Meal-synchronized CEA in rats: effects of meal size, intragastric feeding, and subdiaphragmatic vagotomy

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White, Wesley, Gary J. Schwartz, and Timothy H. Moran. Meal-synchronized CEA in rats: effects of meal size, intragastric feeding, and subdiaphragmatic vagotomy. Am. J. Physiol. 276 (Regulatory Integrative Comp. Physiol. 45): R1276–R1288, 1999.—Within a feeding schedule of intermittent food access, large meals have the ability to induce activity at the same time the next day [circadian ensuing activity (CEA)]. In these experiments, we evaluated the minimum meal size necessary to induce CEA and whether oral-pharyngeal factors and afferent vagal activity played necessary roles in the induction of the underlying process. In experiment 1, every 33 h rats were given two meals separated by a 2-h interval. The size of the first meal was varied, while total intake every feeding cycle was held constant. When the initial meal was <10 g (34 kcal) CEA occurred later, indicating that such a meal size was subthreshold for inducing CEA. In experiment 2, rats were given intragastric (IG) meals every 33 h, before and after complete subdiaphragmatic vagotomy. IG nutrient meals induced CEA, indicating that extensive oral-pharyngeal experience was not necessary for CEA induction. CEA occurred in vagotomized rats but, compared with intact rats, appeared to occur later relative to nutrient infusion, indicating that afferent vagal activity may be sufficient but not necessary to induce CEA.

circadian ensuing activity; food intake; transduction; food-entrainable oscillator; food-anticipatory activity; circadian rhythm; entrainment

When access to food is limited to a single time daily [restricted feeding (RF)], many animals anticipate the time of food availability both behaviorally and physiologically. In the hours just before food availability, a rat will increasingly run in a wheel, approach a feeding site, contact a food cup, and (when food access is contingent on lever pressing) make unreinforced lever presses. Core temperature, serum corticosterone, and a variety of gastrointestinal system variables also increase. These results suggest that RF has the capacity to attach a food-related motive state to a specific time of day (11).

Wheel turning in rats is the best-studied measure of this food-related motive state. Enhanced wheel turning in anticipation of food availability [food-anticipatory activity (FAA)] appears to be an entrained circadian rhythm: FAA has a circadian time course, is contiguous with meal receipt only when meals are presented at a circadian interval, gradually resynchronizes to a new feeding time when food availability is abruptly and markedly shifted to a new fixed time of day, and recurs at circadian intervals during a prolonged fast. However, it occurs in animals with complete lesions of the suprachiasmatic nucleus (SCN) of the hypothalamus, the major circadian pacemaker in the circadian timing system of mammals (11).

Evidently, feedings can engage the circadian timing system. Studying the processes and mechanisms involved in the development of meal-elicited circadian activity patterns is likely to reveal 1) novel ways in which the circadian timing system can be modified and 2) novel determinants of hunger and foraging. Many well-established procedures exist for assessing the contribution of different parts of the nervous system and various compartments of the gastrointestinal system to food-related phenomena. Also, much is known about the substrates and signals involved in the control of food intake (9). These facts are likely to facilitate the study of the ways in which feedings affect circadian patterns.

Recently, White and Timberlake (21) described a new procedure for studying the processes and mechanisms responsible for food-dependent circadian activity patterns. The procedure involved maintaining animals in a standard 12:12-h light-dark cycle and presenting meals with a period (T) of ≈31 h (long-T schedule). When food is presented with a T of 31 h, to pick a specific example, the start of food availability is delayed 7 h every day, meals occur at novel and markedly different times across days, and, after twenty-four 31-h cycles, meals have occurred during each hour of the day.

Meals of a long-T feeding schedule produce some of the same effects that meals presented at 24-h intervals do: each meal of a long-T schedule tends to produce heightened activity ~24 h subsequent to meal initiation. Furthermore, like FAA, the pattern of enhanced activity develops within several feeding cycles, can be manifested at any time of day, appears to oscillate during deprivation, and is evident in rats having complete SCN lesions.

However, a long-T feeding schedule reveals that continuously delaying the time of food availability by many hours every day produces a corresponding abrupt daily displacement in the peak of enhanced activity. In other words, a single experience with food at a particular time of day appears to induce a food-related motive state at that time on subsequent days. The enhanced activity that tends to begin ~24 h after food receipt on a long-T feeding schedule will be called circadian ensuing activity (CEA). This terminological convention was adopted by White and Timberlake (21) because FAA and CEA may have incompatible properties.

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The outcome suggests that daily temporal programs can be significantly rewritten by experience from one day to the next and that circadian processes sometimes appear to be as labile as learning processes. This view contrasts with the current general tendency to regard daily temporal programs as "read-only" programs that modulate rather than reorganize behaviors (17). Understanding CEA might produce additional insights into the nature of circadian processes.

The present research had two major goals: 1) to provide further evidence for the capacity of shifting meal times to organize CEA and 2) to begin a mechanistic analysis of factors that might underlie this capacity. The study involved two experiments. In experiment 1 we examined how the distribution of food intake within a feeding cycle affected the distribution of activity the next day. This study provided information about the meal size necessary to induce CEA. In experiment 2 we determined whether extensive oral-pharyngeal experience and an intact vagus were necessary for the expression of CEA.

**EXPERIMENT 1: DISTRIBUTION OF FOOD INTAKE ON LONG-T SCHEDULES AND CEA**

Some aspect or aspects of food intake appear to induce CEA. Meals have a variety of properties, including taste, volume, calorific density, total caloric content, and macronutrient composition, and one or more of these might be critical. The purpose of the present study was to investigate the role of meal size in the induction of CEA.

FAA develops only when meals of a sufficient size are eaten. Mistlberger and Rusak (12) gave free-feeding rats access to supplemental palatable mash. Rats exhibited FAA when they ate a 10.0-g nutritive meal but not when they ate a 5.0-g nutritive meal. Stephan (20) fed SCN-lesioned rats at one fixed time and then shifted the time of food availability to a later time. FAA developed at the new time only if ≥10 g of food were available.

Our approach was to provide two feeding opportunities every 33 h. The amount of food available at the first feeding varied from 5.0 to, on average, 13.4 g. A first meal of sufficient size to induce CEA was expected to produce enhanced activity ~24 h later, whereas a subthreshold meal was not. Providing a subthreshold first meal would provide the second meal the opportunity to induce CEA. Consequently, a subthreshold first meal would be indicated by a right shift in the distribution of activity in the circadian range relative to the start of meals. This design enabled animals to maintain total cycle intake and gave us the opportunity to show that induction could be accomplished every cycle.

**Method**

Subjects. The subjects were six experimentally naive, young-adult male Sprague-Dawley rats (Charles River). Before the start of the study, they were housed in individual wire mesh hanging cages in a testing room. A 12:12-h light-dark cycle was in effect (lights on at 0700), and temperature (~24°C) and humidity were controlled. The animals had ad libitum access to Purina rat chow and water. They weighed ~250 g at the start of the study.

Apparatus. During the study the animals were continuously housed in individual stations. Each station had a running wheel compartment and an immediately adjoining nest box. The running wheel was 36 cm in diameter and 11 cm wide, and it had one metal wall and a grid floor. Each revolution of the running wheel activated a microswitch. One of the walls enclosing the wheel was a Plexiglas partition that contained openings to a food recess, a water recess, and the nest box. An infrared emitter and detector at the bottom of the food recess indicated when a 45-mg food pellet (BioServ rodent grain-based diet) was in the feeding bin. Pellets were dropped from a dispenser. Tap water was available from a spout within the water recess. The nest box was made of black opaque Plexiglas, had a grid floor, and was 15 × 25 × 15 cm.

Each station was placed in a separate compartment of a sound- and light-attenuating cabinet. Each compartment contained a fluorescent lamp (7 W) connected to an appliance timer: these were used to create a light-dark cycle. Compartments also contained a 2,500-Hz sonalert used to signal food availability and a ventilating fan used to aerate the compartment and provide masking noise. Station devices were connected to an interface and a computer located in an adjacent room. CONMAN (Contingency Management Software) Spyder Systems, Bloomington, IN) arranged contingencies and collected data.

Measures. The number of pellets removed from the feeding bin and the number of running wheel switch activations were recorded every 5 min.

Procedure. The study was ~3 mo in duration, and it consisted of five conditions. **CONDITION 1: ACCLIMATION.** In this and all subsequent conditions, the animals were maintained on a 12:12-h light-dark cycle with lights on at 0700 local time, and they had free access to the running wheel and water. In only this condition the animals had ad libitum access to food pellets: whenever a pellet was not detected in the feeding bin another one was automatically dropped. The condition was in effect until rats reliably took pellets from the feeding bin, drank from the water spout, ran in the wheel, and slept during the light period in the nest. The condition was in effect for 6 days.

**CONDITION 2: RF ON LONG-T SCHEDULE WITH AD LIBITUM ACCESS AT EARLY MEAL.** In this and all subsequent conditions, animals had two feeding opportunities every 33 h. Each 33-h feeding cycle began with a food access period of 1 h, followed by, in order, a 2-h fast, a 4-h food access period, and a 26-h fast. The first meal in the cycle (the meal preceded by the long fast) will be called the early meal (EM), and the second meal (the meal preceded by the short fast) will be called the late meal (LM). In this condition, the amount of food that could be eaten during the EM was not limited. In this and all subsequent conditions, food intake at the LM was unrestricted. The first EM in this condition began...
at 0800 after a 14-h deprivation. Because the feeding schedule had a T substantially >24 h, we will call it a long-T feeding schedule.

When feedings are provided at 33-h intervals, they occur at markedly different times from day to day. Furthermore, at the end of eight 33-h cycles, they will have begun at a representative sample of day times. Specifically, EMs, in this and all subsequent conditions, will have begun every third hour of the day at local times 0800, 1100, 1400, 1700, 2000, 2300, 0200, and 0500 (circadian times 2, 5, 8, 11, 14, 17, 20, and 23, respectively, where time 0 is the time of light onset). In a condition where the first EM began at 0800, EMs would occur, in order, at 0800, 1700, 0200, 1100, 2000, 0500, 1400, and 2300. At the end of eight cycles, the times at which the feedings occurred would begin to repeat.

The initial condition was in effect for eleven 33-h cycles, and food intake and activity during the last eight cycles were analyzed.

**CONDITION 3**: 7.5-g EM. Access to food at the time of the EM was limited to 7.5 g. The first EM was presented at 1000, nine 33-h cycles were provided, and intake and activity during the first eight feeding cycles were analyzed. Equipment maintenance was performed during cycle 9; activity records from this cycle were deemed unreliable and were not analyzed.

**CONDITION 4**: 10.0-g EM. Access to food at the time of the EM was limited to 10.0 g. The first EM was presented at 2000, nine 33-h cycles were provided, and food intake and activity during the first eight feeding cycles were analyzed. Maintenance during the last cycle resulted in incomplete activity records.

**CONDITION 5**: 5.0-g EM. Access to food at the time of the EM was limited to 5.0 g. The first EM was presented at 0500, ten 33-h cycles were provided, and activity and food intake during the first eight feeding cycles were analyzed. During cycle 9 the feeding schedule and the light-dark cycle were advanced 1 h to bring them into conformity with the fall change in daylight savings time. Consequently, the last two cycles of the condition were not analyzed.

The time of the first meal changed across conditions, as did the specific mealtime sequence. Employing precisely the same mealtime sequence in each condition would have been ideal. Unfortunately, maintenance requirements prolonged some conditions beyond eight cycles. In such cases, beginning the next condition with a mealtime that had initiated the prior condition would have involved 1) altering deprivation levels going into the next condition, and 2) overrepresenting certain mealtimes. To avoid these potential confounds, from the start of condition 1 to the end of condition 5, meals were presented every 33 h.

**Data analysis.** Two analyses were used to assess the effects of manipulations on the distribution of next-day activity. The first analysis was used to determine whether a manipulation altered the time at which activity peaked the next day. For each rat, we found the average number of turns in each 5-min bin across the eight 33-h feeding cycles of a condition. We then smoothed each function with a 36-bin moving average. Within each function, the peak or acrophase occurring 22–33 h post-EM was located. Acrophase distributions resulting from each condition were then compared with repeated-measures t-tests.

The second analysis was used to reveal whether the level of activity in the vicinity of an acrophase was significantly greater than the level of activity in neighboring hours. Each rat’s mean condition acrophase was designated as the center of a 3-h bin. Mean activity within this bin and within the four neighboring 3-h bins was calculated. For each condition, the mean level of activity within these successive 3-h bins was compared with one-way repeated-measures ANOVAs and t-tests.

**Results**

Figure 1 shows the effects a long-T feeding schedule tends to have on activity (see Ref. 21 for further examples). Wheel-turning performance of rat 1A during the last eight feeding cycles of condition 2, the condition involving ad libitum access to pellets at the EM, is shown.

Figure 1, left, shows activity from a 24-h frame of reference, the period of the light-dark cycle. The actogram (top left) shows activity across successive days, and the average waveform (bottom left) shows the mean level of hourly activity across the 11 days depicted in the actogram. Activity tended to be concentrated in the dark, especially in the hours just after lights off, although considerable light-period running can also sometimes be observed (Fig. 1, left).

Figure 1, right, shows the same data from a 33-h frame of reference, the period of the feeding schedule. Generally, if data from animals in a 24-h period light-dark cycle are averaged across eight 33-h cycles, then, in the absence of other synchronizing events, a relatively flat function should be obtained. This is the case because light-entrained activity will have occurred 9 h later every cycle and would have been distributed evenly across the average 33-h period function. On the other hand, any systematic patterns that are observed in a 33-h period function can be ascribed to the effects of additional 33-h period synchronizers. Activity tended to peak ~24 h after EMs (Fig. 1, right). The peak can be ascribed primarily to the effects of the feeding schedule.

Figure 1 shows that the activity of this animal was simultaneously controlled by two different events having different periods. This pattern is the most common response to long-T feeding schedules.

The capacity of food intake to elicit circadian activity patterns was the phenomenon we wanted to understand. The continuous displacement of activity promoted by a long-T feeding schedule seemed like an ideal baseline against which to evaluate the effects of manipulations that might produce the pattern.

Figure 2 shows the effect of varying EM size. Figure 2, left, shows how mean hourly food intake was distributed when access at the EM was unrestricted (condition 2) and when it was limited to 10.0, 7.5, and 5.0 g (conditions 4, 3, and 5, respectively). As intake at the EM was progressively limited, rats compensated by
increasing intake during the first hour of the LM; as a result, average total cycle intake did not vary across conditions.

The effect that these distributions of food availability had on activity the next day is indicated in Fig. 2, right. A rather impressive sensitivity of next-day activity to the distribution of food intake on the prior day is revealed: when food intake at the EM was limited to \( \leq 10 \text{ g} \), the distribution of activity the next day occurred later. The right shift in activity with limited EM size (and increased LM size) was significant. Paired t-tests indicated that the mean peak of activity occurring 22–33 h post-EM occurred later in 5.0-, 7.5-, and 10.0-g EM conditions relative to the ad libitum (13.4 g) EM condition \( [t(5) = 5.335 - 3.410, P = 0.018] \). For the 5.0-g EM condition, mean activity in the acrophase bin was greater than the level of activity in the three preceding 3-h bins \( [t(5) = 4.348 - 5.423, P = 0.0074 - 0.0029] \). However, the level of activity in the acrophase bin was not different from the level of activity in the 3-h bin after it \( [t(5) = 2.371, P = 0.0639] \), perhaps because insufficient time elapsed between the acrophases and the next meal in the cycle to allow activity to bottom out. In addition to showing that the feeding conditions enhanced activity after a circadian interval, the analysis also suggested that the elevation in activity was probably at least several hours in duration.

Each panel in Fig. 3 contains data for an individual subject and shows the average distribution of activity across the entire feeding cycle for ad libitum EM and 5.0-g EM conditions. Activity tended to peak \( \approx 24 \text{ h} \) subsequent to the larger meal whether that meal occurred at the early mealtime (13.4-g EM) or at the late mealtime (5.0-g EM). The data in Fig. 3 demonstrate...
strate that the trends depicted in Fig. 2 can be readily observed in the performance of individual subjects.

Figure 4 reveals the implications of meal-engendered circadian activity patterns for the pattern of activity across the day-night cycle, on the basis of data from condition 2 (ad libitum EM). Each panel shows mean group performance during two consecutive feeding cycles.

Several important points are illustrated in Fig. 4. First, the time at which food is available has a profound effect on the distribution of activity observed the next day. This is especially clear when one looks at the effects of meals occurring at different times in the dark. As meals occur progressively later in the dark, a peak of activity tends to occur progressively later in the next dark period.

Second, some meals of a long-T schedule have the capacity to produce oscillating activity patterns. Again, this is evident from the effects of meals occurring in the dark. These meals produce a peak of activity at the same time of day both the next day and, remarkably, the next day after that. The second manifestation occurs even though sizable meals have occurred between the first and second manifestations.

Third, the effect that meals have on subsequent circadian activity patterns tends to be modulated by the time of day at which the meals occur. Meals occurring in the dark produce high-amplitude peaks that are well focused on the prior feeding time, whereas meals occurring in the light period are followed by a more distributed, lower amplitude pattern (though see EXPERIMENT 2: INTRAVENOUS INFUSION, SUBDIAPHRAGMATIC VAGOTOMY, AND CEA for a different pattern).

Fig. 3. Each panel shows a particular rat's average hourly feeding cycle activity in 13.4- and 5.0-g EM conditions. For each condition, hourly totals were averaged across feeding cycles, smoothed with a 3-h moving average, and expressed as percentage of maximum value obtained.

Fig. 4. Each panel shows mean hourly activity subsequent to EM occurring at a particular time of day. Access at EM was not restricted (condition 2). For each rat, hourly activity over 2 cycles was expressed as percentage of maximum hourly value observed during those 2 cycles. Hourly values were then averaged across rats. Panels are not arranged according to order in which meals were actually presented; instead they are arranged according to time at which initial meal occurred relative to dark period. Dark bars at top of each panel indicate when lights were off. ●, Time at which EM was presented and same time 1 and 2 days later. ○, Time of next EM in series and same time following day.
Discussion

Threshold. In experiment 1 we varied the amount of food that was available at the first meal of a two-meal series. As intake at the EM was progressively restricted, intake during the first hour of the LM increased. This pattern might be expected because a rat on a long-T schedule appears to try to eat to the capacity of its stomach when food becomes available. We reasoned that as the size of the initial meal decreased, it would be less likely to induce CEA. A threshold meal size for CEA induction was defined as a meal size that, when presented at the initial mealtime, produced a peak of activity ≈24 h subsequent to receipt. By this criterion a 13.4-g meal was above threshold, and 5.0- and 7.5-g meals were below threshold. The distribution of activity produced by a 10.0-g EM encompassed much of the range covered by distributions of activity produced by the other conditions; a 10.0-g meal may have been above threshold at some times of day and below threshold at others. According to Stephan (20), a 10.0-g meal is threshold for producing FAA in SCN-lesioned rats. The threshold for the establishment of FAA and the induction of CEA appear to be similar.

We expected a subthreshold initial meal to be followed by sufficient intake at the later meal to produce CEA, resulting in an activity peak ≈24 h subsequent to the later meal. Because the start of the initial meal and the start of the later meal were separated by 3 h, we expected the peak of activity to be delayed 3 h in subthreshold initial meal conditions relative to threshold initial meal conditions; this is precisely the result we observed.

In general, meals had the capacity to bring about abrupt changes in subsequent patterns of circadian activity, but the threshold for this effect appeared to be rather high. Induction of CEA would appear to involve an accumulator (which monitors the level of a single variable) and/or an integrator (which combines two or more variables), which determine and/or predict whether a threshold has been achieved.

Necessary stimulus. Meals of a long-T schedule appear to induce a circadian activity pattern. The specific aspect or aspects of a meal that must rise to threshold to produce induction are not clear. Necessary signals could be produced in a variety of alimentary/vascular compartments (mouth, stomach, duodenum, liver, blood) and could be related to a variety of effects that meals have (taste sensation, gastric distension, nutrient detection, metabolism).

The probable time course with which ingested food moved through the gastrointestinal tract made it unlikely that the inducing signal originated from only one of a variety of sites. In all meal size conditions, gastric emptying, flow of nutrient in the duodenum, and liver metabolism were probably more or less continuous, and therefore rather similar, from shortly after the start of an EM until several hours after the end of the LM. The lack of a highly differentiable signal at the gastric sphincter, the duodenum, and the liver suggests that simple accumulation of some variable at any one of these points might not have been sufficient to produce the differential results we observed between conditions.

The inducer may have been a function of simple accumulation of an event more highly correlated with the amount of food consumed, such as cephalic stimulation or gastric distension. The inducer could also have been an integrated function of more than one of the variables already mentioned, such as gastric distension and nutrient concentration in the duodenum.

An alternative to the possibility that CEA is induced by a meal having an absolute threshold size is the idea that CEA is induced by the larger meal of a two-meal series. Our design did not rule out this possibility, but the possibility does not seem parsimonious. To employ this strategy a rat would have to register the levels of the critical property in the EM and in the LM (this would involve sorting out the effects that the two meals had), compare the critical properties (this would involve making a comparison between events beginning hours apart), and completely discount the effects of one meal as a result of the comparison.

Oscillation. When food availability at the EM was not limited, EMs occurring during the dark period tended to be followed by peaks in activity both 24 and 48 h later; meals of a long-T schedule sometimes appear to produce oscillating activity patterns, demonstrating that a single experience with food at a particular time of day can have surprisingly long-lasting effects on activity patterns.

RF schedules provide a unique opportunity for studying circadian oscillations because they appear to induce oscillations. Light-dark cycles, in contrast, synchronize, but do not induce, the rest-activity cycle (18). The typical way to determine whether a meal-dependent circadian activity pattern can oscillate is to observe whether the pattern persists in a condition lacking periodic changes in environmental events (5). White and Timberlake (21) used this kind of approach to provide initial evidence for oscillation of CEA. The current results bolster that demonstration. More importantly, the results suggest that long-T schedules may provide a good means for studying oscillation of meal-gendered activity patterns. The procedure seems to entail several advantages: prolonged deprivation and special illumination conditions are not used, and a temporal segregation of CEA induction and expression occurs.

Modulation by time of day. Finally, the effect that meals had on subsequent levels of activity seemed to depend on the time of day at which meals occurred, with meals occurring during the light producing smaller effects than meals occurring in the dark. A light-period meal might produce a smaller effect on activity for at least two reasons: 1) because the meal occurs at a time when transduction of meal-related information is poor and induction is not accomplished; or 2) because, though induction has been accomplished, the activity occurs at a time of day when expression is not favored. In the first case the time of meal occurrence is never.
well registered, and in the second case the information the animal had previously acquired is not revealed in the dependent measure.

EXPERIMENT 2: IG NUTRIENT INFUSION, SUBDIAPHRAGMATIC VAGOTOMY, AND CEA

Experiment 1 suggested that some consequence of food intake accumulated to a critical level to induce CEA. The nature of this consequence was not evident. The purpose of this study was to begin to assess whether signals arising from several compartments of the digestive tract were necessary or sufficient for the induction of CEA.

The experiment involved two major manipulations. First, rats were fed intragastrically on a long-T schedule to determine whether odor, taste, and motor stimulation arising from eating were necessary for the emergence of CEA. If the accumulation of one or more oral-pharyngeal experiences were necessary for the induction of CEA, then preventing these forms of stimulation during the administration of large, nutrient meals would be expected to prevent the induction of CEA. The necessity of oral-pharyngeal experience in the emergence of meal-engendered circadian activity patterns has not previously been studied. Second, rats with complete subdiaphragmatic vagotomies were fed on a long-T schedule to determine whether afferent signals conveyed by the vagus nerve from the gastrointestinal tract to the brain were necessary for the emergence of CEA. The vagus nerve transmits information regarding a variety of events, including stomach distension, duodenal chemosensation, and liver metabolism (3, 10). If vagally mediated afferent information related to meal receipt is necessary for CEA induction, then subdiaphragmatic vagotomy would be expected to prevent the induction of CEA. FAA persists and perhaps can be shifted from one fixed time of day to another after vagal transection (4).

Method

Subjects. The same six male Sprague-Dawley rats from experiment 1 were used.

Materials. APPARATUS. Animals were housed in the same experimental stations.

DIETS. Rats received two types of diet. During the critical portion of each condition, they were given IG infusion of STAT (PRN Pharmacal), a high-calorie (5.5 kcal/g) diet containing 45% fat, 33% carbohydrate, and 3% protein by weight and supplemented with vitamins. Between critical conditions rats generally received standard Purina rat chow.

Procedure. The study was 2.5 mo in duration, and included three major conditions. CONDITION 1: IG NUTRIENT AT THE EM. Each feeding cycle consisted of a 1-h EM, a 4-h fast, a 1-h LM, and a 27-h fast. The period of food availability was 33 h. IG infusions were given during mealtimes. When an infusion was scheduled, each rat was removed, in turn, from the apparatus, and the end of a polyethylene tube was inserted into its mouth and advanced down its esophagus into its stomach. Solution was then infused into the stomach over a 15- to 30-s interval. Near the beginning of each EM, each rat was infused with 12.5 ml of STAT, and near the end of each EM, each rat was infused with an additional 2.5 ml of STAT. Fifteen milliliters of STAT contained 82.5 kcal, which is the caloric equivalent of ~25 g of grain-based Bioserv pellets. Because nutrient gastric infusions contained the calories needed to sustain an animal for an entire feeding cycle, no other nutrients were provided. Near the beginning and the end of the LMs, rats received IG saline infusions of corresponding volumes. The first feeding cycle began at 2300, at the end of the last cycle in experiment 1, and followed a 26-h deprivation. Nine infusion cycles were given, and activity during the last eight cycles was analyzed.

Beginning 1 h after the end of the last cycle, each rat was given ad libitum access to chow for 7 days. Protein content of the STAT diet is very low, and these ad libitum food-access days enabled animals to redress any protein deficits.

CONDITION 2: IG NUTRIENT AT LM. The procedure was similar, except that IG saline was given at the EM and IG STAT was given at the LM. The first cycle started after a 26-h deprivation at 1100. The condition was in effect for eight feeding cycles, and activity during these eight cycles was analyzed. Immediately after the end of the last cycle, animals were placed on ad libitum food availability.

CONDITION 3: SUBDIAPHRAGMATIC VAGOTOMY AND IG NUTRIENT AT EM. After nine days of ad libitum food availability, rats were removed from the apparatus, placed in hanging cages, deprived of food overnight, and then given complete subdiaphragmatic vagotomies.

Subdiaphragmatic vagotomy. The ventral and dorsal subdiaphragmatic vagal trunks were exposed by gently teasing them apart from the descending esophagus. The hepatic, celiac, and gastric branches were identified but not disturbed, and a 1-cm vagal segment well above (central to) these branches was identified for ligation. Each trunk was ligated with two ligatures ≥1 cm apart using 5-0 silk suture, and a 1-cm nerve segment was then cut between the ligatures. Each ligated end was then cauterized. The abdominal muscles and skin were then sutured in two layers, and all rats received 30,000 U ampicillin intramuscularly. This subdiaphragmatic vagotomy disconnected all subdiaphragmatic vagal afferents and efferents from the dorsal and ventral vagal trunks.

Surgery and initial recovery occurred from 0800 to 2000. Rat 1B did not survive the surgery. The remaining animals were returned to the experimental stations at 2000, at which time cyclic IG feeding was resumed. Infusions were given at the EM for nine 33-h cycles. Activity was monitored, but, not surprisingly given the recent surgery, patterns of activity were highly variable. Data from this portion of the condition will not be presented. During the remaining 9 days of the recovery period, rats had ad libitum access to chow.

In preparation for the final series of IG infusions, several steps were taken to clear residual matter from

SUBDIAPHRAGMATIC VAGOTOMY, AND CEA

EXPERIMENT 2: IG NUTRIENT INFUSION,
the rats' stomachs. Three and one-half days before infusions, Kat-A-Lax (Pitman-Moore) was rubbed on the shoulders of each rat; the preparation, which was subsequently ingested by the rats during grooming, breaks down inert matter in the stomach and otherwise clears the digestive tract. After a 31-h food deprivation, rats were given 46 h of access to Ensure (Ross), a nutritionally complete liquid diet. Seven hours before the first infusion, each rat was deprived of food, and 3 h before the first infusion, each rat's stomach was rinsed until clear of particulate matter with a series of 5.0-ml warmed saline washes, which were first infused and then withdrawn from the stomach through a polyethylene orogastric tube.

Finally, IG infusions were given as in condition 1. Nutrient was given at the EM for nine 33-h cycles, with the first EM in the series starting at 2300. Activity during the last eight cycles was analyzed.

Histological vagotomy verification. All rats were killed 5 wk after the end of the last condition (10 wk postsurgery) for histological verification of vagotomies. To verify the subdiaphragmatic vagal deafferentations, we used a fluorescent tracer strategy (19). Briefly, each animal received two intraperitoneal injections (0.5 ml) of Fluorogold (Fluorochrome, Englewood, CO) solution (2 mg/ml of saline). Three days after the Fluorogold injections, rats were anesthetized with pentobarbital sodium and perfused with two solutions into the left ventricle of the heart. The first solution consisted of 250 ml of PBS delivered over a period of 15 min. The second solution contained 500 ml of 4% paraformaldehyde dissolved in PBS.

The brain and brain stem were then removed and cryoprotected in 20% sucrose in PBS. The medulla was blocked and sectioned at 40 µm on a sliding microtome. Sections for the Fluorogold analysis were thaw-mounted on slides, air dried, dehydrated, cleared in alcohols and xylene, and placed under a coverslip with Permount (Fisher Scientific). Fluorogold label in the brain stem was examined with a Zeiss epifluorescence microscope. Because Fluorogold is taken up and transported retrogradely in intact neurons but not in neurons with transected axons, a successful subdiaphragmatic vagotomy was confirmed by the absence of label in the dorsal motor vagal nucleus, where the cut dorsal subdiaphragmatic vagal trunks would project. All rats were determined to have successful, complete vagotomies.

Results

Figure 5, top, shows the group-mean activity for intact rats given IG nutrient at the EM or the LM. Figure 5, bottom, compares intact and vagotomized rats given IG nutrient at the EM. Activity within the circadian range subsequent to IG nutrient receipt, whether IG infusion of nutrient was given at the EM or at the LM. The distribution of activity of vagotomized rats given IG nutrient at the EM seemed to be slightly delayed compared with intact rats receiving infusions at EM.

Mean acrophase during the LM-nutrient condition occurred significantly later than mean acrophase during either EM-nutrient condition [t(5) = 5.269, P = 0.0033; t(4) = 4.210, P = 0.0136]. Delaying the time of nutrient administration within a feeding cycle also delayed the time at which the peak of activity occurred. Statistically, mean acrophase was the same in intact and vagotomized rats given nutrient at the EM [t(4) = 1.916, P = 0.1279], even though four of five rats had a later acrophase in the vagotomized condition. Only rat 2A, an obvious outlier in two other conditions, failed to follow this general pattern.

In all conditions, the level of activity in the 3-h block centered around the acrophase was significantly greater than the level of activity in neighboring 3-h blocks. In both EM-nutrient conditions, mean activity during the 3-h acrophase block was significantly greater than the mean level of activity in the two neighboring blocks before and after the acrophase block [t(5) = 18.701–32.180, P = 0.0453–0.0072; t(4) = 3.911–4.922, P =
In the LM-nutrient condition, the acrophase block had a mean level of activity significantly greater than the level of activity in three of the four prior blocks \([t(5) = 8.846, P = 0.0003]\). In this condition no 3-h block ensued the acrophase block, because acrophases tended to occur late in this condition. Overall, in each condition the level of activity in the vicinity of an acrophase reflected an actual enhancement of activity.

Figure 6 depicts average activity across feeding cycles for individual intact rats given nutrient at either the EM or the LM. When nutrient was shifted from the EM to the LM, each rat exhibited a peak of activity later the next day. In addition to these activity peaks in the circadian range subsequent to meal receipt, several rats (rats 1A, 1B, and 2A) also manifested peaks that occurred earlier in the feeding cycle. At least some of these peaks could be ascribed to the capacity of CEA to oscillate.

Figure 7 shows average feeding cycle activity for each vagotomized rat receiving IG nutrient at the EM. Each rat had an activity peak that occurred \(\sim 24\) h after nutrient infusion.

Figure 8 shows how nutrient infusion at a particular time of day affected subsequent average patterns of daily activity. Data are from condition 1, which involved nutrient infusion at the EM. The distribution of daily activity seemed to be very sensitive to the most recent feeding time, with a peak of activity occurring at the last time that nutrient was received, whether nutrient infusion had last occurred in the light or in the dark. Oscillation is not evident in the group mean performance.

Acrophases occurring 22- to 33-h post-EM for each rat and condition of experiments 1 and 2 appear in Fig. 9.

**Discussion**

Experiment 2 represented an initial attempt to assess whether two classes of food-related stimulation played an important role in the early stages of CEA induction. We bypassed or minimized cephalic factors by giving IG infusions, and we assessed the contribution of vagal afferents from the gut by monitoring rats with complete subdiaphragmatic vagotomies. Neither manipulation prevented the appearance of CEA.
Intact rats and early nutrient. Intact rats given unsigaled IG nutrient at the EM manifested a peak of activity 24 h afterward. CEA could be elicited by nutrient even after elimination or minimization of meal-related taste, odor, and tongue and mouth movements. The result suggests that CEA induction depends on stimulating a site that is in the stomach or onward.

In experiment 1, 13.4 g (45 kcal) of food at the EM was sufficient to produce a peak of activity ~24 h later. In experiment 2, though the total nutrient infused at the EM had a much higher caloric content (82.5 kcal), the time course of the activity pattern was not significantly advanced. The time course of meal-elicited activity appears to be calorie compensated; once a particular caloric threshold is achieved, the pattern tends to have the same prepared circadian time course.

Each nutrient infusion in experiment 2 contained ~2.5 kcal of protein. A 5.0-g EM of experiment 1 contained 4.4 kcal of protein (and 16.7 kcal total) and was unable, by itself, to induce CEA; consequently, protein does not seem to play a necessary role in inducing CEA.

Intact rats and late nutrient. The activity of intact rats was not elevated 24 h after saline infusion at the EM. The result indicates that two classes of events present at the time of IG nutrient infusion at EM were not sufficient to induce CEA. First, these infusions involved special handling (removing an animal from the apparatus, restraining it, inserting an infusion tube, etc.). Enhanced activity 24 h later could conceivably have been a handling-induced stress reaction that had been linked to the time of feeding. Second, the infusions had probably generated a sudden, large, gastric distension signal. Pronounced gastric distension could conceivably be a necessary or sufficient signal for CEA induction. The fact that enhanced activity did not occur 24 h subsequent to the infusion of saline at the EM indicates that handling and acute distension did not play important roles in CEA induction.

Because saline solutions empty from the stomach much more rapidly than fat solutions (7), the duration of significant gastric distension was shorter in our saline condition than in our nutrient condition. Our results do not evaluate the possibility that an extended gastric distension signal is sufficient to induce CEA. The possibility could be evaluated by confining a saline load to the stomach with a pyloric cuff (16, 22). Gastric distension could also act in combination with nutrient-related signals to induce CEA.

Activity of intact rats was accentuated ~24 h subsequent to nutrient infusion at the LM. Shifting the availability of nutrient from the EM to the LM caused activity to peak 6 h later in the feeding cycle, a shift that agrees well with the 5-h difference in mealtime onsets.

Vagotomized rats. Distension in the stomach, nutrient in the duodenum, metabolism in the liver, and a variety of events in the gut produce afferent activity...
that is transduced and carried by the vagus nerve to the brain (3, 10). The information enables the brain to coordinate efferent activity involved in the processing of food (2, 14, 15). The vagus nerve would also seem to be well positioned to convey critical information to structures involved in the induction of CEA. Consequently, complete subdiaphragmatic vagotomy might have been expected to have some dramatic effect on the expression or time course of CEA.

Interestingly, rats given IG meals after complete subdiaphragmatic vagotomy clearly manifested CEA. The vagus nerve is not necessary for the induction of the circadian activity pattern. The results suggest that information necessary for the induction of CEA can reach critical substrates via a nonvagal pathway. One obvious possibility is that signals are blood borne. After vagotomy, four of five rats had right-shifted acrophases, suggesting that the vagus was somehow involved in altering the time course of CEA. The trend could not be statistically confirmed because of the small sample size. Further experiments using larger samples sizes will be necessary to determine whether vagotomy does in fact right-shift the acrophase of CEA. Further studies will also have to investigate the basis of such a shift. A delayed time course in vagotomized rats might simply reflect slowed gastric emptying; all essential signals and pathways might be intact, but, because of slowed transit in the gut, critical signals may get to critical substrates more slowly. Alternatively, a slowed time course may reflect a shift in the factors that induce CEA; in intact rats CEA may be induced by vagally mediated preabsorptive events such as gastric distension or nutrient detection in the duodenum, whereas in vagotomized rats CEA may be induced by later-occurring postabsorptive factors.

Oscillation and time of day modulation. As a group, intact rats manifested evident CEA in response to EM nutrient infusions occurring at all times of day. They did not manifest oscillating activity patterns in response to infusions occurring in the dark. The overall pattern is quite different from that observed in experiment 1. In the present study, the fact that nutrient infusions had roughly equivalent effects may be related to the high degree of control that we had over nutrient delivery; the time, duration, and amount of nutrient receipt varied very little across animals and feeding cycles. Alternatively, the different patterns observed between experiments 1 and 2 might have reflected an experience-dependent change in behavioral allocation that would have occurred in the absence of a change in nutrient delivery.

We have not yet encountered a convincing case where an individual rat simultaneously showed CEA in response to meals occurring at all times of day and oscillation in response to meals occurring in the dark.

GENERAL DISCUSSION

Methodological Issues

The most common procedure used to study meal-engendered circadian activity patterns schedules a single feeding opportunity at the same time daily. Such procedures produce FAA. Properties of the meal or characteristics of the animal are then varied. How such manipulations affect the expression of FAA can reveal factors that are critical for its development. Typically, investigators examine the effect a manipulation has on the presence or absence of FAA, its onset time, or its amplitude just before the next meal in the cycle (11).

The present studies used a different methodology. Several aspects of the procedure deserve note. First, meals were presented on a long-T schedule. In the two studies described here, meals were presented at 33-h intervals. Meals of a long-T schedule tend to produce an activity peak at approximately the same time the next day. In other words, the incessantly delaying mealtimes appear to continuously reinitialize the time course of the circadian pattern. We call the activity circadian ensuing activity or CEA. The importance a factor had in the emergence of CEA could be evaluated by examining the role that factor played in inducing the pattern.

Second, the period of the feeding schedule was 33 h. Because a 33-h period provided sufficient time for CEA to rise, peak, and diminish every cycle, we were able to use the acrophase of the pattern to assess what effect a meal manipulation had on the induction of the pattern. Furthermore, because meals occurred at eight representative times of day over a block of feeding cycles, we were able to average across a block and discern how meals affected activity independently of the effects produced by the light-dark cycle.

Third, we manipulated mealtime conditions such that one meal was likely to induce CEA, whereas the other meal was not. The practice allowed us to show that one meal within a cycle could induce CEA, even when, as a result of some manipulation, the other meal could not. As a result, the absence of CEA after the manipulation of some aspect of a meal could not readily be ascribed to some nonspecific effect of the manipulation. We did not systematically investigate situations where either both meals or neither meal had properties necessary to induce CEA. The practice also enabled us to stabilize food intake. Most of our experimental conditions restricted food availability at one of the feeding times. But total nutrient intake remained relatively constant from one cycle or condition to the next, because nutrient was available at the other feeding time.

Together, the results suggest further potentially powerful methodologies that could be used to investigate CEA induction. First, subthreshold intragastrically administered EMs could be given together with hypothetical inducing factors. Second, IG meals sufficient to induce CEA could be given just after administration of hypothetical blocking factors.

Properties of CEA

White and Timberlake (21) showed that the pattern of activity elicited by meals presented at 31- or 34-h intervals had a circadian time course, underwent large, abrupt daily displacements, developed quickly, did not extinguish, could be elicited by meals at any time of
day, and oscillated. All of these properties were observed again in this study. In addition, the present study showed that the induction of the process appeared to depend, at least in part, on receipt of a large nutrient meal, but did not depend on either a variety of oral-pharyngeal factors or afferent information arising from the vagus nerve.

Components Involved in CEA Synchronization

Much research has examined the manner in which light-dark cycles affect activity of the suprachiasmatic nucleus of the hypothalamus and synchronize circadian patterns. Much of what we know about the circadian timing system of mammals is based on this animal model (1, 8, 13). However, the model may not be a good source of hypotheses regarding the mechanisms by which food intake synchronizes circadian activity patterns. The synchronization of meal-dependent patterns involves a different zeitgeber, and the synchronization does not depend on the suprachiasmatic nucleus of the hypothalamus. A better source of hypotheses regarding mechanisms by which meals synchronize circadian patterns might be the digestive system.

The feeding system is very complicated. Food has a variety of potentially critical zeitgeber properties, including volume, nutrient concentration, total nutrient content, macronutrient composition, palatability, viscosity, pH, and so forth. Nutrient comes in contact with a variety of compartments, including the oral-pharyngeal compartment, the stomach, the duodenum, the liver, and the blood, and it stimulates afferent activity related to taste, gastric distension, duodenal chemosensation, and liver metabolism. It also produces a host of secondary effects (9). The induction of CEA could be related to effects food intake has on a variety of processes, including hunger, satiety, digestion, and metabolism.

Sketching a model of the factors involved in the induction and the expression of CEA is not yet possible. However, some of the possible conceptual components can be indicated. Experiment 2 indicated that neither extensive oral-pharyngeal stimulation nor vagal afferent activity were necessary for the induction of CEA. The result suggests that induction may be mediated by sympathetic activity or a blood-borne factor and/or may occur at a peripheral site.

Experiment 1 indicated that CEA was induced only by rather large meals. The result suggests that induction depends on some kind of accumulating or integrating process or mechanism. Vagotomized rats manifested CEA, indicating that the process did not depend on vagally mediated afferent activity related to gastric distension, chemosensation in the duodenum, or metabolism in the liver.

Experiment 2 showed that, although rats with complete subdiaphragmatic vagotomies manifested CEA, the peak of activity tended to be right shifted. Although the shift was not statistically established, it occurred in four of five rats. Although we did not investigate the basis of the shift, it was consistent with the possibility that a vagally mediated signal, acting early in the digestive process, was sufficient to induce CEA, and that, in the absence of this signal, induction was accomplished by some signal occurring later in the digestive episode. The result raises the possibility that induction can be carried out via multiple pathways.

Finally, experiment 1 revealed that meals presented during the dark period could induce a peak in activity both 24 and 48 h subsequent to receipt. The induction and expression of CEA appear to be mediated by a circadian oscillator.

CEA and Circadian Synchronization

The development of meal-engendered circadian activity patterns has been ascribed to entrainment and to zeitgedächtnis. Neither process can readily account for the labileness of CEA. Synchronization of a pattern is ascribed to an entrainment process when the pattern has a circadian time course, oscillates, and manifests corollary properties, such as the capacity to be synchronized only by events having a circadian period (18). FAA appears to have these properties (11). FAA and CEA share a variety of properties, including a 24-h time course, the capacity to oscillate, independence of the suprachiasmatic nucleus, the tendency, as the present studies showed for CEA, to be elicited only by large meals, and the capacity to be manifested in the absence of vagal afferent activity. However, a pattern resulting from an entrainment process would not be expected to manifest large daily displacements of the kind that CEA does.

Zeitgedächtnis or “time-of-day learning” is the capacity to use the circadian phase of an event to schedule functionally appropriate behavior at that time on subsequent days. Anticipating an event using a zeitgedächtnis process is thought to depend on experiencing that event at approximately the same time of day more than once (6). Because CEA is elicited by a single experience with food at a particular time of day, it would not seem to be the result of a process of zeitgedächtnis.

More generally, CEA raises questions about the nature of circadian synchronization and the function of the circadian timing system. Zeitgebers such as the light-dark cycle tend to be viewed as slow-acting events, and the rhythms that they synchronize have often been viewed as modulators of behavior (1, 8, 13). The present results indicate that some zeitgebers can significantly reorganize patterns of activity from one day to the next.

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