

Clock Gene Expression in Adult Primate Suprachiasmatic Nuclei and Adrenal: Is the Adrenal a Peripheral Clock Responsive to Melatonin?

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The circadian production of glucocorticoids involves the concerted action of several factors that eventually allow an adequate adaptation to the environment. Circadian rhythms are controlled by the circadian timing system that comprises peripheral oscillators and a central rhythm generator located in the suprachiasmatic nucleus (SCN) of the hypothalamus, driven by the self-regulatory interaction of a set of proteins encoded by genes named clock genes. Here we describe the phase relationship between the SCN and adrenal gland for the expression of selected core clock transcripts (Per-2, Bmal-1) in the adult capuchin monkey, a New World, diurnal nonhuman primate. In the SCN we found a higher expression of Bmal-1 during the h of darkness (2000–0200 h) and Per-2 during daytime h (1400 h). The adrenal gland expressed clock genes in oscillatory fashion, with higher values for Bmal-1 during the

day (1400–2000 h), whereas Per-2 was higher at nighttime (about 0200 h), resulting in a 9- to 12-h antiphase pattern. In the adrenal gland, the oscillation of clock genes was accompanied by rhythmic expression of a functional output, the steroidogenic enzyme 3 β -hydroxysteroid dehydrogenase. Furthermore, we show that adrenal explants maintained oscillatory expression of Per-2 and Bmal-1 for at least 36 h in culture. The acrophase of both transcripts, but not its overall expression along the incubation, was blunted by 100 nM melatonin. Altogether, these results demonstrate oscillation of clock genes in the SCN and adrenal gland of a diurnal primate and support an oscillation of clock genes in the adrenal gland that may be modulated by the neurohormone melatonin. (*Endocrinology* 149: 1454–1461, 2008)

IN MOST MAMMALIAN species, plasma glucocorticoid concentration shows a strong circadian rhythm. It is well established that the circadian production of glucocorticoids involves the orchestrated action of the circadian rhythm of ACTH, adrenal innervation, and local adrenal factors (1–4). However, there is evidence suggesting the involvement of additional regulatory factors that may include the neurohormone melatonin and an intrinsic adrenal clock (5, 6). The capuchin monkey has a clear ACTH and plasma cortisol rhythm (4) showing maximal values in the early morning as in other diurnal nonhuman primates and the human (7–9). Recently we showed that *in vivo* suppression of ACTH by dexamethasone decreased cortisol production to one tenth but did not abolish a circadian rhythm of plasma cortisol in the capuchin, similar to findings in the rat (2, 4). This suggests that a combination of intrinsic adrenal cortex oscillatory properties and neural inputs (2) may participate in the cir-

cadian rhythm of adrenal cortisol secretion and in the circadian cortisol response to ACTH in the capuchin monkey (4).

Circadian rhythms are controlled by the circadian timing system (10–12). In mammals, this system comprises oscillators located in most tissues of the body (peripheral oscillators) and a central rhythm generator located in the suprachiasmatic nucleus (SCN) of the hypothalamus. The integral network of signals linking the SCN and peripheral oscillators is presently not well understood. At the cell level, circadian rhythms are driven by the self-regulatory interaction of a set of proteins encoded by genes named clock genes (Bmal-1, Per 1–2, Cry 1–2, Clock, and other second and third order components). Ultimately the circadian oscillation of core clock gene transcription, translation, and posttranslational modifications contribute to regulate the expression of downstream (*i.e.* clock controlled) genes involved in multiple cellular functions in 24 h in most tissues, resulting in the overt circadian rhythms in the individual (10–12). Recent studies showed circadian *in vivo* expression of clock genes in the adrenal gland of adult rhesus monkey and mice (8, 13–16) and fetal capuchin monkey (17). According to Yoo *et al.* (18), most peripheral tissues show circadian oscillation of clock genes under *in vitro* conditions; however, this possibility has not been investigated for the adrenal gland.

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Abbreviations: 3 β -HSD, 3 β -Hydroxysteroid dehydrogenase; SCN, suprachiasmatic nucleus; ZT, Zeitgeber time.

Melatonin may convey photoperiodic information from the SCN to the peripheral oscillators. As an internal zeitgeber, melatonin should be able to shift clock gene expression in peripheral tissues. There is evidence of melatonin effects on *in vivo* clock gene expression in mice, rat, hamster, and sheep pars tuberalis, which express melatonin receptors at a high density (19–22). The adrenal gland of the adult and fetal capuchin monkey expresses functional MT1 melatonin receptors (5, 23). Our hypothesis is that the adult capuchin monkey adrenal gland is a peripheral clock, displaying oscillatory clock gene expression not only *in vivo* but also *in vitro*. *In vivo*, we investigated the oscillatory expression of clock genes in the SCN and clock genes and 3 β -hydroxysteroid dehydrogenase (3 β -HSD; a key enzyme for cortisol synthesis) in the adrenal gland. *In vitro* we investigated oscillatory clock gene expression in the adrenal gland and the modulation of clock gene expression by the neurohormone melatonin.

Materials and Methods

Animals and tissues

Adrenals and, when feasible, tissue blocks containing the SCN were dissected under sterile conditions from adult capuchin monkeys (*Cebus apella*) at necropsy. Animals had been maintained in individual cages in a room with controlled temperature and humidity and water available *ad libitum* at the Chilean Primate Center, Pontificia Universidad Católica de Chile. Food was administered twice a day. Light-dark cycle in the facility was 1400 and 1000 h (lights on at 0700 h). Animal handling and care was performed following the recommendations of the National Institute of Health Guide for Animal Experimentation Care. Animals were sedated with ketamine (Ketaset, 10 mg/kg im; Wyeth-Ayerst, Madison, NJ) and euthanized with an overdose of sodium thiopental (100 mg/kg iv; Drag Pharma Invetec, Santiago, Chile). The Commission on Bioethics and Biosafety of the Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, approved the study protocol.

To dissect out a hypothalamic block that included the SCN, we relied on our previous experience with the neuroanatomy of the capuchin monkey fetal SCN (17), SCN from different species (24, 25) and on the atlas of the *Cebus apella* by Manocha *et al.* (26). Roughly, the block was 5.5 mm in the anteroposterior plane, 10 mm wide, and 5 mm in the dorsoventral plane. A piece of adrenal gland and the whole SCN block were preserved in TRIzol (Invitrogen Corp., Carlsbad, CA) and stored at -20°C . Clock gene expression was measured in 13 adrenal glands and eight SCNs to assess *in vivo* clock gene expression. Of these, eight adrenal glands and three SCNs, collected as described and stored frozen at -80°C , were obtained from our tissue bank. *In vivo* and *in vitro* clock gene expression was measured in fresh adrenal tissue obtained from five animals.

Experiments

Analysis of clock genes and 3 β -HSD expression by semiquantitative RT-PCR. Expression of the clock genes Bmal-1, Per-2, Cry-2, and Clock was detected in adrenal gland and SCN samples obtained fresh and from the tissue bank. Bmal-1, Per-2, and Clock expression was measured in SCN obtained at 0800 h ($n = 1$), 1400 h ($n = 3$), 2000 h ($n = 3$), and 0200 h ($n = 1$) and in adrenal glands obtained at 0800 h ($n = 5$), 1400 h ($n = 4$), 2000 h ($n = 3$), and 0200 h ($n = 1$). In addition 3 β -HSD expression was measured in the same adrenal samples.

Five adrenal glands (two obtained at 1400 h and three at 2000 h) obtained immediately after necropsy were cut in explants of about 15 mg under sterile conditions. Explants were preincubated with DMEM-F12 medium (Life Technologies, Inc., Grand Island, NY) plus 0.1% BSA (Life Technologies) during 6 h and then further incubated in triplicate in separated tubes with 2 ml medium at 37 $^{\circ}\text{C}$, 100% humidity, 5% CO_2 -95% air. The aim of the protocol was to measure clock gene expression at as close intervals as possible for the longest incubation time in two pro-

ocols. The weight of the adrenals glands allowed to collect explants every 6 h for 36 h. The incubation medium was changed every 6 h. A second set of explants from four of the adrenals was incubated using the previous protocol but replacing the medium between 0800 and 2000 h with DMEM-F12 containing 100 nM melatonin. Medium alone was used at the other clock time intervals. In the control and melatonin protocols, sets of three explants were harvested every 6 h, weighed, and immediately processed with TRIzol (Invitrogen). Bmal-1 and Per-2 expression was measured in all explants. The limited amount of RNA precluded measurement of 3 β -HSD in these samples.

Total RNA was extracted with TRIzol following the manufacturer's instructions. The RNA concentration was measured by absorbance at 260 nm and the sample integrity was assessed by electrophoresis in 4% formaldehyde and 1% agarose gel and stained with ethidium bromide. The transcripts Bmal-1, Per-2, Cry-2, Clock, 3 β -HSD, and the housekeeping gene 18S-rRNA were amplified. The sequence of the primers, size of the expected PCR products, location between exons (according to the clock gene structures reported for human), GenBank accession numbers of the partial cDNA sequences, and the PCR conditions were all previously described by us (17). The homology of each sequenced RT-PCR product obtained from capuchin monkey with the corresponding human clock gene sequence was determined using the BLASTN 2.2.1 tool. Percentage identity was 93% for Per-2, 97% for Bmal-1, 98% for Cry-2, and 97% for Clock. Briefly, cDNAs from all samples were analyzed in triplicate and in several dilutions (equivalent to 0.5–150 ng RNA per tube) to assess linearity. PCR products were separated by electrophoresis on a 2% agarose-ethidium bromide gel, the gel image was captured with a digital camera (Camedia Master 4.1; Olympus, Tokyo, Japan) using DocIt software (UVP, Inc., Upland, CA), and the density of the band was measured using the software Scion Image (Scion Corp., www.scioncorp.com) and corrected by the density of 300 bp standard (12.5 ng). All adrenal and SCN samples were analyzed in at least two assays. Mean coefficient of variation was 15.7 ± 3.6 .

We did not detect oscillatory changes in 18S-rRNA throughout the day in samples obtained at necropsy or during incubation of explants. However, to ascertain that the pattern of Per-2 mRNA to 18S-rRNA and Bmal-1 mRNA to 18S-rRNA observed *in vivo* and *in vitro* was due to changes in these genes and not to undetected minor changes in 18S-rRNA, we assessed the ratio Bmal-1 to Per-2 in each cDNA sample.

The semiquantitative RT-PCR method used in the present report was validated by correlation analyses against real-time PCR for Bmal-1. For this purpose, capuchin monkey adrenal gland ($n = 6$) and SCN ($n = 5$) samples were used. The correlation equation was: $y = 2.54 \times x - 1.81$, where $x =$ semiquantitative PCR and $y =$ real-time PCR, $r^2 = 0.69$ ($P = 0.002$), and $n = 11$ (Fig. 1).

Data analysis

Data are expressed as mean \pm SEM. The mean ratio of Bmal-1 to 18S-rRNA, Per-2 to 18S-rRNA, and Clock to 18S-rRNA *in vivo* in the SCN

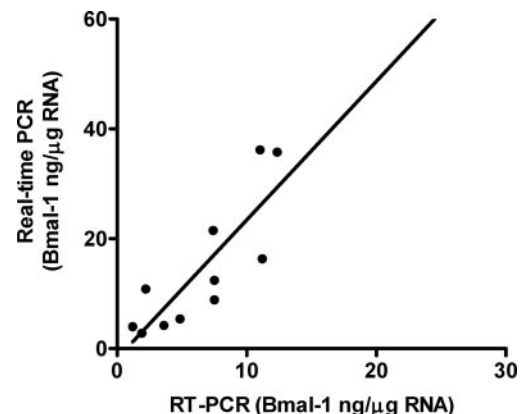


FIG. 1. Correlation between Bmal-1 levels measured by real-time PCR and semiquantitative RT-PCR in capuchin monkey adrenal gland ($n = 6$) and SCN ($n = 5$). The correlation equation was: $y = 2.54 \times x - 1.81$, where $y =$ real-time PCR and $x =$ semiquantitative PCR, $r^2 = 0.69$ ($P = 0.002$), $n = 11$.

at 1400 and 2000 h was transformed to arcsin (27) and analyzed by Student *t* test. The ratios of Bmal-1 to 18S-rRNA, Per-2 to 18S-rRNA *in vivo*, and in culture of adrenal explants and that of 3 β -HSD to 18S-rRNA *in vivo* in the adrenal gland was transformed to arcsin and analyzed by ANOVA and the *post hoc* Newman-Keuls. The ratios in cultured explants were analyzed by ANOVA for repeated measures and the *post hoc* Newman-Keuls test before and after being normalized. Normalization considered the highest value within the experiment as 1 and the lowest as 0. Additionally, data were fitted to a theoretical cosine function. Statistical analyses were performed using GraphPad Prism software (version 3.02; GraphPad Software Inc., San Diego, CA). Results were considered significant when $P < 0.05$.

Results

Expression of the clock gene mRNAs Bmal-1, Per-2, Cry-2, and Clock was detected in the eight SCN samples and 13 adrenal glands obtained at necropsy and in all adrenal explants in culture. Inspection of the Bmal-1 to 18S-rRNA and Per-2 to 18S-rRNA ratio at the clock times available *in vivo* suggested that this mRNA peaked at different clock time intervals in the SCN and adrenal *in vivo* and also that there was a difference between the phase of the expression in adrenal *in vivo* and in cultured adrenal explants.

In vivo expression of clock genes in the SCN

Samples obtained at necropsy showed clock time differences in Bmal-1 to 18S-rRNA and Per-2 to 18S-rRNA ratios (Fig. 2, A and B). Expression of Bmal-1 was about 3-fold higher at 2000 h than at 1400 h (1.45 ± 0.03 , $n = 3$, and 0.41 ± 0.06 , $n = 3$, respectively, $P < 0.05$, Student *t* test). In contrast, the expression of Per-2 was 3-fold lower at 2000 h than at 1400 h (0.39 ± 0.04 , $n = 3$, and 1.11 ± 0.05 , $n = 3$, respectively, $P < 0.05$, Student *t* test). Expression of Clock (Fig. 2C) showed a tendency to higher values at 1400 than at 2000 h (0.53 ± 0.14 , $n = 3$, and 0.22 ± 0.037 , $n = 3$, $P = 0.07$, Student *t* test). The ratio Bmal-1 to Per-2 of each cDNA sample for every time point (measured independently of 18S-rRNA) reproduced the observed changes in the gene to 18S-rRNA ratios (data not shown). The single values of Bmal-1 to 18S-rRNA and Per-2 to 18S-rRNA at 0200 and 0800 h (0.71 and 0.3 and 0.41 and 0.52, respectively) are consistent with a maximal expression of Bmal-1 in the evening and Per-2 in the middle of the day in the SCN.

In vivo expression of clock genes and 3- β HSD in the adrenal gland

Bmal-1, Per-2, and 3 β -HSD ratios to 18S-rRNA were measured at three clock time intervals and showed clock time-related changes in expression by ANOVA and Newman-Keuls (Fig. 3). As shown in Fig. 3A, the expression of Bmal-1 was similar at 1400 and 2000 h and was about 2-fold higher than at 0800 h (0.82 ± 0.12 , $n = 4$, and 0.61 ± 0.04 , $n = 3$ vs. 0.34 ± 0.02 , $n = 5$, respectively, $P < 0.05$, ANOVA and Newman-Keuls), whereas the expression of Per-2 was different at the three clock times, showing a 3-fold change between 1400 and 2000 h (Fig. 3B). The lowest Per-2 values occurred at 1400 h and the higher at 2000 h, intermediate values being attained at 0800 h (0.36 ± 0.08 , $n = 4$; 1.09 ± 0.08 , $n = 3$; and 0.72 ± 0.04 , $n = 5$, respectively, $P < 0.05$, ANOVA and Newman-Keuls). The ratio Bmal-1 to Per-2 of each cDNA sample for every time point (measured independently of

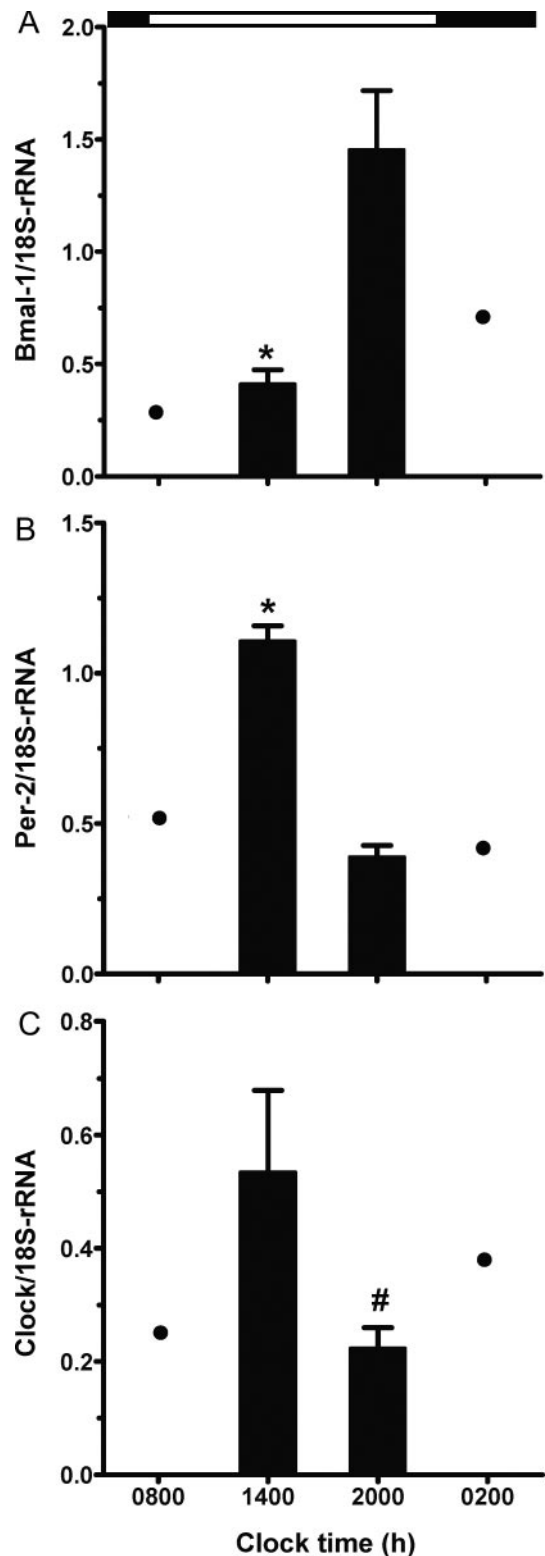


FIG. 2. Expression of clock genes in the capuchin monkey SCN at different clock times. Mean \pm SE of Bmal-1 to 18S-rRNA (A), Per-2 to 18S-rRNA (B), and Clock to 18S-rRNA (C) expression measured by RT-PCR at 0800 h ($n = 1$), 1400 h ($n = 3$), 2000 h ($n = 3$), and 0200 h ($n = 1$). *, Different from 2000 h, $P < 0.05$, Student's *t* test. #, 1400 vs. 2000 h, $P = 0.07$, Student's *t* test. The black bars indicate lights-off hours. Please note differences between the scales used.

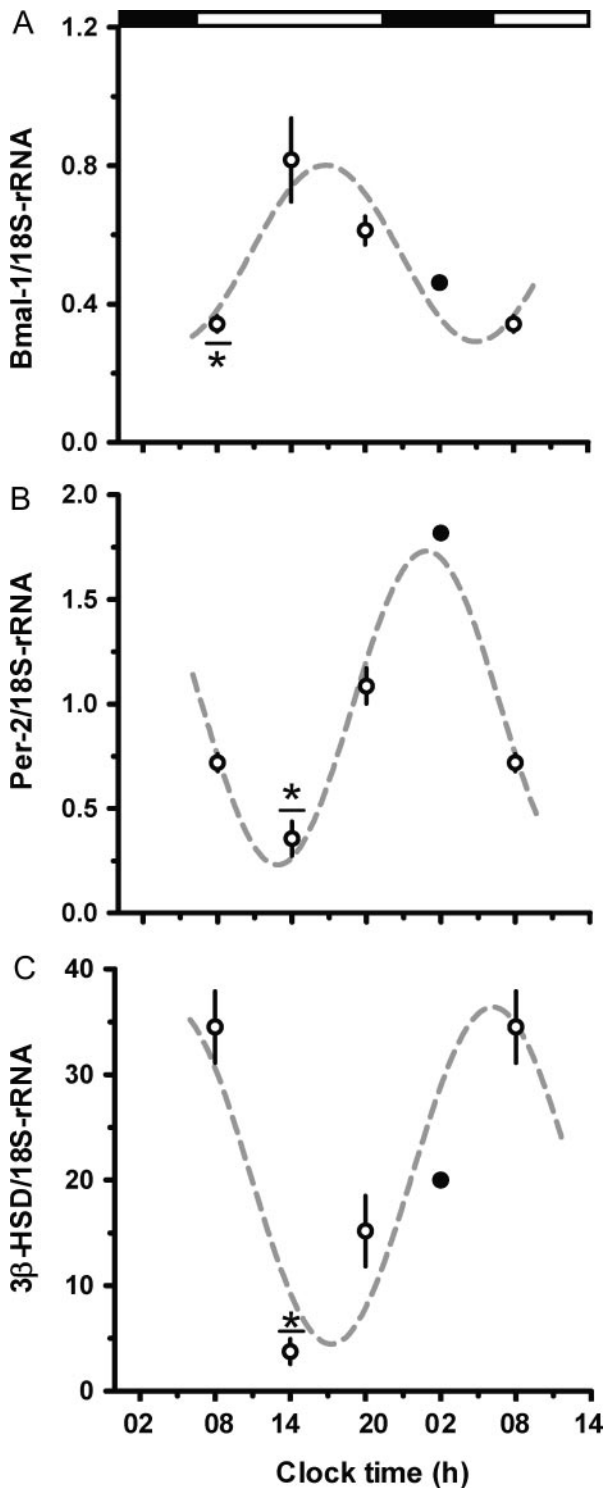


FIG. 3. Oscillatory expression of clock genes Bmal-1 and Per-2 and 3β -HSD in the capuchin monkey adrenal gland. Mean \pm SE of Bmal-1 to 18S-rRNA (A), Per-2 to 18S-rRNA (B), and 3β -HSD to 18S-rRNA expression measured by RT-PCR at 0800 h (n = 5), 1400 h (n = 4), 2000 h (n = 3), and 0200 h (n = 1). The value at 0800 was plotted twice. *, A, Different from 1400 and 2000 h; B, different from 0800 and 2000 h; C, different from 0800 and 2000 h; $P < 0.05$, ANOVA and Newman-Keuls. The dashed line represents the theoretical cosine function for Bmal-1, Per-2, and 3β -HSD ($r^2 = 0.85$, 0.97 , and 0.83 , respectively). The black bars indicate lights-off hours. Please note differences between the scales used.

18S-rRNA) reproduced the observed changes in the gene to 18S-rRNA ratios (data not shown). The single values of Bmal-1 to 18S-rRNA and Per-2 to 18S-rRNA at 0200 h (0.46 and 1.81, respectively) are consistent with a maximal expression of at midday-early evening and Per-2 in the evening in the adrenal. The data fit a theoretical cosine function, suggesting a maximum between 1400 and 2000 h for Bmal-1 and at 0200 h for Per-2 ($r^2 = 0.85$ and 0.97 , respectively).

The expression of the steroidogenic enzyme 3β -HSD showed pronounced clock time-related changes of about 10 times between 0800 and 1400 h. In keeping with known oscillatory function of glucocorticoid synthesis by the adrenal cortex, we found maximal expression of 3β -HSD (Fig. 3C) at 0800 h (34.51 ± 3.38 , n = 5, $P < 0.05$, ANOVA and Newman Keuls). Expression decreased about 10-fold at 1400 h (3.75 ± 1.19 , n = 4) to reach intermediate expression at 2000 h (15.17 ± 3.34); however, values at 1400 and 2000 h were not statically different. The single sample obtained at 0200 h had values in the range of those at 2000 h. The data fit a theoretical cosine function, suggesting a maximum near 0800 h ($r^2 = 0.83$).

In vitro expression of clock genes in the adrenal gland

Bmal-1 and Per-2 mRNAs and 18S-rRNA were expressed in adrenal explants in culture incubated for 36 h with medium alone. Expression of Cry-2 and Clock mRNAs was detected but not quantified. The ratio Bmal-1 to 18S-rRNA and Per-2 to 18S-rRNA measured every 6 h changed during the 36 h of incubation with medium alone. These changes were apparent when analyzing the data against clock time. To decrease the interanimal variation, in each experiment the Bmal-1 to 18S-rRNA and Per-2 to 18S-rRNA ratio obtained at the different clock times was normalized considering the highest individual value within the experiment as 1 and the lowest as 0. The normalized ratio of these clock genes showed a synchronized oscillatory pattern (Fig. 4, A and B). The level of Bmal-1 increased at 0200 h (Fig. 4A; $P < 0.05$, ANOVA for repeated samples and Newman-Keuls test), whereas the level of Per-2 mRNA peaked at 1400 h, reaching a trough value at 0800 h (Fig. 4B; $P < 0.05$, ANOVA for repeated samples and Newman-Keuls test). The normalized mean ratio Bmal-1 to Per-2 of each cDNA sample for every time point displayed a peak at 0200 h and a trough at 1400 h, consistent with the observed changes in the gene to 18S-rRNA ratios (Fig. 4C; $P < 0.05$, ANOVA for repeated samples and Newman-Keuls test), supporting that both genes indeed oscillate in antiphase. The phase of the oscillation of Bmal-1 and Per-2 in cultured adrenal explants is different from that found in the samples obtained at necropsy (shown in Fig. 3, A and B). The level of Bmal-1 to 18S-rRNA and Per-2 to 18S-rRNA ratios without normalization in control incubations is also depicted in Fig. 5. Control incubations showed a significant increase in expression of Bmal-1 to 18S-rRNA at 0200 h and Per-2 to 18S-rRNA at 1400 h.

Effect of melatonin on adrenal clock gene expression in vitro

Addition of melatonin to the incubation from 0800 to 2000 h did not change the mean levels of expression of Bmal-1, Per-2, and 18S-rRNA. However, melatonin abol-

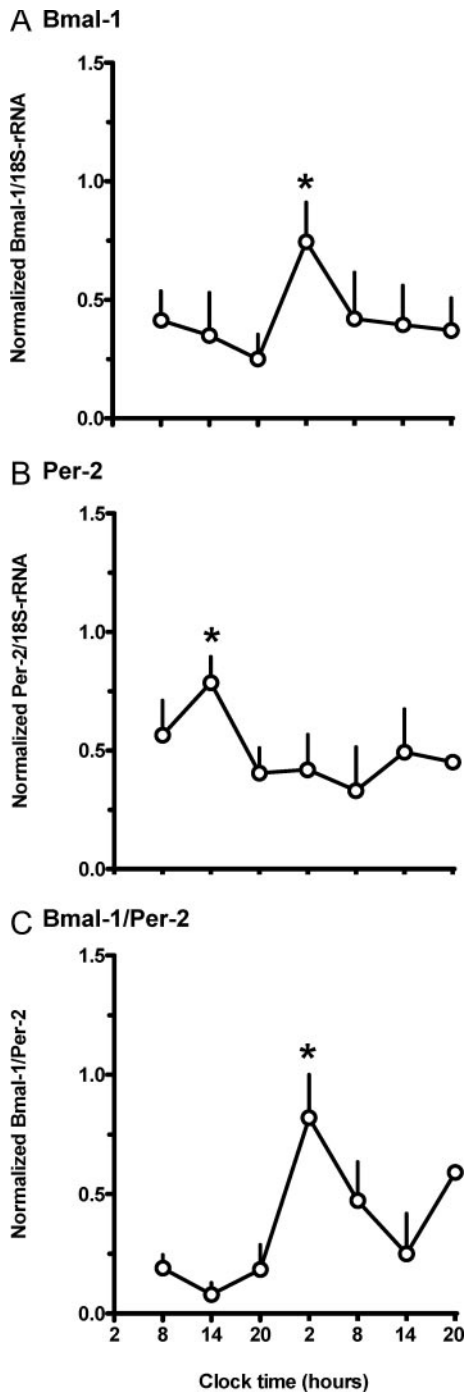


FIG. 4. Time course of Bmal-1 and Per-2 expression in adult adrenal gland explants in culture. Mean \pm SE (n = 5) of Bmal-1 to 18S-rRNA (A), Per-2 to 18S-rRNA (B), and Bmal-1 to Per-2 (C) expression from explants cultured with DMEM-F12. Data were normalized as described in *Materials and Methods*. *, Different from other time points ($P < 0.05$; ANOVA and Newman-Keuls).

ished the peaks of Per-2 to 18S-rRNA and Bmal-1 to 18S-rRNA observed in the control explants (Fig. 5, A and B). In the experiment, adrenals explants were exposed to 12 h of melatonin followed by 12 h of culture medium; therefore, the effect of melatonin on Per-2 to 18S-rRNA occurred during melatonin exposure, whereas the effect on Bmal-1 to 18S-

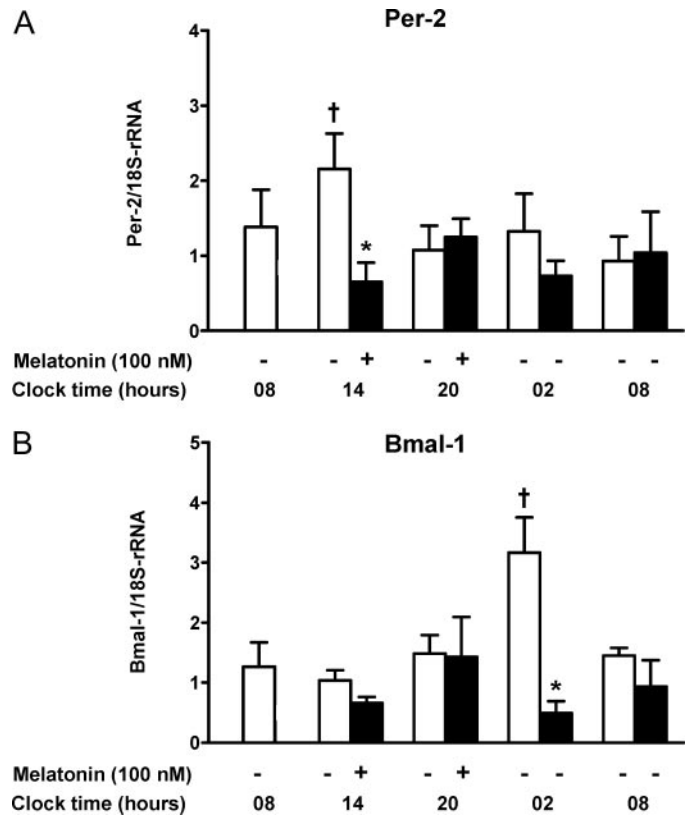


FIG. 5. Effect of melatonin treatment on expression of Per-2 and Bmal-1 in capuchin monkey adrenal explants in culture. Mean \pm SEM (n = 4). Time course of Bmal-1 to 18S-rRNA (A) and Per-2 to 18S-rRNA (B) in adrenal gland explants cultured with DMEM-F12 alone (white bars) or exposed for 12 h to DMEM-F12 plus 100 nM melatonin (black bars). Melatonin was added between 0800 and 2000 h, immediately after harvesting the explants at 0800 h. The clock times at which the explants were exposed to melatonin are indicated with a + symbol. [†], Different from the other hours ($P < 0.05$; ANOVA, and Newman-Keuls); *, different from control condition (medium alone) at the same time point ($P < 0.05$; ANOVA and Newman-Keuls).

rRNA was apparent 6 h after cessation of melatonin exposure (Fig. 5B).

Discussion

Adrenocortical rhythms are regulated by the SCN through regulation of circadian ACTH secretion and a direct autonomic innervation pathway, which has been demonstrated in the rat (28). The capuchin monkey has a clear ACTH and plasma cortisol rhythm (4), showing maximal values in the early morning as in other diurnal species including human. Nevertheless, suppression of ACTH leads to low cortisol values but does not abolish the rhythm of cortisol secretion, suggesting that other factors, most likely SCN dependent, and intrinsic oscillatory capacity of the adrenal gland may contribute to the rhythmic cortisol secretion found *in vivo*. The present study, the first addressing clock gene oscillation in the SCN of adult primates, detected oscillatory expression of Bmal-1 and Per-2 in this tissue, which was compared with the capuchin adrenal gland. In addition, this study addressed whether clock gene oscillation in the adrenal was maintained *in vitro* (*i.e.* in the absence of SCN input) and whether the

neurohormone melatonin had direct effects on adrenal clock gene expression.

The adult capuchin SCN demonstrated a higher expression of Bmal-1 during the hours of darkness [2000–0200 h; equivalent to Zeitgeber time (ZT) 13–21] and Per-2 at 1400 (ZT 7) during daytime hours. It is of interest to note that the temporal pattern of Per-2 in the adult SCN differs from that found in the fetal capuchin monkey (17), suggesting developmental differences in the expression of clock genes in the SCN of primates. A question in the circadian field is the origin of difference in phase in overt circadian rhythms between diurnal and nocturnal species. Similar to our findings in the capuchin, the available data show a similar phase of oscillation of Bmal-1 and Per-2 in the SCN of diurnal and nocturnal rodents and in sheep, a diurnal mammal (29–32). The only difference reported between nocturnal and diurnal species to date is oscillatory expression of the Clock transcript in sheep SCN, which is constitutively expressed in rats (33). Our data suggest that Clock most likely oscillate in the primate SCN as found in the sheep (32). The relevance of this observation for circadian SCN function in diurnal mammals is presently unknown.

The adrenal gland of the adult capuchin monkey expressed clock genes in oscillatory fashion, with a phase delay relative to the SCN. At variance with the SCN, adrenal expression of the Bmal-1 mRNA was higher in daytime, between 1400 and 2000 h (ZT 7–13), and that of Per-2 was higher at nighttime (0200 h, ZT 19), resulting in a 9- to 12-h antiphase pattern. Bmal-1 peak is near the clock time of the peak reported in the rhesus whole adrenal gland by microarrays (ZT 12; Ref. 8) and in mice by quantitative PCR (14). The maximal expression of Bmal-1 and Per-2 in the capuchin monkey adrenal gland was delayed by 18 h with respect to expression in the SCN (14). Delays in clock gene expression between the SCN and adrenal of about 6–10 h have been reported for Bmal-1 and about 12 h for Per-2 in mice (14, 16). Delays averaging 6 h for clock gene expression between the SCN and other peripheral organs have been found in hamster, sheep, and mice (31–34). Microarray studies in the rhesus showed oscillatory expression of two steroidogenic enzymes involved in cortisol and dehydroepiandrosterone sulfate synthesis, Cyp 11A1 and Cyp 17A, with maximal expression at night. In the present study, we investigated the expression of the enzyme 3β -HSD involved in cortisol synthesis as a functional output rhythm. The mRNA of this enzyme showed a maximal expression at 0800 h and minimal values at 1400 h, following closely the temporal patterns of plasma ACTH and cortisol reported in the capuchin (4). 3β -HSD is induced by ACTH (35), which increases at 0200 h in the capuchin, whereas cortisol secretion shows a maximum at 0800 h and a minimum at 1400 and 2000 h (4). Because maximal cortisol secretion implies maximal amount/activity of steroidogenic enzymes, the temporal correspondence between 3β -HSD mRNA and cortisol may represent a short time delay between 3β -HSD transcription and translation.

The link between clock gene oscillation and adrenal cortisol secretion cannot be established from the present studies. However, mutant mice (double Per2/Cry1 knockout) are defective in adrenal clock genes and show loss of hypothalamus-pituitary-adrenal axis rhythmicity. In these mice, the

transplant of adrenals from wild-type to mutant mice, rescued the corticosterone rhythm in light-dark conditions, suggesting the participation of clock genes in glucocorticoid secretion (6). Transcriptome studies in the rhesus and mice adrenal show oscillatory function of a number of transcription factors that play a role controlling steroidogenic enzymes, which may be the link between clock genes and adrenal function (6, 8). The importance of glucocorticoid signaling for normal physiology highlights the need identify the link between clock genes and adrenal function.

As discussed above, *in vivo* the SCN and adrenal in the capuchin display clock gene oscillation, and it is reasonable to assume that the SCN coordinates adrenal clock genes. However, as mentioned, several lines of evidence suggest that the adrenal gland may have intrinsic oscillatory properties. To investigate whether the clock genes detected in the capuchin adrenal oscillate *in vitro*, we measured the time course of Bmal-1 and Per-2 expression for 36 h in control adrenal explants cultured in the absence of ACTH. We found oscillatory expression of Per-2, Bmal-1, and changes in the ratio Bmal-1 to Per-2. Explants were incubated with medium alone, devoid of serum and of ACTH, to ensure that oscillation of Per-2 and Bmal-1 was intrinsic to the adrenal because serum induces clock gene expression in several cell lines including PC12 cells derived from the adrenal medulla (36–38). Cry-2 and Clock mRNAs were also expressed in culture, but we did not have enough RNA to quantify whether they oscillate. We detected a peak of Per-2 at 1400 h and a peak of Bmal-1 12 h later, at 0200 h, suggesting an oscillation in antiphase. However, we did not detect a rise of Per-2 on the second 1400 h. Continuous luminescence measurement of the expression of Per-2-luc reporter gene showed sustained oscillation in various mouse tissues cultured in absence of serum for several days. Depending on the tissue, the amplitude of Per-2-luc oscillation decreased along incubation time and the period of the Per-2-luc oscillation was shortened or lengthened. Adrenal Per-2-luc oscillation was not tested in this study (18). From our experimental design in which measurements were done every 6 h, we cannot determine whether changes in amplitude or period of clock gene oscillation in the capuchin adrenal account for not finding a second peak of Per-2. Early evidence showed that the hamster adrenal gland can maintain rhythmic glucocorticoid production for several days when cultured with serum (39). We could not ascertain in the capuchin adrenal explants whether clock gene expression was accompanied by a rhythm of cortisol production because deliberately we omitted the use of serum or ACTH in the culture. Nevertheless, the present results provide the first evidence that the primate adrenal gland may have the intrinsic capacity to maintain clock gene oscillatory expression *in vitro*.

An interesting observation is that *in vitro*, there is a phase shift of Bmal-1 and Per-2 with regard to the phase observed *in vivo*. This could indicate that the manipulation of the tissue to set up the culture may have a synchronizing effect or that an *in vivo* factor synchronizing clock gene expression was missing in our culture conditions. Effects of manipulation of the tissue are unlikely because the adrenals used in this experiment were obtained at two different clock times. SCN inputs to the adrenal are diverse, including control of both

rhythmic secretion of ACTH and input from the autonomic system to the adrenal (28). Experiments in Per-1-luc mice show that stimulation of the splanchnic nerve induces Per-1-luc in the adrenal cortex, which is accompanied by an increase in corticosterone release, without changes in ACTH (13). Another factor that contributes to regulate adrenal function in the capuchin monkey is the neurohormone melatonin (5), whose daily rhythm is controlled by the SCN as well (40). In the capuchin monkey, the adrenal displays functional melatonin receptors (5). Therefore, we took advantage of our experimental *in vitro* preparation to test direct effects of melatonin on Bmal-1 and Per-2 expression.

Exposure to melatonin inhibited the peaks of Bmal-1 and Per-2: Per-2 during melatonin treatment and Bmal-1 after cessation of the melatonin treatment, suggesting that melatonin had early effects on the expression of both genes or, most likely, that lack of Per-2 resulted in inhibition of Bmal-1. As shown by others (41), Per-2 protein is required to remove the negative effect of Bmal-1 over its own transcription. The changes in Per-2 and Bmal-1 RNA levels most likely reflect effects of melatonin in the adrenal cortex because melatonin binding sites are not present in the capuchin monkey adrenal medulla (5). Whether these effects are mediated through the MT1 melatonin receptor is currently under investigation. Considering that, we did not use a melatonin receptor antagonist in these *in vitro* experiments, the possibility of melatonin acting through a nonreceptor mechanism cannot be ruled out. Of note, our *in vitro* results are in line with *in vivo* studies in the pars tuberalis of the sheep, another diurnal species. In sheep exposed to constant light to suppress endogenous melatonin secretion, treatment with melatonin implants decrease the levels of Per-2 at 6 h and Bmal-1 at 12 h after the initiation of melatonin treatment (42). This time course is remarkably similar to that observed in the present report for direct effects of melatonin in the capuchin adrenal gland. A different picture emerges from studies in nocturnal species, in which melatonin seems to be required for clock gene expression *in vivo* in adrenal cortex and pars tuberalis (15, 43–45). It is difficult to establish whether these differences in the action of melatonin on clock gene expression derive from species specific, study protocol, or actual differences between diurnal and nocturnal animals (46). Nevertheless, the present data provide the first indication of direct actions of melatonin on clock gene expression in a tissue.

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