CIRCADIAN CLOCKS FOR ALL MEALTIMES: ANTICIPATION OF MULTIPLE DAILY MEALS IN RATS

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ABSTRACT

Behavioral studies show that rodents can anticipate a daily mealtime by entrainment of a circadian oscillator separate from the circadian clock responsible for light-dark entrained rhythms. Less is known about how rats anticipate more than one daily meal. The objective of the experiments reported here is to gain insight into the formal mechanisms by which multiple meal anticipation is achieved. Rats were maintained on two daily meals, 7 h or 10 h apart in the light period, and anticipation of each meal was measured during meal omission, meal shift and constant dark tests. The results rule out interval timing as a means for predicting mealtimes, and provide some support for the proposal that separate food-entrainable oscillators may control each bout of anticipatory activity. Characterizing the strategies employed by rats under scheduled access to multiple daily meals may yield important insights into the mechanisms underlying anticipatory behaviour.

Keywords: food anticipation; multiple meal schedules; circadian rhythms suprachiasmatic nucleus; immunocytochemistry; FOS.

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GLOSSARY

c-fos	An immediate early gene. Expression of c-fos correlates with depolarizing neurons and is therefore a neural correlate indicative of neuronal activation
FOS	Protein product of c-fos
Food anticipatory activity (FAA)	The food seeking behaviour and physiology demonstrated by animals under daily schedules of restricted food availability
Entrainment	The process of entraining the period and phase of an endogenous rhythm with an environmental cycle.
Immuncytochemistry	A histological technique that applies antibodies to visualize cellular components
LD	Light/Dark, refers to the scheduled daily light dark cycle employed during an experiment
LL	Constant light
DD	Constant dark
Period	The duration of one cycle in a rhythm
Phase	Any point in a cycle
SCN	Suprachiasmatic nucleus, a in the hypothalamus that exerts control over circadian rhythms of mammalian behaviour and physiology
Subjective Day	Phase during which an animal behaves as if it is day in constant conditions.
Subjective Night	Phase during which an animal behaves as if it is night in constant conditions

Zeitgeber	An environmental time cue
ZT (zeitgeber time)	A time scale based on the period of an external cycle

GENERAL INTRODUCTION

Animals are thought to employ a variety of timing mechanisms to predict the recurrence of environmental stimuli crucial for survival. Much animal timing work has focused on rats and their ability to anticipate food availability. At short intervals between food rewards, in the seconds to minutes range, food anticipation in rats exhibits properties of so-called interval timing (Buhusi and Meck, 2005). Interval timers are conceptualized as mechanisms that enable animals to measure the amount of time between two events, such as between a light or auditory stimulus and a food reward, or between successive food rewards. Interval timers behave like stopwatches, and must be reset by the target event (e.g., a food reward) in order to time the next interval. If the event occurs early or late, the interval timer is instantly reset. Therefore, two formal properties that define interval timing are instantaneous reset and failure to time more than one interval without resetting. A third empirically derived property is proportionality between the duration of the interval being timed and the duration of the bout of anticipation; the longer the interval, the earlier the onset of anticipatory activity, and the longer it persists if the food reward is omitted. This so-called scalar property of interval timing is thought to reflect stochastic error in the operation of the timer (the longer the interval, the more error can accumulate).

At circadian (~24 h) intermeal intervals, food anticipation in rats exhibits properties of a rhythm driven by an entrained circadian oscillator (Boulos and Terman, 1980; Mistlberger, 1994; Stephan, 2002). These properties include persistence for several cycles if daily meals are omitted, gradual resetting if mealtimes are shifted, and failure to anticipate meals that fall outside of a circadian range of intervals (~22-31 h). Although a bona fide circadian pacemaker has been localized to the hypothalamic suprachiasmatic nucleus (SCN), this pacemaker is entrained predominantly by LD cycles, and is not necessary for food anticipatory circadian rhythms (Stephan, Swann and Sisk, 1979; Boulos and Terman, 1980).

The ability of rats to anticipate food at intermediate intervals, such as several hours or more, is not as well characterized, and evidence for both interval timing and oscillator entrainment has been reported (Crystal, 2009). Intermediate intervals are created when rats are provided with two or three daily meals, separated by 6-12 h. Rats exhibit robust anticipation to each of two daily meals 6 or more hours apart, in either the day or night (Bolles and Moot, 1973; Boulos and Logothetis, 1990; Mistlberger, de Groot, Bossert and Marchant, 1996; White and Timberlake, 1994; Aragona, Curtis, Davidson, Wang, and Stephan, 2000; Davidson, Poole, Yamazaki, and Menaker 2003). Rats may fail to anticipate more than 2 of 3 daily meals, when running wheels are used to measure anticipation (Stephan, 1989). Whether this reflects a limitation of the timekeeping mechanism or constraints imposed by the energy costs of wheel running is uncertain, as data on other activity measures are not available.

Some multiple meal studies have added spatial or response contingencies, such that food at one time of day is found in one location (e.g., the left arm of a T-maze) or requires one response (e.g., turning left in a T-maze), while food at another time of day is found in another location (e.g., the right arm of a T-maze), or requires a different response (e.g., turning right in a T-maze). A simple solution to time-place or time-response tasks is to alternate between places or responses (if correct on meal one, then switch on meal 2; e.g., Thorpe and Wilke, 2007). Meal omission tests have ruled out this strategy in at least some studies (Mistlberger et al, 1996). This indicates that rats can both anticipate feeding time and discriminate between two circadian phases to optimize foraging decisions. Although evidence is limited, discrimination, like anticipation, appears to be independent of the SCN pacemaker (Mistlberger et al, 1996).

Conceptually, there are at least three possible mechanisms by which rats could anticipate two daily meals. One possibility is that food-entrainable oscillators can be dissociated into two cohorts, each entrained to a different meal and driving activity in anticipation of that meal. In this model, each bout of food anticipation would represent the active phase of a distinct circadian oscillator. A similar 2-oscillator structure has been proposed for the SCN pacemaker, to explain bimodal or 'split' activity rhythms (Pittendrigh and Daan, 1976), and antiphase oscillations observed within each SCN or between left and right SCN in some experimental preparations (de la Iglesia, Meyer, Carpino, and Schwartz, 2000). A challenge for multiple oscillator models is to explain how each oscillator can couple to one mealtime, and avoid interference from signals related to the

other mealtime, or the other food-entrained oscillators. This challenge is magnified if rats can anticipate more than two meals, although the ability of rats to do this, using circadian timekeeping, has not been established yet.

A second possible mechanism by which rats could anticipate more than one meal per day is based on the evidence that rats can discriminate circadian phase. Phase discrimination suggests that brain systems guiding foraging behaviour receive a continuously updated representation of circadian phase that can be recognized and remembered. The use of a circadian clock in this way is postulated to explain memory for multiple mealtimes in honeybees, and time-compensated sun compass orientation in animals that navigate or migrate over significant distances using the sun as a landmark. To explain multiple meal anticipation in rats, only a single continuously-consulted clock would be necessary. In principle, the LD-entrained SCN pacemaker could be exploited for this purpose, but the available evidence indicates that SCN-ablation does not impair anticipation or discrimination of two daily feeding times in rats (Mistlberger et al, 1996). Therefore, there must be at least one other circadian oscillator that can be consulted. This clock is likely to be food-entrained, so that its phase represents local environmental time and thus can be used to predict mealtimes. If the clock also drives a rest-activity cycle (analogous to the SCN), then in a rat anticipating 2 daily meals, it is possible that one bout of anticipation represents the active phase of a rest-activity cycle, while the other bout of anticipation represents a learned association between the second mealtime and another phase of the same entrained clock. Continuous consultation of a food-entrainable

clock as a mechanism for food anticipation would be both parsimonious (requiring only one entrained clock) and powerful (conceivably many mealtimes could be discriminated and remembered).

A third possible mechanism for anticipating multiple daily meals is to combine an entrained circadian oscillator with an interval timer. A food-entrainable circadian oscillator could drive anticipation of one meal, and an interval timer triggered each day by delivery of the first meal or a LD transition could drive anticipation of the second meal. It is also conceivable that oscillator and interval timing participate in timing both daily meals, with separate entrained oscillators promoting arousal prior to each meal, and interval timers (counting hours between meals or between LD transitions and meals) adding precision or modulating the onset or rate of change of activity as mealtime approaches. One study has shown that a light cue prior to a daily scheduled meal can delay the onset of anticipatory activity (Terman, Gibbon, Fairhurst, and Waring, 1984). Studies of single meal anticipation that included one or more days of constant dark have not reported obvious effects on meal timing, but these studies have generally not included precise measures of the onset or rate of change of anticipatory activity.

A modest number of studies of two meal timing in rats have been published since the first report by Bolles and Moot (1973). Several of these studies have shown that anticipation of two daily meals can persist during meal omission tests (i.e., 1 or more days of total food deprivation; Bolles and Moot, 1973; Boulos and Logothetis, 1990; White and Timberlake, 1994; Aragona et al.,

2002), and does not require LD cues (Edmonds and Alder, 1977; White and Timberlake, 1994; Aragona, 2002) or an intact SCN (Stephan et al., 1979). A limitation of these studies is that analyses were qualitative, and provided evidence only for the presence or absence of two bouts of anticipatory activity during meal omission tests. Anticipation onset times were not quantified, and changes in the timing of one or both bouts were not assessed. Other experiments, in which SCN-ablated rats received two daily meals recurring at different intervals in the circadian range (e.g., one at 24 h intervals and the other at 25 h intervals), yielded some evidence that rats can anticipate both for at least a few cycles, although this was unstable, and during meal omission tests, only a single bout of FAA was evident (possibly because the two mealtimes were too closely spaced during the food deprivation tests; Stephan 1983, 1989a, 1989). Anticipation of two meals recurring with different periodicities (so-called ‘T-cycle’ studies) suggests independent entrainment of two separable oscillators (Stephan, 1983). However, it is possible that a single food-entrainable, continuously consulted clock could produce these anticipation patterns, if the association between phase and mealtime can be adjusted (updated) each day to accommodate the systematically changing phase difference between the two meals. Failure to demonstrate two distinct bouts of anticipation during meal omission tests in 2-meal T-cycle studies also leaves open the possibility that the rats used a combination of entrainment and interval timing, with delivery of the first meal providing the resetting signal for an interval timer used to predict delivery of the second daily meal. Again, for this to accommodate a

systematically advancing or delaying second meal, the target interval would have to be modified each day.

The available evidence provides some support for a two oscillator model, without ruling out a single continuously consulted clock model, or some contribution from interval timing. Ultimately, the validity of a two oscillator model will require confirmation at the cellular level, by the observation of independently phased circadian oscillations in one or more neuronal groups. This task is complicated by continued uncertainty about the location of circadian oscillators critical for food anticipatory rhythms, and about the role of known circadian clock genes in the generation of these rhythms (Davidson, 2009; Pendergast, Nakamura, Friday, Hatanaka, Takumi, and Yamazaki, 2009; Storch and Weitz, 2009). Prior to initiating a search for neural correlates of two meal timing, using clock gene and immediate early gene expression patterns in the brains of rats anticipating two (or more) daily meals, we conducted additional behavioral experiments to further test predictions of entrained oscillator and hybrid models of two meal timing, and to identify two meal schedules that yield strong anticipatory activity rhythms to both meals.

GENERAL METHODS

Animals and Apparatus

In each experiment, adult male sprague dawley rats (400-500 g; Charles River, PQ) were housed individually in clear plastic cages (40.6 cm x 50.8 cm x 21 cm) equipped with a 35.5 cm running wheel and a metal cage top holding a water bottle and food hopper (Lafayette, IN). Each cage was housed in an isolation box (Lafayette, IN) with controlled lighting (LD 14:10, white LEDs, ~30 Lux within the cages) and an exhaust fan. After the first experiment, the cages were moved to larger open cabinets, 2 cages per shelf. Also, a food bin was attached to the outside of each cage, accessed via a 4 cm square window. Temperature in the vivarium was maintained at ~22c. Wheel revolutions were detected by magnetic switches monitored using a commercial interface and data acquisition system (Clocklab, Actimetrics, IL).

Immunocytochemistry

To quantify the number of FOS immunoreactive cells in selected brain regions, rats were euthanized using 1.5cc of sodium pentobarbital I.P. and perfused transcardially with 60cc of 0.01M phosphate buffered saline (PBS, pH7.3) followed by 60cc of 4% paraformaldhyde in 0.01M PBS (PFA, pH7.3). Brains were then harvested and post-fixed overnight in PFA and subsequently

cryoprotected using 20% sucrose in PBS for 48 hrs. The tissue was sectioned at 40 mm on a cryostat into PBS and was stored at 4°C in 0.01% sodium azide in PBS until immunocytochemical staining.

To visualize cells containing FOS protein tissue was treated as follows. All tissue was washed in PBSX (3x5 min rinses, 0.03% Triton X-100, Sigma Aldrich PBS) between incubations, which were done at room temperature unless otherwise specified. Tissue was first washed in PBSX and then transferred to 0.3% H_20_2 for 30 min to reduce endogenous peroxidase. It was then blocked in 10% normal donkey serum (NDS, Jackson ImmunoReseach Laboratories Inc., West Grove, PA, USA; cat. no. 017 000 121) for 2 h. The tissue was incubated in rabbit anti-fos IgG (1:2000, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA; cat. no. CS-52) overnight, followed by a biotinylated donkey anti rabbit antibody (1:250, Jackson ImmunoResearch Laboratories Inc.; cat. no. 711 065 152) with 0.03% NDS for 2 h. The tissue was incubated with an avidin biotin horseradish peroxidase kit (1:200, ABC; standard vectastain elite kit, Vector Laboratories. Burlington, ON, Canada; cat. no. PK-1000) for 1 h. The stain was developed by pre-incubating in diaminobenzidine (DAB) for 5 min and adding H_20_2 for 95 seconds (DAB; 0.04% in 0.5 Tris Buffer with 0.3% H_20_2 and 0.03% $NiCl_2$).

Cell counting

An anterior, medial, and posterior section were selected from the SCN (~0.48, 0.72, and 0.96mm anterior to bregma, Paxinos and Watson, 2007). These sections were digitized using a standard light microscope at 10X (Nikon

Eclipse 80i) attached to a digital camera (Retiga 200R CCD; Q imaging, Surrey, BC, Canada). Pictures encompassing the N.Acc and the DMH were merged using Photoshop CS4 (Adobe Systems Inc., San Jose, CA, USA) to produce high-resolution images. Images were uploaded in Image-J (NIH) and overlays of the respective structures were superimposed on the image to delineate which cells should be quantified. The Image-J automatic counting system was utilized with the threshold set to match counts achieved manually by an experienced cell counter.

Data analysis

For visual inspection, activity data were plotted in the standard 'actogram' format and as 24 h waveforms averaging one or more days across animals within groups. For group mean waveforms, activity data for each rat were first normalized relative to their daily means. Food anticipatory onset was defined as the first 10 min bin within 4h of mealtime in which activity exceeded 30 or 40 counts after an interval of 120 min during which this threshold was not exceeded. Differences in the timing of food anticipatory activity across days were evaluated statistically by repeated measures analysis of variance (ANOVA), and post hoc t-tests with Bonferroni corrections. In keeping with chronobiological conventions for nocturnal animals, time of day relative to the LD cycle is reported as 'Zeitgeber Time' (ZT), with lights-off designated as ZT12. In a 14:10 LD cycle, lights on is therefore designated ZT22. Group means are reported in the text and graphed with standard errors.

Procedures

Overview of Strategies:

1. In these experiments, we used intact rats entrained to a 24 h LD cycle, rather than SCN-ablated rats in constant dark or light, to enable the rats to use whatever strategies and mechanisms they might normally invoke when faced with timing multiple meals at fixed intervals.
2. We used two meals in the light period, rather than in the dark period, because rats anticipate one or two daily meals at any time of day, and anticipation to daytime meals can be detected even at low levels, by comparison with minimal daytime activity characteristic of nocturnal animals, when food is available ad-lib.
3. We used running wheels to measure anticipatory behavior, because wheel running (unlike general locomotor activity) is virtually absent during the day when food is available ad-lib, and because the onset of food anticipatory wheel running is typically more precise than general locomotor activity measures.
4. We used a long-day photoperiod, with 14 h of light. This enabled us to examine wide intermeal intervals, with both meals in the light period. Also, a recent study reported higher amplitude food anticipatory rhythms in mice maintained long-day photoperiods (up to 18h; Pendergast et al., 2009). Sprague Dawley rats are the most commonly used rat strain in restricted feeding studies, and do not exhibit metabolic or reproductive responses to changes in photoperiod.

Strategies and criteria for distinguishing among interval timer, multiple oscillator and continuously consulted clock mechanisms:

1. Constant dark (or light) test: If rats use interval timing to learn that one meal occurs at some fixed interval after a LD transition, then keeping the lights off for one day should eliminate anticipation of one or both meals that day. If rats use interval timing to modulate ('fine-tune') the onset or amount of food anticipation driven by a food-entrained oscillator, then omission of the LD transitions may affect the timing of anticipation (mean onset time, variability of onset time or rate of change) without eliminating anticipation.
2. Single meal omission test: If rats use interval timing to learn that one meal occurs at some fixed interval after another meal, then omitting one meal should eliminate, or substantially modulate the timing of anticipation to the second meal.
3. Meal shift test: If rats use a single food-entrainable circadian oscillator as a continuously consulted clock to anticipate two daily mealtimes, then shifting the clock should shift both bouts of anticipation in parallel. It should in principle be possible to shift the clock by altering one mealtime, and then examine the timing of anticipation to the second mealtime.
4. Adlib feeding with light-pulse test: When stably entrained to a 24 h LD cycle, the SCN pacemaker generating the day-night rest-activity rhythm is typically not shifted by a single mid-day feeding time in rats, based on measures of clock-gene rhythms in the SCN (Damiola, Le Minh, Preitner, Konmann, Fleury-Olela, and Schilber, 2000; Stokkan, Yamazali, Tei, Sakaki, and

Menaker, 2001). It is not known if SCN phase might be altered by feeding schedules that provide two daytime meals. If the SCN were phase advanced or phase delayed sufficiently, then anticipation of a meal late or early in the day could conceivably reflect activity driven by the SCN. It is therefore necessary to assess SCN phase immediately following a 2 meal feeding schedule. This can be done by returning the animals to ad-lib food access, leaving the lights off, and measuring the time of 'nocturnal' activity onset (the rat's subjective night) on the first day of constant dark. If the SCN clock has been shifted by daytime feeding, then activity onsets should begin significantly earlier or later, relative to onsets following weeks of adlib food access, or in a control group not previously subjected to restricted feeding. If activity is shifted in the food restriction group, an additional test of SCN shifting is to expose the rats to a light pulse (e.g., 30 min long) on the first day of constant dark, at the time of day that food anticipation was evident. SCN neurons express the immediate early gene c-fos when exposed to light during their subjective night (which normally corresponds to environmental night), but not during their subjective day. If the SCN has been phase advanced or delayed sufficient to account for daytime anticipatory activity, then it should exhibit sensitivity to light.

EXPERIMENT 1: TWO DAYTIME MEALS 10 HOURS APART: MEAL OMISSIONS IN LD AND DD

Introduction

It has been posited that food anticipatory activity observed to daily meal schedules may be based on an interval timing mechanism. Evidence suggests food anticipatory activity is characteristic of behaviour under the control of a circadian oscillator or network. Whether food anticipatory activity is the output of one or multiple brain regions remains to be elucidated. To examine properties of food anticipatory activity to multiple meals a series of control conditions were employed. These include, single and multiple meal omission tests, removal of light-dark transitions (utilizing constant darkness), and shifting of one mealtime. Results indicate that the food anticipatory activity observed was not dependent on an interval timing strategy. Further, behaviour observed in response to a shift in mealtime favours a multiple oscillator model of food anticipatory activity.

Procedures

The rats (N=15) were acclimated to the LD cycle for 6 weeks and to the running wheels for the last 3 of these weeks. The rats were then food deprived for 37 h, beginning at lights-off. Food (12 g of 5001 rodent chow pellets) was provided twice daily, 3h after lights-on (ZT1, designated meal 1or the morning meal) and 1 h before lights-off (ZT11, designated meal 2 or the afternoon meal). Any remaining pellets were removed after 1-h. On day 13 of restricted feeding

(RF13) meal 1 was omitted. On day RF16, both meals were omitted and the lights-remained off. On day RF20, meal 1 was provided 3-h early for that day only. On day RF23, both meals were omitted again, in LD. The sequence of conditions are illustrated in Figure 1.

Results

Group mean onsets for each bout of food anticipatory activity on each day of the experiment are illustrated in Figure 1. Activity prior to mealtime changed significantly across days, for both meal 1 ($F_{(27,378)}$ =8.68, p<.0001) and meal 2 ($F_{(26, 364)}$ =10.1, p<.0001). Prior to food restriction, wheel running activity was predominantly nocturnal (Fig. 2), with a gradual rise beginning at ~ ZT10, and a peak at dark onset (Fig. 3a). When food was removed for 37 h, the timing and level of running in the day and night were not significantly altered (Fig. 3a). When food was provided for 1h twice daily, at ZT1 and ZT11, running was evident in all rats prior to both meals by day RF3. Group mean activity waveforms generated by averaging across days RF8-12, prior to the first meal omission test, reveal two prominent bouts of food anticipatory running (Fig. 3b), with near identical peaks, positive slopes, and significantly different durations (68 $\pm$ 8 min Vs 101 $\pm$ 9 min, for meals 1 and 2, respectively; $t_{(14)}$=6.51, p<.0001; Fig.4).

When meal 1 was omitted on day RF13, all 15 rats exhibited anticipation of the second meal. The onset of anticipatory activity was on average 26 $\pm$ 8 min late relative to the previous 5 day average (paired $t_{(14)}$=3.13, p<.05), but this was

apparently only in a subgroup of rats (n=9, Fig. 3c), while the remaining rats (n=6, Fig. 3d) exhibited differences of $< \pm$ 10 min in, and no aggregate delay. This indicates little or no role for meal 1 in timing meal 2 in these rats. Also evident in the waveforms is a precipitous drop of activity in many of the rats at the end of the usual mealtime when meal 1 was omitted (e.g., Fig. 3d). This suggests, that rats may time both the beginning and the ending of mealtimes.

On day RF16, after two days of receiving both meals, the lights remained off and both meals were omitted. Meal 1 anticipation began slightly early (27± 21 min) and meal 2 anticipation slightly late (13 $\pm$ 8 min) relative to the previous day, but neither difference was statistically significant (Fig. 3e). This indicates little or no role for lights-on as a timing signal for either meal. Again, the morning bout of running dropped precipitously at the time normally associated with the end of the morning meal.

On the day following the double meal omission test the LD and feeding schedules were reinstated. Despite > 1.5 days (37 h) without food, neither the duration nor the peak level of anticipation to meal 1 was altered by contrast with the previous day (before meal omissions had occurred; Fig. 1, 3f). Unexpectedly, the onset of meal 2 anticipation on this day (RF) was advanced in 11 of 15 cases (day; group mean change = 64 $\pm$ 15 min, $t_{(14)}$=4.32, p<.003). Over the next 2 days, meal 2 anticipation shifted back toward its previous position (Fig. 1, days RF18-19). An earlier onset of meal 2 anticipation is not predicted by either interval timing or clock consultation models. It could be explained if meal 1 on the first day of LD, after a day without food, had acutely phase advanced an

oscillator driving anticipation of meal 2. The susceptibility of food entrainable oscillators to phase resetting by an early meal may be increased following a day without food.

On day RF20, the morning meal was delivered 3 h early, at light onset. Despite the early feeding, most of the rats exhibited some activity at the clocktimes normally associated with meal 1 onset and end (Fig. 3g). The timing of anticipation to meal 2 on this day was not significantly altered, relative to the preceding day RF19 (mean change = 17 $\pm$ 14 min, $t_{(14)}$= 1.20, p= 0.25; Fig. 3e). On the next day (RF21, Fig. 1), the original morning mealtime was reinstated. The morning bout of anticipation on that day was significantly advanced (107 $\pm$ 15 min, $t_{(14)}$= 7.16, p<.0001), whereas there was again no change in the timing of anticipation to the afternoon meal (mean change = - 3 $\pm$ 11 min, Fig. 3f). Over the next 2 days (Fig. 1, RF22-23), the onset of meal 1 anticipation shifted back towards its original position, while meal 2 anticipation maintained a stable phase. These results demonstrate that meal 1 anticipation can be phase advanced by a single early meal, and that anticipation of meal 2 is not timed relative to the onset of meal 1 anticipation.

To minimize disturbances, the rats were weighed only twice during the study. On the last day of ad-lib food access, the rats weighed on average 539 $\pm$ 9 gms. After 9 days of restricted feeding, body weights were reduced on average by 4 $\pm$ 1 %. Body weights taken on day 9 of restricted feeding were negatively correlated with the duration of food anticipation on that day (Fig. 5). The

correlation was significant for meal 1 ($r = -.58$, $p=.02$) but not meal 2 ($r = -.30$, $p>.05$).

Discussion

The results of Experiment 1 rule out a conventional interval timing model as a basis for the food anticipatory activity observed. Interval timing inferred from food-anticipatory behaviour in rats on short-interval feeding schedules is dependent on an external trigger, such as a light, a tone, or the previous food reward. Anticipatory activity observed in Experiment 1 persisted when one or both meals were omitted, in either LD or DD, leaving no other external cues available. There was no evidence that the timing of anticipation of either meal was affected by lighting. Omission of meal 1 was associated with a small delay in the onset of activity prior to meal 2 in some rats, but not in others. This indicates that anticipation of two daily scheduled meals is principally under the control of a circadian clock, and does not involve interval timers that might enable rats to learn that meal 1 occurs 3h after lights-on, or that meal 2 occurs 10 h after delivery of meal 1.

Other results of Experiment 1 tend to favour a 2-oscillator model of 2-meal anticipation over a single continuously consulted clock model. On two occasions, anticipation of one meal was acutely shifted without a change in the timing of the next bout of anticipation. A rapid shift of meal timing after a single event (e.g., one early meal) is predictable if meal timing is based on an oscillator that is

entrained (and thus can be acutely shifted) by a scheduled meal, analogous to resetting of the SCN pacemaker by a single light pulse. If anticipation of both meals was based on the same entrained oscillator (functioning as a continuously consulted clock), then both bouts of anticipation should shift in lock step on the first cycle after one bout has shifted.

Figures

Figure 1. The group mean (± sem) time at which wheel running began, expressed in minutes prior to lights-off during ad-lib food access (B1-B4) and prior to mealtime 1 (red dotted line) and mealtime 2 (solid line) during the food restriction schedule (RF1-22) in Experiment 1.

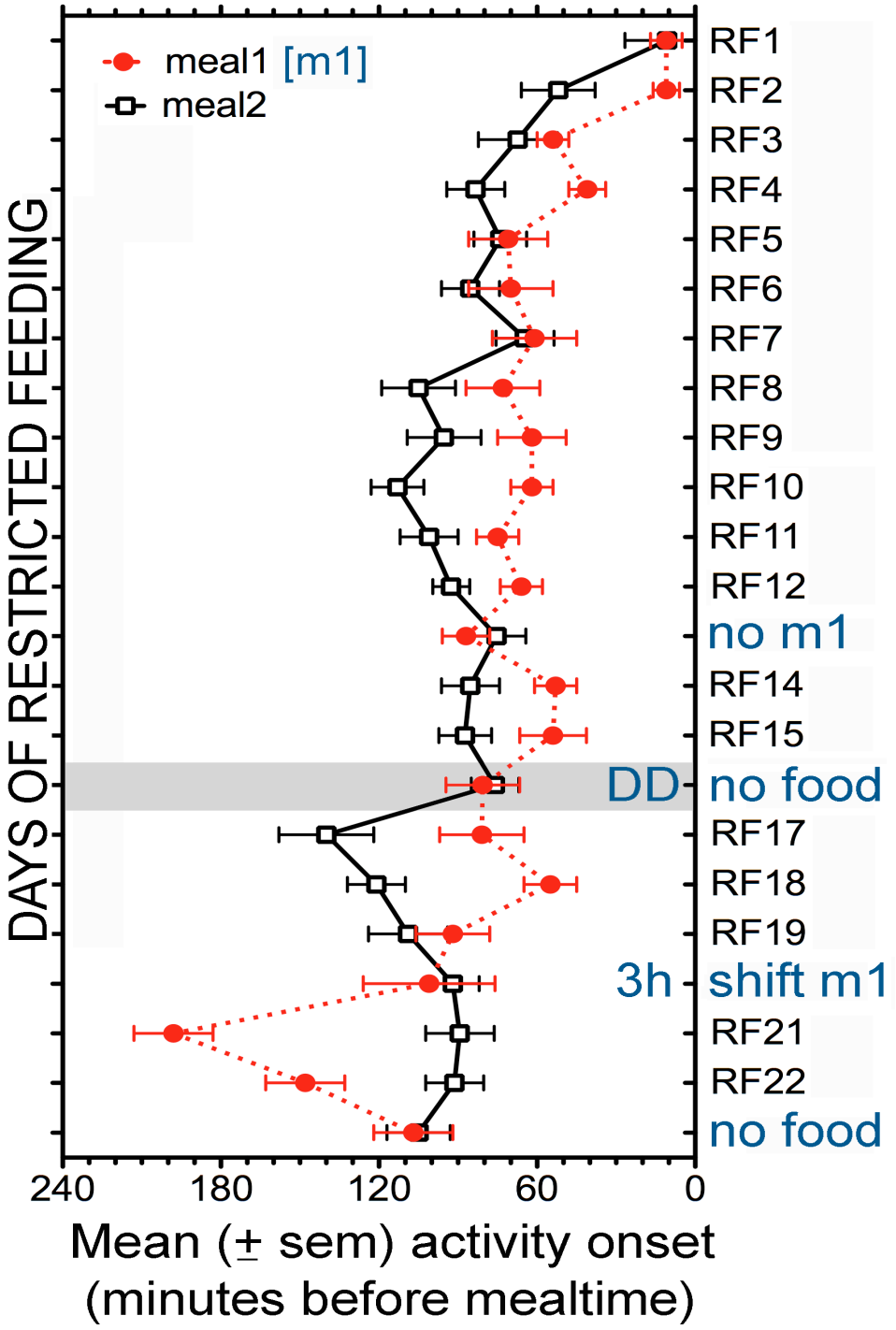


Figure 2. Representative actograms from Experiment1. Each line represents 24 h, with time of day plotted left to right in 10 min bins. Time bins during which wheel counts were registered are denoted by heavy bars. Meals are indicated by opaque bars. Experimental conditions: 1 = 37 h food deprivation, 2 = restricted feeding days, 3 = meal 1 omitted, 4 = both meals omitted in constant dark, 5 = meal 1 provided 3 h early.

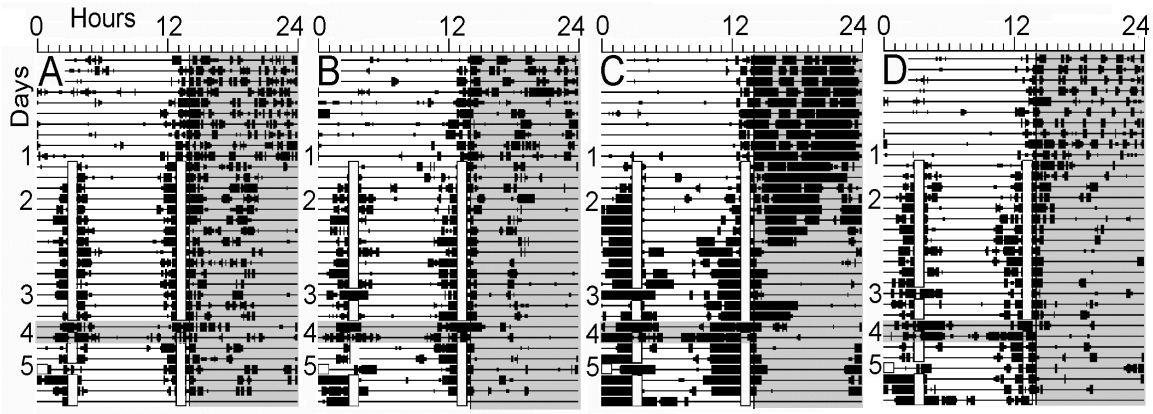


Figure 3. Group mean (± sem) waveforms of wheel running activity in Experiment 1, in 10 min bins starting with lights-on (hour 0, yellow bar). Meal times are denoted by green vertical bars (hollow when meals were skipped). A. Last 4 days of ad-lib food access (dotted line, shaded) and 37-h food deprivation (red solid line) prior to initiation of a 2-meal schedule. B. Adlib (shaded) and days 8-12 of restricted feeding (blue, solid line). C. Restricted feeding (RF) day 12 (blue line) and morning meal omission day (red line), n=9 rats showing delayed onset of anticipation to meal2. D RF day 12 (blue line) and morning meal omission day (red line), n=6 rats showing no change in FAA onset. E. RF day 15 (blue line) and total food deprivation day with lights off (DD, red line). F. Total food deprivation day in DD (red line) and first day of RF after food deprivation. G. RF day 19 (blue line) and 3 h shift of the early meal (red line). H. RF day 19 (black line), 3 hr shift of the early meal (red line), and day after 3 h shift of meal (RF21, blue line).

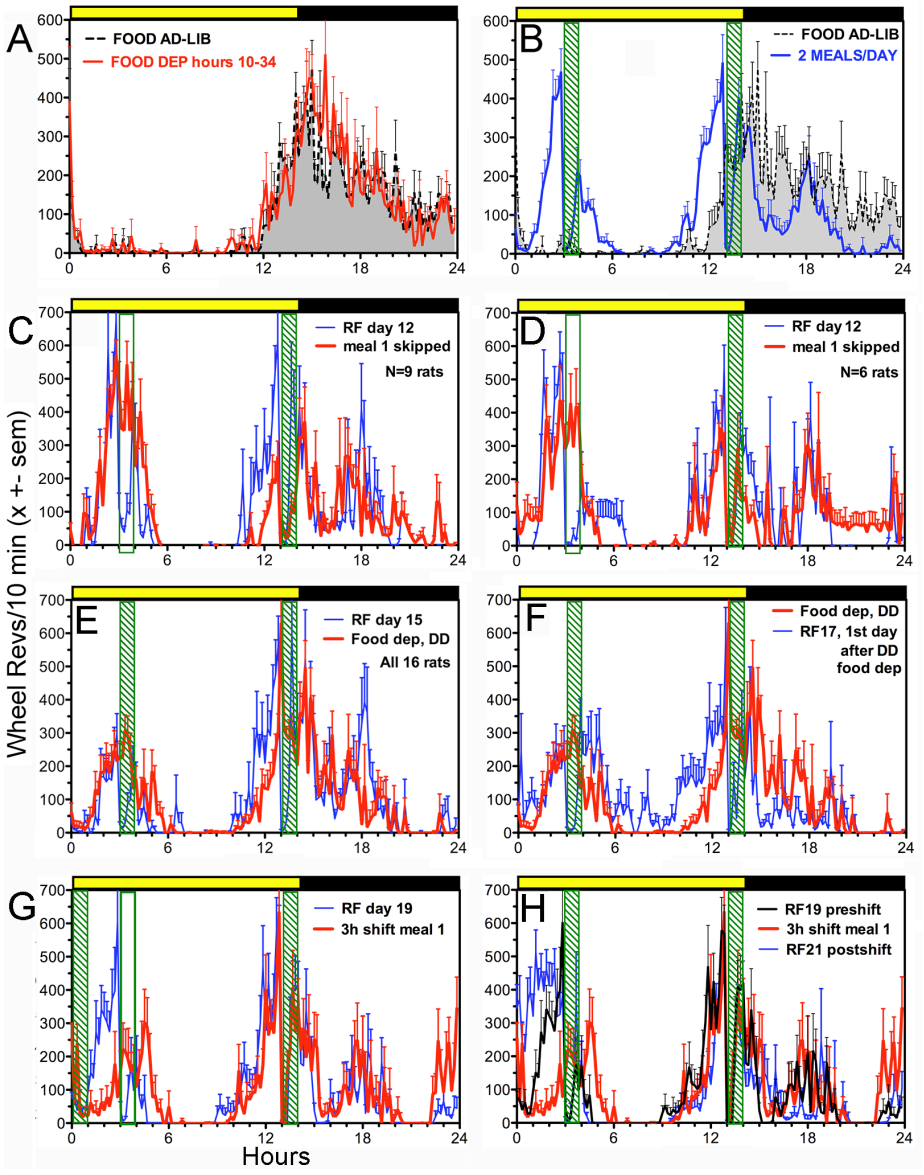


Figure 4. Group mean wheel running during the 4-h prior to the morning meal (solid line) and afternoon meal (dotted line) averaged over restricted feeding days 8-12. Mealtime is indicated by the green vertical lines. This is an expanded view of data in Figure 3a.

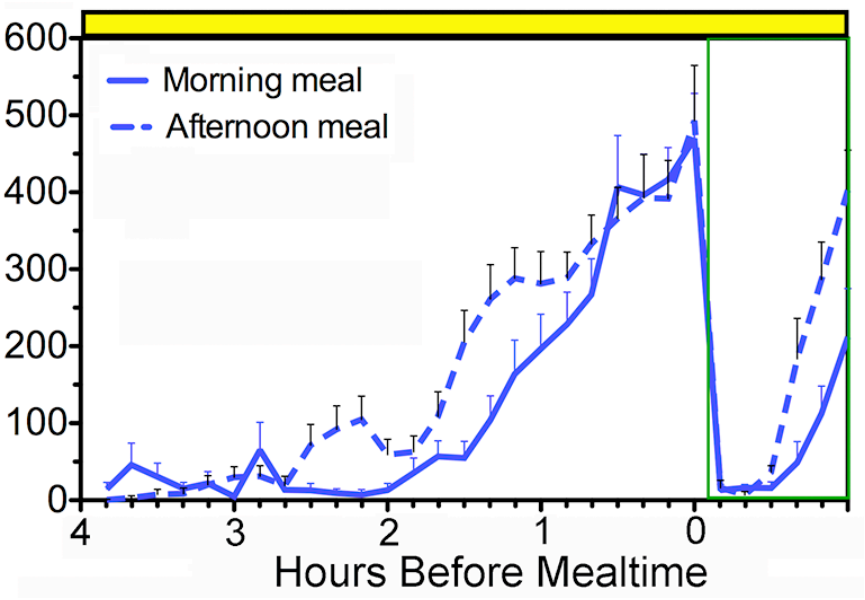
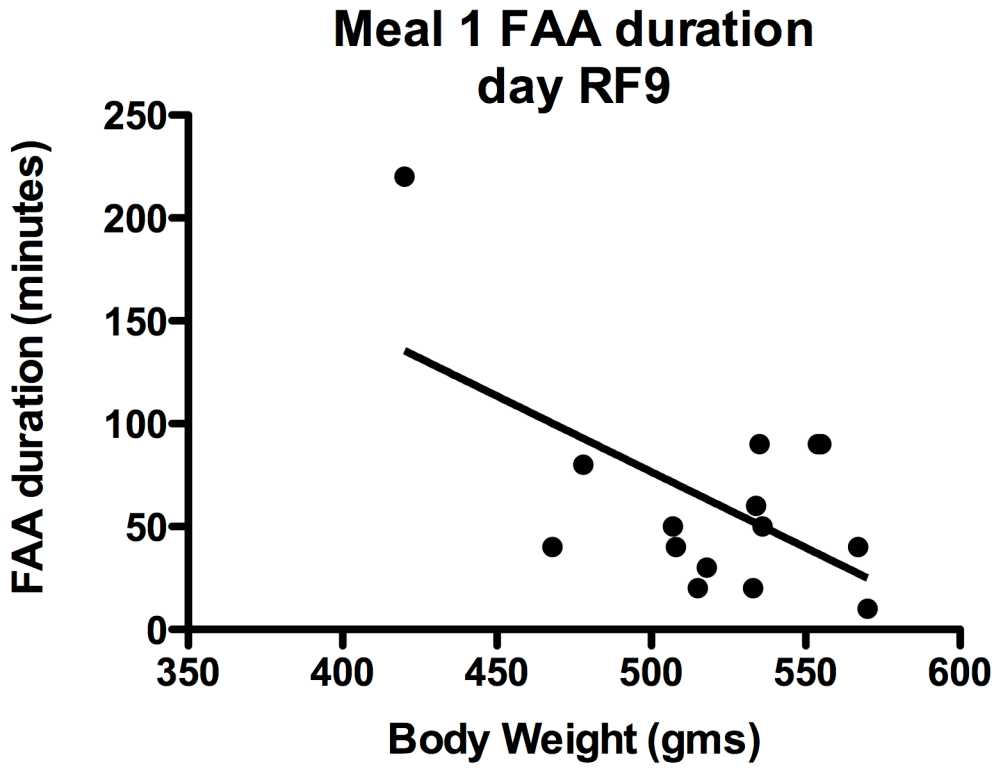
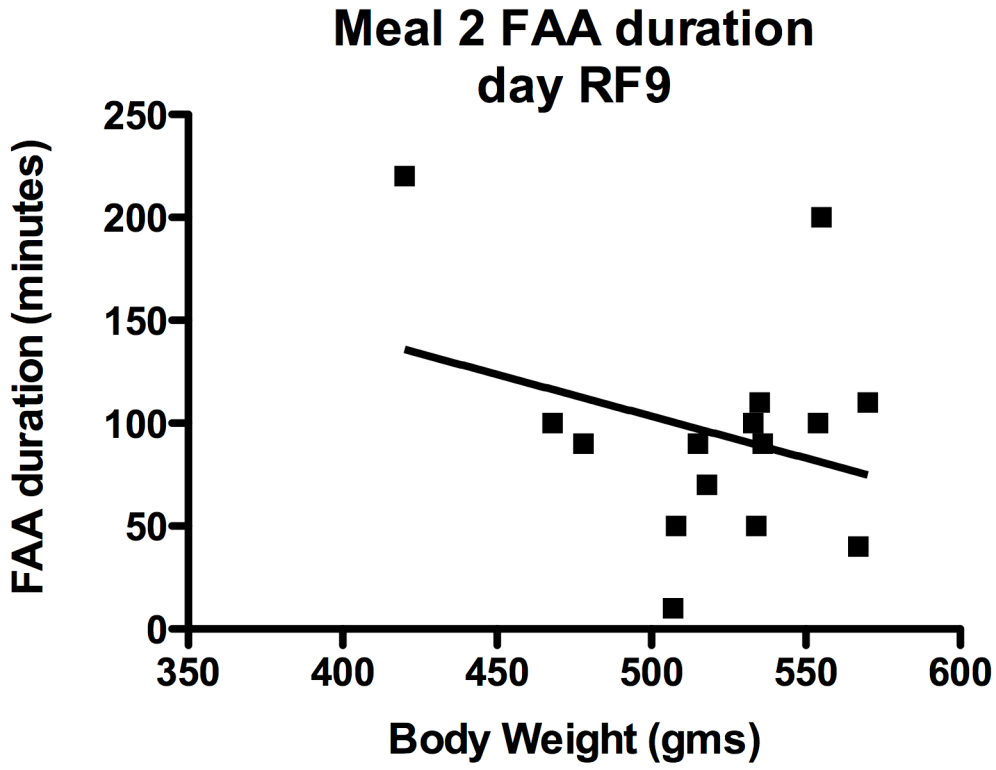


Figure 5. Regression of body weight in grams on day 9 of restricted feeding with FAA duration in minutes





EXPERIMENT 2: TWO DAYTIME MEALS 10 HOURS APART: ASSESSMENT OF SCN PHASE

Introduction

Behavioral and clock gene studies have shown repeatedly that the SCN pacemaker in rats entrained to a 24 h LD cycle is not shifted when food is restricted to the middle of the light period (eg., Damaola et al, 2000, Stokkan et al., 2001; Waddington Lamont, Harbour, Barry-Shaw, Renteria Diaz, Robinson, Stewart and Amir, 2007; Verwey, Khoja, Stewart and Amir, 2007). However, this has not yet been assessed in rats fed two daytime meals. The afternoon meal in Experiment 1 was close enough to lights-off that an assessment of SCN phase was warranted, to determine if its phase might have been advanced sufficiently to account for anticipation of the afternoon meal. Consequently, in Experiment 2, rats were subjected to the same 2-meal feeding schedule, to assess SCN phase using behavioral and cellular phase markers. The behavioral phase assessment was based on the timing of nocturnal activity onset measured on the first day of ad-lib food access in constant dim red light (DDr) after two weeks of restricted feeding or ad-lib food access in LD. The cellular phase assessment was based on the circadian gating of light-inducible FOS protein in SCN neurons. Light exposure during the ‘subjective night’ (when the LD-entrained SCN pacemaker promotes arousal and activity in nocturnal rodents) induces the immediate early gene cFos, while light in the subjective day (when the SCN promotes rest) is less

effective (Rusak, Robertson, Wisden, and Hunt, 1990; Antle and Mistlberger, 2000. If the 2-meal daytime feeding schedule shifted SCN phase forward, then a light pulse prior to the afternoon meal should induce FOS in the SCN of food restricted rats but not ad-lib fed rats.

Procedures

Sixteen male Sprague Dawley rats naïve to restricted feeding schedules were randomly assigned to a food restriction group (N=8) or an ad-lib food access group (N=8). The rats were first acclimated to the running wheel cages for 2 weeks with ad-lib food access. The food restriction group was then food deprived for 37 h, and fed twice daily for 1 h, beginning at ZT1 and ZT11, for the next 15 days. After the last meal at ZT11, food was provided ad-lib in constant dim red light (DDr, <1 lux) for 10 days. Activity onsets on each of the first 3 days of DDr were compared with onsets averaged over the week prior to restricted feeding, and over the last 7 days of restricted feeding.

After 10 days of DDr, the LD cycle was reinstated. Food was available ad-lib for another 14 days, after which the food restriction group was limited to 2 daily meals, at ZT1 and ZT11. After the last ZT11 meal, food was provided ad-lib and LD was replaced by DDr. The next day at ZT9.5, half of the rats from each group were exposed to a 15 min bright light pulse, provided by fluorescent light fixtures (400 Lux). The rats were then euthanized for quantification of FOS immunoreactivity in the SCN.

Results and Discussion

By contrast with the results of Experiment 1, food anticipatory wheel running was less robust in most rats in Experiment 2 (e.g., Fig. 6). Nonetheless, all 8 food restricted rats exhibited some anticipation of meal 1, and 5 of 8 also showed significant anticipation of meal 2. The duration of anticipation to meal 2 was similar to that evident in Experiment 1 (108 $\pm$ 8 min), although the peak level, in normalized units, was about half the magnitude (Fig. 7a).

To assess the effects of the feeding schedule on the phase of the LD entrained activity rhythm, average waveforms were generated for the 5 rats that anticipated meal 2 and were compared across conditions (Fig. 7a). The waveform for the first day of ad-lib food access in DDr shows a rapid rise of activity at the usual time of meal 2, which appears to be about 1 h later than during the feeding schedule, and about 1 h early compared to ad-lib food access prior to restricted feeding. Quantification of activity onsets for each day, using the same algorithm as used in Experiment 1, confirmed the first impression, but not the second. In this subgroup of food restricted rats, the onset of activity on the first day of ad-lib food access in DDr was significantly delayed relative to the food restriction days ($t_{(4)}$= 4.43, $p<.05$), but did not differ from the onsets of activity averaged over the last week of ad-lib food access days prior to restricted feeding ($t_{(4)}$=1.23, p=.30) (Fig. 7c). The onsets of activity during the first 3 days of DDr, expressed as differences from the onsets prior to restricted feeding, also did not differ between the food restricted group (all 8 or the subgroup of 5) and the ad-lib

fed group. These results therefore do not support a hypothesis that anticipation of meal 2 was mediated by a phase advance of the LD-entrained SCN pacemaker. The appearance activity during mealtime 2 (and mealtime 1) on the first day of ad-lib food access in DD is more likely to represent persistence of a food entrained timing mechanism independent of the SCN pacemaker.

After reentrainment to LD, and another 15 days of restricted feeding, half of the rats were exposed to a 15 min light pulse just prior to the afternoon mealtime, to assess FOS immunoreactivity in the SCN. The total number of FOS+ cells, counted bilaterally in anterior, medial and posterior sections of the SCN, did not differ significantly across groups, although there was a weak trend for more labelled cells in the food restricted and ad-lib fed groups exposed to light. To examine this more closely, cells were counted bilaterally in the ventrolateral portion of the posterior SCN sections, where retinohypothalamic innervation is most dense and photic induction of FOS is normally the greatest (Rusak et al.,1990). Within this region, a significant difference across groups was detected ($F(3,9)=5.81$, $p= .017$; Fig. 8). The food restricted rats exposed to light had significantly more FOS+ cells compared to food restricted rats not exposed to light ($p<.05$). However, the food restricted and ad-lib fed rats exposed to light did not differ. Therefore the data provide no evidence that the SCN was phase advanced in the food restricted rats relative to the control rats. Rather, the results indicate that in rats entrained to LD 14:10, the photosensitive phase for the SCN c-fos induction begins prior to the daily onset of the subjective night.

Photic induction of SCN c-fos has not previously been described in rats entrained to LD 14:10.

Figures

Figure 6. Wheel running activity of 4 representative rats in Experiment 2. Panels A-C. Rats restricted to 2 daily meals. D. Rat with ad-lib food access, housed in the same recording room. Mealtimes are indicated by opaque vertical bars and lights-off is indicated by shading. For other plotting conventions, see Figure 2.

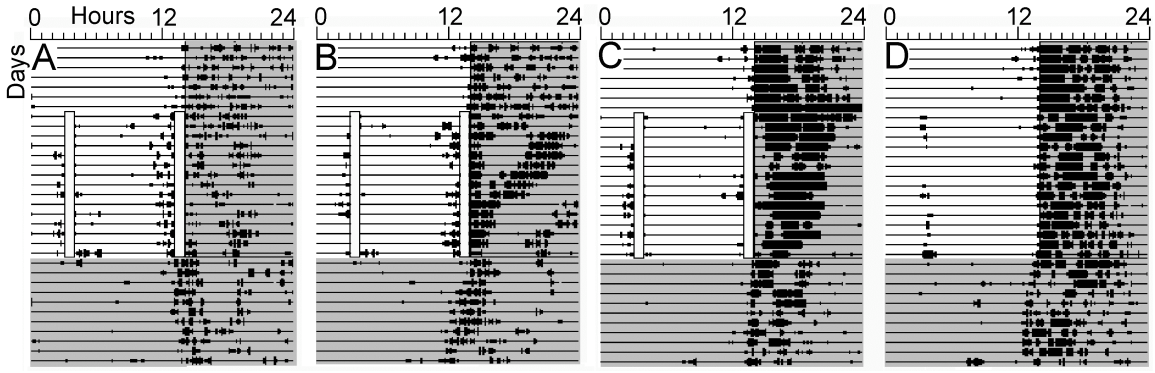


Figure 7. Average waveforms of wheel running activity in Experiment 2. A. Food restriction group (N=5 rats) during the last week of ad-lib food access (grey shaded waveform), the last week of restricted feeding (blue line), and the first day of constant dark with ad-lib food access (black line). Mealtimes denoted by green shaded columns. B. Rats with ad-lib food access throughout the experiment (N=8), same sets of days as in Panel A. C. Group mean (± sem) time of activity onset in minutes relative to the average time of nocturnal activity onset during ad-lib food access prior to restricted feeding, in the food restricted group (solid circles, 2m) and in the adlib fed group (squares, AL). RF = last week of restricted feeding in LD. DD1-3 = constant dark days 1, 2 and 3, with adlib food access. AL base 2 = last week of ad-lib food access in LD.

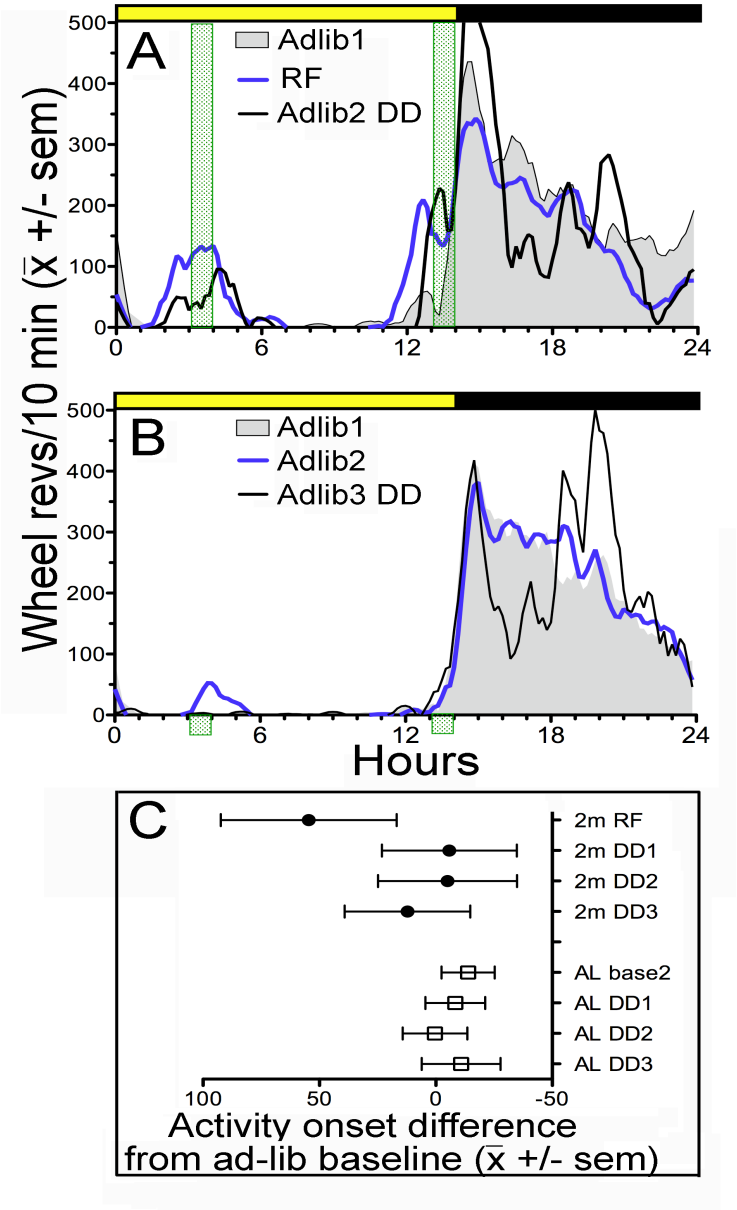
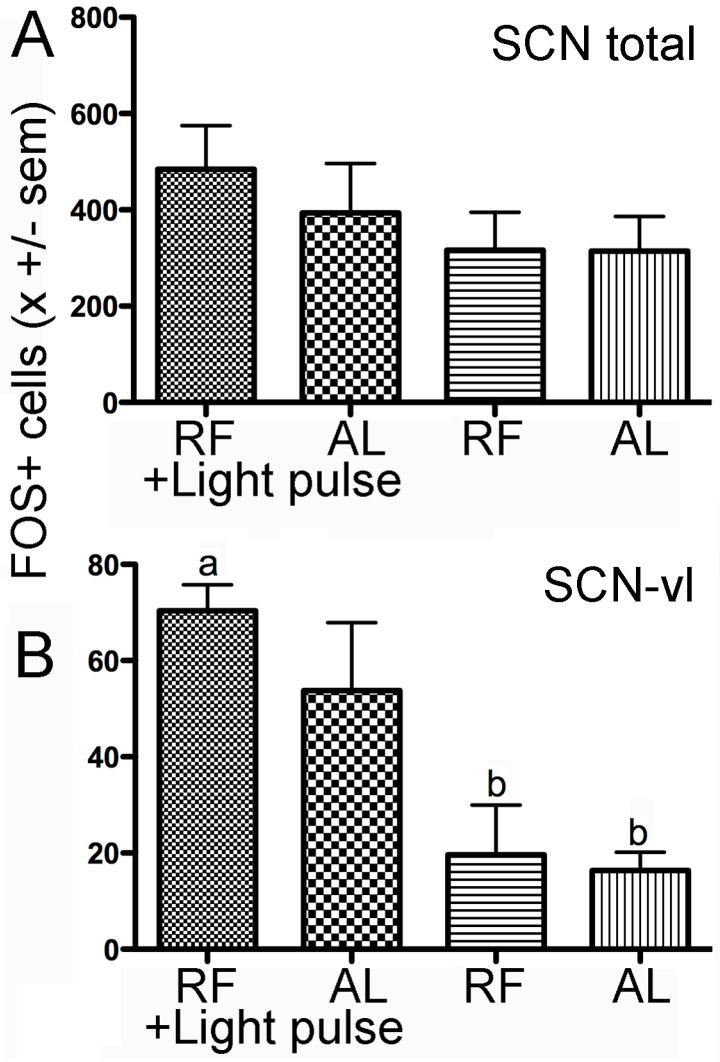


Figure 8. Group mean (±sem) counts of FOS positive cells in A. total SCN, and B. ventral lateral portion of the SCN, in food restricted (RF) and ad-lib fed (AL) groups. '+Light pulse' denotes those groups exposed to 15 min of light 60 min before the afternoon meal, during a day in constant dark.



EXPERIMENT 3: TWO DAYTIME MEALS, 7 HOURS APART

Introduction

The possibility that anticipation of meal 2 might be mediated by the LD-entrained SCN pacemaker was not supported by the results of Experiment 2. If the afternoon bout of food anticipation represented the onset of the SCN-driven daily active phase, then the onset of free-running activity in DDr, with food available ad-lib, should correspond to the time of day that anticipation of meal 2 began during the restricted feeding schedule. Instead, the phase of the free-run was not significantly different from the phase of nocturnal activity onset prior to restricted feeding. Light exposure was associated with an increased number of FOS+ cells in the SCN but there was no difference between food restricted and ad-lib fed rats. Therefore, the data provide no evidence that the SCN was phase advanced by daytime feeding.

The 10 h interval between meal 1 and meal 2 in Experiments 1 and 2 was chosen to minimize ambiguity in identifying distinct bouts of activity associated with each mealtime during meal omission tests, which is the most important criterion for determining whether the two bouts are under independent circadian control. A wide interval should also facilitate detection of differently phased circadian clock gene rhythms in different brain regions or peripheral organs, if separate bouts of anticipation are mediated by different groups of circadian clock

cells, functioning as independently entrained clocks. In earlier work, total food deprivation tests conducted when 2 daily meals were 5 h apart were associated with a single bout of activity extending across the two mealtimes (Stephan, 1989). In Experiment 3 we examined an interval of 7 h, toward defining discrimination limits for 2-meal timing. We also explored the use of 2 day food deprivation tests as a means of revealing covert meal timing in rats that fail to exhibit anticipatory activity. Finally, we examined SCN FOS immunoreactivity in response to light exposure at mealtimes early and late in the subjective day.

Procedures

Two cohorts of rats entrained to LD 14:10 were housed in wheel running cages as in Experiment 2. Both cohorts consisted of a food-restriction group and an ad-lib fed group (N=8 each in cohort A, N=16 each in cohort B). After two weeks of ad-lib food access, the food restriction groups were food deprived for 37 h and then received food (powdered chow in corn oil) twice daily, 3 h (ZT1) and 10 h (ZT8) after lights-on. Cohort A was food restricted for 41 days, during which the morning meal was omitted once (RF day 22), both meals were omitted for one day in LD (RF25) and for two days in DD (RF29-30), and the morning meal was advanced by 3 h once (RF34). The rats were euthanized and perfused on day RF41 without feeding, in groups of 4 at either mealtime 1 or mealtime 2. Matched groups of ad-lib fed rats were perfused at the same times.

Cohort B was food restricted for 33 or 34 days, during which the afternoon meal was omitted once (RF7), the morning meal once (RF17), both meals for 2

days in DD (RF21-22) and both meals for 1 day in LD (RF31). On day RF42 or RF43, the lights were left off, half of the rats were exposed to a 15 min light pulse 1 h prior to mealtime 1 or mealtime 2, and the rats were euthanized and perfused 60 min later at either mealtime 1 or mealtime 2, yielding 8 groups of 4 rats (food restricted or ad-lib fed, exposed to a light pulse or dark, perfused at mealtime 1 or mealtime 2).

Results and Discussion

Food anticipatory wheel running in the rats of Cohort A was again weak by contrast with the rats in Experiment 1. Actograms of the two best cases, and one representative of the remaining 6 cases, are provided in Figure 9(a-c). Meals were provided at ZT1 and ZT8. The number of days in which wheel revolutions occurred in at least one 10 min bin during the hour preceding each mealtime (i.e., ZT0-1 and ZT7-8) were counted separately for the last 7 days of ad-lib food access, the 37 h food deprivation test, and the first 21 days of restricted feeding. During the last 7 days of ad-lib food access, wheel activity was rare during these 2 hours, occurring on 0 of 7 days in the ZT0-1 hour, and on 0.8 $\pm$ .5 of 7 days (range 0 to 1 day) in the ZT7-8 hour. During the 37 h food deprivation test, no wheel counts were registered during the ZT0-1 h in any rat, and only 1 rat showed one 10 min bin with wheel counts during the ZT7-8 hour. During the first 21 days of restricted feeding, the two best cases showed activity on 16 (Fig. 9a) and 17 (Fig. 9b) days, respectively, in the ZT0-1 hour, and on 15 (Fig. 9a) and 7 (Fig. 9b) days, respectively, in the ZT7-8 hour. The remaining 6 rats showed

activity in the hour prior to meal 1 and meal 2 on only 7.3 ± 2.1 and 3 ± 3 of 21 days, respectively (eg.,. Fig. 9c)

Despite the low levels of anticipatory activity in most rats, at least some mealtime-associated activity was evident in the majority of rats during the meal omission tests. This was quantified categorically, by scoring whether any wheel revolutions occurred from ZT0-3 and ZT 7-10, i.e., 3 h blocks spanning each mealtime by 1 h in each direction. On the day preceding omission of meal 1 (day RF22), 4 of 8 rats showed activity in the hour prior to meal 1 (ZT0-1), whereas on the meal omission day, 5 rats showed activity in this hour, and the other 3 became active within the hour that food was previously delivered (i.e., ZT1-2). When both meals were omitted in LD (day RF25), 5 rats showed mealtime-associated activity to meal 1, and 5 to meal 2. When both meals were omitted for 2 days with the lights off, mealtime-associated activity was evident in all 8 rats to meal 1, and in 6 rats to meal 2.

Average waveforms of activity from the two rats with the strongest anticipation confirm that the timing of meal anticipation was not significantly altered during the 2 day food deprivation test in DD despite the absence of environmental time cues (Fig. 10b). Group average waveforms for the other 6 rats (Fig. 10c) further illustrate the utility of 2 day food deprivation tests for revealing timing behaviour in rats that show little anticipatory activity to scheduled meals on most days. Notably, the waveforms reveal a distinct peak of activity centred near the middle of the expected mealtime, which fell off rapidly after the expected meal hour passed without feeding. This is particularly striking at

mealtime 1 in the 6 rats with weak or no anticipatory activity during the preceding 3 days (Fig. 10c).

Similar results were obtained for the rats in Cohort B. Of the 16 food restricted rats, 10 showed anticipation of one or both meals on a majority of days (e.g., Fig. 9d,e), while 6 showed little running in the hour prior to either meal (e.g., Fig. 9f). Despite the overall low levels of pre-meal running, categorical scoring of activity during the 3 h blocks spanning each mealtime confirm at least some mealtime associated activity in the majority of rats during most meal omission tests. This was particularly striking for the 2 day deprivation test in DD, during which 14 rats were active during mealtime 1 on one (N= 4 rats) or both days (N=10 rats), and all 16 were active during mealtime 2 on one (N=6 rats) or both days (N=10 rats). Group average waveforms generated using data from the 10 rats that showed consistent anticipatory activity again showed a spike of wheel running centred at the two mealtime hours during the 2 day (Fig. 10e) and 1 day (Fig. 10f) food deprivation tests.

On the last day of restricted feeding the lights remained off and half of the food restricted and ad-lib fed rats were exposed to a light pulse 1h prior to either the first or the second mealtime, and were euthanized at mealtime. The remaining food restricted and ad-lib fed rats were euthanized at mealtime without light exposure. At ZT8 (mealtime 2), the number of FOS+ cells in the ventrolateral (but not dorsomedial) SCN was significantly greater in the food restricted ($t=4.95$, $P = 0.0026$) and ad-lib fed ($t=6.97$, $P= 0.0009$) rats exposed to light (Fig. 11a), compared to matched groups not exposed to light. At ZT1

(mealtime 1) FOS+ cell counts did not differ by feeding condition or light exposure. In rats not exposed to light, there were significantly fewer FOS+ cells in the SCN of food restricted rats, when data for both SCN regions and both mealtimes were combined. This effect was not evident when only the ventrolateral SCN cell counts alone were compared.

Figures

Figure 9. **Wheel running of representative rats in Experiment 1. Panels A-C. Rats from cohort A. Experimental conditions: (1) 37h food deprivation, (2) two daily meals (3) omission of the early meal, (4) omission of both meals in LD, (5) omission of both meals for 2 days in DD, (6) early meal advanced by 3 hours. Panels D-F. Rats from cohort B. Experimental conditions: (1) 37 h food deprivation, (2) two daily meals, (3) afternoon meal omitted (4), both meals omitted in DD for 2 days, and (5) both meals omitted in LD. An asterisk denotes a 15 min light pulse. X denotes euthanization time. Mealtimes are denoted by opaque vertical bars and lights-off by shading**

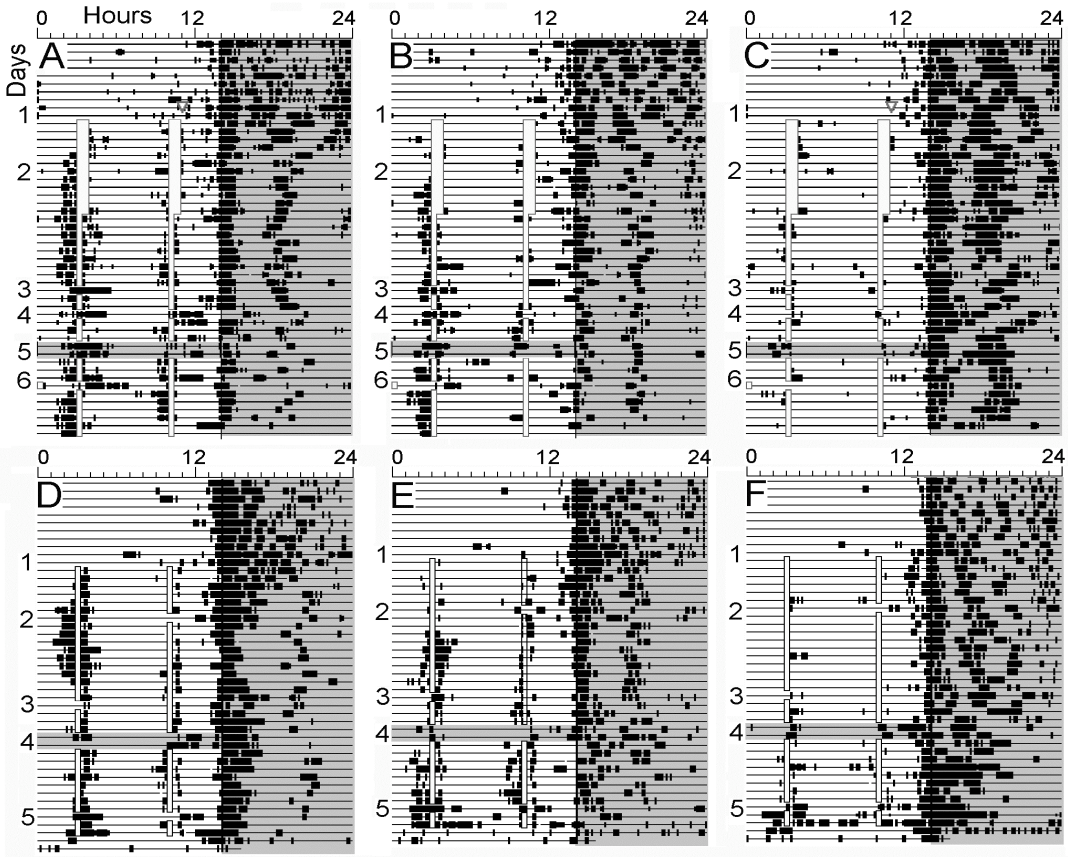


Figure 10. Average waveforms of wheel running activity in Experiment 3 (A-C = cohort A; D-F = cohort B. Raw data were smoothed using Prism (Graphpad) software (see methods section). (A) Last week of ad-lib food access (shaded waveform) and 37 h food deprivation (red waveform). (B) Restricted feeding days 25-28 (black curve) and 2 days of total food deprivation in constant dark (red curve), in two rats that anticipated both meals. (C) Same days as in Panel B, in 6 rats that showed little or no food anticipation on most days during restricted feeding. (D) Last week of ad-lib food access (shaded waveform) and 37 h food deprivation (red waveform), N=16 rats in Cohort B. (E) Restricted feeding days 18-21 (black waveform) immediately prior to 2 days of total food deprivation in DD (red waveform), in 10 rats that showed some anticipation of both meals. (F) Restricted feeding days 23-29 (black waveform) immediately prior to 1 day of total food deprivation in LD (red waveform), in 10 rats that showed some anticipation of both meals.

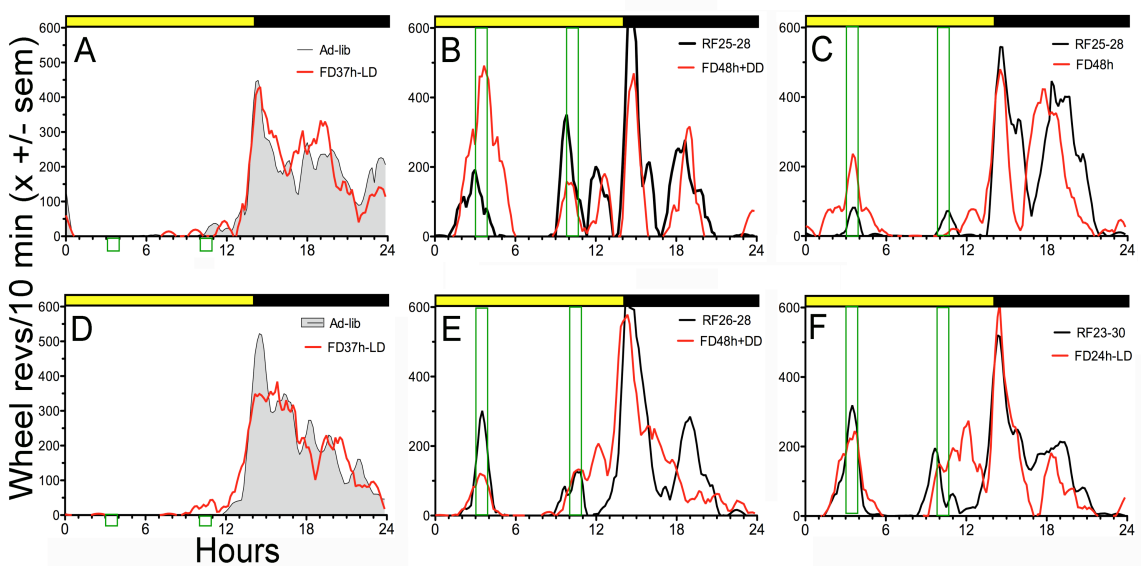
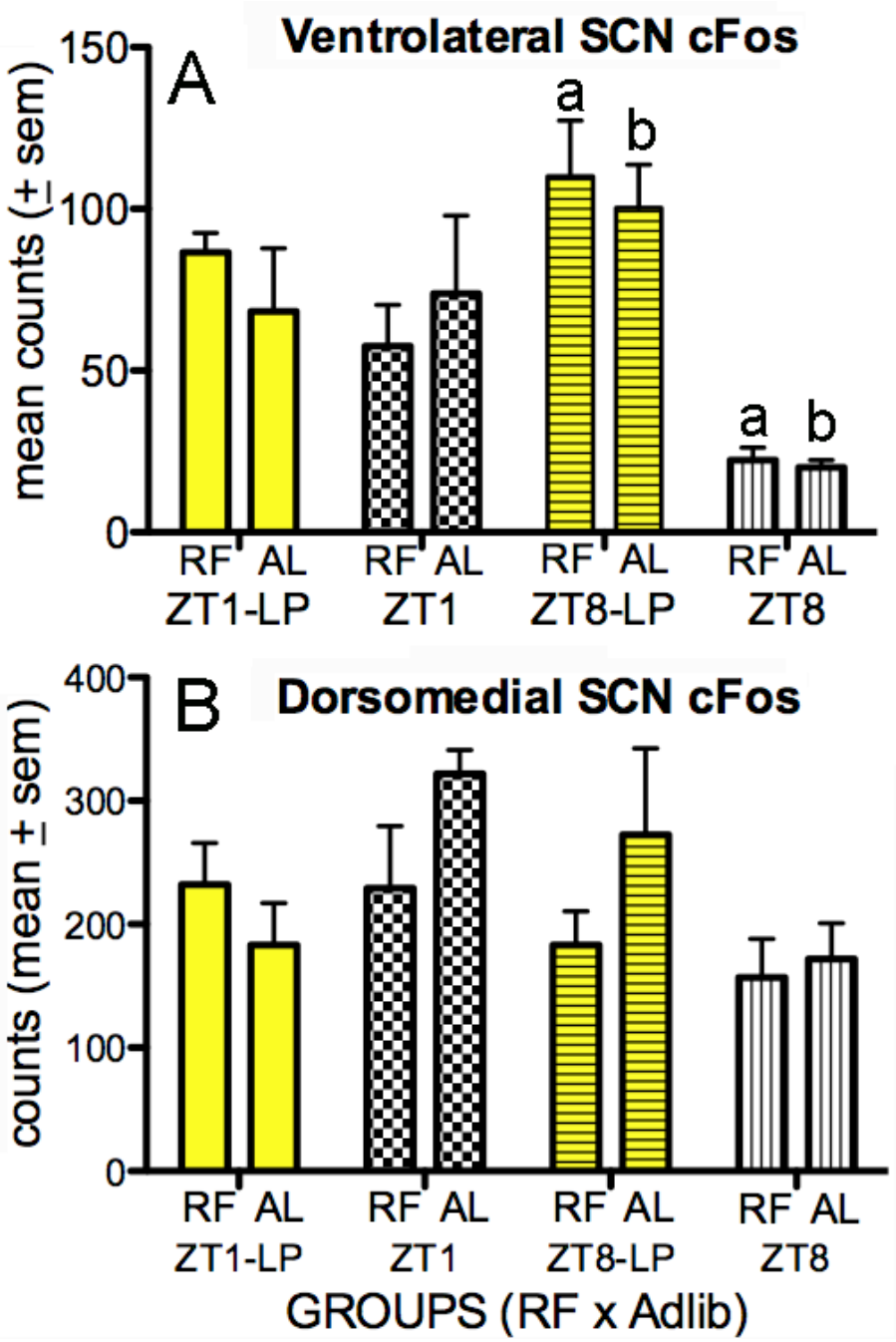


Figure 11. Group mean (± sem) FOS+ cell counts in A. the ventrolateral suprachiasmatic nucleus (SCN) and B. dorsomedial SCN. RF, food restricted rats; AL, ad-lib fed rats; LP, light pulse at either ZT0 or ZT7, 1 h prior to euthanization at the next scheduled mealtime.



GENERAL DISCUSSION AND CONCLUSIONS

The experiments reported here were designed to probe the formal properties of food anticipatory rhythms in rats maintained on two daily meals separated by 7 or 10 h in the light period. Despite variability within groups and across experiments in the amount of food anticipatory wheel running, the results favour a 2-oscillator model of 2 meal timing over a single food-entrainable consulted clock model, and provide no evidence for a contribution of interval timers, if interval timers rely on environmental events to predict the next mealtime. Persistence (or appearance) of food anticipatory activity at scheduled mealtimes during meal omission tests (Experiments 1 and 3) is consistent with predictions of 2-oscillator and consulted clock models, but not interval timing models. Shifting of one bout of food anticipation without parallel shifting of anticipatory running to the next meal (Experiment 1) is consistent with predictions of a 2-oscillator model but not a single consulted clock model. Therefore, although the consulted clock model has theoretical advantages (e.g., it is more parsimonious, especially if it can be shown that rats can anticipate more than 2 meals), the behavioural results lead to a prediction that food-entrainable oscillators can segregate into at least two independently entrained groups, which may be detectable by mapping of circadian clock gene expression at intervals throughout the day, in brain regions already known to express food-entrained clock gene rhythms.

Rest-activity states in mammals are thought to be regulated by two processes, a circadian process that modulates thresholds for sleep and wake onsets across the day, and a homeostatic process that representing a physiological drive for sleep that is assumed to accumulate during waking and dissipate during sleep (Daan et al, 1984). This 2-process model can accommodate other factors that might affect the threshold for wake or sleep onset, including environmental stimuli (e.g., light, noise, temperature) and, interoceptive cues (e.g., pain, or neuro-endocrine correlates of seasonal migration, hibernation, water or food deprivation). Intuitively, food deprivation would be expected to reduce the threshold for expression of clock-controlled activity. In long-term food deprivation studies (e.g., 4-7 days), increased daytime running is typically not evident during the first few days without food (Mistlberger, 1994) and emerges only later (Challet, Le Maho, Pevet, Nobelis, and Malan, 1996). Increased daytime running is evident on the first or second day of total food deprivation only in rats that have previously been fed during the day. Consistent with those findings, rats in Experiments 1-3 did not show a significant alteration in the timing of nocturnal activity onset, or in the total amount of running during 37 h food deprivation tests prior to restricted feeding. Similarly, neither the onset nor the peak level of food anticipatory running were significantly altered following single or double meal omission tests, despite the increased deprivation intervals. These results reinforce the interpretation that the daily onset of both food anticipatory activity, and nocturnal activity, predominantly reflect the phase of circadian clocks entrained by food or light, respectively, with

relatively little contribution from homeostatic factors related to food deprivation (eg., neural or endorcrine correlates of hunger), at least to the extent that these factors vary over 2 days without food. It seems likely that homeostatic factors would make a contribution if food deprivation was extended for more days (increasing the duration and/or peak level of anticipatory activity). Homeostatic factors may have contributed to the appearance of mealtime associated activity during meal omission tests in rats that exhibited little or no premeal activity. In many cases, however, activity revealed by these meal omission tests appeared during or shortly after the scheduled mealtime, which could reflect the phase at which an oscillator is entrained to that meal rather than an amplifying effect of increased hunger on the expression of clock controlled activity.

Rats restricted to one or two daily meals can anticipate food at any time of day, and removal of the SCN by neural ablation if anything enhances food anticipatory rhythms. Consequently, there was no reason a priori to expect that the SCN would be involved in the timing of anticipation to one or both meals. Nonetheless, because meal 2 in Experiment 1 began only 1 before lights-off, in Experiment 2 we evaluated whether the feeding schedule might have shifted the SCN pacemaker forward, thereby conflating SCN-driven activity with activity controlled by an independent food-entrainable oscillator. We used both behavioral and immediate early gene phase markers. The behavioral phase marker (activity onset on the first day of ad-lib food access in DD) showed that activity onset controlled by the SCN was at least 1 h later than the onset of food anticipatory activity, and did not differ from onset times during ad-lib food access

prior to restricted feeding. This argues against a role for the SCN in timing meal 2. The use of light-induced FOS immunoreactivity in SCN neurons did not prove as useful in addressing the issue, because even the control rats exhibited cFos induction in response to light exposure prior to meal 2. It appears that in rats entrained to LD 14:10, the SCN is sensitive to light well before the onset of the subjective night (the daily active period driven by the SCN pacemaker, which in rats occurs at night).

In Experiment 1, all of the rats exhibited robust anticipation of both meals, but in the follow-up experiments, anticipation was notably weaker, in the number of rats anticipating one or both meals, and in the amount of premeal running in those that did anticipate. The reason for this is unclear, but could be physiological or environmental. Rats made obese by high fat diets show attenuated food anticipatory activity, and under sedentary housing conditions, rats become obese even with standard diets. The rats in the latter studies were on average heavier when the feeding schedules were initiated, and this may have contributed to low anticipatory running. Although the running wheel cages were the same in all of the experiments, in Experiment 1 the cages were housed in individual isolation boxes, whereas in the other experiments the cages were in open cabinets. Whether this played any role in the amount of anticipatory running is unknown. One previous study found that mice in isolation boxes failed to anticipate a daily meal, whereas the same strain of mouse did anticipate meals when housed in open racks (deGroot and Rusak, 2004).

The results reported here set the groundwork for further work to elucidate the neurobiological mechanisms by which rats time daily meals. A next step will be to determine if separate daily bouts of food anticipation to different meals are mirrored by differently phased rhythms of circadian clock genes in one or more brain structures, analogous to the differently phase clock gene rhythms in left and right SCN, in rodents with split activity rhythms (e.g., de la Iglesia, et al, 2000).

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