Mise en évidence de l'activité constitutive des récepteurs MT_1 et MT_2 et identification d'agonistes inverses

Introduction

L'étude du récepteur oMT$_2$, aussi bien d'un point de vue pharmacologique, fonctionnel et structurel, nous laisse imaginer que ces récepteurs possédent une activité constitutive. D'une part, en absence de mélatonine, le niveau basal d'AMPc des cellules CHO-oMT$_2$ est élevé. D'autre part, le récepteur oMT$_2$ contrairement aux autres récepteurs mélatoninergiques possède un motif DRY. Ce motif DRY situé entre le troisième domaine transmembranaire et la seconde boucle intracellulaire, est hautement conservé au cours de l'évolution. Cette séquence de trois acides aminés joue un rôle primordial dans la régulation des états d'activation des RCPG. Il est acquis que le motif DRY est impliqué dans l'activité constitutive de plusieurs récepteurs, comme les récepteurs α_{1B} et β2B-adrénergiques (Ballesteros et al., 2001; Rasmussen et al., 1999; Scheer et al., 1996), le récepteur à l'histamine H_2 (Alewijnse et al., 2000), la rhodopsine (Acharya and Karnik, 1996; Cohen et al., 1993; Franke et al., 1992), les récepteurs à la vasopressine de type II (Morin et al., 1998), les récepteurs μ-opioides (Li et al., 2001), et les récepteurs à l'ocytocine (Favre et al., 2005) (Pour revue: Rovati et al., 2007).

L'activité constitutive des récepteurs MT est très peu décrite dans la littérature. Roka et ses collaborateurs ont montré que le récepteur hMT$_1$ exprimé dans la lignée cellulaire HEK293 serait constitutivement actif (Roka et al., 1999). Cette équipe conclut que le luzindole, connu comme étant un antagoniste des récepteurs mélatoninergiques, se comportait comme un agoniste inverse pour ce récepteur. De plus, le récepteur hMT$_1$ formerait de façon spontanée un complexe avec une protéine Gi et une protéine régulatrice de la signalisation des protéines G (RGS20) (Maurice et al., 2010). Ce pré-couplage avec les protéines Gi est un bon argument en faveur de la possible activité constitutive de ce récepteur. De plus, dans des cellules COS co-transfectées avec le récepteur MT$_1$ et le récepteur à la TSH, l'expression du récepteur MT$_1$ diminue l'activation de la voie AMPc induite par la TSH, même en absence de mélatonine dans le milieu. Ceci implique que les récepteurs MT$_1$ non liés peuvent induire une diminution de la signalisation AMPc (Dupre et al., 2011).

Nos travaux se sont portés initialement sur l'activité constitutive des récepteurs mélatoninergiques MT$_1$ et MT$_2$ de trois espèces (humain, rat et ovin). L'activité constitutive a

été mise en évidence en mesurant le niveau d'activité des protéines G en absence de ligand endogène (test de liaison du GTPγS). Nous nous sommes alors interrogés d'une part sur la nature du(des) sous-type(s) de protéines G impliquées dans cette activité constitutive et d'autre part sur les facteurs pouvant moduler le niveau de celle-ci. Deux molécules initialement connues comme des antagonistes des récepteurs à la mélatonine, UCM 549 et UCM 724, ont été identifiées comme étant des agonistes inverses des récepteurs humains MT_2 (Spadoni et al., 2007). Dans la première étude, seuls les résultats concernant les récepteurs MT_1 et MT_2 humains exprimés de façon stable dans les lignées cellulaires CHO et Neuro2A sont présentés[2]. Dans la seconde étude, l'activité constitutive des récepteurs de rat et ovin seront exposés[3].

[2] L'ensemble des résultats concernant l'identification de l'activité constitutive des récepteurs hMT_1 et hMT_2, ainsi que l'identification d'agonistes inverses est rapporté dans l'article inclus page 79-90.

[3] L'ensemble des résultats concernant l'identification de l'activité constitutive des récepteurs ovins et de rat est rapporté dans le paragraphe inclus page 91-93.

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Description of the constitutive activity of cloned human melatonin receptors hMT_1 and hMT_2 and discovery of inverse agonists

Abstract: Melatonin receptors have been described to activate different G protein–dependent signaling pathways, both in laboratory, heterologous, cellular models and in physiological conditions. Furthermore, the constitutive activity of G protein–coupled receptors has been shown to be key in physiological and pathological conditions. In the case of melatonin receptors, information is rather scare and concerns only MT1 receptors. In the present report, we show that the G protein–coupled melatonin receptors do have a constitutive, nonmelatonin-induced signaling activity using two cellular models of different origins, the Chinese hamster ovary cell line and Neuro2A, a neuroblastoma cell line. Furthermore, we show that this constitutive activity involves mainly Gi proteins, which is consistent with the common knowledge on the melatonin receptors. Importantly, we also describe, for the first time, inverse agonist properties for melatonin ligands. Although it is clear than more in-depth, biochemistry-based studies will be required to better understand by which pathway(s) the constitutively active melatonin receptors transfer melatonin information into intracellular biochemical events; our data open interesting perspectives for understanding the importance of the constitutive activity of melatonin receptors in physiological conditions.

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1 Introduction

Melatonin is a neurohormone produced in mammals mainly during the dark period by the pineal gland. This compound, derived from tryptophan and closely related to serotonin, produces a paramount of effects that are both central and peripheral. These actions relate to many physiological functions of the body and include the control of sleep, digestion, circadian rhythm, etc. A vast literature provided evidence that melatonin can, although at high concentrations (micromolar and beyond), modulate physio-pathological situations such as inflammation, cancer progression, and immunological responses. Two melatonin agonists have reached the market: Ramelteon® for the treatment of sleep disorders and agomelatin for the treatment of depression. This later compound has the particularity to have a slight $5\text{-}HT_{2C}$ component [1].

Melatonin acts through at least three targets, namely the binding site MT_3 that is the enzyme quinone reductase 2, and two 7-trans-membrane domain, G protein–coupled receptors (GPCR), MT_1 and MT_2 [2–4]. More and more is also reported in the large variety of signaling pathways associated with these receptors. Depending on the tissue and species, melatonin can activate different second messenger cascades acting on the same receptor subtype. MT_1 and MT_2 receptors are primarily coupled in an inhibitory manner to the adenylate cyclase, via a pertussis toxin–sensitive Gi protein [reviewed in Ref. 5]. Activation of MT_1 receptor inhibits cAMP accumulation and then modulates the PKA signaling pathway [6]. Reports have also shown a coupling of MT_1 receptor with Gq [7]. Activated MT_1 receptors, in addition to inhibition of cREB phosphorylation [8–10], can also inhibit the formation of immediate early gene products, such as c-Fos and jun B [11]. Further,

stimulation of MT_1 and MT_2 receptors may activate phospholipase C (PLC), with a concomitant increase in inositol-(1,4,5)-trisphosphate (IP3)/Ca^{2+} and 1,2-diacylglycerol [reviewed in Ref. 5,12]. In COS-7 cells expressing human MT_1 or MT_2 receptors, it has been demonstrated that activation of these receptors stimulates the c-Jun N-terminal kinase activity *via* pertussis toxin–sensitive and pertussis toxin–insensitive G proteins [13]. Stimulation of the MT_1 receptor has also been associated with an increased phosphorylation of mitogen-activated protein kinase MEK1/2 and extracellular signal-regulated kinase ERK1/2 [13, 14]. In addition, MT_1 melatonin receptors increase potassium conductance by activating Kir3 (GIRK) inward rectifier potassium channels [15] and potentiate prostaglandin F2α and ATP-mediated stimulation of PLC activity [16, 17]. Both processes may involve activation of membrane-bound $\beta\gamma$-subunits released by Gi proteins. In rat microvascular endothelial cells, melatonin inhibits stimulated nitric oxide production. This effect was mediated by the suppression of Ca^{2+} mobilization from intracellular stores [18]. In human benign prostate cells, melatonin inhibits cGMP and DNA synthesis [19]. Modulation of intracellular cGMP level by cloned MT_1 and MT_2 receptors has also been reported [20].

More recently, studies have shown that the melatonin receptor subtypes form both homo- and heterodimers [21, 22]. Moreover, the melatonin-related orphan receptor, gpr50, has been reported to antagonize MT_1 by heterodimerization [23]. The existence of these dimers in native tissues and their physiological significance await further detailed analyses.

Despite all the information on the signaling pathways of melatonin receptors, one observation remains poorly documented, that is, the constitutive activity of the MT_1 receptor. Indeed, only three publications, spanned on almost 10 yr, were published on the subject, each with various levels of details [16, 24, 25]. Incidentally, no observation concerning the MT_2 receptor exists in the literature. It becomes increasingly obvious that receptors are not single signaling units. Receptors should be considered as integrated in a far more complex signaling system [26–28]. Indeed, in this 'new' view of the receptor-as-a-signaling-unit, besides the nature itself of this unit in terms of number of proteins acting in a coordinating manner, the state of the receptor itself, without ligand, is also the subject of studies. In this case, as described by Kenakin [29], constitutive activity corresponds to the 'spontaneous production of a receptor conformation that interacts with cellular components (i.e., G proteins) to initiate signaling in the absence of agonist'. It is clear, then, that what was often taken for inappropriate experimental conditions, during which a receptor activity was observed in the absence of its ligand, was indeed corresponding to a state of the receptor spontaneously active. Revisiting many experiments could bring, at this stage, interesting new observations, as many receptor systems have a state corresponding to this spontaneous constitutive activity.

Indeed, while doing so, it was interesting to note that in the heterologous cellular system used to express cloned receptors, some situations might correspond to constitutive activity, particularly with human melatonin receptors MT_1 and MT_2. Because these observations were reported in two different cellular systems (i.e., CHO-K1 and Neuro2A cell lines) used for expression of the receptors, we could deduce, upfront, that it was not an expression system-mediated artifact. Therefore, we provide in this report the molecular description of human melatonin receptor systems in which not only the constitutive state of the receptors are described and dissected but also the finding of new compounds with inverse agonist properties susceptible to help us decipher these systems, beside the two already described, but poorly documented luzindole and 4P-PDOT [24, 25].

Materials and methods

Materials

Guanosine 5′-[γ-^{35}S]-triphosphate ([^{35}S]-GTPγS; specific activity: 1000 Ci/mmol) was purchased from Perkin-Elmer (Courtaboeuf, France). Suramin was from Calbiochem (EMD Chemicals, Gibbstown, NJ, USA). Luzindole (*N*-Acetyl-2-benzyltryptamine) and 4P-PDOT (4-Phenyl-2-propionamidotetralin) were from Tocris (Ellisville, MI, USA). GTPγS (Guanosine 5′-[γ-thio]triphosphate), Guanosine 5′-diphosphate (GDP), Melatonin, PTX (Pertussis toxin, from Bordetella pertussis), GDP-β-S (guanosine 5′-[β-thio]-diphosphate) were from Sigma-Aldrich (Saint-Quentin-Fallavier, France). Compounds UCM 549 (*N*-(8-Methoxy-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-10-yl-methyl)acetamide) and UCM 724 ((8-Methoxy-10, 11-dihydro-5H-dibenzo[a,d]cyclohepten-10-yl-methyl) urea) were a kind gift of Spadoni group [30]. All culture media were from Invitrogen (Carlsbad, CA, USA). Fetal bovine serum was from Lonza (Levallois-Perret, France).

CHO-K1-hMT, CHO-K1-hH3, and Neuro2A-hMT cell culture

CHO-K1 cells stably expressing hMT_1, hMT_2 [31], rMT_1, rMT_2 [32], oMT_1 [33], oMT_2 [34], or hH_3 receptors were grown in Ham F12 Glutamax medium supplemented with 10% fetal bovine serum and 400 μg/mL geneticin. Neuro2A cells stably expressing hMT_1 and hMT_2 were grown in modified Eagle's medium/Glutamax supplemented with 10% fetal bovine serum, 1% nonessential amino acids, 1 mM sodium pyruvate, and 400 μg/mL geneticin. When mentioned, CHO-K1-hMT and Neuro2A-hMT were treated overnight with *pertussis* toxin (PTX) at 100 ng/mL. Cells were then used for the activity assays (CDS and GTPγS binding).

Membrane preparations

Melatonin receptors were used as previously cloned and described in our laboratory [31–33]. CHO-K1 and Neuro2A cell lines stably expressing human MT_1 and MT_2 receptors were grown to confluence and harvested in phosphate buffer containing 5 mM EDTA and centrifuged at 2500 g for 20 min (12°C). The resulting pellet was suspended in 5 mM Tris/HCl, pH 7.4, containing 2 mM EDTA, and homogenized using a Kineatica Polytron (30 s, in ice 13,000 rpm). The homogenate was then centrifuged

(20,000 g, 30 min, 4°C), and the resulting pellet was suspended in 75 mM Tris/HCl, pH 7.4, containing 2 mM EDTA and 12.5 mM $MgCl_2$. Determination of protein content was performed according to Lowry using the Biorad kit (Bio-Rad SA, Ivry-sur-Seine, France). Aliquots of membrane preparations were stored in binding buffer (75 mM Tris/HCl, pH 7.4 containing 12.5 mM $MgCl_2$ and 2 mM EDTA) at −80°C until use. The density of binding sites Bmax and the dissociation constant of the radioligand (K_D) values were calculated according to the method of Scatchard in 2-[^{125}I]-iodo-melatonin binding assay [31]. The density of receptors was determined as follows: 454 fmol/mg in CHO-hMT1, 1560 fmol/mg in CHO-hMT2, 124 fmol/mg in CHO-rMT1, 2462 fmol/mg in CHO-rMT2, 439 fmol/mg in CHO-oMT1, 203 fmol/mg in CHO-oMT2, 186 fmol/mg in Neuro2A-hMT1, 393 fmol/mg in Neuro2A-hMT2.

[^{35}S]-GTPγS binding assay

Membranes, drugs, GTPγS, and [^{35}S]-GTPγS were diluted in binding buffer (25 mM HEPES, pH 7.4, 75 mM NaCl, 2.5 mM $MgCl_2$, 5 μM GDP). Incubation was started by the addition of 0.2 nM [^{35}S]-GTPγS to membranes and ligands and carried on for 60 min at room temperature in a final volume of 250 μL. To test for inverse agonist activity, membranes were incubated with 5 μM melatonin and 50 μM of the tested compound. Nonspecific binding was assessed using unlabeled GTPγS (10 μM), and maximal receptor activity was assessed by the use of a saturating concentration of agonist, 5 μM melatonin for MT receptors, and 10 μM histamine for hH3 receptor. All reactions were stopped by rapid filtration through GF/B unifilters presoaked with distilled water, followed by three successive washes with ice-cold buffer. Data were analyzed by using the program PRISM to yield [^{35}S]-GTPγS binding in the presence of melatonin, tested compound, or binding buffer. Data were analyzed also by using the program PRISM to yield EC_{50} and E_{max} values for inverse agonists.

Cellular dielectric spectroscopy (CellKey)

CHO-K1 cells expressing human MT_1 and MT_2 receptors and Neuro2A cells expressing human MT_1 and MT_2 were plated at the density of 40,000 cells per well onto MDS Analytical 96-well assay plates with embedded electrodes and were incubated at 37°C, CO_2 6% for 48 hr. Prior to the measurement, cells were washed three times with Hank's balanced salt solution, 0.1% BSA, 20 mM HEPES, pH 7.4, and CHO-K1 and Neuro2A were left to equilibrate at 28–37°C, respectively for 30 min. The impedance measurement was performed on a CellKey system (MDS Analytica, Concord, ON, Canada), where the signal was recorded for 5 min before online addition of melatonin or tested compound and 15 or 25 min for CHO-K1 and Neuro2A, respectively, thereafter. The cells in each well were stimulated once with a single concentration of compounds. The resulting data are expressed as the maximal signal corrected for the baseline and represented as a percentage of agonist effect. Obviously, naïve CHO-K1 cells were used as control in these CDS experiments and do not show any signal after melatonin treatment.

Results

MT receptors display constitutive activity

The activity of melatonin heterotrimeric GPCR can be measured by a [^{35}S]-GTPγS binding assay without the natural agonist. In these conditions, the radioligand titrates the number of sites of active G protein, thus revealing the extent of receptor-activated form of the G protein. In the absence of melatonin, the assay shows the basal, or constitutive, activity of the receptor, while the use of melatonin allows the measurement of the maximal response of the G protein to the melatonin receptors. In these conditions, CHO-K1 cells stably expressing hMT_1 or hMT_2 showed a [^{35}S]-GTPγS binding of 265.2 ± 5.0 and 324 ± 3.3 fmol/mg of protein, respectively, while native CHO-K1 cells yielded 40.4 ± 0.4 fmol/mg of protein, suggesting that hMT_1 and hMT_2 receptors have a spontaneous coupling to G proteins in the absence of agonist (Fig. 1). In the presence of 5 μM melatonin, the same assay gave a value of 970.0 ± 64.5 fmol/mg of protein for CHO-K1-hMT_1 and 650.0 ± 68.0 fmol/mg of protein for CHO-K1-hMT_2. These results show that human MT receptors display constitutive activity when expressed in CHO cells, and that, this activity covers 25% of hMT_1 maximal activity and 46% of hMT_2 maximal activity.

The level of constitutive activity of human melatonin receptors could be compared with that of the human histamine H_3 receptor, which has been described with a strong constitutive activity in orthologous as well as in natural systems [35, 36]. Using [^{35}S]-GTPγS binding assay and the same cellular model CHO-K1 cells, the hH_3 receptor constitutive activity was measured to 35 ± 1.13% of maximal, histamine-stimulated G protein activity. This value compares well with the basal activities of MT receptors mentioned earlier, 25% for hMT_1 and 46% for hMT_2, confirming that melatonin receptors show an important constitutive activity when expressed in CHO cells.

To further document the level of constitutive activities of human MT_1 and MT_2 receptors in alternative cellular models, to be sure that the observation was not artifactually linked to the genetic background of the cell, we used Neuro2A, a mouse cell line derived from a neuroblastoma. In Neuro2A stably expressing hMT_1 or hMT_2, the basal activity of G proteins was 1273.0 ± 150.9 and 1267.0 ± 34.0 fmol/mg of protein, respectively, while native Neuro2A showed a [^{35}S]-GTPγS binding of 657.7 ± 15.5 fmol/mg of protein. In these cells, the maximum melatonin-mediated activation of hMT_1 and hMT_2 was 1459.0 ± 62.8 and 1509.0 ± 37.6 fmol/mg of protein, respectively. These results show that the constitutive activities of human MT_1 and MT_2 receptors seem to be higher in Neuro2A cells than in CHO-K1 cells (Fig. 1), reaching between 80% and 90% for both hMT_1 and hMT_2.

Finally, we wanted to see whether this observation was restricted to the human receptors, or whether it was more generally applying to the melatonin receptors. As we

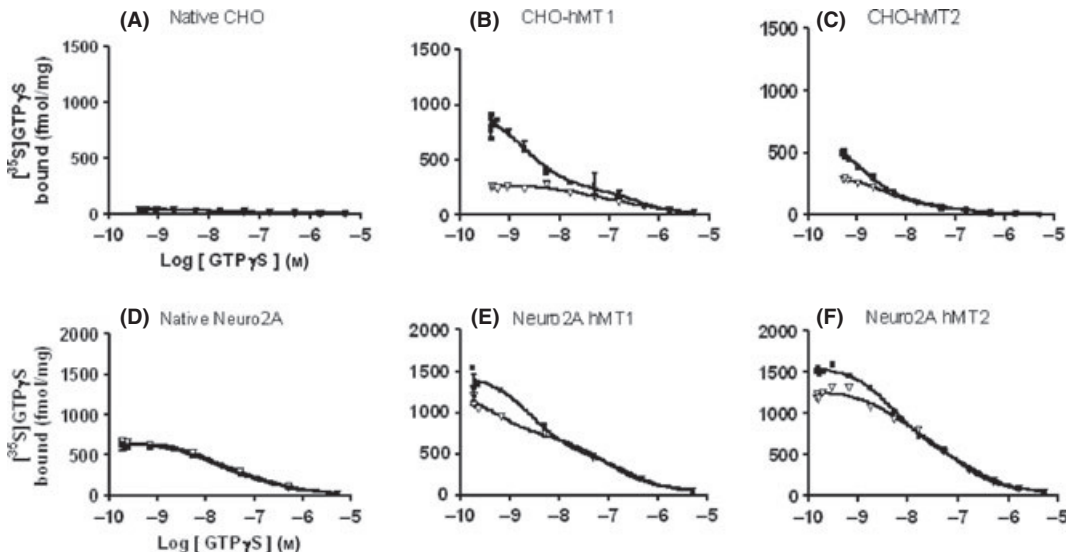

Fig. 1. Constitutive activity of human melatonin receptors expressed in CHO (A, B, C) and Neuro2A (D, E, F) cell lines. Activity of G protein, presented as guanosine 5′-[γ-^{35}S]-triphosphate ([^{35}S]-GTPγS) bound (fmol/mg), was assessed in the absence (△) or presence (■) of 5 μM melatonin and varying concentrations of unlabeled GTPγS. (A) native CHO-K1; (B) CHO-K1-hMT1; (C) CHO-K1-hMT2; (D) Neuro2A; (E) Neuro2A-hMT1; and (F) Neuro2A-hMT2. The data on this figure are representative of two independent experiments in triplicates. The data points and error bars represent the mean of triplicate and standard error, respectively.

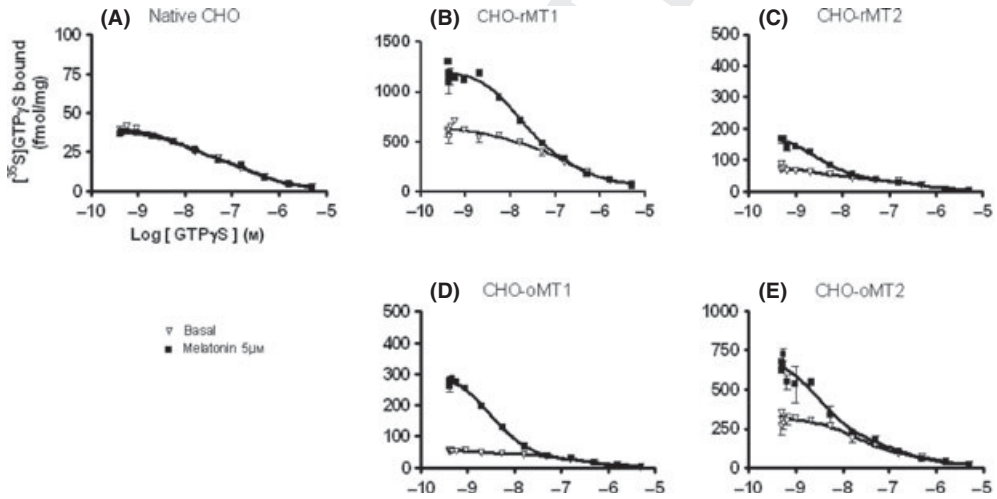

Fig. 2. Constitutive activity of melatonin receptors cloned from ovine (D and E) and rat (B and C) expressed in CHO cell lines. Activity of G protein, presented as guanosine 5′-[γ-^{35}S]-triphosphate ([^{35}S]-GTPγS) bound (fmol/mg), was assessed in the absence (△) or presence (■) of 5 μM melatonin and varying concentrations of unlabeled GTPγS. (A) native CHO-K1; (B) CHO-K1-rMT1; (C) CHO-K1-rMT2; (D) CHO-K1-oMT1; (E) CHO-K1-oMT2. The data on this figure are representative of two independent experiments in triplicate. The data points and error bars represent mean of triplicate and standard error, respectively.

previously cloned the ovine and rat receptors, we applied the same experimental conditions and obtained the results shown in Fig. 2. It can be seen from it that all the recombinant melatonin receptors, from whatever species assessed, were constitutively active to some extent. Besides using stable cell lines, it was not possible to correlate the level of constitutive activity of melatonin receptors with their level of expression (see density values in Materials and methods). This observation suggests that when selected as a stable cell line, cells may adapt to the constitutive elevated or lowered amount of some of their signaling metabolites, making a quantitative comparison difficult between cell lines.

Close coupling between G proteins and melatonin receptors

As constitutive activity mainly results from the strength of coupling between the receptor and the G protein, it would be interesting to quantify this interaction to compare the

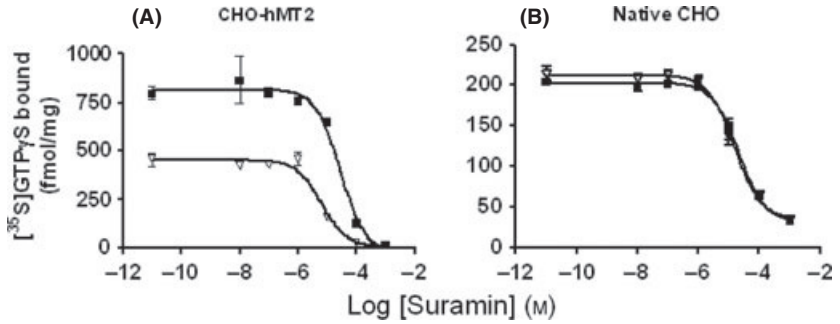

Fig. 3. Effect of suramin on GTPγS binding at CHO-hMT$_2$ (A) and native CHO (B). Activity of G protein, presented as guanosine 5′-[γ-^{35}S]-triphosphate bound (fmol/mg), was assessed in the absence (△) or presence (■) of 5 μM melatonin and increased concentrations of suramin. The data on the figure are representative of three independent experiments in triplicate. The data points and error bars represent the mean of a triplicate and standard error, respectively.

receptors and the cellular models described before. We investigated this possibility using suramin, a polysulfonated naphthylurea, which acts as an uncoupling agent of the receptors from G proteins [37]. Although it is not a selective and potent compound to this end, it is the only one at hand, and it was key in analyzing the constitutive activity of MT$_1$ in the pioneer work of Roka et al. [16]. Suramin acts by blocking the rate-limiting step of G protein activation, hence inhibiting the binding of GTPγS to G proteins, i.e., the dissociation of GDP prebound to the G protein α subunit [38]. Fig. 3 shows the dose–response effect of suramin on the [^{35}S]-GTPγS binding of CHO-K1-hMT$_2$. The IC$_{50}$ of suramin derived from these experiments was 6.8 ± 1.2 μM in basal conditions and 30.6 ± 1.2 μM under melatonin-stimulated conditions. Similar experiments were conducted with CHO-K1-hMT$_1$, CHO-K1-hH$_3$, native CHO-K1, and Neuro2A expressing either hMT$_1$ or hMT$_2$. Overall, the experiments consistently showed an IC$_{50}$ of suramin ranging from 10 ± 1.57 μM in Neuro2A-hMT$_2$ to 93.5 ± 1.21 μM in CHO-K1-hH$_3$ (see Table 1). These data are in line with the data obtained by Roka et al. [16] on the recombinant human MT1 receptor, but do not show a receptor dependency that allows to quantify the strength of the coupling between MT receptors and G proteins.

Melatonin receptors are commonly designated as Gi-coupled receptors [7, 39], although MT$_1$ has also been described with a Gq signaling pathway [7, 39]. As a next step, we aimed at understanding the role of the different G proteins involved in the constitutive activity of melatonin receptors. In this respect, pertussin toxin (PTX), a specific Gi protein activity inhibitor, is an excellent tool to demonstrate the involvement of Gi protein in the signaling of a GPCR. Consequently, CHO-K1-hMT$_1$ and hMT$_2$ cells as well as Neuro2A-hMT$_1$ and hMT$_2$ cells were treated overnight with PTX (100 ng/mL), and cellular membranes were prepared for [^{35}S]-GTPγS binding assay. Fig. 4 shows that after a treatment with PTX, the level of constitutive activity in CHO-K1-hMT$_1$ decreased by 60%, from 862 ± 30 to 353 ± 12 fmol/mg of protein. This is consistent with a recent report, which showed, using biochemical approaches, that hMT$_1$ was precoupled to a Gi protein in resting HEK-hMT$_1$ cells [40]. In CHO-K1-hMT$_2$, the [^{35}S]-GTPγS binding decreased even more drastically by 95%, from 884.8 ± 28.7 to 49.2 ± 3.8 fmol/mg of protein. These results suggest that, indeed, Gi proteins are involved in the constitutive activity the MT receptor signaling. Interestingly and consistent with the literature, an additional signaling component, PTX insensitive, seems to be present for MT$_1$. These results, obtained with the CHO-K1 cell line, were confirmed in the Neuro2A cell line, where Neuro2A-hMT$_1$ [^{35}S]-GTPγS binding decreased by 37%, from 2804 ± 183 to 1777 ± 32 fmol/mg of protein, while in Neuro2A-hMT$_2$ cells the difference was 73%, with a binding of 1091 ± 68 moving to 294 ± 12 fmol/mg after PTX treatment (Fig. 4).

Identification of inverse agonists

Melatonin receptors display constitutive activity in heterologous systems. One of the most demonstrative ways of further documenting this property is to make use of inverse agonists as pharmacological tools. In the Monod-Wyman-Changeux model of equilibrium of a receptor between

Table 1. IC$_{50}$ of suramin in different receptors. The IC$_{50}$ of suramin, presented as guanosine 5′-[γ-^{35}S]-triphosphate bound (fmol/mg), was assessed in the absence (basal conditions) or presence of melatonin and increased suramin concentrations. Values of IC$_{50}$ are expressed in molar with standard error. Values of IC$_{50}$ were representative of one experiment (in duplicate) repeated three times

IC$_{50}$ suramin (M)	CHO-K1				Neuro2A	
	Native	hH$_3$	hMT$_1$	hMT$_2$	hMT$_1$	hMT$_2$
Basal	1.56×10^{-5} ± 1.13	9.31×10^{-6} ± 1.21	2.98×10^{-5} ± 1.21	6.78×10^{-6} ± 1.24	2.51×10^{-5} ± 1.78	9.88×10^{-6} ± 1.57
Natural agonist	2.17×10^{-5} ± 1.12 melatonin	6.56×10^{-6} ± 1.12 histamine	7.60×10^{-5} ± 1.65 melatonin	3.06×10^{-5} ± 1.22 melatonin	2.44×10^{-5} ± 1.21 melatonin	1.18×10^{-5} ± 1.21 melatonin

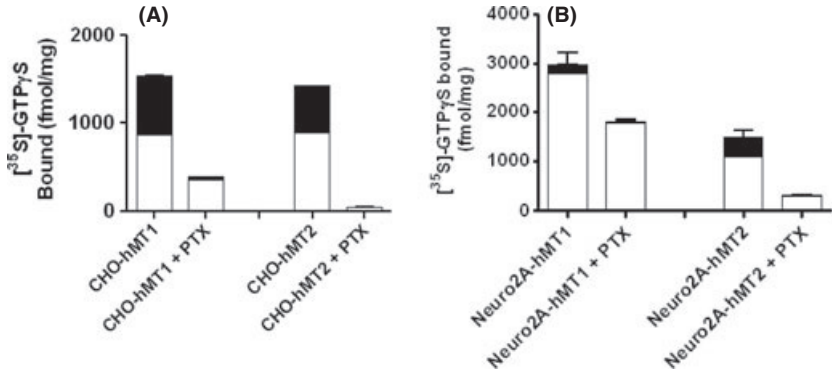

Fig. 4. Involvement of Gi proteins in the constitutive activity of human melatonin receptors expressed in CHO-K1 and Neuro2A cell lines. Cells were treated overnight with PTX (100 ng/mL). Activity of G protein, presented as guanosine 5′-[γ-^{35}S]-triphosphate bound (fmol/mg), was assessed in the absence (□) or presence (■) of 5 μM melatonin in CHO-K1 cells expressing hMT_1 and hMT_2 receptors (A) and in Neuro2A cells expressing hMT_1 and hMT_2 receptors (B). The data on the figure are representative of three independent experiments in triplicate. The data points and error bars represent the mean of a triplicate and standard error, respectively.

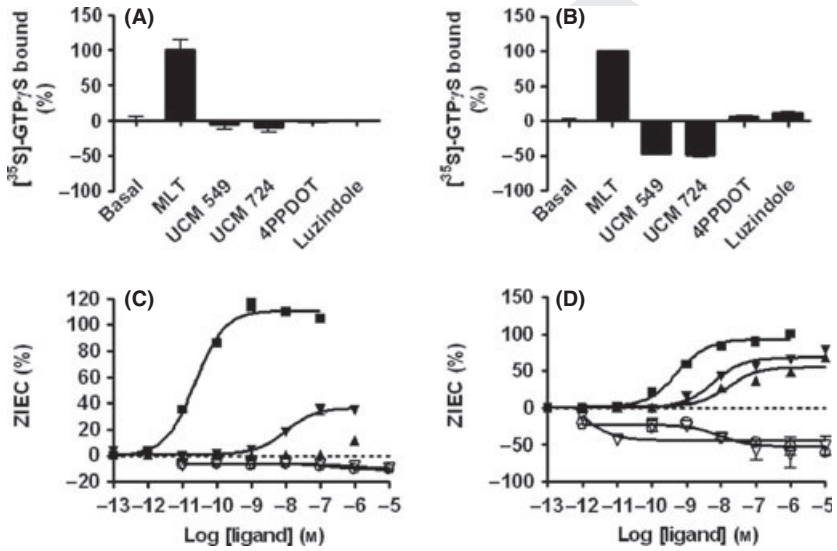

Fig. 5. Identification of inverse agonist of hMT receptors using guanosine 5′-[γ-^{35}S]-triphosphate ([^{35}S]-GTPγS) binding (A, B) and cellular impedance measurement (C, D). The activity of G proteins, presented as [35S]-GTPγS bound (%), was assessed in CHO-K1-hMT1 (A) and CHO-K1-hMT2 (B), in the absence or presence of ligands: 5 μM melatonin or 0.1 μM UCM 549, UCM 724, luzindole or 4-P-PDOT. Raw data were normalized with basal level of activity of G proteins corresponding to 0% and the MLT-stimulated activity of G proteins corresponding to 100%. A and B histograms represent each of one experiment repeated three times. The data points and error bars represent mean of triplicate and standard error, respectively. The cellular impedance response (ZIEC) was measured on CHO-K1-hMT1 (C) and CHO-K1-hMT2 (D), in the presence of increased concentration of melatonin (■), UCM 549 (○), UCM 724 (□), luzindole (▲), or 4-P-PDOT (▼). C and D graphs represent each of one experiment repeated three times. The data points and error bars represent mean of duplicate and standard error, respectively.

several conformational states [41], related to different levels of activation of the receptor, one can consider positive agonists as ligands stabilizing the active conformation of the receptor, while inverse agonists stabilize the inactive state of the receptor. Therefore, such compounds are valuable tools for characterizing the constitutive activity of a receptor. We revisited part of our melatonin-oriented chemical library and noticed a series of intriguing results that escaped our attention without the present context. Two heterotricyclic ligands recently reported with antagonistic activity, UCM 549 and UCM 724 [30], were re-investigated and revealed, indeed, an inverse agonist effect at the melatonin receptors (Fig. 5). These compounds decreased basal [^{35}S]-GTPγS binding of CHO-K1-hMT_2 cells by 35%. To further document and consolidate this activity, the compounds were evaluated using cellular dielectric spectroscopy, a technique which has not made its place to a large number of laboratories, yet, but that allows to measure GPCR signaling [42, 43]. Using this technology, UCM 549 and UCM 724 showed a decrease in 42% of the basal signal. Hence, our data consistently show that both UCM 549 and UCM 724 behave as inverse agonists at hMT_2 receptor. Very interestingly, these compounds were not able to have any significant effect on hMT_1 constitutive

activity, using either [^{35}S]-GTPγS binding or CDS readouts. These results suggest that UCM 549 and UCM 724 behave as antagonists at hMT_1, while they display inverse agonist properties at hMT_2.

So far, only 4P-PDOT and luzindole were reported as inverse agonists at hMT_1 receptor [16, 24, 44]. They were used here as controls of our experimental conditions. Unfortunately, although, they did not show any inverse agonist behavior at either our MT_1 and MT_2 preparations, as shown in Fig. 5.

Discussion

The constitutive activity of MT receptors is poorly documented. A close examination of the three experimental papers dealing with this subject leads to a different conclusion. Indeed, human MT_1 (not MT_2) receptor has been described in 1999 by Roka et al. [16] in full mechanistic details, including some data on luzindole inverse agonism. In a second paper [24], it is reported that in isolated rat caudal arteries, in which MT_1 receptors are expressed, the treatment of the system with 4P-PDOT decreases the basal GTP binding by ~30%. Interestingly, the inhibition is inversely proportional to the concentration of the compound (while clearly seen at 100 nM, it is more modest at 1 μM and not significant at 10 μM). A feature that shade some concerns onto the conclusion, which stated that 1/MT_1 is constitutively active and 2/4P-PDOT is an MT_1 inverse agonist, while initially described as a MT_2 full antagonist [4]. Finally, the third and most recent publication deals with a further description of two human MT_1 cell lines expressing either high (158 fmol/mg prot) or low (88 fmol/mg prot) concentrations of receptors and that show MT_1 receptor constitutive activity or not, respectively [25]. The paper further described the internalization of the receptor that rendered the exact quantification and qualification of the process difficult.

In summary, various reports state that MT_1 was constitutively active, including, indirectly, in native tissues, but no report exists on the status of MT_2. On the other hand, 4P-PDOT, a former MT_2 receptor antagonist, and luzindole, a MT_1/MT_2 receptor ligand, were both reported as MT_1 inverse agonists. With these data available, we felt that there was some place for clarification of the constitutive activity of melatonin receptors, including a search for inverse agonists, despite a statement in the most recent reference review on the subject [4], which postulates that it is now well accepted. This view seems, therefore, a little ahead of our time, because there is still some place for experiments to further document this subject.

In our CHO system, but also when expressed in Neuro2A cells, we confirm that MT_1 is constitutively active, and, for the first time, we show that MT_2 receptors are also constitutively active when expressed in the same cells. The level of their constitutive activity is in line with what has been reported in the literature for MT_1, and, for the sake of orthogonal comparison, for H_3.

As discussed before in this section, the experimental data supporting an inverse agonism of 4P-PDOT and luzindole, all come from a single laboratory, were scarce and, at least, arguable, in particular because the experimental models used were complex, and the readout not specific of melatonin receptors [24, 45, 46]. Besides published results [47], one of our own reports [34] showed that these compounds are in fact partial agonists at melatonin receptors, using either [^{35}S]-GTPγS or the CDS technology. To identify new compounds with inverse agonism, we relied on the long-standing activity of medicinal chemistry of melatoninergic ligands, which yielded a variety of chemical series, bearing either agonist or antagonist activities. Noteworthily, the inverse agonist property of a ligand appears only when dedicated experimental set-up are used, i.e., where the receptor does show constitutive activity, and when the data processing can adequately detect reverse effects of the compounds on the basal signal. If these experimental requirements are not met, a genuine inverse agonist will appear with simple antagonist properties. Indeed, a retrospective analysis of marketed drugs with attached antagonist properties showed that 70% of them did have, in fact, an inverse agonist profile, when evaluated in the appropriate conditions [reviewed in Ref. 48]. Our prospection led to the identification of two compounds that behave as inverse agonist to MT_2, while they have a simple antagonist profile on MT_1 receptors. These compounds can now be considered as new tools to investigate the constitutive activity of melatonin receptors in different experimental systems.

From the perspective of pharmacological and/or physiological pertinence, constitutive activity of receptors are important both at the drug discovery level, which makes intensive use of heterologous systems for screening drug candidates, and at the physiological level, for understanding the fundamental function of a given receptor and its mechanism of action as a target in a therapeutic project such as the melatonin field. Therefore, the in vitro observation of a constitutive activity takes a broader meaning when it is confirmed in physiological conditions. The in vivo constitutive activity of receptors has been documented with, for instance, histamine receptor H_3 [35], the constitutive activity of which is important for the control of histamine functions in the brain. Other classical examples for constitutive GPCR activity in native systems are the β-adrenergic receptor in turkey erythrocyte membranes and intact cardiomyocytes [49, 50] as well as the muscarinic receptor M_2 in cardiac atrial membranes [51]. In this context, reports on GPCR constitutive activity in intact organs are of particular importance. For example, constitutive activity has been observed for β-adrenergic receptor expressed in normal heart tissues [52], α_{1A} and α_{1B}-adrenergic receptor in intact rat aorta [53], and the histaminic receptor H_3 in rat hypothalamus [54]. Many of the constitutively active GPCRs are receptors for neurotransmitters. These findings suggest that constitutive activity of neurotransmitter GPCRs provides a tonic support for basal neuronal activity [26].

In the cases of melatonin receptors, one intriguing question is whether constitutive activity could have a physiological role in reading the rhythm of melatonin secretion. One critical parameter of melatonin secretion is the duration of its daily presence. Could the level of constitutive activity be modulated in vivo by the duration of exposition of receptors to melatonin? If it is the case,

could this regulation be involved in the effects of melatonin? The identification of inverse agonists (which suppress constitutive activity) described in the present work provides valuable tools to address these questions and to determine whether constitutive activity is implicated in the way animals read day/night transition of melatonin secretion.

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L'article était centré sur les récepteurs humains et seule une partie des résultats obtenus chez le rat et l'ovin ont été inclus. L'objectif général est de déterminer si l'activité constitutive est un phénomène général des récepteurs MT. Cette partie a donc pour objet de compléter cet article d'une part en décrivant l'activité constitutive des récepteurs MT de rat et ovin et, d'autre part, en identifiant des agonistes inverses.

Le niveau d'activité constitutive des récepteurs couplés aux protéines G est mesuré par le test de liaison du [^{35}S]-GTPγS. En absence de mélatonine, ce test montre l'activité basale, ou constitutive du récepteur, alors que l'utilisation de la mélatonine permet de mesurer la réponse maximale des protéines G couplés aux récepteurs MT. Dans ces conditions, les cellules CHO-K1 exprimant de façon stable les récepteurs rMT_1 et rMT_2 montrent une liaison du [^{35}S]-GTPγS de 637.1 ± 23.47 et 79.74 ± 4.24 fmol.mg^{-1} de protéines, respectivement, alors que les cellules CHO-K1 natives montrent une liaison du [^{35}S]-GTPγS de 40.41 ± 0.62 fmol.mg^{-1} de protéines. Ceci suggère que les récepteurs rMT_1 et rMT_2 possèdent une activité spontanée en absence de mélatonine. Pour les récepteurs oMT_1 et oMT_2 exprimés stablement dans la lignée CHO-K1, la liaison du [^{35}S]-GTPγS s'élève à 59.08 ± 2.97 et 319.6 ± 9.63 fmol.mg^{-1} de protéines, respectivement. Ces valeurs suggèrent que les récepteurs oMT_1 et oMT_2 comme les récepteurs de rat possèdent une activité constitutive. En présence de 5µM de mélatonine, la liaison du [^{35}S]-GTPγS s'élève à 1218 ± 29.97 et 189.4 ± 7.095 fmol.mg^{-1} de protéines pour les récepteurs rMT_1 et rMT_2 et à 316.3 ± 6.64 et 728.1 ± 41.77 fmol.mg^{-1} de protéines pour les récepteurs oMT_1 and oMT_2 (Figure 2 de l'article). L'ensemble de ces résultats montrent que les récepteurs MT de rat et ovin possèdent une activité constitutivement et que celle-ci recouvre 52%, 42%, 18% et 44% de l'activité maximale des récepteurs rMT_1, rMT_2, oMT_1 et oMT_2, respectivement.

Il est clairement établi que l'activité constitutive des récepteurs couplés aux protéines G est portée par le couplage puissant entre les protéines G et les récepteurs. La force de ce couplage est mesurée par l'IC_{50} de la suramine, agent découplant les récepteurs des protéines G (Roka, 1999). La figure 6 montre l'effet dose-dépendant de la suramine sur la liaison du [^{35}S]-GTPγS sur les cellules CHO-K1 exprimant le récepteur rMT_2. L'IC_{50} de la suramine pour ces expériences est de 10.2 ± 3.24 µM en conditions basales et de 13.0 ± 2.01 µM en conditions de stimulation par la mélatonine. Des expériences similaires ont été conduites sur les CHO-K1-rMT_1, CHO-K1-oMT_1, CHO-K1-oMT_2 et CHO-K1 natives. Ces expériences montrent des valeurs d'IC_{50} allant de 80.7 ± 5.47 µM pour CHO-K1-oMT_1 à 10.2 ± 3.24 µM

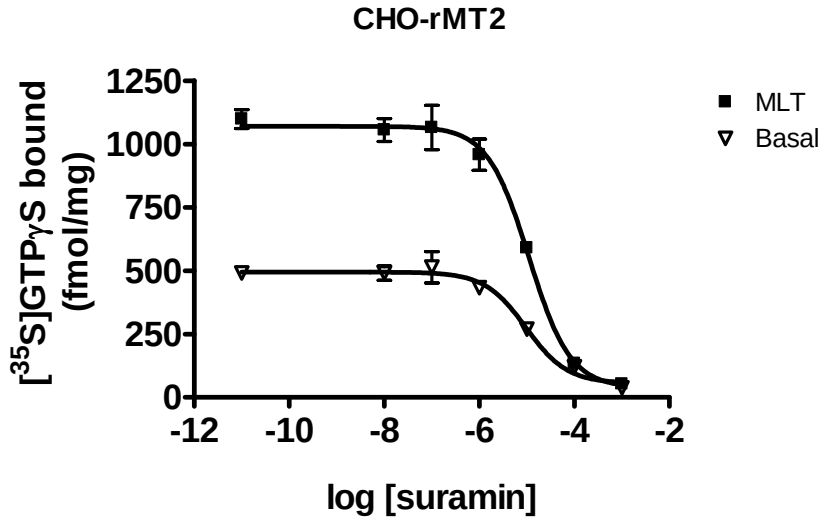

Figure 6 : Effet dose-dépendant de la suramine sur la liaison du [^{35}S]-GTPγS sur CHO-K1-rMT$_2$. L'activité des protéines G, représentée par la liaison du [^{35}S]-GTPγS (fmol/mg), est évaluée en absence (symbole ∇) ou en présence (symbole ■) de 5 µM de mélatonine.

IC$_{50}$ de la suramine (M)	**Native**	**hH$_3$**	**rMT$_1$**	**rMT$_2$**	**oMT$_1$**	**oMT$_2$**
Basal	$1.56.10^{-5}$ ± 1.13	$1.04.10^{-5}$ ± 4.87	$7.46.10^{-5}$ ± 2.81	$1.02.10^{-5}$ ± 3.24	$8.07.10^{-5}$ ± 5.47	$4.76.10^{-5}$ ± 1.60
Agoniste naturel	$2.17.10^{-5}$ ± 1.12	$6.57.10^{-6}$ ± 3.71 (histamine)	$1.22.10^{-4}$ ± 1.76 (mélatonine)	$1.30.10^{-5}$ ± 2.01 (mélatonine)	$1.90.10^{-4}$ ± 3.19 (mélatonine)	$5.02.10^{-5}$ ± 1.34 (mélatonine)

Table 2: Valeurs des IC$_{50}$ de la suramine pour différents récepteurs. Les valeurs d'IC$_{50}$ de la suramine, représentées par la liaison du [^{35}S]-GTPγS, sont évaluées en absence (conditions basales) et en présence de mélatonine et avec des concentrations croissantes de suramine. Les valeurs d'IC$_{50}$ sont exprimées en molaire avec l'erreur standard. Les valeurs d'IC$_{50}$ sont représentatives d'une expérience (en duplicat) répétée trois fois.

pour CHO-K1-rMT_2. Les valeurs d'IC_{50} de la suramine pour le récepteur à l'histamine H3 est de 10.4 ± 4.87 µM en conditions basales et de 6.57 ± 3.71 µM en conditions de stimulation par l'histamine (Table 2). Les valeurs d'IC50 de la suramine obtenues pour les récepteurs MT de rat et ovin sont du même ordre de grandeur d'une part, que les valeurs des récepteurs MT humaines et d'autre part, que les valeurs obtenues pour le récepteur H3, qui est connu pour être constitutivement actif. Cette approche nous permet d'établir l'existence d'un couplage puissant des récepteurs MT avec les protéines G associées. Ceci est un argument supplémentaire dans l'existence d'une activité constitutive des récepteurs oMT et rMT.

Deux molécules UCM 549 et UCM 724, initialement connues pour être des antagonistes du récepteurs hMT_1 (Spadoni et al., 2007), ont été identifiées comme des agonistes inverses du récepteur hMT_2 (Figure 5 de l'article). UCM 549 et UCM 724 diminue la liaison basale du [^{35}S]-GTPγS des cellules CHO-K1-rMT_2 de 9% et de 10%, respectivement. Ces composés diminuent également la liaison du [^{35}S]-GTPγS des cellules CHO-K1-oMT_2 de 10% et de 32%, respectivement. Ces molécules ne semblent pas être des agonistes inverses du récepteur rMT_1. En revanche, elles se comportent comme des agonistes inverses sur le récepteur oMT_1, en diminuant la liaison basale du [^{35}S]-GTPγS de 35% et de 43%, respectivement (Figure 7). Avec la figure 8, nous confirmons par spectroscopie diélectrique cellulaire que les molécules UCM 549 et UCM 724 présentent des propriétés agonistes inverses sur les récepteurs oMT_2 et rMT_2.

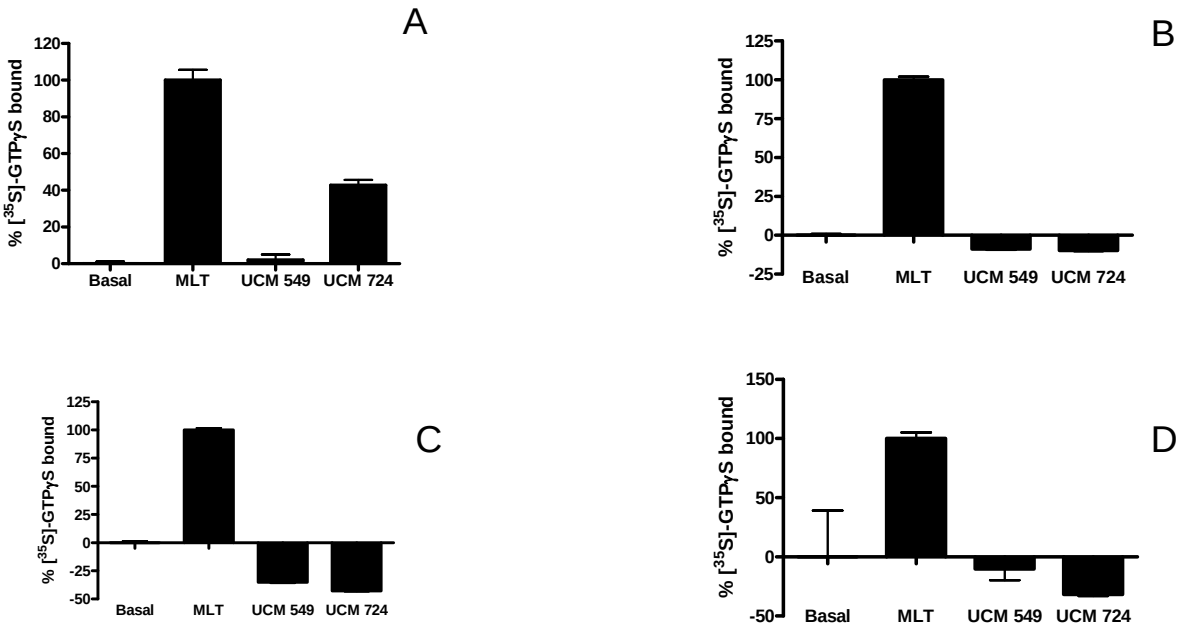

Figure 7: Identification des agonistes inverses des récepteurs MT de rat et ovin exprimés dans la lignée CHO-K1. L'activité des protéines G, représentée par le pourcentage de [35S]-GTPγS lié, est évaluée en absence ou en présence de plusieurs ligands : 5 µM de mélatonine, 0.1 µM de UCM 549 et 0.1µM de UCM 724. Les données brutes sont normalisées avec le niveau basal de l'activité des protéines G comme 0% et l'activité des protéines G stimulées par la mélatonine comme 100%. Les histogrammes représentent chacun une expérience répétée trois fois. Les points de données et les barres d'erreur représentent la moyenne des triplicats et l'erreur standard, respectivement. A) CHO-K1-rMT_1; B) CHO-K1-rMT_2; C) CHO-K1-oMT_1 ; D) CHO-K1-oMT_2.

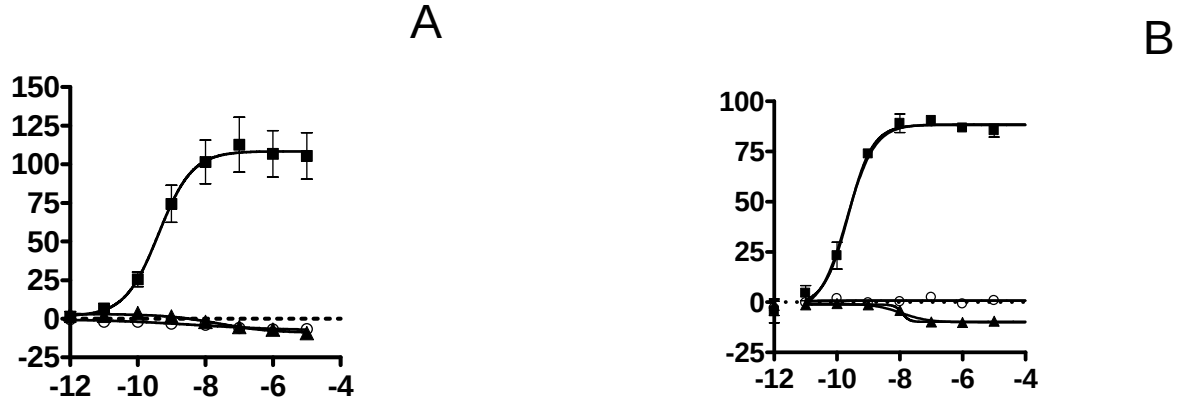

Figure 8 : Identification des agonistes inverses des récepteurs MT_2 de rat and ovin exprimés dans la lignée CHO-K1. L'activité des proteins G, représentée par la liaison du [35S]-GTPγS (fmol/mg), est évaluée en absence et en présence des concentrations croissantes de plusieurs ligands: mélatonine (symbole ■), UCM 549 (symbole ○) et UCM 724 (symbole ▲). A) CHO-K1-rMT_2; B) CHO-K1-oMT_2.

Conclusion

Dans nos conditions expérimentales, nous avons réussi à mettre en évidence l'existence d'une activité constitutive des récepteurs hMT_1 et hMT_2 exprimés de façon stable dans les lignées cellulaires CHO-K1 et Neuro2A. Les récepteurs MT_1 et MT_2 de rat et ovin exprimés de façon stable dans la lignée CHO-K1 présentent également la capacité à s'activer spontanément en absence de mélatonine. D'une façon générale, l'activité constitutive des récepteurs mélatoninergiques serait portée essentiellement par un couplage des récepteurs avec les protéines Gi. Nous avons montré que plusieurs facteurs pouvaient moduler le niveau d'activité constitutive. D'une part, le niveau de cette activité constitutive est influencé par la nature de la lignée cellulaire, puisqu'il est plus important pour les récepteurs hMT exprimés dans la lignée Neuro2A que dans la lignée CHO-K1. D'autre part, le niveau d'expression du récepteur influence le degré d'activité constitutive. Ainsi, l'environnement cellulaire est essentiel pour l'établissement de l'activité constitutive des récepteurs de la mélatonine.

L'identification d'agonistes inverses permet de corroborer l'existence d'une activité constitutive pour les récepteurs MT_2 humain, ovin et de rat. Deux molécules, UCM 549 et UCM 724, se comportent comme des agonistes inverses pour ces récepteurs.