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Melatonin differentially modulates the expression and function of the hMT_1 and hMT_2 melatonin receptors upon prolonged withdrawal

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Abstract

Melatonin is synthesized and released following a circadian rhythm and reaches its highest blood levels during the night. It relays signals of darkness to target tissues involved in regulating circadian and seasonal rhythms. Here, we report the expression of human melatonin receptors type 1 and 2 (hMT_1 and hMT_2, respectively) in Chinese hamster ovary (CHO) cells following exposure to melatonin treatments mimicking the amplitude (400 pM) and duration (8 hr) of the nightly melatonin peak and upon withdrawal. Exposure of CHO-MT_1 cells to melatonin (400 pM) for 0.5, 1, 2, 4, and 8 hr significantly increased specific 2-[^{125}I]iodomelatonin (500 pM) binding to hMT_1 melatonin receptors upon 16-hr withdrawal. However, the same treatment did not affect the expression of hMT_2 melatonin receptors. The increase in specific 2-[^{125}I]iodomelatonin (500 pM) binding ($162 \pm 29\%$, N = 3, $P < 0.05$) 16 hr after melatonin withdrawal was parallel to increases in hMT_1 melatonin receptor mRNA ($231 \pm 33\%$, N = 4, $P < 0.05$). This effect was due to an increase in the total number of hMT_1 receptors [B_{max} 833 ± 97 fmol/mg protein (N = 3), control; 1449 ± 41 fmol/mg protein (N = 3), treated], with no change in binding affinity. The melatonin-mediated increase in MT_1 melatonin receptor expression upon withdrawal was not mediated through either a direct effect of the hormone in the promoter's vector or in the rate of mRNA degradation. In conclusion, melatonin differentially regulates the expression of its own receptors, which may have important implications in the transduction of dark signals *in vivo*.

Keywords: MT_1 melatonin receptors; CHO cells; mRNA; G-proteins

1. Introduction

The synthesis and release of the hormone melatonin from retinal photoreceptors and pineal pinealocytes follow a circadian rhythm with high levels during the hours of darkness. The duration of this nocturnal peak depends upon the length of the dark period, providing circadian and seasonal rhythms of melatonin exposure [1,2]. In mammalian native tissues, melatonin modulates retina, circadian, and vascular function by the activation of membrane-bound receptors that belong to a subfamily of G-protein-coupled receptors [3–6]. Two molecularly and pharmacologically distinct mammalian melatonin receptors have been identified: MT_1 and MT_2 [7]. Activation of either receptor inhibits forskolin-stimulated cAMP formation through a pertussis toxin-sensitive G-protein [8–10]. However, differential signaling pathways for both receptors have also been observed [7]. Additionally, MT_1 and MT_2 melatonin receptors are differentially regulated by short-term exposure to the agonist [11].

Activation of G_i-coupled receptors is known to induce supersensitization of the cAMP signaling pathways in recombinant systems as well as in native tissues. Exposure of primary cultures of ovine pars tuberalis cells to 1 nM melatonin for 16 hr results in an enhanced cAMP response to forskolin (sensitization) by a mechanism involving

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Abbreviations: CHO, Chinese hamster ovary; cAMP, cyclic AMP; SCN, suprachiasmatic nucleus; hMT_1 and hMT_2, human melatonin receptors type 1 and 2, respectively; SSPE, 0.15 M sodium chloride, 10 mM $Na_2H_2PO_4$, 1 mM EDTA; SSC, 0.15 M sodium chloride, 0.015 M sodium citrate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; and CMV, cytomegalovirus.

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tyrosine phosphorylation [12]. Similarly, exposure of the hMT_1 melatonin receptor to a physiological melatonin concentration (400 pM, 8 hr) significantly potentiates the forskolin-stimulated cAMP signal transduction cascade in a time-dependent manner after withdrawal, reaching a maximal effect at 4 hr that is maintained, at least up to 16 hr [13]. In the present study, we investigated whether a similar phenomenon occurs in cells expressing the MT_2 melatonin receptor.

G-protein-coupled receptor systems desensitize, internalize, and down-regulate upon exposure to agonists, as classically shown for the β_2-adrenergic, M_2 acetylcholine, or D_1 dopamine receptors, among numerous other examples (for review, see Ref. [14]). The density of native melatonin receptors is also regulated by exposure to the hormone. In the rat SCN, 2-[^{125}I]iodomelatonin binding sites probably of the MT_1 type [15] are down-regulated during subjective night, when melatonin levels are high [16]. Treatments that reduce plasma melatonin, such as constant light or pinealectomy, increase the expression of melatonin receptors in the pars tuberalis and the SCN, while administration of melatonin reverses this effect [17]. In ovine pars tuberalis cells, exposure to melatonin for 24 hr reduces specific 2-[^{125}I]iodomelatonin binding [18] and represses the transcription of MT_1 receptor mRNA via a cAMP-independent signal transduction pathway [19] determined after a 15-min wash. Paradoxically, for other G-protein-coupled receptors, prolonged exposure to their respective agonists induces an increase in receptor protein (or both protein and mRNA) expression, immediately following exposure. This phenomenon was observed for the 5-hydroxytryptamine$_2$ receptor [20], the β_3-adrenergic receptor [21], the D_2 dopamine receptor [22,23], and the D_3 dopamine receptor [24]. The goal of this study was to assess the role of melatonin in regulating the expression of the MT_1 and MT_2 melatonin receptors immediately following prolonged exposure to melatonin and upon withdrawal. We demonstrated that prolonged treatment (8 hr) with physiological concentrations of melatonin did not affect the adenylyl cyclase system upon withdrawal in CHO cells expressing the hMT_2 melatonin receptor, and that this treatment increased the expression of the hMT_1, but not that of the hMT_2 melatonin receptor. We conclude that exposure to physiological concentrations of melatonin differentially regulates the expression and function of the MT_1 and the MT_2 melatonin receptors upon withdrawal of the hormone.

2. Materials and methods

2.1. Development of CHO cell lines stably expressing human melatonin receptors

CHO cell cultures were grown as monolayers in F12 medium supplemented with 10% fetal bovine serum, penicillin (100 units/mL), and streptomycin (100 μg/mL) in 5% CO_2 at 37°. Complementary DNAs expressing the coding region of either the human MT_1 (subcloned into pcDNAI) or the human MT_2 (subcloned into pcDNA3) melatonin receptor genes were provided by Dr. Steven Reppert, Department of Neurobiology, University of Massachusetts Medical School [8,10]. The vectors contain a CMV promoter for high levels of expression in eukaryotic cells. CHO cells were co-transfected with pcDNAI-hMT_1 or pcDNA3-hMT_2 and pSV-NEO plasmids using lipofectamine (Life Technologies, Gibco BRL), as already described [9]. Cells expressing the melatonin receptor were selected for their resistance to the antibiotic G418 at 300 μg/mL (Life Technologies, Gibco BRL) and their ability to bind specifically 2-[^{125}I]iodomelatonin (2200 Ci/mmol, NEN/DuPont). The CHO-MT_1 and CHO-MT_2 cell lines used in this study, expressing a density of ~800 fmol/mg protein of melatonin receptor, were originated from a single cell selected using the limited dilution protocol.

2.2. Melatonin treatment and withdrawal

Figure 1 shows a schematic representation of the experimental protocol. CHO cells expressing hMT_1 receptors were plated either on 24-well dishes for the measurement of 2-[^{125}I]iodomelatonin binding and cAMP accumulation

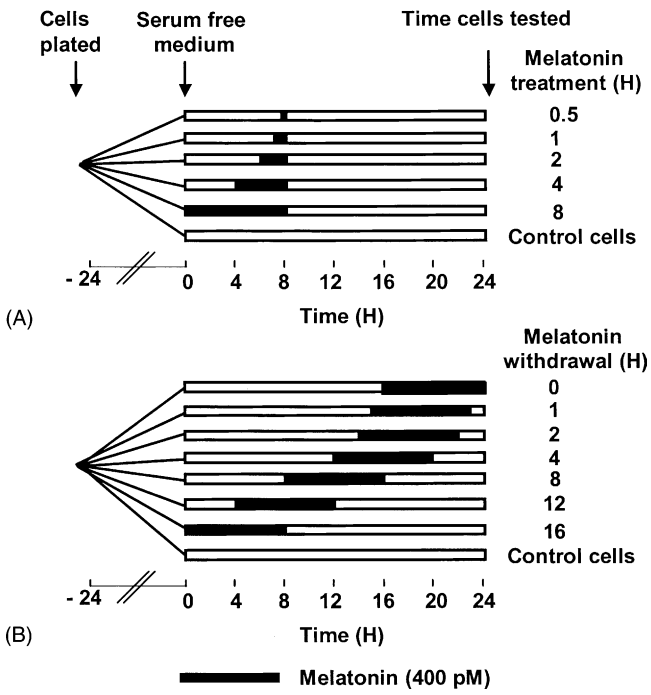

Fig. 1. Schematic representation of the experimental protocol for melatonin treatment and withdrawal. CHO-MT_1 cells were grown in F12 medium supplemented with 10% fetal bovine serum for 24 hr. Subsequently, all the cells were grown in serum-free F12 medium for 24 hr. To account for differences in cell growth, treatments with melatonin (400 pM, 8 hr) were staggered so that all the cells completed the withdrawal period at the same time.

assays or on 10-cm dishes for RNA isolation. Cells were grown for 24 hr in F12 medium supplemented with 10% fetal bovine serum and then in serum-free F12 medium for 24 hr. The concentration of melatonin in the stock fetal bovine serum used in these experiments was 40 pM, as determined by the Buhlmann (ALPCO) melatonin radioimmunoassay kit. The final melatonin concentration in the incubation medium containing 10% fetal bovine serum, therefore, was 4 pM. Cells exposed to this medium were washed before incubation in serum-free medium, suggesting that any residual melatonin might be below the necessary concentration to activate the MT_1 melatonin receptor. The effect of the length (0.5, 1, 2, 4, or 8 hr) of melatonin exposure on specific 2-[^{125}I]iodomelatonin binding to CHO-MT_1 and CHO-MT_2 cells was determined 16 hr after the withdrawal of melatonin (Fig. 1A). To study the effect of melatonin withdrawal, cells were treated with 400 pM melatonin for 8 hr and after that were washed twice with PBS and grown in F12 medium without serum for different periods (0-, 1-, 2-, 4-, 8-, 12-, or 16-hr withdrawal). To account for differences in cell growth, the treatments were staggered so that all the cells completed the withdrawal period at the same time (Fig. 1B). The vehicle for melatonin (400 pM) was ethanol (0.000001%). Treatment of CHO-MT_1 cells with vehicle alone did not affect any of the parameters tested (data not shown).

2.3. cAMP accumulation assays

cAMP accumulation assays were carried out as described [13]. Briefly, forskolin-induced [^{3}H]cAMP accumulation was measured in CHO-MT_1 and CHO-MT_2 cells. Cells, grown to confluence, were incubated with F12 medium containing 2 μCi/mL of [^{3}H]adenine (26.9 Ci/mmol; NEN/DuPont) in the absence (containing vehicle) or presence of melatonin (400 pM) for 8 hr. The medium containing melatonin was aspirated, and cells were incubated with F12 medium containing 1 μCi/mL of [^{3}H]-adenine. Sixteen hours after the withdrawal of melatonin, cAMP formation was stimulated by the addition of F12 medium containing 100 μM forskolin (Sigma) and 30 μM rolipram (RBI). The plates were incubated for 10 min at 37°, and the reaction was terminated by aspiration of the medium and the addition of 1 mL of ice-cold (5%) trichloroacetic acid (16 hr at 4°) to release [^{3}H]cAMP into the solution. [^{3}H]cAMP was separated from [^{3}H]ATP using Dowex (AG50W-X4, Bio-Rad) and alumina (Sigma) column chromatography. [^{3}H]cAMP was quantified by liquid scintillation counting.

2.4. Radioligand binding assays

Binding assays were performed using the radioligand 2-[^{125}I]iodomelatonin on intact cells grown to confluence in 24-well dishes. After completion of melatonin treatment and withdrawal periods, cells were washed twice with PBS (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 0.62 mM KH_2PO_4, pH 7.4). Binding assays were initiated upon the addition of 2-[^{125}I]iodomelatonin (500 pM) in 0.5 mL of Krebs solution (100 mM NaCl) in the absence or presence of melatonin (1 μM). Cells were incubated at 37° for 90 min, and washed four times with 0.5 mL of PBS to remove unbound radioactivity, detached in LIFT buffer (0.25 M sucrose, 10 mM potassium phosphate, 1 mM EDTA, pH 7.4). Radioactivity was assessed in a gamma counter. B_{max} and K_d were determined by saturation binding of 2-[^{125}I]iodomelatonin to membranes obtained from CHO-MT_1 cells treated with or without melatonin. After melatonin treatment, cells were washed with 3 mL of PBS, collected in LIFT buffer, and pelleted by centrifugation (25,000 *g*, 10 min, 4°). Membrane pellets were resuspended in Tris–HCl buffer (50 mM Tris–HCl, pH 7.4; 10 mM $MgCl_2$) to give a final concentration of ~2–5 μg protein/tube. A volume of 200 μL of membrane suspension was incubated with different concentrations of 2-[^{125}I]iodomelatonin in a total volume of 260 μL and incubated for 90 min at 25°. Reactions were terminated by the addition of ice-cold Tris–HCl buffer (50 mM) and rapid filtration over glass-fiber filters (Schleicher & Schuell) soaked in 0.5% polyethylenimine solution. Each filter was washed twice with 5 mL of ice-cold Tris–HCl buffer and counted in a gamma counter. Non-specific binding was determined with 10 μM melatonin. Scatchard plot analysis was performed using commercial software (GraphPad PRISM®).

2.5. Northern blot analysis

Following melatonin treatment and withdrawal, total RNA was isolated from the cells using TRIzol reagent (GIBCO BRL, Life Technologies), according to the protocol of the manufacturer; the pellet was dissolved in diethylpyrocarbonate-treated water and stored at −80° until used. Twenty micrograms of RNA was denatured in formaldehyde/formamide and separated by electrophoresis in the presence of formaldehyde on a 1.2% agarose gel, transferred to a nylon-supported nitrocellulose membrane (Duralon-UV, Stratagene), and then UV crosslinked. As a probe, we used a randomly primed ^{32}P-labeled full-length transcript of the hMT_1 or hMT_2 melatonin receptors, excised from the corresponding vector by *Hin*dIII-*Xho*I digest. Hybridization conditions were 50% formamide, 6 × SSPE, 5 × Denhardt's solution, 100 μg/mL of salmon sperm DNA, and 0.5% SDS for 18 hr at 42°, according to standard procedures. Membranes were washed in 2 × SSC, 0.1% SDS for 30 min at room temperature, 1 × SSC, 0.1% SDS for 30 min at 37°, with a final wash in 0.1 × SSC, 0.1% SDS for 30 min at 55°. Under these conditions, two main transcripts were observed for the hMT_1 receptor (1.9 and 1.3 kb) and for the hMT_2 receptor (1.4 and 0.8 kb). To control for variability in loading, blots stripped of the melatonin receptor probe were then hybridized with a

^{32}P-labeled GAPDH cDNA probe [25] that hybridized with a unique 1.3 kb mRNA. Blots were exposed to Fuji plates at room temperature for 18 hr and quantified using a Fuji BAS2000 PhosphorImaging Analysis System (Fuji Photo Film Co.). The expression level of the hMT_1 melatonin receptor mRNA relative to that of the GAPDH was used for quantification. The level of expression for hMT_1 melatonin receptor mRNA for each treatment was determined in each experiment by dividing the signal intensity of the 1.9 kb hMT_1 band by that of the GAPDH band (hMT_1/GAPDH). To determine RNA stability, actinomycin D (20 μg/mL) was added to the cells at the time of melatonin withdrawal. RNA was isolated from samples of cells taken at several times after the addition of actinomycin D, and RNA blot analysis was performed as described above. To determine the mRNA decay constants, data were graphed as the percent of RNA remaining versus the time after the addition of actinomycin D, and the slopes of the linear portions of the decay curves were calculated by linear regression.

2.6. Transient transfections and β-galactosidase activity

The effect of melatonin on the CMV promoter of the pcDNAI plasmid was studied on CHO-MT_1 cells transiently transfected with a β-galactosidase plasmid driven by a CMV promoter. CHO-MT_1 cells were grown as monolayers in F12 medium supplemented with 10% fetal bovine serum, penicillin (50 units/mL), and streptomycin (100 μg/mL) in 5% CO_2/95% air at 37°. Cells were transfected using the DEAE-dextran method as previously described [26]. Twenty-four hours after transfection, cells were treated with 400 pM melatonin for 8 hr. After 16 hr of melatonin withdrawal, the medium was removed, monolayers were washed with PBS, and the cells were collected in lysis buffer (0.1 M KPO_4, 1% Triton X-100, 1 mM dithiothreitol, and 2 mM EDTA). β-Galactosidase activity was determined using ONPG (*o*-nitrophenyl-β-D-galactopyranoside) as substrate following a standard procedure [27]. Protein content was determined as described by Bradford [28].

2.7. Statistical analyses

All experiments were performed at least three times. Quantitative results are expressed as means ± SEM. Statistical analyses were performed using GraphPad PRISM®. Differences in forskolin-stimulated cAMP formation between treated and control cells for each cell line were assessed by two-way ANOVA, using the Bonferroni test as *post hoc* analysis. Comparison of the effect of melatonin treatment on forskolin stimulation between the two cell lines was assessed using the unpaired *t*-test. Differences in 2-[^{125}I]iodomelatonin binding were assessed by ANOVA of repeated measures. Significant variation in mRNA levels was assessed by one-way ANOVA (time dependency), and *post hoc* analysis was performed using the Newman–Keuls test. The results were considered significant if the probability of error was lower than 5%.

3. Results

The effect of a physiological concentration of melatonin (400 pM) on the regulation of the hMT_1 and hMT_2 melatonin receptors expressed in CHO cells was studied during withdrawal. The concentration of melatonin used corresponds to approximately the highest level measured in human blood during the night [2,29]. Before exposure to melatonin, cells were serum-starved to minimize the effects of growth factors, estrogen, melatonin, and other serum components.

We previously reported that exposure of CHO-MT_1 cells to 400 pM melatonin for a period of 8 hr significantly potentiated forskolin-stimulated cAMP formation upon withdrawal [13]. Here, we have extended this finding to compare the effect of prolonged treatment (8 hr) with physiological concentrations of melatonin (400 pM) after 16 hr of withdrawal on forskolin-stimulated cAMP formation from CHO cells expressing either hMT_1 or hMT_2 melatonin receptors. Forskolin stimulation increased cAMP formation in both CHO-MT_1 cells [basal: 2060± 30; forskolin: 4670 ± 270 cpm/well (N = 6)] and CHO-MT_2 cells [basal: 1130 ± 250; forskolin: 3090 ± 320 cpm/well (N = 6)]. Exposure of CHO-MT_1 cells to melatonin treatment and withdrawal resulted in a significant potentiation of forskolin stimulation [basal: 2060 ± 50; forskolin: 8130 ± 950 cpm (N = 6)] (Fig. 2A; [13]). Two-way ANOVA revealed a significant interaction between forskolin stimulation and melatonin treatment ($F_{1,20} = 12.1$, $P < 0.005$). By contrast, the same treatment did not affect forskolin-stimulated cAMP formation in CHO-MT_2 cells (Fig. 2A).

Specific 2-[^{125}I]iodomelatonin binding to membranes from CHO cells expressing the hMT_1 or hMT_2 receptors was first studied at 16 hr upon withdrawal from exposure to melatonin (400 pM) for 0.5, 1, 2, 4, or 8 hr (see Fig. 1A). This treatment induced a time-dependent increase in specific 2-[^{125}I]iodomelatonin binding to the hMT_1 but not to the hMT_2 melatonin receptor (Fig. 2B). Upon withdrawal, the maximal increase in specific 2-[^{125}I]iodomelatonin binding to CHO-MT_1 cells was observed following treatment with melatonin for 1 hr (193.1 ± 13.1%, N = 4; $F_{5,18} = 5.75$, $P < 0.005$). Under the same experimental conditions, melatonin treatment (400 pM, 0.5, 1, 2, 4, or 8 hr) of CHO-MT_2 cells did not affect 2-[^{125}I]iodomelatonin binding 16 hr after melatonin withdrawal (Fig. 2B). In all subsequent experiments, cells were exposed to melatonin for 8 hr, mimicking the length of the nocturnal melatonin peak.

Next, we investigated the time course of 2-[^{125}I]iodomelatonin binding to hMT_1 melatonin receptors in CHO

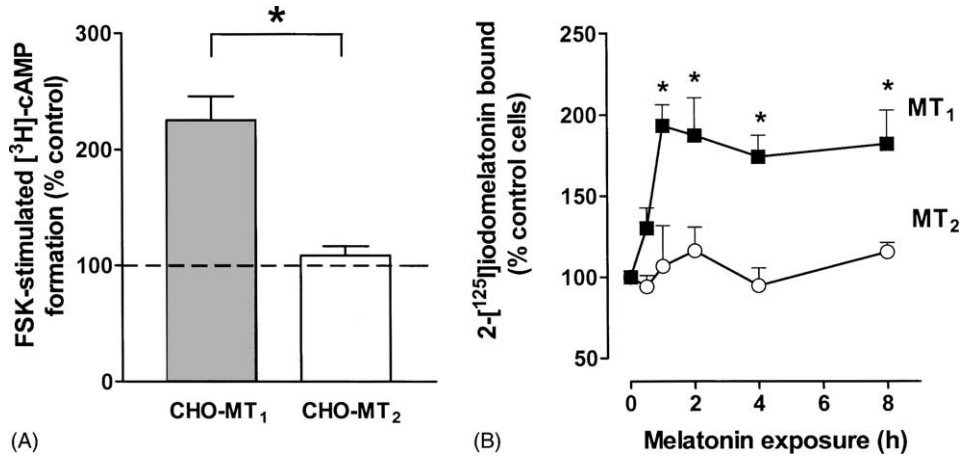

Fig. 2. Differential effects of melatonin (400 pM) treatment on CHO-MT_1 and CHO-MT_2 cells 16 hr after melatonin withdrawal. (A) Effect of melatonin treatment (8 hr) and withdrawal (16 hr) on forskolin-stimulated [^{3}H]cAMP formation in CHO-MT_1 and CHO-MT_2 cells. Cells, attached to plates, were treated with 2 μCi/mL of [^{3}H]adenine for 8 hr as described in "Section 2." Forskolin (100 μM)-stimulated [^{3}H]cAMP formation was increased significantly by melatonin treatment and withdrawal in CHO-MT_1 cells, but not in CHO-MT_2 cells. $^*P < 0.001$ when compared with the percent increase in CHO-MT_1 cells (*t*-test). (B) Effect of length of melatonin exposure on 2-[^{125}I]iodomelatonin binding to CHO-MT_1 and CHO-MT_2 cells 16 hr after melatonin withdrawal. CHO-MT_1 (closed squares) and CHO-MT_2 (open circles) cells were treated with melatonin (400 pM) for 0.5, 1, 2, 4, or 8 hr, then washed with PBS, and maintained in serum-free medium for an additional 16 hr. After the melatonin withdrawal period, cells were incubated in Krebs solution (100 mM NaCl) containing 2-[^{125}I]iodomelatonin (500 pM) for 90 min. Data points represent means ± SEM of three independent experiments performed in duplicate. Time-dependent (0–8 hr) exposure to melatonin (400 pM) significantly increased 2-[^{125}I]iodomelatonin binding upon 16-hr withdrawal in CHO-MT_1 ($F_{5,18} = 6.99$, $P < 0.01$) but not CHO-MT_2 cells ($F_{5,12} = 0.7$, $P > 0.05$). $^*P < 0.05$ when compared with CHO-MT_1 control cells.

cells following exposure to melatonin that mimicked the amplitude (400 pM) and length (8 hr) of the dark phase (see Fig. 1B). Withdrawal from melatonin induced a gradual increase in specific 2-[^{125}I]iodomelatonin binding that reached a plateau 4 hr after withdrawal, which was maintained for up to 16 hr ($F_{6,12} = 3.53$, $P < 0.05$) (Fig. 3). Saturation analysis with 2-[^{125}I]iodomelatonin revealed that the increase in specific binding was due to an increase in the total number of binding sites ($B_{max} = 832 \pm 97$ and 1449 ± 41 fmol/mg protein in control and melatonin-treated cells, respectively) without changes in binding affinity for the radioligand ($K_D = 128 \pm 30$ and 117 ± 37 pM in control and melatonin-treated cells, respectively) (Fig. 4).

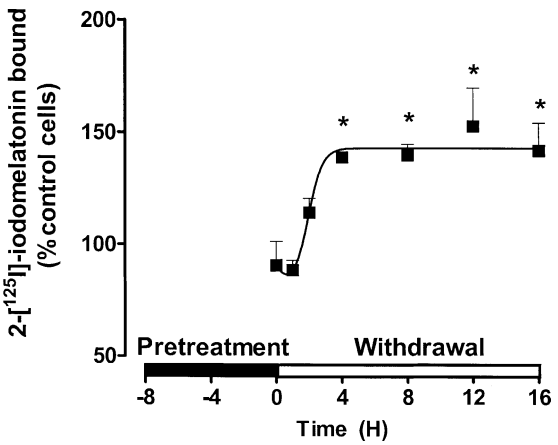

Fig. 3. Time course of 2-[^{125}I]iodomelatonin binding to hMT_1 melatonin receptors following melatonin withdrawal. Cells, attached to plates, were treated with 400 pM melatonin for 8 hr. At different times after withdrawal, the cells were washed with PBS and incubated in Krebs solution (100 mM NaCl) containing 2-[^{125}I]iodomelatonin (500 pM) for 90 min. Data points represent means ± SEM of three independent experiments performed in duplicate. 2-[^{125}I]Iodomelatonin binding increased in a time-dependent manner reaching ~145% of untreated control cells by 16 hr (one-way ANOVA, $F_{7,23} = 3.53$, $P < 0.05$). $^*P < 0.05$ in comparison with control.

The time course (0, 1, 16 hr) of MT_1 melatonin receptor mRNA expression in CHO-MT_1 cells following withdrawal from melatonin exposure (400 pM, 8 hr) was determined by Northern blot analysis. MT_1 Melatonin receptor mRNA expression increased in a time-dependent fashion after cessation of the treatment, reaching 231 ± 33% (N = 4) of control values, 16 hr after melatonin withdrawal ($F_{3,12} = 3.72$, $P < 0.05$) (Fig. 5A and B). MT_2 mRNA expression was not modified following melatonin exposure (400 pM for 8 hr) and withdrawal (16 hr) (Fig. 5C).

We next assessed whether the melatonin-mediated increase in hMT_1 melatonin receptor expression was mediated by a direct effect of melatonin on the CMV promoter of the pcDNAI plasmid. CHO cells were transfected with a plasmid expressing β-galactosidase driven by a CMV promoter and treated with vehicle or melatonin (400 pM) for 8 hr. The β-galactosidase activity was not affected upon withdrawal (16 hr) of melatonin treatment (102.3 ± 5.2%, N = 4 compared with vehicle-treated cells).

The effect of withdrawal (16 hr) from melatonin (8 hr, 400 pM) treatment on the rate of mRNA degradation was measured following inhibition of RNA transcription with actinomycin D (20 μg/mL). The calculated rate of hMT_1 mRNA degradation in vehicle (slope: -0.098 ± 0.027 hr^{-1}) and melatonin-treated cells (slope: -0.095 ± 0.024 hr^{-1}) was not significantly different (Fig. 6).

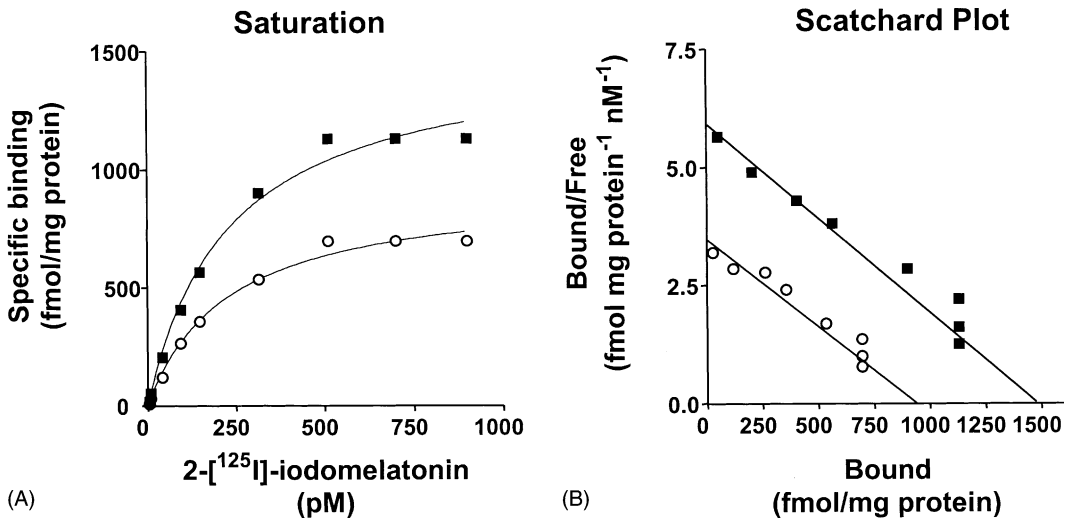

Fig. 4. Saturation analysis of 2-[^{125}I]iodomelatonin binding to CHO-MT_1 cells, 16 hr after melatonin (400 pM) withdrawal. CHO-MT_1 cells were treated with (closed squares) or without (open circles) 400 pM melatonin for 8 hr, washed twice with PBS, and then grown in F12 medium without serum for 16 hr. Membranes were then prepared and incubated with various concentrations of 2-[^{125}I]iodomelatonin for 1 hr at 25°. Non-specific binding was determined in the presence of 10 μM melatonin. Specific binding is defined as total binding minus non-specific binding. Similar results were obtained in two other independent experiments performed in duplicate.

4. Discussion

In the present study, we have demonstrated that activation of hMT_1 melatonin receptors by physiological concentrations of melatonin for a length of time that mimics the nocturnal secretion of the hormone induced a time-dependent increase in hMT_1 melatonin receptor density and mRNA expression following withdrawal. This effect was selective for the hMT_1 melatonin receptor, as it was not observed in cells expressing the hMT_2 receptor. Taken together these results suggest that melatonin may differentially regulate MT_1 and MT_2 melatonin receptor expressions and, therefore, this differential effect on its own receptors may play a role in the regulation of circadian function.

Melatonin exposure appears to differentially regulate the expression of MT_1 and MT_2 melatonin receptors in CHO cells as only the density of hMT_1 receptors was increased

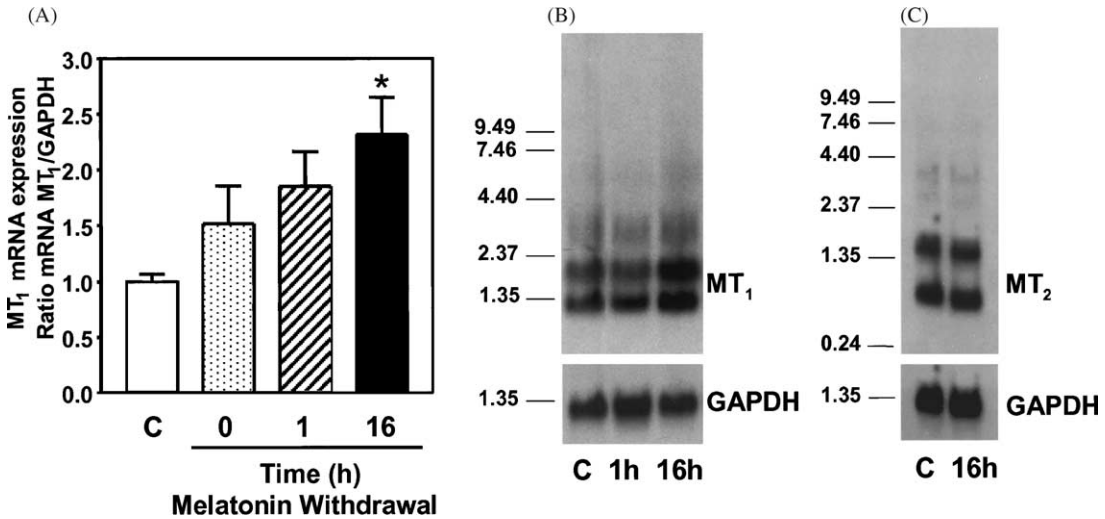

Fig. 5. hMT_1 and hMT_2 melatonin receptor mRNA expressions after melatonin withdrawal. CHO-MT_1 or CHO-MT_2 cells were treated with melatonin (400 pM) followed by 0 hr, 1 hr (MT_1), or 16 hr (MT_1 and MT_2) withdrawal. Control, untreated cells (C) were run in parallel. At the end of the experiment, the medium was removed, total RNA was isolated, and 20 μg of total RNA was separated by electrophoresis as indicated in "Section 2." (A) The amount of hMT_1 melatonin receptor mRNA was compared with the abundance of GAPDH mRNA and expressed as the hMT_1 melatonin receptor/GAPDH ratio. Data represent the means ± SEM of four independent experiments performed in duplicate [one-way ANOVA: $F_{3,12} = 3.72$, (*) $P < 0.05$ in comparison with control cells]. (B) Representitive autoradiograms showing MT_1 melatonin receptor mRNA expression in comparison with the abundance of GAPDH in untreated cells (control: C), and at 1 or 16 hr after withdrawal from melatonin treatment. (C) Representative autoradiograms showing MT_2 mRNA expression in untreated cells (control: C) or 16 hr after withdrawal from melatonin treatment.

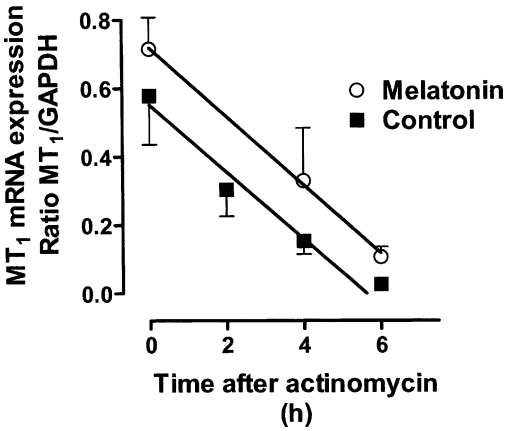

Fig. 6. Rate of degradation of hMT_1 melatonin receptor mRNA following inhibition of mRNA transcription with actinomycin D. CHO-MT_1 cells were treated in the absence (control) or presence of melatonin (400 pM) for 8 hr. After melatonin withdrawal, cells were exposed to 20 μg/mL of actinomycin D, and RNA was isolated after 0, 2, 4, and 6 hr. Total RNA (20 μg) was separated by electrophoresis as indicated in "Section 2." The amount of hMT_1 melatonin receptor mRNA was compared with the abundance of GAPDH mRNA and expressed as the hMT_1 melatonin receptor/GAPDH ratio. Data represent the means ± SEM of four independent experiments performed in duplicate.

upon 16-hr withdrawal. Similarly, differences in the regulation of hMT_1 and hMT_2 melatonin receptors immediately after agonist exposure have also been reported. Specific 2-[^{125}I]iodomelatonin binding to the hMT_1 receptor was not affected by exposure to 1 μM melatonin for different periods of time (1, 6, 18, and 24 hr) [9] or showed only a small but significant decrease following treatment with 100 nM or 1 μM melatonin for 10 min [11]. On the other hand, hMT_2 melatonin receptors are readily internalized by melatonin at concentrations as low as 10 nM [11] with no change in affinity. However, in spite of the rapid internalization of hMT_2 melatonin receptors, no up-regulation was observed after melatonin withdrawal, supporting the idea that the up-regulation observed for the hMT_1 receptors is not a compensatory mechanism to desensitization. Differential regulation of the $D_{2\text{-long}}$ and the D_1 dopamine receptor was also observed. The $D_{2\text{-long}}$ dopamine receptor is up-regulated [23,30,31], while the D_1 dopamine receptor is down-regulated by treatment with an agonist [31]. The use of chimeric constructs between the D_1 and $D_{2\text{-long}}$ dopamine receptors allowed the identification of the transmembrane domain V and the third cytoplasmatic loop as the regions involved in the differential regulation of the $D_{2\text{-long}}$ and D_1 dopamine receptors [31]. Significant differences in the third intracellular loop's amino acid sequence and, therefore, in the presence of putative phosphorylation sites between hMT_1 and hMT_2 melatonin receptors, as well as activation of different signaling pathways [7], may account for the differential regulation by melatonin. In previous reports, melatonin has been shown to decrease 2-[^{125}I]iodomelatonin binding to hMT_1 melatonin receptors [11], in contrast to the lack of effect observed in the present study immediately after melatonin removal. Methodological differences can explain these results, since here we determined 2-[^{125}I]iodomelatonin binding to whole cells attached to plates, instead of a membrane preparation [11]. As melatonin binds with very high affinity to the MT_1 melatonin receptor [9], it is possible that the lack of a detectable increase in 2-[^{125}I]iodomelatonin binding immediately after removing melatonin was due to the presence of the hormone still bound to the receptor.

Prolonged activation of melatonin receptors leading to the supersensitization of the adenylyl cyclase system also differentiates hMT_1 from hMT_2 melatonin receptors. Increased response to adenylyl cyclase activation has been demonstrated in ovine pars tuberalis cells (expressing endogenous MT_1 melatonin receptors) after an 8-hr exposure to 1 μM melatonin [18], in neonatal rat pituitary cells after a 12-hr exposure to 2 nM melatonin [32], and in CHO cells expressing the hMT_1 melatonin receptor after an 8-hr exposure to 400 pM melatonin and at least a 4-hr withdrawal (present study; [13]). However, here we demonstrated that prolonged activation of the hMT_2 melatonin receptor did not result in increased forskolin-stimulated cAMP formation. Activation of different signaling pathways by the two receptors may differentially affect the responsiveness of the cell to a stimulatory input to the cAMP-mediated pathway.

Melatonin exposure increased hMT_1 melatonin receptor mRNA expression and receptor density upon withdrawal in a time-dependent manner. An increase in G-protein-coupled receptor expression immediately after prolonged exposure to an agonist has been reported for other G-protein-coupled receptors, i.e. 5-hydroxytryptamine$_2$ [20], β_3-adrenergic [21], D_{2L} dopamine [22,23,30,31], and D_3 dopamine [24] receptors. However, the mechanism(s) by which melatonin receptor expression (2-[^{125}I]iodomelatonin binding and hMT_1 mRNA) increased following prolonged exposure to melatonin differs, in that it requires the withdrawal of the hormone to be observed (Figs. 3 and 5 of the present study). We did not observe any significant changes immediately after withdrawal. However, the effects of melatonin on the expression of its own receptor seem to be cell- or tissue-specific since in cultured ovine pars tuberalis cells, 24 hr of melatonin exposure reduces specific 2-[^{125}I]iodomelatonin binding to cell membranes [18] and represses the transcription of MT_1 receptor mRNA via a cAMP-independent signal transduction pathway [19] determined immediately after melatonin exposure (15-min wash). A different milieu of G-proteins or different signal transduction mechanisms activated by melatonin receptors in both cell types could account for the different results. Furthermore, in the ovine pars tuberalis cells, the presence of different types of alleles for the MT_1 receptor [33], as well as other not yet determined melatonin receptor types, could account for the different results. Additionally, melatonin could be affecting gene transcription directly through interactions with regulatory

regions of the promoter of the hMT_1 receptor in the pars tuberalis, while the hMT_1 receptor expressed in the CHO-MT_1 cells is lacking the promoter.

Agonist-dependent up-regulation of G-protein-coupled receptors has been attributed to increases in mRNA stability or transcription [20,21,30] or to an increase in translation or protein stability [23]. In the present study, CHO cells were transfected with an hMT_1 melatonin receptor cDNA under the control of a strong viral CMV promoter possessing several elements that could respond to changes in cellular cAMP levels (cAMP response elements, CRE) [34]. However, the possibility that increased cAMP levels resulting from prolonged stimulation and withdrawal of melatonin [13] could be responsible for the up-regulation of hMT_1 receptors after melatonin withdrawal seems unlikely. Melatonin treatment and withdrawal did not increase β-galactosidase activity in cells transfected with a β-galactosidase cDNA under the control of a CMV promoter or alter the expression of the hMT_2 melatonin receptor under the same promoter. We cannot exclude the possibility, however, that the coding region of the melatonin receptor gene contains responsive elements that are involved in cell line-specific up-regulation of the hMT_1 melatonin receptor mRNA, as demonstrated for the androgen receptor [35]. On the other hand, the rate of degradation of the MT_1 melatonin receptor mRNA is not modified by exposure to melatonin, as demonstrated for the ovine pars tuberalis receptor [19] and for the recombinant hMT_1 receptor expressed in CHO cells in the present study. It needs to be taken into account, however, that actinomycin D, used to determine the rate of mRNA degradation, may have inhibited the transcription of putative stability factors induced by melatonin treatment, therefore, obscuring any change in the rate of melatonin receptor mRNA degradation. In conclusion, melatonin directly or through induction of factors within the cell may regulate the transcription of either the hMT_1 melatonin receptor mRNA or mRNA-stabilizing factors, resulting in the observed increases in mRNA levels.

Increased stability of the receptor protein could also potentially underlie the increases in 2-[^{125}I]iodomelatonin binding after melatonin exposure. A high proportion of MT_1 melatonin receptors are constitutively active [7,36,37], and it is known that exposure to an agonist or an inverse agonist, by stabilizing the free form of the receptor, can decrease the rate of constitutively active receptor degradation [38,39]. Therefore, if the presence of melatonin for 8 hr decreased hMT_1 melatonin receptor degradation, then the net result would be an increase in the level of melatonin receptor protein. Although this is an attractive possibility, it may not be the only mechanism responsible for the increase in 2-[^{125}I]iodomelatonin binding reported here, since this phenomenon was not observed immediately but appeared gradually after withdrawal, reaching a maximum at 4 hr that persisted for up to 16 hr.

In conclusion, the results presented here may add to the understanding of the circadian mechanisms of melatonin action. Long exposure to melatonin (as occurs during the night) induces sensitization of the cAMP-mediated signaling following melatonin withdrawal [13]. As melatonin is released in a circadian fashion with high levels during the night, this effect could prepare the cell to receive a stimulatory signal to the adenylyl cyclase system during the day. We have also now demonstrated that withdrawal from melatonin treatment may increase the expression of MT_1 melatonin receptor mRNA and protein. Consistent with this idea, we found that both diurnal and circadian rhythms of 2-[^{125}I]iodomelatonin binding in the C3H/HeN mouse SCN peak at the beginning of the day, after the drop in melatonin levels [40]. Another factor acting during the day may determine the decrease in receptor density, allowing the generation of the rhythm. These effects of melatonin on its own receptor expression appear to be cell- or tissue-specific as well as melatonin receptor-type selective, and may begin to explain the diverse physiological effects of melatonin.

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