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Revisión

Melatonin Makes Me Feel Awake! Seven Years of Lab Experience (2000-2007)

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ABSTRACT

The research on the effect of melatonin on intraocular pressure (IOP) is reviewed from the hystorical point of view of our laboratory. The original idea of melatonin modulating intraocular pressure has been improved by using selective compounds for MT_2 and specially melatonin MT_3 receptors. The selective compound 5-methoxyamino N-acetyltryptamine (5-MCA-NAT) has been an attractive compound due to its ability to reduce IOP about 40%, therefore being a good candidate to the treatment of the ocular hypertension linked to glaucoma. More compounds have been developed and tested permitting us to have a more accurate panorama of those receptors controlling the relevant process of intraocular pressure.

Key words: Melatonin, melatonin receptors, intraocular pressure, MT3 receptor.

RESUMEN

¡La melatonina me hace sentir despierto! Siete años de experiencia en el laboratorio (2000 – 2007)

La investigación sobre el efecto de la melatonina sobre la presión intraocular (PIO) es revisada desde la perspectiva histórica de nuestro laboratorio. La idea

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original sobre la melatonina modulando la presión intraocular ha sido mejorada gracias al empleo de nuevos compuestos selectivos para el receptor MT_2 y especialmente para el MT_3. El compuesto selectivo 5-metoxiamino N-acetil-triptamina (5-MCA-NAT) se ha convertido en un compuesto muy atractivo debido a su capacidad de reducir la PIO hasta un 50%, convirtiéndose en un compuesto muy interesante para el tratamiento de la hipertensión ocular asociada al glaucoma. Se han desarrollado y ensayado más compuestos que nos han permitido tener un panorama más preciso acerca de como estos receptores controlan el proceso fisiológico de la presión intraocular.

Palabras clave: Melatonina, presión intraocular, receptores de melatonina, receptor MT3.

MY HISTORICAL PERSPECTIVE

In May 1999, when I was flying back to Spain from an Ophthalmology meeting in Florida (USA) I started to talk with a colleague about those persons who take *melatonin* to avoid *jet-lag* in long distance trips. Some people comment that this neurohormone helps to recover the light/night cycles faster than when you allow your body to do it without any help.

When I was back in the lab, I had a look to melatonin in the internet looking for some references regarding jet-lag, and I was surprised when I saw a graph in which the concentrations of melatonin along 24 hours was presented. That plot showed that this indole presented higher concentrations in the night and lower during the day. There was something funny about this plot: it was just opposite to one I saw in one of the most popular Ocular Physiology books (Adler's Physiology of the Eye) in which intraocular pressure (IOP) was analysed along 24 hours. If you overlap both graphs you may see that when melatonin was higher IOP was lower and vice versa (Figure 1). Did that mean that melatonin levels modify IOP?

At that moment we were working on the effect of nucleotides on IOP in New Zealand rabbits (1), so it would not be difficult to check whether or not melatonin was able to change IOP. We performed that single experiment and indeed melatonin significantly reduced IOP, but its effect was transitory lasting no more than one hour. This result was interesting and in some way indebted me to carry on working on this topic, but not immediately since I was involved in

the studies regarding nucleotides and dinucleotides as modulators of IOP I wanted to finish.

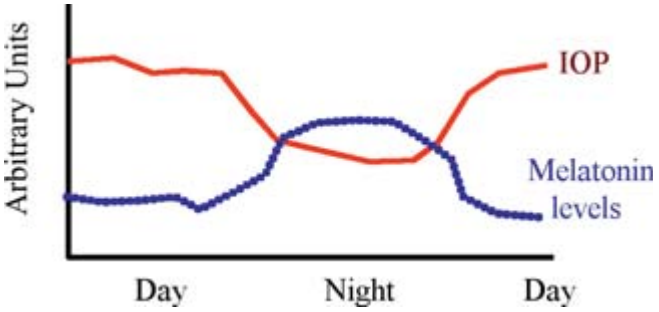

FIGURE 1. ***Comparison between the concentration of melatonin and the changes in IOP along day and night.***

This delay was positive since in the months that passed from the moment I did the experiments with melatonin to the one I started to continue it, January 2000, a paper appeared describing some new compounds which selectively activate the three different subtypes of melatonin receptors, MT_1, MT_2 and MT_3 (2) (Figure 2). Basically my idea was to repeat that melatonin experiment and then to assay different agonists and antagonists of the melatonin receptors in order to pharmacologically characterise the receptor reducing IOP in rabbits.

So, after getting a positive hypotensive effect with melatonin, the next decision was to choose which one was going to be the following compound to be assayed. At that moment I had bought a battery of compounds so it was at the beginning a bit difficult to decide how to start. I thought that assaying the newest compounds would provide an attractive starting point if, of course, they work. The newest substance was 5-methoxyamino N-acetyltryptamine (5-MCA-NAT) and this was the first I assayed. When the experiments were performed with this compound the results were amazing: IOP was reduced almost 40 % and the duration of the effect was longer than 8 hours, just with a single dose of 100 μM 5-MCA-NAT. Compared to melatonin, this agonist was much better than the naturally occurring substance (Figure 3). Moreover, 5-MCA-NAT was a selective melatonin MT_3 receptor (3).

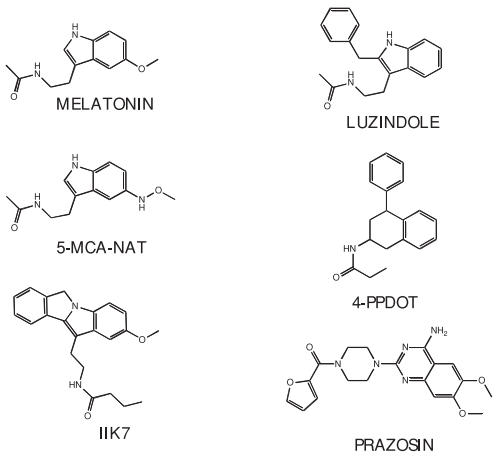

FIGURE 2. ***Chemical structure of agonists and antagonists of melatonin receptors.*** *Melatonin is the naturally occurring agonist of melatonin receptors, while 5-MCA-NAT and IIK7 are MT_3 and MT_2 selective agonists. On the other hand antagonists are represented by luzindole (non selective), 4-PPDOT (MT_2) and Prazosin (MT_3 and α1-adrenergic).*

A review of the existing literature in the field brought some interesting papers but none of them reported the existence of melatonin MT_3 receptors modulating intraocular pressure. In that moment, melatonin and its receptors became an important part of the research developed in the laboratory.

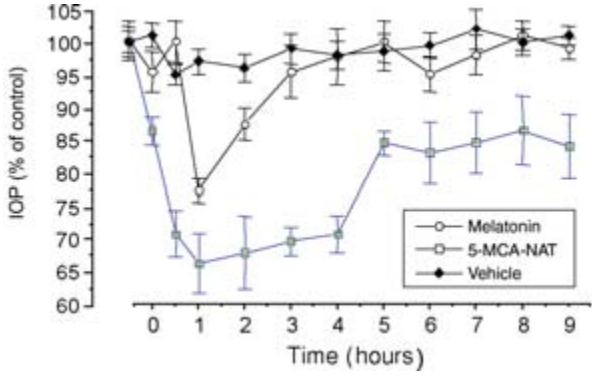

FIGURE 3. ***Changes in IOP produced by melatonin and 5-MCA-NAT.*** *The selective MT_3 agonist presented a more profound and long lasting effect when compared to melatonin.*

LOOKING FOR MELATONIN ANALOGUES FOR THE TREATMENT OF OCULAR HYPERTENSION AND GLAUCOMA

Working on the MT_3 melatonin receptor

Most of the pharmacological approaches for the treatment of ocular hypertension and glaucoma lie in the reduction of IOP that is, in general, abnormally elevated in these patients. In this sense all the approaches reduce IOP by acting on different targets (tissues) that are relevant for the correct physiology of the aqueous humour dynamics. These compounds, independently of their chemical nature can be classified into three classes based on the mechanism of action. Class I drugs act by reducing the production of the aqueous humour and these include carbonic anhydrase inhibitors and beta-blockers, among others. Class II drugs improve trabecular meshwork outflow and these include cholinergics and epinephrines. Class III drugs work by enhancing the uveoscleral outflow being the most representative the prostaglandins (4).

Can we add a new group of compounds to the existing list of pharmaceutical compounds for the treatment of glaucoma? It is a bit soon to provide an answer to this question, nevertheless progress has been carried out to, at least to suggest *melatonins* as candidates for the treatment of this pathology (5).

Our interest in that moment was to fully characterise the effect of melatonin and 5-MCA-NAT in New Zealand white rabbits. Therefore, single dosage, dose-response studies and preliminary antagonist studies permitted us to publish our first paper in the field, paper which was cited as one of the breakthrough in the glaucoma literature in 2001 (3). This experimental work permitted not only to introduce *melatonins* as possible new drugs for the treatment of glaucoma, but also allowed us to fill a patent which open us some gates that permitted us to grow as a competitive laboratory.

Both paper and patent were an excellent starting point to «make business» with pharmaceutical companies, due to their interests in finding new approaches to the treatment of glaucoma. Nevertheless there were lots of things to do in order to clarify what receptors for

melatonin are in the eye and how they can modulate IOP. So, next was to fully characterise the changes in IOP by using all the available melatonin compounds. Studies with agonists and antagonists depicted a clear panorama: 5-MCA-NAT was the most potent and effective compound reducing IOP, but not the only one, since all the tested agonist presented effects, but no so profound to that produced by 5-MCA-NAT. This was suggesting a major effect through MT_3 receptors but never discarded the presence of the other two (MT_1 and MT_2). Antagonist demonstrated a predominant effect mediated by the MT_3, but again they did not discard other melatonin receptors. This work was developed during 2001-2003 and was published finally in 2003 (6). Another interesting result that was obtained was the fact of blocking melatonin effect by means of cholinergic and adrenergic receptor antagonists. These aspects revealed the close relation between melatonin receptors and the neural components that control both the production and the drainage of the aqueous humour. We will come back to this point later.

Designing and synthesising analogues for the MT_3 receptor

In the meantime we were carrying out the experiments previously reported, and at the end of 2000, we started a research project with the company INSPIRE PHARMACEUTICALS in which they improved the pharmacological properties of these compounds by modifying the chemical structure of the indole ring. During the following couple of years we tested a big number of melatonin analogues that provided interesting information on the structure-function of the different melatonin structures regarding IOP changes in our experimental model. Among the 50 or so tested analogues, 3 were particularly interesting since they reduced IOP to values closer to that obtained with 5-MCA-NAT. Together with INSPIRE we filled a US patent as well as a PCT one (worldwide). Also, INSPIRE and other researchers were testing whether 5-MCA-NAT was useful in a different model: The glaucomatous monkey. The group of Dr. Janet Serle in Mount Sinai Hospital demonstrated that the topical application of 5-MCA-NAT during two consecutive weeks significantly reduced IOP in glaucomatous monkeys (7). These fantastic results animated us to continue investigating with the analogues

provided by INSPIRE. Therefore, dose-response analysis and anatagonism experiments were performed with the best three synthetic compounds. Dose-response analysis was OK, as expected, but surprisingly antagonism analysis demonstrated that at least two of the three tested compounds were acting through MT_2 receptors rather than on MT_3 as expected. Another couple of questions arise: Do we have more than the MT_3 melatonin receptor controlling IOP? Is there a MT_2 as well?

MT_2 receptors and IOP

It was quite evident by our previous results that a MT_2 melatonin receptor may exist and that this receptor would control IOP together with the MT_3 melatonin receptor. So, acting in a similar way as we proceeded with 5-MCA-NAT we looked for a selective MT_2 agonist, N-butanoyl-2-(2-methoxy-6H-isoindolo [2,1-α]indol-11-yl) ethanamine, abbreviated IIK7. The application of this compound to the experimental animals reduced IOP, and this reduction was avoided by the MT_2 antagonists 4-PPDOT and DH-97.

The discovery of a receptor for melatonin that belongs to the MT_2 subtype permitted us to expand our knowledge on the melatonin physiology in the eye since this receptor has been cloned and there are antibodies raised against it. In this sense out studies using MT_2 receptor antibodies permitted to localise this receptor in the ciliary processes, which is the place where the aqueous humour is synthesised (Figure 4). So combining the fact that melatonin and IIK7 reduce IOP and that the receptors are located in the ciliary body it seems clear that MT_2 receptors should reduce the production of the aqueous humour and that reduction may be the reason for a decrease in IOP. This makes sense since MT_2 receptors are negatively coupled to adenylate cyclase. Beta-adrenergic receptors are typically antagonised in the pharmaceutical treatments for glaucoma, and they are positively coupled to adenylate cyclase. Altogether the topical application of IIK7 or melatonin finally inhibits PKA with the concomitant fall in the IOP, which at the end is the same ultimate effect beta-receptor antagonists produce.

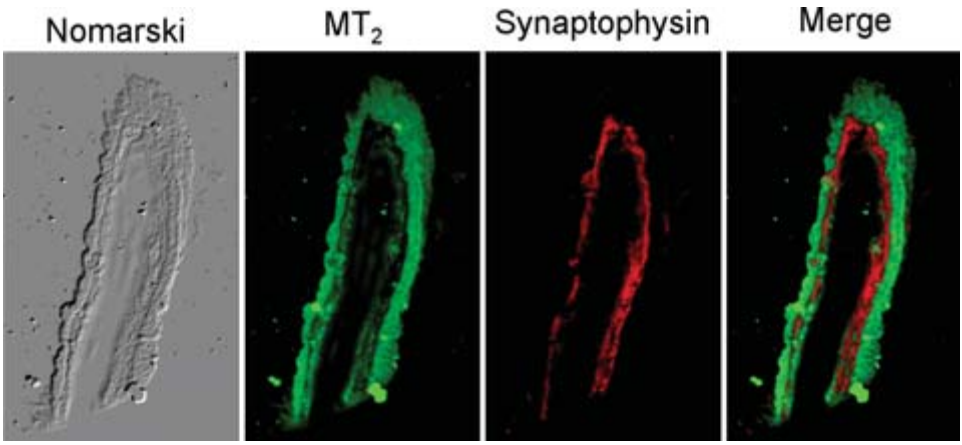

FIGURE 4. ***Immunohystochemical localization of MT_2 melatonin receptors.*** *Four different views are presented in the present figure, Nomarski interferential contrast, MT_2 labelling in green, nervous system (synaptophysin) and a combination MT_2/synaptophysin (merge).*

Melatonin receptors and the nervous system

In our preliminary studies about the changes in IOP by melatonins, we were able to demonstrated that the neural components that naturally modulate the dynamics of the aqueous humour. Apparently both the sympathetic, the parasympathetic components and melatonin (its receptors) are connected since the use of cholinergic and adrenergic receptor antagonists diminished the hypotensive effect of melatonin and 5-MCA-NAT.

Very recently we have demonstrated the importance of the sympathetic component on the effects of melatonin and 5-MCA-NAT. When New Zealand rabbits were treated with reserpine or 6-hydroxydopamine (6-OHDA), the effects almost completely disappeared (Figure 5) (8). We still do not know how these chemical denervation abolishes the effect of indoles. Two main hypothesis can be drawn, either MT_3 melatonin receptors are located in the noradrenergic terminals innervating the cilliary processes or melatonin receptors need the presence of noradrenaline to be fully functional. In the latter, noradrenaline by acting on beta receptors may modulate melatonin receptors by phosharylation.

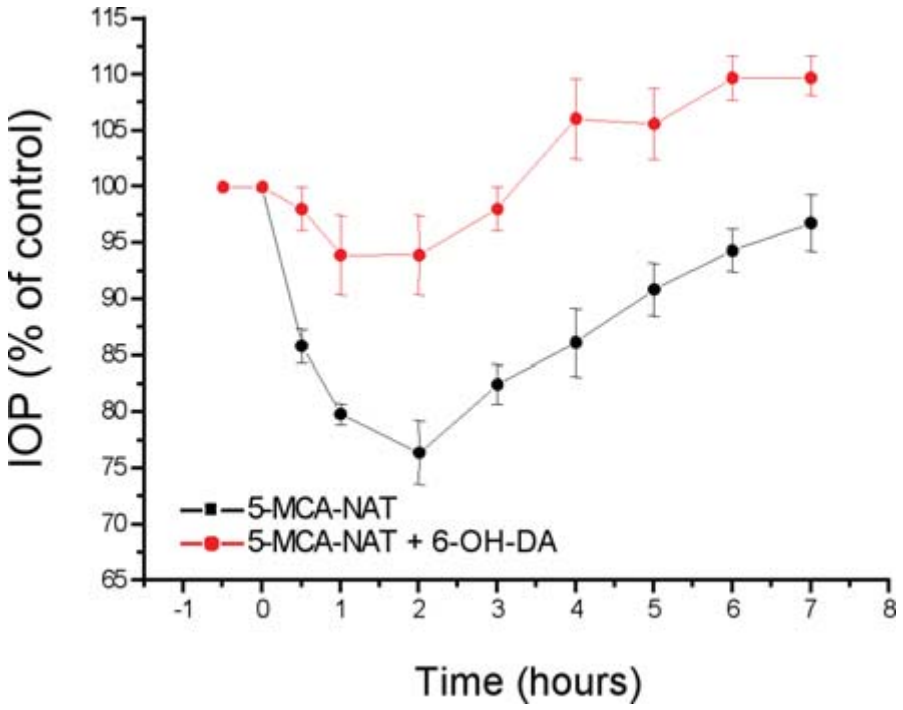

FIGURE 5. ***Effect of the chemical denervation after treatment with 6-OH dopamine.*** *The application of this chemical destroys noradrenergic terminals and this is reflected in a clear reduction in the effect of 5-MCA-NAT.*

MELATONIN ON THE OCULAR SURFACE

Melatonin and its role in corneal wound healing

Corneal wound healing experiments we performed in New Zealand white rabbits, demonstrated that after a superficial wound, melatonin at low micromolar concentrations, significantly accelerate the rate of healing closing the wounds faster than in the absence of this neurohormone (9). This effect is antagonised by luzindole, this is suggesting the presence of MT_1 or MT_2 melatonin receptors. We jumped to a primary rabbit corneal epithelial cell culture in order to perform more detailed biochemical studies. In the in vitro model the same behaviour carried out by melatonin and luzindole was

observed. It has been possible to demonstrate that melatonin effect is to increase the rate of cell migration rather than mitosis. Moreover, immunocytochemical staining of these cells demonstrate the presence of MT_2 melatonin receptors (5). So, till date both MT_1 and MT_2 receptors are present in the corneal epithelium but it is not still clear if both contribute to acelarate the rate of epithelial cell migration or if they combine sequencially: one to facilitate cell migration and other triggering mitosis to complete corneal re-epithelialisation.

Melatonin and diadenosine polyphosphates two good teammates

Very recently, it has been possible to investigate the role of melatonin on the ocular surface and in particular on tear secretion. Melatonin alone, topically applied to New Zealand rabbits, inhibits tear production. This is an interesting aspect since melatonin is elevated in the night and the rate of tear production during the nigh, specially when sleeping is significantly reduced. So, it seems that the general scheme fits well, when less tear is necessary melatonin is increased therefore reducing tear production.

On the other hand the dinucleotide diadenosine tetraphoshate, Ap_4A, enhances tear production. Surprisingly, when melatonin and Ap_4A are both applied simultaneously, the amount of tear production is enhanced 34 %, by means of a mechanism which is sensitive to luzindole, therefore melatonin receptor dependent (10). The cross-talk between melatonin and purinergic receptors may be the reason for such an increase in tear production, although the detailed mechanism is still under study.

OTHER RELEVANT ASPECTS OF MELATONIN IN THE EYE

The controversy: MT_3 or QR2?

Some authors discuss on the possibility that the MT_3 receptor is not an independent melatonin receptor and it is the enzyme quinone reductase 2. Uniquely, the MT_3 has been identified as quinone

reductase 2 (QR2), an enzyme involve in detoxification in mouse (11). QR2 seems to have antioxidants properties although is difficult to establish a clear role in detoxification on the basis of the currently available experiments. Furthermore, many tissues deprived of QR2 genes lack melatonin binding sites. These data indicate that in many cases is indeed QR2 the MT_3 melatonin receptor (12). Nevertheless, there are other cases in which it has not been possible to match the presence of QR2 and the putative MT_3 melatonin receptor (11). Moreover unexpectedly, the binding of melatonin and derivatives to QR2 does not inhibit the activity of the enzyme when the natural substrate is present (menadione), although it occupies the enzyme active site (12). The notion that arises is that binding approaches for melatonin and derivatives on the QR2/MT_3 receptor is not enough to discard the existence of a real receptor with a clear-cut physiological action. Moreover, in both mammals and non-mammals there is a correlation between the pharmacological profile of the putative MT_3 receptor and the generation of IP_3 and diacylglycerol (13). Nevertheless, it is necessary investigate the possibility of cloning a different protein from QR2 that may match with the putative MT_3 receptor to fully dissipate this controversial point.

Cloning the MT_3 receptor

This is indeed one of our main tasks at the moment. Based on the sequences of QR2, MT_1 and MT_2 receptors, we are trying to hunt the putative MT_3 receptor. We have started to dive in ocular tissues and we have cloned a DNA sequence which presents the sequence of the MT_2 melatonin receptor but which has an insert of 90 pb suggesting it is a new specie. It is clear that it can be either a splice variant or the MT_3 receptor. The insert is not only increasing the length of the protein encoded by the gene but also is altering the reading frame that probably will generate a different protein from the cloned MT_2. This interesting discovery is more significant because the ocular tissue where it has been obtained, the cornea, lacks of the QR2 gene. We need to conclude this research since either we have the MT_3 receptor or we have a MT_2 receptor (splicing variant) with a different selectivity for *melatonins,* in particular for 5-MCA-NAT.

CONCLUSIONS

There is still a long way until we fully understand the relevance of melatonin in all the ocular structures. We hope that with our modest contribution along the recent years, we may established the foundations for the development of new pharmacological treatments for the ocular hypertension associated to glaucoma. A good review on melatonin in the eye, including our findings on IOP, has been recently published (14). The introduction of new compounds for the treatment of glaucoma is especially relevant if we take into account the various side effects that happen with some of the commercially available compounds. Additionally we wish to get deeper into the molecular mechanism underlying all these physiological effects, including the cloning of the MT_3 receptor.

ADDENDUM

Melatonin receptors overview

The actions of melatonin in tissues are carried out by several receptors. The melatonin receptors are referred to by the letters MT. «MT» represents melatonin receptors with a well defined functional pharmacology in a native tissue, as well as known molecular structure, followed by a number subscript (15).

Three mammalian melatonin receptors have been cloned so far: MT_1 (16, 17), MT_2 (17), and MT_3 (18). Two first are classified as unique subtypes based on their molecular structure and chromosomal localization, and the third have been affinity-purified from Syrian hamster kidney (18).

It is particularly interesting to note that non-mammalian melatonin receptors have been deeply investigated. We are aware that mammalian melatonin receptors are indeed the ones reported and classified by the IUPHAR, nonetheless many works originally were developed in non-mammalian species providing clues for the search of the corresponding orthologues in mammalian tissues.

Non-mammalian melatonin receptors are classified depending on their pharmacology and kinetics. Therefore, the ML_1 melatonin

receptor is defined as a high-affinity (picomolar) site and a ML_2 as a low-affinity, (nanomolar) site (15). Originally, the nomenclature ML_1 / ML_2 was the one used for both mammals and non-mammals before the receptors were cloned. It is currently used in non-mammalian experimental models, for that reason we consider of interest to illustrate them.

ML_1 includes the non-mammalian melatonin receptor isoforms Mel1a, Mel1b and Mel1c. The Mel1a receptor sequence is homologous to the mammalian MT_1 receptor while the Mel1b is equivalent to the mammalian MT_2 receptor. There is another subtype without a corresponding orthologue in mammals termed Mel1c.

Mammalian melatonin receptors types MT_1 and MT_2 belong to class A rhodopsin-like $GPCR_s$ and present seven transmembrane hydrophobic helices. MT_1 and MT_2 melatonin receptors are formed from 350 and 362 aminoacids, respectively, with molecular weights of 39-40 kDa (19). These two melatonin receptors are encoded by genes located in different chromosomes (20). The gene for the MT_1 receptor is located in position 4q35-1 (21) and the gene for the MT_2 receptor in 11q21-22 (20). Both share approximately 60% homology with each another (17). The existence of three extracellular loops alternating with three intracellular loops to link the seven transmembrane regions, indicate the presence of potential sites for phosphorylation and glycosylation. The MT_1 melatonin receptor has two potential glycosylation sites in the N-terminal region (19), and it may exist in more than one glycosylated form (17), while MT_2 has one potential glycosylation site in the N-terminal region (17, 19).

The signal transduction system associated to the activation of MT_1 or MT_2 in target cells results in the inhibition of adenylate cyclase activity (Figure 6) (22). Activation of these receptors inhibits forskolin-induced cAMP formation with a subsequent decrease in activated protein kinase A (23). This is the common rule in the biochemical pathways for these two receptors, but it is not the only mechanism in signal transduction they can trigger. Depending on the location, organ and species, melatonin by acting on the same receptor can activate different second messenger cascades. This fact provides a rich variability of mechanism that are probably related to the physiological processes that are biochemically modulated.

Concerning the MT_3 melatonin receptor, some authors have indicated that after receptor stimulation the intracellular concentration of IP_3 and diacylglycerol in increased in hamster melanoma cells and in *Xenopus* melanophores (13).

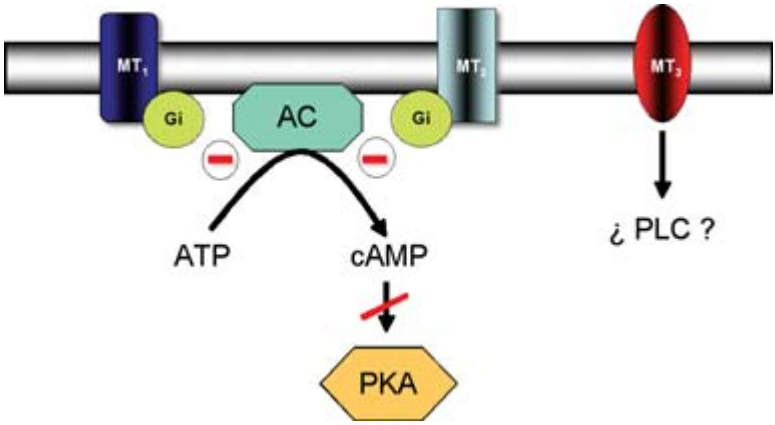

FIGURE 6. ***Schematic diagram showing the main melatonin receptors and the second messengers system to which they are coupled.***

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