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REVIEW ARTICLE

Phototransduction mediated by melanopsin in intrinsically photosensitive retinal ganglion cells

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Abstract

Melanopsin is the most recent photopigment described. As all the other opsins, it attaches in the retina as chromophore. Its amino acid sequence resembles more invertebrate opsins than those of vertebrates. The signal transduction pathway of opsins in vertebrates is based on the coupling to the G protein transducin, triggering a signaling cascade that results in the hyperpolarization of the plasma membrane. On the contrary, the photoreceptors of invertebrates activate the Gq protein pathway, which leads to depolarizing responses. Phototransduction mediated by melanopsin leads to the depolarization of those cells where it is expressed, the intrinsically photosensitive retinal ganglion cells; the cellular messengers and the ion channel type(s) responsible for the cells´ response is still unclear. Studies to elucidate the signaling cascade of melanopsin in heterologous expression systems, in retina and isolated/cultured intrinsically photosensitive retinal ganglion cells, have provided evidence for the involvement of protein Gq and phospholipase C together with the likely participation of an ion channel member of the transient receptor potential-canonical family, a transduction pathway similar to invertebrate photopigments, particularly Drosophila melanogaster. The intrinsically photosensitive retinal ganglion cells are the sole source of retinal inferences to the suprachiasmatic nucleus; thus, clarifying completely the melanopsin signaling pathway will impact the chronobiology field, including the clinical aspects. (Gac Med Mex. 2015;151:709-20)

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Introduction

The eyes are the sensory organs responsible for vision, by means of which, by means of which we are able to perceive objects and their movements, contours and colors. In vertebrates, the eyeball consists of the sclera, the cornea, the pupil, the iris, the lens, the ciliary body and the choroid membrane. Among all these tissues, one of the most important is the retina[1], which is comprised by six classes of nervous cells: photoreceptors, rods and cones, and retinal interneurons: horizontal, bipolar, amacrine and ganglion cells (RGC)[2], which are the neurons of projection towards the encephalon. There is a subset of RGCs produces melanopsin, an opsin that confers these cells the capacity to capture photons through the retinal chromophore bound to bound to it[3]. This way, the cells act as an aditional photoreceptor to the classical rods and cones. They are intrinsically photosensitive RGCs (ipRGCs) because they respond to light stimuli even when cone and rod-activated synaptic signals coming from the intra-retinal neuronal network are inhibited[4], and even once isolated from the retina. ipRGCs have

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been found in all vertebrates studies so far; five subtypes (M1-M5) have been described in the mouse, and three in the rat[5]. Their activites as a third class of retiinal photoreceptor do not lead to image formation and thus are known as retinal non-image forming responses (NIF)[3]. Opsins belong to the class of receptors that couple with trimeric G proteins for signal transduction[6] (GPCR). In the animal kingdom, opsins activate two types of G proteins; those of the classical photoreceptors couple to transducin (Gt), which belongs to the $G_{i/o}$ (G_i) subfamily and decreases the levels of cyclic nucleotides[7]. Opsins of invertebrates activate the G_q class ($G_{q/11}$), which is characterized for stimulating the phospholipase C (PLC) enzyme and for generating ion currents mediated by changes in intracellular Ca^{2+} ($[Ca^{2+}]_i$)[8,9] and are, therefore, depolarizing, in opposition to what happens in cones and rods. The melanopsin amino acid sequence is more similar to invertebrate than to vertebrate opsins[10]. This homology assumes that its transduction mechanism is similar to that of photoreceptors in invertebrates. Several studies in heterologous expression systems, in cell cultures and in the retina suggest that melanopsin uses the phosphoinositide system to carry out the light signal transduction; however, the participation of different molecules, including the signaling cascade final effector, the channel(s) responsible for generating a $(Ca_{2+})_i$ increase underlying the generation of action potentials, remains to be proven or disregarded. The purpose of this article is to review the studies in order to elucidate the melanopsin-mediated phototransduction mechanism.

Melanopsin

In 1998, Provencio et al. were working with melanophores of the *Xenopus laevis* frog in order to identify the opsin involved with melanosomes migration to the cell surface, a phenomenon produced as a consequence of light incidence. They were able to identify an opsin, which they named melanopsin due to its isolation from these melanophores. They also found that it was expressed in the amphibian's retinal cells. In addition, they determined that its amino acid sequence was more similar to that of invertebrates' opsins than to visual opsins, such as those occurring in vertebrates' cones and rods[11]. Therefore, their results indicated that this photopigment was expressed in both non-ocular and ocular tissues and thus, it was then suggested that it might be implied in both visual and non-visual photoreceptive functions[3,11]. Two years later, the same team discovered that, in humans, melanopsin did only express in the eye, particularly in the retina; there, its expression occurred more likely in amacrine and ganglion cell layers[12].

Melanopsin structure

Melanopsin is a 534-amino acid membrane integral protein; it has 7 trans-membrane domains; the amino-terminus end is found at the extracellular region, while the carboxyl-terminus is found in the cytoplasm; it has 3 extracellular loops, with the latter 2 containing some cysteine residues, the function of which is to stabilize the tertiary structure by means of disulfur bonds. It also shows 3 loops in the cytoplasmic region where different amino acids are candidate as intracellular phosphorylation sites, and in the seventh trans-membrane domain, interaction occurs with the chromophore, retinaldehyde. Although melanopsin was identified in vertebrates, their amino acid sequence and structural features are more alike to invertebrate opsins; for example, in the third cytoplasmic loop there is an Asn-225 amino acid inclusion for a Gly-223, an insertion that determines the class of G protein activated by GPCRs[13].

Functions

The impulse from rod and cones is relayed through synatic signals onto retinal ganglion cells (RGC), the axons of which form the optic nerve that projects towards the encephalon for the formation of images. Conversely, melanopsin-expressing ipRGCs project their axons towards the suprachiasmatic nucleus (SCN) in order to regulate the circadian rhythm synchronization in response to the environmental light levels (photosynchronization)[14]. Other functions related to photic responses include melatonin suppression[15,16], pupillary light response (PLR)[17-20] and modulation of sleep[21]. ipRGCs are involved in functions which are related to environmental luminosity-dependent processes but do not lead to image, and thus have been called retinal non-image forming responses (NIF) (Fig. 1).

Signal transduction mechanism (phototransduction)

Visual phototransduction is the mechanism by means of which photons are absorbed by retinal cones and rods to transduce them into a nerve impulse that the brain is able to interpret[22]. Melanopsin is expressed in

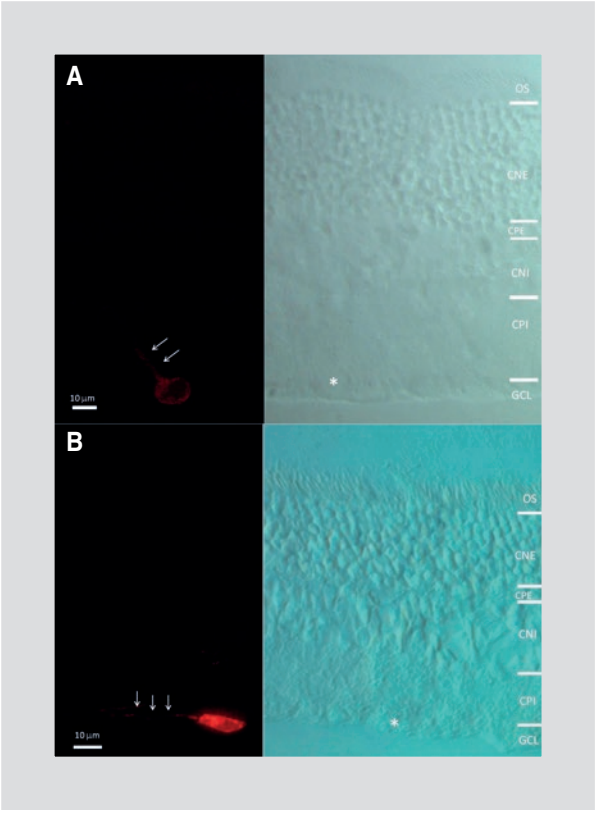

Figure 1. *Melanopsin-containing ganglion cells in the rat retina. Retinal vertical sections processed for melanopsin localization by immunofluorescence.* ***A:*** *M1-type cell with dendritic branching towards the CPI upper strata.* ***B:*** *M2-type cell showing dendrites spreading along the CPI lower strata. Clasification according to (5). Image from the authors. OS: photoreceptor outer segments; CNE: outer nuclear layer; CPE: outer plexiform (synaptic) layer; CNI: inner nuclear layer; CPI: inner plexiform layer; GCL: ganglion cell layer. Rabbit polyclonal antibody against melanopsin from Affinity Bioreagent; fluorescent antibody: goat vs. rabbit IgG coupled with ALEXA488 from Molecular Probes.*

all vertebrates that have been studied[23], both in the retina and outside the eye, as in the case of the *X. laevis* frog, where it is found in dermal melanophores and in the retina; in mammals, it has been found only in retinal tissue[12,24], which with no doubt is determined by the fact that mammals are the only vertebrates with photoreception restricted to the eye balls. Based on this evidence, it could be assumed that this photopigment would have closer structural and functional resemblance with vertebrates' cone and rod opsins than with those of invertebrates' retina; however, the opposite occurs, since melanopsin has a closer phylogenic relationship with invertebrates' opsins, including its phototransduction mechanism[25,26].

In the animal kingdom, two types of photoreceptors occur: rhabdomeric and ciliary. Rhabdomeric photoreceptors are primarily found in invertebrates, while the ciliary type belongs to vertebrates[27]. Both types of photoreceptor cells use opsins belonging to the G-protein-coupled receptors (GPCR) superfamily, whose characteristics have been outlined in previous paragraphs[28]. In response to light, ciliary opsins activate the trimeric G protein transducin (Gt), which activates the effector enzyme phosphodiesterase (PDE) of cyclic guanosine monophosphate (GMP), thus reducing its levels in cytosol and closing the closure of cyclic nucleotide (CNG)-activated ion channels, with the resulting hyperpolarization of the plasma membrane. In contrast, visual transduction occurring in rhabdomeric photoreceptors uses the phosphoinositide system. Rhabdomeric rodopsin activates a G_q-type protein, which in turn activates PLC. PLC hydrolyzes the membrane lipid, phosphatidylinositol 4,5 bisphosphate (PIP_2), thus generating two second messengers: sn-1,2 diacylglycerol (DAG), which remains bound to the membrane, and inositol 1,4,5-trisphosphate (IP_3), which is released into the cytosol[29]. These products act as second messengers direcly or indirectly on a number of proteins[22,30]. In ipRGCs, light provokes membrane depolarization and an increase in intracellular [Ca2++], the access pathway of which remains to be defined, not without debate[3,24,31,32]. It has been proposed to be the result of a TRPC-type ion channel activation[3,24,31,32] or by voltage-gated Ca^{2+} channels (VGCC) activation[33]. The melanopsin signal transduction mechanism has been compared to that of *Drosophila* due to homology with this invertebrate's rhodopsin. In *Drosophila,* light absorption by the rhabdomeric photoreceptor initiates the prototypical signaling cascade by the G_q protein, PLC, and $[Ca^{2+}]_i$ increase and the participation of transient potential channels, even defined by research of this phenomenon in the fly (transient receptor potential [TRP] and TRPL)[27,30].

In mammals, the TRP ion channel subfamily was discovered due to the structural similarity with the TRP channels in *Drosophila*[34,35], which are cation channels that, when activated by physical stimuli, metabolites or GPCR, depolarize the plasma membrane[36,37]. TRPs are organized in three main domains: they contain six transmembrane a-helices that form the ion channel; their amino-terminus group is found in the cytosol and is implied in protein-protein interactions, and their carboxyl-terminus end is also a protein-protein interaction site. In turn, TRPs are further classified in the C, V, M, A, P and ML subfamilies. Most of these are expressed in sensory neurons dendrites and play an important

Table 1. Studies in heterologous expression systems with the OPN4

Author (year)	Objective	Heterologous expression system	Melanopsin source	Method to measure iLR	Results
Newmann et al. (2003)	whether determine melanopsin is a functional photopigment	COS-1 cell line	Mouse cDNA	In vitro biochemical assay	G transducin protein activation
Melyan et al. (2005)	whether determine if melanopsin induces phototransduction in other cell systems	Neuro-2a cell line	Human cDNA	Whole cell patch clamp	Melanopsin is a photopigment that activates a G protein along with the activation of a ion channel
Qui et al. (2005)	whether determine if melanopsin is a functional sensorial photopigment	HEK293 cells (co-expression with TRPC3)	Mouse cDNA	Whole cell patch clamp	G_q, PLC and TRPC3 activation
Panda et al. (2005)	whether characterize the melanopsin function in response to light	*X. laevis* oocytes (co-expression with TRPC3)	Injected mouse mRNA	Voltage clamp	$G_{q/11}$, PLC and TRPC3 activation
Kumbalasiri et al. (2007)	whether investigate the source of $(Ca^{2+})_i$ in melanopsin photoactivation	HEK293 cells (co-expression with TRPC3)	Human cDNA	Calcium imaging	The increase of $(Ca^{2+})_i$ is mediated by Ca^{2+} stores rather than by TRPC3 channels
Bayles and Lucas (2013)	whether determine spectral sensitivity and selectivity to G proteins	HEK293 cells	Human cDNA	Reporter genes	Absorption peak at 479 nm. Selectivity for $G_{q/11} > G_i/_0$

Cos-1: monkey kidney tissue-derived fibroblast-type cell line; cDNA: complementary DNA; neuro-2a: mouse neuronal cell line; HEK293: human embryonic kidney cells.

role in sensory transduction processes, such as vision and pain, among others. TRPCs (known as canonical or classical) differ from the rest in that they are implied in two major functions: $[Ca^{2+}]_i$ increase regulation and membrane depolarization. Several TRPCs have been discovered, which are termed TRPC1 to TRPC7. In addition, members of this subfamily have been found to be homologously closer to the *Drosophila* TRP channels[38-40].

Heterologous expression studies

The number of melanopsin-expressing ganglion cells accounts for 1 to 3% out of the total population of ganglion cells in different species[2], Which makes them difficult to study. One way to counteract this problem is by using heterologous expression systems that allow for the protein under study to be overexpressed. For this, three basic elements are required: a gene that codifies for the foreign protein, a vector that allows for it to be transported and the host where the protein of interest is to be expressed[41]. Table 1 shows the works performed in heterologous expression systems in order to clarify the melanopsin phototransduction mechanism.

In the first heterologous expression study with melanopsin (Table 1), C SV O-rigin S-V40 (COS-1) cell lines were transfected with melanopsin (OPN4) and bovine G_t genes, and biochemical assays were performed in cell membranes. Melanopsin was clearly shown to be a functional photopigment able to interact and activate a G protein[42], in spite of it being normally used by rhodopsin. Of note, melanopsin signal transduction was then thought would be more related to G_q protein activation due to its homology with invertebrates' opsins. However, Weng et al. reported that rhodopsin itself is activated by the human retina metabotropic glutamate receptor (hmGluR6), which is unrelated to the opsins family[43], thus implying that relationships between GPCR and G proteins in heterologous expression systems might be promiscuous.

Two years later, in 2005, melanopsin function as a photopigment was demonstrated again in a heterolo-

gous cell system. Instead of using membrane preparations[42], intact cells were used in this research (Table 1), where melanopsin was found to be able to interact with a G protein and cause the ensuing activation of an ion channel. The response to light stimuli was eliminated by the effect of G-protein inhibitors such as suramin and the non-hydrolyzable analogue guanosine triphosphate gamma sulfur (GTP gamma S); however, the type of G-protein family that interacted was unknown, since the cell line used in the study (neuro-2a) doesn't express the classical G-proteins transducins ($G_i/_o$). In addition, the authors used PLC and kinase proteins A and B antagonists, with responses to light stimuli remaining unchanged. What they did demonstrate was an increase in $[Ca^{2+}]_i$ as a result of melanopsin photoactivation, which was overriden on a Ca^{2+}-exhausted environment or by effect of thapsigargin, an inhibitor of Ca^{2+} intracellular channels. In order to know the identity of the ion channel, Ca^{2+} was substituted with Na^+, together with VGCC inhibitors, and the responses to light weren't affected[44]. These results implied the mobilization of Ca^{2+} from intracellular stores rather than currents through the membrane in the generation of the response to light by melanopsin phototransduction (intrinsic light response [iLR]).

In 2005, two studies were also reported demonstrating for the first time the participation of proteins G_q, PLC and TRPC3 in the melanopsin-mediated signal transduction (Table 1)[45,46]. Human embryonic kidney cells HEK293, transfected with mouse OPN4 and stimulated with light, generated membrane voltage changes, which were decreased or suppressed by using specific G_q protein or PLC antagonists. Presumably, there was an activation of TRPC3 associated with this signaling pathway[46]. Similar results were observed in another heterologous expression system, where mouse mRNA was injected to stores *X. laevis* oocytes (Table 1). In this case, specific $G_{q/11}$ protein and PLC inhibitory antibodies were used, which resulted in the intrinsic light response (iLR) being eliminated, whereas in the presence of the *Pertussis* toxin (specific G_o and G_i inhibitor), the responses were not affected. This way, the participation of the ciliary photoreceptors' G proteins in melanopsin-mediated phototransduction was ruled out. iLR was attributed to TRPC3 channel activation since, in the presence of a Ca^{2+} chelating agent and a TRPC inhibitor, the ion currents were eliminated[45]. Both studies provided the first evidences that melanopsin uses a signaling cascade based on the phosphoinositide system, similar to that used by rhabdomeric photoreceptors.

In spite of the robustness of these evidences, a study carried out by Kumbalasiri's group showed conflicting results (Table 1), since the use of lanthanum (La^{3+}) to inhibit the TRPCs failed to eliminate the iLR; neither did extracellular Ca^{2+} removal with a chelating agent alter intracellular Ca^{2+} flow, whereas the application of thapsigargin (specific inhibitor of the sarco/endoplasmic reticulum Ca^{2+} pumps), which inhibits $[Ca^{2+}]_i$ flow, eliminated the response. With these results, the authors ruled out at least the TRPC3 channel as responsible of the $[Ca^{2+}]_i$ increase in melanopsin-mediated phototransduction, and it was attributed to sarco/endoplasmic reticulum intracellular[47], a conclusion similar to that of Melyan et al. with the neuro-2a cells[43].

One of the most recent investigations in heterologous expression systems is the study conducted by Bayles and Lucas in 2013 (Table 1). The authors tried to determine if human melanopsin was able to activate proteins of the G_s, $G_{i/o}$ or G_q families in HEK293 cells. Using cyclic AMP and Ca^{2+} luminescent indicators, they measured the responses to light. In the assays they observed that the levels of the nucleotide were decreased as a result of luminous stimulation, thus indicating that melanopsin was able to interact with the $G_{i/o}$ protein, while the cyclic adenosine monophosphate (cAMP) levels were not increased, this way ruling out G_s protein activation. The authors measured the $[Ca^{2+}]_i$ increase in order to verify G_q protein activation. Transfected cells responded to luminous stimuli with an increase, indicating G_q protein activation in the human melanopsin signaling pathway in HEK293 cells[48].

Most results obtained in heterologous expression systems to elucidate the melanopsin phototransduction mechanism have arrived to the conclusion that the transduction pathway most probably involves a protein of the $G_q/_{11}$ type, which in turn activates the PLC effector enzyme, ending up with the activation of a TRPC, which allows Ca^{2+} intracellular flow and depolarizes the plasma membrane[44-46,48]. Although melanopsin has more structural and functional resemblance with invertebrates' rhodopsin than with those in vertebrates[3,11,12], which has suggested the previously described[49] phosphoinisides common signaling pathway[27], two studies in heterologous expression systems have demonstrated that melanopsin is able to activate the family of the G transducin ($G_i/_o$) proteins[42,48]. Without being conclusive, there are evidences to consider that melanopsin can share the phototransduction pathway of the rhabdomeric photoreceptors; the data that imply similarities with phototransduction in vertebrates maybe

are a reflection of the fact that GPCRs can promiscuously couple with one or different G-protein subtypes[50]. Furthermore, in vitro association and activation of a G-protein does not imply that the same will occur *in situ*.

Phototransduction studies in ipRGC primary cultures

The investigations to decipher the melanopsin phototransduction mechanism are not limited to studies in heterologous expression systems, but also comprise cell culture systems, studies in the retina and in enriched cultures of isolated ipRGC cells. The investigations conducted in the study of this topic are described next (Table 2).

The entire sequence of molecular events underlying phototransduction by melanopsin was originally described in *X. laevis*-isolated melanophores, where this opsin was precisely discovered. Simultaneous to the performance of several studies in heterologous expression systems by different groups (described in previous paragraphs), in 2005, the Provencio group, using *X. laevis* melanophore cultures (Table 2), measured the melanopsin iLR by the dispersion of melanosomes. By applying by applying PLC, Protein Kinase C (PKC) antagonists and the presence of a Ca^{2+} chelating agent, the dispersion of melanosomes light stimulation was eliminated. In addition, IP_3 levels increased more than 100% in response to light, in comparison with controls in the dark. These data were the first conclusive evidence of the phosphoinositides system participation in the melanopsin-mediated iLR in wild type cells, and the possibility of their presence in retinal ipRGCs was left open[51].

The first study with ipRGC isolated from non-mammalian vertebrate and maintained in culture was conducted by Contín et al. in 2006, who used primary cultures of ganglion cells obtained from chicken embryos by immunopanning with an antibody against Thy1 to measure the effect of light on melatonin synthesis. They demonstrated that this is a process dependent of ligth and that involves Ca^{2+} release from intracellular stores. In these cultures, the expression of mRNAs of the G_q protein, of melanopsin and of genes that are expressed in rhabdomeric photoreceptors *(Pax6* and *Brn3)* could be detected[52]. Thus was obtained the first evidence in retinal neurons that cells with melanopsin express the enzymes of the G_q/PLC transduction pathway.

Later, the same group[53] demonstrated the participation of the phosphoinositide (PIP) cycle in the phototransduction mechanism. By assessing the activity of different enzymes (DAGK, PIK and PIPK) involved in PIP_2 regeneration, they observed that ipRGCs activate the PIP cycle in response to light. They also described the participation of PLC, increases in the IP_3 levels and $[Ca^{2+}]_i$ mobilization[53].

Hartwick et al. (2007)[33] also applied the immunopanning technique, alternatively using an antibody against melanopsin and antibodies targeted against the Thy1 protein, to obtain rat ipRGC enriched cultures, where they looked for the source of $[Ca^{2+}]_i$ increase in the iLR. The authors proposed three possibilities: TRPC, endoplasmic reticulum and VGCC. When the ipRGCs were stimulated in the presence of different TRPC-antagonist including flufenamic acid (Table 2), the $[Ca^{2+}]_i$ increase was reduced, which yielded evidence of TRPCs participating in the phototransduction mechanism. The investigators were even able to detect TRPC3, 6 and 7 mRNAs; notably, they found that TRPC7 showed higher expression in cells immunopanned with the antimelanopsin antibody than in those immunopanned with the anti-Thy1 antibody. In order to confirm if the Ca^{2+} flow came from Ca^{2+} intracellular stores or from VGCCs, they used a Ca2+ free-medium, under which the responses were eliminated. The iLR persisted in the thapsigargin-treated ipRGCs, which ruled out the participation of endoplasmic reticulum stores. This data contrasts with the results obtained by Kumbalasiri et al[46], who found evidence for these stores participating in transfected cells. Finally, in these isolated ipRGCs, Hartwick et al.[33] demonstrated that 90% of the Ca^{2+} flow in the iLR was due to Na^+ and Ca^{2+} voltage-gated channels (VGC) sequential activation.

In another study conducted in ipRGCs isolated from rat retina, Graham et al. (2008)[54] found that these cells use the signalling cascade of the phosphoinositide system similar to the rhabdomeric opsins cascade of invertebrates. By recording the ion currents in complete cells or their membrane fractions, they found that, in response to light, ipRGCs activated G_q/11, which subsequently activated PLCb4. Furthermore, they confirmed the presence of these proteins by retrotranscription and immunofluorescense. In electrophysiological records, the light activated current persisted under treatment with IP_3 receptor antagonists, or with thapsigargin, and it could even be recorded in ipRGC membrane fragments (inside out and outside out patch clamp). The treatment with wortmannin, a drug that inhibits PIP_2 hydrolysis, was the only one that decreased the current, leading the authors to conclude that all molecules involved in the phototransduction

Table 2. Experiments of the intrinsic response to light in isolated cells and *in situ*

Objective	Study model	Method to measure the iLR	mRNA detection	Phosphoinositide system antagonists and/or G proteins	TRPC blockers and Ca^{2+} chelating agents	Detection by immunofluorescence	Results	Authors
Investigate if the phototransduction mechanism in ipRGCs is based on the phosphoinositide system	*X. laevis* melanophores dermal cultures	Melanosome dispersion	OPN4	U73122	BAPTA-AM	N/A	PLC activation, IP_3 production, $[Ca^{2+}]_i$ increase and PKC activation	Graham et al. (2008)
Determine if chicken RGC are intrinsicly photosensitive and the type of phototransduction mechanism	Enriched chicken RGC cultures	Radioactive melatonin synthesis	OPN4 and G_q	Neomicin and U73122	La^{3+}, BAPTA-AM	N/A	Phototransduction mediated by G_q, PLC and $[Ca^{2+}]_i$ increase by a TRP channel and/or Ca^{2+} release from intracellular storages	Contín et al. (2010)
Investigate the identity of the ion channel activated in RGC response to light and the intracellular signaling pathway leading to its activation	Rat (Sprague-Dawley) retina	Whole cell patch clamp	N/A	GTPgS and GDPbS	Ruthenium red, SK&F 96365, La^{3+} and gadolinium (Gd^{3+}), BAPTA	TRPC6	ipRGCs use a G protein to activate a TRPC, probably TRPC6	Pérez-Leight et al. (2011)
Investigate the three possible sources of $[Ca^{2+}]_i$ increase in iRL: TRPC, VGCC and intracellular storages	Rat (Long-Evans) ipRGC enriched cultures	Calcium imaging and perforated patch clamp	OPN4, TRPC 3, 6 and 7	N/A	2-APB, SK&F 96365, flufenamic acid, lanthanides, inhibitors of Ca^{2+} and Na^+ voltage-activated channels	Melanopsin	$[Ca^{2+}]_i$ increase is mainly mediated by VGCCs (90%) and to a lesser extent by TRPC7 (10%)	Xue et al. (2011)
Identify an antagonist that inhibits ipRGC iRL	Mouse (C3H) retina and strains mixture (129/sv and C57BL/6)	Calcium imaging	N/A	N/A	La^{3+}, 2-APB, SK&F 96365, flufenamic acid	TRPC6, 7	TRPC channels mediate conductance in ipRGCs. 2-APB is an ipRGC inhibitor in vitro and in vivo	Isoldi et al. (2005)

(Continues)

Table 2. Experiments of the intrinsic response to light in isolated cells and *in situ* (Continued)

Objective	Study model	Method to measure the iLR	mRNA detection	Phosphoinositide system antagonists and/or G proteins	TRPC blockers and Ca^{2+} chelating agents	Detection by immunofluo-rescence	Results	Authors
Investigate the melanopsin phototransduction mechanism	Rat (Sprague-Dawley) ipRGC enriched cultures	Whole cell patch clamp	OPN4, $G_{q/11}$ family (Ga14, Gaq, Ga11 and Ga15). PLCb family (PLCb1, 2, 3 and 4)	GDPbS, GPAnt-2a and U73122	BAPTA	Melanopsin and PLCb4 (in the retina)	Phototransduction in ipRGCs is based on the plasma membrane-associated phosphoinositide system	Contin et al. (2006)
Investigate the participation of the phosphoinositide cycle in the ipRGC iLR and whether it generates changes in IP_3 activity and in the kinase PIP	Chicken embryonic RGC enriched cultures	Calcium imaging	OPN4m and OPN4x	U73122	N/A	Melanopsin	ipRGCs activate the PLC, there are IP_3 increases, the PIP cycle is activated and there is $[Ca^{2+}]_i$ mobilization observed	Warren et al. (2006)
Identify if the TRPC 3, 6 or 7 channels are involved in the iLR of ipRGCs	Mutant mouse lines retina (C57BL/6 and 1297Sv) $TRPC3^{-/-}$, $TRPC6^{-/-}$ and $TRPC7^{-/-}$	MAE and whole cell patch clamp	TRPC3, 6 and 7	N/A	N/A	Melanopsin	Depolarization in ipRGC is not mediated by homomeric TRPC3, 6 or 7 channels. TRPC6 can be implied forming a heteromeric channel	Hartwick et al. (2007)
Investigate if mammals iris is intrinsicly photosensitive and if PLR are local	Mouse double mutant lines retina (TRPC6–/–TRPC7–/–)	Perforated patch clamp	OPN4 (iris and retina)	N/A	N/A	Melanopsin	TRPC3, 6 ot 7 as homomeric channels are not implicated in iLR. TRPC6 and 7 can be involved in iLR as heteromeric channels	Sekaran et al. (2007)

mRNA: messenger RNA; U73122: 1-(6-((17b-3-methoxyestra 1, 3, 5(10)-trien-17-yl)amino)hexyl-1H-pyrrole-2,5-dione; BAPTA-AM: [1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (acetoxymethyl ester)]; GTPgS: guanosine 5'-O-(3-thiotriphosphate); GDPbS: guanosine 5'-O-(2-thio-diphosphate); BAPTA: 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid; SK&F 96365: 1-[2-(4-methoxyphenil)-2-[3-(4-methoxyphenil)propoxy]ethyl]imidazole, 1[β-(3-(4-methoxyphenyl)propoxyl)-4-methoxyphenil]-1H-imidazole hydrochloride; GP Ant-2a: pGlu-Gln-D-Trp-Met-NH_2.

mechanism are inserted in the plasma membrane or are strongly associated with it[54].

Phototransduction studies in retinal preparations

The heterologous expression systems provide some clues on the interaction of molecules in melanopsin-mediated phototransduction; the studies in isolated ipRGCs corroborate the findings and make them extensive to naturally occurring systems, but the recording from ipRGCs *in situ* provides the data in the setting where these photoreceptors act, weighing the phototransduction mechanism even under interaction with other cell types.

Berson et al. initiated the study of ipRGCs in the retina and managed to establish the nature of these cells as photoreceptors by demonstrating the change of voltage and membrane current underlying the iLR, but didn't elaborate much into the melanopsin phototransduction mechanism. This kind of experiments is largely limited by the difficulty to distinguish the low numbers of ipRGCs among the ganglion cell population, since these have no distinctive morphological features. Some researchers have resorted to the breed of transgenic mice where the OPN4 promoter is fusioned with the coding region of a fluorescent protein gene. A more refined strategy has been to use the combinase response element (CRE) recombinase to induce the precise expression of these genetic constructs. In either case, ipRGCs have one copy less of melanopsin, the effect of which is still impossible to assess in comparison with wild type animal cells. In some cases, expression of reporter proteins is found in cells where melanopsin cannot be detected by immunohistology, which should question whether ipRGC cells are actually being recorded[32].

The alternative to the use of transgenic animals is the labeling of ipRGCs by injection and retrograde transport from the SCN, with large technical limitations and restricted access to significant numbers of cells, but with no doubt this remains the safest method to unequivocally record ipRGCs. The data that are discussed below come from works where either method was adopted.

In 2006, Warren et al.[55] investigated which molecules and what type of ion channel could be involved in the ipRGC phototransduction mechanism of the rat retina. They considered two types of channels as candidates: CNG and TRP. Using a retrograde tracer, whole-cell patch-clamp recording and immunohistochemistry, they were able to demonstrate that the iLR of ipRGC *in situ* depends on G-proteins, but other than G_t (Table 2). CNG channels specific inhibitors did not affect the light-activated current (LAC). Additionally, in the immunohistochemistry analysis, these channels were not identified in the RGCs, not even in those expressing melanopsin. With regard to TRPCs, when specific inhibitors were used (Table 2), light-activated current (LAC) was observed to be partially or completely inhibited, which supports the hypothesis that this response in ipRGCs is mediated by TRPCs. The immunocytochemical experiments revealed the presence of TRPC6 in ganglion cells, including ipRGCs. Thus, the authors concluded for the first time that ipRGCs *in situ* respond to light by a G-protein-dependent process and that TRPC6 may be the ion channel that causes the cell response[56].

In mice lacking functional cones and rods, using the calcium imaging technique, Sekaran et al.[56] measured the iLR in ipRGC and assessed the effect of different drugs (Table 2). 2-aminoethoxydiphenyl borate (2-APB), previously reported as a TRPC7 antagonist, was the most effective in iLR inhibition. 2-APB inhibitory effect in photosensitive ganglion cells was tested in vivo by measuring the PLRs, and when significant attenuation was observed on it, the action of the drug on ipRGCs was corroborated. In addition to increasing the evidence on TRPCs as the conductance pathway of LAC, the authors were pioneers by manipulating the ipRGCs activity and obtaining a behavioral effect[55].

As in other fields of neuroscience, mutant mice strains lacking some gene have been widely used to define the phototransduction mechanism of melanopsin. Perez-Leighton et al. worked with mice lacking channels TRPC3, 6 and 7 in order to determine the light-activated current pathway in ipRGCs (Table 2). By means of simultaneous multi-electrode recording array with extracellular electrodes (MAE) and whole-cell patch-clamp, they measured the iLR in the retina. In the MAE recordings, they found that ipRGCs of all mutant strains in perinatal stage (P6-8) maintained their response in a similar manner than the wild type. In the electrophysiological recordings of adult mutants (ages P22-P-50), of the $TRPC3^{-/-}$ and $TRPC7^{-/-}$ strains, similar responses to those in wild type animals have been found, but iLR attenuation was detected in $TRPC6^{-/-}$ mutants. As an explanation of their findings, these investigators concluded that both types, TRPC5 and TRPC7, could be the final effector in the melanopsin-mediated signaling pathway, but by forming heteromeric channels[57]. That same year (2010), Yau's group

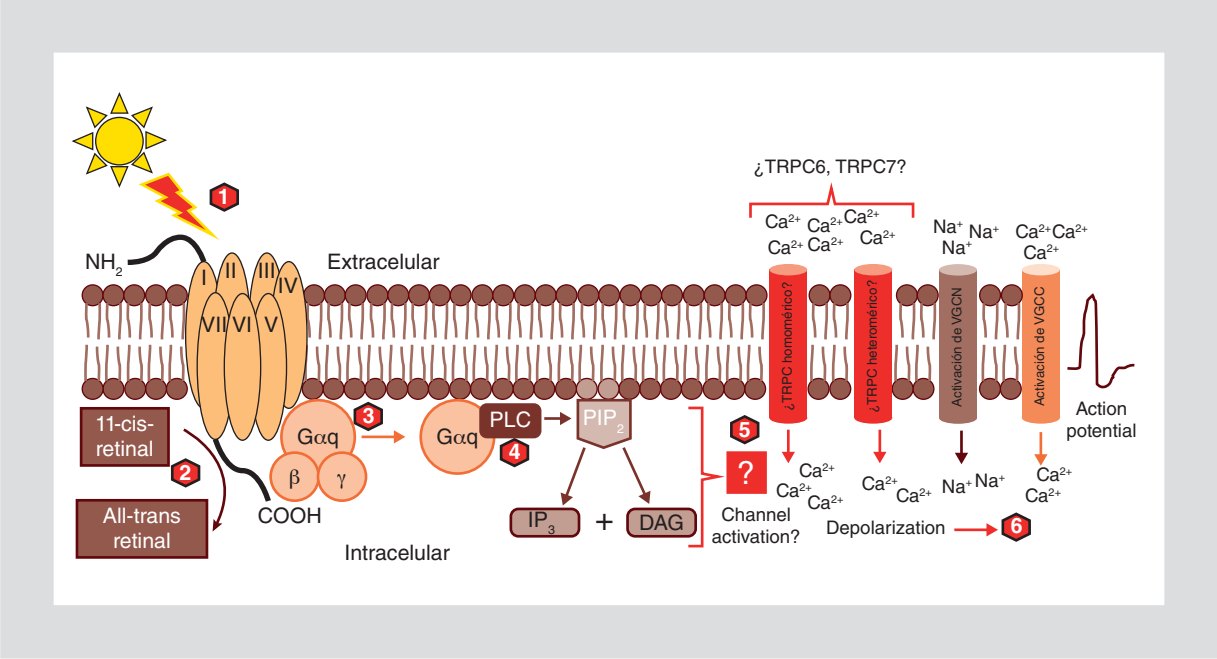

Figure 1. *Phototransduction mechanism in ipRGCs.* ***1:*** *a photon acts upon melanopsin, which is a photopigment of the GPCR superfamily.* ***2:*** *the chromophore 11-cis-retinal is isomerized into all-trans-retinal.* ***3:*** *the melanopsin-associated G protein is a* G_q. ***4:*** *its stimulation activates the PLC, which in turn hydrolyzes* PIP_2 *and DAG and* IP_3 *are produced.* ***5:*** *this system has been proposed to lead to the activation of a TRPC6 or TRPC7 channel, or both, which are the final effectors of the mechanism and cause depolarization of the cell.* ***6:*** *depolarization is amplified by* Na^+ *and* Ca^{2+} *voltage-sensitive channels, generating an action potential. Evidences for this mechanism are discussed in the text.* NH_2 *and COOH: indicate melanopsin amine and carboxyl extremes, respectively.*

arrived to similar conclusions[58] when they used TRPC6/7$^{-/-}$ double mutants, with an additional contribution: they found that the iris has also melanopsin-containing cells that work as photoreceptors responsible for PLR, and that in this species (the mouse and perhaps rodents in general) PLR does not require projection to the encephalon; but the iLR in the iris is not mediated by any of the TRPs so far described. In addition, this group provided conclusive evidence that PLCb4 is effector of iLR, but the identity of the G-protein could not be determined.

A more recent work suggests that the M-type transient potential channels (TRPM1) may also participate in melanopsin-mediated phototransduction in ipRGCs[59]. Hughes et al. demonstrated that mutant mice lacking this channel do not exhibit PLR. By β-galactosidase expression under the TRPM1 promoter, these researchers demonstrated that the channel is expressed in ganglion cells with melanopsin.

So far, the evidence suggests that the final effector in melanopsin-mediated phototransduction is a channel of the TRP superfamily, which, depending on the ipRGC subtype, can be a homo- or heteromeric complex, even composed by different subclasses of these channels (Fig. 2).

Therapeutic aspects of OPN4

In mammals the ipRGCs project directly to the SCN, the biological synchronizer anatomical site. Their exclusivity in the composition of the retinohypothalamic tract has already been established[31], and thus they form the afference of both cones and rods phototransduction and melanopsin iLR; this way, even when cones and rods and melanopsin phototransduction are complementary for SCN photosynchronization, only the ipRGCs conduct the nerve impulse required for such regulation. It is then understandable that a large number of investigations have focused on the relationship of melanopsin and ipRGCs in behavioral disorders of the circadian rhythm.

Different behavioral studies in transgenic mice have demonstrated that melanopsin-mediated phototransduction is necessary for photosynchronization of the circadian rhythm and for sleep induction by light stimuli[19].

ipRGCs also conduct the nerve impulse responsible for behavioral disturbances induced by exposure to light. In mice lacking these cells, Hattar's group[18], using a model that induced depressive states and learning disturbance by exposure to light, demonstrated that

its effects are lost when transmission by ipRGC cells is lacking.

In humans, the participation of melanopsin has already been demonstrated in different behavioral disorders; for example, the P10L point mutation, which is able to interfere with the correct distribution of melanopsin on the plasma membrane, has been found to occur only in patients with seasonal affective disorder[61], a condition where depressive states are experienced in autumm and winter, when days are shorter.

Psychophysics experiments in humans have concluded that the light within the wave length range that stimulates the ipRGCs (480-490 nm) has the most beneficial influence on the cognitive function, on mood, on synchronization for changes in time zones and on the relief of migraine and other conditions associated with light stimulus stimulation[62,63].

Investigations have been initiated aimed at the development of molecules that selectively alter phototransduction by melanopsin, without affecting that of cones and rods. Jones et al. identified a group of sulfonamide compounds, molecular analogues to retinol, which inhibit melanopsin photoactivation without affecting cones and rods phototransduction. By applying these molecules on mice, undergoing photophobia assays, melanopsin-mediated response could be inhibited. This opens the possibility to pharmacologically manipulate the melanopsin function in order to treat photophobia-producing conditions in humans, such as migraine[64].

Conclusions

ipRGCs clearly play a major role in extravisual physiological responses, i.e., in the responses to light as an environmental factor that regulates endocrine, behavioral and mood activities of living organisms; therefore, fully understanding the phototransduction process by melanopsin has exceeded the interest of neurobiology to enter into the fields of therapeutics and, in general, of advantageous use of light energy in all aspects of modern life.

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