THE TONIC RESPONSES TO LIGHT OF THE RAT CIRCADIAN SYSTEM ARE AFFECTED BY THE LIGHTING CONDITIONS DURING LACTATION

Resum

Objectiu: Per tal de discernir quin és l'efecte de les condicions d'il·luminació en què una rata ha crescut sobre la resposta tònica del sistema circadiari, hem estudiat l'efecte de la intensitat de llum en el ritme circadiari d'activitat motora en condicions de llum constant de 3 grups de rates que diferien en les condicions d'il·luminació durant l'alletament.

Material i mètodes: Sis rates Wistar femella van arribar al nostre laboratori el dia 15 de gestació. El dia del naixement, les cries es van barrejar, de manera que cada mare alimentava grups de 12 cries provinents de diverses ventrades. Dos grups es van sotmetre a foscor constant (DD), 2 a llum constant (LL) i els 2 restants, a cicles de llum-foscor (LD) de 24h de període. El dia del deslletament (24 dies després del naixement) les cries es van separar de la mare i es van posar en gàbies individuals amb accés a l'aigua i al menjar *ad libitum* i l'activitat motora es va enregistrar amb actímetre d'infraroig. Aquests animals es van sotmetre a cicles de llum-foscor de 24h de període, per tal de disminuir els postefectes provocats per les diferents condicions d'il·luminació durant l'alletament. Al cap de 16 dies, totes les rates es van passar a condicions de foscor constant (etapa DD). El dia 34 del registre les rates van ser sotmeses a llum blanca constant de 0.02 lux d'intensitat, al cap de 21 dies la intensitat de llum es va pujar fins a 0.14 lux; el dia 76 del registre la intensitat de llum es va pujar fins a 1.04 lux, el dia 96 del registre la intensitat de llum es va pujar fins a 6.90 lux i finalment, el dia 117 del registre les rates es van sotmetre a les mateixes condicions de llum rebudes pel grup LL durant l'alletament (al voltant de 300 lux).

Resultats: Independentment del grup, en totes les rates el període del ritme d'activitat motora incrementa paral·lelament a l'increment de la intensitat de llum. En cada etapa de l'experiment, el valor de tau és similar en els 3 grups. Pel què fa a la manifestació del ritme, aquesta es va debilitant a mesura que augmenta la intensitat de llum. Aquest debilitament del ritme és més pronunciat en les rates DD, que en les LD i el d'aquestes ho és més que en les LL. Aquesta pèrdua en la força del ritme es reflecteix en el nombre d'animals arrítmics en cadascuna de les etapes: quan la intensitat de llum arriba a 6.90 lux, la majoria d'animals DD són arrítmics, mentre que la totalitat de les rates LL són rítmiques. No és fins a 300 lux que algunes rates LL esdevenen arrítmiques.

Conclusions: Les rates LL, LD i DD responen igual a intensitats creixents de llum pel què fa al tau, però en canvi responen diferentment pel què fa a potència del ritme. Això ens fa pensar que la sensibilitat a la llum és la mateixa en els dos grups d'animals i que l'origen d'aquesta diferència en la potència del ritme rau en el mateix *pacemaker* circadiari.

THE TONIC RESPONSES TO LIGHT OF THE RAT CIRCADIAN SYSTEM ARE AFFECTED BY THE LIGHTING CONDITIONS DURING LACTATION

ABSTRACT

Previous experiments showed that the phasic responses of the rat to light depend on the lighting conditions in which it has been reared. The aim of the present experiment was to study whether the tonic responses also depend on the light history. Three groups of rats were reared under various lighting conditions: constant darkness (DD group), constant light of 300 lux (LL group) and 24h-period light-dark cycles (LD group). After weaning, they were first placed under LD, to reduce the after-effects produced by the previous lighting conditions. They were then placed under DD and under LL of increasing intensities (0.02, 0.14, 1.04, 6.90 and 300 lux), and their motor activity rhythm was studied. The tau increased gradually with light intensity, but it was similar in all the rats at each stage of the experiment. We would like to highlight that the strength of the rhythm (expressed as the power content of the first harmonic and the percentage of variance explained by the highest peak in the periodogram) decreased progressively when light intensity increased, especially in the DD rats, than in the LD rats, followed by the LD rats. We hypothesise that the sensitivity of the rats to low light intensities is similar, and that the differences detected are due to differences in the circadian pacemaker, resulting from the lighting conditions during lactation.

INTRODUCTION

The alternation between light and darkness is one of the main cues for the entrainment of several circadian rhythms in rodents, and thus light can be considered the principal *Zeitgeber*. When a rat is placed in an environment without time cues, it shows a free-running rhythm with a period named "tau". The tau under constant darkness (DD) is close to 24 hours. Its value depends on individual and species factors, and light intensity (Pittendrigh and Daan 1976). Under constant light (LL) of low intensity, the rats free-run with a tau longer than 24 hours. Following the first Aschoff's rule, when increasing the light intensity, the tau also increases, but at high light intensities, a large number of ultradian components appear, and even the circadian rhythm may be lost (Deprés-Brummer et al. 1995, Honma et al. 1996, Takeo 1984, Eastman and Rechtschaffen 1983). However, when a rat has been reared under constant light of high intensity since birth, it develops a circadian rhythm under LL which is maintained throughout its life span (Cambras and Díez-Noguera 1991, Cambras et al. 1998). Moreover, the phasic responses to light of rats reared under LL (i.e. the response to a light pulse) and those of rats reared under DD differ in the magnitude of the phase shifts (Canal-Corretger et al. 2000, Canal-Corretger et al. 2001).

To further analyse the effect of light history on the manifestation of the circadian rhythms of rats, we focused on the tonic effect of light. Therefore, the circadian rhythm of motor activity of three groups of rats reared under distinct lighting conditions was studied, first under a DD environment, and then under LL of progressively increasing intensity.

MATERIALS AND METHODS

Six pregnant Wistar rats were provided by Criffa (St. Germain-sur-l'Arbresle, France). They were placed in individual transparent cages (50x25x12 cm) under a light-dark cycle (LD 12:12h) until the day of delivery, four days later. On the day of birth, the pups were cross-fostered so that each rat fed 12 pups, half males and half females. Two dams with their respective pups were then placed under constant light (LL group, around 300 lux of white bright

light), two dams were placed under constant darkness (DD group, less than 0.01 lux of dim red light) and the remaining dams were placed under light-dark cycles (LD group, 12:12h, light of around 300 lux, darkness of less than 0.01 lux) during the lactation period, which lasted 24 days.

On the day of weaning, the pups were separated from the dams and placed in individual transparent cages (25x25x12 cm) under a 24h-period LD cycle (LD stage) to reduce the after-effects of the previous lighting regimen. The light applied during the LD stage and lactation consisted of two fluorescent lamps (Mazdafluor TF36 W/BI) hanging on a white wall 65 cm from the front of the cages.

The rats had free access to food pellets and tap water. On the day of weaning, we started to record the motor activity rhythm of the rats through activity-meters of crossed-infrared beams. Therefore, the weaning day was considered day 0 of the recording period. After 16 days in these conditions (LD stage), the rats were transferred to complete darkness (DD stage) to study their free-running rhythm.

From day 34 of the recording period, the rats were placed under constant light of several intensities. To ensure uniform illumination and the constancy of the light spectral composition at all intensities, light was applied as follows. In each room, 9 cages were regularly arranged in 3 shelves facing a white wall at 65 cm from the front of the cages. Supported in the edges of the shelves, an squared array of 5 x 5 white LEDs was placed (LEDs were between and aside the cages, 40 cm spaced) pointing to the white wall, and covered by a plastic piece that was not visible from inside the cages. LEDs were feed with a 2.15 KHz squared wave tension, allowing the fine control of light intensity through the duty cycle of the wave. Therefore, when the LEDs were on, they always received the same voltage, producing a constant temperature colour of 8000 K (emission colour x=0.31, y=0.32). Light intensity was measured with a high precision digital luxmeter Mavolux 5032B. The first light intensity was of 0.02±0.01 lux (LL1 stage). After 21 days, it was increased to 0.14±0.02 lux (LL2 stage) and on day 76, to 1.04±0.02 lux (LL3 stage). After 20 days, the rats were transferred to LL of 6.90±0.02 lux (LL4 stage), and on day 117 of the recording period, the light was increased to around 300 lux (LL5 stage), the same light intensity and with the same characteristics as the light received by the LL group during the lactation stage.

Mathematical and statistical analysis

In each stage of the experiment, the period of the motor activity rhythm was calculated with the Sokolove&Bushell periodogram (Sokolove and Bushell 1978). The following days of the recording period were used for the calculations: days 1 to 15 (LD stage), days 16 to 34 (DD stage), days 35 to 53 (LL1 stage), days 56 to 74 (LL2 stage), days 77 to 95 (LL3 stage), days 97 to 115 (LL4 stage) and days 118 to 136 (LL5 stage). 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.

A Fourier's analysis was also applied to data at each stage, from which the mesor (mean daily activity) and the power content of the first harmonic (PC1H) were obtained. In addition, in the LD stage the psi value was calculated: time -in minutes- between the onset of activity (when the mean wave form rises above the median) and the onset of darkness. Positive psi values indicate that the onset of activity occurs after lights off, whereas negative psi values indicate that activity starts before lights off.

For the statistical analysis, an ANOVA of several linear models was carried out at each of the experiment stages. The independent variables were the lighting conditions during lactation (LL, LD or DD) and the sex of the animal, and the dependent variables were, one at each time, the period, PVE, mesor, PC1H and the psi value in the LD stage.

RESULTS

The double-plotted actograms showed that all the rats entrained to the LD cycle (Fig.1). Although the characteristics of

the entrainment to the LD cycles have been studied elsewhere (see Experiment 4), we would like to add that the PVE and the PC1H of the circadian rhythm in the LD stage both were higher in the DD than in the LL group ($p<0.05$).

Regarding only the tonic effect of light, all groups of rats followed the first Aschoff's rule in that their tau increased with light intensity (Fig.1). Note that the value of tau was similar in all the groups and for each stage (Fig.2a). The PVE and PC1H tended to decrease when the light intensity was increased (Fig.2b,c). Interestingly, they differed depending on the group of rats: in the DD stage, the group of rats born and raised under DD manifested a stronger rhythm (had higher PVE and PC1H) than those born and raised under LL; but when light intensity was increased, the strength of the rhythm decreased in all the rats, especially in the DD group, which thus showed a weaker circadian rhythm above 0.14 lux. The mesor tended to decrease with increasing light intensity, but there were no differences between DD, LD and LL groups ($p>0.05$, Fig.2d).

Up to 1.04 lux of light intensity, all the rats manifested a circadian rhythm of motor activity, whereas above this value some rats became arrhythmic (Fig.1,2e). Specifically, more than 60% of the DD rats became arrhythmic at 6.90 lux and this percentage increased at 300 lux; 100% of the LL rats manifested a circadian rhythm at 6.90 lux and more than 80% did at 300 lux; the rats of the LD group showed intermediate values (Fig.2e).

DISCUSSION

Tonic responses to light on the circadian system have been classically studied through the tau values under various light intensities. Here, we study this response using other variables. We consider that the tonic response can also be measured by the strength of the rhythm, i.e. the amplitude of the rhythm, and also by the number of rats that become arrhythmic at a given light intensity. The interpretation of all these variables shows that the lighting conditions during lactation modify the tonic responses of the circadian system to light.

At 1.04 lux, all the rats maintain the circadian rhythm and when the intensity increases, DD-reared rats are the first in having a disruption of the circadian rhythm, in such a way that at 300 lux only 10% show a circadian rhythm, which is very weak. In contrast, most of the rats reared under LL still manifest a circadian rhythm at 300 lux. However, in spite of the arrhythmicity caused by the light intensity, it has to be taken into account that the value of tau in the rhythmic rats does not depend on the lighting conditions during lactation. Tau increases in all the rats according to the first Aschoff's rule, following a curve that reaches an asymptote at 6.9 lux, and it is about 25.6 hours. In contrast to the tau values, the PVE and PCH1 (which indicate how "clear" is the manifestation of the rhythm) vary according to the group of rats and the light intensity. Note that the LD shows intermediate values regarding the number of rats that lose the rhythm, PVE and PCH1. This is noticeable, since this group received "half the quantity" of light that the LL group received. Thus, it seems that the quantity of light that the rat receives during lactation determines the light intensity at which the animal lose the circadian rhythm.

Therefore, we propose that the intensity of the rhythm (PVE or PCH1) reflects the internal amplitude of the pacemaker and that it should be added to tau and phase, as another fundamental property of the circadian system. In this way, the circadian pacemaker would be characterized not only by the velocity of the clock (tau) or its phase, but also by the strength of its oscillation. We should differentiate between the amplitude of the output, which is certainly conditioned by the efferent structures, and the amplitude of the output from the clock, which we regard as a property of the pacemaker. The latter indicates the intensity of the rhythmic signal supplied by the circadian system to the organism.

The fact that the animals of the three groups respond similarly to the very low levels of illumination, eliciting similar changes in their tau values, suggests that

there are no differences in the sensitivity to light between the groups. However, the number of arrhythmic rats and the intensity of the rhythm when the animals are exposed to high light intensities (6.9 and 300 lux) depends on the lighting conditions during lactation. This suggests that the different response is not due to differences in the sensitivity to light (afferents of the circadian system) but to a different functionality of the central pacemaker of the circadian system.

One possible explanation involves a population of photoreceptors. There may be two populations of photoreceptors in the retina: one responding to very low levels of light and another responding to high levels. Thus, the receptors with a high threshold of sensitivity would be affected by light during lactation, being therefore, the cause of the different responses to bright light observed in the adults. However, the hypothesis that during development there is a selective alteration of a concrete type of photoreceptors is difficult to sustain. It is more likely that the whole population of receptors is involved in the response to the whole range of illuminations sending information to the circadian system.

Assuming the multioscillatory nature of the circadian system, it can be easily demonstrated by computer simulation that an increase in the number of functional oscillators does not modify the tonic responses to changes in the light intensity (simulated by changes in the internal coupling), but increases the amplitude of the rhythm (Díez-Noguera personal communication). This indicates that the lighting conditions during weaning may affect the number of functional oscillators in the circadian pacemaker, which is a key point to interpret the differences between LL and DD groups.

The arrhythmicity produced by LL may result from the uncoupling of the oscillators that form the circadian system (Aschoff 1981, Díez-Noguera 1994). However, when the animal grows under bright light intensities, its circadian system must adapt, which involves a specific development of the circadian system. The circadian system is forced to develop in environmental conditions that usually produce arrhythmicity in the rat. Hence, the circadian system that has developed under LL should be less sensitive to the disruptive effects of light. This may be due to the fact that the oscillators are less sensitive to light (the neurones of the SCN change their firing rate according to light intensity (Meijer et al. 1986)) or are strongly coupled and thus this coupling is less sensitive to light. Moreover, the intensity of the rhythm under DD is lower in LL rats than in DD rats, suggesting that the circadian system of the latter has fewer functional oscillators.

These data indicate that under constant darkness, the circadian systems of the LL and DD groups function as two systems equally coupled but with a different number of functional oscillators, which produces an overt rhythm with equal tau but distinct amplitude. Therefore, up to 1.04 lux, the increase in light intensity modifies the coupling in both groups in the same way, which alters the tau. However the oscillators of the DD group (sensitive to light) are more affected than those of the LL group, which may explain the marked decrease in the amplitude of their rhythm. We hypothesise that at high light intensities, the number of functional oscillators is higher and they are more strongly coupled in the LL group than in the DD group. The final consequence is that the LL rats are less affected by constant light and that at 300 lux their rhythm is more clearly manifested and has a higher amplitude than that of the DD group.

In conclusion, the lighting conditions during lactation condition the tonic response of the circadian system to light. The differences between the LL, LD and DD groups cannot be explained on the basis of a different photoreception capacity. The explanation of the differences has to be sought in the circadian pacemaker. Although we do not know whether light during lactation affects at the level of the coupling between oscillators or the sensitivity of the oscillators to light, our experiment strongly suggests that it modifies the structure and functionality of the circadian system.

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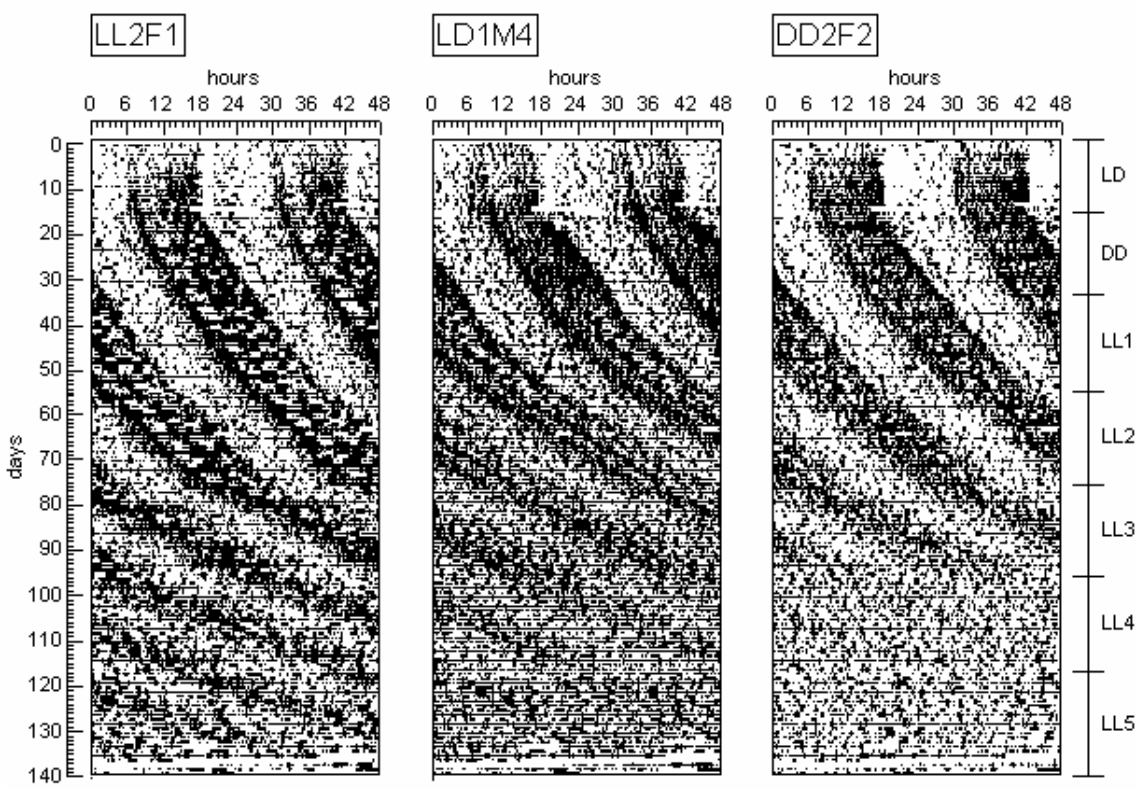

Fig.1.- Double-plotted actograms at modulo 24 hours of one representative animal of the LL, LD and DD groups (from left to right). The horizontal axis indicates the time of the day in hours; the vertical axis on the left indicates the recording days of the experiment, starting on the day of weaning, and the vertical axis on the right indicates the lighting conditions throughout the experiment. LD=light-dark cycles (12:12h), DD=constant darkness, LL1=constant light ≈0.02 lux, LL2=constant light ≈0.14 lux, LL3=constant light ≈1.04 lux, LL4=constant light ≈6.90 lux, LL5=constant light ≈300 lux.

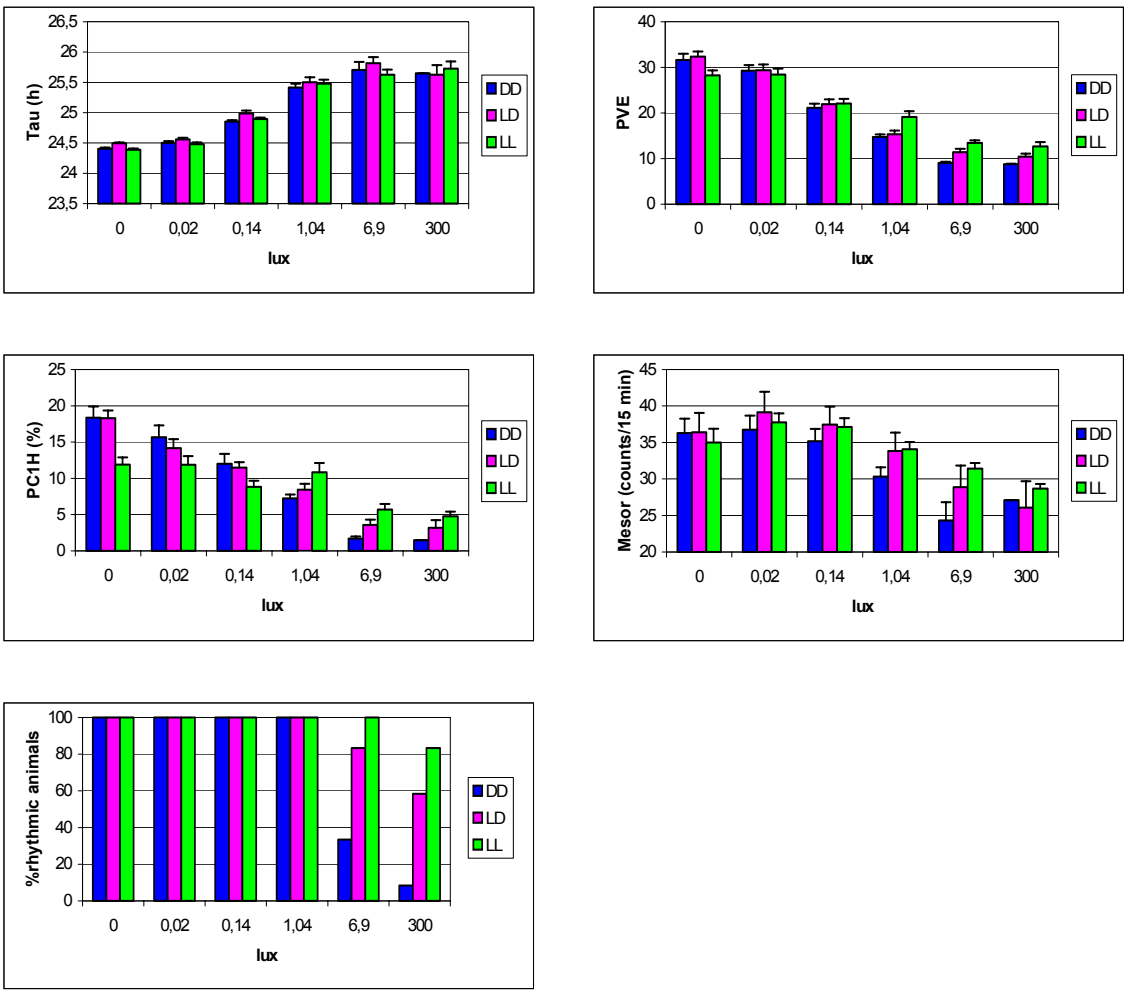

Fig.2.- For each group of rats at each stage of the experiment (0 lux=DD stage): **a.-** Period of the circadian rhythm of motor activity. **b.-** Percentage of variance explained (PVE) by the highest peak in the periodogram. **c.-** Power content of the first harmonic (PC1H). **d.-** Mesor (mean motor activity level). **e.-** Percentage of rhythmic animals (animals with a significant peak in the periodogram). Error bars indicate the standard error.

3.7.- EXPERIMENT 6

THE LIGHTING CONDITIONS DURING LACTATION AFFECT THE FUNCTIONAL CHARACTERISTICS OF THE CIRCADIAN SYSTEM OF ADULT BLINDED RATS

Resum

Objectiu: Les rates que s'han criat en llum constant, comparat amb les que s'han criat en foscor constant, presenten diferències en quant a la manifestació del ritme d'activitat motora sota llum constant de diferents intensitats, als canvis de fase produïts per un pols de llum i a les característiques d'encarrilament a cicles de llum-foscor de diferent període. El nostre objectiu és aclarir si aquestes diferències són degudes només a modificacions a nivell de retina produïdes per la llum durant l'alletament, o bé si és independent de la capacitat visual de l'animal, cosa que suggeriria canvis en el *pacemaker* circadiari.

Material i mètodes: Cinc rates Wistar femella van arribar al nostre laboratori el dia 15 de gestació. El dia del naixement, les cries es van barrejar, de manera que cada mare alimentava lots d'11 cries provinents de diverses ventrades. Tres lots es van transferir a foscor constant (grup DD) i els 2 restants, a llum constant (grup LL). El dia del deslletament (24 dies després del naixement) les cries es van separar de la mare i es van posar en gàbies individuals amb actímetre d'infraroig, per tal de registrar-ne la seva activitat motora, amb accés a l'aigua i al menjar *ad libitum*. Per a aquest experiment es van utilitzar només 6 mascles i 5 femelles del grup DD i 14 mascles i 7 femelles del grup LL. El dia del deslletament aquests animals es van sotmetre a cicles de llum-foscor de 24h de període, per tal de disminuir els post-efectes provocats per les diferents condicions d'il·luminació durant l'alletament. Al cap de 29 dies, totes les rates del grup DD i 6 mascles i 5 femelles del grup LL es van cegar per enucleació òptica dels dos ulls. Totes les rates (cegues o no) es van passar a condicions de foscor constant. Al cap de 21 dies se'ls va administrar una injecció subcutània de melatonina (dosi: 1μg melatonina/kg pes corporal) a l'hora circadiària 10 (CT10). Al cap de 13 dies les rates es van passar a condicions de LL i després de 10 dies en aquestes condicions es va donar una altra injecció de melatonina de la mateixa dosi, però aquest cop a CT12.

Resultats: En l'etapa DD, el període del ritme en curs lliure és similar en totes les rates. Tot i això, les rates DD manifesten un ritme molt més marcat (el contingut de potència del primer harmònic i la variable %Aα/%tα tenen valors més alts) que les rates LL, independentment de si són cegues o no. Les injeccions de melatonina no han produït cap canvi evident en el ritme d'activitat motora de les rates.

Conclusions: El fet que les rates DD cegues i les rates LL cegues presenten diferències en la manifestació del ritme d'activitat motora indiquen que les condicions d'il·luminació en què una rata s'ha criat afecten el *pacemaker* circadiari, independentment de l'efecte que aquestes poguessin tenir a nivell retinal.

THE LIGHTING CONDITIONS DURING LACTATION AFFECT THE FUNCTIONAL CHARACTERISTICS OF THE CIRCADIAN SYSTEM OF BLIND ADULT RATS

ABSTRACT

In previous experiments we observed that the lighting conditions in which a rat was reared affected the manifestation of the circadian rhythm of motor activity under constant light (LL) of different intensity, the response to a light pulse in constant darkness (DD) and the entrainment characteristics to light-dark cycles (LD) of different period. Our aim has been to clarify whether these observations are due to differences at the retinal level, between the rats produced by the light history, or whether they are due to differences at the circadian pacemaker level. Therefore, we reared two groups of rats under different lighting conditions (one group under LL and the other group under DD). After weaning they were placed under 24h-period LD cycles, to eliminate the after-effects produced by the previous lighting conditions. All the animals of the DD group and half the animals of the LL group were then binocularly enucleated. All the animals (blind and intact) were placed under DD. During the DD stage, a single melatonin injection was administered at CT10 to study the effect of a non-photic stimulus on the circadian system of the LL and DD rats. Finally, they were transferred to LL and a second injection of melatonin was administered at CT12. Results revealed that in the DD stage, differences in the tau of all the rats were indistinguishable. However, we found that the manifestation of the rhythm was stronger in the DD than in the LL rats, independently of whether they were blind or intact. The melatonin injections produced no visible effects on the circadian rhythm of motor activity. Hence, there is evidence to believe that the lighting conditions in which a rat has been reared affect the circadian pacemaker, independently of the effect exerted at the retinal level.

INTRODUCTION

Previous experiments in our laboratory have demonstrated that the lighting conditions in which an animal has been reared determine the future responses of its circadian system to light. Specifically, we have shown that animals reared under constant light (LL), compared with animals reared under light-dark cycles (LD) or constant darkness (DD), show a circadian rhythm of motor activity when placed under LL in the adulthood (Cambras and Díez-Noguera 1991, Cambras et al. 1997, Cambras et al. 1998) and also respond differently to a light pulse when kept under DD (Canal-Corretger et al. 2000, Canal-Corretger et al. 2001). To date, only the responses of the animals to photic stimuli have been studied and so we cannot discern whether the observed differences merely reflect a modification at the retinal level or in the central pacemaker.

Therefore, a group of rats were reared in various lighting conditions during lactation and enucleated after weaning to avoid the retinal input to the suprachiasmatic nuclei of the hypothalamus (SCN), which are believed to be the principal pacemaker in mammals. We studied their free-running motor activity rhythm and their response to a non-photic phase-shifting stimulus to test whether the functioning of their circadian pacemaker was affected by the previous lighting environment.

The non-photic stimulus used was a single injection of melatonin, since there is evidence that the rhythm of this pineal hormone regulates the SCN (Gillette and McArthur 1996).

MATERIALS AND METHODS

Five pregnant Wistar rats from Criffa (St. Germain-sur-l'Arbresle, France) on the 15th day of gestation were used. Eight days later, after all the pups were born, they

were cross-fostered so that each dam fed 11 pups. Each dam was kept with her pups in a transparent cage of 50x25x15 cm in an isolated room, with controlled ambient conditions. Three dams were kept under LL (LL group, around 300 lux of white bright light), and the remaining dams were placed under DD (DD group, less than 0.1 lux of dim red light).

Twenty-four days after birth, the pups were weaned and placed in individual cages (25x25x15 cm) under a 24-hour light-dark cycle (LD 12:12h, LD stage), so that all the rats entrained to the same period, thus avoiding the after-effects of the lactation stage. Only 6 males and 5 females of the DD group, and 14 males and 7 females of the LL group were used for this experiment.

From the day of weaning (day 0 of the recording period) and until the end of the experiment, the motor activity of each rat was detected through an activity-meter of crossed infrared beams and recorded every 15 minutes. Throughout the experiment, the rats had free access to tap water and food pellets.

On day 29 of the recording period, all the rats of the DD group, and 6 males and 5 females of the LL group were binocularly enucleated under deep anaesthesia. All the rats (blind and intact) were then placed into constant darkness (less than 0.1 lux of dim red light, DD stage) to study their free-running rhythm. After 21 days, a subcutaneous injection of melatonin (dose: 1 μg melatonin/kg body weight, N-Acetyl-5-methoxytryptamine (Sigma©, Steinheim, Germany) dissolved in ethanol 5% and saline) was given to the rats at circadian time 10 (CT10). To calculate the CT10, two circadian hours were subtracted to CT12, which corresponds to the time of motor activity onset.

On day 63 of the recording period, all the rats were transferred to constant light (around 300 lux of white bright light, LL stage) to facilitate handling of the animals. Ten days later, the blind rats received another subcutaneous injection of melatonin of the same characteristics as the previous one, except for the time of administration, which was at CT12 (time of activity onset).

This experiment is in accordance with the Ethic Committee for Animal Experimentation of the University of Barcelona.

Data analysis

In all the stages, the period of the motor activity rhythm was calculated through the Lomb and Scargle periodogram (Van Dongen et al. 1999, Ruf 1999). The amplitude of the rhythm and power content of the first harmonic (PC1H) were obtained through a Fourier's analysis, but as they went in parallel, only the results for the PC1H are shown. Through the mean wave form (smoothed ±6 hours), the characteristics of the alpha phase (activity phase) were studied in each animal, and for each of the experiment stages. The alpha phase was considered the time during which the motor activity was above the median. The duration (%tα), amount of motor activity (%Aα) and variable %Aα/%tα, which was regarded as an indicator of the "intensity" of the rhythm, were calculated. In the LD stage, the data used ranged from days 9 to 29 of the recording period; in the DD stage, from days 30 to 50; and in the LL stage, from days 63 to 73.

In addition, the psi value was calculated in the LD stage. The psi is the phase relation between the *Zeitgeber* and the rhythm studied. To calculate it, the time (in minutes) between lights off and the onset of activity (when the variable rose above the median) was measured from the mean wave form plotted at modulo 24 hours for each rat. Positive values indicated that the activity started after the lights went off, and negative values that the activity began before the lights went off.

In the DD and LL stages, a line on the onset of motor activity was drawn for the 10 days prior and the 10 days posterior to the injection of melatonin to calculate the phase shifts. The distance (in hours) between the two lines was the phase shift value.

For the statistical analysis, several general linear models were used. The independent variables were the lighting conditions during lactation (LL or DD) and the sex of the animal, and for the DD and LL stages, also the fact of being blind or intact; the dependent variables were the mesor,

PC1H, psi, and %tα, %Aα and %Aα/%tα of the alpha phase in the LD stage, and the period, mesor, PC1H, %tα, %Aα, %Aα/%tα and the phase shift produced by the injection of melatonin in the DD and LL stages.

RESULTS

The double-plotted actograms show the evolution of the motor activity rhythm of a representative animal of each group throughout the experiment (Fig.1).

In the LD stage, no significant difference was observed in the mesor values, so that the amount of activity was similar in the LL and the DD group. However, the PC1H in the DD group was significantly higher than in the LL group ($p<0.005$), which indicates that the DD group manifested a stronger rhythm than the LL group (Fig.3a, 3b). Regarding the mean wave form, as all the rats were still intact, only two means were calculated: one for the DD group and another for the LL group (Fig.2). No significant differences were found in the psi values (mean±se =-21.8±2.2 min.), showing that the onset of the alpha phase was around 20 minutes before the onset of darkness for all the rats. Although the duration of the alpha phase (%tα) was similar in all the groups, the %Aα and the %Aα/%tα were significantly higher in the DD than in the LL group ($p<0.005$, Fig.3c, 3d).

All the rats free-ran, in the DD stage, with a similar period (mean±se =24.30±0.02 hours, Fig.4a), regardless of the lighting conditions they had during lactation and of whether they were blind or intact. Males had a significantly higher mesor than females ($p<0.005$). In addition, DD animals had a stronger rhythm than LL animals (they had higher values of PC1H and %Aα/%tα) ($p<0.05$, Fig.4c, 4e). The duration of the alpha phase (%tα) in the LL group was longer than in the DD group ($p<0.05$, Fig. 4d). The injection of melatonin induced no significant phase shift (Fig.1).

Obviously, differences between blind and intact animals were observed in the LL stage: intact rats had a significantly longer period (mean±se = 25.02±9.70 hours) than the blind ones (mean±se = 24.38±2.25 hours) (Fig.4a). If only the latter are considered, they maintain the same characteristics as in the DD stage.

Significant differences due to sex (males had a higher mesor and females showed higher %Aα/%tα) were also observed in the LL stage. As in the DD stage, no significant phase shifts after the injection of melatonin were detected (see Fig.1).

DISCUSSION

Daily intraperitoneal injections of supraphysiological concentrations of melatonin into rats in DD entrain their locomotor activity (Cassone et al. 1986, Redman et al. 1983). Moreover, in these and other studies, it has been shown that melatonin administration between CT9 and CT12 induces permanent phase advances of locomotor rhythms, whereas it is largely ineffective at other times tested (Armstrong 1989). In the present experiment, although melatonin was administered in this range of times, no consistent effect was observed in the phase of the motor activity rhythm. In C3H/Hen mice, simple injections of melatonin are also ineffective in phase shifting free-running circadian rhythms (Benloucif and Dubocovich 1996). There may be two possible explanations for the inefectiveness of single injections of melatonin: 1) external melatonin is too weak a chronobiotic to disturb the circadian rhythms in a single administration and 2) rat may be less sensitive to melatonin than other animal species. As neither the blind nor the intact rats phase shifted after the injection, we may think that the ineffectiveness of melatonin does not depend on the retinal input.

Although the study of the effect of a non-photic stimulus on LL and DD rats has not been possible, the primary objective of the present experiment (to study the circadian rhythm of DD and LL rats without the influences of the retinal input) has been largely achieved. We would like to highlight some results: the finding that on the one hand, the free-running period in the DD

stage does not depend on the lighting conditions in which the rat has been reared, and on the other hand, the absence of differences in the psi values at the LD stage may reflect a similar functioning of the circadian clocks of DD and LL rats, as the period and the phase relation to a given *Zeitgeber* are two fundamental properties of any pacemaker (Daan and Pittendrigh 1976). Moreover, in a previous experiment with mice reared under different LD cycles, the free-running rhythms were independent of the postnatal lighting environment (Davis and Menaker 1971). Nevertheless, we have here found that the circadian rhythm was stronger in the blinded rats reared under DD than in those reared under LL during lactation. Therefore, if the manifestation of the rhythm of DD and LL blinded rats differs, it could be suggested that there are some differences in their circadian system, which are not related to the visual system and that depend on the early lighting conditions.

It is well known that visual functions are affected by exposure to LL and that previous light history strongly determines the susceptibility to light damage (Terman et al. 1991). In addition, enucleation studies in rats have shown that during the first postnatal weeks, the necessary connections to reach the adult pattern are established in the visual system (Nicoll et al. 1991). Similarly, some essential structures of the central circadian system of new-born pups (like size, number of synapses, and neural morphology of the suprachiasmatic nuclei of the hypothalamus -site of the principal pacemaker-, the retinohypothalamic tract -responsible for the photic entrainment-, and the geniculohypothalamic tract -which brings photic and non-photic information-) maturate during the first postnatal weeks (Moore 1991, Speh and Moore 1993, Moore et al. 1989). Therefore, exposure to light during this period may indirectly affect the future responses of the adult animal to light and the manifestation of the circadian rhythms.

To date, evidence indicated that at least the system that deals with photic information is affected by postnatal lighting experience. However, our results with blinded rats suggest that early lighting conditions condition the circadian pacemaker. The study of the effects of non-photic stimuli other than melatonin may be useful to test the functioning of the circadian pacemaker *per se*, regardless of the retinal input, and thus discern whether the visual system and/or the central circadian system are responsible for the differences between DD and LL rats.

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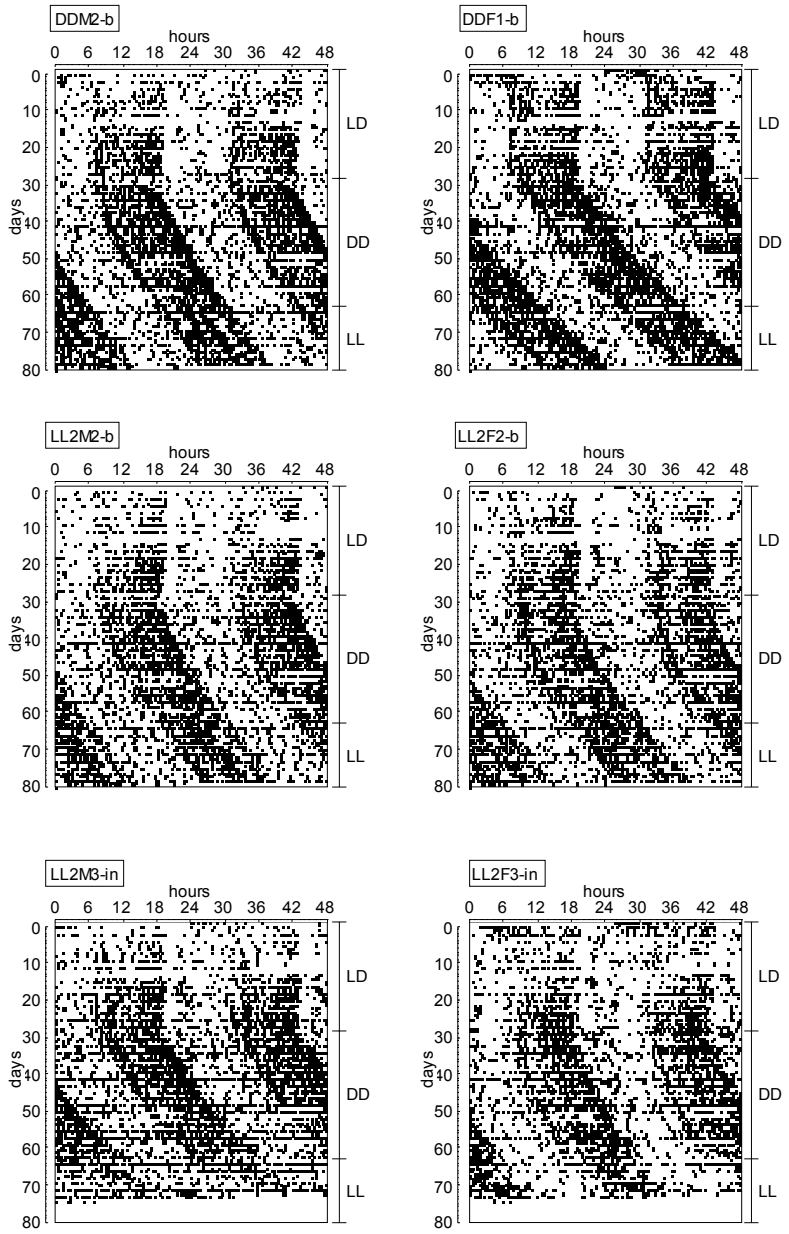

Fig.1.- Double-plotted actograms at modulo 24h of two representative rats (a male -left- and a female -right-) of each group. The horizontal axis indicates the time of the day in hours; the left vertical axis indicates recording days of the experiment, starting on the day of weaning; and the right vertical axis indicates the lighting conditions throughout the experiment. The labels on the top of the actograms indicate the characteristics of the animal: group (DD or LL), sex (M -male- or F -female-) and whether they were blind (b) or intact (in) after the LD stage.

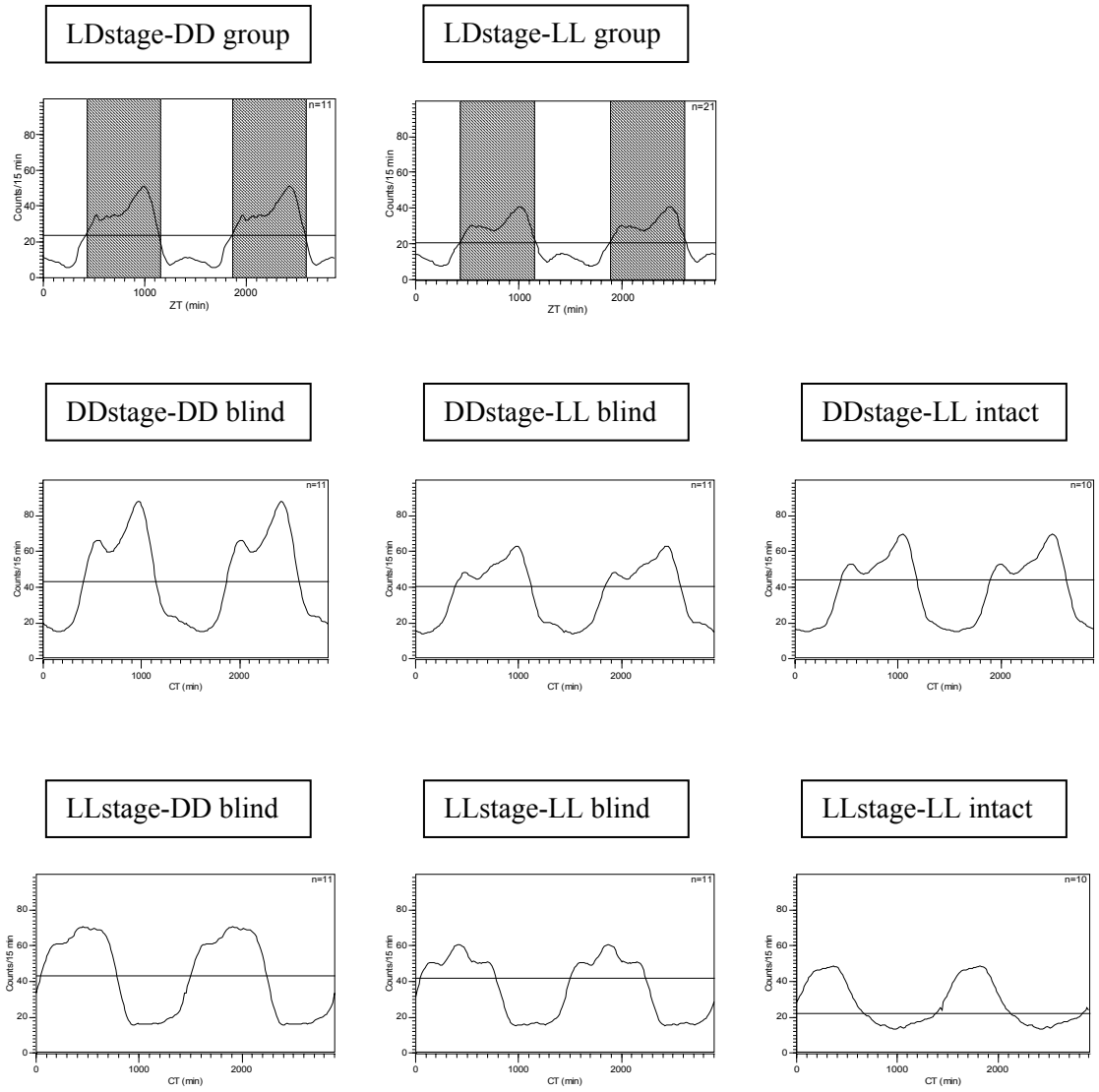

Fig.2.- Double-plotted mean wave forms of the animals of the LL and the DD groups during the LD, DD and LL stages. The horizontal axis indicates *Zeitgeber* time in the LD stage, and circadian time in the DD and LL stages. The vertical axis indicates arbitrary units of motor activity of the animal. The horizontal line in the graph is the median. In the LD stage, the shadowed area indicates the dark phase of the light-dark cycle. In the DD and LL stages, b=blinded animals, in=intact animals

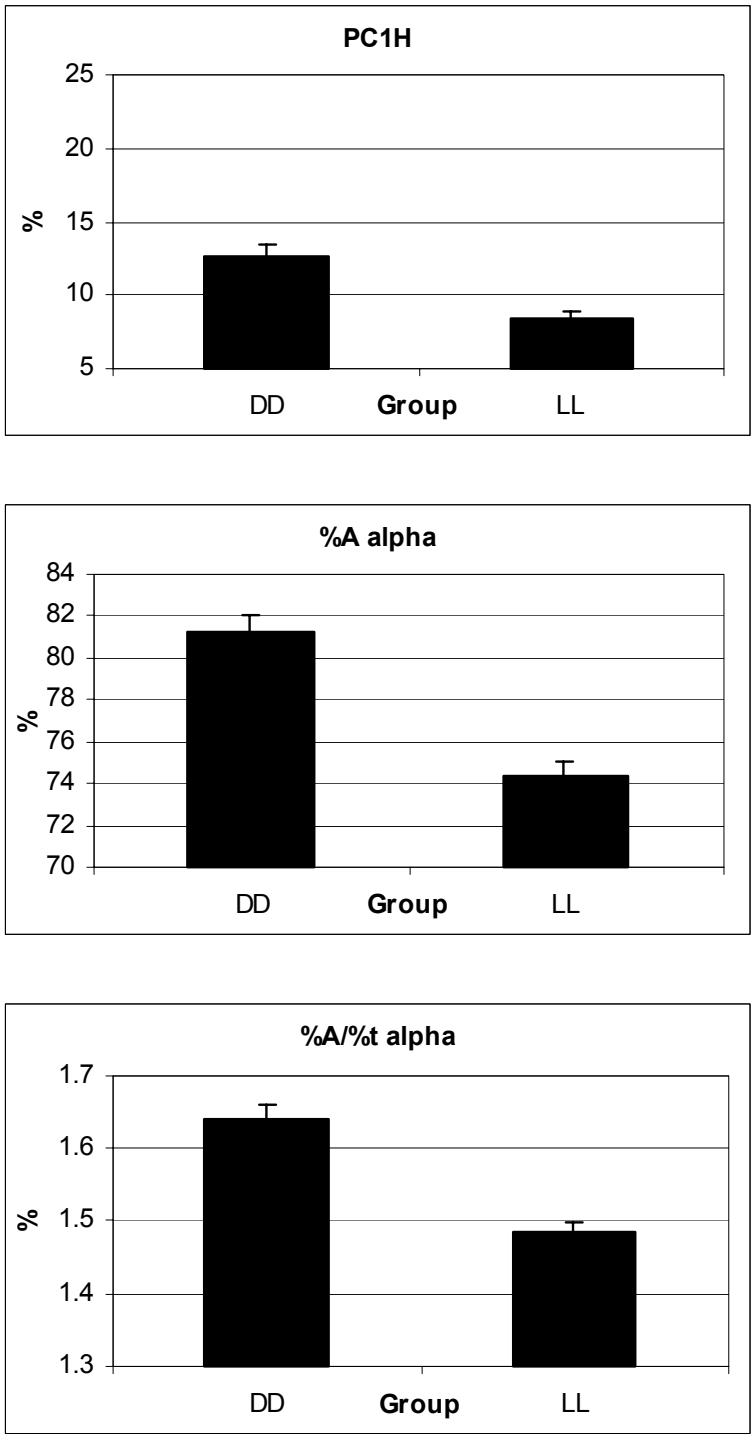

Fig.3.- a.- Power content of the first harmonic of the circadian rhythm of motor activity in the LD stage, for the DD and the LL groups (mean+se).

b.- Percentage of activity during the alpha phase in the LD stage, for the DD and the LL groups (mean+se).

c.- %A/%t of the alpha phase in the LD stage, for the DD and the LL groups (mean+se).

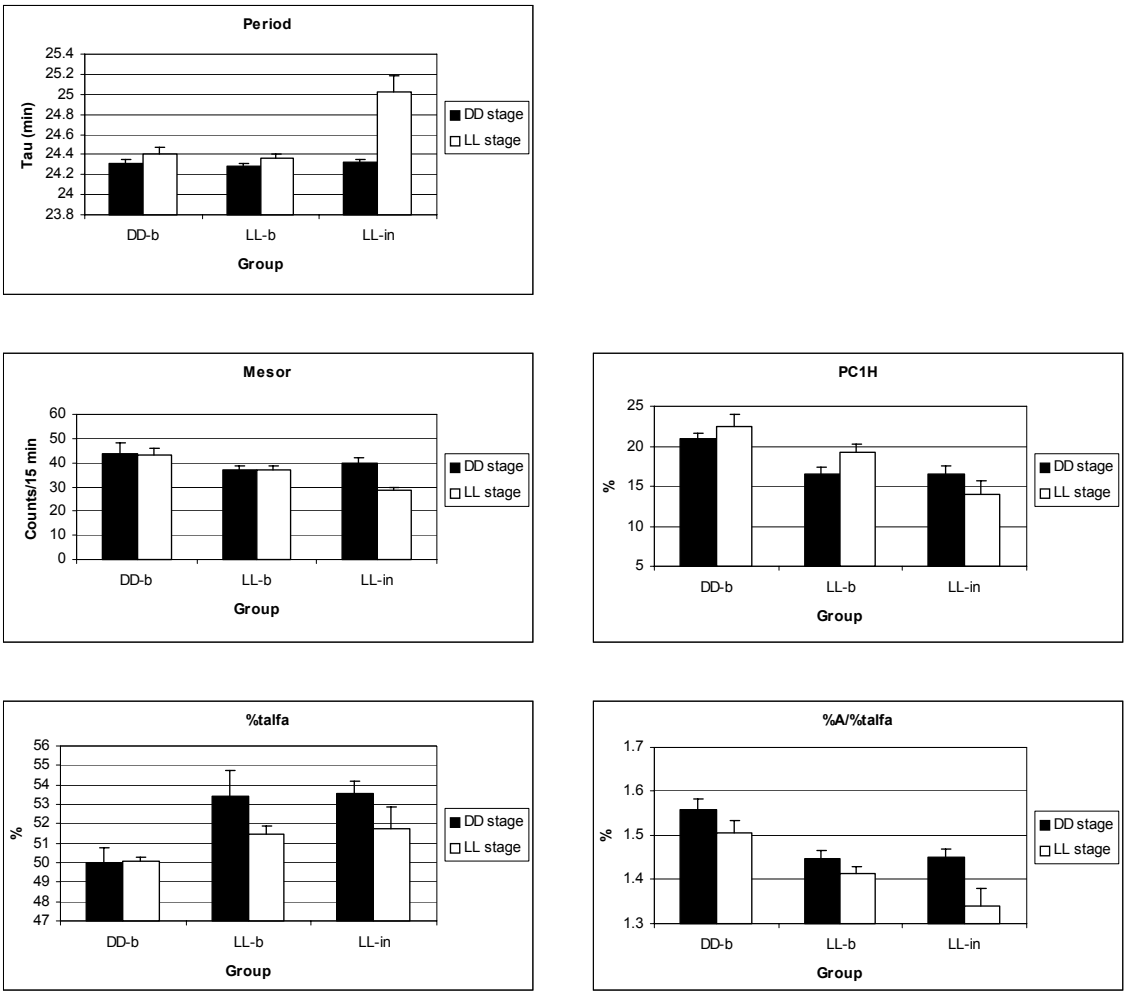

Fig.4.- a.- Period of the circadian rhythm of motor activity in the DD and LL stages, for the DD and the LL groups (mean+se). b=blinded animals, in=intact animals.

b.- Mesor of the circadian rhythm of motor activity in the DD and LL stages stage, for the DD and the LL groups (mean+se). b=blinded animals, in=intact animals.

c.- Power content of the first harmonic of the circadian rhythm of motor activity in the DD and LL stages, for the DD and the LL groups (mean+se). b=blinded animals, in=intact animals.

d.- Duration of the alpha phase in the DD and LL stages, for the DD and the LL groups (mean+se). b=blinded animals, in=intact animals.

e.- %A/%t of the alpha phase in the DD and LL stages, for the DD and the LL groups (mean+se). b=blinded animals, in=intact animals.

3.8.- EXPERIMENT 7

PHASE AND TAU RESPONSE CURVES TO NON-PHOTIC STIMULI IN THE RAT

Resum

Objectiu: Estudiar l'efecte d'estímuls no fòtics sobre la fase i el període del ritme d'activitat motora de la rata, per tal de disposar d'una eina per estudiar la resposta del sistema circadiari independentment de la llum.

Material i mètodes: Es van utilitzar 39 rates Wistar (20 mascles i 19 femelles) de 2 mesos d'edat. Aquestes es van posar en gàbies individuals amb actímetre d'infraroig, per tal d'enregistrar-ne la seva activitat motora, amb accés a l'aigua i al menjar *ad libitum*. Després d'estar 12 dies sota cicles de llum-foscor de 24 hores de període, els animals es van cegar per enucleació òptica dels dos ulls (dia 0 de l'experiment) per tal d'eliminar qualsevol interferència òptica. El dia 11 de l'experiment van ser sotmeses a un pols de calor (2h a 33ºC), el dia 25 van rebre una injecció subcutània de dexametasona (dosi: 10 mg/kg pes corporal), el dia 52 de l'experiment van rebre un pols de fred (2h a 6ºC) i finalment, els dies 66 i 87 es van posar en gàbies amb roda giratòria durant 3 hores. Els polsos es van donar aleatòriament en el temps i posteriorment es va calcular l'hora circadiària corresponent. Després de cada pols es van estudiar els canvis de fase i de tau produïts en el ritme d'activitat motora i es van calcular la Corba de Respostes de Fase i la Corba de Respostes de Tau per a cadascun dels estímuls i per a tots els estímuls agrupats.

Resultats: Cap dels estímuls no fòtics estudiats produeix un canvi evident en la fase o el període del ritme d'activitat motora de la rata. De tota manera, les corbes que agrupen tots els estímuls mostren la següent tendència: es produeixen avenços de fase i allargaments de període al final del dia subjectiu i principi de la nit subjectiva, mentre que al final de la nit subjectiva i principi del dia subjectiu es produeixen endarreriments de fase i escurçaments de període.

Conclusions: Les rates són poc sensibles als estímuls no fòtics. Tot i això, sembla que les zones de més sensibilitat a aquest tipus d'estímul es troben en les zones de transició d'activitat a repòs i de repòs a activitat.

PHASE AND TAU RESPONSE CURVES TO NON-PHOTIC STIMULI IN THE RAT

ABSTRACT

Although light, and therefore photic stimuli, are the principal *Zeitgebers* in mammals, various non-photic stimuli can also affect the properties of a circadian pacemaker. In the present experiment we tested the effect of four types of non-photic stimuli (heat and cold pulses, dexamethasone injection and access to a running-wheel) on the circadian rhythm of the motor activity of Wistar rats. The Phase Response Curve (PRC) and the Tau Response Curve (τRC) for each stimulus, and a global PRC and a global τRC for non-photic stimuli were obtained. Non-photic stimuli have a much weaker effect on the circadian rhythm of the rat than photic stimuli, as no clear results were obtained for any of the studied stimuli. However, regarding the global PRC and τRC, phase advances and tau lengthenings tend to occur at the end of the subjective day and beginning of the subjective night, whereas phase delays and tau shortenings occur at the end of the subjective night and beginning of the subjective day. In conclusion, although rats are scarcely sensitive to non-photic stimuli, the transition zones in the rest-activity cycle appear to be the time when the rats are more sensitive to these stimuli.

INTRODUCTION

Living organisms in the earth have adapted to the surrounding cyclic environment thanks to their circadian system, which will entrain the circadian rhythms of the organism to the external cycle. One of the main important signals or *Zeitgebers* capable of entraining the circadian rhythms is light, and hence light stimuli have been thoroughly studied. Light has phase-resetting affects on the circadian clock, which can be studied through a phase response curve (PRC). However, light is not the only *Zeitgeber*, since other stimuli like temperature, novelty-induced wheel-running and certain drugs or chemicals, which can be described as "non-photic" stimuli, can also able to produce phase changes in several circadian rhythms (Johnson 1992). The PRC for light and non-photic pulses differ. For example in the hamster, light produces phase delays around the onset of activity (CT12) and phase advances during the subjective night, whereas novelty-induced wheel-running and triazolam injections produce phase advances during the subjective day (Mrosovsky 1996). Apart from the effect of novelty-induced wheel-running on hamsters, the effect of other non-photic stimuli on this and other species has seldom been studied.

A *Zeitgeber* can not only affect the phase of the rhythms, but also their period, being both changes a reflection of the function of the clock mechanism. Although tau changes have been observed concomitant to a phase shift after a specific stimulus (Pittendrigh and Daan 1976, Joy et al. 1989, Reebs et al. 1989), very few Tau Response Curves (τRC) have been published for light pulses (Kramm and Kramm 1980, Pohl 1982, Gerkema et al. 1993), and even fewer for non-photic stimuli (Mrosovsky 1993). Several types of non-photic stimuli have been described:

1.- Temperature-related stimuli: temperature provides information about season and time of the day (Balzer and Hardeland 1988). Although temperature is a weaker *Zeitgeber* than light-dark changes (Bünning 1973), temperature cycles can synchronise, in constant conditions, the endogenous circadian rhythms of several species of plants and animals, including nocturnal marsupials (Francis and Coleman 1990), macaques (Tokura and Aschoff 1983), Syrian hamsters (Pohl 1988), blind mole rats (Goldman et al. 1997) and rats (Francis and Coleman 1988). Phase shifts due to cold exposure of several hours have been repeatedly observed in various organisms such as bees, cockroaches, fiddler crabs, various higher plants (Bünning 1973) and even in hamsters (Mistlberger 1996). Moreover, the phase response curve (PRC)

to ambient temperature pulses in rats has been recently reported (Francis and Coleman 1997), supporting the significant synchronising effects of ambient temperature on the circadian rhythm of mammalian species.

2.- Chronobiotics: these chemical substances can therapeutically reentrain non-synchronised circadian rhythms or profilactically prevent their disruption by an ambient effect. Chronobiotics include antidepressive drugs, indoleamines, melatonin, serotonin, various peptides, corticosteroids, etc. (Dawson and Armstrong 1996). Regarding the latter, glucocorticoids have been shown to be a circadian *Zeitgeber*. Specifically, laboratory rats injected with dexamethasone show shifts in the phase of their temperature rhythm, from which a typical PRC has been drawn (Horseman and Ehret 1982).

3.- Novelty-induced wheel-running phase-shifting: this stimulus has been widely studied in hamsters. The phase shifts caused by novelty-induced wheel-running depend on the circadian time (CT) at which the pulses are administered (Mrosovsky 1996, Mrosovsky 1999) and the amount of activity (number of wheel revolutions during the pulse) (Janik and Mrosovsky 1993, Bobrzynska and Mrosovsky 1998). The duration of running is not as critical as expected: although most experiments with hamsters consist of a three-hour period exercise (Van Reeth et al. 1994, Mrosovsky 1993), at certain CTs, one-hour pulses are sufficient to produce the maximal shifts obtainable at those CTs (Bobrzynska and Mrosovsky 1998).

In this study, we tested several non-photic stimuli to get more insight about their action mechanism in the circadian system of the rat, as a tool to study the functioning of the circadian system independently of light. For this purpose, we used two temperature-related stimuli, a heat and a cold pulse, a chemical agent, dexamethasone and a novelty stimulus, access to a running-wheel. We examined their effects on the circadian rhythms of rats. In all cases, both PRC and τRC were obtained.

MATERIALS AND METHODS

Thirty-nine two-month-old Wistar rats (20 males and 19 females) were supplied by Charles River (Criffa, Barcelona, Spain). The rats were then housed in individual cages (25x25x12 cm) with water and food *ad libitum*, under a light-dark cycle (LD 12:12 hours), with white bright light of around 300 lux in the light phase, and dim red light of less than 0.1 lux in the dark phase, at 21±1ºC. Activity-meters of crossed infrared beams were used to detect the motor activity rhythm of the rats. Every 15 minutes, the data collected were stored in a computer for further analysis.

After 12 days under LD cycles, the animals were binocularly enucleated under deep anaesthesia to make sure that photic stimuli did not interfere with non-photic stimuli. The day of the enucleation was considered day 0 of the experiment. On day 11, a 2 hour-heat pulse of 33±1ºC was applied to the rats. For the heat pulse, each cage with one rat was removed from its normal location and placed in another chamber pre-heated to the required temperature. On day 25, the rats were given an intraperitoneal injection of dexamethasone (Dexamethasone 21-phosphate disodium salt, Sigma®) dissolved in saline 0.9% at a dose of 10 mg/kg of body weight. Twenty-seven days later (on day 52 of the experiment), the rats were transferred to a cooled room (6±1ºC) for two hours. On day 66, the rats were placed into cages with running-wheels for three hours. Finally, on day 87, as the rats were already trained with the running-wheel, they were placed, for the second time, in a cage with a running-wheel for three hours. All the non-photic stimuli were applied to the free-running rats, at specific clock times, and for each rat, we determined the circadian time at which the stimuli was applied, and the response to the stimuli in terms of phase shift and tau changes.

All procedures were performed in accordance with the Ethic Committee for Animal Experimentation of the University of Barcelona.

Mathematical and statistical analysis

To determine the circadian time (CT) at which the animal had received the stimulus, a line was drawn on the onset of activity (CT12) during the days previous to the stimulus and the distance (in circadian hours) between this line and the midpoint of the stimulus was then calculated; twelve hours were then added to this value to obtain the exact CT.

After each stimulus, the phase shifts were calculated as follows: two lines were drawn along the activity onset, one for the 10 days before and the other for the 10 days after the stimulus. The difference (in circadian hours) between the two lines was the value of the phase shift.

The period of the motor activity rhythm before and after each stimulus was also calculated by means of the Sokolove&Bushell's periodogram (Sokolove and Bushell 1978), using the data corresponding to 10 days before and 10 days after the stimulus.

As the CTs at which the stimuli were applied were scattered all over the circadian cycle, we grouped the results into two-hour bins, i.e. CT1 included the CTs between CT0 and CT2, CT3 included the CTs between CT2 and CT4, and so on. Thereafter, the PRCs and τRCs were drawn. The results for each CT are expressed as mean ± standard error, unless otherwise stated.

RESULTS

Although no statistically significant difference was observed when each bin was studied individually, we found a tendency in the response to non-photic stimuli.

The heat pulse produced phase advances during the subjective day but no phase delays (Fig.2a). The τRC showed tau shortenings during the subjective day, being the maximum changes at CT7 and CT9, paralleled by the maximal phase advances (Fig.2b).

After the injection of dexamethasone, phase advances were detected at the beginning of the subjective day (Fig.2c) and a phase delay was observed at CT11. The main important tau changes were tau lengthenings at the end of the subjective night (Fig. 2d).

The cold pulse produced no clear phase shifts (Fig.2e), but a tau lengthening at CT11 and tau shortenings during the late subjective night and early subjective day were observed (Fig.2f).

The rats were applied two pulses of running-wheel in order to allow the animal to be accustomed to the wheel. However, although the number of revolutions of the second pulse of running-wheel was significantly higher (Student's T-test) than the first pulse, the phase shifts and tau changes produced by both stimuli were indistinguishable. Therefore, the results of the two wheel pulses were joined, revealing that the most evident phase shifts were the phase advances at CT9 and CT11 and the phase delays at CT23 and CT1 (Fig.2g). The τRC had a similar shape to the PRC: lengthening of the tau occurred after wheel pulses in the mid part of the cycle (between CT11 and CT17), whereas shortening of the tau occurred at the end of subjective night and the beginning of the subjective day, i.e. from CT23 to CT3 (Fig.2h).

To test whether there was any global tendency in the non-photic stimuli, we combined the phase shifts and tau changes produced by the "environmental" pulses (heat, cold and running-wheel pulses) in the same curves and a global PRC and a global τRC for non-photic stimuli in blinded rats were thus obtained. The global PRC showed phase advances during the last part of the subjective day, between CT9 and CT13, a "dead zone" during most of the subjective night, and phase delays in the late subjective night and early subjective day, i.e. between CT23 and CT1 (Fig.3a). The τRC showed tau lengthenings around the onset of activity, between CT11 and CT17, and tau shortenings in the late subjective night and early subjective day (between CT23 and CT3, Fig.3b).

In neither of the stimuli, nor in the global results there was a significant correlation between the direction of the tau changes and phase shifts.

DISCUSSION

Although photic stimuli have been widely studied in the rat, scarce data are available on non-photic stimuli. Non-photic stimuli include different stimuli; from stimuli that indirectly evoke phase shifting indirectly via behavioural activation, such as novel running-wheel or triazolam injections in hamsters, to others that are independent of induced activity, for instance the phase shifts induced by neuropeptide Y (NPY) or restraint-induced phase shifts (Van Reeth et al. 1991). It has been also described that not all non-photic stimuli produce the same PRC: novelty, triazolam and dark pulses PRCs in hamsters, though similar, are not identical (Rosenwasser and Dwyer 2001). The three stimuli produce phase advances during mid subjective day and phase delays during the late subjective night, but only the PRC for dark pulses shows an advance region extending through the first half of the subjective night. The mechanism by which these stimuli affect the circadian clock seems quite complex, as a clear response for non-photic pulses cannot be observed. The intergeniculate leaflet (IGL) through NPY is a key mediator of the phase-shifting effects of activity to the SCN (Kuroda et al. 1997, Janik and Mrosovsky 1994, Janik et al. 1995). However, lesions of the IGL block the phase shifting effects of both induced activity and benzodiazepines (Biello et al. 1991, Janik and Mrosovsky 1994, Johnson et al. 1988, Wickland and Turek 1994), but fail to abolish the phase shifting effects of dark pulses (Harrington and Rusak 1986). Thus, the effects of non-photic stimuli are not exclusively dependent on the activity of the animal.

Here, we assayed various stimuli that, except wheel running, are not necessarily related to the activity of the rat. However, before interpreting the results, a general consideration should be made about methodology. The motor activity of rats, especially when measured through activity-meters with infrared beams, is highly scattered, making the onset of activity sometimes difficult to measure and thus, hindering the detection of phase changes. In all cases, the calculation of tau through the periodogram is more precise because it is an objective method. Therefore, we trust that in terms of methodology and in general, τRCs are more reliable than PRCs in the rat. We would also like to highlight that our blinded rats showed significantly higher daily levels of motor activity than rats of the same strain and age kept under DD in our laboratory for other experiments. This may be a consequence of enucletation. Hence, the abnormally high levels of motor activity may interfere with the effect of the stimuli tested.

Regarding the temperature-related stimuli (heat and cold pulses), clear effects were not observed. Warm ambient temperature pulses produced discrete phase advances during the mid part of the cycle (CT6-CT8), but no delays. The τRC also showed shortenings of about 10 minutes at CT6 and CT8. Neither the direction nor the magnitude of the phase shifts observed agree with those reported in Long-Evans rats by Francis (Francis and Coleman 1997), who observed phase delays of about 3 hours when calculated through the steady state shifts and of near 6 hours when the initial shifts were used. It is difficult to interpret this discrepancy, but it may be related to the difficulty of the determination of the onset of activity in rats for the calculation of the phase shifts. After the cold pulse, no clear results are observed in the PRC, whereas the τRC shows modifications in the tau when the stimulus is applied near the transitions between the subjective day and night.

On the one hand, temperature may directly affect the circadian system. Although a functional prerequisite for circadian pacemakers is tau being temperature-compensated so that time keeping remains accurate over a range of physiological temperatures, this does not imply that the SCN is insensitive to temperature changes. Indeed, ambient temperature may affect the circadian system directly: heat exposure modifies hypothalamic temperature (Sakurada et al. 1994), and the peak firing rates of cultured rat suprachiasmatic nucleus are affected by temperature (Ruby and Heller 1996). There is even a PRC to heat pulses in the rat SCN (Ruby et al. 1999). Moreover, it has been found that the amplitude of the rat circadian rhythm of firing rate in SCN neurones is

highly temperature-sensitive (Ruby et al. 1999). On the other hand, temperature may indirectly affect the circadian system via hormonal alterations, e.g. through variations of plasma thyroid hormone levels (Shido et al. 1993), or through the pineal gland and therefore its hormone melatonin (Zatz et al. 1994, Barrett and Takahashi 1995). However, it is clear that temperature is a weaker *Zeitgeber* in homeotherms than light (Aschoff and Tokura 1986, Francis and Coleman 1988, Francis and Coleman 1990, Tokura and Aschoff 1983). In addition, while studying rats, it must be taken into account that temperature is a more effective *Zeitgeber* in diurnal than in nocturnal species (Rajaratnam and Redman 1998).

In the temperature rhythm of rats, dexamethasone induces phase delays of about 3 hours during the late subjective day and phase advances of about 3 hours during the subjective night (Horseman and Ehret 1982). In contrast, our phase shifts were never higher than 0.5 hours and we found phase advances at the beginning of the subjective day and end of the subjective night. This discrepancy may be due to the fact that the rats of our experiment were enucleated. The levels of plasmatic corticosterone of blinded rats are higher than those of control rats, and they tend to increase in such a way that by 60 days after the surgical operation, they have duplicated (Scafarczyk et al. 1980). Moreover, as mentioned above, in the present experiment the motor activity levels of the blinded rats were higher than normal, which may also be related with an increase in cortisol levels (Weinberg and Wong 1986). Therefore, if our rats had abnormally high corticosteroid levels when they were injected with dexamethasone, the injection would have probably been less effective than in intact rats, which may explain the observed lack of a clear response of the circadian system to this chemical.

Among the non-photic stimuli assayed, the wheel running pulse clearly implies an increase in motor activity. The responses to this stimuli have been widely studied in hamsters. It has been found that a 3h-wheel running pulse produces phase advances during the subjective day (with a maximum delay of nearly 3 hours) and that it has no effect during the subjective night, when animals are already active (Mrosovsky 1992). Moreover, the novelty has a strong effect on the response to the stimuli, because hamsters placed in a running-wheel for the first time show stronger effects on the pacemaker, because exposure to a wheel is a particularly novel event (Mrosovsky 1996).

In the present experiment, running in a wheel was a novelty, at least in the first running-wheel pulse. However, since the rat requires more training than the hamster (Cornish and Mrosovsky 1995), a second wheel pulse was applied. Nevertheless, although in the second exposure to the wheel the number of wheel revolutions was higher than the first time, the magnitude of both the phase shifts and the tau changes were similar to those of the first pulse. In contrast, correlations have been obtained in hamsters between size of shifts and amount of activity (Mrosovsky 1996). Hence, in rats the phase shifts or tau changes are much smaller than of hamsters, and do not apparently dependent on the motor activity level. Therefore, we joined the results obtained in the two pulses to reduce the individual dispersion and to examine the effect of the running-wheel on rats. Here, the tau changes and phase shifts are not confined to the subjective day, as expected in a nocturnal animal, but seem to occur at CTs corresponding to the transition between day and night.

Owing to the weak responses of the rats to the stimuli and the high dispersion between individuals, we trust that the most important result from our paper is that obtained when the responses to the various stimuli are joined. We did not include the results obtained with the dexamethasone injection in the global analysis, because this is a chemical agent and it cannot be related to the environment like the other stimuli. From the global analysis, we can study not only the responses to the non-photic stimuli in general, but also how the clock faces alterations in the environment by changing its tau or its phase.

One of the main targets of natural selection of timing is the accuracy with which the phase angle differences of the circadian systems are maintained constant with respect to the rotation of the earth

(Beersma et al. 1999). Following this principle, whenever a stimulus interacts with a circadian system, the accuracy of the pacemaker improves if both the phase position and the velocity of the system (related to tau) are adjusted. Therefore, when studying the effect of a specific stimulus on the circadian system, both the PRC and the τRC provide useful information. Our results also point to the relevance of the simultaneous study of the PRCs and τRCs when analysing the effect of a given stimulus on the clock.

The global PRC and τRC are indicative of the response of the clock to the non-photic stimuli in general. Our results agree with the model proposed by Beersma et al suggesting that the PRC and τRC have the same shape, indicating that the accuracy of the pacemaker can be increased if it is allowed to respond to stimuli not only by phase shifts, but also by tau changes. However, tau lengthening and phase delays were not correlated, as predicted by the model. This can be due to the fact that we studied non-photic stimuli. Our results agree with those of Mrosovsky (Mrosovsky 1993), who found different relationships between tau changes and phase shifts, suggesting that phase shifts are not a simple consequence of particular tau changes. However, it is difficult to explain why a stimulus that should entrain the circadian clock produces distinct responses in tau and in phase shifts.

In the rat, the magnitude of the phase shifts produced by the non-photic stimuli is much lower than that produced by the photic stimuli. Although the rat is more sensitive to photic stimuli during the subjective night, it is not more sensitive to non-photic stimuli during the subjective day as it would be expected. However, it appears to be specially sensitive to *Zeitgebers* applied around the transition zones of the rest-activity cycle, i.e. around CT9-CT13 and CT23-CT3.

Thus, the transition zones in the rest-activity cycle and the light-dark cycle are crucial to the behaviour of the rat and its adaptation to the rhythmic environment. Indeed, circadian entrainment is better achieved when the LD cycle has simulated twilight transitions than when it has abrupt transitions (Boulos et al. 1996), and there is evidence that, at least in mice, there is an enhanced response to light in the twilight range (Mrosovsky et al. 2000). The higher sensitivity of an animal to the transition zones of the cycle may contribute to restrict its activity and its rest to the correct part of the cycle, what may, in the last instance, improve entrainment.

In conclusion, although rats are little sensitive to non-photic stimuli, the simultaneous consideration of the PRC and the τRC is useful for studying the responses of animals to possible *Zeitgebers*. However, it is difficult to give a biological explanation to the rhythm alterations induced by non-photic stimuli. Actually, we should take into account that these stimuli in the natural environment never take place without the corresponding day and night cycle, which makes them much less important as *Zeitgebers* than light. We suggest that the adaptation to the environment improves when the combination of several *Zeitgebers* (regardless of their capacity to change the fundamental properties of the circadian pacemaker) in the transition zones of the light-dark cycle provides the animal with the ideal environment to achieve the best entrainment.

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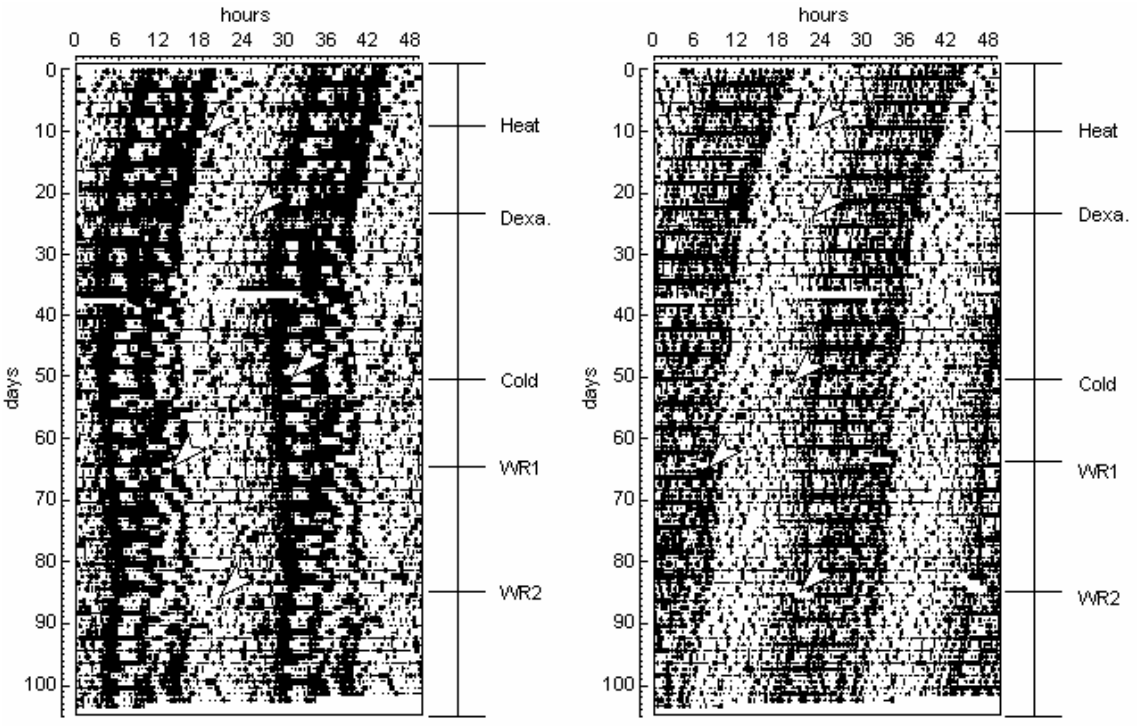

Fig. 1.- Double-plotted actograms at modulo 24.5 hours of the motor activity rhythm of two representative rats. The horizontal axis indicates the daily hours and the vertical axis on the left indicates the days of the experiment, starting on the day of enucleation. The vertical axis on the right indicates the days on which non-photic stimuli were applied. The four arrows in the actograms indicate, in temporal order, the time when heat, dexamethasone (dexa.), cold, first running-wheel (WR1) and second running-wheel pulses (WR2), respectively, were applied.

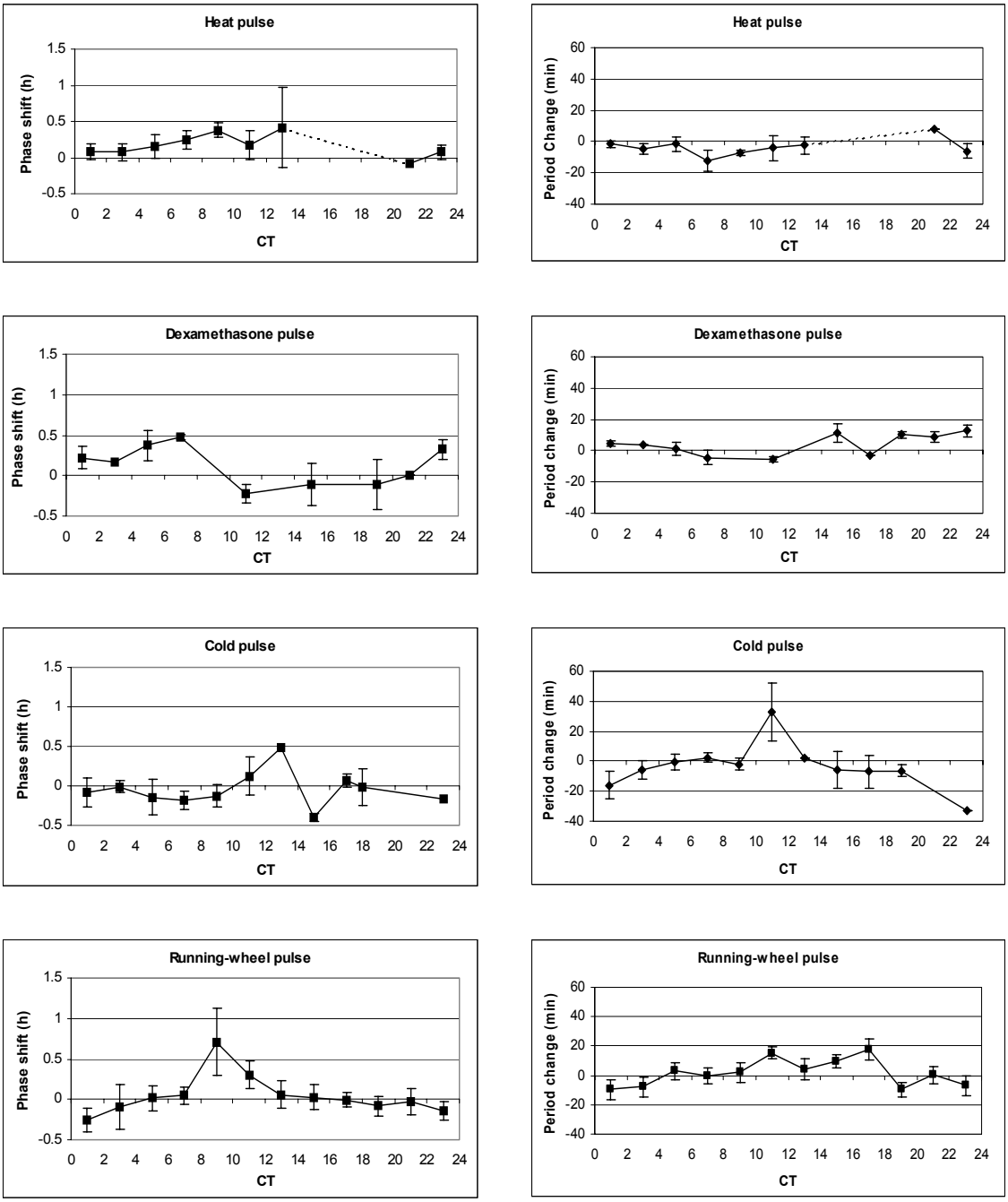

Fig. 2.- On the left, Phase Response Curves of blinded rats, and on the right, Tau Response Curves.

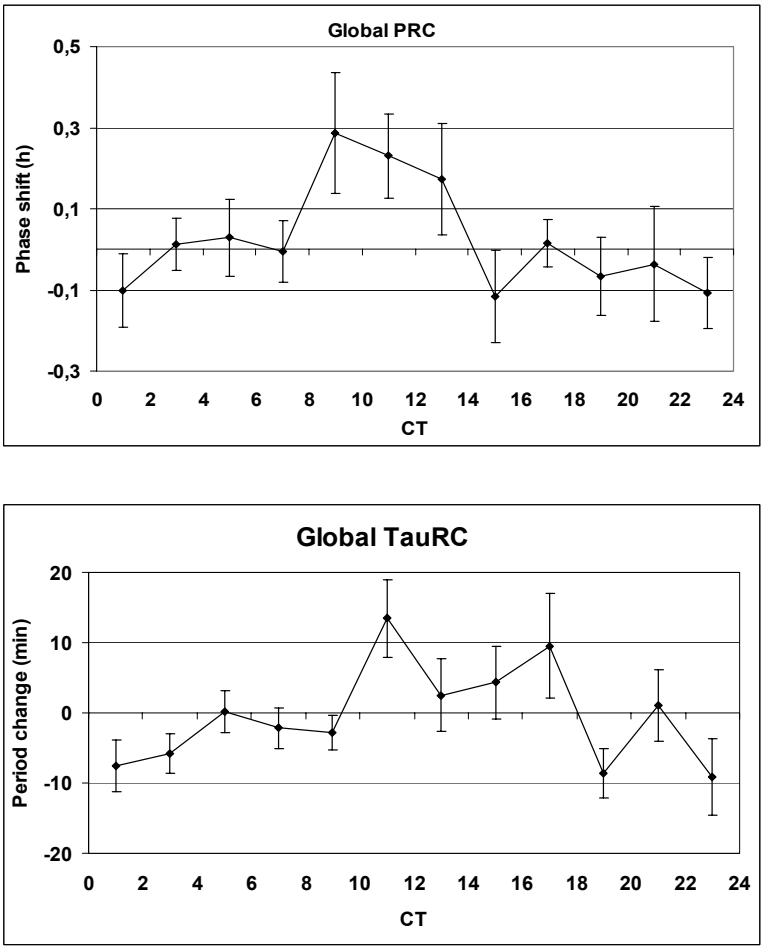

Fig. 3.- (a) Global Phase Response Curve of blinded rats, including the results obtained with the heat, cold, and running-wheel pulses.

(b) Global Tau Response Curve of blinded rats, including the results obtained with the heat, cold, and running-wheel pulses.

3.9.- EXPERIMENT 8

EFFECT OF LIGHT ON THE DEVELOPMENT OF THE CIRCADIAN RHYTHM OF MOTOR ACTIVITY IN THE MOUSE

Chronobiology International, acceptat, 2001.

Resum

Objectiu: Estudi dels efectes de les condicions d'il·luminació postnatals en el desenvolupament del ritme d'activitat motora de ratolins.

Material i mètodes: Sis ratolins Swiss femella van arribar al laboratori el dia 17 de gestació. El dia del naixement, les cries es van barrejar, de manera que cada mare alimentava 6 mascles i 6 femelles de diverses ventrades. Dos lots de mares i cries es van transferir a foscor constant (grup DD), dues a cicles llum-foscor de 24 hores de període (grup LD) i les dues restants es van sotmetre a il·luminació constant (grup LL). Vint-i-dos dies després, les cries es van deslletar. 2 mascles i 2 femelles de cada mare es va posar en gàbies individuals sota condicions de llum constant d'alta intensitat (uns 300 lux de llum blanca, etapa LL). En total es van utilitzar 24 ratolins per aquest experiment, 4 mascles i 4 femelles per grup (DD, LD i LL). A partir del dia del deslletament (dia 0 del registre) es va enregistrar l'activitat motora dels ratolins mitjançant actímetres de feixos infraroigs. Al cap de 53 dies en llum constant, els ratolins es van sotmetre a cicles LD de 24 hores de període (etapa LD), per estudiar l'encarrilament. El dia 75 del registre, es van passar tots els ratolins a condicions de foscor constant (etapa DD) i es va estudiar el seu ritme endogen. Durant l'etapa de foscor i per tal d'estudiar la resposta fàsica dels animals a la llum, es va aplicar un pols de llum de 30 minuts de durada i d'aproximadament 700 lux d'intensitat, a l'hora circadiària 15 del dia 96 del registre. Finalment, el dia 146 del registre es van sotmetre els ratolins a condicions de llum constant de baixa intensitat (uns 2 lux de llum blanca, etapa dLL), per estudiar la resposta tònica d'aquests animals a la llum.

Resultats: El ritme circadiari del grup DD presenta un nombre més elevat de components ultradiaris que el grup LL en l'etapa LL. A més, com més alta és la intensitat de la llum, el període del ritme dels ratolins del grup DD augmenta molt més que el dels altres grups i també s'observa que el seu contingut de potència varia molt més que el dels altres grups en canviar les condicions externes d'il·luminació. Pel que fa als canvis de fase produïts pel pols de llum en l'etapa DD, no s'han observat diferències significatives entre els tres grups d'animals.

Conclusions: Les condicions d'il·luminació a les que els ratolins han estat sotmesos durant les primeres setmanes de vida afectaran les respostes futures d'aquests animals a la llum. A diferència de les rates, però, no tenim prou indicis com per pensar que en els ratolins l'exposició a la llum en edats primerenques alteri el funcionament del seu rellotge circadiari, sinó que sembla que només la seva sensibilitat a la llum estigui afectada.

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EFFECT OF LIGHT ON THE DEVELOPMENT OF THE CIRCADIAN RHYTHM OF MOTOR ACTIVITY IN THE MOUSE

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ABSTRACT

In previous experiments, we found that rats raised in constant light (LL) manifested a more robust circadian rhythm of motor activity in LL and showed longer phase shifts after a light pulse in constant darkness (DD) than those raised under constant darkness. In addition, we observed that the effects produced by constant light differed depending on the time of postnatal development in which it was given. These results suggest that both sensitivity to light and the functioning of the circadian pacemaker of the rat could be affected by the environmental conditions experienced during postembryonic development. Thus, the present experiment aimed to study whether postnatal exposure to light could also affect the circadian system of the mouse. Three groups of mice were formed: One group was raised under constant darkness during lactation (DD group), the second under constant light (LL group), and the third under light-dark cycles (LD group). After lactation, the three groups were submitted first to constant light of high intensity, then to LD cycles, and finally to constant darkness. In the DD stage, a light pulse was given. Finally, mice were submitted to constant light of low intensity. We observed that the circadian rhythm of the DD group was more disturbed under constant light than the rhythm of the LL group, and that, when light intensity increased, the period of the rhythm of the DD group lengthened more than that of the LL group. No significant differences among the groups were found in

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the phase shift induced by the light pulse. Therefore, it appears that DD mice are more sensitive to light than their LL counterparts. However, at present there is no evidence to affirm that the light environment experienced by the mouse during postnatal development affects the circadian pacemaker. (*Chronobiology International, 18(4), 683–696, 2001*)

Key Words: Circadian rhythm; Development; Light; Mice; Motor activity

INTRODUCTION

Light-dark (LD) cycles are one of the most important zeitgebers for the circadian system. However, despite the entraining effect of light, several studies in various animal species show that a constantly illuminated environment causes disturbances in circadian rhythms, such as splitting (1–4) and loss of rhythm (5–7).

We have previously demonstrated that rats reared under constant light (LL) manifested a circadian rhythm of motor activity when subjected to constant light in adulthood (8,9). Moreover, we observed that, in rats, there is a critical window of time in the middle of the lactation period in which the effect of constant light on the circadian system is more pronounced (10). Therefore, it appears that when light is applied during development, it can induce some changes in the organization of the circadian system, and that these changes are long lasting (9) and affect the response of the adult to light (11,12). These experiments suggested that the effect of light on the circadian system depends on the lighting conditions under which a rat has grown. Therefore, we studied whether this was also true in another animal species and whether a general rule about the effect of light on the development of the circadian system could be inferred.

The mouse was chosen because it has been intensively studied in relation to its circadian system (13–15), genetics (16,17), circadian rhythms (18,19), responses to light (20,21), and phase-response curve (22–24). Furthermore, the development of the circadian rhythm of motor activity has been described in mice reared under 12h light-dark (LD 12:12) cycles (25) and under constant light (26), but the effect of the light environment in which a mouse is reared on the response of the adult to light is not clear. Here, we reared three groups of mice under distinct lighting conditions, and after weaning, we studied their responses to various light stimuli.

MATERIALS AND METHODS

Six pregnant mice of the Swiss strain purchased from Criffa (St. Germain-sur-l'Arbresle, France) arrived at our laboratory on the 17th day of gestation. They were placed in Makrolom® cages of $50 \times 25 \times 15$ cm under LD 12:12 cy-

cles until the day of delivery. Once the pups were born, they were cross-fostered to reduce family differences, and each dam was left with 6 males and 6 females. Three groups were formed, each with 2 dams and their respective pups. The first group was reared under LD 12:12 cycles (LD group), the second under constant light (LL group), and the third group under constant darkness (DD group). About 300 lux of bright white light was given, and darkness was less than 0.1 lux of red light. The pups remained with the dams throughout lactation, which lasted 22 days.

On the day of weaning (day 0 of the recording period), 2 male and 2 female pups were taken from each dam and placed in individual cages ($25 \times 25 \times 15$ cm) under constant light (constant light of around 300 lux) for 52 days (LL stage). Thus, a total of 24 mice were used, 4 males and 4 females in each group of mice (LD, LL, and DD).

The motor activity rhythm was recorded through activity meters, which consisted of two crossed infrared beams situated on a plane 7 cm above the floor of the cage. On day 53 of the recording period, the mice were transferred to LD 12:12 cycles for 22 days (LD stage) to entrain them to a 24h period rhythm. On day 75, the mice were transferred to constant darkness (DD stage), and the free-running rhythm was studied. During the DD stage, a 30-minute light pulse of around 700 lux was given at circadian time 15 (CT15) on day 96 to study the phasic response of the mice to light. CT15 was calculated by adding three circadian hours to CT12 (time of the onset of the activity phase), which was visually estimated from the double-plotted actograms by five researchers. Mean values were used.

Finally, to study the tonic effect of light, on day 146 of the recording period, mice were submitted to constant dim white light (around 2 lux intensity, dLL stage) for 24 days.

Throughout the experiment, the temperature of the animal room was maintained around 22°C, and mice had free access to food (Rodent Toxicology Diet, B&K Universal, Molins de Rei, Spain) and water.

To study the period of the motor activity rhythm, the periodogram of Lomb and Scargle (27,28) was calculated using 11 days of the recording period of each stage: days 41 to 52 (LL stage), days 63–74 (LD stage), days 85 to 96 (DD stage), and days 157 to 168 (dLL stage). In the LL stage, some mice changed their free-running period after each cage change, and therefore only 11 days could be used to calculate the period. The same number of days was studied in all the other stages to enable a comparison of the results. For the same range of days, a Fourier analysis was performed, from which the amplitude and power content (PC) of the first harmonic were obtained.

In the LL stage (days 0 to 52 of the recording period), the evolution of the PC of the first 14 harmonics (with a fundamental harmonic of a period of 24h) over the days was studied through the graphic matrix (29). To study the entrainment characteristics of each group in the LD stage, the psi (ψ, difference in minutes between the onset of darkness and the onset of activity) was calculated

from the mean waveform, which had previously been smoothed plus or minus 6h to eliminate reactive peaks to light. Positive values meant that the activity started after the lights went off, while negative values indicated that the activity began before the lights went off.

Graphs and calculations were made using the integrated package for chronobiology analysis El Temps (copyright A. Díez-Noguera, University of Barcelona, Spain, 1999).

The phase shift after the light pulse at CT15 was calculated in the following way: A line was drawn through the daily onsets for the 10 days before and the 10 days after treatment. The difference between the two eye-fitted lines on the day after the light pulse was the value given to the phase shift. The mean value obtained from the responses of five researchers was used for the statistical analysis.

For statistical analysis, a linear model for each of the stages was built. In these models, the independent variables were always sex and lighting conditions during lactation, while the dependent variables (one at each time) were period, mesor (midline estimating statistic of rhythm), amplitude, or PC of the first harmonic in all the stages plus the value of psi in the LD stage and the value of the phase shift produced by the light pulse in the DD stage. In addition, a global linear model to study the effect of light intensity was built using as independent variables sex, lighting conditions during lactation (DD, LD, or LL), and stage of the experiment (LL, DD, or dLL). The dependent variables, one at each time, were period, mesor, amplitude, or PC.

RESULTS

Constant Light Stage

In the LL stage, most mice manifested a significant circadian rhythm of motor activity; however, some mice showed a change in the free-running period of this activity each time they were placed into clean cages (Fig. 1, mouse LLF1). Therefore, only the last 11 days of the LL stage (time from the last cage change until the transfer to LD cycles) were used to calculate the period of the rhythm and to study the characteristics of this rhythm because, at this time, the circadian rhythm is fully developed and has had time to stabilize (8). During these 11 days, most mice had a significant circadian rhythm; only two mice of the DD group were arrhythmic, and one mouse of the LD group showed splitting. The DD group had a significantly longer period (mean ± SEM = 26.54h ± 0.03h) than the LD (mean ± SEM = 26.05h ± 0.22h) and LL (mean ± SEM = 25.02h ± 0.65h) groups (Fig. 2a). No significant differences due to lighting conditions during lactation were found in the amplitude of the first harmonic of the rhythm (Fig. 2b), whereas the PC of the first harmonic was higher in the LL group than in the other groups ($P < .05$, Fig. 2c).

Given the period changes observed during this stage, the periodogram could

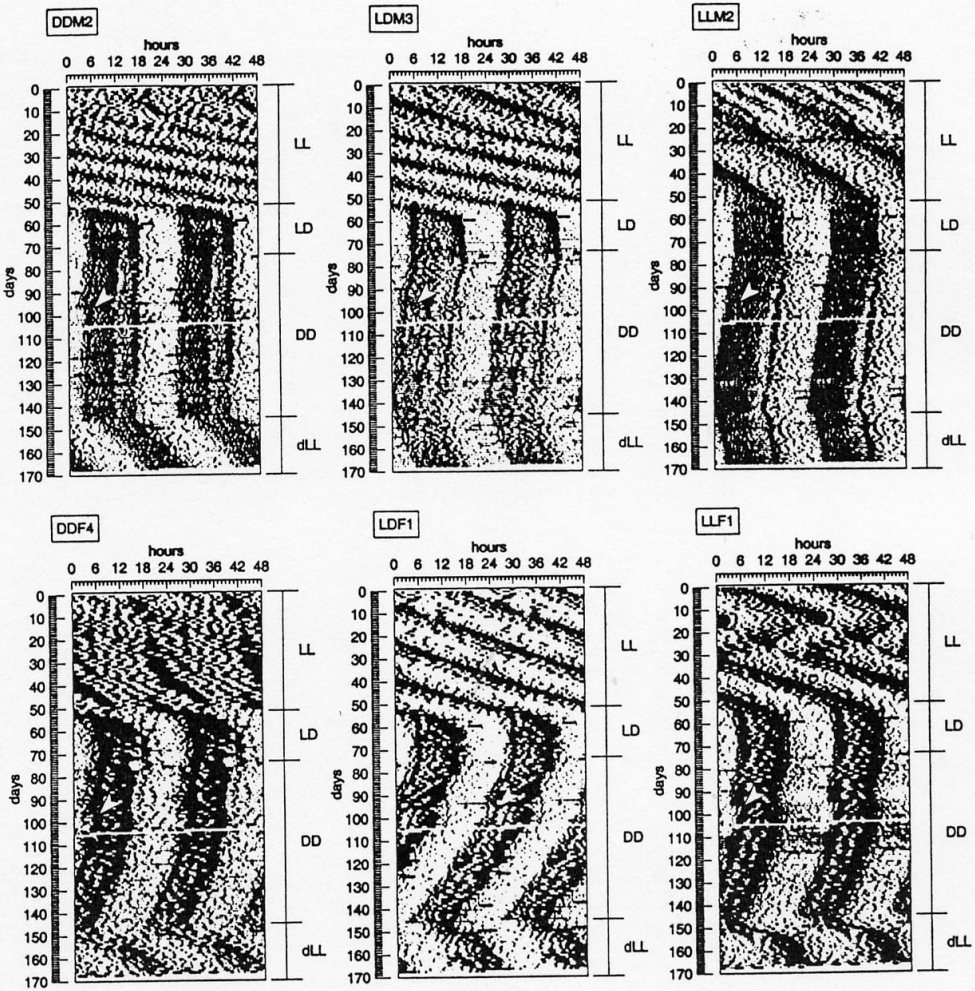

Figure 1. Double-plotted actograms at modulo 24h of six representative mice, a male and a female of each group. The two actograms on the left correspond to the DD group, the two in the middle to the LD group, and the two on the right correspond to the LL group. The left vertical axis indicates the days in which the motor activity rhythm was recorded, starting on the day of weaning. The right vertical axis indicates the stage of the experiment: LL (constant light of 300 lux), LD (light-dark cycles of a 24h period), DD (constant darkness), and dLL (constant dim light of 2 lux). The arrow indicates the position of the 30-minute light pulse (see Materials and Methods for a further explanation).

not be calculated taking into account all the days of the LL stage; therefore, to study the circadian rhythm of motor activity of each mouse in the whole stage and to compare it between groups, the evolution of the first 14 harmonics throughout the LL stage (Fig. 3) was calculated. The importance or PC of the first harmonic (the circadian one) and the sum of the PC of harmonics 4 to 9 (harmonics within an ultradian range of frequencies) were compared between

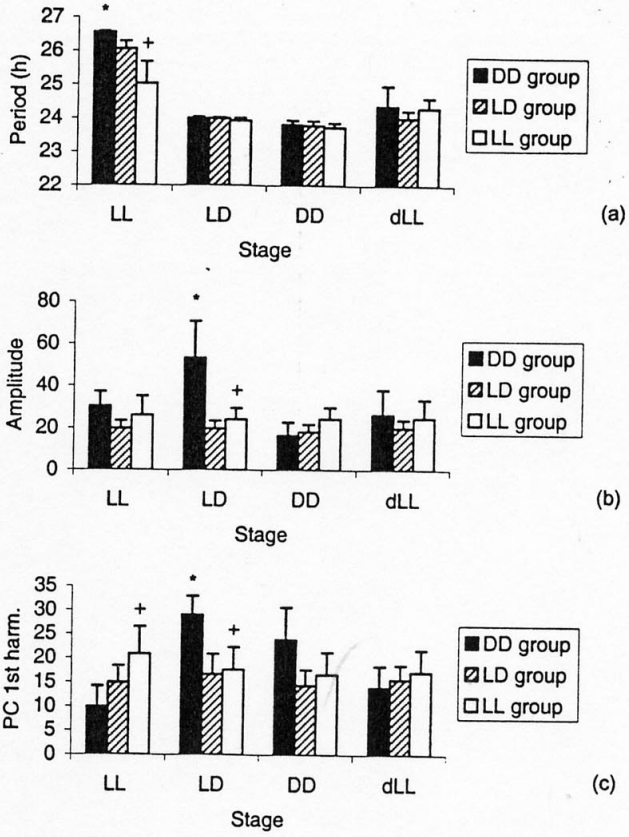

Figure 2. Graph representing (a) the period, (b) the amplitude, and (c) the power content of the first harmonic of the motor activity rhythm in each of the stages of the experiment and for each group of mice. Error bars represent the standard error. $*P < .05$ (DD group compared to LD group); $+P < .05$ (DD group compared to LL group).

groups. Only harmonics 4 to 9 of the ultradian range were used because these harmonics have been observed to be quite relevant in the development of the circadian rhythm of motor activity of rats (8). In both males and females of the LL group, the first harmonic was greater (had a higher PC) than the ultradian harmonics, whereas mice from the DD group showed a higher PC of ultradian components. The same result can be observed in Fig. 4, which shows that, in the LL stage, the PC of the circadian harmonic (harmonic 1) rose with the amount of light received during lactation (LL group > LD group > DD group), while the sum of the PC of the ultradian harmonics (harmonics 4 to 9) showed the opposite tendency (DD group > LD group > LL group). In the LD and DD groups, the sum of the PC of the ultradian harmonics had a significantly higher value than the PC of the circadian harmonics.

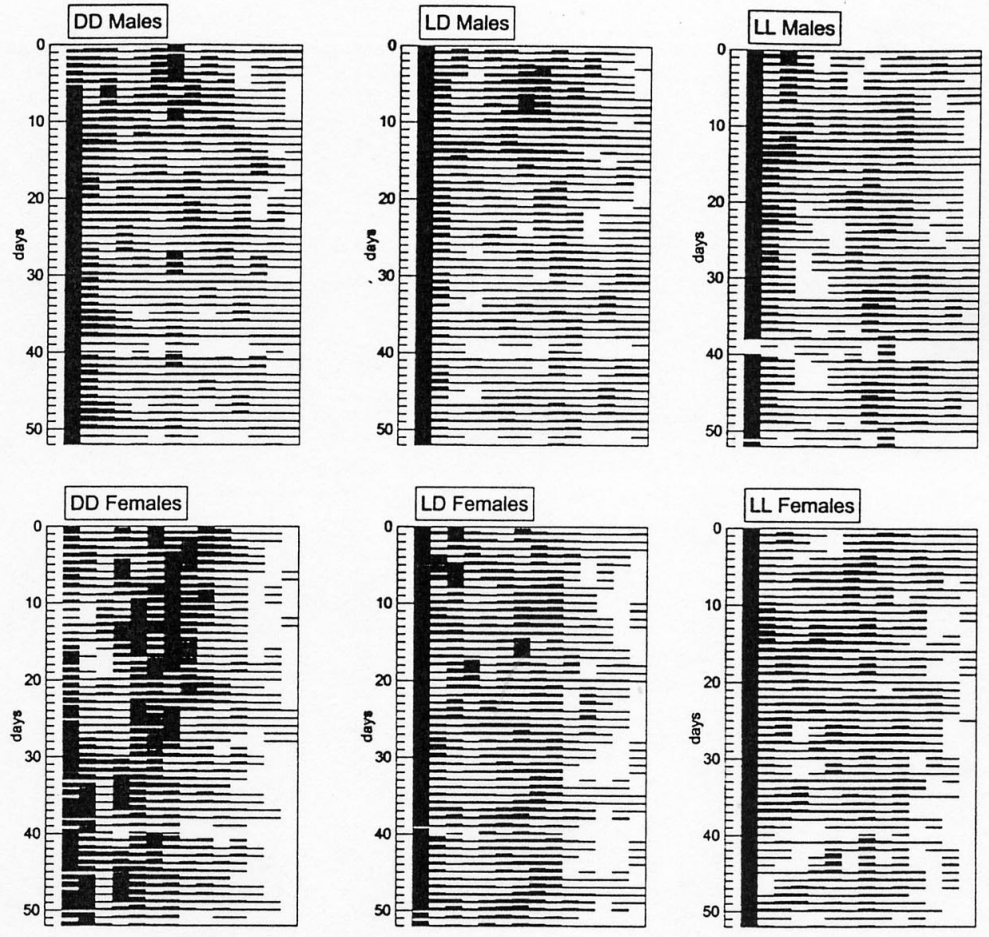

Figure 3. Mean graphic matrices of each group of mice for the LL stage. The vertical axis indicates the days of the recording period. In each column of the graphic matrix, the power content of one harmonic (from 1 to 14, from left to right) of the motor activity rhythm is represented throughout the days (see Materials and Methods for a further explanation).

Light-Dark Stage

In the LD stage, all the mice were entrained to the external LD cycle (Fig. 1), and thus they all had a 24h period rhythm (Fig. 2a). The DD group had the highest amplitude and PC ($P < .05$, Figs. 2b, 2c). No significant differences due to the lighting conditions during lactation were found in the psi, which had a mean value of 69.9 ± 14.3 minutes (mean $\pm$ SEM) for all the groups, thereby indicating that all the mice started moving about 1h after lights off, and that they all entrained with the same phase relationship to the LD cycle.

significant differences due to lighting conditions during lactation were observed in the amplitude or the PC of the rhythm (Figs. 2b, 2c).

General Analysis (Constant Darkness, Constant Dim Light, Constant Light Stages)

The general analysis of the DD, dLL, and LL stages aimed to check the effect of the increase in light intensity (i.e., the tonic effect of light) (DD stage, null intensity; dLL stage, low intensity; LL stage, high intensity) on the variables of the rhythm studied.

In the amplitude of the first harmonic, only differences due to sex (females had higher values than males), and not to the lighting conditions during lactation, were detected ($P < .05$). The PC of the first harmonic was also higher in females than in males, and we observed that, in the DD group, there was a significant tendency ($P < .05$) that the higher the light intensity, the lower the PC of the first harmonic (PC in the LL stage < PC in the dLL stage < PC in the DD stage).

Despite the high interindividual differences, the three groups of mice followed Aschoff's rule; that is, the higher the light intensity was, the longer the tau (tau in the LL stage > tau in the dLL stage > tau in the DD stage; Fig. 1). The finding that, in the LL stage, the period of the mice of the DD group increased to a greater extent than the period of the mice of the other groups is noteworthy ($P < .05$, Fig. 2a).

DISCUSSION

Most anatomical studies of the development of the suprachiasmatic nuclei (SCN) of the hypothalamus, the site of the principal pacemaker in mammals, seem to indicate that neurogenesis is completed prior to birth (30,31), and that the firing rate of SCN neurons already has a clear circadian rhythm in the newborn pup (32). The retinohypothalamic tract (which brings environmental light information to the SCN), the geniculohypothalamic tract (which brings nonphotic information to the SCN), and the pineal gland (which synthesizes the hormone melatonin) develop postnatally and do not reach the adult pattern until postnatal day 16 (33,34). This coincides with the time in which the number of photically induced Fos-like positive cells in the SCN of mice reach adult values (35). In addition, given that long-term light exposure induces some changes in the synapses of optic afferences in the SCN (36), it is possible that the pacemaking system might be susceptible to environmental light manipulation during the early postnatal period. Some experiments in cockroaches (37) and in rats (10–12) also support this hypothesis. However, our results are not so clear.

The phase shift induced by a light pulse under constant conditions, as part of the phase response curve of the animal, has been proposed as an indicator of

confirm that there are differences between both species with respect to the light effects on the circadian system during the first days of life. Although the rat and the mouse are closely related phylogenetically, and despite similar circadian systems and specifically similar SCN (13,41,42), some differences in the circadian systems of both rodents have been reported, for example, in the ontogeny of vasoactive intestinal peptide (VIP) cells (43,44) or in the number of AVP-immunoreactive cells of the SCN (44,45). Although AVP is not required for circadian behavior in rats (46), changes in period length have been correlated in a certain strain of mouse and of hamster with fewer AVP cells (47,48). Differences between rats and mice have not only been found in the central circadian system itself, but also in light reception at the retinal level. It has been reported that, among several laboratory animals, the rat is the most sensitive species to the damaging effects of light (49). There is also a distinct photoreceptor degenerative pattern between rats and mice when submitted to constant bright light (50).

Taking all these considerations into account, we conclude that light applied during the first weeks of life of the mouse affects its future responses to light, and in contrast to rats, there is no evidence that early exposure to light alters the functioning of the circadian clock, but rather the mouse's sensitivity to light. Hence, the lighting conditions perceived during the ontogeny of the SCN may affect the animals of distinct species differently, which prevents the formulation of a general rule on the effect of light on the developing circadian system. Further experiments with different light intensities to characterize the tonic and phasic effect of light, both with mice and with other animal species, are thus required.

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3.10.- DISCUSSIÓ

3.10.- DISCUSSIÓ

A la literatura es poden trobar molts casos descrits sobre l'arritmicitat induïda per la llum constant sobre diversos ritmes circadiaris en diverses espècies animals (Deprés-Brummer et al. 1995, Honma et al. 1996, Takeo 1984, Eastman i Rechtschaffen 1983). Curiosament, però, en el nostre laboratori s'havia observat que els animals que havien estat des del dia del naixement sota condicions de llum constant (LL), desenvolupaven un ritme circadiari d'activitat motora en condicions de LL (Cambras i Díez-Noguera 1991).

El primer que ens vam proposar, doncs, va ser estudiar el desenvolupament del ritme d'activitat motora en rates sotmeses a LL o a foscor constant (DD) des del naixement i veure fins a quin punt la ritmicitat de la mare estava relacionada amb la manifestació del ritme en LL que prèviament havíem observat. Dels resultats de l'Experiment 1 se'n desprèn que, independentment de si la mare era rítmica o arrítmica, el fet que un animal hagués crescut sota condicions de LL feia que pogués manifestar un ritme circadiari d'activitat motora quan se sotmetia a LL. A més a més, vam veure que aquesta capacitat no era passatgera, sinó que es mantenia al llarg de la vida de l'animal, tal i com mostren els resultats de l'experiment que es troba a l'Annex. A partir d'aquí ens vam formular quatre preguntes:

1) Cal que els animals estiguin sotmesos a LL durant tota l'etapa d'alletament per a manifestar posteriorment un ritme circadiari sota LL, o bé amb menys dies és suficient?
2) La diferència entre un animal que s'ha criat en DD i un que s'ha criat en LL està només en la capacitat d'aquests últims de manifestar un ritme circadiari en LL, o bé es poden observar d'altres diferències?
3) Quin tipus de canvis produeix la llum rebuda durant les primeres setmanes de vida sobre el sistema circadiari i quins elements en són afectats?
4) I finalment, l'efecte de la llum constant durant el desenvolupament observat en rata es dóna en d'altres espècies animals?

Per respondre a la primera pregunta vam dissenyar l'Experiment 2. Amb aquest experiment vam veure que els animals no havien d'estar tot l'alletament sota LL per manifestar posteriorment un ritme circadiari sota LL, sinó que 12 dies són suficients. A més amb aquest experiment vam observar que l'expressió d'aquest ritme circadiari depenia no només del nombre de dies en què l'animal havia estat sotmès a LL, sinó també de la posició d'aquests dies dins l'etapa d'alletament. D'aquí va sorgir doncs, l'Experiment 3, en el qual es van acotar més les finestres de LL durant l'alletament i es van aplicar en diversos moments d'aquesta etapa. Els resultats van mostrar que cap al voltant del dia 16 després del naixement és quan el sistema circadiari de la rata és més sensible a la llum ambiental. De fet, quan la rata neix encara no té el

sistema nerviós completament desenvolupat. Així per exemple, pel què fa a estructures relacionades amb el sistema circadiari, se sap que les característiques morfològiques de les poblacions neuronals dels NSQ (Moore et al. 1989, Van den Pol 1980), la mida i forma dels NSQ (Moore 1991), la sinaptogènesi dins els NSQ (Moore i Bernstein 1989), les projeccions del TRH cap als NSQ i cap a d'altres àrees del cervell (Speh i Moore 1993) i la projecció del TGH cap als NSQ (Moore et al. 1989) no arriben als nivells d'adult fins a la segona o tercera setmanes de vida. Així doncs no resulta estrany trobar que la llum exerceix el seu major efecte sobre el sistema circadiari quan és aplicada en el moment en què les estructures més importants d'aquest sistema estan acabant de madurar i d'organitzar-se. El fet que la llum aplicada ben al principi o bé ben al final de l'alletament no eviti la posterior arritmicitat per LL indicaria que en el desenvolupament del sistema circadiari hi ha un període crític durant el qual aquest seria més sensible -o es deixaria influenciar més- pels estímuls externs. Aquest període crític es trobaria en un moment en què el sistema circadiari estaria prou desenvolupat com per rebre i transmetre la informació que li arriba de l'exterior, però alhora, encara estaria madurant per arribar al nivell d'organització de l'animal adult. De fet, aquest període crític o sensible del sistema circadiari podria assemblar-se al descrit en el desenvolupament de l'escorça visual, és a dir, que seria un període de dies durant les primeres setmanes de vida en les quals una experiència visual anormal podria interferir en els processos de desenvolupament genèticament preestablerts, ajustant la seva funcionalitat (Barlow 1975).

La resposta a la segona pregunta plantejada inicialment, ve contestada pels resultats dels Experiments 2, 3, 4, 5 i 6, dels quals se'n pot extreure que les condicions d'il·luminació experimentades durant l'alletament no només influencien la manifestació del ritme d'activitat motora en LL, sinó que també afecten la manifestació del ritme sota condicions de DD i sota cicles LD de diferent període, els canvis de fase produïts per un pols de llum i la resposta de l'animal a un increment progressiu de la intensitat de llum ambiental. Més concretament, hem observat que:

1. Sota condicions de LL, els animals que s'han criat en LL, a diferència dels que han crescut en DD, manifesten majoritàriament un ritme circadiari d'activitat motora. Tot i que no hi ha diferències en quant al període d'aquest ritme, sí que n'hi ha en quant a la seva manifestació: el ritme és més marcat (té una amplitud, un contingut de potència del primer harmònic i un percentatge de variança explicada pel ritme més elevats) en els animals que han crescut sota condicions de LL que els que ho han fet en DD.

   Sota condicions de LL en què la intensitat de llum va incrementant gradualment, hem observat que, seguint la primera regla d'Aschoff, el període del ritme d'activitat motora incrementa juntament amb la

intensitat de llum. Tot i això, aquest increment és similar tant en els animals LL com en els DD, de manera que no hi ha diferències en el període del ritme. Tot i això, la importància d'aquest ritme (expressada per l'amplitud, el contingut de potència del primer harmònic i el percentatge de variança explicada pel ritme) no és pas la mateixa entre els animals DD i LL per a cada intensitat de llum i a més, l'efecte de l'increment de la intensitat de llum sobre aquestes variables també difereix entre els animals LL i els DD.

2. Sota condicions de DD passa el mateix que en LL però a la inversa: el tau del ritme d'activitat motora és el mateix tant en els animals LL com en els animals DD, però el ritme és més marcat en els animals DD que en els animals LL. El canvi de fase produït per un pols de llum a CT15 és més gran en els animals DD que en els LL.
3. Sota cicles LD de període de 22 a 27 hores el ritme d'activitat motora dels animals DD continua sent més manifest en els animals DD que en els LL. Pel què fa a les característiques de l'encarrilament, quan el període del cicle extern és de 24 o 25 hores, no hi ha diferències en el psi degudes a les condicions d'il·luminació durant l'alletament, mentre sí que n'hi ha en els altres períodes.

Aquests experiments també ens han permès observar que les rates que s'han criat sota cicles LD de 24 hores de període tenen unes característiques intermèdies de les rates que s'han criat en LL o DD pel què fa a la fase d'encarrilament sota cicles LD de diferent període, a la importància del ritme sota cicles LD i sota condicions de LL d'intensitat creixent de llum i finalment, en la proporció d'animals rítmics sota diverses intensitats de llum. Sembla doncs que la llum durant l'alletament té un efecte "acumulatiu", és a dir, que part dels resultats obtinguts dependrien de la "quantitat" total de llum rebuda al llarg de l'etapa d'alletament.

Tots aquests resultats ens poden ajudar a contestar, en part, la tercera pregunta formulada inicialment sobre quins canvis ha produït la llum a l'organisme i a quin nivell es poden trobar. Podem considerar que, funcionalment, el sistema circadiari està constituït per tres nivells: unes vies d'entrada d'informació o *input*; pel *pacemaker* circadiari, que és l'element principal i generador dels ritmes; i per unes vies de sortida o *output*. Com s'ha dit anteriorment, durant les primeres setmanes de vida les estructures més importants del sistema circadiari s'estan acabant de desenvolupar i seria doncs aquest el moment en què la llum podria afectar la seva estructuració i/o organització funcional. Ja hem dit també que la importància del ritme d'activitat motora depèn de les condicions d'il·luminació en què un animal ha estat criat, és a dir,

que l'*output* és diferent. L'origen d'aquesta diferència pot trobar-se a nivell d'*input* o bé a nivell de *pacemaker* circadiari.

Fixem-nos primer en l'*input* del sistema. Se sap que sotmetre un animal a llum constant pot danyar-li la retina, ja que la major part de cèl·lules fotoreceptores són destruïdes (Terman et al. 1991). Tot i això, els animals LL podrien no ser els més afectats per la llum en l'etapa adulta, ja que hi ha diversos estudis en rata que demostren que tant la composició i funcionament dels fotoreceptors (Penn i Anderson 1987, Penn et al. 1989), com la quantitat i activitat d'antioxidants a la retina (Penn et al. 1987) depenen de les condicions d'il·luminació en què una rata ha crescut, de manera que les que han crescut sota ambients d'intensitat elevada de llum estan més protegides front la llum que les rates que han crescut sota condicions de baixa intensitat de llum. A més a més, si l'exposició a la llum ocorre abans dels 20 dies de vida, la retina es torna resistent al dany produït per la llum i l'electroretinograma d'aquestes rates mostra respostes normals (Malik et al. 1986). Seguint aquesta idea, en el nostre cas les rates DD serien probablement les més afectades per dany retinal degut a la llum. Per tant, si les diferències que hem observat entre els animals DD i LL fossin degudes només a diferències en la sensibilitat a la llum, lligada a la funcionalitat retinal, els animals DD serien els menys sensibles a la llum. Seguint aquesta hipòtesi, com s'explicaria el fet que precisament aquests animals tinguin un canvi de fase més marcat que les rates LL després d'un pols de llum? Com s'explicaria que els canvis de període deguts a canvis d'intensitat de llum siguin similars en rates DD i LL? I com s'explicaria que sota cicles LD el ritme de les rates DD sigui més manifest que el de les LL? Sembla doncs que les diferències entre els dos grups de rates s'han de buscar a un altre nivell.

Així doncs, l'últim que ens queda per veure és el propi *pacemaker* circadiari. Amb aquesta finalitat es va dissenyar l'Experiment 6, que ens ha aportat una de les troballes més importants: el fet que les rates DD cegues continuen tenint un ritme més marcat que les rates LL cegues, demostra clarament que la diferència entre aquests dos grups de rates va més enllà de la sensibilitat a la llum o *input* fòtic. Cal tenir en compte, a més, d'altres punts. Tradicionalment s'ha dit que les dues característiques principals que descriuen el funcionament d'un *pacemaker* circadiari són el seu període d'oscil·lació i el canvi de fase que li produeix un determinat estímul (Daan i Pittendrigh 1976). Tot i que en cap dels experiments realitzats no hem trobat diferències en el període de rates LL i rates DD, sí que n'hem trobades en la corba de respostes de fase d'aquests animals (tant en els canvis de fase deguts a un pols de llum, com en la relació de fases o psi adoptat per l'animal sota cicles LD de diferent període). Cal afegir a més, que en tots els experiments realitzats hem trobat que la importància del ritme (expressada per l'amplitud, el contingut de potència del primer harmònic i el percentatge de variança explicada pel ritme) depèn de les condicions d'il·luminació durant l'etapa d'alletament. Aquest fet ens fa pensar que hauríem d'incloure la força o amplitud d'oscil·lació com una tercera propietat fonamental dels *pacemakers* circadiaris.

Pel què fa a com la llum podria afectar el *pacemaker* circadiari, diversos estudis demostren que l'exposició a llum constant produeix canvis en les sinapsis entre les neurones del TRH i dels NSQ (Güldner et al. 1997), augmenta la resposta general de les neurones dels NSQ a melatonina (Yu et al. 1993), altera la ritmicitat dels NSQ (Yu et al. 1993) i indueix grans canvis en els nivells de *glial fibrillary acidic protein* (proteïna relacionada amb el nombre de cèl·lules glials) tant en els NSQ com en el FIG (Moriya et al. 2000, Lavialle et al. 2001). En definitiva, sembla ser que la llum pot afectar directament el *pacemaker* circadiari. Tot i que els estímuls lluminosos produeixen uns efectes molt més forts en el ritme circadiari de la rata que els produïts per estímuls no fòtics (Experiments 6 i 7), els resultats d'aquest Experiment 7 ens poden ser d'utilitat per aclarir en quins aspectes difereix el *pacemaker* circadiari de rates DD i rates LL. Només caldria aplicar un d'aquests estímuls no fòtics en rates DD i LL i estudiar-ne les seves conseqüències sobre el ritme, tant a través de canvis de fase, com a través de canvis de període.

Finalment, l'última pregunta que ens queda per respondre és si l'efecte de la llum sobre el desenvolupament del sistema circadiari que hem observat en rata també es dóna en d'altres espècies animals, com és el cas dels insectes (Barret i Page 1989, Page i Barrett 1989, Tomioka i Chiba 1989a, b, Tomioka i Chiba 1992). Els resultats de l'Experiment 8 suggereixen que la manifestació del ritme circadiari d'activitat motora dels ratolins també es veu afectada per les condicions d'il·luminació rebudes durant l'alletament: com en les rates, els ratolins LL tenen un ritme més marcat en llum constant que els ratolins DD; sota cicles LD de 24 hores de període els ratolins DD tenen un ritme més marcat que els ratolins LL i no existeixen diferències en quant a psi; i en DD, no hi ha diferències en quant a tau i els ratolins DD tenen un ritme més marcat que els ratolins LL. Tot i amb això, hi ha tres diferències importants respecte les rates: 1) sota LL d'alta intensitat el tau dels ratolins DD és més llarg que el dels ratolins LL; 2) quan s'incrementa la intensitat de llum, l'allargament de període és molt més pronunciat en els ratolins DD que en els LL, mentre que en les rates DD i LL és similar i 3) en el canvi de fase produït per un pols de llum a CT 15 no hem trobat diferències entre els dos grups de ratolins. Tot i que la rata i el ratolí són espècies properes i per tant, tenen molts punts en comú, les discrepàncies que hem trobat en quant a la manifestació del ritme circadiari confirmen que hi ha diferències en les dues espècies respecte els efectes de la llum en el sistema circadiari. De fet, s'han trobat diferències entre les dues espècies tant a nivell de sistema circadiari (Herzog et al. 2000), com a nivell d'efectes de la llum sobre la retina (LaVail et al. 1987). Sigui com sigui, la llum aplicada durant el desenvolupament del sistema circadiari del ratolí sembla afectar almenys dues de les propietats prèviament descrites dels *pacemakers* circadiaris, com són el període i l'amplitud o força del ritme.

En conseqüència, així com en insectes, en mamífers –o com a mínim en rata i ratolí- hi ha evidència per creure que les condicions ambientals de llum durant les primeres etapes de la vida són capaces d'afectar les propietats del *pacemaker* circadiari.

3.10.- DISCUSSION

The disrupting effects of constant light on several rhythms of various species have been extensively reported (Deprés-Brummer et al. 1995, Honma et al. 1996, Takeo 1984, Eastman and Rechtschaffen 1983). However, we have observed that animals born and raised in constant light conditions (LL) develop a circadian rhythm of motor activity under LL (Cambras and Díez-Noguera 1991).

Therefore, we studied the development of the motor activity rhythm of rats reared under LL or constant darkness (DD) since the day of birth and examined the extent to which the dam rhythmicity was associated with the reported manifestation of the rhythm under LL. The results of Experiment 1 reveal that, regardless of whether the dam was rhythmic or not, all the rats reared under LL will manifest a circadian rhythm of motor activity under LL. Moreover, this was not a temporary property, but lasted throughout the life span of the animal, as shown in the Annex *section. Hence, four questions arose:*

1. *Is it necessary to maintain the animals under LL throughout the lactation stage, or fewer days are sufficient?*
2. *Is the manifestation of the circadian rhythm in LL the only difference between one animal reared under DD and one animal reared under, or are there other observable differences?*
3. *What kind of changes produces the light received during the first weeks of life, and which elements are thus affected?*
4. *Does the effect of constant light during development observed in rats occur in other animal species?*

To answer the first question, Experiment 2 was designed. The results showed that it was not necessary for the animal to remain under LL throughout the lactation stage to manifest later a circadian rhythm of motor activity under LL, but 12 days are enough. Furthermore, with this experiment we also observed that the expression of the circadian rhythm under LL not only depended on the number but also on the position of the LL days received during lactation. This was the starting point of Experiment 3, in which the windows of days under LL were more exactly determined and placed at several moments of the lactation stage. Results revealed that around postnatal day 16, the circadian system of the rat is more sensitive to environmental light. In fact, when the rat is born, its nervous system is still immature. For example, regarding the structures related to the circadian system, it is known that the morphological characteristics of neural populations of the SCN (Moore et al. 1989, Van den Pol 1980), the size and shape of the SCN (Moore 1991), the synaptogenesis within the SCN (Moore and Bernstein 1989), the RHT projections to the SCN and other areas of the brain (Speh and Moore 1993) and the GHT

projection to the SCN (Moore et al. 1989) do not reach the adult level until the second or third postnatal weeks. Therefore, it is not surprising to find that light exerts its major effect on the circadian system when the main structures of this system are completing their maturation and organisation. The finding that light applied few days after birth or few days before weaning does not avoid future arrhythmicity by LL suggests that there is a critical period during the development of the circadian system in which it is more sensitive to –or is more prone to be influenced by- external stimuli. This critical period would be placed in a moment when the circadian system is sufficiently developed to receive and transmit information from the environment, but still maturates to reach the organisation level of the adult. This critical or sensitive period in the development of the circadian system is reminiscent of that of the visual cortex development: during the first postnatal weeks, abnormal visual experience interferes with the developing processes genetically established, adjusting their functionality (Barlow 1975).

The second question can be answered from the results of Experiments 2, 3, 4, 5 and 6, which show that the lighting conditions experienced during lactation are not only reflected in the manifestation of the motor activity rhythm under LL, but they also affect the manifestation of the rhythm in DD and LD of various periods, the phase changes produced by a light pulse and the response of the animal to increasing environmental light intensity. In particular, we observed that:

1. *Under LL, the animals reared in LL, in contrast to those reared in DD, show a circadian rhythm of motor activity. Although there are no differences in the period of this rhythm, the manifestation of the rhythm differs: it is more marked (has a higher amplitude, power content of the first harmonic and percentage of variance explained by the rhythm) in LL than in DD animals.*

   Under LL of increasing light intensity, following the first Aschoff's rule, the period lengthens with the increase in light intensity. However, this increase in period is similar in LL and DD rats for each light intensity, and so the periods are indistinguishable. We would like to highlight that the importance of the rhythm (expressed by the amplitude, power content of the first harmonic and percentage of variance explained by the rhythm) differs between DD and LL animals at each light intensity. Moreover, the effect of increasing light intensity also differs between LL and DD animals.
2. *Under DD, we obtained the same results as in LL but vice versa: the tau of the motor activity rhythm is similar in LL and DD animals, but the rhythm is more marked in DD than in LL animals. The phase shift*

produced by a light pulse at CT15 was higher in DD than in LL animals.

3. *Under LD cycles of 22h- to 27h-periods, the motor activity rhythm of DD animals is more marked than that of LL animals. Regarding the phase relation with the LD cycle, when the period of the LD cycle is of 24 or 25 hours, there are no differences in the psi due to the lighting conditions during lactation, whereas differences in the psi are found at the other periods tested.*

These experiments have also revealed that rats reared under 24h-period cycles have intermediate characteristics compared with LL and DD rats in the psi to LD cycles of various periods, in the importance of the rhythm under LD cycles and under LL of increasing intensity, and in the proportion of rhythmic animals under LL of several intensities. Thus, light during lactation may have an "accumulating" effect, so that part of the results obtained depend on the total "quantity" of light received during lactation.

These results allow us to answer, in part, the third question previously formulated about the type of changes that light has produced on the organism and at which level they can be found. We can consider that the circadian system consists of three functional levels: the input pathways, the circadian pacemaker (principal element of the system and the generator of the rhythms) and the output pathways. As mentioned above, the main structures of the circadian system maturate during the first weeks of light and hence, at this time light can affect the structure and the functional organisation of the system. We have also reported that the importance of the motor activity rhythm depends on the lighting conditions in which an animal is reared, and so the output differs. The origin of this difference could be at the input or at the pacemaker level.

Let's focus first in the input to the system. It is well known that when an animal is placed under constant light, it suffers from retinal damage, as most photoreceptor cells are destroyed (Terman et al. 1991). However, LL rats may not be the most affected by light in the adulthood, as several studies demonstrate that both the composition and functioning of the photoreceptors (Penn and Anderson 1987, Penn et al. 1989) and the quantity and activity of the retinal antioxidants (Penn et al. 1987) depend on the lighting conditions in which a rat was reared, in such a way that those reared under a high light intensity environment are more protected against the negative effects of light than those reared under a low intensity environment. Moreover, if light exposure occurs before postnatal day 20, the retina becomes resistant to light damage and the electroretinogram of these rats shows normal responses (Malik et al. 1986). Following this idea, in our case DD rats would be the most affected by light-induced retinal damage. Therefore, if the differences observed between DD and LL animals were only caused by light sensitivity (associated with retinal function), DD animals

would be the least sensitive to light. According to this hypothesis, how could it be explained that the DD animals have the greatest phase shifts after a light pulse? How could it be explained that period changes due to light intensity modifications are indistinguishable between DD and LL rats? And finally, how could it be explained that the rhythm of DD rats is more marked than that of LL rats under LD cycles? Therefore, the differences between the two groups of rats should be sought at other levels.

The last hypothesis involves the circadian pacemaker. Hence, we designed Experiment 6, from which we have obtained one of the most important results: the fact that blind DD rats still show a more marked rhythm than blind LL rats, supporting that the difference between the two groups is beyond light sensitivity or photic input. Some other points have to be taken into account too. Traditionally it has been proposed that the two fundamental properties that describe the functioning of a pacemaker are the period of its oscillation and the phase shift produced by a specific stimulus (Daan and Pittendrigh 1976). Although we found no period differences between DD and LL animals, the phase response curve of these animals (both in the phase shifts produced by a light pulse, and in the phase relation or psi adopted by the rhythm under a specific LD cycle) differed. Moreover, we found that the importance of the rhythm (expressed by the amplitude, power content of the first harmonic and percentage of variance explained by the rhythm) depended on the lighting conditions during lactation in all the experiments. This suggests that the strength or amplitude of oscillation should be included as a third fundamental property of circadian pacemakers.

Regarding the way light can affect the circadian pacemaker, several studies show that exposure to constant light produce changes in the optic synapses at the SCN level (Güldner et al. 1997), increases the general response of SCN neurones to melatonin (Yu et al. 1993), alters the rhythmicity of the SCN (Yu et al. 1993) and largely modifies the levels of glial fibrillary acidic protein (which is linked to the number of glial cells) both in the SCN and the IGL (Moriya et al. 2000, Lavialle et al. 2001). Light can thus directly affect the circadian pacemaker. Although photic stimuli have greater effects on the circadian rhythm of the rat than non-photic stimuli (Experiments 6 and 7), the results of Experiment 7 may be useful to identify the aspects that differ in the SCN of DD and LL rats. Non-photic stimuli should be applied to DD and LL rats and study their effect on the circadian rhythm, through both phase shifts and period changes.

Finally, the last question is whether the effect of light on the developing circadian system observed in the rat also occurs in other animal species, as is the case of insects (Barret and Page 1989, Page and Barrett 1989, Tomioka and Chiba 1989a, b, Tomioka and Chiba 1992). The results of Experiment 8 suggest that the manifestation of the circadian rhythm of motor activity in mice is also affected by the lighting conditions received during lactation: like rats, LL mice also manifest a stronger rhythm under LL than DD mice; under 24h-preriod LD

cycles, DD mice have a more marked rhythm than LL mice and there are no differences in the psi values, and under DD there are no differences in tau and DD mice have a stronger rhythm than LL mice. However, there are three main differences with respect to rats: 1) under LL of high intensity, most DD mice show a circadian rhythm whith a longer period than LL mice; 2) when light intensity is increased, the rise in the tau of DD mice is more pronounced than that of LL mice (whereas the increase is similar in DD and LL rats) and 3) no differences in the phase shift produced by a light pulse at CT15 have been found between DD and LL mice. Although rat and mice are close species and so they have many things in common, the discrepancies that we have found related to the manifestation of the circadian rhythm confirm that they differ in the effects of light on the circadian system. In fact, differences at the circadian system level (Herzog et al. 2000) and in the light effects at the retinal level (LaVail et al. 1987) have been reported elsewhere. Anyway, the light applied during the development of the circadian system of the mouse affects at least two of the above described fundamental properties of the circadian pacemaker: the period and the amplitude of oscillation.

In conclusion, we show that the lighting conditions during the first weeks of life affect the properties of the circadian pacemaker in rats and mice.