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Unpredictable feeding schedules unmask a system for daily resetting of behavioural and metabolic food entrainment

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Abstract

Restricted feeding schedules (RFS) are a potent Zeitgeber that uncouples daily metabolic and clock gene oscillations in peripheral tissues from the suprachiasmatic nucleus (SCN), which remains entrained to the light–dark cycle. Under RFS, animals develop food anticipatory activity (FAA), characterized by arousal and increased locomotion. Food availability in nature is not precise, which suggests that animals need to adjust their food-associated activity on a daily basis. This study explored the capacity of rats to adjust to variable and unpredictable feeding schedules. Rats were exposed either to RFS with fixed daily meal (RF) or to a variable meal time (VAR) during the light phase. RF and VAR rats exhibited daily metabolic oscillations driven by the last meal event; however, VAR rats were not able to show a robust adjustment in the anticipating corticosterone peak. VAR rats were unable to exhibit FAA but exhibited a daily activation pattern in phase with the previous meal. In both groups the dorsomedial nucleus of the hypothalamus and arcuate nucleus, involved in energy balance, exhibited increased c-Fos expression 24 h after the last meal, while only RF rats exhibited low c-Fos expression in the SCN. Data show that metabolic and behavioural food-entrained rhythms can be reset on a daily basis; the two conditions elicit a similar hypothalamic response, while only the SCN is inhibited in rats exhibiting anticipatory activity. The variable feeding strategy uncovered a rapid (24-h basis) resetting mechanism for metabolism and general behaviour.

Introduction

In addition to the suprachiasmatic nucleus (SCN), the master biological clock, diverse tissues and cerebral structures express clock genes that oscillate with a period similar to 24 h; they are therefore classified as peripheral oscillators. Peripheral clock genes as well as digestive and metabolic functions under physiological conditions oscillate in synchrony with the SCN but preferentially adjust their daily oscillations to feeding schedules (Damiola *et al*., 2000; Stokkan *et al*., 2001; Hastings *et al*., 2003).

Nocturnal rodents ingest their meals and produce the main digestive and food-related signals during the night, which maintains the oscillations of peripheral tissues coupled to the SCN and adjusted to the light–dark (LD) cycle. When nocturnal rodents are forced to eat during the light phase by scheduling food access during the day, peripheral oscillators uncouple from the SCN, which maintains its phase coupled to the LD cycle (Stokkan *et al*., 2001; Damiola *et al*., 2001). Thus under restricted feeding schedules (RFS), food becomes a potent Zeitgeber that overrides the influence of the SCN on peripheral oscillators. The mechanism by which the feeding schedule provides the entraining signal to set the phase of the peripheral oscillators is not clear but may involve catabolic–anabolic cycles and the induction of cycles in the redox state in the cells (Díaz-Muñoz *et al*., 2000; Rutter *et al*., 2001; Cardona, 2004). It has been suggested that, as metabolites, hormones and temperature modify the metabolic state of cells, they may play a relevant role as entraining signals (Balsalobre *et al*., 2000; Damiola *et al*., 2001; Rutter *et al*., 2001; Brown *et al*., 2002; Hirota *et al*., 2002).

Single pulses of hormones and metabolites are sufficient to initiate oscillations in cultured fibroblasts (Balsalobre *et al*., 1998) or to produce a phase change of clock gene products in peripheral oscillators (Balsalobre *et al*., 2000; Le Minh *et al*., 2001; Shibata, 2004). Such findings support the possibility that peripheral oscillators are capable of a fast phase shift.

Neuronal activity in diverse brain structures is also entrained to feeding schedules and becomes uncoupled from the SCN (Wakamatsu *et al*., 2001; Lamont *et al*., 2005). Food-entrained patterns of c-Fos and clock gene expression have been reported in hypothalamic and brain stem areas that are involved in energy balance and arousal (Angeles-Castellanos *et al*., 2004, 2005; Gooley *et al*., 2006; Mieda *et al*., 2006; Waddington-Lamont *et al*., 2006) and are also found in limbic structures involved in motivational and affective responses (Mendoza *et al*., 2005; Angeles-Castellanos *et al*., 2007). After 4–5 days of RFS, brain structures exhibit food-entrained patterns of c-Fos expression (Inzunza *et al*., 2000; Meynard *et al*., 2005).

In consequence, RFS also entrains behavioural patterns; rats restricted to food access during the light phase develop a pattern of activity known as food anticipatory activity (FAA), which is characterized by increased movement and foraging starting 1–2 h prior to the scheduled meal time (Mistlberger, 1994; Stephan, 2002). Similar to c-Fos expression, FAA can be observed after 3–5 days of

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RFS. The capacity to adjust FAA to phase shifts in the feeding schedule has been explored in rats bearing a bilateral lesion of the SCN: a phase delay of 4–10 h resulted in transitory cycles (Stephan, 1984, 1992) while in some cases the rapid re-entrainment did not show transitory cycles (Stephan, 1992). The shifting speed was reported to be faster in rats bearing a bilateral lesion of the SCN than in intact rats. There are no further reports on the shifting properties of FAA.

The present study was designed to explore the speed with which oscillations in metabolic variables, brain structures and FAA adapt to shifts in meal time. Because we assumed that they depend on different mechanisms we hypothesized that metabolism would adapt to daily challenges and thus oscillations in metabolites and hormones would shift on a daily basis, while FAA and the activity pattern of brain structures would need several cycles to adjust to shifts in meal time. Therefore, this study characterized metabolic oscillations, c-Fos expression (as marker of neuronal activity) in the SCN and hypothalamic nuclei involved in energy balance, and behavioural patterns of rats exposed to RFS with food provided at fixed or at variable and unpredictable hours daily.

Materials and methods

Animals and general housing conditions

Adult male Wistar rats weighing between 250 and 300 g at the beginning of the experiment were maintained in a 12 : 12 h LD cycle [lights on at 07.00 h, Zeitgeber time (ZTO)], constant temperature (22 ± 1 °C) and free access to food (Rodent Laboratory Chow 5001) and water, unless otherwise stated. Experimental procedures were approved and conducted according to the ethical committee at the Medical Faculty in accordance with the institutional guide for care and use of animal experimentation (Universidad Nacional Autónoma de México), which conforms to international guidelines for animal handling.

Experiment I

A first set of rats was used to characterize metabolic rhythms under fixed or unpredictable feeding schedules, as well as to examine the possible persistence of food-entrained rhythms for two cycles in fasting conditions. Rats were randomly assigned to one of the three feeding conditions and maintained in this situation for 2 weeks. After the last scheduled meal rats remained in total food deprivation for 24 h. (i) Fixed restricted feeding (RF) schedules: for this group food was available for 2 h daily from ZT5 to ZT7. (ii) Variable feeding schedules (VAR12): food was available once, mainly in and around the light period, for 2 h at variable and unpredictable times. The interval between meals was never shorter than 18 h and never longer than 36 h (one example of such a schedule is as follows: 09.00, 13.00, 08.00, 16.00, 11.00, 20.00, 19.00, 10.00, 13.00, 21.00, 18.00, 09.00, 15.00 and 12.00 h). The last meal for this group before starting blood sampling was at ZT5 (12 h). (iii) Variable restricted feeding schedules (VAR18): this group was treated in the same way as the VAR12, but the last meal was delivered at 18.00 h (one example of such a schedule is as follows: 11.00, 17.00, 13.00, 12.00, 16 19.00, 10.00, 20.00, 09.00, 14.00, 18.00, 08.00, 15.00, 21.00 and 18.00 h).

For the three groups the last meal time was defined as MT0 to allow comparson of food-related oscillations. Before the last meal rats were weighed. Blood sampling was started at MT3, which represents 3 h after the last scheduled meal and was continued every 3 h to complete a 24-h cycle (n = 4–6 rats per group and time point).

In order to determine the persistence of food-entrained metabolic rhythms a second set of rats was entrained as described for groups RF and VAR12 and then maintained in fasting for two 24-h cycles. Blood sampling was performed for the interval between 42 and 58 h after the last meal (MT42–58) and for corticosterone determinations for the interval MT42–55.

Serum and tissue sampling

For each temporal point and each group 4–6 rats were decapitated and trunk blood (3–4 mL) was collected in 10-mL test tubes containing a clot-forming gel (Vacutainer; Becton Dickinson). Tubes were centrifuged at 2500 r.p.m. for 15 min to obtain blood serum. Aliquots of 250 and 700 μL were coded and frozen at −70 °C for subsequent determination of serum corticosterone, leptin and free fatty acids (FFA).

After blood collection the stomach was dissected out at the level of the lower gastroesophageal sphincter and the pylorus, and wet stomach weight was registered.

Determination of serum hormones and FFAs

Serum corticosterone and leptin were determined in duplicate with a standard ^{125}I radioimmunoassay kit (Medidores Industriales TKRC1 for corticosterone, and Linco Res, Inc., St Charles, MO, USA for leptin). Assays were performed with a sensitivity of 0.5 ng/mL, and intra- and interassay coefficients of variation of 4%.

Serum FFAs were processed by using a colourimetric method, as described elsewhere (Escobar *et al*., 1998).

Statistical analysis

Data are reported as mean ± SEM and are represented as temporal waveforms. Body weight after each feeding protocol was compared with a one-way ANOVA. Metabolic variables were analysed with a two-way ANOVA for the factors group and time. Temporal effects for each group were tested with a one-way ANOVA followed by a Tukey multiple comparison *post hoc* test with α set at $P < 0.05$. Statistical analysis was performed with the package Statistica (StatSoft, Inc. 1993).

Experiment II

A different set of rats was placed in an automated activity monitoring system in order to determine temporal behavioural organization under a fixed or a variable unpredictable schedule (n = 16 per group) and to determine the possible persistence of food-entrained patterns in fasting conditions.

Behavioural monitoring

Rats were placed in individual cages (45 × 30 × 35 cm) positioned on plates with movement sensors in soundproof lockers with controlled lighting conditions and with a regulated temperature of 24 ± 1 °C and a 12 : 12-h LD cycle (lights on at 07.00 h). This system was developed in our group with contributions from Nico Bos in Amsterdam the Netherlands and the Mexican biomedical company Omnialva. Behavioural events were collected with a digitized system and stored at 10-min intervals for further analysis with an analysis system developed for our laboratory in Matlab (SPAD9).

For baseline, rats were monitored for 14 days with free access to food and water; on day 15 food was removed and starting on day 17 food was delivered for 2 h under an RF schedule. A first group of rats was assigned to an RF schedule with daily food access from 12.00 to

14.00 h local time. A second group was assigned to a variable and unpredictable feeding schedule (VAR12) with random daily food access as described for experiment 1. At the end of each feeding protocol rats were left for 2 days in total food deprivation to evaluate persistence of the food-entrained behavioural rhythm. This protocol was performed in two series of eight animals each, giving a total of 16 rats per group.

Actograms were obtained in order to visualize temporal organization of behaviour. In each 10-min period, the duration of detected movement was measured, normalized and converted to a percentage of total daily activity. Mean activity profiles were obtained for baseline and entrainment interval for each group. In order to determine FAA and persistence of entrained patterns for the two conditions, fixed and unpredictable meal schedules, a daily analysis was performed comparing the activity 2 h prior to, 2 h during and 2 h after meal time. In addition, the activity in the same intervals the following day was analysed. For the VAR group, analysis days were chosen such that in the first day food access had been scheduled early and in the following day food was delivered later, in order to observe persistence of behavioural patterns. Days used for this later analysis were days 1 (meal at 08.30 h) and 2 (meal at 13.00 h), days 5 (meal at 08.30 h) and 6 (meal at 18.00 h) and days 11 (meal at 12.00 h) and 12 (no food). Total activity counts in the 2-h intervals for both groups were compared with the mean activity for the same interval during the baseline. A two-way ANOVA was used for the factors group and 2-h intervals followed by a Tukey multiple comparison *post hoc* test with α set at $P < 0.05$.

Experiment III

A third set of rats was used to characterize neuronal activity during FAA in rats entrained by fixed feeding schedules or exposed to variable and unpredictable daily food restriction. Rats were randomly assigned to and maintained for 2 weeks in one of three groups ($n = 6$ per group). (i) *Ad libitum* (control), in which animals had free access to food and water during the 24-h cycle. (ii) Fixed restricted feeding (RF); food was available for 2 h daily from 12.00 to 14.00 h (ZT5–7). (iii) Variable feeding schedules (VAR); food was available daily for 2 h at variable and unpredictable times of the day as described for experiment 1. The last meal for this group was at 12.00 h.

In order to determine neuronal activity associated with anticipatory activity, rats were perfused the day after termination of the entraining protocols 1 h after the expected meal (i.e. perfused at 13.00 h) and their brains were removed and processed.

Immunohistochemistry

Rats were anaesthetized with an overdose of sodium pentobarbital (Sedal-Vet 65 mg/mL), and were perfused transcardially with 150 mL of 0.9% saline followed by 250 mL of fixative: 4% paraformaldehyde in phosphate-buffered saline (PBS; 0.1 M, pH 7.2). Brains were removed, post-fixed for 18 h and cryoprotected in 30% sucrose for 3–4 days. Brains were frozen and cut into sections of 40 μm at −18 °C. Sections were serially collected in four sets; one set was stained with cresyl violet acetate (Sigma Chemical Company) and a second set was processed for cFos immunohistochemistry. Free-floating sections were incubated in c-Fos antibody raised in rabbit (1 : 2500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 72 h. This was followed by incubation in secondary antibody, processed using the avidin–biotin method for immunohistochemistry as previously described (Angeles-Castellanos *et al.*, 2004). Tissues were reacted in diaminobenzidine with hydrogen peroxide, mounted, dehydrated and coverslipped for light-microscopical analysis.

Cell count and analysis

Fos-immunoreactive cells were evaluated in the suprachiasmatic nucleus (SCN), the arcuate nucleus (ARC) and the dorsomedial nucleus of the hypothalamus (DMH).

Two representative sections for each nucleus were selected in accordance with the stereotaxic atlas of Paxinos and Watson (1998). Bilateral images of selected sections were digitized at a 200× magnification using a computerized image analysis system (Meta Vue series 4.5; Universal Imaging Corporation) attached to a Nikon light microscope (Nikon Eclipse E600). To minimize the number of false positives, background optic density was established in a nearby region lacking Fos immunoreactivity; stained nuclei that reached or surpassed 2× the background optic density were considered positive and were included, whereas cells below this staining threshold were discarded. A single examiner, who was blinded to treatment conditions, performed all counts.

The mean cell number for the two representative sections were classified by group and given as mean ± SEM. Data between groups (three levels) were compared with a one-way ANOVA for independent measures followed by a Tukey *post hoc* test with significant values set at $P < 0.01$. Statistical analysis was performed with the program Statistica version 4.5 (StatSoft, Inc. 1993).

Results

Experiment I

On the last day of restricted feeding, rats in the two variable feeding groups (VAR12 and VAR18) showed a significantly lower body weight than rats fed at a fixed feeding schedule (RF, 330.3 ± 5.1; VAR12, 311.9 ± 6.9, VAR18, 312 ± 3.1 g; $F_{2,119} = 4.49$, $P < 0.03$).

Stomach weight and serum determinations

Stomach weight of the three food-restricted groups (RF, VAR12 and VAR18) showed a daily oscillation associated with the scheduled meal time (two-way ANOVA: $F_{7,99} = 107.6$, $P < 0.0001$). Single one-way ANOVAs performed per group confirmed a significant effect of time for the three groups (RF, $F_{7,24} = 47.78$, $P < 0.0001$; VAR12, $F_{7,32} = 71.18$, $P < 0.00001$; VAR18, $F_{7,32} = 20.79$, $P < 0.0001$). In all groups, the highest values were observed at the first sampled time point after meal time (MT3) and stomach weight decreased slowly to attain statistically significantly lower values 15 h after the last meal. Empty stomachs were observed 21 h after feeding, thus 3 h before the expected meal (Fig. 1, top). Although the temporal pattern was similar for the three groups, the two groups exposed to variable feeding schedules attained lower values after the meal; two-way ANOVA showed a significant effect among groups ($F_{2,88} = 40.96$; $P < 0.001$) and a significant group × time interaction ($F_{14,88} = 8.19$; $P < 0.001$). After two cycles of fasting, RF and VAR12 exhibited low values indicating an empty stomach and no statistical difference was observed between groups [$F_{1,60} = 3.49$; not significant (NS)] or due to time ($F_{5,60} = 2.19$; NS).

Serum leptin of the three food-restricted groups showed a daily oscillation associated with meal time (two-way ANOVA: $F_{7,117} = 57.77$, $P < 0.0001$), highest values were observed 3–6 h after meal time and decreased slowly to attain the lowest values 12 h after the last meal (Fig. 1, right top).The single one-way ANOVA per

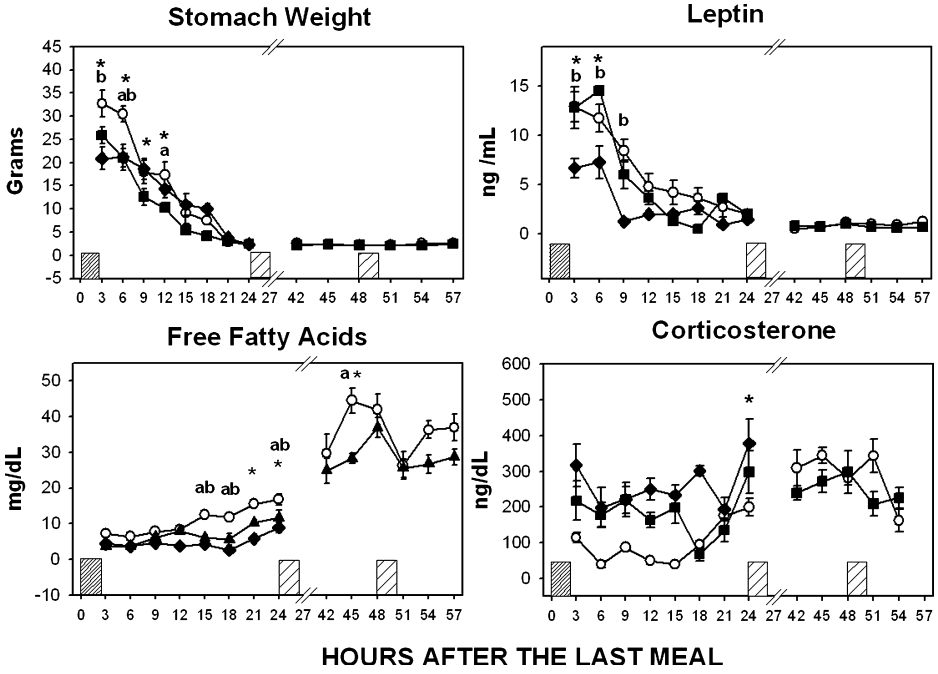

FIG. 1. Stomach weight and serum determinations of rats maintained under fixed (RF; white circles) or variable (VAR12, black squares; VAR18, black diamonds) feeding schedules. The three groups exhibited daily rhythms adjusted to the last meal. Asterisks indicate time points where high values for the 3 groups were statistically significantly different from lowest values of the same group (*$P < 0.01$, Tukey). Letters indicate significant difference between (a) RF and the VAR12 groups and (b) RF and the VAR18 groups. The solid bar indicates last meal and hatched bars indicate expected meal time during the fasting interval.

group confirmed a significant effect of time for the three groups (RF, $F_{7,40} = 16.64$, $P < 0.0001$; VAR12, $F_{740} = 37.04$, $P < 0.00001$; VAR18, $F_{7,37} = 11.71$, $P < 0.0001$). Highest values were observed 3–6 h after meal time and decreased slowly to the lowest values 12 h after the last meal (Fig. 1, right top). At 3, 6 and 9 h after meal time the RF and VAR12 groups exhibited significantly higher values than the VAR18 group; however, at the subsequent time points leptin levels were similar for the two groups. The two-way ANOVA indicated a significant difference among groups ($F_{2,117} = 33.91$; $P < 0.0002$) and interaction group × time ($F_{14,117} = 4.40$; $P < 0.0002$. During fasting both RF and VAR12 exhibited low values and no oscillations of serum leptin; no statistical difference was observed between groups ($F_{1,66} = 0.07$; *NS*) or due to time ($F_{5,66} = 1.68$; NS).

In serum FFAs the three groups showed a similar pattern associated with meal time ($F_{7,114} = 24.69$; $P < 0.001$), characterized by low values for the 9 h following the last meal and an increasing tendency to reach peak values 3 h before and at the moment of the next expected meal (Fig. 1, left bottom). The single one-way ANOVA performed per group confirmed a significant effect of time for the three groups (RF, $F_{7,40} = 17.53$, $P < 0.0001$; VAR12, $F_{7,37} = 9.71$, $P < 0.0001$; VAR18, $F_{7,37} = 4.02$, $P < 0.002$). The anticipatory increase was more evident in the RF group and was significantly different from that of the variable groups. The two-way ANOVA indicated main differences among groups ($F_{1,114} = 81.58$; $P < 0.0001$) and for the interaction group × time ($F_{14,114} = 3.16$; $P < 0.001$). During fasting there was a gradual increase in FFA due to 2 days fasting. Remarkably, under these conditions the RF and the VAR12 groups showed an increase in serum FFA before the expected meal time followed by a sharp decrease after the expected (but not given) feeding. Main effects were observed between groups ($F_{1,48} = 17.10$; $P < 0.0001$) and for the factor time ($F_{5,48} = 4.40$; $P < 0.002$), but not for their interaction ($F_{5,48} = 1.21$; NS). This later effect was confirmed by one-way ANOVA per group, which indicated a significant effect of time for the RF group ($F_{5,24} = 2.96$; $P < 0.03$) but not for the VAR12 group ($F_{5,24} = 2.47$; $P = 0.06$).

Serum corticosterone for the three food-restricted groups showed a daily oscillation associated with the last meal time ($F_{7,78} = 3.98$; $P < 0.001$), highest values were observed 24 h after the last meal (Fig. 1, right bottom). In both groups exposed to variable meals mean values were higher than in the RF group and the two-way ANOVA indicated a significant difference among groups ($F_{2,78} = 30.27$; $P < 0.0001$). It also indicated a significant effect of time ($F_{7,78} = 3.98$; $P < 0.001$), but not due to the interaction of the two factors ($F_{14,78} = 1.31$; NS). This was further confirmed with the one-way ANOVA per group for which a significant effect of time was statistically significant for the RF group ($F_{7,24} = 19.79$; $P < 0.0001$) but not for the VAR12 ($F_{7,24} = 2.03$; $P = 0.09$) or for the VAR18 ($F_{7,30} = 1.47$; NS) groups. After 2 days fasting, the RF group exhibited high values before the expected meal; these decreased after the expected meal interval. This food-entrained pattern was not observed in the VAR12 group. The two-way ANOVA indicated no statistically significant difference between groups ($F_{1,35} = 2.13$; NS), due to time ($F_{4,35} = 2.13$; NS) or due to their interaction ($F_{4,35} = 1.81$; NS). The one-way ANOVA per group confirmed a significant effect of time for the RF group ($F_{4,19} = 3.66$; $P < 0.02$) but not for the VAR12 group ($F_{4,19} = 0.67$; NS).

Experiment II

During baseline all rats exhibited a daily rhythm with low activity during the light phase (22%) and high activity during the night (78%; Fig. 2, right top). During food restriction RF rats developed anticipatory activity characterized by increased movement starting ~2 h prior to food access, with peak values at the moment of meal time (Fig. 2, left top and right middle). This activity was clearly observed on days 4–5 of food restriction and promoted an increase in

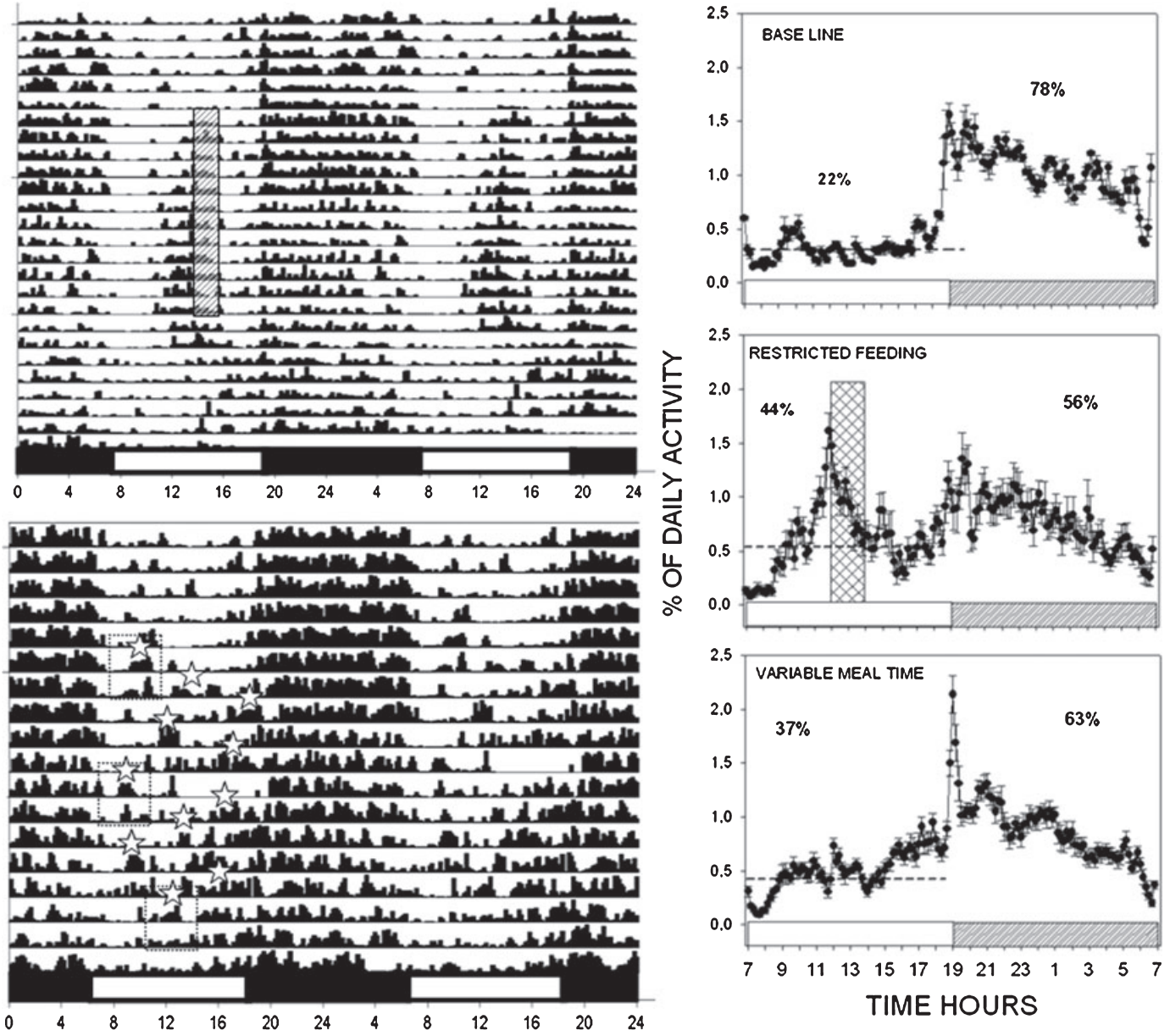

FIG. 2. (Left) Actograms for general activity of individual rats synchronized to (top) a restricted feeding schedule or to (bottom) a variable feeding schedule. Mean activity graphs of 16 rats per group are shown on the right; vertical axis, percentage of total daily activity occurring in 10-min time bins. The total percentages of activity in the light and the dark phases are indicated above each plot. The mean expected activity for the light phase as observed during the baseline is indicated with the dotted line. In the actogram the vertical bar and the stars for the VAR rat indicate meal time. The three squares indicate the consecutive days that were further analysed.

activity during the light phase to 44%. VAR rats did not exhibit anticipatory activity; however, they showed increased general activation at the moment that food was delivered (Fig. 2, left bottom). Similar to the RF group, their mean activity in the light phase was increased, to 37% (Fig. 2, right bottom).

The detailed quantitative analysis for specific days allowed visualization of mean activity 2 h before, 2 h during and 2 h after meal time for both groups. After 2 days of total food deprivation the first meal event (day 1) produced in rats of both groups, RF and VAR, increased movement counts during the 2 h-feeding episode (Fig. 3, top). Activity counts for both groups were statistically significantly different from that observed at this phase during baseline ($F_{2,122} = 13.44$; $P < 0.0005$). The two-way ANOVA also indicated a significant difference among time points ($F_{2,122} = 11.48$; $P < 0.001$) and for the interaction group × time ($F_{4,122} = 4.45$; $P < 0.002$). On day 2, both groups exhibited a slight increase in activity during the 2 h prior to the expected meal (22 h after the previous feeding event); however, this was not statistically significantly different from the baseline activity. Both groups exhibited significantly increased activity during the 2-h interval of the expected meal time compared with the baseline activity. The proportion of activity exhibited by the VAR group was similar to that observed the previous day during food presentation and was significantly different from baseline. Because the RF group received food at this time, the activity displayed was significantly higher than and different from the VAR group (Fig. 3, bottom). The two-way ANOVA indicated a statistically significant

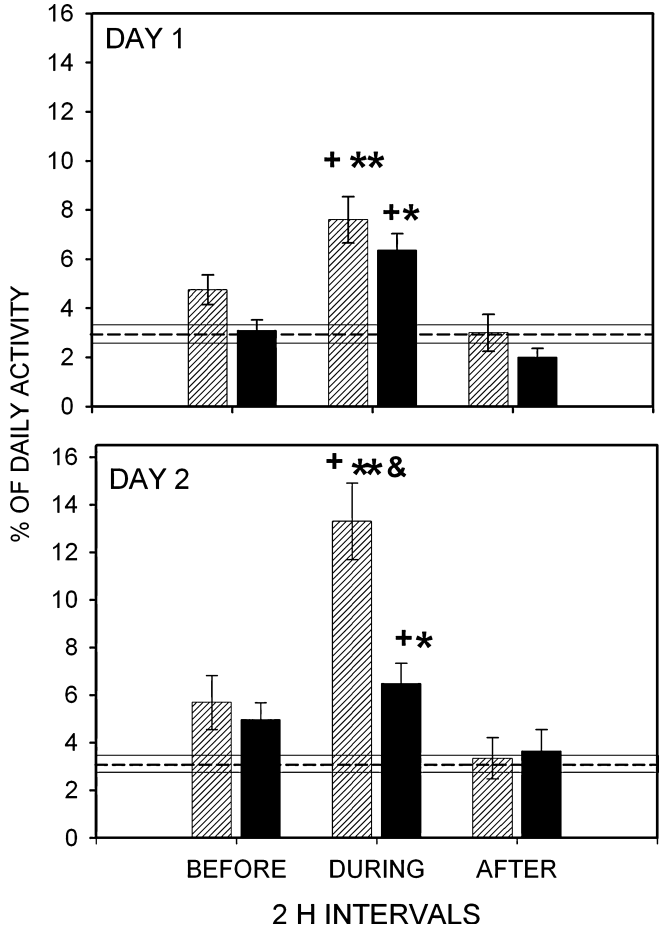

FIG. 3. Percentage of the total daily activity for 2 h intervals before, during and after the first meal episode for the RF (hatched bars) and the VAR (black bars) group ($n = 16$ rats per group). On day 2 only the RF group received food, in accordance with the 24-h schedule. The dotted horizontal line represents mean values obtained for the same phase during baseline, while continuous horizontal lines represent SEM; $^{+}P < 0.05$ vs. the baseline values, $^{**}P < 0.05$ between the highest point and the two lower points in the same group, and $^{*}P < 0.05$ between the highest and the lowest point in the same group and between RF and VAR rats (Tukey's *post hoc* test). $^{\&}P < 0.01$, RF vs. VAR group.

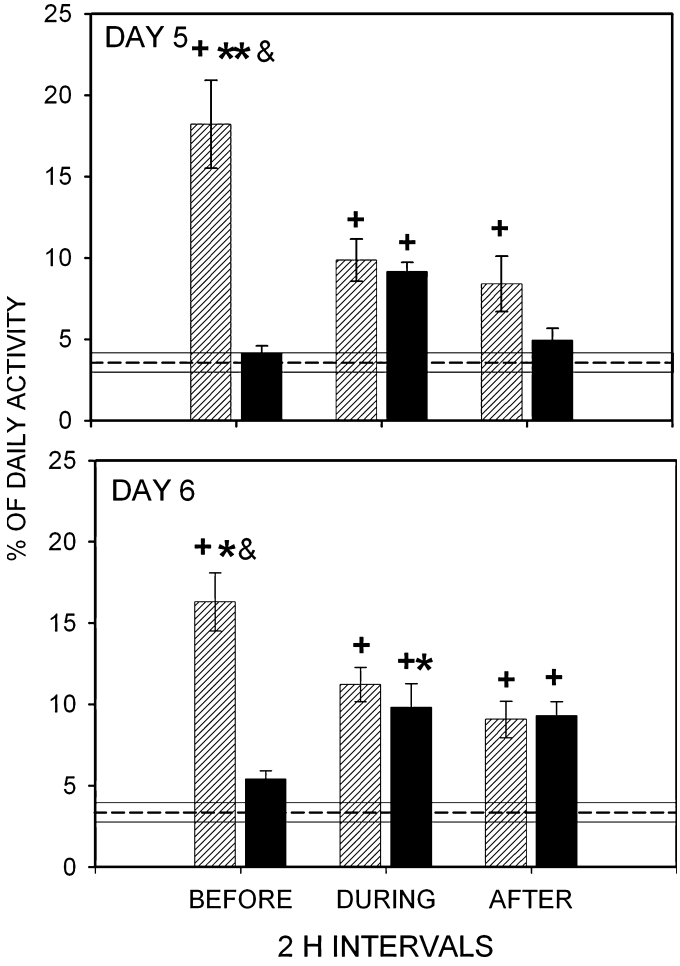

FIG. 4. Percentage of total daily activity for 2-h intervals before, during and after the fifth meal episode for the RF (hatched bars) and the VAR (solid bars) group ($n = 16$ per group). On the following day (day 6) only the RF group received food, in accordance with the 24-h schedule. Other indications as in Fig. 3.

difference among groups ($F_{2,101} = 33.79$; $P < 0.0001$), among time points ($F_{2,101} = 15.30$; $P < 0.0001$) and due to the interaction group × time ($F_{4,101} = 8.90$; $P < 0.0001$).

The analysis for days 5 and 6 under fixed or variable restricted feeding schedules exemplifies the development of anticipatory activity in the RF group with highest values of activity during the 2 h prior to meal time and also high activity counts during food access compared to the baseline activity (Fig. 4). In contrast, VAR rats did not show increased activity before food access; however, they showed significant increased values during meal time on day 5. The following day (day 6) VAR rats did not receive food for 24 h; however, during and after the expected meal time (estimated 24 h after the last meal) they showed increased activity at the time of the previous meal, although no food was delivered (Fig. 4, bottom). The two-way ANOVA indicated significant differences between groups ($F_{2,105} = 41.22$, $P < 0.0001$ for day 5 and $F_{2,105} = 51.62$, $P < 0.0001$ for day 6), a main effect of time ($F_{2,105} = 6.42$, $P < 0.002$ for day 5 and $F_{2,105} = 3.30$, $P < 0.04$ for day 6) and due to the group × time interaction ($F_{4,105} = 6.62$, $P < 0.0001$ for day 5 and $F_{2,105} = 7.41$, $P < 0.0001$ for day 6).

On day 11 of restricted feeding the RF group again exhibited high activity values in anticipation of and during meal time, while the VAR group exhibited increased values only during food access (Fig. 5, top). On day 12 neither group received food at the expected time. The RF group again exhibited increased activity in anticipation and both the RF and VAR groups exhibited activation during and after the expected meal (Fig. 5, bottom). The two-way ANOVA indicated significant difference among groups ($F_{2,81} = 28.28$, $P < 0.0001$ for day 11 and $F_{2,81} = 35.51$, $P < 0.0001$ for day 12), due to time ($F_{2,81} = 11.20$, $P < 0.0001$ for day 11 and $F_{2,81} = 11.16$, $P < 0.001$ for day 12) and due to the group × time interaction ($F_{4,105} = 9.70$, $P < 0.0001$ for day 11 and $F_{2,105} = 3.88$, $P < 0.006$ for day (12).

Experiment III

The brains of rats of the two food-restricted groups (RF and VAR) were obtained 25 h after the last meal scheduled at 12 h (assuming that c-Fos protein expression takes at least 1 h). Rats were expecting food but had not been fed at the time of perfusion. Evaluation of c-Fos-positive cells in the SCN was performed separately for the ventral and dorsal regions. In both regions the VAR group showed similar values as the *ad libitum* control, while the RF group showed

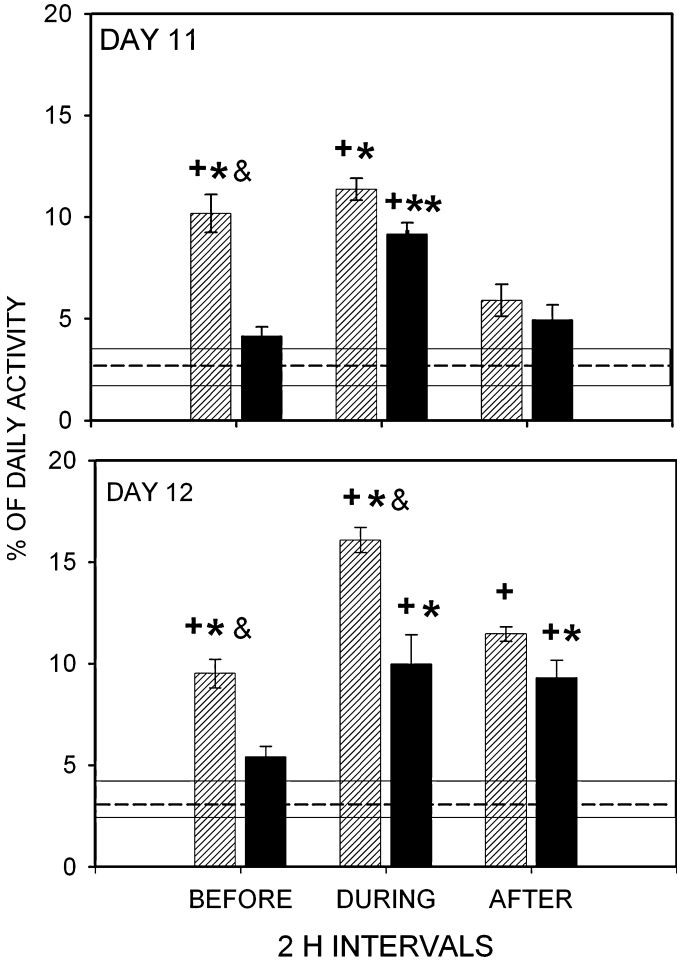

FIG. 5. Percentage of the total daily activity for 2-h intervals before, during and after the 11th meal episode for the RF (hatched bars) and the VAR (solid bars) group ($n = 16$ per group). On the following day (day 12) neither of the groups received food at the expected meal time. Other indications as in Fig. 3.

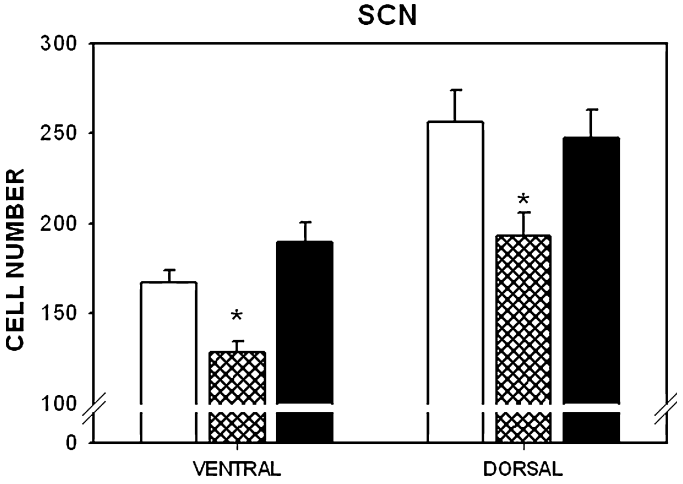

FIG. 6. Number of c-Fos-positive cells in the suprachiasmatic nucleus (SCN). Bilateral cell counts for a medial section in the SCN are represented for control *ad libitum* rats (open), RF rats (cross-hatched) and VAR group (solid). *$P < 0.01$vs. control and VAR groups (Tukey's *post hoc* test).

significantly lower values (Fig. 6) than both groups. The one-way ANOVA indicated significant differences among groups ($F_{2,14} = 6.40$, $P < 0.01$ for the ventral and $F_{2,14} = 8.46$, $P < 0.003$ for the dorsal region.

In the ARC and DMH (Fig. 7) the two food-restricted groups showed similar mean c-Fos-positive cell numbers independently of whether the meal schedules had been predictable or not. In both structures c-Fos-positive cells in RF and VAR groups were significantly higher than in their *ad libitum* controls (ARC: $F_{2,12} = 4.45$, $P < 0.02$; DMH: $F_{2,12} = 4.75$, $P < 0.03$).

Discussion

The present data show that metabolism, behaviour and neuronal activity respond to variable and unpredictable feeding schedules with daily phase adjustments, whereby the phase is set by the last meal. The present data show that animals under a variable meal schedule were unable to exhibit FAA but showed increased activity during the expected time of the previous meal, suggesting that food expectation is reset on a daily basis. Moreover, the behavioural data indicate that food-associated activity was already apparent the day after the first daily scheduled interval.

Metabolic entrainment

Rats exposed to fixed predictable schedules (RF) as well as rats receiving food at variable schedules (VAR12 and VAR18) developed similar strategies to ingest a large amount of food in the 2-h interval of food availability. Both groups exhibited slow rates for gastric emptying and only attained empty stomachs 3 h prior to the next meal. Although the temporal pattern was similar, the capacity for food (and water) ingestion was significantly lower in the VAR than in the RF groups, which can explain their lower weight gain during the 2 weeks of restricted feeding. The enormous stomach distention attained by RF rats, up to seven times the capacity of *ad libitum* animals, followed by a slow emptying rate has an evident adaptive function to endure the long fasting intervals (Escobar *et al.*, 1998; Martinez-Merlos *et al.*, 2004; Baez-Ruiz *et al.*, 2005). Evidently a fixed feeding schedule provides better conditions for predicting a feeding event than the variable schedules and is in agreement with a previous study which also described deficient metabolic adaptations in rats maintained under irregular feeding schedules (Bazotte *et al.*, 1989).

FFAs and leptin, both variables directly related with metabolic responses, also showed temporal patterns elicited by the last meal in both RF and VAR groups, suggesting a daily response to metabolic needs. Restricted feeding schedules induce daily oscillations in digestive and hormonal variables (Krieger, 1974; Saito *et al.*, 1976; Davidson & Stephan, 1999; Díaz-Muñoz *et al.*, 2000). Other daily adaptive changes elicited by RFS are observed in liver functions, e.g. the storage and depletion of glycogen during the feeding and fasting intervals and the increase in the oxidative capacity of the mitochondria (Escobar *et al.*, 1998; Baez-Ruiz *et al.*, 2005). Hereby the daily metabolic oscillations may be adaptive responses essential in preparing animals for cycles of 24 h meals and timed metabolic adjustments may enable animals to cope with the 22-h intervals of fasting and the short period for food intake associated with daily meals (Lima *et al.*, 1981). Data here presented suggest that metabolic oscillations have the capacity to rapidly follow the feeding events, an adaptive strategy that could allow an organism to subsist in a world where food is not always abundant. Under fasting conditions we observed that food-entrained patterns persisted for two cycles only in RF rats, with the phase imposed by the last meal. Thus, although rats may react on a daily

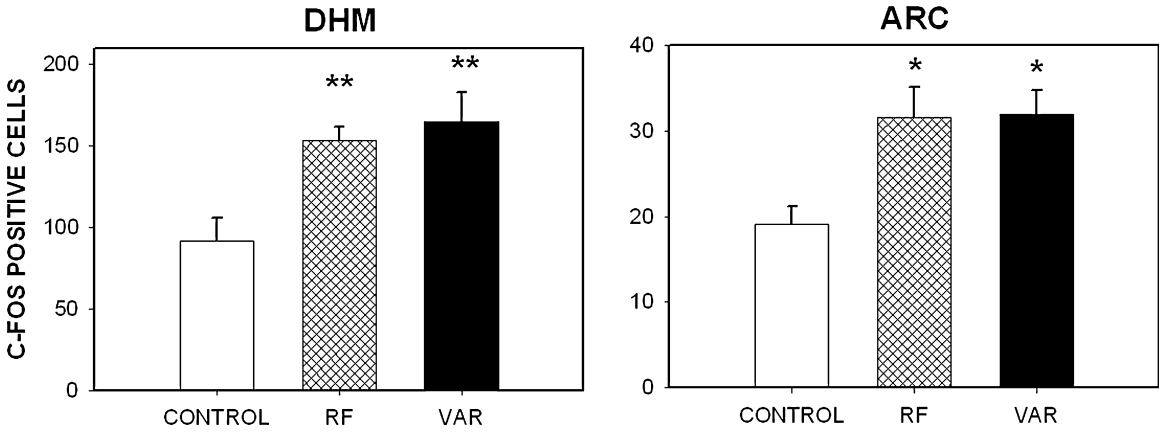

FIG. 7. Number of c-Fos-positive cells in the arcuate nucleus (ARC) and the dorsomedial nucleus of the hypothalamus (DMH). Bilateral cell counts for a representative section of both regions is represented for control *ad libitum* (open), RF (cross-hatched) and VAR (solid) rat groups. $*P < 0.01$, $**P < 0.001$ vs. control.

basis to feeding schedules, a strong entraining effect that can persist for more than two cycles can only be attained after a regular feeding schedule.

In contrast to metabolic responses, the premeal corticosterone peak did not attain complete adjustment under variable schedules as observed for both VAR12 and VAR18 groups. It is well established that the circadian corticosterone rhythm depends strongly on SCN regulation and that under RFS an additional peak anticipating meal time can be elicited (Honma *et al*., 1984); moreover, this premeal corticosterone peak under RF conditions is influenced by the SCN (Kalsbeek *et al*., 1998) Data here presented suggest that variable feeding schedules do not exert the same power to produce the significant food anticipatory peak observed under RFS (Krieger, 1974) and thus possibly cannot override signals elicited by the SCN. However, Honma *et al*. (1983) showed that after a similar fasting interval as used in the present study the onset of a corticosterone peak was observed after the first meal cycle 'as if anticipating the next cycle', indicating a critical role of the fasting state in development and maintenance of this oscillation.

A rapid shifting capacity seems to be tissue-dependent (Davidson *et al*., 2003) and mainly expressed in digestive and feeding-related functions. A recent report indicates that oxygen consumption rate shifts after the first cycle of diurnal feeding, while such a shift takes up to 6 days for clock genes in the liver (Satoh *et al*., 2006).

Metabolic oscillations can constitute time-keeping systems similar to an hour glass and can explain that 24 h daily oscillations can be easily reset. On the other hand, persistence of entrained metabolic patterns for at least two cycles in intact animals (Martinez-Merlos *et al*., 2004) and of clock gene expression in isolated tissue explants for longer intervals (Stokkan *et al*., 2001; Yoo *et al*., 2004) suggests the influence of an oscillatory mechanism that can keep time memory of the phase imposed by the last meal. This mechanism may depend on local oscillations maintained in the tissue by clock genes (Yamazaki *et al*., 2000; Yoo *et al*., 2004) and/or can be driven in intact animals by metabolic signals or by central nervous signals (Buijs & Kalsbeek, 2001; Terazono *et al*., 2003; Cailotto *et al*., 2005; Guo *et al*. 2005). Present data support the existence of oscillatory mechanisms, which, however, are rapidly reset; the fact that clock gene-entrained patterns rapidly adapt to a new cycle (Stokkan *et al*., 2001) supports this proposal.

Food-entrained behavioural patterns

Rats under RF and VAR rats showed already at day 2 an increase in activity at the expected meal time. Rats under RF exhibited consistent anticipatory activity on day 5 and the following days while visual inspection of actograms indicated that most RF animals exhibited FAA as early as day 3. Because in contrast to VAR, RF animals received food at the expected time, behavioural activity during food presentation was always associated with the given food. In the VAR animals, however, already at day 2 a significant increase in activity could be seen at the time when the animals had received food on the previous day. Similarly, the following days' food-related activity in the VAR animals increased even more and maintained significantly higher values than the daily activity observed during baseline.

The use of movement detectors to monitor general activity in this study provided relevant advantages in order to observe food expectancy. Other movement indicators such as wheel running and approaches to a food bin have been shown to be strong markers for FAA (Mistlberger, 1994); however, they do not provide information about other behavioural activity during the feeding interval.

There is no previous study in the literature that has tried to determine the capacity to react to changing feeding schedules. Studies performed by Stephan (1984, 1992) showed that, in rats bearing bilateral SCN lesions, the FAA can shift in 2–3 cycles to a new meal time; this is faster than in intact rats. For these studies FAA was used as a measure of food entrainment; however, present data indicate that, without exhibiting FAA, rats are able to change their food expectancy activity on a daily basis to the expected meal time.

Studies using palatable treats that do not seem to challenge metabolic functions have shown that the regularity of a stimulus that can produce high motivational state and arousal is sufficient to elicit FAA (Mistlberger & Rusak, 1988; Mendoza *et al*., 2005). This evidence combined with data here presented confirms that FAA is expressed when feeding events are regular and predictable. Also, several studies have provided evidence that FAA is elicited when meal intervals are maintained in a circadian range of 19–29 h (Bolles & Moot, 1973; Stephan, 1981). When the interval between meals exceeds or is shorter than this circadian range, animals are unable to exhibit FAA. The feeding program for the VAR group exceeded this range during certain days, because feeding intervals fluctuated between 18 and 36 h. Although this was not the case on a daily

basis, this factor could have hindered the VAR rats in developing FAA.

On the other hand, this study allowed the observation that FAA and activation at the moment of food expectancy can depend on two different systems, as this behavioural expectation was expressed in VAR but not FAA rats: Food expectancy as observed in the VAR rats, defined as the behavioural activation at the expected meal time, may represent the initial phase of FAA behaviour. This food expectation may rely on the interaction of hormonal, metabolic or digestive signals with brain structures involved in energy balance, including the ARC and DMH, in order to promote foraging and food-seeking behaviours after a long fasting interval. Clearly, the present data show that after just one single meal (under the right fasting conditions) rats exhibit an increase in behavioural activity the next day at the previous meal time. This agrees with the early observation of Honma *et al*. (1983) that, after fasting, animals who had received a meal show a peak in corticosterone 24 h later.

The biological clock

It has been thought that the SCN is not involved in food entrainment because FAA can be elicited after SCN lesion. (Stephan *et al*., 1979; Clarke & Coleman, 1986). However, this does not rule out the possibility that under regular conditions the SCN is participating or reacting during FAA.

The present study demonstrates that, when rats were exhibiting FAA, neuronal activity, as observed by c-Fos expression, was decreased in the SCN. This effect was observed exclusively in rats that exhibited FAA in the fixed feeding schedules and not in rats that did not anticipate because of the unpredictable meal time. In this experimental design, rats were perfused 1 h after the expected meal time, which was 24 h after the previous meal, when the c-Fos protein corresponding to meal time expectation was reaching high levels of expression (Morgan & Curran, 1989). It is, however, possible that in the VAR rats the behavioural activation at the moment of feeding had a lower intensity than that observed in RF rats exhibiting FAA. In addition, the differential activation during FAA and food expectation due to variable feeding may underlie different brain processes; whilst FAA may require time-keeping signals and probably a time-learning process, food expectation, as observed in VAR rats, may rely on behavioural activation by metabolic or hunger signals.

Previous data from our group did not show inhibition of the SCN during FAA; this could be due to the fact that horizontal sections were used and only small parts of the SCN could be evaluated (Angeles-Castellanos *et al*., 2004) In the present study coronal sections allowed better analysis of the SCN and enabled differentiation of the ventral and dorsal areas. This observation agrees with an earlier study (Kalsbeek *et al*., 1998) which showed that vasopressin secretion from SCN terminals is modified in an RFS, consistent with a modification of corticosterone secretion. In the present study the inhibition of c-Fos expression observed in the SCN suggests that arousal associated with FAA can influence the activity in the SCN.

The cellular response in the SCN due to nonphotic stimuli that produce arousal is not well understood; some studies report the induction of c-Fos expression (Janik *et al*., 1995; Amir *et al*., 1999) and others the lack of c-Fos expression (Mead *et al*., 1992). It has also been suggested that photic and nonphotic stimuli could have opposing effects on the SCN activity (Mistlberger & Antle, 1998; Maywood *et al*., 1999; Yi *et al*., 2006); however, other studies do not support this opposing effect (Edelstein & Mrosovsky, 2001). Taken together it seems that arousal during FAA can be considered a nonphotic stimulus for the SCN, and it is reported that under certain conditions it can influence the SCN activity (for a review, see Mendoza, 2007). Other studies have tried to determine the influence of RFS on the SCN using the phase of clock gene products as activity markers. All studies have reported consistently that clock genes in the SCN are not shifted with meal time (Damiola *et al*., 2000; Hara *et al*., 2001; Wakamatsu *et al*., 2001; Mieda *et al*., 2006). However, c-Fos (the activity marker in our study) and clock gene oscillations reflect two different cellular processes; c-Fos reflects acute responses to stimuli (Morgan & Curran, 1989) while clock gene products result from 24-h oscillatory mechanisms (Reppert & Weaver, 2001).

Our results are consistent with the idea that, during FAA, neuronal activity in the SCN can be modulated, probably allowing the expression of behavioural and hormonal parameters associated with FAA.

Other hypothalamic structures

The DMH and ARC nuclei are structures involved in functions of metabolic integration (Horvath & Diano, 2004). The two structures showed similar neural activity when rats were expecting food, 24 h after the last meal, and this response was different from the rats fed *ad libitum*. Because the two groups, RF and VAR, exhibited similar metabolic responses 24 h after the last meal, it is possible that the neuronal responses of both hypothalamic structures are related to signals originating in the periphery, induced by the prolonged fasting interval. In the ARC, increased levels of c-Fos expression have been reported in rats during fasting (Johnston *et al*., 2006; Scott *et al*., 2007), and increased c-Fos has been reported in the DMH in rats anticipating a meal after 22 h of fasting (Angeles-Castellanos *et al*., 2004; Gooley *et al*., 2006).

The ARC nucleus receives hormonal signals related to energy balance and plays a relevant role transmitting these metabolic signals to other hypothalamic areas, including the DMH and SCN (Yi *et al*., 2006). The DMH also receives signals from the periphery and can be considered an integrative area linking feeding-related processes with arousal (Saper, 2006; Sakurai, 2007). It is well documented that the DMH exhibits increased c-Fos when rats are displaying FAA and while they are feeding (Angeles-Castellanos *et al*., 2004; Gooley *et al*., 2006). Also, RFS entrain the clock genes per1 and per2 in the DMH, confirming the influence of feeding schedules on the cellular cycles on this structure (Mieda *et al*., 2006). It remains to be established whether in addition to inducing c-Fos expression, variable feeding schedules can also influence clock gene oscillations in hypothalamic structures or whether this requires a regular and predictable schedule.

Conclusions

Altogether, the present data illustrate a strong adaptive capacity of rats to adjust to meal time on a daily basis. This fast daily response was observed on metabolism and on activity of hypothalamic structures and behaviour, and may be a relevant adaptive strategy for coping with a changing environment in which food is not presented daily at a precise time.

Metabolic signals associated with an empty stomach and depletion of energy stores may trigger the hypothalamic activity reported here. The activity of this and other brain areas may underlie the general activation observed in rats 24 h after the last meal. This process represents a rapidly resetting clock on a 24 h basis to alert and activate organisms for the next meal. This system, however, proved to be different from a system promoting FAA, as rats under variable feeding

schedules were not anticipating. Thus, the functional systems involved in producing anticipatory activity require regular and predictable feeding schedules which are different from but may be expressed in parallel with metabolic signals.

In contrast to the LD cycle, which is strongly predictable, food access in nature is not necessarily precise and time of food availability can differ from one day to another. The present experiments indicate that this condition has induced functional systems that respond to changes in food availability and suggest that they can adjust their function on a daily basis.

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Abbreviations

ARC, the arcuate nucleus; DMH, dorsomedial nucleus of the hypothalamus; FAA, food anticipatory activity; FFA, free fatty acids; LD, light–dark; MT, time (h) since last meal; NS, not significant; RF, fixed restricted feeding; RFS, restricted feeding schedule(s); SCN, suprachiasmatic nucleus; ZT, Zeitgeber time.

References

Amir, S., Cain, S., Sullivan, J., Robinson, B. & Stewart, J. (1999) Olfactory stimulation enhances light-induced phase shifts in free-running activity rhythms and Fos expression in the suprachiasmatic nucleus. *Neuroscience*, **92**, 1165–1170.

Angeles-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**, R158–R165.

Angeles-Castellanos, M., Mendoza, J., Díaz-Muñoz, M. & 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.

Angeles-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.

Baez-Ruiz, A., Escobar, C., Aguilar-Roblero, R., Vázquez-Martínez, O. & Díaz-Muñoz, M. (2005) Metabolic adaptations of liver mitochondria during restricted feeding schedules. *Am. J. Physiol. Gastrointest. Liver Physiol.*, **289**, G1015–G1023.

Balsalobre, A., Brown, S.A., Marcacci, L., Tronche, F., Kellendonk, C., Reichardt, H.M., Schutz, G. & Schibler, U. (2000) Resetting of circadian time in peripheral tissues by glucocorticoid signaling. *Science*, **289**, 2344–2347.

Balsalobre, A., Damiola, F. & Schibler, U. (1998) A serum shock induces circadian gene expression in mammalian tissue culture cells. *Cell*, **93**, 929–937.

Bazotte, R.B., Curi, R. & Hell, N.S. (1989) Metabolic changes caused by irregular-feeding schedule as compared with meal-feeding. *Physiol. Behav.*, **46**, 109–113.

Bolles, R.C. & Moot, S.A. (1973) The rat's anticipation of two meals a day. *J. Comp. Physiol. Psychol.*, **83**, 510–514.

Brown, S.A., Zumbrunn, G., Fleury-Olela, F., Preitner, N. & Schibler, U. (2002) Rhythms of mammalian body temperature can sustain peripheral circadian clocks. *Curr. Biol.*, **12**, 1574–1583.

Buijs, R.M. & Kalsbeek, A. (2001) Hypothalamic integration of central and peripheral clocks. *Nat. Rev. Neurosci.*, **2**, 521–526.

Cailotto, C., La Fleur, S.E., Van Heijningen, C., Wortel, J., Kalsbeek, A., Feenstra, M., Pevet, P. & Buijs, R.M. (2005) The suprachiasmatic nucleus controls the daily variation of plasma glucose via the autonomic output to the liver: are the clock genes involved? *Eur. J. Neurosci.*, **22**, 2531–2540.

Cardona, F. (2004) Periodic dip of lipidperoxidation in humans: a redox signal to synchronize peripheral circadian clocks? *Med. Hypotheses*, **63**, 841–846.

Clarke, J.D. & Coleman, G.J. (1986) Persistent meal-associated rhythms in SCN-lesioned rats. *Physiol. Behav.*, **36**, 105–113.

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, A.J., Poole, A.S., Yamazaki, S. & Menaker, M. (2003) Is the food-entrainable circadian oscillator in the digestive system? *Genes Brain Behav.*, **2**, 32–39.

Davidson, A.J. & Stephan, F.K. (1999) Plasma glucagon, glucose, insulin, and motilin in rats anticipating daily meals. *Physiol. Behav.*, **66**, 309–315.

Díaz-Muñoz, M., Vázquez-Martínez, O., Aguilar-Roblero, R. & Escobar, C. (2000) Anticipatory changes in liver metabolism and entrainment of insulin, glucagon, and corticosterone in food-restricted rats. *Am. J. Physiol. Regul. Integr. Comp. Physiol.*, **279**, R2048–R2056.

Edelstein, K. & Mrosovsky, N. (2001) Behavioral responses to light in mice with dorsal lateral geniculate lesions. *Brain Res.*, **918**, 107–112.

Escobar, C., Diaz-Munoz, 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.*, **274**, R1309–R1316.

Gooley, J.J., Schomer, A. & Saper, C.B. (2006) The dorsomedial hypothalamic nucleus is critical for the expression of food-entrainable circadian rhythms. *Nat. Neurosci.*, **9**, 398–407.

Guo, H., Brewer, J.M., Champhekar, A., Harris, R.B. & Bittman, E.L. (2005) Differential control of peripheral circadian rhythms by suprachiasmatic-dependent neural signals. *Proc. Natl. Acad. Sci. USA*, **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.

Hastings, M.H., Reddy, A.B. & Maywood, E.S. (2003) A clockwork web: Circadian timing in brain and periphery, in health and disease. *Nat. Rev. Neurosci.*, **4**, 649–661.

Hirota, T., Okano, T., Kokame, K., Shirotani-Ikejima, H., Miyata, T. & Fukada, Y. (2002) Glucose down-regulates Per1 and Per2 mRNA levels and induces circadian gene expression in cultured Rat-1 fibroblasts. *J. Biol. Chem.*, **277**, 44244–44251.

Honma, K.I., Honma, S. & Hiroshige, T. (1983) Critical role of food amount for prefeeding corticosterone peak in rats. *Am. J. Physiol.*, **245**, R339–R344.

Honma, K.I., Honma, S. & Hiroshige, T. (1984) Feeding-associated corticosterone peak in rats under various feeding cycles. *Am. J. Physiol.*, **246**, R721–R726.

Horvath, T.L. & Diano, S. (2004) Opinion: The floating blueprint of hypothalamic feeding circuits. *Nat. Rev. Neurosci.*, **5**, 662–667.

Inzunza, O., Seron-Ferre, M.J., Bravo, H. & Torrealba, F. (2000) Tuberomammillary nucleus activation anticipates feeding under a restricted schedule in rats. *Neurosci. Lett.*, **293**, 139–142.

Janik, D., Mikkelsen, J.D. & Mrosovsky, N. (1995) Cellular colocalization of Fos and neuropeptide Y in the intergeniculate leaflet after nonphotic phase-shifting events. *Brain Res.*, **698**, 137–145.

Johnstone, L.E., Fong, T.M. & Leng, G. (2006) Neuronal activation in the hypothalamus and brainstem during feeding in rats. *Cell Metab.*, **14**, 313–321.

Kalsbeek, A., Van Heerikhuize, J.J., Wortel, J. & Buijs, R.M. (1998) Restricted daytime feeding modifies suprachiasmatic nucleus vasopressin release in rats. *J. Biol. Rhythms*, **13**, 18–29.

Krieger, D.T. (1974) Food and water restriction shifts corticosterone, temperature, activity and brain amine periodicity. *Endocrinology*, **95**, 1195–1201.

Lamont, E.W., Diaz, L.R., Barry-Shaw, J., Stewart, J. & Amir, S. (2005) Daily restricted feeding rescues a rhythm of period2 expression in the arrhythmic suprachiasmatic nucleus. *Neuroscience*, **132**, 245–248.

Le Minh, N., Damiola, F., Tronche, F., Schutz, G. & Schibler, U. (2001) Glucocorticoid hormones inhibit food-induced phase-shifting of peripheral circadian oscillators. *EMBO J.*, **20**, 7128–7136.

Lima, F.B., Hell, N.S., Timo-Iaria, C., Scivoletto, R., Dolnikoff, M.S. & Pupo, A.A. (1981) Metabolic consequences of food restriction in rats. *Physiol. Behav.*, **27**, 115–123.

Martinez-Merlos, M.T., Angeles-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.

Maywood, E.S., Mrosovsky, N., Field, M.D. & Hastings, M.H. (1999) Rapid down-regulation of mammalian period genes during behavioral resetting of the circadian clock. *Proc. Natl. Acad. Sci. USA*, **96**, 15211–15216.

Mead, S., Ebling, F.J.P., Maywood, E.S., Humby, T., Herbert, J. & Hastings, M.H. (1992) A nonphotic stimulus causes instantaneous phase advances of the light-entrainable circadian oscillator of the syrian hamster but does not induce the expression of c-fos in the suprachiasmatic nuclei. *J. Neurosci.*, **12**, 2516–2522.

Mendoza, J. (2007) Circadian clocks: setting time by food. *J. Neuroendocrinol.*, **19**, 127–137.

Mendoza, J., Angeles-Castellanos, M. & Escobar, C. (2005) A daily palatable meal without food deprivation entrains the suprachiasmatic nucleus of rats. *Eur. J. Neurosci.*, **22**, 2855–2862.

Meynard, M.M., Valdes, J.L., Recabarren, M., Seron-Ferre, 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, S.C., Richardson, J.A., Tanaka, K. & Yanagisawa, M. (2006) The dorsomedial hypothalamic nucleus as a putative food-entrainable circadian pacemaker. *Proc. Natl. Acad. Sci. USA*, **103**, 12150–12155.

Mistlberger, R.E. (1994) Circadian food-anticipatory activity: Formal models and physiological mechanisms. *Neurosci. Biobehav. Rev.*, **18**, 171–195.

Mistlberger, R.E. & Antle, M.C. (1998) Behavioral inhibition of light-induced circadian phase resetting is phase and serotonin dependent. *Brain Res.*, **786**, 31–38.

Mistlberger, R.E. & Rusak, B. (1988) Food-anticipatory circadian rhythms in rats with paraventricular and lateral hypothalamic ablations. *J. Biol. Rhythms*, **3**, 277–291.

Morgan, J.I. & Curran, T. (1989) Stimulus-transcription coupling in neurons: role of cellular immediate-early genes. *Trends Neurosci.*, **12**, 459–462.

Paxinos, G. & Watson, C. (1998) *The Rat Brain in Stereotaxic Coordinates*, 4th edition, San Diego, USA, Academic Press.

Reppert, S.M. & Weaver, D.R. (2001) Molecular analysis of mammalian circadian rhythms. *Annu. Rev. Physiol.*, **63**, 647–676., 647–676.

Rutter, J., Reick, M., Wu, L.C. & McKnight, S.L. (2001b) Regulation of clock and NPAS2 DNA binding by the redox state of NAD cofactors. *Science*, **2001**, 510–514.

Saito, M., Murakami, E., Nishida, T., Fujisawa, Y. & Suda, M. (1976) Circadian rhythms of digestive enzymes in the small intestine of the rat. II. Effects of fasting and refeeding. *J. Biochem. (Tokyo)*, **80**, 563–568.

Sakurai, T. (2007) The neural circuit of orexin (hypocretin): maintaining sleep and wakefulness. *Nat. Rev. Neurosci.*, **8**, 171–181.

Saper, C.B. (2006) Staying awake for dinner: hypothalamic integration of sleep, feeding, and circadian rhythms. *Prog. Brain Res.*, **153**, 243–252., 243–252.

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**, R1276–R1283.

Scott, V., McDade, D.M. & Luckeman, S.M. (2007) Rapid changes in the sensitivity of arcuate nucleus neurons to central ghrelin in relation to feeding status. *Physiol. Behav.*, **1**, 180–185.

Shibata, S. (2004) Neural regulation of the hepatic circadium rhythm. *Anat. Rec. A*, **280**, 901–909.

Stephan, F.K. (1981) Limits of entrainment to periodic feeding in rats with suprachiasmatic lesions. *J. Comp. Physiol.*, **143**, 401–410.

Stephan, F.K. (1984) Phase shifts of circadian rhythms in activity entrained to food access. *Physiol. Behav.*, **32**, 663–671.

Stephan, F.K. (1992) Resetting of a feeding-entrainable circadian clock in the rat. *Physiol. Behav.*, **52**, 985–995.

Stephan, F.K. (2002) The 'other' circadian system: food as a Zeitgeber. *J. Biol. Rhythms*, **17**, 284–292.

Stephan, F.K., Swann, J.M. & Sisk, C.L. (1979) Entrainment of circadian rhythms by feeding schedules in rats with suprachiasmatic lesions. *Behav. Neural Biol.*, **25**, 545–554.

Stokkan, K.A., Yamazaki, S., Tei, H., Sakaki, Y. & Menaker, M. (2001) Entrainment of the circadian clock in the liver by feeding. *Science*, **291**, 490–493.

Terazono, H., Mutoh, T., Yamaguchi, S., Kobayashi, M., Akiyama, M., Udo, R., Ohdo, S., Okamura, H. & Shibata, S. (2003b) Adrenergic regulation of clock gene expression in mouse liver. *Proc. Natl. Acad. Sci. USA* **100**, 6795–6800.

Waddington-Lamont, E., Harbour, V.L., Barry-Shaw, J., Renteria, D.L., Robinson, B., Stewart, J. & Amir, S. (2006) Restricted access to food, but not sucrose, saccharine, or salt, synchronizes the expression of Period2 protein in the limbic forebrain. *Neuroscience.*, **144**, 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 and mPer2 mRNA in the cerebral cortex and hippocampus but not in the suprachiasmatic nucleus of mice. *Eur. J. Neurosci.*, **13**, 1190–1196.

Yamazaki, S., Numano, R., Abe, M., Hida, A., Takahashi, R., Ueda, M., Block, G.D., Sakaki, Y., Menaker, M. & Tei, H. (2000) Resetting central and peripheral circadian oscillators in transgenic rats. *Science*, **288**, 682–685.

Yi, C.X., Van, D.V., Dai, J., Yin, G., Ru, L. & Buijs, R.M. (2006) Ventromedial arcuate nucleus communicates peripheral metabolic information to the suprachiasmatic nucleus. *Endocrinology.*, **147**, 283–294.

Yoo, S.H., Yamazaki, S., Lowrey, P.L., Shimomura, K., Ko, C.H., Buhr, E.D., Siepka, S.M., Hong, H.K., Oh, W.J., Yoo, O.J., Menaker, M. & Takahashi, J.S. (2004) PERIOD2: LUCIFERASE real-time reporting of circadian dynamics reveals persistent circadian oscillations in mouse peripheral tissues. *Proc. Natl. Acad. Sci. USA*, **102**., 608–613.