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Diurnal Rhythms of Paraventricular Hypothalamic Norepinephrine and Food Intake in Rats

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MORIEN, A., P. J. WELLMAN AND J. FOJT. *Diurnal rhythms of paraventricular hypothalamic norepinephrine and food intake in rats.* PHARMACOL BIOCHEM BEHAV 52(1) 169–174, 1995.—Extracellular levels of endogenous norepinephrine (NE) within rat paraventricular hypothalamus (PVN) vary across the diurnal cycle, with a peak in NE level noted at the onset of the dark cycle, at which time feeding occurs in a burst. The present experiment further examined the relationship between food intake and extracellular levels of NE within the PVN and within sites located outside of the hypothalamus. Adult male rats were implanted with concentric microdialysis probes aimed at either the PVN or brain sites outside the PVN. Measures of food intake and of extracellular NE were collected every hour over a 24-h period. Rats with PVN probes exhibited two peaks in extracellular NE. The first peak in PVN NE occurred within 1 h before the onset of the dark phase (ZT11) and was significantly correlated ($p < 0.02$) with a marked burst of feeding during the first hour of the dark phase. In addition, a second NE peak occurred 8 h into the dark phase (ZT19) but was not accompanied by feeding. Rats bearing non-PVN probes did not exhibit alterations in extracellular NE yet did show a pattern of feeding similar to that noted in the PVN rats. These data support the hypothesis that the burst of feeding evident at the onset of dark is related, in part, to an increase in PVN extracellular NE. The presence of a second NE peak evident later in the dark phase, which was not accompanied by an increase in feeding, suggests that fluxes in NE secretion within the PVN may modulate feeding early in the dark phase and may be involved in the other functions later in the dark phase.

Microdialysis Feeding Norepinephrine Diurnal cycle Paraventricular hypothalamus Rat Adrenergic receptors

MICROINJECTION of exogenous norepinephrine (NE) into the hypothalamic paraventricular nucleus (PVN) stimulates feeding (2,6,14). The feeding evident after intra-PVN injection of exogenous NE is thought to arise from the activation of postsynaptic α_2-adrenoceptors (7). This view is supported by intra-PVN injection studies that document feeding after intra-PVN injection of drugs that activate α_2-adrenoceptors, such as NE and clonidine, and by studies in which NE-induced feeding is inhibited by intra-PVN administration of an α_2-receptor antagonist, such as yohimbine or rauwolscine (2,7).

The PVN is heavily innervated by ascending NE fibers that arise from the hindbrain (10,16). Presumably, these PVN terminals release NE that in turn induces feeding via activation of postsynaptic α_2-adrenoceptors. Although a variety of studies have examined the effects of microinjection of *exogenous* NE within the PVN on feeding, few studies have sought to relate changes in *endogenous* PVN NE to changes in feeding (8). Stanley and colleagues (15), for example, used the microdialysis technique (17) to determine whether the level of extracellular NE systematically changes within the PVN during the day–night cycle. In their study, rats were prepared with PVN microdialysis probes, and dialysate samples were quantitated every 2 h. Stanley et al. (15) reported that extracellular levels of NE within the PVN exhibited a marked increase (about 200%) at the onset of the dark cycle. Their study, however, did not simultaneously measure feeding with each microdialysate sample.

If this rhythm in extracellular NE within the PVN is to be

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viewed as a prominent factor in the induction of feeding at the start of the dark cycle, it is important to simultaneously record NE and feeding. It is also critical to assess whether other brain sites that are innervated by noradrenergic terminals, yet are not related to feeding, exhibit similar (or dissimilar) rhythms in the level of extracellular NE and food intake. Therefore, the present study examined the diurnal changes in extracellular NE, using the microdialysis technique, within the PVN and within non-PVN brain regions. In addition, food intake was measured hourly and compared with changes in extracellular NE over the 24-h period.

METHOD

Animals and Housing

Sixteen adult male albino Sprague–Dawley rats (350–400 g, Harlan Industries) were individually housed in polycarbonate cages in a temperature-controlled room (23 ± 1°C). The rats were placed on a 12L : 12D cycle, with lights on at 0700 h and off at 1900 h, and were allowed continuous access to tap water and to food (Teklad pelleted diet) except as noted below.

Surgical Procedures

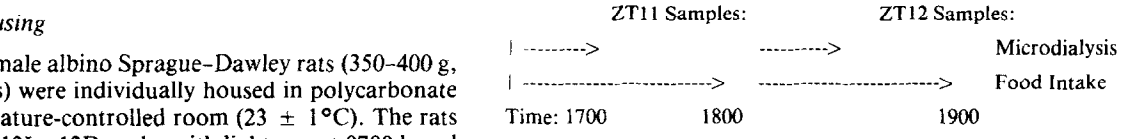
Each rat was pretreated with 0.4 mg/kg (IP) of atropine sulfate to minimize bronchial secretions during anesthesia. A combination of ketamine (Ketaset: 60 mg/kg, IP) and sodium pentobarbital (20 mg/kg, IP) was used to induce anesthesia (injections were spaced 5 min apart). Each rat underwent stereotaxic surgery in which a 20-ga stainless steel guide cannula (18), 17 mm in length, was implanted dorsal to the PVN. With the upper incisor bar positioned 3.3 mm below the interaural line, each cannula was implanted 1.7 mm posterior and 0.3 mm lateral to bregma and 7.5 mm below the surface of the skull. The cannula and associated T-bar were affixed to the rat skull using cyanoacrylate cement, stainless steel screws, and dental acrylic. The guide cannula was sealed using a stainless steel obturator. Rats were treated with penicillin (200,000 units, IM) after surgery and were allowed to recover for 7 days before undergoing microdialysis testing.

Microdialysis Procedures

Microdialysis probes similar to those described by Robinson and Wishaw (13) and Wellman (18) were prepared using a 250-μm diameter cellulose fiber (Spectrum membrane: 6000 molecular weight cutoff). The active length of the probe fiber was 2 mm with a 0.5-mm epoxy plug at the probe tip. Each probe was inserted into the PVN at either 1700 h or at 0500 h on the day prior to microdialysis testing and fixed in place within the guide shaft using a locking acorn cap and rubber o-rings (18). When inserted into the guide cannula, the stainless steel probe body extended for 1 mm beyond the guide cannula tip. This positioned the 2-mm active length of the probe through and slightly lateral to the magnocellular region of the PVN.

The microdialysis apparatus consisted of a Plexiglas chamber (30 × 30 × 60 cm high) with a wire floor and open top (3). A single-channel fluid swivel (Stoelting Model 50500) and associated stainless steel leash was mounted at the top of the chamber. An alligator clip soldered at the end of the leash served to connect the leash to the T-post mounted on the rat's head. PE tubing served to connect the swivel to a syringe pump (BAS model 100) equipped with a 10-ml gas-tight syringe (Hamilton) and through the leash to the inlet of the microdialysis probe.

Ringers solution (1.2 mm CaCl, 4 mm KCl, 145.8 mM NaCl) was pumped through each probe at a rate of 2.0 μl/min. The perfusate was collected in a plastic 400-μl Ependorph tube clipped to the swivel leash just above the rats head. Each rat was provided continuous access to food and water during the acclimation period and during actual testing. To establish the diurnal rhythm of endogenous NE within each rat, a 20-min dialysate sample was collected and quantitated every 60 min for 24 h. Half of the rats started microdialysis sampling at 0600 h (zeitgeber time, ZT23) and the other half started at 1800 h (ZT12). The temporal relation between hourly food intake measures and microdialysis sampling is depicted below:



Food intakes were recorded to the nearest 0.1 g with each intake sample starting at the hour and finishing 59 min later. Thus, food intake for ZT11 began at 1700 h and terminated at 1800 h. Microdialysis sampling for each hour began at the top of the hour and terminated 20 min later. Thus, the microdialysis sample for ZT11 began at 1700 h and terminated at 1720 h.

HPLC Technique

Each dialysate sample was analyzed by reverse-phase high pressure liquid chromatography with electrochemical detection (HPLC-EC, Model 400, BAS Co.). This system consisted of a 20-μl sample loop, a 10-cm column (3.2 mm bore, 3 μm particles, and C-18 packing) and two pulse-dampeners. The mobile phase consisted of 1.25 mM NaOH, 0.58 mM MCAA, 1.07 mM acetonitrile, 250 mg/l SOS, 33 mg/l EDTA, and HPLC-grade water, adjusted to pH 3.1.

Dialysate NE was oxidized with a glassy carbon electrode at an applied voltage of 0.699 V, and a retention time of 1.9 min. The minimal detectable level of NE (0.5 pg/20 μl) within the dialysate was three times baseline noise. The presence of NE within dialysate samples was verified by two methods: comparison of sample NE peak with a known NE standard (Sigma Chemical), and at the end of some testing sessions, tyramine (17.1 mg/10 ml Ringers Solution) was counterinfused through the probe membrane into the PVN. Addition of this compound to the Ringer's solution caused NE to be released from PVN storage vesicles within synaptic terminals, thus resulting in a substantial increase in the amount of NE within the dialysate (n = 4 rats, 81% increase over baseline).

Histology

At the completion of behavioral testing, each rat was removed from the microdialysis testing chamber and returned to the home cage. Each probe was left in place after testing to allow for development of gliosis around the probe membrane (this aided visualization of the probe track during histology). Two days later, each rat was deeply anesthetized with sodium pentobarbital (60 mg/kg, IP) and intracardially perfused with 0.9% saline followed by 10% formalin. Brains were removed and further fixed in 10% formalin for 72 h, and frozen sections were photographically enlarged (×7). The location of each probe track was determined by comparing photographs to the plates of the Paxinos and Watson (12) rat atlas.

Statistical Analyses

The histological analyses were used to form two groups of rats. The first group (group PVN) consisted of six rats for which the probes were found to lie within the boundaries of the PVN. In addition, a second group of rats (group CON: $n = 5$) was formed using rats with probe placements outside the PVN such as the septal nuclei and the ventromedial thalamus. These CON rats exhibited detectable levels of NE within the dialysate and thus these rats served as an anatomical control group. The remaining five rats had probe placements outside the PVN and did not exhibit detectable levels of NE within the dialysates.

The dependent measures of this study were hourly NE levels (pg/20 μl) and food intake (g) recorded every hour during a 24-h period. Average NE ($\pm$ SEM) and FI ($\pm$ SEM) per group during the light phase and dark phase are reported. Differences between groups were analyzed using a 2 (group: PVN vs. CON) $\times$ 24 (time: 24 h) mixed model ANOVA (1). Duncan's Multiple Range Test ($p < 0.05$) determined whether groups differed at each hour (comprising the hours ZT0–23). In addition, separate ANOVAs were computed for both NE and FI within each group (PVN, CON) across the 24-h cycle. The specific times at which NE (or FI) significantly differed over time was determined by comparing the average NE (or FI) to NE (or FI) to all time points (SAS User's Guide: Statistics, Version 5.0, 1985). Difference probabilities of less than 0.05 were deemed statistically significant.

RESULTS

Figure 1 (top panel) depicts the average extracellular level of NE during the light phase (ZT0–ZT11) and the dark phase (ZT12–ZT23) for rats in group PVN and group CON. The average level of extracellular PVN NE during the light phase was 18.1 $\pm$ 1.6 pg/20 μl whereas the average level of PVN NE during the dark phase was 20.4 $\pm$ 2.4 pg/20 μl. In contrast to PVN NE measurements, the average level of the control group's extracellular NE was relatively unchanging across time. The average level of extracellular NE of the CON group during the light phase was 10.4 $\pm$ 4.0 pg/20 μl whereas the dark phase average was 9.9 $\pm$ 3.7 pg/20 μl. During the day, there was no significant difference between CON and PVN groups in average group level of extracellular NE ($p < 0.1136$) whereas group PVN exhibited significantly higher levels of NE relative to group CON during the night ($p < 0.04$).

Figure 1 (bottom panel) depicts the average food intake consumed by rats in group PVN and group CON during the light phase and during the dark phase. The PVN group ate an average of 0.4 $\pm$ 0.07 g/h during the light phase whereas the average FI during the dark phase was 0.8 $\pm$ 0.1 g/h. For rats in group CON, FI during the light phase was 0.35 $\pm$ 0.08 g/h whereas the dark phase average was 0.78 $\pm$ 0.21 g/h. There were no significant differences in food intake between groups CON and PVN during the day or the night ($p > 0.05$). Thus, the day/night pattern of feeding evident in group CON was similar to the PVN group's feeding pattern during the light/dark cycle.

The top panel of Fig. 2 displays the hourly changes in extracellular NE for rats in group PVN and group CON. Rats in group PVN exhibited two peaks in extracellular NE over the 24-h period. The first peak rose from a basal value of approximately 19 pg/20 μl to nearly 28 $\pm$ 4.4 pg/20 μl at 1 h before the onset of the dark cycle (ZT11) whereas a second peak of NE reached an asymptote of 26.2 $\pm$ 4.6 pg/20 μl some 8 h into the dark phase (ZT19). An ANOVA computed

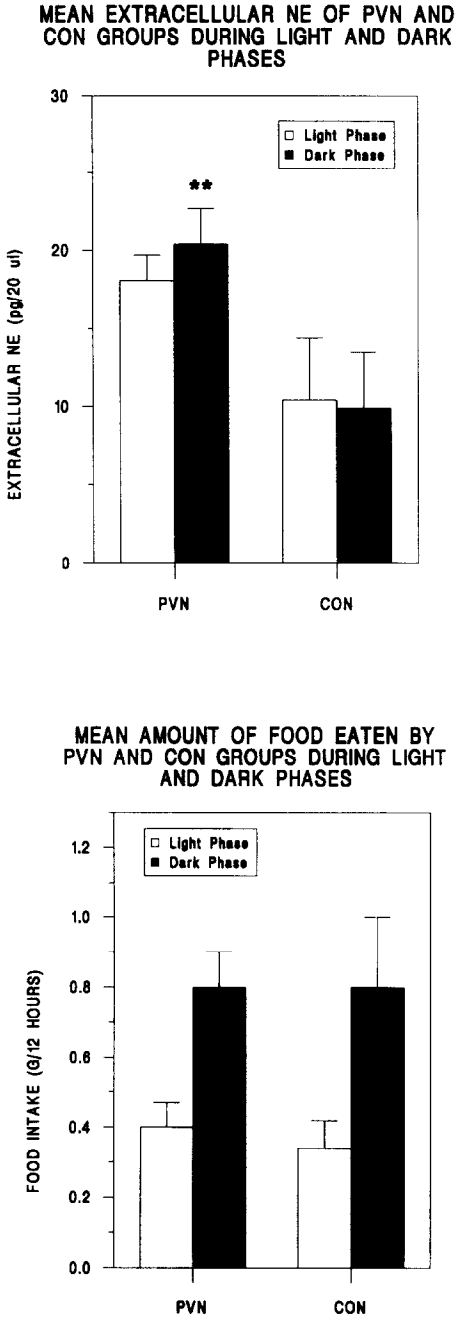


FIG. 1. Mean group values of extracellular NE (top panel) and food intake (bottom panel) for rats with microdialysis probe placements within the PVN (group PVN) or outside the PVN (group CON) during the light phase (open bar) and the dark phase (filled bar). The line above each bar represents the standard error of the mean. **Significant difference between light and dark phases for a group.

for changes in NE in group PVN across the 24-h period revealed a significant effect of time, $F(23, 115) = 1.7$, $p < 0.04$. Subsequent contrasts of each hourly NE value with average NE revealed that NE at ZT3 was significantly less than average NE ($p < 0.04$), that NE at ZT11 was significantly greater than average NE ($p < 0.04$), but NE at ZT19 was not significantly greater than average NE ($p < 0.07$). In contrast,

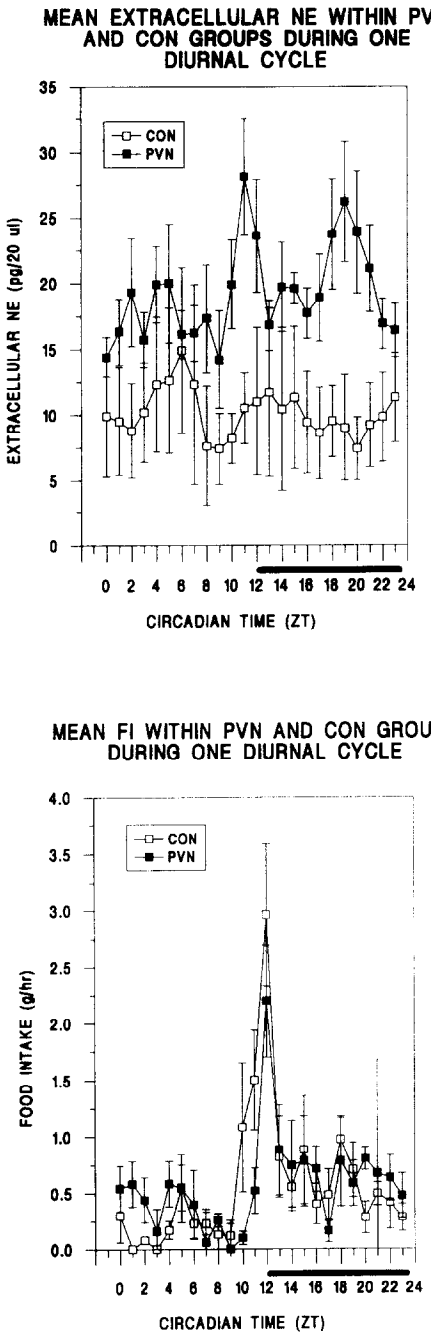


FIG. 2. Mean group hourly extracellular NE (top panel) and hourly group food intake (bottom panel) over a 24-h period for rats in group CON (open square) and for rats in group PVN (filled square). The line above each symbol represents the SEM. The horizontal dark bar extending from ZT12 to ZT24 represents the dark phase.

rats in group CON exhibited a stable level of extracellular NE over the 24-h diurnal cycle. A final ANOVA computed using NE across the 24-h period revealed no significant difference in group CON across time, $F(23, 92) = 1.07, p < 0.39$.

Figure 2 (bottom panel) depicts the changes in hourly food intake for rats in groups PVN and CON. Hourly food intakes within both groups fluctuated in a similar pattern, with the greatest increase in feeding evident at ZT12. Further analysis of the food intakes noted in the PVN and CON groups over a 24-h period using split-plot ANOVA revealed no significant effect of group, $F(1, 9) = 0.0, p < 0.99$, nor an interaction, $F(23, 207) = 1.2, p < 0.22$; however, a significant effect of time was evident, $F(23, 207) = 6.9, p < 0.0001$. Subsequent contrasts revealed significant increases in food intake from average food intake in group PVN ($p < 0.009$) and group CON ($p < 0.037$) only at ZT12. These results suggest that the pattern of feeding is not significantly different between the PVN and CON group. The finding that PVN and CON rats did not differ in feeding during the 24-h time period suggests that the placement of a probe near the PVN does not interrupt normal circadian feeding.

A final set of correlational analyses was computed for NE and food intake using data from periods at which NE peaked within the PVN. In the first analysis, extracellular NE level within the PVN at ZT11 was correlated with food intake during the periods of ZT11, ZT12, and ZT13. This analysis revealed a correlation significantly greater than zero between NE at ZT11 and food intake at ZT12 ($r = 0.89, p < 0.02$) but no significant relation between NE at ZT11 and food intake at ZT11 ($r = 0.65, p < 0.156$) or NE at ZT11 and food intake at ZT13 ($r = 0.34, p < 0.509$). A second analysis examined whether there were any correlations evident between NE and food intake at the second peak of NE during the dark phase. This analysis between NE level at ZT19 and food intakes at ZT19, ZT20, and ZT21 resulted in nonsignificant rs of -0.22, -0.8, and 0.17, respectively. Thus, only the increase in extracellular NE within the PVN at the start of the night phase was significantly correlated with increased food intake.

DISCUSSION

The present experiment examined the changes in extracellular NE within the PVN for rats over a 24-h diurnal cycle. These results confirm the original findings of Stanley et al. (15) in which a marked peak in extracellular NE within the PVN was noted at the onset of dark. However, the precise timing of the first NE peak relative to dark onset differs between these two studies. Our findings indicate that PVN NE reached an asymptote 1 h prior to the onset of the dark phase whereas Stanley et al. (15) noted that asymptotic levels of extracellular NE within the PVN were attained within an hour after dark onset. These differences in timing of an NE peak at the onset of dark may, in part, reflect the different sampling intervals (hourly vs. every 2 h), slightly different testing procedures (a fixed start for sampling vs. the counterbalanced start time used in the present study), or may reflect differences in the ionic composition of the Ringers used in each study (3.37 mM $CaCl_2$ vs. 1.2 mM $CaCl_2$ used in the present study) or a combination of these factors.

In addition to confirming the presence of a rise in extracellular NE at the onset of dark, the present study also noted a second PVN NE peak, equivalent in magnitude to that noted at the onset of dark, which occurred approximately 7 h after the onset of the dark phase. This second NE peak is also evident in the report of Stanley et al. (15), who noted a second peak in PVN NE that occurred 8 h after onset of dark (ZT19) but that was slightly smaller in magnitude than the early NE peak evident at the onset of dark. These results suggest the existence of a reliable rhythm in PVN extracellular NE with

two peaks evident: one at the onset of dark and another some 8 h into the dark cycle.

Because the present study assessed hourly food intake as well as extracellular NE, it was possible to determine the covariation of these measures across the 24-h cycle. In the present study, food intake measures were recorded every 60 min whereas microdialysis measures were recorded for a 20-min period that occurred at the beginning of the 60-min food intake sampling period. The present results indicate that a significant increase in food intake was noted at ZT12 and was preceded by a peak in NE at ZT11. Correlational analyses revealed that the peak in NE at ZT11 was significantly correlated with the peak in food intake occurring at ZT12. In contrast, although NE within the PVN increased over baseline at ZT19, this increase was not significant nor was it accompanied by a significant increase in food intake.

These data make clear that a rise in PVN NE alone is not sufficient to stimulate food intake in that, although the increases in PVN NE noted at ZT11 and at ZT19 were similar in magnitude, food intake increased significantly only at the onset of the dark cycle. One possible explanation for this dissociation is that endogenous NE secretion and food are independent phenomena that exhibit coincidental covariation at the start of the dark cycle. This notion, however, would suggest that these processes are coupled in time but are otherwise uncorrelated. However, a correlational analysis computed using extracellular NE and FI at ZT11 revealed that increases in NE at ZT11 reliably predicted increases in food intake at ZT12 ($r = 0.89$, $p < 0.02$). Moreover, in a recent experiment, Paez et al. (11) examined the association between PVN NE levels and the size of meals noted during the first 2 h of the dark phase. Their results document that PVN NE levels were greater when rats consumed a large meal than when these rats consumed a small meal. If PVN NE and food intake were truly independent phenomena, one might expect a random relation between NE level and the magnitude of a meal during the onset of the dark phase.

Another explanation for the dissociation between food intake levels and the two PVN NE peaks may be a function of the quantity of NE and the sensitivity of postsynaptic PVN α_2-adrenoceptors. Prior studies have suggested that NE interacts with these PVN receptors to stimulate feeding (2,7). A distinct diurnal rhythm in PVN α_2-adrenoceptor binding was described by Jhanwar-Uniyal, Roland, and Leibowitz (4). They reported a significant increase in the number of α_2-adrenoceptor binding sites at the onset of the dark phase, whereas less binding was evident 7 h into the dark phase. In addition, far less binding occurred several times within the light phase. This suggests that a large meal may occur at the onset of the dark cycle because the increase in extracellular NE level coincides with an increased number of α_2-adrenoceptors within the PVN. Later in the dark, an equivalent pulse of NE may not evoke another large meal because the number of α_2-adrenoceptors binding sites within the PVN has declined, and because other feeding-inhibitory mechanisms may come into play after consumption of that initial meal at dark onset.

The present study also examined whether sites outside the PVN exhibit this rhythm in extracellular NE. An anatomical control group was used in our study that consisted of rats with probes sampling from sites outside the PVN (primarily the septal nuclei) but that exhibited detectable levels of NE. These rats did not exhibit day/night variation in NE secretion, yet displayed a feeding pattern comparable to that of the PVN group. The anatomical control procedure used by Paez et al. (11) also noted a similar lack of correspondence between NE levels within non-PVN sites and food intake.

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