A Circadian Clock Entrained by Melatonin Is Ticking in the Rat Fetal Adrenal

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The adrenal gland in the adult is a peripheral circadian clock involved in the coordination of energy intake and expenditure, required for adaptation to the external environment. During fetal life, a peripheral circadian clock is present in the nonhuman primate adrenal gland. Whether this extends to the fetal adrenal gland like the rat is unknown. Here we explored *in vivo* and *in vitro* whether the rat fetal adrenal is a peripheral circadian clock entrained by melatonin. We measured the 24-h changes in adrenal content of corticosterone and in the expression of clock genes *Per-2 and Bmal-1* and of steroidogenic acute regulatory protein (StAR), Mt1 melatonin receptor, and early growth response protein 1 (Egr-1) expression. In culture, we explored whether oscillatory expression of these genes persisted during 48 h and the effect of a 4-h melatonin pulse on their expression. *In vivo*, the rat fetal adrenal gland showed circadian expression of Bmal-1 and Per-2 in antiphase (acrophases at 2200 and 1300 h, respectively) as well as of Mt1 and Egr-1. This was accompanied by circadian rhythms of corticosterone content and of StAR expression both peaking at 0600 h. The 24-h oscillatory expression of Bmal-1, Per-2, StAR, Mt1, and Egr-1 persisted during 48 h in culture; however, the antiphase between Per-2 and Bmal-1 was lost. The pulse of melatonin shifted the acrophases of all the genes studied and restored the antiphase between Per-2 and Bmal-1. Thus, in the rat, the fetal adrenal is a strong peripheral clock potentially amenable to regulation by maternal melatonin. **(*Endocrinology* 152: 1891–1900, 2011)**

The adrenal gland in the adult is a peripheral circadian clock (1–4) that by interaction with the master clock located in the suprachiasmatic nucleus (SCN) of the hypothalamus generates the circadian rhythm of glucocorticoids (5, 6) and is involved in the circadian coordination of energy intake and expenditure, response to stress, and adaptation to the external environment (7). 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, Per1–3, Cry1–2, and Clock)* that regulate the expression of downstream genes (*i.e.* clock-controlled genes) involved in multiple cellular functions in 24 h, resulting in the overt circadian rhythms in the individual (8–10).

In the rat and hamster, in which several circadian rhythms (locomotor activity and drinking behavior) and the rhythm of plasma corticosterone are detected 2 wk after birth, it is well established that the phase of these rhythms and the synchronization between litters of the same dam is set by the maternal circadian system during gestation (11–14). Maternal melatonin may exert such chronobiotic action as shown in the rat (15), given that the fetal pineal gland does not produce melatonin (16), but maternal melatonin, which crosses the placenta without being altered (17), exposes the fetus to a circadian rhythm of melatonin (18). Melatonin receptors are widely distributed in several rat fetal tissues that potentially may be involved in melatonin chronobiotic actions (19).

The fetal adrenal gland, through the secretion of glucocorticoids (cortisol or corticosterone in rodents) orchestrates maturational processes central for the successful transition from fetus to newborn (20). In fetuses of nonhuman pri-

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Abbreviations: AUC, Area under the curve; BMAL-1, brain and muscle aryl hydrocarbon receptor nuclear translocator like protein 1; ΔΔCt, comparative cycle threshold; Egr-1, early growth response protein 1; qRT-PCR, quantitative RT-PCR; SCN, suprachiasmatic nucleus; StAR, steroidogenic acute regulatory protein.

mates, the hallmarks of circadian clocks (clock genes), present a circadian expression in SCN and adrenal gland. In the latter, such rhythms are accompanied by a circadian rhythm of dehydroepiandrosterone sulfate, suggesting that an early maturation of a functional adrenal clock occurred in this species (21). Functional melatonin receptors are present in the sheep and capuchin monkey fetal adrenal and follow a circadian pattern in the latter (21–23). Less is known in the fetal rat. In this species, fetal SCN neurogenesis is completed close to birth, much later than in nonhuman primates and sheep (10–11), and oscillatory expression of clock genes and metabolic activity in the SCN is present at 90% of gestation (20 d; term is 22 d) (24, 25). However, circadian oscillatory expression of the clock gene *Per-1* in whole uterine content is detected from d 12 of gestation in the rat and at 18 d in the fetal mice pars tuberalis (26, 27). These results support that some fetal organs may start circadian oscillation before the fetal SCN.

Here we explored *in vivo* and *in vitro* whether the rat fetal adrenal is a peripheral circadian clock entrained by melatonin at 18 d of gestation. At this age, in contrast to the fetal SCN, the rat fetal adrenal is functional, producing corticosterone (28, 29). We measured the 24-h changes in adrenal content of corticosterone and in the expression of clock genes Per-2 and Bmal-1 and of steroidogenic acute regulatory protein (StAR), Mt1 melatonin receptor, and early growth response protein 1 (Egr-1) expression. Per-2 and Bmal-1 are two of the core elements of the clockwork rhythm that in general is expressed in antiphase in circadian clocks, giving a broad picture of circadian clock expression (8–10). StAR is a key protein in the corticosterone synthesis positively regulated by brain and muscle aryl hydrocarbon receptor nuclear translocator like protein 1 (BMAL1) (30–33), and Egr-1 is an early gene regulated by melatonin and involved in several cellular responses (34, 35). In culture, we explored whether oscillatory expression of these genes persisted during 48 h and the effect of a 4-h melatonin pulse on their expression. The present report demonstrates that the rat fetal adrenal gland is a functional peripheral clock, active at 18 d of gestation.

Materials and Methods

Animals

Animal handling and care was performed following the National Institutes of Health Guide for Animal Experimentation Care recommendations, and the protocols were approved for the Bioethics Commission from Facultad de Medicina, Universidad de Chile.

Timed-pregnant female Sprague Dawley rats were obtained after mating (embryonic d 0 correspond to the day in which spermatozoa were observed in the smear of the vaginal contents) from Bioterio Central, Facultad de Medicina Universidad de Chile. The dams were maintained in a 12-h light, 12-h dark cycle (lights on at 0700 h) under controlled temperature (18–20 C) with food and water *ad libitum* until 18 d of gestation. Dams were weighed at 10 and 18 d of gestation. At 18 d of gestation, the dams were euthanized with an overdose of sodium thiopental (150 mg/kg), and their fetuses were dissected in sterile conditions. Dams included in the experiments had a mean increase in maternal weight of 54.8 ± 5.9 g (n = 100) between 10 and 18 d of gestation, weighed 380.0 ± 10.5 g (n = 100) at 18 d of gestation, carried eight to 14 fetuses evenly distributed in each uterine horn, and presented no signs of fetal reabsorption.

Daily rhythms in the fetal adrenal gland *in vivo*

We euthanized pregnant rats every 2 h around the clock (four to five mothers per clock time; n = 55 mothers), maternal blood samples were collected at each clock time (for melatonin and corticosterone measurement), the fetuses were delivered, and the fetal adrenals were dissected. Experiments at nighttime were performed under red light. At each clock time, six adrenal glands (from three fetuses per mother) were kept at −20 C for corticosterone measurement. About 18–12 adrenal glands from the remaining fetuses were kept in lysis buffer (SV Total RNA Isolation System; Promega, Madison, WI) until RNA extraction. In addition, placentas were collected in lysis buffer from the experiments performed at 0800 h to be used as calibrator sample in the real time measurement.

Circadian rhythms in the fetal adrenal gland *in vitro*: effect of a melatonin pulse

In three separate experiments, 15 dams were euthanized between 2000 and 2200 h under red light, and about 300 fetal

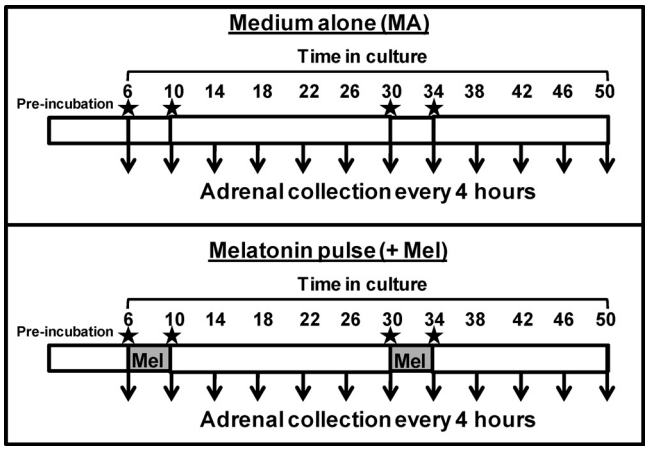

FIG. 1. Experimental design for the study of circadian rhythms *in vitro* in the rat fetal adrenal gland. Fetal adrenals were collected between 2000 and 2200 h. Equal numbers of fetal adrenals were separated into two groups (n = 180 per group): medium alone (MA; *upper panel*) or medium plus 100 nM melatonin (+Mel, *lower panel*). Each group was incubated for 6 h with medium alone (2200–0400 h preincubation) and the first set of adrenals was collected from each group. Then the medium was removed and replaced with medium alone (*upper panel*) or with medium containing 100 nM melatonin (+Mel; *lower panel*). At 0800 h, a second set of fetal adrenals was collected from both groups, and the medium was changed to medium alone, continuing fetal adrenal collection every 4 h until 0400 h. At this time, the medium was changed to medium alone or medium plus melatonin. The procedure was continued as described above to complete a total of 48 h in culture. The *stars* indicate the clock times at which the medium was replaced.

TABLE 1. qRT-PCR primers for gene studied

Gene	Primer sequence (sense/antisense)	Size (bp)	Annealing temperature (°C)	*E*		CV (%)
				Placenta	Adrenal	
18S rRNA (44)	gtaacccgttgaaccccatt/ccatccaatcggtagtagcg	150	60	2.022 ± 0.005	2.033 ± 0.007	13.3
Per-2 (45)	caccctgaaaagaaagtgcga/caacgccaaggagctcaagt	148	62	2.031 ± 0.008	2.044 ± 0.010	11.7
Bmal-1b (45)	ccgatgacgaactgaaacacct/tgcagtgtccgaggaagatagc	215	64	2.014 ± 0.006	2.049 ± 0.004	7.4
Mt1 (36)	tttactatcgtggtggacatcc/gcactaacttgacaatgcagatatc	206	60	2.019 ± 0.011	2.028 ± 0.013	12.5
Egr-1 (46)	cacgtcttggtgcctttg/ctcagccctcttcctcatc	143	64	2.023 ± 0.003	2.042 ± 0.004	8.5
StAR (47)	agaaggaaagccagcaggaga/tctcccatggcctccatg	147	60	2.071 ± 0.003	2.132 ± 0.002	13.8

CV, Coefficient of variation (interassay calculated in the calibrator sample); *E*, assay efficiency.

adrenals were collected per experiment, pooled in 15 ml DMEM-F12 (GIBCO, Grand Island, NY), preincubated for 6 h at 37 C, and then further incubated in triplicate (six fetal adrenals per tube) in two groups of separated tubes with 1.5 ml medium at 37 C, 100% humidity, 5% CO_2, and 95% air. The incubation conditions (Fig. 1) were 6 h with medium alone from 2200–0400 h (preincubation), and after collection of the first set of adrenals, the medium was replaced with medium alone (control) or in the other group of adrenals with medium containing 100 nM melatonin. We chose this dose because of previously reported actions of 100 nM in clock gene expression in the adult capuchin monkey adrenal (3) and over glucocorticoid production in the adult rat adrenal (36).

At 0800 h, adrenals were collected from both treatments, and the media were changed to medium alone, continuing adrenal collection every 4 h until 0400 h (28 h in culture). The procedure was repeated for the next 24 h to complete a total of 48 h in culture. Collected fetal adrenals were preserved in lysis buffer until RNA extraction.

Real-time quantitative RT-PCR (qRT-PCR)

Fetal adrenals total RNA from *in vivo* and *in vitro* protocols and from the placenta pool (calibrator sample) were extracted using SV total RNA isolation system (Promega) according to the manufacturer's instructions. To assure that genomic DNA was absent in the final reaction, the kit includes a step with deoxyribonuclease. About 1.0 μg of total RNA was reverse transcribed at 37 C in a reaction mixture that contained 1.0 μl random primers (50 ng; Promega), 1.0 μl 10 mM dNTPs mix (Promega), 4.0 μl 5× first-strand buffer (Promega), 2.0 μl 0.1M dithiothreitol (Promega), and 200 IU Maloney murine leukemia virus reverse transcriptase (Invitrogen Corp., Carlsbad, CA) in a final volume of 20 μl. The expression levels of the mRNA of Per-2, Bmal-1, Mt1, Egr-1, and StAR and of the housekeeping gene 18S rRNA, were measured by real-time qRT-PCR.

The primers used in the present work were described previously by others and designed to include at least one intron (see Table 1). Using semiquantitative PCR all the genes studied were amplified in the calibrator sample (placenta pool). The sqPCR products were purified by chromatography (DNA Wizard PCR Preps; Promega) and sequenced in the Ecology Department of Facultad de Ciencias Biologicas, Pontificia Universidad Catolica de Chile. The homology degree of each semiquantitative PCR product with the corresponding rat gene sequence reported in GenBank was determined using the BLASTN 2.2.1 tool (www.ncbi.nlm.nih.gov). Percentage identity was 100% for Per-2, 100% for Bmal-1, 99% for Mt1, 99% for StAR, and 100% for Egr-1.

The qRT-PCRs were performed in a final reaction volume of 12.5 μl containing 7.5 μl SYBR Green Master Mix (Applied Biosystems, Foster City, CA) and 0.15 μM of each sense and antisense primer, H_2O PCR grade, and cDNA. The qPCR was carried out in an Applied Biosystems StepOne real-time PCR system thermocycler (Applied Biosystems) and consisted of an initial denaturation step at 95 C for 5 min, followed by 50 cycles of 95 C for 30 sec, primer annealing temperature for 30 sec, and 72 C for 30 sec (Table 1).

Relative amounts of all mRNAs were calculated by the comparative cycle threshold (ΔΔCt) method (37) using the equation $2^{-\Delta\Delta Ct}$. To validate the quantification using the ΔΔCt method, in all genes analyzed, a dilution curve (range 50–0.25 ng of total RNA) was performed in the calibrator and in a pool of fetal adrenal gland, the Ct obtained *vs.* RNA concentration was plotted, and the slope (m) of the lineal regression was calculated, the efficiency (*E*) in each curve for each gene was determined as $E = 10^{-1/m}$. Efficiencies were from 2.01–2.10 (equivalent 101–110%, respectively; Table 1). The first ΔCt is the difference in the sample of the Ct values between the gene of interest and its respective 18S rRNA, whereas the second ΔCt is the difference in the calibrator sample between the gene of interest and its respective 18S rRNA. The sample and the calibrator were assayed simultaneously. All the samples were amplified in duplicate in at

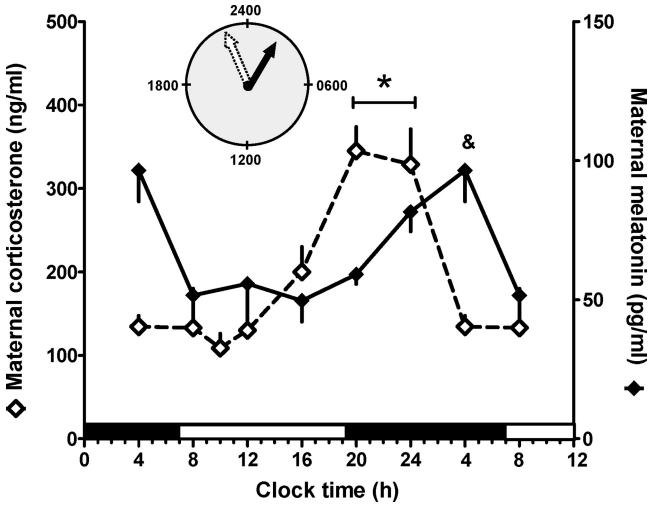

FIG. 2. A daily rhythm of plasma melatonin and corticosterone concentration is present in pregnant dams at 18 d of gestation. The results are expressed as mean ± SE. Plasma melatonin (◆; n = 5 per clock time) and corticosterone concentration (◇; n = 5 per clock time). *Inset*, Representative distribution of the acrophase of melatonin (*black arrow*) and corticosterone (*white arrow*). *, Different from other time points ($P < 0.05$, ANOVA and Newman-Keuls); &, different from 0800–1600 and 2000 h ($P < 0.05$, ANOVA and Newman-Keuls). The *dark bars* indicate lights-off hours.

least three mRNA concentrations (range 100–2.5 ng). A melting curve analysis was performed on each sample after the final cycle to ensure that a single product was obtained, and agarose gel electrophoresis confirmed that the single PCR product was of the expected size.

Hormone assays

Corticosterone

Rat fetal adrenal corticosterone content was measured using the protocol described by Wotus *et al.* (28). Briefly, fetal adrenals were homogenized with 20% ethanol in PBS and then centrifuged at 1200 × *g* at 4 C for 5 min, and the supernatants were collected for corticosterone analysis. Fetal adrenal corticosterone content and plasma maternal corticosterone concentration were measured by RIA using a commercial kit (Coat-a-Count rat corticosterone kit; Siemens Healthcare Diagnostics, Plainfield, IN) following the manufacturer's instructions. The inter- and intraassay coefficients were less than 10%.

Melatonin

Maternal melatonin concentration in plasma was measured by RIA. Melatonin antiserum (G/S/70408483; Stockgrand Ltd., Guldford Surrey, UK) and [*O*-methyl-^{3}H]melatonin (85 Ci/mmol; Amersham Bioscience AB, Uppasala, Sweden) as a tracer were used following the manufacturer's recommendations. The inter- and intraassay coefficients were less than 15%.

Statistical analysis

Data are expressed as mean ± SEM. The 24-h changes in maternal plasma corticosterone and melatonin levels, fetal corticosterone content, and the values of $2^{-\Delta\Delta Ct}$ for each gene measured were analyzed by one-way ANOVA using Newman-Keuls as a *post hoc* test. Additionally, mean data were fitted to a theoretical cosine function. To assess circadian gene expression *in vitro*, the data were normalized for each 24-h interval. Normalization considered the highest value within the experiment as 1 and the lowest as 0. Additionally, mean data were fitted to a theoretical cosine function. The effect of melatonin on the expression of all the genes studied was analyzed by two-way ANOVA using Dunn as a *post hoc* test. Integrated expression in 24 h for each gene in the fetal adrenal *in vivo*, *in vitro*-control (medium alone), and *in vitro* plus melatonin was calculated as area under the curve (AUC) from a 24-h interval between two peaks; for *in vivo* conditions, the first peak was repeated. The results were analyzed by one-way ANOVA using Newman-Keuls as a *post hoc* test. Statistical analyses were performed using GraphPad Prism software (version 3.02; GraphPad Software Inc., San Diego, CA). Results were considered significant when $P < 0.05$.

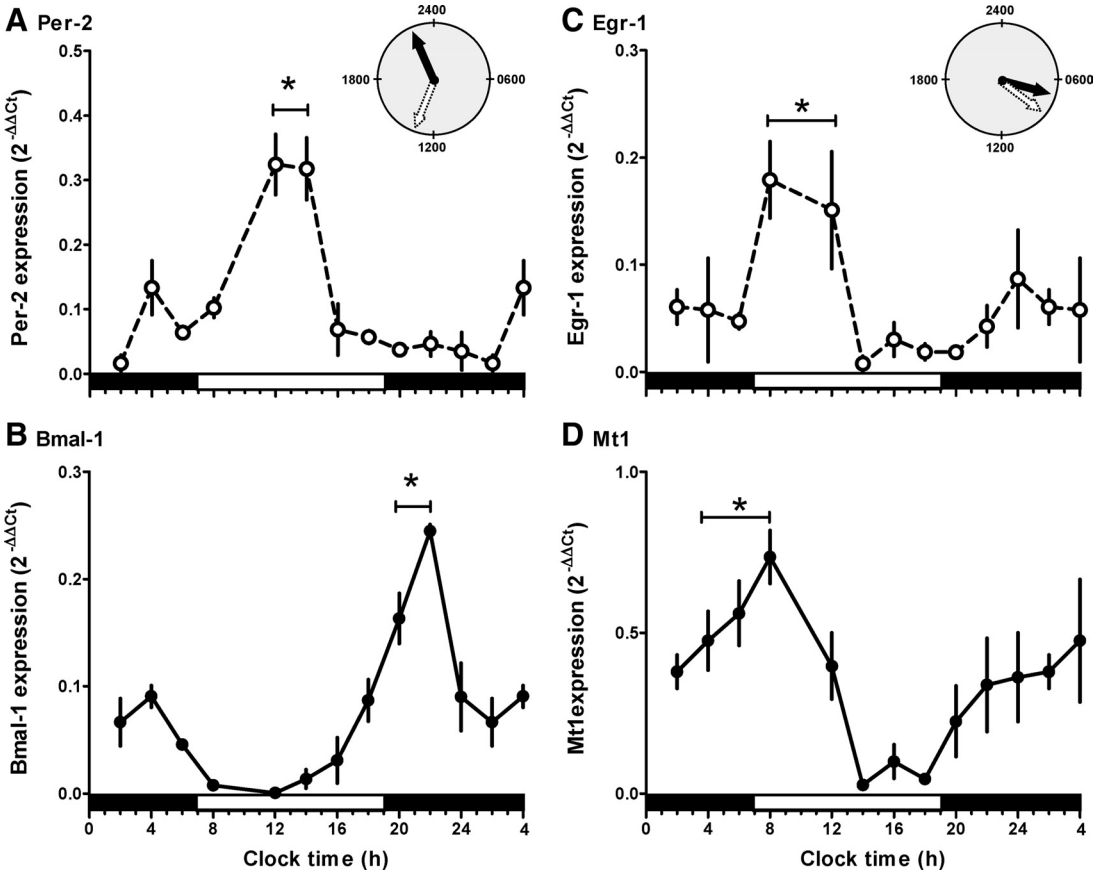

FIG. 3. Oscillatory expression of Per-2 (A), Bmal-1 (B), Egr-1 (C), and Mt1 (D) in the rat fetal adrenal gland at 18 d of gestational age. The results are expressed as mean ± SE of $2^{-\Delta\Delta Ct}$. Fetal adrenal glands were collected every 2 h from fetuses obtained from pregnant dams maintained in 12:12 photoperiod. *Inset* in A, Representative distribution of the acrophase of Per-2 (*white arrow*) and Bmal-1 (*black arrow*); *inset* in C, representative distribution of the acrophase of Egr-1 (*white arrow*) and Mt1 (*black arrow*) around 24 h. *, Different from other time points ($P < 0.05$, ANOVA and Newman-Keuls). The *dark bars* indicate lights-off hours.

Results

The pregnant dams showed daily rhythms of plasma melatonin and corticosterone by ANOVA and Newman-Keuls (Fig. 2), as reported by others in the nonpregnant rat (38, 39). The mean plasma concentration of melatonin and corticosterone fitted a theoretical cosine function with an acrophase at 0212 h for melatonin and at 2041 h for maternal corticosterone ($r^2 = 0.712$ and 0.695, respectively).

Daily rhythms in the fetal adrenal gland *in vivo*

Daily rhythms of clock gene expression and of the Mt1 melatonin receptor expression

All the genes analyzed in the fetal adrenal gland showed clock time-related changes in their expression.

Per-2 and Bmal-1 presented oscillatory expression in the fetal adrenal gland ($P < 0.05$ by ANOVA and Newman-Keuls). As shown in Fig. 3, A and B, these clock genes oscillated in opposition, with a phase delay of 9 h. The expression of both clock genes fitted a theoretical cosine function with a maximum at 1323 h for Per-2 and at 2217 h for Bmal-1 ($r^2 = 0.789$ and 0.745, respectively).

To examine whether the potential clock contained in the adrenal is a target for melatonin, we measured the expression of the melatonin receptor Mt1 as well as the expression of Egr-1. We found that Mt1 and Egr-1 oscillate in the fetal adrenal gland *in vivo* ($P < 0.05$, ANOVA and Newman-Keuls; Fig. 3, C and D). As shown in Fig. 3D, Mt1 mRNA expression presented an acrophase at 0718 h ($r^2 = 0.742$) preceding the acrophase of Egr-1 expression (0949 h; $r^2 = 0.643$) by about 3 h.

Daily rhythm of fetal adrenal corticosterone content and StAR mRNA expression

The content of corticosterone in the fetal adrenal gland followed an oscillatory pattern by ANOVA and Newman-Keuls (Fig. 4) and fitted a theoretical cosine function with an acrophase at 0548 h ($r^2 = 0.817$). This rhythm was accompanied by a daily rhythm of StAR expression ($P < 0.05$, ANOVA and Newman-Keuls; Fig. 4) with an acrophase at 0607 h ($r^2 = 0.632$), coincident with that of the fetal daily rhythm of adrenal corticosterone content. The rhythm of fetal adrenal corticosterone content and of StAR expression was almost opposite to the rhythm of corticosterone in maternal plasma (Fig. 2).

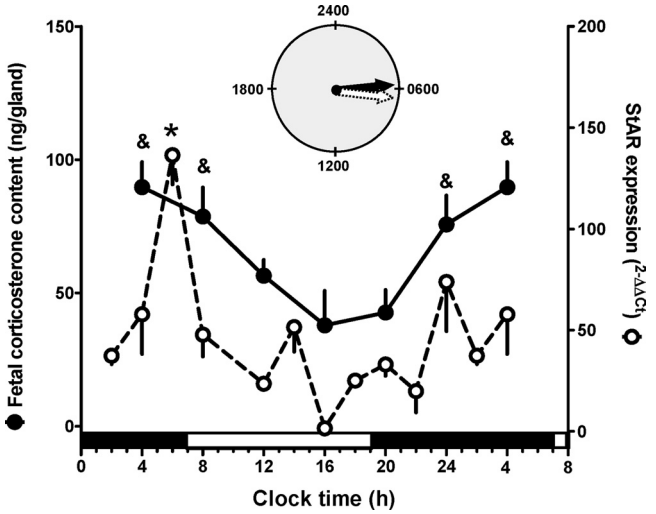

FIG. 4. A daily rhythm of corticosterone content and StAR expression is present in the rat fetal adrenal gland at 18 d of gestational age. The results are expressed as mean ± SE of fetal corticosterone content (○; n = 5 per clock time) and $2^{-\Delta\Delta Ct}$ of StAR (●, n = 5 per clock time). *Inset*, Representative distribution of the acrophase of fetal adrenal corticosterone content (*black arrow*) and StAR (*white arrow*) around 24 h. * and &, Different from other time points ($P < 0.05$, ANOVA and Newman-Keuls). The *dark bars* indicate lights-off hours.

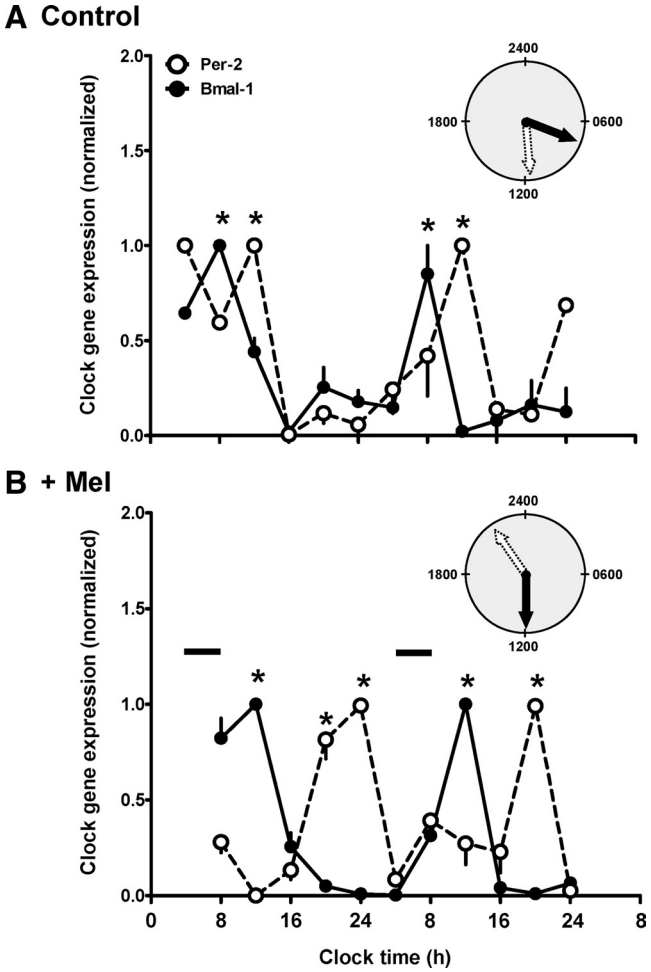

FIG. 5. Normalized oscillatory expression (mean ± SE) of Per-2 (○) and Bmal-1 (●) in rat fetal adrenal glands cultured with medium alone (A) and treated for 4 h every 24 h with a pulse of 100 nM melatonin (B). Data were normalized as described in *Materials and Methods*. Fetal adrenal glands were collected every 4 h during 48 h. *Insets*, Representative distribution of the acrophase of Per-2 (*white arrow*) and Bmal-1 (*black arrow*) around 24 h. *, Different from other time points ($P < 0.05$, ANOVA and Newman-Keuls); + Mel different from control ($P < 0.05$, two way ANOVA and Dunn). The *black line* in B indicates the 4-h pulse of melatonin.

Circadian rhythms in the fetal adrenal gland *in vitro*: effect of a melatonin pulse

The expression of Bmal-1, Per-2, Mt1, Egr-1, and StAR in the fetal adrenal continued in culture. To assess the presence of a circadian oscillation, the data were normalized within each 24-h interval. Fetal adrenal gene expression presented 24-h oscillation in culture, with maximums occurring at the same clock time hours during the first and second 24 h in culture in control and melatonin-treated fetal adrenal explants ($P < 0.05$, ANOVA and Newman-Keuls). Nevertheless, there were differences in the timing of the acrophases of the different rhythms between the fetal adrenals with medium alone (control) or treated with a melatonin pulse ($P < 0.05$, two-way ANOVA and Dunn).

In control conditions, the acrophase of Per-2 and of Bmal-1 occurred at 1147 h and 0753 h, respectively ($r^2 = 0.663$ and 0.714, respectively), resulting in a phase difference between these genes of only 4 h (Fig. 5A). The melatonin treatment delayed differentially the acrophases of both clock genes, Per-2 by 10 h (acrophase at 2133 h, $r^2 = 0.794$) and Bmal-1 by 4 h (acrophase at 1207 h, $r^2 = 0.698$) compared with control conditions, resulting in an antiphasic pattern (Fig. 5B).

In control conditions, the acrophases of StAR, Mt1, and Egr-1 occurred at 0725; 0751, and 1129, respectively ($r^2 = 0.644$, 0.657, and 0.596, respectively; Fig. 6, A–C). The melatonin pulse induced a phase delay of about 4 h of StAR expression (acrophase at 1113 h, $r^2 = 0.813$) and of

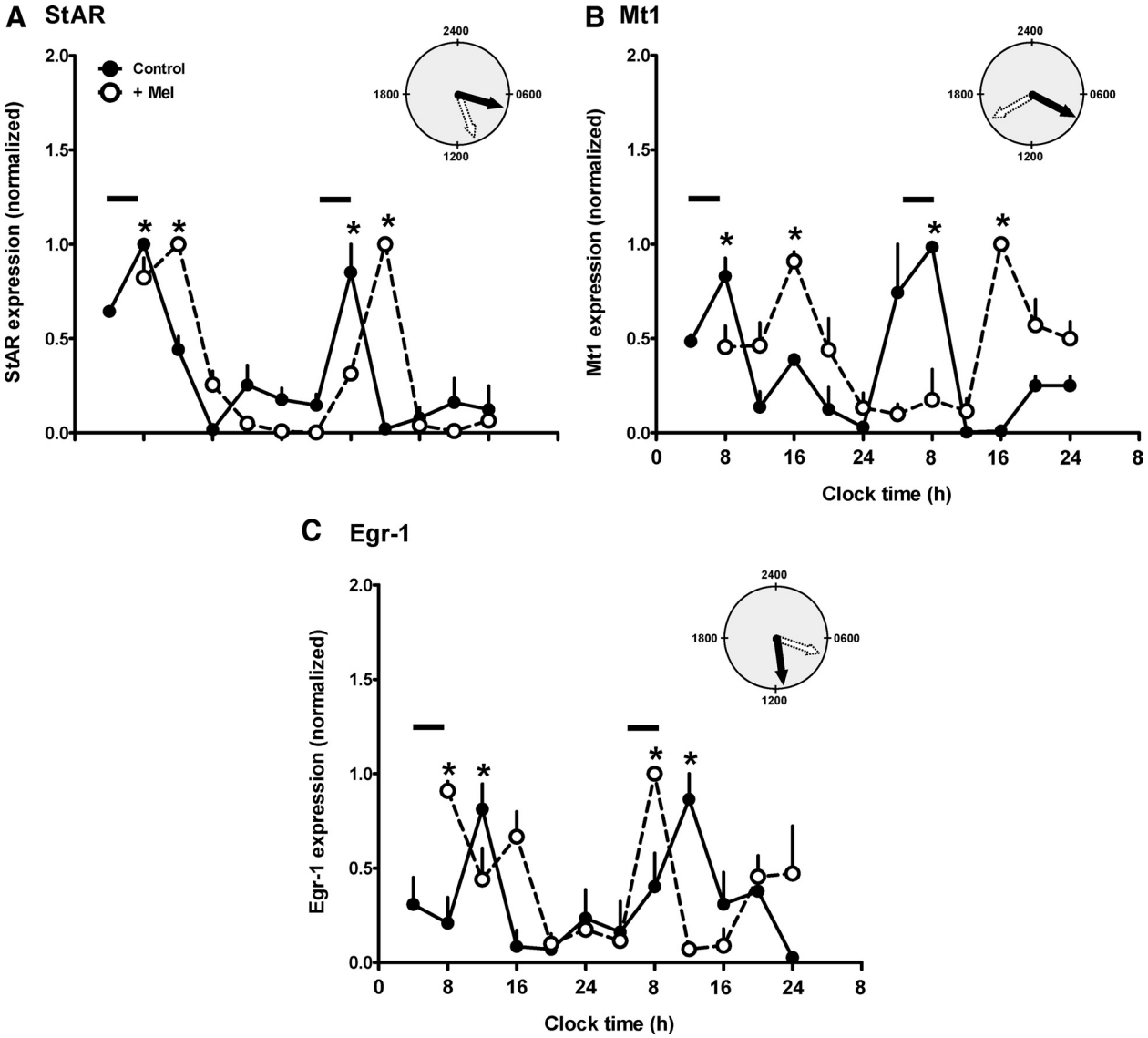

FIG. 6. Normalized oscillatory expression (mean ± SE) of StAR (A), Mt1 (B), and Egr-1 (C) in rat fetal adrenal glands cultured with medium alone (*solid line*, ●) and treated with a pulse of 100 nM melatonin (*dotted line*, ○). Data were normalized as described in *Materials and Methods*. Fetal adrenal glands were collected every 4 h during 48 h. *Insets*, Representative distribution of the acrophase for each gene measured in medium alone (*white arrow*) and treated with a pulse of melatonin (*black arrow*) around 24 h. *, Different from other time points ($P < 0.05$, ANOVA and Newman-Keuls); + Mel different from control ($P < 0.05$, two way ANOVA and Dunn). The *black line* in each graph indicates the 4-h pulse of melatonin.

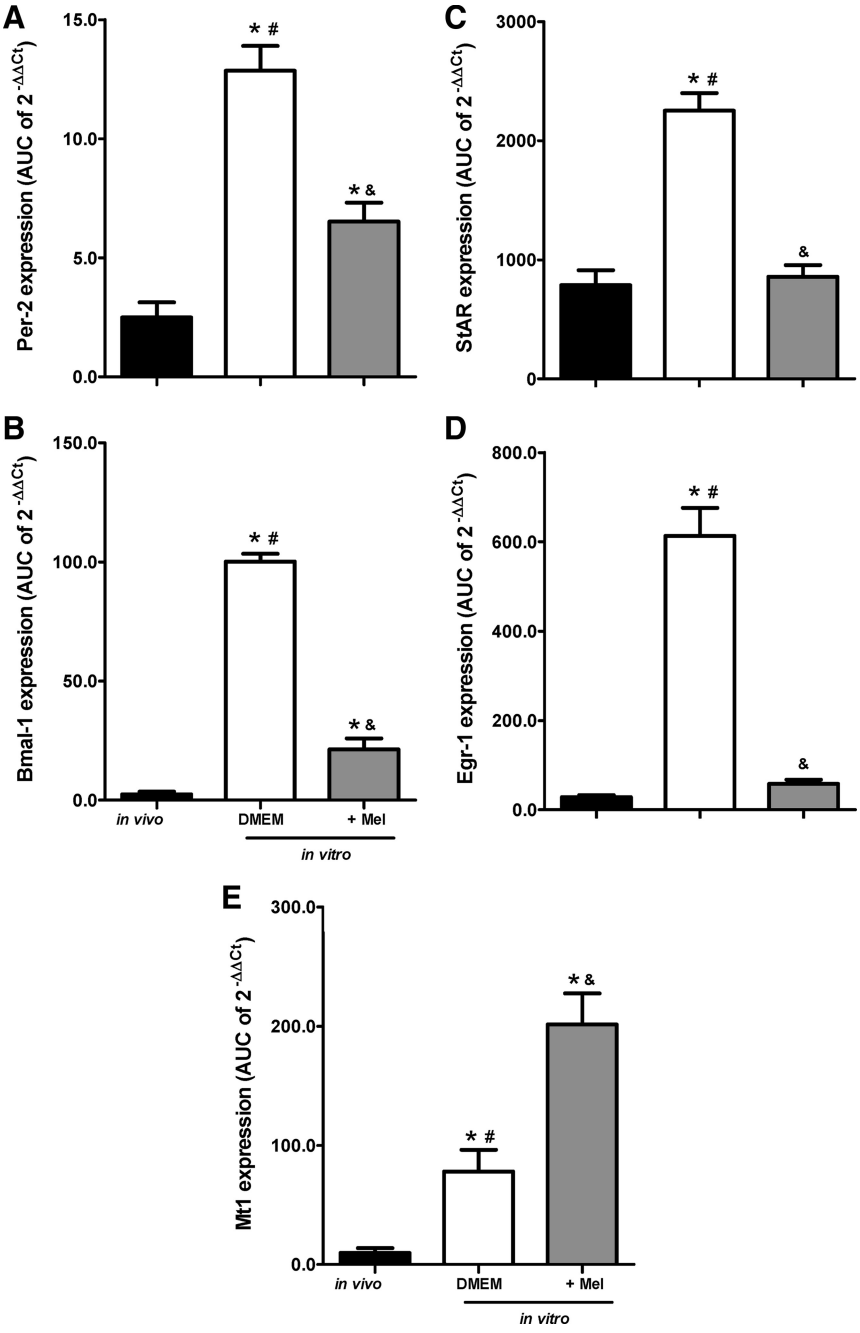

FIG. 7. Integrated mRNA expression of Per-2 (A), Bmal-1 (B), StAR (C), Egr-1 (D) and Mt1 (E) in the rat fetal adrenal glands in culture. The total amount of each gene was calculated as AUC in 24 h $2^{-\Delta\Delta Ct}$ *in vivo* (*black bars*), fetal adrenal gland cultured with medium alone (*white bars*), and fetal adrenal gland cultured with a 4-h pulse of 100 nM melatonin (*gray bars*).*, Different from *in vivo* ($P < 0.05$; ANOVA and Newman-Keuls); &, different from medium alone ($P < 0.05$, ANOVA and Newman-Keuls); #, different from + Mel ($P < 0.05$, ANOVA and Newman-Keuls).

8 h of Mt1 expression (acrophase at 1544 h, $r^2 = 0.744$) compared with control conditions (Fig. 6, A and B). In contrast, melatonin treatment advanced the phase of Egr-1 by 4 h (acrophase at 0712 h, $r^2 = 0.681$; Fig. 6C).

Comparing the integrated expression of Bmal-1, Per-2, Mt1, Egr-1, and StAR (AUC of $2^{-\Delta\Delta Ct}$) in culture and the corresponding expression of these genes in the fetal adrenal *in vivo*, we observed that when the fetal adrenal was cultured with medium alone (control), there was a higher expression in culture than *in vivo* (Fig. 7). The treatment with a pulse of melatonin reduced expression of Per-2, Bmal-1, StAR, and Egr-1 and increased expression of Mt1 in comparison with the medium alone (Fig. 7). We took care to eliminate the sampling bias by calculating AUC from a 24-h interval between two peaks in each *in vitro* condition. For *in vivo* conditions, the first peak was repeated; thus, in the three cases, the same amount of hours and peaks were considered. We did not find differences in the expression of 18S rRNA between *in vitro* (medium alone or treated with melatonin) and *in vivo* conditions (AUC Ct in 25 ng of RNA = 243.9 ± 15.7, 272.3 ± 21.1, and 291.8 ± 19.9, respectively).

Discussion

Regardless of the degree of maturity achieved at birth, in all species studied, the fetal adrenal gland is a key fetal endocrine organ that through an active glucocorticoid production orchestrates maturational processes central for the transition to newborn. Here we demonstrate by means of *in vivo* and *in vitro* experiments that at 18 d gestational age (term 22 d), the rat fetal adrenal gland is a peripheral circadian clock.

In vivo, the rat fetal adrenal expressed a robust daily oscillation of the clock genes Bmal-1 and Per-2. These genes oscillated in antiphase, Per-2 peaking in the middle of subjective day and Bmal-1 about 9 h later. In addition, the clock-controlled gene *StAR* involved in glucocorticoid production, the Mt1 melatonin receptor, and the early gene Egr-1 (two melatonin targets) also showed daily rhythms. The clock-time pattern of Bmal-1 expression was opposite to that reported in adult rat and mice adrenal glands, whereas the phase delay between Bmal-1 and StAR acrophases was similar to that of the rat adult adrenal gland (33, 39). The rat fetal adrenal has a prominent corticosterone content, and at 18 d of gestation, fetal plasma corticosterone concentration is

higher than the mother's (28, 29), indicating active steroidogenesis. Supporting the involvement of the adrenal circadian clock in this activity, corticosterone content showed a high-amplitude circadian rhythm, being maximal at 0600 h, 8 h later than the acrophase of the maternal corticosterone rhythm. In addition, StAR circadian pattern of expression presented an acrophase close to the acrophase of the rhythm of fetal adrenal corticosterone content, as in the adult mice and rat adrenal gland (33, 39). Thus, at 18 d of gestational age, the rat fetal adrenal gland shares the characteristics of the circadian clock found in the adult rat adrenal and the adrenal of other species (1–4).

Demonstrating an intrinsic oscillatory capacity of the rat fetal adrenal gland, the expression and circadian oscillation of the clock genes Per-2 and Bmal-1 and of StAR, Egr-1, and Mt1 persisted for 48 h in cultures with medium alone. In this condition, there was a general increase in expression of Per-2 and Bmal-1 and of StAR, Egr-1, and Mt1 compared with the levels found *in vivo*, whereas 18S rRNA expression was maintained. Such increase was evident during the first and second 24 h of culture despite a reduction of gene expression in the latter. In addition, the antiphasic relation between Per-2 and Bmal-1 present in the rat fetal adrenal *in vivo* was lost in culture, the acrophases of these genes becoming separated by only 4 h. These changes in clock gene relationship had minor effects on Mt1 receptor StAR, and Egr-1 acrophases. A similar observation has been reported in mice preadipocytes in culture, in which a circadian rhythm of metabolic genes and leptin expression is present, although there is no antiphasic relationship between Per-2 and Bmal-1 (40). Examination of our previous work in the adult capuchin monkey adrenal (3) and the reports by others in cultured rat heart (41) also reveal an increase in clock gene expression *in vitro vs. in vivo*. Altogether, in the rat fetal adrenal, the differences in the amount of expression of the clock genes and in their phase relationship and the changes in Mt1 receptor, StAR, and Egr-1 expression between *in vivo* and *in vitro* conditions suggests that factors acting upon these genes *in vivo* were missing in culture.

The circadian expression of the Mt1 receptor in the rat fetal adrenal *in vivo* and *in vitro* suggests that melatonin may be one of the factors missing in culture. Melatonin is a lipophilic hormone produced by the mother that crosses freely the placenta and thus exposes fetal organs to the maternal melatonin rhythm (16–18). We subjected rat fetal adrenal cultures to a melatonin pulse given during the second half of the subjective night. Melatonin increased Mt1 expression while down-regulating the expression of the clock genes Per-2 and Bmal-1 and of StAR and Egr-1. A second effect of melatonin in the rat fetal adrenal in culture was to modify the phase of the rhythm of clock gene expression, which by delaying Per-2 and Bmal-1 acrophases restored the antiphasic relationship found *in vivo*. In addition to shifting the clock genes, melatonin shifted the Mt1 rhythm and had minor effects upon the phase of StAR and Egr-1 rhythms. To date, only a few studies have explored acute effects of melatonin on circadian oscillation of circadian genes in culture, and results are inconsistent. Melatonin treatment in adult capuchin monkey adrenal explants in culture abolished the peaks of Per-2 and Bmal-1, present in control conditions (3), whereas in PC3 cells (human prostate carcinoma cell lines), melatonin induced a circadian expression of Per-2, which was absent in control conditions (42). Likewise, there is no information on acute effects of melatonin in the expression of Mt1 in the fetal or adult adrenal gland, whereas the melatonin-induced decrease in StAR could be linked to the decrease in Bmal-1 expression, because StAR is a clock-controlled gene regulated by Bmal-1 (31–33). On the other hand, Egr-1 has been shown to be negatively regulated by melatonin in sheep pars tuberalis (34, 35). Altogether, our results demonstrate that melatonin acts in the autonomous peripheral circadian clock contained in the fetal adrenal. In view of the fact that melatonin reaching the fetus comes from the mother, the rat fetal adrenal clock at 18 d of gestation is potentially amenable to regulation by maternal melatonin.

In adult mammals, completeness of the circadian system, with a pacemaker (SCN) that commands peripheral oscillators, is an essential biological system involved in the regulation of almost all physiological functions (10, 43). Less is known about the role of the circadian system during fetal life and its organization, particularly in species like the rat whose newborns are very immature at birth. Considering that in the fetal rat at 18 d of gestational age, the circadian metabolic activity in the fetal SCN has not been established yet (24, 25), the rat fetal adrenal may be an important circadian clock during fetal life synchronized by maternal melatonin. An intriguing possibility is that the circadian production of glucocorticoids by the rat fetal adrenal gland, in addition to its known role on maturation of the lung and other fetal organs (20), contributes to organize a fetal internal temporal order appropriate for adequate fetal development.

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