Daily rhythms in Fos activity in the rat ventrolateral preoptic area and midline thalamic nuclei

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Novak, Colleen M., and Antonio A. Nunez. Daily rhythms in Fos activity in the rat ventrolateral preoptic area and midline thalamic nuclei. Am. J. Physiol. 44): R1620–R1626, 1998.—The present experiment investigated the expression of the nuclear phosphoprotein Fos over the 24-h light-dark cycle in regions of the rat brain related to sleep and vigilance, including the ventrolateral preoptic area (VLPO), the paraventricular thalamic nucleus (PVT), and the central medial thalamic nucleus (CMT). Immunocytochemistry for Fos, an immediate-early gene product used as an index of neuronal activity, was carried out on brain sections from rats perfused at zeitgeber time (ZT) 1, ZT 5, ZT 12.5, and ZT 17 (lights on ZT 0–ZT 12). The number of Fos-immunopositive (Fos+) cells in the VLPO was elevated at ZT 5 and 12.5 (i.e., during or just after the rest phase of the cycle). Fos+ cell number increased at ZT 17 and ZT 1 in the PVT and CMT, 180° out of phase with the VLPO. A positive correlation was found between the numbers of Fos+ cells in the PVT and CMT, and Fos expression in each thalamic nucleus was negatively correlated with VLPO Fos+ cell number. The VLPO, PVT, and CMT may integrate circadian and homeostatic influences to regulate the sleep-wake cycle.

circadian rhythms; sleep; paraventricular thalamus; central medial thalamus; vigilance

Most organisms show circadian rhythms in physiology and behavior, and environmental cues modify the phase and period of these endogenous rhythms. In mammals, the sleep-wake cycle is a salient behavioral rhythm controlled by the circadian clock, which is located in the suprachiasmatic nucleus (SCN) and entrained by the light-dark cycle (18). The dissociation between homeostatic control of sleep (i.e., amount or intensity of sleep) and circadian control of sleep (i.e., timing of sleep throughout the day) can be seen in studies using lesions of the SCN. These lesions abolish the circadian control of sleep without substantially altering the amount of sleep or other aspects of its homeostatic regulation (8, 14, 45, but also see Ref. 9). The circadian modulation of sleep is most likely mediated by outputs of the SCN to brain structures involved in the regulation of sleep homeostasis. One group of cells important for the induction of sleep has been localized in the basal forebrain and preoptic area/anterior hypothalamus (POAH). Lesions of these areas disrupt slow-wave sleep in rats (53) and cats (26, 29, 50). Electrical stimulation of these structures induces behavioral sleep and cortical slow waves (42, 48, 49); similar effects are found when the POAH is warmed (28, 37). Furthermore, activity of a subpopulation of neurons in the basal forebrain anticipates non-REM sleep onset (16, 27, 32, 51). These brain areas may integrate circadian and homeostatic influences on the daily display of sleep.

A specific cell group within the preoptic area appears to play a role in the onset and maintenance of slow-wave sleep. Immunoreactivity for the immediate-early gene product Fos, used as an index of neuronal activity, was employed to identify a sleep-active group of cells in the ventrolateral preoptic area (VLPO) of rats (41). More Fos expression was seen in the morning than at night in these cells. However, after sleep deprivation uncoupled the display of sleep from the light-dark cycle, Fos expression in the VLPO still increased during sleep. These data are consistent with the claim that VLPO cells are involved in the regulation of homeostatic aspects of sleep. Cells of the VLPO project to the posterior hypothalamic tuberomammillary nuclei (TMN; see Ref. 41), and the VLPO may be one source of GABAergic inhibitory inputs to the histaminergic neurons of the TMN that regulate vigilance (i.e., alertness and attention to environmental stimuli) and cortical arousal (41).

Midline and intralaminar thalamic nuclei also play an important role in the control of the sleep-wake cycle and may be affected by the circadian clock (11, 30, 35, 46). The SCN is reciprocally connected with one of the midline thalamic nuclei, the paraventricular thalamic nucleus (PVT; see Refs. 30, 54, 55). In fact, the SCN sends a heavier projection to the PVT than to any other nucleus outside the hypothalamus (55). Thus the PVT may function to relay circadian information to other forebrain regions, such as the central amygdala and the nucleus accumbens (NAC; see Refs. 10, 30, 35). In rats, Fos immunoreactivity in the PVT shows a day-night difference with greater expression in the dark period compared with the light period (35).

The thalamic intralaminar nuclei also participate in the control of sleep and arousal. This nuclear complex shows a decrease in electrical activity during slow-wave sleep (11). The intralaminar nuclei, including the central medial thalamic nucleus (CMT), are considered an extension of the midbrain reticular formation (MRF), relaying information from the MRF to the caudate nucleus and neocortex (11, 46). Evidence suggests that projections from the intralaminar nuclei to the cortex mediate electroencephalogram (EEG) desynchronization, especially in relation to attention and vigilance (17, 46). The circadian modulation of neural activity in the CMT has not been investigated.
Expression of the immediate-early gene products such as Fos and Fos-related proteins has been used as an index of neural activity in specific brain areas (39) and to investigate how this neural activity relates to sleep and wakefulness (2, 12, 13, 19, 23, 41). The present study extends previous findings by determining Fos expression across the 24-h light-dark cycle in the VLPO, PVT, and CMT of rats. The goal of the study was to further document the circadian modulation of neural activity in areas of the brain associated with sleep and vigilance by sampling at four different times of day and to examine the relationship that exists among these brain areas.

METHODS

Thirty-two adult male Sprague-Dawley rats (obtained from Charles River breeders) were perfused at different times in their 12:12-h light-dark cycle: zeitgeber time (ZT) 1, ZT 5, ZT 12.5, and ZT 17, with lights on at ZT 0 and dim red light present after lights off (at ZT 12). These times were chosen to sample changes at dawn and dusk as well as the middle of the light and dark periods. At each ZT point, a group of animals (8 animals/time point) was injected with Equithesin (1.5 ml) in the animal room. The animals were then perfused with 0.01 M PBS followed by 4% paraformaldehyde mixed with 0.2% sodium periodate and 1.3% lysine in 0.1 M phosphate buffer. Brains were postfixed for 24 h and then transferred to 20% sucrose in PBS. The brains were sectioned at 30 µm using a freezing microtome. The sections were divided into three sets and stored in cryoprotectant.

Every third section was processed for Fos immunocytochemistry using a rabbit anti-Fos primary antibody (Santa Cruz); all incubations and rinses were done with rotation at room temperature unless otherwise noted. The tissue was rinsed in 0.1 M PBS for 2 h and 40 min and then was incubated in normal goat serum (NGS; 5%; Vector Laboratories) in PBS with 0.3% Triton X-100 (PBS-TX) for 1 h and 45 min. The sections were then washed (3 x 10 min) and incubated in the primary antibody (1:1,000) with 3% NGS in PBS-TX overnight at 4°C. They were then rinsed and incubated in the biotinylated secondary antibody (goat anti-rabbit; Vector Laboratories), 1:250, with 3% NGS, in PBS-TX overnight at 4°C. The sections were washed and incubated in avidin-biotin solution (7.5 µl avidin and biotin/ml PBS; ABC elite kit, Vector Laboratories) for 2 h and 10 min. After rinsing, the chromagen 3,3'-diaminobenzadine was added to Trizma buffer, and the sections were incubated in the chromagen for 2.5 min; hydrogen peroxide (2%) was added, and the incubation was continued for an additional 6 min. The sections were then mounted onto gelatin-coated slides, and the number of cells showing Fos immunoreactivity (Fos+) was determined using a Zeiss microscope.

The VLPO was identified following the description in Sherin et al. (41). An area (200 µm2) in the VLPO was counted bilaterally at ×100 magnification. The data were averaged to give a mean number of Fos+ VLPO cells per section. The number of Fos+ cells in the PVT was counted at ×250 magnification. The PVT was divided into anterior, middle, and posterior, using the landmarks described in published reports (35); the anterior PVT was defined by the stria medularis and the third ventricle dorsally; the middle and posterior PVT were located ventral to the habenula; the posterior PVT was also more distinctly bilateral (2 nuclei as opposed to 1 midline nucleus) than the middle PVT. A grid was not used to count Fos+ cells in the PVT because the anatomical boundaries of the PVT were distinct. The CMT was identified using the Paxinos and Watson rat brain atlas (34), and an area of 300 µm2 centered over the nucleus was
Fig. 3. Mean number of Fos<sup>+</sup> cells/section in the paraventricular nucleus of the thalamus (PVT), collapsed across all subregions (anterior, middle, posterior). Fos<sup>+</sup> cell number differed across the light-dark cycle (P < 0.0001). Animals at ZT 1 had significantly more Fos<sup>+</sup> cells in the PVT when compared with animals at ZT 5 (P < 0.0001), ZT 12.5 (P < 0.0001), and ZT 17 (P = 0.0099). The PVT of animals taken at ZT 17 also contained more Fos<sup>+</sup> cells when compared with animals taken at ZT 5 (P = 0.0006) or ZT 12.5 (P = 0.0001).<sup>a</sup> Different from ZT 5 and ZT 12.5; <sup>b</sup> different from ZT 17.

Fig. 4. Fos<sup>+</sup> cells in the PVT (middle subregion) of animals taken at ZT 1 and ZT 12.5. Arrows point to the PVT. 3V, third ventricle.

Fig. 5. Mean number of Fos<sup>+</sup> cells/section of the central medial thalamic nucleus (CMT). An overall effect of ZT was found (P < 0.0001). Animals at ZT 1 had significantly more Fos<sup>+</sup> cells in the CMT when compared with animals at ZT 5 (P < 0.0001), ZT 12.5 (P < 0.0001), and ZT 17 (P = 0.0066). The CMT of animals taken at ZT 17 also contained more Fos<sup>+</sup> cells when compared with animals taken at ZT 5 (P = 0.0131) or ZT 12.5 (P = 0.0331).<sup>a</sup> Different from ZT 5 and ZT 12.5; <sup>b</sup> different from ZT 17.

Fig. 6. Fos<sup>+</sup> cells in the CMT of animals taken at ZT 1 and ZT 12.5. Boxes indicate the area where Fos<sup>+</sup> cells were counted.
counted at ×250 magnification. All data were analyzed using a one-way ANOVA for each brain region, with the mean number of Fos+ cells per section per animal as the dependent variable and the ZT as the independent variable, followed by Fisher’s protected least significant difference test for pairwise comparisons. The numbers of sections used for the analysis of each region were counted and subjected to an ANOVA; no differences were found for any area between any of the ZTs. Correlation coefficients (Pearson’s r) were computed to determine the relationships between the Fos+ cell numbers in the different brain regions.

RESULTS

Overall ZT significantly affected the number of Fos+ cells (P = 0.0001) in the VLPO of rats (see Fig. 1) such that more Fos+ cells were found in the VLPO of animals taken at ZT 5 and ZT 12.5 than at ZT 1 and ZT 17 (P < 0.001). Examples of the distribution of Fos+ cells in the VLPO at ZT 1 (fewer Fos+ cells) and ZT 5 (more Fos+ cells) are shown in Fig. 2.

Within each ZT, there were no differences across the three levels of the PVT; therefore, the data were collapsed across subregions; data for the whole PVT are shown in Fig. 3. There was a significant overall effect of ZT in the PVT (P < 0.0001). Fos activity in the PVT at ZT 1 was significantly different from all other time points (P < 0.01); Fos cell number from animals at ZT 17 was also significantly different from ZT 5 and ZT 12.5 (P < 0.001). Figure 4 illustrates the differences in PVT Fos+ cell number seen between animals taken at ZT 1 (more Fos+ cells) and ZT 12.5 (fewer Fos+ cells). The pattern of Fos expression was the opposite of that of the VLPO (see Figs. 1 and 3); a significant negative correlation was found between the number of Fos+ cells in the VLPO and the PVT (r = −0.527, P < 0.01). This negative correlation held for all regions of the PVT (P < 0.05).

DISCUSSION

More Fos+ cells were found in the VLPO in the middle and just at the end of the light period, when rats are (or recently have been) inactive and frequently asleep (4, 45). It has been shown that, after sleep deprivation, the VLPO becomes active during sleep, regardless of the time of day (41). However, when the animals are left undisturbed, the VLPO shows a rhythm of Fos expression over the light-dark cycle (41 and present data). Taken together, these results suggest that the VLPO may integrate circadian and homeostatic variables affecting sleep onset.

The rhythm seen in the VLPO suggests that the circadian clock of the SCN may modulate neuronal activity in the VLPO. Efferent projections from the SCN have been traced to the POAH (55). If some of
these fibers terminate in the VLPO, they could provide direct circadian information to the sleep-active cells of the VLPO. In turn, the VLPO may contribute to the circadian modulation of other brain regions via inhibitory connections with the histaminergic cells of the TMN (41). The TMN projects to the cortex as well as to subcortical brain regions (1, 33). Confirmation of SCN projections to the VLPO is needed to support this functional model.

The rhythm of Fos expression in the VLPO may also be influenced by retinal inputs to that area (25). Experiments with animals kept in constant darkness are needed to differentiate between true circadian modulation of the VLPO from effects due to direct retinal inputs. Therefore, the conclusion that the 24-h pattern of Fos expression seen in the VLPO is due to SCN input has to remain tentative at this time. The same qualification applies to the rhythm seen in the PVT, since retinal projections also reach that structure in the rat (44).

In contrast to the pattern seen in the VLPO, the two thalamic nuclei investigated here showed enhanced Fos expression during and immediately after the active period. Neuronal Fos activity in the PVT and CMT showed a remarkably strong positive correlation and a pattern that was 180° out of phase with the VLPO rhythm. Previous work indicates that both thalamic regions become active during situations incompatible with the onset and maintenance of sleep. For example, neuronal activity in the PVT is associated with consummatory behaviors (38) and increased dopamine utilization in the NAC (15), which is associated with reward attainment (36). Activity in the intralaminar nuclei increases during wakefulness, enhanced vigilance (11, 47), and, in humans, during tasks that require attention (17). In addition, enhanced activity in both the PVT and CMT is associated with stress (6, 7, 40). The rhythms in Fos expression described here for the PVT and CMT are consistent with previous reports (11, 35).

Therefore, the circadian system may modulate motivation, attention, and vigilance by acting on these thalamic areas. Although the circadian influence on the PVT can easily be attributed to direct projections from the SCN, the possible neural circuit responsible for rhythms in the CMT is not as evident, given that the SCN efferent fibers seen in the CMT do not appear to terminate there (55). One possibility is that the CMT neurons may respond to a diffusible factor, first identified in SCN neural transplants, that exerts a rhythmic influence on behavior after SCN lesions (20, 21, 22, 43). Alternatively, the SCN may project to POAH hypogonic areas (55), which in turn inhibit MRF neurons that send excitatory input to the intralaminar nuclei (3, 24, 52). In this way, the SCN could indirectly affect activity in the intralaminar nuclei, as well as the display of sleep.

In summary, the present findings show how areas of the brain important for the regulation of the sleep-wake cycle are modulated by the circadian system and/or the light-dark cycle. These data are useful to develop models concerning the integration of circadian and homeostatic influences on sleep by different brain structures and to describe how these regions interact to orchestrate the sleep-wake cycle.

The available data support the idea that the thalamic intralaminar and midline nuclei serve functions that are incompatible with the display of sleep. The elevated expression of Fos in the PVT and CMT during the active phase of the daily cycle (present findings), as well as during stressful situations (6, 7, 40), suggests that these thalamic nuclei share common functional features. The mechanisms responsible for the coupling of activity between the PVT and CMT, and the circuits involved in the circadian modulation of neural activity in these thalamic areas, deserve further attention from researchers in the fields of sleep and circadian biology.

The patterns of activity seen in the brain areas studied here are likely to be imposed by outputs of the SCN, and this circadian modulation then determines the overt activity pattern of the animal over the day-night cycle. In rats, a night-active species, the neural activity of the VLPO has a different phase relation to the light-dark cycle than do the activity patterns of the PVT and CMT. Through comparative studies, knowledge about these phase relationships provides an avenue for investigating the neural basis for the differential organization of sleep and activity in diurnal and nocturnal species. Within a species, the study of how phase relationships between the activity of specific brain structures change as a result of aging should provide important insights concerning the causes of sleep disorders that accompany normal aging, as well as pathologies such as Alzheimer’s disease (5, 31, 56).

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REFERENCES


6. Chastrette, N., D. W. Pfaff, and R. B. Gibbs. Effects of daytime and nighttime stress on Fos-like immunoreactivity in the paraventricular nucleus of the hypothalamus, the habenula, and


