Short-Term Exposure to Melatonin Differentially Affects the Functional Sensitivity and Trafficking of the hMT₁ and hMT₂ Melatonin Receptors

M.J. Gerdin, M.I. Masana, D. Ren, R.J. Miller & M.L. Dubocovich

Department of Molecular Pharmacology and Biological Chemistry (MJG, MIM, DR, RJM, MLD)

Department of Psychiatry and Behavioral Sciences (MLD)

Northwestern University Institute for Neuroscience (MJG, MIM, RJM, MLD)

Northwestern Drug Discovery Program (MJG, MIM, RJM, MLD)

The Feinberg School of Medicine of Northwestern University

Chicago, Illinois, 60611 USA
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Address Correspondence To:

Dr. Margarita L. Dubocovich

Department of Molecular Pharmacology and Biological Chemistry (S215)

The Feinberg School of Medicine

Northwestern University

303 East Chicago Avenue

Chicago, IL 60611-3008, USA

Tel.: (312) 503-8005; Fax: (312) 503-2334

E-mail: mdubo@northwestern.edu

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Abbreviations: GFP (green fluorescent protein); PBS (phosphate buffered saline); CHO (Chinese hamster ovary); GPCR (G protein-coupled receptor); SCN (suprachiasmatic nucleus); GRK (G protein-coupled receptor kinase); cAMP (adenosine 3’5’ cyclic monophosphate); cGMP (cyclic guanosine 5’ monophosphate).
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ABSTRACT

The hormone melatonin mediates a variety of physiological functions in mammals through activation of pharmacologically distinct MT₁ and MT₂ G protein-coupled melatonin receptors. We therefore sought to investigate how the receptors were regulated in response to short melatonin exposure. Using 2-[¹²⁵I]-iodomelatonin binding, cAMP functional assays, and confocal microscopy, we demonstrated robust differences in specific 2-[¹²⁵I]-iodomelatonin binding, receptor desensitization and cellular trafficking of hMT₁ and hMT₂ melatonin receptors expressed in CHO cells following short (10 min) exposure to melatonin. Exposure to melatonin decreased specific 2-[¹²⁵I]-iodomelatonin binding to CHO-MT₂ cells (70.3 ± 7.6 %, n=3) compared to vehicle controls. The robust decreases in specific binding to the hMT₂ melatonin receptors correlates both with the observed functional desensitization of melatonin to inhibit forskolin-stimulated cAMP formation in CHO-MT₂ cells pretreated with 10 nM melatonin (EC₅₀: 159.8 ± 17.8 nM, n=3, p<0.05) versus vehicle (EC₅₀: 6.0 ± 1.2 nM, n=3), and with the arrestin-dependent internalization of the receptor. In contrast, short exposure of CHO-MT₁ cells to melatonin induced a small decrease in specific 2-[¹²⁵I]-iodomelatonin binding (34.2 ± 13.0 %, n=5) without either desensitization or receptor internalization. We conclude that differential regulation of the hMT₁ and hMT₂ melatonin receptors by the hormone melatonin could underlie temporally regulated signal transduction events mediated by the hormone in vivo.
In mammals, the hormone melatonin regulates a myriad of physiological functions including visual, circadian, cardiovascular, and neuroendocrine, through activation of high-affinity membrane receptors belonging to the G protein-coupled receptor (GPCR) superfamily (Brzezinski, 1997; Masana and Dubocovich, 2001). These physiological functions are mediated through activation of at least two molecularly and pharmacologically distinct melatonin receptors, the MT1 and MT2 (Dubocovich et al., 2000). Activation of the MT1 receptor mediates inhibition of neuronal firing in the mouse suprachiasmatic nucleus (SCN) (Liu et al., 1997) and vasoconstriction in rat vascular smooth muscle (Doolen et al., 1998; Masana et al., 2002). Activation of the MT2 receptor mediates phase shifts of both the circadian rhythm of wheel running activity in C3H/HeN mice (Dubocovich et al., 1998) and the peak of the circadian rhythm of neuronal firing rate in the rat SCN (Hunt et al., 2001), inhibition of dopamine release in the rabbit retina (Dubocovich et al., 1997), and vasodilatation in rat vascular smooth muscle (Doolen et al., 1998; Masana et al., 2002).

Activation of both recombinant and endogenous MT1 and MT2 melatonin receptors by the hormone melatonin induces a variety of signal transduction cascades (Masana and Dubocovich, 2001). Melatonin inhibits forskolin-stimulated cAMP production through a pertussis toxin (PTX)-sensitive G protein (Carlson et al., 1989; Witt-Enderby and Dubocovich, 1996) and stimulates protein kinase C (PKC)-dependent processes in recombinant and native tissues through activation of either receptor (Godson and Reppert, 1997; Hunt et al., 2001). Melatonin potentiates PGF$_{2\alpha}$ induced phospholipase C stimulation and arachidonate release through a PTX-sensitive G protein and PKC-dependent pathway via activation of the MT1 melatonin receptor (Godson and Reppert, 1997). In the SCN, activation of the MT2 receptor
phase shifts the circadian rhythm of the peak of neuronal firing through a PKC-dependent mechanism (Hunt et al., 2001). In contrast, activation of the MT1 and MT2 melatonin receptors exhibits different coupling mechanisms to the cGMP-signaling pathway. Activation of the MT2 melatonin receptor expressed in human embryonic kidney (HEK-293) cells decreases cGMP levels in a dose-dependent manner via the soluble guanylyl cyclase pathway, however, melatonin did not affect cGMP levels in HEK-293 cells expressing the MT1 melatonin receptor (Petit et al., 1999).

Desensitization of GPCRs is the waning of receptor responsiveness in the presence of persistent agonist challenge. Both MT1 and MT2 recombinant melatonin receptors expressed in mammalian cells desensitize following prolonged exposure to high concentrations of melatonin as shown by the attenuation of cAMP formation and phosphoinositide hydrolysis (MacKenzie et al., 2002). Desensitization of endogenous melatonin receptors has also been reported in ovine pars tuberalis cells using cAMP functional assays (Hazlerigg et al., 1993). Regulation of melatonin binding sites by endogenous melatonin is ambiguous. In the rat SCN and pars tuberalis, there is an inverse relationship between melatonin receptor density and serum melatonin levels (Gauer et al., 1993; Tenn and Niles, 1993), while in the hamster SCN no such correlation was observed (Recio et al., 1996). Together, these studies suggest that melatonin can negatively regulate its own receptors following long term exposure (> 5hr) to either endogenous or exogenous melatonin. With the exception of one study (MacKenzie et al., 2002), however, these regulatory effects could not be attributed to either the MT1 or MT2 melatonin receptors.
Melatonin regulation of the MT₁ and MT₂ melatonin receptors by short-term exposure to melatonin has not been reported. Therefore, the goal of the present study was to assess the effects of short melatonin exposure on the functional sensitivity and cellular trafficking of the MT₁ and MT₂ melatonin receptors using cAMP accumulation assays, confocal microscopy, and radioligand binding. We demonstrated that the hMT₂ melatonin receptor stably expressed in CHO cells desensitized and internalized through an arrestin-dependent mechanism following short exposure to melatonin, but these effects were not observed in CHO cells expressing the hMT₁ melatonin receptor.
Materials and Methods

Materials. cDNA containing the complete coding region of the hMT₁ (human Mel₁a cloned into pcDNAI) and hMT₂ melatonin receptor (human Mel₁b cloned into pcDNA-3) were provided by Dr. S.M. Reppert (Department of Neurobiology, University of Massachusetts Medical School, Worcester, MA) (Reppert et al., 1995; Reppert et al., 1994). Effectene transfection and plasmid DNA purification kits were obtained from Qiagen (Valencia, CA). Cell culture products were obtained from Gibco BRL (Grand Island, NY). 2-[¹²⁵I]-Iodomelatonin (SA: 2000 Ci/mmol) and [³H]-adenosine 3’,5’, cyclic monophosphate (SA: 23 Ci/mmol) were purchased from Amersham (Piscataway, NJ). Melatonin and other general reagents were obtained from Sigma (St. Louis, MO). Luzindole (2-benzyl-N-acetyltryptamine) and 4P-PDOT (4-phenyl-2-propionamidotetraline) were obtained from Tocris (Ballwin, MO), and 2-iodomelatonin from RBI (Natick, MA). Rat β-arrestin 1 (pCMV5) and β-arrestin 2 (pCR3.1) were provided by Dr. Richard J. Miller (Northwestern University, Chicago, IL).

FLAG and GFP Epitope Tagging. The FLAG peptide (DYKDDDDK) coding sequence was fused to the coding region of the hMT₁ and hMT₂ melatonin receptors by subcloning the corresponding receptor cDNA into the vector pFLAG-CMV-2 (Sigma). These constructs were used for expression of N-terminal FLAG-tagged hMT₁ and FLAG-tagged hMT₂ melatonin receptor fusion proteins in CHO cells (CHO-MT₁ and CHO-MT₂). The GFP coding sequence was fused to the carboxy-terminus of FLAG-tagged hMT₁ and FLAG-tagged hMT₂ melatonin receptors by subcloning the corresponding receptor cDNA into the vector pEGFP-N1 (Clontech, Palo Alto, CA). These constructs were used for expression of N-terminal FLAG-tagged/ C-terminal GFP-tagged hMT₁ or hMT₂ melatonin receptor fusion proteins in CHO cells (CHO-
MT₁-GFP and CHO-MT₂-GFP). The successful construction of carboxy-terminus GFP-tagged and/or amino-terminus FLAG-tagged hMT₁ and hMT₂ receptors was confirmed by DNA sequencing (Northwestern University Biotechnology Facility).

Cell Culture, Transfections, and Stable Melatonin Receptor Expression in 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% CO₂ at 37°C. CHO cells stably expressing the hMT₁-FLAG or hMT₂-FLAG receptors were generated by transfecting pcDNAI-hMT₁ or pcDNA3-hMT₂ with pSV-NEO plasmids using Effectene (Qiagen). Cells expressing the melatonin receptor were selected for their resistance to the antibiotic G418 at 300 µg/ml (Life Technologies, Gibco BRL) and the ability of these G418 resistant clones to specifically bind 2-[¹²⁵I]-iodomelatonin (2000 Ci/mmol, Amersham) in whole cell lysates. The CHO-MT₁ and CHO-MT₂ cell lines used in this study originated from a single cell selected using the limited dilution protocol. For transient expression of the hMT₁-FLAG-GFP or hMT₂-FLAG-GFP receptors, CHO cells at 40-50% confluency were transiently transfected with the corresponding constructs using Effectene (Qiagen).

Melatonin and Drug Treatments. CHO-MT₁ and CHO-MT₂ cells were cultured 2.5 days to 80-90% confluence. The cells were then incubated in serum free media for 5 hours and washed once with phosphate buffered saline (PBS). Cells were then treated with vehicle, melatonin, luzindole or 4P-PDOT in serum free media at 37°C as appropriate. In some studies, this was followed by short vehicle or melatonin treatment for 10 min in the absence or presence of the drugs as appropriate.
Membrane Preparation and 2-[^125]I-Iodomelatonin Binding Studies. Cells were washed twice with (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) and then pelleted by centrifugation (13,800 x g). Pellets were resuspended in Tris-HCl (50 mM, pH 7.4; MgCl₂ 10 mM) and pelleted by centrifugation (13,800 x g). Membrane pellets were stored at –80°C until used. 2-[^125]I-Iodomelatonin binding was determined in cell membranes (10-25 µg and 15-40 µg protein/assay for MT₁ and MT₂, respectively) as previously described (Witt-Enderby and Dubocovich, 1996). Briefly, binding reactions were started by adding cell membranes to tubes containing binding buffer (50 mM Tris-HCl, pH 7.4, 10 mM MgCl₂), 2-[^125]I-iodomelatonin (102.6 ± 0.6 pM, n=8, unless otherwise indicated) and appropriate concentrations of vehicle or competing agents in a total assay volume of 0.26 ml. Following incubations for 1 hour 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. Non-specific binding was determined in the presence of 1 µM melatonin. Homologous competition assays were performed by competition of 2-iodomelatonin (0.2 pM to 1 µM) for 2-[^125]I-iodomelatonin (33.3 ± 6.7 pM, n=3) binding to cell membranes. Saturation analyses were performed with 2-[^125]I-iodomelatonin (1 – 4000 pM) and non-specific binding to cell membranes defined with 1 µM melatonin.

cAMP Assay. CHO-MT₁ or CHO-MT₂ cells were seeded in 12 well plates and grown for 24 hours. Cells were then serum starved for 5 hours and treated with 100 nM (hMT₁) or 10 nM (hMT₂) melatonin or vehicle (F12 media) for 10 minutes. Cells were washed twice with PBS for 5 minutes at room temperature and then incubated with rolipram (30 µM) for 50 minutes at 37°C. Cells were stimulated with forskolin (20 µM) for 10 min in the absence or presence of melatonin (0.1 nM to 1µM). The amount of cAMP in the cells was determined by radioreceptor
binding (~65,000 cpm [3H]cAMP/tube) using cAMP as standard and the purified regulatory subunit of protein kinase A as binding protein. After incubation at 0°C for 2 hours, the reaction was terminated by vacuum filtration using glass fiber filters (Schleicher & Schuell, Keene, NH, USA) soaked in 0.5% polyethyleneimine (v/v). Filters were washed with ice-cold 50 mM Tris-HCl and the radioactivity counted by liquid scintillation.

Confocal Microscopy. CHO cells in culture at 40-50% confluence were transiently transfected with the hMT1-FLAG-GFP or hMT2-FLAG-GFP melatonin receptors and/or co-transfected with rat β-arrestin 1 and 2 using the Effectene transfection kit (Qiagen). After 24 hours, cells were lifted with trypsin and seeded on poly-D-lysine coverslips for another 24 hours. Cells were then incubated in serum free media for 5 hours, washed once in PBS, and treated with vehicle or melatonin at 37°C in serum free media. Cells were then washed twice with PBS for 5 minutes and fixed with 4% paraformaldehyde for 7.5 minutes. Coverslips were then mounted with a 40% glycerol/PBS solution and visualized on a Zeiss LSM 510 confocal microscope.

Data Analysis and Statistics. Percent decreases in specific 2-[125I]-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 control. IC50 Values from homologous competition analyses were converted to affinity constants using the equation IC50 = [Radioligand]+K_D. These parameters were calculated using the Prism program (GraphPad Software Inc., San Diego, CA). Saturation and competition curves were generated through nonlinear regression analysis. Statistical significance was determined by paired Student t-test for comparisons between two groups, or by
one-way analysis of variance (ANOVA) repeated measures with Dunnett post-test or two-way ANOVA with Bonferroni post-test for comparisons between more than two groups. A value of p<0.05 was taken as statistically significant.
Results

Short Exposure to Melatonin Decreased Specific 2-[\(^{125}\)I]-Iodomelatonin Binding to Both the hMT\(_2\) and the hMT\(_1\) Melatonin Receptors.

Melatonin-mediated regulation of specific 2-[\(^{125}\)I]-iodomelatonin binding sites was studied in CHO cell lines stably expressing either the hMT\(_1\) or hMT\(_2\) melatonin receptors with an N-terminal FLAG epitope: CHO-MT\(_1\) (B\(_{\text{max}}\): 604 ± 227 fmol/mg protein, n=3) and CHO-MT\(_2\) (B\(_{\text{max}}\): 320 ± 111 fmol/mg protein, n=3). The FLAG epitope did not alter the affinity of 2-[\(^{125}\)I]-iodomelatonin for the receptor or receptor function (Table 1).

CHO-MT\(_1\) cells were pretreated in serum free media with various concentrations of melatonin (1 nM - 1 \(\mu\)M) for 10 min (Fig. 1A). Cells were washed and specific binding was determined in cell membranes using 2-[\(^{125}\)I]-iodomelatonin (102.6 ± 0.7 pM, n=5) and defined with 1 \(\mu\)M melatonin. A small but significant decrease in specific 2-[\(^{125}\)I]-iodomelatonin binding was observed following pretreatment of CHO-MT\(_1\) cells for 10 min with melatonin concentrations of 100 nM (34.2 ± 13.0 %, n=5) and 1 \(\mu\)M (32.4 ± 9.2 %, n=5) compared to vehicle-treated control (Fig.1A), (vehicle treated: 135.2 ± 22.3 fmol/mg protein, n=5, vs. melatonin treated: 89.2 ± 22.9 fmol/mg protein, n=5, for 100 nM and 89.5 ± 16.4 fmol/mg protein, n=5, for 1 \(\mu\)M). In addition, pretreatment with 100 nM melatonin for 10 min did not significantly affect specific 2-[\(^{125}\)I]-iodomelatonin (33.3 ± 6.7 pM, n=3) binding to the hMT\(_1\) when defined with 2-iodomelatonin (1 \(\mu\)M) (Fig. 2A) (vehicle treated: 51.9 ± 8.2 fmol/mg protein, n= 3, vs. melatonin treated: 46.3 ± 7.2 fmol/mg protein, n= 3, ~11 % reduction).
Melatonin (1 nM to 1 µM) pretreatment of CHO-MT\textsubscript{2} cells induced a robust and statistically significant decrease (70.3 % ± 7.6, n=3) in specific 2-\textsuperscript{\textsuperscript{[125]}I}-iodomelatonin (103.3 ± 0.3 pM, n=3) binding beginning at a concentration as low as 10 nM (vehicle treated: 52.2 ± 10.4 fmol/mg protein, n=6, vs. melatonin treated: 14.4 ± 3.6 fmol/mg protein, n=3) compared to vehicle-treated controls (Fig. 1B). Specific 2-\textsuperscript{\textsuperscript{[125]}I}-iodomelatonin (33.3 ± 6.7 pM, n=3) binding defined with 2-iodomelatonin was also decreased when CHO-MT\textsubscript{2} cells were pretreated with melatonin (10 nM) (Fig 2B), (vehicle treated: 36.6 ± 7.22 fmol/mg protein (n=3), vs. melatonin treated 8.3 ± 1.7 fmol/mg protein (n=3), ~77 % reduction).

**Short Exposure to Melatonin did not Affect the Affinity of the hMT\textsubscript{1} and hMT\textsubscript{2} Melatonin Receptors for 2-\textsuperscript{\textsuperscript{[125]}I}-Iodomelatonin.**

The effect of melatonin pretreatment on the affinity (K\textsubscript{D}) of 2-\textsuperscript{\textsuperscript{[125]}I}-iodomelatonin for the CHO-MT\textsubscript{1} and CHO-MT\textsubscript{2} melatonin receptors was assessed in homologous competition experiments using 2-iodomelatonin (0.2 pM – 1 µM) (Fig 2). Competition curves were analyzed by non-linear regression and best fitted to one site. In vehicle treated cells, 2-iodomelatonin (0.2 pM – 1 µM) competed for 2-\textsuperscript{\textsuperscript{[125]}I}-iodomelatonin (33.3 ± 6.7 pM, n=3) binding to the hMT\textsubscript{1} and hMT\textsubscript{2} melatonin receptors with an affinity (K\textsubscript{D}) of 262 ± 0.03 pM, (n=3) and 331 ± 0.07, (n=3), respectively (Fig. 2A-D). Melatonin pretreatment did not affect the affinity (K\textsubscript{D}) of 2-iodomelatonin for competition with 2-\textsuperscript{\textsuperscript{[125]}I}-iodomelatonin binding to either the hMT\textsubscript{1} (202 ± 0.02 pM, n=3) or hMT\textsubscript{2} (360 ± 0.09 pM, n=3) when compared with results obtained in vehicle treated cells (Fig. 2 A-D).
Luzindole and 4P-PDOT Differentially Affected Specific 2-[\textsuperscript{125}I]-iodomelatonin Binding to CHO-MT\textsubscript{1} and CHO-MT\textsubscript{2} Cells.

The indole ligand luzindole shows 15-25 times higher affinity for the MT\textsubscript{2} than the MT\textsubscript{1} melatonin receptor, while the amidotetraline 4P-PDOT is a selective MT\textsubscript{2} receptor ligand (Dubocovich et al., 1997; Dubocovich et al., 1998). Short pretreatment (10 min) of CHO-MT\textsubscript{1} cells with luzindole (10 nM-10 \(\mu\)M) or 4P-PDOT (1 nM-10 \(\mu\)M) did not affect specific 2-[\textsuperscript{125}I]-iodomelatonin binding (Fig. 3A and 3C). Pretreatment of CHO-MT\textsubscript{2} cells with luzindole (1 nM – 10 \(\mu\)M) significantly decreased specific 2-[\textsuperscript{125}I]-iodomelatonin binding only at concentrations higher than 10 \(\mu\)M (Fig. 3B). By contrast, short pretreatment of CHO-MT\textsubscript{2} cells with 4P-PDOT (1 nM - 1 \(\mu\)M) induced a concentration dependent inhibition of 2-[\textsuperscript{125}I]-iodomelatonin binding, which was significant at concentrations of 10 nM and higher (Fig. 3D). 4P-PDOT also inhibited in a concentration-dependent manner forskolin-stimulated cAMP formation in CHO-MT\textsubscript{2} cells (pEC\textsubscript{50}: 7.5 \(\pm\) 0.1 nM, efficacy 51.7 \(\pm\) 7.7 \%, n=3, for 1 \(\mu\)M).

Luzindole and 4P-PDOT Blocked the Melatonin-Mediated Decreases in Specific 2-[\textsuperscript{125}I]-Iodomelatonin Binding to the hMT\textsubscript{2} Melatonin Receptor.

To assess whether the melatonin-induced decreases in specific 2-[\textsuperscript{125}I]-iodomelatonin binding to the hMT\textsubscript{2} melatonin receptor was a receptor-mediated event, we used the competitive melatonin receptor ligands luzindole and 4P-PDOT. Pretreatment of CHO-MT\textsubscript{2} cells with luzindole (100 and 1000 nM) alone did not affect specific 2-[\textsuperscript{125}I]-iodomelatonin binding to the hMT\textsubscript{2} melatonin receptor (Fig. 4A). However, there was a significant interaction (p<0.05).
between luzindole and melatonin. In the presence of luzindole, the melatonin-mediated (10 nM) decrease in specific 2-[125I]-iodomelatonin binding was completely antagonized (Fig. 4 A). In contrast, pretreatment of CHO-MT2 cells with 4P-PDOT (30 nM) alone reduced specific 2-[125I]-iodomelatonin binding (Fig. 4B). There was, however, a significant interaction (p<0.001) between 4P-PDOT and melatonin. Furthermore, pretreatment with 4P-PDOT (30 nM) antagonized the decrease in specific 2-[125I]-iodomelatonin binding reduced by pretreatment with melatonin (Fig. 4B).

Short Exposure to Melatonin Desensitized the hMT2 receptor and not the hMT1 Receptor.

The functional sensitivity of the hMT1 and hMT2 melatonin receptors following short term exposure to melatonin was assessed by determining the potency of melatonin to inhibit forskolin-stimulated cAMP formation in vehicle and melatonin-treated CHO-MT1 and CHO-MT2 cells. In vehicle treated CHO-hMT1 (Fig. 5A) and CHO-hMT2 (Fig. 5B) cells, melatonin (0.1 – 1000 nM) inhibited forskolin-stimulated cAMP formation in a dose-dependent manner. Forskolin stimulation was similar in vehicle (81.4 ± 12.0 pmol/well cAMP, n=5) and melatonin pretreated (82.8 ± 21.6 pmol/well cAMP, n=5) CHO-MT1 cells. Pretreatment of CHO-MT1 cells for 10 minutes with melatonin (100 nM) did not affect the potency of melatonin to inhibit forskolin stimulated cAMP formation compared to vehicle control (Fig 5A). In contrast, while melatonin pretreatment of CHO-MT2 cells did not affect forskolin-stimulated cAMP formation (59.4 ± 14.8 pmol/well cAMP, n=3) compared to vehicle control (80.0 ± 17.2 pmol/well cAMP, n=3), the potency of melatonin was significantly decreased in CHO-MT2 cells pretreated with
melatonin (10 nM) for 10 minutes (EC$_{50}$: 159.8 ± 17.8 nM, n=3, p<0.05) compared to vehicle control (EC$_{50}$: 6.0 ± 1.2 nM, n=3) (Fig. 5B).

**Short Exposure to Melatonin Internalized the hMT$_2$ but not the hMT$_1$ Melatonin Receptors when Arrestins Were Overexpressed.**

To investigate the cellular localization of the hMT$_1$ and hMT$_2$ melatonin receptors following pretreatment with melatonin, CHO cells were transiently transfected with hMT$_1$ or hMT$_2$ melatonin receptor cDNA constructs containing a N-terminal FLAG epitope and a C-terminal GFP (CHO-MT$_1$-GFP or CHO-MT$_2$-GFP). Expression of the GFP sequence on the melatonin receptor C-terminus did not affect the binding affinity of 2-[${}^{125}$I]-iodomelatonin for the melatonin receptors or receptor function (Table 1).

Figures 6A and 6E show confocal images of CHO-MT$_1$-GFP or CHO-MT$_2$-GFP cells pretreated with vehicle. The hMT$_1$ melatonin receptor localized to the cell plasma membrane, while the hMT$_2$ appeared more diffusely distributed within the perinuclear regions. Short exposure with melatonin (100 nM) for 10 min did not affect the membrane localization of hMT$_1$ melatonin receptor (Fig. 6B) compared to vehicle treated control (Fig. 6A). In contrast, pretreatment with melatonin (10 nM) appeared to increase the perinuclear fluorescence of the hMT$_2$ melatonin receptor (Fig. 6F) compared to vehicle treated control (Fig. 6E). Overexpression of arrestin 1 and 2 did not affect the cellular localization of the hMT$_1$ melatonin receptors treated with vehicle or melatonin (Fig. 6C and 6D). However, overexpression of arrestin 1 and 2 led to the formation of more defined intracellular puncta following acute
melatonin (10 nM) treatment of the hMT$_2$ melatonin receptor (Fig. 6H) compared with vehicle treated control (Fig. G).
Discussion

This study demonstrated that short exposure to melatonin differentially affects the functional sensitivity and cellular trafficking of hMT\(_1\) and hMT\(_2\) melatonin receptors under heterologous expression in CHO cells. Short melatonin pretreatment desensitized and internalized the hMT\(_2\) melatonin receptor, but not the hMT\(_1\) melatonin receptor, through an arrestin-dependent mechanism. We conclude that the differential regulation of the hMT\(_1\) and hMT\(_2\) melatonin receptors by short melatonin exposure may be a mechanism by which the hormone regulates temporally-mediated physiological events in vivo.

Short exposure to melatonin decreased 2-[\(^{125}\)I]-iodomelatonin binding to both recombinant hMT\(_1\) and hMT\(_2\) melatonin receptors. This decrease in specific 2-[\(^{125}\)I]-iodomelatonin binding was more robust for hMT\(_2\) melatonin receptors (~70%) compared to hMT\(_1\) melatonin receptors (~30%) suggesting different regulatory mechanisms. Changes in specific binding following agonist exposure has been shown for other GPCRs. Ko et al. (2002) reported a decrease in specific binding to D2 dopamine receptors following dopamine treatment (1hr) to be representative of internalization and Boundy et al. (1995) observed a loss of D2\(_L\) dopamine high affinity binding sites following exposure to quinpirole (1.5hr) compatible with receptor desensitization. Thus, the differential decreases in specific 2-[\(^{125}\)I]-iodomelatonin binding to the hMT\(_1\) and hMT\(_2\) melatonin receptors following short exposure to melatonin maybe mediated by different mechanisms including receptor desensitization/internalization and/or residual melatonin binding to the receptor.
Short exposure to melatonin functionally desensitized the hMT₂ melatonin receptor. Desensitization of GPCRs can involve uncoupling of receptor and G protein, receptor internalization, or receptor down-regulation (Ferguson, 2001). Desensitization of other Gᵢ-coupled receptors including the δ-opioid (Lowe et al., 2002), κ opioid (Appleyard et al., 1999), and A3 adenosine receptors (Palmer et al., 1996) occurs within minutes. Initially, receptor phosphorylation by second messenger kinases, G protein-coupled receptor kinases (GRKs), and other kinases including casein kinase 1α results in the uncoupling of receptor and G protein (Ferguson, 2001). Subsequent binding of arrestins to phosphorylated receptors targets GPCRs for endocytosis (Ferguson, 2001). Internalization as a mechanism of receptor desensitization has been shown for endogenous Gᵢ/Gₒ-coupled somatostatin receptors in NG108-15 cells (Beaumont et al., 1998). Using a similar melatonin treatment paradigm that functionally desensitized the hMT₂ melatonin receptor we observed that overexpression of arrestin 1 and 2 led to a marked enhancement of melatonin-mediated internalization of the hMT₂ melatonin receptor. Thus the robust decrease in specific 2-[¹²⁵I]-iodomelatonin binding following short melatonin exposure of hMT₂ melatonin receptors appears consistent with rapid receptor desensitization followed by internalization.

The hMT₁ melatonin receptors did not desensitize or internalize following short melatonin exposure even when co-expressed with arrestins 1 and 2. In contrast, Roy et al. (2001) found a rapid arrestin-dependent internalization of the MT₁ melatonin receptor in GT1-7 neurons following acute exposure to melatonin. GT1-7 cells express endogenous MT₁ melatonin receptors and thus the presence of endogenous signalling partners, which may be absent in CHO cells, may have facilitated MT₁ melatonin receptor internalization. In addition, there is a high
proportion of constitutively active MT₁ melatonin receptors expressed in non-neuronal mammalian cells (Dubocovich and Masana, 1998; Roka et al., 1999). Constitutively active GPCRs are thought to be constitutively phosphorylated and desensitized, however, constitutively active mutant AT₁A receptors do not show elevated basal phosphorylation (Thomas et al., 2000) and α₁b adrenergic receptors do not internalize due to an inability to activate arrestins (Mhaouty-Kodja et al., 1999). Therefore constitutively active hMT₁ melatonin receptors expressed in CHO cells may exist in a receptor conformation which does not exhibit elevated basal phosphorylation and is therefore unable to interact with arrestins to mediate internalization. Additionally, melatonin-mediated receptor internalization could be species specific as our study uses the human melatonin receptor while Roy et al. (2001) used the mouse melatonin receptor. Species specific patterns of internalization have been observed for the opioid receptors as the human κ-opioid receptor internalizes while the rat κ-opioid does not (Li et al., 1999).

The small decrease in 2-[¹²⁵I]-iodomelatonin binding to the hMT₁ melatonin receptor following acute melatonin exposure (~30%) is likely due to residual melatonin on the receptor given that the receptor did not desensitize or internalize following short melatonin exposure and has slightly higher affinity for melatonin than the hMT₂ melatonin receptor. Tight melatonin binding to the super high affinity state of the hMT₁ melatonin receptor was suggested by Witt-Enderby and Dubocovich (1996) where melatonin (1 μM) pretreatment of CHO cells stably expressing hMT₁ melatonin receptors for 1hr reduced binding of 2-[¹²⁵I]-iodomelatonin to the super high affinity site in whole cell lysates. However, we propose that the decrease in specific 2-[¹²⁵I]-iodomelatonin binding following melatonin pretreatment of CHO-MT₁ cells represents
residual melatonin bound to the high affinity site of the hMT₁ melatonin receptor. In the present study, competition of 2-iodomelatonin for the radioligand 2-[¹²⁵I]-iodomelatonin to crude washed membranes from either vehicle and melatonin treated CHO-MT₁ cells resulted in monophasic curves with identical affinity for a single site. Similarly, 2-[¹²⁵I]-iodomelatonin binds to a single high affinity site in crude washed CHO or HEK cell membranes expressing the hMT₁ receptor (Roka et al. 1999; Browning et al. 2000). Melatonin binding to the high affinity state is supported by the fact that melatonin (100 nM) pretreatment did not significantly affect specific binding to the MT₁ melatonin receptor when defined with 2-iodomelatonin (1µM), whereas a significant decrease in specific binding was observed when defined using melatonin (1µM). This difference is due to 2-iodomelatonin being able to compete for 2-[¹²⁵I]-iodomelatonin with 10 times higher affinity compared to melatonin (Browning et al., 2000).

The mechanism through which melatonin differentially affects desensitization and internalization of the hMT₁ and hMT₂ melatonin receptors is not well understood. In COS-7 and HEK-293 cells, activation of the D₂, but not the D₃ dopamine receptor results in internalization following receptor phosphorylation and translocation of arrestins to the membrane (Kim et al., 2001). Similarly, the β₂-adrenergic receptor undergoes agonist-mediated internalization, while the β₁-adrenergic receptor does not internalize due to its low affinity for β-arrestins (Shiina et al., 2001). The δ-opioid receptor activates arrestin more efficiently than the µ-opioid receptor resulting in faster desensitization kinetics (Lowe et al., 2002). Thus differences in affinity and/or activation of arrestins between the hMT₁ and hMT₂ melatonin receptors may underlie their different patterns of desensitization and internalization. Recently, MacKenzie et al. (2002) reported desensitization of both hMT₁ and hMT₂ melatonin receptors following exposure to 1
μM melatonin for 5 hours. The observations in the present study were made after acute (10 min) exposure of hMT₁ and hMT₂ melatonin receptors to melatonin. Therefore desensitization and internalization of the hMT₁ melatonin receptor may require longer periods of exposure. It is also possible that the GFP on the hMT₁ melatonin receptor C-terminus may have compromised contact between the putative PDZ domain on the receptor and potential trafficking partners (Stricker et al., 1997).

Luzindole and 4P-PDOT, two well characterized competitive melatonin receptor ligands, were used to determine if the decreases in specific 2-[¹²⁵I]-iodomelatonin binding to CHO-hMT₂ melatonin receptors was receptor-mediated. Luzindole is an MT₂ melatonin receptor antagonist in both recombinant systems (Browning et al., 2000) and in native tissue (Dubocovich et al., 1997; Hunt et al., 2001). Pretreatment of CHO-MT₂ cells with luzindole did not affect specific 2-[¹²⁵I]-iodomelatonin binding determined in cell membranes, except at concentrations higher than 10 μM. However, pretreatment with luzindole followed by co-incubation with melatonin blocked the melatonin-mediated decrease in specific 2-[¹²⁵I]-iodomelatonin binding to the hMT₂ melatonin receptor induced by short exposure suggesting a receptor-mediated event. In contrast, 4P-PDOT is a competitive MT₂ melatonin receptor antagonist in native tissue (Dubocovich et al., 1998; Hunt et al., 2001), but also shows partial agonist/antagonist efficacy in recombinant systems (Browning et al., 2000; Nonno et al., 1999; MacKenzie et al., 2002) and partial agonist efficacy on leukocyte rolling in the rat microcirculation (Lotufo et al., 2001). In the present study, 4P-PDOT acted as a partial agonist to inhibit forskolin-stimulated cAMP formation via activation of the MT₂-FLAG melatonin receptor with a potency (pEC₅₀: 7.5 ± 0.09) and intrinsic activity (0.79 ± 0.03) similar to that reported by Browning et al. (2000) at MT₂ melatonin
receptors (pEC50: 8.7 ± 0.29; intrinsic activity 0.86 ± 0.15). Thus, consistent with 4P-PDOT’s efficacy as a partial agonist, pretreatment of CHO-MT2 cells with 4P-PDOT decreased specific 2-[125I]-iodomelatonin binding and blocked the melatonin-mediated decrease in specific 2-[125I]-iodomelatonin binding.

Overall, these results suggest that signal transduction events mediated through activation of the hMT1 and hMT2 melatonin receptors are differentially regulated by acute melatonin exposure. Melatonin induced rapid desensitization and arrestin-mediated internalization of the hMT2 melatonin receptor and not the hMT1. Whether this differential regulation of MT1 and MT2 melatonin receptors by diurnal physiological concentrations (~1-400 pM) of the hormone in a native tissue in the presence of native signalling partners is observed is still an open question. The present findings, however, highlight that administration of supraphysiological doses of melatonin to humans (above 0.3 mg orally) may desensitize the MT2 melatonin receptor thereby affecting MT2 melatonin receptor-mediated physiological responses including exacerbating vasoconstriction (Doolen et al., 1998; Masana et al., 2002) and impairing phase shifts of circadian rhythms (Dubocovich et al., 1998; Hunt et al., 2001).
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Footnotes

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Reprint Requests:

Dr. Margarita L. Dubocovich

Department of Molecular Pharmacology and Biological Chemistry (S215)

The Feinberg School of Medicine

Northwestern University

303 E. Chicago Ave.

Chicago, IL 60611
TABLE 1. Affinity and Potency of Melatonin at WT and Epitope Tagged hMT<sub>1</sub> and hMT<sub>2</sub>

Melatonin Receptors.

The affinity constants (K<sub>D</sub>) of 2-[<sup>125</sup>I]-iodomelatonin binding to the WT, FLAG, and FLAG/GFP tagged MT<sub>1</sub> or MT<sub>2</sub> receptors were determined using homologous competition with 2-iodomelatonin. The potency and efficacy of melatonin to inhibit forskolin-stimulated cAMP formation was determined in CHO cells stably expressing WT or FLAG-tagged or transiently expressing FLAG/GFP-tagged MT<sub>1</sub> or MT<sub>2</sub> melatonin receptors. The potency of melatonin (0.1 – 1000 nM) was determined by non-linear regression analysis and the maximal efficacy determined at a melatonin concentration of 100 nM. Values represent the mean ± SEM (3-5) independent experiments.

<table>
<thead>
<tr>
<th>Receptor Construct</th>
<th>2-[&lt;sup&gt;125&lt;/sup&gt;I]-Iodomelatonin Binding</th>
<th>Forskolin-Stimulated cAMP Accumulation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K&lt;sub&gt;D&lt;/sub&gt; (nM)</td>
<td>EC&lt;sub&gt;50&lt;/sub&gt; (nM)</td>
</tr>
<tr>
<td>WT MT&lt;sub&gt;1&lt;/sub&gt;</td>
<td>0.15 ± 0.03</td>
<td>0.6 ± 0.3</td>
</tr>
<tr>
<td>MT&lt;sub&gt;1&lt;/sub&gt; FLAG</td>
<td>0.24 ± 0.02</td>
<td>1.7 ± 0.4</td>
</tr>
<tr>
<td>MT&lt;sub&gt;1&lt;/sub&gt; FLAG/GFP</td>
<td>0.18 ± 0.01</td>
<td>0.7 ± 0.3</td>
</tr>
<tr>
<td>WT MT&lt;sub&gt;2&lt;/sub&gt;</td>
<td>0.36 ± 0.06</td>
<td>1.4 ± 0.3</td>
</tr>
<tr>
<td>MT&lt;sub&gt;2&lt;/sub&gt; FLAG</td>
<td>0.45 ± 0.09</td>
<td>5.0 ± 1.5</td>
</tr>
<tr>
<td>MT&lt;sub&gt;2&lt;/sub&gt; FLAG/GFP</td>
<td>0.33 ± 0.07</td>
<td>2.5 ± 0.9</td>
</tr>
</tbody>
</table>
**Fig 1.** Short exposure to melatonin decreased specific 2-[125I]-iodomelatonin binding to the hMT2 and hMT1 melatonin receptors. CHO-MT1 (A) or CHO-MT2 (B) cells grown in culture to ~80% confluency were serum starved for 5 h and then pretreated *in situ* for 10 min at 37°C with either vehicle or melatonin (1-1000nM). Cells were harvested and membranes were prepared as described in *Materials and Methods*. Specific binding was defined with 1 µM melatonin. The ordinate represents specific 2-[125I]-iodomelatonin (102.9 ± 0.4 pM, n=8) binding expressed as fmol/mg protein. Data represent mean ± SEM of 3-5 independent experiments performed in duplicate. Data were analyzed using one-way ANOVA repeated measures with Dunnett post-test (**p< 0.01 when compared with vehicle treated). MLT: Melatonin; V: Vehicle.

**Fig. 2.** Short exposure to melatonin did not affect the affinity of 2-iodomelatonin for competition with 2-[125I]-iodomelatonin binding to either the hMT1 or hMT2 melatonin receptors. CHO-MT1 (A,C) and CHO-MT2 (B,D) cells grown in culture to ~80% confluency were serum starved for 5 h and pretreated *in situ* for 10 min at 37°C with either vehicle (square, diamond) or melatonin (■, ◆) [100 nM (A,C); 10 nM (B,D)]. Cells were harvested and membranes were prepared as described in *Materials and Methods*. 2-Iodomelatonin (0.2 pM - 1 µM) competed for 2-[125I]-iodomelatonin (33.3 ± 6.7 pM, n=3) binding to membranes from either cell line. The ordinate represents 2-[125I]-iodomelatonin binding expressed as fmol/mg protein (A, B) and as percentage of total binding (C, D). Data represent the mean ± SEM of 3 independent experiments performed in duplicate.

**Fig 3.** Effect of pretreatment with luzindole and 4P-PDOT on specific 2-[125I]-iodomelatonin binding to the hMT2 or hMT1 melatonin receptors. CHO-MT1 (A,C) and CHO-MT2 (B,D) cells
in culture were serum starved for 5 h and then pretreated *in situ* for 10 min at 37°C with either vehicle, luzindole [1 nM - 10 μM (A,B)] or 4P-PDOT [1 nM - 10 μM (C,D)]. Cells were harvested and membranes were prepared as described in *Materials and Methods*. Specific binding was defined with 1 μM melatonin. The ordinate represents specific 2-[125I]-iodomelatonin (100.8 ± 0.8 pM, n=9) binding expressed as fmol/mg protein. Data represent mean ± SEM of 3-4 independent experiments performed in duplicate. Data were analyzed using one-way ANOVA repeated measures with Dunnett post-test (*p<0.05, **p<0.01 when compared with vehicle control). V:Vehicle.

**Fig. 4.** Luzindole and 4P-PDOT antagonized the decrease in 2-[125I]-iodomelatonin binding to the hMT2 receptors induced by short pretreatment with melatonin. CHO-MT2 (A,B) cells in culture were serum starved for 5 h and then pretreated *in situ* for 10 minutes at 37°C with either vehicle or melatonin (10 nM) in the absence or presence of luzindole [100 nM, 1000 nM (A)] or 4P-PDOT [30 nM (B)]. Luzindole or 4P-PDOT was added 10 min before acute pretreatment with melatonin. Cells were harvested and membranes were prepared as described in *Materials and Methods*. Specific binding was defined with 1 μM melatonin. The ordinate represents specific 2-[125I]-iodomelatonin (100.2 ± 1.3 pM, n=5) binding expressed as fmol/mg protein. Data represent mean ± SEM of 2-3 independent experiments performed in duplicate. Data were analyzed using two-way ANOVA with Bonferroni post-test (**p<0.01 when compared with vehicle control). MLT: Melatonin; V: Vehicle.

**Fig. 5.** Short exposure to melatonin functionally desensitized the hMT2 melatonin receptor. CHO-MT1 (A) and CHO-MT2 (B) cells were serum starved 5 hours and treated with either
vehicle (□) or melatonin (■), 100 nM MT₁ and 10 nM MT₂. The ordinate (A,B) represents the percent of forskolin-stimulated cAMP (pmol). Curves represent the percent of forskolin-stimulated cAMP (pmol) inhibited by increasing concentrations of melatonin (0.1 – 1000 nM). In figure 5A, melatonin potently inhibited forskolin-stimulated cAMP formation in both vehicle (EC₅₀: 1.7 ± 0.4 nM, n=5) and melatonin treated (EC₅₀: 3.1 ± 1.4 nM, n=5) CHO-MT₁ cells. In figure 5B, melatonin potently inhibited forskolin-stimulated cAMP formation in vehicle (EC₅₀: 6.0 ± 1.2 nM, n=3), but not melatonin treated (EC₅₀: 159.8 ± 17.8 nM, n=3, *p<0.05) CHO-MT₂ cells. Data represents mean ± SEM of 3-5 independent experiments performed in duplicate.

**MLT:** Melatonin.

**Fig. 6.** Short exposure to melatonin induced internalization of the hMT₂ but not of the hMT₁ melatonin receptor. CHO cells were transfected with MT₁-GFP (A,B) or MT₂-GFP (E,F) or with MT₁-GFP (C,D) or MT₂-GFP (G,H) and arrestin 1 & 2. Shown are confocal microscopic images of CHO-MT₁-GFP cells pretreated for 10 minutes with vehicle (A,C), or 100 nM melatonin (B,D), CHO-MT₂-GFP cells pretreated for 10 minutes with vehicle (E,G), or 10 nM melatonin (F,H). Images are representative of 3-4 independent experiments captured at 100x magnification. Scale bar represents 5 µM. **MLT:** Melatonin.
Figure 1

A. hMT₁

B. hMT₂

Specific 2-[125I]-iodomelatonin binding (fmol/mg protein)

Log [MLT] (M)
Figure 3

A. hMT1

B. hMT2

C. hMT1

D. hMT2

PRETREATMENT

Log [LUZINDOLE] (M)

SPECIFIC 2-[3H]-IODOMELATININ BINDING (fmol/mg protein)

PRETREATMENT

Log [4P-PDOT] (M)

SPECIFIC 2-[125I]-IODOMELATININ BINDING (fmol/mg protein)

*  

**
Figure 4

A. Pretreatment with MLT or luzindole affects specific 2-[125I]-iodomelatonin binding (fmol/mg protein).

B. Pretreatment with 4P-PDOT also affects specific 2-[125I]-iodomelatonin binding (fmol/mg protein).
Figure 5

A. hMT$_1$

B. hMT$_2$

Log Melatonin (M)

Vehicle

MLT Treated

Log Melatonin (M)
Figure 6

hMT$_1$

hMT$_2$

Vehicle  MLT  Vehicle  MLT

β-arrestin 1 and 2