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PER2 rhythms in the amygdala and bed nucleus of the stria terminalis of the diurnal grass rat (*Arvicanthis niloticus*)

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ABSTRACT

The suprachiasmatic nucleus (SCN) of the hypothalamus is the central pacemaker that controls circadian rhythms in mammals. In diurnal grass rats (*Arvicanthis niloticus*), many functional aspects of the SCN are similar to those of nocturnal rodents, making it likely that the difference in the circadian system of diurnal and nocturnal animals lies downstream from the SCN. Rhythms in clock genes expression occur in several brain regions outside the SCN that may function as extra-SCN oscillators. In male grass rats PER1 is expressed in the oval nucleus of the bed nucleus of the stria terminalis (BNST-ov) and in the central and basolateral amygdala (CEA and BLA, respectively); several features of PER1 expression in these regions of the grass rat brain differ substantially from those of nocturnal species. Here we describe PER2 rhythms in the same three brain regions of the grass rat. In the BNST-ov and CEA PER2 expression peaked early in the light period Zeitgeber time (ZT) 2 and was low during the early night, which is the reverse of the pattern of nocturnal rodents. In the BLA, PER2 expression was relatively low for most of the 24-h cycle, but showed an acute elevation late in the light period (ZT10). This pattern is also different from that of nocturnal rodents that show elevated PER2 expression in the mid to late night and into the early day. These results are consistent with the hypothesis that diurnal behavior is associated with a phase change between the SCN and extra-SCN oscillators.

In mammals, the suprachiasmatic nucleus of the hypothalamus (SCN) is the primary pacemaker that controls circadian rhythms in physiology and behavior. It is entrained to the 24-h day/night cycle by the light information from melanopsin containing retinal ganglion cells that reach the SCN via the retino-hypothalamic tract [18]. At the cellular level, the SCN generates 24 h rhythms via molecular mechanisms of transcriptional and translational feedback loops involving a set of clock genes, which includes two period genes and their protein products (PER1 and PER2 [12]). When behavioral rhythms are entrained to a light–dark cycle, some species are most active during the day, i.e., diurnal species like us, while nocturnal species show the opposite phase preference and are most active at night. We have used the diurnal grass rat (*Arvicanthis niloticus*) to investigate features of the circadian system that may contribute to diurnality in some mammalian species. One consistent finding of our work with grass rats is that many features of the SCN appear to be very similar when grass rats are compared to nocturnal rodents; these similarities extend to the phase of rhythms in PER1 and PER2 in the SCN with respect to the light–dark cycle [19,25]. Observations reported by others [13] also support the general principle that the SCN functions in a similar fashion in diurnal and nocturnal species. Based on these results we and others have hypothesized that the fundamental differences in the circadian system of diurnal and nocturnal mammals reside downstream from the SCN [24,25].

Molecular oscillators are present not only in the SCN, but also in other brain regions and peripheral tissues (reviewed by [10]). These extra-SCN oscillators appear to control region specific functions [11] and in most cases depend upon circadian signals from the SCN in order to sustain their oscillations [1,3]. Several areas of the mammalian brain that contain extra-SCN oscillators are involved in the control of behavioral and physiological functions that are temporally inverted in diurnal and nocturnal rodents [4,5,10,20,21]. The central extended amygdala, which includes the oval nucleus of the bed nucleus of the stria terminalis (BNST-ov), and the central amygdala (CEA; [2]), and the basolateral amygdala (BLA) are of particular interest because they show rhythms in the expression of PER proteins [5] and play important roles in the control of autonomic functions [22] and emotional learning and memory [16]. We have identified salient differences in the expression of PER1 in the BNST-ov, CEA and BLA when male grass rats are compared to males of nocturnal species [21], and we now extend our observations to the rhythmic expression of PER2 in these three regions of the brain

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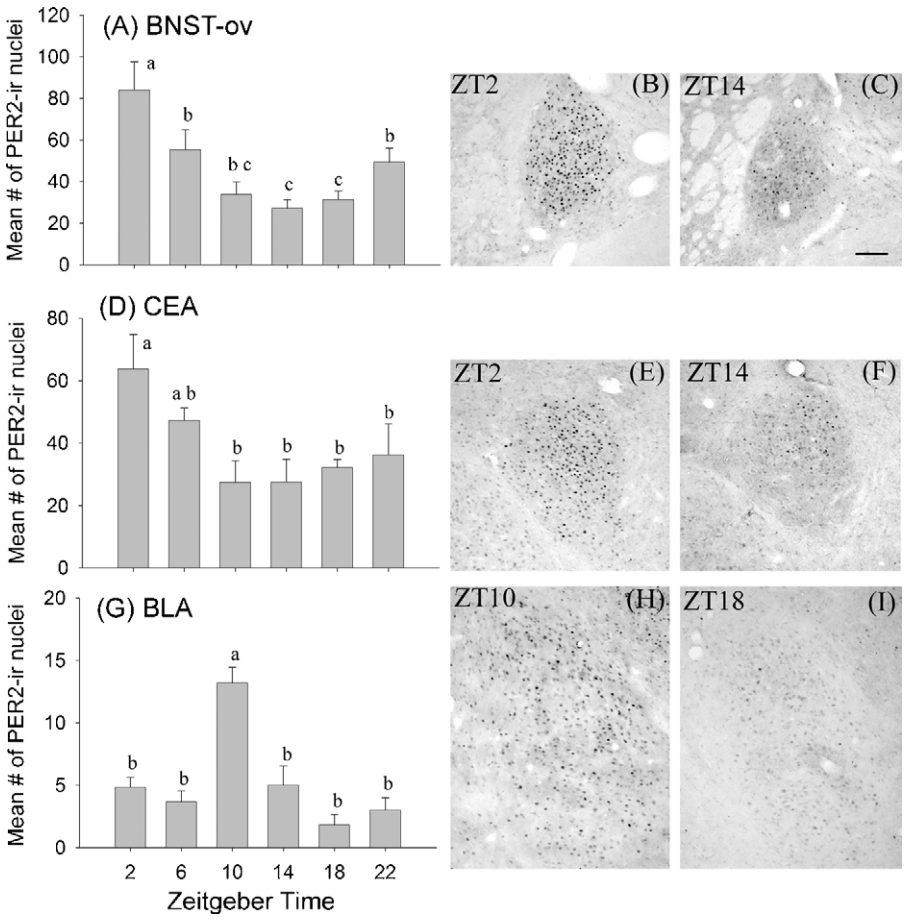

Fig. 1. Left: bar graphs showing the mean (±standard error of the mean) number of PER2-immunoreactive cells as a function of Zeitgeber time (ZT) in (A) the oval nucleus of the bed nucleus of the stria terminalis (BNST-ov), (D) the central amygdala (CEA), and (G) the basolateral amygdala (BLA). Right: photomicrographs of the BNST-ov (B, C), CEA (E, F), and BLA (H, I) of representative animals perfused at times of high and low PER2 expression. Scale bar = 100 μm for the photomicrographs. ac anterior commissure.

of grass rats. Collecting data on PER2 rhythms in our diurnal animal model was motivated by our interest in comparing data from grass rats to those obtained from nocturnal species, and by the facts that in nocturnal laboratory rats, only PER2 is expressed rhythmically in the amygdala [5,6] and only PER2 data are available for the BNST-ov of nocturnal laboratory mice [23].

Adult male grass rats ($n = 36$) (*A. niloticus)* were obtained from our institution's breeding colony and were housed individually in Plexiglass cages (34 × 28 × 17 cm), under a 12:12 h light/dark (LD) cycle [lights on at 06:00 h; Zeitgeber time (ZT) 0]. Animals had free access to food (PMI Nutrition Prolab RMH 2000, Brentwood, MO, USA) and water. All experiments were performed in compliance with guidelines established by the Michigan State University All University Committee on Animal Use and Care, and the National Institute of Health guide for the Care and Use of Laboratory Animals.

Animals were perfused at 4-h intervals from Zeitgeber time (ZT) 2-22. At each of the six ZTs, groups of grass rats ($n = 6$/ZT) were deeply anesthetized with sodium pentobarbital and perfused transcardially with 0.01 M phosphate buffered saline (PBS; pH 7.2) followed by 4% paraformaldehyde in 0.1 M phosphate buffer (Sigma, St Louis, MO USA). Brains were removed and post-fixed for 4 h and then transferred to 20% sucrose overnight and then the brains stored in cryoprotectant at −20 °C until they were sectioned (30 μm; coronal plane) using a freezing microtome. Immunocytochemical (ICC) procedure for PER2 was performed on every other section exactly as previously described [19] using the primary antibody against PER2 (made in rabbit, 1:10,000, and graciously provided Dr. D. R. Weaver, University of Massachusetts, MA, USA).

For counts of PER2-immunoreactive cells, pictures of the BNST-ov, CEA, and the BLA regions were obtained using a digital camera (MBF Bioscience Inc, 2007) attached to a Zeiss light microscope (Carl Zeiss, Gottingen, Germany). We used NIH ImageJ software (National Institute of Health [NIH], Bethesda, MD) to count immunopositive cells. Cells in the BNST-ov and the CEA were bilaterally counted every other section for three sections and average cell counts from these were used for analysis; one section was used to count cells bilaterally in the BLA. Cell counts were made by an individual blind to the times at which animals were perfused. For all three regions, one-way ANOVAs were used to evaluate the effects of ZT and significant F ratios were followed by Fisher's LSD tests for individual group comparison. In all comparisons, differences were considered significant when $P < 0.05$.

The distribution of PER2 positive cells in the BNST-ov, CEA, and BLA was very similar to that previously seen for PER1 in grass rat. In the BNST-ov, there was a significant main effect of time on PER2 expression ($F = 6.45$, df = 5, $P < 0.001$). In this region, the number of labeled cells was highest at ZT2 and decreased progressively until ZT18 and then rose from ZT18 to ZT22 (Fig. 1 top panel). In the CEA there was a significant main effect of time on PER2 expression ($F = 3.22$, df = 5, $P = 0.02$). Pair-wise comparison revealed that the number of PER2 labeled cells peaked at ZT2 and declined from ZT2 to ZT10 and were unchanged from ZT10 to ZT22 (Fig. 1 middle panel). In the BLA, there was a significant main effect of time on PER2 expression ($F = 12.86$, df = 5, $P < 0.001$). Pair-wise comparison revealed that ZT10 was significantly different from all other ZTs (Fig. 1 bottom panel).

The main finding of this study was that the temporal patterns of PER2 expression in the extra-SCN oscillators of the extended amygdala of grass rats were substantially different from those of nocturnal species, as previously reported for the expression of *Per2* mRNA in the peripheral oscillator of the liver [14]. These results provide further evidence for the hypothesis that the fundamental differences in the circadian systems of diurnal and nocturnal species reside in neural circuits downstream from the SCN. More specifically, they suggest that a change in phase between the oscillator in the SCN and those that reside in other brain regions may contribute to the fundamental differences between chronotypes.

In the BNST-ov of the grass rat, the peak of the PER2 rhythm occurred early in the light period, which is similar to the pattern of PER1 expression of this species [21]. These PER1 and PER2 rhythms in the BNST-ov of grass rats are 180° out of phase with those seen in the BNST-ov of the laboratory rat, in which PER1 and PER2 both peak early in the dark period [3,6]. In mice, the pattern of PER2 expression in this region resembles that seen in laboratory rats, but the available data are limited to two sampling times (ZT1 and ZT13; [23]); no information about PER1 is available for the BNST-ov of this species. As in the BNST-ov, PER2 peaked early in the light period in the CEA of grass rats, and again, the pattern of PER2 seen here resembled that seen earlier for PER1 in this species [21]. In the CEA of laboratory rats, there is a PER2 rhythm that peaks early in the dark period [15] and is, again, reversed relative to that of grass rats, but there is no PER1 rhythm [6]. In mice, PER1 and PER2 in the CEA start to rise early in the night, and while PER1 expression diminishes over the next few hours, that of PER2 is sustained across most of the dark period [9]. In the BLA of the grass rat, there is an acute increase in PER2 expression late in the light period, whereas PER1 expression is high throughout the light period in males [21], but not females [20], of this species. In the BLA of laboratory rats [15] and mice [9], PER2 production starts to rise mid to late in the night and most studies report continued elevated levels into the early light period [5]; there is no PER1 rhythm in the BLA of lab rats [6] and no PER1 expression in the BLA of mice [9]. In summary, our results and the available literature indicate that the PER2 rhythms in the central extended amygdala and BLA in diurnal grass rats are 12 h out of phase with those of nocturnal rodents, except the BLA of mice which is 6 h out of phase with the grass rats.

The BNST-ov, the CEA and the BLA play distinct and important roles in emotional learning and memory [16,26] and autonomic regulation [17,22]. It is likely that the phase of oscillators in these brain regions influences functions that are regulated by neuronal circuits that include them. The current data suggest that the daily rhythms of these functions may be quite different in diurnal and nocturnal rodents. For example, species differences in the phase of oscillators in the BLA could promote differences in the circadian modulation of long term retention of learned fear responses [8]. If that is the case, then rhythms in retention of acquired fear responses should differ in grass rats and lab rats in ways that might be predicted from patterns of clock gene expression seen here in the BLA and related structures.

Work using nocturnal rodents has shown that most extra-SCN oscillators in the brain and in peripheral tissues depend upon the SCN, and that they dampen in its absence [1,3]. However, other factors such as hormones and feeding schedules can also affect extra-SCN oscillators and change their phase with respect to that of the SCN [4,5]. These observations suggest at least three potential mechanisms that could account for the differences between grass rats and nocturnal species with respect to the phase of extra-SCN oscillators. One possibility is that direct or indirect targets of the SCN of diurnal and nocturnal species respond differently to common signals originating in the SCN [25]. A second possibility is that these differences are secondary to a reversal in rhythms in hormone secretion. The rhythms in PER2 expression in the BNST-ov and the CEA of laboratory rats and mice depend upon circadian rhythms in circulating glucocorticoids from the adrenal gland [4,23], which are inverted in diurnal and nocturnal mammals [7]. A phase reversal in the adrenal gland could thus be responsible for the species differences in the temporal patterns of PER2 expression in the BNST-ov and the CEA (present data and [5]). Finally, grass rats eat more frequently during the light period (unpublished observations), while laboratory rats have larger and more frequent meals at night [27]. Since feeding schedules can shift the phase of extra-SCN oscillators [5,6,9], including those of the BNST-ov and the amygdala [5], it is possible that different feeding patterns are responsible for the differences in phase in the extra-SCN oscillators of diurnal and nocturnal species.

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