Rhythms in Fos expression in brain areas related to the sleep-wake cycle in the diurnal Arvicanthis niloticus

Colleen M. Novak, Laura Smale, and Antonio A. Nunez. Rhythms in Fos expression in brain areas related to the sleep-wake cycle in the diurnal Arvicanthis niloticus. Am J Physiol Regulatory Integrative Comp Physiol 278: R1267–R1274, 2000.—Most mammals show daily rhythms in sleep and wakefulness controlled by the primary circadian pacemaker, the suprachiasmatic nucleus (SCN). Regardless of whether a species is diurnal or nocturnal, neural activity in the SCN and expression of the immediate-early gene product Fos increases during the light phase of the cycle. This study investigated daily patterns of Fos expression in brain areas outside the SCN in the diurnal rodent Arvicanthis niloticus. We specifically focused on regions related to sleep and arousal in animals kept on a 12:12-h light-dark cycle and killed at 1 and 5 h after both lights-on and lights-off. The ventrolateral preoptic area (VLPO), which contained cells immunopositive for galanin, showed a rhythm in Fos expression with a peak at zeitgeber time (ZT) 17 (with lights-on at ZT 0). Fos expression in the paraventricular thalamic nucleus (PVT) increased during the morning (ZT 1) but not the evening activity peak of these animals. No rhythm in Fos expression was found in the centromedial thalamic nucleus (CMT), but Fos expression in the CMT and PVT was positively correlated. A rhythm in Fos expression in the ventral tuberomammillary nucleus (VTM) was 180° out of phase with the rhythm in the VLPO. Furthermore, Fos production in histamine-immunoreactive neurons of the VTM cells increased at the light-dark transitions when A. niloticus show peaks of activity. The difference in the timing of the sleep-wake cycle in diurnal and nocturnal mammals may be due to changes in the daily pattern of activity in brain regions important in sleep and wakefulness such as the VLPO and the VTM.

ventrolateral preoptic area; sleep; suprachiasmatic nucleus; galanin; histamine

The suprachiasmatic nucleus (SCN), the primary circadian pacemaker in mammals, is critical for the expression of circadian rhythms in physiology and behavior (12). Although physiological and behavioral variables follow predictable circadian patterns, animals differ in how their daily activity relates to the light-dark cycle; namely, some animals are active during the dark phase (nocturnal) and some are active during the light phase (diurnal). Little is known about the differences between neural structures controlling the circadian systems of diurnal and nocturnal species, and much of what we know about circadian physiology comes from studies using only nocturnal rodents.

Although species differences exist in how light pulses affect the SCN of nocturnal and diurnal animals (13, 24), other observations have identified similarities in SCN function across species that show very different activity patterns with respect to the light-dark cycle. For example, taken as a whole, the SCN is metabolically more active during the light phase of the cycle in both nocturnal and diurnal mammals (29, 30); but see Ref. 25). Similarly, when animals are kept on a 12:12-h light-dark cycle, the pattern of Fos protein expression in the SCN of a diurnal rodent Arvicanthis niloticus (Nile grass rat) is similar to that seen in the rat (10, 21).

In both species, Fos immunoreactivity is high in the light and low during darkness (21). Last, the SCN neuronal firing rates peak in the light phase regardless of the phase in which the animals are active (9, 14, 26, 39).

Given these common features, it is possible that differences in the phase of behavioral rhythms across species result from the differential responsiveness of SCN targets to clock signals received via axonal and/or humoral outputs of the SCN (34, 37, 38).

In rats, forebrain regions important in sleep and wakefulness show rhythms in Fos expression over a 12:12-h light-dark cycle (19). These rhythms are predictable from the daily activity pattern of these nocturnal rodents. In the ventrolateral preoptic area (VLPO), a brain region important in slow-wave sleep initiation and maintenance (33, 36), Fos expression increases in the middle of the light phase, at the time when these animals sleep. A mechanism through which the VLPO is believed to induce sleep is via the inhibition of monoaminergic brain regions important in arousal, through the actions of GABA and galanin (Gal); one such region is the histaminergic tuberomammillary nucleus (TMN) of the posterior hypothalamus (32, 33, 35).

In rats, the rhythms in Fos expression are 180° out of phase in the VLPO relative to both the rhythms seen in the paraventricular thalamic nucleus (PVT) and centromedial thalamic nucleus (CMT) (19). Fos expression in these nuclei increases during the night when rats are active and show less sleep. These rhythms in Fos expression are also predictable from the activity patterns of these animals, because neural activity in the CMT and PVT increases during behavioral arousal and vigilance (1, 3, 5, 6, 8, 11, 23, 31).

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The difference between the sleep-wake cycles of diurnal and nocturnal animals is presumably associated with a change in the phase of the daily patterns of neural activity in brain areas that control sleep and wakefulness. Recent observations support that claim. Thus, using the diurnal murid rodent A. niloticus, we demonstrated an increase in Fos expression in the VLPO that coincided with the display of behavioral correlates of sleep in that species (20). This elevation in Fos expression was seen 8 h into the dark phase of a 12:12-h light-dark cycle, a time associated with low levels of Fos production in the VLPO of the nocturnal rat (19). Different from rats, A. niloticus sleep during the dark phase of the illumination cycle and are awake during the day and also awake and active at dawn and dusk (10, 20).

In this study, we present data on some anatomic features of the sleep-active area of the VLPO of A. niloticus. We also report on the patterns of Fos expression in the VLPO of this diurnal rodent across the light-dark cycle. Finally, we compare the patterns seen in the VLPO with those of brain regions involved in the support of wakefulness (i.e., the PVT, CMT, and TMN).

METHODS

Experiment 1: Anatomic Features of the VLPO of A. niloticus

Animals. The A. niloticus used in these experiments were born in the laboratory and were descended from a group of animals trapped in Kenya in 1993. Animals were group housed (2–5 per box, in clear polycarbonate cages, 35.7 x 3 x 17 cm) under a 12:12-h light-dark cycle, with lights on at zeitgeber time (ZT) 0; a dim red light was on all the time. Animals were fed and watered ad libitum with Harlan 8640 Teklad 2215 rodent diet and tap water. Two female A. niloticus were used in the first experiment.

Galanin immunostaining. Two female A. niloticus were injected with Nembutal (pentobarbital sodium, Abbott Laboratories; 0.9 ml/kg ip) and placed in a stereotaxic device (Stoelting), and a local anesthetic was given under the skin of the head (0.01 ml 2% lidocaine, 1:1 with saline, Elkins-Sinn). Two holes were drilled in the skull using a dental drill, and the head (0.01 ml 2% lidocaine, 1:1 with saline, Elkins-Sinn). The animals were anesthetized with ketamine (50 mg/kg) and xylazine (7 mg/kg, ip). The animals were then removed from the stereotaxic apparatus, and the exposed brain was transferred to ice-cold sucrose in PBS. The brains were sectioned into three sets at 30 µm using a freezing microtome and the sections were stored in cryoprotectant for later use.

One set of sections (every 3rd section) was processed using immunocytochemistry (ICC) for Gal. Sections were rinsed for 1 h in 0.01 M PBS before incubation in the primary antiserum, rabbit anti-Gal (Cambridge), at a 1:1,000 dilution in 5% normal goat serum (NGS) and 3% Triton X-100 (tx) for 18 h at 4°C. Sections were then rinsed and incubated in the secondary antiserum (biotinylated goat anti-rabbit, 1:200; Vector Laboratories) in PBS-tx. Tissue was then placed in the avidin-biotin complex (Vector Laboratories). Sections were then reacted with 3,3’-diaminobenzidine with 0.3% hydrogen peroxide for 2 min, then rinsed, mounted onto gelatin-coated slides, placed under a coverslip, and observed under the microscope. An optical grid (190 µm²) was placed over the VLPO, and Gal+ cells within this area were counted on each side of the brain in four sections at ×400 magnification using a Zeiss microscope. The number of Gal+ cells in each VLPO (unilateral) was averaged across sections. Additionally, preabsorption with Gal peptide (Sigma; 50 µg Gal peptide in 500 µl PBS, with 5 µl anti-Gal primary antibody) and deletion of the primary antiserum for Gal both resulted in tissue devoid of immunostaining.

Experiment 2: Daily Rhythms of Fos Expression in the Forebrain of A. niloticus

Animals and tissue collection. Twenty-four male A. niloticus housed on a 12:12-h light-dark cycle were used in this experiment. Animals were singly housed and fed and watered ad libitum as described above.

The animals were perfused with PBS and PLP as described earlier. Brains were postfixed for 24 h and then transferred to 20% sucrose in PBS. The brains were sectioned into three sets at 30 µm using a freezing microtome and the sections were stained with cresyl violet.

Fos and histamine immunostaining. Every third section was processed for Fos ICC using a rabbit anti-Fos primary antibody (Santa Cruz). Separately, the posterior half of the hypothalamus was subjected to double-labeled ICC for Fos and histamine. The standard protocol (described above) was used with some modifications. For Fos, the primary antiserum used was sheep anti-Fos (Cambridge, 1:1,000) with normal donkey serum, the secondary antiserum was donkey anti-sheep (1:200, Vector Laboratories), and DAB with nickel intensification was the chromagen. For histamine ICC, rabbit antihistamine (Sigma, 1:1,000) was used with NGS; the secondary antiserum was goat anti-rabbit, and DAB was the chromagen (3-min incubation for each chromagen). No immunostaining for histamine was detected after primary antibody deletion or preabsorption with histamine (100 µg/ml diluted serum; Sigma).

Analysis. The number of Fos-immunoreactive (Fos+) cells in the VLPO was counted at ×400 magnification using a 190 µm² optical grid (20). The PVT, CMT, and TMN were identified with the aid of the rat brain atlas (22). The PVT was divided into anterior, middle, and posterior using the landmarks described in Peng et al. (23): the anterior PVT was bounded 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. The number of Fos+ cells was determined for each region at ×250 magnification. The number of Fos+ cells in the CMT was determined at ×250 magnification using an optical grid (300 µm²).
Two subdivisions of the TMN were examined for Fos expression \(7, 22\). The dorsal TMN (DTM) was located in the posterior hypothalamus, on either side of the third ventricle; the number of Fos\(+\) cells in one section containing the DTM was counted at \(\times 100\) in an area of 120 \(\mu m^2\). The ventral TMN (VTM) was located caudal to the DTM, along the edge of the brain bilaterally in the posterior hypothalamus; the number of Fos\(+\) cells in a 150 \(\mu m^2\) grid was counted at \(\times 250\). This grid encompassed the magnocellular region of the VTM, the size of which differed from the size of the DTM.

The data (number of Fos\(+\) cells) for each brain region were used to obtain the average number of Fos\(+\) cells per section. For each brain region, one-way ANOVAs were used for data analysis, with the number of Fos\(+\) cells per section as the dependent variable and the ZT as the independent variable; Fisher's protected least significant difference (PLSD) test was used for pairwise comparisons. Correlations between brain regions with respect to the number of Fos\(+\) cells per section were also determined using Pearson's \(r\). Differences and correlations were considered significant if \(P < 0.05\).

For the histamine double-labeled ICC, the number of histamine-immunopositive (Hist\(+\)) cells as well as the number of Hist\(+\) cells containing Fos were counted in the lateral group of Hist\(+\) cells (including but not confined to the area used to count Fos\(+\) cells in the VTM described above) using an Olympus BX 60 microscope at \(\times 400\). The percent of Hist\(+\) cells containing Fos was calculated for each animal. Because the data were expressed as percentages, they were subjected to an arc sin transformation to normalize the distribution. The data were then subjected to a one-way ANOVA, with ZT as the independent variable and percent Hist\(+\) cells containing Fos as the dependent variable; Fisher's PLSD tests were used for pairwise analyses.

**RESULTS**

**Experiment 1**

Gal. As shown in Fig. 1, many Gal\(+\) cells were found in the area identified as the VLPO in *A. niloticus*. The location of the cluster (Fig. 1) is similar to that described in rats \(32\). This area is found just dorsal to the lateral edge of the optic chiasm and medial to the nucleus of the horizontal limb of the diagonal band, with the caudal end of the VLPO in the same section as the rostral pole of the SCN. The cluster of cells is pyramidal in shape, with the base near the edge of the optic chiasm and flaring out dorsally and laterally. In the set of sections processed for Gal-ICC (every 3rd section), a mean of 34.63 (SE = 3.63) Gal\(+\) cells per section (unilateral) was found in the VLPO. Gal\(+\) cells were concentrated in a cluster in the VLPO, with scattered Gal\(+\) cells in the preoptic area (Fig. 2). Cells heavily labeled for Gal were also seen in the paraven
tricular hypothalamic nucleus and supraoptic nucleus.
Experiment 2

As shown in Fig. 3, ZT significantly affected the number of Fos+ cells in the VLPO ($F_{3,20} = 3.486$, $P < 0.05$). The number of Fos+ cells at ZT 17 was significantly greater than the number at ZT 5 and ZT 13 ($P < 0.05$; see Fig. 4). As shown in Fig. 5, Fos+ cell number also varied significantly across time of day in the PVT ($F_{3,20} = 8.439$, $P < 0.001$; see Fig. 6). There were more Fos+ cells in the PVT at ZT 1 than at any other time point ($P < 0.05$); also, more Fos+ cells were found in the PVT at ZT 5 than at ZT 13 ($P < 0.05$).

When the PVT subregions were analyzed, a significant rhythm of Fos expression was found in the middle PVT ($F_{3,20} = 4.605$, $P < 0.05$) and posterior PVT ($F_{3,20} = 7.861$, $P < 0.01$), but not in the anterior PVT ($F_{3,20} = 1.782$, $P = 0.183$). The daily patterns in Fos expression were similar in each PVT subdivision; the data for the PVT subdivisions were pooled, and the data on Fos expression for the entire PVT Fos expression were used for the correlational analyses (see below).

No significant differences in Fos+ cell number across the light-dark cycle were found in the CMT ($F_{3,20} = 1.251$, $P = 0.318$; see Fig. 5). However, as illustrated in Fig. 7, a highly significant correlation was found between Fos+ cell number in the PVT and the CMT ($r = 0.794$, $P < 0.0001$). A significant rhythm in the number of Fos+ cells was found in the VTM ($F_{3,20} = 4.330$, $P < 0.05$), where Fos cell number at ZT 17 was less than any other time point ($P < 0.05$; see Figs. 8 and 9). No effect of ZT was found in the DTM ($F_{3,19} = 0.646$, $P = 0.595$). Fos+ cell numbers in the CMT were also positively correlated with those for the VTM ($r = 0.414$, $P < 0.05$).

As shown in Fig. 10, some Hist+ cells in and around the VTM contained Fos immunoreactivity. There was a significant rhythm in Fos expression within Hist+ cells of the VTM ($F_{3,18} = 5.965$, $P < 0.01$). Specifically, more Hist+ cells contained Fos at both ZT 1 and ZT 13 than at ZT 17 and more double labeling was seen at ZT 13 than at ZT 5 ($P < 0.05$; see Fig. 11).

DISCUSSION

The location of the VLPO of A. niloticus is similar to that described for the rat (20, 32, 33). It is comprised of a cluster of cells adjacent to the nucleus of the horizontal limb of the diagonal band in the lateral preoptic area. Furthermore, as in the rat (32), neurons of this
area are Gal+. Together with data showing that Fos expression in the VLPO increases at times when these animals sleep (19), these findings suggest that the VLPO serves a similar function in nocturnal rats and in the diurnal A. niloticus.

The results showed that the VLPO of the diurnal A. niloticus displays a daily rhythm in Fos expression in animals kept in a 12:12-h light-dark cycle. Fos+ cell number increased in the middle of the night at ZT 17. Observations of behavioral indexes of sleep showed that A. niloticus are most likely to be sleeping in the middle of the dark phase and least likely to be sleeping at the light-dark transitions and during the light phase (20). The pattern of Fos expression seen in the VLPO of A. niloticus is, therefore, correlated with these animals' sleep patterns: at ZT 17, when VLPO Fos expression is high, grass rats are likely to be sleeping, whereas they are unlikely to be sleeping when VLPO Fos expression is low, at ZT 1–ZT 13. Taken together with previous data (20), these results indicate that VLPO Fos expression increases throughout the middle and late night in A. niloticus and decreases just before lights-on. The presence of a rhythm in the VLPO raises the possibility that the SCN may directly or indirectly influence the activity of the VLPO. Given the presence of very few direct projections from the SCN to the VLPO in rats (4, 18), this circadian regulation may be imposed via relay sites or via humoral rather than axonal SCN outputs (34).

This study also showed that the PVT of A. niloticus displays a daily rhythm in Fos expression. For the most part, the pattern seen in the PVT is predictable from the activity pattern of this species: at the time of the morning activity bout of these animals (ZT 1), Fos expression is highest in the PVT. The pattern seen

Fig. 6. Photomicrographs of Fos+ cells in PVT (A) and CMT (B) at different ZTs (hours after lights-on) in A. niloticus. More Fos+ cells were seen in PVT at ZT 1 than at any other time point, including ZT 13 as illustrated here. No significant rhythm in Fos expression was seen in CMT (300 µm × 300 µm box).

Fig. 7. Correlation between Fos+ cell number in PVT (horizontal axis) and CMT (vertical axis) at all time points. r = 0.794, P < 0.0001.

Fig. 8. Fos+ cell number in ventral tuberomammillary nucleus (VTM; A) showed a rhythm over light-dark cycle, with Fos+ cell number lowest at ZT (hours after lights-on) 17 compared with all other time points. No rhythm in Fos+ cell number was seen in dorsal TMN (DTM; B). *Significantly different from ZT 17, P < 0.05.
during the dark phase, however, does not match expectations: Fos expression in the PVT does not increase at ZT 13, after these animals show a moderate activity peak, and it does not decrease at ZT 17, when they are likely to be sleeping and when Fos expression is elevated in the VLPO. These observations raise the possibility that the PVT is more important in maintaining wakefulness early in the light phase than during the rest of the cycle. In this diurnal species, other brain regions may be responsible for inducing wakefulness later in the day.

Although no rhythm was found in the DTM in A. niloticus, a significant rhythm in Fos expression was seen in the VTM, the region of the TMN shown to receive GABAergic and galaninergic projections from the VLPO in rats (32). Fewer Fos+ cells were found in the VTM of A. niloticus taken at ZT 17 than at any other time point. A. niloticus are most likely to be sleeping in the middle of the night (20), when VTM Fos expression is lowest. This pattern is the opposite of that seen in the VLPO, where Fos+ cell number was increased at ZT 17; given that the VTM and VLPO have mutually inhibitory connections (32), the decrease in Fos expression in the VTM at ZT 17 is not surprising. Because no rhythm in Fos expression was found in the rat TMN (unpublished data), the circadian modulation of Fos expression in the grass rat TMN may represent a species difference in the mechanism through which the circadian clock controls the activity cycle. The reduction in Fos expression in the VTM in A. niloticus seen during the middle of the night (ZT 17) may reflect a circadian signal to the TMN in A. niloticus that is weak or absent in rats. Reduced activity in the TMN may facilitate the display of sleep in this diurnal species.

The importance of histamine in the peaks of locomotor activity in grass rats is supported by data from this experiment showing that Fos expression in histaminergic cells within the VTM peaks at ZT 1 and ZT 13. Therefore, histaminergic cells are most active at the times of the day when this species shows the strongest activity, at the light-dark transitions (10, 20). This may contribute to the crepuscular nature of the A. niloticus activity pattern. Furthermore, because Fos expression in Hist+ cells did not significantly decrease from ZT 1 to ZT 5 in grass rats, histamine may also promote wakefulness during the daytime and therefore contribute to the diurnal portion of its activity cycle.

No significant rhythm in Fos expression was found in the CMT of A. niloticus, which suggests that there is a stronger circadian modulation of the VTM and PVT.
than the CMT in this species. This is in contrast to the significant pattern of Fos expression seen in the rat. CMT and represents further evidence that wakefulness is supported by different brain arousal systems in the two species. Although no rhythm was seen in the CMT of A. niloticus, a significant positive correlation was seen between Fos expression in the PVT and CMT. Similar to the rat, because the PVT and CMT are activated together in A. niloticus, this correlation supports the assertion that these two brain regions contribute to wakefulness. Additionally, there was a significant correlation between Fos expression in the CMT and VTM. Both the activity of intralaminar nuclei and histamine release in the cortex are associated with desynchronization of the electroencephalogram (8, 28). Together, the activity of the CMT, PVT, and histaminergic nuclei may account for different components of arousal and wakefulness in this species.

Finally, the possible effects of light on Fos expression in the VLPO must be taken into account when evaluating the data from these two species. For example, the presence of significant retinal projections to VLPO in the rat (15), nearly absent in A. niloticus (20), implies that light may directly stimulate activity of the VLPO to contribute to its rhythmic expression of Fos in rats, but not in A. niloticus. In rats, but not diurnal chipmunks, the light-dark cycle has a powerful effect on daily activity even after SCN lesions (2, 27). The present findings suggest that this effect of light may be absent or diminished in the diurnal rodent examined here.

In summary, both nocturnal rats and diurnal A. niloticus show rhythms in brain areas affecting sleep and wakefulness, and these rhythms differ between the two species in ways that often mirror their activity patterns. Furthermore, species differences may extend to the differential involvement of brain arousal systems in the control of wakefulness and on the direct effects of light in the daily control of sleep patterns.

Perspectives

As observed in hypothalamic targets of the SCN (21), the daily pattern of Fos expression seen in the A. niloticus VLPO differs considerably from that seen in the rat VLPO (i.e., increased Fos production at ZT 5 and ZT 13). For the VLPO, there are several possible explanations for the species difference in the circadian control of Fos expression. The critical difference may lie in 1) a different circadian signal emanating from the SCN, such as a different neurotransmitter; 2) the same signal emitted at a different phase, in which case subpopulations of SCN cells that affect the VLPO would be expected to have different patterns of activity over the circadian cycle in the two species; and/or 3) there may be a species-specific alteration of the circadian signal by an intermediary brain region. Finally, 4) there may be differential responsiveness of the VLPO and other targets of the SCN to similar circadian signals. If this were the case, then the process of modifying the timing of activity of a mammalian species over evolution (i.e., from nocturnal to diurnal, after a nocturnal bottleneck) would have involved a change in responsiveness of various SCN target regions to a common signal.

When the combined patterns of Fos expression in the PVT and VTM are considered, it is possible that, in A. niloticus, these two brain regions contribute in distinct but complementary ways to the overall activity pattern of these animals. This introduces a possible difference between diurnal and nocturnal animals not previously considered: that species differences in activity patterns may involve differential contributions of distinct arousal systems to overall activity and that the strength of circadian modulation (along with the daily pattern of circadian modulation) of these arousal systems differs between species. Additional work is needed to establish causal links between species differences in patterns of neural Fos expression and species differences in the distribution of sleep and wakefulness across the day-night cycle.

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REFERENCES