Feeding-entrained circadian rhythms in hypophysectomized rats with suprachiasmatic nucleus lesions

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Davidson, Alec J., and Friedrich K. Stephan. Feeding-entrained circadian rhythms in hypophysectomized rats with suprachiasmatic nucleus lesions. Am J. Physiol. 277 (Regulatory Integrative Comp. Physiol. 46): R1376–R1384, 1999.—Several pituitary hormones are important in the regulation of metabolism, and their release appears to be controlled by a circadian clock. Consequently, they may be involved in feeding-entrained circadian rhythms. Hypophysectomized (Hypox) and sham-operated male Sprague-Dawley rats had access to food for 4 h each day. Food-anticipatory activity (FAA) and core body temperature (Tb) were monitored. Both groups entrained to the daily meal with an increase in activity in the 4 h preceding meal access and quickly reentrained after an 8-h phase advance of food access. FAA was not disrupted in either group after suprachiasmatic lesions were added. Core Tb increased in the sham-operated subjects before mealtime, but Hypox rats failed to show this effect. Rather, Tb declined during anticipation and throughout the food access period. Respiratory quotient (RQ), an indirect measure of metabolic rate, was measured for 24 h in some subjects. Sham-operated rats showed a dramatic downturn in RQ 1 h before mealtime, whereas Hypox rats showed a steadily decreasing RQ throughout the day. The results show that the pituitary hormones are not necessary for FAA and that in Hypox rats the anticipatory rise in Tb and changes in RQ become dissociated from anticipatory behavior, indicating that these functions are separate outputs of the food-entrainable circadian oscillator.

Despite many attempts to identify the biological substrate for the FEO, its location has not yet been established (for review, see Ref. 20). Lesion studies targeting the paraventricular and lateral hypothalamic regions fail to abolish FAA (23). Lesions of hippocampus, amygdala, and nucleus accumbens yielded the same result (21). Although ventromedial hypothalamic lesions transiently eliminate FAA (22) and recent studies have suggested a possible role for these nuclei in food anticipation (7), the eventual recovery of FAA implies that the ventromedial nuclei are not the focus of the FEO but rather may be involved in its input or output pathways.

Neither subdiaphragmatic vagotomy (9, 25) nor peripheral deafferentation with capsaicin (10) attenuates feeding-associated rhythms. Therefore, it is unlikely that neural communication between the gastrointestinal (GI) system and the central nervous system is necessary for the expression of FAA. Because gut-brain communication is necessary for the expression of FAA, endocrine mechanisms are likely to be involved.

Serum concentrations of several humoral factors have been shown to change during the 2–3 h preceding mealtime. For example, serum glucagon levels are lower during anticipation than before FAA onset (11). Free-fatty acids and ketone bodies increase dramatically during anticipation in concert with a decrease in triacylglycerides (12). A causal connection between these substances and the FEO remains to be firmly established. Taken together, these studies suggest the importance of hormonal signaling in anticipatory activity and related circadian rhythms.

Links between circadian rhythms and pituitary function are well established. For example, the female reproductive cycle is heavily dependent on circadian output from the pituitary gland. Cyclic luteinizing hormone release is triggered by gonadotropin releasing hormone from the hypothalamus, which is under circadian control. Therefore, ovaries do not develop normally in female rats that are given SCN lesions 2 days after birth (26). Also, estrous cycles are disrupted by SCN ablation (19). Another example is the circadian rhythm of adrenal corticosterone release. Corticosterone release is regulated by ACTH from the pituitary, and both hormones show circadian fluctuations. ACTH release is controlled by the circadian release of corticotropin releasing hormone (CRH) from the hypothalamus (for review, see Ref. 17). The circadian corticosterone rhythm in the rat is abolished by SCN lesions (1) and by serotonergic deafferentation of the SCN (6). Thus the pituitary serves as an important output pathway of the SCN.
Circadian rhythms in GI function are also well documented. The gastric mucosa displays rhythms in mitotic activity in the rat (2). Mouse small intestine also shows 24-h rhythms in mitotic activity and DNA synthesis (27). However, these studies do not separate effects due to the light/dark cycle (i.e., SCN efferent activity) from food intake as entraining signals for these oscillations. This is important because nocturnal ad libitum-fed rodents essentially restrict food intake to the dark phase. However, after SCN lesions, circadian rhythms in small intestinal cell proliferation persist in mice (31). Furthermore, when food is restricted to the daytime, disaccharidase activity in the rat small intestine increases before mealtime (29).

Intestinal crypt cell villus length and cell number also increase before daily meals in rats (39). Because these functions become entrained to daily meals and are also influenced by metabolic hormones, these hormones could be an important component of feeding entrained circadian rhythms.

Some pituitary hormones may be important for food anticipatory rhythms. Corticosterone release is entrained by restricted feeding schedules. However, it is not yet clear whether release of hypophysial ACTH or CRH also peak before mealtime (14, 16). Thyrotropin releasing hormone (TRH) release, leading to thyroid hormone release, causes an increase in metabolic rate and core temperature (T_b). Because T_b is elevated during food anticipation (20), TRH release may correlate with or be causally related to FAA. Because adrenalectomy does not abolish FAA, the anticipatory rise in corticosterone does not trigger FAA (36). However, growth hormone (GH) and TRH are both major metabolic hormones that could be oscillatory outputs that are in pathways leading to behavioral activation. Additionally, feedback from peripheral metabolic hormones may modulate other clock outputs even if they are not causally related to their fluctuation.

One prior experiment showed that hypophysectomy severely suppressed anticipatory wheel running in rats (37). Although it appeared that the hypophysis was not necessary for the entrainment of FAA, the loss of its hormones seemed to have a profound effect on the level of FAA. In a follow-up study, one hypophysectomized (Hyphox) rat with an SCN lesion expressed low levels of FAA, whereas another did not (unpublished observation). Because of the small number of subjects, their low body weights (~80 g), and low levels of wheel running, these results were considered inconclusive.

The purpose of the present study was to reinvestigate the role of the hypophysis on FAA. Approach behavior to a feeder was used instead of wheel running because this type of behavior should be less affected by the small size of Hyphox rats compared with sham-operated controls. SCN lesions were added in the second part of the experiment. Subsequently, the entrainment of T_b was investigated. Finally, the respiratory quotient (RQ) was measured in metabolic chambers to gain a better understanding of metabolic rate in rats fed a restricted daily meal.

**METHODS**

**Subjects**

Ten adult male hypophysectomized Sprague-Dawley CD rats were purchased from Charles River Laboratories (Wilmington, MA). The subjects underwent pituitary removal at 45 days of age. Sham surgical controls (n = 9) were obtained from the same source and underwent identical procedures without removing the pituitary. All subjects were 52 days old on arrival at our lab, and mean body weights were 164.8 ± 1.9 g for the Hyphox group and 230.7 ± 2.9 g for the sham-operated group.

**Housing and Procedures**

Phase I: FAA. Eight Hyphox and eight control subjects were randomly chosen and transferred to plastic boxes covered with a gabled superstructure. The remaining three subjects (2 Hyphox, 1 control) were housed in stainless steel hanging cages, fed ad libitum, and kept as spare animals. A compartment attached to the side of the box provided access to food in a stainless steel tray. The feeder was mounted on a sliding carrier that was operated by air pressure under computer control. An infrared beam was mounted in front of the feeder. To access the food tray, rats had to break the photobeam and thereby activate a timer. This compartment was accessible whether the food tray was in the available or unavailable position. Approaches to the feeder (number of seconds the beam was broken) were recorded continuously by computer, stored on disk in 10-min time bins, and are presented by day of the year 1998 (see Fig. 2). The only activity in the room was the daily refilling of the food tray and water jar (between 0900 and 1100) and bedding changes twice per week. Initially, the room was kept on a 12:12-h light-dark cycle with lights on at 0800.

The food trays were loaded with ~30 g of a mash consisting of powdered chow and 5% sucrose in water. This mixture was used initially to ensure a sufficient caloric intake for the Hyphox rats. Additionally, the drinking water contained 5% sucrose. Subjects were later fed dry powdered chow (during restricted feeding) without any apparent change in behavior or loss of body weight. Also, sucrose was removed from the drinking water after 2 wk of restricted feeding without any reduction in intake.

A restricted feeding schedule was implemented on day 75 (see Fig. 2). Food access occurred at 1200 and lasted 4 h. Food was replenished each day after the end of food access (1600–1800). Both the light/dark cycle and the daily meal were phase advanced by 8 h on day 90. After the phase shift, food was replenished between 0800 and 0900.

Beginning on day 105 and continuing through day 108, subjects were given lesions aimed at the SCN (see SCN Lesions below). Subjects were returned to the approach boxes after surgery, and the lighting condition was changed to constant light (day 106). Restricted feeding was not interrupted.

Two Hyphox and two control subjects died during SCN lesions. They were replaced with the extra two Hyphox rats and one control rat for the second phase of the study. One additional control subject (SCN lesioned) was added to the experiment from another set of animals. This rat was obtained from the same source and had an age and weight similar to that of the other controls.

Phase II: Core T_b and FAA. Four subjects from each group were transferred to plastic chambers equipped for telemetry during restricted feeding. A stainless steel gate in front of the feeder was automatically raised and lowered slowly by air...
pressure for access to daily meals. Contact with the gate in the closed position was monitored by computer and stored in 30-s time bins. The computer sampled gate contacts at a rate of 20 Hz. If the animal was in contact with the gate, 50 ms was added to the 30-s time bin. The eight remaining subjects were kept in the apparatuses from phase I, and the feeding schedule was maintained.

One Hypox rat sustained a leg injury from the food gate and was therefore killed. Hypox rats had difficulty learning to retreat from the feeder when the gates lowered to block food access. To prevent further injuries, gates were lowered manually for the remainder of the experiment.

After 6 days of acclimation, the seven remaining subjects in the group received implants of radiotransmitters (Barrows, Magalia, CA; model T) in the abdominal cavity under methoxyflurane anesthesia (Metofane, Mallinckrodt Veterinary, Mundelein, IL). The calibrated transmitters had a pulse rate of ~500 Hz at 37°C, and changes in pulse rate were processed by software to provide mean T_b every 30 s with high resolution (about ±0.01°C). Transmitter signals were picked up by three antennae located under, behind, and in front of each chamber. A three-channel receiver combined the signals and sent the pulse rate to a computer once per second. Outlying pulse rates were removed by the software before the average T_b for each 30 s was stored. Data collected over each 24-h period were copied to the hard drive at midnight, thereby interrupting data collection for 2 min. The daily meal was 4 h in duration and began at 0500. This was a continuation of the post-phase shift mealtime, but the computer clocks were changed to daylight savings time.

After 2 wk of monitoring T_b and FAA, the seven subjects were removed from the boxes and killed. The remaining eight subjects were then transferred to the telemetry apparatuses and the same procedures were carried out. Because one Hypox subject died of an unknown cause and one sham-operated subject died of respiratory arrest during transmitter implantation, three rats from each group remained.

Phase III: Metabolic measures. After at least 6 days of T_b and behavioral measurement, the three remaining subjects in each group were transferred to a metabolic chamber, which is fully described elsewhere (28). Briefly, the chamber was individually housed inside an environmental chamber with precisely controlled ambient temperature (23 ± 0.1°C), T_b, O_2 consumption, and CO_2 production were calculated offline for every 2.5-min segment of a 24-h period beginning after the morning meal. Only one subject was studied each day. Subjects were not fed the scheduled meal the following morning until after they were removed from the chamber.

One week after the first run, the controls were returned to the metabolic chamber for a second 24-h period. Because of limited availability of the metabolic chambers, not all subjects from the experiment could be tested.

SCN Lesions and Histology

On days 105–108, all rats received bilateral electrolytic lesions aimed at the SCN (incisor bar +5 mm, anterior to bregma 1.3 mm, lateral ±0.2 mm, ventral to dura 9.5 mm). An anodal current (1.8 mA) was passed for 20 s through a stainless steel electrode with an uninsulated tip of ~0.5 mm. A mixture of ketamine HCl (62% Ketaset, Fort Dodge Animal Health, Fort Dodge, IA) and xylazine (38%; Xyla-ject, Phoenix Scientific, St. Joseph, MO) was used for general anesthesia (0.13 ml/100 g body wt).

After completion of the experiment, animals were deeply anesthetized with pentobarbital sodium and perfused with saline followed by 10% Formalin. Brains were carefully removed and inspected with a dissecting microscope for pituitary tissue. The brains were then postfixed in 10% Formalin overnight and cryoprotected in 30% sucrose-10% Formalin until they sank.

Serial sections through the hypothalamus were taken at a thickness of 40 µm on a freezing microtome. Sections were mounted on subbed slides and stained with cresyl violet for light microscopic inspection of the lesion damage.

RESULTS AND DISCUSSION

Hypophysectomy

Inspection with a dissecting microscope revealed no pituitary remnants in the hypophyseal fossa or on the ventral surface of the brain for any Hypox subjects. Lack of weight gain in the Hypox group relative to the sham-operated rats also confirmed successful hypophysectomy. Lack of growth hormone prevents weight gain after hypophysectomy (8). During the first 19 days in our laboratory, the Hypox group mean body weight dropped 4.9 g, whereas the sham-operated rats gained 73.9 g. None of the Hypox rats gained significant weight for the duration of the experiment in ad libitum or restricted feeding situations.

SCN Lesions

Light microscopic examination revealed that of the eight Hypox SCN-lesioned rats, four had complete bilateral ablation of the SCN. Two rats had partial lesions on one or both sides, whereas the last two had most of the SCN spared. Six rats in the sham-operated group had complete lesions, one had one-third of the nucleus spared unilaterally, and one had the complete SCN spared. Hypothalamic brain sections from four subjects with complete suprachiasmatic ablation are shown in Fig. 1. Because there were no differences in the measures of interest between subjects with complete and partial lesions, all subjects were included in the data analysis.

Phase I: Food Anticipatory Activity

After initiation of restricted feeding, most subjects in both groups displayed anticipatory behavior within a few days. Figure 2 shows representative event records for two Hypox rats (Fig. 2, A and B) and two controls (Fig. 2, C and D). Before restricted feeding, most controls and some Hypox subjects already showed elevated activity between 0800 and 1200, most likely because feeders were refilled daily during that time. During restricted feeding, this activity became elevated and less variable in onset. Figure 3A shows a distribution of activity averaged for each group over 5 days just before the phase shift. Both groups are clearly anticipating the daily meal: there is no difference in time of onset or amplitude of FAA. However, the sham-operated group displays anticipatory behavior within a 4-h period preceding food access and behavior during the rest of the 24-h period. Although...
the amount of time spent in front of the feeder within the anticipatory time period did not differ between the groups, there was an increase in total daily approach time in the sham-operated group (t-test, \( P < 0.03 \)). This difference is accounted for by the increased approach time during the non-FAA period (\( P < 0.05 \)).

Phase shift. Food access and the light/dark cycle were both phase advanced by 8 h on day 91. A phase shift was used to determine the ability of the Hypox subjects to reentrain to a new feeding time. The number of days required to reentrain was determined by visual inspection of event records. Reliability of the judgments was high between two raters blind to the treatment conditions (\( r = 0.97 \)). As has been reported before (33, 35), advancing transients are more difficult to discern than delaying transients. In the controls shown in Fig. 2, the onset of FAA advances for 3 or 4 consecutive days (group mean 3.25 ± 0.41 days) before reaching a phase position comparable to that before the phase shift. The Hypox rats entrained to the new mealtime slightly faster (2.25 ± 0.45 days; \( P > 0.05 \)). Two rats (1 in each group) did not fully reentrain to the new mealtime (Fig. 5). Instead, a “free running” rhythm, or delaying transients, with a period longer than 24 h is evident in food bin approaches for both rats during the 3 wk after the phase shift. The Hypox rat (rat 2) shows anticipation with a short phase angle simultaneously with delaying transients, whereas the control rat (rat 12) displayed no FAA at all until day 110, i.e., after SCN ablation. In SCN-lesioned rats, phase advances often result in delaying transients or split transients (33). Also, these subjects display delaying transients that reflect an endogenous period (\( \tau \)) that is too long to be mediated by the SCN but is consistent with FEO activity (32). A long
relative to the 24-h period of the feeding schedule would result in a decreased or negative phase angle, thus eliminating FAA. It is also possible that the SCN interfered with reentrainment of the FEO (34), because both animals resumed robust anticipation immediately after SCN lesions.

After SCN lesions, all subjects continued to anticipate the daily meal (see Figs. 2 and 3B). Indeed, many subjects increased FAA after lesions due to the reduction in nocturnal behavior. Figure 4B shows food bin approach behavior for the two groups after SCN ablation. The overall increase in approach activity in the sham-operated group that was evident before SCN ablation was no longer statistically different. Figure 1 shows the suprachiasmatic region for the four subjects used for the event records shown in Fig. 2. This comparison shows that complete SCN ablation is compatible with robust FAA in both Hypox and sham-operated rats.

Phase II: Core Tb and FAA

The SCN-lesioned rats were transferred to a different apparatus to record Tb and food gate contacts as a measure of FAA (see METHODS). Figure 6 shows gate contacts for the two groups averaged over 2 days. Again, anticipation of mealtime is evident in both groups, starting ~3 h before the daily meal. As before, controls displayed more succeeding activity than did the Hypox subjects. Mean group Tb for the same 2 days is shown in Fig. 7. The sham-operated subjects had an overall mean Tb of 36.8°C compared with a mean of 35.0°C for the Hypox group. An increase in Tb normally accompanies FAA (20) and is present in the sham-operated group beginning ~4 h before mealtime. How-
ever, the Hypox group shows a steady decline in \( T_b \) before the meal, and \( T_b \) did not rise until after the Hypox rats had started to consume food.

Although it is notable that hypophysectomy dissociated two common measures of feeding-associated circadian rhythms, the effect is most likely downstream from the site of the oscillator. The Hypox subjects were much smaller in size and therefore had less capacity for energy storage. It seems reasonable to assume that the Hypox rats did not have sufficient energy reserves to produce an elevation in \( T_b \). The increase in \( T_b \) immediately after some food had been consumed supports this notion.

Phase III: Metabolic Measures

The rats were studied in metabolic chambers to see whether the onset of FAA was accompanied by a shift from carbohydrate to fat metabolism as would be indicated by a reduction in the RQ. Figure 8 shows the mean RQ (\( \text{CO}_2/\text{O}_2 \)) and the mean \( T_b \) for three animals in each group during one circadian cycle. Core \( T_b \) in the Hypox rats did not decrease as dramatically in the metabolic chamber during FAA as was observed in the open-air boxes (Fig. 7). This could be due to a slightly higher ambient temperature in the metabolic chambers (23 ± 0.1 vs. 22 ± 0.5°C). The sham-operated group again showed the expected increase in \( T_b \) beginning ~3.5 h before the 0500 meal. This rise in \( T_b \) continued throughout the previously scheduled mealtime, although no food was presented in the metabolic chamber. In the Hypox rats, mean \( T_b \) decreased slightly during the “subjective” mealtime, suggesting a lack of energy reserves.

The RQ is an indirect measure of metabolic rate (13). Under ad libitum feeding conditions, rats have RQs of 0.85–1.0. An unexpected (1 time) food deprivation...
The sudden drop in RQ just before mealtime has been shown once before by Sugano (40), but that study was performed with intact rats kept on a light/dark cycle. Our findings indicate that neither the SCN nor lighting cues are important factors in this RQ shift before a scheduled meal. Instead, it can be concluded that the feeding schedule is responsible for this diurnal rhythm.

Escobar et al. (12) recently showed that there is an increase in the concentrations of circulating free fatty acids and ketone bodies and a concurrent decrease in circulating triacylglycerides during anticipatory activity in rats. These findings are consistent with the current results suggesting a shift in substrate utilization during food anticipatory activity. Also, we have shown recently that glucagon, a hormone that increases carbohydrate availability to systemic tissues, is reduced during FAA (11). This is also consistent with a model that predicts a depletion of stored carbohydrates a few hours before the scheduled meal.

Important questions remain regarding the relationship between the switch to lipid metabolism and the trigger(s) of FAA. First, the timing of the shift relative to FAA onset and mealtime is important to understanding any directional relationships between the two phenomena. It is possible that the metabolic shift is an entraining signal to the clock. This would require the clock to have a short $\tau$, because FAA and the temperature rise precede the metabolic shift. However, this seems unlikely because the Hypox rats anticipate behaviorally while not showing the metabolic shift before feeding.

Second, it remains to be shown that the FAA-related metabolic changes that are suggested in this study and others (e.g., 12, 40) persist during food deprivation after restricted feeding in rats with SCN lesions. Persistence of rhythmicity during constant conditions is an important feature of any variable that is under circadian control. Because the SCN is not necessary for the expression of FAA, the shift to lipid metabolism should also persist after SCN ablation if this shift is related to FAA.

It seems most likely that the shift in substrate utilization is an adaptive response to predictable daily fasting that is not under circadian control. Pigeons on restricted feeding show a similar pattern of RQ decrease before feeding concurrent with other common measures of circadian food anticipation (28). When the animals were fasted for 1 day, $T_b$ and $O_2$ consumption both followed normal patterns of anticipation indicative of circadian control, but RQ did not recover. Clearly, carbohydrate stores were depleted and could not be restored without caloric intake.

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**Fig. 8.** Mean profiles for respiratory quotient (RQ) and core $T_b$ for 3 subjects in each group. Although daily meal was not presented in metabolic chambers, dashed box indicates preceding food access time. Two days of data were used for each sham-operated subject (A), whereas only 1 day was used for each Hypox subject (B).

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 lowers the RQ to $\sim 0.75$. This decrease in RQ is indicative of a shift in substrate utilization away from carbohydrates and toward lipids. Under daily restricted feeding, subjects with sham hypophysectomies had an interesting temporal pattern of RQ (Fig. 8A). During 24-h food deprivation, intact ad libitum-fed rats show a steadily decreasing RQ throughout the day (15). This reflects the gradual depletion of stored carbohydrates and the resulting increase in lipid metabolism. Our food-restricted rats show a steady decrease in RQ from the time they were put into the metabolic chamber until $\sim 2300$. The mean RQ then levels off at $\sim 0.9$ until 0400. About 2 h after the anticipatory increase in core $T_b$ and 1 h before expected meal onset, RQ begins to drop dramatically. This occurs presumably because carbohydrate stores in liver and muscle tissue have been used up and lipid metabolism is providing most of the energy to the animals. Because no food was presented, the RQ continues to fall during the subjective mealtime. This pattern was different in Hypox rats (Fig. 8B). Hypox subjects showed a steady decrease in RQ during the entire day in the metabolic chamber.

The mean RQ never dropped <0.8 in either group. This is presumably due to an adaptation that allows rats on restricted feed to increase metabolic efficiency (18). Rats on scheduled meal feeding gain more weight and deposit more fat than do rats that nibble the same amount of food ad libitum. If the food deprivation had continued for another 24 h, it is expected that RQ would eventually reach levels that are seen in ad libitum-fed rats deprived of food for 24 h.

It is interesting that the Hypox group failed to demonstrate the sudden shift in substrate utilization. The steady decline in RQ results in a drop below 0.9 approximately 6 h sooner than in the sham-operated subjects. This is probably due to the lack of stored carbohydrate fuel available to the Hypox rats. The plateau that is present in the sham-operated animals reflects a period during which new fuel is not being taken in, but there is ample stored carbohydrate (e.g., muscle and liver glycogen) that can be mobilized to maintain a steady supply of metabolic fuel to the organism.

The sudden drop in RQ just before mealtime has been shown once before by Sugano (40), but that study was performed with intact rats kept on a light/dark cycle. Our findings indicate that neither the SCN nor lighting cues are important factors in this RQ shift before a scheduled meal. Instead, it can be concluded that the feeding schedule is responsible for this diurnal rhythm.

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Perspectives

F AA does not require pituitary hormones to be entrained or expressed. Although the T, increase preceding feeding is absent in Hypox rats, anticipatory approaches to the food cup are not affected when compared with sham-lesioned subjects. However, it is not yet clear if this disruption in T, is due to reduced capacity for energy storage or a regulatory deficit.

This finding rules out important metabolic hormones (i.e., GH, ACTH, TSH) as potential candidates for signaling behavioral anticipation in the feeding-entrained clock system. Evidence implicating GI physiology and energy homeostatic mechanisms in F AA is growing. A peripheral locus for the F EO is consistent with the notion that clocks are located near the site of action of their zeitgeber. This is true for the SCN and retinal clocks. Remaining potential signaling molecules to be investigated are gut peptides that have effects in the central nervous system. These include glucagon, motilin, substance P, vasoactive intestinal peptide, and cholecystokinin.

Further evidence suggesting a potential peripheral clock locus is the discovery of clock genes and clock-controlled gene expression in tissues of the digestive system and other organs (e.g., liver, kidney; Refs. 5, 30). Rhythmic gene expression in peripheral tissues of rats with SCN lesions is currently under investigation.

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