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# mt1 Melatonin Receptor in the Primate Adrenal Gland: Inhibition of Adrenocorticotropin-Stimulated Cortisol Production by Melatonin

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**The pineal hormone melatonin participates in circadian, seasonal, and reproductive physiology. The presence of melatonin binding sites in human brain and peripheral tissues is well documented. However, in the mammalian adrenal gland, low-affinity melatonin binding sites have been detected only in the rat by some but not all authors. Conflicting evidence for a regulatory role of melatonin on adrenal cortisol production, prompted us to investigate this possibility in a New World primate, the capuchin monkey. Expression of melatonin receptors in the adrenal cortex was demonstrated through pharmacological characterization and autoradiographic localization of 2-[$^{125}$I]iodomelatonin binding sites (dissociation constant = 96.9 ± 15 pM; maximal binding capacity = 3.8 ± 0.4 fmol/mg protein). The mt1 identity of these receptors was established by cDNA sequencing. Melatonin treatment of dispersed cells and explants from adrenal gland did not affect basal cortisol production. However, cortisol production stimulated by 100 nM ACTH was significantly inhibited by low melatonin concentrations (0.1–100 nM); this inhibitory effect was reversed by the mt1/MT2 melatonin antagonist luzindole. Melatonin also inhibited dibutyril-cAMP-stimulated cortisol production, suggesting that melatonin acts through a cAMP-independent signaling pathway. The present data demonstrate that the primate adrenal gland cortex expresses functional mt1 melatonin receptors and shows that melatonin inhibits ACTH-stimulated cortisol production. (*J Clin Endocrinol Metab* 88: 450–458, 2003)**

THE PINEAL HORMONE melatonin is a rhythmically secreted signaling molecule that participates in circadian and seasonal physiology, including reproductive behavior (1). Melatonin acts through two high affinity G protein-coupled receptors, termed mt1 and MT2 (2, 3). As in other species (4), in the human these receptors are present in brain and in a variety of peripheral tissues. High affinity binding sites for 2-[$^{125}$I]iodomelatonin are present in the human and rhesus monkey suprachiasmatic nucleus (SCN) and pituitary (5), human cerebellum (6), prostate (7), and kidney (8). Recent work detected mt1 receptors by immunohistochemistry in hippocampus (9) and cerebral arteries (10). The mRNA encoding for mt1 melatonin receptor isoform is expressed in human SCN (11), cerebellum (12, 13), and fetal kidney (14), whereas mRNA of isoform MT2 is expressed in cerebellum and fetal kidney (13, 14). Melatonin receptors are also present in steroidogenic tissues. Human granulosa cells and capuchin monkey testis show high affinity binding sites for 2-[$^{125}$I]iodomelatonin and express mRNA for melatonin receptor isoforms (15–18). In these tissues, melatonin has direct effects, increasing progesterone and decreasing testosterone production stimulated by human chorionic gonadotropin (16, 17), respectively.

In the human, there is evidence showing an inverse relationship between plasma melatonin and cortisol circadian rhythms. Melatonin is secreted with a 24-h pattern that peaks during the night, declines in the early morning and stays low during daytime (19). The 24-h pattern of plasma cortisol concentration peaks in the early morning (before lights on), to decline in the afternoon, remaining low most of the night (20). Under normal day/night conditions, the quiescent part of the cortisol rhythm coincides with the onset of the daily melatonin rhythm. This relationship remains phase-locked in subjects working night shifts despite circadian phase shifts of the cortisol and melatonin rhythms (21). This phase relationship is also preserved after exposure to a 3-h bright light pulse at the end of the night; treatment that abruptly suppresses plasma melatonin concentration, whereas cortisol concentration increases (22). Because multisynaptic pathways communicate the SCN, circadian pacemaker, with the pineal gland (23) and the adrenal cortex (24), phase locking and responses to light are interpreted as due to SCN control of both rhythms (21, 22).

The relationship between the rhythms of plasma melatonin and cortisol, together with the wide distribution of melatonin receptors in the human, as well as the evidence for direct effects of melatonin on steroidogenesis; prompted us to investigate whether melatonin may directly inhibit cortisol production by the primate adrenal gland. For this purpose, we used adrenal glands from adult capuchin monkey (*Cebus*

Abbreviations: $B_{max}$, Maximum binding capacity; $(Bu)_2$cAMP, *N*,*O*′-dibutyryl cAMP; DNase, deoxyribonuclease; GTP, guanosine 5′-triphosphate; $K_d$, dissociation constant; mt1 and MT2, high affinity G protein-coupled melatonin receptors; SCN, suprachiasmatic nucleus.

*apella*), to assess: 1) the presence, and 2) the localization of 2-[$^{125}$I]iodomelatonin binding sites, 3) the expression of melatonin receptor mRNAs, and 4) the effect of melatonin upon ACTH-stimulated cortisol production.

## Materials and Methods

### *Animals*

Tissues were obtained from eight adult capuchin monkeys (*Cebus apella*) from the Chilean Primate Center, Pontificia Universidad Católica de Chile (weight, 3.105 ± 0.381 kg; age, 16.31 ± 2.85 yr). In the colony, animals are kept on a 14-h light, 10-h dark schedule, controlled temperature and humidity, and food and water available *ad libitum* (25). Three sets of frozen adrenal glands and kidneys and one hypothalamus and diaphragm were obtained from the colony Tissue Bank; the tissues belonged to four animals that were killed for reasons unrelated to the present experiments. These tissues were used for membrane preparation, autoradiography, and RNA extraction. Fresh adrenal glands were obtained from four animals killed as part of the present experiments. Before processing, a piece of each adrenal gland was stored in RNA*later* (Ambion, Inc., Austin, TX) for RNA isolation. The rest of the tissues were incorporated into the Colony Tissue Bank.

In all cases, the animals were anesthetized with ketamine (Ketaset, 10 mg/kg of weight; Wyeth-Ayerst, Madison, NJ) and killed by an overdose of sodium thiopental (100 mg/kg of weight). Tissues were removed under sterile conditions, and either quickly frozen in liquid nitrogen and stored at −80 C or used fresh. Animal handling and care was performed following the recommendations of the NIH Guide for Animal Experimentation Care. The study protocol was approved by the Animal Experimentation Ethical Committee of the Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile.

### *Methods*

*Binding and competition studies.* Membranes were prepared by homogenizing the tissues in 20 vol of ice-cold Tris buffer (0.1 M, pH 7.4), containing 0.1 M cocktail of protease inhibitors (Sigma-Aldrich, St. Louis, MO). The homogenates were centrifuged for 30 min at 8,000 × *g* at 4 C, the pellets were discarded and the supernatants were centrifuged for 90 min at 40,000 × *g* at 4 C. The membrane pellet was resuspended in Tris-Ca buffer (25 mM Tris-HCl; 25 mM $CaCl_2$; and 0.2% BSA, pH 7.5) by sonication. Protein concentration was measured by spectrophotometry at 280 nm using 1 mg/ml albumin solution (Sigma-Aldrich) as standard.

The radioligand binding assay was performed as described by Song and Pang (26). Triplicate aliquots of membrane preparations (50–400 μg of protein) were incubated at 4 C for 4 h with 25–300 pM 2-[$^{125}$I]iodomelatonin (NEN Life Science Products, Boston, MA; specific activity 2200 Ci/mmol), in presence or absence of 1 μM melatonin (Sigma-Aldrich), in a final volume of 200 μl. The reaction was stopped by adding ice-cold Tris-Ca buffer (2 ml) and the membranes were separated by immediately filtering through borosilicate microfiber membrane filters (pore size: 1 μm, GC50; Advantec MFS Inc., Pleasanton, CA). The amount of 2-[$^{125}$I]iodomelatonin retained in the filter was measured in a γ-counter. Specific binding was calculated by substracting the nonspecific binding from the total binding. We tested the effect of melatonin and the related indols 6-hydroxymelatonin, serotonin, tryptamine, D-L-tryptophan, of luzindole (mt1 and MT2 receptor antagonist) and of guanosine 5′-triphosphate (GTP)γ-S (nonhydrolyzable GTP analog) upon 2-[$^{125}$I]iodomelatonin binding. All the compounds were purchased from Sigma-Aldrich. Triplicate aliquots of membrane preparations (50–400 μg) were incubated with 125 pM of 2-[$^{125}$I]iodomelatonin, and 1 pM to 1 μM of each compound in a final volume of 200 μl. The maximum number of 2-[$^{125}$I]iodomelatonin binding sites ($B_{max}$) and dissociation constant ($K_d$) were determined by Scatchard analysis using GraphPad Software, Inc. (San Diego, CA). Prism software, version 3.02. $IC_{50}$ was determined by analysis of competition curves using the method of Cheng and Prusoff (27).

### *Autoradiography*

Adrenal frozen sections (20 μm) were thaw-mounted on superfrost slides (Thomas, Swedesboro, NJ). Sections were stored at −70 C until processed. The sections were preincubated with Tris-Ca buffer for 15 min at 4 C and then incubated with 600 μl of 125 pM 2-[$^{125}$I]iodomelatonin for 4 h at 4 C. Nonspecific binding was determined in adjacent sections incubated in presence of 1 μM melatonin. To investigate whether the binding sites were coupled to G protein, we incubated simultaneously with 125 pM 2-[$^{125}$I]iodomelatonin and 1 μM GTPγ-S. We also tested the effect of 1 μM luzindole upon the binding of 2-[$^{125}$I]iodomelatonin. After incubation, the sections were washed 5 times with Tris-Ca buffer and dried at room temperature. The sections were left in contact with $^{125}$I-Hyperfilm (Amersham Pharmacia Biotech, Buckinghamshire, UK) in a x-ray cassette for 3 d, at −70 C. After exposure, the films were developed using D-72 solution (Eastman Kodak, Rochester, NY) for 3 min and immediately fixed by immersing in U-3 solution (Kodak) for 6 min. The autoradiographic images were scanned using a digital densitometer (GS-700 Imaging Densitometer, Bio-Rad Laboratories, Inc., Hercules, CA). The sections were stained with hematoxylin and eosin and scanned. Autoradiographic and histological staining images were adjusted to the same size.

### *Detection and identification of mt1 and MT2 melatonin receptor mRNAs by RT-PCR and sequencing*

SuperScript II RNaseH$^-$ reverse transcriptase, *Taq* DNA polymerase and deoxyribonuclease (DNase) I were all purchased from Life Technologies, Inc. (Rockville, MD). Random hexamers, Wizard DNA Purification System, and 500-bp DNA ladder were purchased from Promega Corp. (Madison, WI), and phenolic Chomczynski solution was from Winkler Ltd. (Santiago, Chile). Primer pairs were designed with the assistance of OLIGO 4.1 (Primer Analysis Software, Plymouth, MN) and BLASTN 2.2.1 tool (Ref. 28; www.ncbi.nlm.nih.gov), and synthesized by Life Technologies, Inc. Custom Primers. The sequence of the primers was chosen after alignment of human, hamster, rat and sheep mt1 and MT2 melatonin receptor sequences available at GenBank. Primers, numbered according to the human mt1 and MT2 mRNAs, (GenBank accession nos.: NM_005958.2 and U25341, respectively) were for mt1 melatonin receptor: forward, bases 477–501 (5′-CAAGTACGACAAACTGTACAGCAG-3′) and reverse, bases 888–912 (5′-CACAAACAGCCACTCTGGGATCCT-3′). The expected amplification product has a size of 435 bp. Primers for MT2 melatonin receptor were: forward, bases 629–653 (5′-TCATCCACTTCCTCCTCCCTATCG-3′) and reverse, bases 1001–1025 (5′-TTGGAAGCATCTTGAATGCAGTGC-3′). The expected amplification product has a size of 396 bp. In addition, we amplified a β-actin 280-bp fragment using primers for rat β-actin available in our laboratory (29).

Tissue samples (about 100 mg) were homogenized with Chomczynski reagent for total RNA purification. Two to 5 μg of each RNA sample were digested with DNase I, reverse transcribed using random primers, and subjected to PCR amplification. The reaction mixtures contained 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each deoxy-NTP, 0.2 μM of each primer, 2.5 U Taq DNA polymerase, and 10% of the reverse transcription product in a total volume of 25 μl. The PCR was carried out in a MJ Research, Inc. (PT-200; Waltham, MA) thermocycler and consisted of an initial denaturation step at 95 C for 5 min, followed by 35 cycles of 95 C for 30 sec 60 C for 1 min, and 72 C for 1 min, with a final extension at 72 C for 10 min. Aliquots of 12–20 μl of the PCR products were analyzed by electrophoresis on a 1.5% agarose-ethidium bromide gel. The mt1 and MT2 PCR products were purified using a Wizard DNA Purification System (Promega Corp.), and sequenced by the sequencing facility of our Faculty at the Ecology Department. The homology degree displayed by the mt1 and MT2 sequences from capuchin monkey to that of human mt1 and MT2 reported sequences was analyzed using BLASTN 2.2.1 tool (www.ncbi.nlm.nih.gov).

### *Effect of melatonin on ACTH-induced cortisol production in dispersed cells and explants*

Immediately after dissection, adrenal glands were weighed and processed. Dispersed cells were prepared from adrenal glands from two

animals, and explants from three animals. In one case, one adrenal was used for cell dispersion and the other for explants.

*Cell dispersion.* Cells were dispersed after Pepe and Albrecht (30), with slight modifications. Briefly, adrenals were cut in small fragments with fine scissors and suspended in DMEM F12 culture medium (Sigma-Aldrich), supplemented with 2% BSA and 0.2% collagenase (type II, Sigma-Aldrich). The cells were mechanically dispersed in a shaker bath for 5 min at 37 C. Dispersion was completed by aspiration and flushing through a 19-gauge needle and the dispersion was filtered through a sterile nylon mesh. The cell suspension was centrifuged at 1500 × *g* for 30 min. The pellet was washed with culture medium without collagenase and placed in an incubator for 2 h at 37 C, 100% humidity, 5% $CO_2$, and 95% air. Cell yield and viability (trypan blue exclusion, Sigma-Aldrich) was determined by counting an aliquot of cells in a hemocytometer. Triplicate aliquots of 100,000 cells/well were incubated during 48 h with 0.1–100 nM human ACTH (1–39) peptide (Sigma-Aldrich), with or without 1–100 nM melatonin. In addition, to confirm that the effect of melatonin is mediated by a membrane-bound receptor, we repeated the same experiment in presence of 1 μM luzindole (a competitive antagonist of melatonin receptors mt1 and MT2; Sigma-Aldrich). Additionally, we investigated whether melatonin triggers a signaling cascade involving an intracellular cAMP decrease by incubating cells with 1 μM *N,O'*-dibutyryl cAMP [$(Bu)_2$cAMP; a nonhydrolyzable cAMP analog] and 1–100 nM melatonin. At the end of the experiments, the supernatants were collected and stored at −20 C until assayed by RIA.

*Explants.* Adrenal glands were cut in small pieces with a sterile razor blade. Explants were mixed and suspended in 6 ml of culture medium (DMEM F12, 0.1% BSA). Triplicate 0.2 ml aliquots, taken using a Gilson pipette with cut off tip were incubated in 2 ml of culture medium (DMEM F12, 0.1% BSA) for 48 h. Aliquots were alternatively incubated with medium alone (control), 100 nM ACTH, 100 nM ACTH, and melatonin (0.1–100 nM) or with 100 nM ACTH and melatonin (0.1–100 nM) plus 1 μM luzindole. At the end of the experiment, the medium was separated and stored at −20 C until assayed by RIA and the explants were weighed. In each experiment, we checked cell viability by trypan blue exclusion after dispersion with collagenase. Cell viability was measured in one explant aliquot at the beginning of the experiment, and one explant aliquot of each treatment at the end of the 48-h incubation. Percentage of dead cells (n = 3) were 7.41 ± 3.0% at the beginning of the incubation. After 48-h incubation, the percentages of dead cells were 4.53 ± 0.59, in the control groups, 3.53 ± 1.17 in the 100 nM ACTH-treated groups, 5.29 ± 0.28 in the 100 nM ACTH + 100 nM melatonin treated groups, and 4.96 ± 0.30 in the 100 nM ACTH + 100 nM melatonin + 1 μM luzindole-treated groups.

### *Cortisol assay*

Cortisol concentration in culture medium was measured by RIA, using the reagents and methodology of the World Health Organization Program for the Provision of Matched Assay reagents for RIA of Hormones in Reproductive Physiology Program. The interassay and intraassay coefficients of variation for a plasma pool of 1.67 ng/ml were 8.1% and 9.9%, respectively.

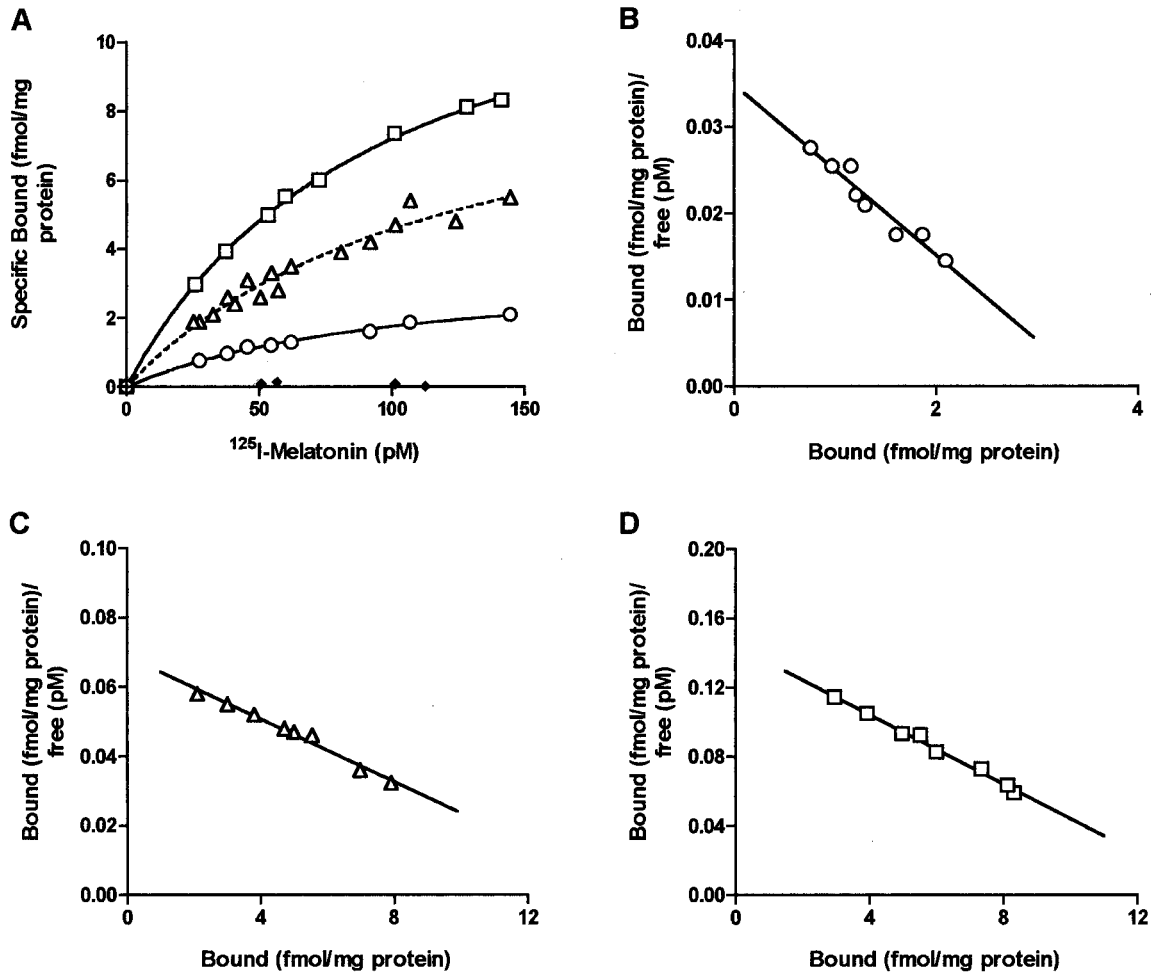


FIG. 1. Specific 2-[$^{125}$I]iodomelatonin saturation binding in membrane preparations from capuchin monkey (A). Scatchard plot analysis of adrenal gland (B), kidney (C), and hypothalamus (D). *Circles,* Adrenal gland; *triangles,* kidney; *squares*, hypothalamus; *rhombuses*, diaphragm.

### *Data analysis*

The data were expressed as mean ± SEM. The effect of melatonin treatment on *in vitro* ACTH-stimulated cortisol production was assessed by Kruskal-Wallis test followed by the post-hoc Dunn's Multiple Comparison test, using GraphPad Software, Inc. Prism (version 3.02). Results were considered significant when $P < 0.05$.

## Results

### *Characterization of melatonin binding sites*

We found specific binding of 2-[$^{125}$I]iodomelatonin in membrane preparations from capuchin monkey adrenal gland, kidney, and hypothalamus. There was no specific binding in the diaphragm (Fig. 1). 2-[$^{125}$I]Iodomelatonin binding in adrenal gland, kidney, and hypothalamus showed a $K_d$ in the picomolar range and was displaced by melatonin > 6-OH melatonin >>> serotonin, tryptamine, and tryptophan. Binding was displaced by the melatonin antagonist luzindole and by GTPγ-S in the three tissues (Table 1). These results indicate that the adrenal gland, kidney, and hypothalamus display specific high affinity binding sites for 2-[$^{125}$I]iodomelatonin, and that these sites most likely represent a membrane-bound receptor coupled to G protein.

**TABLE 1.** Binding affinity ($K_d$), receptor density ($B_{max}$) and $IC_{50}$ displayed by melatonin and compounds competing with the 2-[$^{125}$I]iodomelatonin binding in membrane preparations from adult capuchin monkey adrenal gland, kidney, and hypothalamus

| | Adrenal gland (n = 3) | Kidney (n = 3) | Hypothalamus (n = 1) |
|---|---|---|---|
| $K_d$ (pM) | 96.96 ± 15.41 | 91.07 ± 8.61 | 99.65 |
| $B_{max}$ (fmol/mg protein) | 3.83 ± 0.38 | 17.70 ± 3.86 | 15.51 |
| $IC_{50}$ (nM) | | | |
| Melatonin | 0.32 ± 0.06 | 5.61 ± 0.95 | 4.24 |
| 6-Hydroxymelatonin | 6.62 ± 1.72 | 1.05 ± 0.14 | 1.51 |
| Serotonin | >1000 | >1000 | >1000 |
| D-L-Tryptophan | >1000 | >1000 | >1000 |
| Tryptamine | >1000 | >1000 | >1000 |
| Luzindole | 24.32 ± 6.76 | 18.87 ± 8.7 | 23.3 |
| GTPγ-S | 1.35 ± 0.14 | 1.47 ± 0.02 | 1.51 |

Mean ± SEM; n, number of animals.

### *Autoradiographic localization of binding sites for melatonin*

Whole-mount contact autoradiography of adrenal gland sections incubated with 125 pM 2-[$^{125}$I]iodomelatonin showed label in the cortex whereas the medulla appeared negative (Fig. 2). The 2-[$^{125}$I]iodomelatonin binding in the cortex was displaced by 1 μM melatonin, 1 μM luzindole (Fig. 2), and 1 μM GTPγ-S (not shown). These results confirmed those obtained in membrane preparations.

### *Expression of mRNAs encoding for mt1 and MT2 melatonin receptors*

The expected 435-bp mt1-PCR-product was efficiently amplified from adrenal gland, kidney and hypothalamus RNA samples. In contrast, when the same RNA samples were subjected to RT-PCR for the expression of the MT2 mRNA, the predicted PCR-product of 396 bp was detected only in kidney and hypothalamus. Neither mt1 mRNA nor MT2 mRNA were detected in diaphragm. A 280-bp β-actin fragment was amplified as RNA loading control (Fig. 3, *middle panels*). Amplification of traces of genomic DNA was ruled out by digesting the RNA samples with DNase I, as well as by omitting the reverse transcriptase step or the RNA template (not shown).

The sequences of the mt1-PCR-product obtained from adrenal gland, and of the MT2-PCR-product from hypothalamus were 90% and 95% homologous, respectively, with the corresponding region of the cDNAs reported for human mt1 and MT2 melatonin receptors (Fig. 3, *lower panels*).

### *Effect of melatonin on ACTH-stimulated cortisol production in vitro*

Cortisol production by dispersed cells from adrenal gland of adult capuchin monkey showed the expected dose-

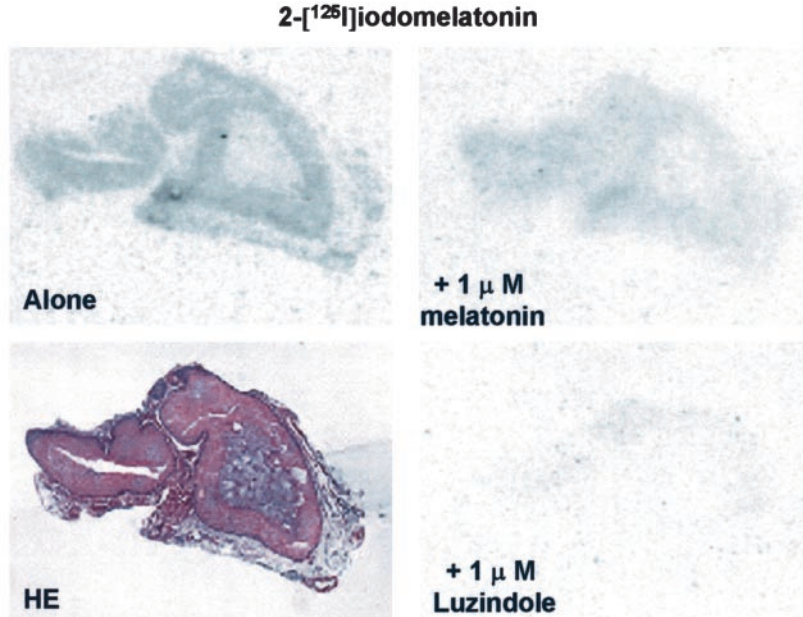


FIG. 2. Autoradiographic images of sections from capuchin monkey adrenal gland incubated with 2-[$^{125}$I]iodomelatonin. Each incubation condition (2-[$^{125}$I]iodomelatonin alone, plus 1 μM cold melatonin and plus 1 μM luzindole), is indicated. The *lower left panel* shows a hematoxylin and eosin (HE) staining of the section treated with 2-[$^{125}$I]iodomelatonin alone.

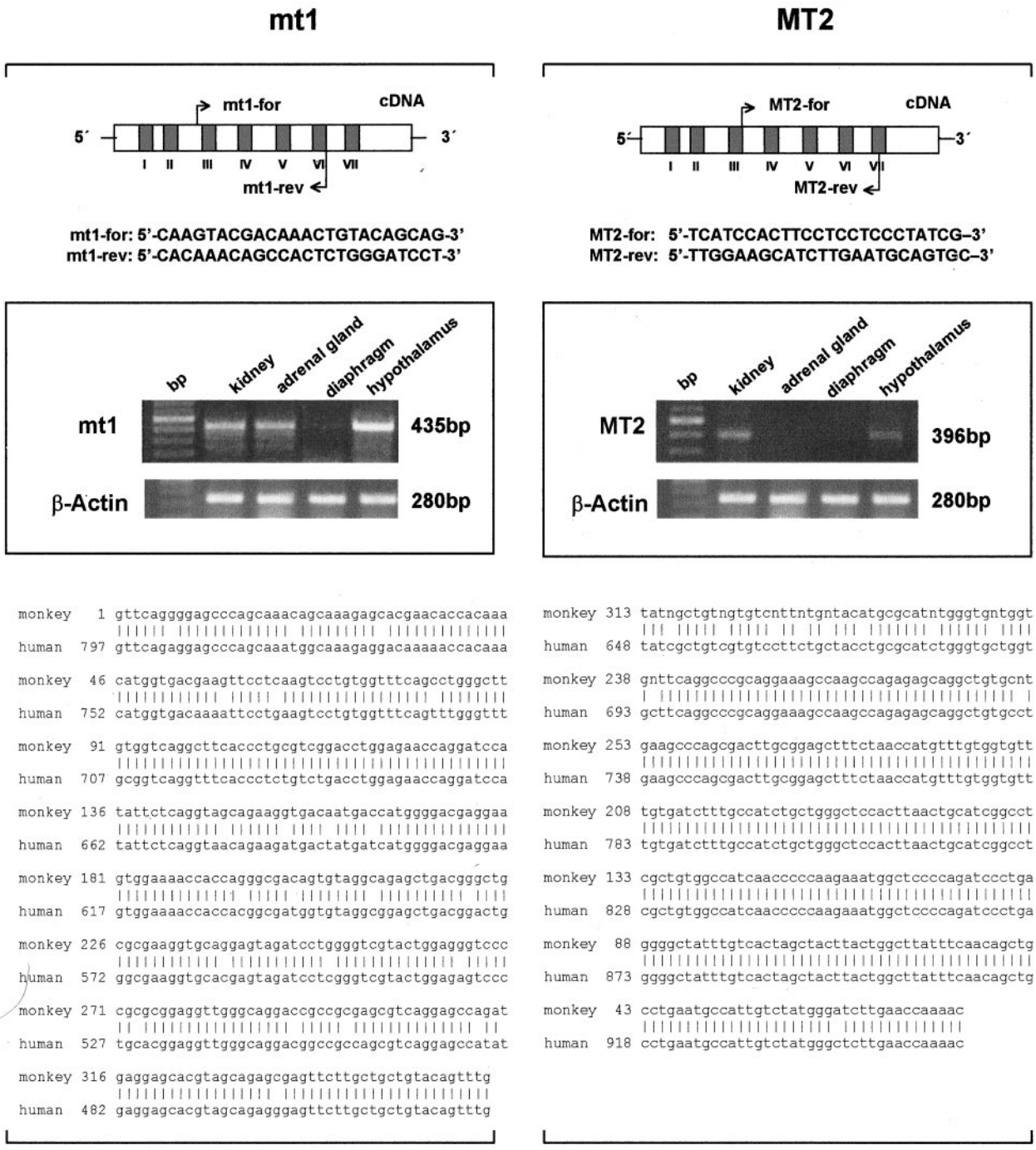


FIG. 3. *Upper panels*, schematic representation of the cDNAs encoding for human mt1 (*left*) and MT2 (*right*) melatonin receptors. *Roman numerals* indicate regions coding for transmembrane segments. The sequence of the primers, deduced from the second exon of the mt1 and MT2 melatonin receptors, is shown. *Middle panels*, mt1 (*left*) and MT2 (*right*) RT-PCR products. A 435-bp mt1 fragment was amplified in kidney, adrenal gland and hypothalamus; no amplification product was detected in diaphragm. A 396-bp MT2-fragment was amplified in kidney and hypothalamus; no amplification product was detected in the adrenal gland and diaphragm (*bp*, DNA size marker). *Lower panels*, homology analyses between the capuchin monkey mt1 (*left*) and MT2 (*right*) partial sequences and the corresponding region of the human mt1 and MT2 cDNA sequences.

response to ACTH (31). Maximal cortisol production (about 3-fold the basal value) was obtained with 10–100 nM ACTH (Fig. 4A), whereas incubation with 0.1–100 nM melatonin did not change basal secretion of cortisol (Fig. 4B). In the following experiments, a 100 nM ACTH dose was used to test the effects of melatonin upon ACTH-stimulated cortisol production in both adrenal dispersed cells and explants (Fig. 5). Melatonin (1, 10, and 100 nM) inhibited the cortisol production induced by 100 nM ACTH in dispersed cells; this effect was reversed by 1 $\mu$M luzindole (Fig. 5, *upper panels*). In the

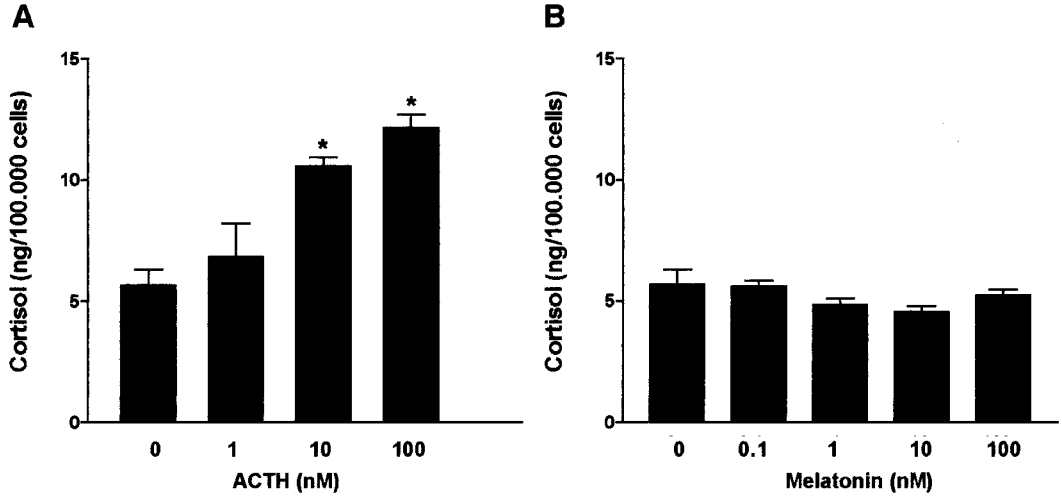


FIG. 4. Cortisol response of capuchin monkey adrenal cells in culture to increasing concentrations of ACTH (A) and melatonin (B). *, $P < 0.05$ *vs.* basal, Kruskal-Wallis test followed by the *post hoc* Dunn's multiple comparison test.

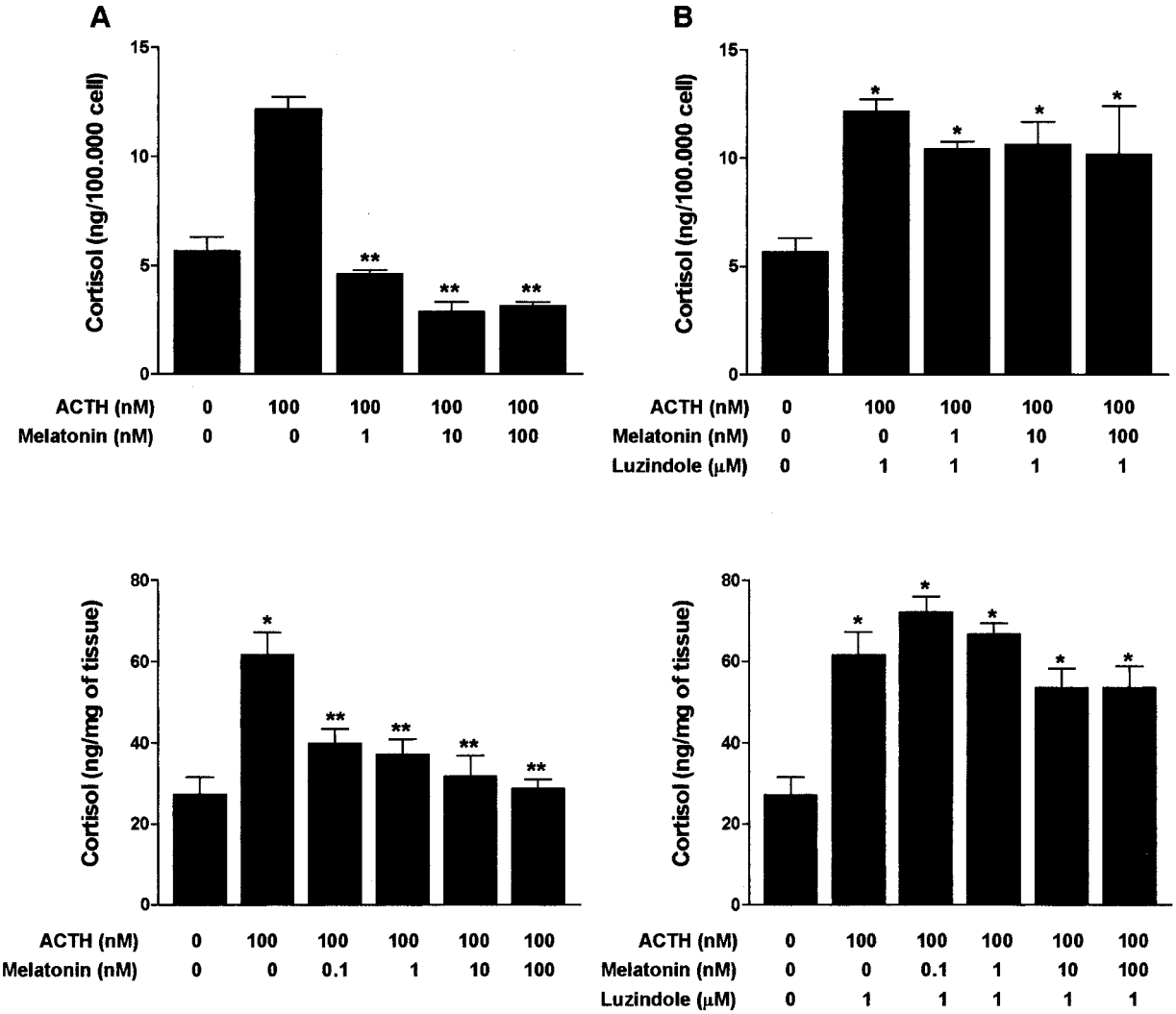


FIG. 5. Effect of melatonin upon 100 nM ACTH-stimulated cortisol production by cultured capuchin monkey adrenal cells (*upper panel*) and explants (*lower panel*). Response to increasing concentrations of melatonin (A). Reversion of the melatonin effect by 1 μM luzindole (B). Note that in explants a 0.1 nM dose of melatonin was also tested. *, $P < 0.05$ *vs.* without ACTH; **, $P < 0.05$ *vs.* with ACTH; Kruskal-Wallis test followed by the *post hoc* Dunn's multiple comparison test.

experiments with explants (Fig. 5A, *lower panel*), we additionally tested a 0.1 nM melatonin concentration. In the three experiments performed, this concentration was also inhibitory. Melatonin effect was reversed by luzindole. The antagonist *per se* had no effect on ACTH-stimulated cortisol production (Fig. 5B, *lower panel*). We used $(Bu)_2$cAMP to investigate whether the inhibitory effect of melatonin could be reversed by increasing the intracellular cAMP content. Indeed, we found that 1 $\mu$M $(Bu)_2$cAMP increased cortisol secretion about 2.5-fold, a pharmacological response that continued to be inhibited by melatonin (Fig. 6). These results show a direct inhibitory effect of low concentrations of melatonin on ACTH-stimulated cortisol production. The reversion of this effect, observed when melatonin was coadministered with luzindole, is consistent with the presence of functional membrane melatonin receptors in the steroidogenic cells of the adrenal cortex.

## Discussion

The present data show that the adrenal cortex of the adult capuchin monkey expresses membrane-bound mt1 melatonin receptors and that stimulation of these receptors by physiological concentrations of melatonin inhibits ACTH-stimulated cortisol production.

The adrenal cortex of adult capuchin monkey showed specific high affinity binding sites for 2-[$^{125}$I]iodomelatonin, with a pharmacological profile and $K_d$ similar to those reported for membrane-bound melatonin receptors in other tissues and species (32). In both whole adrenal gland membrane preparations and adrenal gland sections, 2-[$^{125}$I]iodomelatonin binding was displaced by luzindole (mt1/MT2 melatonin receptor antagonist; Ref. 33) and by GTP$\gamma$-S, a nonhydrolyzable analog of GTP (32). Moreover, 6-hydroxymelatonin was a weak competitor for 2-[$^{125}$I]iodomelatonin, whereas serotonin, D-L-tryptophan and tryptamine showed no effect (32). The $K_d$, about 100 pM, is within the range reported in human hypothalamus, kidney, and granulosa cells (5, 8, 15), ovine pars tuberalis (34), rat and capuchin monkey Leydig cells (16, 35), and for human mt1 and MT2 receptors and sheep and mouse mt1 receptors in transfected cell lines (2, 3, 36–38). There is no clear evidence for melatonin binding sites of high affinity in the adrenal gland of other mammals. No binding sites were detected in adult sheep (39), whereas sites of low affinity were detected (40) but not confirmed (41) in the rat.

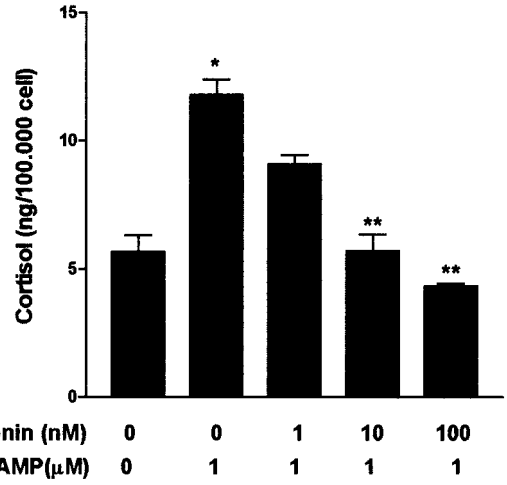


FIG. 6. Effect of increasing concentrations of melatonin upon 1 $\mu$M $(Bu)_2$cAMP-stimulated cortisol production by capuchin monkey adrenal cells in culture. *, $P < 0.05$ *vs.* basal; **, $P < 0.05$ *vs.* with (Bu)2cAMP; Kruskal-Wallis test followed by the *post hoc* Dunn's multiple comparison test.

The isoform of melatonin receptor present in the adrenal gland of the adult capuchin monkey was investigated using PCR primers designed for mt1 and MT2 receptors. In the adrenal gland, we detected mt1 but not MT2 mRNA expression. The sequence of the 435 bp mt1-PCR product was 90% homologous with bases 477–912 of the full-length human mt1 mRNA, encoding the transmembrane domains 3–6 of the protein (2, 3). The 396-bp sequence of the MT2-PCR-product was 95% homologous with the sequence of the corresponding region of the cDNA reported for human MT2 melatonin receptor (3). We detected expression of the mt1 and the MT2 isoform in hypothalamus and kidney, tissues that also showed high affinity binding sites for 2-[$^{125}$I]-iodomelatonin with the pharmacological profile of melatonin receptors. Expression of the mRNAs encoding for mt1 receptor has been reported in human SCN (11), and for mt1 and MT2 receptors in human fetal kidney (14). All together, these data indicate consistency between the expression of mRNA encoding for melatonin receptor isoforms and the presence of specific high affinity binding sites for melatonin.

We next tested whether melatonin had a direct action upon the adrenal gland. As expected, adrenal dispersed cells and explants increased cortisol production in response to ACTH (31). Melatonin completely inhibited the cortisol response to ACTH, without affecting cell viability. The fact that this effect was also observed using a 0.1 nM concentration (in the $K_d$ range found for 2-[$^{125}$I]iodomelatonin binding) and that it was reverted by luzindole, supports melatonin activation of a membrane-bound receptor. However, luzindole, in the concentration used in the present report, does not discriminate between mt1 and MT2 receptors (33). As we found that the adrenal gland expresses only the mt1 isoform mRNA, we conclude that the inhibitory effect of melatonin upon ACTH-stimulated cortisol production is exerted through a functional mt1 receptor.

In all species studied, ACTH stimulation of the adrenal cortex involves an increase of intracellular cAMP content (42). Activation of the endogenous mt1 receptor by melatonin lowers GnRH-stimulated cAMP production in rat Leydig and pituitary cells (1, 43) and forskolin-stimulated cAMP production in Syrian hamster SCN and ovine pars tuberalis (44, 45), as well as in mt1 transfected cell lines (2, 36). However, the inhibitory activity of melatonin was maintained in capuchin monkey adrenal cells cultured in presence of $(Bu)_2$cAMP, suggesting an action of melatonin downstream cAMP production. We have preliminary data showing increased production of progesterone after ACTH plus melatonin treatment, suggesting an effect similar to that reported in rat testis, in which melatonin increases 17-OH progesterone production (43). Mechanisms involving transcriptional control are also possible. Melatonin modifies the expression of several mRNAs encoding for peptide receptors in human granulosa cells, up-regulating LH receptor and down-regu-

lating GnRH receptor mRNAs (17). Thus, further investigation is required to elucidate the mechanisms by which melatonin inhibits ACTH-stimulated cortisol production.

Our experiments show for the first time the presence of a melatonin receptor in the adrenal gland of a primate. In the capuchin monkey, this receptor is located in the adrenal cortex. Moreover, we demonstrated a direct inhibitory effect of melatonin upon ACTH-stimulated cortisol production, in concentrations within the physiological range reached at night time hours in the capuchin monkey (unpublished) and the human (20–100 pg/ml, 0.086–0.436 nM; Ref. 46). The contribution of melatonin to adrenal function *in vivo* remains to be investigated. In the human, melatonin receptors have not been reported in the adrenal gland, and the results of experiments in which exogenous melatonin was given are conflicting. No effect of melatonin treatment at 1700 h upon cortisol concentrations was reported (47), whereas in other studies the same melatonin treatment schedule phase shifts the cortisol rhythm with increases in cortisol (48). Melatonin treatment at 0800 h increased midday cortisol in postmenopausal women but not in follicular phase women (49). These authors also found that, in young volunteers, 4-h light exposure at the end of the night suppresses melatonin and advances the acrophase of the cortisol rhythm decreasing its amplitude, effects that were reverted by simultaneous administration of melatonin during the light pulse (50). However, two recent reports highlight the inverse temporal relationship of the start of the quiescent part of the cortisol rhythm with the onset of the daily melatonin rhythm (21) and the inverse relationship between plasma cortisol and melatonin after exposure to a 3-h bright light pulse at the end of the night (22). Whether direct effects of melatonin upon the adrenal contribute to these responses is presently unknown.

Adrenal cortex regulation is complex, involving the concerted action of the SCN, ACTH, adrenal innervation, and intradrenal factors to produce a circadian rhythm of cortisol secretion and the appropriate reactive responses to sustain homeostasis against internal and external stressors (51). In diurnal mammals, as the capuchin monkey and the human, nighttime is the phase of sleep and reduced physical activity. Considering that melatonin signals nighttime, it is conceivable that its direct action upon the adrenal gland (reported here), fine tunes cortisol production for the physiological requirements of sleep.

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