Deciphering the Function of the Blunt Circadian Rhythm of Melatonin in the Newborn Lamb: Impact on Adrenal and Heart

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Neonatal lambs, as with human and other neonates, have low arrhythmic endogenous levels of melatonin for several weeks until they start their own pineal rhythm of melatonin production at approximately 2 weeks of life. During pregnancy, daily rhythmic transfer of maternal melatonin to the fetus has important physiological roles in sheep, nonhuman primates, and rats. This melatonin rhythm provides a circadian signal and also participates in adjusting the physiology of several organs in preparation for extrauterine life. We propose that the ensuing absence of a melatonin rhythm plays a role in neonatal adaptation. To test this hypothesis, we studied the effects of imposing a high-amplitude melatonin rhythm in the newborn lamb on (1) clock time–related changes in cortisol and plasma variables and (2) clock time–related changes of gene expression of clock genes and selected functional genes in the adrenal gland and heart. We treated newborn lambs with a daily oral dose of melatonin (0.25 mg/kg) from birth to 5 days of age, recreating a high-amplitude melatonin rhythm. This treatment suppressed clock time–related changes of plasma adrenocorticotropic hormone, cortisol, clock gene expression, and functional genes in the newborn adrenal gland. In the heart, it decreased heart/body weight ratio, increased expression of *Anp* and *Bnp*, and resulted in different heart gene expression from control newborns. The interference of this postnatal melatonin treatment with the normal postnatal pattern of adrenocortical function and heart development support a physiological role for the window of flat postnatal melatonin levels during the neonatal transition. **(*Endocrinology* 158: 2895–2905, 2017)**

Neonatal lambs, as with human and other neonates, have low constant plasma melatonin levels throughout the 24-hour cycle that do not show circadian rhythmicity (1–3). This situation persists for a number of weeks until the neonate starts its own rhythmic pineal melatonin production. This is in marked contrast with the adult and fetal physiological context in which a circadian rhythm of plasma melatonin is present, although it originates in a different way. In the adult, the plasma melatonin rhythm is generated by rhythmic pineal gland secretion (4), whereas in the fetus, this rhythm is imposed by transfer of the maternal melatonin rhythm through the placenta (5). Birth interrupts this maternal melatonin transfer; therefore, the myriad physiological changes conducing to a successful postnatal adaptation occurs in the absence of a melatonin rhythm (6). We hypothesize that the absence of a melatonin rhythm plays a key role in this early neonatal adaptation.

Melatonin is a pleiotropic hormone that, in the fetal and adult physiological contexts has effects on endocrine,

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in USA

Received 12 March 2017. Accepted 17 July 2017.
First Published Online 20 July 2017

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Abbreviations: ACTH, adrenocorticotropic hormone; ANOVA, analysis of variance; HW/BW, heart weight/body weight; mRNA, messenger RNA; PCR, polymerase chain reaction; SCN, suprachiasmatic nucleus; T3, triiodothyronine; ZT, Zeitgeber.

metabolic, cardiovascular, and circadian systems through membrane receptors or as a scavenger of free radicals, which contribute to homeostasis (4). A well-established melatonin function provides clock time and seasonal information to circadian clocks. At the cell level, the molecular engine of circadian clocks is an interconnected stimulatory and inhibitory transcriptional–translational feedback loop of the clock genes *Bmal1*, *Clock*, *Per1-3*, and *Cry 1-2*. This loop drives genes involved in major cellular functions resulting in a 24-hour oscillation of the transcriptome in practically every tissue (7). In adult mammals, light/dark information received by the suprachiasmatic nucleus of the hypothalamus (SCN; master clock), is conveyed through neural and/or humoral signals to circadian clocks in other organs (named peripheral circadian clocks), generating circadian rhythms of heart rate, temperature, melatonin, and glucocorticoid, among others, entrained to the external light/dark cycle (8–10). Among the humoral signals, it is well established that the circadian rhythms of melatonin (11) and glucocorticoid (12) act as internal time-givers [Zeitgebers (ZTs)] for other peripheral clocks.

Melatonin plays important roles during fetal life. We and others have shown that melatonin is important for fetal adrenal function, accrual of brown fat, vascular function, and preparation for newborn life. In addition, it plays the classical chronobiotic role for fetal circadian rhythms (13). The current model suggests that development of fetal circadian clocks in organs as SCN, adrenal, and heart are part of the genetic fetal program (14); nonetheless, synchronization of these clocks to external clock time is provided by signals controlled by the maternal SCN, one of them being the maternal melatonin rhythm (15–19). As the fetus prepares to be a newborn, an important function of the maternal melatonin rhythm is to set the phase of overt newborn circadian rhythms (20–22), allowing behavioral synchronization with the mother and the environment. Whether postnatal manifestation of this programming requires absence of a plasma melatonin rhythm in the postnatal period is unknown. After birth, the newborn experiences major changes in all physiological systems. It is unknown whether the postnatal absence of a melatonin rhythm facilitates these changes. Answering these questions, besides contributing to the understanding of normal neonatal physiology, may be important because several melatonin therapies have been proposed in the early neonatal period (23).

We hypothesize that the absence of a diurnal melatonin rhythm plays a role in early neonatal adaptation. Within the first week of life, as most other newborn organs, the lamb adrenal and heart experience pronounced changes. Adrenal responsiveness to adrenocorticotropic hormone (ACTH), prostaglandin E, and α–melanocyte-stimulating hormone decreases and plasma cortisol decreases from high prepartum levels at the end of gestation to low plasma levels characteristic of adult sheep (24–26). Postnatal changes in the lamb heart are striking, and well known and similar to those experienced by the human newborn. Immediate changes take place at birth and during the days that follow, leading to heart hemodynamic, morphological, and metabolic remodeling. The heart changes from the fetal to the neonatal-adult circulatory pattern (27) and shifts energy production from glucose to fatty acids, taking advantage of the high-fat diet supplied by maternal milk (28, 29). This is accompanied by changes in expression of genes associated with fatty acid and carbohydrate metabolism among others (30). Of note, the adrenal gland and heart in several species, during fetal life and in the adult, contain peripheral circadian clocks. Moreover, the adrenal clock responds to melatonin (12, 16, 17, 31). We hypothesize that the absence of a diurnal melatonin rhythm plays a role in early neonatal adaptation of the adrenal gland and heart. To test this hypothesis, we selected to study the effects of maintaining a high-amplitude melatonin rhythm in the newborn lamb on: (1) clock time–related changes in cortisol and plasma variables and (2) clock time–related changes in gene expression of circadian clock genes and selected functional genes in the adrenal gland and heart.

Materials and Methods

Animals

All experimental protocols were reviewed and approved by the Committee for Animal Bioethics, Faculty of Medicine, University of Chile (CBA no. 0287), and Fondecyt (Project Fondecyt 1090381). Animal care, maintenance, procedures, and experimentation were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (Publication No. 85-23, revised 1996) and adheres to the American Physiological Society's Guiding Principles in the Care and Use of Animals.

Experimental protocols

Twenty-six term newborn lambs were studied. Experiments were performed during a 6-week interval (from the end of May to the first week July, autumn-winter in the southern hemisphere). Gestation and postnatal photoperiods were 12 hours of light, 12 hours of dark, with lights on at 07:00 and off at 19:00. Considering the light/dark cycle as a ZT, clock time 07:00 corresponds to ZT 0 and 19:00 to ZT 12. Birth occurred before 08:00 (ZT1) in all cases. All newborns were fed by their mothers. Twelve newborns (five males, seven females) received daily oral doses of 1 mg of melatonin in 1 mL 0.9% NaCl at 17:00 (ZT10) from the day of birth until 5 days of age. The melatonin solution was prepared by dissolving 100 mg of melatonin (Sigma-Aldrich, Quimica Limitada, Santiago, Chile) in 10 mL ethanol and then diluted 1 to 10 in 0.9% NaCl. Control newborns (six males and eight females) received the vehicle.

Newborns were weighed and clinically examined at birth and daily until the last day of the experiment by one of the team´s veterinarians. Lambs were euthanized at 5 days of age using an overdose of sodium thiopentone 100 mg kg^{-1} intravenously (Tiopental; Laboratorio Biosano, Santiago, Chile). Euthanasia was performed at 08:00 (ZT1) and 14:00 (ZT7) in two groups of four controls and four melatonin-treated newborns and at 20:00 (ZT13) in six controls and four melatonin-treated newborns.

A blood sample was taken by jugular puncture and plasma was separated and stored at –20°C to measure plasma hormones and metabolites. Heart, adrenal glands, and remaining organs were sterilely dissected and weighed. Free wall pieces of the right and left atrium, ventricles, and adrenal glands were stored in TRIzol at 4°C to measure messenger RNA (mRNA) expression. Remaining tissues were collected for other experiments.

Assays

Plasma assays

Melatonin concentration in plasma was measured using melatonin antiserum (AB/S/02, Stockgrand Ltd., Guildford Surrey, UK) and [O-methyl-3H] melatonin (TRK798, Buckinghamshire, UK) as a tracer, following the manufacturer's recommendations. Inter- and intra-assay coefficients were <15%. Glycerol was measured by the glycerol oxidase method as previously reported (32). Glucose and triglycerides were measured using kits (Valtek Diagnostics, Santiago, Chile) following the manufacturer's recommendations. Plasma cortisol, ACTH, triiodothyronine (T3), and thyroxine were measured by radioimmunoassay and plasma epinephrine, norepinephrine, and dopamine were measured by high-performance liquid chromatography in a commercial laboratory (Barnafi Krause Diagnóstica, Santiago, Chile).

mRNA extraction and quantitative reverse transcription polymerase chain reaction

Heart samples (about 100 mg) were homogenized in TRIzol. The RNA fraction was subjected to DNase treatment using the SV Total RNA Isolation System (PROMEGA, Madison, WI). RNA obtained was resuspended in nuclease-free water and the absorbance was measured at 260 and 280 nm. The ratio of 260 to 280 was 1.9 to 2.05. The RNA was stored at −20°C. RNA reverse transcription was performed from approximately 2 μg of RNA with 100 ng of random primers and 200 U M-MLV RT (200 U/μL) in a final volume of 20 μL. Gene expression was measured by quantitative reverse transcription polymerase chain reaction (PCR). Primers reported in the literature were used to measure *Pgc-1α, StAR, Pparγ, Pparα, Per1,* and *Per2* (33); *Bmal1, Clock,* and *Cry2* (34); *Anp* and *Bnp* (35); *glucocorticoid receptor* (*Nr3c1*) and *Gapdh* (36); *Rlp0* (37); *Cd36* and *hFABP* (38); *Glut1(Slc2a1), Hk* (39), and *Mc2r* (forward: 5-TCC-TTC-TGG-CTG-TGG-CCA-AGA-ATA-3; reverse: 5-AGG-CTG-CCC-AGC-ATA-TCG-GAA-ATA-3). Reverse transcription PCR conditions were 65°C for 5 minutes, 4°C for 5 minutes, 37°C for 2 minutes, 25°C for 10 minutes, 37°C for 50 minutes, and 70°C for 15 minutes. Assays were performed in a StepOne thermal cycler from Applied Biosystems, CA, USA, Fast SYBER Green Master Mix (Applied Biosystems, Carlsbad, CA) or 5x Hot FIREPol Eva Green HRM Mix (Solis BioDyne, Riia, Tartu, Estonia) following the manufacturer's instructions. The quantification of samples was performed using a standard curve constructed with serial dilutions of known quantities of PCR products for each gene. These products were prepared by conventional PCR, purified, quantified by spectrophotometry, and stored in aliquots at −20°C. Nontemplate controls were included in every PCR reaction and three complementary DNA pools were included to assess inter-assay variability. The threshold cycle of each sample and the internal control was interpolated in the respective standard curve. *RLP0, 18S rRNA, Gapdh*, and *β-actin* were tested as housekeeping genes, but were found to change with clock time or treatment precluding their use in this regard. *Anp* and *Bnp* expression was much higher in the atrium than in ventricles; thus, data for these genes were expressed only for atrium. For the remaining genes, data for the four heart chambers were combined. Gene expression was measured as femtogram per nanogram RNA.

Data analysis

Data are expressed as means ± standard error of the mean. Lamb body weight and adrenal gland and heart weight at 5 days of age were compared by Student *t* test. Clock time–related changes in plasma variables and gene expression within each group, were analyzed by one-way analysis of variance (ANOVA), using Newman-Keuls as a *post hoc* test. The effects of melatonin treatment in plasma variables and gene expression were analyzed by two-way ANOVA and the *post hoc* Bonferroni test. Statistical analyses were performed using GraphPad Prism 6.00 (GraphPad Software Inc.). Differences were considered significant when $P < 0.05$.

Results

Melatonin treatment did not affect newborn clinical health. As depicted in Fig. 1, control newborns display low, steady melatonin values, reaching about 20 pg/mL at the three clock times studied. In contrast, newborns receiving daily doses of melatonin at 17:00 had higher plasma concentrations of this hormone than control newborns at all clock times tested. Plasma melatonin daytime values were twofold of those of control newborns, whereas at nighttime, values were much higher than of those of control newborns that did not have nighttime increase in melatonin. Taken as a whole, daily melatonin administration resulted in rhythmic changes in plasma concentrations with the highest values (about 800 pg/mL) at 20:00 (Fig. 1). As shown in Table 1, newborn weight was similar at birth and at 5 days of age. However, absolute heart weight and change to ratio between heart weight/body weight (HW/BW) was lower in melatonin-treated newborns than in control newborns (Table 1). There were no effects of melatonin on adrenal weight or the weight of other organs.

In control newborns, plasma concentrations of cortisol, ACTH, T3, norepinephrine, epinephrine, glycerol, and triglycerides showed clock time changes (Fig. 2). Cortisol and ACTH values were higher at 08:00 than at 20:00. A different pattern was observed for T3, norepinephrine, glycerol, and triglycerides, with the highest values were observed at 20:00 and at 14:00 for epinephrine. No significant clock time changes were observed in

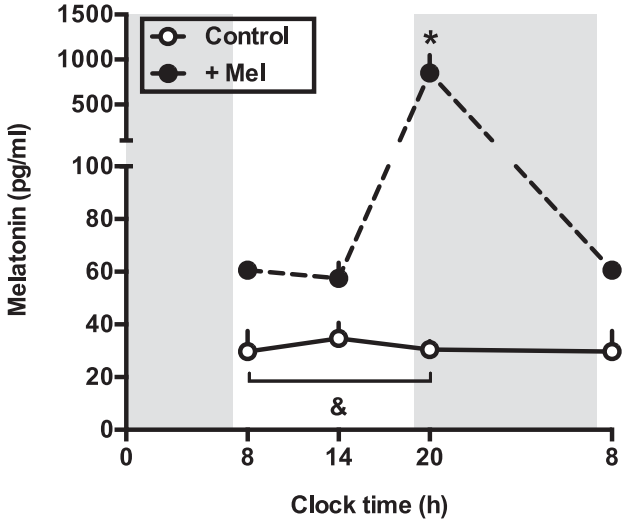

Figure 1. Plasma levels of melatonin (Mel) in control (○) and melatonin-treated newborns (●). Data are means ± standard error of the mean. Lambs were given 1 mg oral melatonin (0.24-0.2 mg/kg) daily at 17:00, starting the day of birth. Control lambs received vehicle. Blood samples were taken at euthanasia at 5 days of age, at the hours indicated in the graph. Values at 08:00 (ZT1) are plotted twice. *Different from 08:00 and 14:00, $P < 0.05$ one-way ANOVA and Newman-Keuls. [&]Different from melatonin treatment, $P < 0.05$, two-way ANOVA and Bonferroni multiple comparisons test (Supplemental Table 1).

plasma dopamine and glucose. Imposing a melatonin rhythm resulted in a different pattern of clock time changes than in control newborns. Clock time–related changes of cortisol, ACTH, T3, norepinephrine, and epinephrine, were no longer detected, whereas glycerol and triglycerides patterns were maintained. Clock time changes were observed for glucose with higher values at 14:00. The concentration of norepinephrine was lower than in control newborns at 20:00, whereas the converse was true for triglycerides. A different effect was observed for norepinephrine with lower values at 20:00 (Fig. 2).

The adrenal glands of control newborns showed clock time–related changes in expression of clock genes and of the functional genes measured (Fig. 3). The clock genes *Per1*, *Per2*, *Cry2*, and *Clock* showed highest values at 08:00 than at 20:00, whereas *Bmal1* had a maximum value at 14:00. Clock time–related changes were also detected for mRNA expression of the steroidogenic enzyme *StAR*, ACTH receptor *Mc2r*, glucocorticoid receptor (*Nr3c1*), and coactivator *Pgc1-α* and *Rlp0*. Expression of these genes was higher at 08:00 than at 20:00, excepting *RLP0*.

Melatonin treatment altered the clock time–related pattern of gene expression in the newborn adrenal gland and reduced the level of expression of most of the genes measured. Clock time–related changes in adrenal expression of *Per1*, *Per2*, *Cry 2*, *Clock*, *StAR*, *Pgc1-α*, *Nr3c1*, and *Rlp0* were not detected in the adrenal of melatonin-treated newborns (Fig. 3). Values of these genes were lower than in adrenals of control newborns at 08:00 and for some at 14:00. Melatonin also affected *Bmal1* expression differently than in other genes, resulting in an apparent clock time–related change with higher values at 20:00 and lower expression at 14:00 (Fig. 3D).

As observed in the adrenal gland, the heart of control newborns showed clock time–related changes in gene expression of clock genes (Fig. 4) and of the other functional genes measured (Figs. 5 and 6). The clock genes *Per1* and *Per2* showed higher expression at 08:00 than at 20:00, whereas the converse was true for *Bmal1* and *Clock*, which were maximally expressed at 20:00. No clock time–related changes were observed for *Cry2* (Fig. 4C). As shown in Fig. 5, clock time–related changes were also detected for transcription factors *Nr3c1*, *Pparα*, *Pparγ*, the coactivator *Pgc1-α* as well as for the intracellular lipid transporter *H-fabp*, glucose transporter *Scl2a1*, and the gene coding for the enzymes *Hk1* and *GAPDH*. These genes were highly expressed at 08:00 than at other clock times. In contrast, no clock time–related changes were detected for the lipid transporter *Cd36* (Fig. 5E).

In marked contrast with the adrenal gland, the overall effect of melatonin on the heart was enhanced expression of some genes at 14:00 or 20:00, clock times in the downward slope of clock time–related changes in control newborns. As depicted in Fig. 4A and 4B, such was the case of *Per1* and *Per2* that, although maintaining the clock time–related changes observed in the heart of control newborns, showed higher expression at 14:00 or 20:00, than in control newborns. *Cry2* values at 14:00 were increased (Fig. 4C). Melatonin neither affected the pattern nor the levels of *Bmal1*, but increased *Clock* levels at 20:00 over the amount found in control newborns. As a result of these increases in expression, *Cry2* and

Table 1. Lamb Body Weight and Adrenal and Heart Weight at 5 Days of Age

Treatment	Control (n = 14)	Melatonin (n = 12)
Body weight at birth (kg)	4.5 ± 0.18	4.3 ± 0.24
Body weight at 5 days of age (kg)	6.0 ± 0.2	5.9 ± 0.3
Adrenal weight at 5 days of age (mg)	1048.0 ± 61.4	1112.0 ± 120.3
Adrenal weight/body weight (mg/kg)	0.18 ± 0.011	0.19 ± 0016
Heart weight at 5 days of age (g)	52.7 ± 2.1	43.0 ± 3.0[a]
Heart weight/body weight (g/kg)	8.7 ± 0.2	7.3 ± 0.4[a]

Lambs were treated daily with an oral dose of 1 mg of melatonin or vehicle at 17:00 from the day of birth. Values are presented as means ± standard error of the mean.

[a] $P < 0.05$ vs control (Student *t* test).

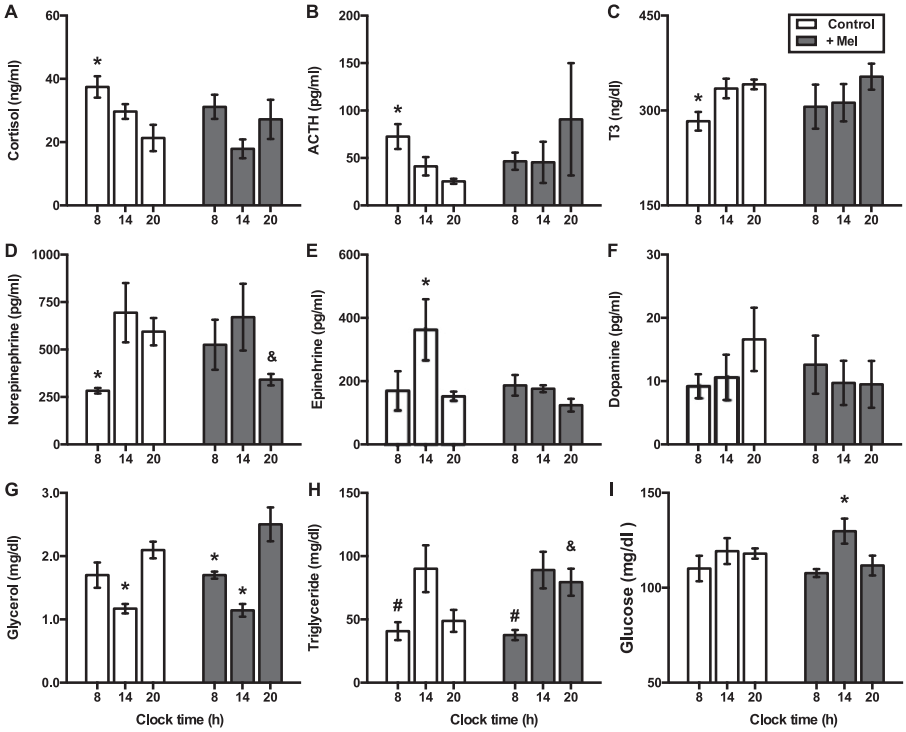

Figure 2. Clock time changes (means ± standard error of the mean) of plasma concentration of hormones and metabolic markers in control (open bars) and melatonin-treated newborns (filled bars) at 5 days of age. (A) Cortisol; (B) ACTH; (C) T3; (D) norepinephrine; (E) epinephrine; (F) dopamine; (G) glycerol; (H) triglycerides; and (I) glucose. *Different from 20:00, $P < 0.05$, one-way ANOVA and Newman-Keuls. $^{\&}$Different from control, $P < 0.05$, two-way ANOVA and Bonferroni multiple comparisons test (Supplemental Table 1). $^{\#}P = 0.06$ vs other clock time, one-way ANOVA and Newman-Keuls.

Clock showed maximal expression at 14:00 in the heart of melatonin-treated newborns. An increase in *RLP0* at 20:00 (Fig. 4F) without changing the pattern was also observed. A trend to higher values in the heart of melatonin-treated newborns than those in the heart of control newborns at several clock times was also observed for some of the transcription factors and metabolic genes depicted in Fig. 5. *Nr3c1*, *Hk1*, and *Gapdh* values were higher than those of control newborns at 20:00, *PPARα* at 14:00 and 20:00, and *Cd36* at 14:00, whereas *Pgc1α* and *Pparγ* had higher values than control newborns at the three clock times tested. *Slc2a1* expression was not modified by melatonin treatment (Fig. 5G). As a result, melatonin treatment apparently induced a shift in clock time related pattern *Nr3c1*, induced a clock time related pattern *of Pparγ* and *CD 36* and maintained high constant levels of *Pparα*, and *Hk1* (Fig. 5C and 5E and Fig. 5D and 5H, respectively.).

A marked effect of melatonin treatment was also observed over heart *Anp* and *Bnp* (Fig. 6A and 6B). In control newborns, only *Bnp* showed a clock time–related change. However, melatonin treatment induced a marked increase in *Anp* at 14:00 and 20:00 to values several fold higher than in control newborns (Fig. 6A). An increase was also observed for *Bnp* (Fig. 6B), with higher values than control newborns at 20:00.

Discussion

In the current study, we postulated that the transition from the diurnal melatonin rhythm experienced in fetal life to low constant postnatal levels, plays a role in early adrenal gland and heart neonatal adaptation. We tested this hypothesis in the lamb by imposing a rhythmic pattern of high melatonin concentrations from birth to 5 days of age. Newborn lambs, as with other newborns, have low constant plasma melatonin levels for several weeks (1–3). Daily oral melatonin administration (0.24–0.2 mg/kg) at 17:00 mimicked a high-amplitude melatonin rhythm. High plasma melatonin concentrations were detected at 20:00 but subsided to constant values about twice that found in control newborns at 14:00 and 20:00. This exogenous melatonin rhythm altered clock time changes in plasma endocrine and metabolic levels, affected adrenal and heart circadian clocks genes, and induced genomic changes, suggesting altered function of these organs. Further, the heart weight and the ratio between heart weight/body were lower in melatonin-treated newborns than in control newborns. In the current study, we cannot discriminate whether the effects observed resulted from the twofold increase of melatonin concentrations during daytime or sharp day-night changes imposed by the melatonin treatment.

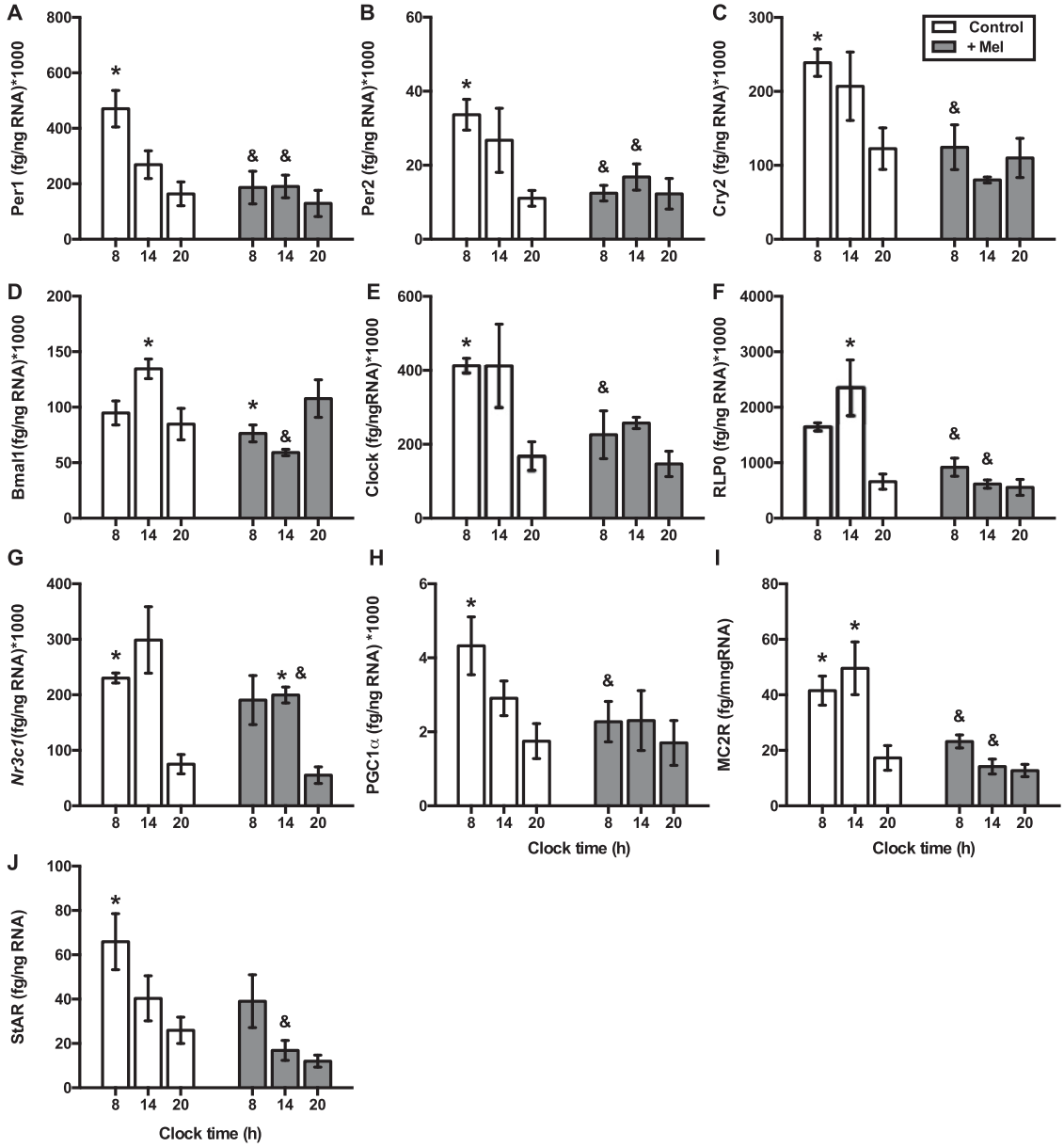

Figure 3. Clock time changes (means ± standard error of the mean) of clock gene and functional gene expression in the adrenal gland of control (open bars) and melatonin-treated newborns (filled bars) at 5 days of age. (A) *Per1*; (B) *Per2*; (C) *Cry2*; (D) *Bmal1*; (E) *Clock*; (F) *RLP0*; (G) glucocorticoid receptor *Nr3c1*; (H) *PGC1α*; (I) ACTH receptor *MC2R*; and (J) the enzyme StAR. *Different from 20:00, $P < 0.05$, one-way ANOVA and Newman-Keuls. [&]Different from control at the same clock time, $P < 0.05$, two-way ANOVA and Bonferroni multiple comparisons test (Supplemental Table 1).

Nevertheless, our data demonstrates that postnatal melatonin administration, mimicking a high-amplitude melatonin rhythm, at an age at which a melatonin plasma circadian rhythm is not present, interfered with postnatal evolution of the adrenal and heart, supporting our hypothesis.

Control newborns showed clock time–related changes in plasma cortisol, consistent with the reported presence of a circadian cortisol rhythm in newborn lambs (40). In addition, ACTH, epinephrine, norepinephrine, and T3 and metabolic variables such as glycerol and triglycerides also showed clock time–related changes, whereas glucose levels were constant. In our experimental design, each newborn contributes with a single clock time point; thus, the detection of clock time–related changes seen in control newborns implies that individual newborns are synchronized to a time signal (ZT). Imposition of an exogenous melatonin rhythm affected plasma endocrine and metabolic variables. Clock time–related changes of cortisol, ACTH, epinephrine, and T3 were not observed. Instead, values were higher or lower than in control newborns at some clock times, as was the case for norepinephrine, glucose, and triglycerides. However, we cannot distinguish whether effects of imposing an exogenous

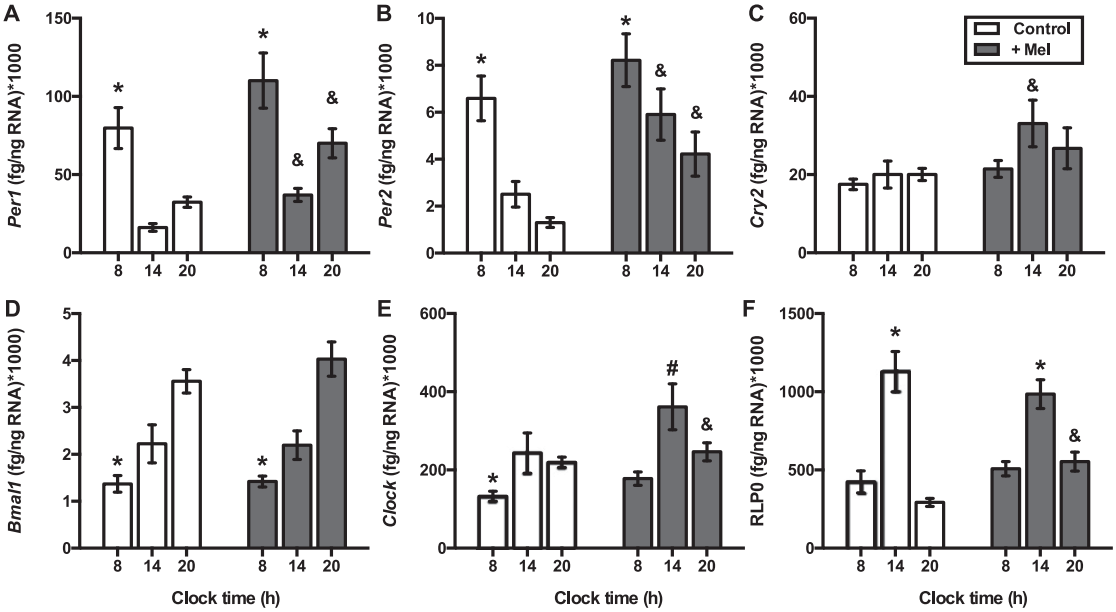

Figure 4. Clock time changes (means ± standard error of the mean) of clock gene expression in the heart of control (open bars) and melatonin-treated newborns (filled bars) at 5 days of age. (A) *Per1*; (B) *Per2*; (C) *Cry2*; (D) *Bmal1*; (E) *Clock*; and (F) *RLP0*. *Different from 20:00, $P < 0.05$, one-way ANOVA and Newman-Keuls. #Different from 08:00 one-way ANOVA and Newman-Keuls. &Different from control at the same clock time, $P < 0.05$, two-way ANOVA and Bonferroni multiple comparisons test (Supplemental Table 1).

melatonin rhythm are due to desynchronization or whether plasma changes represent other effects of melatonin. For example, there is evidence that in rats intracerebral ventricular infusion of melatonin decreases plasma ACTH (41). Moreover, the reduction in plasma norepinephrine concentration found at 20:00 in melatonin-treated newborns may be an acute response, as reported in the adult human and rats and fetal sheep (33, 42–44). Direct effects of melatonin on the adrenal gland through a MT1 receptor have been demonstrated by *in vitro* studies in fetal sheep (32), human (45), nonhuman primates (31), and the rat (16), altering cortisol production in response to ACTH. Likewise, effects of melatonin on thyroid hormones and metabolic variables, such as triglyceride and glucose, have been reported (46). Melatonin is a pleiotropic hormone that, in the fetal and adult physiological context in which a melatonin circadian rhythm is present, acts on endocrine, metabolic, cardiovascular, and circadian systems through membrane receptors or as a scavenger of free radicals, contributing to homeostasis (4). The situation is different in the early newborn context, in which plasma melatonin is low and a nighttime increase is absent. The alterations in plasma levels of endocrine and metabolic variables induced by submitting newborns to a high-amplitude melatonin rhythm, suggest wide effects on several organ systems in the newborn. To address this question, we studied gene expression in the newborn adrenal gland and heart. The lamb adrenal gland was markedly affected in melatonin-treated newborns. Control newborns showed marked clock time–related changes of clock gene expression as well as of genes related to adrenal steroidogenic function. This concurs with reports on the adrenal gland being a peripheral circadian clock in fetuses and adults of other species (12, 16, 17, 31). In view of the fact that the adrenal gland expresses a functional melatonin receptor and the compelling evidence about the role of melatonin in this organ, which includes effects on clock genes expression and steroidogenic enzymes (12, 16, 17, 31), we expected adrenal effects when imposing a high-amplitude melatonin rhythm on lamb newborns (13, 16, 32). Indeed, clock time–related changes in adrenal clock genes *Per1*, *Per2*, *Cry2*, and *Clock* were suppressed by the imposed melatonin rhythm. In contrast, *Bmal1* maintains clock time–related changes, but the higher values occur at different clock times than control newborns, suggesting a shift in the circadian rhythm of this gene, as has been reported in the rat fetal adrenal gland (16). In keeping with a disturbed adrenocortical function, expression of the ACTH receptor (*Mc2r)* was decreased, as was that of *StAR*, enzyme encoding for the first step in steroidogenesis, and cholesterol entrance in the mitochondria. Moreover, the expression of the cofactor *Pgc1-α*, involved on adrenal steroidogenesis (47), was also decreased by melatonin and the expression of the glucocorticoid receptor, involved in intra-adrenal negative feedback on cortisol production (48). Adrenal cortisol production and cortisol clearance determine plasma cortisol levels; however, plasma cortisol was maintained in melatonin-treated newborns. From our experimental design, we cannot elucidate if cortisol production by the adrenal gland was indeed changed or if cortisol clearance was affected, because melatonin receptors are present in the kidney (31, 49). Regardless of the mechanism, our data suggest that imposing a melatonin

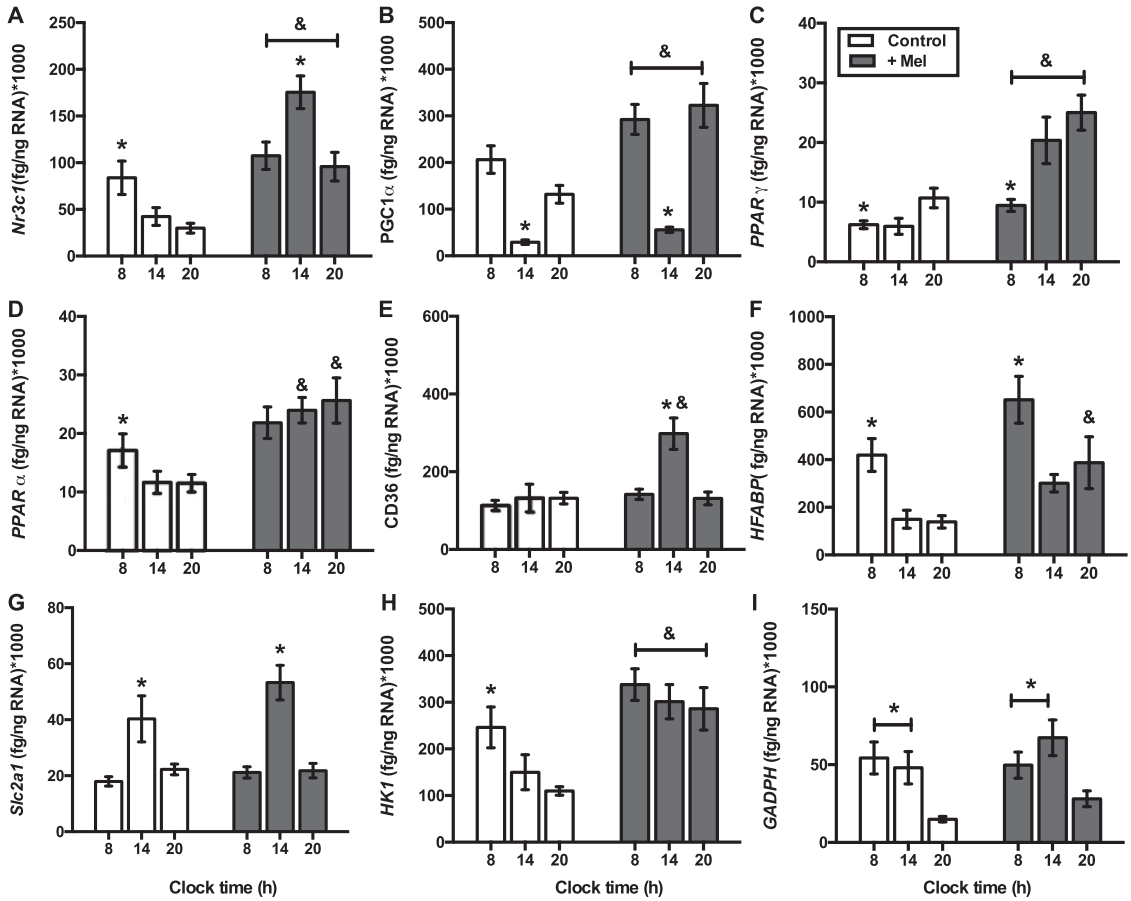

Figure 5. Clock time changes (means ± standard error of the mean) of gene expression in the heart of control (open bars) and melatonin-treated newborns (filled bars) at 5 days of age. (A) Glucocorticoid receptor *Nr3c1*; (B) *PGC1α*; (C) *PPARγ*; (D) *PPARα*; (E) lipid transporter *CD36*; (F) intracellular lipid transporter *HFABP*; (G) glucose transporter *Slc2a1*; (H) hexokinase *HK1*; and (I) glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). *Different from 20:00, $P < 0.05$, one-way ANOVA and Newman-Keuls. &Different from control, $P < 0.05$, two-way ANOVA and Bonferroni multiple comparisons test (Supplemental Table 1).

rhythm in the postnatal period causes a marked disarray of adrenocortical function, potentially contributing to altering clock time–related changes in plasma cortisol. Because circulating glucocorticoids act in all organs of the body, a disarrangement of clock time–related changes plasma glucocorticoid, may have a marked impact on other physiological functions, such as that of the heart, a known target of glucocorticoid (50), as we will discuss in the following section.

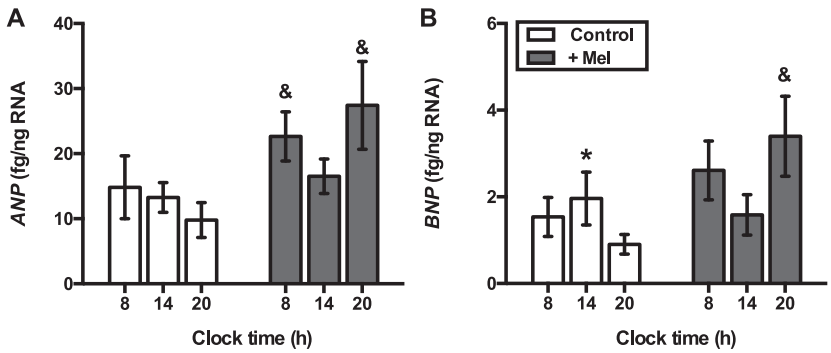

Figure 6. Clock time changes (means ± standard error of the mean) of gene expression of natriuretic peptides in the atrium of control (open bars) and melatonin-treated newborns (filled bars) at 5 days of age. (A) Atrial natriuretic peptide (*ANP*) and (B) brain natriuretic peptide (*BNP*). *Different from other clock times, $P < 0.05$, one-way ANOVA and Newman-Keuls. &Different from control at the same clock time, $P < 0.05$, two-way ANOVA and Bonferroni multiple comparisons test (Supplemental Table 1).

In the control lamb heart, canonical clock genes and other genes tested show clock time–related changes as demonstrated in adult rat heart (51). Consistent with the observation in the adult rat indicating that 10% of the heart transcriptome oscillates (51), we found clock time–related changes in the mRNA of transcription factors (glucocorticoid receptor, *Pparα*, *Pparγ*, and the coactivator *Pgc1a*), enzymes (*Hk1* and *Gapdh*), intracellular lipid transporter (*Hfabp*), and *Bnp* in the lamb heart. *Glut1* (Slc2a1), *Cd36*, and *Anp* did not show diurnal changes in the heart of control newborns. As for plasma cortisol and ACTH, and the

deranged adrenal gene expression discussed previously, imposing a high-amplitude melatonin rhythm had effects upon lamb heart. These newborns were purposely non-instrumented; therefore, cardiovascular function was not studied. Nevertheless, we found indications of disturbed heart function. The heart of newborns exposed to higher melatonin during the night showed decreased heart weight/body weight ratio and increased heart expression of the natriuretic peptides *Anp* and *Bnp*. In addition, other genes were increased, demonstrating an impact of a high-amplitude melatonin rhythm on the neonatal lamb heart. This or the abnormal physiological environment created by it changed expression of genes linked to several heart functions. For example, in our experiments, the rhythmic exposure to melatonin enhanced heart expression of the circadian clock genes *Per1, Per2*, *Clock*, and *Cry2*, which other studies have linked to heart fatty acid and carbohydrate metabolism (52, 53). In keeping with metabolic heart alterations, as a consequence of an overimposing of melatonin rhythm, expression of glucocorticoid receptor *Nr3c1*, *Pparα, Pparγ*, and *Pgc1-α*, the transporter *Cd36*, and the enzymes *Hk1* and *Gapdh* was increased. *Pparα* and *Pgc1-α* participate in the normal heart transition from carbohydrate to lipid as metabolic fuel by increasing mitochondria number and lipid utilization (28, 29). This could be favored further by increased expression of *Cd36*, augmenting lipid uptake. *Pparγ* may also play a role in this regard because one effect of heart *Pparγ* overexpression is increased CD36. Of note, heart *Pparγ* overexpression also leads to enhanced apoptosis (54). Finally, *HK1* and *Gapdh*, besides being involved in carbohydrate metabolism, have moonlighting functions, one of them in the case of *Gapdh* being apoptosis (55).

Melatonin treatment, besides inducing the changes in expression of the genes discussed previously, increased heart expression of *Anp, Bnp*, and glucocorticoid receptor and decreased the HW/BW ratio. The combination of increased *Anp* and *Bnp* expression and decreased HW/BW ratio is surprising. To date, this combination has been reported in an experimental preparation in which the heart is unloaded by substantially reducing the preload and afterload (56–58). Following this line of thought, the decrease in HW/BW induced by high-amplitude melatonin rhythm may reflect slight decreases in pulmonary and systemic vascular resistance, eliciting less cardiac work and therefore less growth stimuli because adult heart and vasculatures express melatonin receptor (59), and we have preliminary evidence of Mt1 and Mt2 expression in lamb heart (not shown). Newborns lambs subjected to high-altitude chronic hypoxia given daily melatonin in the evening from 3 to 12 days of age, show a decreased pulmonary artery resistance for the first 3 days of treatment, although no systemic cardiovascular changes were found *in vivo*. Furthermore, these effects are accompanied by an important reduction of oxidative stress (60). Additionally, the increased glucocorticoid receptor expression in the heart may affect a wide variety of functions. For instance, in neonatal rat cardiomyocytes *in vitro*, dexamethasone, acting through the glucocorticoid receptor, augmented the expression of genes involved in lipolysis, antilipogenic, and apoptosis (61). In the current study, some of the effects of imposing a high-amplitude melatonin rhythm could relate to glucocorticoid action, either by increasing glucocorticoid-regulated genes (*Pgc-1α, Per1, Per2*, and *Anp*) (62–66) or genes that modulate the activity of the glucocorticoid receptor, by enhancing *Pparγ* (67) or decreasing it (*Clock* and *Cry*) (67, 68). These observations are of concern because treatment of rat newborns with dexamethasone results in a dysfunctional hypertrophic heart in the adult (69–71). Altogether, the changes at 5 days of age in the heart of melatonin-treated newborns point to alterations in normal remodeling processes occurring in the newborn heart. These alterations are possibly mediated by the combined direct effects of an unusual increase of melatonin on the heart and vasculature, plus the known melatonin effects on sympathetic tone, melatonin antioxidant effects, and last but not least, the abnormal pattern of glucocorticoid on the circulation induced by our treatment. It is well-accepted that the circadian melatonin rhythm is important in organ and system homeostasis; however, the natural history of melatonin in several species includes an age window, neonate, at which a melatonin circadian rhythm is absent. The present findings demonstrate that imposing a high-amplitude melatonin rhythm in early neonatal period in the lamb results in marked changes in heart and adrenal, both central for fetal-to-neonatal transition. The treatment with melatonin in the newborn also may affect directly or indirectly other organs not studied by us, potentially altering the phenotype later in life. Thus, our studies suggest a word of caution in the therapeutic use of melatonin in the neonatal period.

Acknowledgments

We are grateful to Juan Carlos Fuenzalida for help with the experiments and Monica Prizant for editorial help.

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This work was supported by Fondecyt Grants 1090-381 (to M.S.-F.) and 1140647 (to A.J.L.), ANILLO ACT-1116 (to C.T.-F. and M.S.-F.), DID Universidad Austral de Chile (to C.T.-F.), and the Women's Health Department, Arrowhead Regional Medical Center, Colton, CA (M.S.-F.).

Author Contributions: M.S.-F., C.T.-F., G.J.V., and A.J.L. conceived and designed the research; N.M., F.J.V., A.R., H.R., and S.C.-G. performed and analyzed the experiments; and M.S.-F., C.T.-F., G.J.V., and A.J.L. interpreted results of experiments and prepared the figures and manuscript.

Disclosure Summary: The authors have nothing to disclose.

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