NOTE *Physiology*

Involvement of Thalamic Paraventricular Nucleus in the Anticipatory Reaction under Food Restriction in the Rat

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(Received 8 April 2004/Accepted 27 May 2004)

ABSTRACT. To investigate which brain regions are involved in the anticipatory activity in rats restricted feeding for 2 hr, we examined *c-Fos* expression before and after feeding. Only the thalamic paraventricular nucleus (tPVN) showed *c-Fos* expression before feeding than after feeding. After the anticipatory locomotor activity rhythm was established, lesioning the tPVN attenuated this rhythm, but not the light-dark entrained rhythm. The anticipatory increase of blood corticosterone levels was not established in long-term tPVN-lesioned rats. These results suggest that the tPVN is involved in the expression of anticipatory reactions under a food-restricted regimen.
KEY WORDS: circadian rhythm, food restriction, suprachiasmatic nucleus.

J. Vet. Med. Sci. 66(10): 1297–1300, 2004

It is well established that the suprachiasmatic nucleus (SCN) is the predominant circadian oscillators in mammals. When rats are restricted to a single feeding time for several hours at a fixed time every day, they begin to show an anticipatory reaction before the time of feeding [2, 7, 11]. The anticipatory reactions involve increases in locomotor activity, plasma corticosterone level, body temperature, and enzyme activity of the digestive organ. These anticipatory behaviors and their underlying physiologies are not erased by lesioning the SCN, suggesting the existence of a time-keeping oscillator other than the SCN under a restricted-feeding regimen [9].

There have been many investigations into the location of the oscillator of the anticipatory reaction. Lesioning the ventromedial hypothalamus (VMH) only temporarily abolished the anticipatory increase in plasma corticosterone levels and body temperature [10, 13]. In addition, food-anticipatory activity persisted after olfactory bulb ablation [5], and in hypophysectomized rats with SCN lesions [7]. Recently, Davidson *et al.* found that feeding-entrained circadian rhythms are attenuated by lesioning the parabrachial region in rats [4]. On the other hand, expression of *mPer1* and *mPer2* (which are mouse period genes) is associated with food entrainment rhythms in the hypothalamic paraventricular nucleus (PVN), cerebral cortex, pyriform cortex, hippocampus, and stratum, but not the SCN [19]. In our preliminary experiment, however, lesioning the hypothalamic PVN did not completely attenuate the food-restriction-induced anticipatory locomotor activity. This suggests that even if the expression of *mPer1* and *mPer2* is associated with the food entrainment rhythm in hypothalamic PVN, this nucleus may not be the oscillator for the anticipatory reaction under a restricted-feeding regimen.

The neuronal activity of the oscillator involved in the anticipatory reaction may change between before and after a restricted feeding time, and hence *c-Fos* expression was compared at these two times as well as during a free-feeding regimen. *c-Fos* expression were used to establish activated neural populations. We then lesioned the candidate regions to examine whether or not this attenuated the anticipatory reaction.

Six-week-old Wistar rats (Charles River Japan, Shiga, Japan) were kept individually under a regimen of 12 hr light and 12 hr darkness (lights on at 0700 hr) and a temperature of 23 ± 1°C. Animals were supplied with standard laboratory chow and water *ad libitum* for 2 weeks, and then some of these animals were subjected to a restricted feeding time for 2 hr beginning at 0900 hr every day. All procedures were performed in accordance with the Japanese Physiological Society's guidelines for animal care.

The anticipatory reactions were monitored by either recording locomotor activity or blood corticosterone levels. The locomotor activity of the animals was measured by a rat locomotor activity recording system (Muromachi, Tokyo, Japan) comprising infrared sensors, an interface, and a computer [14]. The infrared sensors were placed above the rat cage, and these detected all movements. The data were collected at 15-min intervals and analyzed by CompACT AMS software (Muromachi). The blood corticosterone levels were determined in individual rats at 4-hr intervals for 24 hr by the tail-tip-incision method described previously [15, 16].

In the first experiment, to compare the *c-Fos* expression in various brain regions, the following three groups of rats were used: free-feeding group (n=8), restricted-feeding group (n=8), and fasting group (n=8). In the restricted-feeding group, 2-hr food restriction was performed for 3 weeks. In the fasting group, food was removed at 1900 hr on the day before sacrifice. All rats were anesthetized by an i.p. injection of pentobarbital at 0900 or 1200 hr and perfused transcardially with 100 m*l* of 0.1 M phosphate buffer (pH 7.4) containing 100 U heparin, and then with 150 m*l* of fixative containing 4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer. The brains were removed and placed in fixative for 4 days at 4°C, and then transferred to 0.1 M phosphate buffer containing 20% sucrose. They were

serially sectioned at 40 μm at –20°C with a cryostat, and every one to four slices were placed in 0.1 M phosphate buffer in a six-well culture plate. The floating sections were treated with 0.3% hydrogen peroxide for 1 hr. The *c-Fos* staining was performed using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA) according to the method described previously with modifications [17]. After blocking the section with diluted normal goat serum, the floating sections were incubated at 4°C for 24 hr with anti-*c-Fos* antiserum (Santa Cruz Biotechnology, Santa Cruz, CA) in 0.3% Triton X-100/phosphate buffer saline (PBS). After washing for 30 min with 0.3% Triton X-100/PBS, the sections were incubated for 2 hr with a biotinylated second antibody and for an additional 2 hr with an avidin-biotin-peroxidase complex. The sections were then stained with 0.02% 3–3'-diaminobenzidine and 0.05% hydrogen peroxide in tris buffer.

From the results of *c-Fos* expression experiments, we proposed the thalamus PVN (tPVN) as a candidate regulator of the anticipatory reaction. In the second experiment, therefore, the effect of tPVN lesioning on the anticipatory locomotor activity or circadian locomotor activity rhythms were examined; SCN lesioning was used as a control. About 3 weeks after food restriction, rats were anesthetized by i.p. injection of pentobarbital, and then mounted in a brain stereotaxic instrument (Narishige, Tokyo, Japan). The brain lesioning was performed using an electrode according to the method described previously with minor modifications [10]. The electrodes were made from stainless steel insulated with Epoxylite (0.6 mm in diameter) except for a 0.4-mm-long tip (lesion for tPVN) or a 0.2-mm-long tip (lesion for SCN). The electrode tip was placed at the following stereotaxic coordinates: 7.3 and 7.6 mm anterior to interaural, 0.35 and 0.28 mm lateral to the midline, and 6.3 and 8.6 mm below the dura for the tPVN and SCN lesions, respectively [18]. The lesions of the tPVN (n=12) and SCN (n=12) were made by passing a 3 and 2 mA anodal current for 15 min, respectively. The same surgical procedure, except without current, was used to produce sham-operated control animals (n=8). The lesioned area was confirmed histologically at the end of experiment in brain sections.

In the third experiment, we examined whether the anticipatory reaction is established in rats whose tPVN has been already destroyed before initiation of food restriction. The tPVN was lesioned using the method described above. One week after the operation, 2-hr food restriction (feeding only during 0900–1100 hr) was started. Three weeks after food restriction, blood samples were collected at 4-hr intervals for 24 hr beginning at 0850 hr using the tail-tip-incision method, and plasma corticosterone levels were measured [15].

c-Fos expression was observed before restricted feeding in septohypothalamic nucleus, SCN, and tPVN in food-restricted, but not in free-feeding and fasted rats. In SCN and tPVN, but not septohypothalamic nucleus, *c-Fos* expression was also observed after restricted feeding, and the expression was more abundant in tPVN before than after the restricted feeding time (Fig. 1). *c-Fos* expression was not detected in hypothalamic PVN, VMH, LH, cerebral cortex, pyriform cortex, parietal cortex, and hippocampus in restricted-feeding rats before and after feeding time.

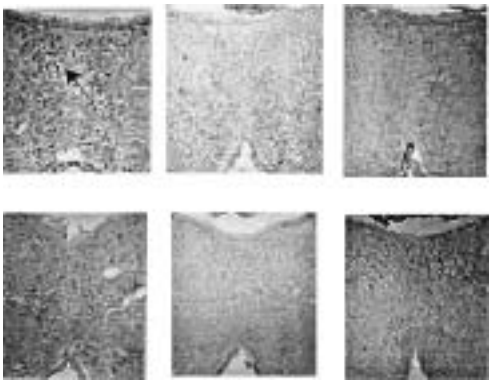

Fig. 1. *c-Fos* expression in tPVN in food-restricted rat. Arrow shows the staining of *c-Fos*. Abundant *c-Fos* expression was observed before the time of food restriction in only food-restricted rats.

Complete lesioning of the tPVN attenuated the anticipatory locomotor activity in all food-restricted rats (Fig. 2). However, the light-dark entrained locomotor activity rhythm was maintained, since the resting period was observed just after light on, and high locomotor activity was recorded during the dark phase (Fig. 2). Lesioning the SCN attenuated the light-dark entrained locomotor activity rhythm, but not anticipatory activity (Fig. 2). To examine the possibility that the attenuation of anticipatory locomotor activity is due to an acute and temporary reaction to the lesioning of the tPVN, and that the other anticipatory reaction is not attenuated by this lesioning, we examined whether food restriction could induce the anticipatory reaction in blood corticosterone levels in rats without tPVN for a long time. The plasma corticosterone levels exhibited a circadian rhythm with the peak just after the dark period in the free-feeding, sham-operated, and tPVN-lesioned rats. Three weeks of food restriction induced an anticipatory corticosterone peak just before the restricted feeding time in sham-operated but not tPVN-lesioned rats (Fig. 3). Although the tPVN-lesioned rats showed increase of corticosterone levels just after restricted feeding time, it may be temporal increase caused by increase of blood glucose. Similarly, no anticipatory reaction was established in locomotor activity rhythm in long-term tPVN-lesioned rats (data not shown).

It is expected that two kinds of oscillators are involved in restricted-feeding-time rats under light-dark conditions: one is the light-dark entrained circadian system, which is regulated by SCN; the other is the food-restriction-induced rhythm, which is driven by unknown oscillators. As in previous studies, lesioning the SCN attenuated the light-dark entrained rhythm, but not the food-restriction-induced anticipatory locomotor activity rhythm [9]. In contrast, lesioning

the tPVN attenuated completely the anticipatory locomotor activity rhythm, but not the light-dark entrained rhythm. In addition, feeding behavior itself was not affected by lesioning the tPVN. The abolishment of anticipatory locomotor activity did not recover over 1 month. Further, an anticipatory plasma corticosterone peak was not established in rats whose tPVN had already been destroyed before initiation of the food-restriction regimen. These results suggest that tPVN plays an important role in the anticipatory reaction under food restriction. From the present study, however, it is unknown whether the tPVN is acting as an oscillator itself or is one of the passages from the other oscillators.

However, the hypothesis that the tPVN is involved in the anticipatory reaction is supported by *c-Fos* expressions, which were used as markers of activated neural populations [2, 6]. *c-Fos* expression was observed in tPVN before the restricted feeding time, suggesting that tPVN neurons were activated before that time. It is unknown which signals activate the tPVN neuron. It is possible that autonomous neu-

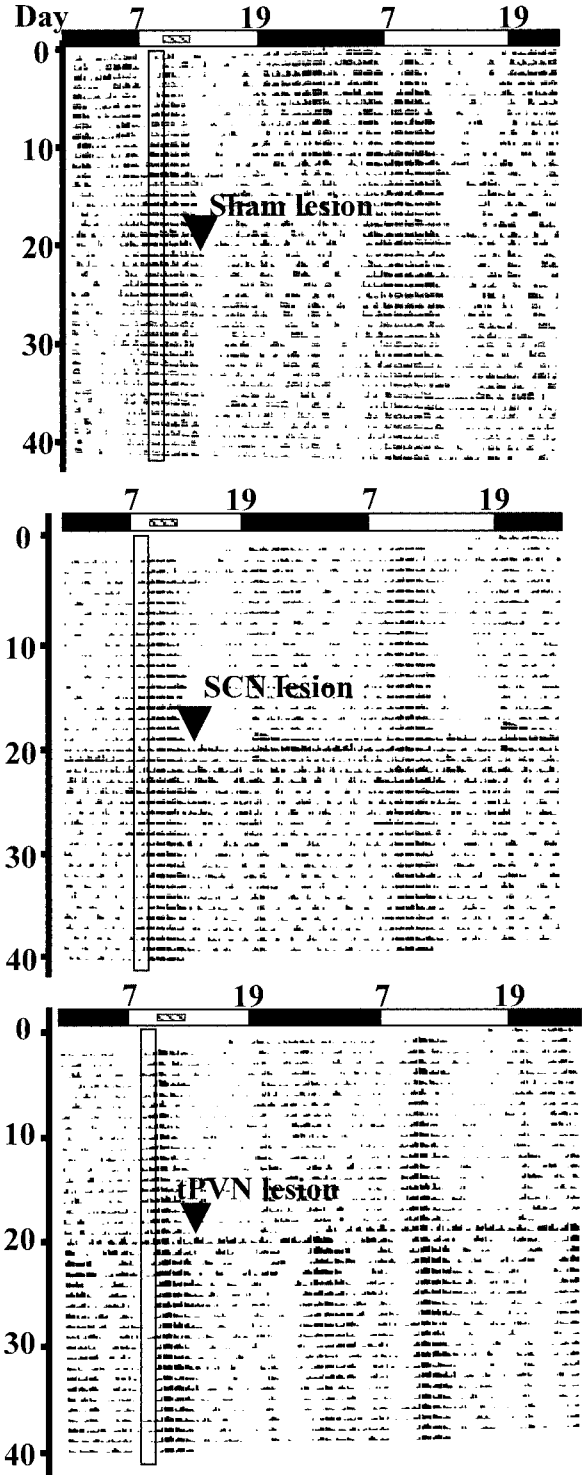

Fig. 2. Effect of lesion of SCN (middle panel) and tPVN (lower panel) on the anticipatory locomotor activity in food-restricted rats. The actograms are displayed using the double-plot method. The white and black bars at the top of each indicate the light-dark period. The black triangle indicates the day of lesioning. A vertical square indicates the expected anticipatory locomotor activity time. Feeding - indicated with a small box - in the food-restricted rats was allowed from 0900 to 1100 hr every day.

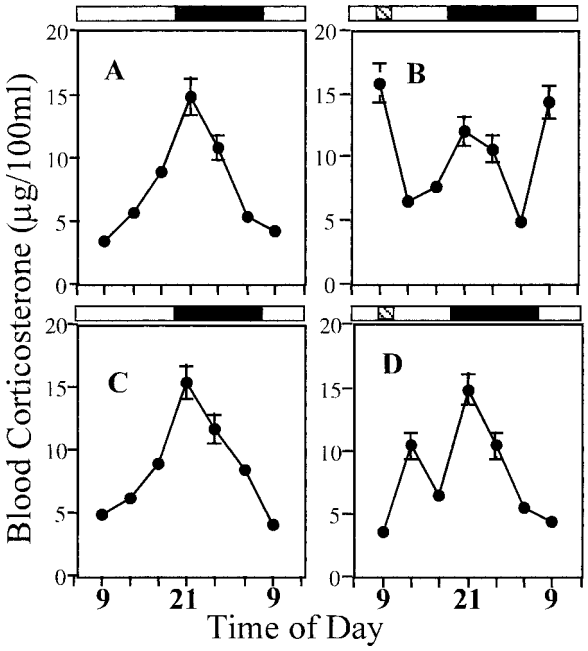

Fig. 3. Twenty-four-hour pattern of blood corticosterone levels in sham-operated (A, B) and tPVN-lesioned (C, D) rats. Two weeks after surgery, blood samples were collected at 4-hr intervals for 24 hr in free-feeding rats (two left-hand panels), after which food intake was restricted to 2 hr every day. Three weeks after food restriction, blood samples were collected again (right panels). The white and black bars and small box at the top of each panel represent the light-dark period and 2-hr food-intake period, respectively.

ronal firing of the tPVN activates neurons each other, as probably those in the SCN. We did not detect a significant increase in *c-Fos* expression before the restricted feeding time in cerebral cortex, pyriform cortex, parietal cortex, and hippocampus, which are candidate regions for the food-restriction-induced oscillator due to *mPer* expression rhythms [19]. However, the present results do not discount the possibility that these regions are acting as the oscillator and that the tPVN is an output passage from them. In addition, it is reported that feeding-entrained circadian rhythms are attenuated by lesions of the parabrachial region in rats [4], and that the limbic and dopamine systems do not play a entrainment of food-anticipatory circadian rhythms [12], and hence further studies are required to elucidate which region acts as the oscillator of the anticipatory reaction in food-restricted rats.

ACKNOWLEDGEMENT. This study was supported by the Nissan Science Foundation to K.N, and Program for Promotion of Basic Research Activities for Innovative Bioscience (PROBRAIN) to N.M.

REFERENCES

1. Boulos, Z., Rosenwasser, A.M. and Terman, M. 1980. *Behav. Brain Res.* **1**: 39–65.
2. Challet, E., Jacob. N., Vuillez, P., Pevet, P. and Malan, A. 1997. *Brain Res.* **770**: 228–236.
3. Davidson, A.J., Aragona, B.J., Werner, R.M., Schroeder, E., Smith, J.C. and Stephan, F.K. 2001. *Physiol. Behav.* **72**: 231–235.
4. Davidson, A.J., Cappendijk, S.L.T. and Stephan, F. K. 2000. *Am. J. Physiol.* **278**: R1296–R1304.
5. Davidson, A.J. and Stephan, F.K. 1999. *Am. J. Physiol.* **277**: R1376–R1384.
6. Dragunow, M. and Robertson, H.A. 1988. *Brain Res.* **440**: 252–260.
7. Krieger, D.T. 1974. *Endocrinology* **95**: 1195–1200.
8. Krieger, D.T. 1980. *Endocrinology* **106**: 649–654.
9. Krieger, D.T. and Hauser, H. 1977. *Science* **197**: 398–399.
10. Kuroda, H., Fukushima, M., Nakai, M., Katayama, T. and Murakami, N. 1997. *Physiol. Behav.* **61**: 633–637.
11. Mistlberger, R.E. 1994. *Neurosci. Biobehav. Rev.* **18**: 171–195.
12. Mistlberger, R.E. and Mumby, D.G. 1992. *Behav. Brain Res.* **47**: 159–168.
13. Mistlberger, R.E. and Rechtschaffen, A. 1984. *Physiol. Behav.* **33**: 227–235.
14. Murakami, N., Marumoto, N., Nakahara, K. and Murakami, T. 1997. *Brain Res.* **775**: 240–243.
15. Murakami, N. and Takahashi, K. 1982. *Neuroendocrinology* **34**: 157–162.
16. Murakami, N., Takahashi, K., Kuroda, H. and Etoh, T. 1986. *Jpn. J. Vet. Sci.* **48**: 29–34.
17. Nakazato, M., Murakami, N., Date, Y., Kojima, M., Matsuo, H., Kangawa, K. and Matsukura, S. 2001. *Nature (Lond.)* **409**: 194–198.
18. Paxinos, G. and Watson, C. 1986. The Rat Brain in Stereotaxic Coordinates, 2nd ed., Academic Press, Australia.
19. Wakamatu, H., Yoshinobu, Y., Aida, R., Moriya, T., Akiyama, M. and Shibata, S. 2001. *Eur. J. Neurosci.* **13**: 1190–1196.