Free Radical Biology and Medicine 53 (2012) 2287–2297

Contents lists available at SciVerse ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original Contribution

Cadmium as an endocrine disruptor: Correlation with anterior pituitary redox and circadian clock mechanisms and prevention by melatonin

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A R T I C L E I N F O

Article history:
Received 13 June 2012
Received in revised form
9 October 2012
Accepted 10 October 2012
Available online 17 October 2012

Keywords:
Cadmium
Prolactin
Redox state
Circadian rhythms
Gene expression
Melatonin
LH
TSH
Corticosterone
Free radicals

A B S T R A C T

To examine the effect of a low dose of cadmium (Cd) as an endocrine disruptor, male Wistar rats received $CdCl_2$ (5 ppm Cd) in drinking water or drinking water alone. After 1 month, the rats were euthanized at one of six time intervals around the clock and the 24-h pattern of adenohypophysial prolactin (PRL) synthesis and release, lipid peroxidation, and redox enzyme and metallothionein (MT) gene expression was examined. Cd suppressed 24-h rhythmicity in expression of the PRL gene and in circulating PRL by increasing them at early photophase only, in correlation with an augmented pituitary lipid peroxidation and redox enzyme expression. $CdCl_2$ treatment effectively disrupted the 24-h variation in expression of every pituitary parameter tested except for MT-3. In a second experiment the effect of melatonin (3 μg/ml in drinking water) was assessed at early photophase, the time of maximal endocrine-disrupting effect of Cd. Melatonin treatment blunted the effect of Cd on PRL synthesis and release, decreased Cd-induced lipid peroxidation, and counteracted the effect of Cd on expression of most redox enzymes. A third experiment was performed to examine whether melatonin could counteract Cd-induced changes in the 24-h pattern of pituitary circadian clock gene expression and plasma PRL, luteinizing hormone (LH), thyrotropin (TSH), and corticosterone levels. Rats receiving $CdCl_2$ exhibited a suppressed daily rhythm of *Clock* expression and a significant disruption in daily rhythms of pituitary *Bmal1*, *Per1*, *Per2*, *Cry1*, and *Cry2*. The coadministration of melatonin restored rhythmicity in *Clock* and *Bmal1* expression but shifted the maxima in pituitary *Per1*, *Cry1*, and *Cry2* expression to the scotophase. Melatonin also counteracted the effect of Cd on 24-h rhythmicity of circulating PRL, LH, TSH, and corticosterone. The results highlight the occurrence of a significant endocrine disruptor effect of a low dose of Cd. Generally melatonin counteracted the effects of Cd and ameliorated partially the circadian disruption caused by the pollutant.

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Introduction

The heavy metal cadmium (Cd) is one of the most toxic industrial and environmental metals and acts as an endocrine disruptor in humans and rodents [1]. Cd is ranked 8th in the top 20 hazardous substances; it is released into water as a by-product of smelting, into air by combustion of coal and oil, and into soils as impurities. The main uses of Cd are for nickel–cadmium battery manufacture, pigments, and plastic stabilizers [2].

Neuroendocrine and neurobehavioral disturbances in animals and humans caused by endocrine disruptors are suspected to be implicated in the recent declining fertility in developed countries [1,3]. Cd is recognized as an endocrine disruptor that modifies, among others, prolactin (PRL) secretion in a number of species, including humans [4–11]. Cd is readily absorbed and retained in the pituitary gland of rats [12,13] and affects lactotroph cell activity, causing biochemical, genomic, and morphological changes [7]. In the rat, the effect of orally administered $CdCl_2$ on PRL release is dose- and time-dependent [14]. A high dose of Cd inhibits PRL release both in vivo and in vitro [8,14–16], whereas low doses of Cd are stimulatory in the early morning hours and inhibitory later on [9].

The objective of this study was to examine the effect of a low dose of Cd as an endocrine disruptor in male rats. Specifically we

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http://dx.doi.org/10.1016/j.freeradbiomed.2012.10.533

aimed to answer the following questions: (i) Is the 24-h variation in pituitary PRL synthesis and release affected by Cd? (ii) Does the effect of Cd on PRL synthesis correlate with pituitary redox status as assessed by measuring lipid peroxidation and redox enzyme and metallothionein (MT) gene expression? (iii) Is the 24-h expression of circadian clock genes in the anterior pituitary modified by $CdCl_2$ treatment? (iv) Can Cd act as an endocrine disruptor to affect 24-h variations in plasma luteinizing hormone (LH), thyrotropin (TSH), and corticosterone levels? (v) Is melatonin effective at palliating the activity of Cd as an endocrine disruptor? The rationale for employing melatonin relied on its demonstrable chronobiotic [17,18] and cytoprotective activities [19]. We had previously reported melatonin efficacy at preventing the stimulatory effect of Cd on anterior pituitary lipid peroxidation and mRNA levels for nitric oxide synthase (NOS)-1 and -2 and heme oxygenase-1 (HO-1), when examined at two time intervals in the 24-h span [20].

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 light/dark schedule; lights on at 0800 hours; Zeitgeber time (ZT), 00:00) and temperature (22 ± 2 °C). Three experiments were carried out as follows.

In Experiment 1 rats received $CdCl_2$ (8 μg/ml, 5 ppm Cd) in drinking water or drinking water alone (control) for 1 month. The dose of Cd was calculated by using the body surface area normalization method [21] taking into consideration the tolerable limit in humans proposed by the World Health Organization (WHO) (1 μg/day) [22]. After 1 month, groups of six to eight rats were euthanized by decapitation under conditions of minimal stress at six time intervals (every 4 h) throughout a 24-h cycle, starting at ZT 01:00. At night intervals, animals were killed under red dim light. The brains were rapidly removed and the adenohypophysis was quickly dissected out. Trunk blood was collected and the plasma was frozen at −70 °C until further processing.

In Experiment 2 rats were divided into four groups and treated for 1 month as follows: (a) $CdCl_2$ (8 μg/ml) in drinking water; (b) $CdCl_2$ (8 μg/ml) plus melatonin (3 μg/ml) in drinking water; (c) melatonin (3 μg/ml) in drinking water; (d) drinking water alone (control). The stock solution of melatonin was prepared in 50% ethanol, the final ethanol concentration in drinking water being 0.015%. Cd-administered animals and controls received 0.015% ethanol in drinking water. Nocturnal water consumption did not differ among the experimental groups in any of the experiments. After 1 month, groups of six to eight rats were euthanized by decapitation under conditions of minimal stress at ZT 01:00. The brains were rapidly removed and the adenohypophysis was quickly dissected out. Trunk blood was collected and the plasma was frozen at −70 °C until further processing.

In Experiment 3, four groups of rats treated for 1 month as for Experiment 2 were euthanized at the same six time intervals as in Experiment 1. The brains were rapidly removed and the adenohypophysis was quickly dissected out. Trunk blood was collected and the plasma was frozen at −70 °C until further processing.

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, Spain). 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* [23].

Real-time quantitative polymerase chain reaction (qPCR)

Total RNA extraction was performed using the RNeasy Protect minikit and RNA was analyzed using the QuantiTec SYBR green kit (Qiagen, Hilden, 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 specific primers for the genes of PRL, NOS-1 and -2, HO-1 and -2, Cu/Zn and Mn superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), glutathione reductase (GRd), and MT-1 and -3, as well as 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.

PCRs were carried out in an Eppendorf RealPlex Mastercycler (Eppendorf AG, Hamburg, Germany). The real-time qPCR 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 [24]. All samples were analyzed in triplicate and in three different

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	
GRd	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	

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 the expression of anterior pituitary β-actin, PCR employing 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 of employing β-actin as a housekeeping gene.

Lipid peroxidation

Lipid peroxidation was measured in the anterior pituitary by the thiobarbituric acid-reactive substances (TBARS) assay as described elsewhere [20]. Supernatant absorbance (535 nm) was measured. Results were expressed as (absorbance/mg of protein in treated sample)/(absorbance/mg of protein in control sample) × 100.

Plasma Hormone levels

Plasma PRL and LH levels were measured by a homologous specific double-antibody radioimmunoassay (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 pg/ml for both hormones using the NIDDK rat PRL RP-3 and rat LH-RP-3, respectively. Results were expressed as ng/ml (PRL) or pg/ml (LH) [25,26]. Plasma testosterone levels were measured using a commercial kit (ICN Pharmaceuticals, Costa Mesa, CA, USA). Sensitivity of the assay was 0.2 ng/ml and the intra-assay coefficient of variation was 5%, as previously described [25]; results were expressed as ng/ml. Plasma corticosterone was assayed by a specific RIA obtained from Labor Diagnostika Nord (Nordhorn, Germany). The intra- and interassay 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 test or by Student's t test, as stated. 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) [27].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 0.05 were taken as evidence of statistical significance.

Results

Fig. 1 depicts the effect of Cd on the 24-h pattern of expression of the anterior pituitary PRL gene and of plasma PRL levels in male rats. When analyzed as a main factor in a factorial ANOVA, Cd augmented expression of the PRL gene by 24% ($p < 0.03$). A significant interaction of Cd × time of day was found ($p < 0.01$), i.e., the stimulatory effect of Cd on gene expression was seen during the photophase, in particular in the morning hours (Fig. 1). The changes in gene expression brought about by Cd were reflected in a significant increase in circulating PRL at early morning only. In controls only a cosinor analysis indicated that the 24-h changes in PRL gene expression and plasma PRL followed a significant sinusoidal pattern, with acrophases at ZT 09:36 and 14:23, respectively (Table 2).

Data from Figs. 2 and 3 summarize the daily changes in gene expression of enzymes in the redox pathway and in lipid peroxidation in rat adenohypophysis after the administration of Cd. When analyzed as a main factor in the factorial ANOVA, $CdCl_2$ treatment augmented expression of adenohypophysial NOS-1, HO-2, and Cu/Zn SOD by 40, 22, and 25% ($p < 0.01$) and lipid peroxidation by 24% ($p < 0.05$), whereas it decreased expression of GPx by 20% ($p < 0.01$). Significant interactions for Cd treatment × time of day were found for pituitary expression of every redox parameter tested ($p < 0.01$) except for MT-3 expression, indicating that Cd effectively disrupted 24-h variation of redox enzyme expression. Generally, Cd treatment augmented the expression of redox enzymes and lipid peroxidation in the early morning with varying effects on gene expression and absence of significant changes in lipid peroxidation at other time intervals

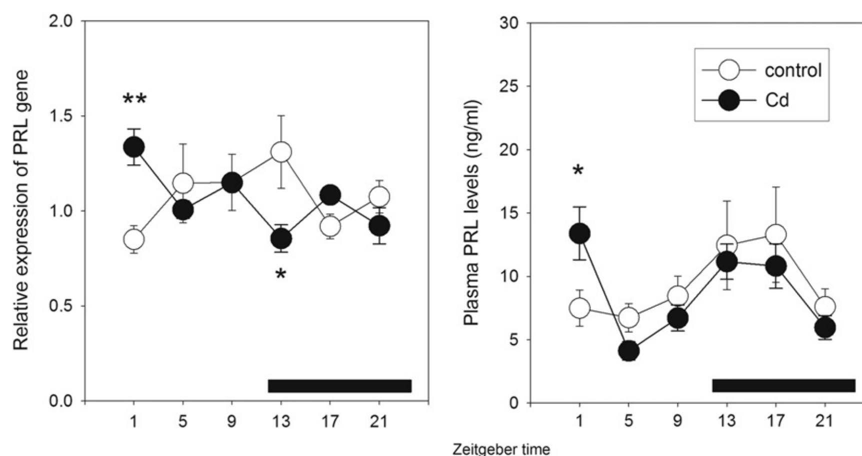

Fig. 1. Effect of cadmium on 24-h pattern in adenohypophysial PRL gene expression and plasma PRL levels in rats. The rats received $CdCl_2$ (8 μg/ml, 5 ppm Cd) in drinking water or drinking water alone for 1 month (control). Groups of six to eight rats were euthanized by decapitation at six time intervals 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 determinations as measured by triplicate real-time PCR analyses of RNA samples and the means ± SEM of plasma PRL levels. *$p < 0.05$, **$p < 0.02$, compared to control in Student's t tests performed at every time interval. The analysis of data by cosinor is summarized in Table 2. For further statistical analysis, see the text.

Table 2
Cosinor analysis of the effect of cadmium on 24-h pattern in adenohypophysial PRL gene expression and plasma PRL levels in rats.

	Mesor	Amplitude	Acrophase (ZT)	R^2, p
Control				
PRL relative gene expression	0.96 ± 0.06	0.17 ± 0.03	09:36 ± 01:13	0.61, < 0.02
Plasma PRL levels (ng/ml)	9.06 ± 1.11	3.36 ± 0.56	14:23 ± 02:18	0.72, < 0.01
Cadmium				
PRL relative gene expression	1.19 ± 0.06*	–	–	0.25, NS
Plasma PRL levels (ng/ml)	8.63 ± 0.98	–	–	0.11, NS

Data are from Fig. 1. Shown are the means ± SEM. R^2 values and their probability are also shown. Mean values at each time series in Fig. 1 (n=6) were used to calculate the acrophase and the 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 the text.

* $p <$ 0.05 vs control, Student's t test.

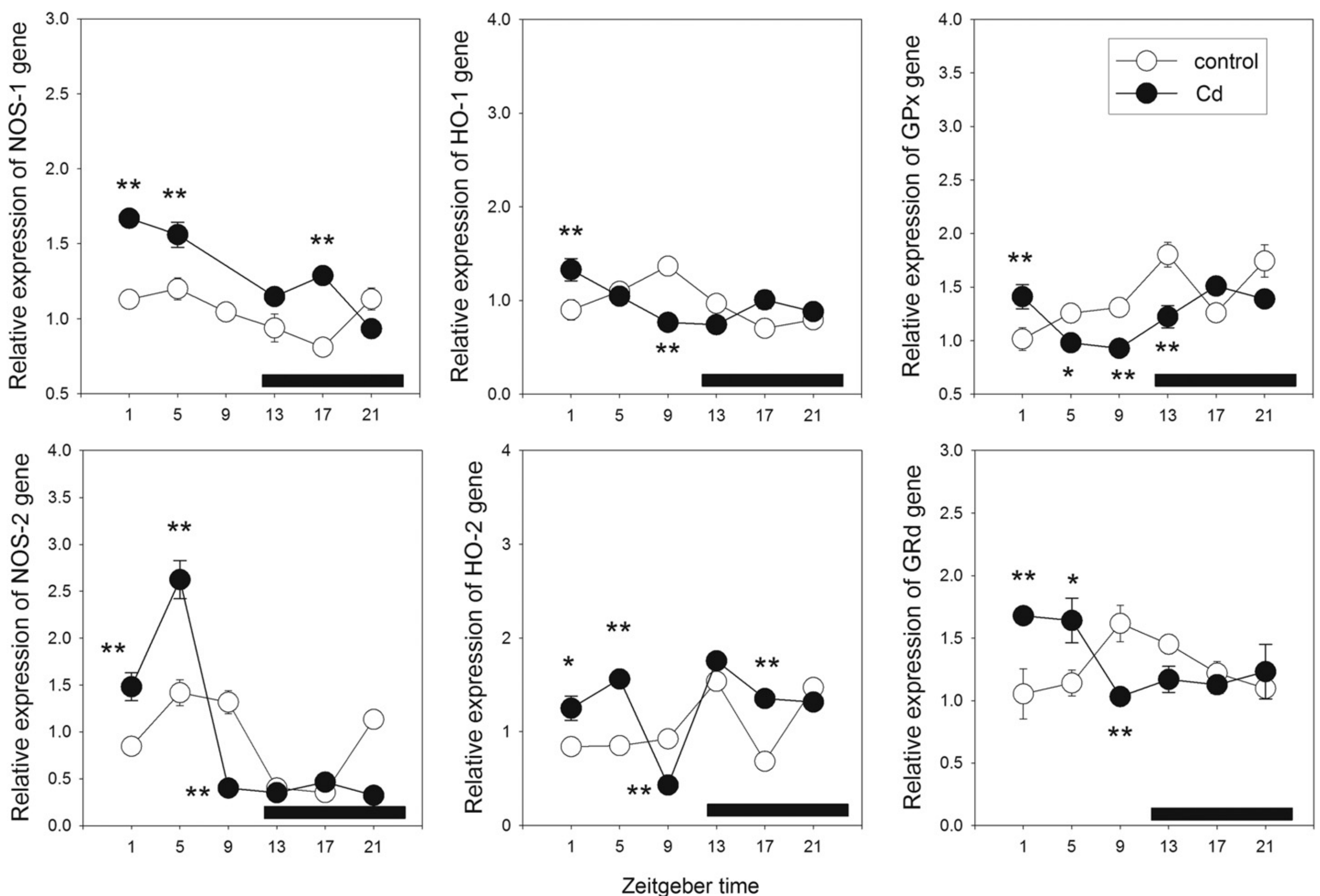

Fig. 2. Effect of cadmium on 24-h pattern in adenohypophysial expression of mRNA for NOS-1, NOS-2, HO-1, HO-2, GPx, and GRd in rats. For experimental details see Fig. 1 legend. mRNA levels encoding the enzymes were measured as described in the text. Shown are the means ± SEM of mRNA determinations as measured by triplicate real-time PCR analyses of RNA samples. *$p < 0.05$, **$p < 0.02$, compared to control in Student's t tests performed at every time interval. The analysis of data by cosinor is summarized in Table 3. For further statistical analysis, see the text.

(Figs. 2 and 3). In the case of MT-1 gene expression, $CdCl_2$ treatment decreased it at late scotophase (Fig. 3).

The data were further analyzed by the cosinor analysis summarized in Tables 3 and 4. $CdCl_2$ treatment phase-advanced HO-1 and GRd mRNA expression by 6–9 h (from late to early photophase; Table 3). whereas it phase-delayed by 9–10 h (from early to late scotophase) that of Cu/Zn SOD and Mn SOD (Table 4). $CdCl_2$ increased significantly the amplitude of NOS-1, NOS-2, and Cu/Zn SOD expression. The 24-h variations in HO-2 (Table 3) and MT-1 expression (Table 4) did not follow a sinusoidal pattern in controls, whereas lipid peroxidation did not follow a sinusoidal pattern in either group (Table 4).

Figs. 4 and 5 summarize the results of Experiment 2, designed to assess whether the concomitant administration of melatonin could counteract $CdCl_2$ effects on PRL synthesis and release and redox parameters when assessed at ZT 01:00, i.e., the time interval of maximal effects of Cd, as per Experiment 1. As shown in Fig. 4, melatonin treatment was effective at blunting the effect of Cd on PRL synthesis and circulating levels. Moreover, a significant effect of melatonin in decreasing PRL synthesis and

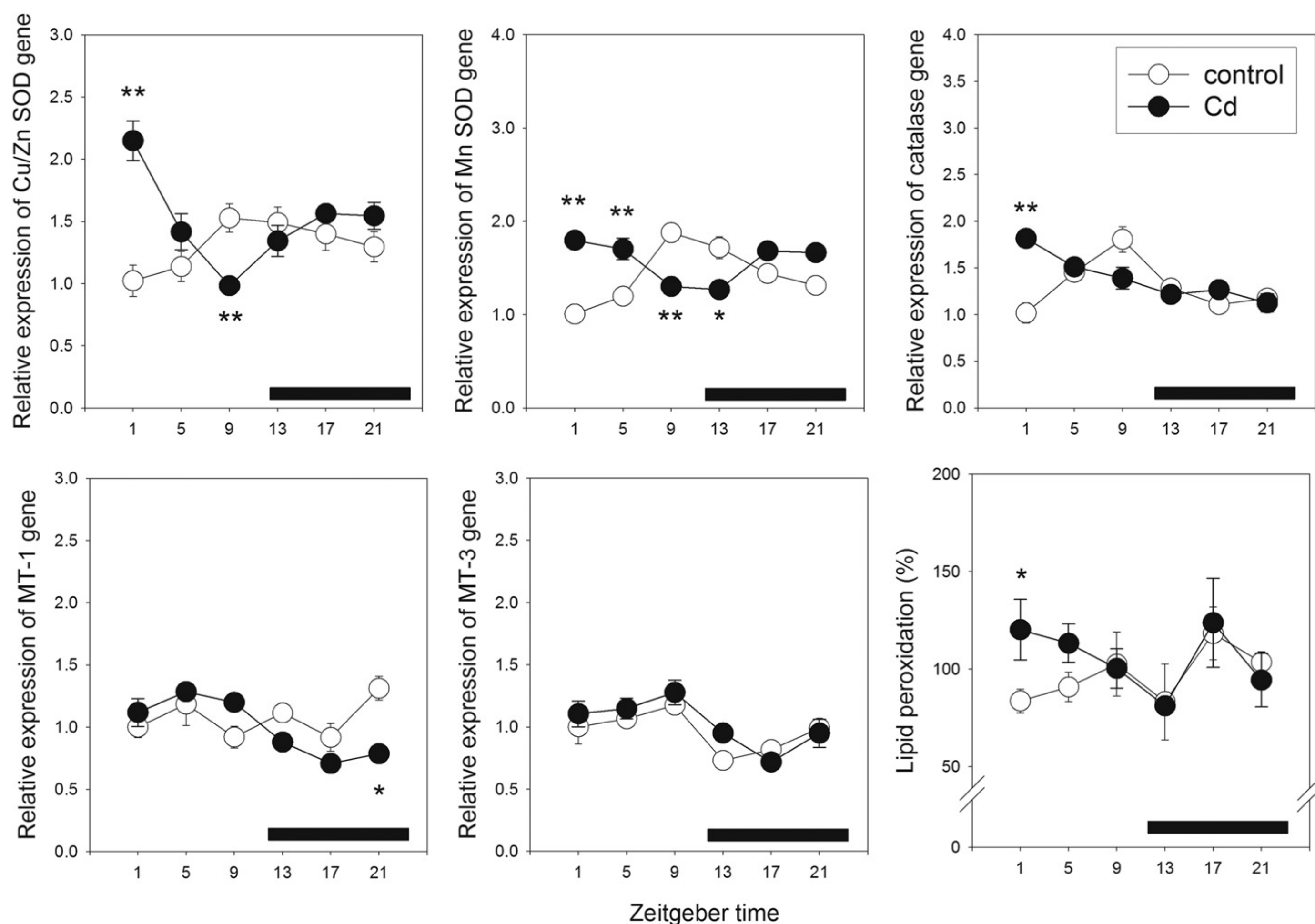

Fig. 3. Effect of cadmium on 24-h pattern of expression of mRNA for Cu/Zn SOD, Mn SOD, catalase, MT-1 and MT-3, and lipid peroxidation in rat adenohypophysis. For experimental details see Fig. 1 legend. Shown are the means ± SEM of mRNA determinations as measured by triplicate real-time PCR analyses of RNA samples. Lipid peroxidation was assessed by the TBARS procedure as described under Materials and methods. *$p < 0.05$, **$p < 0.02$, compared to control in Student's t tests performed at every time interval. The analysis of data by cosinor is summarized in Table 4. For further statistical analysis, see the text.

Table 3
Cosinor analysis of the effect of Cd on 24-h changes in adenohypophysial expression of mRNA for NOS-1, HO-1, GPx, NOS-2, HO-2, and GRd in rats.

	Mesor (relative gene expression)	Amplitude (relative gene expression)	Acrophase (ZT)	R^2, p
Control				
NOS-1	1.04 ± 0.20	0.17 ± 0.02	03:05 ± 02:12	0.81, < 0.001
HO-1	0.97 ± 0.10	0.28 ± 0.05	07:44 ± 01:10	0.86, < 0.001
GPx	1.43 ± 0.09	0.23 ± 0.04	15:15 ± 02:16	0.32, < 0.05
NOS-2	0.91 ± 0.12	0.47 ± 0.06	04.23 ± 01:34	0.62, < 0.02
HO-2	1.10 ± 0.07	–	–	0.18, NS
GRd	1.26 ± 0.12	0.27 ± 0.03	11:06 ± 01:05	0.84, < 0.001
Cadmium				
NOS-1	1.46 ± 0.20**	0.47 ± 0.10**	05:40 ± 02:43	0.79, < 0.01
HO-1	0.95 ± 0.11	0.21 ± 0.05	01:19 ± 02:34*	0.52, < 0.02
GPx	1.16 ± 0.10**	0.26 ± 0.03	19:32 ± 02:56	0.77, < 0.01
NOS-2	0.95 ± 0.08	0.96 ± 0.07**	03:50 ± 02:14	0.63, < 0.01
HO-2	1.34 ± 0.08*	–	–	0.11, NS
GRd	1.31 ± 0.11	0.31 ± 0.05	02:11 ± 01:37*	0.72, < 0.01

Shown are the means ± SEM. R^2 values and their probability are also shown. Mean values at each time series in Fig. 2 (n=6) were 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 the text.

* $p < 0.05$ vs control, Student's t test.

** $p < 0.01$ vs control, Student's t test.

Table 4
Cosinor analysis of the effect of Cd 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 (ZT)	R^2, p
Control				
Cu/Zn SOD	1.27 ± 0.11	0.24 ± 0.03	13:18 ± 01:12	0.85, < 0.001
Mn SOD	1.42 ± 0.14	0.33 ± 0.07	13:13 ± 02:45	0.83, < 0.001
Catalase	1.31 ± 0.15	0.31 ± 0.04	08:43 ± 02:43	0.71, < 0.01
MT-1	1.07 ± 0.11	–	–	0.16, NS
MT-3	0.96 ± 0.08	0.16 ± 0.03	04:22 ± 01:58	0.57, < 0.02
Lipid peroxidation (%)	91 ± 7	–	–	0.28, NS
Cadmium				
Cu/Zn SOD	1.57 ± 0.10*	0.39 ± 0.04**	22:56 ± 01:17*	0.64, < 0.01
Mn SOD	1.58 ± 0.31	0.26 ± 0.07	23:26 ± 02:32*	0.81, < 0.001
Catalase	1.40 ± 0.32	0.27 ± 0.07	03:33 ± 02:56	0.69, < 0.01
MT-1	0.99 ± 0.02	0.33 ± 0.08	05:54 ± 02:12	0.98, < 0.001
MT-3	1.04 ± 0.22	0.21 ± 0.06	05:57 ± 01:54	0.84, < 0.001
Lipid peroxidation (%)	113 ± 7*	–	–	0.21, NS

Shown are the means ± SEM. R^2 values and their probability are also shown. Mean values at each time series in Fig. 3 ($n=6$) were 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 the text.

* $p < 0.05$ vs control, Student's t test.
** $p < 0.01$ vs control, Student's t test.

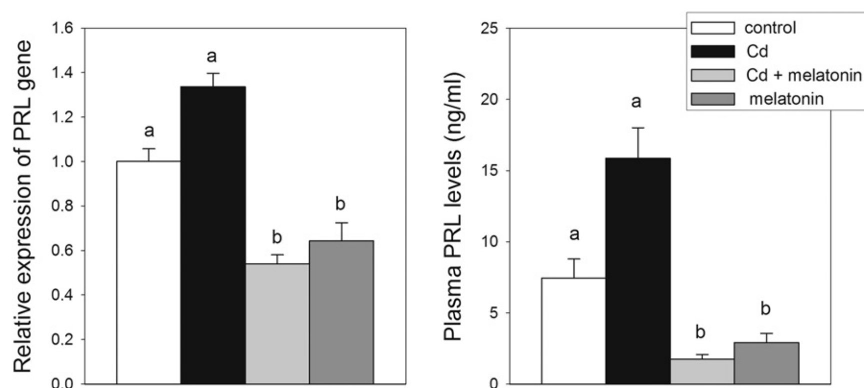

Fig. 4. Effect of melatonin on Cd-induced adenohypophysial PRL gene expression and plasma PRL levels in rats. Rats were divided into four groups and treated for 1 month as follows: (i) $CdCl_2$ (8 μg/ml drinking water); (ii) $CdCl_2$ (8 μg/ml drinking water) plus melatonin (3 μg/ml drinking water); (iii) melatonin (3 μg/ml drinking water); (iv) drinking water alone (control). Groups of six to eight rats were euthanized by decapitation at ZT 01:00. mRNA levels encoding the PRL gene and plasma PRL levels were measured as described in the text. Shown are the means ± SEM of mRNA determinations as measured by triplicate real-time PCR analyses of RNA samples and the means ± SEM of plasma PRL levels. [a]$p < 0.01$ vs the remaining means, [b]$p < 0.01$ vs control and Cd-administered animals, one way ANOVA, Bonferroni tests. For further statistical analysis, see the text.

release was shown when it was examined as a main factor in a factorial ANOVA ($p < 0.001$, Fig. 1). Melatonin was also effective at counteracting the effect of Cd on expression of NOS-1, NOS-2, Cu/Zn SOD, Mn SOD, and catalase and on lipid peroxidation when assessed at ZT 01:00 (Fig. 5). The analysis of melatonin as a main factor in the factorial ANOVA indicated a significant effect in decreasing NOS-2 expression and in augmenting HO-2, catalase, GRd, MT-1, and MT-3 expression ($p < 0.02$; Fig. 5).

Figs. 6 and 7 and Tables 5 and 6 summarize the results of Experiment 3, aimed at examining the effects of melatonin on Cd-induced changes in the 24-h pattern of pituitary circadian clock gene expression and plasma PRL, LH, TSH, and corticosterone levels. Significant time-related changes in clock gene expression in the anterior pituitary were found in controls, *Clock* and *Bmal1* expression peaking during scotophase (acrophases at ZT 18:53 and 19:11, respectively), whereas that of *Per1* and *Per2*, and of *Cry1* and *Cry2*, peaked at the beginning and the middle of photophase (ZT 02:10 and 02:16 and ZT 10:20 and 11:34, respectively, Table 5). As indicated by the significant interactions Cd × time of day in the factorial ANOVA, rats receiving $CdCl_2$ showed a suppression of daily rhythm in *Clock* expression and a significant disruption in daily rhythms of the remaining circadian genes ($p < 0.01$). The cosinor analysis indicated that the expression of only *Bmal1*, *Per1*, and *Cry2* fit a sinusoidal pattern in the anterior pituitary of $CdCl_2$-treated rats, showing acrophases at late scotophase (ZT 00:03), late photophase (ZT 11:44), and middle scotophase (ZT 19:33; Table 5).

The coadministration of melatonin restored the sinusoidal pattern of circadian genes, resembling controls, only for *Clock* and *Bmal1* expression (acrophases at ZT 19:28 and 21:42, respectively). Maxima in pituitary expression of *Per1*, *Per2*, *Cry1*, and *Cry2* shifted to the scotophase in Cd-melatonin-treated rats (acrophases at ZT 13:43, 13:58, 16:08, and 18:34, Table 5). Compared to controls, rats receiving melatonin alone showed significant changes in acrophases of *Per1* and *Per2* expression (from early to late photophase) and of *Cry1* expression (from late photophase to early scotophase).

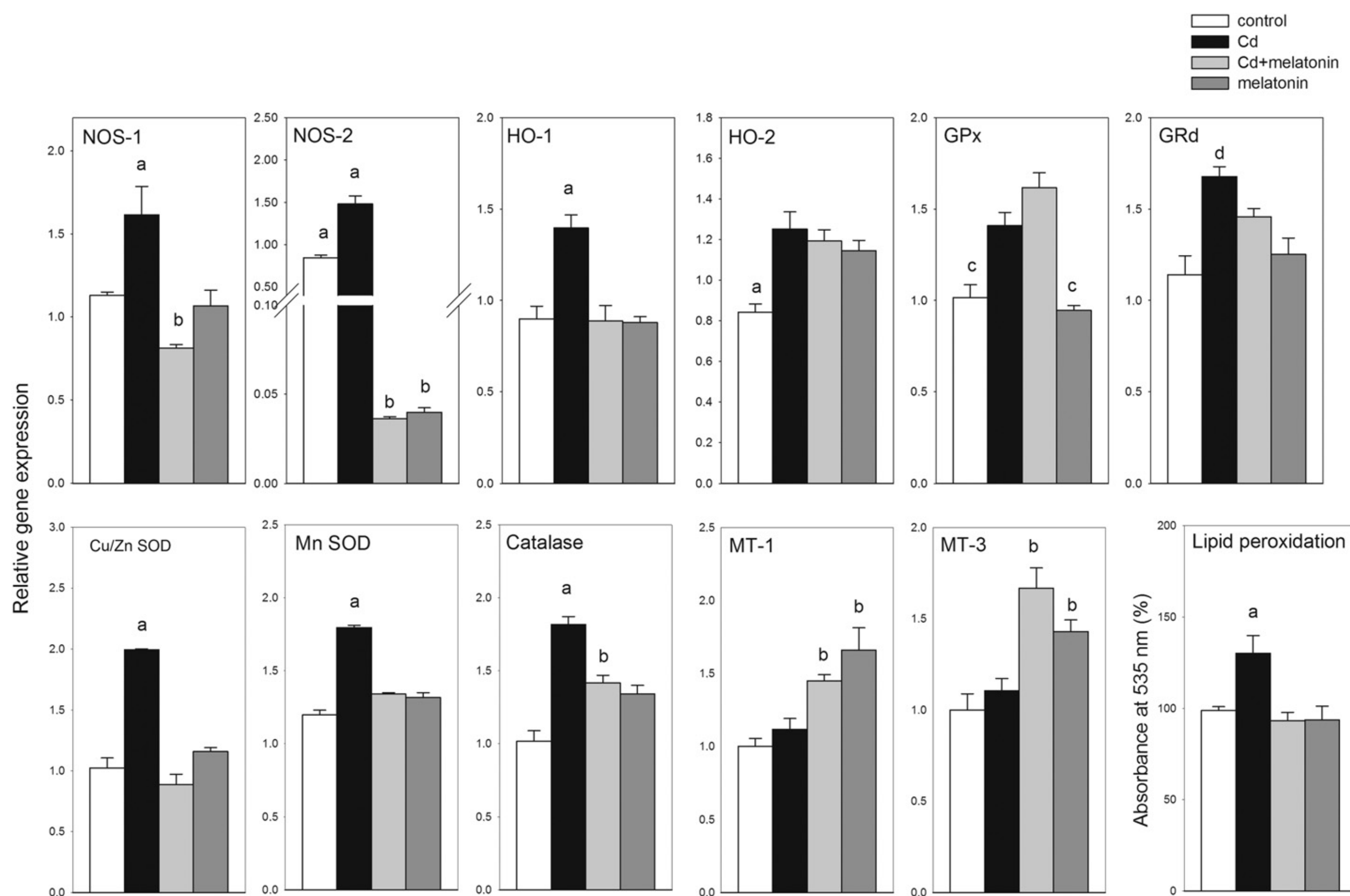

Fig. 5. Effect of melatonin on Cd-induced changes in expression of mRNA for NOS-1, NOS-2, HO-1, HO-2, Cu/Zn SOD, Mn SOD, catalase, GPx, GRd, MT-1, and MT-3 and in lipid peroxidation in rat adenohypophysis. The experimental details are given in the Fig. 4 legend. Shown are the means ± SEM of mRNA determination as measured by triplicate real-time PCR analyses of RNA samples. Lipid peroxidation was measured by the TBARS assay as described under Materials and methods. $^{a}p < 0.01$ vs the remaining means, $^{b}p < 0.01$ vs control and Cd-administered animals, $^{c}p < 0.01$ vs Cd and Cd+melatonin groups, $^{d}p < 0.01$ vs control and melatonin groups, one way ANOVA, Bonferroni tests. For further statistical analysis, see the text.

The changes in the daily variations of PRL, LH, TSH, and corticosterone are depicted in Fig. 7. In controls the four hormones tested exhibited significant 24-h variations with acrophases at ZT 15:40 (PRL), 19:06 (LH), 05:59 (TSH), and 08:22 (corticosterone). $CdCl_2$ treatment disrupted the 24-h rhythmicity of the four hormones examined as indicated by the significant interactions Cd × time of day in the factorial ANOVA ($p < 0.01$). The cosinor analysis (Table 6) indicated that the acrophases of TSH and corticosterone rhythms were almost inverted in $CdCl_2$ rats from midphotophase to midscotophase compared to controls. The daily variations in plasma PRL and LH did not follow a sinusoidal pattern. The coadministration of melatonin was effective to restore rhythmicity of plasma PRL, LH, and TSH, with acrophases similar to those found in controls, whereas in the case of corticosterone a phase delay at about 7 h was observed (from ZT 08:22 to 15:10, Table 6). For every hormone, the amplitude of the rhythm was significantly higher in Cd+melatonin-treated rats than in controls or Cd-treated rats ($p < 0.01$, Table 5). Rats receiving melatonin alone showed a significant delay in acrophase of plasma corticosterone rhythm (from ZT 08:22 to 15:10) and significant increases in amplitude of LH, TSH, and corticosterone rhythms (Table 6).

Discussion

The questions posed in the introduction may now be answered. A low dose of Cd has a demonstrable endocrine disruptor effect in rats as regards PRL synthesis and release, as shown by the suppression of 24-h variations in both parameters. $CdCl_2$ treatment also resulted in the disruption of 24-h rhythmicity of plasma LH, TSH, and corticosterone. In the anterior pituitary, Cd increased significantly expression of the PRL gene during the photophase, particularly at morning hours. Concomitantly, Cd augmented pituitary lipid peroxidation and expression of a number of pituitary redox enzyme genes, again at early photophase. Cd treatment had a significant disruptive effect on pituitary circadian clock gene expression, making it feasible that genomic effects of the pollutant on cellular circadian clock mechanisms may underlie some or all of the abovementioned changes. The coadministration of melatonin counteracted the effect of Cd on the 24-h rhythmicity of plasma PRL, LH, TSH, and corticosterone levels. Melatonin was also effective at counteracting the promoting effect of Cd on lipid peroxidation and expression of redox enzymes, when assessed at the time of maximal expression of pituitary effects (ZT 01:00), and at restoring partially Cd-induced changes in pituitary circadian clock gene expression.

In this study, and with the aim of mimicking human exposure, we gave Cd in the drinking water for 1 month at a low concentration (5 ppm). A single dose of Cd, rather than a dose–response design, was chosen because of the experimental design and the number of animals employed. The dose of Cd was selected by the body surface area normalization method [21] to resemble the acceptable limit put forth by the WHO (1 μg/day for a life span of 60 years) [22]. It mimicked exposure levels in moderately to heavily polluted areas or occupational exposure conditions [2].

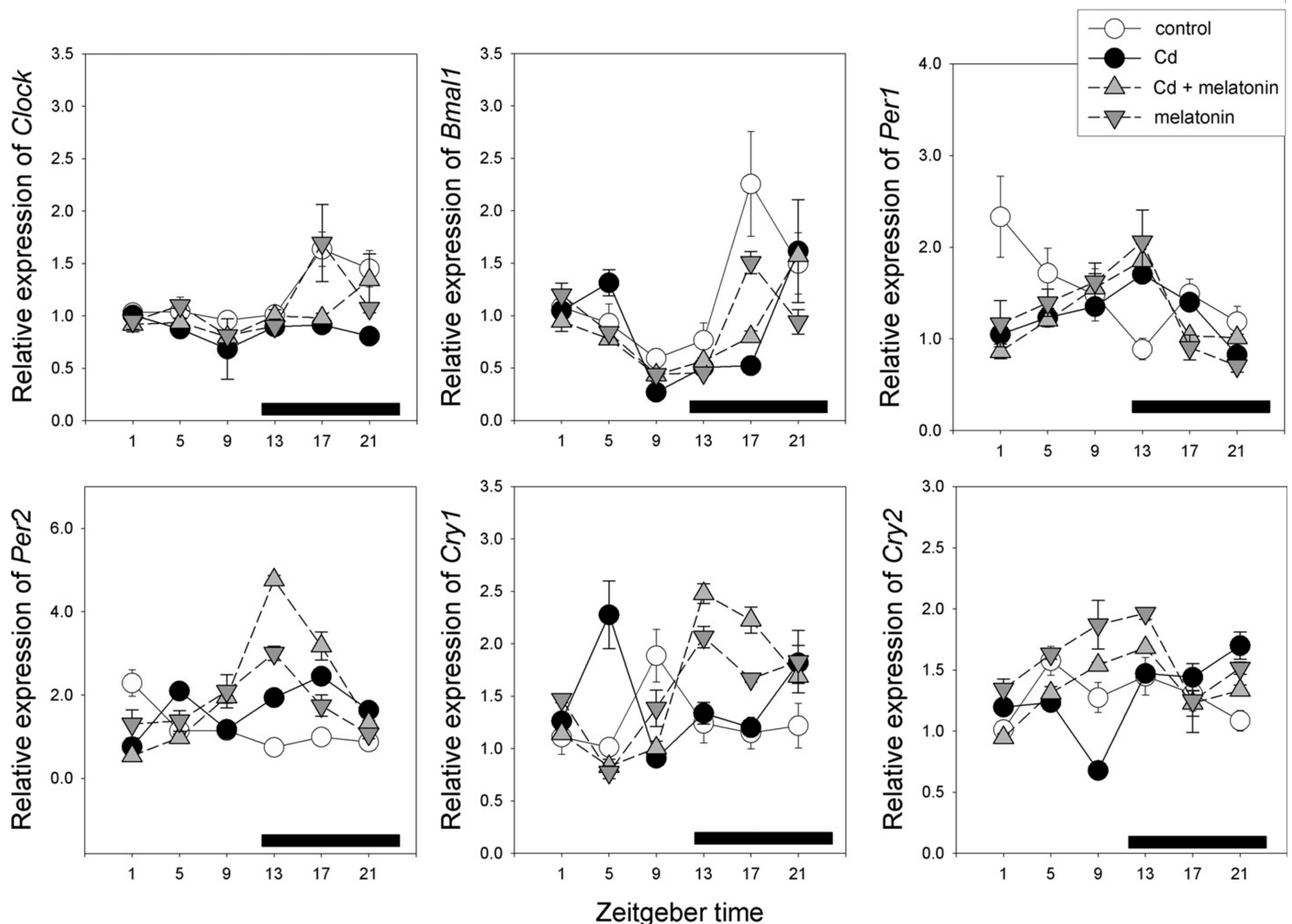

Fig. 6. Effect of melatonin on Cd-induced changes in 24-h pattern in adenohypophysial expression of mRNA of *Clock*, *Bmal1*, *Per1*, *Per2*, *Cry1*, and *Cry2*. The rats were divided into four groups and treated for 1 month as follows: (i) $CdCl_2$ (8 μg/ml drinking water); (ii) $CdCl_2$ (8 μg/ml drinking water) plus melatonin (3 μg/ml drinking water); (iii) melatonin (3 μg/ml drinking water); (iv) drinking water alone (control). Groups of six to eight rats were euthanized by decapitation at six time intervals throughout a 24-h cycle. Shown are the means ± SEM of mRNA determinations as measured by triplicate real-time PCR analyses of RNA samples. The analysis of data by cosinor is summarized in Table 5. For further statistical analysis, see the text.

The effect of low doses of Cd on plasma PRL levels had been reported previously [9], but the mechanisms by which Cd alters PRL secretion remained unsettled. In vitro, Cd causes oxidative damage of lactotrophs and inhibits PRL release by increasing radical oxygen species (ROS) in a mitochondrial-dependent way and by inducing apoptosis [15,16,28]. These in vitro effects of Cd on pituitary cells were prevented by antioxidants.

In this study Cd treatment augmented significantly lipid peroxidation presumably by augmenting nitric oxide (NO), which is also a relevant modulator of pituitary hormone release [29,30]. The expression of both NOS isoforms NOS-1 and NOS-2 increased in Cd-treated rats, particularly during the photophase. Indeed, Cd affects NO production in various tissues [31,32] and several hypotheses have been entertained about the role of NO in Cd toxicity, ranging from mediation of toxicity [33] to no effect [34] or a protective effect [35,36].

Because ROS generation is a continuous and physiological phenomenon, cells possess efficient antioxidant systems that protect them from oxidative damage (for reviews, see [37,38]). HO has an important role in controlling the redox state of the cell by functioning as a rate-limiting enzyme in the heme degradation process, which results in the formation of equimolar amounts of two potent antioxidants, i.e., carbon monoxide and biliverdin [37]. In this study, and concomitant with the disrupting effect that Cd has on PRL synthesis during the photophase, an increase in HO-1 and HO-2 expression was observed. The results suggest that an overexpression of mRNA for HO-1 and HO-2 may counteract the oxidative damage caused by excess NO.

The detoxification of ROS in cells involves the cooperative action of intracellular antioxidant enzymes like Cu/Zn SOD, Mn SOD, and catalase [39]. In addition, GPx and GRd help to maintain adequate levels of reduced glutathione, a major antioxidant defense of the cells. The changes in redox enzyme mRNA expression brought about by $CdCl_2$ treatment in this study can be interpreted in terms of a compensatory increase caused by the augmented oxidative load. Because ROS play a role in cellular signaling processes, including transcription factor activities, such NF-κB and AP-1, the increase in free radicals caused by Cd would allow regulation of gene transcription via modulation of redox-sensitive transcription factors [39,40].

Melatonin, the major secretory product of the pineal gland, participates in diverse physiological functions not only signaling the length of the night (the chronobiotic effect [17,18]) but also enhancing ROS scavenging, the immune response, and cytoprotection [19]. At least two previous observations supported a protective role of melatonin on Cd-induced pituitary changes in rats.

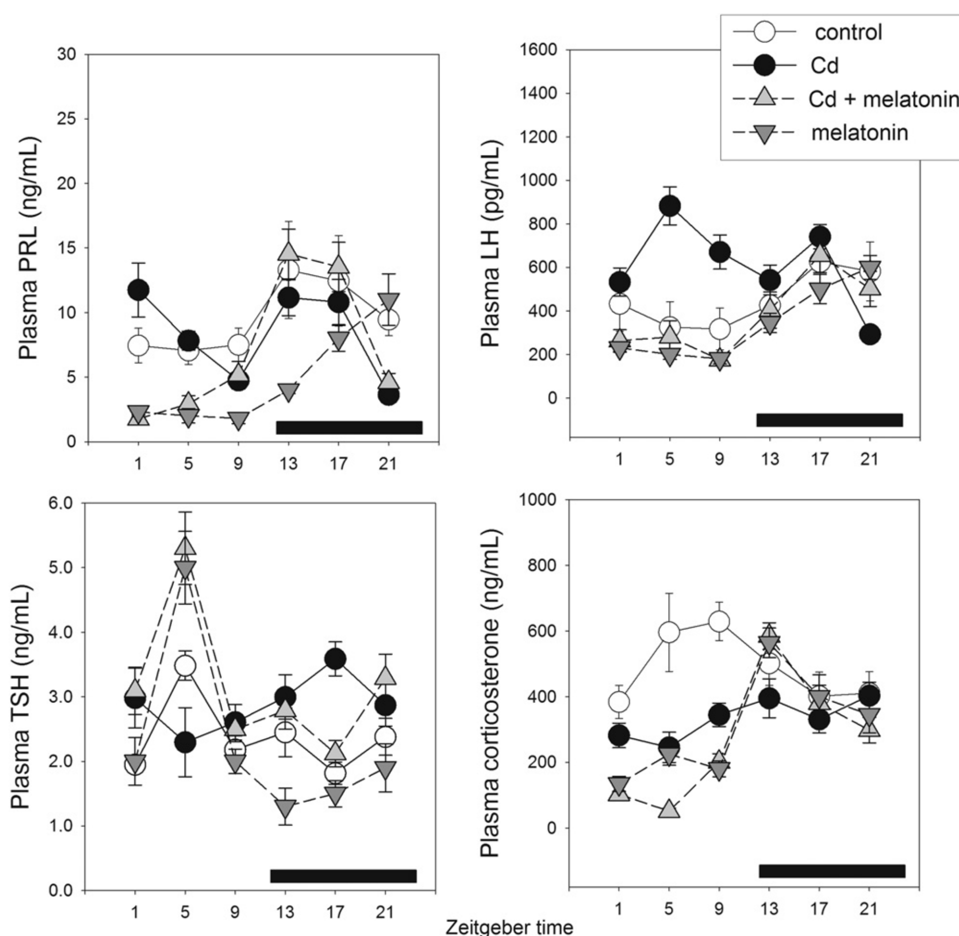

Fig. 7. Effect of melatonin on Cd-induced changes in 24-h pattern of circulating PRL, LH, TSH, and corticosterone. The experimental details are given in the Fig. 6 legend. Shown are the means ± SEM. The analysis of data by cosinor is summarized in Table 6. For further statistical analysis, see the text.

Poliandri et al. [20] demonstrated that in the anterior pituitary, melatonin administration prevented Cd-induced increases in lipid peroxidation and in mRNA levels for NOS-1, NOS-2, and HO-1. Miler et al. [6] reported, in a study aimed to assess whether Cd-induced oxidative stress in pituitary gland was reversed by removing the pollutant source, that in rats exposed to 5 ppm Cd the augmented expression of pituitary HO-1 was prevented by the concomitant administration of melatonin. In both studies, the dose of melatonin used was similar to that employed herein.

When assessed at early morning, i.e., at the time interval at which Cd treatment showed maximal effects, melatonin treatment was effective at suppressing the effect of Cd on PRL synthesis and circulating levels. Melatonin was also effective at counteracting the promoting effect of Cd on lipid peroxidation and the concomitant expression of NOS-1, NOS-2, Cu/Zn SOD, Mn SOD, and catalase. Moreover, a significant effect of melatonin in decreasing PRL synthesis and release and in augmenting HO-2, catalase, GRd, MT-1, and MT-3 expression was found. Because the binding of Cd to MTs facilitates the tissue deposit of the metal, any toxic effect of Cd could be modified by compounds able to modulate the synthesis of MT proteins [41]. It should be noted that the effects of melatonin depicted in Figs. 5 and 6 could be due, rather than to actual blunting, to a phase-shifting effect of the methoxyindole. Further studies are needed to clarify this point.

In this study the effect of melatonin on the expression of antioxidant enzymes given by Cd was generally inhibitory except for GPx. Thus the beneficial effect of melatonin to counteract the augmented lipid peroxidation induced by $CdCl_2$ seems to be more related to the decrease in expression of NOS-1 and NOS-2 and to the augmentation of MT-1 and MT-3, because most of the Cd in the body is bound to small, cysteine-rich, MTs [42]. Further studies are needed to verify whether the actual levels of MTs are also increased by melatonin.

In this study significant time-related changes in circadian clock gene expression were found in the anterior pituitary of control rats. The peaks of pituitary *Clock* and *Bmal1* expression were in antiphase with those of *Per1* and *Per2* expression. *Per1 and Per2* peaked at the beginning of the light phase and *Clock* and *Bmal1* peaked during scotophase. In the case of *Cry1* and *Cry2* their maximal expression took place in the middle of photophase, with a phase delay of 4–8 h compared to *Per1* or *Per2*. Rats receiving $CdCl_2$ exhibited a disruption in expression of every pituitary clock gene tested as well as suppression of rhythmicity of *Clock* and *Bmal1* expression. In addition to the daily variations in PRL, those in LH, TSH, and corticosterone became disrupted by Cd.

The coadministration of melatonin restored rhythmicity in *Clock* and *Bmal1* expression but shifted the maxima in *Per1*, *Cry1*, and *Cry2* expression to the scotophase. Melatonin was also effective at restoring the rhythmicity of plasma PRL, LH, and TSH, with acrophases similar to those found in controls, whereas in the case of corticosterone a phase delay of about 7 h was observed. In addition, melatonin had a significant effect by itself, i.e., for every hormone the amplitude of the rhythm was significantly higher in melatonin-treated rats. Collectively, these observations are compatible with the chronobiotic role of melatonin proposed by others [17,18]. However, it is important to realize that findings

Table 5
Cosinor analysis of the effect of melatonin on Cd-induced changes in the 24-h pattern of adenohypophysial expression of mRNA of *Clock*, *Bmal1*, *Per1*, *Per2*, *Cry1*, and *Cry2* in rats.

	Mesor (relative gene expression)	Amplitude (relative gene expression)	Acrophase (ZT)	R^2, p
Control				
Clock	1.22 ± 0.13	0.31 ± 0.03	18:53 ± 01:24	0.74, < 0.01
Bmal1	1.22 ± 0.16	0.64 ± 0.07	19:11 ± 01:45	0.67, < 0.01
Per1	1.51 ± 0.21	0.49 ± 0.05	02:10 ± 02:14[a]	0.61, < 0.01
Per2	1.24 ± 0.16	0.56 ± 0.08	02:16 ± 01:12[a]	0.60, < 0.01
Cry1	1.30 ± 0.15	0.24 ± 0.05	10:20 ± 01:04	0.36, < 0.05
Cry2	1.35 ± 0.14	0.29 ± 0.04	11:34 ± 01:56	0.39, < 0.05
Cadmium				
Clock	0.88 ± 0.06	–	–	0.31, NS
Bmal1	0.87 ± 0.08	0.56 ± 0.08	00:03 ± 01:45[a]	0.75, < 0.01
Per1	1.30 ± 0.26	0.35 ± 0.04	11:44 ± 02:04	0.84, < 0.001
Per2	1.72 ± 0.24	–	–	0.29, NS
Cry1	1.50 ± 0.21	–	–	0.25, NS
Cry2	1.31 ± 0.22	0.35 ± 0.05	19:33 ± 01:50[b]	0.62, < 0.01
Cadmium + melatonin				
Clock	1.01 ± 0.13	0.25 ± 0.04	19:28 ± 01:32	0.63, < 0.01
Bmal1	0.98 ± 0.32	0.46 ± 0.05	21:42 ± 01:59	0.79, < 0.001
Per1	1.33 ± 0.21	0.53 ± 0.07	13:43 ± 01:23	0.72, < 0.01
Per2	2.11 ± 0.21[c]	1.76 ± 0.31[d]	13:58 ± 02:04	0.94, < 0.001
Cry1	1.61 ± 0.24	0.82 ± 0.11[d]	16:08 ± 02:09[d]	0.87, < 0.001
Cry2	1.27 ± 0.27	0.35 ± 0.05	18:34 ± 01:14[b]	0.53, < 0.04
Melatonin				
Clock	1.11 ± 0.08	0.26 ± 0.04	18:23 ± 01:12	0.40, < 0.05
Bmal1	0.85 ± 0.11	0.49 ± 0.07	20:30 ± 02:44	0.93, < 0.001
Per1	1.28 ± 0.21	0.45 ± 0.08	10:28 ± 02:14	0.83, < 0.001
Per2	1.88 ± 0.25	0.88 ± 0.11	11:43 ± 01:03	0.83, < 0.001
Cry1	1.55 ± 0.27	0.40 ± 0.05	16:37 ± 01:12[d]	0.66, < 0.01
Cry2	1.37 ± 0.18	0.29 ± 0.05	11:56 ± 01:23	0.75, < 0.01

Shown are the means ± SEM. Superscripts denote significant differences in a one-way ANOVA and post hoc Bonferroni tests as follows:

[a] $p < 0.05$ vs the remaining groups.
[b] $p < 0.05$ vs control and melatonin groups.
[c] $p < 0.05$ vs control.
[d] $p < 0.01$ vs control. R^2 values and their probability are also shown. Mean values at each time series in Fig. 6 ($n = 6$) were 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 the text.

from laboratory rats cannot be directly extrapolated to humans, because in a nocturnally active species such as the rat high circulating melatonin is associated with neuronal and locomotor activity, the opposite to what is seen in a diurnally active species such as the human.

One important limitation of this descriptive study is that gene expression needs to be completed with Western blotting analysis of the proteins assessed to obtain a better understanding of Cd–melatonin interactions on pituitary redox enzymes and circadian clock genes. It is also necessary to establish whether the changes in amplitude and timing of 24-h rhythms in gene expression are due to effects exerted on the hypothalamic suprachiasmatic nuclei or at the level of some of their output. Previous results from this laboratory indicated that Cd administration disrupted the 24-h rhythmicity and overall expression of redox enzyme and clock genes in rat medial hypothalamus, an effect partly counteracted by melatonin administration [43,44]. As reported herein for PRL, LH, and TSH and corticosterone daily rhythms, the chronic exposure of rats to similar low doses of Cd affected the circadian variation of pituitary hormone release [11,45,46]. Collectively this information would allow prediction of the occurrence of changes in behavioral and other physiological rhythms after Cd. However, as there is no recent information on this point, this important subject remains unanswered.

Table 6
Cosinor analysis of the effects of melatonin on Cd-induced changes in 24-h pattern of circulating PRL, LH, TSH, and corticosterone levels.

	Mesor	Amplitude	Acrophase (ZT)	R^2, p
Control				
PRL	9.53 ± 1.11	3.30 ± 0.41	15:40 ± 02:45	0.87, < 0.001
LH	409 ± 37	163 ± 13	19:06 ± 02:17	0.96, < 0.001
TSH	2.35 ± 0.32	0.50 ± 0.04	05:59 ± 01:45	0.43, < 0.03
Corticosterone	490 ± 43[a]	121 ± 13	08:22 ± 02:02[a]	0.83, < 0.001
Cadmium				
PRL	8.27 ± 0.91	–	–	0.07, NS
LH	615 ± 44[a]	–	–	0.21, NS
TSH	2.79 ± 0.32	0.34 ± 0.01	16:56 ± 01:59[a]	0.54, < 0.03
Corticosterone	337 ± 45	65 ± 8	16:10 ± 01:19	0.64, < 0.02
Cadmium + melatonin				
PRL	7.33 ± 1.13	6.70 ± 0.87[a]	14:45 ± 01:40	0.91, < 0.001
LH	393 ± 47	208 ± 23	18:15 ± 01:57	0.86, < 0.001
TSH	3.16 ± 0.32	1.11 ± 0.23[b]	03:52 ± 01:45	0.50, < 0.03
Corticosterone	278 ± 32	234 ± 15	15:10 ± 01:23	0.88, < 0.001
Melatonin				
PRL	4.23 ± 0.51[a]	2.72 ± 0.16	17:08 ± 02:15	0.84, < 0.001
LH	349 ± 42	213 ± 32	18:52 ± 02:58	0.83, < 0.001
TSH	2.28 ± 0.42	1.31 ± 0.21[b]	04:30 ± 02:54	0.56, < 0.03
Corticosterone	309 ± 34	187 ± 12[b]	15:02 ± 02:05	0.72, < 0.01

Mesor and amplitude values are expressed as ng/ml (TSH, testosterone, corticosterone) or pg/ml (LH). Shown are the means ± SEM. Superscripts denote significant differences in a one-way ANOVA and post hoc Bonferroni tests as follows.

[a] $p < 0.01$ vs the remaining groups.
[b] $p < 0.01$ vs control and cadmium groups. R^2 values and their probability are also shown. Mean values at each time series in Fig. 7 ($n = 6$) were 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 the text.

Acknowledgments

This work was supported by grants from the Ministerio de Educación y Ciencia, Spain (SAF2008-00442), and Agencia Nacional de Promoción Científica y Tecnológica, Argentina (PICT 2007-01045), and Universidad de Buenos Aires, Argentina (M 048). D.P.C. is a Research Career Awardee from the Argentine Research Council and Professor Emeritus, University of Buenos Aires.

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