorylation in human ciliary body epithelial cells.

“Don't confuse symmetry with balance.”

Tom Robbins

Introduction

The ciliary body, together with the iris and choroid comprise the vascular coat or uvea of the eye. The ciliary body is composed of the ciliary muscle and ciliary processes, the latter being approximately 70 in number in the human eye. Ciliary processes consist of a central core of connective tissue stroma, covered by a double layered epithelium, the pigmented and non-pigmented epithelium which is in direct contact with the aqueous humor (Do and Civan, 2004). In fact, the non-pigmented epithelium of the ciliary processes is where the aqueous humor is secreted by a process of active transport, through diffusion and ultrafiltration (To et al., 2002). The aqueous humor bathes the avascular structures of the eye, such as the crystalline lens and the posterior surface of the cornea, and this constant flow supplies these structures with the necessary nutrients and carries out their metabolic waste. The dynamic of this is also very important to maintain the intraocular pressure of the eye balanced (Anshu et al., 2011; Mark, 2010).

The ciliary body is considered the third organ which secretes melatonin after the retina and the pineal gland (Martin et al., 1992). This discovery took place after observing that intraocular pressure follows a circadian variation, together with the fact that the ciliary epithelium shares an embryonic origin with the pineal gland and the retina (Martin et al., 1992). These findings, opened a window of investigation to link melatonin and IOP.

Melatonin synthesis starts from 5 hydroxy-tryptamine (5-HT or serotonin), which is transformed into melatonin in two steps, being catalysed by the enzyme aralkylamine N-acetyltransferase (AANAT) and acetylserotonin O-methyltransferase (HIOMT) (Blomeke et al., 2008; Coon et al., 1996; Klein et al., 1997; Roseboom et al., 1996). The rate limiting step in melatonin synthesis seems to depend on AANAT activity since it shows circadian

fluctuations throughout the day (Klein, 2007). However, AANAT could be regulated by other environmental factors rather than only the light/darkness changes, such as other hormones, food or alcohol intake (Garcia-Marin et al., 2015; Kashani et al., 2015; Peres et al., 2011).

AANAT has two known ways of regulation, first, a fast process of phosphorylation which depends on the binding of AANAT to 14-3-3 protein. This process of phosphorylation protects AANAT from proteosomal degradation and has been related to the activation of PKA after cAMP generation (Baler et al., 1997; Kleppe et al., 2014). However, cyclic AMP/protein kinase A pathway may also activate gene expression. In rodents, transcriptional activation of Aanat gene is the classical mechanism to induce melatonin biosynthesis. It involves PKA-dependent phosphorylation of the transcription factor cAMP response element binding protein (CREB) and binding of phosphorylated CREB in the promoter region of Aanat gene (Stefulj et al., 2001).

Melatonin synthesis in the ciliary epithelial cells is of great interest, since melatonin has many functions in the eye, besides working as a scavenger for free radicals, it also regulates intraocular pressure. These actions are mainly mediated by melatonin membrane receptors MT_1 and MT_2 (Zlotos et al., 2014) and the putative MT_3 (Pintor et al., 2003; Serle et al., 2004) which have been also identified in some tissues as the enzyme quinone reductase-2 (Nosjean et al., 2000).

Intraocular pressure is normally balanced by the aqueous humour production and its outflow. When this equilibrium is affected by any reason, this could lead to elevated IOP which is a significant risk factor leading to glaucoma pathology (Acott et al., 2014; Anshu et al., 2011; Paycha, 1992). Glaucoma is a multifactorial disease called “the silent thief of sight” (1995) because it shows no symptoms and, if not detected at an early stage,

could lead to permanent blindness (Abdull et al., 2016). All current treatments for glaucoma pathology aim to decrease and stabilize IOP levels, in such a way as to protect retinal damage and ganglion cells death. Melatonin and its analogues have been investigated in the search for new treatments for elevated IOP since commercial compounds present several side effects, which can sometimes be a reason to stop the medication. Melatonin taken orally has been proven to reduce IOP in human patients who underwent cataract surgery (Ismail and Mowafi, 2009) and it was tested on normotensive and hypertensive animal models as well (Martinez-Aguila et al., 2013; Martinez-Aguila et al., 2016). However, a previous study (refer to chapter 1) showed higher levels of melatonin in the aqueous humor of patients with elevated IOP. Here emerges the need to understand the mechanism in which melatonin levels rise under high pressure. A search was carried out to find a sensor in the non-pigmented ciliary epithelial cells which reacts to such stress.

The relatively recent discovery of the transient receptor potential (TRP) superfamily of cation channels allowed a further understanding of the mechanisms by which cells are able to sense various stimuli such as osmolarity, mechanical loading, heat and cold (Montell, 2005; Mutai and Heller, 2003; Peier et al., 2002a; Peier et al., 2002b). This discovery led to more studies, where the transient receptor potential vanilloid 4 (TRPV4), a non-selective cation channel that regulates osmo-, thermo-, mechanosensation (Loukin et al., 2010; Mutai and Heller, 2003) was seen to play an important role in the ciliary body epithelium cells after performing some experiments to confirm its presence and function (refer to chapter 1). This channel activation caused melatonin levels to increase in the extracellular medium, which raised the question about its mechanism of action and the reason behind its increment.

The present work has been undertaken to answer the above questions, to conduct a more in-depth study to discover the role of TRPV4 in the non pigmented ciliary epithelium cells and its relation to melatonin in the case of mechanical stress when IOP is elevated.

Material and methods

Cells

Non-pigmented ciliary epithelial cells (59HCEsv), a human immortalized cell line was kindly supplied by Dr. Coca-Prados (Yale University). Cells were grown in high glucose Dulbecco's modified Eagle's medium (Gibco/Invitrogen, Carlsbad, CA) containing 10% foetal bovine serum (Sigma-Aldrich, St. Louis, MO) and 0.05 penicillin/ streptomycin (Gibco/Invitrogen) at 37°C in humidified atmosphere 5% CO_2–95% air. After the culture reached the confluence, cells were seeded in 6 well plates and/or to 4 well chamber slides respectively (for more details, see chapter 2).

Human eye tissues

Donor human eyes were obtained from the Fundación Banco de Sangre y Tejidos de las Islas Baleares (Blood and tissue bank Foundation from Baleares Islands). Six donor eyes were used for this assay, two of a healthy normal subject and another four from glaucoma patients. Eyes were enucleated and collected without the cornea in sterile tubes and maintained in 4% paraformaldehyde in 0.1 M phosphate buffer (PB) (pH 7.2-7.4) at 4ºC until posterior processing. Eyes were dissected under a stereo-microscope (Zeiss) and with 0.8 mm tip curved forceps and sterile dissecting scissors, the iris and ciliary processes were collected. Several washes in PBS1X were performed, each for 10 minutes under constant stirring, and then, the specimen were cryoprotected in a sucrose gradient. Initially, eye structures were submerged in a solution of 11% sucrose in PBS 1X for a minimum of 12 hours at 4 °C; then transferred to 22% sucrose solution overnight, and finally sections were incubated with 33% of sucrose solution. In this way, we preserve the tissue from

deterioration caused by the formation of water crystals during the freezing process. Tissues were embedded in a tissue freezing medium (Tissue-Tek© OCT), maintaining the proper orientation taking into account the desired cutting plane. They were rapidly frozen with in liquid nitrogen. All included biological material was stored at -20 ° C.

The blocks were introduced into the cryostat chamber for about 1 hour to reach the optimum cutting temperature (about -27 ° C). Vertical sections of 10 µm thickness were obtained using a cryostat (Leica, Nussloch, Germany). Sections were adhered to slides (pre-treated with poly-L-lysine) and stored at -20 ° C until further use.

Immunolabelling Asseys

Immunodetection was performed using histological sections of the ciliary body, as well as in immortalized non-pigmented epithelial cells.

Frozen sections were used for imumunodetection following the described procedure. First, sections were hydrated with PBS1X for 10 minutes each before permeabilization. Cell membranes were permeabilized with with a solution of Triton X-100- 0.05% - PBS for 30 minutes, followed by blocking. In order to remove inspecific labelling, the sections were incubated in a PBS 1X-0.1% Triton X-100 solution, and 10% normal donkey serum (NDS, Jackson Immunoresearch, West Grove, PA) was used and sections were incubated for 1 hour at room temperature. Sections were incubated in 0.1% Triton X-100 and 1% normal donkey serum together with the primary antibody rabbit anti-serotonin N-acetyltrasnferase (AANAT, ab3505, Abcam, Cambrigde, UK) at a 1:500 dilution at 4ºC overnight.

Next day, three washes with PBS1X-Triton X-100-0.1% were performed for 5 minutes each. Followed by the secondary antibody; donkey anti-immunoglobulin IgG

rabbit antibody conjugated with fluorescein isothiocyanate (FITC; green, Jackson ImmunoResearch, West Grove, PA, USA) at 1:100 dilution in PBS1X-Triton X-100-0.1% for one hour in a dark chamber at room temperature. Several washes with PBS1X were done. The nuclei were stained with propidium iodide (red, Sigma-Aldrich, St. Louis, MO) diluted 1:500 in PBS for 10 min. Finally, sections were rinsed and mounted in Vectashield fluoromount (Vector Laboratories, Palex Medical, Barcelona, Spain) and coverslipped.

For ciliary epithelial cells immunostaining, cells were treated with a specific TRPV4 agonist GSK1016790A for 18 hours before performing the immunocytochemistry. Cells were washed with PBS1X three times for 5 minutes each, and they were incubated with 4% paraformaldehyde diluted in PBS1X during 15 minutes, followed by several washes and 15 minutes of incubation with a solution of Triton X-1000- 0.05%. Experiments were done afterwords in the same way as the above procedure with the tissue sections. Immunolabelling showing negative controls were performed by substitution of the primary antibodies with normal or isotype specific immunoglobulins serum of the same specie at a similar working concentration.

For the analysis, the images were acquiered using a laser scanning microscope, a confocal microscope (Zeiss LSM 5, Jena, Germany) at 40X magnification.

TRPV4 experiments

Long-term studies

Cells were seeded in 6-well multiwells and observed until they reached 100% confluence, a density of $1.2x10^6$ cells. A TRPV4 agonist GSK1016790A (Tocris Bioscience, Bristol, United Kingdom) was applied at a concentration of 10nM and left for different durations of 1, 3, 6, 18, 24, 48 hours to establish the adequate time course. The

supernatants at each time were collected for melatonin and AANAT activity measurements by HPLC as described below. Posterior to collecting the medium, cells were homogenized for western-blot assays.

In different multiwells, after deciding the best time to see the desired effect, a dose-response curve was obtained by incubating the cells for 18 hours with GSK1016790A at different concentrations: 1nM, 10nM, 100nM, 1μM, and 10μM. Cell lysis was done for AANAT quantification.

For antagonists studies, the compounds ruthenium red (RR) and RN-1734 (RN) (Vincent et al., 2009), were assayed at concentrations of 1 nM and 10 nM respectively, either alone or 30 min before the application of the TRPV4 agonist GSK1016790A. Cell lysis was done afterwards.

Short term assays

Experiments were designed equally as the above assays in terms of cell density and TRPV4 agonist and antagonists. A short-term assays were done to study AANAT phosphorylation effect. A time-course was done incubating cells with GSK1016790A for different durations starting from 1, 5, 10, 15, 30, and 60 minutes at 10 nM concentration. A dose-response curve assayed with the same concentration range but only incubating the cells with the agonist for 5 minutes. Finally, for antagonist studies, the same protocol was applied taking into account the agonist time of incubation (5 minutes).

Supernatants were collected and cells were homogenized for HPLC and western-bolt analysis, respectively.

Silencing TRPV4 channel

Cells were grown in 6-well multiwells. Transfection took place using a transfection reagent (sc-29528, Santa Cruz, CA, USA) and a transfection medium (sc-36868). For some wells, TRPV4 siRNA (sc-61726, Santa Cruz, CA, USA) was incubated, and in others the scramble was incubated as a control (sc-36869, Santa Cruz, CA, USA). Incubation was for 5 hours for the transfection with either siRNA or scramble, followed by several washes and finally, cells were incubated with a fully supplemented medium with 10% foetal serum and 0.05% of antibiotics for 48 hours. Cells were homogenized after TRPV4 silencing. A western blot assay was done using rabbit polyclonal to TRPV4 primary antibody (ab94868), and the medium was collected for melatonin analysis by HPLC.

A different set of cell culture was prepared posteriorly, and transfected in the same way. Cells transfected with the TRPV4 siRNA were incubated with GSK1016790A for 5 minutes, the same experiment in wells with no siRNA was done. Cell lysis was performed for p-AANAT quantification by western-blot.

Second messengers inhibition

For second messenger studies, different agents were applied for 30 minutes to assure the inhibitory effect later. TRPV4 agonist GSK1016790A was applied for 5 minutes to study the effect of these inhibitors on p-AANAT expression by means of western-blot. An inhibitor of adenylyl cyclase (SQ 22536) was used at a concentration of 50μM, a protein kinase A inhibitor (KT 5720) at 600 nM, a calmodulin-dependent protein kinase II inhibitor (Autocamid-2-related inhibitory peptide) was used at a concentration of 500nM,

and calmodulin antagonist (Calp2) at 20μM. All the inhibitors were purchased from Tocris Bioscience, Bristol, United Kingdom.

Western-blot studies

Cells were removed and homogenized in ice with radioimmunoprecipitation assay (RIPA) buffer (1:5 v/v) containing 50 mM HEPES, pH 8, 150 mM NaCl, 1% NP-40 (w/v), 0.5% sodium deoxicolate, 0.1% SDS and Halt Protease and Phosphate Inhibitor Cocktail (Thermo Fisher Scientific). lysates were centrifuged at 15,000 g for 15 minutes at 4ºC. the supernatant were transferred to a chilled 1.5 ml eppendorf tubes and stored at -20ºC until use.

The evaluation of the protein concentration present in the cell extracts was performed by the commercial kit (Pierce BCA Protein Assay kit, Thermo Scientific), based on the copper ion reaction of bicinchoninic acid with the proteins of the biological sample, according to the manufacturers instructions. This kit allows us to evaluate amounts of protein between 20 μg / ml and 2000 μg / ml to construct the standard curve. In this assay the protein samples, whose concentration was intended to be titrated, were diluted (1:10) and applied in triplicate to a 96-well plate. To discover the protein concentration, a calibration line was generated from known concentrations of albumin. The WR compound, provided with the kit, was then added, containing copper ions which form coloured complexes with the proteins to be titrated. After 30 minutes of incubation at 37 ° C the absorbance of the colored compounds obtained in a plate reader (Power Wave XS2, Biotek) at a wavelength of 562 nm was measured.

In the case of AANAT detection, 60 μg proteins were used for western blotting, while 80μg proteins were used for p-AANAT. Gels for electrophoresis were always

prepared with a stacking gel of 15% and separator at 4% SDS-PAGE. Samples were diluted with Laemmli's sample buffer, pre-stained ladders were loaded onto a well in the same gel (referencia patron). Electrophoresis took place at 150V for around 1 hour.

For electro-transfer, a transfer sandwich was prepared, still wet with the transfer buffer as follows, foam pad, 3 blot papers, gel SDS-PAGE, nitrocellulose membrane, 3 blot papers, and a foam pad,on the black side on the cassette, ensuring there were no bubbles trapped between layers. Transference of the separated proteins was run at 150mA for 80 minutes at 4ºC, and covered by the transference buffer. A frozen cooling unit was then inserted in the rear of the cassettes to keep the transfering unit cool during the proccess.

After the electrotransfer, in the case of AANAT detection, membranes were blocked with 5% of non-fat milk (Bio-Rad) dissolved in TBS x 0.1% Tween20 for 1 hour using an orbital shaker, while in p-AANAT experiments, blots were blocked with 5% Albumin bovine serum (Sigma-Aldrich) for 1 h at room temperature. After discarting the washing solutions, blots were incubated with primary antibodies AANAT primary antibody (ab3505, 1:1000, Abcam) and p-AANAT primary antibody (ab3439, 1:1000, Abcam). Mouse monoclonal glyceraldehyde-3-phosphate dehydrogenase (anti-GAPDH, sc-166574, 1:500, Santa Cruz, CA, USA) served as a loading control diluted in the blocking solution and incubated at 4°C overnight on a shaker.

Blots were washed 3 times, each for 5 minutes with TBS x 0.1% Tween 20, and membranes were incubated with a goat anti rabbit IgG-HRP-conjugated or a goat mouse anti-IgG-HRP-conjugated with horseradish peroxidase secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA) diluted in 0.25% of non-fat milk in TBS x 0.1%

Tween20 for 1 h at room temperature. All steps were performed with the membrane on a shaker. Several washes were done before visualization of the desired protein.

Proteins were revealed by chemiluminiscence, using enhanced chemiluminiscence (ECL) detection kit (Amersham Phamacia Biotech, Barcelona, Spain) in a dark room. Films were scanned with Gel Logic 200 Imaging System (Kodak, USA). The densitometric analysis was performed by using Kodak Molecular Imaging software (Kodak, Rochester, NY, USA). The densitometry values of each sample were normalized to respective densitometric GAPDH values.

HPLC analysis

The chromatographic studies was performed to detect melatonin following the protocol previously described (for more details, see chapter 2). Briefly, supernatants of cells after the different experimental conditions were processed for injecting in the HPLC system. For melatonin detection, the system was equilibrated with 40% methanol and 60 % water. Measurements were taken at a flow rate of 0.8 ml/min fixing the detector at a wavelength of 244 nm.

HPLC analysis to detect the product of AANAT, N-acetyl serotonin (NAS), was performed with a SunFire18 (5 µ, 25 cm in length, 0.4 cm inner diameter) from Waters (Milford, MA) equilibrated with a mobile phase consisting of 15 % acetonitrile, 0.1 % acetic acid and at a flow rate of 0.75 ml/min, detecting NAS at the wavelength of 244 nm.

Quantification of melatonin and NAS was examined by comparing the samples with external standard provided by Sigma (st. Louis, MO, USA).

Statistical analysis

The data represent the mean s.e.m. of 4-6 independent experiments (indicated in each case). Statistical significant was calculated by student t-test or ANOVA test when necessary. GraphPad Prism (GraphPad Software Inc., San Diego, CA) was used to obtain the plots and to fit nonlinear regression curves in order to obtain the pD_2 value (EC_{50}).

Results

Presence and changes of AANAT in the human ciliary body

Human ciliary body tissues were dissected from whole human eyes for immunofluorescent labelling. Six samples were compared, two of healthy donors and 4 from patients who had glaucoma. Ciliary body epithelium showed a positive staining mostly in the cytoplasm. However, the ciliary body of the glaucomatous donor presented stronger fluorescent labelling (Fig. 3.1.).

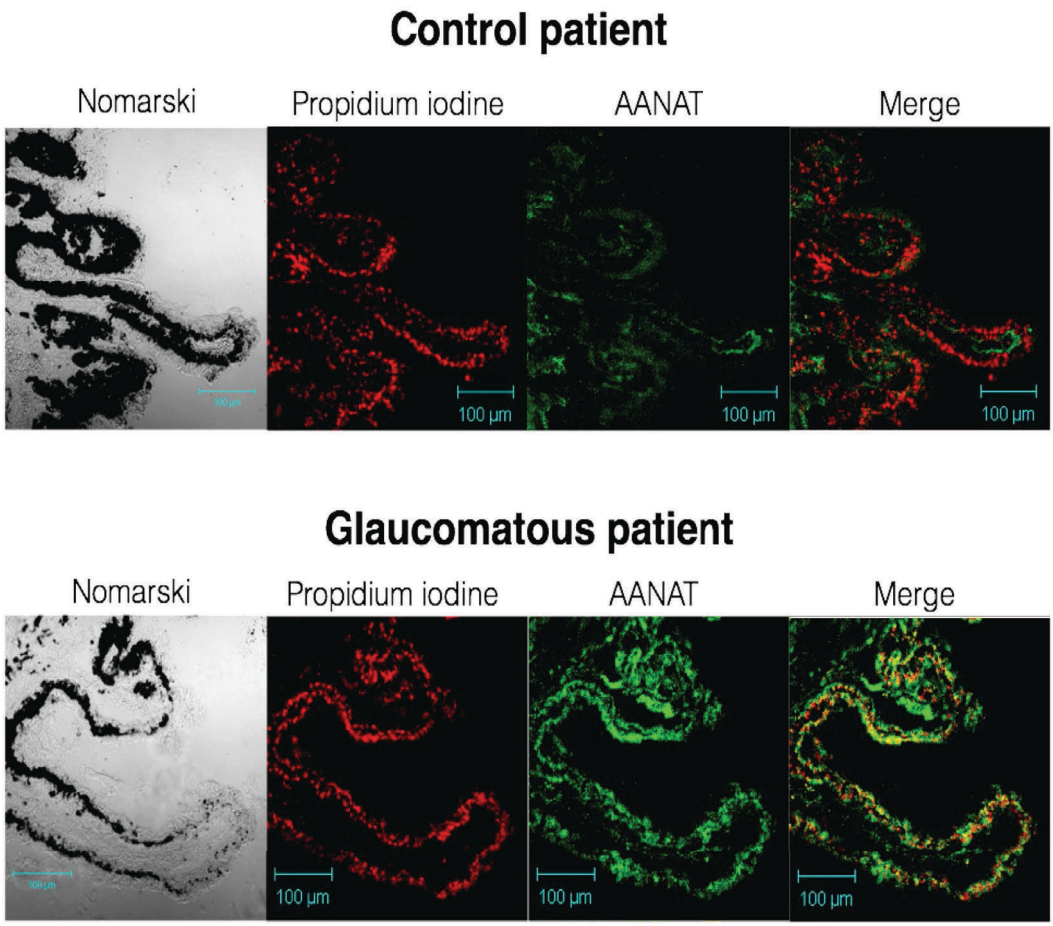

Figure 3.1. Presence and changes of AANAT in human ciliary body tissue. A: Representative pictures of human ciliary processes of a non-glaucomatous individual. From left to right DIC image, nuclei (in red, propidium iodine), AANAT (in green) and merge image.B: Representative image of human ciliary processes of a glaucomatous individual. From left to right DIC image, nuclei (in red, propidium iodine), AANAT (in green) and merge image.

Experiments were performed in isolated non-pigmented epithelial cells of the ciliary body as well. Cells were grown in multiwells, and some were treated with TRPV4 agonist GSK1016790A to mimic an elevated pressure since this channel is sensitive to pressure among other stimulus (Ramsey et al., 2006; Thorneloe et al., 2008). The results showed AANAT labelling in both control and TRPV4 stimulated cells. Interestingly, stronger fluorescent labelling was observed in cells treated with GSK1016790A (fig.3.2.). These results were consistent with the observed differences between healthy and glaucomatous donor eyes.

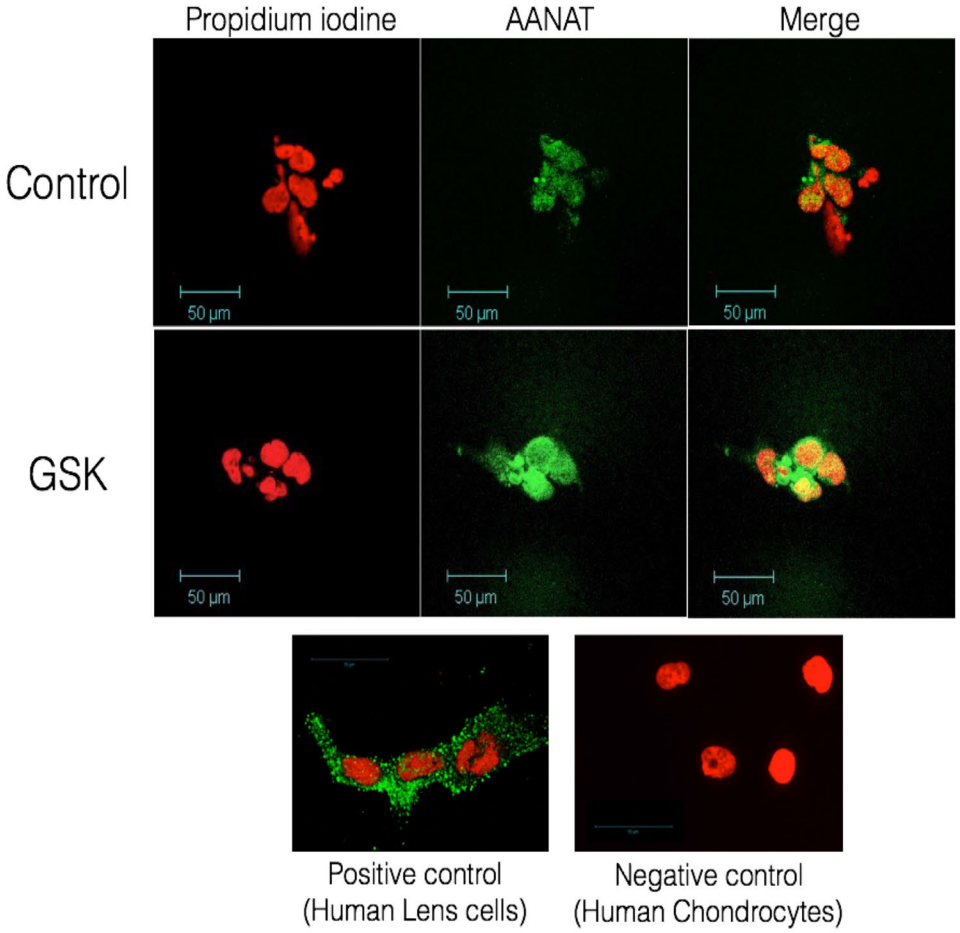

Figure. 3.2. Presence and changes of AANAT in human ciliary body epithelial cells. A: Untreated human ciliary body epithelial cells showing the expression of AANAT (in green) and the nuclei (in red). B: Human ciliary epithelial cells after treatment with 10 nM GSK1016790A for 18 h. AANAT expression can be seen in green while nuclei appear in red. C: Positive and negative controls for AANAT performed with human lens epithelial cells (positive) and human chondrocytes (negative).

TRPV4 activation increases AANAT and its phosphorylation: time-dependent behavior

TRPV4 agonist GSK1016790A was applied to non-pigmented epithelial cells at different times. For the study of the long- term effect of TRPV4 stimulation on AANAT expression, assay durations took place from 1 hour up to 48 hours. Short term experiments were also performed to see the phosphorylation of AANAT. Short-term experiments started for 1 minute gradually increasing in time to 1 hour. All experiments were done at 10nM concentration of GSK1016790A. Both AANAT and p-AANAT expression can be observed in figure 3.3.

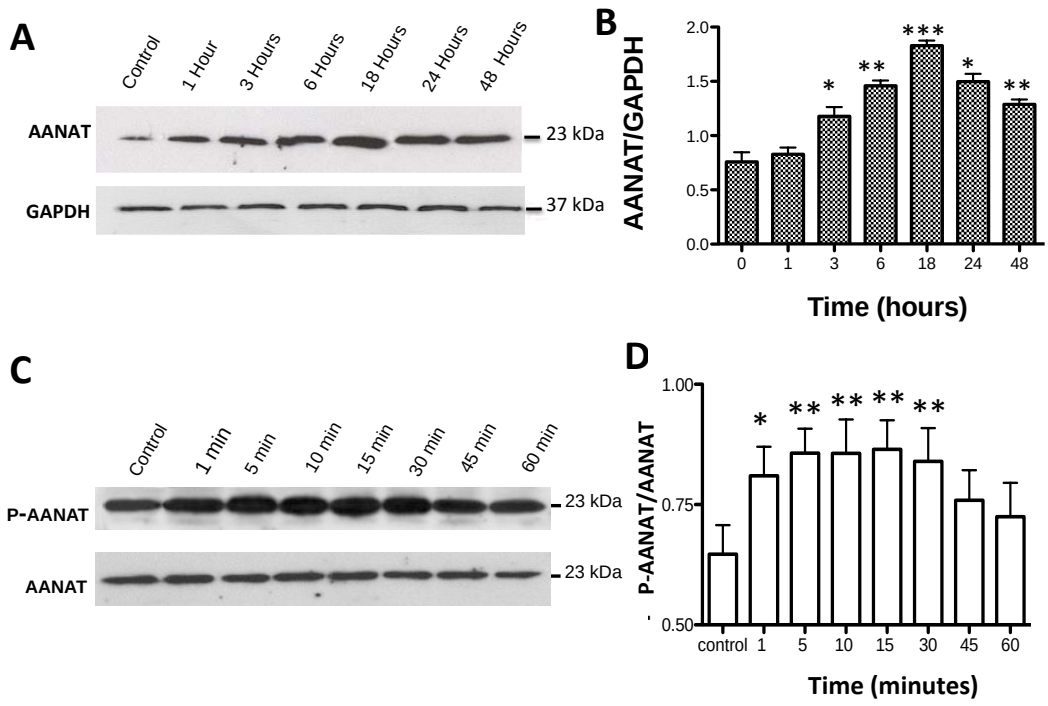

Figure 3.3. Time-course of the effect of GSK on AANAT protein synthesis. A) Representative western-blot showing the changes in AANAT during a maximal period of 48 hours after cell treatment with 10 nM GSK1016790A. B) Column plot showing the relative quantification of the western-blots band intensities. Values represent the mean s.e.m of four independent experiments (*p < 0.05 and **p < 0.01 versus time 0). C) Representative western-blot showing the changes in p-AANAT during a maximal period of 60 min after cell treatment with 10 nM GSK1016790A (GSK). D) Column plot showing the relative quantification of the western-blots band intensities. Values represent the mean s.e.m of six independent experiments (**p < 0.01 versus time 0).

Results showed a maximal peak of AANAT after 18 hours of incubation with TRPV4 agonist, AANAT increment reached 2.4 folds above the control values (**p<0.000, n=4). AANAT values decreased after 48 hours (Fig. 3.3 B). When the TRPV4 channel was activated by GSK1016790A at short durations for the phosphorylation of AANAT, pAANAT increased after 5 minutes of the agonist application. The maximal increase corresponded to 60% above the control values (**p<0.01, n=6). Phosphorylation values of AANAT returned to control ones after one hour (Fig. 3.3 D).

Since AANAT is responsible for melatonin synthesis by producing the precursor N-acetyl serotonin (NAS), HPLC studies were performed to investigate if there was a correlation between the expression levels of AANAT shown in figure 3 and the production of both, NAS and melatonin. As can be seen in figure 3.4, both the levels of NAS and melatonin presented maximal concentrations at 18h, presenting concentrations of 40.24 ± 1.82 nM in the case of NAS and 21.36 ± 1.83 nM for melatonin (n=4, ###p<0.001 vs control for NAS and **p<0.005 vs control for melatonin). As happened in the case of AANAT expression, after 18h, both NAS and melatonin returned to their initial values.

The experiments performed to measure NAS and melatonin production showed an increase which was similar to that observed for AANAT phosphorylation (figure 2A). A maximal elevation in NAS concentration was observed at 5 min with a value of 39.4 ± 3.7 nM which was different from the initial NAS concentration (19.2 2.0 nM, ***p<0.001, n=6, figure 3.4 A)

The changes in melatonin concentration were observed between 5 and 10 min with values of 23.9 ± 3.0 and 21.9 ± 4.1 nM, which were statistically different from the initial melatonin concentration (8.8 ± 1.3 nM, ##p<0.01, n=6, figure 3.4 B).

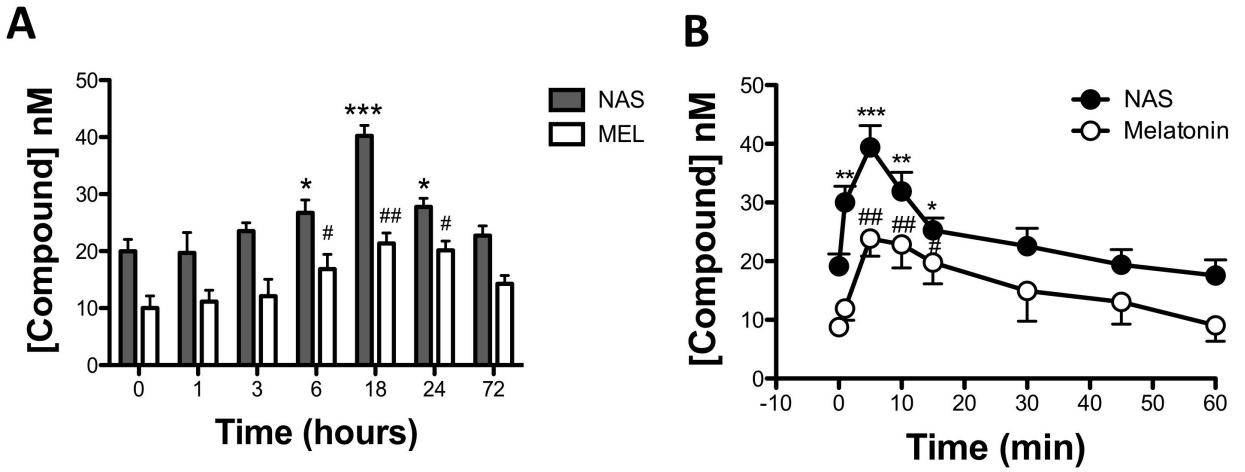

Figure 3.4. Time-course on the effect of GSK on melatonin levels. A) Columns presenting the concentrations of NAS and melatonin calculated as described in material and methods. Values represent the mean s.e.m. of six independent experiments (*p < 0.05 and **p < 0.01 versus time 0). B) Line graphs presenting the concentrations of NAS (filled circles) and melatonin (open circles) calculated from the chromatographic studies. Values represent the mean s.e.m. of six independent experiments (*p < 0.05, **p < 0.01, ***p<0.001 versus time 0 for NAS and #p < 0.05, ##p < 0.01 versus time 0 for melatonin).

Concentration-response effect of GSK1016790A over AANAT and its phosphorylation

After the observation of the time required to promote the maximal AANAT expression on a long term assay after activating TRPV4 channel for 18 hours, and the demonstration that 5 minutes is enough to maximize AANAT phosphorylation, a dose-response analysis was performed both at the long term and short term activation by TRPV4 agonist.

Different graded concentrations were applied to ciliary non-pigmented epithelial cell cultures ranging from 1nM to 10 μM. After 18 hours incubation of GSK1016790A, AANAT expression was the highest at 10^{-8} M (10 nM) concentration of TRPV4 agonist (Fig. 3.5). Transformation of western-blots into a dose-response curve allowed the observation of a sigmoidal pattern which provided a pD_2 value for GSK of 8.34 ± 0.302, which was equivalent to an EC_{50} value of 4.57 nM (n=3, figure 3.5 A).

Figures 3.5C and D, shows the same behavior observed in the case of AANAT phosphorylation, it was possible to observe that the phosphorylation of AANAT reached a maximum at a concentration of GSK of 10^{-8} M (10 nM). And the transformation of western-blots into a concentration-response curve permitted us to observe a sigmoidal pattern which provided a pD_2 value for GSK of 8.5 ± 0.3, which was equivalent to an EC_{50} value of 2.87 nM (n=6, figure 3.5 D).

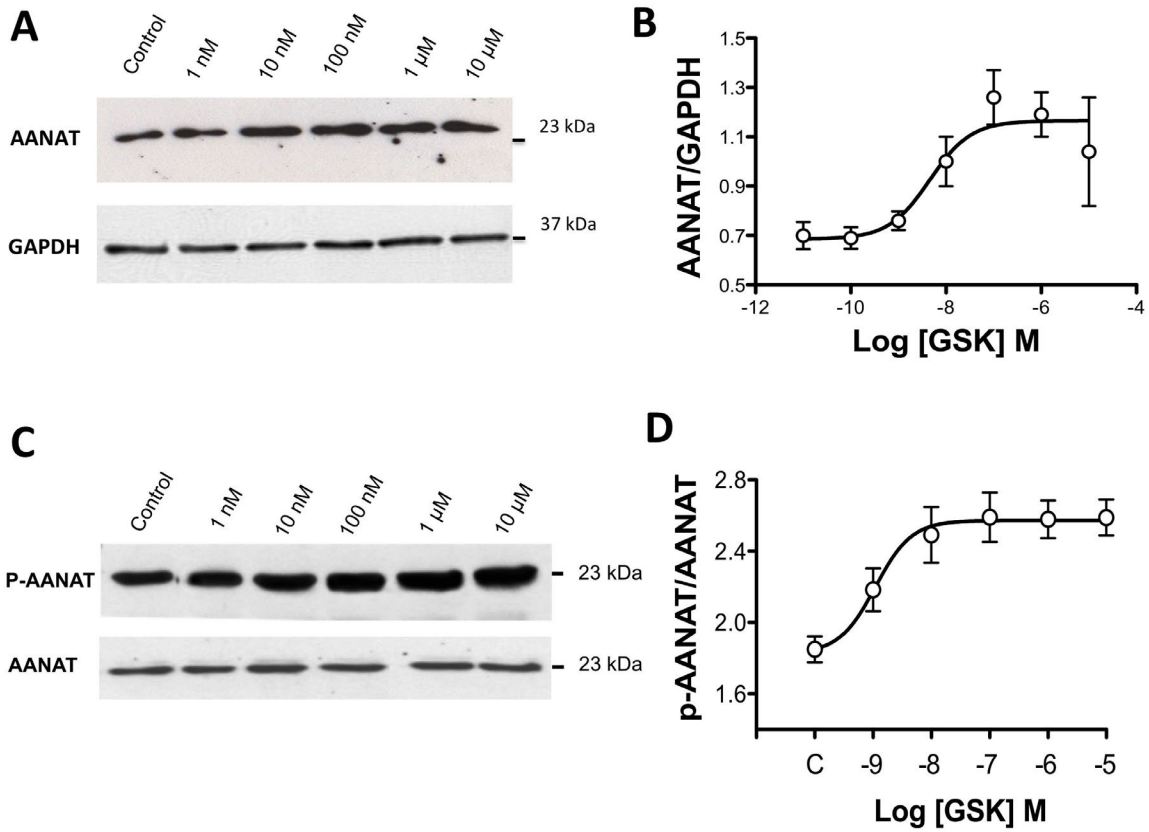

Figure 3.5. A) Representative western-blot study showing the concentration dependency of AANAT when cells are challenged with GSK1016790A ranging from 1 nM to 10 M. B) Concentration-response curve plotted with the relative quantification of the western-blot band intensities. The values represent the mean s.e.m. of five independent experiments. C) Representative western-blot study showing the concentration dependency of AANAT phosphorylation when cells are challenged with GSK1016790A (GSK) ranging from 1 nM to 10 M. D) Concentration-response curve plotted with the relative quantification of the western-blot band intensities. The values represent the mean s.e.m. of six independent experiments.

TRPV4 antagonist effect

The application of GSK1016790A showed an increase of both AANAT and pAANAT in a long and short period of time, 18 hours and 5 minutes for AANAT and pAANAT, respectively. This increment was blocked after applying a non-selective TRPV1/ TRPV4 antagonist RutheniumRed (RR) and by the selective antagonist of the TRPV4; RN-1734, ($P<0.01$ for both compound vs. GSK alone; n=5, Fig. 3.6 B.

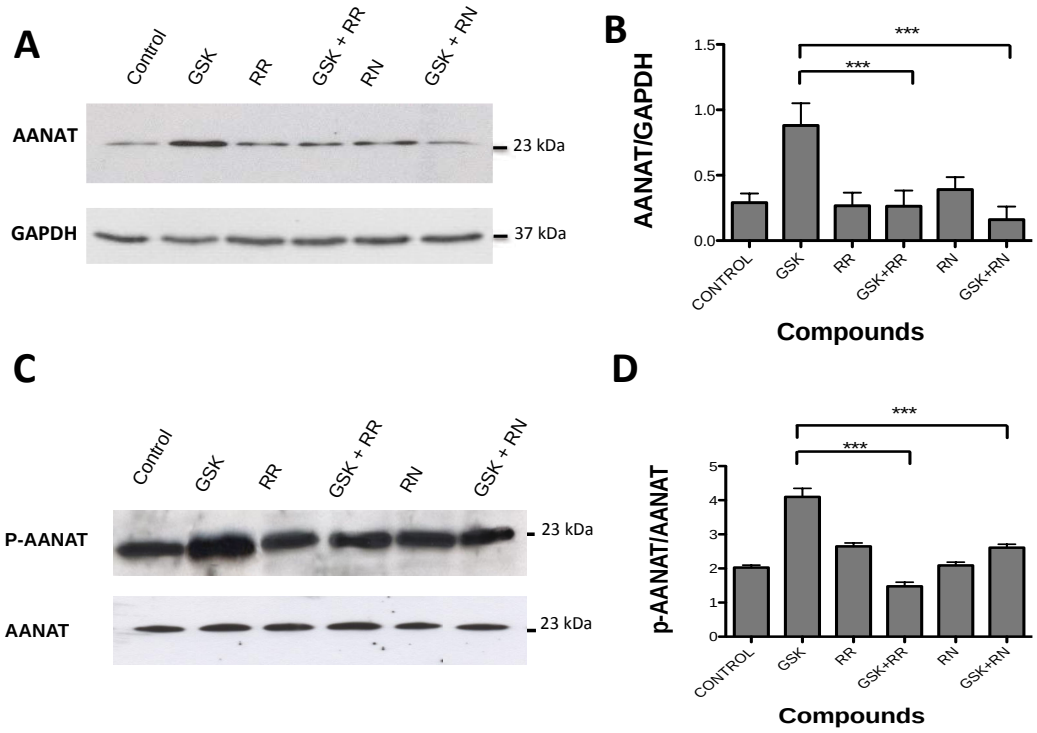

Figure. 3.6. Effect of TRPV4 antagonists on AANAT triggered by GSK1016790A. A) Representative western-blot study showing the activity of TRPV4 antagonist Ruthenium Red (RR) and RN-1734 (RN), both alone and together with GSK1016790A, following the protocol described in methods. B) Column plot showing the relative quantification of the western-blots band intensities. Values represent the mean s.e.m. of five independent experiments (***$p < 0.001$ versus GSK1016790A effect). C) Representative western-blot study showing the activity of TRPV4 antagonists. D) Column plot showing the relative quantification of the western-blots band intensities. Values represent the mean s.e.m. of six independent experiments (***$p < 0.001$ versus GSK1016790A effect).

As shown in figure 3.6, 10 nM GSK (5 min), produced a significant increase compared to non-treated cells. This rise in AANAT phosphorylation was blocked by Ruthenium Red (RR) and by the selective antagonist of the TRPV4 RN-1734 (***p<0.001 for both compounds vs. GSK alone; n=6, figure 3.6 D). Interestingly, when applied alone, neither RR nor RN-1734 was able to change AANAT phosphorylation (n=6).

Silencing TRPV4

To fully confirm the involvement of a TRPV4 channel in the phosphorylation of AANAT, studies were performed with siRNA for this protein. As can be seen in figure 5A, the incubation of cells with the siRNA for TRPV4 produced a very significant reduction in the expression of this membrane protein with a decrease of 85.8 % (***p<0.001, n=4, figure 3.7).

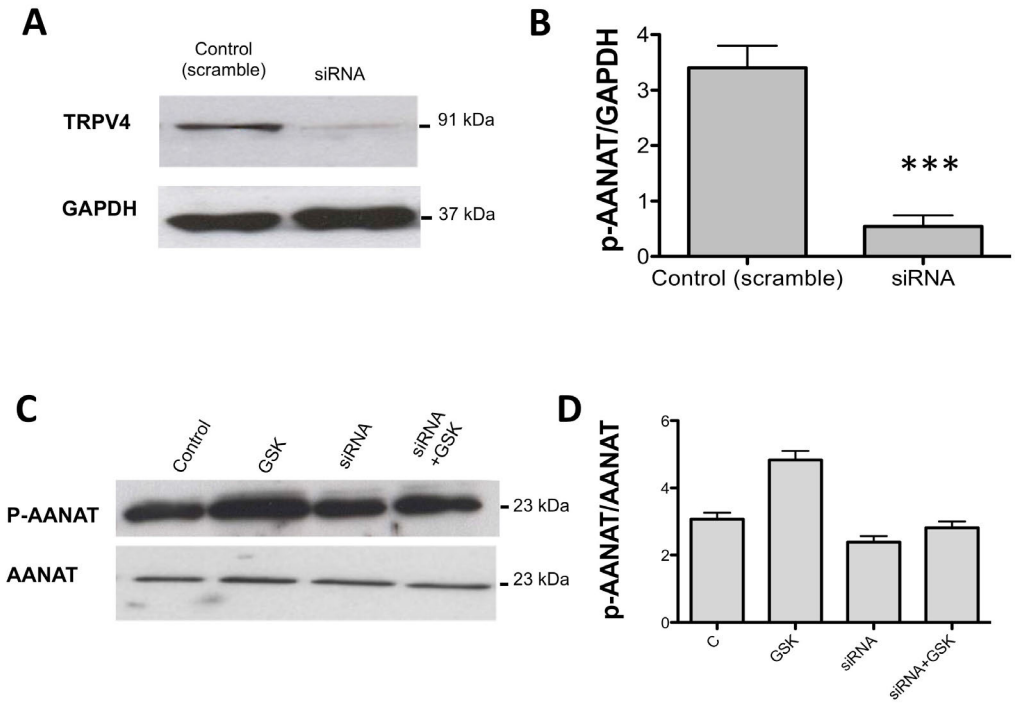

Figure. 3.7. A) Representative western-blot showing the reduction in TRPV4 expression after the incubation with a siRNA against this protein. B) Relative quantification of the western-blots band intensities presented in A. Values represent the mean s.e.m. of four independent experiments (***p < 0.001 versus control). C) Western-blot showing AANAT

phosphorylation and how TRPV4 silencing abolishes this process. B) Relative quantification of the western-blots band intensities presented in A (***p<0.001, n=4).

When the silencing conditions previously demonstrated were obtained, cells were challenged with the TRPV4 agonist GSK. As observed in figure **, western-blots indicated that GSK was practically unable to induce AANAT phosphorylation when compared to GSK in untreated cells. Quantification of western-blots confirmed that TRPV4 siRNA blocked the effect of GSK on AANAT phosphorylation (***p<0.001, n=4, figure 6B), and that this silencing effect was also measurable in the melatonin levels (figure 3.8). The effect of the siRNA changed melatonin levels from 24.2 ± 3.2 nM (GSK in non-treated cells), to 10.4 ± 2.7 nM (***p<0.001, n=4, figure 3.8).

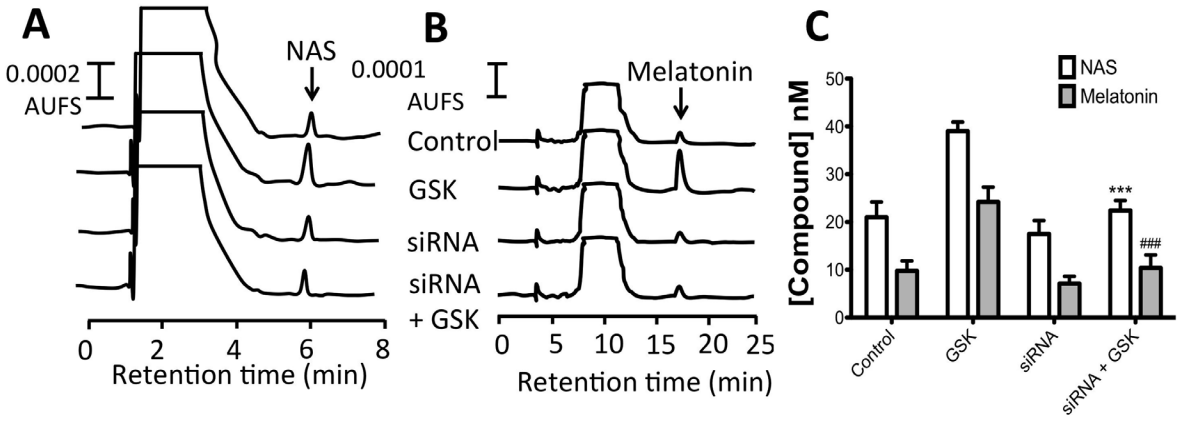

Figure. 3.8. Effect of TRPV4 silencing on AANAT phosphorylation on NAS and melatonin concentrations. A) Representative chromatograms showing the variations in NAS. B) melatonin chromatograms with GSK, siRNA and GSK + siRNA. B) Bar plot presenting the concentrations of NAS and melatonin calculated from the chromatographic studies. Values represent the mean s.e.m. of four independent experiments (***p < 0.001 versus control for NAS and ###p < 0.001 versus control for melatonin).

Effect of the second messenger inhibitors on AANAT phosphorylation triggered by GSK

In order to see how the activation of the TRPV4 was able to induce AANAT phosphorylation, several inhibitors of the adenylate cyclase and calmodulin pathways were tested in their ability to reverse the effect produced by GSK. As presented in figure 3.9,

four different inhibitors were assayed: SQ 22536 (an adenylyl cyclase inhibitor), KT 5720 (a protein kinase An inhibitor), Calp2 (an inhibitor of Ca-calmodulin) and Autocamid-2 related inhibitory peptide (inhibitor calmodulin dependent protein kinase II, CMPKII). In figure 3.9 A, it was possible to observe that the increase of phosphorylation induced by 10 nM GSK was not significantly affected by either, the adenylate cyclase inhibitor SQ 22536 or the protein kinase inhibitor KT 5720 (n=4). When applied alone, both inhibitors were able to reduce slightly but significantly control phosphorylation levels of AANAT (results not shown).

On the contrary, the application of the inhibitor of calmodulin, Calp2 and the inhibitor of CMPKII, Autocamid-2, significantly abolished the effect triggered by GSK (figure 3.9A). The effect of both inhibitors returned GSK values to those close to the control (in the absence of GSK, figure 3.9 B) (***p<0.001 for both inhibitors vs. GSK; n=4). As happens with the inhibitors of adenylate cyclase and PKA, when applied alone, Calp2 and Autocamid-2, reduced slightly but significantly the level of phosphorylation of control AANAT (Results not shown).

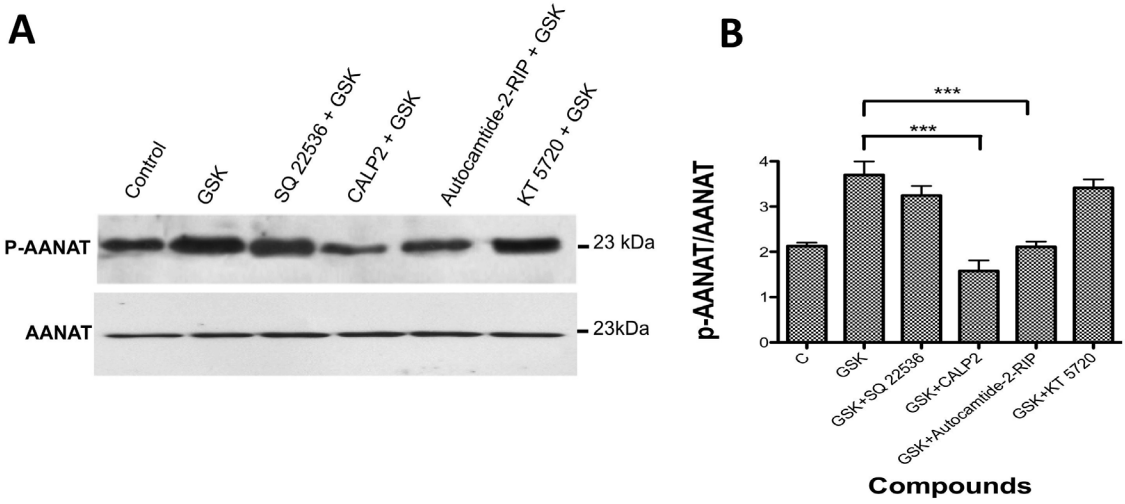

Figure. 3.9. Effect of cAMP-PKA and calmodulin-CAMPKII inhibitors on GSK1016790A induced AANAT phosphorylation.A) Representative western-blot showing the lack of inhibition of SQ 22536 (an adenylyl cyclase inhibitor), KT 5720 (a protein kinase A

inhibitor), on the phosphorylation effect of GSK1016790A (GSK) on AANAT, and the inhibition conducted by Calp2 (an inhibitor of Ca-calmodulin) and Autocamid-2 (Auto) related inhibitory peptide (an inhibitor calmodulin dependent protein kinase II, CMPKII) following the protocol described in methods. B) Column plot showing the relative quantification of the western-blots band intensities. Values represent the mean s.e.m. of four independent experiments (***p < 0.001 versus GSK1016790A effect).

In a parallel way, the levels of NAS and melatonin were investigated when cells were treated with the indicated compounds. As can be seen in figure 9A, NAS and melatonin chromatographic peaks changed in a similar way to what occurred with AANAT phosphorylation. The quantification of such peaks indicated a strong inhibition of NAS and melatonin concentration by Calp-2 (**p<0.01 for NAS, $^{\#\#}$p<0.01 for melatonin, n=4) and Autocamid-2 (***p<0.001 for NAS, $^{\#\#\#}$p<0.001 for melatonin, n=4), while the other agents did not significantly change the effect triggered by GSK (n=4, figure 9B).

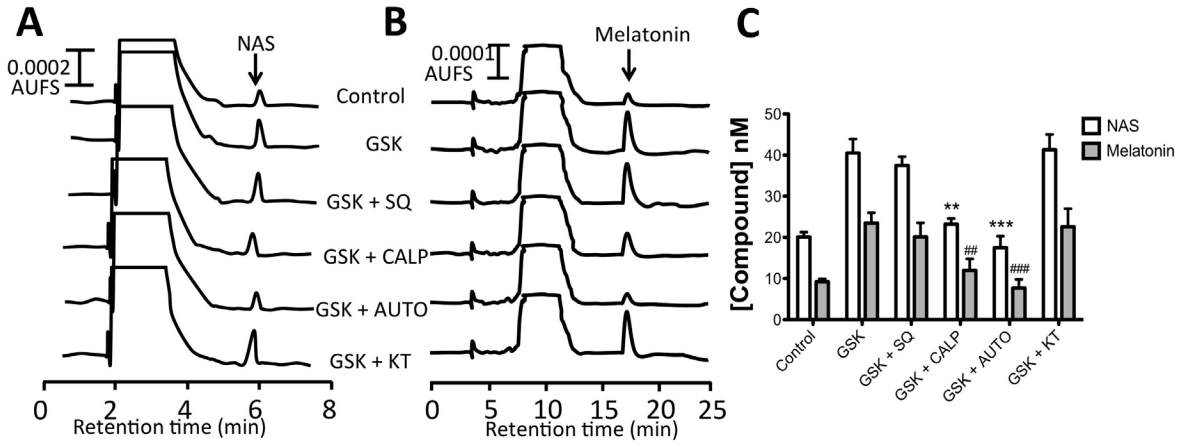

Figure. 3.10. Effect of cAMP-PKA and calmodulin-CAMPKII inhibitors on GSK1016790A induced NAS and melatonin concentrations. A) Representative HPLC profiles showing the changes in NAS (left set of chromatograms) and melatonin levels (right chromatograms) depending on the second messenger inhibitors tested. B) Bar plot presenting the concentrations of NAS and melatonin calculated from the chromatographic studies. Values represent the mean s.e.m. of four independent experiments (**p<0.01, ***p < 0.001 versus GSK1016790A effect for NAS and $^{\#\#}$p<0.01, $^{\#\#\#}$p < 0.001 versus GSK1016790A effect for melatonin).

Discussion

In the present chapter, TRPV4 channel stimulation has been described in the human non-pigmented ciliary body epithelial cells. TRPV4 activation was able to increase the rate limiting enzyme of melatonin synthesis aralkylamine N-acetyltransferase (AANAT) after a long period of incubation by a specific agonist, as well as its ability to phosphorylate AANAT after 5 minutes of stimulation. Both AANAT and pAANAT maximal increment were discovered to be time and dosage dependent by the TRPV4 specific agonist GSK1016790A (Vincent et al., 2009). This effect was blocked by TRPs antagonist Ruthenium Red and with a selective TRPV4 antagonist RN-1734 (Thorneloe et al., 2008). Abolishing TRPV4 by using siRNA specific for TRPV4 has been experimented to confirm the observed effect of TRPV4 channel on AANAT and its phosphorylation. It has also been possible to demonstrate that, after the activation with GSK1016790A for a short period of time, a cascade of intracellular events involves the participation of calmodulin and calcium-calmodulin dependent protein kinase II. Interestingly, when measuring the concentrations of melatonin, it was possible to observe that in all the cases where AANAT was phosphorylated a concomitant rise in melatonin levels was observed.

In a previous chapter (refer to chapter 1), the presence of the TRPV4 channel was demonstrated in human non-pigmented epithelium cells, and that TRPV4 stimulation increases melatonin levels. In order to understand the significance of this channel stimulation in the eye, measuring melatonin levels in the aqueous humor of healthy and elevated IOP subjects showed an increase of melatonin concentration in patients with high IOP. Hence, the existence of a TRPV4 able to detect the rise in pressure or variations in the aqueous humor osmolarity, may act as a sensor to reduce IOP by increasing the

extracellular concentrations of melatonin. This substance will act through melatonin receptors reducing aqueous humor formation (Huete-Toral et al., 2015).

TRPV4 channel was first discovered as an osmosensor; a strech-gated channel. Currently, it is recognized that the TRPV4 function extends remarkably beyond its role as an osmoregulator and that this ion channel is involved in mediating numerous arrays of physiological and pathological conditions (Liedtke, 2007). In fact, the polymodal TRPV4 channel is believed to be essential for osmoregulation and Ca^{2+} homeostasis in the mammalian ciliary body. A recent study hasidentified TRPV4 presence in mice non-pigmented ciliary epithelial cells (pigmented C57BL/6J, non-pigmented B6.Cg-Tg(Thy1-YFP)HJrs/J) and it has been discovered that calcium ions potentiate non-pigmented epithelium swelling, beside the observation of differential osmoregulation and Ca^{2+} homeostasis mechanisms in NPE vs. PE (Jo et al., 2016). Investigations have shown that TRPV4 channel is expressed in secretory and absorptive epithelia, regulating Ca^{2+} signaling, volume changes, cytoskeletal remodeling, and responses to shear flow and mechanical stress (Harteneck and Reiter, 2007; Krizaj et al., 2014; Mamenko et al., 2015; Sokabe et al., 2010).

The physiological importance of the tandem TRPV4-melatonin in the regulation of IOP has already been demonstrated (refer to first chapter). However, little is known about the mechanism connecting TRPV4 and melatonin. In the present chapter, TRPV4 activation induced typical Ca^{2+} transient. However, Ca^{2+} regulation of TRP channels is complex, they often show Ca^{2+} dependent desensitization to many stimuli by multiple mechanisms including the action of Ca^{2+} calmodulin (Rosenbaum et al., 2004; Zhu, 2005). In the case of TRPV4, Ca^{2+} increases the channel response to hypotonicity (Loukin et al., 2015). The presence of the cation in the cytosol may bind to calmodulin to further activate

calcium-calmodulin dependent protein kinase II, as can be seen in figures 3.9 and 3.10. This is an interesting mechanism by which AANAT is phosphorylated, and it is an alternative to the one produced by cAMP and PKA already described (Ganguly et al., 2001) or the one mediated to PKC (Choi et al., 2004).

The study of PKA phosphorylation of AANAT deserves special attention, since AANAT phosphorylation stimulates the formation of a complex with 14-3-3 proteins, this mechanism plays an important role in regulating melatonin diurnal synthesis. Indeed, it has been demonstrated that in darkness, when the complex is formed, the amount of the produced melatonin can rise 10 times in the pineal gland (Jones et al., 1995; Klein et al., 2002). In the present work, melatonin concentrations could rise 2.5 times when AANAT is phosphorylated. This is clearly indicating that the mechanism triggered by the TRPV4 in the ciliary body is not so robust as the one present in the pineal gland, however it may produce melatonin in the aqueous humor in concentrations which are sufficient to reduce IOP, since melatonin receptors present in the ciliary are activated by nanomolar concentrations (Alarma-Estrany et al., 2008).

The regulation of AANAT by a phosphorylation dependent of CAMPKII is interesting and offers another pathway used by many receptors and channels to regulate the synthesis of melatonin, at least in the eye, and in this cellular model in particular. The analysis of the human AANAT sequence (GenBank: AAH92430.1), shows a sequence which fits in the consensus sequence for CAMPKII as previously indicated (Pearson and Kemp, 1991; Songyang et al., 1996), supporting the findings described in the present work.

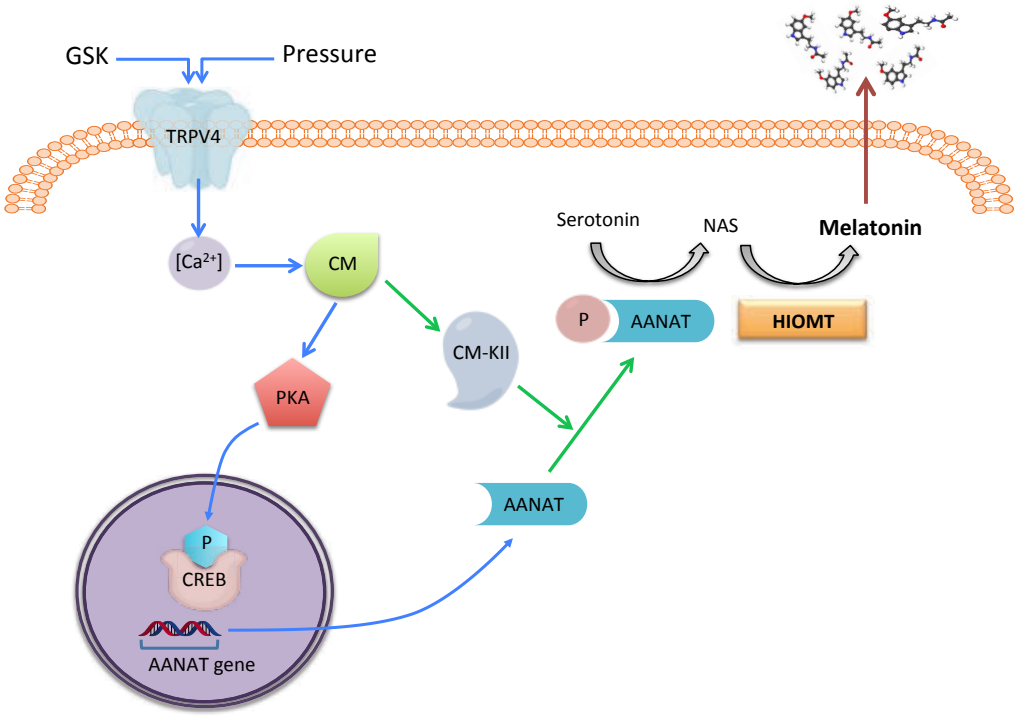

Figure 3.11. Possible mechanism of acttion of GSK acting on the TRPV4 channel in the ciliary body. After the activation of the TRPV4 the influx of Ca^{2+} will bind to calmodulin (CM) which from on side activates adenylate cyclase and later protein kinase A (PKA). This kinase will finally produce the phosphorylation of CREB (cAMP response element-binding) then may stimulate the synthesis of the enzyme AANAT. From the other hand, CM binds to calcium-calmodulin dependant kinase II to phosphorylate AANAT. This protein together with HIOMT will finally augment the production of melatonin in ciliary body epithelial cells. TRPV4 channel can be stimulated by the abnormal elevation in the IOP that often occur in glaucoma pathology.

Reducing IOP is a very important target for patients who present glaucoma, this being the main risk factor for developing glaucoma. This ocular neurophathy, progressively and irreversibly damages the retinal cells. The aqueous humor is essential for the regulation of intraocular pressure, if its formation increases in an abnormal manner, elevated intraocular pressure occurs. However, a different case is also possible, when aqueous humor drainage is decreased through the uveoscleral outflow pathway or the conventional way through the trabecular meshwork (Acott et al., 2014; Stamer and Acott, 2012). Searching for new treatments to lower IOP is a serious challenge, because all current treatments present several adverse effect which can even result in patients

eventually discarding the treatment (Lo et al., 2016). In previous studies, in the search for new treatments for glaucoma, TRPV4 was linked to trabecular meshwork cells. Authors have studied the role of TRPV4 mediating Ca^{2+} influx in the trabecular meshwork, the inhibition of TRPV4 by using an antagonist in glaucoma mice resulted in a decrease of the IOP (Ryskamp et al., 2016) suggesting that this could protect the retinal ganglion cells from mechanical stress by TRPV4 inhibition (Krizaj, 2016; Krizaj et al., 2014). However, different studies showed the opposite effect on IOP after TRPV4 activation. In lowe syndrome patients, TRPV4 activation actually lowered IOP by acting on the cilia present in the trabecular meshwork (Luo et al., 2014) These findings can be explained by understanding the mutations that this class of patients have.

From a pharmacological perspective, the fact of TRPV4 synthetic agonists being able to trigger a mechanism that produces a melatonin-induced reduction of IOP, opens the possibility to look for new agents to fight against those cases of glaucoma related with an abnormal increase of IOP. Indeed, it has been already demonstrated that the application of GSK1016790A in mice can reduce IOP by acting on TRPV4 located in the trabecular meshwork (Luo et al., 2014), one of the drainage site for the aqueous humor. The development of new selective compounds, to add to the already available (Vincent et al., 2009), suitable to activate the TRPV4 present in these two ocular locations, may join the pharmaceutical compounds commercially available for the treatment of ocular hypertension and glaucoma.

Chapter IV: Melatonin and α_1-adrenergic receptor-receptor interaction: A new pharmacological approach for glaucoma treatment

“What is now proved was once only imagined.”

William Blake

Introduction

Glaucoma, a pathology characterized by visual field loss, is associated with the damage of the optic nerve (Casson et al., 2012b). The main risk factors are *inter alia* aging, genetic conditions, intraocular pressure (IOP) and myopia (Quigley, 2011). Without treatment glaucoma leads to blindness, being the second cause of blindness in the world. In this sense, it has been estimated that about 61 million people in the world suffer from glaucoma, and the afflicted individuals may increase to about 80 million in year 2020 (Quigley and Broman, 2006). Nearly three quarters of all glaucoma occurs in individuals with irido-corneal open angles, and open angle glaucoma (OAG) is the most common form of glaucoma in nearly all countries. While some forms of OAG occur secondary to other phenomena, the vast majority is idiopathic and therefore is referred to as primary open angle glaucoma (POAG) (Quigley and Broman, 2006).

Since IOP is the only modifiable risk factor known to date, glaucoma treatment has focused on decreasing IOP, which is proven to slow the progression of the disease, decrease the rate of visual field loss, thus, protecting against visual function loss and blindness (2009; Boland et al., 2013). Although IOP measurments may fluctuate, IOP values in adult humans are established from 10 till 20 mm Hg (normotensive IOP). When the value exceeds 21 mm Hg ocular hypertension is diagnosed. A high and persistent ocular hypertension results in damage of the optic disc, causing the degeneration of the ganglion cells and subsequent blindness. The relationship between ocular hypertension and glaucomatous pathology has been well known for many years. Even today with the current and effective therapeutic arsenal to decrease IOP, ocular hypertension is still considered

the most important risk factor for the development of this disease (Rossetti et al., 2015; Tamm et al., 2015).

Many different agents have been used to reduce the ocular hypertension that presents glaucoma patients. These include parasympathomimetics, adrenergic receptor antagonists, carbonic anhydrase inhibitors or prostaglandins, the later the most popular nowadays (Hommer, 2010; Lee, 2005). These drugs are prescribed depending on the patients' characteristics, mainly because all present notorious side effects. Looking for better and safer anti-hypertensive drugs, pharmaceutical companies have started to combine the aforementioned agents, providing combinations that may be more effective at reduced doses and with reduced side effects (Polo et al., 2001; Sakai et al., 2005).

On this regard, anti-glaucomatous compounds can act by reducing IOP in the ciliary body, which participate in aqueous humor formation by means of a strict control carried out, in the healthy eye, by a wide variety of neurotransmitters and messengers (Delamere, 2006). Adrenoreceptor ligands, in particular, α-adrenergic agonists and β-adrenergic antagonists, decrease IOP due to the presence of both types of adrenergic receptors in the ciliary body (Kiuchi et al., 1992; Murray and Leopold, 1985; Naito et al., 2001). Concerning α-adrenergic receptors, and in particular α_1-adrenoceptors, studies in different animal models indicated that phenylephrine produce a reduction in IOP which can be blocked by a selective antagonist, prazosin.

Adrenergic-receptor family are members of GPCRs, which possesses an extracellular N-terminal domain and an intracellular C-terminal domain and different intracellular and extracellular loops. Intracellular loops allow adrenergic receptors to interact with cascade signaling proteins such as β-arrestin and dinamine (Cotecchia, 2010;

Small et al., 2006; Tan et al., 2009); while the extracellular loops form a structural "pocket" which allows ligand binding to the receptor.

The α1 adrenergic receptor is the first member of the α-receptor subfamily, and it consists of three subtypes receptors called α_{1A}, α_{1B} and α_{1D} that differentiate among them by their pharmacological properties and localization in the organism. In general, α_1 adrenergic receptors are coupled to Gq protein, which activation induced by the exchange of GDP by GTP produces the activation of phospholipase C (PLC) and the consequent phosphorylation of protein kinase C (PKC). This phospholipase hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP2) to inositol triphosphate (IP3) and diacylglycerol (DAG). These secondary metabolites allow the opening of the calcium channels of the endoplasmic and sarcoplasmic reticulum thus increasing intracellular calcium levels, with the activation of a cascade of phosphorylation that leads to the induction of several transcription factors (Johnson and Liggett, 2011; Maronde and Stehle, 2007; Schmitz et al., 1981).

A different approach in the treatment of glaucoma is by the use of melatonin and/or its analogues which are proven to decrease IOP as well as working as a neuroprotectant (Belforte et al., 2010; Crooke et al., 2012; Ismail and Mowafi, 2009). Interestingly, α1 adrenergic antagonist prazosin interacts with melatonin receptors by inhibiting melatonin's action in normotensive model (Dubocovich, 1995; Pintor et al., 2003). Such an observation opened the window of the possible interaction between melatonin receptors and α_1 adrenergic in the ciliary body. The present section of this thesis describes a novel sight of melatonin-α_1 adrenergic receptors heteromerization with a profound work using biochemical techniques together with the use of actual glaucomatous model and human samples of healthy and diseased subjects to confirm our hypothesis.

Material and methods

Drugs and animals

Melatonin, IIK7 (a selective MT2 receptor agonist) (Sigma, St. Louis, USA), Prazosin (α_{1A}-adrenergic receptor antagonist), (R)-(-)-Phenylephrine hydrochloride (α_{1A}-adrenergic receptor agonist), Luzindole (non-selective melatonin antagonist), 4-phenyl-2-propionamidotetralin (4PPDOT, MT_2 selective antagonist) were purchased from Tocris Bioscience (Bristol, UK). All drugs were formulated in isotonic saline containing 1% DMSO (Sigma, St. Louis, USA) at a concentration of 100 μM.

Experiments were performed on female C57BL/6J (n=5) (control animals) and DBA/2J (n=5) (glaucomatous animals) mice obtained from the European distributor of Jackson Laboratories Mice (Charles Rivers Laboratories). All animal maintenance and experimental procedures followed Spanish and European guidelines for animal care in the laboratory and animal research (Guide for the Care and Use of Laboratory Animals) and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Mice were housed (1-4 mice per cage) in temperature and light-controlled rooms maintained according to a 12-h light/dark cycle; all animals were fed ad libitum. DBA/2J and C57BL/6J mice were studied at 3 and 12 months of age.

Cells

A human non-pigmented ciliary epithelial (59HCE) immortalized cell line was kindly supplied by Dr. Coca-Prados (Yale University). Cells were grown in high glucose Dulbecco's modified Eagle's medium (Gibco/Invitrogen, Carlsbad, CA) containing 10% foetal bovine serum (Sigma-Aldrich, St. Louis, MO) and 0.05 mg/ml penicillin/

streptomycin (Gibco/Invitrogen) at 37°C in humidified atmosphere 5% CO_2–95% air. After the culture reached the confluence, cells were detached with 0.25% trypsin and seeded into 6 well plates and/or to 4 well chamber slides respectively. All the experiments were performed using cells comprised numbers 10-15 passages to assure assays reproducibility.

HEK-293T cells were grown in DMEM (Invitrogen) supplemented with 2mM L-glutamine, 100 U/ml penicillin/streptomycin, and 5% (v/v) heat inactivated fetal bovine serum (all supplements were from Invitrogen). Cells were maintained at 37ºC in an atmosphere of 5% CO_2 and were passaged when they were 80-90% confluent (i.e., approximately twice a week).

Fusion proteins and expression vectors

The human cDNAs for the MT_1, MT_2 and α_{1A} receptors was 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 (α_{1A}). The fragments were then subcloned to be inframe with *Renilla* luciferase (Rluc) into the EcoRI and BamHI (α1A) restriction sites of an Rluc-expressing vector (pRluc-N1, PerkinElmer, Wellesley, MA), or into the BamH1 and HIND III (MT1, MT2) restriction sites of a yellow fluorescence protein YFP expressing vector, to give the plasmids that express MT1 ,MT2, and α_{1A} receptors fused to Rluc or YFP on the C-terminal end of the receptor (α1A –Rluc, MT1–YFP or MT2–YFP).

Transient transfection and sample preparation for BRET and FRET experiments

HEK-293T cells growing in 6-well dishes were transiently transfected with the corresponding fusion protein cDNA by the PEI (PolyEthylenImine, Sigma, Steinheim, Germany) method. Cells were incubated (4h) with the corresponding cDNA together with PEI (5.47 mM in nitrogen residues) and 150 mM NaCl in a serum-starved medium. After 4 hours, the medium was changed to a fresh complete culture medium. Forty-eight hours after transfection, cells were washed twice in quick succession in HBSS with 10 mM glucose, detached, and resuspended in the same buffer containing 1mM EDTA. To control the cell number, sample protein concentration was determined using a Bradford assay kit (Bio-Rad, Munich, Germany) using bovine serum albumin dilutions as standards. Cell suspension (20 µg of protein) was distributed into 96-well microplates; black plates with a transparent bottom were used for FRET and fluorescence determinations, while white plates with white bottom were used for BRET.

BRET and FRET assays

HEK-293T cells were transiently co-transfected with a constant amount of the cDNA encoding for receptor fused to Rluc and with increasingly amounts of the cDNA corresponding to receptors fused to YFP. To quantify receptor-YFP expression, cells (20 µg protein) were distributed in 96-well microplates (black plates with a transparent bottom), and fluorescence was read in a Fluo Star Optima Fluorimeter (BMG, Lab Technologies) equipped with a high-energy xenon flash lamp, using a 10 nm bandwidth excitation filter at 400nm reading. Receptor-fluorescence expression was determined as fluorescence of the sample minus the fluorescence of cells expressing the BRET donor

alone. The equivalent of 20 µg of cell suspension was distributed in 96-well microplates (Corning 3600, white plates; Sigma), and 5 (Corning 3600, white plates; Sigma), and 5µM coelenterazine H (Invitrogen) was added. After 1 min of adding coelenterazine H, the readings were collected using a Mithras LB 940 that allows the integration of the signals detected in the short-wavelength filter at 485 nm (440-500nm) and the long-wavelength filter at 530nm (510.590nm). To quantify receptor-Rluc, luminescence readings were also performed after 10 min of adding 5 µM coelenterazine H. The net BRET is defined as [(long-wavelength emission)/(short-wavelength emission)] for the donor construct expressed alone in the same experiment. BRET is expressed as milli-BRET units (mBU = net BRET X 1000). The net BRET is defined as [(long-wavelength emission)/(short-wavelength emission)]-Cf where Cf corresponds to [(long-wavelength emission)/(short-wavelength emission)] for the Rluc construct expressed alone in the same experiment.

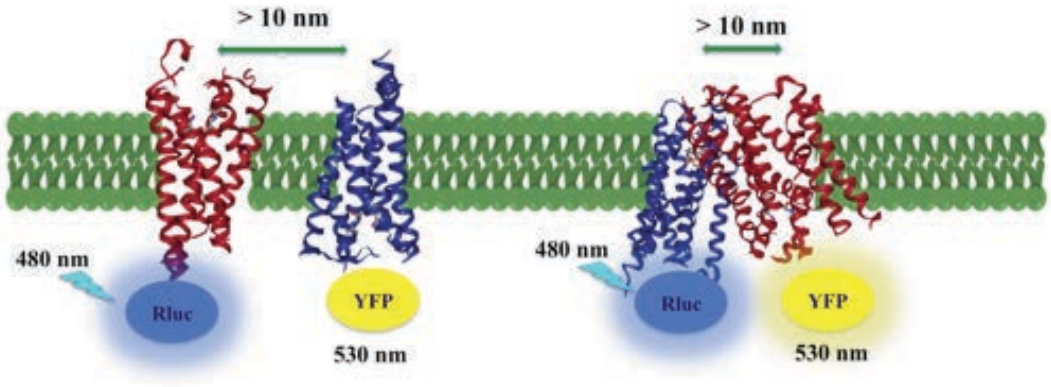

Figure 4.1. Illustration of BRET technique to study protein-protein interaction. For instance, two fusion proteins with Rluc and YFP must be coexpressed, and luminescent signals are measured at 480 nm (Rluc light emission) and 530 nm (YFP light emission) upon addition of the Rluc substrate coelenterazine. If one protein does not interact with the other and if Rluc and YFP are not at a BRET-permissive distance (>10nm) and orientation, non-radioactive light emission is mainly measured at 480 nm. On the contrary, if both proteins are in close proximity and interacting together, placing Rluc and YFP at a BRET-permissive distance (<10nm) non-radioactive energy transfer can be measured at an increased light emission at 530 nm.

Label-free dynamic mass redistribution (DMR) assay

The global cell signaling profile was measured using an EnSpire Multimode Plate Reader (Per- kinElmer, Waltham, Massachusetts, US). This label-free approach uses refractive waveguide grating optical biosensors, integrated into 384-well Epic® (Corning®, NY, USA) sensor microplates. Changes in local optical density are measured in a detection zone up to 150 nm above the surface of the sensor. Cellular mass movements induced upon receptor activation are detected by illuminating the underside of the biosensor with polychromatic light and measured as changes in the wavelength of the reflected monochromatic light. These changes are a function of the refraction index. The magnitude of this wavelength shift (in picometers) is directly proportional to the amount of DMR. Briefly, 24 h before the assay, cells were seeded at a density of 10,000 cells per well in 384-well sensor microplates with 30 µl growth medium and cultured for 24 h (37°C, 5% CO2) to obtain 70%–80% confluent monolayers. Previous to the assay, cells were washed twice with assay buffer (HBSS with 20 mM HEPES, pH 7.15) and incubated for 2 hours in 30 µl per well of assay-buffer with 0.1% DMSO in the reader at 24°C. Hereafter, the sensor plate was scanned, and a baseline optical signature was recorded before adding 10 µl of test compound dissolved in assay buffer containing 0.1% DMSO. Data were normalized and expressed as % of maximum activation induced by test compound. For analysis of DMR data, the AUC values of DMR signals between the 0 and 3600 s time points with mean and SEM were used to calculate agonist activity. AUC values were transformed into relative AUC units to give equivalent baseline optical recordings for all dose–response curves from any given assay plate. Data were then normalized and expressed as % of maximum (100%) activation induced by a saturating concentration of

LPI. Data were additionally analysed by determining the slopes of tangents to the origin of the real-time traces to capture the steepness of the initial ascending parts. Kinetic results were analyzed using EnSpire Workstation Software v 4.10.

cAMP determination

Two hours before initiating the experiment, 59HCE cells or HEK-293T cell-culture medium was exchanged to serum-starved DMEM medium. Then, cells were detached and resuspended in growing medium containing 50 μM zardaverine 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.

Readings were performed after 15 min incubation at 25º. 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).

Intracellular calcium release

Cells were co-transfected with the cDNA for the indicated receptors and 3 μg of GCaMP6 calcium sensor (Chen et al., 2013) using lipofectamine 2000 (Thermo Fisher Scientific). 48 hours after transfection, cells (150.000 HEK-293T cells/well in 96-well black, clear bottom microtiter plates) were incubated with 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 and receptor ligands were added as indicated. Fluorescence emission intensity of GCaMP6 was recorded at 515 nm upon

excitation at 488 nm on the EnSpire® Multimode Plate Reader for 335 s every 15 s and 100 flashes per well.

Arrestin recruitment assays

Arrestin recruitment was determined using BRET experiments as described above in HEK-293T cells expressing the cDNA corresponding to β-arrestin-2-Rluc (1 µg transfected), MT_1-YFP (1,5 µg transfected) or MT_2-YFP (1,5 µg transfected) alone or with α_{1A} (0.05 to 0,5 µg transfected) after the indicated treatment with ligands.

Intraocular pressure measurements

Mice were anesthetized by inhalation of isoflurane, by means of the Matrx VIP 3000 Calibrated Vaporizer (Midmark, OH, USA). Oxygen is mixed with isoflurane and sent to two outflows at 500cc/ minute, delivering 2.5% of isoflurane in oxygen to the animal. One outflow enters a box where mice were placed into for initial sedation. After about 2 min, the sedated animal was positioned for IOP measurement and clinical examination with a nose cone delivering the isoflurane gas/oxygen mixture. The nose cone permitted access to the eyes.

The above-mentioned substances were applied in drops to the cornea at a fixed volume of 2 µl in both eyes. IOP was measured by means of a TonoLab® non-invasive rebound tonometer supplied by Tiolat Oy (Danias, Kontiola et al. 2003, Wang, Millar et al. 2005). The tonometer is placed in a fixed position and the sensor aligns with the optical axis of the eye between 1-4 mm apart. Both eyes of each animal were measured immediately after the application of the anesthesia. Six consecutive measurements were taken for each reading, and three readings were obtained on each eye. To avoid the

putative effect of the circadian rhythm, the IOP was always tested at the same time of the day (Aihara, Lindsey et al. 2003). Finally, to study the effect of the different antagonists tested, they were instilled 2 µl 30 min before the agonist, at a concentration of 100 µM, measuring IOP in the same way as previously described.

Human eye tissues

Donor Human eyes were obtained from the Fundación Banco de Sangre y Tejidos de las Islas Baleares (Blood and tissue bank Foundation from Baleares Islands). Three eyes of healthy normal subjects and two of glaucoma patients were used for these assays. Eyes were enucleated and collected without the cornea in sterile tubes and maintained in 4% paraformaldehyde in 0.1 M phosphate buffer (PB) (pH 7.2-7.4) at 4ºC until posterior processing. Eyes were dissected under stereo microscope (Zeiss) and with the 0.8 mm tip curved forceps and sterile dissecting scissors, the iris and ciliary processes was collected. Several washes in PBS were performed and then, the specimens were cryoprotected in a sucrose gradient (from 11% to 33%) and were embedded in tissue freezing medium (Tissue-Tek© OCT) until frozen with liquid N_2. Vertical sections of control and glaucomatous human samples (10 µm thick) were collected using a cryostat (Leica, Nussloch, Germany) and mounted from the same region. Samples were maintained in a -20 ºC until use.

Immunofluorescent studies

Frozen sections of healthy and glaucomatous subjects were rinsed in phosphate buffer saline (PBS) 1X and permeabilized with PBS-0.05% Tx-100 solution for 30 min. Afterward, to avoid non-specific staining, sections were incubated in a blocking solution

with a 10% normal donkey serum (NDS, Jackson Immunoresearch, West Grove, PA) during 1 hour at room temperature. Then, primary antibodies were incubated in each section overnight at 4ºC. The antibody raised against MT1 receptor was used at (1:200) dilution (Santa Cruz, sc-13179), MT2 antibody at (1:1000, ABIN122307, antibodies-online) and the -adrenergic antibody purchased from Abcam (1:500, ab3462). Sections were washed in PBS1X-0.1% Tx-100 and incubated with donkey anti-immunoglobulin IgG rabbit antibody conjugated with fluorescein isothiocyanate (FITC; green, Jackson ImmunoResearch, West Grove, PA, USA) at 1:100 dilution in the case of MT2 and -adrenergic antibodies, and donkey anti-immunoglobulin IgG goat antibody conjugated with fluorescein isothiocyanate (FITC; green, Jackson ImmunoResearch, West Grove, PA, USA) at 1:100 dilution in the case of MT1 receptor, antibodies were diluted in PBS-0.1% Tx-100 for 1 hour in a dark chamber at a room temperature. The nuclei were stained with propidium iodide (red, Sigma-Aldrich, St. Louis, MO) diluted 1:500 in PBS for 10 min. Finally, sections were rinsed with PBS1X and mounted in Vectashield (Vector Laboratories, Palex Medical, Barcelona, Spain) and coverslipped. The samples were examined under a confocal microscope (Zeiss LSM 5, Jena, Germany) at 40X magnification. For ciliary epithelial cells immunostaining, similar protocol was done for immunostaining of ciliary epithelium cells after incubating them with TRPV4 agonist GSK1016790A at 10μM for 18 hours.

In situ and In vitro proximity ligation assay (PLA)

The proximity ligation assay (PLA) allows the detection of molecular interactions between two endogenous proteins *ex vivo*. PLA requires both receptors to be sufficiently close to allow the two antibodies DNA probes to form double stranded segments (<16 nm),

a signal that is further amplified in the presence of fluorescent nucleotides. By PLA, the heteromerization of MT1 α_{1A}-adrenergic and MT2 α_{1A}-adrenergic was detected in non-pigmented epithelial ciliary body cells and, *in situ*, in human ciliary body sections of healthy and glacomatous donors.

59HCE cells were treated with a selective TRPV4 agonist GSK1016790A at different concentrations graded from 10nM, 100nM, 1µM, to 10µM during 18 hours for the performance of proximity ligation assay.

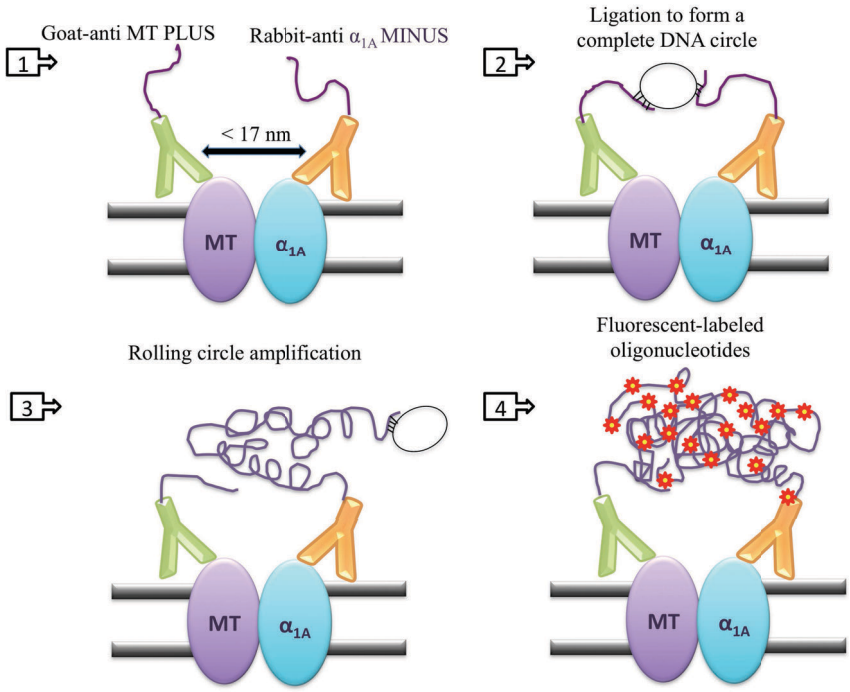

Figure 4.2. Schematic representation of the PLA assay. The direct method uses antibody pairs with primary conjugation. Following incubation with primary antibodies linked to PLA PLUS and MINUS probes (1), Hybridize connector oligos and use ligase to complete the DNA circle (2). Roling circle amplification (3). Hybridize fluorescence-labeled oligonucleotides for signal detection (4).

The PLA technique was carried out in paraformaldehyde-fixed human ciliary body sections and non-pigmented epithelial ciliary body cells. The presence/absence of receptor-receptor molecular interactions in the samples was detected using the Duolink II *in situ*

PLA detection kit (developed by Olink Bioscience, Uppsala, Sweden; and now distributed by Sigma-Aldrich as Duolink® using PLA® Technology). To create our PLA probes we linked the primary MT1 and MT2 antibodies to PLUS oligonucleotide (DUO92009, Sigma, St. Louis, USA) and the α_{1A}-adrenergic antibody to MINUS oligonucleotide (DUO92010, Sigma, St. Louis, USA). Sections were washed in PBS 1X pH 7.4 buffer, permeabilized with PBS containing 0.05% Triton X- 100 for 30 min and finally washed with PBS. After permeabilization, sections were washed at room temperature, incubated in a preheated humidity chamber for 1 hour at 37°C with the blocking solution provided with the PLA kit and then incubated overnight with the PLA probe-linked antibodies (1:100 dilution for all antibodies) at 4°C. After washing with buffer A (Wash buffer A, DUO82047, Sigma-Aldrich, St. Louis, MO, USA) at room temperature, sections were incubated with the ligation solution for 1 hour at 37°C in a humidity chamber. Following washes with buffer A, sections were incubated with the amplification solution for 100 min at 37°C in a humidity chamber and then washed in buffer B (Wash buffer B, DUO82048, Sigma-Aldrich, St. Louis, MO, USA), followed by another wash with buffer B x0.01. Finally, samples were incubated with 1:1000 dilution of SYTOX green stain (S7020, Thermo fisher scientific) and then washed with buffer B x0.01 and mounted using amounting medium vectashield (Vector Laboratories, Palex Medical, Barcelona, Spain). Appropriate negative control assays were carried out by incubating only one antibody to ensure lack of nonspecific labeling and amplification. Finally, sections were rinsed and mounted in Vectashield (Vector Laboratories, Palex Medical, Barcelona, Spain) and coverslipped. The samples were examined under a confocal microscope (Zeiss LSM 5, Jena, Germany) at 40X magnification.

For each field of view, a stack of two channels (one per staining) and 13 to 22 Z stacks with a step size of 0.7 µm were acquired. A quantification of cells containing one or more red spots versus total cells (green nucleus) was determined considering a total 2200-2500 cells from 18 different fields. Nuclei and red spots were counted on the maximum projections of each image stack. After getting the projection each channel was processed individually. The nuclei were segmented by filtering with a median filter, subtracting the background, enhancing the contrast and finally applying a threshold to obtain the binary image and the regions of interest (ROI) around each nucleus. Red spots images were also filtered and thresholded to obtain the binary images. Red spots were counted in each of the ROIs obtained in the nuclei images.

Results

Identification of an Interaction between both MT_1, MT_2 and α_{1A} adrenergic receptor

To identify potential direct interactions between receptors, a FRET biophysical approach was used using HEK-293T cells. Cells were transfected with a constant amount of cDNA for α_{1A} -Rluc and increasing amounts of cDNA for MT_1-YFP. In co-transfected cells, a saturable FRET curve was obtained ($BRET_{max}$ 48.9 $BRET_{50}$ 43.5) (Fig. 4.4A), indicating the existence of for α_{1A}-MT_1 heteroreceptor complexes. Similar experiments using increasing amounts of cDNA of MT_2-YFP also provided a saturable BRET curve with $BRET_{max}$ 168.5 and $BRET_{50}$ values of 128.2 ,thus demonstrating that both melatonin receptors may form heteromers with α_{1A}-adrenergic receptors (Fig. 4.4B).

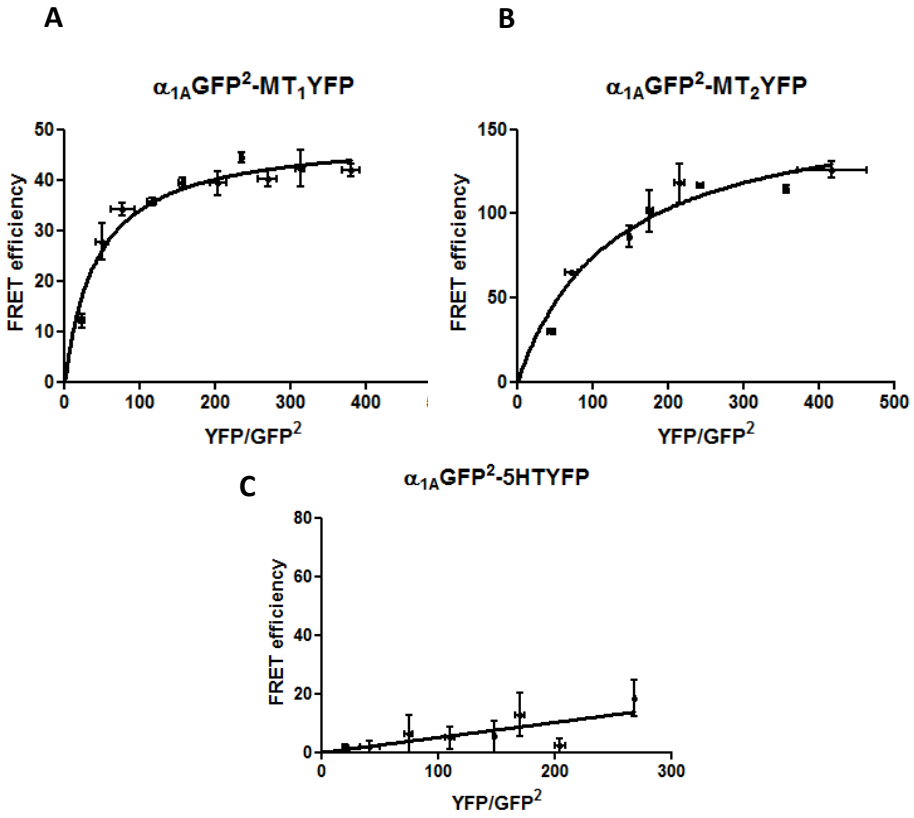

Figure 4.4. BRET saturation experiments showing α_{1A} –MT1 and α_{1A} –MT2 heteromerization were performed using cells transfected with 0.5 µg of cDNA

corresponding to α_{1A}-Rluc and increasing amounts of cDNA (0–3 µg cDNA) corresponding to either MT1-YFP or MT2-YFP. As negative control, cells were also transfected with cDNA corresponding to α_{1A} (0.5 µg) and serotonin 5HT-YFP (0 to 4 µg cDNA). Both fluorescence and luminescence for each sample were measured before each experiment to confirm similar donor expressions (approximately 150 000 bioluminescence units) while monitoring the increase in acceptor expression (100 to 80 000 net fluorescence units). The relative amount of BRET is given as the ratio between the fluorescence of the acceptor minus the fluorescence detected in cells expressing only the donor and the luciferase activity of the donor. BRET data are expressed as the mean ± SEM of 4–8 different experiments; data are grouped according to the signal provided by the BRET acceptor. mBU, mili BRET units.

Co-expression of MTs and α_{1A} causes cell signaling via G_s

Signaling was investigated in both, single transfected and co-transfected cells. First of all, one of the main findings was the G_i-protein-coupling when melatonin or α_{1A}-adrenergic receptors were individually expressed (Fig 4.5A-C). In fact, the application of either melatonin agonists or α_{1A}-agonist reduced cAMP levels induced by forskolin. Activation of receptors in single transfected cells also provided significant DMR read-outs (Fig 4.5 D-F). Finally, BRET assays using -arrestin-2-Rluc and receptors fused to YFP, showed β-arrestin recruitment only induced by the selective agonists of either α_{1A}, MT_1 or MT_2 receptors (Fig 4.6 A-C).

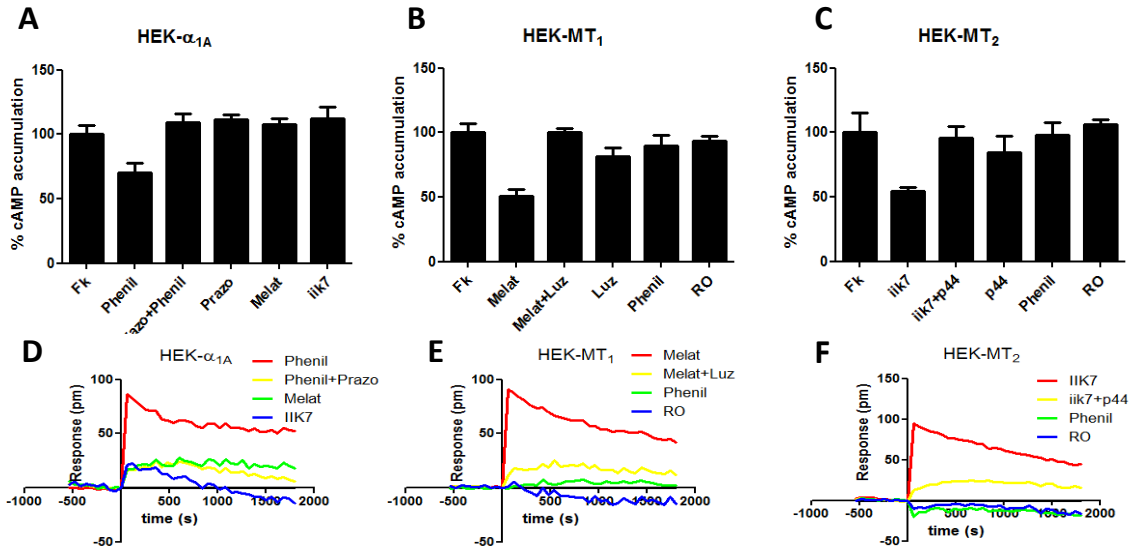

Figure 4.5 α_{1A} and MTs alone are associated to Gi and G_q signaling respectively when expressed alone. HEK-293T cells expressing α_{1A} ,MT_1 or MT_2 receptors were used. In A,B, and C cAMP production was determined in cells stimulated with 5 µM forskolin. Cells were stimulated with 10 µM melatonin or 10 µM IIK7 or 10 µM phyneliphrine. Values (cAMP produced in each condition minus basal stimulation in the absence of forskolin or agonists) represent mean ± SEM of n = 3–4 and are expressed as the percentage of the forskolin-treated cells in each conditions (120–150 pmols cAMP/106 cells). One-way ANOVA followed by a Dunnett's multiple comparison test showed a significant effect over the forskolin-alone effect in each condition (* $p < 0.05$, ** $p < 0.01$). In (D,E,F) label free real time DMR analysis of each receptor expressed alone in HEK-293T cell, in D cells were challenged with phyneliphrine, E, with melatonin and F with IIK7, and wavelength shift was monitored over time as a measure of receptor activation. . Each curve is the mean of a representative optical trace experiment carried out in triplicates

As it has been reported that α_{1A}-adrenoceptor is generally coupled to $G_{q/11}$, experiments on Ca^{2+} mobilization were performed using a calmodulin-based biosensor (GCaMP6 sensor). The results in cells individually expressing the α_{1A}-adrenergic receptor showed $G_{q/11}$ coupling, with a robust increase in the Ca^{2+} biosensor (Fig.4.6 D). The effect of phenylephrine in these cells was antagonized by prazosin, but not by the melatonin antagonists luzindole or 4-PPDOT. Neither MT_1 nor MT_2 receptors were coupled to $G_{q/11}$ in HEK-293T cells (Fig. 4.6E and 4.6F).

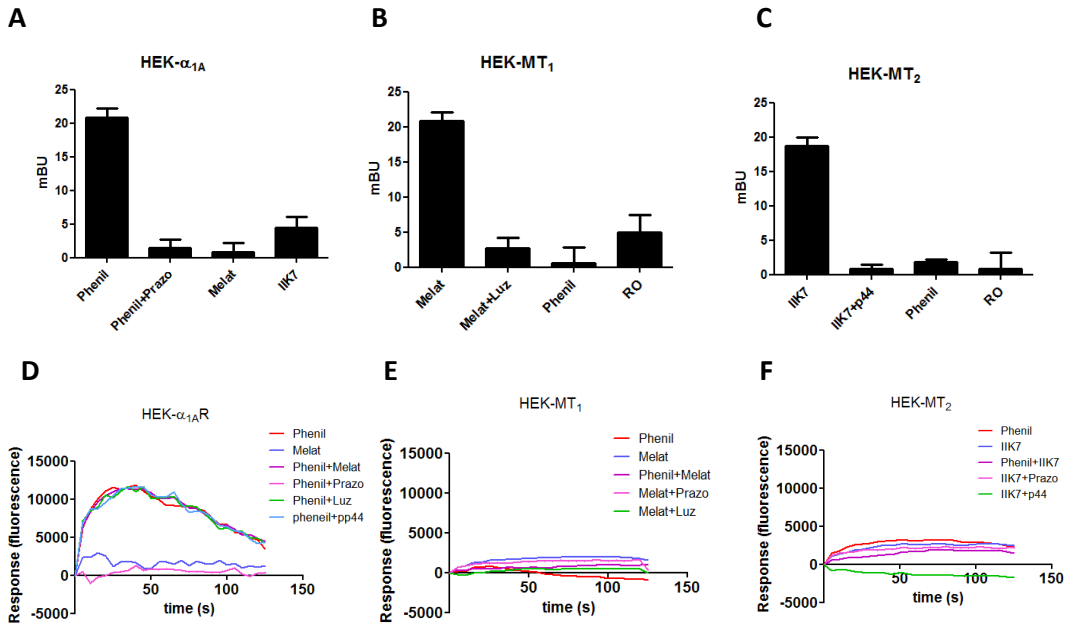

Figure 4.6. A, B and C: BRET-based β-arrestin recruitment assay in HEK293T cells transiently expressing with either 2 µg of MT_1, MT_2, or α_{1A} –YFP cDNA, 0.2 µg of β-arrestin II-Rluc cDNA. Each agonist showed a positive reaction which was diminished by its corresponding agonist. Values represent mean ± SEM of n = 4–6. One-way ANOVA. D: robust increase in the Ca^{2+} biosensor in cells expressing α_{1A} receptor, and the effect was antagonized by prazosin. F and K confirmed that melatonin receptors MT_1 nor MT_2 receptors were negatively coupled to $G_{q/11}$ in HEK-293T cells

Qualitatively different results were obtained in the signaling experiments performed in co-transfected cells (Fig. 4.7, 4.8 and 4.9). Actually, the cAMP determination data in co-transfected cells were very similar to the results obtained in HCE cells. In this sense, the activation of receptors in HEK-293T cells expressing α_{1A}-MT_1 heteromers or α_{1A}-MT_2 heteromers produced an increase in forskolin-induced cAMP levels, in clear contrast to what happened in single-transfected cells (Fig. 4.7 A and 4.7 B). Also, cross-antagonism reappeared in co-transfected cells.

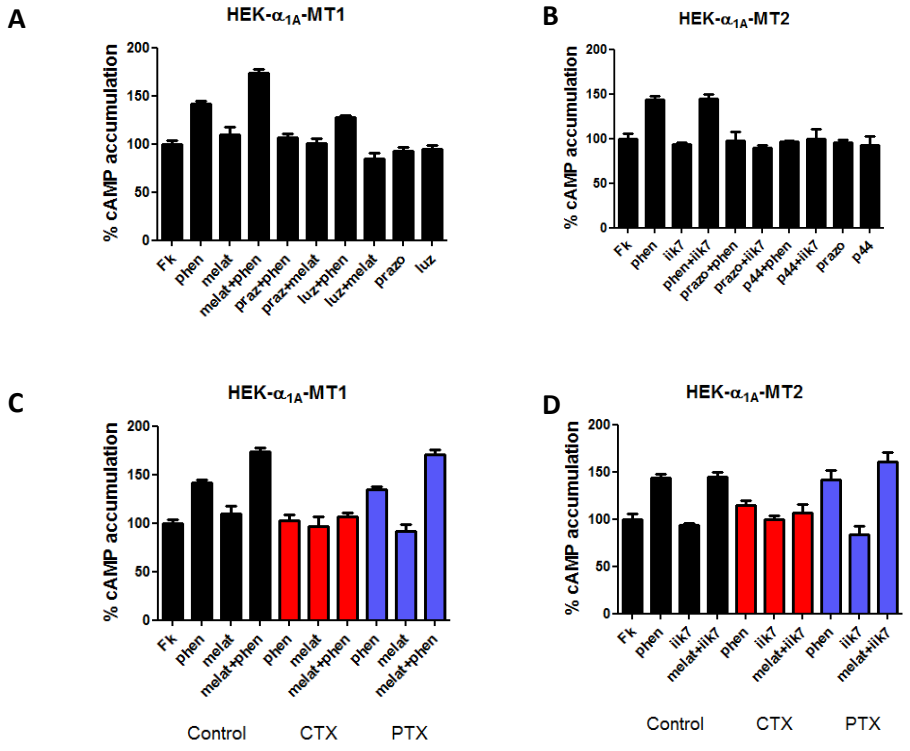

Figure 4.7 cAMP experiments in HEK-293T expressing either α_{1A}-MT_1 heteromers or α_{1A}-MT_2 heteromers (A and B). Stimulation with melatonin and phyneliphrine caused an increment in forskolin-induced cAMP levels, same results obtained with $^{\alpha}{}_{1A}$-MT_2 with

the activation by IIK7 and phyneliphrine. As shown in C and D cross-talk of melatonin and $^{\alpha}{}_{1A}$-agonists was blocked by cholera but not by pertussis toxin.

Analogies between HCE and co-transfected KEK-293T cells were further found in experiments with toxins; in KEK-293T cells expressing melatonin-adrenergic receptors, the cross-talk of melatonin and α_{1A}-agonists was blocked by cholera but not by pertussis toxin (Fig. 4.7C and 4.7D). Also, matching the results obtained in HCE cells, a potentiation of the label-free DMR signal upon co-activation, cholera toxin sensitivity and a cross-antagonism were detected (Fig 4.8A-4.8D).

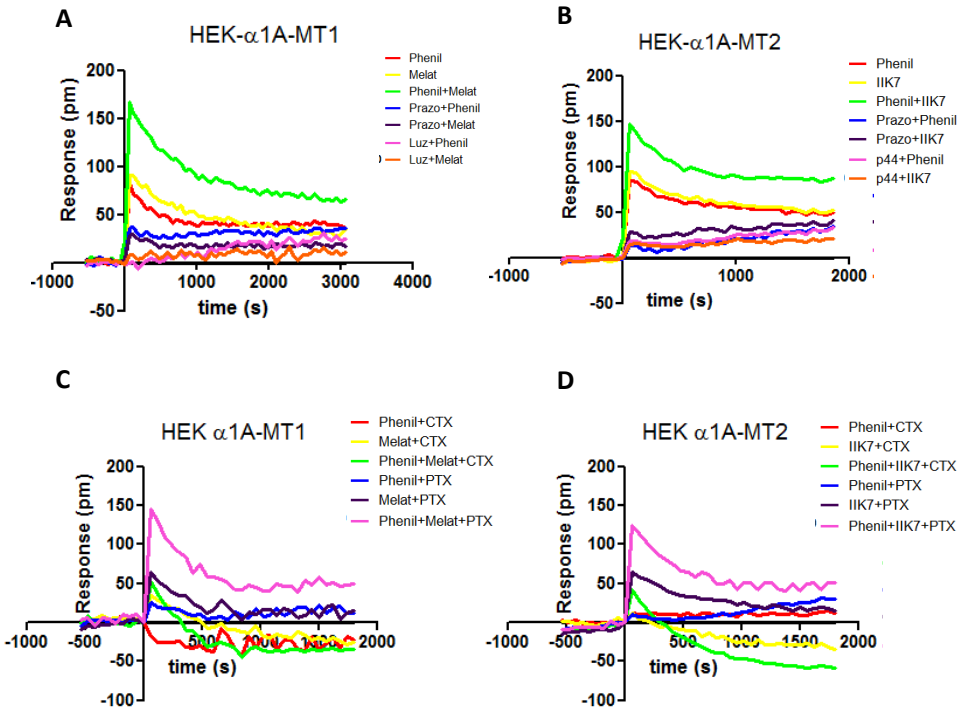

Figure 4.8 label free real time DMR analysis of receptors coexpressed in HEK-293T cell confirms response of a cross talk between phyneliphrine and melatonin (A) and phyneliphrine and IIK7 (B). in C and D this effect was blocked by cholera but not by pertussis toxin.Each curve is the mean of a representative optical trace experiment carried out in triplicates.

Remarkably, phenylephrine did not produce any Ca^{2+} response in cells expressing either α_{1A}-MT_1 or α_{1A}-MT_2 heteroreceptor complexes (Fig. A 4.9A and 4.9B). These results indicate a lack of productive coupling of receptor heteromers with $G_{q/11}$ or G_i, and a

dependence on G_S-proteins. Cross-modulation was also detected in G-protein independent signaling investigated in co-transfected cells. Indeed, β-arrestin recruitment occurred. Not only α_{1A}-receptor activation but the activation of MT_1 melatonin receptor recruited β-arrestin to the α_{1A}-receptor, co-activation resulting in a stronger signal (Fig. 4.9C and 4.9D). The recruitment was abolished by the α_{1A}-receptor antagonist as well as by the MT_1 melatonin receptor antagonist. Fully similar results were obtained in cells co-expressing α_{1A}-MT_2 heteromers using the selective MT_2 agonist and antagonist (Fig. 4.9C and 4.9D).

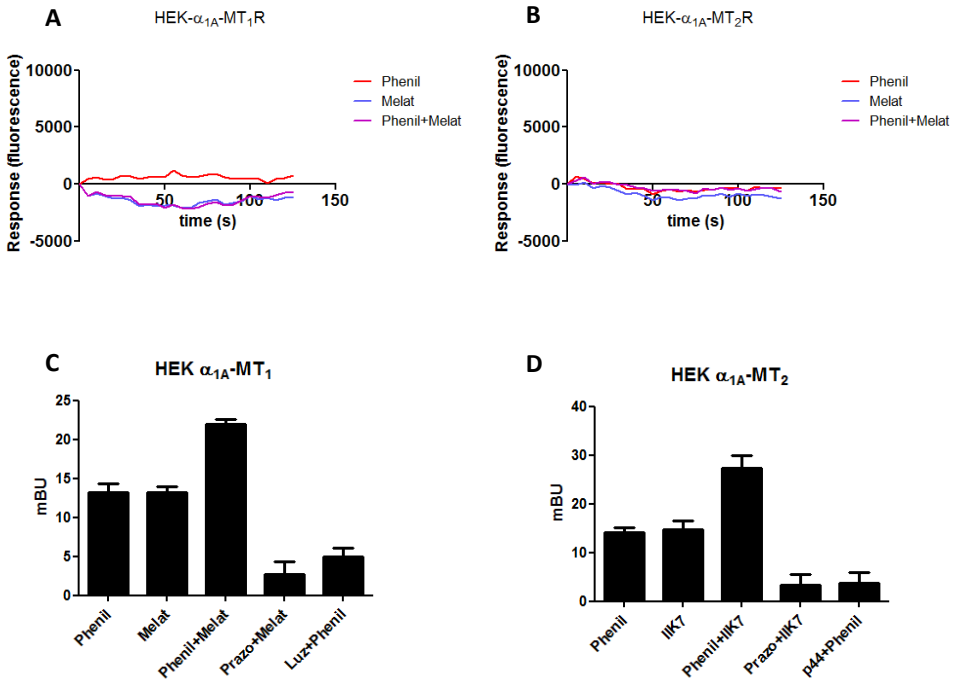

Figure 4.9. Ca^{2+} response after stimulation with α_{1A} and MTs agonist was deleted when cells were cotransfected with both receptors (A and B). BRET-based β-arrestin recruitment assays showed a stronger signal when cells were challenged with both receptors aginsts, this effect was abolished by antagonists of both receptors. Values represent mean ± SEM of n = 4–6. One-way ANOVA.

Altogether, these results indicate the formation of heteromeric complexes in the heterologous expression system, and that the receptor-receptor interactions condition intracellular signaling. Since the results obtained in HEK-293T cells in terms of signaling

are identical to those obtained in HCE cells, we presume that human HCE cells present heteromers that are functionally coupled to G_s but not to G_i or $G_{q/11}$ proteins.

Important role of C-terminal tail of the α_{1A} adrenergic receptor

Looking at the primary structure and membrane topological domains of the GPCR receptors here studied, we hypothesized that some of the intracellular domains might be responsible of the differential G coupling to heteromers versus to individual receptors. On the one hand, the area projected by a G protein on the membrane plane almost doubles that of a GPCR molecule. On the other hand, the "clam-shell like" opening of the small globular domain in G_α subunits (also known as the α-helical domain), which occurs in every GTP/GDP exchange, has been structurally elucidated (Chung et al., 2011, Westfield et al., 2011). With all this information and with the restricted transmembrane interface possibilities for heteromer formation we noticed that the long C-terminal domain of the α_{1A} adrenergic receptor could lead to steric hindrance in the heteromer-G protein macromolecular complex (Cordomi et al., 2015). It is predicted that the human α_{1A} adrenergic receptor has 137 amino acids in the cytoplasmic C-terminal domain (www.uniprot.org/uniprot/P35348), and that human MT_1 and MT_2 receptors have C-terminal ends of, respectively, 55 (www.uniprot.org/uniprot/P48039) and 54 (www.uniprot.org/uniprot/P49286) amino acids. For the three receptors, we prepared mutants lacking most of the C terminal domain; some amino acids after the las transmembrane helical domain are needed to link the FRET/BRET donor/acceptor proteins (see Methods for details).

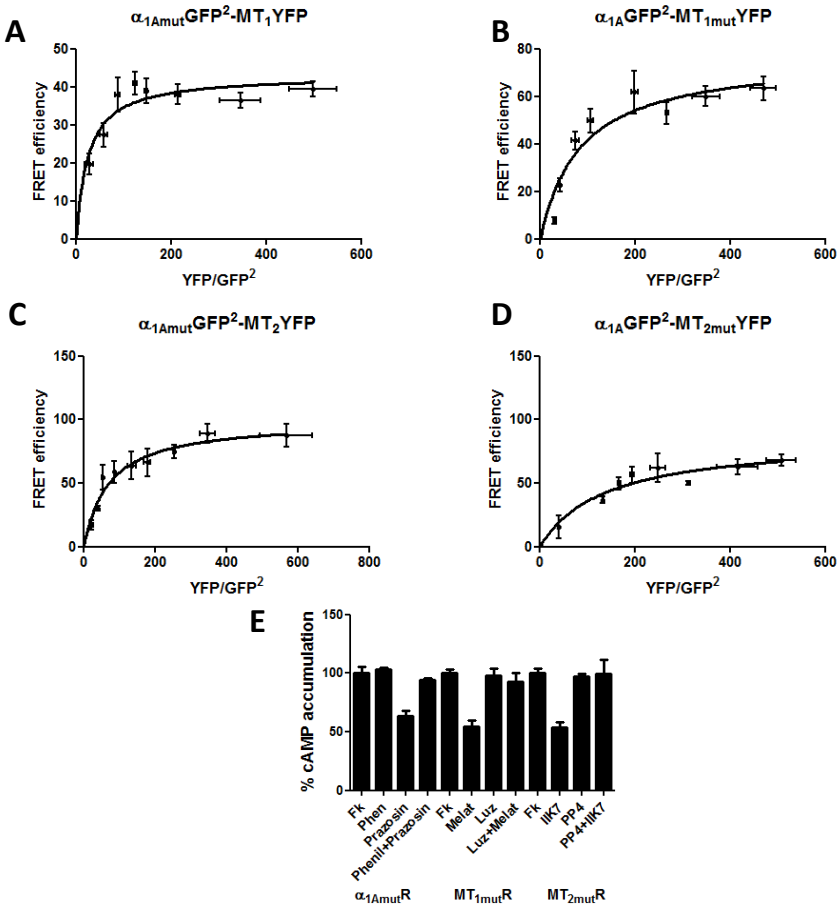

Figure 4.10 BRET assays in cells transfected with α_1, MT_1 and MT_2 mutant receptors resulting from C-terminal deletion. However, BRET showed a positive interaction between receptors. E: cAMP experiments in cells transfected with only one of the mutant receptors confirmed that truncated receptors are functional and all coupled to G_i.

Biophysical energy transfer assays showed that truncated melatonin receptors were able to interact with α_1-adrenergic receptors and that truncated α_1-adrenergic receptors were able to interact with melatonin receptors (Fig. 4.10A-D). BRET parameters in the case of α_1-MT_{1mut} resulted in $BRET_{max}$ 77.1 and $BRET_{50}$ 84.8 and for α_1-MT_2 $BRET_{max}$ 85.4 and $BRET_{50}$ 137.7. For the α_1 mutant BRET curve was saturated when cells were cotransfected with α_{1mut}-MT_1, $BRET_{max}$ 43.2 $BRET_{50}$ 24.7 and finally α_{1mut}-MT_2 resulted in $BRET_{max}$ 99.7 $BRET_{50}$ 70.4. This indicate that efficiency was variable thus reflecting either less dimer formation or a different conformation that in turn leads to changes in the

donor-acceptor distance. Truncated receptors, when expressed individually, were functional and, as the full length version, were all coupled to G_i (Fig. 10E). Functional assays of Ca^{2+} mobilization in cells co-expressing truncated melatonin and full length α_{1A} adrenergic receptors, or full length melatonin and truncated α_{1A} adrenergic receptors, showed that truncation of melatonin receptors did not lead to any significant effect (Fig. 4.11A and 4.11B). In sharp contrast, removal of the C-terminal domain of α_1-adrenergic receptors results in adrenergic-receptor mediated Ca^{2+} mobilization (Fig. 4.11C and 4.11D). Thus, the truncated α_1-adrenergic receptor even in an heteromeric context, is able to mobilize Ca^{2+}, as it occurred when the full length receptors was individually expressed.

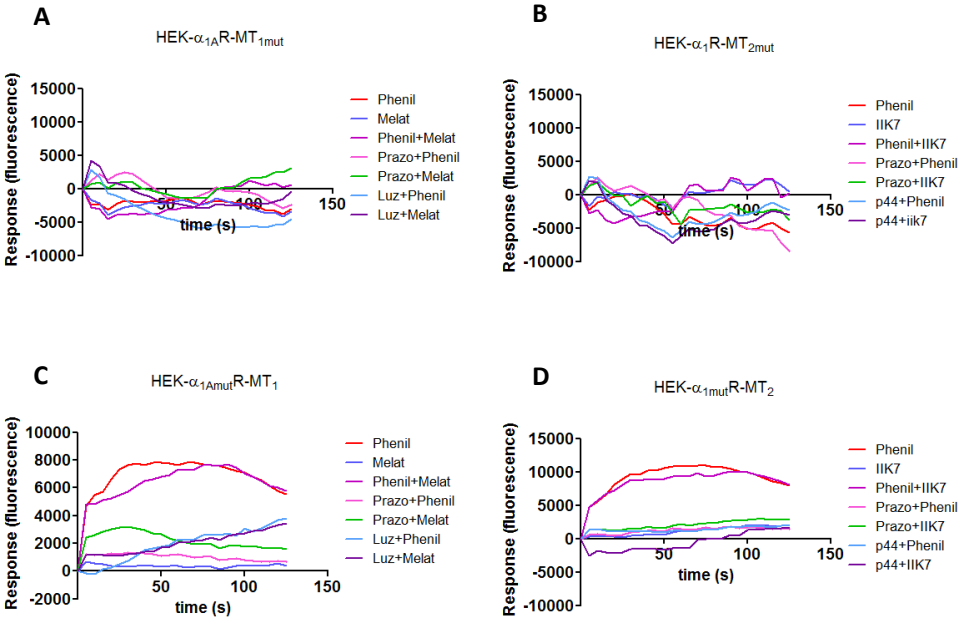

Figure 4.11. A and B: Both melatonin receptors mutations had no effect on Ca^{2+} mobilization. C and D: Truncated α_1-adrenergic was able to mobilize Ca^{2+} even when it is coexprssed with either MT_1 or MT_2.

Relevant results were also obtained in cAMP determination assays. Truncated melatonin receptors interacting with full-length α_{1A} adrenergic receptors are still coupled to G_s protein (Fig 4.12A and 4.12B), whereas truncated α_{1A} adrenergic receptors

interacting with melatonin receptors, behaved as the full length receptor when individually expressed, i.e. they coupled to G_i (Fig. 4.12C-F). In summary, the C-terminal tail of the α_{1A} adrenergic receptor is important for the change of coupling, from G_i to G_s, when it forms heteromers with MT_1 or MT_2 receptors.

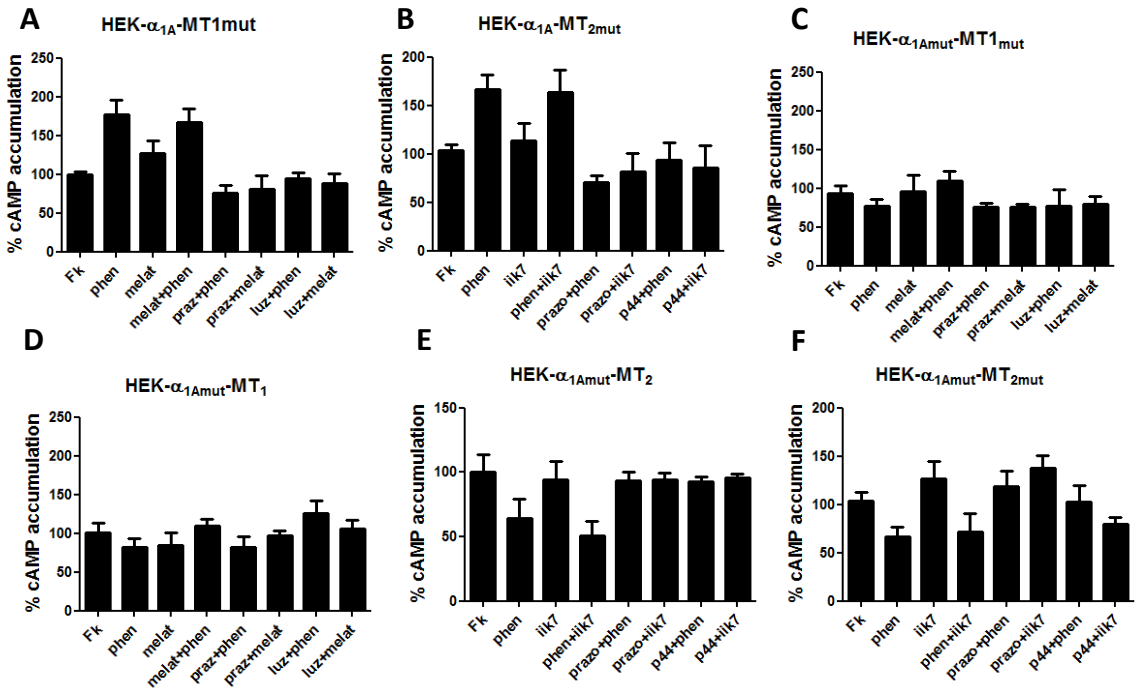

Figure 4.12. A and B: cAMP assays done in cells co-transfected with α_{1A} adrenergic receptor and either truncated MT_1 or MT_2 receptors showed similar response when agonists applied; cAMP levels increased indicating a G_s protein coupling. whereas truncated α_{1A} adrenergic receptors interacting with melatonin receptors, showed a clear reduction in cAMP levels confirming that they are coupled to G_i (C-F).

Pharmacology of receptors expressed in HCE cells

The pharmacological characterization of melatonin and α_{1A}-adrenergic receptors was performed in HCE cells paying particular attention to variations within the canonical signalling pathways reported for these receptors. In fact, the reported cognate G proteins are G_i for both melatonin receptors and $G_{q/11}$ for α_{1A}-adrenergic receptors (www.guidetopharmacology.org). We measured cAMP intracellular levels and dynamic

mass redistribution (DMR) upon receptor activation. The effect of 100 μM melatonin produced a further increase in the forskolin-induced intracellular cAMP levels, effect which was reversed by the melatonin MT_1 receptor antagonist, luzindole (Fig. 4.13A). When the MT_2 melatonin receptor selective agonist, IIK7, was applied, a rise in forskolin-induced cAMP levels was also observed (Fig. 4.13B) that was reversed by the receptor MT_2 selective antagonist, 4-PPDOT (Fig. 4.13B). When cells were challenged with the α_1-adrenergic agonist phenylephrine (1 μM) a remarkable elevation of cytosolic forskolin-induced cAMP was achieved. The antagonist prazosin abolished the cAMP elevation produced by phenylephrine. These results demonstrate that human ciliary epithelial cells express functional MT_1 and MT_2 melatonin, and α_{1A}-adrenergic receptors, none of which was coupled to the reported cognate G protein.

To further confirm such finding, similar assays were performed in the presence of cholera toxin, which disrupts G_s-mediated signaling or pertussis toxin which disrupts G_i-mediated signaling. The results show that cholera but no pertussis toxin is inhibiting the action of all the agonists indicating that a G_s protein is involved in signal transduction afforded by activation of any of the assayed receptors (Fig. 4.13C, 4.13D). Interestingly, when melatonin or IIK7 action on cAMP levels were studied in the presence of prazosin, this α_1-adrenergic antagonist was able to abolish the effect of each melatonergic agent. Remarkably, when phenylephrine was assayed in the presence of either luzindole or 4-PPDOT, the α_1-adrenergic agonist was unable to rise cAMP levels in the way it did when applied alone.

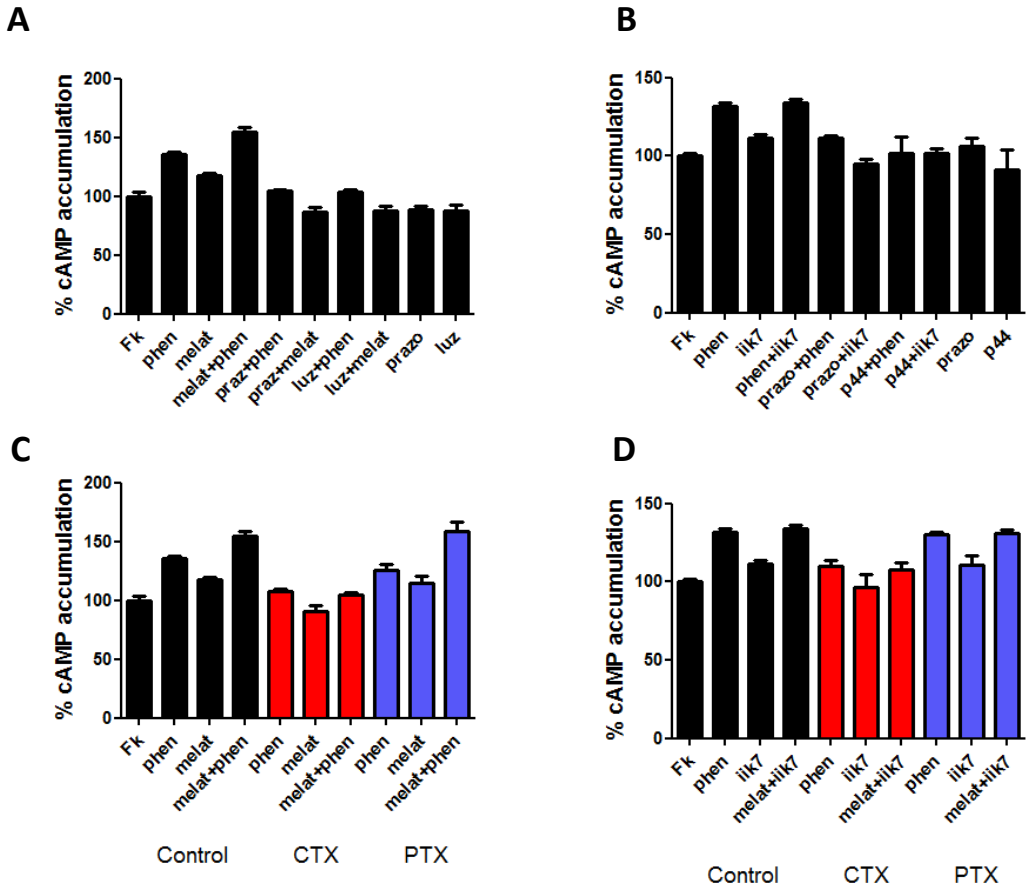

Figure 4.13 cAMP experiments in 59HCEsv cells. Stimulation with melatonin and phyneliphrine caused an increment in forskolin-induced cAMP levels, same results obtained with the activation by IIK7 and phyneliphrine. As shown in C and D cross-talk of melatonin and α_{1A}-agonists was blocked by cholera but not by pertussis toxin.

The label-free DMR induced by adrenergic or melatonergic compounds was relatively high. The combination of melatonin and phenylephrine (Fig 4.14A), or IIK7 and phenylephrine (Fig 4.14B), provided a more robust DMR response than single treatments.When the experiments were carried out in the presence of cholera and pertussis toxins, we again found that only cholera toxin was able to abolish the DMR responses triggered by all the agonists (Fig 4.14C and 4.14D).

In the same way as we found in cAMP determination assays, it was possible to observe cross-antagonism between melatonin and α_1-adrenergic receptors. Taken together these results suggest the possible crosstalk between melatonergic and α_1-adrenergic

signalling. Also, and importantly finding was the cross-antagonism, which up to date is a feature only displayed by GPCR heteromers.

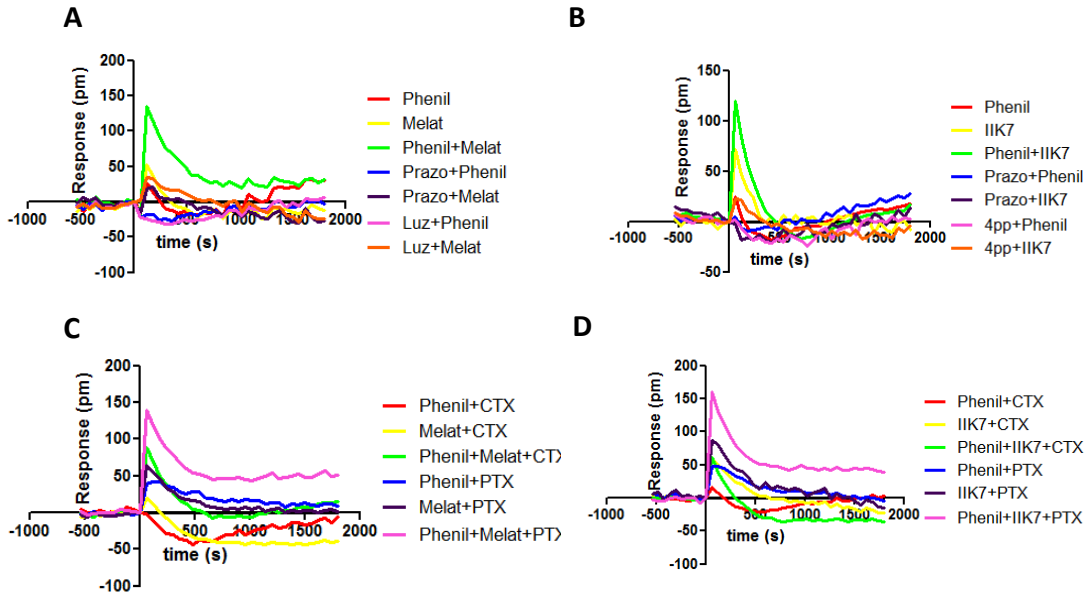

Figure 4.14 label free real time DMR analysis of receptors natively expressed in 59HCEsv cells confirms response of a cross talk between phyneliphrine and melatonin (A) and phyneliphrine and IIK7 (B). in C and D this effect was blocked by cholera but not by pertussis toxin.Each curve is the mean of a representative optical trace experiment carried out in triplicates.

Identification of α_{1A}-MT_1 and α_{1A}-MT_2 heteromer complexes in healthy and glaucomatous conditions

Proximity ligand assay (PLA) is being instrumental as a complementary technique to detect receptor-receptor interaction in a native system. PLA assays using specific antibodies against the receptors of interest, proved the occurrence of both α_{1A}-MT_1 and α_{1A}-MT_2 heteromer complexes in HCE cells. Red dots, which reflect the existence of heteromers were clearly visible for both receptor pairs. The analysis of the PLA labeling provided values of 65±10 dots/nucleus in the case of the $MT_1/\alpha1$ heteromer (negative control value: 5±1 dots/nucleus), and 73±8 dots/nucleus in the case of the MT_2/α_1

heteromer (negative control value: 2±2 dots/nucleus (n=5, P<0.005). The percentage of cells that presented positive PLA in the case of the MT_1/α_1 receptor heteromer was 56±4, while in the case of MT_2/α_1 receptor heteromer the percentage was 57±5 (n=150).

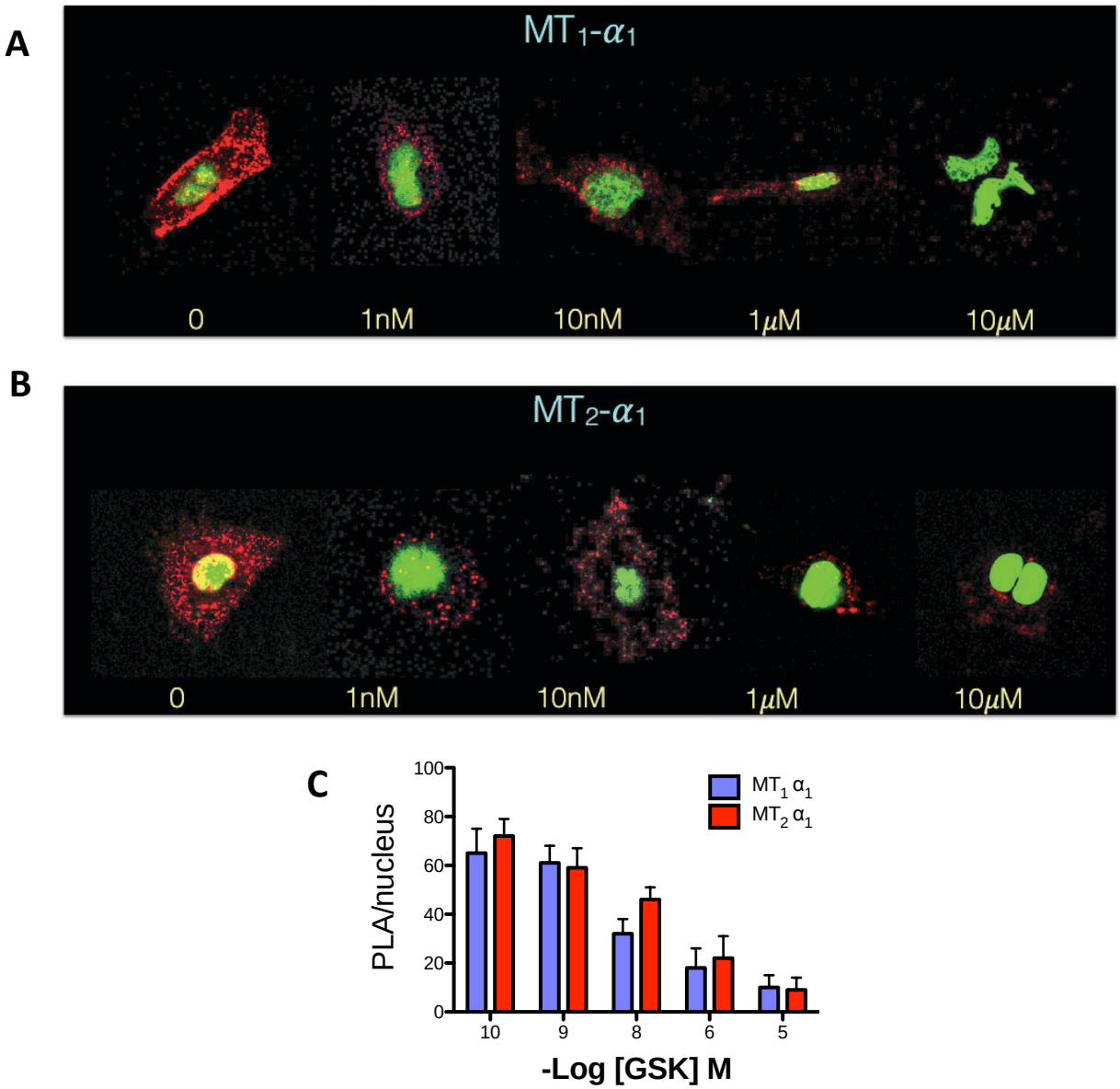

Figure 4.15. PLA α_{1A}-MT_1 heteromers or α_{1A}-MT_2 in human non-pigmented ciliary body epithelial cells (59HCE). A dose –response effect was observed when activating TRPV4 channel by using a selective agonist GSK1016790A. From left to right, increased dosis of the agonist was used after observing a positive PLA with no treatment (first at left). α_{1A}-MT_1 heteromers or α_{1A}-MT_2 close proximity are detectable as a punctate red fluorescent signal by confocal microscopy. Cell nuclei were stained with SYTOX green stain. C: Quantitavie analysis revealed a decrease in PLA with higher concentrations of GSK1016790A (n=5, P<0.005).

In order to check whether a glaucomatous condition modifies the amount of heteromeric complexes, cells were subjected to stimulation of the transient receptor potential vanilloid 4 (TRPV4) channel. As previously reported (for more details, see chapter 2), activation of the channel mimics the changes in the hydrostatic and osmotic pressure that occur in the glaucomatous eye. The application of the TRPV4 agonist, GSK1016790A, to HCE cells modified the PLA signal in a dose-dependent manner. As shown in Fig. 4.15, the higher the concentration of GSK1016790A, the lower the PLA signal. Therefore, when a glaucomatous condition is reproduced in HCE cells, a reduction in the expression of MT_1/α_1 or MT_2/α_1 receptor heteromers occurred.

Studies to check the expression of heteromers in samples from human eyes were also performed. The analysis carried out in ciliary body samples of glaucomatous individuals and age-matched healthy controls, presented relevant findings. In the healthy eye, the presence of melatonin receptors, MT_1 and MT_2, as well as $_{1A}$-adrenergic receptors were confirmed by immunohistochemical assays using validated antibodies, showing a clear immunoreactivity across the human ciliary body (Fig. 4.16.A,4.16B,4.16C).

A strong labeling for the MT_1 melatonin receptor was present in the non-pigmented epithelial cells, while the labeling for the MT_2 melatonin receptor was observed in the basal membrane of the non-pigmented epithelium (Fig. 4.16D and 5F). No immunoreactivity for any of the two melatonin receptors was observed in the stromal part of the ciliary body. Concerning the $_{1A}$-adrenergic receptor, a positive labeling was observed in the pigmented and non-pigmented epithelial cells as well as in the stromal part of the ciliary body (Fig 4.16B).

When a similar immunohistochemical study was carried out in samples obtained from patients with glaucoma, we observed that the expressions of MT_1 and MT_2 melatonin

receptors were markedly reduced compared to healthy individuals, with decreases of 81.2 15.6 % and 54.1 13.2 % for MT_1 and MT_2 respectively (normalized values to non-glaucomatous patients, n=4, **P<0.005, ***P<0.001). On the contrary, the α_1-adrenergic receptor showed a trend (to increase) that was not statistically significant (Fig. 4.16.D-F).

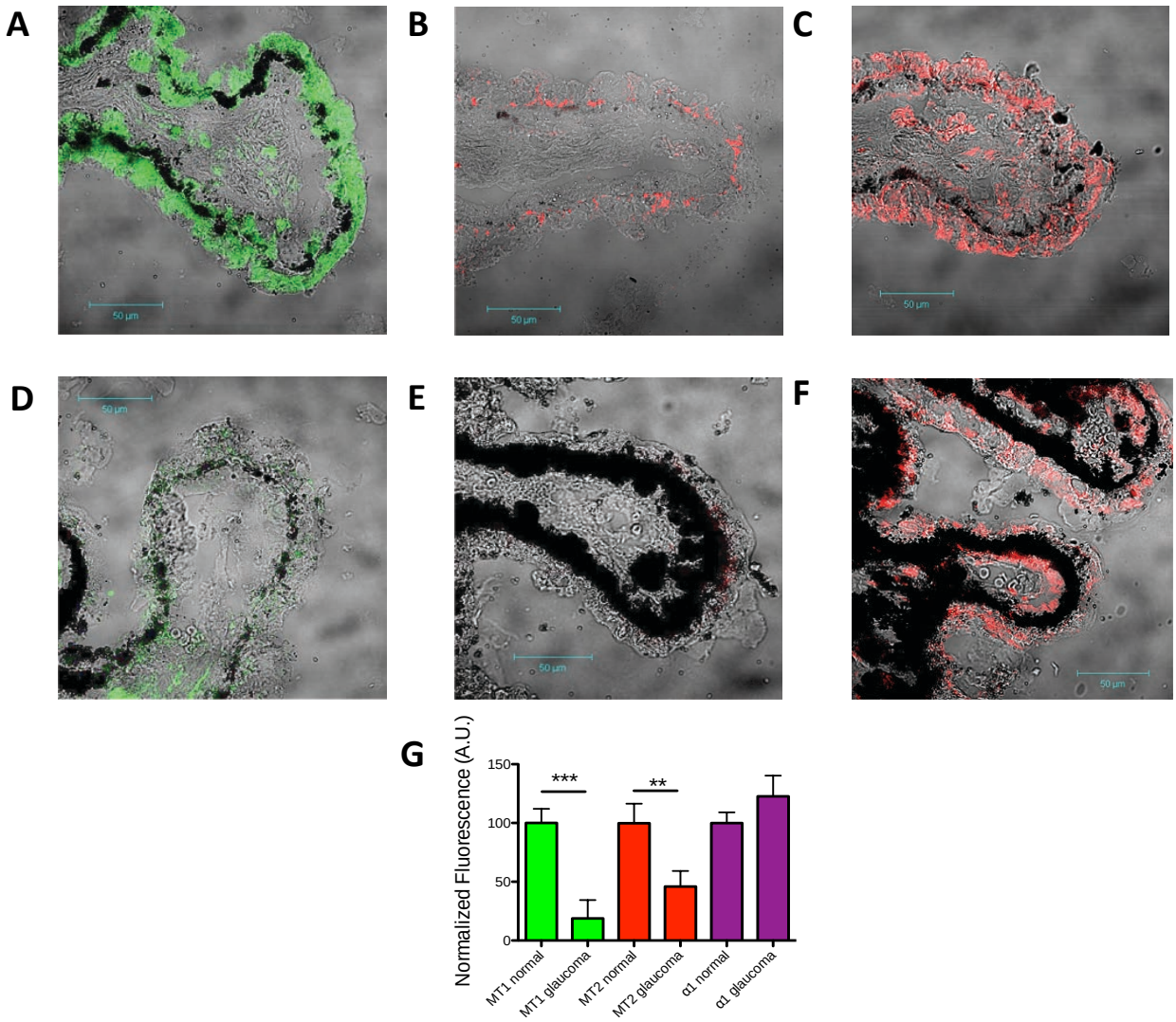

Figure 4.16. Immunohistochemistry analysis of human ciliary body tissue. A and D: Comparison of MT_1 labelling in healthy and glaucomatus donor showing a clear decrease in recptor presence (D: glaucomatous subject). B and E: Similar results were obtained regarding MT_2 receptor, clear labelling was observed in healthy donors (B). C and F: α_{1A} immunolabelling showing no statistically segnificant differences between healthy and diseased subjects. G: normalized values to non-glaucomatous patients, n=4, **P<0.005, ***P<0.001.

We then reasoned that the marked reduction in melatonin receptor expression would also lead to a decrease in the expression of melatonin-adrenergic receptor heteromers. Accordingly, we investigated by PLA in the ciliary body from the healthy and the glaucomatous eye, the ability of MT_1 and MT_2 receptors to form heteroreceptor complexes with the α_1-adrenergic receptor. For both MT_1/α_1 and MT_2/α_1 receptor pairs, a marked PLA positive labeling was observed in samples from healthy individuals (Fig. 4.17A and 4.17C), Remarkably, when PLA was performed in samples from glaucoma patients, PLA labeling for the two pair of receptors was strongly reduced as observed in Fig. 4.17B, 4.17D and 4.17E .

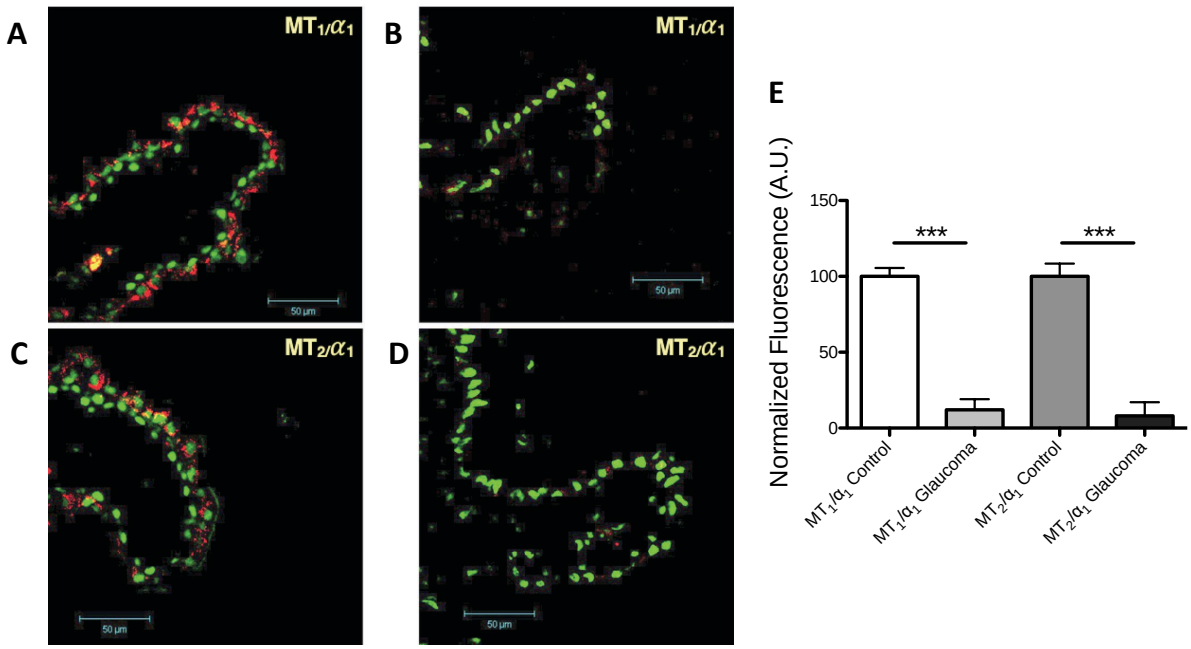

Figure 4.17. A and C showing PLA of $^{\alpha}{}_{1A}$-MT_1 heteromers and $^{\alpha}{}_{1A}$-MT_2 , respectivly, in human ciliary body tissue of healthy subjects. B and C: Same PLA experiments done in glaucomatous tissue sample, indicating a decline in PLA labelling. E: Quantitavie of PLA dots (***P<0.001).

Once more, these results match with the results obtained in HCE cells treated with a TRPV4 activator to mimic a glaucomatous condition. Taken together, these results indicate that the number of heteromers are reduced in glaucoma, mainly because a

reduction in the presence of melatonin receptors in a context of robust expression of the α_1-adrenergic receptor. Considering these results, one would expect that the use of a α_1-adrenergic receptor antagonist in the presence of melatonin may help to recover the lack of effect of the indoleamine in a glaucoma condition. We next moved to a well-established murine model of glaucoma.

MTs and α_{1A} Heteromers are involved in glaucoma pathology: in vivo evidences

The glaucoma DBA/2J mouse model presents most of the features of the human pathology. IOP andmelatonin levels normal and undistinguishable from the control mouse C57BL/6 at 3 months of age, while these physiological parameters change when the pathology is established at 9 months of age (see chapter 2).

We predicted in 3-month-old DBA/2J and control animals, the existence of heteromers and, consequently, a marked cross-antagonism. Indeed, the results, measuring the IOP in the eye, proved the assumption. In this sense, the effect of melatonin or IIK7 were blocked by, respectively, luzindole and 4-PPDOT, but also by the α_1-adrenergic receptor antagonist, prazosin (Fig. 4.18A and 4.18D).

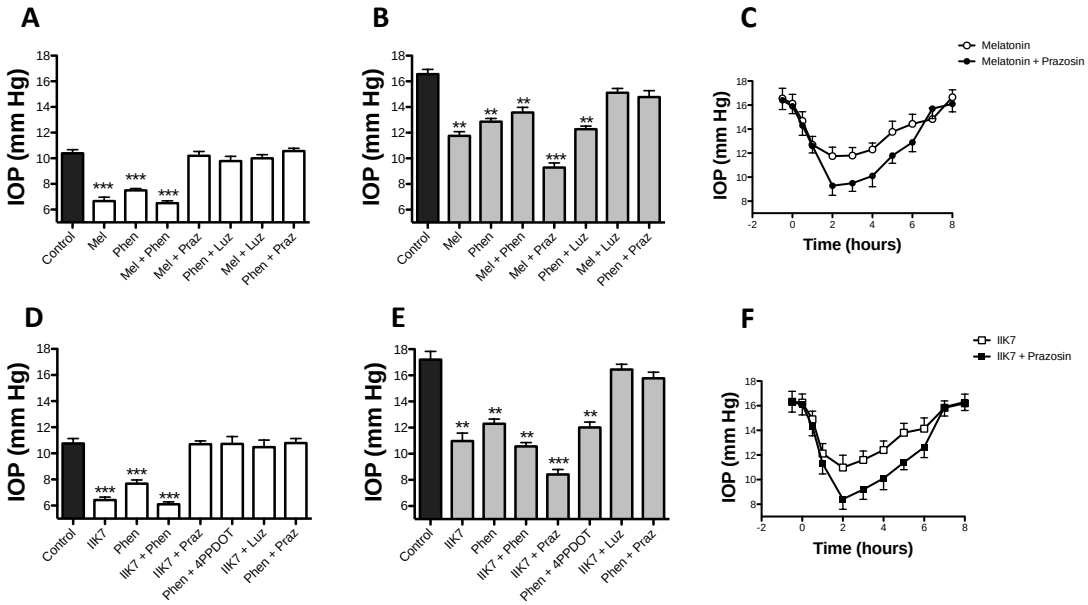

Figure 4.18. Effect of of α_{1A},MT_1 and MT_2 activation over IOP in both C57BL/6J and DBA/2J mice models. A: Both melatonin and phyneliphrine reduced IOP, Interestingly, melatonin effect was antagonized by prazosin (α_{1A} antagonist). D: Same effect was observed when IIK7 was instilled. B and E demonstrate the effect of same compound onDBA/2J mice models at 12 months old. Prazosin allowed melatonin and IIK7 to act more effectively instead of antagonizing melatonins effect. IOP was lower in mice treated with prazosin and afterwards, with melatonin or IIK7. C and F represent a time course effect of melatonin+prazosin and IIK7+prazosin respectivly. **P<0.005, ***P<0.001versus control (two-way ANOVA with Tukey post-test, $n = 4$).

The prediction in 12-month-old animals was the opposite, i.e. that cross-antagonism was markedly reduced due to the negligible level of melatonin-α_1-adrenergic receptor heteromers. Indeed, when IOP was measured in 12-month-old glaucomatous animals, all the agonists tested reduced IOP, and when assaying the antagonists for the three receptors, no cross-antagonism was detected, i.e. the antagonists worked only in blocking the effect mediated by their respective receptors (Fig. 4.18B and 4.18E). Remarkably, the hypotensive effect of melatonin receptor activation was further enhanced by the application of prazosin. Moreover, the effect of prazosin lasted more than 6 hours, this indicating a very appropriate therapeutic time window for therapy (Fig. 4.18C and 4.18F).

Discussion

The present work introduce the concept of heterodimerization between both melatonin receptors and α_{1A}-adrenirgic receptor. A growing body of biochemical and biophysical evidences indicates that some GPCRs are able to form both homodimers and heterodimers with their existence at this time widely accepted (Angers et al., 2002; Jordan and Devi, 1999).

The concept that GPCR heterodimerization could have a role in pharmacological diversity was first indicated by studies on the δ- and κ-opioid receptors when co-expression of both receptors led to the formation of a stable heterodimer with a very low affinity for either the δ- or the κ-selective ligand alone. However, high affinity was restored following the combination of the two ligands, suggesting the occurrence of positive cooperativity (Jordan and Devi, 1999). Such an observation led to more discoveries although the the direct link between heterodimerization itself and the changes in pharmacological properties has not been formally established, positive or negative ligand-binding cooperativity that occurs after receptor co-expression has been interpreted as resulting from receptor heterodimerization for many other GPCRs. These include the metabotropic GbR GbR1/GbR2 (Galvez et al., 2001), opioid δ/μ (Gomes et al., 2000), and adenosine A2A /dopamine D1 (Franco et al., 2000) receptors. If this is a general phenomenon, such heterodimerization between pharmacologically distinct receptors could underlie a level of pharmacological diversity that would have far-reaching implications for drug development. In particular, it could provide new opportunities for the development of more selective compounds that would target specific heterodimers without affecting the individual protomers (George et al., 2002).

An interesting molecule that modulates aqueous humor production by acting in the ciliary body is melatonin. This indoleamine has emerged as a good candidate for the treatment of ocular hypertension, mainly because it is a natural substance already present in the aqueous humor and because melatonin MT_1 and MT_2 receptors are expressed in the ciliary body (Alarma-Estrany and Pintor, 2007). It is well known the existing inverse correlation between melatonin presence in blood and intraocular pressure (IOP). In the same way, when melatonin is administered to patients during the daytime their IOP get clearly reduced[1]. Paradoxically, when melatonin is measured in the aqueous humor of glaucoma patients its concentration is higher than in normotensive individuals. In fact, the mean concentration in the aqueous humor of healthy subjects was 14.62 ng/ml (range: 5.4-38.0; n=23) whereas in glaucoma patients it was 46.63 ng/ml (range: 10.3-167.3; n = 14). It appears as if the system is unbalanced in such a way that the 3.2-fold increase in the concentration of melatonin is totally insufficient to achieve normotension (for more detail, see chapter 2). Accordingly, melatonin-receptor mediated signalling is impaired in glaucoma.

Melatonin performs its hypotensive action on the ciliary body and in particular on its epitheliums, which express MT_1 and MT_2 melatonin receptors. Its action on IOP is mediated by regulation of Cl^- efflux from ciliary body epithelial cells (Huete-Toral et al., 2015). Interestingly, one of the molecules that block the effect of melatonin on reducing IOP is prazosin, the α_1-adrenoceptor antagonist. Melatonin should reduce IOP in glaucoma patients since its levels are abnormally high, nevertheless, patients with this pathology exhibit elevated IOPs. It could be the case that melatonin receptors may interact with α_{1A}-adrenergic receptors according to prazosin actions on melatonin effects. This evidence points to some sort of interaction between melatonin and α_1-adrenergic receptors. Ciliary

body non-pigmented epithelial cells (HCE) present MT_1 and MT_2 melatonin receptors as well as α_{1A}-adrenergic receptors. Interestingly, we have been repeatedly trying to find the canonical coupling of α_{1A}-adrenergic receptor to increases in the concentration of cytosolic Ca^{2+} with no success whatsoever. In fact, as shown in Fig. 1 α_{1A}-adrenergic receptor activation using phenylephrine did not trigger any intracellular Ca^{2+} mobilization in HCE cells. The relation between melatonin receptors and α_{1A}-adrenergic receptors may exist not only because they are present in the same cells but because some of melatonin actions can be blocked by the α_{1A}-adrenergic receptors antagonist prazosin as observed in fig 1.

Since Neufeld et al. first reported that adrenergic agents, including epinephrine and phenylephrine have the ability to increase cAMP concentration in the aqueous humor (Neufeld et al., 1972), the attention on the adrenergic control of IOP and the therapeutic potencial of the cAMP signalling pathway in glaucoma treatment has been taken into consideration. Since that moment, several studies have identified an adrenergic receptor-AC complex in the ciliary processes (Caprioli and Sears, 1984) supporting the functional role of cAMP in aqueous humor formation. The activation of ACs-linked receptors by several endogenous or exogenous factors not only increases intracellular cAMP level, but also decreases net aqueous humor flow and lowers IOP (Eakins and Eakins, 1964; Lee, 1958; Neufeld et al., 1972; Neufeld and Sears, 1974). Furthermore, an increase of the cAMP level by a topical suspension of 1% forskolin lowered IOP in rabbits and monkeys, as well as in normal human volunteers (Caprioli and Sears, 1983) suggesting that increasing cAMP may decrease the net rate of aqueous humor inflow (Caprioli and Sears, 1984). Our findings in this regards suggest a reduction in cAMP in the case of cells transfected with melatonin receptors or α_{1A}-adrenergic receptor (Fig 2F-H). However, cAMP ditermination in co-transfected cells with α_{1A}-MT_1 or α_{1A}-MT_2 heteromers showed

an increase in forskolin-induced cAMP levels, such results were very similar to the experiments done in HCE cells supporting the results obtained from native ciliary body tissue; we were able to visualize heteromer labelling through PLA technique and it was clearly reduced in the case of glaucomatous subjects. These results suggest a change of intracellular signalling when melatonin receptors form heteromers with α_{1A}-adrenergic receptor, nevertheless, when heteromerization is reduced due to glaucoma pathology, and receptors are separated, the cross-antagonism is missed as it can be shown in IOP measurements of 12-month-old glaucomatous DBA/2J mice. Remarkably, the hypotensive effect of melatonin receptor activation was further enhanced by the application of prazosin (Fig. 6E and 6H).

Here we propose the important role of melatonin receptors interaction with α_{1A}-adrenergic receptor in the physiology of the ciliary body and the role it has in reducing IOP. It can be a great possibility to suggest a potential pharmacological approach by using prazosin in combination with melatonin for the treatment of glaucoma.

Chapter V: Presence of melanopsin in human crystalline lens epithelial cells and its role in melatonin synthesis

Sensing the light through the crystalline lens

Light brings us the news of the Universe.

Sir William Bragg

Introduction

One of the most ancient phenomena of nature is light. It provides all living creatures with the information about the time of day in order for them to be able to adapt their physiology to their internal clock (Do and Yau 2010). In this sense, the ability to receive light is essential to regulate the circadian system. In mammals, including humans, the ocular system is responsible for light detection, beside its visual function, such as object recognition (vision), it is responsible for other non-image forming tasks like photoentrainment. In this sense, melanopsin emerges as a photoreceptor protein which has different functions from the classically known photoreceptors, rods and cones (Fu et al., 2005; Schmidt and Kofuji, 2011; Zele et al., 2011).

Melanopsin, expressing photosensitive retinal ganglion cells, represents a third class of photoreceptors, called intrinsically photosensitive retinal ganglion cells (ipRGCs), and it mediates several non-image forming responses to light, but can also contribute to visual pathways (Brown et al., 2012). Melanopsin is blue light sensitive photopigment which regulates pupil constriction, circadian entrainment and melatonin production (Foster and Bellingham, 2002).

Melanopsin is a member of the G-protein-coupled receptor family, a vitamin A-based opsin in the vertebrate retina (Zhao et al., 2014). It is specifically sensitive to blue light (Hattar et al., 2002; Provencio et al., 2000), in the way in which they capture this specific wavelength and send signals through several areas of the brain, such as the suprachiasmatic nucleus until reaching the pineal gland to suppress melatonin synthesis during the day (Vimal et al., 2009; Viola et al., 2008).

Since the discovery of this new opsin, it has been known to exclusively exist in the ipRGCs. However, with the development of new techniques, recent studies showed that melanopsin is present in several cerebral regions in the human brain (Nissila et al., 2016).

Melatonin is a molecule which is involved in the regulation of the circadian rhythm, it was classically known to be synthesized in the pineal gland. However, in recent decades, it has been possible to investigate this substance and come to numerous conclusions. Melatonin presence, its synthesis, as well as melatonin receptors have been found in several organs, besides the pineal gland. These findings allow us to see that melatonin has many different functions, apart from regulating the circadian rhythm (for more details, see general introduction) It is present and synthesized by several ocular structures, such as in the ocular surface, ciliary body, crystalline lens, aqueous humour and the retina (Alarma-Estrany and Pintor, 2007; Alkozi et al., 2016; Itoh et al., 2007).

Melatonin is synthesized from serotonin through two steps. First, serotonin is catalysed by an enzyme called aralkymine N-acetyltransferase (AANAT) and transformed to N-acetylserotonin. Melatonin is then synthesized by the final enzyme Hydroxindole O-methyltransferase (HIOMT) (Klein et al., 1997; Menendez-Pelaez et al., 1987). Both enzymes are necessary for melatonin synthesis; nevertheless, AANAT seems to be the rate limiting step of melatonin synthesis (Blomeke et al., 2008; Coon et al., 1996; Izawa et al., 2009; Klein, 2007).

In the eye, and specifically, in the anterior segment of the eye, melatonin has been measured in the aqueous humour , which is in contact with the crystalline lens and is responsible for several functions, such as maintaining the intraocular pressure, as well as nourishing the crystalline lens, because it is a transparent structure with no access to nutrients from the blood (Alkozi et al., 2016; Chiquet et al., 2006). Melatonin in the

aqueous humour can have an autocrine/paracrine effect on the trabecular meshwork, ciliary body and the lens, since all mentioned structures are in contact with the aqueous humour and present melatonin receptors (Alarma-Estrany and Pintor, 2007; Osborne and Chidlow, 1994).

Little is known about melatonin function and regulation in the crystalline lens, despite the importance it could have related to homeostatic processes controlling the lens transparency and its possible role as an antioxidative (Bai et al., 2013; Tok et al., 2014). In this sense, this work has been performed to study the possiblity of melanopsin being present in the lens and the possible regulation of melatonin synthesis in the lens through melanopsin receptor by studying melatonin levels under light, darkness and different light wavelengths.

Material and methods

Cells

Crystalline epithelial cells (HCL), a human immortalized cell line was kindly supplied by Dr. Coca-Prados (Yale University). Cells were grown in high glucose Dulbecco's modified Eagle's medium (Gibco/Invitrogen, Carlsbad, CA) containing 10% foetal bovine serum (Sigma-Aldrich, St. Louis, MO) and 0.05 penicillin/streptomycin (Gibco/Invitrogen) at 37°C in humidified atmosphere 5% CO2–95% air. After the culture reached the confluence, cells were detached with 0.25% trypsin and seeded into 6 well plates and/or to 4 well chamber slides respectively. All the experiments were performed using cells comprised numbers 10-15 passages to assure assays reproducibility.

Human eye tissue

Donor Human eyes were obtained from the Fundación Banco de Sangre y Tejidos de las Islas Baleares (Blood and tissue bank Foundation from Baleares Islands). Eyes were enucleated and collected without the cornea in sterile tubes and maintained in 4% paraformaldehyde in 0.1 M phosphate buffer (PB) (pH 7.2-7.4) at 4ºC until posterior processing. Eyes were dissected under a stereo microscope (Zeiss) and with the 0.8 mm tip curved forceps and sterile dissecting scissors, the crystalline lens was collected. Several washes in PBS were performed and then, the specimen were cryoprotected in a sucrose gradient (from 11% to 33%) and were embedded in tissue freezing medium (Tissue-Tek© OCT) until frozen with liquid N_2. Vertical sections of control and glaucomatous human samples (10 µm thick) were collected using a cryostat (Microm, Walldorf, Germany) and mounted from the same region. Samples were maintained in a -20 ºC until use.

Immunofluorescent studies

In the case of the cells, when cells reached 60% confluence, they were washed with PBS1X and then fixed in 4% parafprmaldehyde in PBS 0.1 M for 15 minutes. Several washes with PBS 1X were done before permeabilization using PBS 1X triton 0.05% during 15 minutes. Afterwards, one hour incubation was done with a blocking solution which contains 10% normal donkey serum (NDS), followed by incubation of CRYAB antibody (1:200, MA5-15383, Thermo Fisher Scientific) in the case of studying alpha crystalline presence and melanopsin antibody (1:100, sc-26957, Santa Cruz Biotechnology).

In a different slide chamber, cells were incubated either in darkness or in light condition for a duration of 8 hours before the immunocytochemistry, then, the same protocol was applied and cells were incubated with AANAT primary antibody (ab3505, 1:500, Abcam). All incubations took place overnight at 4˚C. Thereafter, an incubation of one hour with secondary antibody labelled with FITC (Jackson ImmunoResearch Laboratories) at a dilution of (1:100) was performed. Samples were examined under a confocal microscope (Axiovert 200M. Carl Zeiss Meditec GmbH, Germany). Similar protocol was applied to the human lens tissue, except the permeabilization duration which was for 30 minutes.

Light/darkness experiments

In order to perform these experiments, cells were seeded in multiwells at a density of $75x10^4$ cells/well and then incubated in the presence of a LED system. Cells were treated with white (WL), blue (BL, 465-480 nm, 2.66 -2.58 eV, 400 Luz, 3.2 W), green

(GL, 520-550 nm, 2.38-2.25 eV, 400 Lux, 3.2 Lux) red (RL, 625-640 nm, 1.98-1.93 eV, 400 Lux, 3.2 W) in total darkness. All experiments were performed at different durations, starting from 2, 4, 8, 12 hours. Afterwards, the medium was collected for HPLC analysis of melatonin levels, whereas the cells were homogenized for western blot assays.

HPLC experiments

HPLC was used to measure both N-acetylserotonin and to measure melatonin, in both cases the supernatant was processed as previously explained (chapter 2).

For N-acetylserotonin measurments, HPLC was performed following the protocol described by (Semak et al., 2008). NAS detection was carried out with SunFire18 (5 µ, 25 cm in length, 0.4 cm inner diameter) from Waters (milford, MA) equilibrated with a mobile phase consisting of 15% acetonitrile, 0.1% acetic acid and at a flow rate of 0.75 ml/ min, detecting NAS at the wavelength of 244 nm.

Melatonin detection was performed as described previously (refer to the second chapter). In brief, the column was a kromaphase C18 column 5.0 mm (25 cm in length, 0.4 cm inner diameter) (Scharlau, Madrid, Spain). The system was equilibrated overnight with 40% methanol, 60% H2O. Measurements were performed at a flow rate of 0.8 ml/min fixing the detector at a wavelength of 244 nm. Quantification of NAS and melatonin was performed by comparing the samples with the corresponding external standard provided by Sigma.

Western-blot studies

Experiments took place to quantify AANAT protein level in each condition and to visualize melanopsin protein by means of western-blot. In the case of AANAT detection,

cells were removed and homogenized with radioimmunoprecipitation assay (RIPA) buffer (1:5 v/v) containing 50 mM HEPES, pH 8, 150 mM NaCl, 1% NP-40 (w/v), 0.5% sodium deoxicolate, 0.1% SDS and Halt Protease and Phosphate Inhibitor Cocktail (Thermo Fisher Scientific). The lysates were centrifuged at 15,000 × g for 15 min at 4 °C. The supernatant was stored at −20 °C until use.

Protein concentration was determined by Pierce BCA Protein Assay Kit (Thermo Scientific). Cell samples (30 µg proteins) were diluted in Laemmli's sample buffer, loaded on a 15% SDS-PAGE gels and transferred to nitrocellulose membrane. Blots were blocked with 5% non-fatty milk (Bio-Rad) for 1 h at room temperature and then they were incubated overnight at 4 °C in TB- S1X 0.1% Tween 20 containing 5% of blocking buffer and AANAT primary antibody (ab3505, 1:1000, Abcam).

For melanopsin detection by western blot, a lysate of lens epithelial cells was used together with 4 homogenized retinas taken from mice (C57BL/6J, 6 months of age) isolated as described previously (Perez de Lara et al., 2015). Briefly, C57BL/6J mice were euthanized through decapitation on a sterile gauze pad. Both eyes were enucleated by a sterile curved scissors to cut the optic nerve. Eyecups were placed in PBS 1X solution and dissected under a stereomicroscope (SteREO Discovery.V8, Zeiss, Madrid, Spain). With 0.8 mm tip curved forceps and sterile fine-angled dissecting scissors, the cornea was severed. The forceps were then gently moved at a slight backward angle to pull up the cornea. Then the lens was removed with slight pressure on the eyecup. Gently, the retina was separated from the sclera, and the retina was cleaned of choroid pigment and vitreous. The mice lung was also dissected to use as a negative control and was homogenized similarly to retinas. Samples were removed and homogenized in ice with radioimmunoprecipitation assay (RIPA) buffer (1:5 v/v) containing 50 mM HEPES, pH 8,

150 mM NaCl, 1% NP-40 (w/v), 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and inhibitor proteases (1 mM phenyl methanesulfonyl fluoride (PMSF), 10 μg/ml leupeptin, 5 μg/ml pepstatin, 10 μg/ml aprotinin, 1 mM sodium fluoride, and 2 mM sodium orthovanadate). The lysates were centrifuged at 15,000 ×g for 15 min at 4 °C. The supernatant was stored at −20 °C until use.

Melanopsin was detected by submitting all samples to electrophoresis and western-blot as previously described, nitrocellulose membrane were incubated with a specific melanopsin antibody (1:50) (sc-26957, Santa Cruz Biotechnology, CA, USA) overnight. Mouse monoclonal glyceraldehyde-3-phosphate dehydrogenase (anti-GAPDH 1:500) (Santa Cruz, CA, USA) served as a loading control.

All the membranes were incubated with a goat anti IgG rabbit or a goat anti IgG mouse conjugated with horseradish peroxidase secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA) for 1h at room temperature. Then, proteins were revealed by chemiluminescence using ECL detection (Amersham Pharmacia Biotech). Films were scanned with Gel Logic 200 Imaging System (Kodak, U.S.). The densitometric analysis was performed by using Kodak Molecular Imaging software (Kodak, Rochester, NY, USA). The densitometry values of each sample were normalized to respective densitometric GAPDH values.

Blocking Melanopsin function

Cells were treated with a selective melanopsin inhibitor AA92593 (prepared in PEG-400, DMSO, SML0865, Sigma, St. Louis, Mo, USA) at a final concentration of 1.5 μM and then were incubated under light for 8 h. For second messenger inhibition, cells were incubated with a phospholipase C inhibitor (also prepared in PEG-400, DMSO,

U73122, Tocris, Bristol, UK) at 3 µM. In different wells, a negative control was incubated at same concentrations with the inactive compound U73343 (prepared in PEG-400, DMSO, Tocris, Bristol, UK), the experiments being performed under 8 h illumination with white light. Thereafter, supernatant was collected and cells were homogenised for HPLC analysis and western blot studies respectively.

Statistical analysis

All the data are the mean ± S.D. of 6 independent experiments. Statistical analysis was performed with ANOVA or Student's t-test when necessary. Statistical analysis and plots were carried out by means of the program Prism Graph Pad (GraphPad Software Inc., San Diego, CA).

Results

Melanopsin presence in human crystalline lens epithelial cells

The characterization of the human lens epithelial cells was done by means of the B chain of alpha crystalline (CRYAB), a representative protein of the lens (Augusteyn, 2004; Maddala and Rao, 2005; Yang et al., 2016). There was an intense labelling of the cytoplasmic area in the cells, indicating the abundance of this protein which is typical in the lens cells as commented (Fig. 5.1A).

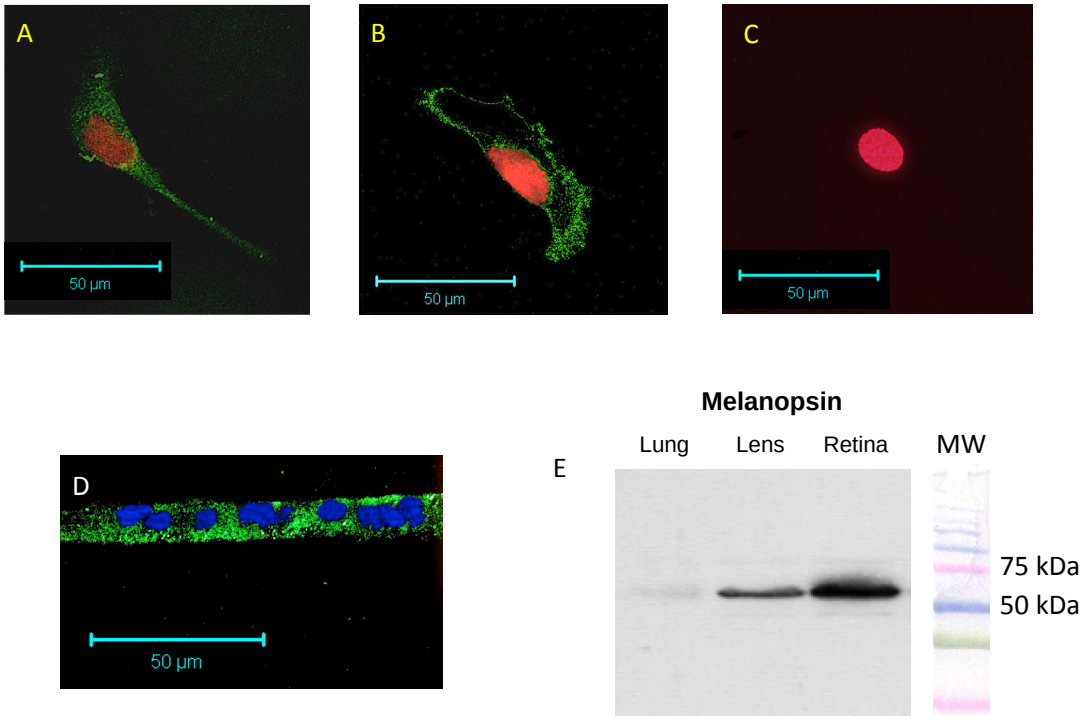

Figure. 5.1. Presence of melanopsin in human lens epithelial cells. A) Expression of the B chain of the alpha crystalline (CRYAB, in green) in human lens epithelial cells (nucleus labelled with propidium iodine in red). B) Localization of melanopsin in human lens epithelial cells (green), nucleus appear in red labelled with proidium iodine. C) Negative control for the presence of melanopsin in human chondrocytes. D) Presence of melanopsin in the epithelial cells of a human lens section (green), nuclei labelled with DAPI in blue. E) Western-blot showing melanopsin band obtained from mice retinas (positive control), human lens epithelial cells and lung negative control.

For melanopsin detection in the lens epithelium, an immunocytochemistry and western-blot experiments were conducted. With regard to immunocytochemical assays, a positive labelling of melanopsin was observed mostly on the cytoplasmic membrane of the lens epithelial cells, as well as in the human epithelial cells obtained from healthy donors (Fig.1B and 1D). As a negative control for melanopsin, human chondrocytes were used as observed in Fig.1C.

Western-blot experiments using the lens epithelial cells homogenate showed a band with a molecular weight of 65 kDa, which is similar to that described in literature (Carr et al., 2011). A lysate of mice retinas was used for a positive control and lung tissue as a negative control as well (n = 3) (Fig. 1E).

NAS and melatonin levels changes under different light conditions

Melatonin synthesis follows a circadian rhythm in the pineal gland and this is regulated by light. Melanopsin is the protein responsible for such task transmitting light information to the pineal gland through a certain pathway starting from the retina. However, in other organs which synthesize melatonin, such as the crystalline lens in this case, it was interesting to see the effect on melatonin after stimulating the lens epithelial cells with light or darkness, together with NAS determination, the precursor of the first enzyme of melatonin synthesis (AANAT). An increasing temporal assay was done in both conditions starting from 0, 2, 4, 8, 12 hours of incubation.

Results of HPLC analysis showed no significant changes neither in NAS nor melatonin levels at all times under light condition (Fig. 2A and C). Nonetheless, when cells were submitted to total darkness for the same period of time, the melatonin level, as well as NAS, increased with a maximal effect occurring after 8 hours (Fig. 2B and D). In the case of NAS it was clear that the peak increased between 4 to 8 hours. Melatonin peaks

increased up to 8 hours and then stabilized up to 12 hours of darkness. Melatonin levels quantification after 2 hours incubation in darkness was 10.32 ± 2.40 pmol/106 cells, while NAS levels were 15.06 ± 2.52 pmol/106 cells. These values changed in the case of NAS from 120.59 ± 11.21 pmol/106 cells at 8 h to 104.29 ± 11.80 pmol/106 at 12 h (***p < 0.001, n = 6). Melatonin values increased from 59.45 ± 15.71 pmol/106 cells to 66.01 ± 22.14 pmol/106 cells after 8 h and 12 h incubation respectively (***p < 0.001, n = 6).

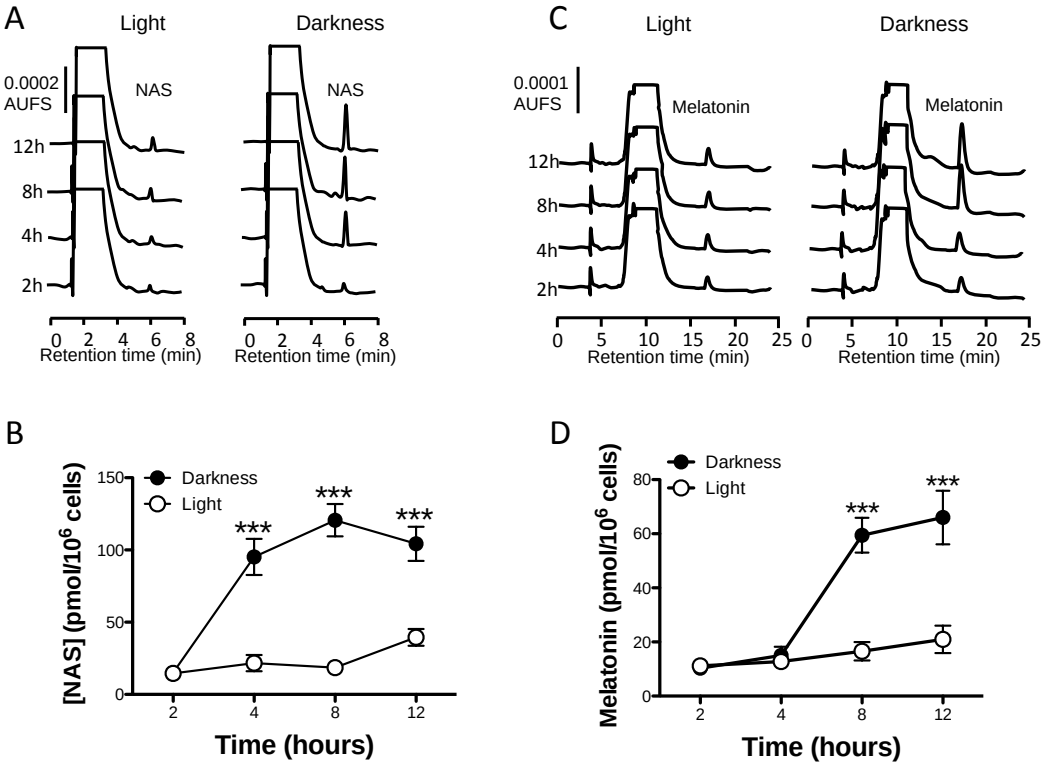

Figure. 5.2. Effect of light and darkness on NAS and melatonin production in human lens epithelial cells. A) Representative HPLC elution profiles showing the changes in NAS levels after cell treatment with light (left panel) and darkness (right panel). B) Line graph presenting the concentrations of NAS calculated from the chromatographic studies under light and darkness conditions shown in A. C) HPLC profiles showing the changes in melatonin levels after cell treatment with light (left panel) and darkness (right panel). D) Line graph presenting the concentrations of melatonin calculated from the chromatographic studies under light and darkness conditions shown in C. Values are the mean ± SD of 6 independent experiments (***p < 0.001).

Light/darkness effect on AANAT

Aralkylamine N-acetyltransferase (AANAT) is considered the key enzyme which regulates melatonin synthesis. After observing that NAS levels were modified under darkness conditions as well as melatonin concentration, the presence of AANAT was verified by western-blotting. A study took place to evaluate and quantify the differences between light and dark conditions after incubating the cells for 8 hours in the corresponding condition.

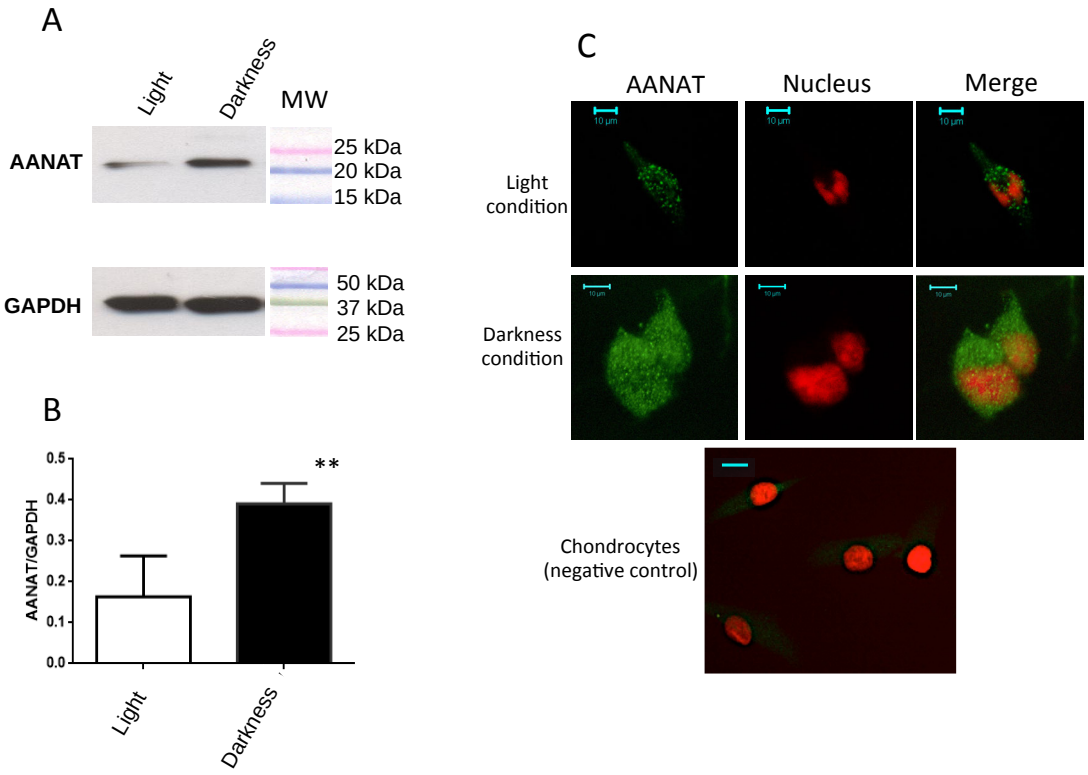

Figure. 5.3. Changes in AANAT expression under light and darkness conditions in human lens epithelial cells. A) Representative western-blot showing the changes in AANAT expression after 8 h of light or darkness. B) Column plot showing the relative quantification of the western-blots band intensities shown in A. Immunohystochemical study performed in human lens epithelial cells showing the changes in cytosolic AANAT (green label) before and after 8 h of darkness (the nuclei appear in red). A negative control was obtained using human chondrocytes. The values represent the mean ± SD of six independent experiments (**$p < 0.01$).

A decrease in AANAT band (23 kDa) could be appreciated when cells were incubated with light (Fig. 5.3A). A clear difference was shown between AANAT levels under light and dark condition. Western-blot showed that AANAT expression increased approximately 2.5 times in darkness compared to light (Fig. 3B, **$p < 0.01$, $n = 6$).

The presence of AANAT in the crystalline epithelial cells was also proven by immunocytochemical analysis as shown in Fig. 3C (n=4). There was an increase in the cytosolic presence of the enzyme when the cells were submitted to darkness during 8 h. On the other hand, human chondrocytes served as a negative control since they did not apparently show the presence of AANAT in their cytoplasm (Fig. 5.3C).

NAS and melatonin sensitivity to wavelength

Melanopsin is a photosensitive protein with a specific sensitivity to 460-480nm, which corresponds to blue light in the visible spectrum. To verify melanopsin functionality and its characteristics regarding its absorbance properties, lens cells were incubated with blue (465–480 nm), green (520–550 nm) and red light (625–640 nm) during 0, 2, 4, 8, and 12 hours. Later, extracellular medium was analyzed to determine NAS and melatonin levels.

Results revealed that blue light did not modify either NAS or melatonin (Fig. 5.4 A, B). On the contrary, red and green lights were able to change NAS and melatonin levels. Analysis of NAS levels showed values of 129.45 ± 9.14 pmol/106 cells at 8 hours and 124.66 ± 26.16 pmol/106 cells after 12 hours incubation with red light. Values obtained from experiments with incubation of 2 hours were 14.58 ± 2.99 pmol/106 cells, significant differences were observed after 8 hours of incubation (***$p < 0.001$, $n = 6$). Green light also stimulated NAS presence at 8 and 12 h, providing values of 113.89 ±

15.44 pmol/ 106 cells and 95.99 ± 14.59 pmol/106 cells respectively (*p < 0.05, ***p < 0.001, n = 6) (Fig. 4A).

Effect of wavelength on melatonin production in human lens epithelial cells. A) Line graphs showing the changes in NAS after cell treatment with blue (465–480 nm), green (520–550 nm) and red light (625–640 nm) during 0, 2, 4, 8, and 12 h. B) Line graph presenting the concentrations of melatonin under the light conditions and time exposure shown in A. The values are the mean ± SD of 6 independent experiments cells (*p < 0.05, **p < 0.01, ***p < 0.001 vs. Blue light).

Melatonin analysis showed an increase after 8 - 12 hours incubation with red light of 51.99 ± 6.51 pmol/106 cells (Fig. 4B, n = 6). However, under blue and green light melatonin levels were 37.61 ± 6.64 pmol/106 cells and 42.31 ± 7.72 pmol/106 cells respectively (n = 6). Differences between 8 h and 12 h were statistically significant in the case of red and green illumination when compared to blue treated cells (**p < 0.01, ***p < 0.001, n = 6).

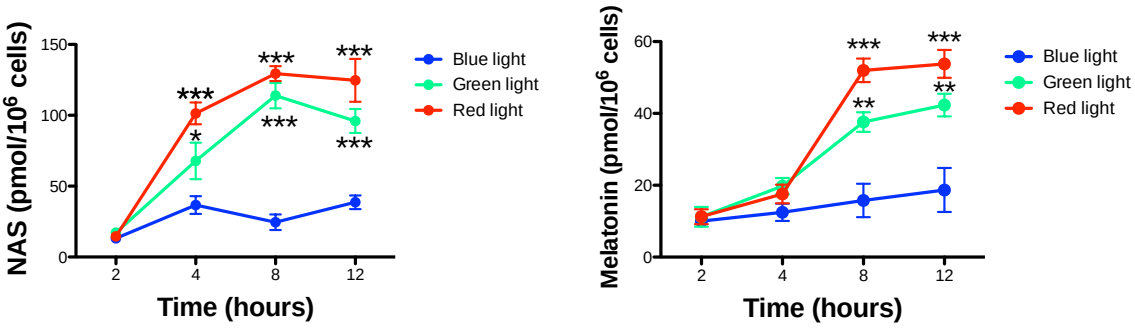

Figure 5.4. Effect of wavelength on melatonin production in human lens epithelial cells. A) Line graphs showing the changes in NAS after cell treatment with blue (465-480 nm), green (520-550 nm) and red light (625-640 nm) during 0, 2, 4, 8, and 12 h. B) Line graph presenting the concentrations of melatonin under the light conditions and time exposure shown in A. The values are the mean ± SD of 6 independent experiments cells (*p < 0.05, **p < 0.01, ***p < 0.001 vs. Blue light).

Lens AANAT levels varies under different light conditions

A study of the expression of AANAT took place at the same time as the identification of melatonin levels by HPLC. After the incubation of the cells under blue, green, and red light during 8 h, as can be observed in Fig. 5.5A, intensities of AANAT expression were different under each mentioned condition. A clear reduction of the expression can be seen in blue light treated cells, while an increment could be observed in the cells submitted to green or red light (Fig. 5.5A).

AANAT expression was found to be 2.5 times more robust under green light in comparison to blue light, and it rose up 3.2 times under red light compared to blue light (Fig. 5.5B, **p < 0.01, n = 6).

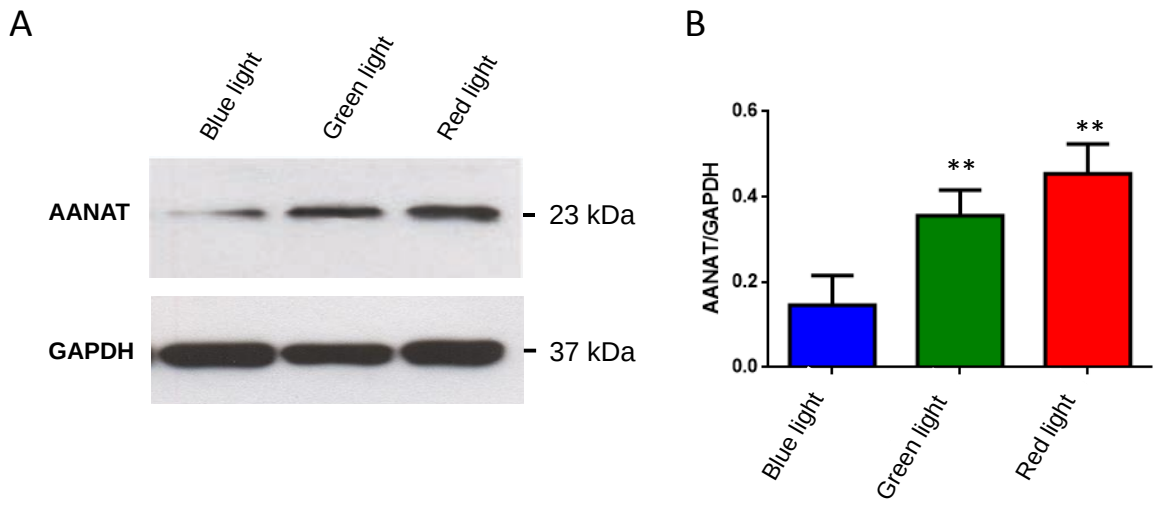

Figure 5.5. Effect of wavelength on AANAT expression in human lens epithelial cells. A) Representative western-blot showing the changes in AANAT expression after 8 h of blue (465e480 nm), green (520e550 nm) and red light (625e640 nm). B) Column plot showing the relative quantification of the western-blots band intensities shown in A. Values represent the mean ± SD of six independent experiments (**p < 0.01). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

NAS, melatonin and AANAT levels after blocking melanopsins' effect

There are only a few melanopsin antagonists commercially available, such as AA92593. Melanopsin antagonist AA92593 was assayed under light condition to verify if NAS and melatonin can be synthesized under light and in the antagonist presence. Also, as melanopsin is coupled via phospholipase C (PLC), the inhibitor U73122 and the inactive analogue of the former, the compound U73343, were also used.

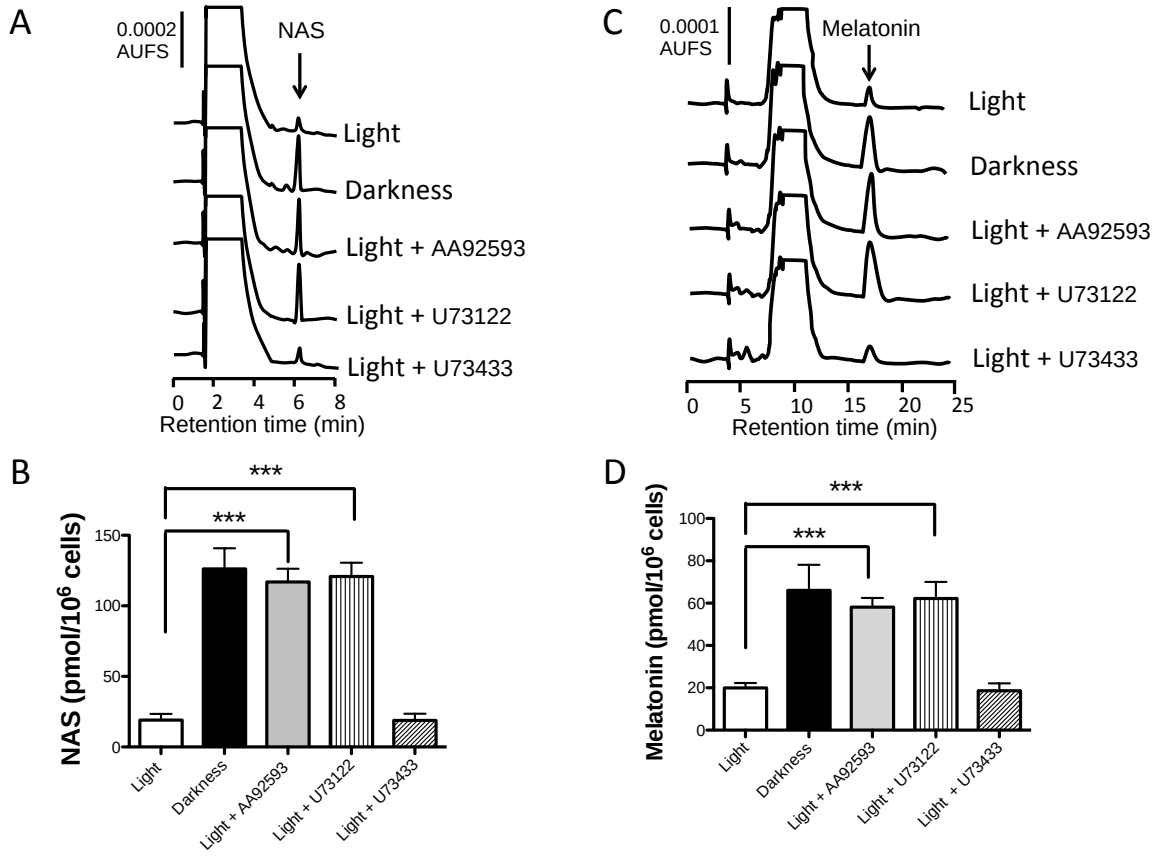

Figure 5.6. Effect of melanopsin agents on melatonin production in human lens epithelial cells. A) HPLC elution profiles showing NAS peaks under 8 h light conditions in the presence of the melanopsin antagonist AA92593 and the PLC inhibitor U73122 and the inactive compound U73343. B) Column plot presenting the concentrations of NAS calculated from the chromatographic studies shown in A. C) HPLC chromatograms showing the changes of melatonin with the agents described in A. D) Column plot presenting the quantification calculated from the chromatograms showed in C. Values are the mean ± SD of four independent experiments (***$p < 0.001$).

As presented in figure Fig. 5.6, cells stimulated with light for 8 h in the presence of melanopsin antagonist AA92593, both NAS and melatonin levels increased. NAS level was 116.94 ± 9.34 pmol/10^6 cells in the presence of AA92593 and 120.75 ± 9.82 pmol/ 10^6 cells with the PLC inhibitor U73122 (n = 4, ***p < 0.001, Fig. 6A and B). The change in melatonin level with AA92593 was 58.16 ± 4.26 pmol/10^6 cells, which is very close to the one obtained in darkness conditions during 8 h, this being 66.01 ± 12.14 pmol/10^6 cells (n = 4, Fig. 5.6C). When the experiments were performed with the PLC inhibitors U73122 melatonin levels were 62.13 ± 7.80 pmol/10^6 cells, while in the case of the inactive compound melatonin presence was 18.66 ± 3.52 pmol/10^6 cells (n = 4, ***p < 0.001, Fig. 5.6D).

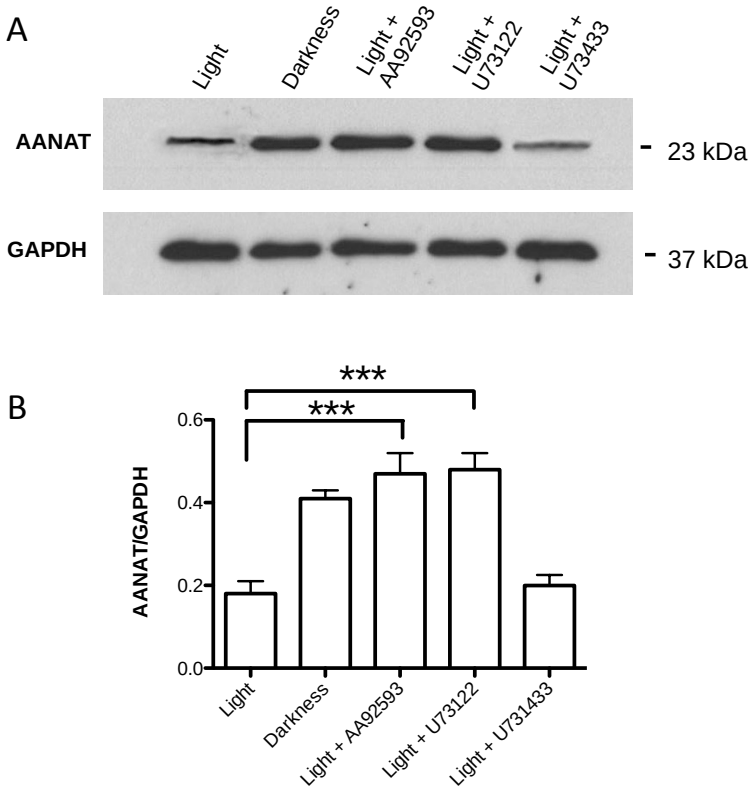

Figure 5.7. Effect of melanopsin agents on AANAT expression in human lens epithelial cells. A) Representative western-blot showing the expression of AANAT under 8 h light conditions in the presence of the melanopsin antagonist AA92593 and the PLC in- hibitor U73122 and the inactive compound U73343. B) Column plot showing the relative

quantification of the western-blots band intensities shown in A. Values are the mean ± SD of four independent experiments (***$p < 0.001$).

Studies of AANAT expression using western-blot of the mentioned assay resulted consistent with the results previously commented (Fig. 7A). Quantification of western-blots showed no significant differences between darkness and light in the presence of melanopsin antagonist or the PLC inhibitor ($n = 4$, ***$p < 0.001$, Fig. 7B).

Discussion

Melanopsin presence was discovered for the first time in the human lens epithelial cells and it has been related to the local synthesis of melatonin. Changes in the extracellular melatonin levels were able to be induced by changing light/darkness conditions, due to the variation of the expression of melatonin synthesizing enzyme AANAT. Light reduced the expression of AANAT, therefore, a concomitant decrease in melatonin production was observed. On the other hand, darkness increased both AANAT and melatonin levels.

Melanopsin presence in the intrinsically photosensitive retinal ganglion cells (ipRGC) shares some features with the one described in this study, for instance, melanopsin sensitivity to blue light was found in the literature, and melanopsin present in the epithelial cells of the lens were mainly stimulated by the blue component of white light, about 480 nm wavelength, as what happens with ipRGC showing similar spectral sensitivity (Bailes and Lucas, 2013; Graham and Wong, 1995; Kumbalasiri and Provencio, 2005). Inhibition of melanopsin action by using a specific antagonist AA92593 was able to abolish the effect of melanopsin and AANAT and melatonin levels followed a very similar pattern to the darkness, an increment in AANAT expression as well as melatonin levels was observed, confirming that the effect observed is due to melanopsin (Bertolesi et al., 2016; Jones et al., 2013). Moreover, melanopsin in the ipRGCs has been shown to be linked to phospholipase C (PLC) membrane protein(Angueyra et al., 2012; Panda et al., 2005; Xue et al., 2011). It was worthwhile investigating this feature of melanopsin pathway in the studied cell model, blocking PLC by an inhibitor U73122 demonstrating that, in the presence of light, there was no inhibition in AANAT expression or in melatonin production.

Light profoundly influences circadian, neuroendocrine, and neurobehavioral regulation in all mammals, it is essential to life on our planet. Daily rhythmic melatonin signals provides temporal coordination of physiological functions, such as chronobiologic rhythms of locomotor activity, sleep-wake cycle, dietary and water intake, hormone secretion and metabolism (Slater, 1983). Melanopsin containing photoreceptors, is very important in many aspects and studies have shown that, although light suppresses melatonin production, which can appear as a negative effect, however, daytime exposure to blue-enriched light resulted very positively on the night-time melatonin amplitude and the circadian regulation (Dauchy et al., 2015; Dauchy et al., 2016).

Light effect on melatonin production has been investigated in the past (Burgess et al., 2015). However, classically, melatonin synthesized in the pineal gland was studied, and regulation was driven through the suprachiasmatic nucleus (Berson et al., 2002). In the current study, the control of AANAT expression by the light was shown to have similar behaviour to the one studied in the pineal gland (Bergstrom et al., 2003), but with no connection to the neural component. Since the crystalline lens is neither innervated nor vascularized, this makes it difficult to receive neuronal messages or vascular melatonin release at night. Therefore, it may synthesize melatonin locally to allow it to follow the same rhythmicity of the pineal melatonin.

Melatonin synthesis in the lens has been discovered in experimental animals, and its preventive role in cataract pathology has been suggested (Abe et al., 1999; Abe et al., 1994). The current research using human epithelial lens cells and human lens tissue added a relevant importance to the role of melatonin and its synthesis and regulation by melanopsin in the human crystalline lens, together with the ciliary body, melatonin is released by both ocular structures to the aqueous humour (Aydin and Sahin, 2016; Chiquet

et al., 2006). Melatonin presence in the aqueous humour plays numerous functions in the anterior segment level of the eye, such as an antioxidant and protectant agent (Khorsand et al., 2016; Sande et al., 2016).

Recent studies have shown a negative effect of crystalline lens removal due to cataract on the circadian rhythm and melatonin levels, all studies are focused on the harmful effect of blue light on the retina and the fact that the lens absorbs UV light and protect the retina from photo-toxicity (Alexander et al., 2014; Brondsted et al., 2015; Erichsen et al., 2015). Besides the afore-mentioned facts occurring due to the lens removal, it could be important to remember that, by removing the lens, a melatonin synthesizing structure is also removed. More studies are definitely needed to assess the importance of melatonin presence in the lens. Moreover, further speculations could also rise by the discovery of melanopsin in the lens.

It would be possible to modify the levels of melatonin in the lens by changing the illumination condition, especially because melatonin has the ability to reduce free radicals (Ianas et al., 1991), and all those damaging products would be diminished, thereby preventing cataract formation, as suggested by other authors (Siu et al., 2006). This may suggest that the use of some filters preventing the light in the range 460-480 nm wavelength may facilitate the production of melatonin and would protect the lens of free radical damage, bearing in mind, of course that the time of usage of such filters since exposing to short wavelength light could be time-dependant beneficial at some moments of the day (An et al., 2009). This technology has been applied in some intraocular lenses used in cataract surgery or also in spectacles to treat some neurological disorders such as migraines (Hoggan et al., 2016; Mainster, 2006). It would be very interesting to test

melatonin levels in the eye for variation regarding what happens in the pineal gland and bloodstream (Casper and Rahman, 2014; Csernus et al., 1999; Sasseville et al., 2006).

On the other hand, melatonin synthesized by the lens would contribute to melatonin levels observed in the aqueous humour as previously studied (for more details, see chapter 2). Since melatonin synthesis can be induced by darkness, it can activate MT_2 and MT_3 receptors located in the ciliary body (Alarma-Estrany and Pintor, 2007). This activation leads to a reduction in the chloride efflux which produces a concomitant decrease in aqueous humour formation and a reduction in IOP (Huete-Toral et al., 2015). This is clearly suggesting a possible role of melatonin and analogues in the treatment of glaucoma. Indeed, recent studies have demonstrated that the applications of melatonin or its analogue 5-MCA-NAT were able to reduce IOP in an animal model of glaucoma (Martinez-Aguila et al., 2013; Martinez-Aguila et al., 2016). Filtering the blue component of light could be useful to trigger the synthesis of melatonin in the lens increasing the concentration of this substance in the aqueous humour with the related reduction in IOP. Experiments are necessary to confirm this possibility.

In conclusion, melanopsin is present in human lens epithelial cells. Its stimulation by light reduces the expression of melatonin synthesis enzyme AANAT, reducing the presence of melatonin in the extracellular milieu. Since melatonin presents interesting features such as a scavenger agent towards free radicals and it has hypotensive properties on IOP, it would be of interest to explore its therapeutic use by naturally stimulating it in the lens using filters or other optical devices.

Chapter VI: General discussion

It's not what you look at that matters, it's what you see

Henry David Thoreau

Discussion

In the present work, several aspects of melatonin in some ocular structures were investigated, mainly in the search for a better understanding of its role on intraocular pressure. First of all, human aqueous humor of patients with ocular hypertension were analyzed and they showed higher levels (around 46.63 ng/ml) of melatonin compared to normotensive patients (14.62 ng/ml). Currently, it is possible to assume that melatonin found in the aqueous humor does not only come from the ciliary body, where the aqueous humor is produced. Also, the crystalline lens, which stand in contact with the aqueous humor, contributes to the aqueous humor melatonin levels as previously described (see chapter 5).

In order to study in depth the phenomena of finding higher melatonin concentrations in patients with elevated intraocular pressure, a cellular model was used. In vitro studies mimicking a glaucomatous condition were carried out by activating a channel sensitive to pressure (TRPV4). Several assays were done to investigate melatonin synthesis and signalling pathways in non-pigmented ciliary body epithelial cells, where the formation of aqueous humor is held. TRPV4 channel is a member of the transient receptor potential channels which are expressed in a range of species from yeast to humans and are widely expressed throughout many different tissues (Clapham, 2003; Shibasaki, 2016). In the current work, the presence of the channel TRPV4 was confirmed in human non-pigmented ciliary epithelial cells, and its role in activating the mechanism of melatonin synthesis was studied. Many TRPV channels are activated by multiple stimuli (Benham et al., 2003; Hardie, 2003), implying a diversity in ion selectivity and the polymodal mechanisms of activation, which necessarily result in equally diverse cell functions (Montell, 2005; Mutai and Heller, 2003; Nilius and Voets, 2004). For instance, TRPV4

channel was first cloned as an osmosensor (Strotmann et al., 2000), however, it can also act as shear stress or fluid flow sensor as well as a temperature sensor (Chung et al., 2003). The presence of TRPV4 in the ciliary body epithelial cells was recently described in the mouse experimental model and it was suggested to play an important role in regulating cell volume, lipid, and calcium signals and therefore it represented a novel and potencial target for antiglaucoma medications (Jo et al., 2016).

Interestingly, our experiments revealed a relevant relationship between TRPV4 and AANAT phosphorylation, a process known to protect AANAT enzyme from proteosomal degradation (Pozdeyev et al., 2006). Not only that TRPV4 activation permits melatonin release into the aqueous humor, but also at a short period stimulation by a selective agonist, a series of intracellular actions occurred with the final goal of permitting the melatonin enzyme (AANAT) to rise and to be phosphorylated.

Our results indicated that activating TRPV4 resulting in AANAT phosphorylation is mediated by calmodulin and calcium-calmodulin dependent protein kinase II. Previous studies using heterologous cellular models expressing TRPV4 channel, showed that intracellular Ca^{2+} potentiates this channels' currents and it accelerate and amplifies the current response to hypotonic solutions and phorpol ester agonists. Moreover, This effect of Ca^{2+} occurs through an action at an intracellular site in the C terminus of the TRPV4 channel protein and is mediated by CaM binding (Strotmann et al., 2003).

Activation or potentiation of Ca^{2+} permeable channels by Ca^{2+} is uncommon, particularly for channels in the plasma membrane, because of the positive feedback effect and the potential damage due to the cellular Ca^{2+} overload. Other Ca^{2+}-permeable channels that are potentiated by Ca^{2+} are those involved in Ca^{2+} release from intracellular stores and include ryanodine receptors and inositol 1,4,5-trisphosphate receptors. As for TRPV4, the

activity of these channels is tightly controlled by Ca^{2+}-dependent negative feedback mechanisms that involve CaM (Rebas et al., 2012). It is likely that Ca^{2+}-CaM, by binding to the C-terminal domain, induces a conformational change in the TRPV4 channel protein resulting in increased channel activity. Similar mechanisms have been proposed for the activation of a number of channel types, including Ca^{2+}-activated K^+ channels and cyclic nucleotide-gated channels, whose activity is controlled by intracellular mediator (Ca^{2+}, Ca^{2+}-CaM, or cAMP/cGMP) binding in the C terminus (Strotmann et al., 2003).

TRPV4 channel has been extensively investigated in several areas and its presence was confirmed in numerous cell types. Concerning the eye, recent studies indicated a special importance of this channel in regulating IOP as a result of its presence in the trabecular meshwork. Results confirmed that TRPV4 in the trabecular meshwork represents a crucial link between membrane stretch, Ca^{2+} signals and cytoskeletal reorganization, moreover, TRPV4 activation is required for persistent IOP elevation in an animal model of glaucoma (Ryskamp et al., 2016). Our results suggests an added value in the search to understand glaucoma for several reasons, TRPV4 presence in both important ocular structures corroborate its role in the regulation of IOP. From the one hand, non-pigmented ciliary body epithelial cells are secretory cells where aqueous humor is formed, and elevated IOP could be due to an excessive formation of aqueous humor, or a decrease of its drainage through the trabecular meshwork. A pressure sensor in essential for both functions to work properly avoiding IOP to raise and TRPV4 could be a good target for the treatment of elevated IOP.

Both in the case of glaucoma patients and in the cellular model, the rise in pressure produces an increase in extracellular melatonin levels. So, the unanswered question is: Why if melatonin levels are high in the aqueous humor IOP is not reduced?

One possibility is that melatonin receptors are not working as they normally do when glaucoma appears. This led us to study melatonin receptors considering the possibility of forming receptor-receptor heteromers with different types of receptors.

In recent years, new strategies have been proposed to consider the complex functioning of GPCRs. Thus, the complexity of the receptors at different molecular levels is considered, so specific drugs are sought for a subtype of GPCR, drugs that recognize an allosteric binding center, drugs that consider the oligomerization of GPCR, drugs that neutralize constitutive activity and drugs targeting other molecular elements that regulate different signalling pathways (Liebmann, 2004). In this sense, a study was conducted to investigate the possibility of melatonin receptors to heteromerize with other receptors present in the ciliary body.

Receptor homomerization is defined as the physical association between identical receptors, whereas heteromerization is the association between distinct receptors. Because to date, the available techniques did not allow the distinction between higher order dimers or oligomers, the term dimer is often used in the sense that it is the simplest form of an oligomeric functional unit; In spite of this, at the moment the techniques to distinguish between dimers, trimers and tetramers are being developed (Carriba et al., 2008; Vidi and Watts, 2009).

The dimers/oligomers have different functional characteristics than the receptors that constitute them, so oligomerization confers new properties to GPCRs, which establishes a possible mechanism to generate new functions in these receptors. This phenomenon has given rise to a new level of complexity that governs the signaling and regulation of these proteins (Ferre et al., 2009).

Concerning melatonin and some of its analogues, it has been possible to suggest an interaction between melatonin and its analog 5-methoxycarbonylamino-N-acetyltryptamine, 5-MCA-NAT and other adrenergic receptors like ß$_2$ and α_{2a} on a pharmacological level. Results showed that pre-treatment with melatonin or its analog 5-MCA-NAT, followed by the application of commercial eye drops of ß$_2$ antagonist or α_2 adrenergic agonist, potentiate ocular hypotensive effect of melatonin or its analogue (Crooke et al., 2013). These results support the possibility of melatonin receptor forming heteromers with different receptors. In addition, studies confirmed that MT_1 and MT_2 receptors form heteromers in transfected HEK293T cells, as well as its presence and functionality in mice photoreceptor cells (Baba et al., 2013). Another interesting pharmacological finding was the use of agomelatine, a commercial antidepressant agent which was proven to restore disrupted circadian rhythm and reduces depression-like behavior. Agomelatine is an agonist for MT_1 and MT_2 receptors as well as an antagonist of serotonergic 5-HT_{2C} receptors. Interestingly, treatment with 5-HT_{2C} antagonists or melatonin alone failed to reproduce these effects (Cardinali et al., 2012; Carney and Shelton, 2011; Racagni et al., 2011). Supporting these findings, melatonin was found to inhibit the ability of 5-HT to phase shift the suprachiasmatic circadian clock (Prosser, 1999). More recently, with the technological advances in the area of GPCRs heteromerization, consistent results have shown that melatonin receptor MT_2 physically interact with serotonin 5-HT_{2C} (Kamal et al., 2015).

A fascinating discovery regarding receptor-receptor interaction, is the fact that in some cases, signalling pathways can change the canonical pathway. In fact, reports are starting to appear that link heteromers to a distinct signalling machinery recruitment. An example of such a phenomena is dopamine receptors D_1 and D_2, these two receptors are

coupled to Gs and Gi respectively (George and O'Dowd, 2007; Rashid et al., 2007a). These receptors were investigated and showed to form heteromers with a selective G_q-protein coupling (Rashid et al., 2007b). Earlier, dopamine receptor-mediated calcium signalling in the central nervous system was assumed to be due to an interaction between Dopamine receptor D_1 and calcyon (Lezcano et al., 2000). Up to date, the only evidence correlating dopamine neurotransmission and calcium signalling is through the D_1-D_2 receptor heteromer (Casado et al., 2009).

Our findings suggests that melatonin receptors MT_1 and MT_2 when forming heteromer with an α_{1A} adrenergic receptor, shifts its signalling to be coupled to G_s-protein (while MT receptors are coupled canonically to Gi). Such a behavior in shifting the signalling pathway was found also in the previous example of MT_1-MT_2 heteromer in photoreceptors, were experiments showed that the effect of melatonin on the photoreceptors is independent of the G_i/cAMP pathway and it involves instead an activation of PLC/PKC pathway (Baba et al., 2013). Actually, there is consensus in considering that a receptor heteromer is an entity with a different pharmacology and a different functionality (Ferre et al., 2009). Therefore, distinctive pharmacological and functional characteristics (a kind of “heteromer fingerprint”) are indeed helpful to detect receptor heteromers in native tissues and it would be predicted to be invaluable for drug development (Casado et al., 2009).

Our findings showed a decrease in melatonin receptors in glaucomatous cases, together with a drop of receptor heteromerization with alpha adrenegic α_{1A} receptor (for more details, see chapter 4). However, antagonizing the effect of alpha adrenergic receptor by applying prazosin seems to permit melatonin action to work. Such a behavior is demonstrated in different heteromer formed cases mentioned above.

Melatonin appears to be helpful together with its effect in decreasing intraocular pressure among other functions, it could be beneficial since it works as an antioxidant among numerous functions (Anderson and Rodriguez, 2015; Tchekalarova et al., 2015).

Another ocular structure bathed with the aqueous humour is the crystalline lens. It is known that the lens synthesize melatonin as well. Accordingly, melatonin synthesis and regulation by the crystalline lens was studied and a search for a light sensor to regulate melatonin synthesis took place and resulted in the discovery of melanopsin receptors in the lens epithelial cells. These findings are of extreme importance from several aspects. In the present work, light is spotted towards the aqueous humor melatonin content and its possible contribution of the crystalline lens in producing enough melatonin to lower IOP. Nevertheless, melanopsin presence in an ocular anterior segment part demand a deeper investigation since it indicates numerous hidden functions which needs to be addressed in the future.

Conclusions

“People think of education as something they can finish.”

Isaac Asimov

Conclusions:

1. Melatonin levels are increased in the aqueous humor of patients with elevated intraocular pressure. A correlation between melatonin and IOP is proved.

2. The TRPV4 channel acts as a pressure sensor in non-pigmented ciliary body epithelial cells. TRPV4 activation triggers melatonin release in a time and dose dependent manner, this effect beingabolished by silencing TRPV4 as well as by the use of antagonists.

3. TRPV4 activation in a short period of time was able to phosphorylate AANAT enzyme, phosphorylation was mediated by calcium-calmodulin dependent protein kinase II, the effect being an activation of this enzyme.

4. Melatonin receptors MT_1 and MT_2 form heteromers with α_{1A} adrenergic receptors in the ciliary body epithelial cells. Also, cross-antagonism was confirmed between both receptors.

5. Melatonin receptors MT_1 and MT_2 when forming heteromers with an α_{1A} adrenergic receptors, shifts its signalling to be coupled to G_s-protein. Stimulation with either receptor agonist produced an increment in cAMP, this effect was blocked by cholera toxin.

6. Glaucomatous model DBA/2J showed a different behaviour depending on the disease development when treated with melatonin or IIK7 together with phenylephrine. Healthy mice showed that both agonists revealed a potential hypontensive effect, however, in glaucomatous mice, blocking α_{1A} by prazosin

prior to melatonin instillation resulted in more potent effect of melatonin in lowering IOP.

7. The discovery of melanopsin in the crystalline lens is an indication of various roles and mechanisms of melatonin regulation in the anterior segment of the eye.

8. Light can regulate melatonin levels in the crystalline lens epithelial cells, this effect is mediated by PLC in the same manner described in the retina.

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Annex I : Reagents and Buffers

Table 1. Phosphate-Buffered Saline (PBS) 10X

Compounds	Concentration
NaCl	1.37 mM
Na_2HPO_4	81 mM
KCl	26.8 mM
KH_2PO_4	14.7 mM

Table 2. Radioimmunoprecipitation assay buffer (RIPA)

Compounds	Concentration
Tris-HCl pH=8	50 mM
SDS	0,1%
NaCl	150 mM
NP-40	1%
Na deoxicolate	0,5%
Protease inhibitors cocktail	1%

Table 3. Electrophoresis Buffer 5X

Compounds	Concentration
Tris	125mM
Glycine	0.96 M
SDS	0,5%

Table 7. SDS-Page stacking gel-1

Compounds	Concentration
Tris-HCl pH=6.8	0.125 mM
SDS	4%
Glycerol	29%
ß-mercaptoethanol	10%
EDTA	15mM
Bromophenol blue	0.006g

Table 5. Reprobing solution

Compounds	Concentration
ß-mercaptoethanol	1%
SDS	10%
Tris pH=6.8	6,25%

Table 6. TBS 10x (concentrated Tris-buffered saline)

Compounds	Concentration
Tris	10mM
NaCl	100mM

Table 7. SDS-Page stacking gel

Compounds	Concentration
Tris-HCl pH=6.8	0.125 mM
SDS	0,1%
Ammonium persulfate	0.25 mg/ml
TEMED	0%
Acrylamide/Bis-acrylamide	4%

Table 8. SDS-Page separating gel

Compounds	Concentration
Tris-HCl pH=6.8	375 mM
SDS	0,1%
Ammonium persulfate	0.25 mg/ml
TEMED	0,05%
Acrylamide/Bis-acrylamide	Specifically determined for each protein%

Table 9. HPLC mobile phase for melatonin detection

Compounds	Concentration
Methanol	40%
H_2O	60%

Table 10. HPLC mobile phase for NAS detection

Compounds	Concentration
Acetonitrile	15%
acetic acid	0,1%

Table 11. 33% Sucrose solution in PBS 1X

Compounds	Concentration
Sucrose	330 g/l
PBS 10X	10% (v/v)

Table 11. 4% Paraformaldehyde Fixative (PFA) in PB 0.1M (pH: 7.4)

Compounds	Concentration
PFA	4% (m/v)
Na_2HPO_4 0.2M	36% (v/v)
NaH_2PO_4 0.2 M	14% (v/v)

Table 13. Hank's Buffered Salt Solution (HBSS)

Compounds	Concentration
NaCl	0.137 M
KCl	5.4 mM
Na_2HPO_4	0.25 mM
Glucose	0.1 g
$CaCl_2$	1.3 mM
$MgSO_4$	1.0 mM
$NaHCO_3$	4.2 mM
KH_2PO_4	0.44 mM

Annex II: Scientific Contributions

“Tell me and I forget, teach me and I may remember, involve me and I learn.”

Benjamin Franklin

Published articles related to the thesis

- **Alkozi HA** and Pintor J. Melatonin and derivatives as promising tools for glaucoma treatment. World J Opthalmol, (2013) 3(4): 32-37.

- Colligris B., **Alkozi HA** and Pintor J. Recent developments on dry eye disease treatment compounds. Saudi J Opthalmol. 2014 Jan; 28(1): 19–30.(2014) .

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- **Alkozi H A**, Sánchez-Naves J, de Lara MJ, Carracedo G, Fonseca B, Martinez-Aguila A, Pintor J. Elevated intraocular pressure increases melatonin levels in the aqueous humour. Acta Ophthalmol. 2016 Sep 6. doi: 10.1111/aos.13253.

- **Alkozi H A**, Xiaoyu Wang, Maria Jesus Perez de Lara, Jesus Pintor. Presence of melanopsin in human crystalline lens epithelial cells and its role in melatonin synthesis. . Exp Eye Res. 2016 Nov 30. doi: 10.1016/j.exer.2016.11.019.

- **Alkozi H A**, Maria Jesus Perez de Lara, Sánchez-Naves J, Jesus Pintor. TRPV4 Stimulation Induced Melatonin Secretion by Increasing Arylalkymine N-acetyltransferase (AANAT) Protein Level. Int J Mol Sci. 2017 Apr 1;18(4). pii: E746. doi: 10.3390/ijms18040746.

- **Alkozi H A**, Maria Jesus Perez de Lara, Jesus Pintor. Melatonin synthesis in the human ciliary body triggered by TRPV4 activation: Involvement of AANAT phosphorylation. Exp Eye Res. 2017 June. doi: 10.1016/j.exer.2017.06.018.

- **Alkozi HA,** Pintor J (2017) Improving Melatonin Delivery Within the Eye. J Bioequiv Availab 9: 516-517. doi: 10.4172/jbb.1000355.

- **Alkozi HA,** Pintor J. Epigenetics in the eye: an overview of the most relevant ocular diseases. Mini Review, Front. Genet.- Epigenomics and epigenetics. In press.

Symposiums and conferences

- Attended and participated in The Association for Research in Vision and Ophthalmology (ARVO) with a poster titled “Melatonin and its synthesising enzyme increases under ocular hypertension and glaucomatous conditions” in Orlando, USA, 2014

- Attended and participated in The 3rd ScienceOne Conference on Environmental Sciences “Melatonin and Derivatives as Promising Tools for Glaucoma Treatment” in Dubai, UAE January 21-23, 2014.

- Attended and participated in The International Congress of Optometry and Visual Sciences with a poster titled “Melatonin and its Analogues as potential intraocular hypotensors for Glaucoma Treatment” in University of Minho, Portugal May, 2014.

- Attended and participated in The International Congress of Optometry and Visual Sciences with a poster titled “TRPV4 activation triggers the release of melatonin from human non-pigmented ciliary body epithelial cells” in University of Minho, Portugal April, 2015.

- Attended and participated in The Vision Science and Eye Research Meeting with a poster titled “¿Son los nucleótidos procedentes del cristalino responsables del desarrollo del glaucoma?” in Santiago de Compostela, Spain October 2014.

- Attended and participated in The Association for Research in Vision and Ophthalmology 2015 with a Poster titled “Melatonin and its synthesising enzyme increases under ocular hypertension and glacomatous conditions” Denver,USA 2015.

- Attended and participated in the 24th International symposium of Optometry, contact lenses and ophthalmology 2016, presented a poster titled “La presión intraocular elevada aumenta los niveles de melatonina en el humor acuoso” Madrid, Spain 2016.

- Participated in The Association for Research in Vision and Ophthalmology (ARVO 2016) with a Poster titled “Elevated intraocular pressure increases melatonin levels in the aqueous humour” Seattle, USA 2016.

- Attended and participated in the 13th meeting of the Association for ocular pharmacology and therapeutics, presented a poster titled “Presence of

melanopsin in human crystalline lens epithelial cells and its role in melatonin synthesis" Florence, Italy. February 2017.

- Accepted Abstract in The Association for Research in Vision and Ophthalmology (ARVO2017) for a poster titled "TRPV4 stimulation induced aralkylamine N-acetyltransferase (AANAT) phosphorylation via Ca-calmodulin pathway in human ciliary body epithelial cells" to be held in Baltimore, USA on May 2017.

- participated in The International Congress of Optometry and Visual Sciences with a poster titled "Melatonin levels in the aqueous humour: connection with intraocular pressure" in University of Minho, Portugal April, 2017.

- participated in The International Congress of Optometry and Visual Sciences with a poster titled "Effects of light on melanopsin and its presence in human crystalline lens epithelial cells" in University of Minho, Portugal April, 2017.

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MINIREVIEWS

Melatonin and derivatives as promising tools for glaucoma treatment

Hanan Awad Alkozi, Jesús Pintor

Hanan Awad Alkozi, Jesús Pintor, Department of Biochemistry and Molecular Biology IV, Faculty of Optics and Optometry, Universidad Complutense de Madrid, E-28037 Madrid, Spain
Author contributions: Alkozi HA and Pintor J contributed equally to this work.
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Correspondence to: Jesús Pintor, Professor, Department of Biochemistry and Molecular Biology IV, Faculty of Optics and Optometry, Universidad Complutense de Madrid, c/Arcos de Jalón 118, E-28037 Madrid, Spain. jpintor@ucm.es
Telephone: +34-91-3946859 Fax: +34-91-3946885
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Core tip: This mini review depicts the main features of melatonin and derivatives as interesting agents for the treatment of the ocular hypertension associated with glaucoma.

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Abstract

Neurohormones melatonin and its analogues are present with an important physiological and pharmacological ability to reduce intraocular pressure (IOP); thus, they are suitable for the treatment of ocular hypertension often associated with glaucoma. It is demonstrated that two of its analogues, 5-MCA-NAT and IIK7, are more effective than melatonin to reduce IOP for a longer period of time. The research for the discovery of better compounds resulted in the development of newer and improved analogues compared to 5-MCA-NAT and IIK7. Furthermore, already commercially available drugs currently used as treatment for other pathologies, presenting a resemblance to the melatonin structure, are being tested as potential glaucoma drugs. In this sense, agomelatine, which is already used as an anti-depressant medicine, is recognized as a worthy candidate since it reduces IOP, even under hypertensive conditions. To sum up, the use of melatonin and its analogues as promising anti-glaucomatous substances is of great importance and should be given serious consideration.

INTRODUCTION

There is a general interest in searching for novel compounds capable of reducing intraocular pressure (IOP) as an improved alternative to the existing drugs. IOP can be lowered through the reduction of aqueous humor production or by increasing its outflow through the trabecular meshwork or uveoscleral pathways. The interest for searching for new compounds relies on the fact that most of the existing drugs produce important side effects, hampering the treatment of certain patients. Side effects are a common issue in glaucoma medications. β-blockers such as timolol can cause bradycardia and hypotension and they are unsuitable for patients suffering from cardio vascular problems[1], asthma, obstructive pulmonary disease or corneal dystrophy[2]. Cholinergic agonists such as pilocarpine produce fixed pupils and induce myopia and cataracts[1], whereas prostaglandins (*e.g.*, latanoprost) cause eyelash growth, iris pigmentation[3], muscle and joint pain[2]. Frequently, ocular redness and ocular surface discomfort obligates patients to abandon the treatment.

Several new compounds and approaches are under development in companies' pipelines or in academic institutions. Among the plethora of substances, the naturally occurring are more attractive as its administration is expected to result in fewer side effects[4]. Among these, the neurohormone melatonin emerges as a promising substance with interesting hypotensive properties[5]. The use of 5-MCA-NAT (a melatonin analogue, see below) when applied to the eye does not produce severe side effects. It does not affect corneal and lens transparency nor cause redness or corneal edema. No negative effects were noticed in general ocular examinations[5]. It is important to bear in mind that most of melatonin intake is not by prescription as it is considered a dietetic supplement. In this case, high dosages and an elevated number of intakes could produce some minor side effects. The Mayo Clinic indicates that the most common side effects are drowsiness, headache and dizziness. Moreover, large doses of melatonin can interfere with some medications, such as anticoagulants, immunosuppressants, diabetes medications and birth control pills.

There are two interesting works describing the melatonin effect and its analogues on reducing IOP. Serle *et al*[6] demonstrated that a melatonin analogue was able to reduce IOP in glaucomatous monkeys, suggesting these molecules as a possible treatment of ocular hypertension related to glaucoma. Additionally, a group of ophthalmologists started to use melatonin during cataract surgery because it reduces IOP substantially, which is recommendable during phacoemulsification[7].

From these two relevant works, the question arises as why these groups decided to use melatonin and its analogues for clinical purposes and mainly for reducing IOP. The present mini review introduces the reader to the basis of why melatonin is an attractive molecule to reduce IOP and why it should be considered in the future as a respectable alternative to the current ocular hypertension and glaucoma therapies.

MELATONIN, MORE THAN A PINEAL GLAND HORMONE

Melatonin is a molecule known by its chemical name N-acetyl-5-methoxytryptamine (Figure 1). It has been traditionally related to a particular area of brain, termed the pineal gland, where it is synthesized in low illumination conditions like during the night[8] and it regulates many day-night processes, called circadian rhythms[9]. It is necessary to emphasize that this substance is also synthesized in other tissues and ocular structures such as the retina, the ciliary body or the lens. This clearly suggests that melatonin can exert some local actions on the tissues where it is synthesized or in surrounding areas. Keeping in mind that melatonin is released by the lens or the ciliary body, its presence in the aqueous humor, modifying the physiology of these structures being bathed in the fluid, can be speculated about. Interestingly, one of the possible physiological processes to be modified is IOP. It is documented that in many animal models there are changes in IOP during the day (high IOP) and night (low IOP). It is possible that both processes are associated considering the circadian pattern of melatonin production. Consequently, we should study what happens if we topically apply melatonin during the day when IOP is high.

MELATONIN REGULATES INTRAOCULAR PRESSURE

When melatonin is topically applied at a single dose of 100 μmol/L in a volume of 10 μL, there is a transient reduction in IOP and values return quickly to initial figures in about 2 h[10]. This effect is similar to that of endogenous melatonin at night which reduces IOP. Despite the acquired hypotensive effect, the rapid return to normal pressure values suggests that either it is necessary to regulate the doses or to look for an alternative compound to produce a more sustained effect[11].

There are several commercially available melatonin analogues depicting similar behavior to melatonin. Two compounds present sharper and long lasting effects on reducing the IOP compared to melatonin. In particular, the compound N-butanoyl-2-2-methoxy-6H-isoindolo ([2,1-a]indol-11-yl) ethanamine (abbreviated as IIK7) has a hypotensive effect that lasts up to 7 hours and the compound 5-methylcarboxyamino-N-acetyltryptamine (also known as 5-MCA-NAT), which can reduce IOP for up to 9 h (Figure 1)[12]. Consequently, 5-MCA-NAT is more interesting since it presents a longer term effect with a significant reduction of IOP for up to 96 h. This remarkable effect has been taken into consideration as we indicate below[11] (Table 1).

5-MCA-NAT was tested in normotensive models as well as under hypertensive conditions, including glaucomatous monkeys (Table 1). Interestingly, the effects on the monkeys, a model closer to the human glaucomatous pathology, were extremely interesting. Compared to vehicle treatment, twice daily administration of 5-MCA-NAT for 5 d reduced IOP from 1 to 5 h after the first dose and the IOP-lowering effects were shown to last at least 18 h following administration, based on IOP measurements made after the fourth and eighth doses[6].

One interesting characteristic to take into account was that the ocular hypotensive effect of 5-MCA-NAT was enhanced by repeated dosing. The maximum reduction of IOP was acquired 3 h after each morning dose and was 10% on day 1, 15% on day 3, and 19% on day 5 (control = 100%). No adverse ocular or systemic side effects were observed during the 5 treatment days, suggesting that this compound could be used perfectly as ocular hypertension treatment[6] (Table 1).

IIK7 reduced intraocular pressure by acting through MT_2 melatonin receptors, presumably decreasing aqueous humor formation. Its effect is concentration dependent and it can reduce IOP 38.5% ± 3.2% when compared to controls (Table 1). It is important to notice that these

Table 1 Hypotensive effects of melatonin analogues: animal models, conditions and receptors involved

Compound species	IOP reduction	Receptor involved	Ref.
Melatonin			
Human	32.0%± 3.2%	Unknown	[7]
Rabbit	22.0% ± 1.6%	MT_2, MT_3	[10,11]
Mouse (glaucomatous)	33.4% ± 2.5%	MT_2	UD
5-MCA-NAT			
Monkey (hypertensive)	19.2% ± 2.1%	MT_3	[6]
Rabbit	42.5% ± 1.6%	MT_3	[10,11]
IIK7			
Rabbit	38.5% ± 3.2%	MT_2	[12]
INS48848			
Rabbit	36.0% ± 2.0%	MT_3	[22]
INS48852			
Rabbit	33.1% ± 1.4%	MT_2	[22]
INS48862			
Rabbit	26.0 V ± 1.3 V	MT_2	[22]
Agomelatine			
Rabbit			
Normotensive	20.8% ± 1.4%	MT_2	[25]
Hypertensive	68.8% ± 5.7%	MT_2	[25]

The values represent the mean ± SEM for the indicated compounds in the respective animal model. IOP: Intraocular pressure; UD: Unpublished data.

experiments have not been performed in glaucomatous monkeys yet but only in rabbits[12].

In summary, it seems that some compounds, such as melatonin, 5-MCA-NAT and IIK7, clearly reduce IOP. But what is the mechanism for this IOP reduction? What receptors activate these substances in order to produce the observed effects?

MELATONIN AND ITS ANALOGUES ACTIVATE MELATONIN RECEPTORS

Melatonin exerts its effect *via* membrane and nuclear receptors. The protein membrane receptors are better understood and until recently three proteins have been cloned. Two of these membrane receptors, termed MT_1 and MT_2, are melatonin receptors belonging to the 7-transmembrane G protein-coupled receptor family (GPCR). There have been claims that a third receptor exists, the MT_3 melatonin receptor, although it has not been cloned yet. Some authors have identified it as quinone reductase 2 (QR2), demonstrating features of a melatonin receptor in some animal models (for a review see[9]).

MT_1, MT_2 and the probable MT_3 melatonin receptors are present in several ocular structures, according to pharmacological, biochemical and immunological studies[13,14]. This evidence suggests that melatonin plays a role in physiological processes in ocular tissues, such as the modulation of IOP, and it has been documented that MT_2 and MT_3 are responsible for IOP reduction.

When melatonin, 5-MCA-NAT and IIK7 are applied to normotensive or hypertensive eyes, they produce a dissimilar IOP reduction, depending on the compound

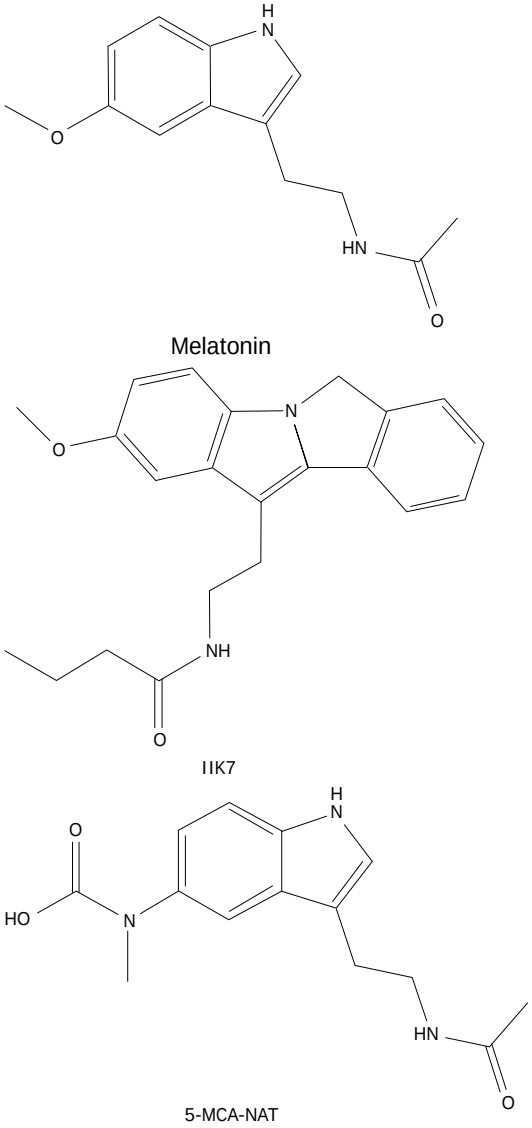

Figure 1 Chemical structure of melatonin and analogues. Melatonin (N-acetyl-5-methoxytryptamine), IIK7 (N-butanoyl-2-(2-methoxy-6H-isoindolo[2,1-a]indol-11-yl)ethanamine) and 5-MCA-NAT (5-methylcarboxyamino-N-acetyltryptamine).

under study. The use of selective antagonists for melatonin receptors has allowed identification of the presence of MT_2 melatonin receptors in the ciliary body of experimental animals, such as New Zealand white rabbits. This has been confirmed through immunohistochemical studies. In these studies it has been possible to verify the presence of MT_2 melatonin receptors on pigmented and non-pigmented ciliary epithelia. Accordingly, the application of melatonin or IIK7, which is a selective MT_2 agonist, results in a reduction in the production of the aqueous humor[12] (Figure 2).

5-MCA-NAT has been suggested as an MT_3 melatonin receptor agonist that reduces IOP. To date, the location of the receptor is unknown. As there is a controversy with the possible identification of the MT_3 receptor which is tentatively identified in some animal models as QR_2, some sophisticated experiments were performed to

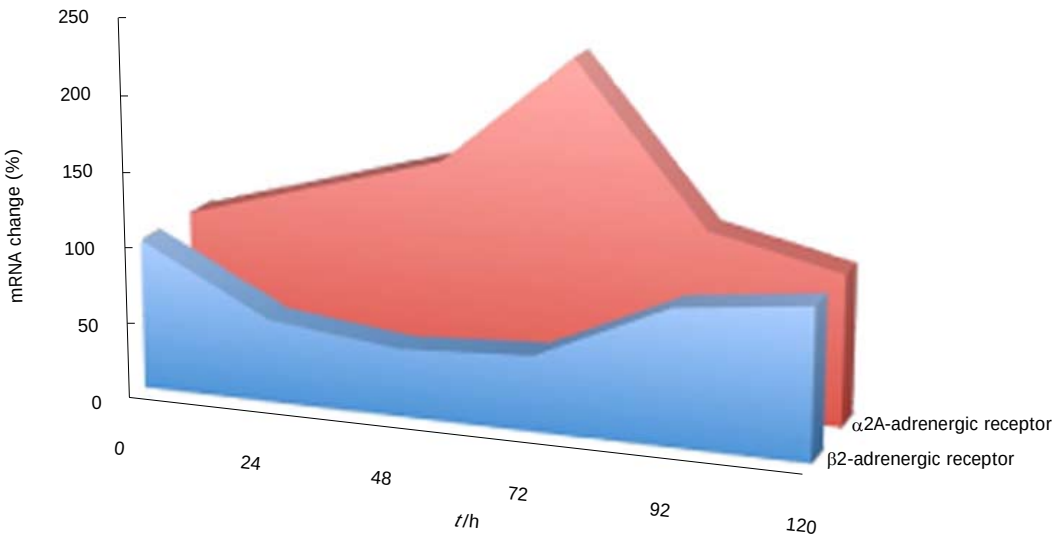

Figure 2 Expression of mRNA levels in ciliary body cells. The amounts of mRNA and concomitant adrenoceptors were changed after the application of 5-MCA-NAT. While there was an increase of α2A-adrenoreceptors (in red), there was a decrease in the levels of β2-adrenoreceptors (in blue).

clarify the issue[15-17]. In New Zealand rabbits, the use of a siRNA silencing QR_2 (therefore avoiding the expression of this enzyme) did not abolish the hypotensive effect of 5-MCA-NAT, clearly indicating that, in this animal model, $MT_3 \neq QR_2$, opening the possibility of speculating about the existence of a receptor that needs to be cloned to fully understand its functioning and location[18].

Apart from melatonin and its derivatives, some other compounds, like 5-MCA-NAT, can keep IOP below normal values for up to 5 d. This long-term effect is mediated by the action of melatonin receptors on the expression of genes expressing proteins important for the homeostasis of the aqueous humor.

To date, it has been possible to demonstrate that the 5-MCA-NAT long-term effect is in part the result of the expression inhibition of carbonic anhydrases. This down-regulation means that 24 h after 5-MCA-NAT application there is a reduction in IOP because the amounts of carbonic anhydrases are severely reduced. In particular, when 5-MCA-NAT is applied, carbonic anhydrase 2 is reduced 32% (protein levels), while carbonic anhydrase 12 is reduced 39% (protein levels). This reduction in protein expression mimics the carbonic anhydrase inhibitor action, such as dorzolamide or acetazolamide[19].

Likewise, the expression of adrenergic receptors is modified by the application of 5-MCA-NAT. Interestingly, this melatonin analogue is able to produce a sequential process consisting of an initial reduction in the β2-adrenoreceptors expression, followed by an increase in α2A-adrenoreceptors[20]. Altogether, these consecutive effects produce a sustained reduction in IOP lasting for at least 96 h[21].

In summary, 5-MCA-NAT, apart from a sharp hypotensive effect, exerts a long term effect, maintaining low IOP for 4 d.

SO, WHAT IS NEXT NOW?

Several aspects need to be studied, taking into account that melatonin and analogues can significantly reduce IOP.

It is clear that it is necessary to research and design new melatonin analogues with more profound and long lasting effects[5]. Inspire Pharmaceuticals Inc. (now absorbed by Merck) has designed several melatonin analogues with interesting hypotensive properties to reduce IOP. In recent studies, melatonin analogues, termed INS48848, INS48852 and INS48862, demonstrated similar behavior to melatonin, 5-MCA-NAT and IIK7[22]. Indeed, these three compounds decreased IOP in a dose-dependent manner similar to melatonin, 5-MCA-NAT and IIK7, confirming their efficiency in decreasing IOP (Table 1). Concerning their selectivity on melatonin receptors, the effects of INS48848 were completely blocked by prazosin, an antagonist of MT_3 melatonin receptors, and were potently inhibited by luzindole, a non-selective antagonist of melatonin receptors. However, DH97, a selective MT_2 receptor antagonist, had a limited effect against INS48848 and the results obtained from INS48862 and INS48852 were contradictory. Luzindole and prazosin had no significant effects against those two compounds, whereas DH97 blocked them completely. These results strongly suggest that INS48848 could be acting through the MT_3 melatonin receptors and that INS48862 and INS48852 could be acting preferentially through MT_2 melatonin receptors. In any case, all these compounds are worthy candidates to reduce IOP, especially when it is abnormally elevated[22].

Another alternative to the development of newly synthesized compounds is to search for melatoninergic compounds already used for other medical purposes. Compounds such as ramelteon ((S)-N-[2-(1,6,7,8-tetrahyd-

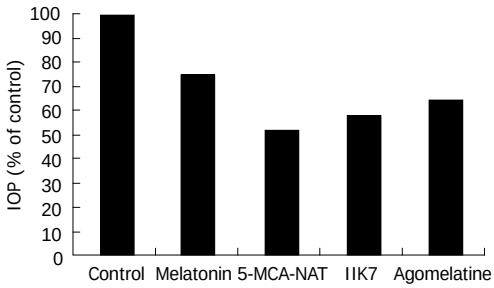

Figure 3 Comparative effects of melatonin and analogues in an animal model. Equal doses of melatonin or the corresponding analogues (100 μmol/L, 10 μL), reduced intraocular pressure in New Zealand white rabbits. Differences among the compounds rely on the activation of different receptors in each case (see text).

ro-2H-indeno-[5,4-b]furan-8-yl)ethyl]propionamide), also known as *Rozerem*, used for sleep disorders[23] or agomelatine (N-[2-(7-methoxynaphthalen-1-yl)ethyl]acetamide), known also by the names *Valdoxan, Melitor, Thymanax*[24], used for the treatment of depression, could be candidates to reduce IOP since their structure is similar to melatonin.

There is a lack of information regarding the use of ramelteon in IOP studies. Agomelatine significantly reduces IOP when topically applied on rabbit eyes. Agomelatine (10 μL, 100 μmol/L) reduced IOP by 20.8% ± 1.4% and its maximal IOP reduction was 180 minutes after the compound application. Interestingly, this compound exhibited an ability to reduce IOP in hypertensive conditions. It is noteworthy to stress that under high IOP the ability of this melatonin analogue to reduce IOP was 68.8% ± 5.7% (Figure 3, Table 1)[25].

There is a clear advantage in using compounds already commercialised for other conditions as the timeline for testing and clinical trials is significantly reduced.

CONCLUSION

It is necessary to perform an exhaustive study on the role of melatonin and its analogues in the different ocular structures since it is very probable that this knowledge will contribute to the discovery of more effective treatments for pathologies like glaucoma, corneal wound healing, cataracts or retinal pathology[26].

Taking into account the importance of the role of melatonin and its analogues in hypertension, often associated with glaucoma, it is quite evident that these compounds should be used as treatment to reduce IOP. Melatonin or agomelatine can simply and rapidly reduce IOP, although further research is required to prove that they can be safely used as treatment for ocular hypertension.

Most of the presented data resulted from experiments assaying melatonin or its analogues on animal models. We still have a long way to go to test these compounds on human beings. Nevertheless, there are a lot of positive points regarding the efficacy of certain melatoninergic compounds. For instance, melatonin itself is able to reduce IOP in normotensive humans, as previously described[7]. These authors reported an approximate 30% reduction in IOP during cataract surgery compared to the initial patient's pressures. This is quite interesting because the IOP reduction has been obtained in normotensive patients and it could be even more substantial in hypertensive (glaucomatous) patients. Several experiments in animal models demonstrated that melatonin and analogues are able to reduce IOP equally in normotensive and hypertensive animals, being more effective in hypertensive than in normotensive animals (Table 1). Also, experiments performed with 5-MCA-NAT on hypertensive monkeys, a step before human clinical trials, have proved that this melatonin analogue reduced IOP.

In conclusion, agomelatine is the compound that we strongly believe should be tested in glaucomatous patients for its ability to reduce IOP. Agomelatine is already used as a depression treatment drug under the commercial name Valdoxan[24]. Since many of the pre-clinical tests have already been completed, we should not be surprised if agomelatine clinical trials start and it becomes the first melatoninergic compound to join the group of glaucoma treatment substances.

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TRPV4 activation triggers the release of melatonin from human non-pigmented ciliary epithelial cells

Hanan Awad Alkozi, Jesús Pintor*

Department of Biochemistry, Faculty of Optometry, Universidad Complutense de Madrid, C/ Arcos de Jalón 118, E281037 Madrid, Spain

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ABSTRACT

Melatonin is a neurohormone mainly produced in the pineal gland; nevertheless, various ocular structures such as the ciliary body, lens and the retina produce it. One of the roles of melatonin in the eye is the modulation of intraocular pressure, although little is known about the mechanisms that causes its presence in the aqueous humour. TRPV4 is a membrane channel which is activated by both physical and chemical stimuli. Therefore, this channel is sensitive to osmotic and hydrostatic pressure. As a consequence, TRPV4 results as an interesting candidate to study the relation between the activation of the TRPV4 channel and the production of melatonin. In this sense we have studied the role of the TRPV4 agonist GSK1016790A to modulate the production of melatonin in a cell line derived from human non-pigmented ciliary epithelial cells. The stimulation of the TRPV4 produced an increase in the extracellular melatonin levels changing from 8.5 ± 0.6 nM/well/30 min (control) to 23.3 ± 2.1 nM/well/30 min after 10 nM GSK1016790A application, this action being blocked by the selective antagonist RN 1734. The activation of the TRPV4 by GSK1016790A permitted to observe a melatonin increase which was concentration-dependent, and provided a pD_2 value of −8.5 ± 0.1 (EC_{50} of 3.0 nM). In conclusion, the activation of the TRPV4 present in human non-pigmented ciliary epithelial cells can modulate the presence of extracellular melatonin, this being of relevance since this substance controls the dynamics of the aqueous humour.

Melatonin is a neurohormone produced by the pineal gland, but also by many ocular structures. These include the ciliary body, lens, the harderian glands and also the retina (Alarma-Estrany and Pintor, 2007). As a consequence of its synthesis, it has been possible to measure melatonin in the aqueous humour of various species including human beings (Rohde et al., 1985). The role of melatonin in the anterior pole has been related to the control of the aqueous humour formation (Osborne, 1994) and therefore with the regulation of intraocular pressure (IOP) (Pintor et al., 2001; Samples et al., 1988).

Although it is known that melatonin is present in the aqueous humour, little is known about what substances and mechanisms may stimulate its presence in the aqueous humour. An interesting candidate is the channel TRPV4. It is a membrane protein that belongs to the transient receptors, which is a non-selective cation channel permeable to Ca^{2+}. In this sense, the Ca^{2+} influx triggered after TRPV4 stimulation is probably able to induce the release of melatonin as it occurs in other cells such as pinealocytes or retinal cells, although recently it has been demonstrated that melatonin could be transported by means of glucose transporters (Hevia et al., 2015).

TRPV4 is a channel that participates in the transduction of both physical (osmotic, mechanical, and heat) and chemical (endogenous, plant-derived, and synthetic ligands) stimuli (Garcia-Elias et al., 2014). Since this channel is sensitive to mechanical as well as osmotic pressure, it results of interest to see whether there is a relation between the activation of the TRPV4 channel and the production of melatonin. In this sense we have studied the role of the TRPV4 selective agonist GSK1016790A (Vincent et al., 2009) to modulate the production of melatonin in non-pigmented human ciliary epithelial cells.

Non-pigmented ciliary epithelial cells (59HCE), a human immortalized cell line was kindly supplied by Dr. Coca-Prados. Cells were grown in high glucose Dulbecco's modified Eagle's medium (Gibco/Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (Sigma–Aldrich, St. Louis, MO) and 0.05 mg/ml Gentamicin (Gibco/Invitrogen) at 37 °C in humidified atmosphere 5% CO_2–95% air.

* Corresponding author.
E-mail address: jpintor@ucm.es (J. Pintor).

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Acta Ophthalmologica

Elevated intraocular pressure increases melatonin levels in the aqueous humour

Hanan Alkozi,[1] Juan Sánchez-Naves,[2] Maria Jesús Perez de Lara,[1] Gonzalo Carracedo,[3] Begoña Fonseca,[1] Alejandro Martinez-Aguila[1] and Jesús Pintor[1]

[1]Department of Biochemistry and Molecular Biology IV, Faculty of Optics and Optometry, University Complutense of Madrid, Madrid, Spain
[2]Department of Ophthalmology, Balear Institut of Ophthalmology, Palma de Mallorca, Spain
[3]Department of Optics II (Optometry and Vision), Faculty of Optics and Optometry, University Complutense of Madrid, Madrid, Spain

ABSTRACT.

Purpose: **To study the levels of melatonin in the aqueous humour of normotensive and hypertensive intraocular pressure (IOP) patients and to compare them to an animal model of glaucoma.**

Methods: **A total of 37 eyes of 37 patients who underwent cataract surgery were included in the study and were divided into normotensive patients, with IOP below 21 mmHg (n = 23), and hypertensive patients, with IOP > 21 mmHg (n = 14). Glaucomatous DBA/2J (n = 6) and control C57BL/6J (n = 6) mice presenting 3 and 12 months of age for each strain were also used. Human and mice aqueous humours were aspirated using a 30-gauge Rycroft cannula on a tuberculin syringe and further processed to quantify melatonin by high-performance liquid chromatography analysis.**

Results: **Melatonin levels in normotensive patients (IOP below 21 mmHg) presented values as medians (first quartile; third quartile) of 14.62 (5.38;37.99) ng/ml (n = 23), while hypertensive patients (IOP above 21 mmHg) showed melatonin concentrations of 46.63 (10.28; 167.28) ng/ml (n = 14; $p < 0.039$). Glaucoma mice presented melatonin values of 0.37 (0.34; 0.59) ng/ml (at 3 months of age, before the pathology starts), which increased to 1.55 (0.94; 1.88) ng/ml (at 12 months of age, when the pathology is fully developed and IOP is maximum; n = 6, $p < 0.001$). Control mice did not significantly modified melatonin concentrations between 3 and 12 months of age.**

Conclusion: **Patients with high IOP present increased concentrations of melatonin in their aqueous humour compared to normotensive patients. This has been confirmed in a glaucomatous animal model in which it has been possible to see a correlation between the development of the pathology, with an increase in IOP, and a concomitant elevation of melatonin in the aqueous humour.**

Key words: aqueous humour – glaucoma – high-performance liquid chromatography – humans – melatonin – mice

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Introduction

Melatonin is an indoleamine that has been classically considered as being only produced by the pineal gland; nevertheless, this neurohormone is also synthesized and released by many ocular structures including the retina, ciliary body, the lens and the harderian glands (Alarma-Estrany & Pintor 2007). Although its implications in ocular physiology is still under investigation, it seems to be clear that this substance is involved in relevant processes such as the control of the aqueous humour dynamics (Osborne 1994). In this sense, melatonin levels are normally elevated during the night (Wurtman et al. 1963), which is the moment in which intraocular pressure (IOP) is lower. This connection between melatonin levels and IOP has been demonstrated on patients that underwent cataract surgery who were treated with melatonin to reduce their IOP (Ismail & Mowafi 2009). Therefore, this control of IOP carried out by melatonin has suggested the use of this compound and its analogues for the treatment of ocular hypertension and glaucoma (Mediero et al. 2009). In this sense, studies performed in animal models and more recently in humans suggest that melatonin or any of its analogues can significantly reduce IOP in normotensive, in hypertensive animals (Martinez-Aguila et al. 2013), and what is more important, in ocular hypertensive human beings (Pescosolido et al. 2015). All the actions performed by melatonin and analogues to reduce IOP are mediated by membrane receptors termed MT_1, MT_2 and MT_3 melatonin receptors present mainly in the ciliary body (Alarma-Estrany & Pintor 2007). After activation of such receptors, mostly MT_2 and MT_3

melatonin receptors, IOP decreases providing normal values of pressure within the eye and therefore avoiding possible ocular complications (Pintor et al. 2001; Alarma-Estrany et al. 2008). The mechanism by which melatonin produces a reduction of IOP in the ciliary body is mostly due to a modification in the intracellular levels of cAMP (Huete-Toral et al. 2015). Melatonin and its analogue 5-MCA-NAT activate MT_2 and MT_3 melatonin receptors that stimulate adenylate cyclase with the concomitant activation of PKA. This activation produces an inhibition in the efflux of chloride (Huete-Toral et al. 2015), the main ion governing the movement of water from the ciliary epithelium to the posterior chamber (Civan 1998). Therefore, the reduction in the chloride efflux causes a reduction in aqueous humour formation (Civan & Macknight 2004).

Melatonin is physiologically present in the aqueous humour, together with other relevant molecules such as dinucleotides (Castany et al. 2011) or more recently vascular endothelial growth factor (VEGF) (Wang et al. 2016); the last two elevated in the aqueous humour of glaucoma patients. Related to melatonin, one interesting question is to understand where and how this substance is produced, and what factors can modulate its presence in the aqueous humour. Recently, a work published by Alkozi and Pintor has demonstrated that human non-pigmented ciliary epithelial cells produce and release more melatonin when the TRPV4 channel, present in the membranes of these cells, is activated (Alkozi & Pintor 2015). This channel is sensitive, among several stimuli, to pressure (Garcia-Elias et al. 2014), and this indicates that an elevation in IOP can modify the melatonin that is released to the aqueous humour (Alkozi & Pintor 2015). This increase can activate melatonin receptors that will reduce IOP (Pintor et al. 2001), as commented above.

Although a connection between intraocular pressure and melatonin seems to be possible, there are not so many works measuring the levels of this substance in normo- and/or hypertensive patients. Moreover, some groups have obtained variable and non-conclusive results indicating that melatonin concentrations can vary from the pg/ml to the ng/ml range (Martin et al. 1992; Chiquet et al. 2006). Under this high variability in the quantification of melatonin in human aqueous humour, in the present experimental work, we have studied the relationship between melatonin concentration in aqueous humour and IOP in non-glaucomatous patients, dividing them in those which present IOP higher than 21 mmHg, and those whose pressure is below this value. Also, trying to clarify what happens in humans, we have performed the same study in a glaucoma animal model, and altogether, we demonstrate that there is an increase in melatonin levels when intraocular pressure is abnormally elevated.

Patients and Methods

Subject recruitment and aqueous humour collection

From a sample of 265 patients who underwent cataract surgery, all hypertensive patients ($n = 14$), with IOP >21 mmHg (Tavares et al. 2006) and 23 normotensive patients, with IOP below 21 mmHg and randomly selected, were included in the study. The study protocol was approved by the clinical research Ethics Committee of Instituto Balear de Oftalmología, Palma de Mallorca, Spain. Signed informed consent to the aqueous humour contribution was obtained from all patients in accordance with the Declaration of Helsinki.

Patients with history of primary acute angle-closure attack, chronic angle-closure glaucoma, any treatment for glaucoma or active inflammatory ocular disease were excluded. Glaucoma was defined as high intraocular pressure associated with optic nerve glaucomatous characteristics consistent with the visual field defect. The IOP measurements were taken before the surgery by means of a Perkins tonometer after instillation of one drop of double anaesthetic Colircusí® that contains tetracaine 0.1% and oxybuprocaine 0.4% (Colircusí, Alcon Cusí SA, Barcelona, Spain). This value should be representative of at least three other previous visits with non-statistically different IOP values from those presented in the manuscript. Patients with variations among visits were not considered in this study.

Each aqueous humour sample was taken in the first step of the cataract surgery with anaesthetic drops (lidocaine 2%). Through standard clear corneal microincision (mics 2.2 mm) under surgical microscope vision, aqueous humour was aspirated using a 30-gauge Rycroft cannula on a tuberculin syringe (1 ml). Aqueous humour (0.1–0.2 ml) was collected, immediately transferred to an Eppendorf and stored at −20°C and kept protected from light until the chromatographic analysis was performed (Castany et al. 2011).

Glaucomatous and non-glaucomatous mice

Aqueous humours were collected from female C57BL/6J (control, $n = 6$) and DBA/2J (glaucomatous, $n = 6$) mice obtained from the European distributor of Jackson Laboratories Mice (Charles Rivers Laboratories, Barcelona, Spain). All animal maintenance and experimental procedures followed Spanish and European guidelines for animal care in the laboratory and animal research (Guide for the Care and Use of Laboratory Animals) and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Mice were housed (1–4 mice per cage) in temperature and light-controlled rooms maintained according to a 12-hr light/dark cycle; all animals were fed *ad libitum*. DBA/2J and C57BL/6J mice were studied at 3 and 12 months of age.

Aqueous humour collection was performed after anesthetizing the mice with an intraperitoneal (i.p.) injection of a mixture of ketamine (95 mg/kg, Imalgene 1000, Merial, Barcelona, Spain) and xylazine (5 mg/kg, Rompún, Bayer, S.A., Barcelona, Spain). Aqueous humour was processed in the same way as with the human samples. Just before aqueous humour collection, IOP was measured by means of a TonoVet® contact tonometer supplied by Tiolat Oy (Vantaa, Finland). The application of this tonometer does not require the use of any anaesthetic.

High-performance liquid chromatography (HPLC) analysis

Samples from the aqueous humour were processed and analysed following

the protocol described elsewhere (Alkozi & Pintor 2015). Briefly, the tubes containing the aqueous humour were heated in a 98°C bath for 2 min and transferred to ice 10 min, to precipitate the proteins. Centrifugation of the tubes at 22 000 g for 10 min at 4°C was then performed to pellet the proteins. Melatonin concentrations were measured by HPLC. The chromatographic system consisted of a Kromaphase C18 column 5.0 μm (25 cm in length, 0.4 cm inner diameter; Scharlau, Madrid, Spain), a 1515 Isocratic HPLC pump, a 2487 dual absorbance detector and a Rheodyne injector, all managed by the software Breeze from Waters (Milford, MA, USA). The system was equilibrated overnight with 40% methanol and 60% H_2O. Measurements were performed at a flow rate of 0.8 ml/min fixing the detector at a wavelength of 244 nm. Quantification of melatonin was performed by comparing the samples with external standards provided by Sigma (St Louis, MO. USA).

Statistical analysis

The presented data were analysed using the statistical software SPSS 22.0 (SPSS, Inc., Chicago, IL, USA). Normal distribution of melatonin concentration was assessed by the Shapiro–Wilk normality test, resulting in no normality due to the heterogeneity of the patients. Then, its values are presented as the median and quartiles (first quartile; third quartile) of the experiments performed. The rest of variables are showed as means ± SD. Therefore, differences between before surgery and after surgery were estimated by non-parametric test of Wilcoxon for paired samples. In the animal experiment, the differences were also evaluated by Wilcoxon for paired samples, comparing between ages of 3 and 12 months. For correlations, Pearson bivariate regression was used. $p < 0.05$ was considered statistically significant.

Results

Melatonin levels in normotensive and hypertensive patients' aqueous humour

All the patients, who underwent cataract surgery, not being treated or diagnosed of glaucoma were distributed in two groups. The normotensive group consisted of all those patients with IOP values below 21 mmHg, and the hypertensive was formed by those with IOP values above 21 mmHg. Demographic details of recruited patients are shown in Table 1.

After collecting and processing aqueous humour samples, the chromatographic analysis clearly showed that those patients exhibiting IOP higher than 21 mmHg presented concomitantly higher concentrations of melatonin (Fig. 1, left panel). While normotensive patients (IOP below 21 mmHg) presented values of 14.62 (5.38; 37.99) ng/ml ($n = 23$), hypertensive patients showed melatonin concentrations of 46.63 (10.28; 167.28) ng/ml ($n = 14$), these values being statistically different ($p < 0.002$). Moreover, a significant correlation was found between the melatonin concentration and IOP presurgery ($p = 0.03$), the Pearson's correlation coefficient being 0.441 (Fig. 2).

Melatonin levels in control and glaucomatous mice's aqueous humour

The levels of melatonin were also studied in glaucomatous (DBA2/J) and non-glaucomatous (C57) mice strains. The IOP values obtained in the DBA2/J were 15.05 ± 0.87 mmHg for 3 months and 30.41 ± 1.35 mmHg for 12 months of age, respectively. For C57 model, the IOP at 3 months was 11.86 ± 0.69 mmHg and 11.13 ± 1.21 mmHg for 12 months of age. As it can be seen in Fig. 3, the development of the glaucomatous pathology in the DBA/2J mice modified melatonin concentration, its values changing from 0.37 (0.34; 0.59) ng/ml (at 3 months of age, before the pathology starts) to 1.55 (0.94; 1.88) ng/ml (at 12 months of age, when the pathology is fully established and IOP is maximum; $n = 6$, $p < 0.001$).

On the contrary, the levels of melatonin in the control mice (C57), analysed at the same ages, did not bring statistically significant differences, being 0.47 (0.42; 0.53) ng/ml and 0.42 (0.32; 0.56) ng/ml at 3 and 12 months, respectively ($n = 6$, $p = 0.418$). Melatonin concentration and IOP showed a strong correlation in mice, the Pearson's correlation coefficient being 0.851 ($p < 0.001$) for all the animals under study (Fig. 4).

Discussion

The present experimental work describes the changes observed in melatonin concentrations in patients presenting normal and high IOP. According to the present results, those patients presenting high IOP had also high melatonin concentrations compared to the melatonin values measured in normotensive patients, this difference being almost threefold. A similar behaviour has been observed in a glaucomatous animal model. In the mouse model, when the pathology has been fully developed and the pressure is the highest (12 months of age), melatonin concentrations were also threefold. Altogether these results indicate that there is a close relationship between the levels of melatonin in the aqueous humour and the IOP values, both in human and in experimental models.

The use of an animal model such as the DBA/2J helps to understand the changes in melatonin levels in the aqueous humour before and when the pathology is established. This is very valuable due to the impossibility to collect the aqueous humour of patients before they develop ocular hypertension.

In the present work, the values of melatonin found in the aqueous humour of hypertensive patients, near 50 ng/ml compared to 15 ng/ml in normotensive, represent an increase of threefold. This rise is higher than the increase that can be observed in humans for melatonin levels in other fluids such as tears. Human tears contain melatonin that changes in a circadian manner, but when comparing melatonin

Table 1. Demographic characteristics of participants in the study.

	Non-glaucomatous patients		
Parameter	Total	<21 mmHg	>21 mmHg
Patients	37	23	14
Mean age (years) ± SD	63.41 ± 13.12	62.13 ± 13.97	66.36 ± 10.81
Age range (years)	[24, 84]	[35, 82]	[35,84]
Gender (male/female)	[21, 16]	[15, 8]	[6, 8]
IOP presurgery (mmHg)	17.70 ± 3.80	15.48 ± 0.48	22.25 ± 0.32

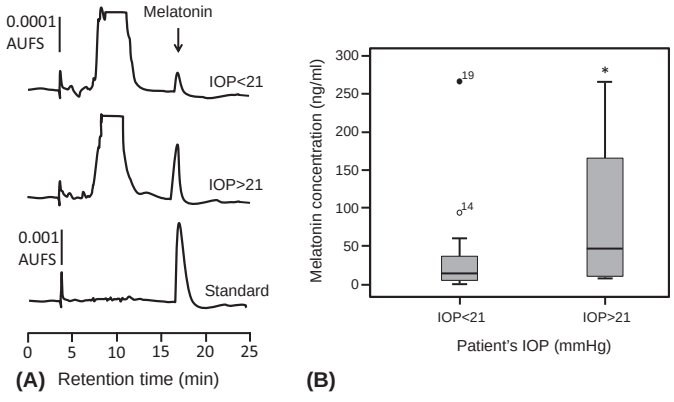

Fig. 1. Presence of melatonin in the aqueous humour of glaucoma patients. (A) representative high-performance liquid chromatography elution profiles of human aqueous humour samples of patients with IOP above 21 mmHg and below 21 mmHg, following the protocol described in methods. (B) bar plot showing the concentrations of melatonin in the two populations of patients. *p < 0.039 (Wilcoxon for paired samples).

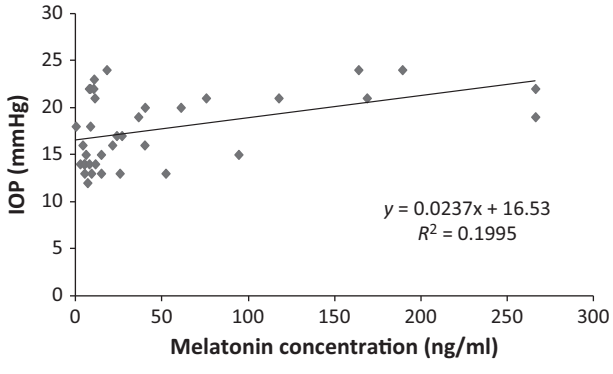

Fig. 2. Relationship between melatonin concentration (ng/ml) in aqueous humour of patients who underwent cataract surgery and the IOP before the surgery. Pearson correlation was statistically significant (p = 0.025; r = 0.441).

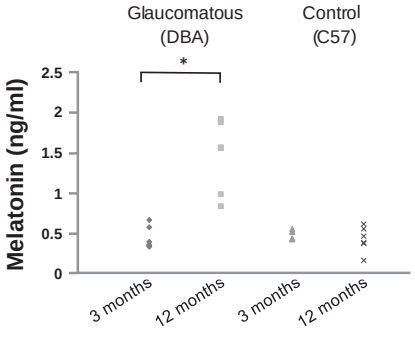

Fig. 3. Melatonin concentrations in the aqueous humour of control (normotensive) and glaucomatous mice. The DBA glaucomatous mice presented a marked increase in their aqueous humour melatonin concentrations when comparing 3 months of age, before the pathology starts and at 12 month of age, when the glaucomatous pathology has been fully established and the pressure in maximal. On the contrary, control mice, C57, did not change their melatonin concentrations with ageing. *p < 0.001 (Wilcoxon for paired samples).

concentrations during the day, with the amount during the night, the values in darkness are 1.6-fold higher than under light conditions (Carracedo et al. 2016). This is clearly suggesting that the melatonin concentration in the hypertensive patients is not only governed by circadian aspects but it may be suggested as also related to the fact of the pressure being abnormally high.

The connection between melatonin and pressure may rely on the existence of a pressure sensor, the TRPV4 channel, that after activation triggers the release of melatonin in the aqueous humour according to the results already observed in human non-pigmented ciliary epithelial cells (Alkozi & Pintor 2015). The activation of the TRPV4 channel produces changes in the extracellular levels of melatonin, but this effect requires a continuous stimulation of this channel. This condition is typical in glaucoma patients before any treatment starts or in those whose treatments are not totally effective. Therefore, finding changes in the aqueous humour of ocular hypertensive patients could be a natural process to reduce IOP, as melatonin is able to reduce IOP as previously described (Crooke et al. 2012). The question that arises is why, if the levels of melatonin are higher, IOP does not return to normal values, as one should expect. This is a real challenge to fully understand the role of melatonin in the glaucoma pathology, and although it deserves further research, some ideas can be suggested. If melatonin levels are high and the IOP does not come down, it might be the case that melatonin receptors are not fully active or their number has significantly reduced their expression with the pathology. The existence of age-related and region-specific changes in the expression of the melatonin receptor subtypes has already been described in animal models (Guo et al. 2015). It could be the case that these changes in expression reducing the presence of melatonin receptors may happen in some humans, and therefore, the increased levels of melatonin are not capable to reduce IOP as one could be expecting. Also, melatonin, besides its function as synchronizer of the biological clock, is a powerful free radical scavenger and wide-spectrum antioxidant, and this role may also appear as the oxidative damage represents a fundamental step in the pathogenesis of glaucoma (Sacca & Izzotti 2014). Therefore, the increase in melatonin levels could also reflect a mechanism of protection carried out in the eye to prevent oxidative damage of high efficiency tissues such as the ciliary body or the trabecular meshwork (Sacca et al. 2016). Moreover, if similar changes regarding melatonin levels were occurring in the retina, this molecule would help to ameliorate the glaucomatous pathology by acting on retinal glutamate clearance, GABA concentrations, NO synthesis and retinal redox status (Belforte et al. 2010). More experiments are necessary to fully confirm all these possibilities.

This work has some limitations as it has focused on patients suffering of ocular hypertension, but it would be worthy to extend it to those suffering from diagnosed glaucoma. Moreover, it would be of great interest to see whether the treatment of such patients with antiglaucomatous medicines may modify aqueous humour melatonin levels in those patients as it happens with other aqueous humour components in experimental animal models (Reyes et al. 1998). This seems to be

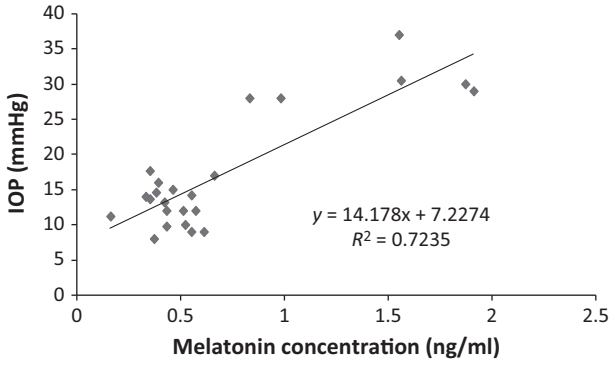

Fig. 4. Relationship between melatonin concentration (ng/ml) in aqueous humour of glaucomatous (DBA2/J) and non-glaucomatous (C57) mice strains and the IOP measured in all times of experiment. Pearson correlation was statistically significant ($p < 0.001$; $r = 0.851$).

interesting because it will help to understand the connection of antiglaucomatous drugs with melatonin and, probably by extension, it will clarify the best moment of the day to apply glaucoma medication (Bron 2004).

In summary, patients with high IOP present higher concentrations of melatonin in their aqueous humours compared to normotensive patients. This elevation in IOP may trigger the activation of a TRPV4 channel, this being the reason of melatonin rise. The lack of effect of the melatonin present in the aqueous humour of hypertensive patients is suggesting an imbalance between the neurohormone and the receptors that should reduce IOP.

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Correspondence:
Jesús Pintor, PhD
Department of Biochemistry and Molecular Biology IV
Faculty of Optics and Optometry
Universidad Complutense de Madrid
C/ Arcos de Jalón, 118
E-28037, Madrid
Spain
Tel: +34 913946859
Fax: +34 913946885
Email: jpintor@ucm.es

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Article

TRPV4 Stimulation Induced Melatonin Secretion by Increasing Arylalkymine *N*-acetyltransferase (AANAT) Protein Level

Hanan Awad Alkozi [1], Maria J. Perez de Lara [1], Juan Sánchez-Naves [2] and Jesús Pintor [1,*]

[1] Department of Biochemistry and Molecular Biology IV, Faculty of Optics and Optometry, University Complutense of Madrid, 28040 Madrid, Spain; Hanan-q1@live.com (H.A.A.); mariajpdl@opt.ucm.es (M.J.P.d.L.)
[2] Department of Ophthalmology, Balear Institut of Ophthalmology, 07011 Palma de Mallorca, Spain; juansanchez.naves@gmail.com
* Correspondence: jpintor@ucm.es; Tel.: +34-91-394-6859; Fax: +34-91-394-6885

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Abstract: Melatonin is a molecule which has gained a great deal of interest in many areas of science; its synthesis was classically known to be in the pineal gland. However, many organs synthesize melatonin, such as several ocular structures. Melatonin is known to participate in many functions apart from its main action regulating the circadian rhythm. It is synthesized from serotonin in two steps, with a rate-limiting step carried out by arylalkymine *N*-acetyltransferase (AANAT). In this report, the role of TRPV4 channel present in human ciliary body epithelial cells in AANAT production was studied. Several experiments were undertaken to verify the adequate time to reach the maximal effect by using the TRPV4 agonist GSK1016790A, together with a dose–response study. An increase of 2.4 folds in AANAT was seen after 18 h of incubation with 10 nM of GSK1016790A ($p < 0.001$, $n = 6$). This increment was verified by antagonist assays. In summary, AANAT levels and therefore melatonin synthesis change after TRPV4 channel stimulation. Using this cell model together with human ciliary body tissue it is possible to suggest that AANAT plays an important role in pathologies related to intraocular pressure.

Keywords: AANAT; ciliary body; eye; melatonin; TRPV4

1. Introduction

Melatonin is an indolamine synthesized by several ocular structures apart from its classical production in the pineal gland. It is originally known to regulate the circadian rhythm, however, many studies have indicated further important functions of melatonin, such as its role as an antioxidant, antidepressant, suppressing carcinogenesis, among other functions [1–4]. Melatonin presence in the eye is fundamental since it participates in numerous functions such as controlling tear secretion [5], accelerating corneal wound healing [6], controlling intraocular pressure (IOP) and regulating retinal physiology [7,8]. All these actions are mediated by melatonin membrane receptors whose presences have previously been described in the eye [9].

Melatonin is well known for following a circadian rhythm, which has higher levels during the night and lower levels at daytime [10]. This pattern matches with the changes observed in IOP, as when melatonin levels rise at night, intraocular pressure comes down [11]. This observation opened a window of investigation to understand the link between IOP and melatonin.

One of the leading causes of irreversible vision loss is glaucoma, a multifactorial optic neuropathy that results in progressive blindness. The only risk factor that can be controlled in glaucoma is the

elevated intraocular pressure. Studies have shown that melatonin and its analogs are able to bring down IOP by exogenous consumption [12,13]. Surprisingly, a recent study analyzing melatonin levels in human aqueous humors has demonstrated that those patients with elevated intraocular pressure present higher melatonin concentrations than healthy subjects [14,15]. These patients should have lower IOP, nonetheless this does not occur. The reasons that cause it are not yet understood.

Melatonin is synthesized from serotonin through two steps. In the first, serotonin is transformed to *N*-acetylserotonin (NAS) through acetylation by an enzyme called arylalkymine *N*-acetyltransferase (AANAT). This enzyme catalyzes the transfer of acetyl group from acetyl-CoA to serotonin. In the second step, to convert NAS to melatonin, the second enzyme called hydroxyindole-*O*-methyltransferase (HIOMT) is responsible for the *O*-methylation [16,17].

The first enzyme in the melatonin synthesis, AANAT, seems to be the key enzyme regulating melatonin synthesis. Studies have shown that AANAT fluctuate following a circadian rhythm [18–20], while HIOMT does not seem to change [21]. In fact, this is critical given that melatonin changes throughout the day; however, it is possible that AANAT is regulated by other environmental factors such as hormones, food or drug intake [22–24]. AANAT seems to have two ways of regulation. One is a quick process to protect the enzyme against degradation that happens through its phosphorylation. This regulatory mechanism also depends on a protein termed 14-3-3 that binds to AANAT and which has been linked to the activation of PKA after cAMP generation [25]. The second regulation mechanism is a long-term one, which is also dependent on cAMP/protein kinase A pathway but which activates gene expression. In rodents, transcriptional activation of *aanat* gene is the classical mechanism to induce melatonin biosynthesis. It involves PKA-dependent phosphorylation of the transcription factor cyclic AMP response element binding protein (CREB) [26] and binding of phosphorylated CREB in the promoter region of *aanat* gene.

Very recently, a transient receptor potential vanilloid 4 (TRPV4), a non-selective cation channel that regulates osmo-, thermo-, mechanosensation was said to play an important role in the ciliary body epithelium cells [27,28]. This channel activation has led to an increment of the extracellular level of melatonin [29]. These findings are pharmacologically relevant in the search of new therapies for glaucoma because melatonin has the ability to lower IOP as previously commented. In this report, we describe the effect of TRPV4 stimulation on the protein levels of AANAT, one of the enzymes responsible for melatonin synthesis, as well as its changes in the ciliary body of normal and glaucomatous patients.

2. Results

2.1. Presence of AANAT in the Human Ciliary Body

Human eyes were first treated for immunflourescent labeling, and the search for possible changes in the AANAT labeling in the ciliary body was undertaken by analyzing samples of ciliary body tissue of healthy subjects and comparing them to glaucomatous donors. Ciliary body epithelium presented a positive labeling in both normal and glaucomatous human samples (Figure 1).

In particular, a stronger fluorescent labeling was observed in the glaucomatous patient sections (Figure 1B $n = 4$) when compared to normal samples (Figure 1A, $n = 2$). This elevation in the expression of AANAT, in the case of the glaucomatous donors, was "in vitro" established using human ciliary body epithelial cells which were stimulated by the TRPV4 agonist GSK1016790A, as previously described [29]. The results obtained with the treated cells were consistent with the human ciliary body sections obtained from the donors. In this sense, the presence of AANAT was detected in both control and treated cells (Figure 2), the labeling being stronger in the GSK-treated cells (Figure 2B), than in the untreated cells (Figure 2A). Positive and negative controls were also performed for AANAT with human lens epithelial cells and human chondrocytes, respectively (Figure 2C) [30].

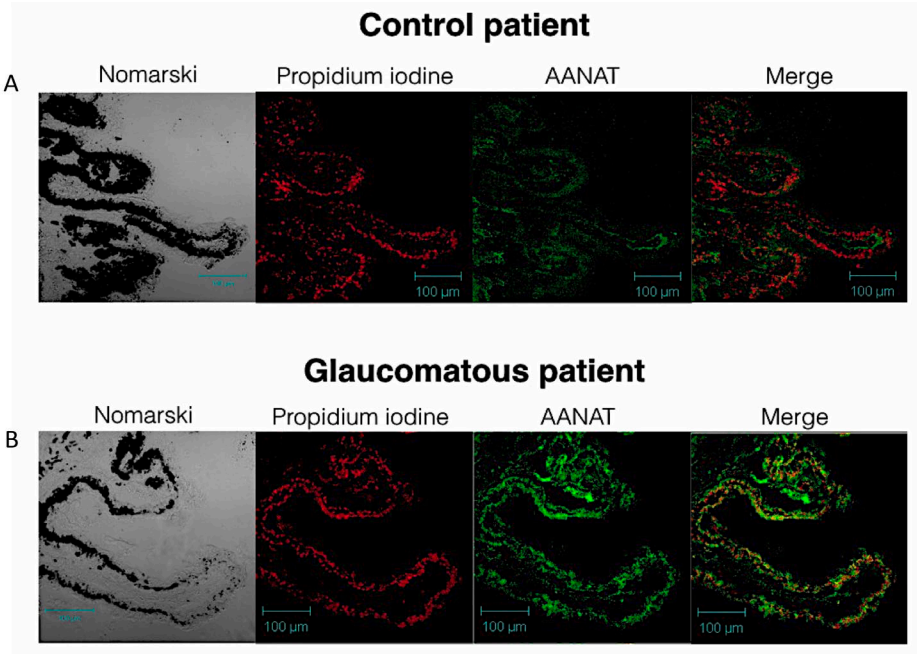

Figure 1. Apparent changes of AANAT in human ciliary body tissue: (**A**) Representative pictures of human ciliary processes (n = 2) of a non-glaucomatous individual (n = 4). From left to right, Differential Interference Contrast (DIC) image, nuclei (in red, propidium iodine), AANAT (in green) and merge image; (**B**) Representative image of human ciliary processes of a glaucomatous individual. From left to right, DIC image, nuclei (in red, propidium iodine), AANAT (in green) and merge image.

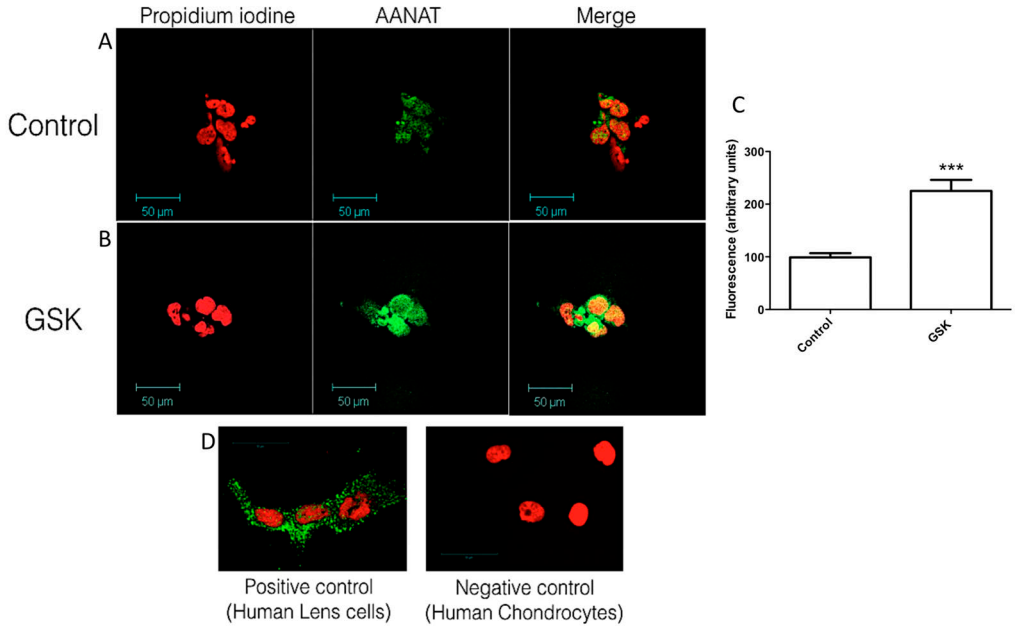

Figure 2. Presence and changes of AANAT in human ciliary body epithelial cells: (**A**) Untreated human ciliary body epithelial cells showing the expression of AANAT (in green) and the nuclei (in red); (**B**) Human ciliary epithelial cells after treatment with 10 nM GSK1016790A for 18 h. AANAT expression can be seen in green while nuclei appear in red; (**C**) Fluorescence quantification of the images shown in **A** and **B** for the AANAT intensity (green), normalized to control values; (**D**) Positive and negative controls for AANAT performed with human lens epithelial cells (positive) and human chondrocytes (negative). The values are the mean ± SEM of six independent experiments (*** $p < 0.001$).

2.2. TRPV4 Activation Increases AANAT Protein Expression in Ciliary Body Epithelial Cells

The application of the selective TRPV4 agonist GSK1016790A during different times up to 48 h at a single dose of 10 nM, showed changes in AANAT expression as observed in Figure 3. The results indicated that AANAT expression has a clear maximal peak of 2.4 folds above the control value after 18 h of incubation with TRPV4 agonist (Figure 3A, *** $p < 0.001$, $n = 6$).

Interestingly, the values of AANAT showed a decrease after that maximal expression of the enzyme, returning towards the initial levels (Figure 3B).

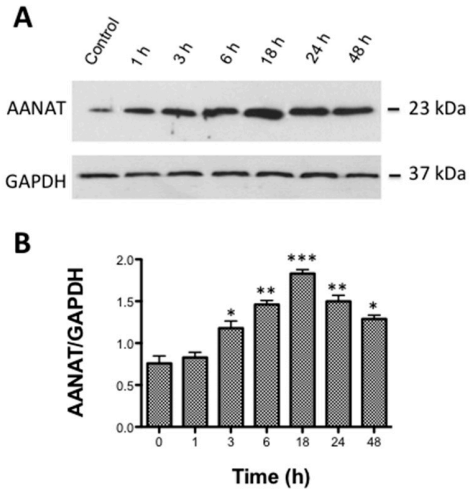

Figure 3. Time-course of the effect of GSK on AANAT protein synthesis: (**A**) representative Western blot showing the changes in AANAT during a maximal period of 48 h after cell treatment with 10 nM GSK1016790A; and (**B**) column plot showing the relative quantification of the Western blots band intensities. Values represent the mean ± SEM of six independent experiments (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus time 0).

2.3. TRPV4 Activation Increases Melatonin Levels in Ciliary Body Epithelial Cells

Since AANAT is responsible for melatonin synthesis by producing the precursor *N*-acetyl serotonin (NAS), HPLC studies were performed to investigate a possible correlation between the expression levels of AANAT showed in Figure 3 and the production of both, NAS and melatonin. As can be seen in Figure 4, both the levels of NAS and melatonin presented maximal concentrations at 18 h, with concentrations of 40.24 ± 1.82 nM in the case of NAS and 21.36 ± 1.83 nM for melatonin ($n = 6$, *** $p < 0.001$ vs. control for NAS and $^{\#\#}$ $p < 0.01$ vs. control for melatonin). As happened in the case of AANAT expression, after 18 h, both NAS and melatonin returned to their initial values.

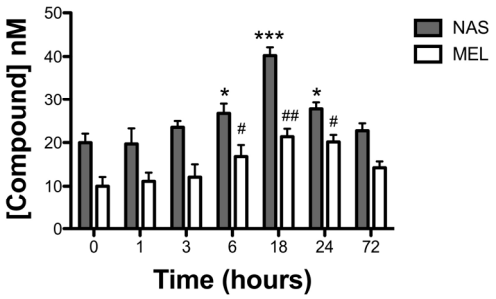

Figure 4. Time-course on the effect of GSK on NAS and melatonin levels. Columns presenting the concentrations of NAS and melatonin calculated as described in material and methods. Values represent the mean ± SEM of six independent experiments (* $p < 0.05$ and *** $p < 0.001$ versus time 0 for NAS and $^{\#}$ $p < 0.05$ and $^{\#\#}$ $p < 0.01$ versus time 0 for melatonin).

2.4. Concentration-Response Study of GSK Effect on AANAT Levels in Ciliary Body Epithelial Cells

After adjusting the time necessary to reach the maximal AANAT expression when activating the TRPV4 channel, a concentration–response assay was performed at this time by applying different graded concentrations of GSK ranging from 1 nM to 10 μM. In this sense, it was possible to observe that AANAT expression reached a maximum at GSK concentration of 10^{-8} M (Figure 5A). Transformation of Western blots into a dose–response curve allowed the observation of a sigmoidal pattern that provided a pD_2 value for GSK of 8.34 ± 0.30, which was equivalent to an EC_{50} value of 4.57 nM (n = 5, Figure 5B).

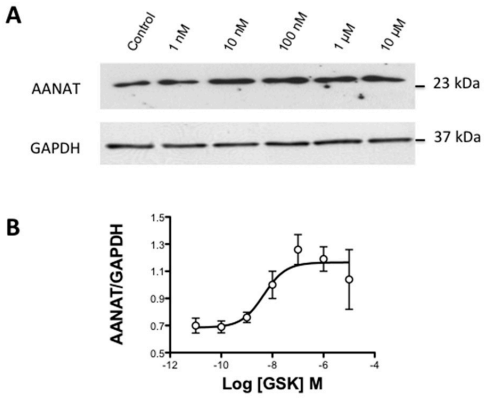

Figure 5. Concentration–response curve for GSK1016790A on AANAT phosphorylation level: (**A**) representative Western blot study showing the concentration dependency of AANAT phosphorylation when cells are challenged with GSK1016790A ranging from 1 nM to 10 M; and (**B**) concentration–response curve plotted with the relative quantification of the Western blot band intensities. The values represent the mean ± SEM of five independent experiments.

2.5. Effect of TRPV4 Antagonists on AANAT Levels in Ciliary Body Epithelial Cells

Different antagonists were used to confirm that the effect shown by GSK1016790A was actually acting on TRPV4 channel. The application of 10 nM of GSK after 18 h produced a significant increase in AANAT compared to non-treated cells (Figure 6A). This increment was blocked after applying a non-selective TRPV1/TRPV4 antagonist Ruthenium Red (RR) and by the selective antagonist of the TRPV4, RN-1734 ($p < 0.001$ for both compounds vs. GSK alone; n = 5, Figure 6B).

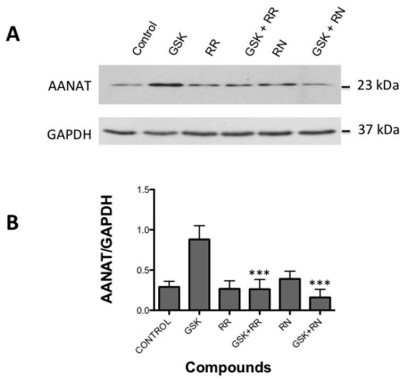

Figure 6. Effect of TRPV4 antagonists on AANAT phosphorylation triggered by GSK1016790A: (**A**) representative Western blot study showing the activity of TRPV4 antagonist Ruthenium Red (RR) and RN-1734 (RN), both alone and together with GSK1016790A, following the protocol described in methods; and (**B**) column plot showing the relative quantification of the Western blots band intensities. Values represent the mean ± SEM of five independent experiments (*** $p < 0.001$ versus GSK1016790A effect).

3. Discussion

In this study, we have described that the TRPV4 channel stimulation in the ciliary body epithelial cells is able to increase the expression of the rate-limiting enzyme of melatonin synthesis aralkylamine *N*-acetyltransferase (AANAT) (Figure 7). This increment of AANAT is both time and dosage dependent, and reaches its maximal effect after 18 h of stimulation with the selective TRPV4 agonist GSK1016790A at 10 nM concentration [31]. This effect was blocked by TRPs antagonist Ruthenium Red and with the selective TRPV4 antagonist RN-1734 [32].

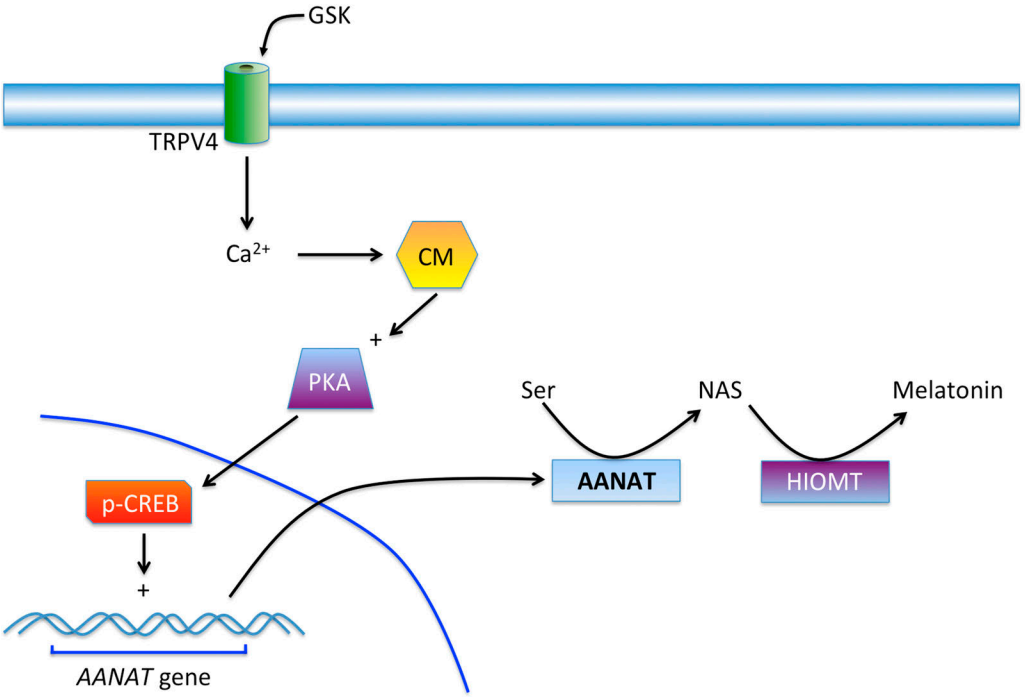

Figure 7. Possible mechanism of action of GSK acting on the TRPV4 channel in the ciliary body. After the activation of the TRPV4 the influx of Ca^{2+} will bind to calmodulin (CM) which activates adenylate cyclase and later protein kinase A (PKA). This kinase will finally produce the phosphorylation of CREB (cAMP response element-binding) than may stimulate the synthesis of the enzyme AANAT. This protein together with HIOMT will finally augment the production of melatonin in ciliary body epithelial cells. TRPV4 channel can be stimulated by the abnormal elevation in IOP that often occur in glaucoma pathology.

In a previous study, it was possible to demonstrate the presence of the TRPV4 channel in human ciliary epithelial cells and the effect, after its stimulation, was an increment of melatonin levels [29]. In fact, in a different study carried out in our lab, it has shown that patients with elevated intraocular pressure actually have higher melatonin levels in the aqueous humor compared to healthy subjects [15]. Moreover, in this study, and supporting the previous observation, it has been possible to visualize the changes in AANAT expression in the ciliary body when comparing normal and glaucomatous individuals (Figure 7). This increase may explain the rise of melatonin in glaucomatous patients and opens the question of why, as melatonin concentrations are abnormally elevated in glaucomatous individuals, this substance cannot reduce IOP as should be expected. In this sense, many studies have reported the hypotensive action of melatonin and analogs either in normotensive and glaucomatous animal models [33,34] as well as in normotensive and ocular hypertensive human beings [12,35]. The reason why the exogenously added melatonin or analogs, produce a reduction in IOP, seems to be related to the dose reached in the aqueous humor. In the glaucomatous patient´s ciliary body and in the human ciliary epithelial cells the elevation of AANAT explains the rise in melatonin in their

aqueous humor as if the eye wanted to counteract the IOP by acting on melatonin receptors, as the melatonin is unable to perform its hypotensive effect [15]. More studies are necessary to understand why melatonin cannot reduce IOP in glaucomatous patients.

Glaucoma pathology is known as the silent thief of sight, as it progressively damages the retinal cells. Nevertheless, the main risk factor of this disease is, in fact, the elevated intraocular pressure which can occur either by an increment of the aqueous humor production from the non-pigmented ciliary body cells or by a decrease in its drainage through the uveoscleral outflow pathway or the conventional way through the trabecular meshwork [36]. Previous studies in search of finding new treatments for glaucoma have shown that TRPV4 is linked to trabecular meshwork cells. TRPV4 activation mediates Ca^{2+} influx in the trabecular meshwork which, after the use of an antagonist for this channel, resulted in a decrease in IOP in a murine model of glaucoma [37]. This would imply that the antagonism of the TRPV4 in the trabecular meshwork could protect the retinal ganglion cells from mechanical stress [38,39]. However, different studies have shown the opposite effect on IOP after TRPV4 activation in Lowe syndrome patients. In this sense, TRPV4 activation lowered IOP by acting on the cilia present in the trabecular meshwork [40].

In this study, together with previous ones [15], it has been possible to establish a clear link between melatonin synthesis and the TRPV4, pressure sensor channel, which could lead the ciliary epithelium cells to produce more AANAT in order to synthesize melatonin, which may modify the physiology of those tissues bathed by the aqueous humor.

4. Materials and Methods

4.1. Cells

Non-pigmented ciliary epithelial cells (59HCE), a human immortalized cell line was kindly supplied by Miguel Coca-Prados. Cells were grown in high glucose Dulbecco's modified Eagle's medium (Gibco/Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA) and 0.05 mg/mL Gentamicin (Gibco/Invitrogen) at 37 °C in humidified atmosphere 5% CO_2–95% air. After the culture reached the confluence, cells were detached with 0.25% trypsin and seeded into 6-well plates and/or to 4-well chamber slides, respectively. All the experiments were performed using cells comprising numbers 10–15 passages to assure assays reproducibility.

4.2. Human Eye Tissues

Donor Human eyes were obtained from the Fundación Banco de Sangre y Tejidos de las Islas Baleares (Blood and tissue bank Foundation from Baleares Islands). This has been approved by the Ethics Committee of the Universidad Complutense de Madrid with reference C.P.-C.I. 16/249-E (21 June 2016). Six donor eyes were used for this assay, two of a healthy normal subject and another four of glaucoma patients. Eyes were enucleated and collected without the cornea in sterile tubes and maintained in 4% paraformaldehyde in 0.1 M phosphate buffer (PB) (pH 7.2–7.4) at 4 °C until posterior processing. Eyes were dissected under stereomicroscope (Zeiss) and with the 0.8 mm tip curved forceps and sterile dissecting scissors, the iris and ciliary processes were collected. Several washes in phosphate buffer saline (PBS) were performed and then, the specimens were cryoprotected in a sucrose gradient (from 11% to 33%) and were embedded in tissue freezing medium (Tissue-Tek® OCT, Qiagen, Barcelona, Spain) until frozen with liquid nitrogen. Vertical sections of control and glaucomatous human samples (10 µm thick) were collected using a cryostat (Microm, Walldorf, Germany) and mounted from the same region. Samples were maintained in a −20 °C until use.

4.3. Immunofluorescent Studies

Frozen sections were rinsed in PBS 1X and permeabilized with PBS-0.05% Tx-100 solution for 30 min. Afterwards, to avoid non-specific staining, sections were incubated in a blocking solution with a 10% normal donkey serum (NDS, Jackson Immunoresearch, West Grove, PA, USA) during 1 h at

room temperature. Then, the primary antibody rabbit anti-serotonin *N*-acetyltrasnferase (AANAT, ab3505, Abcam, Cambrigde, UK) was incubated at a 1:500 dilution at 4 °C overnight. Sections were washed in PBS1X-0.1% Tx-100 and incubated with donkey anti-immunoglobulin IgG rabbit antibody conjugated with fluorescein isothiocyanate (FITC; green, Jackson ImmunoResearch, West Grove) at 1:100 dilution in PBS-0.1% Tx-100 for 1 h in a dark chamber at a room temperature. The nuclei were stained with propidium iodide (red, Sigma-Aldrich, St. Louis, MO, USA) diluted 1:500 in PBS for 10 min. Finally, sections were rinsed and mounted in Vectashield (Vector Laboratories, Palex Medical, Barcelona, Spain) and coverslipped. The samples were examined under a confocal microscope (Zeiss LSM 5, Jena, Germany) at 40× magnification. For ciliary epithelial cells immunostaining, similar protocol was done for immunostaining of ciliary epithelium cells after incubating them with TRPV4 agonist GSK1016790A using a rabbit anti-TPRV4 (ab94868, 1:1000 Abcam) primary antibody.

4.4. TRPV4 Experiments

Non-pigmented ciliary body epithelial cells were seeded in multiwells of 6 at a density of 1.2×10^6 cells and then treated with the TRPV4 agonist GSK1016790A (Tocris Bioscience, Bristol, UK), for different durations starting from 1, 3, 6, 18, 24, and 48 h to establish a time course at a concentration of 10 nM. The supernatants at the indicated times were collected for melatonin quantification by HPLC as described below. After the corresponding times cells were submitted to lysis as described below for Western blot assays.

In different multiwells, after choosing the best time to see an increase of AANAT, a dose–response curve was obtained by incubating the cells with GSK1016790A at different concentrations ranging from 1 nM to 10 μM, for 18 h (according to the time-course results). Then, cell lysis was performed to quantify AANAT (see below).

For antagonists studies, the compounds ruthenium red (RR) and RN-1734 (RN) [29], were assayed at concentrations of 1 nM and 10 nM respectively, either alone or 30 min before the application of the TRPV4 agonist GSK1016790A. The supernatants at the indicated times were taken for melatonin quantification by HPLC as described below, as well as lysed cells for AANAT detection.

4.5. AANAT Western-Blot Studies

Cells were removed and homogenized in ice with RIPA buffer (1:5 v/v) containing 50 mM HEPES, pH 8, 150 mM NaCl, 1% NP-40 (w/v), 0.5% sodium deoxicolate, 0.1% SDS and Halt Protease and Phosphate Inhibitor Cocktail (Thermo Fisher Scientific, Madrid, Spain). The lysates were centrifuged at 15,000× g for 15 min at 4 °C. The supernatant was stored at −20 °C until use.

Protein concentration was determined by Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Madrid, Spain). Cell samples (60 μg proteins) were diluted in Laemmli´s sample buffer, loaded on a 15% SDS-PAGE gels and transferred to nitrocellulose membrane. Blots were blocked with 5% non-fat dry milk (Bio-Rad, Madrid, Spain) for 1 h at room temperature and then they were incubated overnight at 4 °C in TBS1X 0.1% Tween 20 containing 5% non-fat milk (Bio-Rad) (blocking buffer) and AANAT primary antibody (ab3505, 1:1000, Abcam). Mouse monoclonal glyceraldehyde-3-phosphate dehydrogenase (anti-GAPDH 1:500) (Santa Cruz, Dallas, TX, USA) served as a loading control. Membranes were incubated with a goat anti Ig G-rabbit or a goat anti-Ig G mouse conjugated with horseradish peroxidase secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA) for 1 h at room temperature. Then, proteins were revealed by chemiluminiscence using enhanced chemiluminiscence (ECL) detection (Amersham Phamacia Biotech, Barcelona, Spain). Films were scanned with Gel Logic 200 Imaging System (Kodak). The densitometric analysis was performed by using Kodak Molecular Imaging software (v 4.0.3, Kodak, Rochester, NY, USA). The densitometry values of each sample were normalized to respective densitometric GAPDH values.

4.6. HPLC Analysis

N-acetyl serotonin measurements were performed by HPLC following the protocol described by Alkozi and co-workers [28]. Before injection, the supernatants were heated in a 98 °C dry bath for 2 min before being transferred to ice for 10 min. To eliminate proteins, tubes were centrifuged at 13,000× *g* for 10 min at 4 °C. The analysis by HPLC to detect NAS was carried out using a SunFire18 column (5 µ, 25 cm in length, 0.4 cm inner diameter) from Waters (Milford, MA, USA) equilibrated with a mobile phase consisting of 15% acetonitrile, 0.1% acetic acid and at a flow rate of 0.75 mL/min, detecting NAS at the wavelength of 244 nm.

Melatonin measurements were carried out by HPLC as previously described. Briefly, the HPLC was connected to column Kromaphase C18 with 5.0 µm particle (25 cm in length, 0.4 cm inner diameter) from Scharlau, Madrid, Spain. The HPLC consisted of a 1515 Isocratic HPLC pump, a 2487 dual absorbance detector, and a Reodyne injector, ruled by the program Breeze from Waters (Milford, MA, USA). The mobile phase was obtained with 40% methanol, 60% H_2O. Chromatograms were obtained at a flow rate of 0.8 mL/min measuring melatonin at a wavelength of 244 nm [27,28].

Quantification of NAS and melatonin was performed by comparing the samples with external standards provided by Sigma (St. Louis, MO, USA).

4.7. Statistical Analysis

The data represent the mean ±SEM of 4–6 independent experiments (indicated in each case). Statistical significance was calculated by student *t*-test or *ANOVA* test when necessary. GraphPad Prism, v 5 for MAC (GraphPad Software Inc., San Diego, CA, USA) was used to obtain the plots and to fit nonlinear regression curves in order to obtain the pD_2 value (EC_{50}).

5. Conclusions

TRPV4 channel activation, which is present in the human ciliary body and ciliary body epithelial cells, increases the expression of the enzyme AANAT, which elevates the concentration of NAS and melatonin. Elevated intraocular pressure can stimulate this channel too promoting the presence of higher concentrations of melatonin in the aqueous humor of patients with glaucoma as previously observed [15]. Altogether, we can suggest that melatonin and AANAT play an important role in pathologies related to intraocular pressure, although more research is necessary to fully understand the role of melatonin in the homeostasis of the aqueous humor.

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Author Contributions: Jesus Pintor conceived and designed the experiments and analyzed the data; Hanan Awad Alkozi and Maria J. Perez de Lara performed the experiments; Juan Sánchez-Naves contributed and prepared human samples; Jesús Pintor and Hanan Awad Alkozi wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest.

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Melatonin synthesis in the human ciliary body triggered by TRPV4 activation: Involvement of AANAT phosphorylation

Hanan Awad Alkozi, María J. Perez de Lara, Jesús Pintor*

Department of Biochemistry and Molecular Biology IV, Faculty of Optics and Optometry, Universidad Complutense de Madrid, Madrid, Spain

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ABSTRACT

Melatonin is a substance synthesized in the pineal gland as well as in other organs. This substance is involved in many ocular functions, giving its synthesis in numerous eye structures. Melatonin is synthesized from serotonin through two enzymes, the first limiting step into the synthesis of melatonin being aralkylamine N-acetyltransferase (AANAT). In this current study, AANAT phosphorylation after the activation of TRPV4 was studied using human non-pigmented epithelial ciliary body cells. Firstly, it was necessary to determine the adequate time and dose of the TRPV4 agonist GSK1016790A to reach the maximal phosphorylation of AANAT. An increase of 72% was observed after 5 min incubation with 10 nM GSK ($^{**}p < 0.05$, $n = 6$) with a concomitant rise in N-acetyl serotonin and melatonin synthesis. The involvement of a TRPV4 channel in melatonin synthesis was verified by antagonist and siRNA studies as a previous step to studying intracellular signalling. Studies performed on the second messengers involved in GSK induced AANAT phosphorylation were carried out by inhibiting several pathways. In conclusion, the activation of calmodulin and calmodulin-dependent protein kinase II was confirmed, as shown by the cascade seen in AANAT phosphorylation ($^{***}p < 0.001$, $n = 4$). This mechanism was also established by measuring N-acetyl serotonin and melatonin levels. In conclusion, the activation of a TRPV4 present in human ciliary body epithelial cells produced an increase in AANAT phosphorylation and a further melatonin increase by a mechanism in which Ca-calmodulin and the calmodulin-dependent protein kinase II are involved.

1. Introduction

Melatonin is the substance that was discovered in the pineal gland and is responsible for the control of those processes related to light/darkness cycles (Reiter et al., 2014). This molecule can also be synthesized in other organs and tissues such as the eye and the gut (Balemans et al., 1980; Bertrand et al., 2010; Rohde et al., 1985). The effects observed in the eye are related to the control of several relevant physiological aspects including tear secretion (Hoyle et al., 2006), corneal wound healing (Crooke et al., 2015), intraocular pressure (IOP) regulation (Pintor et al., 2001; Samples et al., 1988) and retinal physiology (Tosini et al., 2012). All these actions are mainly mediated by membrane melatonin receptors that have been classified as MT_1, MT_2 (Zlotos et al., 2014) and the putative MT_3 (Pintor et al., 2003; Serle et al., 2004) which have been also identified in some tissues such as the enzyme quinone reductase-2 (Nosjean et al., 2000).

Melatonin synthesis depends on 5 hydroxy-tryptamine (5-HT or serotonin) levels. 5-HT is transformed in two steps catalysed by the enzymes aralkylamine N-acetyltransferase (AANAT) and acetylserotonin O-methyltransferase (HIOMT) (Bernard et al., 1999). Although both enzymes are necessary for the synthesis of melatonin, AANAT seems to be the limiting step in terms of regulation (Kim et al., 2007; Tosini et al., 2006). This is a critical aspect when considering the circadian fluctuations of melatonin throughout the day, but this does not discard the possibility of AANAT being regulated by other environmental factors different from the light/darkness changes such as other hormones, food or even alcohol intake (Garcia-Marin et al., 2015; Kashani et al., 2015; Peres et al., 2011; Schomerus and Korf, 2005).

Independently of the cause, regulation of AANAT occurs in two main ways, by regulating the expression of the gene that encodes for this enzyme or by a quick process, which involves

* Corresponding author. Dpto Bioquímica y Biología Molecular IV, Facultad Óptica y Optometría, Universidad Complutense de Madrid, C/ Arcos de Jalón, 118, E-28037, Madrid, Spain.
E-mail address: jpintor@ucm.es (J. Pintor).

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Research article

Presence of melanopsin in human crystalline lens epithelial cells and its role in melatonin synthesis

Hanan Awad Alkozi, Xiaoyu Wang, Maria J. Perez de Lara, Jesus Pintor*

Department of Biochemistry and Molecular Biology IV, Faculty of Optics and Optometry, Universidad Complutense de Madrid, Madrid, Spain

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ABSTRACT

Melanopsin is a non-image forming photoreceptor known to be present in the retina and it is considered to have light regulated tasks among other functions. In the present work, melanopsin presence in human lens epithelial cells as well as in human lens tissue is described for the first time. Moreover, studying the concentration of melatonin and its synthesising enzyme AANAT proved a clear link between melanopsin activation and the suppression of melatonin synthesis. Melanopsin sensitivity to specific wavelength (465–480 nm, blue) was confirmed after making temporal studies incubating lens epithelial cells under light, red, green, blue and total darkness for 2, 4, 8, 12 h and analysing the concentration of both melatonin and its synthesising enzyme AANAT, discovering that melatonin levels after submitting cells to total darkness are significantly higher to ones submitted to white or specifically blue light (***$p < 0.001$, $n = 6$). The involvement of melanopsin in the regulation of melatonin was also determined by using a specific inhibitor AA92593 and by inhibiting melanopsin-induced phospholipase C activation. Under this situation neither AANAT nor melatonin levels changed under light conditions ($n = 4$, ***$p < 0.001$). The discovery of melanopsin in the lens opens the possibility of regulating melatonin synthesis with the corresponding implication as an antioxidant substance.

1. Introduction

The most important environmental changes for almost any living organism are associated with earth's daily rotation. Thus, the ability to grab occurring changes is critical to survival. Accordingly, light is considered to provide the most reliable source of information about time of the day in order for the organism to adapt its physiology in accordance with the internal clock (Arbab, 2009). In such a way, photoreception is essential for the regulation of the circadian system (Hanifin and Brainard, 2007).

Beside object recognition, eyes serves as the sensory organ which detects the presence or absence of light and eye loss in human or other mammals results in a failure of photoentrainment (Bellingham and Foster, 2002). In this sense, melanopsin emerges as a non-image forming photoreceptor protein in which one of its functions is a light-regulated task (Provencio et al., 1998).

Melanopsin is a member of the G-protein-coupled receptor family, a vitamin A-based opsin in the vertebrate retina that has been shown to be involved in the synchronization of the circadian rhythms, pupillary light reflex, and melatonin suppression (Foster and Bellingham, 2002; Gooley et al., 2003; Ruby et al., 2002). It is localized in the intrinsically photosensitive retinal ganglion cells (ipRGC) and it is known to be specifically sensitive to blue light (Hattar et al., 2002). Melanopsin receptors capture this specific wavelength and send signals through the suprachiasmatic nucleus until reaching the pineal gland to supress the synthesis of the hormone melatonin during the day (Baver et al., 2008; Wurtman et al., 1963).

Melatonin is synthesized from serotonin through two steps. In the first, serotonin is catalysed by the enzyme Aralkylamine N-acetyltransferase (AANAT), then the product, N-acetyl-serotonin (NAS) is taken by the last enzyme in the melatonin synthesis, Hydroxyindole O-methyltransferase (HIOMT) (Bernard et al., 1999; Ganguly et al., 2002; Menendez-Pelaez et al., 1987). Both enzymes are necessary for melatonin formation; nevertheless, AANAT appears to be the limiting step for its synthesis (Ganguly et al., 2001, 2005).

Melatonin is a neurohormone mainly synthesised by the pineal gland as indicated, but it is also present in many ocular structures

* Corresponding author.Dpto Bioquímica y Biología Molecular IV, Facultad Óptica y Optometría, Universidad Complutense de Madrid, C/ Arcos de Jalón, 118, E-28037 Madrid, Spain.
E-mail address: jpintor@ucm.es (J. Pintor).

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Short Commentary OMICS International

Improving Melatonin Delivery Within the Eye

Alkozi HA* and Pintor J

Department of Biochemistry and Molecular Biology IV, University Complutense of Madrid, Madrid, Spain

Short Commentary

Melatonin is a neurohormone synthesized in the pineal gland as well as in other organs and it plays an important role in many ocular functions, as it is synthesized in numerous eye structures. Melatonin found to reduce the intraocular pressure and in serves as an antioxidant preventing against free radicals, hence cataract formation as well as retinal damage due to glaucoma, among other functions. Ocular pharmacology is a challenging field giving the difficulties of drug delivery inside the eye due to its low bioavailability. In this sense, the present brief commentary is summarizing the latest advances in non-invasive ocular drug delivery focused on the effect of melatonin in different ocular diseases.

Melatonin is an ancient molecule which was first identified in the late 1950s [1]. It exists in almost every organism starting from the primitive ones as prokaryotes to the very complex organisms as the humans. Melatonin is known as a circadian rhythm regulator [2]. This indoleamide is a neurohormone considered classically to be secreted by the pineal gland, nonetheless, it is currently known to be synthesized in other organs and tissues such as the retina and cerebellum [3,4], iris, ciliary body [5], crystalline lens [6,7], Harderian gland [8] and the lacrimal gland [9], spleen, heart, skeletal muscle, liver, stomach, gut, placenta, testes, ovaries, cerebral cortex and striatum [10].

Melatonin levels in the body are variable giving the time of the day, it increases at night and when light enters the eye and reach the retina, melatonin is suppressed [11], such photoreception being due to specific ganglion cells in the retina containing the pigment melanopsin [12]. This neurohormone is of great importance in the eye, apart from the retina it has numerous functions, such as working as an antioxidant protecting ocular structures against free radicals, for instance, melatonin intraperitoneal injection on rats instantly following an oxidative stress has shown to protect the lens against cataract [13]. Melatonin is inversely related to intraocular pressure since IOP decreases at night while melatonin increases [14]. It also has been proved to reduce intraocular pressure (IOP) by decreasing the rate of aqueous humour secretion by the non-pigmented ciliary epithelium, resulting in a modulation of IOP [15]. More importantly, melatonin is important for the cornea, studies showed that melatonin accelerate corneal wound healing, and it has been possible to demonstrate that the effect of melatonin is to increase the rate of cell migration rather than mitosis [16]. Besides, melatonin has the ability to potentiate the effect of diadenosine tetraphosphate, a tear secretion inducer, being suitable to treat one of the most prevalent ocular conditions: Dry eye [17].

Treating ocular conditions have many challenges due to poor drug delivery because of effective multiple barriers to drug entry, comprising nasolacrimal drainage, epithelial drug transport barriers and clearance from the vasculature in the conjunctiva [18]. While topical ocular bioavailability is extraordinary poor, in the order of 5% or less, sustained delivery systems for diseases of the posterior segment such as various vitreoretinal disorders through intraocular delivery systems are used *via* implantable devices or injections [19]. However, independently of the fact that intraocular drug delivery systems are invasive, up to date, long-term drug delivery for diseases of the anterior segment of the eye does not exist.

There are several ways for drugs to reach the ocular system, for instance, melatonin orally administered at a concentration of 10 mg to patients before performing cataract surgery have shown to lower intraocular pressure, consequently, they had better operating condition [20]. Melatonin was also investigated for the treatment of uveitis in hamsters, experiments were done by injecting 5 mg of melatonin before the induction of uveitis, and results suggested that melatonin prevents the clinical and biochemical consequences of this disorder [21].

Among several factors leading to poor bioavailability of drug administered topically, the cornea is the primary barrier for anterior segment due to lipophilicity and tight junctions which restrain the entrance of pharmacological substances [22]. In this sense, experiments have demonstrated that when melatonin or any of its analogues is topically applied, the amount that appears within the eye was between 3 and 4 orders of magnitude lower than the instilled amount [23]. This relevant fact is suggesting that it is necessary to improve the delivery ways to permit better results with less amount of melatonin.

There are three main ideas that can be highlighted: One can be to facilitate the entrance through the cornea by modifying the barrier effect. Second, to permit melatonin to remain longer on the ocular surface to allow a slow but a sustained entrance of this substance. Third to induce the intraocular synthesis of melatonin by modulating light wavelength.

Concerning the first idea, an interesting study showed that the molecule diadenosine tetraphosphate (Ap4A), has the ability to make transiently disappear the corneal tight junctions permitting efficient drug delivery to the eye. A study showed that 5-MCA-NAT, a melatonin analog, had more hypotensive effect when instilled topically on New Zealand white rabbits when diadenosine tetraphosphate was applied two hours before the melatonin analogue. Indeed, when 5-MCA-NAT was topically applied after using diadenosine tetraphosphate, the amount of this melatonin analogue found within the eye was 3-fold the one measured when it was instilled alone [24]. This is indicating that the transient elimination of the corneal tight junctions is an effective mechanism to permit the entrance of molecules into the eye [25].

The second idea consists of permitting melatonin to remain longer on the ocular surface. A possible alternative for a long-term drug delivery is the use of contact lenses, in such a way, ocular bioavailability will be improved considering reduced tear mixing between the lens and the cornea besides the extended drug release [26]. The traditional method is to soak the lens in drug solution in order for the drug to be

***Corresponding author:** Alkozi HA, Faculty of Optics and Optometry, Department of Biochemistry and Molecular IV, Complutense University of Madrid, C/Arcos de Jalón 118, E-28037 Madrid, Spain, Tel: +34674467489; E-mail: Hawadh@ucm.es

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absorbed into the polymeric lenses, this way permit limited and slow release of the drugs into the post-lens lacrimal fluid [18,27]. Melatonin and analogues can be soaked in contact lenses overnight and the lenses can be fitted to obtain a sustained release of these substances. In this way, when melatonin is topically instilled it lasted no more than 2 min on the ocular surface as happens with all compounds applied in this way [18] (Kompella, Kadam et al. 2010). When melatonin and analogues are released from contact lenses the maximal release occurs 2 hours after the lens fitting their presence being measurable for more than 300 min, as it happens with other naturally occurring substances [27]. This slow but sustained release of melatonin will permit the entry of this substance and therefore a more robust intraocular effect.

The third idea is based on modifying the light that enters the eye in order to induce the natural production of melatonin in the lens, instead of applying it exogenously. Recently, it has been possible to describe the presence of melanopsin in the lens epithelium [28]. This pigment abolishes the synthesis of melatonin when the lens is illuminated with blue light (including the blue component of white light). Therefore, it is possible to induce the synthesis of melatonin by reducing the blue component of white light (460-490 nm wavelength) by means of filters. Interestingly, white light permits a discrete synthesis of melatonin in lens epithelial cells (about 20 pmol/10^6 cells), but the blockade of blue light permits levels which are 3-fold higher (60 pmol/10^6 cells) [28]. This regulation of the local synthesis of melatonin by filtering light, could be an interesting approach to induce melatonin synthesis intraocularly and to help in processes such as the reduction of IOP and cataract prevention.

In summary, melatonin has proven effective for several ocular disorders and it could be interesting to investigate the best possible way for its delivery giving the challenges ophthalmologists are facing due to limited non-invasive drug delivery systems to the eye.

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