Research Article

Specificity and nanomolar potency of melatonin on G-protein coupled melatonin MT_1 and MT_2 receptors expressed in HEK-293T human embryo kidney cells

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ABSTRACT

This is a pre-registered study, i.e. a study whose hypotheses and experiments designed to address these hypotheses has been deposited in a database before starting the experiments. The study aims at assessing the G_s versus G_i coupling and the potency of melatonin in the human version of melatonin MT_1 and MT_2 G-protein-coupled receptors expressed in HEK-293T cells. The results show that these receptors are G_i but not G_s coupled. By using a standard procedure of modulation of 0.5 µM forskolin-induced cAMP levels, it was found that the potency of MT_2 receptor-mediated actions is in the low nanomolar range, but the potency of MT_1 is in the high nanomolar range. The potency of melatonin to stimulate the MT_2 receptor is similar to that of a selective agonist, N-[2-(2-methoxy-6H-isoindolo[2,1-a]indol-11-yl)ethyl]butanamide (IIK7). Overall, the new data on the potency of melatonin on its receptors will provide a new look for melatonin research. It is important to consider this finding for appropriately addressing physiological or therapeutical effects based on melatonin potency. Thus, the low doses of melatonin used in the existing prolonged release preparations or in other supplements should be revisited.

Key words: Melatonin receptor, melatonin, sleep, cAMP, signal transduction, binding, pharmacokinetics.

1. INTRODUCTION

Aiming at finding substances to treat vitiligo, a skin disease characterized by the occurrence of depigmentation areas, Lerner and Case reported, in 1959, that a substance produced by the mammalian pineal gland, caused the aggregation of melanin near the nucleus of the amphibian melanocytes (1). Later, they identified the active molecule, named it (melatonin) and further deciphered its chemical structure: N-acetyl-5-methoxytryptamine (2). Since then many functions of melatonin have been discovered. At present, melatonin is very popular and is recommended as a supplement for a variety of uses, the most common being sleep regulation (3-9); it is even available via Amazon. In Europe, it is the active component of a medicine, Circadin^{R}, consisting of a pharmaceutical prolonged release preparation, prescribed for sleep disturbances, that contains 2 mg of melatonin (10). However, no melatonin-based medicine has been approved by the US Food and Drug Administration (FDA), which has instead approved a non-selective melatonin receptor agonist, ramelteon (sold as Rozerem^{R}) (11, 12). It is accepted that melatonin provides benefits via its putative antioxidant action or via activation of specific melatonin receptors. MT_1 and MT_2 are the two primary melatonin membrane receptors, which belong to the superfamily of G-protein-coupled receptors (GPCRs). In both receptors, the cognate heterotrimeric protein is $G_{i.}$ The biological consequence of G_i activation is inhibition of adenylate cyclase, reduction of cytosolic cAMP levels and inhibition of protein kinase A signaling pathway (13). Interestingly, other signaling pathways have also been assigned to melatonin receptor activation; one of them is just the opposite to the canonical one, i.e. G_s coupling, activation of adenylyl cyclase and increases of cytoplasmic cAMP levels (14). In addition, it has also been suggested that MT_1 receptors may couple to G_q or other G proteins, and activate protein kinase C, inositol-phosphate- and calcium ion-mediated signaling (15, 16).

Quite surprisingly, a substantial number of reports show that the potency of melatonin acting on its receptors is in the picomolar range, something that it is not usual for endogenous compounds acting on GPCRs. ~~However, by~~ using high concentrations of the adenylyl cyclase activator, forskolin, many studies have shown that the potency of melatonin to its receptors is in the subnanomolar range. Furthermore, any GPCR-mediated action, if specific, must be blocked by an antagonist. Very few studies contemplate the experiment of antagonist treatment to confirm specificity and selectivity of melatonin receptor-mediated actions. In addition, atypical outputs and seemingly pleiotropic signaling [see (3, 17-20) for review] led to hypothesize the existence of a third melatonin receptor (21), which was later identified as an enzyme rather than a melatonin receptor. The enzyme, human quinone reductase 2 (22), seems to be allosterically regulated by melatonin and other endogenous compounds (e.g. N-acetylserotonin); however, its role as potential mediator of melatonin physiological effects is under discussion (23-25).

Radioligand binding assays have led to fairly low K_D values of melatonin binding to its receptors. Many studies were performed with iodinated-labeled melatonin-related compounds and it is known that iodine may unspecifically bind to membranes. To our knowledge, the initial study concerning 2-[^{125}I]iodomelatonin binding to hamster brain membranes was reported in 1986 by Duncan *et al*. (26). The binding potency calculated by kinetic association/dissociation data, Scatchard plot analysis and competition assays led to monophasic curves and the estimated K_D value for iodomelatonin was in the low nanomolar range (3.1 to 4.9 nM). In this study the reported K_i value for melatonin was 8 nM, whereas in a subsequent study using a similar preparation the reported value was 10.8 ± 2.1 nM (27). The same authors in further studies reported that the K_D value of a 2-[^{125}I]iodomelatonin binding site in the hypothalamus was 43 ± 5 pM, postulating that the hamster brain tissue shows nanomolar and picomolar affinities corresponded to, “ML-2” and “ML-1” sites, respectively (28).

A recent study reports K_D values of 332 pM and 289 pM for melatonin binding to preparations of cells expressing MT_1 and MT_2, respectively (29). In Chinese hamster ovary CHO cells expressing either MT_1 or MT_2, the significant inhibitory effects of 1 nM melatonin on 100 µM forskolin-induced cAMP cytosolic levels were observed while, surprisingly, the EC_{50} values in functional studies to assess phosphoinositide signal transduction cascade were in the micromolar range. Also unusual is ~~that such a~~ high concentration (1 M) of the MT_2 specific antagonist, cis-4-phenyl-2-propionamidotetralin (4-P-PDOT), ~~being~~ used to block MT_2 mediated action while ~~it is lack of study to use a~~ MT_1 specific antagonist (29). All of these data are very intriguing from a pharmacological point of view.

It has been reported that rabbit gastrointestinal smooth muscle only expresses MT_1 that ~~only~~ couple to G_q but not to G_i. For example, by use of [^{35}S] GTPgammaS labeling prior to immunoprecipitation of α subunits of G proteins Ahmed *et al*. (30) ~~have~~ observed that a very high concentration of melatonin (1 µM) induces an increase in the radioactivity associated to α_q while the radioactivity associated to α_{i1}, α_{i2} and α_{i3} (also to α_s) was not significantly altered. Although melatonin promotes phosphoinositide turnover in a dose-dependent fashion with an EC_{50} of 4 ± 1 nM, other functional responses (cytosolic calcium mobilization or IP hydrolysis in the presence of minigenes) require 1 µM concentration of melatonin (30). In summary, these data indicated that melatonin receptors may not couple to G_i proteins and that high concentrations of melatonin are required to afford receptor functionality (K_D values in the picomolar range but EC_{50} values for PI hydrolysis in the low nanomolar range). Melatonin at the concentration of 1 µM decreases muscle contraction while the effect is reversed by a MT_1 antagonist, luzindole, at a concentration of only 100 nM. If the potency of melatonin is in the nanomolar range (<10 nM according to dose-response curve illustrated in Fig. 5 of reference (30), it is difficult to believe that 100 nM luzindole will significantly inhibit the effect of melatonin at a concentration of 1 µM. In brief the MT_1 receptor is expressed in the muscle cells and melatonin acts via G_q and not via G_i; however, the involvement of the receptors in the G_q-mediated effects is dubious as the conditions of the assays are not standard from a pharmacological point of view. In this regard, activation of melatonin receptors in a heterologous expression system does not lead to immediate Ca^{2+} mobilization as it occurs in the case of other GPCRs that are coupled to G_q (31).

Pre-registering is a recently developed instrument aimed at improving the reliability of results from experimental research. Pre-registered studies were first used for clinical trial implementation, but now this option is open, and convenient, for any type of scientific research. It consists of uploading detailed information of the hypothesis and the experimental designs in a database before starting the experiments. Individuals who are interested can have free access to such information. When, based on the experimental approaches, the results are obtained, they are mainly interpreted in terms of confirming or rejecting the initial hypotheses. These experimental approaches should match as much as possible to those that were *a priori* registered. One of the main resources is provided within the Open Science Framework (OSF), where pre-registered studies are deposited in https://osf.io. As it is stated by Foster and Deardoff (32): "*Registration is a major feature of the OSF and its efforts to preserve, provide access to, and promote transparency in research. Any OSF project can be registered, which means that a time-stamped version of the project is created that cannot be edited or deleted and is intended to act as a preserved version of a project*".

As it has been already demonstrated that melatonin receptors do not couple to G_q in the HEK-293T cell heterologous expression system (31), this pre-registered study (available at (33), by using the HEK-293T cell expression system, will evaluate whether i) MT_1 or MT_2 receptors can couple to G_s and/or G_i proteins, ii) G_s/G_i-coupled melatonin receptors are sensitive to subnanomolar concentrations of melatonin and iii) melatonin potency is similar to that previously reported by using other methods to measure cAMP levels.

2. MATERIALS AND METHODS

2.1. Chemicals.

N-Acetyl-5-methoxytryptamine (melatonin), N-acetyl-2-benzyltryptamine (luzindole: non-selective MTR antagonist), cis-4-phenyl-2-propionamidotetralin (4-P-PDOT, a selective MT_2R antagonist) and forskolin were purchased from Tocris Bioscience (Bristol, UK). N-[2-(2-methoxy-6H-isoindolo[2,1-a]indol-11-yl)ethyl]butanamide (IIK7, a selective MT_2 receptor agonist) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Cell Culture and Transient Transfection.

A heterologous system consisting of human HEK-293T cells was used in this study. These immortalized cells come from Human Embryonic Kidney (34) and are used in many laboratories for heterologous expression of proteins. Previous heterologous expression systems were not of human origin and, accordingly, the development of HEK-293T cells was a of paramount relevance for biomolecular research; they are currently used in biochemistry, pharmacology, electrophysiology and biotechnology approaches aimed at advancing knowledge into protein structure/function relationships (35-37). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM L-glutamine, 100 U/ml penicillin/streptomycin, and 5% (v/v) heat inactivated fetal bovine serum (FBS) (Invitrogen, Paisley, Scotland, United Kingdom). Cells were maintained in a humid atmosphere of 5% CO_2 at 37˚C. Cells were transiently transfected with the polyethylenimine (PEI, Sigma, St. Louis, MO, United States) method (38-40). Briefly, cells were incubated (4h) in a serum-starved medium with the corresponding cDNA and with PEI (5.47 mM in nitrogen residues) and 150 mM NaCl. After 4 hours, the medium was replaced by a fresh complete culture medium. The cDNAs used were obtained from the cDNA resource Center (Ref. #MTNR1A0000 for the MT_1 and #MTNR1B0000 for the MT_2). Transfection efficiency (>60% of cells expressing each of the receptors) was checked using specific antibodies and immunocytochemical staining.

2.3. cAMP determination.

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

2.3. Statistical Analysis

Data were analyzed using Prism 7 (GraphPad Software, Inc., San Diego, CA, United States). The data in graphs are the mean ± SEM. Significance was analyzed by one-way ANOVA, followed by Bonferroni's multiple comparison *post hoc* test. Significant differences were considered when $p < 0.05$.

3. RESULTS

Although G_i is the cognate heterotrimeric protein coupled to melatonin receptors, as classified by the International Union of Pharmacology and British Society of Pharmacology (13) (https://www.guidetopharmacology.org/), there have been reports on coupling to G_s, so we first tested whether activation of melatonin receptors increases cAMP production. Results in Figure 1A and 1C show that neither melatonin treatment on MT_1-expressing HEK-293T cells or on MT_2-expressing HEK-293T cells led to any significant increase in cytosolic cAMP levels. Therefore, in a heterologous expression system, the human versions of MT_1 and MT_2 are likely not G_s-coupled. In contrast, in the same experimental system, melatonin treatment significantly decreased the cAMP levels which previously increased upon 0.5 µM forskolin treatment.

Forskolin treatment increased cytosolic cAMP levels in both cell lines (also in untransfected cells) and this increase was reduced by cotreatment with melatonin at the concentration of 100 nM in both MT_1-expressing and MT_2-expressing cells. In addition, it is evidenced that melatonin membrane receptors are coupled to G_i in a specific manner, as the cAMP-inhibitory effect is completely blocked by the non-selective antagonist, luzindole (N-acetyl-2-benzyltryptamine, 1 µM), in MT_1-expressing cells and by the MT_2 selective antagonist, 4-P-PDOT (0.5 µM), in MT_2-expressing cells. These antagonists in the absence of melatonin did not significantly affect the forskolin effect on cAMP production (Figure 1B and D). The results confirmed that both MT_1 and MT_2 were specifically coupled to G_i (Figure 1B and D).

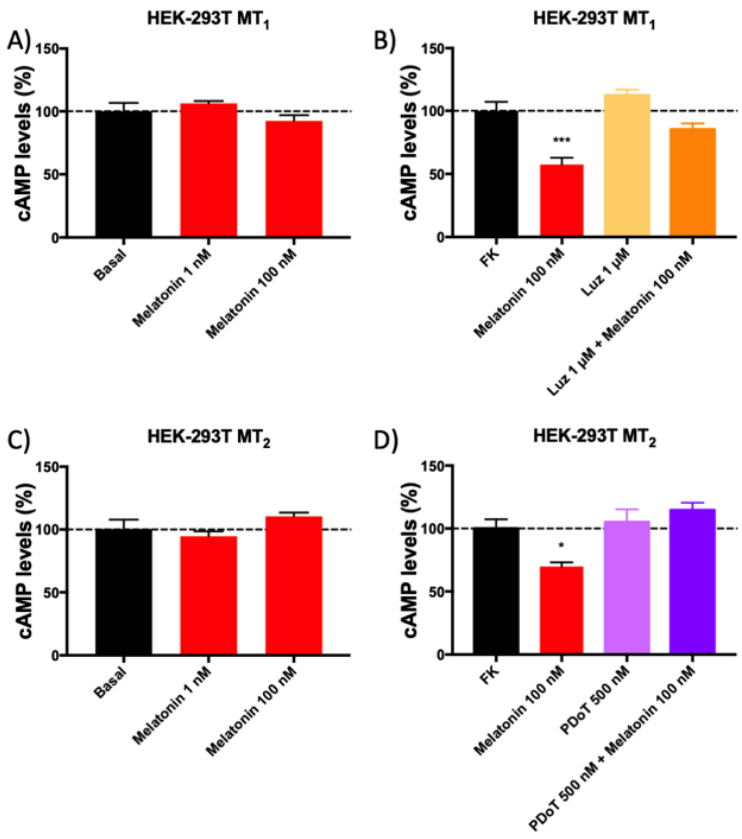

Fig. 1. Assessment of G_s and G_i coupling.

HEK-293T cells expressing MT_1 (A, B) or MT_2 (C, D) treated with vehicle or with either 1 or 100 nM melatonin. G_s coupling (A, C) was assessed by measuring the increase of cytosolic cAMP levels whereas G_i coupling (B, D) was assessed by simultaneous treatment ~~of melatonin~~

*with 0.5 µM forskolin. Cytosolic cAMP levels were determined by TR-FRET as described in Methods. Specificity was assessed by preincubating cells with antagonists (for 15 min): the melatonin receptor nonselective antagonist luzindole, in ~~the~~ MT_1-expressing cells (B) and MT_2 selective antagonist, 4-P-PDOT, in ~~the~~ MT_2-expressing cells (D). Values are the mean ± SEM. of 6 independent experiments performed in triplicates. One-way ANOVA followed by Bonferroni's multiple comparison post hoc test were used for statistical analysis (*p < 0.05, ***p < 0.001 versus forskolin treatment).*

To further assess receptor functionality, dose response assays were performed. At ~~the~~ subnanomolar levels, no significant inhibitory effect of melatonin (MT_1 expressing cells) or of melatonin or IIK7 (MT_2 expressing cells) was detected in forskolin-induced cAMP determination experiments (Figure 2A and B). The calculated IC_{50} value of melatonin on the MT_1 was 58.0 nM (pIC_{50}=7.24, SD 0.35) and IC_{50} values of melatonin and IIK7 on the MT_2 were 3.9 nM (pIC_{50}=8.4, SD 0.22) and 7.3 nM (pIC_{50}=8.1, SD 0.22), respectively (Figure 3A and B).

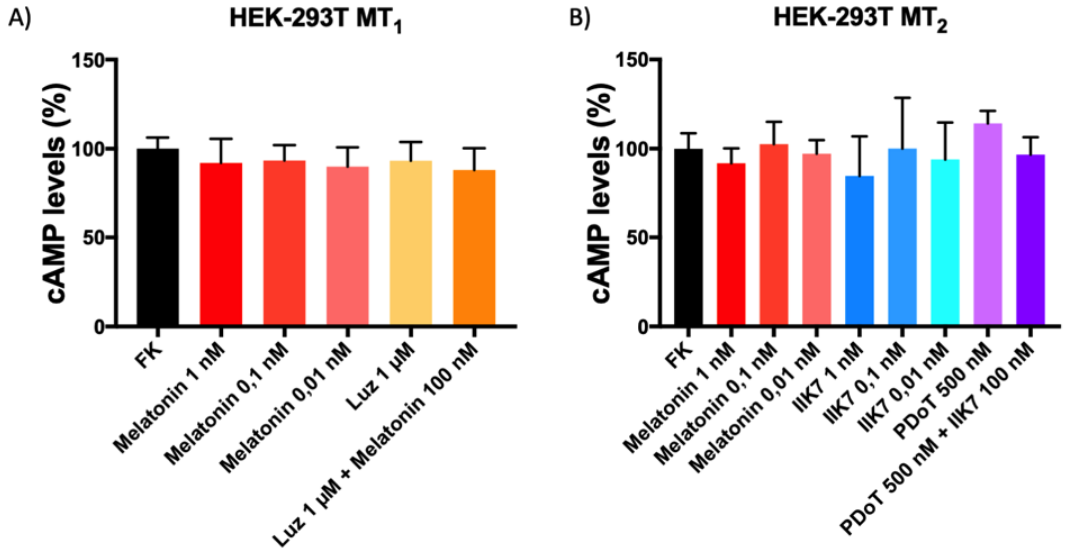

Fig. 2. Effects of melatonin and IIK7 on forskolin-induced cAMP production in MT_1 or MT_2 expressing cells.

HEK-293T cells expressing MT_1 (A) or MT_2 (B) were treated with 0.5 µM forskolin and melatonin and/or IIK7 (selective MT_2 agonist) at the indicated concentrations. In parallel, assays with cells pretreated (15 min) with antagonists: luzindole or 4-P-PDOT, were also performed. Cytosolic cAMP levels were determined by TR-FRET as described in Methods. Values are the mean ± SEM of 6 independent experiments performed in triplicates. No statistically significant differences were observed in any of the treatments (versus the forskolin treatment).

The antagonistic assays were carried out in MT_2-expressing cells treated with melatonin or with the selective MT_2 agonist IIK7 (100 nM) plus selective MT_2 antagonist, 4-P-PDOT, and in MT_1-expressing cells with melatonin plus luzindole. The results showed that the effect of 100 nM melatonin was blocked by 1 µM luzindole (Figure 3C) and both the effects of 100 nM melatonin or 100 nM IIK7 were completely blocked by 0.5 µM 4-P-PDOT (Figure 3D). Taken together, the data suggest that i) the effect was specifically due to action on MT_1 or MT_2, ii)

the potency of melatonin was lower in MT_1 than in MT_2 and ii) the potency of the endogenous (melatonin) and the synthetic (IIK7) agonists is similar (in the low nM) range for MT_2.

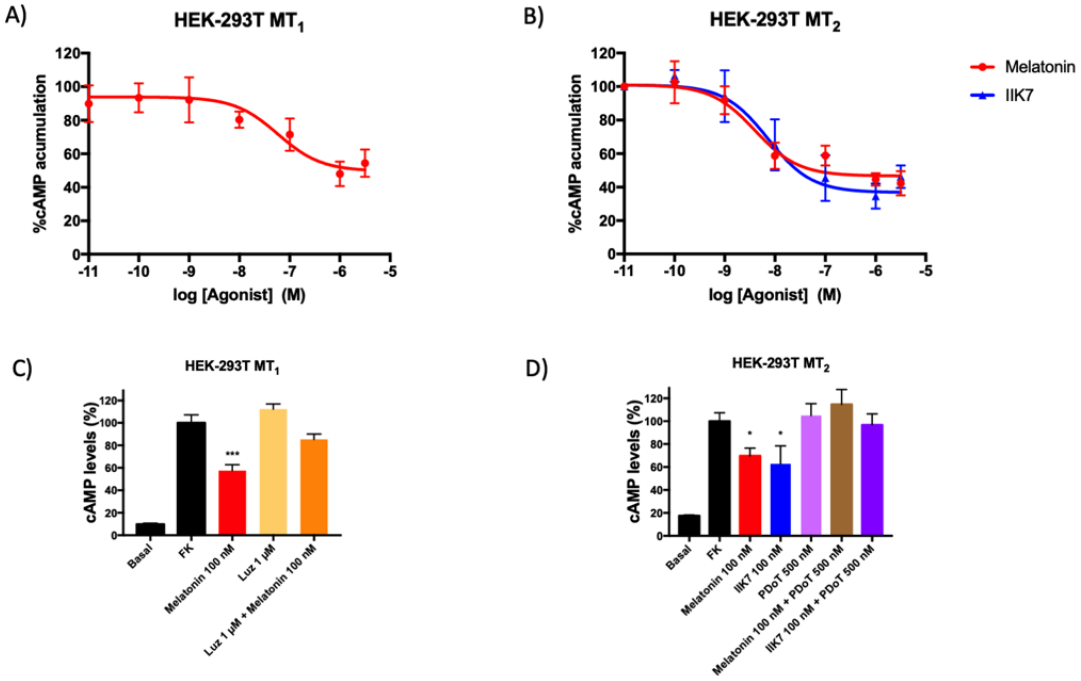

Fig. 3. Dose-response curves and selectivity of antagonists of MT_1 and of MT_2.

*Melatonin and/or IIK7 dose-response curves in HEK-293T cells expressing MT_1 (A) or in cells expressing MT_2 (B). The conditions of the assay to measure effects on forskolin-induced cAMP levels were similar to those described in figure 1. Specificity of the effect ~~on the MT_1~~ was shown using luzindole in MT_1-expressing cells (C) and 4-P-PDOT in MT_2-expressing cells (D). Panels C-D: Values are the mean ± SEM. of 6 independent experiments performed in triplicates. One-way ANOVA followed by Bonferroni's multiple comparison post hoc test were used for statistical analysis (*p < 0.05, ***p < 0.001 versus forskolin treatment).*

4. DISCUSSION

The results presented ~~here~~ corroborate the hypothesis of the pre-registered study, i.e. the potency of melatonin does not lie in the pM but in the low nM range for MT_2 and in the high nM range for MT_1. This seems different from previous concepts on the melatonin receptor potency. Anyway, the data will provide a new look and new vistas as to the melatonin's biology and on the role of melatonin receptors in melatonin physiological functions (41).

Melatonin receptors when expressed in HEK-293T cells specifically couple to G_i and not to G_s (observed in the present study) or to G_q (31). This fits well with the canonical pathway defined by the International Union of Pharmacology and British Society of Pharmacology (https://www.guidetopharmacology.org/). The possibility of melatonin receptors coupling to G_s or G_q (even to G_{16}) proteins reported in CHO cells, in cell lines or in intact tissues (16, 30, 42-44) was not contemplated in this pre-registered study. However, data from our own laboratory have shown that G_s or G_q coupling may occur by formation of complexes involving melatonin receptors and other GPCRs [31]. It seems that the real potency of melatonin on the MT_1 is lower than that previously described but such potency in terms of EC_{50} or IC_{50} values for proximal signaling is similar to that of other endogenous compounds acting on the

populated GPCR superfamily. In addition, the data related to MT_2 were consistent with the data reported in hamster brain [26–28].

There are two main inconsistencies brought about by this pre-registered study. One is related to MT_2 ~~in which~~ our results are consistent with those of Duncan *et al*., who reported 8-10.8 nM K_D values for melatonin, but not with other laboratories reporting picomolar K_D values. Another is related to MT_1. ~~As to this receptor,~~ almost all laboratories have claimed the potency of melatonin on it is in the range of pM for both K_D of radioligand binding and IC_{50} of G_i-mediated effects. In a study ~~by~~ using the same cells employed here, HEK-293 cells, forskolin-induced cAMP determinations in cells expressing MT[illegible] MT2 led to IC_{50} values for melatonin being 7.7 and 117 pM, respectively (45). Thus, the differences between this report and ours seem ~~not being~~ due to the melatonin receptor expression system. However, in our study, the receptor specificities are investigated and this is not the case reported by Conway *et al*. (45). In sharp contrast, the effect that we have demonstrated was, on either melatonin receptor, specifically blocked by selective receptor antagonists (Figure 3C and D).

The sequences of the plasmids used in our study are the canonical ones: GeneBank accession number NM005958 for MT_1 and AY521019 for MT_2, which are the same as the ones used by Conway *et al*. (45). Thus, the differences between their study and ours may come from the concentration of forskolin, the method of cAMP level determination, which is now more reliable than before, and also from the approach for data acquisition and analysis.

Two logical questions ~~are~~ raised ~~for~~ the high potency ~~with the~~ K_D in the pM range found using either MT_1 or MT_2 in ~~the~~ previously published articles: i) the specificity is (often) not confirmed by antagonism and ii) if the potency and K_D are picomolar why melatonin is used at micromolar concentrations when assessing its physiological effects?

In summary, in this pre-registered study t[illegible] important issues on melatonin research have been confirmed, that are, 1. both MT[illegible] nd MT2 are directly coupled with G_i but not with G_s and G_q (may be ~~indirectly~~ a[illegible]ciated with them as mentioned in the text); 2, the melatonin potency ~~to~~ both MT1 and MT2 ~~are~~ significantly lower (nM) than that ~~of~~ previously reported (pM). We believe that these new data, especially on the potency of melatonin on its receptors will provide a new perspective in melatonin research. It is important to consider that the amount of melatonin needed to achieve its physiological or therapeutic effects may be much higher than that of previously thought.

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In memoriam of Jesús (Suso Pintor) an outstanding scientist and yet a good person.

AUTHOR CONTRIBUTIONS

RF and late JP designed the study. RF, RRS, IRR, HAA and GN pre-registered the study. RRS with the help of IU in terms of cell culturing and reagent preparation performed the experiments designed by GN, HAA and RF. RF, RRS, IRR, HAA and GN analyzed the results. RF supervised the work in relation to adherence to pre-registration terms. RRS and GN wrote the Methods section and RF prepared a first version of the manuscript that was further edited by RRS, IRR, HAA and GN. All authors have read and approved the submitted version of the manuscript.

CONFLICT OF INTERESTS

Authors declare no conflict of interest

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