

## EXPECTANCY FOR FOOD OR EXPECTANCY FOR CHOCOLATE REVEALS TIMING SYSTEMS FOR METABOLISM AND REWARD

M. ÁNGELES-CASTELLANOS,<sup>a</sup>  
R. SALGADO-DELGADO,<sup>a</sup> K. RODRÍGUEZ,<sup>a</sup>  
R. M. BUIJS<sup>b</sup> AND C. ESCOBAR<sup>a\*</sup>

<sup>a</sup>Departamento de Anatomía, Edificio B 4° Piso, Fac de Medicina Universidad Nacional Autónoma de México, México DF 04510, Mexico

<sup>b</sup>Departamento de Biología Celular y Fisiología, Instituto de Investigaciones Biomédicas Universidad Nacional Autónoma de México, México DF 04510, Mexico

**Abstract**—The clock gene protein Per 1 (PER1) is expressed in several brain structures and oscillates associated with the suprachiasmatic nucleus (SCN). Restricted feeding schedules (RFS) induce anticipatory activity and impose daily oscillations of c-Fos and clock proteins in brain structures. Daily access to a palatable treat (chocolate) also elicits anticipatory activity and induces c-Fos expression mainly in corticolimbic structures. Here the influence of daily access to food or chocolate was explored by the analysis of the oscillatory patterns of PER1 in hypothalamic and corticolimbic structures. Wistar rats were exposed to RFS or to daily access to chocolate for 3 weeks. Persistence of food or chocolate entrained rhythms was determined 8 days after cessation of the feeding protocols. RFS and chocolate induced a phase shift in PER1 rhythmicity in corticolimbic structures with peak values at zeitgeber time 12 and a higher amplitude in the chocolate group. Both RFS and chocolate groups showed an upregulation of PER1 in the SCN. Food and chocolate entrained rhythms persisted for 8 days in behavior and in PER1 expression in the dorsomedial hypothalamic nucleus, accumbens, prefrontal cortex and central amygdala.

The present data demonstrate the existence of different oscillatory systems in the brain that can be activated by entrainment to metabolic stimuli or to reward and suggest the participation of PER1 in both entraining pathways. Persistence and amplification of PER1 oscillations in structures associated with reward suggest that this oscillatory process is fundamental to food addictive behavior. © 2008 IBRO. Published by Elsevier Ltd. All rights reserved.

**Key words:** food-entrainment, reward, clock genes, suprachiasmatic nucleus, hypothalamus, addiction.

Circadian rhythms are driven by the suprachiasmatic nucleus of the hypothalamus (SCN), and are coupled to the light dark cycle (Klein et al., 1991). Time keeping mecha-

nisms in the SCN are driven by a set of clock genes (*per1*, *per2*, *per3*, *cry1*, *cry 2*, *clock* and *bmal1*), which transcription/translation cycles produce oscillations with a 24 h cycle (Dunlap, 1999; Okamura, 2004). Other brain structures and peripheral tissues also express clock genes in a circadian manner and their oscillations are mainly driven directly or indirectly by the SCN (Guo et al., 2005; Buijs and Kalsbeek, 2001). The detection of clock genes or the resulting proteins in the brain as well as in peripheral organs has indicated that the circadian system consists of multiple oscillators. Interestingly, when food is restricted to a few hours daily, mealtime not only exerts a powerful entraining force on metabolic functions (Escobar et al., 1998; Satoh et al., 2006) but also entrains clock gene expression in peripheral oscillators and uncouples their daily rhythm from the SCN (Damiola et al., 2000; Hara et al., 2001; Stokkan et al., 2001). Moreover this, restricted feeding schedule (RFS) at a predictable time elicits food anticipatory activity (FAA) characterized by behavioral arousal and increased locomotor activity 2–3 h prior to mealtime.

RFS induces c-Fos expression in hypothalamic regions involved in mediating metabolic and arousal signals to the rest of the brain (Ángeles-Castellanos et al., 2004; Gooley et al., 2006; Meynard et al., 2005). In addition, c-Fos is induced in corticolimbic structures involved in hedonic and motivational processes (Ángeles-Castellanos et al., 2007; Mendoza et al., 2005). RFS entrain daily cycles of *per1* and *per2* products in the dorsomedial nucleus of the hypothalamus (DMH) (Mieda et al., 2006) and in structures of the corticolimbic system (Ángeles-Castellanos et al., 2007; Verwey et al., 2007; Waddington Lamont et al., 2007; Wakamatsu et al., 2001) indicating that RFS is able to induce daily oscillations in brain systems involved in energy homeostasis and motivation for food.

Motivational factors involved in food entrainment can be dissected from homeostatic factors by providing to rats fed *ad libitum* daily at a fixed time a piece of chocolate, sucrose or sweet milk (Mistlberger and Rusak, 1987; Abe and Rusak, 1992; Mendoza et al., 2005). This procedure does not induce a negative metabolic state in the rats or a cycle of daily fasting–feeding alternation. Daily scheduled access to chocolate elicits anticipatory activity of short duration and elicits c-Fos expression in corticolimbic structures and not in hypothalamic nuclei (Mendoza et al., 2005), indicating that a daily rewarding stimulus is sufficient to elicit behavioral expectations and to induce strong anticipatory neuronal activation in corticolimbic structures.

The present study was designed to determine whether a daily predictable palatable treat entrains oscillations in

\*Corresponding author. Tel: +55-5623-2422; fax: +55-5623-2425.

E-mail address: [escocarolina@gmail.com](mailto:escocarolina@gmail.com) (C. Escobar).

**Abbreviations:** Acc, nucleus accumbens; Acc-Core, nucleus accumbens sub-region core; Acc-Shell, nucleus accumbens sub-region shell; AL, *ad libitum* group; ARC, arcuate nucleus; BLA, basolateral amygdala; CeA, central amygdala; CH, chocolate entrainment group; DMH, dorsomedial hypothalamic nucleus; FAA, food anticipatory activity; FE, food entrainment group; LD, light/dark; PeF, perifornical area; PER1, protein Per1; PFC, prefrontal cortex; PVT, paraventricular thalamic nucleus; RFS, restricted feeding schedules; SCN, suprachiasmatic nucleus; VMH, ventromedial nucleus; ZT, zeitgeber time.

similar brain areas as those entrained by RFS. Therefore expression of the clock gene protein Per1 (PER1) was used as indicator of such daily temporal activation. In order to demonstrate the endogenous nature of this oscillation and to reject that temporal patterns were elicited by the daily stimulus, persistence of the temporal patterns was explored in food and chocolate entrained rats 8 days after interruption of the entraining protocol. The present data indicate that corticolimbic structures contain circadian oscillators mainly driven by the reward of chocolate, while hypothalamic oscillations are specifically driven by food.

## EXPERIMENTAL PROCEDURES

### Animals and general housing conditions

Adult male Wistar rats weighing between 250 and 300 g at the beginning of the experiment were housed in individual transparent acrylic cages and were maintained in isolated lockers in a sound-proof monitoring room with a 12-h light/dark (LD) cycle (lights-on at 06:00 h defined as zeitgeber time (ZT) 0), constant temperature ( $24 \pm 1$  °C), circulating air and free access to food (Rodent Laboratory Chow 5001; Purina, Minnetonka, MN, USA) and water, unless otherwise stated. Experimental procedures were approved and conducted according to the ethical committee at the Medical Faculty UNAM, Mexico. Experiments conformed to international guidelines on the ethical use of animals; procedures were aimed at minimizing the number of animals used and their suffering.

### Groups and food entrainment

After 2 weeks' acclimatization in the monitoring cages rats were randomly assigned to one of three groups: *ad libitum* feeding control (AL;  $n=24$ ), food entrainment (FE;  $n=24$ ) or chocolate entrainment (CH;  $n=24$ ). The FE group had access to food for 2 h/day, from 12:00 to 14:00 h (ZT6–ZT8). Rats assigned to the CH group, had always free access to food and received daily at ZT6, 5 g of a commercial chocolate bar containing 10% of proteins, 51% of carbohydrates and 34% fat for a caloric value of 550 kcal/100 g. After 2 weeks in the feeding schedule rats were anesthetized and perfused at one of four temporal points to complete a 24 h cycle: ZT0, ZT6, ZT12 and ZT18 (ZT0 denotes time of lights on). Four of the control and FE subjects included were used for a prior study (Ángeles-Castellanos et al., 2007), however two additional subjects were included for each group and temporal point to replicate our previous findings, thus resulting six subjects per temporal point.

In order to explore persistence of the food and chocolate entrained patterns 16 additional rats were randomly assigned to a food (FE-P;  $n=8$ ) or chocolate (CH-P;  $n=8$ ). Entrainment group and were exposed to the same monitoring conditions and entrainment protocol, as described. After 2 weeks' RFS, rats were left *ad libitum* for 5 days followed by 3 days in fasting and for CH rats the delivery of the palatable treat was interrupted and rats were left *ad libitum*. On day 8 after cessation of entraining protocol rats were perfused at two time points ZT6 and ZT12 (low and high point of the daily curve, respectively).

### General activity monitoring system

For behavioral monitoring rats were placed in individual transparent acrylic cages (45×30×35 cm) positioned on plates with movement sensors in soundproof lockers housing eight cages and controlled lighting conditions. Experimental groups were maintained in separate lockers in order not to influence mutual behavior.

The detection system was developed in our group with the contributions from Nico Bos in Amsterdam the Netherlands and

the Mexican biomedical company Omniaiva. Sensors placed under the cages detect continuously general activity. Behavioral events were collected with a digitized system and automatically stored every minute in a PC for further analysis with a system developed for our laboratory SPAD9 based on Matlab.

Double-plotted actograms were obtained by organizing activity counts in 15 min intervals with the SPAD9. Due to different detection threshold among sensors, movement counts were normalized to the proportional percentage of the daily activity. Mean activity waveforms were constructed for the baseline and for the last 8 days of food or chocolate entrainment. In order to determine persistence of behavioral patterns activity, wave forms were constructed for the FE group with data for the 2 days in fasting (persistence) or for the CH group with data of the following 5 days in *ad libitum* feeding after interrupting chocolate delivery.

The intensity of FAA was calculated by totaling the normalized counts of activity displayed by the rats 1 h before meal or chocolate time. The intensity of persistence was estimated in fasting (FE; 2 days) or *ad libitum* (CH; 5 days) for the same 1 h interval. Activation in both conditions was compared with the expected activity during basal conditions.

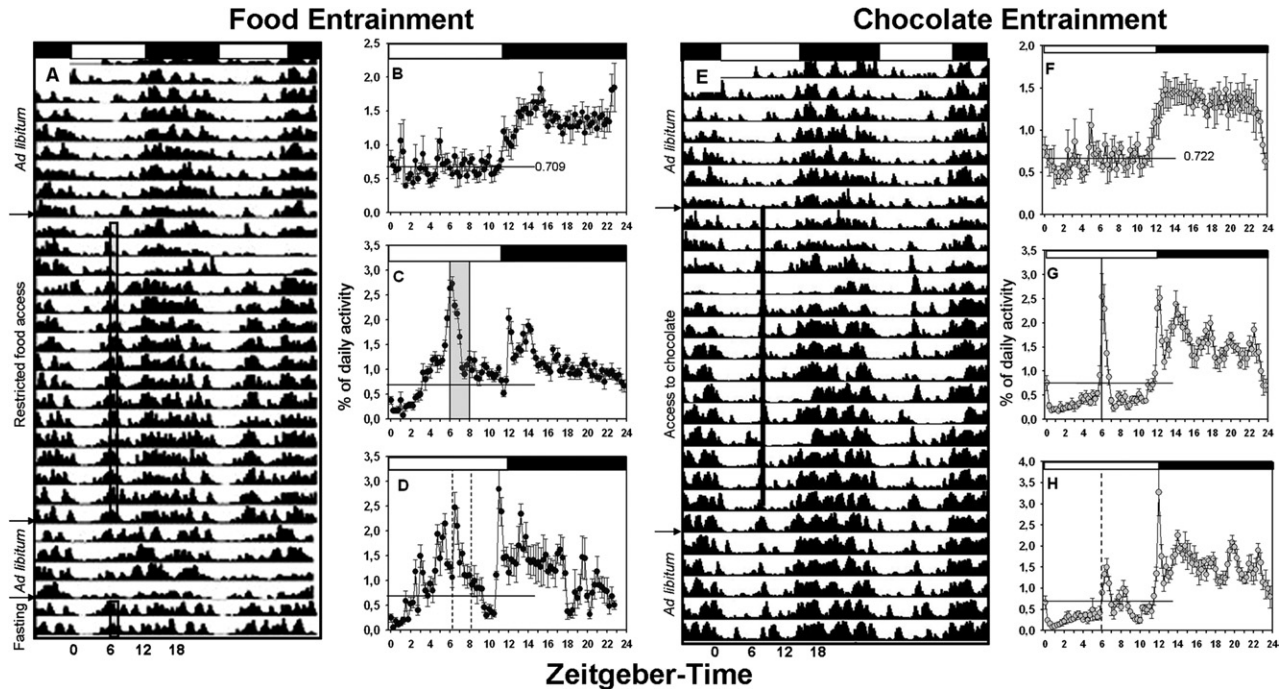
### Immunohistochemistry

Rats were anesthetized with an overdose of sodium pentobarbital (Sedal-Vet 65 mg/ml), and were perfused transcardially with 250 ml of 0.9% saline followed by 250 ml of fixative 4% paraformaldehyde in phosphate buffer saline (PBS, 0.1 M, pH 7.2). Brains were removed, postfixed for 24 h and cryoprotected in 30% sucrose for 3–4 days. Brains were frozen and cut in sections of 40  $\mu\text{m}$  at  $-18$  °C. Sections were serially collected in four series. The first series was processed for PER1 immunohistochemistry. Free floating sections were incubated in PER1 antibody raised in goat (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 72 h at 4 °C. This was followed by incubation in secondary antibody (rabbit anti-goat; Vector Laboratories) 1:200 in PBSGT for 2 h at room temperature, followed by incubation in avidin–biotin complex (0.9% avidin and 0.9% biotin solutions; Vector Laboratories) in PBSGT for 2 h at room temperature. Tissue was then reacted in diaminobenzidine (0.5 mg/ml, in Trizma buffer 7.2) with hydrogen peroxide (35  $\mu\text{l}$ , 30%  $\text{H}_2\text{O}_2$ ), mounted, dehydrated and coverslipped with mounting medium for microscopy (Estellanew; Merck cat. HX614429). Brains of the three groups and for different time points were processed simultaneously in order to standardize the intensity of the staining and background.

### Cell count

In order to quantify PER1 positive cells in hypothalamic and forebrain areas, one representative section for each structure was selected in accordance with the stereotaxic atlas from Paxinos and Watson (1998). In the hypothalamus the expression of PER1 in the SCN was quantified at the level of bregma  $-1.30$ , while the number of PER1 positive cells in the DMH, the ventromedial nucleus (VMH), the perifornical area (PeF) and the arcuate nucleus (ARC) was quantified in a posterior section (bregma  $-3.30$ ). For corticolimbic areas we analyzed the prefrontal cortex (PFC) at the level of bregma 2.70 and the nucleus accumbens (Acc). Subdivided and evaluated in two sub-regions core (Acc-Core) and shell (Acc-Shell) at bregma 2.20. The paraventricular nucleus of the thalamus (PVT) was analyzed in two levels bregma  $-1.40$  and  $-1.60$ ; and the amygdala subdivided in basolateral (BLA) and central (CeA) was analyzed at the level of bregma  $-2.80$ .

Images of selected sections were obtained with a 20× ocular using a computerized image system (Image-Pro plus 5.1; mediaCibernetica) attached to a light microscope (Olympus BX41). Cells positive to PER1 were counted bilaterally in the selected section with the image processing program (ImageJ) of the National Institutes of Health (NIH Image). To minimize the number of false



**Fig. 1.** Representative double-plotted general activity actograms for a food entrained (A) and chocolate entrained (E) rat. Mean activity profile of eight rats in *ad libitum* (B, F), 2 weeks under food restriction (C) or under chocolate entrainment (G), show the differential response in intensity and duration for anticipatory activity. Lower graphs show persistence of entrained activity after 5 days in *ad libitum* and 2 days in fasting (D) or for 5 days in *ad libitum* after chocolate entrainment (H). White and black bars represent the LD cycle, horizontal line represents the mean activity during the light phase. The rectangle and line in ZT6 represent food or chocolate access (respectively).

positives, background optic density was established for each section in a nearby region lacking PER1. Stained cells that reached or surpassed  $2\times$  the background optic density were considered positive and were included, whereas cells under this staining threshold were discarded. A single examiner, who was blind to the treatment conditions, performed all counts.

### Data analysis

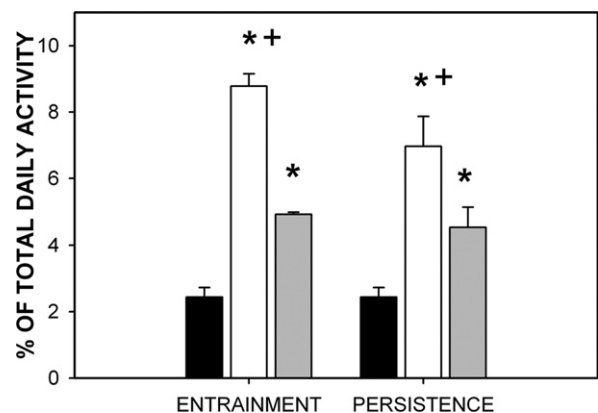
Behavioral data for the intensity of FAA and persistence were classified by group and were compared with a one-way ANOVA followed by a Tukey post hoc test with significant values set at  $P<0.05$ .

The number of PER1 positive cells for each structure was classified for group and time and is represented as mean  $\pm$  standard error of the mean. Data for the entrained groups were compared with the *ad libitum* control with a two-way ANOVA for independent measures with a factor for group and a factor for time. The two-way ANOVA was followed by a Tukey post hoc test with significant values set at  $P<0.01$ . The number of PER1 positive cells for the persistence groups was compared between temporal points (ZT6 and ZT12) with a one-way ANOVA. Statistical analysis was performed with the program Statistica version 4.5 (StatSoft, Inc. 1993).

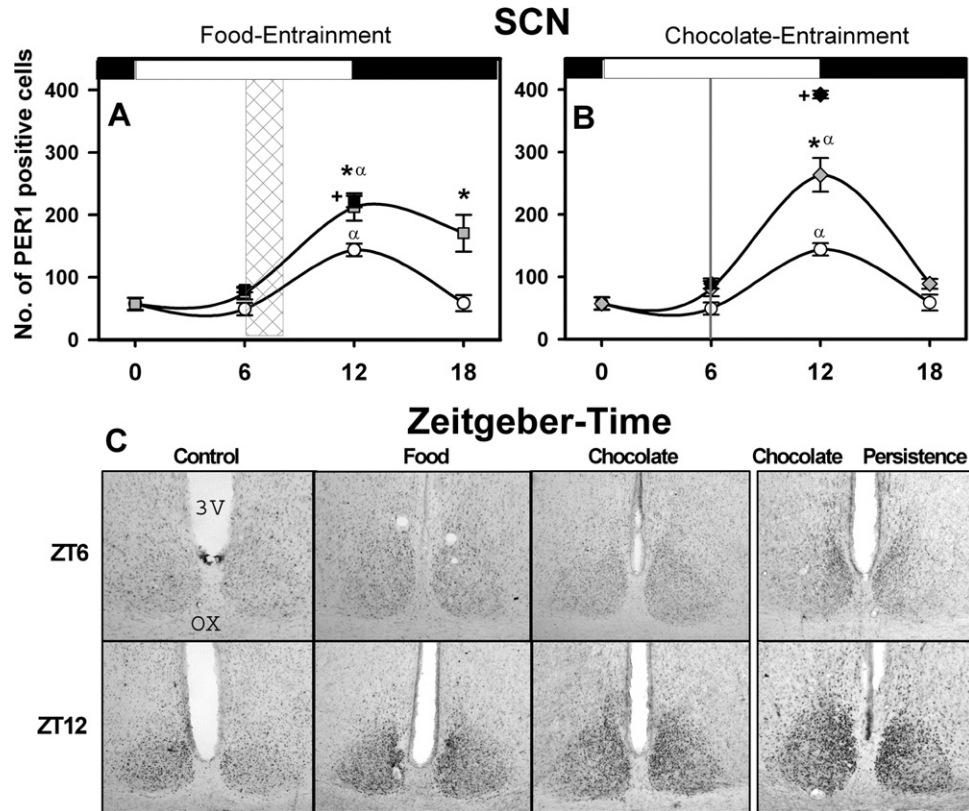
## RESULTS

Visual inspection of actograms indicated that scheduled food access produced anticipatory activity in all the rats, characterized by increased general activity at least 1 h prior to and at the moment of mealtime (Fig. 1A). Scheduled chocolate access produced increased activity briefly prior and at the moment of chocolate delivery (Fig. 1E).

Average activity waves confirmed the expression of FAA in the FE group and a brief activation just before the moment of chocolate delivery for the CH group (Fig. 1C and 1G). After cessation of the entraining protocol a clear persistence of the entrained behavioral pattern at the expected mealtime was observed 7 days later in fasting for the FE-P group and at least 7 days in *ad libitum* for the CH-P group (Fig. 1A, D, E, H). Further analysis indicated that during entrainment as well as during persistence the intensity of activity for the 3 h prior to mealtime was significantly dif-



**Fig. 2.** Total normalized activity of rats for 1 h prior to mealtime for the *ad libitum* baseline (black). The food entrained group (white) and the chocolate group (gray). \* Significant difference vs. the *ad libitum* rats ( $P<0.01$ ); + statistical difference between food entrained and chocolate group ( $P<0.01$ ).



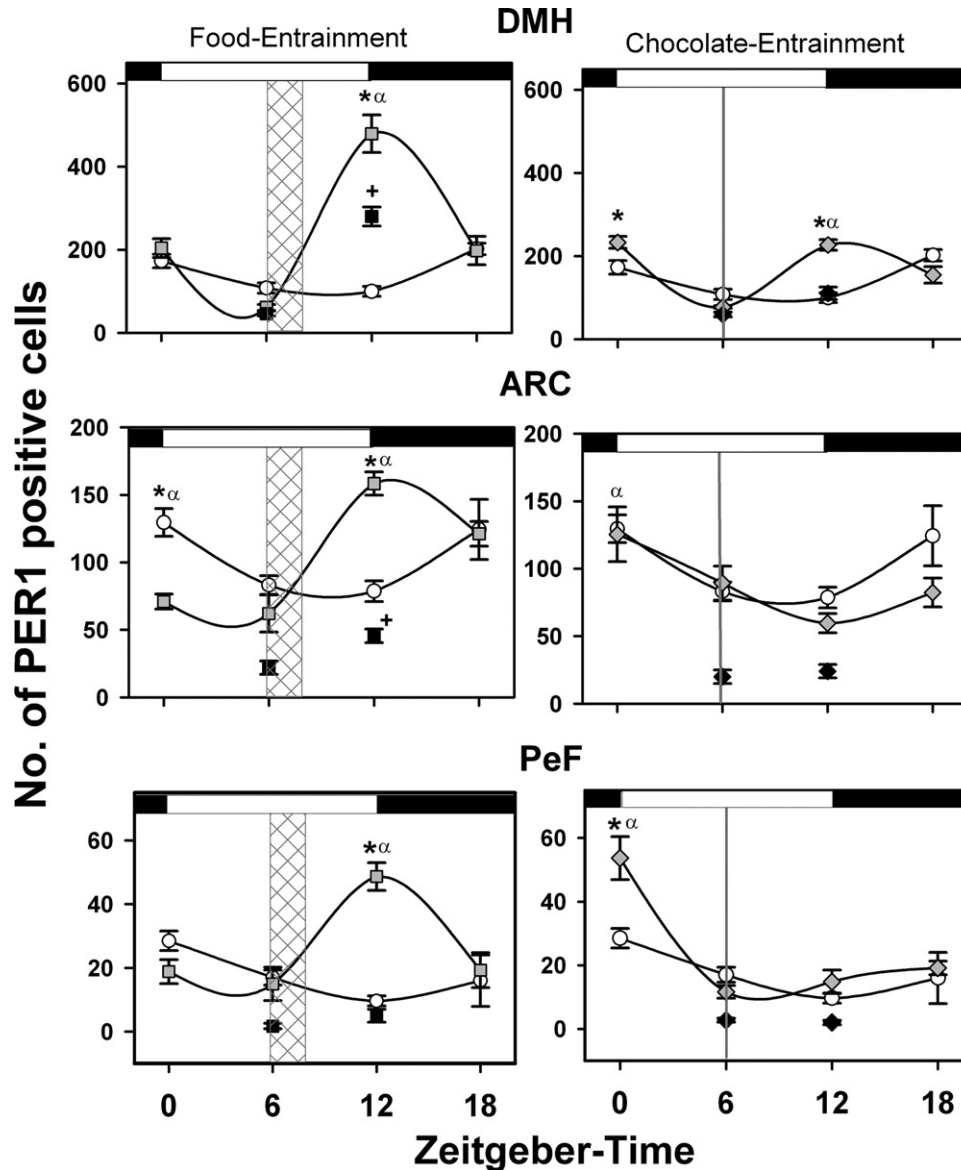
**Fig. 3.** Total cell number expressing PER1 protein in the SCN of *ad libitum* controls (white circles), food entrained (gray squares, A) and chocolate entrained (gray rhombus, B) rats at four temporal points and in persistence for FE-P group (black squares) and CH-P group (black rhombus). ZT0 represents time of lights on, the white and black bars represent the L/D cycle. Food or chocolate was delivered at ZT6 indicated by the rectangle and vertical line respectively on the x axis. \* Statistical difference between FE or CH and the *ad libitum* rats ( $P < 0.01$ );  $\alpha$  significant difference between the peak and lower temporal points of the same group; + statistical difference between two temporal points in the persistence group ( $P < 0.01$ ). In C representative microphotographs at ZT6 and ZT12 of PER1 expression in the SCN. 3V=Third ventricle; OX=Optic chiasm.

ferent from the activity observed in the baseline (Fig. 2). The one-way ANOVA indicated significant difference among groups (baseline vs. FAA  $F(2,19)=57.87$ ;  $P < 0.00001$ ), and during the persistence interval (baseline vs. persistence  $F(2,19)=16.04$ ;  $P < 0.00001$ ).

The *ad libitum* group showed a diurnal pattern of PER1 protein expression in the SCN, with peak values at ZT12 (Fig. 3). Neither food nor chocolate entrainment modified the phase of PER1 expression in the SCN, however both conditions induced up-regulation of the daily peak and this effect was enhanced in the CH group. Statistical analysis indicated significant difference between AL and FE groups ( $F(1,40)=25.68$ ;  $P < 0.0001$ ), a significant effect of time ( $F(3,40)=30.92$ ;  $P < 0.0001$ ) and for the interaction group $\times$ time ( $F(3,40)=5.89$ ;  $P < 0.001$ ) and between AL and CH groups ( $F(1,40)=27.52$ ;  $P < 0.0001$ ) due to time ( $F(3,40)=64.85$ ;  $P < 0.0001$ ) and the interaction of both factors ( $F(3,40)=8.94$ ;  $P < 0.0001$ ). In the FE-P group, after the refeeding–fasting protocol, PER1 expression maintained the same levels and temporal pattern as the FE group. (Fig. 3 left). The one-way ANOVA confirmed a significant temporal difference between ZT6 and ZT12 ( $F(1,6)=893.67$ ;  $P < 0.00001$ ). In the CH-P group, after 8 days without chocolate access PER1 expression in the SCN maintained the same phase but with increased peak

levels at ZT12 (Fig. 3 right), statistically different from ZT6 ( $F(1,6)=215.77$ ;  $P < 0.0006$ ). The increased levels of PER1 expression were mainly due higher cell counts in the dorsomedial SCN. In this region at ZT12 the control group had a mean of  $110 \pm 11.8$  positive cells, the FE  $136.8 \pm 6.7$ , the CH  $177.9 \pm 12.9$ , the FE-P  $148.5 \pm 20.5$  and the CH-P  $350.7 \pm 30.7$ .

In the DMH no daily rhythmicity in the expression of the PER1 protein was observed for the *ad libitum* group (Fig. 4). FE induced a rhythm with peak values at ZT12 (6 h after mealtime), significantly higher than values observed along the daily cycle in *ad libitum* condition. Statistical analysis indicated significant difference between groups ( $F(1,40)=35.95$ ;  $P < 0.0001$ ), a significant effect of time ( $F(3,40)=31.43$ ;  $P < 0.0001$ ) and for the interaction group $\times$ time ( $F(3,40)=42.67$ ;  $P < 0.0001$ ). In the FE-P group the food-entrained pattern was maintained with lower values, but keeping a significant statistical difference between ZT6 and ZT12 ( $F(1,6)=96.95$ ;  $P < 0.0001$ ). Daily chocolate access also modified the temporal pattern from that observed in the *ad libitum* controls (Fig. 4 right), with high values at ZT0 and ZT12 (6 h after chocolate access). The two-way ANOVA indicated significant difference between groups ( $F(1,40)=10.21$ ;  $P < 0.0001$ ) due to time ( $F(3,40)=31.07$ ;  $P < 0.0001$ ) and the interaction of both



**Fig. 4.** Total cell number expressing PER1 protein in the DMH, ARC and PeF. The effect of food entrainment is shown on the left and chocolate entrainment on the right. The *ad libitum* controls (white circles), FE group (gray squares) and CH group (gray rhombus) at four temporal points and in persistence for FE-P group (black squares) and CH-P group (black rhombus) \* statistical difference between FE or CH and the *ad libitum* rats ( $P < 0.01$ ); α significant difference between the peak and lower temporal points of the same group; + statistical difference between two temporal points in the persistence group ( $P < 0.01$ ). Other indications as in Fig. 3.

factors group $\times$ time indicated statistical differences ( $F(3,40)=23.04$ ;  $P < 0.0001$ ). This pattern did not persist after interruption of chocolate entrainment.

The ARC, which is a structure involved in receiving humoral and metabolic signals from the periphery exhibited a diurnal rhythm of PER1 expression in the *ad libitum* control, with high values during the night, when rats usually feed (Fig. 4). In the FE group the daily peak was shifted toward ZT12 (6 h after meal access). The two-way ANOVA indicated no difference between groups ( $F(1,40)=0.01$ ;  $P = \text{NS}$ ) but significant difference due to time ( $F(3,40)=9.48$ ;  $P < 0.0001$ ) and due to the interaction of both factors group $\times$ time ( $F(3,40)=15.56$ ;  $P < 0.0001$ ). After 5 days *ad*

*libitum* and 3 days in fasting, PER1 expression in the FE-P group exhibited low amplitude but still significant higher levels at ZT12 ( $F(1,6)=169.92$ ;  $P < 0.0001$ ). Daily access to chocolate did not modify the temporal pattern of PER1 expression, which remained similar to the AL group and no statistical difference was obtained between groups ( $F(3,40)=2.97$ ;  $P = \text{NS}$ ), a significant effect was obtained for the factor time ( $F(3,40)=8.52$ ;  $P < 0.001$ ), and not for the interaction group $\times$ time ( $F(3,40)=1.50$ ;  $P = \text{NS}$ ). After 8 days without chocolate access PER1 did not express a temporal difference ( $F(1,6)=4.8$ ;  $P = \text{NS}$ ; Fig. 6 right).

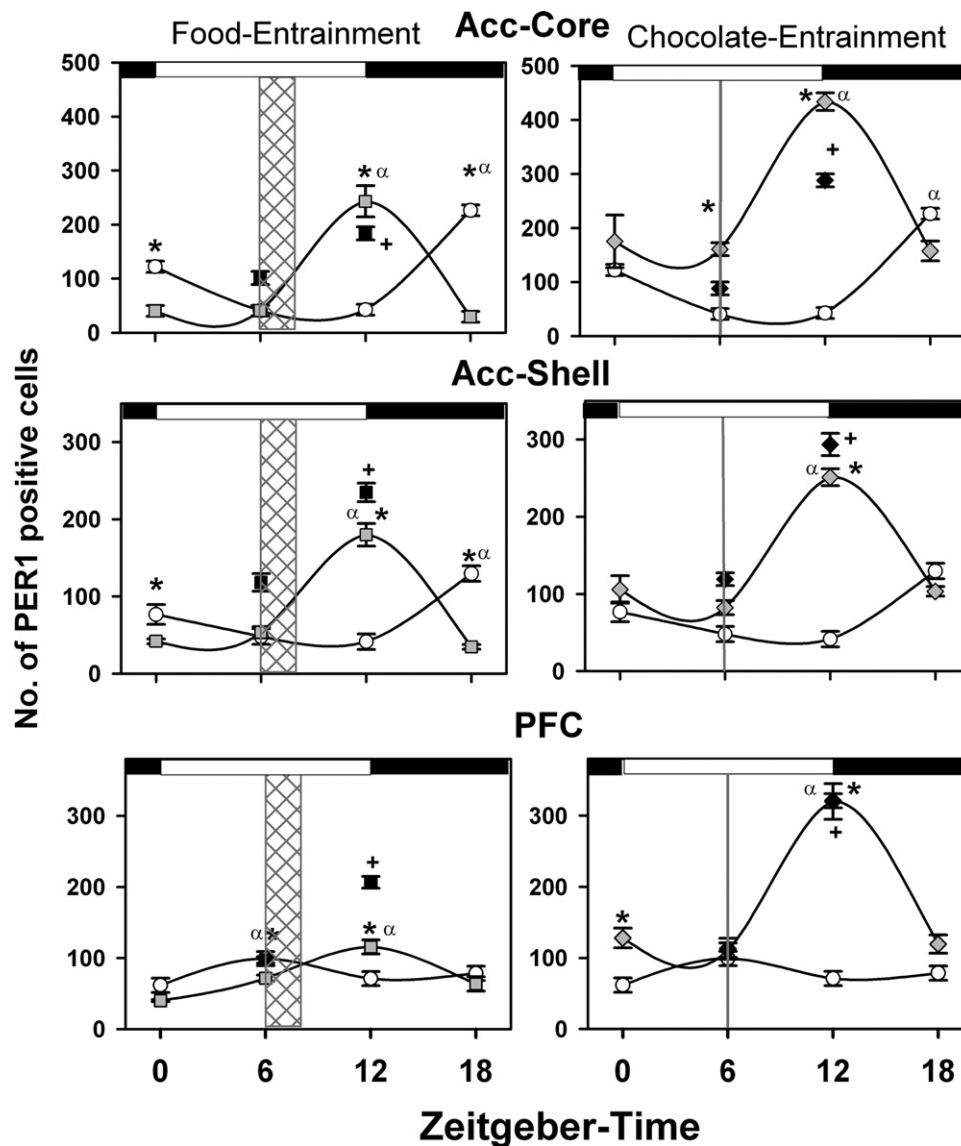
In the PeF no daily oscillation in the expression of the PER1 protein was observed (Fig. 4 bottom), but RFS

induced a significant rhythm with peak values at ZT12 (6 h after mealtime), as observed in the DMH and ARC. Statistical analysis indicated significant difference between *ad libitum* and RFS groups ( $F(1,40)=6.74$ ;  $P<0.01$ ), a significant effect of time ( $F(3,40)=4.14$ ;  $P<0.01$ ) and for the interaction group $\times$ time ( $F(3,40)=13.33$ ;  $P<0.0001$ ). After the refeeding–fasting protocol the food-entrained temporal pattern was not maintained, thus no difference was observed between ZT6 and ZT12 ( $F(1,6)=2.15$ ;  $P=NS$ ). Daily chocolate delivery produced a peak at ZT0 (6 h before chocolate access) in the PeF (Fig. 4 right). The two-way ANOVA indicated significant difference between *ad libitum* and CH group ( $F(1,40)=6.55$ ;  $P<0.01$ ) due to time ( $F(3,40)=23.58$ ;  $P<0.0001$ ) and the interaction of both factors group $\times$ time indicated statistical differences

( $F(3,40)=5.50$ ;  $P<0.002$ ). After 8 days without chocolate values of PER1 expression were lower than those observed in the *ad libitum* and RFS groups. Due to the sampling it was not possible to define the persistence of the peak observed at ZT0.

In the VMH the expression of PER1 was very scarce and in some cases nonexistent, no temporal pattern could be determined for the different groups (data not shown).

In both Acc subregions (Acc-Core and Acc-Shell) a daily temporal pattern in PER1 expression was observed in the *ad libitum* group with peak values at ZT18, RFS shifted the daily peak to ZT12 (Fig. 5 left). Statistical analysis indicated a significant difference between groups ( $F(1,38)=77.10$ ;  $P<0.001$  for the Acc-Core;  $F(1,39)=0.56$ ;  $P<0.0001$  for the Acc-Shell), a significant effect of



**Fig. 5.** Total number of cells expressing PER1 protein in the two sub-regions of Acc and PFC. The *ad libitum* controls (white circles), FE group (gray squares) and CH group (gray rhombus) at four temporal points and in persistence for FE-P group (black squares) and CH-P group (black rhombus) \* statistical difference between FE or CH and the *ad libitum* rats ( $P<0.01$ );  $\alpha$  significant difference between the peak and lower temporal points of the same group; + statistical difference between two temporal points in the persistence group ( $P<0.01$ ). Other indications as in Fig. 3.

time ( $F(3,38)=425.29$ ;  $P<0.00001$  for the Acc-Core;  $F(3,39)=29.86$ ;  $P<0.00001$  for the Acc-Shell) and for the interaction group $\times$ time ( $F(3,38)=1.41$ ;  $P<0.00001$  for the Acc-Core;  $F(3,39)=101.65$ ;  $P<0.00001$  for the Acc-Shell). After the refeeding–fasting protocol a significant temporal difference persisted between ZT6 and ZT12 in both Acc subregions ( $F(1,6)=39.04$ ;  $P<0.0007$ , for Acc-Core;  $F(1,6)=75.76$ ,  $P<0.0001$ , for Acc-Shell). Chocolate entrainment also shifted the daily peak (acrophase) to ZT12 and, increased significantly the amplitude of the rhythm in both Acc subregions (Fig. 5 right). The two-way ANOVA indicated significant difference between AL and CH groups ( $F(1,39)=8.94$ ;  $P<0.0001$  for Acc-Core;  $F(1,39)=96.97$ ,  $P<0.00001$  for Acc-Shell), due to time ( $F(3,39)=1.97$ ;  $P<0.0001$  for Acc-Core;  $F(3,39)=30.11$ ,  $P<0.0001$  for Acc-Shell) and the interaction of both factors group $\times$ time ( $F(3,39)=5.65$ ;  $P<0.0001$  for Acc-Core;  $F(3,39)=68.21$ ,  $P<0.0001$  for Acc-Shell). After interruption of chocolate delivery for 8 days the chocolate entrained temporal pattern persisted in both Acc subregions (Fig. 5), and the temporal difference between ZT6 and ZT12 remained statistically significant ( $F(1,6)=231.66$ ;  $P<0.0005$  for Acc-Core;  $F(1,6)=110.96$ ,  $P<0.0001$  for Acc-Shell).

The PFC exhibited daily PER1 protein oscillations in control *ad libitum* with a peak at ZT6, RFS induced a shift of the PER1 peak to ZT12 (Fig. 5 bottom) as observed in the Acc. Statistical analysis did not indicate a significant difference between groups ( $F(1,40)=2.51$ ;  $P=NS$ ). But significant effect by time ( $F(3,40)=38.01$ ;  $P<0.0001$ ) and for the interaction group $\times$ time ( $F(3,40)=30.54$ ;  $P<0.0001$ ). In the FE-P group PER1 expression maintained the same temporal pattern as the FE group with even higher amplitude between ZT6 and ZT12, the one-way ANOVA indicated significant difference between the two time points ( $F(1,6)=136.82$ ;  $P<0.0001$ ). In the CH group the acrophase of PER1 expression was also shifted to ZT12 and with increased amplitude (Fig. 5). The two-way ANOVA confirmed significant difference between AL and CH groups ( $F(1,40)=153.40$ ;  $P<0.0001$ ), due to time ( $F(3,40)=41.98$ ;  $P<0.0001$ ) and the interaction of both factors group $\times$ time ( $F(3,40)=51.88$ ;  $P<0.0001$ ). After 8 days without chocolate access PER1 expression maintained the temporal pattern imposed by daily chocolate access and with the same peak level at ZT12 ( $F(1,6)=684.08$ ;  $P<0.0001$ ).

In the CeA and BLA no daily temporal pattern in PER1 expression was observed under *ad libitum* conditions (Fig. 6). In the CeA, RFS induced a low amplitude oscillation with high values at ZT12, however statistical analysis indicated no significant difference between groups ( $F(1,40)=2.96$ ;  $P=NS$ ), a significant effect of time ( $F(3,40)=9.30$ ;  $P<0.0001$ ) and no effect due to the interaction group $\times$ time ( $F(3,40)=1.11$ ;  $P=NS$ ). After 8 days the temporal pattern induced by RFS persisted with higher amplitude and thus was statically significant ( $F(1,6)=81.84$ ;  $P<0.0001$ ). In the BLA food entrainment did not induce any effect on PER1 expression. Statistical analysis confirmed no significant effects among groups ( $F(1,40)=0.33$ ;  $P<NS$ ), in the time ( $F(3,40)=1.36$ ;  $P=NS$ ) and due to the interaction group $\times$ time ( $F(3,40)=0.81$ ;  $P=NS$ ). In contrast chocolate entrainment induced a significant oscillation in both CeA

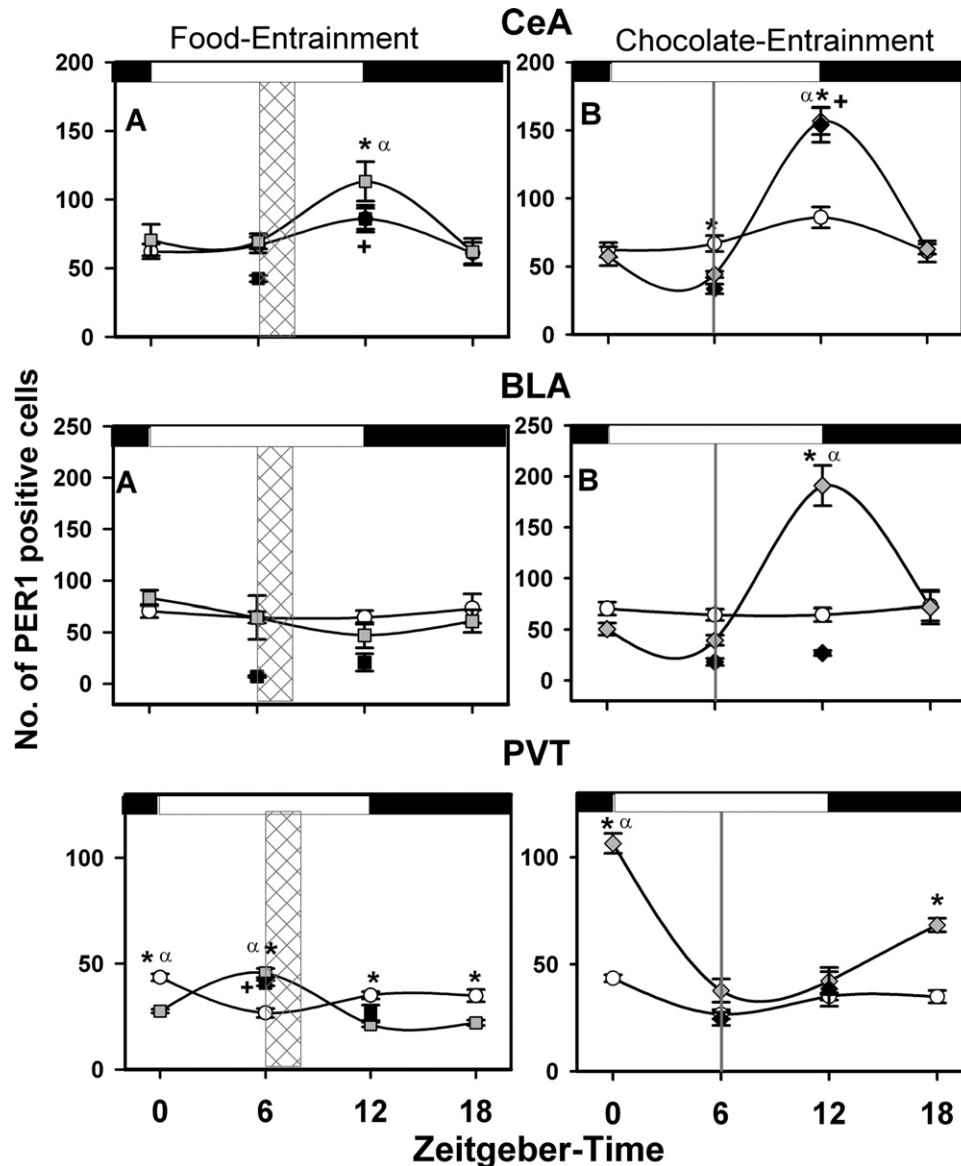
and BLA with acrophase at ZT12 (Fig. 6 right). The two-way ANOVA indicated a significant difference between AL and CH groups ( $F(1,40)=7.28$ ;  $P<0.01$  for CeA;  $F(1,40)=7.78$ ,  $P<0.008$  for BLA), due to time ( $F(3,40)=56.24$ ;  $P<0.00001$  for CeA;  $F(3,40)=22.36$ ,  $P<0.0001$  for BLA) and the interaction of both factors group $\times$ time ( $F(3,40)=24.12$ ;  $P<0.00001$  for CeA;  $F(3,40)=24.68$ ,  $P<0.0001$  for BLA). In CeA the chocolate entrained temporal pattern persisted with the same amplitude as during the entrained condition (Fig. 6 right), the temporal difference between ZT6 and ZT12 was statistically significant ( $F(1,6)=81.84$ ;  $P<0.0001$ ). In contrast in the BLA the chocolate induced rhythm did not persist ( $F(1,6)=2.59$ ;  $P=NS$ ).

The PVT exhibited daily PER1 protein oscillations in *ad libitum* with a peak at ZT0. RFS induced a shift of the PER1 peak to ZT6 (Fig. 6), statistical analysis indicated a significant difference between groups ( $F(1,40)=26.23$ ;  $P<0.0001$ ) by time ( $F(3,40)=14.06$ ;  $P<0.0001$ ) and for the interaction group $\times$ time ( $F(3,40)=50.79$ ;  $P<0.0001$ ). In the FE-P group PER1 expression maintained the same temporal pattern as the FE group. The one-way ANOVA indicated significant difference between time points ZT6 and ZT12 ( $F(1,6)=13.10$ ;  $P<0.01$ ). In the CH group the acrophase of PER1 expression remained at ZT0, however with increased amplitude (Fig. 5). The two-way ANOVA confirmed significant difference between groups ( $F(1,40)=136.08$ ;  $P<0.00001$ ), due to time ( $F(3,40)=59.28$ ;  $P<0.00001$ ) and the interaction of both factors group $\times$ time ( $F(3,40)=27.27$ ;  $P<0.00001$ ). After 8 days without chocolate access PER1 expression remained similar to the AL and to the CH group. The one-way ANOVA did not indicate significant difference between ZT6 and ZT12 ( $F(1,6)=2.6$ ;  $P=NS$ ).

## DISCUSSION

The present study shows that RFS as well as daily chocolate entrainment produce behavioral activation that persists for 7 days after cessation of the feeding protocol. It also provides evidence that RFS and not chocolate induces daily PER1 oscillations in the hypothalamus, indicating that this structure is not essential for the expression of chocolate anticipating behavior. Food and especially daily chocolate entrainment induces pronounced oscillations in structures of the corticolimbic system, resetting the acrophase to 6 h after the stimulus. After interruption of the entraining protocols, the food entrained PER1 oscillations persisted for at least eight cycles in the DMH. Likewise chocolate and food entrained patterns persisted in the Acc (Acc-Core and Acc-Shell), PFC and CeA, which are areas involved in the motivation for feeding and arousal, moreover chocolate induced oscillations attained higher amplitude.

In rats expecting a daily meal, FAA starts about 1–2 h earlier than in rats expecting a daily sweet treat. We have reported this different anticipatory response in a previous study (Mendoza et al., 2005) and we have speculated that the longer and more intense FAA associated with food entrainment might be due to the catabolic state and empty



**Fig. 6.** Total number of cells expressing PER1 protein in the CeA, BLA and PVT. The *ad libitum* controls (white circles), FE group (gray squares) and CH group (gray rhombus) at four temporal points and in persistence for FE-P group (black squares) and CH-P group (black rhombus) \* statistical difference between FE or CH and the *ad libitum* rats ( $P < 0.01$ );  $\alpha$  significant difference between the peak and lower temporal points of the same group; + statistical difference between two temporal points in the persistence group ( $P < 0.01$ ). Other indications as in Fig. 3.

stomach that rats endure several hours previous to meal-time (Escobar et al., 1998; Martínez-Merlos et al., 2004). In contrast, access to a sweet treat produces a short but timely activation at the expected treat time, suggesting a precise 24 h timing system, independent of metabolic factors, at the basis of such activation. A recent study also found differential activation between food and chocolate entrained rats (Verwey et al., 2007). After interrupting the entrainment protocols both entrained behavioral patterns persisted for at least seven cycles demonstrating the endogenous nature of these oscillations. In *ad libitum* conditions the behavioral activation at the expected chocolate time could be observed with a brief, timely and precise bout of activity, while persistence of the food entrained

rhythm lasted longer and was observed in fasting conditions only, as described previously (Mistlberger, 1994; Stephan, 1981, 2001). Both conditions indicate the presence of a timekeeping system that once entrained, leads subjects to search for food at the conditioned time for many cycles. This system may underlie addictive feeding behavior and the observation that PER1 oscillation did not persist in the hypothalamus in chocolate entrained animals may suggest that mainly accumbens, PFC and amygdala are involved in this type of behavior.

*Ad libitum* fed rats exhibited a clear diurnal rhythm of PER1 in the SCN with a peak at ZT12 as previously described (Lamont et al., 2005; Amir et al., 2004). This temporal organization was not modified by RFS or by

chocolate entrainment, which is in agreement with previous reports describing that rats when kept in a LD cycle, food or chocolate entrainment do not modify the phase of c-Fos or clock gene products in the SCN (Damiola et al., 2000; Hara et al., 2001; Waddington Lamont et al., 2007; Wakamatsu et al., 2001; Mendoza et al., 2005). However, the present data indicate that both RFS and daily chocolate access induced an up-regulation of PER1 expression at ZT12. This peak was mainly enhanced in the chocolate group, suggesting that arousal or the motivational state associated with chocolate entrainment provides information to the SCN and also influences the activity of the SCN. A possible input of limbic information to the SCN could be the PVT, which shows anticipatory PER1 expression in food and chocolate entrained rats. It is well described that arousal is a non-photic stimulus and produces inhibition of c-Fos activity in the SCN (Escobar et al., 2007; Mistlberger et al., 2003; Mistlberger and Skene, 2004; Mrosovsky, 1996), the upregulation of PER1 (mainly in the dorsomedial part) argues for a role of the SCN in this anticipatory activity.

RFS specifically induced oscillations in hypothalamic structures involved in energy balance and feeding behavior. RFS set the daily rhythm of PER1 expression in the ARC, DMH and PeF to ZT12, 6 h after mealtime, this effect was exclusive for RFS and was not produced by chocolate delivery. The fact that chocolate did not produce this activation and that after the refeeding–fasting protocol the food entrained pattern did not persist or was dampened in the ARC and PeF, suggests that oscillations observed during RFS were a response to the daily meal and depend on an hourglass or a dampening oscillator. During this entrainment process the ARC and PeF may play a relevant role in transmitting food-related signals to other brain regions. In addition to the ARC, metabolic signals also enter the brain by the autonomic system and brain stem nuclei. This dual pathway explains why food entrainment is not prevented in rats bearing subdiaphragmatic vagotomy, bearing lesions of the vagal complex (Comperatore and Stephan, 1990; Davidson et al., 2000, 2001) or of the ARC (Mistlberger and Antle, 1999).

Interestingly other structures like the VMH that are known to play a role in metabolic functions and feeding behavior exhibited only very limited expression of PER1, which did not allow quantitative evaluation. In contrast with other hypothalamic structures, food entrained oscillations persisted in the DMH for up to eight cycles. Persistence for two cycles of food entrained PER1 oscillations was previously reported by Mieda et al. (2006). The role of the DMH on food entrainment is controversial (Landry et al., 2007; Gooley and Saper, 2007). The DMH is proposed as an integrator, and key regulator for the expression of food-entrained circadian rhythms (Gooley et al., 2006; Saper et al., 2005) because RFS entrain cellular activity in the DMH (Ángeles-Castellanos et al., 2005; Gooley et al., 2006; Mieda et al., 2006). Contrasting with these data, lesions in the DMH do not completely abolish FAA (Gooley et al., 2006; Landry et al., 2006, 2007). Apparently the relevance of the DMH as integrator of food entrained rhythms relies

on its projections to different brain areas, promoting the sleep–wake cycle, arousal, feeding, endocrine, and body temperature rhythms (Thompson et al., 1996; Aston-Jones, 2005; Harris and Aston-Jones, 2006). The fact that the present data show that chocolate hardly entrains PER1 oscillation in the DMH indicates that the effect of RFS on the DMH is probably triggered by signals of the animal's metabolic state.

The present results demonstrate that daily scheduled access to chocolate had a powerful but selective influence on areas of the brain that are known to be involved with the motivational and reward systems for feeding (Berthoud, 2007; Kelley et al., 2005) and did not modify the systems involved in regulating homeostasis. The same structures entrained by daily chocolate access (Acc-Core and Acc-Shell, CeA and PFC) were also entrained by RFS albeit with much lower amplitude. A similar effect by both, chocolate and RFS, on these structures indicates that not only chocolate but also RFS access induces a motivational or reward state. Herein the present data show chocolate, to have special strong effects. A previous study using PER2 as reporter of rhythmicity did not find entrainment of limbic structures by daily Ensure access (Verwey et al., 2007). The main difference with the present study is that animals were allowed to ingest the nutritional complex to satiety and that authors explored different structures with a different reporter. Present data are in agreement with the evidence that Acc-Core is a constituent of a circuit mediating anticipatory actions, better known as “wanting” response (Berridge and Robinson, 2003; Cardinal and Everitt, 2004) and that the Acc-Shell contributes to the hedonic response and motivation for sweet and palatable food (Berthoud, 2004). Also, the Acc-Core and PFC are related with the expectancy of reward by an attractive diet especially with high concentrations of carbohydrates and fat, leading to changes in dopamine release (Bassareo and Di Chiara, 1999; Berridge and Robinson, 2003). In this respect it is remarkable that both Acc-Shell and PFC show an undiminished or increased PER1 expression respectively in RFS or chocolate persistence indicating a special role for these structures. The present study demonstrates that a rewarding treat can start or entrain daily oscillations within fore-brain structures that are known to mediate addictive behavior (Kelley et al., 2005). Further studies need to explore the role of these oscillations on palatable treat expectation in relation with the dopaminergic reward system in order to understand the temporal regulation of addictive processes, especially since such food addictive processes may underlie the development of obesity and uncontrolled eating (Trinko et al., 2007).

## CONCLUSION

In conclusion, the present data demonstrate that scheduled food or chocolate access entrains daily oscillations of the clock gene PER1 in structures involved in homeostasis and reward, which may lead to the identification of motivational feeding systems. These oscillations remained for at least 8 days after the interruption of the feeding sched-

ule. The persistence and amplification of these oscillations in structures associated with reward suggest that this oscillatory process may form part of temporal addictive behavior. The present data support our hypothesis that (food) anticipatory behavior depends on a multi-oscillatory system. Whether the induction of PER1 oscillation really signifies autonomous oscillations in these brain regions or is a reflection of the activation of a larger neuronal circuit will be a matter of future investigation.

*Acknowledgments*—This study was supported by grants DGAPA PAPIIT: IN-203803 and 203907, CONACYT 43950-M and ECOS NORD MÉXICO-FRANCIA M04S02.

## REFERENCES

- Abe H, Rusak B (1992) Anticipatory activity and entrainment of circadian rhythms in Syrian hamsters exposed to restricted palatable diets. *Am J Physiol* 263:R116–R124.
- Amir S, Waddington E, Robinson B, Stewart J (2004) A circadian rhythm in the expression of PERIOD2 protein reveals a novel SCN-controlled oscillator in the oval nucleus of the bed nucleus of the stria terminalis. *Neuroscience* 24:781–790.
- Ángeles-Castellanos M, Aguilar-Roblero R, Escobar C (2004) c-Fos expression in hypothalamic nuclei of food-entrained rats. *Am J Physiol Regul Integr Comp Physiol* 286:R159–R165.
- Ángeles-Castellanos M, Mendoza J, Escobar C (2005) Food entrainment modifies the c-Fos expression pattern in brain stem nuclei of rats. *Am J Physiol Regul Integr Comp Physiol* 288:R678–R684.
- Ángeles-Castellanos M, Mendoza J, Escobar C (2007) Restricted feeding schedules phase shift daily rhythms of c-Fos and protein Per1 immunoreactivity in corticolimbic regions in rats. *Neuroscience* 144:344–355.
- Aston-Jones G (2005) Brain structures and receptors involved in alertness. *Sleep Med Rev Suppl* 1:S3–S7.
- Bassareo V, Di Chiara G (1999) Modulation of feeding-induced activation of mesolimbic dopamine transmission by appetitive stimuli and its relation to motivational state. *Eur J Neurosci* 11(12):4389–4397.
- Berridge KC, Robinson TE (2003) Parsing reward. *Trends Neurosci* 26:507–513.
- Berthoud HR (2004) Mind versus metabolism in the control of food intake and energy balance. *Physiol Behav* 81:781–793.
- Berthoud HR (2007) Interactions between the “cognitive” and “metabolic” brain in the control of food intake. *Physiol Behav* 91(5):486–498.
- Buijs RM, Kalsbeek A (2001) Hypothalamic integration of central and peripheral clocks. *Nat Rev Neurosci* 2(7):521–526.
- Cardinal RN, Everitt BJ (2004) Neural and psychological mechanisms underlying appetitive learning: links to drug addiction. *Curr Opin Neurobiol* 14:156–162.
- Comperatore CA, Stephan FK (1990) Effects of vagotomy on entrainment of activity rhythms to food access. *Physiol Behav* 47:671–678.
- Damiola F, Le Minh N, Preitner N, Kornmann B, Fleury-Olela F, Schibler U (2000) Restricted feeding uncouples circadian oscillators in peripheral tissues from the central pacemaker in the suprachiasmatic nucleus. *Genes Dev* 14:2950–2961.
- Davidson AJ, Cappendijk SL, Stephan FK (2000) Feeding-entrained circadian rhythms are attenuated by lesions of the parabrachial region in rats. *Am J Physiol Regul Integr Comp Physiol* 278:R1296–R1304.
- Davidson AJ, Aragona BJ, Stephan FK (2001) Persistence of meal entrained circadian rhythms following area postrema lesions in the rat. *Physiol Behav* 74:349–354.
- Dunlap JC (1999) Molecular bases for circadian clocks. *Cell* 96:271–290.
- Escobar C, Díaz-Muñoz M, Encinas F, Aguilar-Roblero R (1998) Persistence of metabolic rhythmicity during fasting and its entrainment by restricted feeding schedules in rats. *Am J Physiol Regul Integr Comp Physiol* 274:R1309–R1316.
- Escobar C, Martínez-Merlos MT, Angeles-Castellanos M, Miñana MC, Buijs RM. (2007) Unpredictable feeding schedules unmask a system for daily resetting of behavioural and metabolic food entrainment. *European Journal of Neuroscience*, Vol. 26, pp 2804–2814.
- Gooley JJ, Schomer A, Saper CB (2006) The dorsomedial hypothalamic nucleus is critical for the expression of food-entrainable circadian rhythms. *Nat Neurosci* 9(3):398–407.
- Gooley JJ, Saper (2007) Is food-directed behavior an appropriate measure of circadian entrainment to restricted daytime feeding? *J Biol Rhythms* 22:479–483.
- Guo H, Brewer JM, Champhekar A, Harris RB, Bittman EL (2005) Differential control of peripheral circadian rhythms by suprachiasmatic-dependent neural signals. *Proc Natl Acad Sci U S A* 102:3111–3116.
- Hara R, Wan K, Wakamatsu H, Aida R, Moriya T, Akiyama M, Shibata S (2001) Restricted feeding entrains liver clock without participation of the suprachiasmatic nucleus. *Genes Cells* 6:269–278.
- Harris G, Aston-Jones G (2006) Arousal and reward: a dichotomy in orexin function. *Trends Neurosci* 29(10):571–577.
- Kelley AE, Schiltz CA, Landry FC (2005) Neural systems recruited by drug- and food-related cues: Studies of gene activation in corticolimbic regions. *Physiol Behav* 86:11–14.
- Klein DC, Moore RY, Reppert SM (1991) *Suprachiasmatic nucleus: the mind's clock*. New York: Oxford University Press.
- Lamont EW, Robinson B, Stewart J, Amir S (2005) The central and basolateral nuclei of the amygdala exhibit opposite diurnal rhythms of expression of the clock protein Period2. *Proc Natl Acad Sci U S A* 102:4180–4184.
- Landry GJ, Simon MM, Webb IC, Mistlberger RE (2006) Persistence of a behavioral food-anticipatory circadian rhythm following dorsomedial hypothalamic ablation in rats. *Am J Physiol Regul Integr Comp Physiol* 290:R1527–R1534.
- Landry GJ, Yamakawa GR, Webb IC, Mear RJ, Mistlberger RE (2007) The dorsomedial hypothalamic nucleus is not necessary for the expression of circadian food-anticipatory activity in rats. *J Biol Rhythms* 22:467–478.
- Martínez-Merlos MT, Ángeles-Castellanos M, Díaz-Muñoz M, Aguilar-Roblero R, Mendoza J, Escobar C (2004) Dissociation between adipose tissue signals, behavior and the food-entrained oscillator. *J Endocrinol* 181:53–63.
- Mendoza J, Ángeles-Castellanos M, Escobar C (2005) Entrainment by a palatable meal induces food-anticipatory activity and c-Fos expression in reward-related areas of the brain. *Neuroscience* 133:293–303.
- Meynard MM, Valdés JL, Recabarren M, Serón-Ferré M, Torrealba F (2005) Specific activation of histaminergic neurons during daily feeding anticipatory behavior in rats. *Behav Brain Res* 158:311–319.
- Mieda M, Williams SC, Richardson JA, Tanaka K, Yanagisawa M (2006) The dorsomedial hypothalamic nucleus as a putative food-entrainable circadian pacemaker. *Proc Natl Acad Sci U S A* 103(32):12150–12155.
- Mistlberger RE, Rusak B (1987) Palatable daily meals entrain anticipatory activity rhythms in free-feeding rats: dependence on meal size and nutrient content. *Physiol Behav* 41:219–226.
- Mistlberger RE (1994) Circadian food-anticipatory activity: formal models and physiological mechanisms. *Neurosci Biobehav Rev* 18:171–195.
- Mistlberger RE, Antle MC (1999) Neonatal monosodium glutamate alters circadian organization of feeding, food anticipatory activity and photic masking in the rat. *Brain Res* 842(1):73–83.
- Mistlberger RE, Antle MC, Webb IC, Jones M, Weinberg J, Pollock MS (2003) Circadian clock resetting by arousal in Syrian hamsters: the

- role of stress and activity. *Am J Physiol Regul Integr Comp Physiol* 285:R917–R925.
- Mistlberger RE, Skene DJ (2004) Social influences on mammalian circadian rhythms: animal and human studies. *Biol Rev Cam Philos Soc* 79:533–556.
- Mrosovsky N (1996) Locomotor activity and non-photoc influences on circadian clocks. *Biol Rev* 71:343–372.
- Okamura H (2004) Clock genes in cell clocks: roles, actions, and mysteries. *J Biol Rhythms* 19:388–399.
- Paxinos G, Watson C (1998) *The rat brain in stereotaxic coordinates*. New York: Academic Press.
- Saper CB, Scammell TE, Lu J (2005) Hypothalamic regulation of sleep and circadian rhythms. *Nat Rev* 437:1257–1263.
- Satoh Y, Kawai H, Kudo N, Kawashima Y, Mitsumoto A (2006) Time-restricted feeding entrains daily rhythms of energy metabolism in mice. *Am J Physiol Regul Integr Comp Physiol* 290(5): R1276–R1283.
- Stephan FK (1981) Limits of entrainment to periodic feeding in rats with suprachiasmatic lesions. *J Comp Physiol* 143:401–410.
- Stephan FK (2001) Food-entrainable oscillators in mammals. In: *Circadian clocks* (Takahashi JS, Turek FW, Moore RY, eds), pp 223–246. New York: Kluwer Academic/Plenum Publishers.
- Stokkan KA, Yamazaki S, Tei H, Sakaki Y, Menaker M (2001) Entrainment of the circadian clock in the liver by feeding. *Science* 291:490–493.
- Thompson RH, Canteras NS, Swanson LW (1996) Organization of projections from the dorsomedial nucleus of the hypothalamus: a PHA-L study in the rat. *J Comp Neurol* 376:143–173.
- Trinko R, Sears RM, Guarnieri DJ, DiLeone RJ (2007) Neural mechanisms underlying obesity and drug addiction. *Physiol Behav* 91(5):499–505.
- Verwey M, Khoja Z, Stewart J, Amir S (2007) Differential regulation of the expression of Period2 protein in the limbic forebrain and dorsomedial hypothalamus by daily limited access to highly palatable food in food-deprived and free-fed rats. *Neuroscience* 147(2):277–285.
- Waddington Lamont E, Harbour VL, Barry-Shaw J, Renteria Diaz L, Robinson B, Stewart J, Amir S (2007) Restricted access to food, but not sucrose, saccharine, or salt, synchronizes the expression of Period2 protein in the limbic forebrain. *Neuroscience* 144(2):402–411.
- Wakamatsu H, Yoshinobu Y, Aida R, Moriya T, Akiyama M, Shibata S (2001) Restricted-feeding-induced anticipatory activity rhythm is associated with a phase-shift of the expression of mPer1 mRNA in the cerebral cortex and hippocampus but not in the suprachiasmatic nucleus of mice. *Eur J Neurosci* 13:1190–1196.

*(Accepted 2 June 2008)*  
*(Available online 7 June 2008)*