RESTRICTED FEEDING REGIME AFFECTS CLOCK GENE EXPRESSION PROFILES IN THE SUPRACHIASMATIC NUCLEUS OF RATS EXPOSED TO CONSTANT LIGHT

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Abstract—The master circadian clock located in the suprachiasmatic nuclei (SCN) is dominantly entrained by external light/dark cycle to run with a period of a solar day, that is, 24 h, and synchronizes various peripheral clocks located in the body’s cells and tissues accordingly. A daily restricted normocaloric feeding regime synchronizes the peripheral clocks but has no effect on SCN rhythmicity. The aim of this study was to elucidate whether feeding regime may affect the molecular mechanism generating SCN rhythmicity under conditions in which the rhythmicity is disturbed, as occurs under constant light. The rats were maintained under constant light for 30 days and were either fed ad libitum during the whole period, or their access to food was restricted to only 6 h a day during the last 2 weeks in constant light. Locomotor activity was monitored during the whole experiment. On the last day in constant light, daily expression profiles of the clock genes Per1, Per2, Bmal1, and Rev-erba were determined in the SCN of both groups by *in situ* hybridization. Due to their exposure to constant light, the rats fed *ad libitum* became completely arrhythmic, while those exposed to the restricted feeding were active mostly during the time of food availability. In the SCN of behaviorally arrhythmic rats, no oscillations in Rev-erba and Bmal1 gene expression were detected, but very low amplitude, borderline significant, oscillations in Per1 and Per2 persisted. Restricted feeding induced significant circadian rhythms in Rev-erba and Bmal1 gene expression, but did not affect the low amplitude oscillations of Per1 and Per2 expression. These findings demonstrate that, under specific conditions, when the rhythmicity of the SCN is disturbed and other temporal entraining cues are lacking, the SCN molecular clockwork may likely sense temporal signals from changes in metabolic state delivered by normocaloric food. © 2011 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: circadian system, suprachiasmatic nucleus, restricted feeding, constant light, rat.

The behavior and many physiological processes of most organisms exhibit daily rhythmic changes. This temporal control is driven by endogenous time-keeping system which, in mammals, consists of central clock in the hypothalamic suprachiasmatic nuclei (SCN) (Ralph et al., 1990) and of peripheral clocks in other brain regions and various peripheral organs like retina, liver, lung, skeletal muscle, and intestine (for review, see Dibner et al., 2010). The system self-sustainingly generates a rhythmic signal with a period of about a day (i.e. circadian) at the cellular level. The molecular mechanism is based on multiple transcriptional-translational feedback loops, which are formed by several clock genes and their protein products (for review, see Takahashi et al., 2008). The basic loop consists of a positive element, the CLOCK:BMAL1 heterodimer, which binds to E-box sequences on the promoters of the clock genes Per1, Per2, Per3, Cry1 and Cry2, and promotes their transcription. The PER:CRY heterodimer functions as negative element by repressing the transcriptional activity of CLOCK:BMAL1, which results in a rhythmic expression of clock genes. These rhythms are fine-tuned by accessory feedback loops involving the genes Rev-erba and Rorα and by post-translational modulations of the clock proteins. Importantly, in the SCN, the robustness of the rhythmicity is reinforced by a web communication among individual cellular oscillators (Hogenesch and Herzog, 2011). This seems to be the crucial feature that distinguishes the central SCN clock from the peripheral clocks.

The circadian period of the clockwork is corrected by external cues every day so that the clock runs with a period of exactly 24 h. The strongest entraining cue is the daily light/dark (LD) cycle, especially its light period (Pittendrigh, 1981). Light stimuli perceived by the retinas are conveyed via retinohypothalamic and geniculohypothalamic tracts to the central clock in the SCN (Moore, 1996), which synchronizes the numerous peripheral clocks in the body accordingly (Schibler, 2005). Due to its effect on the molecular clock mechanism, light entrains the circadian rhythms in such a way that exposure to light during the first part of the night phase delays and during the second part of the night phase advances the clock (Pittendrigh, 1981). The light-sensitive components of the mechanism are the Per genes whose expression is induced by light during the night, but not during the day (Shearman et al., 1997; Zylka et al., 1998). Prolonged exposure to constant light (LL) results in species-dependent alternations of circadian rhythmicity. In rats, LL results in the gradual loss of overt rhythms in locomotor activity and body temperature as well as in suppression of melatonin secretion (Eastman and Rechtschaffen, 1983; Depres-Brummer et al., 1995; Widman and Murphy, 2009; Chiesa et al., 2010). Rhythmicity
is likely disrupted due to mutual desynchronization of individual neurons in the SCN (Ohta et al., 2005) that consequently are not able to generate a synchronized high-amplitude output signal for the rest of the body. However, the mechanism by which LL disrupts the intercellular synchrony is still unknown.

Apart from light, circadian rhythms can also be reset by temporally restricted access to food. Restriction of food availability to only the resting part of the day, that is, to its light phase in nocturnal rodents, is a potent entraining cue for the peripheral clocks, while the SCN clock remains dominantly synchronized with the LD cycle (Damiola et al., 2000; Hara et al., 2001; Stokkan et al., 2001; Wakamatsu et al., 2001; Schibler et al., 2003). However, under specific conditions, when the signal is of significant relevance and affects the motivational state, for example, palatable food reward (Mendoza et al., 2010), malnutrition, or caloric restriction, the SCN-driven behavioral rhythms can be entrained by food availability (Challet et al., 1996; Andrade et al., 2004; Caldelas et al., 2005; Mendoza et al., 2007). This suggests that under certain circumstances the molecular clockwork in the SCN might be sensitive to the timing of food availability. Indeed, expression of Per genes in the SCN and light-induced behavioral phase-delays are reduced when mice are maintained in constant darkness (DD) and fed by palatable food at the time of activity onset (Mendoza et al., 2010). This finding suggests that signals of food availability may compete with light to entrain the central clock. Apparently, under conditions when normocaloric feeding is allowed, albeit at the incorrect time of day, the SCN maintains daily time and runs in synchrony with the LD cycle. However, when the food supply is related to a change in motivation state, the signal becomes strong enough to entrain the central clock.

Several pieces of evidence suggest that normocaloric food may affect the SCN under conditions when the central clock is disturbed and/or any other entraining cue is lacking, like in the case of prolonged exposure to LL (Castillo et al., 2004; Lamont et al., 2005) or in DD (Caldelas et al., 2005). Therefore, the aim of the current study was to elucidate whether normocaloric food may play a residual role in the entrainment of the molecular core clockwork in the SCN. The daily expression profiles of the clock genes Per1, Per2, Rev-erba, and Bmal1 in the SCN were determined in rats maintained in LL and fed either ad libitum or exposed to the normocaloric restricted feeding (RF) regime for 6 h a day.

**EXPERIMENTAL PROCEDURES**

**Experimental animals**

Two-month-old male Wistar rats (Bio Test, Konarovice, Czech Republic) were maintained at a temperature of 21 ± 2 °C under a regime of 12 h of light and 12 h of darkness per day. Light was provided by overhead 40-W fluorescent tubes, illumination was between 50 and 300 lux, depending on the cage position in the animal room. The animals had free access to food and water. On the day of the experiment, the animals were released into LL, that is, the light was turned off at 18:00 h, the usual time of the light-to-dark transition. The animals were maintained in LL for 30 days. The control group was fed ad libitum during the whole interval in LL, while the experimental group was fed ad libitum only for the first 15 days in LL and had food available only for 6 h a day (from 9:00 h to 15:00 h) during the next 14 days in LL. On the 30th day in LL, five animals from each group were sampled every 4 h throughout the 24-h period to determine daily profiles of clock gene expression in the SCN. Sampling started at the time of the lights-on of the previous LD cycle (i.e. at 6:00 h). Rats were killed by decapitation in deep anesthesia (thiopental; 50 mg per kg), their brains were removed, immediately frozen on dry ice, and kept frozen at −80 °C until processed for *in situ* hybridization. In addition to the brains, peripheral tissues including the liver, duodenum, and colon, were also sampled. Data on clock gene expression profiles in these tissues have been published elsewhere (Polidarová et al., 2011).

All experiments were conducted under license no. A5228–01 with the US National Institutes of Health and in accordance with the Animal Protection Law of the Czech Republic (license no. 42084/2003–1020).

**Locomotor activity monitoring**

The locomotor activity of rats from both control and experimental groups was monitored throughout the experiment. The rats were maintained individually in cages equipped with infrared movement detectors attached above the center of the cage top enabling detection of spontaneous locomotor activity across the entire cage. A circadian activity monitoring system (Dr. H.M. Cooper, INSERM, France) was used to measure activity every minute, and the resulting data were analyzed using the ClockLab toolbox (Actimetrics, IL, USA). Double-plotted actograms were generated for visualization of data. The data were also analyzed by chi square periodogram using ClockLab toolbox (Actimetrics).

**In situ hybridization**

The brains were sectioned throughout the whole rostrocaudal extent of the SCN into 5 series of 12-μm-thick slices in an alternating order. Sections were further processed for *in situ* hybridization to determine daily profiles of Per1, Per2, Bmal1, and Rev-erba mRNAs in the SCN. Brain sections from rats of control and experimental groups were processed simultaneously under identical conditions. The cDNA fragments of rat Per1, Per2, Bmal, and Rev-erba were used as templates for *in vitro* transcription of complementary RNA probes. Probes were labeled using 35S-UTP, and the *in situ* hybridizations were performed as previously described (Sládek et al., 2004). After the hybridization (20 h in 60 °C) and posthybridization wash, slides were exposed to BIO-MAX MR film (Kodak) for 10 days and developed using the ADEFO-MIX-S developer and ADEFOFIX fixer (ADEFO-CHEMIE Gmbh, Germany).

Autoradiographs of sections were analyzed using an image analysis system (Image Pro, Olympus, New Hyde Park, NY, USA) to detect the relative optical density (OD) of the specific hybridization signal. In each animal, mRNA was quantified bilaterally at the mid-caudal SCN section containing the strongest hybridization signal. Each measurement was corrected for nonspecific background by subtracting OD values from the same adjacent area in the hypothalamus. The background signal of that area served as an internal standard; it was consistently low and did not exhibit marked changes with the time of day. Finally, slides were counterstained with cresyl violet to check the presence and mid-caudal position of the SCN in each section. The ODs for each animal were calculated as a mean of values for the left and right SCN.

**Statistical analysis**

Daily profiles of mRNA levels were analyzed by a one-way analysis of variance (ANOVA) for time differences, with P < 0.05 being
and Rev-erb/Rev-erbA/H11001 (amplitude*cos[2*pi*(X-acrophase)/wavelength]) with a constant wavelength of 24 h. The least-squares regression method implemented in Prism 5 software (GraphPad, La Jolla, USA) was applied. Amplitude, acrophase, and coefficient of determination $R^2$ (i.e. goodness of fit) were calculated. Rhythms were considered to be present when the one-way ANOVA revealed a significant effect of time and, at the same time the cosine curve was fit with $R^2$>0.25. $R^2$ values <0.25 were considered too low to fit a cosine curve confidently enough for determination of the amplitude and acrophase.

The data are expressed as means of five animals ± standard error of the mean (SEM) per each time point, and a fitted single cosine curve is shown.

RESULTS

Effect of LL on locomotor activity

Releasing the rats into LL affected their locomotor activity, as previously demonstrated (Polidarová et al., 2011). The rats started to run with a period of 25.5±0.3 h during the first 14 days of LL (data not shown), and thereafter the rhythmicity gradually deteriorated. Finally, after 30 days in LL, rhythmicity was completely lost. All rats fed ad libitum became completely arrhythmic on the 30th day in LL, as confirmed by periodogram analysis (data demonstrated in the study by Polidarová et al., 2011). Fig. 1 (upper part) depicts a representative activity profile of a control rat maintained in LL for 30 days.

Effect of RF under LL conditions on locomotor activity

Exposure of rats to the RF regime during the last 14 days in LL (for details see Experimental procedures) affected the locomotor activity rhythm, and the rats became active mostly during the time of food availability. Periodogram analysis confirmed rhythmicity with approximately 24 h (data not shown). As expected, they also exhibited food- anticipatory activity, becoming active shortly before the time when food was provided (see the representative activity profile of one animal of the experimental group in Fig. 1, lower part).

Effect of LL on clock gene expression profiles in the SCN

24-h profiles of Per1, Per2, Bmal1, and Rev-erbA expression are depicted in Fig. 2. One-way ANOVAs revealed significant effects of time for the 24-h profiles of Per1, Per2, and Rev-erbA (P=0.008; <0.001; and 0.01, respectively), but not of Bmal1 (P=0.313), mRNA levels. The $R^2$ values and amplitudes of the fitted cosine curves were very low for all clock genes studied (Table 1), suggesting that the variation of mRNA levels exhibited only weak circadian rhythms; $R^2$ values for Per1 and Per2, but not for Bmal1 and Rev-erbA, expression profiles exceeded the threshold arbitrarily set for significance (see Experimental procedures). Therefore, in the SCN of animals exposed to LL for 30 days, attenuated and low amplitude rhythms were indicated in the expression of Per1 and Per2, but not in the expression of Bmal1 and Rev-erbA.

Effect of RF under LL conditions on gene expression profiles

RF affected the 24-h clock gene expression profiles in the SCN of animals maintained under LL in a gene-dependent manner (Fig. 2). One-way ANOVAs revealed significant effects of time for the 24-h profiles of Per1, Per2, Bmal1, and Rev-erbA mRNA (P=0.049; 0.012; 0.008; and <0.001, respectively). For the Per1 mRNA profile, the $R^2$ value of the fitted cosine curves was too low to be considered as varying in a circadian manner, but for the Per2, Bmal1, and Rev-erbA profiles, the cosine fits proved circadian rhythmicity (Table 1). Thus, according to the $R^2$ values, expression of Per1 was not rhythmic, and the expressions of Per2, Bmal1, and Rev-erbA exhibited weak, intermediate, and robust circadian rhythms, respectively (Fig. 2, Table 1).
The two-way ANOVA comparisons between the clock gene expression profiles in the SCN of animals from both groups, that is, those fed ad libitum and those exposed to RF, revealed significant effect of group for Per1 and Rev-erbα (P < 0.004 and <0.001, respectively), but not for Per2 (P = 0.791). For Per1 profiles, post hoc analysis revealed that they differed only at one time point, 6:00 h (P < 0.001). In contrast, for Rev-erbα profiles, significant differences were found at 18:00, 22:00, and 2:00 h (all P < 0.05). For expression of Bmal1, the effect of group was nonsignificant (P = 0.904), but the highly significant interaction effect suggested that the profiles of Bmal1 expression differed significantly between the control and experimental groups. Post hoc analysis confirmed that the Bmal1 expression differed significantly at 22:00 h (P < 0.05).

Due to RF, the R² values of profiles found in animals maintained under LL increased slightly for Per2, moderately for Bmal1, and substantially for Rev-erbα, while they decreased for Per1 (Table 1). Thus, these data suggest that in the SCN of rats maintained under LL, RF only slightly increased circadian organization of the Per2 rhythm and induced rhythms in Bmal1 and Rev-erbα expression. The acrophases of the RF-induced rhythms were mutually phased according to the current model of circadian molecular clockwork (i.e. expression of Rev-erbα peaked 7 h and 15 h before Per2 and Bmal1, respectively).

Table 1. Circadian characteristics of the cosine fitted profiles of gene expression

<table>
<thead>
<tr>
<th></th>
<th>Per1</th>
<th>Per2</th>
<th>Bmal1</th>
<th>Rev-erbα</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LL</td>
<td>LL + RF</td>
<td>LL</td>
<td>LL + RF</td>
</tr>
<tr>
<td>R²</td>
<td>0.31</td>
<td>0.20</td>
<td>0.26</td>
<td>0.36</td>
</tr>
<tr>
<td>Amplitude</td>
<td>0.017</td>
<td>—</td>
<td>0.012</td>
<td>0.015</td>
</tr>
<tr>
<td>SE amplitude</td>
<td>0.005</td>
<td>—</td>
<td>0.004</td>
<td>0.004</td>
</tr>
<tr>
<td>Acrophase</td>
<td>16.6</td>
<td>—</td>
<td>16.2</td>
<td>17.5</td>
</tr>
<tr>
<td>SE acrophase</td>
<td>1.2</td>
<td>—</td>
<td>1.3</td>
<td>1.1</td>
</tr>
</tbody>
</table>

Goodness of the fit (R²), amplitude, and acrophase of the profiles of clock genes Per1, Per2, Bmal1, and Rev-erbα in the SCN of the rats maintained under constant light and fed ad libitum (LL) or subjected to restricted feeding regime (LL + RF) are summarized. Data for amplitudes and acrophases are depicted only for the profiles with R² being considered as significant; for further details, see Experimental procedures. SE amplitude and SE acrophase mean standard error of deviation for amplitude and acrophase.

Fig 2. The 24-h clock gene expression profiles in the SCN of rats maintained under constant light and fed ad libitum (open triangles, dashed line) or exposed to restricted feeding (full circles, full line). Per1, Per2, Bmal1, and Rev-erbα mRNA levels are depicted as the mean ± SEM of five animals per time point. On the x-axis, time is expressed as real time in hours. For details about experimental design, see Experimental procedures.
DISCUSSION

These data demonstrate that in the SCN of rats that are behaviorally completely arrhythmic due to 30-day-exposure to LL, not all rhythms in clock gene expression are completely abolished. Although the *Bmal1* and *Rev-erba* expression did not exhibit circadian rhythmicity, attenuated low amplitude rhythms of *Per1* and *Per2* expression were suggested. Exposing the rats maintained under LL to RF did not affect the weak rhythm of *Per1* and *Per2* expression, but affected significantly the rhythms of *Bmal1* and *Rev-erba* expression.

Gradual loss of circadian rhythmicity in the locomotor activity of animals fed *ad libitum* and exposed to LL has been repeatedly documented (Depres-Brummer et al., 1995; Beaule et al., 2003; Wideman and Murphy, 2009; Chiesa et al., 2010; Nováková et al., 2010; Polidarová et al., 2011). In our study, the circadian rhythm of locomotor activity free-ran with a long period of about 25.5 h during the first 2 weeks in LL. Thereafter, it progressively deteriorated such that during the next 2 weeks in LL, the activity gradually became completely arrhythmic. Moreover, in our previous study we found that apart from the locomotor activity, the feeding rhythms were also lost in these rats (Polidarová et al., 2011). Thus, these data confirm that in our experimental arrangement, the SCN finally loses its ability to drive at least some of the output rhythms due to exposure to LL. This effect has previously been attributed to the fact that under LL, the individual SCN cells that harbor circadian oscillators might become mutually desynchronized (Ohta et al., 2005). In our study, clock gene expression profiles exhibited faint, if any, circadian rhythms. However, a certain level of synchrony still persisted, because *Per1* and *Per2* expression profiles exhibited low amplitude, but borderline significant, rhythms. Obviously, our data reflect the rhythmicity of the whole population of SCN neurons, but not that of individual cells. Therefore, no conclusion regarding the effect of LL on intercellular synchrony can be drawn. Nevertheless, our data suggest that under disturbing conditions such as exposure to LL synchrony for at least a subpopulation of the SCN cells might persist *in vivo*. Apparently, this rhythmicity was not sufficient to drive rhythm in behavior. However, the possibility that the SCN drives other output signals to the rest of the body cannot be excluded. For example, circadian rhythms in clock gene expression in the rat duodenum persist under LL, while those in the liver and colon are mostly abolished (Polidarová et al., 2011). Therefore, whether the duodenal rhythm reflects a self-sustained persistence of the peripheral clock or is facilitated by putative rhythmic signaling emanating from a subpopulation of SCN cells remains to be elucidated. If these cells remain synchronized under LL, they might convey the rhythmic signals to the peripheral clocks, for example via the autonomic neuronal system or by any other pathways. However, due to the low amplitude of the SCN rhythmicity, this possibility seems to be less plausible. Moreover, the rhythms in gene expression are likely not reflected in rhythms of protein products because circadian rhythmicity in a number of *PER2* immunoreactive cells is lost in the SCN of behaviorally arrhythmic animals maintained under LL (Beaule et al., 2003; Sudo et al., 2003). Also, *PER2* levels are constitutively elevated in the SCN of mice exposed to LL for 50 days in spite of the fact that *Per2* mRNA levels were rhythmic and locomotor activity still exhibited a free-running rhythm (Munoz et al., 2005). However, it is necessary to point out that quantification of mRNA levels by *in situ* hybridization is more precise than quantification of protein levels by immunocytochemistry and thus low-amplitude protein rhythms might likely be hidden.

Restriction of food availability to only 6 h a day significantly affected the behavior of the animals. The SCN is likely not necessary for the effect of RF on the behavior, because RF has been shown to entrain locomotor activity rhythms in animals that were arrhythmic due to SCN lesion (Stephan et al., 1979; Stephan et al., 1981). The rats became active mostly during the time when food was provided, and shortly before due to the food anticipatory activity described previously (Mistlberger, 1993, 1994). In the SCN, RF induced circadian rhythms in *Bmal1* and *Rev-erba* expression that were arrhythmic due to exposure to LL. The amplitude of the rhythm in *Rev-erba* expression was the most prominent due to RF. Importantly, the expression rhythms of these two genes were in antiphase, in accordance with the current model of molecular clockwork (Takahashi et al., 2008). Hence, it is plausible that under LL, RF was able to affect SCN cells to synchronize them. Interestingly, the *Per1* and *Per2* mRNA profiles were unaffected and only marginally affected, respectively, in our study. This finding does not support the effect of RF on *PER2* immunoreactivity profiles previously reported in the SCN of rats maintained under LL (Lamont et al., 2005). However, we cannot exclude the possibility that this discrepancy is due to different experimental designs (e.g. time window of food availability was 3 h in the study by Lamont et al., 2005, but it was 6 h in our study).

The mechanism by which the SCN clock gene expression rhythms are restored by RF is unknown. The SCN has been found to be sensitive to various nonphotic cues (Hastings et al., 1998). One possibility is that the activity rhythm restored by RF might feedback on the SCN and thus provide a temporal cue to the desynchronized SCN cells. Another theoretical possibility is that pineal melatonin might also affect the SCN function via its receptors present on the SCN cells (Vaněček et al., 1987), because melatonin rhythm has been found to be re-established by RF in animals arrhythmic due to SCN lesion (Feillet et al., 2008). However, under LL the melatonin levels are likely suppressed in animals fed *ad libitum* as well as in those subjected to the RF. Moreover, it is not clear whether the activity and/or melatonin rhythms might affect synchrony among the individual SCN cells under LL. Our finding that the RF-induced rhythm in *Rev-erba* expression exhibited the highest amplitude of all studied clock genes allows us to speculate that signaling related to the metabolic state of the organism might impinge directly on the molecular clockwork by affecting the *Rev-erba* expression; the gene has been recognized as a sensor of the metabolic state in...
peripheral tissues (Yin et al. 2010; Torra et al., 2000; Liu et al., 2007). Apparently, the metabolic signal is of no significance for the SCN when the clock is synchronized with an external LD cycle (Damiola et al., 2000; Sládek et al., 2007). However, theoretically, the signal might become relevant when the SCN function is distorted and any entraining cues are lacking as likely happens in animals maintained under LL and fed ad libitum.

CONCLUSION
In conclusion, our data demonstrate that in the SCN of rats behaviorally arrhythmic due to prolonged exposure to LL, low amplitude oscillations in Per1 and Per2 gene expression may be detected. These rhythms, though only borderline significant, may reflect a persistence of synchrony among a small subpopulation of the SCN cells. Moreover, our data demonstrate for the first time that under conditions of prolonged exposure to LL, the RF may synchronize Rev-erba and Bmal1 gene expression profiles in the SCN. These findings suggest the possibility that under specific conditions in which the SCN rhythmicity is disturbed and temporal entraining cues are lacking, the SCN molecular clockwork may sense changes in metabolic state delivered by normocaloric food, likely via the regulation of Rev-erba.

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