

Nature's food anticipatory experiment: entrainment of locomotor behavior, suprachiasmatic and dorsomedial hypothalamic nuclei by suckling in rabbit pups

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Abstract

In nature and under laboratory conditions, dams nurse rabbit pups once daily for a duration of fewer than 5 min. The present study explored neural mechanisms mediating the timing of nursing in this natural model of food anticipatory activity, focussing on the suprachiasmatic nucleus (SCN), the locus of the master circadian clock and on the dorsomedial hypothalamic nucleus (DMH), a region implicated in timing of food-entrained behavior. Rabbit pups are born in the dark, with eyelids closed. Nursing visits to the litters also occurs during the dark phase. To explore the effect of the timing of feeding, pups were maintained in constant darkness, while females housed in a light–dark cycle were permitted to nurse their pups either during the night (night-fed group) or day (day-fed group). All pups exhibited anticipatory locomotor activity before daily nursing. In the SCN, PER1 and FOS peaked during the night in both groups, with a longer duration of elevated protein expression in the night-fed group. In contrast, DMH peak PER1 expression occurred 8 h after pups were fed, corresponding to the shift in timing of nursing. Comparison of nursed and 48 h fasted pups indicates that the timing of PER1 expression was similar in the SCN and DMH, with fewer PER1-positive cells in the latter group. The results indicate that rabbit pups show food anticipatory activity, and that timing of nursing differentially affects PER1 expression in the SCN and DMH.

Introduction

Rabbit pups provide an ideal model system for examining food anticipatory activity (FAA) as they suckle up to 35% of their body weight once every 24 h for a mere 3–5 min (Hudson & Distel, 1989; Jilge, 1993; Caba *et al.*, 2003b). Pups are born in a dark burrow and remain in darkness for about 2 weeks (Broekhuizen & Mulder, 1983) but the dam leaves the nest and is exposed to light–dark (LD; Zarrow *et al.*, 1965) cycles. While photic cues are the most salient environmental zeitgeber that entrain the suprachiasmatic nucleus (SCN) to the light–dark cycle (Moore & Card, 1985), the response of the pups is not synchronized by retinal input and photic stimuli, as they remain in the burrow and their eyelids do not open until postnatal day 10 (Rapisardi *et al.*, 1975). However, it is clear that they are exposed to temporal cues.

Many studies indicate that the daily nursing visit of the mother is a powerful zeitgeber for the pups during the first week after birth, serving to synchronize circadian rhythms of locomotor activity (Jilge, 1993, 1995), temperature (Jilge *et al.*, 2000) and plasma corticosterone (CORT; Rovirosa *et al.*, 2005). Around 2 h before the arrival of the mother, pups become increasingly active, and emerge from the nest material (Hudson & Distel, 1989). Following nursing, they become less active and cover themselves with nest material for about 21 h, the cycle repeating thereafter (Jilge, 1993, 1995). Laboratory controlled experiments suggest that this behavior is under circadian control

(Jilge, 1993). Furthermore, 7-day-old rabbit pups also have an anticipatory rise in core body temperature (Jilge *et al.*, 2000) and CORT (Rovirosa *et al.*, 2005), which persists during a fasting period.

The above studies indicate that rabbit pups express anticipatory responses before daily nursing, similar to FAA seen in rodents under an experimentally restricted feeding schedule (Stephan, 1983; Gooley *et al.*, 2006). Rabbit pups thus provide a naturally occurring model of food restriction. Under laboratory conditions, regardless of time of birth, nursing occurs during the dark phase (Jilge, 1993). However, time of nursing can be controlled without disrupting the mother–pup dyad (Jilge, 1993, 1995; Escobar *et al.*, 2000; Caba *et al.*, 2003b).

In the present study we explored circadian changes in the SCN – locus of a brain circadian clock, and the dorsomedial hypothalamic nucleus (DMH) – an area implicated in the control of FAA (Gooley *et al.*, 2006; Mieda *et al.*, 2006) (but see Landry *et al.*, 2006; Gooley & Saper, 2007; Landry & Mistlberger, 2007; Landry *et al.*, 2007). To this end we examined PER1 expression in the brains of 7- to 8-day-old rabbit pups, nursed at two different time points, 10:00 and 02:00 h. Using FOS protein as a marker of stimulus-induced cellular activation and of circadian oscillation in the SCN, we also explored whether the rabbit pup's SCN has a circadian rhythm of FOS protein and if this too is affected by the two schedules of nursing.

Materials and methods

Animals and housing

New Zealand white female rabbits bred in the colony in Xalapa, México, were housed under a controlled light cycle (12/2 h LD; lights

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on at 07:00, lights off at 19:00 h), and provided with rabbit pellets (Purina) and water *ad libitum*. Females were mated and housed individually in stainless steel cages in the colony at room temperature of 23 ± 1 °C and were monitored daily from day 28 of pregnancy until delivery. The nest compartment in which pups were born was kept in constant darkness (DD) and pups were undisturbed for the entire experiment.

Each cage had three compartments, one for the mother and one for the nest (0.60 cm wide \times 0.50 cm long \times 0.40 cm high), with a tunnel (0.25 cm wide \times 0.50 long \times 0.40 cm) between them. A sensor between the tunnel and the nest box detects the mother's entrance to the nest. General locomotor activity of pups was monitored with a thermal radiation detector sensitive to infrared radiation (λ , 5–14 μ m) located on the ceiling of the nest box. The tunnel has a sliding door on each end, permitting the experimenter to control the mother's access to the tunnel and nest. When the dam crosses one of the doors the other closes automatically. In this way light cannot reach the nest compartment and pups are in continuous dark. Before parturition the mother has free access to the nest compartment where she builds a nest with straw that we provide. Pups were born in the nest and, on the day of parturition, the litter was adjusted to 4–5 and the sliding door was locked.

All experimental procedures were approved and conducted according to the Statement of Assurance with Standards for Humane Care and Use of Laboratory animals approved by the National Institutes of Health (NIH) to the Universidad Veracruzana.

Experimental design

The mother's access to the nest was regulated starting on postnatal day 0 (PD0), the day of births. The door giving access to the tunnel and nest was opened at either 02:00 or 10:00 h. The 10:00 h time point is the nursing timing often selected in laboratory conditions (Jilge, 1993, 1995; Escobar *et al.*, 2000; Caba *et al.*, 2003b). In unrestrained conditions, rabbits nurse at a range of times. Thus, Jilge (1993) studied ten litters and reported that they nurse at 07:00–09:00 h ($n = 2$), 01:00–02:00 h ($n = 2$), 03:00–04:00 h ($n = 1$), 04:00–05:00 h ($n = 4$) and 05:00–06:00 h ($n = 1$). The mother entered the nest immediately and the mean daily duration of nursing was 224 ± 3 (mean \pm SEM) and 235 ± 8 s for pups nursed at 02:00 and 10:00 h, respectively, similar to previous reports from our laboratory (Caba *et al.*, 2003b).

Experimental groups were established based on time of nursing. Pups (in DD) were nursed at 02:00 h (restricted feeding–nursed at 02:00 h; RF-N 02:00) or 10:00 h (restricted feeding–nursed at 10:00 h; RF-N 10:00). Pups ($n = 4$ at each time point) were killed at PD7 starting just before their scheduled time of nursing (02:00 or 10:00 h) and then at 4-h intervals. Another two groups were treated as above, and fasted (restricted feeding–fasted; RF-F) on PD8 and killed at the beginning of their respective scheduled nursing time and then every 4 h, similar to nursed pups ($n = 48$ total number of fasted subjects). Pups were weighed at the end of the experiment, prior to being killed.

Immunohistochemistry

Rabbits were anesthetized with an overdose of sodium pentobarbital (20 mg per pup, i.p.) and were perfused transcardially with saline solution (0.9%), followed by 4% paraformaldehyde in phosphate buffer (PB, pH 7.4). The brains were removed immediately after perfusion, cryoprotected successively in 10, 20 and 30% sucrose in

PB and were then sectioned coronally at 50 μ m with a cryostat (Microm). Serial sections were collected in PB from the level of the opening of the organum vasculosum of lamina terminalis to the mammillary bodies. Every two of four sections were used for labeling of FOS or PER1 as described below, following protocols previously established for FOS protein (Caba *et al.*, 2003b; Toledo *et al.*, 2005) in rabbit pup brain. Tissue was washed in PB four times, 5 min each, to remove excess aldehydes and then exposed for 10 min in 0.5% hydrogen peroxide solution to eliminate endogenous peroxidase activity. Non-specific tissue antibody reactions were blocked by placing the sections in 3% normal horse serum (Vector Laboratories) for 1 h at room temperature. Sections were then incubated for 48 h at 4 °C in the polyclonal PER1 (sc-7724) or FOS antibody (sc-52) diluted at 1 : 5000 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in 3% normal horse serum with 0.3% Triton X-100 (Sigma). Tissue was placed in biotinylated horse anti-goat serum (1 : 200, Vector Laboratories) for 1 h and after three washes in PB it was incubated in avidin–biotin–HRP complex (1 : 250, Vector Laboratories) for 1 h. FOS and PER1 antibody–peroxidase complex was stained with a solution of 0.05% diaminobenzidine (Polysciences) in the presence of nickel sulfate (10 mg/mL, Fisher Scientific), cobalt chloride (10 mg/mL, Fisher scientific) and 0.01% hydrogen peroxide, which produced a black–purple precipitate. After 10 min, tissue was transferred to PB to stop the reaction. Tissue was mounted onto gelatin-subbed slides, dehydrated and cleared in Hemo-De (Fisher Scientific) and then coverslipped with Permount. In all cases, tissue sections from subjects of each of the different time points were processed together. We used two immunohistochemistry controls. First, control sections were processed as above but with the primary antibody omitted. Second, we performed an adsorption control. The antibodies for PER1 and FOS were preadsorbed in dilutions of 1 : 1 (0.04 μ g/mL), 1 : 10 (0.4 μ g/mL) and 1 : 20 (0.8 μ g/mL) of the blocking peptides sc-7724P (Santa Cruz Biotechnology) for PER1 and sc-52P (Santa Cruz Biotechnology) for FOS. In all cases the PER1 and FOS immunostaining disappeared in preadsorbed sections, but not in the adjacent sections that were run at the same time in which the primary antibody was not preadsorbed. To select the appropriate concentration of PER1 and FOS antibodies we ran a dilution series of 1 : 1000, 1 : 5000 and 1 : 20 000 and concluded that a dilution of 1 : 5000 for 48 h was optimal as it gives very strong staining of nuclei with little background staining.

Quantitation of immunostaining

FOS and PER1 immunoreactivity (IR) was identified as a black–purple precipitate from the diaminobenzidine–nickel/cobalt reaction in the cell nucleus. To determine an immunoreactive nucleus we determined the background optical density in a nearby region lacking immunoreactivity with the Image-Pro Plus program, v. 5 (Media Cybernetics, Silver Spring, MD, USA). Immunoreactive cells that reached five times the optical density of background level were considered positive. Cells below this staining level were considered negative. The number of PER1- and FOS-positive nuclei were counted bilaterally by two observers blind to the experimental conditions with an Olympus BX41 microscope. The localization of brain structures and nuclei was determined using the terminology of the rabbit brain of Gerhard (1968) and Girgis & Shih-Chang (1981) and our previous experience in the forebrain of this species (Caba *et al.*, 2003a). The SCN was examined from its rostral to caudal extent, and the sections at the middle level were quantitated. For the SCN, we examined the Nissl stain in the mid-SCN to delineate its extent. We then used an ocular grid for

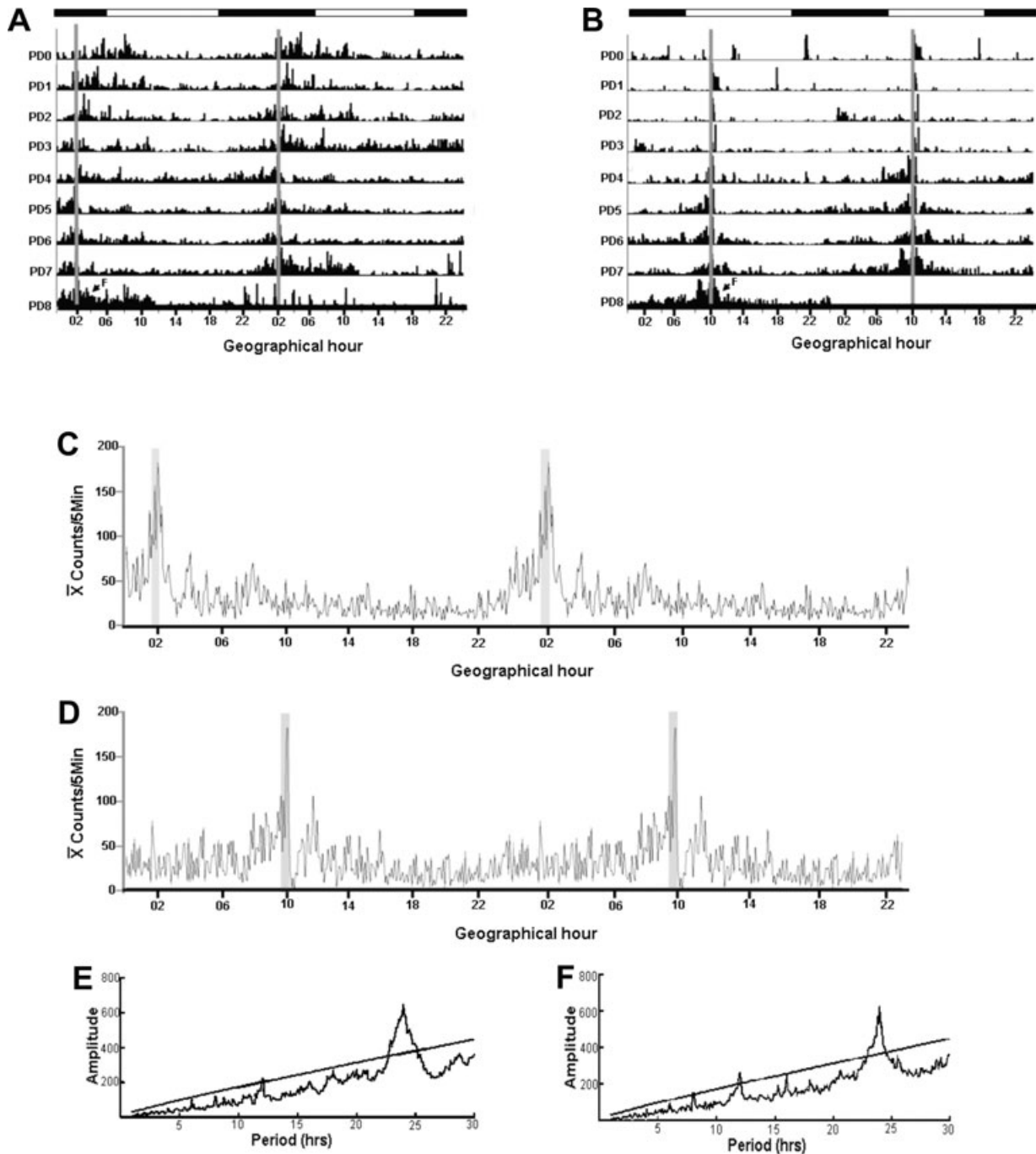


FIG. 1. Description and analysis of activity of two representative litters nursed at 02:00 (A, C, E) and at 10:00 h (B, D, F). (A, B) Double plotted actograms. Black and white bar at top represents LD cycle for the mother, but pups were in constant darkness since birth. Each line represents a postnatal day (PD) of recording. The intensity of activity is represented by the black vertical lines. On PD8 the doe was not permitted to nurse (F, fasting). (C, D) Double plotted waveforms are mean counts per 5 min over 24 h from PD5 to PD7 when anticipatory activity is well established. Shaded area represents time of nursing. (E, F) Periodograms. Locomotor activity from actograms; in both cases there is a circadian component of 24 h (χ^2 , $P < 0.01$).

quantitation, using an area of $208 \times 416 \mu\text{m}$ (width \times height), divided into 32 squares (4×8 squares). The grid was placed above the optic chiasm and lateral to the third ventricle. Immunostaining outside the grid limits was not considered. The region analyzed corresponds to the antero-posterior coordinate NA1 of the stereotaxic rabbit atlas of Girgis & Shih-Chang (1981). For analysis of the DMH, we analysed both the pars compacta (DMHc) and the pars diffuse (DMHd). In order to delineate these regions consistently among groups, we constructed a

template based on the region of pars compacta in animals with high PER1 expression, and marked the location of the fornix and the arcuate nucleus. This template was superimposed on captured images of the DMH for the analysis of pars compacta and diffuse. Pars compacta was readily delineated as it occurred at the level of the fornix, and staining with PER1 clearly delineated this region. In the pars compacta, we did not see differences among groups (data not shown). The analysis of the DMH was made at level NP 2.5 of the above-mentioned atlas. Images

of the sections were captured with a Cool snap Pro digital camera and processed with a computerized image analysis system (Image-Pro Plus v. 5; Media Cybernetics).

Statistical analysis

Locomotor data were collected and stored in 15-s bins and double-plotted actograms and periodograms were generated with the SPAD9 (Omnialva, México) circadian recording system. Locomotor activity was analysed by an Enright periodogram (SPAD9) and 2-h bins of activity prior to daily nursing were compared with the remaining 22-h bins after nursing by using a Student's *t*-test for every day starting at PD1. For FOS-IR and PER1-IR, one-way analysis of variance (ANOVA) was used to determine whether there were differences in cell number across different time points. This was followed by a *post-hoc* analysis using the Tukey–Kramer test (GB-STAT v. 6.0). Comparisons between nursed and fasted pups and body weight of pups were performed using a Student's *t*-test. Probability levels of $P < 0.05$ were considered to be significant. Values given are mean \pm SEM. PER1- and FOS-IR expression in the SCN and DMH were evaluated by a cosinor analysis (MATLAB, v. 6.5) to determine

protein rhythmicity with the hypothesis that rhythm amplitude is different from 0 ($P < 0.05$).

Results

Locomotor activity

Pups developed FAA by PD5 as shown in representative litters (Fig. 1A and B). Litters were entrained by the time of nursing, as indicated based on activity (Fig. 1C and D) and periodogram analysis of locomotor activity (χ^2 , $P < 0.001$; Fig. 1E and F).

FAA develops gradually and it is established by PD3–PD4 (Fig. 2A and B). At this time activity prior to nursing was significantly elevated compared with the remaining 22-h bins. Locomotor activity was higher in the RF-N 10:00 group than in the RF-N 02:00 group both 2 h prior to nursing on PD6–PD7 (Student's *t*-test, $P < 0.001$; Fig. 2C) and after nursing from PD4 to PD7 (PD4, $P < 0.01$; PD5–PD7, $P < 0.001$; Fig. 2D). It is noteworthy that the FAA of the day-fed animals before nursing was significantly higher than all other groups.

Mean body weight of groups at the time the animals were killed was: RF-N 10:00 = 108.3 \pm 12.9 g; RF-N 02:00 = 101.2 \pm 11.2 g;

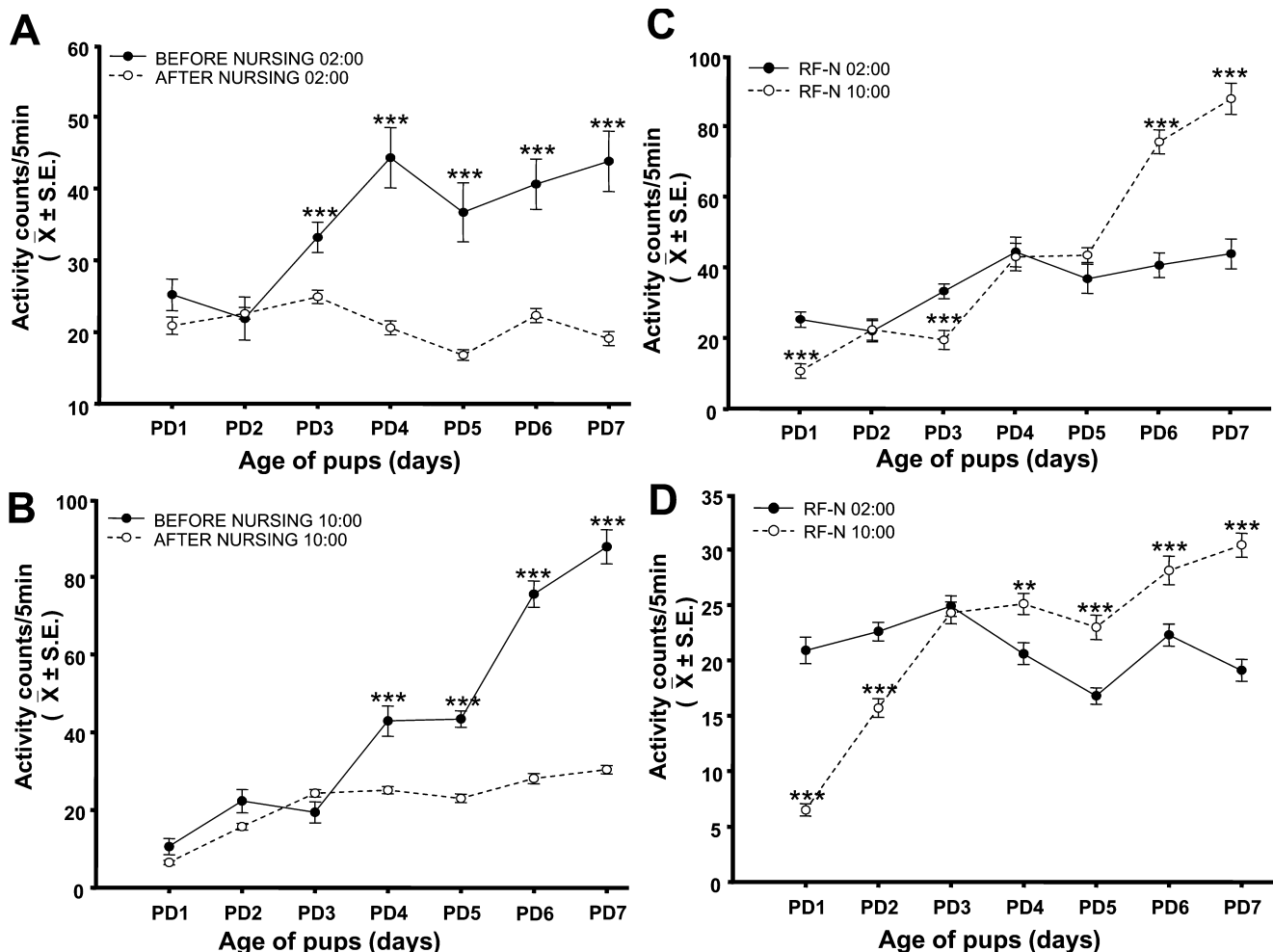


FIG. 2. Locomotor activity of pups from PD1 to PD7 in animals nursed at 02:00 h (RF-N 02:00) and at 10:00 h (RF-N 10:00). Locomotor activity of pups 2 h prior to and 22 h after nursing in night- (A) and day-fed (B) pups. Comparison of locomotor activity between RF-N 02:00 vs. RF-N 10:00 before (C) and after (D) nursing. Values are mean \pm SEM and were plotted from six litters nursed at 02:00 and six nursed at 10:00 h. ** $P < 0.01$ and *** $P < 0.001$ against their corresponding time point group. Data of panels C and D are replotted from A and B.

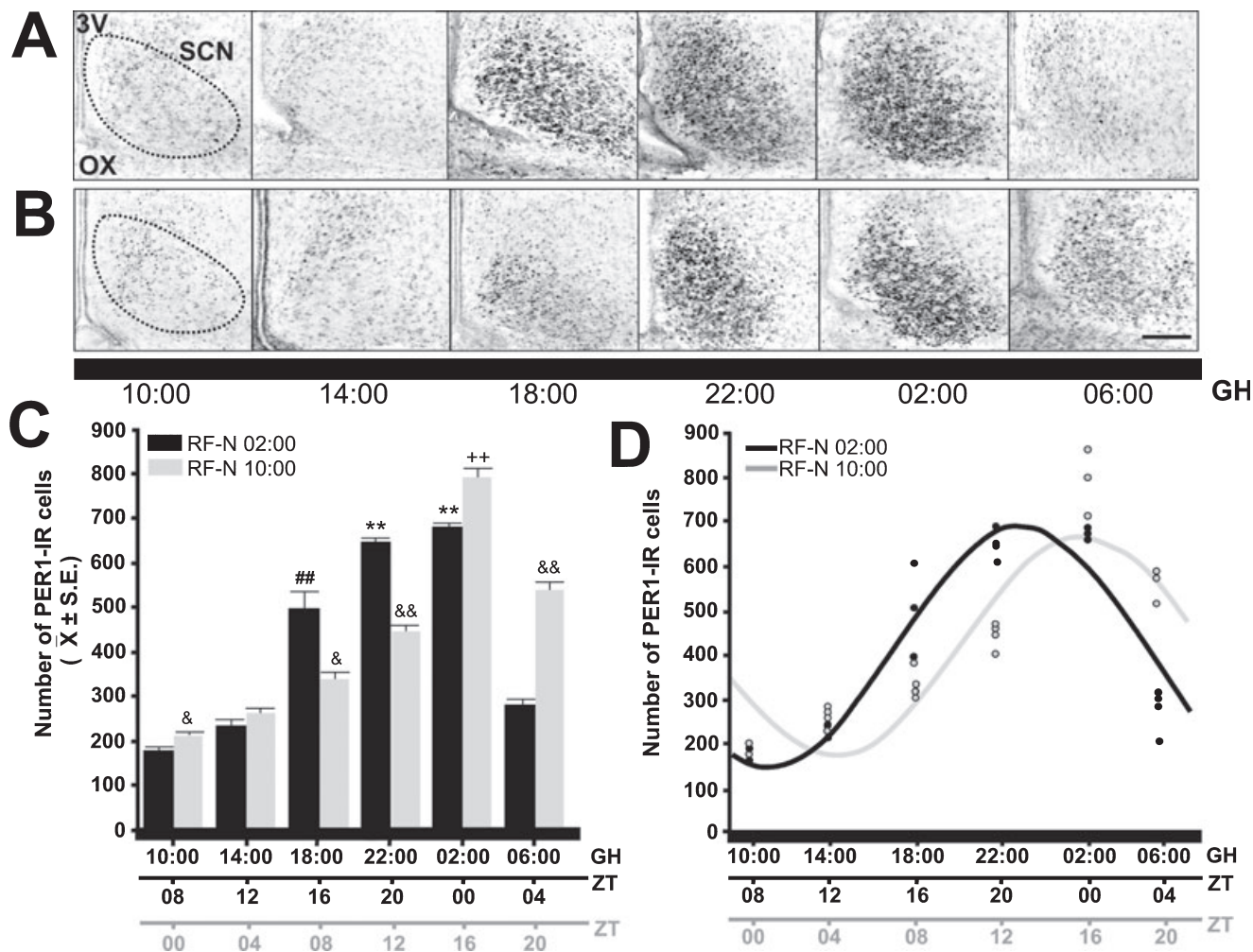


FIG. 3. Rhythmic expression and phase difference of PER1 in the suprachiasmatic nucleus (SCN) in subjects nursed at 02:00 h (RF-N 02:00) and 10:00 h (RF-N 10:00) from PD1 to PD7. (A, B) Photomicrographs of representative sections illustrating the expression of PER1 protein at the level of the middle portion of the SCN at six different time points throughout a complete 24-h cycle at PD7 in RF-N 02:00 pups (A) and RF-N 10:00 pups (B). Dotted line delimits SCN. 3V, third ventricle; OX, optic chiasm. Scale bar, 100 μ m. (C) PER1-IR cells in RF-N 02:00 (black bars) and RF-N 10:00 (grey bars) groups. For the RF-N 02:00 group values at 18:00 (##), 22:00 (**) and 02:00 h (**) were significantly higher than the lowest values. For the RF-N 10:00 group, the value at 02:00 h (++) was significantly different from other values (see text for details). & denotes significant differences between corresponding time point groups. Values are mean \pm SEM. &, $P < 0.05$, &&, ##, **, $P < 0.01$. Black bar at bottom represents DD condition of pups and geographical hours (GH). Black and grey lines represent zeitgeber time (ZT; ZT0 = time of nursing). (D) Cosinor analysis. There is a phase difference of PER1-IR of 2.54 h in subjects nursed at 02:00 against those at 10:00 h (see text for details).

RF-F 10:00 = 71.3 ± 7.1 g; RF-F 02:00 = 74.7 ± 9.6 g ($P > 0.05$ RF-N 02:00 vs. RF-N 10:00).

PER1-IR in the SCN

Figure 3A and B show PER1 protein at six different time points throughout a complete 24-h cycle at PD7 in pups nursed at 02:00 and 10:00, respectively. Quantitative analysis indicates that the SCN showed a robust circadian rhythm in expression of PER1 in nursed pups in both night- and day-fed groups (Fig. 3C). The lowest values were between 10:00 and 14:00 h in both groups. Pups nursed at RF-N 02:00 had more PER1-positive cells at earlier time points, namely 18:00 ($P < 0.05$) and 22:00 h ($P < 0.001$) than those nursed at RF-N 10:00. ANOVA revealed a significant difference across time in PER1-IR in pups nursed at RF-N 02:00 ($F_{5,18} = 104.4$, $P < 0.0001$) and RF-N 10:00 ($F_{5,18} = 158.3$, $P < 0.0001$). Quantitative analysis of PER1 in the SCN of the RF-N 02:00 group showed that maximal values at 22:00 and 02:00 h were significantly different from values at 06:00, 10:00,

14:00 and 18:00 h ($P < 0.01$ in all cases), and the value at 18:00 h was significantly higher than values at 06:00, 10:00 and 14:00 h. In the RF-N 10:00 group, maximal PER1 value at 02:00 h was significantly different from values at 06:00, 10:00, 14:00, 18:00 and 22:00 h ($P < 0.01$ in all cases). Cosinor analysis revealed a significant phase difference of 2.54 h in PER1-IR in SCN – RF-N 02:00 group: acrophase 22:54 h, rhythmicity $P < 0.001$, compared with RF-N 10:00 group: acrophase 01:48 h, rhythmicity $P < 0.001$ (Fig. 3D).

FOS-IR in the SCN

Because the results suggested a phase difference between groups in PER1, we explored this effect with a second index of SCN phase using FOS expression. Figure 4A and B show expression of FOS protein in the SCN at six different time points throughout a complete 24-h cycle at PD7 in the RF-N 02:00 and RF-N 10:00 groups, respectively. As seen in Fig. 4C, quantitative analysis indicates a rhythmic expression of FOS-IR in the SCN of the RF-N 02:00 ($F_{5,18} = 47.2$, $P < 0.0001$) or

10:00 h groups ($F_{5,18} = 83.6$, $P < 0.0001$). Both groups have their lowest values at between 22:00 and 10:00 h, but the elevated number of FOS-IR-expressing cells lasted longer and started earlier (at 14:00, $P < 0.01$) in the RF-N 02:00 group than in the RF-N 10:00 group. In RF-N 02:00 pups maximal FOS-IR values at 14:00 and 18:00 h were significantly different from those at 22:00, 02:00, 06:00 and 10:00 h ($P < 0.01$ in all cases). In the RF-N 10:00 group maximal FOS-IR value at 18:00 was significantly different from values at 22:00, 02:00, 06:00, 10:00 and 14:00 h ($P < 0.01$). Cosinor analysis revealed a significant phase difference of 55 min in FOS-IR in SCN – RF-N 02:00 group: acrophase 16:10 h, rhythmicity $P < 0.001$, compared with RF-N 10:00 group, acrophase 17:05 h, rhythmicity $P < 0.001$ (Fig. 4D).

PER1-IR in the SCN in fasted pups

Figure 5A and B show the expression of PER1 protein at six different time points throughout a complete 24-h cycle at PD7 in RF-F groups previously nursed at RF-N 02:00 and at RF-N 10:00, respectively. As seen in Fig. 5C, quantitative analysis of the number of immunostained cells indicates that PER1 protein showed a circadian expression in the

SCN of RF-F subjects. ANOVA revealed a significant difference across time in PER1-IR expression between the RF-F 02:00 ($F_{5,18} = 121.2$, $P < 0.0001$; Fig. 5C) and RF-F 10:00 groups ($F_{5,18} = 165.4$, $P < 0.0001$; Fig. 5C). For the RF-F 02:00 group maximal values of PER1-IR cells in the SCN at 22:00 and 02:00 h were significantly different from those at 10:00, 14:00, 18:00 and 06:00 ($P < 0.01$ in all cases; Fig. 5C). For the RF-F 10:00 group, maximal number of PER1-IR cells at 02:00 h was significantly different from those at 06:00, 10:00, 14:00, 18:00 and 22:00 ($P < 0.01$ in all cases; Fig. 5C). Note that the RF-F 02:00 group had a greater number of cells at 18:00 ($P < 0.01$) and 22:00 h ($P < 0.05$) than the RF-F 10:00 group. Cosinor analysis revealed a phase difference of 01.19 h in PER1-IR in SCN – RF-F 02:00 group: acrophase 24:00 h, rhythmicity $P < 0.001$, compared with RF-F 10:00 group, acrophase 1:19 h, rhythmicity $P < 0.001$ (Fig. 5D).

Comparison of PER1-IR in nursed and fasted pups

The temporal pattern of maximal and minimal PER1-IR was similar between nursed and fasted subjects, yet RF-F showed lower PER1 protein expression in the SCN compared with RF-N group (Fig. 6).

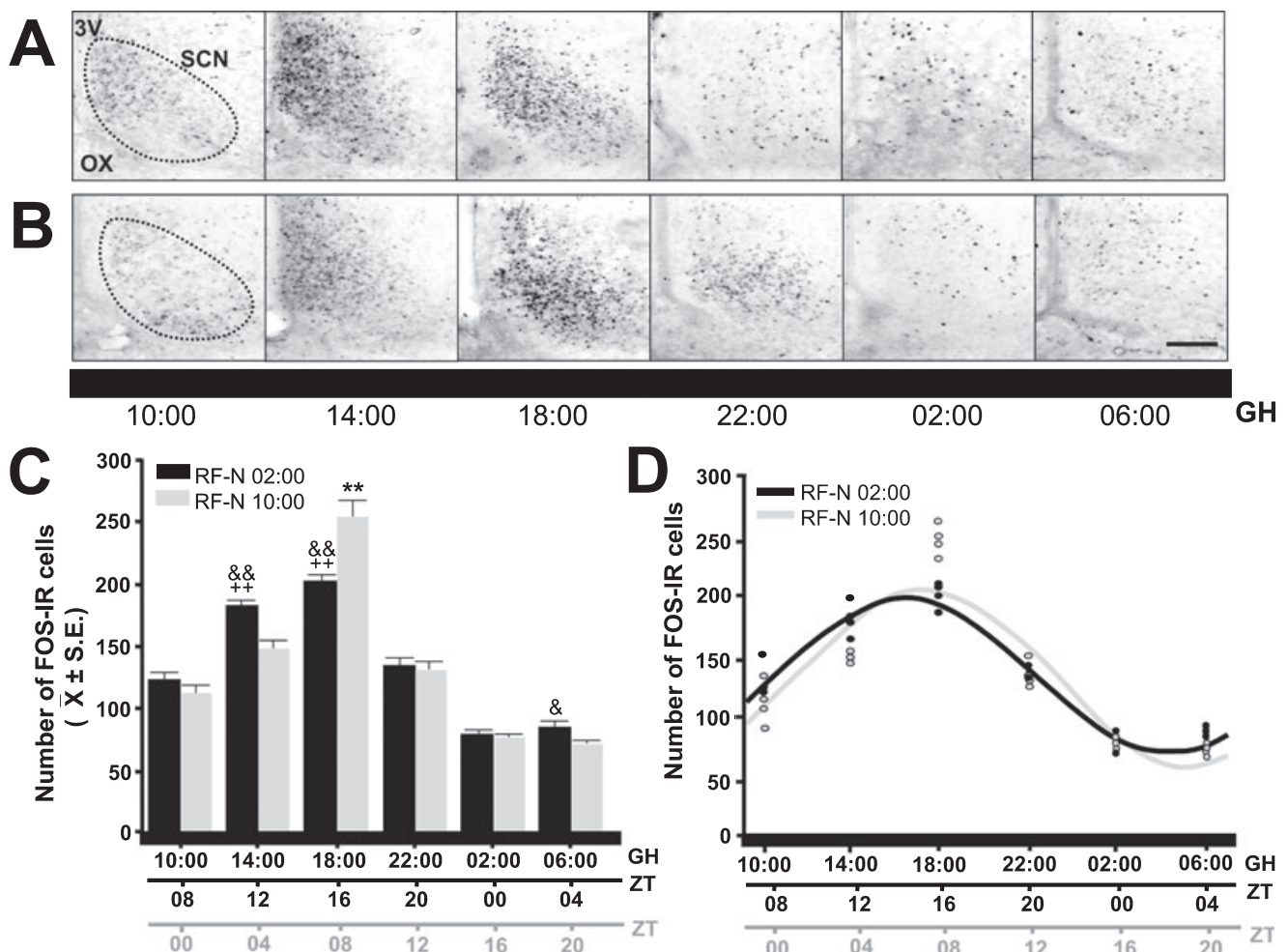


FIG. 4. Rhythmic expression and phase difference of FOS in the SCN in subjects nursed at 02:00 (RF-N 02:00) and 10:00 h (RF-N 10:00) from PD1–PD7. (A, B) Photomicrographs of representative sections illustrating the expression of FOS in the SCN at six different time points throughout a complete 24-h cycle at PD7 in pups nursed at 02:00 (A) and 10:00 h (B). (C) Pups nursed (RF-N) at 02:00 (black bars) and at 10:00 h (grey bars). For pups nursed at 02:00 values at 14:00 and 18:00 h (++) were significantly higher than lowest values. For pups nursed at 10:00, the value at 18:00 h (**) was significantly different from other values (see text for details). & denotes significant differences between corresponding time point groups. Values are mean \pm SEM. &, $P < 0.05$, &&, ++, **, $P < 0.01$. (D) Cosinor analysis. There is a phase difference of FOS-IR of 0.55 h in subjects nursed at 02:00 against those at 10:00 h (see text for details). Abbreviations and legends as in Fig. 3.

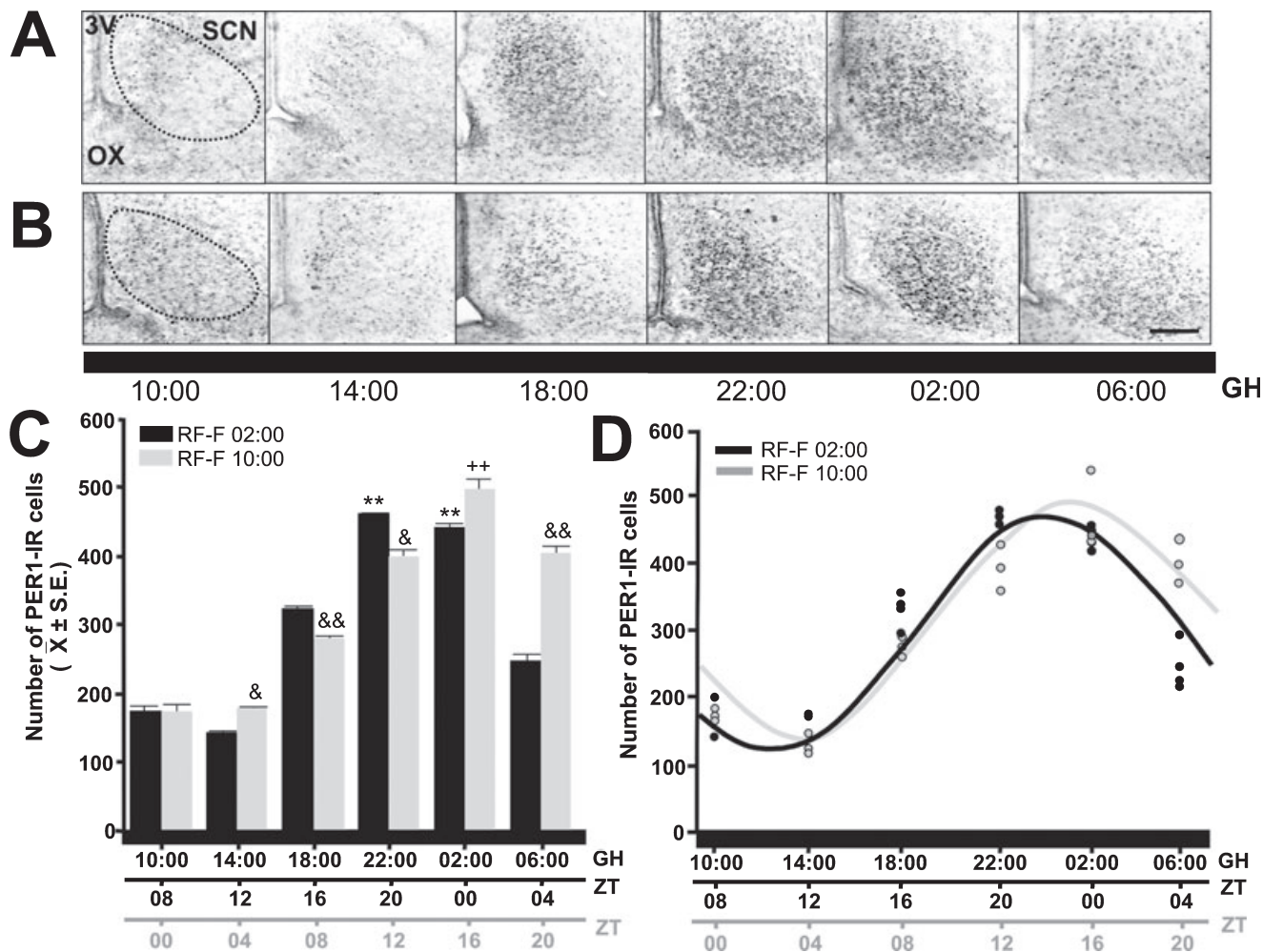


Fig. 5. Rhythmic expression and phase difference of PER1 in the SCN of fasted pups previously nursed at 02:00 (RF-F 02:00) and 10:00 h (RF-F 10:00) from PD1 to PD7. (A, B) Photomicrographs are representative sections illustrating the expression of PER1 protein in the SCN at six different time points throughout a complete 24-h cycle at PD7. (C) For fasted pups previously nursed at 02:00 h values at 22:00 (***) and 02:00 h (***) were significantly higher than lowest values. For fasted pups previously nursed at 10:00 h, the value at 02:00 h (++) was significantly different from other values (see text for details). & denotes significant differences between corresponding time point groups. Values are mean \pm SEM. &, $P < 0.05$, &&, ++, **, $P < 0.01$. (D) Cosinor analysis. There is a phase difference of PER1-IR of 1.19 h in subjects nursed at 02:00 against those at 10:00 h (see text for details). Abbreviations and legends as in Fig. 3.

This difference held true for groups nursed during both subjective night (Fig. 6A) and day (Fig. 6B).

PER-1 in the dorsomedial hypothalamic nucleus

Figure 7A and B reveal a robust rhythm in PER1-IR cells in the DMH synchronized to nursing time. There are few PER-IR cells before and at the time of suckling (Fig. 7C), and their numbers increase thereafter. RF-N 02:00 pups showed a significant difference in PER1-IR along the 24-h cycle ($F_{5,18} = 18.7$, $P < 0.0001$). Maximal levels were observed at 10:00 and were significantly higher than those at 18:00 ($P < 0.01$), 22:00 ($P < 0.01$) and 02:00 h ($P < 0.01$). RF-N 10:00 pups showed a significant difference along the 24-h cycle ($F_{5,18} = 11.82$, $P > 0.0001$), with maximal significant levels of PER1-IR cells at 18:00 and lowest levels at 02:00 ($P < 0.01$), 06:00 ($P < 0.01$) and 10:00 ($P < 0.01$; Fig. 7C). Cosinor analysis revealed a phase difference of 7.3 h in PER1-IR in DMH – RF-N 02:00 group: acrophase 10:00 h, rhythmicity $P < 0.001$, compared with RF-N 10:00 group, acrophase 17 : 30 h, rhythmicity $P < 0.001$ (Fig. 7D).

In the RF-F 02:00 group there was a significant rhythm of PER1-IR ($F_{5,18} = 22.12$, $P < 0.0001$); maximal PER1 expression was at 10:00 (similar to the nursed pups), which was significantly different from subjects at 14:00 ($P < 0.01$), 18:00 ($P < 0.01$), 22:00 ($P < 0.01$) and 02:00 h ($P < 0.01$). In the RF-F 10:00 group there was a significant peak of PER1 (ANOVA $F_{5,18} = 28.6$, $P < 0.0001$) at 18:00 h (as in nursed pups) and this was significantly different from all remaining time points ($P < 0.01$ in all cases; Fig. 8C). Cosinor analysis revealed a phase difference of 08.36 h in PER1-IR in DMH of fasted pups – RF-F 02:00 group: acrophase 07:04 h, rhythmicity $P < 0.001$, RF-F 10:00 group, acrophase 15:40 h, rhythmicity $P < 0.001$ (Fig. 8D).

In both RF-F groups there were fewer PER1-IR cells at most time points compared with their nursed counterparts, although the former still showed rhythmic changes (Fig. 9). In RF-F 02:00 pups there was a significant decrease in PER1 at 10:00 ($P < 0.05$) and 14:00 h ($P < 0.01$) compared with their corresponding nursed groups (Fig. 9A). In RF-F 10:00 pups there was a significant decrease in PER1-IR at 14:00 ($P < 0.05$), 18:00 ($P < 0.01$) and 22:00 h ($P < 0.01$) compared with their corresponding nursed groups (Fig. 9B).

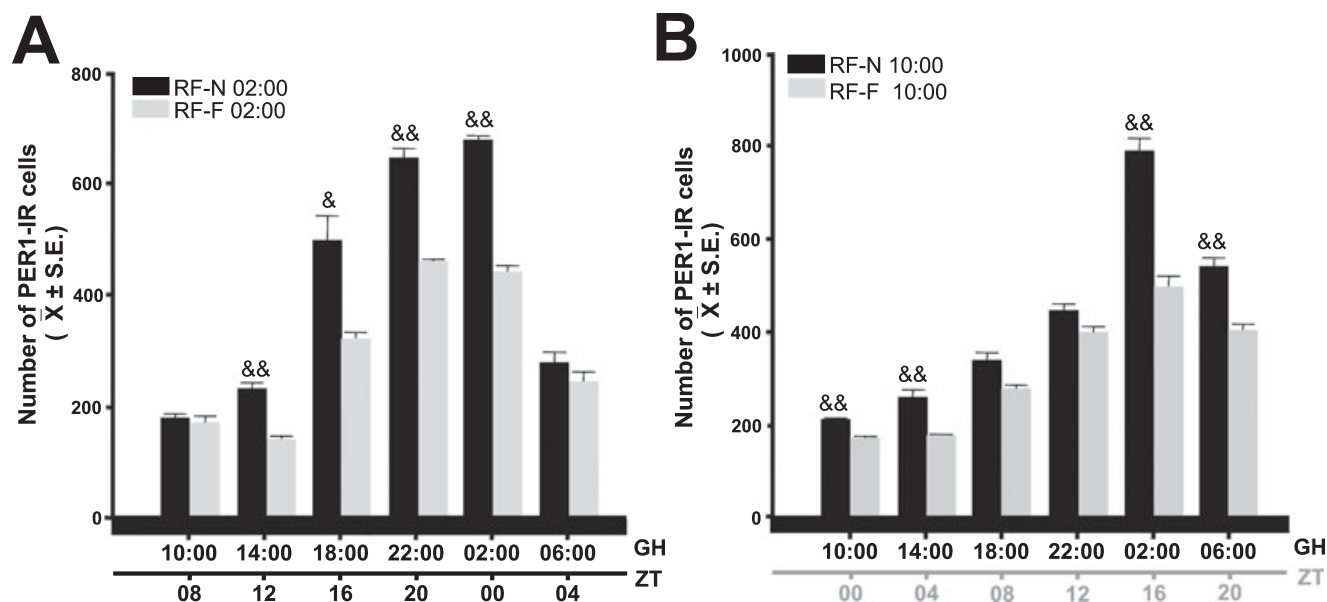


FIG. 6. Comparative distribution of PER1 in the SCN in nursed (RF-N) and in fasted (RF-F) subjects scheduled to nurse at 02:00 (A) and 10:00 h (B). Values are mean \pm SEM. &, $P < 0.05$, &&, $P < 0.01$. & denotes significant differences between corresponding time point groups. Additional legends as in Fig. 3. Data are re-plotted from Figs 3 and 5.

Figure 10 provides a comparison of the number of PER1 cells in the SCN (Fig. 10A) and DMH (Fig. 10B) in the RF-N 02:00 and RF-N 10:00 groups in relation to time of nursing. In the SCN, pups nursed at 10:00 h had low cell numbers at time of nursing and highest 16 h later, while pups nursed at 02:00 had highest PER1-IR cells at time of nursing. In contrast, in the DMH, there was a clear effect of nursing time on PER1-IR as cell numbers are low at time of nursing and reach highest values 8 h after nursing following a similar pattern in both day- and night-fed subjects.

Discussion

We explored the neural mechanisms of circadian timing in the rabbit pup, a naturally occurring model of temporally restricted feeding. The present results indicate that the locomotor behavior of the rabbit pup is synchronized to the daily time of the nursing bout, and that the rabbit pup shows FAA. In the SCN, there is little difference in the timing of PER1 and FOS expression in animals fed at 02:00 vs. 10:00 h whether they have been nursed or have fasted prior to being killed. In contrast, the timing of PER1 expression in the DMH changes in proportion to the shift in feeding time.

At the behavioral level, rabbit pups display locomotor FAA prior to the time of feeding, as in other animals (Mistlberger, 1994; Stephan, 2001). The pups fed at 10:00 h show much greater activity than those fed at 02:00 h. The rabbit is a nocturnal animal (Jilge, 1991; Szeto *et al.*, 2004), and regardless time of birth dams nurse their pups during the dark period (Jilge, 1993). In the present study pups nursed at 02:00 h were exposed to the mother when she was in her active phase, but those at 10:00 h were nursed at her inactive period. It is of interest to understand how this might produce changes in the pup's activity levels. In rats the timing of the mother's presence with the litter is a critical factor that affects locomotor behavior and stress response in pups. Maternal deprivation at different times of day, 3 h in the morning or 3 h in the afternoon, during the first six postnatal days in rats induces significant differences in circadian rhythms of pups after weaning. At 8 weeks old, maternal deprivation in the first group induced increases in

corticosterone plasma levels and locomotor behavior over control but not in subjects deprived for 3 h a day in the afternoon (Yoshihara *et al.*, 2005). Although researchers do not know the reason for this difference it has been reported that rat maternal care and nutritional supply are more abundant in the early light phase than in the dark (Levin & Stern, 1975). In the present study, there was no difference in body weight between groups fed at different times of day, which suggests that nutritional factors are not associated. However, we consider that it is possible that dams are stressed at 10:00 h, though we do not know how this might be communicated to the pups during the brief visit to the nest.

Surprisingly we found an effect of timing of nursing on the SCN, corroborated by changes in acrophase in both PER1 and FOS in nursed and fasted subjects. Extensive experiments in adult rodents demonstrate that clock genes and proteins in the SCN do not shift as a consequence of a restricted feeding schedule (Damiola *et al.*, 2000; Hara *et al.*, 2001; Stokkan *et al.*, 2001). However, under particular conditions food cues can affect the SCN in adult rodents. Timed hypocaloric food restriction, but not normocaloric diet, induces a phase advance of *Per1* mRNA in the SCN of mice (Mendoza *et al.*, 2005), similar to the present results in PER1 protein. However, a scheduled feeding without caloric restriction also entrains PER2 protein expression in the SCN of adult mice exposed to 4–6 months of constant darkness (Castillo *et al.*, 2004). These studies demonstrate that food-related cues can also act as zeitgeber for the SCN. Again, there is no evidence that caloric effects are involved in the present studies. Future studies should explore the peptidergic identity of PER1- and FOS-expressing cells, and the possibility of regional differences in the SCN in response to various schedules of nursing.

To our knowledge this is the first study exploring entrainment of the SCN in neonatal subjects under a restricted feeding paradigm. However, by exposing the mother to the pups at specific times it has been shown that the SCN of immature subjects can be synchronized to non-photic stimuli. Cross-fostered newborn pups, blinded immediately after birth, by mothers on a reversed light–dark cycle from that of their biological mothers, show a phase difference of

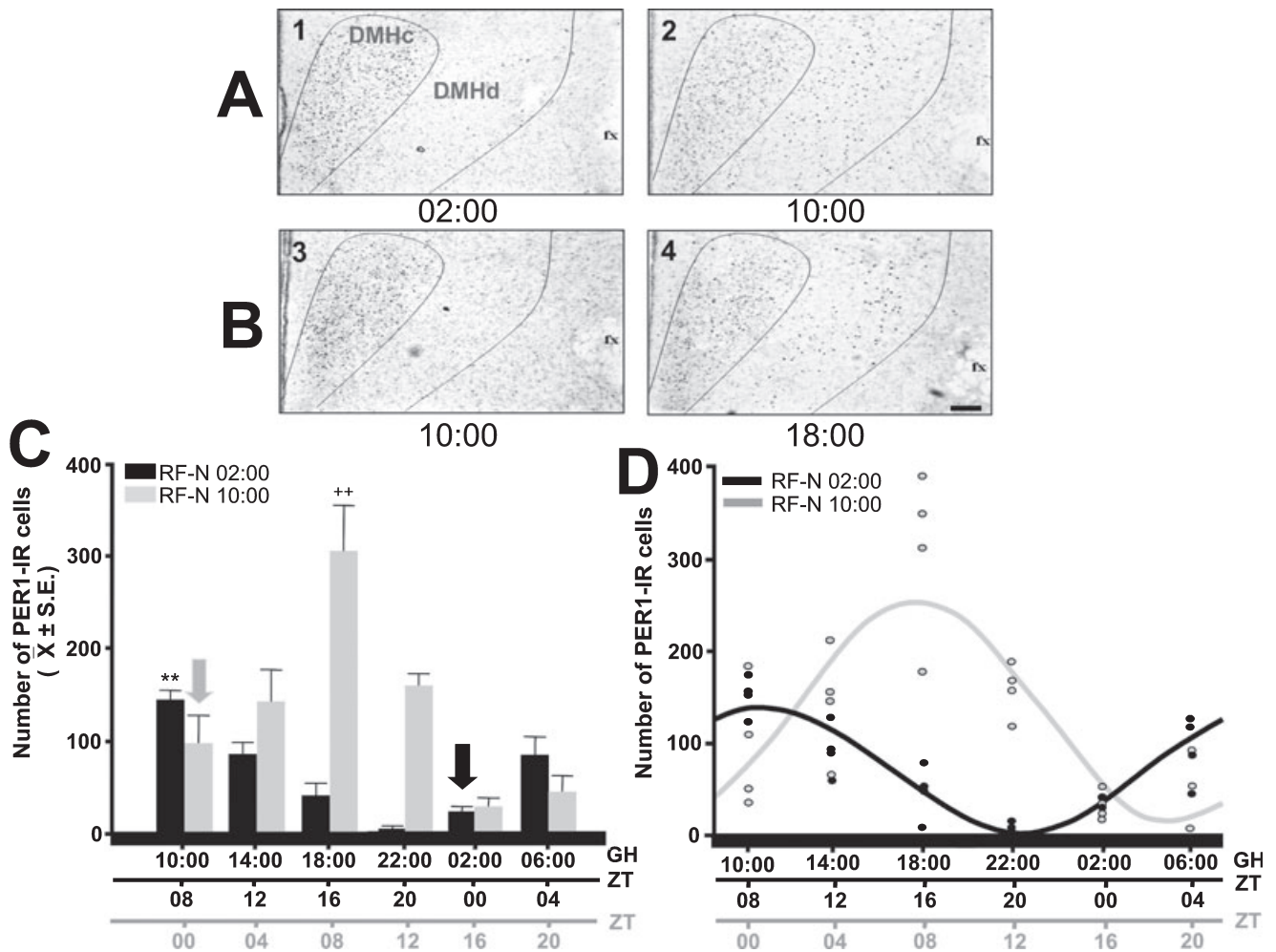


FIG. 7. Rhythmic expression and phase shift of PER1-IR in the dorsomedial hypothalamus (DMH) in subjects nursed at 02:00 (RF-N 02:00) and 10:00 h (RF-N 10:00) from PD1-PD7. (A, B) Photomicrographs of representative sections illustrating the induction of PER1 protein in the DMH pars diffuse (DMHd) by nursing. Nursing was scheduled at 02:00 (A1) and 10:00 h (B3), and PER1 was maximally induced 8 h later at 10:00 (A2) and 18:00 h (B4), respectively. DMHc, dorsomedial hypothalamus pars compacta; fx, fornix. (C) Rhythmic expression of PER1-IR. At time of nursing (arrows) subjects have low numbers which reach highest levels 8 h later. Values are mean \pm S.E. ++, **, $P < 0.01$. For pups nursed at 02:00 h the value at 10:00 h (++) was significantly higher than lowest values. For pups nursed at 10:00 h, the value at 18:00 h (**) was significantly different from lowest values (see text for details). (D) Cosinor analysis. There is a phase difference of PER1-IR of 7.30 h in subjects nursed at 02:00 against those at 10:00 h (see text for details). Additional legends as in Fig. 3.

~ 2 h in the rhythm of *mPer1* in the SCN (Ohta *et al.*, 2002). These authors further reported that periodic maternal deprivation for 12 h completely phase-reversed the circadian rhythm of *mPer1* in the pup's SCN at postnatal day 6 (Ohta *et al.*, 2003). It is not clear which factor is responsible for the phase differences of clock gene expression in the SCN, as deprived pups are potentially exposed to cold, dehydration, starvation and lack of maternal contact. In our rabbit model, however, these factors did not likely affect pups as they normally remain alone in the nest most of the time.

We also found that the DMH exhibits a circadian pattern of PER1 protein both in nursed and in fasted pups synchronized to a daily nursing bout, but unlike the SCN, the DMH shows a complete phase shift in parallel to scheduled nursing. PER1 induction was restricted to the diffuse region of the DMH. Rhythmic expression in DMH is not simply due to energy depletion or food availability as this pattern persists in subjects after a fasting period at the same expected hour as in nursed subjects.

The DMH receives neural and humoral inputs from pathways implicated in the regulation of feeding (Thompson & Swanson,

1998) and contains high levels of glucose receptors (Berthoud, 2002). DMH destruction produces alterations in feeding behavior and, based on lesioning studies, it has been established that it plays a main role in the homeostatic control of ingestive behavior and body weight regulation (Bernardis & Bellinger, 2002). Moreover the DMH is recognized as important for the expression of circadian rhythms of feeding, locomotor activity and serum corticosteroid hormone levels in adult rats, as revealed by excitotoxic lesions (Chou *et al.*, 2003). In addition, recently it has been proposed that the DMH is critical for the regulation of food-entrainable circadian rhythms. Under a restricted food schedule, cells in the DMH synchronize their activity as measured by FOS protein expression and *mPer1*, in anticipation of food availability and during food presentation, and recently has been proposed as a putative food-entrainable circadian pacemaker (Angeles-Castellanos *et al.*, 2004; Gooley *et al.*, 2006; Mieda *et al.*, 2006).

Our results in rabbit pups indicate that circadian expression of PER1 in the DMH is synchronized both by nursing and free-runs during a 48-h fasting period, although in the latter we observed in

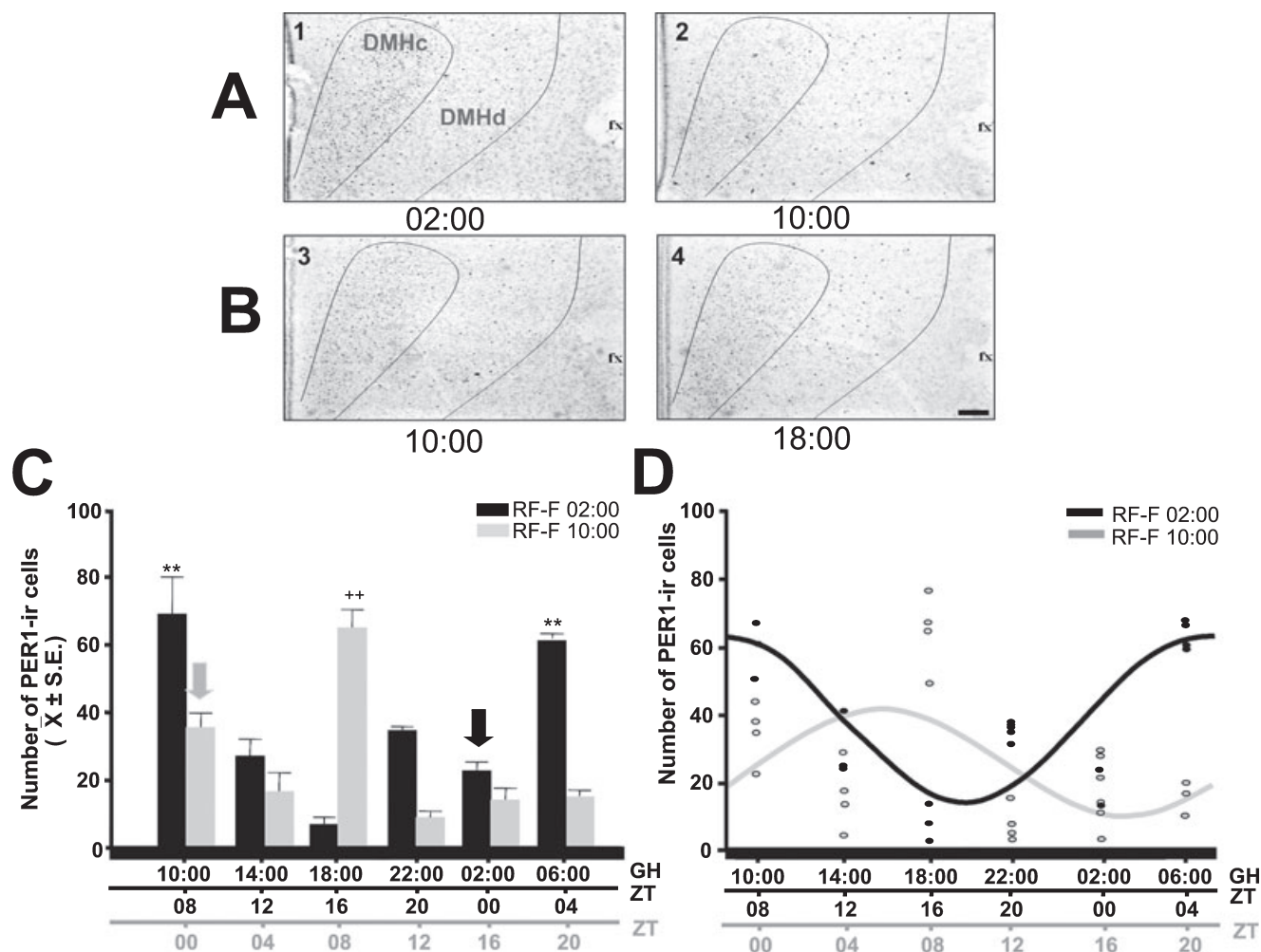


FIG. 8. Rhythmic expression and phase shift of PER1-IR in the DMH in fasted subjects previously nursed at 02:00 (RF-F 02:00) and 10:00 h (RF-F 10:00) from PD1 to PD7. (A, B) Photomicrographs of representative sections illustrating the persistence of PER1 protein in the DMH pars diffuse (DMHd) in absence of nursing. At the time of the expected scheduled nursing, 02:00 (A1) and 10:00 h (B3), there are few PER1-IR cells, which increase their numbers 8 h later at 10:00 (A2) and 18:00 h (B4), respectively. (C) At the time of nursing (arrows) subjects have low numbers which reach highest levels 8 h later. Values are mean \pm SEM. ++, **, $P < 0.01$. For fasted pups previously nursed at 02:00 h, the values at 06:00 and 10:00 h (**), were significantly higher than lowest values. For pups nursed at 10:00 h, the value at 18:00 h (++) was significantly different from lowest values (see text for details). (D) Cosinor analysis. There is a phase difference of PER1-IR of 8.36 h in fasted subjects previously nursed at 02:00 h against those at 10:00 h (see text for details). Additional legends as in Fig. 3.

general a significantly lower number of PER1-IR cells than in nursed subjects. This result is in agreement with that of Mieda *et al.* (2006), who also found a reduced peak expression of *mPER1* in the DMH of fasted mice under a restricted feeding schedule. On this basis, we consider that this nucleus is entrained by nursing and may have a key role in the circadian anticipatory activity to daily nursing in the rabbit. However, it is unlikely that the DMH is the neural locus of the food entrainable oscillator given that partial and total lesions of this nucleus do not affect FAA in rats (Landry *et al.*, 2006; Landry *et al.*, 2007).

Which is the entraining signal? Although the identity of the specific synchronizer is unknown, the large ingestion of milk, up to 35% of body weight at PD7 (Caba *et al.*, 2003b), which produces a strong gut distention (Escobar *et al.*, 2000), has been proposed as the zeitgeber (Jilge *et al.*, 2000) as already mentioned. Besides food and its rewarding/nutritional properties, during nursing pups are exposed to tactile, acoustic and olfactory/pheromonal stimuli. For example, rabbit pups show prandial and circadian variations in their response to the mammary pheromone when this is presented throughout a 24-h cycle (Montigny *et al.*, 2006).

We have explored the role of CORT as a physiological signal in the pups. Rabbit pups are unusual, as compared with neonatal rodents, in that they show circadian variations in CORT at an early age when rats lack CORT secretion and experience a stress-hyporesponsive period (Levine, 1994). In contrast, rabbit pups 7–9 days old have a circadian rhythm of CORT which peaks at the time of the scheduled nursing. After nursing, values steadily decrease to a nadir 12 h later and then rise again in advance of the next scheduled nursing (Rovirosa *et al.*, 2005). Furthermore, recently we found that if one period of nursing in 8-day-old rabbit pups is omitted, CORT remains high around the time of the expected nursing; however, CORT concentration drops, but around 60 h after the last nursing levels starts to increase again in anticipation of the next period of nursing (Morgado *et al.*, 2006).

There are several candidate metabolic or humoral signals that might act directly or indirectly on the DMH and SCN. In the neonatal rat the SCN and DMH express high levels of glucocorticoid receptors during the first week of life (Van Eekelen *et al.*, 1987; Morimoto *et al.*, 1996). In 7-day-old rabbit pups we detected a rhythm of ghrelin, a hormone produced and released primarily by the

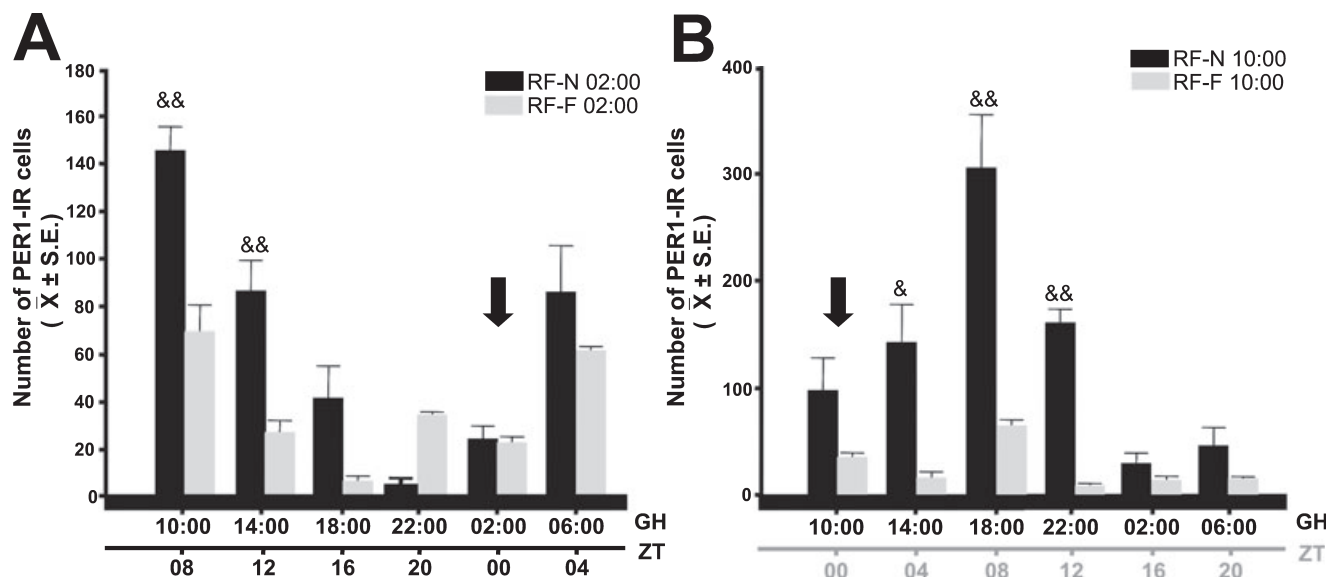


FIG. 9. Comparative distribution of PER1 in the DMH in nursed (RF-N, black bars) and in fasted (RF-F, grey bars) subjects scheduled to nurse at 02:00 (A) and 10:00 h (B). Arrows denotes time of nursing. Values are mean \pm SEM. &, $P < 0.05$, &&, $P < 0.01$. & denotes significant differences between corresponding time point groups. Additional legends as in Fig. 3. Data are re-plotted from Figs 7 and 8.

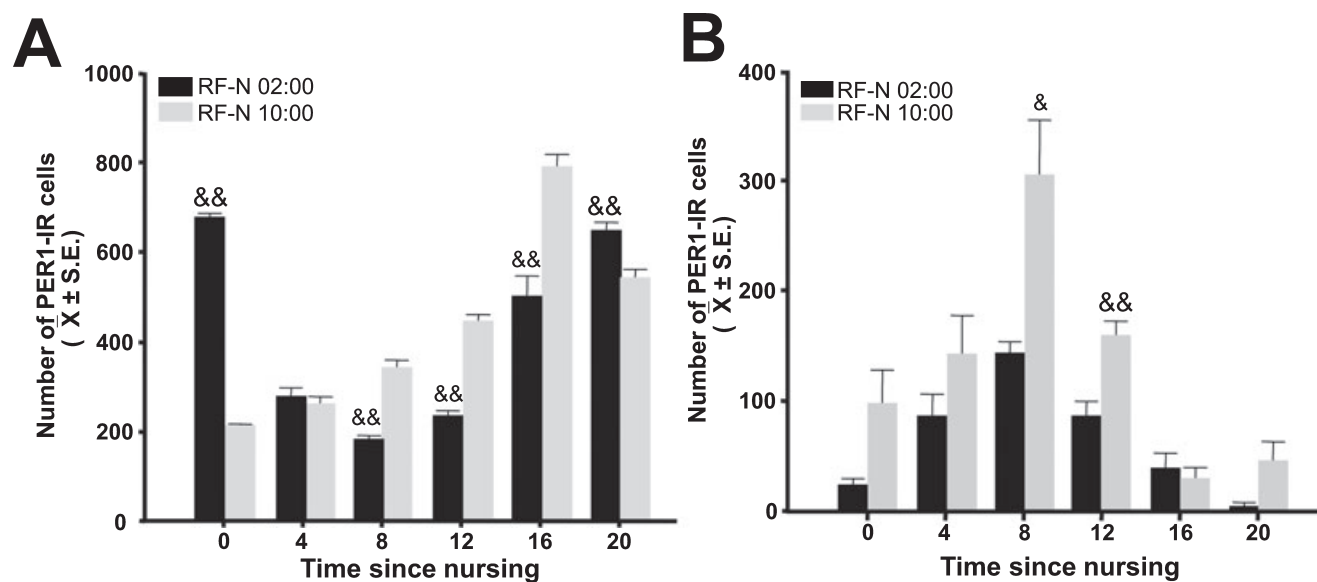


FIG. 10. Comparative distribution of PER1-IR in the SCN (A) and DMH (B) in pups scheduled to nurse at 02:00 (RF-N 02:00) and 10:00 h (RF-N 10:00) in relation to time since nursing. &, $P < 0.05$, &&, $P < 0.01$. & denotes significant differences between corresponding time point groups. Additional legends as in Fig. 3. Data for panel A are replotted from Fig. 3 and for panel B from Fig. 7.

oxyntic cells in the gut (Date *et al.*, 2000), which has receptors in several hypothalamic nuclei, including the SCN and DMH (Zigman *et al.*, 2006). In this regard it is very interesting that in the rabbit pup, ghrelin secretion reaches a peak at the middle of the cycle, 12 h after nursing (unpublished results from our laboratory). Future studies should determine whether CORT and ghrelin participate as synchronizing signals for the observed phase changes in the SCN and DMH.

Taken together, the evidence indicates that periodic nursing synchronizes locomotor rhythm (Jilge, 1993; present contribution), physiological parameters such as temperature (Jilge *et al.*, 2000) and corticosterone (Rovirosa *et al.*, 2005), and affects clock genes and

FOS expression (present contribution). In conclusion, the rabbit pup is a unique natural model of food restriction which offers the possibility to explore the neural and physiological basis of this phenomenon without excessive manipulations, unlike in rodents, as the dyad mother–pup come together once a day for a short period of time.

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Abbreviations

CORT, corticosterone; DD, constant dark; DMH, dorsomedial hypothalamic nucleus; DMHc, dorsomedial hypothalamus, compact part; DMHd, dorsomedial hypothalamus, diffuse part; FAA, food anticipatory activity; FOS, c-fos protein; FOS-IR, c-Fos immunoreactivity; LD, light/dark; PB, phosphate buffer; PER1, PERIOD1 protein; PER1-IR, PERIOD1 immunoreactivity; PD, postnatal day; RF-F, restricted feeding, fasting group; RF-N, restricted feeding, nursed group, SCN, suprachiasmatic nucleus.

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