Feeding-entrained circadian rhythms are attenuated by lesions of the parabrachial region in rats

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It is well established that many species can entrain circadian rhythms to restricted daily meals. Wheel running, food-bin approach behavior, unreinforced bar pressing, serum corticosterone, core body temperature, and liver enzyme activity increase before a daily meal. Rats anticipate daily restricted meals with increased approaches to a feeder and an increase in core body temperature. Food anticipatory activity (FAA) is thought to be under the control of a feeding-entrained circadian oscillator. Although numerous forebrain lesions have failed to permanently abolish FAA, the hindbrain has not been investigated. The parabrachial nuclei (PBN) integrate information from visceral and gustatory afferents. This region is also innervated by neurons in the area postrema that have access to the peripheral circulation. Therefore, it is possible that this region plays a role in triggering FAA. In two experiments, a total of 19 rats were given ibotenic acid or electrolytic lesions targeted at the PBN. The PBN-lesioned animals showed a marked attenuation in anticipatory approaches to the food bin relative to sham-operated controls. Some animals did not anticipate the meal at all. In addition, the expected increase in core body temperature was severely attenuated in the PBN-lesioned animals compared with controls. The most likely interpretation of these data is that the PBN serve as a relay for information about the zeitgeber (food in the gut) or as a clock output pathway, but not as the site of the feeding-entrained circadian oscillator.

hindbrain; food restriction; core body temperature

Attempts to identify a biological substrate for the rat feeding-entrainable oscillator (FEO) have been focused primarily on the forebrain. Once SCN lesions were found to have no effect on FAA (9) or other food-entrainable rhythms (12), other hypothalamic nuclei were the next most likely site because of their involvement in feeding and energy regulation. Ventromedial nucleus (VMH) lesions were at first believed to abolish FAA (11), but it was later shown that after the active phase of weight gain was completed, anticipation recovered (15). The anticipatory rise in corticosterone was absent for 2–4 wk but recovered 8–10 wk after VMH lesion in rats fed a 4-h daily meal (10). When the meal duration was shortened to 1 h, the VMH lesions did not abolish the corticosterone peak before the meal. Radio-frequency lesions in the paraventricular hypothalamus (PVN) and ibotenic acid lesions in the lateral hypothalamic nuclei failed to abolish food-anticipatory circadian rhythms (17). Large lesions of the limbic system (e.g., hippocampus, amygdala, nucleus accum-bens) also did not prevent FAA (14).

Although the dopamine antagonist haloperidol did not affect FAA when injected systemically just before the daily meal (14), 6-hydroxydopamine injected into the ventral ascending noradrenergic fibers or into the PVN did eliminate the anticipatory rise in serum corticosterone in rats (9). This finding may suggest a role for the hindbrain in food-anticipatory processes, since this region is the source of the ventral ascending noradrenergic fibers. However, no behavioral measures of anticipation were taken in this study.

Other lesion studies have focused on the gut-brain pathways that might be critical for FAA. If the FEO is located in the brain, information about food must be transmitted from the gastrointestinal (GI) system to the brain to entrain the dock. It has been shown that caloric intake, but not volume or taste, determines the magnitude of phase-shifting transients when a restricted daily meal is presented 8 h later than it was presented previously (26). Other studies also indicate that olfactory (4) and gustatory cues (16, 27) are not sufficient or necessary for entrainment to daily meals. On the other hand, if the FEO is located in a peripheral structure, such as the liver or small intestine, the dock must signal the brain to trigger anticipatory activity. In either case, FAA requires gut-brain communication.
Subdiaphragmatic vagotomy removes a large proportion of afferent and efferent fibers from the GI tract but has no effect on FAA (5) or the anticipatory rise in corticosterone (18). Visceral deafferentation by injection of capsaicin into the peritoneal cavity also does not attenuate FAA (6). Given these results and the fact that the gut releases many peptides that have identified receptors in the brain, it is likely that the gut-brain communication necessary for FAA uses hormonal mechanisms. These mechanisms have not been identified.

One brain region that has not received much attention with regard to entrainment to meals is the hindbrain. Several nuclei that are important in feeding and hunger as well as regulatory processes are located in the hindbrain. The nucleus of the solitary tract (NTS) receives afferent information from the gut as well as the tongue. Nearby is the area postrema (AP), a region that lacks the blood-brain barrier, allowing blood-borne signals to act on the central nervous system (CNS). This could be an important location for the reception of humoral signals by the CNS.

The parabrachial nucleus (PBN), also located in the dorsal hindbrain, receives dense input from the NTS and the AP. One striking example of the integrative properties of the PBN is the elimination of taste aversion conditioning after PBN lesions (20). Conditioned taste aversion learning requires integration of taste and visceral information.

Given the convergence of sensory and humoral information in the PBN and the importance of the brain stem nuclei in basic regulatory processes, it seems reasonable that this area may be important to FAA. The present study used rats to investigate the effect of parabrachial lesions on FAA and the entrainment of core Tb to daily meals.

**METHODS**

**Experiment I**

Animals. Animals for experiment I were generously provided by the laboratories of Ralph Norgren at the Pennsylvania State University and James Smith at Florida State University. The animals were used for a conditioned food texture aversion experiment before they were used in this study (22). Briefly, 14 male Sprague-Dawley rats were obtained from Charles River Laboratories (Wilmington, MA). They were initially housed individually in stainless steel cages. A temperature-controlled (21°C vivarium, Hershey, PA) on a 12:12-h light-dark schedule, with food and water available ad libitum. After surgery (see below), the rats were shipped to Florida State University, where they were housed in similar conditions.

The rats were trained to consume their daily water within a 10-min period by successive reduction in the period of time it was available each day. Some rats were allowed to drink a mixture of sucrose and corn oil and then were injected with lithium chloride (0.6 M, 5 µl/kg body wt ip). Controls were given injections of isotonic saline. After the conditioning trial, animals underwent two-bottle preference tests for several days to determine whether taste aversions had developed to the sucrose and/or fat. After the two-bottle tests, the rats were transferred to the apparatus described below to participate in the study.

Surgery. Eight rats were given injections of ibotenic acid (0.2 µl, 20 µg/µl) bilaterally into the gustatory zone of the PBN. The pipette tip was targeted in the PBN by electrophysiological recording of field potentials while the tongue was washed with saline. The lesion procedure is described in detail elsewhere (20). A sham group (n = 3) was given saline injections into the PBN region, and a nonsurgical control group (n = 3) was also used. The rats were given 2 wk to recover from surgery before they were transferred to Florida State University.

Apparatus and procedure. Eight PBN-lesioned (PBNx), three sham-lesioned, and three intact rats were individually housed in feeder-approach boxes that are fully described elsewhere (27). Briefly, two compartments attached to one side of the plastic boxes provided access to food and water in a stainless steel tray and glass jar, respectively. The floor was mounted on a pneumatic slider that was under computer control, allowing for automatic delivery and removal of the floor tray. To access food and water, the rats had to place their front paws on a hinged pedal that was 12 cm above the cage floor. Pressure on the pedal activated a timer that was monitored by computer. The number of seconds of pedal contact per 10 min was stored on disk. Food and water were changed daily after the scheduled mealtime, and bedding was changed twice per week. The room was otherwise undisturbed. A 12:12-h light-dark cycle (lights on at 0700) was maintained in the room.

After 11 days of ad libitum feeding, food restriction (FR) was initiated. Food was available for 3 h beginning at 1400 (Fig. 1). Approximately 20 g of powdered rat chow were provided each day. At 1700, any remaining food was removed and the trays were refilled for automatic delivery on the next day. After 13 days of FR, the rats were deeply anesthetized and perfused with saline and then with 10% Formalin. The fixed brains were dissected out and returned to Pennsylvania State University for histology.

Coronal sections were cut through the pons on a freezing microtome. Alternate sections were stained with the cresyl violet and Weil procedures. The percentage of damage to the parabrachial region was estimated by light-microscopic examination.

**Experiment II**

Animals and surgery. Sixteen adult male Sprague-Dawley rats were obtained from Charles River Laboratories. The animals were housed in stainless steel hanging cages, with food and water provided ad libitum for several weeks. The room was maintained on a 12:12-h light-dark cycle (lights on at 0800). Average body weight before surgery was ~400 g. Ten rats were given bilateral electrolytic lesions aimed at the PBN (~3.3 mm incisor bar, 0.3 mm caudal to lambda, ± 1.8 mm lateral, 3.0 mm dorsal from interaural line). A mixture of ketamine hydrochloride (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 ip). Anodal current (1.5 mA) was passed for 20 s through a tungsten electrode with an uninsulated tip of ~0.5 mm. Some animals showed postsurgical motor deficits that subsided within 3–4 days. The six remaining rats underwent identical procedures without the current being applied to the electrode.

After recovery from the lesions, four PBNx and four sham rats received implants of radio transmitters (model T, Barrows, Magalia, CA) in the abdominal cavity under methoxyflurane anesthesia (Metafane, Mallinckrodt Veterinary, Mun-
mean $T_b$ every 30 s with high resolution (about changes in pulse rate were processed by software to provide chial nucleus-lesioned (PBNx) animal.

Timing of daily meal. Light-dark cycle is shown in open (light) and solid (dark) horizontal bars at top. Arrows, beginning of food restriction (FR). Solid horizontal bars at bottom indicate timing of daily meal. A: control; B: parabra-chial nucleus-lesioned (PBNx) animal.

delein, IL). Average body weight during transmitter implantation of all animals used in the experiment was 487.8 g.

Immediately after transmitters were implanted, the eight rats 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 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 were added to the 30-s time bin.

Core $T_b$ was monitored by telemetry. The calibrated abdominal transmitters had a pulse rate of $\sim 500$ 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 $\pm 0.01°C$). 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 room was maintained on a 12:12-h light-dark cycle with lights on at 0800. After 6 days of acclimation and baseline monitoring, an FR schedule was begun, with meal access between 1400 and 1800. Approximately 15 g of rat chow were provided each day. Food tray refilling was automated and occurred 5 min after the gates closed. The water and bedding in the chambers were changed twice per week.

Data were copied to floppy disk approximately every 3 days. Because of a flaw in the computer software, data were not properly saved on the mornings that copying occurred, yielding several half-days of gate approach and $T_b$ data. This problem was solved before the second group was put into the boxes. Analysis was performed using only data with consecutive complete days.

After 5 wk of FR, transmitters were removed from the first eight rats and implanted into the remaining eight rats (6 PBNx, 2 shams) 1 wk later. Animals were immediately transferred to the experimental boxes. Procedures identical to those described for the first eight rats were followed. All animals were killed 6 wk after initiation of FR for the second group. Final body weights were 381.8 g (controls) and 382.4 g (PBNx).

Histology. After completion of the experiment, animals were deeply anesthetized with pentobarbital sodium and perfused with saline and then with 10% Formalin. Brains were removed and postfixed in 10% Formalin overnight. They were then cryoprotected in 10% Formalin containing 30% sucrose until they sank.

Serial sections through the brain stem were taken at a thickness of 40 µm on a freezing microtome. Sections were mounted onto subbed slides and stained with cresyl violet for light-microscopic inspection of the lesion damage. Alternate sections were processed with a myelin stain (Mahon) to clarify the site of the lesion relative to the superior cerebellar peduncle.

RESULTS

Experiment I

Histology. Lesions were assessed bilaterally and scored according to the following criteria: complete, 100% destroyed; nearly complete, $\geq 80%$ destroyed; partial, 50–80% destroyed; miss, <50% destroyed. One rat had a complete bilateral ablation of the PBN. Two others had nearly complete lesions bilaterally. Three more had a complete lesion on one side and a partial lesion on the other side. The remaining two rats had partial lesions bilaterally or a partial lesion on one side with a miss on the other side. Because all rats showed a substantial reduction in FAA, none were omitted from the analysis.

Food bin approach behavior. Figure 1 shows event records of two representative animals from experiment I. Development of FAA in the control rat (Fig. 1A) is apparent by the 4th day of FR and is fully expressed by the 7th day. However, the PBNx rat (Fig. 1B) shows no anticipation for the full 2 wk of FR. Visual inspection of the event records indicated that six of the eight PBNx rats showed no tendency toward FAA and the remaining two rats exhibited a reduced magnitude of FAA relative to the controls. Figure 2 shows group averages of the pedal approach behavior during the last 3 days of restricted feeding. The controls show the expected increase in approach time beginning at about 1000. This rise is greatly reduced in the PBNx rats. Figure 3

Fig. 1. Representative event records of food bin approach time per 10 min from experiment I. Day of year is shown at left; light-dark cycle is shown in open (light) and solid (dark) horizontal bars at top. Arrows, beginning of food restriction (FR). Solid horizontal bars at bottom indicate timing of daily meal. A: control; B: parabra-chial nucleus-lesioned (PBNx) animal.
shows the mean approach time during the anticipatory “window” (4 h before meal access) and the approach time during the rest of the day (excluding mealtime) for the two groups (day 21, see Fig. 1). FAA was significantly reduced in the PBNx group (P < 0.02, t-test) compared with controls. Although the data from the PBNx animal depicted in Fig. 1B show a marked reduction in overall feeder approaches relative to Fig. 1A, there was no significant difference between the groups in daily approach time outside the period of FAA (Fig. 3). However, there was a significant correlation between FAA and non-FAA food-directed activity (r = 0.87 and 0.96 for PBNx and controls, respectively).

Experiment II
Lesions. All brain sections were traced onto paper with use of a projector, and lesion area and location were estimated visually by two experimenters. Each rat was assigned an arbitrary score between 1 (total bilateral miss) and 5 (=80% destroyed bilaterally) that indicated the completeness of the lesion. The interobserver correlation for the scores was 0.77. Of 10 PBNx rats, 2 received a score of 1 and were subsequently omitted from further data analysis. Three animals scored 2, two scored 3, two scored 4, and one scored 5. Figure 4 depicts representative sections including one (Fig. 4D) from a rat that was omitted from analysis. Although the lesions in the remaining animals were not complete, their data were analyzed because they showed a substantial attenuation of FAA.

Gate approach behavior. Figure 5 shows event records of gate contact time for three representative animals from experiment II. The control rat (Fig. 5A) showed some anticipatory activity just 4 days after the initiation of FR. Full FAA, along with the expected decrease in nocturnal food-directed activity, was evident after ~8 days. A PBNx rat (Fig. 5B) took longer to show anticipation (~7 days), displayed a much lower amplitude of FAA, and did not exhibit a decrease in nocturnal behavior for the first 20 days of FR (although the bulk of activity does seem to shift from late night to early night). Another PBNx rat (Fig. 5C) showed no indication of anticipation of the daily meal.

Figure 6 shows the mean gate contact time for 2 days during FR. The control group again displayed robust anticipation of the daily meal, whereas the PBNx rats showed a near-total absence of the behavior. The average nocturnal gate contact time looks similar between the groups. Figure 7 shows the mean contact time...
during anticipation and the remaining gate approach time. The anticipatory activity was significantly reduced because of the lesions ($P < 0.002$, t-test). This reduction in FAA caused a reduction in total daily gate contact time in the PBNx group ($P < 0.003$, t-test; not shown in Fig. 7). Gate-directed activity outside the period of FAA was not different between the groups ($P > 0.05$).

$T_b$. Visual inspection of the $T_b$ profiles for the PBNx rats indicated that five of the eight animals showed no anticipatory rise in $T_b$. The remaining three animals showed a rise preceding the meal with a reduced

Fig. 5. Representative event records of gate contact time per 30 s from experiment II. See Fig. 1 legend for explanation of horizontal bars at top and bottom. *, Days used for analysis. Gates did not come up on day 105, and there are missing data on days 106 and 107 because of computer malfunction. A: control; B: PBNx; C: PBNx.
amplitude and slope compared with controls. The two rats with poor lesions that were omitted from analysis showed a premeal rise in $T_b$ that was robust and comparable to that of control rats. Figure 8 depicts mean $T_b$ every 30 s for the two groups averaged over 2 days during FR. The overall mean $T_b$ was slightly lower for the PBNx group than for the controls ($35.95 \pm 0.01$ and $36.36 \pm 0.01$°C, respectively, $P < 0.01$). The premeal rise in $T_b$ for the controls was typical for rats fed a single daily meal. Area under the curve (AUC) was determined for the two mean profiles by first calculating the difference between each $T_b$ value and the nadir value. These differences were then summed for the time period between the nadir (for each profile) and the onset of the meal to yield AUC. The nadir for both groups occurred $-4.5$ h before the meal. For control rats, the AUC from the nadir to the beginning of food access was 539.86. The PBNx group showed a greatly reduced premeal rise with an AUC of 114.41, a nearly fivefold reduction in $T_b$ change. The nocturnal peak in $T_b$ for the two groups was similar in amplitude (37.09 and 37.12°C for PBNx and control, respectively) and phase (2208 and 2225 for PBNx and control, respectively). The rapid rise in $T_b$ during the 1st h of food access is evident in both groups but is more pronounced in the PBNx rats.

**DISCUSSION**

Lesions of the parabrachial region reduced, and in some animals abolished, FAA in the rat. The rat with the most complete lesion in experiment II (Fig. 4B) is the same rat that shows the least FAA (Fig. 5C). In addition, these lesions abolished or severely attenuated the increase in core $T_b$ before a daily meal but left the nocturnal peak unaffected.

Because ibotenic acid lesions resulted in a deficit similar to that observed in the animals with electrolytic lesions, it is unlikely that the destruction of fibers of passage (e.g., superior cerebellar peduncle, ventral spinocerebellar tract) can explain these results. One potential concern regarding the results was the possibility that PBNx rats would not eat as much and, therefore, would experience a less salient zeitgeber. Because the controls actually lost slightly more weight during FR than the PBNx animals, it is unlikely that food intake was a factor in the group differences.

In both experiments, the effect of the lesions appeared to be almost totally specific to the approach behavior to the feeder during anticipation (Figs. 3 and 7). Although group differences in non-FAA approaches were not statistically significant, PBNx rats appeared to show slightly less approach behavior overall than controls. Not surprisingly, within groups, non-FAA is positively correlated with FAA. However, although
there is considerable overlap between groups in non-FAA, there is no overlap in FAA, suggesting that the attenuation or abolishment of FAA is not simply the result of inactivity. Furthermore, because our measure of FAA is time spent at a location, and not activity, this relationship does not suggest that a general reduction in locomotor activity underlies our findings. Also, we found that hypophysectomized rats, although much smaller in size, far less active, and less able to regulate \( T_b \), still showed anticipatory approaches to the food bin (7). Gate touches and approaches to the feeder are not metabolically expensive behaviors. As indicated above, post-FR body weights did not differ between the groups, suggesting that food intake was similar and that PBN lesions probably do not reduce feeding motivation once food is accessible.

The small average premeal increase in \( T_b \) that is still present in the PBNx group (Fig. 8) was due to a premeal rise in \( T_b \) in only three of the eight animals. The smaller peak after meal onset may be the result of the digestive phase of diet-induced thermogenesis (2). Because the control rats were at a much higher \( T_b \) already at meal onset, the digestive phase of thermogenesis may not be expressed as readily in this group.

The duration of restricted feeding and activity monitoring postsurgery was an important consideration in this study because of the earlier finding that VMH lesions initially abolished FAA (11; see introduction). The duration of FR for experiment II was only 2 wk, but the lesions were made 14 wk before FR was initiated. In experiment II, the first group was kept on FR for 6 wk after surgery, whereas FR was initiated for the second group 9 wk after surgery and the days used for analysis were 13 wk after surgery. Because there was no indication of recovery of anticipation in the animals with the best lesions and body weight was not changing during the experiment except for the minimal weight loss that accompanies FR, it is unlikely that FAA varies as a function of elapsed time since surgery in these rats.

The large attenuation of FAA, despite the variability in size and location of the lesions, was somewhat surprising. Determining a specific location that caused the most disruption of FAA is not possible on the basis of the present data. Smaller lesions targeted throughout the PBN will be necessary to specify a critical region, if one exists.

It is not clear what these findings imply about the physiological mechanisms underlying FAA. The PBN may be a region where a clock output signal is received and relayed in the CNS to trigger the various secondary outputs that are mediated by the brain. In this case, the FEO could reside in a peripheral structure such as the liver or GI tract. Alternatively, an entraining signal...
may be coming from the gut after a meal and passing through the dorsal brain stem on its way to the FEO located somewhere in the brain that has not yet been the target of a lesion study. The third possibility is that the PBN, or a nearby structure, is the locus of the FEO. Although circadian clocks tend to be located near the source for their zeitgeber, such as the avian pineal, the SCN, and the retina, this third possibility cannot be ruled out.

Perspectives

The goal in the study of circadian rhythms that are synchronized by meal feeding is the identification of the biological substrate(s) responsible for this phenomenon. This has proven to be a frustrating goal for the past 20 years. The present study helps narrow the scope of experiments designed to achieve this goal.

Lesions targeting the NTS and the AP are underway in an attempt to establish the pathways that are involved in FAA. Because the PBN is heavily innervated by these closely related nuclei, it seems reasonable to assume that if a signal is being transmitted from the gut, whether it be an input to or an output from the FEO, it probably passes through these structures.

Clock genes and clock-controlled genes have been found in a number of mammalian structures not thought of as circadian clocks (8, 21, 29). In fact, rhythmic expression of several rat mRNAs (e.g., rev-erbα, rper2, DBP) has been measured in liver cells in vivo and in vitro (1). However, no studies have specifically identified high levels of any of these genes in the brain stem. Current studies in our laboratory attempt to measure expression of a number of genes at different times relative to food access in the rat brain and peripheral organs. The discovery of rhythmic expression of known circadian genes in SCN-ablated rats on a restricted feeding schedule would represent a leap forward in the study of feeding-entrained circadian rhythms.

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