Melatonin desensitizes endogenous MT_2 melatonin receptors in the rat suprachiasmatic nucleus: relevance for defining the periods of sensitivity of the mammalian circadian clock to melatonin

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ABSTRACT **The hormone melatonin phase shifts circadian rhythms generated by the mammalian biological clock, the suprachiasmatic nucleus (SCN) of the hypothalamus, through activation of G protein-coupled MT_2 melatonin receptors. This study demonstrated that pretreatment with physiological concentrations of melatonin (30–300 pM or 7–70 pg/mL) decreased the number of hMT_2 melatonin receptors heterologously expressed in mammalian cells in a time and concentration-dependent manner. Furthermore, hMT_2-GFP melatonin receptors heterologously expressed in immortalized SCN2.2 cells or in non-neuronal mammalian cells were internalized upon pretreatment with both physiological (300 pM or 70 pg/mL) and supraphysiological (10 nM or 2.3 ng/mL) concentrations of melatonin. The decrease in MT_2 melatonin receptor number induced by melatonin (300 pM for 1 h) was reversible and reached almost full recovery after 8 h; however, after treatment with 10 nM melatonin full recovery was not attained even after 24 h. This recovery process was partially protein synthesis dependent. Furthermore, exposure to physiological concentrations of melatonin (300 pM) for a time mimicking the nocturnal surge (8 h) desensitized functional responses mediated through melatonin activation of endogenous MT_2 receptors, i.e., stimulation of protein kinase C (PKC) in immortalized SCN2.2 cells and phase shifts of circadian rhythms of neuronal firing in the rat SCN brain slice. We conclude that in vivo the nightly secretion of melatonin desensitizes endogenous MT_2 melatonin receptors in the mammalian SCN thereby providing a temporally integrated profile of sensitivity of the mammalian biological clock to a melatonin signal.—Gerdin, M. J., Masana, M. I., Rivera-Bermúdez, M. A., Hudson, R. L., Earnest, D. J., Gillette, M. U., Dubocovich, M. L. Melatonin desensitizes endogenous MT_2 melatonin receptors in the rat suprachiasmatic nucleus: relevance for defining the periods of sensitivity of the mammalian circadian clock to melatonin. *FASEB J.* 18, 1646–1656 (2004)**

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THE HORMONE MELATONIN is released primarily from the pineal gland following a circadian rhythm with high levels at night and low levels during the day. The synthesis of melatonin is regulated by the biological clock in the suprachiasmatic nucleus (SCN) of the hypothalamus. In turn, pineal melatonin modulates clock function through a direct action on G-protein-coupled MT_1 and MT_2 melatonin receptors in the SCN (1). Both the mRNA and protein for MT_1 melatonin receptors have been reported in the mammalian SCN (2–5). Physiologically, melatonin acutely inhibits neuronal firing in the SCN through the MT_1 melatonin receptor, as this action is absent in mice with targeted deletion of the MT_1 melatonin receptor (6). Similarly, both the MT_2 melatonin receptor mRNA and protein have been localized in the mammalian SCN (3–5). In contrast to the MT_1 melatonin receptor, the MT_2 receptor protein in the SCN is expressed at such a low level that it is undetectable by 2-[^{125}I]-iodomelatonin binding (3, 6, 7). There is, however, a clear physiological role for this receptor in the mammalian SCN.

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Melatonin through activation of the MT_2 melatonin receptor phase-shifts circadian rhythms generated within the SCN both in vivo and in vitro as this response is blocked by the selective MT_2 melatonin receptor antagonist 4P-PDOT (3, 4). Furthermore, in the rat SCN and immortalized SCN2.2 cells activation of the MT_2 melatonin receptor by melatonin stimulates PKC activity (4, 5). Taken together these studies demonstrate the existence of functionally active MT_1 and MT_2 melatonin receptors in the mammalian SCN.

Several studies reported direct melatonin-mediated regulation of endogenous MT_1 melatonin receptors. In the rat SCN, specific 2-[^{125}I]-iodomelatonin binding, primarily to MT_1 receptors, follows a diurnal rhythm with low levels during the night, while melatonin directly decreases the density of MT_1 melatonin receptors (8, 9). In GT1-7 neurons, endogenous MT_1 melatonin receptors internalize after short-term exposure to melatonin (10). Melatonin-mediated regulation of endogenous MT_2 melatonin receptors has not been examined because the low level of expression of this receptor in native tissues makes analysis difficult. As such, melatonin-mediated regulation of the MT_2 melatonin receptor has been restricted to recombinant systems in which the receptor is overexpressed. Melatonin desensitizes and internalizes MT_2 melatonin receptors after exposure to supraphysiological concentrations of melatonin (11, 12). The role of physiological concentrations of melatonin regulating MT_2 melatonin receptors has not been elucidated.

Evidence suggests that melatonin may in fact desensitize endogenous MT_2 melatonin receptors in the SCN. In a rat in vitro SCN brain slice model, melatonin through activation of endogenous MT_2 melatonin receptors phase-shifts circadian rhythms of neuronal firing when administered either at dawn or dusk (3, 4, 13). In contrast, melatonin phase-shifts the circadian rhythm of rat locomotor activity in vivo only when administered at dusk (14). The reason for this discrepancy is unknown, but the lack of a phase shift by melatonin at dawn in vivo could be attributed to desensitization of MT_2 melatonin receptors by the nocturnal surge of endogenous melatonin, which is not present in the in vitro SCN brain slice model.

In the present study, we investigated melatonin-mediated regulation of MT_2 melatonin receptors by physiological concentrations of melatonin. Neuronal and non-neuronal mammalian cells expressing recombinant hMT_2 melatonin receptors were used to determine the mechanism(s) by which physiological concentrations of melatonin regulate receptor internalization and recovery. In parallel, both the native rat SCN brain slice and immortalized rat SCN2.2 cells expressing endogenous MT_2 melatonin receptors were used to determine changes in the functional sensitivity of this receptor upon pretreatment with physiological concentrations of melatonin mimicking the nocturnal surge.

MATERIALS AND METHODS

Materials

cDNA containing the complete coding region of the hMT_1 or hMT_2 melatonin receptor (human Mel_{1a} cloned into pcDNAI or human Mel_{1b} cloned into pcDNA-3) was provided by Dr. S. M. Reppert (Department of Neurobiology, University of Massachusetts Medical School, Worcester, MA, USA). The Effectene transfection kit was obtained from Qiagen (Valencia, CA, USA) and Lipofectamine 2000 from Invitrogen (Carlsbad, CA, USA). Cell culture products were obtained from Invitrogen. 2-[^{125}I]-Iodomelatonin (SA: 2000 Ci/mmol) was purchased from Amersham (Piscataway, NJ, USA). Melatonin, cycloheximide and other general reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA). 4P-PDOT was obtained from Tocris (Ballwin, MO, USA). Wheat germ agglutinin/Texas Red conjugate and the Slowfade antifade kit were from Molecular Probes (Eugene, OR, USA). SCN2.2 cells were developed by Dr. D. J. Earnest (15).

Cell culture

Chinese hamster ovary (CHO) cells

CHO cell cultures were grown as monolayers in F12 media supplemented with 10% fetal calf serum, penicillin (100 units/mL) and streptomycin (100 μg/mL) in 5% CO_2 at 37°C. Generation of hMT_1 or hMT_2 melatonin receptor fusion proteins expressing an amino-terminal FLAG epitope and in some cases a carboxyl-terminal green fluorescence protein (GFP) epitope, as well as CHO cells stably expressing hMT_2-FLAG receptors (CHO-MT_2) has been described previously (12). The density of MT_2 melatonin receptors in the CHO-MT_2 cell line was 320 ± 111 fmol/mg protein (n=3). For transient expression of hMT_2-FLAG-GFP receptors, CHO cells at 40–50% confluency were transiently transfected with the corresponding constructs using Effectene (Qiagen).

Rat immortalized SCN2.2 cells

SCN2.2 cells were maintained in minimal essential medium (Mediatech, Herndon, VA, USA) supplemented with 10% (vol/vol) fetal bovine serum, 2 μg/mL glucose, penicillin (100 U/mL) and streptomycin (0.1 mg/mL). Cells were propagated continuously at 37°C under a humidified 5% CO_2: 95% air mixture, grown to confluence and passed every 3 days. Media was changed every 48 h. Experiments were carried out on cells between the 17th and 35th passage. For transient expression of hMT_1-FLAG, hMT_2-FLAG, or hMT_2-FLAG-GFP receptors, SCN2.2 cells at 80–90% confluency were transfected with Lipofectamine 2000 (Invitrogen).

Membrane preparation and 2-[^{125}I]-iodomelatonin binding studies

CHO or SCN2.2 cells were washed twice with phosphate-buffered saline (PBS) on ice for 5 min, lifted in potassium phosphate buffer (10 mM, pH 7.4) containing sucrose (0.25 M) and EDTA (1 mM), then pelleted by centrifugation. Cell pellets were then resuspended in Tris-HCl (50 mM, pH 7.4; $MgCl_2$ 10 mM) and collected by centrifugation (13,800 *g*). Membrane pellets were stored at −80°C until used. 2-[^{125}I]-Iodomelatonin binding was determined in cell membranes (10–40 μg protein/assay) as described previously (16). 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, unless otherwise stated) and appropriate concentrations of vehicle or competing agents in a total assay volume of 0.26 mL. After incubations for 1 h at 25°C, reactions were terminated by rapid vacuum filtration through glass fiber filters (Schleicher and Schuell No. 30) soaked in 0.5% polyethylenimine solution. Nonspecific binding was determined in the presence of 1 μM melatonin.

Confocal microscopy

CHO cells in culture at 40–50% confluency were transiently transfected with hMT_2-FLAG-GFP melatonin receptors using the Effectene transfection kit (Qiagen). SCN2.2 cells in culture at 80–90% confluency were transiently transfected with hMT_2-FLAG-GFP melatonin receptors using Lipofectamine 2000 (Invitrogen). Twenty-four hours after transfection, CHO or SCN2.2 cells were plated on poly-D-lysine-coated coverslips. One day later, cells were incubated in serum-free media (F12 media for CHO and N2 media for SCN2.2) for 5 h before and during treatments, washed once in PBS, then treated with vehicle or melatonin, as appropriate. Cells were then washed once with PBS for 5 min, fixed with 4% paraformaldehyde for 7.5 min, and incubated with wheat germ agglutinin/Texas Red conjugate (2 μg/mL) for 30 min at room temperature. Subsequently, 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 (Molecular Probes) and visualized on an Olympus confocal microscope.

PKC assay

SCN2.2 cells were plated (4.0×10^4 cells/cm^2) at circadian time (CT) 10 on 12-well plates precoated with poly-D-lysine. The time of plating was denoted as CT10 based on previous comparisons between the rhythms of glucose utilization and brain-derived neurotrophic factor content in immortalized SCN2.2 cells and the SCN in vivo (17, 18). After 54.5 h (~80% confluency), cells were serum starved for 1 h, then pretreated in serum-free media for 8 h (CT17.5–CT1.5) with either vehicle or 300 pM melatonin in the absence or presence of 4P-PDOT (10 nM). After the pretreatment, cells were washed with PBS and incubated for 0.5 h in serum-free media. Cells were then stimulated with vehicle or melatonin (1 nM) at CT2 for 10 min. PKC activity was determined by incorporation of [γ^{32}P]ATP into a synthetic substrate peptide corresponding to amino acids residues 4-14 of myelin basic protein in accordance with manufacturer's protocol (Upstate Biotechnology, Lake Placid, NY, USA).

Extracellular recordings from the rat SCN brain slice

SCN brain slices were prepared and extracellular recordings performed following procedures described by Hunt et al. (4). Male Long-Evans rats (8- to 12-wk-old) were maintained in a 12/12 light/dark cycle with free access to food and water. Rats were decapitated during the light period (between CT1-CT9), and the brain quickly dissected and blocked. A 500 μm coronal brain slice containing the SCN was placed in a recording chamber and continuously perfused with Earle's balanced salt solution supplemented with 24.6 mM glucose, 26.2 mM sodium bicarbonate and 5 mg/L gentamicin and saturated with 95% O_2–5% CO_2 at 37°C. A glass electrode filled with 5 M NaCl was used to sample cells throughout the SCN. Each cell was monitored for 4 min, the action potentials grouped into 10 s bins, and the mean firing rate determined for each cell. Mean firing rates were grouped into 2 h bins and smoothed by plotting the data as 15 min running averages. Data were collected using customized LabVIEW software (National Instruments, Austin, TX, USA). The peak in the circadian rhythm of neuronal firing was visually determined. Brain slices were pretreated for 8 h (CT14.5–CT22.5) with either vehicle or melatonin (300 pM) in the absence or presence of 4P-PDOT (100 nM), which was added from CT14 to CT22.5. After a 0.5 h washout period, slices were stimulated at CT23 with either vehicle or melatonin (1 nM) applied by a 1 μL microdrop for 10 min.

Data analysis and statistics

Percent decreases in specific 2-[^{125}I]-iodomelatonin binding were calculated by dividing the average specific binding (fmol/mg protein) after treatment with a defined melatonin concentration by the average specific binding (fmol/mg protein) of the vehicle-treated control. Statistical significance was determined by either an unpaired Student's t test for comparisons between two groups, or 1- or 2-way ANOVA with Bonferroni post hoc test for multiple comparisons. A value of $P<0.05$ was taken as statistically significant.

RESULTS

Physiological concentrations of melatonin decreased the number of hMT_2 melatonin receptors heterologously expressed in mammalian cells

Pretreatment of CHO-MT_2 cells with melatonin significantly decreased specific 2-[^{125}I]-iodomelatonin binding in a time- (10–480 min) and concentration- (30 pM–10 nM) dependent manner (**Fig. 1**). A physiological diurnal concentration of melatonin (30 pM or 7 pg/mL) mimicking daytime levels significantly decreased specific 2-[^{125}I]-iodomelatonin binding after 8 h by 44.6 ± 8.3% (n=3, $P<0.05$), while a melatonin concentration (300 pM or 70 pg/mL) mimicking nighttime levels significantly decreased binding after 1 h by 63.0 ± 1.3% (n=3, $P<0.001$) compared with vehicle-treated, time-matched controls (Fig. 1). Supraphysiological concentrations of melatonin (1–10 nM or 0.23–2.3 ng/mL) significantly decreased specific 2-[^{125}I]-iodomelatonin binding after 1 h and 10 min exposure, respectively (Fig. 1).

The time course of MT_2 melatonin receptor recovery after exposure to melatonin was determined in CHO-MT_2 cells pretreated with a physiological (300 pM for 1 h) or a supraphysiological (10 nM for 10 min) concentration of melatonin. After pretreatment of CHO-MT_2 cells with 300 pM melatonin, incubation in melatonin-free media led to a significant recovery in specific 2-[^{125}I]-iodomelatonin binding after 8 h washout (97.5±16.4%, n=5, $P<0.01$) and continued until reaching a plateau at 24 h (121.8±3.8%, n=3, $P<0.001$) (**Fig. 2*A***). Similarly, a significant recovery after pretreatment with 10 nM melatonin was observed after 8 h washout (56.6±0.7%, n=3, $P<0.05$), but recovery was only 86.7 ± 2.8% (n=3, $P<0.001$) after 24 h (Fig. 2*B*).

To assess whether recovery of specific 2-[^{125}I]-iodomelatonin binding sites after exposure of CHO-MT_2 cells

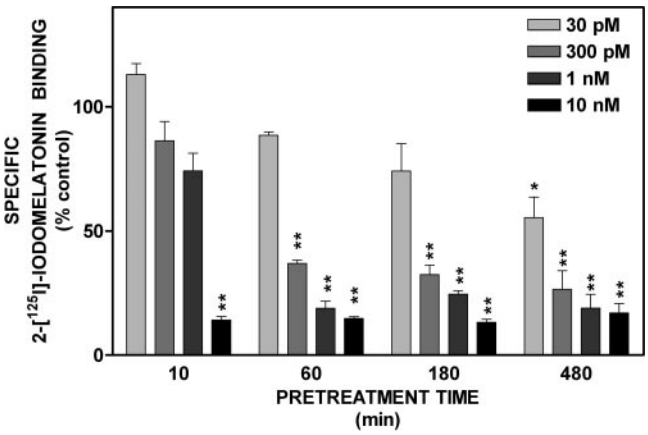

Figure 1. Reductions in specific 2-[^{125}I]-iodomelatonin binding to recombinant MT_2 melatonin receptors is time and concentration dependent. CHO-MT_2 cells were cultured to ~80% confluency, serum starved for 5 h, then pretreated in situ with either vehicle or melatonin 30, 300, 1000, or 10,000 pM for 10–480 min. Cells were harvested, membranes were prepared, and specific 2-[^{125}I]-iodomelatonin binding defined with 1 μM melatonin was determined as described in Materials and Methods. The ordinate represents specific 2-[^{125}I]-iodomelatonin binding expressed as % of the appropriate time-matched, vehicle-treated control. Data represent the mean ± SEM of 3–5 independent experiments performed in duplicate. Data were analyzed using 2-way ANOVA with a Bonferroni post hoc test for multiple comparisons. There was a significant effect of pretreatment time ($F_{4,41}$=49.48) as well as of melatonin concentration used during pretreatment ($F_{3,41}$=42.32). *P <0.05, **P <0.001 compared with vehicle-treated control.

to melatonin was dependent on new protein synthesis, the protein synthesis inhibitor cycloheximide (20 μg/mL) was added into the recovery media during a single 16 h recovery period. Pretreatment of CHO-MT_2 cells with 300 pM melatonin for 1 h reduced specific 2-[^{125}I]-iodomelatonin binding by 75.0 ± 0.5% (n=3) (Fig. 2*C*). Specific 2-[^{125}I]-iodomelatonin binding fully recovered after 16 h (122.2±14.0%, n=3, P<0.001), but recovery was significantly reduced in the presence of cycloheximide (73.3±3.4%, n=3, P<0.05) (Fig. 2*C*). Similarly, after pretreatment with 10 nM melatonin, specific 2-[^{125}I]-iodomelatonin binding was reduced by 85.9 ± 3.0% (n=4), but recovery after 16 h reached only 48.3 ± 8.0% (n=6, P<0.01) (Fig. 2*D*). In the presence of cycloheximide, specific 2-[^{125}I]-iodomelatonin binding recovered to only 27.0 ± 3.5% (n=6, P<0.05) (Fig. 2*D*).

Effect of melatonin pretreatment on the trafficking of hMT_2 melatonin receptors heterologously expressed in mammalian cells

CHO cells were transiently transfected with hMT_2 melatonin receptors containing an amino-terminal FLAG epitope and a carboxyl-terminal GFP sequence (MT_2-GFP). The FLAG or GFP epitopes did not affect receptor binding or function (12). Cells were serum starved for 5 h, then treated with 10 nM melatonin for 10, 60, 180, or 480 min (**Fig. 3**). Receptors were localized by overlay of the GFP-tagged MT_2 melatonin receptors with cells co-stained with wheat germ agglutinin/Texas Red (labels cell surface plasma membrane). In vehicle-treated cells (0 min), the MT_2 melatonin receptors were localized to the cell surface plasma membrane and perinuclear regions (Fig. 3). Melatonin (10 nM) treatment for 10 min appeared to increase the intracellular fluorescence (Fig. 3). Furthermore, pretreatment with this concentration for 1, 3 and 8 h not only appeared to increase the intracellular fluorescence but also induced the formation of large intracellular puncta compared with vehicle-treated controls, with a concomitant decrease in MT_2-GFP receptor membrane localization (Fig. 3). More important, pretreatment with a physiological concentration of melatonin (300

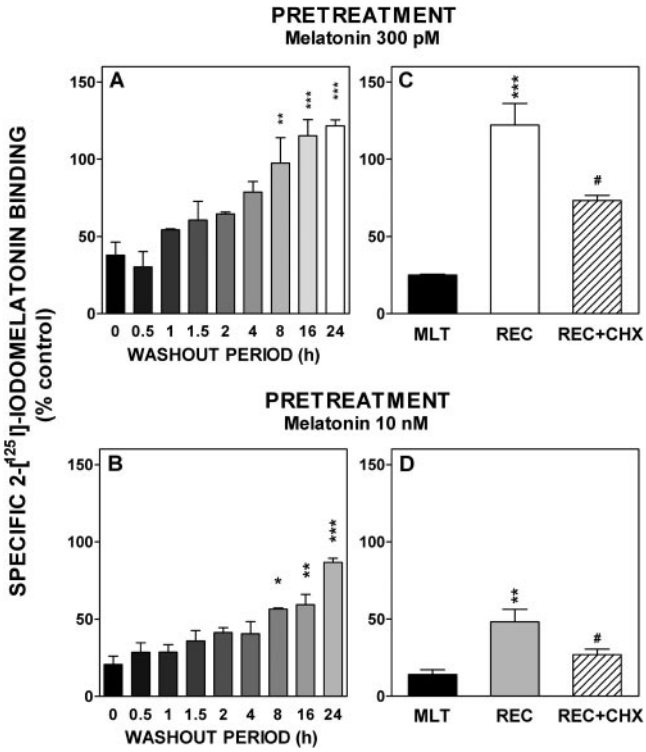

Figure 2. Recovery of specific 2-[^{125}I]-iodomelatonin binding to recombinant MT_2 melatonin receptors after melatonin removal. CHO-MT_2 cells were cultured to ~80% confluency, serum starved for 5 h, then treated in situ with 300 pM melatonin for 1 h (*A, C*) or 10 nM melatonin for 10 min (*B, D*). After removal of the melatonin containing media, cells were incubated in serum-free media for 0.5–24 h (*A, B*) or for 16 h in the absence or presence of 20 μg/mL cycloheximide (*C, D*). At various time points, cells were harvested, membranes prepared, and specific 2-[^{125}I]-iodomelatonin binding defined with 1 μM melatonin determined as described under Materials and Methods. The ordinate represents specific 2-[^{125}I]-iodomelatonin binding expressed as % of the appropriate time-matched, vehicle-treated control. Data represent the mean ± SEM of 3–6 independent experiments performed in duplicate. Data were analyzed using 1-way ANOVA (*A*: $F_{8,23}$=8.294, P<0.0001; *B*: $F_{8,21}$=11.78, P<0.0001; *C*: $F_{2,6}$=34.03, P<0.001; *D*: $F_{2,13}$=8.101, P<0.01) with a Bonferroni post hoc test for multiple comparisons. *P < 0.05, **P < 0.01, ***P < 0.001 compared with melatonin pretreatment; #P < 0.05 compared with REC. MLT: melatonin; REC: recovery; CHX: cycloheximide.

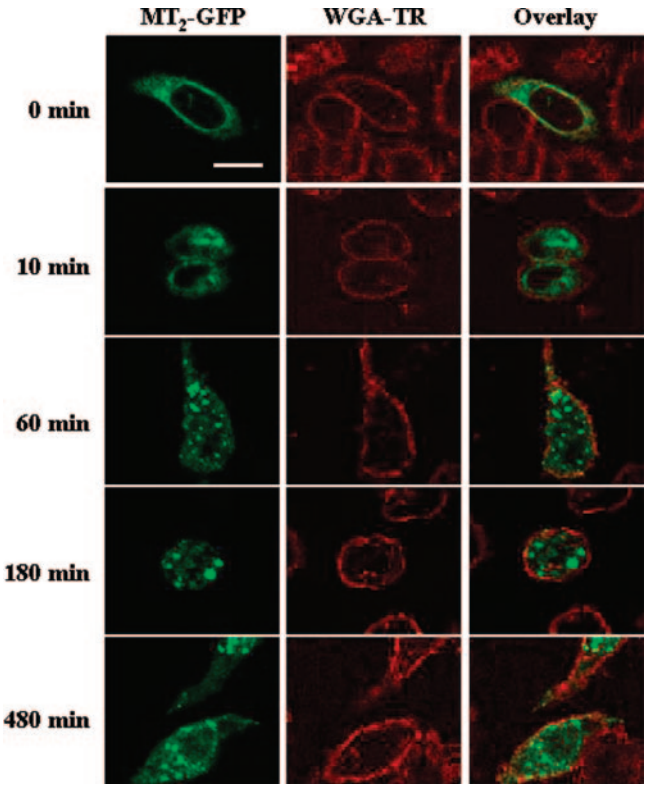

Figure 3. Supraphysiological concentrations of melatonin internalized recombinant MT_2 melatonin receptors in CHO cells in a time-dependent manner. CHO cells transiently expressing FLAG-hMT_2-GFP melatonin receptors were exposed to vehicle or 10 nM melatonin for 10, 60, 180, or 480 min. Cells were also co-stained with wheat germ agglutinin/Texas Red conjugate (WGA-TR) to label the cell surface plasma membrane. Images were captured using an Olympus confocal microscope and are representative of 3 independent experiments. Scale bar: 20 μm.

pM for 8 h) increased the intracellular fluorescence and induced the formation of large intracellular puncta compared with vehicle-treated controls (**Fig. 4**).

Effect of melatonin pretreatment on the density and trafficking of hMT_2 melatonin receptors heterologously expressed in immortalized rat SCN2.2 cells

SCN2.2 cells were transiently transfected with MT_1-FLAG or MT_2-FLAG receptors, serum starved for 1 h, then treated with vehicle or 300 pM melatonin for 8 h. Changes in receptor density were determined in crude washed membranes using a single saturating concentration of 2-[^{125}I]-iodomelatonin (511.9±12.0 pM, n=7) and specific binding defined with 1 μM melatonin. No decrease in specific 2-[^{125}I]-iodomelatonin binding after melatonin pretreatment was observed in SCN2.2 cells transfected with MT_1-FLAG receptors (3096.0±593.2 fmol/mg protein, n=5) compared with vehicle-treated controls (2903.0±629.8 fmol/mg protein, n=5) (**Fig. 5*A***). In contrast, melatonin pretreatment of SCN2.2 cells transfected with MT_2-FLAG receptors significantly reduced specific 2-[^{125}I]-iodomelatonin binding (75.1±9.7

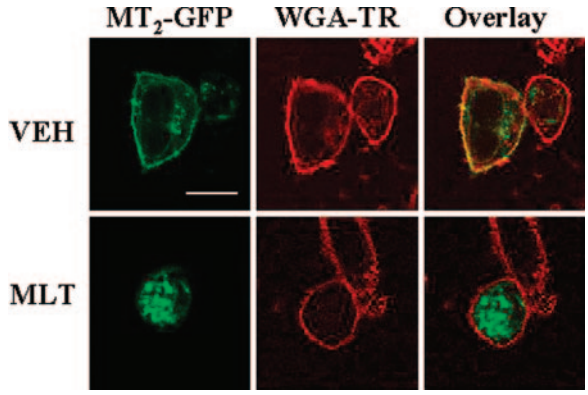

Figure 4. Physiological concentrations of melatonin mimicking the nocturnal duration of hormone secretion internalized MT_2 melatonin receptors in CHO cells. CHO cells transiently expressing FLAG-hMT_2-GFP melatonin receptors were exposed to vehicle or 300 pM melatonin for 480 min. Cells were also costained with wheat germ agglutinin/Texas Red conjugate (WGA-TR) to label the cell surface plasma membrane. Images were captured using an Olympus confocal microscope and are representative of 3 independent experiments. Scale bar: 10 μm.

fmol/mg protein, n=6, P<0.01) compared with vehicle-treated controls (147.6±18.8 fmol/mg protein, n=6) (Fig. 5*B*). No specific binding was detected in untransfected SCN 2.2 cells (data not shown).

In SCN2.2 cells, the decreases in MT_2 melatonin receptor density appeared to correlate with receptor internalization. Transient transfection of SCN2.2 cells with MT_2-FLAG-GFP receptors showed receptor expression in two morphologically distinct populations of immortalized SCN2.2 cells, small rounded neuronal-

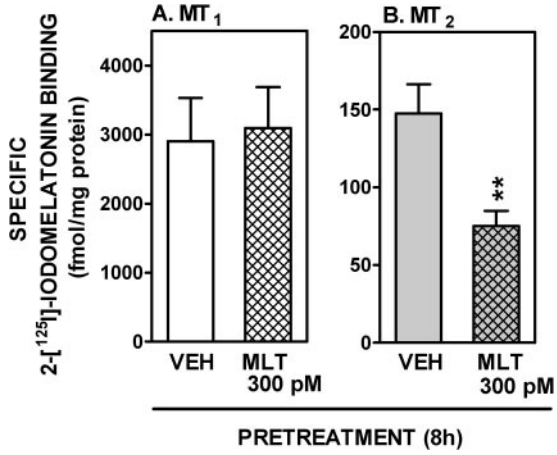

Figure 5. Exposure to a physiological concentration of melatonin decreased MT_2 but not MT_1 melatonin receptor density in SCN2.2 cells. SCN2.2 cells transiently expressing MT_1-FLAG or MT_2-FLAG receptors were serum-starved for 1 h, then treated with vehicle or 300 pM melatonin for 8 h. Changes in receptor density were determined in crude washed membranes using a single saturating concentration of 2-[^{125}I]-iodomelatonin. Specific binding was determined in the presence of 1 μM melatonin. Data were analyzed using unpaired Student's t test (**P<0.01 compared with vehicle-treated control). VEH: vehicle; MLT: melatonin.

like and large glial-like cells (**Fig. 6**) (15). MT_2 melatonin receptors were expressed on the cell surface and in perinuclear regions of both neuronal-like and glial SCN2.2 cells, as observed by colocalization of MT_2-FLAG-GFP receptors with wheat germ agglutinin/Texas Red (Fig. 6A, *C*). Pretreatment with a concentration of melatonin (300 pM) mimicking the nocturnal hormonal surge (8 h) increased the intracellular fluorescence and induced the formation of large intracellular puncta with a concomitant reduction of cell surface residency (Fig. 6*B, D*). Pretreatment of SCN2.2 cells with 10 nM melatonin for 1 h also internalized MT_2 melatonin receptors in both glial and neuronal-like cells (data not shown).

Effect of pretreatment with nocturnal physiological levels of melatonin on the functional sensitivity of endogenous MT_2 melatonin receptors in rat immortalized SCN2.2 cells

In rat immortalized SCN2.2 cells, basal PKC activity shows a diurnal rhythm with nadirs during subjective

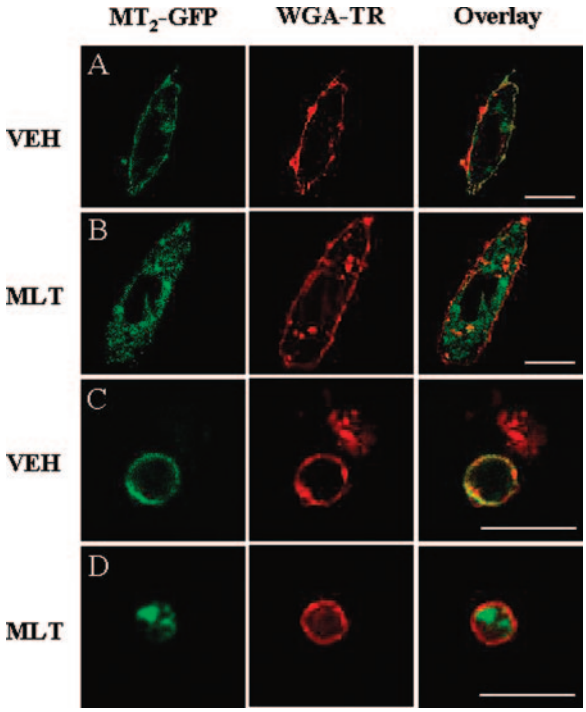

Figure 6. Physiological concentrations of melatonin mimicking the nocturnal duration of melatonin secretion internalized MT_2 melatonin receptors in SCN2.2 cells. SCN2.2 cells transiently transfected with hMT_2-GFP receptors were maintained in N2 medium and treated with vehicle (*A, C*) or 300 pM melatonin for 8 h (*B, D*). Cells were then fixed with 4% paraformaldehyde and costained with wheat germ agglutinin/Texas Red conjugate (WGA-TR) to label the cell surface plasma membrane. Colocalization of the receptor with the cell surface plasma membrane is shown in yellow (overlay). *A, B)* Representative cells with glial morphology; *C, D)* Representative cells with neuronal-like morphology. Shown are representative confocal images selected from 3 independent experiments. Scale bars: 10 μm.

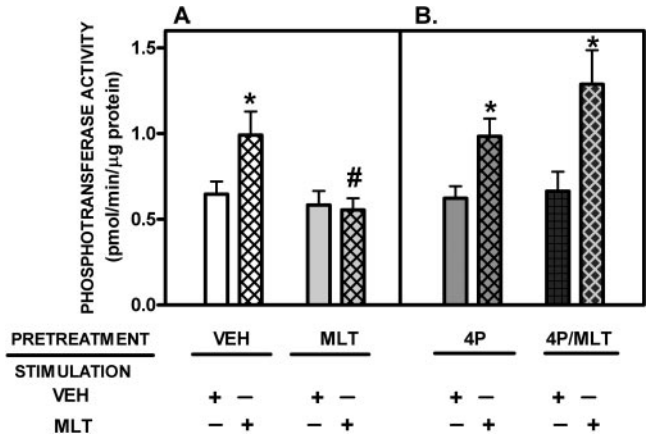

Figure 7. Desensitization of endogenous MT_2 melatonin receptors in the rat immortalized SCN2.2 cell line. SCN2.2 cells were cultured 54.5 h to ~80% confluency, serum starved for 1 h, then pretreated for 8 h (CT17.5–CT1.5) in serum-free media with vehicle, 300 pM melatonin (*A*), or 10 nM 4P-PDOT or melatonin and 4P-PDOT (*B*), as represented on the abscissa. After a 0.5 h washout in serum-free media, cells were stimulated with either vehicle or 1 nM melatonin for 10 min. The ordinate represents phosphotransferase activity (pmol·min^{-1}·μg $protein^{-1}$). Data represent the mean ±SEM of at least 3 independent experiments performed in triplicate and were analyzed using 2-way ANOVA (*A*: $F_{1,36}=5.683$, $P<0.05$; *B*: $F_{1,15}=1.267$, $P>0.05$) with Bonferroni post hoc test. $*P<0.05$ compared with corresponding vehicle; $\#P<0.05$ compared with melatonin stimulation. VEH: vehicle; MLT: melatonin; 4P: 4P-PDOT.

day at CT10 and CT2 (19). At these periods of sensitivity, when PKC activity is at the lowest (19) melatonin stimulates PKC activity through activation of MT_2 melatonin receptors (4, 19). We used this model to assess whether physiological concentrations of melatonin mimicking the nocturnal duration in vivo, desensitized the MT_2 melatonin receptor-mediated activation of PKC at CT2. A melatonin (1 nM) pulse at CT2 stimulated PKC phosphotransferase activity to 0.94 ± 0.14 pmol·min^{-1}·μg $protein^{-1}$ ($n=13$, $P<0.05$) compared with vehicle control (0.61±0.08 pmol·min^{-1}·μg $protein^{-1}$, $n=13$) (**Fig. 7*A***). Melatonin-mediated stimulation of PKC phosphotransferase activity at CT2 was significantly reduced ($P<0.05$) after pretreatment with 300 pM melatonin for 8 h (CT17.5–CT1.5) (Fig. 7*B*). We next used the selective and competitive MT_2 melatonin receptor antagonist 4P-PDOT (10 nM) (3, 4) to assess whether the melatonin-mediated desensitization was mediated through activation of the MT_2 receptor. 4P-PDOT when used alone (CT17.5-CT1.5) or in combination with melatonin (300 pM) for 8 h (CT17.5–CT1.5) did not affect basal phosphotransferase activity at CT2 (Fig. 7*A, B*). Pretreatment with 4P-PDOT (10 nM) alone did not affect melatonin-mediated stimulation of phosphotransferase activity (Fig. 7*B*). After combined pretreatment of 4P-PDOT (10 nM) and melatonin (300 pM) for 8 h, a melatonin pulse (1 nM, CT2) significantly stimulated phosphotransferase activity (Fig. 7*B*).

Effect of pretreatment with nocturnal physiological levels of melatonin on the functional sensitivity of endogenous MT_2 melatonin receptors in the rat SCN brain slice

We next studied the functional sensitivity of endogenous MT_2 melatonin receptors in the rat SCN brain slice after exposure to physiological concentrations of melatonin mimicking the nocturnal duration of hormone release in vivo. The functional sensitivity of the MT_2 receptor after various pretreatments was assessed by the ability of melatonin to shift the peak of the circadian rhythm of neuronal firing in the rat SCN brain slice (4, 13, 20). In controls, a vehicle pulse at CT23 after pretreatment with vehicle during subjective night did not affect the circadian rhythm of neuronal firing as the peak was observed at CT 6.8 ± 0.1 (n=3) (**Fig. 8*A*, Fig. 9**). In contrast, a melatonin (1 nM) pulse applied as a microdrop to the SCN slice at CT23 advanced the peak of the circadian rhythm of neuronal firing to CT 3.2 ± 0.2 (n=3) (Fig. 8*B* vs. *A*, Fig. 9). A vehicle pulse at CT23 after pretreatment with 300 pM melatonin for 8 h (CT14.5–CT22.5) did not affect the circadian rhythm of neuronal firing (Fig. 8*C* vs. *A*; Fig. 9). However, in SCN slices pretreated with 300 pM melatonin for 8 h (CT14.5–CT22.5) the phase advance induced by melatonin (1 nM) at CT23 was prevented as the peak position (CT 6.6±0.2, n=3) (Fig. 7*D*) was not significantly different from control (Fig. 8*C*, Fig. 9). In addition, preincubation with 1 nM melatonin during 8 h also prevented melatonin-mediated phase advance by 1 nM melatonin applied at CT23 (data not shown).

To determine whether the lack of phase shift after pretreatment with physiological concentrations of melatonin during subjective night was due to activation of MT_2 melatonin receptors we used the MT_2 selective and competitive melatonin receptor antagonist, 4P-PDOT (3, 4). Incubation with the selective MT_2 antagonist 4P-PDOT (100 nM) alone for 8.5 h (CT14–CT22.5) did not affect the position of the peak of neuronal firing upon a vehicle pulse at CT23 (CT 6.8±0.15, n=3) (Fig. 8*E* vs. *A*, Fig. 9) or the phase advance induced by melatonin (3.5±0.15 h) (Fig. 8*F* vs. *B*, Fig. 9). Coincubation with the selective MT_2 antagonist 4P-PDOT during pretreatment with 300 pM melatonin for 8 h (CT14.5–CT22.5) did not affect the position of the peak of neuronal firing after a vehicle pulse (Fig. 8*G* vs. *E*, Fig. 9). This treatment, however, did not prevent the phase advance induced by melatonin (1 nM) at CT23 as the peak position was at CT 2.8 ± 0.1 (n=3) (Fig. 8*H* vs. 8*F*, Fig. 9).

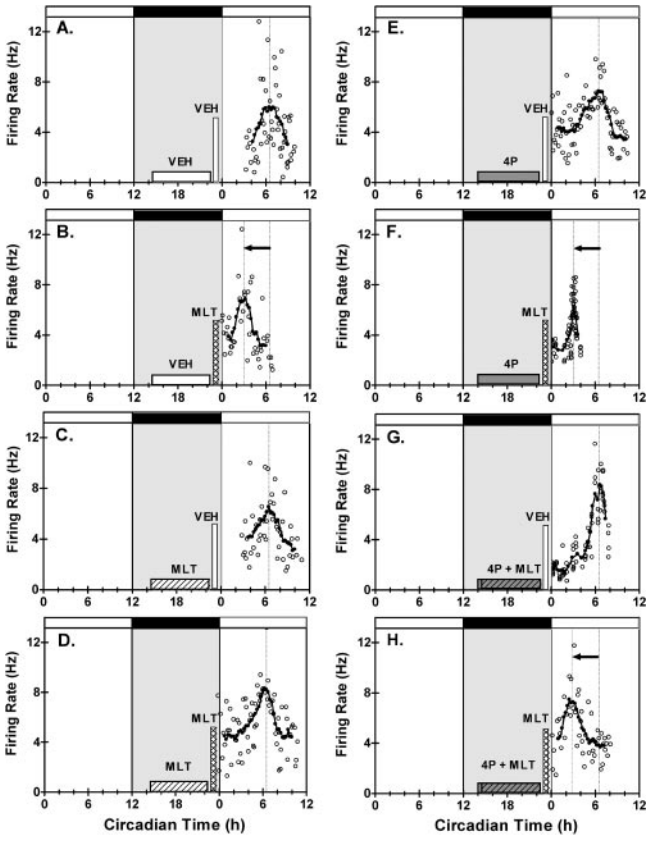

Figure 8. Desensitization of endogenous MT_2 melatonin receptors in the rat SCN impaired the melatonin-mediated phase advance at CT23. Shown are representative rhythms of neuronal firing assessed by extracellular recording in rat coronal brain slices containing the SCN. The horizontal bar indicates pretreatment during subjective night with vehicle (CT14.5-CT22.5), melatonin (300 pM, 8 h) (CT14.5–CT22.5), 4P-PDOT (100 nM, 8.5 h) (CT14-CT22.5), or combined 4P-PDOT and melatonin. The vertical bar represents stimulation with either vehicle or melatonin (1 nM) applied by a 1 μL microdrop for 10 min at CT23. Firing rates from individual cells in the SCN (open circles) are shown as well as the mean firing rates presented as running averages (filled circles). Phase shifts were visually determined with reference to the peak of the circadian rhythm of neuronal firing at CT 6.8 ± 0.1 (n=3) (A) obtained after pretreatment with vehicle for 8 h and a vehicle pulse at CT 23. VEH: vehicle; MLT: melatonin; 4P: 4P-PDOT.

DISCUSSION

This study demonstrates that physiological concentrations of melatonin mimicking the nocturnal surge desensitize endogenous MT_2 melatonin receptors expressed in the rat SCN. The melatonin-mediated internalization of recombinant MT_2 melatonin receptors expressed in mammalian cells was time and concentration dependent. This decrease in MT_2 melatonin receptor number was partially recoverable and dependent on new protein synthesis. Similarly, pretreatment with physiological concentrations of melatonin functionally desensitized endogenous MT_2 melatonin receptors in an immortalized SCN2.2 cell line as determined by the activation of PKC stimulation at CT2 and in the rat SCN, as determined by the ability of melatonin to phase-shift the circadian rhythm of neuronal firing at CT23. We conclude that physiological concentrations of melatonin mimicking the nocturnal secretion of the hormone in vivo desensitize endogenous MT_2 melatonin receptors in the rat SCN, possibly through receptor internalization, thereby contributing to the

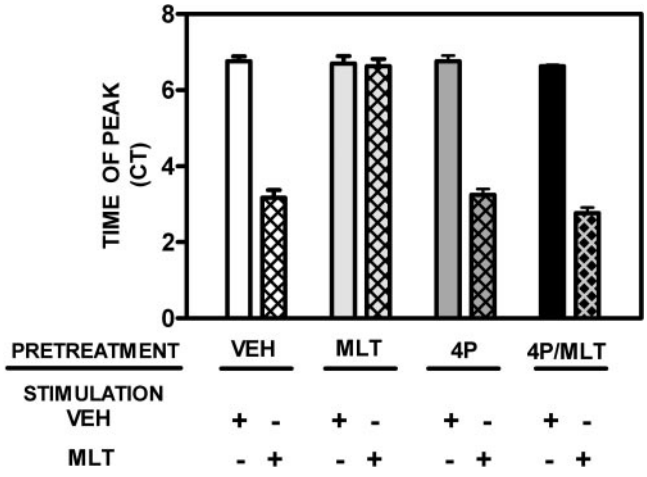

Figure 9. Summary of melatonin-mediated phase shifts of the circadian rhythm of neuronal firing after pretreatment during the subjective night with melatonin and/or the competitive receptor antagonist 4P-PDOT. Mean values of peak time of the circadian rhythm of neuronal firing induced by stimulation with vehicle or melatonin (1 nM) applied by a 1 μL microdrop for 10 min at CT 23 after pretreatment during subjective night with vehicle (VEH), melatonin (MLT, 300 pM), 4P-PDOT (4P, 100 nM) or 4P-PDOT and melatonin (4P/MLT). Values represent mean ±SEM.

temporal specificity of melatonin in modulating circadian rhythms.

Physiological concentrations of melatonin mimicking the nocturnal surge decreased the number and internalized recombinant MT_2 melatonin receptors expressed in neuronal- and glial-like immortalized rat SCN2.2 cells and in non-neuronal CHO cells. Reductions in MT_2 receptor number after exposure of CHO cells to either a physiological or supraphysiological concentration of melatonin were recoverable, but a portion of this recovery was dependent on new protein synthesis. A correlation between reductions in specific 2-[^{125}I]-iodomelatonin binding and receptor internalization after exposure to melatonin was also observed in both the CHO and rat SCN2.2 cells. The fact that a portion of the recovery was protein synthesis-dependent suggests that some of the internalized MT_2 melatonin receptors were targeted for degradation, while another portion of the receptors were directed to a recycling pathway. Furthermore, the observation that the recovery was greater after exposure to 300 pM melatonin compared with 10 nM suggested that this higher concentration may have served as a stronger stimulus targeting a larger proportion of the receptors for degradation. After internalization, many G-protein-coupled receptors (GPCR) are targeted to endosomes, then degraded or recycled back to the cell surface; however, the molecular mechanisms mediating this sorting process remains to be determined (21). GPCRs are often degraded after prolonged exposure to agonists (i.e., hours) and is associated with slow or incomplete recovery (22). For example, recovery of ^{125}I-ET1 binding to the endothelin B receptor after 30 min exposure to ET1 (100 nM) reaches control values after 4 h and is cycloheximide dependent (23). Tight binding of endothelin to the receptor (K_i ~59 pM) after internalization may target the receptor for degradation (23). Melatonin also has picomolar affinity for the MT_2 melatonin receptor (3, 11, 24) and therefore may remain bound after internalization of the receptor and in some way target a portion of the internalized receptor pool to degradation. Internalization induced by supraphysiological concentrations of melatonin could contribute to prolonged MT_2 receptor desensitization when nonphysiological doses of exogenous melatonin are used for the treatment of circadian disfunctions (25). G_i-coupled receptors, however, show wide variability in their ultimate fates after internalization. For example, the D2 dopamine receptor is rapidly internalized ($t_{1/2}$ ≤5 min) and recycled back to the plasma membrane ($t_{1/2}$≤30 min) (26). Both M_2 muscarinic and δ-opioid receptors are rapidly internalized but the M_2 muscarinic receptor requires several hours to recover and a portion of this recovery is protein synthesis dependent (27), while the δ-opioid is down-regulated by at least 50% (28). Recovery of 2-[^{125}I]-iodomelatonin binding to MT_2 melatonin receptors after exposure to melatonin takes at least 8 h to reach control levels and appears to involve receptor internalization followed by receptor degradation and recycling back to the membrane. This dynamic internalization and subsequent recycling suggests physiologically that the MT_2 melatonin receptor may follow a time-dependent diurnal rhythm of desensitization during long exposure to the hormone at night and a resensitization during the day when melatonin levels are at their lowest. Maximum receptor responsiveness would then coincide with the periods of melatonin sensitivity to phase-shift circadian rhythms at the beginning of the night (29, 30).

A physiological concentration of melatonin mimicking the nocturnal secretion of the hormone decreased MT_2 melatonin receptor density, but not MT_1, in SCN2.2 cells transiently expressing either receptor. Immortalized SCN2.2 cells express components of the mammalian circadian clock (31), circadian rhythms of metabolic activity and gene expression (18, 32), and functional melatonin receptors (5). This cell line is endowed with endogenous melatonin receptors that are functionally coupled to the inhibition of forskolin-stimulated cAMP formation and protein kinase stimulation (5, 19, 31). Thus, the SCN2.2 cell line appears to be an appropriate in vitro model of the native SCN. In SCN2.2 cells, the differential regulation of MT_1 and MT_2 melatonin receptor number and trafficking by physiological concentrations of melatonin mirrors that observed in non-neuronal mammalian cells (33). This finding is significant because it suggests that when the mammalian melatonin receptors are expressed in a cell in which endogenous MT_1 and MT_2 melatonin receptors are present, regulatory mechanisms may mediate receptor desensitization and internalization similar to

what is observed in the CHO-MT_1 and CHO-MT_2 cells (34). Specific 2-[^{125}I]-iodomelatonin binding to recombinant MT_1 and MT_2 melatonin receptors in the SCN2.2 cell line, however, is most likely to both neurons and glia. In fact, transient expression of MT_2-GFP melatonin receptors in SCN2.2 cells showed expression in both neuronal and glial-like cells (present results). Rivera-Bermudez et al. (5) localized MT_1 and MT_2 melatonin receptor mRNA only to neuronal-like cells in the SCN2.2 cells, but we cannot exclude low level of expression in glia as melatonin receptors have been localized to human cerebellar Bergmann glia and astrocytes (35) and in cultured chick astrocytes (36).

Prolonged exposure of endogenous MT_2 melatonin receptors to a physiological concentration of melatonin mimicking the nocturnal secretion of the hormone desensitized endogenous MT_2 melatonin receptors in the native rat SCN and in an immortalized SCN2.2 cell line. In the rat native SCN in vitro, activation of MT_2 melatonin receptors, through a PKC-dependent signaling cascade, phase-shift the peak of the circadian rhythm of neuronal firing both at dawn and dusk (4, 13, 20). In the rat in vivo, however, melatonin only phase-shifts the circadian rhythm of locomotor activity at dusk (14). It is likely that the lack of a melatonin-mediated phase shift at dawn in the rat in vivo is due to desensitization of MT_2 melatonin receptors in the SCN by circulating endogenous melatonin secreted during the night. Thus, when the in vivo situation was recapitulated in the rat SCN brain slice in vitro by administration of exogenous melatonin mimicking the concentration and length of the nocturnal secretion in vivo, the phase shift at dawn was prevented. A similar treatment paradigm in the SCN2.2 cells also desensitized the MT_2-mediated activation of PKC. In this cell line, there is a rhythmic oscillation of basal PKC activity, and melatonin activates PKC activity via MT_2 receptors at windows of sensitivity equivalent to the periods of sensitivity described for melatonin (i.e., dawn ~ CT 9–11; dusk ~23–2) (19). These windows of sensitivity roughly correlate with the specific times when melatonin phase-shifts the circadian rhythm of neuronal firing in the rat SCN in vitro (i.e., dawn and dusk) (4, 13). Together these results suggest that the nightly secretion of melatonin in vivo likely desensitizes endogenous MT_2 melatonin receptors in the rat SCN shaping the periods of sensitivity and determining when the biological clock is sensitive to a melatonin signal.

Melatonin, the ubiquitous hormone of darkness, should also induce desensitization of endogenous MT_2 melatonin receptors by physiological nocturnal levels and contribute to defining the periods of sensitivity of the biological clock in other mammals, including humans. Our results suggest that reduced sensitivity of the biological clock to exogenous melatonin at dawn should correlate with nocturnal plasma melatonin levels, which are known to differ among mammalian species. In humans, nocturnal circulating serum melatonin levels are similar to those reported in the rat (~50–60 pg/mL) (8). In support of our results, administration of a single oral dose of melatonin to humans at dawn does not phase-shift circadian rhythms of core body temperature, heart rate, or dim light melatonin onset/offset (37). Melatonin administration to humans for 4 consecutive days at dawn did phase-shift the circadian rhythm of dim light melatonin onset/offset, but the magnitude of this shift was smaller than the one observed when the hormone was given at dusk (30). Although the low efficacy of melatonin to phase-shift circadian rhythms in humans when given at dawn may be attributed to the relatively weak zeitgeber properties of this hormone, we cannot exclude melatonin receptor desensitization due to nocturnal serum melatonin which is known to vary among individuals (~72 to 269 pM or 17 to 62 pg/ml) (38). This receptor desensitization may manifest itself as either diminished or abolished phase-shifting responses at dawn. In C3H/HeN mice, melatonin phase advances and phase delays by 1 h circadian rhythms of wheel running activity when administered at dusk (CT 9–11) and dawn (CT24–CT2) (29), respectively. The apparent lack of desensitization of the melatonin-mediated delays at dawn is possibly related to the low levels of circulating nocturnal melatonin in this species (~10–20 pg/mL) (39) compared with the levels in the rat and human (8, 38). The functional sensitivity of endogenous MT_2 melatonin receptors in the mammalian SCN, and hence the effectiveness of exogenous melatonin to phase-shift circadian rhythms, may reflect individual differences in circulating melatonin levels.

In conclusion, our results are the first to demonstrate that physiological concentrations of melatonin desensitized endogenous MT_2 melatonin receptors, possibly without affecting the sensitivity of endogenous MT_1 receptors (34). Physiologically, we propose that MT_2 melatonin receptor desensitization in the SCN may contribute to determining the temporal sensitivity profile of the mammalian biological clock to a melatonin signal (30). It follows that variations in the amplitude of the circadian rhythm of melatonin secretion could desensitize endogenous MT_2 melatonin receptors at inappropriate times, preventing desired melatonin-mediated circadian phase shifts or entrainment (40). Together, these results suggest that therapeutic doses of melatonin reaching supraphysiological circulating levels could desensitize endogenous MT_2 melatonin receptors at critical periods of sensitivity (i.e., dusk and/or dawn), and therefore affect phase shifts by subsequent doses and/or produce detrimental side effects (41). This could potentially prevent desired circadian phase shifts by exogenous melatonin for the treatment of circadian disorders as those observed in advance or delay sleep phase syndrome, jet lag, shift work, seasonal affective, and other mood disorders (25, 37, 40). Thus, our finding of feedback regulation by melatonin in the SCN to mediate phase shifts through MT_2 melatonin receptors has important implications for a range of physiological and pharmacological contexts. FJ

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