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Melatonin Supplementation Decreases Prolactin Synthesis and Release in Rat Adenohypophysis: Correlation With Anterior Pituitary Redox State and Circadian Clock Mechanisms

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In the laboratory rat, a number of physiological parameters display seasonal changes even under constant conditions of temperature, lighting, and food availability. Since there is evidence that prolactin (PRL) is, among the endocrine signals, a major mediator of seasonal adaptations, the authors aimed to examine whether melatonin administration in drinking water resembling in length the exposure to a winter photoperiod could affect accordingly the 24-h pattern of PRL synthesis and release and some of their anterior pituitary redox state and circadian clock modulatory mechanisms. Melatonin (3 μg/mL drinking water) or vehicle was given for 1 mo, and rats were euthanized at six time intervals during a 24-h cycle. High concentrations of melatonin (>2000 pg/mL) were detected in *melatonin-treated rats* from beginning of scotophase (at 21:00 h) to early photophase (at 09:00 h) as compared with a considerably narrower high-melatonin phase observed in controls. By cosinor analysis, *melatonin-treated rats* had significantly decreased MESOR (24-h time-series average) values of anterior pituitary PRL gene expression and circulating PRL, with acrophases (peak time) located in the middle of the scotophase, as in the control group. Melatonin treatment disrupted the 24-h pattern of anterior pituitary gene expression of nitric oxide synthase (NOS)-1 and -2, heme oxygenase-1 and -2, glutathione peroxidase, glutathione reductase, Cu/Zn- and Mn-superoxide dismutase, and catalase by shifting their acrophases to early/middle scotophase or amplifying the maxima. Only the inhibitory effect of melatonin on pituitary NOS-2 gene expression correlated temporally with inhibition of PRL production. Gene expression of metallothionein-1 and -3 showed maxima at early/middle photophase after melatonin treatment. The 24-h pattern of anterior pituitary lipid peroxidation did not vary after treatment. In *vehicle-treated rats*, *Clock* and *Bmal1* expression peaked in the anterior pituitary at middle scotophase, whereas that of *Per1* and *Per2* and of *Cry1* and *Cry2* peaked at the middle and late photophase, respectively. Treatment with melatonin raised mean expression of anterior pituitary *Per2*, *Cry1*, and *Cry2*. In the case of *Per1*, decreased MESOR was observed, although the single significant difference found between the experimental groups when analyzed at individual time intervals was increase at early scotophase in the anterior pituitary of *melatonin-treated rats*. Melatonin significantly phase-delayed expression of *Per1*, *Per2*, and *Cry1*, also phase-delayed the plasma corticosterone circadian rhythm, and increased the amplitude of plasma corticosterone and thyrotropin rhythms. The results indicate that under prolonged duration of a daily melatonin signal, rat anterior pituitary PRL synthesis and release are depressed, together with significant changes in the redox and circadian mechanisms controlling them. (Author correspondence: danielcardinali@uca.edu.ar; danielcardinali@fibertel.com.ar)

Keywords: Free radicals, Corticosterone, LH, Metallothioneins, Seasonality, Testosterone, TSH

INTRODUCTION

Animals inhabiting temperate areas where there are drastic changes in the environment show dramatic adaptative seasonal changes in physiology. One example is reproduction, occurring within limited time periods in seasons to ensure young are born and raised in optimal environmental conditions for survival (Chemineau et al., 2008; Lehman et al., 2010; Scherbarth & Steinlechner, 2010; Yoshimura, 2010). The role of melatonin in regulating this seasonal variation in animal physiology

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and behavior is supported by considerable scientific evidence (Reiter, 1980; Reiter et al., 2009). Experiments on long-day breeders such as the Syrian hamster, in which a prolonged melatonin phase induces gonadal regression (Bartness et al., 1993), and on short-day breeders such as the sheep, in which a prolonged melatonin signal elicits gonadal activity (Bittman & Karsch, 1984), indicate that melatonin is the primary neuroendocrine timing signal encoding information on length of the night.

When domesticated, the lack of a well-defined breeding season arises presumably because selective pressures for seasonal breeding have been minimized. However, data have accumulated indicating that seasons continue to influence several neuroendocrine parameters in domesticated species, even under constant conditions of temperature, lighting, and food availability. This is the case for the laboratory rat albino *Rattus norvegicus* (Cohen & Mann, 1979; Mock & Frankel, 1978). When housed under a controlled environment, rats display seasonal variation in reproductive capability (Lee & McClintock, 1986), pituitary hormones and sex steroids, and sexual maturation (Díaz et al., 2012; Shishkina et al., 1993; Utembaeva et al., 2009; Wallen & Turek, 1981; Wong et al., 1983). Several other physiological parameters exhibit circannual rhythmicity in the rat, among them hypothalamic content of corticotrophin-releasing hormone (CRH), thyrotropin-releasing hormone (TRH), neurotensin, and neuromedin (Bissette et al., 1995); plasma levels of thyrotropin (TSH) and triiodothyronine (T3) (Wong et al., 1983; corticosteroid-binding globulin levels (Tinnikov & Oskina, 1994); pineal somatostatin content and somatostatin and somatostatin receptor gene expression (Mato et al., 1997); pineal phosphodiesterase (Spiwoks-Becker et al., 2011); mammary carcinogenesis (Kubatka et al., 2002); reserpine pseudopregnancy (De Scremin & Scremin, 1972); endocrine sequels of pituitary grafts (Esquifino et al., 1999); striatal glutamate, aspartate (Parrot et al., 2001), and substance P content (Díaz et al., 2011); glial fibrillary acidic protein in suprachiasmatic nucleus (SCN) (Gerics et al., 2006); resistance to hypoxia (Khachatur'yan & Panchenko, 2002); stress-induced systolic hypertension (Weinstock et al., 1985); endothelial free-radical production (Konior et al., 2011); membrane properties of heart mitochondria (Mujkosova et al., 2008); and plasma polyunsaturated fatty acid and lipoprotein cholesterol levels (Masumura et al., 1992).

Among the endocrine signals, there is ample evidence that prolactin (PRL) is a major mediator of seasonal adaptations (Duncan, 2007; Steger & Bartke, 1995). Many mammals that exhibit photoperiodically induced seasonal changes in reproduction also exhibit photoperiodically induced seasonal changes in circulating levels of PRL, which not only regulate lactation, but also modulate gonadal activity and seasonal molts. It is likely, therefore, that the seasonal pattern of PRL secretion participates in neuroendocrine sensitivity to changing photoperiod as well as in seasonally related changes in animals kept under regular light (L):dark (D) cycles (Lincoln et al., 2003).

Since administration of melatonin in drinking water is an equivalent to exposure of animals to short daily photoperiod in terms of prolonged duration of the melatonin signal (Bartness et al., 1993; Bittman & Karsch, 1984), a goal of this study was to investigate in the laboratory rat some circadian clock–related mechanisms that may modulate PRL synthesis and release from the adenohypophysis under melatonin supplementation. Because redox enzymes are involved in PRL secretory mechanisms (Quinteros et al., 2007; Velárdez et al., 2000), we wished to correlate changes in 24-h PRL synthesis and secretion with pituitary lipid peroxidation, gene expression of enzymes, and proteins related to redox balance and circadian clock mechanisms. Specifically, our aims were to (i) examine the effect of melatonin administration in the drinking water to rats for 1 mo on the correlation of the 24-h patterns of PRL gene expression and PRL release by the anterior pituitary; (ii) analyze the 24-h changes in PRL synthesis and release in correlation with the 24-h pattern of anterior pituitary lipid peroxidation and of redox enzyme, metallothionein (MT), and circadian clock gene expression; and (iii) correlate the 24-h changes in PRL synthesis and release with the daily pattern in circulating TSH, luteinizing hormone (LH), testosterone, and corticosterone levels. 24-Hour changes of these hormones were examined because of the reported seasonal variation in plasma levels of reproductive hormones (Esquifino et al., 1999; Wallen & Turek, 1981; Wong et al., 1983), TSH (Wong et al., 1983), and corticosteroid-binding globulin (Tinnikov & Oskina, 1994), as well as in hypothalamic content of CRH and TRH (Bissette et al., 1995).

MATERIALS AND METHODS

Animals and Experimental Design

Male Wistar rats (45 d of age) were kept under standard conditions of a controlled (12 L:12 D schedule; lights-on at 08:00 h) and temperature (22°C ± 2°C). Half of the animals received melatonin (3 μg/mL) in drinking water. The stock solution of melatonin was prepared in 50% ethanol, with final ethanol concentration in drinking water being .015%. Vehicle-treated controls received .015% ethanol in drinking water. Drinking water bottles were changed every other day. Since rats drank ~20 mL/d, with 90–95% of this total daily water taken up during the dark period, the melatonin dosage used provided ~60 μg melatonin/d. This dose is ~10-fold higher than that needed to maintain physiological circulating melatonin levels in pinealectomized rats (Cardinali et al., 2004). Nocturnal water consumption did not differ between melatonin- and vehicle-treated groups.

After 1 mo, groups of 6–8 rats were euthanized by decapitation under conditions of minimal stress at six different time intervals, every 4 h throughout a 24-h

cycle, starting at 09:00 h. At night, animals were killed under dim red light. Brains were rapidly removed, and the adenohypophysis free of the pituitary stalk was quickly dissected out from the sella turcica. Blood was collected from the cervical wound for plasma hormone assays. Samples were kept frozen at −70°C until assayed.

The experiment was performed between January and March, 2009 in Madrid (40°26′N, 3°42′W). The care and use, as well as all procedures involving animals, were approved by the Institutional Animal Care Committee, Faculty of Medicine, Complutense University, Madrid. The experimental protocol conformed to international ethical standards (Portaluppi et al., 2010).

Real-Time Quantitative Polymerase Chain Reaction (qPCR)

Total RNA extraction was performed using the RNeasy protect mini kit and was analyzed using QuantiTec SYBR green kit (Qiagen, Hielden, Germany). The iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Madrid, Spain) was used to synthesize cDNA from 1 μg of total RNA, according to the manufacturer's protocol. The housekeeping gene β-actin was used as a constitutive control for normalization. Reactions were carried out in the presence of 200 nM of specific primers for genes of nitric oxide (NO) synthase (NOS)-1 and -2, heme oxygenase (HO)-1 and -2, Cu/Zn and Mn superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), glutathione reductase (GSR), MT-1, and MT-3, and for the circadian genes *Clock*, *Bmal1, Per1*, *Per2, Cry1*, and *Cry2*. Primers were designed using Primer3 software (The Whitehead Institute, http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and are shown in Table 1.

PCR reactions were carried out in an Eppendorf RealPlex Mastercycler (Eppendorf, Hamburg, Germany). The real-time qPCR reaction program included a 94°C enzyme activation step for 2 min, followed by 40 cycles of 95°C denaturation for 15 s, 60°C annealing for 30 s, and 72°C extension for 30 s. Detection of fluorescent product was carried out at the end of the 72°C extension period.

Serial dilutions of cDNA from control adenohypophysis were used to perform calibration curves to determine amplification efficiencies. For the primers used, there were no differences between transcription efficiencies, the amount of initial cDNA in each sample being calculated by the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001). All samples were analyzed in triplicate and in three different measures. Fractional cycle at which the amount of amplified target becomes significant (C_t) was automatically calculated by the PCR device. To estimate whether treatment or time-of-day modified expression of anterior pituitary β-actin, PCR with serial dilutions of this housekeeping gene was performed. In this study, C_t did not vary significantly as a function of treatment or of time-of-day, indicating the validity to employ β-actin as the housekeeping gene.

Lipid Peroxidation

Lipid peroxidation was measured in the anterior pituitary by thiobarbituric acid–reactive substance (TBARS) assay as described elsewhere (Poliandri et al., 2006). Supernatant absorbance (535 nm) was measured. Results are expressed as (absorbance/mg of protein in treated sample)/(absorbance/mg of protein in control sample) × 100.

Hormone Assays

Plasma melatonin levels were measured by enzyme-linked immunosorbent assay (ELISA) (Immuno Biological Laboratories, Hamburg, Germany). The intra- and interassay coefficients of variation were 7–8%. Sensitivity of the assay was 10 pg/mL. Results are expressed as pg/mL. Plasma PRL, TSH, and LH levels were measured by a homologous specific double-antibody radioimmunoassay (RIA), using materials kindly supplied by the National Institute of Diabetes and Digestive and Kidney Diseases' (NIDDK's) National Hormone and Pituitary Program. Intra- and interassay coefficients of variations were 6–9%. Sensitivities of the RIAs were 45, 190, and 45 pg/mL for PRL, TSH, and LH—using the NIDDK rat PRL-RP-3, rat TSH-RP-3, and rat LH-RP-3, respectively. Results are expressed as ng/mL (PRL, TSH) or pg/mL (LH) (Castrillón et al., 2001; Garcia Bonacho et al., 2000). Plasma testosterone levels were measured using a commercial kit (ICN Pharmaceuticals, Costa Mesa, CA, USA). Sensitivity of the assay was .2 ng/mL, and intra-assay coefficient of variation was 5%, as previously described (Garcia Bonacho et al., 2000); results are expressed as ng/mL. Plasma corticosterone was assayed by a specific RIA obtained from Labor Diagnostika Nord (Nordhorn, Germany). Intra- and interassay coefficients of variation were 6% and 8%, respectively. Sensitivity of the RIA was 25 ng of corticosterone/mL; results are expressed as ng/mL.

Data Analysis

After verifying normality of data distribution, statistical analysis was performed by one-way or two-way factorial analysis of variance (ANOVA) followed by Bonferroni's multiple comparison tests or by Student's *t* tests, as stated. Cosinor analysis of the mean values at each time series (n = 6) was performed to calculate the acrophase (maximum of the cosine function fit to the experimental data with the period = 24 h) and amplitude (half the difference between maximal and minimal values of the approximated cosine curve) of the 24-h rhythms. Statistical significance of the derived cosine curves was tested against the null hypothesis, i.e., amplitude = 0 (Nelson et al., 1979). To calculate the MESOR (the statistical

TABLE 1. Sequence of the primers used for real-time PCR

Gene		Primers	Product size (bp)
β-Actin	Forward	ctctcttccagccttccttc	99
	Backward	ggtctttacggatgtcaacg	
PRL	Forward	ttcttggggaagtgtggtc	86
	Backward	tcatcagcaggaggagtgtc	
NOS-1	Forward	atcggcgtccgtgactactg	92
	Backward	tcctcatgtccaaatccatcttcttg	
NOS-2	Forward	tggcctccctctggaaaga	93
	Backward	ggtggtccatgatggtcacat	
HO-1	Forward	tgctcgcatgaacactctg	123
	Backward	tcctctgtcagcagtgcc	
HO-2	Forward	agcaaagttggccttaccaa	84
	Backward	gtttgtgctgccctcacttc	
Cu/Zn-SOD	Forward	ggtggtccacgagaaacaag	98
	Backward	caatcacaccacaagccaag	
Mn-SOD	Forward	aaggagcaaggtcgcttaca	94
	Backward	acacatcaatccccagcagt	
Catalase	Forward	gaatggctatggctcacaca	100
	Backward	caagtttttgatgccctggt	
GPx1	Forward	tgcaatcagttcggacatc	120
	Backward	cacctcgcacttctcaaaca	
GSR	Forward	atcaaggagaagcgggatg	96
	Backward	gcgtagccgtggatgactt	
MT-1	Forward	gttgctccagattcaccaga	105
	Backward	gcatttgcagttcttgcag	
MT-3	Forward	ctgctcggacaaatgcaaa	96
	Backward	ttggcacacttctcacatcc	
Clock	Forward	tgccagctcatgagaagatg	98
	Backward	catcgctggctgtgttaatg	
Bmal1	Forward	ccgtggaccaaggaagtaga	102
	Backward	ctgtgagctgtgggaaggtt	
Per1	Forward	ggctccggtacttctctttc	106
	Backward	aataggggagtggtcaaagg	
Per2	Forward	acacctcatgagccagacat	99
	Backward	ctttgactcttgccactggt	
Cry1	Forward	cagttgcctgtttcctgacc	91
	Backward	cagtcggcgtcaagcagt	
Cry2	Forward	attgagcggatgaagcagat	103
	Backward	ccacagggtgactgaggtct	

estimate of the 24-h time-series mean) and R^2 (statistical validity of the cosine fit to the data), the total number of individual values was considered. p values < .05 were taken as evidence for statistical significance of the amplitude being non-zero and thus rhythmicity.

RESULTS

As shown in Figure 1, peak melatonin level in plasma of *vehicle-treated rats* was 350 ± 79 pg/mL at 01:00 h, with photophase and early scotophase values <30 pg/mL. In the case of rats drinking the melatonin solution, concentrations attained at the three scotophase time intervals examined did not differ significantly (means ± SEM: 4310 ± 715, 4120 ± 798, and 5230 ± 699 pg/mL at 21:00, 01:00, and 05:00 h, respectively). In melatonin-treated animals, values >2000 pg/mL were detected from ~20:00 to 09:00 h (Figure 1). The acrophases derived from the cosinor analysis did not differ significantly between groups (Table 2).

Figure 2 depicts the effect of melatonin on 24-h pattern of expression of anterior pituitary PRL gene and plasma PRL levels. When analyzed as a main factor in a factorial ANOVA, melatonin had significant depressive effect on expression of PRL gene (mean inhibition: 40%; $F = 70.8$, $p < .001$), by decreasing it significantly at every time point tested (Figure 2). Melatonin treatment also decreased plasma PRL by 31% ($F = 7.97$, $p < .01$), with a significant "melatonin treatment × time-of-day" interaction ($F = 2.31$, $p < .05$) in the factorial ANOVA; changes in circulating PRL becoming significant in the photophase only (Figure 2). In a cosinor analysis, acrophases for pituitary PRL gene expression and plasma PRL levels occurred in the middle of the scotophase, at approximately the same time intervals in both experimental groups (Table 3).

Figures 3 and 4 and Tables 4 and 5 summarize the daily changes in anterior pituitary gene expression of enzymes in the redox pathway, MT-1 and MT-3, and pituitary lipid peroxidation in rats administered melatonin. When analyzed as a main factor in the factorial ANOVA,

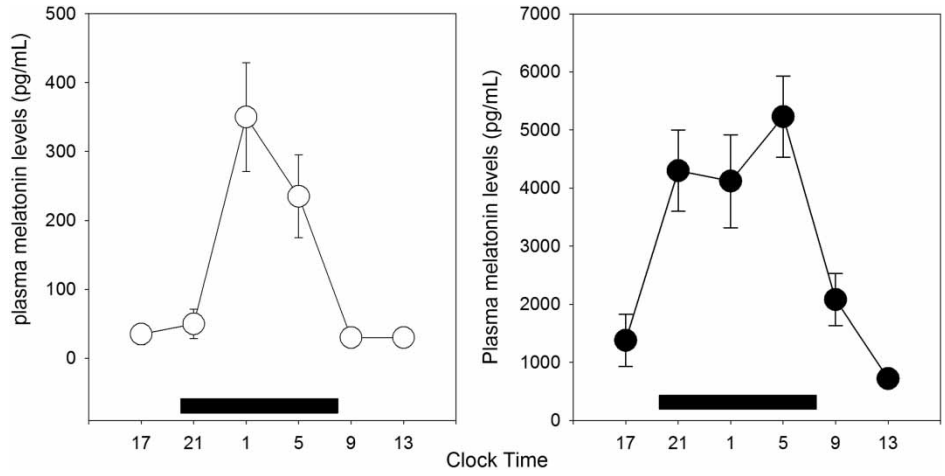

FIGURE 1. Melatonin levels in plasma of rats receiving melatonin (3 μg/mL) (right panel) or vehicle (left panel) in drinking water for 1 mo. Groups of 6–8 rats were euthanized by decapitation at six different time intervals throughout a 24-h cycle. Melatonin was measured by ELISA as described in Materials and Methods. Shown are the means ± SEM (n = 6–8/group). Bars indicate scotophase duration.

TABLE 2. Cosinor analysis of plasma melatonin levels in rats receiving melatonin (3 μg/mL) in drinking water for 1 mo

Rats	MESOR (pg/mL)	Amplitude (pg/mL)	Acrophase (h, min)	R^2, p value
Melatonin-treated	3055 ± 201*	2252 ± 557*	01:33 ± 00:58	.84, .001
Vehicle-treated	122 ± 16	151 ± 52	02:19 ± 01:19	.74, .001

Shown are the means ± SEM. *$p < .001$ vs. *vehicle-treated rats*, Student's t test. R^2 values and their p values are also shown. Mean values of each time series in Figure 1 (n = 6) were used to calculate the acrophase (peak time) and amplitude of rhythms. To calculate the MESOR and R^2 statistical validity, the total number of individual values was considered (n = 44–48).

melatonin treatment decreased NOS-2 by 95% and HO-1 mRNA levels by 16% ($F = 567$ and 12.8, respectively, $p < .001$; Figure 3) and augmented by 18%, 42%, 16%, 17%, 14%, and 11% NOS-1, HO-2, GPx, GSR, Mn-SOD, and MT-3 mRNA levels ($F = 10.9$, $p < .01$; $F = 46.1$, $p < .001$; $F = 13.1$, $p < .001$; $F = 10.3$, $p < .001$; $F = 16.9$, $p < .001$; and $F = 7.04$, $p < .02$, respectively; Figures 3 and 4). Except for lipid peroxidation, significant "melatonin treatment × time-of-day" interactions were found for every anterior pituitary redox parameter tested in the factorial ANOVA (NOS-1: $F = 2.51$, $p < .04$; HO-1: $F = 17.5$, $p < .001$; GPx: $F = 7.71$, $p < .001$; NOS-2: $F = 2.84$, $p < .01$; HO-2: $F = 7.71$, $p < .001$; GSR: $F = 2.78$, $p < .05$; Cu/Zn-SOD: $F = 3.03$, $p < .03$; Mn-SOD: $F = 4.73$, $p < .01$; catalase: $F = 17.3$, $p < .001$; MT-1: $F = 2.71$, $p < .04$; MT-3: $F = 7.73$, $p < .001$; Figures 3 and 4). Results of the cosinor analyses are summarized in Tables 4 and 5. Consistently, melatonin treatment disrupted the 24-h pattern in gene expression of redox enzymes by shifting or amplifying the maximum to early/middle scotophase, and in gene expression of MT-1 and MT-3 by showing maxima in early/middle photophase (Figures 3 and 4). Among the several parameters tested, only the effect of melatonin on NOS-2 gene expression resembled the concomitant inhibition of PRL gene expression depicted in Figure 2.

Figure 5 and Table 6 show the effect of melatonin on the 24-h pattern of anterior pituitary circadian clock gene expression. Significant time-related changes in clock gene expression were found in controls. The peak of *Clock* and *Bmal1* expression occurred at middle scotophase (acrophases at 02:52 and 03:14 h, respectively), whereas that of *Per1* and *Per2*, and of *Cry1* and *Cry2*, occurred at middle and late photophase (10:11 and 11:14 h, and at 18:20 and 19:03 h, respectively). As analyzed as a main factor in the factorial ANOVA, treatment with melatonin increased mean expression of *Per2*, *Cry1*, and *Cry2* by 86%, 20%, and 15% ($F = 4.71$, $p < .03$; $F = 6.54$, $p < .02$; and $F = 8.92$, $p < .001$, respectively). It also decreased mean expression of *Per1* by 18% ($F = 3.83$, $p < .05$), although the single significant difference found between melatonin and vehicle groups at the individual time intervals was an increase at 21:00 h in the anterior pituitary of *melatonin-treated rats* ($p < .01$) (Figure 5). In the cosinor analysis, melatonin treatment phase-delayed expression of anterior pituitary *Per1*, *Per2*, and *Cry1* by ~6–10 h to attain acrophases at 17:19, 21:11, and 23:55 h, respectively (Table 6).

The effect of melatonin on the 24-h pattern of rat plasma TSH, LH, testosterone, and corticosterone concentration is shown in Figure 6 and Table 7. Mean values of TSH were augmented by 37%, and those of LH and testosterone decreased by 41% and 33% ($F = 14.3$, $p < .001$; $F = 7.57$, $p < .01$; and $F = 3.45$, $p < .05$, respectively; main factor analysis, factorial ANOVA).

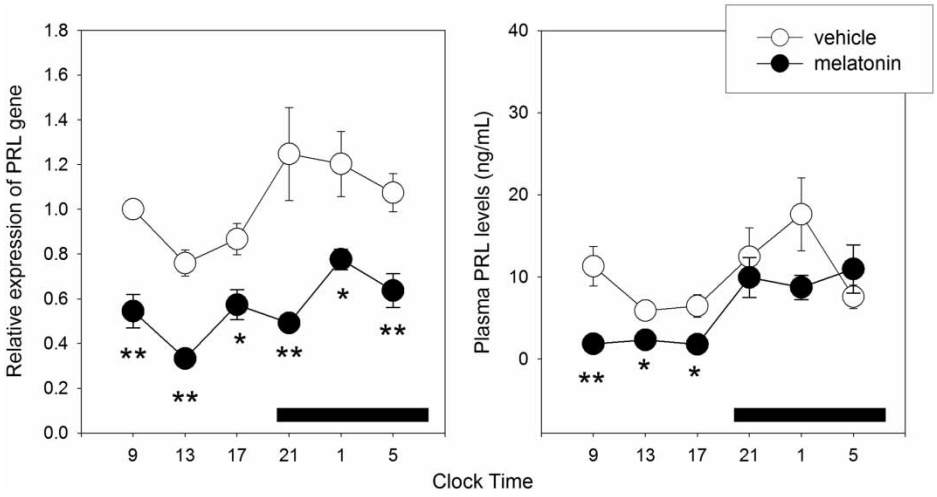

FIGURE 2. Effect of melatonin on 24-h pattern in adenohypophysial PRL gene expression and plasma PRL levels in rats. Rats received melatonin (3 μg/mL) or vehicle in drinking water for 1 mo. Groups of 6–8 rats were euthanized by decapitation at six different time intervals throughout a 24-h cycle. mRNA levels encoding the PRL gene and plasma PRL levels were measured as described in the text. Shown are the means ± SEM of mRNA determination as measured by triplicate real-time PCR analyses of RNA samples and the means ± SEM of plasma PRL levels. Bars indicate scotophase duration. Asterisks denote significant differences as compared with vehicle by Student's *t* tests performed at every time interval (*$p < .05$, **$p < .01$). One-way ANOVAs within each experimental group indicated significant time-related changes in PRL gene expression (melatonin: $F = 5.76$, $p < .003$; vehicle: $F = 2.72$. $p < .04$) and in plasma PRL levels (melatonin: 3.62, $p < .01$; vehicle: $F = 2.59$, $p < .04$). For further statistical analysis, see text.

TABLE 3. Cosinor analysis of the effect of melatonin on 24-h changes in pituitary PRL gene expression and circulating PRL levels

	MESOR	Amplitude	Acrophase (h, min)	R^2, *p* value
Melatonin-treated rats				
PRL relative gene expression	.56 ± .03*	.16 ± .07	01:51 ± 01:36	.65, <.01
Plasma PRL levels (ng/mL)	6.58 ± .73*	5.10 ± 1.49	01:11 ± 01:08	.79, <.001
Vehicle-treated rats				
PRL relative gene expression	.92 ± .03	.22 ± .06	00:48 ± 00:58	.84, <.001
Plasma PRL levels (ng/mL)	9.57 ± .28	4.28 ± 2.24	01:00 ± 02:00	.55, <.01

Shown are the means ± SEM. *$p < .001$ vs. *vehicle-treated rats*, Student's *t* test. R^2 values and *p* values are also shown. Mean values of each time series in Figure 2 (n = 6) were used to calculate the acrophase (peak time) and amplitude of rhythms. To calculate the MESOR and R^2 statistical validity, the total number of individual values was considered (n = 43–46). For further statistical analysis, see text.

As shown by cosinor analysis, the acrophase of the plasma corticosterone rhythm in controls (17:28 h) was phase-delayed ~4 h by melatonin to 21:10 h (Table 7). In addition, melatonin treatment augmented significantly the circadian rhythm amplitude of plasma corticosterone and TSH (Figure 6 and Table 7).

DISCUSSION

In the present study, we administered melatonin via drinking water to rats as a way to resemble the prolonged duration of the melatonin signal presumably found in the natural environment for wild *Rattus norvegicus* during winter. Doses of melatonin employed (~60 μg/d) resulted in peak plasma values of melatonin that were ~15-fold higher than those found in vehicle-treated controls. More importantly, high concentrations of melatonin (>2000 pg/mL) were detected in *melatonin-treated rats* from the late photophase to early photophase as compared with the considerably narrower high-melatonin phase observed in controls (Figure 1).

Under these experimental conditions, profound inhibitory effect on pituitary PRL gene expression and circulating PRL levels was observed in melatonin-treated animals, with a reduction of MESOR values by about half (cosinor analysis). In contrast, the acrophase for pituitary PRL gene expression and plasma PRL levels occurred at approximately the middle of scotophase in both experimental groups. As analyzed within each time interval, inhibition of PRL gene expression was significant throughout the 24-h cycle in melatonin-treated rats, whereas circulating PRL decreased significantly during the photophase only. These results support the view that the laboratory rat, regardless of being housed under constant conditions of temperature, lighting, and food availability, did maintain the capability

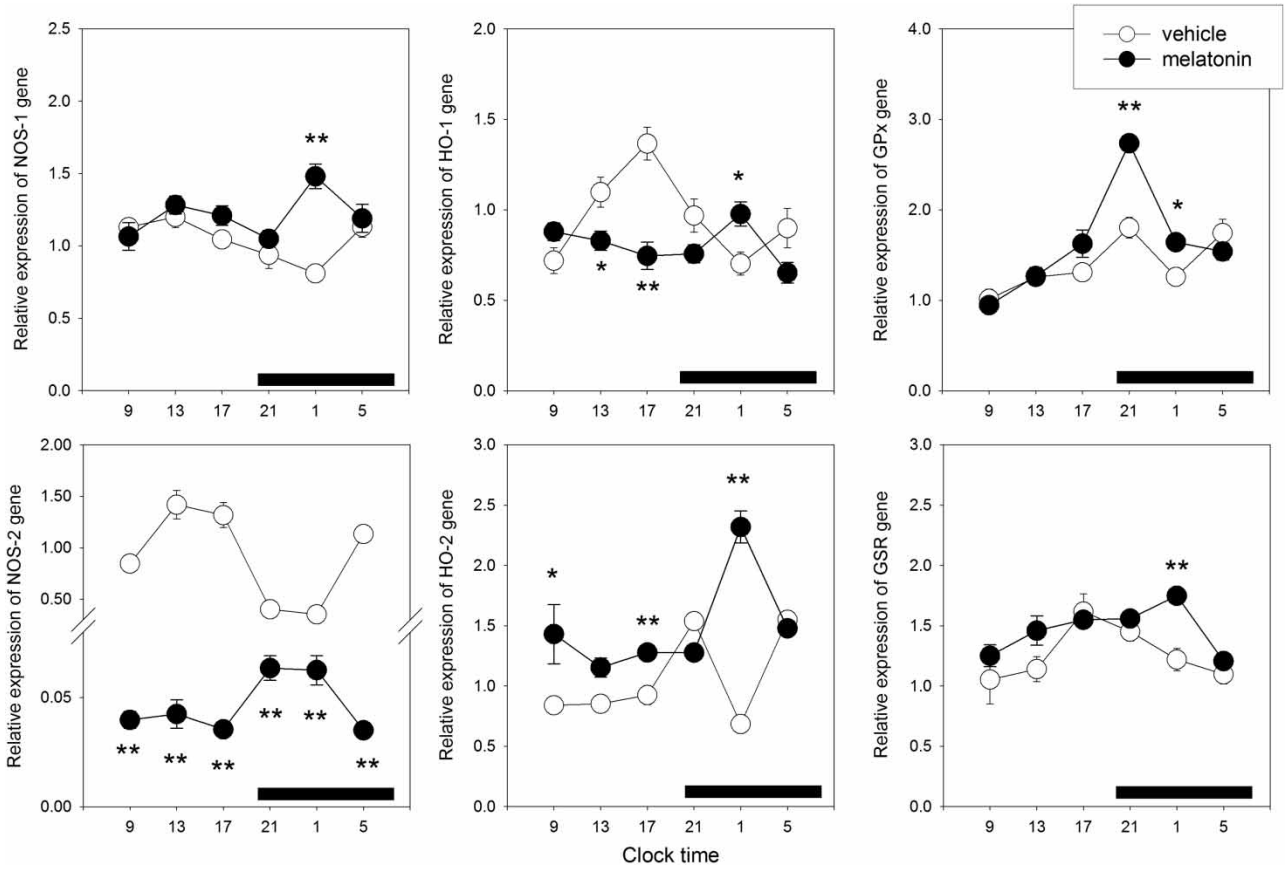

FIGURE 3. Effect of melatonin on 24-h pattern in adenohypophysial expression of mRNA for NOS-1, HO-1, GPx, NOS-2, HO-2, and GSR in rats. For experimental details see legend to Figure 2. mRNA levels encoding the enzymes were measured as described in the text. Shown are the means ± SEM of mRNA determination as measured by triplicate real-time PCR analyses of RNA samples. Bars indicate scotophase duration. Asterisks denote significant differences as compared with vehicle by Student's *t* tests performed at every time interval (*$p < .05$, **$p < .01$). One-way ANOVAs within each experimental group indicated significant time-related changes in NOS-1, HO-1, GPx, NOS-2, HO-2, and GSR gene expression as follows: Vehicle: $F = 4.56$, $p < .01$; $F = 8.14$, $p < .001$; $F = 8.53$, $p < .001$; $F = 27.4$, $p < .001$; $F = 36.3$, $p < .001$; and $F = 2.73$, $p < .05$, respectively. Melatonin: $F = 3.54$, $p < .03$; $F = 3.68$, $p < .02$; $F = 34.6$, $p < .001$; $F = 6.09$, $p < .003$; $F = 10.7$, $p < .001$; and $F = 5.44$, $p < .001$, respectively. See text for further statistical analysis.

to respond to increase in the duration and magnitude of the melatonin signal with an inhibition of PRL synthesis and release, as shown in long-day breeders such as the Syrian hamster (Bartness et al., 1993).

Kamberi et al. (1971) were the first to provide evidence that in the rat melatonin does not act directly on the anterior pituitary, but centrally at the hypothalamic level to modify PRL release. Studies involving targeted administration of melatonin in diverse areas of the brain of white-footed mice indicated that these effects were exerted at a region within or surrounding the hypothalamus (Glass & Lynch, 1981). Exact sites in the brain responsible for the seasonal effects of melatonin were first unraveled by the autoradiographic description of 2-[^{125}I]-iodomelatonin binding sites. Although these binding sites were characterized in the suprachiasmatic nucleus (SCN) and other regions of the hypothalamus, the binding observed in these areas was lower than that found in the pars tuberalis, a thin layer of the adenohypophysis that surrounds the pituitary stalk and extends rostrally along the ventral surface of the median eminence.

There is evidence indicating that in order to regulate the seasonal PRL rhythm, the melatonin signal has to target the pars tuberalis, and not the pars distalis or the hypothalamic tuberoinfundibular dopaminergic neurons (see for reference Dardente, 2007; Dupre, 2011). Melatonin does not directly regulate PRL gene expression or secretion in pars distalis cultures (Stirland et al., 2001). Despite transient expression of melatonin receptors in gonadotrophs of neonatal rat pars distalis (Johnston et al., 2003), co-localization studies have shown that these receptors are only expressed in the pars tuberalis (Morgan et al., 1994) of specific thyrotroph cells in adult rodents (Klosen et al., 2002). Thyrotroph cells in the pars tuberalis may act as master factors regulating seasonal reproduction via retrograde TSH effect on the ependymal cells of the mediobasal hypothalamus to induce expression of type II thyroid hormone deiodinase and subsequent increase of hypothalamic T3 levels (Hanon et al., 2010; Yasuo et al., 2010). This is thought to trigger gonadotropin-releasing hormone secretion from the hypothalamus of long-day breeders, whereas it terminates reproductive activity in short-day breeders. Pars tuberalis cells also control

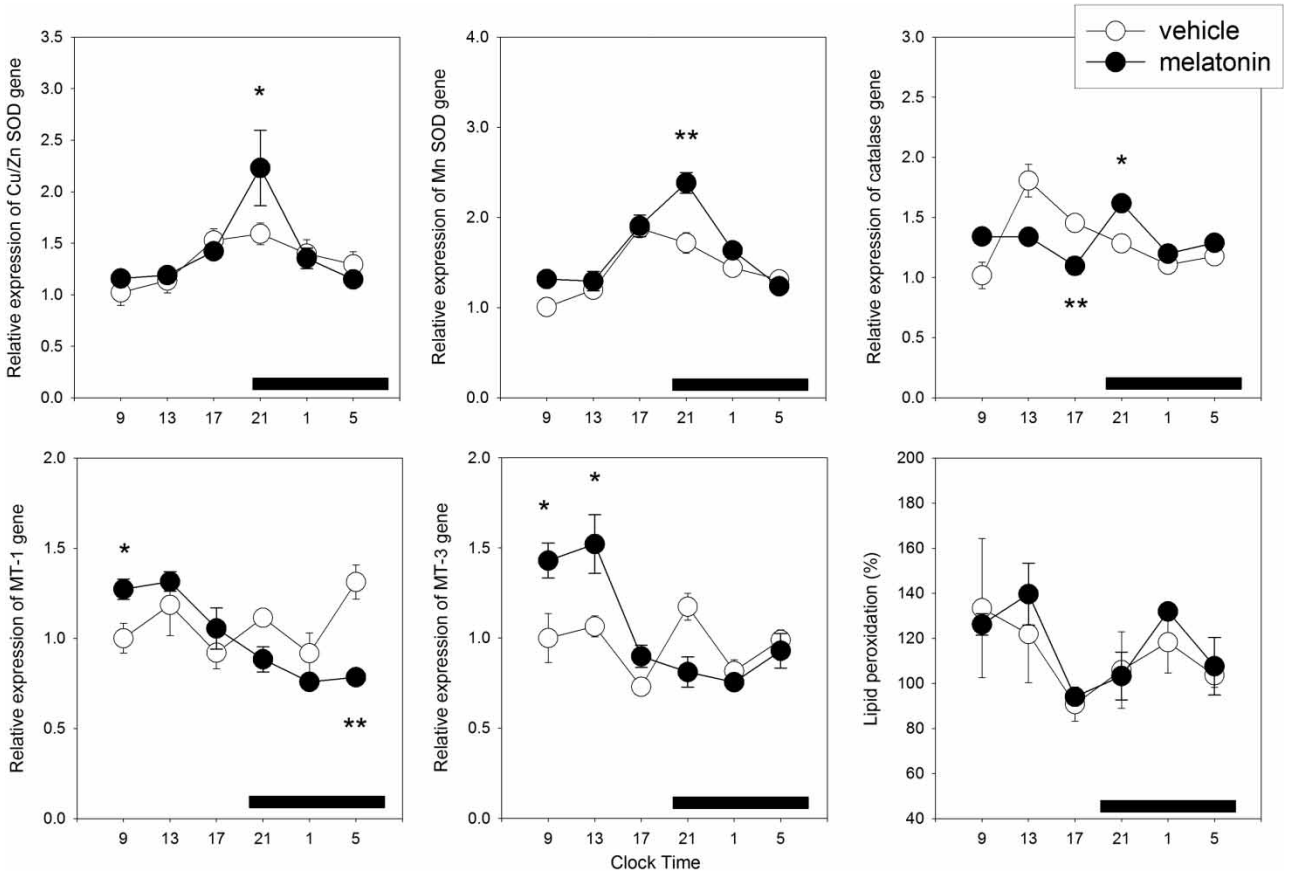

FIGURE 4. Effect of melatonin on 24-h pattern in expression of mRNA for Cu/Zn-SOD, Mn-SOD, catalase, MT-1, and MT-3, and in lipid peroxidation in rat adenohypophysis. For experimental details see legend to Figure 2. Lipid peroxidation was assessed by the thiobarbituric acid–reactive substances procedure as described in Materials and Methods. Shown are the means ± SEM. Bars indicate scotophase duration. Asterisks denote significant differences as compared with control by Student's *t* tests performed at every time interval (*$p < .05$, **$p < .01$). One-way ANOVAs within each experimental group indicated significant time-related changes in Cu/Zn-SOD, Mn-SOD, catalase, and MT-3 gene expression for *vehicle-treated rats* ($F = 3.44$, $p < .03$; $F = 14.4$, $p < .001$; $F = 12.5$, $p < .001$; and $F = 4.32$, $p < .01$, respectively) and in Cu/Zn-SOD, Mn-SOD, catalase, MT-1, and MT-3 gene expression for *melatonin-treated rats* ($F = 8.27$, $p < .001$; $F = 25.7$, $p < .001$; $F = 9.65$, $p < .001$; $F = 13.7$, $p < .001$; and $F = 12.6$, $p < .001$, respectively). See text for further statistical analysis.

TABLE 4. Cosinor analysis of the effect of melatonin on 24-h changes in adenohypophysial expression of mRNA for NOS-1, HO-1, GPx, NOS-2, HO-2, and GSR in rats

	MESOR (relative gene expression)	Amplitude (relative gene expression)	Acrophase (h, min)	R^2, *p* value
Melatonin-treated rats				
NOS-1	1.23 ± .06**	—	—	.06, N.S.
HO-1	.81 ± .04*	.01 ± .09**	02:34 ± 22:30**	.96, <.001
GPx	1.62 ± .03*	.68 ± .23	21:37 ± 01:16*	.75, <.01
NOS-2	.05 ± .001**	.01 ± .01**	22:45 ± 02:05	.53, <.02
HO-2	1.49 ± .07**	.41 ± .02	01:59 ± 01:54**	.57, <.01
GSR	1.48 ± .06**	.21 ± .09	20:50 ± 01:43	.63, <.01
Vehicle-treated rats				
NOS-1	1.04 ± .03	.17 ± .05	11:07 ± 01:06	.81, <.001
HO-1	.96 ± .06	.27 ± .11	16:26 ± 01:31	.68, <.01
GPx	1.40 ± .08	.23 ± .19	23:12 ± 03:09	.33, <.05
NOS-2	.93 ± .05	.46 ± .22	12:24 ± 01:46	.61, <.01
HO-2	1.06 ± .09	—	—	.12, N.S.
GSR	1.26 ± .05	.27 ± .07	19:06 ± 00:57	.84, <.001

Shown are the means ± SEM. *$p < .02$, **$p < .01$ vs. *vehicle-treated rats*, Student's *t* test. R^2 values and *p* values are also shown. Mean values at each time series in Figure 3 (n = 6) was used to calculate the acrophase (peak time) and amplitude of rhythms. To calculate the MESOR (24-h time series mean) and R^2 statistical validity, the total number of individual values was considered (n = 42–48). For further statistical analysis, see text.

TABLE 5. Cosinor analysis of the effect of melatonin on 24-h changes in expression of mRNA for Cu/Zn-SOD, Mn-SOD, catalase, MT-1, and MT-3 and in lipid peroxidation in rat adenohypophysis

	MESOR (relative gene expression)	Amplitude (relative gene expression)	Acrophase (h, min)	R^2, p value
Melatonin-treated rats				
Cu/Zn-SOD	1.43 ± .08	.43 ± .18	20:46 ± 01:33	.67, <.02
Mn-SOD	1.63 ± .06*	.51 ± .13	20:24 ± 00:59**	.84, <.01
Catalase	1.31 ± .09	—	—	.03, N.S.
MT-1	1.01 ± .06	.31 ± .06	12:34 ± 00:44	.90, <.001
MT-3	1.06 ± .04*	.40 ± .08	11:07 ± 00:48	.88, <.001
Lipid peroxidation (%)	118.3 ± 8.2	—	—	.24, N.S.
Vehicle-treated rats				
Cu/Zn-SOD	1.33 ± .04	.27 ± .05	21:07 ± 00:42	.91, <.001
Mn-SOD	1.43 ± .06	.39 ± .10	02.03 ± 01:00	.83, <.001
Catalase	1.31 ± .10	.28 ± .14	15:14 ± 01:54	.58, <.02
MT-1	1.07 ± .08	—	—	.16, N.S.
MT-3	.96 ± .02	—	—	.02, N.S.
Lipid peroxidation (%)	112.2 ± 6.3	11.9 ± 8.89	08:15 ± 02:51	.37, <.04

Shown are the means ± SEM. *p < .04, **p < .01 vs. *vehicle-treated rats*, Student's t test. R^2 values and p values are also shown. Mean values of each time series in Figure 4 (n = 6) were used to calculate the acrophase (peak time) and amplitude of rhythms. To calculate the MESOR and R^2 statistical validity, the total number of individual values was considered (n = 42–47). For further statistical analysis, see text.

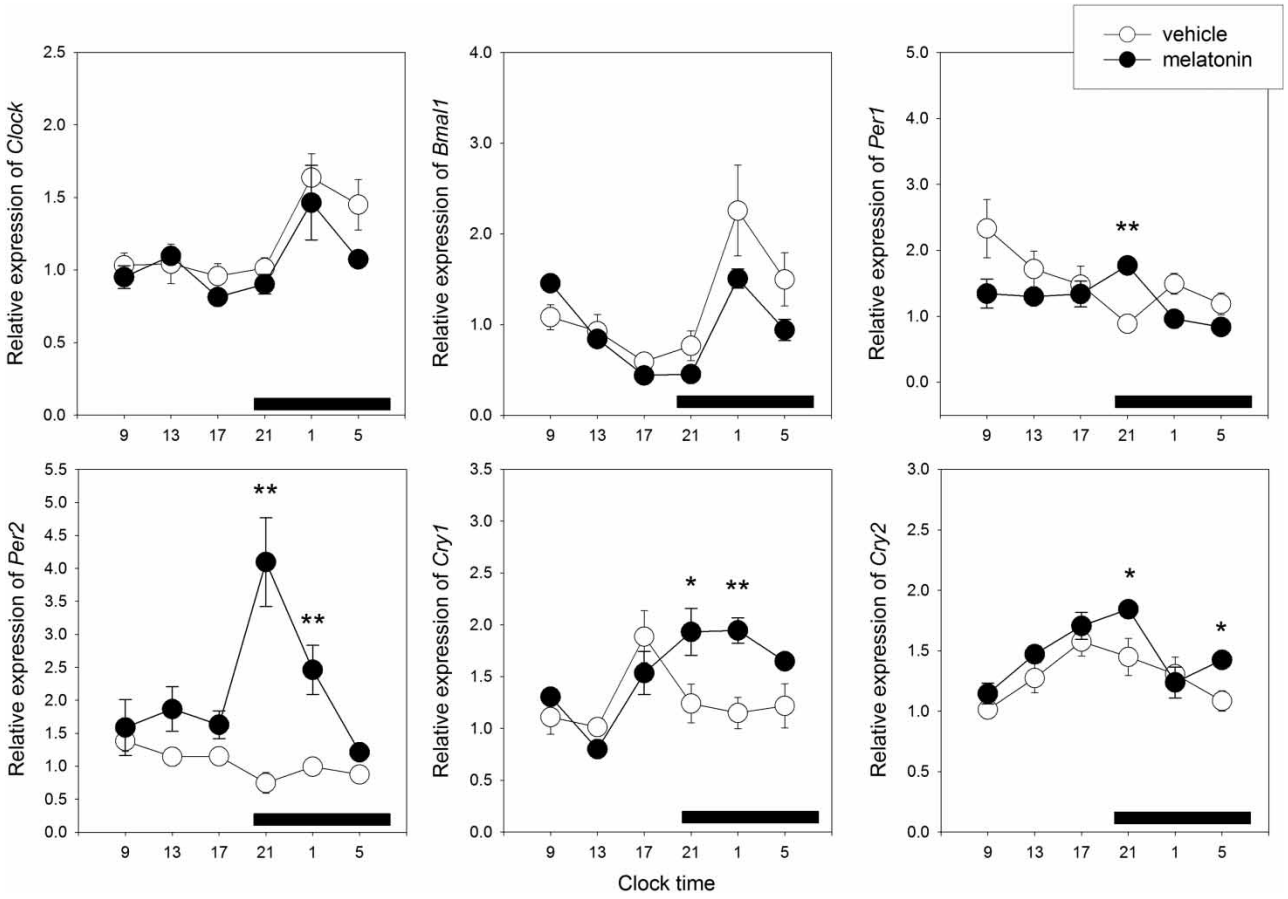

FIGURE 5. Effect of melatonin on 24-h pattern in adenohypophysial expression of mRNA of *Clock*, *Bmal1*, *Per1*, *Per2*, *Cry1*, and *Cry2* in rats. Experimental details are given in the legend to Figure 2. Adenohypophysial mRNA levels were measured as described in text. Shown are the means ± SEM of mRNA determination as measured by triplicate real-time PCR analyses of RNA samples. Bars indicate scotophase duration. Asterisks denote significant differences as compared with vehicle by Student's t tests performed at every time interval (*p < .05, **p < .01). One-way ANOVAs within each experimental group indicated significant time-related changes in *Clock*, *Bmal1*, *Per1*, *Per2*, *Cry1*, and *Cry2* expression: Vehicle: F = 5.09, p < .001; F = 5.56, p < .001; F = 3.18, p < .01; F = 3.28, p < .01; F = 2.57, p < .04; and F = 3.39, p < .01, respectively. Melatonin: F = 3.92, p < .01; F = 44.5, p < .001; F = 4.64, p < .004; F = 6.24, p < .001; F = 9.04, p < .001; and F = 8.61, p < .001, respectively. See text for further statistical analysis.

TABLE 6. Cosinor analysis of the effect of melatonin on 24-h changes in adenohypophysial expression of mRNA of *Clock*, *Bmal1*, *Per1*, *Per2*, *Cry1*, and *Cry2* in rats

	MESOR (relative gene expression)	Amplitude (relative gene expression)	Acrophase (h, min)	R^2, p value
Melatonin-treated rats				
Clock	1.06 ± .08	.19 ± .13	02:56 ± 02:41	.41, <.03
Bmal1	.99 ± .06	.44 ± .23	05:44 ± 02:00	.55, <.02
Per1	1.26 ± .07**	.31 ± .17	17:19 ± 02:09*	.51, <.02
Per2	2.14 ± .16**	.99 ± .51	21.11 ± 01:58*	.56, <.02
Cry1	1.53 ± .07**	.52 ± .11	23:59 ± 00:46*	.89, <.001
Cry2	1.47 ± .05**	.29 ± .11	18:59 ± 01:27	.71, <.01
Vehicle-treated rats				
Clock	1.19 ± .09	.31 ± .11	02:52 ± 01:19	.74, <.01
Bmal1	1.16 ± .08	.64 ± .26	03:14 ± 01:31	.68, <.01
Per1	1.52 ± .08	.49 ± .23	10:11 ± 01:47	.61, <.02
Per2	1.15 ± .07	.23 ± .11	11.14 ± 01:47	.60, <.02
Cry1	1.27 ± .06	.24 ± .19	18:20 ± 03:05	.34, <.05
Cry2	1.28 ± .03	.27 ± .04	19:03 ± 00:31	.95, <.001

Shown are the means ± SEM. *$p < .03$, **$p < .01$ vs. *vehicle-treated rats*, Student's t test. R^2 values and their p values are also shown. Mean values of each time series in Figure 5 (n = 6) were used to calculate the acrophase (peak time) and amplitude of rhythms. To calculate the MESOR and R^2 statistical validity, the total number of individual values was considered (n = 42–45). For further statistical analysis, see text.

TABLE 7. Cosinor analysis of the effect of melatonin on 24-h changes in circulating TSH, LH, testosterone, and corticosterone levels

	MESOR	Amplitude	Acrophase (h, min)	R^2, p values
Melatonin-treated rats				
TSH	3.17 ± .17**	1.08 ± .33*	15:00 ± 01:52	.58, <.01
LH	275.5 ± 13.5**	86.4 ± 33.2	03:11 ± 01:28	.69, <.01
Testosterone	.60 ± .04**	.16 ± .15	12:52 ± 03:31	.28, <.05
Corticosterone	456.6 ± 25.8	310.6 ± 46.8*	21:10 ± 00:48*	.88, <.001
Vehicle-treated rats				
TSH	2.31 ± .08	.32 ± .08	14:04 ± 03:23	.30, <.04
LH	462.0 ± 22.0	182.4 ± 31.1	02:41 ± 00:39	.92, <.001
Testosterone	.89 ± .06	—	—	.21, N.S.
Corticosterone	567.7 ± 24.7	168.5 ± 35.6	17:28 ± 00:48	.86, <.001

Shown are the means ± SEM. *$p < .05$, **$p < .01$ vs. *vehicle-treated rats*, Student's t test. MESOR and amplitude values are expressed as ng/mL (TSH, testosterone, corticosterone) or pg/mL (LH). R^2 values and p values are also shown. Mean values of each time series in Figure 6 (n = 6) were used to calculate the acrophase (peak time) and amplitude of rhythms. To calculate the MESOR and R^2 statistical validity, the total number of individual values was considered (n = 44–46). For further statistical analysis, see text.

anterogradely anterior pituitary lactotrophs, presumably via tachykinins affecting PRL release (Dupre, 2011). Although circadian and melatonin-dependent control of TSH appears to link the circadian clock and the photoperiodic response in mammals, their exact mechanism of action for these effects remains still unraveled (Revel et al., 2009).

Because reactive oxygen species (ROS) generation is a continuous and physiological phenomenon, cells possess efficient antioxidant systems that protect them from oxidative damage (see for reference Jacob, 2011; Locasale & Cantley, 2011; Mancuso et al., 2007; Winyard et al., 2011). Detoxification of ROS in cells involves cooperative action of intracellular antioxidant enzymes, among them Cu/Zn-SOD (which is cytosolic), Mn-SOD (which is mitochondrial), and catalase (which is present in peroxisomes). In addition, GPx and GSR help maintain adequate levels of reduced glutathione, a major antioxidant defense of the cells. These defense systems are thought to prevent free radicals from causing irreparable damage by reacting with lipids, proteins, and nucleic acids and are controlled in vivo by a wide spectrum of enzymatic and nonenzymatic systems (Jacob, 2011; Locasale & Cantley, 2011; Winyard et al., 2011).

In the anterior pituitary gland, almost all redox pathway enzymes have been described; however, there is scarce information on circadian regulation of enzyme activity or gene expression in this tissue. We previously reported that in rats fed a high-fat diet, the correlation

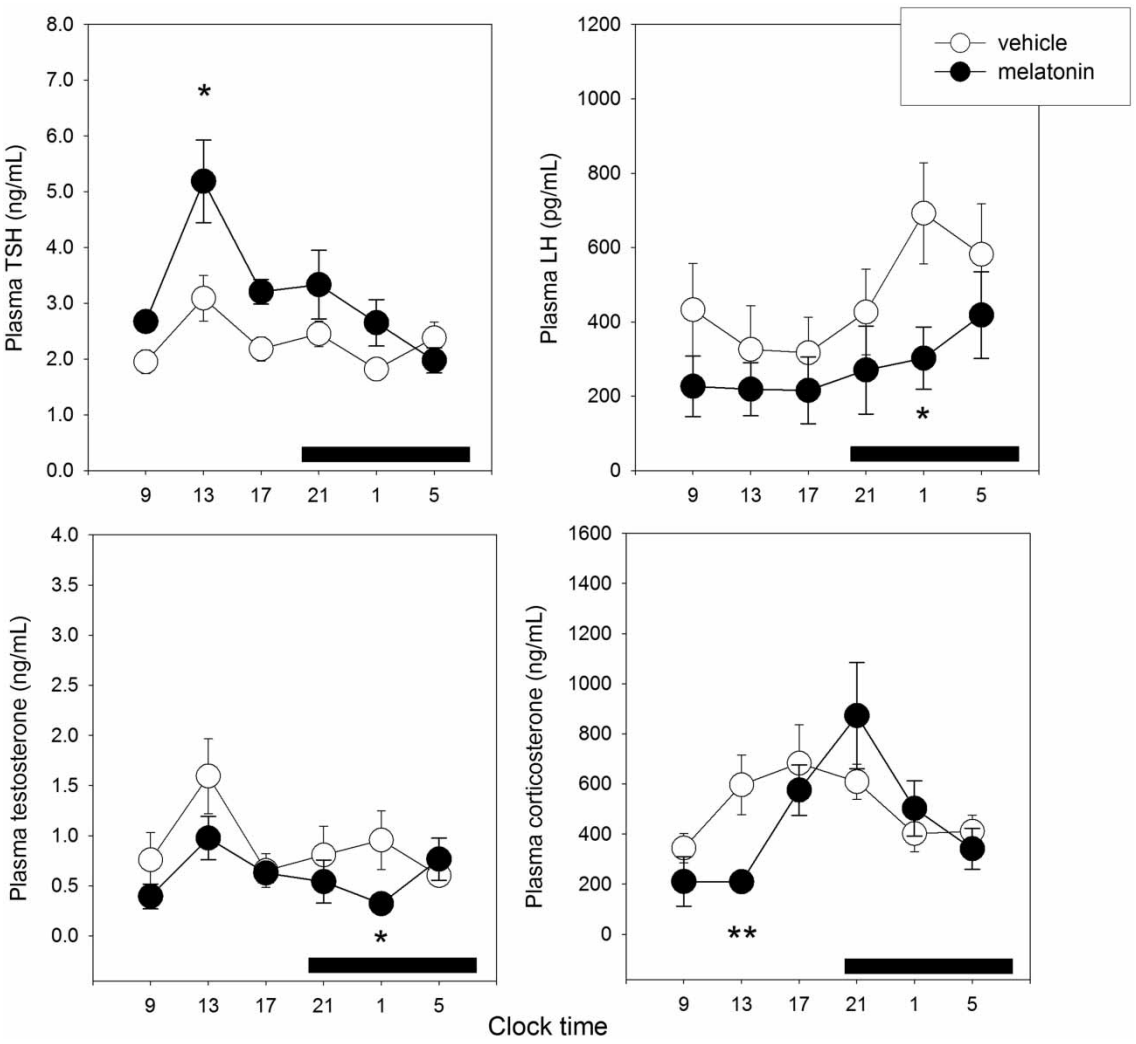

FIGURE 6. Effect of melatonin on 24-h pattern in rat plasma TSH, LH, testosterone, and corticosterone levels. Rats received melatonin (3 μg/mL) or vehicle in drinking water for 1 mo. Groups of 6–8 rats were euthanized by decapitation at six different time intervals throughout a 24-h cycle. Plasma hormone levels were measured by RIA as described in the text. Shown are the means ± SEM. Bars indicate scotophase duration. Asterisks denote significant differences as compared with vehicle by Student's *t* tests performed at every time interval (*$p < .05$, **$p < .01$). One-way ANOVAs within each experimental group indicated significant time-related changes in TSH and corticosterone in vehicle-treated rats ($F = 3.05$, $p < .03$; and $F = 2.52$, $p < .05$, respectively) and *melatonin-treated rats* ($F = 6.02$, $p < .001$; and $F = 3.84$, $p < .01$, respectively). See text for further statistical analysis.

of 24-h changes in expression of pituitary PRL gene and plasma levels of PRL was lost, as was the 24-h rhythmicity in pituitary gene expression of HO-2, Cu/Zn- and Mn-SOD, catalase, GPx, and GSR (Cano et al., 2010). In the present study, among the several pituitary redox enzyme mRNAs tested, only the inhibition of NOS-2 gene expression correlated temporally with inhibition of PRL gene expression seen in melatonin-fed rats. In the case of HO-1 gene expression (inhibited by melatonin) and of NOS-1, HO-2, GPx, GSR, and Mn-SOD gene expression (which augmented after melatonin), a phase delay in the acrophase to early/middle scotophase or amplification of its maximum at the scotophase was detected. Even the very low levels of expression of pituitary NOS-2 gene showed a similar 24-h profile in melatonin-treated rats, with a shift in acrophase from the middle afternoon to early scotophase. These significant changes in gene expression of several components of intracellular redox defense did not reflect in any significant change of pituitary lipid peroxidation, which remained essentially at the same levels in melatonin- and *vehicle-treated rats*. Whether other anterior pituitary oxidative markers, e.g., oxidative DNA damage measured by 8-hydroxy-2'-deoxyguanosine, remain also unaffected by melatonin deserves to be explored.

It is clear that melatonin participates in diverse physiological functions, signaling not only the length of the night, but also enhancing ROS scavenging and cytoprotection (Galano et al., 2011; Hardeland et al., 2011). Regulation of redox enzyme gene expression by melatonin probably includes receptor-mediated and receptor-independent phenomena. Among the latter, inhibition of ROS generation is attractive. Inasmuch as ROS play a role in cellular signaling processes, including transcription

factors such as nuclear factor-κB, decrease of free radical production by melatonin would lead to repression of redox-sensitive transcription factors regulating gene transcription (Beni et al., 2004; Lezoualc'h et al., 1998; Rodríguez et al., 2004). Collectively, the present results on redox enzyme gene expression underline the significant effect of melatonin on expression of pituitary redox enzymes involved in PRL secretion. In this respect, NO presumably plays a major role (Quinteros et al., 2007; Velárdez et al., 2000). NO is synthesized by NOS-1 (constitutive) and NOS-2 (inducible), both enzymes being expressed in the pituitary gland. NOS-1 is responsible for low and persistent concentrations of NO, typical of normal situations. Melatonin-induced down-regulation of pituitary NOS-2 expression could presumably result in very low levels of NO, perhaps instrumental for the disruption of PRL release mechanisms herein reported. Indeed, whereas long-term exposure of pituitary cells to high concentrations of NO induces cellular damage, submicromolar concentrations of NO can protect anterior pituitary cells (Benarroch, 2011). Further studies are needed to assess histologically whether PRL-secreting cells are homogeneously less active or there are less lactotroph cells under melatonin treatment.

Mammalian MTs are intracellular low-molecular-weight, cysteine-rich, proteins involved in protecting cells against ROS and heavy metal toxicity (Namdarghanbari et al., 2011). Melatonin has been reported to affect MT expression in a number of tissues, including the anterior pituitary (Alonso-González et al., 2008; Miler et al., 2010; Mukherjee et al., 2011). In rats receiving melatonin, significant increase in pituitary MT-3 gene expression and significant 24-h changes with maxima for MT-1 and MT-3 at early/middle photophase were observed. To what extent these changes are related to redox-mediated mechanisms in the anterior pituitary affected by melatonin is presently not known.

In mammals, the circadian system is composed of many individual, tissue-specific cellular clocks whose phases are synchronized by the master circadian pacemaker located in the SCN (Lincoln et al., 2006). Circadian rhythms are driven by the self-regulatory interaction of a set of clock genes and their protein products (Levi et al., 2010). Positive transactivator gene products, such as *Clock* and *Bmal1*, induce the expression of their own repressors (*Per1–3* and *Cry1–2*) with a cycle of 24 h. The present results in the anterior pituitary of vehicle-treated rats indicate that the acrophases of *Clock* and *Bmal1* expression and those of *Per1*, *Per2*, *Cry1*, and *Cry2* expression were in antiphase. *Per1* and *Per2* and *Cry1* and *Cry2* peaked at dawn and late photophase, respectively, whereas acrophases of *Clock* and *Bmal1* expression were at the middle scotophase. Such a reciprocal relation was reported in the SCN as well as in some peripheral tissues (Levi et al., 2010; Lincoln et al., 2006). In the rodent anterior pituitary, however, published data are conflicting. In tissue explants of pituitary glands from *Per1*-luciferase transgenic rats, maximum in *Per1*-luciferase expression occurred, as hereby reported, during the light phase (Lundkvist et al., 2010), whereas in mice kept under 12 L:12 D photoperiods, maximal *Per1* and *Per2* expression occurred at the L/D transition and those of *Bmal1*, *Cry1*, and *Cry2* during the scotophase (Bur et al., 2010). In another study, in rats euthanized at six time intervals during the 24 h cycle, rhythmic expression of *Per2* and *Bmal1*, but not *Per1*, was detected (Girotti et al., 2009). As in the present study, *Per2* and *Bmal1* expression in the rat anterior pituitary were in antiphase with each other, *Per2* expression showing its maximum at middle photophase whereas *Bmal1* expression showing its maximum at middle scotophase. As suggested by Bur et al. (2010), the pituitary gland is presumably a compendium of the complexity of peripheral circadian oscillators, since the circadian clockwork, expressed in most pituitary cells, may pace a wide number of functions through the various hormones released by the gland.

Treatment with melatonin augmented mean expression of *Per2, Cry1*, and *Cry2*. In the case of *Per1*, decreased MESOR was observed, although the single significant difference found between the melatonin and vehicle groups when analyzed at individual time intervals was an increase at 21:00 h in *melatonin-treated rats*. Melatonin treatment phase-delayed expression of *Per1*, *Per2*, and *Cry1* by ~6–10 h. These changes fit well the phase-delayed gene expression of most redox enzymes detected in the anterior pituitary as well as with the phase delay found in the plasma corticosterone rhythm.

Among the other hormones assessed in the present study and from which there is evidence of seasonality in laboratory rats, the amplitude of the plasma corticosterone and TSH rhythms was augmented after melatonin administration, whereas plasma LH and testosterone concentration decreased significantly. The results indicate that the inherent transcription, translation, and post-translational modifications that give the clock its own natural rhythmicity are modified by an extended daily exposure to melatonin in rats.

A number of limitations must be considered in the analysis and interpretation of the present results. First, further experiments are needed to shed light on the mechanisms that explain the effect of prolonged administration of melatonin on redox enzyme gene expression. In particular, Western blotting analysis of enzyme protein levels will be useful in this respect. Photoperiodic changes in PRL secretion are accompanied by robust changes in pituitary PRL mRNA expression (Hegarty et al., 1990; Stirland et al., 2001), which may result from changes in either gene transcription or mRNA degradation (Khodursky & Bernstein, 2003). Studies including transfection of PRL-luciferase reporter into lactotroph cells could be helpful to determine whether transcriptional regulation is a key factor driving the effect of melatonin on rat PRL expression. This is particularly important because dissociation of PRL gene expression and PRL release was observed in melatonin-treated

rats. It resembled the previously reported disrupted coordination between PRL gene expression and PRL release found in obese rats (Cano et al., 2010). Another limitation is given by the method of sampling. A pulsatile pattern exists for PRL release, and such ultradian variations are not measurable with the experimental approach used. Additionally, further examination is needed to assess whether acute administration of melatonin immediately disrupted the rhythmicity of PRL. An earlier measurement of gene expression shortly after melatonin supplementation could be useful in this respect. Finally, the data only draw a correlation, and the causality of the changes observed can only be speculated upon. In future studies it will be important to establish the level of actual ROS production in the anterior pituitary of melatonin-supplemented rats, and whether by blocking or stimulating ROS an alteration in circadian profile of PRL secretion is obtained.

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The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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