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Diurnal Changes in Paraventricular Hypothalamic α_1 and α_2-Adrenoceptors and Food Intake in Rats

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MORIEN, A., M. V. CASSONE AND P. J. WELLMAN. *Diurnal changes in paraventricular hypothalamic α_1- and α_2-adrenoceptors and food intake in rats*. PHARMACOL BIOCHEM BEHAV **63**(1) 33–38, 1999.—The prominent feeding rhythm evident in rats may reflect circadian variation in activity of feeding-relevant adrenoceptors within the hypothalamic paraventricular nucleus (PVN). In the present study, separate groups of rats were sacrificed at six time points (ZT0, ZT4, ZT8, ZT12, ZT16, ZT20) over a diurnal cycle. Food intakes were recorded during the 4-h period prior to sacrifice in each group. Brain sections were incubated with either an α_1-adrenoceptor ligand (^{3}H)-prazosin [(^{3}H)-PRZ] or an α_2-adrenoceptor ligand (^{3}H) para-aminoclonidine [(^{3}H)-PAC] prior to autoradiography analyses. Binding of (^{3}H)-PRZ within the PVN varied as a function of the diurnal cycle, with significantly greater binding evident during the light phase of ZT0 (first 4 h of the light phase) and at ZT4, compared to nadir binding during the dark phase at ZT16 (first 4 h of the dark phase). Binding of (^{3}H)-PAC within the PVN also varied as a function of the diurnal cycle, with significantly greater binding evident during the first 8 h of the dark phase (ZT16 and ZT20) than during the light phase. Food intake and α_1-adrenergic binding were inversely related across the diurnal cycle. These results support the hypothesis that PVN adrenergic systems may be organized in an antagonistic fashion so as to modulate feeding in the rat. © 1999 Elsevier Science Inc.

Prazosin *para*-Amino-clonidine Autoradiography

ADRENOCEPTORS localized on neurons within the hypothalamic paraventricular nucleus (PVN) modulate feeding in the rat (24). Microinjections into the PVN of α_1-adrenoceptor agonists result in dose-dependent reductions in food intake (3–5,22), whereas intra-PVN microinjections of an agonist at the α_2-adrenoceptor stimulate feeding (6,15). These mutually antagonistic effects on feeding induced by adrenoceptor agonists are likely due to changes in cell membrane potential brought on by interactions with adrenoceptors within the PVN. In support of this notion, Kow and Pfaff (14) noted the induction of excitation in a subpopulation of PVN cells following bath application of the α_1-adrenoceptor agonist phenylephrine, whereas neuronal inhibition was evident after application of an α_2-adrenoceptor agonist. Further support is derived from studies in which the effects of intra-PVN injections of α_2-adrenoceptor agonists on feeding are reversed by prior treatment with an α_1-adrenoceptor antagonist such as prazosin (23).

That PVN adrenoceptors modulate feeding leads to consideration of whether these receptors might vary across the day/night cycle in such a way as to contribute to the prominent circadian feeding rhythm evident in the rat (1,19,20). Prior studies reveal a marked increase in α_2-adrenoceptor number within rat PVN just at the onset of the dark phase (10,15). Limited information is presently available as to whether brain α_1-adrenoceptors vary over a diurnal cycle. The intent of the present study was thus to examine potential changes in α_1-adrenoceptor number over a 24-h diurnal cycle in rats using the autoradiographic technique (16). Of particular interest were potential changes in α_1-adrenoceptors within the PVN, and the relation of these changes to feeding and drinking. Accordingly, food and water intakes were assessed every hour during the 4 h preceding sacrifice. To compare these results with earlier studies, α_2-adrenoceptor activity was also assessed in the same animals. Finally, to determine if the rhythms of α_1- and α_2-adrenoceptors are specific to the PVN,

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adrenoceptor ligand binding was also assessed in a variety of brain sites outside the PVN.

METHOD

Animals

Adult male Sprague–Dawley rats were maintained on a commercially available pelleted dry-food diet (Teklad) and tap water. The rats were individually housed in hanging polycarbonate cages in a temperature-controled room (23 ± 1°C). Each cage contained a wire floor over a paper pad (used to collect food spillage). The rats were placed on a 12:12 light:dark (LD) cycle with lights on at 0600 h (ZT0) and off at 1800 h (ZT12).

General Autoradiographic Procedures

Thirty rats were randomly assigned to one of six possible groups (i.e., five rats per time period: ZT0, ZT4, ZT8, ZT12, ZT16, and ZT20). Food pellets were placed on the wire floor, and tap water was available from a drinking spout positioned on the mesh top of each cage. Pellet intakes were measured (to the nearest 0.1 g) every hour for 4 h for each group prior to sacrifice, and were corrected for spillage.

Each rat was sacrificed via decapitation, and the brain was quickly removed and frozen in −40°C isopentane. Each brain was transversely sectioned in a cryostat at −20° C, and each 20-micron section was then thaw mounted on a gelatin-coated slide. Individual slides were placed on a warming plate for at least an hour. The total number of sections per rat was approximately 216 (36 slides).

Alternate sections were incubated with either (^{3}H)-prazosin [(^{3}H)-PRZ; α_1-adrenoceptor ligand] or (^{3}H)-*para*-aminoclonidine [(^{3}H)-PAC; α_2-adrenoceptor ligand]. Prazosin is a highly selective α_1-adrenoceptor antagonist with low affinity for α_2-adrenoceptors (7). Likewise, PAC selectively binds to α_2-adrenoceptor binding sites (25). Autoradiographic procedures and ligand concentrations were based on methods described by Levin (16), Levin and Hamm (17), Huguet, Comoy, Piriou, and Bohuon (9), and Jhanwar-Uniyal and Leibowitz (11).

Sections were exposed to tritium-sensitive Ultra Film (LBK, Leica) for 8 weeks for (^{3}H)-PRZ and 13 weeks for (^{3}H)-PAC. Films were developed for 5 min using D19 (Kodak), placed for 30 s in a stop-water bath and and then fixed for 15 min (Kodak fix). Each film was then allowed to dry overnight.

Autoradiographs were quantified using a computer-assisted image analysis densitometry system (JAVA System, Jandel Scientific). Cresyl violet-stained sections were superimposed upon the corresponding autoradiographic image for the purpose of defining anatomical boundaries according to Paxinos and Watson (18). An average of six to nine density readings were taken in each area and automatically converted to binding activity (nCi/mg tissue) using a best-fit equation based on the radioactivity of the appropriate standard [(^{3}H) embedded polymer standards; American Radiochemical Co.]. The optical density readings (nCi/mmol) were converted into fmol/mg tissue (total binding).

Procedures for Obtaining Relative Receptor Densities

α_1-Adrenoceptor binding. Binding of (^{3}H)-PRZ to high-affinity α_1-adrenoceptor sites was assessed by the following procedure. Slides were preincubated with 50 mM Tris buffer (5 min), 10 nM Na_2 EDTA (pH 7.4), and then incubated with 1 nM (^{3}H)-PRZ (72.2 Ci/mmol; Amersham International, UK) for 60 min at 23–25°C. Slides were taken through two 15-min washes in ice-cold buffer, then dipped in ice-cold distilled water and allowed to dry. Nonspecific binding was defined as the binding of 1 nM (^{3}H)-PRZ in the presence of 100 μM phentolamine (excess unlabeled competing ligand). Specific binding for (^{3}H)-PRZ was determined by subtracting nonspecific binding from total binding.

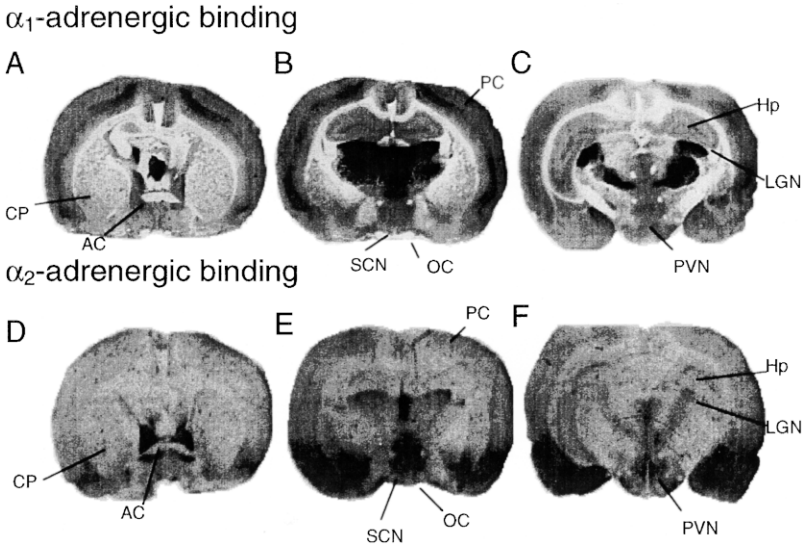

FIG. 1. Representative autoradiograms of α_1-adrenoceptor ligand binding (A, B, and C) and of α_2-adrenoceptor ligand binding (D, E, and F) within the rat brain. Abbreviations: CP = caudate/putamen, PC = pyriform cortex, SCN = suprachiasmatic nucleus, OC = optic chiasm, AC = anterior commissure, HP = hippocampus, PVN = paraventricular hypothalamic nucleus, and LGN = lateral geniculate nucleus.

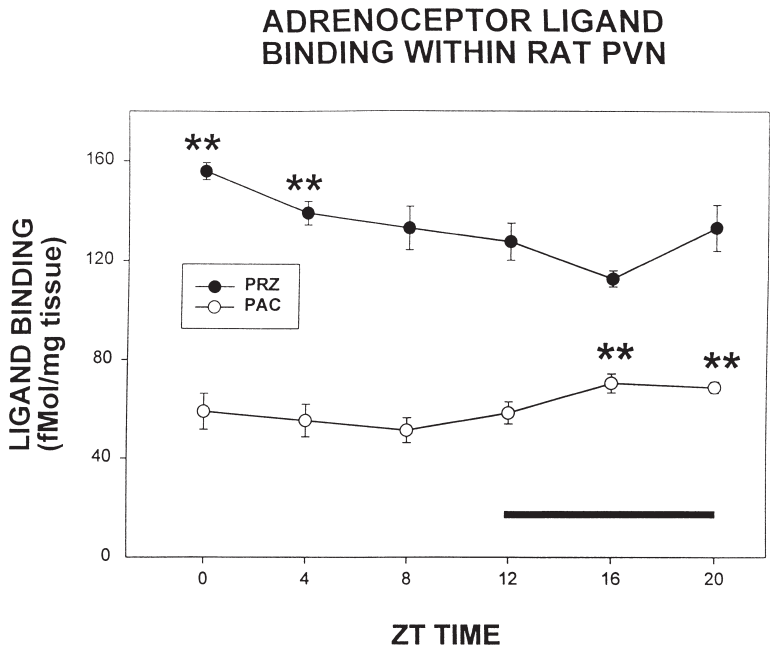

FIG. 2. Mean group PVN [^{3}H]-PRZ or (^{3}H)-PAC binding (fMol/mg tissue) at each of six time periods. A double star indicates that a value is significantly greater than nadir values ($p < 0.05$). The dark phase is indicated by a thick horizontal bar.

α_2-Adrenoceptor binding. Binding of (^{3}H)-PAC to high-affinity α_2-adrenoceptor sites was assessed by the following procedure: slides were preincubated for 30 min in 170 mM Tris buffer, 10 mM $MgCl_2$, 0.01% ascorbic acid, 10 μM pargyline, pH 7.6. Sections were then incubated with 1 nM (^{3}H). PAC (50.0 Ci/mmol; Amersham) for 45 min at 23–25°C followed by two 10 min washes in ice-cold buffer, then dipped in ice-cold distilled water and allowed to dry. Nonspecific bind-

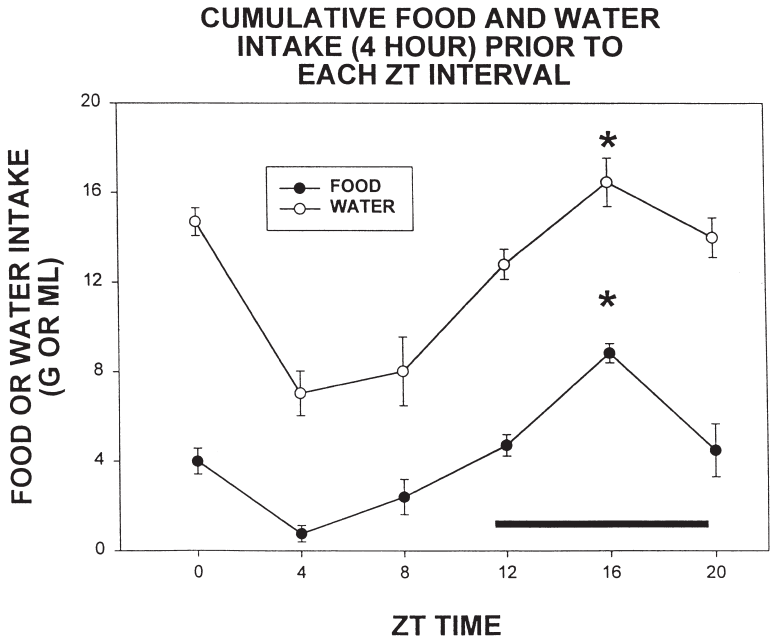

FIG. 3. Mean group cumulative food (FI) and water intakes (WI) (g or ml) recorded during 4-h periods over six portions of a 24-h period. A value at ZT8 represents the intake of food or water during the 4-h period preceding sacrifice at ZT8. The line above and below each symbol represents the standard error of the mean. A double star indicates that a value is significantly greater than nadir values ($p < 0.05$). The dark phase is indicated by a thick horizontal bar.

ing was defined as 1 nM (^{3}H)-PAC binding in the presence of 100 μM norepinephrine (excess unlabeled competing ligand). Specific binding for (^{3}H)-PAC was determined by subtracting nonspecific binding from total binding.

In addition to the measurement and analysis of (^{3}H)-PRZ and (^{3}H)-PAC binding within the PVN, binding was also measured within various other brain regions, i.e., suprachiasmatic nucleus, thalamic nucleus, lateral geniculate (dorsal and ventral), ventromedial nucleus, lateral hypothalamic area, and cortical area (outer and middle). These sites served as a "positive control" or comparison groups for the relative changes in α_1- and α_2-adrenoceptor binding noted in the PVN.

Statistical Analyses

Of the 30 animals used at the start of the experiment, 29 brains were used in the final analyses (one brain was inadvertently destroyed during perfusion). The optical (grain) densities of the rat brain tissue were within the linear response range of the known standards.

The design of the present experiment represents a hierarchical factorial in which the factor of time (ZTO, ZT4, ZR8, and ZT12, ZT16, ZT20) is nested within the factor of phase (light vs. dark). The measures of binding and of ingestion were subjected to General Linear Models analysis using the between-group factor of phase and the nested factor of time within phase. Differences among groups were explored using Duncan's Multiple Range Test ($p < 0.05$).

RESULTS

Autoradiographic analyses revealed detectable binding of (^{3}H)-PRZ within rat brain. Figure 1 depicts representative autoradiograms of (^{3}H)-PRZ binding within a variety of brain structures. Figure 2 depicts mean group (^{3}H)-PRZ binding determined at each of six time points. Average (^{3}H)-PRZ binding within the PVN was significantly greater during the light phase than the dark phase, $F(1) = 10.24$, $p < 0.0047$. The factor of time nested within phase was not significant, $F(4) = 2.39$, $p < 0.0871$. A subsequent analysis using Duncan's procedure (alpha = 0.05, $df = 19$, MSE = 196.4) revealed that PRZ binding at ZT0 and at ZT4 was significantly higher than PRZ binding at ZT8–ZT20.

As expected, food intake (expressed as cumulative g/4 h period) exhibited significant diurnal variation (depicted in Fig. 3). Average feeding during the dark phase was significantly greater than that evident during the light phase, $F(1) = 40.6$, $p < 0.0001$. Contrasts using Duncan's procedure (alpha = 0.05, $df = 24$, MSE = 2.46) revealed that feeding was significantly elevated during the 4-h period following the offset of lights (designated as ZT16) compared to all other time points. Moreover, food intake was significantly less than all other time points at ZT4 (the first 4 h of the light phase) and at ZT8 (the middle 4-h period of the light phase). Water intakes (depicted in Fig. 3) followed the same general pattern as did food intake over the diurnal cycle.

Detectable binding of (^{3}H)-PAC was noted in many regions of rat brain (see Fig. 1), including the PVN. Mean group (^{3}H)-PAC binding density determined at six time points during a diurnal cycle is depicted in Fig. 2. Average (^{3}H)-PAC binding within the PVN was significantly greater during the dark phase than during the light phase, $F(1) = 6.47$, $p < 0.0185$. Duncan's procedure (alpha = 0.05, $df = 22$, MSE = 120.8) determined that (^{3}H)-PAC binding within the PVN was significantly greater at ZT16 and at ZT20, compared to PAC binding at all other time points.

α_1- and α_2-adrenoceptor Binding in Other Brain Regions

Table 1 summarizes mean (^{3}H)-PRZ binding in eight brain regions including the suprachiasmatic nucleus (SCN), the dorsal and ventral aspects of the lateral geniculate (LGN), the ventromedial hypothalamus (VMH), the lateral hypothala-

TABLE 1

AVERAGE (±SEM) GROUP BINDING (FMOL/MG TISSUE) OF (^{3}H)-PRZ OR (^{3}H)-PAC WITHIN NON-PVN BRAIN REGIONS OVER SIX TIME POINTS DURING A DIURNAL CYCLE

Region	Time	PRZ (SEM)	PAC (SEM)
Suprachiasmatic nucleus	ZT0	33.1 (1.8)	33.0 (3.3)
	ZT4	16.8 (3.2)	29.7 (1.7)
	ZT8	47.1 (0.5)	29.4 (3.5)
	ZT12	31.8 (9.4)	31.7 (8.2)
	ZT16	39.2 (8.3)	23.9 (4.9)
	ZT20	55.7 (3.2)	30.7 (6.7)
Thalamic nucleus	ZT0	327.5 (10.9)	ND
	ZT4	255.4 (30.4)	ND
	ZT8	308.5 (41.1)	ND
	ZT12	377.1 (53.2)	ND
	ZT16	347.1 (23.7)	ND
	ZT20	391.0 (70.9)	ND
Lateral geniculate (dorsal)	ZT0	308.9 (39.0)	ND
	ZT4	253.4 (15.1)	ND
	ZT8	247.9 (20.7)	ND
	ZT12	266.6 (38.7)	ND
	ZT16	261.0 (29.4)	ND
	ZT20	249.4 (20.5)	ND
Lateral geniculate (ventral)	ZT0	54.5 (3.2)	ND
	ZT4	46.9 (3.1)	ND
	ZT8	38.3 (1.0)	ND
	ZT12	47.5 (5.8)	ND
	ZT16	52.3 (3.7)	ND
	ZT20	45.6 (6.4)	ND
Ventromedial hypothalamic nucleus	ZT0	109.7 (10.6)	21.3 (4.4)
	ZT4	91.9 (8.4)	16.9 (3.0)
	ZT8	91.8 (3.3)	14.7 (1.5)
	ZT12	98.8 (9.9)	16.3 (1.1)
	ZT16	106.1 (9.5)	19.4 (2.5)
	ZT20	96.2 (9.5)	13.9 (1.5)
Lateral hypothalamic area	ZT0	59.2 (8.0)	15.6 (4.1)
	ZT4	49.5 (7.3)	14.5 (3.2)
	ZT8	63.2 (8.5)	12.8 (2.4)
	ZT12	62.6 (5.3)	21.5 (3.9)
	ZT16	54.7 (7.1)	10.0 (1.4)
	ZT20	60.7 (7.9)	14.3 (1.1)
Outer cortical area	ZT0	164.0 (2.7)	33.7 (5.2)
	ZT4	187.5 (9.3)	21.7 (4.2)
	ZT8	163.9 (18.6)	23.5 (4.5)
	ZT12	176.9 (8.4)	21.7 (1.9)
	ZT16	170.1 (15.6)	19.0 (2.6)
	ZT20	176.5 (12.0)	26.9 (4.6)
Middle cortical area	ZT0	381.2 (61.7)	8.2 (0.9)
	ZT4	376.5 (22.4)	6.2 (1.2)
	ZT8	291.5 (46.2)	6.1 (1.4)
	ZT12	385.1 (44.4)	4.9 (0.5)
	ZT16	336.6 (23.6)	6.1 (1.1)
	ZT20	412.0 (91.0)	6.3 (0.9)

ND = nondectable

mus (LH), outer and medial aspects of cortex, and the thalamic nucleus. Of these brain regions, only the SCN exhibited significant variation in (^{3}H)-PRZ binding. Although the factor of PHASE was not significant, $F(1) = 2.34$, $p < 0.12$, there was evident a significant effect of time within phase, $F(4,18) = 3.53$, $p < 0.04$, for (^{3}H)-PRZ binding within the SCN. The effect appeared to be due to a nadir level of binding at ZT4 relative to all other time periods. In contrast, other brain regions that bound (^{3}H)-PRZ did not exhibit a discernable diurnal variation in binding density (Table 1).

Table 1 also summarizes mean group (^{3}H)-PAC binding in six brain regions including the SCN, the VMH and LH, and the outer and medial aspects of the cortex. Although (^{3}H)-PAC binding was detected in each of these regions, none of these brain regions exhibited significant diurnal variation in (^{3}H)-PAC binding.

DISCUSSION

The purpose of the present experiment was to determine whether α_1- and α_2-adrenoceptor ligand binding within the PVN varies significantly over a diurnal cycle, and to assess potential relationships between subreceptor binding density and feeding. As hypothesized, binding of (^{3}H)-PRZ within the PVN varied significantly over the diurnal cycle. The greatest level of PRZ binding occurred during the light phase, whereas less binding was observed during the dark phase. Specifically, (^{3}H)-PRZ binding density was greatest at ZT0 and ZT4, whereas the least amount of binding was evident at ZT16. These results confirm and extend the work of Kafka et al. (13), who examined binding of (^{3}H)-WB-4104 (an α_1-adrenoceptor antagonist) within whole hypothalamus across a 24-h period. Their results indicated a significant increase in hypothalamic α_1-adrenoceptor binding during the light phase, with maximal binding evident at ZT3 and ZT7, and decreased binding (minimal binding at ZT15) evident during the dark phase.

The present experiment suggests that the variation in α_1-adrenoceptors within the PVN may contribute to the feeding rhythm evident in the rat. PVN α_1-adrenoceptor binding reached a maximum during the early portion of the light phase, when feeding was at a nadir. PVN α_1-adrenoceptor binding then declined to a nadir during the first 4 h of the dark phase, when the rate of feeding was at a maximum. Note that in Fig. 3, a value of food intake at ZT16 actually represents the cumulative intake during the first 4 h of the dark phase, whereas a value of food intake at ZT4 actually represents the cumulative intake during the first 4 h of the light phase. In the present study, (^{3}H)-PAC binding was lowest during the light phase and highest during the dark phase. PVN binding of (^{3}H)-PAC PVN was at a nadir value at ZT8, whereas peak binding was evident at ZT16 and ZT20. These results generally support the pattern of increased α_2-adrenoceptor binding during the early portion of the dark phase noted in earlier studies (10). A minor discrepancy is that the peak point of binding was evident at ZT16 in the present study, whereas earlier studies reported peak α_2-binding at ZT12 (15).

Of interest here is the observation that the α_1- and α_2-adrenoceptor binding rhythms in the present study were complementary such that peak α_2-binding occurred at a time point at which α_1-binding reached a nadir. These findings parallel earlier studies suggesting that α_1- and α_2-adrenoceptor systems within the PVN may be organized in an antagonistic and complementary fashion (24). Not only do these systems produce opposite actions on feeding, these systems may interact within the PVN. For example, the capacity of intra-PVN norepinephrine, a mixed agonist at α_1- and α_2-adrenoceptors, to elicit feeding can be enhanced by a pretreatment with an α_1-adrenoceptor antagonist such as corynanthine (6).

The present study also sought to determine whether α_1-adrenoceptors vary in number across the diurnal cycle in brain regions other than the PVN. Of particular interest here were hypothalamic regions such as the VMH or LH that also play a role in the modulation of feeding. As noted in Table 1, no evidence was obtained in the present study for a signficiant diurnal variation (^{3}H)-PRZ binding within either the VMH or LH regions. Of the remaining areas assessed in the present study, only the SCN exhibited significant variation in (^{3}H)-PRZ binding. Interestingly, the pattern of binding evident in the SCN was opposite to that evident in the PVN in that (^{3}H)-PRZ binding was lower during the light phase and higher during the dark phase. These findings suggest a relationship between α_1-adrenoceptor activity within the SCN and within the PVN. It is interesting to note that cells of the SCN and retinal ganglion project to the region ventral to the PVN (2,12,21). Direct neural connections between the SCN and PVN are suggested by the report of Hermes and Reynold (8), in which stimulation of the SCN produced excitation of PVN parvocellular cells. Further evidence linking adrenoceptor systems within the SCN and PVN comes from the work of Kafka et al. (13), who noted that ablation of the SCN eliminated diurnal variation in adrenoceptor binding within the hypothalamus. Neuronal activity within the SCN may play a role in the modulation of PVN α_1-adrenoceptors, which in turn, may contribute to a diurnal feeding rhythm.

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