Biometals (2010) 23:327–337
DOI 10.1007/s10534-010-9292-6

Effect of cadmium on 24-hour pattern in expression of redox enzyme and clock genes in rat medial basal hypothalamus

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Received: 11 September 2009 / Accepted: 13 January 2010 / Published online: 27 January 2010

Abstract The effect of cadmium (Cd) in the brain has been attributed to an increase in reactive oxygen species in cells, particularly when high amounts of the metal are given. In this study we examined the effect of a low dose of Cd (7.5 μg/day) on 24-h changes in expression of redox pathway enzyme and circadian genes in rat medial basal hypothalamus (MBH). Rats receiving $CdCl_2$ (5 ppm in drinking water) or tap water for 1 month were killed at six different time intervals throughout a 24 h cycle. MBH mRNA levels were measured by real-time PCR analysis. In $CdCl_2$ treated rats a disruption of 24-h pattern of hypothalamic gene expression of nitric oxide synthase (NOS)-1 and -2, heme oxygenase (HO)-1 and -2, Mn- superoxide dismutase (SOD), catalase, glutathione peroxidase and glutathione reductase was detectable. Mean levels of MBH mRNA for HO-2, Mn-SOD and catalase augmented after Cd intake, whereas those of NOS-2 decreased. After $CdCl_2$ intake rats the 24-h pattern of clock gene expression in MBH seen in controls was significantly suppressed (*Bmal1*) or changed in phase (*Per1*, *Per2, Cry2*) while in the case of *Clock* significant 24-h variations were induced. The results are compatible with the view that a low amount of Cd given in tap water brought about significant changes in circadian expression of redox enzyme and clock genes in rat MBH.

Keywords Cadmium · Circadian rhythms · Gene expression · Medial basal hypothalamus · Nitric oxide synthase · Heme oxygenase · Superoxide dismutase · Catalase · Glutathione peroxidase · Glutathione reductase · Clock genes

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Introduction

Cadmium (Cd) is a nonessential element widely used in industrial applications and is an important side contaminant of agricultural products (WHO 1995).

Cd in soil and water is taken up by plants and is concentrated and transferred to upper links of the food chain, including humans (WHO 1995; Satarug et al. 2003). Due to the long biological half-life of Cd (i.e., 10–30 years) its accumulation in the body can increase the risk of toxicity (Sugita and Tsuchiya 1995). The principal determinants of human Cd exposure are smoking habits, diet and to a certain extent, occupational exposure. According to WHO (Wakabayashi et al. 1987) one cigarette (containing 0.5–3 μg Cd per gram of tobacco) can result in up to 3 μg daily Cd absorption via the lungs. Chronic exposure to these low doses of Cd causes neuroendocrine and neurobehavioral disturbances in animals and humans (Viaene et al. 2000; Lafuente et al. 2003, 2004, 2005; Leret et al. 2003).

The effects of Cd in high concentrations have been attributed to an excessive increase in reactive oxygen species (ROS) in cells. Such an effect is particularly evident in kidney and liver and has been also demonstrated in vitro in neurons (Lopez et al. 2006) and glial cells (Yang et al. 2007). ROS play a dual role in biological systems, since they can be either harmful or beneficial to cells (Valko et al. 2006). Beneficial effects of low amounts of ROS involve physiological roles in cellular responses to noxa, as for example in defense against infectious agents and in the function of a number of cellular signalling systems. In contrast, at high concentrations, ROS can be important mediators of damage to cell structures, including lipids and membranes, proteins and nucleic acids (oxidative stress). While some metals, like iron, copper, chromium, vanadium or cobalt undergo redox-cycling reactions, a second group of metals including Cd, mercury and nickel cause toxicity mainly by depleting glutathione and by binding to sulfhydryl groups of proteins (Wright and Baccarelli 2007). Metal-mediated formation of free radicals causes various modifications to DNA bases and may alter calcium and sulfhydryl homeostasis.

At a high concentration Cd increases oxidative damage in the brain (Lopez et al. 2006; Yang et al. 2007). Less information is available on the mediation by redox mechanisms of the effect of a low concentration of Cd on the rat brain. The objective of the present study was to examine whether Cd intake in low amounts (i.e. 5 ppm $CdCl_2$) modified circadian expression of redox enzyme and clock genes in the medial basal hypothalamus (MBH) of rats. Treatment with these doses may resemble real exposure level in active tobacco smokers, in moderately to heavily polluted areas or under occupational exposure conditions (Brzóska and Moniuszko-Jakoniuk 2005).

Materials and methods

Animals and experimental design

Male Wistar rats (45 days of age) were maintained under standard conditions with controlled light (12:12 h light/dark schedule; lights on at 0800 hours) and temperature (22 ± 2°C). Rats received $CdCl_2$ (5 ppm) or tap water. Since rats at this age drank about 20 mL/day with 90–95% of this total daily water taken up during the dark period, a relatively constant circadian intake of $CdCl_2$ occurred throughout the experiment. After 1 month groups of 6–8 rats were sacrificed by decapitation under conditions of minimal stress at six different time intervals (6–8 rats per group), every 4 h throughout a 24-h cycle, starting at 0900 hours. At night intervals animals were killed under red dim light.

The brains were rapidly removed and the MBH was dissected out following the landmarks of Szentagothai et al. (1968). Tissues 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, CoLS NRC 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-1, NOS-2, heme oxygenase (HO)-1, HO-2, Cu/Zn- superoxide dismutase (SOD), Mn-SOD, catalase, glutathione peroxidase (GPx), glutathione reductase (GSR), *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 AbiPrism 7300 (Applied Biosystems, Foster City, CA). 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 MBH 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

Table 1 Sequence of the primers used for real-time PCR

Gene	Primers		Product size (bp)
β- Actin	Forward	ctctcttccagccttccttc	99
	Backward	ggtctttacggatgtcaacg	
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	
Clock	Backward	ggtctttacggatgtcaacg	98
	Backward	gcgtagccgtggatgactt	
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	

cycle at which the amount of amplified target becomes significant (Ct) was automatically calculated by the PCR device.

To estimate whether $CdCl_2$ treatment or time of day modified the expression of β-actin, PCR with serial dilutions of this housekeeping gene was performed. 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.

Data analysis

Statistical analysis of results was performed by a factorial analysis of variance (ANOVA). Generally, the ANOVA included assessment of treatment effect (i.e., the occurrence of differences in mean values between Cd-treated and control rats), of time-of-day effects (the occurrence of daily changes) and of the interaction between treatment and time, from which inference about differences in timing and amplitude between the experimental groups could be obtained. Post-hoc Bonferroni's multiple comparisons tests in a one-way ANOVA were employed to show which time points were significantly different within each experimental group to define the existence of peaks. *P*-values lower than 0.05 were considered evidence for statistical significance.

Results

As shown in Fig. 1 the 24-h changes in MBH NOS and HO gene expression were changed significantly by $CdCl_2$ administration. Cd affected the 24-h pattern of expression of MBH NOS-1 and NOS-2 genes by inducing a maximum at late scotophase (0500 hours, NOS-1) or at midday (1300 hours, NOS-2) ($F = 7.25$ and 14.9 for the interaction "treatment × time" in the factorial ANOVA, $P < 0.001$, respectively) (Fig. 1, left upper and lower panels). $CdCl_2$ intake also disrupted the 24-h pattern of expression of MBH HO-1 and HO-2 genes by inducing a maximum at early scotophase (0100 hours, HO-1) or by augmenting the maximum found at early photophase (0900 hours, HO-2) ($F = 16.9$ and 6.34 for the interaction "treatment × time" in the factorial ANOVA, $P < 0.0001$, respectively) (Fig. 2, right upper and lower panels). As shown by main factor analysis of the data in the factorial ANOVA, $CdCl_2$ intake decreased by 51% expression of the NOS-2 gene ($F = 26.4$, $P < 0.0001$) and augmented by 74% that of HO-2 gene ($F = 32.1$, $P < 0.0001$).

The effect of Cd on 24-h pattern of expression of SOD and catalase genes is depicted in Fig. 2. In control rats only Cu/Zn SOD mRNA expression exhibited significant 24-h variations while following $CdCl_2$ treatment a peak at early scotophase in expression of Mn-SOD and catalase genes was observed ($P < 0.05$). As shown by main factor analysis of the data, $CdCl_2$ intake increased significantly MBH mRNA levels for Mn-SOD and catalase genes by 21 and 24%, respectively ($F = 5.61$, $P < 0.02$ and $F = 11.6$, $P < 0.002$, factorial ANOVA) (Fig. 2).

After $CdCl_2$ the 24 h pattern of GPx and GSR gene expression became disrupted, with suppression of the maximum seen at midday in the case of GPx ($P < 0.01$) and of minimum found at late scotophase for GSR ($P < 0.05$) (Fig. 3). Mean levels of MBH mRNA levels of the antioxidant enzymes GPx and GSR were not affected by $CdCl_2$ intake.

Figures 4 and 5 summarize the effect of $CdCl_2$ on MBH hypothalamic mRNA levels for the circadian genes examined. $CdCl_2$ treatment significantly suppressed *Bmal1* circadian rhythmicity and changed the phase of that of *Per1*, *Per2* and *Cry2*, while in the case of *Clock* significant 24-h variations were induced ($P < 0.01$) (Fig. 4). A significant effect of treatment was found in the factorial ANOVA for *Clock*, *Cry1* and *Cry2*, $CdCl_2$ augmenting their expression significantly ($F = 31.6$, 11.7 and 52.7, $P < 0.001$, respectively, main factor analysis, factorial ANOVA).

Discussion

Although metals have multiple effects on biological systems, an understudied effect is their role in programming gene expression. A growing body of evidence suggests that metals may influence epigenetic phenomena which regulate the expression of genes and ultimately their protein products (Wright and Baccarelli 2007). Foregoing results indicate that the administration of a low amount of $CdCl_2$ in drinking water for a month to young rats disrupted 24-h rhythmicity and overall expression of redox enzyme and clock genes in MBH. In particular, a disruption of 24-h pattern of hypothalamic expression

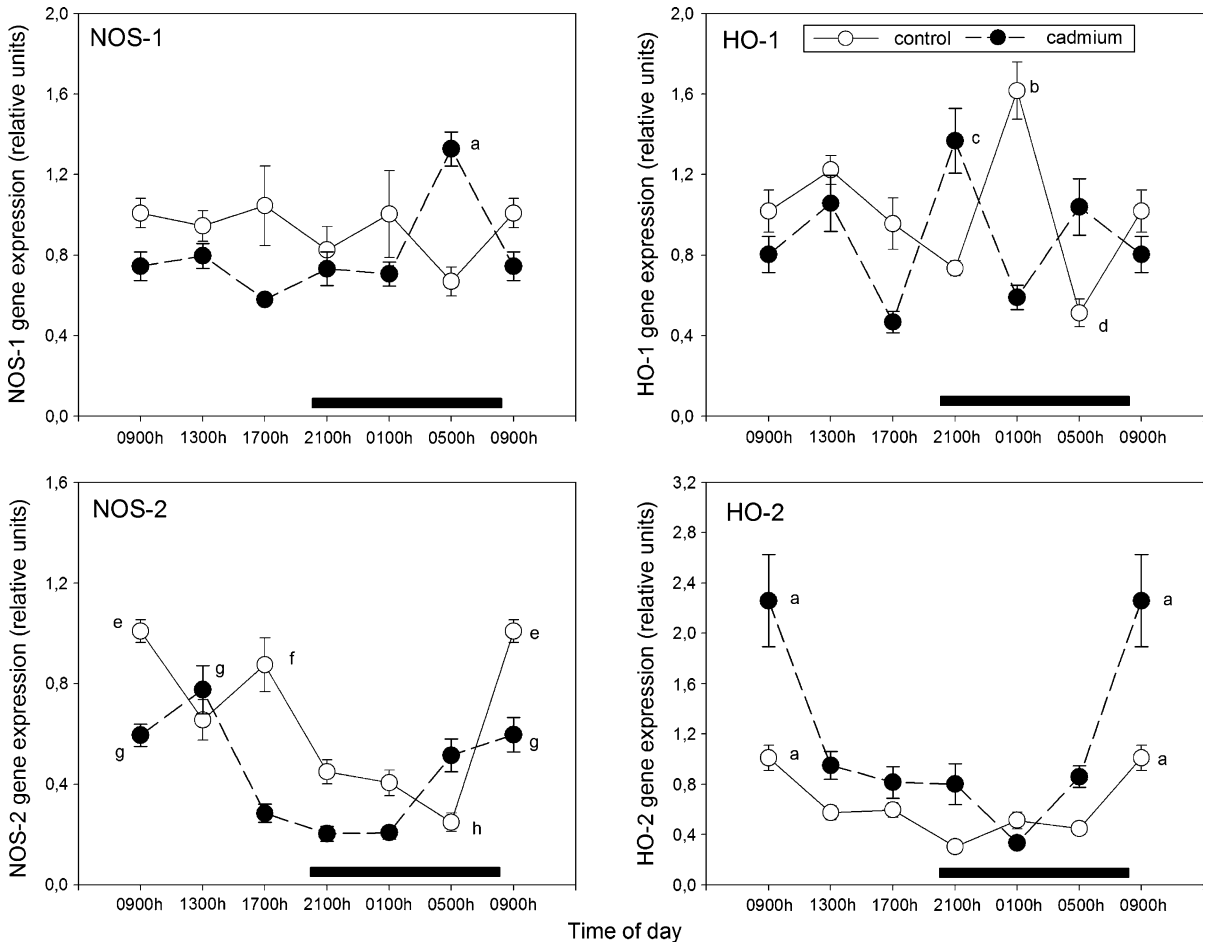

Fig. 1 Effect of Cd on 24-h changes in expression of mRNA for NOS-1, HO-1, NOS-2 and NOS-2 in rat medial basal hypothalamus. Groups of 6–8 rats receiving $CdCl_2$ (5 ppm in drinking water) or tap water for 1 month were killed by decapitation at six different time intervals throughout a 24 h cycle. Values at 0900 hours are repeated on the "second" day. 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. *Letters* denote significant differences in a one-way ANOVA followed by a Bonferroni's multiple comparison test, as follows: [a] $P < 0.01$ versus all other time points; [b] $P < 0.01$ versus 0900, 1700, 2100 and 0500 hours; [c] $P < 0.01$ versus 1700 and 0100 hours; [d] $P < 0.01$ versus 0900, 1300 and 0100 hours; [e] $P < 0.01$ versus 1300, 2100, 0100 and 0500 hours; [f] $P < 0.01$ versus 2100, 0100 and 0500 hours; [g] $P < 0.01$ versus 1700, 2100 and 0100 hours; [h] $P < 0.01$ versus 0900, 1300 and 1700 hours. For further statistical analysis, see text

of NOS-1 and -2, HO-1 and -2, Mn-SOD, catalase, GPx and GSR genes, and *Bmal1*, *Per1*, *Per2* and *Cry2* was seen in $CdCl_2$-treated rats.

In the brain ROS generation is a continuous and physiological phenomenon and therefore nerve cells possess efficient antioxidant systems that protect them from oxidative damage [for reviews, see (Hardeland et al. 2003; Mancuso et al. 2007; Radak et al. 2007; Reynolds et al. 2007)]. These defense systems are comprised by a wide spectrum of enzymatic (Inal et al. 2001) and non-enzymatic systems (Wang et al. 2006). Circadian variations of brain redox pathway enzymes have been described, including NOS (Tunctan et al. 2002; Ayers et al. 1996; Clemens et al. 2005), HO (Artinian et al. 2001; Rubio et al. 2003), SOD (Diaz-Munoz et al. 1985; Schaper et al. 1986; Martin et al. 2003) and catalase (Sani et al. 2006). In many cases rhythms in enzyme activity and gene expression coincide but in others they are out of phase, e.g. NOS (Ayers et al. 1996; Clemens et al. 2005). The present study indicates that in the MBH a significant 24-h variation in gene expression of NOS-2, HO-1 and HO-2, Cu/Zn-SOD, GPx and GSR is detectable in control rats.

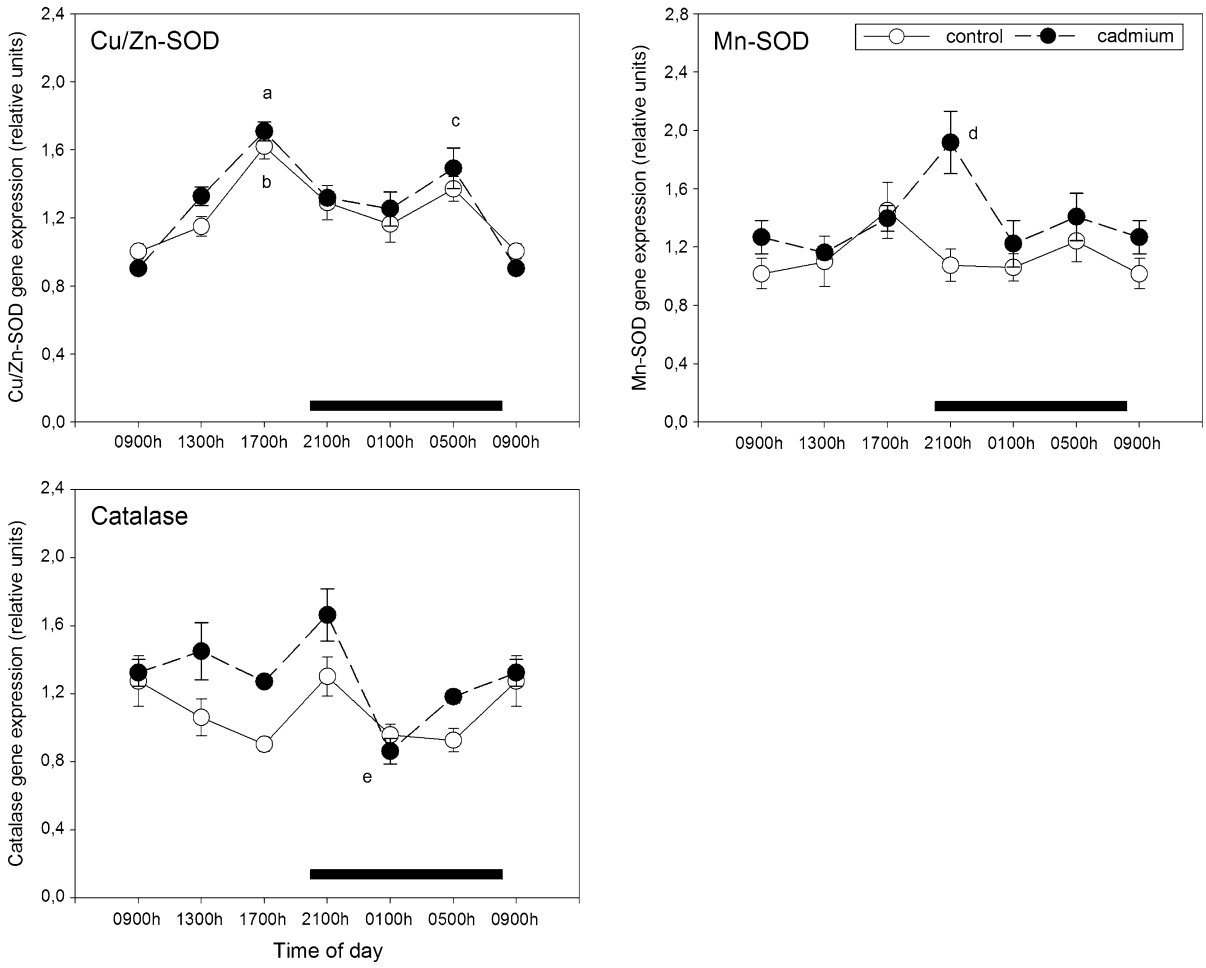

Fig. 2 Effect of Cd on 24-h changes in expression of mRNA for Cu/Zn-SOD, Mn/SOD and catalase in rat medial basal hypothalamus. Groups of 6–8 rats receiving $CdCl_2$ (5 ppm in drinking water) or tap water for 1 month were killed by decapitation at six different time intervals throughout a 24 h cycle. Values at 0900 hours are repeated on the "second" day. mRNA levels encoding Cu/Zn-SOD, Mn/SOD and catalase 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. *Letters* denote significant differences in a one-way ANOVA followed by a Bonferroni's multiple comparison test, as follows: [a] $P < 0.01$ versus 0900 and 0100 hours, $P < 0.05$ versus 2100 hours; [b] $P < 0.01$ versus 0900 hours, $P < 0.05$ versus 1300 and 0100 hours; [c] $P < 0.01$ versus 0900 hours; [d] $P < 0.05$ versus 0100 hours; [e] $P < 0.01$ versus 2100 hours. For further statistical analysis, see text

A number of studies examined the toxicity of Cd using a single (acute) dose of the metal in the range of 1–5 mg/kg (Barbier et al. 2004; Majumder et al. 2003). In the present study, and to mimic human exposure, we gave $CdCl_2$ in the drinking water to young rats for 1 month at a low concentration (5 ppm). Under this condition Cd intake (about 7.5 μg/day) was close to that proposed by the World Health Organization as a tolerable limit for humans [1 μg/day for a life span of 60 years (WHO 1995)]. It should be noted that because Cd absorption in the gastrointestinal tract of rats is lower than in humans, rat models simulating human exposure need to increase exposure doses to be higher than the real daily human intake of Cd (Rogalska et al. 2009).

Two possible interpretations can be entertained to explain the present observations in animals receiving low doses of $CdCl_2$: (1) the changes observed after Cd indicate homeostatic mechanisms related to excessive ROS generation; (2) the low levels of Cd given fall below the threshold needed to cause oxidative damage in MBH and affect gene programming, probably via a

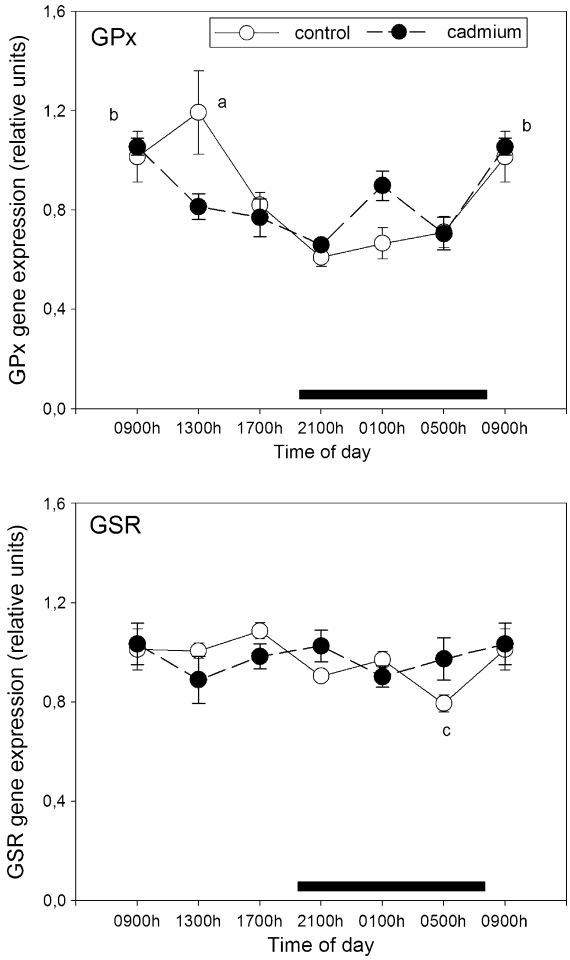

Fig. 3 Effect of Cd on 24-h changes in expression of mRNA for GPx and GSR in rat medial basal hypothalamus. Groups of 6–8 rats receiving $CdCl_2$ (5 ppm in drinking water) or tap water for 1 month were killed by decapitation at six different time intervals throughout a 24 h cycle. Values at 0900 hours are repeated on the "second" day. mRNA levels encoding GPx and GSR 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. *Letters* denote significant differences in a one-way ANOVA followed by a Bonferroni's multiple comparison test, as follows: [a] $P < 0.05$ versus 2100, 0100 and 0500 hours; [b] $P < 0.01$ versus 1700, 2100 and 0500 hours; [c] $P < 0.05$ versus 0900. 1300 and 1700 hours. For further statistical analysis, see text

moderate increase of ROS (Wright and Baccarelli 2007)- Clearly our results are not a consequence of an acute intoxication with Cd since body weight was not modified as we have previously reported using higher doses of the metal (Lafuente and Esquifino 1999). In a previous study (Poliandri et al. 2006) we found that a Cd intake similar to that used herein did not induce lipid peroxidation in the liver as it was reported using a similar experimental approach with 15 ppm of Cd (Koyu et al. 2006). Indeed, the blood brain barrier effectively prevents Cd uptake into the brain. For example, in a study investigating $CdCl_2$ absorption, distribution, and retention in rats after oral administration, 0.013% of the dose was taken up by brain tissue (Lazarus et al. 2009).

There is controversy about the role played by NO in Cd toxicity (Misra et al. 1996; Harstad and Klaassen 2002). The foregoing results indicate that a low Cd dose differentially affects NOS-1 and NOS-2 expression in MBH by disrupting their 24-h pattern and by decreasing the mRNA for the inducible isoform NOS-2. These results are compatible with the view that the toxic effect of a low dose of Cd is independent on excessive NO formation. It must be noted that in a previous publication using the same low dose of $CdCl_2$ at single time points, Cd increased expression of NOS-1 gene at 0900 hours, while having no effects on that of NOS-2 in the anterior hypothalamus (Poliandri et al. 2006). Since both the MBH and anterior hypothalamic blocks used to perform the studies are heterogenous, differences in neuronal nuclei composition may explain this regional difference. Further studies including in situ-hybridization of specific protein products could be helpful to answer this query.

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 (Mancuso et al. 2007). HO-1 is an inducible isoform that is responsive to various stimuli, including oxidative stress. HO-2 is a constitutive isoform not inducible by oxidative stress. In the present study increases in HO-2 expression, but not in HO-1 expression, were observed in MBH of $CdCl_2$-treated rats suggesting that the low levels of the heavy metal given fall below the threshold needed to cause oxidative damage in this brain region. In a previous study using anterior hypothalamic blocks (Poliandri et al. 2006), a similar dose of $CdCl_2$ increased expression of HO-1 gene at 0900 hours. Again, in situ-hybridization studies of the specific protein products could be helpful to support regional differences in responses to Cd.

The detoxification of ROS in cells involves the cooperative action of intracellular antioxidant

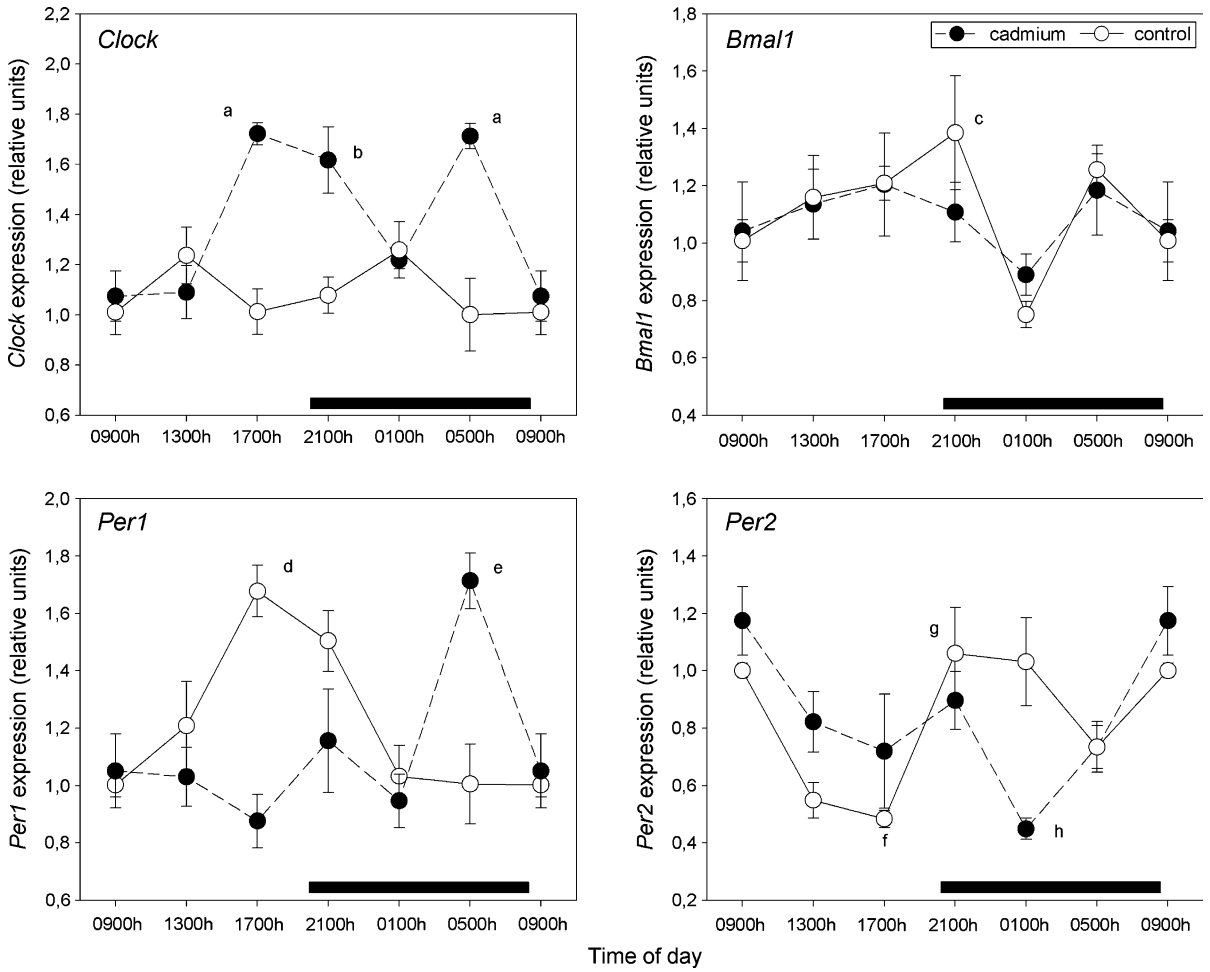

Fig. 4 Effect of Cd on 24-h changes in expression of *Clock*, *Bmal1*, *Per1* and *Per2* in rat MBH. Half of the rats received $CdCl_2$ (5 ppm in drinking water); controls were given water alone. After 1 month, groups of 6–8 rats were killed by decapitation at six different time intervals throughout a 24 h cycle. Values at 0900 hours are repeated on the "second" day. mRNA levels encoding circadian clock genes 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. Significant time-of-day variations within each experimental group, as detected by a one-way ANOVA, were as follows: *Clock*: cadmium, $F = 13.3$, $P < 0.01$. *Bmal1*: control, $F = 3.54$, $P < 0.02$. *Per1*: control, $F = 4.84$, $P < 0.01$; cadmium, $F = 7.38$, $P < 0.01$. *Per2*: control, $F = 6.37$, $P < 0.01$; cadmium, $F = 5.09$, $P < 0.01$. *Letters* denote significant differences in a one-way ANOVA followed by a Bonferroni's multiple comparison test as follows: [a] $P < 0.01$ versus 0900, 1300 and 0100 hours; [b] $P < 0.01$ versus 0900 and 1300 hours, $P < 0.05$ versus 0100 hours; [c] $P < 0.02$ versus 0100 hours; [d] $P < 0.05$ versus 0900 hours; [d] $P < 0.05$ versus 0900 hours; [e] $P < 0.01$ versus 0900, 1300, 1700 and 0100 hours; [f] $P < 0.01$ versus 0100 and 2100 hours, $P < 0.03$ versus 0900 hours; [g] $P < 0.01$ versus 1700 hours, $P < 0.03$ versus 1300 hours; [h] $P < 0.01$ versus 0500 hours. For further statistical analysis, see text

enzymes, among them Cu/Zn-SOD that is cytosolic, Mn-SOD that is mitochondrial, and catalase that is present in peroxisomes (Rodriguez et al. 2004). In addition, GPx and GSR help to maintain adequate levels of reduced glutathione. $CdCl_2$intake increased slightly Mn- SOD and catalase mRNA expression and did not affect that of Cu/Zn-SOD, GPx or GSR, therefore not supporting the notion that this is a compensatory increase caused by the augmented oxidative load.

It is known that the mammalian circadian timing system comprises peripheral oscillators located in almost every cell of the body together with a central rhythm generator located in the hypothalamic

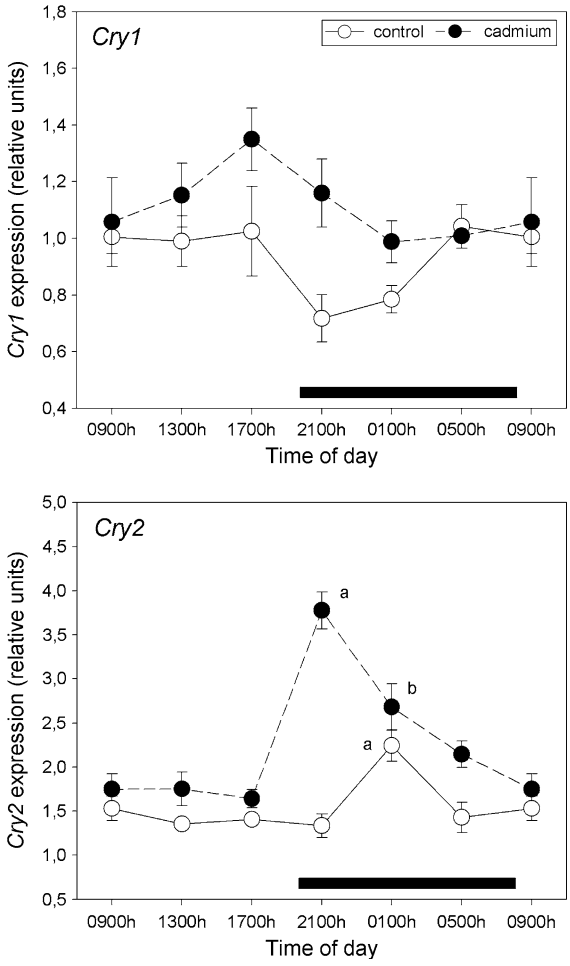

Fig. 5 Effect of Cd on 24-h changes in expression of *Cry1* and *Cry2* in rat MBH. Half of the rats received $CdCl_2$ (5 ppm in drinking water); controls were given water alone. After 1 month, groups of 6–8 rats were killed by decapitation at six different time intervals throughout a 24 h cycle. Values at 0900 hours are repeated on the "second" day. mRNA levels encoding circadian clock genes 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. Significant time-of-day variations within each experimental group, as detected by a one-way ANOVA, were as follows: *Cry2*: control, $F = 7.29$, $P < 0.01$; cadmium: $F = 18.8$, $P < 0.01$. *Letters* denote significant differences in a one-way ANOVA followed by a Bonferroni's multiple comparison test as follows: [a] $P < 0.01$ versus all remaining time intervals; [b] $P < 0.05$ versus 0900, 1300, 1700 and 100 hours. For further statistical analysis, see text

suprachiasmatic nucleus (SCN) (Richter et al. 2004; Lincoln 2006). At the cell level, circadian rhythms are driven by the self-regulatory interaction of a set of genes named clock genes and their protein products. Among these *Bmal1, Per1, Per2, Cry1, Cry2, and Clock* play a major role. The heterodimer of the proteins Clock:Bmal1 binds E-box elements at the promoter region of *Per1, Per2, Cry1 and Cry2,* inducing their transcription. Conversely, Per1–2 and Cry1–2 proteins, by interacting with the Clock:Bmal1 heterodimer operate as negative regulators inhibiting their own transcription (Richter et al. 2004; Lincoln 2006). As shown herein, $CdCl_2$ administration significantly suppressed (*Bmal1*) or disrupted 24-h pattern of expression (*Per1*, *Per2, Cry2*) while in the case of *Clock* significant 24-h variations were induced. The results indicate that the inherent transcription, translation, and post-translational modifications that give the clock its own natural rhythmicity (Ramsey et al. 2007) is disrupted in rats drinking a low amount of $CdCl_2$ in tap water. Previous studies form this laboratory indicated that chronic exposure of rats to low doses of Cd affected significantly the circadian variation of pituitary hormone release (Lafuente et al. 2003, 2004, 2005). Presumably, the changes in clock gene expression in MBH, a key region in hormone regulation, play a role in the circadian hormone disruption.

There are a number of limitations to the present study. One important is that additionally studies employing Western blotting analysis of enzyme protein levels and measuring enzyme activity are needed to understand $CdCl_2$ effect on redox enzyme gene expression in MBH. Additionally, studies employing Western blotting analysis of clock protein levels are needed to further describe the effect of $CdCl_2$ on circadian gene expression. It should be also important to assess whether the changes in amplitude as well in timing of 24-h rhythm of gene expression discussed herein can be attributed to an effect on the circadian master clock or to a masking effect on some output(s) of the clock. In any event the present results are compatible with the view that a low amount of Cd given in tap water brought about significant changes in circadian expression of redox enzyme and clock genes in rat MBH.

Acknowledgments This work was supported by grants from Fondo de Investigaciones Sanitarias (FIS-PI05-0163), Madrid, Spain, Programa de Creación y Consolidación de Grupos de Investigación, Universidad Complutense-Comunidad de Madrid (CCG08-UCM/SAL-4188) and Agencia Nacional de Promoción Científica y Tecnológica, Argentina (PICT 2007-01045). DPC is a Research Career Awardee and PAS is a postdoctoral fellow from the Argentine Research Council (CONICET).

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