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Melatonin-mediated regulation of human MT_1 melatonin receptors expressed in mammalian cells

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Abstract

In mammals, the pineal hormone melatonin activates G protein-coupled MT_1 and MT_2 melatonin receptors. Acute exposure of recombinant MT_1 and MT_2 melatonin receptors to supraphysiological concentrations of melatonin differentially regulates these two receptors with the MT_2, but not the MT_1, exhibiting rapid desensitization and internalization. In the present study, we sought to determine whether prolonged exposure to supraphysiological and physiological concentrations of melatonin desensitized and/or internalized the MT_1 melatonin receptor. Using a Chinese hamster ovary (CHO) cell line stably expressing MT_1-FLAG or transiently expressing MT_1-green fluorescent protein (GFP) melatonin receptors, we found that prolonged exposure (8 h) to supraphysiological concentrations of melatonin (100 nM) significantly increased the number of MT_1 melatonin receptors and decreased the affinity (K_i) of melatonin for competition for 2-[^{125}I]iodomelatonin. A similar treatment also desensitized the MT_1 melatonin receptor-mediated stimulation of [^{35}S]GTPγS binding, but did not internalize the receptor. In contrast, prolonged exposure to a concentration of melatonin mimicking nocturnal levels (400 pM) did not affect the number of MT_1 melatonin receptors, the affinity for melatonin, or the functional sensitivity of the receptor. We conclude that in vivo endogenous melatonin does not significantly affect the functional sensitivity of MT_1 melatonin receptors, however, exogenous melatonin taken therapeutically at doses above physiological levels could desensitize the receptor thereby affecting physiological responses mediated following activation of MT_1 melatonin receptors.

Keywords: Melatonin; MT_1 melatonin receptor; G protein-coupled receptor; Desensitization; Internalization; Circadian rhythms

1. Introduction

The pineal hormone melatonin is synthesized and released following a circadian rhythm with high levels at night (~400 pM) and low levels during the day (~40 pM) in humans and rodents [1–3]. In mammals, melatonin activates two high affinity G protein-coupled membrane receptors—the MT_1 and MT_2. These receptors are molecularly and pharmacologically distinct, signal through pertussis-toxin-sensitive G proteins to inhibit forskolin-stimulated cAMP formation, and mediate a myriad of functional responses [3–5]. Understanding the mechanism(s) of melatonin receptor regulation in target tissues is important to elucidate the role of both endogenous and exogenously applied melatonin on receptor function.

In the rodent suprachiasmatic nucleus (SCN), melatonin receptor density follows a diurnal rhythm, however, the shape of this rhythm varies among laboratories and species [2,6,7]. Changes in specific 2-[^{125}I]iodomelatonin binding in the rodent SCN is attributed to MT_1 melatonin receptors, as the radioligand does not detect MT_2 binding sites in native tissues [8–10]. Tenn and Niles [2] suggested

Abbreviations: CHO, Chinese hamster ovary; GFP, green fluorescent protein; h, human; PBS, phosphate-buffered saline; SCN, suprachiasmatic nucleus

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that this diurnal rhythm of MT_1 melatonin receptor density is regulated directly by melatonin as specific 2-[^{125}I]iodomelatonin binding was highest towards the end of the light cycle when serum melatonin levels are the lowest. In support of this view, it has been reported that a single dose of melatonin decreased MT_1 melatonin receptor density in the SCN of pinealectomized rats or rats maintained in constant light [11]. Several studies, however, suggest that this diurnal rhythm is regulated by mechanisms other than melatonin. In the SCN of rat and mouse, specific 2-[^{125}I]iodomelatonin binding is highest at the night/day transition when serum melatonin levels are the highest suggesting a regulatory mechanism independent of melatonin [6,7]. Interestingly, it has been suggested that the diurnal rhythm of MT_1 melatonin receptor density in the SCN is regulated by photic input as a light pulse during the subjective night increased specific binding [12,13]. These discrepancies regarding the mechanism by which MT_1 melatonin receptor density in native tissues is regulated may be attributed to a number of non-exclusive factors, including sex, age, species, tissue, environmental conditions, and/or clock regulation [13] (for detailed review, see Masana et al. [7]).

Evidence suggests that regulation of MT_1 melatonin receptors by melatonin depends on cellular milleu, time of exposure, and concentration [14–16]. In recombinant systems, short-term exposure of MT_1 melatonin receptors to supraphysiological concentrations of melatonin (100 nM) decreased the number of specific 2-[^{125}I]iodomelatonin binding sites, however, this treatment did not induce receptor desensitization or internalization [16]. MacKenzie et al. [15] reported, however, that a 5 h exposure to a supraphysiological concentration of melatonin (1 μM) desensitizes MT_1 melatonin receptor-mediated inhibition of forskolin-stimulated cAMP accumulation and stimulation of phosphoinositide hydrolysis. Whether the desensitization of recombinant MT_1 melatonin receptors is associated with receptor internalization has not been reported. Endogenously expressed MT_1 melatonin receptors in mouse hypothalamic GT1–7 neurons are rapidly internalized by melatonin (10 nM) exposure (10 min) through a beta-arrestin 1-dependent mechanism [14].

Treatment of circadian and sleep disorders with melatonin often raises the blood levels of the hormone to supraphysiological concentrations, which might alter the functional sensitivity of MT_1 melatonin receptors. This is important as the MT_1 melatonin receptor is involved in the regulation of neuronal firing in the SCN [8], vasoconstriction [17,18], and neuroendocrine function [19]. Thus, here we use a heterologous expression system, MT_1 melatonin receptors expressed in CHO cells, to determine the effect of prolonged exposure of MT_1 melatonin receptors to supraphysiological (100 nM) or physiological (400 pM) concentrations of melatonin for a time mimicking the nocturnal surge of the hormone on specific 2-[^{125}I]iodomelatonin binding, receptor desensitization and/or internalization.

2. Materials and methods

2.1. Materials

The hMT_1 ($hMel_{1a}$) melatonin receptor cDNA (cloned into pcDNAI) containing the complete coding region of the receptor was provided by Dr. S.M. Reppert (Department of Neurobiology, University of Massachusetts Medical School, Worcester, MA, USA) [20]. Effectene transfection and plasmid DNA purification kits were obtained from Qiagen. Cell culture products were obtained from Invitrogen. 2-[^{125}I]Iodomelatonin (SA: 2000 Ci/mmol) was purchased from Amersham and [^{35}S]GTPγS from PerkinElmer Life Sciences. Melatonin and other general reagents were obtained from Sigma. Wheat-germ agglutinin/Texas red conjugate and Slowfade antifade reagent were purchased from Molecular Probes.

2.2. Melatonin treatment and membrane preparation for 2-[^{125}I]iodomelatonin binding studies

CHO cells stably expressing hMT_1 melatonin receptors with an N-terminal FLAG epitope (CHO-MT_1) (B_{max}: 640 ± 111 fmol/mg protein, $n = 3$) were cultured to 80–90% confluency in serum-containing media. Cells were then incubated in serum-free media for 5 h and washed once with phosphate-buffered saline (PBS). Cells were then treated with vehicle or melatonin (100 nM or 400 pM) in serum-free media at 37 °C for 10, 60, 180, or 480 min. In time course experiments, treatments were staggered so that all cells were harvested at the same time. Therefore, cells were exposed to serum-free media for a total of 13 h. Cell membranes were harvested and specific 2-[^{125}I]iodomelatonin binding was determined as previously described [16]. Briefly, binding reactions were started by adding cell membranes to tubes containing binding buffer (50 mM Tris–HCl, pH 7.4, 10 mM $MgCl_2$), 2-[^{125}I]iodomelatonin (∼100 pM), and ∼15–30 μg of crude washed membranes in a total assay volume of 0.26 ml. After incubating for 1 h at 25 °C, reactions were terminated by rapid vacuum filtration through glass fiber filters (Schleicher & Schuell No. 30) soaked in 0.5% polyethylenimine solution. Nonspecific binding was determined in the presence of 1 μM melatonin. Radioactivity was determined in a gamma counter.

2.3. [^{35}S]GTPγS binding assays

Binding assays were performed as previously described with modification [21]. CHO-MT_1 cells were serum starved for 5 h and treated with vehicle or melatonin

(100 nM or 400 pM) for 8 h. Cells were harvested in membrane buffer (50 mM Tris–HCl, pH 7.4, 3 mM $MgCl_2$, 1 mM EGTA) and pelleted by centrifugation at 4 °C (23,400 × *g*). Cell pellets were resuspended in membrane buffer and pelleted by centrifugation at 4 °C (13,800 × *g*). Cell pellets were stored at −80 °C. Membranes (~20–30 μg protein) were incubated in assay buffer (50 mM Tris–HCl, pH 7.4, 3 mM $MgCl_2$, 150 mM NaCl, 0.2 mM EGTA) in the presence of 10 μM GDP, 0.04 nM [^{35}S]GTPγS (~80,000–100,000 cpm/tube) in a total volume of 520 μl. [^{35}S]GTPγS binding was stimulated by melatonin (10 nM to 1 μM). Assays also included a blank (no membranes) and a total (no GDP). Assays were incubated at 37 °C for 1 h and the reaction terminated by dilution with 50 mM Tris–HCl (pH 7.4) and vacuum filtration through glass fiber filters (Schleicher & Schuell No. 30). Filters were washed twice with ice-cold 50 mM Tris–HCl (pH 7.4). Bound [^{35}S]GTPγS was determined using liquid scintillation counting.

2.4. Confocal microscopy

CHO cells in culture at 40–50% confluence were transiently transfected with hMT_1 melatonin receptors containing an N-terminal FLAG epitope and a C-terminal GFP epitope (CHO-MT_1-GFP). The FLAG or GFP epitopes did not interfere with melatonin binding or receptor function [16]. Cells were then incubated in serum-free media for 5 h, washed once in PBS, and treated with vehicle or 100 nM melatonin at 37 °C in serum-free media for 10–480 min. Cell treatments were staggered so all cells were washed once with PBS for 5 min and fixed with 4% paraformaldehyde for 7.5 min at the same time. Cells were then incubated with wheat-germ agglutinin/Texas red conjugate (2 μg/ml) for 30 min at room temperature. Cells were incubated with 4% paraformaldehyde for 20 min at room temperature and washed twice with PBS. Coverslips were then mounted with Slowfade antifade reagent and visualized on an Olympus confocal microscope.

2.5. Data analysis and statistics

Percent changes in specific 2-[^{125}I]iodomelatonin binding relative to control were calculated by dividing the average specific binding (fmol/mg protein) defined by a particular melatonin concentration by the average specific binding (fmol/mg protein) of the vehicle-treated time-matched control. K_i values were calculated from IC_{50} values in competition experiments using the Cheng and Prusoff equation [22]. The K_D value used in this equation was previously reported [16]. Statistical significance was determined by paired Student's *t*-test for comparisons between two groups, or by one-way ANOVA with Dunnett's post-test for comparisons between more than two groups. A value of $P < 0.05$ was taken as statistically significant.

3. Results

3.1. Time-dependent regulation of 2-[^{125}I]iodomelatonin binding to CHO-MT_1 cell membranes following melatonin pretreatment

CHO-MT_1 cells kept in culture were pretreated with 100 nM melatonin for 10, 60, 180, or 480 min. Melatonin pretreatment induced a time-dependent increase in specific 2-[^{125}I]iodomelatonin binding to CHO-MT_1 cell membranes measured immediately after exposure to melatonin. Specific binding increased in a time-dependent manner and was significantly higher after 8 h pretreatment (Fig. 1). The affinity (K_i) of melatonin to compete for 2-[^{125}I]iodomelatonin binding in CHO-MT_1 cells pretreated for 8 h with melatonin was slightly but significantly decreased (0.84 ± 0.05 nM, $n = 3$, $P < 0.05$, paired Student's *t*-test) compared to vehicle-treated cells (0.38 ± 0.01 nM, $n = 3$) (Fig. 2A and B).

Pretreatment of CHO-MT_1 cells with a physiological concentration of melatonin (400 pM) for a time mimicking the nocturnal surge of the hormone (8 h) did not affect specific 2-[^{125}I]iodomelatonin binding (172.6 ± 27.1 fmol/mg protein, $n = 5$) compared to vehicle control (175.9 ± 27.4 fmol/mg protein, $n = 5$) (Fig. 2C and D). This treatment also did not affect the affinity (K_i) of melatonin for competition for 2-[^{125}I]iodomelatonin (vehicle: 0.30 ± 0.06 nM versus melatonin: 0.44 ± 0.07 nM, $n = 5$) (Fig. 2C and D).

3.2. Functional sensitivity of MT_1 melatonin receptors following melatonin pretreatment

The potency of melatonin to stimulate [^{35}S]GTPγS binding was determined in crude CHO-MT_1 cell mem-

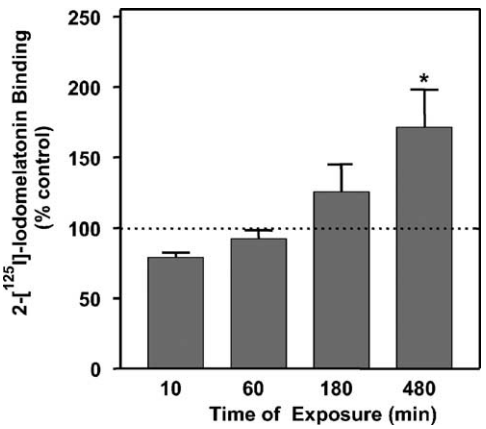

Fig. 1. Specific 2-[^{125}I]iodomelatonin binding to CHO-MT_1 cells following exposure to melatonin. CHO-MT_1 cells were serum starved for 5 h and then treated in situ with vehicle or 100 nM melatonin for 10, 60, 180, or 480 min. Cells were harvested and membranes prepared as described under Section 2. The ordinate represents specific [2-^{125}I]iodomelatonin binding expressed as a percent of the appropriate vehicle-treated time-matched control. Data represent the mean ± S.E.M. of three to six independent experiments performed in duplicate.

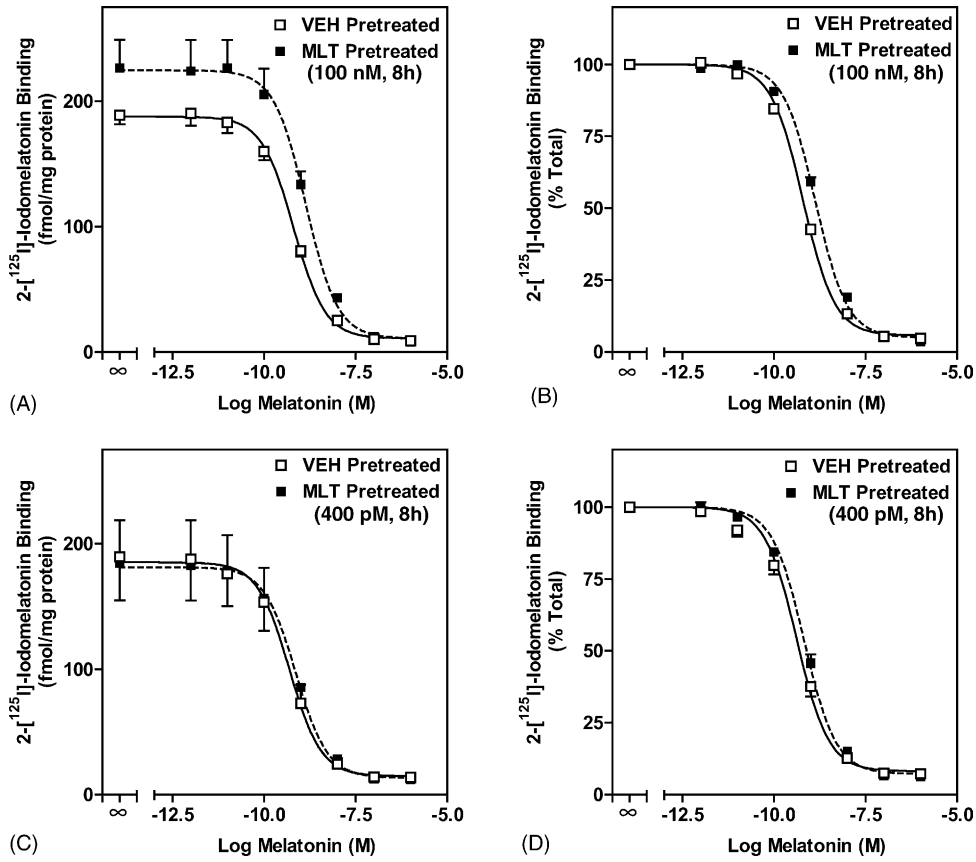

Fig. 2. The affinity of melatonin to compete for 2-[^{125}I]iodomelatonin binding in CHO-MT$_1$ cells following exposure to melatonin. CHO-MT$_1$ cells were serum starved for 5 h and then treated in situ with vehicle or melatonin [(100 nM, panels A and B) or (400 pM, panels C and D)] for 8 h. Cells were harvested and membranes prepared as described under Section 2. The affinity of melatonin was determined in competition (1 pM to 1 μM) for [2-^{125}I]iodomelatonin. The ordinate represents specific [2-^{125}I]iodomelatonin binding expressed as fmol/mg protein (A, C) and percent of total (B, D). Data represent the mean ± S.E.M. of three to five independent experiments performed in duplicate. Data were analyzed using a paired Student's *t*-test. VEH, vehicle. MLT, melatonin.

branes following vehicle or melatonin (100 nM or 400 pM) pretreatments for 8 h. Basal [^{35}S]GTPγS binding was not affected following pretreatment with 100 nM (30.8 ± 7.1 fmol/mg protein, $n = 4$) or 400 pM melatonin (27.0 ± 3.3 fmol/mg protein, $n = 5$) compared to vehicle controls (29.8 ± 6.8 fmol/mg protein, $n = 4$, or 30.4 ± 3.6 fmol/mg protein, $n = 5$, respectively). In vehicle pretreated cells, melatonin stimulated [^{35}S]GTPγS binding with an EC_{50} of 0.56 ± 0.15 nM ($n = 4$) (Fig. 3A). Pretreatment of CHO-MT$_1$ cells with 100 nM melatonin

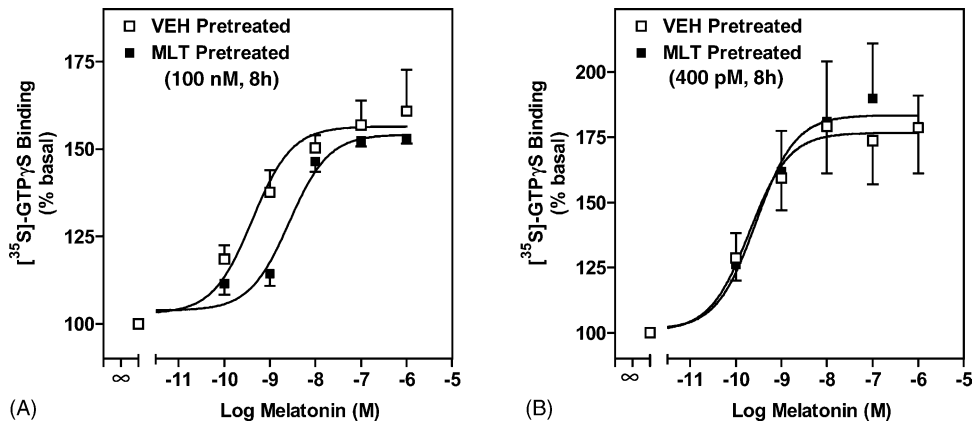

Fig. 3. [^{35}S]GTPγS binding to CHO-MT$_1$ cell membranes following exposure to melatonin. CHO-MT$_1$ cells were serum starved for 5 h and then treated in situ with either vehicle or melatonin (100 nM, panel A) or (400 pM, panel B) for 8 h. Cells were harvested and membranes prepared as described under Section 2. The ordinate represents [^{35}S]GTPγS binding expressed as percent of basal incorporation. [^{35}S]GTPγS binding was stimulated with melatonin (10 nM to 1 μM). Data represent the mean ± S.E.M. of four to five independent experiments performed in duplicate. VEH, vehicle. MLT, melatonin.

significantly reduced the potency of melatonin to stimulate [^{35}S]GTPγS binding (EC_{50}: 3.0 ± 0.91 nM, $n = 4$, $P < 0.05$, compared to vehicle-treated control, paired Student's *t*-test) (Fig. 3A). The maximal efficacy of 1 μM melatonin to stimulate [^{35}S]GTPγS binding was similar in vehicle ($160.9 \pm 11.8\%$, $n = 4$) and melatonin-treated cells ($153.1 \pm 1.5\%$, $n = 4$) (Fig. 3A). By contrast, the potency of melatonin to stimulate [^{35}S]GTPγS binding in CHO-MT_1 cells pretreated for 8 h with a physiological concentration of melatonin (400 pM) (EC_{50}: 0.53 ± 0.22 nM, $n = 5$) was not significantly different from vehicle-treated cells (EC_{50}: 0.25 ± 0.06 nM, $n = 5$) (Fig. 3B). The maximal efficacy of 1 μM melatonin to stimulate [^{35}S]GTPγS binding was similar in vehicle ($178.6\% \pm 17.4$, $n = 4$) and melatonin-treated cells ($179.0 \pm 12.0\%$, $n = 4$) (Fig. 3B).

3.3. Cellular trafficking of MT_1 melatonin receptors following exposure to melatonin

MT_1 melatonin receptor trafficking following melatonin pretreatment was assessed in CHO cells transiently expressing MT_1-GFP receptors. CHO-MT_1-GFP cells were pretreated with vehicle or melatonin and stained with

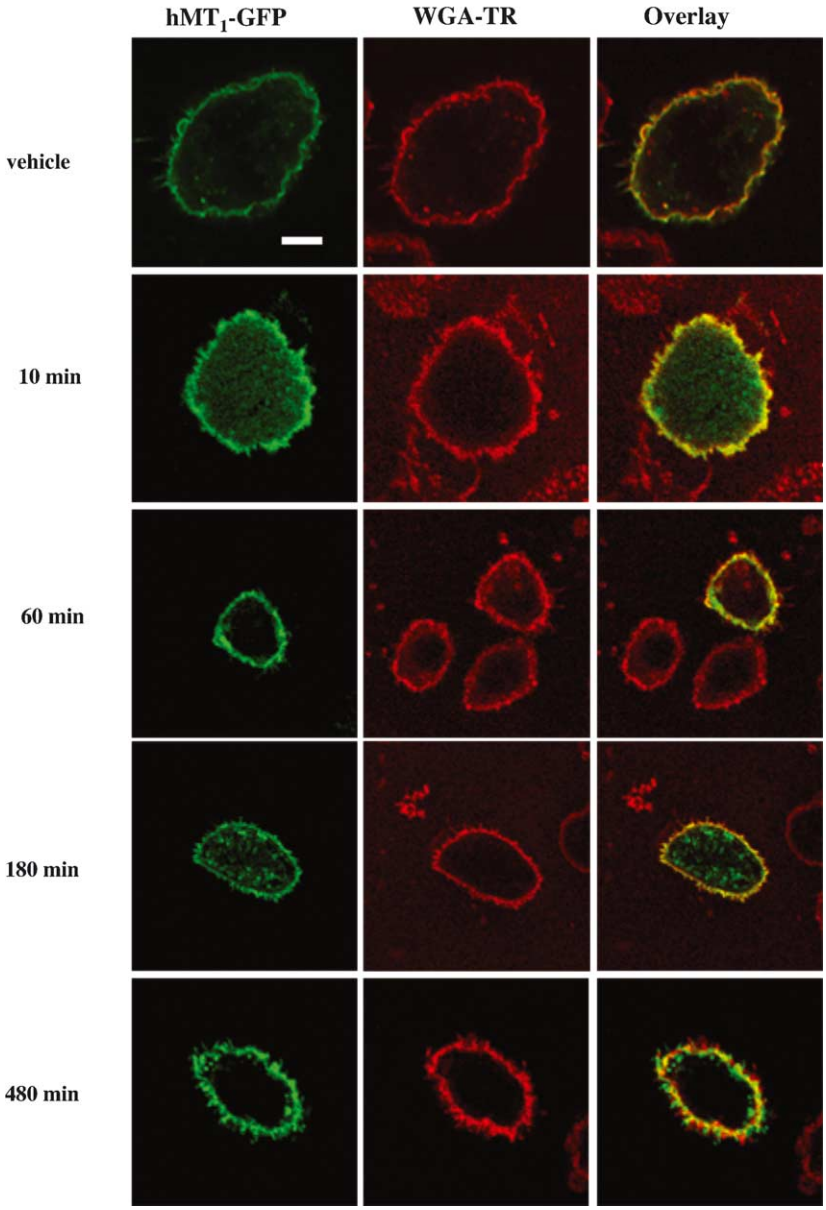

Fig. 4. Trafficking of MT_1 melatonin receptors following exposure to melatonin. CHO cells transiently transfected with MT_1-GFP receptors were serum starved for 5 h and treated in situ with vehicle or 100 nM melatonin for 10, 60, 180, or 480 min. Cells were then fixed with 4% paraformaldehyde and co-stained with wheat-germ agglutinin/Texas red conjugate (WGA-TR) to label the cell surface plasma membrane. Co-localization of the receptor with the cell surface plasma membrane is shown in yellow (overlay). Shown are representative confocal images from three independent experiments. Scale bar represents 10 μm.

wheat-germ agglutinin/Texas red to label the cell surface plasma membrane. In vehicle pretreated cells, the MT_1 melatonin receptor co-localized with the cell surface plasma membrane as determined by overlay of MT_1-GFP melatonin receptors and wheat-germ agglutinin/Texas red (Fig. 4). Pretreatment with 100 nM melatonin for 10, 60, 180, and 480 min did not affect membrane localization of the MT_1-GFP melatonin receptors (Fig. 4).

4. Discussion

The present study demonstrated that exposure to a supraphysiological concentration of melatonin for a time mimicking the nocturnal surge (8 h) significantly increased specific 2-[^{125}I]iodomelatonin binding to CHO-MT_1 cells and decreased melatonin binding affinity. Furthermore, this treatment functionally desensitized the MT_1 melatonin receptor, without inducing receptor internalization. In contrast, pretreatment of CHO-MT_1 cells with a concentration of melatonin mimicking endogenous nocturnal hormone levels did not alter receptor number, the affinity for melatonin or the functional sensitivity of the MT_1 melatonin receptor. This is the first study to investigate trafficking of recombinant MT_1 melatonin receptors and regulation of receptor function by physiological concentrations of melatonin.

Pretreatment with supraphysiological concentrations of melatonin significantly increased the number of MT_1 melatonin receptors in a time-dependent manner. Similarly, an increase in D2 dopamine receptor density following prolonged exposure to the agonist *N*-propylnorapomorphine was observed in HEK-293 cells with a $t_{1/2}$ value of 6 h [23]. The density of D2 dopamine receptors expressed in C_6 glioma cells pretreated for 14 h with the agonist *N*-propylnorapomorphine exhibited both a time- and concentration-dependent increase [24]. This increase in D2 dopamine receptor density was not due to modulation of second messengers or protein synthesis but rather to stabilization of the receptor possibly by decreasing receptor degradation [24]. Similarly, constitutively active β_2-adrenergic receptors, which are viewed as structurally unstable, are stabilized by ligands of various efficacies, including agonists, leading to an increase in receptor expression [25]. A structurally unstable α_{2a}-adrenergic receptor mutant is stabilized by an agonist leading to an increase in receptor density [26]. MT_1 melatonin receptors stably expressed in CHO cells are constitutively active [27,28]. Taken together, we suggest that prolonged exposure to supraphysiological concentrations of melatonin may stabilize inherently unstable constitutively active MT_1 melatonin receptors leading to the increase in specific 2-[^{125}I]iodomelatonin binding.

The affinity and functional sensitivity of the MT_1 melatonin receptor was decreased upon prolonged pretreatment with supraphysiological concentrations of melatonin. The decrease in melatonin affinity for the MT_1 melatonin receptor following melatonin pretreatment cannot be attributed to changes in 2-[^{125}I]iodomelatonin affinity (K_D) since this parameter was not modified following pretreatment with supraphysiological concentrations of melatonin [15,16]. Rather the decrease in melatonin affinity appears related to MT_1 melatonin receptor desensitization. Desensitization of MT_1 melatonin receptor-mediated cAMP accumulation and phosphoinositide hydrolysis was also reported following prolonged exposure to melatonin (1 μM) [15]. Similarly, exposure of 5-HT_2 receptors to agonist led to receptor desensitization by receptor/G protein uncoupling with a concomitant increase in radioligand binding [29]. We suggest that prolonged exposure of MT_1 melatonin receptors to supraphysiological concentrations of melatonin leads to receptor/G protein uncoupling thereby decreasing the affinity for the agonist melatonin and inducing receptor desensitization. We propose that the increases in specific 2-[^{125}I]iodomelatonin binding by prolonged melatonin exposure may be a compensatory mechanism protecting against sustained receptor desensitization as suggested for the 5-HT_2 receptors [29].

MT_1 melatonin receptors did not internalize upon prolonged exposure to supraphysiological concentrations of melatonin which was compatible with the observed increase in MT_1 melatonin receptor number. Although internalization mediates desensitization of G protein-coupled receptors, the two processes are not necessarily consequential [30]. For the δ opioid receptor, internalization is only part of the overall mechanism of receptor desensitization [31] while for the D1 dopamine receptor internalization is completely distinct from desensitization [32]. Prolonged exposure to supraphysiological concentrations of melatonin desensitized the MT_1 melatonin receptor, however, internalization does not appear to significantly contribute to this process. Internalization, however, of endogenous MT_1 melatonin receptors has been reported in GT1–7 neurons [14]. Our confocal microscopic images may suggest trafficking of a small receptor pool or at least loose association of MT_1 melatonin receptors with the plasma membrane particularly after 8 h exposure to melatonin. The somatostatin (sst_5) receptor rapidly internalizes, however, cell surface receptor density is maintained due to rapid recycling and recruitment of intracellular receptor stores [33]. Therefore, we cannot rule out that a small MT_1 receptor population may internalize upon melatonin exposure but does not affect receptor function perhaps due to rapid receptor recycling or receptor recruitment. The increase in specific 2-[^{125}I]iodomelatonin binding following exposure for 8 h to supraphysiological concentrations of melatonin may in fact be representative of some receptor recruitment in addition to receptor stabilization.

Prolonged exposure of MT_1 melatonin receptors to physiological concentrations of melatonin did not affect MT_1 melatonin receptor number, affinity, or functional sensitivity. This is the first study to investigate regulation

of recombinant MT_1 melatonin receptors by physiological concentrations of melatonin. These results are compatible with several findings related to regulation of endogenous MT_1 melatonin receptors by melatonin in vivo. First, the diurnal rhythm of specific 2-[^{125}I]iodomelatonin binding to endogenous MT_1 melatonin receptors expressed in the SCN appears unrelated to endogenous melatonin [6,7,13,34]. Interestingly, the diurnal variation in MT_1 melatonin receptor density as determined by specific 2-[^{125}I]iodomelatonin binding in the rat SCN was absent when rats were maintained in constant darkness suggesting a direct role of light in modulating MT_1 receptor density [10]. A similar result was found in our CHO-MT_1 cell line, which is unresponsive to photic input, following exposure to physiological concentrations of melatonin. Second, functional studies in hamsters in vivo that received daily administration of melatonin (1.0 mg/kg) for 14 days showed no desensitization of the MT_1 melatonin receptor-mediated inhibition of neuronal firing in the SCN [35]. In vitro, prolonged exposure of endogenous MT_1 melatonin receptors in ovine pars tuberalis cells to physiological concentrations of melatonin (100 pM for 16 h) significantly reduced the potency of melatonin to inhibit forskolin-stimulated cAMP formation with no change in efficacy [36]. This apparent receptor desensitization, however, correlated with supersensitization of the cAMP signaling cascade [36]. Thus, the current results in vitro and the in vivo studies suggest that physiological concentrations of melatonin do not negatively regulate the MT_1 melatonin receptor.

Desensitization of recombinant MT_1 melatonin receptors by supraphysiological concentrations of melatonin requires prolonged exposure. While the desensitization is significant, it is neither robust nor associated with any observable receptor internalization or reduction in the number of specific 2-[^{125}I]iodomelatonin binding sites. This is in contrast to recombinant MT_2 melatonin receptors which following exposure to supraphysiological concentrations of melatonin are rapidly desensitized and internalized [16]. As the MT_1 and MT_2 melatonin receptors appear to be differentially regulated by desensitization and internalization, both exogenous melatonin and the circadian production of melatonin could differentially affect physiological responses mediated by these mammalian melatonin receptors following exposure to nocturnal levels of the hormone.

Acknowledgments

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