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Rhythmic Expression of Functional MT1 Melatonin Receptors in the Rat Adrenal Gland

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We previously demonstrated that melatonin is involved in the regulation of adrenal glucocorticoid production in diurnal primates through activation of MT1 membrane-bound melatonin receptors. However, whether melatonin has a similar role in nocturnal rodents remains unclear. Using an integrative approach, here we show that the adult rat adrenal gland expresses a functional MT1 melatonin receptor in a rhythmic fashion. We found that: 1) expression of the cognate mRNA encoding for the MT1 membrane-bound melatonin receptor, displaying higher levels in the day/night transition (1800–2200 h); 2) expression of the predicted 37-kDa MT1 polypeptide in immunoblots from adrenals collected at 2200 h but not 1000 h; 3) no expression of the MT2 melatonin receptor mRNA and protein; 4) specific high-affinity 2-[^{125}I]iodomelatonin binding in membrane fractions and frozen sections from adrenals collected at 2200 h but not 0800 h (dissociation constant = 14.22 ± 1.23 pM; maximal binding capacity = 0.88 ± 0.02 fmol/mg protein); and 5) *in vitro* clock time-dependent inhibition of ACTH-stimulated corticosterone production by 1–100 nM melatonin, which was reversed by 1 μM luzindole (a melatonin membrane receptor antagonist). Our findings indicate not only expression but also high amplitude diurnal variation of functional MT1 melatonin receptors in the rat adrenal gland. It is conceivable that plasma melatonin may play a role to fine-tune corticosterone production in nocturnal rodents, probably contributing to the down slope of the corticosterone rhythm. (*Endocrinology* 149: 995–1003, 2008)

IN MOST SPECIES, plasma glucocorticoid concentration shows a robust circadian rhythm. The phase of the plasma glucocorticoid rhythm is opposite in nocturnal and diurnal mammals. In the rat, a nocturnal mammal, the peak of plasma corticosterone is found at the onset of nighttime (1). It is well established that circadian production of glucocorticoid involves the orchestrated action of the circadian rhythm of plasma ACTH, adrenal innervation, and local adrenal factors (2–4). However, there is new evidence suggesting the involvement of additional regulatory factors such as the intrinsic oscillatory capacity of the adrenal gland as a peripheral clock (5–9) and the neurohormone melatonin (10–12).

In mammals, plasma melatonin is produced by the pineal gland (13). Circadian plasma melatonin levels are characterized by high concentrations during nighttime, signaling day/night transition and also the season of the year. Indeed, the role of melatonin as a seasonal signal is clearly established in both diurnal and nocturnal mammals (14). Melatonin binds to two high-affinity G protein-coupled membrane-bound receptor isoforms (MT1 and MT2) (15, 16). Recently we demonstrated the expression of melatonin receptors in the adrenal gland of a diurnal mammal, the capuchin monkey, a new-world nonhuman primate. In this species, we showed that binding of 2-[^{125}I]iodomelatonin was restricted to the adrenal cortex and identified the melatonin receptors expressed as the MT1 isoform. This functional receptor displays dissociation constant (Kd) and maximum number of melatonin binding sites (Bmax) values in the range reported in other tissues; it mediates a direct inhibitory effect of melatonin on ACTH-stimulated cortisol production (10–12) and shows marked circadian changes in the capuchin fetal adrenal gland (17).

The rat, at variance of diurnal mammals, displays concurrent plasma melatonin and corticosterone peaks in the 24 h. It has been reported that the MT1 melatonin receptor mRNA is expressed in the rat adrenal gland (18), consistent with earlier reports describing putative melatonin binding sites in this tissue (19, 20). However, the evidence for direct actions of melatonin on adrenal glucocorticoid production is highly conflicting in nocturnal rodents like the rat (21). These authors studied several *in vivo* experimental conditions and concluded that there is no evident coupling between plasma melatonin and corticosterone. This conclusion is in line with that of Gromova *et al.* (22), Malendowicz (23), and Persengiev *et al.* (24). However, evidence for melatonin inhibition of adrenal glucocorticoid production and release has been obtained by several authors through a wide arrange of exper-

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Abbreviations: Bmax, Maximum number of melatonin binding sites; C_p, crossing point; Kd, dissociation constant; MT, melatonin receptor isoform; qPCR, quantitative PCR; SCN, suprachiasmatic nucleus.

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imental approaches (25–32), including study of sex (33) and time-of-day-specific differences (34, 35). According to Hajak *et al.* (21), these discrepancies may arise from the use of unphysiological experimental conditions, such as high doses of melatonin. However, undeniable evidence accounting for the expression of functional membrane-bound melatonin receptors in the rat adrenal gland has yet to be shown. Furthermore, daily variations of melatonin receptors have to be investigated because in the rat there is compelling evidence for circadian expression of melatonin receptors in both suprachiasmatic nucleus (36, 37) and pancreas (38).

To shed light on the discrepancies about possible direct actions of melatonin in the rat adrenal, here we ascertained the expression and 24-h changes of MT1 mRNA and protein as well as specific binding of melatonin in this tissue and whether this melatonin receptor is functional under *in vitro* conditions. In addition, we investigated whether the MT2 melatonin receptor mRNA and protein are also expressed in the rat adrenal gland.

Materials and Methods

Animals

The animals used in the present study were male Sprague Dawley rats (8 wk old; about 210 g body wt), obtained from the animal facility of the Facultad de Ciencias Biologicas, Pontificia Universidad Catolica de Chile. Groups of four rats per cage were kept under a photoperiod of 14 h light, 10 h dark (lights on at 0700 h), with food (chow) and water *ad libitum*. Temperature and humidity were controlled. Lights-off samples were obtained under red light (<0.2 lux). Animal handling and care was performed following the recommendations of the National Institute of Health Guide for Animal Experimentation Care. The study protocol was approved by the local ethics committee of our university.

Conventional RT-PCR for MT1 and MT2 receptors and quantitative PCR (qPCR) for MT1 receptor

After decapitation, total RNA of the adrenals from each rat (sampled at 0200, 0600, 1000, 1400, 1800, and 2200 h; n = 3 rats per time point), was extracted with RNAqueous-Midi large scale phenol-free total RNA isolation kit (code 1911; Ambion Inc., Austin, TX) following the manufacturer's instructions. The RNA concentration was measured by absorbance at 260 nm, and the sample integrity was assessed by electrophoresis in 1.5% agarose gel, prestained with ethidium bromide. Residual genomic DNA contained in the total RNA samples was digested with DNase I (Invitrogen Corp., Carlsbad, CA), according to the manufacturer's instructions. First-strand cDNA was synthesized from 1.5 μg of total RNA using SuperScript II RnaseH$^-$ reverse transcriptase (Invitrogen) and random hexamers and deoxynucleotide triphosphates from Promega Corp. (Madison, WI).

MT1 (expected size: 264 bp), MT2 (expected size: 230 bp), and the housekeeping gene β-actin (expected size: 352 bp) PCR fragments were amplified using conventional PCR primers and amplification conditions reported previously [Pozo *et al.* (39) for melatonin receptors and Torres-Farfan *et al.* (10) for β-actin].

For quantitative purposes, relative expression of the mRNA encoding for MT1 melatonin receptor was measured by qPCR (*i.e.* real-time PCR). To this end, we slightly modified the sequence of intron-flanking primers published for mouse MT1 to make them fit the corresponding rat MT1 sequence and β-actin primers published for rat (see Table 1 for details and references). The qPCRs were accomplished in a LightCycler instrument (Roche Applied Science, Mannheim, Germany), using 2.0 μl of cDNA sample, 0.3 μM each primer (Table 1), 3 mM $MgCl_2$, and the LightCycler FastStart DNA Master SYBR Green I kit (Roche) in a final volume of 10 μl. PCR conditions were: 10 min at 94 C followed by 40 cycles of denaturation at 94 C for 8 sec, annealing at 57 C for 6 sec, and extension at 72 C for 11 sec. The specificity of the PCR products and absence of primers-dimers was verified by electrophoresis in agarose/ethidium bromide gels (1.5% for MT1 and 1.8% for β-actin) against a 500-bp DNA ladder (Promega) and also by melting curve analysis. The expression of the MT1 gene was calculated relative to the expression of β-actin (an endogenous reference gene), according to a previously described method (40). For every PCR run, a standard curve was performed using serial dilutions of the corresponding purified PCR product. The slope of the standard curve was used to calculate the reaction efficiency, $E = 10^{-(1/\text{slope})}$. From the crossing point (C_p) values of each sample, E^{-Cp} was calculated for MT1 and β-actin and then used to obtain the ratio $E^{-Cp,\,MT1}$ to $E^{-Cp,\beta\text{-actin}}$. The C_p values for β-actin in each sample were just equal, validating its use as reference gene. All cDNAs were assayed in triplicate. Ratios MT1 to β-actin per adrenal set were calculated. Mean ratio for each clock time were calculated as described below.

To identify the qPCR products obtained as MT1 partial cDNA, the corresponding amplification product was purified by chromatography (DNA Wizard PCR Preps; Promega) and sequenced in the Ecology Department of the Faculty of Biological Sciences of the Pontificia Universidad Catolica de Chile. The identity of the qPCR product obtained from rat adrenal with the published rat MT1 mRNA sequence was determined using the BLASTN 2.2.1 tool [Altschul *et al.* (41); www.ncbi.nlm.nih.gov].

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

Membrane preparations. Rats were decapitated at 0800 (n = 15) and 2200 h (n = 15) to dissect the adrenal and hypothalamus (positive control) for membrane preparation. For each tissue, five pools were assembled from three animals at each time point to prepare membrane fractions as previously reported (10). In brief, the tissues were homogenized in Tris-Ca buffer [25 mM Tris-HCl; 25 mM $CaCl_2$; and 0.2% BSA (pH 7.5)], containing protease inhibitors. Four membrane preparations of adrenal and hypothalamus were selected on the basis of their electrophoretic pattern (10% SDS-PAGE) and spectrophotometric parameters (absorbance at 260 nm). The Bmax and Kd values were assessed as described below, running independent assays for the selected membrane preparations at both 0800 and 2200 h.

2-[^{125}I]iodomelatonin binding assay was performed as previously described (10), with slight modifications. In brief, triplicate aliquots of membrane preparations (500 μg protein) were incubated at 37 C for 2 h with 5–300 pM 2-[^{125}I]iodomelatonin (NEN Life Science Products, Boston, MA; specific activity 2200 Ci/mmol) in the presence or absence of 1 μM melatonin (Sigma-Aldrich Corp., St. Louis, MO), 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

TABLE 1. Sequence of the qPCR primers, exon location (according to the melatonin receptor gene structure reported for mouse), size of the expected qPCR product, annealing temperature, and number of cycles used to amplify partial cDNAs for MT1 and β-actin in rat adrenal gland

Gene	Primers (5′–3′)	Location exons	bp	Ta	Cycle no.	GenBank accession (reference)
MT1	Forward, tttactatcgtggtggacatcc Reverse, gcactaacttgacaatgcagatatc	1–2	206	57	>40	AF130341.1 (modified from Ref. 59)
β-Actin	Forward, gctcctcctgagcgcaag Reverse, catctgctggaaggtggaca	ND	75	60	40	NM_031144 (60)

Ta, annealing temperature (C); bp, base pairs of the expected qPCR product; ND, not determined.

was calculated by subtracting the nonspecific binding from the total binding. We tested the effect of luzindole (a melatonin membrane receptor antagonist) and GTPγ-S (nonhydrolyzable GTP analog) on 2-[^{125}I]iodomelatonin binding. All the compounds were purchased from Sigma-Aldrich. The binding assay was performed using three different adrenal pools for each time point assayed (0800 and 2200 h), whereas the effect of GTPγ-S and luzindole was assayed separately using one adrenal pool for each time point. The Bmax and Kd values were determined by Scatchard analysis using Prism software (version 3.02; GraphPad Software, Inc., San Diego, CA).

Contact autoradiography. Three rats were decapitated at 2200 and 0800 h. Immediately after dissection, the adrenals were fixed by immersion in 4% paraformaldehyde solution for 1 h, cryopreserved in sucrose gradient, and stored at −80 C. Afterward, 20-μm cryostat sections were obtained and thaw mounted on Superfrost slides (Thomas, Swedesboro, NJ). Adrenal frozen sections were preincubated with Tris-Ca buffer for 15 min at 37 C and then incubated with 100 μl of 25 pM 2-[^{125}I]iodomelatonin for 2 h at 37 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 25 pM 2-[^{125}I]iodomelatonin and 1 μM GTPγ-S. We also tested the effect of 1 μM luzindole on the binding of 2-[^{125}I]iodomelatonin. After incubation, the sections were washed five 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 an x-ray cassette for 1 d at −70 C. After exposure, the films were developed using an Image Station XP-100 system (Kodak, Rochester, NY).

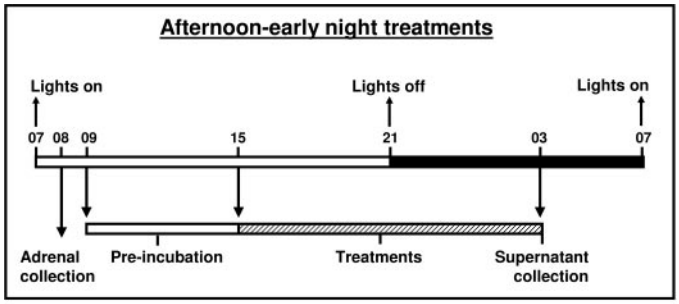
Immunoblotting for MT1 and MT2 receptors

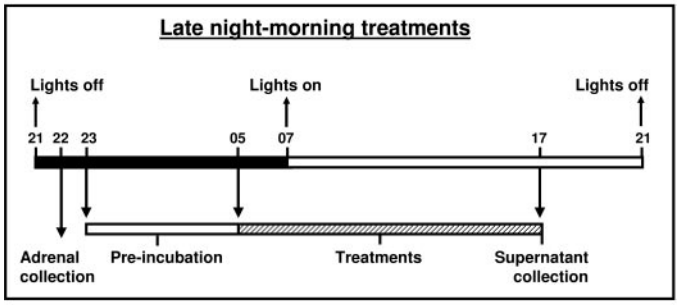
Thirty-microliter aliquots, containing 200 μg of membrane proteins from rat adrenal, kidney, and diaphragm (extracted at 1000 and 2200 h as described before; see membrane preparations), were separated on 10% SDS-PAGE and then blotted into polyvinyl difluoride sheets by standard techniques. After blocking nonspecific protein binding sites, immunoreactive polypeptides were detected using an enhanced chemiluminescence system (Super Signal; Pierce Biotechnology, Rockford, IL), as previously described (42). The primary antibody was anti-MT1 (dilution 1:100; Santa Cruz Biotechnology Inc., Santa Cruz, CA; code MEL-1A-R/R18: sc-13186, affinity purified goat polyclonal antibody raised against a peptide mapping near the C terminus of rat MT1) incubated for 18 h at 37 C, followed by rabbit antigoat IgG (dilution 1:50,000; Sigma-Aldrich), for 40 min at 37 C and then goat antirabbit IgG conjugated with horseradish peroxidase, dilution 1:50,000, for 30 min at 37 C. After washing, the membranes were incubated with Super Signal substrate (Luminol/enhancer solution-stable peroxidase solution, 1:1), for 10 min, in darkness. The blots were immediately exposed to BioMax MR-1 film (8G; Kodak) from 5 sec to 5 min. The films were developed in D-72 (Kodak), fixed in U3 solution (Kodak), dried, and digitalized.

We carried out the same experiment but using anti-MT2 as primary antibody (dilution 1:250; Santa Cruz Biotechnology; code MEL-1B-R/T18: sc-13177, affinity purified goat polyclonal antibody raised against a peptide mapping within an internal region of MT2 of mouse origin).

Effect of melatonin on ACTH-stimulated corticosterone production in vitro

Seven rats were decapitated at 0800 and 2200 h, the adrenal glands were dissected out, and their surrounding fat tissue was quickly removed. Immediately afterward, the adrenal glands of each animal were separately immersed in 15 ml of ice-cold sterile physiological serum and transferred to the tissue culture facility for further dissection to obtain rat adrenal quarters, based in our previous experience working with primate adrenal explants (10–12). In brief, each rat adrenal gland was cut in four roughly equivalent pieces (quarters), containing adrenal cortex and medulla, which were suspended in culture medium (DMEM/F12, 0.1% BSA; Life Technologies, Inc., Grand Island, NY). Each quarter (about 5 mg of tissue) was preincubated in 1 ml of culture medium for 6 h before any further treatment. Next, the eight adrenal quarters rendered by each rat were subjected to a full set of treatments applied during 12 h (incubation volume: 500 μl; see Fig. 1), as follows:





Treatment	1	2	3	4	5	6	7	8
ACTH (nM)	-	100	100	100	100	100	100	-
Melatonin (nM)	-	-	1	10	100	10	100	10
Luzindole (μM)	-	-	-	-	-	1	1	-

FIG. 1. Experimental design for study of melatonin/ACTH interaction to regulate corticosterone production by rat adrenal quarters at two different circadian stages. *Upper panel*, Adrenal quarters were harvested 1 h after lights on (0800 h), preincubated by 6 h (0900–1500 h), and then subjected to different treatments at the ensuing afternoon-early night transition (1500–0300 h). *Middle panel*, Adrenal quarters were harvested 1 h after lights off (2200 h), preincubated by 6 h (2300–0500 h), and then subjected to different treatments at the ensuing late night-morning transition (0500–1700 h). *Lower panel*, The set of treatments arises from eight different combinations of DMEM, ACTH, melatonin, and/or luzindole. Of note, given that adrenals quarters were used in these experiments, the end point corticosterone response of each single rat to the complete set of treatments could be tracked.

1) medium alone (basal) or medium containing 2) 100 nM ACTH; 3) 100 nM ACTH and 1 nM melatonin; 4) 100 nM ACTH and 10 nM melatonin; 5) 100 nM ACTH and 100 nM melatonin; 6) 100 nM ACTH, 10 nM melatonin, and 1 μM luzindole; 7) 100 nM ACTH, 100 nM melatonin, and 1 μM luzindole; and 8) 10 nM melatonin. We selected 100 nM ACTH to achieve maximal corticosterone production based on preliminary experiments and also published data (43). The adrenal explants were exposed to the different treatments at two clock times. The adrenal quarters collected at 0800 h were subjected to treatment during the ensuing afternoon-early nighttime interval (1500–0300 h). Those collected at 2200 h were treated during the ensuing late night-morning time interval (0500–1700 h).

Melatonin solutions were prepared from a 10-mM stock solution in absolute ethanol and kept at −20 C. Working solutions were prepared by successive dilutions (between 10 million- and 100,000-fold) in DMEM/F12 to reach the 1 nM concentration used in the experiments. Fresh dilutions of melatonin (as well as ACTH and luzindole) in DMEM/F12 were prepared for every experiment. At the end of each experiment, the incubation medium was separated and stored at −20 C, and the explants were weighed. Immediately afterward, the explants from each treatment were pooled and subjected to total RNA extraction to be analyzed by means of qPCR for MT1 mRNA expression as described above.

Corticosterone was measured by RIA in the incubation medium of each adrenal quarter and corticosterone production was expressed per

mg of tissue. For RIA measurement, we used Rat Corticosterone (^{125}I) Biotrak assay system with Amerlex-M magnetic separation (Amersham Biosciences, Piscataway, NJ; code RPA 548), following the manufacturer's instructions.

Data analysis

Data were expressed as mean ± SEM. The mean ratio of MT1 to β-actin of each adrenal set was transformed to arcsin (44) and analyzed by ANOVA and the *post hoc* Newman-Keuls test. The effect of melatonin treatment on *in vitro* ACTH-stimulated corticosterone production was assessed by ANOVA followed by the *post hoc* Newman-Keuls test. The effect of the clock time on ACTH and melatonin *in vitro* effects was compared by two-way ANOVA followed by the *post hoc* Bonferroni test. Statistical analyses were performed using GraphPad Prism software (version 3.02; GraphPad Software). Results were considered significant with $P < 0.05$.

Results

The mRNA encoding for the MT1 melatonin membrane receptor is expressed in the rat adrenal in a rhythmic fashion

The rat adrenal expressed the MT1 melatonin receptor mRNA. The 206-bp MT1 qPCR product was identified by means of automated sequencing. The forward and reverse sequences (bases 6–188 and 8–180, respectively) of the purified MT1-qPCR product obtained from adrenal gland were identical with the corresponding regions of the cDNA reported for rat MT1 melatonin receptor (bases 118–301 and 96–272, respectively; GenBank accession no. AF130341.1).

There were readily apparent changes of MT1 mRNA levels during the day (Fig 2). Thus, if one analyzes the expression level of this transcript starting from lights on, the time interval 1000–1400 h showed the lowest relative transcription levels, which were strongly increased at the 1800- to 2200-h time interval. Finally, the MT1 mRNA relative expression levels were again low at the 0200- to 0600-h time interval ($P < 0.05$; ANOVA and Newman-Keuls test).

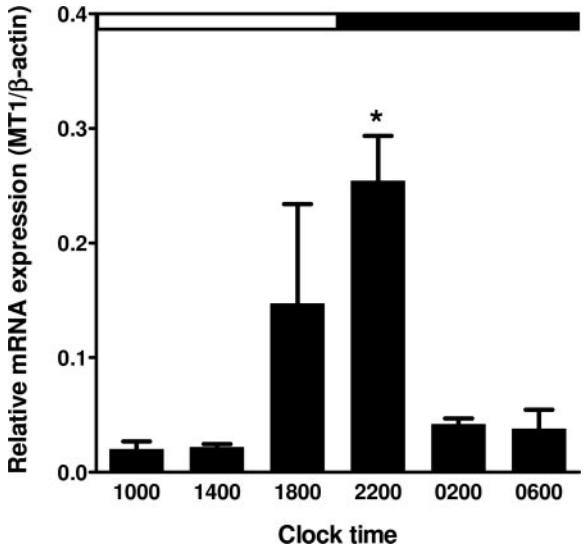


FIG. 2. Time course of MT1 melatonin receptor mRNA relative expression in rat adrenal gland. Each mean *column* and *error bar* (± SE) represents three adult male rats. Adrenals were harvested every 4 h along the 24 h to obtain total RNA, which was subjected to reverse transcription followed by qPCR. *, Different from other clock times ($P < 0.05$; ANOVA and Newman-Keuls). The *top white* and *black horizontal bar* indicates lights on and off, respectively.

The rat adrenal displays high-affinity binding sites for melatonin at 2200 but not 0800 h

We detected specific binding of 2-[^{125}I]iodomelatonin in membrane preparations from rat adrenal gland (Fig. 3A and Table 2) and hypothalamus (positive control; Table 2). 2-[^{125}I]iodomelatonin binding in adrenal gland showed Kd and Bmax values of 14.22 ± 1.23 pM and 0.88 ± 0.02 fmol/mg protein, respectively (Table 2). As shown in Fig. 3B, specific binding was displaced by the melatonin antagonist luzindole (Kd = 111.8 ± 14.25 pM; Bmax = 0.865 ± 0.171 fmol/mg protein) and GTPγ-S (Kd = 80.74 ± 7.06 pM; Bmax = 0.517 ± 0.080 fmol/mg protein). These results indicate that the rat adrenal gland and, as expected, the 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. Of note, in the rat adrenal, we detected 2-[^{125}I]iodomelatonin-specific binding only at 2200 but not 0800 h. On the other hand, we have only partial binding data at other clock times because during standardization we found no 2-[^{125}I]iodomelatonin binding at 1000 and 1400 h (not shown). Contact autoradiography of rat adrenal gland sections incubated with 25 pM 2-[^{125}I]iodomelatonin at 2200 h showed label in the whole adrenal (Fig. 3C), whereas no binding was detected at 0800 h (data not shown). The 2-[^{125}I]iodomelatonin binding in the adrenal sections was displaced by 1 μM melatonin, 1 μM luzindole, and 1 μM GTPγ-S (Fig. 3C).

In addition, we investigated the expression of the MT1 melatonin receptor by Western blot at 1000 and 2200 h. We

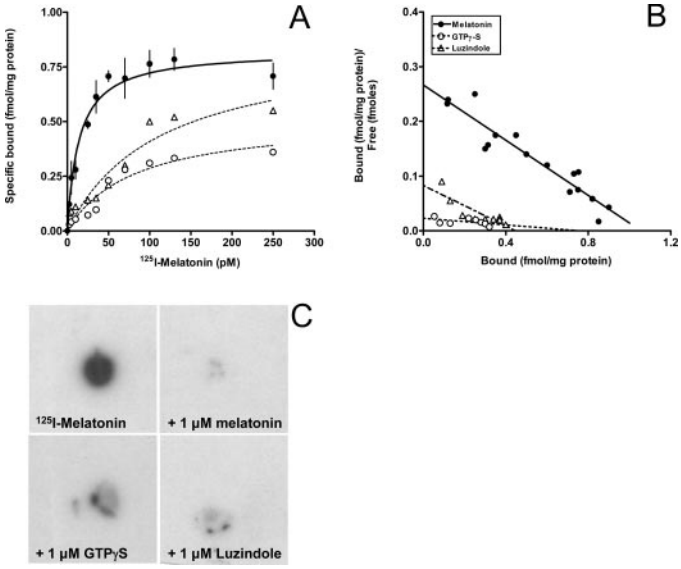


FIG. 3. 2-[^{125}I]iodomelatonin binding and competition studies in rat adrenal gland sampled at 2200 h. A, Specific 2-[^{125}I]iodomelatonin saturation binding in membrane preparations. B, Scatchard plot analysis. *Closed circles*, Melatonin; *open circles*, GTPγ-S; *triangles*, luzindole. C, Autoradiographic images of sections incubated with 2-[^{125}I]iodomelatonin. Each incubation condition (25 pM 2-[^{125}I]iodomelatonin alone, plus 1 μM cold melatonin, plus 1 μM GTPγ-S, and plus 1 μM luzindole), is indicated.

TABLE 2. K_d and receptor density (Bmax), determined by melatonin competition with 2-[^{125}I]iodomelatonin binding in membrane preparations from adrenal and hypothalamus pools (n = 3 animals per pool)

Tissue	Clock time (h)	K_d (pM)	Bmax (fmol/mg protein)
Adrenal gland	0800	ND	ND
	2200	14.22 ± 1.23	0.88 ± 0.02
Hypothalamus	0800	33.79 ± 12.10	16.57 ± 2.02
	2200	44.23 ± 9.43	25.48 ± 6.52

K_d and Bmax values were assessed running four independent assays at both 0800 and 2200 h.
ND, Not detected.

found the expected band of 37 kDa for MT1 in adrenal sampled at 2200 but not at 1000 h (Fig. 4A), and kidney sampled at 2200 h (positive tissue control; Fig. 4A). No MT1 protein could be detected in the diaphragm sampled at 1000 or 2200 h (negative tissue control; Fig. 4A). Controls without primary antibody did not render any signal (not shown). These results agree with those obtained using specific binding assay and demonstrate a diurnal variation of MT1 melatonin receptor polypeptide. On the other hand, using a commercial polyclonal antibody raised against an internal peptide of MT2, no band was detected at 1000 or 2200 h in adrenal membrane proteins, whereas immunopositive bands of 37 and 40 kDa were detected at 2200 h in kidney membrane proteins (Fig. 4A). Finally, the rat adrenal RNA samples used for MT1 receptor identification/quantification (see above), were subjected to MT1/MT2 receptor expression analyses by conventional RT-PCR. The expected MT1 amplification product was detected at 2200 but not 1000 h, whereas no MT2 PCR product could be amplified (Fig. 4B). Meanwhile, both MT1 and MT2 mRNAs were detected in kidney total RNA (Fig. 4B).

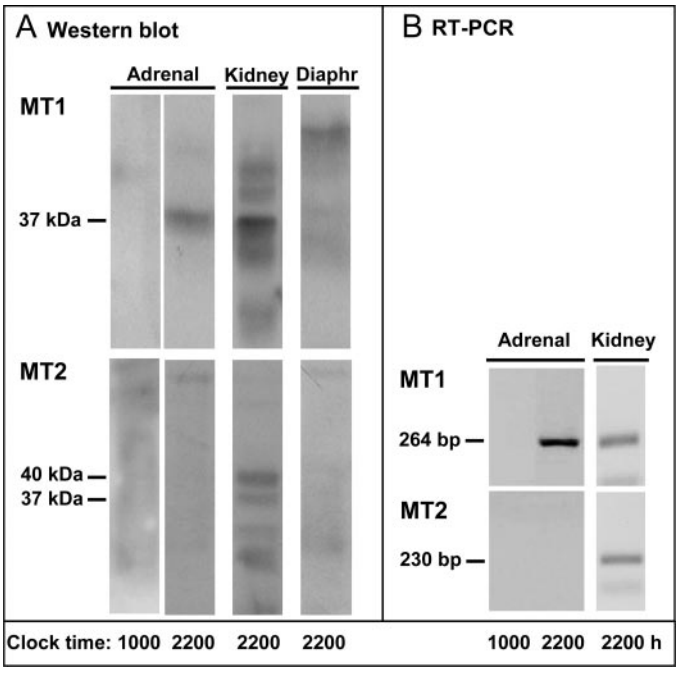


FIG. 4. Detection of MT1 and MT2 melatonin receptor transcripts and proteins in the rat adrenal gland. A, Immunoblot of rat adrenal gland obtained at 1000 and 2200 h and control tissues obtained at 2200 h (kidney, positive; diaphragm, negative), using anti-MT1 and -MT2 polyclonal antibodies. For MT1, a 37-kDa band was detected in membrane protein extracts from both adrenal gland and kidney sampled at 2200 h, whereas no polypeptides were detected in either rat adrenal sampled at 1000 h or diaphragm sampled at 2200 h. No MT2 polypeptide was detected at 1000 or 2200 h in adrenal membrane proteins. Meanwhile, two MT2-immunopositive bands of 37 and 40 kDa were detected at 2200 h in kidney but not diaphragm membrane proteins. Diaphr, Diaphragm. B, The expected MT1 amplification product (264 bp) was detected by conventional RT-PCR in adrenal total RNA samples obtained at 2200 but not 1000 h, whereas the expected PCR product for MT2 (230 bp) was not detected at either time point. Both MT1 and MT2 mRNAs were detected in kidney total RNA, used as positive tissue control. Negative image of ethidium bromide-stained agarose gel.

Melatonin inhibits in vitro ACTH-stimulated corticosterone production in the rat adrenal only during the afternoon-early nighttime interval

We found that ACTH increased corticosterone production at both clock times studied, *i.e.* rat adrenal quarters collected at 0800 h and stimulated during the ensuing afternoon-early nighttime interval, as well as those collected at 2200 h and stimulated during the ensuing late night-morning time interval (Fig. 5, *upper* and *lower panel*, respectively; $P < 0.05$; ANOVA and Newman-Keuls test). However, the effects of the different treatments were clearly determined by the clock time at which the rat adrenal quarters had been collected. First, the basal production of corticosterone was markedly higher in adrenal quarters collected at 0800 h and assayed in the ensuing afternoon-early nighttime interval ($P < 0.05$; two-way ANOVA and Bonferroni test). Second, the response to ACTH was higher during stimulation at the afternoon-early nighttime interval (about 3-fold *vs.* 2-fold during late night-morning time interval; $P < 0.05$, two-way ANOVA and Bonferroni test). Finally, we determined that melatonin inhibited corticosterone production induced by ACTH only when quarters collected at 0800 h were subjected to treatment during the ensuing afternoon-early nighttime interval (Fig. 5, *upper panel*). Incubation with 10 nM melatonin alone did not change basal secretion of cortisol (Fig. 5). The inhibitory effect of 10 and 100 nM melatonin on ACTH-induced cortisol production was reversed by 1 μM luzindole (Fig. 5, *upper panel*). We obtained similar results for 1 nM melatonin plus 1 μM luzindole (not shown). Taken together, these results indicate the presence of functional melatonin receptors in the rat adrenal gland and show a clock time-dependent direct inhibitory effect of low concentrations of melatonin on ACTH-induced cortisol production.

Discussion

In the present study, we used an integrative approach to demonstrate the expression of a functional MT1 melatonin receptor in the rat adrenal. We also detected 24-h oscillation in the level of MT1 melatonin receptor mRNA as well as in the respective protein level. On the other hand, we did not detect the MT2 mRNA or the encoded protein in this tissue. Melatonin had inhibitory effects on ACTH-stimulated corticosterone production *in vitro*. These effects related to clock time, depending on the time at which the adrenal harvesting occurred.

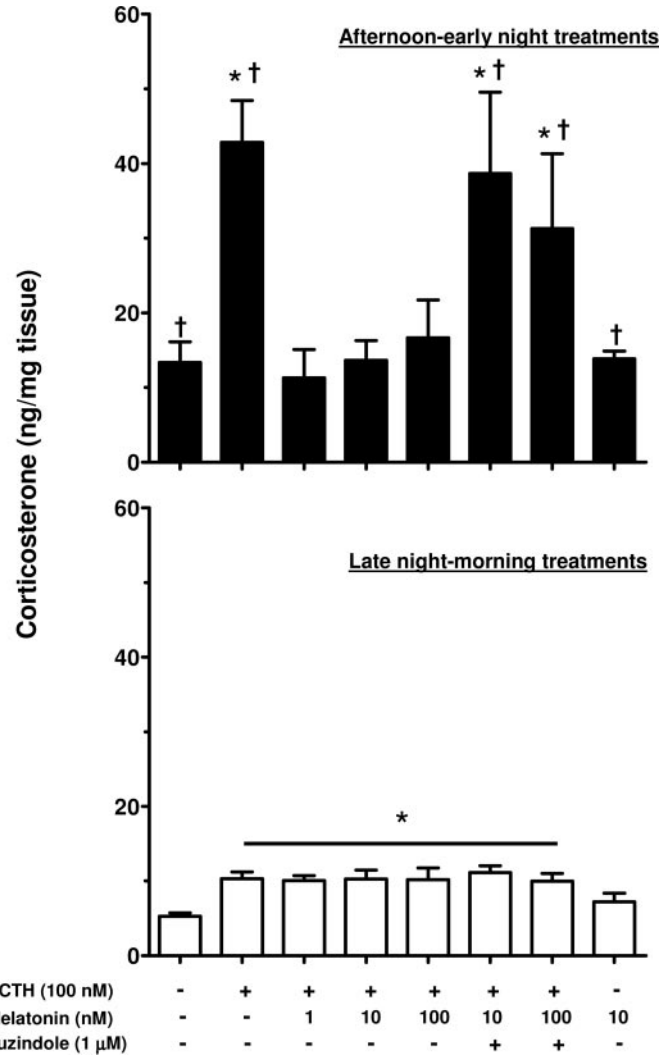


FIG. 5. Effect of increasing concentrations of melatonin on 100 nM ACTH-stimulated corticosterone production by cultured rat adrenal. *Upper panel*, Adrenal quarters obtained at 0800 h, preincubated from 0900 to 1500 h, and then treated during the afternoon-early nighttime interval (1500–0300 h). *Lower panel*, Adrenal quarters obtained at 2200 h, preincubated from 2300 to 0500 h, and then treated during the late night-morning time interval (0500–1700 h). Note that reversion of the melatonin effect by 1 μM luzindole was significant for both 10 and 100 nM melatonin treatment in the afternoon-early nighttime interval (*upper panel*). Melatonin *per se* had no effect on corticosterone production. Treatments are indicated below each *bar* in the *lower panel*. *, Different from basal concentration ($P < 0.05$, ANOVA and Newman-Keuls test); †, different from late night-morning treatments ($P < 0.05$, two-way ANOVA and Bonferroni test).

The present results indicate that the rat adrenal gland transcribes the MT1 melatonin receptor isoform, in agreement with a previous report (18). This transcript is rhythmically expressed in the rat adrenal, displaying markedly higher levels at 1800–2200 h than at other clock times. At 2200 h we found specific high-affinity binding sites for 2-[^{125}I]iodomelatonin, with a Kd about 15 pM, within the range reported for membrane-bound melatonin receptors in other cells and tissues from different species (10, 45). In both whole adrenal membrane preparations and frozen sections, 2-[^{125}I]iodomelatonin binding was displaced by luzindole [a melatonin membrane receptor antagonist (46)] and GTPγ-S, a nonhydrolyzable analog of GTP (45). We were not able to determine whether the binding sites are located exclusively in the cortex or in both cortex and medulla because the rat adrenal gland is a small organ (about 20 mg each). In line with the diminished mRNA expression at 0800 h, no melatonin binding was detected at this clock time in membrane preparations or by contact autoradiography. Our results agree with those of Pang *et al.* (20), who found low density (<0.2 fmol/mg protein) of high-affinity 2-[^{125}I]iodomelatonin binding sites in the rat adrenal; notwithstanding no Kd value or clock time of tissue harvesting was provided in that study. These authors also examined melatonin binding in duck adrenal sampled at midday determining a Kd value of about 28 pM, very close to the one determined in the present report. Low affinity melatonin binding sites (Kd 541 pM), exhibiting a Bmax of 3.23 fmol/mg protein, were reported in rat adrenal sampled between 0930 and 1100 h (19). Nonetheless, the Kd value calculated by this author is 38-fold higher than that found by us and at the same time higher than any other report for melatonin binding sites (10, 45). Upon this, the specific binding curve reported by Persengiev (19) does not seem to actually saturate with increasing picomolar concentrations of 2-[^{125}I]iodomelatonin.

Consistent with the results indicating expression of the cognate mRNA encoding for the MT1 melatonin receptor isoform, we detected a 37-kDa polypeptide in extracts of rat adrenal membrane proteins. Interestingly, despite this experiment being carried out several times using adrenal glands from different pools of animals, the MT1 polypeptide was consistently detected at 2200 but not 1000 h. Although the molecular mass calculated for MT1 is 39–40 kDa (47), several authors have found a 37-kDa band for MT1 in immunoblots probed with different antibodies (48–51). In contrast, we did not find MT2 mRNA or protein expression, strongly suggesting that MT2 is not expressed in the rat adrenal. This agrees well with the finding of transcription of MT1 but not MT2 in the adrenal gland of mouse (8) and capuchin monkey (10) as well as hamster Leydig cells (52).

We and others have previously reported circadian changes of MT1 expression in the fetal capuchin monkey suprachiasmatic nucleus (SCN) and adrenal (17) and adult rat SCN (53). Moreover, in a recent paper, a nocturnally elevated MT1 mRNA expression was also found in the adult rat pancreas (38). Our finding that MT1 transcription begins to rise quickly from 1800 h onward and then decreases sharply at 2200 h is consistent with protein expression and melatonin binding being readily detected at 2200 h because MT1 translation and sorting may have started about 3–4 h before the MT1 protein was detected by Western blot and 2-[^{125}I]iodomelatonin binding in membrane fractions and frozen sections at 2200 h. Circadian variations of high-affinity 2-[^{125}I]iodomelatonin binding have been previously described in the brain of rats (36, 53) and chickens (54). In agreement with these reports, here we show that in the rat hypothalamus, Bmax increased from 16.57 ± 2.02 at 0800 h to 25.48 ± 6.52 fmol/mg protein at 2200 h. An interesting observation is that the maximal expression of the adrenal MT1 receptor precedes the peak of plasma

melatonin. In the rats used in the present report, the melatonin peak was reached at 0200 h (data not shown). The decrease in MT1 mRNA and protein expression levels at 0800 h agrees with the evidence of melatonin binding down-regulation by plasma melatonin itself (55). The strong diurnal variation of MT1 melatonin receptor clearly suggests that particular care must be taken to accurately study the effects of melatonin in the rat adrenal.

The functional capacity of the MT1 receptor in the rat adrenal was investigated taking into account the marked daily variation for MT1 mRNA and protein levels. Thus, rat adrenals were obtained at 0800 and 2200 h (1 h after lights on and off, respectively), preincubated for 6 h, and then subjected to treatment, which lasted for the next 12 h (Fig. 1). Low melatonin doses inhibited the response to ACTH only when given in the 1500- to 0300-h interval, whereas none of the melatonin doses tested at late night-morning inhibited the weak corticosterone response to ACTH observed at this time interval. Melatonin alone did not change basal secretion of corticosterone, indicating that melatonin modulates the action of ACTH rather than having effect *per se*. The reversal observed for melatonin plus luzindole is in agreement with melatonin acting on membrane-bound melatonin receptors present in the steroidogenic cells of the rat adrenal cortex.

The clock time dependency of the inhibitory effect of melatonin on the corticosterone response to ACTH may be due to intraadrenal factors related to clock time changing during the incubation. Indeed, we found that time of adrenal collection and the ensuing time of treatment resulted in differences in basal corticosterone production and corticosterone response to ACTH. Thus, basal corticosterone production in the time interval 1500–0300 h was about 3-fold higher than at the interval 0500–1700 h. In addition, the adrenal quarters stimulated from 1500 to 0300 h had a larger response to ACTH (3-fold over basal *vs.* 2-fold over basal).

It is unlikely that the lower basal values and diminished corticosterone response to ACTH found in the interval 0500–1700 h represent decreased viability of the adrenal quarters collected at 2200 h because procedures were identical with those applied to the adrenals collected at 0800 h. The observation that basal corticosterone production was higher in the afternoon-early night interval than in the late night-morning treatment is reminiscent of the changes in plasma corticosterone found *in vivo*. In addition, there is evidence accounting for *in vivo* rat adrenal afternoon/morning sensitivity differences to ACTH (2, 56). Early studies by Andrews and Folk (57) in hamster adrenal culture showed that rhythmic glucocorticoid production is maintained over several days. Overall, the data suggest that intraadrenal factors related to clock time changed during the incubation, playing a critical role not only in the basal corticosterone production and corticosterone responses to ACTH observed in the present study but also in the effect of melatonin on ACTH-stimulated corticosterone production.

In view of our findings of daily variations of MT1 melatonin receptor mRNA and protein expression *in vivo*, it is conceivable that the adrenal samples followed their internal clock to reach a rise in MT1 mRNA/protein toward the afternoon-early night transition and a decrease toward the late night-morning transition, thus explaining the clear cut inhibition by melatonin of the corticosterone response to ACTH in one protocol and the lack of response in the other. This interpretation is supported by qPCR detection of MT1 transcripts in adrenal quarters subjected to afternoon-early night treatments but not in those subjected to late night-morning treatments (data not shown). Otherwise, the fact that the intrinsic responsiveness to ACTH was different in adrenals harvested at 0800 and 2200 h may in turn determine the response to melatonin. In this context, it can be tentatively suggested that for melatonin to interfere with corticosterone production, it is necessary that the adrenal response to ACTH to go beyond a threshold settled by the circadian stage. Thus, no effect of melatonin would take place at lower ACTH-stimulated corticosterone production rates and vice versa. There is only one report in which *in vitro* adrenal clock time dependency of melatonin/ACTH interactions was studied. In mouse bisected adrenals, Sanchez de la Pena *et al.* (35) found that corticosterone production induced by ACTH was either attenuated or amplified by isophasic aqueous pineal homogenate addition at 2 and 14 h after light onset, respectively. It is interesting that Sanchez de la Pena *et al.* (35) also concluded that coadministration of melatonin and ACTH affects the *in vitro* corticosterone production by nocturnal rodent's differently at diverse adrenal harvest times.

Altogether, our findings indicate not only expression but also high amplitude diurnal variation of functional MT1 melatonin receptors in the rat adrenal gland *in vivo*. Moreover, our results using *in vitro* rat adrenal suggest that melatonin directly inhibits ACTH-stimulated corticosterone production in a clock time-dependent manner. Although our experiments were not designed to address to what extent this potential novel action of melatonin in nocturnal rodents contributes to adrenal physiology, our results allow a speculation. In the rat, plasma corticosterone increases 2–3 h before lights off (58), whereas melatonin rises slowly, reaching a maximum about 4 h after lights off (Ref. 14 and data not shown), coinciding with the reported down slope of the corticosterone peak (58). If the 3-h delay between mRNA peak values and protein accumulation reported in the rat SCN (37) is present in the rat adrenal gland, the increase in plasma melatonin would encounter functional adrenal receptors and could contribute to the down slope of the corticosterone rhythm.

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