Bright light during lactation alters the functioning of the circadian system of adult rats

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Canal-Corretger, M. M., T. Cambras, J. Vilaplana, and A. Díez-Noguera. Bright light during lactation alters the functioning of the circadian system of adult rats. Am. J. Physiol. Regulatory Integrative Comp. Physiol. 278: R201–R208, 2000.—To examine the role of light in the maturation of the circadian pacemaker, twelve groups of rats were raised in different conditions of exposure to constant bright light (LL) during lactation: both duration and timing of LL were varied. We studied the motor activity rhythm of the rats after weaning, first under LL and then under constant darkness (DD). In DD, two light pulses [at circadian time 15 (CT15) and CT22] were applied to test the response of the pacemaker. Greater exposure to LL days during lactation increased the number of rhythmic animals and the amplitude of their motor activity rhythm in the LL stage and decreased the phase delay due to the light pulse at CT15. The timing of LL during lactation affected these variables too. Because the response of the adult to light depended on both the number and timing of LL days during lactation, the exposure to light at early stages may influence the development of the circadian system by modifying it structurally or functionally.

The circadian system provides organisms with temporal organization. In mammals, the circadian system consists mainly of the suprachiasmatic nuclei (SCN) of the hypothalamus but also of structures such as the retina (26). The circadian system generates circadian rhythms, synchronizes them to environmental factors such as light, and transmits this rhythmic pattern to physiological processes and behavior. As light-dark alternation is the main zeitgeber for the circadian system, many physiological variables under natural lighting conditions show a 24-h rhythm that synchronizes to the external zeitgeber. Under constant conditions, such as constant darkness (DD), daily rhythms usually deviate slightly from 24 h. The period of this free-running rhythm, which is very stable, is known as τ and varies according to species and individuals. However, under constant bright light (LL), the circadian rhythm of most animals is not that established, because splitting (4, 8) and many ultradian components in the pattern of motor activity rhythm appear. In rats, after a long exposure to LL, the circadian periodicity disappears, as do rhythms of motor activity (9, 15, 18), plasma melatonin (18), sexual hormones (34), and body temperature (9, 11, 15).

Previous experiments indicate that the arrhythmicity under LL can be prevented: rats reared under LL during all their lactation period showed, after weaning, a circadian rhythm of motor activity under LL that emerged from an ultradian pattern (5, 6). The period of this rhythm under LL is much longer than that under DD and lasts most of the life span of the animal, especially in females (7). These results reveal the influence of the lighting conditions during lactation, when the circadian system matures. This is not surprising, taking into account that although SCN neurons are already formed in the embryo (1) and are rhythmic before birth (25), various studies demonstrate that the general maturation of the SCN is postnatal. It develops rapidly from the time when the neurons are formed until postnatal day 10 (P10), and more slowly to adulthood. The synaptogenesis increases until P10, when the number of synapses per unit of SCN area is the same as in adulthood. However, the volume of the SCN grows between P10 and adulthood, which may be due to the extension of some dendritic processes (see Ref. 21 for review). Moreover, the pathways that bring light information to the SCN are present at P4 but develop until P10 (19, 29). Thus, in the early life of the rat, during lactation, the SCN reaches its full outgrowth, which suggests that its functionality might be modified at distinct development stages by the effect of external factors such as light.

The purpose of this experiment was to find out whether the prevention of arrhythmicity due to LL exposure during the lactation period was related to the amount of light (no. of days in LL) that the animal had received during this period or whether it was due to the effect of LL during some critical days of development. We therefore subjected rats to a fixed number of days of constant bright light during the lactation stage. In the adult rats, the motor activity pattern under LL and under DD and also the phase shifts induced by a light pulse were studied.

Materials and Methods

Twelve pregnant Wistar rats (Criffa, France) were brought to our laboratory on day 16 of gestation. The rats were housed in individual transparent Makrolom cages (50 × 25 × 12 cm) under a 12:12-h light-dark (LD) cycle (with a light intensity of ~300 lx). They remained in these conditions until delivery, 5 days later. When all the pups were born, they were cross-
fostered so that each dam fed one group of rats, made up of five males and five females [except groups 12LA and 8LB (see Fig. 1), which each had 6 males and 4 females] belonging to different litters.

The new litters (each one was an experimental group of pups) were subjected to DD (−0.1 lx of dim red light) or LL (−300 lx) for a different number of days during lactation, which lasted 24 days. Thus pups of each group were called after the number (0, 4, 8, 12, 16, 20, or 24) and timing (A groups, LL close to birth; B groups, LL close to day of weaning) of those LL days during lactation (see Fig. 1). For instance, group 4LA remained under LL the first 4 days of lactation and then was under DD until day 24; group 8LB remained under DD the first 16 days of lactation and then was under LL the last 8 days of lactation.

At 25 days old (day 1 of the experiment), the pups were weaned and isolated in individual cages (25 × 25 × 12 cm). From this day on, and until the end of the experiment, motor activity was detected by means of an actimeter using two crossed perpendicular infrared beams situated on a plane 3 cm above the floor of the cage. Motor activity counts were automatically recorded every 15 min in a personal computer by means of a data-acquisition system developed in our department.

Rats were fed commercial chow (Rodent Toxicology Diet, B&K Universal) and tap water ad libitum. Approximately every 10 days, the cages were cleaned, and until experimental day 78 the rats were weighed.

After weaning, all the rats were maintained under LL for 55 days to examine the appearance of a circadian rhythm under this condition. They were then all shifted to DD, to study the free-running rhythm. Rats remained under DD until day 160 of the experiment. The DD situation was also used to test the responsiveness of the circadian system of each animal to a light pulse. Thus, on day 103 of the experiment, all the rats received a 1-h light pulse of a mean intensity of 345 lx, at circadian time 15 (CT15); on day 131, a second light pulse of the same intensity and duration was given at CT22. The phase shifts were calculated in both cases.

To determine the exact hour (local time) when the light pulses were to be applied, the time of activity onset or CT12 was independently estimated from the actograms for each animal by four investigators by visual estimation of the rest-activity transition; mean values were used. CT15 was the result of adding 3 circadian hours to CT12; for CT22, we added 10 circadian hours to CT12.

Mathematical and statistical analysis. The circadian rhythm was separately studied for the LL stage and for the DD stage. To determine the presence of motor activity rhythm and its period, we used Sokolove and Bushell’s periodogram (28), with a low global level of significance (P = 0.01) to reject spurious peaks.

In the LL stage, the rhythm was determined using data from day 15 to day 55; for the DD stage, data were from day 74 to day 102. The first days of each stage were excluded to ensure a stable motor activity pattern in the analyzed data. An animal was considered to be rhythmic only when the period of its circadian rhythm in the periodogram was statistically significant. The percentage of variance explained (PVE) by the highest peak (significant or not) obtained in the periodogram was used as an indicator of the importance of the motor activity rhythm. Moreover, in the LL stage, a Fourier analysis was applied to the data, using the period of the highest peak of the periodogram of each rat as the period of the fundamental harmonic. The amplitude of the first harmonic, and the sum of the power content of the first five harmonics (PC5H), also showed the importance of the circadian rhythm.

Phase shift responses due to a 1-h light pulse in the activity rhythm were determined by drawing eye-fitted lines through the daily onsets and offsets (calculated separately) of activity for the 10 days before and the 10 days after treatment. The phase shifts resulted from the difference between the two lines at the day after the light pulse. These phase shifts were calculated independently by four researchers, and the mean values for each pulse were used for further statistical analyses.

Statistical analysis was carried out by ANOVA of several linear models (Systat). In all the models, the independent variables were sex, number of days under LL (considered categorical), and five other variables used to estimate differences within the groups that had 4, 8, 12, 16, or 20 days of light, depending on the timing of the LL days in the lactation stage (A and B groups). In this way, we tested the influence of the different number of LL days and determined whether the timing of the LL days in the lactation period was significant. The dependent variables were body weight increase; period, PVE, amplitude, and PC5H in the LL stage; period and PVE in the DD stage; and the delays and advances in the onset and offset occurring after the light pulses at CT15 and CT22, respectively. Each dependent variable was analyzed in a separate model.

Moreover, to determine whether the variables studied had a linear relation with the number of LL days, we applied the above model, but using the number of days under LL during the lactation as a quantitative independent variable.
Graphs and calculations were carried out using the integrated package for chronobiology analysis, El Temps (A. Díez-Noguera, Universitat de Barcelona, 1999).

RESULTS

The double-plotted actograms (see Fig. 2) show the general traits of the activity of the rats. At first sight, three distinct patterns of motor activity can be differentiated in the LL stage, independently of the group: some rats (e.g., Fig. 2A) developed a clear circadian rhythm; other rats (e.g., Fig. 2C) showed an initially weak circadian rhythm, which gradually became completely arrhythmic; finally, a third group of rats (e.g., Fig. 2, B and D) did not show a circadian rhythm of motor activity. In the DD stage, all the rats manifested a clear circadian rhythm, a phase delay after the light pulse at CT15 and a phase advance after the light pulse at CT22.

On studying the manifestation of rhythmicity under LL, it can be seen that the number of rhythmic rats (rats with a statistically significant peak in the periodogram) and nonrhythmic rats differs between groups, depending on the number of LL days during lactation. The rats from groups subjected to less than 12 LL days...
were mainly arrhythmic (8 rhythmic, 42 arrhythmic), whereas rats that received 12 or more LL days were mainly rhythmic (64 rhythmic, 6 arrhythmic) (Fig. 3, A and B).

The PVE of the highest peak in the periodogram of the LL-stage data was different for each group. It is related to the number of days under LL during the lactation period ($P < 0.001$) but does not depend on the sex of the rat. Thus, the higher the number of LL days, the higher the PVE. We also found differences between groups 16LA and 16LB; the latter had a higher PVE ($P < 0.001$) (see Fig. 3, C and D). Amplitude and PC5H behave like the PVE, that is, they rise with an increasing number of LL days during lactation and do not depend on the sex of the animal, and there are also differences between groups 16LA and 16LB (data not shown).

The period of the rhythm under LL did not depend on sex, but it did depend on the number of days of LL during lactation (more days of light imply a longer period under LL) (see Fig. 3, E and F). It should be borne in mind that for the study of this variable, we only included the values of the rats whose rhythm was statistically significant. As a result, there were few rhythmic rats (8 of 50) in the groups that had 8 or fewer days of LL during lactation. Thus, care must be taken when interpreting the correlation of this variable with the number of LL days, because there were no differences between groups with 12 or more LL days during lactation (64 rhythmic rats of 70). The mean value of the period in LL for all of the rhythmic animals was $25 \pm 2.75$ min (mean $\pm$ SE).

When transferred to DD all rats generated a stable circadian rhythm. Most of the rats acquired this stable rhythm immediately after being moved to DD (Fig. 2, A and B), but in some of the rats that were arrhythmic in the last days of the LL stage (Fig. 2, C and D), the
appearance of their rhythm under DD was delayed for more than 3 days after the lights were switched off. Under DD, period values of the motor activity rhythm (24 h, 39 ± 0.69 min) were not dependent on sex or on the number of days of LL during the lactation stage (see Fig. 4, A and B). However, the animals that received more than 12 LL days during the lactation showed a longer period (24 h, 42 ± 0.94 min) than the others (24 h, 38 ± 0.91 min); the difference was statistically significant (Student's t-test, P < 0.05). PVE under DD

Fig. 4. Variables studied in DD stage (mean ± SE). A and B: period of motor activity rhythm. C: PVE. D and E: phase delays after light pulse at CT15. F: phase advances after light pulse at CT22. Left: value of variable for each group of rats. Right: variable related to no. of days under LL (graph is missing when no correlation was found).
only depends on the sex of the animal (P < 0.001); the females had a higher PVE than the males (see Fig. 4C).

After the light pulse at CT15, a phase delay (mean ± SE = 2 h, 51 ± 4 min) was observed in all the animals, in both onset and offset of motor activity rhythm. A correlation between the phase delay and the number of LL days was found (see Fig. 4, D and E): more LL days during the lactation stage implies a smaller phase delay (P < 0.05). Differences can also be seen between groups 4LA and 4LB (P < 0.005) as well as between groups 8LA and 8LB (P < 0.005): the ones that started the lactation period under LL (groups 4LA and 8LA) have greater phase shifts than their corresponding group. Anyway, no significant differences were found between the phase delays calculated using the onset or the offset of the rhythm, and so only the onset is shown in Fig. 4E.

Light pulse given at CT22 (see Fig. 4F) caused a phase advance (mean ± SE = 2 h, 42 ± 4 min) of the motor activity rhythm in all the rats, but there was no correlation with the number of LL days during lactation. In this case, there were differences between the values of the phase advance calculated using the onset and the offset (P < 0.001).

At day 50 of the experiment we found no differences in body weight due to the lighting conditions during lactation; differences were only related to the sex of the animal.

**DISCUSSION**

Constant light provokes the loss of circadian rhythmicity in adult rats in some variables such as motor activity (9, 15, 18). However, in past experiments we observed that adult rats exhibited a circadian rhythm of motor activity under LL if previously subjected to LL throughout their entire lactation period (5–7). Therefore it appears that, at least in rats, the lighting environment at the age when the circadian system is developing plays a critical role in the manifestation of the rhythm of the adult animal. In the present study, we have not only corroborated the former results but have also demonstrated that the number of LL days is critical; in particular, 12 or more days of LL during the lactation period seem to be needed to elicit a circadian rhythm of motor activity under LL in adult rats. Rats that had fewer than 12 LL days during lactation were mainly arrhythmic and showed a lower amplitude of rhythm under LL. The present study also suggests that there is a critical stage during the lactation in which light affects the circadian system.

Taking into account that the circadian system is formed by coupled individual oscillators (3, 10, 20, 35), the arrhythmicity observed in some adult rats subjected to LL could be interpreted as the loss of the coupling between the oscillators. In our experiment this can explain why most of the rats that are arrhythmic under LL, although they all developed a circadian rhythm under DD, take some time after lights off to manifest the endogenous rhythm. Probably the absence of light forced the oscillators to couple and to oscillate synchronically. In the case of rats that are rhythmic under LL, we may assume that if light is given when the oscillators are establishing their coupling, this coupling will become strong enough to generate and maintain a circadian rhythmicity. We can thus suggest that in this last group, the pacemaker is robust enough to endure the effects of light, and therefore their oscillators remain coupled.

Although the nature of the oscillators remains unknown, some hypotheses regard neurons as the principal candidates and suggest that the glial cells could be responsible for the coupling among these oscillators (35). Hence, as the complete morphological and functional maturation of these cells, as well as the synaptogenesis and the development of the SCN afferences [i.e., from the optic chiasm (retina) and the intergeniculate leaflet] take place during lactation (see Ref. 21 for review), this stage becomes decisive for the normal development of the circadian system. In fact, our findings indicate that LL during lactation results in a more robust pacemaker, which is less susceptible to the inhibiting effects of light on the manifestation of the circadian rhythm. More precisely, greater exposure to LL during lactation implies 1) fewer arrhythmic adult animals under LL, 2) higher amplitude of the circadian rhythm under LL, and 3) smaller phase shifts.

Despite the differences between groups in the phase shift after a light pulse, the values of the phase advances and delays (−3 h each) observed here fit with the values found previously by Honma et al. (16) in Wistar albino rats, taking into account that in our experiment the duration and intensity of the light pulse were higher. In fact, Gander et al. (14) demonstrated that the higher the duration of the light pulse, the higher the phase shift.

In this experiment we have found sexual differences in the manifestation of the rhythm in the DD stage (in PVE), but not in the LL stage. This lack of sexual differentiation in the rhythm under LL seems to disagree with our previous experiments (5), in which the PVE of females was significantly higher than that of males. We trust that the reason for this disagreement is that in the present experiment the number of rats per group was smaller than in the previous one and those sexual differences in the rhythm could not be detected. Under DD, as the rats are older and consequently sexual maturation has been achieved, the sexual differences in the manifestation of the rhythm should be more pronounced and, thus, detected.

The motor activity rhythm is influenced not only by the number of LL days during lactation but also by the timing of these days. This hypothesis is supported by the differences found between A and B groups (e.g., differences between groups 16LA and 16LB in PVE, amplitude, and PC5H of the rhythm in the LL stage). If the development of the circadian system is a continuous process, the effect of a certain number of days of LL may change, according to the stage of development at which it is applied. Because groups 4LA, 8LA, and 0L have a similar behavior, it can be assumed that light applied the first 8 days of the rat's life does not affect the later expression of the circadian pacemaker. We
may consider that before postnatal day 8, the circadian system is too immature to perceive, transmit, or manage the surrounding light information. Likewise, as groups 20LA and 4LB compared with groups 24L and 0L, respectively, show a similar pattern in the manifestation of the rhythm, we may assume that from 20 days of age the development of the circadian system is not influenced either by darkness or by light. Therefore, a window of effectiveness of light on the biological clock could be placed between day 8 and day 20 after birth.

Actually, in rats, the synaptogenesis between the SCN cells and the expansion of the geniculohypothalamic tract (GHT) take place between postnatal day 4 (P4) and P10 (22), and the number of projections of the retinohypothalamic tract (RHT) to the SCN increases gradually from P1, achieving the adult pattern between P10 and P15 (29). Also, on P20 the vasoactive intestinal peptide mRNA signals produced by the SCN neurons (2) and the number of neuropeptide Y-immunoreactive fibers originating from the GHT (33) reach their adult stage. This indicates that before P20 the main events of the development of the circadian system take place, and thus this period may be more influenced by light.

However, light is not the only zeitgeber for the biological clock at this early age. Honma et al. (17), through the use of restricted feeding as a zeitgeber to the mother and by analyzing the pups’ locomotor pattern, suggested that the first postnatal week is ineffective for maternal entrainment and that the critical period extends until the end of the second postnatal week. In a similar way, Takahashi et al. (32) proposed that for the blinded pups to be entrained by a foster mother, nursing had to start before 10 days of age and be continued for more than 10 days. Thus there is general agreement that the critical period for sensitivity and ability to adapt to external factors may be located, at least in rats, in the middle of the lactation stage.

The retina is a component of the circadian system that plays an important role in the sensitivity and ability of the circadian pacemaker to adapt to the external environment, as it is the only photoreceptor organ in mammals (loss of the eyes blocks all the circadian responses to light) (23). The retinal input may be important for a normal morphological formation of the SCN during development (31). In fact, the SCN of the hereditary microphthalmic rat (the retina of which is seen as a cyst and lacks the optic nerve) has a shorter length, lower total volume, and fewer neurons than the SCN of normal rats, but this does not prevent it from generating circadian and ultradian rhythms (30). Therefore, if the pacemaker itself is altered, the sensitivity of the circadian system to light may also be affected. Moreover, Foster and co-workers (13, 24) found that despite extensive damage of their visual photoreceptors and loss of visual function, rd/rd mice (mice whose rods and cones suffer a massive degeneration) still showed circadian responses to light that are indistinguishable from those of mice with normal retinas. On the other hand, rdta mice, whose rods degenerate during ontogeny because of a fusion gene integrated in the genome (12), showed 2.5-fold greater shifts than wild-type mice, at irradiances that produce saturating phase shifts in the wild-type mice. The only difference between the two strains of retina-degenerated mice is the time at which the onset of rod ablation takes place (1 wk earlier in rdta mice than in rd/rd mice). It has been suggested (27) that the earlier loss of rods in the rdta mice alters the amplitude of clock responses to light but does not change the sensitivity of the clock to light, possibly because the loss of photoreceptors occurs when the retina and/or its central projections are still plastic enough to permit some reorganization. In our experiment, we have observed, on the one hand, that both the manifestation of the rhythm under LL and the value of the phase shifts vary depending on the lighting conditions during lactation. These two variables are related to both the functionality of the pacemaker and its sensitivity to light. On the other hand, the rhythm manifested in the DD stage (a variable that permits one to study exclusively the effect of light on the functionality of the clock) does not seem to depend on the lighting conditions during lactation. A possible effect of light, however, appears in the period under DD, as two levels of this variable (groups with less or groups with more than 12 LL days during lactation) can be differentiated. On the basis of our results, we cannot know whether a change in the sensitivity of the circadian system to light, an increase in the circadian responses to light due to a change in the functioning of the circadian system, or both have occurred. Thus further clarifying experiments that would test separately these two effects of light on the pacemaker are needed to find out which is responsible for our findings.

It is clear that the lighting conditions to which newborn animals are exposed are of great significance, as they will affect the circadian system and condition the further adaptation of the adult animal to the external conditions in which it lives. Therefore, it is crucial for the organism to adapt to the coming circumstances as early as possible, while the system is still sufficiently plastic.

Perspectives

The present experiment shows the importance of lighting conditions during the development of the circadian system. This suggests that some responses, and probably the functionality of the circadian system of the adult animal, depend on specific environmental conditions during the early stages of life. This has several implications for further lines of research. First, we wonder whether the biological clock of species other than rats, including humans, would respond in the same way constant light affects the final structure of the circadian system in adulthood. If this were the case, would the responses of the circadian system be similar to those generated by light? Moreover, the way constant light affects the final structure of the circadian pacemaker when applied during the first days of light also remains to be elucidated. Light may
induce changes in the distribution and secretion of some neurotransmitters in the SCN or in its affereces. Light could also act on the connection between SCN cells, for instance, by influencing synaptogenesis or the development of glial cells. Further experiments that will determine the structure of the SCN, the distribution of the neurotransmitters, and its affereces under different lighting conditions may answer some of these questions.

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