Effect of Long-Term Exposure to Constant Dim Light on the Circadian System of Rats

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Key Words

Suprachiasmatic nucleus · Retina · Pineal gland · Arylalkylamine N-acetyltransferase · Melatonin · Desynchronization, internal · *Period 1* gene

Abstract

The newly discovered multi-oscillatory nature of the mammalian circadian clock system and the cloning of the genes involved in the molecular mechanism that generates circadian rhythmicity have opened new approaches for understanding how mammals are temporally organized and how the mammalian circadian system reacts to the lack of normal synchronization cues. In the present study we investigated the effects of long-term exposure to constant red dim light on the pattern of the expression of *Period 1* in the suprachiasmatic nuclei of the hypothalamus and of *Arylalkylamine N-acetyltransferase (Aa-nat)* in the retina and pineal gland. Our data demonstrate that *Period 1* mRNA expression in the suprachiasmatic nuclei of the hypothalamus was not affected by exposure to constant red dim light for 60 days, whereas *Aa-nat* mRNA expression in the retina and in the pineal gland was significantly affected, since in some animals (20–30%) *Aa-nat* mRNA levels were found

to be higher during the subjective day. A circadian rhythm of serum melatonin and locomotor activity was present in all the animals tested. In 4 animals serum melatonin levels were high during the subjective day. Our data suggest that long-term exposure to constant red dim light may induce desynchronization between the circadian rhythm of locomotor activity and serum melatonin levels.

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Introduction

Daily rhythmicity is a ubiquitous feature of living systems. Generally, these rhythms are not just a passive consequence of cyclic fluctuations in the environment, but rather they originate within the organisms: they are generated and controlled by endogenous physiological oscillators. The fundamental adaptive function of an endogenously programmed rhythmicity is to provide an optimal and anticipatory temporal organization of physiological processes and behavior in relation to the environment [1]. Synchronization of endogenous circadian rhythms to the 24-hour environment occurs through daily adjustment of the clock by external time cues or zeitgebers. The most

important and reliable cue for synchronization is the daily light-dark cycle [2].

The newly discovered multi-oscillatory nature of the mammalian circadian clock system [3] and the cloning of the genes involved in the molecular mechanisms that generate circadian rhythmicity [4] have opened new approaches for understanding how mammals are temporally organized, and how the mammalian circadian system reacts to the lack of normal synchronization cues.

The primary goal of the present study was to determine how the circadian rhythms in the suprachiasmatic nuclei (SCN) of the hypothalamus, retina and pineal are affected by long-term exposure to constant red dim light (LL_{dim}). Previous work has demonstrated that a clear phase relationship exists among the pattern of expression (in the SCN and elsewhere) of the clock genes belonging to the *Period* family and the locomotor activity rhythm [5–8]. In addition, other studies have demonstrated that the pattern of expression of *Arylalkylamine N-acetyltransferase* (*Aa-nat*, i.e., the gene that is responsible for the synthesis of the key forming enzyme in melatonin synthesis) in the retina and the pineal gland is under circadian control, and its expression shows a fixed and dependable phase relationship with the circadian rhythm of locomotor activity [for review see 5, 9, 10]. This experimental evidence suggested that the pattern of expression of clock genes (e.g. *Period 1* [*Per1*]) and clock-controlled genes (e.g., *Aa-nat*) could be used as reliable markers of the circadian phase in different organs, and thus could be utilized to investigate how exposures to constant conditions affect the circadian system.

In the present study we investigated how circadian rhythms in the SCN (by assessing locomotor activity and *Per1*) mRNA levels, in the retina and in the pineal (by measuring *Aa-nat* mRNA and serum melatonin levels) are affected by prolonged exposure to constant LL_{dim}.

Materials and Methods

Experimental Animals

Three groups (n = 36 in each group) of male Wistar (Charles River Laboratory) rats, 8 weeks old at the start of experiment, were used in this study. During the experiment, the rats had free access to food and water.

Monitoring of Wheel-Running Activity

Animals were housed in cages equipped with a running wheel and a magnetic sensor system to detect wheel rotation (Mini-Mitter Co., Sunriver, Oreg., USA). Wheel-running data were recorded in 5-min bins saved on a PC using specific software (Tau, Mini-Mitter Co.) and were analyzed with specific software (VitalView, Mini-Mitter

Co.) to determine rhythmicity and the free-running period. Period was estimated by χ^2 periodogram analysis.

Housing and Tissue Collection and Assessment of the Circadian Phase

Animals were housed in a temperature controlled (23–24°C) ventilated lightproof room. The animals were kept in a light:dark (LD) cycle or in constant dim illumination (LL_{dim}, 1 μ W/cm², 5 Lux). In LD the onset of light was defined as zeitgeber time (ZT) 0, and the onset of darkness as ZT12. In LL_{dim} light was provided by a special fluorescent fixture (Litho light No. 2, lower wavelength cutoff at 640 nm). In all the experiments when animals were sacrificed during the dark phase, the procedure was carried out in LL_{dim}. Retinas and pineal glands were removed under LL_{dim} (except for the sampling during the light phase).

After 0 (LD), 5, and 60 days of exposure to LL_{dim}, animals (6 individuals for each time point) were sacrificed at six different circadian times (CTs): 3 during the subjective day (CT4, CT8 and CT12) and 3 during the subjective night (CT16, CT20 and CT24), and the SCN, retinas and pineal glands were collected and immediately frozen on dry ice. In order to ensure that each animal was sacrificed at the exact circadian time, the following procedure was adopted. For each animal, the time of sacrifice was determined based upon each animal's locomotor activity rhythm. CT12 was defined as the time at which an animal began its daily bout of wheel-running activity. Wheel-running records were plotted, and a line was drawn through activity onsets in order to calculate CT12 on the day of sacrifice. All other circadian times were calculated relative to CT12.

Semiquantitative in situ Hybridization

Blocks of brain containing the SCN region were obtained using razor blades, immediately frozen using dry ice, and kept at –80°C until sectioning. Pieces of brain were then sliced into 20- μ m-thick sections. For hybridization at least 5 sections from the medial part of the SCN were used for each animal. A 982-bp fragment of rat *Per1* cDNA cloned in the pGEM-T easy vector (Promega) was linearized by *SalI* or *SacII* for antisense or sense probe. [³⁵S]-UTP-labeled antisense and sense probes were obtained by in vitro transcription using T7 or SP6 RNA polymerase. Hybridization was performed overnight at 55°C. Following 30 min of RNase A treatment at 37°C, sections were washed, dehydrated by ethanol, and air-dried. The slides were then exposed to Kodak MR film with ¹⁴C standards (Amersham Life Science) for 4–6 days at room temperature.

Each autoradiography was subjected to quantification. Image of autoradiography films was scanned using a high-resolution scanner (U-MAX, PowerLook III) and optical density (OD) values for sense and antisense probes were determined using NIH image (version 1.62) on a Macintosh (G 3) computer. OD values for the sense probe were subtracted from OD values of the anti-sense probe. At least 5 sections from 1 animal were used and averaged OD values were represented as one animal's OD value. All the slides were stained using toluidine blue for evaluation of the SCN region and tissue conditions [for further details see 11, 12].

Real-Time Quantitative RT-PCR

Total RNA from the retina and pineal gland was extracted using Trizol reagent (Gibco, BRL) following sonication. DNA was degraded by DNase I (Boehringer Mannheim). One set of sample (ZT or CT 4, 8, 12, 16, 20, 24) was subjected to all the procedures (RNA extraction, DNase I treatment, cDNA synthesis, and PCR) at the

same time. Real-time quantitative RT-PCR was carried out using an *iCycler* (Biorad). Standards that contained a known amount of *Aa-nat* or *Gapdh* plasmid DNA were used to generate standard curves during each run. We used the following primers: *Gapdh* (5'-AGACAGCCGCATCTTCTTGT-3', 5'-TGATGGCAACAATG-TCCACT-3'), and *Aa-nat* (5'-TCACTGAGCTGCAATGTTCC-3', 5'-GATGTCAGCCTGGCCTAGAG-3'). Forty cycles of amplification were carried out following 15 s of denaturation at 95°C. Once the temperature reached 95°C, it was decreased to 60°C, maintained for 45 s, and raised to 72°C for 30 s. Fluorescence (Sybr Green) was measured at melting temperature after each cycle, rather than at the end of 40 cycles of amplification, which allows comparison of fluorescence intensities among samples while their increase is within linear regression. Melting temperatures used were 86°C for *Gapdh* and 89°C for *Aa-nat*.

After RT-PCR, PCR products were run on the agarose gel to verify the quality of products. The amount of RNA was estimated based on the standard by computer software (The LightCycler Software Version 3). *Aa-nat* mRNA levels were normalized by *Gapdh* [13].

Blood Sampling and Radioimmunoassay

Thirteen rats were exposed to constant LL_{dim} for 60 days, then blood samples (80–100 µM) were collected every 4 h from the tail artery in heparinized tubes, stored overnight at 4°C, and then centrifuged at 1,500 rpm. For each animal, the time of sampling was determined based upon each animal's locomotor activity rhythm. CT12 was defined as the time at which an animal began its daily bout of wheel-running activity. Wheel-running records were plotted, and a line was drawn through activity onsets in order to calculate CT12 on the day of sacrifice. All other circadian times were calculated relative to CT12. Control samples were obtained from 2 rats kept in LD for 60 days. After that the serum was stored at –80°C until the samples were processed for radioimmunoassay. Melatonin was extracted from the serum (50 µM) using chloroform and then melatonin levels were measured using a commercially available kit (Alpco Diagnostics). The sensitivity of the assay was 0.2 pg/ml. Intra-assay variability was 7% and the inter-assay variability was 11%.

Results

Wheel-Running Activity

In all animals tested, clear circadian rhythms in locomotor activity were observed. As expected, the free-running period was longer than 24 h (for all the animals tested the mean ± SEM was 24.9 ± 0.12). There was no noticeable difference in the locomotor activity pattern (i.e., in the free-running period or in the duration of the activity) during the duration of the whole experiment (fig. 1).

Per1 Expression in the SCN

Using in situ hybridization we investigated the pattern of *Per1* gene expression in the SCN. *Per1* expression in the SCN showed robust rhythmicity in animals kept in LD cycles (fig. 2; Kruskal-Wallis, $p < 0.05$), the rhythmicity persisted in animals exposed to LL_{dim} for 5 days (fig. 2;

Kruskal-Wallis, $p < 0.05$), and in animals that have been exposed to LL_{dim} for 60 days (fig. 2; Kruskal-Wallis, $p < 0.05$). In all three conditions the *Per1* expression peaked during the middle of the day (subjective day for the animals held in constant conditions) around CT4–8. The only noticeable differences between LD and 60 days in LL_{dim} were a small reduction in the amplitude of the rhythm and an increase in the overall variability (fig. 2).

Aa-nat Expression in the Retina

In LD conditions, *Aa-nat* mRNA levels showed marked day-night variations (fig. 3; Kruskal-Wallis, $p < 0.05$). The retinal *Aa-nat* mRNA rhythm persisted after 5 days of LL_{dim} conditions (fig. 3; Kruskal-Wallis, $p < 0.05$). After 60 days in LL_{dim} *Aa-nat* mRNA levels showed an increase in the overall variability at each CT analyzed (fig. 3). In some animals *Aa-nat* mRNA levels were found to be high during the subjective day (CT 4–12) or low during the subjective night (CT16–24).

Aa-nat Expression in the Pineal

In LD conditions, pineal *Aa-nat* mRNA levels showed a robust day-night variation (fig. 4; Kruskal-Wallis, $p < 0.05$). *Aa-nat* mRNA levels were very low during the day and higher at night. Pineal *Aa-nat* mRNA rhythm persisted after 5 days of LL_{dim} conditions (fig. 4; Kruskal-Wallis, $p < 0.05$); *Aa-nat* signals were observed throughout the day with the highest level at CT16–20 and the lowest at CT12. After 60 days in LL_{dim} (fig. 4), in a few animals *Aa-nat* mRNA levels were high during the subjective day (CT4–12) or low during the subjective night (CT16–24; fig. 4).

Serum Melatonin

In figure 5 are shown the serum melatonin profiles over a 24-hour period for 2 controls (i.e., animals exposed to LD) and 13 experimental animals (i.e., animals exposed to LL_{dim} for 60 days). All the animals showed significant circadian melatonin rhythms ($p < 0.01$). In controls melatonin levels were high during the night and low during the day (fig. 5A, B), the same profile was also observed in the majority ($n = 9$) of the experimental animals (fig. 5C–E, G–I, K, M, N). In 4 animals serum melatonin levels were higher during the subjective day (CT4–12) than during the subjective night (fig. 5F, J, L, O).

Fig. 2. Circadian variation of *Per1* mRNA levels in the SCN measured using semiquantitative in situ hybridization. 12L:12D; after 5 days in LL_{dim} and after 60 days in LL_{dim}. The white circles represent individual values; the black circles represent the mean ($\pm$ SEM) values ($n = 6$ for each time point).

Fig. 3. Circadian variation of *Aa-nat* mRNA levels in the retina. 12L:12D; after 5 days in LL_{dim} and after 60 days in LL_{dim}. The white circles represent individual values, while the black circles represent the mean ($\pm$ SEM) values ($n = 6$ for each time point).

Fig. 4. Circadian variation of *Aa-nat* mRNA levels in the pineal. 12L:12D; after 5 days in LL_{dim} and after 60 days in LL_{dim}. The white circles represent individual values, while the black circles represent the mean ($\pm$ SEM) values ($n = 6$ for each time point).

Fig. 1. Representative double plotted activity records (from day 20 to day 60) for 2 rats exposed to LL_{dim} for 60 days. In these animals, as in all the others, a clear circadian rhythm in locomotor activity was present for entire duration of the experiment. Small changes in the free-running period were observed (see periodogram analysis next to each actogram; The lines indicate the onset of activity and the interval of time in which the periodogram was calculated). The serum melatonin levels for these animals are shown in figure 5D (A) and 5J (B).

Fig. 5. Serum melatonin profile in animals exposed to LD (**A, B**) and after 60 days of exposure to LL_{dim} (**C–O**). In the control animals and in most of the experimental animals serum melatonin levels were high during the subjective night (ZT16–24), whereas in 4 animals serum melatonin levels were high during the subjective day (**F, J, L** and **O**).

Discussion

At the core of the system that controls and regulates circadian rhythms in mammals there are three structures, i.e., the retina, the pineal gland and the SCN, that together with interconnection form a central circadian axis [14]. The present work demonstrates that long-term exposure to LL_{dim} has profound effects on this axis, since it affects the circadian transcription of *Aa-nat* mRNA in the retina and pineal gland. However it is important to note that all the animals tested showed a robust circadian rhythm in the levels of circulating melatonin and in the locomotor activity rhythm. Interestingly in a few animals ($n = 4$) melatonin levels peaked during the daytime (CT4–8). Such a result suggests that exposure to LL_{dim} may induce desynchronization between the circadian rhythm of locomotor activity and the circadian rhythm of serum melatonin.

Our data are consistent with previous investigations that have demonstrated that *Per1* mRNA transcription in the SCN is rhythmic in LD and this rhythm is maintained when the animals are released in constant conditions [6–8]. The data presented here expand these previous results by demonstrating that circadian expression in *Per1* mRNA in the SCN is maintained in animals that have been exposed to LL_{dim} for 60 days (fig. 2). Such a finding suggests that the expression of this gene in the SCN is not significantly affected by long-term exposure to LL_{dim}. This is not surprising, since a previous study has shown that *Per1* expression in cultured SCN can be maintained for at least 32 cycles [15].

Previous investigations have also shown that *Aa-nat* mRNA expression in the pineal gland and retina is rhythmic in LD, and after few days in a constant lighting condition [5, 10]. Our data agree well with these previous reports. Different from what we have observed in the SCN, *Aa-nat* mRNA in the retina and pineal gland was significantly affected by long-term exposure to LL_{dim}, since in some animals *Aa-nat* mRNA levels were found to be high in the subjective day and low in the subjective night (fig. 3, 4).

An important question that arises from these data is the physiological significance of the results obtained in this study with respect to the relationship between locomotor activity and *Aa-nat* mRNA. Several studies have shown that in the rat *Aa-nat* mRNA is the rate-limiting enzyme in the melatonin biosynthetic pathway and that changes in the transcription rate will induce changes in the protein levels and activity, and thus in the levels of melatonin [for review see 9]. Therefore, it is likely that the

observed alteration of *Aa-nat* mRNA levels may induce in significant changes in the circadian profile of the circulating melatonin.

To further support our inference, we investigated the circadian rhythms of serum melatonin in rats exposed to LL_{dim} for 60 days. As shown in figure 5C–O melatonin was detected in the serum and showed a robust circadian rhythm in all the animals tested, thus demonstrating that exposure to LL_{dim} does not abolish melatonin synthesis. The levels of circulating serum melatonin were similar to those observed in previous studies [16] and no significant differences were observed between control and experimental animals (fig. 5). In most of the animals the phase of the melatonin rhythm was similar to that observed in the control animals. In 4 animals the melatonin rhythm was uncoupled from the locomotor activity rhythm since it peaked during the subjective day (fig. 5E, F, J, O), thus confirming the results obtained with pineal *Aa-nat* mRNA (fig. 4).

Such a result further indicates that in some animals long-term exposure to LL_{dim} may uncouple the circadian of locomotor activity from the circadian rhythm of *Aa-nat* mRNA and serum melatonin levels. Furthermore, our data suggest that the uncoupling of the melatonin circadian from the locomotor activity rhythm occurs without affecting the overall pattern of activity and *Per1* expression in the SCN.

Alteration in the rhythm of melatonin may produce important effects on the organism. For example, altering melatonin levels during the daytime has a significant effect on sleep, mood, and performance [17], and motor performance [18].

Internal desynchronization among circadian rhythms has been reported only by a few studies. For example, spontaneous internal desynchronization between the body temperature rhythms and locomotor activity rhythms was observed in reptiles [19] and in the squirrel monkey [20]. In the owl monkey internal desynchronization between circadian activity and the feeding patterns has been also observed [21]. A recent study has reported that exposure to dim illumination may uncouple several (e.g., sleep, body temperature, locomotor activity and drinking) circadian rhythms in the rat [22]. Internal desynchronization has also been reported in humans [23, 24], and it is believed to be the cause of several pathologies [25, 26]. The data presented demonstrate that desynchronization may occur between the locomotor activity rhythm and the molecular mechanisms controlling melatonin synthesis.

Since it is well established that the mammalian retina contains an autonomous circadian clock that directly controls *Aa-nat* mRNA [27] and melatonin synthesis [28], it is likely that the desynchronization observed in some animals between retinal *Aa-nat* mRNA and locomotor activity rhythm is the consequence of the uncoupling between the retinal clock and the SCN clock. On the other hand an explanation of the results obtained in the pineal gland is more complicated. *Aa-nat* mRNA in the rat pineal gland is directly regulated by the SCN via a multisynaptic pathway [29]. Although recent experimental evidence has suggested that the mammalian pineal may also contain a circadian clock [30], it is unlikely that *Aa-nat* mRNA is regulated by this endogenous clock since the mechanism by which the circadian clock directly controls the *Aa-nat* gene is not functional in the mammalian pineal gland [27]. Therefore, we believe that the mechanisms responsible for the desynchronization of *Aa-nat* mRNA in the pineal gland may reside within the SCN and/or in the pathway connecting the SCN and the pineal gland. This belief is based on the fact that the SCN is composed of different populations of circadian oscillators that constitute regional pacemaker-controlling specific circadian outputs. For example, the circadian rhythm of arginine vasopressin and vasoactive intestinal polypeptide release in the rat SCN is regulated by different populations that can desynchronize from each other [31, 32]. Indeed a recent study has revealed that splitting of the locomotor activity rhythm may be the consequence of desynchronization of populations between the SCN [33], and another study using a 'forced desynchronization' protocol has shown the presence of two oscillators in the anatomically defined ventrolateral and dorsomedial SCN subdivisions [34]. Our autoradiography data did not show any obvious evidence of desynchronization between the two SCN subdivisions in each animal (data not shown). However, we cannot categorically exclude that a desynchronization be-

tween the two SCN subdivisions has occurred in our animals. Indeed, an increase in the overall variability of *Per1* mRNA was observed in the SCN after 60 days in LL_{dim} (fig. 2). In fact, in the present study *Per1* expression was only examined in sections obtained from the middle part of the SCN. Another limiting factor in our analysis is represented by the technique we employed (autoradiography followed by a densitometry analysis) that is not suitable to detect small changes in the hybridization signal. Further studies are necessary to test the hypothesis that long-term exposure to LL_{dim} may induce desynchronization of a subpopulation of SCN neurons that is responsible for the control of pineal melatonin synthesis.

In conclusion, our data demonstrate that exposure to LL_{dim} did not affect the circadian rhythms of *Per1* mRNA in the SCN and locomotor activity, whereas *Aa-nat* mRNA expression in the pineal gland and the retina of some animals was somewhat affected by exposure to LL_{dim} for 60 days. Finally, we found that in all the animals examined melatonin levels showed robust circadian rhythms, although in a few (30%) animals the serum melatonin rhythm was uncoupled from the circadian rhythms of locomotor activity and *Per1* mRNA expression in the SCN. Finally, it is worthwhile to mention that we observed that pineal *Aa-nat* mRNA levels showed an altered pattern of expression (i.e., high level during the subjective day or low levels during the subjective night) in a similar percentage of animals that showed altered serum melatonin rhythms.

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