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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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Running title: melatonin and pituitary prolactin synthesis

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ABSTRACT

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, we 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 pituitary redox state- and circadian clock modulatory mechanisms. Melatonin (3 μg/mL drinking water) or vehicle was given for a month and rats were eutanized at 6 time intervals during a 24-h cycle. High concentrations of melatonin (more than 2000 pg/mL) were detected in melatonin-treated rats from the beginning of scotophase (at 21:00 h) to early photophase (at 09:00 h) as compared to a considerably narrower high melatonin phase observed in controls. In a cosinor analysis, melatonin-treated rats had significantly decreased mesor values of pituitary PRL gene expression and circulating PRL levels with acrophases at middle of scotophase as in the control group. Melatonin treatment disrupted the 24-h pattern in 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 dismutases and catalase by shifting their acrophases to early/middle scotophase or by amplifying the maxima found. Only the inhibitory effect of melatonin on pituitary NOS-2 gene expression correlated temporally with the inhibition of PRL production. Gene expression of metallothionein-1 and -3 showed maxima at early/middle photophase after melatonin treatment. 24-Hour pattern of pituitary lipid peroxidation did not vary after treatment. In vehicle-treated rats, *Clock* and *Bmal1* expression peaked at mid scotophase while that of *Per1* and *Per2*, and of *Cry1* and *Cry2,* peaked at mid and late photophase, respectively. Treatment with melatonin decreased mean expression of *Per1* and raised that of *Per2*, *Cry1* and *Cry2*. Melatonin significantly phase-delayed expression of *Per1*, *Per2* and *Cry1*. Melatonin also phase-delayed plasma corticosterone rhythm and increased the amplitude of plasma corticosterone and TSH rhythms. The results indicate that under a prolonged duration of a daily melatonin signal, rat pituitary PRL synthesis and release are depressed together with significant changes in the redox and circadian mechanisms controlling them.

Key words: Seasonality; Free radicals; Corticosterone; TSH; LH; Testosterone; Metallothioneins

INTRODUCTION

Animals inhabiting temperate areas where there are drastic changes in the environment show dramatic adaptative seasonal changes in physiology. One example is reproduction, confined to occur within limited time periods to ensure that young are born and raised in optimal environmental conditions (Chemineau et al., 2008; Lehman et al., 2010; Scherbarth and Steinlechner, 2010; Yoshimura, 2010). The role of melatonin in regulating this seasonal variation in animal physiology and behavior is supported by considerable scientific evidence (Reiter, 1980; Reiter et al., 2009). Experiments on long day breeders like the Syrian hamster, in which a prolonged melatonin phase induced gonadal regression (Bartness et al., 1993), and on short day breeders like the sheep, in which a prolonged melatonin signal elicits gonadal activity (Bittman and Karsch, 1984), indicate that melatonin is the primary neuroendocrine timing signal encoding information on the length of the night.

When domesticated, the lack of a well defined breeding season arises presumably because the 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*) (Mock and Frankel, 1978; Cohen and Mann, 1979). When housed under a controlled environment, rats display seasonal variation in reproductive capability (Lee and McClintock, 1986), pituitary hormones and sex steroids and sexual maturation (Wallen and Turek, 1981; Wong et al., 1983; Shishkina et al., 1993; Utembaeva et al., 2009; Díaz et al., 2012). Several other physiologic parameters exhibit circannual rhythmicity in the rat, among them hypothalamic content of CRF, TRH, neurotensin, and neuromedin (Bissette et al., 1995) and plasma levels of TSH and tri-iodothyronine (Wong et al., 1983), corticosteroid-binding globulin levels (Tinnikov and Oskina, 1994), pineal somatostatin content, 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 and 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 the suprachiasmatic nucleus (SCN) (Gerics et al., 2006); resistance to hypoxia (Khachatur'yan and 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 acids and lipoprotein cholesterol levels (Masumura et al., 1992).

There is ample evidence that prolactin (PRL) is, among the endocrine signals, a major mediator of seasonal adaptations (Steger and Bartke, 1995; Duncan, 2007). 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 a seasonal pattern of PRL secretion

participates in neuroendocrine sensitivity to changing photoperiod as well as in the seasonal-related changes in animals kept under regular light/dark (L/D) cycles (Lincoln et al., 2003).

Since the administration of melatonin in drinking water is an equivalent to expose the animals to short daily photoperiod in terms of a prolonged duration of the melatonin signal (Bittman and Karsch, 1984; Bartness et al., 1993), 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 (Velárdez et al., 2000; Quinteros et al., 2007) we wished to correlate the 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: 1) to examine the effect of melatonin administration in the drinking water to rats for a month on the correlation of the 24-h patterns of PRL gene expression and PRL release by the anterior pituitary; 2) to analyze the 24-h changes in PRL synthesis and release *viz-a-viz* the 24-h pattern of pituitary lipid peroxidation and of redox enzyme, metallothionein (MT) and circadian clock gene expression; 3) to correlate the 24-h changes in PRL synthesis and release with the daily pattern in circulating TSH, LH, testosterone and corticosterone levels.

MATERIALS AND METHODS

Animals and Experimental Design

Male Wistar rats (45 days of age) were kept under standard conditions of controlled light (12:12 h L/D schedule; lights on at 08:00 h) and temperature (22 ± 2 C). Half of the animals received melatonin (3 μg/mL) in drinking water. The stock solution of melatonin was prepared in 50 % ethanol; final ethanol concentration in drinking water was .015 %. Vehicle-treated controls received .015 % ethanol in drinking water. Drinking water bottles were changed every other day. Since rats drank about 20 mL/day with 90-95% of this total daily water taken up during the dark period, the melatonin dosage used provided approximately 60 μg melatonin/day. This dose was about 10 times 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 month, groups of 6-8 rats were euthanized by decapitation under conditions of minimal stress at 6 different time intervals, every 4 h throughout a 24-h cycle, starting at 09:00 h. At night intervals animals were killed under red dim light. The 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 further assayed.

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 study was in accordance with the guidelines of the

Institutional Care and Use Committee of the National Institute on Drug Abuse, National Institutes of Health and the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996).

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 SA; Madrid) was used to synthesize cDNA from 1 μg of total RNA, according to the manufacturer's protocol. The house keeping 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 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 genes 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 AG, 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 in order 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 and Schmittgen, 2001). All samples were analyzed in triplicate and in three different measures. Fractional cycle at which the amount of amplified target becomes significant (Ct) was automatically calculated by the PCR device.

To estimate whether treatment or time of day modified the expression of anterior pituitary β-actin, PCR with serial dilutions of this housekeeping gene was performed. In this study Ct did not vary significantly as a function of treatment or of time of day, indicating the validity to employ β-actin as a housekeeping gene.

Lipid Peroxidation

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

Hormone Assays

Plasma melatonin levels were measured by 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 were expressed as pg/mL.

Plasma PRL TSH and LH levels were measured by a homologous specific double antibody RIA, using materials kindly supplied by the NIDDK's National Hormone and Pituitary Program. The 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, and using the NIDDK rat PRL RP-3, rat-TSH-RP-3 and rat LH-RP-3, respectively. Results were expressed as ng/mL (PRL, TSH) or pg/mL (LH) (García Bonacho et al., 2000; Castrillón et al., 2001). Plasma testosterone levels were measured using a commercial kit (ICN Pharmaceuticals, Inc., Costa Mesa, CA, USA). Sensitivity of the assay was .2 ng/mL and the intraassay coefficient of variation was 5%, as previously described (García Bonacho et al., 2000); results were expressed as ng/mL. Plasma corticosterone was assayed by a specific RIA obtained from Labor Diagnostika Nord GmbH & Co., Nordhorn, Germany. The intra- and inter-assay coefficients of variation were 6 and 8%, respectively. Sensitivity of the RIA was 25 ng of corticosterone/ml; results were expressed as ng/mL.

Data Analysis

After verifying normality of distribution of data, the statistical analysis of the results was performed by a one-way or a two-way factorial analysis of variance (ANOVA) followed by Bonferroni´s multiple comparison tests or by Student´s t tests, as stated. A cosinor analysis of mean values of the several parameters examined (N=6 in each case) was used. A cosinor analysis of the mean values at each time series (n= 6) was performed to calculate the acrophase (the maximum of the cosine function fit to the experimental data) and amplitude (half the difference between maximal and minimal values of the derived 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 estimate of the 24-h time series mean) and R^2 statistical validity the total number of individual values was considered. P values lower than .05 were taken as evidence for statistical significance.

RESULTS

As shown in Fig. 1, peak melatonin level in plasma of vehicle-treated rats was 350 ± 79 pg/mL at 0100 h, with photophase and early scotophase values < 30 pg/mL. In the case of rats drinking the melatonin solution, concentrations attained at the 3 scotophase time intervals examined (4310 ± 715, 4120 ± 798 and 5230 ± 6990 pg/mL at 2100, 0100 and 0500 h, respectively) did not differ significantly among them. In melatonin-treated animals values higher than 2000 pg/mL were detected from *ca.* 20:00 to 09:00 h (Fig. 1). Acrophases in 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 on plasma PRL levels. When analyzed as a main factor in a factorial ANOVA, melatonin had a 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 (Fig. 2). Melatonin treatment also decreased plasma PRL by 31 %

(F= 7.97, p< .01) with a significant interaction "melatonin x time of day" (F= 2.31, p< .05) in the factorial ANOVA, the changes in circulating PRL becoming significant at the photophase only (Fig. 2). In a cosinor analysis, acrophases for pituitary PRL gene expression and plasma PRL levels occurred at middle of scotophase at approximately the same time intervals in both experimental groups (Table 3).

Data from Fig. 3 and 4 and Tables 4 and 5, summarize the daily changes in pituitary gene expression of enzymes in the redox pathway and of MT-1 and MT-3, and in pituitary lipid peroxidation in rats administered with melatonin. When analyzed as a main factor in the factorial ANOVA, melatonin treatment decreased by 95 and 16 % NOS-2 and HO-1 mRNA levels (F= 567 and 12.8, p< .001, respectively, Fig. 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) (Fig. 3 and 4). Except for lipid peroxidation, significant interactions "melatonin treatment x time of day" were found for every pituitary redox parameter tested in the factorial ANOVA (NO-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, respectively) (Fig. 3 and 4). Cosinor analysis is 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 (Fig. 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 Fig. 2.

Figure 5 and Table 6 show the effect of melatonin on 24-h pattern of pituitary circadian clock gene expression. Significant time-related changes in clock gene expression were found in controls. *Clock* and *Bmal1* expression peaked at mid scotophase (acrophases at 02:52 and 03:14 h, respectively) while that of *Per1* and *Per2*, and of *Cry1* and *Cry2,* peaked at mid and late photophase (acrophases at 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 decreased mean expression of *Per1* by 18 % (F= 3.83, p< .05) and rise mean expression of *Per2, Cry1* and *Cry2* by 86, 20 and 15 % (F= 4.71, p< .03; F= 6.54, p< .02; F= 8.92, p< .001, respectively). In the cosinor analysis, melatonin treatment phase-delayed expression of *Per1*, *Per2* and *Cry1* by approximately 6-10 h to attain acrophases at 17:19, 21.11 and 23:55 h, respectively (Table 6).

The effect of melatonin on 24-h pattern of rat plasma TSH, LH, testosterone and corticosterone concentration is shown in Fig. 6 and Table 7. Mean values of TSH augmented by 37 %, and those of LH and testosterone decreased by 41 and 33 % (F= 14.3, p< .001; F= 7.57, p< .01; F= 3.45, p< .05, respectively, main factor analysis, factorial ANOVA). As shown by cosinor analysis the acrophase of plasma corticosterone rhythm in controls (17:28 h) was phase-delayed approximately 4 h by

melatonin (21:10 h) (Table 7). In addition, melatonin treatment augmented significantly rhythm amplitude of plasma corticosterone and TSH (Fig. 6 and Table 7).

DISCUSSION

In the present study we administered melatonin in 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. The doses of melatonin employed (*ca.* 60 μg/day) resulted in peak plasma values of melatonin that were about 15 times higher than those found in vehicle-treated controls. More importantly, high concentrations of melatonin (more than 2000 pg/mL) were detected in melatonin-treated rats from the late photophase to early photophase as compared to a considerably narrower high melatonin phase observed in controls (Fig. 1).

Under these experimental conditions a profound inhibitory effect on pituitary PRL gene expression and circulating PRL levels was observed in melatonin-treated animals, with a reduction of about half in mesor values in the cosinor analysis. In contrast, acrophases 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, the inhibition of PRL gene expression was significant throughout a 24-h cycle in melatonin-treated rats, while circulating PRL decreased significantly during the photophase only. The results support the view that the laboratory rat, regardless of being developed under constant conditions of temperature, lighting and food availability, did maintain the capability to respond to an increase in the duration and magnitude of the melatonin signal with an inhibition of PRL synthesis and release, as shown in long day breeders like 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 a 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 and Lynch, 1981). The exact site of action of melatonin in brain responsible for its seasonal effect was 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 much 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.

Strong evidence now supports the concept that in order to regulate the seasonal PRL rhythm, the melatonin signal does target the pars tuberalis, and not the pars distalis or the hypothalamic tuberoinfundibular dopaminergic neurons [see for ref. Dardente (2007), Dupre (2011)]. Despite transient expression of melatonin receptors in rat neonatal pars distalis gonadotrophs (Johnston et al., 2003), co-localization

studies have shown that these receptors are only expressed in the pars tuberalis (Morgan et al., 1994) at specific thyrotroph cells in adult rodents (Klosen et al., 2002). The restriction of melatonin receptor expression to the pars tuberalis in the adult pituitary, together with the inability of melatonin to directly regulate either PRL gene expression or secretion in pars distalis cultures (Stirland et al., 2001), are consistent with the hypothesis that melatonin drives changes in PRL secretion via altered secretion of a pars tuberalis-derived PRL secretagogue(s) (Dardente, 2007; Dupre, 2011).

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 ref. Mancuso et al. (2007). Locasale and Cantley (2011), Jacob (2011), Winyard et al. (2011)]. The detoxification of ROS in cells involves the cooperative action of intracellular antioxidant enzymes, among them Cu/Zn-SOD that is cytosolic, Mn-SOD that is mitochondrial, and catalase that is present in peroxisomes. In addition, GPx and GSR help to 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 non-enzymatic systems (Locasale and Cantley, 2011; Jacob, 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 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 the 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 the 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 middle afternoon to early scotophase. These significant changes in gene expression of several components of the 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.

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 (Hardeland et al., 2011; Galano 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 like nuclear factor-κB, a decrease of free radical production by melatonin would lead to repression of redox-sensitive transcription factors regulating gene transcription (Lezoualc'h et al., 1998; Rodríguez et al., 2004; Beni et al., 2004). Collectively, the present results on redox enzyme gene expression underline a significant effect of melatonin, either direct or pars tuberalis-mediated on expression of pituitary redox enzymes involved in PRL secretion. In this respect, NO presumably plays a major role (Velárdez et al., 2000; Quinteros et al., 2007). 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. The 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, while 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).

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 a 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 a 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, like *Clock* and *Bmal1*, induce the expression of their own repressors (*Per1-3* and *Cry 1-2*) 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, while acrophases of *Clock* and *Bmal1* expression were at middle scotophase. Such a reciprocal relation was reported in SCN as well as in some peripheral tissues (Lincoln et al., 2006; Levi et al., 2010).

Treatment with melatonin decreased mean expression of *Per1* and raises that of *Per2, Cry1* and *Cry2.* Melatonin treatment phase-delayed expression of *Per1*, *Per2* and *Cry1* by approximately 6-10 h. These changes fit well the phase-delayed gene expression of most redox enzymes detected in anterior pituitary as well as with the phase-delay found in plasma corticosterone rhythm. The amplitude of plasma corticosterone and TSH augmented after melatonin 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 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 and Bernstein, 2003). Studies including trasfection 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 a 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 release 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 diurnal 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.

Declaration of Interest

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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	

Table 2. Cosinor analysis of plasma melatonin levels in rats receiving melatonin (3 μg/ml) in drinking water for 1 month.

	Mesor (pg/mL)	**Amplitude (pg/mL)**	**Acrophase (hour, minutes)**	**R^2, probability**
Melatonin-treated	3055 ± 201 *	2252 ± 557 *	01:33 ± 00:58	.84, p< .001
Vehicle-treated	122 ± 16	151 ± 52	02:19 ± 01:19	.74, p< .001

Shown are the means ± SEM. * p< 0.001 vs. vehicle-treated rats, Student´s t test. R^2 values and their probability are also shown. Mean values at each time series in Fig. 1 (n= 6) was used to calculate the acrophase and amplitude of rhythms. To calculate the mesor and R^2 statistical validity the total number of individual values was considered (n= 44-48).

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

	Mesor	**Amplitude**	**Acrophase (hour, minutes)**	**R^2, probability**
Melatonin-treated rats				
PRL relative gene expression	.56 ± .03 *	.16 ± .07	01:51 ± 01:36	.65, p< .01
Plasma PRL levels (ng/mL)	6.58 ± 0.73 *	5.10 ± 1.49	01:11 ± 01:08	.79, p< .001
Vehicle-treated rats				
PRL relative gene expression	.92 ± .03	.22 ± .06	00:48 ± 00:58	.84, p< .001
Plasma PRL levels (ng/mL)	9.57 ± 0.28	4.28 ± 2.24	01:00 ± 02:00	.55, p< .01

Shown are the means ± SEM. * p< 0.001 vs. vehicle-treated rats, Student´s t test. R^2 values and their probability are also shown. Mean values at each time series in Fig. 2 (n= 6) was used to calculate the acrophase 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.

Table 4. Cosinor analysis of the effect of melatonin on 24-hour 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 (hour, minutes)**	**R^2, probability**
Melatonin-treated rats				
NOS-1	1.23 ± .06 **	-	-	.06, N.S.
HO-1	.81 ± .04 *	.01 ± .09 **	02:34 ± 22:30 **	.96, p< .001
GPx	1.62 ± .03 *	.68 ± .23	21:37 ± 01:16 *	.75, p< .01
NOS-2	.05 ± .001 **	.01 ± .01 **	22:45 ± 02:05	.53, p< .02
HO-2	1.49 ± .07 **	.41 ± .02	01:59 ± 01:54 **	.57, p< .01
GSR	1.48 ± .06 **	.21 ± .09	20:50 ± 01:43	.63, p< .01
Vehicle-treated rats				
NOS-1	1.04 ± .03	.17 ± .05	11:07 ± 01:06	.81, p< .001
HO-1	.96 ± .06	.27 ± .11	16:26 ± 01:31	.68, p< .01
GPx	1.40 ± .08	.23 ± .19	23:12 ± 03:09	.33, p< .05
NOS-2	.93 ± .05	.46 ± .22	12:24 ± 01:46	.61, p< .01
HO-2	1.06 ± .09	-	-	.12, N.S.
GSR	1.26 ± .05	.27 ± .07	19:06 ± 00:57	.84, p< .001

Shown are the means ± SEM. * p< .02, ** p< .01 vs. vehicle-treated rats, Student´s t test. R^2 values and their probability are also shown. Mean values at each time series in Fig. 3 (n= 6) was used to calculate the acrophase and amplitude of rhythms. To calculate the mesor 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-hour 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 (hour, minutes)**	**R^2, probability**
Melatonin-treated rats				
Cu/Zn SOD	1.43 ± .08	.43 ± .18	20:46 ± 01:33	.67, p< .02
Mn SOD	1.63 ± .06 *	.51 ± .13	20:24 ± 00:59 **	.84, p< .01
Catalase	1.31 ± .09	-	-	.03, N.S.
MT-1	1.01 ± .06	.31 ± .06	12:34 ± 00:44	.90, p< .001
MT-3	1.06 ± .04 *	.40 ± .08	11:07 ± 00:48	.88, p< .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, p< .001
Mn SOD	1.43 ± .06	.39 ± .10	02.03 ± 01:00	.83, p< .001
Catalase	1.31 ± .10	028 ± .14	15:14 ± 01:54	.58, p< .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, p< .04

Shown are the means ± SEM. * p< .04, ** p< .01 vs. vehicle-treated rats, Student´s t test. R^2 values and their probability are also shown. Mean values at each time series in Fig. 4 (n= 6) was used to calculate the acrophase 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.

Table 6. Cosinor analysis of the effect of melatonin on 24-hour 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 (hour, minutes)**	**R^2, probability**
Melatonin-treated rats				
Clock	1.06 ± .08	.19 ± .13	02:56 ± 02:41	.41, p< .03
Bmal1	.99 ± .06	.44 ± .23	05:44 ± 02:00	.55, p< .02
Per1	1.26 ± .07 **	.31 ± .17	17:19 ± 02:09 *	.51, p< .02
Per2	2.14 ± .16 **	.99 ± .51	21.11 ± 01:58 *	.56, p< .02
Cry1	1.53 ± .07 **	.52 ± .11	23:59 ± 00:46 *	.89, p< .001
Cry2	1.47 ± .05 **	.29 ± .11	18:59 ± 01:27	.71, p< .01
Vehicle-treated rats				
Clock	1.19 ± .09	.31 ± .11	02:52 ± 01:19	.74, p< .01
Bmal1	1.16 ± .08	.64 ± .26	03:14 ± 01:31	.68, p< .01
Per1	1.52 ± .08	.49 ± .23	10:11 ± 01:47	.61, p< .02
Per2	1.15 ± .07	.23 ± .11	11.14 ± 01:47	.60, p< .02
Cry1	1.27 ± .06	.24 ± .19	18:20 ± 03:05	.34, p< .05
Cry2	1.28 ± .03	.27 ± .04	19:03 ± 00:31	.95, p< .001

Shown are the means ± SEM. * p< .03, ** p< .01 vs. vehicle-treated rats, Student´s t test. R^2 values and their probability are also shown. Mean values at each time series in Fig. 5 (n= 6) was used to calculate the acrophase 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-hour changes in circulating TSH, LH, testosterone and corticosterone levels.

	Mesor	**Amplitude**	**Acrophase (hour, minutes)**	**R^2, probability**
Melatonin-treated rats				
TSH	3.17 ± .17 **	1.08 ± .33 *	15:00 ± 01:52	.58, p< 0.01
LH	275.5 ± 13.5 **	86.4 ± 33.2	03:11 ± 01:28	.69, p< 0.01
Testosterone	.60 ± .04 **	.16 ± .15	12:52 ± 03:31	.28, p< 0.05
Corticosterone	456.6 ± 25.8	310.6 ± 46.8 *	21:10 ± 00:48 *	.88, p< 0.001
Vehicle-treated rats				
TSH	2.31 ± .08	.32 ± .08	14:04 ± 03:23	.30, p< 0.04
LH	462.0 ± 22.0	182.4 ± 31.1	02:41 ± 00:39	.92, p< 0.001
Testosterone	.89 ± .06	-	-	.21, N.S
Corticosterone	567.7 ± 24.7	168.5 ± 35.6	17:28 ± 00:48	.86, p< 0.001

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 their probability are also shown. Mean values at each time series in Fig. 6 (n= 6) was used to calculate the acrophase 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.

FIGURE LEGENDS

FIGURE 1

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

FIGURE 2

Effect of melatonin on 24-h pattern in adenohypophysial PRL gene expression and plasma PRL levels in rats. The rats received melatonin (3 μg/ml) or vehicle in drinking water for 1 month. Groups of 6-8 rats were euthanized by decapitation at 6 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 to vehicle in 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.

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 Fig. 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 to vehicle in 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: $F = 4.56$, $p < .01$; $F = 8.14$, $p < .001$; $F = 8.53$, $p < .001$; $F = 27.4$, $p < .001$; $F = 36.3$, $p < .001$; $F = 2.73$, $p < .05$, respectively. Melatonin: $F = 3.54$, $p < .03$; $F = 3.68$, $p < .02$; $F = 34.6$, $p < .001$; $F = 6.09$, $p < .003$; $F = 10.7$, $p < .001$; $F = 5.44$, $p < .001$, respectively). For further statistical analysis, see text.

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 Fig. 2. Lipid peroxidation was assessed by the thiobarbituric acid reactive substances procedure as described in Methods. Shown are

the means ± SEM. Bars indicate scotophase duration. Asterisks denote significant differences as compared to control in 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 and 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). 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. The experimental details are given in the legend to Fig. 2. Adenohypophysial mRNA 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. Bars indicate scotophase duration. Asterisks denote significant differences as compared to vehicle in 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 as follows: 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; 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; F= 8.61, p< .001, respectively). For further statistical analysis, see text.

FIGURE 6

Effect of melatonin on 24-h pattern in rat plasma TSH, LH, testosterone and corticosterone levels. The rats received melatonin (3 µg/ml) or vehicle in drinking water for 1 month. Groups of 6-8 rats were euthanized by decapitation at 6 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 to vehicle in 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- (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). For further statistical analysis, see text.

Figure 1

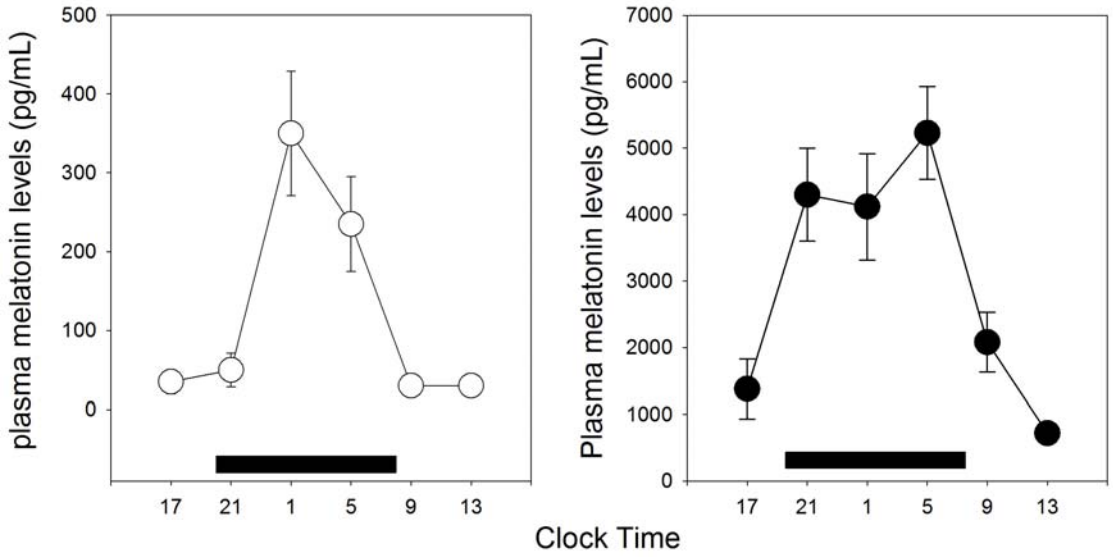

plasma melatonin levels (pg/mL)
500
400
300
200
100
0
17
21
1
5
9
13
Plasma melatonin levels (pg/mL)
7000
6000
5000
4000
3000
2000
1000
0
17
21
1
5
9
13
Clock Time

Figure 2

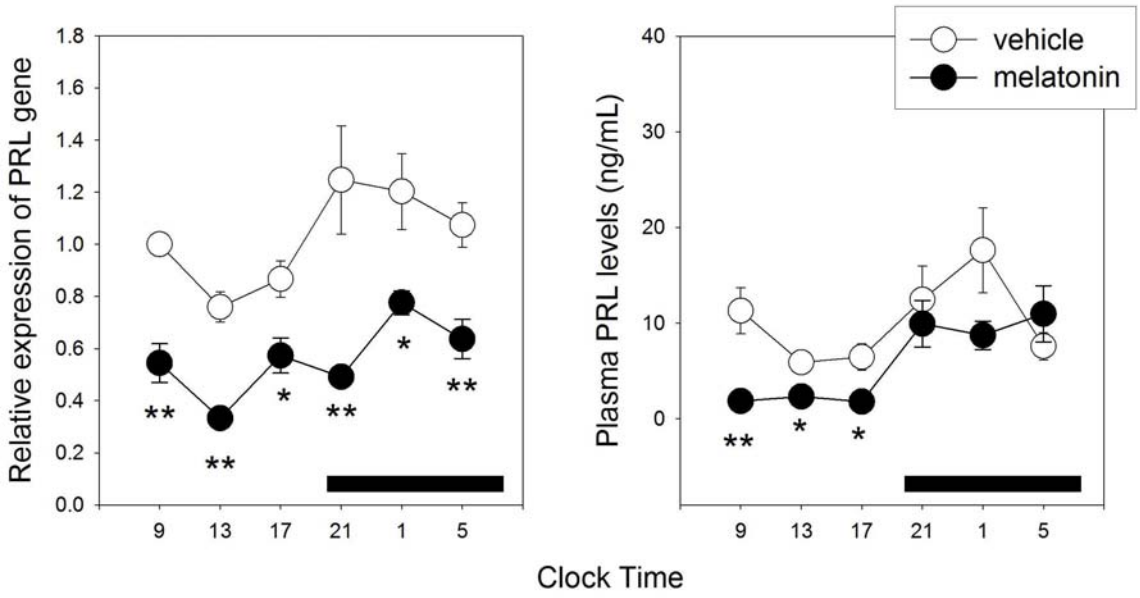

Relative expression of PRL gene
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
9
13
17
21
1
5
**
**
*
**
*
**
Plasma PRL levels (ng/mL)
40
30
20
10
0
vehicle
melatonin
**
*
*
Clock Time

Figure 3

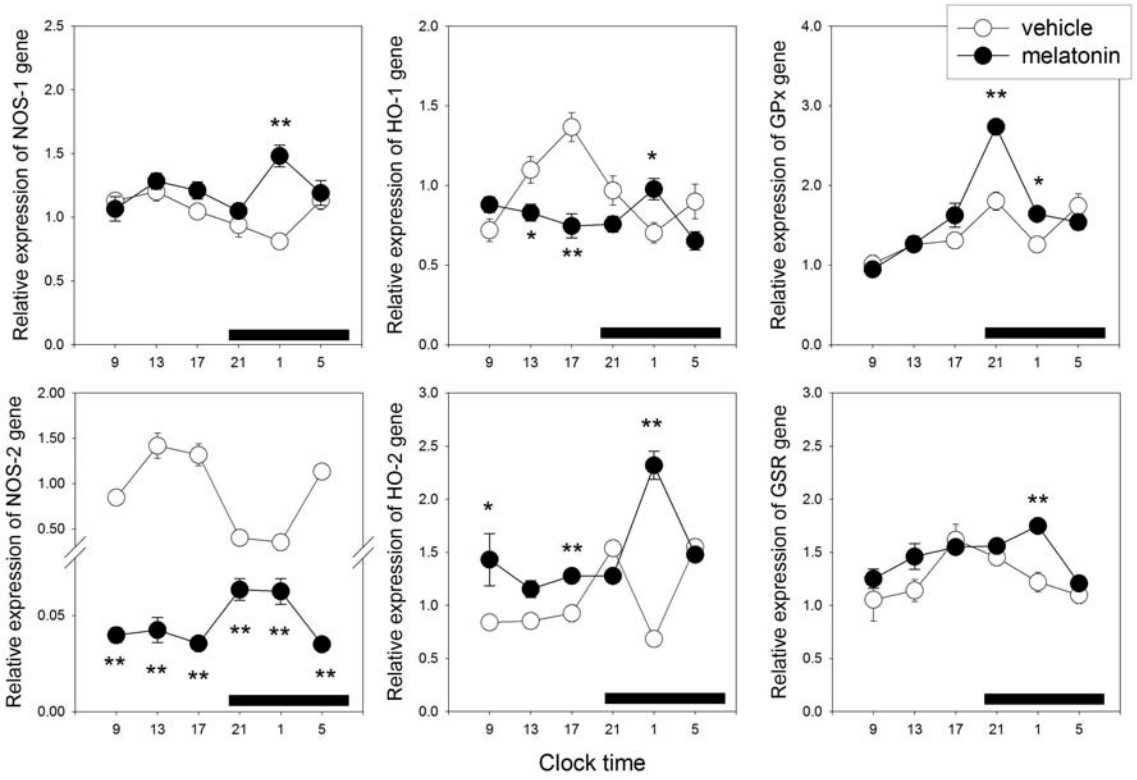

vehicle
melatonin
Relative expression of NOS-1 gene
Relative expression of HO-1 gene
Relative expression of GPx gene
Relative expression of NOS-2 gene
Relative expression of HO-2 gene
Relative expression of GSR gene
Clock time

Figure 4

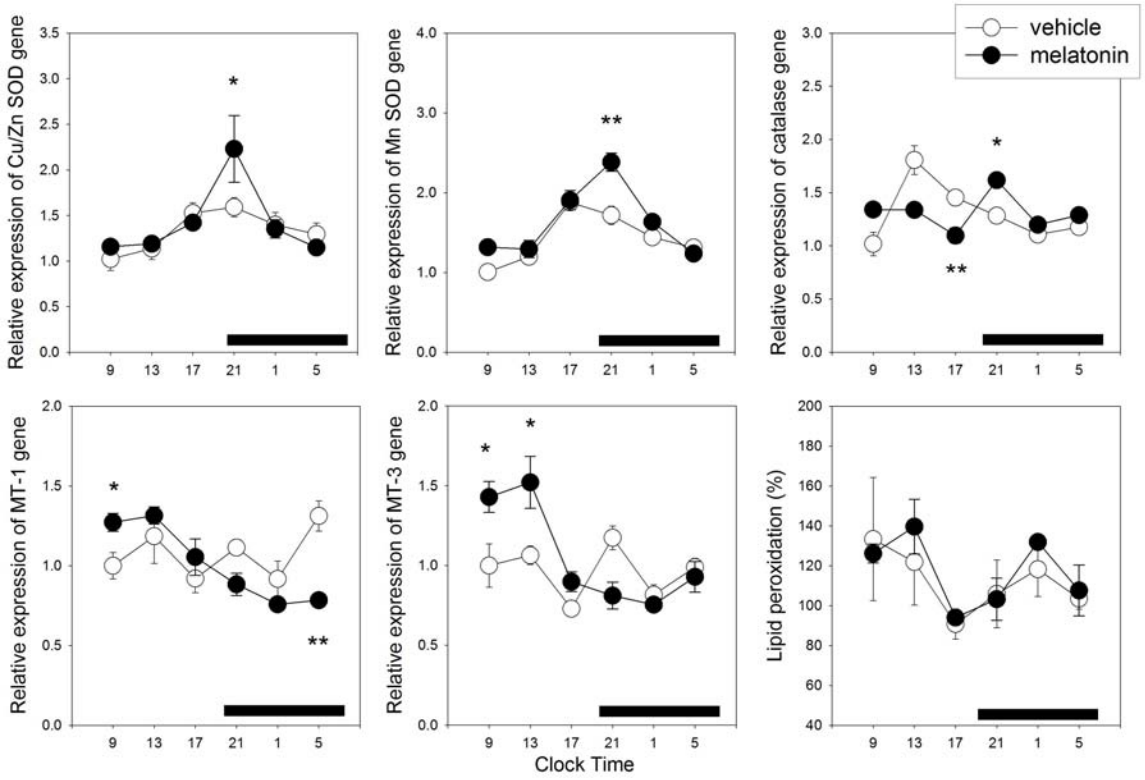

vehicle
melatonin
Relative expression of Cu/Zn SOD gene
Relative expression of Mn SOD gene
Relative expression of catalase gene
Relative expression of MT-1 gene
Relative expression of MT-3 gene
Lipid peroxidation (%)
Clock Time
9
13
17
21
1
5

Figure 5

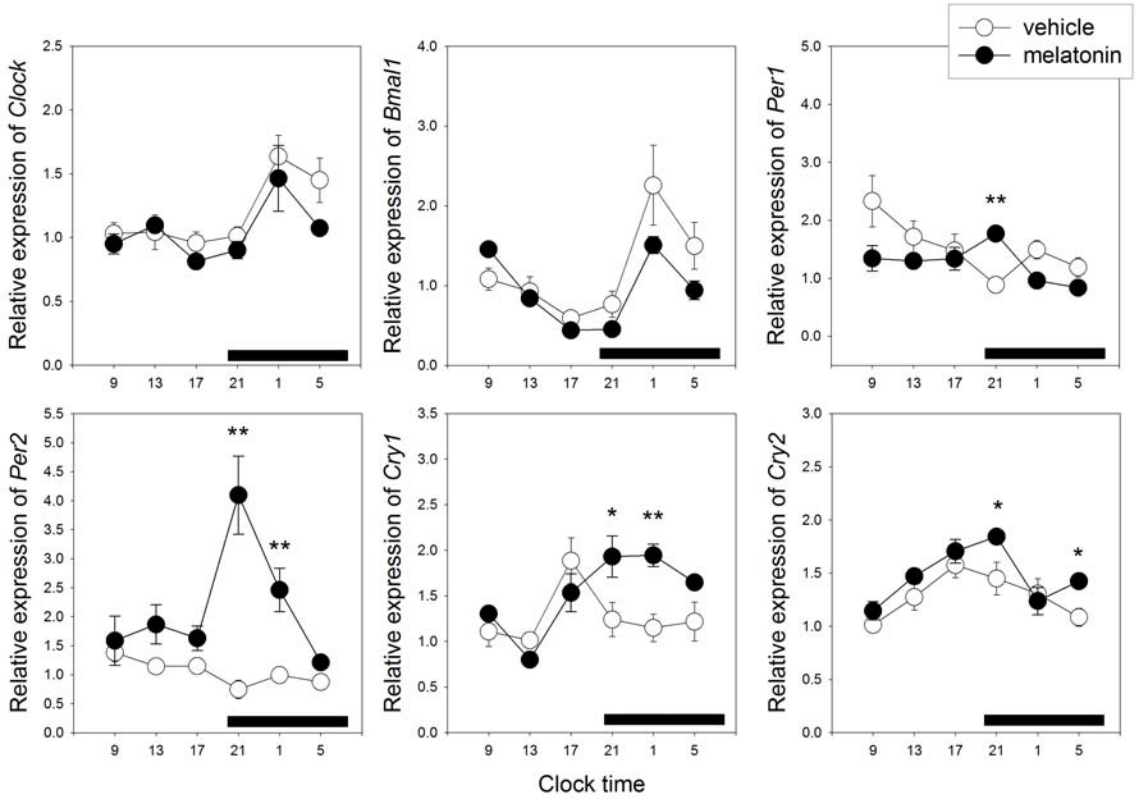
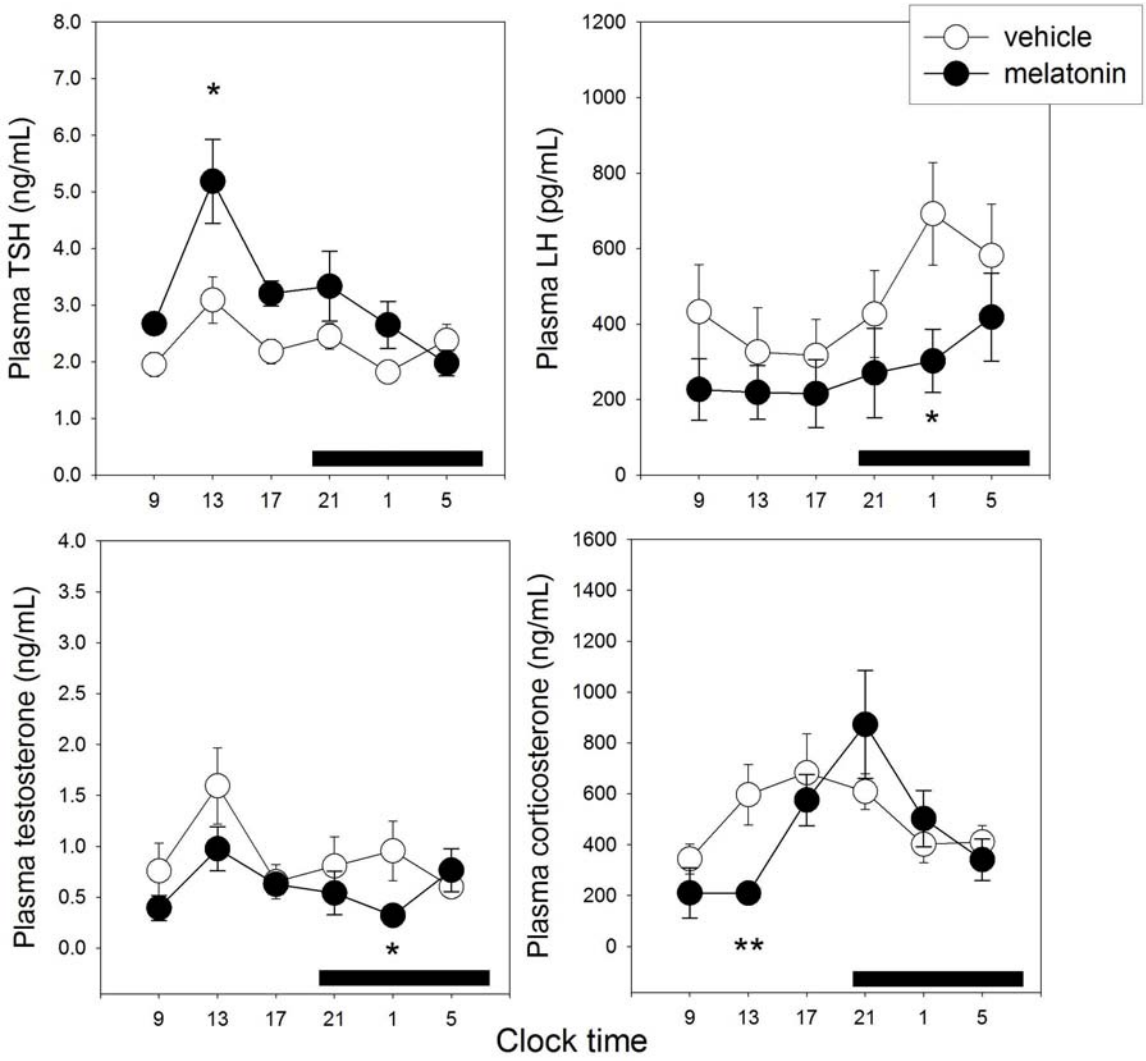

vehicle
melatonin
Relative expression of Clock
Relative expression of Bmal1
Relative expression of Per1
Relative expression of Per2
Relative expression of Cry1
Relative expression of Cry2
Clock time
9
13
17
21
1
5
*
**

Figure 6


vehicle
melatonin
Plasma TSH (ng/mL)
Plasma LH (pg/mL)
Plasma testosterone (ng/mL)
Plasma corticosterone (ng/mL)
Clock time
9
13
17
21
1
5
*
**