

# C-terminal domains within human MT<sub>1</sub> and MT<sub>2</sub> melatonin receptors are involved in internalization processes

**Abstract:** Melatonin, a molecule implicated in a variety of diseases, including cancer, often exerts its effects through G-protein-coupled melatonin receptors, MT<sub>1</sub> and MT<sub>2</sub>. In this study, we sought to understand further the domains involved in the function and desensitization patterns of these receptors through site-directed mutagenesis. Two mutations were constructed in the cytoplasmic C-terminal tail of each receptor subtype: (i) a cysteine residue in the C-terminal tail was mutated to alanine, thus removing a putative palmitoylation site, and a site possibly required for normal receptor function (MT<sub>1</sub>C7.72A and MT<sub>2</sub>C7.77A) and (ii) the C-terminal tail in the MT<sub>1</sub> and MT<sub>2</sub> receptors was truncated, removing the putative phosphorylation and  $\beta$ -arrestin binding sites (MT<sub>1</sub>Y7.64 and MT<sub>2</sub>Y7.64). These mutations did not alter the affinity of 2-[<sup>125</sup>I]-iodomelatonin binding to the MT<sub>1</sub> or MT<sub>2</sub> receptors. Using confocal microscopy, it was determined that the putative palmitoylation site (cysteine residue) did not play a role in receptor internalization; however, this residue was essential for receptor function, as determined by 3',5'-cyclic adenosine monophosphate (cAMP) accumulation assays. Truncation of the C-terminal tail of both receptors (MT<sub>1</sub>Y7.64 and MT<sub>2</sub>Y7.64) inhibited internalization as well as the cAMP response, suggesting the importance of the C-terminal tail in these receptor functions.

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## Introduction

Melatonin is released from the pineal gland during the hours of darkness. Melatonin's levels in the body are impaired in a variety of diseases, e.g. breast, prostate, lung and colorectal cancer, insomnia, seasonal affective disorder and Alzheimer's disease [1]. Melatonin often exerts its effects via G-protein-coupled melatonin receptors, MT<sub>1</sub> and MT<sub>2</sub> [2, 3]; however, it can modulate quinone reductase-2 enzyme activity via a melatonin binding site, MT<sub>3</sub> [4]. MT<sub>1</sub> and MT<sub>2</sub> receptors mainly couple to G<sub>i</sub> proteins [5] and inhibit intracellular 3',5'-cyclic adenosine monophosphate accumulation (cAMP) [6, 7]. An orphan receptor (GPR50) was also identified, which has a 45% homology to the melatonin receptors [8, 9].

The structure of MT<sub>1</sub> and MT<sub>2</sub> receptors is similar to other G-protein coupled receptors (GPCRs), in that it consists of seven transmembrane (TM) domains, connected by three extracellular and three intracellular loops [10]. Homology models of the melatonin receptors are based on the crystal structure of rhodopsin. The extracellular loops may be involved in ligand recognition, and the intracellular loops may participate in G-protein binding [9, 11]. Based on the knowledge of the rhodopsin structure, we sought to understand the function and regulation of melatonin receptors through construction of two mutants in the C-terminal tails of the MT<sub>1</sub> and MT<sub>2</sub> receptors (Fig. 1).

In the first mutation, a cysteine residue in the C-terminal tail was mutated to alanine (MT<sub>1</sub>C7.72A and MT<sub>2</sub>C7.77A).

This site is essential for the G-protein binding of the rhodopsin receptor. The cytoplasmic surface of rhodopsin consists of four intracellular loops. The first, second and third intracellular loops connect adjacent TM helices. The fourth intracellular loop is formed by the attachment of palmitoyl groups to two cysteine residues (Cys 322 and Cys 323) in the C-terminal region and inserting it into the lipid bilayer [12]. It has been suggested that the fourth intracellular loop interacts directly with G<sub>i</sub> and plays a role in the regulation of G<sub>t $\beta$  $\gamma$</sub>  subunit [13]. These palmitoylated cysteine residues have also been reported for other GPCRs like human luteinizing hormone receptor, where the palmitoylation state of the receptor governs the accessibility of the receptor to  $\beta$ -arrestins to regulate receptor internalization [14]. Palmitoylation of the cysteine residues of the C-tail also occurs in  $\beta$ -adrenergic receptors [15] and  $\alpha$ -adrenergic receptors [16]. The C-terminal tails of the MT<sub>1</sub> and MT<sub>2</sub> receptors contain a single cysteine residue, which could be a possible palmitoylation site and may form a fourth intracellular loop. Mutation of this cysteine within the melatonin receptors might prevent the fourth intracellular loop from forming, which may affect receptor function.

In the second mutation, the C-terminal tail in the MT<sub>1</sub> and MT<sub>2</sub> receptors was truncated (MT<sub>1</sub>Y7.64 and MT<sub>2</sub>Y7.64). The role of the C-terminal tail in receptor phosphorylation and subsequent  $\beta$ -arrestin binding is well known.  $\beta$ -Arrestins are proteins that are involved in the desensitization of most GPCRs [17], including MT<sub>1</sub> [18] and MT<sub>2</sub> receptors [19]. For most GPCRs, upon agonist

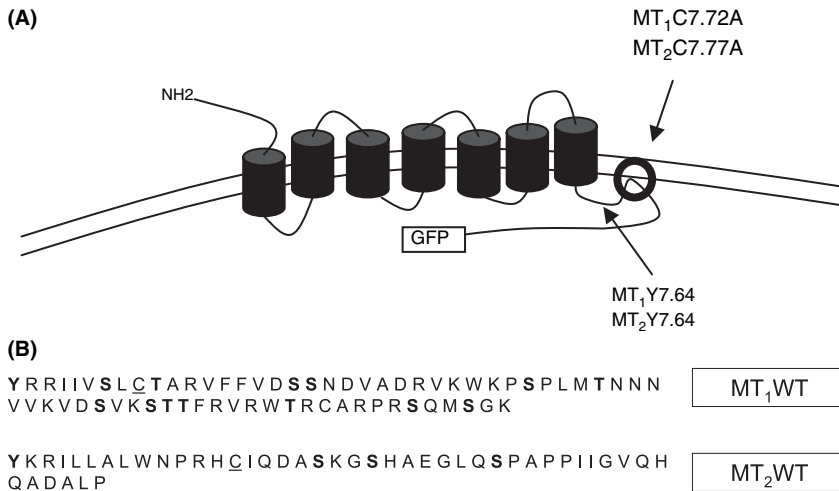


Fig. 1. (A) Diagram representing the site of the C-terminal mutations in MT<sub>1</sub> and MT<sub>2</sub> melatonin receptors. (B) Amino acid sequence of the C-terminal tails of the MT<sub>1</sub>WT and MT<sub>2</sub>WT receptors. In mutants MT<sub>1</sub>Y7.64 and MT<sub>2</sub>Y7.64, all the above amino acids were removed in their respective receptor type to remove the putative phosphorylation sites in the C-tail (the amino acids represented in bold). In mutants MT<sub>1</sub>C7.72A and MT<sub>2</sub>C7.77A, a cysteine residue in the respective C-tails was mutated to alanine (the underlined cysteines in the above sequence).

stimulation, the serine, threonine and tyrosine residues in the C-terminal tail are phosphorylated by the G-protein kinases.  $\beta$ -Arrestin recruitment to these phosphorylated amino acids forms a complex which is transferred to clathrin-coated pits for endocytosis, recycling and degradation [17]. Based on this information, it was thought that the truncation of the C-terminal tail of the melatonin receptors would remove a majority of these phosphorylation sites and impair receptor internalization.

The mutations were named according to the scheme described elsewhere [20]. Because it has been shown that desensitization/internalization processes drive cellular events induced by melatonin [18, 21] and  $\beta$ -arrestin scaffolds modulate melatonin receptor function [18, 19], the focus of this study was to determine if the above-mentioned domains were involved in melatonin receptor desensitization and internalization processes.

## Materials and methods

### Cell culture and transfections

COS-7 cells were grown as monolayers in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS) and 2 mM glutamine. The cells were grown to 60–70% confluence and transiently transfected with MT<sub>1</sub> or MT<sub>2</sub> wild-type (WT) or mutant constructs using genePORTER transfection reagent (Genlantis, San Diego, CA, USA), as per manufacturer's instructions.

### Materials

COS-7 cells were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). Geneporter transfection reagents were obtained from Genlantis. Cell culture products were obtained from GIBCO and Invitrogen (Carlsbad, CA, USA). Plasmid DNA purification kits were obtained from Qiagen (Valencia, CA, USA). Iodomelatonin was purchased from NEN/DuPont (Boston, MA, USA). cAMP kits were obtained from Assay Designs (Ann Arbor, MI, USA). Cell proliferation kits were obtained from Promega (Madison, WI, USA). 9,10-<sup>3</sup>H]

palmitic acid was obtained from PerkinElmer (Boston, MA, USA). Melatonin was obtained from Sigma-Aldrich (St Louis, MO, USA).

### Construction of mutant plasmids

Two mutations were made to the genes encoding human MT<sub>1</sub> or MT<sub>2</sub> receptors (Fig. 1) using site-directed mutagenesis (Quikchange II; Stratagene, La Jolla, CA, USA), according to manufacturer's instructions. In the first mutation, a cysteine residue in the C-terminal tail of each receptor was converted to alanine using primer pairs (MT<sub>1</sub>C7.72A: forward = GAATTATAGTCTCGC TCGCTACAGCCAGGGTGTTTC; reverse = GAACAC-CCTGGCTGTAGCGAGCGAGACTATAATTC) and (MT<sub>2</sub>C7.77A: forward = GAACCCACGGCAGCCAT-TCAAGATGCTTC; reverse = GAAGCATCTTGAAT-GGCGTGCCGTGGGTTTC). This substitution removes the putative palmitoylation site of the C-terminal tail, an important component for signaling in some other GPCRs. In the second mutation, the C-terminal tail of each receptor was truncated, deleting all potential phosphorylation sites in the C-terminal tail using primer pairs (MT<sub>1</sub>Y7.64: forward = CCAAATTTTCAGGAAGGAATAAAGGA-GAATTATAGTCTCGCTC; reverse = GAGCGAGACTATAATTCTCCTTTATTCCTTCTGAAATTTTGG) and (MT<sub>2</sub>Y7.64: forward = CCAAACCTCCGCAGG-GAATAAAGAGGATCCTCTTG; reverse = CAAGA-GGATCCTCTTTTATTCCTGCGGAAGTTTGG). All constructs included a green fluorescent protein (GFP) tag at the C-terminal region. Gene products for WT MT<sub>1</sub>, MT<sub>2</sub>, MT<sub>1</sub>Y7.64 and MT<sub>2</sub>Y7.64 were each cloned into pcDNA3 (Invitrogen), and the gene products for MT<sub>1</sub>C7.72A and MT<sub>2</sub>C7.77A were each cloned into pcDNA1 (Invitrogen). All mutants were confirmed by sequencing.

### Radioligand binding assays

Saturation binding assays were performed using 2-[<sup>125</sup>I]-iodomelatonin on whole cell lysates. The COS-7 cells were grown and transfected with the MT<sub>1</sub> or MT<sub>2</sub> WT or mutant

constructs in 10-cm<sup>2</sup> culture dishes, as described in ‘Cell culture and transfections’ section. On the third day after transfection, cells were washed twice with 2 mL of phosphate-buffered saline (PBS) and collected in buffer (0.25 M sucrose, 10 mM potassium phosphate, 1 mM EDTA, pH 7.4) and subjected to saturation binding analysis as already described [7]. Saturation binding curves were analyzed using commercial software (GRAPHPAD PRISM<sup>®</sup>; GraphPad Prism, Inc., San Diego, CA, USA) and  $K_D$  and  $B_{max}$  values were calculated and compared with WT melatonin receptors.

### cAMP accumulation assays

The cAMP accumulation assays were carried out by enzyme immuno-antibody according to manufacturer’s instructions. COS-7 cells were transfected with MT<sub>1</sub> or MT<sub>2</sub> WT or mutant constructs in 12-well plates. On the third day after transfection, the cell medium was aspirated, and the cells were incubated in serum-free media containing either 30  $\mu$ M rolipram alone (basal), 30  $\mu$ M rolipram and 100  $\mu$ M forskolin (maximal accumulation) or 30  $\mu$ M rolipram, 100  $\mu$ M forskolin and 10 nM melatonin. cAMP accumulation was expressed as a percentage of forskolin response within each group. Statistical analysis was performed using commercial software (GRAPHPAD PRISM<sup>®</sup>, GraphPad Prism, Inc.), whereby the maximal efficacy of melatonin to inhibit forskolin-induced cAMP accumulation was compared against the maximal forskolin response within each group.

### Confocal microscopy

The cells were grown and transfected with MT<sub>1</sub> or MT<sub>2</sub> WT or mutant constructs, as described in ‘Cell culture and transfections’ section. On the third day after transfection, the cells were lifted with trypsin and seeded on sterile coverslips for 24 hr in 6-well plates. Cells were incubated in serum-free media for 5 hr at 37°C and incubated with 1  $\mu$ M melatonin or vehicle (0.001% ethanol) for 1 hr. The cells were then washed twice with PBS and fixed with 2% paraformaldehyde for 15 min. Coverslips were mounted on slides and then visualized under oil immersion at 1000 $\times$  using a Leica TCS-SP2 confocal laser microscope (Leica Microsystems, Inc., Confocal Division, Exton, PA, USA).

### Radiolabeling with [<sup>3</sup>H] palmitic acid

Radiolabeling of WTMT<sub>1</sub> and WTMT<sub>2</sub> receptors with [<sup>3</sup>H] palmitic acid was carried out as previously described [22]. COS-7 cells were grown in six-well plates until the cells reached 60–80% confluence. Cells were then transfected with the MT<sub>1</sub> or MT<sub>2</sub> WT constructs. Twenty-four hours after transfection, cells were labeled with 50  $\mu$ Ci/mL 9,10-[<sup>3</sup>H] palmitic acid for 16 hr in the same media with reduced serum (3% FBS). Cells were then washed once with PBS, and scraped in cold RIPA buffer (Boston Bioproducts, Worcester, MA, USA). Aliquots of cell lysate were added to scintillation vials and counted in a liquid scintillation counter or used in a cell proliferation assay. The amount of [<sup>3</sup>H] palmitic acid counts were expressed as fmol/total cell

count. Cell proliferation assay was performed on the scraped cells using one solution cell proliferation assay kit (Promega) as per manufacturer’s instructions.

## Results

Saturation binding analysis revealed that the affinity and density of 2-[<sup>125</sup>I]-iodomelatonin for the mutant receptors was similar to the affinity and density obtained for WT type receptors (Table 1).

To determine if the mutations affected melatonin-mediated decreases in forskolin-induced cAMP accumulation, cAMP assays were carried out in naive cells (i.e. cells never exposed to melatonin). As shown in Fig. 2, melatonin (10 nM) inhibited forskolin-induced cAMP accumulation in COS-7 cells expressing the WT melatonin receptors. However, no inhibition of forskolin-induced cAMP accumulation by melatonin (10 nM) occurred in COS-7 cells expressing either of the MT<sub>1</sub> or of the MT<sub>2</sub> receptor mutants.

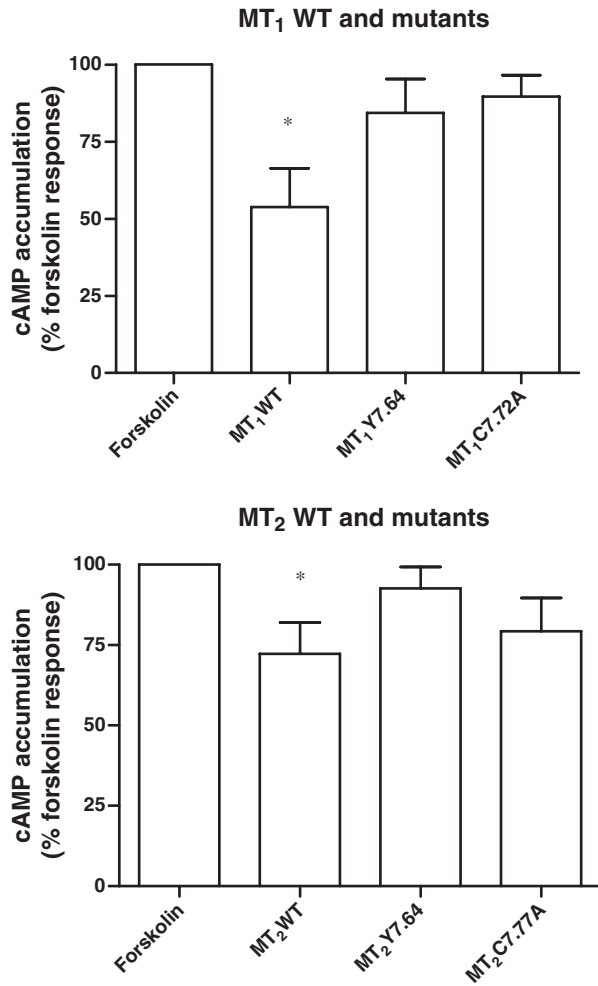
To determine if the C-terminal mutations affected the desensitization patterns of MT<sub>1</sub> and MT<sub>2</sub> receptors, COS-7 cells expressing either the WT or mutant forms of the receptors were first pretreated with 1  $\mu$ M melatonin for 1 hr, washed and then rechallenged with 10 nM melatonin for 20 min. Results show that pretreatment with melatonin desensitized WT MT<sub>2</sub> receptors reflected by attenuation in melatonin-induced inhibition of forskolin-induced cAMP accumulation. The selected mutations within the MT<sub>2</sub> receptors were without effect (Fig. 3). As for the WT MT<sub>1</sub> receptors, a 1-hr pretreatment with melatonin did not desensitize the receptor reflected by no attenuation in melatonin-mediated inhibition of forskolin-induced cAMP accumulation. The C-terminal mutations were without effect on modifying the responses of the MT<sub>1</sub> or MT<sub>2</sub> receptors to this melatonin exposure (Fig. 3).

All mutants and WT constructs used in this study were tagged with GFP, enabling visualization of the receptors through confocal microscopy. The WT MT<sub>1</sub> and MT<sub>2</sub> receptors internalized following melatonin pretreatment, as shown by a punctate and cytosolic distribution of the GFP-tagged receptor (panels B and H) when compared with their vehicle controls (panels A and G). The cysteine mutants

Table 1. Saturation binding analysis of 2-[<sup>125</sup>I]-iodomelatonin binding to the mutant and wild-type MT<sub>1</sub> and MT<sub>2</sub> receptors

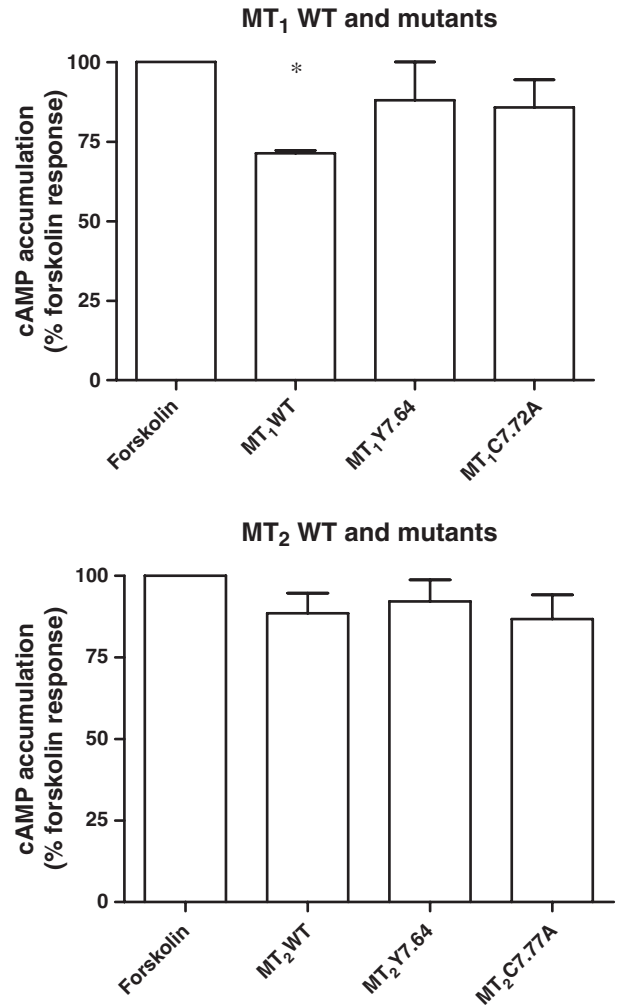
Receptor	$K_D$ (pM)	$B_{max}$ (fmol/mg protein)
MT <sub>1</sub> WT	80 $\pm$ 20	0.5 $\pm$ 0.2
MT <sub>1</sub> Y7.64	76 $\pm$ 16	0.5 $\pm$ 0.1
MT <sub>1</sub> C7.72A	60 $\pm$ 23	14 $\pm$ 11
MT <sub>2</sub> WT	73 $\pm$ 8	0.9 $\pm$ 0.4
MT <sub>2</sub> Y7.64	56 $\pm$ 8	0.3 $\pm$ 0
MT <sub>2</sub> C7.77A	75 $\pm$ 38	0.7 $\pm$ 0.2

Saturation binding was performed on whole cell lysates of COS-7 cells transiently expressing wild-type MT<sub>1</sub> and MT<sub>2</sub> receptors as well as their respective mutants. The affinity ( $K_D$ ) or  $B_{max}$  values for 2-[<sup>125</sup>I]-iodomelatonin binding were not significantly different between wild-type and mutant receptors. Each value represents the mean  $\pm$  S.E.M. of three to five independent experiments.



**Fig. 2.** Melatonin (10 nM)-mediated inhibition of forskolin-induced 3',5'-cyclic adenosine monophosphate (cAMP) accumulation in COS-7 cells transiently expressing wild-type or mutant melatonin receptors. As shown, a 20-min exposure to 10 nM melatonin inhibited forskolin-induced cAMP accumulation when compared with the forskolin response alone. By contrast, expression of mutant MT<sub>1</sub> (MT<sub>1</sub>C7.72A, MT<sub>1</sub>Y7.64) or MT<sub>2</sub> (MT<sub>2</sub>C7.77A, MT<sub>2</sub>Y7.64) receptors within COS-7 cells followed by a 20 min exposure to melatonin did not result in an inhibition of cAMP accumulation induced by forskolin. Each bar graph represents the mean  $\pm$  S.E.M. of six to eight individual experiments performed in duplicate. Significance (\*) is defined as  $P < 0.05$  when compared with the forskolin response alone.

(MT<sub>1</sub>C7.72A and MT<sub>2</sub>C7.77A) also internalized in response to melatonin (panels D and J) when compared with their controls (panels C and I) similar to the wild type receptors, indicating the lone cysteine residue in the C-terminal tails of these receptors did not play a role in receptor internalization (Fig. 4). The truncation mutants (MT<sub>1</sub>Y7.64 and MT<sub>2</sub>Y7.64) did not internalize after melatonin treatment (panels F and L) when compared with their vehicle controls (E and K). As shown in Fig. 4, the location of the GFP-tagged receptors remained at the plasma membrane even after melatonin exposure, suggesting that the C-terminal tail is essential for melatonin receptor internalization.



**Fig. 3.** Melatonin (10 nM)-mediated inhibition of forskolin-induced 3',5'-cyclic adenosine monophosphate (cAMP) accumulation in melatonin-pretreated COS-7 cells transiently expressing wild-type or mutant melatonin receptors. As shown, a rechallenge with 10 nM melatonin inhibited forskolin-induced cAMP accumulation in COS-7 cells expressing the wild-type MT<sub>1</sub> receptor (MT<sub>1</sub>WT) even after a 1-hr prior exposure to melatonin. By contrast, no melatonin-mediated inhibition of forskolin-induced cAMP accumulation occurred in COS-7 cells expressing wild-type MT<sub>2</sub> receptors (MT<sub>2</sub>WT). The C-terminal mutations for each of the melatonin receptor subtypes, MT<sub>1</sub> (MT<sub>1</sub>C7.72A, MT<sub>1</sub>Y7.64) or MT<sub>2</sub> (MT<sub>2</sub>Y7.64 and MT<sub>2</sub>C7.77A), were without effect when compared with wild-type receptors exposed to the same melatonin pretreatment. Each bar graph represents the mean  $\pm$  S.E.M. of three to five individual experiments performed in duplicate. Significance (\*) is defined as  $P < 0.05$  when compared with forskolin response alone within each group.

To determine if the WTMT<sub>1</sub> and MT<sub>2</sub> melatonin receptors were palmitoylated, COS cells, transiently transfected with WTMT<sub>1</sub> and WTMT<sub>2</sub> melatonin receptors, were labeled in culture with 9,10-[<sup>3</sup>H] palmitic acid for 16 hr, and the incorporated radioactivity was measured. WTMT<sub>1</sub>, but not WTMT<sub>2</sub> receptors, incorporated significantly higher levels of [<sup>3</sup>H] palmitic acid when compared with untransfected COS-7 cells (Fig. 5).

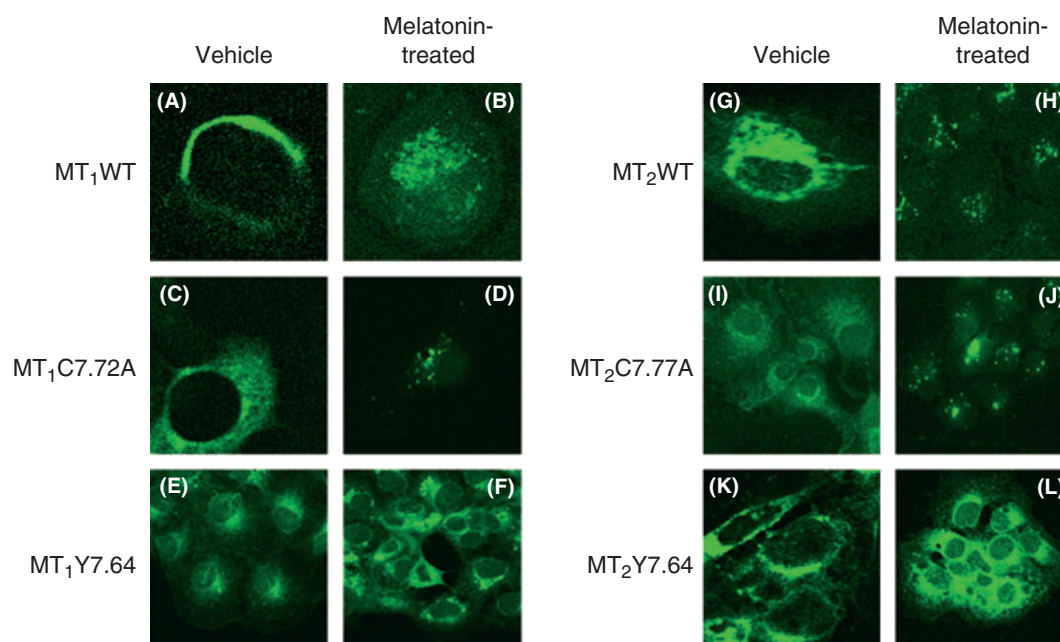


Fig. 4. Internalization patterns of green fluorescent protein-tagged wild-type and mutant receptors following exposure to melatonin. As shown, a 1-hr exposure to 1  $\mu$ M melatonin (B and H) induced internalization of wild-type MT<sub>1</sub> and MT<sub>2</sub> receptors expressed in COS-7 cells when compared with vehicle controls (A and G). Mutation of the cysteine residue in each of the melatonin receptor subtypes, MT<sub>1</sub> (MT<sub>1</sub>C7.72A) and MT<sub>2</sub> (MT<sub>2</sub>C7.77A) receptors internalized in response to melatonin (D and J) similar to wildtype (C and I). However, truncation of the C-terminal tail in each of the receptor subtypes, MT<sub>1</sub> (MT<sub>1</sub>Y7.64) and MT<sub>2</sub> (MT<sub>2</sub>Y7.64) prevented this internalization response to melatonin (F and I) when compared with their vehicle controls (E and K). Images are representative of six to seven independent experiments. Cells were visualized under oil immersion using a 100 $\times$  objective.

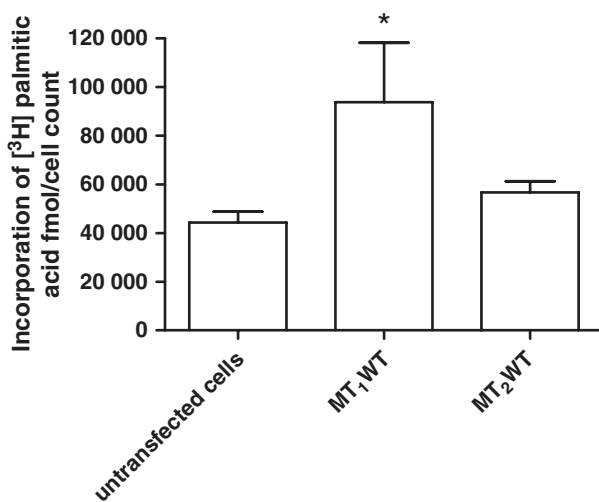


Fig. 5. Incorporation of 9,10-<sup>3</sup>H palmitic acid in COS-7 cells transiently transfected with WT MT<sub>1</sub> and WT MT<sub>2</sub> receptors as compared with untransfected COS-7 cells. The incorporation of [<sup>3</sup>H] palmitic acid was significantly increased in MT<sub>1</sub>WT constructs compared with untransfected COS-7 cells. Each bar graph represents the mean  $\pm$  S.E.M. of three to four individual experiments. Significance (\*) is defined as  $P < 0.05$  when compared to untransfected cells.

## Discussion

The results from this study demonstrate the importance of the C-terminal domains within each of the MT<sub>1</sub> and MT<sub>2</sub>

melatonin receptors as it relates to modulating receptor function and desensitization responses. In the first type of mutation, the cysteine residue in the C-terminal tail of each receptor was mutated to alanine. This cysteine is a palmitoylation site in many other GPCRs, and serves to form a fourth intracellular loop by embedding the palmitoyl groups into the membrane. This loop is important in G-protein binding and activation of the rhodopsin receptor [13], and the absence of this loop in some receptors has led to G-protein uncoupling [15, 23]. In this study, mutation of the cysteine residue within the C-terminal domain of each of the melatonin receptor subtypes, MT<sub>1</sub> and MT<sub>2</sub>, blocked the transfer of signal from the receptor to inhibit cAMP production. It is possible that the absence of this potential palmitoylation site within each of the melatonin receptor subtypes disrupts the formation of the fourth intracellular loop: a loop that may bind directly to G<sub>i</sub> proteins. This disruption in receptor/G<sub>i</sub>-protein coupling may result in attenuated cAMP inhibitory responses coming from their respective receptors.

Uncoupling of melatonin receptors from G<sub>i</sub> proteins using either GTP $\gamma$ S or pertussis toxin results in attenuated cAMP inhibitory responses in CHO cells [7]. Although not tested in this study, it is also possible that the cysteine mutations make the melatonin receptors more prone to desensitization by somehow allowing easier access of G-protein receptor kinases to these phosphorylation sites. This is merely speculative and more studies are required to support or refute this idea. The truncation mutants also confirm these findings because they also lack these impor-

tant cysteine residues. Taken together, the findings from both C-terminal mutations suggest that cysteine 7.72 in MT<sub>1</sub> receptors and 7.77 in MT<sub>2</sub> receptors play an important role in transducing cAMP responses throughout the cell.

Essential to the study of receptor regulation using mutagenesis approaches is the requirement that the mutations do not affect the binding affinity of the agonist to its receptors. As revealed through saturation binding analysis using the agonist radioligand, 2-[<sup>125</sup>I]-iodomelatonin, shows that no changes in the affinity ( $K_D$ ) of 2-[<sup>125</sup>I]-iodomelatonin for these mutated receptors occurred compared with their respective WT receptors (Table 1) or to those values reported in the literature [1]. It was expected that the mutation of the C-terminal tails of each of the melatonin receptor subtypes would not affect agonist binding affinity because the ligand binding site for melatonin receptors is thought to lie between TM 5 and 7 [24, 25], TM 3 [25, 26] for MT<sub>1</sub> receptors and TM 4 and 5 for MT<sub>2</sub> receptors [24, 27].

Desensitization is a process in which a receptor becomes refractory to its agonist over time. Many reports have shown that melatonin receptors become desensitized following exposure to melatonin either directly [18, 19, 28] or during the night when melatonin levels are highest [29]. In this study, WT MT<sub>2</sub> receptors but not MT<sub>1</sub> receptors desensitized after a 1-hr pretreatment with 1  $\mu$ M melatonin. These data are consistent with previous studies [7, 30]. As shown in this study, neither of the C-terminal mutations modulated the responses of each of the receptor subtypes (MT<sub>1</sub>, MT<sub>2</sub>) to a 1-hr exposure to 1  $\mu$ M melatonin. These data suggest that the lack of signal transfer through each of the melatonin receptors because of the cysteine 7.72 (MT<sub>1</sub>) or 7.77 (MT<sub>2</sub>) mutation prevented these receptor mutants from responding to the melatonin exposure like their WT counterparts.

Besides desensitizing through uncoupling, melatonin receptors, in response to agonist exposure, also desensitize by internalization of the receptors [18, 19]. As revealed through this study, truncation of the C-tail of each of the melatonin receptor subtypes (MT<sub>1</sub>Y7.64 or MT<sub>2</sub>Y7.64) prevented melatonin-induced internalization of MT<sub>1</sub> and MT<sub>2</sub> receptors when compared with their WT counterparts. By contrast, the cysteine point mutations at position 7.72 for MT<sub>1</sub> receptors or position 7.77 for MT<sub>2</sub> receptors displayed internalization patterns similar to what was observed for their WT receptors. These data suggest that these amino acids are not involved in melatonin-induced internalization processes.

Overall, the findings from the internalization assays show that the C-terminal tails within the MT<sub>1</sub> and MT<sub>2</sub> melatonin receptors function in melatonin receptor internalization. As such, removal of these sites through truncation mutagenesis prevented melatonin-induced internalization responses. Because these C-terminal tails contain many putative phosphorylation sites, these tails may modulate melatonin receptor internalization by providing the binding site to which  $\beta$ -arrestins can bind similar to what occurs for other GPCRs [31].

Palmitoylation assays demonstrate that the WTMT<sub>1</sub> receptors are palmitoylated when compared with nontransfected COS-7 cells. The palmitoylation of WTMT<sub>2</sub>

receptors was not significantly different than untransfected cells, which may suggest that palmitoylation occurs in MT<sub>1</sub> receptors (perhaps in the C-tail) but not in MT<sub>2</sub> receptors. Even though no significant difference in [<sup>3</sup>H] palmitate incorporation occurred in MT<sub>2</sub> receptors, this could be due to a limitation to the assay conditions (i.e. transiently transfected cells). More experiments need to be performed in this area to support or refute this idea.

In conclusion, this study reveals many important findings regarding those domains within the melatonin receptor that underlie desensitization processes. Considering that desensitization events may underlie melatonin-induced cellular differentiation [18, 21], then understanding the role that specific domains play in melatonin receptor function may lead to novel melatonin therapies targeted to enhance cellular differentiation such as for the treatment of cancer and osteoporosis (as reviewed [32]). Additionally, the knowledge gained from this study could lead to novel therapeutic targets as it relates to melatonin and melatonin-related disorders including insomnia and depression.

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